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Enhanced Wnt signaling improves bone mass and strength, but not brittleness, in the *Col1a1*$^{+/mov13}$ mouse model of type I Osteogenesis Imperfecta

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ABSTRACT

Osteogenesis Imperfecta (OI) comprises a group of genetic skeletal fragility disorders. The mildest form of OI, Osteogenesis Imperfecta type I, is frequently caused by haploinsufficiency mutations in *COL1A1*, the gene encoding the α_1(I) chain of type 1 collagen. Children with OI type I have a 95-fold higher fracture rate compared to unaffected children. Therapies for OI type I in the pediatric population are limited to anti-catabolic agents. In adults with osteoporosis, anabolic therapies that enhance Wnt signaling in bone improve bone mass, and ongoing clinical trials are determining if these therapies also reduce fracture risk. We performed a proof-of-principle experiment in mice to determine whether enhancing Wnt signaling in bone could benefit children with OI type I. We crossed a mouse model of OI type I (*Col1a1*$^{+/Mov13}$) with a high bone mass (HBM) mouse (*Lrp5*$^{+/p.A214V}$) that has increased bone strength from enhanced Wnt signaling. Offspring that inherited the OI and HBM alleles had higher bone mass and strength than mice that inherited the OI allele alone. However, OI + HBM and OI mice still had bones with lower ductility compared to wild-type mice. We conclude that enhancing Wnt signaling does not make OI bone normal, but does improve bone properties that could reduce fracture risk. Therefore, agents that enhance Wnt signaling are likely to benefit children and adults with OI type 1.

1. Introduction

1.1. Osteogenesis Imperfecta (OI) represents a genetically heterogeneous group of disorders that have skeletal fragility as a principal component

The clinical severity of OI ranges from mild to severe. Newborns with the most severe forms of OI have prenatal-onset skeletal deformities and do not survive the neonatal period, whereas adults with the mildest forms of OI typically have no deformity and normal life expectancy [1]. Mutations in *COL1A1* or *COL1A2*, which encode the α_1(I) and α_2(I) chains of type 1 collagen, are the most common cause of OI. A specific class of mutations in *COL1A1* is a frequent cause of mild OI (OI type I) [2]; this class of mutations result in functional haploinsufficiency for the α_1(I) chain and includes *COL1A1* nonsense, frameshift, and whole-gene deletion mutations.

OI type I is the most prevalent form of OI and ~40% of patients with OI type I have functional haploinsufficiency [3]. Despite being called "mild," patients with OI type I have significant skeletal involvement. In a cohort of 86 individuals with *COL1A1* haploinsufficiency, 70 of whom were ≤21 years old, the mean lumbar spine bone mineral density was −3 standard deviations (SD) and the mean height was ~ − 1 SD compared to controls [3]. Data from children in this cohort were used to determine an annualized fracture rate of 0.77 fractures/child/year, which is a 95-fold increase compared to the fracture rate in controls. Finally, 41 of 58 (71%) pediatric participants with spine radiographs in this study had vertebral compression fractures, with a mean of 3

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vertebral fractures/individual [3]. Therefore, children and adults with OI type 1 would benefit from therapies that increase their skeletal strength.

1.2. Current therapies for OI include bisphosphonates

Bisphosphonates that increase bone mass, and presumably bone strength, by reducing osteoclast-mediated bone resorption have been given to patients with mild OI. The efficacy of bisphosphonate therapy in reducing fracture rates in all types of OI is unclear, although some pediatric patients benefit [4–6]. Since the principal effect of bisphosphonate therapy is anti-catabolic, the effectiveness of bisphosphonates for reducing fracture risk in adults with OI is uncertain [7].

Anabolic therapeutic agents that increase osteoblast activity in bone could benefit children and adults with OI. Recombinant human parathyroid hormone (teriparatide) therapy has been shown to increase bone mass and reduce fracture risk in men and women with age-related bone loss [8,9]. Although teriparatide is presently contra-indicated in children with OI, two studies tested teriparatide efficacy in adults with OI. An 18-month-long, open label intervention trial was performed in 13 post-menopausal women with OI type I and significant increases in spine areal BMD (aBMD) and the bone anabolic biomarker P1NP were observed [10]. Among participants with OI type I in an 18-month long, double-blind, placebo-control trial, the 24 participants who received teriparatide had significantly increased spine and hip aBMD and spine volumetric BMD compared to those who receive placebo [11]. Unfortunately neither study was sufficiently powered to determine if teriparatide therapy affected fracture rates.

