Journal of Bone Marrow Research
ISSN: 2329-8820

Chang CF et al., J Bone Marrow Res 2014, 2:2
DOI: 10.4172/2329-8820.1000144

Research Article Open Access

Enrichment and Characterization of Two Subgroups of Committed Osteogenic Cells in the Mouse Endosteal Bone Marrow with Expression Levels of CD24

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Abstract

Primary osteogenic cells have been known to reside within the CD45$^-$CD31$^-$Ter119$^-$Sca-1$^-$ cell fraction, particularly in the CD51$^+$ subpopulation. However, detailed determination of the frequency of osteogenic cells within this Sca-1$^-$ cell population remains yet to be determined. In addition, it is not clear that other cell surface markers can be used to further sub-fractionate this Sca-1$^-$CD51$^+$ osteogenic cell population and to define their developmental stages. In this report, both Sca-1$^-$CD24^{med} and Sca-1$^-$ CD24$^{-/lo}$ cells have been shown to be two small subsets of the Sca-1$^-$CD51$^+$ cell fraction. These two cell fractions show subtle difference in the expression level of osteogenic marker genes such as *Osx* and *Opn*, and *in vitro* proliferate rate. All these observations suggest that they may be at different developmental stages of osteogenesis. The Sca-1$^-$CD24^{med} cell fraction is enriched for the more mature osteolineage cells than the Sca-1$^-$CD24$^{-/lo}$ counterpart. In contrast, most of the Sca-1$^-$CD24^{hi} and Sca-1$^+$CD24$^{-/lo}$ cells do not contain CFU-ALP nor express osteogenic gene markers. The high proliferation ability and osteo-adipogenic differentiation potentials confirm that the Sca-1$^+$CD24$^{-/lo}$ cells are the multipotential mesenchymal stromal cells. The determination of individual stromal cell subpopulations will lead to a better understanding in the hierarchical organization of these osteolineage cells.

Keywords: Osteolineage cells; Osteogenic subpopulation; Fluorescence-activated cell sorting; CD24; Alkaline phosphatase

Introduction

Bone is a highly organized tissue, comprised of a calcified connective tissue matrix and specific bone cells, including bone progenitors, osteoblasts, and osteocytes. Osteoblasts are derived from multipotent marrow stromal cells (MSCs) through a series of proliferation and differentiation steps before expressing recognizable specific osteoblast marker genes. Although much has been learned about the cellular identity and differentiation potential of MSCs [1-3], little is known about the hierarchical relationship between cells of the osteogenic lineage and those with other related cell lineages within the bone marrow. The specific surface markers, which can distinguish MSCs and their osteogenic or adipogenic progeny, are necessary. Recently, MSCs have been identified as Lin$^-$CD45$^-$CD31$^-$Sca-1$^+$CD51$^+$ [4,5], CD45$^-$Ter119$^-$Sca-1$^+$ PDGFR-α$^+$ [6], and CD45$^-$CD31$^-$Ter119$^-$Sca-1$^+$ ALCAM$^-$ [7]. These cells have high proliferation capacity (self-renewal); multiple cell lineage differentiation potential (multipotential), and the lack of gene expression for osteogenic differentiation, such as *Runt related transcription factor 2 (Runx2), Osteocalcin (Ocn), Osteopontin (Opn).*

Primary osteolineage cells are usually isolated from Sca-1$^-$ [6,7], or Sca-1$^-$CD51$^+$ cell fraction [4,5,8] within CD45$^-$CD31$^-$Ter119$^-$ non-hematopoietic and non-endothelial cell compartment. These osteoblast-enriched cell fractions have been characterized to contain Alkaline Phosphatase (ALP) activity and expressed a high level of intracellular osteoblast-specific genes (*Runx2, Ocn, Opn*). However, the percentage of ALP$^+$ osteolineage cells within these Sca-1$^-$ cell populations has not been quantitated, and detailed determination of the frequency of these osteogenic cells remains yet to be determined. In addition, it is not clear whether other cell surface markers can be used to further sub-fractionate the Sca-1$^-$CD51$^+$ osteogenic cell subpopulation.

Stem cell antigen-1 (Sca-1) is a mouse glycosylphosphatidylinositol (GPI) -anchored cell surface protein of the Ly6 gene family [9]. It is the most commonly used cell surface marker for the enrichment of adult murine hematopoietic stem cells [10-12] and MSCs [4-7]. Cluster of differentiation 24 (CD24, also known as heat stable antigen) is another mouse GPI-linked sialoglycoprotein, which has been used as a marker for the isolation of neuron stem cells [13], mammary gland stem cells [14], and *in vivo* white adipose progenitors [15]. As adipocytes also exist in the bone marrow, we speculate that the use of CD24 antigen, as in white adipose tissue, may allow us to discriminate with osteogenic, adipogenic or other lineage cells within the mouse bone marrow.

Although primary osteogenic cells have previously been enriched in the CD45$^-$CD31$^-$Sca-1$^-$ or its CD51$^+$ sub-fraction from the mouse bone marrow, these cells are heterogeneous and detailed determination of the frequency of osteogenic cells within these cell populations remains yet to be investigated. Because the current method using CD51$^+$ to identify osteogenic cells is not ideal, we attempt to characterize the endosteal stromal cell component through a combined use of the Sca-1 and CD24 monoclonal antibodies (MoAbs) in this study.

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Received May 09, 2014; **Accepted** September 01, 2014; **Published** September 03, 2014

Citation: Chang CF, Hsu KH, Shen CN, Li CL, Lu J (2014) Enrichment and Characterization of Two Subgroups of Committed Osteogenic Cells in the Mouse Endosteal Bone Marrow with Expression Levels of CD24. J Bone Marrow Res 2: 144. doi: 10.4172/2329-8820.1000144

Materials and Methods

Preparation of the central bone marrow and bone associated cell fractions from animals

C57BL/6JNarl mice, 4-6 weeks of age, were purchased from the National Laboratory Animal Center (Taiwan). After cervical dislocation, both femurs and tibiae were removed and collected in phosphate buffered saline (PBS) containing 5% fetal bovine serum (FBS, Biological industries, Israel) (PBS/FBS buffer). We first punched through both ends of tibiae and femurs with 21-gauge needles, and then flushed out the bone marrow cells using 3 ml syringes. These flushed-out bone marrow cells were pooled and labelled as the central bone marrow (CBM) cell fraction (Supplementary Figure 1A, upper panel). The remaining long bones (Supplementary Figure 1A, lower panel) were then cut with a pair of scissors into 1-2 mm fragments, which were then incubated at 37°C with 0.2% type I collagenase in PBS/FBS buffer for 2 hours (Sigma-Aldrich, USA). The dissociated cells collected each hour after enzyme digestion. This CBM-depleted cell preparation contains endosteal cells from both cortical and trabecular bone regions and hemopoietic cells from the epiphyseal cavity (Supplementary Figure 1B and 1C). It is also coined as endosteal bone marrow (EBM) cell fraction as only the $(CD31/CD45/Ter119)^-$ (triple negative) stromal cells are gated for subsequent flow analysis and cell sorting. Both CBM and CBM-depleted cell preparations were then filtered through 40-μm cell strainers (BD Falcon 2350, USA). Lastly, the erythrocytes present in both cell fractions were lysed in hypotonic buffer solution (0.156M NH_4Cl, 0.1M EDTA and 0.01M $NaHCO_3$).

