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Methods and insights from the characterization of osteoprogenitor cells of bats (Mammalia: Chiroptera)

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ABSTRACT

Osteoprogenitor cells contribute to the development and maintenance of skeletal tissues. Bats are unique model taxa whose cellular processes are poorly understood, especially in regards to skeletal biology. Forelimb bones of bats, unlike those of terrestrial mammals, bend during flight and function in controlled deformation. As a first step towards understanding the molecular processes governing deposition of this flexible bone matrix, we provide the first method for isolation and differentiation of cell populations derived from the bone marrow and cortical bone of bats, and compare results with those harvested from C57BL/6J mice. Osteogenic capacity of these cells was assessed via absolute quantitative real-time PCR (qPCR) and through quantification of in vitro mineral deposition. Results indicate the differentiated bone cells of bats display significantly lower gene expression of known osteogenic markers (Runt-related transcription factor (*RUNX2*), osteocalcin (*BGLAP*) and osterix (*SP7*)), and deposit a less-mineralized matrix compared with murine controls. By characterizing the in vitro performance of osteoprogenitor cells throughout differentiation and matrix production, this study lays the ground work for in vitro manipulations of bat stem and osteoprogenitor cells and extends our understanding of the cellular diversity across mammals that occupy different habitats.

1. Introduction

Osteoprogenitor cells are ubiquitous, multipotent stromal cells capable of differentiating into multiple cellular lineages [e.g. osteoblasts (responsible for secretion of the extracellular bone matrix) and osteocytes (mature bone cells)], and replenish existing local cell populations (Pittenger et al., 1999; Rahaman and Mao, 2005; Krampera et al., 2006; Maxson et al., 2012; Lee et al., 2013). Their self-renewal and immunosuppressive properties contribute to their importance in the development and maintenance of various tissue types (Nombela-Arrieta et al., 2011; Lee et al., 2013) and their growing potential as therapeutic agents in the field of regenerative medicine (Paquet-Fifield et al., 2009; Williams and Hare, 2011; Maxson et al., 2012; Voswinkle et al., 2013; Reinders and Hoogdujin, 2014).

Cell populations derived from mammalian bone marrow were first isolated from >40 years ago (Friedenstein et al., 1970), and since then marrow cells have been successfully cultured from a wide variety of tissue sources and from an increasing number of species (e.g., Pittenger et al., 1999; Erices et al., 2000; Zvaifler et al., 2000; Zuk et al., 2001; Hatzistergos et al., 2010; Nardi and Camassola, 2011; Baer and Geiger, 2012). Protocols for differentiating osteoprogenitor cell populations into osteoblasts, have been well characterized in model organisms (i.e. mice, rats, and guinea-pigs) (e.g., Caplan, 1991; Pereira et al., 1995; Bruder et al., 1997; Jaiswal and Haynesworth, 1997; Franceschi, 1999). The process of differentiation requires three steps: 1) commitment to the osteoblast lineage, 2) proliferation of the committed cells and, 3) committed cell maturation into matrix-secreting osteoblasts (Yamaguchi et al., 2000). Many questions remain regarding the extent of naturally occurring variation in osteoprogenitor cell biology found in alternative species (e.g., Friedenstein et al., 1970; Pittenger et al., 1999; Di Nicola et al., 2002; Nardi and Camassola, 2011). This study characterizes the in vitro performance of differentiated osteoprogenitor cells of the only flying mammals, bats, and therefore extends our understanding of mammalian cellular diversity.

Bats are the only mammals capable of powered flight. The forelimb wing bones of bats are compliant and capable of withstanding the controlled deformations produced during wingbeats (Swartz and Middleton, 2008; Lucas et al., 2014). Gene and protein sequences of mice and bats share high levels of homology [e.g., bat *FGF8* demonstrates a 97% amino acid sequence conservation the laboratory mouse (Cretekos et al., 2007)]. As a result, past studies have focused on

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characterizing spatiotemporal variations in expression of key limb development genes (e.g., fibroblast growth factor 8 (*FGF8*), bone morphogenic protein 2 (*BMP2*), sonic hedgehog (*SHH*) and *HOXD13*) during wing development (Minina et al., 2001; Sun et al., 2002; Yoon and Lyons, 2004; Chen et al., 2005; Sears et al., 2006; Cretekos et al., 2007; Pajni-Underwood et al., 2007; Cretekos et al., 2008; Behringer et al., 2009; Rasweiler et al., 2009; Cooper and Sears, 2013 and Wang et al., 2014). Beyond characterizing gene expression, some studies have also characterized in vitro performance of organs and fibroblasts (De Luca et al., 2001; Minina et al., 2001; Moratelli et al., 2002; Sears, 2007 & Weatherbee et al., 2006). However, the osteoprogenitor and/or stem cells of bats have yet to be studied in a culture system. This study documents the methods necessary for assays of bat stem cells and may lay the foundation for future comparative studies to delineate unique cellular properties of skeletal development and maintenance in bats.