1.3. Wnt signaling is important for bone mass and strength

Studies in humans and mice indicate that the Wnt signaling pathway is anabolic in bone [12]. Mutations that interfere with Wnt signaling through the low density lipoprotein related protein 5 (LRP5) receptor reduce bone mass and bone strength, whereas these bone properties are improved by mutations that increase Wnt signaling. Two monoclonal antibodies, blosozumab and romosozumab, that increase Wnt signaling by interfering with Sclerostin, an endogenous LRP5 inhibitor in bone, are in clinical trials for age-related osteoporosis (www.clinicaltrials.gov), and could be considered for patients with OI. Therefore, we and other investigators have been performing proof-of-principle studies looking at the effect of enhancing Wnt signaling in mouse models of OI [13–15]. Improved bone mass and bone strength have been observed in mice with moderate forms of OI treated with Sclerostin neutralizing antibodies [13,14], and in mice that have moderate OI and an allele of the Wnt co-receptor LRP5 ($Lrp5^{p.A214V}$) that makes the receptor resistant to Sclerostin [13].

1.4. Enhancing Wnt signaling may improve bone properties in OI type 1

The present study examines the effect of using the $Lrp5^{p.A214V}$ allele to enhance Wnt signaling in the $Col1a1^{+/Mov13}$ mouse strain, which models the most common type (type I) of OI in humans. $Col1a1^{+/Mov13}$ mice are heterozygous for a retroviral insertion in *Col1a1* gene that prevents mRNA expression in osteoblasts, thereby causing haploinsufficiency [16]. The type 1 collagen in the bone of the mice is qualitatively normal, but reduced in quantity [17]. Bone quality is also abnormal, with $Col1a1^{+/Mov13}$ mouse bones having lower matrix to mineral ratios and lower post-yield deformation compared to wild-type mice [17,18]. Herein, we compare the bone properties of littermate mice with wild-type ($Col1a1^{+/+}$;$Lrp5^{+/+}$), OI ($Col1a1^{+/mov13}$;$Lrp5^{+/+}$), OI + HBM ($Col1a1^{+/mov13}$;$Lrp5^{+/p.A214V}$), or HBM ($Col1a1^{+/+}$;$Lrp5^{+/p.A214V}$) alleles.

2. Materials and methods

2.1. Mouse strains and genotyping

All experimental techniques involving animals were approved by the Boston Children's Hospital Institutional Animal Care and Use Committee. $Col1a1^{+/Mov13}$ mice have been previously described [16], and were obtained on a fixed C57BL/6 background (JAX Stock # 000664) from The Jackson Laboratory (Bar Harbor, ME) [16]. $Lrp5^{+/p.A214V}$ mice have been previously described [19], and have been maintained on a fixed 129/SvJ background. Tail snip DNA was recovered for PCR genotyping using the HotSHOT method [20]. Genotyping was performed as previously described [19](www.jax.org).

2.1.1. Mouse care and handling

Male $Col1a1^{+/Mov13}$ mice were mated with female $Lrp5^{+/p.A214V}$ mice to generate wild-type (WT), OI, OI + HBM, and HBM offspring. Offspring of both sexes were genotyped between 8 and 14 days-of-age, weaned before 28 days-of-age, and then group housed as same-sex littermates. At 7-weeks-old, mice of both sexes were given an intraperitoneal (IP) injection of calcein green (Sigma-Aldrich, St. Louis, MO; 10 mg/kg) followed 4 days later by an IP injection of alizarin complexone (Sigma-Aldrich, St. Louis, MO; 20 mg/kg). When 8-weeks-old, measures of bone mineral density (BMD) and bone mineral content (BMC) were obtained with the Piximus II dual energy X-ray absorptiometer (GE Lunar, Madison, WI, USA) and the mice were euthanized. Femurs and the 5th lumbar vertebra were immediately removed and dissected free of soft tissues. Each animal's left femur and vertebra were fixed in 10% formalin and the right femur was wrapped in sterile phosphate buffered saline (PBS)-soaked gauze (Gibco-Life Technologies, Grand Island, NY), and stored frozen at −20 °C.

2.2. Assessment of bone properties

MicroCT (μCT) measurements of the left femur (midshaft cortical bone and distal trabecular bone) and 5th lumbar vertebra from both male and female animals were performed as previously described [13,19]. After μCT measurements were obtained, the left femur from male mice was embedded in methyl methacrylate, sectioned, and imaged for quantitative histomorphometry as previously described [19,21].

