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Mesenchymal Progenitors Residing Close to the Bone Surface Are Functionally Distinct from Those in the Central Bone Marrow

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Abstract

Long bone is an anatomically complicated tissue with trabecular-rich metaphyses at two ends and cortical-rich diaphysis at the center. The traditional flushing method only isolates mesenchymal progenitor cells from the central region of long bones and these cells are distant from the bone surface. We propose that mesenchymal progenitors residing in endosteal bone marrow that is close to the sites of bone formation, such as trabecular bone and endosteum, behave differently from those in the central bone marrow. In this report, we separately isolated endosteal bone marrow using a unique enzymatic digestion approach and demonstrated that it contained a much higher frequency of mesenchymal progenitors than the central bone marrow. Endosteal mesenchymal progenitors express traditional mesenchymal stem cell markers and are capable of multi-lineage differentiation. However, we found that mesenchymal progenitors isolated from different anatomical regions of the marrow did exhibit important functional differences. Compared to their central marrow counterparts, endosteal mesenchymal progenitors have superior proliferative ability with reduced expression of cell cycle inhibitors. They showed greater immunosuppressive activity in culture and in a mouse model of inflammatory bowel disease. Aging is a major contributing factor for trabecular bone loss. We found that old mice have a dramatically decreased number of endosteal mesenchymal progenitors compared to young mice. Parathyroid hormone

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(PTH) treatment potently stimulates bone formation. A single PTH injection greatly increased the number of endosteal mesenchymal progenitors, particularly those located at the metaphyseal bone, but had no effect on their central counterparts. In summary, endosteal mesenchymal progenitors are more metabolically active and relevant to physiological bone formation than central mesenchymal progenitors. Hence, they represent a biologically important target for future mesenchymal stem cell studies.

Keywords

mesenchymal progenitors; endosteal bone marrow; immunosuppression; aging; parathyroid hormone

1 Introduction

Mesenchymal stem cells (MSCs) are multipotent stem cells capable of differentiating into multiple cell lineages including osteoblasts, chondrocytes, adipocytes, smooth muscle cells, myocytes, endothelial cells, and neurons. Together with their homing ability to sites of injury and immunoregulatory effects, they are a promising therapeutic tool for tissue engineering and regeneration [1]. MSCs reside in bone marrow and many other tissues and organs, such as placenta, peripheral blood, cord blood, cord Wharton's jelly, adipose tissue, amniotic fluid, periosteum, synovial membrane, synovial fluid, articular cartilage, and muscle. Due to a lack of specific cell surface markers to unequivocally identify these cells, currently there is no commonly accepted method to isolate true MSCs. The standard approach to study MSCs is to isolate and culture bone marrow cells from rodent long bones for plastic-adherent and clonogenic fibroblastoid mesenchymal progenitors, which is a heterogeneous population containing not only true MSCs but also their proliferative progeny. Bone marrow from the central part of the rodent diaphyseal bone can be easily separated from the surrounding cortical shell by flushing and this source of bone marrow is commonly used to make mesenchymal progenitor cultures. On the other hand, bone marrow in the metaphyseal region is evenly distributed within honeycombed or spongy-shaped trabecular bone and therefore, is not easily isolated.

The bone marrow is also the home of another important stem cell, the hematopoietic stem cell (HSC). Interestingly, recent studies demonstrate that HSCs, particularly the long-term HSCs, residing in the endosteal region have a superior proliferative capacity and homing efficiency compared to their counterparts in the diaphyseal bone [2, 3], indicating that the microanatomical location of stem cells might have a great influence on their behavior. Previous publications [4–6] described the endosteal cells as those cells within a 12 cell distance from the bone surface and proposed that all bone marrow cells within mouse trabecular bone are in the endosteal region [4]. These data imply an importance to isolate the mesenchymal progenitors within the endosteal region and compare their stem cell properties and biological functions with their counterparts derived from the central region of diaphyseal bone.

Aging is one of the most important risk factors for osteoporosis. A number of studies using the conventional colony forming unit-fibroblast (CFU-F) assay to access mesenchymal progenitor numbers and cultured mesenchymal progenitors for functional studies have indicated that aging may decrease the number and activity of mesenchymal progenitors in the central bone marrow of rodents [7–11] and humans [12–16], suggesting that mesenchymal progenitors play an important role in age-related osteoporosis. However, there are also reports demonstrating no effect or a marginal effect of aging on mesenchymal progenitor numbers or differentiation potential in rodents [17, 18] and humans [19, 20].

Parathyroid hormone (PTH) is the only approved anabolic therapy for the treatment of postmenopausal women and men with osteoporosis who are at a high risk of fractures. Intermittent injection of PTH1-34 (teriparatide) induces an anabolic effect on bone, increasing trabecular bone volume, connectivity, plate-like microarchitecture [21, 22], periosteal bone mass [23], cortical cross-sectional area [24], and reducing fractures [25]. PTH injection greatly stimulates bone formation and increases osteoblast numbers, which involves activating bone lining cells, inhibiting osteoblast apoptosis, and enhancing osteoblast maturation through suppressing the expression of sclerostin from osteocytes [26, 27]. Mendez-Ferrer et al. recently reported that a 5-week injection of PTH doubles the number of nestin⁺ MSCs in bone marrow [6]. However, CFU-F assays yield conflicting results, with an increase [28–30], decrease [31], or no change [32, 33] in the number of mesenchymal progenitors after PTH injections. All these experiments were performed with a flushed bone marrow population and therefore, the effects of aging and PTH on the mesenchymal progenitors within the endosteal region of bone has not yet been investigated.

Several enzymatic digestion approaches, together with chopping and crushing bones into small pieces, have been reported to obtain mesenchymal progenitors either from rodent whole bone [34, 35], pre-flushed long bone [36, 37], or cortical bone [38, 39]. While several of these reports [34–36] showed a dramatic increase in the number of CFU-F colonies from the cells isolated by collagenase digestion methods, the anatomical location and biological functions of the mesenchymal progenitors isolated by these approaches has not been analyzed. In this report, we used enzymatic digestion following bone marrow flushing to separately isolate mesenchymal progenitors from the endosteal and central regions of bones. We show that, while they are phenotypically identical to the central counterparts in terms of stem cell surface marker expression and multi-differentiation ability, endosteal mesenchymal progenitors exist at a higher frequency and display important functional differences, such as an elevated proliferative rate and greater immunomodulatory ability. Most interestingly, we demonstrate that endosteal mesenchymal progenitors, but not central mesenchymal progenitors, are highly responsive to aging and PTH treatment, suggesting that they are a major target of the anabolic PTH therapy.