This study augments existing in vivo molecular methods for the study of stem cells by providing a protocol for the isolation and osteogenic differentiation of bone marrow and cortical bone-derived cells harvested from two bat taxa: Seba's short-tailed bat (*Carollia perspicillata*) and the big brown bat (*Eptesicus fuscus*). *Carollia* is frugivorous, while *Eptesicus* is insectivorous. Our overall goal was to compare the in vitro performance of cells harvested from the cortex and marrow of the forelimb bones of male bats to those harvested from the same bones of male C57BL/6J laboratory mice selected for its well-documented and relatively low bone-mass skeletal phenotype compared to other strains (Beamer et al., 1996; Halloran et al., 2002 and Ferguson et al., 2003).We first isolated cells populations from the cortex and marrow to compare species-specific proliferation rates. After inducing osteogenic differentiation, we harvested cells at three time points (Days 7, 14, and 21) to assess the composition of the extracellular matrix as well as quantify expression levels of genes known to play a role in mineralization of that matrix including bone-specific alkaline phosphatase (*ALPL*), osterix (*SP7*), osteonectin (*ON*), Runt-related transcription factor 2 (*RUNX2*), osteocalcin (*BGLAP*), and osteoprotegrin (*OPG*). Results showed that bats and mice shared similar proliferation rates. In vitro, the cells of bats differed compared to cells of C57BL/6J mice by producing a less mineralized extracellular matrix, and significantly decreasing expression of *RUNX2*, osteonectin, osteocalcin and osterix. Taken together, results suggest the differentiated osteoblasts of bats and mice differ in expression of several genes essential for mineral deposition.

2. Methods

2.1. Isolation and culture of bat osteoprogenitor cells from bone marrow

The Cooper laboratory at Northeast Ohio Medical University (NEOMED; Rootstown, Ohio) maintains colonies of Seba's short-tailed bat (*Carollia perspicillata*), the big brown bat (*Eptesicus fuscus*), and C57BL/6J laboratory mice. *Carollia* breed well in captivity and were transferred from a colony at SUNY Downstate maintained by Drs. Mark Stewart and Rena Orman (Rasweiler and Badwaik, 1996). *Eptesicus* were originally transferred from a colony maintained by Dr. Ellen Covey at the University of Washington. To avoid complications associated with pregnancy-related bone-loss, this study focused on males only. Radii were obtained post-mortem from four week old C57BL/6J mice ($n = 4$). Only adult *Carollia* ($n = 2$) and *Eptesicus* ($n = 2$) were used as known-aged pups were unavailable for both taxa. Cell populations containing osteoprogenitor cells from bone marrow were isolated using previously published protocols (Safadi et al., 2002; Abdelmagid et al., 2007; Moussa et al., 2014). In brief, surrounding tissues were removed and epiphyses of radii were severed with sterile razor blades (Fig. 1). Marrow was flushed with basal growth media consisting of Minimum Essential Medium (MEM) Alpha Medium (Corning Cellgro) with 10% FBS (ThermoScientific), 1% penicillin-streptomycin (Corning Cellgro) and 0.1% Amphotericin B (Corning Cellgro). Supernatant was removed after marrow flush and centrifuged at 1200 rpm at 4 °C for 12 min. Cells were re-suspended in basal growth medium and plated in a 100 mm cell culture dish incubated in humidified incubator at 37 °C with 5% CO_2. Adherent cells were cultured for an additional 7–10 days until confluent with fresh media being added every third day.

2.2. Isolation and culture of bat osteoprogenitor cells from cortical bone

Radii were isolated and marrow flushed as described above. Cortical bone was then crushed and diced into 2–5 mm pieces and digested with a buffered Collagenase B (Roche) medium (PBS, 0.1% Collagenase B, 0.25% FBS) at 37 °C with horizontal shaking for 50 min. The supernatant was collected and filtered through a 70 μm cell strainer (Fisher Scientific) (Fig. 1). Remaining bone fragments were rinsed twice with washing buffer (PBS, 0.25% FBS) and the supernatants collected as described above and pooled. Cells were centrifuged at 1200 rpm at 4 °C for 12 min. Cell pellets were then re-suspended in 10 ml of basal growth

