CHAPTER II

BACKGROUND AND RELATED LITERATURES

1. Hematopoietic stem cells (HSCs)

Hematopoietic stem cells (HSCs) are the best characterized adult stem cells which responsible for blood cell production throughout life. HSCs were first discovered in 1961 (Till and McCulloch, 1961) as a cell that has long term repopulate activity, the capacity for regeneration of hematopoietic system in lethally irradiated mice after transplantation. By transplantation assay, HSCs are divided into two types, long-term HSCs (LT-HSCs) and short-term HSCs (ST-HSCs) (Figure 1). LT-HSCs have ability to produce blood cells for at least 6 months and can be reconstituting bone marrow of secondary lethally irradiated recipient in serial transplantation assay, but ST-HSCs cannot. ST-HSCs are limited potential for self-renewal, they proliferate rapidly and then undergo differentiation.

By asymmetric division, HSCs could generate two daughter cells with different cell fates, one remain the same potential as parental cell, so called self-renewal, and another one give rise to a progenitor cell or differentiation. Self-renewal is important for maintaining stem cell number, whereas differentiation is crucial for hematopoiesis. Both HSCs properties are controlled by cytokines and signaling molecules, which provided by their niche. So, nowadays, the aim of HSCs research is to purify homogenous population of LT-HSCs and find the way to maintain their properties *ex vivo*.

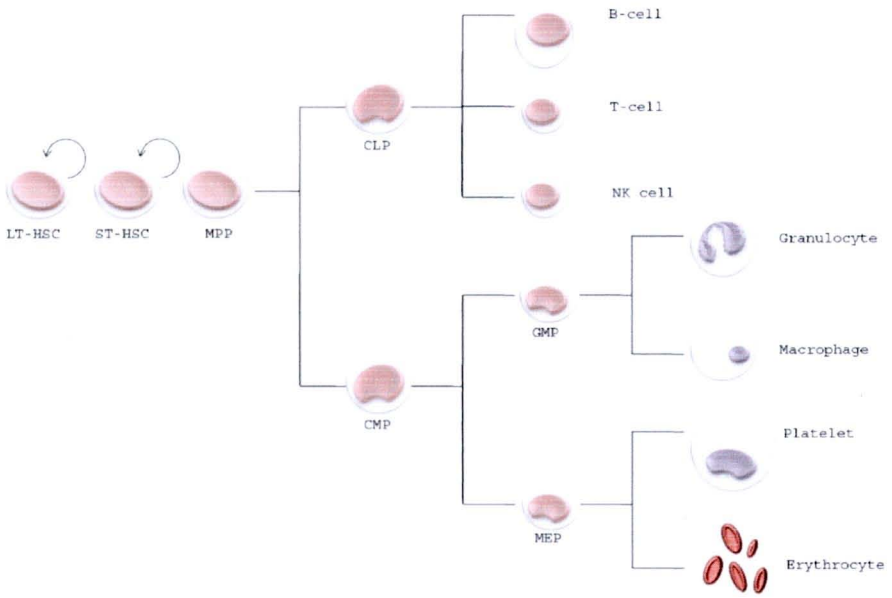

Figure 1 The hematopoietic hierarchy. The HSC compartment contain LT-HSCs, ST-HSCs, multipotent progenitors (MPP) and each subpopulation of which, common lymphoid progenitors (CLP); common myeloid progenitor (CMP); granulocyte/macrophage progenitor (GMP); and megakaryocyte/erythrocyte progenitor (MEP).

