

Original Research Report

Origin and Characterization of Multipotential Mesenchymal Stem Cells Derived from Adult Human Trabecular Bone

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ABSTRACT

Much of the knowledge regarding the regulatory pathways for adult stem cell self-renewal and differentiation has been obtained from the results of *in vitro* cultures. However, it is unclear if adult stem cells are controlled in the same way under physiological conditions. We examined this issue with respect to the migration of stem cells to tissue injury and how switch from a migratory state to one of proliferation wherein they participate in development. Building on our previous identification of multipotent stem cells in trabecular bone, we have examined the *in vitro* behavior of these cells within the bone milieu. We found that cell proliferation is inhibited within the trabecular bone niche as cells migrate out of the trabecular bone prior to proliferation. Additionally, multiple cell types were detected in adult trabecular bone, including osteoblasts, osteoclasts, endothelial cells, and Stro-1-positive mesenchymal stem cells. Furthermore, we demonstrated that Stro-1-positive cells migrated out of their native bone niche to generate multipotential stem and progenitor cells during *in vitro* culture. We conclude that self-renewal and differentiation of adult stem cells in connective tissues are tightly controlled and separately orchestrated processes. A regulatory network of extrinsic factors and intrinsic signals acts to stimulate the exit of stem cells from their niche so that they can localize to sites of wound healing, where they participate in development after functional differentiation.

INTRODUCTION

MULTIPOTENTIAL STEM CELLS have been isolated from numerous postnatal tissues and organs such as bone marrow, epithelia, skeletal muscle, and trabecular bone (1–7). These cells are capable of extensive self-renewal and multilineage differentiation during *in vitro* culturing. At present, much of the knowledge regarding the regulation of the maintenance, proliferation, and differentia-

tion of adult stem cells has been obtained from studies using *in vitro*-expanded cells. Although *in vitro* cell culture systems provide invaluable insight into the nature of adult stem cells, it is largely unknown if stem cells are controlled in the same way in their native tissues and organs *in vivo*.

In tissues and organs that undergo high turnover, such as the hematopoietic system, the skin, and the intestine, adult stem cells proliferate to maintain the stem cell reser-

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voir as well as to generate progenitor cells, which in turn give rise to various differentiated cells during normal homeostasis as well as during tissue regeneration and repair upon damage. Adult stem cells are localized in a defined *in vivo* microenvironment, i.e., niche (8), that provides regulatory signals for cell proliferation and differentiation. For example, the long-term hematopoietic stem cells (HSCs) in murine bone marrow are located on the endosteal surface of long bone, a niche consisting of cavity-lining osteoblasts (9,10). Epithelial stem cells reside in the hair follicle bulge in the skin (2,11–13) and at the base of the crypt in the intestine (14). Although tissue-specific stem cells reside in a quite distinct microenvironment, they share a common characteristic by remaining in a mitotically quiescent state inside the niche, and only start to undergo asymmetric division upon exiting their niche (13,14). Growth factors, extracellular matrix (ECM) molecules, and proteins that mediate the direct cell–cell interactions have been implicated to play key roles in the process.

In addition to HSCs and epithelial stem cells, adult stem cells can be isolated from several connective tissues, such as the mesenchymal stem cells (MSCs) derived from bone marrow (15), umbilical cord blood (16,17), adipose tissues (18,19), and trabecular bone (4,6), and muscle stem cells from skeletal muscle (3). Unlike HSCs of the bone marrow and epithelial stem cells, the *in vivo* functions of the stem cells that reside in connective tissues that have a low remodeling rate are not completely clear. For example, do they possess the same regenerative function as HSCs, or are they merely the residual cells left from embryonic development? Such questions can only be answered fully after the anatomical location and identity of these stem cells are identified. However, a number of challenges exist. MSCs and muscle stem cells are isolated on the basis of their preference to adhere to tissue culture plastic (3,15) and are defined largely by their capability to proliferate and differentiate subsequently into cells of multiple lineages *in vitro*. The lack of definitive cell-surface markers and relatively low cell numbers also hinder the attempt to localize these stem cells *in vivo*.

Adult human trabecular bone, cultured as explants of dissociated bone chips, has shown promising potential as an alternative source for multipotential MSCs and progenitor cells (4–6,20). We propose that trabecular bone provides an *in vivo* niche for MSCs, and can be used as an experimental model to study the maintenance and proliferation of connective tissue-derived stem cells. In the present study, we have examined adult human trabecular bone to determine the origin of stem cells, identify their location within the tissue, and to characterize their migration out of their microenvironment. We found that multiple cell types, including a rare Stro-1-positive stem cell population, exist in adult trabecular bone *in situ* and

exhibit the ability to differentiate subsequently into several cell types of mesenchymal lineage. We also demonstrate that cells located in the trabecular bone niche are mitotically quiescent, and that cells migrate out of the niche prior to division and subsequent differentiation. Additionally, we demonstrate that outgrowth of trabecular bone cells is independent of cell proliferation.

