STUDY OF LYMPHOCYTE DEVELOPMENT FROM EMBRYONIC STEM CELLS *IN VITRO*

by

NOOSHIN TABATABAEI-ZAVAREH

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Abstract

Embryonic stem (ES) cells have been shown to differentiate to all hematopoietic lineages. However, lymphocyte development from murine ES cells has not been fully investigated. In this thesis, the developmental pathway of natural killer (NK) lymphocytes from ES cells was investigated, using a multi-step *in vitro* ES cell differentiation system. ES cells were induced to differentiate into embryoid bodies (EBs). $CD34^+$ EB cells were isolated and cultured on OP9 stroma with a cocktail of cytokines to generate cells; termed ES-derived hematopoietic progenitors (ES-HPs). EB cell subsets as well as ES-HPs were tested for NK, T, B, and myeloid lineage potentials using lineage specific differentiation cultures. ES-HPs derived from $CD34^+$ EB cells appeared to be heterogeneous and contained NK, T, B and myeloid potentials. At the EB level, lymphoid potential was found in $CD34^+CD45^-$ EB cell subset, while $CD34^+CD45^+$ EB cells had only myeloid but not lymphoid potential. $CD34^+CD45^-$ EB cells gave rise to $CD45^+Mac\text{-}1^-Ter119^-$ (Lin^-) ES-HPs, which were highly enriched for NK progenitors, but also had other lineage potentials. The NK progenitors among ES-HPs lacked CD122, a marker for NK lineage committed precursors, but they acquire CD122 as they differentiate along the NK lineage. To further enrich lymphoid progenitors among $CD45^-$ EB cells, EB cell populations were dissected according to several surface markers and tested for myeloid and lymphoid lineage potentials. Hematopoietic progenitors with lymphoid potential in EBs were found to be $CD45^-CD34^+c\text{-}kit^+CD41^+CD31^+Flk\text{-}1^+$. As they differentiate *in vitro* into more mature hematopoietic progenitors, they slowly acquire CD45 and lose Flk-1 and CD41 expression while retaining erythroid/myeloid and lymphoid potentials. This study suggests that hematopoietic progenitors in EBs are similar to immature embryonic hematopoietic stem cells (HSCs) and they differentiate into more mature type HSCs *in vitro*.

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AGM: aorta, gonads, mesonephros
BCR: B cell receptor
BFU-E: burst-forming unit-erythroid
BL-CFC: blast colony forming cell
BM: bone marrow
CBF: core biding factor
CFU-GEMM: colony forming unit- granulocyte, erythrocyte, monocyte, megakaryocyte
CFU-GM: colony-forming unit-granulocyte macrophage
CLP: common lymphoid progenitor
CMP: common myeloid progenitor
D: diversity
DAPI: 4'-6-Diamidino-2-phenylindole
DC: dendritic cell
DL: Delta-like
DMEM: Dulbecco's modified eagle's medium
DN: double negative
dNTP: Deoxyribonucleotide triphosphate
DP: double positive
DSL: Delta-Serrate-Lag2
EB: embryoid body
EGF: epidermal growth factor
ELP: earliest lymphocyte progenitor
ED: embryonic day
ES: embryonic stem
ES-HP: embryonic stem cell-derived hematopoietic progenitor
ES-NK: ES-derived NK cell
ETP: earliest T lineage progenitor
FBS: fetal bovine serum
FL: fetal liver
Flt3: Fms-like tyrosine kinase 3
Flt3-L: Flt3 ligand
FTOC: fetal thymic organ culture
GFP: green fluorescent protein
GM-CSFR: granulocyte macrophage cell-stimulating factor receptor
HES: Hairy/Enhancer of split
HPC: hematopoietic progenitor cell
HSC: hematopoietic stem cell
ICN: intracellular Notch
IL: interleukin
J: joining
Lin: lineage
LSK: lineage$^-$Sca-1$^+$c-kit$^+$
LT: long-term
MAML: Mastermind-like

M-CSF: macrophage-colony stimulating factor
MEM: Minimum essential medium eagle
MHC: major histocompatibility complex
Mll: mixed leukemia lineage
MPP: multipotent progenitor
MTG: monothioglycerol
NK: natural killer
NKP: NK precursor
OC: ontogenic counterpart
OP9-DL1: OP9 cells expressing Delta-like 1
PCR: polymerase chain reaction
PM: Pharmingen
P-Sp: para-aortic splanchnopleura
RAG-1: recombination-activating gene 1
RT-PCR: reverse transcription polymerase chain reaction
SP: single positive
Sp: splanchnopleura
ST: short-term
SCF: stem cell factor
TCR: T cell receptor
T/NKP: bipotent T/NK progenitor
TRAIL: TCF-related apoptosis-inducing ligand
V: variable
VEGF: vascular endothelial growth factor
V_H: variable heavy chain
YS: yolk sac

γc: common γ chain

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Chapter 1: Introduction

1.1. Embryonic stem cells and lymphopoiesis

Embryonic stem (ES) cells are totipotent cells originating from the inner cell mass of developing blastocycts. When placed in culture, ES cells retain their totipotential capacity and contribute to the formation of all the tissues after being introduced into the host blastocysts. In addition, ES cells are able to differentiate to various cell types under appropriate conditions *in vitro*[1]. The properties of ES cells have made them useful in at least two areas: First, ES cells provide a powerful *in vitro* model system to address questions in cell and developmental biology. Second, ES cell are a valuable source for cell replacement therapies[2].

Hematology, in particular, has taken advantage of ES cells as a research and discovery tool to dissect the process of hematopoiesis (e.g. generation of blood cells from stem cells). The blood-forming potential of ES cells has been shown by multiple studies. Blood lineages including erythroid, myeloid and lymphoid lineages can be produced from ES cells in a predictable fashion[1,3-6], however developmental processes from ES cells to blood cells, in particular to lymphocytes, are still unclear.

The purpose of this study was to identify the developmental pathway from ES cells to lymphocytes in culture. A multi-step culture system to induce differentiation of ES cells into cells of lymphoid lineage was developed. Using antibodies to cell surface markers,

intermediates with lymphoid potential among ES differentiated cells were characterized. Therefore, a lymphocyte developmental pathway was constructed that can be used to study lineage commitment. In addition, this work resulted in isolation of a population of hematopoietic progenitors within differentiated ES cells, which harbor both myeloid and lymphoid potentials, and may be useful in obtaining hematopoietic stem cells (HSCs) for transplantation treatments. I will therefore review the current understanding of hematopoiesis and lymphopoiesis in the adult mouse, followed by the early embryonic and fetal hematopoietic development. Hematolymphoid potential of ES cell subsets will also be reviewed and compared with their counterparts during ontogeny.

1.2. Adult hematopoiesis

The hematopoietic system is generated and maintained throughout life through the regulated proliferation and differentiation of hematopoietic stem cells (HSCs). HSCs are clonogenic precursors capable of generating more stem cells, as well as differentiated blood cells. It is well established that hematopoiesis is a stepwise developmental process, in which HSCs lose self-renewal potential and multipotency and give rise to downstream oligopotent and unipotent lineage-restricted progenitors. The lineage commitment is defined as irreversible and gradual loss of developmental potential to other lineages and maturing to a functionally and phenotypically distinct set of progeny. HSCs in mouse bone marrow (BM) have been identified according to cell surface characteristics. These cells are negative for lineage markers (Lin^-) and positive for Sca-1 (Ly6A/E), and c-kit receptor tyrosine kinase (receptor for stem cell factor), (referred to as LSK phenotype). The properties of HSCs have been

demonstrated by *in vitro* clonal assays for all major hematolymphoid cell lineages as well as by *in vivo* transplantation assays. A subset of LSK HSCs (which are $CD34^{-/lo}Flt3^{-}$) is known as long-term (LT) HSCs, because they give rise to long-term multi-lineage reconstitution and self-renewal in irradiated mice. According to the described model of hematopoiesis, the LT-HSCs initially differentiate into sort-term (ST) HSCs with limited self-renewal activity and subsequently to multipotent progenitors (MPPs) with no detectable self-renewal potential *in vivo*[7-9]. MPPs, which are also included in LSK population, coexpress CD34 and Fms-like tyrosine kinase 3 (Flt3). $Flt3^{+}$ MPPs have multi-lineage potential; but have lost their megakaryocyte and erythrocyte lineage potentials (Figure 1.1)[10-12]. Multipotent cells (ST-HSCs/MPPs) then pass through the next developmental step and commit to either lymphoid or myeloid lineages. Two kinds of oligolineage-restricted cells: common lymphocyte progenitors (CLPs) and common myeloid progenitors (CMPs) have been found in bone marrow. CLPs ($Lin^{-}IL\text{-}7R\alpha^{+}Sca\text{-}1^{lo}c\text{-}kit^{lo}$) are the clonal precursors of the lymphocyte lineage (T lymphocytes, B lymphocytes, and natural killer (NK) cells)[13], while CMPs are restricted to the myeloid lineages (granulocytes, monocytes, erythrocytes, and megakaryocytes)[14]. CLPs and CMPs can be distinguished by two cell surface receptors for cytokines, namely interleukin (IL)-7 receptor and granulocyte macrophage cell-stimulating factor receptor (GM-CSFR). While CMPs express GM-CSFR and do not express IL-7R, CLPs express cell surface IL-7Rα but lack GM-CSFR.

1.3. Adult lymphopoiesis

Change in chromatin status and gene expression initiates the process of lineage specification, even though it does not always signal a firm commitment to a particular lineage[15]. Expression of RAG-1 (recombination-activating gene 1) and concomitant rearrangement of antigen receptor genes is the key developmental event specific to lymphocyte lineage[16]. CLPs are long considered to be the most immature lymphoid-committed progenitors. However, recent studies have identified progenitors upstream of CLPs with lymphoid potential (Figure 1.1). In a study by Igarashi *et al.*, RAG-1/GFP (green fluorescent protein) knock-in mice were used to isolate the earliest lymphocyte progenitors (ELPs), which have activated RAG-1 locus. ELPs were found in a non-self renewing LSK $Flt3^{+}CD27^{+}$ subset of BM cells. These cells could sustain T, B, and NK cell production, but had minimal potential for myelo-erythroid differentiation[17]. In addition, cells with similar properties have been described in the bloodstream[18]. The evidence for a common lineage relationship between ELPs and CLPs was obtained with immunoglobulin D_H-J_H rearrangement products (i.e. heavy-chain diversity gene segment linked to joining gene segment) in purified ELPs as well as CLPs[17]. Consistent with these findings, in transgenic recombination substrate mice, recombination activity (or evidence of previous activity) was found in CLPs, as well as downstream progeny B, T, NK and dendritic (DC) cells. However, RAG expression in CLPs was shown to be regulated by a B cell specific enhancer, *Erag*, suggesting that CLPs are associated with the B lineage and do not substantially contribute to T cell production *in vivo* despite a residual T cell potential[19]. This study supported earlier work by Allman *et al.* which showed that a defect in hematopoietic cell-specific transcription factor, Ikaros, affects CLP,

B and NK lineages, while sparing the T lineage, presenting CLPs as a separate lineage from T cells[20]. 40% of GFP^+ cells in $Lin^-c\text{-}kit^{lo}$ population in RAG-1/GFP mice do not express the CLP marker, IL-7Rα, suggesting lymphocyte progenitors may be heterogeneous with regard to their surface markers[21]. Kouro *et al.*[22] examined $Lin^-c\text{-}kit^{lo}$ fraction of BM cells, which also includes CLPs, for lineage potentials. $Lin^-c\text{-}kit^{lo}$ cells included NK and B cell progenitors (pro-lymphocytes), and were depleted of both progenitors in response to estrogen treatment, suggesting a common origin for both lineages. Single cell assays confirmed the presence of B-restricted, NK-restricted and bipotent progenitors in $Lin^-c\text{-}kit^{lo}$ pro-lymphocytes. However, NK and B differentiation from $Lin^-c\text{-}kit^{lo}$ cells was shown to follow distinct pathways. For example, while B cell precursors cross an IL-7Rα^+ stage, the IL-7R is either not expressed or transiently expressed by developing NK-lineage cells. In addition, cells with B cell potential never displayed CD122 (IL-2Rβ), whereas NK differentiation took place via a $CD122^+B220^+$ stage, followed by the $CD122^+B220^-$ pathway. Moreover, NK precursors were more estrogen resistant than B cell progenitors. Even though the contribution of $Lin^-c\text{-}kit^{lo}$ pro-lymphocytes to T lymphopoiesis is not clear, it does seem to represent an important intermediate in B and NK cell development (Figure 1.1)[22].

In an effort to identify the earliest lymphocyte progenitors with T cell potential in bone marrow, a transgenic mice model was developed in which a T-lineage specific pre-TCRα (pTα) promoter directed expression of a hCD25 surface marker. $hCD25^+c\text{-}kit^-B220^+$ BM cells were found to be bipotent precursors of T and B cells in clonogenic assays, and efficiently home to the thymus after intravenous injection into recipients. $c\text{-}kit^-B220^+$ cells could be generated from the CLP cells and were called CLP-2[23]. Briefly, one can define the

lineage pathway of lymphocyte progenitors as ELP→CLP/pro-lymphocytes→CLP-2 (Figure 1.1).

1.3.1. T cell development

T cells developing in the thymus fall into two major categories according to the type of antigen-specific receptors displayed on the surface. αβ T cells express the T-cell receptor (TCR) made up of the α and β chains, while the second population of T cells bears a heterodimeric γδTCR. The TCR chains are encoded by gene segments that undergo rearrangement during development. Random recombination of variable (V) TCR gene segments with joining (J) and, in some cases, with diversity (D) gene segments provides the T cell population with a diverse repertoire of TCR molecules such that each T cell expresses a TCR with a single specificity. This enables T cells to distinguish antigens derived from virtually any kind of pathogen. αβ T cells produce adaptive immune response in response to infections. One group of αβ T cells (CD8 T cells) can differentiate into cytotoxic T cells that lyse infected cells, while a second population of αβ T cells known as CD4 T cells activate macrophages or induce B cells for antibody production. CD8 and CD4 αβ T cells recognize peptide ligands in combination with major histocompatibility complex (MHC) class I and MHC class II molecules, respectively[16]. γδ T cells are non-MHC restricted and function in immune responses to infections, immunoregulation and tumor immunosurveilance. They recognize protein (e.g. nonclassical MHC molecules, and heat shock proteins) and non-protein antigens[24]. In functional T cells, both types of TCR are associated with invariant

accessory CD3 molecules (such as CD3γ, CD3δ, CD3ε) on the cell surface[25]. αβTCR complex also includes intracytoplasmic CD3ζ homodimers. CD3 proteins are involved both in the surface expression of T cell receptors and in signal transmission to the inside of the cells[16].

The thymus is required for the generation of T cells. However, progenitors are not generated in the thymus and have to enter the thymus from the bone marrow through the bloodstream[12]. Immature T cell progenitors in the thymus are negative for the expression of CD4 and CD8 and referred to as double-negative (DN) cells (these cells are also $CD3^-$). DN cells are heterogeneous and change their phenotype at different stages as they develop along the T lineage pathway (Figure 1.1). $CD44^+CD25^-$c-kit^+ DN cells represent the earliest precursor stage termed as DN1 stage. DN2 stage is characterized by CD25 expression and initiation of TCR gene rearrangement. DN3 ($CD44^-CD25^+$) cells continue rearrangement and express either pre-TCR, consisting of a rearranged TCRβ chain and an invariant pre-TCR (pTα) chain, or the γδTCR depending on which gene rearrangements have been successful. Signaling through pre-TCR is required for the progression of DN3 cells to $CD4^+CD8^+$ double-positive (DP) stage in αβ T cells, which enables cells to activate TCRα rearrangement. Alternatively, signaling via the γδTCR results in commitment of cells to the γδ T lineage. An additional step in αβ T cell development is positive and negative selection, which allows the generation of MHC-restricted and self-tolerant single positive (SP) CD8 and SP CD4 T cells[16,25-27]. Even though specification, which refers to onset of expression of T-lineage genes, occurs at the DN1 and DN2 stages, commitment to the T lineage as defined

by loss of alternative choices (B, myeloid, NK and DC) does not happen until DN3 stage[26]. In addition, within the T lineage, commitment to the γδ T lineage appears to occur at least one full stage earlier than that of αβ T lineage. TCRγ (e.g. Vγ2-Jγ1) and TCRδ rearrangements are clearly observed at DN2 stage, while TCRβ locus is still in the germline form[28].

Despite the well defined intra-thymic T differentiation, the pathway from BM derived HSC to precursors in thymus is not well characterized. HSCs and downstream progenitor population (including ELP, CLP and CLP-2) have been shown to have T cell potential. The contribution of each progenitor population to the steady-state T cell development is not clear. To better resolve thymic-seeding progenitors, it is conceivable to compare them with their counterparts in the thymus. A study by Allman, *et al.* showed that the earliest T lineage progenitors (ETPs) among DN1 cells are $Lin^{-}c\text{-}kit^{+}Sca\text{-}1^{+}$ and develop independently of CLPs, suggesting that progenitors other than CLPs may seed the thymus[20]. A later study reported that ETPs express Flt3, and have B-cell lineage potential[29]. NK, DC and myeloid potential of ETPs have also been reported[30,31]. Taken together these data suggest that ETPs resemble BM MPPs ($LSKFlt3^{+}$), and therefore, MPPs may be the progenitors entering the thymus at the times needed (Figure 1.1). On the other hand, an additional BM precursor population with $LSKCD62L^{+}$ $(L\text{-selectin})^{+}$ phenotype has been identified. $LSKCD62L^{+}$ cells contain robust T progenitor activity with minimal B and myeloid differentiation potential, similar to ETPs. This data suggested that $CD62L^{+}$ cells selectively home to the thymus and give rise to thymic ETPs, which also express $CD62L$[32]. In addition, this study raised the

possibility that commitment to the T lineage may happen before contact with the thymic environment.

1.3.1.1. Notch signals and T cell development

The Notch pathway regulates developmental choices and cellular differentiation across species. Notch regulation is mediated through a conserved signaling pathway in which transmembrane Notch receptors undergo sequential proteolytic cleavage followed by nuclear translocation of the intracellular domain and activation of transcription. There are four mammalian Notch receptors. The extracellular portion of Notch contains tandem epidermal growth factor (EGF-like) repeats, which binds the extracellular domain of DSL ligands (Delta-Serrate-Lag2). Mammals have two Serrate-like ligand (Jagged 1 and 2) and three Delta-like ligands (DL-1, -3 and -4). The intracellular domain of Notch (ICN, intracellular Notch) consists of domains involved in signal transduction and protein stability including the RAM domain, ankyrin repeats and PEST domains. Ligand binding results in two cleavage events that dissociate ICN from the membrane. The first cleavage occurs extracellularly and uses metalloprotease, while the second happens within the transmembrane domain and is γ-secretase dependent. γ-secretase inhibitors block Notch signaling. ICN travels to the nucleus, where it binds the helix-loop-helix transcription factor CSL/RBP-J via its RAM and ankyrin repeat domains. Ankyrin repeats of ICN also interact with Mastermind-like proteins (MAMLs), which recruit transcriptional coactivators. Notch signaling activates transcription of a bHLH-type transcriptional repressor, known as Hairy/Enhancer of split (HES), which inhibits transcription of HES target genes. Other tissue specific targets have been proposed[33].

During lymphoid development, signaling through Notch plays an important role in T cell fate determination. It has been reported that the inactivation of Notch signals in hematopoietic progenitors selectively blocks T cell differentiation with an excess of B cells in the thymus. Conversely, HSCs with constitutive active Notch1 adopt T cell fate and lose B cell potential. Relevant to thymopoiesis is Notch1 and Delta-like family members. Absence of Notch1 results in complete suppression of T cell development, indicating a lack of compensation by other Notch family members. Also, expression of the Delta-like family (DL-1 or DL-4) of Notch ligands in BM as well as thymic stromal cells is sufficient to induce the differentiation of hematopoietic progenitors, even embryonic stem cells, into the T cell lineage *in vitro*[12,33,34]. This data supports a model for T lymphopoiesis in which incoming multipotent progenitors first encounter Delta-like ligands in the thymus, which in turn triggers Notch signaling and specification to the T lineage. In fact, T specification of ETPs and suppression of B cell potential are mediated through the Notch pathway[29,35].

1.3.2. B cell development

B cells, the other players of adaptive immunity, recognize antigens derived from a great variety of pathogens with their antigen-specific receptors, termed the B cell receptor (BCR). The BCR is composed of two immunoglobulin (Ig) heavy chains and two immunoglobulin light chains. Each BCR has a single specificity and is produced through two series of gene rearrangements, one for each receptor-chain locus. The secreted form of BCR is the antibody proteins, which are produced by terminally differentiated B cells, plasma cells. The secretion of antibodies, which provides the humoral immune response, is the main effector function of

B cells[16]. B cells develop in bone marrow from HSCs. The stages of B cell development are defined by the sequential rearrangement and expression of immunoglobulin genes as well as the cell surface expression of protein markers for the B cell lineage (Figure 1.1). The earliest B linage cells are known as pre-pro-B cells and express the B cell lineage marker B220 but lack other lineage markers including CD19. B220 expression is not restricted to the B lineage as some cells in the NK cell lineage also express this molecule. Most pre-pro B cells have not initiated rearrangements. Recent data have shown that pre-pro B cells are heterogeneous and generate other cell types such as NK, T and DC cells. B cell precursors undergo D_H-J_H rearrangement, followed by heavy chain variable (V_H) to DJ_H rearrangement at pro-B cell stage. This stage is also accompanied by the expression of CD19 which serves to characterize all later B lineage cells. Productive rearrangement of VDJ_H leads to the expression of the μ heavy chain which is expressed on the cell surface in combination with a surrogate light chain to form the pre-B cell receptor. Expression of the pre-B cell receptor is the hallmark of the pre-B cell stage, and occurs prior to the rearrangement and expression of the light chain. Subsequently, immature B cells that express IgM but not IgD are generated[16,36,37].

1.3.3. NK cell development

Natural killer (NK) cells are large granular lymphocytes that play a significant role in innate immunity [38]. These cells are important in eliminating viruses and possibly other pathogens during the initial phase of infections, before an adaptive immune response begins. They also contribute to immune responses against cancer cells, and transplanted allogenic bone marrow cells. Immunization or pre-activation of NK cells is not a requirement for NK cell

responses[39]. The major effector mechanisms employed by NK cells are direct killing of infected cells or tumor cells and cytokine production. Target killing is mainly mediated through the exocytosis of granules containing perforin and granzyme. NK cells can also induce cell death via FasL and TNF-related apoptosis-inducing ligand (TRAIL)[40]. Besides direct cytotoxicity against aberrant target cells, NK cells are also responsible for the regulation of adaptive immune responses and the action of other innate cells, such as macrophages, through cytokine production[41]. Interaction between NK and DC cells also regulates innate and adaptive immune responses[42].

Unlike T or B cells, NK cells do not express antigen-specific receptors that are the end product of somatic gene rearrangements. Instead, they use an intricate system of co-expression of multiple receptors in various combinations to distinguish aberrant target cells from normal cells. The NK receptor repertoire consists of activating and inhibitory receptors that interact with several ligands on target cells. Inhibitory receptors, which recognize self MHC class I molecules on normal cells and deliver negative signals to NK cells, protect normal host cells from being lysed by NK cells, thus ensuring self-tolerance. Cells that lack sufficient MHC class I expression as a result of infection, transformation or mutation are susceptible to NK killing. Activating receptors, upon ligation, send stimulating signals. These receptors enable NK cells to respond to microbial products or molecules that are induced upon stress or transformation. The balance between stimulating and inhibitory signals governs the fate of NK-target cell interactions. The murine NK receptors that regulate NK cell functions mostly belong to two families of C-type lectin-like receptors, Ly49 homodimers and CD94/NKG2 heterodimers. These receptors are expressed in a clonal

fashion on overlapping subsets of mature NK cells[43-46]. However, some NK receptors are uniformly expressed among NK cells. Examples of these receptors are activating NKG2D[47], and NK1.1 receptors (in the C57BL/6 strain of mice)[48], as well as integrins, such as Mac-1 and DX5[49].

