J Musculoskelet Neuronal Interact 2007; 7(2):166-173

Original Article

Hylonome

Gender specific LRP5 influences on trabecular bone structure and strength

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Abstract

A mutation in LRP5 (low-density lipoprotein receptor-related protein 5) has been shown to increase bone mass and density in humans and animals. Transgenic mice expressing the LRP5 mutation (G171V) demonstrate an increase in bone mass as compared to non-transgenic (NTG) littermates. This study evaluated LRP5 gene and gender-related influences on the structural and biomechanical strength properties of trabecular and cortical bone in femurs and vertebrae (L5) of 17-week-old mice. Micro-computed tomography was used to evaluate the trabecular bone structure of distal femurs and vertebrae *ex vivo*. Mechanical testing of the trabecular bone in the distal femur was done to determine biomechanical strength. Differences due to genotype and gender were tested using two-way ANOVA at a significance level of $p<0.05$. Trabecular bone structural parameters (BV/TV, trabecular thickness, number, etc.) at the distal femur, femoral neck, and vertebral body sites were greater in the transgenic as compared to the NTG mice. In addition, vertebral cortical thickness and trabecular strength parameters (ultimate and yield loads, stiffness, ultimate and yield stresses) in the distal femur were greater in the transgenic mice as compared to NTG. The increasing trends of cortical thickness were also noted in the transgenic mice as compared to NTG. Within LRP5 (G171V) mutant mice, there were significant gender-related differences in some of the trabecular bone structural parameters at all the sites (distal femur, femoral neck, and vertebral body). However, unlike trabecular structural parameters, the gender-specific differences were not found in the trabecular strength of LRP5 transgenic mice. In summary, these findings suggest that the LRP5 (G171V) mutation results in greater trabecular bone structure and strength at both the distal femurs and vertebral bodies as compared to NTG. In addition, only the trabecular structure parameters were affected by gender within the LRP5 (G171V) mutation.

Keywords: LRP5, Bone, Femur, Vertebral Body, Stiffness, Stress

Introduction

Patients with osteoporosis typically have low bone mass and develop skeletal fragility, leading to low-trauma fractures, especially in the hip, femur, wrist and vertebrae. It is estimated that 50% of women over the age of 50 are at significant risk for developing osteoporosis-related fractures[1-4]. Over 300,000 hip fractures are attributed to osteoporosis in the USA alone[1]. The critical factors that determine bone strength include bone size, density, and genetics. Genetic effects play a large role in osteoporosis-related fractures[5-9].

LRP5 (low-density lipoprotein receptor-related protein 5) has been considered a key regulator[10,11] in bone homeostasis. The LRP5 gene is responsible for both the loss and gain of bone mineral density[12-21]. A single nucleotide mutation in the LRP5 gene leading to an amino acid substitution (G171V) is associated with a high bone mass (HBM) phenotype in humans[12]. Animal models of the LRP5 gene developed to study the various bone phenotypes that include size, mass, density and bone strength suggest that the mutation is responsible for increases in bone tissue strength[16,22-24].

LRP5 is expressed in many tissues and cell types, including osteoblasts[25]. Prior studies show that LRP5 over-expression in LRP5-transgenic mice causes a modest increase in bone mass, whereas over-expression in the G171V mutant causes a substantial increase in bone mass; this suggests a direct link between LRP5 and osteoblast function[22,26-28]. This also supports the hypothesis that it is a structural change in the receptor protein rather than its simple over-expression

The authors have no conflict of interest.

