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Functionally improved bone in Calbindin-D28k knockout mice

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Abstract

In vitro studies indicate that Calbindin-D28k, a calcium binding protein, is important in regulating the life span of osteoblasts as well as the mineralization of bone extracellular matrix. The recent creation of a Calbindin-D28k knockout mouse has provided the opportunity to study the physiological effects of the Calbindin-D28k protein on bone remodeling in vivo. In this experiment, histomorphometry, μ CT, and bend testing were used to characterize bones in Calbindin-D28k KO (knockout) mice. The femora of Calbindin-D28k KO mice had significantly increased cortical bone volume ($60.4\% \pm 3.1$) compared to wild-type (WT) mice ($45.4\% \pm 4.6$). The increased bone volume was due to a decrease in marrow cavity area, and significantly decreased endosteal perimeters ($3.397 \text{ mm} \pm 0.278$ in Calbindin-D28k KO mice, and $4.046 \text{ mm} \pm 0.450$ in WT mice). Similar changes were noted in the analysis of the tibiae in both mice. The bone formation rates were similar in the femoral and tibial cortical bones of both mice. μ CT analysis of the trabecular bone in the tibial plateau indicated that Calbindin-D28k KO mice had an increased bone volume ($35.2\% \pm 3.1$) compared to WT mice ($24.7\% \pm 4.9$) which was primarily due to increased trabecular number ($8.99 \text{ mm}^{-1} \pm 0.94$ in Calbindin-D28k KO mice compared to $6.75 \text{ mm}^{-1} \pm 0.85$ in WT mice). Bone mineral content analysis of the tibiae indicated that there is no difference in the calcium or phosphorus content between the Calbindin-D28k KO and WT mice. Cantilever bend testing of the femora demonstrated significantly lower strains in the bones of Calbindin-D28k KO mice ($4135 \text{ } \mu\text{strain/kg} \pm 1266$) compared to WT mice ($6973 \text{ } \mu\text{strain/kg} \pm 998$) indicating that the KO mice had stiffer bones. Three-point bending demonstrated increased failure loads in bones of Calbindin-D28k KO mice ($31.6 \text{ N} \pm 2.1$) compared to WT mice ($15.0 \text{ N} \pm 1.7$). In conclusion, Calbindin-D28k KO mice had increased bone volume and stiffness indicating that Calbindin-D28k plays an important role in bone remodeling.

Keywords

Calbindin-D28k knockout; Bone histomorphometry; μ CT; Biomechanics

Introduction

Calbindin-D28k is a vitamin D-dependent intracellular calcium binding protein. Studies of Calbindin-D28k knockout (KO) mice have demonstrated that they have impaired motor coordination likely due to deficits in cerebellar pathways [1]. Calbindin-D28k has also been shown to participate in calcium uptake in the distal tubule in vitro [2]. This result correlates with in vivo studies indicating that Calbindin-D28k KO mice have significant hypercalciuria when fed a high calcium diet [3]. Aside from being expressed in neuronal and kidney cells, Calbindin-D28k is also expressed in osteoblast cells and has been shown to regulate their

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apoptosis in vitro [4]. Cell culture studies have also demonstrated that Calbindin-D28k is important in regulating calcium uptake during mineralized matrix formation in vitro [5].

Recently, our laboratory demonstrated that the KO mice have a decreased level of serum osteocalcin compared with the wild-type mice [6]. Osteocalcin is a protein uniquely synthesized by osteoblasts and secreted into the bone extracellular matrix [7,8]. Osteocalcin has been shown to correlate with bone formation rate [9] and serum osteocalcin levels are used as a clinical indicator of bone metabolism [10]. Lower serum osteocalcin levels suggest that bone formation may be slower in Calbindin-D28k KO mice and result in altered biomechanical characteristics of bones in these mice.

Another recent study in Calbindin-D28k KO and vitamin D receptor KO mice demonstrated that Calbindin-D28k plays an important physiologic role in calcium homeostasis; however, its function can be mostly compensated for by Calbindin-D9k [11]. This study provides the most direct in vivo evidence to date that Calbindin-D28k is important in bone; however, previous studies [1,11] have not found any significant changes in the bones or growth plates of Calbindin-D28k KO mice.