NK cell development depends on the bone marrow environment. Indeed, bone marrow ablation by ^{89}SR[50] or by oestradiol[51] results in NK cell deficiencies. A similar defect is observed in osteopetrotic animals[51]. NK cell development from BM precursors has only recently been examined. NK lineage committed precursors (NKPs) that differentiate into NK cells but not other hematopoietic cells have been identified in adult mouse BM by the surface phenotype of Lin^-CD122^+ (Figure 1.1)[52]. CD122 is the β subunit of IL-15R, a cytokine receptor chain shared by IL-2R and IL-15R[53]. These two receptors are dependent on signaling through a third subunit, known as common γ chain (γc)[54]. NKPs lack many cell-surface receptors for mature NK cells (NK1.1, DX5, and Ly49 receptors) and do not show any lytic activity or cytokine production[52]. Williams *et al.* demonstrated that culture of HSCs in the presence of cytokines such as stem cell factor (SCF), Flt3 ligand (Flt3-L), IL-7, and IL-6 generates $CD122^+$ cells that presumably are NKPs. These cells give rise to NK cells in response to IL-15[55]. Collectively, this data indicates that the acquisition of CD122 by NKPs, marks their commitment to the NK lineage[56].

Even though the stages of NKP differentiation from HSCs are still unclear, the developmental processes from NKPs to mature NK cells in BM have been described in detail. As for B and T lymphocytes, stages of NK cell maturation can be defined according to

the expression pattern of cell surface molecules and functional characteristics (Figure 1.1). The first developmental stage represents $CD122^{+}NK1.1^{-}DX5^{-}$ NKPs[49]. Further characterization of these cells by Vosshenrich *et al.*[57] showed that they are γc^{+}, but CD25 (IL-2Rα) negative with a subset expressing IL-7Rα, c-kit, and Flt3. It was also shown that NKG2D is expressed at this stage[57], while the earlier studies reported its expression at stage II[49]. Acquisition of NK-lineage markers; $NK1.1^{hi}$ (high expression), $DX5^{lo}$ (low expression), and CD94/NKG2 receptors occurs at the stage II of development. The stage II cells resemble mature fetal/neonatal NK cell which lack Ly49 receptors. These cells also express the integrin α_v^{+} and are $Mac\text{-}1^{lo}CD43^{lo}c\text{-}kit^{-}$. Developing NK cells acquire Ly49 receptors and become $c\text{-}kit^{+}$ while in transit through stage III. This step is then followed by an upregulation of DX5 and extensive proliferation of NK cells in BM in stage IV. The proliferation of NK cells is slowed and they upregulate Mac-1 and CD43 as they mature in the final step. These mature NK cells are capable of proliferation in response to pathogens and exhibit full functional competence, including natural killing and an ability to secrete cytokines[49].

BM is often considered as the main site for generating mature NK cells in the adult, as all introduced developmental intermediates including ELPs, CLPs, NKPs and immature NK cells are found in BM. The finding of bipotent T/NKPs in adult thymus suggested the existence of other types of NK precursors and possibly pathways contributing to the NK cell pool[30]. Moreover, recent studies have provided evidence for a thymic-dependent pathway of NK cell development. Vosshenrich *et al.*[58] demonstrated that thymic NK cells are unique, as in contrast to BM-derived splenic NK cells, most of them are $IL\text{-}7R\alpha^{+}$, and express Gata-3, the transcription factor essential for the development of the earliest T cell progenitors in the

thymus[59]. The generation of thymic NK cells was IL-7 dependent, while BM NK cell development is not[58]. The IL-7Rα$^+$ NK cells were represented as a sizeable proportion (15-30%) in lymph nodes compared to other tissues, suggesting the possible export of thymic NK cells to lymphoid organs. The engraftment of *Rag2*$^{-/-}$ recipient mice with thymus from *Tcra*$^{-/-}$ mice confirmed selective homing of thymic NK cells to the lymph nodes. *Gata3*$^{-/-}$ hematopoietic precursors did not generate IL-7Rα$^+$ NK cells in the thymus and lymph node. From these observations, they conclude that IL-7Rα$^+$ NK cells develop intrathymically from, perhaps early T cell precursors, in a process dependent on Gata-3, and are exported to the peripheral lymphoid tissues. Functional characteristics of thymic and lymph node NK cells differed from splenic NK cells. These cells showed higher cytokine production and lower cytotoxicity[58]. The work by Veinotte *et al.* also confirmed the contribution of thymic pathway in generation of NK cells. They showed that TCRγ genes are rearranged in at least half of thymic NK cells, but in a very small fraction of BM NK cells (less than 5%), suggesting that a subpopulation of NK cells derives from thymic T/NK bipotential precursors[60]. They proposed that the thymic NK cells develop from DN2 cell stage since this is the stage when TCRγ rearrangement initiates[28].

Studies of human NK cell development have also indicated an involvement of tissues other than BM in the generation of at least subsets of NK cells. Initially, the NK potential was noted in human BM-derived CD34$^+$ hematopoietic progenitor cells (HPC) cultured with IL-2 and IL-15 in liquid cultures or in combination with stromal cells, suggesting the existence of a NKP population among CD34$^+$ HPCs[61-64]. However, CD122 expression on freshly isolated CD34$^+$ HPCs was undetectable by flow cytometry, making it difficult to identify a human

equivalent of NKPs, even though $CD122^+$ NKPs were generated in *in vitro* cultures[63-65]. Human NK cells are characterized as $CD3^-CD56^+$ cells and can be divided into two subsets. One subset, defined by $CD56^{dim}$ has potent cytolytic functions, with little ability to produce cytokines. The other subset, $CD56^{bright}$ has poor cytolytic properties but are potent cytokine producers[66]. Currently, the developmental relationship between these two subsets is unclear. A recent report from the Caligiuri laboratory has discovered a novel $CD34^{dim}CD45RA^+$integrin $\alpha_4\beta_7^{hi}$ hematopoietic precursor in human lymph node which differentiates to $CD56^{bright}$ NK cells. These cells were located in the T cell-rich regions of human lymph nodes near $CD56^{bright}$ NK cells[64]. A follow up study showed that all developmental intermediates spanning from $CD34^+$ progenitors to $CD56^{bright}$ NK cells can be found in lymph nodes[65]. NK cell differentiation in lymph node progressed through four consecutive stages: stage 1, $CD34^+$c-kit^-CD94^-; stage 2, $CD34^+$c-kit^+CD94^-; stage 3, $CD34^-$c-kit^+CD94^-; stage 4, $CD34^-$c-$kit^{+/-}CD94^+$. NK receptors were not expressed at stage 1, but were progressively acquired throughout the next developmental stages. A high-density expression of CD56 was observed within stage 4, and was associated with the acquisition of capacities for both cytotoxicity and cytokine production. Surface expression of CD122 was first detected at stage 3. B cell potential was absent in all stages, whereas T and DC potentials were seen in stage 1 and 2 but not in stage 3 and 4. Therefore, commitment to the NK lineage seems to occur at stage 3 of development, when CD122 appears. Nevertheless, the ability to respond to IL-15 begins at stage 2 of differentiation, which correlates with CD122 mRNA expression at this stage[65].

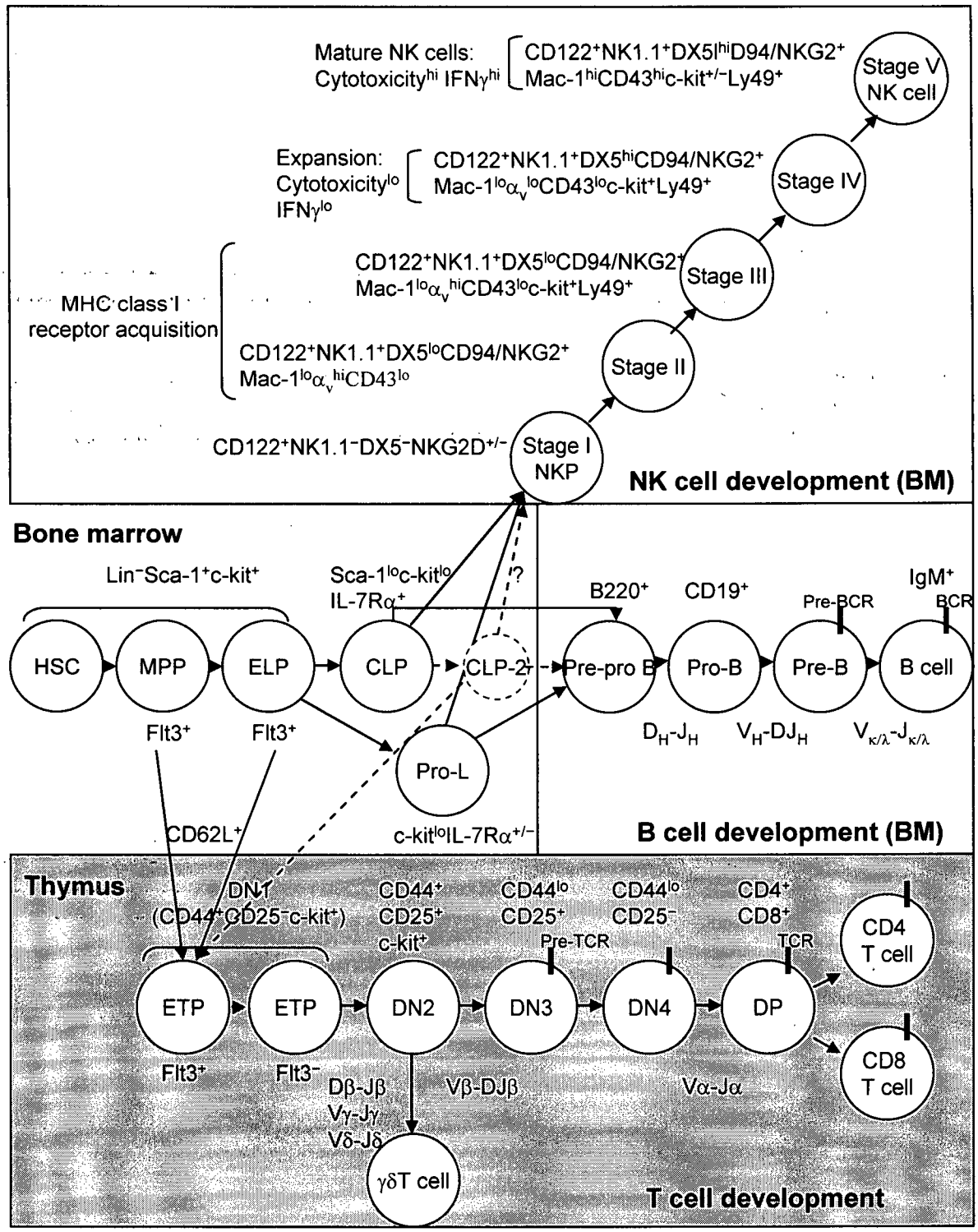


Figure 1.1. The scheme of lymphopoiesis in the adult mouse.

Figure1.1. The scheme of lymphopoiesis in the adult mouse.
Progenitors with lymphoid potential including MPPs, ELPs, CLPs, CLP-2 cells and pro-lymphocytes (Pro-L) are continuously generated from HSCs in BM. NK and B lineage derive from CLPs, Pro-L, or possibly CLP-2 cells, while T lineage arise from progenitors upstream CLPs such as MPPs and ELPs, which express Flt3 and/or CD62L. CLP-2 cells derived from CLPs also efficiently generate T cells. Steps for individual lymphocyte lineage differentiation have been illustrated: Differentiation of NK lineage-committed precursor (NKP) to mature NK cells in BM involves 5 stages, each defined by indicated surface markers. B cell development proceeds from less mature pre-pro B cells to immature B cells. Gene rearrangements associated with each stage are shown below the relevant stage. The earliest T lineage progenitors (ETPs) in thymus are also $Flt3^{+}CD62L^{+}$. These cells, which fall into DN1 category of thymic progenitors, undergo differentiation into following steps marked by differential expression of CD44, CD25, CD4 and CD8. TCR rearrangements take place from DN2 stage onwards. Figure is from Ref.[12,25,35,37,49,67].

1.3.4. Cytokines essential for lymphocyte development

1.3.4.1. SCF and Flt3 Ligand: Stem/progenitor cell factors

c-kit ligand (SCF) and Flt3-ligand (Flt3-L) interact with receptor tyrosine kinases known as c-kit and Flt3, respectively. Flt3 and c-kit are expressed by HSCs, ELPs, CLPs, and NKPs as well as various subsets of lineage-committed cells including those for B, T, NK, erythroid, megakaryocyte and mast cells[56,68]. SCF and Flt3-L have synergistic interactions with each other or with other early acting cytokines to exert their hematopoietic activities, although they also play distinct roles in the differentiation process of discrete cell lineages. SCF is critical for maintenance and self-renewal of HSCs, whereas both SCF and Flt3-L are essential for optimal production of mature hematopoietic cells from HSCs[68]. In addition, c-kit and Flt3 receptors are known to be required for the early events in hematopoiesis, as it is evidenced from the reduction of hematopoietic precursors, including CLPs, in the absence of signaling through either receptor[69,70]. This defect becomes more severe when both c-kit and

Flt3 are inactivated[71]. While c-kit is redundant in B and T cell development in fetal, neonatal and young mice, it is indispensable in adult mice. The number of pro-B cells and pro-T (DN2) progenitors are severely reduced in adult c-kit mutant mice[69,72]. Mice lacking Flt3 or Flt3-L manifest deficiencies in B cell precursors[71,73]. In competitive repopulation experiments, $Flt3^{-/-}$ stem cells fail to reconstitute the hematopoietic system, particularly the T-lymphoid lineage[71]. A severe reduction in ETP number but only a small reduction in more differentiated thymocytes has been reported in $Fl3L^{-/-}$ mice[29,70]. By contrast, NK cell development is only mildly affected with defective c-kit or Flt3-L signaling[73,74]. The absolute number of NKPs is normal in c-kit mutants and only slightly reduced in $Flt3\text{-}L^{-/-}$ mice[75,76]. These observations have lead to the conclusion that c-kit and Flt3-L play a role in lymphocyte developmental pathway at the transition from HSCs to CLP, since the generation of CLPs is dependent on both receptors signaling[69,70]. In fact, SCF and Flt3-L may promote CLP survival and/or expansion. However, it is not clear whether developing NK cells traverse through CLP stage, as NKPs develop without either receptor. Since a functional redundancy between these two receptors has been suggested, analysis of NK cells in double-mutant mice may reveal the role of these receptors in NK cell commitment.

In vitro studies have indicated that SCF and Flt3-L may have influence on individual lymphoid lineage differentiation pathways. The i*n vitro* differentiation of T cells has been made possible by a recently developed OP9-DL1 (OP9 cells expressing Notch ligand Delta-like-1) culture system which could replace the fetal thymic organ cultures (FTOC) to assess the effect of cytokines in T cell proliferation and differentiation[77]. In the presence of Flt3-L and IL-7, progenitors derived from embryonic stem cells[78], fetal liver[77] or adult thymus[78]

differentiate into T cells on OP9-DL1 stroma. Also, c-kit and IL-7 signaling induce *in vitro* proliferation and differentiation of DN1 and DN2 cells along the T lineage pathway[79]. Flt3-L synergizes with IL-7 to stimulate the growth of B cell progenitors[80]. Stimulating mouse and human HSCs with SCF and Ft3L renders them responsive to IL-15, which in turn promotes NK cell differentiation[55,63].

1.3.4.2. IL-7

The receptor for IL-7 consists of two chains: IL-7Rα and γc. The importance of IL-7 in lymphocyte development has been shown by IL-7 and IL-7Rα-deficient mice. In these mice, the development of B and T cells (including γδ T cells) has been severely compromised, even though pre-pro-B and pro-T cells are still produced. Most NK cells develop normally in IL-$7^{-/-}$ and IL-$7\alpha^{-/-}$ mice. However, a unique subset of NK cells that is thymus-derived and expresses IL-7R, is deficient in IL-$7^{-/-}$ mice[37,58,81-85]. Further studies have revealed that signals delivered through IL-7 receptor mediate gene rearrangement events in the immunoglobulin heavy chain (IgH) and TCR-γ loci[86,87]. In a stromal-free culture system, IL-7R signaling is sufficient to specify B cell fate in CLPs, confirming a central role for IL-7 in B cell development[88]. IL-7 seems to play an important role in survival and proliferation of B and T cell precursors through induction of anti-apoptotic factors such as Bcl2 or Mcl1[89-91]

1.3.4.3. IL-15

IL-15 is a critical cytokine for NK cell development. IL-15 binds to the IL-15R complex, which is composed of IL-2/IL-15Rβ (CD122), IL-15Rα, and the γc[92]. IL-15 and IL-15Rα-deficient mice exhibit NK cell deficiencies, as well as deficiencies in NKT cells, intestinal epithelial cells, and memory $CD8^+$ T cells[93,94]. γc mutations/absence cause similar defects in NK cell development, although these defects are accompanied by X-linked severe combined immune deficiency[95-98]. Overall, these data suggest an essential role for IL-15 in NK cell generation. Further investigations showed that the commitment to the NK lineage does not occur through γc-dependent cytokine stimulation, since NKPs exist in normal numbers in γc-deficient mice. Conversely, IL-15 functions in subsequent stages of NK differentiation by maintaining normal numbers of immature and mature NK cells[57]. IL-15 signaling acts on the upstream of the anti-apoptotic factor Bcl-2. Normal numbers of NK cells are obtained in IL-2Rβ-deficient mice through the overexpression of Bcl-2[99]. Bcl-2 transgenic NK cells persist after adoptive transfer in IL-15 deficient hosts[100,101]. Collectively, these data implicate IL-15 in the survival and proliferation of developing NK cells. A candidate downstream target of IL-15 signaling has been proposed through studies of human NK cells. The expression of Ets-1, a winged helix-turn-helix transcription factor, increases in response to signaling via IL-2 and IL-15. The exact role of Ets-1 in NK function and the target genes regulated by Ets-1 is yet to be determined[102]. However, lack of Ets-1only impairs NK cell numbers and their cytolytic activity without affecting T and B cells, suggesting an essential role for Ets-1 in NK cell development[103].

1.4. Ontogeny of hemato/lymphopoietic system

1.4.1. Embryonic stage

The first hematopoietic cells observed in the mouse embryo are erythroid cells emerging from yolk sac (YS) blood islands at embryonic day 7 (ED7). These cells are nucleated and express embryonic hemoglobin (ε and βH1). This first wave of erythropoiesis in the YS is known as primitive wave, as opposed to the production of definitive erythrocytes, which are enucleated and express adult forms of hemoglobin (β-major and α). Primitive erythropoiesis in the YS is associated with production of macrophage and megakaryocyte lineages[104]. A second wave of definitive erythroid progenitors has been detected in the YS beginning at ED8.25. These progenitors seed the liver where they begin to mature. The YS also provides multiple committed myeloid progenitors including mast cell progenitor, granulocyte-macrophage progenitors and megakaryocyte progenitors during the definitive phase of hematopoieisis[105]. Characterization of hematopoietic progenitors in the YS have indicated that the primitive erythroid as well as definitive hematopoietic progenitors express CD41 as an early marker (Figure 1.2)[106,107]. The definitive myeloerythroid progenitors also express c-kit, but lack the pan-hematopoietic marker CD45 (Ptprc; protein tyrosine phosphatase receptor C), even though they turn on CD45 during their progressive maturation[108]. Despite the abundant production of myeloerythroid progenitors in the YS, YS cells cannot generate lymphoid progeny[109,110], and lymphoid progenitors previously detected in the YS have been obtained from the YS after the establishment of circulation and could have originated in intra-embryonic sites[104,107,111]. Precirculation YS cells may need a maturation step to acquire

the properties of adult definitive HSCs. Conditioning of YS cells such as injecting into the liver of newborn mice or co-culturing with AGM stromal cells provides them with log-term reconstitution potential in the adult[112-114].

The earliest HSCs, which are able to generate long-term multilineage engraftment in the adult recipient, have been identified in the intra-embryonic sites, known as splanchnopleura (Sp), as early as ED7.5 before the onset of circulation[110]. Para-aortic splanchnopleura (P-Sp), which comprises the dorsal aorta and surrounding splanchnic mesoderm (ED8.5-10), also displays hematopoietic progenitor activity[115,116]. After ED10, P-Sp region which develops to the aorta, gonads, and mesonephros, is called AGM. AGM is the source of definitive HSCs[117,118]. The AGM region does not support expansion and differentiation of hematopoietic progenitors. These AGM-derived HSCs are thought to colonize the fetal liver and ultimately bone marrow to provide long-term adult hematopoiesis[105,119]. HSCs isolated from AGM exhibit a phenotype that is characteristics of immature HSCs (e.g. $CD41^{+}$c-$kit^{+}CD45^{-}CD31^{+}AA4.1^{+}$ (CD93)[120], or $CD34^{+}$c-kit^{+}[121,122]) (Figure 1.2).

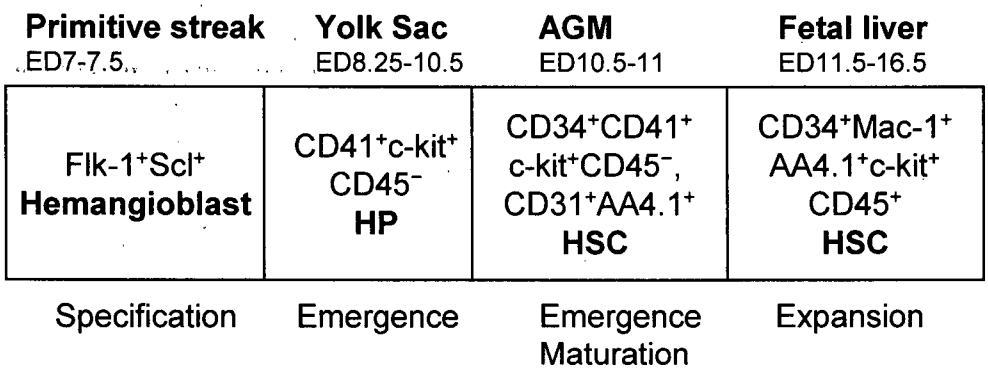


Figure 1.2. HSCs and their precursors during mouse ontogeny

Hematopoietic potential is specified initially in mouse primitive streak in hemangioblasts. Hemangioblasts are thought to be the precursors of YS hematopoietic progenitors (HP) and AGM HSCs. Markers attributed to HSCs in each location are displayed. While YS and AGM generates HSCs de novo, FL is site of HSC expansion and differentiation. Figure is adapted from Mikkola *et al.*[111]

1.4.2. Fetal stage

Although fetal liver (FL) is the main site of hematopoiesis in the fetus, it does not produce HSCs de novo, and has to be seeded by circulating hematopoietic cells. FL is first seeded by hematopoietic progenitors from YS at ED9.5-10.5, followed by HSCs derived from AGM and placenta beginning at ED11.5. After ED12.5, the FL becomes the main fetal organ for HSC expansion and differentiation. HSCs expand rapidly by ED15.5-16.5, after which the expansion is slowed. FL hematopoiesis changes according to the needs of the fetus. At earlier stages, proerythroblasts reflective of definitive erythropoiesis are dominant, whereas myeloid and lymphoid progenitors accumulate later on[111]. FL HSCs differ from adult HSCs in the cell surface marker expression. For example, unlike adult HSCs, FL HSCs express CD34 and lineage markers such as the monocyte/macrophage marker Mac-1 (CD11b) and B cell marker AA4.1 (Figure 1.2)[121,123,124]. These differences might be related to the difference in cell-cycle

status between FL and adult HSCs. FL HSCs are actively cycling, whereas most HSCs in adult BM are quiescent. Both intrinsic and environmental factors are attributed to the observed differences between adult and fetal HSCs[111].