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Accepted 1 November 2006

Variables (Mean±SD)	F-NTG	F-HET	M-NTG	M-HET
Distal Femur (trabecular bone)				
BV/TV	0.177±0.023	0.378±0.125[a]	0.168±0.032	0.362±0.139[a]
Conn. D [1/mm^3]	164±31.4	166±49.6	135±30.5	245±73.1[ab]
Tb. N [1/mm]	3.25±0.153	5.20±1.23[a]	3.99±0.357	6.46±1.43[ab]
Tb. Th [mm]	0.064±0.004	0.084±0.011[a]	0.059±0.004	0.072±0.010[ab]
Tb. Sp [mm]	0.327±0.017	0.206±0.061[a]	0.260±0.025[b]	0.156±0.047[ab]
Cortical Shell thickness (mm)	0.227±0.035	0.249±0.046	0.218±0.020	0.243±0.038
Femoral Neck				
BV/TV	0.752±0.050	0.876±0.066[a]	0.700±0.053	0.864±0.085[a]
Conn. D [1/mm^3]	210±119	171±135	168±87.0	88.2±116
Tb. N [1/mm]	8.22±0.679	7.47±1.13	8.30±0.715	7.45±1.28
Tb. Th [mm]	0.148±0.015	0.234±0.061[a]	0.132±0.017	0.216±0.068[a]
Tb. Sp [mm]	0.102±0.012	0.060±0.018[a]	0.104±0.010	0.069±0.015[a]
Average cortical thickness (mm)	0.224±0.015	0.283±0.016[a]	0.198±0.022	0.240±0.016[ab]
Femoral neck width (mm)	0.694±0.041	0.83±0.071[a]	0.698±0.047	0.803±0.048[a]
Vertebral body (trabecular bone)				
BV/TV	0.26±0.05	0.55±0.05[a]	0.21±0.03	0.42±0.1[ab]
Conn. D [1/mm^3]	201±54	342±98[a]	175±27	364±80[a]
Tb. N [1/mm]	5.00±0.73	7.96±0.63[a]	5.24±0.34	7.56±0.97[a]
Tb. Th [mm]	0.060±0.007	0.077±0.009[a]	0.054±0.004	0.067±0.009[ab]
Tb. Sp [mm]	0.206±0.036	0.119±0.013[a]	0.189±0.015	0.126±0.022[a]
Length (mm)	2.58±0.18	2.61±0.21	2.38±0.24	2.54±0.25
Cross-sectional area (mm^2)	1.24±0.25	1.39±0.22	1.22±0.27	1.28±0.22
Average cortical shell thickness (μm)	93±11	115±27	81±10	104±20[a]

[a] Different ($P<0.05$) from non-transgenic littermates (NTG) of the same gender.
[b] Different ($P<0.05$) from females (F) of the same genotype.

Table 1. Bone architecture (micro-CT).

that results in increased bone mass[22].

Cortical thickness does contribute to the overall vertebral body and femoral neck strength[29]. Turner et al.[29] have shown greater cortical thickness in the femoral neck of C3H/HeJ mice, which have been reported to have high bone mass[30] as compared to C57BL/6J which have low bone mass. Although greater whole bone strength has been reported in LRP5 transgenics heterozygotes (HET) as compared to NTG mice[22,23], we do not know the gender-related structural contribution of either trabecular or cortical bone to vertebral body, femoral neck, and distal femur.

The most significant changes in bone density have been reported in bone associated with the load-bearing sites of the LRP5 G171V high bone mass (HBM) kindred[12,19,21,31]. Therefore, it has been suggested that a more sensitive bone adaptation mechanism enables LRP5 G171V transgenic mice to attain greater bone density and strength as compared to the non-transgenic littermates (NTG)[22,23].

The expression of mutant LRP5 in transgenic mice has led to higher bone mass, enhanced strength, and increased bone mineral density compared to littermate controls[22,23]. Previous studies have shown that LRP5 (G171V) mice have an increased response to skeletal loading[32] with an increase in trabecular thickness of the distal femoral metaphysis and increased cortical bone thickness of the femoral diaphysis[22,23]. Although the whole skeleton is expected to be sensitive to mechanical stimulus, the influence of the LRP5 gene on the cortical shell thickness and trabecular bone strength of the distal femur and vertebral body is not known with respect to gender.

