Bone 84 (2016) 194–203

Contents lists available at ScienceDirect

Bone

journal homepage: www.elsevier.com/locate/bone

Original Full Length Article

Deficiency of circadian clock protein BMAL1 in mice results in a low bone mass phenotype

William E. Samsa [a], Amit Vasanji [b], Ronald J. Midura [b], Roman V. Kondratov [a,*]

[a] Center for Gene Regulation in Health and Diseases, BGES, Cleveland State University, 2121 Euclid Ave., Cleveland, OH 44115-2214, USA
[b] Department of Biomedical Engineering, Cleveland Clinic, 9500 Euclid Ave., Cleveland, OH 44195, USA

ARTICLE INFO

Article history:
Received 9 November 2015
Revised 1 January 2016
Accepted 5 January 2016
Available online xxxx

Keywords:
Biological clock
Mesenchymal stem cell
Aging
Osteoblast
Osteogenesis, bone

ABSTRACT

The circadian clock is an endogenous time keeping system that controls the physiology and behavior of many organisms. The transcription factor Brain and Muscle ARNT-like Protein 1 (BMAL1) is a component of the circadian clock and necessary for clock function. *Bmal1*$^{-/-}$ mice display accelerated aging and many accompanying age associated pathologies. Here, we report that mice deficient for BMAL1 have a low bone mass phenotype that is absent at birth and progressively worsens over their lifespan. Accelerated aging of these mice is associated with the formation of bony bridges occurring across the metaphysis to the epiphysis, resulting in shorter long bones. Using micro-computed tomography we show that *Bmal1*$^{-/-}$ mice have reductions in cortical and trabecular bone volume and other micro-structural parameters and a lower bone mineral density. Histology shows a deficiency of BMAL1 results in a reduced number of active osteoblasts and osteocytes in vivo. Isolation of bone marrow derived mesenchymal stem cells from *Bmal1*$^{-/-}$ mice demonstrate a reduced ability to differentiate into osteoblasts in vitro, which likely explains the observed reductions in osteoblasts and osteocytes, and may contribute to the observed osteopenia. Our data support the role of the circadian clock in the regulation of bone homeostasis and shows that BMAL1 deficiency results in a low bone mass phenotype.

1. Introduction

Age-related deterioration of trabecular and cortical bones, coupled with disruption in normal mineral deposition and accumulation, causes alterations in bone architecture and a reduction in bone strength [9,45]. Bone remodeling requires strict control of two processes in which bone formation by osteoblasts and bone resorption by osteoclasts is necessary to maintain a healthy skeleton. Another bone cell, the osteocyte, is an important regulatory cell influencing and participating in bone remodeling through orchestrating both osteoblast and osteoclast activity [40]. Importantly, osteocytes regulate mechanotransduction, as targeted ablation of osteocytes in mice results in increased cortical porosity and trabecular bone reduction, with resistance to unloading-induced bone loss [44]. Thus, osteocytes are a third bone cell necessary for maintenance of the structural integrity of bone. Several signaling pathways are implicated in the regulation of bone homeostasis and the circadian clock is one of these systems [16,41]. Over a quarter of murine calvarial bone genes are expressed with 24-hour periodicity [48]. Additionally, there is a high bone mass phenotype present in mice lacking *Cry* or *Per* because of a dysregulation of leptin, through sympathetic nervous system signaling, ultimately controlling bone formation mediated by circadian clock signaling [16]. Furthermore, genes involved in osteoblast differentiation are under circadian control; indeed, genes involved in mesenchymal stem cell differentiation and mineral deposition occur in a circadian fashion and are under direct control of the BMAL1:CLOCK complex [11,17,30,48].

