ผลของ N-cadherin ที่มีการแสดงออกบน osteoblast ที่สร้างจาก stromal cell ต่อคุณสมบัติของ Hematopoietic stem cell.

นางสาวแพรวพรรณ อิงรุ่งเรืองเลิศ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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THE EFFECT OF N-CADHERIN EXPRESSED IN STROMAL CELL DERIVED OSTEOBLAST ON HEMATOPOIETIC STEM CELL PROPERTIES.

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แพรวพรรณ อิงรุ่งเรืองเลิศ : ผลของ N-cadherin ที่มีการแสดงออกบน osteoblast ที่สร้างจาก stromal cell ต่อคุณสมบัติของ Hematopoietic stem cell. (THE EFFECT OF N-CADHERIN EXPRESSED IN STROMAL CELL DERIVED OSTEOBLAST ON HEMATOPOIETIC STEM CELL PROPERTIES) อ. ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ.ดร.นพ.นิพัญจน์ อิศรเสนา, 62 หน้า.

การเพิ่มจำนวนเซลล์ต้นกำเนิดเม็ดเลือด (Hematopoietic Stem Cells; HSC) โดยการเพาะเลี้ยงภายนอกร่างกาย นับว่าเป็นวิธีการหนึ่งในการเอาชนะข้อจำกัดของจำนวนเซลล์ต้นกำเนิดเม็ดเลือดในการทำการปลูกถ่ายทางด้านคลินิก อย่างไรก็ตาม ยังไม่มีระบบในการเพาะเลี้ยงที่ช่วยในการเพิ่มจำนวนอย่างรวดเร็วของเซลล์ต้นกำเนิดเม็ดเลือดพร้อมกับการรักษาสภาพของเซลล์ไว้ ในการศึกษาครั้งนี้ เราได้สร้างระบบการเพาะเลี้ยงภายนอกร่างกายโดยการใช้ เซลล์ตัวอ่อนของกระดูกที่สร้างจากเซลล์ stromal (mouse marrow stromal cell-derived osteoblast; MOB) รวมกับเซลล์ stromal (3T3 noggin) ที่มีการแสดงออกอย่างมาก ของ noggin (เป็นการยับยั้งการส่งสัญญาณของ BMP; BMP antagonist) เรียกว่า M3B feeder โดย feeder นี้จะมีคุณสมบัติในการช่วยการเพิ่มจำนวนอย่างรวดเร็วของเซลล์เม็ดเลือด โดยการเพิ่มจำนวนเซลล์ชนิด $CD45^{+}$ได้มากกว่า 20 เท่า เมื่อเปรียบเทียบกับการใช้เซลล์ตัวอ่อนของกระดูกที่สร้างจากเซลล์ stromal (MOB) เพียงอย่างเดียว นอกจากนี้ เรายังพบว่าเซลล์ที่ได้จากการเพาะเลี้ยงเซลล์บน M3B feeder จะมี long-term culture initiating cell (LTC-IC) ใกล้เคียงกับการใช้เซลล์ตัวอ่อนของกระดูกที่สร้างจากเซลล์ stromal (MOB) ที่น่าสนใจก็คือชนิดของ colony forming cell (CFC) ที่มี LTC-IC จะมีการเปลี่ยนโคโลนีชนิดจาก CFU-M (colony forming unit macrophage) ไปเป็น CFU-GEMM (colony forming unit granulocyte, erythrocyte, macrophage and megakaryocyte) เมื่อเพาะเลี้ยงเซลล์ LSK บน M3B feeder จากการศึกษานี้แสดงให้เห็นว่าการรวมกันของเซลล์สองชนิดสามารถสร้างสภาพแวดล้อมขนาดเล็กที่เหมาะสมสำหรับการเพิ่มจำนวนอย่างรวดเร็วของเซลล์ต้นกำเนิดเม็ดเลือดพร้อมกับการรักษาสภาพของเซลล์ไว้ได้อีกด้วย นอกจากนี้เราได้ใช้ระบบการเพาะเลี้ยงแบบใหม่เพื่อศึกษาบทบาทของ N-cadherin ใน osteoblast niche เราพบว่าการแสดงออกของ N-cadherin ใน osteoblast ไปจำกัดจำนวนการสร้างของ $CD45^{+}$ จาก M3B feeder และเพิ่มจำนวนของ CFC ในสัปดาห์ที่สองของการเพาะเลี้ยง จากผลการศึกษาครั้งนี้ เป็นการสนับสนุนบทบาทที่สำคัญของ N-cadherin ในการรักษาสภาพของเซลล์ต้นกำเนิดเม็ดเลือด

สาขาวิชาวิทยาศาสตร์การแพทย์ ลายมือชื่อนิสิต.แพรวพรรณ อิงรุ่งเรืองเลิศ.....

ปีการศึกษา 2552 ลายมือชื่ออ.ปรึกษาวิทยานิพนธ์หลัก.....

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PRAEWPHAN INGRUNGRUANGLERT: THE EFFECT OF N-CADHERIN EXPRESSED IN STROMAL CELL DERIVED OSTEOBLAST ON HEMATOPOIETIC STEM CELL PROPERTIES. ADVISOR: ASST. PROF. NIPAN ISRASENA M.D. Ph.D., 62 pp.

Ex vivo expansion of Hematopoietic stem cells (HSCs) has been suggested as a way to overcome the limitation of hematopoietic cell number for clinical transplantation. However, there is no culture system that could promote both HSCs proliferation and maintenance. Here, we generate the ex vivo culture system using mouse marrow stromal cell-derived osteoblasts (MOBs) combine with noggin (BMP antagonist) overexpressed stromal cells (3T3 noggin), so called M3B feeder. This feeder system shows their ability to promote hematopoietic cell proliferation by increasing $CD45^+$ cell more than 20 fold compared with MOBs alone. Moreover, we found that the LTC-IC number of cells derived from cell cultured on M3B feeder was comparable to MOBs. Interestingly, type of CFC of LTC-IC was changed from CFU-M (colony forming unit macrophage) to CFU-GEMM (colony forming unit granulocyte, erythrocyte, macrophage and megakaryocyte) when cultured LSK cells on M3B feeder. This study proposes that combination of two cell types could generate proper microenvironments which both promote HSC maintenance and proliferation. We also used this new culture system to investigate the role of N-cadherin in osteoblastic niche. We found that overexpression of N-cadherin in osteoblasts limits number of $CD45^+$ produced from M3B feeder and increases the number of CFC at week 2 of culture. This finding supports the important role of N-cadherin in HSC maintenance.

Field of Study: Medical science ..... Student's Signature Praewphan Ingrungruanglert

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LIST OF ABBREVIATIONS

HSCs	=	Hematopoietic stem cells
MSCs	=	Marrow stromal cells
MOBs	=	Marrow stromal cell-derived osteoblasts
CFC	=	Colony forming cell
LTC-IC	=	Long term culture initiating cell
BMP	=	Bone Morphogenic Protein

CHAPTER I

INTRODUCTION

1. Background and rationale

Hematopoietic stem cells (HSCs) are the self-renewing multipotent progenitors that have ability to differentiate into all blood cell types. By their ability to reconstitute bone marrow of lethally irradiated mice after transplantation, HSCs are divided into two distinct populations, long-term HSCs (LT-HSCs) and short-term HSCs (ST-HSCs). LT-HSCs can sustain their properties, self-renewal and differentiation, throughout life and after serial transplantation whereas ST-HSCs are limited in their self-renewal activity. Therefore, the aim of HSCs research is to purify homogeneous population of LT-HSCs and maintain their properties *ex vivo*. Several methods have been established to identify HSCs. Based on immunophenotypical analysis, lineage-negative $(Lin)^-$ stem-cell antigen $1(SCA1)^+KIT^+$ (LSK) markers (Morrison and Weissman 1994) are commonly used for purifying HSCs containing population. Additional markers such as side population (SP) (Goodell et al. 1996), CD34 (Osawa et al., 1996) and Flt3 (CD135) (Adolfsson et al., 2001; Yang et al., 2005), have been used for further purify highly enriched HSC populations

HSCs were shown to localize to endosteum after homing assay (Xie et al., 2009). Osteoblasts were shown to secrete factors that regulate HSC properties in vivo and in vitro (Taichman et al. 1996; Taichman and Emerson 1998). It has been proposed that, by directly contact with osteblasts, HSCs is regulated by factors providing by these cells. While there are the numbers of research studies address the role of Wnt signaling in HSC maintenance, there is still limited data on the role of BMP and its antagonists, which are known as important regulators of hematopoiesis, in osteoblastic niche. In mutant mice with conditional inactivation of BMP receptor type IA (BMPRIA), an increase in the number of HSCs was observed in correlation with an increase in the number of N-cadherin$^+$CD45$^-$ osteoblastic cells (Zhang et al., 2003). The adhesion of HSCs to

osteoblast has been thought to be mediated by N-cadherin (Zhang et al. 2003). Consistent with these data, activation of Tie2/Ang1 signaling by Ang1 produced in osteoblasts, promote HSCs quiescence and also upregulate N-cadherin expression level (Arai et al., 2004). Increasing osteoblast number correlated with the increasing HSCs number (Calvi et al., 2003; Zhang et al., 2003). These data supported the role of osteoblasts in maintaining HSCs via N-cadherin mediated cell-cell adhesion in osteoblastic niche. Results from recent studies, however, raise the question of whether N-cadherin and osteoblast are really crucial in HSCs maintenance. HSCs identified by SLAM markers do not express N-cadherin and the deletion of N-cadherin in HSCs did not effect on HSC maintenance and hematopoiesis (Kiel et al., 2009).A better understanding of how each niche molecules combined to regulate HSC function will provide fundamental knowledge for creating effective ex vivo culture system for HSC expansion for clinical uses.

In this study, we generate the new feeder system that combines osteoblasts and cell engineered to secrete noggin. We demonstrated that noggin can counter effect of osteoblastic signals that limit HSC proliferation. Importantly, HSC cultured on the new feeder system produce more primitive colonies than osteoblast alone in long term culture assay. We also test the effect of N-cadherin directly expressed in mouse marrow stromal cell (mMSC) derived osteoblasts (MOBs) on HSC properties. Overexpression of N-cadherin in mMSCs showed greater capability to maintain CFC number of culture HSCs (LSK cells). In contrast, MOBs could maintain higher CFC number than mMSCs so the overexpression of N-cadherin in these cells did not cause the different in HSCs maintenance. Nevertheless, N-cadherin overexpressed MOBs in our new feeder system showed the effect on hematopoietic cell proliferation by reducing CD 45^{+} number and maintaining more CFC number than control. Therefore, our data demonatrated that N-cadherin expressed in niche cells, mMSCs and MOBs, increase their ability to regulate HSCs maintemance and function. Our data implicate the role of N-cadherin in maintaining HSCs in osteoblastic niche. Lastly, we also show the effect of IFNα treatement on reduction of *Dickkopf-1 (Dkk1)*, a soluble inhibitor of Wnt/beta-catenin signaling, expressed in MSCs but not in osteoblast. This result provided indirect

evidence that IFNα also has an effect on MSCs to induce HSCs proliferation. Knowledge of signaling generated by niche cells which control HSCs properties is very useful to apply for further ex vivo expansion and gene therapies in hematological diseases.

2. Research Questions

1. BMP and BMP antagonist involves in maintenance of HSCs self-renewal and differentiation or not

2. N-cadherin involves in maintenance of HSCs self-renewal and differentiation or not

3. Objectives

1. To develop ex vivo culture system for HSCs expansion and maintenance ex vivo
2. To study the effect of N-cadherin expressed in stromall cell-derived osteoblast on HSCs properties

4. Hypothesis

1. Balance between BMP and BMP antagonist regulate HSCs maintenance.
2. N-cadherin has the effect on HSCs maintenance by encouraging tightly adhesion of HSCs to niche cell and involve in regulating signaling in HSCs.

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*.

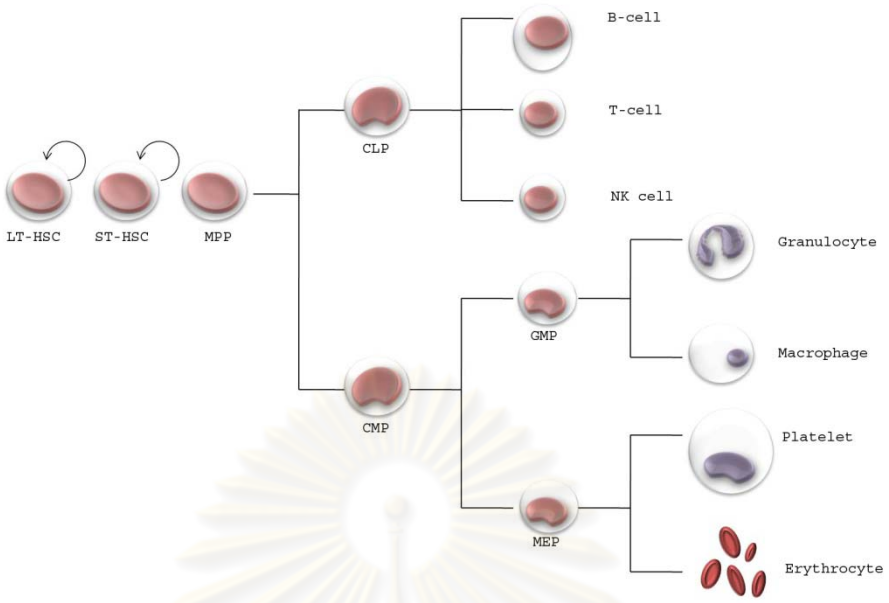

Figure 1 The hematopoietic hierarchy. The HSC compartment contain LT-HSCs, ST-HSCs, multipotent progenitors (MPP) and each subpopulation of which, common lymphoid progenitors (CLP); common myeloid progenitor (CMP); granulocyte/macrophage progenitor (GMP); and megakaryocyte/erythrocyte progenitor (MEP).

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.

