CHAPTER II

REVIEW OF RELATED LITRATURE

Blood cell formation occurs as a result of a series of maturation cell divisions. The hematopoietic system, which gives rise to all circulating blood cells, can be envisioned as a series of *overlapping functional compartments. The stem cell* compartment is made up of rare primitive cells that are multipotential and have a high self-renewal capacity. Progenitor cell compartments are comprised mainly of cells with the capacity to differentiate along one lineage, with lower frequencies of bipotential and multipotential primitive cells. Progenitor cells are generally defined functionally, that is, by the capacity of the cell to form colonies in *in vitro* assays; *they demonstrate little* self-renewal capacity. Most cells in the BM compose of the precursor compartment. Little self-renewal capacity exists in this compartment, but because of the large number of precursor cells and high mitotic activity of these less primitive cells, considerable amplification in absolute cell numbers occurs within these compartments.

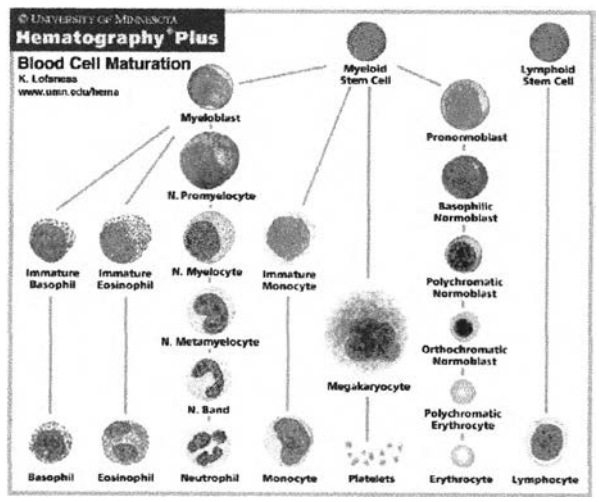

Figure1 Hematopoietic cells hierarchy

The most commonly used marker to identify human HSCs is the CD34 antigen. This antigen is expressed on primitive cells and is down *regulate* upon

differentiation into more mature cells. $CD34^+$ cell comprise about 1-3% of the normal adult bone marrow cells and approximately 1% of cord blood mononuclear cells (MNCs)[15]. The $CD34^+$ population is quite heterogeneous consisting of progenitor cells with different cloning, survival, differentiation ability and long-term cloning capacity

It is possible to distinguish among HSCs, progenitors, and more mature cells within the $CD34^+$ population by their different patterns of expression of other markers. For example, the CD38 antigen is expressed at intermediate to high level on >90% of $CD34^+$ cells from most sources of hematopoietic cells, including most CFCs and approximately 60% of the LTC-ICs[16].

The $CD34^+CD38^-$ immunophenotype defines a rare, quiescent subpopulation in both CB and BM It was found that $CD34^+$ cells that lacked CD38 were highly enriched for the most primitive hematopoietic progenitors[17, 18]. In cord blood, $CD34^+CD38^-$ cells comprise 0.05% of the MNCs and enriched for long-term culture-initiating cells (LTC-IC; cells able to generate colony-forming unit-cells after 35 to 60 days of coculture with bone marrow stroma)[19]. $CD34^+CD38^-$ cells have the ability for sustained multilineage hematopoietic reconstitution after transplantation into immunodeficient mice. Although the $CD34^+CD38^-$ immunophenotype defines a highly primitive population in both BM and CB, important function differences exist between the two sources. $CD34^+CD38^-$ CB cells have a higher cloning efficiency, proliferation more rapidly in response to cytokine stimulation, and generate more progenitor than do their counterpart in BM[19].

Other markers that are absent or only weakly expressed on HSCs and primitive progenitor cells but highly expressed on certain types of lineage-committed progenitor cells include CD33, CD71, HLA-DR and CD45[16].

