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Similarities and Differences between Porcine Mandibular and Limb Bone Marrow Mesenchymal Stem Cells

Brandon Lloyd^a, Boon Ching Tee^b, Colwyn Headley^c, Hany Emam^d, Susan Mallery^e, and Zongyang Sun^{a,*}

^aDivision of Orthodontics, College of Dentistry, The Ohio State University, Columbus, OH, USA.

^bDivision of Biosciences, College of Dentistry, The Ohio State University, Columbus, OH, USA.

^cBiomedical Sciences Graduate Program, College of Medicine, The Ohio State University, Columbus, OH, USA.

^dDivision of Oral and Maxillofacial Surgery, College of Dentistry, The Ohio State University, Columbus, OH, USA.

^eDivision of Oral and Maxillofacial Pathology and Radiology, College of Dentistry, The Ohio State University, Columbus, OH, USA.

Abstract

Objective—Research has shown promise of using bone marrow mesenchymal stem cells (BMSCs) for craniofacial bone regeneration; yet little is known about the differences of BMSCs from limb and craniofacial bones. This study compared pig mandibular and tibia BMSCs for their *in vitro* proliferation, osteogenic differentiation properties and gene expression.

Design—Bone marrow was aspirated from the tibia and mandible of 3–4 month-old pigs (n=4), followed by BMSC isolation, culture-expansion and characterization by flow cytometry. Proliferation rates were assessed using population doubling times. Osteogenic differentiation was evaluated by alkaline phosphatase activity. Affymetrix porcine microarray was used to compare gene expressions of tibial and mandibular BMSCs, followed by real-time RT-PCR evaluation of certain genes.

Results—Our results showed that BMSCs from both locations expressed MSC markers but not hematopoietic markers. The proliferation and osteogenic differentiation potential of mandibular BMSCs were significantly stronger than those of tibial BMSCs. Microarray analysis identified 404 highly abundant genes, out of which 334 genes were matched between the two locations and annotated into the same functional groups including osteogenesis and angiogenesis, while 70

* **Corresponding author:** Zongyang Sun, Division of Orthodontics, College of Dentistry, The Ohio State University, Rm 4088 Postle Hall, 305 W 12th Ave, Columbus, OH 43210, USA. Tel: 614-247-7944; Fax: 614-688-3077; sun.254@osu.edu. Lloyd and Tee contributed equally to this work

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AUTHOR DISCLOSURE STATEMENT

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genes were mismatched and annotated into different functional groups. In addition, 48 genes were differentially expressed by at least 1.5-fold difference between the two locations, including higher expression of cranial neural crest-related gene BMP-4 in mandibular BMSCs, which was confirmed by real-time RT-PCR.

Conclusions—Altogether, these data indicate that despite strong similarities in gene expression between mandibular and tibial BMSCs, mandibular BMSCs express some genes differently than tibial BMSCs and have a phenotypic profile that may make them advantageous for craniofacial bone regeneration.

Keywords

marrow stromal cells; DNA microarray; craniofacial bone regeneration; BMP-4; osteogenesis

INTRODUCTION

Bony defects of the face and jaws may result from congenital malformations, infections, neoplastic processes, trauma, or from surgical treatment such as dental extraction. Appropriate reconstruction of these defects is functionally necessary and esthetically important, but clinically challenging. The current standard treatment approach, autogenous bone grafts, is effective to a certain degree, but often comes with donor site morbidity. The finite amount of donor bone also greatly limits its practicality (Bauer & Muschler, 2000).

Mesenchymal stem cell (MSC)-based tissue engineering has been extensively studied in recent years as an alternative treatment for craniofacial defects. Although extensive work has confirmed that adult MSCs can be derived from a number of tissues (including periodontium, dental pulp, fat, etc.), bone marrow derived mesenchymal stem cells (BMSCs) were the first described MSCs and remain among the most reliable and relevant sources of MSCs for skeletal regeneration (Friedenstein, Gorskaja, & Kulagina, 1976; Gronthos et al., 2003; Knight & Hankenson, 2013). For years, the iliac crest or long bones such as the tibia and femur have been most commonly utilized to obtain BMSCs for craniofacial regeneration (Cerruti et al., 2007; Kaigler et al., 2013; Kawaguchi et al., 2004; Li, Yan, Lei, Li, & Xiao, 2009; Marei, Nouh, Saad, & Ismail, 2005; Soltan, Smiler, Prasad, & Rohrer, 2007; Xie et al., 2010), but it remains uncertain whether BMSCs from craniofacial bones are more potent for this purpose. Several recent studies isolated BMSCs from the maxilla and mandible by rinsing bone fragments or by flushing cells through the extraction sockets, and compared their properties with those from long bones or iliac crest so far (Aghaloo et al., 2010; Akintoye et al., 2006; Dong et al., 2014). These studies revealed that in both rats (Aghaloo et al., 2010; Dong et al., 2014) and humans (Akintoye et al., 2006), craniofacial BMSCs had greater proliferative and osteogenic capacities than BMSCs from long bones (Aghaloo et al., 2010; Dong et al., 2014) or from the iliac crest (Akintoye et al., 2006).