2 Material and Methods

2.1 Isolation and culture of rodent endosteal and central mesenchymal progenitors

All animal experiments in this study were performed under the institutionally approved protocols for the use of animal research either at the University of Pennsylvania or at the University of South California.

Sprague-Dawley rats and C57Bl/6 mice (Charles River) were euthanized by CO₂ inhalation and the bilateral femora and tibiae were dissected free of surrounding tissues under sterile conditions and washed in α MEM. Both ends of the long bones were removed at growth plate sites and the bone marrow was flushed out of the bone with α MEM using a 25-gauge needle (Fig. 1A step 1). Flushed cells (the central bone marrow) were passed through a 70-micron cell strainer and then either used directly for flow cytometry, or plated at 3×10^6 /T25 flask for CFU-F assays and $30\text{--}50 \times 10^6$ /100 mm dish for expanding. To obtain endosteal mesenchymal progenitors, the outer surface of flushed long bones was scraped several times and then digested with a protease solution (2 mg/ml collagenase A and 2.5 mg/ml trypsin in PBS) for 20 min to remove the periosteum and periosteal progenitors. Next, bones were longitudinally cut into two halves (step 2), gently washed to remove loosely attached bone marrow (step 3), and then digested in the protease solution for 1 h (step 4). Cells within the supernatant (endosteal bone marrow) were collected, washed one time with culture media, passed through a cell strainer, and then either used directly for flow cytometry, or plated at 1×10^6 /T25 flask for CFU-F assays and $3\text{--}5 \times 10^6$ /100 mm dish for expanding. For rat

mesenchymal progenitors, the growth media was α MEM with 15% fetal bovine serum (FBS), 100 IU/ml penicillin and 100 μ g/ml streptomycin. For mouse mesenchymal progenitors, the growth media was α MEM with 20% FBS, 55 μ M β -mercaptoethanol, 2 mM glutamine, 100 IU/ml penicillin, and 100 μ g/ml streptomycin. After reaching 80–90% confluence, adherent mesenchymal progenitors were detached from flasks by trypsin and EDTA and subcultured at a dilution of 1:3 to 1:5. Only passages less than five were used in the experiments.

For PTH treatment, 1-month-old Sprague Dawley rats or C57Bl/6 mice were injected subcutaneously with various PTH peptides [1-34 (human), 1-31 (human), and 3-34 (bovine), Bachem] at 80 μ g/kg or vehicle (saline). Twenty-four h later, central and endosteal bone marrow cells were isolated as described above and plated for CFU-F assays. In addition, rats were injected with PTH(1-34) 80 μ g/kg or vehicle daily for 12 days. Both central and endosteal bone marrow cells were isolated 1 day after the last injection for CFU-F assay.

2.2 CFU-F Assays

Endosteal and central bone marrow cells in growth medium were allowed to form colonies for 5 and 7 days, respectively. Cells were fixed and stained for alkaline phosphatase (ALP) activity using a leukocyte ALP kit (Sigma-Aldrich). The number and diameter of ALP-positive colonies were counted and measured under a microscope. The flasks were then stained with 3% crystal violet in methanol and total CFU-F numbers and diameters were quantified. Only the colonies composed of more than 50 cells were counted.

2.3 Cell sorting

To sort for GFP-positive and negative cells, endosteal bone marrow cells were isolated from 1-month-old transgenic mice expressing GFP under the control of the 2.3-kb fragment rat α 1(I) collagen gene promoter [40] as described above. Cells were washed, re-suspended in PBS containing 0.5% BSA, and sorted into GFP⁺ and GFP⁻ populations using a FACSDiva Cell Sorter (BD Biosciences). GFP⁻ cells were seeded at 1×10^6 /T25 flask for CFU-F assay. GFP⁺ cells were either seeded alone at about 2×10^4 /well in a 24-well plate or co-cultured at about 2×10^4 /T25 flask together with 1×10^6 central bone marrow cells from wild-type mice. The co-cultured cells were switched to osteogenic medium (α MEM with 10% FBS, 10 nM dexamethasone, 10 mM β -glycerophosphate and 50 μ g/ml ascorbic acid) at day 7 and constantly monitored for GFP signal under a fluorescent microscope (Eclipse T62000-U, Nikon) until day 21.

2.4 Identification and characterization of mesenchymal progenitors by flow cytometry

All flow cytometric analyses in this study were performed with either FACS Calibur or LSRII flow cytometers (BD Biosciences). To characterize cell surface marker expression, rat mesenchymal progenitors were suspended in PBS containing 0.5% BSA, blocked with anti-CD32 (BD Biosciences) antibody for 10 min at 4°C, and then incubated with anti-CD90-APC (LSBio), anti-CD49e-PE (Biolegend), anti-CD45-FITC (Abcam), or anti-CD34 (Santa Cruz) antibodies for 30 min at 4°C. To detect CD34, cells were additionally stained with goat anti-mouse IgG2A-APC (Southern Biotech) antibody for 30 min at 4°C. To detect intracellular nestin expression, rat mesenchymal progenitors were fixed and permeabilized using Invitrogen's intracellular staining kit. Cells were incubated with anti-nestin-PE (R&D Systems) or normal mouse IgG2a-PE isotype control (Santa Cruz) for 30 min at 4°C. For mouse MSCs, the cell surface marker expression of CD45, CD29, Sca-1, and CD105 was identified using the Mouse Multipotent Mesenchymal Stromal Cell 4-Color Flow Kit (R&D Systems) according to the manufacturer's instruction. To detect mouse CD166, CD34, CD44, CD73, and CD71, mouse mesenchymal progenitors were suspended in PBS containing 0.5% BSA, blocked with FcR blocking reagent (Miltenyi Biotec) for 10 min at

4°C, and then incubated with anti-CD166-PE (Ebioscience), anti-CD34-PE (BD Pharmingen), anti-CD44-PE (BD Pharmingen), anti-CD73-PE (eBioscience), or anti-CD71-PE (BD Pharmingen) for 30 min at 4°C. Unstained cells or staining with corresponding isotype controls were used as the negative control.