Monoclonal antibodies (MoAbs)

APC-Sca-1, APC/eFluor780-CD45.2, APC/eFluor780-Ter119, Biotin-mCD51, Biotin-mCD105, Biotin-PDGFR-α, FITC-CD24, PE-Cy7-mCD29, PerCP/Cy5.5-Ter119, Streptavidin-PE-Cy7, FITC-CD90.2 and PerCP/Cy5.5-CD24 were used in this study. Most of the fluorochrome-conjugated antibodies were purchased from eBioscience except the last two, which were from Biolegend. All these antibodies have first been pre-titrated to determine the working concentration using a panel of positive and negative cells or cell lines. Detailed information of these fluorochrome-conjugated MoAbs, including the name of hybridoma clones and pre-determined working concentrations are listed in Supplementary Table 1.

Flow cytometric analysis and fluorescence activated cell sorting (FACS)

For cell sorting, both CBM and CBM-depleted cell preparations were

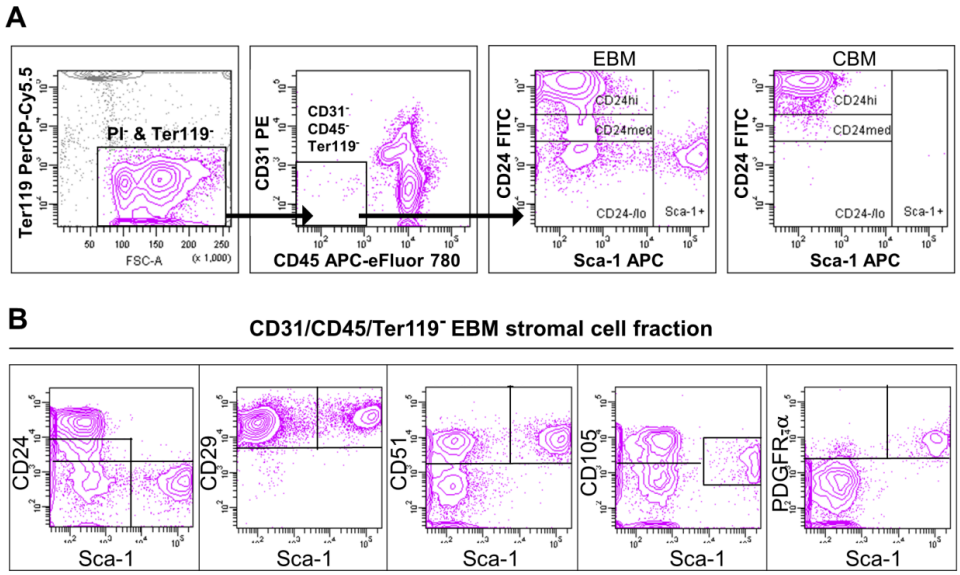


Figure 1: The CBM-deplted cell preparation is enriched for both $Sca\text{-}1^-CD24^{-/lo}$ cells and $Sca\text{-}1^+CD24^{-/lo}$ cells (A) Representative flow cytometric profiles of CD24 and Sca-1 expression in the CD31/CD45/Ter119 triple negative cells in both EBM and CBM cell fractions. The CD31/CD45/Ter119 triple negative cells in EBM cell fraction can be subdivided into four fractions: (i) $Sca\text{-}1^-CD24^{hi}$, (ii) $Sca\text{-}1^-CD24^{med}$, (iii) $Sca\text{-}1^-CD24^{-/lo}$, and (iv) $Sca\text{-}1^+CD24^{-/lo}$. Note that there is an apparent absence of $Sca\text{-}1^-CD24^{-/lo}$, and $Sca\text{-}1^+CD24^{-/lo}$ cells in the CBM cell fraction. (B) A contour-dot blot analysis of the $CD31/CD45/TER119^-$ triple negative stromal cells co-stained with anti-Sca-1 MoAb and individually with anti-CD24, -CD29, -CD51, -CD105 and -PDGFR-α MoAbs. The plots show that there are only one population of $Sca\text{-}1^+$ cells and one to three $Sca\text{-}1^-$ cell sub-populations can be readily identified.

cell fraction	cell number per femur/tibia pair ($\times 10^4$)					
	Total	$CD31/CD45/Ter119^-$	$Sca\text{-}1^-CD24^{hi}$	$Sca\text{-}1^-CD24^{med}$	$Sca\text{-}1^-CD24^{-/lo}$	$Sca\text{-}1^+CD24^{-/lo}$
CBM	3016.7 ± 635.3 [%]	16.8 ± 13.2 [0.60 ± 0.52]	16.6 ± 13.1 [0.59 ± 0.52]	0.14 ± 0.07 [0.005 ± 0.003]	0.011 ± 0.006 [< 0.001]	0.0016 ± 0.000 [< 0.001]
EBM	231.8 ± 124.2 [%]	3.83 ± 2.55 [2.15 ± 1.81]	3.48 ± 2.57 [1.96 ± 1.79]	0.11 ± 0.05 [0.063 ± 0.051]	0.11 ± 0.06 [0.063 ± 0.054]	0.13 ± 0.06 [0.068 ± 0.033]

Table 1: Relative frequency of various cell sub-populations in the central and endosteal BM cell fractions

A summary shows the absolute cell number and percentage of individual cell sub-populations in the CBM and EBM cell fractions. Note that the frequency and the number of $Sca\text{-}1^-CD24^{-/lo}$ and $Sca\text{-}1^+CD24^{-/lo}$ fraction was significantly different between the CBM and EBM cell fractions, and the majority of $Sca\text{-}1^-CD24^{-/lo}$ and $Sca\text{-}1^+CD24^{-/lo}$ cells existed in the EBM harvest. Data represent means ± S.D. The data was calculated from 3 experiments for CBM cell fraction and 5 experiments for EBM cell fraction (number of mice used for each experiment, n=6).

first stained with Höechst33342 (Hö, Invitrogen, USA) at 5.5 μg/ml in PBS-FBS for 1 hour at 37^{0}C. After washing, cells were then incubated on ice for 15 min with a mixture of antibodies: FITC-CD24, PE-CD31, PerCP/Cy5.5-Ter119, APC-Sca-1, APC/eFluor 780-CD45.2. Cells were washed again, centrifuged and re-suspended in PBS/FBS buffer containing 2 μg/ml propidium iodide (PI). Lastly, cells were analyzed and sorted using a standard operation protocol of FACSAria I cell sorting system (BD BioScience, USA) equipped with three lasers at positions 1, 2, and 3 with wavelengths of 488, 633, and 365 nm, respectively.

The PI and Hö fluorescence were measured which was served as a gate for live cells (excluding those that are positive for PI) and another gate for cell debris (excluding those that are negative for Hö) respectively. In addition, the Hö staining profile can allow the detection of Hö sub-populations and also serve as a surrogate marker for the cell cycle status of individual cell populations within the EBM cell fraction. The CBM cell fraction with an additional depletion of $CD45^+$ cells is served as a reference control and was stained as described above for the EBM cell fraction. The $CD45^+$ cells were depleted using the CD45 MicroBeads (Miltenyi Biotec) following the manufacturer's procedure.

For analysis of stromal cell subpopulations, cells were incubated on ice for 15 min with each of following antibodies combining with above-mentioned mixture of antibodies. We used antibodies: PE-Cy7-mCD29, Biotin-mCD51, Biotin-PDGFR-α, Biotin-mCD105, and Streptavidin-PE-Cy7. In addition, different fluorescence dyes, APC/eFluor 780-Ter119, PerCP/Cy5.5-CD24, and FITC-CD90 combining with a mixture of PE-CD31, APC-Sca-1, and APC/eFluor 780-CD45.2 were also used for stromal cell analysis. After antibody staining, cells were then washed, re-suspended in 2 μg/ml propidium iodide (PI). Finally, cells were analyzed using a FACSAria I cell sorting system (BD BioScience, USA).

