BONE MARROW MICROENVIRONMENT IN ACUTE MYLEOID LEUKEMIA

by

PRIYA CHANDRAN

B.Sc., University of Kerala, 2010

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ABSTRACT

Acute myeloid leukemia (AML) often remains refractory to current chemotherapy and transplantation approaches despite many advances in our understanding of mechanisms in leukemogenesis. The bone marrow "niche" or microenvironment, however, may be permissive to leukemia development and studying interactions between the microenvironment and leukemia cells may provide new insight for therapeutic advances. Mesenchymal stem cells (MSCs) are central to the development and maintenance of the bone marrow niche and have been shown to have important functional alterations derived from patients with different hematological disorders. The extent to which MSCs derived from AML patients are altered remains unclear. The aim of this study was to detect changes occurring in MSCs obtained from human bone marrow in patients with AML by comparing their function and gene expression pattern with normal age-matched controls.

MSCs expanded from patients diagnosed with acute leukemia were observed to have heterogeneous morphological characteristics compared to the healthy controls. Immunohistochemistry and flow data confirmed the typical cell surface immunophenotype of $CD90^+$ $CD105^+$ $CD73^+$ $CD34^-$ $CD45^-$, although MSCs from two patients with AML revealed reduced surface expression of CD105 and CD90 antigens respectively. Differentiation assays demonstrated the potential of MSCs from AML patients and healthy donors to differentiate into bone, fat and cartilage. However, the ability of MSCs from AML samples to support hematopoietic function of $CD34^+$ progenitors was found to be impaired while the key hematopoietic genes were found to be differentially expressed on AML-MSCs compared to nMSCs.

These studies indicate that there exist differences in the biologic profile of MSCs from AML patients compared to MSCs derived from healthy donors. The results described in the thesis provide a formulation for additional studies that may allow us to identify new targets for improved treatment of AML.

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LIST OF ABBREVIATIONS

HSC	Hematopoietic stem cells
CFC	Colony forming cells
LTC-IC	Long term culture initiating cells
CD	Cluster of differentiation
SCF	Stem cell factor
IL	Interleukin
VCAM	Vascular adhesion molecule
ANGPT	Angiopoietin
VEGF	Vascular endothelial growth factor

bFGF	Basic fibroblast growth factor
VCAM	Vascular adhesion molecule
Spp	Osteopontin
SDF1-α/CXCL12	Stromal cell-derived factor 1α/ C-X-C motif chemokine 12
RGMB	Repulsive guidance molecule B
AML	Acute Myeloid Leukemia
CML	Chronic Myelogenous Leukemia
MDS	Myelodysplastic syndrome
MM	Multiple Myeloma
LIC	Leukemia initiating cells

MSC	Mesenchymal stromal cells
CFU-F	Colony forming units-Fibroblast
GvHD	Graft versus host disease
ISCT	International Society for Cellular Therapy
P/S	Pencillin/Streptomycin
ml	Milliliter
µm	Micrometer
µl	Microlitre
ng/ml	Nanograms/Milliliter

U/ml	Units/Milliliter
mm	Millimeter
µg	Micrograms
cm	Centimeters
s	Seconds
rpm	Rotations per minute
UCB	Umbilical cord blood
BFU-E	Burst Forming Unit-Erythrocytes
CFU-M	Colony Forming Unit-Monocytes
CFU-G	Colony Forming Unit-Granulocytes
CFU-GEMM	Colony Forming Unit-Granulocyte, Erythrocyte, Monocyte, Megakaryocyte

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I. INTRODUCTION

1.1 BACKGROUND

1.1.1 HEMATOPOIESIS AND ACUTE MYELOID LEUKEMIA

Hematopoiesis is the process in which different blood cells are formed in the bone marrow [1, 2]. Development of blood cells is organized as a cellular hierarchy derived from a common precursor, the hematopoietic stem cell (HSCs) which is capable of self renewal and is able to continually produce all the lineages of blood cells [3, 4].

In 1961, Till and McCulloch demonstrated by transplanting immunodeficient mice with human marrow cells, the existence of stem cells within the hematopoietic system. These cells were shown to have the ability to generate the different lineages of blood cells [5]. Since then several *in vivo* models have been developed to study the repopulation of the entire hematopoietic system by HSCs [6, 7].

Further studies of HSCs was performed using *in vitro* clonogenic assays including two week long colony forming cell assay (CFC) that test the ability of hematopoietic progenitor cells, in culture, to proliferate and give rise to colonies of heterogeneous populations and a five week, long term cell culture initiating assay (LTC-IC) that measure the ability of primitive hematopoietic cells to give rise to different progenitors [7, 8]. These *in vitro* assays and the *in vivo* repopulation assay, an important assay function that more accurately predicts the blood cells with long term repopulating abilities, have provided knowledge about the proliferative capacity, differentiation potential and the identification of different lineages of HSCs.

More recently, the identification of cell surface markers specific for HSCs has allowed for the selection and enrichment of the HSC population. These markers include CD34, an antigen that is selectively expressed by the primitive progenitor populations of hematopoietic and endothelial stem cells, CD45RA and CD38 that are present on more committed and differentiated populations of HSCs and CD90 (Thy1), a surface marker that is specific for a number of stem cell populations including HSCs. Combinations of cell surface markers have been used by different groups to isolate HSCs with an expression profile of $CD34^{+}CD45RA^{-}CD38^{-}Thy1^{+}$ [9, 10]. Among other markers, CD49f stem cell antigen expressed by cells with long term repopulating ability may help to correctly and better identify HSCs [11]. These and other markers have improved in distinguishing HSCs and their successful isolation from heterogeneous pools of cells. Thus, hematopoietic stem cells are mutlipotent, self renewing progenitor cells that are capable of differentiating into various blood lineages and are important for lifelong production of blood cells [1, 4].

Functionally, hematopoietic stem cells can divide to form two daughter cells or self renew to maintain the stem cell pool. According to the model of hematopoiesis, HSCs, gives rise to both myeloid and lymphoid progenitors. The myeloid lineage includes erythroid cells that transport oxygen, granulocytes and monocytes that provide immune protection against pathogens and megakaryocytes involved in the production of platelets. The lymphoid lineage gives rise to T-cells, NK cells, dendritic and B-cells that are responsible for providing cell mediated immunity within the body, **Figure 1-1** [12]. HSCs function to replenish the blood cells and maintain homeostasis within the hematopoietic system. But the decision of HSCs to maintain balance between self renewal and differentiation depends on many factors including complex gene regulatory networks

which are controlled by transcription factors and external signals from the bone marrow microenvironment where stem cells reside.

Studies have shown that several genes and transcription factors function together to either maintain the "stemness" of the hematopoietic cells or to give rise to mature committed progeny. For example HoxB4, a homeodomain transcription factor, is expressed at high levels to maintain the stem cell state of HSC progenitors, while c-myc and scl are up regulated during differentiation of hematopoietic cells [13, l4]. In addition, HSC fate is also determined by the cell-cell and cell-growth factor interactions within the hematopoietic microenvironment. Crosstalk between endothelial cells, osteoblasts, stromal cells and other cell types with HSCs present within the microenvironment is crucial for maintaining normal hematopoiesis. The signaling by cytokines and growth factor further mediates HSC survival, proliferation and expansion. Examples include stem cell factor (also referred to as c-kit ligand or SCF), a growth factor essential for the generation of blood cells from HSCs, interleukin 7 (IL-7), a hematopoietic growth factor responsible for the development of B-cell precursors and activation of mature T-cells. And vascular cell adhesion molecule 1 (VCAM1), a type 1 membrane protein involved in cell adhesion and signal transduction of leukocytes and endothelial cells [11, 14, 15]. It is the interplay between all these factors that influences the fate of HSCs and dictates whether they remain quiescent, enter a phase of self renewal or differentiate in a lineage-specific manner to maintain homeostasis [16].

Defects in the regulation of hematopoiesis within the marrow can disrupt the balance maintained in the hematopoietic niche and lead to profound changes in the blood system, including the development of disorders such as acute myeloid leukemia (AML).

AML is characterized by the accumulation of inefficient, dysfunctional leukemic blasts that inhibit the production of healthy blood cells resulting in marrow failure [17]. It is an uncommon but life-threatening blood malignancy with high rates of mortality. About 80% of patients with acute myeloid leukemia are adults, 60-70% of which can be expected to attain first complete remission following induction chemotherapy but only 25% of such patients have survival rates of three or more years [18, 19]. AML is currently classified into different sub groups based on morphology of leukemic cells present at the time of diagnosis (**Table 1-1**), chromosome abnormalities (**Table 1-2**) and molecular defects.

The accumulated research in AML confirms that it is clinically, molecularly and cytogenetically heterogeneous [20, 21]. For example, mutations in nucleophosmin-1(NPM1), a molecular chaperone, involved in cell regulation and transport of ribosomal proteins and mutations in fms related tyrosine kinase 3 (FLT3), which is functionally related to cell proliferation are some of the typical high frequency mutations or molecular defects that together account for just 20-27% of AML cases [22]. Therefore, even though gene expression profiling has yielded insights regarding chromosomal aberrations and gene abnormalities that correlate to certain sub types of AML, they are implicated in only small number of cases [22]. And despite modest increase in survival rates in recent years AML continues to be associated with low survival and high treatment related morbidity especially in elderly patients and patients with specific sub-types of AML [23]. Hence more knowledge is needed regarding mechanisms of leukemogenesis in order to improve the outcomes of patients with AML.

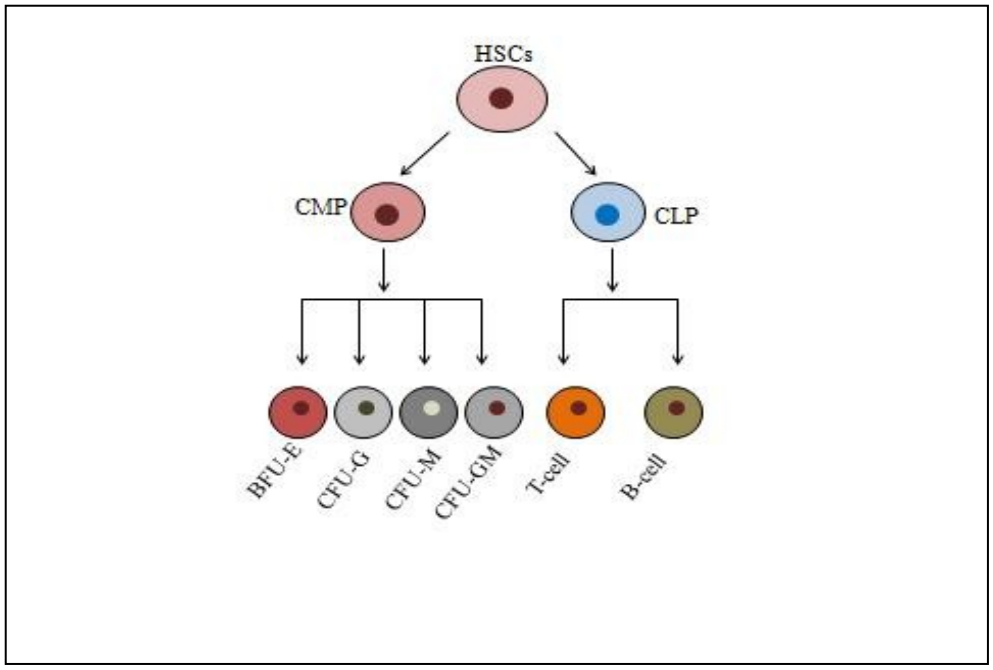


Figure 1-1: Model of hematopoiesis.

Hematopoietic stem cells branch into two main lineages: the common myeloid lineage (CMP) and common lymphoid lineage (CLP). The myeloid lineage further branches into burst forming units-erythrocytes (BFU-E), colony forming unit-granulocytes (CFU-G), colony forming unit-monocytes (CFU-M) and multilineage colony forming unit-granulocyte-monocyte (CFU-GM). The lymphoid lineage includes T-cell and B-cell.

FAB Subtype	Name	Prognosis compared to average for AML
M0	Undifferentiated acute myeloblastic leukemia	Unfavorable
M1	Acute myeloblastic leukemia with minimal maturation	Intermediate
M2	Acute myeloblastic leukemia with maturation	Favorable
M3	Acute promyelocytic leukemia	Favorable
M4	Acute myelomonocytic leukemia	Intermediate
M4(eos)	Acute myelomonocytic leukemia with eosinophilla	Favorable Better
M5	Acute monocytic leukemia	Intermediate
M6	Acute erythroid leukemia	Unfavorable

Table 1-1: The French-American-British (FAB) classification of AML.

This classification is based on the morphology of leukemic cells observed under the microscope after routine staining. The table was modified from reference 24.

Favorable abnormalities	Unfavorable abnormalities
Translocation between chromosomes 8 and 21(seen most often in patients with M2)	Deletion (loss) of part of chromosome 5 or 7 (no specific AML type)
Inversion of chromosome 16 (seen most often in patients with M4 eos)	Complex changes, those involving several chromosomes (no specific AML type)
Translocation between chromosomes 15 and 17 (seen most often in patients with M3)	____

Table 1-2: Classification according to the chromosome abnormalities
Common chromosomal changes occurring in AML. They are used as a tool for prognosis. The table was modified from reference 24.

Insight into the biology of human leukemic cells was gained using transplantation studies of human leukemic cells into immunosuppressed NOD-SCID mice and has allowed the identification of SCID leukemia-initiating cells (SL-ICs) [25]. Leukemia initiating cells were shown to have phenotypes similar to normal hematopoietic stem cells, isolated based on the surface profile of $CD34^{+}CD38^{-}$ [25]. These $CD34^{+}CD38^{-}$ stem cells from AML samples when transplanted into mice gave rise to different blood cells that were organized in a similar hierarchical fashion as their normal counterparts. The resulting leukemic cells recapitulated the morphology and phenotypes of the disease into secondary recipient mice thereby establishing the self-renewal and differentiation potential of leukemic stem cells [26]. The inherent ability of leukemic stem cells to give rise to different colonies of AML-CFUs and to self renew as demonstrated in serial transplantation assays suggest that

leukemogenesis parallels similar developmental processes in normal hematopoietic development [26, 27].

In addition some of the molecular studies on healthy blood cells have shed light on the regulation of hematopoietic cellular behavior. Studies by Chan et al demonstrated that the expression of oncogenic KRAS resulted in the transformation of normal HSCs into leukemia initiating cells with indefinite self replicating potential. Comparable results were obtained in other studies where activation of KRAS and inhibition of the tumor suppressing factor p53, increased proliferation and altered differentiation of normal HSCs to acquire a leukemic phenotype when transplanted in mice [28, 29].

But despite the cytogenetic and molecular insight, additional factors that initiate AML or cause progression of the disease remain largely unknown [22, 30, 31]. Therefore it is essential to consider key alternatives such as the role of bone marrow microenvironment in supporting the emergence of leukemia and the relevance of the stem cell niche in the pathophysiology of the disease to help better understand the underlying causes of AML and its progression.

1.1.2 BONE MARROW MICROENVIRONMENT: INTERACTIONS WITH HSCS AND ROLE IN LEUKEMOGENESIS

Bone marrow is a spongy tissue located inside the large bones, and is the major site of hematopoiesis in adult humans [32]. The bone marrow niches are dynamic microenvironments that support the growth, maturation and maintenance of hematopoietic stem and progenitor cells.

In particular the marrow environment includes a complex network of extracellular matrix proteins such as collagens, proteoglycans; soluble growth factors and cytokines including stromal derived growth factor1 (SDF1α), angiopoietin 1 (Ang1) etc and two definitive niches, the **endosteal** (or osteoblastic) niche and the **vascular** niche. The endosteal niche is comprised of osteoblasts, mesenchymal progenitor cells along with endosteal fibrocytes while the vascular niche is defined by its proximity to vascular endothelial cells, as shown in **Figure 1-3** [33, 34, 35]. The endosteal niche maintains HSC quiescence while enabling self-renewal and the perivascular niche promotes the activation and maturation of hematopoietic cells and controls the influx and efflux of circulating bio-elements into and out of the niche [36].

Thus the components of the microenvironments are interconnected forming a complex network along with interactions with neighboring cells and soluble factors and is governed by signaling pathways that allow crosstalk to maintain and regulate the HSCs *in vivo*, **Figure 1-4** [37, 38].

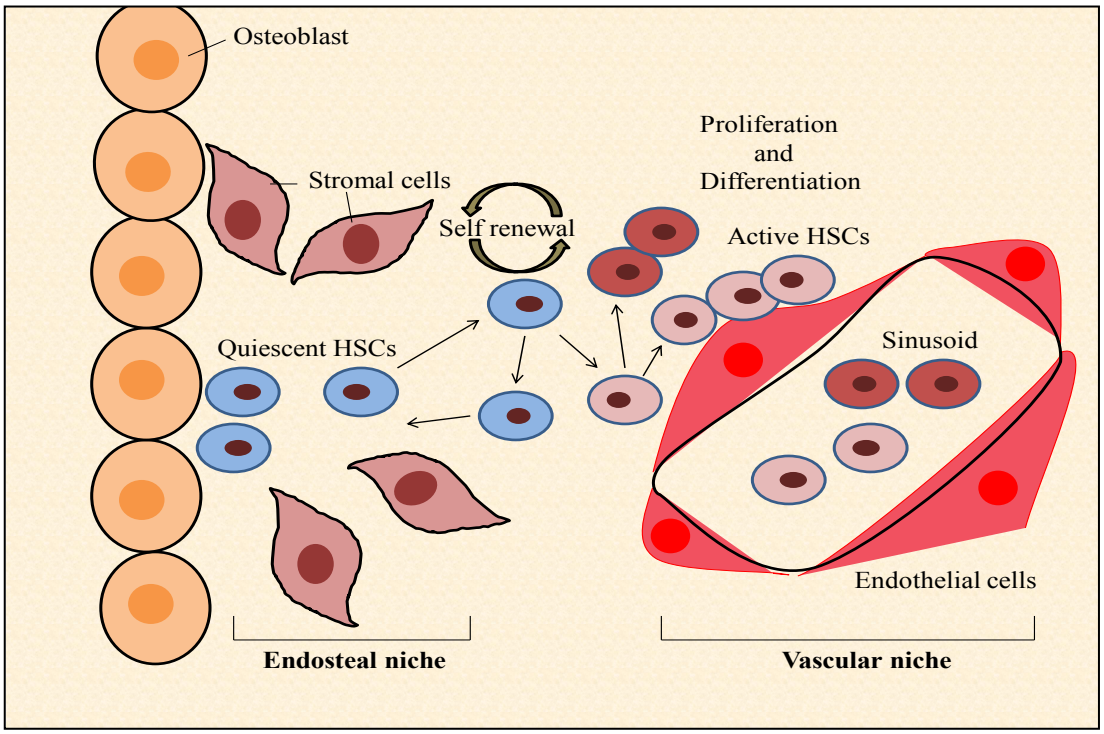


Figure 1-2: Stem cell niche in bone marrow.

Bone marrow contains two distinct niches: the endosteal niche and the peri-vascular niche. In the endosteal niche, stromal cells including mesenchymal stem cells, together with osteoblasts contribute to maintain the HSCs in quiescent state. The perivascular niche contains sinusoidal endothelial cells that maintains and controls HSC proliferation, differentiation and recruitment to the vascular niche. Concepts for the figure adapted from reference 39.

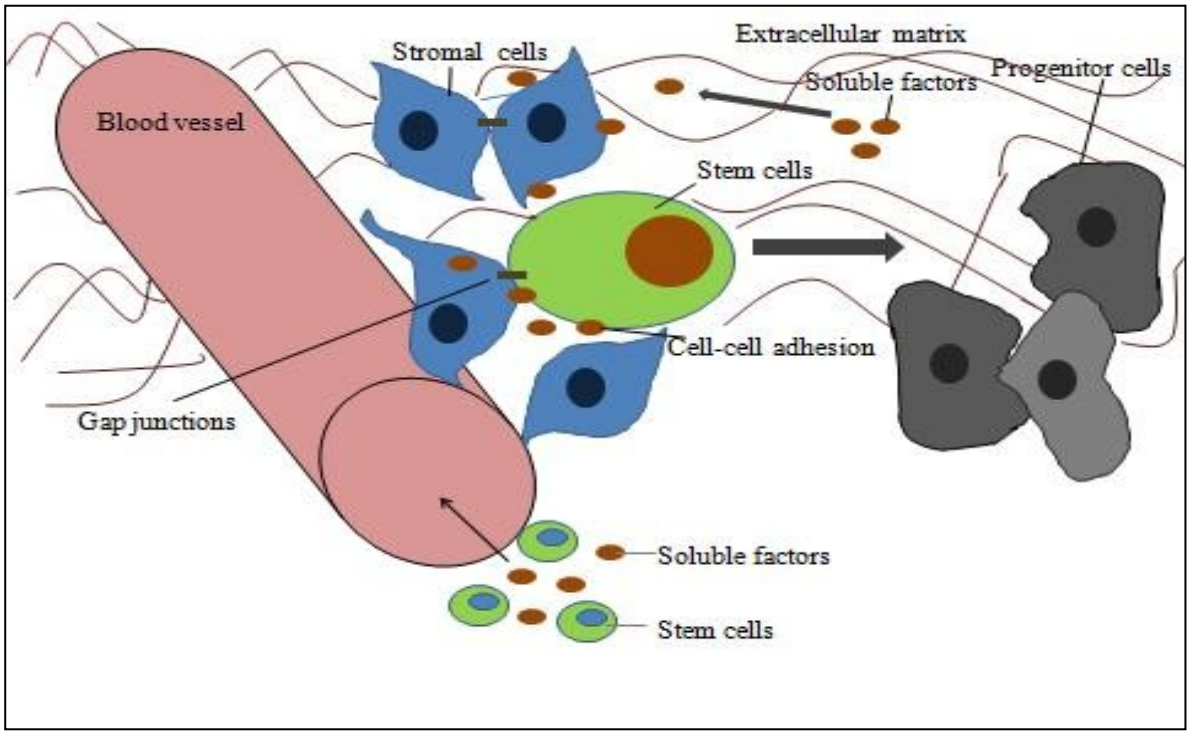


Figure 1-3: Components and functions of stem cell niches.
A hypothetical niche is composed of stem cells, soluble factors and extracellular matrix proteins that provide the structure to the niche, blood vessels that carry signals from outside the cells and adhesion molecules and growth factors together that maintains the stem cells in the niche. Concepts for the figure modified from reference 36.

