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Histone deacetylase 3 is required for maintenance of bone mass during aging

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ABSTRACT

Histone deacetylase 3 (Hdac3) is a nuclear enzyme that removes acetyl groups from lysine residues in histones and other proteins to epigenetically regulate gene expression. Hdac3 interacts with bone-related transcription factors and co-factors such as Runx2 and Zfp521, and thus is poised to play a key role in the skeletal system. To understand the role of Hdac3 in osteoblasts and osteocytes, Hdac3 conditional knockout (CKO) mice were created with the osteocalcin (OCN) promoter driving Cre expression. Hdac3 CKO_{OCN} mice were of normal size and weight, but progressively lost trabecular and cortical bone mass with age. The Hdac3 CKO_{OCN} mice exhibited reduced cortical bone mineralization and material properties and suffered frequent fractures. Bone resorption was lower, not higher, in the Hdac3 CKO_{OCN} mice, suggesting that primary defects in osteoblasts caused the reduced bone mass. Indeed, reductions in bone formation were observed. Osteoblasts and osteocytes from Hdac3 CKO_{OCN} mice showed increased DNA damage and reduced functional activity in vivo and in vitro. Thus, Hdac3 expression in osteoblasts and osteocytes is essential for bone maintenance during aging.

Introduction

Post-translational modifications of histones are important contributing factors in the epigenetic regulation of gene expression. Acetylated histones facilitate an open chromatin conformation that allows transcription factors, co-factors, and RNA polymerase II-complexes to access the DNA. In contrast, deacetylated histones encourage a condensed chromatin conformation and transcriptional repression. Histone acetyltransferases (HATs) and histone deacetylases (Hdacs) are counteracting enzymes that control histone acetylation levels. HATs and Hdacs also target lysine residues in many other proteins, such as transcription factors, altering their activity, cellular localization or stability [1], thus adding another layer of complexity to the mechanisms by which these enzymes regulate gene expression.

Hdacs regulate the activity of transcription factors important to bone development and maintenance and are crucial for skeletal development [2–7]. Germline deletion of Hdac8 causes craniofacial ossification defects [8], whereas germline deletion of Hdac4 or Hdac6 enhances endochondral ossification and bone mineral density [9,10]. Hdacs also play important roles in the postnatal skeleton. The pan-Hdac inhibitor, vorinostat, depletes the immature osteoblast population and causes trabecular bone loss [11,12]. Another pan-Hdac inhibitor, valproate, causes loss of both cortical and trabecular bone mass [13]. Biological responses to Hdac inhibition vary because Hdac expression changes during cellular differentiation. For example, Hdac1 and Hdac2 are abundantly expressed in osteoblast progenitors, but not in mature osteoblasts, whereas expression levels of Hdac4 and Hdac6 increase during osteoblastic differentiation [14]. Osteoblast progenitors appear to be particularly sensitive to Hdac inhibition, as stromal cells treated early in the time course of osteoblastic differentiation fail to form mature, matrix-producing osteoblasts, but cells treated with Hdac inhibitors at later culture stages are unaffected [11].

The role of Hdac3 in bone is of interest because it is strongly expressed in osteoblasts at all stages of differentiation [3,14,15] and it interacts with the master osteoblast transcription factor Runx2 [3,4]. Germline deletion of Hdac3 causes embryonically lethality prior to skeleton formation [16], and thus its effects on bone must be assessed using conditional knockout models. Mice deficient in Hdac3 under the control of the Osterix (Osx) promoter (Hdac3 CKO_{OSX}) have a striking developmental phenotype of reduced body size, decreased bone length, and deficits in both cortical and trabecular bone mass [17]. This phenotype is explained, in part, by a deleterious impact on osteoblasts, as osteoblast numbers and bone formation rates were lower in Hdac3 CKO_{OSX} compared to wildtype mice [17]. However, Osterix (Sp7) is also expressed by chondrocytes, and indeed, Hdac3 CKO_{OSX} mice have a