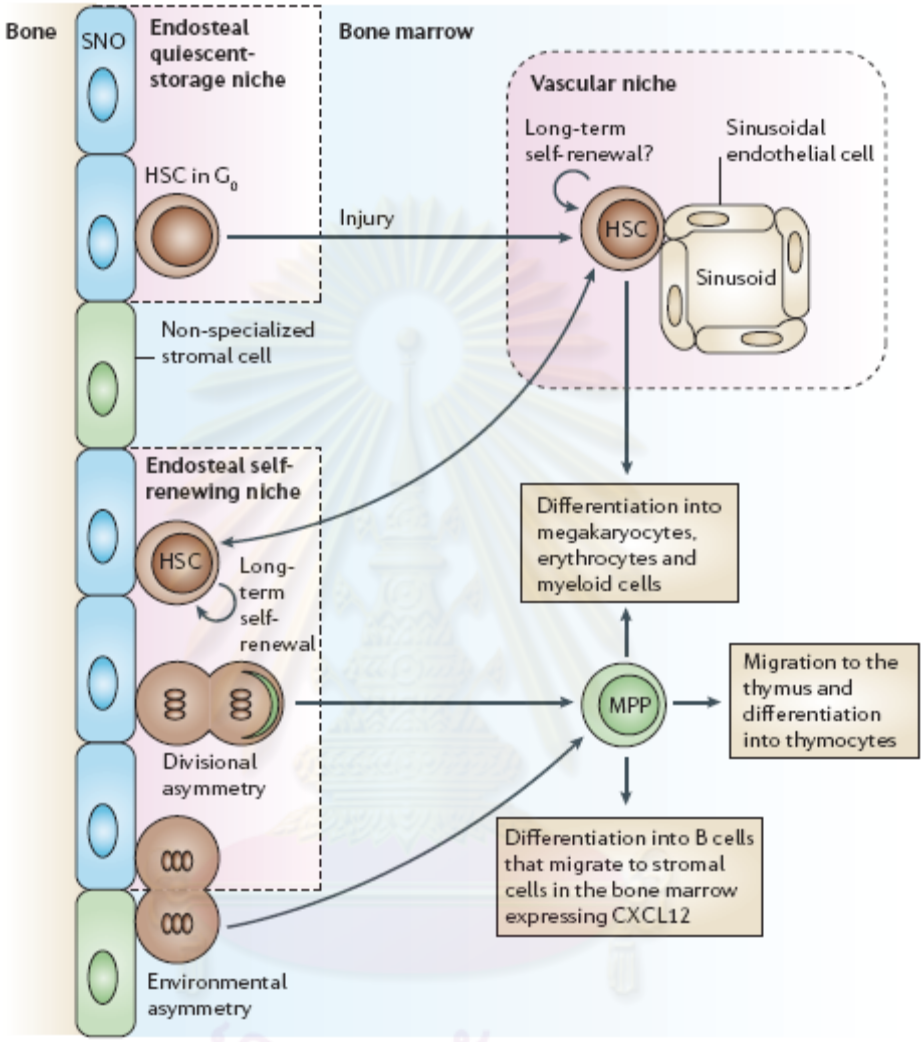

Figure 2 Bone-marrow HSC niches. Endosteal bone surfaces are lined with stromal cells. Spindle-shaped N-cadherin-expressing osteoblasts (SNOs) serve as niche cells to maintain quiescence and prevent differentiation of attached haematopoietic stem cells (HSCs). In response to injury, quiescent HSCs might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

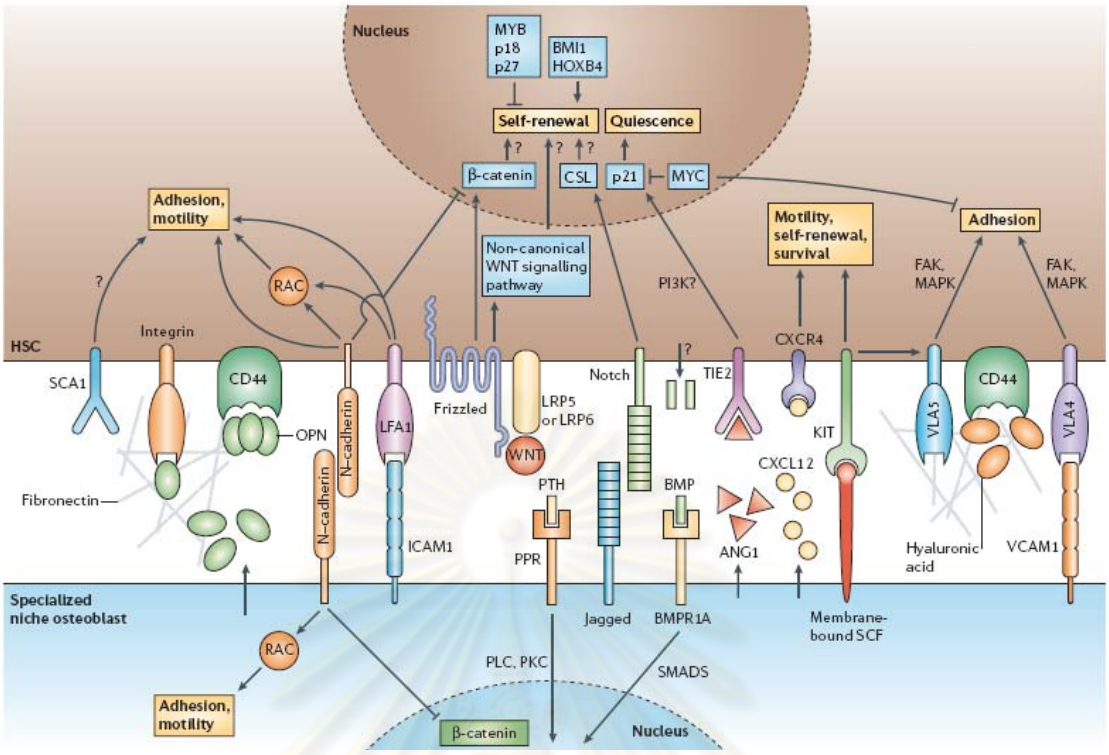

Figure 3 A model of the endosteal niche–stem-cell synapse. Schematic diagram of the endosteal niche–stem-cell synapse showing putative ligand–receptor interactions and adhesion molecules, as well as some of the intracellular pathways that are activated following signalling. (Anne and Andreas, 2006)

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.

CHAPTER III

MATERIALS AND METHODS

1. Hematopoietic stem cells (HSCs) isolation

8-12 week C57BL/6 mice were purchased from National Laboratory Animal Centre (Nlac), Mahidol University. For HSCs isolation (LSK cells), 3-5 mice were anesthetized by using 80 mg/kg Ketamine before cervical dislocation. Tibias and femurs were sacrificed under aseptic technique by sterile scissors and forceps. Both ends of the long bones were cut with scissors and bone marrow cells were flushed with Hank's buffered salt solution (HBSS) without calcium or magnesium (Invitrogen), supplemented with 5% heat-inactivated bovine serum (Hyclone) using a sterile 21 gauge needle attached to 5 ml syringe. Bone marrow cells were gently aspirated through the same syringe and needle to obtain single cell suspension and filtered through 70 μm nylon mesh (BD Falcon). Red blood cells were lysed with 1X ammonium chrolide lysis solution for 5 min at room temperature and centrifuged at 1000 rpm for 5 min. Cells were washed twice with 1XPBS. Then cells were counted and pre-enriched by lineage depletion using immunomagnetic cell separation (MACS, Miltenyi) according to the manufacturer's instruction. Briefly, cells were resuspended in 40 μl of lineage depletion buffer (Appendix B) per $1x10^7$ total cells. Then $1x10^7$ cells were incubated with 10 μl of biotinylated antibodies cocktail against a panel of so-called "lineage" antigens (CD5, CD45R (B220), CD11b, Anti-Gr-1 (Ly-6G/C), 7-4, and Ter-119 antibodies) for 10 min and 20 μl of Anti-Biotin MicroBeads for 15 min at 4 °C. Cells were washed by adding 5 ml of lineage depletion buffer and centrifuged at 1000 rpm for 5 min. Cells were resuspended in 500 μl of lineage depletion buffer. Then $lineage^+$ cell were depleted by using magnetic column. Firstly, MS column were prepared by rinsing with 500 μl of lineage depletion buffer. Secondly, cell suspension was applied onto the column and washed three times with 500 μl of lineage depletion buffer. Finally, the effluent were collected and centrifuged at 1000 rpm for 5 min. This fraction represents the enriched lineage negative cells. Next, $lineage^-$ cells were then incubated 30 min with 0.5 μg of FITC-

conjugated anti-Sca-1 and PE-conjugated anti-c-kit (Pharmingen, BD) per 10^6 cells in 100 µl of HBSS+5%FBS. Cells were washed twice with HBSS and centrifuged at 1000 rpm. Then cells were resuspended in HBSS+5%FBS containing 2 µg/ml propidium iodine (PI). Stained cells were analyzed and sorted by using BD FACSAriaII (Becton Dickinson, BD) (Appendix C)

2. Marrow stromal cells (MSCs) isolation

Flushed bone marrow cells were filtered through 70 µm nylon mesh (BD Falcon) and red blood cells were lysed with 1X ammonium chrolide lysis solution for 5 min at room temperature. Cells were washed twice with 1XPBS and centrifuged at 1000 rpm for 5 min. Then cells were seeded on 10 cm dish (Corning) in Dulbecco's Modified Eagle's Medium (Hyclone) supplemented with 10% FBS, 1% Penicillin-Streptomycin (GIBCO). Cells were left to settle for 3-4 day and media were changed to remove non-adherent cells. Adhering cell were trypsinized by using 0.25%Trypsin-EDTA (invitrogen) when reach 50-80% confluence.

3. Osteoblastic differentiation

MSCs were seeded on 0.1% gelatin (Sigma) coated 6-well plate at $1x10^5$ cells/well in 2 ml completed growth media (Dulbecco's Modified Eagle's Medium (Hyclone) supplemented with 10% FBS, 1% Penicillin-Streptomycin (GIBCO)) supplemented with 10 mM glycerol-2-phosphate, 0.2 mM ascorbic acid 2- phosphate and 0.1 µM dexamethasone. Cells were incubated at 37°C in humidified incubator with 5% CO2 in air. Media were changed every 2 days for 7 days.

4. Alizarin Red staining

Osteoblastic differentiation were performed in 24 well plate by seeding cells at $2x10^4$ cells/well in 500 µl completed growth media supplemented with 10 mM glycerol-2-phosphate, 0.2 mM ascorbic acid 2- phosphate and 0.1 µM dexamethasone. Cells were incubated at 37°C in humidified incubator with 5% CO2 in air. Media were changed every 2 days for 7 days. At day 7, the media from each well were aspirated and added with 1 ml 70% alcohol for 1 hour at room temperature. Alcohol was aspirated and cells

were rinsed twice with water for 10 min. 1 ml Alizarin Red Solution was added to cover the cell and incubated at room temperature for 30 min. After 30 minutes, Alizarin Red Solution was removed and washed four times with 1 ml water. 1 – 1.5 ml water was added to each well and visualized by using inverted microscope (Nikon).

5. N-cadherin overexpression

MSC were seeded at 1.5 x 10^6 cells in T25 flask one day before transfection. Next day, culture media were change to completed media without antibiotic. Preparation of a transfection complex, pCDH2 (Origene) were diluted with Opti-MEM to a concentration of 6 µg plasmid DNA/300 µl Opti-MEM. 24 µl of FuGENE® HD Transfection Reagent was added to diluted DNA to form transfection complex and incubate 15 min at room temperature. The transfection complex was added to the cells in a drop-wise manner and mixed well by swirling flask. Culture vessel was cultured at 37°C in humidified incubator with 5% CO2 in air. After 24 hour, media was removed and washed with 1 ml PBS. Cells were trypsinized by adding 1ml of 0.25% Trypsin-EDTA and incubated at 37°C for 2 min. Completed media was added to flask and cell suspension were transferred into 15 ml centrifuge tube. Cells were centrifuged at 1000 rpm for 5 min. Finally, cells were resuspened in 1 ml completed media and seeded into 35 mm dish as feeder cells and used for osteoblastic differentiation.

6. Cell culture

3T3 cell lines were purchased from American Type Culture Collection (ATCC). 3T3 were maintained in DMEM supplemented with 10% FBS, 1% Penicillin-Streptomycin (GIBCO) and incubated at 37°C in humidified incubator with 5% CO2 in air. Media were changed every 2-3 days and passage by trypsinization when they reached 70-80% confluent. For 3T3 noggin, 3T3 cell lines were transfected with pcDNA3.1-noggin by using FuGENE® HD Transfection Reagent. Next day, media was chaged to completed media and cultured for another one day. Then cells were trypsinized and seeded on 10 cm dish in completed media supplemented with 300 µg/ml geneticin. Geneticin resistance colonies were selected and tested for noggin expression level.

7. Long-term culture initiating cell assay (LTC-IC)

LTC-IC assay was preformed essentially as previously described with some modifications (Sutherland et. al., 1989; 1990). Briefly, all feeder cells were mitotically inactivated by irradiation at 5000 Gy. KLS cells were sorted and cultured at 1500 cells/35 mm dish on feeder cells in long term culture media (M5300, Stem Cell Technologies) supplemented with 10^{-6}M hydrocortisone. Half of media was changed every week for 4 weeks. After cultivation, cells were trypsinized by using 025% trypsin-EDTA (invitrogen). Total cells were resuspended in 500 µl PBS. 300 µl of suspended cells were added to 3.3 ml of methycellulose media (GF3434, StemCell Technologies) and vortex. Then, 1.1 ml of methycellulose containing cells were plated per 35 mm petri dish in duplicate using 3 cc syringe attached to 16 gauge blunt-end needle. Methylcellulose cultures were incubated for 12 to 14 days at 37°C in humidified incubator with 5% CO2 in air. The total number of colonies per dish was scored at day 12 to 14.