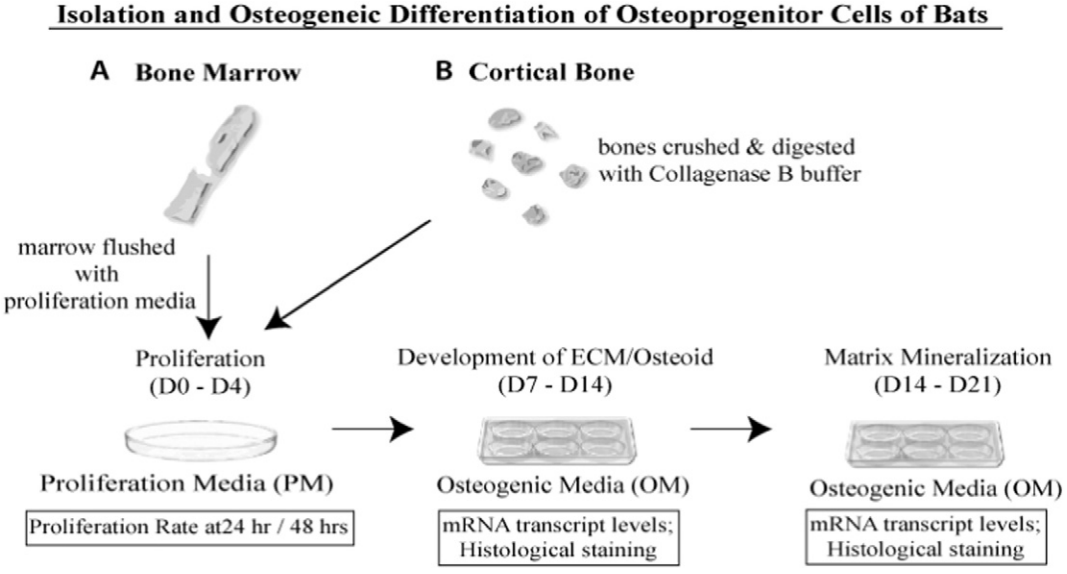

Fig. 1. Protocol for the successful isolation and osteogenic differentiation of osteoprogenitor cells located in the marrow (A) or cortex (B) of radii from two species of bats and C57BL/6J mice.

Table 1
Gene and species-specific qPCR primers.

Gene	Primer	Sequence (5′-3′)	Amplicon size (bp)	Function
Alkaline phosphatase (*ALPL*)	Alp 490F	GTGACTACCACTCGGGTGAAC	95	Indicator of early bone formation.
	Alp 585R	CTCTGGTGGCATCTCGTTATC		
Osterix (*SP7*)	Sp7 28F	ATGGCGTCCTCCCTGCTTG	113	Osteoblast differentiation; bone formation
	Sp7 141R	TGTTGTTGAGTCCCGCAGAG		
Runt-related transcription factor 2 (*RUNX2*)	RUNX2 127F	CCAACCGAGTCATTTAAGGCT	206	Osteoblast differentiation.
	RUNX2 333R	GCTCACGTCGCTCATCTTG		
Osteoprotegerin (*OPG*)	OPG 95F	ACCCAGAAACTGGTCATCAGC	156	Osteoclast differentiation.
	OPG 251R	CTGCAATACACACACTCATCACT		
Osteonectin (*ON*)	ON 728F	ACCTGGACTACATCGGACCA	194	Cell proliferation; cell-ECM interaction.
	ON 922R	CCAGGCGCTTCTCATTCTCA		
Osteocalcin (*BGLAP*)	BGLAP 74F	CCAGTGGTGCAGAGTCTGAG	189	Inorganic ECM formation.
	BGLAP 263R	ATGTGGTCAGCCAGTTCGTC		

medium and incubated in a humidified incubator at 37 °C with 5% CO_2. Non-adherent cells were removed after 24 h, and adherent cells were cultured for an additional 5–7 days, with fresh basal growth medium added every third day, until cells became confluent.

2.3. Differentiation of osteoprogenitor cells

Once confluent, osteoprogenitor cells of both species harvested from bone marrow and cortical bone were passaged. Plates were treated with trypsin EDTA (Corning Cellgro) in PBS and incubated at 37 °C with 5% CO_2 until adherent cells began to detach. Trypsin was then deactivated with the addition of 10 ml of basal growth medium (MEM Alpha medium with 10% FBS (ThermoScientific), 1% penicillin-streptomycin (Corning Cellgro) and 0.1% Amphotericin B (Corning Cellgro)) and centrifuged at 1200 rpm at 4 °C for 12 min. Cell pellets were then resuspended in 10 ml of basal growth medium and counted using a hemocytometer. Cells were plated in 24-well cell culture plates (cell density of 5×10^3 cells/cm^2 (Baer and Geiger, 2012)), incubated at 37 °C with 5% CO2 and allowed to adhere overnight. The basal growth medium was then removed and replaced with osteogenic medium (OM; basal growth medium supplemented with 0.1 μM dextamethasone (Sigma Aldrich), 10 mM β-glycerophosphate (Sigma Aldrich) and 50 μg/ml ascorbic acid (Fisher Scientific; Fig. 1). Cells were incubated in a humidified incubator as described above with fresh OM provided every third day until termination on Days 7, 14 and 21 (Safadi et al., 2002; Abdelmagid et al., 2007; Moussa et al., 2014; Fig. 1).

