T.L. Aghaloo[1], T. Chaichanasakul[1], O. Bezouglaia[1], B. Kang[1], R. Franco[1], S.M. Dry[2], E. Atti[1], and S. Tetradis[1,3]*

[1]Division of Diagnostic and Surgical Sciences, UCLA School of Dentistry, 10833 LeConte Ave., CHS Rm. 53–068, Los Angeles, CA 90095-1668, USA; [2]Department of Pathology and Laboratory Medicine, David Geffen School of Medicine at UCLA, Los Angeles, CA 90095, USA; and [3]Molecular Biology Institute, UCLA, Los Angeles, CA 90095, USA; *corresponding author, stetradis@dentistry.ucla.edu

Osteogenic Potential of Mandibular *vs.* Long-bone Marrow Stromal Cells

ABSTRACT

Although fundamentally similar to other bones, the jaws demonstrate discrete responses to developmental, mechanical, and homeostatic regulatory signals. Here, we hypothesized that rat mandible *vs*. long-bone marrow-derived cells possess different osteogenic potential. We established a protocol for rat mandible and long-bone marrow stromal cell (BMSC) isolation and culture. Mandible BMSC cultures formed more colonies, suggesting an increased CFU-F population. Both mandible and long-bone BMSCs differentiated into osteoblasts. However, mandible BMSCs demonstrated augmented alkaline phosphatase activity, mineralization, and osteoblast gene expression. Importantly, upon implantation into nude mice, mandible BMSCs formed 70% larger bone nodules containing three-fold more mineralized bone compared with long-bone BMSCs. Analysis of these data demonstrates an increased osteogenic potential and augmented capacity of mandible BMSCs to induce bone formation *in vitro* and *in vivo*. Our findings support differences in the mechanisms underlying mandible homeostasis and the pathophysiology of diseases unique to the jaws.

KEY WORDS: marrow stromal cells, BMSCs, mandible, long bone, osteogenic.

INTRODUCTION

Despite being seemingly similar to other bones in the body, the maxilla and mandible serve distinct functions and demonstrate discrete responses to developmental, mechanical, and homeostatic stimuli (Sodek and McKee, 2000). Developmentally, the jaws, similar to other craniofacial bones, but distinct from the axial and appendicular skeleton, arise from neural crest cells of the neuroectoderm germ layer, and not from the mesoderm (Chai and Maxson, 2006), and undergo intramembranous instead of endochondral ossification (Karaplis, 2002). The mandible in particular is formed primarily by intramembranous ossification, while secondary cartilage at its proximal end contributes endochondral components at later stages. Meckel's cartilage, which precedes mandible formation but mainly disappears as the mandible develops, plays an important role in mandibular morphogenesis (Ramaesh and Bard, 2003; Tsutsui *et al.,* 2008). Although the same key regulators of osteoblastic differentiation, such as Runx2 and osterix, determine precursor commitment in intramembranous and endochondral bones (Ducy *et al.,* 1997; Komori *et al.,* 1997; Otto *et al.,* 1997; Nakashima *et al.,* 2002), several growth factors, receptors, and associated signaling cascades play distinct roles in the craniofacial *vs.* axial and appendicular skeleton (Abzhanov *et al.,* 2007; De Coster *et al.,* 2007; Kimmel *et al.,* 2007). Systemic diseases, such as osteoporosis, hyperparathyroidism, or Paget's disease, affect all bones, including the jaws (White and Pharoah, 2004). Indeed, there appears to be a correlation between mandibular bone density and osteoporosis (Kribbs *et al.,* 1989; Jeffcoat *et al.,* 2000; Lerner, 2006; Mavropoulos *et al.,* 2007). However, rat mandibles lose significantly less trabecular bone and bone mineral density at a lower rate than tibiae primary spongiosa after ovariectomy and malnutrition (Mavropoulos *et al.,* 2007), suggesting different homeostatic mechanisms of the two bones.