The stem cell niche hypothesis was first proposed by R. Schofield in 1978 and was based on the observation that hematopoietic stem cells in the bone marrow environment compared to those in the spleen were less restricted in their proliferation capacity because they were associated with the cells that support their stem cell activity [40]. These initial observations demonstrated the need of a supportive environment for HSCs to retain their proliferative potential and maintain stem cell activity. Further characterization studies in *Drosophila* and *C.elegans* provided a means to better identify and distinguish different niches and their constituents [41]. Use of functional assays and surface markers to confirm the identity of stem and other cell types present in the niche, real time imaging to visualize

the interactions between the cells and studying the functions of different soluble factors contributed to better understanding of the functioning of the bone marrow microenvironment under normal conditions. But it was only in the last decade that the function of the niche in providing a permissive microenvironment to the malignant cells in different hematologic disorders, were studied more extensively [42].

Studies using a xenograft model of human pre-B acute lymphoblastic leukemia (ALL) in severe immunodeficient (SCID) mouse showed that ALL cells altered the bone marrow niche by disrupting the normal microenvironment and were capable of attracting normal human $CD34^+$ cells to the altered niche but failed to preserve the overall pool of $CD34^+$ cells. The leukemic cells were shown to have recruited the $CD34^+$ cells by secreting chemo-attractant molecules including SCF. Neutralization of SCF resulted in normalization of $CD34^+$ cell numbers and their activity [43]. Leukemic cells in B-cell and T-cell ALL xenograft models, have also been implicated in promoting angiogenesis in the marrow microenvironments through the release of proangiogenic molecules including VEGF, bFGF, interuleukin-6 and interleukin-8, thus influencing the microenvironment and establishing a more hospitable niche for their survival [44]. Altered profiles of growth factors and adhesion molecules have been associated with hematopoietic malignancies. Bhatia et al showed that CML $CD34^+$ cells had poor adhesive properties and hence impaired growth regulation. However when treated with interferon-α, the adhesion interactions of tumor cells with the stromal populations were restored and the proliferation potential of otherwise rapidly multiplying CML tumor cells was normalized [45].

Another study demonstrated that the leukemia initiating cells (LIC) from AML human samples engrafted within the endosteal region of the bone marrow in NOD/SCID

mice and were protected from chemotherapeutic agent arabinoside. This suggested that the tumor cells recruited to the endosteum received anti-apoptotic signals for their survival against treatment [46]. Deletion of Dicer1, a miRNA processing endonuclease, in osteoprogenitors from mice resulted in alterations in proliferation, growth and differentiation of the hematopoietic cells inducing myelodysplastic syndrome (MDS) and subsequently gave rise to acute myeloid leukemia when the tumor cells were transplanted into secondary mice recipients. While transplanted HSCs from mutant mice reverted to a normal phenotype in wild type mice, the transplantation of cells from wild type to mutant mice resulted in MDS suggesting the importance of the bone marrow microenvironment in tumor progression [47]. Additional insight from the literature suggests that tumor-stromal interactions play a vital role in cancer progression. Demonstrated by Karnoub et al, human bone marrow mesenchymal cells increase the metastatic potential of breast carcinoma cells by secreting chemokine CCL5 which acts in a paracrine fashion on cancer cells and increases their motility and invasiveness [48]. These experimental observations support the existence of crosstalk between the microenvironment and the malignant cells present in the milieu [49, 50].

Several concepts have emerged regarding the role of the microenvironment in tumorigenesis in the context of AML. First, leukemic cells can compete with normal hematopoietic cells for the existing niches and make use of the regulatory signals and functions of the cells present within the niche, for their own survival and maintenance within the microenvironment. Secondly, tumor cells may modify the environment including stromal and endothelial cells in such a way that the environment supports tumor progression. Thirdly, disrupting the regulatory network of normal hematopoiesis within the

niche may contribute to leukemia initiation [51]. One model that has been previously proposed in the context of marrow diseases is that tumor-microenvironment interactions are permissive for the selection and expansion of leukemic cells [34]. The leukemic cells in turn manipulate the microenvironment through cell-cell communication mediated by proteins that include growth factors and cytokines such as osteopontin (Spp1) which is a highly acidic phosphoprotein involved in regulation of inflammation, cell adhesion and angiogenesis. Angiopoietin1 (Ang1), a protein growth factor required for the formation of mature blood vessels and stromal derived growth factor1 (SDF1α) which retain progenitors within the niche. These bio-factors contribute to the maintenance of the pool of leukemic cells in the niche and their survival, proliferation and metastasis to the nearby tissues and organs [52, 53].

Despite the increasing knowledge about the environment in the bone marrow, its components and functionality, many questions remain including whether changes in the bone marrow niches are induced by tumor cells to support their growth and survival or from aberrant adult stem cells present in the niche or both. Much of the knowledge about the role of bone marrow in promoting leukemia suggests their importance in disease initiation and progression. Hence investigating key cellular components of the niche and the way in which those populations support and sustain a permissive microenvironment for AML may contribute to better understanding of leukemogenesis.

1.1.3 MESENCHYMAL STROMAL CELLS

Mesenchymal stromal cells (MSCs) are adult progenitor cells with the capacity to differentiate and contribute to different mesenchymal as well as non mesenchymal tissues such as bone, fat, cartilage, ligament, tendon, both *in vivo* and *in vitro*, and to recapitulate bone marrow stroma that supports hematopoietic function [54-57]. The first experimental evidence of the MSCs were provided by Friedenstein and his colleagues, with the development of an ex vivo assay (colony forming unit-fibroblast, CFU-F) to examine MSC clonogenic ability [58]. They described the cells as colony-forming unit of fibroblast origin (CFU-F) that were spindle shaped, adherent to plastic and clonogenic in culture conditions. The technique of simple plastic adherence was subsequently used by several groups to successfully isolate and differentiate the stromal cell populations [59, 60].

Although the cells have been originally isolated from bone marrow, they are also found in other tissues and have been isolated from umbilical cord blood, adipose tissue, skin and fetal tissues [61]. Mesenchymal stromal cells have high capacity for proliferation *in vitro* and also possess potent immunosuppressive capacity. They have demonstrated the ability, to inhibit the proliferation of T and B lymphocytes, dendritic cells and natural killer (NK) cells and have low immunogenicity [62, 63]. MSCs migrate to sites of injuries and inflammation and secrete growth factors and cytokines that stimulate tissue repair *in vivo* [64]. Based on their biologic properties to maintain homeostasis of organs and tissues and secrete various growth factors, MSCs are being explored therapeutically to enhance hematopoietic engraftment after HSC transplantation and in regenerative therapy to rebuild damaged or diseased organs [65, 66]. Several clinical trials involving Crohn's disease, diabetes, graft versus host disease (GvHD), stroke, myocardial infarction (MI) and

osteogenesis imperfecta, have been conducted using mesenchymal stromal cells showing promising results [67, 68, 57]. However concerns about the efficacy and safety of the cells and their detailed mechanism in the context of therapeutic applications need to be addressed before their routine use in cell therapy trials.

MSCs have also been investigated as support cells for the culture of HSCs and have been shown to improve the engraftment when infused along with HSCs in the bone marrow of mice [69, 70]. Many studies have also reported the use of mesenchymal cells in expanding $CD34^+$ hematopoietic progenitor cells from cord blood [71, 72].

Because MSC identity *in vivo* remains incompletely defined, several methods have been used to isolate these cells and expand them in culture giving rise to heterogeneous sub-populations of MSCs. Phenotypically, MSCs express a number of markers such as CD105 (SH2), CD73 (SH3/4), CD90 (Thy1), CD44, CD71, SSEA-4 and Stro-1 [73, 74]. They are devoid of expression of hematopoietic markers CD45, CD34, CD14, HLA-1or CD11. They also do not express co-stimulatory molecules CD40a, CD40b, CD80, CD86 or the adhesion molecules CD31 and CD18 [75]. Considering the fact that these markers are not specific for the identification of mesenchymal stromal cells and due to lack of general agreement on the nomenclature and isolation of these cells, minimal criteria to define mesenchymal stromal cells have been assigned. According to the minimal criteria set by International Society for Cellular Therapy (ISCT), MSCs must be plastic adherent, should express CD73, CD90, CD105 antibodies, lack the expression of hematopoietic markers CD34, CD45 and CD14 and demonstrate *in vitro*, ability to differentiate into adipocytes, osteocytes and chondrocytes [76].

MSCs are the central components of hematopoietic microenvironment and have been shown to regulate hematopoiesis directly regulating HSCs or indirectly through production and secretion of growth factors and cytokines [77]. Several studies have observed important functional and quantitative alterations in MSCs obtained from patients with different hematologic disorders [78, 79]. In some studies, such as by Corre et al, distinct gene expression profile was observed for mesenchymal cells derived from bone marrow of normal and multiple myeloma (MM) [80, 81]. These differences were found in the expression of cell adhesion molecules, extracellular matrix proteins and cytokines such as interleukin 6 (IL-6) an inflammatory cytokine important for multiple myeloma, dickkopf related protein 1 (Dkk1) a wnt inhibitor, amphiregulin (AREG) growth factor for fibroblasts and angiopoietin4 (Angpt4) involved in vascular growth and angiogenesis. Other groups have documented the existence of genomic alterations including copy number variations, mutations, loss of heterozygosity and presence of leukemic fusion genes in MSCs from patients with multiple myeloma (MM), myelodysplastic syndrome (MDS) and pediatric leukemia patients [82, 83]. Few other studies showed the presence of cytogenetic abnormalities in MSCs derived from a significant number of acute myeloid leukemia patients [77, 84, 85].

MSCs also seem to have a relevant role in survival of tumor cells and protecting them from the effects of adverse conditions within the microenvironment [86]. Wei et al observed a marked decrease in apoptosis of chronic myelogenous cell (CML) lines when the serum deprived tumor cells were cultured with MSCs [87]. In addition, MSCs have been shown to confer resistance to drugs in tumor cells. Chronic lymphocytic cells lines (CLL) when treated with forodesine (purine phosphorylase inhibitor), a potential drug for

the treatment of CLL, showed increased apoptosis of cancer cells but displayed reduced apoptosis when cultured with human and murine mesenchymal stromal cells [88]. The mesenchymal co-culture system also protected CLL lines thereby making it resistant to drugs. Similar properties of MSCs were observed when ALL cells that normally express low levels of asparagine synthase (ASNS) became resistant to asparaginase treatment (which depletes asparagine present in the circulation and kills tumor cells) when grown in proximity to MSCs, as the mesenchymal cells have been shown to express high levels of ASNS [89]. Furthermore mesenchymal stem cells have also been demonstrated to play a role in tumor progression by their ability to secrete SDF-1/CXCL12 that helped to promote tumor growth [90]. But the extent of functional changes in MSCs, in these syndromes including AML and their impact on progression or relapse of the disease is still yet to be determined.

Moreover the extent to which MSCs revert back to normal phenotype following treatment with induction chemotherapy or other treatments is unknown. Recent reports have provided new insight regarding the existence of multiple clones in patients with acute leukemia, suggesting that the marrow microenvironment is particularly permissive to the emergence of pre-leukemic and dysplastic cells with more dominant and proliferative clones emerging as frank leukemia [91]. Although leukemic stem cells acquire changes that allow them to proliferate more readily in the stem cell niche, the extent to which changes occur in adult stem cells of the microenvironment that facilitate the emergence of leukemia remains unclear.

Taken together these observations provide a rationale for the potential role of mesenchymal cells being critical for the support of HSCs in the marrow niche, a basis for studying the role of the marrow microenvironment in supporting the survival of leukemic cells. Investigating the biologic functions of mesenchymal stromal cells from marrow of AML patients could offer an approach to understand the role of the marrow niche in AML.

Therefore, in this thesis we aim to study the biologic changes that occur in MSCs from bone marrow of patients with AML by comparing their function and gene expression pattern with MSCs from healthy individuals. Studying the functional characteristics and the expression pattern of genes important in maintaining hematopoiesis including SCF, IL-7, VCAM1, Spp1, Ang1, RGMB and SDF1α, in MSCs derived from AML patients would provide insight on MSCs function in the leukemic niche, compared to MSCs from healthy controls. Observations from these studies should aid in detecting alterations, if any, present in mesenchymal stromal cells derived from marrow of leukemic patients and should provide a foundation to further our understanding about the role of the marrow microenvironment in the development and expansion of leukemic cells.

1.2 HYPOTHESIS, RATIONALE AND OBJECTIVES OF THE RESEARCH PROJECT

1.2.1 HYPOTHESIS: Mesenchymal stromal cells derived from acute myeloid leukemia patients harbor functional alterations compared to MSCs from healthy individuals which may contribute to the development of AML.

1.2.2 RATIONALE: Extensive research into the field of acute leukemia has revealed that leukemic cells may arise due to a permissive environment for leukemogenesis in the bone marrow. Characterizing the biologic changes that occur in cells of the marrow niche in patients with AML will provide a first step towards investigating the extent to which current treatments can normalize the marrow microenvironment to ensure sustained remission and eradication of leukemia.

1.2.3 OBJECTIVES:

To address our hypothesis we propose the following objectives:

AIM 1: Characterize a "normal" and "leukemic" biologic profile of marrow-derived MSCs from healthy controls and from patients with AML.

AIM 2: Develop leukemic and normal gene expression profile for MSCs by studying the expression pattern of genes associated with hematopoietic supportive function.

II. MATERIALS AND METHODS

2.1 MATERIALS

Medium used for Mesenchymal cell cultures was Dulbeco's Modified Eagle Medium, low glucose containing L-glutamine and 110mg/l Sodium Pyruvate purchased from Gibco Invitrogen, USA. Characterized Fetal Bovine Serum (Hyclone) was used as a supplement to the medium. Antibodies for immunohistochemistry including CD34 (FITC), CD45 (PE), CD90 (Purified) and CD105 (Purified), mouse antibodies raised against human, were bought from BD Pharmingen, CA, USA. The secondary antibodies were PE and FITC flurochrome conjugated raised against mouse species (Invitrogen). Stained cells were observed under inverted microscope (Zeiss) and images obtained using Axiovison software. Flow analysis was done on Becton Dickson Biosciences (LSR) and data recorded and analyzed using cell quest software and FLOWJO™ software (TreeStar Inc., Ashland, OR). CD105 (APC), CD90 (PerCp-Cy 5.5), CD73 (PE), CD45 (FITC) and C34 (PE-Cy7) brought from BD Biosciences were used for flow phenotyping. Differentiation kit for MSCs was obtained from R&D systems, Minneapolis, MN. Secondary antibodies for adipocytes, chondrocytes and osteocytes were IgG donkey conjugated raised against goat (Cat No: NL001) and mouse (Cat No: NL007) respectively, purchased from Northern Lights. The chemical stain for differentiation assays, Oil Red O-Isopropanol solution (Cat NO: 26503-02) and Alizarin Red solution (Cat NO: 26202-01) were obtained from Electron Microscopy Sciences, Cedarlane, Canada. $CD34^+$ isolation kit, cytokines for co-culture assay namely GSCF, SCF, and EPO and Methocult medium (H4434 Classic) for colony forming cell assay were purchased from Stem Cell Technologies, Vancouver, Canada.

The antibodies for sorting and immunophenotyping hematopoietic stem cell samples were provided by our collaborator Dr Michael Rosu-Myles. Flow sorting was performed by Paul Olyenik (StemCore laboratories, OHRI) using Beckman Coulter MoFlo sorter. Iscove's modified Dulbeco's medium (IMDM) was brought from Sigma Aldrich. RNA extraction from cells was performed using Rneasy Mini kit by qiagen and cDNA extraction kit was purchased from Invitrogen. Rnase inhibitor and buffer were brought from Biolabs (New England). SyBGR for qPCR was brought from Quanta Biosciences. Primers were designed using primer express software and purchased from Integrated DNA Technologies (IDT, Coraville, USA). cDNA synthesis and PCR reaction was carried out on eppendorf thermocycler and Rotor Gene Q (Qiagen) respectively.

2.2 METHODS

2.2.1 ISOLATION AND CULTURING OF MESENCHYMAL STROMAL CELLS FROM BONE MARROW

Bone marrow samples obtained from healthy donors (n=5; age: 20-50; female: male ratio 3:2) were collected in standard filter collection bags after informed consent in accordance with the institutional review board (OHREB protocol #1997509-01H). Total nucleated cells were obtained after washing the filter bag with buffer (PBS/2%FBS) and collected in 50ml tubes. Cells were centrifuged at 2000 rpm for 10 minutes. Cells were Ficoll separated and high density centrifugation done at speed of 2000g for 20 minutes at 22°C. Mononuclear cells (buffy coat formed between plasma and red blood cells) were obtained and washed in buffer (PBS/2%FBS). After two washes, the mononuclear cell

number was counted using a hematocytometer with trypan blue staining. Cells were seeded at approximately $1.3x10^5$ cells/cm^2 in DMEM (low glucose) supplemented with 15%FBS.

In case of bone marrow aspirates from diagnostic samples of AML patients (n=11; age: 20-75; female: male ratio 8:3), samples were collected and experiments conducted, following ethical approval from institutional review board (OHREB protocol #2011885-01H) and informed consent from the patients. The cells were density centrifuged and washed twice in buffer before seeding them between $1.2x10^5$-$1.4x10^5$ cells/cm^2 of mononuclear cells in DMEM/15%FBS. Cell culture underwent medium change once a week and maintained in the same culture dish until plastic adherent spindle shaped cells were observed within the culture. On attaining 75-80% confluency, the cells were trypsinized and harvested for further passage and culture. Cell numbers and viability were checked after every passage of cells using trypan blue.

2.2.2 IMMUNOPHENOTYPING BY FLOW CYTOMETRY AND IMMUNOHISTOCHEMISTRY

Flow Cytometry

Mesenchymal stem cells were harvested and counted, then aliqoted in 6 tubes at 1 $x10^5$cells (could stain $3x10^4$– $5x10^5$ cells) in 5ml polystyrene FACS tubes and washed with 3-4ml of phosphate buffered saline containing 2% FBS (PBS/2%FBS). One tube was considered as "unstained" and the others as "sample". Cells were centrifuged at 1000-1200 rpm for 6 minutes at 4°C. Cells were resuspended in 100µL ($1x10^5$ cells per 100µl) PBS/2% FBS and kept at 4°C until ready for staining. 1µl of appropriate flurochrome conjugated monoclonal antibodies was added to corresponding tubes and included human

CD105 Allophycocyanin (APC), CD90 PE-Cy5.5, CD73 PE, CD34 Phycoerythrin-Cy7 (PE-Cy7) and CD45 Fluorescein iso-thiocyanate (FITC). No antibodies were added to the unstained tube. Tubes were mixed well and incubated at 4°C in the dark for ~20 minutes and then further with 3-4ml of PBS/2%FBS added to each of the tubes, tightly capped and inverted to mix. Cells were given a final centrifuge at 1000-1200 rpm at 4°C for 6 minutes and resuspended in 400-500µl of PBS/2%FBS. Before flow analysis, cells were filtered through a 70µm cell strainer to remove cell clumps. Tubes were kept at 4°C in the dark until analysis.

Immunohistochemistry

Cover slips were prepared by sterilizing them in 95% EtOH for 10 minutes and exposed to UV for 15 minutes. The cover slips were then inserted into each well of 24-well plate and 0.5ml of sterile PBS added to each well. The plates were incubated at 37° C until needed. Approximately $1x10^5$ cells/well were seeded on the cover slips with 0.5ml of DMEM/15%FBS. Confluent cells were fixed with 4 % paraformaldehyde for about 20 minutes. The cells were then washed with PBS/1%BSA, 5 minutes for a total of three washes and permeabilized using PBS/1%BSA/10%FCS (fetal calf serum) for another 20 minutes. Primary antibody solutions were prepared by adding CD34 (FITC), CD45 (PE), CD105 (Purified) and CD90 (Purified) antibodies in the ratio of 1:100 dilution to the buffer solution of PBS/1%BSA/2%FCS. Primary antibody solution was added to the cover slips and incubated overnight at 4° C. On the following day, cover slips with fixed cells were washed thrice with PBS/1%BSA for each wash of 5 minutes duration and incubated in PBS/1%BSA with secondary antibody conjugated with PE and FITC flurochrome for an

hour. Cells were given a final wash with PBS/1%BSA and incubated in DAPI for one minute. The cover slips were then mounted on microscopic slides with DAKO medium and viewed under microscope.

2.2.3 DIFFERENTIATION

Cover slips for immunostaining of differentiated cells were prepared as described above.

2.2.3.1 ADIPOGENESIS DIFFERENTIATION

Mesenchymal cells were seeded at a density of $2.1x10^4$ cells/cm^2 in a 24 well plate in 0.5 ml DMEM/10%FBS. Cells were incubated overnight in 37° C and 5% CO_2. When the cells were 100% confluent, the medium was replaced by 0.5ml of adipogenic differentiation medium containing α-MEM/10%FBS and adipogenic supplement (50µl of supplement in 5ml of medium) to induce adipogenesis. Every three days, medium was changed and after 7 – 16 days, adipocytes were fixed and saved for immuno and chemical staining.

Fixing and Staining Procedure: Immunocytochemistry of Adipocytes

The cells were washed twice with 0.5 ml of PBS and fixed with 0.5 ml of 4% paraformaldehyde for 20 minutes at room temperature. Cells were given a wash three times with 0.5 ml of 1% BSA/PBS for each wash of 5 minutes and permeabilized with 0.5 ml of 1% BSA in PBS containing 10% normal donkey serum and 0.3% Triton X-100 at room temperature for 45 minutes. Primary antibody goat anti-mouse FABP-4 antibody was

reconstituted in 1% BSA in PBS containing 10% normal donkey serum to a final concentration of 10 µg/ml. The cells were incubated with 300 µl/well of goat anti-mouse FABP-4 antibody solution overnight at 4° C.