We hypothesize that the LRP5 gene is responsible for greater bone mass and structure of both trabecular and cortical bone at the distal femur and vertebral body as compared to NTG mice within each gender. In this paper, we present a more thorough examination of HBM phenotypic effects on the femur and vertebral body by analyzing both cortical and trabecular structural and apparent material strength properties, as well as the distribution of cortical and trabecular bone. We examined the influences of the LRP5 gene on the distribution and strength of trabecular and cortical bone within the distal femur, femoral neck, and vertebral body using microcomputed tomography (micro-CT) analysis and mechanical testing by indentation. In addition, gender-related differences within the LRP5 transgenic and NTG were also examined.

Materials and methods

Femurs and vertebral bodies from 60 adult mice (17-week-old) were used to evaluate trabecular bone mass, distribution, and cortical thickness in the femoral distal end and vertebral bodies (lumbar-5, L5) *ex vivo* using microcomputed tomography (micro-CT). The study was equally divided into LRP5 transgenics heterozygotes (HET, n=15/gender, with C57BL/6Tac background) and non-transgenic littermates (NTG, n=15/gender). At the time of necropsy, body weights were measured and femur/vertebral body specimens were collected and placed in a saline solution and subsequently frozen at -20˚C for structural and biomechanical strength analyses at a later time.

Microcomputed tomography (micro-CT)

All femurs and vertebral bodies were evaluated for cortical and trabecular bone architecture using high-resolution (9 micron [μm] slice increments, at 55 kEv) microcomputed tomography (μCT-20; Scanco Medical AG, Basserdorf, Switzerland) to capture details of the trabecular architecture at the distal femur and vertebral bodies. Morphometric analyses were performed using reconstructed micro-CT images with an isotropic voxel size of 9 μm and a constrained 3-D Gaussian filter (σ=1.2, support=2). Contours were drawn manually on approximately 168 slices for distal femur, 150 slices for femoral neck, and 300 slices for vertebral body. A threshold of 275 was used for bone structural analyses. Trabecular bone contouring was conducted in such a way as to maintain at least a 0.5 mm distance between the cortical shell and the trabecular bone[33].

Morphometric analysis at the distal femoral end began at the growth plate, and extended proximally through the metaphysis for 168 slices (~1.5 mm). Analysis at the femoral neck started at the origin of the femoral head and extended distally through the femoral neck just prior to the beginning of the shaft. Whole vertebral bodies (L5) were analyzed for trabecular architecture.

For both the distal femurs and vertebral bodies, bone volume fraction (BV/TV), connectivity density (Conn. D [1/mm^3]), trabecular number (Tb. N [1/mm]), trabecular thickness (Tb.Th [mm]), and trabecular separation/spacing (Tb.Sp [mm]) were evaluated. Average cortical shell thickness (Th. [mm]) was also computed for the distal femur and vertebral bodies.

Mechanical testing

Ex vivo mechanical indent testing was performed using a mechanical testing device (Instron 5543, Canton, MA). Tests were performed at room temperature (22^{o}C) and at a standard testing rate of 3 mm/minute. Each mechanical test generated a load-displacement diagram, which was then used to determine structural strength parameters (ultimate load, yield load, and stiffness)[34]. These structural strength parameters, along with the bone size measurements taken from micro-CT, were used to calculate the apparent material strength properties (ultimate stress, and yield stress) at the distal femur.