The small size and anatomic complexity of the maxilla and mandible render bone cell isolation a challenge (Sodek and McKee, 2000). Thus, little is known about the cellular and particularly molecular basis for mandibular bone *vs.* long-bone divergent homeostasis. Most of our knowledge on mandible cell function and differentiation is derived from experimental models with cells from other skeletal sites (Lerner, 2006). However, caution should be exercised in the extrapolation of such data to mandibular cell function. Indeed, human mandibular or maxillary marrow stromal cells demonstrate increased cell proliferation, delayed senescence, and stronger expression of osteoblastic markers compared with iliac-crest-derived marrow cells from the same patients (Akintoye *et al.,* 2006), suggesting distinct functions and differentiation potential.

Here, we sought to compare the *in vitro* osteoblastic differentiation and capacity for *in vivo* bone formation of bone marrow stromal cells (BMSCs) derived from rat mandible *vs.* BMSCs derived from rat tibiae. We hypothesized that these marrow populations, derived from two distinct skeletal sites in the rat, would display diverse osteogenic potential. Indeed, mandible BMSCs demonstrated a more robust osteoblastic differentiation and induced significantly greater bone formation than their long-bone counterparts. Our findings demonstrate differences in osteogenic potential of mandible *vs.* long-bone BMSCs, and suggest an increased capacity of mandible BMSCs to induce bone formation *in vitro* and *in vivo*.

MATERIALS & METHODS

Isolation and Culture of Mandible and Long-bone BMSCs

All animals were handled in accordance with guidelines of the Chancellor's Animal Research Committee at UCLA. Soft tissue was dissected from one-month-old Sprague-Dawley rat mandibles, and the third molar was extracted. Using a 26-gauge needle on the buccal cortex, we flushed bone marrow from the superior alveolar ridge with α-MEM (Mediatech, Herndon, VA, USA) and collected it through the extraction socket. Long-bone BMSCs were isolated from the tibiae of the same animals (Javazon *et al.,* 2001; Yoshimura *et al.,* 2007). Harvested cells were pooled from 10 rats, filtered through a 40-μm strainer, counted (Vi-CELL™ cell viability analyzer, Beckman Coulter, Fullerton, CA, USA), and plated at 1×10^6 cells/mL (2.6×10^5 cells/cm^2). Confluent cells were supplemented with osteogenic differentiation media (α-MEM+10% FBS with 50 μg/μL ascorbic acid and 4 mM β-glycerophosphate), which was replaced every 3-4 days.

