Daily leptin blunts marrow fat but does not impact bone mass in calorie-restricted mice

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Abstract

Starvation induces low bone mass and high bone marrow adiposity in humans, but the underlying mechanisms are poorly understood. The adipokine leptin falls in starvation, suggesting that hypoleptinemia may be a link between negative energy balance, bone marrow fat accumulation, and impaired skeletal acquisition. In that case, treating mice with leptin during caloric restriction (CR) should reduce marrow adipose tissue (MAT) and improve bone mass. To test this hypothesis, female C57Bl/6J mice were fed a 30% CR or normal (N) diet from 5 to 10 weeks of age, with daily injections of vehicle (VEH), 1 mg/kg leptin (LEP1), or 2 mg/kg leptin (LEP2) ($N=6-8/\text{group}$). Outcomes included body mass, body fat percentage, and whole-body bone mineral density (BMD) via peripheral dual-energy X-ray absorptiometry, cortical and trabecular microarchitecture via microcomputed tomography (μCT), and MAT volume via μCT of osmium tetroxide-stained bones. Overall, CR mice had lower body mass, body fat percentage, BMD, and cortical bone area fraction, but more connected trabeculae, vs N mice ($P<0.05$ for all). Most significantly, although MAT was elevated in CR vs N overall, leptin treatment blunted MAT formation in CR mice by 50% vs VEH ($P<0.05$ for both leptin doses). CR LEP2 mice weighed less vs CR VEH mice at 9–10 weeks of age ($P<0.05$), but leptin treatment did not affect body fat percentage, BMD, or bone microarchitecture within either diet. These data demonstrate that once daily leptin bolus during CR inhibits bone marrow adipose expansion without affecting bone mass acquisition, suggesting that leptin has distinct effects on starvation-induced bone marrow fat formation and skeletal acquisition.

Key Words

- ▶ leptin
- ▶ caloric restriction
- ▶ marrow adiposity
- ▶ bone
- ▶ mouse

Journal of Endocrinology
(2016) **229**, 295–306

Introduction

Bone marrow adipose tissue (MAT) is a complex and dynamic depot that likely includes both constitutive and regulated cell populations (Devlin & Rosen 2015,

Scheller *et al.* 2015). MAT accumulation is a normal component of skeletal aging in healthy individuals, but is also observed in metabolic disease, including anorexia

nervosa, diabetes, and osteoporosis, and in skeletal unloading, including paralysis and bedrest (Yeung *et al.* 2005, Bredella *et al.* 2009, Trudel *et al.* 2009, Ecklund *et al.* 2010, Tuljapurkar *et al.* 2011, Baum *et al.* 2012, Schwartz *et al.* 2013). As has been widely noted, MAT may influence bone mass via interactions with adjacent osteoblasts in the marrow, as adipocytes and osteoblasts derive from a common mesenchymal progenitor (Muruganandan *et al.* 2009, Bianco *et al.* 2010). Although bone–fat interactions involve multiple hormones and growth factors, several lines of evidence suggest a role for the adipokine leptin in mediating the balance between bone and fat in the endosteal niche. First, leptin promotes the differentiation of bone marrow mesenchymal stem cells *in vitro* to the osteoblast rather than the adipocyte lineage, leading to the hypothesis that low leptin could lead to a relative increase in fat vs bone cells (Thomas *et al.* 1999). Secondly, leptin replacement in hypoleptinemia decreases skeletal fragility in animal models (Cornish *et al.* 2002) and increases bone formation markers in humans (Welt *et al.* 2004). Finally, the adipokine leptin falls in starvation, raising the possibility that hypoleptinemia contributes to marrow fat expansion and impaired skeletal acquisition (Ahima *et al.* 1996, Boden *et al.* 1996). Interactions between bone and fat may be particularly important in young, rapidly growing animals, as CR in young mice is associated with both low bone mass and high marrow adiposity (Devlin *et al.* 2010), whereas CR in older mice has more modest effects on bone and may not alter marrow adiposity (Hamrick *et al.* 2008, Tatsumi *et al.* 2008). Thus, here we test whether daily leptin bolus during caloric restriction (CR) reduces marrow fat and improves bone mass, using the established system of CR in young, rapidly growing mice to model human energy restriction (Devlin *et al.* 2010).

Materials and methods

Mouse model and experimental protocol

This study used female C57Bl/6J mice (Jackson Laboratories), studied from 5 to 10 weeks of age. Mice were weighed at baseline and assigned to groups of equivalent body mass (mean $\pm$ S.D.). Mice were housed individually and fed a normal (N) diet *ad libitum* (Research Diets 12450B, 10% kcal/fat) or a 30% CR diet with supplemental micronutrients, which provided equivalent vitamins and minerals when fed at 70% of caloric intake *ad libitum* (Research Diets D10012703, 10% kcal/fat). Food allotments were placed into CR cages daily

at 18:00 h. Body mass was measured daily on a digital scale. Body length was measured at 5 and 10 weeks of age. Mice were killed by CO₂ inhalation at approximately 10:00 h. Experimental protocols were approved by the Beth Israel Deaconess Medical Center Institutional Animal Care and Use Committee.

Leptin administration

At 18:00 h daily, all mice received subcutaneous injection of recombinant murine leptin (R&D Systems) at 1 mg/kg/day (LEP1), leptin at 2 mg/kg/day (LEP2), or vehicle (VEH) (N = 6–8/group). Dosages were adjusted daily based on the body mass. We used a single late-day bolus in order to replicate the murine leptin peak that occurs in the dark cycle, during the period of greatest food intake (Ahima *et al.* 1996, Ahren 2000). The two doses tested, 1 or 2 mg/kg/day, correspond to roughly 20–40 μ g leptin/day for a 20 g mouse and were intended to produce serum leptin levels in the physiological range of 4–8 ng/mL in C57Bl/6J mice, as previously reported (Ahima *et al.* 1996, Ahren 2000, Surwit *et al.* 2000).

Peripheral dual-energy X-ray absorptiometry

In vivo measurements of whole-body (excluding the head) bone mineral density (BMD, g/cm²), bone mineral content (BMC, g), and body composition (% body fat) were obtained at 5 and 10 weeks of age using peripheral dual-energy X-ray absorptiometry (pDXA, PIXImus II, GE Lunar Corporation, Madison, WI, USA), as previously described (Bouxsein *et al.* 2009).

Serum hormones

At the time of killing, blood was collected by cardiac puncture to measure serum leptin by ELISA (Crystal Chem, Downers Grove, IL, USA) (Devlin *et al.* 2010). The assay sensitivity was 0.2–12.8 ng/mL.

Specimen collection and preparation

After the animals were killed, gonadal white adipose depots were dissected and weighed. Femurs, tibiae, and fifth lumbar vertebrae were excised and cleaned. The right femur and L5 vertebral body were wrapped in saline-soaked gauze and frozen at –20°C for imaging analysis (Devlin *et al.* 2010). The left tibia was prepared for histology in 70% ethanol at 4°C until processing.

The right tibia was fixed in 10% neutral buffered formalin at 4°C overnight and stored in PBS for osmium tetroxide staining (Scheller *et al.* 2014).