1.1. HSCs isolation

HSCs are defined by their long term multilineage reconstitution (LTMR) activity and ability to form colony forming unit spleen (CFU-S) after transplanted into lethally irradiated recipient *in vivo*. HSCs activity can also access *in vitro* by long term culture initiating cell (LTC-IC) and cobblestone area forming cell (CAFC) assays. In order to purification, firstly HSCs were separated based on size and density. Isolated cells were assayed for their functions (Iscove, 1972, 1990). Until the set up of B-lineage colony assay (Whitlock and Wittle, 1982), the clonal precursor of Whitlock-Witte culture B lineage-engrafting cells were defined. Interestingly, these B-lineage precursor cells did not express B220, B-lineage marker (Coffman and Weissman, 1981; Muller-seiburg et al., 1986). They hypothesized that precursor cells might not express all committed lineage markers. This finding leads to an establishment of new method to enrich HSCs population by depletion of lineage positive cells from marrow cell. Lin^- cells have ability to form all lineage colonies and contained hematopoietic reconstituting cells. Next, Lin^- were used in the combination of others markers to purified highly enriched HSCs

population. In mouse, Thy1 (CD90), Sca1 (a CD59/Ly6 family member) and c-kit (CD117) has been identified as a marker of HSCs (Morrison and Weissman, 1994). Thy1.1^{lo}, Lin^{-}, Sca1^{+}, c-kit^{+} (KTLS) population contained LT-HSC, one in 5 cells yielded long term multilineage reconstitution. Because of the complexity of KTLS cell purification, using combination of 10-12 surface markers, HSCs isolation method has been continually studied to find the simple methods. Side population (SP) is a marrow cell fraction weakly labeled with the DNA dye Hoechst 33342 (Goodell et al., 1996). This is thought to be the mechanism to avoid toxicity of quiescence HSCs by ATP-binding cassette (ABC)/G2, a cell surface transporter. SP cells are enriched 1000 folds for HSCs activity. Combination of KTLS with SP could isolate nearly homogenous subset of HSCs. Furthermore, SLAM family receptors have been identified as an effective marker to purify HSCs (Kiel et al., 2005). Simple combination of these receptors, CD150^{+} and CD48^{-}, yield HSCs enrichment that equal to KTLS cells, 20% of these cells yield LTMR in irradiated mice. The percentage of LTMR cells were increased to 47% when combined CD150^{+} CD48^{-} with Lin^{-} Sca1^{+} c-kit^{+} and to 45% when used CD150^{+} CD48^{-} CD41^{-} markers. Using SLAM family markers, CD150^{+} CD48^{-} CD41^{-}, is simplify and enhance HSCs purification. But these cells are mostly found on sinusoid endothelial cells which differ from KTLS cells that are found on endosteal surface, this results in a controversial of HSCs niche.

1.2. HSCs niche

HSCs are quiescence in specialized microenvironment within bone marrow, so-called niche. HSCs niche was hypothesized in 1978 (Schofield, 1978) as a place that control HSCs self-renewal, mobilization, differentiation and death by interaction between HSCs and niche cells. However, the exactly place where HSCs located within bone marrow and molecular mechanisms that control HSCs properties remain largely unclear. Nowadays two HSCs niches, osteoblastic niche and vascular niche, were proposed. Generally, HSCs were thought to be proximity to bone surface, which composed of osteoblast-lining cells, within bone marrow. Osteoblastic niche has been shown to play an important role in maintaining HSCs quiescence whereas vascular niche was known as a place for HSCs mobilization and differentiation. However, using SLAM markers, CD150^{+} CD48^{-} CD41^{-}, these cells are found mostly in vascular niche which contrast to

former hypothesis. So molecular mechanisms of both niches that could maintain HSCs quiescence has been extensively studied.