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relative expansion of the hypertrophic zone in the growth plate cartilage, suggesting that aberrant chondrocyte hypertrophy contributes to the skeletal phenotype during development [17]. Recent studies confirmed that chondrocytes from Hdac3 CKO_{OSX} mice undergo accelerated hypertrophy but are impaired in their ability to produce a cartilaginous matrix [18], contributing to the growth plate defects in these mice. Thus, the phenotype of Hdac3 CKO_{OSX} mice is likely due to the combined effects of Hdac3 depletion in chondrocytes and osteoblasts.

To better understand the effects of Hdac3 in mature osteoblasts and osteocytes, independent of cartilage defects, we crossed Hdac3-floxed animals with mice containing the Osteocalcin (OCN)-Cre transgene [19]. Here we show that Hdac3 depletion in mature osteoblasts causes a progressive postnatal decrease in cortical and trabecular bone mass with age as compared to age-matched control mice. In vitro and in vivo studies attribute this osteopenic phenotype to increased DNA damage and an overall decline in osteoblast and osteocyte function, particularly in cortical bone.

Materials and methods

Animal studies

Mice containing LoxP sites flanking exon 7 of Hdac3 (*Hdac3$^{fl/fl}$* mice) [16,20] were crossed with transgenic mice expressing Cre recombinase under control of a segment (approximately 4 kb) of the human OCN promoter [19,21] to create Hdac3$^{fl/fl}$, Cre+ conditional knockout mice (CKO; also referred to as Hdac3 CKO_{OCN} mice). All mice were maintained on a mixed CD1:C57Bl/6 background. The OCN-Cre mouse features an 88% excision index in osteoblasts and osteocytes [19,22]. Mice were genotyped for Hdac3 alleles and the OCN-Cre transgene with PCRs using tail DNA as a template as previously reported [17,19]. Because transgene and LoxP insertions may cause unpredictable and unknown consequences, Hdac3 CKO_{OCN} mice were compared to two different control groups when possible. These groups were: Hdac3$^{fl/fl}$ mice that did not harbor the OCN-Cre transgene (hereafter referred to as Hdac3$^{fl/fl}$, Cre− control (Ctrl) mice), and mice expressing the OCN-Cre transgene but wildtype at the Hdac3 locus (hereafter referred to as Hdac3$^{+/+}$, Cre+ Ctrl mice). The latter group was not maintained past 12 weeks of age because at the early ages the two control groups were largely indistinguishable from each other in skeletal phenotype. Data from the larger control group is shown in the figures. All animal research was conducted according to guidelines provided by the National Institutes of Health and the Institute of Laboratory Animal Resources, National Research Council. The Mayo Clinic Institutional Animal Care and Use Committee approved all animal studies. Animals were housed in an accredited facility under a 12-h light/dark cycle and provided water and food (PicoLab Rodent Diet 20, LabDiet) ad libitum.

Dual-energy X-ray absorptiometry analyses and tissue collection

Bone mineral density (BMD) was assessed longitudinally via dual-energy X-ray absorptiometry (DXA) scanning (PIXImus, GE Healthcare) of live mice at 4, 12, and 24 weeks of age. Three regions of interest were monitored: lower body (including the lumbar spine, pelvis, and hindquarters), lumbar vertebrae, and femoral midshaft. Body weights were recorded and serum was collected via cardiac puncture at sacrifice. To assess protein expression of Hdac3, long bones were removed from 5- to 6-week-old mice and dissected free of soft tissues. Epiphyses were removed from both femurs and one tibia from each mouse and marrow was flushed with saline; cortical bone segments were subsequently flash frozen in liquid nitrogen prior to protein isolation. For mRNA analyses, demarrowed cortical bone segments from one femur and one tibia were prepared from 2-week-old mice as described above and flash frozen in liquid nitrogen prior to mRNA extraction. To permit dynamic histomorphometry studies of bone remodeling, adult mice were administered subcutaneous injections of calcein (10 mg/kg) on days 4 and 1 prior to death as previously described [17]. Femurs and tibias were collected from mice at 4, 6, 12, and 24 weeks of age for quantification of bone architecture, material properties, and remodeling indices. One femur from each of the 12-week-old mice was cleaned of soft tissue and stored at −20 °C in saline-soaked gauze for preservation of mechanical properties. All remaining bones were fixed in 70% ethanol (for Fourier transform infrared (FT-IR) imaging) or 10% neutral buffered formalin (for static and dynamic histomorphometry) and stored in 70% ethanol prior to microCT analysis and histological preparation.