Next day, the cells were given a wash three times with 0.5 ml of PBS containing 1% BSA for 5 minutes each. The secondary antibody was diluted 1:200 in PBS containing 1% BSA. The cells were incubated with secondary antibody (working solution at 300 µl/well) in the dark for 60 minutes at room temperature. The cells were washed three times with 0.5 ml of 1% BSA/PBS for 5 minutes. 1ml of DAPI solution added to the cells for a minute and the cover slips removed with forceps and mounted on microscopic slides with cell side down onto a drop of mounting medium (DAKO) on the glass slide. Slides were observed under the microscope.

Chemical Staining of Adipocytes

Fixed cells with 4% PFA were washed with 1ml (for 24-well plates) dH_2O/ well and further with 0.5ml (for 24-well plates) of 60% Isopropanol to each well and left for 2-5 minutes. 0.5ml of filtered oil red o working solution (3ml of ORO solution and 2ml of distilled water) was added onto each well and incubated for 15 minutes.

Cells were washed with 1ml of dH_2O till the aspirate came off clear and kept in 1ml of PBS until observed under microscope.

2.2.3.2 OSTEOGENIC DIFFERENTIATION

Cells were seeded at a density of $4.2x10^3$ cells/cm^2 in a 24 well plate in 0.5 ml of DMEM/10%FBS and incubated overnight at 37° C and 5% CO_2. When the cells were

100% confluent, the medium was replaced by 0.5ml of osteogenic differentiation medium containing α-MEM/10%FBS and osteogenic supplement (250µl of supplement in 5ml of medium) to induce osteogenesis. Every three days, medium was changed and after 14-27days, osteocytes were fixed and saved for immuno and chemical staining.

Fixing and Staining Procedure: Immunocytochemistry of Osteocytes

Same procedure as described for adipocytes. The primary antibody mouse anti-human osteocalcin was reconstituted in 1% BSA/PBS containing 10% normal donkey serum to a final concentration of 10 µg/ml.

Chemical Staining of Osteocytes

Fixed cells were washed with 2ml (1ml for 24-well plates) dH_2O/ well twice. 0.5ml (for 24-well plates) of alizarin red solution added onto each well and incubated for an hour. Cells were washed with 1ml of dH_2O till the aspirate came off clear and kept in 1ml of PBS until observed under microscope.

2.2.3.3 CHONDROGENIC DIFFERENTIATION

$2.5x10^5$ cells were taken in to a 15 ml conical tube and centrifuged at 200g for 5 minutes at room temperature. Medium was removed and cells resuspended with 0.5 ml of DMEM basal medium. Cells were centrifuged at 200 g for 5 minutes and resuspended in 0.5 ml of chondrogenic differentiation medium containing DMEM basal medium and chondrogenic supplement (50µl supplement in 5ml). Cells were centrifuged further and the tubes were incubated without discarding the medium, at 37° C and 5% CO_2 with caps

loosened to allow gas exchange. Medium was replaced every three days being careful not to fix the pellet. After 21 days, chondrocytes were fixed for immuno and chemical staining.

Cryosectioning Pellets

The cells were washed twice with 0.5 ml of PBS and fixed with 0.5 ml of 4% paraformaldehyde in PBS for 20 minutes at room temperature. Pellets were aspirated from formalin and dropped in the center of a cryomold disposable vinyl specimen mold. Duplicate pellets were added to the same specimen mold so that they could be cryosectioned together. Residual formalin was removed and prior to cryosectioning, the mold was filled with OCT (Optimal Cutting Temperature) solution. After 10 minutes of mold freezing, a layer of OCT solution added to the upper section of the mold and a plastic knob pressed onto it so that the OCT sticks to the base of the knob. The knob was leveled on the block by placing the weight apparatus over it. Once frozen, block was removed from the vinyl specimen mold along with the plastic knob and placed it in the sample holder of the cryosection machine. The sectioning increment was placed at 10um and the pellet sectioned one at a time. A gelatin covered slide was placed over the section and pressed down gently so that the section adhered to the slide. Mounted sections were stored at 4°C until analysis.

Fixing and Staining Procedure: Immunocytochemistry of Chondrocytes

Same procedure as described for adipocytic staining. Primary antibody for chondrocytes, goat anti-human aggrecan was reconstituted in 1% BSA/PBS containing

10% normal donkey serum to a final concentration of 10 µg/ml. Secondary antibody same as used for adipocyte staining.

Chemical Staining of Chondrocytes

Slides were washed with distilled water and placed in alcian blue stain for 15 minutes. Residual stain was rinsed off with water, slides air dried and aqueous mounting medium was placed on top of the sections and covered with cover slips.

2.2.4 HEMATOPOIETIC CO-CULTURE

Fresh umbilical cord blood samples obtained after informed consent from mothers were processed using similar procedures for the bone marrow processing to obtain mononuclear cells. Cells were counted using trypan blue and numbers obtained. A total of $2x10^7$ cells and above were resuspended in 1ml of PBS/2%FBS/1Mm EDTA and for lower cell counts suspended in 100µl-1000µl of the same buffer. The cells were then transferred to a 5ml polystyrene tube and Easy Sep R Positive Selection Cocktail added at 100µl/ml of cell sample. Cells were mixed well and incubated for 15 minutes at room temperature. Easy sep magnetic nanoparticles was next added at a concentration of 50µl/ml of the cell sample, mixed well and incubated for 10 minutes at room temperature. The cell suspension was then made up to a total volume of 2.5ml and tube placed into purple Easy Sep magnet for 5 minutes. Magnet along with the tube was inverted pouring off the supernatant fraction and held for 2-3 seconds and then turned to upright position. The tube was removed from the magnet and 2.5ml of medium PBS/2%FBS/1mM EDTA added. The cell suspension was mixed thoroughly and placed in the magnet for next five minutes. This procedure was repeated for a total of 5x5 minute magnetic separations. Tube was removed and

magnetically labeled $CD34^+$ cells resuspended in 1-2ml of with cytokines medium (DMEM,10% FBS, P/S antibiotics (1:100).

Cells were counted and separated for co-culture with MSCs. 25ml of with cytokines medium containing 10% FBS, 20ng/ml SCF, 1U/ml EPO, 20ng/ml GSCF and DMEM was prepared. About $1x10^5$ $CD34^+$ isolated cells was suspended into each well of a 6 well plate containing 2ml of medium (with cytokines) on top of a confluent layer of MSCs ($3x10^5$cells/well). Cells for flow were washed with PBS/2% FBS buffer, centrifuging at 1200 rpm for 8 minutes. Depending on the cell count, for every $1x10^5$ cells, 100µl of buffer was added and mixed with the pellet obtained. One tube of cells was used as unstained and other tubes as sample stained with CD34 (PE-Cy7) and CD38 (APC). Two compensation tubes were also prepared with cells or beads stained with PE-Cy7 and APC flurochrome conjugated antibodies. Tubes were incubated for 15 minutes at 4°C and given a spin at 1100 rpm for 6 minutes. Cells resuspended in 500µl of PBS/2% FBS and kept in dark until flow analysis.

For colony forming cell assay on $CD34^+$ cells, a total of $1x10^3$ cells-$2x10^3$ cells ($1x10^4$-$2X10^4$ cells/ml of cell concentration) obtained from the isolation of $CD34^+$ were diluted in 1ml of IMDM/2%FBS . 0.3ml of the diluted cells was added to 3ml methocult medium to perform duplicate assay. Tubes were vortexed to mix the contents thoroughly and allowed to stand for 5 minutes to let the bubbles rise the top of the tube. 1.1 ml of the resulting medium was then drawn up using 3ml syringe fitted with a 16 gauge blunt end needle and about 1.0 ml of the contents dispensed into the 35mm dish. The medium was distributed evenly across the 35mm dish by gently tilting and rotating the dish to allow the medium to attach the walls of the dish on all sides. Approximately 3ml of sterile water was

added to the uncovered dish. The 2 covered 35mm dish was then placed along with uncovered 35mm dish containing distilled water into a 100mm dish and incubated at 37º C in 5% CO_2 for 12-14 days. The colonies were enumerated at 14^{th} day from plating.

For the co-cultured cells of MSCs and $CD34^+$, the media (with cytokines) was replenished every 3-4 days to an overall of 8 days. On day 8, the suspended cells along with MSCs were trypsinized (1.0-1.5ml) and the weakly attached hematopoietic cells recovered by adding 2ml-2.5ml of DMEM/10%FBS to each well. Cells were spun at 1200 rpm for 8 minutes at room temperature and supernatant discarded. Pellet was resuspended in with cytokines medium and the cells were counted and viability checked with trypan blue. Cells were then prepared for sorting using hematopoietic marker CD45 (FITC). Post sorted cells from with cytokines conditions were plated for CFU. On day 14^{th} of plating, CFCs obtained from both the culture conditions were enumerated. An experimental model of the hematopoietic co-culture is shown in **Figure 2-1**.

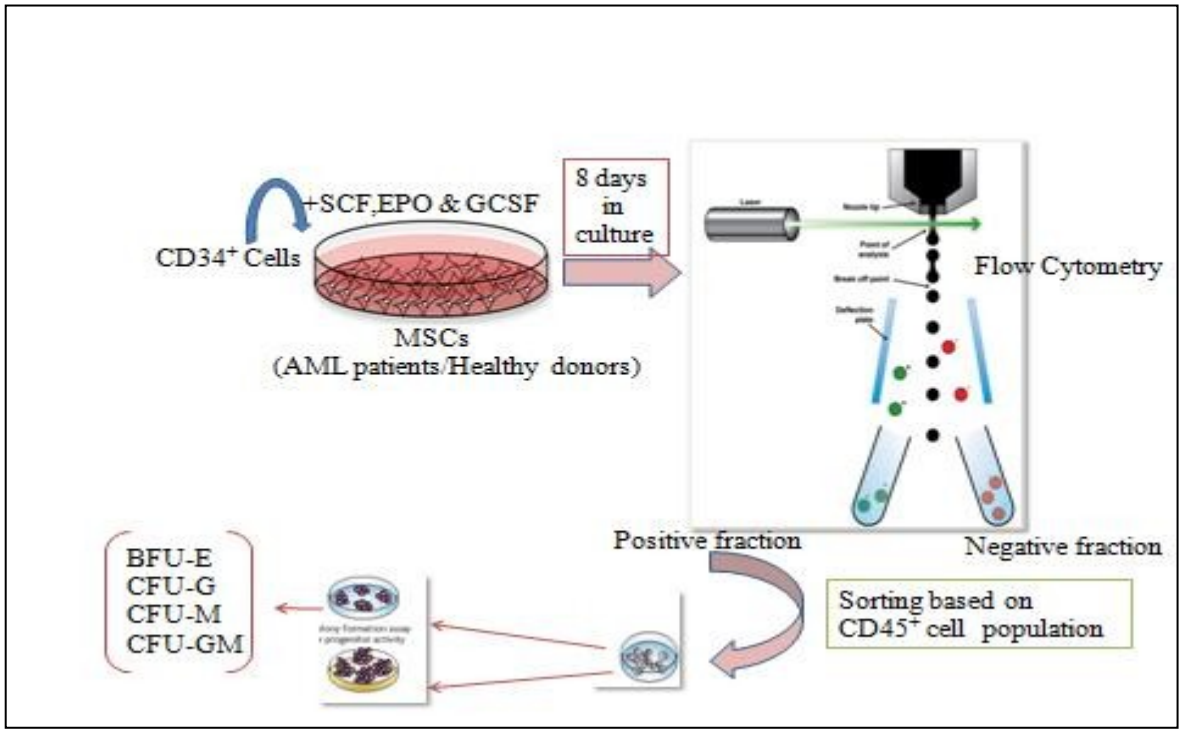


Figure 2-1: Experimental model for assessing the ability of mesenchymal stromal cells to support hematopoiesis *in vitro*.

UCB derived $CD34^+$ cells are seeded on top of a confluent layer of AML-MSCs or nMSCs and maintained in cultures for eight days. On eighth day, cells are sorted based on $CD45^+$ expression of hematopoietic stem cells. Sorted HSCs are further assayed *in vitro* to enumerate the colony forming potential of cells.

2.2.5 GENE EXPRESSION PROFILING

2.2.5.1 EXTRACTION OF RNA

Mesenchymal stem cells from confluent culture of passages three and four were trypsinized and washed with RLT buffer and B-mercaptoethanol. The lysate obtained was pipetted directly into a QIA shredder spin column placed in a 2 ml collection tube, and centrifuged for 2 minute at 13,000 rpm. Equal volume of 70% ethanol was added to the

homogenized lysate, and mixed well by pipetting. The total volume was made up to 700ul of the sample and transferred to an RNeasy spin column and centrifuged for 15s at 10,000 rpm. Flow through was discarded. 350 µl of RW1 buffer was added to the RNeasy spin column and centrifuged for 15s at 10,000 rpm to wash the spin column membrane. Flow through discarded.

To eliminate the genomic DNA contamination, 80 µl of DNase I incubation mix (10 µl of DNase I to 70 µl Buffer RDD) was added to the RNeasy spin column membrane, and kept at room temperature for 15 minutes. 350 µl of buffer RW1 was further added to the sample and centrifuged for 15s at 10,000 rpm. Flow through was discarded. 500 µl of RPE buffer added to the RNeasy spin column and centrifuged for 15s at 10,000 rpm to wash the spin column membrane. Flow through was discarded. Same step repeated with spin specifications of 2 minute at 10,000 rpm. RNeasy spin column was placed in a new 2 ml collection tube, and old collection tube discarded with the flow-through. The tubes were given a spin at full speed for 1 minute to eliminate any traces of RPE buffer. The RNeasy spin column was finally placed in a new 1.5 ml collection tube and 30–50 µl RNase-free water added directly to the spin column membrane. Lid closed and centrifuged for 1 minute at 10,000 rpm to elute the RNA. Previous step was repeated again if the expected RNA yield was >30 µg, using the elute from previous step. RNA concentration was measured using nanophotometer and recorded.

2.2.5.2 REVERSE TRANSCRIPTASE POLYMERASE CHAIN REACTION (RT-qPCR) ANALYSIS

1.5ug of DNase treated RNA was gently mixed with RT reaction mix (includes random primers and distilled water). The reaction mix was then treated in the thermocycler at 70°C for 10 minutes and 4°C for 2 minute for transcribing cDNA from RNA sample. After the first reaction, dNTPs and RT enzyme mix was added (including RNA inhibitor, M-MuLV RT and M-MuLV RT buffer and distilled water) to the existing sample. Reverse transcriptase process was resumed in the thermocycler at 42°C for 1 hour, 70°C for 10 minutes and 4°C for 2 minute, for a total of 1hour 15minutes to obtain cDNA from the samples.

Real time RT-PCR reaction was then performed using 2µl of cDNA (sample), 0.4µl of forward and reverse primers, 10µl of SYBR Green (Quanta Biosciences) and 7.2ul of distilled water, for a total volume of 20µl loaded for each reaction. The samples were run in duplicates. An initial denaturation of cDNA was carried out at 50°C for 2 minute and 95°C for 2 minute followed by annealing and extension at 95°C for 10s, 60°C for 20s and 72°C for 34s. The amount of cDNA for each of the target genes was quantified using relative standard curve method. Control cDNA obtained from normal MSC samples was diluted at 5x with concentrations of 1.169 ug/ml, 0.234 ug/ml, 0.0468 ug/ml, 0.0098 ug/ml, and 0.000198 ug/ml and standard curve generated for the analysis. GAPDH (IDT) was used as endogenous control for the reaction: Forward Primer: 5'-CCACCCAGAAGACTGTGGAT-3' and Reverse Primer: 5'-CCCTGTTGCTGTAGCCAAAT-3'. The following primer sequences of target used were: RGMB Forward primer: 5'-CCTTTTTTGTGGCTTGTTTGG-3',

RGMB Reverse Primer: 5'-CAGGCCCCTTCTACTTTGCAT-3',

Spp1ForwardPrimer: 5'-AACCGAAGTTTTCACTCCAGTTG-3',

Spp1 Reverse Primer: 5'-CCTCAGTCCATAAACCACACTATCAC-3',

SCF Forward Primer: 5'-GTCCCCGGGATGGATGTT-3',

SCF Reverse Primer: 5'-GATCAGTCAAGCTGTCTGACAATTG-3',

IL-7 Forward Primer: 5'-CTGTTGCCAGTAGCATCATCTGA-3',

IL-7 ReversePrimer: 5'-TTGATCGATGCTGACCATTAGAA-3',

Angpt1 Forward Primer: 5'-GTCGGAGATGGCCCAGATAC-3' and

Angpt1 Reverse Primer: 5'-TGAGAGAGGAGGCTGGTTCCT-3'.

2.2.6 STATISTICAL ANALYSIS

All results were expressed as mean ± standard error mean (mean±s.e.m). A minimum of two duplicates were performed for all studies, though in flow cytometry representative samples were presented. Statistical evaluations were done using Graphpad Prism 6.0 software. All p values reported were calculated using unpaired two-tailed *t-test* with Welch's correction and statistical significance was determined by $p \leq 0.05$.

III. RESULTS

3.1 BONE MARROW MESENCHYMAL STROMAL CELL MORPHOLOGY AND GROWTH CHARACTERISTICS

Human bone marrow derived MSCs from patients with AML and from healthy donors was expanded in T-75 cm^2 or T-25 cm^2 plastic tissue culture flasks in standard MSC culture conditions (see Methods). While no adherent cells were obtained from primary cultures of two AML patients, MSCs were generated from nine samples with varying growth rates. Cells was harvested at 80% confluency (18±3 days post seeding mononuclear cells in culture) in samples of normal (n=5) and in a similar time period for five AML samples. Cells from the remaining four AML samples took longer time (54±5 days) to achieve the 80% confluency despite seeding these at the same initial plating concentration of $1.3x10^5$ cells/cm^2. MSC cultures from passage one (P0) were further expanded *in vitro* to test and compare the biologic function and gene expression pattern of mesenchymal cells derived from acute myeloid leukemia patients. Characteristics of patients used for obtaining bone marrow samples are shown in **Table 3-1**.

Mesenchymal cells from healthy donors at early passages (P2-P4) appeared spindle shaped, flattened and spread out forming networks with adjacent cells, **Figure 3-1A**. All control samples had uniform morphology with a gradual increase in cell size over time in culture. MSCs from AML patients (P2-P4) displayed heterogeneous morphology including small round-shaped cells with or without projections and fibroblast like cells with cytoplasmic projections of variable length. Cells from four leukemic samples were spindle shaped fibroblasts that were similar in appearance to MSCs from healthy controls.

The viability of adherent MSCs in cell culture was evaluated by trypan blue exclusion dye at each passage. The viability of adherent MSCs from AML patients was lower compared to the healthy controls at each passage (P1-P4), **Figure 3-1B**.

SAMPLE#	AGE*	GENDER	DIAGNOSIS	DISEASE STATUS	PRIOR TREATMENT
1	52	M	AML	REFRACTORY	CHEMOTHERAPY & BMT
2	64	M	MDS	DIAGNOSIS	NONE
3	61	F	AML	REFRACTORY	INDUCTION CHEMOTHERAPY
4*	56	M	AML	DIAGNOSIS	NONE
5	75	M	AML	DIAGNOSIS	NONE
6*	56	M	AML	REFRACTORY	INDUCTION CHEMOTHERAPY
7	44	F	AML	REFRACTORY	INDUCTION CHEMOTHERAPY
8	41	M	AML	REFRACTORY	INDUCTION CHEMOTHERAPY
9	59	M	MDS	DIAGNOSIS	NONE
10	66	M	AML	COMPLETE REMISSION	INDUCTION CHEMOTHERAPY
11	22	F	AML	COMPLETE REMISSION	INDUCTION CHEMOTHERAPY

Table 3-1: Characteristics of patients used for obtaining bone marrow samples.
Nine of the eleven patients were diagnosed with AML. Four patients were in their initial diagnosis and untreated at the time of study. AGE* at time of study. *Samples from same patient before and after treatment. AML: Acute myeloid leukemia, MDS: Myelodysplastic syndrome.

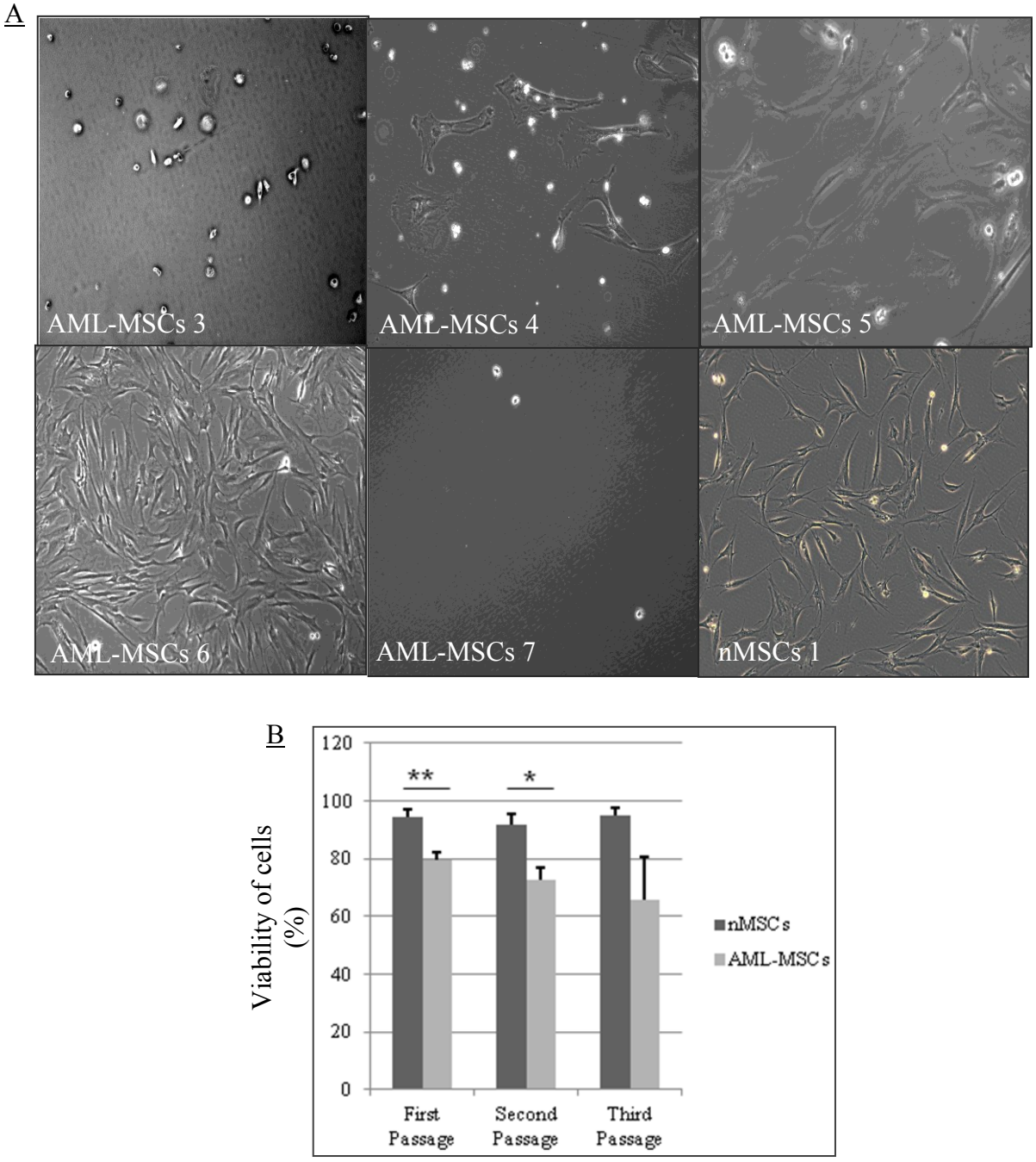


Figure 3-1: Mesenchymal stromal cells derived from AML patients exhibit heterogeneous morphology with reduced viability over time in culture.

