Deletion of a Single β-Catenin Allele in Osteocytes Abolishes the Bone Anabolic Response to Loading

Behzad Javaheri,1 Amber Rath Stern,2,3,4 Nuria Lara,2 Mark Dallas,2 Hong Zhao,2 Ying Liu,2 Lynda F Bonewald,2 and Mark L Johnson2

1The Royal Veterinary College, London, UK
2UMKC School of Dentistry, Department of Oral and Craniofacial Sciences, Kansas City, MO, USA
3UMKC School of Computing and Engineering, Department of Civil and Mechanical Engineering, Kansas City, MO, USA
4Engineering Systems Incorporated, Charlotte, NC, USA

ABSTRACT

The Wnt/β-catenin signaling pathway is essential for bone cell viability and function and for skeletal integrity. To determine if β-catenin in osteocytes plays a role in the bone anabolic response to mechanical loading, 18- to 24-week-old osteocyte β-catenin haploinsufficient mice (Dmp1-Cre × β-catenin fl/++; HET cKO) were compared with their β-catenin fl/fl (control) littermates. Trabecular bone volume (BV/TV) was significantly less (58.3%) in HET cKO females versus controls, whereas male HET cKO and control mice were not significantly different. Trabecular number was significantly less in HET cKO mice compared with controls for both genders, and trabecular separation was greater in female HET cKO mice. Osteoclast surface was significantly greater in female HET cKO mice. Cortical bone parameters in males and females showed subtle or no differences between HET cKO and controls. The right ulnas were loaded in vivo at 100 cycles, 2 Hz, 2500 μe, 3 days per week for 3 weeks, and the left ulnas served as nonloaded controls. Calcein and alizarin complexone dihydrate were injected 10 days and 3 days before euthanization, respectively. Micro-computed tomography (μCT) analysis detected an 8.7% and 7.1% increase in cortical thickness in the loaded right ulnas of male and female control mice, respectively, compared with their nonloaded left ulnas. No significant increase in new cortical bone formation was observed in the HET cKO mice. Histomorphometric analysis of control mice showed a significant increase in endocortical and periosteal mineral apposition rate (MAR), bone-formation rate/bone surface (BFR/BS), BFR/BV, and BFR/TV in response to loading, but no significant increases were detected in the loaded HET cKO mice. These data show that deleting a single copy of β-catenin in osteocytes abolishes the anabolic response to loading, that trabecular bone in females is more severely affected and suggest that a critical threshold of β-catenin is required for bone formation in response to mechanical loading. © 2014 American Society for Bone and Mineral Research.

KEY WORDS: β-CATENIN; MECHANICAL LOADING; OSTEOCYTE; BONE FORMATION

Introduction

Bone adapts its mass and architecture in response to mechanical loading, but the exact cellular mechanisms that mediate this adaptive response are not fully understood. Studies of human mutations that result in altered bone mass phenotypes first suggested an important role for the Wnt/β-catenin signaling pathway in bone mass accrual1–3 and the possible role of this pathway in bone response to mechanical loading.4 There is now clear evidence that Wnt/β-catenin signaling is one of the pathways that is activated and involved in bone cells’ response to mechanical load.5–13 We have proposed a model in which osteocytes initially activate β-catenin signaling in response to mechanical loading in part through an Akt-dependent–Lrp5/6-independent mechanism.14 In vitro evidence using osteoblastic and osteocytic cells supports this model.13,14,17

The Wnt/β-catenin signaling pathway also regulates basal bone mass through a number of different mechanisms including renewal of stem cells,18 stimulation of osteoblast differentiation and proliferation,19 enhancement of osteoblast activity,19,20 and inhibition of osteoblast and osteocyte apoptosis.21 Conditional deletion of β-catenin in osteoblasts in vivo using alpha 1 type I collagen Cre22 or osteocalcin-Cre23 showed that osteoblast regulation of osteoclast differentiation was dependent on Wnt/β-catenin signaling. The mechanism underlying the fragile bone phenotype in these mice is apparently owing to increased osteoclastic bone resorption as a result of decreased expression of osteoprotegerin.22,23 Interestingly, mice with targeted deletion in osteocytes are born normal but display

