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.

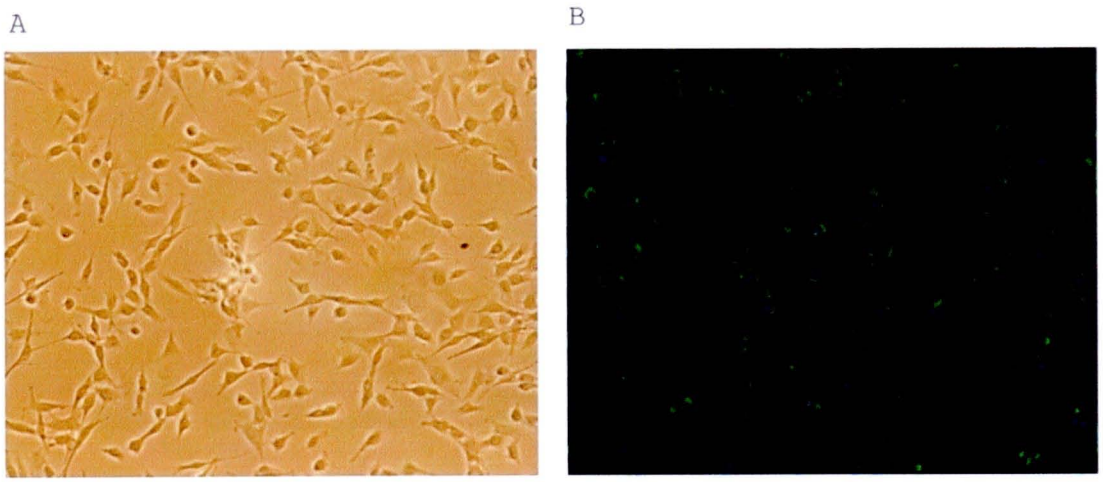

Figure 4 Morphology of isolated mouse marrow stromal cells (mMSCs). (A). Morphology of isolated mMSCs using phase contrast microscope. (B). mMSCs were stained with a mouse monoclonal antibody against STRO1 (Chemicon, Millipore). Magnification :x10.

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.

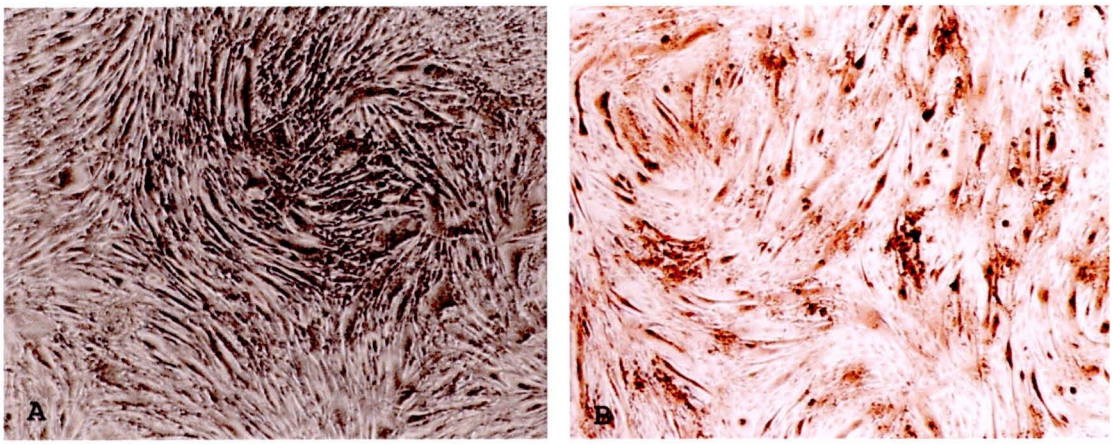

Figure 5 Morphology of mMSC-derived osteoblasts (MOBs). (A) mMSCs were induced with osteogenic media for 7 days (B) After osteob;astic differentiation, cells were stained with Alizarin Red. Magnification: x4

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.

