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The glucocorticoid receptor in osteoprogenitors regulates bone mass and marrow fat

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Abstract

Excess fat within bone marrow is associated with lower bone density. Metabolic stressors such as chronic caloric restriction (CR) can exacerbate marrow adiposity, and increased glucocorticoid signaling and adrenergic signaling are implicated in this phenotype. The current study tested the role of glucocorticoid signaling in CR-induced stress by conditionally deleting the glucocorticoid receptor (*Nr3c1*; hereafter abbreviated as GR) in bone marrow osteoprogenitors (Ox1-Cre) of mice subjected to CR and *ad libitum* diets. Conditional knockout of the GR (GR-CKO) reduced cortical and trabecular bone mass as compared to WT mice under both *ad libitum* feeding and CR conditions. No interaction was detected between genotype and diet, suggesting that the GR is not required for CR-induced skeletal changes. The lower bone mass in GR-CKO mice, and the further decrease in bone by CR, resulted from suppressed bone formation. Interestingly, treatment with the β -adrenergic receptor antagonist propranolol mildly but selectively improved metrics of cortical bone mass in GR-CKO mice during CR, suggesting interaction between adrenergic and glucocorticoid signaling pathways that affects cortical bone. GR-CKO mice dramatically increased marrow fat under both *ad libitum* and CR-fed conditions, and surprisingly propranolol treatment was unable to rescue CR-induced marrow fat in either WT or GR-CKO mice. Additionally, serum corticosterone levels were selectively elevated in GR-CKO mice with CR, suggesting the possibility of bone–hypothalamus–pituitary–adrenal crosstalk during metabolic stress. This work highlights the complexities of glucocorticoid and β -adrenergic signaling in stress-induced changes in bone mass, and the importance of GR function in suppressing marrow adipogenesis while maintaining healthy bone mass.

Key Words

- ▶ skeleton
- ▶ marrow adiposity
- ▶ osteoprogenitor
- ▶ osteoblast
- ▶ nutrition
- ▶ metabolic stress
- ▶ corticosterone
- ▶ sympathetic tone
- ▶ β -adrenergic

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Introduction

The bone marrow niche houses stem cell populations that give rise to blood, immune, bone and fat cells within the skeletal microenvironment. The fate of bone marrow progenitor cells can be influenced by hormones and nutrient signals from the circulation, establishing a relationship between whole-body physiology and bone health (Li *et al.* 2016). A vital component of the bone marrow microenvironment is the bone marrow stromal cell (BMSC), which is a multipotent progenitor capable of differentiating into osteogenic and adipogenic cell populations within bone (Baker *et al.* 2015). The differentiation fate of the BMSCs can be influenced by genetic predispositions, dietary and lifestyle factors, stress and injury responses and epigenetics. Osteoblastogenesis is influenced by pathways including Wnt/ β -catenin, transforming growth factor- β (TGF- β), bone morphogenetic proteins (BMPs) and glucocorticoid signaling, among others (Day *et al.* 2005, Bennett *et al.* 2007, Kang *et al.* 2007, Zhang *et al.* 2012). There is a delicate balance between these mechanisms, and interruptions to the homeostasis of the bone marrow microenvironment can dramatically shift BMSC differentiation patterns at the expense of tissue function and bone health.

Recent studies have uncovered an intricate relationship between caloric intake and bone. For example, chronic caloric deficit induces high bone marrow adipogenesis accompanied by osteopenia in humans and mice (Devlin *et al.* 2010, Fazeli *et al.* 2013, Periyasamy-Thandavan *et al.* 2015, Singhal & Bredella 2019). It has also been reported that a low-calorie diet reduces bone mass in part by decreasing cortical bone mass while largely sparing trabecular bone in mice (Hamrick *et al.* 2008). There is evidence that the phenotype produced by caloric restriction (CR) is mediated in part by glucocorticoid signaling (Cawthorn *et al.* 2016). The metabolic stress of CR can stimulate counter-regulatory hormone secretion, including glucocorticoids, that may interfere with osteoblast function and drive marrow adipose tissue expansion (Devlin *et al.* 2010, Cawthorn *et al.* 2016). The enzyme 11 β -hydroxysteroid dehydrogenase type 1 (Hsd11b1) (which converts inactive glucocorticoids to their active forms in the periphery) also mediates upregulated glucocorticoid metabolism throughout the body and locally within bone as a result of CR (Tomlinson *et al.* 2004, Gathercole *et al.* 2013). Additionally, it has been reported that *in vitro* pharmacological inhibition of Hsd11b1 activity protects osteoblasts from glucocorticoid-induced cellular dysfunction (Wu *et al.* 2013).

These data suggest a potential role for aberrant glucocorticoid signaling in the CR-induced bone phenotype, but the mechanistic role of modulators downstream of Hsd11b1 remain largely unknown.

Activated glucocorticoids bind the glucocorticoid receptor (GR; also known as *Nr3c1*) within target tissues and drive downstream stress response signaling cascades (Almeida *et al.* 2011). Once bound to glucocorticoids, the GR translocates to the nucleus to bind glucocorticoid response elements (Nixon *et al.* 2013, Lim *et al.* 2015) or associate with transcription factors that act on genomic regulatory elements (Hua *et al.* 2016a,b). While physiological levels of endogenous glucocorticoids are necessary for osteoblastogenesis and bone formation (Zhou *et al.* 2013), an excess of circulating glucocorticoids can compromise bone mass by inhibiting osteoblast differentiation and inducing osteoblast and osteocyte apoptosis (Weinstein *et al.* 1998, O'Brien *et al.* 2004, Rauch *et al.* 2010, Weinstein 2012). Downstream glucocorticoid signaling may also drive marrow adipogenesis, as it has been reported that glucocorticoids induce adipocyte commitment and contribute to the fatty infiltration of soft tissues (e.g., liver and skeletal muscle) as well as bone (Canalis *et al.* 2004, Kuo *et al.* 2013, Hamrick *et al.* 2016, Papanastasiou *et al.* 2017). These findings further support a role for the GR in mediating the bone loss and increased marrow fat caused by CR and suggest that the GR is a key in the response of bone to this facet of metabolic stress.

