Supplementary information

Differential bone metabolism and protein expression in mice fed a high-fat diet versus Daurian ground squirrels following natural pre-hibernation fattening

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Materials and methods

Pathological obesity model

All animal experiments were approved by the Experimental Animal Protection Committee of the Ministry of Health of the People's Republic of China (Approval Number: SL-2012-42). Four-week-old male Kunming mice were purchased from the Chengdu Dashuo Experimental Animal Company. The initial weight of the mice ranged from 23 to 25 g. The mice were kept in plastic cages in the animal room and provided with food and water ad libitum. The animal room was maintained at a temperature of 18–25 °C, and lighting was changed daily to coincide with local sunrise and sunset. After a week of normal diet feeding, the mice were randomly divided into two groups (n=6): a CON group, control mice fed a normal diet for 3 months; and an OB group, obesity mice fed a high-fat diet for 3 months. After the 3 months of dietary intervention, all mice were sacrificed.

Healthy obesity model

Twelve Daurian ground squirrels of both sexes were caught from the Weinan city in Shaanxi, a Province in China. The squirrels were raised in cages of the animal room and provided with food and water ad libitum. The squirrels were divided into two groups (n=6): a summer active (SA) control group that were captured and sacrificed at the end of June, and a pre-hibernation (PRE) group that were captured and sacrificed at the end of September after natural fattening. Both groups acquired natural foods before being caught. After returning to the lab, they were fed rat chow with the same composition as the normal diet in Table S1. To ensure nutritional requirements were satisfied during the fattening period, appropriate amounts of high-fat and high-protein nuts (such as peanuts) were added to the diet of the PRE ground squirrels.

Table S1 Compositions of normal diet and high-fat diet for mice

Components	Content
Normal diet	
Corn	25.4 g
Wheat	30.6 g
Soybean	13 g
Fish meal	6 g
Rice bran	6 g
Wheat bran	10 g
Soybean meal	5 g
Other	4 g
Total	100 g
High-fat diet	
Patterned animal base	50 g
Soy flour	5 g
Fish meal	5 g
Milk powder	10 g
Peanut	6 g
Egg yolk powder	5 g
Lard	12 g
Salt	2 g
Sucrose	5 g
Total	100 g
Fat calorie percentage	61%

Sample collection

After body weight was recorded, mice were anesthetized with 60 mg/kg sodium pentobarbital (1 mg/mL, Sigma, Kenilworth, NJ, USA) intraperitoneally, and ground squirrels with a 90 mg/kg dose. Then after thirty minutes of anesthesia induction time, the femurs and tibias from both legs were carefully dissected free of associated connective tissue, weighed quickly, and immediately placed in sealed containers with lactated 70% absolute ethanol, followed by freezing in liquid nitrogen and storage at −70 °C. We also sampled adipose tissues, including mesenteric adipose, perirenal adipose and back scapula subcutaneous adipose. Similarly, adipose tissues were weighed, then frozen in liquid nitrogen and storage at −70 °C. Animals were euthanized by an overdose injection of sodium pentobarbital after sampling.

Biomechanical testing

Mechanical properties of the femurs were determined by loading the left femurs to failure in a 3-point bending test, exactly as described previously with female mice (Lloyd et al., 2012). Left femurs were prepared for biomechanical testing by wrapping gauze soaked in normal saline, freezing at −70 °C and mechanically tested with three-point bending using an electronic tensile machine (CMT4304, MTS, USA). Three parameters (i.e., ultimate bearing capacity, stiffness, and ultimate bending energy) were determined as described previously (Lloyd et al., 2014). Stiffness was defined as the slope of the linear region of the pre-yield load displacement curve, and the yield point was defined as the point where the load-displacement curve intersected with a regression line that was 10% lower than that used to define stiffness. Maximum load was defined as the load at which the bone catastrophically failed.

Micro-computed tomography

Micro-computed tomography (micro-CT) scanner (L-SP, GE, USA) was used to scan the right femurs and tibias of mice and ground squirrels by an X-ray tube potential of 70 kVp and tube current of 114 µA in air. Then the captured images were analyzed by GEHE Micro View V2.1.2 (GE, USA), and images with voxel size of 43.305 µm were obtained. Next, a region of interest (ROI_L) at the longitudinal plane was then analyzed; this was the coronal central region (1-mm

thick) of the distal femur and proximal tibia. Similarly, the other region of interest (ROI_T) at the transverse plane was then analyzed, which was defined as between 0.95 and 1.05 mm from the metaphyseal growth plate of the distal femur and the proximal tibia. A top-down tomographic scan was performed starting at 3% of the bone length, 0.5 mm at a time, and cycled three times. In short, ROI_L is used to analyze trabecula and cortical bones, and ROI_T is used to analyze trabecula. The chosen ROIs of trabecular and cortical bones were analyzed for the parameters shown in Table S2 (Parfitt et al., 1987).