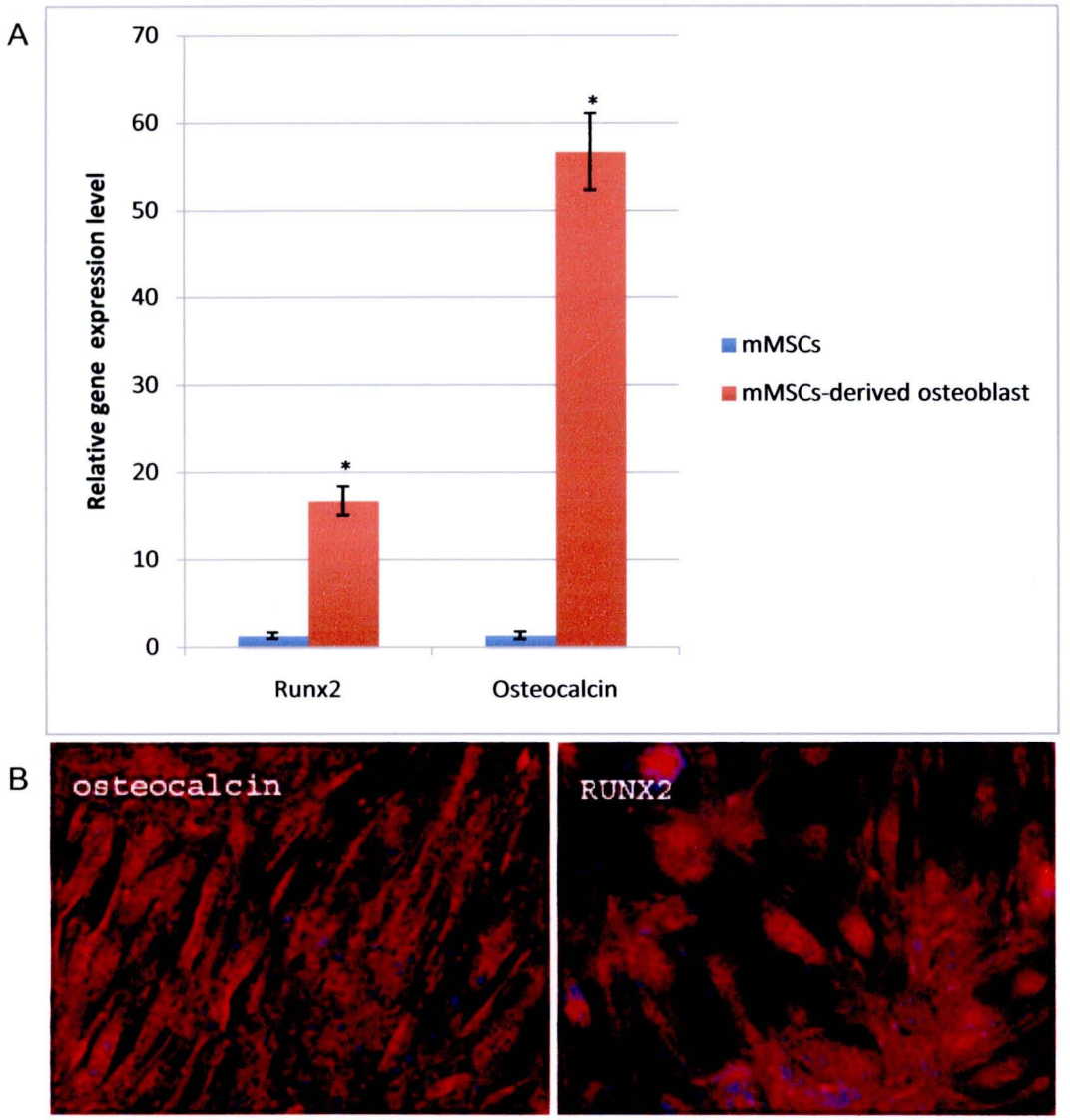

Figure 6 Osteoblastic gene expression of MOBs. (A) Quantitative RT-PCR analysis for the expression of osteoblast specific markers. The data are presented as average ± SD, (*$p < 0.005$, $n = 3$). (B) Immunostaining confirming in vitro differentiation of mMSCs into osteoblast. Secondary antibodies were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

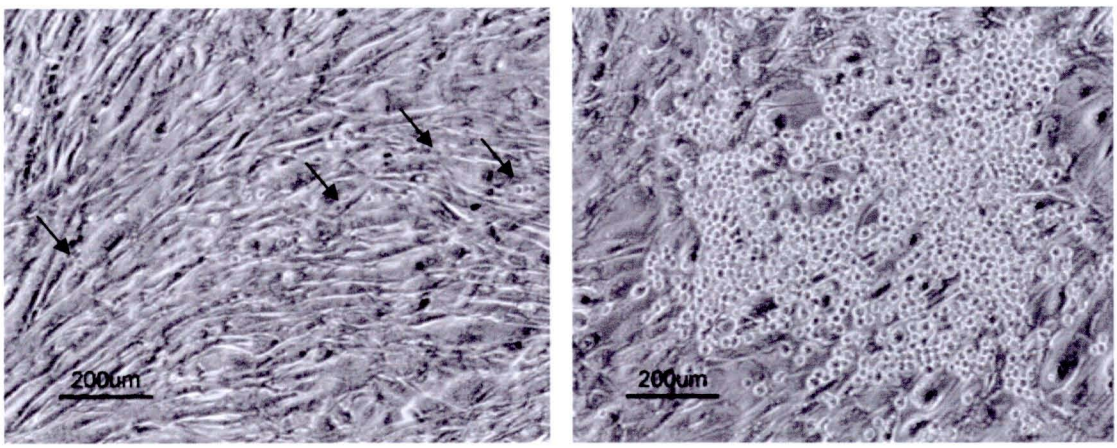

Figure 7 Growth phenotype of LSK cells cultured on MOBs and mMSCs. (A) Cobblestone area (arrow) of LSK cells underneath MOBs without any output cells. (B) Differentiated colony of LSK cells on mMSC. Magnification: x10.

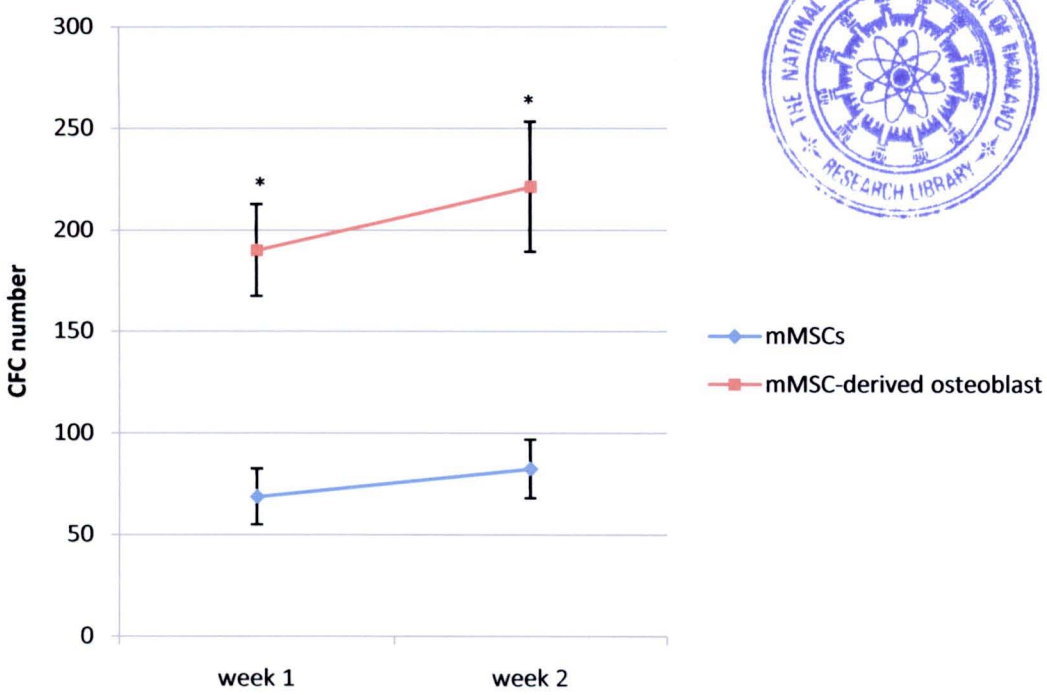

Figure 8 Effect of MOBs on the output of colony forming cell (CFC). Data shown are the mean number (± SD) of CFC derived from 1,000 input LSK cells (*$p < 0.01$, n=4).

