ORIGINAL ARTICLE

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Hdac3 Deficiency Increases Marrow Adiposity and Induces Lipid Storage and Glucocorticoid Metabolism in Osteochondroprogenitor Cells

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ABSTRACT

Bone loss and increased marrow adiposity are hallmarks of aging skeletons. Conditional deletion of histone deacetylase 3 (Hdac3) in murine osteochondroprogenitor cells causes osteopenia and increases marrow adiposity, even in young animals, but the origins of the increased adiposity are unclear. To explore this, bone marrow stromal cells (BMSCs) from Hdac3-depleted and control mice were cultured in osteogenic medium. Hdac3-deficient cultures accumulated lipid droplets in greater abundance than control cultures and expressed high levels of genes related to lipid storage (Fsp27/Cidec, Plin1) and glucocorticoid metabolism (Hsd11b1) despite normal levels of Pparγ2. Approximately 5% of the lipid containing cells in the wild-type cultures expressed the master osteoblast transcription factor Runx2, but this population was threefold greater in the Hdac3-depleted cultures. Adenoviral expression of Hdac3 restored normal gene expression, indicating that Hdac3 controls glucocorticoid activation and lipid storage within osteoblast lineage cells. HDAC3 expression was reduced in bone cells from postmenopausal as compared to young women, and in osteoblasts from aged as compared to younger mice. Moreover, phosphorylation of S424 in Hdac3, a posttranslational mark necessary for deacetylase activity, was suppressed in osseous cells from old mice. Thus, concurrent declines in transcription and phosphorylation combine to suppress Hdac3 activity in aging bone, and reduced Hdac3 activity in osteochondroprogenitor cells contributes to increased marrow adiposity associated with aging. © 2015 American Society for Bone and Mineral Research.

KEY WORDS: LIPID DROPLETS; HSD11B1; AGING; OSTEOPOROSIS; RUNX2

Introduction

Bone loss increases during aging and the resulting osteopenia and osteoporosis are underlying causes of millions of skeletal fractures each year. The sudden loss of independence and costs of treating fractures in the elderly place enormous economic and psychological burdens on patients, caretakers, and healthcare systems. Although fracture risk can be reduced with therapies that either prevent bone loss (eg, bisphosphonates) or stimulate new bone formation (eg, PTH, anti-sclerostin antibodies), long-term use of these drugs causes adverse events in some patients. Thus, a better understanding of bone biology is needed to improve skeletal health, prevent fracture-associated morbidities, and reduce healthcare expenditures.

Intriguing relationships exist between bone mass, fracture risk, and marrow adiposity.[1,2] Bone mineral density and marrow fat abundance are inversely correlated in children and adults,[3] and skeletal fracture risk is associated with bone mass and the amount of marrow fat present.[4] Marrow adipose tissue (MAT) accumulates throughout life and is generally linked with poor bone quality.[5–7] Disuse increases marrow adiposity, whereas osteoanabolic stimuli that require energy including exercise and mechanical loading reduce MAT abundance.[8–13] MAT likely evolved as a means to store excess energy and protect bone from free fatty acids that can negatively impact hematopoiesis and osteoblast differentiation through lipotoxicity,[14,15] but the origin and function of MAT are complex and incompletely understood. Numerous studies support the presence of a reciprocal relationship between bone marrow stromal cell (BMSC) commitments into osteoblast versus adipocyte lineages during aging.[16–19] For example, BMSCs from aged mice express the adipogenic transcription factor, Pparγ2, at nearly 10-fold greater levels than BMSCs from young animals; they also express

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more adipocytic genes and fewer osteoblastic transcripts.[17] A variety of endogenous modulators and signaling pathways, including Wnts,[20] bone morphogenic proteins,[21] Hedgehog factors,[22] and glucocorticoids[23] influence this process in one direction or the other. Infiltration of adipocytes from non-osseous tissues has also been proposed as a mechanism to explain the formation of MAT; however, the origin and route of such migrating cells are unclear.[5]

Histone deacetylases (Hdacs) influence the fate of differentiating mesenchymal progenitor cells by removing acetyl groups from lysine residues in histones and other proteins and altering chromatin structure and gene expression, as well as the activity of numerous biochemical and signal transduction pathways.[24] Activation of sirtuin 1 (Sirt1), a class III Hdac, in C3H10T1/2 stromal cells or in primary BMSCs promotes osteoblastic differentiation at the expense of adipogenesis.[25] In contrast, Hdac1 and Hdac2 are required for adipocyte differentiation, because genetic deletion of both Hdac1 and Hdac2 robustly blocks the effects of pro-adipogenic stimuli.[26] Hdacs may also influence BMSC lineage specification through interaction with cofactors. For example, the zinc finger protein Zfp467 requires an Hdac-associated co-repressor complex to promote Pparγ2 transactivation and adipocyte differentiation,[27] and the transcriptional co-repressor protein transducin-like enhancer of split 3 (TLE3), requires Hdac activity to repress Runx2 and inhibit osteoblastic differentiation of BMSCs.[28] Similarly, Hdac3 is recruited to a co-repressor complex by small leucine zipper protein (sLZIP) to negatively regulate Pparγ2 transcriptional activity, altering the balance between adipogenic as compared to osteogenic differentiation of mesenchymal progenitor cells.[29] We became interested in the origins of marrow adiposity while studying the role of Hdac3 in skeletal development.[30] Conditional deletion of Hdac3 in osteoblast lineage cells at early and late stages of differentiation with Osx1-Cre or OCN-Cre, respectively, caused osteopenia owing to defects in osteoblast activity and survival.[30,31] However, only Hdac3 deletion from osteochondroprogenitor cells (with Osx1) produced a concomitant increase in bone marrow adiposity in young mice. The goal of this study was to delineate molecular mechanisms by which Hdac3 insufficiency in osteochondroprogenitor cells increase MAT to produce a progeroid skeletal phenotype.

Materials and Methods

Mice

All mice were maintained on a C57BL/6 background and genotyped as described.[30,31] Animals were housed in an accredited facility under a 12-hour light/dark cycle and provided water and food (PicoLab RodentDiet20; LabDiet, St. Louis, MO, USA) *ad libitum*. All animal research was conducted according to guidelines provided by the National Institute 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. Mice with two copies of the Hdac3 allele carrying loxP sites in introns surrounding exon 7 were crossed with mice expressing Cre recombinase under control of the osterix (Osx1) promoter, eventually yielding two groups of progeny that were studied: Hdac3-conditional knockout animals (Hdac3 $CKO_{Osx} = Hdac3^{fl/fl;}$ Osx1-Cre+) and control littermates (Control = $Hdac3^{fl/fl}$; Osx1-Cre–) as described.[30] The $Hdac3^{fl/fl}$ mice were alternatively crossed with mice expressing Cre recombinase under control of a tamoxifen inducible type 2 collagen alpha 1 (Col2ERT) promoter. Pups received a single injection of tamoxifen (1 mg; Sigma-Aldrich, St. Louis, MO, USA) to induce conditional deletion of Hdac3 (Hdac3 $CKO_{Col2ERT} = Hdac3^{fl/fl;}$ Col2ERT-Cre+ tamoxifen injected) or with corn oil (Control$_{Col2ERT}$ = $Hdac3^{fl/fl}$; Col2ERT-Cre+ oil injected) on postnatal day 5 (P5) as described.[32] For experiments designed to assess tissue specificity of the Osx1-Cre and Col2ERT-Cre transgenes, Cre-expressing mice were bred to Rosa26 reporter mice (R26R, B6.129S4-Gt (ROSA) 26Sortm1Sor/J; Jackson Laboratories, Bar Harbor, ME, USA; Strain #003474). The Hdac3-CKO_{OCN} mice were described in another publication.[31] For aging studies, 14-month-old to 26 month-old wild-type C57Bl/6 animals were obtained from the National Institute on Aging (NIA) aged rodent colony or in-house colonies, and 1-month-old to 2 month-old wild-type C57Bl/6 mice were obtained from in-house laboratory colonies. Both males and females were used and combined for in vitro studies. Mice were euthanized at specified ages by carbon dioxide asphyxiation. Long bones (tibias, femurs) were aseptically harvested immediately after euthanasia.