Osteoblast Phenotype Assays

To evaluate CFU-F, we counted colonies (at least 50 cells *per* group) on BMSC cultures stained with Giemsa (Ladd Research, Williston, VT, USA). Alkaline phosphatase (ALP) staining and activity and von Kossa staining were measured (Pirih *et al.,* 2008; Liu *et al.,* 2009). To evaluate ^{45}Ca incorporation, we added 1 μCi/mL $^{45}CaCl_2$ (ICN Biomedicals, Irvine, CA, USA) for 48 hrs. Then, cells were collected, and ^{45}Ca was measured by scintillation counting. To assess gene expression, we collected total RNA at days 3 and 7 using Trizol (Invitrogen, Carlsbad, CA, USA). qPCR was performed in triplicate for at least 3 independent experiments, with iQ SYBR Green supermix (Bio-Rad, Hercules, CA, USA) and rat gene-specific primers, including: ALP (NM_013059) 5′-GGACGGTGAACGGGAGAAC-3′ (forward, 1424–1442), 5′-TGAAGCAGGTGAGCCATAGG-3′ (reverse, 1548–1567); osteocalcin(NM_013414)5′-GGACCCT-CTCTCTGCTCACTC TG-3′ (forward, 54–80), 5′-ACCTTACTGC-CTCCTGCTTGG-3′ (reverse, 156–177); DSPP (NM_ 012790) 5′-CGGTCCCTCAGT-TAGT-3′ (forward, 64–80), 5′-TACGTCCTCGCGTTCT-3′ (reverse, 336–355); and GAPDH (NM_017008) 5′-CGGCAAGTTCAA-CGGCACAGTCAAGG-3′ (forward, 229–254) and 5′-ACGACA-TACTCAGCACCAGCATCACC-3′ (reverse, 332–357). Gene expression normalized to GAPDH was expressed as fold induction.
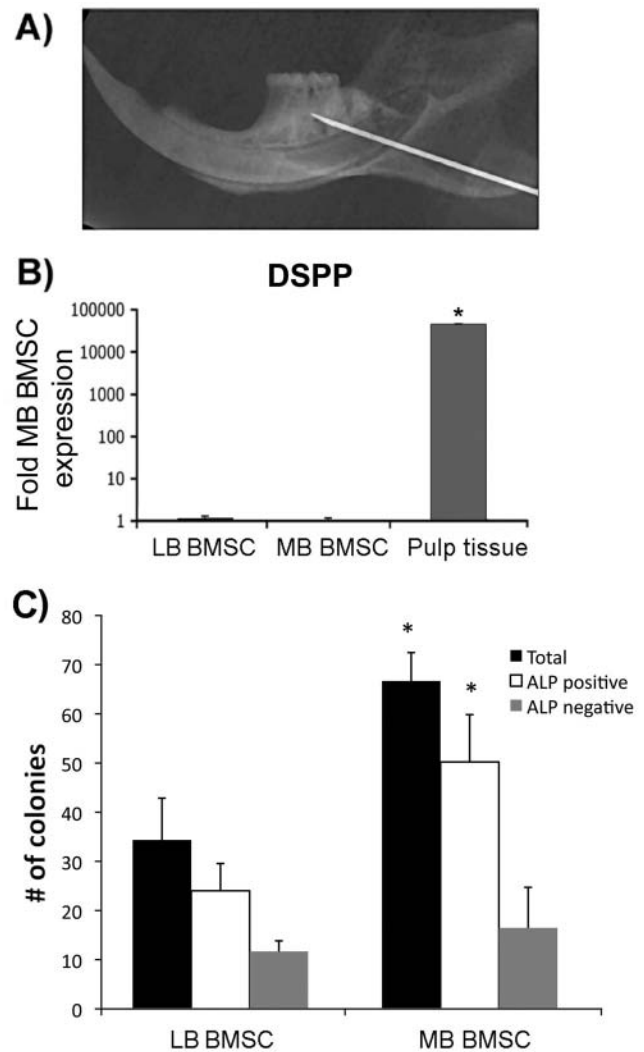

Figure 1. Isolation and culture of mandible BMSCs. **(A)** Radiograph of hemimandible showing the final position of the needle in the alveolar ridge superior to the incisor. The third molar has been extracted. **(B)** DSPP expression of long-bone (LB) BMSCs, mandible (MB) BMSCs, and pulp tissue by qPCR representative of more than 6 independent experiments. **(C)** Quantification of total, ALP-positive, and ALP-negative colonies formed by long-bone (LB) *vs.* mandible (MB) BMSC cultures (average of 3 independent experiments). *p < 0.05; error bars represent standard error of the mean.