The right femur from male and female animals was used for biomechanical testing in a three-point bending assay as previously described [13,19]. Briefly, the frozen femur was brought slowly to room temperature (~1.5 h) in a saline bath, positioned with the posterior side down across the two bottom supports of the three-point bending apparatus and mounted in a Bose Electroforce 3200 electromagnet test machine (Bose, Eden Prairie, MN), which has a force resolution of 0.01 N. Each femur was loaded to failure in monotonic compression using a cross-head speed of 0.2 mm s^{-1}, during which force and displacement measurements were collected every 0.005 s.

Three longitudinal sections were cut from the left femur of male mice previously embedded in methyl methacrylate and mounted directly onto BaF2 windows for the Fourier transform infra-red spectroscopic imaging (FTIRI). Using a Spectrum 300 (Perkin Elmer, CT, USA) infrared spectrometer and microscope, images were acquired at 3 different trabecular and cortical sites for each bone as previously described [22].

2.3. Statistical analyses

Bone density and strength properties were tested for normality using the Shapiro-Wilk W statistic and no significant departures or outliers were detected for males or females on any parameter [23]. Therefore, two-way mixed model analysis of variance (ANOVA) with genotype and treatment as factors was applied with Bonferroni correction to compare groups and assess genotype effects [24]. The sample

sizes of 8–16 animals per group for each sex provided 80% power ($\alpha = 0.05$, $\beta = 0.2$) for detecting mean differences of 30% (moderate effect sizes of 0.8 or larger) at 8 weeks of age (version 7.0, nQuery Advisor, Statistical Solutions, Saugus, MA). All data, except where indicated, are presented as mean ± 1 SD with two-tailed $p < 0.05$ considered statistically significant. FTIR cortical and trabecular measures were compared using ANOVA based on a generalized linear modeling approach with a repeated-measures within-subjects factor in order to account for the triplicate measurements per animal and the Wald chi-square test to assess differences between the OI + HBM and OI groups. Statistical analysis was performed using SPSS software (version 19.0, SPSS Inc./IBM, Chicago, IL).

3. Results

3.1. An Lrp5 HBM allele improved bone density in mild OI caused by a *Col1a1*^{Mov13} allele

Total body bone mineral density did not differ between WT and OI littermates, consistent with the mild phenotype of this mouse model, but was significantly increased in OI + HBM mice for both sexes (Fig. 1A). MicroCT studies also detected no significant differences between OI and WT littermates with respect to femoral trabecular bone volume/total volume (BV/TV) for female mice or midshaft cortical volume for both sexes (Fig. 1B, D); data which are consistent with prior studies that suggested *Col1a1*$^{+/Mov13}$ mouse long bones undergo adaptive changes that reduce the effect of *Col1a1* haploinsufficiency on long bone mass and strength [18,25,26]. In contrast, the vertebral trabecular BV/TV was significantly lower in female OI compared to WT mice ($p < 0.01$) and trended toward being lower in the male OI mice ($p = 0.09$) (Fig. 1C). Importantly, mice of both sexes with OI + HBM had significant increases in femoral and vertebral trabecular BV/TV and femoral cortical volume when compared to same-sex OI or WT mice (Figs. 1 and 2). Trabecular number and thickness was increased in both male and female mice at the lumbar spine and in male mice at the distal femur. Trabecular thickness, but not number, was increased in female mice at the distal femur. Increased polar moment of inertia (pMOI) was seen in bones from mice of both sexes with the HBM mutation with or without an OI allele (Table 1).

3.2. Mineralizing surface ratios were increased in OI+HBM mice compared to WT mice

Quantitative histomorphometry was performed on femur sections from at least 3 male WT, OI, OI+HBM and HBM mice (Table 1). Mice with OI + HBM had significantly increased periosteal and endocortical mineralizing surface/total surface ratios compared to WT mice, but not compared to OI mice. The latter result may reflect the adaptive bone growth changes that were previously described in *Col1a1*$^{+/Mov13}$ mice [26].

3.3. Bone strength was increased in OI + HBM mice

Three point bending studies revealed that OI femurs tended to have significantly higher stiffness (for males), ultimate force (for both sexes), and energy to ultimate force (for females) values compared to WT femurs (Fig. 3). Again, these changes likely reflect adaptive long bone changes. The OI + HBM male mice exhibited further increases in ultimate force and energy to ultimate force values compared to OI mice, and male and female mice with the OI + HBM allele had significantly