Isolation of murine BMSCs and cortical bone osteoblasts

Bone marrow was flushed from femurs and tibias of mice at specified ages and pooled between like groups. Cells were immediately seeded into six-well plates at 1×10^7 cells per well, 12-well plates on type 1 collagen–coated glass coverslips at 4×10^6 cells per well, or 96-well plates at 5×10^5 cells per well in basal culture medium (α-MEM, 20% FBS, 1% antibiotic/antimycotic [Invitrogen, Carlsbad, CA, USA; #15240-062], 1% nonessential amino acids), osteogenic culture medium (basal culture medium + 50 μg/mL ascorbic acid, 10 mM beta glycerol phosphate, $\pm 10^{-7}$ M dexamethasone [Dex], as indicated), or adipogenic culture medium (basal culture medium + 1 μM rosiglitazone, Sigma-Aldrich). Media were changed every 3 days after seeding, and BMSC were selected by their ability to adhere to the plate after the first 3 days in culture. BMSC were cultured for up to 21 days in osteogenic medium to promote osteoblastic differentiation. In a subset of experiments, cells were exposed to 5 μM carbenoxolone during osteogenic culture, as described.[33,34] Demarrowed cortical bone diaphyses were digested with collagenase as described to isolate cortical bone osteoblasts.[35] Cells were grown for 6 days in culture medium (α-MEM, 5% FBS, 5% calf serum [Hyclone Laboratories, Logan, UT, USA], 1% penicillin/streptomycin [Gibco, Grand Island, NY, USA]) before protein extracts were made.

MTS assays

Cell metabolism in BMSC cultures was quantified after 7 days of osteogenic stimulation with a commercial MTS assay (CellTiter 96 AQueous Non-Radioactive Cell Proliferation Assay [MTS]; Promega, Madison, WI, USA).

Adenoviral transduction

BMSCs were transduced once with Hdac3- or GFP-expressing adenoviruses (SignaGen Laboratories, Gaithersburg, MD, USA; multiplicity of infection [MOI] = 10) at the time of cell seeding. Media were changed every 3 days. Total RNA was harvested after 7 days in culture with TRIzol reagent (Invitrogen).

Western blotting

Cortical osteoblasts were placed in radioimmunoprecipitation assay (RIPA) buffer on ice and sonicated to generate protein extracts. Lysates were cleared by centrifugation at 12,000 × g for 15 min at 4°C. Thirty micrograms (30 μg) of total protein from each sample were resolved by SDS-PAGE. Western blotting was performed with antibodies recognizing phosphorylated (p) S424 in Hdac3 (pS424; 1:1000; Cell Signaling Technology, Beverly, MA, USA; #3815), Hdac3 (1:20,000, Abcam, Cambridge, MA, USA; #ab7030), Hsd11b1 (1:1000, Abcam; #ab39364), V5 (Sigma V8137, 1:5000; Sigma-Aldrich) or actin (1:10,000; Sigma-Aldrich; #A5316).

Immunofluorescence and microscopy

Murine BMSCs were cultured on glass coverslips for 7 days and fixed with 0.1 M piperazine-N,N′-bis(2-ethanesulfonic acid) (PIPES), pH 6.95, 1 mM EGTA, 3 mM $MgSO_4$, and 3% formaldehyde for 15 min at room temperature. After three washes with Dulbecco's PBS (DPBS), cells were stained with a 60% solution of Oil Red O (Sigma-Aldrich), or a 1:1000 dilution of AUTOdot (Abgent, San Diego, CA, USA) for imaging lipid droplets in the red and blue channels,[36] respectively. Cells were then permeabilized using a 0.1% solution of Triton X-100 in DPBS for 2 min and incubated in immunofluorescence (IF) blocking buffer (5% goat serum, 5% glycerol, 0.1% NaN_3, pH 7.2) for 1 hour at 37°C. Primary antibodies to Runx2 (mouse, 1:200; MBL International, Woburn, MA, USA) or Hdac3 (rabbit, 1:700; Abcam) were applied in IF blocking buffer for 2 hours at 37°C, followed by a 1-hour incubation at 37°C with Alexa-488 or Alexa-594-conjugated goat secondary antibodies (Life Technologies, Grand Island, NY, USA). Fixed and stained cells were mounted for microscopy in ProLong Gold Antifade reagent (Invitrogen).

Wide-field epifluorescence images were acquired using an Axiovert 200 epifluorescence microscope (Carl Zeiss, Thornwood, NY, USA) fitted with a Carl Zeiss 40× Plan-Neofluar (0.75 NA) or 63× Plan-Apochromat objective (1.4 NA oil) at room temperature. Digital images were captured with an Orca II-ERG CCD camera (Hamamatsu Photonics, Bridgewater, NJ, USA) using the iVision software package (BioVision Technologies, Exton, PA, USA). Confocal images were acquired using a Zeiss LSM 780 confocal microscope (Carl Zeiss Microscopy, Peabody, MA, USA) fitted with a 63X/1.2NA water immersion lens. The Zen 2012 software package (Carl Zeiss) was used for image capture.

For standard staining of cell phenotype and matrix production, cells were cultured for 14 or 21 days, fixed in 10% neutral buffered formalin, and stained with 2% Alizarin Red to detect matrix calcification or with 60% Oil Red O to detect lipid droplets.[37]

Gene expression studies

Cells were cultured in six-well plates or 10-cm dishes for 7 days, after which total RNA was harvested with TRIzol reagent (Invitrogen). For microarray studies, RNA was reverse transcribed using Qiagen's Quantitect Reverse Transcription Kit (Qiagen, Valencia, CA, USA) and transcript levels were determined with the Illumina MouseRef-8 BeadChip array (Illumina, San Diego, CA, USA). Five independent experiments on cells from paired Hdac3 CKO_{Osx} mice and control littermates were analyzed. Raw data were pre-processed using Beadstudio (Illumina) and submitted to the Minimum Information About a Microarray Experiment (MIAME)-compliant Gene Expression Omnibus (GEO) database (NCBI; http://www.ncbi.nlm.nih.gov/geo; Accession number: GSE64432). Genes that were not expressed (detection $p \geq 0.05$) were eliminated from analysis. A standard ANOVA model (Partek software; Partek, Inc., St. Louis, MO, USA) was applied to calculate the differentially expressed genes for each comparison. Selected genes were subjected to reverse transcription (RT) and real-time semi-quantitative PCR (qPCR) analyses for confirmation as described.[30] RT was performed using the SuperScript III First-Strand Synthesis System (Invitrogen), and PCRs 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 (Bio-Rad Laboratories, Hercules, CA, USA). Transcript levels were normalized to the reference gene Gapdh. Gene expression levels were quantified using the comparative threshold cycle ($2^{-\Delta\Delta Ct}$) method.[31] Gene-specific primer sequences are as follows: Gapdh_F: 5′-GGGAAGCCCATCACCATCTT-3′, Gapdh_R: 5′-GCCTCACCCCATTTGATGTT-3′; Hdac3_F: 5′-CCCGCATCGAGAATCAGAAC-3′, Hdac3_R: 5′-TCAAAGATTGTCTGGCGGATCT-3′; Pparγ_F: 5′-CCCACCAACT TCGGAATCAG-3′, Pparγ_R: 5′-AATGCGAGTG GTCTTCCATCA-3′; Fasn_F: 5′-GTGATAGCCGGTATGTCGGG-3′, Fasn_R: 5′-TAGAGCCCAGCCTTCCATCT-3′; Plin1_F: 5′-TGCTGCA CGTGGAGAGTAAG-3′, Plin1_R: 5′-TGGGCTTCTTTGGTGCTGTT-3′; Cidec_F: 5′-TCCAAGCCCTGGCAAAAGAT-3′, Cidec_R: 5′-CGGAGCATCTCCTTCACGAT-3′; Hsd11b1_F: 5′-ACTCAGACCTCGCTGT CT CT-3′, Hsd11b1_R: 5′-TGGGTCATTTTCCCAGCCAA-3′; Pnpla2 _F: 5′-GCAATCTCTACCGCCTCTCG-3′, Pnpla2_R: 5′-TTGGTTCAGTAGGCCATTCCTC-3′; Lipe_F: 5′-AGAAGGATCGAAGAACCGCA-3′; and Lipe_R: 5′-GTGTGAGAACGCTGAGGCTTT-3′.