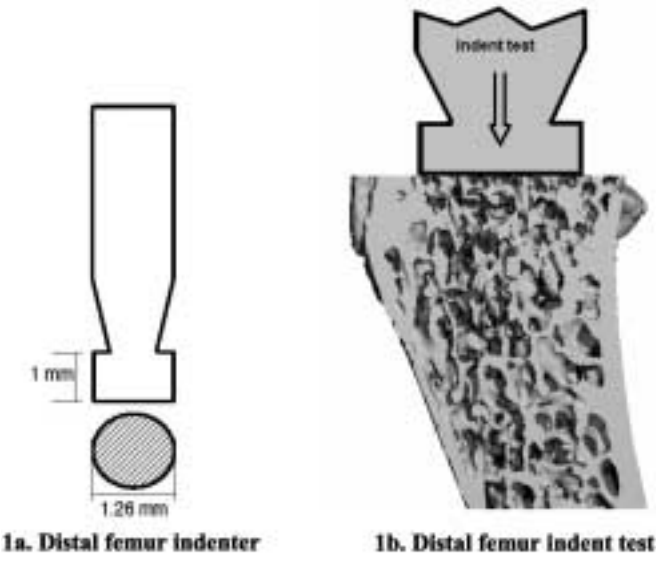

Figure 1. Trabecular bone strength test using indenter "a" (1.26 mm diameter). The indenter contacts the trabecular bone in the distal femur "b" (metaphysis, just below the growth plate).

Distal Femur Trabecular Bone

The growth plate was removed from each specimen at the distal femoral end using an Ecomet-3 grinding device (Buehler, IL). Grinding was administered perpendicular to the femoral shaft at the distal end until the disappearance of the growth plate, in order to correlate with the area of interest for micro-CT analysis. Each specimen was set in a fixture, with the distal end surface perpendicular to the indenter (Figure 1), using an acrylic dental paste (Bosworth Fastray manufactured by The Bosworth Company®).

An indentation compression test was done on the trabecular bone of the distal femoral end using a mechanical testing system (Instron-5543, MA). Force was applied to the trabecular bone via a stainless steel indenter (diameter of 1.26 mm and height of 1 mm, Figure 1a) until it peaked on the load-displacement diagram. During each test, the indenter was centered on the trabecular bone while avoiding contact with the cortical bone (Figure 1b). The average stiffness of the indenter was 1470 N/mm, which was approximately 6 times greater than the trabecular bone in the distal femur (Table 2)[35].

Structural strength measurements (ultimate load, yield load, and stiffness) were taken directly from the load-displacement diagrams for each mechanical test. Apparent material strength properties from the distal femur were calculated using the following equation:

$$\text{Ultimate/Yield Stress } (\sigma_{df}) = F/A$$

Variables (Mean±SD)	F-NTG	F-HET	M-NTG	M-HET
Ultimate load (N)	3.77±1.93	24.9±17.3[a]	4.13±1.84	27.1±19.2[a]
Yield Load (N)	2.54±1.18	18.9±16.2[a]	2.86±1.65	16.3±14.3[a]
Stiffness (N/mm)	64.9±37.9	245±135[a]	57.9±37.9	195±151[a]
Ultimate stress (N/mm^2)	2.82±1.68	19.9±13.8[a]	3.31±1.48	21.7±15.3[a]
Yield stress (N/mm^2)	1.90±1.05	15.1±13.0[a]	2.29±1.32	13.0±11.5[a]
[a] Different ($P<0.05$) from non-transgenic littermates (NTG) of the same gender.				

Table 2. Trabecular bone strength in the distal femur.

σ_{df} represents the stress (ultimate or yield) at the trabecular bone of the distal femur, F is either the ultimate or yield load, A is the area of the "indenter" instrument compressing the trabecular bone. Stiffness of the distal femur and represents the slope [force (N)/displacement (mm)] of the linear portion of the load-displacement diagrams[23].

Statistical analysis

Differences in all measured variables corresponding to genotype and gender were tested using two-way ANOVA. The level of statistical significance was set at $p \leq 0.05$, however, trends where $p<0.1$ were also indicated. The data are reported as a mean ± standard deviation (SD). Statistical analyses were performed using the statistical package for Windows v. 13 (SPSS Inc., Chicago, IL, USA).

Results

No significant differences in body weight (average 22.6±1.3) existed due to genotype within each gender. Males had 18% greater body weight (average 26.6±3.3) than the female mice within each genotype.