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Address correspondence to: Mark L Johnson, PhD, Department of Oral and Craniofacial Sciences, UMKC School of Dentistry, 650 East 25th Street, Kansas City, MO 64108, USA. E-mail: johnsonmark@umkc.edu
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progressive bone loss with age accompanied by growth retardation and premature lethality. Cancellous bone mass is almost completely lost and cortical bone thickness is severely reduced. The low bone mass correlates with increased osteoclast number and activity owing to an increased osteocyte receptor activator of NF-κB ligand/osteoprotegerin (RANKL/OPG) ratio. Given that the osteocyte is now recognized as an important source of RANKL, in bone, this raises the intriguing question of how much of the observed phenotype in the osteoblast targeted deletions of β-catenin might have been the result of osteocyte loss of β-catenin signaling in those mice.

The osteocyte is widely believed to be the main mechanosensory cell in bone, but there is little in vivo evidence demonstrating a clear role for the β-catenin signaling pathway in osteocytes with regards to anabolic loading–induced bone formation. The targeted homozygous deletion of β-catenin in osteocytes results in a severely fragile skeleton, and these mice do not live much beyond 8 weeks of age, making it impossible to perform in vivo mechanical loading studies with these mice. However, the skeletal phenotype of the heterozygous mice with the loss of a single β-catenin allele is relatively normal at 2 months of age with the exception that cancellous bone volume is decreased by about 10% in heterozygous male and about 25% in heterozygous female mice relative to control littermates.

Therefore, to determine whether β-catenin is required for the anabolic bone-formation response to mechanical loading, we conditionally deleted a single allele of β-catenin in osteocytes (designated HET cKO) by crossing β-catenin fl/fl (designated control) mice with Dmp1-Cre mice. We report our findings on the characterization of the adult skeleton and response to anabolic loading of these heterozygous mice and their fl/fl control littermates at 18 to 24 weeks of age. Our loading studies demonstrate that deletion of a single allele of β-catenin in osteocytes abolishes anabolic load-induced new bone formation. Our studies also revealed major gender differences in trabecular bone phenotypes in HET cKO mice with a more severe effect of osteocyte β-catenin haploinsufficiency observed in females.

### Materials and Methods

#### Mouse lines and breeding

The β-catenin fl/fl (exon 2–6) mice (control) were purchased originally from Jackson Laboratories (B6.129-Catnb1tm2Kem/J; Jackson Laboratories, Bar Harbor, ME, USA). The β-catenin fl/fl mice were crossed with the 10Kb Dmp1-Cre mice to produce mice with a heterozygous deletion of β-catenin in osteocytes. All mice were 18 to 24 weeks old. The studies described in this article were approved by the UMKC Institutional Animal Care and Use Committee.

#### Tartrate-resistant acid phosphatase (TRAP) staining

Femurs from male (n = 3) and female (n = 3) β-catenin fl/fl (control) and HET cKO (male n = 3 and female n = 5) were excised, cleaned of soft tissue, placed in ice-cold 4% paraformaldehyde for 24 hours, and decalcified in EDTA for 1 week. Paraffin-embedded histological sections were stained for TRAP. TRAP-positive osteoclasts were quantified using the OsteoMeasure Histomorphometry System (Osteometrics Inc., Decatur, GA, USA) in a standard zone of 1 mm starting 200 μm below the growth plate (to exclude primary spongiosa) and 100 μm from the cortical bone (to avoid trabecular area associated with cortical bone). Osteoclast numbers were expressed as N.Oc/BPm and Oc.S/BS according to accepted histomorphometric standards.