Sympathetic tone may also be involved in glucocorticoid-induced marrow adiposity (Eleftheriou 2005). Osteoblasts express β -adrenergic receptors (β -AR) that, when activated, initiate signaling cascades that result in decreased osteoblast number and activity as well as loss of bone mass. Importantly, the sensitivity of osteoblast to adrenergic signaling is also increased by glucocorticoid treatment (Bonnet *et al.* 2008, Ma *et al.* 2011). β -Adrenergic receptor activity also stimulates adipogenesis and mediates CR-induced marrow adiposity, whereas treatment with the β -adrenergic antagonist propranolol in a chronic caloric restriction model partially rescues the trabecular bone loss and marrow fat phenotype in mice and rats (Baek & Bloomfield 2012, Baek *et al.* 2014b). Moreover, propranolol protects against the loss of bone mass associated with a high-calorie diet (Baek *et al.* 2014a). Despite these findings, there is still a knowledge gap regarding the functional role of the GR in bone marrow lipid storage in the CR model when β -blockade occurs.

The current study sought to test the role of the GR in caloric restriction-induced changes in bone and marrow

fat. Within this model, we also sought to deduce the effect of sympathetic tone on the phenotype of metabolically stressed GR-insufficient bone through the use of the β -blocker propranolol.

Materials and methods

Animals and diet administration

All experiments followed NIH guidelines and were approved by the Institutional Animal Care and Use Committee at Augusta University. As described previously, GR was inactivated through Cre recombinase (Cre)-mediated excision of exons 1C and 2 of the *GR* gene (Fig. 1A). This modification deletes 50% of the mature GR protein, encompassing the translation start site and the tau1 transactivation domain and has previously been shown to effectively disrupt GR action (Brewer *et al.* 2003, Kim *et al.* 2006). Osteoprogenitor-specific inactivation of GR was accomplished through *Osx1* promoter-driven Cre expression as described previously (Rodda & McMahon 2006, McGee-Lawrence *et al.* 2016) to generate GR conditional knockout mice (GR-CKO; GR^{fl/fl}:*Osx1*-Cre+) and control littermates (GR-WT; GR^{fl/fl}:*Osx1*-Cre-) for this study ($n=7-8$ mice/group; Fig. 1A). While inclusion of an additional 'Cre+ control' (i.e., GR^{+/+}:*Osx1*-Cre+ for this study) is preferred for knockout models generated with the *Osx1*-Cre line, due to the presence of a mild, early skeletal phenotype arising from the *Osx1*-Cre itself, in pilot studies of 6-month-old male mice, we observed that marrow fat was elevated specifically in GR^{fl/fl}:*Osx1*-Cre+ mice, whereas there were no differences observed between GR^{fl/fl}:*Osx1*-Cre- (i.e., 'Cre- control') mice and GR^{+/+}:*Osx1*-Cre+ (i.e., 'Cre+ control') mice (Supplementary Methods and Supplementary Fig. 1, see section on [supplementary data](#) given at the end of this article). Furthermore, the skeletal phenotype of *Osx1*-Cre+ control mice is reported to resolve by 12 weeks (~3 months) of age, and all studies reported here were only initiated at 6 months of age (Davey *et al.* 2012). Consequently, we focused the current study on GR conditional knockout mice (GR-CKO; GR^{fl/fl}:*Osx1*-Cre+) and Cre- control littermates (GR-WT; GR^{fl/fl}:*Osx1*-Cre-). While the *Osx1*-Cre used does possess a doxycycline-suppressible Tet-off element, the mice used here were not treated with doxycycline to suppress Cre because studies were initiated after the *Osx1*-Cre skeletal phenotype was expected to resolve. Consequently, mice used for these studies experienced constitutive expression

of Cre in *Osx1*-expressing cells throughout their development. Female mice were specifically selected for study because in pilot work, we observed similar trends of elevated marrow adiposity in both male and female GR-CKO mice (Supplementary Fig. 1), but trends were much more pronounced in females, reducing the number of animals needed for study. Mice were genotyped by PCR amplification of tail biopsy DNA isolates with appropriate negative and positive controls. Mice were housed in standard rodent cages on a 12h light/12h darkness schedule. At 6 months of age, female GR-WT and GR-CKO mice were randomly assigned to either *ad libitum* (AL) or CR feeding for 9 weeks (Fig. 1B). Caloric intake was determined by the average weight of food pellets consumed daily, and CR was defined by a given fraction of the calculated average caloric intake provided to individual AL-fed mice daily. Mice subjected to CR were provided a 10% calorie deficit for one week followed by a 25% calorie deficit for 8 weeks. A subset of CR mice received drinking water with 0.01% propranolol (PRO, 0.1 mg/ml; Sigma-Aldrich #P0884) for the duration of the experiment, whereas other mice received only normal water (Vehicle, VEH); drinking water was available to both groups AL. This dosage of propranolol was chosen to match a previous study, where administration of ~0.1 mg/mL propranolol in the drinking water (equating to a dosage of approximately 6 mg propranolol per kg body mass) was sufficient to prevent accumulation of bone marrow adipocytes with CR in female rats (Baek & Bloomfield 2012). Calcein injections (10 mg/kg) were administered at 5 and 2 days before killing for measurements of cortical bone mineralization. Mice were killed via carbon dioxide inhalation and were not fasted the evening prior to killing.

Body composition and bone mineralization

Whole-body dual energy X-ray absorptiometry (DXA; Kubtec Digimus, KUB Technologies, Milford, CT, USA) was performed on isoflurane-anesthetized mice 2 days before killings. Bone mineral content (BMC) and bone mineral density (BMD) for whole mouse and femur mid-diaphysis regions of interest were calculated using the manufacturer's software. All DXA analyses excluded heads and ear tags. Percent fat mass was calculated as the fraction of the total body pixels interpreted by the manufacturer's software as fat tissue via densitometry. Final body mass measurements were recorded 5 days before killing.

Figure 1

Confirmation of GR knockdown in GR-CKO mice. (A) Model for glucocorticoid receptor (GR) conditional deletion with Osterix1-Cre (Osx1-Cre) to generate an osteoprogenitor-specific knockout model. (B) Timeline for *ad libitum* (AL), caloric restriction (CR) and CR+ propranolol (PRO) treatment conditions. (C) Expression of GR and Cre RNA was measured by qPCR in BMSC subjected to osteogenic culture for 7 days. $n = 4$ biological replicates per group, $*P < 0.05$ vs GR-CKO WT for each primer pair. (D) GR protein expression was detected via immunohistochemistry in the tibia; the inset label on each image indicates which antibody was used (GR, glucocorticoid receptor; IgG, mouse IgG isotype control). GR-CKO mice demonstrated reduced intensity of GR staining in the growth plate and in cortical bone (e.g., osteocytes). Images are representative of staining patterns observed across the entire group for each genotype ($n = 7$ animals per group); each row of images is from a different mouse. Growth plate images were captured with a 20× objective, whereas cortical bone images were captured with a 40× objective, as indicated.