For three-color detection of mouse mesenchymal progenitors in the endosteal and central bone marrow, bone marrow cells were suspended in red blood cell lysing buffer (Sigma-Aldrich), blocked with FcR blocking solution for 10 minutes at 4°C, and then stained with anti-CD45-PerCP, anti-Sca-1-APC, and anti-CD29-PE (R&D Systems) antibodies for 30 minutes at 4°C. Staining with isotype controls (R&D Systems) was used as the negative control.

2.5 Multi-lineage differentiation assays

Freshly isolated central and endosteal mesenchymal progenitors were seeded at 3 and $0.4 \times 10^6/35$ mm dish, respectively, and reached confluency at almost the same time after 10–14 days. The cultures were then incubated with either osteogenic medium or adipogenic medium (α MEM with 10% FBS, 0.5 mM isobuthylmethyl-xanthine, 10 mM indomethacin, 1 μ M dexamethasone and 10 μ g/ml insulin). A micromass culture system was used for chondrocyte differentiation [41, 42]. A 20 μ l droplet of 4×10^5 viable cells was seeded in the center of a well in a 24-well plate. Two h later, 0.5 ml chondrogenic medium (DMEM with 10% FBS, 0.1 μ M dexamethasone, 50 μ g/ml L-ascorbic acid, 40 μ g/ml L-proline, 100 μ g/ml sodium pyruvate, 1xITS+, and 10 ng/ml TGF β 3) was added. The control micromass pellets were cultured in 10% DMEM with 1xITS+ only.

2.6 RNA isolation and qRT-PCR analysis

Total RNA was isolated from cells using Tri Reagent (Sigma-Aldrich). A Taqman Reverse Transcription kit (Applied Biosystems) was used to reverse transcribe mRNA into cDNA. Following this, qRT-PCR was performed using a Power SYBR Green PCR Master Mix kit (Applied Biosystems). The primer sequences for the genes used in this study are listed in the supplementary table.

2.7 Cell proliferation assays

For bromodeoxyuridine (BrdU) incorporation assays, cells seeded in 96-well plates were incubated with a BrdU labeling solution for 4 h before being assayed with a Cell Proliferation BrdU-colorimetric ELISA kit (Roche). For MTT assays, cells seeded in 96-well plates were incubated with 0.1 mg/ml MTT (Sigma-Aldrich) for 4 h before addition of DMSO and measurement of absorbance at 570 nm. To calculate the cell doubling time, 25,000 endosteal and central mesenchymal progenitors were plated in 6-well plates and the numbers of cells were counted using a hemocytometer at different times of culture. Doubling time was calculated as: $\text{time interval} / [3.3 * \log(\text{number of cells at end of time interval} / \text{number of cells at beginning of time interval})]$. For cell cycle analysis, cells were fixed in 70% ethanol overnight at 4°C and then stained with a solution of 0.02 mg/ml propidium iodide, 0.2 mg/ml DNase-free RNase A, and 0.1% triton X-100 (Sigma-Aldrich) in PBS for 15 minutes at 37°C. Samples were then analyzed by a FACSCalibur to determine the percentage of cells in G0/G1, G2/M, and S phase.

2.8 T cell apoptosis assay with mesenchymal progenitors

T cells from C57BL/6J (Jackson Laboratory) mouse spleen were stimulated with anti-CD3e (3 mg/ml) and anti-CD28 antibodies (2 mg/ml) for 2 days and loaded into 24-well culture plates that had been pre-seeded with endosteal or central bone marrow mesenchymal progenitors for overnight. After 3 days, floating cells were collected with soft pipetting and

apoptotic T cells were detected by staining with anti-CD4-APC antibody, followed by Annexin-V-PE Apoptosis Detection Kit I (BD Pharmingen) staining and flow cytometry analysis.

2.9 Allogenic bone marrow MSC transplantation into acute colitis mice

Acute colitis was induced by administering 3% (w/v) dextran sulfate sodium (DSS, molecular mass 36,000 – 50,000 Da; MP Biochemicals) into female C57BL/6J mice through drinking water, which was fed *ad libitum* for 10 days. Endosteal or central mesenchymal progenitors were infused (1×10^6 cells/animal) into disease model mice (n=4 each) intravenously at day 3 after feeding DSS water. In the disease control group, mice received PBS only (n=4). Non DSS water-treated C57BL6 (n=3) mice were used as negative control. Colitis severity (disease activity index) was scored by evaluating the clinical disease activity through daily monitoring of weight loss, stool consistency/diarrhea, and presence of fecal bleeding as previously described [43, 44]. All mice were euthanized at day 10 after feeding DSS water. Colon samples were collected for histopathological analyses and induced colitis was evaluated as previously described [43, 44]. Peripheral blood was used for flow cytometric analyses of Treg and Th17. For Foxp3 intracellular staining to identify regulatory T cells (Tregs), T cells were stained with anti-CD4-PerCP, CD8a-FITC, and CD25-APC antibodies for 30 min on ice, followed by anti-Foxp3-PE antibody using Foxp3 staining buffer (eBioscience). For IL17 staining to identify T helper 17 cells (Th17), T cells were stained with anti-CD4-PerCP antibody and then stained with anti-IL17-PE and anti-IFN γ -APC antibodies using Foxp3 staining buffer.

2.10 Statistical analysis

All results are expressed as means $\pm$ sem of triplicate measurements with all experiments being repeated independently at least three times unless otherwise stated. Unpaired Student's t test was used to evaluate the statistical difference between control and treated groups. In cases of multiple groups, differences were first analyzed with one-way analysis of variance (ANOVA) with Bonferroni post test. Values of $p < 0.05$ were considered significant.