Cell culture

Initially, 10^3 FACS-sorted cells of individual sub-populations were plated into a well of 6-wells plate with standard stromal cell culture medium, Minimum Essential Medium alpha medium (MEM-α) containing 25 μM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffer supplemented with 20% FBS at 37°C, 10% CO_2 and 5% O_2. After 14 days culture, adherent cells derived from individual cell fraction were harvested by trypsin-EDTA digestion, and then cells were sub-cultured at the density, ~1.5 x 10^3 cells per cm^2. However, due to the very low plating efficiency of Sca-1$^-$ $CD24^{hi}$ cells, these cells were cultured at a higher cell density. Over 10^5 FACS-sorted Sca-1$^-$$CD24^{hi}$ cells were plated in one well of 6-wells plate with standard stromal cell culture medium.

In vitro differentiation assays

7,500 culture-expanded cells from each FACS-sorted cell fractions were seeded into each well of 96 well plates. For osteogenic differentiation, cells were exposed to induction medium, which contained 60% (v/v) DMEM-LG, 40% (v/v) MCDB201 (Gibco) supplemented with 10% FBS and an inducer cocktail (ABD): 50 μM ascorbate-2 phosphate, 10 mM β-glycerol phosphate disodium and 10^{-7} M dexamethasone. After 14 days of incubation, cells were first fixed with 4% para-formaldehyde (PFA) and then stained with ALP. Freshly prepared ALP staining solution, which contains 0.5 mg/ml Fast Red violet LB salt, and 0.5 mg/ml Naphthol AS-MX phosphate disodium salt, and 10% 0.56 M Amp buffer in water were added and incubated for 30 min at room temperature in darkness. After incubation, the ALP staining solution was removed, and the stained cell preparation was washed twice with Milli-Q-qualified water. For calcium deposition analysis, cells were fixed with ice-cold 70% ethanol for 1hour. After PBS washing, cells stained with 40 mM Alizarin Red (pH 4.2) for 15 min. For adipogenic differentiation assay, cells were exposed to induction medium, which contained MEM-α medium supplemented with 10% FBS and inducer cocktail (DIXIn): 10^{-6} M dexamethasone, 10 μg/ml insulin, 0.45 mM 3-isobutyl-1-methylxanthine, and 50 μM indomethacin. After 14 days of incubation, cells were fixed with 4% PFA for 1 hour. After PBS washing, cells stained with 0.3% Oil Red O in 60% isopropanol. The whole well pictures of the ALP or Alizarin Red stain were taken with the Olympus E330 digital single-lens reflex camera with 50 mm digital lens. The analysis of adipocyte drops was used an optical inverted microscope (Olympus CK40). All chemicals are from Sigma-Aldrich, USA.

Real time RT-PCR

Total RNA was extracted from the FACS-isolated cells using TRIzol reagent. For cDNA synthesis, random hexamers were used in the presence of SuperScriptTM III Reverse Transcriptase (Invitrogen). For real time PCR reaction, master mix of the following components was prepared to the indicated working concentration: 4 μl LightCycler® TaqMan® Master Mix (5X, Roche), 1 μl forward primer (0.5 μM), 1 μl reverse primer (0.5 μM), 0.2 μl hydrolysis probe (Roche, 0.1 μM), and 11.8 μl water. Reagents (18 μl) were filled in the LightCycler glass capillaries and 2 μl cDNA was added as PCR template. Each Capillary was sealed with a stopper and placed the adapters in a standard benchtop microcentrifuge. Capillaries were centrifuged at 3000 rpm for 5 sec, and then were transferred to the sample carousel of the LightCycler® 1.5 instrument. The parameters for a LightCycler® carousel-Based System PCR run with the LightCycler® TagMan® Master are as follows: pre-incubation, 95°C for 10 min; denaturation, 95°C for 10 sec; annealing, 60°C for 10 sec, extension, 72°C for 1 sec; The amplification processes included denaturation, annealing, extension for 35 cycles, and lastly cooling at 40°C for 30 sec. Each of the primer and the probe number was listed as follows: mouse *runt related transcription factor 2 (Runx2)* (Probe No. 60), forward: CCACAAGGACAGAGTCAGATTACA, reverse: TGGCTCAGATAGGAGGGGTA; mouse *Osterix (Osx)* (Probe No.1), forward: CGGCCACGCTACTTTCTTTA, reverse: TATCGCCGTCTCCATTTCT; mouse *Alkaline phosphatase (Alp)* (Probe No.31), forward: CGGATCCTGACCAAAAACC, reverse: TACTGATGTCCGTGGTCAAT; mouse *Parathyroid hormone 1 receptor (pth1r)* (Probe No.66), forward: GCTGCTCAAGGAAGTTCTGC, reverse: CGTCCACCCTTTGTCTGACT; mouse *Osteocalcin (Ocn)* (Probe No.32), forward: AGACTCCGGCGCTACCTT, reverse: CCCGGTGAAAGTGACTGATT; mouse *Osteopontin (Opn)* (Probe No.82), forward: CCCGGTGAAAGTGACTGATT, reverse: TTCTTCAGAGGACACAGCATTC; mouse *60S ribosomal protein L32-like (RPL32)* (Probe No.46), forward: GCTGCCATCTGTTTTACGG reverse: TTTTGACGATCTTGGGCTTC. The relative expression level was normalized to mouse *RPL32* expression level.

Colony forming unit (CFU) assay

It was performed by plating 10^3 individual subpopulation cells in 6 well plates contain with standard stromal cell culture medium. After 14 days of incubation, the cultures were stained with ALP staining solution and followed by staining with 0.5% methylene blue solution. The number of ALP^+ and ALP^- colonies (over 50 cells) was counted using an optical inverted microscope (Olympus CK40). The clonogenic cells that form these ALP^+ and ALP^- colonies are termed as CFU-ALP and CFU-F respectively.

Immunofluorescence analysis of the sorted cells

Individual FACS-sorted cell fractions were collected and re-suspended in PBS-FBS; 100 µl aliquots of individual cell suspension were centrifuged onto ethanol-cleaned glass slides in a Shandon Cytospin 4 Cytocentrifuge (Thermo Electron Corp., USA). The dried, spun-down cells were then fixed with 4% PFA, and then washed with Milli-Q-qualified water twice (Milli-Q integral water purification system, Merck Millipore, USA). For Runx2 staining, anti-mouse Runx2 (working concentration is 1:100, Cat no. SC-10758, Santa Cruz Biotechnology, USA) and Alexa Fluor 488 goat anti-rabbit IgG (working concentration: 1:200, Cat no. A11008, Invitrogen) antibodies were used. The ALP staining solution, which contains 0.5 mg/ml Fast Red violet LB salt, 0.5 mg/ml Naphthol AS-MX phosphate disodium salt and 10% 0.56 M Amp buffer in water (all chemicals are from Sigma-Aldrich) was freshly prepared each time. After incubation for 30 min at room temperature in complete darkness, the staining solution was removed, and the stained cell preparation was washed twice with Milli-Q-qualified water. Lastly, all slides were mounted in mounting medium, (VECTASHIELD®) containing DAPI (4,' 6-diamidino-2-phenylindole, Vector laboratories, USA), and cells were examined under an inverted fluorescence microscope (Axiovert 200M, Zeiss). The excitation and emission wavelength for DAPI is 358 nm and 461 nm; Alexa Fluor 488 is 495 nm and 519 nm, and the ALP stained product is 522 nm and 628 nm [16].