2.4. Cell proliferation assay

Cell proliferation was examined by DNA synthesis cell proliferation method using CyQUANT® NF Cell Proliferation Assay Kit (Invitrogen). Cell populations from bat and mouse marrow and cortical bone were plated in 96-well plates at a density of 1000 cells per well and allowed to grow under the conditions of 37 °C with 5% CO_2 for 24 and 48 h. Cell proliferation was assessed with the CYQUANT® kit (Invitrogen) following manufacturer recommended protocols for adherent cells. Briefly, growth medium was removed and cells were incubated with 100 μl of the dye binding solution (22 μl of Dye in 10 ml 1X HBSS buffer) at 37 °C with 5% CO_2 for 1 h. Fluorescence intensity was measured at an excitation wavelength of 485 nm with emission at 530 nm using a BioTek plate reader.

2.5. Mineral staining

Matrix mineralization was assessed with Von Kossa and Alizarin Red methods. Von Kossa and Alizarin Red stains identified mineral deposits in the osteoprogenitor cell-secreted extracellular matrix (Wang et al., 2006). For Alizarin Red staining, cells were fixed with formalin (Fisher Scientific) for 1 h. Cells were washed twice with dH_2O and incubated with a 10% Alizarin Red solution (2 g per 100 ml, pH 4.2; Sigma Aldrich) in the dark for 2–5 min. The Alizarin Red solution was aspirated, cells were rinsed three times with dH_2O and a treated with a final wash with 1% sterile PBS (Amresco®). Alizarin Red stained wells were rinsed twice more with dH_2O and imaged. Cells stained using Von Kossa were fixed with formalin as described above and washed three times with dH_2O. Cells were then incubated for 45 min under UV light in a 5% $AgNO_3$ solution (Fisher Scientific). Silver nitrate solution was then aspirated and the cells washed three times with dH_2O. Von Kossa stain was developed using 5% Na_2CO_3 (in 10% formalin; Fisher Scientific) solution for 4 min and the cells were rinsed twice with dH_2O. The stain was fixed with 5% $Na_2S_2O_3$ (Fisher Scientific) for 2 min and the cells rinsed twice with dH_2O. Images of Alizarin Red and Von Kossa stained cells were taken on a Nikon Eclipse Ti microscope with NIS Element software (Nikon).

2.6. RNA extractions and qPCR analysis

Differentiated osteoprogenitor cells harvested from cortex and bone marrow of bats and mice were terminated at Days 7, 14 and 21 and immediately fixed in RNAlater® (Ambion) and stored at −80 °C. Total RNA was isolated under RNAse-free conditions (RNAse OUT™, GBiosciences) following recommended TRI-Reagent® (Ambion) protocols with Turbo DNA-*free*™ treatment (Ambion). RNAs were quantified with a Nanodrop® 2000c spectrophotometer (Nanodrop) and integrity assessed using an ethidium bromide stained 1% agarose gel. cDNA and no reverse transcriptase controls were synthesized following recommended protocols for the High Capacity cDNA Reverse Transcription kit (Applied Biosystems) and products normalized to 50 ng total RNA.

Concentrations of osteoblast-specific mRNA target genes alkaline phosphatase (*ALPL*), osterix (*SP7*), osteonectin (*ON*), Runt-related transcription factor 2 (*RUNX2*), osteocalcin (*BGLAP*), and osteoprotegerin (*OPG*) were amplified from synthesized *Carollia*, *Eptesicus* and C57BL/6J mouse culture cDNAs using in-house designed gene-specific primers and quantified via qPCR analyses (Tables 1 and 2). All target genes demonstrated nucleotide sequence homology of 95% or greater between species. Although relative quantitative real-time PCR (qPCR) is the

Table 2
Accession numbers of *Eptesicus fuscus* and *Carollia perspicillata* sequences used for validation of designed qPCR primers.

Gene	Taxon	Accession numbers
Alkaline phosphatase (*ALPL*)	*Eptesicus fuscus*	KX218235
	Carollia perspicillata	KX218236
Osterix (*SP7*)	*Eptesicus fuscus*	KX228246
	Carollia perspicillata	KX228247
Runt-related transcription factor 2 (*RUNX2*)	*Eptesicus fuscus*	KX218249
	Carollia perspicillata	KX218250
Osteoprotegerin (*OPG*)	*Eptesicus fuscus*	KX218247
	Carollia perspicillata	KX218248
Osteonectin (*ON*)	*Eptesicus fuscus*	KX218245
	Carollia perspicillata	KX218246
Osteocalcin (*BGLAP*)	*Eptesicus fuscus*	KX218237
	Carollia perspicillata	KX218238

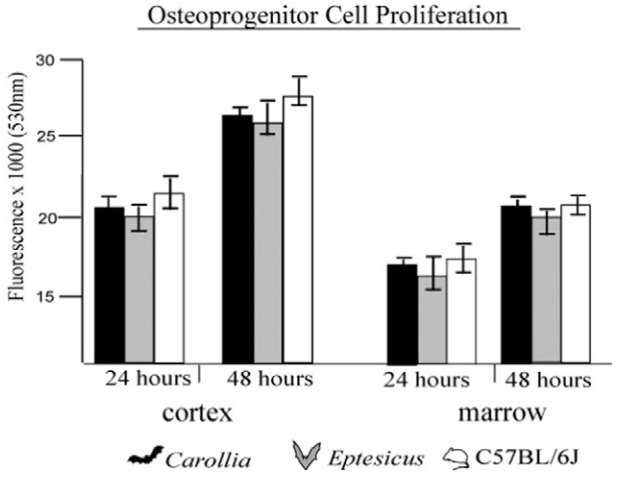

Fig. 2. Cells harvested from cortices of all species proliferate faster in vitro compared to those from bone marrow. Proliferation rates of cortex and marrow osteoprogenitor cells from *Carollia* (black), *Eptesicus* (grey) and C57BL/6J (white) radii, measured by CYQUANT® dye fluorescence.