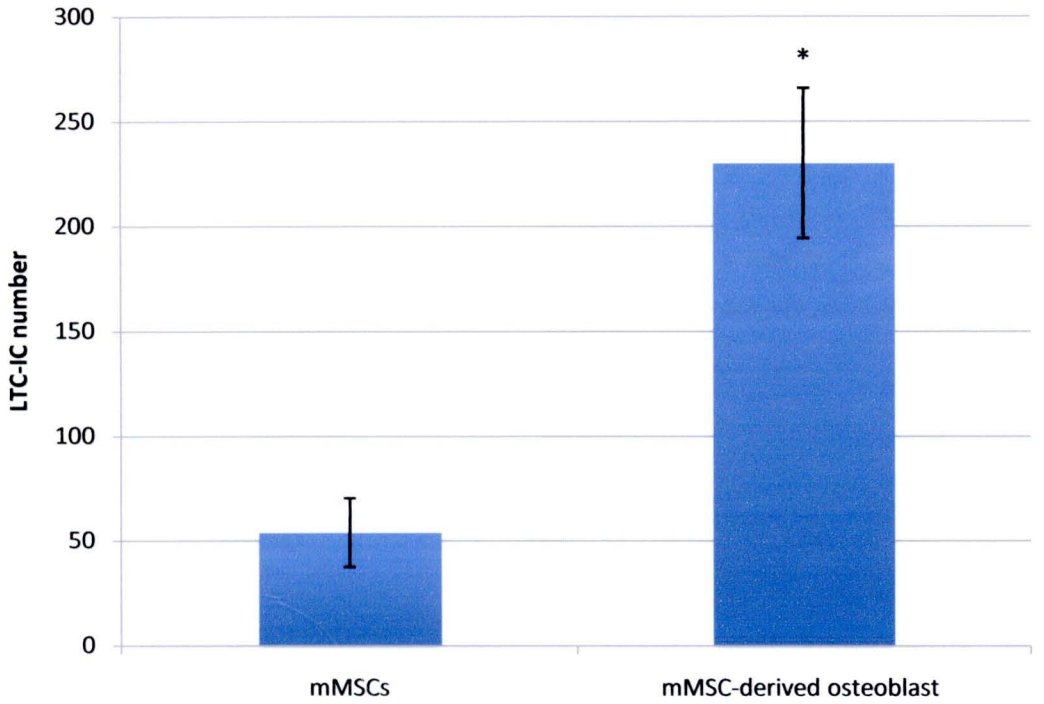

Figure 9 Effect of MOBs on the output of long-term culture initiating cell (LTC-IC). Data shown are the mean number (± SD) of LTC-IC derived from 1,000 input LSK cells (*$p < 0.005$, n=4).

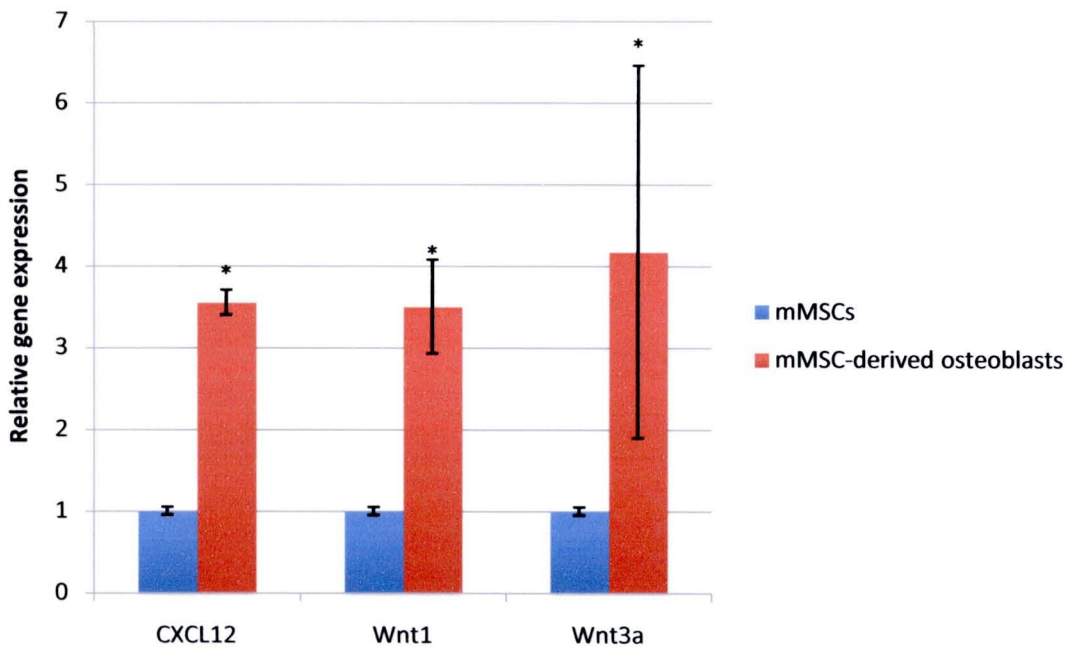

Figure 10 Quantitative RT-PCR analysis of CXCL12, Wnt1 and Wnt3a expression in MOBs and mMSCs. The data are presented as average (± SD), (*$p < 0.05$, n = 3).

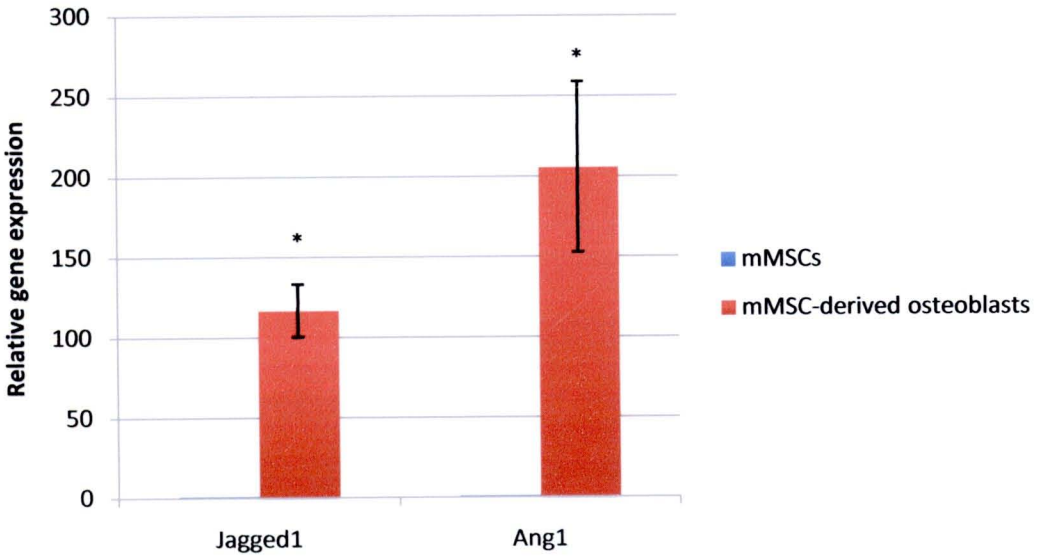

Figure 11 Quantitative RT-PCR analysis of Jagged1 and Angiopoietin1 (Ang1) expression in MOBs and mMSCs. The data are presented as average (± SD), (*$p < 0.01$, $n = 3$).

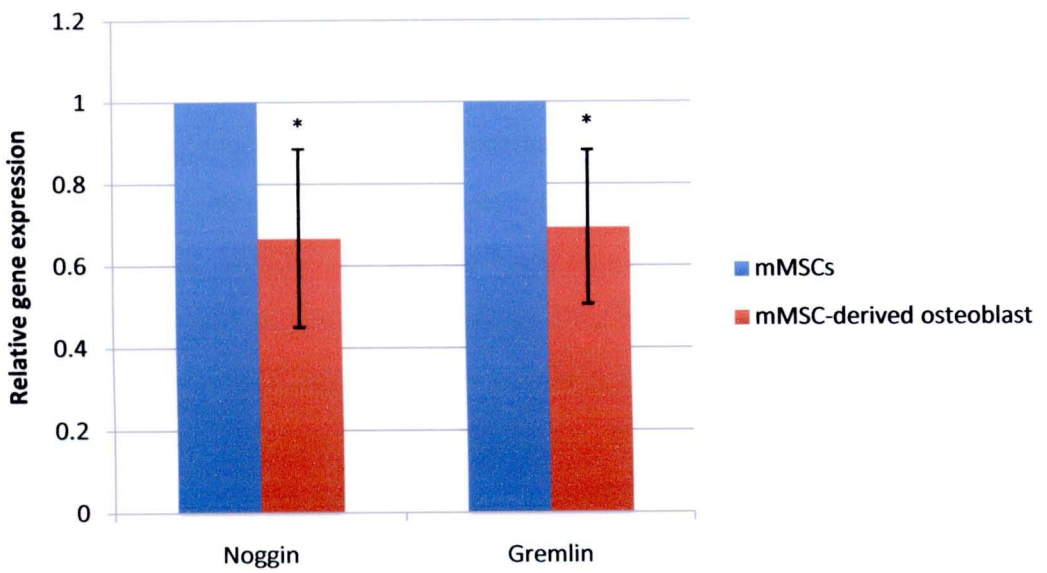

Figure 12 Quantitative RT-PCR analysis of Noggin and Gremlin expression in MOBs and mMSCs. The data are presented as average (± SD), (*$p < 0.005$, $n = 3$).