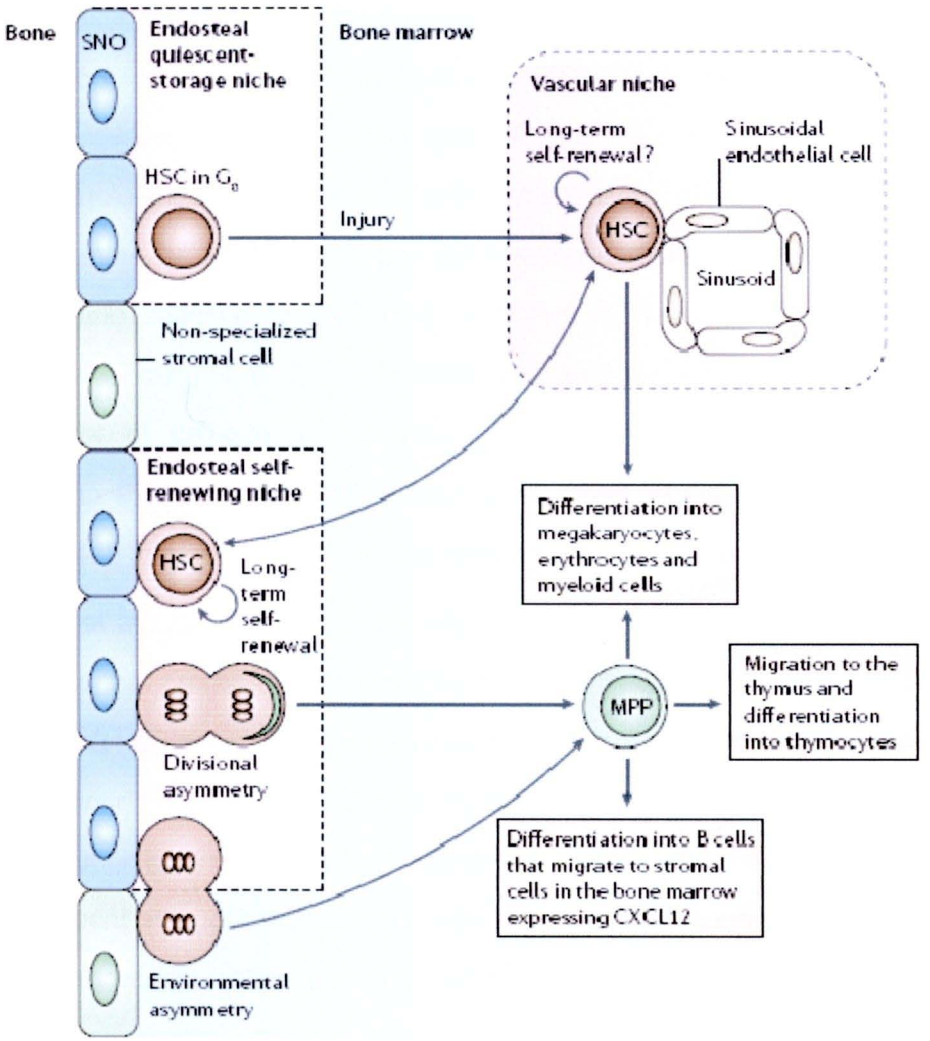

Figure 2 Bone-marrow HSC niches. Endosteal bone surfaces are lined with stromal cells. Spindle-shaped N-cadherin-expressing osteoblasts (SNOs) serve as niche cells to maintain quiescence and prevent differentiation of attached haematopoietic stem cells (HSCs). In response to injury, quiescent HSCs might be activated and recruited to the vascular niche. (Anne and Andreas, 2006)

1.2.1. Osteoblastic niche

Osteoblasts, bone-forming cells derived from Mesenchymal stem cells, were defined as a crucial component of HSCs niche. Firstly, osteoblasts were shown to secrete cytokines and growth factors that promote HSCs proliferation *in vitro* (Taichman