Surgical BMSC Implantation

We collected 20×10^6 long-bone or mandible BMSCs, then centrifuged and incubated them with gelatin sponges (Gelfoam, Pfizer, New York, NY, USA) for 3 days. Sponges alone were utilized as controls. Sponges were subcutaneously implanted into nude mice at the intrascapular area (Pettway *et al.,* 2005). After 6 wks, transplants were placed in 10% formalin for 48 hrs and stored in 70% ethanol. μCT imaging was performed at 12 μM isotropic voxel resolution, and tissue and bone volume analysis was performed. We used Dolphin Software (Dolphin Imaging, Chatsworth, CA, USA) to generate 3D and multiplanar
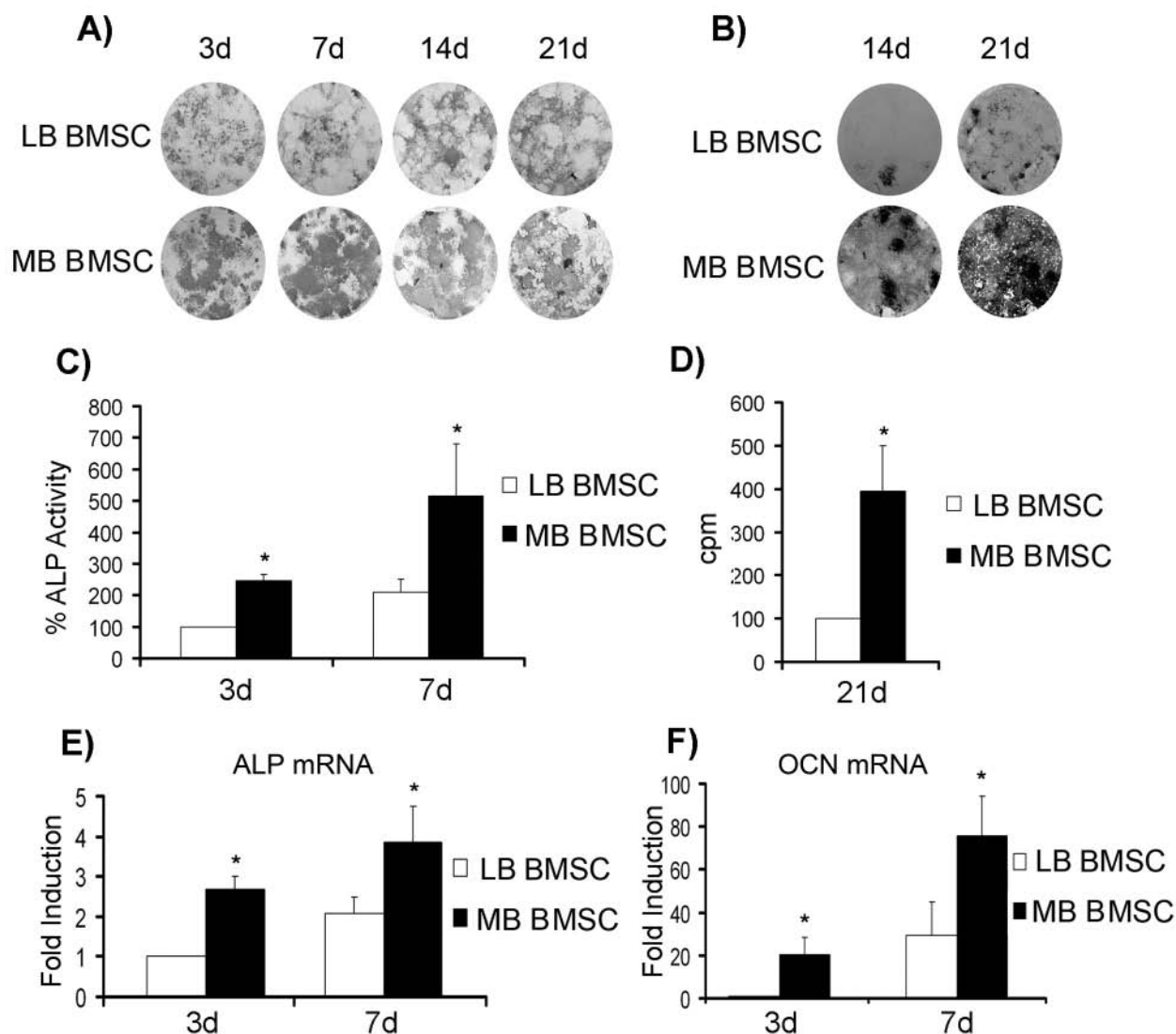
Figure 2. *In vitro* characterization of mandible *vs.* long-bone BMSCs. **(A)** Representative of 5 independent experiments of ALP staining at 3-, 7-, 14-, and 21-day cultures of long-bone (LB) *vs.* mandible (MB) BMSCs in osteogenic media. **(B)** Representative of 4 independent experiments of von Kossa staining at 14- and 21-day cultures of long-bone (LB) *vs.* mandible (MB) BMSCs in osteogenic media. **(C)** ALP activity assay at 3- and 7-day cultures of long-bone (LB) *vs.* mandible (MB) BMSCs in osteogenic media (average of 3 independent experiments). **(D)** [45]Ca assay at 21 days' culture of long-bone (LB) *vs.* mandible (MB) BMSCs in osteogenic media (average of 3 independent experiments). **(E)** ALP and **(F)** OCN mRNA expression determined by qPCR of long-bone (LB) *vs.* mandible (MB) BMSCs cultured in osteogenic media for 3 and 7 days (average of 4 independent experiments). *$p < 0.05$; error bars represent standard error of the mean.