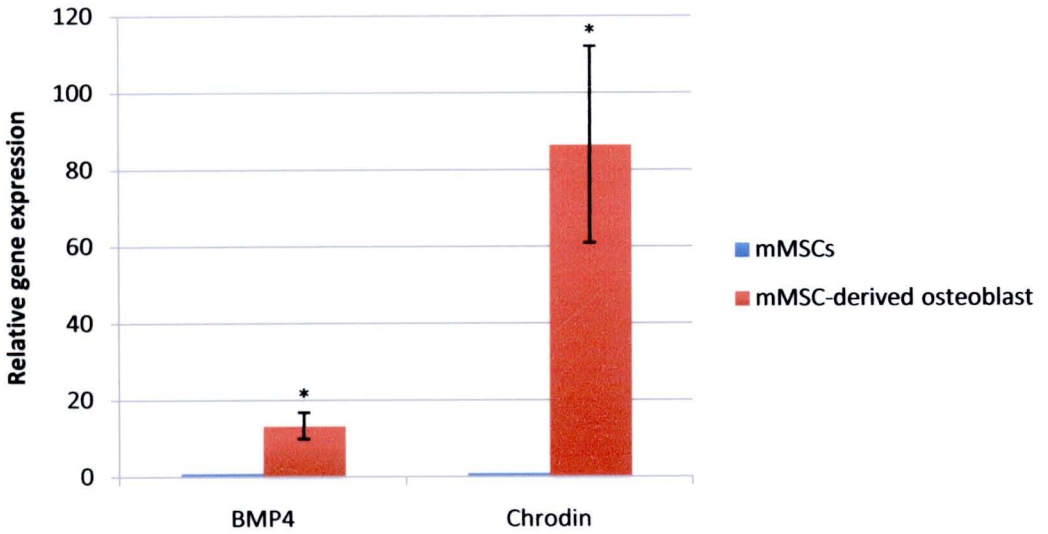

Figure 13 Quantitative RT-PCR analysis of BMP4 and Chordin expression in MOBs and mMSCs. The data are presented as average (± SD), (*$p < 0.001$, $n = 3$).

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.

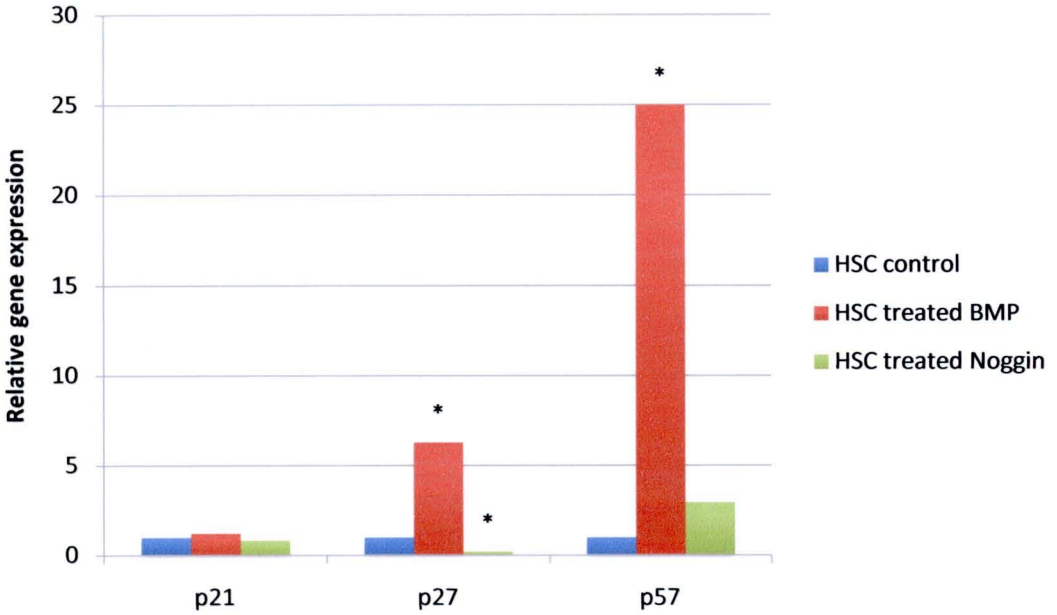

Figure 14 Effect of BMP2 and noggin treatment in HSCs. Cyclin-dependtdent kinase inhibitor expressed in HSCs measured by change in p21, p27 and p57 expression. The data are presented as average, (*$p < 0.05$, $n = 3$).

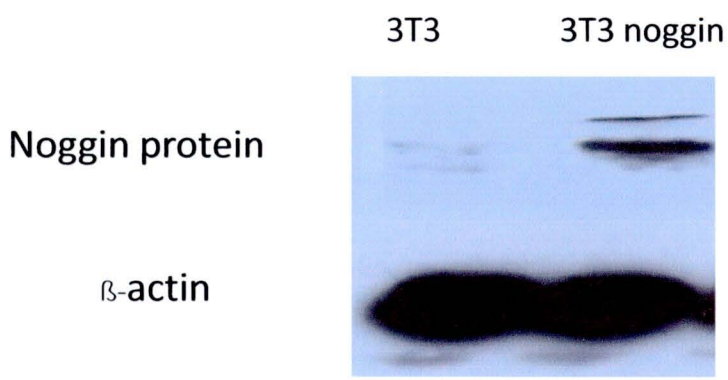

Figure 15 Western blot analysis of noggin protein in 3T3 noggin cells. ß-actin was used as a control for loading.