et al., 1996; Taichman and Emerson, 1998). Many signaling molecules were found to be expressed on osteoblasts such as Jagged1, Angiopoietin1 which their pathway has been shown to play an important role in maintaining HSCs quiescence (Calvi et al., 2003; Arai et al., 2004). In co-culture experiments, osteoblasts have been shown to support hematopoiesis (Taichman and Emerson, 1994). Moreover, from *in vivo* studies, conditional inactivation of BMP receptor type IA (BMPRIA) reveal an increase in the number of spindle-shape N-cadherin^{+} CD45^{-} osteoblastic cell (SNO cells) and also resulted in an increase of HSCs number. By immunofluorescence analysis, the LTMR HSCs were found attached to SNO cells mediated by N-cadherin in N-cadherin/ß-catenin complex (Zhang et al., 2003). Similarly, over expression of parathyroid hormone (PTH), regulator of calcium homeostasis and bone formation, and constitutively activation of the PTH/PTHrP (PTH1R) under type I collagen α1 promoter (osteoblast specific promoter) induced osteoblasts proliferation correlated with an increase in HSCs number (Calvi et al., 2003). Ablation of osteoblasts by GCV-treated Col2.3ΔTK mice, transgenic mice that express the herpesvirus thymidine kinase (TK) under the control of type I collagen α1 promoter, led to decreases in number of hematopoietic cell and bone marrow cellularity (Visnjic et al., 2004). Together, these data support the role of osteoblasts in maintenance of HSCs. In addition, osteoblasts were shown to improve HSCs engraftment after cotransplantation into allogenic mouse strains (El-Badri et al., 1998). Furthermore, there are not only osteoblasts within osteoblastic niche, non-osteoblast marrow stromal cells has been shown as a key regulator in HSCs niche. For example, stromal cells express membrane-bound c-KitL that was not expressed on mature osteoblasts *in vitro* (Taichman et al., 1996). Membrane-bound c-KitL was found to be a crucial molecule for localization of c-Kit^{+} HSCs in osteoblastic niche (McCulloch et al., 1965 and Driessen et al., 2003).

1.2.2. Vascular niche

Vascular niche has been identified as microenvironment generated by endothelial cells that affects the behavior of adjacent cells (Nikolova et al., 2006).By *in vitro* experiments, endothelial cells were shown to have the effects on many cell types such as neural cell, epithelial cell (Lammert et al., 2001; Shen et al., 2004) and

hematopoietic cells (Avecilla et al., 2004). As from embryonic development, both hematopoietic and endothelial cells are derived from common precursor named as hemagioblast (Choi et al., 1998), these cells are closely connected from yolk sac to bone marrow, places for hematopoiesis. Additionally, within bone marrow, sinusoidal endothelial cells are defined as crucial component of vascular niche for HSCs by the ability to support HSCs proliferation whereas other vascular endothelial cells cannot (Li, W. et al. 2003). Initially, vascular niche were defined as a place for HSCs differentiation and mobilization (Kopp et al., 2005). For example, sinusoidal endothelial cells express CXCL12 and fibroblast growth factor-4 (FGF4) that induce megakaryocyte localization and adhesion to these endothelial cells (Avecilla et al., 2004). By direct contact, endothelial cells induce maturation and platelets release of megakaryocyte through VE-cadherin and VLA-4/VCAM-1 axis (Avecilla et al., 2004).Not only thrombopoiesis, erythroid and B lymphoid progenitors have also been shown to reside in vascular niche (Ryan, 1993; Barbe et al.,1996). These data correspond to the idea that vascular niche serve as a place that allows HSCs differentiation and readily to mobilization to the peripheral circulation after stress, via the detachment of quiescence HSCs from osteoblastic niche and migrate towards the vascular niche ,then re-create hematopoiesis. However, the discovery of SLAM family receptors for HSCs purification, $CD150^{+}$ $CD48^{-}$ $CD41^{-}$, they found that $CD150^{+}$ HSCs are mostly attached to sinusoidal endothelial cell in vascular niche (Kiel et al., 2005). This finding showed the role of vascular niche in supporting HSCs. Several studies suggest that HSCs in difference niches might be in different state of quiescence (Shiozawa et al., 2008). HSCs in an osteoblastic niche may be truly quiescence than in vascular niche (Shiozawa et al., 2008). So the existence of these two distinct niches and their different roles in maintaining HSCs are extensively studied.

1.3. Regulation of HSCs within the niche

As previously described, HSCs are controlled by signaling molecules and cytokines that produced by niche cells (Figure 3). During embryonic development there are many signaling pathways that influence the structure of embryo, cell fate determination, identity and these signaling also play an important role in adult stem cells (Blank et al., 2008).