Isolation of murine BMSC, cell culture and analysis of gene expression

Primary murine BMSC from the AL-fed groups were harvested from one femur and two humeri via bone marrow flush with basal medium composed of minimum essential media (MEM)- α , 20% fetal bovine serum (Atlanta Biologicals, Flowery Branch, GA, USA), 1% non-essential amino acids (Gibco #11140-050), and 1% antibiotic/antimycotic (Gibco #15240-062) as previously described (McGee-Lawrence *et al.* 2016). BMSC-derived osteoblasts were generated via culture in osteogenic induction medium (basal medium+50 μ g/mL ascorbic acid, 10 mM β -glycerophosphate and 10^{-7} M dexamethasone), which began at seeding (Day 0) and were maintained under osteogenic conditions until harvest after 7 days in culture. Media changes for all cells in culture were performed every 3 days until harvest. Total RNA was isolated from the cultures in TRIzol (Invitrogen) following the manufacturer's instructions. RNA quality was assessed by absorbance at 260 and 280 nm (NanoDrop, Thermo Scientific). RNA (1000 ng) was reverse-transcribed from four biological replicates per genotype (where each replicate represented an independent well of a six-well plate) into cDNA using a Superscript III cDNA synthesis kit (Superscript III First Strand synthesis (#18080-051), Life Technologies) and a programmable thermal cycler (Bio-Rad). Expression levels of mRNA for the GR (official gene name: nuclear receptor subfamily 3, group C; Nr3c1) were quantified by subjecting cDNA to real-time PCR amplification (37.5 ng cDNA per 15 μ L reaction volume reaction, run in triplicate) using a Bio-Rad CFX Connect system, SYBR green reagent (Quanta Biosciences #95054-500) and primers designed to bind within the floxed region of the GR Exon 2. Expression levels of Cre mRNA were also assessed as previously described (McGee-Lawrence *et al.* 2013, 2016). Gene expression levels were quantified using the comparative threshold cycle ($2^{-\Delta\Delta C_t}$) method (McGee-Lawrence *et al.* 2016). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the

internal control (housekeeping gene) for normalization. Primer sequences are shown in Table 1.

Micro-computed tomography, histology, histomorphometry and immunohistochemistry

Femora and tibiae were harvested at killing and fixed in 10% neutral buffered formalin for 24 hours followed by storage in 70% EtOH. Femora were scanned by micro-computed tomography (microCT, 10.5 μ m isotropic voxel size; Scanco VivaCT 40) with settings of 70 kV and 300 ms for analysis of cortical and trabecular bone architecture. The cortical bone region of interest was equal to 5% of total bone length, with a proximal starting point at 50% of total bone length. The trabecular bone region of interest was equal to 7% of total bone length, with a proximal starting point at 82% of total bone length. Architectural parameters (e.g., cortical bone area, trabecular number and spacing, trabecular volume) were calculated with the manufacturer's software.

Following microCT analysis, femora were embedded in methyl methacrylate (MMA) for microtome sectioning and mounting (5 μ m sections; Leica Biosystems). Calcein-labeled sections were imaged on a fluorescence microscope (20 $\times$ objective, Olympus IX70; Olympus Life Science) with a digital camera attachment (Qicam; QImaging) for dynamic histomorphometry measurements in the distal femoral metaphyseal trabecular bone with Bioquant Osteo. Mineralizing surface (MS/BS, %) was quantified as $\frac{1}{2}$ single labeled surface+double labeled surface normalized to bone surface. Mineral apposition rate (MAR, μ m/day) was quantified as inter-label width divided by time between calcein injections. Bone formation rate (BFR/BS, μ m/day) was quantified as the product of MAR and MS/BS. Tibiae were decalcified for 2 weeks in 15% EDTA followed by paraffin embedding and microtome sectioning. Sections for each tibia were stained with either hematoxylin and eosin (H&E; Sigma #HHS16, #H110316) or a commercially available tartrate-resistant acid phosphatase kit

Table 1 Primer sequences used for PCR analyses of mRNA expression.

Primer pair name	Target/amplicon size	Forward primer, 5'-3'	Reverse primer, 5'-3'
GR primer pair 1	GR Exon 2, 293 bp	CTCCCCCTGGTAGAGACGAA	TCCCATGGACAGTGAAACGG
GR primer pair 2	GR Exon 2, 174 bp	GGGGAATGACTTGGGCTACC	AATCCTTCTGTGCGGGGT
GR primer pair 3	GR Exon 2, 78 bp	CTCAATAGGTCGACCAGCCG	CTTCTGTGTCGGGGTAGCAC
GR primer pair 4	GR Exon 2, 150 bp	CATGGCGTGAGTACCTCTGG	TCCAGACCCTTGGCACCTAT
Cre	Cre, 200 bp	ACCAGCCAGCTATCAACTCG	TTACATTGGTCCAGCCACC
Gapdh	Gapdh, 59 bp	GGAAGCCCATCACCATCT	GCCTACCCCATTTGATGTT

Note that 'GR' refers to the gene Nr3c1, the murine glucocorticoid receptor.

(TRAP; Sigma-Aldrich #386A) and mounted using a xylene-based mounting medium. Images were captured with 10× (H&E) and 20× (TRAP) objectives on a brightfield microscope (Olympus IX70). Bone marrow adiposity was measured from the H&E-stained sections as adipocyte ghost area normalized to marrow area (Ad.Ar/Ma.Ar, %) and adipocyte density (N.Ad/Ma.Ar, #/mm²). Osteoclast number (N.Oc/BS, #/mm) and osteoclast surface (Oc.S/BS, %) were quantified from the TRAP-stained sections in the proximal tibia metaphyseal trabecular bone using Bioquant Osteo software. All histological data were collected in a blinded fashion.