reconstructed images. Then, transplants were decalcified (Fisher, Pittsburgh, PA, USA), paraffin-embedded, sectioned, and H&E-stained. Photomicrographs were taken with a Leica DMLB microscope (Leica Microsystems, Wetzlar, Germany) with BioQuant software (R&M Biometrics, Nashville, TN, USA). Two independent experiments, utilizing cells from different animals and performed at a different time, were performed, for a total of 8 transplants for each group.

Statistical Analysis

Data are expressed as mean ± standard error of the mean (SEM) from at least 3 independent experiments. One-way ANOVA and unpaired *t*

tests were used to determine statistical significance between 3 and 2 groups, respectively. A $p < 0.05$ was considered as significant.

RESULTS

Isolation, Culture, and Characterization of Mandible BMSCs

To avoid contamination of bone marrow cells with incisor pulp, we inserted a 26-gauge needle into the buccal cortex, at the ret-romolar area and above the external oblique ridge, away from the central incisor root, and directed toward the alveolar ridge. A plain radiograph demonstrates the needle position, superior to the incisor
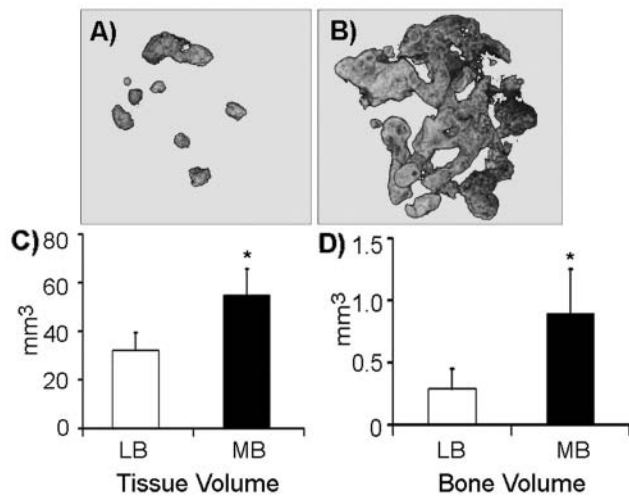
Figure 3. 3D-reconstructed **(A,B)** microCT images of representative gelatin sponges seeded with **(A)** long-bone- or **(B)** mandible-derived marrow cells. **(C)** Tissue volume (TV) and **(D)** bone volume (BV) of long-bone (LB) *vs.* mandible (MB) marrow cell-seeded sponges, quantified by µCT (average of 8 individual transplants from 2 independent experiments). *$p < 0.05$; error bars represent standard error of the mean.

root and inferior to the molars (Fig. 1A). Marrow was flushed from the mandible and collected through the extraction socket.

To confirm the absence of contamination from dental pulp/odontoblasts, we extracted total RNA from confluent BMSC cultures, and determined dentin sialophosphoprotein (DSPP) expression by qPCR using incisor pulp RNA as a positive control. DSPP, a precursor of dentin phosphoprotein (DPP) and dentin sialoprotein (DSP), is highly expressed by odontoblasts and has been used widely to demonstrate odontogenic differentiation (Iohara *et al.*, 2006; Yang *et al.*, 2007). Bone cells express DSPP, but at a much lower level than odontoblasts (Qin *et al.*, 2002). Pulp tissue expressed high DSPP levels that were > 42,000-fold than those in mandible BMSC cultures. In contrast, long-bone BMSCs expressed DSPP levels (1.12-fold) very similar to those in mandible BMSCs (Fig. 1B). Analysis of these data suggests that mandible BMSC contamination with pulp cells, if any, was minimal. For all remaining experiments, DSPP levels were determined.