Embryonically, the development of craniofacial bones differs from that of long bones by having cranial neural crest contribution (Chai et al., 2000). Whether this difference is retained in adult BMSCs and influences their regenerative potential are largely unknown. The aforementioned studies explored differences in the expression of some neural crest,

osteogenic and angiogenic genes (Akintoye et al., 2006; Dong et al., 2014), but no studies have assessed genome-wide differences between craniofacial and long bone BMSCs.

The pig has become a useful preclinical model for studying MSC-assisted mandibular bone regeneration (Liu et al., 2008; Ringe et al., 2002; Sun et al., 2013; Zhou et al., 2007) due to its similarity in mandibular size, anatomy, function and bone metabolism to those of humans (Herring, 1976; Litten-Brown, Corson, & Clarke, 2010). Whether the differences between limb and mandibular BMSCs found from rats and humans are also present in this animal model, is an important question yet to be answered for better choosing BMSC sources. In addition, while previous studies demonstrated that mandibular BMSCs can be obtained from tooth extraction sites, no attempted have been made to obtain BMSCs through direct bone marrow aspiration from the mandible, which would be more practical and provide a greater yield. Therefore, the purposes of this study were to examine the feasibility of bone marrow aspiration from pig mandibles and compare BMSCs from the tibia and the mandible regarding their phenotypic characteristics and gene expressions.

MATERIALS AND METHODS

Animals and bone marrow aspiration

All live animal procedures conducted in this study were carried out according to an animal protocol (2009A0189) approved by The Ohio State University Institutional Laboratory Animal Care and Use Committee (IACUC). Female, 3–4 months of age, Yorkshire pigs were used in this study. Pigs at this age are comparable to pre-teen humans in craniofacial skeletal maturity.

To determine mandibular locations possible for direct bone marrow aspiration, computed tomography (CT) images and histological sections of 6 pigs from a previous independent study (IACUC protocol 2008A0080) were observed (Price et al., 2015). The inferior third of the symphysis, characterized by well-corticated labial and lingual plates housing a large quantity of trabecular bones in between, present a likely source for direct bone aspiration (Fig. 1A and B). Following this observation, a trial bone marrow aspiration was performed at the symphyseal region of a pig, which had been used for another unrelated research project and was about to be sacrificed. This trial session confirmed that bone marrow aspiration from this region is feasible (Fig. 1C).

Subsequently, bone marrow samples were obtained from the mandibular symphyses and the tibiae of 4 pigs following procedures of an approved IACUC protocol (2009A0189). The pigs were utilized by university medical students for training of endoscopic abdominal surgery immediately before bone marrow aspiration. With the pigs maintained under general anesthesia (2–3% isoflurane with 2–5% oxygen through an endotracheal intubation) and placed at a supine position, bone marrow aspirates were first obtained from the mandibular symphyseal region. Briefly, after disinfecting the symphyseal area, a 16-gauge Monoject Illinois needle (Covidien, Mansfield, MA) attached to a 10 mL syringe containing 1 mL heparin (1000 U/mL) was inserted at a location 1 cm above the inferior symphyseal border, and 1 cm away from the symphyseal midline (Fig. 1D). After gaining access to the bone marrow space, bone marrow characterized by a thick, grainy appearance, was aspirated to

the syringe. The syringe was changed after aspirating 4 mL of bone marrow. The angle and depth of the aspiration needle were adjusted one to two times for access of bone marrow. Typically, 16–24 mL bone marrow was aspirated from a single needle insertion. Then, bone marrow was aspirated from the tibia following a previously established procedure (Swindle & Smith, 2015). Briefly, bone marrow at the medial aspect of the proximal end of tibia was accessed using a sterile 16-gauge Monoject Illinois needle. By adjusting the angle and depth of the needle, a total of 16–24 mL bone marrow was aspirated. After bone marrow aspiration, with general anesthesia still maintained, the pigs were euthanized using 125 mg/mL potassium chloride intravenously dosed at 0.5 mg/kg.

Isolation and culture of BMSCs

Following aspiration, bone marrow samples were processed in the laboratory using a technique adapted from an established method for human postnatal skeletal MSCs (Bianco, Kuznetsov, Riminucci, & Gehron Robey, 2006). Each aspirate was combined with alpha-minimum essential medium (α -MEM, Life Technologies, Carlsbad, CA) and then centrifuged. The pellet was re-suspended in growth media that consisted of α -MEM supplemented with 20% heat-inactivated fetal bovine serum (FBS), 1% penicillin-streptomycin, and 1% L-glutamine. All culture supplements were from Life Technologies (Carlsbad, CA) unless stated otherwise. The suspension was passed through a 16 gauge needle twice and then through a cell strainer (70 μ m, BD Biosciences, Bedford, MA) to obtain a single cell suspension. The mixture was then plated in flasks as Passage-0 (P0) cells to incubate at 37 °C with 5% CO₂. On day 4 after aspiration, cell cultures were washed with Dulbecco's phosphate buffered saline (D-PBS) and provided with fresh media. Fresh media was replaced every 3–4 days until the culture reached 70–80% confluence, at which time the cells were passaged using TrypLE to dissociate the cells from the flasks.