Histology

Tibias from 8-week-old animals were fixed in 10% neutral buffered formalin, decalcified in 15% EDTA for 7 days, paraffin-embedded, sectioned at a thickness of 5 μm, and stained with Fast Green and Safranin O as described.[38] Adipocyte volume fraction (AV/TV, %) and adipocyte number (N.Ad/T.Ar, $1/mm^2$) were quantified with image analyses software (Bioquant Osteo, Nashville TN, USA). To trace expression and activity of the Cre transgene, bone specimens were fixed in 0.2% glutaraldehyde, cryoprotected in 30% sucrose (dissolved in PBS, pH 7.4) at 4°C for 48 hours, frozen in embedding medium (Tissue-Tek O.C.T.), and sectioned at 8-μm thickness on a cryotome using either the Cryofilm IIc system[39] or the CryoJane Tape-Transfer System (Leica Biosystems). Sections were incubated in X-gal reaction buffer overnight, counterstained with eosin, dehydrated through graded ethanols and xylenes, and mounted with Permount medium on glass slides. Tibias from Hdac3 CKO_{Osx} mice, Hdac3 CKO_{OCN} mice, and control littermates were histologically prepared with Goldner's trichrome and used for gross observation of bone marrow architecture as described.[30,31]

Transcription assays

For analyses of glucocorticoid-related transcriptional activity, C2C12 cells were transfected with Lipofectamine (Invitrogen) and a DNA mixture consisting of 200 ng of a glucocorticoid-sensitive luciferase reporter construct (murine mammary tumor virus [MMTV]-Luc),[40] promoterless Renilla luciferase (pRL-null) (10 ng), and either pCMV-Hdac3 expression or control plasmids (300 ng) in 12-well plates. Following an overnight incubation at 37°C, cells were treated with 100 nM Dex or vehicle (ethanol) for 24 hours.

Luciferase activity in 20 μL of cell lysate was measured using the dual luciferase assay system (Promega) and a Glomax 96 microplate luminometer (Promega). An additional set of luciferase reporter constructs were used to quantify the effects of Hdac3 on Hsd11b1 transcriptional activity. Hsd11b1 transcripts arise from two different promoters, which show tissue-specific activity patterns[41]: the more widely expressed proximal (P2) promoter, and a more recently identified distal (P1) promoter. C2C12 cells were transfected with 200 ng of luciferase reporter constructs encompassing either DNA sequences 2.1 kb upstream from the transcription start site (P1 promoter) or 1.8 kb upstream from the transcription start site (P2 promoter) (both kind gifts from Dr. Karen Chapman[41]), along with pRL-null and pCMV-Hdac3 as described (earlier in this paragraph). Luciferase activity in 20 μL of cell lysate was measured using the dual luciferase assay system (Promega) and a BioTek Synergy HT plate reader (BioTek, Winooski, VT, USA). All transfections were performed in triplicate, and data were normalized to the activity of Renilla luciferase.

Human tissue samples and mRNA analysis

The collection and processing of human bone cores (containing trabecular and cortical bone) from the posterior iliac crest of young (22 to 40 years old, $n = 10$) and postmenopausal (64 to 88 years old, $n = 10$) women were described previously.[42] All samples were collected with informed consent under an IRB-approved protocol. For qPCR analysis, primers were designed using the Primer Express program (Applied Biosystems, Inc., Foster City, CA, USA) and PCR reactions were run in the ABI Prism 7900HT Real time System (Applied Biosystems) using SYBR Green (Qiagen) as the detection method. Normalization for variations in input RNA was performed using a panel of 10 housekeeping genes with the geNorm algorithm[43] used to select the three most stable housekeeping genes on the plate (POLR2a, RPL13A, and TUBA1B), which were used for every sample. The PCR Miner algorithm[44] was used to correct for variations in amplification efficiencies.

Statistics

Statistics were performed with JMP 11 statistical analyses software (SAS Institute Inc., Cary, NC, USA). Murine data were compared between groups within each experiment with Student's unpaired *t* tests or analyses of variance (ANOVA) followed by post hoc tests as appropriate. Gene expression data in the human bone biopsies were not normally distributed, and therefore compared with nonparametric statistics (Wilcoxon-Mann-Whitney test). Values of *p* are noted in the figure legends.

Results

Hdac3 insufficiency in osteochondroprogenitor cells increases marrow adiposity in vivo

Mice deficient in Hdac3 in Osx1-expressing progenitor cells have increased bone marrow adiposity as compared to littermate controls,[30] but mice with conditional deletion of Hdac3 in mature osteoblasts (OCN-Cre) do not have increased bone marrow fat[31] (Fig. 1*A*). These results suggested that Hdac3 expression in preosteoblasts affects marrow adiposity. To further test this hypothesis, we deleted Hdac3 postnatally in osteochondroprogenitor cells using a tamoxifen-inducible Col2-Cre^{ERT} driver, which is highly expressed in growth plate

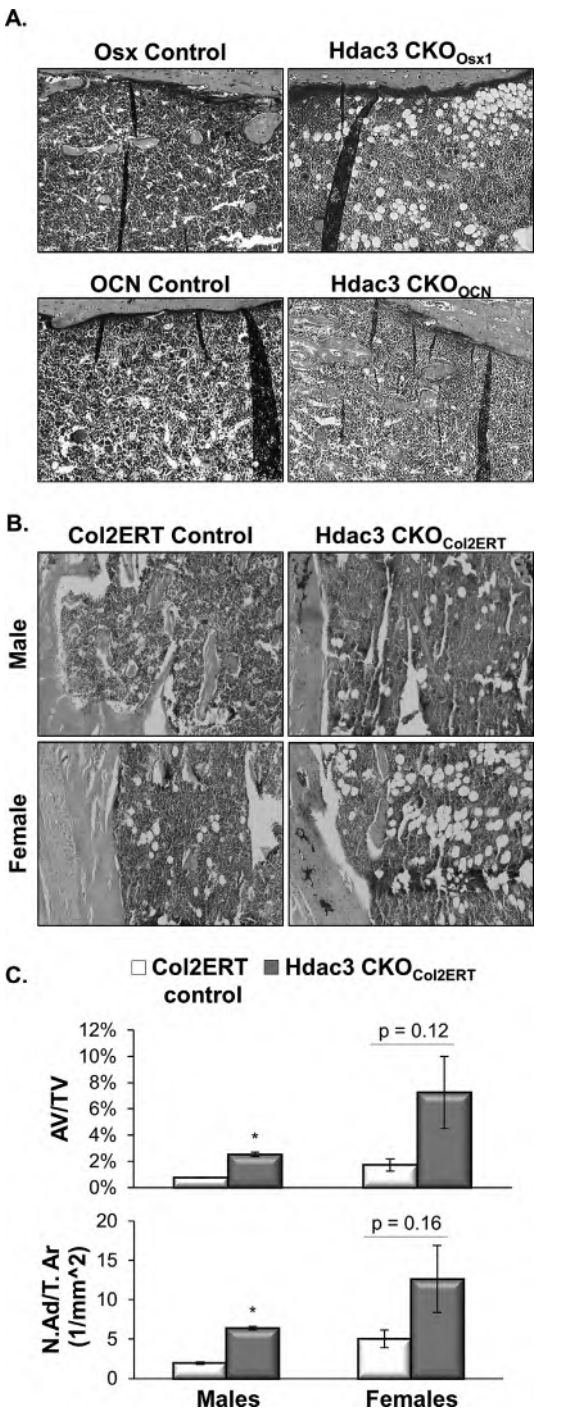

Fig. 1. Mice lacking Hdac3 in osteochondroprogenitor cells have high bone marrow fat. (*A*) Sections of tibias from 5-week-old to 6-week-old Hdac3 CKO$_{Osx}$, Hdac3 CKO$_{OCN}$, and corresponding control mice were stained with Goldner's trichrome. (*B*) Tibias from 8-week-old male and female Hdac3 CKO$_{Col2ERT}$ mice and control littermates were histologically prepared with Safranin O/Fast Green for observation of bone architecture. (*C*) AV/TV (%) and N.Ad/T.Ar were quantified in histologic sections from Hdac3 CKO$_{Col2ERT}$ and control mice. Means ± SE are shown ($n = 4$ female control, 5 female Hdac3$_{Col2ERT}$ CKO, 3 male control, 6 Hdac3$_{Col2ERT}$ CKO) $^{*}p \leq 0.05$ versus control. Other *p* values are indicated. AV/TV = adipocyte volume per tissue volume; N.Ad/T.Ar = number of adipocytes per tissue area.

chondrocytes, but is also present in bone marrow mesenchymal progenitor populations, particularly those in the endocortical region[45–47] (Supporting Fig. 1). Similar to that observed in the Hdac3 CKO_{Osx} line, MAT was elevated in both male and female Hdac3 $CKO_{Col2ERT}$ mice as compared to littermate controls (Fig. 1*B*). The fraction of total marrow tissue that contained adipose ghosts (AV/TV) and the number of adipose ghosts per tissue area (N.Ad/T.Ar) were significantly increased in the male Hdac3 $CKO_{Col2ERT}$ mice as compared to controls, and tended to be higher in female Hdac3 $CKO_{Col2ERT}$ mice compared to controls as well, although the comparison in females did not reach statistical significance (Fig. 1*C*). These results demonstrate that conditional deletion of Hdac3 in osteochondroprogenitor cells promotes marrow adiposity.