3 Results

3.1 Bone marrow cells from the endosteal region contain higher frequency of mesenchymal progenitors than those from the central region of bone

The standard method to isolate bone marrow cells from rodent long bones by flushing only released the bone marrow from the central part of the diaphyseal bone. After flushing, bone marrow within the metaphyseal bone remained entrapped within the trabeculae (Fig. 1B, left panel) and the bone marrow close to the endosteum remained within the bone (Fig. 1C, left panel). To isolate these endosteal cells, we established an enzymatic digestion approach to process the flushed long bone as described in “Material and Methods”. As shown in the right panel of Fig. 1B, we observed a dramatic decrease in the bone marrow cells remaining within the trabeculae of the metaphyseal bone after digestion. At the cortical site, a pre-digestion of uncut bone depleted the periosteal progenitors and the final digestion released the bone marrow cells close to the endosteum (Fig. 1C). Note that digestion removed bone lining cells and osteoblasts from the trabecular bone surface but kept osteocytes within the trabecular bone (Fig. 1D). This is confirmed by the increased expression of osteoblast markers, such as osterix, osteopontin, bone sialoprotein and osteocalcin in the freshly isolated endosteal bone marrow cells compared to the central bone marrow cells (Supplemental Fig. 1).

When cultured in growth medium at a low density, endosteal bone marrow cells generated 3.0-fold more CFU-F colonies per million cells compared to central bone marrow cells (Fig.

2A). From 2 femurs and 2 tibiae of one rat, we typically obtained 280 and 14 million cells from central and endosteal bone marrow cells, respectively, which contained a total of about 11,000 and 2,000 mesenchymal progenitors, respectively. Note that this is a great underestimation of the number of endosteal mesenchymal progenitors because only a portion of endosteal bone marrow cells is released by digestion. A similar result was also obtained with mouse long bones (Supplemental Fig. 2A). Since the endosteal population contains cells released from the endosteal region of the cortical bone and the trabecular area, we further cut the halved bones (Fig. 1A, bone between steps 3 and 4) into trabecular-rich metaphyseal and cortical-rich diaphyseal fragments, digested them separately, and seeded these cells for CFU-F assays. As shown in Fig. 2A, both populations contained a higher frequency of mesenchymal progenitors compared to the central bone marrow but, the majority of endosteal mesenchymal progenitors was located within the trabecular bone region.

We noted that endosteal bone marrow cells formed larger colonies compared to central cells (Fig. 2B and C). To obtain CFU-F colonies large enough to count but still maintain them at a single colony level, we stained the CFU-F colonies from the endosteal bone marrow cells after 5 days of culture, while it took at least 7 days to count the CFU-F numbers from central bone marrow cells. Colonies stained positive for ALP are considered to have osteogenic potential. We observed no difference in the percentage of CFU-F ALP⁺ cells between central and endosteal cells (Figure 2D), indicating that both populations contain mesenchymal progenitors with a similar differentiation status. Both endosteal and central mesenchymal progenitors exhibited a fibroblastic morphology in culture (Fig. 2E). Similar results were also confirmed with mouse mesenchymal progenitors (Supplemental Fig. 2B and C).

Since bone histology showed that osteoblasts and bone lining cells were removed from the bone surface during the digestion process, it is possible that those cells could have de-differentiated and then proliferated in culture to form CFU-F colonies. However, we think it is highly unlikely because it usually requires 1–2 weeks for de-differentiation to occur. To exclude the possibility that osteoblasts were the source of the endosteal CFU-F colonies, we harvested endosteal bone marrow cells from mice expressing GFP in mature osteoblasts/osteocytes under the control of a 2.3-kb fragment of the rat $\alpha 1(I)$ collagen gene promoter (2.3kb col-GFP, [40]). A previous histology analysis demonstrated that in these mice, the majority of cells on the trabecular bone surface are GFP positive [37]. Endosteal bone marrow cells derived from these mice contained both GFP-positive and negative populations (Fig. 2F). Upon monitoring adherent cells during CFU-F colony formation using a fluorescence microscope, we found a few GFP⁺ cells but they did not form fibroblastic colonies (Supplemental Fig. 3A). We confirmed this by sorting for GFP-positive and –negative endosteal cells and plating them separately. Only the negative cells were capable of forming typical fibroblastic CFU-F colonies in culture. We observed that some positive cells attached to the plate but never formed colonies (Fig. 2G, H). To rule out the possibility that supporting cells were required for the GFP⁺ cells to form colonies, we co-cultured sorted GFP⁺ cells with bone marrow cells derived from wild-type mice at normal seeding density and found that no CFU-F colonies formed were GFP⁺ (Supplemental Fig. 3B). Since GFP should be expressed at a high level in mature osteoblasts, we induced osteogenic differentiation in this co-culture but none of cells close to bone nodules were GFP⁺ (Supplemental Fig. 3C). Overall these data confirms that mature osteoblasts are not the source of the proliferative mesenchymal progenitors observed in the endosteal bone marrow cells.

3.2 Characterization of endosteal mesenchymal progenitors

Next, we used flow cytometry to detect specific cell surface markers on endosteal mesenchymal progenitors. Similar to their central counterparts, plate-adherent cells derived from endosteal bone marrow were 99.0% CD90⁺, 90.1% CD49e⁺, 84.4% Nestin⁺, 88.6% CD45⁻, and 99.8 % CD34⁻ (Fig. 3A), suggesting that those cells are indeed mostly composed of mesenchymal progenitors. Mouse endosteal and central mesenchymal progenitors also share similar cell surface marker expression pattern (Supplemental Fig. 4A).

In addition to their plastic adherent property, mesenchymal progenitors are also identified by their ability to differentiate into multiple mesenchymal lineage cells including osteoblasts, adipocytes, and chondrocytes. Similar to central cells, rat endosteal mesenchymal progenitors readily differentiated into osteoblasts and adipocytes when cultured in their respective differentiation medium and identified by positive von Kossa and oil red O staining, respectively, at the end of culture (Fig. 3B and D). We obtained similar results with mouse endosteal MSCs (Supplemental Fig. 4B and C). qRT-PCR analyses demonstrated strong increases in the expression of osteoblast marker genes (osteocalcin, bone sialoprotein, runx2, and osterix, Fig. 3C) in endosteal mesenchymal progenitors cultured in osteogenic medium and in the expression of adipocyte marker genes (lipoprotein lipase, PPAR γ , and CEBP α , Fig. 3E) in those cells cultured in adipogenic medium. Three weeks of culture in a chondrocyte differentiation micromass assay led to positive staining for alcian blue (Fig. 3F) and up-regulation of aggrecan, type II collagen, and Sox9 mRNA (Fig. 3G). Moreover, the increased levels of the marker gene expression were comparable to that observed in central mesenchymal progenitors.