Serial sections from the decalcified tibiae were immunohistochemically stained to detect the localization of the GR, using methodology as previously described (McGee-Lawrence *et al.* 2013, Hagan *et al.* 2019). Briefly, paraffin sections were deparaffinized in xylene and rehydrated through graded alcohols to water. Tissue sections were heated in a citrate buffer solution for 10 min for antigen retrieval followed by incubation for 2 h at room temperature with a GR monoclonal antibody (BuGR2; 1:50 dilution, #MA1-510, Invitrogen) or mouse IgG isotype control (1:50 dilution, I-2000; Vector Laboratories). The sections were then incubated in chromogens and were detected with a polyvalent secondary HRP kit (Abcam, ab93697) and 3,3'-diaminobenzidine (DAB, 10-min incubation; Sigma-Aldrich, D3939) as per manufacturer's instructions. Images were captured using a microscope (Olympus IX-70) and digital camera (QImaging QICam and QCapture software).

Enzyme-linked immunosorbent assays

Serum samples were obtained by cardiac puncture at killing followed by centrifugation in serum separator tubes (BD Microtainer #365967, Franklin Lakes, NJ, USA) and stored at −80°C until analysis. A mouse TRAcP-5b ELISA (MouseTrap™; #SB-TR103, Immunodiagnostic Systems, Gaithersburg, MD, USA) was used to measure the circulating bone resorption marker TRAP 5b. Additional aliquots of serum were analyzed in a rat/mouse procollagen I intact N-terminal (P1NP) enzyme immunoassay (#AC-33F1, Immunodiagnostic Systems) for quantification of bone formation markers. Serum glucocorticoids were measured using a corticosterone ELISA (#ab108821, Abcam) using a 100-fold dilution of mouse serum and provided protocols. All ELISA plates were measured with a microplate reader (BioTek Synergy HT, Winooski, VT, USA) using Gen5 software and readings at 405 (P1NP) and 450 nm (TRAcP-5b, corticosterone)

wavelengths, as recommended for each assay. Serum data were normalized to calibrants and standards provided in respective ELISA kits.

Statistical analyses

After checking for normality and appropriate distribution, the effects of diet were compared between WT and GR-CKO mice by two-factor (2 genotype × 2 diet) analysis of variance (ANOVA) with interaction using JMP Pro 14 statistical software (SAS Institute). The effects of propranolol intervention on the CR diet background were compared between WT and GR-CKO mice with two-factor ANOVA (2 genotype × 2 treatment) with interaction. Histomorphometric measurements of osteoclasts (N.Oc/BS and Oc.S/BS) were rank-transformed prior to analysis to stabilize the variance across groups and to reduce the influence of outliers. For gene expression analyses, GR-WT and GR-CKO groups were compared via *t*-tests. Statistical significance was defined by 95% confidence ($P < 0.05$). Box plots in figures show median, quartiles and outlier fences for each dataset, where outlier fences represent first quartile − 1.5 × (interquartile range) and third quartile + 1.5 × (interquartile range).

Results

Confirmation of GR knockdown in GR^{fl/fl}:Osx1-Cre⁺ mice

BMSC and skeletal tissue were harvested from the GR-CKO and GR-WT mice on AL diets to assess the efficiency of GR knockdown in the GR-CKO mice. BMSC from the GR-CKO mice subjected to osteogenic culture demonstrated an approximately 90% knockdown of GR mRNA levels as compared to cells from GR-WT mice (Fig. 1C). These same BMSC cultures expressed high levels of Cre mRNA (+55 fold signal as compared to GR-WT cultures). To confirm these results at the protein level in bone tissue, we performed immunohistochemistry against the GR. These studies demonstrated robust knockdown of the GR in Osx1-Cre-targeted regions including the pre-hypertrophic chondrocytes and osteo-lineage cells (Fig. 1D). These studies confirmed that the GR-CKO mice were deficient in skeletal expression of the glucocorticoid receptor.

GR deletion in Osx-expressing cells reduced bone mass under AL and CR feeding conditions

GR-WT and GR-CKO mice were subjected to AL or CR feeding conditions for 9 weeks. Body mass was reduced

by both GR deficiency (-10.6% , $P=0.0002$) and by caloric restriction (-17.6% , $P<0.0001$), but there was not a significant interaction between these factors ($P=0.453$), meaning that CR decreased body mass similarly in GR-WT and GR-CKO mice (Fig. 2A). Percent fat mass was not different between groups (data not shown; $P>0.203$), perhaps because female mice resist loss of fat mass during CR (Shi *et al.* 2007, Li *et al.* 2010b, Cawthorn *et al.* 2016). While no differences were observed in whole-body BMD or BMC between groups ($P>0.516$, data not shown), both genotype and CR diet administration negatively impacted cortical bone mineralization in the femur as measured by DXA (Fig. 2B-D). Specifically, femur BMD and BMC were reduced by GR deficiency (-5.3 and -7.6% respectively;

$P<0.004$) and by CR (-10.4 and -13.9% respectively, $P<0.0001$) with no interaction effect ($P>0.535$, Fig. 2C and D). Cortical bone geometry was similarly affected; for example, cortical bone thickness was reduced in GR-CKO mice (-7.6% , $P=0.0001$) and by CR (-15.9% , $P<0.0001$), but no interaction effects were observed ($P>0.736$; Fig. 2E and Table 2). Taken together, these data support the idea that both GR deficiency and caloric restriction are deleterious for cortical bone, but CR effects are not exacerbated in GR-CKO mice.

With regards to trabecular bone, although trabecular bone volume fraction was not different between groups ($P>0.130$), subtle but statistically significant differences were observed in the trabecular bone properties Tb.N and

Figure 2

GR-CKO mice are smaller and have less bone mass than GR-WT mice. (A) Body mass of animals at the conclusion of study ($n=7$ to 8 per group) and (B) representative images of body composition dual X-ray absorptiometry scans for GR-WT and GR-CKO mice (white = bone mass, blue = lean mass, green = fat mass). Larger white boxes on representative images outline area of exclusion from analysis. Smaller red boxes outline area of analysis for (C) femur bone mineral density (BMD) and (D) femur bone mineral content (BMC). Micro-computed tomography analyses for femur (E) cortical bone thickness and (F) trabecular bone separation are shown quantitatively. Boxes show median, quartiles and outlier fences for each dataset. Each data point represents one mouse. Statistical analyses for comparing the influence of diet (blue text) and propranolol intervention (red text) between GR-WT and GR-CKO mice are shown beneath each graph.

Table 2 Micro-computed tomography (microCT) analysis of bone architecture, comparing the effects of diet (AL vs caloric restriction) and genotype (WT vs GR-CKO).