Trabecular and cortical bone morphology by microcomputed tomography

Bone microarchitecture of the femoral midshaft, distal femur, and vertebral body was measured via high-resolution microcomputed tomography (μ CT40, Scanco Medical, Brüttisellen, Switzerland), as previously described (Glatt *et al.* 2007). Scans were acquired using a $12\mu\text{m}^3$ isotropic voxel size, 70 kVP, 114 mA, and 200 ms integration time, and were subjected to Gaussian filtration and segmentation. In the distal femur, transverse CT slices were evaluated beginning $360\mu\text{m}$ proximal to the growth plate, extending $1800\mu\text{m}$ proximally. At the femoral mid-diaphysis, transverse μ CT slices spanning $600\mu\text{m}$ were obtained, whereas in the fifth lumbar vertebra, transverse CT slices spanning the entire vertebra, excluding growth plates, were obtained ($2400\text{--}3000\mu\text{m}$). Thresholds of 287, 708, and $365\text{ mg hydroxyapatite (HA)/cm}^3$ were used to segment the bone from the soft tissue for the distal femur, femoral diaphysis, and vertebral body, respectively. Terminology and units followed the recommendations of the American Society for Bone and Mineral Research (Bouxsein *et al.* 2010).

Histology and quantitative histomorphometry

To examine bone formation rates, calcein (15 mg/kg) was injected intraperitoneally at 9 and 2 days before killing the animals. Undecalcified bones were dehydrated and cleared on a tissue processor using an alternating vacuum/pressure cycle and, once cleared, infiltrated under vacuum for 3 days at 4°C with 85% methylmethacrylate (MMA) and 15% dibutyl phthalate (DP). Following infiltration, bones were embedded in 85% MMA, 15% DP, and 2.5% benzoyl peroxide (catalyst), and polymerized in a radiant heat oven at 37°C. Once polymerized, bones were cut on a Leica 2265 microtome using a 160 mm D-profile tungsten-carbide knife (Dorn and Hart, Loxley, AL, USA). Unstained sections for dynamic histomorphometry were coverslipped directly with a UV inert mountant. Sections for static histomorphometry were deplastified and stained with toluidine blue (Fisher), pH 4.2. Microscopic analysis of static and dynamic parameters was performed using an Olympus BX40 microscope interfaced with the Osteomeasure system software and hardware (Osteometrics, Atlanta, GA, USA). All static parameters

were measured on trabecular bone beginning immediately under the growth plate excluding endosteal surfaces. The field size for static parameters was $290\times 290\mu\text{m}$, and the area measured under the growth plate extended $580\mu\text{m}$ under the growth plate where most of the trabecular bone is located. Dynamic parameters of bone formation were read similarly using a Nikon Labophot scope equipped with epifluorescence to visualize pulse-labeled calcein on the trabecular surfaces. Mineralizing surface was calculated using double labels plus half the single-labeled surface. The field size for dynamic parameters was $350\times 350\mu\text{m}$ and extended $700\mu\text{m}$ under the growth plate. Terminology and units followed the recommendations of the American Society for Bone and Mineral Research (Dempster *et al.* 2013).

Osmium tetroxide staining

Left tibiae were fixed in 10% neutral buffered formalin at 4°C and decalcified in EDTA. Marrow fat was stained with a 1:1 solution of 2% aqueous osmium tetroxide (OsO_4) and 5% potassium dichromate (Turello *et al.* 1984, Fretz *et al.* 2010, Scheller *et al.* 2014). Fat volume in the proximal and distal tibia medullary compartments was quantified using high-resolution microcomputed tomography as described previously (μ CT40, Scanco Medical, Brüttisellen, Switzerland). For the proximal tibia, CT slices were evaluated beginning 1 mm below the growth plate and extending $2400\mu\text{m}$ distally. In the distal tibia, a $2400\mu\text{m}$ region was evaluated beginning at the tibiofibular junction and extending distally. The medullary canal was identified by manually tracing the endocortical border. Osmium-stained marrow fat was segmented from the adjacent marrow using a fixed threshold (1134 mg HA/cm^3). Adipose volume (mm^3), marrow volume (mm^3), and adipose/marrow volume fraction (%) were measured.

Statistical analyses

Standard descriptive statistics were computed for all outcome variables, and data checked for normality. We used a General Linear Model in SPSS 22 (IBM) to test for the effect of diet, leptin, and leptin–diet interactions on body composition and bone variables. All tests were two tailed, with the significance level for major effects set at $\alpha=0.05$. If there was an overall significant effect of diet, leptin, or leptin–diet interaction for a given variable, we performed *post hoc* Tukey tests to identify pairwise differences. As long bone cortical cross-sectional geometry is influenced by body mass, we also adjusted femoral

cortical variables (Ct.Th, Ct.Ba, Ct.TA, Imax, Imin, pMOI) by regressing these variables against body mass and testing for significance using the residuals (Lang *et al.* 2005).

Results

CR mice have lower body mass, body fat, serum leptin, and BMD

Body mass was lower in CR vs N mice from 6 to 10 weeks of age ($P<0.0001$), and in CR LEP2 vs CR VEH mice

at 9–10 weeks of age ($P<0.03$ for both, Fig. 1A). From 5 to 10 weeks of age, the total body fat mass increased by 216% in N mice vs 6% in CR mice ($P<0.03$); within the CR group, fat mass in VEH mice increased by 24% despite overall weight loss, whereas LEP1 and LEP2 maintained and lost body fat, respectively ($P<0.04$, Fig. 1B). Lean mass decreased in CR by 22% and increased in N mice by 9.5% ($P<0.001$), Fig. 1D). At 10 weeks of age, CR mice had shorter femurs, smaller bodies, and smaller perigonadal fat pads compared with N ($P<0.0001$ for all, Table 1). Fasting serum leptin, measured 16 h after

Figure 1

(A) CR weighed less than N at 6–10 weeks of age, and CR LEP2 weighed less vs CR VEH at 9–10 weeks of age ($P<0.0001$ for diet at 6–10 weeks, $P<0.03$ for dose at 9 and 10 weeks). (B) From 5 to 10 weeks of age, CR gained less body fat (g) vs N ($P<0.0001$ for diet and $P=0.023$ for dose), and within CR, LEP1 and LEP2 gained less body fat vs VEH ($P<0.04$ for both). (C) From 5 to 10 weeks of age, CR lost lean mass, whereas N gained lean mass ($P<0.0001$ for diet) (D) At 10 weeks of age, CR had markedly lower leptin vs N irrespective of treatment group ($P<0.0001$ for diet). Daily leptin injections at 18:00h did not alter fasting serum leptin ~16h postinjection, consistent with short half-life. (E) From 5 to 10 weeks of age, CR exhibited smaller gains in BMC vs N mice irrespective of leptin treatment ($P=0.001$ for diet).

the final daily injection for all mice and the final daily feeding for CR, was markedly lower in CR vs N mice (2.5 vs 10.5 ng/mL, $P < 0.05$) and did not differ across leptin treatments within either diet (Fig. 1D). BMD and BMC at 10 weeks of age and the gain in BMC from 5 to 10 weeks of age were lower in CR vs N overall ($P < 0.0004$, Fig. 1E and Table 1), but did not differ within either diet across leptin treatments.