Statistical analysis

One way AVONA analysis (GraphPad Prism) was performed to determine the statistical significance. Results are expressed as mean ± standard deviation (SD).

Results and Discussion

The CBM-depleted Cell Preparation is Enriched for Both Sca-1^{-}CD24$^{-/lo}$ Cells and Sca-1^{+}CD24$^{-/lo}$ Cells

The standard method to isolate bone marrow cells from long bones, by flushing, only released the bone marrow cells from the central part of the diaphyseal bone and is named as the Central Bone Marrow (CBM) cell fraction. The CBM-depleted BM preparation contains, in addition to the hematopoietic cells from the trabecular bone cavity, the osteogenic cells and stromal cells from the trabecular and cortical endosteum and periosteum where active bone formation and remodeling is occurring in young mice. In this study, we collected not only the CBM cell fraction from the cortical bone central cavity, but also the CBM-depleted cell fraction. During flow analysis and cell sorting, the cellular subpopulations within this CD31/CD45/Ter119^{-} (triple negative) stromal cell fraction were focused. This stromal cell fraction was also coined as endosteal bone marrow (EBM) cell fraction throughout the study. Flow cytometric analysis of both CBM and EBM cells showed that there was a significant difference between these two cell fractions when the triple negative stromal cells were analyzed (Figure 1A and Supplementary Figure 2). Interestingly, the CBM cell preparation was mainly enriched for the Sca-1^{-}CD24^{hi} cells (0.59%) while the EBM cell fraction has four cell subpopulations: Sca-1^{-}CD24^{hi} (1.96%), Sca-1^{-}CD24^{med} (0.063%), Sca-1^{-}CD24$^{-/lo}$ (0.063%), and Sca-1^{+}CD24$^{-/lo}$ (0.068%). Of note, there is a 10-fold more Sca-1^{-}CD24$^{-/lo}$ cells (1.1×10^{2} versus 1.1×10^{3}) and a 100-fold more Sca-1^{+}CD24$^{-/lo}$ cells (1.6×10^{1} versus 1.3×10^{3}) in the EBM cell fraction (Table 1). In addition, the FSC/SSC profile and cell cycle status of these four individual cell subpopulations have also been analyzed, and the results are shown in Supplementary Figure 2 and summarized in the figure legend.

To confirm enzyme digestion treatment did not affect the result, we analyzed the CBM cell fraction after collagenase treatment and found that enzyme treatment does not increase the number of Sca-1^{-}CD24$^{-/lo}$ or Sca-1^{+}CD24$^{-/lo}$ cells as it was in EBM cell fraction (data not shown). These results indicate that the isolation procedure did not affect the experimental results.

CD24 antigen is a new marker for the sub-fractionation of osteogenic cell population

It has been reported that osteogenic cells are mostly resided in the Sca-1^{-} or Sca-1^{-}D51^{+} cell fraction, whereas MSCs are enriched in the Sca-1^{+}CD51^{+} or Sca-1^{+}PDGFR-α^{+} cell fraction [4-6]. We then analyzed the triple negative stromal cell population using the Sca-1 together with the CD24, CD29/Integrin β1 [17], CD51, CD105 [18], and PDGFR-α MoAbs individually. The contour dot plot analysis showed that a single Sca-1^{+} cell population could be readily identified. Using this Sca-1^{+} cell population as a reference to define the level expression of other markers in Sca-1^{-} cell fraction, a single PDGFR-α^{-} and CD29^{+} cell population, two (CD51^{+}/CD51^{-} and CD105^{+}/CD105$^{-/lo}$) and three (CD24^{hi}, CD24^{med}, CD24$^{-/lo}$) cell sub-populations can be readily recognized within the Sca-1^{-} cell fraction (Figure 1B).

Although the Sca-1^{-}CD51^{+} cell fraction has previously been identified as a cell fraction highly enriched for osteoblast lineage cells, our analysis shows that it is a heterogeneous population and can be sub-fractionated into three cell sub-populations (CD24^{hi}, CD24^{med}, CD24$^{-/lo}$). The percentage of the three cell sub-populations (CD24^{hi}, CD24^{med}, CD24$^{-/lo}$) within Sca-1^{-}CD51^{+} cell fraction is 77.3%, 10.4% and 12.7% respectively (Figure 2A). We next examined the expression of these previously identified cell surface markers, such as CD51, CD90 [19], CD105, and PDGFR-α in these four individual cell fractions (Figure 2B) and found the Sca-1^{-}CD24^{hi} cell fraction contained obviously two sub-populations of CD51 and CD105 cells. Both of the Sca-1^{-}CD24^{med} and Sca-1^{-}CD24$^{-/lo}$ cells displayed a similar profile and most of these cells expressed CD51. However, in contrast, the majority of the Sca-1^{+}CD24$^{-/lo}$ cells expressed CD51, CD90 and PDGFR-α (Figure 2B).

Enrichment of osteogenic cells in the Sca-1^{-}CD24^{med} and Sca-1^{-}CD24$^{-/lo}$ cell populations

During osteogenesis, three sequential steps have been recognized: proliferation, matrix maturation, and minimization. Conceptually, progenitor cells would lose their proliferation ability when these cells progress through the osteogenic differentiation [20]. To examine the cellular properties of these stromal cell subpopulations, 10^{3} FACS-sorted cells were plated into a well of 6-well plate with a stromal cell culture condition. After 14 days of culture, adherent cells derived from individual cell subpopulations were harvested and further expanded at a low cell density culture (~1.5×10^{3} cells per cm^{2}) for another two weeks. To our surprise, after culture expansion, 85.3% and 77.5% ALP^{+} cells were found in the cultures of Sca-1^{-}CD24^{med} and Sca-1^{-}CD24$^{-/lo}$ cell fractions respectively (Figure 3A). These ALP^{+} osteolineage cells were present in FBS-containing medium without any exogenous osteogenic inducers (ABD, including ascorbate-2 phosphate, β-glycerol phosphate disodium and dexamethasone). It may provide a methodology to generate abundant ALP^{+} osteolineage cells in culture for further investigative studies using advanced genomics and proteomics techniques. In contrast, few or none ALP^{+} cells were observed in both cultures of the Sca-1^{-}CD24^{hi} and Sca-1^{+}CD24$^{-/lo}$ cell fractions with standard culture medium (Figure 3A). We next monitored the proliferation ability and differentiation potential of individual cell subpopulations after culture expansion. The Sca-1^{+}CD24$^{-/lo}$ cells can proliferate over 50 days and