Flow cytometry

Surface markers indicative of MSC identity were examined by flow cytometry. Two million cells from each culture were suspended in FACS buffer containing cold D-PBS supplemented with 2% FBS and 0.01% sodium azide and then divided into 200,000 cell samples for staining/analysis. Samples from each site were then stained with the following fluorescent-conjugated monoclonal antibodies specific for mesenchymal stem cell markers: phycoerythrin (PE) anti-CD105 (Acris Antibodies Inc, San Diego, CA), peridinin chlorophyll protein-cyanine (PerCP) anti-CD44 (Biolegend, San Diego, CA), and fluorescein isothiocyanate (FITC) anti-CD90 (Biolegend). Corresponding cell samples were also stained with the following antibodies specific for hematopoietic cell markers: allophycocyanin (APC) anti-CD11b (Biolegend) and PE anti-CD45 (AbD Serotec, Raleigh, NC). Staining occurred in the dark for 45 minutes at 4 °C. Unstained tibia and mandible cell samples were used as negative controls. Expression of markers was accessed using BD LSR II flow cytometer system (BD Biosciences) and FlowJo software (Tree Star, Inc., Ashland, OR). At least 10,000 events were counted for each sample.

Cell proliferation

Mandible and tibia-derived BMSCs (Passage 1–4) were seeded on 12-well tissue culture-treated plates (Corning, NY) at 5,000 cells per well and resuspended in regular growth

media as described above. Cultures were then incubated at 37 °C and 5% CO₂ and designated for harvest according to predetermined time points. Cells were then detached using TrypLE dissociation reagent every two days for two weeks and enumerated using a hemacytometer to record the final cell number. Triplicate measurements of each time point were used and two measurements were made of each sample to minimize measurement error. Remaining wells were fed with fresh growth media every two days until the time point at which they were designated for enumeration. The population doubling time for each culture was then calculated based on the longitudinal cell counting values up to 13 days by using an online calculator (http://www.doubling-time.com/compute_more.php) which uses a least squares fitting exponential regression.

Assessment of osteogenic differentiation

Qualitative assessment—Individual 22 × 22 mm glass microscope coverslips were placed at the bottom of each partition of a 6-well plate (Corning). Tibial and mandibular BMSCs were then seeded at a density of 100,000 cells per well. The cultures were given 2 mL growth media supplemented with fibroblast growth factor 2 (FGF-2; Peprotech, Rocky Hill, NJ), a growth factor reported to be stimulatory for MSC osteogenic differentiation (Ito, Sawada, Fujiwara, & Tsuchiya, 2008; Sun et al., 2013) and proliferation (Yamachika et al., 2012), at 0 ng/mL, 5 ng/mL, or 10 ng/mL. Cells were replaced with fresh corresponding media every three days and kept at 37 °C and 5% CO₂. On day 5 or 10, cells grown on the glass slides were evaluated using the Leukocyte Alkaline Phosphatase Kit (85-L2, Sigma Aldrich®, St Louis, MO) following the manufacturer's protocol.

Quantitative assessment—Similar to the qualitative assessment, tibia and mandible-derived BMSCs were seeded on a tissue-culture treated 6-well plate at 100,000 cells per well, and treated with FGF-2 at 0 ng/mL, 5 ng/mL, or 10 ng/mL. On day 5 or 10, cell samples were rinsed, scraped, and then lysed using 300 µL of 0.2% Triton X-100 solution (Thermo Fisher Scientific, Waltham, MA). The collected cells were vortexed, placed on a shaker for 20 minutes, and then frozen. The mixture was later thawed and alkaline phosphatase (ALP) activity was quantified using the QuantiChrome™ Alkaline Phosphatase Kit (BioAssay Systems, Hayward, CA) according to the manufacturer's protocol.

RNA isolation

RNA extraction was performed on P0 cells cultured in growth media from both mandible and tibia-derived BMSCs isolated from three pigs. After rinsing twice with D-PBS, the cells were detached from the flask using TrypLE. After inactivating trypsin with growth media containing FBS, the cells were centrifuged and counted. One million cells were separated from each culture and were again centrifuged directly afterward so that the media could be aspirated. RNA extraction was performed using the RNeasy Mini Kit according to the manufacturer's protocol (Qiagen, Valencia, CA). The concentration of total RNA was then quantified for each sample by Nanodrop ND-1000 (Thermo Fisher Scientific) according to the manufacturer protocol. RNA quality was also evaluated by assessing the 260/280 and 260/230 ratios on the same machine.

Microarray assay

Three pairs of RNA samples extracted from P0 tibial and mandibular BMSC cultures were used for this assay. The quality of total RNA was confirmed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA) to ensure an RNA integrity number (RIN) above 9. Subsequently, double-stranded cDNA was generated from total RNA, labeled and fragmented with the GeneChip WT Plus reagent kit (Affymetrix, Santa Clara, CA), then hybridized to the Affymetrix GeneChip® Porcine Genome Array which contains 23,937 probe sets to evaluate 20,201 genes. The array was scanned with the GeneChip Scanner 3000, normalized using the RMA algorithm in the Expression Console, and analyzed with the Transcriptome Analysis Console 3.0 (TAC 3.0) (Affymetrix).

Analyzed data collected from TAC 3.0 was sorted for Tukey's bi-weight average signals (log2) to obtain the highly abundant transcriptomes, which have a cut-off point of 10.57, 75% of the highest gene expression of 14.09. Same set of data was then sorted to obtain the differentially expressed genes, which was characterized by the ANOVA. The genes that reached statistical significance ($p < 0.05$) in differential expression between mandibular and tibial BMSCs were further analyzed using a 1.5 fold-of-difference as the cut-off point. The gene symbols were converted to Entrez Gene ID using the Gene Accession Conversion Tool from Database for Annotation, Visualization, and Integrated Discovery (DAVID) v. 6.7. Functional annotation was performed on the highly abundant transcriptomes and differentially expressed genes using the clustering tool in DAVID. Our microarray data are accessible at NCBI Gene Expression Omnibus, <http://www.ncbi.nlm.nih.gov/geo/>, accession number GSE81430.