Hdac3-insufficiency promotes lipid storage in osteoblastic cultures of BMSCs

A limitation of using standard paraffin-based or plastic-based histology as a means to study bone marrow adiposity is that the chemical processing removes lipids and destroys cells, leaving voids (ghosts) where cells once resided and making it difficult to identify the cell type that contained lipids. These ghosts are routinely described as adipocytes, but many cell types in addition to adipocytes can store lipids.[48] Thus, to determine how Hdac3 depletion in osteochondroprogenitor cells impacts mesenchymal cell fate, primary BMSCs from Hdac3 CKO_{Osx} and control mice were cultured in osteogenic medium in vitro. In as little as 14 days, osteogenic cultures of Hdac3 CKO_{Osx} BMSCs contained more lipid droplets than control cultures, with a macroscopic difference in the number of lipid-positive colonies by day 21 in culture (Fig. 2*A*). Lipid droplet formation was similar between control Cre-expressing animals ($Hdac3^{+/+}$:Osx1-Cre+) and Cre-negative animals ($Hdac3^{f/f}$:Osx1-Cre–); therefore, the Cre-negative animals were used as controls for all in vitro studies (Supporting Fig. 2). Notably, no differences were seen in lipid droplet formation between osteogenic cultures of BMSCs from Hdac3 CKO_{OCN} mice and littermate controls at either day 21

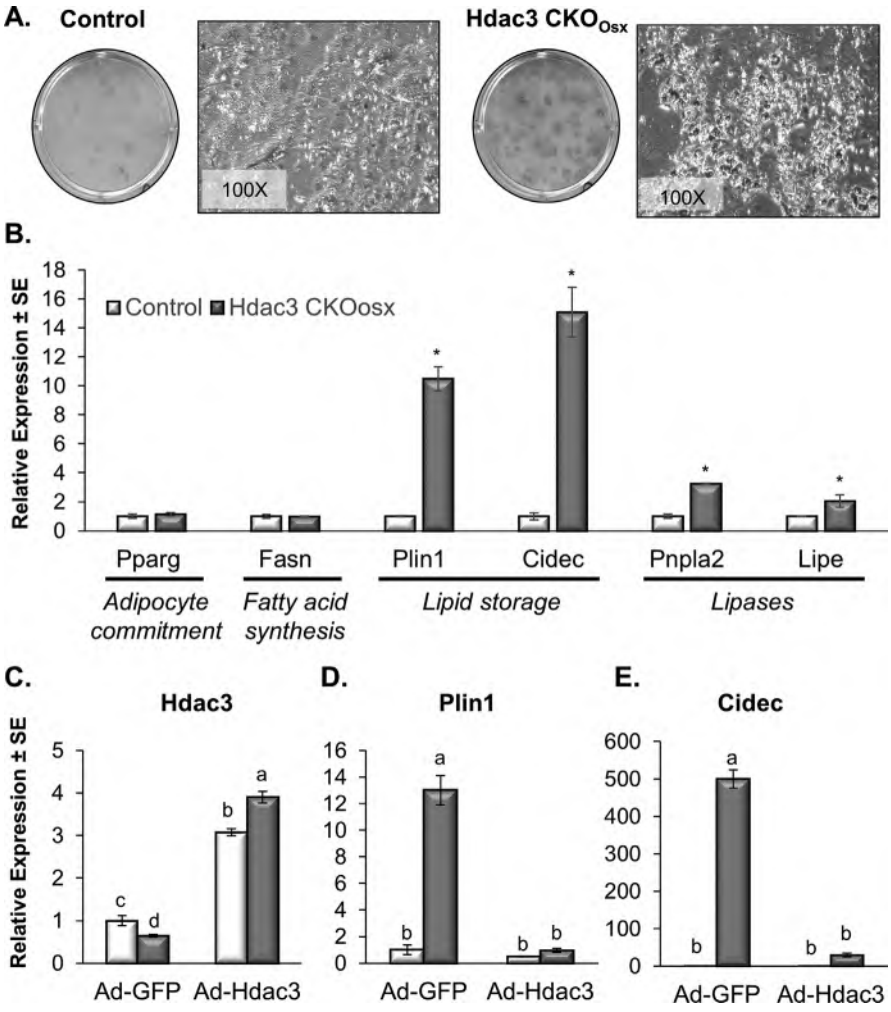

Fig. 2. Hdac3-deficient BMSC form lipid droplets when grown in osteogenic medium. (*A*) BMSCs from 8-week-old Hdac3 CKO_{Osx} or control animals were cultured in osteogenic medium for 21 days and stained with Oil Red O. (*B*) BMSCs from 4-week-old Hdac3 CKO_{Osx} animals were cultured in osteogenic medium for 7 days. The abundance of mRNA transcripts for the indicated genes was determined by qPCR. Data represent means ± SE of 3 replicates. $^{*}p \leq 0.05$ versus control. (*C–E*) BMSCs from 4-week-old Hdac3 CKO_{Osx} mice and control littermates were transduced once (at the time of seeding) with adenoviruses expressing either V5-tagged Hdac3 (Ad-Hdac3) or GFP (Ad-GFP). Cells were grown in osteogenic medium for 7 days and the indicated transcripts were measured by qPCR. Data represent means ± SE of three replicates. Bars with different letters are significantly ($p \leq 0.05$) different from one another.

(data not shown) or day 28 in culture (Supporting Fig. 2), which is consistent with the observation that Hdac3 CKO_{OCN} mice do not develop more marrow fat than controls.[31] No striking differences in MTS activity, fibroblastic potential via fibroblastic colony-forming unit (CFU-F) assays, or response to adipogenic stimuli in adipogenic CFU (CFU-Ad) assays were observed between control and Hdac3 CKO_{Osx} cultures (Supporting Fig. 3).

Lipid droplets are organelles where excess triglycerides are stored and can form in many cell types.[48] Lipid droplet density, like bone density, is a balance of formation and resorption.[49] Lipid droplet formation is dependent on the activity of molecules involved in fatty acid synthesis (eg, FASN) and storage (eg, Plin1, Fsp27/Cidec), whereas lipolysis is mediated by lipases (eg, Pnpla2, Lipe) and autophagic processes.[49,50] In Hdac3 CKO cultures, we observed substantial increases in lipid storage genes within 7 days (eg, Plin1, Fsp27/Cidec), but relatively little change in Pparγ2 or fatty acid synthesis (Fasn) and small increases in lipase genes (Pnpla2, Lipe) as compared to control BMSCs (Fig. 2*B*). The levels of Plin1 and Fsp27/Cidec were restored (repressed) after adenoviral delivery of V5-Hdac3 (Ad-Hdac3) into Hdac3 CKO_{Osx} cells (Fig. 2*C–E*). This response was unique to culture in osteogenic medium, because gene expression of Plin1 and Cidec were comparable between control and Hdac3 CKO_{Osx} cultures grown in adipogenic medium (Supporting Fig. 3). These data show that Hdac3 depletion increases the ability of bone marrow–derived cells to store lipids.