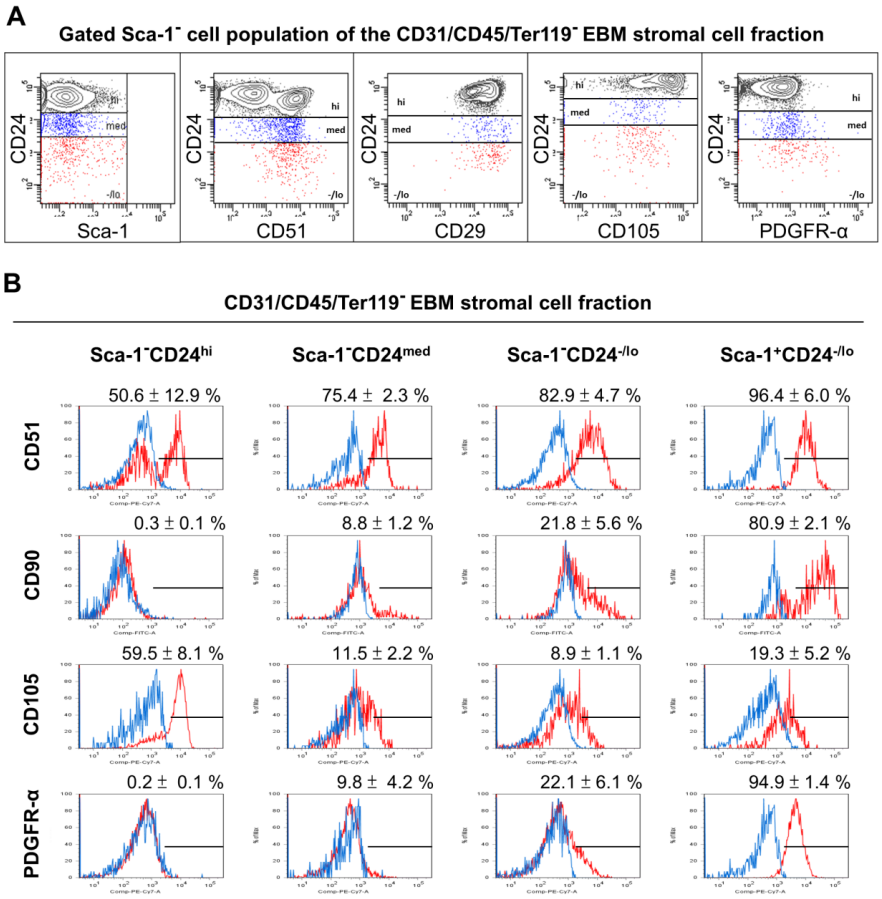


Figure 2: CD24 antigen as a new marker for the sub-fraction of committed osteogenic cells
(A) A contour-dot plot analysis of the gated Sca-1⁻ cell populations of the CD31/CD45/Ter119 triple negative cell fraction showing relative distribution of the $CD24^{hi}$ (displayed as black contour plot), $CD24^{med}$ (as blue dots), and $CD24^{-/lo}$ cells (as red dots) in plots co-stained with CD51, CD29, CD105 and PDGFR-α. The two $CD24^{med}$ and $CD24^{-/lo}$ committed osteogenic cell sub-populations are $CD29^{+}$, $CD51^{+}$, $CD105^{-/lo}$ and PDGFR-α⁻. The $CD24^{hi}$ cell subpopulation contains a single $CD29^{+}$, PDGFR-α⁻ fraction and each two fractions of CD51 ($CD51^{+}/CD51^{-}$) and CD105 ($CD105^{+}/CD105^{-/lo}$). The percentage of three populations ($CD24^{hi}$, $CD24^{med}$ and $CD24^{-/lo}$) within $CD51^{+}$ cells is 77.3 ± 5.8%, 10.4 ± 1.0% and 12.7 ± 4.8% respectively. (B) Representative results of flow cytometric analysis for the expression of MSC markers (CD51, CD90, CD105 and PDGFR-α) in the four cell sub-populations fractionated based on the differential staining pattern with the Sca-1 and CD24 antibodies. Red line represents the histogram profile of cells staining with the respective specific MoAbs, whereas blue line is the isotype control profile. The numerical number (expressed as means ± SD) represented the percent positive cells obtained from 3 independent experiments.

generate > 10^9 cells from the initially seeded 10^3 cells. In contrast, three sub-populations within Sca-1⁻ cell fraction showed a relative lower growth rate. The Sca-1⁻$CD24^{med}$ and Sca-1⁻$CD24^{-/lo}$ cells, but not the Sca-1⁻$CD24^{hi}$ could proliferate at early passage. The Sca-1⁻$CD24^{med}$ and Sca-1⁻$CD24^{-/lo}$ cells showed slight difference in proliferation ability in three biological replicates. However, the results between individual experiments showed a consistent pattern. The Sca-1⁻$CD24^{med}$ cells stopped cell growth at an earlier time point when compared with those the Sca-1⁻$CD24^{-/lo}$ cells. The Sca-1⁻$CD24^{hi}$ cells hardly grew and senescent rapidly during culture expansion (Figure 3B).

Conventional *in vitro* differentiation assay was then performed within 30 days of culture. The culture-expanded cells from Sca-1⁻$CD24^{med}$, Sca-1⁻$CD24^{-/lo}$ and Sca-1⁺$CD24^{-/lo}$ cells were incubated with inducing agents at confidence states (~2.5×10^4 cells per cm^2) for both osteogenic and adipogenic differentiation assays. The culture-expanded cells from these three cell fractions all displayed similar levels of ALP activity when incubated in FBS-containing medium alone (Figure 3C). In addition, the presence of osteogenic inducer cocktail (ABD) did not further increase the intensity of ALP activity in the Sca-1⁻$CD24^{med}$ and Sca-1⁻$CD24^{-/lo}$ cells (Figure 3C). These observations suggest that FBS alone is sufficient for continuing the ALP^{+} cell growth in the culture within these two cell fractions. Compared to the ALP^{-} cells in standard culture condition, the Sca-1⁺$CD24^{-/lo}$ cells could differentiate into ALP^{+} osteolineage cells after 14 days of incubation with FBS containing stromal cell culture medium (Figure 3A and C). These observations suggest that FBS contains sufficient osteogenic inducer(s) for the expression of ALP. The late stage of osteogenic differentiation, calcium deposition, of these three sub-populations requires the presence of inducers (ABD) (Figure 3C).

In adipogenic differentiation assay, only short-term culture expanded Sca-1⁺$CD24^{-/lo}$ cells, but not the Sca-1⁻$CD24^{med}$ or Sca-1⁻$CD24^{-/lo}$ cells, efficiently differentiated into adipocytes after DIXIn (a mixture of dexamethasone, insulin, 3-isobutyl-1-methylxanthine, and indomethacin) induction (Figure 3C). These observations suggest that the MSCs are enriched in the Sca-1⁺$CD24^{-/lo}$ cell population, whereas both Sca-1⁻$CD24^{med}$ and Sca-1⁻$CD24^{-/lo}$ cells are enriched for the committed osteogenic cells.