Leptin treatment did not affect trabecular or cortical microarchitecture

In the distal femur trabecular bone, CR mice had lower structural model index (SMI) and higher Conn.D vs N ($P < 0.0001$ for both), but Tb.BV/TV, Tb.N, and Tb.Th did not differ between CR and N mice (Fig. 2A and Table 2). There were no differences in trabecular bone variables across leptin treatments within either diet group (Fig. 2A and Table 2).

At the femoral midshaft, cortical bone cross-sectional geometric properties, including Ct.Th, Ct.BA, Ct.TA, Imax, Imin, and pMOI, were lower in CR vs N ($P < 0.0001$ for all, Fig. 2B, C, D and Table 2), but these differences no longer reached statistical significance after body mass adjustment (Lang *et al.* 2005). There were no differences

in cross-sectional geometric properties across leptin treatments within either diet group.

In the fifth lumbar vertebra, N LEP2 mice had lower Tb.BV/TV vs N VEH ($P = 0.033$, Table 2). CR mice had higher Tb.N and Conn.D, and lower Tb.Th and Tb.Sp vs N ($P < 0.03$ for all, Table 2), but there were no differences across leptin treatments within each diet.

Leptin blunted MAT expansion in CR mice

Daily leptin bolus during CR partially suppressed the expansion of MAT observed in CR VEH mice (Fig. 3A). In the proximal tibia, adipose volume/marrow volume as quantified by μ CT scanning of bones stained with osmium tetroxide was higher in CR vs N overall ($P < 0.0001$), but 50% lower in CR LEP1 and CR LEP2 vs CR VEH ($P < 0.05$ for both, Fig. 3B). Quantitative histomorphometry confirmed this pattern, with N.Ad/T.Ar higher in CR compared with N overall ($P < 0.002$), but lower in CR LEP1 and CR LEP2 compared with CR VEH ($P = 0.046$, Table 3). BV/TV (%) of the proximal tibia did not differ in CR vs N (Table 3). N.Ob/T.Ar and N.Ob/BS were lower in CR compared with N overall ($P < 0.02$ for both, Table 3), but did not differ across leptin treatments within either diet. Dynamic markers of bone formation, including sLS/BS, dLS/BS,

Figure 2

(A) Distal femur Tb.BV/TV (%) did not differ by diet or by leptin treatment. (B) Ct.TA (mm²), (C) Ct.BA/TA (%), and (D) Ct.pMOI were lower in CR vs N before body mass adjustment ($P < 0.0001$ for diet), but did not differ by leptin treatment.

Table 1 Body size and body composition at 10 weeks of age in female C57BL/6J mice fed 30% CR diet or N diet *ad libitum* from 5 to 10 weeks of age with daily injection of leptin (1–2 mg/kg/day) or vehicle.

	CR				N				Overall P-value		
	Vehicle		1 mg/kg/day		2 mg/kg/day		Vehicle		1 mg/kg/day		Diet–Leptin
	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	
ANL (mm)	80.75 ^a	2.49	81.50 ^a	1.41	79.17 ^a	1.33	85.57	1.99	86.88	1.55	<0.0001
Femur length (mm)	12.50 ^a	0.20	12.62 ^a	0.08	12.51 ^a	0.28	13.44	0.19	13.53	0.32	<0.0001
Perigonadal WAT (g/g body weight)	0.073 ^a	0.046	0.036 ^a	0.018	0.037 ^a	0.041	0.324	0.134	0.295	0.152	<0.0001
Lean mass (g)	9.91 ^a	0.64	9.64 ^a	0.23	8.98 ^a	0.59	13.18	0.95	13.33	0.76	<0.0001
Fat mass (g)	2.60 ^a	0.35	2.10 ^{a,b}	0.13	1.91 ^{a,b}	0.16	5.10	1.47	4.31	1.36	<0.0001
WB BMD (g/cm ³)	0.042 ^a	0.002	0.042 ^a	0.002	0.043 ^a	0.001	0.046	0.002	0.045	0.001	0.008
WB BMC (g)	0.283 ^a	0.022	0.300 ^a	0.025	0.313 ^a	0.018	0.320	0.024	0.324	0.022	<0.0001

^aSignificant overall difference in CR vs N mice with leptin treatments pooled; ^bSignificant difference from vehicle treated within diet.

ANL, anus-to-nose length; WAT, white adipose tissue (g/g body weight); WB BMD, whole-body bone mineral density; WB BMC, whole-body bone mineral content.

Table 2 Cortical and trabecular microarchitecture at 10 weeks of age in female C57BL/6J mice fed 30% CR diet or N diet *ad libitum* from 5 to 10 weeks of age with daily injection of leptin (1–2 mg/kg/day) or vehicle.

	CR				N				Overall P-value		
	Vehicle		1 mg/kg/day		2 mg/kg/day		Vehicle		1 mg/kg/day		Diet–Leptin
	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	
Distal femur trabecular bone											
Tb.N (/mm)	4.27	0.62	4.38	0.85	4.14	0.53	4.23	0.24	4.16	0.26	NS
Tb.Th (mm)	0.047	0.001	0.047	0.002	0.045	0.001	0.049	0.002	0.047	0.002	NS
Tb.Sp (mm)	0.236	0.041	0.233	0.064	0.240	0.036	0.231	0.016	0.237	0.018	NS
Conn.D (/mm)	164.2 ^a	42.5	166.9 ^a	55.0	140.9 ^a	45.7	115.5	16.7	102.3	17.6	<0.0001
SMI	2.40 ^a	0.26	2.34 ^a	0.39	2.54 ^a	0.25	2.73	0.11	2.84	0.18	<0.0001
Midshaft femur cortical bone											
BA	0.480	0.031	0.479	0.025	0.465	0.033	0.571	0.030	0.577	0.032	NS
Ct.Th (mm)	0.111 ^{a,b}	0.006	0.113 ^{a,b}	0.005	0.109 ^{a,b}	0.005	0.131	0.007	0.131	0.009	NS
Imax (mm ⁴)	0.142 ^{a,b}	0.020	0.141 ^{a,b}	0.016	0.135 ^{a,b}	0.021	0.175	0.010	0.183	0.013	NS
Imin (mm ⁴)	0.077 ^{a,b}	0.009	0.071 ^{a,b}	0.008	0.071 ^{a,b}	0.009	0.091	0.008	0.094	0.008	NS
Lumbar vertebra trabecular bone											
BV/TV (%)	24.53	1.50	24.77	1.53	25.21	1.68	26.15	1.85	24.03	1.76	NS
Tb.N (/mm)	5.34 ^a	0.27	5.27 ^a	0.11	5.29 ^a	0.16	5.00	0.13	4.88	0.27	NS
Tb.Th (mm)	0.048 ^a	0.001	0.049 ^a	0.002	0.048 ^a	0.002	0.054	0.002	0.052	0.002	<0.0001
Tb.Sp (mm)	0.180 ^a	0.011	0.180 ^a	0.004	0.180 ^a	0.006	0.192	0.008	0.198	0.013	NS
Conn.D (/mm)	226.1 ^a	26.5	197.1 ^a	16.5	210.5 ^a	19.0	200.3	12.6	195.4	29.7	<0.0001
SMI	0.88	0.15	0.82	0.20	0.75	0.22	0.73	0.17	0.93	0.19	NS

^aSignificant overall difference in CR vs N mice with leptin treatments pooled; ^bNo longer significant after adjustment for body mass; ^cSignificant difference from vehicle treated within diet.