Real-time reverse-transcription polymerase chain reaction (real-time RT-PCR)

To further test the differential expression of two neural crest related genes (bone morphogenetic protein-4 (BMP-4) and Nestin), real-time RT-PCR was conducted on the RNA samples. Forward/reverse primer sets were designed using Primer-BLAST (NCBI, Bethesda, MD). For Nestin (fragment size 94 base pair (bp), the primer set was: forward, 5'-TCTCTCAGCATCTTGACCCTA-3'; reverse, 3'-TAGGACTCAGGACAGAGAGCAA-5'). For the housekeeping gene β -actin (fragment size 179 bp), the primer set was: forward, 5'-TCCCTGGAGAAGAGCTACGA-3', reverse, 5'-TAGAGGTCCTTGCGGATGTC-3'). The primer set for porcine BMP-4 was commercially available from Qiagen. Using these primer sets, real-time RT-PCR was performed using iQ-SYBR Green Supermix (Bio-Rad, Hercules, CA) and the iCycler (Bio-Rad, Hercules, CA) according to the manufacturer's protocols. Three pairs of cDNA samples from three animals were tested and for each sample, duplicate trials were completed. For each of the genes investigated, the expression was calculated using the comparative C_T method (or 2^{-C_T} method) using β -actin for normalization. The average C_T value obtained from the PCR reaction of each mandible-derived sample was further normalized to the average C_T value obtained from the tibia-derived sample corresponding to the same animal. Gene expression comparison was then calculated between sites (mandible :tibia ratio) for the fold-of-difference.

Statistical analysis

One-way ANOVA tests were used to compare the data collected from microarray. Pearson's tests were used to calculate the coefficient of correlation between mandibular and tibial microarray data. Cell proliferation data (doubling times) were compared using the Mann-Whitney U test. The real-time PCR fold-of-difference (mandible/tibia) data of BMP-4 and Nestin was examined by one-tailed one-sample t-tests against a critical value of 1. All statistical analyses were performed using SPSS v. 20 software (IBM, Chicago, IL) and a significance level of $\alpha < 0.05$ was used.

RESULTS

BMSC isolation, culturing and verification

From both mandibular and tibial bone marrow, cells showing MSC features were isolated and cultured uneventfully. Passage-0 (P0) cells isolated from mandibular bone marrow aspirates were visibly less abundant during the early days of culturing than cells isolated from tibial bone marrow aspirates (Fig. 2A and B). Flow cytometry demonstrated that cells isolated from both locations expressed the MSC surface markers tested, CD105, CD44, and CD90, but not hematopoietic surface markers CD11b and CD45 (Fig. 2C and D).

Proliferation of tibial and mandibular BMSCs

BMSCs isolated from both sites demonstrated active proliferation during early passages (P1-P4) of subculture of the same initial seeding density. Generally, noticeable slowdown in proliferation was not observed until approximately passage 5 for tibial BMSCs and passage 7 for mandibular BMSC cultures. In subpopulations seeded for proliferation measurements, considerable variability was noted between subjects for both mandible and tibia-derived BMSCs. Nevertheless, by calculating the average cell doubling times of subcultures without over-confluence, we found that mandibular BMSCs proliferated consistently faster than that of tibial BMSCs (Fig. 3A). The average cell doubling time calculated based on serial cell number counts during the 13 days of subculture was significantly lower for mandibular BMSCs than tibial BMSCs (Mann-Whitney U test, $p < 0.01$) (Fig. 3B).

Osteogenic differentiation of tibial and mandibular BMSCs with and without FGF-2

With only standard growth media for BMSCs, the cultured cells demonstrated a certain degree of osteogenic differentiation as reflected by positive ALP activity. Qualitatively, the staining for ALP showed that it was stronger at day 10 than day 5, with positive BMSCs forming clusters. At the periphery where fewer BMSCs were present, ALP-positive clusters were less abundant. Regardless of FGF-2 concentration, the ALP staining of mandibular BMSCs (Fig. 4A–C) was stronger than that of tibial BMSCs (Fig. 4D–F). For all trials and at all time points, quantitative measurement of ALP activity was higher for mandibular BMSCs than tibial BMSCs, regardless of presence or absence of FGF-2 (5 ng/mL and 10 ng/mL) (Fig. 4G). Combined, mandibular BMSC ALP activities were significantly higher (factorial ANOVA, $p < 0.001$) than those of tibial origin (Fig. 4H).

Comparison of gene (mRNA) expression through microarray assay

The overall coefficient of correlation between gene expression of BMSCs isolated from both locations was strong and significant (Pearson's test, $r = 0.99$, $p < 0.001$). When correlation tests were conducted on differentially expressed genes, the degree of correlation reduced with the increase of the cut-off point for the fold-of-difference. More specifically, the correlation was 0.84 ($p < 0.001$), and 0.48 ($p = 0.82$) for cut-off criterion for 1.5, and 2.0 fold of difference, respectively (Fig. 5A).