While there may be no gross changes in the bones of Calbindin-D28k KO mice, quantitative histological and biomechanical testing has not been performed to confirm these observations. The intention of this study was to quantify the histological properties, bone formation rates, biomechanical properties, and mineral content of the bones in Calbindin-D28k KO mice. In this study, bone structure was evaluated in Calbindin-D28k KO (knockout) and wild-type (WT) mice using histology and μ CT. Bone formation was analyzed using histomorphometry, and the mechanical properties of the bones were compared in cantilever bending and in three-point bending tests. Bone mineral content was analyzed using atomic emission spectroscopy and a fluorescent assay.

Materials and methods

National Institutes of Health guidelines for the care and use of laboratory animals (NIH publication #82-23, Rev. 1985) were followed during this study. Calbindin-D28k KO mice developed by Airaksinen et al. [1] were purchased from the Jackson Laboratories (Bar Harbor, ME) and maintained at the University of Arizona animal facilities. C57BL/6 mice were used as controls in this experiment. Six male C57BL/6 mice and six male Calbindin-D28k knockout mice which were 90 days old when they were sacrificed, were skeletally immature. Thirteen and three days prior to sacrifice, six of each type of mouse (12 total) were labeled with calcein (Sigma, St. Louis, MO) subcutaneously at a dose of 15 mg/kg. Following sacrifice, the right femora and tibiae were explanted and cleaned of soft tissue. The femora were immediately frozen at -20°C , and the tibiae were placed in a 70% ethanol solution.

Histology and histomorphometry

Following sacrifice, the tibiae of the calcein-labeled mice were immediately dehydrated in a series of ethanol solutions, and then embedded in polymethylmethacrylate using a previously reported technique [12]. One cross-section was cut perpendicular to the long axis of the bone, two and a half millimeters distal to the tibia-fibular junction. The cross-sections were ground to a thickness of approximately 10 μm and polished with 0.5 μm alumina using a Leco GP-25 Grinder/Polisher (Leco Corp, St. Joseph, MI). The ground and polished sections were stained using mineralized bone stain (MIBS) following a published procedure [13]. Images of the bones were collected using an Optronics digital imaging system that consists of an Olympus camera and Magnafire software (Optronics, Goleta, CA) coupled to a Machintosh G4 computer. Image analysis was performed using Image J software for the Machintosh OSX operating system, developed by the National Institutes of Health (Research Services Branch, NIMH). The

quantitative histological measurements included the following parameters: bone, marrow cavity, and osteoid areas (mm^2), as well as endosteal and periosteal perimeters (mm). The tissue area was calculated as the sum of the bone, marrow, and osteoid areas. The bone volume (BV/TV, %) was calculated as the bone area divided by the tissue area, multiplied by 100 to convert it into percent. Marrow cavity volume (MaV/TV, %) and osteoid volume (OS/TV, %) were calculated the same way. The length and distance of the calcein labels were measured under fluorescent light. The mineral apposition rate (MAR, $\mu\text{m}/\text{day}$) was calculated as the distance between the calcein double labels divided by the days between administration of the calcein. The mineralizing surface (MS) was calculated as the sum of the length of the double labels plus one half of the length of single labels. The labeled surface (%) was calculated by dividing the MS by the bone surface (the sum of the periosteal and endosteal perimeters). The bone formation rate was found with respect to both a bone surface and a bone volume reference. BFR/BA ($\mu\text{m}/\text{day}$) was calculated as the product of MAR and MS, divided by the bone area. BFR/BS ($\mu\text{m}^3/\mu\text{m}^2/\text{day}$) was established using the product of MAR and MS, divided by the bone surface (the bone surface is the sum of the endosteal and periosteal perimeters). All histomorphometry parameters were determined using the guidelines set forth by the American Society of Bone and Mineral Research [14]. Histology and histomorphometry were also performed on one perpendicular cross-section through the sensing element of a strain sensor attached to the femoral diaphysis, and on the trabecular bone in a coronal section cut through the femoral head.

μCT imaging

A Scanco Medical μCT 20 (Scanco, Switzerland) was used to scan the tibias. The trabecular bone in the tibial plateau, extending 0.9 mm below the subcondral plate, was analyzed. In addition to trabecular bone, the cortical bone was analyzed two and a half millimeters distal to the tibia-fibular junction in the same place that histomorphometry was performed on the contralateral tibia. The resolution of the scans was $9 \mu\text{m}$. The trabecular bone was analyzed for bone volume (BV/TV, %), trabecular number (mm^{-1}), trabecular thickness (mm), and trabecular spacing (mm). The cortical bone was analyzed for bone volume (BV/TV, %), cortical bone thickness (mm), and the ratio of bone surface to bone volume (BS/BV, mm^{-1}). In addition, the two-dimensional cortical bone scans were analyzed for bone area (mm^2).