Figure 3

(A) Leptin treatment during CR blunted MAT expansion compared with CR VEH, as indicated by extent of OsO_4 staining (shown in dark gray). (B) Proximal tibia adipose volume/marrow volume in OsO_4 stained bones was higher in CR vs N, and 50% lower in CR LEP1 and CR LEP2 vs CR VEH ($P < 0.0001$ for diet, $P = 0.016$ for dose).

MAR, MS/BS, and BFR/BS, were 36–108% lower in CR vs N overall, but did not differ across leptin treatments within either diet ($P < 0.0001$ for all, Table 3).

Discussion

In this study, we tested the effects of a daily leptin bolus of 1–2 mg/kg/day during CR in young, rapidly growing mice. We hypothesized that leptin treatment would attenuate the deleterious effects of CR on bone mass and bone marrow adiposity. To test this hypothesis, we evaluated BMD, cortical and trabecular bone properties, and marrow adipose expansion in female C57Bl/6J mice from 5 to 10 weeks of age. VEH-treated CR mice lost body mass, particularly lean mass, and exhibited smaller gains in body fat and BMD during the experiment compared with VEH-treated N controls. The skeletal phenotype of VEH-treated CR mice included lower whole-body BMD and lower cross-sectional geometric properties in the midshaft femur before adjustment for body mass, with no effects observed on the trabecular bone microarchitecture. Static and dynamic histomorphometry in the proximal tibia showed that the bones of CR mice had fewer osteoblasts, more adipocytes, and lower indices of bone formation compared with N mice.

Our first hypothesis, that leptin treatment during CR would increase bone mass acquisition, was not supported

by the data. Daily leptin injection of 1–2 mg/kg/day did not increase BMD, alter cortical or trabecular bone architecture, or increase bone formation indices in CR mice, nor did it alter skeletal phenotype in N mice fed *ad libitum*. There was support for our second hypothesis that leptin would blunt the increase in marrow adiposity observed with exposure to a CR diet. Specifically, VEH-treated CR mice had a fourfold increase in marrow adiposity compared to VEH-treated N mice. Leptin treatment reduced the CR-induced expansion of marrow adipose in the proximal tibia by 50%, although the MAT volume was still markedly higher than in N mice. These data support the hypothesis that hypoleptinemia contributes to MAT expansion during CR.

Comparison to prior studies of leptin and bone

Hypoleptinemia is associated with low bone mass in humans with anorexia nervosa (Hebebrand *et al.* 1997) and in animal models (Devlin *et al.* 2010). The skeletal effects of leptin are complex and include both central nervous system pathways via the hypothalamus and peripheral pathways involving direct effects of leptin on bone cells. Studies in wild-type mice reported lower trabecular bone volume fraction in the vertebra following intracerebroventricular (ICV) leptin infusion (Ducy *et al.* 2000, Takeda *et al.* 2002, Sato *et al.* 2007).

Table 3 Number of osteoblasts, osteoclasts, and adipocytes at 10 weeks of age in the proximal tibia of female C57BL/6J mice fed 30% CR diet or N diet *ad libitum* from 5 to 10 weeks of age with daily injection of leptin (1–2 mg/kg/day) or vehicle.

	CR						N						Overall P-value								
	Vehicle			1mg/kg/day			2mg/kg/day			Vehicle						1mg/kg/day			2mg/kg/day		
	Mean	S.D.		Mean	S.D.		Mean	S.D.		Mean	S.D.		Mean	S.D.		Mean	S.D.		Diet	Leptin	Diet-Leptin
BV/TV (%)	21.3	2.9		20.8	3.7		19.1	2.4		20.9	4.9		21.3	2.9		20.8	3.7		NS	NS	NS
N.Ob/T.Ar (mm ²)	79.9 ^a	53.0		61.4 ^a	38.1		52.2 ^a	8.9		100.2	42.2		98.6	86.8		118.1	46.7		0.013	NS	NS
N.Ob/BS (mm)	6.7 ^a	4.5		5.3 ^a	3.5		4.7 ^a	1.2		10.0	4.4		10.3	7.7		12.8	4.7		0.001	NS	NS
N.Oc/T.Ar (mm ²)	20.2	11.1		18.1	8.1		21.2	5.2		21.9	8.3		17.9	8.9		18.9	7.2		NS	NS	NS
N. Oc/BS (mm)	1.7	0.8		1.5	0.7		1.9	0.6		2.2	1.0		2.0	0.7		2.0	0.6		NS	NS	NS
N.Ad/T.Ar (mm ²)	112.0 ^a	52.2		85.4 ^{ab}	46.8		60.5 ^{ab}	20.6		26.5	20.8		21.3	16.4		15.3	4.2		<0.002	0.046	NS
sLS/BS (%)	13.7 ^a	5.24		14.28 ^a	3.89		13.62 ^a	2.84		18.34	4.06		22.20	4.94		20.93	6.85		<0.0001	NS	NS
dLS/BS (%)	9.63 ^a	4.75		10.86 ^a	4.71		12.76 ^a	3.27		18.45	3.65		16.37	4.90		16.06	6.79		<0.0001	NS	NS
MS/BS (%)	16.5 ^a	6.1		18.0 ^a	5.4		19.6 ^a	3.7		27.6	3.4		27.5	4.8		26.5	5.0		<0.0001	NS	NS
MAR (μm/day)	1.39 ^a	0.28		1.31 ^a	0.24		1.36 ^a	0.23		1.71	0.16		1.89	0.23		1.90	0.39		<0.0001	NS	NS
BFR/BS (mm ³ /mm ² *year)	86.5 ^a	41.0		85.3 ^a	29.9		98.6 ^a	30.2		172.2	27.2		199.3	36.7		183.8	51.5		<0.0001	NS	NS

^aSignificant overall difference in CR vs N mice with leptin treatments pooled; ^bSignificant difference from vehicle treated within diet.

By contrast, in the hypoleptinemic *ob/ob* mouse, ICV leptin improved bone formation and reduced MAT (Hamrick *et al.* 2007, Bartell *et al.* 2011), and hypothalamic leptin gene therapy increased femur length, total femur volume, and circulating osteocalcin but reduced trabecular bone volume in the distal femur and vertebra (Iwaniec *et al.* 2007, 2009, Kalra *et al.* 2009).