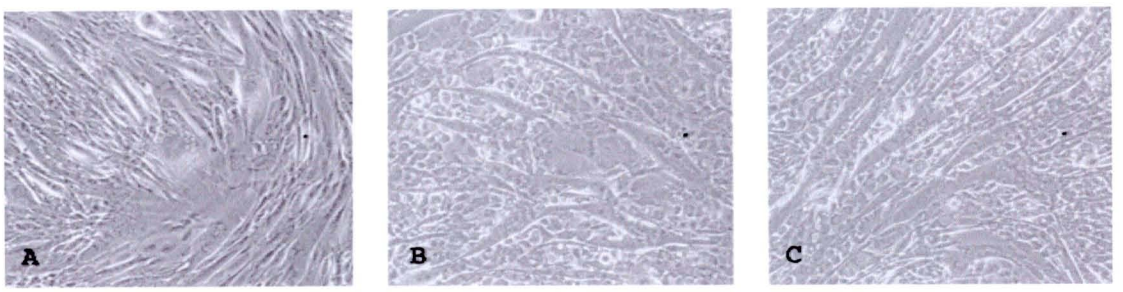

Figure 16 Morphology of M3B feeder. MOBs (A) and M3B system which combine of two cell types, mMSC and 3T3 noggin cells in ratio 1:1 (B) and 5:1 (C) after inducion with osteogenic media for 7 day

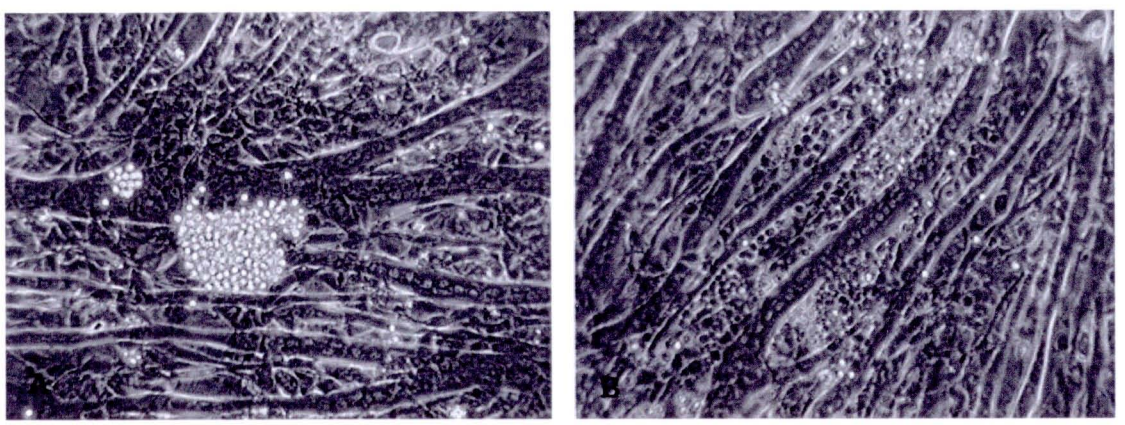

Figure 17 The effect of M3B feeder on LSK growth phenotype. Daughter cells were produced on M3B feeder (A) output colonies (B) Cobblestone-like colonies.

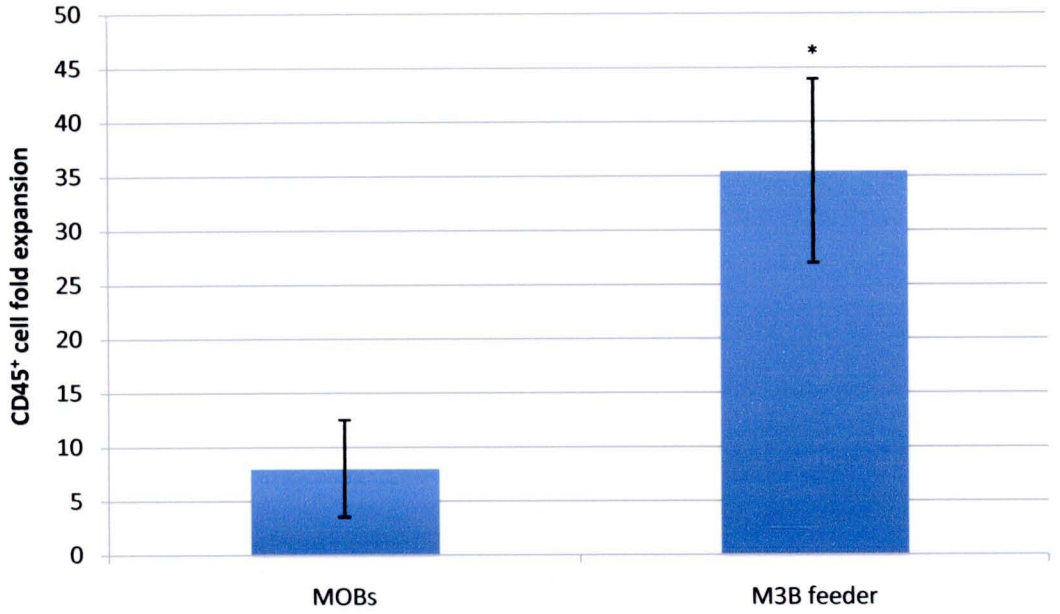

Figure 18 Effect of M3B feeder on the expansion of $CD45^+$ cell. Data shown are the mean number (± SD) of CFC derived from 1,000 input LSK cells (*$p < 0.05$, n=3).

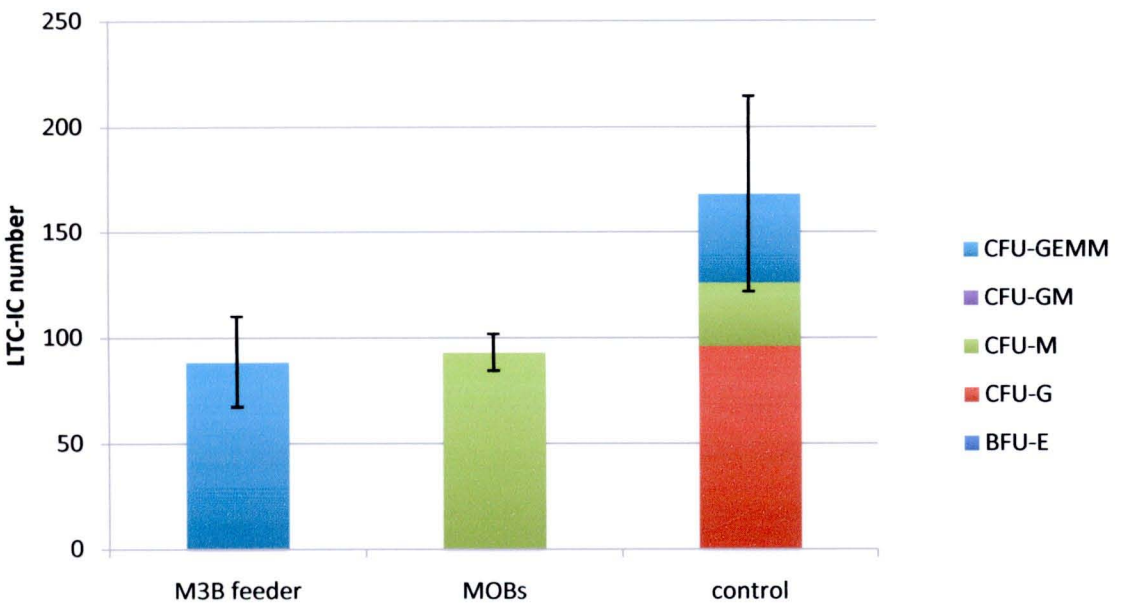

Figure 19 Effect of M3B feeder on the output of long-term culture initiating cell (LTC-IC). Control is the number of CFC derived from 1,000 LSK cells at day 0. Data shown are the mean number (± SD) of LTC-IC derived from 1,000 input LSK cells (n=4).