(A) MSCs derived from leukemia patients (AML-MSCs, passage 2, P2) are round shaped with or without projections (AML-MSC-3, 4 and 7) and fibroblasts-like with spindle shaped (AML-MSC 5 and 6). Control samples of MSCs derived from healthy donors (nMSCs, P2) have the typical spindle shaped morphology. B) AML-MSCs have reduced viability (grey bars) compared to the cells from healthy donors (black bars) harvested at different passages. All error bars indicate s.e.m. *$p \leq 0.05$, **$p \leq 0.005$. Phase contrast images were taken at 10X magnification.

3.2 PHENOTYPE OF MESENCHYMAL STROMAL CELLS

Mesenchymal stromal cells obtained from primary cultures were further characterized for specific surface antigen expression. The cells were immunophenotyped in accordance with the criteria established by the ISCT [76].

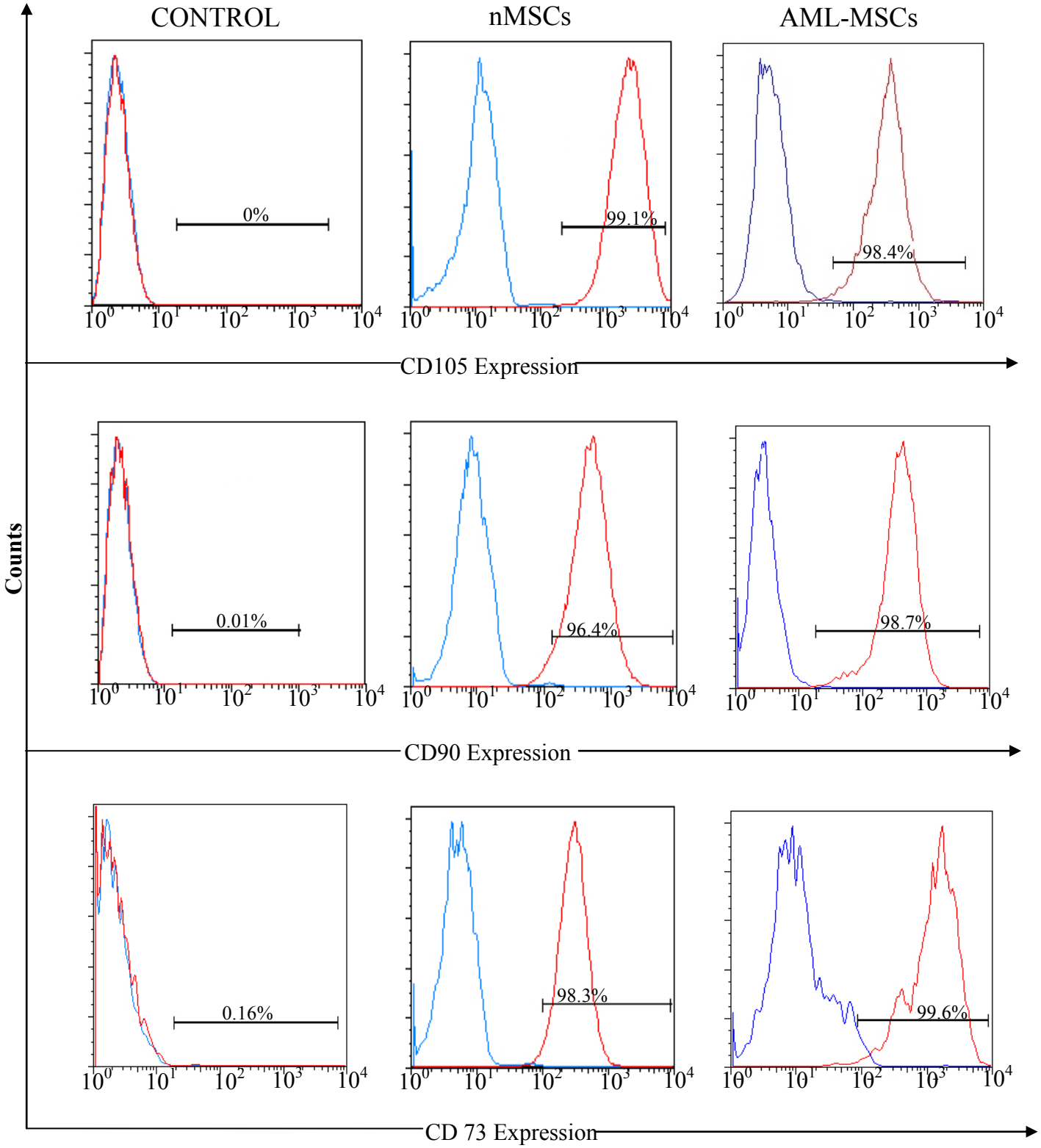
MSCs from healthy donors (P4) expressed surface markers CD105, CD90 and CD73 as shown by the histogram plots in **Figure 3-2**. Unstained cells were used as the primary control to set the threshold for analyzing the stained cells by flow cytometry as represented by the blue curve in **Figure 3-2**. Mononuclear and $CD34^+$ selected cells from peripheral blood and umbilical cord blood respectively were used as controls for flow cytometry analysis, **Figure 3-2**. Although a percentage of cells from early passage MSCs (P1-P3) derived from healthy donors and AML patients expressed hematopoietic markers CD45 and CD34 on their surface, this expression was absent at later passages (P4 onwards, **Figure 3-3**). These data confirmed the absence of detectable hematopoietic cells within the cultures beyond P3 and suggests that a more homogenous population of mesenchymal stromal cells were obtained.

Sufficient number of MSC cells from AML patients were obtained from five samples (P4) and three of the AML samples exhibited antigen expression profile of $CD105^+CD90^+CD73^+CD34^-CD45^-$ similar to the MSCs from healthy control, (**Figure 3-2**). Additional immunophenotyping on mesenchymal cells using immunohistochemistry confirmed the surface marker expression profile observed by flow cytometry for healthy controls and AML MSCs, **Figure 3-4**. However, reduced expression of CD105 and CD90 was observed in samples of AML6 and AML7 respectively, obtained from cultures of passage four and the differences in expression were confirmed by immunohistochemistry,

Figure 3-5. Samples were re-analyzed for CD105 and CD90 antigen expression at a later passage (P6) but MSCs from both the samples had lower expression for the markers. As control, AML samples positive for CD105 and CD90 antigen were used, **Figure 3-5**.

Quantification of the flow data showed that despite reduced expression of some of the mesenchymal markers for two AML samples, MSCs from normal and AML patients had similar extent of expression for surface markers CD105 (98.1±0.7% vs 72.2±18.8%), CD90 (87.4±4.3% vs 83.0±10.3%), CD73 (99.3±0.3% vs 81.2±8.7%), CD34 (0.06±0.03% vs 0.2±0.1%) and CD45 (0.3±0.07% vs 0.4±0.09%), as shown by the bar graph in **Figure 3-6**.

Thus, marrow derived mesenchymal cells obtained from healthy controls displayed a typical immunophenotype defined by the ISCT protocol. No differences were observed in three AML samples that underwent extensive characterization. Two AML samples had reduced surface expression of CD105 and CD90.


CONTROL
nMSCs
AML-MSCs
0%
99.1%
98.4%
CD105 Expression
Counts
0.01%
96.4%
98.7%
CD90 Expression
0.16%
98.3%
99.6%
CD 73 Expression

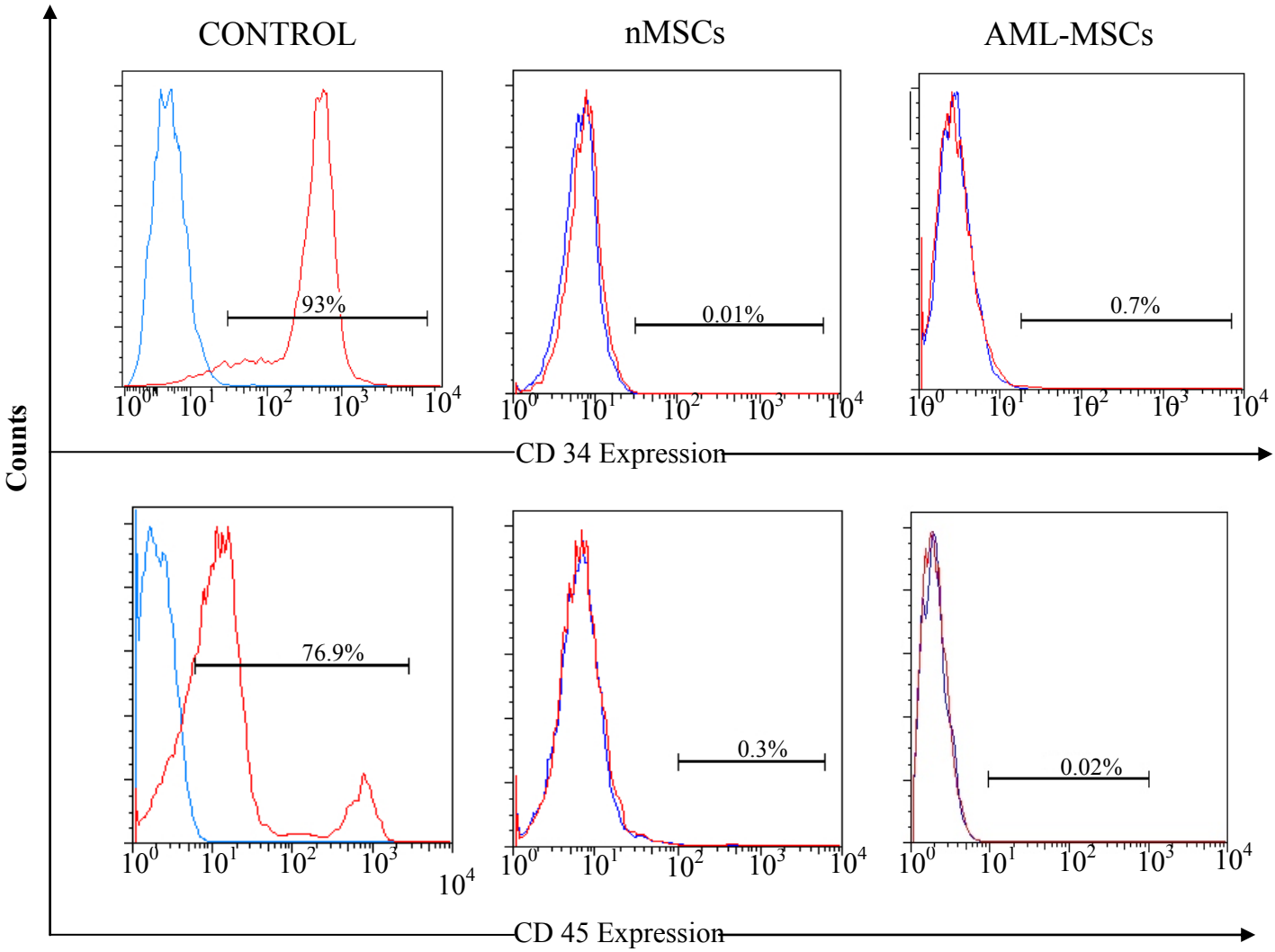
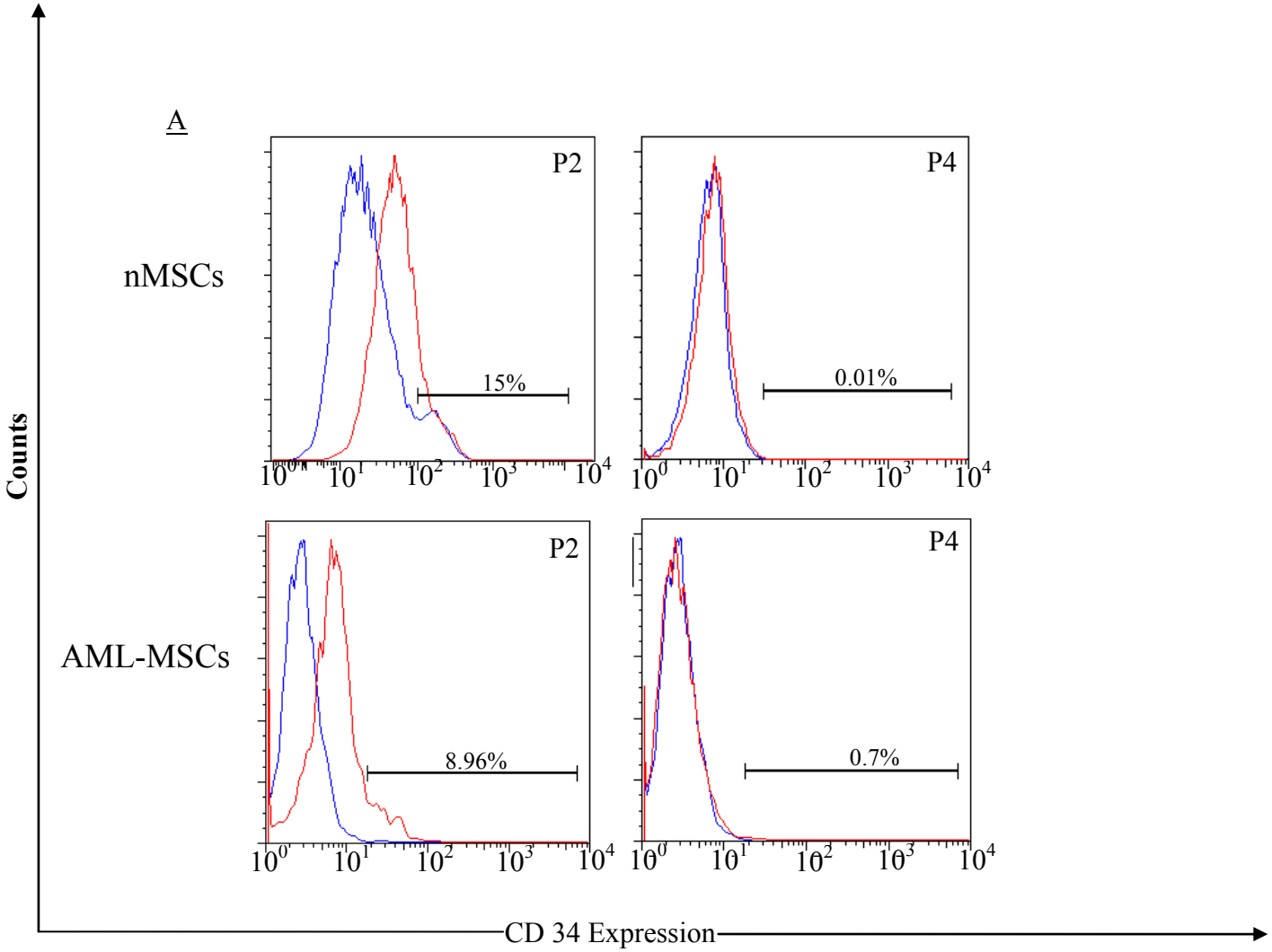


Figure 3-2: AML derived MSCs display phenotype similar to mesenchymal stromal cells derived from healthy donors.

Cell surface phenotypic characterization of human bone marrow MSCs at passage 4 was determined by flow cytometry. Surface phenotype of mononuclear cells (for CD105, CD90, CD73 and CD45) obtained from peripheral blood and CD34 selected cells (for CD34) from cord blood as controls, MSCs from healthy donors (nMSCs) and MSCs from AML patients (AML-MSCs) were performed. The percentage of cells expressing the indicated surface marker has been indicated in each graph. Unstained controls in each graph are blue.


A
nMSCs
AML-MSCs
P2
P4
15%
0.01%
8.96%
0.7%
Counts
CD 34 Expression

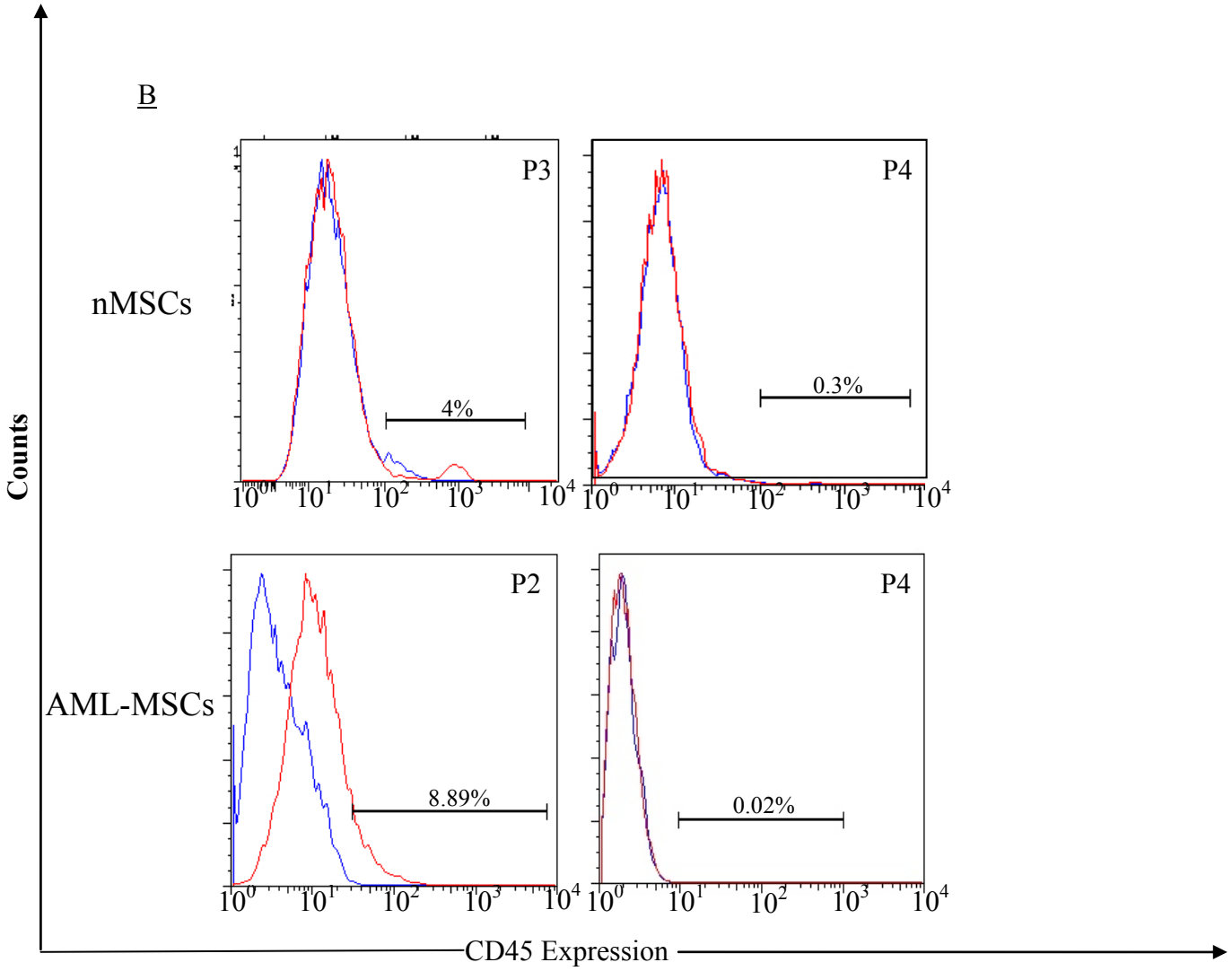


Figure 3-3: Culture of early passage cells contains some hematopoietic cells.

Expression profile of hematopoietic markers (A) CD34 and (B) CD45 by mesenchymal stromal cells from healthy donors (nMSCs) and AML patients (AML-MSCs) over time in culture. Unstained controls in each graph have been indicated as blue.