Microcomputed tomography - distal femur and femoral neck

Trabecular bone in the distal femur showed a greater BV/TV (40%), Tb. N (60%), and Tb.Th (22-31%) for the HET as compared to NTG strains within each gender (Table 1). Although no differences due to genotype were found in females, connectivity density (Conn. D) was greater (80%) in HETs as compared to NTG within the male gender. A decrease in Tb. Sp (37-40%) was apparent in the HETs vs. NTGs of the whole distal femur within each gender (Table 1). In addition, gender-related differences were also apparent in HETs with increased Conn. D (48%) and Tb.N. (24%) and decreased Tb.Th (14%) and Tb.Sp (27%) in male as compared to female distal femur (Table 1). A decrease in Tb. Sp (25%) is evident in male vs. female HETs at the distal femur (Table 1). Average cortical shell thickness in distal femurs was not different due to either genotype or gender (Table 1). Trabecular structure in the femoral necks (neck and head) showed similar trends to that of the distal femur. The femoral neck showed (Figure 2) greater BV/TV (16-23%), Tb. Th (58-63%), average cortical thickness (~20%), and width (15-20%) in HET as compared to NTG mice in both genders (Table 1). A decrease in Tb. Sp (34-41%) was apparent in the HET vs. NTG at the femoral neck (Table 1). There was no difference in the Conn. D and Tb. N between HET and NTG mice at the femoral neck (Table 1).

Microcomputed tomography - vertebral body

Morphometric analysis of vertebral bodies revealed multiple differences in trabecular bone microarchitecture due to genotype, with denser trabeculae in HETs as compared to NTG mice (Figure 3). Bone volume fraction (115-100%), connectivity density (70-108%), trabecular number (59-44%), and trabecular thickness (28-24%) of trabecular bone were significantly greater, while trabecular spacing (73-50%) was lower in HET mice of both genders as compared to NTG (Table 1). Average cortical shell thickness was greater (28%) for HET mice within the male gender only, although a trend of greater cortical thickness was evident for HET mice within the female gender as well. In addition, there was a decreased trabecular bone volume fraction (32%) and trabecular thickness (15%) of males with respect to females within the HET genotype only. A trend of lower bone volume fraction in NTG male mice compared to NTG females was also observed.

Mechanical testing

For the distal femur, the structural strength variables (ultimate load, yield load, and stiffness) in the trabecular bone were greater in the HET (550-560%, 470-640%, 240-280%, respectively) than the NTG mice within each gender (Table 2). The apparent material strength properties (ultimate stress and yield stress) were greater in HET (560-600%, 470-690%, respectively) as compared to NTG mice within each gender (Table 2). However, similar to BV/TV in distal femur, there were no significant differences in trabecular bone strength properties due to gender (Table 2).