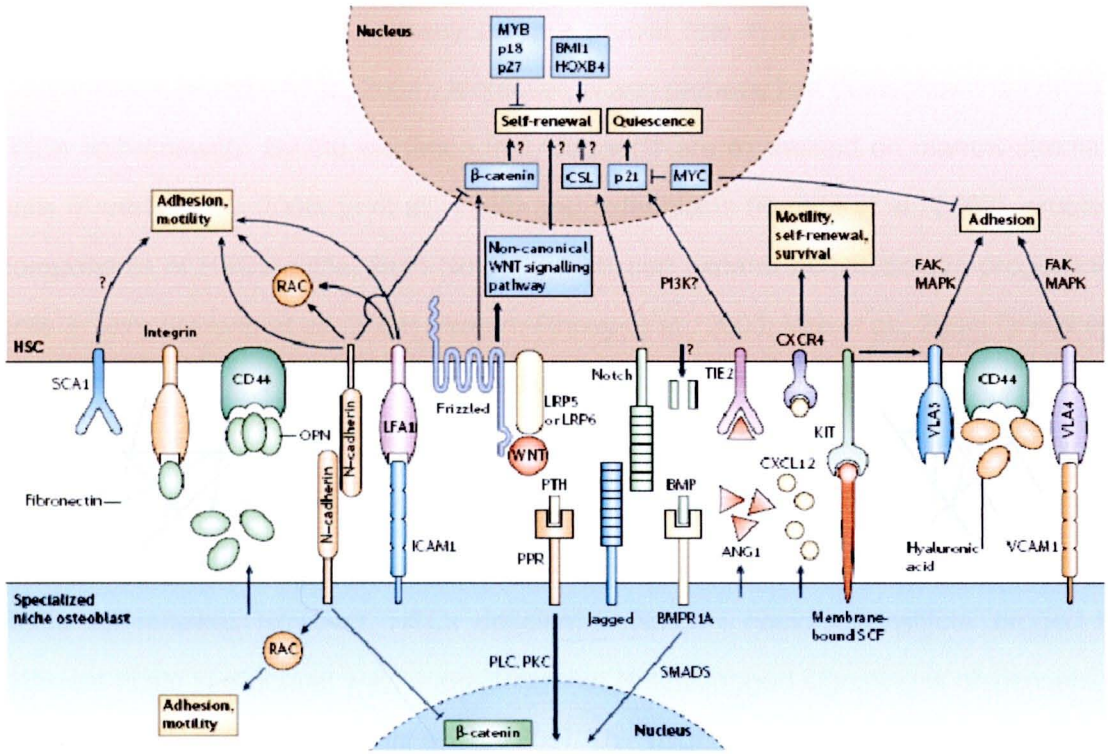

Figure 3 A model of the endosteal niche–stem-cell synapse. Schematic diagram of the endosteal niche–stem-cell synapse showing putative ligand–receptor interactions and adhesion molecules, as well as some of the intracellular pathways that are activated following signalling. (Anne and Andreas, 2006)

1.3.1. Notch pathway

Notch is a single spanning transmembrane protein, which has a modular architecture including many repeats of a protein such as epidermal growth factor (EGF), membrane-proximal Lin12/Notch/Glp-1 (LNG) repeats (Baron, 2003). The intracellular domain has four distinct regions, the RAM domain, the ankyrin repeats, a transcriptional activator domain (TAD) and the PEST (proline-, glutamate-, serine-, threonine-rich) sequence. Two nuclear localisation sequences are present prior to and following the ankyrin repeats (Baron, 2003). By binding to its ligands, Jagged and Delta, led to Notch activation result in proteolytic cleavage, release the intracellular domain (NICD) from its transmembrane domain. NICD is translocated to nuclease via its nuclear localisation sequences and act as transcription factor, activating their target genes such as *Hes* and *Hrt*. The Notch signaling pathway regulates cell fate specification in various systems. In