Although the Sca-1⁻$CD24^{hi}$ cell fraction is the major cell population and high proportion of cycling cells within the CD45/CD31/Ter119⁻

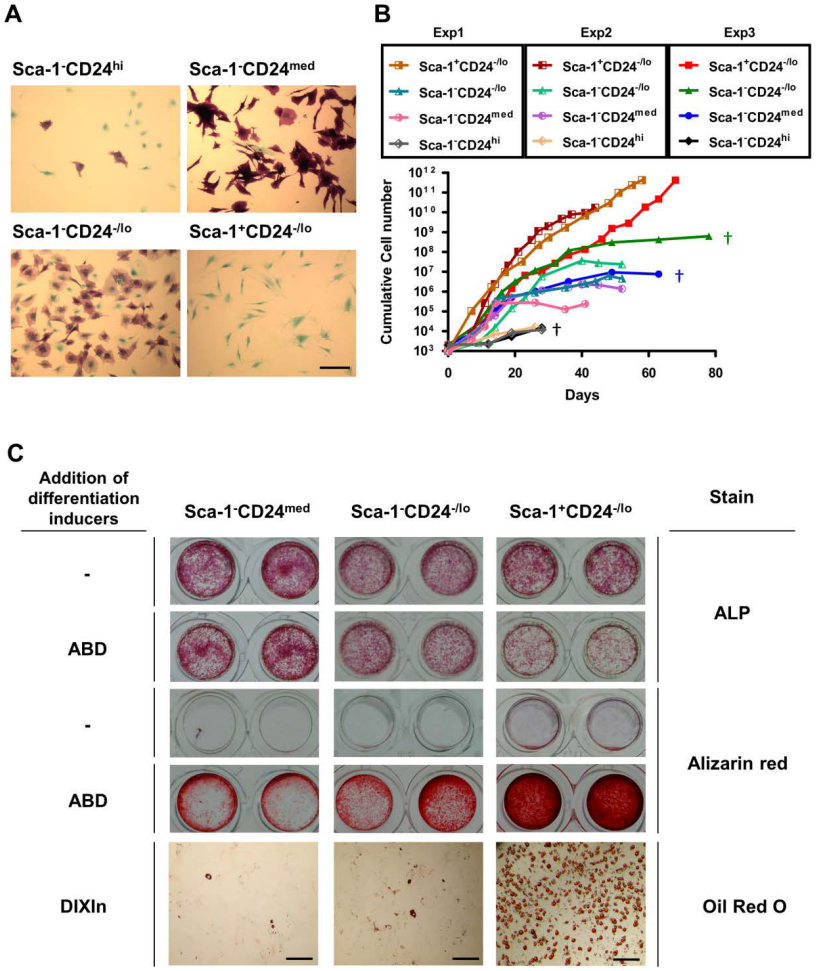


Figure 3: Enrichment of Sca-1^{+}CD24$^{-/lo}$ MSCs, Sca-1^{-} CD24^{med} and Sca-1^{-}CD24$^{-/lo}$ osteogenic cells in culture respectively. (A) Photomicrographs of the cells grown from individual cell fractions taken at 30 days of culture and after staining with ALP (red) and methylene blue (blue). Note the presence of 85.3 ± 4.9% and 77.5 ± 2.4% ALP^{+} cells in the cultures of Sca-1^{-}CD24^{med} and Sca-1^{-}CD24$^{-/lo}$ cells respectively. In contrast, 5.1 ± 1.5% and 0% ALP^{+} cell in the cultures of Sca-1^{-}CD24^{hi} and Sca-1^{+}CD24$^{-/lo}$ cells, respectively (B) Growth curves of representative cell fractions derived from 10^{3} cells plated. The Y axis represents cumulative cell number and the X axis as the number of days in culture. († denotes cell senescence). The results between individual experiments were showed, and individual subpopulations in each experiment were labelled as different color and symbols. (C) *In vitro* differentiation assay performed on the culture-expanded cells from the Sca-1^{-}CD24^{med}, Sca-1^{-}CD24$^{-/lo}$ and Sca-1^{+}CD24$^{-/lo}$ cell populations. Left hand panel lists the type of inducing agents in culture medium, whereas the right hand panel represents the types of stain for detecting osteogenic and adipogenic differentiation after 14 days of induction. Onset of osteogenesis and calcium deposition was indicated by ALP and Alizarin red staining respectively. The adipocytes were indicated by the presence of neutral lipid vacuoles that were stained with Oil red O. In conclusion, the Sca-1^{-}CD24^{med} and Sca-1^{-}CD24$^{-/lo}$ committed to osteogenic cells while Sca-1^{+}CD24$^{-/lo}$ cells were MSCs that can differentiate into both osteogenic and adipogenic cells. Scale bar: 100μm. Data are representative of three independent experiments. The number of mice used for one experiment, n=6.

stromal cell fraction (Figure 1A and Supplementary Figure 2), their plating efficiency was quite low. The low plating efficiency may reflect low frequency of stromal progenitors in this cell fraction, high density cultures were then set up for this *in vitro* differentiation experiments. Adherent stromal-like cells did grow out, but there is no significant increase in cell number over 30 days of culture (Supplementary Figure 3A). However, enough number of these stromal-like cells could be harvested and was subjected to the *in vitro* differentiation assay. The results, shown in Supplementary Figure 3B, indicate that these cultured cells do not have *in vitro* osteogenic or adipogenic differentiation capacity. It remains to be determined what other lineage cell types are present in this cell fraction.

Combined with the results of Figure 2A and supplementary Figure 3, Sca-1^{-}CD24^{hi} cells, which are over 70% of Sca-1^{-}CD51^{+} cells contained non-osteogenic differentiation potential. Only small subsets of the Sca-1^{-}CD51^{+} cells, which are CD24^{med} and CD24$^{-/lo}$ cells committed to osteogenic lineage. It indicated that CD24 is a new and useful marker for the sub-fractionation of the Sca-1^{-}CD51^{+} osteogenic cell population.

Sca-1^{-}CD24^{med} and Sca-1^{-}CD24$^{-/lo}$ cell fractions are at two different developmental stages of osteogenesis

Both of the culture expanded Sca-1^{-}CD24^{med} and Sca-1^{-}CD24$^{-/lo}$ cells are committed osteogenic cells, but their CD24 antigen density were different (Figure 1A). To address whether these two cell populations are at different developmental stage of skeletogenesis, primary CFU-assay as it reflects the ability of a cell to grow in a density-insensitive manner and generate colonies from single cells when plated in culture [3]. This detection method allows the simultaneous identification of both ALP^{-} colony (CFU-F) and ALP^{+} colony (CFU-ALP) with minimal influence of culture conditions.

As shown in the figure 4A, CFU-F has a fibroblast-like morphology, whereas the CFU-ALP shows a relative irregular morphology with round ALP$^+$ cells (Figure 4A). Consistent with the osteogenic cell enrichment, CFU-ALP was significant increased in the cultures of both Sca-1$^-$CD24^{med} and Sca-1$^-$CD24$^{-/lo}$ cells (Figure. 4B). In addition, the number of CFU-F in Sca-1$^-$CD24$^{-/lo}$ cells and Sca-1$^-$CD24^{med} cells was higher than in Sca-1$^-$CD24^{hi} and Sca-1$^+$CD24$^{-/lo}$ cells. It indicates that majority of CFU-F in bone marrow was also enriched by two