FL is rich in intermediate progenitors derived from the first waves of stem cell differentiation. These intermediate progenitors are also distinct from the multipotent and committed precursors described in adult stage. The developmental potential of FL cells has been investigated with a novel multilineage progenitor assay. In this assay, unipotent progenitors (p)-T and p-B, but not bipotent p-T/B were detected in IL-7R$^+$ population of FL cells, which corresponds to BM-derived CLPs. T cell precursors are enriched at ED12, while B cell precursors predominate on ED14. Moreover, P-T and p-B are generated through the intermediate p-myeloid/T and p-myeloid/B stages (Figure 1.3)[125]. CLP-like precursors with myeloid differentiation potential have also been reported by other groups[126]. These observations lead to the conclusion that the dichotomy between lymphoid and myeloid lineages as seen in adult does not hold true in fetal development.

Committed B cell precursors have been identified in FL at ED12. These cells also retain the ability to differentiate to macrophages, confirming that commitment to lymphoid precursors in FL does not result in loss of macrophage differentiation potential[127,128]. The first CD19$^+$ B cells become apparent in FL by ED14[129], after which developing B cells accumulate in a fashion similar to adult B cell development, although later stages only appear closer to birth[130]. Some differences exist between adult and fetal B lymphopoiesis. For instance, CD5$^+$ B1 B cells, with a unique repertoire of immunoglobulin V_H genes, are prevalent during fetal

life[131]. In addition, while IL-7 is essential for B cell development in adult mice, it is dispensable for fetal B cell development[129].

Since the number of T cell precursors in FL declines between ED12 and ED14, and this temporally coincides with the appearance of T cell precursors in blood, it has been proposed that T cell precursors migrate from the FL to fetal thymus through bloodstream during this time period[132]. DN1 cells first appear in the thymus at ED12, followed by a successive generation of DN2, DN3 and DN4 cells over the next 3 days. The number of T cell precursors increase dramatically by ED15. This increase is due to both expansion and import of cells from FL[129]. A bipotent T/NK progenitor (T/NKP), which has the Lin^{-}c-$kit^{+}NK1.1^{+}$ phenotype, has been detected in fetal thymus[133]. Further reports have shown that the DN1 and DN2 populations contain bipotent T/NKPs, which produce p-NK and p-T. $CD122^{+}$ NK-lineage committed progenitors (NKP) were identified within $CD44^{+}CD25^{-}$ DN cells (Figure 1.3)[134,135]. Prethymic T cell precursors in ED15 FL have been identified as $Lin^{-}B220^{lo}$c-$kit^{+}CD19^{-}$ (Figure 1.3). These cells that comprise 70% of total T cell progenitor activity in FL are also restricted to T and NK lineage and do not exhibit B or myeloid lineage potentials as shown in a limiting dilution fetal thymic organ culture (FTOC) assay. When injected into $Rag2^{-/-}\gamma c^{-/-}$ mice, they reconstitute the NK and T cell compartments, but not B-cell or myeloid compartments[136]. These results indicate that commitment to the T/NK lineage and loss of B cell potential can occur before thymic entry. Bipotent T/NKPs that display a CLP-type phenotype (Lin^{-}c-kit^{+}IL-$7R^{+}$) have also been found in fetal blood between ED11 to ED14 (Figure 1.3)[137]. The conclusion from these data is that contact with thymic stroma to induce Notch signaling, as needed in the adult, is not a prerequisite for commitment to the T

cell lineage in fetal environment. The first thymic immigrants residing in the mesenchymal region of the ED11 fetal thymic anlage have been shown to express similar surface markers to fetal blood T/NKPs and are restricted to T, NK and DC lineages, suggesting a selective colonization of T/NKPs to thymic anlage[138]. Furthermore, it has been shown that Notch signaling is not activated in precursors located in perithymic mesenchyme, yet they have already lost B cell potential and committed to the T lineage[139,140]. Hence, these data strongly suggest that Notch signaling is not involved in determining T cell versus B cell fate during the first wave of thymic colonization. Nonetheless, Notch signaling is required for induction and maintenance T cell specification and differentiation at both the DN1 and DN2 stages[135].

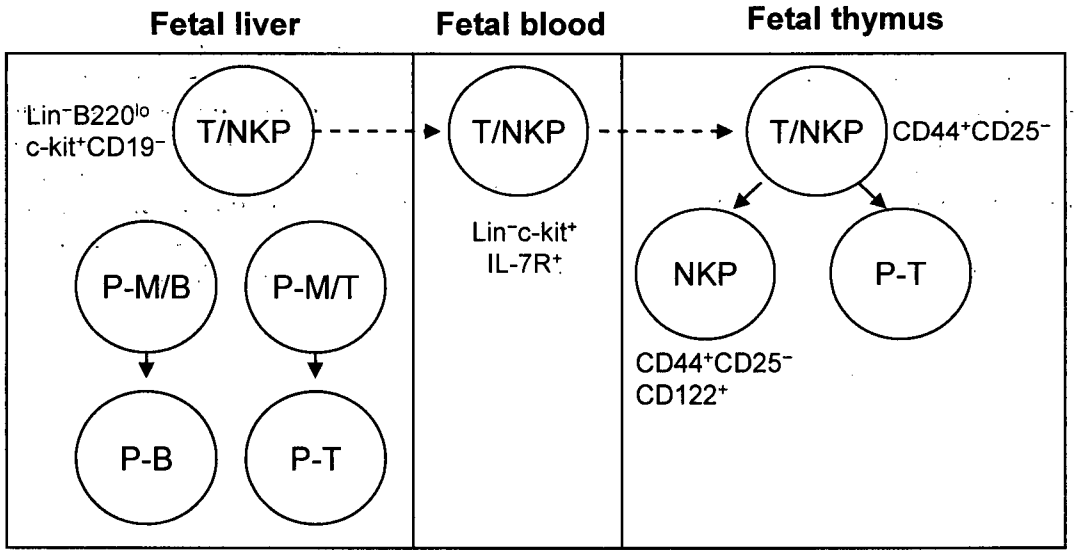


Figure 1.3. Lymphocyte development at fetal stage

Bipotent T/NK cell progenitors (T/NKP) are isolated from fetal liver, blood, and thymus. A bipotent T/B stage could not be found, but p-myeloid/B (p-M/B), p-M/T and unipotent p-B, and p-T are present in fetal liver. Revised from Tabatabaei *et al.*[141] and Katsura[125].

As with fetal B cells, fetal T cells are less dependent on IL-7R signaling to develop, since all major thymocyte subpopulations are generated, though in reduced numbers, in IL-7R$^{-/-}$ animals. A similar situation to B1 category of B cells also exists for Vγ3^{+} $\gamma\delta$ T cells. These cells originate more frequently from FL stem cells than from adult BM HSCs[131].

Evidence suggests that there are differences between fetal and adult NK cell development. From studies of fetal bipotent T/NKPs, it appears that fetal NK cells share a close developmental pathway with T cells, whereas a common precursor for B and NK cells seems to be more relevant in adult BM. In addition to progenitor differences, fetal and neonatal NK cells are phenotypically distinct from adult NK cells such that they lack surface expression of Ly49 receptors normally present on adult NK cells[142]. In contrast, the majority of fetal NK cells express a member of Ly49 family, Ly49E, which is almost absent on adult NK cells[143].

1.4.3. Markers of developing HSCs

Developmental stages of HSC differentiation have been identified using cell surface markers. These markers which have been mentioned throughout this thesis are listed in Table 1.1. CD41 is the first marker used for separation of committed hematopoietic progenitors from other mesodermal cell types in early embryo (Figure 1.2)[111]. CD41 (GPIIb), an αIIb integrin, is an adhesion molecule restricted to cells of megakaryocytic lineage in the adult. However, a number of studies have demonstrated the expression of CD41 on hematopoietic progenitors of YS, AGM, fetal liver and adult bone marrow[106,144-147]. Yet, it appears that CD41 expression is more limited to early hematopoiesis, as it gradually declines in the fetal liver

stage[106,108]. Moreover, adult HSCs do not express CD41[148]. Conversely, CD45 the panhematopoietic marker, is absent on immature HSCs and appears by ED11.5[122]. At this stage, an additional marker of adult HSCs, Sca-1, is also upregulated[122]. Several markers that are not restricted to hematopoietic cells are consistently expressed by both immature and mature fetal HSCs. These markers include CD34, CD31 (PECAM-1), and c-kit[121,149,150]. CD34 is a sialomucin expressed on cells of the hematopoietic and the endothelial lineages throughout murine ontogeny[151]. Mice deficient in CD34 have decreased numbers of YS and FL hematopoietic precursors, while adult hematopoiesis is less affected[152]. This is consistent with the expression pattern of CD34 on fetal and adult HSCs. CD34 expression on HSCs is downregulated by 10 weeks of mouse development[153].

Table 1.1. Cell surface molecular markers described in this thesis

Marker	Description/ Family relationship	Cell types expressing the marker	Biological function
Sca-1 (Ly6A/E)	Phosphatidylinositol-anchored protein, encoded by a member of interferon-inducible Ly6 family of genes	HSCs, MPPs, ELPs in adult BM[8,67], HSCs in fetal liver[123], subsets of B, T, myeloid, and non-hematopoietic lineages[154-156]	
c-kit (CD117)	Tyrosine receptor kinase	HSCs, CLPs and Pro-Ls[67], subpopulation of hematopietic cells, germ cells, melanocytes[68]	Growth-promoting and survival factor for hematopoietic stem/progenitors[68,157]
CD34	Sialmucin	Endothelial cells, hematopoietic progenitors in mouse, HSCs in human[158]	Binds CD62L and plays a role in adhesion in endothelial cells; also involved in adhesion and homing of hematopoietic progenitors[158,159].
Flt3	Tyrosine kinase receptor	Hematopoietic progenitors (MPPs, ELP, CLP, ETPs)[12]	Growth promoting and survival factor for lymphoid progenitors[68,70]
IL-7Rα (CD127)	Cytokine chain receptor for IL-7	CLPs, early T and B progenitors[160]	Plays a role in development, survival and proliferation of B and T cell precursors[160]

Marker	Description/ Family relationship	Cell types expressing the marker	Biological function
CD27	TNF receptor	Medullary thymocytes, T, NK and some B cells[16]	Binds CD70; a co-stimulator for T and B cells[16]
CD122 (IL-2Rβ)	Cytokine chain receptor for IL-2 and IL-15	NK lineage, resting T cell subsets, some B cell lines[16]	Involved in NK cell development via interaction with IL-15[141], activates NK cells through binding IL-2[161]
B220 (CD45R)	Tyrosine phosphatase, B cell specific form of CD45	B cells, some NK lineage[36]	Functions in signaling through BCR[16]
αβ TCR	antigen receptor	αβ T cells	Recognizes antigen in a complex with MHC molecules[16]
CD4	Immunoglobulin	T_H1 and T_H2 T cells, monocytes, marophages[16]	Co-receptor for MHC class II molecules[16]
CD8	Immunoglobulin	Cytotoxic T cells	Co-receptor for MHC class I molecules[16]
CD3	Immunoglobulin	T cells	Associated with the TCR; necessary for cell surface expression and signal transduction by the TCR[16]
CD44	Link protein	Leukocytes, erythrocytes	Binds hyaluronic acid, mediates leukocyte adhesion[16]
CD25 (IL-2Rα)	Cytokine chain receptor	Activated T cells, B cells, and monocytes	In combination with IL-2Rβ and γc binds IL-2, (IL-2 is T cell growth factor)[16]
γδ TCR	Antigen receptor	γδ T cells	Recognizes protein and nonprotein ligands[24]
CD62L (L-selectin)	C-type lectin	B, T,and NK cells, monocytes	Leukocyte adhesion molecule, binds CD34, GlyCAM, mediates rolling on the endothelium[16]
CD19	Immunoglobulin	B cells	Co-receptor for B cells[16]
NKG2D	C-type lectin	NK cells, subset of T cells	Forms homodimers; NK stimulating receptor implicated in immunity against viruses and tumors[162]
NK1.1 (NKR-P1C)	C-type lectin	NK cells, subset of T cells	NK cell stimulating receptor[162]
DX5 (CD49b)	α2 Integrin	NK cells	CD49b binds collagen or laminin, its function in NK cells is unknown[163]
Mac-1 (CD11b)	Integrin α	Myeloid and NK cells	Associates with CD18; binds CD54, complement component and extracellular matrix[16]
Ly49	C-type lectin	NK cells, subset of memory T cells	Activates or inhibits NK cells based on the type of receptor[162]

Marker	Description/ Family relationship	Cell types expressing the marker	Biological function
CD94	C-type lectin	T-cell subsets, NK cells[16]	Associates with NKG2A/C/E receptors to form NK receptors, plays a role in activation or inhibition of NK cells[43]
NKG2A/C/E	C-type lectin	NK cells, subsets of T cells	Associates with CD94, activates or inhibits NK cytotoxicity depending on the type of NKG2 receptors (A/C/E)[43]
NKRP1A (CD161)	C-type lectin	NK cells, T cells	Regulates NK cytotoxicity[16]
Granzyme B	Serine proteinase	NK cells, cytotoxic T cells[164]	Involved in cytolysis by triggering apoptosis[40]
CD43 (Leukosialin)	Mucin	Leukocytes, except resting B cells	Anti-adhesive role due to its extended structure[16]
CD56	Immunoglobulin	Human NK cells[66]	Isoform of human neural-cell adhesion molecule (NCAM), adhesion molecule[16,66]
CD45RA	Isoform of CD45	B cells, T cell subsets (naïve T cells), monocytes	See CD45
Integrin $\alpha_4\beta_7$		Fetal lymphoid tissue inducer (LTi) cells, human NK progenitors in lymph nodes[64]	Adhesion ($\alpha4\beta7$), Involved in hematopoiesis, development of placenta, heart ($\alpha4$), and gut lymphocytes ($\beta7$)[165]
Common γ chain (γc) (CD132)	Cytokine chain receptor used by IL-2, IL-4, IL-7, IL-9, IL-15, IL-21[57]	B cells, T cells, NK cells, mast cells, neutrophils[16]	Essential for development of T, B and NK cells[97,98]
CD45 (leukocyte common antigen, LCA)	Tyrosine phosphatase, multiple isoforms resulting from alternative splicing	All hematopoietic cells, except platelets and erythrocytes[11]	Augments signaling through BCR and TCR[16]
CD31 (PECAM-1)	Immunoglobulin	Monocytes, platelets, granulocytes, T cell subsets, endothelial cells	Adhesion molecule, mediates both leukocyte-endothelial and endothelial-endothelial interactions[16]
AA4.1 (CD93)		Early B cell progenitors, fetal liver cells, endothelial cells, neutrophils, monocytes[16,36,124]	unknown
CD41 (GPIIb)	α Integrin	Platelets, megakaryocytes, embryonic hematopoietic progenitors[16,111]	αIIb integrin, associates with CD61 to form GPIIb, mediates platelet functions: binds fibrinogen, fibronectin, von willebrand factor, and thrombospondin[16]
CD5	Scavenger receptor	Thymocytes, T cells, subset of B cells[16]	Possibly negative regulator of BCR[36]
Flk-1 (VEGF-	Tyrosine kinase receptor	Extraembryonic and	Binds VEGF, plays a role in

Marker	Description/ Family relationship	Cell types expressing the marker	Biological function
R2)		embryonic mesoderm, endothelial cells, hemangioblasts[166]	hematopoiesis and vasculogenesis[166,167]
VE-cadherin (CD144)	Cadherin	Endothelial cells	Organizes adherens junction in endothelial cells[16]
Ter119	Glycophorin A	Erythroid lineage[168]	Erythroid lineage marker
Gr-1	GPI-linked protein	Myeloid lineage (granulocytes)	Myeloid lineage marker[169]

1.5. Hematopoiesis from ES cells

Doetschman *et al.* initially noted the hematopoietic potential of ES cells differentiated in suspension cultures. In these cultures, ES cells formed cystic multilayered structures, known as embryoid bodies (EB), which resembled the egg-cylinder-stage embryos, and developed hemoglobinized blood islands in a reproducible fashion[170]. A sequential development of hematopoietic precursors in EB was observed. Primitive erythroid precursors appear first by day 4 of differentiation. Shortly afterwards, precursors of macrophage and definitive erythroid lineage arise, followed by bipotential neutrophil, macrophages, multilineage precursors, and finally mast cell precursors. The time course of EB blood cell development as well as the growth factor responsiveness of the EB-derived hematopoietic precursors suggests that these early cells are more similar to blood precursors of embryonic YS and early FL than those in the adult BM[171].

The ES/EB differentiation system has been used to investigate the developmental precursors to the hematopoietic lineage by dissecting EB cell populations prior to the initiation of hematopoietic development. Keller and colleagues identified a population of cells in day-3

EB, which is able to form blast colonies in methylcellulose culture in response to vascular endothelial growth factor (VEGF) (designated as blast-colony-forming cell, BL-CFC). Cells from these blast colonies were replated into other conditions, and were shown to be capable of differentiating into both primitive and definitive erythroid and myeloid hematopoietic precursors, as well as into endothelial cells. These studies supported the notion that the hematopoietic and the endothelial lineage share a common ancestor termed hemangioblast[172,173]. Comparable BL-CFC displaying hematopoietic and vascular endothelial potential was found in the posterior region of the primitive streak of ED7-ED7.5 mouse embryos, thereby providing evidence for the *in vivo* existence of hemangioblast (Figure 1.2)[174]. BL-CFC in both mouse embryo and EB co-express Flk-1 and Scl[174-176]. Gene-targeting studies have demonstrated that Flk-1 and Scl play an important role in hematopoietic and endothelial cell development. Flk-1 is a receptor tyrosine kinase that is stimulated by VEGF[167]. Mice deficient in *Flk-1* shows defects in the blood vessels and yolk sac blood islands[177]. In chimeric aggregation experiments between *Flk-1*$^{-/-}$ ES cells and wild type embryos, *Flk-1*$^{-/-}$ ES cells fail to contribute to primitive and definitive hematopoiesis or vasculogenesis[178]. Homozygous mutations in *Scl*, a basic helix-loop-helix transcription factor, causes death at around ED10.5 due to defective embryonic hematopoiesis[179,180]. An analysis of chimeric mice derived from *Scl*$^{-/-}$ ES cells demonstrated that Scl is required for definitive hematopoietic development[181] as well as endothelial cell development[166,182]. *Scl*$^{-/-}$ ES cells are unable to give rise to hemangioblast-derived blast colonies[183]. The aforementioned data is in agreement with a role for both Flk-1 and Scl in hemangioblast formation. Using knock-in ES cells carrying a non-functional human CD4 gene in one allele of *Scl*, Chung *et al.* showed that hemangioblasts within days 2.75-3 EBs co-express Flk-1

and Scl. As they further differentiate, these cells downregulate Flk-1 expression to become Flk-1$^-$Scl$^+$ cells. Only hematopoietic cells, but no endothelial cells, developed from Flk-1$^-$Scl$^+$ cells[176]. Endothelial cells were generated from Flk-1$^+$Scl$^+$ and Flk-1$^+$Scl$^-$ cell populations. The conclusion from these observations is that commitment to the hematopoietic lineage is accompanied by loss of Flk-1 expression and maintenance of Scl expression.

Mikkola *et al.* presented CD41 as the marker indicating divergence of definitive hematopoietic precursors from the hemangioblasts during EB differentiation, since endothelial cells only developed from CD41$^-$ cells. In this study, erythroid and myeloid precursors were enriched in CD41$^+$c-kit$^+$CD45$^-$ fraction in day-6 EBs, while the lymphoid potential of this population was not tested[108]. The phenotype described for hematopoietic progenitors from EBs is similar to embryonic HSCs. However, it is unclear whether these cells represent YS or AGM-derived hematopoietic precursors.

Several groups have reported the potential to repopulate irradiated adult mice with cells differentiated from ES cells *in vitro*. One study demonstrated that ES cells after at least 4 days of EB differentiation obtain repopulating capacity, as assessed by PCR 12 weeks after transplantation[184]. Another study documented transient lymphoid reconstitution in *Rag-1*$^{-/-}$ mice injected with CD45$^+$, AA4.1$^+$, B220$^-$ cells from day-15 EBs[185]. Very low lymphoid repopulation in immunodeficient SCID neonates has also been reported with day-11 to day-13 EB cells[186]. In both studies, EB cells used for engraftment had been differentiated long beyond the time point at which the hematopoietic lineage is initially specified. Thus, even though blood formation in early EBs recapitulates yolk sac hematopoiesis, the later stages

may represent AGM-type hematopoietic differentiation, which includes lymphopoiesis. Moreover, c-kit$^+$CD45$^+$ cells taken from day-7 to day-10 EBs differentiated in a cocktail of cytokines were shown to contribute to lymphomyeloid engraftment in irradiated mice[187]. This latter experiment has not been reproduced, and adult repopulation with ES-derived cells still remains a big challenge. As with YS HSCs, the difficulty of engraftment with ES/EB HSCs may reflect their immaturity. The molecular mechanisms responsible for directing HSCs differentiation from ES cells have not been defined[112]. However, genetic modifications of EB cells with the oncoprotein Bcr/Abl[188], the activated form of its signaling target Stat5[189], and the homeobox proteins HoxB4[190], and Cdx4[191] have resulted in reproducible multilineage hematopoietic repopulation in irradiated adult recipients.

Nakano *et al.* showed that co-culture with OP9 stromal line allows the generation of lymphohematopoietic progenitors from ES cells[192]. OP9 stromal cells were generated from newborn calvaria of *op/op* mice that are deficient in macrophage colony-stimulating factor (M-CSF)[193,194]. Unlike other stromal cell lines which preferentially support monocyte-macrophage generation from ES cells, M-CSF deficient OP9 cells promoted lymphoid differentiation[192]. Furthermore, B and T cells were efficiently generated from ES cells using ES/OP9 co-culture[195,196]. In these studies, the intermediate hematopoietic progenitors were not identified. Nakayama *et al.* reported that CD34$^+$ cells from day-6 to 7 of EBs differentiate into B and NK cells when co-cultured with OP9 stroma. These cells also had myeloid differentiation potential[197]. They also demonstrated that erythro-myeloid CFCs and lymphoid progenitors are enriched in CD34$^+$CD31^{lo} and CD34$^+$CD45$^-$ fractions, respectively[198]. The enrichment for both myeloid and lymphoid progenitors in the same population was not

shown. Since, both OP9 co-culture system and conventional EB system are used for the formation of hematopoietic cells from ES cells, Zhang e al. compared hematopoietic development from ES cells in these two systems. While similar kinetics of Scl and Flk-1 expression was observed in both methods, OP9 culture promoted development of endothelial ($Flk1^+VE\text{-}cadherin^+$) cells, whereas $CD45^+$ and $Ter119^+$ hematopoietic cells preferentially developed within EBs[199].

Using the EB system, Lian *et al.* devised a multi-step differentiation culture to recapitulate NK cell development from hematopoietic precursors. In this model, ES cells were induced to form EBs. $CD34^+$ cells from day-8 EBs were cultured on OP9 with SCF, Flt3-L, IL-7 and IL-6 to differentiate into cells of hematopoietic lineage. In a final step, these cells became NK cells in presence of IL-2 and IL-15[200].

1.6. Factors regulating definitive hematopoietic development

As previously explained, definitive hematopoiesis refers to the development of all lineages other than primitive erythroid and encompasses definitive erythroid, myeloid, and lymphoid lineages[105]. The genes known to specify blood fate in the embryo are discussed.

1.6.1. Scl

Scl is a transcription factor, which can be used to track early hematopoietic commitment[199]. Scl null EBs do not form BL-CFC hemangioblasts[183]; instead they form blast-like colonies

with endothelial but not hematopoietic potential[175]. Knockout studies have shown that lack of Scl impairs hematopoietic development, while endothelial cells can still develop, indicating a pivotal role for Scl in hematopoietic commitment but not in the early stage of vasculogenesis. Scl expression extends from all hemogenic embryonic tissues to adult HSCs[112]. However, the deletion of Scl from adult BM has no effect on HSC engraftment, self-renewal, and differentiation into myeloid and lymphoid lineages, and only proper differentiation to erythroid and myeloid lineages is impaired. This indicates that the sustained expression of Scl is not essential for HCS functions[112,201]. Scl interacts with other transcription factors such as Lmo2 and Gata-2[112].