The femoral shafts of the bones tested in three-point bending were also imaged using μCT prior to mechanical testing. The femora were thawed prior to imaging and were wrapped in saline-soaked gauze and then paraffin to prevent dehydration. The femora were scanned at a resolution of $18 \mu\text{m}$. Scanning at this resolution resulted in scan times of less than 12 h. Immediately following acquisition of the μCT image, the femurs were frozen at -20°C until the day they were three-point bend tested. A 0.9 mm section of the mid-diaphysis of the femur was used to measure bone volume, bone thickness, and bone surface to volume ratio. In addition, the second moment of inertia (mm^4) was calculated for the 6.00 mm segment of bone that was loaded in three-point bending.

Cantilever bend testing

On the day of cantilever bend testing, the femora were removed from the freezer and allowed to warm to room temperature prior to additional sample preparation. Cantilever bend testing was conducted according to a previously published procedure [15]. Briefly, one single element EA-06-015DJ-120 strain gauge (Measurements Group Inc., Raleigh, NC) was trimmed to $3.5 \text{ mm} \times 1.5 \text{ mm}$ and attached to the posterior surface of each femur using M-Bond 200 (Measurements Group Inc., Raleigh, NC). The gauges were placed parallel to the long axis of the bone, along a line running from the center of the condyles to a point on the posterior aspect of the proximal femur (Fig. 1A). The sensing element of the gauge was placed approximately $6 \pm 0.3 \text{ mm}$ above the condyles of each femur, near the center of the femoral mid-shaft. The

point at which the loading pin contacted the bone surface was marked on both the tension and compression sides of the bones, and was 10 ± 0.5 mm above the bottom of the condyle. For each test, the femoral condyle was potted in a fixture using a low-melting-point alloy, Cerrobend® (Scottsdale Tool, AZ) that held the specimen perpendicular to the loading pin of the materials testing machine (Series 810 Materials Testing System, MTS Systems Corporation, Minneapolis, MN). The bones were potted such that the Cerrobend® embedded 4 mm of the distal end of each femur (Fig. 1A). Great care was taken when potting the femora to meticulously position the bones so that the alignment of the femora was exactly the same between each test. The femora were loaded four times at a rate of 6.5 N/s to a peak load of 2.5 N with the strain-gauged surface of the bone being placed in compression (Fig. 1A) while load, stroke, and strain measurements were recorded using a custom made virtual instruments written in Labview 5. Measurements were collected through a National Instruments data acquisition board into a Macintosh G5 computer (Apple Computer, Cupertino, CA). The potting fixture was then rotated 180° and strain values were recorded for four tests while the strain-gauged surface was placed in tension.

Following cantilever bend-testing, the femora were embedded in polymethylmethacrylate [12] and one cross-section was taken through the sensing element of each gauge perpendicular to the long axis of the bone. Images were analyzed for the second moments of inertia using a modified version of Image 1.52β, a software package provided by the National Institutes of Health (Research Services Branch, NIMH). The elastic moduli were calculated using $E = Mc / I\varepsilon$, where M is the moment in N-m, c is the distance from the centroid to the strain gauge in meters, I is the second moment of inertia about the bending axis in m^4 , and ε is the strain.

Three-point bend testing

All femora tested in three-point bending were imaged using μ CT prior to testing. On the day of testing, the bones were removed from the freezer and allowed to warm to room temperature. Three-point bending was performed with a support span of 6.0 mm. The bones were loaded at the midshaft of the femur, perpendicular to the long axis of the bone, with the load applied in the anterior to posterior direction (Fig. 1B). The specimens were loaded to failure at a displacement rate of 10 mm/min, while collecting load and ram displacement measurements using the same data acquisition system described for cantilever bend testing. Stiffness (N/mm) was calculated as the slope of the linear elastic portion of the load–displacement curve. The elastic modulus (GPa) was calculated using $E = (FL^3) / (48dI)$, where F is the applied load in Newtons, L is the span of the supports in meters (0.006 m), d is the ram displacement in meters, and I is the second moment of inertia about the bending axis in m^4 . Breaking force (N) was defined as the load at failure. Breaking stress was defined as the stress at failure and was calculated using $\sigma = FLC / 4I$, where F is the force, L is the span of the supports, C is the distance from the centroid to the bone surface in meters, and I is the second moment of inertia about the bending axis.