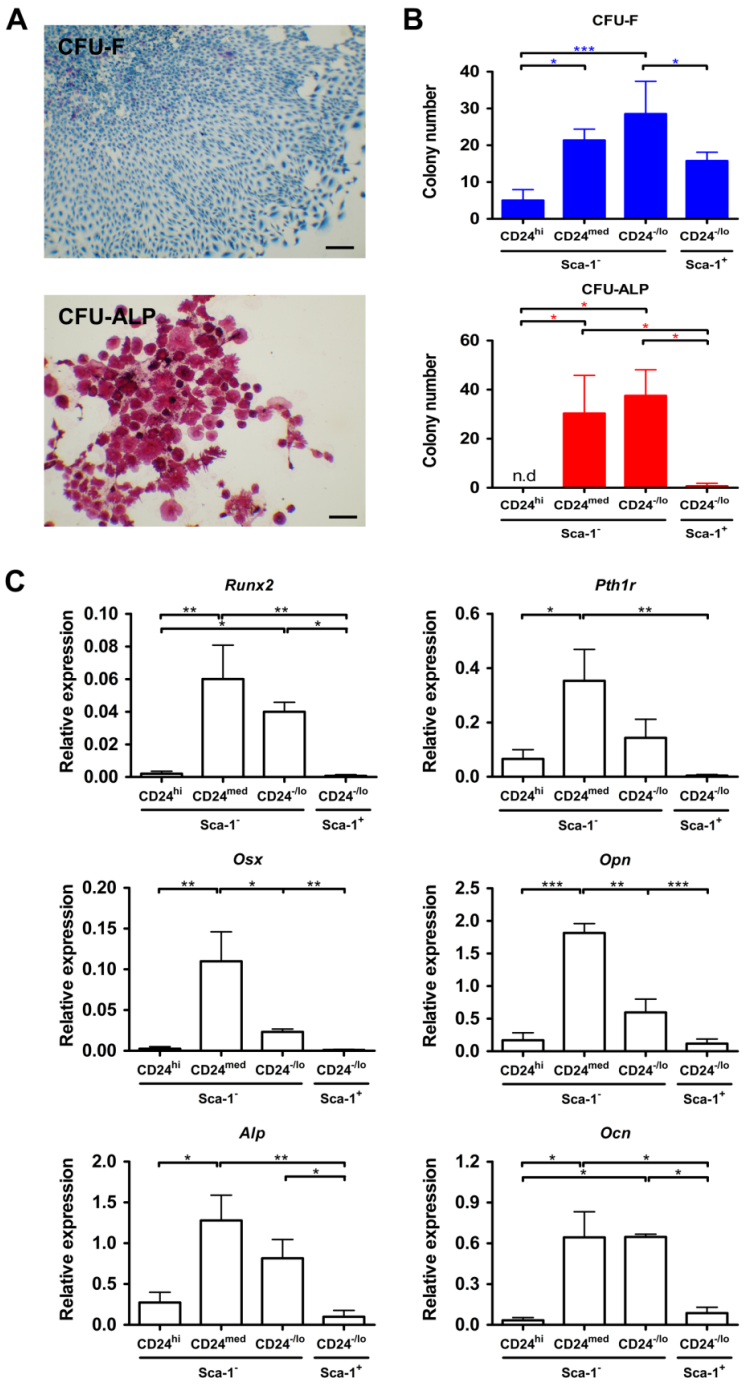


Figure 4: The FACS-isolated Sca-1$^-$CD24^{med} and Sca-1$^-$CD24$^{-/lo}$ cells express osteogenic markers and form ALP-positive colonies in primary cultures. (A) Colony-forming unit assay was performed using FACS-sorted cells isolated from individual sub-populations. After 14 days of incubation, individual cultures were fixed, stained with ALP, and then counterstained with methylene blue. CFU-ALP and CFU-F were identified and scored under the Olympus inverted microscope using a 10x objective. The upper and lower bright field photomicrographs show the appearance of CFU-F (blue) and CFU-ALP (red). Scale bar: 200 μm. (B) A graphic presentation of quantification data obtained from 3 independent experiments of CFU-F (upper panel) and CFU-ALP (lower panel). The number of CFU-ALP or CFU-F was significant higher in cultures plated with Sca-1$^-$CD24^{med} or Sca-1$^-$CD24$^{-/lo}$ cells than other cell fractions. (C) Real time RT-PCR analysis of the osteoblastic markers, including *Runx2, Osx, Alp, Opn, Pth1r*, and *Ocn* in the four freshly FACS-sorted sub-populations were shown. Relative expression was normalized to the mouse RPL32 level. Data represent means ± SD (*P < 0.05, **P < 0.01, ***P < 0.001). Representative data from 3 independent experiments are show.

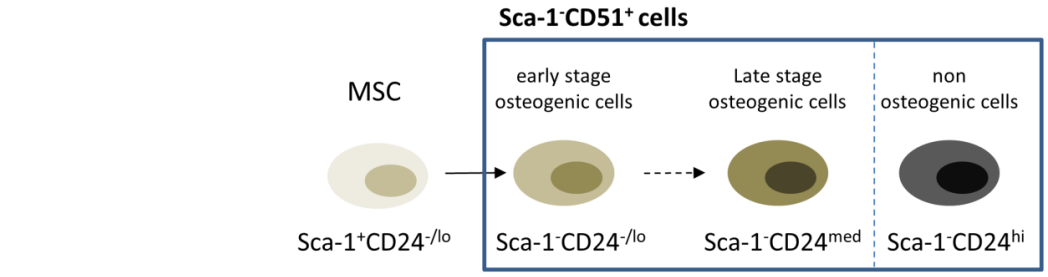
osteogenic populations, Sca-1$^-$CD24^{med} and Sca-1$^-$CD24$^{-/lo}$ (Figure 4B). However, there is no difference in the number or ratio of CFU-ALP or CFU-F between two osteogenic populations.

Osteogenic cells at different stages of cell maturation can be defined by the expression level of various bone intracellular gene markers [21]. Thus, we next performed immune-staining and real-time RT-PCR analysis of osteogenic marker gene expression on freshly isolated individual CD24 subpopulations to investigate their developmental stage. The results showed that ALP$^+$ or Runx2$^+$ cells presented in both Sca-1$^-$CD24^{med} and Sca-1$^-$CD24$^{-/lo}$ cell populations, but the frequency of ALP or Runx2 showed no difference (data not showed). In contrast, ALP$^+$ or Runx2$^+$ cells was virtually absent in the Sca-1$^-$CD24^{hi} and Sca-1$^+$CD24$^{-/lo}$ cell fractions (Supplementary Figure 4).

In osteogenic gene expression analysis of individual fresh isolated cells, the Sca-1$^-$CD24^{med} and Sca-1$^-$CD24$^{-/lo}$ cell populations were constant to express osteogenic genes, including *Runx2, Osx, Alp, Opn, Pthr1,* and *Ocn* (Figure 4C). In contrast, there is almost no CFU-ALP and no osteogenic marker expressions in Sca-1$^-$CD24^{hi} and Sca-1$^+$CD24$^{-/lo}$ sub-populations. These results further support the notion that these two sub-fractioned cells are not committed osteogenic cells. Notable, the Sca-1$^-$CD24^{med} cell population had a significantly higher level of *Osx* and *Opn* gene expression when compared to the Sca-1$^-$CD24$^{-/lo}$ cell fraction. It was reported that *Osx* may act downstream of *Runx2* and regulate osteogenic genes, including *Ocn* and *Opn* [22]. Therefore, the increase of *Osx* and *Opn* expression in Sca-1$^-$CD24^{med} cells may indicate they are more mature osteolineage cells than Sca-1$^-$CD24$^{-/lo}$ cells.