### High-resolution micro-computed tomography (μCT)

μCT analysis of femur/tibia (n = 7 for male and female controls and male HET cKO; n = 9 for female HET cKO) for baseline characterization and right/left ulnas for loading studies (n = 6 for male and female controls and n = 5 for male and female HET cKO) was conducted using a Scanco vivCT40 (Scanco Inc., Basel, Switzerland) at a resolution of 10 μm for femurs and tibia and 15 μm for ulna, 55 kV voltage, 145 μA current, and 200 ms integration time following recommended guidelines. Analysis was performed on postmortem femurs/tibias that were fixed in 70% ethanol or once embedded in plastic in the case of the ulnas. Using our standard approach for adult bones, a high-resolution scan of the region of interest was performed. For ulna, the 1 mm between the 2- to 3-mm distal to midshaft was analyzed. The threshold used for trabecular and cortical bone was set to 300 and 350, respectively. The region of analysis is shown in Fig. 1 and comprised 30 slices of secondary spongiosa beginning just beneath the region of primary spongiosa. Three-dimensional images were generated using the following values: gaussian filter: σ = 0.8; support = 1; and threshold as noted above. Three-dimensional analyses were performed to determine bone volume/tissue volume, trabecular number, trabecular thickness, and trabecular separation at the proximal femur and distal tibia. Cortical bone was measured at the midshaft region of the bone. Quantitation of BMD of the bone samples was determined by comparison against a hydroxyapatite standard (Scanco Inc.) that was scanned weekly for instrument calibration.

#### Strain gaging and in vivo loading

For ex vivo strain gaging analysis, the gages were applied to the ulnas at 2 mm distal to the ulna midshaft on the medial surface and at a site 5 mm distal to the end of the olecranon process on the lateral surface. Uniaxial strain gages (EA-06-015DJ-120-option, Vishay Micro-Measurements, Raleigh, NC, USA) were glued (Bond 200 kit, Vishay Micro-Measurements) to the lateral surface and parallel to the longitudinal axis. The forearms (n = 3 per gender and genotype) were placed in the loading system (EnduraTEC ELF 3200 Loading device, BOSE Corp., Minnetonka, MN, USA) and the device cycled at least four times to ascertain that a stable waveform was obtained before starting data collection. Strain measurements were made using an electronic bridge conditioner Model 7000-32-SM (Vishay Micro-Measurements) and digitally displayed using StrainSmart software (Vishay Micro-Measurements). After application of strain gages, loading was conducted on the right forearm at 2 Hz, using a haversine waveform for 15 cycles. Loading was conducted at −0.5, −1, −1.5, −2, −2.5, −3, and −3.5 N for the intact forearm. Strains in the last five cycles were averaged.

In vivo loading was performed on the right ulna of each mouse under compression using the EnduraTEC ELF 3200 Loading device (BOSE Corp.). Each loading session was conducted for 100 cycles at 2 Hz. The magnitude of load applied was 2.25 N, sufficient to produce a global strain of 2500 με. The left ulnas serve as a contralateral nonloaded control. Loading was performed 3 days per week (MWF) for 3 weeks.

#### Bone-formation analysis

During the 3-week loading regimen, mice were injected with calcine (5 mg/kg) and alizarin red (20 mg/kg) (Sigma Chemical...
Fig. 1. Bone phenotype of control and HET cKO femurs. (A) Representative 3D μCT images of femoral trabecular bone. Ex vivo high-resolution analyses of distal femurs to determine (B) trabecular bone mineral density (BMD), (C) trabecular bone volume/total volume (BV/TV), (D) trabecular number, (E) trabecular thickness, (F) trabecular separation, (G) percentage ash content, (H) cortical BMD, (I) cortical BV/TV, and (J) cortical thickness in control (white bars) and HET cKO mice (gray bars). (B–G) Bar graphs represent means plus SD. Group sizes were n = 7 for male and female controls and male HET cKO mice; n = 9 for female HET cKO mice. Statistical comparisons: a, p < 0.05 between male and female of same genotype; b, p < 0.05 comparing control with HET cKO.
Company, St. Louis, MO, USA) 10 and 3 days, respectively, before euthanization and collection of bone samples. The forearms were dissected, fixed in 4% paraformaldehyde (Alfa Aesar Inc., Ward Hill, MA, USA), methyl methacrylate embedded, and sectioned at a thickness of 20 μm. Sections were taken from the blocks containing bone 3 mm distal to midshaft. Standard bone histomorphometry was performed using the OsteoMeasure Histomorphometry System. This software follows ASBMR nomenclature for bone histomorphometry. Mineral apposition rate (MAR) and bone-formation rate (BFR) on the endocortical (Ec) and periosteal (Ps) surfaces were calculated from comparable sections obtained in the midshaft region of the loaded and nonloaded ulnas. BFR was normalized against either the bone surface (BS), bone volume (BV), or the tissue volume (TV) as determined by the software per standard convention. Ps and Ec single-label (SL) surfaces (S) were also determined as a function of total bone surface.