Hdac3 deficiency enhances lipid droplet formation in Runx2-expressing osteochondroprogenitor cells

To better understand which bone marrow cells were storing lipid droplets, BMSC cultures were examined by immunofluorescence microscopy. After 14 days in osteogenic medium, a substantial number of cells in both control and Hdac3 CKO_{Osx} cultures expressed either Runx2 (a marker of the osteoblastic lineage) or Pparγ2 (a marker of the adipocyte lineage) (Fig. 3*A*). The Pparγ2-positive cells contained numerous lipid droplets. A subpopulation of Runx2-expressing cells also contained cytosolic lipid droplets surrounded by Perilipin 1 (Fig. 3*B*). Consistent with previous studies,[51] this subpopulation accounted for approximately 5% of the control cultures; however, the number of Runx2-positive/lipid-positive cells was threefold higher in cultures from Hdac3 CKO_{Osx} mice (Fig. 3*C*). These data indicate that Runx2+ cells can form lipid droplets, and that Hdac3 deficiency enhances formation of these Runx2+ lipid-storing cells in osteogenic BMSC cultures.

Glucocorticoids promote lipid droplet formation in Hdac3-insufficient BMSC cultures

To further define the molecular signature of the osteogenic BMSC cultures, a microarray analyses of gene expression was performed. A total of 806 genes were differentially regulated ($p < 0.05$) in the Hdac3 CKO_{Osx} BMSCs as compared to control cultures. Twenty-one genes demonstrated an absolute fold change of 1.4-fold or greater (Table 1), including Leptin and an additional gene involved in lipid storage, G0S2. Notably, Pparγ2 was not identified as being differentially regulated in the Hdac3 CKO_{Osx} cultures. Interestingly, the most consistently and highly induced gene in the Hdac3 CKO_{Osx} cultures was 11β-hydroxysteroid dehydrogenase type 1 (Hsd11b1) (Table 1), which encodes an enzyme involved in the intracellular activation of glucocorticoids and promotion of glucocorticoid receptor (GR)-based signaling mechanisms. Elevated expression of Hsd11b1 in Hdac3 CKO cultures was validated by qRT-PCR and Western blotting assays and was restored to normal levels by adenoviral delivery of Hdac3 (Fig. 4*A*, *B*). Hdac3 repressed activation of Hsd11b1 promoters by approximately 60% (Fig. 4*C*). Carbenoxolone, an Hsd11b1 inhibitor, greatly reduced Cidec and Plin1 expression in Hdac3 CKO cultures, but had no effect on Pparγ levels (Fig. 4*D*). These data indicate that Hsd11b1 contributes to the elevation of lipid storage genes in Hdac3-deficient BMSCs.

Given this gene profile, we next determined if glucocorticoids influenced lipid droplet formation in Hdac3-insufficient osteogenic BMSC cultures. Similar to other groups,[28] our standard osteogenic culture medium contains a low concentration of dexamethasone, a common osteogenic additive which is

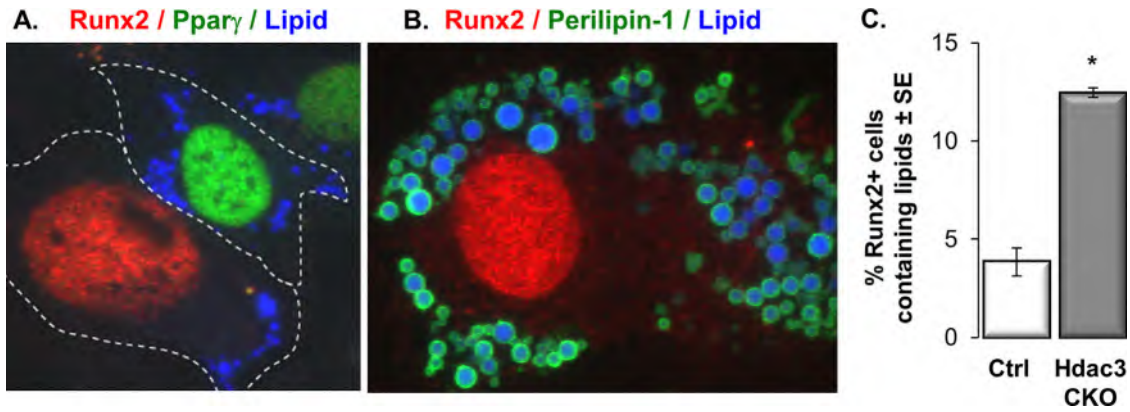

Fig. 3. Runx2-positive cells can contain cytosolic lipid droplets. (*A*, *B*) BMSC from 4-week-old Hdac3 CKO_{Osx} mice or control littermates were grown in osteogenic medium for 14 days and stained with antibodies against Runx2 (red) and Pparγ2 (green) (*A*) or antibodies against Runx2 (red) and Perilipin 1 (green), with subsequent staining with monodansylpentane (blue), a neutral lipid stain, to highlight lipid droplets (*B*). Cells from Hdac3 CKO_{Osx} cultures demonstrated prevalence of Runx2-positive cells that also showed positive staining for intracellular lipid droplets. (*C*) The number of Runx2-positive/lipid droplet-positive cells in cultures from Hdac3 CKO_{Osx} and control mice were counted. Data were collected from over 100 cells in each culture and represent the means ± SE of two independent experiments. $^{*}p \leq 0.05$ versus control.

Table 1. Genes Identified in Microarrays as Differentially Regulated >1.4-fold (±3 SD) in Hdac3 CKO_{Osx} BMSCs Compared to Control BMSCs Grown for 7 Days in Osteogenic Medium

Gene ID	Gene name	Control (mean)	Hdac3 CKO_{Osx} (mean)	*p*	Fold-change Hdac3 CKO_{Osx} versus control
Hsd11b1	11-beta hydroxysteroid dehydrogenase 1	171	457	0.001	2.7
Mrap	Melanocortin 2 receptor accessory protein	272	524	0.012	1.9
G0s2	G0/G1 switch gene 2	231	410	0.027	1.8
Lep	Leptin	108	186	0.023	1.7
Sort1	Sortilin 1	701	1148	0.026	1.6
4930486L24Rik	RIKEN cDNA 4930486L24 gene	248	405	0.009	1.6
Stard8	START domain containing 8	639	982	0.017	1.5
Ltc4s	Leukotriene C4 synthase	193	296	0.011	1.5
Slc4a8	Solute carrier family 4, member 8	162	243	0.036	1.5
Eno3	Enolase 3, beta muscle	347	503	0.006	1.5
Cygb	Cytoglobin	141	200	0.011	1.4
Col4a1	Collagen, type IV, alpha 1	2925	4153	0.020	1.4
Apoc1	Apolipoprotein C-I	92	129	0.050	1.4
Col4a2	Collagen, type IV, alpha 2	1415	1977	0.033	1.4
Angptl4	Angiopoietin-like 4	519	723	0.041	1.4
Mgl1	Macrophage galactose N-acetyl-galactosamine specific lectin 1	177	242	0.021	1.4
P2ry6	Pyrimidinergic receptor P2Y, G-protein coupled, 6	5157	7048	0.041	1.4
Txnip	Thioredoxin interacting protein	1125	1526	0.020	1.4
Arhgap29	Rho GTPase activating protein 29	718	973	0.046	1.4
Tnn	Tenascin N	602	438	0.003	−1.4
Brinp3	Bone morphogenetic protein/retinoic acid inducible neural specific 3	247	152	0.004	−1.6

Control (mean) and Hdac3 CKOOsx (mean) represent the average gene expression within each group, as measured by the Illumina MouseRef 8 assay, from 5 independent experiments using cells from control and Hdac3 CKOOsx littermate mice.

required for osteogenic differentiation of human BMSCs[52] and that is routinely included in osteoblast cultures from other species as well (although not strictly required for murine stromal cells[53]). Genes associated with lipid droplet formation (Plin1 and Cidec) were not elevated in osteogenic Hdac3 CKO_{Osx} cultures when dexamethasone was excluded from the culture medium (Fig. 4*E*). These results suggest that Hdac3 insufficiency sensitizes differentiating osteoblasts to the effects of glucocorticoids. Similar expression patterns and glucocorticoid sensitivity were seen in osteogenic BMSC cultures from the Col2ERT-Cre line, although the dexamethasone-induced expression of lipid storage genes was less in Hdac3 $CKO_{Col2ERT}$ mice than in the Hdac3 CKO_{Osx} line (Supporting Fig. 4).