Comparison of gene expression between mandibular and tibial BMSCs is presented in Fig. 5B and C. When the 1.5-fold of difference criterion was used, the expression profiles of BMSCs isolated from the mandible and the tibia could be categorized into distinctive clusters (Fig. 5B). Although a total of 372 annotated genes reached statistical difference in expression ($p < 0.05$), at a 1.5 fold-of-difference cut-off criterion, only a total of 48 genes of them remained. They consisted of 25 down-regulated and 23 up-regulated genes. Two neural crest-related genes BMP-4 and Nestin showed significantly higher expression in mandibular BMSCs than in tibial BMSCs, but only BMP-4 had a fold of difference (1.79) that is above the 1.5-fold criterion (Fig. 5C). Real-time PCR confirmed the tendency of higher expression of BMP-4 and Nestin in mandibular BMSCs, but the difference only reached significance for BMP4 (Fig. 5D).

DAVID functional annotation of gene expression is shown in Table 1 and 2. First, highly abundant transcriptomes, defined as genes with an average log2 expression above 10.57, were categorized into functional groups (Table 1). There were 404 highly abundant transcriptomes expressed in mandibular and tibial BMSCs. Among these genes, only 17.33% (70/404 genes) were mismatched. Using DAVID's functional annotation clustering tool, it was found that both mandibular and tibial BMSCs were significantly enriched in the same biological processes. In addition to those essential for cell function including protein translation, extracellular matrix organization, cellular respiration, cytoskeleton organization and cells motion, genes related to skeletal and blood vessel development were also highly expressed (Table 1). All of these genes were significantly enriched even after Benjamin-Hochberg FDR correction (EASE score = 0.05, $FDR < 0.05$).

Next, the differentially expressed genes between mandibular and tibial BMSCs were analyzed (Table 2). Compared to the highly abundant transcriptomes described above, more functional groups were annotated but fewer genes were found for each functional group. Combined, the enrichment scores for genes associated with these different functional groups between mandibular and tibial BMSCs were barely below 0.05, which became insignificant after Benjamini-Hochberg FDR correction.

DISCUSSION

The purposes of this study were to examine the feasibility of bone marrow aspiration from pig mandibles and to compare BMSCs from the tibia and the mandible regarding their phenotypic characteristics and gene expression. While bone marrow aspiration from pig tibiae or iliac crests has been routinely and consistently performed by many researchers, this study for the first time established a method to aspirate bone marrow from the pig mandible.

Various studies have described isolating MSCs from a number of craniofacial sources including the dental pulp (Gronthos et al., 2002; Yasui et al., 2016) and the periodontal ligament (Prateetongkum, Klingelhoff, Muller, Ettl, & Morsczeck, 2016; Seo et al., 2004), but only three studies so far have attempted to obtain MSCs from craniofacial bone marrow (Aghaloo et al., 2010; Akintoye et al., 2006; Dong et al., 2014), and none of them was through direct bone marrow aspiration. This is likely because of the small size and irregular shape of craniofacial bones relative to long bones have led to the belief that marrow volume in the jaw bones is sparsely distributed and hard to aspirate. Thus previous investigators who attempted to isolate BMSCs from craniofacial bones have done so by rinsing trabecular bone fragments recovered at human third molar extractions sites (Akintoye et al., 2006) or by flushing bone marrow from the superior alveolar ridge through extraction sockets (Aghaloo et al., 2010; Dong et al., 2014) in rats.

Our data demonstrated that 10–18 mL bone marrow can be readily aspirated from the mandibular symphyseal region of 3- to 4-month-old female pigs through a single needle insertion. Although the abundance of bone marrow in the mandibular symphyseal region appears to be smaller than that from the tibia, 10–18 mL of bone marrow was adequate for subsequent isolation and expansion of BMSCs. In another project (data not shown), which involved 3 bone marrow aspirations on 1-week intervals from the mandibular symphysis of the same pigs, demonstrated no complications developed around the aspiration sites, further confirming that bone marrow in this region can be quickly replenished and the procedure is safe. Recently, we have found bone marrow aspiration from the symphysis in 5-month-old pigs became much more difficult and less productive because of substantial thickening of the labial cortical bone, suggesting that for female pigs, this approach may be only applicable to 4 months or younger pigs. For male pigs, this age limit needs to be ascertained. Additionally, compared to the pig mandibular symphysis, the human counterpart has similar size, anatomy as well as radiographic and histological features, but the feasibility and age limit of bone marrow aspiration from human mandibular symphysis are yet to be investigated.

Through flow cytometry analysis, we confirmed that the cells isolated from the mandibular bone marrow are indeed MSC, similar to those isolated from the tibia. More specifically, both mandible and tibia BMSCs strongly expressed CD105/endoglin, CD90, and CD44 (surface markers of MSCs) and lacked expression of CD11b and CD45, surface markers expressed by hematopoietic stem cells. These data corroborate findings from other investigators who isolated BMSCs from pig and human iliac crest and long bones and found them to be positive for CD105, CD44, and CD90 (Bruckner et al., 2013; Noort et al., 2012), while negative for CD11b (Colter, Class, DiGirolamo, & Prockop, 2000) and CD45 (Bruckner et al., 2013; Colter et al., 2000; Noort et al., 2012; Pittenger et al., 1999). Unfortunately, previous studies that collected mandibular BMSCs from extraction sites (Aghaloo et al., 2010; Akintoye et al., 2006; Dong et al., 2014) did not examine these markers, thus no direct comparison can be made.