3.3 Endosteal mesenchymal progenitors have superior proliferative potential

The increase in CFU-F colony size provided the first line of evidence that endosteal mesenchymal progenitors are more proliferative than central mesenchymal progenitors. When these two populations were cultured in vitro, we noticed that endosteal mesenchymal progenitors reached confluence in a much shorter time period than central cells. BrdU incorporation assays showed that endosteal mesenchymal progenitors incorporated 3.0-fold more BrdU than central cells (Fig. 4A). MTT assays revealed that there were significant increases in viable cell numbers in endosteal mesenchymal progenitor culture compared to central cells after 5 days of culture even when the initial seeding densities were the same (Fig. 4B). The population doubling times were calculated to be 15.6 $\pm$ 2.6 and 45.3 $\pm$ 8.7 h for endosteal and central mesenchymal progenitors, respectively. A similar accelerated growth rate was also seen in mouse endosteal mesenchymal progenitors (data not shown). Furthermore, cell cycle analysis showed increased percentages of cells in G2/M and S phase and a decreased percentage of cells in G0/G1 phase in endosteal mesenchymal progenitors (Fig. 4C). To understand the underlying mechanism, qRT-PCR analyses were performed to determine the mRNA levels of cell cycle inhibitors. Interestingly, we observed significant decreases in the expression of major regulators of G1 to S phase, p15 (INK4b), p16 (INK4a), and p21 (Cip1/WAF1), but not p27 (Kip1), in endosteal mesenchymal progenitors compared to central mesenchymal progenitors (Fig. 4D). It is worthwhile to note that rat endosteal mesenchymal progenitors maintain this increased proliferative ability even after 10 passages. This growth difference is more remarkable in mouse cells since, in most experiments, mouse central mesenchymal progenitors ceased proliferation after 5–10 passages but, endosteal mesenchymal progenitors could be easily passaged beyond 10 passages.

3.4 Endosteal mesenchymal progenitors display greater immunosuppressive ability

Bone marrow mesenchymal progenitors have an important immunomodulatory function and systemic infusion of these cells has been proposed as a treatment for a variety of immune disorders [45–48]. To compare the immunosuppressive capacity of endosteal mesenchymal progenitors with that of central cells, we co-cultured T cells pre-stimulated with anti-CD3e and anti-CD28 antibodies with mesenchymal progenitors. Apoptosis assays revealed that both central and endosteal mesenchymal progenitors induced T cell death but endosteal mesenchymal progenitors had a significantly greater effect (Fig. 5A). To test the immunosuppressive ability of the mesenchymal progenitors in vivo, we took advantage of a mouse model of inflammatory bowel disease that uses the administration of DSS to mice to induce an inflammatory response in the colon (acute colitis, Fig. 5B). DSS-treated mice develop colon tissue damage characterized by a decreased epithelial layer and infiltration of inflammatory cells into the colon. When mice administered with DSS were transplanted with mesenchymal progenitors, the endosteal mesenchymal progenitors decreased the disease activity index much more (76%) than central cells (44%, Fig. 5C). Both the central and endosteal mesenchymal progenitors reduced the epithelial tissue damage and inflammatory cell invasion induced by DSS with a trend toward more reduction in damage after endosteal mesenchymal progenitor transplantation (Fig. 5D). Mice with colitis also had decreased Tregs and increased Th17 levels. Transplantation of either central or endosteal mesenchymal progenitors up-regulated Treg levels and down-regulated Th17 levels but endosteal mesenchymal progenitors showed a greater improvement in T cell levels (Fig. 5E and F).

3.5 The number of endosteal mesenchymal progenitors decreases with aging

To investigate whether endosteal mesenchymal progenitors numbers are affected by aging, we harvested both endosteal and central bone marrow cells from young (1–2-month-old) and old (10–12-month-old) mice for CFU-F assays. Interestingly, we found that there was a 51% decrease in the number of endosteal CFU-F colonies in old mice compared to young mice, while no decrease was detected in the number of central CFU-F colonies (Fig. 6A).

Sca-1 and CD29 are mouse bone marrow mesenchymal progenitor surface markers and CD45 is a hematopoietic lineage marker. A combination of these markers has been proposed to detect mouse mesenchymal progenitors in bone marrow [49–53]. To further explore the relationship between the endosteal mesenchymal progenitors and aging, we obtained endosteal and central bone marrow cells from both young and old mice and detected the percentage of Sca-1⁺CD29⁺CD45[−] cells by flow cytometry. Consistent with the aforementioned CFU-F assays, we found that in young mice, the percentage of these cells in endosteal bone marrow was much higher than those in central bone marrow (Fig. 6B). Furthermore, there was a significant decrease (57%) in the percentage of Sca-1⁺CD29⁺CD45[−] cells (Fig. 6B), in particular in the cells with high surface expression of Sca-1 and CD29 (81%) (Fig. 6C), in the endosteal bone marrow of aging mice compared to those in young mice. On the contrary, there was no difference in the percentage of Sca-1⁺CD29⁺CD45[−] cells in the central bone marrow cells of young and old mice. Note that central bone marrow cells contained a very low percentage of Sca-1^{high}CD29^{high}CD45[−] cells, much lower than those observed in the endosteal bone marrow.