traditional method for analyzing gene expression variation, known fluctuations in housekeeping gene expression, sample rarity, and difficulties evaluating across species make this method of analysis unreliable (Thellin et al., 1999; Bustin, 2002; Fernandes et al., 2008). Here, we employ validated absolute qPCR techniques utilizing gene-specific standard curves which permit comparisons of transcript copy number within an individual and across species (Ball et al., 2013). All qPCR reactions were run in triplicate on an ABI 7900-HT system (Applied Biosystems)with no reverse transcriptase and primer controls using SYBR® Select Mastermix for CFX (Applied Biosystems).

2.7. Statistical analyses

Gene-specific dilution curves and linear regression equations were generated for each target gene and utilized in analyses of bat target gene expression. Significance of threshold cycle (Ct) values and comparisons of copy number expression were calculated using analysis of variance calculations (ANOVA) and Wald and Wolfowitz tests (Wald and Wolfowitz, 1940). Amplification efficiency was calculated for all standard curve assays (Yuan et al., 2007). Percent area fraction analyses for Von Kossa were performed using ImageJ associated software (NIH), where data are presented as mean ± SEM and $p \leq 0.05$ (Schneider et al., 2012).

3. Results

3.1. Osteoprogenitor cells of bats and mice proliferate at approximately the same rate

Differences in cell proliferation of cortical- and marrow-derived cell populations were assessed at the same passage. No species-specific differences in proliferation rate were detected in samples derived from

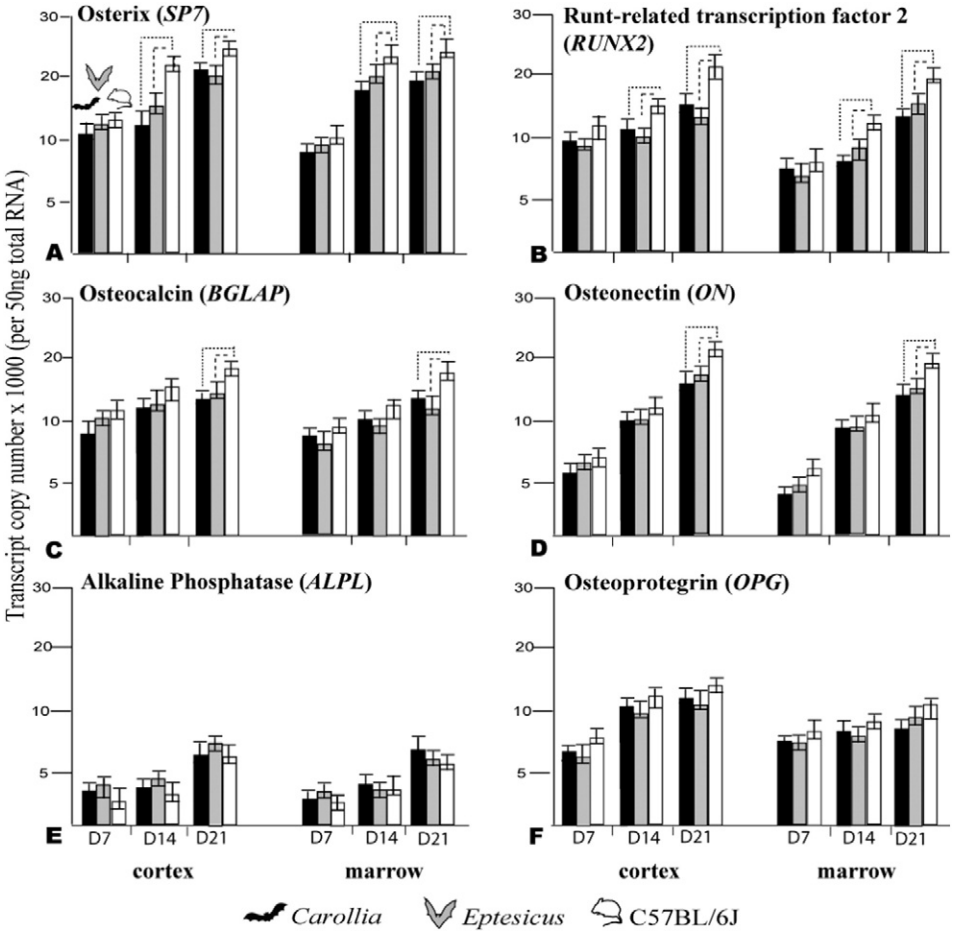

Fig. 3. Cells harvested from bats express significantly lower numbers of transcripts of key osteogenic markers than those of mice. qPCR analyses of differentiated osteoblasts from cortex and marrow of *Carollia* (black), *Eptesicus* (grey) and C57BL/6J (white) radii. mRNA expression was assessed at Days 7, 14 and 21 for A) osterix (*SP7*), B) Runt-related transcription factor 2 (*RUNX2*), C) osteocalcin (*BGLAP*), D) osteonectin (*ON*), E) bone-specific alkaline phosphatase (*ALPL*) and F) osteoprotegerin (*OPG*). Dashed lines denote significant differences (where $p \leq 0.05$) compared to C57BL/6J control.