To begin characterizing CFU-F numbers in BMSCs from mandible *vs.* long bone, we grew cells for 1 wk in α-MEM and counted colonies. Mandible BMSC cultures displayed significantly more total colonies and more ALP-positive colonies than their long-bone counterparts (Fig. 1C), suggesting increased CFU-F numbers in mandible marrow.

Mandible BMSCs Have a Higher Osteoblastic Potential Compared with Long-bone BMSCs

To evaluate the osteogenic differentiation potential of mandible *vs.* long-bone BMSCs, we cultured confluent cells in osteogenic media for 0-21 days. ALP staining was stronger in mandible BMSCs from the beginning of the culture (3 days), continuing through the entire experiment (21 days; Fig. 2A). Von Kossa assay showed significant mineral deposition in mandible BMSCs compared with long-bone BMSCs (Fig. 2B). ALP activity paralleled ALP staining, demonstrating significantly higher levels for

mandible *vs.* long-bone BMSCs (Fig. 2C). Mineralization quantification was also tested by [45]Ca assays that, similar to von Kossa, showed higher calcium deposition in mandible BMSC cultures (Fig. 2D). For further investigation of BMSC osteoblastic potential, ALP and osteocalcin (OCN) mRNA levels were examined. Mandible BMSCs demonstrated significantly higher ALP and OCN expression at 3 and 7 days (Figs. 2E, 2F). Analysis of these data, collectively, demonstrates that mandible BMSCs possess higher osteogenic potential compared with their long-bone counterparts.

In vivo-increased Osteogenic Potential of Mandible *vs.* Long-bone BMSCs

For determination of whether *in vitro* differences are recapitulated *in vivo,* BMSCs seeded on gelatin sponges for 3 days or gelatin sponges alone were implanted subcutaneously into nude mice for 6 wks (Pettway *et al.,* 2005). At the end of the experiment, sponges alone did not produce a radiographic image (data not shown), suggesting absence of mineralization. Representative 3D-reconstructed microCT images of long-bone (Fig. 3A) and mandible (Fig. 3B) BMSC-seeded sponges are shown. Mandible BMSC sponges were consistently larger and more calcified than long-bone BMSC sponges. Tissue volume (TV) and bone volume (BV) quantification further demonstrate the significantly greater ability of mandible BMSCs to form larger and more calcified nodules than long-bone BMSCs *in vivo* (Figs. 3C, 3D).

Then, transplants were decalcified, and H&E sections were performed (Fig. 4). Sponges without BMSCs demonstrated a thin fibrous capsule only, without any indication of bone formation (data not shown). All sponges seeded with long-bone or mandible marrow cells supported bone formation. Mandible BMSC sponges showed increased and more mature lamellar bone (Fig. 4D) compared with long-bone BMSC sponges (Fig. 4A). Marked osteoblastic rimming of bony trabeculae and bone marrow was seen only in mandible BMSC sponges (Fig. 4E). On high magnification, orderly lines of lamellar bone formation with osteocytes were also observed (Fig. 4F). In contrast, long-bone BMSC sponges showed a primarily cartilaginous matrix (purple in Figs. 4B, 4C), with only peripheral bone formation. Analysis of these *in vivo* data further complements the *in vitro* data demonstrating that mandible BMSCs have higher osteoblastic potential compared with long-bone BMSCs.