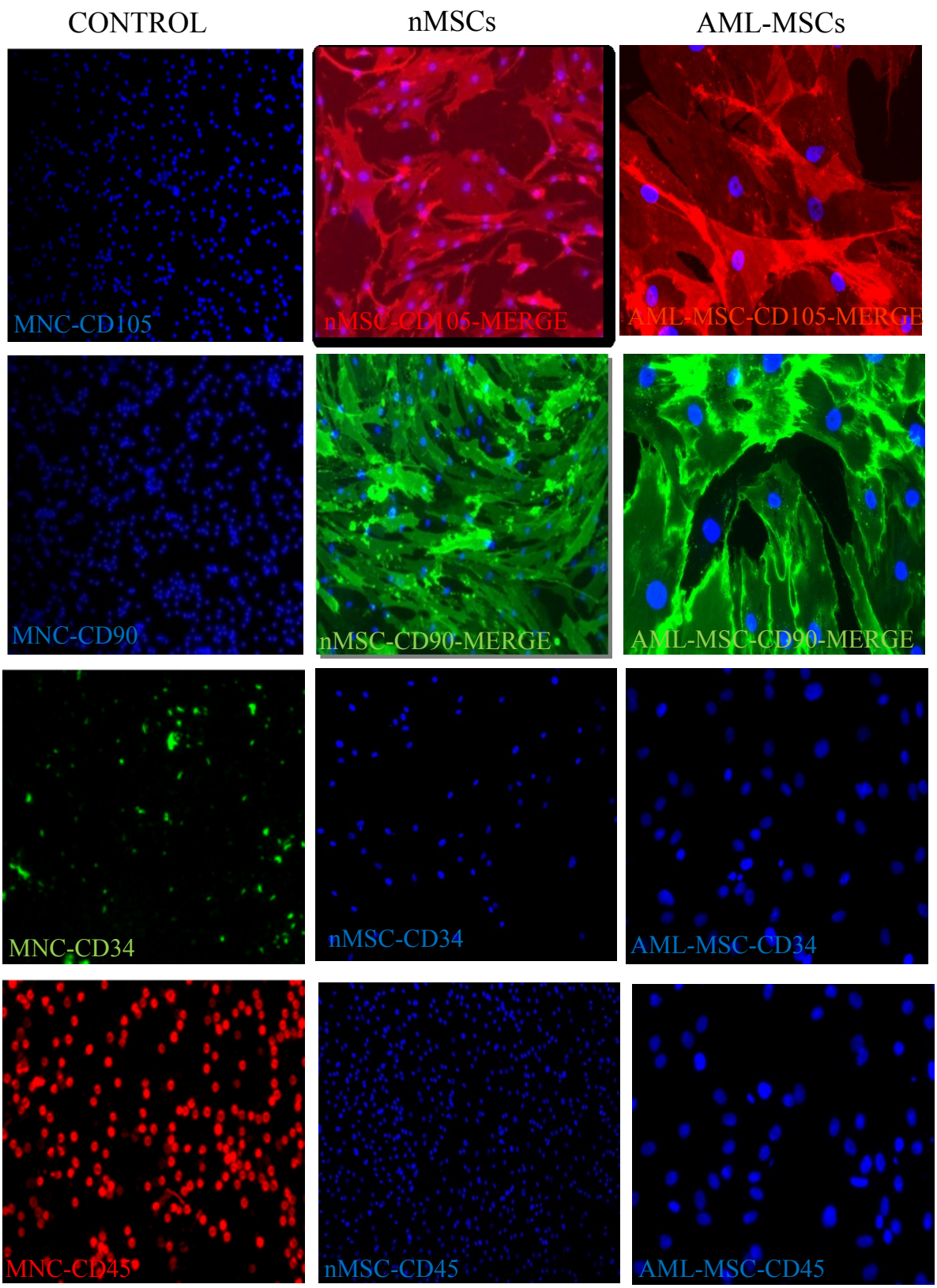

CONTROL
nMSCs
AML-MSCs
MNC-CD105
nMSC-CD105-MERGE
AML-MSC-CD105-MERGE
MNC-CD90
nMSC-CD90-MERGE
AML-MSC-CD90-MERGE
MNC-CD34
nMSC-CD34
AML-MSC-CD34
MNC-CD45
nMSC-CD45
AML-MSC-CD45

Figure 3-4: AML derived cells have a typical immunophenotype of mesenchymal stromal cells.

Immunohistochemical staining of MSCs derived from healthy donors (nMSCs) and AML patients (AML-MSCs) at passage three (P3) with antibodies against Rhodamine labeled CD105 (red), GFP labeled CD90 (green). Nuclei have been stained with DAPI (blue). Both nMSCs and AML-MSCs lacked expression for CD34 and CD45 marker. Mononuclear cells were used as control for the experiment. Images were obtained at 20X magnification.

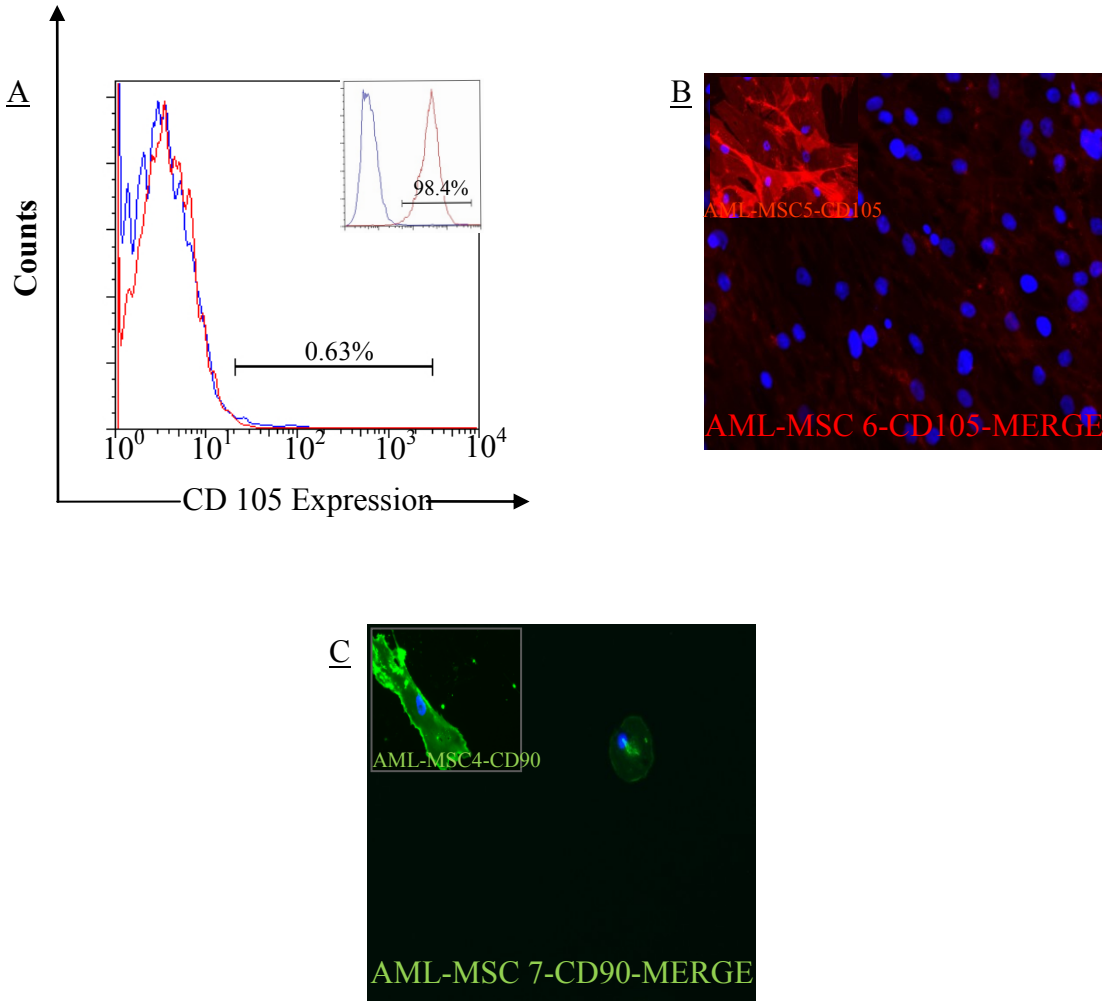


Figure 3-5: Few samples of AML derived mesenchymal stromal cells exhibit differential marker expression.

Differences in phenotype observed by (A) CD105 expression (red) in AML-MSCs sample 6, (B) corresponding fluorescence staining of the same cells with CD105 (red) and (C) CD90 staining (green) in AML-MSCs sample 7. Inset images are controls (A) AML-MSC sample 9 for CD105 antigen expression (red), (B) AML-MSC sample 5 for CD105 expression (red) and (C) AML-MSC sample 4 for expression of CD90 antigen (green). Immunohistochemistry images were obtained at 20X magnification. Unstained sample for flow cytometry has been denoted as blue. Nuclei for immunostaining have been stained with DAPI (blue).

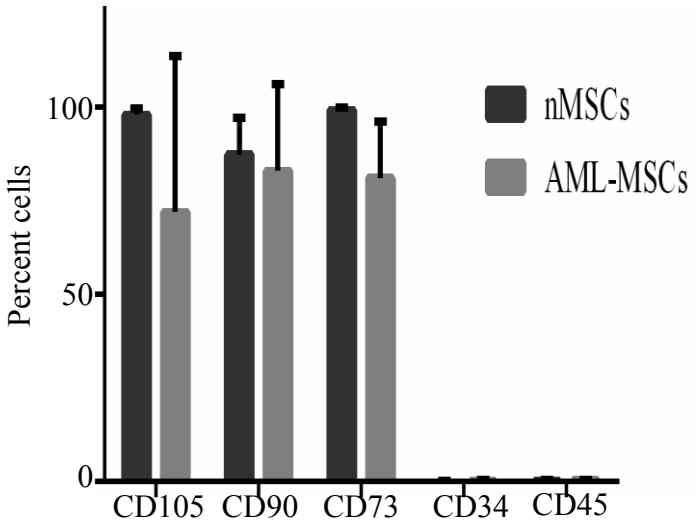


Figure 3-6: Quantification of surface marker expression on mesenchymal stromal cells obtained from healthy donors and AML patients.

Percent AML (AML-MSCs, n=5) and normal MSC cells (nMSCs, n=5) expressing various MSC surface markers as detected by fluorescent antibody binding and flow cytometry. Data obtained from phenotypic characterization of MSCs from AML patients (AML-MSCs) and healthy donors (nMSCs). Values are represented as mean and error bars indicate s.e.m.

3.3 DIFFERENTIATION POTENTIAL OF MESENCHYMAL STROMAL CELLS

MSCs from five AML patients and healthy controls were expanded *in vitro* to evaluate their biologic property to differentiate into mesenchymal tissues, including the AML sample that displayed low surface expression of CD105. The adherent cells were cultured in differentiation medium to test the potential of these cells to differentiate into adipocytes, osteocytes and chondrocytes.

Adipogenic differentiation: Adherent mesenchymal stromal cells derived from the healthy donors as well as AML samples, were grown in adipogenic medium and after one week in culture gave rise to lipid vacuoles, **Figure 3-7**. Their ability to form fat was demonstrated by the immuno-staining with FABP4 antibody (fatty acid binding protein antibody 4) that specifically binds to lipid vacuoles. The results were confirmed qualitively using Oil Red O chemical stain of the vacuoles formed *in vitro* culture. Lipid vacuoles generated by the AML-MSC cells appeared larger compared to the vacuoles observed in normal MSCs, **Figure 3-8**.

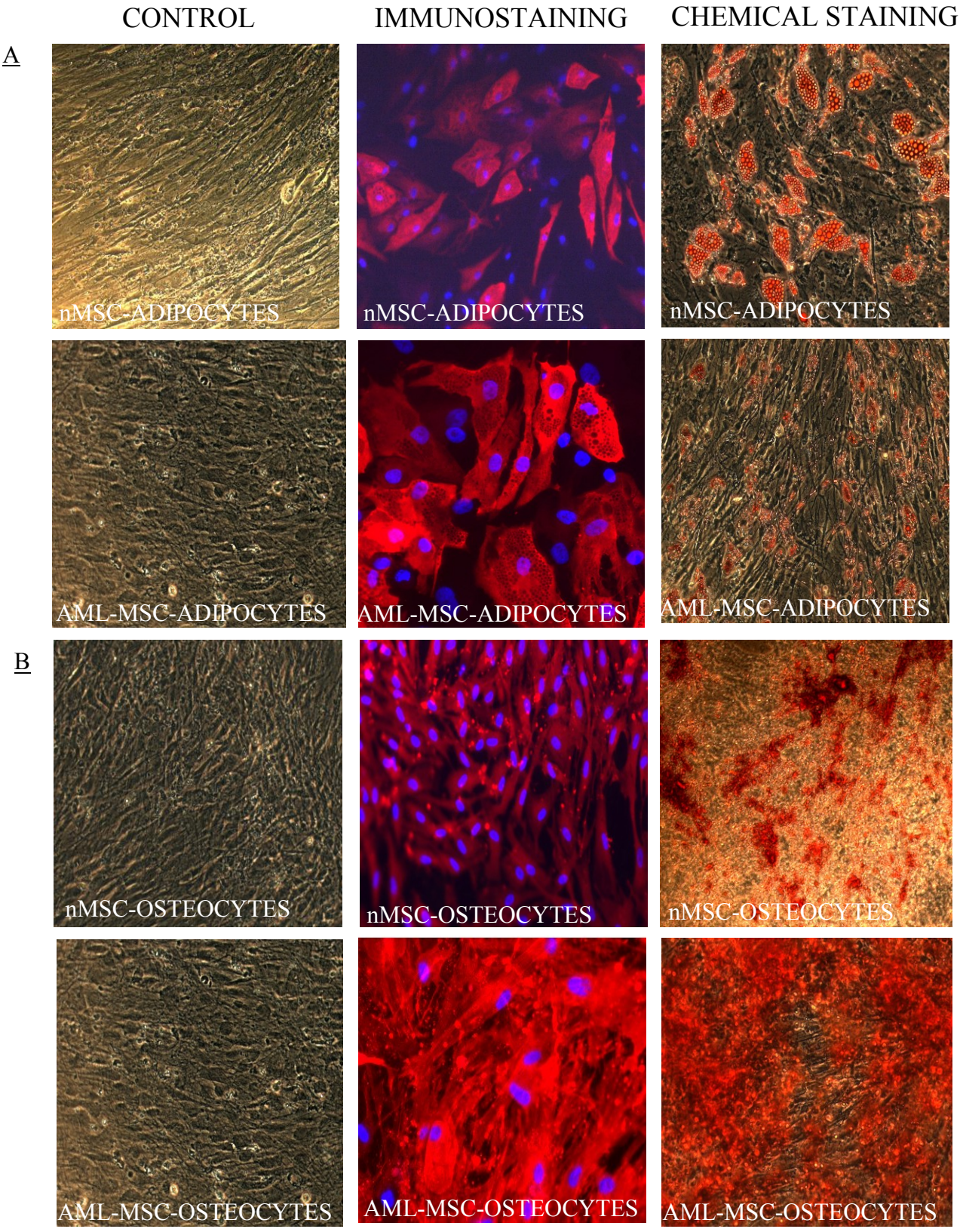
Osteogenic differentiation: Cultured MSCs from normal as well as AML samples formed aggregates of calcium deposits when kept in osteogenic medium for three weeks. The presence of calcium deposits was confirmed by alizarin staining of osteocytes, **Figure 3-7**. The results were also verified by immunostaining of osteocalcin that has been previously shown to regulate mineralization in bones.

Chondrogenic differentiation: For a third mesenchymal lineage, cartilage formed by the mesenchymal stromal cells was stained with aggrecan antibody as well as alcian-blue stain that selectively binds to the carbohydrates present on the cells. AML samples and healthy

human MSCs demonstrated their ability to differentiate into cartilage as shown in **Figure 3-7**. Mesenchymal stromal cells derived from healthy donors and AML patients grown in normal culture medium were used as controls for the experiment.

Hence, differentiation assays performed on bone marrow derived human MSCs from AML patients and healthy donors exhibited similar ability to differentiate into three lineages: adipocytic, osteocytic and chondrocytic as confirmed by immunofluorescence and chemical staining.

Adherence of AML and healthy donor derived MSC cells to the tissue culture flasks, their phenotypic profile and the ability to differentiate, fulfilled the three minimum criteria required to define mesenchymal cells *in vitro*, thereby confirming that the isolated populations of cells were mesenchymal stem cells generated from healthy donors and leukemic patients.


CONTROL
IMMUNOSTAINING
CHEMICAL STAINING
A
nMSC-ADIPOCYTES
nMSC-ADIPOCYTES
nMSC-ADIPOCYTES
AML-MSC-ADIPOCYTES
AML-MSC-ADIPOCYTES
AML-MSC-ADIPOCYTES
B
nMSC-OSTEOCYTES
nMSC-OSTEOCYTES
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AML-MSC-OSTEOCYTES
AML-MSC-OSTEOCYTES
AML-MSC-OSTEOCYTES

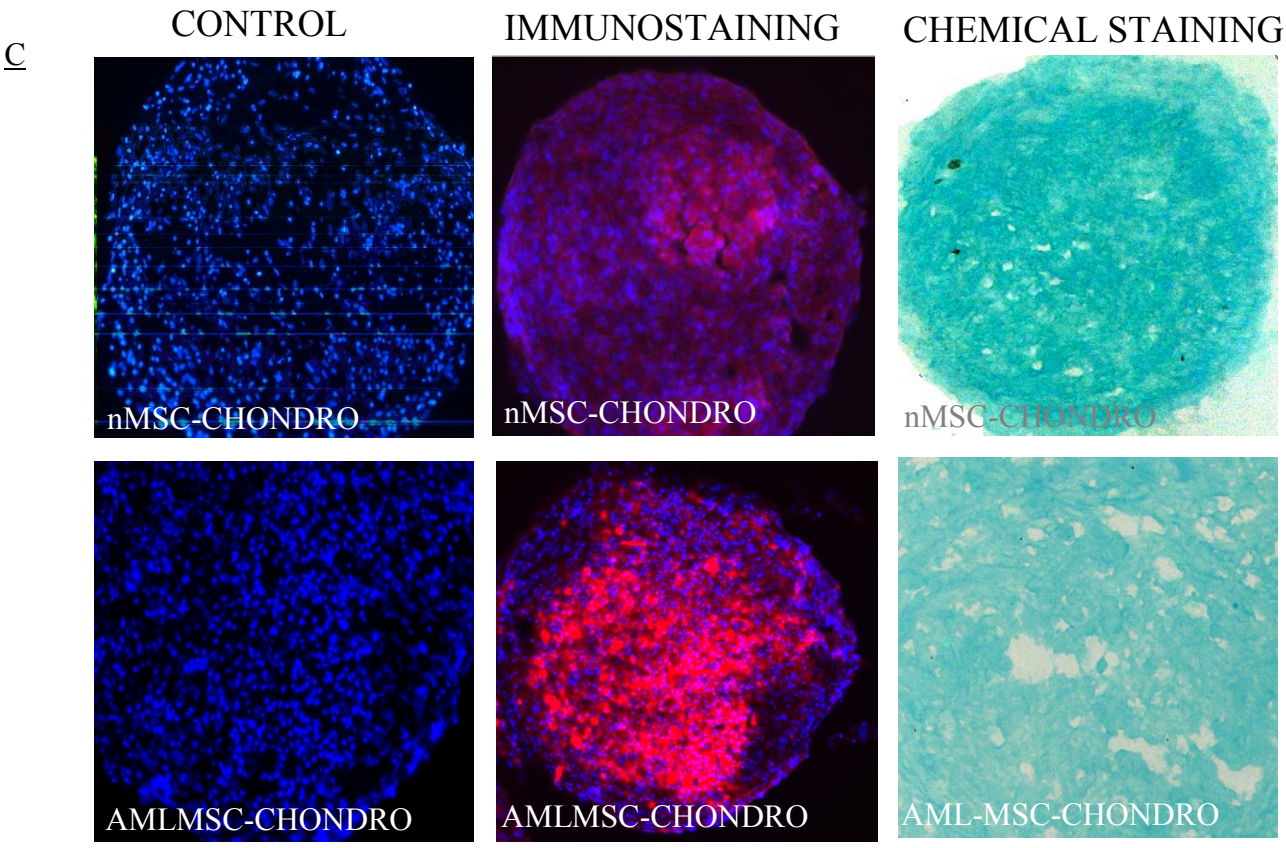


Figure 3-7: AML derived mesenchymal stromal cells exhibit a predicted multipotent differentiation potential.

The differentiation potential of mesenchymal stromal cells from healthy donors (nMSCs) and AML patients (AML-MSCs) were verified for (A) adipogenesis by immunostaining adipocytes with antibody against FABP4 (red) and chemical staining with Oil Red O (red), (B) osteogenesis by staining osteocytes for osteocalcin (red) and chemical staining with alizarin (red), (C) chondrogenesis by staining for chondrocytes using antibody against aggrecan (red) and chemical staining with alcian-blue stain (blue). Nuclei for immunostaining have been stained with DAPI (blue). MSCs not induced for differentiation were used as control for both chemical and immunostaining. Images were obtained at 20X magnification.