8. Colony forming cell (CFC) assay

All hematopoietic progenitor assays were performed according to the manufacturer's instructions (Stem Cell Technologies; Vancouver, Canada). All feeder cells were mitotically inactivated by irradiation at 5000 Gy. KLS cells were sorted and cultured at 1500 cells/35 mm dish on feeder cells in long term culture media (M5300, Stem Cell Technologies) supplemented with 10^{-6}M hydrocortisone. After cultivation at week 1 and week 2, cells were trypsinized by using 025% trypsin-EDTA (invitrogen). Total cells were resuspended in 500 µl PBS. 300 µl of suspended cells were added to 3.3 ml of methycellulose media (GF3434, StemCell Technologies) and vortex. Then, 1.1 ml of methycellulose containing cells were plated per 35 mm petri dish in duplicate using 3 cc syringe attached to 16 gauge blunt-end needle. Methylcellulose cultures were incubated for 12 to 14 days at 37°C in humidified incubator with 5% CO2 in air. The total number of colonies per dish was scored at day 12 to 14.

9. $CD45^+$ cells analysis

All feeder cells were mitotically inactivated by irradiation at 5000 Gy. KLS cells were sorted and cultured at 1500 cells/35 mm dish on feeder cells in long term culture media (M5300, Stem Cell Technologies) supplemented with 10^{-6}M hydrocortisone. Half of media was changed every week for 4 weeks. After cultivation, cells were trypsinized by using 025% trypsin-EDTA (invitrogen). Total cells were resuspended in 500 µl PBS. all feeder cells were mitotically inactivated by irradiation at 5000 Gy. KLS cells were sorted and cultured at 1500 cells/35 mm dish on feeder cells in long term culture media (M5300, Stem Cell Technologies) supplemented with 10^{-6}M hydrocortisone. Half of media was changed every week for 4 weeks. After cultivation, cells were trypsinized by using 025% trypsin-EDTA (invitrogen). Total cells were resuspended in 100 µl PBS and stained with 0.2 µg APC-conjugated anti-mouse CD45 (Biolegend) per 10^6 cells for 30 min at room temperature in dark. Cells were washed twice with HBSS and centrifuged at 1000 rpm. Then cells were resuspended in PBS and analyzed by using FACS caliber (Becton Dickinson, BD).

10. RNA extraction

Total RNA was extracted using TRI reagent (Molecular Research Center; MRC) according to the manufacturer's instructions. Briefly, cells were lysed directly in 6 well plate by using 1 ml of TRI reagent per well. The lysate was homogenized by pipetting and incubated at room temperature for 5 min. 0.1 ml of BCP (Molecular Research Center; MRC) was added to the lysate and shaked vigorously for 15 seconds. The resulting mixture was stored at room temperature for 2-15 minutes and centrifuged at 12,000 g for 15 minutes at 4 °C. Following centrifugation, the mixture was separated into a lower red phenol-chloroform phase, interphase and the colorless upper aqueous phase. The aqueous phase was transfered to a fresh tube and mixed with 0.5 ml isopropanol. Samples were stored at room temperature for 5-10 minutes and centrifuged at 12,000 g for 8 minutes at 4 °C. The supernatant was removed and the RNA pellet was washed with 75% ethanol and subsequent centrifugation at 7,500 g for 5 minutes at 4°C.

The ethanol was removed and the RNA pellet was briefly air-dry for 3 - 5 min. RNA was dissolved in The RNA storage solution (Ambion).

11. Complementary DNA (cDNA) synthesis

Isolated RNA was reverse transcribed by using RevertAid™ H Minus M-MuLV (Fermentus) according to the manufacturer's instruction. Briefly, 1 µg of total RNA in 11 µl of DNase/Rnase free water was incubated with 1 µl of 0.5 µg/µl Oligo dT at 70 °C for 5 min and incubated on ice. Then, 7 µl of mastermix containing 4 µl of 5X reaction buffer, 2 µl of 10mM dNTP and 1 µl of RiboLock™ RNase Inhibitor (20 u/µl) was added into RNA template tube and incubated at 37°C for 5 min. Finally, 1 µl of RevertAid™ H Minus M-MuLV Reverse Transcriptase (200 u/µl) was added into reaction tube and cDNA was transcribed at 42°C for 60 min. The reaction was terminated by incubating at 70°C for 5 min. Complementary DNA was kept at -20°C until used for measuring gene expression by real-time PCR.

12. Quantitative Real-Time PCR Analysis

A quantitative real-time PCR (qPCR) assay was performed on an ABI 7500 Fast Real-Time PCR System in a final volume of 25 µl. Each reaction was performed using reagents from the using Maxima SYBR (Fermentus), with 0.3 µM of primer, and 2 µl of cDNA. Amplification consisted of 50 cycles of denaturation at 94°C for 10 seconds, annealing at 50°C - 60°C, depending on the primer pair, for 10 seconds, and extension at 72°C for 40 seconds. Appendix A. shows the sequence of primer pairs (purchased from Operon, Hawthorne, NY USA). After the processes were completed, the real-time PCR results were automatically reported by Applied Biosystme software version 7500 and analyzed by relative quantification method (comparative Ct method). All experiments were done in triplicates.

13. Immunofluorescence staining

Cells were fixed with 4% paraformaldehyde for 10 minutes at room temperature, permeabilized with PBS supplemented with 0.3% Triton X-100 for 15 minutes, and blocked in blocking buffer (10% goat serum and 0.3% Triton X-100 in PBS) for 30 min at

room temperature. After each step, cells were washed twice with PBS for 5 minutes. Primary antibodies, goat anti mouse RUNX2 (santa cruz) and rabbit anti mouse osteocalcin (Takara) were diluted in diluents (5% goat serum, 0.3% Triton X-100 [Sigma], and PBS) at dilution 1:100 and incubated overnight at 4°c in humidified conditions. Cells were washed with PBST (PBS + 0.3% TritonX-100) supplemented with 0.1% BSA three times for 5 min. Secondary antibodies, Alexaflour546 conjugated donkey anti goat antibody and Alexaflour546 conjugated goat anti rabbit antibody were also diluted in diluents at dilution 1:200 and incubated for 1 h at room temperature in dark. For nuclear staining, 0.1 µg/mL DAPI (Molecular Probes) was used and incubated for 5 min at room temperature. Fluorescence images were obtained using Fluorescence microscope (Carl Zeiss).

14. Western blotting

Nuclear and cytoplasmic proteins were extracted by using M-PER® Mammalian Protein Extraction Reagent (PIERCE, Thermo) according to the manufacturer's instruction. Total protein was electrophoresed on 12% SDS-polyacrylamode gel at 150 V for 60 min and transferred to nitrocellulose at 150 V for 60 min. To detect noggin protein, nitrocellulose was blocked with 5% non-fat dry milk in PBS for 1 hour at room temperature with constant agitation, and incubated with goat anti-noggin antibody (Santa cruz), diluted in 5% non-fat dry milk in TBST (0.2% Tween 20 in Tris buffer saline) (1:500) at 4°C for overnight. Next day, the nitrocellulose was washed with TBST for 5 min three times and incubated with donkey anti-goat HRP conjugated in 5% non-fat dry milk in TBST (1:2000) for 60 min at room temperature with constant agitation. Finally, the nitrocellulose was visualized by chemiluminescence. ß-actin protein level was used as a control for equal protein loading. For ß-actin protein detection, total protein was electrophoresed on 12% SDS-polyacrylamode gel at 150 V for 60 min and transferred to nitrocellulose at 150 V for 60 min. After blocking with 5% non-fat dry milk in PBS, nitrocellulose membrane was incubated with mouse anti-mouse ß-actin diluted in 5% non-fat dry milk in TBST (1:500). Subsequently, the nitrocellulose was incubated with goat anti-mouse HRP conjugated IgG in 5% non-fat dry milk in TBST and visualized by chemiluminescence.

15. Wright-Giemsa staining

LTC-IC derived colonies were picked by using Pipetman P20-100 (Gilson) attached to 100µl fine tip. Colony was resuspended in 500 µl PBS and centrifuged at 1000 rpm for 5 min. Cells were diluteded in 100 µl PBS. Slides and filters were place into appropriate slots in the cytospin (Shandon Cytospin 3) with the cardboard filters facing the center of the cytospin. 100 µl of each sample was aliquot into the appropriate wells of the cytospin and centrifuged at 600 rpm for 5 min. The filters were removed from their slides without contacting the smears on the slides and slides were air dried for 5 min. Cells were stained using Wright Giemsa Stain for 5 min and added with water for 10 min. Slides were washed with water and air dried for 5 min. Images were obtained using microscope (Nikon).

16. Statistical analysis

Statistical significance was assayed by Student's t-test. The results were considered significant if $p < 0.05$.

CHAPTER IV

RESULTS

The role of osteoblasts in HSCs maintenance has been proposed for decade. Evidences of osteoblasts in supporting HSCs mostly came from *in vivo* study. Isolated osteoblasts have been used for in vitro studies and showed their ability to support hematopoietic cell development in vitro (Taichman and Emerson 1998; Shiozawa et al. 2008). However, state of osteoblasts that proper to maintain HSCs is unclear. It has been known that osteoblasts are derived from marrow stromal cells, mesenchymal progenitors, within bone marrow. So we used mMSC-derived osteoblasts to directly examine the role of these cells on HSC properties. First of all, mouse marrow stromal cells (mMSCs) were isolated from bone marrow of C57BL/6 mice. Bone marrow cells were cultured in completed growth media and adhered cells were primarily selected as mMSCs (Figure 4A). Selected mMSCs were tested for their specific marker, STRO1, expression. STRO1 is widely used MSCs marker. As shown in Figure 4B, using immunofluorescence staining assay, these cells are STRO1 positive.

Next, we evaluated their osteogenic potential using chemical induction. mMSCs were cultured in osteogenic media for 7 days. After 7 days, the morphology of mMSCs was changed into compact-structure (Figure 5A). To assess whether there were osteoblasts in these culture system, we performed Alizarin Red staining. As expected, these cells are Alizarin Red positive (Figure 5B). Furthermore we also analyzed the expression of osteoblast specific transcripts, Runx2 and osteocalcin, by these cells using real-time PCR and immunofluorescence staining assay. Both Runx2 and osteocalcin were produced at increased levels in induced cells (Figure 6). These data confirmed the differentiation of mMSCs into osteoblastic cells. So now on we called these cells as mouse marrow stromal cell-derived osteoblasts (mMSC-derived osteoblasts, MOBs) for further experiments.

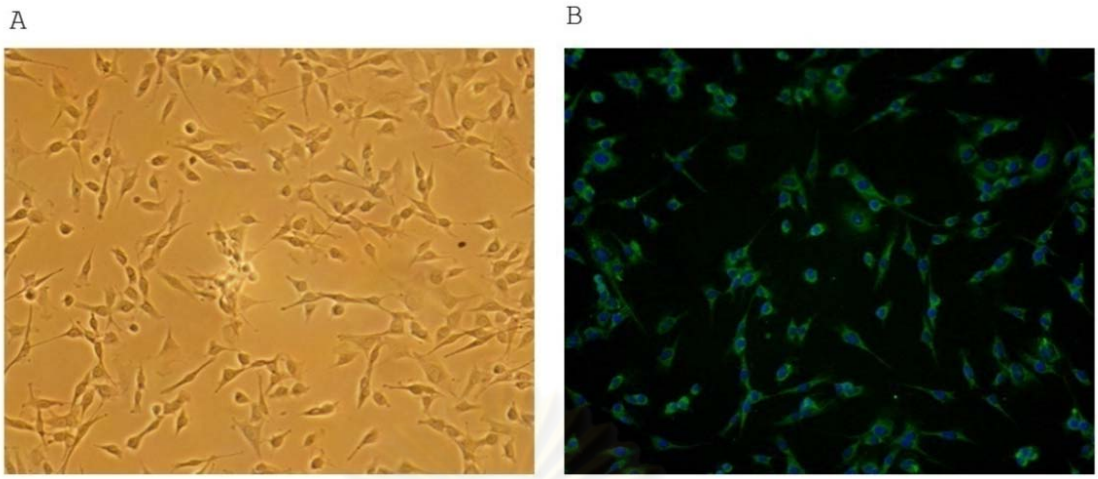

Figure 4 Morphology of isolated mouse marrow stromal cells (mMSCs). (A). Morphology of isolated mMSCs using phase contrast microscope. (B). mMSCs were stained with a mouse monoclonal antibody against STRO1 (Chemicon, Millipore). Magnification :x10.

We tested whether MOBs have ability to maintain colony formation after long-term culture using long-term cultured initiating cell (LTC-IC) assay. Lineage^{-} SCA1^{+} C-KIT^{+} cells (LSK cells), HSCs containing population, were culture on mitotically inactivated mMSC-derived osteoblasts compared with mMSCs. By microscopic observation, LSK cells formed a cobblestone area underneath MOBs whereas only differentiated cells were found on mMSCs (Figure 7). The colony forming cell (CFC) number derived from cells cultured on MOBs were higher than the cells cultures on mMSCs (Figure 8). Furthermore, MOBs showed greater number of LTC-IC than mMSCs (Figure 9). Taken together, these results support the role of osteoblasts in supporting HSCs and confirmed the differentiation of mMSCs into osteoblast-like population by their functional studies.