Ash content
The right and left femurs were processed to determine final mineral content by measurement of ash fraction. Dry mass was obtained by drying in an oven at 100°C for 24 hours followed by 600°C for 24 hours in a muffle furnace. Ash weight was measured, and ash content was expressed as percentage of dry ash mass/dry mass.

Three-point bending to failure
Displacement-controlled three-point bending to failure tests were conducted on tibias and femurs ex vivo to characterize the biomechanical properties of the bones. Tibias and femurs were harvested from freshly euthanized mice. The associated soft tissue was removed, and the bones were individually wrapped in saline-soaked gauze and stored at −20°C until testing. Before biomechanical testing, samples were allowed to thaw and reach room temperature. Samples were kept hydrated with saline-soaked gauze and not allowed to dry out before or during testing. Span lengths of 7.6 mm were used for the samples, and the bones were placed into the fixture so they would be impacted in what would be the anterior to posterior direction in vivo. A crosshead displacement rate of 0.1 mm/s was used. Crosshead displacement and axial load were recorded at a rate of 70 Hz. The stiffness and ultimate force were calculated from the resulting load versus displacement curves for each bone. The Young’s modulus (E) for each bone was calculated using the following equation:

$$E = \frac{S/I}{48I}$$

where S is the stiffness, I is the span length, and I is the area moment of inertia. The area moment of inertia was calculated across the midspan at the fracture location using 10 slices midspan of the realigned μCT scans using the BoneJ plug-in for the image-processing program ImageJ (BoneJ, NIH, Bethesda, MD, USA).

Statistical analysis
Two-way analysis of variance (ANOVA) was used to investigate the effect of gender and treatment. A least square t test was applied to identify significant differences between control and HET cKO within the same gender and to test for significance between female and male within each treatment level. A repeated measurements test was utilized to investigate the effect of treatment (parameter measured) and loading and gender in which loading was designated as the within-subject variable with two levels (load and nonload) and two between-subject variables: gender and treatment. If a significant interaction was detected, then a simple effect test was applied to identify the significant treatment effect within the same experimental condition. Data analysis was performed with SPSS (Statistical Package for Social Science, version 20; SPSS Inc., Chicago, IL, USA). A p < 0.05 was considered significant.

Results
Basal bone properties of HET cKO mice
Homozygous deletion of β-catenin in osteocytes results in a skeleton that is too frail to withstand in vivo forearm loading. We, therefore, used a heterozygous deletion of β-catenin strategy in which β-catenin fl/fl mice were crossed with the 10kb Dmp1-Cre mice, which results in the loss of one allele of β-catenin principally in osteocytes (HET cKO). These mice are long-lived and able to undergo forearm compression loading without evidence of fracture. μCT analysis revealed no significant differences (ns) in femoral trabecular bone mineral density (BMD) (Fig. 1B) or cortical BMD (Fig. 1H), although percentage ash content was lower in the HET cKO mice compared with their gender controls (p < 0.05). Three-dimensional images (Fig. 1A) show decreased trabecular content in female HET cKO mice, and quantitative analysis demonstrated that femoral trabecular BV/TV was 58.3% less (p < 0.05) in HET cKO females and 19.1% less (not significant, ns) in HET cKO males compared with male controls (Fig. 1C). Both control and HET cKO females showed lower BV/TV (p < 0.05) compared with their male counterparts (Fig. 1C). Significant decreases in trabecular number were observed in both genders with females significantly lower than their male counterparts (Fig. 1D). Trabecular separation was significantly (p < 0.05) increased in females versus males and in female HET cKO versus female controls (Fig. 1F). Female HET cKO mice also had significantly decreased trabecular thickness compared with male HET cKO. Female cortical HET cKO BV/TV was significantly lower compared with female control mice, but cortical thickness was not different (Fig. 1F). Similar findings were observed in the μCT analysis of the proximal tibia (Supplemental Fig. S1).