The circadian clock is an endogenous system present in most organisms that synchronizes their physiology and behavior with the earth's rotation [6]. It was previously shown that clock genes play a role in bone remodeling [16,28]. Using the *Per1,2*$^{-/-}$ double knockout mouse model of circadian disruption, Fu et al. demonstrated that the circadian clock is involved in leptin dependent regulation of osteoblast proliferation, and that the absence of the clock resulted in increased proliferation and bone formation [16]. At the same time, it was reported that shift work (circadian disruption) results in lower bone mineral density and increases the risk of osteoporosis in humans [15,37]. Therefore, these lines of evidence are seemingly at odds and suggest the connection

Abbreviations: BMAL1, Brain and Muscle ARNT-Like Protein 1; MSCs, Mesenchymal Stem Cells; CLOCK, Circadian Locomotor Output Cycles Kaput; Per1, Period 1; Per2, Period 2; Cry1, Cryptochrome 1, Cry2, Cryptochrome 2; Rev-Erb α, Nuclear Receptor Subfamily 1, Group D, Member 1; CCGs, Clock Controlled Genes; Micro-CT, Micro Computed Tomography; ROI, Region of Interest; Tb.Th., Trabecular Thickness; Tb.N., Trabecular Number; BV/TV, Bon Volume per Tissue Volume; BMD, Bone Mineral Density; BMC, Bone Mineral Content; ARS, Alizarin Red S; VK, Von Kossa; ALP, Alkaline Phosphatase; CV, Crystal Violet; HBSS, Hank's Balanced Salt Solution.

* Corresponding author at: Center for Gene Regulation in Health and Disease, BGES, Cleveland State University, 2121 Euclid Ave., Science and Research Building, Cleveland, OH 44115-2214, USA.
E-mail addresses: samsa.william@gmail.com (W.E. Samsa), avasanji@image-iq.com (A. Vasanji), midurar@ccf.org (R.J. Midura), r.kondratov@csuohio.edu (R.V. Kondratov).

http://dx.doi.org/10.1016/j.bone.2016.01.006
8756-3282/

between the circadian clock and bone homeostasis is more complicated than first thought arguing for the need of additional study. BMAL1 is a transcriptional factor playing a key role in the circadian clock mechanisms, and mice deficient in BMAL1 display an accelerated aging phenotype associated with non-orthotropic ossifications and lower bone weights often observed with older age [7,25]. However, a definitive role of BMAL1 in regulating bone formation over an organism's lifespan is as yet undetermined.

Therefore, we hypothesized that BMAL1 is involved in the regulation of osteoblast differentiation and bone deposition, and decided to examine the bone phenotype of *Bmal1*$^{-/-}$ mice at varying ages and not just at an adolescent age [16]. In this study, we used micro-computed tomography (micro-CT) scanning to show that BMAL1 deficiency in mice results in a low bone mass phenotype characterized by bone loss in both cortical and trabecular bone compartments. Micro-CT scans of *Bmal1*$^{-/-}$ mouse long bones show lower bone volume fraction, decreased trabecular number and decreased cortical thickness compared to wild type littermates. Using conventional histomorphometry, we measured a lower number of active osteoblasts per bone surface and a significantly reduced number of osteocytes per bone area, the terminal differentiation product of osteoblasts, in *Bmal1*$^{-/-}$ mice as compared to wild type mice. Reductions in active osteoblast and osteocyte numbers likely contribute to the low bone mass phenotype. Follow-up data revealed an impaired ability of bone marrow derived mesenchymal stem cells (MSCs) isolated from *Bmal1*$^{-/-}$ bone marrow to differentiate into osteoblasts in cell culture. This impaired osteogenic differentiation in vitro correlates with the reduced number of active osteoblasts and osteocytes in vivo. These *Bmal1*$^{-/-}$ mice data validate our hypothesis that the circadian clock is involved in age related bone homeostasis, and suggest another model of circadian disruption that may be used for studying bone homeostasis.

2. Experimental procedures

2.1. Animals

Bmal1$^{-/-}$ mice were previously generated in Dr. C. Bradfield's laboratory (University of Wisconsin) [8], and backcrossed to the C57BL/6J inbred strain (The Jackson Laboratory, Bar Harbor, ME, USA) for 12 generations. Wild type and knockout mice were generated by breeding of heterozygous parents. Genotypes were determined using a PCR-based method as previously described [8]. All animal studies were conducted in accordance with the regulations of the Committee on Animal Care and Use at Cleveland State University and Roswell Park Cancer Institute.