cortical bone (CB, $p = 0.711$) or marrow (BM, $p = 0.553$). However, differences were seen in the proliferation rates between cortical and medullary derived cells across all taxa. Proliferation rate of cortical bone (CB) osteoprogenitor cells was significantly greater (*Carollia* $p = 0.0386$; *Eptesicus* $p = 0.0374$; C57BL/6J $p = 0.0411$) than those derived from bone-marrow (BM, Fig. 2) across all samples.

3.2. Osteoprogenitor cells of bats display significantly lower transcripts of matrix related genes

Expression levels of matrix related genes in marrow-derived cells that were plated to a final concentration of 5 x 10(Ball et al., 2013) cells per well were assessed at three termination time points (Days 7, 14, and 21). No species-specific differences were found in expression of matrix related genes in these marrow-derived cells until Day 14 (Fig. 3). Absolute qPCR assays at Day 7 showed no significant species-specific differences, and transcript numbers were the lowest of the three termination points (*ALPL* $p = 0.703$, *SP7* $p = 0.694$, *ON* $p = 0.218$, *RUNX2* $p = 0.667$, *BGLAP* $p = 0.361$, *OPG* $p = 0.471$; Fig. 3). Furthermore, no significant differences were seen in the expression of bone-specific alkaline phosphatase (*ALPL*; Day 14 $p = 0.668$ and Day 21 $p = 0.901$) and osteoprotegerin (*OPG*; Day 14 $p = 0.633$ and Day 21 $p = 0.597$) in bone marrow derived cells of bats and mice of in all three sample times (Fig. 3). At Day 14, species-specific differences in expression were not seen in osteocalcin (*BGLAP*, $p = 0.296$) and osteonectin (*ON*, $p = 0.407$). Significant differences in expression of these two genes was seen at Day 21 (*BGLAP*, $p = 0.042$ and *ON*, $p = 0.038$; Fig. 3) with higher transcript levels detected in mice. At Days 14 and 21 significantly lower transcript numbers were detected in the cells harvested from bats and mice in both osterix (*SP7*, $p = 0.047$ and $p = 0.0481$, respectively) and *RUNX2* ($p = 0.043$ and $p = 0.036$, respectively; Fig. 3).

Similarly expression of cortical-bone derived cells (CB) differed between species began at 14 days in some matrix-related genes, and was most pronounced at 21 days. At Day 7 no species-specific differences in expression were detected (*ALPL* $p = 0.618$, *Sp7* $p = 0.602$, *ON* $p = 0.341$, *RUNX2* $p = 0.611$, *BGLAP* $p = 0.220$, *OPG* $p = 0.371$). Species-specific differences in transcript levels were not found between bone-specific alkaline phosphatase (*ALPL*) or osteoprotegrin (*OPG*) on Day 14 ($p = 0.510$ and $p = 0.302$, respectively) or Day 21 ($p = 0.490$ and $p = 0.419$, respectively; Fig. 3). Although transcript numbers of osteocalcin (*BGLAP*) and osteonectin (*ON*) lacked significant differences Day 14 ($p = 0.359$ and $p = 0.407$, respectively), results showed significant differences were detected in both at Day 21 (*BGLAP* $p = 0.031$, *ON* $p = 0.026$; Fig. 3). At Day 21, the differentiated osteoblast of bats displayed significantly lower transcript numbers relative to C57BL/6J murine controls at Days 14 and 21 in both *SP7* ($p = 0.041$ and $p = 0.040$, respectively) and *RUNX2* ($p = 0.045$ and $p = 0.045$, respectively; Fig. 3). Taken together, these data suggest expression associated with mineral deposition in osteoprogenitor cells of bats is reduced compared to that of our murine controls.

3.3. Of bats secrete a less mineralized matrix relative to those of C57BL/6J

Osteoprogenitor-derived osteoblasts secreted mineralized nodules across all samples (Fig. 4A). At Day 14 same-passage cultures, mineralized nodules were visualized using Von Kossa and Alizarin Red stains. The number and size of stained nodules were then compared between taxa. At Day 14, the C57BL/6J-derived cells (controls) showed significantly greater numbers and sizes of mineralized nodules compared to both species of bats, regardless of cite of origin (e.g., marrow, $p = 0.031$; cortical, $p = 0.043$; Fig. 4A). Area fractions of Alizarin Red stained mineral deposits of mouse-derived osteoprogenitor cells were also significantly greater than those of both species of bats, regardless of cite of origin (marrow, $p = 0.043$; cortical, $p = 0.046$; Fig. 4B).