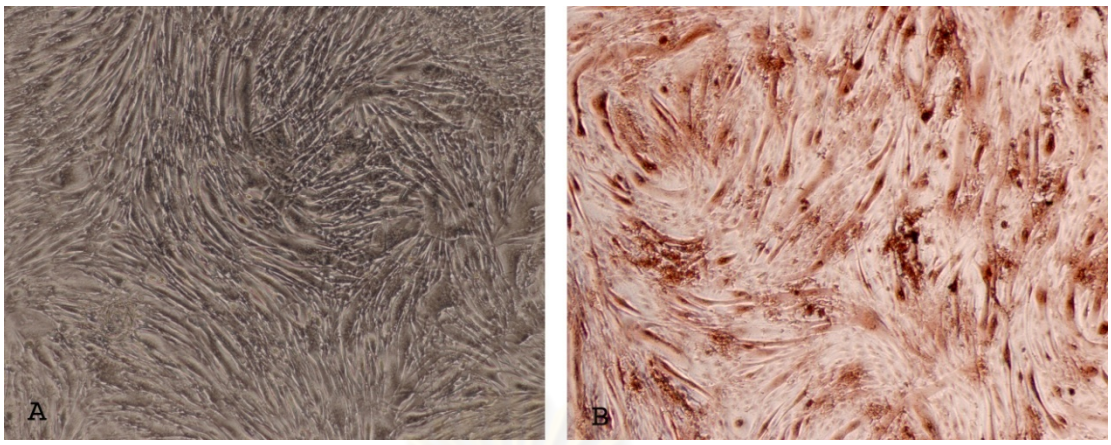

Figure 5 Morphology of mMSC-derived osteoblasts (MOBs). (A) mMSCs were induced with osteogenic media for 7 days (B) After osteob;astic differentiation, cells were stained with Alizarin Red. Magnification: x4

We further analyzed the gene expression profile of MOBs compared to mMSCs using Real-time PCR in order to dissect the different molecular mechanisms in controlling HSCs. As previous paper reported, Angiopoietin1 (Ang1) (Figure 10), CXC-chemokine ligand 12 (CXCL12) (Figure 10) and Jagged1 (Notch ligand) (Figure 11) expression levels were increased in MOBs. We also found upregulation level of Wnt molecules, Wnt1 and Wnt3a, in mMSCs-derived osteoblasts. These factors have been known to play an important role in HSCs maintenance. Upregulation of these factors correlated with their higher ability to supporting HSCs than mMSCs.

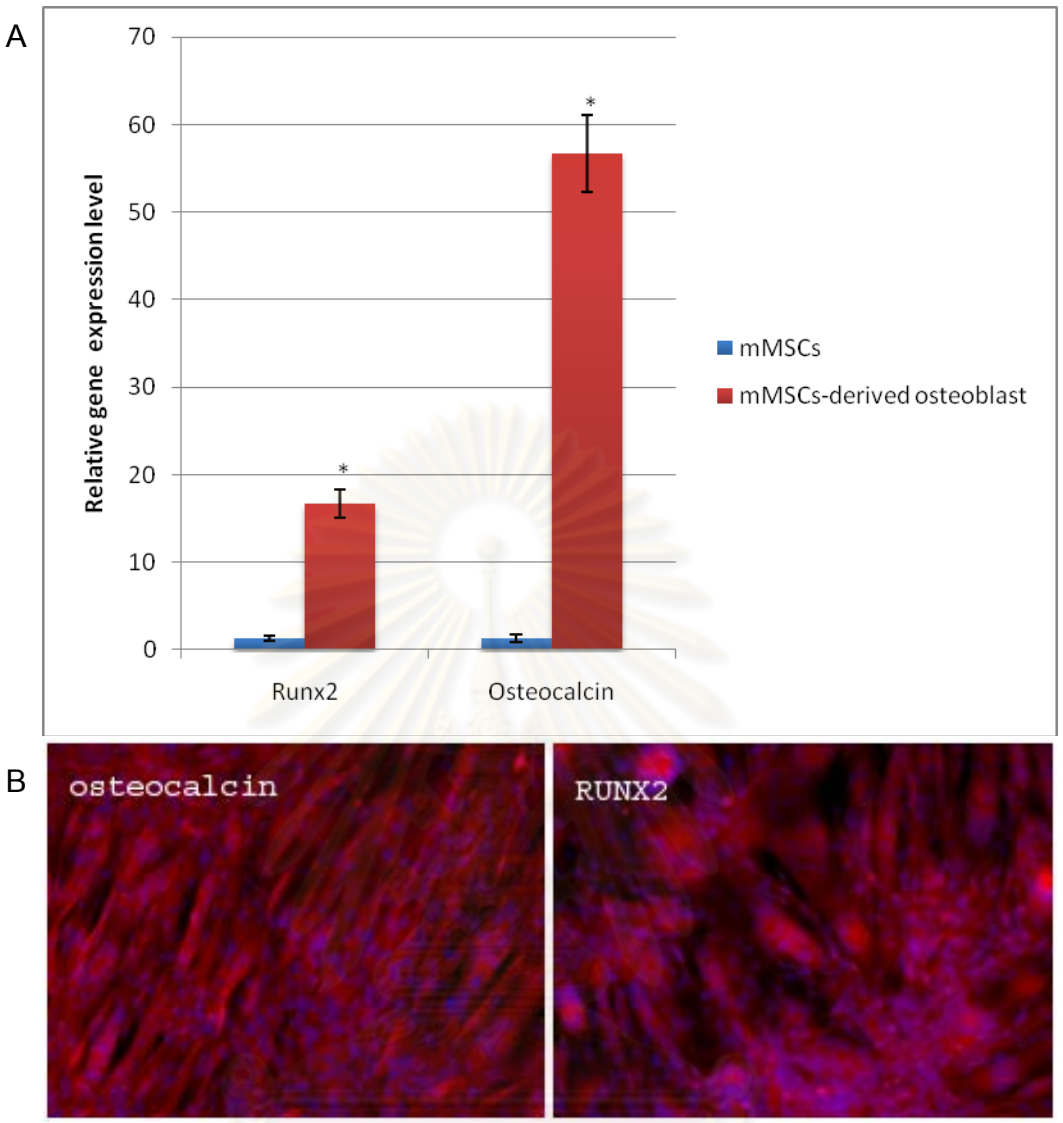

Figure 6 Osteoblastic gene expression of MOBs. (A) Quantitative RT-PCR analysis for the expression of osteoblast specific markers. The data are presented as average ± SD, (*$p < 0.005$, $n = 3$). (B) Immunostaining confirming in vitro differentiation of mMSCs into osteoblast. Secondary antibodies were labeled with Alexa flour 546 (red). Magnification: x10.

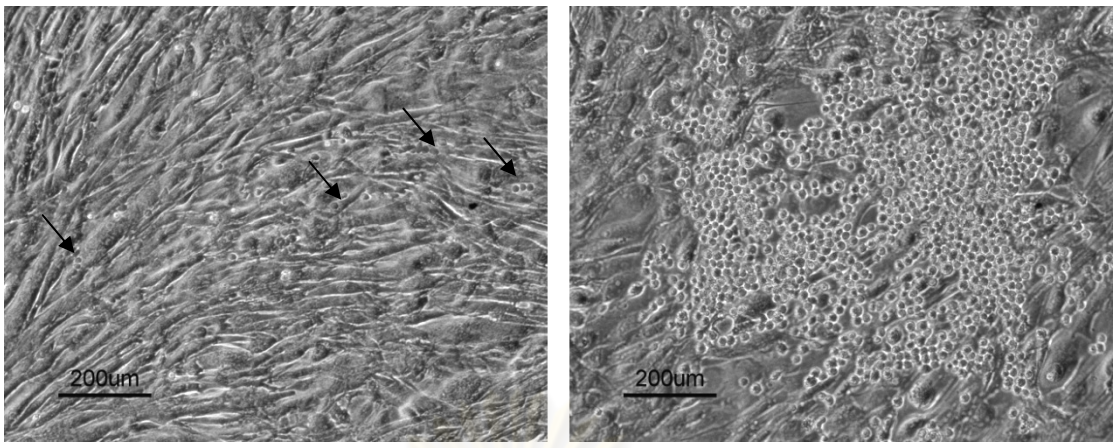

Figure 7 Growth phenotype of LSK cells cultured on MOBs and mMSCs. (A) Cobblestone area (arrow) of LSK cells underneath MOBs without any output cells. (B) Differentiated colony of LSK cells on mMSC. Magnification: x10.

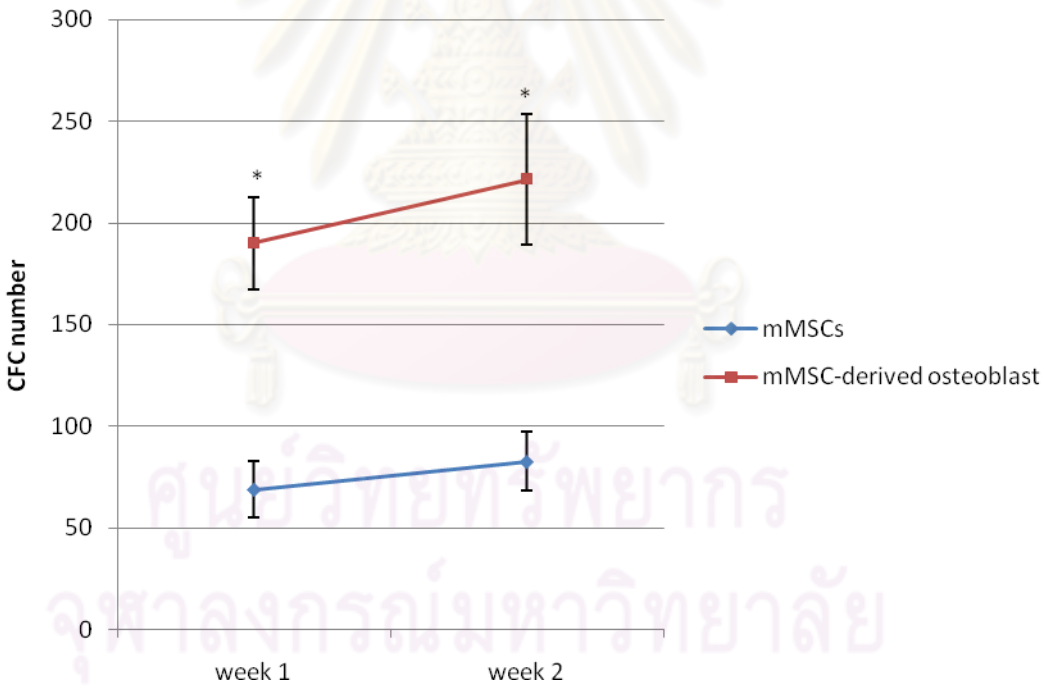

Figure 8 Effect of MOBs on the output of colony forming cell (CFC). Data shown are the mean number (± SD) of CFC derived from 1,000 input LSK cells (*$p < 0.01$, n=4).

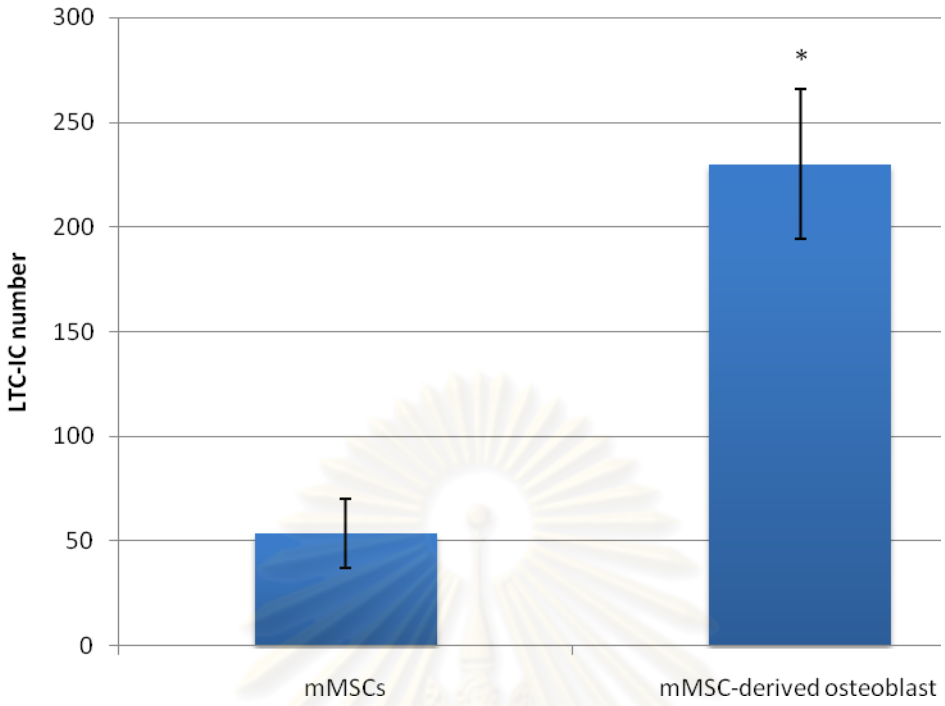

Figure 9 Effect of MOBs on the output of long-term culture initiating cell (LTC-IC). Data shown are the mean number (± SD) of LTC-IC derived from 1,000 input LSK cells (*p < 0.005, n=4).

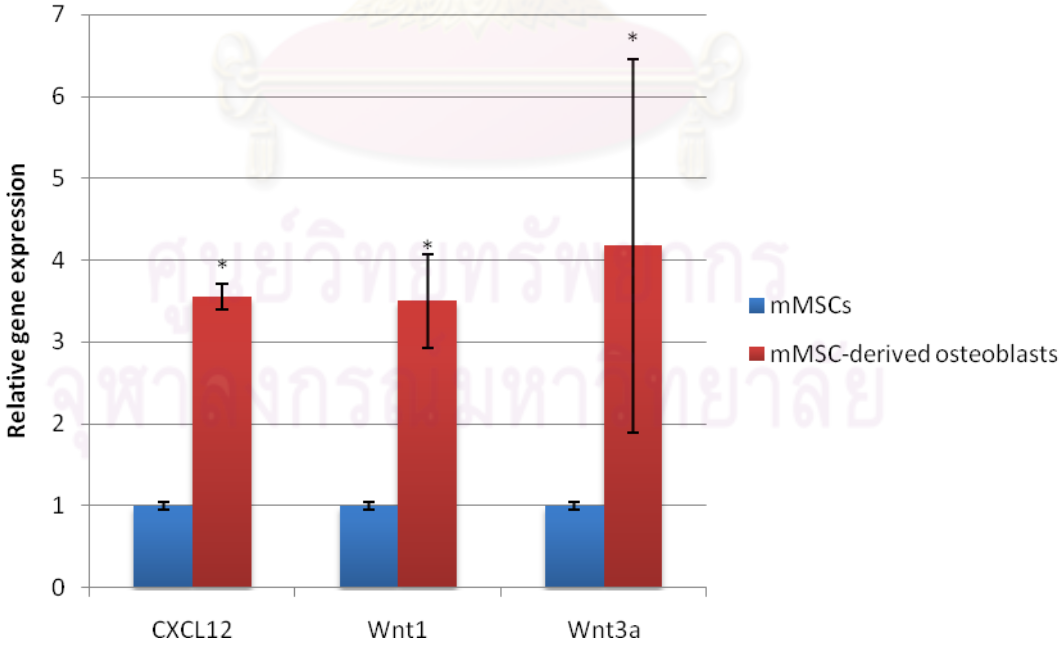

Figure 10 Quantitative RT-PCR analysis of CXCL12, Wnt1 and Wnt3a expression in MOBs and mMSCs. The data are presented as average (± SD), (*p < 0.05, n = 3).