Table S2 Parameters analyzed by micro-CT

Name	Abbreviation	Unit
Bone surface density	BS/BV	mm^{-1}
Trabecular number	Tb.N	mm^{-1}
Trabecular thickness	Tb.Th	mm
Trabecular separation	Tb.Sp	mm
Bone volume fraction	BV/TV	%
Average cortical thickness	Ct.Th	mm
Marrow area	Ma.Ar	mm^2
Cortical bone area	Ct.Ar	mm^2
Total cortical bone area	Tt.Ar	mm^2
Tissue mineral density	TMD	mg/cm^3
Tissue mineral content	TMC	mg
Bone mineral density	BMD	mg/cm^3
bone mineral content	BMC	mg

Western blots

Methods were those described by Chang et al. (2020). Briefly, total protein was extracted from frozen femurs of mice and ground squirrels by homogenization and put into sample buffer (pH 6.8, 100 mmol/L Tris, 4% SDS, 5% glycerol, 5% 2-β-mercaptoethanol, and bromophenol blue). Then, the bone protein extracts were separated by SDS-PAGE (10% Laemmli gels with an acrylamide/bisacrylamide ratio of 29:1). After electrophoresis, total proteins bands were visualized by putting the gel on a UV transilluminator and irradiating the gel for 2 min. A Syngene G:BOX system (Syngene, Frederick, MD, USA) was used to take photographs of the gel. Proteins in the gels were then transferred to PVDF membranes (0.45 μm pore size, Millipore, IPVH00010, Darmstadt, Germany) using a Bio-Rad semi-dry transfer apparatus. Membranes were blocked with 5% (0.05 g/mL) skim milk (P0216, Beyotime, Shanghai, China) dissolved in TBST (10 mmol/L Tris-HCl, 150 mmol/L NaCl, 0.05% Tween-20. pH 7.6) for 2 h at room temperature and then incubated with a primary antibody. The primary antibodies (diluted 1:1000 (v:v) before use) were ALP, OPG, Ptch1, P-β-catenin, cathepsin K, GSK-3β, MMP9, OCN, RANKL and RunX2. Membranes were incubated at 4 ℃ overnight in TBST containing 0.1% bovine serum albumin (BSA) and the chosen antibody. They were then washed with TBST for 4×10 min each followed by incubation with HRP-conjugated anti-mouse secondary antibody (1:10000) or HRP-conjugated anti-rabbit secondary antibody (1:5000) for 2 h at room temperature. The specific information of primary and secondary antibodies was shown in Table S3. Then each PVDF membrane was washed with TBST for 3×10 min and bands were visualized using enhanced chemiluminescence reagents (Thermo Fisher Scientific, NCI5079, Waltham, MA, USA). NIH ImageJ software (NIH, Bethesda, MD, USA) was used to carry out quantification analysis. The density of the target immunoblot band in each lane was standardized against the summed densities from a group of total protein bands in the same lane that were well separated from the band of interest and present in all lanes.

Table S3 Primary and secondary antibodies used in western blots

Antibodies	Source	Identifier
Primary antibody		
ALP	Abcam (Cambridge, UK)	81283
OPG	Affinity Biosciences (Melbourne, AUS)	DF6824
Ptch1	Affinity Biosciences (Melbourne, AUS)	AF5202
P-β-catenin	Sigma (Kenilworth, NJ, USA)	4504476
Cathepsin K	Cell Signaling Technology (CST, Boston, MA, USA)	9205S
GSK-3β	CST (Boston, MA, USA)	2855S
MMP9	CST (Boston, MA, USA)	2556S
OCN	CST (Boston, MA, USA)	2539S
RANKL	CST (Boston, MA, USA)	4146S
RunX2	Sigma (Kenilworth, NJ, USA)	T6277
Secondary antibody		
HRP-conjugated anti-mouse secondary antibody	Thermo Fisher Scientific (Waltham, MA, USA)	A28177
HRP-conjugated anti-rabbit secondary antibody	Thermo Fisher Scientific (Waltham, MA, USA)	A27036

ALP: alkaline phosphatase; OPG: osteoprotegerin; Ptch1: patched protein homolog 1; GSK-3β: glycogen synthase kinase-3β; MMP9: matrix metallopeptidase 9; OCN: osteocalcin; RANKL: receptor activator of nuclear factor-κB ligand; RunX2: Runt-related transcription factor 2; HRP: horseradish peroxidase.

Statistical analyses

All data of the results expressed as mean±standard deviation (SD) were analyzed using the SPSS 24 (IBM, Armonk, NY, USA). Differences between the OB and CON groups or between the PRE and SA groups were determined by the independent-samples *t*-test, and $P<0.05$ was considered to be statistically significant.

References

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