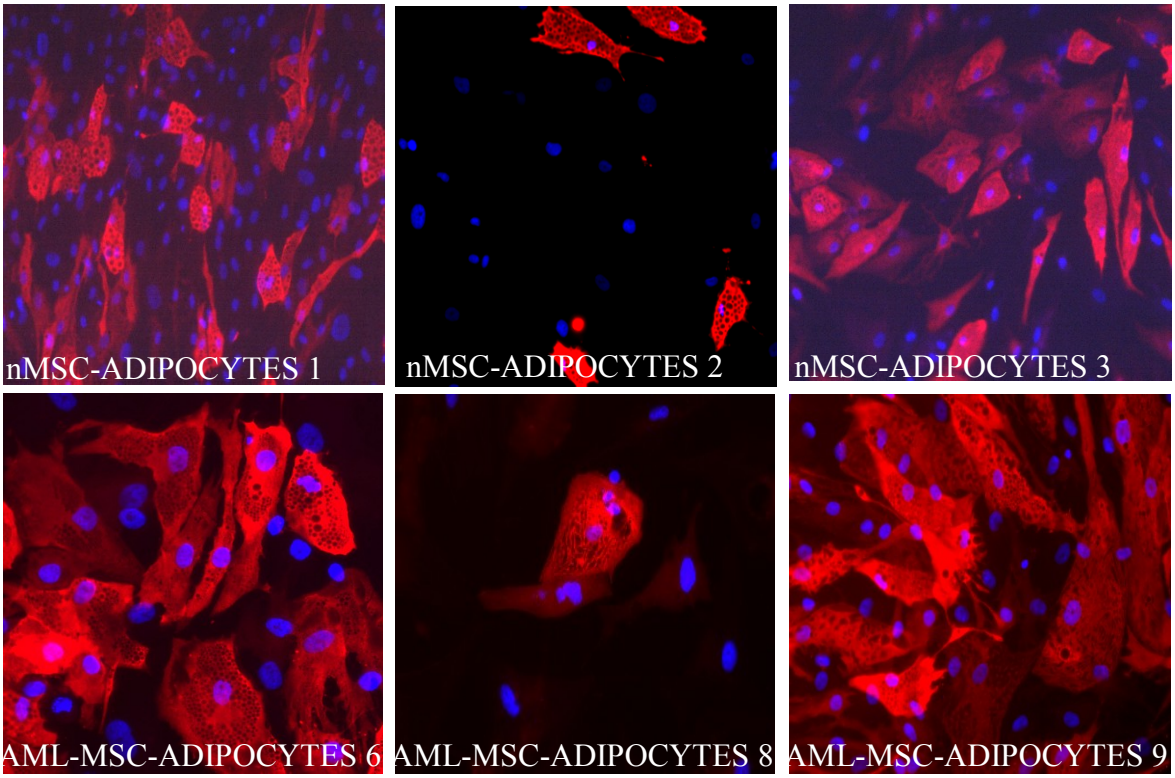


Figure 3-8: Adipocytes formed from AML derived mesenchymal stromal cells appear larger compared to mesenchymal stromal cells from healthy donors.

Immunostaining of adipocytes generated from healthy donors (nMSCs, n=3) and AML samples (AML-MSCs, n=3) with antibody against FABP4 (red). Nuclei have been stained with DAPI (blue). All images were obtained at 20X magnification.

3.4 HEMATOPOIETIC SUPPORTIVE FUNCTION OF MESENCHYMAL STROMAL CELLS

The ability of MSCs to support hematopoiesis *in vitro* was investigated with $CD34^+$ enriched cells. This study was important as it allowed assessing the differences in the functional ability of MSCs from AML patients to support hematopoiesis.

$CD34^+$ hematopoietic cells freshly isolated from umbilical cord blood (UCB) had a purity of 84±4.6% as detected by flow cytometry and were cultured for eight days on top of a confluent layer of MSCs derived from healthy donors (n=5) and patients with AML (n=3). A total of five cord blood units with mean of $(2.52\pm0.69)x10^6$ $CD34^+$ cells were obtained and utilized in the assessment of MSCs in hematopoietic supportive function.

The cord blood derived $CD34^+$ cells were co-cultured with MSCs and cytokines (nMSCs+cytokines) for eight days and were observed to be viable and proliferating in all assays using MSCs from healthy controls, **Figure 3-9A**. Following the eight days of co-culture, cells were sorted based on $CD45^+$ expression to analyze the expansion of hematopoietic population. A 6.0±1.5 fold (p=0.04) expansion of $CD45^+$ hematopoietic cells was observed by day eight in comparison to the starting number of $CD34^+$ cells seeded, **Figure 3-9B**. When $CD34^+$ cells were co-cultured with MSCs obtained from AML patients (AML-MSCs+cytokines), only a 1.9±1.47 fold (p=0.61) change in number of blood cells expressing hematopoietic $CD45^+$ cells was observed, **Figure 3-9C**. However, the cells appeared healthy and proliferating in culture similar to nMSCs+cytokines.

The sorted hematopoietic progenitor populations of $CD45^+$ cells were further analyzed for CD34 and CD38 antigen expression, **Figure 3-10**. Different sub-populations of $CD34^+$ cells were considered to address the expansion of more primitive ($CD34^+CD38^-$)

and more committed ($CD34^+CD38^+$) progenitors. More differentiated ($CD34^-CD38^+$) cells were also enumerated. Compared to the number of cells at the start of co-culture, a trend towards increased committed progenitor $CD34^+CD38^+$ cells (9.2±3.1 fold, p=0.07) and differentiated $CD34^-CD38^+$ cells (33.3±12.3 fold, p=0.07) was observed over the eight day culture period with nMSCs+cytokines. Absolute number for more primitive $CD34^+CD38^-$ progenitor cells remained unchanged (1.4±0.6 fold, p=0.5), **Figure 3-11A**. For co-culture experiments with AML-MSCs+cytokines, a possible increase in committed $CD38^+CD34^+$ cells was observed in comparison to the initial number of cells seeded, (8.8 fold±4.6 fold, p=0.2). More differentiated populations of $CD34^-CD38^+$ cells remained unchanged in contrast to the observations made for nMSCs+cytokines co-cultures, (0.8±0.4 fold, p=0.2). Absolute number for primitive population of $CD34^+CD38^-$ cells also remained unchanged (2.3±2.2 fold, p=0.6), **Figure 3-11A**. Taken together, data from the flow cytometry revealed that while primitive progenitor $CD34^+CD38^-$ population remained unchanged for both nMSCs+cytokines and AML-MSCs+cytokines co-culture system, samples from AML-MSCs provided reduced support for the expansion of differentiated progenitors $CD34^-CD38^+$ cells compared to nMSCs (33.3±12.2 fold vs 0.8±0.4 fold, p=0.07), **Figure 3-11B**.

Additionally, the ability of hematopoietic $CD45^+$ cells to give rise to different hematopoietic progenitor colonies was evaluated using CFC assays. In comparison to normal $CD34^+$ cells freshly isolated from UCB unit, $CD45^+$ cells isolated following co-culture with nMSCs+Cytokines formed a similar frequency of total colonies (82.8±13.8 colonies per $1x10^3$ $CD34^+$ cells vs 99.0±13.0 colonies per $1x10^3$ $CD45^+$ cells, p=0.2), **Figure 3-12A**. Given the overall expansion of hematopoietic cells, absolute number of CFC colonies increased significantly following co-culture with nMSCs+cytokines

compared to freshly isolated $CD34^+$ cells from UCB unit, ((28.0±8.0)x10^4 colonies vs (3.7±1.4)x10^4 colonies, p=0.04), **Figure 3-12B**. The plating efficiency of $CD45^+$ cells from co-culture with AML-MSC+cytokines, appeared lower compared to $CD34^+$ cells freshly isolated from UCB unit (21.0±18.5 colonies per 1x10^3 $CD45^+$ cells vs 82.8±13.8 colonies per 1x10^3 $CD34^+$ cells, p=0.06). Plating efficiency of $CD45^+$ cells from AML-MSC+cytokines also appeared lower when compared to nMSCs+cytokines (21.0±18.5 colonies per 1x10^3 $CD45^+$ cells vs 99.0±13.0 colonies per 1x10^3 $CD45^+$ cells, p=0.04), **Figure 3-12A**. The absolute number of total CFCs from expanded $CD45^+$ cells was similar to the number of colonies obtained from unexpanded $CD34^+$ cells ((2.5±2.4)x10^4 colonies vs (3.7±1.4)x10^4 colonies, p=0.6). When compared to nMSCs+cytokines, the mean CFC colonies obtained from expanded $CD45^+$ cells following co-culture with AML-MSCs was reduced significantly ((28.0±8.0)x10^4 colonies vs (2.5±2.4)x10^4 colonies, p=0.03), **Figure 3-12B**.

Evaluation of different sub-types of colonies generated from $CD45^+$ cells following co-culture with nMSCs+cytokines demonstrated a trend towards increased absolute number of BFU-E colonies compared to colonies arising from day 0 ((2.5±0.7)x10^4 colonies vs (1.1±0.3)x10^4 colonies, p=0.07). However, absolute number of CFU-GEMM colonies were similar ((0.6±0.2)x10^4 colonies vs (0.4±0.03)x10^4 colonies, p=0.1). A greater number of more differentiated colonies were formed from $CD45^+$ cells following co-culture compared to freshly isolated $CD34^+$ cells, including CFU-G ((4.7±1.5)x10^4 colonies vs (0.4±0.2)x10^4 colonies, p=0.04), CFU-M ((7.5±1.7)x10^4 colonies vs 0.6±0.2)x10^4 colonies, p=0.02) and CFU-GM ((14.7±4.5)x10^4 colonies vs (1.1±0.3)x10^4 colonies, p=0.03), **Figure 3-12C**. For AML-MSCs+cytokines, absolute number for each colony subtype remained unchanged in

comparison to freshly isolated $CD34^+$ cells. However, when compared to nMSCs+cytokines, the absolute number of colonies from $CD45^+$ cells following co-culture with AML-MSCs+cytokines significantly decreased. The colonies included CFU-GEMM ($(0.6\pm0.2)x10^4$ colonies vs $(0.08\pm0.08)x10^4$ colonies, p=0.05), CFU-G ($(4.7\pm1.5)x10^4$ colonies vs $(0.1\pm0.1)x10^4$ colonies, p=0.04), CFU-M ($((7.5\pm1.7)x10^4$ colonies vs $0.7\pm0.7)x10^4$ colonies, p=0.02) and CFU-GM ($(14.7\pm4.5)x10^4$ colonies vs $(1.0\pm1.0)x10^4$ colonies, p=0.04), **Figure 3-12C**. Additionally for $CD45^+$ cells from AML-MSCs+cytokines, a suggested decrease in absolute number was observed for primitive colonies of BFU-E compared to nMSCs+cytokines ($(0.6\pm0.6)x10^4$ colonies vs $(2.5\pm0.7)x10^4$ colonies, p=0.08). The type and morphology of CFCs formed by $CD45^+$ cells from nMSCs+Cytokines and AML-MSCs+cytokines were similar, as shown in **Figure 3-12D**. A table summarizing the results obtained from assessing the hematopoietic supportive function of mesenchymal cells derived from healthy donor and AML patients in presence of cytokines is shown in **Table 3-2**.

Therefore, MSCs from healthy donors supported the expansion of normal selected CD34 cord blood cells. An expansion of differentiated ($CD34^-CD38^+$) cells was observed while more primitive progenitors ($CD34^+CD38^-$) remained stable suggesting that nMSCs may mediate differentiation of blood cells rather than the maintenance of more primitive $CD34^+$ progenitor cells. Increased number of committed colonies was also generated in CFC assay. Co-culture of normal CD34 selected cells with MSCs derived from patients with AML led to possible reduction in differentiated ($CD34^-CD38^+$) cells and fewer committed CFCs *in vitro* compared to co-culture with nMSCs. However, for both nMSCs and AML-MSCs, a trend towards increased expansion of more committed progenitors

($CD34^+CD38^+$) was observed while the pool of the earlier progenitors ($CD34^+CD38^-$) appeared to remain stable, thereby suggesting that the ability of AML-MSCs to support hematopoiesis was impaired in terms of reduced expansion of differentiated hematopoietic populations.

CATEGORY	CO-CULTURED $CD34^+$ CELLS WITH nMSCs+CYTOKINES vs FRESHLY ISOLATED $CD34^+$ CELLS		CO-CULTURED $CD34^+$ CELLS WITH AML-MSCs+CYTOKINES vs FRESHLY ISOLATED $CD34^+$ CELLS		CO-CULTURED $CD34^+$ CELLS WITH AML-MSCs+CYTOKINES vs CO-CULTURED $CD34^+$ CELLS WITH nMSCs+CYTOKINES	
	ABSOLUTE NUMBER	p VALUE	ABSOLUTE NUMBER	p VALUE	ABSOLUTE NUMBER	p VALUE
$CD34^+CD38^-$	↔	0.5	↔	0.6	↔	0.7
$CD34^+CD38^+$	↑↔	0.07	↑↔	0.2	↔	0.9
$CD34^-CD38^+$	↑↔	0.07	↔	0.2	↓↔	0.07
TOTAL CFCs	↑	0.04	↔	0.6	↓	0.03
BFU-E	↑↔	0.07	↔	0.8	↓↔	0.08
CFU-G	↑	0.04	↔	0.9	↓	0.04
CFU-M	↑	0.02	↔	0.6	↓	0.02
CFU-GM	↑	0.03	↔	0.6	↓	0.04
CFU-GEMM	↔	0.1	↓↔	0.07	↓	0.05

Table 3-2: Summary of the hematopoietic supportive function of mesenchymal stromal cells from healthy donors and AML patients.

The table summarizes the flow cytometry analysis on sub-populations of $CD34^+$ cells pre and post co-culture with nMSCs and AML-MSCs in presence of cytokines (G-CSF, SCF and EPO) and the CFCs of hematopoietic cells generated before and after eight days of co-culture with MSCs in comparison to freshly isolated $CD34^+$ cells. An increase (↑), decrease (↓) or no change (↔) in absolute numbers compared to unexpanded $CD34^+$ cells is indicated. A trend towards an increase (↑↔) or a decrease (↓↔) that was not statistically significant ($p<0.1$ but $p>0.05$) is also indicated.

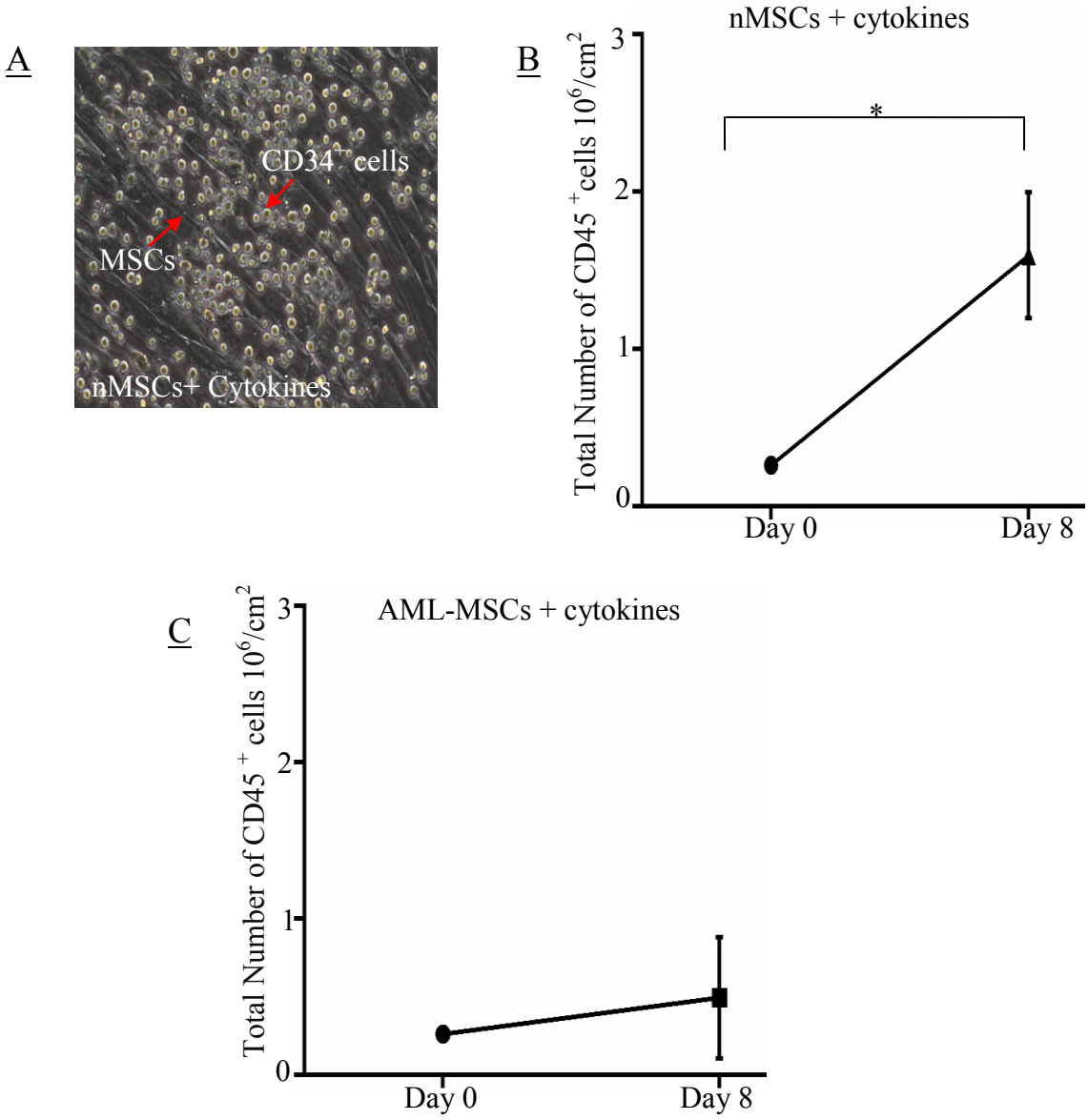


Figure 3-9: CD34$^+$ cells exhibit normal viability but differential expansion profiles when co-cultured with mesenchymal cells derived from healthy donor and AML patients in presence of cytokines.

Co-culture of UCB derived CD34$^+$ cells with (A) healthy donor derived MSCs (nMSCs) proliferate in presence of cytokines. (B) Increase in total number of CD45$^+$ cells harvested after eight days in culture of nMSCs+cytokines, compared to the initial CD45$^+$ cells seeded at day 0. (C) Total number of CD45$^+$ cells obtained at day 8 slightly increase for AML-MSCs. Image was obtained at 10X magnification. All values are represented as mean and error bars indicate s.e.m. *p≤0.05.

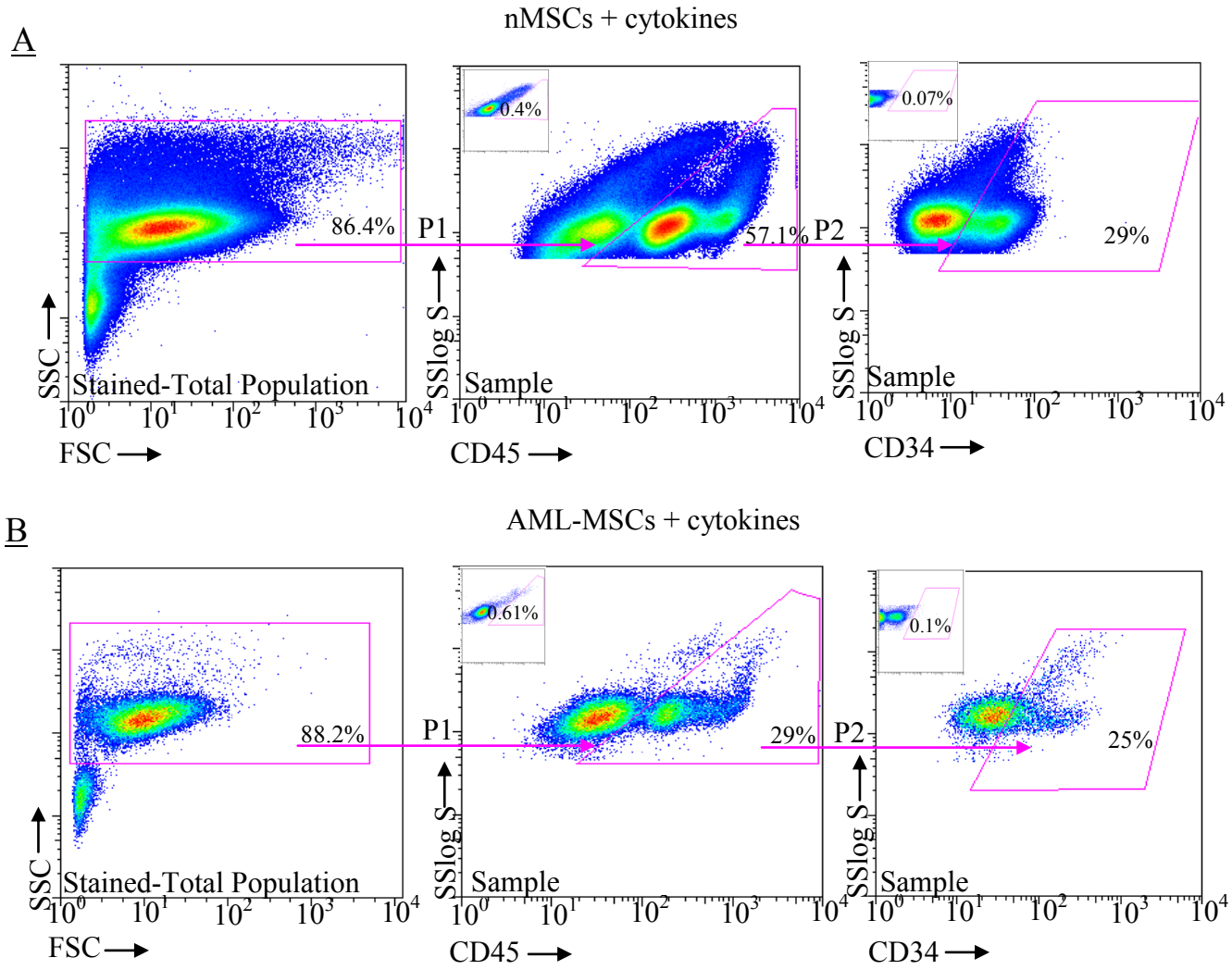
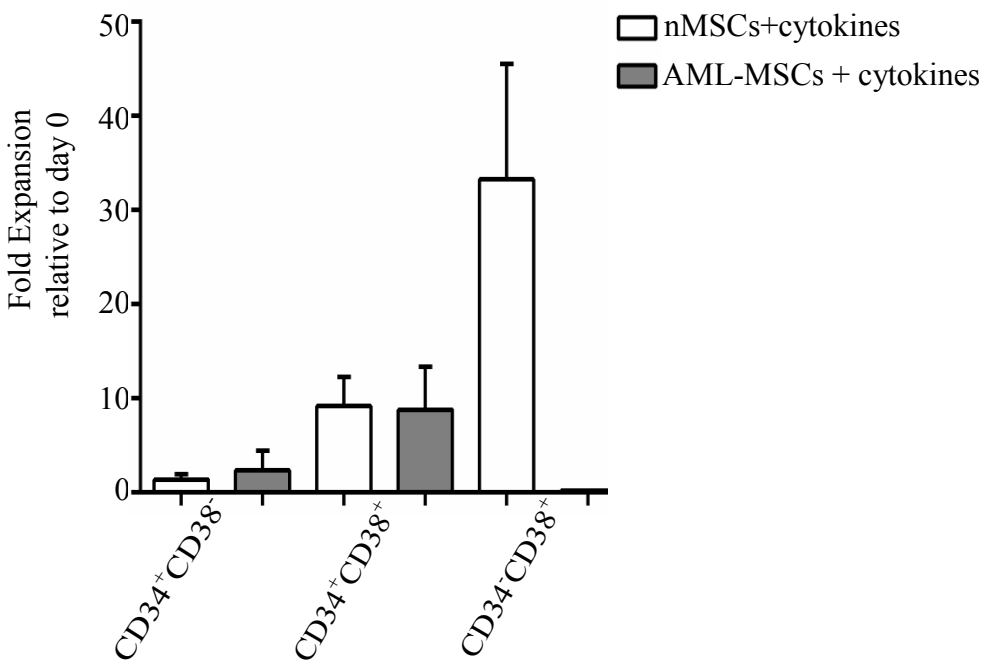


Figure 3-10: Phenotypic analysis of hematopoietic populations after 8 days of co-culture with mesenchymal stromal cells derived from healthy donor and AML patients.