Parameter	Mean $\pm$ s.d.				P_{genotype}	P_{diet}	$P_{\text{genotype} \times \text{diet}}$
	WT _{AL}	CKO _{AL}	WT _{CR}	CKO _{CR}			
Ct. B.Ar (mm ²)	0.751 $\pm$ 0.046	0.681 $\pm$ 0.030	0.647 $\pm$ 0.055	0.560 $\pm$ 0.043	<0.0001	<0.0001	0.613
Ct. T.Ar (mm ²)	1.542 $\pm$ 0.106	1.459 $\pm$ 0.093	1.516 $\pm$ 0.142	1.349 $\pm$ 0.069	0.004	0.094	0.286
Ct. B.Ar/T.Ar	0.488 $\pm$ 0.017	0.468 $\pm$ 0.015	0.427 $\pm$ 0.016	0.415 $\pm$ 0.020	0.021	<0.0001	0.527
Ct. I.max (mm ⁴)	0.180 $\pm$ 0.026	0.161 $\pm$ 0.019	0.163 $\pm$ 0.030	0.123 $\pm$ 0.018	0.002	0.005	0.240
Ct. I.min (mm ⁴)	0.104 $\pm$ 0.012	0.089 $\pm$ 0.010	0.089 $\pm$ 0.014	0.070 $\pm$ 0.007	0.0003	0.0005	0.690
Ct. Th (mm)	0.194 $\pm$ 0.007	0.180 $\pm$ 0.004	0.163 $\pm$ 0.008	0.152 $\pm$ 0.010	0.0001	<0.0001	0.736
Ct. T.BMD (mg/cm ³)	1030.2 $\pm$ 19.9	1008.1 $\pm$ 14.2	1016.3 $\pm$ 25.5	979.8 $\pm$ 38.55	0.007	0.044	0.474
Tb. BV/TV	0.012 $\pm$ 0.007	0.007 $\pm$ 0.004	0.013 $\pm$ 0.005	0.011 $\pm$ 0.006	0.130	0.237	0.371
Tb. Th (mm)	0.040 $\pm$ 0.006	0.041 $\pm$ 0.005	0.037 $\pm$ 0.005	0.036 $\pm$ 0.006	0.924	0.068	0.721
Tb. N (1/mm)	2.110 $\pm$ 0.182	1.573 $\pm$ 0.271	2.250 $\pm$ 0.371	1.997 $\pm$ 0.348	0.002	0.020	0.221
Tb. Sp (mm)	0.480 $\pm$ 0.043	0.668 $\pm$ 0.122	0.459 $\pm$ 0.079	0.517 $\pm$ 0.080	0.0007	0.012	0.052

Parameter means and standard deviations are presented. P values for variables and interactions assessed by two-factor ANOVA are shown.

Tb.Sp between groups. Trabecular number was decreased by GR deficiency (-17.5% , $P=0.002$) and increased by CR ($+14.9\%$, $P=0.020$), whereas trabecular separation was increased by GR-deficiency ($+25.2\%$, $P=0.0007$) but decreased by CR (-14.7% , $P=0.012$) (Fig. 2F). Taken together, these data suggest that loss of GR in *Osx*-expressing cells is mildly detrimental for trabecular bone, but CR minimally impacts trabecular bone properties, as previously reported (Hamrick *et al.* 2008).

Propranolol treatment differentially affected cortical bone mass in GR-WT and GR-CKO mice subjected to CR

Propranolol treatment had little to no impact on body mass, whole-body DXA measurements, and trabecular bone in the calorically restricted GR-WT and GR-CKO mice, except for stimulating a mild decrease in trabecular bone thickness (-11.2% , $P=0.023$) that was comparable between GR-WT and GR-CKO mice (Fig. 1C, F and Table 3). In cortical bone, however, interaction effects between genotype and drug administration were observed for several properties including cortical bone moment of inertia, cortical bone area and femur BMC (Fig. 1E, F, G and Table 3). These data suggest that propranolol treatment differentially affected cortical bone in the calorically restricted GR-WT and GR-CKO mice, with a mild detrimental effect on cortical bone in GR-WT mice but a mild stimulatory effect on cortical bone mass in GR-CKO mice.

Bone formation was impaired by CR and GR-CKO *in vivo*

To determine how bone remodeling activity contributed to the skeletal phenotypes observed with GR-CKO and CR, ELISAs were performed on sera isolated from the mice to quantify markers of bone formation and resorption. Circulating markers were verified in a site-specific fashion through histomorphometric measurements of metaphyseal trabecular bone in the proximal tibia (TRAP staining) and distal femur (calcein measurements). Bone formation activity was suppressed by both GR deficiency and by CR, as seen by changes in serum P1NP levels (-41.3 in GR-CKO vs GR-WT, -42.8% in GR vs AL) and trabecular bone dynamic histomorphometry (Fig. 3A, B, C and D). No interaction effects were observed, suggesting that CR suppressed bone formation similarly between GR-WT and GR-CKO mice ($P>0.184$). Histomorphometric measurements of trabecular bone osteoclasts suggested a

Table 3 Micro-computed tomography (microCT) analysis of bone architecture, comparing the effects of drug (vehicle vs propranolol) and genotype (WT vs GR-CKO).