Histological analysis (Fig. 2) revealed a significant increase in the number of TRAP-positive osteoclasts in female HET cKO compared with male HET cKO and to female control (p < 0.05) (Fig. 2B). Osteoclast surface per total bone surface in female HET cKO was significantly higher compared with female control and male HET cKO (p < 0.05) (Fig. 2C).

Biomechanical properties of osteocyte β-catenin HET cKO mice
We next characterized the biomechanical properties of the femurs and tibias from these mice using ex vivo three-point bending to failure. The stiffness, ultimate force, and Young’s modulus were determined. There were no significant differences found between the control and the HET cKO mice for the femurs and tibias isolated from either males or females (Fig. 3 and Supplemental Fig. S2). We observed a statistically significant increase in the femoral Young’s modulus (Fig. 3) in the female mice compared with their male counterparts (p < 0.05) but no
difference in the HET cKO mice of either gender compared with their gender controls. Tibial mechanical properties showed reduced ultimate load in female mice compared with their male counterparts.

In vivo bone-formation response to loading

Before mechanical loading, the strain:load relationship of the ulnas from HET cKO and control mice was determined using strain gage measurements. As shown in Fig. 4, there were no statistically significant differences in the strain:load curves between males and females and/or between HET cKO compared with control ulnas. A load of 2.25 N was applied to the right forearms of all mice producing a global strain level of \( \sim 2500 \mu \varepsilon \). As expected, there was a significant increase in new bone formation in the control groups in the loaded compared with the nonloaded ulnas of the control group (Fig. 6A, B and Table 1). In contrast to controls, male and female HET cKO loaded ulnas’ MAR and BFRs on both the endocortical and periosteal surfaces were not significantly different from the nonloaded ulnas (Fig. 6A, B and Table 1). µCT analysis of loaded and nonloaded ulnas obtained at the end of the 3-week loading regimen also demonstrated significant increases in control mice cortical thickness (8.7% in males and 7.1% in females) at the midshaft of the ulna corresponding to the region assessed by dynamic histomorphometry (Fig. 6C and Supplemental Fig. S3).

Discussion

The Wnt/β-catenin signaling pathway plays an important role in bone mass regulation. In addition, the osteocytes play an important role in the regulation of osteoblast and osteoclast activity and their role in mechanosensation and responsiveness in bone. Previous work by Kramer and colleagues demonstrated that homozygous deletion of β-catenin targeted to osteocytes resulted in a severely compromised skeleton by 5 to 8 weeks of age, whereas the deletion of a single allele had little or no effect at this young age. Therefore, to better understand the role of β-catenin in the adult skeleton, we examined the basal bone phenotypes and new bone formation response to anabolic loading in male and female mice, lacking a single allele of β-catenin in osteocytes.
Characterization of the basal bone phenotype revealed major gender differences as a result of the targeted deletion of a single allele of β-catenin in osteocytes in trabecular bone of female compared with male mice yet no differences in cortical bone. We performed μCT measurements on 30 slices proximal to the primary spongiosa of the distal femur and immediately distal to the primary spongiosa of the proximal tibia. Trabecular BV/TV was significantly reduced in female mice by 58.3% (p < 0.05), whereas trabecular BV/TV in male mice was decreased 19.1% but was not statistically significant from controls (p = 0.745). However, the 3D images suggest that there is less trabecular content in the male HET cKO toward the femur midshaft but is still less evident compared with female mice. The data from Kramer and colleagues\(^24\) indicated a ~25% reduction in cancellous bone volume in female mice and a nonsignificant reduction in male mice at 8 weeks of age. Thus, our data indicate that the trabecular bone phenotype of the HET cKO mice continues to progressively worsen with aging. The decrease in trabecular BV/TV appears to be the result of increased osteoclast activity as observed by histological analysis, supporting the concept that β-catenin in osteocytes regulates osteoclastogenesis.\(^{25-27}\) Given that RANKL expression in the osteocyte is also regulated by Wnt/β-catenin signaling,\(^{22,23}\) our findings are consistent with this concept. Importantly,
overall BV/TV was significantly higher in male mice compared with their female counterparts in both control and HET cKO genotypes.