The peripheral or direct effects of leptin on bone are similarly heterogeneous. The finding that leptin receptor (*Lepr*) deletion in osteoblasts had no effect on murine skeletal phenotype implied little direct effect of leptin on osteoblasts (Shi *et al.* 2008). However, Zhou and coworkers recently reported that bone marrow mesenchymal stromal cells expressing the leptin receptor can differentiate into the bone, cartilage, and adipocytes, both *in vivo* and *in vitro*, and showed by fate mapping that in adult bone marrow, these leptin receptor-positive cells represent the primary source of bone and adipocytes (Zhou *et al.* 2014). *In vitro*, leptin increased osteoblast and chondrocyte proliferation, and inhibited osteoclast formation, and in adult male wild-type mice, peripheral injection of leptin (43 μg/day) increased the bone strength, suggesting anabolic effects of leptin on cortical bone (Cornish *et al.* 2002). Leptin treatment in mice subjected to 40% CR prevented the CR-induced decrease in longitudinal bone growth (Gat-Yablonski *et al.* 2004). In the *ob/ob* mouse, peripheral leptin improved bone mass and bone formation rate and reduced MAT (Steppan *et al.* 2000, Hamrick *et al.* 2005, Turner *et al.* 2013). Continuous infusion of supraphysiological leptin doses (100 or 200 mg/day) in female rats increased serum leptin by 14- to 33-fold and decreased body mass, but while the 100 mg/day dose led to lower whole-body BMD, femoral cortical bone volume, femoral bone strength, and femoral polar moment of inertia, the higher dose did not, suggesting that bone loss was prevented despite weight loss (Stunes *et al.* 2012). High-dose leptin (2.5 mg/kg 2×/day) also prevented the fasting-induced decrease in osteocalcin in BALB/c males (Goldstone *et al.* 2002).

Taken together, these studies suggest that both centrally and peripherally administered leptin can be anabolic, neutral, or deleterious to bone mass. Although some of this variability likely reflects the different background strains, dosages, routes of administration, sexes, and ages at onset and offset of treatment used in different studies, it appears that in general, leptin is anabolic to bone in hypoleptinemic animals, but has less effect in leptin-replete animals unless delivered at supraphysiological doses (Kawai *et al.* 2009). In this study, the lack of skeletal

effects of leptin likely reflects an insufficient dose and/or route of administration (see below).

Comparison to prior studies of leptin and MAT

Previous studies have demonstrated that leptin decreases MAT in genetic models of hypoleptinemia (Hamrick *et al.* 2005, Turner *et al.* 2013) and in leptin-replete wild-type rats (Hamrick *et al.* 2007). Humans with anorexia nervosa similarly exhibited attenuation of marrow adiposity with weight recovery (Fazeli *et al.* 2012). The inverse relationship of leptin and marrow adiposity may reflect direct as well as indirect mechanisms. Bone marrow adipocytes, osteoblasts, and myocytes derive from a common mesenchymal progenitor (Bianco *et al.* 2008, 2010, Zhou *et al.* 2014), and leptin directly promotes osteoblastogenesis and suppresses adipogenesis, such that hypoleptinemia could both increase marrow adiposity and decrease bone mass (Muruganandan *et al.* 2009, Evans *et al.* 2011). In addition, there may be cross talk between leptin, estrogen, and the GH-IGF1 axis, both of which are suppressed in CR in humans and animal models (Mobbs *et al.* 2001, Devlin *et al.* 2010, Misra & Klibanski 2011). Postmenopausal estrogen deficiency in women is associated with MAT expansion, and estrogen replacement increases serum leptin and decreases marrow adiposity (Syed *et al.* 2008, Limonard *et al.* 2015). GH deficiency leads to expansion of MAT that is attenuated by GH replacement in dwarf (*dw/dw*) rats (Gevers *et al.* 2002) and in hypophysectomized mice and rats (Menagh *et al.* 2010). In humans, vertebral MAT is inversely associated with circulating IGF1 (Bredella *et al.* 2010). Thus, in addition to direct action on osteoblast and adipocyte progenitors, leptin replacement during CR may decrease MAT by increasing GH and/or estrogen levels (Casanueva & Dieguez 1999, Ahima *et al.* 2000, Chou *et al.* 2011), a possibility that should be tested in future studies. Another possibility is that glucocorticoids are key to MAT expansion. A new study of male and female C57BL/6J mice exposed to CR from 9 to 15 weeks found CR-induced hypoleptinemia and loss of WAT in males but not females (Cawthorn *et al.* 2016). Further, CR in rabbits from 6–13 or 15–22 weeks of age induced hypoleptinemia, lower bone mass, and loss of WAT, but not MAT expansion (Cawthorn *et al.* 2016). These intriguing findings demonstrate that bone loss, hypoleptinemia, and MAT expansion are not inevitable consequences of CR and may occur independently of one another depending on age, sex, and species. The authors suggest that a CR-induced increase in

glucocorticoids observed in mice but not rabbits may be the driver of MAT expansion (Cawthorn *et al.* 2016).

Limitations and future directions

Our study has several limitations that should be addressed in future work. First, and most importantly, we used a leptin dosage of 1–2 mg/kg/day, or 20–40 µg leptin/day, in a single daily bolus timed to coincide with the nocturnal peak in leptin following food intake (Ahima *et al.* 1996, Ahren 2000). This dosage and administration route may have induced transiently normal or high leptin levels but were insufficient to achieve sustained normalization of serum leptin in CR mice, as measured 16 h following injection. Leptin has a short half-life, and it is clear that higher doses given via multiple daily injections or continuous leptin infusion will be needed to fully correct hypoleptinemia in CR animals. For example, daily leptin injection at 8 µg/g was sufficient to reverse inhibition of longitudinal bone growth in CR, although serum leptin levels were not reported (Gat-Yablonski *et al.* 2004). Secondly, these data reflect only female mice, and there may be sex differences in the response to CR and/or effects of leptin on the bone and MAT. Thirdly, we studied mice during a single interval of 5–10 weeks of age. The effects of CR on the bone mass show marked age-related variation (Hamrick *et al.* 2008, Tatsumi *et al.* 2008, Devlin *et al.* 2010), and its effects on leptin levels and MAT expansion may also vary with age. Fourthly, here we focused solely on leptin and did not assess other bone-active hormones such as adiponectin or osteocalcin that are known to change in CR (Cawthorn *et al.* 2014) and to contribute to skeletal homeostasis in humans (Gravenstein *et al.* 2011). Finally, we studied only a single strain, C57BL/6J, and it is clear that the relationships between the bone mass and MAT vary tremendously across strains (Fazeli *et al.* 2013, Scheller *et al.* 2015). Thus, future studies should include males; incorporate different ages and durations of CR exposure; consider additional hormonal factors; and include strains such as C3H/HeJ, which exhibits both high bone mass and high MAT on diet *ad libitum* (Motyl & Rosen 2011, Scheller *et al.* 2015).

Conclusion

In this study, we found that once daily leptin treatment of 1–2 mg/kg/day is sufficient to blunt CR-induced MAT expansion, but does not alter the serum leptin level or cortical or trabecular bone architecture or bone formation

in young female C57Bl/6J mice. These results suggest that bone MAT and bone tissue may have different sensitivities to small and/or transient changes in circulating leptin level. Such subtle changes may be sufficient to reduce MAT without inducing a corresponding increase in bone mass during CR in young female mice.