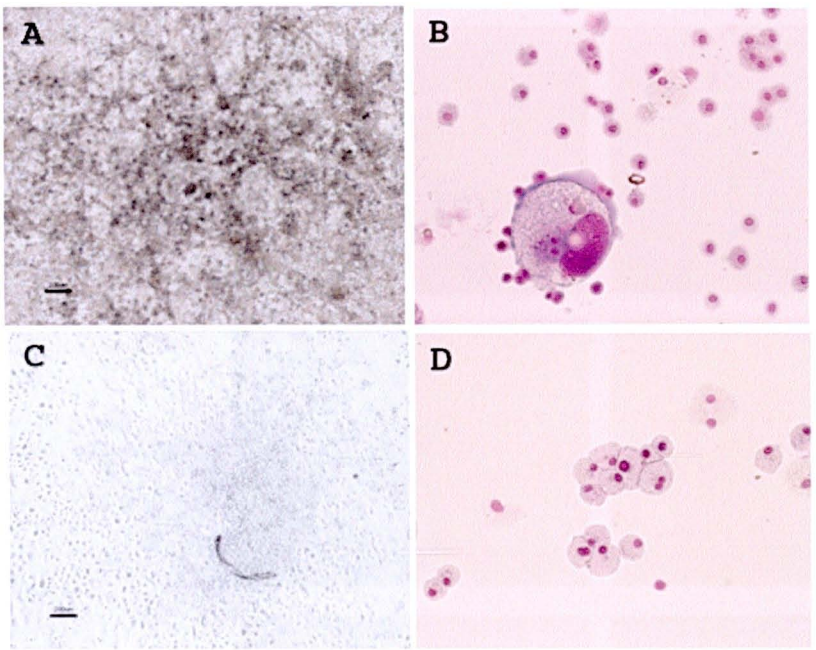

Figure 20 Wright-Giemsa stains of cytospin preparations of LTC-IC derived colonies. (A) CFU-GEMM consists of erythrocyte, macrophage, granulocyte and megakaryocyte (B). (C) CFU-M consist only macrophage (D)

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).

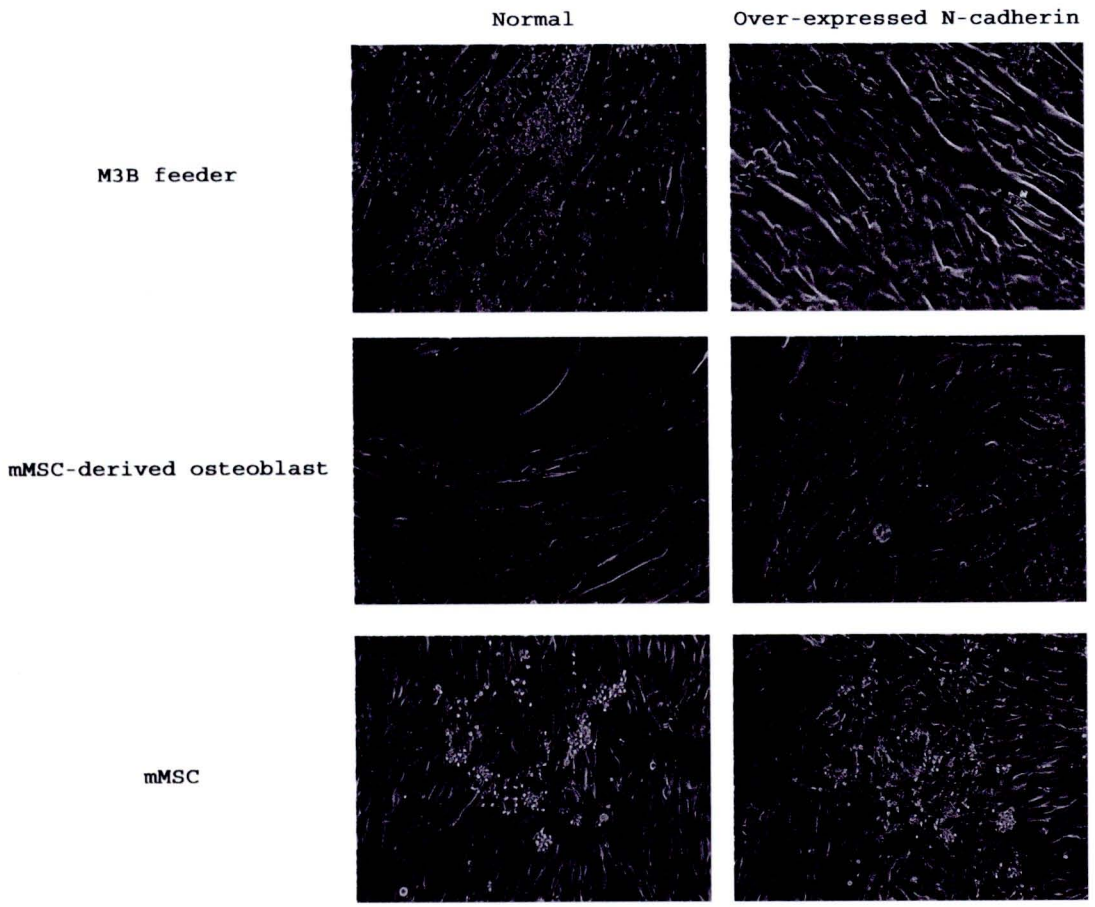

Figure 21 The effect of N-cadherin on the growth phenotype of LSK cells. LSK cells were cultured on normal mMSCs, MOBs and M3B feeder and N-cadherin overexpressed conditions in long-term culture media.