To determine if Hdac3 influences glucocorticoid-responsive transcriptional activity, we tested its ability to regulate transcription from a glucocorticoid-responsive MMTV-luciferase reporter construct.[40] Dexamethasone strongly activated the MMTV-luciferase reporter more than 10-fold, but Hdac3 attenuated this activation by 60% (Fig. 4*F*). Together, these results demonstrate that Hdac3 is a crucial regulator of glucocorticoid-induced lipid droplet formation in osteoprogenitor cells.

Hdac3 levels are suppressed in bone cores from aged humans and in osteogenic cultures of BMSCs from aged wild-type mice

The high marrow adiposity and low bone mass phenotype of young Hdac3 CKO_{Osx} mice mimics that of an aged skeleton.[30] To unify this observation with our molecular findings in Hdac3 CKO animals, we determined if aging affects Hdac3 expression levels in bone and isolated osteoblasts from mice and humans. Bone cores from the ilia of postmenopausal (mean age: 72.9 years old) women expressed less HDAC3 mRNA than those from young (mean age: 30.0 years old) women (Fig. 5*A*). Previous analyses of these same samples reported a lipogenic gene expression profile in old as compared to young bone samples, where both Leptin and PLIN1 were elevated in the bone samples from the older women, but PPARγ2 levels were unchanged,[42] similar to gene expression patterns observed in osteochondroprogenitor cultures from Hdac3 CKO mice. In osteogenic cultures of murine BMSCs from 22-month-old animals, Hdac3 mRNA levels were also reduced 20% and Hdac3 protein levels were reduced by 50% as compared to 2-month-old mice (Fig. 5*B*, *C*). We also assessed the phosphorylation of S424 in Hdac3 (pHdac3) because this posttranslational modification is required for Hdac3 enzymatic activity.[54] Osteoblasts from 14-month-old mice expressed 60% less pHdac3 than osteoblasts isolated from 2-month-old mice (Fig. 5*C*); similar results were obtained comparing BMSC-derived osteoblasts from 26-month-old to 2-month-old mice (Fig. 5*D*). Osteogenic BMSC cultures from old mice contained sixfold more lipid droplets as compared to cells from young mice (Fig. 5*E*; ratio of lipid containing to total cells: old = 26.5% ± 2.4%, young = 4.3% ± 1.1%). Moreover, the lipid droplets were approximately 60% larger in aged mice (average lipid droplet area older

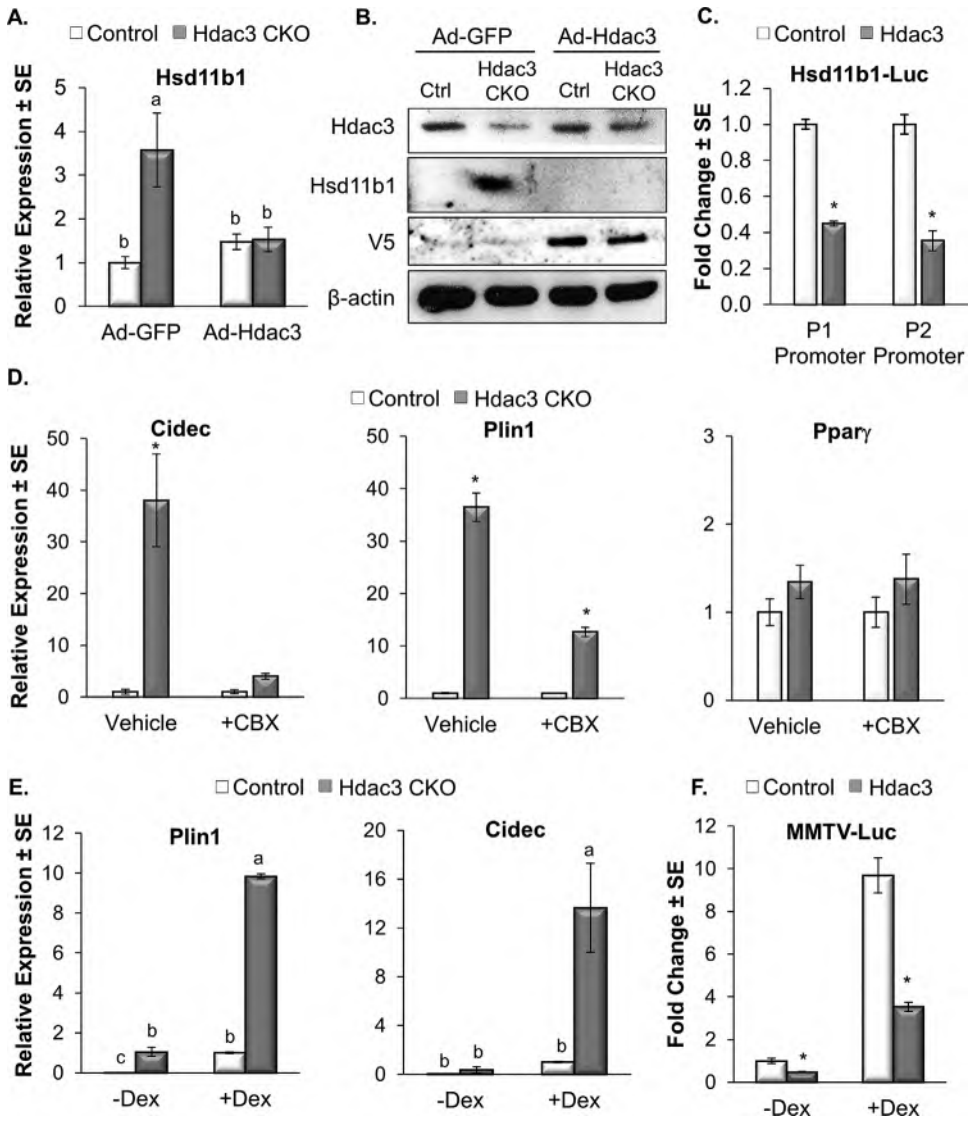

Fig. 4. Lipid droplet formation in Hdac3-insufficient cells is dependent upon glucocorticoid signaling. (*A*, *B*) BMSCs from 4-week-old Hdac3 CKO_{Osx} mice and control littermates were transduced once (at the time of seeding) with either Ad-GFP or Ad-Hdac3 and cultured in osteogenic medium for 7 days. Hsd11b1 expression was measured by qPCR (*A*) and Western blotting (*B*). (*C*) C2C12 cells were transfected with Hsd11b1 reporter constructs and 300 ng pcDNA3 (control) or Hdac3 cDNA. Firefly luciferase values were normalized to Renilla luciferase. Data represent the mean change ± SE of three replicates from each respective control. Results are representative of at least three experiments; $^{*}p \leq 0.05$ versus control. (*D*) BMSC from 4-week-old Hdac3 CKO_{Osx} mice and control littermates were cultured in osteogenic medium with and without 5 μM carbenoxolone (CBX) for 7 days. Expression levels of Plin1, Cidec, and Pparγ were measured by qPCR. Data represent the means ± SE of three replicates. $^{*}p \leq 0.05$ versus Control. (*E*) BMSCs from 4-week-old Hdac3 CKO_{Osx} mice and control littermates were cultured in osteogenic medium with and without dexamethasone (Dex) for 7 days. Expression levels of Plin1 and Cidec were measured by qPCR. Data represent the means ± SE of three replicates. Bars with different letters are significantly ($p \leq 0.05$) different from one another. (*F*) C2C12 cells were transfected with glucocorticoid-responsive MMTV-luciferase reporter construct and 300 ng pcDNA3 (Control) or Hdac3 cDNA. Cells were treated with 100 nM Dex or vehicle (ethanol) for 24 hours. Firefly luciferase values were normalized to Renilla luciferase. Data represent the mean change ± SE of three replicates from the vehicle-treated control. Results are representative of at least three experiments. $^{*}p \leq 0.05$ versus control.

mice = 506 ± 51 pixels, younger mice = 285 ± 53 pixels), and 7% were Runx2+ (Fig. 5*F*). Like Hdac3-depleted mice, the osteogenic BMSC cultures from older mice did not have elevated levels of Pparγ2 or Fasn as compared to cells from young mice despite their propensity to form lipid droplets, but did demonstrate multifold increases in the expression levels of lipid storage genes, Plin1 and Cidec/Fsp27, in the presence of dexamethasone (Fig. 5*G*). Collectively, these data demonstrate that reductions in Hdac3 transcript levels and phosphorylation combine to suppress overall Hdac3 activity in aging bone.