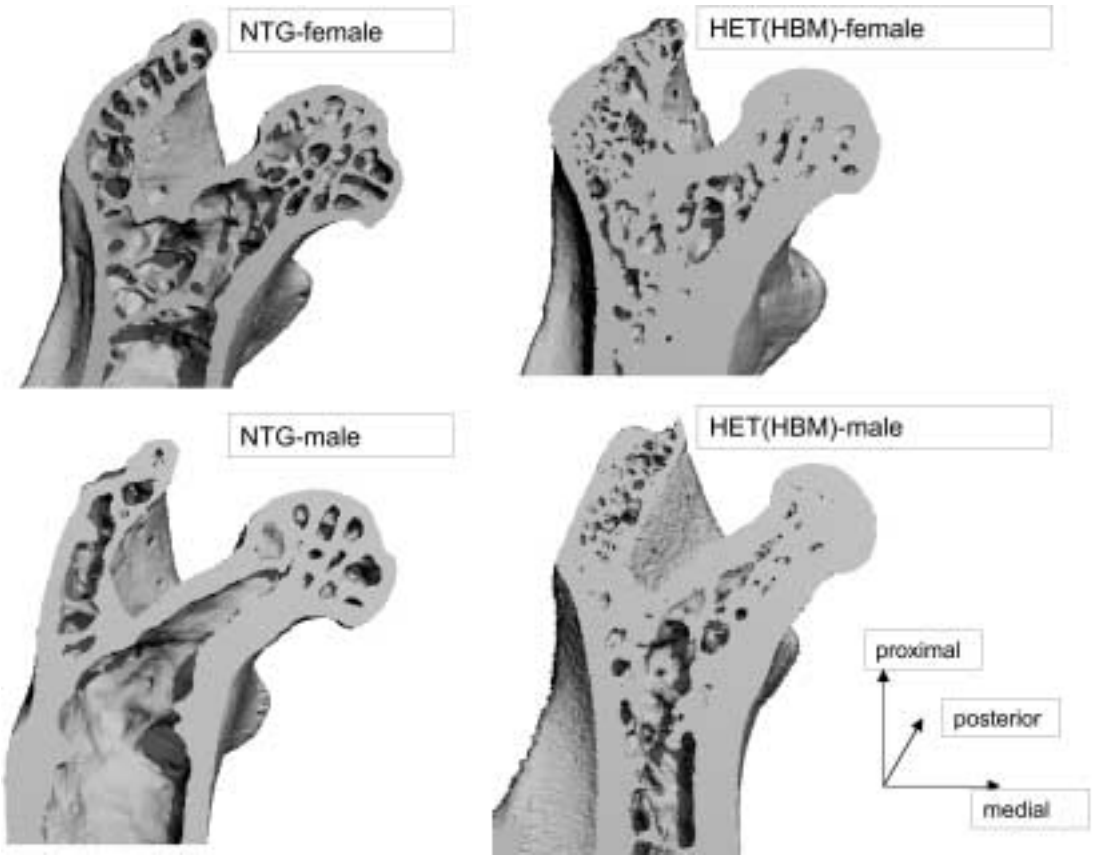

Figure 2. Micro-CT images of the femoral neck. The femoral neck cortical and trabecular bone with thicker structure in HET (HBM) as compared to the NTG within both genders.

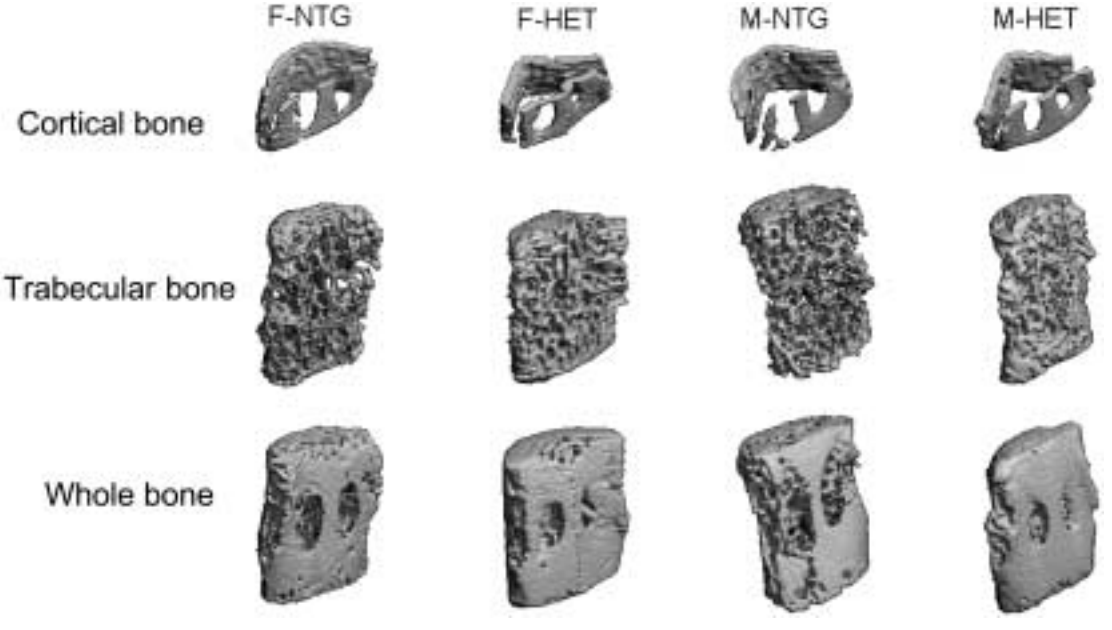

Figure 3. Micro-CT images of the vertebral body. The greater cortical shell thickness and denser trabecular structure with higher bone fraction in HET (HBM) as compared to NTG within both genders.