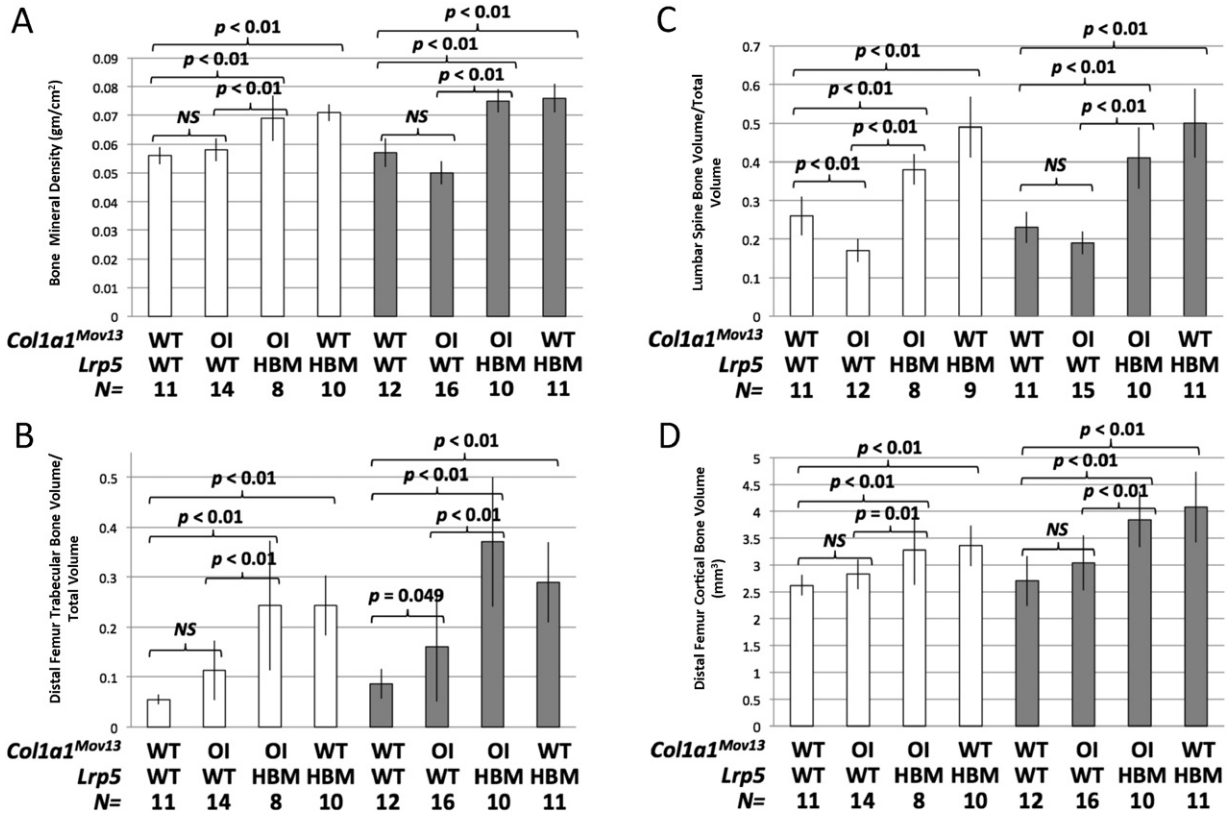

Fig. 1. An *Lrp5* HBM allele increases bone density in mice with a *Mov13* OI allele. Graphs depicting mean (±SD) measures of total body bone mineral density (A), distal femur trabecular bone volume/total volume (B), lumbar spine bone volume/total volume (C), and midshaft femur cortical volume (D), in female (open bars) and male (shaded bars) 8-week-old WT, OI, OI with HBM and HBM mice. Genotypes, WT or heterozygous mutant (OI or HBM, with respect to *Col1a1 and Lrp5*), are indicated, as is the number (*N*) of animals with each genotype that were studied. Brackets indicate comparisons and *p* values between mice with the indicated genotypes. *NS* – not significant.