Rapid and reproducible proliferation is an important property of MSCs needed for tissue engineering. Through a series of cell culturing and passaging experiments, we found that mandibular BMSCs proliferated significantly faster than tibial BMSCs. The mandibular BMSCs retained proliferative growth for longer durations than the tibial BMSC through

later passages. These findings are consistent with those reported by other groups (Aghaloo et al., 2010; Akintoye et al., 2006; Dong et al., 2014), who phenotypically compared craniofacial and lower extremities BMSCs from humans or rats. Specifically, Akintoye's group evaluated the proliferative capacity of human BMSCs isolated from the mandible, maxilla, and iliac crest cells over the same time period (14 days) and showed that both maxillary and mandibular BMSCs proliferated faster than iliac crest BMSCs (Akintoye et al., 2006). They also reported delayed senescence of orofacial-derived BMSCs compared to iliac crest BMSCs. Similarly, rat mandibular BMSCs exhibited stronger proliferation and anti-apoptotic potentials as compared to long bone BMSCs (Aghaloo et al., 2010; Dong et al., 2014). Morphologically, the iliac crest is a flat bone, while the tibia is a long bone. Anatomically, however, both the iliac crest and the tibia are bones of the lower extremity, and embryonically they share the same mesoderm origin. On the other hand, the jaws are craniofacial bones with both the mesoderm and cranial neural crest cells contributing to their embryonic development (Chai et al., 2000). More importantly, cells expressing neural crest markers are known to be more pluripotent, a feature believed to contribute to the survival of MSCs in hypoxia after transplantation (Millman, Tan, & Colton, 2009; Pan, Cai, Li, Liu, & Izpisua Belmonte, 2013). Therefore, despite the difference in research subjects between the present study and the Akintoye et al.'s and Dong et al.'s studies (Akintoye et al., 2006; Dong et al., 2014), the combined data suggest that the cranial neural crest-derived BMSCs have a stronger proliferation potential than those from their mesodermal-derived counterparts in the limbs.

The ability of osteogenic differentiation is another important property of BMSCs for skeletal tissue engineering. In the present study, osteogenic capacity was compared by assessing ALP activity, an indicator for early osteogenic differentiation. Previous studies including those from our lab have evaluated osteogenic capacity of BMSCs in varied ways (Aghaloo et al., 2010; Fiorentini, Granchi, Leonardi, Baldini, & Ciapetti, 2011; Sun et al., 2013; Wei et al., 2012). Typically, the cultured cells were induced by supplementing the culturing media with ascorbic acid, dexamethasone, and β -glycerophosphate or using other commercially developed kits. As we previously found, however, even without osteogenic induction, the cultured BMSCs demonstrated a certain degree of ALP activity (Sun et al., 2013), suggesting that some of the replicated cells may be committed to the osteogenic lineage during *in vitro* expansion. This was confirmed in the current study, and the mandibular BMSCs appear to be stronger ALP producers than tibia BMSCs. We also found that these site-related differences were not changed by the addition of FGF-2. Previously we showed that when FGF-2 was used together with osteogenic media, osteogenic differentiation measured by ALP activity and Runx-2 expression was enhanced (Sun et al., 2013). Other investigators have also shown that FGF-2 at concentrations as low as 1 ng/mL can increase ALP activity in human MSCs placed in osteogenic media (Ito et al., 2008). The present data, however, seem to suggest that FGF-2 by itself does not act as an osteogenic inducer for either mandibular or tibial BMSCs. This may indicate that osteogenic inducers are required for FGF-2 to augment ALP activity in these cells and additional studies are warranted to evaluate this speculation. In a related study on rats, Aghaloo et al. and Dong et al. observed significantly greater ALP activity and osteocalcin expression in mandibular BMSCs than long bone BMSCs (Aghaloo et al., 2010; Dong et al., 2014). The same trend of enhanced

osteogenic potential of craniofacial-derived BMSCs was also reported in humans using small marrow samples collected by flushing third molar extraction sites (Akintoye et al., 2006). Therefore, although we tested ALP activity without the addition of common osteogenic inducers while the other two groups did use osteogenic media, our findings are consistent with theirs. Combined, these data suggest that craniofacial BMSCs are likely advantageous to long bone BMSCs in terms of the ability of osteogenic differentiation. It is worth noting that our present data only reveal a small part of the osteogenic capacity of BMSCs as we only studied ALP activity. More studies are needed to examine the expression of markers indicative of middle and late stage osteogenic differentiation such as osteopontin and osteocalcin, respectively (Uchiyama et al., 2011; Weinreb, Shinar, & Rodan, 1990; Yoshikawa et al., 1992), as well as type I collagen and mineral production.