1.6.2. Notch1

The Notch receptors regulate a wide range of developmental processes including hematopoiesis. YS primitive hematopoiesis occurs normally in the absence of Notch1, but *in vivo* definitive repopulating potential of *Notch1*$^{-/-}$ cells derived from P-Sp is severely impaired. Furthermore, preventing Notch signaling by γ-secretase inhibitors abolishes *in vitro* hematopoietic activity from P-Sp explants (ED9.5) but not from AGM explants (ED10.5). Therefore, Notch1 regulates definitive hematopoiesis during a narrow time window possibly around P-Sp stage, and is not required at the later stages of hematopoietic development[112]. Interactions with other key factors, such as Scl, Aml1 and Mll need to be discovered. Adult HSC's self-renewal is promoted by ligand-mediated Notch stimulation, or expression of constitutively active forms of Notch[33].

1.6.3. Aml1

The Aml1 (Runx1/Cbfα) gene encodes the DNA-binding subunit of a transcription factor of the core binding factor (CBF) family[202]. Targeting studies have demonstrated that Aml1 is essential for the establishment of the definitive but not primitive hematopoiesis. *Aml1*$^{-/-}$ embryos reveal intact primitive YS hematopoiesis but die at ED12 – 13[203,204]. Before death, the liver rudiment is devoid of all definitive erythroid, myeloid, and megakaryocytic cells but contains primitive erythrocytes, indicating a complete block in the development of definitive hematopoietic potential. *Aml*$^{-/-}$ ES cells were also unable to differentiate into definitive hematopoietic cells[203]. *Aml1* hemizygous mouse display a milder phenotype, suggesting a gene dosage effect in the process of definitive hematopoiesis[112,205]. While reducing HSC activity in the AGM region, haploinsufficiency of *Aml1* causes an earlier appearance of HSCs in the normal position in AGM and also in YS[206]. Aml1 is expressed in precursors with hemangioblast properties in EBs. Consistent with the *in vivo* findings, *Aml1*$^{-/-}$ EBs give rise to normal numbers of primitive erythroid colonies but are unable to differentiate to definitive hematopoietic lineages[207]. Haploinsufficiency of *Aml1* in EBs causes the acceleration of mesodermal commitment and hemangioblast development[208].

1.6.4. Mll

The Mll (All-1/Hrx1) gene is disrupted in leukemic cells displaying both lymphoid and myeloid differentiation programs (mixed lineage leukemia), suggesting the involvement of an early progenitor. The requirement for Mll in definitive hematopoiesis was shown in

Rag2$^{-/-}$ chimeric animals reconstituted with *Mll*$^{-/-}$ or heterozygous ES cells. In adult chimeric animals, Mll-deficient cells could not generate lymphocytes. Analysis of fetal chimeras showed a deficiency in fetal liver HSC/progenitor populations, which is preceded by a reduction of HSC activity in AGM. Mll is thought to regulate Hox genes[112,209].

1.6.5. Hox genes

Homeobox-containing Hox genes are believed to be the target of signaling pathways involved in anteroposterior (AP) patterning and mesoderm specification during development. Hox genes show an overlapping and graded expression pattern along the AP axis, and possibly direct the position of tissue formation[112]. Several Hox genes are expressed in definitive HSCs but not in YS, including HoxB3, B4, A4 and A5[210,211]. Overexpression of HoxB4 in adult BM enhances hematopoietic repopulation without inducing leukemia or interrupting normal hematopoietic differentiation. Hox B4 seems to regulate self-renewal of definitive HSCs[212]. Moreover, Hox B4 increases the formation of mixed hematopoietic colonies from ES cells *in vitro*[213]. Finally, ectopic expression of HoxB4 in ES cells and YS hematopoietic progenitors confers definitive lympho-myeloid engraftment potential[190].

1.6.6. Cdx genes

Cdx genes are the target of morphogens, such as retinoic acid, fibroblast growth factor, and Wnt family members, which mediate positional patterning, and are thought to convey positional information from these pathways to the Hox gene family[112]. Genetic analysis in the

zebrafish has elucidated a link between Cdx genes, Hox genes and hematopoiesis. Cdx4 was shown to be mutated in *Kgg* zebrafish mutants, which display a defect in hematopoiesis, abnormal anterposterior patterning and aberrant Hox gene expression. The hematopoietic deficiency could be rescued by overexpression of HoxB7a and HaxA9a, but not Scl, indicating that the Cdx regulation of Hox genes make the posterior mesoderm competent for blood development. Ectopic expression of Cdx4 promotes hematopoietic colony formation of ES cells during EB development[214]. In addition, expression of several hematopoietic genes, including Scl, Aml1, and Gata1 is reduced in Cdx4 null zebrafish, whereas overexpression of Cdx4 results in ectopic blood formation and upregulation of HoxB4[214]. Furthermore, overexpression of Cdx4 during early EB development was shown to enhance lymphomyeloid engraftment potential of ES-derived hematopoietic progenitors[191]. Therefore, it seems that a genetic pathway involving Cdx and Hox genes plays an essential role in specification of definitive hematopoiesis in the EB system.

1.7. Thesis objectives and hypotheses

The main objective of my thesis was to study the differentiation pathway of lymphocytes, in particular NK cells from ES cells. Even though it is possible to derive lymphocytes from ES cells, the developmental pathway leading to lymphocytes and the intermediate progenitors along the pathway have not been described. Since the EB system is more efficient in hematopoiesis than other methods of hematopoietic differentiation from ES cells, this method was chosen to identify lymphocyte precursors from ES cells. The culture system developed by Lian *et al.*[200] was modified and used to characterize NK cell progenitors at different steps

in development and the relationship between the NK and other hematopoietic lineages was examined. My hypotheses were that: 1) Co-culture with OP9 and cytokines induces development of NK progenitors from $CD34^+$ EB cells. 2) These NK progenitors may have other lymphoid potentials similar to CLPs or bipotent T/NKPs. 3) $CD34^+$ EB cells are heterogeneous and may include non-hematopoietic lineages. The following objectives were formulated to test these hypotheses: 1) to identify NK intermediate progenitors within cells differentiated from $CD34^+$ EB on OP9 by testing NK potential of various populations at different time points in limiting dilution assays. 2) to test other lineage potentials of these NK progenitors in *in vitro* lineage-specific cultures. 3) to purify hematopoietic progenitors in $CD34^+$ EB population using cell surface markers.

Chapter 2. Materials and methods

2.1. Cell lines

OP9 cells were obtained from RIKEN (Tokyo, Japan). OP9 cells transduced with Delta-like1 and green fluorescent protein (OP9-DL1) were kind gift from Dr. J-C Zuniga Pflucker (Toronto, Canada). R1 ES cell line (129/SvJ strain) was provided by Dr. RK Humphries (Vancouver, Canada). OP9 and OP9-DL1 cells were cultured in Minimum essential medium eagle, alpha modification with nucleosides (αMEM) with 10% FBS (HyClone), penicillin, and streptomycin.

2.2. Antibodies

Antibodies used in this thesis are listed in Table 2.1.

Table 2.1. List of antibodies

Antibody	Clone	Animal	Isotype	Conjugate	Company
NKG2A/C/E	20d5	mouse	Rat IgG_{2a}, κ	biotin	PM
CD94	18d3	mouse	Rat IgG_{2a}, κ	biotin	PM
NKG2D	C7	mouse	Ar Ham IgG	biotin	Biolegend
CD3ε	145-2C11	mouse	Ar Ham IgG1, κ	biotin	PM
CD8a (LY-2)	53-6.7	mouse	Rat IgG_{2a}, κ	PE	Boehringer
CD4	GK1.5	mouse	Rat IgG_{2b}, κ	biotin	PM
gamma-delta TCR	GL3	mouse	Ar Ham IgG2, κ	biotin	PM
TCR beta chain	H57-597	mouse	Ar Ham IgG2, λ1	biotin	PM
CD44	1M7	mouse	Rat IgG_{2b}, κ	biotin	PM
CD25 (IL-2 R alpha chain)	3C7	mouse	Rat IgG_{2b}, κ	PE	PM
CD19	1D3	mouse	Rat IgG_{2a}, κ	biotin	PM
B220	RA3-6B2	mouse	Rat IgG_{2a}, κ	biotin	homemade
Mac-1	M1/70	mouse	Rat IgG_{2b}, κ	biotin	homemade
Gr-1	RB6-8C5	mouse	Rat IgG_{2b}, κ	biotin	homemade
TER-119	Ter-119	mouse	Rat IgG_{2b}, κ	biotin	PM
CD127 (IL-7 R alpha chain)	A7R34	mouse	Rat IgG_{2a}, κ	biotin	eBioscience

Antibody	Clone	Animal	Isotype	Conjugate	Company
CD122 (IL-2R beta chain)	TM-β1	mouse	Rat $IgG_{2b,\kappa}$	PE	PM
common gamma chain (γc)	TUGm2	mouse	Rat $IgG_{2b,\kappa}$	biotin	PM
CD45.2 (Ly5.2)		mouse	mouse IgG_{2a}	purified	homemade
CD45.2 (Ly5.2)		mouse	mouse IgG_{2a}	FITC	homemade
CD45	30-F11	mouse	Rat $IgG_{2b,\kappa}$	APC	PM
CD34	RAM34	mouse	Rat $IgG_{2a,\kappa}$	biotin	eBioscience
CD34	RAM34	mouse	Rat $IgG_{2a,\kappa}$	FITC	PM
Flk-1	Avas 12α1	mouse	Rat $IgG_{2a,\kappa}$	PE	PM
c-kit	2B8	mouse	Rat $IgG_{2b,\kappa}$	PE-CY7	PM
c-kit	2B8	mouse	Rat $IgG_{2b,\kappa}$	biotin	PM
Sca-1 (Ly6A/E)	E13-161.7	mouse	Rat $IgG_{a,\kappa}$	PE	PM
CD31	MEC-13.3	mouse	Rat $IgG_{2a,k}$	biotin	PM
CD31	MEC-13.3	mouse	Rat $IgG_{2a,k}$	purified	PM
CD41	MWReg30	mouse	Rat IgG1, k	FITC	PM
Streptavidin (SA)				FITC	PM
Streptavidin (SA)				APC	PM
Streptavidin (SA)				PE	PM
Streptavidin (SA)				PE-Texas Red	PM
Goat anti-mouse IgG				Alexa Flour 647	Molecular Probes
Goat anti-rat IgG				Alexa Flour 647	Molecular Probes

PM: Pharmingen

2.3. Cell culture

2.3.1. ES cell culture

The ES cell line R1 was maintained on gelatin-coated tissue culture flasks in Dulbecco's modified Eagle's medium (with 4500 mg D-glucose/L) (DMEM) containing 15% FBS, 2 mM L-glutamine, 0.1 mM nonessential amino acids (StemCell Technologies, Vancouver, Canada), 1,000 U/ml leukemia inhibitory factor (Chemicon International, Temecula, CA) and 100 μM monothioglycerol (MTG) (Sigma-Aldrich, Oakville, Canada)[200]. Two days before initiation of differentiation, ES cells were passaged in Iscove's modified Dulbecco's medium (IMDM) supplemented with the same reagents used in the maintenance medium. On the day

of differentiation, cells were harvested, resuspended at 1,500-2,500 cells/ml in the differentiation medium consisting of IMDM, 15% FBS, 1% methylcellulose, 2 mM L-glutamine, 150 μM MTG, 50 ng/ml mouse stem cell factor (SCF), 30 ng/ml mouse IL-3, and 30 ng/ml human IL-6, and 1 ml of the cell suspension was dispensed into 35 mm petri dish (StemCell Technologies, Vancouver, Canada)[187,200]. The cells were incubated at 37°C and 5% CO_2 for 8 days for EB formation. EB cells were then harvested, trypsinized and made into single-cell suspension by passing them through a 21-gauge 1½-inch needle three times. Subsequently, the cells were stained with biotinylated anti-CD34 followed by streptavidin-PE and FITC conjugated anti-CD45.2 or purified CD45.2 followed by Alexa Flour 647-conjugated goat anti-mouse IgG, and sorted on FACSVantage^{TM} SE (BD Biosciences, San Jose, CA) for isolation of $CD34^+$, $CD34^+CD45^-$ and $CD34^+CD45^+$ EB cells. In other experiments, to fractionate $CD34^+CD45^-$ EBs according to c-kit and CD41 expression, EB cells were stained with biotinylated anti-CD34 followed by streptavidin-PE-Texas Red, APC-conjugated anti-CD45, PE-Cy7-conjugated anti-c-kit and FITC-conjugated anti-CD41 mAbs and sorted into $CD34^+CD45^-$c-kit^+CD41^+, $CD34^+CD45^-$c-kit^-CD41^-, $CD34^+CD45^-$c-kit^+CD41^-, and $CD34^+CD45^-$c-kit^-CD41^+ fractions. Sorted EB cells were then either directly transferred into lineage differentiation cultures or into ES-HP culture (see below).

2.3.2. ES-HP culture

Sorted EB cells were seeded onto OP9 stroma in 24-well plates at a concentration of $4x10^4$ cells/well and cultured for 7 days in αMEM with 5% FBS, 30 ng/ml human IL-6, 4 ng/ml mouse IL-7, 40 ng/ml mouse SCF, 100 ng/ml human Flt3-L and 100 μM MTG (Stem Cell Technologies) to generate ES-HPs[200]. After the first 3-4 days of incubation, half the media

was replaced with fresh medium containing the same cytokines. ES-HPs were harvested on day 7 by vigorous pipetting, washed, and passed through a 45 μm filter. Cells were then stained for FACS analysis or sorted and transferred into lineage differentiation cultures.

2.3.3. Lineage differentiation cultures

OP9 and OP9-DL1 cells used in lineage differentiation cultures were cultured for 2 days in 24 well plates to pre-form stroma layers. Cells to be tested for lineage-specific differentiation were plated onto the pre-formed stroma and cultured. If the culture period was longer than one week, stromal cells were replaced with the new cells. To do so, cells were harvested by pipetting, passed through 45 μm filter, and mixed with new media and plated on new pre-formed stroma.

2.3.3.1. NK cell culture

EB cells or ES-HPs were plated on OP9 layers in OP9 media containing 1,000 U/ml human IL-2 (PeproTech, Rock Hill, NJ) and 5 ng/ml human IL-15 (StemCell Technologies)[200]. Cells were cultured for 7-14 days before FACS analysis.

2.3.3.2. T cell culture

EB or ES-HP cells were seeded onto OP9-DL1 stroma with 5ng/ml IL-7, and 10 ng/ml Flt3-L and cultured for 8-14 days. After this period, media was removed from the culture and replaced with media containing Flt3-L and 1 ng/ml IL-7[77,215,216]. Cells were cultured for additional 2-6 days and harvested for FACS analysis or RNA extraction.

2.3.3.3. B cell culture

To show the B cell potential of EB cells, EB cells were cultured on OP9 with 5 ng/ml IL-7, 10 ng/ml Flt3-L and 1,000 U/ml IL-2 for 7-8 days. At this time, cells were transferred onto

fresh OP9 with only IL-7 and Flt3-L and cultured for additional 6-7 days[197]. For differentiation of ES-HPs into B cells, ES-HP cells were cultured on OP9 stroma with 5 ng/ml IL-7 and 10 ng/ml Flt3-L for one week[195].

2.3.3.4. Erythroid/Myeloid colony forming

To test the myeloid colony forming capacity of EB or ES-HP cells, cells were resuspended in IMDM, transferred onto Methocult™ GF M3434 media (StemCell Technologies) at the density of 1000/plate or 5000/plate in duplicate and cultured for 7 – 14 days. The numbers of all colonies including erythroid burst-forming units (BFU-E), granulocyte-macrophage colonies (CFU-GM/CFU-G/CFU-M), and erythrocyte-containing mixed colony-forming units (CFU-GEMM) were scored.

2.4. Staining and FACS sorting or analysis

Cells were first preincubated with 2.4G2 hybridoma supernatant or human γ globulin (Sigma-Aldrich) to block Fc-receptors, and then stained with mAbs. All incubations were performed on ice for 30 minutes. Propidium iodide (at the final concentration of 5 μg/ml) or 4'-6-Diamidino-2-phenylindole (DAPI) (at the final concentration of 100 ng/ml) was added to stain dead cells. Stained cells were analysed on a FACSCalibur (BD Biosciences, San Jose, CA) with CellQuest Pro software (BD Biosciances). Cell sorting was carried out on a FACSVantage™ SE (BD Biosciences).

2.5. RT-PCR

RNA from bulk cells was isolated with QIAGEN's RNeasy® Mini Kit and reverse transcribed into cDNA using QIAGEN's Omniscript Reverse Transcription kit. For samples

from limiting dilution cultures, cells in 96-well plates were lysed with 50 µl guanidinium isothiocyanate solution, RNA was isolated (as described[210]) and reverse transcribed using a 18-mer oligo(dT) primer (1 µg/µl) (New England BioLabs, Pickering, OT) and Superscript™ II (Invitrogen, Carlsbad, CA). Aliquots (1/5) of cDNA thus generated were used in PCR reaction. Forward primers from TCR Vγ2, 3, 4, and 5, and reverse primer from the TCRγ constant region have been described [60]. The primer sequences are listed in Table 2.2. The 50µl reaction volume contained 5µl of 10x PCR buffer, 1.5 µl of 50mM $MgCl_2$, 1 µl of 10mM dNTPs, 1 µl of each 10 µm forward and reverse primers, and 0.5 µl of 5 U/µl *Taq* DNA polymerase. The PCR thermocycling conditions for TCRγ RT-PCR were as follows: 5 min at 96°C followed by 40 cycles of 96°C for 15 s, 50°C for 40 s, 72°C for 1 min, and a final 10 min extension at 72°. The following condition was used for granzyme B RT-PCR: 1 min at 94°C followed by 40 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s. 18 µl of PCR products from bulk cultures were mixed with 2 µl of 10x loading buffer and analyzed on 1.2% agarose gel.

Table 2.2. List of primers

RT-PCR:
Vγ2: CCTTGGAGGAAGAAGACGA
Vγ3: CATCGGATGAAGCCACGTA
Vγ4: AGTGACAGAAGAGGACACG
Vγ5: CGATTCTGCTCTGTACTACT
C-region: CTTATGGAGATTTGTTTCAGC
Granzyme B(5'): CAAAGGCAGGGGAGATCATC
Granzyme B(3'): CTCTTCAGCTTTAGCAGCATG
CD3ε(5'): GCCTCAGAAGCATGATAAGC
CD3ε(3'): AGACTGCTCTCTGATTCAGG
RAG-1(5'): TGCAGACATTCTAGCACTCTGG
RAG-1(3'): ACATCTGCCTTCACGTCGAT
GAPDH(5'): TCAACGACCCCTTCATTGACCTC

RT-PCR:
GAPDH(3'): AGACTCCACGACATACTCAGCAC
Oligonucleotide probe:
Jγ1: TGCAAATACCTTGTGAAAACCTGAG

2.6. Southern blot

The gel from TCRγ RT-PCR from bulk cultures was alkaline blotted to ZetaProbe membranes (Bio-Rad, Hercules, CA). In limiting dilution experiments, the entire TCRγ PCR products from individual wells were transferred onto ZetaProbe membranes using Bio-Dot® Microfiltration apparatus (Bio-Rad) according to manufacturer's protocol. The membrane was probed with biotin-labeled oligonucleotides and visualized by North2South® Chemiluminescent Nucleic Acid hybridization and Detection Kit (Pierce, Rockford, IL). The 3' end of oligonucleotide was labeled with biotin-14-dATP according to the manufacturer's (Invitrogen) protocol. The probe sequence is shown in Table 2.2.

2.7. Limiting dilution analysis

OP9 and OP9-DL1 cells were X-ray irradiated (30Gy) and cultured for 2 days in 96 well plates to pre-form stroma layers. Limiting numbers of cells were deposited into 96-well plates containing the pre-established stroma using the CloneCyt Plus option of FACSVantage™ SE cell sorter. To determine NK progenitor frequency, cells were cultured for 10-12 days on OP9 stroma in IL-2 and IL-15. For T progenitor frequency, cells were cultured on OP9-DL1 stroma with 5 ng/ml IL-7 and 10 ng/ml Flt3-L for 10-12 days. Cells were then cultured with 1 ng/ml IL-7 for additional 3-5 days. After this culture period, RNA was isolated from cells as described in RT-PCR section. RNA thus generated was reverse

transcribed into cDNA and used in PCR reaction. The presence of NK cells was confirmed by granzyme B RT-PCR (Table 1.1). Since γδ T cell lineage seems to arise earlier than αβ T cells during T cell development[28], TCRγ RT-PCR was used to detect cells of T lineage. The Cγ1 cluster of the TCRγ locus, which contains four Vγ2, 3, 4 and 5 segments, is frequently rearranged to Jγ1 segment and expressed as Vγ2/3/4/5-Jγ1Cγ1 products by the fetal and/or adult γδ T cells[217]. Therefore, a mixture of Vγ2, 3, 4, and 5 primers was paired with a reverse primer from TCRγ constant region (a sequence shared by Cγ1, 2, and 4)[60]. Rearranged TCRγ transcripts were detected by southern blotting using a probe to Jγ1. Progenitor frequency was determined using Poisson statistics and the method of maximum likelihood using L-Calc™ software (StemCell Technologies).

Chapter 3: Characterization of developmental pathway of natural killer cells from embryonic stem cells *in vitro*[1]

3.1. Introduction

The NK cell developmental pathway includes the formation of committed NKPs from multipotent progenitors. Currently, a number of intermediate progenitors derived from HSCs are thought to generate $CD122^+$ NKPs. These progenitors include CLPs or $Lin^-c\text{-}kit^{lo}$ pro-lymphocytes in the adult and bipotent T/NKPs in the fetus. In the ES cell differentiation model used by Nakayama *et al.*[197], it was shown that $CD34^+$ hematopoietic progenitors from EB cells give rise to NK and B cells. However, the developmental stages and intermediate progenitors were not characterized. Furthermore, using a multi-step culture system, Lian *et al.*[200] showed that the first stage of NK development from $CD34^+$ EB cells involves differentiation on OP9 stroma with SCF, Flt3-L, IL-7 and IL-6 cytokines, which generates cells we called ES-derived hematopoietic progenitors (ES-HPs). In the second step, ES-HPs gave rise to cells of NK lineage. No other lymphocytes were generated in this culture system.

In this study, the ES cell culture system was used to determine the differentiation pathway from ES cells to NK cells. $CD34^+$ EB cells were isolated and differentiated into ES-HPs. These ES-HPs had NK, T, and very low myeloid potentials. Further characterization of $CD34^+$ EBs showed that NK and T potentials were contained in a $CD45^-$ subset, whereas

[1]A version of this chapter has been published. Tabatabaei-Zavareh N, Vlasova A, Greenwood CP, Takei F. Characterization of developmental pathway of natural killer cells from embryonic stem cells in vitro. PLoS ONE. 2007;2:e232.

$CD45^+$ EB cells had myeloid but not lymphoid potential. Limiting dilution analysis of ES-HPs generated from $CD34^+CD45^-$ EB cells showed that $CD45^+Mac\text{-}1^-Ter11^-$ ES-HPs are highly enriched for NK progenitors but they also had T, B and myeloid potentials.