MATERIALS AND METHODS

Reagents

Cell culture media and reagents were purchased from Invitrogen (Carlsbad, CA). All chemicals were obtained from Sigma-Aldrich (St. Louis, MO) unless specified otherwise.

Isolation and culture of trabecular bone and trabecular bone derived cells

Trabecular bone chips were obtained as described previously (4,5,7) from the femoral heads of patients (3 females, aged 54, 63, and 74; 1 male, 70 years) undergoing total hip arthroplasty with Institutional Review Board approval from George Washington University. The bone chips were digested with 0.2 mg/ml collagenase XI at 37°C for 3 h followed by extensive rinsing with phosphate-buffered saline (PBS) to remove adventitious tissues and cells. The freshly isolated bone chips were divided into two fractions. One part was fixed immediately with 4% paraformaldehyde in PBS (FD NeuroTechnologies, Inc, Baltimore, MD) for histological analysis. The other part was cultured in basal medium (BM) containing Dulbecco's modified Eagle medium (DMEM), 10% fetal bovine serum (FBS), 10 units/ml penicillin G sodium, 10 µg/ml streptomycin sulfate, and 0.25 µg/ml Fungizone in a 37°C incubator with 5% CO₂. For some experiments, aphidicolin, a cell cycle inhibitor, was added to the trabecular bone culture at a final concentration of 1 µg/ml to block cell proliferation. Human MSCs were also isolated from bone marrow aspirate (15) and cultured as described below.

Cells that grew out from cultured trabecular bone chips were further expanded in BM with a medium change every 2–3 days until they reached 90% confluence. Cells were then removed from the culture plates with 0.25% trypsin-EDTA, rinsed with PBS, and induced to differentiate into osteoblasts, adipocytes, and chondrocytes as described previously (21).

Preparation of trabecular bone chips for histological analysis

Fixed trabecular bone chips were decalcified with 14.3% ethylenediamine tetraacetic acid (EDTA) in wa-

ter for 14 days, rinsed with PBS, dehydrated in a graded series of ethanol, infiltrated with xylene, and embedded in paraffin. Histological sections were prepared at 9- μ m thickness.

Immunofluorescence analysis

Before staining, paraffin sections were rehydrated and digested with Digest-All™ 3 pepsin solution for 10 min at 37°C (Zymed, S. San Francisco, CA). To identify various cell types in the trabecular bone, sections were blocked with 1% bovine serum albumin (BSA) in PBS for 30 min at room temperature, rinsed with PBS, incubated with 1 μ g/ml of primary antibody (Table 1) in PBS for 1 hr at 37°C, rinsed with PBS, and incubated with 1 μ g/ml of secondary antibody in PBS for 1 h at 37°C followed by several rinses in PBS. Nuclei were counterstained with 1 μ g/ml of 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) (Molecular Probes, Eugene, OR). The following secondary antibodies were used: Alexa Fluor 568-conjugated goat anti-mouse immunoglobulin M (IgM) (A-21043), Alexa Fluor 488-conjugated donkey anti-rabbit IgG (A-21206), Alexa Fluor 488-conjugated goat anti-mouse IgG (A11029) (Molecular Probes, Eugene, OR), and fluorescein isothiocyanate (FITC)-conjugated anti-goat IgG (F2016, Sigma-Aldrich, St. Louis, MO). Slides were mounted with VECTASHIELD (Vector Laboratories, Inc., Burlingame, CA) and kept at -20°C in dark until analysis. Sections were also double stained for Stro-1 and one of several other differentiated cell markers. To detect 5-bromo-2-deoxyuridine (BrdU)-labeled, proliferative cells (see below), sections were stained with biotin-labeled mouse anti-BrdU antibody (Zymed, S. San Francisco, CA), followed by Alexa Fluor 568-conjugated streptavidin (Molecular Probes, Eugene, OR). To quantify the number of total cells and cells expressing specific antigens, a minimum of 200 DAPI-stained cells were counted per sample, and data were averaged from triplicate samples from three separate experiments. Statistical analysis was per-

formed using paired Student's *t*-test at a significance level of $p < 0.05$.

Preparation of trabecular bone-derived cells for immunostaining

To identify the various cell types present in the out-growth monolayer cell culture, bone chips were removed from day-14 cultures, and the monolayer cells were fixed with 4% paraformaldehyde, and then stained with the various markers as described above.

Labeling of proliferative cells

To identify proliferative cells, bone chips were cultured in the presence of BrdU (1:100 dilution in BM) (Zymed, S. San Francisco, CA) for 24 h at days 1, 3, 7, 14, or 21, respectively. Bone chips were then prepared for immunohistochemistry as described above.