We did not observe a significant difference in trabecular or cortical bone mineral density (material density by μCT) in these mice at 18 to 24 weeks of age compared with controls. We interpret this as indicating that the bone that is being made has the same material density in HET cKO mice versus controls, but the reduction in BV/TV indicates there is less bone present. The decrease in percentage of ash content suggests that there is overall modestly reduced mineral in the combined cortical and trabecular compartments, which is likely because of reductions in the trabecular compartments.

Analysis of the biomechanical properties by three-point bending of control and HET cKO mice showed no significant differences in male or female mouse femurs and their controls. The control female group had a significantly higher Young’s modulus compared with male control mice, which was not evident in the HET cKO group. Perhaps this relates to the greater decrease in BV/TV in female versus male HET cKO mice. The fact that the biomechanical properties were not different between male versus female HET cKO in female versus male HET cKO mice. The deletion of a single allele of β-catenin in osteocytes mainly appears to affect trabecular bone at 18 to 24 weeks of age. It will be interesting to determine

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**Fig. 5.** β-catenin heterozygous mice have decreased response to loading. Representative images of double fluorochrome labeling using calcein and alizarin red of loaded and nonloaded ulnas 3 mm distal to the midshaft region. (A) Male control and HET cKO mice. (B) Female control and HET cKO mice. Right top corner shows a magnification of similar regions to better visualize double labeling. Sections were viewed under fluorescent light to observe mineral deposition resulting from loading. Control females and males showed clear double labeling after loading; this response was absent in β-catenin heterozygous mice.

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**Fig. 6.** Histomorphometric analysis of mineral apposition rates on periosteal and endocortical surfaces of loaded and nonloaded ulnas. (A) Endocortical and (B) periosteal surface analysis. Group sizes were n = 5 for males and n = 4 for females. (C) Representative percent change in cortical thickness comparing loaded to nonloaded ulnas using μCT analysis. Statistical comparisons: b, p < 0.05 comparing control to HET cKO; c, p < 0.05 comparing nonloaded versus loaded.
Table 1. Dynamic Histomorphometry Indices of Loading Response in HET cKO and Control Mice Ulnas

<table>
<thead>
<tr>
<th></th>
<th>Male Control</th>
<th>HET cKO</th>
<th>Female Control</th>
<th>HET cKO</th>
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<tr>
<td></td>
<td>Nonloaded</td>
<td>Loaded</td>
<td>Nonloaded</td>
<td>Loaded</td>
</tr>
<tr>
<td>BFR/BS (mcm³/mcm²/d)</td>
<td>0.01 ± 0.01</td>
<td>1.43 ± 0.19&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td>ND</td>
<td>0.03 ± 0.02</td>
</tr>
<tr>
<td>BFR/BV (%/y)</td>
<td>0.01 ± 0.01</td>
<td>0.35 ± 0.06&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td>ND</td>
<td>0.01 ± 0.01</td>
</tr>
<tr>
<td>Ec.sLS/B.Pm (%)</td>
<td>14.0 ± 10.3</td>
<td>51.40 ± 13.50</td>
<td>7.48 ± 6.83</td>
<td>8.80 ± 8.30</td>
</tr>
<tr>
<td>BFR/BS (mcm³/mcm²/d)</td>
<td>0.01 ± 0.01</td>
<td>0.49 ± 0.13&lt;sup&gt;a,b,c&lt;/sup&gt;</td>
<td>ND</td>
<td>0.07 ± 0.06</td>
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<tr>
<td>BFR/BV (%/y)</td>
<td>0.01 ± 0.01</td>
<td>0.44 ± 0.13&lt;sup&gt;a,b,c&lt;/sup&gt;</td>
<td>ND</td>
<td>0.07 ± 0.05</td>
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<tr>
<td>Ps.sLS/B.Pm (%)</td>
<td>5.44 ± 4.44</td>
<td>43.36 ± 9.75&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td>1.78 ± 2.05</td>
<td>8.58 ± 3.19</td>
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<tr>
<td>Ps.dLS/B.Pm (%)</td>
<td>1.52 ± 3.41</td>
<td>25.09 ± 7.94&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td>ND</td>
<td>3.35 ± 4.07</td>
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</table>

