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 $p < 0.05$.