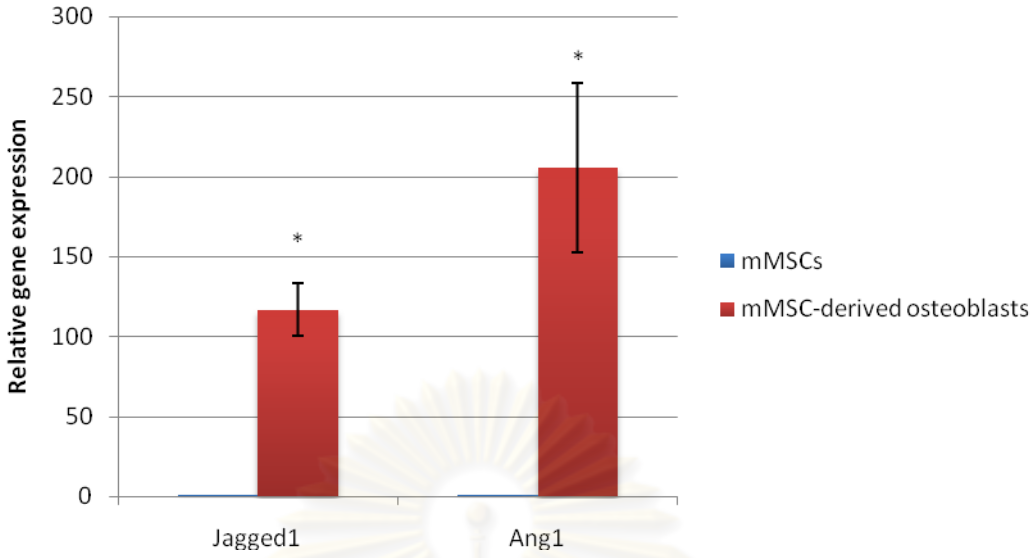

Figure 11 Quantitative RT-PCR analysis of Jagged1 and Angiopoietin1 (Ang1) expression in MOBs and mMSCs. The data are presented as average (± SD), (*$p < 0.01$, $n = 3$).

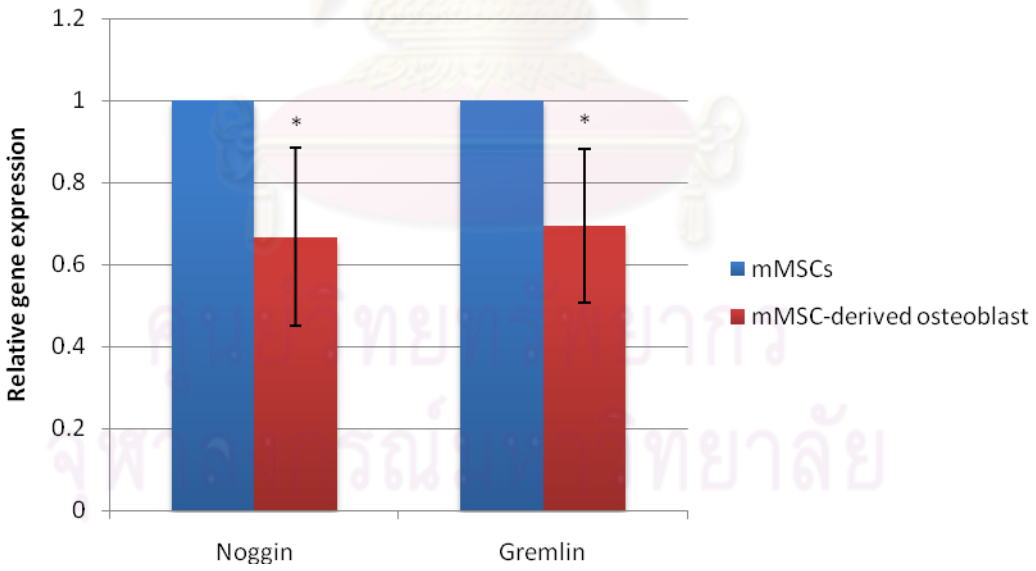

Figure 12 Quantitative RT-PCR analysis of Noggin and Gremlin expression in MOBs and mMSCs. The data are presented as average (± SD), (*$p < 0.005$, $n = 3$).

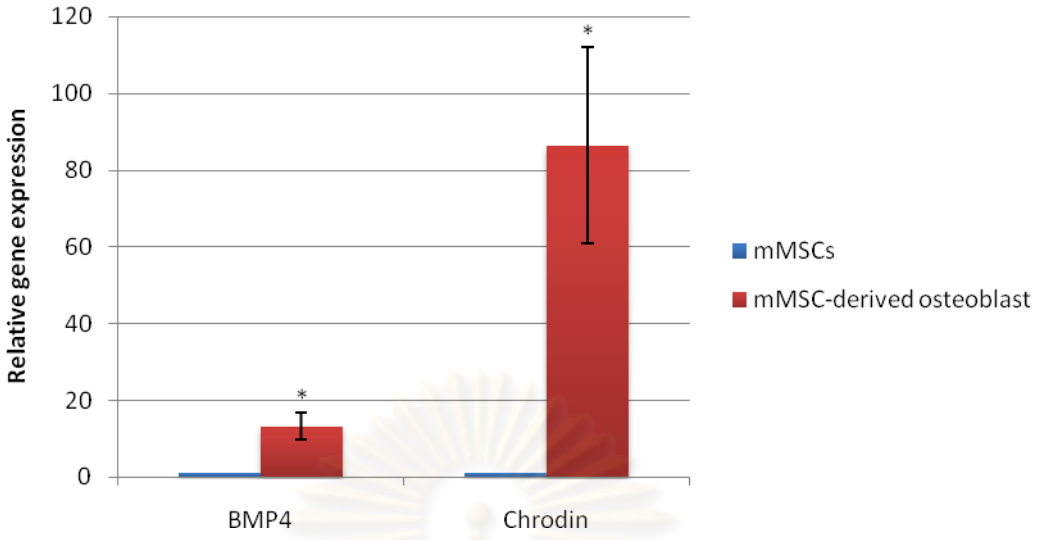

Figure 13 Quantitative RT-PCR analysis of BMP4 and Chordin expression in MOBs and mMSCs. The data are presented as average (± SD), (*$p < 0.001$, $n = 3$).

To expand our knowledge of the role of signaling crosstalk in maintaining HSC in osteoblastic niche, we study the expression of BMP and its antagonists in MOBs and mMSCs. We found that BMP antagonists Noggin and gremlin are mainly expressed in mMSCs (Figure 12) whereas BMP4 and one of its antagonists, Chordin, are highly expressed in MOBs (Figure 13). It has been proposed that BMP signaling may play a role in HSC maintenance and lower concentration of BMP promote HSCs proliferatiojn and differentiation. We next tested the effect of BMP on cell cycle regulators expressed in HSCs. LSK cells were treated with BMP and noggin for 24 hour. Then the expression of p21, p27 and p57 in HSCs were analyzed by quantitative RT-PCR. We found that BMP treatment increased the expression level of p27 and p57 whereas noggin treatment showed the down regulation of p27 (Figure 14). These data showed the role of BMP signaling in HSCs maintenance.

Table1 Expression profile of mMSC and MOBs

Signaling Pathway	Marrow stromal cells (MSCs)	MSC bone induction
Wnt pathway		
Wnt molecules	+	++
Wnt1	+	++
Wnt3a		
Wnt antagonist	+	++
Dkk1		
TGF pathway		
BMP molecules		
BMP2	+	++
BMP4	+	++++
BMP7	-	-
TGF molecules		
TGF1	++	+
TGF3	+	+++
BMP antagonists		
Noggin	++	+
Chrodin	+	+++++
Gremlin	++	+
Notch pathway		
Jagged1	+	+++++
Tei-Ang pathway		
Angiopoietin1	+	+++++
CXCR4-CXCL12 pathway		
CXCL12	+	++

+ = baseline level of gene expression ++ = 1.01-5 folds

+++ = 5.01-10 folds ++++ = 10.01-50 folds

+++++ = > 50.01 folds

To evaluate the effect of noggin on HSC maintenance, we generate cell line that overexpressing noggin, BMP antagonist, from 3T3 cell line, named as 3T3 noggin. These cells showed higher level of noggin protein expression than 3T3 cell line (Figure 15). We hypothesized that noggin may influence ability of osteoblasts which expressed BMP4 in HSCs maintenance. Therefore, we first tested whether we can combine 3T3 noggin cells with mMSC to generate *ex vivo* culture system for HSCs. We found that mixing mMSCs:3T3 noggin ratio 5:1 can be reproducibly induced to become to osteoblasts with area of stroma cells in between (Figure 16). The feeder system is stable for long-term period. So we used M3B feeder ratio 5:1 for further experiments. Then we compared ability of M3B with MOBs in maintain HSC by coculturing with LSK cells. We observed both differentiated cells and cobblestone area of LSK cells on this feeder (Figure 17). Whereas MOBs shown limited ability to support cell proliferation, M3B have higher ability to support hematopoiesis. This system can produced more than 20 folds of $CD45^{+}$ cell than MOBs after 2 week of culture (Figure 18). Interestingly, when we assessed M3B ability to maintain long term HSCs by LTC-IC assay, we found that the LTC-IC number of cells derived from cell cultured on M3B feeder was comparable to mMSCs-derived osteoblast. Moreover type of CFC of LTC-IC was changed from CFU-M (colony forming unit macrophage) to CFU-GEMM (colony forming unit granulocyte, erythrocyte, macrophage and megakaryocyte) when cultured LSK cells on M3B feeder (compared with mMSCs-derived osteoblast) (Figure 19) and (Figure 20). CFU-GEMM is the most immature CFC whereas CFU-M has been thought to produce by granulocyte-macrophage progenitor (GMP), more mature cell. This result suggested that M3B feeder could maintain HSCs in more immature state than mMSCs-derived osteoblast. Together, these results demonstrated that combination of mMSC-derived osteblasts with 3T3 noggin could promote cells proliferation as well as HSCs maintenance.

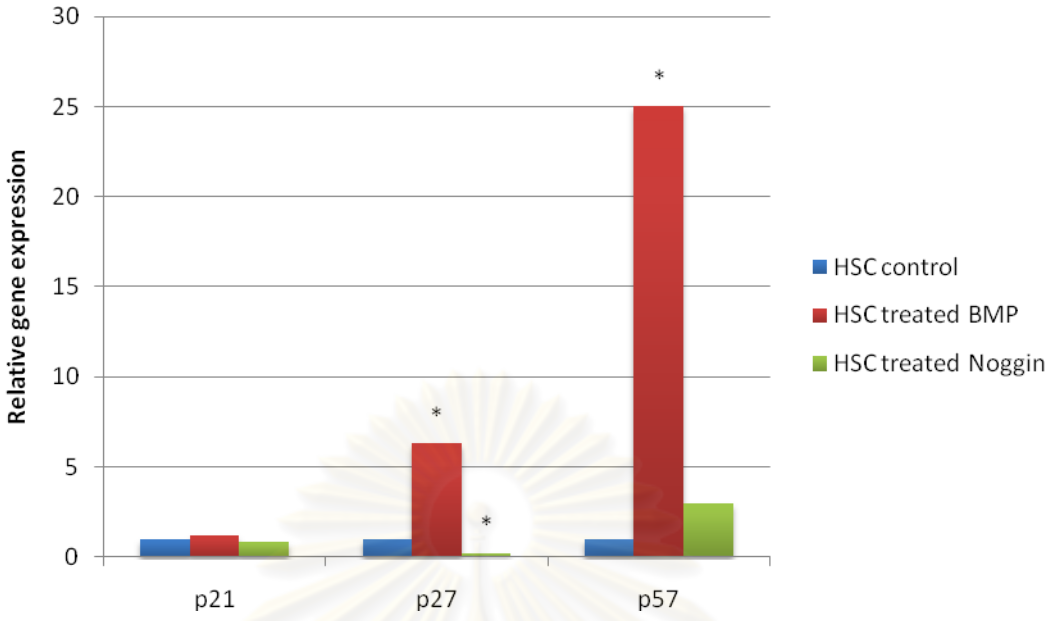

Figure 14 Effect of BMP2 and noggin treatment in HSCs. Cyclin-dependtdent kinase inhibitor expressed in HSCs measured by change in p21, p27 and p57 expression. The data are presented as average, (*$p < 0.05$, $n = 3$).

Figure 15 Western blot analysis of noggin protein in 3T3 noggin cells. ß-actin was used as a control for loading.