Light and fluorescence microscopy

Samples were observed with phase-contrast or epifluorescence optics using an Axiovert S100 TV microscope (Carl Zeiss Microimaging, Inc., Thornwood, NY). Fluorescence images were captured with a SENSYS CCD camera using IPLab software (Scanalytics, Inc, Fairfax, VA). All images were processed using Adobe Photoshop 6.0 (Adobe Systems, Inc., San Jose, CA).

RESULTS

Postnatal trabecular bone retains multipotential MSCs

Trabecular bone chips isolated from the femoral heads appeared uniform in size (~100 μ m in diameter) (Fig. 1A, a) and stripped of any surface-associated fibrous tissues or cells as a result of extensive collagenase diges-

TABLE 1. MARKERS FOR DETECTION OF MSCs AND DIFFERENTIATED CELLS IN TRABECULAR BONE

Cell type	Marker	Antibody used
Mesenchymal stem cells	Stro-1 (1,34)	STRO-1 ¹
Osteoblasts	PEBP2- β (35)	sc-20693 ²
	Osteopontin (OPN) (36)	sc-10591 ²
Osteoclasts	RANK (37)	sc-9072 ²
Pericytes	α -smooth muscle actin (38,39)	IMMH2 ³
Endothelial cells	Flk-1 (40)	sc-5040 ²
	von Willebrand's factor (VWF) (41)	sc-14014 ²

Sources: ¹Developmental Studies Hybridoma Bank (The University of Iowa, IA); ²Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); ³Sigma-Aldrich (St. Louis, MO).

tion. Cells became visible after an average of 14 days of *in vitro* culturing (Fig. 1A, b). Similar to MSCs derived from bone marrow (Fig. 1A, c), cells derived from trabecular bone chips appeared fibroblastic in shape and showed extensive cell proliferation potential *in vitro*. In addition, as reported previously (4,5,7), these cells were capable of differentiating into several cell types of mesenchymal origin, such as osteoblasts, adipocytes, and chondrocytes, in response to various inducing factors (Fig. 1B), a typical characteristic of MSCs.

MSCs migrate out of trabecular bone prior to proliferation

To characterize the process by which cells contained within the trabecular bone chips gave rise to multipotential MSCs, we analyzed their migration and outgrowth from the trabecular bone chips. The number of cells located within the bone lacunae and those at the periphery were determined during a 21-day culture period. We found that initially the majority of DAPI-stained cells (86.8%) were located inside the lacunae of freshly isolated trabecular bone chips, and only a small percentage of cells (13.2%) were found associated with the periphery of the bone chips. The percentage of cells in the lacunae gradually decreased over time, as only 13% of total cells were retained inside the bone chips by day 21 (Fig. 2B). At the same time, the amount of DAPI-stained cells appearing at the periphery of the bone chips increased from 13.2% on day 0 to 87% on day 21.

To determine whether the cells residing in the trabecular bone could proliferate, BrdU was included in the culture medium to label proliferating cells. No cell proliferation was detected inside the trabecular bone, because none of the cells located in the lacunae incorporated BrdU. Cells that incorporated BrdU were found exclusively at the periphery of the bone chips or outside the chips altogether (Fig. 2A, i), suggesting that cells only proliferated after having migrated out of the bone structure, and that cell proliferation was likely inhibited inside the trabecular bone. To examine the relationship between cell proliferation and cell outgrowth, trabecular bone chips were cultured in the presence of the cell cycle inhibitor, aphidicolin. Interestingly, cells still migrated out of the trabecular bone in the absence of cell division. As expected, without aphidicolin treatment, a dramatically higher number of cells was observed outside the bone chips, likely due to extensive cell proliferation over the 5-week culture period. However, there was no significant difference in the number of cells retained in the bony lacunae, with or without aphidicolin treatment (Fig. 3). Approximately 10% of cells were detected in the lacunae in the presence of the inhibitor, whereas 6.5% were retained in the absence of aphidicolin after 5

weeks. Taken together, these results suggest that migration or outgrowth of trabecular bone cells is independent of cell proliferation.