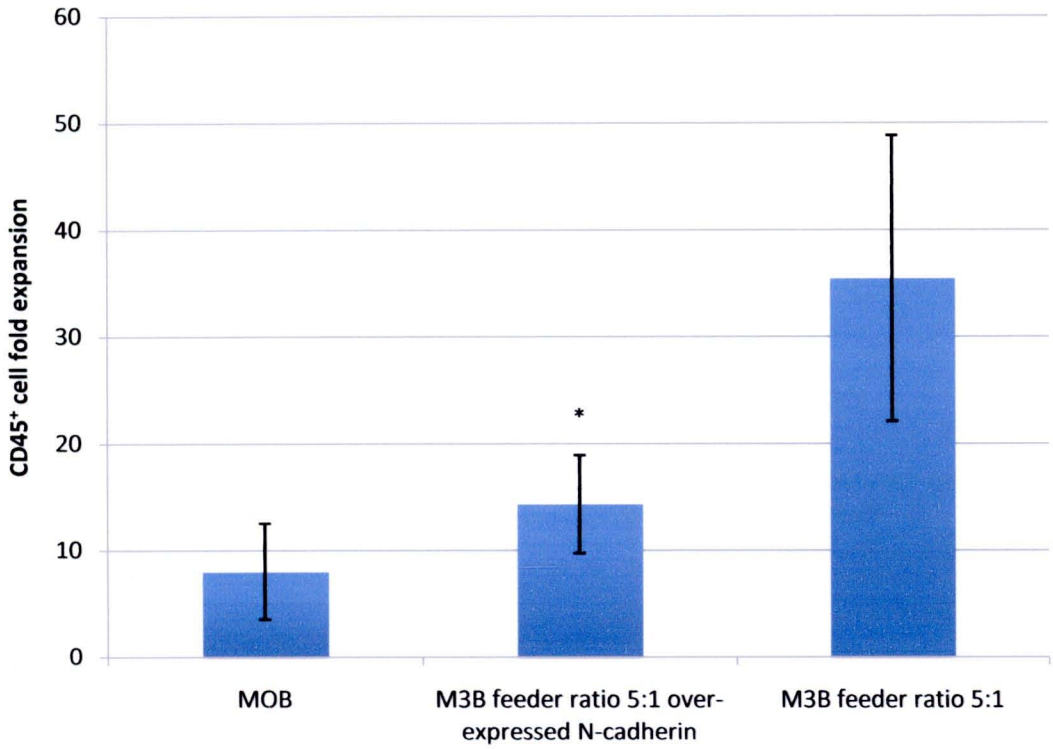

Figure 22 Effect of N-cadherin overexpressed M3B feeder on the expansion of $CD45^+$ cell. Data shown are the mean number (± SD) of $CD45^+$ fold expansion derived from 1,000 input LSK cells (*$p < 0.05$, n=3).

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.

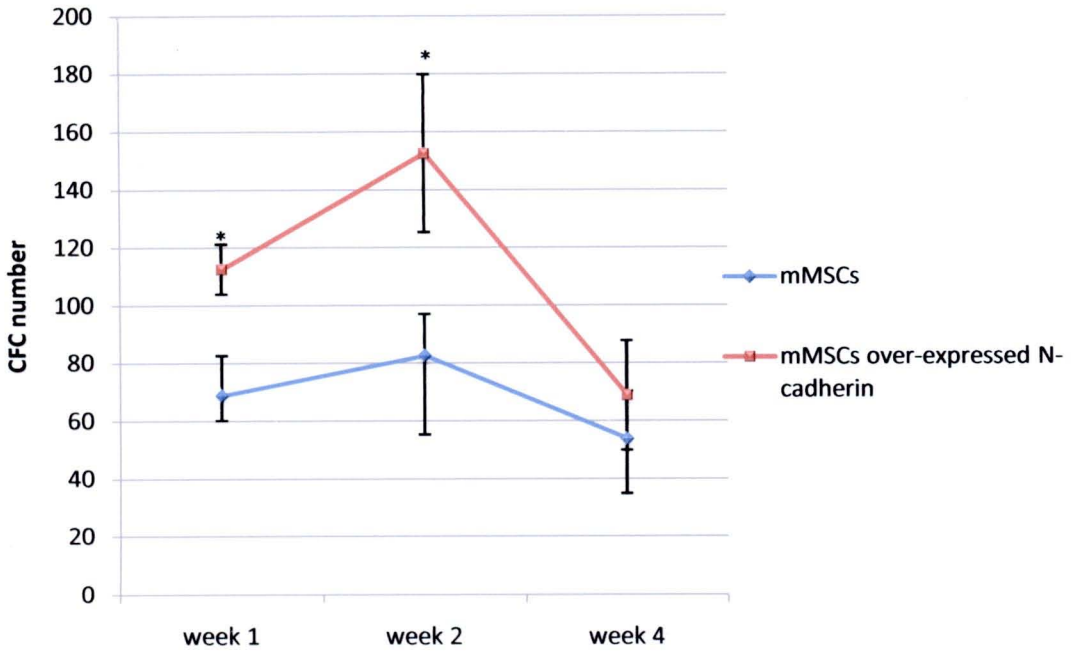

Figure 23 Effect of N-cadherin overexpressed mMSCs on the output of colony forming cell (CFC). Data shown are the mean number (± SD) of CFC derived from 1,000 input LSK cells (*$p < 0.05$, n=4).

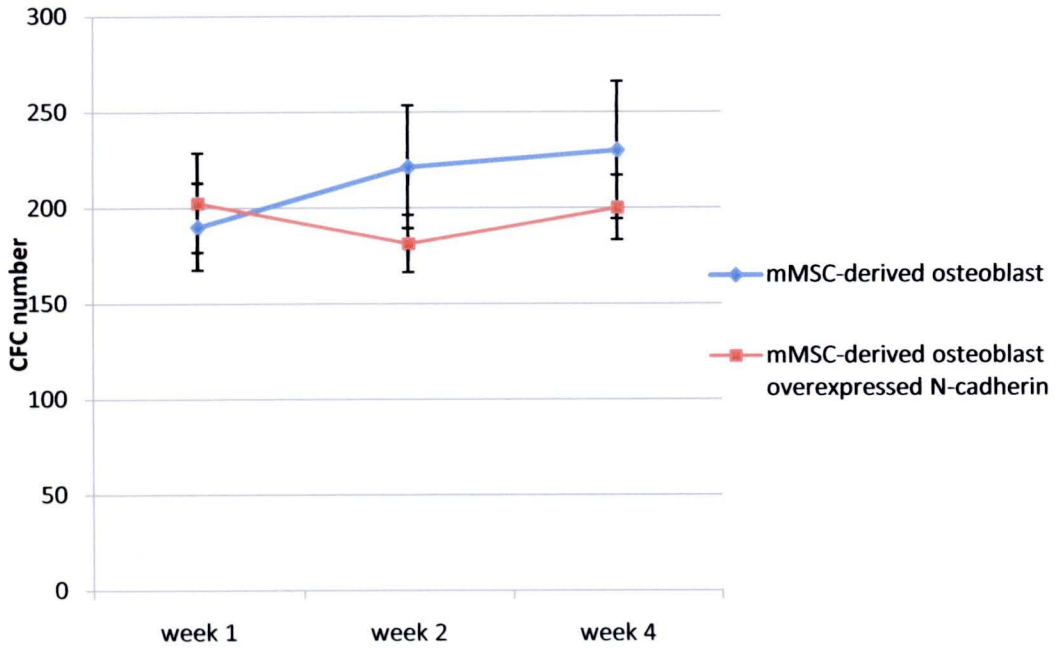

Figure 24 Effect of N-cadherin overexpressed MOBs on the output of colony forming cell (CFC). Data shown are the mean number (± SD) of CFC derived from 1,000 input LSK cells (n=4).