Bone mineral content analysis

Following μ CT imaging of the cleaned tibiae, the bones were dried for 2 days at 100°C and then weighed. Following this, the tibiae were ashed at 800°C for 8 h. The ashed bones were weighed and then dissolved in 7.19 mL of concentrated HNO_3 for trace metal analysis (Sigma Fluka, St. Louis, MO). The solutions were diluted to 500 mL with deionized water using volumetric glassware. A 1 mL aliquot of the solution was analyzed for calcium using a calcium assay kit (587-M, Sigma Diagnostics, St. Louis, MO). Following preparation of the standard solutions in the kit, the pH of the dissolved bone solution was adjusted to match the pH of the standard solutions prior to determining Ca concentration. A 5 mL aliquot of the dissolved bone solution was used to determine the amount of phosphorus in the samples using an ICP-MS (ELAN DRC-II, Perkin Elmer, Shelton, CT). The standard solutions for the ICP-MS analysis

were prepared using the same concentration of HNO_3 that was used to prepare the experimental samples. All concentrations were calculated as the mass of the element (Ca or P) divided by the dry bone mass and multiplied by 100 to convert into a percent. To test the accuracy and precision of the mineral content analysis, seven aliquots of a standardized bone meal (standard reference material 1486, National Institute of Standards and Technology, Gaithersburg, MD) with known concentrations of Ca and P were prepared and tested as described above. The concentrations of Ca and P that were measured in the bone meal aliquots were within 5% of the values reported by the National Institute of Standards and Technology.

Statistical analysis

Kurtosis was noted in some of the mechanical testing and histological measurements, so determination of statistical significance for all data was carried out using a Mann–Whitney U test ($P < 0.05$).

Results

Histology and histomorphometry

One bone from a Calbindin-D28k KO and one bone from a WT mouse broke during preparation. As such, 5 bones from each type of mouse were analyzed. The histology and histomorphometry for the cortical bone in the femora and tibia (Table 1) of Calbindin-D28k KO mice demonstrate similar changes at both sites. Calbindin-D28k KO mice have significantly increased cortical bone areas (23% and 29.5% higher for the femora and tibiae, respectively) and decreased marrow cavity areas compared to WT mice. This is reflected by a greater bone volume (33% and 12.4% higher for the femora and tibiae, respectively) and decreased marrow cavity volume (27.5% and 32.1% lower for the femora and tibiae, respectively) in the long bones of Calbindin-D28k KO mice. The enlarged bone volume seen in Calbindin-D28k KO mice is primarily due to increased bone on the endosteal surface as is demonstrated by the similar periosteal perimeters and decreased endosteal perimeters when compared to WT mice (16% and 22.9% lower for the femora and tibiae, respectively). Despite the changes in bone area and volume, the dynamic parameters did not differ significantly between the Calbindin-D28k KO and WT mice.

The results for the histology of the trabecular bone were similar to those observed in cortical bone (Table 2). The trabecular bone in the femoral head demonstrated a 32.4% increase in bone volume and a 36.6% decrease in marrow volume (Fig. 2). While the MAR and BFR/BS were not significantly different in the trabecular bone of Calbindin-D28k KO and WT mice, the BFR/BV was significantly reduced in Calbindin-D28k KO mice (Table 2).

μCT imaging

The cortical bone in the femora and tibiae of Calbindin-D28k KO mice demonstrated an increased bone volume, thicker cortical bone, and a decreased bone surface to bone volume ratio compared to WT mice (Fig. 3). The μCT data for the cortical and trabecular bone are summarized in Table 3. The trabecular bone also demonstrated a significantly increased bone volume in Calbindin-D28k KO mice (Fig. 4). In the tibial plateau, the increased bone volume in Calbindin-D28k KO mice was due to a 33.2% increase in trabecular number. However, in the femoral head, the increased bone volume in Calbindin-D28k KO mice was due to a 41.3% increase in trabecular thickness and a 30.7% decrease in the space between the trabeculae, as the number of trabeculae was 20.5% lower in the femoral head of Calbindin-D28k KO mice (Table 3).