3.2. Results

3.2.1. NK and T cell potentials of ES-HPs

The previously described ES cell culture system[200] was slightly modified and used in this study (depicted in Figure 3.1). Briefly, R1 ES cells were cultured in methylcellulose for 8 days for EB formation. $CD34^+$ EB cells were sorted and cultured on OP9 stromal cells with SCF, Flt3-L, IL-7 and IL-6 for 7 days. As previously reported[200], the cells generated in this culture, termed ES-HPs, further differentiated into NK cells upon co-culturing with OP9 in the presence of IL-2 and IL-15. Typically, from 4×10^4 EB cells, 10^5 ES-HP cells were generated per well. From those ES-HP cells, 5×10^5 NK cells were produced per well. The NK cells derived from ES cells (ES-NK cells) expressed CD94, NKG2A/C/E receptors on the surface as well as granzyme B transcripts (Figure 3.2A, Figure 3.3D). It has previously shown that similar to fetal NK cells, ES-NK cells lack Ly49 expression but express NKRP1A (CD161) mRNA and are capable of differentially killing some tumor cell lines and MHC class I-deficient lymphoblasts[200]. To test whether ES-HPs have potential for the T cell lineage, they were co-cultured with OP9-DL1 cells, which promotes T cell differentiation from progenitors[218], in the presence of IL-7 and Flt3-L for 8-12 days. RT-PCR analysis of the resultant cells showed the expression of rearranged TCRγ mRNA as well as CD3ε and RAG1 mRNA (Figure 3.2B, C). Rearranged TCRγ mRNA was undetectable in ES-derived NK cells

(Figure 3.2B, bottom row). Moreover, flow cytometric analysis showed the surface expression of TCRβ and TCRγ (Figure 3.2D). Thus, ES-HPs included T cell progenitors. ES-HPs were also tested for the myeloid differentiation potential by *in vitro* colony forming cell assays. The ES-HP population contained very low frequency of erythroid/myeloid colony forming cells (Figure 3.2E).

To further characterize lymphoid progenitors among the ES-HP population, we analyzed the expression of markers associated with BM lymphoid progenitors. Flow cytometric analysis showed that most ES-HPs expressed c-kit whereas only very small fractions expressed IL-7Rα (CD127), common γ chain (γc, CD132), IL-2Rα (CD25) and IL-2Rβ (CD122) (Figure 3.3A). Furthermore, only 10% of ES-HPs expressed CD45, suggesting that the majority of the ES-HPs are non-hematopoietic or mature erythroid cells. As expected, cells expressing IL-7Rα and γc were enriched in the $CD45^+$ subset of ES-HPs (Figure 3.3B). Limiting dilution culture analysis for NK progenitors showed that on average 0.5% (1 in 202) of the bulk ES-HPs differentiate into NK cells. As expected, NK progenitors were more enriched in $CD45^+$ ES-HPs (1 in 53 or 1.9%) than in $CD45^-$ ES-HPs (1 in 286) (Figure 3.3C,D).

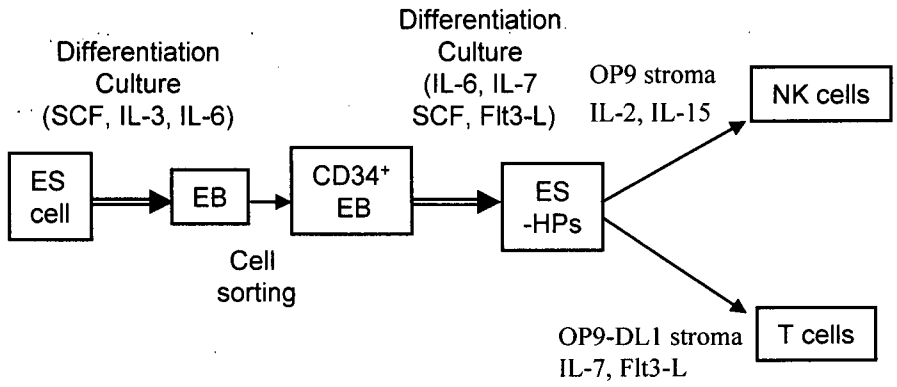
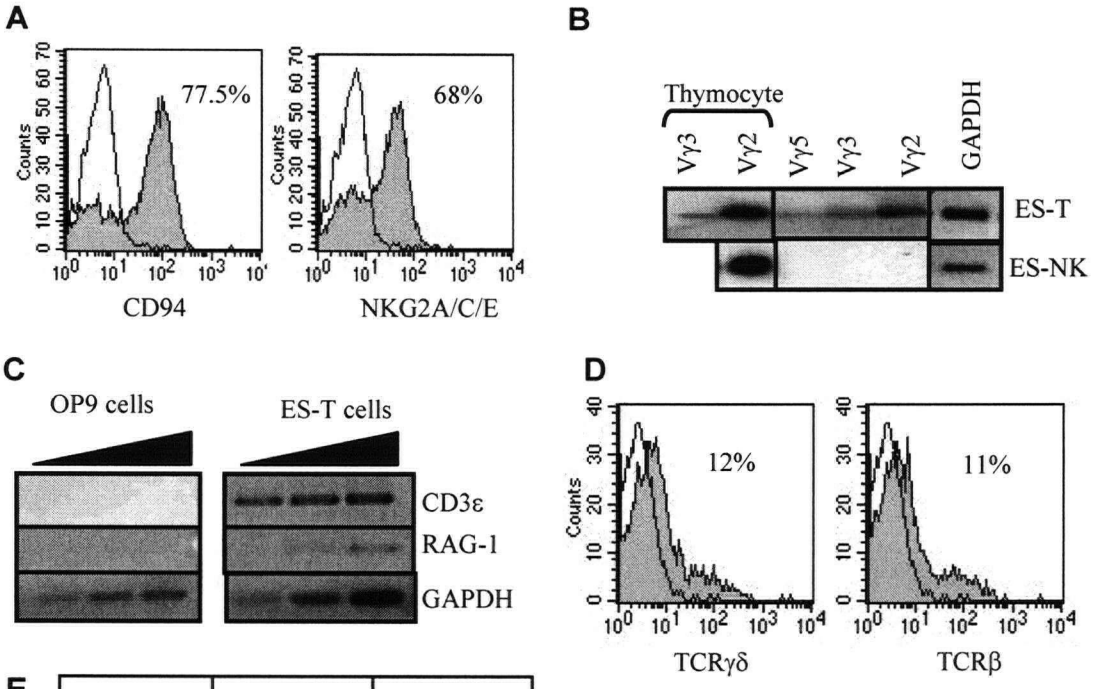


Figure 3.1. Schematic representation of ES cell differentiation culture.

ES culture system to generate ES-HPs is illustrated. ES cells are induced to form EBs in methylcellulose. $CD34^+$ EB cells are sorted and cultured for one week with OP9 stroma in the presence of indicated cytokines to generate ES-HP cells. ES-HPs are cultured on OP9 stroma with IL-2 and IL-15 for NK cell differentiation or on OP9-DL1 stroma with IL-7 and Flt3-L for T cell differentiation.



E

Expt. No.	CD34$^+$ EB	ES-HP
1	2.3 /1000	5 /1000
2	5.9 /1000	10.6 /1000

Figure 3.2. Lineage potential of ES-HPs generated from CD34$^+$ EBs.

ES-HPs derived from CD34$^+$ EB cells were incubated in NK and T culture as described in Figure 3.1. **(A)** NK cells generated from ES-HPs were stained for the indicated receptors and analyzed by flow cytometry. Filled histograms show staining with the appropriate mAbs and open histograms show isotype-matched control antibody staining. Percentages of positively stained cells over the control staining are shown. Dead cells were stained with propidium iodide and gated out. Residual OP9 cells were also gated out by their high scatter profile. **(B)** RNA was isolated from 2×10^5 bulk T cells and 2×10^6 bulk NK cells derived from ES-HPs (ES-T and ES-NK, respectively) and converted into cDNAs. Aliquots (1/30) of cDNAs were subjected to PCR for rearranged TCRγ genes. The PCR products were blotted and hybridized to a Jγ1 probe. Thymocytes were used as positive control for rearranged Vγ2 and Vγ3 TCR mRNA (top) and Vγ2 TCR mRNA (bottom). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) RT-PCR was also used as control. **(C)** cDNA was generated from ES-T cells as in (B). One microlitre of undiluted, 1/10 and 1/100 diluted cDNA was subjected to PCR for CD3ε and RAG-1. OP9 cells were used as negative control. **(D)** ES-T cells were harvested on day 7, stained for TCRγδ and TCRβ and analyzed using a flow cytometer as in (A). OP9-DL1 cells expressed green fluorescent protein and were gated out by green fluorescence. **(E)** One thousand CD34$^+$ EBs and ES-HP cells were plated in methylcellulose media for myeloid and erythroid colony formation and the total number of myeloid and erythroid colonies were counted as described in materials and methods.

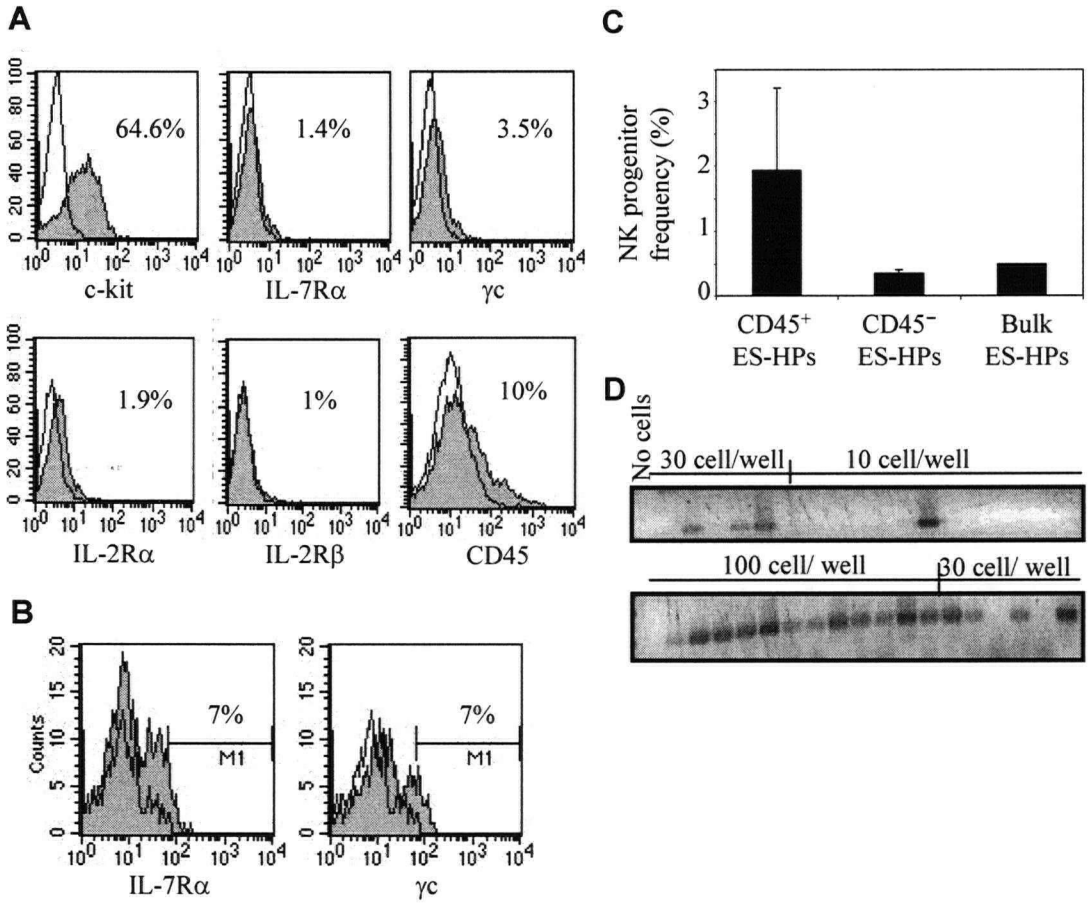


Figure 3.3. Characterization of NK progenitors within ES-HPs.

(A) ES-HP cells derived from $CD34^+$ EBs were stained for the indicated markers and analyzed by flow cytometry as in Figure 3.2A. **(B)** ES-HPs were co-stained with anti-CD45.2 and IL-7Rα mAbs (left panel), or anti-CD45.2 and γc mAbs (right panel) and analyzed by flow cytometry. CD45 positive cells were gated and analyzed for the expression of indicated receptors. **(C)** Irradiated OP9 cells were cultured for 2 days in 96 well plates. ES-HPs were stained with anti-CD45.2 mAb and $CD45^+$ and $CD45^-$ ES-HPs were sorted by FACS into the wells with pre-formed OP9 stroma layers. For $CD45^+$ ES-HPs, 10, 30 and 100 cells per well were sorted into 12 wells each. For $CD45^-$ and bulk ES-HPs, 30, 100 and 300 cells per well were sorted into 12 wells each. After culturing with appropriate cytokines for NK cell differentiation, cells in individual wells were harvested and analyzed for the presence of NK cells by granzyme B RT-PCR. Statistical analysis was performed using L-Calc™ software. The results are means ± SD of three independent experiments, except for bulk ES-HPs, which is average of two experiments. **(D)** One example of RT-PCR for granzyme B expression in limiting dilution experiments is shown. cDNA is from sorted $CD45^+$ ES-HPs cultured in NK condition as in (C). Specificity of RT-PCR products was confirmed by size and southern hybridization to granzyme B-specific oligo probe.

3.2.2. Heterogeneity of $CD34^+$ EB cells: $CD34^+CD45^-$ EB cells have lymphoid potential

The above results showed that the ES-HP population was very heterogeneous, containing hematopoietic and possibly non-hematopoietic (e.g. endothelial) cells, and the frequency of NK progenitors among ES-HPs was low. To determine whether this is due to heterogeneity of $CD34^+$ EB cell population that gave rise to ES-HPs, we analyzed the expression of various hematopoietic cell markers on $CD34^+$ EB cells (Figure 3.4A). Large fractions of $CD34^+$ cells expressed the hematopoietic progenitor-associated markers c-kit (65%) and Sca-1 (35%), and almost all expressed CD31, a marker for endothelial cells[219] and erythroid progenitors[150]. No significant levels of IL-7Rα (CD127) and mature hematopoietic lineage markers, including Mac-1, Gr-1, Ter119, B220, CD19, CD3 and CD8 were detected on $CD34^+$ EB cells. Approximately 15-25% of $CD34^+$ EB cells expressed the pan-leukocyte marker CD45.

We fractionated $CD34^+$ EB cells into $CD45^+$ and $CD45^-$ subsets by FACS sorting and analyzed for myeloid/erythroid and lymphoid potentials. Both populations contained comparable frequencies of myeloid/erythroid colony forming cells (Figure 3.5A). When they were cultured on OP9 stroma in the presence of SCF, Flt3-L, IL-7 and IL-6 for 7 days to generate ES-HPs and examined for the myeloid potential, the frequency of myeloid/erythroid colony forming cells in $CD45^+$ EB-derived ES-HPs was significantly higher than that of $CD34^+CD45^-$ EB-derived cells. However, $CD45^+$ EB cells or those cultured for ES-HP generation did not differentiate into NK, T or B cells upon co-culturing with OP9 or OP9-DL1 in the presence of appropriate cytokines (see materials and methods). In contrast, $CD34^+CD45^-$ EB cells cultured under the same conditions differentiated into NK and T cells (Figure 3.5B, C). Limiting dilution analysis showed that on average 1 in 633 $CD34^+CD45^-$

EB cells differentiated into NK cells, while frequency of T progenitors was 1 in 1266 (Figure 3.5D). No NK or T cell progenitors were detected among $CD45^+$ EB cells by limiting dilution analysis (data not shown). These results indicate that lymphoid potential is contained in the $CD45^-CD34^+$ EB cell population whereas $CD45^+$ EB cells have erythroid/myeloid but not lymphoid potential. Analysis of Mac-1 expression on $CD34^+$ EB cells showed that in contrast to $CD45^-$ subset, $CD45^+$ subsets are Mac-1^+, suggesting that the latter are committed myeloid precursors (Figure 3.4B).

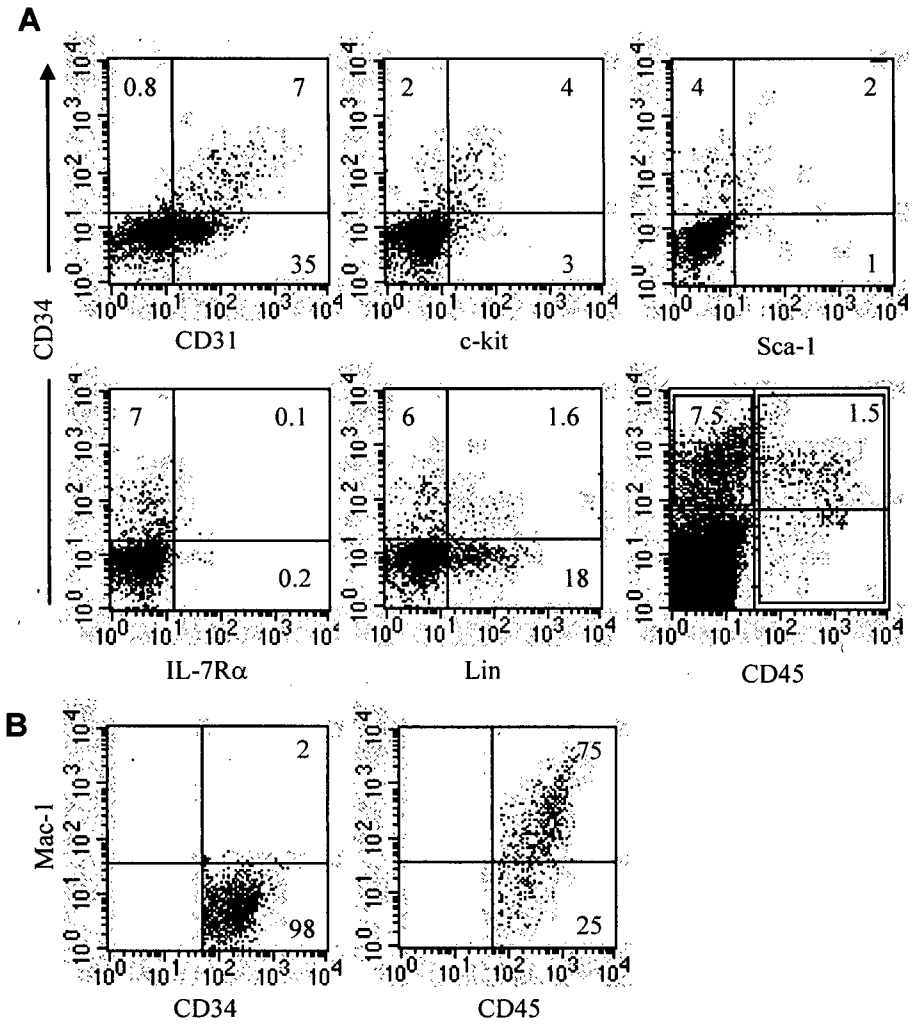


Figure 3.4. Surface marker analysis of EB cells.

(A) EBs were harvested on day 8 and single cell suspension was prepared. Cells were stained with anti-CD34 mAb together with mAb to the indicated markers and analyzed by flow cytometry. **(B)** $CD34^{+}CD45^{-}$ (left) and $CD45^{+}$ EB cells (right) in (A) were gated and anlaysed for Mac-1 expression.

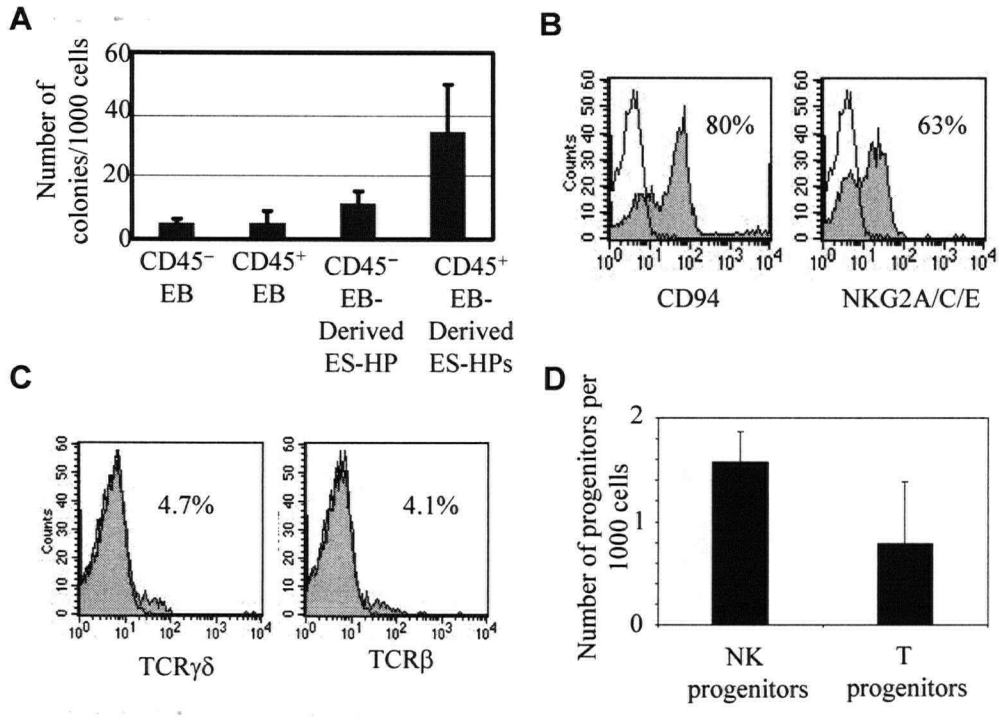


Figure 3.5. $CD34^+CD45^-$ EB cells contain lymphoid potential while $CD34^+CD45^+$ EB cells are committed to myeloid lineage.

(A) $CD34^+CD45^-$ and $CD45^+$ EB cells were isolated by FACS sorting and cultured to generate ES-HPs. The sorted EB cells and the ES-HPs generated from them were plated for myeloid and erythroid colony formation as in Figure 3.2E. **(B)** $CD34^+CD45^-$ EB cells were directly cultured on OP9 stroma with IL-2 and IL-15 for NK cell differentiation. Cells were harvested after 2 weeks, stained for NK markers and analyzed by flow cytometry. **(C)** $CD34^+CD45^-$ EB cells were directly cultured onto OP9-DL1 stroma with proper cytokines for T cell differentiation for 3 weeks. Expression of T cell markers was analyzed by flow cytometry. **(D)** Limiting numbers (100, 300 and 1,000 cells per well) of $CD34^+CD45^-$ EB cells were sorted into each well of 96 well plates. For NK progenitor assays, wells contained OP9 stroma, IL-2, and IL-15. For T progenitor assays, wells contained OP9-DL1 cells, IL-7 and Flt3-L as described in materials and methods. NK cells were detected by granzyme B RT-PCR and T cells detected by TCRγ RT-PCR followed by southern blotting. The number of progenitors in 1000 plated cells was calculated as in Figure 3.3C.

3.2.3. NK cell progenitors among ES-HPs derived from $CD34^+CD45^-$ EB cells

The above results showed $CD34^+CD45^-$ EB cells have lymphoid potentials but the frequencies of NK and T cell progenitors were rather low. Therefore, we generated ES-HPs

from $CD34^+CD45^-$ EB cells and analyzed them for cell surface marker expression and lymphoid differentiation potentials. Flow cytometric analysis showed that the cells generated *in vitro* from $CD34^+CD45^-$ EB cells were again heterogeneous and included large fractions expressing c-kit, Sca-1, and CD31 whereas IL-7R was almost undetectable. Cells expressing the erythroid marker Ter119 and the myeloid cell marker Mac-1 (CD11b, CD18) were also detected (Figure 3.6A). Only 13% of the cells were $CD45^+$, which included cells co-expressing c-kit and Sca-1 and those expressing Mac-1 and/or Ter119 (Figure 3.6B).

NK potential of ES-HPs generated from $CD34^+CD45^-$ EB cells was first tested by bulk cultures. NK cells expressing CD94 and NKG2A/C/E were readily generated in cultures on OP9 stroma (data not shown). Limiting dilution analysis showed that NK progenitors are highly enriched in the $CD45^+Lin^-$ ($Mac\text{-}1^-Ter119^-$) subset of ES-HPs. In three experiments, the frequency of NK progenitors in this population was on average about 10% (1 in 10), ranging from 14% (1 in 7) to 4% (1 in 24) in three experiments (Figure 3.7), which is comparable to the frequency of NK progenitors in the BM NKP (Lin^-CD122^+) population[52], whereas the NK progenitor frequency of the bulk ES-HP population was on average 0.4% (1 in 230). As the $CD45^+Lin^-$ subset is about 5% of the total ES-HP population, most NK progenitors seemed enriched in this subset of ES-HPs.