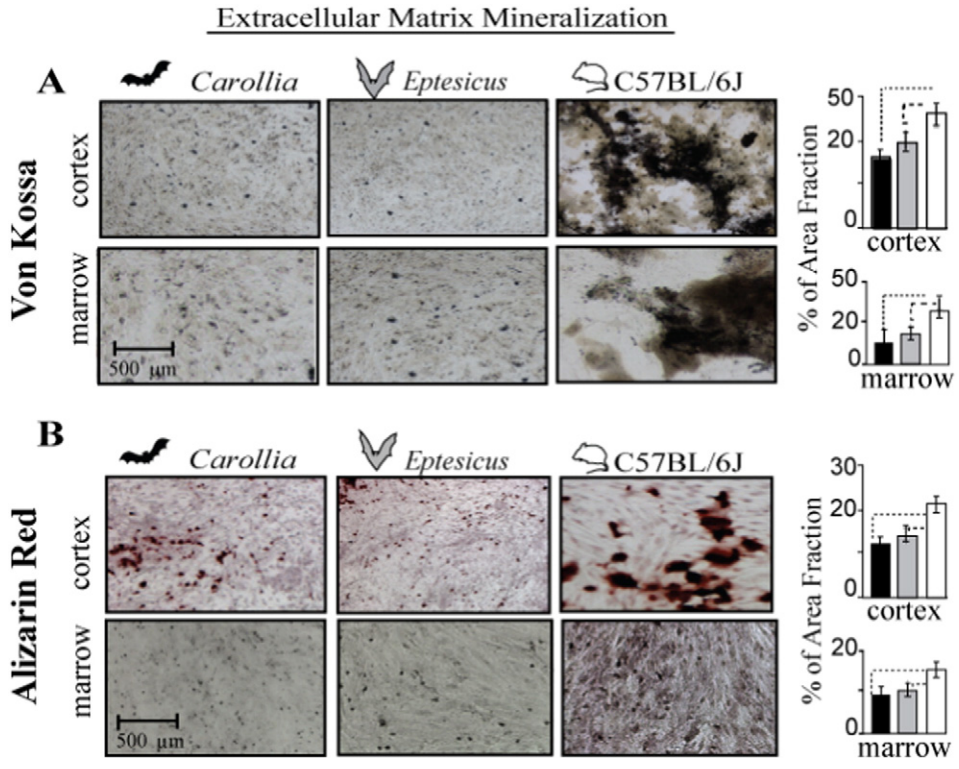

Fig. 4. Von Kossa stain and Alizarin Red staining of extracellular matrix mineralization indicate at Day 14 bats deposit a less mineralized matrix compared to mice. Photomicrographs, taken at 10×, of Von Kossa (A; black indicative of phosphate and calcium deposition) and Alizarin Red (B; red indicative of calcium deposition) staining show more intense staining for mineral in differentiated osteoblasts of mice compared to those of bats. ImageJ analysis quantified percent area fraction for each stain based on three independently sampled experiments of each species (*Carollia*, black; *Eptesicus*, grey and C57BL/6J, white). Data presented as mean + SEM with dashed line denoting $p \leq 0.05$ when compared to same day C57BL/6J controls.

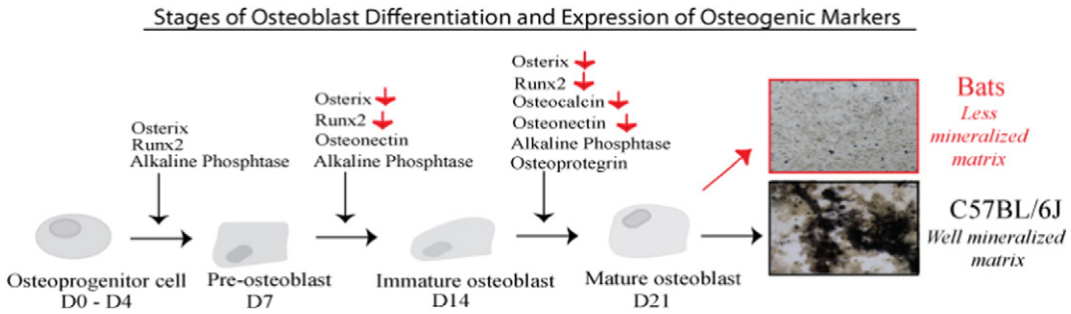

Fig. 5. Bats and mice differ in expression of osteoblast markers and extracellular matrix mineralization. Transcript copy numbers of known osteogenic markers are significantly lower (indicated in red) in bats compared to mouse controls and correlate with a reduction in matrix mineralization demonstrated by 10× magnification photomicrograph of Von Kossa stained Day 14 same passage cultures.