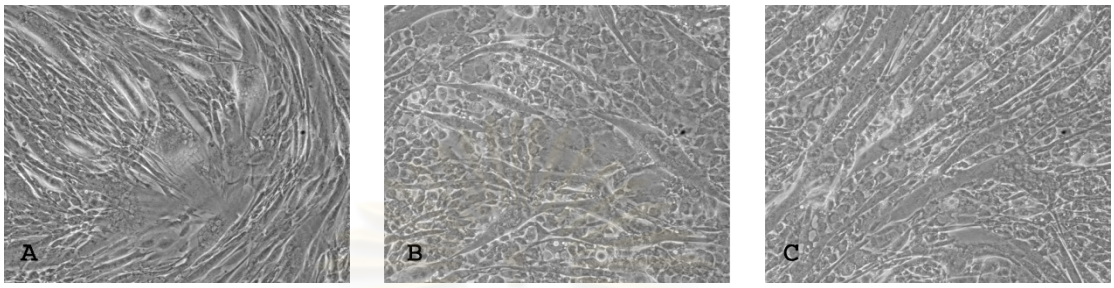

Figure 16 Morphology of M3B feeder. MOBs (A) and M3B system which combine of two cell types, mMSC and 3T3 noggin cells in ratio 1:1 (B) and 5:1 (C) after inducion with osteogenic media for 7 day

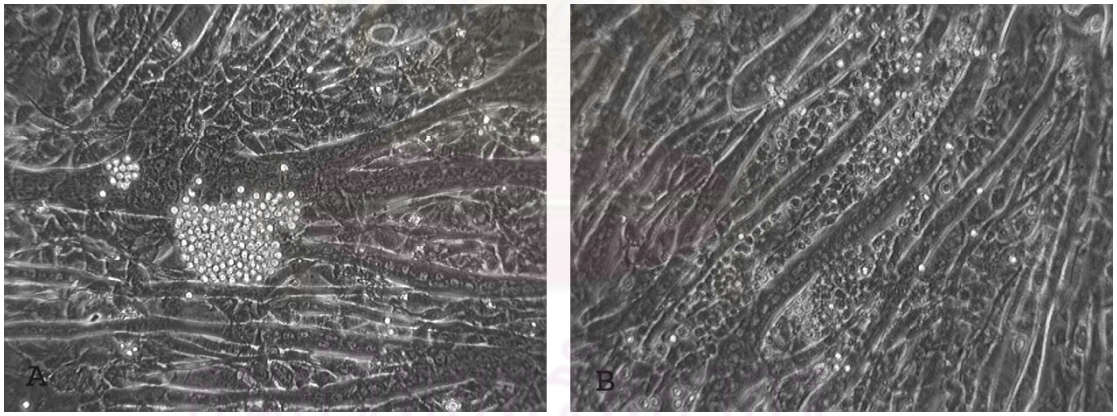

Figure 17 The effect of M3B feeder on LSK growth phenotype. Daughter cells were produced on M3B feeder (A) output colonies (B) Cobblestone-like colonies.

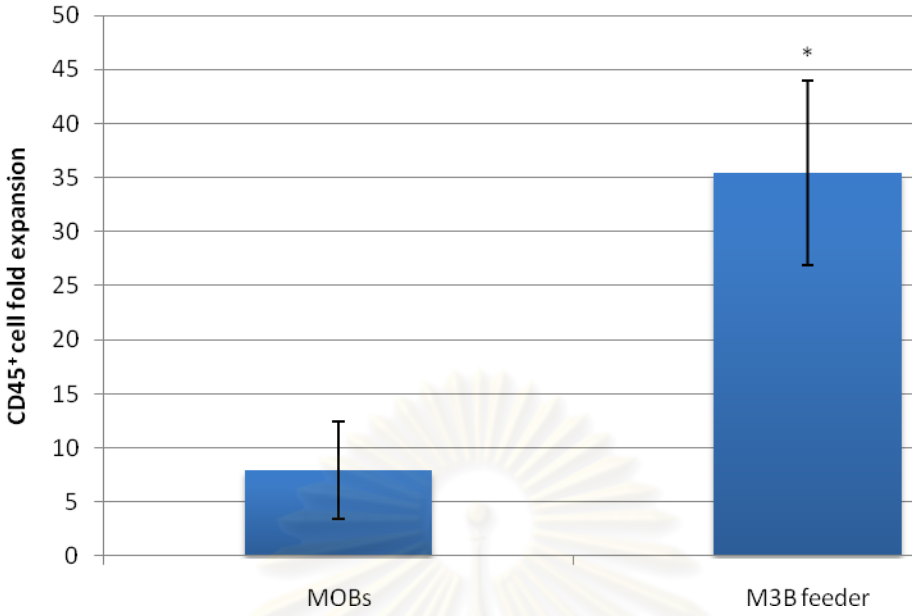

Figure 18 Effect of M3B feeder on the expansion of $CD45^{+}$ cell. Data shown are the mean number (± SD) of CFC derived from 1,000 input LSK cells (*$p < 0.05$, n=3).

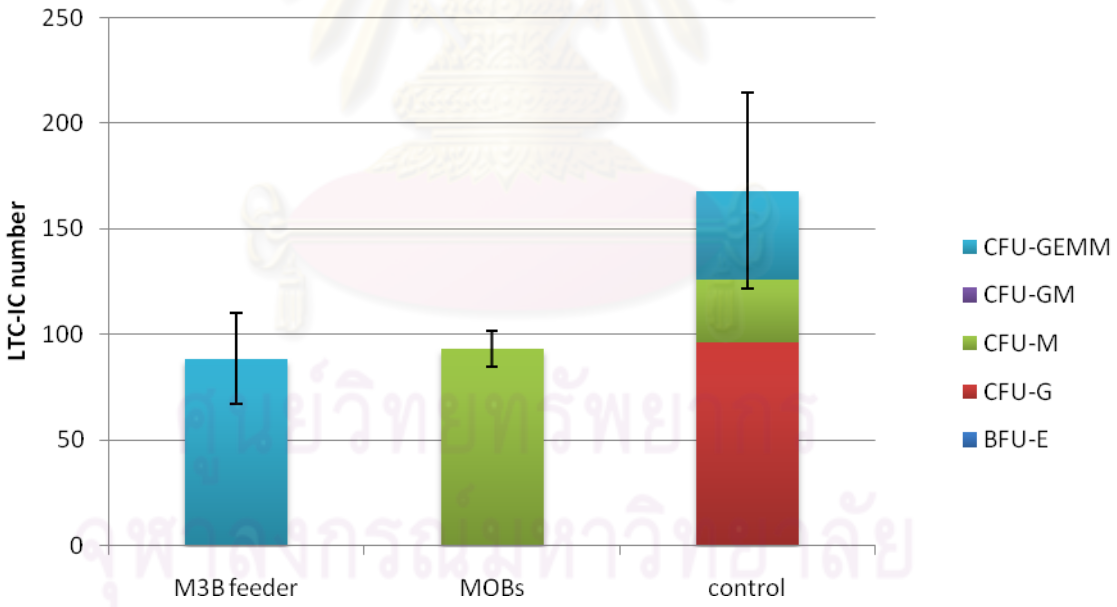

Figure 19 Effect of M3B feeder on the output of long-term culture initiating cell (LTC-IC). Control is the number of CFC derived from 1,000 LSK cells at day 0. Data shown are the mean number (± SD) of LTC-IC derived from 1,000 input LSK cells (n=4).

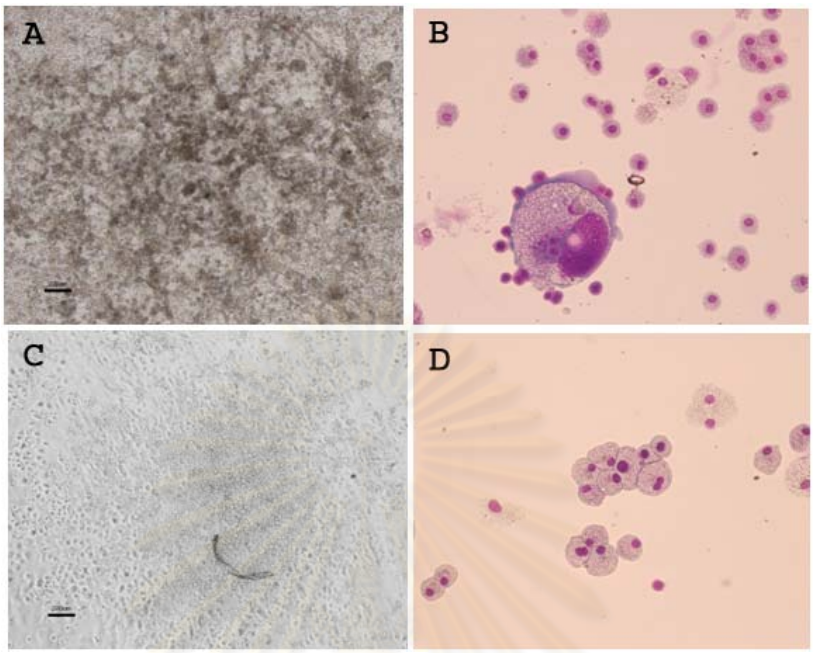

Figure 20 Wright-Giemsa stains of cytospin preparations of LTC-IC derived colonies. (A) CFU-GEMM consists of erythrocyte, macrophage, granulocyte and megakaryocyte (B). (C) CFU-M consist only macrophage (D)

To assess whether over-expression of N-cadherin in mMSCs-derived osteoblasts could have the effect on their HSCs supporting activity. mMSCs were transiently transfection with pCDH2 before osteogenic induction. LSK cells cultured on N-cadherin over-expressed feeder compared with normal feeder. Growth phenotype of LSK cell on both conditions of mMSCs and MOBs were not different (Figure 21) In contrast, in M3B feeder, we did not found proliferated cells on N-cadherin overexpressed M3B feeder compared to normal condition (Figure 21). These finding correlated with the reduction of $CD45^{+}$ cells in N-cadherin overexpressed condition (Figure 22).

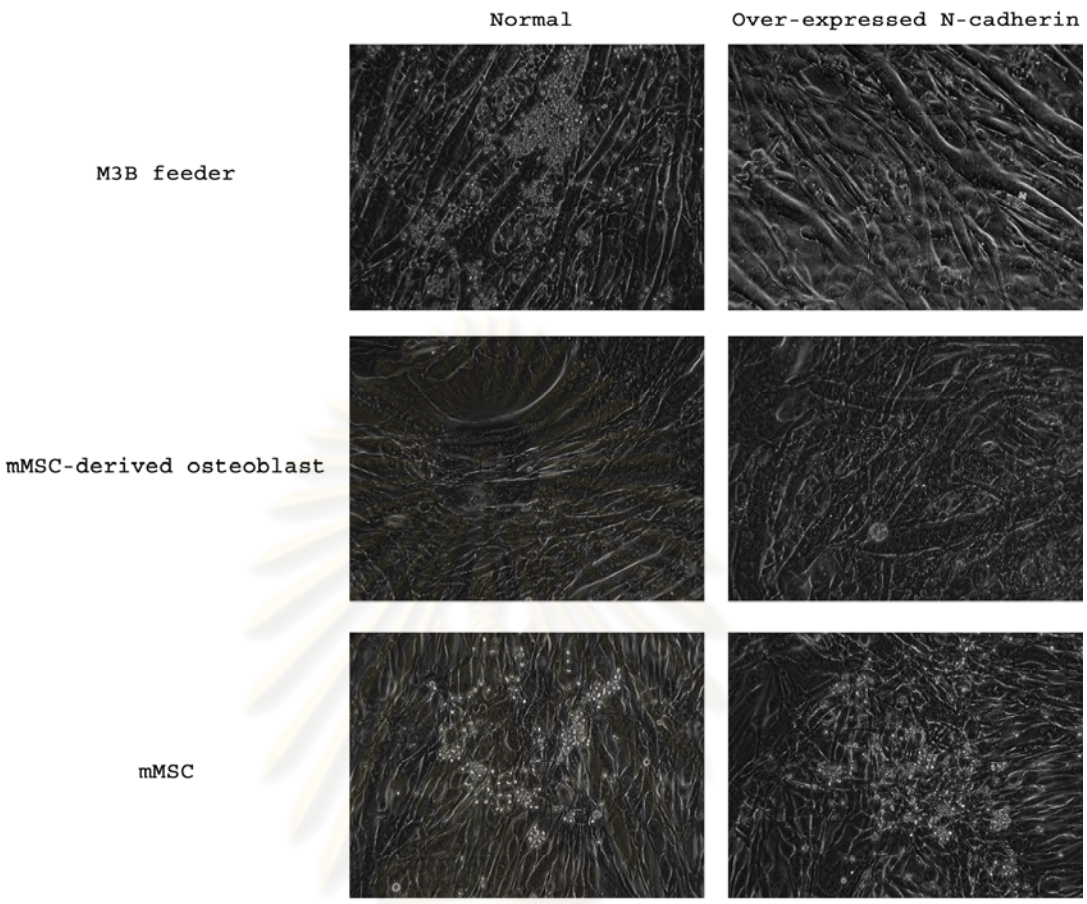

Figure 21 The effect of N-cadherin on the growth phenotype of LSK cells. LSK cells were cultured on normal mMSCs, MOBs and M3B feeder and N-cadherin overexpressed conditions in long-term culture media.

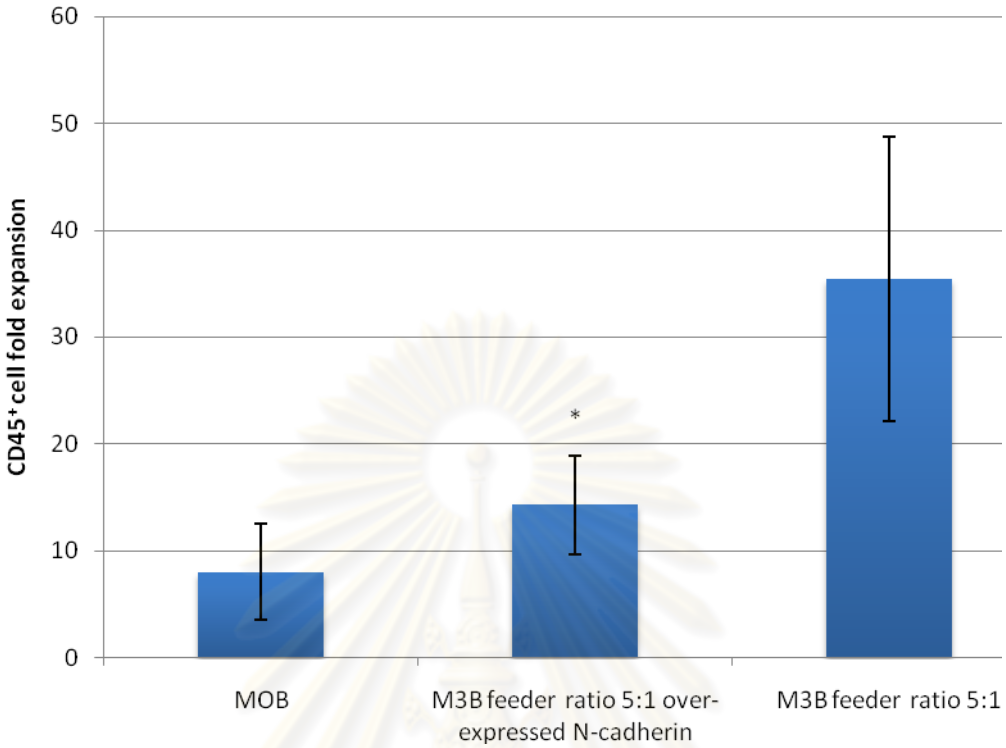

Figure 22 Effect of N-cadherin overexpressed M3B feeder on the expansion of $CD45^+$ cell. Data shown are the mean number (± SD) of $CD45^+$ fold expansion derived from 1,000 input LSK cells (*$p < 0.05$, n=3).