Discussion

Hdac3 is essential for skeletal development and a key regulator in cellular lipid metabolism. In previous studies, we showed that mice with conditional deletion of Hdac3 in mature osteoblasts

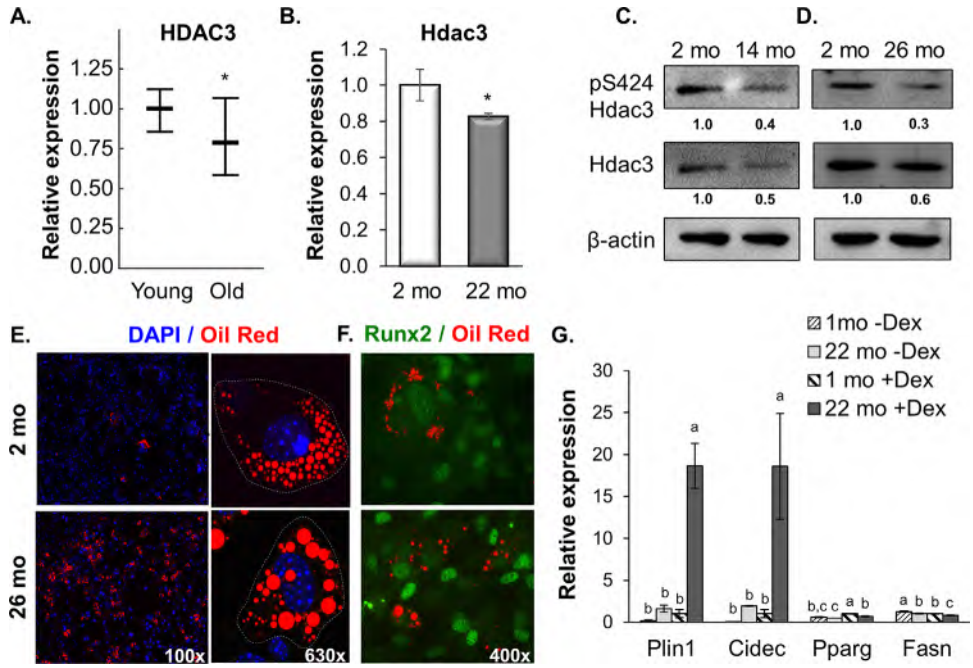

Fig. 5. Osteoblastic Hdac3 expression decreases with age. (*A*) HDAC3 mRNA levels were measured in bone cores from the ilium of old (64 to 88 years old) and young (22 to 40 years old) women by qPCR. Plots show the median ± interquartile range of 10 independent samples per group. $^{*}p = 0.045$. (*B*) Hdac3 mRNA levels were measured in BMSCs from 22-month-old and 1-month-old mice that were cultured in osteogenic medium for 7 days. Data represent the means ± SE of three replicates, and are representative of aging-related changes in at least three independent experiments. $^{*}p \leq 0.05$ versus younger mice. (*C*, *D*) Cortical bone-derived osteoblasts (*C*) or BMSC-derived osteoblasts cultured for 14 days in osteogenic medium (*D*) were lysed for Western blotting with antibodies recognizing pS424-Hdac3, Hdac3, and beta-actin. Relative band intensity (normalized to beta-actin) is expressed beneath each band. (*E*, *F*) After growth in osteogenic medium for 14 days, BMSC cultures from 26-month-old and 2-month-old mice were stained with Oil Red O (red), DAPI (blue), and/or antibodies to Runx2 (green) and imaged by epifluorescence and confocal microscopy. (*G*) BMSCs from 22-month-old and 1-month-old mice were cultured in osteogenic cell culture medium in the presence or absence of dexamethasone. Data represent the means of triplicate samples ± SE. Bars with different letters are significantly ($p \leq 0.05$) different from one another.

(with OCN-Cre) or osteoblast progenitor cells (Osx1-Cre) develop osteopenia and are more prone to fracture,[30,31] but only the latter model developed a concomitant phenotype of increased bone marrow adiposity.[30] Here we show that postnatal in vivo deletion of Hdac3 with Cre recombinase driven by another promoter active in osteochondroprogenitor cells (Col2ERT-Cre) also increases marrow adiposity. BMSCs isolated from mice lacking Hdac3 in osteochondroprogenitor cells formed lipid droplets within 7 days when cultured in osteogenic medium containing dexamethasone. A small fraction (~5%) of the cells containing lipid droplets in control cultures were Runx2-positive; however, this number increased threefold in Hdac3-depleted BMSCs. These data suggest that Runx2-expressing osteoblast lineage cells can form lipid droplets and constitute a distinct component of marrow adipose tissue.

Adipose tissue is abundant throughout the adult human skeleton, accounting for up to 70% of bone marrow volume and approximately 7% of total body fat content.[6] The fraction of bone MAT increases with age, ranging from approximately 20% before the third decade in life to nearly 50% by the ninth decade.[4] The origins of MAT are complex and incompletely understood. Adipocytes, chondrocytes, and osteoblasts originate from mesenchymal progenitors, and a prevailing view is that adipogenesis is a default program when osteogenesis is interrupted, as occurs in Runx2-deficient cells.[18,19] Infiltration of adipocytes from non-osseous tissues and transdifferentiation of osteoblasts into adipocytes have also been proposed[5]; however, the technologies used in these studies have numerous limitations. For example, traditional paraffin-embedding or plastic-embedding procedures make it difficult to histologically identify bone marrow cell types that contain lipids because chemical processing removes lipids, leaving behind voids (ghosts) in tissue sections. In vitro studies on BMSC cultures measure gene expression levels of the entire population, allowing majority subsets to obscure changes within smaller subsets or single cells in the cultures. Thus, in addition to employing these techniques, we used fluorescent probes and antibodies to observe lipid droplets in BMSCs cultured in osteogenic medium at the single-cell level. Some Runx2+ cells in control cultures had lipid droplets, but threefold more Runx2+ cells in Hdac3-CKO$_{Osx}$ cultures contained lipids. Recent data from other groups show that Osx1+ osteoprogenitor cells harbor lipid droplets and were interpreted as evidence of transdifferentiation.[20,55,56] Although we cannot rule out transdifferentiation in our system, we did not observe any cells that simultaneously expressed Runx2, Pparγ2, and lipid droplets. Previous studies suggested that BMSC-derived adipocytes are capable of inducing cocultured osteoblasts to develop adipocyte-like characteristics,[57] possibly through the release of extracellular vesicles carrying adipogenic RNA contents that target the osteoprogenitor cells.[58] These mechanisms could be related to our observations, because Pparγ2-expressing cells

were also observed in the osteogenic cultures via immunofluorescence microscopy. Lineage tracing experiments will be required to test the transdifferentiation hypothesis, but we propose an alternative possibility based on our immunofluorescence studies with Hdac3 CKO mouse cells that some Runx2-expressing osteoprogenitors are capable of storing lipids when Hdac3 levels decline because cellular and biochemical pathways which regulate lipid metabolism are consequently induced by transcriptional derepression. Studies characterizing the molecular profile of lipid-containing bone marrow cells in situ will be necessary to test whether this mechanism persists in vivo and whether it contributes directly to bone marrow adiposity.

Molecular profiling indicated that lipid droplets accumulate in Hdac3-depleted osteogenic BMSC cultures along with increased expression of genes involved in lipid storage and intrinsic glucocorticoid processing. Osteoprogenitor cultures from 14-month-old and 26-month old mice also had reduced expression of total Hdac3 and Hdac3 phosphorylated on serine residue 424 (which may be reflective of its enzymatic activity) that coincided with higher numbers of lipid droplets and elevated levels of lipid storage genes and Hsd11b1 as compared to cells from young animals. These results are comparable to the Hdac3 mRNA reductions found in raw bone marrow from aging C57BL/6 mice in the Atlas of Gene Expression in Mouse Aging Project (AGEMAP) study.[59] HDAC3 mRNA levels also tended to be lower in bone from postmenopausal women as compared to young women. The 20% decreases in Hdac3 mRNA levels in old mice and humans are modest; however, it is important to consider that we typically only detect 30% to 50% reductions in Hdac3 transcripts in the CKO bones and BMSC cultures because Hdac3 is required for cell survival,[30,31] and therefore the surviving cells are possibly hypomorphic for Hdac3, rather than fully Hdac3-deficient. Thus, a prolonged 20% reduction in Hdac3 mRNA in aging progenitor cell populations may be functionally impactful. Combined with our published results,[30] these data indicate that mice deficient in Hdac3 in osteoprogenitor cells are models of premature skeletal aging, and that reductions in Hdac3 activity and expression levels in bone cells during aging can contribute to reduced bone formation, higher marrow adiposity, and increased fragility.