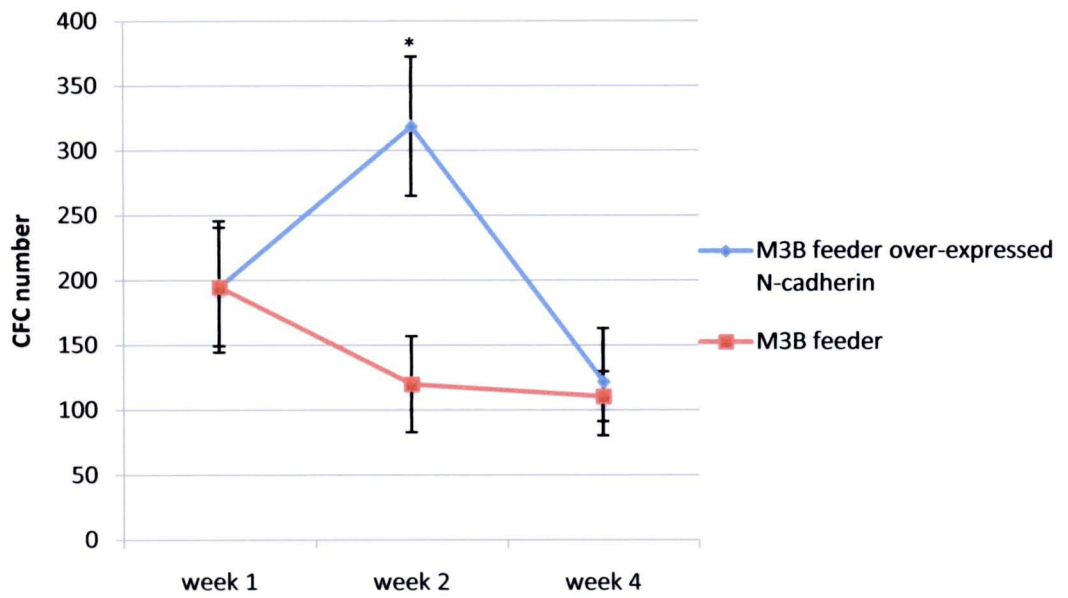

Figure 25 Effect of N-cadherin overexpressed M3B feeder on the output of colony forming cell (CFC). Data shown are the mean number (± SD) of CFC derived from 1,000 input LSK cells (*$p < 0.05$, n=4).

Recently, it was reported that Interferon alpha (IFN**α**) has ability to promote HSCs proliferation *in vivo* by activating STAT and Wnt pathway in HSCs. But the effect of IFN**α** 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 IFN**α**, 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 IFN**α** on HSCs activation may be also mediated indirectly through down regulation of Wnt antagonist secreted from niche cells.

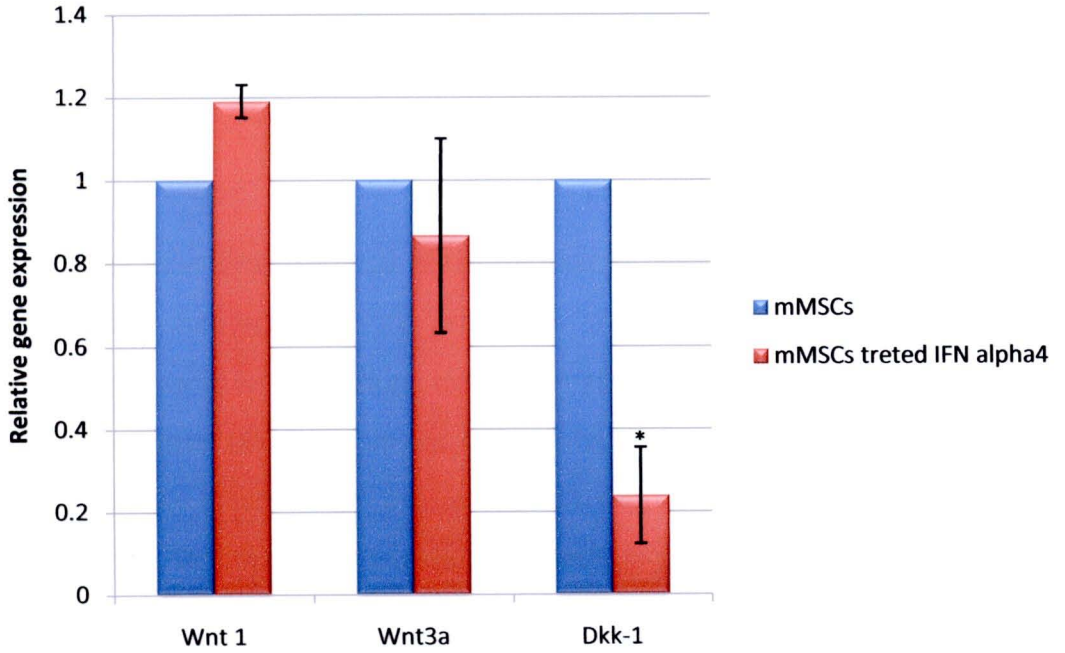

Figure 26 The effect of IFN-alpha 4 treatment on Wnt1, Wnt3a and Dkk1 expression in mMSCs. The data are presented as average (± SD), (*$p < 0.05$, n = 3).

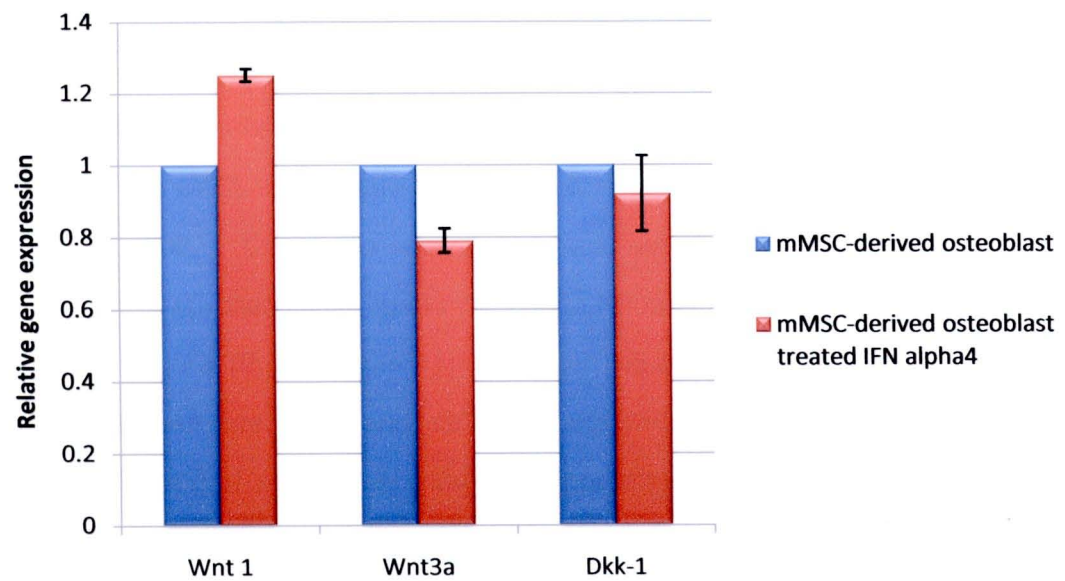

Figure 27 The effect of IFN-alpha 4 treatment on Wnt1, Wnt3a and Dkk1 expression in MOBs. The data are presented as average (± SD), n = 3.