hematopoietic cells, Notch pathway plays a crucial role in lymphopoiesis and T cell differentiation (Radtke et al., 2004). Moreover, Notch pathway has been shown to control HSCs self-renewal. By the evidence that, Jagged1 are expressed on marrow-stromal cells (Karanu et al., 2000; Li et al., 1998) and osteoblasts (Pereira et al., 2002), crucial components of HSCs niche. Both Notch ligands can expand hematopoietic progenitor cells *in vitro* (Karanu et al., 2001; Varnum-Finney et al., 2003; Vas et al., 2004; Ohishi et al., 2002; Delaney et al., 2005). The activation of the Notch signaling pathway or its downstream target, Hes1, has been shown to increase the self-renewal capacity of LTMR cells (Kunisato et al., 2003; Stier et al., 2002). Together, these data illustrated that Notch/Jagged signaling activated by osteoblasts might be essential for regulation of HSCs self-renewal. However, HSCs deficient in Notch-1 could reconstitute Jagged-1 deficient hosts in a normal way, suggesting that Notch/Jagged signaling is dispensable for in vivo HSC function (Mancini et al., 2005). This might be the result from redundancy of other Notch receptors and ligands.

1.3.2. Wnt pathway

Wnts are a large family of secreted lipid-modified glycoproteins that are expressed in a wide variety of tissues. Wnt pathway has been known to regulate multiple processes of development. Three different signaling pathways are typically described for Wnt proteins. The canonical Wnt/ß-catenin pathway, through which ß-catenin-dependent activity occurs; the noncanonical pathways, which include the polar cell polarity pathway that involves activation of AP1 through c-jun N-terminal kinase and ß-catenin-independent; and the Wnt-$Ca2^{+}$ pathway, which activates protein kinase C and affects cell adhesion (Widelitz, 2005; Scoyk et al., 2007). Activation of Wnt signaling, Wnt ligands bind to its receptor Frizzleds (Fzds), a family of 7 transmembrane spanning receptors, which are involved in both canonical and noncanonical (Schulte and Bryja, 2007).In the absence of Wnt ligands, for canonical pathway, the cytosolic level of ß-catenin is kept low by the degradation complex which is produced by the active serine-threonine kinase glycogen synthase kinase-3ß (GSK3ß), the tumor suppressor proteins APC (Adenomatous Polyposis coli) and Axin/Conductin. ß-catenin is phosphorelated by GSK3ß and leads to its ubiquitinylation via ß-TrCP (ß-Transducin repeat Containing Protein) and degradation by the proteasomal degradation machinery. After binding of

Wnt ligand with its co-receptor LRP5/6 (low-density lipoprotein receptor related protein) result in hyperphosphorylation of Dishevelled (Dsh) by the activated casein kinase 2 (CK2) leads to the inhibition of GSK3ß (Willert et al., 1997). Increasing of cytosolic ß-catenin also increase nuclear ß-catenin that interacts with T-cell factor/Lymphoid enhancer factor (TCF/Lef). The ß-catenin/TCF complex activates transcription of their target genes. Wnt pathway has been shown to regulate hematopoiesis and HSCs properties. The expression of Wnt molecules were discovered at the site of hematopoiesis. Wnt5a was shown to encourage self-renewal and proliferation of fetal HSCs in vitro (Austin et al., 1997). Moreover, treatment of Wnt3a was shown to increase self-renewal of murine HSCs (Willert et al., 2003). Likewise, Wnt molecule also expanded the number of human $Lin^{-}CD34^{+}$ in vitro (Van Den Berg et al., 1998). Wnt proteins have been reported to be expressed in both HSCs and bone marrow stromal cells (Van Den Berg et al., 1998). This finding suggest the important of Wnt signaling and these molecules act on HSC through both paracrine and autocrine mechanism (Blank et al., 2008). It has been studied that overexpressing *Dkk1*, Wnt inhibitor expressed in niche cells, in bone marrow impairs HSCs long-term reconstitution activity (Fleming et al., 2008). Activation of Wnt signaling in HSCs by overexpressing ß-catenin increases HSCs self-renewal (Reya et al., 2003).On the other hand, excessive activation of ß-catenin reduces cell-cycle quiescence and blocks the differentiation of HSCs resulting in HSC exhaustion (Scheller et al., 2006; Kirstetter et al., 2006). So, the fine-tuned modulation of Wnt/ß-catenin signaling intensity is required for the proper control of HSC quiescence (Suda and Arai, 2008). Therefore, the control of Wnt signaling is driven by intrinsic factors within HSCs itself and extrinsic factors produced by niche cells.