MSCs represent a rare cell population in trabecular bone

Cells derived from trabecular bone chips possessed extensive proliferative ability and multilineage differentiation potential, similar to MSCs derived from bone marrow (4,5,7). To determine the location of these MSCs in trabecular bone, freshly isolated trabecular bone chips were immunostained for markers characteristic of various cell types, including MSCs, osteoblasts, osteoclasts, endothelial cells, and pericytes (see Table 1). The trabecular bone chips did not contain pericytes as no cells were found to express α -smooth muscle actin. As shown in Fig. 4, the bone chips contained cells positive for STRO-1, PEBP2- β , RANK, Flk-1, and VWF antibodies (Table 2), suggesting the presence of MSCs, osteoblasts, osteoclasts, and endothelial cells, respectively. Surprisingly, the total percentage of cells that were positive for these five markers was in excess of 100% (Fig. 5A), suggesting the existence of cells that expressed more than one cell type marker. To characterize the MSCs present in trabecular bone further and to distinguish them from other cell types, cells were co-stained with Stro-1 antibody in combination with another antibody of the differentiated cell markers. As shown in Fig. 5B, the majority of Stro-1-positive cells (which made up approximately 9.4% of total cells) did express other markers: 5% for PEBP2- β , 22% for RANK, 27% for Flk-1, and 22% for VWF. However, the remainder (approximately 2.2% of total trabecular cells) was positive only for Stro-1, indicating the existence of true MSCs in adult trabecular bone.

Stro-1-positive trabecular bone cells give rise to multipotent stem and progenitor cells

To identify which trabecular bone cell type migrated out and subsequently gave rise to the multipotent MSC population, trabecular bone chips were cultured for 14 days *in vitro*. More than 50% of the cells migrated out of the bone chips. Cells that remained in the bony lacunae and cells that migrated out and gave rise to the monolayer outside of the bone chips were immunostained. Compared to freshly isolated trabecular bone chips, Stro-1-positive cells were not detected within the bone chips after 14 days of culture (Table 2). The percentage of cells positive for differentiated cell markers also decreased slightly (Table 2), suggesting that some of these cells had also migrated out of the bone chips. On the other hand, 47% of the monolayer cell population outside of the bone chips was Stro-1 positive, a five-fold increase in abun-

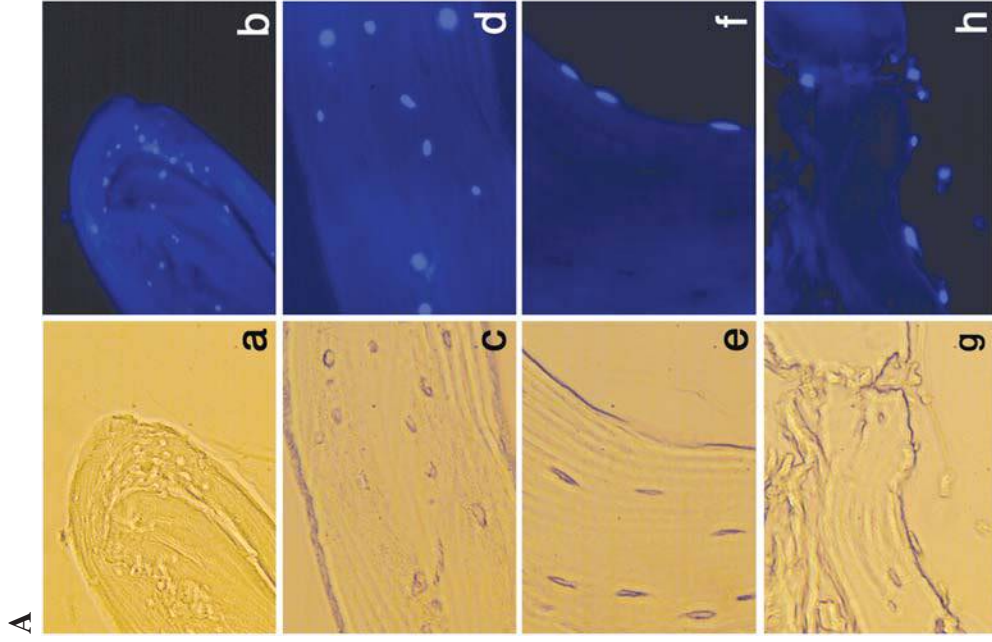


FIG. 2. Migration and proliferation of trabecular bone cells. (A) Trabecular bone chips were cultured in vitro for 0 (a, b), 7 (c, d), 14 (e, f), and 21 (g-i) days. Total cells were detected by DAPI staining (b, d, f, h) and proliferating cells were determined by BrdU incorporation (i). (a, c, e, g) Phase-contrast; (b, d, f, h, i) epifluorescence micrographs. Scale bar, 50 μm . (B) Bar graph showing the percentage of cells located in lacunae versus periphery of the bone chips over a 21-day in vitro culture period.