UCB derived $CD34^+$ populations after co-culture with (A) nMSCs+cytokines (B) AML-MSCs+cytokines demonstrates the profile of co-cultured hematopoietic cells at day 8 stained with CD45 and CD34 antibodies. Percentages of each population have been indicated in the graph. Inset graphs are unstained controls of the sample used for gating the desired population.

A


nMSCs+cytokines
AML-MSCs + cytokines
Fold Expansion relative to day 0
50
40
30
20
10
0
CD34+CD38-
CD34+CD38+
CD34-CD38+

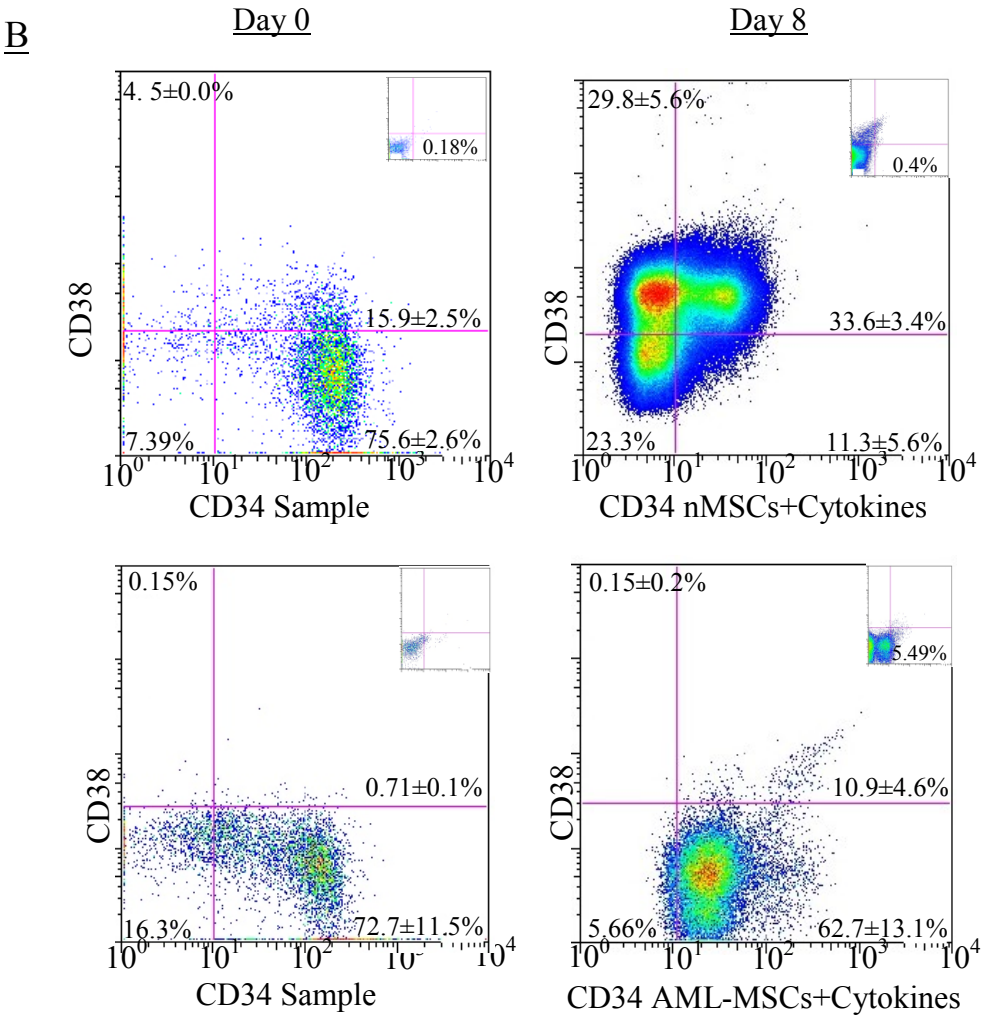
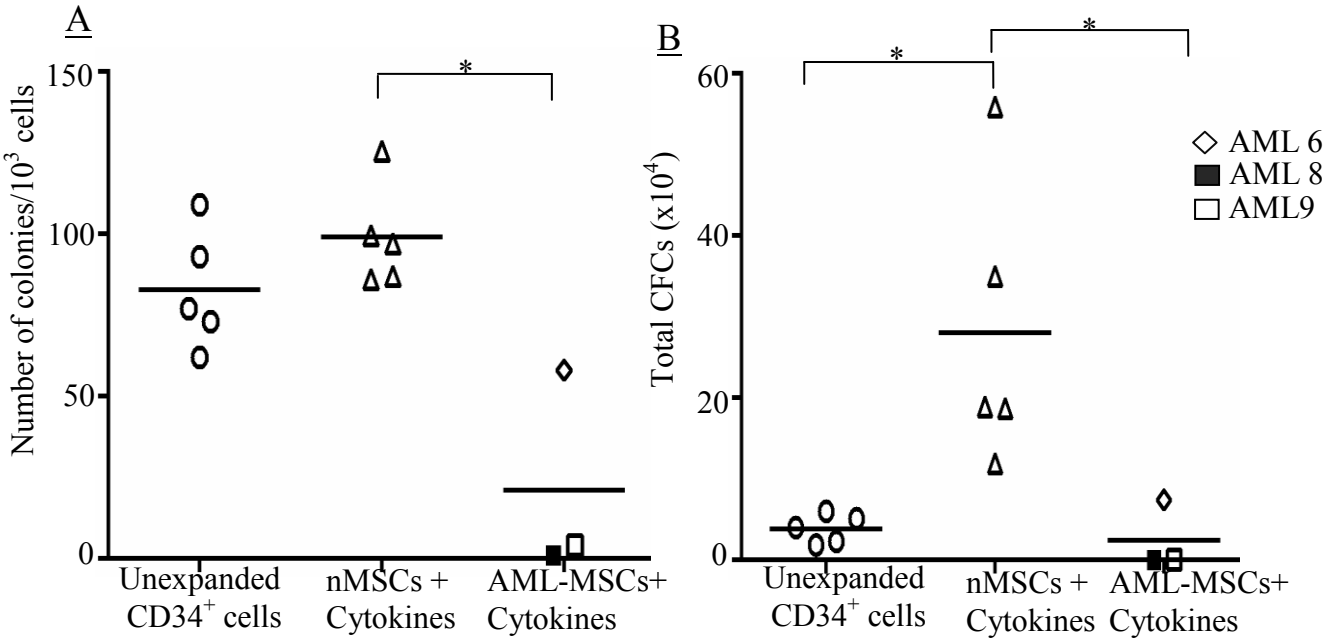
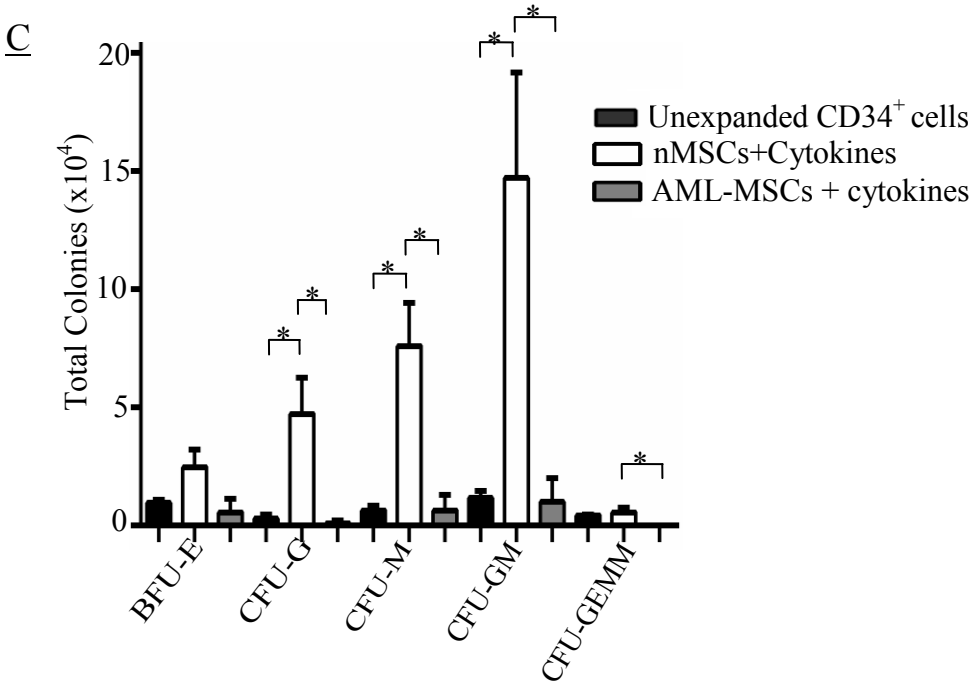


Figure 3-11: AML derived mesenchymal stromal cells have reduced hematopoietic support of CD34$^+$ cells.

Overall fold expansion of hematopoietic progenitor cells expressing (A) CD34$^+$CD38$^-$, CD34$^+$CD38$^-$ and CD34$^+$CD38$^-$ expressing cells after 8 days of co-culture with MSCs from healthy donors (nMSCs) and AML patients (AML-MSCs). (B) Two parameter histogram plot to quantify the percentages of CD34 and CD38 antigen expressing populations within the expanded hematopoietic cells from nMSCs and AML-MSCs in presence of cytokines. All error bars indicate s.e.m. Percentages of each population from nMSCs (n=5) and AML-MSCs (n=3) have been indicated in the graph as mean±s.e.m. Overall fold expansion of cells expressing CD34 sub-population were calculated based on the percentage from the flow data multiplied by total yield of CD34$^+$ cells (day 0) and CD45$^+$ cells (day 8).


A
Number of colonies/10^3 cells
150
100
50
0
*
Unexpanded CD34+ cells
nMSCs + Cytokines
AML-MSCs+ Cytokines
B
Total CFCs (x10^4)
60
40
20
0
*
*
AML 6
AML 8
AML9
Unexpanded CD34+ cells
nMSCs + Cytokines
AML-MSCs+ Cytokines


C
Total Colonies (x10^4)
20
15
10
5
0
Unexpanded CD34+ cells
nMSCs+Cytokines
AML-MSCs + cytokines
BFU-E
CFU-G
CFU-M
CFU-GM
CFU-GEMM

D

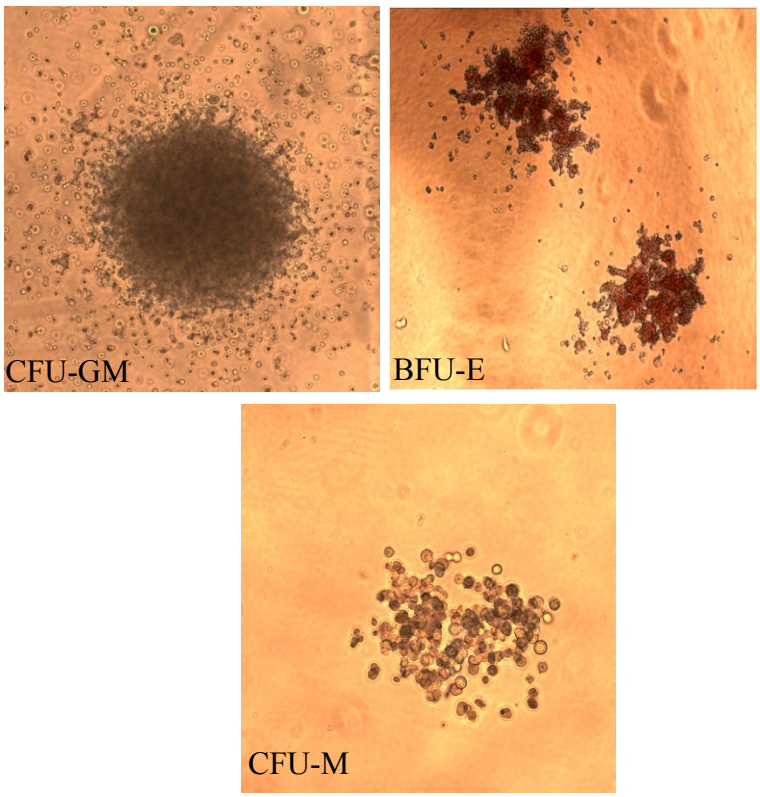


Figure 3-12: Decrease in the number of progenitor colonies generated by $CD45^+$ cells grown with AML derived mesenchymal stromal cells *in vitro*.

CFCs formed by $CD45^+$ cells in the presence of mesenchymal cells from healthy donors (nMSCs) and AML patients (AML-MSCs) calculated based on (A) number of colonies per $1x10^3$ cells seeded and (B) absolute number of colonies generated by total $CD45^+$ cells at day 8 of co-culture. (C) Different types of colonies formed by $CD45^+$ cells previously co-cultured with nMSCs+cytokines and AML-MSCs+cytokines. (D) Representative microscopy images of different colonies. The arithmetic mean is represented by a straight line. Freshly isolated $CD34^+$ primitive cells were used for CFC assay as a control. All error bars indicate s.e.m.*$p \leq 0.05$. Sample from each AML patient is represented with a different symbol. Images were obtained at 10X magnification. Absolute number of total and subtype of colonies was calculated based on number of colonies obtained per $1x10^3$ cells seeded multiplied by total yield of $CD34^+$ cells (day 0) and $CD45^+$ cells (day 8).

3.5 EXPRESSION PATTERN OF HEMATOPOIETIC SUPPORTIVE GENES BY MESENCHYMAL STROMAL CELLS

Given the apparent reduced potential of AML-MSCs to support overall hematopoietic expansion, in particular, the reduced expansion of differentiated $CD34^-$ $CD38^+$ cells and generation of fewer committed CFCs compared with nMSCs, the expression pattern of certain key genes was studied using quantitative RT-qPCR. The genes included SDF1-α, IL-7, SCF, VCAM1, SPP1 and ANGPT1, previously identified to be involved in maintaining and regulating hematopoietic stem cells [14, 15, 35]. Additionally, expression of RGMB, a bone morphogenetic protein co-receptor that promotes cell adhesion and is up regulated in MSCs from MDS patients as well as in elderly healthy controls, was studied in MSCs [92, 93].

The expression level of ANGPT1 and SDF1-α in AML-MSCs increased compared to MSCs from healthy donors (4.2±1.7 fold expression relative to GAPDH vs 1.1±0.4 fold expression relative to GAPDH, p=0.14) and (2.8±0.9 fold expression relative to GAPDH vs 1.32±0.4 fold expression relative to GAPDH, p=0.18) respectively, **Figure 3-17A**. A trend towards increased gene expression was observed for SPP1 in AML-MSCs compared to MSCs from healthy donors (5.1±1.7 fold expression relative to GAPDH vs 1.2±0.5 fold expression relative to GAPDH, p=0.08). While expression level of RGMB in AML-MSCs suggested a possible increase compared to MSCs from healthy donors (7.2±5.2 fold expression relative to GAPDH vs 2.4±1.1 fold expression relative to GAPDH, p=0.40). Expression levels of SCF and VCAM1 however remained unchanged in samples of AML-MSCs compared to MSCs from healthy donors (1.6±0.9 fold expression relative to GAPDH vs 1.6±0.7 fold expression relative to GAPDH) and (3.5±2.2 fold expression

relative to GAPDH vs 4.0±2.6 fold expression relative to GAPDH) respectively, **Figure 3-17A**. Expression level of IL-7 in AML-MSCs also remained same compared to MSCs from healthy donors (0.1±0.07 fold expression relative to GAPDH vs 0.3±0.1 fold expression relative to GAPDH, p=0.2), **Figure 3-17A**.

Although there were no significant differences in the expression of genes studied, between AML-MSCs and nMSCs, a clustering based on the expression level was observed among AML samples. In particular, two patient samples (AML8 and AML9) were observed to have the highest expression level for ANGPT-1, SDF1-α, and SPP1 compared to the expression level from all other samples of AML and healthy donors. AML9 also had higher expression levels for RGMB and VCAM1. For some patient samples, a low or no expression was detected for the genes studied highlighting the wide variance of gene expression in MSCs from marrow of AML patients. Variations were also observed among MSCs from healthy donors especially for expression of VCAM1 and IL-7, **Figure 3-17A**.

Overall, four key hematopoietic maintenance genes namely ANGPT1, SDF1-α, SPP1 and RGMB demonstrated a possible trend in differential expression between mesenchymal cells derived from AML patients and healthy donors. Expression of SCF, VCAM1 and IL-7 remained unchanged for AML-MSCs and nMSCs, **Figure 3-17B**.

Hence, modest changes in the expression of selected genes implicated in the support of hematopoiesis in MSCs from AML patients compared to MSCs from healthy donors was demonstrated. Wide variances in expression levels were observed in mesenchymal cells derived from different AML patients. Two such AML samples had highest expression for three of the genes studied. Variations in expression levels were also observed among nMSCs for two of the genes studied.

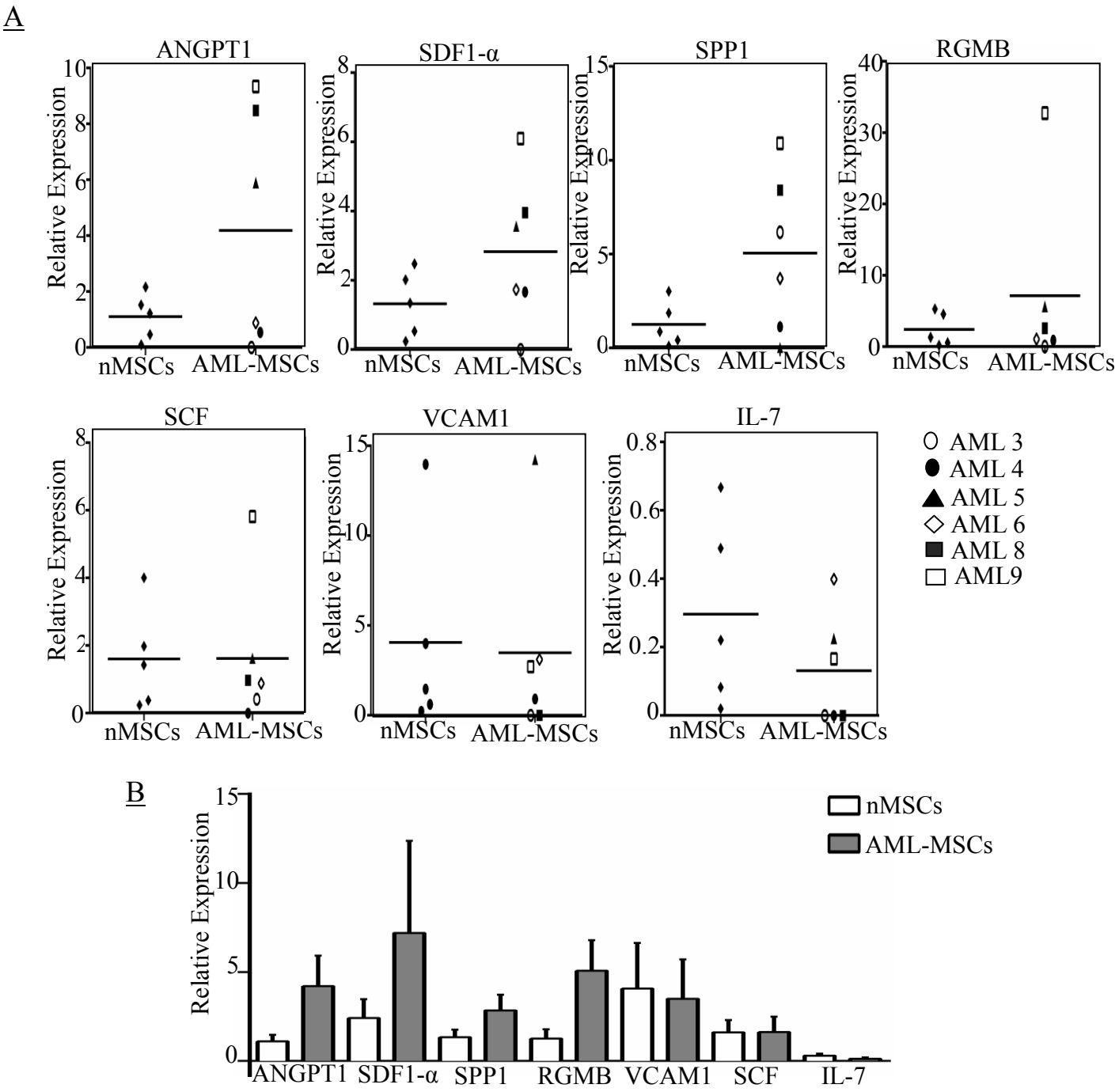


Figure 3-17: Differential expression of genes that regulate the HSC niche.

Expression of core hematopoietic maintenance genes by MSCs derived from healthy donors compared with leukemic patients. RT-qPCR results for (A) ANGPT1, SDF1-α, SPP1, RGMB, SCF, VCAM-1, IL-7 and (B) Overall differences in expression of seven hematopoietic genes. Relative expression was obtained based on the housekeeping gene Gapdh. Points for each sample represent average of at least two replicates. $p \geq 0.05$. All error bars indicate s.e.m. Sample from each AML patient is represented with a different symbol.