After the successful hematopoietic reconstitution of baboons with selected $CD34^+$ baboons BM cell by Berenson et al, CD34 expression became the hallmark of murine and human HSCs[20]. However, there are several results indicated the existence of $CD34^-$ cell population. These $CD34^-$ HSCs have been detected by transplantation and multilineage engraftment of human cells in both NOD/SCID mice

and pre-immune fetal sheep. First, Osawa et al. provided the first experiment evidence of the existance of $CD34^-$ HSCs that has a marked capacity for long-term repopulation and may be a more primitive precursor of $CD34^+$ cells[21]. Sato et al. demonstrated that CD34 expression on murine HSCs is a reversible process that reflects their activation state[22]. By using a murine BM stromal cell line, Nakamura et al. were able to expand cell fractions that did not express CD34 and lineage marker ($CD34^-Lin^-$ cells) and to convert them to $CD34^+$ cells; these results demonstrate that $CD34^-$ cells can be precursor of $CD34^+$ cells[23]. Study performed by Goodell et al also raised serious questions about CD34 expression by HSCs. They reported that murine BM stem cells are highly enriched in a cell population defined by Hoechst 33342 dye as side population (SP)[24, 25]. Adult BM of many species also contain a rare population of $Ho^{-/lo}$ cells that have been designated as SP cells because they form a characteristic cluster of events off to the lower left side in dual wavelength FACS dot-plot profiles of Ho-stained cells.

Recently, a novel antigen expressed on human hematopoietic stem and progenitor cells was reported[26]. The surface antigen AC133 (CD133) was discovered in the same year of prominin-1 which is one of two members of a pentaspan transmembrane glycoprotein family by generating a monoclonal antibody to $CD34^+$ HSCs isolated from FL, BM and CB[27]. After cDNA encoding the protein was cloned, CD133 was recognized to be the human homologue of mouse Prominin-1[26]. The CD133 epitope of human Prominin-1 was discovered as a marker that is specific for HSCs.

CD34 and CD133 antigens are coexpress on primitive hematopoietic progenitor and some leukemic cells. CD133 antigen is expressed only in the $CD34^{bright}$ subset of human hematopoietic progenitors[27]. The CD133 selected cells engrafted successfully in a fetal sheep transplantation model in both primary and secondary recipients, indicating the presence of long-term repopulating cells[27]. Furthermore, SCID-repopulating cells are found exclusively in the $CD133^+CD34^+$ fraction of human CB cells[28]. These findings suggest that CD133 antigen could be a specific marker for

primitive progenitor cells. Cell-surface and metabolic markers of human hematopoietic stem and progenitor cells are shown in Table1.

Table1 Immunophenotyping of human hematopoietic stem / progenitor cells

Marker	Expression / remark	Refferences
Positive marker		
CD34	Positive	[29]
Thy-1	Positive	[30]
CD133	Positive	[27]
Negative / low marker		
CD38	Negative / low	[31]
HLA-DR	Negative to low	[32]
Mature lineage marker, Lin^-		
CD2	T-cell lineage	[33]
CD3	T-cell lineage	[33]
CD19	B-cell lineage	[33]
CD16	NK-cell lineage	[33]
CD14	Myeloid lineage	[33]
CD15	Myeloid lineage	[33]
Glycophorin A	Erythroid lineage	[33]
Metabolic marker		
Rhodamine 123 (Mitochondria-binding dye)	Low	[34]
Hoechst 33342 (DNA-binding dye)	Low	[35]
Pyronin Y (RNA-binding dye)	Low	[35]
Propidium iodide (Dead-cell exclusion)	Negative to low	

The low frequency of this cell in the nucleated cell population, the lack of reagents to distinguish the stem cell from other immature cell types have made their purification and characterization a highly challenging goal.

HSCs have been reported to be purified by two mathod : positive and negative selection. Selective recovery of cell with one or more antibodies to antigens expressed on the surface of the desired cells is termed positive selection. Removal of unwanted cells using antibodies to cell surface antigens not expressed on the desired cells is termed negative selection. High purity is the main advantage of positive selection method. The disadvantage of positive selection historically has been that the desired cells are labeled with antibody must be removed from the separation matrix (e.g. columns, flasks or large micron-size magneric particle). However, recent advances in magnetic cell separation have overcome the requirement for removing the desired cells from the separation matrix, by using very small submicron magnetic particles in a column-free system. The advantage of such small magnetic particles are improved delivery to cells in suspension and that the particle-antibody complexs do not interfere with susbequent flow cytometric analysis.