Many cell types, including hepatocytes, cardiac myocytes, pancreatic beta cells, and lymphocytes, can form lipid droplets, particularly in disease states,[60] where lipid droplet formation and utilization in these cells can be linked to important biological processes such as autophagy.[61] Interestingly, chondrocytes were shown to store lipids as early as 1965.[62] An important role for fatty acid oxidation in osteoblast biology has been reported as well.[63,64] At present, it is unknown if cellular bioenergetics are affected in Hdac3 CKO osteochondroprogenitors, but ongoing and future studies will address this important question. Conditional deletion of Hdac3 in liver[65,66] and cardiac muscle[67,68] promoted lipid accumulation. Because osteoblasts and adipocytes share a common mesenchymal progenitor cell, the abundant MAT found in the Osx1-Cre Hdac3 CKO mice raises the question of whether Hdac3 deletion would promote adiposity in peripheral adipose depots. Surprisingly, however, the opposite is true; our recent studies indicate that Hdac3 CKO mice present with a leaner body mass and lower fasting glucose levels as compared to control littermates.[69] Hdac3 and its cofactors, NCoR1/2 (nuclear hormone co-receptors), influence metabolic pathways and lipid droplet formation in several tissues, including liver and skeletal muscle, in both deacetylase-dependent and deacetylase-independent manners, by binding transcription factors and altering gene expression networks regulating cellular metabolism.[65–67,70–73] The enzymatic (deacetylase) activity of Hdac3 is dependent on the phosphorylation of S424 and inversely correlated with the serine/threonine phosphatase, PP4c.[54] In preliminary experiments, Ppp4c levels appear to be inversely proportional to pHdac3 in BMSCs. Future studies will be needed to address the mechanistic role of Hdac3 in the formation of lipid droplets in Runx2-positive cells, and whether these processes are linked to biochemical or epigenetic events. Thus, Hdac3 is emerging as a crucial regulator of lipid metabolism in bone as well as in other organs.

Bone marrow adipose tissue and skeletal fragility are linked in a number of physiological conditions and disease states besides aging. For example, disuse or inactivity increases marrow adiposity and reduces bone strength, whereas osteoanabolic stimuli such as exercise and mechanical loading reduce marrow adipose tissue.[8–12] A multitude of other stimuli, including exogenous glucocorticoid therapy,[74] thiazolidinediones,[75] and severe caloric restriction,[16,76] increase marrow adipose tissue. Importantly, the Hdac3 CKO_{Osx} animals consume as much chow on a per-bodyweight basis as littermate mice[69]; thus, the increased bone marrow fat in Hdac3 CKO_{Osx} animals is not due to nutritional deficiencies. Bone marrow fat is not quiescent; rather, this tissue is a primary producer of adiponectin during caloric restriction, consequently affecting the metabolic activity of nearby muscle,[16] and is also an insulin-sensitive tissue.[77] Thus, skeletal expression of Hdac3 (and its subsequent regulation of bone marrow fat) is positioned to play a key role in other metabolic models.

Transcripts for proteins associated with lipid storage were highly expressed in Hdac3 CKO BMSCs cultured in osteogenic medium containing the glucocorticoid dexamethasone, but the most consistently induced gene in these cultures was 11β-hydroxysteroid dehydrogenase (Hsd11b1). The role of Hdac3 in regulating transcriptional activity of Hsd11b1 was confirmed in transcription assays (Fig. 4*C*), and lipid storage gene expression patterns in Hdac3 CKO cells were partially attenuated by carbenoxolone, a pharmacological inhibitor of Hsd11b1 activity (Fig. 4*D*). Hsd11b1 is an important enzyme that drives glucocorticoid action within osteoblasts at the pre-receptor level by converting biologically inactive glucocorticoids (eg, prednisone or cortisone in humans, 11-dehydrocorticosterone in rodents) to active forms (prednisolone or cortisol in humans, corticosterone in rodents) through reductase activity.[78] Hsd11b1 levels and activity increase in both human and murine bone cells during aging, which suggests that cells from older individuals may be exposed to higher intrinsically active glucocorticoid levels even if circulating glucocorticoid concentrations do not change.[79–82] Genetic evidence is aligned with these biological data because HSD11B1 polymorphisms associate with low bone mineral density.[83–86]

In conclusion, our data show that suppression of Hdac3 activity in osteochondroprogenitor cells contributes to bone loss and increased marrow adiposity associated with aging and long-term glucocorticoid treatment. We propose a model for interaction between Hdac3, Hsd11b1, and glucocorticoid signaling that primes osteochondroprogenitors to store lipids (Fig. 6*A*), which expands our knowledge of the role of Hdac3 in osteoblast differentiation (Fig. 6*B*). A mechanistic link between Hdac3, aging, and glucocorticoids is important because aging

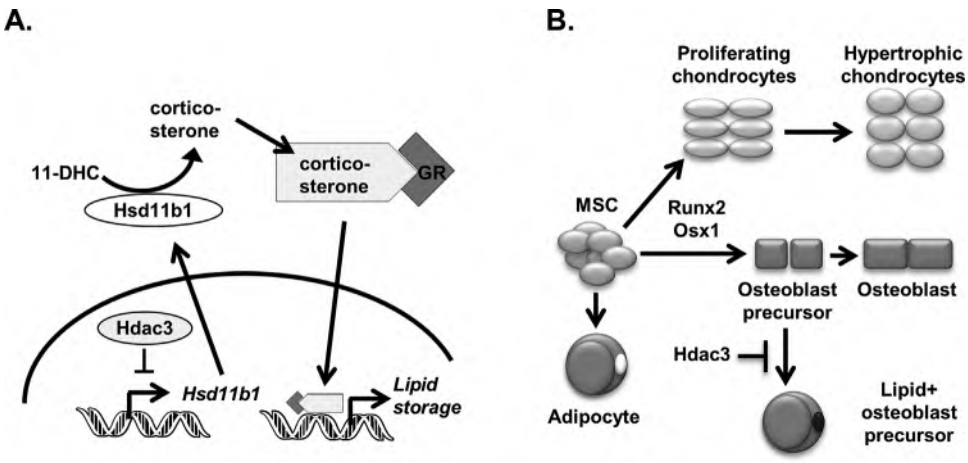

Fig. 6. (*A*) Proposed model for interaction between Hdac3, Hsd11b1, glucocorticoid signaling, and lipid storage mechanisms in osteoblasts. Hdac3 suppresses Hsd11b1 expression to control intracellular glucocorticoid activation and lipid metabolism. (*B*) Model for Hdac3's role in the differentiation patterns of skeletal cell populations. Hdac3 is required for proper osteoblast and chondrocyte maturation and inhibits the formation of lipid droplets in Runx2+ osteoblast progenitor cells. 11-DHC = 11-dehydrocorticosterone.

and prolonged glucocorticoid treatments are two of the leading causes of osteoporosis and fractures.[87,88] Better knowledge of how Hdac3 expression levels and activity are controlled, and in turn, how Hdac3 suppresses lipid storage, will improve bone health and prevent debilitating fractures.

Disclosures

All authors state that they have no conflicts of interest.

Acknowledgments

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Authors' roles: Study design: MEML and JJW. Study conduct: MEML, LRC, RJS, JNF, and JLP. Data collection: MEML, LRC, RJS, JNF, and JLP. Data analysis: MEML, LRC, RJS, JNF, JLP, and JJW. Data interpretation: MEML, LRC, RJS, SK, and JJW. Drafting manuscript: MEML and JJW. Revising manuscript content: MEML, LRC, RJS, JNF, JLP, SK, MJO, and JJW. Approving final version of manuscript: MEML, LRC, RJS, JNF, JLP, MJM, SK, MJO, and JJW. MEML and JJW take responsibility for the integrity of the data analysis.

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