DISCUSSION

Systemic diseases, such as osteoporosis, hyperparathyroidism, and Paget's disease, affect all bones, including the jaws (White and Pharoah, 2004). Oral bone metabolism is affected in ovariectomized rodent models of osteoporosis and osteopenia, showing parallel responses in long bones and mandible (Hsieh *et al.,* 1995; Miller *et al.,* 1997). But in response to external stimuli, including ovariectomy and malnutrition, the mandible loses significantly less bone than the proximal tibia (Mavropoulos *et al.,* 2007). In addition, cherubism (Ueki *et al.,* 2001), hyperparathyroid jaw tumor syndrome (Simonds *et al.,* 2002), and, more recently, bisphosphonate-related osteonecrosis of the jaws (BRONJ) (Ruggiero *et al.,* 2004) affect only the maxilla and mandible, suggesting different homeostatic mechanisms between the jaws
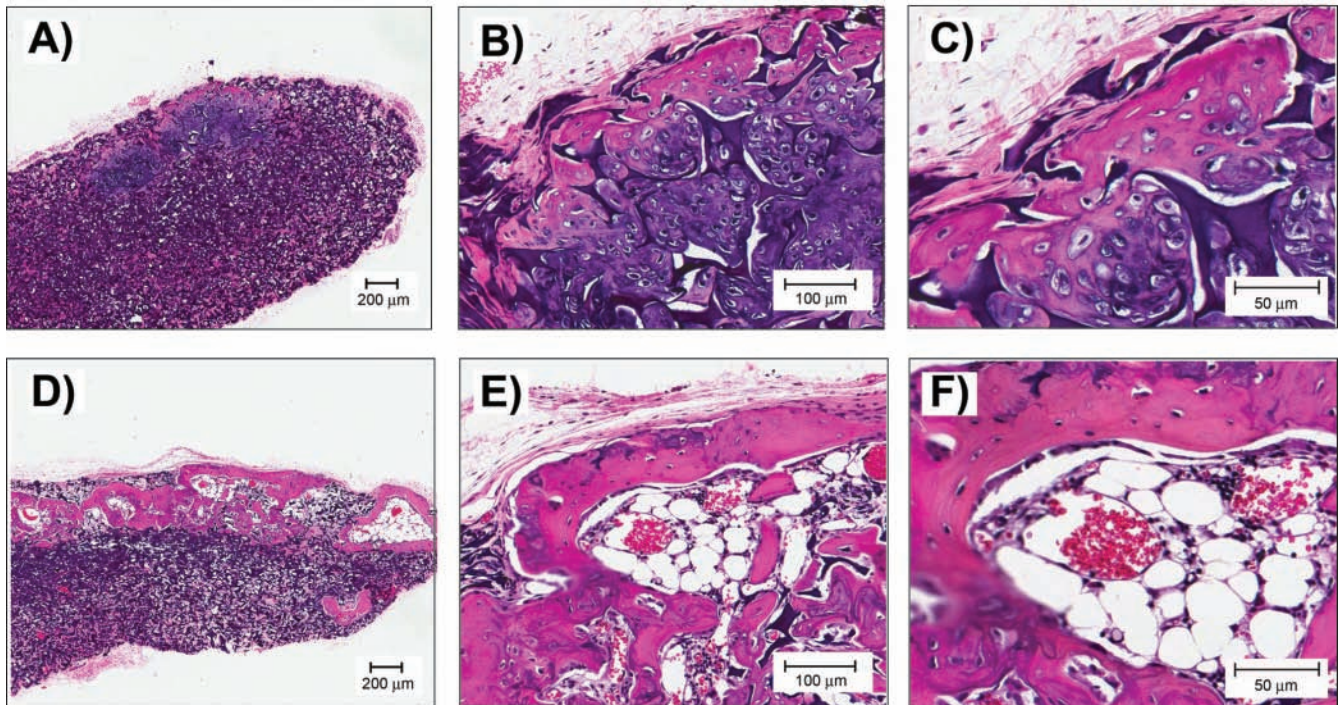
Figure 4. H&E sections at 2X **(A,D)**, 10X **(B,E)**, and 20X **(C,F)** of sponges seeded with long-bone marrow cells **(A,B,C)** or sponges seeded with mandible marrow cells **(D,E,F)** and implanted at the intrascapular area of nude mice.

and long bones. This is further demonstrated by differences in mandibular mechanical loading during mastication (Mavropoulos *et al.,* 2004). In fact, forces generated during walking are almost half those to which alveolar bone is exposed during mastication (Knoell, 1977; Daegling and Hylander, 1997).