3.6 Endosteal mesenchymal progenitors are highly responsive to anabolic PTH injection

To investigate the effect of PTH on endosteal mesenchymal progenitors, we injected rats with PTH and harvested both endosteal and central bone marrow cells from long bones 1 day later for CFU-F assays. Interestingly, a single injection of PTH significantly increased the number of endosteal mesenchymal progenitors by 66%, while it had no effect on the central mesenchymal progenitors (Fig. 7A). Furthermore, those PTH-responsive endosteal

mesenchymal progenitors mainly resided in trabecular bone since PTH stimulated the number of CFU-F colonies (77%) in bone marrow cells digested from the metaphyseal region but not those from the cortical region (Fig. 7A). Meanwhile, both numbers of ALP⁺ and ALP⁻ CFU-F colonies derived from endosteal bone marrow cells was elevated by PTH with a large increase observed in the number of ALP⁻ colonies (Fig. 7B). The size of ALP⁻ colonies was normally larger than that of ALP⁺ colonies. PTH further significantly enlarged ALP⁻ colonies but had little effect on ALP⁺ colonies (Fig. 7C and D). It is worthwhile to point out that the increase in the number of endosteal mesenchymal progenitors persisted after 12 days of daily PTH injections (Fig. 7E), a period required for detecting the potent anabolic effects of PTH on rat trabecular bone [54]. The same effect of PTH on the number of total, ALP⁻, and ALP⁺ CFU-F colonies was also observed in mouse bone (Supplemental Fig. 5A and B). However, in mice, PTH increased the size of both the ALP⁻ and ALP⁺ colonies (Supplemental Fig. 5C, D). Finally, in vitro differentiation assays demonstrated that PTH did not alter the osteogenic and adipogenic differentiation potential of endosteal mesenchymal progenitors (Fig. 7F and Supplemental Fig. 5E).

Binding of PTH(1-34) to its receptor PTH1R initiates two major intracellular signaling pathways: cAMP/PKA and PLC/PKC pathways, which can also be activated independently by two truncated PTH analogs 1-31 and 3-34 [55, 56]. To investigate which pathway is utilized by PTH to regulate the number of endosteal mesenchymal progenitors, rats were injected with different PTH peptides and endosteal cells were harvested for CFU-F assays. Consistent with previous reports that the anabolic action of PTH is mainly associated with the cAMP/PKA pathway [54], we found that only PTH(1-31) but not PTH(3-34) increased CFU-F numbers (Fig. 7F), suggesting that the PKA pathway plays an important role in mediating this effect of PTH on endosteal mesenchymal progenitors.

4 Discussion

The pioneering work of Alexander Friedenstein and coworkers in the 1960s and 1970s established the standard method to isolate rodent bone marrow cells by flushing out the central region of rodent hind long bones using a needle and a syringe [57, 58]. Since then, this approach has been commonly used to generate and characterize plastic-adherent and clonogenic bone marrow mesenchymal progenitors. However, due to the small size and high connectivity of rodent trabeculae, bone marrow within the metaphyseal region cannot be isolated by this approach. Recently, emerging evidence suggests that, while they all express typical mesenchymal markers, mesenchymal progenitors isolated from different tissue sources (bone marrow, placenta, adipose tissue, umbilical cord, and skin) exhibit different growth rates, differentiation abilities, and molecular phenotypes [59–62]. Here, we report that, even within the same long bone, mesenchymal progenitors located within different anatomical locations are functionally distinct. Using a collagenase/trypsin digestion method, we are able to isolate endosteal bone marrow mesenchymal progenitors and demonstrate that, while they share similar stem cell characteristics with their central counterparts, endosteal mesenchymal progenitors are much more proliferative and metabolically active in terms of immunomodulation and reactivity toward aging and PTH stimulation.

There is a concern that protease digestion itself might regulate the cell surface protein pattern and consequently, affect the plastic adhesive property or growth rate of mesenchymal progenitors. To exclude this possibility, we treated the flushed bone marrow cells with the same digestion protocol as described for obtaining endosteal cells and then seeded them for CFU-F assays. We did not observe an increase in the number of CFU-F colonies in those enzymatic-treated flushed bone marrow cells (data not shown). Moreover, treating the cultured mesenchymal progenitors with proteases did not stimulate their proliferation (data not shown).

In this report, we first performed CFU-F assays to demonstrate that endosteal bone marrow contains a higher concentration of mesenchymal progenitors than central bone marrow. We also used a combination of cell surface antigens (Sca-1, CD29, and CD45) to directly detect the progenitors in the freshly isolated bone marrow cells. The cell surface marker combination Sca-1⁺CD29⁺CD45⁻CD11b⁻ has been used to detect mouse mesenchymal progenitors within the bone marrow [49–53]. Levaot et al. found that the Sca-1⁺CD29⁺CD45⁻CD11b⁻ comprised about 0.15% of nucleated central bone marrow cells [51], whereas in our study the percentage of Sca-1⁺CD29⁺CD45⁻ cells is around 0.05% in the central bone marrow. Interestingly, we consistently observed about a 3-fold increase in the number of this population of cells in the endosteal bone marrow cells, providing a direct piece of evidence that there are more mesenchymal progenitors in the endosteal population than in the central one. To eliminate the possibility that enzymatic digestion might modify the immunoreactivity of mesenchymal progenitors and thus affect the flow cytometry results, we performed a time course experiment to detect the amount of Sca-1⁺, CD29⁺, or CD45⁺ cells in the central bone marrow cells treated with collagenase/trypsin. No progressive loss of these antigens was observed after up to 1 h of incubation with proteases (data not shown).

Here, we have shown that endosteal mesenchymal progenitors proliferate at a much quicker rate and maintain this proliferative ability longer than central cells in vitro. However, quiescence is known to be a critical feature of stem cells to maintain their long-term reconstituting capacity [63]. Therefore, an intriguing explanation for the differences in growth rate between these two populations of mesenchymal progenitors is that endosteal mesenchymal progenitors may represent a less primitive progenitor than central mesenchymal progenitors. Since the skeleton is constantly undergoing remodeling which requires the continuous replenishment of osteoblasts at the bone surface, a less primitive, more actively dividing mesenchymal progenitor may be important for the rapid supply of osteoblasts at the bone surface. Our recent data revealed that osteoblastic cells secrete potent chemotactic factors for mesenchymal progenitors [64]. These factors could stimulate the migration of central mesenchymal progenitors toward the bone surface, resulting in a higher frequency of mesenchymal progenitors in the endosteal bone marrow. Another explanation for the differences between the endosteal and central mesenchymal progenitors could be the effect of the changed environment. Once mesenchymal progenitors migrate toward the bone surface, the proximity to the bone surface lining cells, such as osteoblasts and osteoclasts, and the growth-factor rich bone matrix could result in the modification of the biological functions of these stem cells, making them more proliferative and reactive toward external stimuli, such as aging and PTH.