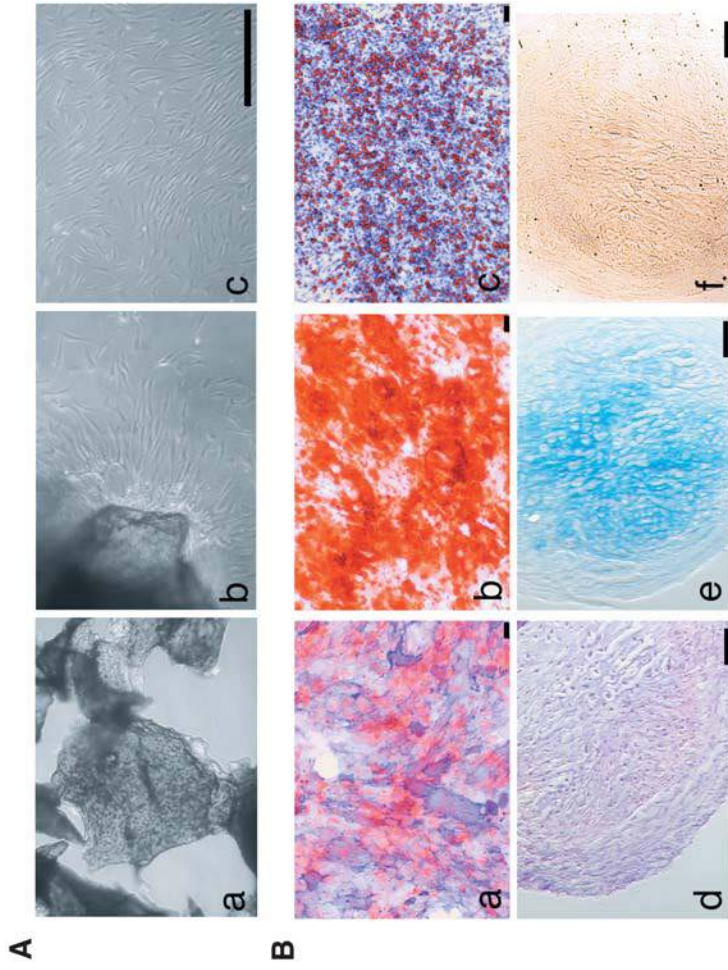


FIG. 1. Trabecular bone-derived MSCs exhibit fibroblastic morphology and multilineage differentiation potential. (A) Phase-contrast micrographs showing freshly isolated trabecular bone chips (a) and in vitro-cultured MSCs isolated from trabecular bone (b) and bone marrow aspirate (c). Scale bar, 50 μm . (B) Trabecular bone-derived MSCs are capable of differentiating into osteoblasts (a,b), adipocytes (c), and chondrocytes (d-f). Fully differentiated osteoblasts stained positive for plasma membrane-bound alkaline phosphatase activity (a, counterstained with Neutral Red) and sequestered an Alizarin Red-positive calcified matrix (b). Differentiated adipocytes were detected by the presence of Oil Red O-stained lipids in the cytoplasm (c, counterstained with hematoxylin). Chondrogenesis was demonstrated by the presence of matrix sulfated proteoglycan stained with Alcian Blue (e) and positive for collagen type II (f). d, Hematoxylin & Eosin staining for cell morphology. Scale bar, 100 μm .

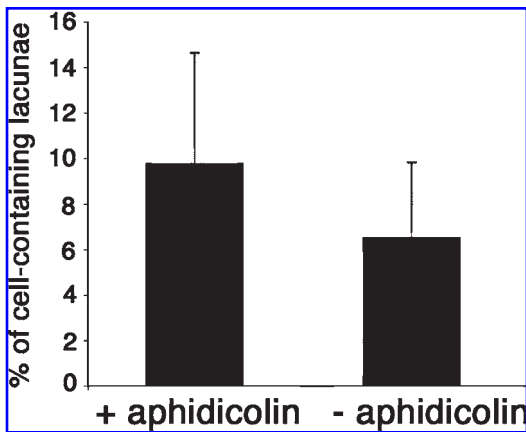


FIG. 3. Percentage of cells retained in trabecular bone after 5 weeks of in vitro culturing in the presence or absence of the cell proliferation inhibitor aphidicolin. No statistical difference was seen, suggesting that cell proliferation was not essential to cell migration and outgrowth.

dance compared to that inside the bone chips (Table 2), suggesting that they migrated out of the bone chips. Unexpectedly, almost all of these Stro-1-positive cells co-expressed PEBP2- β , RANK, Flk-1, and VWF (Table 2). This finding is consistent with our earlier observation of the presence of similar, co-stained cells inside the trabecular bone chips. Taken together, these results suggest that Stro-1-positive trabecular bone cells are likely to be the cell source that gives rise to the multipotent stem and progenitor cells.