However, $CD45^+Lin^-$ ES-HPs were different from BM-derived NKPs, as they do not express detectable level of IL-2Rβ (CD122) (Figure 3.6B, right panel). When $CD45^+Lin^-$ ES-HPs were sorted and cultured with IL-2 and IL-15 for three days, IL-2Rβ was detected on the cultured cells (Figure 3.6C). $CD45^+Lin^-$ ES-HP cells became mature $CD94^+$ and $NKG2A/C/E^+$ NK cells after one week in the NK differentiation culture (Figure 3.6D).

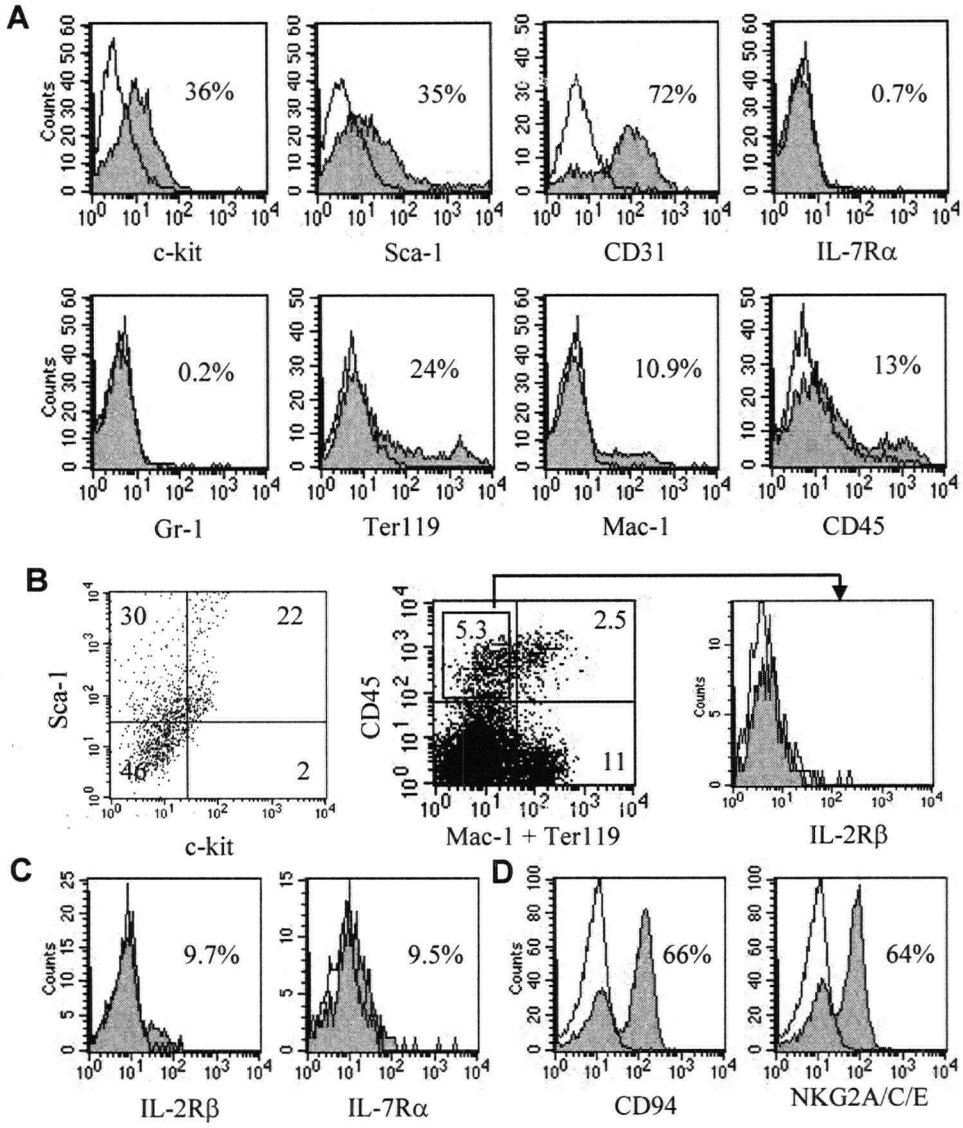


Figure 3.6. Characterization of ES-HP cells derived from CD34$^+$CD45$^-$ EBs for surface markers and NK potential.

(A) ES-HP cells derived from CD34$^+$CD45$^-$ EB cells were stained with mAbs to indicated markers and analysed by flow cytometry. **(B)** ES-HP cells were co-stained with anti-CD45, c-kit and Sca-1 mAbs. Cells were then gated on CD45$^+$ and analyzed for the expression of c-kit and Sca-1 (left panel). ES-HP cells were stained with a combination of purified anti-CD45.2 mAb followed by Alexa Flour 647-goat anti-mouse IgG, and biotinylated anti-Mac-1 and Ter119 mAbs followed by streptavidin-PE (middle panel). CD45$^+$Mac-1$^-$Ter119$^-$ cells were gated and analyzed for the expression of IL-2Rβ (right panel). **(C)** CD45$^+$Lin$^-$ ES-HPs were sorted and cultured on OP9 stroma with IL-2 and IL-15. After 3 days, cells were harvested and subjected to flow cytometry analysis after staining with anti-IL-2Rβ and anti-IL-7Rα. **(D)** Cells in (C) are analysed for the expression of NK markers after one week.

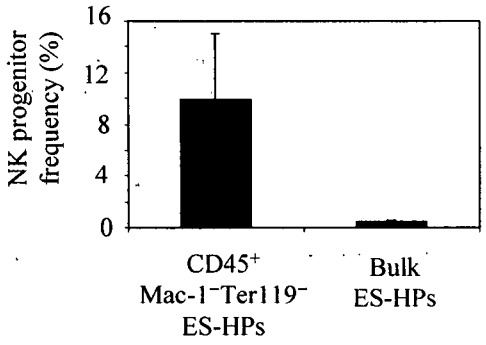


Figure 3.7. NK progenitor frequency of ES-HPs derived from $CD34^+CD45^-$ EBs.
The bulk and sorted $CD45^+Lin^-$ ES-HP cells were analyzed for NK progenitor frequency by limiting dilution cultures as in Figure 3.3C. For the former 30, 100 and 300 cells per well and for the latter 3, 10 and 30 cells per well were plated. The results are mean ± SD of three independent experiments.

3.2.4. ES-HPs derived from $CD34^+CD45^-$ EB cells have T and B cell potentials

To test whether ES-HPs derived from $CD34^+CD45^-$ EB cells have T cell differentiation potential, they were cultured on OP9-DL1 stromal cells in the presence of IL-7 and Flt3-L. We first analyzed the expression of CD44 and CD25 at different time points during the culture (Figure 3.8A) as these markers are commonly used to define the early steps of immature double negative (DN, $CD4^-CD8^-$) thymocyte differentiation[25]. The cells initially resembled the most immature thymocyte population DN1 ($CD4^-CD8^-CD44^+CD25^-$). After one week of culture, some (~8%) of the cells acquired CD25 and resembled DN2 ($CD4^-CD8^-CD44^+CD25^+$) thymocytes. After two weeks, the level of CD44 on DN2-like cells decreased and they resembled DN3 ($CD4^-CD8^-CD44^-CD25^+$) thymocytes. Prolonged culture did not induce a further differentiation into the DN4 ($CD4^-CD8^-CD44^-CD25^-$) population. Although $CD44^-CD25^-$ cells were detected early in the cultures (week one and two), they seemed to directly derive from the DN1-like cells without going through the DN2

and DN3 stages. Very few cells expressing CD4, CD8 or CD3 were generated in this culture (data not shown). It appeared that T cell development from ES-HPs was impaired at the transition from DN3 to DN4. This may be due to a high dose of IL-7, which has been reported to block differentiation of adult progenitors into T cells[215,216]. Therefore, after 8-14 days of culture, IL-7 concentration was reduced to 1 ng/ml, and the cells were cultured for an additional 4-6 days with OP9-DL1 cells. Cells expressing CD4, CD8 and the TCR were detected in the low-dose IL-7 culture (Figure 3.8B, C). However, the expression levels of CD4 and CD8 were significantly lower than those on thymocytes (data not shown), and very few double positive ($CD4^+CD8^+$) and single positive ($CD4^+CD8^-$ and $CD4^-CD8^+$) cells were generated.

Limiting dilution analysis showed that the frequency of T cell progenitors was approximately 0.3% (1 in 297) in the bulk ES-HP population and 0.9% (1 in 115) in the $CD45^+Lin^-$ subset of ES-HPs (Figure 3.9). Thus, T cell progenitors were enriched in the $CD45^+Lin^-$ subset, which contained most of the NK progenitors. However, the $CD45^+Lin^-$ subset was about 5% of the bulk ES-HPs while the enrichment of T cell progenitors was only about three fold, suggesting that T cell progenitors were also contained in other subsets of ES-HPs.

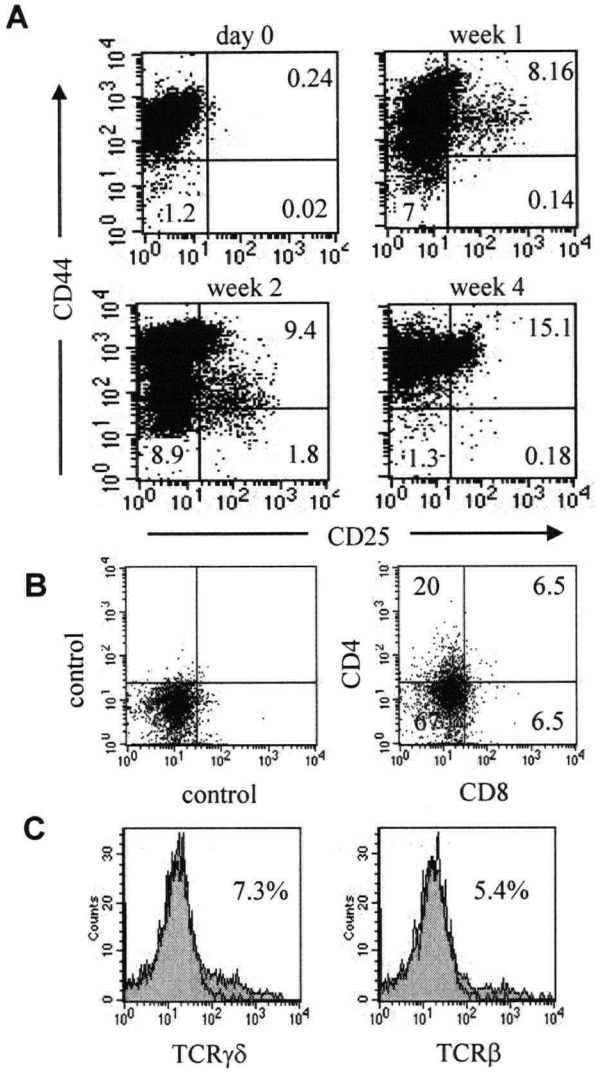


Figure 3.8. T cell potential of ES-HPs derived from CD34$^+$CD45$^-$ EB cells.

(A) CD34$^+$CD45$^-$ EB cells were sorted and cultured with OP9 and cytokines to generate ES-HPs. They were then cultured on OP9-DL1 stroma with cytokines for T cell differentiation. At different time points, cells were harvested and stained for CD44 and CD25 and analyzed using a flow cytometer. The numbers show the percentages of cells in the quadrants. **(B)** ES-HP cells were cultured for T cell differentiation for 8 days with 5 ng/ml IL-7 as in (A). On day 8, IL-7 concentration was reduced to 1 ng/ml and cells were cultured for an additional 6 days. Cells were harvested and analyzed by flow cytometry for CD4 and CD8 expression. **(C)** ES-HP cells were cultured for T cell differentiation with 5 ng/ml IL-7 for 2 weeks and an additional 4 days with 1 ng/ml IL-7, stained for TCRγδ and TCRβ and analyzed using a flow cytometer.

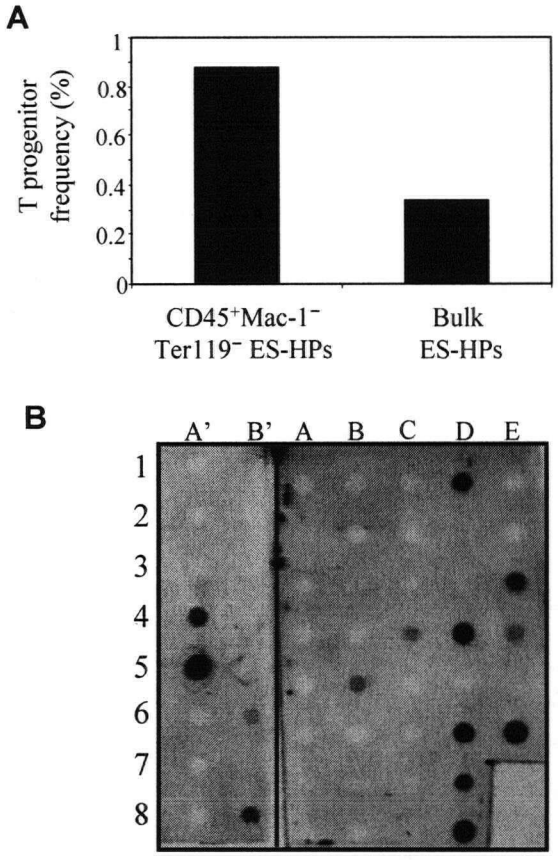
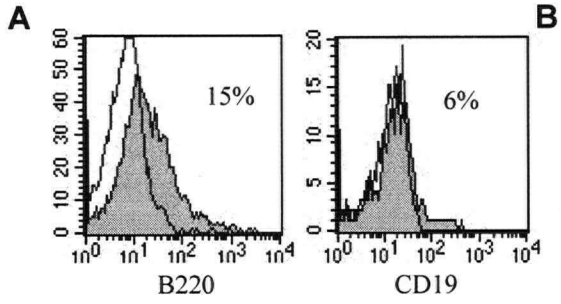


Figure 3.9. T progenitor frequency of ES-HPs derived from $CD34^+CD45^-$ EBs.

(A) ES-HPs were generated from $CD34^+CD45^-$ EB cells as described in materials and methods. The bulk and sorted $CD45^+Lin^-$ ES-HP cells were analyzed for T progenitor frequency by limiting dilution cultures as in Figure 3.5D, except that 30, 100, 300 and 900 cells per well for bulk and 3, 10, 30 and 100 cells per well for $CD45^+Lin^-$ cells were plated. The results are average of two independent experiments. **(B)** Southern blot of TCRγ RT-PCR of $CD45^+Lin^-$ ES-HPs created in one of two cloning assays in (A) is shown. The number of cells used in each well with their corresponding location in the blot are as follows: 3 cells/well: A1-A8, B1-B4; 10 cells/well:A'1-A'8, B'1-B'4; 30 cells/well: B5-B8, C1-C8; 100 cells/well: D1-D8, E1-E4; positive controls (thymocytes): B'6, B'8, E6; negative controls (OP9-DL1 cells): B'7, E5. Pictures of two blots are separated by a line.

To test the B cell potential of ES-HPs, $CD45^+Lin^-$ ES-HPs were sorted and cultured on OP9 with IL-7 and Flt3-L for one week. B cells were detected in the culture by the expression of B220 and CD19 (Figure 3.10A). Therefore, $CD45^+Lin^-$ ES-HPs contained B cell potential. To test whether these cells also have myeloid differentiation potential, sorted $CD45^+Lin^-$ ES-

HPs were transferred into methylcellulose for myeloid colony formation. On average, 5.7% of $CD45^+Lin^-$ ES-HPs formed erythro/myeloid colonies (Figure 3.10B). These results indicate that ES-HPs have NK, T, B and myeloid/erythroid potentials.



B

Expt. No.	$CD45^+Lin^-$ ES-HPs
1	54.3/1000
2	60.9/1000

Figure 3.10. B and myeloid potentials of $CD45^+Lin^-$ ES-HPs derived from $CD34^+CD45^-$ EBs.

(A) $CD45^+Lin^-$ ES-HPs were sorted and cultured on OP9 cells with IL-7 and Flt3-L for B cell generation. After one week, cells were harvested and analysed for the expression of B220 and CD19 by flow cytometry. **(B)** One thousand sorted $CD45^+Lin^-$ ES-HPs were transferred into myeloid differentiation media as in Figure3.2E. The number of myeloid and erythroid colonies were scored.

3.3. Discussion

We have characterized NK cell progenitors at various stages of development from ES cells *in vitro*. The most immature progenitors with NK cell potential are found among EB cells expressing CD34 but not the pan-leukocyte marker CD45. When cultured on OP9 stroma in the presence of IL-7, IL-6, Flt3-L and SCF, $CD34^+CD45^-$ EB cells differentiate into a heterogeneous cell population, termed ES-HP (ES cell-derived hematopoietic progenitors). Among ES-HPs, NK cell progenitors are highly enriched in a subpopulation expressing CD45 but not the mature erythroid and myeloid cell lineage markers Ter119 and Mac-1.

Thus, $CD34^+CD45^-$ immature hematopoietic progenitors in EBs differentiate into more mature $CD45^+$ NK progenitors in this culture. Since $CD34^+CD45^-$ EB cells are heterogeneous, the relationship between NK progenitors in the EB cell population and those among ES-HPs is still unclear. However, the difference in CD45 expression indicates that NK progenitors among ES-HPs are not products of simple amplification of NK progenitors in EB but rather they are differentiation products derived from immature hematopoietic progenitors in EBs.

The overall ES-HP cell population is rather heterogeneous and contains myeloid and erythroid cells at various stages of development as well as $CD45^-Lin^-$ cells, which mostly includes immature hematopoietic progenitors (see next chapter). Most, if not all, NK progenitors are in the $CD45^+Lin^-$ ($Ter119^-Mac\text{-}1^-$) subset. The frequency of NK progenitors in this subset, as determined by limiting dilution assay, is about 10%, which is comparable to that of adult mouse BM NKPs defined by $CD122^+Lin^-$[52]. However, there are some differences between NKP in BM and those in ES-HPs. Most notably, BM NKPs express $CD122^{52}$ whereas it is undetectable on those among ES-HPs (Figure3.6B, right panel). It is likely that NK progenitors at the ES-HP stage are more immature than BM NKPs. $CD122^-$ NK progenitors, which mature into $CD122^+$ progenitors, have been found in human lymph nodes as well[65]. As expected, NK progenitors in the ES-HP population rapidly acquire CD122 during their maturation to NK cells (Figure 3.6C).

Although $CD45^+Lin^-$ ES-HPs also include T cell progenitors, it is still unclear whether ES-HPs include bipotent T/NK progenitors. Both T and NK cells are generated in bulk cultures to induce differentiation of $CD45^+Lin^-$ ES-HPs into T cells. However, the results with limiting dilution analysis show that the frequency of T cell progenitors among $CD45^+Lin^-$ ES-HPs is low (less than 1%). Our attempt to determine the frequency of progenitors that give rise to both NK and T cells by limiting dilution assays have resulted in very variable frequencies (data not shown). One of the difficulties in determining T cell progenitor frequency in this study is that most T cell progenitors do not differentiate into mature T cells expressing the TCR, CD3, CD4 or CD8 in our cultures as their differentiation is blocked at the DN2 stage before TCRγ gene rearrangement, which was used to detect T cells in the limiting dilution analysis. Thus, the frequency of T cell progenitors is likely very much underestimated in this study, making it difficult to detect bipotent T/NK progenitors. Nevertheless, this study has shown that $CD45^+Lin^-$ ES-HPs include both NK and T cell progenitors. We also showed that $CD45^+Lin^-$ ES-HPs have B cell and myeloid potentials. Thus, $CD45^+Lin^-$ ES-HPs likely comprise of multiple committed progenitors. However, it is also possible that $CD45^+Lin^-$ ES-HPs include multi-potent hematopoietic progenitors which are downstream progeny of $CD45^-$ progenitors.

Our study has also shown that $CD34^+$ EB cells are rather heterogeneous and include both $CD45^+$ and $CD45^-$ cells. Although the relationship between these two subsets of $CD34^+$ EB cells is still unclear, the former includes erythroid/myeloid colony forming cells but no lymphoid progenitors whereas the latter has both myeloid/erythroid and lymphoid potentials.

Since $CD34^+CD45^+$ EB cells express Mac-1 (Figure 3.4B), it is possible that these cells are myeloid precursors that have derived from $CD45^-$ EB cells generated earlier in the EBs. $CD45^+$ macrophage precursors have been described during early embryonic development in AGM region[120]. On the other hand, $CD45^-$ EB cells resemble precursors found in P-Sp and AGM region which generate lymphocytes and also lack CD45[107,120]. This work demonstrates, for the first time, a differentiation pathway in which these cells acquire CD45 during ES-HP stage before subsequent differentiation to individual hematopoietic lineages. It will be interesting to compare the reconstitution capacity of $CD45^-$ EBs and that of $CD45^+Lin^-$ ES-HPs. While EB cells have been shown to reconstitute adult BM only by overexpression of *HoxB4*[190] and/or *Cdx4*[191], $CD45^+$ ES-HPs may be developmentally closer to adult HSCs and give rise to multilineage engraftment without a need for genetic manipulation.

Chapter 4: Characterization of hematopoietic progenitors with lymphoid and myeloid potentials generated *in vitro* from embryonic stem cells[2]

4.1. Introduction

A number of groups including our group have reported the hematopoietic potential of $CD34^+$ EB cells[197,198,200]. The results in my previous chapter showed that $CD34^+$ EBs are quite heterogeneous and can be divided into two subsets based on the pan-leukocyte marker CD45. $CD34^+CD45^+$ cells in day-8 EBs have erythroid/myeloid but not lymphoid potential, whereas the $CD34^+CD45^-$ EB cells have both. CD34 is found on non-hematopoietic lineages including endothelial cells[151]. $CD34^+$ cells arise from ES-derived $Flk\text{-}1^+$ endothelial progenitors[220]. For the purposes of HSC transplantation treatments, it is important to distinguish hematopoietic progenitors with lymphoid potential (definitive hematopoietic progenitors) within $CD34^+CD45^-$ EBs. CD41 and c-kit have been used to identify progenitors with definitive hematopoietic potential in EBs[108].

The work in this chapter was initiated based on the hypothesis that $CD34^+CD45^-$ EBs should contain $CD41^+c\text{-}kit^+$ subsets that bear the myeloid/lymphoid progenitors. Differential expression of CD41 and c-kit on $CD34^+CD45^-$ EB cells elucidated four fractions in this subset. Using cell separation strategies, four different fractions (namely double positive, single positive and double-negative) were isolated and tested for erythroid/myeloid and

[2] A version of this chapter has been submitted for publication. Tabatabaei-Zavareh N and Takei F. Characterization of hematopoietic progenitors with lymphoid potential generated in vitro from embryonic stem cells.

lymphoid potentials. As expected, erythroid/myeloid and lymphoid progenitors were only found in double positive fraction. $CD34^+CD45^-$c-kit^+CD41^+ EBs were further characterized and also differentiated into ES-HPs (as described in chapter 3). The hematopoietic potential of various ES-HP subpopulations was also examined *in vitro*.

4.2. Results

4.2.1. Day-8 $CD34^+CD45^-$ EB cells are heterogeneous

The culture system used in this study to induce differentiation of ES cells to hematopoietic cells is delineated in Figure 4.1. In the first step, ES cells differentiated to form EBs. EB cell subsets were isolated by cell sorting and cultured with OP9 stroma and a cytokine cocktail for hematopoietic differentiation, which resulted in a heterogeneous cell population we termed ES-HPs. ES-HP subsets were isolated by cell sorting and cultured for further differentiation. Cells at each step were analyzed by flow cytometry, and individual subpopulations were sorted and tested for differentiation into myeloid/erythroid, NK, T and B cells.