β-catenin heterozygous knockout mice do not respond to loading. Data represent mean ± SD for bone-formation rate/bone surface (BFR/BS), bone-formation rate/bone volume (BFR/BV), bone-formation rate/total volume (BFR/TV), single-labeled surface per bone perimeter (sLS/B.Pm), and double-labeled surface per bone perimeter (dLS/B.Pm) in the endocortical and periosteal region. Group sizes were n = 5 for male controls and HET cKO, n = 4 for female controls and HET cKO. Statistical significance was determined using a two independent sample t tests (control and HET cKO) and matched paired t tests for left and right (loaded, nonloaded). ND = Not detected.<br/><br/><sup>a</sup>Statistically significantly different comparing male to female.<br/><br/><sup>b</sup>Statistically significantly different comparing control versus HET cKO.<br/><br/><sup>c</sup>Statistically significantly different between nonloaded and loaded ulna.
if older aged mice (up to 2 years of age) will eventually display cortical effects or if the changes will remain restricted to trabecular bone.

Our findings of gender differences related to β-catenin signaling has been observed in previous studies involving other components associated with the pathway, albeit not always discussed in detail. Yao and colleagues demonstrated systemic overexpression of sFRP1, a negative regulator of the Wnt/β-catenin signaling pathway, resulted in an osteopenic skeleton in which distal femur BV/TV was decreased by 22% in females and 51% in males and midshaft cortical thickness was 13% lower in females and 21% lower in male mice. They also reported increased bone resorption in male mice, which appears to be a common finding in terms of the consequences of altered β-catenin signaling in bone that has been reported by others. Data from the Sost null mouse demonstrated a more pronounced BMD increase in the female mice compared with male mice, but was not discussed, most likely because the data were not specifically analyzed for gender differences. In a loading study performed with male and female Lrp5 null mice, Sawakami and colleagues noted greater suppression of load-induced bone formation in female Lrp5 null mice compared with males. Saxon and colleagues also reported gender-related differences in load and disuse studies of Lrp5 null and LRP5 high bone mass (LRP5G171V) mice expressing human LRP5 transgene containing the G171V activating mutation. The female LRP5G171V mice were more responsive to lower magnitudes of loads and had less bone loss in response to disuse compared with males. Those authors also puzzled over a possible explanation for their observed gender differences but speculated that a possible explanation could be the higher Lrps mRN expression level compared with controls. Javaheri and colleagues reported gender-related differences in proliferation and apoptosis in primary osteoblast-like cells isolated from Lrp5 null and LRP5G171V mice compared with cells from wild-type littermates with cells isolated from female Lrp5−/−and LRP5G171V mice being more affected by LRPS-β-catenin signaling. Interestingly, the bone biomechanical properties of female and male LRP5G171V mice did not display gender differences in the study by Akhter and colleagues, which further supports the complex nature of gender influences on β-catenin signaling and the need for more study. The mechanism underlying this gender difference and the greater sensitivity of the female skeleton to osteocyte β-catenin haploinsufficiency is not clear. The estrogen receptor-α has been shown to be important in β-catenin signaling in osteoclasts, whereas estrogen receptor-β appears to regulate sclerostin production. It is reasonable to speculate that sex hormone differences could underlie this gender difference, but until studies with gonadectomy are performed in HET CkO mice, the exact mechanism remains unknown.