Cantilver bend testing

The stress-strain curves generated while loading the femora were all linear, showing r^2 values in excess of 0.9513 in all tests. This indicated that the bones of both WT and Calbindin-D28k KO mice respond to loading in a linear elastic fashion. The strains generated in the femora during cantilever loading were 40.7% and 37% lower in Calbindin-D28k KO bones compared to the bones of WT mice, during cantilver loading with the gauged surface of the bone in compression and tension, respectively (Table 4). This indicates that bones in Calbindin-D28k KO mice are stiffer than WT mice. This difference is likely due to the distribution of the bone, as the Calbindin-D28k KO mice had a significantly larger second moment of inertia about the bending axis, but a similar elastic modulus (Table 4).

Three-point bend testing

The bones of the Calbindin-D28k KO mice demonstrated more than a 100% increase in both stiffness and failure load in three-point bending (Table 4). The average second moments of inertia, determined using μ CT imaging, in the bones tested in three-point bending were similar to the moments determined from optical images of cross-sections of the contralateral bones tested in cantilver bending ($P = 0.4$). The failure stress of the bones and the elastic moduli were not significantly different between the Calbindin-D28k KO and WT mice in three-point bending (Table 4). The elastic modulus measured in three-point bending was significantly lower than the elastic modulus measured in cantilever bending ($P < 0.001$).

Bone mineral content analysis

Despite having a significantly higher % ashed weight, the amount of calcium and phosphorus was not significantly different between the WT and Calbindin-D28k KO mice (Table 5).

Discussion

The significantly altered histological and biomechanical properties of bones in Calbindin-D28k KO mice provide the first direct evidence that Calbindin-D28k KO mice have a quantitative difference in bone distribution and stiffness compared to WT mice. It should be noted that the mice used in this study were 90 days old. Since mice are not skeletally mature until 4 months [16], these mice were still growing and their bones have not reached steady state. This leaves open the possibility that further changes in the histological and biomechanical properties of the bones can occur prior to the mice reaching skeletal maturity. Although this possibility exists, the point of this study was to quantify the histological and biomechanical properties of the bones of Calbindin-D28k KO mice at this commonly studied time point because prior analysis of the bones in Calbindin-D28k KO mice was qualitative. This time point was chosen specifically because previous studies that have characterized the bones in Calbindin-D28k KO mice have been done in growing mice [6,11], and this provided information about the properties of mice used in published studies.

Previous studies [1,11] have indicated that the bones in Calbindin-D28k KO mice are grossly similar to WT mice and there are no abnormalities in serum Ca or PTH in Calbindin-D28k KO mice. The results of this study are in agreement with those studies showing that the bones of Calbindin-D28k KO and WT mice are grossly the same dimensions and weight. However, quantification of the bone structure using both histology and μ CT measurements demonstrated an increased amount of bone in Calbindin-D28k KO mice. In cortical bone, the additional bone is located on the endosteal surface (indicated by the decreased endosteal perimeter). The similar periosteal perimeters of Calbindin-D28k KO and WT mice explain why the bones appear grossly similar.

The change that results in an increased cortical bone volume also occurs in trabecular bone as noted in both the femoral head and tibial plateau, and indicates the systemic nature of the histological increase in bone volume. Despite the apparent systemic increase in bone volume, there appears to be a difference in the way in which the increased trabecular bone volume is distributed in different sites. In the femoral head of Calbindin-D28k KO mice, an increase in the thickness of the trabeculae and decreased spacing between them resulted in higher bone volume despite a reduction in the number of trabeculae. In the tibial plateau of Calbindin-D28k KO mice, the increased bone volume is primarily due to an increased number of trabeculae and the trabecular thickness was not significantly different. This difference in trabecular structure may be due to the different strain environments in the different sites, as the amount of load passing through the femoral head is similar to that passing through the tibial plateau, but the load is distributed over a larger area in the tibia.