DISCUSSION

The existence of adult stem cells in connective tissues has been under debate, owing to the lack of both unique cell markers and valid in vivo functional analysis, such as the transplantation and rescue assay widely used to confirm the existence of HSCs (as reviewed in ref. 22). However, numerous studies have demonstrated the existence of multipotential stem and progenitor cells in various adult tissues, on the basis of their capability for extensive self-renewal and multilineage differentiation in vitro. However, it is difficult to identify the location of these stem cells in vivo, and/or to elucidate how they migrate to the tissue remodeling and injury site from their native microenvironment.

In this study, we have postulated that one type of connective stem cells, MSCs, exist in postnatal trabecular bone, and that bone could be used as a model to study the migration and homing process of adult stem cells. Several lines of evidence support our hypothesis. First of all, cells derived from trabecular bone exhibit two fundamental characteristics of MSCs. First, they show ex-

tensive proliferation as well as the ability to differentiate into multiple cell types (4–6), consistent with their being stem or progenitor cells. Second, no cell proliferation occurs inside the trabecular bone, similar to observations made on the germ cell niche in *Drosophila* reproductive systems (23–26), and in the hematopoietic stem cell (HSC) niche in murine bone marrow (9,10). For example, HSCs are maintained in a very slow cycling state by the neighboring osteoblasts through Notch/Jagged1 and N-cadherin-mediated cell interactions in their niche at the endosteal surface of long bones (as reviewed in ref. 27). Once HSCs detach from the osteoblastic niche, either by matrix metalloproteinase activity or via asymmetric cell division, they begin to proliferate and replenish the hematopoietic cell populations. Similarly, MSCs stay in a mitotically quiescent state until they have migrated out of the trabecular bone. Therefore, this observation strongly suggests that trabecular bone functions as one of the MSC niches.

Using immunostaining, we have demonstrated the existence of multiple cell types in adult trabecular bone, including cells with the characteristics of osteoblasts, osteoclasts, endothelial cells, and MSCs. We did not detect any pericytes in trabecular bone, although they have been suggested as candidate cells to give rise to multipotential MSCs and progenitor cells in various connective tissues (28–30). Our results show that pericytes are not the major cell type contributing to the multipotent MSCs. On the other hand, our study does not exclude the possibility that pericytes may contribute to the generation of progenitor cells in other, nontrabecular bone sources, such as umbilical cord blood (31). Surprisingly, we did not identify any cells positive for OPN, a marker for mature osteoblasts. Because osteoblasts are usually found lining the bone surface, they may have been excluded from the trabecular bone chips used here, which have been prepared by extensive enzymatic digestion. A portion of Stro-1-positive cells in the trabecular bone chips express markers of other differentiated cells, implying the mesodermal origin of these cells. Compared to the low abundance of MSCs in bone marrow (32,33), umbilical cord blood (31), and adipose tissue (18), adult trabecular bone is a relatively rich source for MSCs, because they comprise 2.2% of the total cell population. The close proximity of these cells to bone marrow implies that trabecular bone might function as a reservoir to supply MSCs to bone marrow, before the cells circulate to the tissue injury sites for regeneration and repair.

Interestingly, Stro-1-positive cells are no longer detected inside the trabecular bone chips after 14 days of culture. On the other hand, close to half of the monolayer cell population outside of the trabecular bone chips are positive for Stro-1, indicating that these cells are derived from the Stro-1-expressing trabecular bone cells, thereby contributing to the generation of multipotential MSCs.