Parameter	Mean $\pm$ s.d.				P_{genotype}	P_{drug}	$P_{\text{genotype} \times \text{drug}}$
	WT _{CR} ^{VEH}	CKO _{CR} ^{VEH}	WT _{CR} ^{PRO}	CKO _{CR} ^{PRO}			
Ct. B.Ar (mm ²)	0.647 $\pm$ 0.055	0.560 $\pm$ 0.043	0.607 $\pm$ 0.068	0.603 $\pm$ 0.043	0.025	0.941	0.041
Ct. T.Ar (mm ²)	1.516 $\pm$ 0.142	1.349 $\pm$ 0.069	1.485 $\pm$ 0.099	1.442 $\pm$ 0.078	0.006	0.394	0.095
Ct. B.Ar/T.Ar	0.427 $\pm$ 0.016	0.415 $\pm$ 0.020	0.408 $\pm$ 0.025	0.418 $\pm$ 0.012	0.902	0.244	0.127
Ct. I.max (mm ⁴)	0.163 $\pm$ 0.030	0.123 $\pm$ 0.018	0.151 $\pm$ 0.025	0.141 $\pm$ 0.017	0.004	0.745	0.079
Ct. I.min (mm ⁴)	0.089 $\pm$ 0.014	0.070 $\pm$ 0.007	0.083 $\pm$ 0.013	0.082 $\pm$ 0.010	0.022	0.538	0.042
Ct.Th (mm)	0.163 $\pm$ 0.008	0.152 $\pm$ 0.010	0.154 $\pm$ 0.014	0.157 $\pm$ 0.008	0.270	0.588	0.051
Ct. T.BMD (mg/cm ³)	1016.3 $\pm$ 25.5	979.8 $\pm$ 38.5	1000.7 $\pm$ 20.8	983.9 $\pm$ 11.8	0.008	0.546	0.301
Tb.BV/TV	0.013 $\pm$ 0.005	0.011 $\pm$ 0.006	0.021 $\pm$ 0.009	0.011 $\pm$ 0.004	0.018	0.111	0.065
Tb.Th (mm)	0.036 $\pm$ 0.005	0.036 $\pm$ 0.006	0.034 $\pm$ 0.003	0.030 $\pm$ 0.005	0.229	0.023	0.371
Tb.N (1/mm)	2.250 $\pm$ 0.371	1.997 $\pm$ 0.348	2.505 $\pm$ 0.385	1.991 $\pm$ 0.191	0.003	0.306	0.282
Tb.Sp (mm)	0.459 $\pm$ 0.079	0.517 $\pm$ 0.080	0.410 $\pm$ 0.063	0.510 $\pm$ 0.045	0.003	0.263	0.396

Parameter means and standard deviations are presented. P values for variables and interactions assessed by two-factor ANOVA are shown.

similar pattern of decreased bone resorption with both GR deficiency and CR, with no interaction effects (Fig. 3E, F and G). In contrast, serum TRAcP5b levels, which serve as a circulating marker of osteoclast number throughout the entire body (Halleen *et al.* 2002, Alatalo *et al.* 2003), were suppressed by GR-deficiency (−41.0% in GR-CKO vs GR-WT, $P=0.006$) but were not impacted by CR in this study (Fig. 3H). Taken together, these data support the assertion that the reduced bone mass observed with GR insufficiency and with CR could be attributed to decreased bone formation activity, rather than high levels of bone resorption. Propranolol treatment had little impact on bone remodeling activity, except for a mild suppression of serum P1NP levels that was similar between genotypes (−24.5%, $P=0.014$; Fig. 3).

GR-insufficient mice accumulated more marrow fat than GR-WT mice *in vivo*

Previous work demonstrated that chronic caloric restriction-induced marrow fat accumulation in WT mice and that β -blockade with propranolol partially rescued this CR-induced marrow fat phenotype (Devlin *et al.* 2010, Baek *et al.* 2014b, Periyasamy-Thandavan *et al.* 2015, Cawthorn *et al.* 2016). To determine whether the GR is a mechanistic factor in caloric restriction-induced marrow adiposity *in vivo*, marrow adipogenesis was measured histologically in long bones harvested from the mice. Interestingly, these studies demonstrated that GR deficiency in osteoprogenitors drove an increase in marrow adiposity, as both marrow adipocyte density and area fraction were greater in GR-CKO as compared to GR-WT mice (+169% adipocyte density, +139% marrow adipocyte fraction, $P<0.003$; Fig. 4). To the best of our knowledge, this phenotype has not been previously reported in mechanistic investigations of the role of the GR in bone biology (Liu *et al.* 2016). Consistent with previous studies, CR was also observed to drive an increase in marrow adiposity (+96% adipocyte density, +396% marrow adipocyte fraction, $P<0.003$; Fig. 4). No interaction effects were observed between genotype and diet, suggesting that the GR is not required for CR-induced marrow adipogenesis. Interestingly, although propranolol was previously reported to ameliorate bone marrow adiposity induced by CR (Baek & Bloomfield 2012, Baek *et al.* 2014b), we observed no effect of propranolol on marrow adiposity in our studies even in WT mice ($P>0.591$, Fig. 4). These data suggest that β -adrenergic signaling might not always be a critical component of CR-induced marrow adipogenesis.

Figure 3

GR conditional deletion and caloric restriction suppress bone remodeling. (A) Representative images of calcein-labeled bone surface from each group. Arrows in representative images highlight areas of active mineralization (original magnification: 10× objective). (B) Mineralizing surface (MS/BS) and (C) bone formation rate (BFR/BS) for each condition. (D) Serum levels of procollagen type I N-terminal (P1NP, ng/mL), a circulating marker of bone formation activity. (E) Representative images of tartrate-resistant acid phosphatase (TRAP) staining on tibiae (original magnification: 20× objective). (F) Quantification of TRAP-positive osteoclast surface and (G) osteoclast number (N.Oc) normalized to trabecular bone surface. (H) ELISA measurement of serum concentrations of TRAcP-5b, a circulating marker of osteoclast number. Boxes on each graph show median, quartiles and outlier fences for each dataset. Each data point represents one mouse. Statistical analyses for comparing the influence of diet (blue text) and propranolol intervention (red text) between GR-WT and GR-CKO mice are shown beneath each graph.

GR-CKO mice have high serum glucocorticoids under CR, which is rescued by propranolol treatment

A canonical physiological response to metabolic stressors such as CR is the upregulated secretion of glucocorticoids – such as corticosterone in mice – from the adrenal cortex into the circulation (Chapman *et al.* 2013, Cawthorn *et al.* 2016, Teich *et al.* 2016). To determine the glucocorticoid response of GR-WT and GR-CKO mice in response to

CR and propranolol treatment, serum samples from the mice were analyzed by a corticosterone ELISA. Under AL conditions, there were negligible differences in circulating corticosterone between GR-WT and GR-CKO mice (Fig. 5). Surprisingly, CR differentially impacted corticosterone levels in the serum between GR-CKO and GR-WT mice, significantly increasing circulating corticosterone only in the GR-CKO animals (genotype×diet interaction P -value=0.012; Fig. 5). Propranolol treatment differentially

Figure 4

GR-CKO mice have more marrow fat than GR-WT mice. (A) Representative H&E-stained tibiae (original magnification: 20× objective) showing 'adipose ghosts' indicative of marrow fat. (B) Adipocyte area fraction (Ad.Ar/Ma.Ar) and (C) adipocyte density (# adipocytes per mm² marrow area) were quantified for each sample. Boxes show median, quartiles and outlier fences for each dataset. Each data point represents one mouse. Statistical analyses for comparing the influence of diet (blue text) and propranolol intervention (red text) between GR-WT and GR-CKO mice are shown beneath each graph.

affected corticosterone levels in GR-WT and GR-CKO mice during CR, suppressing corticosterone levels in GR-CKO mice while having no impact on GR-WT mice (genotype×drug interaction P value=0.001; Fig. 5). Together, these data suggest an aberrant stress response of GR-insufficient animals to CR that is sensitive to β -blockade, and a potential role for bone-specific GR activity in regulating whole-body glucocorticoid signaling.