To assess whether this difference in proliferation and osteogenic differentiation is relevant to variation in gene expression we conducted a microarray assay. No previous study has directly compared gene expression between mandibular and long bone BMSCs using microarray, thus our data shed the first light on the similarity and difference of gene expression between these two sources of cells. As for the similarity, our data demonstrated that BMSCs from these two sources are strongly correlated (Fig. 5A) in gene expression. Notably, the highly abundant transcriptomes expressed in mandibular and tibial BMSCs were mostly matched, with only 17.33% genes mismatched. More importantly, both mandibular and tibial BMSCs were enriched in genes controlling processes of ossification and angiogenesis in addition to those needed for cell function such as extracellular matrix organization and cytoskeletal functions. Notably, they remain significant after Benjamini–Hochberg false discovery rate (FDR) corrections (Table 1). These findings thus confirm that both mandibular and tibial BMSCs, at least at the transcriptome level, strongly express genes related to osteogenesis and angiogenesis which are crucial processes needed for bone regeneration. Our findings of gene expression in tibial BMSCs are also similar to those reported by Monaco et al. (Monaco, Bionaz, Rodriguez-Zas, Hurley, & Wheeler, 2012), who conducted microarray analysis to compare porcine BMSCs isolated from long bones and adipose tissues. Specifically, they found that long bone BMSCs have higher angiogenic, osteogenic, migratory, and neurogenic capacity compared to adipose-derived MSCs (Monaco et al., 2012).

As for the difference in gene expression, only 48 genes demonstrated >1.5 fold of differences, and 14 genes demonstrated >2 fold of differences, between the two locations. This again confirmed that gene expression of mandibular BMSCs and tibial BMSCs only have relatively minor differences at the transcriptome level. Nevertheless, with the 1.5-fold difference criterion, the hierarchical clustering revealed some incongruence between these two sources of cells (Fig. 5B). DAVID analysis further categorized the gene profile into functional groups, which showed some moderate differences between mandibular and tibial BMSCs (Table 2). Specifically, mandibular BMSCs tended to be enriched in neurological processes and protein dimerization activity. In particular, two of the four genes involved in the protein dimerization activity, BMP-4 and activating transcription factor (ATF6), are both related to cell survival (Teodoro, Odisho, Sidorova, & Volchuk, 2012; Ueki & Reh, 2012) and osteogenesis (Jang et al., 2012; Valcourt & Moustakas, 2005). BMP-4 is also specifically a neural-crest gene, and was found critical for stem cell renewal and maintaining

pluripotency. Nestin was another noteworthy upregulated neural crest marker in mandibular BMSCs, but only has a fold change of 1.23. It is also essential for cell proliferation and migration, while maintaining the stemness of the cells (Suzuki, Namiki, Shibata, Mastuzaki, & Okano, 2010; Xue & Yuan, 2010). In contrast, tibial BMSCs were enriched in cellular component lysosome, which is an organelle involved in the terminal steps of apoptosis (Ivanova et al., 2008). These distinctive clusters, although categorized at a less stringent criterion (1.5 fold instead of 2 fold of difference), at least demonstrate a tendency that mandibular BMSCs have a higher chance of survival and osteogenic potential, while tibial BMSCs may be more prone to apoptosis. Combined, these differential gene expression, may contribute to the difference in proliferation and osteogenic differentiation found between mandibular and tibial BMSCs.

While microarray analysis is highly efficient in screening thousands of genes, precautions are warranted for the findings and interpretations. One of such precautions is that the data only reflect gene expression at the transcription level, thus the phenotypical differences shown in proliferation and osteogenic differentiation may be contributed by different or additional genes shown by microarray assay. The other precaution is that only a limited number of genes were retested by using real-time RT-PCR. Although the results indeed confirmed the trend that was reflected by the microarray assay, that is, both BMP-4 and Nestin tended to have stronger expression in mandibular BMSCs than tibial BMSCs, we cannot rule out that there may be other genes involved. This is especially true given that the differences of gene expression between the two sources were not significant based real-time RT-PCR tests. Lastly, although BMP-4 and Nestin have been used as neural crest markers, their unique function in human development continues to be researched. BMP-4 is essential for differentiation of mesoderm, including development of craniofacial and appendicular bone, and knockouts of this gene result in embryonic lethality (Wang et al., 2014). Nestin, an intermediate filament protein, is expressed predominantly in rapidly dividing cells of developing and regenerating tissues and is understood to play an important role in central and peripheral nervous system development (Chen et al., 2006; Michalczyk & Ziman, 2005). However, the exact role and the extent that these genes regulate mandibular BMSC proliferation and osteogenic differentiation need further clarification, likely by transgenic techniques.

In conclusion, this study confirmed that BMSCs are readily obtainable from the pig mandible and demonstrates that MSCs from this location have superior proliferation and osteogenic differentiation capacity to those of tibial BMSCs, which suggests that they may be a better cell source for craniofacial bone regeneration. Our data demonstrated that although gene expression at the transcription level is generally similar between mandibular and tibial BMSCs, there are a number of genes that are differentially expressed, including cranial neural crest-related genes BMP-4 and Nestin, which tend to be more strongly expressed in mandibular BMSCs. Their exact contributions and mechanisms attributable for phenotypical differences are yet to be ascertained.

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Abbreviations

BMSCs	bone marrow mesenchymal stem cells
IACUC	Institutional Laboratory Animal Care and Use Committee
α-MEM	alpha-minimum essential medium
P	Passage
ALP	alkaline phosphatase
CT	computed tomography
FGF-2	fibroblast growth factor 2
BMP-4	bone morphogenetic protein-4
PE	phycoerythrin
PerCP	peridinin chlorophyll protein-cyanine
FITC	fluorescein isothiocyanate
APC	allophycocyanin
TAC	Transcriptome Analysis Console

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HIGHLIGHTS

- We established the feasibility/method to aspirate bone marrow from pig mandibles.
- Mandibular BMSCs proliferate faster than tibial BMSCs *in vitro*.
- Mandibular BMSCs demonstrate stronger osteogenic differentiation than tibial BMSCs.
- Overall mandibular and tibial BMSCs have highly similar gene expression profiles.
- Mandibular BMSCs express higher *BMP4* and *Nestin* than tibial BMSCs.