Next we examined the effect of N-cadherin on maintenance of colony formation ability. In mMSCs, overexpressing N-cadherin significantly increased the CFC number of culture at week 1 and week 2 and slightly increased at week 4 (Figure 23). In contrast, we did not detected the different of CFC number derived from LSK cells cultured on N-cadherin overexpressed MOBs compared with mMSCs-derived osteoblast control (Figure 24). However, N-cadherin overexpressed MOBs in M3B feeder showed an increase in number of CFC derived from LSK cells compared with M3B feeder only in week 2 (Figure 25). But the number of LTC-IC was not different in both conditions. This might be caused by transiently transfection of N-cadherin. Taken together, these data showed the role of N-cadherin in HSCs proliferation and differentiation.

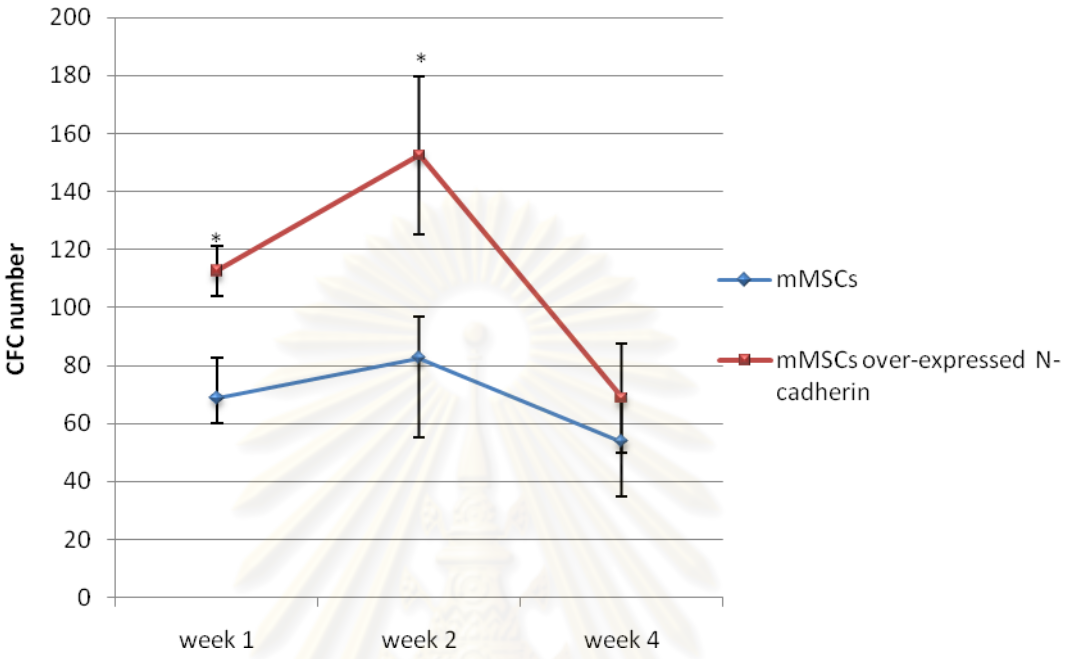

Figure 23 Effect of N-cadherin overexpressed mMSCs on the output of colony forming cell (CFC). Data shown are the mean number (± SD) of CFC derived from 1,000 input LSK cells (*$p < 0.05$, n=4).

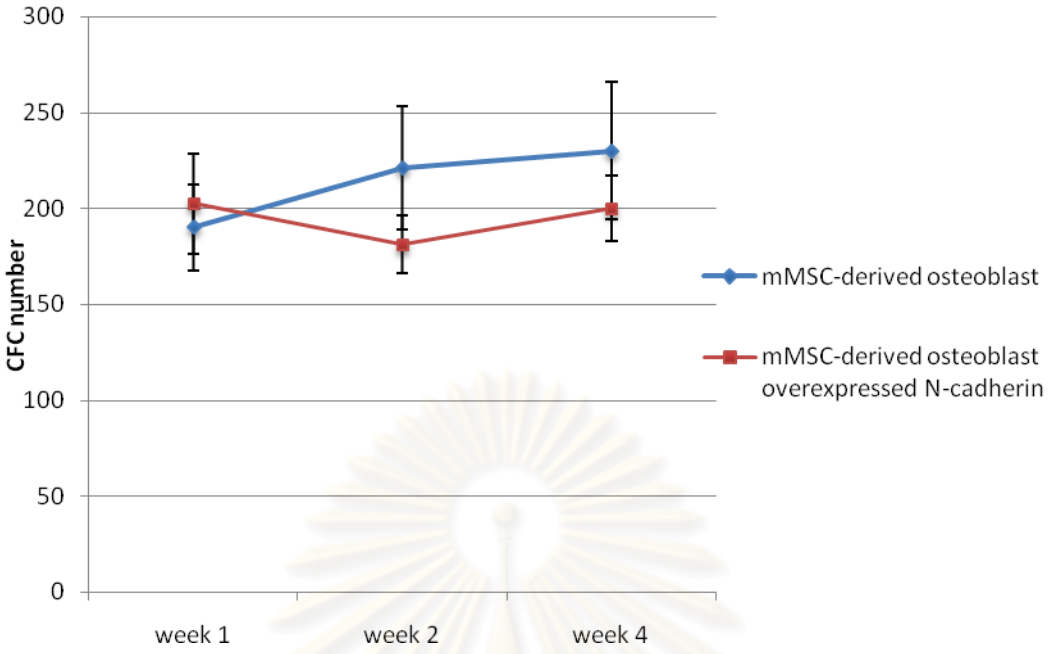

Figure 24 Effect of N-cadherin overexpressed MOBs on the output of colony forming cell (CFC). Data shown are the mean number (± SD) of CFC derived from 1,000 input LSK cells (n=4).

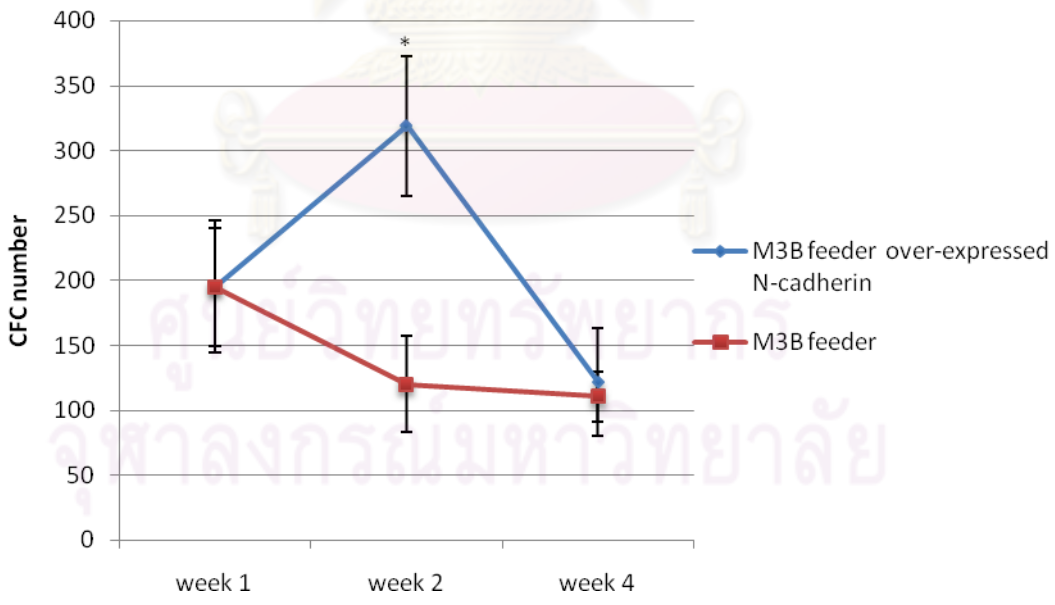

Figure 25 Effect of N-cadherin overexpressed M3B feeder on the output of colony forming cell (CFC). Data shown are the mean number (± SD) of CFC derived from 1,000 input LSK cells (*$p < 0.05$, n=4).

Recently, it was reported that Interferon alpha (IFNα) has ability to promote HSCs proliferation *in vivo* by activating STAT and Wnt pathway in HSCs. But the effect of IFNα on niche cells has not been studied. We next studied the effect of IFN alpha on Wnt and Wnt antagonists expressed on niche cells. It has been shown that Wnt signaling components, Wnt1, Wnt3a and its inhibitor, Dkk1, are normally expressed by osteoblast and MSCs. To assess the effect of IFNα, mMSCs and mMSCs-derive osteoblast were treated with IFN alpha 4. After 8 hours, total RNA were extracted and RT-PCR were preformed. We observed that the level of *Wnt1* amd *Wnt3a* mRNA expression was not significantly different in both mMSCs and osteoblasts. Interestingly, we found selective down regulation of Wnt antagonist, *Dkk1* in mMSCs (Figure 26) but not in osteoblasts (Figure 27). It has been studied that *Dkk1* expressed in niche cells regulates Wnt signaling in HSCs and fine-tuned modulation of Wnt/ß-catenin signaling intensity is required for the proper control of HSC quiescence. Our data suggests that the effect of IFNα on HSCs activation may be also mediated indirectly through down regulation of Wnt antagonist secreted from niche cells.

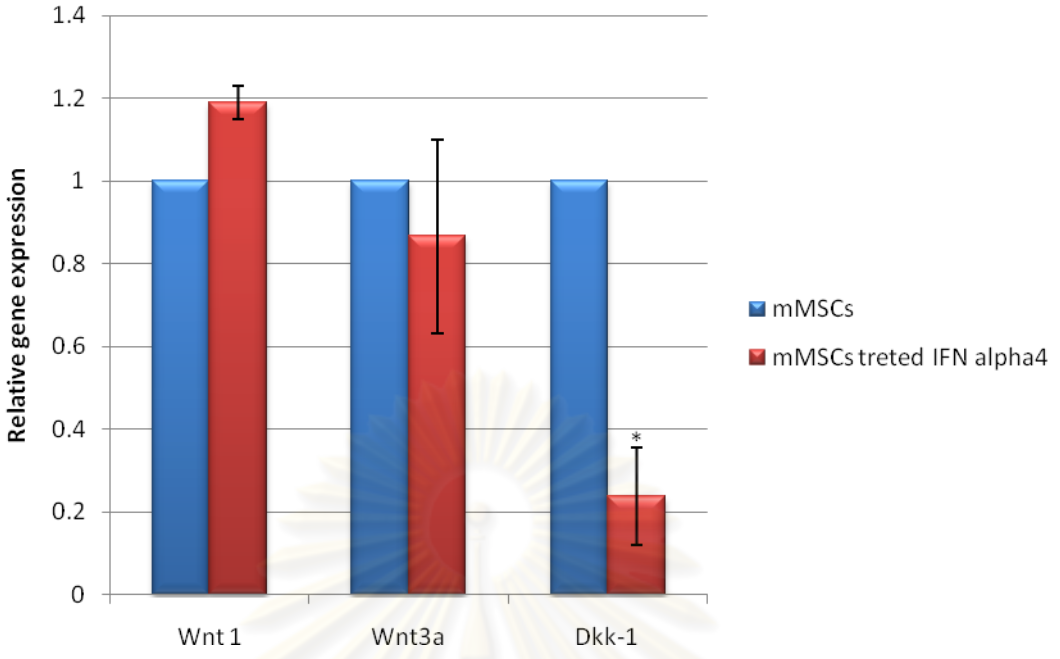

Figure 26 The effect of IFN-alpha 4 treatment on Wnt1, Wnt3a and Dkk1 expression in mMSCs. The data are presented as average (± SD), (*$p < 0.05$, $n = 3$).

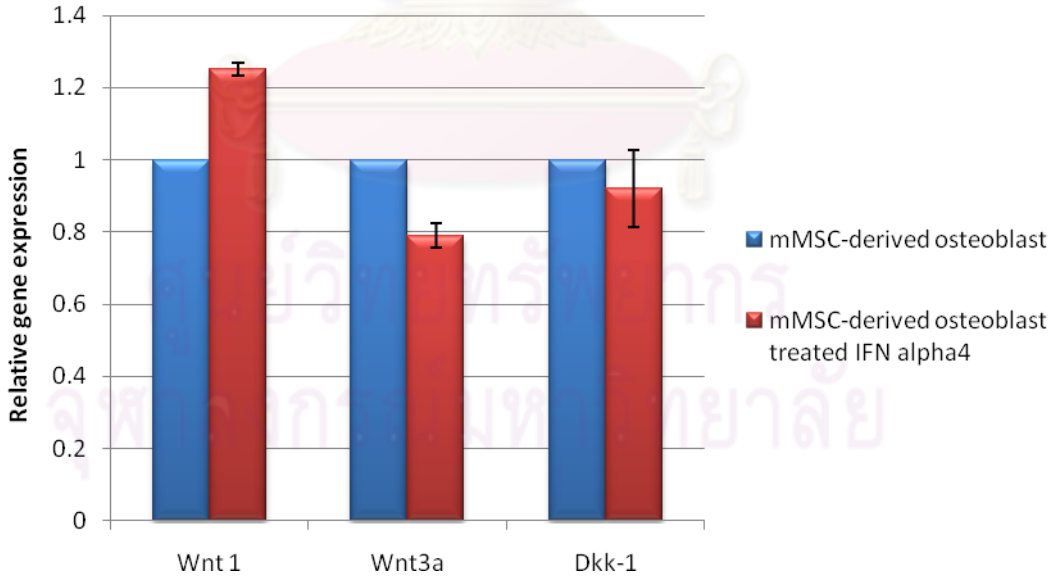

Figure 27 The effect of IFN-alpha 4 treatment on Wnt1, Wnt3a and Dkk1 expression in MOBs. The data are presented as average (± SD), $n = 3$.