Previously, we determined that $CD34^+CD45^-$ EB subsets contain lymphoid as well as myeloid precursors. In this study, we further dissected the $CD34^+CD45^-$ cell population in day-8 EBs. At this stage, all $CD34^+$ EB cells expressed CD31 and 6% of total EB cells were $CD34^+CD45^-$ (Figure 4.2A). We first analyzed expression of several hematopoietic progenitor markers including Flk-1, c-kit (CD117) and CD41 on $CD34^+CD45^-$ EB cells. Flk-1 has been shown to be expressed by hemangioblasts, the common progenitors of

hematopoietic and endothelial lineages. Hemangioblasts are identified in mouse ED7.5 embryos as well as in day 2.5 to 3.5 EBs as blast colony-forming cells (BL-CFCs)[166]. The majority (approximately 70%) of $CD34^+CD45^-$ EB cells expressed high level of Flk-1 while some expressed low level (Figure 4.2A). Similarly, most (~80%) $CD34^+CD45^-$ EB cells expressed high level of c-kit. As the expression patterns of c-kit and Flk-1 among $CD34^+CD45^-$ EB cells were similar, staining for the combination of c-kit and Flk-1 did not reveal distinct subsets. The majority of them were c-kit^+Flk-1^+ and only minor single positive and double negative populations were detected. On the other hand, combination of c-kit and CD41 divided $CD34^+CD45^-$ EB cells into four subsets, namely double positive, single positive and double negative. Our attempt to further dissect the double positive subset by Flk-1 expression was not successful as most of the cells expressed Flk-1 (Figure 4.2B).

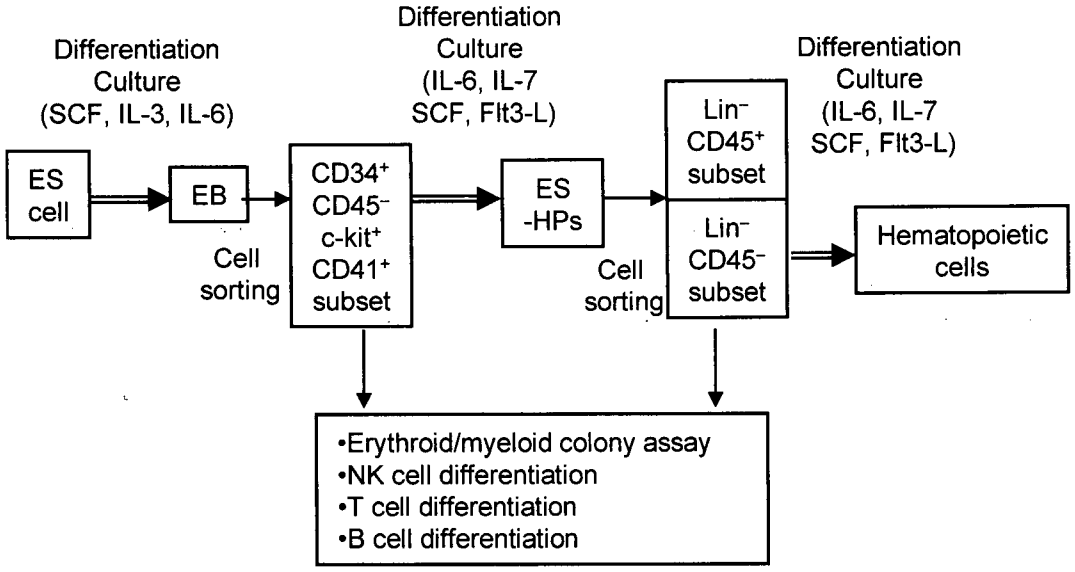


Figure 4.1. Scheme of ES cell culture used in this study for hematopoietic differentiation

ES cells were cultured in methylcellulose to induce EB formation. $CD34^{+}CD45^{-}c\text{-}kit^{+}CD41^{+}$ EBs were sorted and cultured for one week with OP9 stroma in the presence of indicated cytokines to generate ES-HP cells. ES-HPs were sorted into $CD45^{+}$Mac-1/$Ter119^{-}$ (Lin^{-}) and $CD45^{-}Lin^{-}$ subsets and cultured in lineage differentiation cultures for erythroid/myeloid, NK, T and B lineages as described in materials and methods. Sorted $CD45^{-}Lin^{-}$ ES-HPs were also incubated on OP9 stroma with the same cytokines used for the primary ES-HP differentiation for one week before FACS analysis.

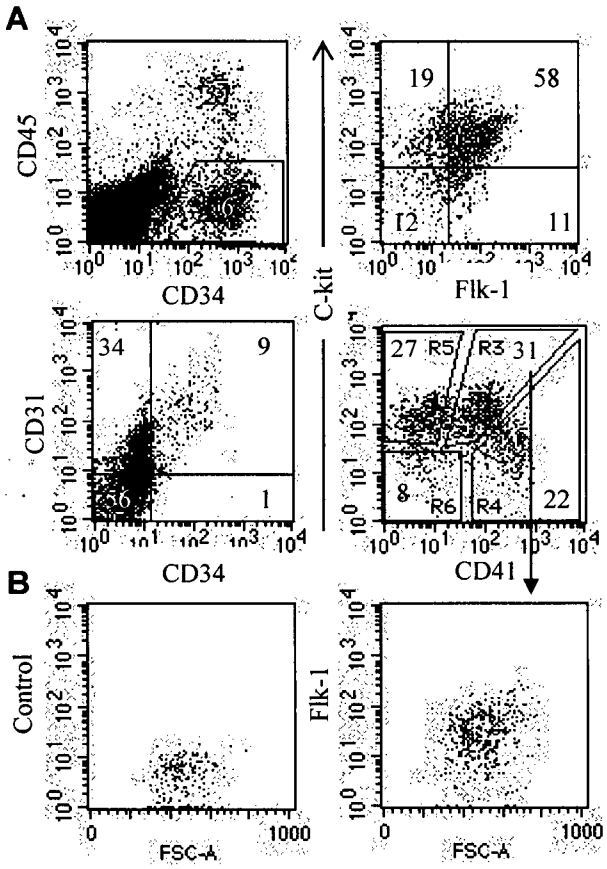


Figure 4.2. Surface expression of hematopoietic markers on day-8 EB cells.

(A) EB cells were harvested at day 8 of differentiation and stained with biotinylated anti-CD34 followed by streptavidin-PE-Texas Red, APC-anti-CD45, FITC-anti-CD41, PE-Cy7-anti-c-kit and PE-anti-Flk-1 mAbs. Cells were analysed on FACSVantage (Diva). Dead cells were stained with 4'-6-Diamidino-2-phenylindole (DAPI) and gated out. $CD34^+CD45^-$ cells were gated (left, upper panel) and analysed for the expression of c-kit and Flk-1 (right upper panel), and c-kit and CD41 (right lower panel). Numbers show percentages of cells in the quadrants or in the gates. For analysis of CD34 and CD31 expression, cells were stained with anti-CD34 and anti-CD31 mAbs and analysed by a FACSCalibur flow cytometer (left lower panel). **(B)** $CD34^+CD45^-c\text{-}kit^+CD41^+$ cells were gated in (A) (R3 gate) and analysed for Flk-1 expression. The x-axis shows forward scatter whereas the y-axis shows fluorescence intensity.

4.2.2. $CD34^+CD45^-c\text{-}kit^+CD41^+$ EB cells have hematopoietic potential

$CD34^+CD45^-$ EB cells as well as the individual subsets identified by c-kit and CD41 expression were sorted and cultured in methylcellulose for erythroid and myeloid colony

formation (Figure 4.3A). Clonogenic erythroid/myeloid progenitor activity was enriched more than three fold in $CD34^+CD45^-$c-kit^+CD41^+ EB cell subset as compared with the whole $CD34^+CD45^-$ EB cell population. Since the $CD41^+$c-kit^+ population is about 31% of $CD34^+CD45^-$ EB cells, most erythroid/myeloid colony forming cells seemed to be contained in the $CD41^+$c-kit^+ subset. We also examined the lymphoid potential of various EB subsets (Figure 4.3B). For B and NK cell differentiation, cells were cultured on OP9 stroma, while OP9-DL1 cells were used to induce T cell differentiation (see chapter 2). $CD34^+CD45^-$c-kit^+CD41^+ EB cells generated B, NK and T cells, while the other subsets did not generate any lymphoid cells with the exception of c-kit^+CD41^- population, which generated small number of lymphocytes *in vitro* (Figure 4.3B and data not shown). These results indicate that both myeloid and lymphoid potentials are enriched in the c-kit^+CD41^+ subset of $CD34^+CD45^-$ EB cells.

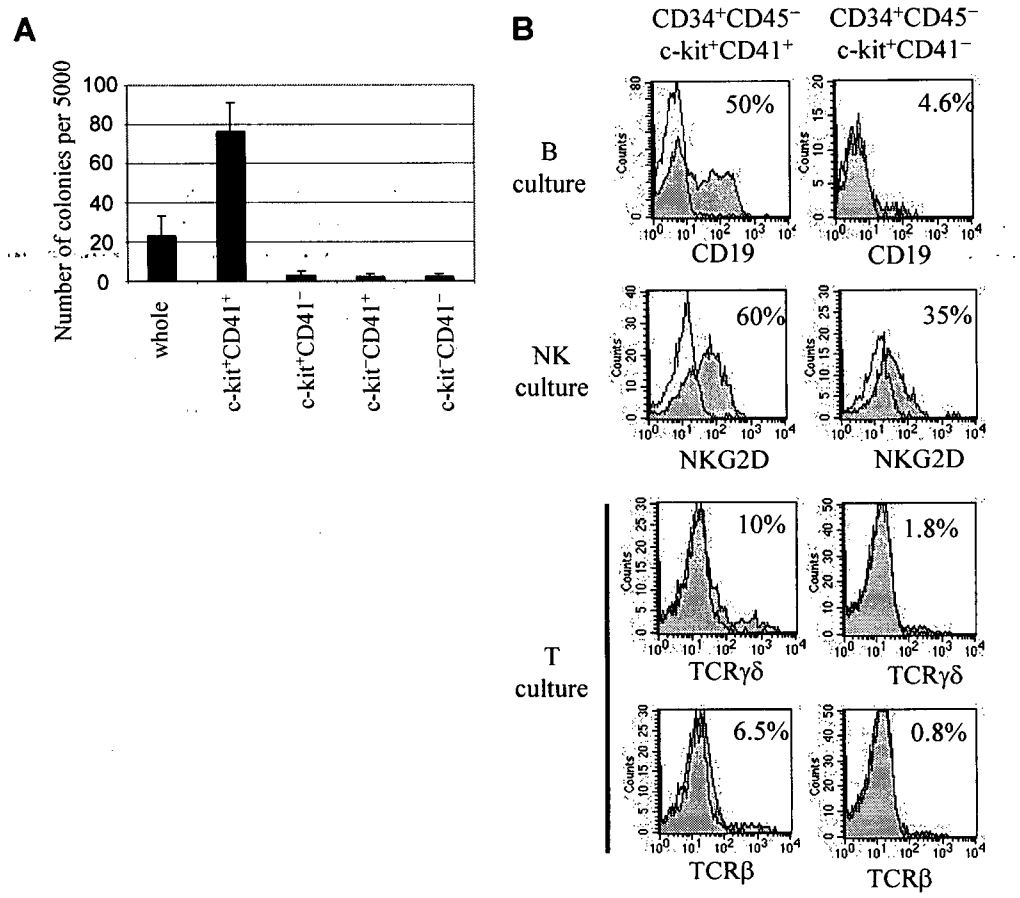


Figure 4.3. Myeloid and lymphoid potential of CD34$^+$CD45$^-$ EB subsets.

(A) CD34$^+$CD45$^-$ EB cells were sorted according to expression of c-kit and CD41. Myeloid differentiation cultures were initiated with 5000 sorted EB cells. Erythroid and myeloid colonies were enumerated as described in chapter 2. **(B)** Sorted EB cells were cultured in B, NK and T differentiation conditions (see chapter 2), stained for CD19 for B cells, NKG2D for NK cells and TCRγδ and TCRβ for T cells and analysed by a flow cytometer. Percentages indicate the frequencies of positively stained cells over the control staining.

4.2.3. Characterization of developmental intermediate progenitors derived from CD34$^+$CD45$^-$c-kit$^+$CD41$^+$ EB cells

In order to further study the developmental stages leading to the generation of hematopoietic cells from EB cells, CD34$^+$CD45$^-$c-kit$^+$CD41$^+$ EB cells were purified and cultured on OP9 stroma with IL-7, Flt3-L, SCF and IL-6 for differentiation along the hematopoietic lineage

for 1 week. The cells thus generated, termed ES-derived hematopoietic progenitors (ES-HPs), were harvested and analyzed for expression of the pan-hematopoietic marker CD45, the myeloid cell marker Mac-1 and the erythroid marker Ter119. Approximately 25% of ES-HPs expressed CD45 (Figure 4.4). $CD45^+$ cells included $Mac\text{-}1^+$ and some $Ter119^+$ cells but were negative for other lineage markers (data not shown). The majority of $CD45^+$ ES-HPs retained expression of CD34, c-kit, CD41 and CD31 suggesting that they were mostly hematopoietic progenitors. The majority of $CD45^-$ ES-HPs also retained CD34, c-kit and CD41 expression and very few expressed Mac-1 or Ter119. However, some of them lost hematopoietic progenitor-associated markers. In particular, Flk-1 was expressed on only 23% of $CD45^-$ ES-HPs as compared to 70% of $CD34^+CD45^-c\text{-}kit^+CD41^+$ EB cells being $Flk\text{-}1^+$. Thus, ES-HPs derived from $CD34^+CD45^-c\text{-}kit^+CD41^+$ EB cells were rather heterogeneous and included $CD45^+$ hematopoietic cells at different levels of development along the hematopoietic lineages as well as $CD45^-$ cells, which were also heterogeneous with respect to expression of CD34, c-kit, CD41 and Flk-1.

Most Erythroid/myeloid potential as determined by *in vitro* colony forming cell assay was found among $CD45^+Lin^-$ ES-HPs (Figure 4.5A). The frequency of the colony forming cells among this subset of ES-HPs was almost 7% and significantly higher than that among $CD34^+CD45^-c\text{-}kit^+CD41^+$ EB cells whereas erythroid/myeloid potential of $CD45^-Lin^-$ ES-HPs was similar to that of the original EB cells they derived from. $CD45^+Lin^-$ (Figure 4.5B, C) and $CD45^-Lin^-$ (Figure 4.5D, E) ES-HPs also showed NK and T cell potentials. There was no obvious difference between the two subsets of ES-HPs in their potentials for NK and T cells, although it was not a quantitative assay. On the other hand, B cell potential of ES-

HPs seemed rather low as very few (2.5 ± 2%, n=4) $CD19^+$ cells were generated in B cell differentiation cultures (data not shown). However, $CD45^-Lin^-$ ES-HPs generated more (5%, n=3) $CD19^+$ cells, when incubated on OP9 with SCF, Flt3-L, IL-7 and IL-6 (Figure 4.6), while less than 1% $CD19^+$ cells were generated from $CD45^+Lin^-$ ES-HPs under same conditions (data not shown). Therefore, $CD45^-Lin^-$ ES-HPs seem to have higher B cell potential than $CD45^+Lin^-$ ES-HPs.

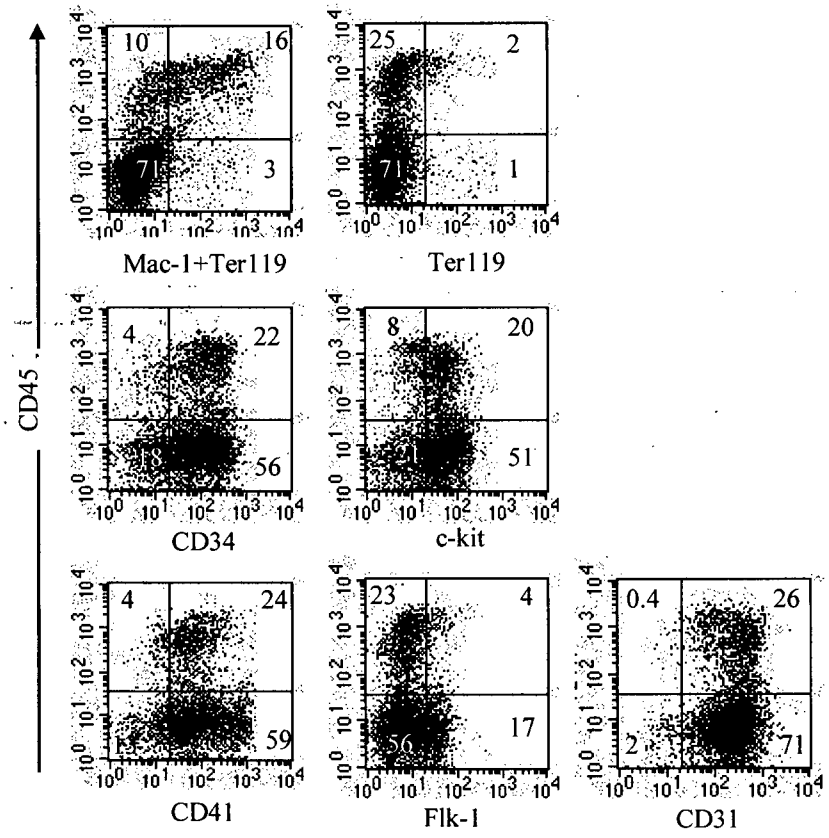


Figure 4.4. Cell surface marker expression of ES-derived hematopoietic progenitors (ES-HPs) derived from $CD34^+CD45^-$c-kit^+CD41^+ EB cells.

$CD34^+CD45^-$c-kit^+CD41^+EB cells were sorted and incubated on OP9 stroma with cytokines for 7 days to generate ES-HPs. Cells were then harvested and co-stained with anti-CD45 and indicated markers and analysed by flow cytometry. Numbers show the percentage of cells in the quadrants.

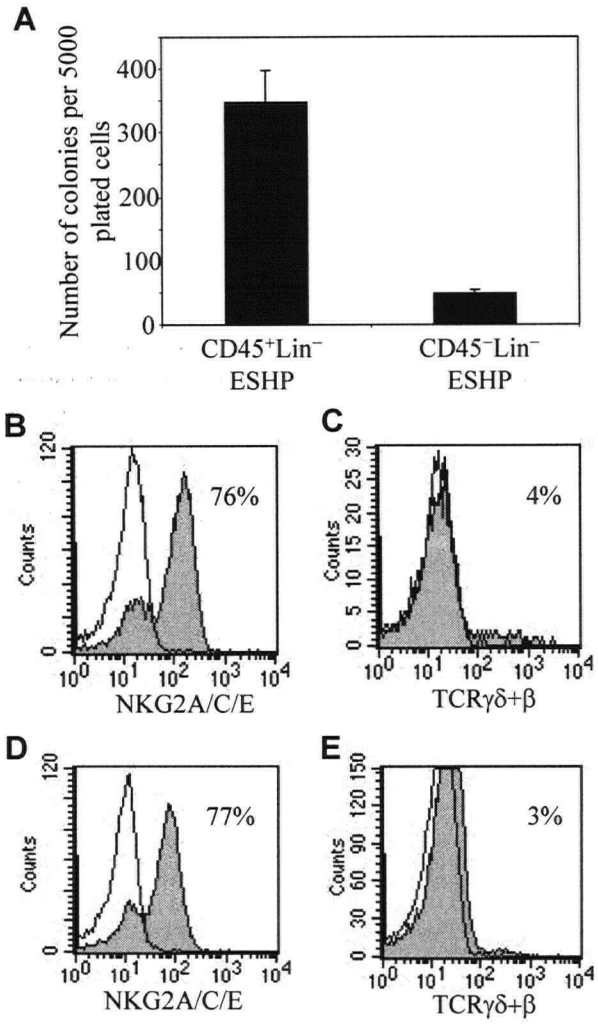


Figure 4.5. Lineage potential of ES-HP subsets.

ES-HPs generated as in Figure 4.1 were stained for CD45, Mac-1 and Ter119 and sorted into $CD45^+$Mac-1/$Ter119^-$ (Lin^-) and $CD45^-Lin^-$ cells. **(A)** $CD45^+Lin^-$ and $CD45^-Lin^-$ ES-HPs were sorted and plated for erythroid and myeloid colony formation as in Figure 4.3A. $CD45^+Lin^-$ ES-HPs **(B** and **C)** and $CD45^-Lin^-$ ES-HPs **(D** and **E)** were cultured for NK **(B** and **D)** and T cell **(C** and **E)** differentiation and analysed for NKG2A/C/E expression for NK cells and TCRγδ/TCRβ expression for T cells by flow cytometry.

4.2.4. Hematopoietic differentiation of $CD45^-$ ES-HPs

The above results showed that the majority of ES-HPs generated from $CD34^+CD45^-$c-kit$^+$$CD41^+$ EB cells were $CD45^-$ and many of them had similar phenotype to that of

progenitors in EBs. Since EB cells were thought to include hemangioblasts, it was possible that $CD45^-$ ES-HPs might be products of EB cell differentiation into non-hematopoietic cell lineages. To test whether they are immature hematopoietic progenitors or non-hematopoietic cells, $CD45^-Mac\text{-}1^-Ter119^-$ (Lin^-) ES-HPs were sorted and cultured in a secondary ES-HP differentiation culture comprised of OP9 stroma and cytokines identical to the primary ES-HP cultures. After one week, the cells were harvested and analyzed by flow cytometry (Figure 4.6). Approximately 80% of the cells recovered from the secondary cultures were $CD45^+$ and the majority expressed the erythroid and myeloid lineage-specific markers Mac-1 and Ter119, respectively. Some (25%) expressed the granulocyte marker Gr-1. Small numbers of B cells ($CD19^+$), and NK cells ($Il\text{-}2R\beta^+$, $NKG2A/C/E^+$) were also detected. Interestingly, the expression of CD34 and c-kit on both $CD45^+$ and $CD45^-$ secondary ES-HPs was similar to that of the original $CD45^-Lin^-$ primary ES-HPs whereas CD41 was significantly down-regulated. These results showed that $CD45^-$ cells in the primary ES-HP cultures were immature hematopoietic cells.

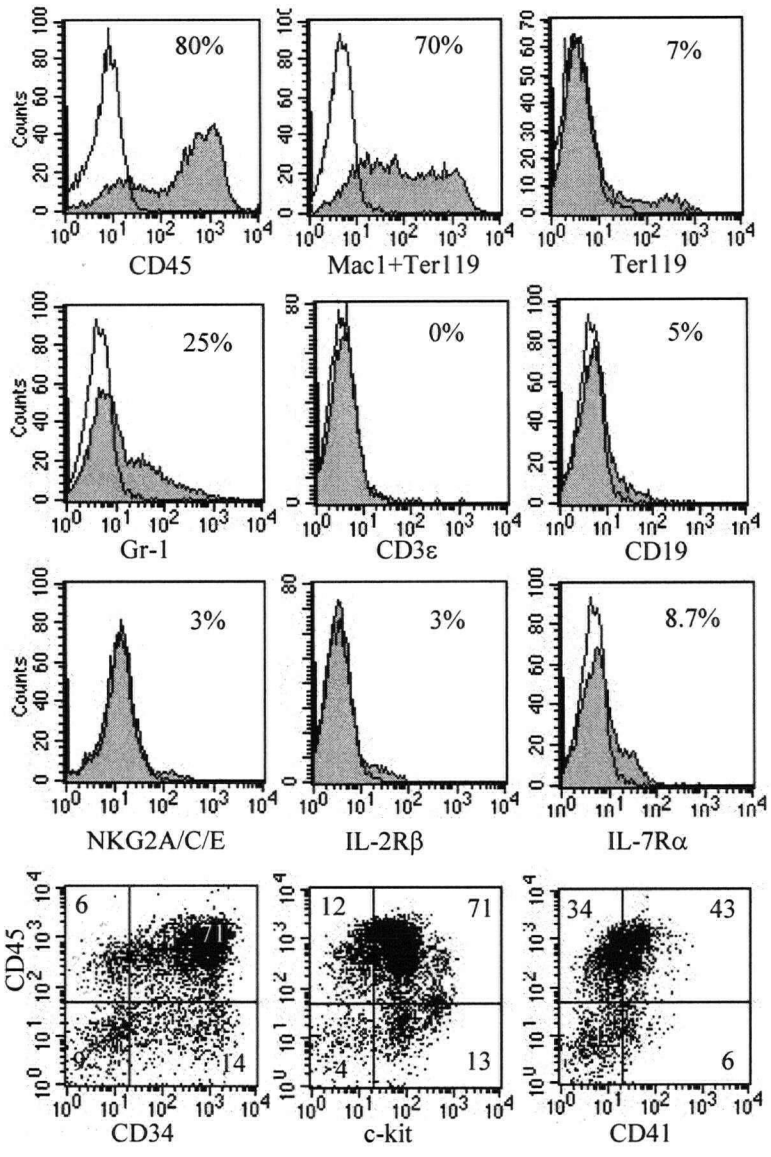


Figure 4.6. Characterization of cells derived from CD45$^-$Lin$^-$ ES-HPs after further differentiation in ES-HP culture.

