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.