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.

Funding

This work was supported by the National Institutes of Health (RC1AR058389, R24DK092759, S10RR017868, and F32HD060419).

Acknowledgments

The authors thank Mark Horowitz, Yale School of Medicine, New Haven, Connecticut, for assistance with bone histomorphometry and osmium tetroxide staining.

References

- Ahima RS, Prabakaran D, Mantzoros C, Qu D, Lowell B, Maratos-Flier E & Flier JS 1996 Role of leptin in the neuroendocrine response to fasting. *Nature* **382** 250–252. (doi:10.1038/382250a0)
- Ahima RS, Saper CB, Flier JS & Elmquist JK 2000 Leptin regulation of neuroendocrine systems. *Frontiers in Neuroendocrinology* **21** 263–307. (doi:10.1006/frne.2000.0197)
- Ahren B 2000 Diurnal variation in circulating leptin is dependent on gender, food intake and circulating insulin in mice. *Acta Physiologica Scandinavica* **169** 325–331. (doi:10.1046/j.1365-201x.2000.00746.x)
- Bartell SM, Rayalam S, Ambati S, Gaddam DR, Hartzell DL, Hamrick M, She JX, Della-Fera MA & Baile CA 2011 Central (ICV) leptin injection increases bone formation, bone mineral density, muscle mass, serum IGF-1, and the expression of osteogenic genes in leptin-deficient ob/ob mice. *Journal of Bone and Mineral Research* **26** 1710–1720. (doi:10.1002/jbmr.406)
- Baum T, Yap SP, Karampinos DC, Nardo L, Kuo D, Burghardt AJ, Masharani UB, Schwartz AV, Li X & Link TM 2012 Does vertebral bone marrow fat content correlate with abdominal adipose tissue, lumbar spine bone mineral density, and blood biomarkers in women with type 2 diabetes mellitus? *Journal of Magnetic Resonance Imaging* **35** 117–124. (doi:10.1002/jmri.22757)
- Bianco P, Robey PG & Simmons PJ 2008 Mesenchymal stem cells: revisiting history, concepts, and assays. *Cell Stem Cell* **2** 313–319. (doi:10.1016/j.stem.2008.03.002)
- Bianco P, Robey PG, Saggio I & Riminucci M 2010 'Mesenchymal' stem cells in human bone marrow (skeletal stem cells): a critical discussion of their nature, identity, and significance in incurable skeletal disease. *Human Gene Therapy* **21** 1057–1066. (doi:10.1089/hum.2010.136)
- Boden G, Chen X, Mozzoli M & Ryan I 1996 Effect of fasting on serum leptin in normal human subjects. *Journal of Clinical Endocrinology and Metabolism* **81** 3419–3423. (doi:10.1210/jcem.81.9.8784108)
- Bouxsein ML, Devlin MJ, Glatz V, Dhillon H, Pierroz DD & Ferrari SL 2009 Mice lacking beta-adrenergic receptors have increased bone mass, but are not protected from deleterious skeletal effects of ovariectomy. *Endocrinology* **150** 144–152. (doi:10.1210/en.2008-0843)
- Bouxsein ML, Boyd SK, Christiansen BA, Guldberg RE, Jepsen KJ & Muller R 2010 Guidelines for assessment of bone microstructure in rodents using micro-computed tomography. *Journal of Bone and Mineral Research* **25** 1468–1486. (doi:10.1002/jbmr.141)
- Bredella MA, Fazeli PK, Miller KK, Misra M, Torriani M, Thomas BJ, Ghomi RH, Rosen CJ & Klibanski A 2009 Increased bone marrow fat in anorexia nervosa. *Journal of Clinical Endocrinology and Metabolism* **94** 2129–2136. (doi:10.1210/jc.2008-2532)
- Bredella MA, Torriani M, Ghomi RH, Thomas BJ, Brick DJ, Gerweck AV, Rosen CJ, Klibanski A & Miller KK 2010 Vertebral bone marrow fat is positively associated with visceral fat and inversely associated with IGF-1 in obese women. *Obesity* **19** 49–53. (doi:10.1038/oby.2010.106)
- Casanueva FF & Dieguez C 1999 Neuroendocrine regulation and actions of leptin. *Frontiers in Neuroendocrinology* **20** 317–363. (doi:10.1006/frne.1999.0187)
- Cawthorn WP, Scheller EL, Learman BS, Parlee SD, Simon BR, Mori H, Ning X, Bree AJ, Schell B, Broome DT, et al. 2014 Bone marrow adipose tissue is an endocrine organ that contributes to increased circulating adiponectin during caloric restriction. *Cell Metabolism* **20** 368–375. (doi:10.1016/j.cmet.2014.06.003)
- Cawthorn WP, Scheller EL, Parlee SD, Pham HA, Learman BS, Redshaw CM, Sulston RJ, Burr AA, Das AK, Simon BR, et al. 2016 Expansion of bone marrow adipose tissue during caloric restriction is associated with increased circulating glucocorticoids and not with hypoleptinemia. *Endocrinology* **157** 508–521. (doi:10.1210/en.2015-1477)
- Chou SH, Chamberland JP, Liu X, Matarese G, Gao C, Stefanakis R, Brinkoetter MT, Gong H, Arampatzis K & Mantzoros CS 2011 Leptin is an effective treatment for hypothalamic amenorrhea. *PNAS* **108** 6585–6590. (doi:10.1073/pnas.1015674108)
- Cornish J, Callon KE, Bava U, Lin C, Naot D, Hill BL, Grey AB, Broom N, Myers DE, Nicholson GC, et al. 2002 Leptin directly regulates bone cell function in vitro and reduces bone fragility in vivo. *Journal of Endocrinology* **175** 405–415. (doi:10.1677/joe.0.1750405)
- Dempster DW, Compston JE, Drezner MK, Glorieux FH, Kanis JA, Malluche H, Meunier PJ, Ott SM, Recker RR & Parfitt AM 2013 Standardized nomenclature, symbols, and units for bone histomorphometry: a 2012 update of the report of the ASBMR Histomorphometry Nomenclature Committee. *Journal of Bone and Mineral Research* **28** 2–17. (doi:10.1002/jbmr.1805)
- Devlin MJ & Rosen CJ 2015 The bone-fat interface: basic and clinical implications of marrow adiposity. *Lancet Diabetes & Endocrinology* **3** 141–147. (doi:10.1016/S2213-8587(14)70007-5)
- Devlin MJ, Cloutier AM, Thomas NA, Panus DA, Lotinun S, Pinz I, Baron R, Rosen CJ & Bouxsein ML 2010 Caloric restriction leads to high marrow adiposity and low bone mass in growing mice. *Journal of Bone and Mineral Research* **25** 2078–2088. (doi:10.1002/jbmr.82)
- Ducy P, Amling M, Takeda S, Priemel M, Schilling AF, Beil FT, Shen J, Vinson C, Rueger JM & Karsenty G 2000 Leptin inhibits bone formation through a hypothalamic relay: a central control of bone mass. *Cell* **100** 197–207. (doi:10.1016/S0092-8674(00)81558-5)
- Ecklund K, Vajapeyam S, Feldman HA, Buzney CD, Mulkern RV, Kleinman PK, Rosen CJ & Gordon CM 2010 Bone marrow changes in adolescent girls with anorexia nervosa. *Journal of Bone and Mineral Research* **25** 298–304. (doi:10.1359/jbmr.090805)
- Evans BA, Bull MJ, Kench RC, Fox RE, Morgan LD, Stevenson AE, Gevers EF, Perry MJ & Wells T 2011 The influence of leptin on trabecular architecture and marrow adiposity in GH-deficient rats. *Journal of Endocrinology* **208** 69–79. (doi:10.1677/JOE-10-0178)
- Fazeli PK, Bredella MA, Freedman L, Thomas BJ, Breggia A, Meenaghan E, Rosen CJ & Klibanski A 2012 Marrow fat and preadipocyte factor-1 levels decrease with recovery in women with anorexia nervosa. *Journal of Bone and Mineral Research* **27** 1864–1871. (doi:10.1002/jbmr.1640)