Human cells with progenitor or HSCs function are identified retrospectively, by their unique behavior in different assays. Colony-forming cell (CFC) assays are used to quatify multi-potential progenitor and single lineage-restricted progenitors of the erythroid, granulocytic, monocyte-macrophage and megakaryocytic pathways.The most commonly used procedure involves the plating of a single cell suspension in semi-solid nutrient medium, supplemented with the appropriate combinations of cytokines, that supports the proliferation and differentiation of individual progenitor cells into discrete colonies containing recognizable progeny. The CFCs are classified and enumerated based on morphologic recognition of mature cells within the colony in situ by light microscopy. The number of colonies obtained should be linearly proportional to CFCs content of the input cell suspension provided that a sufficiently low number of cells are plated. More immature progenitors generate larger colonies and require a longer period of time in culture to allow maturation of the cells within the colony.

In contrast, human HSCs function can be assayed only *in vivo*, most commonly using a xenotransplantation assay. The nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice is widely accepted as a model to assess the *in vitro* optimization of human progenitor expansion before proceeding to human trials[36].This assay identifies the rare cells that support long-term multilineage hematopoiesis in immune-deficient NOD/SCID mice (termed SCID-repopulating cells, SRC).

A number of laboratories have attempted to develop ex vivo culture condition that mediate extensive numerical expansion of HSCs with maintenance of HSC potential for proliferation and multilineage differentiation. Many approaches have been reported during the last decade, and they can be divided into 2 categories.

The first category is treatment of HSCs with various combinations of cytokines. Proliferation, self-renewal, and differentiation of normal as well as malignant hematopoietic cells is regulated by a number of soluble and membrane-bound factors such as the colony-stimulating factors (CSFs), multiple interleukins (ILs), erythropoietin (Epo), c-kit ligand (KL), and flt3 ligand (FL), The most primitive progenitors have been shown to proliferate in vitro only when stimulated by multiple growth factors[37, 38]. In contrast, committed progenitor cells can be stimulated to proliferate by a single cytokine, although the synergistic effect of a number of growth factors can cause enhanced growth[37, 38]. The optimal choice of cytokines for the ex vivo expansion of human hematopoietic stem/progenitor cells has not yet been determined. During the last two decades, investigators in a number of laboratories used murine transplantation models to characterize the cytokines regulating the human HSCs. Various combinations of cytokines were examined. The representative combinations of cytokines attempted are listed in Table2.

Table2 Ex vivo generation of cord blood primitive hematopoietic stem / progenitor cells from human $CD34^+$ cells

Cytokines	Feeder layers	Test	Fold increase	Reference
IL-1, IL-3, SCF	-	LTC-IC	15-20	[39]
FL, IL-3, IL-6, SCF	-	LTC-IC	Increase	[40]
FL, TPO	-	LTC-IC	>200,000	[41]
FL, TPO, SCF, IL-6	-	LTC-IC	280	[42]
SCF, FL, TPO, G-CSF	-	LTC-IC	47	[43]
SCF, FL, TPO, IL-6/sIL-6R	-	CFU-Mix	Increase	[44]
TPO, FL	Murine stromal cell line MS-5	CAFC 8 week	13	[45]
FL, SCF, TPO,IL-3	-	LTC-IC	16	[46]
TPO, FL, SCF	Human marrow stromal cells	CAFC 4 week	60	[47]
TPO, IL-6, FL,PDGF	-	CFU-Mix LTC-IC	11 7	[48]
SCF, TPO, FL	Human hTERT-transfected human stromal cell	CFU-Mix	79	[49]
SCF, bFGF, LIF, FL	Human stromal cells	CFU-Mix	137	[50]

Two cytokines (FL and TPO) have been shown to significantly improve the generation of stem/progenitor cells ex vivo. FL strongly potentiates the clonogenic capacity of immature progenitor/stem cells when combined with other growth

factors[40, 51-55]. TPO, in addition to its effects on the megakaryocytic cell lineage, enhances proliferation of stem/progenitor cells[41, 56, 57].