Discussion

The purpose of this experiment was to investigate the trabecular and cortical bone distribution and its relation to bone strength in the distal femur, femoral neck, and vertebral body (L5) sites in the mouse model (LRP5 (G171V) transgenic and NTG), as well as to further establish the significance of LRP5 gene in regulating bone mechanical properties in both female and male mice. Trabecular structure was assessed using micro-CT scans. Distal femur trabecular strength was measured using an indent test. LRP5 (G171V) transgenic mice (HET) demonstrated a notable increase in bone trabecular structure (BV/TV, trabecular thickness and number) and strength (ultimate and yield loads, stiffness, ultimate and yield stresses) as compared to NTG at most measured sites. The increased trabecular bone structure and indentation strength in the distal femur of HET genotype also agree with the increased mid-shaft femoral cortical bone area and thickness as reported earlier[22,23]. The LRP5 mutation (G171V) is responsible for the increased trabecular bone mechanical properties (current study) and therefore affects both cortical and trabecular bone in a manner that is similarly observed in humans[12,19]. Similar to the structural distribution of the mid-shaft femoral bone with a greater second moment of area (greater AP and ML widths)[22,23], the trabecular bone volume fraction was also greater in HET, resulting in the greater indention strength properties as compared to NTG. The lack of difference in the femoral cortical shell (distal femur) thickness between HET and NTG bone in the distal femur suggests that the G171V mutation mostly affected trabecular bone. Greater trabecular bone structure (BV/TV and Tb.Th) in the femoral neck of HET mice is further evidence of greater femoral neck structural strength, as was reported previously[23]. In addition, while some of the trabecular bone structural parameters were different due to gender at all the sites (distal femur, femoral neck, and vertebral body), the gender specific differences were not found in the trabecular strength within LRP5 transgenic mice.

Cortical thickness and femoral neck width also made major contributions to the HET's greater femoral neck strength as compared to the NTG mice (Figure 2). Within each gender it has been shown that both the structural strength of the femoral mid-shaft and the femoral neck bending strength are greater in LRP5 transgenics as compared to NTG mice[23]. The femoral neck mechanical test represents both the trabecular and cortical bone components. However, the trabecular bone could not be tested separately (without cortical bone) in the femoral neck. Therefore, the contribution towards increased strength from either the cortical or trabecular bone could not be quantified[23].

Morphometric analysis shows that HET mice have a higher total bone volume ratio and a greater trabecular density (due to an increase in Tb. N. and Tb. Th., as well as a decrease in Tb. Sp.) in all femoral sites (distal femur and femoral neck), thus making a stronger femur as compared to NTGs. It has previously been reported that an increase in cortical thickness was a common finding in both the human phenotype and in the transgenic mice containing the LRP5 mutation[12,19,22,23]. The data in the current study, however, show that the genotype had a lack of effect on the cortical thickness at the distal femur, suggesting that the mutant LRP5 gene affects certain bones differently. It may be the case that the LRP5 gene regulation of the cortical bone is different from that of the trabecular bone at the distal femoral end.

The significantly greater BV/TV, Tb.Th, Conn. D, and Tb.N in vertebral body trabecular bone of HETs were most likely responsible for the superior vertebral compressive strength (both structural and apparent material strength) properties as reported previously[23]. The HET mice showed greater vertebral stiffness (structural strength), ultimate stress, and yield stress (apparent material strength)[23]. The vertebral strength represented bone strength from both cortical (shell) and the trabecular bone[22,23]. Although cortical shell thickness was greater in male HETs, no differences were noted due to the genotype in female mice. Therefore, the overall vertebral body strength is enhanced by cortical bone in male HETs while the major contribution to the increased vertebral body strength of female HETs mice was due to trabecular bone alone.