Discussion

Chronic CR interrupts the metabolic homeostasis of bone. Glucocorticoid signaling, which is upregulated by stress and inflammation, is implicated in decreasing bone mass and mediating fat accumulation in the marrow. The current study sought to test the role of glucocorticoids in CR-induced marrow adipogenesis and bone turnover through conditional deletion of the glucocorticoid receptor in osteoprogenitors, revealing that loss of GR function in osteoprogenitors reduced bone mass and impaired bone formation as measured by histomorphometry and serum P1NP levels. The low bone mass phenotype of the GR-insufficient animals highlights the importance of physiological levels of glucocorticoid signaling and GR function for proper osteogenesis. Our results suggest that sympathetic tone has complex roles in bone maintenance during CR. Propranolol-treated GR-CKO mice were slightly better protected against the CR-induced loss of femur cortical bone than WT littermates, but showed no such improvement in trabecular bone mass. It is plausible that CR-induced cortical bone loss is promoted

by activation of the β 2AR, which can be upregulated by circulating glucocorticoids and leptin (Ma *et al.* 2011, Baek & Bloomfield 2012). Further work is needed to better understand these stress response mechanisms in bone.

The GR-CKO model used in this study conditionally deleted the transactivation domain of the GR in Osterix1-expressing osteoprogenitor cells, rendering the

Figure 5

Circulating corticosterone levels are elevated by GR-CKO and caloric restriction. ELISA measurement at the conclusion of the study of the active glucocorticoid corticosterone (ng/mL) in serum harvested from GR-WT and GR-CKO mice subjected to AL, CR or CR+Prop treatment conditions. Boxes show median, quartiles and outlier fences for each dataset. Each data point represents one mouse. Statistical analyses for comparing the influence of diet (blue text) and propranolol intervention (red text) between GR-WT and GR-CKO mice are shown beneath the graph.

GR inactive through dimerization but not completely deleted by Cre-LoxP recombination. Previous work using a Runx2-Cre-mediated GR conditional deletion model confirmed that the monomeric GR is still functional and can attenuate osteoblast function when activated (Rauch *et al.* 2010). Moreover, the monomeric GR induces genetic transrepression via downstream genomic elements – described as negative glucocorticoid response elements (nGREs) – and the recruitment of mediating transcription factors such as AP-1, NF- κ B and nuclear receptor corepressors 1 and 2 (NCoR1 and SMRT) (Hudson *et al.* 2013, Oakley & Cidlowski 2013, Lim *et al.* 2015). As an example, there is evidence that activation of an nGRE upstream of a Runx2-binding site on the osteocalcin promoter is a mechanism by which glucocorticoids can repress the Egr2/Krox20 enhancer that is required for osteoblast function and trabecular bone formation (Leclerc *et al.* 2005). A GR dimerization mutation in mice also showed that the monomeric GR can inhibit bone resorption by downregulating retinoic acid receptor-dependent RANKL production (Conaway *et al.* 2011). These findings raise the possibility that the upregulated serum corticosterone resulting from CR in GR-CKO animals could act via the monomeric GR to induce bone loss through impaired osteogenesis rather than increased resorption. Additional work will be necessary to determine the activity of the GR in our model and its potential interaction with transcription factors and genomic elements during metabolic stress responses. Moreover, whether this mechanism contributes to CR-induced marrow adiposity in GR-CKO mice remains in question.

We were surprised to observe that a caloric restriction-induced increase in bone marrow adiposity was not prevented by propranolol administration in either GR-WT or GR-CKO mice. This may be due in part to the dosage of propranolol used to modulate sympathetic tone in this study. We selected a dose of 0.1 mg/mL propranolol administered in drinking water based on a previous study demonstrating efficacy of this dosage in limiting marrow adipogenesis during CR (Baek & Bloomfield 2012). However, we recognize that several other investigations into the role of sympathetic tone in regulation of bone and marrow adiposity have used higher dosages of approximately 0.5 mg/mL propranolol in drinking water (Takeda *et al.* 2002, Motyl *et al.* 2013, Baek *et al.* 2014a,b). Accordingly, the limited impact of propranolol on bone and marrow fat in the current study may have been due to incomplete blockade of adrenergic signaling. However, it is important to note that propranolol administration did induce several biological changes in the current

study, including blunting the upregulation of serum corticosterone in GR-CKO mice subjected to CR (Fig. 5) and inducing a mild decrease in both circulating PINP levels and trabecular bone thickness similarly between genotypes (Fig. 3D and Table 3). Based on our data, we hypothesize that β -adrenergic signaling might not always be a critical component of CR-induced marrow adipogenesis, but further studies are needed to thoroughly test this hypothesis.

Loss of GR function in the murine CR model presented here was not protective against excess marrow adipogenesis. Recent work in rats demonstrated that treatment with the GR antagonist mifepristone was protective against adiposity rebound in rats following the completion of chronic exercise and CR conditions (Teich *et al.* 2016). In this study, however, GR conditional deletion did not protect against CR-driven marrow fat, and instead resulted in a high marrow fat phenotype even under AL feeding conditions. This is somewhat surprising, as recent work suggested that the GR augments adipose tissue formation, as GR-deficient mouse embryonic fibroblasts were impaired in their ability to form adipocytes both *in vitro* and *in vivo* (Bauerle *et al.* 2018). Our results suggest alternative mechanisms by which marrow fat accumulates in this osteoprogenitor-specific GR-deficient model. One possibility is that systemic rather than local glucocorticoids in the GR-CKO mice bind the GR to drive its activation of the master adipogenic transcription factor, peroxisome proliferator-activated receptor γ (PPAR γ), via known interactions of the GR with PPAR γ -transcription regulating CCAAT/enhancer-binding proteins (C/EBPs) (Grontved *et al.* 2013, Li *et al.* 2013, Zhao *et al.* 2013). However, little is known regarding whether the monomeric GR interacts with C/EBPs or the potential for C/EBP-mediated transrepression (rather than activation) to stimulate BMSC-derived adipogenesis. Another possibility is that the binding and activation of the mineralocorticoid receptor (MR) is involved in the phenotype, as corticosterone also binds this receptor (Li *et al.* 2005); indeed, MR has high affinity for glucocorticoids and in the absence of the inactivating enzyme, 11 β -hydroxysteroid dehydrogenase-2 may be occupied by these steroids. Moreover, the MR has implications in facilitating adipogenesis, and adipocyte-specific MR activity is associated with metabolic syndrome and glucocorticoid-induced lipid accumulation (Hirata *et al.* 2009, Hoppmann *et al.* 2010, Caprio *et al.* 2011, Nguyen Dinh Cat *et al.* 2016). These findings also highlight a potential limitation to the GR conditional deletion model used in the current study, as Osx1-Cre fate mapping