IV. DISCUSSION

4.1 OVERVIEW

In this study we characterized mesenchymal stromal cells from marrow of AML patients and healthy donors. MSCs from AML patients were found to have heterogeneous morphology with lower proliferation capacity compared to MSCs from healthy donors. Importantly, we observed reduced ability of AML-MSCs to support the expansion and differentiation of hematopoietic progenitors *in vitro* and modest but not significant change in expression of genes associated with maintenance of hematopoiesis in atleast some samples of AML-MSCs. These findings suggest the presence of important functional alterations within MSCs from leukemia patients which may provide basis for additional studies to help better understand the underlying mechanisms regarding leukemogenesis and its treatment.

The bone marrow microenvironment is a complex network of stem and progenitor cells influenced by a multitude of factors, and is known to provide support for normal hematopoiesis. Studies in the past few decades have led to better understanding of the regulatory interactions that occur within the marrow niches and have provided some insight regarding the interplay of the niche with tumor cells that favors malignant hematopoiesis within the marrow [94]. Although the role of bone marrow microenvironment in mediating the survival and progression of tumor cells is now well accepted, the role played by mesenchymal stromal cells in contributing towards the disease has remained less clear [95, 77].

MSCs are an essential component of bone marrow microenvironment and are precursors to osteoblasts, adipocytes and other mesenchymal tissues present within the niche. The cells have been demonstrated to promote the growth and survival of cancer cells from different tissues by

providing drug resistance and protecting them from growth inhibitory signals [89]. Other studies have also reported the presence of genomic clonal alterations in MSCs from pediatric ALL leukemia patients suggesting the potential involvement of MSCs in leukemia [84]. But whether MSCs play a similar role in AML has not been previously studied.

4.2 CHARACTERISATION OF MESENCHYMAL STROMAL CELLS

We adopted the criteria of ISCT to perform standard and uniform characterization of MSCs from bone marrow of human samples [76]. MSCs were adherent to plastic for all the samples including from AML patients and healthy donors. The cells were generated from all but two AML samples and AML-MSCs took significantly longer to expand from marrow mononuclear cells in comparison to MSCs from healthy donors. Sufficient cells were obtained from five AML samples and five healthy donors for more extensive biologic characterization.

AML-MSCs exhibited greater heterogeneity in cell morphology compared to healthy donor derived MSCs and had decreased viability after each harvest of cells. Immunophenotpying by flow cytometry confirmed a typical MSC phenotype in three samples of AML-MSCs, however two other samples (AML 6, AML 7) demonstrated reduced marker expression for CD105 and CD90 antigen respectively. Of note, higher levels of soluble CD105 (used as a marker for tumor prognosis) was observed in serum samples from patients with breast cancer, colorectal cancer and other tumors and correlated with metastasis while patients that underwent chemotherapy had reduced serum CD105 levels [96]. In this context it is interesting that the AML-MSC sample showing lower CD105 expression was derived from a patient who had received chemotherapy, which may explain the observation of reduced CD105 expression. Furthermore, we also expanded MSCs (AML 4) from the same patient before treatment and the

MSC cells were positive for CD105. The pre and post treated AML samples also had similar morphology to that of nMSCs and differentiated into all the three lineages of MSCs, thereby supporting the notion that the treatment might be the reason for reduced levels of surface CD105 observed in the sample. For the AML-MSC sample with lower levels of expression for CD90 antigen (AML 7), further experiments could not be performed to characterize the mesenchymal stromal cells due to poor growth of cells.

For mesenchymal differentiation, all five samples of AML-MSCs and nMSCs tested were able to differentiate into the three mesenchymal lineages. In the case of adipocytic differentiation, lipid vacuoles from samples of AML-MSCs appeared larger compared to adipocytes formed from nMSCs. We cannot exclude age as a contributing factor as all the AML samples were obtained from more elderly patients compared to healthy donors.

4.3 HEMATOPOIETIC SUPPORTIVE FUNCTION OF MSCs

MSCs are known to regulate hematopoiesis by secreting chemokines and soluble growth factors and offer cell-cell contact to support and preserve HSC cell numbers within the marrow. The addition of MSCs as feeder layers has been shown to augment the maintenance and expansion of HSCs including cord blood-derived $CD34^+$ hematopoietic progenitors *in vitro* [72, 73]. Greater expansion of HSCs and increased number of CFC colonies can be observed when blood cells are co-cultured in direct contact with MSCs compared to non-contact co-culture systems [97].

Based on these reports, we developed a co-culture technique using MSCs from healthy donors and from patients with AML and co-cultured them in direct contact with $CD34^+$ progenitors from healthy cord blood samples in presence of cytokines. Our study demonstrated a

modest increase in early $CD34^+CD38^-$ hematopoietic progenitors following co-culture with nMSCs+cytokines compared to the starting population of $CD34^+$ cells alone while more marked expansion of committed $CD34^+CD38^+$ and differentiated $CD34^-CD38^+$ cells was observed. We also observed an overall increase in absolute number of total colonies from CFC assays especially more differentiated colonies such as CFU-G, CFU-M and CFU-GM, following co-culture of $CD34^+$ $CD45^+$ cells with nMSCs compared to the absolute number of freshly isolated $CD34^+$ cells. The absolute number of more primitive progenitor colonies (BFU-E and CFU-GEMM) had a modest increase or remained same. These data suggest a degree of differentiation at the expense of maintaining more primitive progenitors. Our findings are comparable with previous reports that demonstrate a similar influence of marrow derived MSCs on the proliferation and differentiation of primitive progenitor HSCs, *in vitro* [70, 98].

In contrast, the mean CFC colonies formed by $CD34^+$ progenitor reduced significantly compared to nMSCs+cytokines. In particular, the absolute number of colonies for each sub-type remained similar compared to absolute number of colonies from freshly isolated $CD34^+$ cells. However when compared to nMSCs+cytokines, the absolute number of differentiated colonies of CFU-G, CFU-M and CFU-GM formed was significantly reduced. This reduced effect of AML-MSCs on proliferation and differentiation of progenitors was consistent with data obtained from flow analysis wherein hematopoietic cells co-cultured with AML-MSCs exhibited less expansion of differentiated $CD34^-CD38^+$ cells compared to nMSCs. While expansion in populations of committed population of $CD34^+CD38^+$ cells and more primitive $CD34^+CD38^-$ cells remained similar. Taken together it is plausible that AML-MSCs compared to their normal counterparts are less able to support the differentiation of more committed hematopoietic precursors while maintaining earlier progenitors in a quiescent state.

The samples used to assess the hematopoietic supportive function were obtained from patients with active disease including a sample from untreated patient at the time of study which might explain the overall observation of reduced formation of committed CFCs and preservation of earlier progenitors. Hence it is intriguing to contemplate whether chemotherapy could normalize the function of MSCs. If the microenvironment within the bone marrow is still defective after having received treatment (chemotherapy) and continues to support the survival of quiescent tumor cells, AML will inevitably spread and explain the high frequency of relapses in AML. More numbers of samples from AML patients, pre and post treatment, is needed to confirm these preliminary observations.

There are several limitations regarding our approach that are worth addressing. We made use of surface markers (CD34 and CD38) to phenotype the hematopoietic progenitor cells. Although these markers provide only partial characterization of true progenitor cells, they have allowed us to draw preliminary insight onto the influence of mesenchymal stromal cells on hematopoietic progenitor stem cells in culture. Additional markers for *in vitro* studies could include CD133 in combination with CD34 and CD38 to be more informative regarding hematopoietic stem cells, as CD133 is expressed by progenitor HSCs with high repopulating capacity in transplant [99].

Importantly, functional assays (CFC) were performed to define the effect of MSCs on the hematopoietic progenitor stem cells that helped corroborate the results obtained from the flow data. We recognize the limitations of the co-culture *in vitro* assay of hematopoietic progenitor cells and acknowledge the need for more definitive tests in future studies to assess the ability of MSCs to support HSCs. Transplantation experiments of HSCs, co-cultured with AML and healthy donor derived MSCs, into model of NOD/SCID mice and assessing the multilineage

engraftment and serial transplantation capacity of hematopoietic cells would provide more conclusive evidence regarding whether MSCs are supporting the self renewal or differentiation potential of HSCs or are maintaining the HSCs in a quiescent state.

4.4 GENE EXPRESSION PATTERN OF MSCs

MSCs are known to secrete a range of soluble growth factors and chemokines some of which play a role in regulating the activity of blood cells and the support of hematopoiesis. Human marrow MSCs have been shown to secrete cytokines such as SDF1a, IL-7, SCF, VCAM1, SPP1 and ANGPT1 among other factors [99, 100]. These active bio molecules have been demonstrated to be key factors involved in maintenance of the HSC niche [14, 35].

Ding et al showed using a mouse model that CreER mediated deletion of SCF resulted in loss of HSC cells within the marrow [104]. Similarly, MSCs have been shown to confer drug resistance to tumor cells through SDF1-α signaling [105]. Also MSCs from patients with different disorders have been shown to differentially express SDF1-α, Angiopoietin and SCF [80, 106]. Apart from these key genes, we also investigated the expression pattern of RGMB in MSCs as it was previously found to be up regulated in MSCs from MDS patients and in elderly healthy donors [92, 93].

To the best of our knowledge this is the first time that the expression pattern of some of the growth factors and cytokines critical to the support of HSC survival, especially the expression of RGMB is being studied in MSCs from AML patients. And our data demonstrated differences in expression of five key hematopoiesis maintaining genes namely SDF1-α, SPP1, ANGPT1, RGMB and IL-7 in MSCs from AML patients. Although these differences were not statistically significant due in part to the wide variation observed among different samples, a

trend towards increased expression was however observed. Interestingly, out of the samples assessed for hematopoietic supportive function and RT-qPCR, AML sample 9 obtained from untreated patient, had highest level of expression for ANGPT1, SDF1-α, SPP1, RGMB and SCF. These biofactors except RGMB modulate HSC quiescence within the niche [102, 103]. The sample AML-9 also displayed a normal MSC phenotype and differentiation ability but generated fewer total CFC colonies and exhibited reduced expansion of most committed $CD34^{-}CD38^{+}$ cells *in vitro*. This observation of increased expression for quiescence maintaining genes could be a possible explanation for reduced support of the AML-MSC towards expansion of differentiated hematopoietic population. Correlating further, AML8, patient sample with refractory AML that had received prior chemotherapy treatment, had similar reduced capacity to hematopoiesis and increased expression for hematopoietic quiescent regulating genes (ANGPT1, SDF1-α and SPP1). Thus it could be possible that MSCs obtained from the marrow of patients with active disease are functionally altered and may secrete essential growth factors in greater quantities to maintain the leukemic cells in quiescent state and further protect them from treatment.

However a more detailed approach is required to understand the underlying molecular mechanisms of the differential regulation of genes observed in relation to leukemogenesis and their relation to the treatment received by the patients. Larger cohorts and more systematic microarray analysis of MSCs would augment the ability to discern distinct gene expression profile and identify genes and potential cellular pathways that are differentially regulated. This in turn would help us better understand potential mechanisms by which MSCs support the survival of leukemic cells within the marrow.

One significant challenge of this study has been the acquisition of sufficient numbers of primary human samples from patients with AML. Throughout our study, human samples were

used to perform the experiment. Procurement of primary human samples introduces many challenges including written consent from patients and volunteers in accordance with the institutional protocol. Obtaining age matched controls for our test samples remains challenging. We are also aware of the biological variations that occur in our results due in part to the variation among human samples but these data provide valuable information about disease status and maintain an important degree of clinical relevance. The greater ease of applicability to clinical purposes or diseases makes the study with human samples extremely important towards our understanding of the relevance of niche within the tumor marrow.

V. CONCLUSION

The main objective of this work was to study the changes that occur in MSCs from human bone marrow in patients with AML by comparing their function and gene expression profile with MSCs from healthy volunteers. In support of the hypothesis of this study, we observed MSCs derived from the bone marrow of leukemic patients to have an abnormal biologic profile including heterogeneous morphology, limited proliferation ability, differences in phenotype and impaired ability to support the expansion and differentiation of hematopoietic cells *in vitro* compared to the biologic functions of MSCs from healthy donors. Some of the key hematopoietic regulating genes were also found to have altered but not significant expression in samples of AML-MSCs.

The difference observed in the functions of AML-MSCs in preserving the primitive population of hematopoietic cells in culture ($CD34^{+}CD38^{-}$) and allowing only fewer cells to differentiate into most committed progenitors ($CD34^{-}CD38^{+}$), may suggest the sheltering property of MSCs towards tumor cells, reported previously by different groups. MSCs, therefore, may be responsible in providing a quiescent niche for tumor cells in the marrow. Our data further suggests that MSCs from some of the patient samples either newly diagnosed or refractory AML, secrete higher amounts of cytokines and growth factors associated with maintenance of hematopoietic stem cells or quiescent leukemic cells, thereby enabling their retention within the marrow and protecting themselves from the effects of adverse conditions such as chemotherapy and radiation.

These observations from the experimental data allow us to propose conceptual model regarding the role of MSCs in the microenvironment and leukemogenesis, **Figure 5-1**. One possible explanation for the observed differences in MSCs could be that tumor cells pattern the

microenvironment through complex cellular and molecular processes and the modified or functionally altered MSCs in turn provide leukemia cells with growth factors, cytokines and other molecules critical for the survival, growth and progression of the disease (**Figure 5-1**).

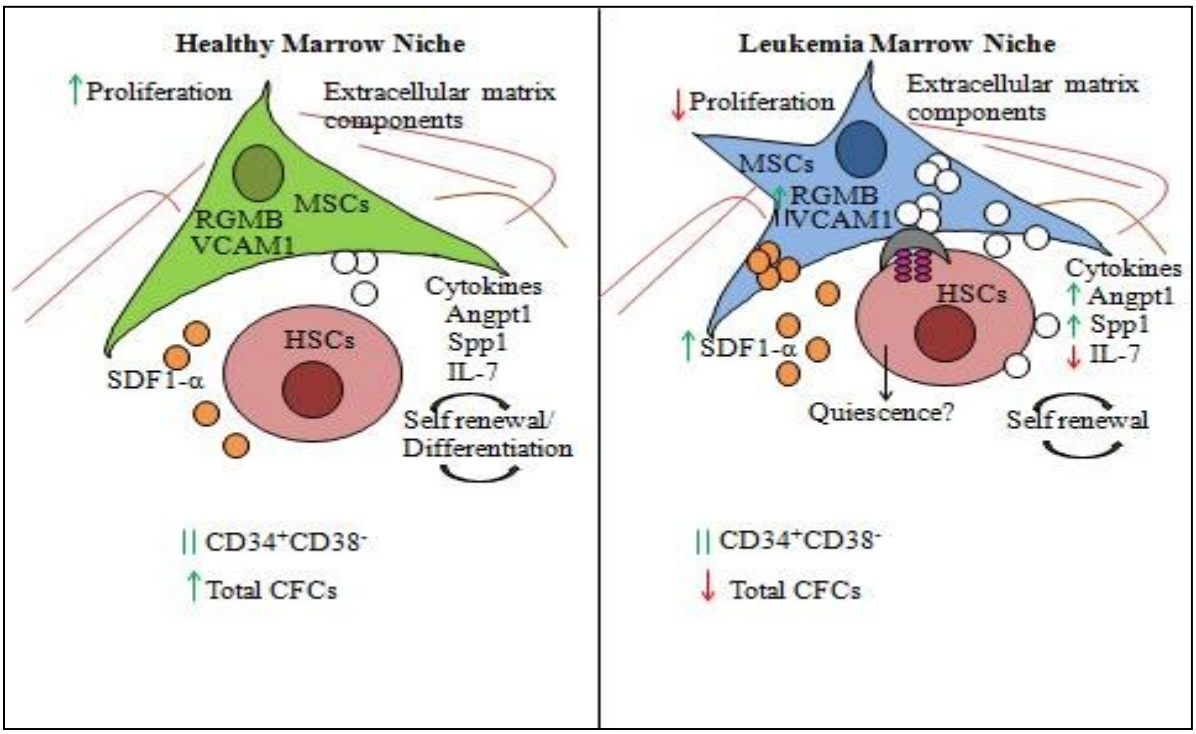


Figure 5-1: Schematic model of regulation of mesenchymal stromal cells and hematopoietic cells within the healthy and leukemia marrow niche.

Hypothetical representation of the differences in interaction occurring between healthy and leukemia marrow niche. Mesenchymal stromal cells (MSCs) proliferate and secrete normal levels of cytokines and growth factors essential for HSC growth and survival in the healthy marrow. The differentiation of HSCs to different lineages happens at the required normal levels. While MSCs in leukemia niche exhibit heterogeneous morphology and reduced proliferation capacity. Increased levels of growth factors are secreted from MSCs to create a supporting niche for HSCs. HSCs respond to the favorable environment by remaining quiescent and with reduced differentiation to most committed lineages. The above model was based on the inferences made from the experiments. Arrows indicate: ↑increase in expression levels, ↓decrease and || similar expression levels.

The present work in the thesis is a preliminary study which allows us to design more definitive functional approaches to further study the contribution of MSCs towards a permissive

bone marrow environment that harbors leukemic cells and lessens the impact of treatments aimed at eradicating AML. Studies in large cohort are needed to validate our findings.

A systematic microarray analysis to attain a distinct gene expression profile of MSCs would be valuable to detail the genes involved in regulating the functions of MSCs in AML. Experiments to test whether treatments provided to AML patients help revert the normal characteristics of MSCs in comparison to the MSCs derived from untreated patients should be envisioned. MSCs from normal donors co-cultured with leukemic cells could also be studied to address the mechanisms that explain how the niche is altered. In addition epigenetic factors such as DNA methylation status of key genes in MSCs could affect the normal function of MSCs and is worthy of considering. Thus, the studies done in the current work and future research would help better understand the role marrow environment plays in harboring leukemic cells and the extent to which current treatments can normalize the marrow microenvironment to ensure sustained remission and eradication of leukemia.

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PRIYA CHANDRAN

EDUCATION:

2011- Present	Master's in Biochemistry (candidate), with specialization in Regenerative Medicine, University of Ottawa, Canada.
2006- 2010	Bachelors in Technology with specialization in Biotechnology and Biochemical Engineering, University of Kerala, India.

EMPLOYMENT:

2011 – 2012	Teaching Assistant (TA), University of Ottawa. (Course: Molecular Biology, 3rd year undergraduate)

PUBLICATIONS AND PRESENTATIONS:

1) Chandran P. Bone marrow microenvironment in acute myeloid leukemia. Master's thesis, University of Ottawa (to be submitted).

2) Chandran P, Rosu-Myles M, Allan DS. Bone marrow microenvironment in acute myeloid leukemia.
   - Poster presentation at the Ottawa Hospital Research Institute, Annual Research Day, 15 November, 2012.
   - Poster presentation at Hematology Trainee Research Day, University of Ottawa, 1st June, 2012.
   - Oral presentation at Department of Biochemistry, Microbiology and Immunology Research Day, University of Ottawa, 22 February, 2012.
   - Poster presentation at Department of Biochemistry, Microbiology and Immunology, University of Ottawa, 19 May, 2011.

Manuscripts in preparation:

3) Chandran P, Li Y, Palii C, Westwood C, Rosu-Myles M, Allan DS. Functional changes in mesenchymal stromal cells from patients with acute myeloid leukemia: role of the marrow microenvironment in leukemogenesis. (Manuscript in preparation).

RESEARCH PROJECTS:
Undergraduate:

2009 – 2010	**"**An Innovative approach to removal of bio-films on polymeric substance using enzyme and detergent pre-treatment**"** at Biogenix, Trivandrum, Kerala, India. Supervisor: Dr. Rajesh Ramachandran

Graduate:

2011 – 2013 **"**Bone marrow microenvironment in Acute myeloid leukemia**"** at University of Ottawa, Ottawa, Ontario.
Supervisor: Dr. David Allan

2012 – 2013 "Human platelet lysate compared to other growth media supplements for mesenchymal stromal cells expansion: a preclinical systematic review", at Ottawa Hospital Research Institute, Ottawa, ON.
Co-supervisors: Drs. Lauralyn McIntyre, Manoj Lalu and David Allan

SKILLS:

Laboratory

- Extensive experience in cell culture technique, histology staining and sectioning of tissues, use of fluorescent microscopy and working with human and blood samples.
- Flow cytometry, RNA extraction, RT-qPCR and primer design.
- Familiar with HPLC, Ion exchange chromatography, Spectrophotometer, DNA extraction and bacterial culture.

Communication:

- Languages: Fluent in English, Hindi, Malayalam and Bengali.
- Written skills: Composed many formal lab reports and wrote thesis for undergraduate as well as for graduate studies.
- Oral skills: Excellent interpersonal skills, excellent organizer, responsible, punctual, hard-working and a student leader.
- Computer: Very well acquainted with Microsoft office, adobe photoshop, flowjo, graphpad Prism software 6.0 and familiar with windows and apple operating system.
- Analysis: Basics in statistics and experience of extrapolating and analyzing experimental data.

Collaboration:

- Dr. Michael Rosu-Myles, Health Canada: Co-culture of $CD34^+$ cells with Mesenchymal stem cells.
- Drs. Lauralyn McIntyre and Manoj Lalu, Ottawa Hospital Research Institute: Human platelet lysate compared to other growth media supplements for mesenchymal stromal cells expansion: a systematic review.

OTHER CONTRIBUTIONS:

2010	Organized an English vocabulary contest "Vocomania", India
2009	Organized seminar conducted by Dr VV GIRI, (Head of Forensic Dept. Amrita Institute of medical Sciences), India
2009	Headed the university team members in organizing Inter-university competition, India
2008	Master of Ceremony for a national level seminar in India, Medico Venturus
2007	Volunteered the programme "Career Trends" for grade 12 students, India

INTERESTS, VOLUNTEER WORK AND COMMUNITY PARTICIPATION:

2008- 2013	Extensive involvement in dance (classical, contemporary), drama, and Indian cultural activities
2007- 2013	Extensive involvement for fundraising activities in Ottawa and India