Histomorphometry did not demonstrate a difference in the cortical bone formation rates. However, one of the bone formation rates was significantly reduced (the trabecular bone of the femur). This decreased bone formation was not due to a difference in the MAR or the amount of labeled surface. Since the bone volume referent of BFR was decreased (BFR/BV) and the BFR/BS was not decreased, the decreased BFR was likely due to the significantly increased trabecular bone volume in Calbindin-D28k KO mice. The decreased BFR/BV seen in the trabecular bone is in agreement with a previous study that showed reduced serum osteocalcin levels in Calbindin-D28k KO mice [6]. There is no standard method to measure bone resorption rates using histomorphometry; however, it can sometimes be inferred from other histological parameters such as marrow cavity area [14,17]. The decreased marrow cavity volume in the cortical bone of Calbindin-D28k KO mice supports the idea that bone resorption may be impaired. Since the cortical bone formation rate was not affected in Calbindin-D28k KO mice, the increased bone volume may be related to impaired bone resorption.

The measurements taken during cantilever and three-point bend testing demonstrated that Calbindin-D28k KO mice have stiffer bones than WT mice. This results in functionally improved bones, as the failure load was more than doubled in Calbindin-D28k KO mice. Both cantilever bend tests and three-point bending demonstrated significant changes in the extrinsic properties of the bones, such as compliance, stiffness, and failure load. These changes are due to the increased amount of bone and the distribution of the bone, as the second moment of inertia about the bending axis is increased in Calbindin-D28k KO mice. Furthermore, there were no significant changes noted in the intrinsic properties of the bones, such as elastic modulus and failure stress, in either cantilever or three-point bending.

Although similar differences between the KO and WT mice were noted in the two types of biomechanical tests, the value measured for the elastic modulus was significantly higher in cantilever bending. The application of a strain gauge to the mouse femur may have caused a stiffening effect that resulted in an overestimation of the elastic modulus in the cantilever bend tests. In addition, the preparation time required to correctly position and attach a strain gauge to the bone may have resulted in some dehydration, which also raises the elastic modulus of bone [18]. This effect was minimized by keeping the bones wrapped in saline-soaked gauze whenever possible; however, gluing the strain gauge to the bone requires drying the surface and the process allowed some time for the bones to dehydrate. While the application of a strain gauge to the bone and potential dehydration have a stiffening effect and result in the overestimation of the elastic modulus, the use of three-point bending in mouse bones results in an overestimation of strain and an underestimation of elastic modulus [18]. Previous studies comparing the elastic modulus of rat bones in three-point bending using ram displacement measurements or a glued strain gauge found that the elastic modulus calculated using ram displacement was 7.6 GPa, while the elastic modulus found using the glued strain gauge was 29.4 GPa [18]. This is similar to the differences noted in this study between the elastic moduli

calculated in cantilever bending using a glued strain gauge, and three-point bending using ram displacement measurements. The true values for the measured biomechanical parameters likely lie between the values measured in cantilever bending and three-point bending. However, since both techniques demonstrated similar changes, the differences noted in the mechanical properties between the Calbindin-D28k KO and WT mice are valid.

This change in stiffness resulting from changes in bone distribution instead of differences in intrinsic material properties is also supported by the similarity of the amounts of calcium and phosphorus noted in the bones of Calbindin-D28k KO and WT mice in this study. Although we did not find a significant change in calcium content in this study, a previous study demonstrated a decreased amount of calcium in the bones of Calbindin-D28k KO mice [11]. This discrepancy is accounted for by the methods of analysis, which determined the amount of calcium per weight in the bones, while the study demonstrating less calcium found a lower amount per volume [11]. Since the volume of the bones is significantly higher in Calbindin-D28k KO mice, the amount of calcium per volume will be lower. For this reason, determination of the calcium content by weight provides an indication that the calcium content in the bone material of Calbindin-D28k KO mice is unaffected.

Calbindin-D28k plays an important role in regulating bone volume and stiffness. Recent studies have demonstrated that Calbindin-D28k acts as both a calcium buffer and a calcium sensor [19,20] that can regulate the activity of *myo*-inositol-monophosphatase. In Calbindin-D28k KO mice, it was noted that purkinje neurons, where Calbindin-D28k is normally expressed, had higher amounts of free cytoplasmic calcium following neuronal excitation [1]. Further evidence in vivo [21] and in vitro [5] have indicated that Calbindin-D28k is expressed by cells producing mineralized matrix, and since Calbindin-D28k is expressed in osteoblasts, a situation similar to purkinje neurons may exist where the amount of calcium within osteoblasts following a stimulus may be higher in the osteoblasts of Calbindin-D28k KO mice. This leaves open the possibilities that the increase in bone volume and stiffness may be due to changes in intracellular signaling within osteoblasts at the level of the bone. Further studies are needed to elucidate the mechanism by which stiffness, failure load, and bone volume increase in these knockout mice. The fact that limiting the function of Calbindin-D28k results in only minor changes in the phenotype of the mouse [1] and that levels of blood calcium and PTH are not significantly altered [11], yet the volume and failure load of bone are significantly improved, makes this protein a good target for future research examining treatments for osteoporosis.