FL belongs to a small family of hematopoietic cytokine, including stem cell factor (SCF) and megakaryocytic-colony stimulating factor (M-CSF), that are specific for class III tyrosine kinase receptors[58]. The receptor for FL, isolated independently by two groups and termed fms-like tyrosine kinase3 (flt3) or fetal liver kinase-2 (flk-2), is structurally related to M-CSF and SCF receptors c-fms and c-kit, respectively[59, 60]. The flt3 and c-kit are expressed predominantly on primitive hematopoietic progenitors, an indication that signaling through these receptors is important in the early stages of hematopoiesis. Flt3 expression is restricted to $CD34^{+}$ cells lacking the lineage-specific markers. Furthermore, within the stem cell pool, expression of flt3 is heterogeneous, found on cells with long- and short-term marrow – reconstituting activity. FL is a cell surface transmembrane protein type I that can be proteolytically processed and released as a soluble protein[37]. In contrast to the restricted distribution of flt3 receptors, FL mRNA is ubiquitously expressed in hematopoietic and non-hematopoietic tissue[37]. Despite the widespread expression of FL mRNA, the FL protein has only been found in stromal fibroblasts present in the BM microenvironment and T lymphocytes[61].

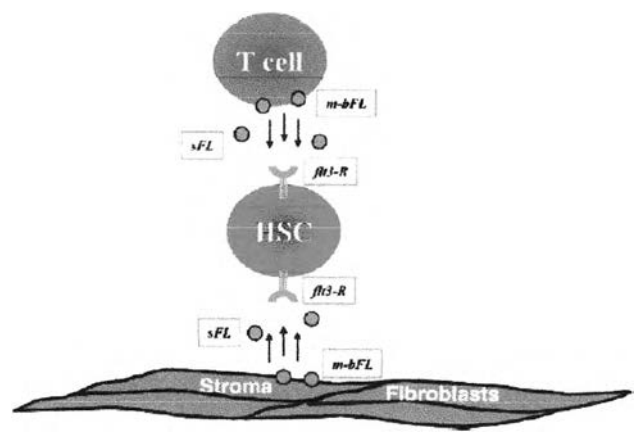

Fig2. Flt-3 ligand (FL)-mediated interactions in the bone marrow microenvironment. HSC, hematopoietic stem cell; mbFL, membrane-bound FL; sFL, soluble FL; flt3-R, flt3 receptor.

Numerous in vitro studies have demonstrated that FL is a recently identified cytokine having a central role in the proliferation, survival and differentiation of early murine and human hematopoietic precursor / stem cells. FL is usually not efficient when used as a single cytokine, but in synergy with other hematopoietic growth factors and interleukins it exerts pleiotropic effects on precursors of myeloid and lymphoid lineages. Both native and recombinant FL induced tyrosine autophosphorylation of the FLT3 receptor. Antisense oligonucleotides directed against FLK2 / FLT3 mRNA were shown to inhibit the growth of human progenitors in long-term BM cultures.

Results of in vivo studies in animals and humans support the notion that FL plays important role in hematopoiesis. Administration of FL into mice results in expansion of HSCs and significant stimulation of hematopoiesis leading to BM hyperplasia, splenomegaly, and enlargement of lymph nodes and liver. These effects are transient, and hematopoietic values return to normal upon cessation of FL treatment[62]. Administration of FL to mice and rabbits subjected to lethal doses of irradiation protected HSCs and allowed rapid hematopoietic recovery[63].

Thrombopoietin (TPO), the recently cloned ligand for c-mpl, is a hematopoietic cytokine regulating megakaryopoiesis and platelet production[64]. The receptor for thrombopoietin, c-mpl , is expressed on HSCs, hematopoietic progenitor cells, megakaryocytes, and platelets. In vitro, TPO increases megakaryocyte proliferation and differentiation[64]. Animals in which TPO or Mpl is deleted are viable, but have significantly decreased platelets as well as megakaryocyte levels[65]. Surprisingly, other committed progenitors that do not express Mpl are also decreased as a result of significant loss of HSCs[64]. Consistant with the notion that TPO affects HSCs is the finding that HSCs are almost exclusively MPL^{+}, and that TPO induces proliferation of primitive progenitors in vitro and has therefore become an important factor in ex vivo HSC expansion schemes[66].