The Wnt/β-catenin signaling pathway is activated in response to mechanical loading. We found that deletion of a single allele of β-catenin in osteocytes has a profound effect on the anabolic loading response. Ulnas for each group were loaded at a global load of ~2500 μN. Surprisingly, the new bone formation observed in response to in vivo mechanical loading in control mice was essentially absent in the HET CkO mice ulnas. This was demonstrated by both dynamic histomorphometry of double labeling and by μCT cortical bone thickness quantitation. These data support the concept that there is a critical level of β-catenin in osteocytes that is needed to develop a normal skeleton, adequately contributed by one allele of β-catenin, but the ability to mount a bone-formation response to anabolic mechanical loading requires a higher level of β-catenin expression that requires both alleles.

Our findings suggest an intriguing, new paradigm for contextualizing bone mass regulation by the Wnt/β-catenin signaling pathway. Namely, there is a critical stoichiometry for all of the key proteins comprising the Wnt/β-catenin signaling pathway that is required for the proper accrual and maintenance of a normal skeleton. The various mouse and human bone phenotypes that result from deletions or mutations of components of the Wnt/β-catenin pathway suggest functional redundancy of its components. As such, it is not clear why deletions or mutations in any of these components produce a bone phenotype. This suggests to us that the stoichiometry of these various components must be critical. For example, the LRP5 G171V or HBM transgenic mouse model expresses the human transgene against the background of two normal mouse alleles producing a high bone mass phenotype, whereas overexpression of the normal LRP5 transgene only results in a modest increase in bone mass. Deletion of the Sost gene produces a high bone mass phenotype but in a background in which the expression of other Wnt pathway inhibitors such as Dkk1 are normal. Similarly, the sFRP1 knockout mouse has an increased bone mass phenotype even though presumably both Sost and Dkk1 expression are normal. Lrp5 homozygous knockout mice have low bone mass, whereas Lrp6 homozygous knockout mice die at birth and have multiple patterning defects, although studies in Lrp5/Lrp6 mutant mice indicate that some amount of adult bone mass is also contributed by Lrp6. Lrp4-deficient mice have a low bone-mass phenotype, yet both Lrp5 and Lrp6 are still present and normal. All of these models suggest that there is a critical, yet unknown, balance between each component of the pathway and that this nominal equilibrium of all components is needed for normal bone growth/mass and accrual. An additional level of complexity that is not understood is the differential expression at specific stages of development or response of individual components of this pathway to stimuli, or relative expression in specific cell types.

Another important consideration is the restricted role of the Wnt/β-catenin signaling pathway in osteocytes. The osteocyte is considered to be a terminally differentiated cell of the osteoblast lineage (for review, see Dallas and colleagues). It resides within the mineralized matrix and lacks the ability to divide when contained in that environment. Hence, the normal proliferative and differentiation associated functions of the pathway are not operative in the osteocyte. Proliferation would appear to be a fatal event for the osteocyte in its lacunae given the space confinement. Therefore, at least two important functions of β-catenin signaling are abrogated in the case of the osteocyte. It appears that the pathway in osteocytes is used predominantly as an anti-apoptosis protective mechanism and in proper response to loading, functions that are observed in many other cells/tissues and tumors. In summary, we have demonstrated that mice with a heterozygous deletion of β-catenin in osteocytes lose the ability to form new bone in response to in vivo mechanical loading. The effects of osteocyte β-catenin haploinsufficiency are more severe in the female skeleton. These findings suggest that there is a threshold level of β-catenin in osteocytes that is required for new bone formation in response to in vivo mechanical loading that is different than the level required to form the skeleton and that regulation of the pathway is influenced by gender. Our data indicate that a better appreciation of the subtle nuances of regulation of the Wnt/β-catenin signaling pathway at the level of

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the stoichiometry and balance of its various components is required. Secondly, a better understanding of the potential mitigating influences of gender is needed as new bone anabolic pharmaceutical agents are being developed that target components of this pathway.

Disclosures

All authors state that they have no conflicts of interest.

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