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Fig. 1.
(A) Calbindin-D28k knockout mouse femur with a strain gauge attached to the posterior surface being loaded in a cantilever bend test. (B) A WT mouse femur being loaded in three-point bending.

Fig. 2. Cross-sectional images through the femoral head. The WT mouse (left) has less trabecular bone volume compared to the CDKO mouse (right). Scale bar = 1 mm.

Fig. 3.
μCT images of the tibia from a WT (top) and CDKO (bottom) mouse. Note the similar periosteal perimeters in both bones and the decreased endosteal perimeter in CDKO mice. Scale bar = 1 mm.

Fig. 4. μ CT images of the trabecular bone (dark grey) inside the tibial plateau (light grey) of a WT (left) and CDKO (right) mouse demonstrating the increased bone volume in CDKO mice. Scale bar = 1 mm.

Table 1

Histology and histomorphometry of cortical bone^a

	Wild type	Calbindin KO	% change	P <
Femora	Cross-sectional cortical bone area (mm ²)	1.109 ± 0.092	↑ 23.0	0.01
	Periosteal perimeter (mm)	5.427 ± 0.190	↑ 3.6	NS (0.4)
	Endosteal perimeter (mm)	3.397 ± 0.278	↓ 16.0	0.05
	BV/TV (%)	60.4 ± 3.1	↑ 33.0	0.01
	MaV/TV (%)	39.5 ± 3.1	↓ 27.5	0.01
	OsV/TV (%)	0.1 ± 0.1	0	NS (0.8)
	Labeled surface (%)	34.9 ± 4.7	↑ 17.1	NS (0.2)
	Mineral apposition rate (µm/day)	1.522 ± 0.392	↓ 11.4	NS (0.3)
	BFR/BS (µm ³ /µm ² /day)	0.527 ± 0.147	↑ 2.3	NS (0.8)
	BFR/BA (µm/day)	3.90 ± 1.24	↓ 28.8	NS (0.2)
Tibiae	Cross-sectional cortical bone area (mm ²)	0.847 ± 0.018	↑ 29.5	0.01
	Periosteal perimeter (mm)	3.797 ± 0.084	↑ 7.5	NS (0.1)
	Endosteal perimeter (mm)	1.669 ± 0.070	↓ 22.9	0.01
	BV/TV (%)	72.0 ± 4.8	↑ 12.4	0.05
	MaV/TV (%)	27.7 ± 4.8	↓ 32.1	0.05
	OsV/TV (5%)	0.3 ± 0.3	0	NS (0.7)
	Labeled surface (%)	38.2 ± 18.2	↑ 36.4	NS (0.2)
	Mineral apposition rate (µm/day)	0.938 ± 0.201	↑ 14.4	NS (0.7)
	BFR/BS (µm ³ /µm ² /day)	0.494 ± 0.187	↑ 56.3	NS (0.2)
	BFR/BA (µm/day)	3.19 ± 1.22	↑ 17.7	NS (0.7)

^aData are expressed as the mean ± standard deviation.

Table 2

Histology and histomorphometry of trabecular bone^a

	Wild type	Calbindin KO	% change	P <
Femoral head	BV/TV (%)	68.2 ± 5.3	↑ 32.4	0.01
	MaV/TV (%)	29.1 ± 6.1	↓ 36.6	0.01
	OsV/TV (5%)	2.7 ± 1.4	0	NS (0.7)
	Labeled surface (%)	44.2 ± 6.0	↓ 12.5	NS (0.5)
	Mineral apposition rate (µm/day)	1.274 ± 0.322	↓ 16.1	NS (0.3)
	BFR/BS (µm ³ /µm ² /day)	0.362 ± 0.100	↓ 22.3	NS (0.5)
	BFR/BA (µm/day)	5.25 ± 2.08	↓ 49.9	0.05

^aData are expressed as the mean ± standard deviation.