However, it is difficult to maintain HSC activity in long-term culture even if the total number of hematopoietic cells could be expanded. Hence, these methods could be improved for use in clinical settings. Historically, most emphasis has been

placed on characterizing the intrinsic cellular and molecular properties of stem cells. In recent years, attention has shifted to the niches or microenvironments where stem cells reside in situ. The second category involves using stromal cells.

The post-natal BM has traditionally been as an organ composed of two main system rooted in distinct lineages-the hematopoietic tissue proper and the associated supporting stroma which together with the extracellular matrix provide growth factors and cell-to-cell and cell-to-matrix interactions essential for the maintenance growth and differentiate of HSCs[67, 68]. This supportive scaffolding termed the BM microenvironment. Bone marrow stroma is a heterogeneous connective tissue which play an important role in regulating hematopoiesis. The cellular components of the marrow microenvironment include reticular endothelial cells, macrophages, adipocytes, fibroblasts, and osteogenic precursor cells which were derive from multipotent stem cell[67, 68]. Figure4 shown mesenchymal stem cell differentiation with multilineage cell types.

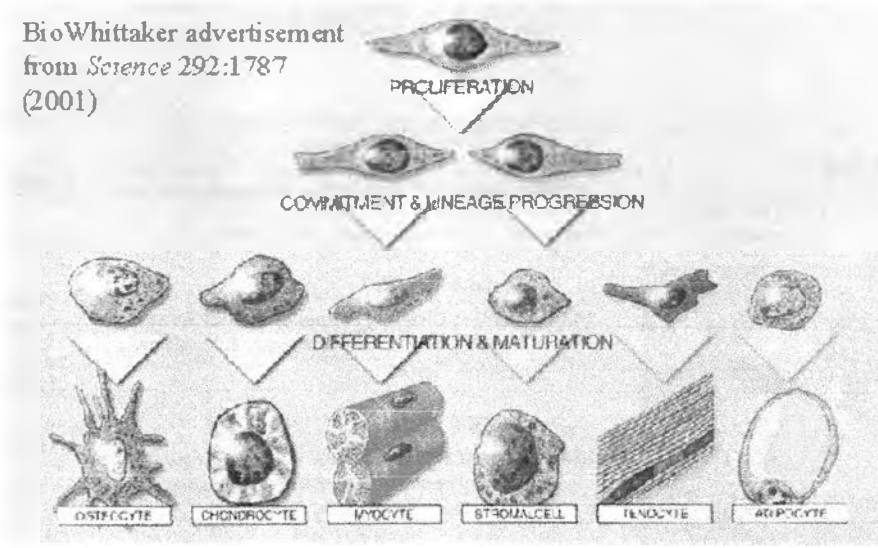

Figure3 Mesenchymal stem cell differentiation

When hematopoietic BM is examined in vivo, hematopoietic cells of different stages of differentiation and lineage commitment can be found in distinct areas throughout the BM space. For instance, cells with an immature morphology can

be found lining the subendosteal region in close proximity with osteoblasts[69, 70]. More differentiated progenitors and precursors of the myeloid, erythroid, and megakaryocytic lineage are located throughout the marrow. The apparent association of lineage-specific progenitors and their more differentiated precursors in island suggests that lineage-specific differentiation may depend on specialized progenitor-stromal cell interactions. This has led to the concept of "stem cell/progenitor niches"[71]. Such niches would consist of specialized stromal cells that produce extracellular matrix components and hematopoietic supportive cytokines that are conductive for the commitment and/or differentiation of progenitor cells at a specific stage of differentiation.