Conclusion

In this report, based on the results of (i) flow cytometric analysis of stromal cell subpopulations with Sca-1 and CD24, (ii) *in vitro* expansion capacity and differentiation assays (iii) primary adherent CFU assay, and (iv) osteogenic marker gene profiling in primary cells, we have identified there are one single MSC population (Sca-1$^+$CD24$^{-/lo}$) and two subsets of committed osteogenic cells (Sca-1$^-$CD24^{med} and Sca-1$^-$CD24$^{-/lo}$) in the mouse endosteal bone (Figure 5). Thus, we have demonstrated that CD24 antigen is a new cell surface marker for the enrichment and identification of committed osteogenic cell sub-populations in the endosteal bone marrow. Only very few of the Sca-1$^-$CD51$^+$ cells, which are Sca-1$^-$CD24^{med} or Sca-1$^-$CD24$^{-/lo}$ committed to osteolineage. Both Sca-1$^-$CD24^{med} or Sca-1$^-$CD24$^{-/lo}$ cells highly enriched CFU-ALP, expressed osteogenic genes, and both of them cannot differentiation into adipocyte. Based on the differential gene expression profiles, and *in vitro* proliferation rate, the Sca-1$^-$CD24^{med} cells are more mature than Sca-1$^-$CD24$^{-/lo}$ in the developmental status. The hypothesis of hierarchical organization of osteogenic lineage were proposed that during osteogenesis, high proliferative Sca-1$^+$CD24$^{-/lo}$ MSCs would lose their proliferation and adipocyte differentiation capacity, and then differentiate into Sca-1$^-$CD24$^{-/lo}$ early stage of osteogenic cells,



Intracellular protein				
Runx2	-	+	+	-
ALP	-	+	+	-
In vitro potential				
Adipocyte	+	-	-	-
Osteoblast	+	+	+	-
Gene signature	-	Osx^{low},Opn^{low},*Runx2* *ALP,Pth1r, Ocn*	Osx^{hi},Opn^{hi},*Runx2* *ALP,Pth1r, Ocn*	-
CFU-ALP	-	+	+	-
Proliferation ability	+++	++	+	±

Figure 5: Summary of osteogenic lineage cell characterization.
In the endosteal stromal cell compartment, Sca-1$^-$CD24$^{-/lo}$ MSCs differentiate into osteogenic cell populations in conceptually. Here, the Sca-1$^-$CD51$^+$ osteogenic cell fraction is a heterogeneous population and contains three different sub-populations: CD24^{hi}, CD24^{med}, and CD24$^{-/lo}$ cells. Only CD24^{med} and CD24$^{-/lo}$ cells committed to osteolineage, they highly enrich CFU-ALP, express osteogenic genes and proteins. In addition, based on the expression level of osteogenic marker genes and *in vitro* proliferation ability, it suggests that the CD24^{med} subpopulation is enriched for the late stage of osteogenic cells, whereas the CD24$^{-/lo}$ subpopulation contains cells at early stage of osteogenesis.

lastly mature into Sca-1^{-}CD24^{med} late stage cells (Figure 5). All these approaches used in the assessment of the FACS-sorted cell fractions give a greater detailed understanding in the hierarchical organization of sub-populations of osteogenic cells during skeletogenesis occurring *in vivo*.

Acknowledgments

The authors thank the excellent technical assistance, service, and advice provided by the flow cytometry central facility of the Division of Medical Biology, Genomics Research Center; the Taiwan Mouse Clinic, which is funded by the National Research Program for Biopharmaceuticals at the National Science Council of Taiwan for technical support in histopathology experiments. This work was supported by intramural grants from Academia Sinica (#5202402020-0) and the National Science Council (NSC-97-2120-M-001-005, NSC-95-2745-B-001-002-MY2, and NSC-102-2321-B001-013).

References

1. Friedenstein AJ, Piatetzky S, II, Petrakova KV (1966) Osteogenesis in transplants of bone marrow cells. J Embryol Exp Morphol 16: 381-390.
2. Tavassoli M, Crosby WH (1968) Transplantation of marrow to extramedullary sites. Science 161: 54-56.
3. Owen ME, Cave J, Joyner CJ (1987) Clonal analysis in vitro of osteogenic differentiation of marrow CFU-F. J Cell Sci 87 (Pt 5): 731-738.
4. Semerad CL, Christopher MJ, Liu F, Short B, Simmons PJ, et al. (2005) G-CSF potently inhibits osteoblast activity and CXCL12 mRNA expression in the bone marrow. Blood 106: 3020-3027.
5. Winkler IG, Sims NA, Pettit AR, Barbier V, Nowlan B, et al. (2010) Bone marrow macrophages maintain hematopoietic stem cell (HSC) niches and their depletion mobilizes HSCs. Blood 116: 4815-4828.
6. Morikawa S, Mabuchi Y, Kubota Y, Nagai Y, Niibe K, et al. (2009) Prospective identification, isolation, and systemic transplantation of multipotent mesenchymal stem cells in murine bone marrow. J Exp Med 206: 2483-2496.
7. Nakamura Y, Arai F, Iwasaki H, Hosokawa K, Kobayashi I, et al. (2010) Isolation and characterization of endosteal niche cell populations that regulate hematopoietic stem cells. Blood 116: 1422-1432.
8. Lundberg P, Allison SJ, Lee NJ, Baldock PA, Brouard N, et al. (2007) Greater bone formation of Y2 knockout mice is associated with increased osteoprogenitor numbers and altered Y1 receptor expression. J Biol Chem 282: 19082-19091.
9. van de Rijn M, Heimfeld S, Spangrude GJ, Weissman IL (1989) Mouse hematopoietic stem-cell antigen Sca-1 is a member of the Ly-6 antigen family. Proceedings of the National Academy of Sciences of the United States of America 86: 4634-4638.
10. Spangrude GJ, Heimfeld S, Weissman IL (1988) Purification and characterization of mouse hematopoietic stem cells. Science 241: 58-62.
11. Okada S, Nakauchi H, Nagayoshi K, Nishikawa S, Miura Y, et al. (1992) In vivo and in vitro stem cell function of c-kit- and Sca-1-positive murine hematopoietic cells. Blood 80: 3044-3050.
12. Li CL, Johnson GR (1992) Rhodamine123 reveals heterogeneity within murine Lin-, Sca-1+ hemopoietic stem cells. J Exp Med 175: 1443-1447.
13. Rietze RL, Valcanis H, Brooker GF, Thomas T, Voss AK, et al. (2001) Purification of a pluripotent neural stem cell from the adult mouse brain. Nature 412: 736-739.
14. Shackleton M, Vaillant F, Simpson KJ, Stingl J, Smyth GK, et al. (2006) Generation of a functional mammary gland from a single stem cell. Nature 439: 84-88.
15. Rodeheffer MS, Birsoy K, Friedman JM (2008) Identification of white adipocyte progenitor cells in vivo. Cell 135: 240-249.
16. Kamalia N, McCulloch CA, Tenenbaum HC, Limeback H (1992) Direct flow cytometric quantification of alkaline phosphatase activity in rat bone marrow stromal cells. J Histochem Cytochem 40: 1059-1065.
17. Zimmerman D, Jin F, Leboy P, Hardy S, Damsky C (2000) Impaired bone formation in transgenic mice resulting from altered integrin function in osteoblasts. Dev Biol 220: 2-15.
18. Park D, Spencer JA, Koh BI, Kobayashi T, Fujisaki J, et al. (2012) Endogenous bone marrow MSCs are dynamic, fate-restricted participants in bone maintenance and regeneration. Cell Stem Cell 10: 259-272.
19. Chen XD, Qian HY, Neff L, Satomura K, Horowitz MC (1999) Thy-1 antigen expression by cells in the osteoblast lineage. J Bone Miner Res 14: 362-375.
20. Lian JB, Stein GS (1992) Concepts of osteoblast growth and differentiation: basis for modulation of bone cell development and tissue formation. Crit Rev Oral Biol Med 3: 269-305.
21. Hughes FJ, Turner W, Belibasakis G, Martuscelli G (2006) Effects of growth factors and cytokines on osteoblast differentiation. Periodontol 2000 41: 48-7
22. Nakashima K, Zhou X, Kunkel G, Zhang Z, Deng JM, et al. (2002) The novel zinc finger-containing transcription factor osterix is required for osteoblast differentiation and bone formation. Cell 108: 17-29.