Although craniofacial and long bones are similar histologically (Leucht *et al.,* 2008), the craniofacial skeleton arises from neural crest cells that migrate ventrolaterally to the branchial arches (Chai *et al.,* 2000). During neural crest cell migration, growth factors and their signaling pathways determine neural crest differentiation into functional cells forming craniofacial structures primarily *via* intramembranous ossification (Chai *et al.,* 2000). In contrast, the axial and appendicular skeleton arises from mesenchymal condensations of mesoderm that undergo chondrocytic differentiation (Mackie *et al.,* 2008). Taken together, *in vivo* evidence of varied responses to external stimuli, coupled with development of the mandible *vs.* long bones from different germ layers, encourages study to evaluate differences between mandible and long bones.

A better understanding of the diseases and processes affecting the jaws would be significantly improved by an animal system to study cellular and molecular processes *in vitro*. Here, we established a protocol for isolation and culture of rat mandible BMSCs, to test our hypothesis that mandible *vs.* long-bone BMSCs possess distinct osteogenic differentiation potential. Isolated mandible BMSCs differentiate, under appropriate conditions, toward an osteoblastic phenotype, expressing osteoblastic markers and forming mineralized nodules. Interestingly, ALP staining and activity were stronger in mandible BMSCs compared with long-bone BMSCs. Von Kossa and [45]Ca also showed increased mineral deposition in mandible *vs.* long-bone BMSCs. Furthermore, mandible BMSCs demonstrated significantly increased ALP and OCN

mRNA expression. Analysis of these data, collectively, demonstrates that mandible BMSCs possess higher osteogenic potential compared with their long-bone counterparts. Our findings are consistent with those of studies utilizing trabecular bone from patient extraction sites, and showing that orofacial BMSCs demonstrate increased osteogenic differentiation compared with iliac-crest-derived BMSCs from the same individuals (Akintoye *et al.,* 2006). Furthermore, a recent study with labeled neural-crest- and mesoderm-derived cells demonstrated bone defect healing through selective recruitment of cells from their specific embryonic origin (Leucht *et al.,* 2008), reinforcing site-specific differences in BMSCs from the craniofacial *vs.* appendicular skeleton.

To investigate whether *in vitro*-observed differences between mandible *vs.* long-bone BMSCs osteogenic differentiation is reproducible *in vivo,* we used an ectopic bone formation model (Krebsbach *et al.,* 1997; Kuznetsov *et al.,* 1997; Pettway *et al.,* 2005; Akintoye *et al.,* 2006). Differences between mandible and long-bone BMSCs *in vitro* were recapitulated *in vivo*. BMSCs implanted into nude mice underwent osteogenic differentiation and developed mineralized nodules. MicroCT qualitatively and quantitatively showed larger and more calcified structures from mandible *vs.* long-bone BMSCs. These data were confirmed by histology, which revealed increased and more mature lamellar bone derived from mandible BMSC sponges.

Analysis of our data strongly supports increased osteogenic potential of mandible *vs.* long-bone BMSCs both *in vitro* and *in vivo*. The diverse osteogenic potential between mandible- and long-bone-derived cells could be due to inherent differences of BMSCs between these two sites. However, marrow cells consist of a variety of cell lineages. Thus, some of the observed differences between these two cell populations could be due to the differential composition of marrow from mandible *vs.* long

bone. Utilizing this *in vitro* mandible BMSC model system, we detected baseline differences and can now explore detailed characterization of mandible marrow composition, as well as evaluate responses to various external stimuli including hormones, growth factors, and signaling cascades as well as potential differences in bone remodeling and healing.

ACKNOWLEDGMENTS

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