4. Discussion

Methodologies exist for the isolation, cultivation and differentiation of mammalian osteoprogenitor cells from a variety of tissue types and with methods best known for murine cells. However, study of osteoprogenitor and other stem cell types from alternative species may provide vital information regarding naturally occurring variation in cell biology and their ability for adaptation under novel conditions. Bats provide a unique model for just such an examination. While ex vivo studies have limitations, they permit the growth of cell populations and characterization of stem cell biological function. Here, we provide the first known method for the isolation and osteogenic differentiation of cell populations from the cortical bone and bone marrow of bats, and characterize how proliferation rates, osteogenic gene expression, and mineral deposition vary compared to a murine control.

Our method for osteoprogenitor cell culture is similar to protocols for mice (Meirelles and Nardi, 2003; Soleimani and Nadri, 2009; Nardi and Camassola, 2011; Abdelmagid et al., 2014; Fig. 1). Marrow and cortex-derived cell populations of bats and mice shared similar proliferation rates. However, proliferation was greater in cells harvested from cortical bone of bats compared to mice (Fig. 2). Histological staining and quantification of mineral deposition showed cells harvested from bats deposited significantly less mineral compared to murine controls, with the lowest amount deposited by bone marrow-derived bat cells (Fig. 4). Significantly lower transcript numbers of genes associated with bone formation (*ALPL* and *SP7*), osteoblast and osteoclast differentiation (*RUNX2*, *OPG* and *SP7*) and extracellular matrix formation and interaction (*BGLAP* and *ON*) were detected in bats-derived cells relative to murine controls (Stein and Lian, 1993; Yao et al., 1994; Bailey et al., 1999; Delany et al., 2000; Harada and Rodan, 2003; Byers and García, 2004; Tai et al., 2004; Cao et al., 2005; Gregory et al., 2005; Stiehler et al., 2009; Zhang et al., 2009; Gramoun et al., 2010; Korostishevsky et al., 2012; Masrour Roudsari and Mahjoub, 2012; Sardiwal et al., 2012; Sroga and Vashishth, 2012; Pekovits et al., 2013; Koide et al., 2013; Krämer et al., 2014; Krege et al., 2014; Fig. 3). These differences in gene expression were associated with a less mineralized extracellular matrix in bats (Fig. 5). Deposition of a less mineralized extracellular matrix, even in vitro, is suggestive of intrinsic, naturally occurring differences in the auto-regulation of bat osteoprogenitor cell function and performance. These physiological differences suggest regulation of osteoprogenitor cell matrix synthesis differs in bats compared to mice.

The wing bones of bats, including the radius studied here, are elongated, complaint bones (Papadimitriou et al., 1996; Swartz, 1997; Swartz and Middleton, 2008; Bergou et al., 2015). The proximal forelimb elements of bats, relative to terrestrial mammals such as mice, display thinner cortices and the greatest mineral content compared to distal elements (Papadimitriou et al., 1996; Dumont, 2010; Cooper and Sears, 2013). These modifications in length, mineral concentration and extracellular matrix may increase flexibility and create specialized skeletal microenvironments. It may be that the unusually flexible bones of bats impart unique micro-loads on constituent osteoprogenitor and stem cells, as documented in other taxa, e.g., rodents, etc. (Gilbert et al., 2010; Miller et al., 2015). Surprisingly, even in a 2D culture system with equivalent treatments and lacking the stressors associated with locomotion, our study shows the performance of osteoprogenitor cells harvested from bats differed significantly from mice both in their gene expression patterns and matrix production. Taken together, these results suggest that the osteoprogenitor cells of bats display different autoregulation of matrix secretion, compared to that of mice, regardless of microenvironment. Until now, the methods required for the study of bat osteoprogenitor cell activity and/or cell-cell interactions in a culture system were unknown. This study establishes a protocol to successfully isolate and differentiate osteoprogenitor cells from bat cortical bone and bone marrow.

Furthermore, this study also partially lays the foundation for broader comparative studies of the molecular cross-talk between bone cells (osteoclasts, osteoblasts). Imbalances in osteoblast and osteoclast crosstalk lead to imbalanced cell activity that ultimately negatively impacts skeletal health, integrity and repair (Sims and Martin, 2014; Weivoda et al., 2015). Some of these imbalances are associated with senescence-related changes that negatively impact osteoprogenitor and stem cell differentiation rates and decrease stem cell populations leading to age-related skeletal fragility (Muraglia et al., 2000; Janzen et al., 2006; Raggi and Berardi, 2012; Yu and Kang, 2013). Our results suggest that osteoprogenitor cells of bats are intrinsically different from mice in their biology and performance. Future work may extend to quantifying similar characteristics in the hematopoietic stem cells (HSCs) of bats and mice, and therefore allow for comparative studies of age-specific bone cell cross-talk. Results may provide novel insights into potential therapeutic targets for human age-related skeletal disorders including osteoporosis, etc.

Conflict of interest

The authors declare that they have no conflict of interest.

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