CHAPTER V

DISCUSSION AND CONCLUSION

It has been proposed that HSCs are directly contacted with osteoblasts, which secrete factors that regulate HSCs properties. In this study, we used mMSC-derived osteoblasts as same as their nature, to test their ability to support HSCs in vitro. mMSC-derived osteoblasts (MOBs) were test for their osteoblast specified phenotypes which confirm their fully differentiation into osteoblasts (Figure 4, Figure 5 and Figure6). We assessed the ability of mMSC-derived osteoblasts in supporting HSCs compared with mMSC. Growth phenotype of LSK cells cultures on mMSCs and mMSC-derived osteoblasts were different. Differentiated cells were found on mMSCs whereas cobblestone area forming cells were formed colonies underneath mMSCs-derived osteoblasts (Figure 7). mMSCs-derived osteoblasts showed greater capability to maintain colony formation after long-term culture using long-term cultured initiating cell (LTC-IC) assay (Figure 9). Osteoblasts have been suggested to express factors that regulating HSCs maintenance in the bone marrow, including angiopoietin (Arai et al., 2004), CXC-chemokine ligand 12 (CXCL12) (Petit et al., 2002) and Jagged1 (Calvi et al., 2003). Functional studies showed that angiopoietin control HSCs quiescence (Arai et al., 2004), whereas CXCL12 regulates HSCs migration and localization (Peled et al., 1999; Petit et al., 2002). Correlated with these data, we detected the upregulation of angiopoietin, CXC-chemokine ligand 12 (CXCL12) and Jagged1 in mMSCs-derived osteoblasts (Figure 10 and Figure 11). Moreover, we also considered in Wnt and BMP signaling between osteoblasts and HSCs. Wnt signaling in HSC niche has been broadly studied. Activation of Wnt signaling by overexpressing ß-catenin increases HSCs self-renewal (Reya et al. 2003). However, constitutive activation of canonical Wnt signaling using stable form of ß-catenin causes multilineage differentiation block and compromised hematopoietic stem cell maintenance (Kirstetter et al., 2006). These findings suggested that fine-tuned modulation of Wnt/ß-catenin signaling intensity is required for the proper control of HSC quiescence. The control of Wnt signaling is driven

by intrinsic factors within HSCs itself and extrinsic factors produced by niche cells. For example, the inhibition of Wnt signaling by *Dickkopf-1 (Dkk1)*, Wnt inhibitor that normally expresses in niche cells, impairs HSCs long-term reconstitution activity (Fleming et al. 2008). Likewise, we also detected the upregulation of *Wnt1*, *Wnt3a* and *Dkk1* in MOBs (Figure10 and Figure11). Conversely from Wnt signaling, the role of BMP signaling, especially in its antagonists, in HSC niche is unclear. There are two evidences from in vitro study. BMP4 treatment 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). These data showed the important of BMP signaling intensity in controlling HSCs properties. As in embryonic development, gradient of BMP signaling activity is crucial for cell fate determination. This gradient is controlled by its antagonists, such as noggin, gremlin and chordin that secreted by surrounding cells. As a result, BMP4 is expressed by MOBs where as its antagonists, noggin and gremlin, except chordin, are mainly expressed in mMSCs (Figure 12 and Figure 13). This finding provides the possibility of marrow stromal cells in regulating BMP signaling activity in HSC niche to promote HSC proliferation and differentiation. Coexpression of BMP4 and chordin in MOBs also demonstrated the tightly regulation of BMP signaling activity by these cells. But chordin is further regulated by Tolloid family proteases (Little and Mullins, 2006). So, the effect of chrodin expressed by MOBs on BMP signaling will be studied further.

However, it has been long known that bone marrow is composed of various cells types which contribute to generate HSCs niche. Using single cell type such as osteoblast or stromal cell for ex vivo culture could not completely clarify the mechanism that control HSCs in vivo. Bone marrow provides complicated microenvironment that promotes both HSC quiescence and differentiation. As we mention above, mMSCs showed to expressed BMP antagonists, noggin and gremlin whereas BMP4 are mostly expressed from MOBs. As from previous study of BMP4 treatment *in vitro* demonstrated that gradient of BMP signaling activity is important for HSCs fate determination (Bhatia et al., 1999). Consequently, our data showed that BMP treatment have the effect on HSCs maintenance (Figure 14). In this study we have established a system that could generate gradient of BMP signaling activity which might be permit HSC maintenance

and proliferation/differentiation. By using 3T3 noggin, 3T3 cells that stably expressed noggin (Figure 15), combined with MOBs so we called this feeder cells as M3B feeder. Noggin secreted from 3T3 noggin could bind to BMP4 that produced from MOBs result in generating BMP gradient between these cells. We used ratio 5:1 (MOBs:3T3 noggin) that generate more well-defined structure (Figure 16). When cultured LSK cells on M3B feeder, differentiated colonies as well as cobblestone area forming cells (CAFC) were found on this feeder (Figure 17). Furthermore M3B feeder was shown to maintain LTC-IC number of cultured LSK cells comparable to MOBs. As we proposed, this feeder cell promotes HSCs proliferation/differentiation (Figure 18) as well as HSCs maintenance (Figure 19). Interestingly, type of colony from LTC-IC assay was changed from CFU-M in MOBs culture to CFU-GEMM in M3B culture (Figure 19 and Figure 20). This might be influenced of signaling provided by different feeder cells.

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). Activation of Tie2-Ang1 signaling promotes tight adhesion of HSCs by increasing N-cadherin expression level, resulting in HSCs quiescence (Arai et al., 2004). Loss of self-renewal activity in HSCs correlated with N-cadherin repression (Wilson et al., 2004). These findings suggested that N-cadherin is required to maintain HSCs in quiescence state. In contrast, Kiel et al. demonstrated that 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). Moreover, using SLAM family marker, most of HSCs are present around sinusoids, some of which are close proximity to endosteum (Kiel et al., 2005, 2007) These data are inconsistent with the former idea. These raise the question that whether N-cadherin expressed by osteoblasts is crucial for maintaining HSCs properties. So, we assessed the effect of N-cadherin expressed in niche cells on HSCs properties. Overexpression of N-cadherin in mMSCs increased CFC and LTC-IC number derived from cultured LSK cells (Figure 22). Slightly increasing of LTC-IC number in N-cadherin overexpressed MOBs might be caused by transiently transfection that results in loss of N-cadherin expression after long-term culture. On the other hand,

overexpression of N-cadherin in MOBs, we did not observe any different in both CFC and LTC-IC number compared with normal condition (Figure 23).

When we used N-cadherin overexpressed MOBs in M3B feeder, differentiated colonies derived from LSK cells were not observed (Figure 21) consistent with the reduction of $CD45^+$ cell on this feeder compared with M3B normal condition (Figure 22). The CFC number, at week 2 of culture, on N-cadherin overexpressed M3B was higher than normal M3B feeder (Figure 25). However there is not significantly different in LTC-IC number in both conditions. These might be the effect of transiently transfection of N-cadherin that could not maintain the over-expression level throughout 4 weeks of LTC-IC assay as we previously proposed. Our results indicated that N-cadherin is involved in HSCs maintenance by it can increased CFC number and reduced cell proliferation/differentiation. There are three possibility of N-cadherin in controlling HSCs. First of all, upregulation of N-cadherin expression promote tightly adhesion of HSCs with niche cells, resulting in protection of HSCs from various stresses. Second of all, upregulation of N-cadherin regulates cytoplasmic level of ß-catenin, resulting in control canonical Wnt signaling preventing cells to entering cell cycle. Lastly, N-cadherin might be signals by its cytoplasmic domain in HSCs. Given that overexpression of N-cadherin in MOBs also results in bone formation that could be cause an indirect effect on HSCs regulation. These results provided an opposite role of N-cadherin in vivo study. Because of the redundancy of signaling that could further regulate HSCs properties to sustain hematopoiesis over times, so deletion of N-cadherin in vivo did not effect to HSC properties. As same as overexpression of N-cadherin in MOBs, we did not observe any different in HSCs properties. To further assess the effect of N-cadherin in LTC-IC, over-expressed N-cadherin or inducible N-cadherin stable cell lines are required as same as cell cycle analysis of $CD45^+$ on each feeder to confirm their maintenance of HSC quiescence.

Lastly, we studied the effect of Interferon alpha (IFNα) on niche cells. IFNα, a cytokine with antiviral activity, had an effect on HSCs proliferation (Sato, T. et al. 2009 and Essers, M.A. et al. 2009). *In vivo* study showed that treatment of IFNα induced quiescent HSCs to undergo proliferation (Essers, M.A. et al. 2009). We found that IFNα

treatement reduced *Dickkopf-1 (Dkk1)* (Figure 26), a soluble inhibitor of Wnt/beta-catenin signaling, expression in MSCs but not in osteoblast (Figure 27). Dkk1 has been shown to play an important role in HSCs properties by controlling level of Wnt signaling in bone marrow. IFNα directly activates STAT pathway and also down regulates Wnt inhibitor in niche cells, primarily in MSCs, encouraging HSCs to enter cell cycle.

Conclusion

Our results support the role of osteoblasts in HSCs maintenance and this mechanism require more than one cell type to modulate the proper signaling intensity. M3B feeder also showed the role of BMP signaling in regulating HSCs within their niche. Moreover, M3B feeder is provided as a tool for molecular studies in HSCs niche. By this feeder can be genetically modified in order to study the effect of molecule that expressed in osteoblasts or stromal cells. Furthermore, overexpression of N-cadherin improved HSCs supportive activity of mMSCs and suppressed proliferation and/or differentiation of $CD45^+$ cells on M3B feeder. For further study, generating N-cadherin over-expressed stable cells is the better way to assess its long-term effect on LTC-IC number. We also found an indirect effect of IFNα on Dkk1 expression level in niche cells, especially in MSCs. Ultimately, the understanding of signaling that controls HSCs properties will lead to HSCs maintenance and expansion *ex vivo*, which benefit for further clinical application.

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APPENDICES

APPENDIX A

Table 2 Primer sequences

Name	sequence
mouse TGF1 F	5'-CTC CCG TGG CTT CTA GTG C-3'
mouse TGF1 R	5'-GCC TTA GTT TGG ACA GGA TCT G-3'
mouse TGF3 F	5'-CCT GGC CCT GCT GAA CTT G-3'
mouse TGF3 R	5'-TTG ATG TGG CCG AAG TCC AAC-3'
mouse Wnt3a F	5'-CCC AAC TTC TGC GAA CCT AA-3'
mouse Wnt3a R	5'-TCT CCG CCC TCA AGT AAG AA-3'
mouseWnt1F	5'-CCG AGA AAC AGC GTT CAT CT-3'
mouse Wnt1R	5'-GCC TCG TTG TTG TGA AGG T-3'
mouse Angpt-1F	5'-CAG TGG CTG CAA AAA CTT GA-3'
mouse Angpt-1R	5'-TCT GCA CAG TCT CGA AAT GG-3'
mouse BMP2 F	5'-ACA CAG GGA CAC ACC AAC CAT-3'
mouse BMP2 R	5'-TGT GAC CAG CTG TGT TCA TCT TG-3'
mouse BMP4 F	5'-CTC CCA AGA ATC ATG GAC TG-3'
mouse BMP4 R	5'-AAA GCA GAG CTC TCA CTG GT-3'
mouse BMP7 F	5'-GAC GCC AAA GAA CCA AGA GG-3'
mouse BMP7 R	5'-GGC GTT CAT GTA GGA GTT CA-3'
mouse Noggin F	5'-CCT CTA CGC CCT GGT GGT GGT C-3'
mouse Noggin R	5'-GCG GCT GCC TAG GTC ATT CCA C-3'
mouse Gremlin1 F	5'-GCA ACA GCC GCA CTA TCA-3'
mouse Gremlin1 R	5'-CCA AGT CGA TGG ATA TGC-3'
mouse Chrodin F	5'-TAC AGT GAT CGA GGG GAA CC-3'
mouse Chrodin R	5'-GGT AGG AGA CAG AGA AGC GTA AAC T-3'
mouse CXCL12 F	5'-GAG CCA ACG TCA AGC ATC TG-3'
mouse CXCL12 R	5'-CGG GTC AAT GCA CAC TTG TC-3'

APPENDIX B

REAGENTS

1. **10X ammonium chrolide lysis solution**

NH4Cl (1.5 M)	80.2 g
NaHCO3 (100 mM)	8.4 g
disodium EDTA (10 mM)	3.7 g
ddH_2O to	900 ml

Adjust pH to 7.4 with 1 N HCl or 1 N NaOH

Add ddH_2O to 1 liter

2. **1X ammonium chrolide lysis solution**

10X ammonium chrolide lysis solution	100 ml
ddH_2O	900 ml

3. **Phosphate Buffered Saline (PBS), 30x conc.**

NaCl	526	g
$NaH_2PO_4H_2O$	82.8	g
5N NaOH	120	ml
ddH_2O maker up to	2000	ml

4. **Working PBS pH 7.4**

30X PBS	70 ml
ddH_2O	2030 ml

5. **10X Tris buffered saline (TBS)**

NaCl	80 g
KCl	2 g
Tris base	30 g
ddH_2O	800 ml

Adjust pH to 7.4 with 1 N HCl or 1 N NaOH

Add ddH_2O to 1 liter

6. **1X Tris buffered saline**

10X TBS	100 ml
ddH_2O	900 ml

7. **Wright-Giemsa stain**

0.3% Wright stain

0.1% Giemsa stain

3% glycerine / 97% Methanol

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