This study showed that the LRP5 mutation has many positive influences on mouse vertebral body microarchitecture and strength while maintaining similar external bone dimensions (Figure 3). This suggests that bone intrinsic material properties may also be greater in HET as compared to NTG.

While the LRP5 mutant phenotype of high bone mass is developed during skeletal growth, the bone adaptation response remained more sensitive to mechanical stimulus as compared to NTG mice tibiae[11]. The increased bone adaptation response sensitivity to loading is attributed to the activation of canonical Wnt signaling pathway by LRP5 G171V mutation in HET mice[11]. Robinson et al.[11] reported similar increases in loading-related (mechanical stimulus) bone adaptation responses (expression of Wnt/β-catenin target genes) in NTG adult (17-week-old) mice when treated with a canonical Wnt pathway activator, Glycogen Synthase Kinase 3-β inhibitor (GSK3βi) as compared to LRP5 G171V mutant mice. This suggests the potential of GSK3βi compound to help increase exercise-related bone adaptation response in mice without the LRP5 G171V mutation.

Large increases (2.4 to 2.8 fold) in the overall trabecular bone stiffness at the distal femur supports thicker and more numerous trabecular data in HBM transgenic mice as compared to the NTG. The increased stiffness may increase the chances of fatigue-related micro-damage; however, we did not quantify the brittleness of bone specimens. In addition, the lack of gender-related differences in the trabecular bone strength at the distal site suggests that the mechanical testing technique may not be sensitive enough to measure the small changes in strength resulting from changes in trabecular bone structure.

The LRP5 mutation generates a high-density bone microarchitecture highlighted by thicker cortical bone (although not shown to be significant within the female gender) and a thicker trabecular meshwork containing an increased number of connections/structural supports. The result is a denser arrangement of trabecular bone in the femoral neck and vertebral body of similar size and shape in HETs as compared to the NTG (wild type, Figure 2 and 3). The denser trabeculae (both femoral neck and vertebral body) and greater cortical thickness (vertebral body [male] and femoral neck) are responsible for the enhanced biomechanical properties in both femoral neck and vertebral bodies[22,23]. Although the gender-related differences within the HET genotype for vertebral body trabecular bone volume fraction and trabecular thickness (greater in females for both) are shown, the same trends are present within the NTG genotype. This suggests that the female mice in this study may have had a greater bone adaptation response sensitivity[11].

Within LRP5 HET (HBM) mutant mice there were significant gender-related differences in a few of the trabecular bone structural parameters at all the sites (distal femur, femoral neck, and vertebral body, Table 1). The gender-related differences suggest that the LRP5 gene affected trabecular bone structure differently within female as compared to male mice. In addition, in a separate study, female LRP5 transgenic mice lost bone due to disuse but not to ovariectomy[36]. The gender-related differences are not understood and need further investigation of the Wnt signaling pathway[37-40]. However, the relationship between signaling mechanism in the estrogen and Wnt pathway may be responsible for the gender-specific influences of LRP5 G171V mutation[37,38]. For instance, Bennet et al.[37] have shown that transgenic mice expressing Wnt 10b were resistant to both age- and ovariectomy-related bone loss. This suggests that the complex interaction of the estrogen[38] and androgen[39,40] receptor with Wnt/β-catenin pathway may cause differences in the trabecular bone architecture of LRP5-transgenic male and female mice and needs additional investigation.

With respect to the phenotypic structural effects of mutant LRP5, future studies may attempt to test trabecular and cortical bone intrinsic material[41] strength properties separately to elucidate the strength contribution of each to the high bone mass (HBM) phenotype.

Acknowledgements

The authors thank Andy Lund, Bryan McGuckin, Gregory Thompson, and Gleb R. Haynatzki for their assistance.

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