Characterization of the role of the BM mocroenvironment in the hematopoietic process became possible with the development of stroma-dependent cultures, initially developed by Dexter and coworkers, also termed Long-Term Culture (LTC). A number of investigators have cloned murine stromal cell lines from either fetal liver or adult BM[33, 72-79]. Some of these cell lines support the ex vivo maintenance and expansion of multipotent HSC population while they support poorly more committed HPC[72, 73].

Since their original description, these BM multipotent stem cell were referred to by different names. The original term "colony forming unit-fibroblast (CFU-F)" or "marrow stromal fibroblast (MSF)"[80-82] has been replaced by diverse denominations like "marrow stromal cell (MSC)"[83], "mesenchymal stem cell (MSC)"[84], or "mesenchymal progenitor cell (MPC)"[85].

Mesenchymal stem cells (MSCs) are a population of pluripotent cells within the bone marrow microenvironment defined by their ability to differentiate into several mesenchymal tissue under defined in vitro or in vivo conditions. Human MSC can be isolated from bone marrow aspirates as cells which grow in a plastic adherent layer following culture in low-glucose medium supplemented with fetal bovine serum. MSC has revealed that they possess integrins, matrix receptors, and secrete cytokines essential for the support of hematopoiesis[86, 87]. Table2 show main characteristics of BM-derived MSCs[88].

Table3 Main characteristic of bone marrow-derived mesenchymal progenitors: Expression of specific antigens, cytokine receptors, adhesion molecules, and production of cytokines and matrix molecules

Marker type	Designation
Specific antigens	SH2, SH3, SH4 STRO-1 α-smooth muscle actin MAB1740
Cytokines and growth factors	Interleukins: 1α, 6, 7, 8, 11, 12, 14, and 15 LIF, SCF, FL GM-CSF, G-CSF, M-CSF
Cytokine and growth factor receptors	IL-1R, IL-3R, IL-4R, IL-6R, IL-7R, LIFR, SCFR, G-CSFR, IFNγR, TNFIR, TNFIIR, TGFβIR, TGFβIIR, bFGFR, PDGFR, EGFR
Adhesion molecules	Integrins: αvβ3, αvβ5 Integrin chains: α1, α2, α3, α4, α5, αv, β1, β3, β4 ICAM-1, ICAM-2, VCAM-1, LFA-3, L-selectin, endoglin, CD44
Extracellular matrix	Collagen type I, III, IV, V, and VI Fibronectin, laminin Hyaluronan, proteoglycans

In 1996, Haynesworth et al. study MSC further to establish the phenotype characteristic of cultured marrow-derided MPCs through identification of a cytokine expression profile[89]. They used immunoassays to identify and measure the levels of expression of cytokines that are known to be important in the regulation of cell division, differentiation, or expression of a variety of mesenchymal and hematopoietic phenotypes. They measured cytokine expression by MPCs under culutre conditions

that they have previously reported for optimal MPC mitotic expression without differentiation. In addition, they measured cytokine expression by MPCs in culture medium supplemented with IL-1α which is secreted into the marrow microenvironment during the inflammatory response, has been reported to enhance the BM stroma's capacity to support hematopoiesis and, thus, may play a role in controlling the differentiation and/or expression of BM-derided MPCs along a postulated stromal cell lineage[90-93]. The data from these analyses show that cultured human marrow-derived MPCs treated with IL-1α alter the profile of MPCs cytokine expression by increased the expression of G-CSF, M-CSF,LIF, IL-6 and IL-11 and induced the expression of GM-CSF, but had no effect on SCF expression and was not observed to decrease the production of any of the cytokine assayed.

Futhermore, study by Majumdar et al. in 2000, they used a RT-PCR to characterize cytokine and growth factor gene expression that occurs during stromal differentiation[94]. MSCs constitutively expressed mRNA for IL-6, IL-11, LIF, M-CSF and SCF. MSCs treated with IL-1α upregulated mRNA levels of IL-6, IL-11, and LIF, and began to express detectable levels of G-CSF, GM-CSF. MRNA levels of M-CSF and SCF did not change. These results suggesting that MSCs treated with IL-1α may provide an excellent ex vivo environment for hematopoiesis during progenitor cell expansion and may be important for in vivo cell therapy.