Tissue-level gene expression

For mRNA analyses, demarrowed cortical bone explants were homogenized in TRIzol using a high-speed disperser (Ultra-Turrax T25, IKA). RNA was extracted and purified from the ground tissue with TRIzol reagent (Invitrogen) and was reverse transcribed into cDNA using the SuperScript III First-Strand Synthesis System (Invitrogen). Relative expression levels of mRNAs for genes of interest, including Hdac3, characteristic osteoblast and osteocyte genes, Rankl, Opg, and genes relating to proliferation (CyclinD1, Cdkn1a) (Table 1) were measured by real-time PCR (qPCR). Reactions were performed using 37.5 ng of cDNA per 15 μl with Bio-Rad iQ SYBR Green Supermix and the Bio-Rad MyiQ Single Color Real-Time PCR Detection System. Transcript levels were normalized to the reference gene Gapdh. Gene expression levels were quantified using the $2^{-\Delta\Delta Ct}$ method [23].

Cortical bone protein expression

Demarrowed cortical bone segments were pulverized with a mortar and pestle in liquid nitrogen, placed in RIPA buffer on ice, and sonicated to generate protein extracts. Lysates were cleared by centrifugation at 15,000 RPM for 15 min at 4 °C. Ten to 40 μg of total protein from each sample were resolved by SDS-PAGE. Western blotting was performed with antibodies recognizing Hdac3 (1:5000, Abcam #ab63353), acetylated histone 3: K9 and K14 (1:10,000, Millipore #06-599), acetylated histone 4: K5, K8, K12, and K16 (1:10,000, Millipore 06–866), histone 3 (1:20,000, Millipore #05-928), and actin (1:5000 or 1:10,000, Santa Cruz, I-19 SC-1616). Relative expression levels of Hdac3 and acetylated histone 3 were quantified from band intensity with image analysis software (Image J).

Serum bone remodeling markers and serum calcium

Circulating levels of procollagen type 1 amino-terminal propeptide (P1NP; bone formation marker) and tartrate-resistant acid phosphatase 5b (TRAcP5b; bone resorption marker) were measured with colorimetric assays (Rat/Mouse P1NP EIA #AC-33 F1, Immunodiagnostic Systems, Fountain Hills AZ; MouseTRAP ELISA #SB-TR103, Immunodiagnostic Systems, Fountain Hills AZ). All samples were tested in duplicate within each assay. For quantification of total serum calcium, serum samples were diluted 1:40 in a solution of 1% lanthanum chloride and 0.1 M HCl, and then analyzed via atomic absorption using a Perkin Elmer AAnalyst 800.

Bone structure and composition in the femur (micro-computed tomography and Fourier transform infrared imaging)

Bone architecture and mineralization were evaluated in femurs of male mice using ex vivo micro-computed tomography (microCT) and FT-IR imaging. For microCT studies, the central portion of the femoral diaphysis and secondary spongiosa in the distal femoral metaphysis of each bone were scanned in 70% ethanol on a μCT35 scanner (Scanco Medical AG, Basserdorf, Switzerland). Cortical and trabecular bone scans were performed with 7 μm voxel size using an energy