1.3.3 BMP pathway

Bone morphogenic protein (BMP) is a secreted molecule which belongs to TGF-ß superfamily. BMPs were initially identified by their capacity to induce endochondral bone formation (Canalis et al., 2003; Chen et al., 2004; Cao and Chen 2005). To date over 20 BMP family members have been isolated and characterized. BMP signals are mediated by type I and II BMP receptors. Upon ligand binding, the type II receptor forms a heterodimer with the type I receptor, and the constitutive kinase of the type II activates the type I receptor and initiates the signal transduction cascade

by phosphorylating downstream nuclear factors, which then translocate to the nucleus to activate or inhibit transcription. After receptor activation, BMP signals via smad 1, 5 and 8. Phosphorylated Smad1, 5 and 8 proteins form a complex with Smad4 and then are translocated into the nucleus where they interact with other transcription factors. BMP signaling is also regulated by its antagonists, secreted polypeptides that limit BMP action by binding BMPs, therefore precluding their binding to specific cell surface receptors. Knowledge of BMP antagonists is derived from the developmental effects of the Spemann organizer. The antagonists Chordin, Follistatin, Noggin, Xnr3, and Cerberus are all locally expressed in Spemann's organizer. The expression of these antagonists is essential to prevent BMPs, which are ubiquitously expressed, from suppressing dorsal differentiation (Zimmerman et al., 1996; Khokha et al., 2005). BMPs have been report as key regulators of hematopoietic development. BMP4 deficiency results in early lethality due to severe mesodermal defects (Winnier et al., 1995). But, there are limited data of BMP signaling in HSC maintenance. Treatment of BMP4 was shown to promote HSCs maintenance in vitro, whereas lower concentrations of BMP4 induced proliferation and differentiation of human hematopoietic progenitors (Bhatia et al., 1999).

1.3.4. N-cadherin

N-cadherin, calcium dependent cell-cell adhesion molecule, belongs to cadherin family. N-cadherin is a transmembrane glycoprotein protein, which composed of extracellular domain, transmembrane domain and intracellular domain. The extracellular part consists of 5 cadherin repeats (EC) which bind to calcium ion to mediated homophilic interactions between cadherins molecule. ß-catenin, α-catenin ann p120 catenin bind to cytoplasmic part of N-cadherin which link cadherin molecule to the actin cytoskeleton. N-cadherin expression is controlled by both gene expression level and protein trafficking. Slug/snail/SIP1 (Conacci-Sorrell et al .2003) and TCF/ß-catenin (Comijn et al., 2001) suppress N-cadherin expression whereas twist stimulates the expression of N-cadherin (Batlle et al., 2000). Transport of synthesized cadherin to the plasma membrane requires binging of ß-catenin (Chen et al., 1999) and further regulated by phosphorylation, ubiquitination and proteolysis at the cell surface. N-cadherin is known as neural-cadherin or cadherin2 that has been first identified by

Grunwald et al. in the chicken retina (Grunwald et al., 1982) and in 1984 A-CAM was identified (now called N-cadherin) as a molecule that was localised at the adherens junctions (Volk and Geiger, 1984). As from embryonic development, N-cadherin is expressed in different tissues. Knock-out mice lacking expression of N-cadherin have been shown to die early during gestation. These embryos display major heart defects and malformed neural tubes and somites. The defect can be rescued partially by re-expression of N-cadherin (Radice et al, 1997; Luo et al, 2001). In addition to a structural role, cadherins have also been implicated in the regulation of signaling events (Aplin et al., 1998; Steinberg and McNutt, 1999). Intracellular part of N-cadherin is linked to ß-catenin, mediators of canonical Wnt signaling pathway. It has been suggested that cadherins can influence the Wnt signaling pathway, essentially by competing for the pool of ß-catenin (Sadot et al. 1998; Orsulic et al., 1999). In addition, N-cadherin interacts with Fibroblast growth factor receptor (FGFR). Binding of N-cadherin inhibits internalization of FGFR, leading to a persistent mitogen activated protein kinase extracellular signal regulate kinase (MAPK-ERK) activation and metrix metaloprotease 9 (MMP9) expression (Suyama et al., 2002).