Recent publications from other groups have already hinted that trabecular bone may be a richer source of mesenchymal progenitors and that these mesenchymal progenitors may be phenotypically different. The Kalajzic group demonstrated that smooth muscle actin α (α SMA) is a marker for bone marrow mesenchymal progenitors. Fluorescent imaging in their article revealed that those cells expressing α SMA-GFP were located more in the trabecular region than in the diaphyseal bone [65]. Xu et al. [35] reported an improved method to harvest mesenchymal progenitors by a combination of flushing long bones, mechanical crushing of long bones and vertebrae, and enzymatic treatment of the resulting bone fragments. They mixed the flushed bone marrow cells and enzymatically released cells together for CFU-F assays and found that this method significantly increased the CFU-F frequency by 70%, implying that the enzymatically released portion of cells contain more mesenchymal progenitors. They further demonstrated that these MSCs have augmented tri-lineage mesenchymal differentiation. However, due to a lack of data about their proliferation rate, it is difficult to conclude whether this increased differentiation is a result of enhanced growth or increased differentiation ability. Studies from other groups also suggest that

collagenase digestion of minced or crushed long bone fragments increases the yield of mesenchymal progenitors [34, 36]. However, all these methods are likely to have contamination of periosteal progenitors because they did not perform pre-digestion of the intact long bones to get rid of periosteal cells. Long bone periosteum is known to contain a much higher frequency of mesenchymal progenitors than central bone marrow and these periosteum-derived cells are normally isolated by a similar collagenase digestion method [66]. Hence, we adopted a pre-digestion step in our unique approach to ensure no contamination of periosteal mesenchymal progenitors in the endosteal bone marrow populations, which is evident from Fig. 1C. Human trabecular bone is much larger than rodent trabeculae. Normally human bone marrow mesenchymal progenitors are cultured from bone marrow aspirates. Recent studies on the collagenase-released (CR) cells from human trabecular bone fragments demonstrated that mesenchymal progenitors derived from those CR cells are phenotypically identical to mesenchymal progenitors from bone marrow aspirates. Furthermore, these CR cells, which are likely to be the endosteal bone marrow cells, contain 15 to 100-fold more CFU-Fs than bone marrow aspirates [67, 68].

Interestingly, studies from the Nilsson group in the past several years revealed a similar finding about HSCs. They found that the properties of HSCs are dependent on their anatomical location inside the bone. After flushing out the central bone marrow, they used a slightly different approach to dissociate the endosteal cells by grinding the remaining long bones and digesting them with collagenase and dispase. They demonstrated that HSCs isolated from the endosteal region have greater proliferative, homing, and hemopoietic potential [2, 3]. Moreover, when stimulated by granulocyte colony stimulating factor (G-CSF) for mobilization, the long-term HSCs are expanded within the central bone marrow region but not in the endosteal region [69]. Considering a recent elegant study demonstrating a close proximity between nestin⁺ MSCs [6] and HSCs and emerging evidence that MSCs and HSCs share a perivascular niche [70, 71], we are not surprised to find that MSCs display similar location-dependent properties. However, the frequency of HSCs at different locations remains the same [3] and the overall blood vessel density and the average width of blood vessels have no difference between the metaphysis and diaphysis [4], suggesting that the increase in the frequency of mesenchymal progenitors within the metaphysis is probably not due to the increase in the niche area.

In osteoporosis patients, fractures most commonly arise in bones with high trabecular volume including the hip, wrist, and spine. Aging is a major contributing factor for developing osteoporosis. As the only approved anabolic therapy for osteoporosis, PTH is known to have more dramatic anabolic effects on the trabecular bone than the cortical site. Therefore, the study of endosteal mesenchymal progenitors is more biologically relevant to the mechanisms of age-related osteoporosis and its treatment than central mesenchymal progenitors. Indeed, our data revealed a significant decrease in the endosteal mesenchymal progenitors, particularly the Sca-1^{high}CD29^{high}CD45⁻ population, in old mice. Moreover, CFU-F assays showed that PTH increases the number of endosteal mesenchymal progenitors while it has no significant effect on the central cells. One possible explanation for this result is that PTH stimulates the growth of endosteal mesenchymal progenitors. However, previous reports show that the percentage of mature osteoblasts incorporated with ³H-thymidine or BrdU, acquired at a proliferative stage, remain the same after PTH treatment [72, 73]. While these data were interpreted that PTH does not affect the proliferation of progenitors, we think that they do suggest a stimulatory effect of PTH because PTH did increase the total number of labeled cells on the bone surface. Another possible explanation for the discrepancy between our data and the previous observations is that PTH might mainly regulate the survival, but not proliferation, of mesenchymal progenitors.

In summary, we demonstrate that endosteal mesenchymal progenitors are a distinct population of mesenchymal progenitors that display different proliferative and immunomodulatory activities and respond differently toward external stimuli, such as aging and PTH, compared to their central counterparts. We therefore suggest that endosteal mesenchymal progenitors are more relevant to age-related diseases of the bone than central mesenchymal progenitors and they represent a biologically important target for future MSC studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Using an enzymatic digestion approach, we separately isolated endosteal and central bone marrow from rodent long bones.
- The endosteal bone marrow contained a much higher number of mesenchymal progenitors than the central bone marrow.
- Endosteal mesenchymal progenitors are able to undergo multi-lineage differentiation and express mesenchymal stem cell surface markers.
- Endosteal mesenchymal progenitors are more proliferative, immunosuppressive, and responsive to aging and parathyroid hormone than central mesenchymal progenitors.
- Therefore, endosteal mesenchymal progenitors represent a distinct population of mesenchymal progenitors that are more metabolically active and relevant to physiological bone formation.

Figure 1. Isolation of endosteal bone marrow cells from rat long bones

(A) Steps to isolate central and endosteal bone marrow cells from long bones and representative images of rat femurs after each step. Details are described in “Material and Methods”.