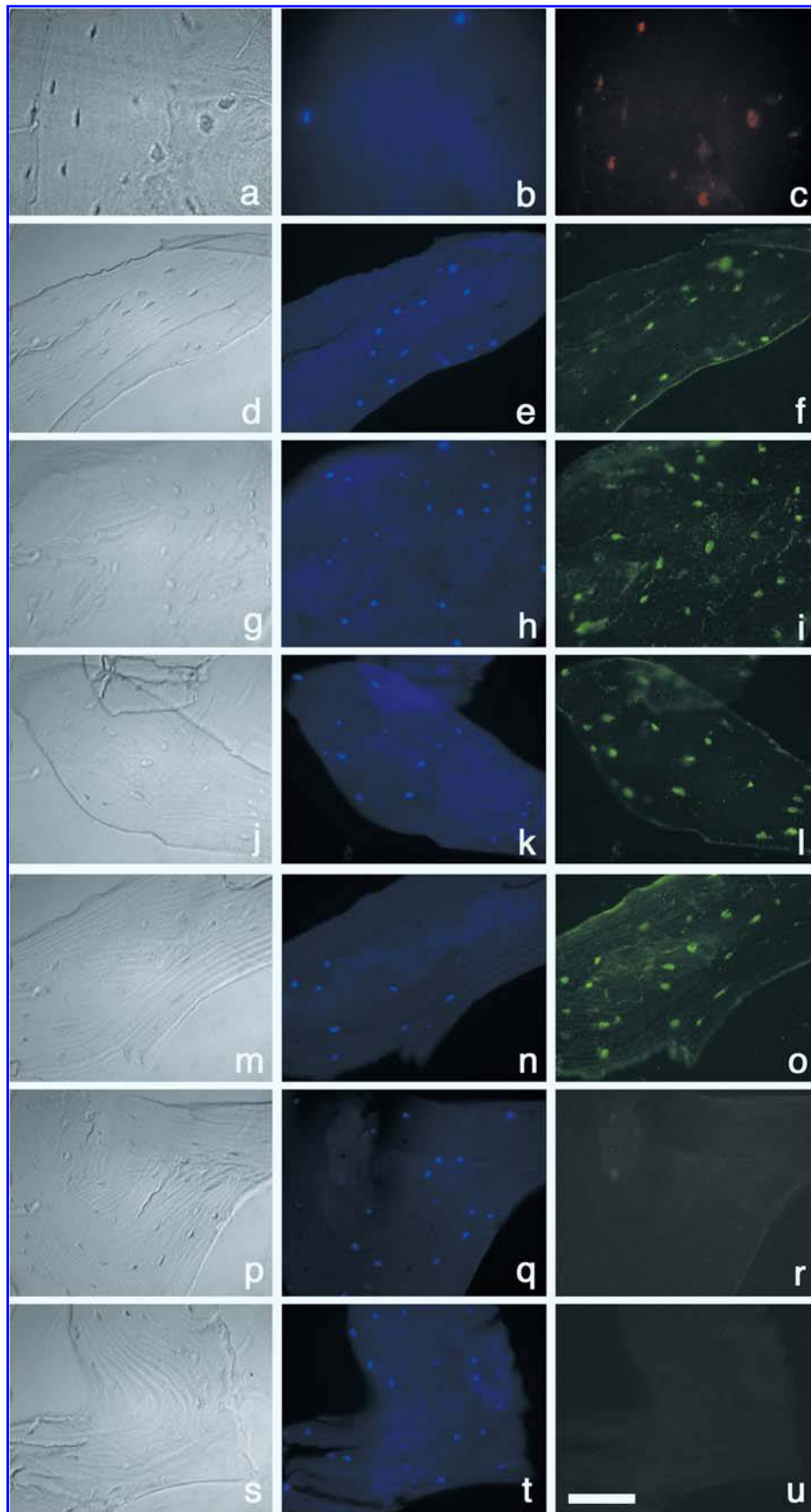


FIG. 4. Existence of multiple cell types, including Stro-1-positive mesenchymal stem cells, in trabecular bone as detected by immunofluorescence. (*Left panels*) Phase-contrast micrographs showing trabecular bone sections. (*Middle panels*) Epifluorescence micrographs showing DAPI-stained cells. (*Right panels*) Epifluorescence micrographs showing antibody-stained cells. (**a–c**) Stro-1; (**d–f**) PEBP2- β ; (**g–i**) RANK; (**j–l**) VWF; (**m–o**) Flk-1; (**p–r**), OPN; (**s–u**), α -smooth muscle actin. Scale bar, 50 μ m.

ORIGIN AND CHARACTERIZATION OF MSCs

TABLE 2. RELATIVE ABUNDANCE OF TRABECULAR BONE-DERIVED CELLS POSITIVE FOR STEM AND DIFFERENTIATED CELL MARKERS

Marker	<i>Percent of positive cells (average ± SD)</i>		
	<i>Within lacunae (day 0)</i>	<i>Within lacunae (day 14)</i>	<i>In monolayer (day 14)</i>
Stro-1	9.4 ± 5.0	0	47.1 ± 5.0
PEBP2-β	31.9 ± 10.4	27.9 ± 14.9	65.3 ± 0.2
RANK	38.4 ± 5.4	27.9 ± 13.1	43.2 ± 1.3
Flk-1	36.7 ± 26.6	16.4 ± 11.8	87.0 ± 2.3
VWF	32.8 ± 8.8	11.7 ± 4.7	76.4 ± 2.9
OPN	N.D.	N.D.	N.D.
α-actin	N.D.	N.D.	N.D.

N.D., Not detected.