In HSCs niche, N-cadherin is expressed in early human hematopoietic cells ($CD34^{+}CD19^{+}$) and is involved in the development and retention of early hematopoietic cells in the bone marrow (Puch et al., 2001). Moreover, N-cadherin is also expressed in all stages of bone formation and in vitro N-cadherin levels increase concomitantly with osteoblast differentiation (Ferrari et al., 2000). In murine system, adhesion between osteoblast and HSCs is thought to mediated by N-cadherin (Zhang et al., 2003; Arai et al., 2004; Wilson et al., 2004; Suda et al., 2005; Wilson and Trumpp, 2006; Haug et al., 2008; Zhang and Li, 2008). N-cadherin was defined as downstream target of Tie/Ang signaling that has been shown to maintain HSC quiescence (Arai et al., 2004). Loss of HSC properties by reactive oxygen species (ROS) and c-myc also showed the reduction of N-cadherin expression (Wilson et al., 2004; Hosokawa et al., 2007). These finding demonstrated the role of N-cadherin in regulating HSC properties. Nevertheless, the expression of N-cadherin in HSCs remains controversial. N-cadherin expression was not detected in highly purified HSCs by polymerase chain reaction, by using commercial anti-N-cadherin antibodies, or by ß-galactosidase staining of N-

cadherin gene trap mice (Kiel et al., 2007). N-cadherin deletion in vivo from HSCs and other hematopoietic cells has no effect on hematopoiesis in the bone marrow, HSC frequency, HSC maintenance or on ability of HSCs to engraftment and reconstitute irradiated mice in serial transplantation (Kiel et al., 2009).

2. Marrow stromal cells (MSCs)

Marrow stromal cells or Mesenchymal stem cells (MSCs) were firstly isolated from bone marrow (Friedenstein et al., 1970). These cells are able to differentiate into multiple cell types such as osteoblasts, chondroblasts and adipocyte under defined condition in vitro (Pittenger et al. 1999). However, MSCs have been isolated from other tissue such as peripheral blood (Zvaifler et al., 2000), cord blood (Rogers et al., 2004), trabecular bone (Noth et al. 2002), adipose tissue (Pittenger et al. 1999), synovium (De Bari et al., 2001), skin (Toma et al., 2001), muscle, and brain (Jiang et al., 2002). MSCs have been identified by both their morphology and immunophenotypes. MSCs are fibroblasts-like morphology, plastic-adhesive and self-renewal properties. STRO1, CD29, CD44, CD90 and CD105 have been used to isolate MSCs which hematopoietic lineage markers negative. However, there is no definitely markers for MSCs isolation.

MSCs have capability to support HSCs by secreting growth factors that regulate hematopoiesis and hematopoietic stem cell function. MSCs have ability to produce cytokines which maintain HSC in quiescence or promote their self-renewal, such as SCF, LIF, SDF- 1, OSM, BMP-4, Flt-3, TGF-ß (Haynesworth et al., 1996; Majumdar et al., 1998; Majumdar et al., 2000). MSC also produce a variety of interleukins (IL-1, IL6, IL-7, IL-8, 1L-11, IL-12, IL-14, IL-15), GM-CSF and G-CSF (Majumdar et al., 1998). Moreover, most of niche cells within bone marrow are derived from MSCs, especially osteoblasts which known to play an important role in HSCs maintenance. Together, these data show the important of MSCs in regulating HSCs properties.