(B, C, D) Hematoxylin and eosin (H&E) staining of femoral trabecular areas at a low magnification (25x, B), cortical (C) and trabecular areas (D) at a high magnification (50x) before and after enzymatic digestion (step 4). Endo: endosteum; peri: periosteum.

Figure 2. Endosteal bone marrow cells contain a high frequency of clonogenic fibroblastic progenitors

(A) CFU-F assays of rat central bone marrow cells or endosteal bone marrow cells derived from whole bone (total), cortical-enriched diaphyseal bone, and trabecular-enriched metaphyseal bone revealed that endosteal cells, particularly those residing within trabecular bone, harbor more mesenchymal progenitors. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$ vs central.

(B, C) Representative CFU-F colonies (B) and quantification (C) demonstrate increased colony size from rat endosteal bone marrow cells compared to central cells. **: $p < 0.01$ vs central.

(D) CFU-F ALP assays suggest similar differentiation status of mesenchymal progenitors from rat central and endosteal bone marrow cells.

(E) Representative morphologies of rat central and endosteal mesenchymal progenitors in culture.

(F) Endosteal bone marrow harvested from 2.3kb col1-GFP mice contained both GFP-positive and negative cells.

(G) Sorted GFP-positive and negative cells from endosteal bone marrow were plated for CFU-F assay. Only GFP⁻ cells formed fibroblastic colonies. Some GFP⁺ cells adhered to the flask but exhibited a round shape and did not expand into colonies.

(H) The number of CFU-F colonies formed by GFP-negative and positive cells. ***: $p < 0.001$ vs GFP-negative.

Figure 3. Characterization of the stem cell properties of rat endosteal mesenchymal progenitors (A) Flow cytometric analyses of mesenchymal progenitor surface profiling of cultured cells derived from rat central and endosteal bone marrow cells. The expression of antigen (shaded curve) is shown together with their corresponding negative control (open curve). (B, C) In vitro osteoblast differentiation assays demonstrated that endosteal mesenchymal progenitors are capable of osteoblast differentiation (OB) as detected by von Kossa staining (B) and qRT-PCR assays (C) for osteoblast marker genes. (D, E) In vitro adipocyte differentiation assays demonstrate that endosteal mesenchymal progenitors are capable of adipocyte differentiation (AD) as detected by oil red O staining (D) and qRT-PCR assays (E) for adipocyte marker genes. (F, G) In vitro micromass differentiation assays demonstrate that endosteal mesenchymal progenitors are capable of chondrocyte differentiation as detected by alcian blue staining of pellets (F) and qRT-PCR assays (G) for chondrocyte marker genes.

Figure 4. Rat endosteal mesenchymal progenitors are more proliferative than central mesenchymal progenitors

(A) BrdU incorporation assay of rat central and endosteal mesenchymal progenitors.
 (B) Central and endosteal mesenchymal progenitors were plated at the same density to allow growth for 5 days. Viable cells were quantified at the indicated times by MTT assay.
 (C) Cell cycle distribution of central and endosteal mesenchymal progenitors (MPs) cultured in growth medium.
 (D) qRT-PCR analyses of the expression of cell cycle inhibitor genes in central and endosteal mesenchymal progenitors. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$ vs central.

Figure 5. Endosteal mesenchymal progenitors show higher immunomodulation property

(A) Flow cytometry of in vitro T cell apoptosis induced by central and endosteal mesenchymal progenitors.

(B) Schema of mesenchymal progenitor transplantation in DSS-induced experimental colitis mice.

(C) Disease activity index reduction in colitis model mice after transplantation of central or endosteal mesenchymal progenitors.

(D) H&E staining showed the infiltration of inflammatory cells (grey arrows) in colon with destruction of epithelial layer (yellow triangles) in colitis mice. Central mesenchymal progenitor-transplanted group showed significant reduction of epithelial damage and infiltration inflammatory cells but its efficacy was lower compare to endosteal mesenchymal progenitor-transplanted group. The histological activity index is shown on the right. Bar = 200 μ m. n=3–4/group.

(E) Flow cytometric assay revealed up-regulation of Tregs level in colitis model mice after mesenchymal progenitor transplantation.

(F) Flow cytometric assay revealed down-regulation of Th17 level in colitis model mice after mesenchymal progenitor transplantation.

*: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.005$. The bar graph represents mean $\pm$ SD.

Figure 6. The number of endosteal mesenchymal progenitors decreases with aging

(A) CFU-F assays were performed to measure the mesenchymal progenitor numbers within the central and endosteal bone marrow cells among young (1–2-month-old) and old mice (10–12-month-old).

(B, C) Flow cytometric analyses were performed to quantify the percentage of mesenchymal progenitors Sca-1⁺CD29⁺CD45⁻ (B) and Sca-1^{high}CD29^{high}CD45⁻ cells (C) within the central and endosteal bone marrow cells in young and old mice. n=3/group. *: p<0.05; **: p<0.01; ***: p<0.001.

Figure 7. A single PTH injection increases the number of endosteal mesenchymal progenitors but not central mesenchymal progenitors in rat

(A) Central and endosteal bone marrow cells from whole bone, trabecular-enriched metaphysis, and the diaphyseal cortical midshaft were harvested from rat femurs and tibiae after 24 h of vehicle or PTH treatment and plated for CFU-F assays. n=3/group.

(B) CFU-F ALP assay of endosteal mesenchymal progenitors after a vehicle or PTH injection.

(C, D) Representative images (C) of the ALP-negative and positive CFU-F colonies formed by endosteal mesenchymal progenitors after a vehicle or PTH injection. The quantification of size is shown in (D).

(E) Rats receiving PTH daily injections for 12 days had increased number of endosteal mesenchymal progenitors. Bone marrow cells were harvested 1 day after the last injection and plated for CFU-F assay. n=3/group.

(F) In vitro osteogenic (OB) and adipogenic (AD) differentiation assays demonstrate that PTH injection does not alter the differentiation potential of endosteal cells as identified by Von Kossa staining for osteoblasts and by oil red O staining for adipocytes.

(G) A single injection of PTH(1-31), but not PTH(3-34), into rats stimulated the increase in CFU-F numbers from endosteal bone marrow cells. n=3/group. *: p<0.05; **: p<0.01; ***: p<0.001.