Table 3

μ CT imaging^a

		Wild type	Calbindin KO	% change	P <
Cortical bone	Tibial shaft	0.672 ± 0.078	0.828 ± 0.117	↑ 23.2	0.05
		70.3 ± 4.9	80.4 ± 2.1	↑ 14.4	0.05
		0.244 ± 0.019	0.299 ± 0.010	↑ 22.5	0.01
		8.22 ± 0.61	6.68 ± 0.23	↓ 18.7	0.01
Femoral shaft		0.925 ± 0.071	1.346 ± 0.098	↑ 45.5	0.005
		46.8 ± 3.5	59.0 ± 2.4	↑ 26.1	0.005
		0.208 ± 0.015	0.303 ± 0.011	↑ 45.7	0.005
		9.64 ± 0.64	6.59 ± 0.25	↓ 31.6	0.005
Trabecular bone	Tibial plateau	24.7 ± 4.9	35.2 ± 3.1	↑ 42.5	0.05
		6.75 ± 0.85	8.99 ± 0.94	↑ 33.2	0.05
		0.056 ± 0.004	0.062 ± 0.006	↑ 10.7	NS (0.1)
		0.150 ± 0.017	0.120 ± 0.017	↓ 20.0	NS (0.1)
Femoral head		70.4 ± 7.0	87.6 ± 1.2	↑ 24.4	0.01
		19.74 ± 1.36	15.69 ± 0.81	↓ 20.5	0.01
		0.080 ± 0.011	0.113 ± 0.006	↑ 41.3	0.01
		0.039 ± 0.005	0.027 ± 0.002	↓ 30.8	0.01

^aData are expressed as the mean ± standard deviation.

Table 4

Mechanical test results^a

	Wild type	Calbindin KO	% change	P <
Cantilever bending	Compliance in compression (μ strain/kg)	4135.1 $\pm$ 1266.0	$\downarrow$ 40.7	0.05
	Compliance in tension (μ strain/kg)	4201.4 $\pm$ 1013.6	$\downarrow$ 37.0	0.05
	$I_{\text{Anterior-Posterior}}$ through strain gauge (mm^4)	0.32 $\pm$ 0.07	$\uparrow$ 21.9	NS (0.2)
	$I_{\text{Medial-Lateral}}$ through strain gauge (mm^4)	0.20 $\pm$ 0.04	$\uparrow$ 53.8	0.05
Three-point bending	Elastic modulus in compression (GPa)	28.8 $\pm$ 9.1	$\uparrow$ 53.2	NS (0.1)
	Elastic modulus in tension (GPa)	28.0 $\pm$ 8.9	$\uparrow$ 40.0	NS (0.1)
	Stiffness (N/mm)	165.5 $\pm$ 24.2	$\uparrow$ 100.8	0.005
	Failure load (N)	31.6 $\pm$ 2.1	$\uparrow$ 110.7	0.005
	$I_{\text{Anterior-Posterior}}$ femoral diaphysis (mm^4)	0.41 $\pm$ 0.09	$\uparrow$ 24.2	NS (0.2)
	$I_{\text{Medial-Lateral}}$ femoral diaphysis (mm^4)	0.23 $\pm$ 0.04	$\uparrow$ 64.3	0.01
	Failure stress (MPa)	91.6 $\pm$ 23.5	$\uparrow$ 14.2	NS (0.5)
	Elastic modulus (GPa)	4.2 $\pm$ 1.6	$\uparrow$ 61.5	NS (0.6)

^aData are expressed as the mean $\pm$ standard deviation.

Table 5Bone mineral content analysis of tibiae^a

	Wild type	Calbindin KO	% change	<i>P</i> <
Weight (dry, mg)	37.30 ± 3.86	42.26 ± 5.86	↑ 13.3	NS (0.2)
Ashed weight percent	59.7 ± 0.6	62.2 ± 0.6	↑ 4.2	0.01
Calcium (%)	26.2 ± 3.84	30.27 ± 8.02	↑ 15.5	NS (0.3)
Phosphorus (%)	10.33 ± 1.87	11.87 ± 2.47	↑ 14.9	NS (0.4)

^aData are expressed as the mean ± standard deviation.