2.2. Micro-CT imaging

Excised tibiae and femora scanned using GE eXplore Locus μCT (GE Healthcare, Piscataway, NJ) and 360 X-ray projections were collected in 1° increments (80 kVp; 500 μA; ~40 min total scan time). Projection images were preprocessed and reconstructed into 3-dimensional volumes (1024 [3] voxels, 20 μm resolution) on a 4PC reconstruction cluster using a modified tent-FDK cone-beam algorithm (GE reconstruction software). Three-dimensional data were processed and rendered (isosurface/maximum intensity projections) using MicroView (GE Healthcare). Tibial segmentation from surrounding bone, volume enhancement, and delineation of regions-of-interest (ROIs) were performed in MicroView. For the tibiae, a ROI extending from below the proximal tibial growth plate to the distal fibular/tibial synostosis was created and then subsequently split into 3 contiguous regions: 0–25%, 25–50%, and 50–100% of the total ROI length. ROI's were created using the same approach for femora with the initial ROI extending from above the distal femoral growth plate to immediately below the femoral neck. For both femora and tibiae, measurements were taken at a distance from the growth plate that excludes the primary spongiosa.

2.3. Dissection of femora and tibiae and isolation of marrow stromal cells

For the isolation of femora and tibiae for histological staining, young (4 week-old) and adult (24, 32 and 36 week-old) wild type and Bmal1$^{-/-}$ mice were euthanized by CO_2 asphyxiation followed by cervical dislocation. Skin was removed from the hind legs, soft tissues were dissected away with forceps and adherent nonosseous tissue was removed with surgical scissors. For isolation of marrow MSCs, femora and tibiae were disarticulated from 7 to 9 month old wild type and Bmal1$^{-/-}$ mice under aseptic conditions and the epiphyses were removed. Total bone marrow was flushed from tibiae and femora with 5 mL of α-MEM by inserting a 22-gauge syringe needle into the end. Cells from the marrow tissue were separated by fluid shear and plated for culture. Three days after plating media was replaced to remove non-adherent cells.

2.4. Cell culture

All cells were maintained in Minimal Essential Medium α (α-MEM) (LRI, Cleveland Clinic, Cleveland, USA) supplemented with 10% Defined FBS (Atlanta Biologicals, USA) and 10,000 units of Penicillin G and 10,000 μg/ml streptomycin (LRI, Cleveland Clinic, Cleveland, USA). Cells were maintained under standard growth conditions at 37 °C in a humidified atmosphere with 5% CO_2. Media was replaced twice a week.

2.5. Decalcification and histology

Femora were disarticulated from 6 to 7 month old wild type and 5–6 month old Bmal1$^{-/-}$ mice and following the removal of soft tissue, decalcified and fixed in a 1:20 tissue:fixative volume ratio of Cal-Rite (Thermo Scientific) for 14 days, with Cal-Rite being replaced every 24 h. All samples were paraffin embedded using the Leica TP1020 tissue processor, after serial dehydration with a graded series of ethanol washes to Clear-Rite (Thermo Scientific). All bones were oriented in such a manner that cuts were made yielding a medial to lateral presentation. Tissue sections (10 μm thick) were cut and placed onto gelatin-coated Superfrost + slides, and then stained with hematoxylin and eosin. The aspect ratio of a cell (and its nucleus) was used to determine if osteoblasts were active, partially active or inactive as described previously [36]. Briefly, images were analyzed in ImageJ to determine the number and aspect ratio of osteoblasts present on the surface of the periosteal bone, endosteal bone, and the trabecular compartment. Total number of osteoblasts, as well as the number of osteoblasts categorized into active, partially active and inactive were normalized to the length of bone surface in ImageJ and quantified as number of cells per millimeter. Using ImageJ, aspect ratio was determined and an aspect ratio of 1:1 to 1:4 were marked as active, 1:5 to 1:9 were marked as partially active, and 1:10 or greater were marked as inactive.

2.6. Osteoblast differentiation in vitro

For osteogenic differentiation, adherent cells were sub-cultured 3 times and plated into 96 well plates at 2.0×10 [3] cells per well. Cells were cultured for 0, 14 and 21 days in either control or differentiation media followed by alizarin red staining. For colony forming unit assay, total bone marrow was flushed and plated directly into 6 well plates at 2.0×10 [6] cells per well. After 72 h, non-adherent cells were removed by replacing media with either fresh media or differentiation media. These primary cell cultures were incubated for 18 days at which time they fixed and stained by alizarin red S, von Kossa, alkaline phosphatase and crystal violet staining procedures.