Figure 1. Bone marrow aspiration from the pig mandibular symphyseal region

(A) A representative CT image in the sagittal view showing a large marrow space and porous low-density bones inside of the bulged symphyseal process. (B) Histological section stained with H&E showing abundant trabecular bone inside of the bulged symphyseal process. (C) With the pig anesthetized and the symphyseal area disinfected, the symphyseal marrow space was accessed and bone marrow was gradually aspirated into syringes prefilled with heparin. (D) Volumetric surface reconstruction of clinical CT images showing an optimal site for inserting the aspiration needle (arrow), which was 1 cm away from the symphyseal midline and 1 cm above the inferior symphyseal border. The boxes in (A) and (B) and the arrow in (D) indicate the approximate site where bone marrow was aspirated.

Figure 2. Confirmation of MSC identity

(A–B) Brightfield microscopy image of cultured Passage-0 (P0) mandibular BMSCs at day 5 and 10, respectively. (C–D) Brightfield microscopy image of cultured P0 tibial BMSCs at day 5 and 10, respectively. Note P0 mandibular BMSCs tended to be fewer than P0 tibial BMSCs at the beginning but quickly proliferated to comparable levels. (E–F) Flow cytometry analysis showed both mandibular (E) and tibial BMSC (F) were positive for MSC markers CD105, CD90, and CD44, but negative for hematopoietic stem cell markers CD11b and CD45. Scale bar, 200 μ m.

Figure 3. Proliferation during *in vitro* expansion (Passages 1–4)

(A) Mandibular BMSCs demonstrated a higher cell proliferation rate than tibial BMSCs. **(B)**

The mean cell doubling time of mandibular BMSCs was significantly shorter than that of tibial BMSCs (*, $p < 0.01$). Error bars, standard deviations.

Figure 4. Osteogenic differentiation capacity

(A–F) Representative images of ALP staining at 10-day culture of mandibular (A–C) and tibial BMSC (D–F) in regular growth media supplemented with 0 ng (A, D), 5 ng (B, E) and 10 ng (C, F) FGF-2. (G) ALP activity assay at 5- and 10-day cultures of mandibular and tibia BMSCs in the absence or presence of FGF-2. Note the mean ALP activity was higher in mandibular BMSCs than in tibial BMSCs regardless of the FGF-2 concentration. Scale bar, 2 mm. Error bars, standard deviations.

Figure 5. Gene expression reflected by microarray assay

(A) Scatter plot of microarray data overall showed strong correlation ($r=0.98$) in gene expression between mandibular and tibial BMSCs. Genes that were differentially expressed between mandibular and tibial BMSCs and genes that were highly expressed are indicated by colored dots. (B) Hierarchical clustering showed that mandibular (label 1; yellow) and tibial (label 2; green) BMSCs (both at passage 0) belonged to distinctive clusters when 1.5-fold of difference in gene expression was set as the cut-off threshold. (C) Volcano plot of the microarray data showed the distribution of differentially expressed genes between mandibular and tibial BMSCs (ANOVA tests, $p < 0.05$). Genes showed above 1.5-fold of difference between mandibular and tibial MSCs are indicated by colored dots. Note neural

crest-related genes BMP4 and Nestin were upregulated in mandibular BMSCs. **(D)** Real-time RT-PCR confirmed stronger BMP4 and Nestin expression in mandibular BMSCs, showing a mandibular BMSCs:tibial BMSCs expression ratio greater than 1 (broken line) . Error bars, standard deviations.

Table 1

Functional annotation of highly expressed genes from different clusters.

The origin of highly expressed genes (number of genes involved)	Biological Processes	Number of genes
Both locations (334)	Translation	27
	Extracellular matrix organization	16
	Cytoskeleton organization	23
	Collagen fibril organization	9
	Blood vessel development	21
	Skeletal system development	18
	Cell motion	29
	Generation of precursor metabolites and energy	20
	Cellular respiration	8
Mandibular BMSCs (35)	Regulation of kinase activity	5
	Response to organic substance	6
	Regulation of phosphorylation	5
	Regulation of translation	3
	Protein complex biogenesis	5
	Regulation of cell proliferation	5
	Cellular response to stress	5
	Regulation of peptidase activity	3
	Positive regulation of catalytic activity	5
	Positive regulation of molecular function	5
Tibial BMSCs (35)	Cell adhesion	6
	Extracellular matrix organization	3
	Immune response	6
	Response to organic substance	6

Table 2

Functional annotation of differentially expressed genes between mandibular and tibial BMSCs.

The origin of differentially expressed genes (number of genes involved)	Category	Gene Ontology terms
Mandibular BMSCs (23)	Molecular Function	Protein dimerization activity
		Identical protein binding
		Protein homodimerization activity
	Biological process	Neurological system process Sensory perception of chemical stimulus
Tibial BMSCs (25)	Cellular Component	Lytic vacuole
		Vacuole