- Fazeli PK, Horowitz MC, MacDougald OA, Scheller EL, Rodeheffer MS, Rosen CJ & Klibanski A 2013 Marrow fat and bone – new perspectives. *Journal of Clinical Endocrinology and Metabolism* **98** 935–945. (doi:10.1210/jc.2012-3634)
- Fretz JA, Nelson T, Xi Y, Adams DJ, Rosen CJ & Horowitz MC 2010 Altered metabolism and lipodystrophy in the early B-cell factor 1-deficient mouse. *Endocrinology* **151** 1611–1621. (doi:10.1210/en.2009-0987)
- Gat-Yablonski G, Ben-Ari T, Shtaf B, Potievsky O, Moran O, Eshet R, Maor G, Segev Y & Phillip M 2004 Leptin reverses the inhibitory effect of caloric restriction on longitudinal growth. *Endocrinology* **145** 343–350. (doi:10.1210/en.2003-0910)
- Gevers EF, Loveridge N & Robinson IC 2002 Bone marrow adipocytes: a neglected target tissue for growth hormone. *Endocrinology* **143** 4065–4073. (doi:10.1210/en.2002-220428)
- Glatt V, Canalis E, Stadmeier L & Bouxsein ML 2007 Age-related changes in trabecular architecture differ in female and male C57BL/6J mice. *Journal of Bone and Mineral Research* **22** 1197–1207. (doi:10.1359/jbmr.070507)
- Goldstone AP, Howard JK, Lord GM, Ghatti MA, Gardiner JV, Wang ZL, Wang RM, Girgis SI, Bailey CJ & Bloom SR 2002 Leptin prevents the fall in plasma osteocalcin during starvation in male mice. *Biochemical and Biophysical Research Communications* **295** 475–481. (doi:10.1016/S0006-291X(02)00697-6)
- Gravenstein KS, Napora JK, Short RG, Ramachandran R, Carlson OD, Metter EJ, Ferrucci L, Egan JM & Chia CW 2011 Cross-sectional evidence of a signaling pathway from bone homeostasis to glucose metabolism. *Journal of Clinical Endocrinology and Metabolism* **96** E884–E890. (doi:10.1210/jc.2010-2589)
- Hamrick MW, Della-Fera MA, Choi YH, Pennington C, Hartzell D & Baile CA 2005 Leptin treatment induces loss of bone marrow adipocytes and increases bone formation in leptin-deficient ob/ob mice. *Journal of Bone and Mineral Research* **20** 994–1001. (doi:10.1359/JBMR.050103)
- Hamrick MW, Della Fera MA, Choi YH, Hartzell D, Pennington C & Baile CA 2007 Injections of leptin into rat ventromedial hypothalamus increase adipocyte apoptosis in peripheral fat and in bone marrow. *Cell Tissue Research* **327** 133–141. (doi:10.1007/s00441-006-0312-3)
- Hamrick MW, Ding KH, Ponnala S, Ferrari SL & Isales CM 2008 Caloric restriction decreases cortical bone mass but spares trabecular bone in the mouse skeleton: implications for the regulation of bone mass by body weight. *Journal of Bone and Mineral Research* **23** 870–878. (doi:10.1359/jbmr.080213)
- Hebebrand J, Blum WF, Barth N, Coners H, Englaro P, Juul A, Ziegler A, Warnke A, Rascher W & Romschmidt H 1997 Leptin levels in patients with anorexia nervosa are reduced in the acute stage and elevated upon short-term weight restoration. *Molecular Psychiatry* **2** 330–334. (doi:10.1038/sj.mp.4000282)
- Iwaniec UT, Boghossian S, Lapke PD, Turner RT & Kalra SP 2007 Central leptin gene therapy corrects skeletal abnormalities in leptin-deficient ob/ob mice. *Peptides* **28** 1012–1019. (doi:10.1016/j.peptides.2007.02.001)
- Iwaniec UT, Dube MG, Boghossian S, Song H, Helferich WG, Turner RT & Kalra SP 2009 Body mass influences cortical bone mass independent of leptin signaling. *Bone* **44** 404–412. (doi:10.1016/j.bone.2008.10.058)
- Kalra SP, Dube MG & Iwaniec UT 2009 Leptin increases osteoblast-specific osteocalcin release through a hypothalamic relay. *Peptides* **30** 967–973. (doi:10.1016/j.peptides.2009.01.020)
- Kawai M, Devlin MJ & Rosen CJ 2009 Fat targets for skeletal health. *Nature Reviews Rheumatology* **5** 365–372. (doi:10.1038/nrrheum.2009.102)
- Lang DH, Sharkey NA, Lionikas A, Mack HA, Larsson L, Vogler GP, Vandenbergh DJ, Blizard DA, Stout JT, Stitt JP, et al. 2005 Adjusting data to body size: a comparison of methods as applied to quantitative trait loci analysis of musculoskeletal phenotypes. *Journal of Bone and Mineral Research* **20** 748–757. (doi:10.1359/JBMR.041224)
- Limonard EJ, Veldhuis-Vlug AG, van Dussen L, Runge JH, Tanck MW, Ender E, Heijboer AC, Fliers E, Hollak CE, Akkerman EM, et al. 2015 Short-term effect of estrogen on human bone marrow fat. *Journal of Bone and Mineral Research* **30** 2058–2066. (doi:10.1002/jbmr.2557)
- Menagh PJ, Turner RT, Jump DB, Wong CP, Lowry MB, Yakar S, Rosen CJ & Iwaniec UT 2010 Growth hormone regulates the balance between bone formation and bone marrow adiposity. *Journal of Bone and Mineral Research* **25** 757–768. (doi:10.1359/jbmr.091015)
- Misra M & Klibanski A 2011 Bone health in anorexia nervosa. *Current Opinion in Endocrinology, Diabetes and Obesity* **18** 376–382. (doi:10.1097/MED.0b013e32834b4bdc)
- Mobbs CV, Bray GA, Atkinson RL, Bartke A, Finch CE, Maratos-Flier E, Crawley JN & Nelson JF 2001 Neuroendocrine and pharmacological manipulations to assess how caloric restriction increases life span. *Journals of Gerontology Series A: Biological Sciences and Medical Sciences* **56** 34–44. (doi:10.1093/gerona/56.suppl_1.34)
- Motyl KJ & Rosen CJ 2011 Temperatures rising: brown fat and bone. *Discovery Medicine* **11** 179–185.
- Muruganandan S, Roman AA & Sinal CJ 2009 Adipocyte differentiation of bone marrow-derived mesenchymal stem cells: cross talk with the osteoblastogenic program. *Cellular and Molecular Life Sciences* **66** 236–253. (doi:10.1007/s00018-008-8429-z)
- Sato S, Hanada R, Kimura A, Abe T, Matsumoto T, Iwasaki M, Inose H, Ida T, Mieda M, Takeuchi Y, et al. 2007 Central control of bone remodeling by neuromedin U. *Nature Medicine* **13** 1234–1240. (doi:10.1038/nm1640)
- Scheller EL, Troiano N, Vanhoutan JN, Bouxsein MA, Fretz JA, Xi Y, Nelson T, Katz G, Berry R, Church CD, et al. 2014 Use of osmium tetroxide staining with microcomputerized tomography to visualize and quantify bone marrow adipose tissue in vivo. *Methods in Enzymology* **537** 123–139. (doi:10.1016/B978-0-12-411619-1.00007-0)
- Scheller EL, Doucette CR, Learman BS, Cawthorn WP, Khandaker S, Schell B, Wu B, Ding SY, Bredella MA, Fazeli PK, et al. 2015 Region-specific variation in the properties of skeletal adipocytes reveals regulated and constitutive marrow adipose tissues. *Nature Communications* **6** 7808. (doi:10.1038/ncomms8808)
- Schwartz AV, Sigurdsson S, Hue TF, Lang TE, Harris TB, Rosen CJ, Vittinghoff E, Siggeirsdottir K, Sigurdsson G, Oskarsdottir D, et al. 2013 Vertebral bone marrow fat associated with lower trabecular BMD and prevalent vertebral fracture in older adults. *Journal of Clinical Endocrinology and Metabolism* **98** 2294–2300. (doi:10.1210/jc.2012-3949)
- Shi Y, Yadav VK, Suda N, Liu XS, Guo XE, Myers MG Jr & Karsenty G 2008 Dissociation of the neuronal regulation of bone mass and energy metabolism by leptin in vivo. *PNAS* **105** 20529–20533. (doi:10.1073/pnas.0808701106)
- Steppan CM, Crawford DT, Chidsey-Frink KL, Ke H & Swick AG 2000 Leptin is a potent stimulator of bone growth in ob/ob mice. *Regulatory Peptides* **92** 73–78. (doi:10.1016/S0167-0115(00)00152-X)
- Stunes AK, Westbroek I, Gordeladze JO, Gustafsson BI, Reseland JE & Syversen U 2012 Systemic leptin administration in supraphysiological doses maintains bone mineral density and mechanical strength despite significant weight loss. *Endocrinology* **153** 2245–2253. (doi:10.1210/en.2011-1848)
- Surwit RS, Edwards CL, Murthy S & Petro AE 2000 Transient effects of long-term leptin supplementation in the prevention of diet-induced obesity in mice. *Diabetes* **49** 1203–1208. (doi:10.2337/diabetes.49.7.1203)
- Syed FA, Oursler MJ, Hefferanm TE, Peterson JM, Riggs BL & Khosla S 2008 Effects of estrogen therapy on bone marrow adipocytes in postmenopausal osteoporotic women. *Osteoporosis International* **19** 1323–1330. (doi:10.1007/s00198-008-0574-6)