studies have confirmed expression of this Cre reporter in bone marrow adipocytes (Liu *et al.* 2013); therefore, at present, it is not possible to discriminate between whether the enhanced adiposity in the GR-CKO mice is a progenitor-driven effect or an adipocyte-driven effect. However, it is important to note that several studies have addressed the role of the GR in adiponectin-expressing adipocytes, and bone marrow adipocytes are an important source of adiponectin during CR (Cawthorn *et al.* 2014). Interestingly, the majority of these reports suggest that GR deletion in adipocytes does not independently promote adipocyte expansion or hypertrophy (Bose *et al.* 2016, Desarzens & Faresse 2016, Shen *et al.* 2017) and may even decrease fat mass (Mueller *et al.* 2017). Thus, if the primary effect of *Osx1*-Cre on marrow adiposity resulted from directly targeting bone marrow adipocytes, it would be logical to expect that marrow fat should be unaffected or decreased in GR-CKO animals. In contrast, the current study found robust increases in marrow adiposity in the GR-CKO animals under both AL and CR feeding conditions. A remaining question is the role of glucocorticoid-activated MR in BMSC-derived osteoblasts and adipocytes – including the role of MR-driven marrow adipogenesis and mechanistic interactions of the GR and MR in BMSC. With both of these possible mechanisms, however, it is important to note that we only observed an increase in circulating corticosterone levels in the GR-CKO mice under conditions of CR. As we only measured serum corticosterone at the time of killing, and glucocorticoids demonstrate substantial circadian variation in their circulating levels (Halberg *et al.* 1959, Gong *et al.* 2015), it is possible that we may have missed the optimal time point at which to compare corticosterone levels across different groups. In future studies, it will be important to conduct a thorough time course of blood sampling to observe how circulating GC levels are impacted by loss of GR in the skeleton throughout periods of activity and dormancy.

It is also important to consider the complex role of β -adrenergic signaling in marrow adipogenesis, as it has been demonstrated that β -AR antagonism with propranolol can positively regulate BMSC adipogenesis as well as C/EBP and PPAR γ expression, while the β -AR agonist isoproterenol inhibits these processes (Li *et al.* 2010a). As previously mentioned, however, β -adrenergic signaling during CR shows differing roles in terms of facilitating bone loss and marrow fat accumulation (Baek & Bloomfield 2012, Baek *et al.* 2014b). It was also recently reported that marrow adipose tissue has a blunted response to adrenergic signaling as compared to other fat

depots like white adipose tissue (Scheller *et al.* 2019). This further demonstrates the complexities of inflammatory and stress responses in bone-resident cell types and the potential for canonical signaling pathways to be pro- or anti-adipogenic depending on mechanistic crosstalk that is yet to be elucidated in the CR stress model.

While the findings in this study highlight the complex effects of glucocorticoid signaling and GR activity in bone during CR, there are limitations to acknowledge in the study design that, when addressed in future work, may further clarify mechanisms of interest. For instance, the presented work was completed in only in female mice; this was done because pilot studies suggested similar trends of increased marrow fat accumulation in both male and female GR-CKO mice as compared to WT controls, but trends were more pronounced in females. We consequently focused on females for further study, but this observation raises the question of whether sex differences exist in the response of marrow adiposity to loss of GR-mediated signaling and CR in bone. In addition, as described earlier, it is important to acknowledge that the preferred control for the *Osx1*-Cre, GR^{+/+}:*Osx1*-Cre+ mice (i.e., 'Cre+ WT' mice), was not included here. In pilot studies, presented in the Supplementary Methods, we observed no difference in marrow adiposity between Cre⁻ and Cre⁺ WT animals, but, these pilot data were from male mice at 6 months of age, whereas mice in the dietary studies presented here were female mice that were 6 months old at the onset of studies and 8 months old at killing. It will be important to age- and sex-match additional control groups in future studies of *Osx1*-Cre: GR-CKO animals in order to draw better conclusions regarding the role of the GR in regulation of bone mass and marrow fat during periods of metabolic stress. Lastly, while we confirmed appreciable reduction in GR expression in bone via immunostaining and in marrow-derived osteoblasts from these mice, we did not perform a comprehensive evaluation of GR expression in potential off-target tissues, such as the brain, that may express both GR and Osterix (Park *et al.* 2011, Madalena & Lerch 2017). From our immunostaining analyses, we note that the intensity of GR staining in the general bone marrow area tended to be reduced in the GR-CKO mice, although the impact of this observation on outcome measurements in the current study is not clear. Interpretation of data in the *Osx1*-Cre: GR-CKO model described here could be strengthened by analyses of GR expression in the whole bone marrow stroma and brain to conclusively establish whether observed effects are mediated directly by loss of GR in bone or influenced via off-target effects in other

tissues. Our future work will address the aforementioned limitations in methodology to better understand the role of the GR in the skeletal response to stressors.

In conclusion, the present study suggests that GR function in osteoprogenitors is necessary to maintain bone mass and inhibit marrow fat accumulation that occurs with the stress of long-term CR. The β -blocker propranolol selectively improved cortical bone mass in GR-CKO mice but had no impact on CR-induced marrow adiposity in either GR-WT or GR-CKO animals. Future work will focus on elucidating the molecular mechanisms driving the osteoporotic phenotype of GR-insufficient bone as well as determining how loss of the GR in osteoprogenitors affects the response of bone to common physiological stressors such as aging.

Supplementary data

This is linked to the online version of the paper at <https://doi.org/10.1530/JOE-19-0230>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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