This could also explain why, in a previous study, only 11 out of 23 colonies of cells (i.e., 48%) derived from trabecular bone are able to differentiate into all three mesenchymal lineages (6). Unexpectedly, almost all of the Stro-1-positive cells in monolayer are found to co-ex-

press other differentiated cell markers, whereas a distinct population (2.2%) of the cells located initially within the trabecular bone are exclusively positive for Stro-1. What was the fate of these exclusively Stro-1-positive cells during in vitro culture? They could have been diluted in the monolayer cell population as a result of their relatively slower proliferation rate than others, thus making their detection difficult if not impossible. Stro-1-positive cells from the trabecular bone could also have acquired differentiated cell markers during the course of in vitro culture, possibly as a result of induction by various growth factors present in the serum. These cells appear to retain an undifferentiated state, because they lack the phenotypic and functional characteristics of differentiated cells. Nonetheless, Stro-1-positive cells appear to be the major cell type that proliferate and populate the monolayer cell culture, regardless of whether they express other markers. Therefore, as suggested by our previous study (5), we postulate that Stro-1-expressing MSCs play a key role in replenishing the stem cell pool as well as in generating differentiated cells. Because committed cells could also dedifferentiate and regain their multipotentiality (21), cells that coexpress the stem cell marker Stro-1 and markers of other differentiated cells could thus function as an alternative source for MSCs in vivo, which may count for the functional heterogeneity of clonal cells observed in vitro (6).

What signals trigger the migration of MSCs from the trabecular bone niche in vitro? Do the same signals play a key role in vivo during tissue homeostasis and damage repair? By blocking cellular division with a cell cycle inhibitor, we have demonstrated that cell migration is independent of cell proliferation, thus excluding the possibility that proliferation is a prerequisite for cell migration and outgrowth. In vitro, trabecular bone is cultured in a serum-supplemented medium, enriched with multiple growth factors and signaling molecules. The extended period of culturing also could deplete the endogenous fac-

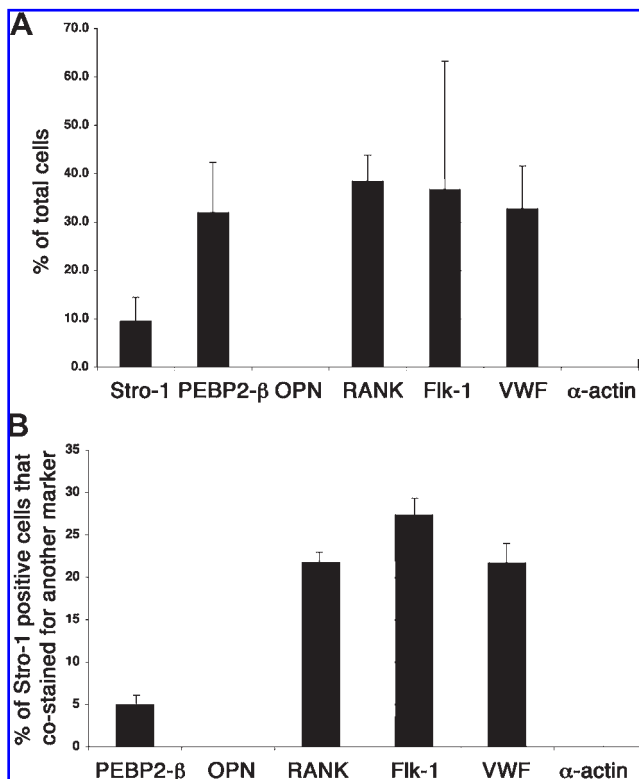


FIG. 5. Abundance of cells expressing various cell markers (A) and co-expressing Stro-1 and other differentiated cell markers (B) in trabecular bone. These results demonstrated the presence of osteoblastic, osteoclastic, and endothelial cells, as well as MSC-like cells in the trabecular bone chips. No pericytes or smooth muscle cells were found. Although most of the Stro-1-positive cells co-expressed differentiated cell markers, 2.2% was exclusively Stro-1 positive.

tors normally sequestered in the extracellular matrix. The combined effects of the gradual loss of endogenous inhibitors of cell proliferation from the matrix and the presence of stimulatory growth factors in the culture medium would result in promotion of the outgrowth of cells from the trabecular bone chips and support subsequent cell proliferation. The fact that outgrowing cells only become visible after an extended culture period supports this theory. In addition, we postulate that similar events are very likely to be involved in stem cell-mediated skeletal tissue regeneration *in vivo*, because the process of bone fracture and damage mimics the dissociative steps used here to prepare trabecular bone chips, whereas subsequent cultivation mimics the tissue repair process. However, the identities and mechanisms of action of extrinsic (from the regulatory niche) and intrinsic (inside the stem cells) factors controlling the renewal and maintenance of MSC in trabecular bone remain to be investigated.

In summary, our study demonstrates the existence of multipotential stem cells in adult connective tissues and, for the first time, provides useful insights into the behavior and function of stem cells during tissue homeostasis and repair. *In vitro* culturing of adult trabecular bone chips presents great potential as a useful system to identify extrinsic factors as well as the intrinsic signaling pathways that stimulate stem cell migration and subsequent proliferation and differentiation in response to tissue injury. Identification and characterization of these factors should enhance our understanding of the fundamental developmental processes and also provide cellular targets for potential pharmacological reagents that would accelerate the fracture healing and bone regeneration processes.

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