- Takeda S, Eleftheriou F, Levasseur R, Liu X, Zhao L, Parker KL, Armstrong D, Ducky P & Karsenty G 2002 Leptin regulates bone formation via the sympathetic nervous system. *Cell* **111** 305–317. (doi:10.1016/S0092-8674(02)01049-8)
- Tatsumi S, Ito M, Asaba Y, Tsutsumi K & Ikeda K 2008 Life-long caloric restriction reveals biphasic and dimorphic effects on bone metabolism in rodents. *Endocrinology* **149** 634–641. (doi:10.1210/en.2007-1089)
- Thomas T, Gori F, Khosla S, Jensen MD, Burguera B & Riggs BL 1999 Leptin acts on human marrow stromal cells to enhance differentiation to osteoblasts and to inhibit differentiation to adipocytes. *Endocrinology* **140** 1630–1638. (doi:10.1210/endo.140.4.6637)
- Trudel G, Payne M, Madler B, Ramachandran N, Lecompte M, Wade C, Biolo G, Blanc S, Hughson R, Bear L, *et al.* 2009 Bone marrow fat accumulation after 60 days of bed rest persisted 1 year after activities were resumed along with hemopoietic stimulation: the Women International Space Simulation for Exploration study. *Journal of Applied Physiology* **107** 540–548. (doi:10.1152/japplphysiol.91530.2008)
- Tuljapourkar SR, McGuire TR, Brusnahan SK, Jackson JD, Garvin KL, Kessinger MA, Lane JT, BJ O'Kane & Sharp JG 2011 Changes in human bone marrow fat content associated with changes in hematopoietic stem cell numbers and cytokine levels with aging. *Journal of Anatomy* **219** 574–581. (doi:10.1111/j.1469-7580.2011.01423.x)
- Turello R, Snyder D & Hartman HA 1984 A modification the ssmium tetroxide post-fixation technique for the demonstration of extracellular lipid in paraffin-embedded tissue sections. *Journal of Histotechnology* **7** 75–77. (doi:10.1179/his.1984.7.2.75)
- Turner RT, Kalra SP, Wong CP, Philbrick KA, Lindenmaier LB, Boghossian S & Iwaniec UT 2013 Peripheral leptin regulates bone formation. *Journal of Bone and Mineral Research* **28** 22–34. (doi:10.1002/jbmr.1734)
- Welt CK, Chan JL, Bullen J, Murphy R, Smith P, DePaoli AM, Karalis A & Mantzoros CS 2004 Recombinant human leptin in women with hypothalamic amenorrhea. *New England Journal of Medicine* **351** 987–997. (doi:10.1056/NEJMoa040388)
- Yeung DK, Griffith JE, Antonio GE, Lee FK, Woo J & Leung PC 2005 Osteoporosis is associated with increased marrow fat content and decreased marrow fat unsaturation: a proton MR spectroscopy study. *Journal of Magnetic Resonance Imaging* **22** 279–285. (doi:10.1002/(ISSN)1522-2586)
- Zhou BO, Yue R, Murphy MM, Peyer JG & Morrison SJ 2014 Leptin-receptor-expressing mesenchymal stromal cells represent the main source of bone formed by adult bone marrow. *Cell Stem Cell* **15** 154–168. (doi:10.1016/j.stem.2014.06.008)

Received in final form 5 April 2016

Accepted 11 April 2016