CD45$^-$Lin$^-$ ES-HP cells were generated and sorted as in Figure 4.1 and cultured in a secondary ES-HP culture on OP9 stroma with IL-6, IL-7, Flt3-L and SCF. After one week, cells were harvested and analysed for the indicated markers by flow cytometry as in Figure 4.3B and Figure 4.4.

4.3. Discussion

In this study, we have generated *in vitro* multiple hematopoietic progenitors with myeloid and lymphoid potentials from ES cells. The most immature hematopoietic progenitors in our cultures are found among $CD34^+$ cells in day-8 EBs, which have been known to contain hematopoietic progenitors. In the previous chapter, $CD34^+$ cells in day-8 EBs were divided into $CD45^+$ and $CD45^-$ populations, and it was found that the former has erythroid/myeloid but not lymphoid potential whereas the latter has both. Here, the $CD34^+CD45^-$ EB cell population were further dissected into four subsets based on the expression of c-kit and CD41. The results indicated that erythroid/myeloid as well as T, B and NK cell potentials are mostly confined among c-$kit^+CD41^+CD34^+CD45^-$ EB cells. Although $CD34^+CD45^-$c-kit^+CD41^- EB cells seem to have some lymphoid potential, generation of T, B and NK cells from these EB cells *in vitro* is significantly less efficient than that from the c-kit^+CD41^+ subset. It should also be noted that the method to determine lymphoid potential in this study is not quantitative. Moreover, the $CD41^+$ and $CD41^-$ EB cell populations are not well separated, and it is possible that the lymphoid potential of the $CD41^-$ population may be due to contamination of $CD41^+$ cells. $CD34^+CD45^-$c-kit^+CD41^+ EB cells are $CD31^+$ and mostly Flk-1^+. Separation of $CD34^+CD45^-$c-kit^+CD41^+ EB cells into Flk-1^{hi} and Flk-1^{lo} subpopulations did not have much effect on erythroid/myeloid or lymphoid potential (data not shown). Thus, the most immature hematopoietic progenitors with erythroid/myeloid and lymphoid potential in day-8 EBs have the phenotype of $CD34^+CD45^-$c-kit^+CD41^+Flk-1^+CD31^+. The expression of Flk-1, the vascular endothelial growth factor receptor, suggests

that they may have potential for the endothelial cell lineage. However, their endothelial potential was not tested, and whether these EB cells include hemangioblasts is unknown.

HSCs have been previously shown to express different surface markers depending on their location and their stage of development. CD41 is expressed by primitive HSCs, but downregulated by HSCs during the transition to fetal liver stage[106,108,122], and adult HSCs lack CD41 expression[148]. In addition, CD41 expression precedes CD45 expression in early hematopoiesis[108]. The phenotype of EB cells in this study is in total agreement with that of embryonic hematopoietic progenitors described in these studies. Since the hematopoietic progenitors in day-8 EBs in our study have lymphoid potential, they differ from yolk sac hematopoietic progenitors. AGM-derived HSCs have been defined as $CD34^{+}$[122] as well as $CD45^{-/lo}c\text{-}kit^{+}CD41^{+}CD31^{+}AA4.1^{+}$[120], which is very similar to the progenitors expressing CD31, CD34, CD41 and c-kit in EBs in our study. However, they differ in Flk-1 expression. In contrast to EB-derived hematopoietic progenitors, AGM-HSCs lack Flk-1 expression. This suggests that hematopoietic progenitors in day 8 EBs are more immature than ED10.5 AGM-HSCs, since the former still express the marker of early hemangioblasts. We assume that these EBs correspond to endothelial like lymphohematopoietic progenitors isolated from caudal half of ED9.5 embryo, which encompass P-Sp region[221].

Culturing $CD34^{+}CD45^{-}c\text{-}kit^{+}CD41^{+}$ EB cells with OP9 stroma and cytokines results in a heterogeneous cell population we termed ES-HPs, some of which express CD45, Mac-1 and/or Ter119 while the majority remains $CD34^{+}CD45^{-}c\text{-}kit^{+}CD41^{+}$. $CD34^{+}CD45^{-}c\text{-}kit^{+}CD41^{+}$ ES-HPs resemble the original EB population in phenotype and lineage potential.

However, the majority of these ES-HPs have lost Flk-1 expression. It has been reported that Flk-1expression is downregulated after hemangioblast stage, and precursors committed only to the hematopoietic lineage are Flk-1^{-}[166,176]. Thus, the expression of Flk-1 on day-8 $CD34^{+}CD45^{-}$c-$kit^{+}CD41^{+}$ EB cells suggests that they may still retain endothelial potential while loss of Flk-1 on ES-HPs suggests that the EB cells become fully committed for the hematopoietic lineage as they differentiate into ES-HPs. In addition, CD41 expression is differentially regulated during EB differentiation. Most cells derived from $CD45^{-}Lin^{-}$ ES-HPs down-regulate CD41 surface expression, while most of the cells at this stage still express c-kit.

The finding of hematopoietic progenitors with lymphoid and myeloid potential is important for identification of HSCs with long-term repopulating capacities in adult recipients. Only genetic modification of EBs has resulted in reproducible engraftment in irradiated adult recipients, which reflects their state of immaturity[112]. Our current study shows that EB cells differentiate *in vitro* into hematopoietic progenitors that are similar to adult hematopoietic progenitors in phenotype (expression of CD45 and loss of CD41 and Flk-1)[111]. It remains to be determined whether ES-HPs, which appear to be more mature than hematopoietic progenitors in EBs, have *in vivo* repopulating capacity.

Chapter 5. General discussion

Presented in this thesis is a novel developmental pathway of lymphocyte differentiation from ES cells *in vitro* (depicted in Figure 5.1). This pathway entails lymphocyte progenitors at different stages of development. Each examined progenitor population represents the known *in vivo* developmental precursors during ontogeny. The earliest lymphocyte progenitors were found among day 8 EB cells with the surface marker expression of $CD34^{+}CD45^{-}$c-$kit^{+}CD41^{+}Flk\text{-}1^{+}CD31^{+}$. These progenitors, which also contain erythroid/myeloid potential are developmentally immature and close to ED9.5 hematopoietic progenitors derived from caudal half of embryo, with the phenotype of VE-$cadherin^{+}Flk\text{-}1^{+}CD31^{+}CD34^{+}CD45^{-}$[221]. The EB hematopoietic precursors undergo differentiation subsequent to co-culture with OP9 cells and form heterogeneous cell population we termed ES-HPs. ES-HPs consist of mature lineage positive cells ($Mac\text{-}1^{+}Ter119^{+}$) and immature lineage negative cells, of which a minor fraction is $CD45^{+}$. It was demonstrated that $CD45^{+}Lin^{-}$ ES-HPs derived from $CD34^{+}CD45^{-}$ EBs are the most immediate NK progenitors along the pathway to NK lineage. The analyses of $CD45^{-}Lin^{-}$ ES-HPs derived from $CD34^{+}CD45^{-}$c-$kit^{+}CD41^{+}$ EB cells indicated that these cells are immature hematopoietic progenitors comparable to the EB progenitor population. However, loss of Flk-1 expression by these cells places them at the same developmental stage as ED10.5 AGM-derived HSCs[120].

The important observation in this model of EB differentiation was step-wise phenotypic changes as EB cells differentiate on OP9 in ES-HP cultures (Figure 5.1). The most significant change during differentiation of EBs is the acquisition of CD45 by ES-HPs. This

change is accompanied by increase in potential for all lineages except B lineage. $CD45^+Lin^-$ ES-HPs derived from $CD34^+CD45^-$ contained the highest frequency of NK progenitors (1 in 10), T progenitors (1 in 115), and erythroid/myeloid progenitors (1 in 17.5) as compared with bulk ESHP (1 in 230 for NK, 1 in 297 for T, and 1 in 92 for myeloid progenitors) or EB populations (1 in 633 for NK, 1 in 1266 for T, and 1 in 222 for myeloid progenitors). $CD45^+Lin^-$ ES-HPs may include multiple committed progenitors, and/or multipotent and bipotent progenitors. High frequency of NK and myeloid progenitors among $CD45^+Lin^-$ ES-HPs implies the existence of a bipotent NK/myeloid precursor, which is expected with respect to identification of other myeloid/lymphoid precursors in the fetus[129]. Since the expression of CD45 on HSCs is first seen on ED11.5 AGM cells[122], $CD45^+Lin^-$ ES-HPs might be counterparts to these progenitors (Figure 5.1). Limiting dilution analysis of $CD45^+Lin^-$ ES-HPs for B cell progenitor frequency was not performed; however, when we compared the B cell generation from EBs and ES-HPs in chapter 3, we always detected higher number of B cells from $CD34^+CD45^-c\text{-}kit^+CD41^+$ EBs. It is likely that B cells develop from a separate population within $CD34^+CD45^-c\text{-}kit^+CD41^+$ EBs, which lose their potential in ES-HP culture. These results are in accordance with the defined scheme of B and T cell development before birth. Evidence has been provided that CLP-type progenitors do not exist during prenatal life, instead bipotent T/NKPs are abundant. This implies a dissociation of the B lineage from more immature hematopoietic precursors before birth. This is in direct contrast to postnatal life in which B cells develop from a more differentiated hematopoietic progenitor like CLPs.

The next phenotypic changes are consecutive downregulation of Flk-1 and CD41 (Figure 5.1). Flk-1 expression diminishes early after the primary ES-HP culture (Figure 4.4) whereas CD41 expression continues until after the secondary ES-HP culture though at half frequency of original EBs (Figure 4.6). While loss of Flk-1 has been linked with loss of endothelial potential and commitment to hematopoietic lineage[176], CD41 expression serves as a marker for hematopoietic restricted progenitors[106,108]. CD41 is a target gene of Scl transcription factor[108], which is essential for blood formation. In the absence of Scl, no blood cell development occurs *in vivo* in chimera mice or *in vitro* in embryoid bodies[181,222]. Our study also showed that CD41 is present on hematopoietic progenitors from day 8 EBs, but its expression decreases with differentiation toward more mature hematopoietic cells. Otani *et al.* analysed expression of CD41 on primitive erythroid progenitors from ES cells and found that $CD41^+$ are precursors to $CD41^+Ter119^+$ erythroid progenitors, which further differentiate to $CD41^-Ter119^+$ erythroblasts[223]. It is possible that CD41 expression is maintained in transition to each lineage, but is eventually lost except in megakaryocyte lineage.

Our results reveal the characteristics of various $CD34^+$ EB subpopulations. Of great interest is $CD45^+$ EB subset. Here, this subset was defined as $Mac\text{-}1^+$ myeloid precursors. Lymphoid progenitors in EBs were $CD45^-$. Multiple studies have documented lack of CD45 expression on definitive hematopoietic progenitors[111]. Burt *et al.* reported lymphoid potential of $CD45^+$ cells from day 7-10 EBs in *in vivo* transplantation experiments[187]. This report is in contrast to our study. One explanation is that they have used later stage EBs, which may contain more mature $CD45^+$ HSCs. The other possibility is that since they had isolated $CD45^+$ cells by sorting, the sorted cells may have been contaminated with other $CD45^{-/lo}$ fractions, which

have lymphoid potential. Finally, the lymphoid potential of $CD45^+$ EBs may be too low to be detected in our *in vitro* assays, but is assessable by *in vivo* assays. On the other hand, $CD45^+$ committed macrophage precursors have been described in embryonic hemogenic sites such as AGM, which may have resulted from $CD45^-$ HSCs[120]. Likewise, $CD45^+$ EB cells in our study may represent in situ differentiation of precursors to lineage-restricted progenitors.

With regard to $CD34^+CD45^-$c-kit^-CD41^+ EB cells, we speculated that they could be erythroid progenitors. The number of $Ter119^+$ erythroid cells among ES-HPs derived from $CD34^+CD45^-$ EB cells are consistently higher than those derived from $CD34^+CD45^-$c-kit^+CD41^+ cells (10-24% versus 3%). Therefore, it is likely that erythroid potential in the $CD34^+CD45^-$ EB cell population is contained in subsets lacking expression of c-kit or CD41, particularly c-$kit^{-/lo}CD41^+$ subset. Erythroid precursors in ES/OP9 co-culture system are $CD41^{+}$[223]. $CD41^+$ EB cell subset may have been formed at a transitional stage in erythropoiesis. Since their colony forming potential is not substantial, they may be in a more mature stage of erythroid differentiation.

Since $CD45^+Lin^-$ ES-HPs derived from EBs display a more mature phenotype and bear all NK, T, B and myeloid lineage potentials, we determined the frequency of myeloid, NK and T progenitors among these cells. The results show that these cells are enriched for NK potential (one in 10 $CD45^+Lin^-$ ES-HPs give rise to NK cells). This NK potential is similar to that of BM-derived NKPs. However, we did not detect CD122 expression on ES-HPs (Figure 3.6) and (Figure 5.1). CD122 expression on human NKPs has been difficult to demonstrate, even though $CD34^+$ HPCs cultured with IL-2, IL-15 or SCF and Flt3-L produce NKPs[63,76]. *In vivo*

stages of NK differentiation from progenitors in lymph node has shown that committed $CD122^+$ NKPs derive from uncommitted $CD122^-$ precursors at stage III of differentiation[65]. We also showed that CD122 appears on NK progenitors in ES-HPs with IL-2 and IL-15 (Figure 5.1). Also, $CD45^+Lin^-$ NKPs in ES-HPs have other lineage potentials and are not restricted to NK lineage. This data suggests that these progenitors are still immature and need signaling via IL-2 and/or IL-15 to upregulate this receptor.

Analysis of NK cells generated from ES cells suggests that ES-derived NK (ES-NK) cells resemble fetal NK cells in phenotype and function. ES-NK cells express CD94/NKG2 but not Ly49 receptors. They are differentially cytotoxic against MHC class I-deficient target cells[200]. This data confirms the notion that ES cell culture system recapitulates fetal NK cell development.

T cell differentiation from EBs appeared to follow the same developmental pathway as NK lineage. The frequency of T cell precursors increases almost 4 fold as $CD34^+CD45^-$ EB cells differentiate into ES-HPs (Figure 5.1). Therefore, a T cell developmental intermediate may be generated in ES-HP culture. However, the enrichment of T cell progenitors in $CD45^+Lin^-$ ES-HP fraction compared to bulk population was much less than that of NK progenitors (three fold versus 25 fold). Nevertheless, these results show that T progenitors co-exist with NK progenitors in the same cell population. Clonal assays using OP9-DL1 cells have shown that fetal DN1 and DN2 cells contain bipotent T/NKPs. These assays showed that NK and T cell potential can be simultaneously explored on this cell line[135]. However, we could not detect bipotent T/NKPs in $CD45^+Lin^-$ ES-HPs on OP9-DL1 stromal cells in limiting dilution

experiments, despite the fact that both NK and T cells could be generated in bulk cultures. This could be due to the very low frequency of these progenitors in ES-HP population. Alternatively, defective T cell differentiation on OP9-DL1 cells may have resulted in the observed low frequency of T progenitors and consequently T/NKPs. Analysis of T cell differentiation from CLPs on OP9-DL1 cells by Porritt *et al.* have revealed a block in DN3/DN4 transition[224]. The block in T cell development on OP9-DL1 cells was also reported from other adult hematopoietic progenitors, but not from fetal thymic progenitors. It was thought that IL-7 used in these cultures may inhibit differentiation of adult progenitors to CD4 and/or CD8 T cells[215,216]. We also reduced IL-7 concentration in the T culture; and were able to detect TCR^+, $CD4^+$ and $CD8^+$ T cells. However, percentage of TCR^+ cells was very variable in different experiments, making it impossible to detect bipotent T/NKPs in OP9-DL1 co-culture. It is also likely that T cell progenitors from ES-HPs are more immature than DN thymic progenitors and need further differentiation beyond the time point when we performed our analysis. Unfortunately, in clonal assays cells die after 2 week of culture and we were not able to extend the culture period beyond that.

The most significant outcome of this thesis research is the identification of hematopoietic progenitors with lymphoid potential generated *in vitro* from ES cells. Although previous studies have shown that mature B, NK and T cells can be generated from ES cells *in vitro*, the progenitors for those have not been characterized. Lymphopoiesis signifies definitive hematopoietic progenitors, which could be used in transplantation treatments. Thus far, ES cell progeny can repopulate adult recipient mice, only after they are differentiated on stromal cell lines[225] or transduced with genes to induce definitive hematopoietic potential[112]. We

have demonstrated that the progeny of EB cells (ES-HPs) possess more definitive hematopoietic properties and will be valuable to compare their *in vivo* repopulating capacity with that of EBs. Several factors play a role in triggering definitive hematopoietic developmental program. These factors include Cdx and Hox family members, as well as Aml1, Mll, and Notch family members. Both Aml1 and Mll specify definitive hematopoiesis at or before the AGM stage, whereas Notch1 is critical for HSC genesis in P-Sp region, and is dispensable afterward[112]. The *in vitro* ES differentiation culture will allow us to further explore the role of these factors in the induction of definitive hematopoiesis in the EB system.

5.1. Future work

Future studies will focus on analysis of *in vivo* hematopoietic repopulation using described hematopoietic progenitors derived from ES cells. In these experiments, the type of recipient mice and route of transplantation are important for successful engraftement. For example, $Rag2^{-/-}\gamma c^{-/-}$ immunodeficient mice that lack NK cells are preferred as recipients, because ES derived progenitors have low-intermediate level of MHC class I molecules and could be destroyed by NK cells[112]. Also, intrafemural injection has resulted in better engraftment compared to intravenous injection, which may improve homing efficiency of ES derived progenitors[112]. Besides transplantation of self-renewing HSCs, the use of more differentiated progenitors such as NKPs or mature immune cells has certain advantages. These cells could potentially be exploited in areas of infectious diseases, cancer and BM transplantation[41]. One interesting example of clinical application of NK cells is in BM transplantation for leukemia

patients. While T cells may cause graft-versus–host disease, NK cells generated from transplanted bone marrow may generate anti-leukemia effects without causing graft-vs-host disease[141,226].

Hematopoietic differentiation of mouse ES cells has set the stage for studies of human ES cell development to hematopoietic cells. Many genetic and malignant diseases of bone marrow is cured by HSC transplantation; and therefore, a protocol to generate hematopoietic progenitors from human ES cells which reconstitute adult recipients will be very beneficial. The current sources of reconstituting HSCs for clinical therapy including human BM, mobilized peripheral blood and umbilical cord blood are limited due to lack of well-matched donors. Deriving HSCs or hematopoietic progenitors from human ES cells could provide unlimited source of these cells in transplantation treatments. Similar to mouse ES cells, human ES cells can differentiate into hematopoietic cells by both co-culture with stromal cells and formation of EBs. Early hematopoietic precursors during human EB formation have been identified as $CD45^{-}CD31^{+}Flk\text{-}1^{+}CD34^{+}$. These cells contain bipotent precursors for hematopoietic and endothelial lineages similar to hemangioblasts. They are also able to minimally contribute to hematopoiesis after transplantation into immunodeficient recipient animals[227]. Our analyses of hematopoietic development from mouse ES cells could be extrapolated into human ES cell system to isolate progenitors with robust myelolymphoid potential capable of multilineage hematopoietic engraftment in recipients.

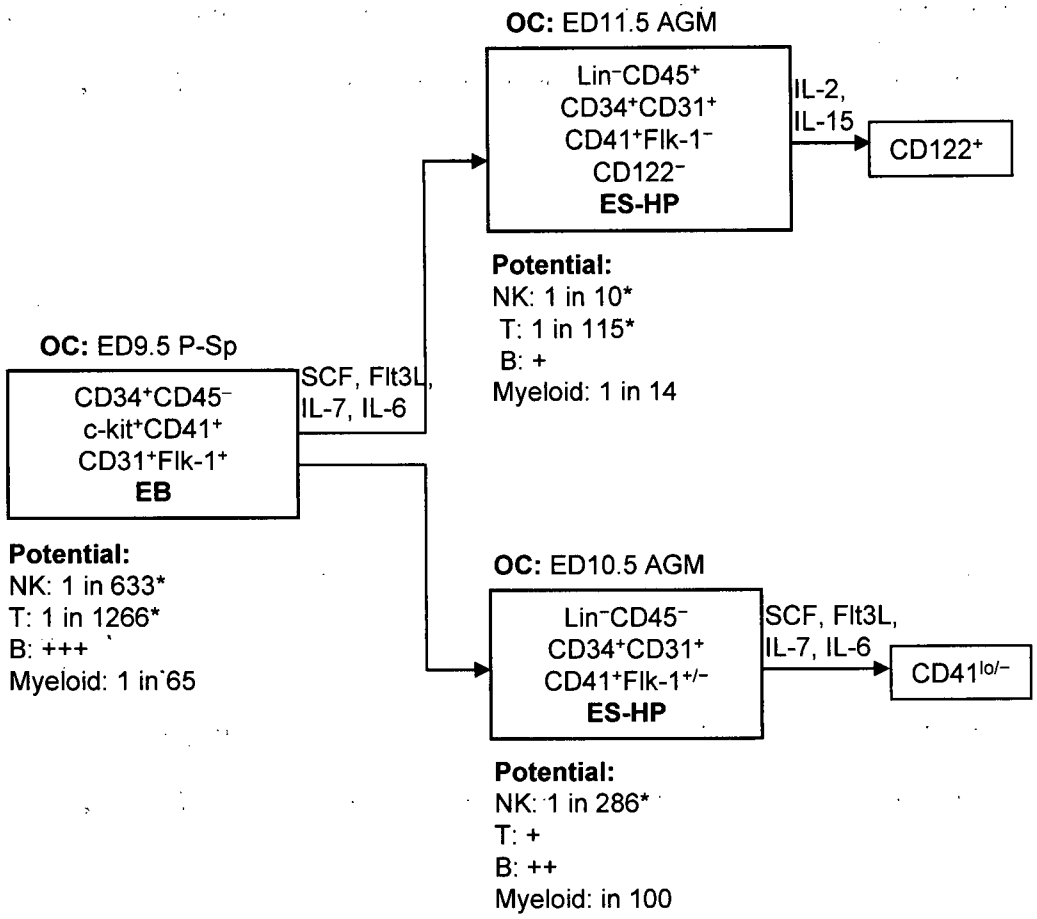


Figure 5.1. Hematopoietic differentiation of EB cells.

EBs differentiate into hematopoietic progenitors (ES-HPs) in presence of the indicated cytokines. Markers that change during differentiation are demonstrated. Transferring ES-HPs into other conditions generates hematopoietic cells. The quantitative hematopoietic lineage potential of each population is shown below it, except for the B lineage. The number of (+) for B potential refers to the estimated yield of B cells from each population. (*) indicates that limiting dilution assay has been performed on cells derived from $CD34^{+}CD45^{-}$ EBs. Ontogenic counterparts **(OC)** for each subset have been proposed.

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