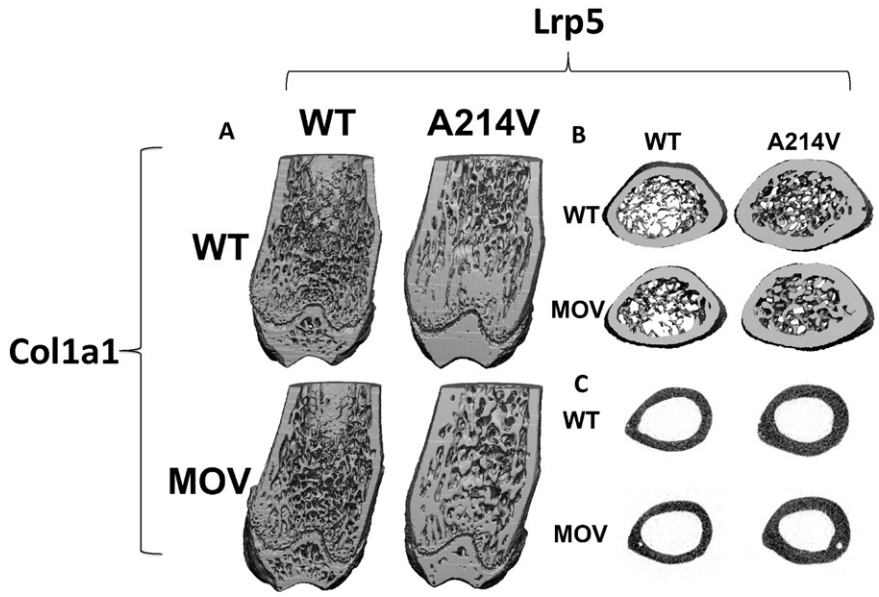

Fig. 2. An *Lrp5* HBM allele increases bone volume in mice with a *Mov13* OI allele. 3D reconstruction of microCT for distal (A and B) and midshaft (C) femurs from 8-week-old WT, OI, OI + HBM, and HBM mice. Note the increased trabecular and cortical bone in OI + HBM mice compared to OI mice.

increased bone stiffness, ultimate force, and energy to ultimate force when compared to their WT counterparts (Fig. 3). As was previously reported [18], we observed that OI mice of both sexes had significantly reduced post-yield displacement compared to WT mice and, importantly, this reduction in post-yield displacement was not improved in OI + HBM mice (Fig. 3D).

3.4. Mineral-to-matrix ratio was unaffected by an Lrp5 HBM allele

FTIRI data from femurs of male mice reveal no significant differences between mice with an OI allele and those with both an OI allele and an *Lrp5* HBM allele at all measures of cortical bone (Table 2). Notably, there was no difference in mineral-to-matrix ratio, which

Table 1
Femur and lumbar spine μCT and quantitative histomorphometry data from mice with different sexes and genotypes.

MicroCT and histomorphometry data				
	WT	OI	OI + HBM	HBM
Femur μCT data				
Females	$N = 11$	$N = 14$	$N = 8$	$N = 10$
Tb. N (1/mm)	3.58 (0.26)	3.28 (0.31)	3.72 (0.47)	4.46 (0.43)**
Tb·Th (μm)	0.04 (0.001)	0.05 (0.12)*	0.09 (0.02)**‡	0.07 (0.01)**‡‡
pMOI	0.34 (0.05)	0.43 (0.11)*	0.56 (0.10)**‡‡	0.47 (0.08)**
Males	$N = 12$	$N = 16$	$N = 10$	$N = 11$
Tb. N (1/mm)	4.08 (0.30)	4.06 (0.58)	4.82 (0.80)**‡‡	5.27 (0.39)**‡‡
Tb·Th (μm)	0.04 (0.01)	0.06 (0.02)**	0.10 (0.10)**‡	0.07 (0.01)**‡‡
pMOI	0.47 (0.18)	0.56 (0.14)	0.70 (0.13)** ‡‡	0.77 (0.12)** ‡‡
Vertebra μCT data				
Females	$N = 11$	$N = 12$	$N = 8$	$N = 9$
Tb. N (1/mm)	4.95 (0.55)	3.72 (0.24)**	4.86 (0.35)‡‡	6.29 (0.64)**‡
Tb. Th (μm)	0.05 (0.004)	0.05 (0.010)	0.08 (0.01)**‡‡	0.08 (0.01)**
Males	$N = 11$	$N = 15$	$N = 10$	$N = 11$
Tb. N (1/mm)	5.10 (0.23)	3.94 (0.27)**	5.07 (0.54) ‡‡	6.28 (0.31)**‡‡
Tb. Th (μm)	0.048 (0.01)	0.06 (0.01)*	0.09 (0.01)**‡‡	0.09 (0.02)**‡‡
Quantitative histomorphometry data				
Males	$N = 3$	$N = 4$	$N = 4$	$N = 4$
PsMS/BS	41.09 (26.98)	60.10 (10.26)	74.55 (19.73)*	73.40 (13.35)
PsMAR (μm/day)	1.81 (0.15)	2.33 (0.75)	2.43 (0.74)	2.50 (0.45)
PsBFR ($\mu m^3/\mu m^2$/year)	0.75 (0.45)	1.48 (0.72)	1.94 (1.07)	1.85 (0.54)
EcMS/BS	51.17 (28.42)	67.90 (5.77)	97.57 (6.61)**	89.54 (5.77)*‡
EcMAR (μm/day)	1.91 (0.94)	1.70 (0.19)	1.39 (0.08)	1.36 (0.15)
EcBFR ($\mu m^3/\mu m^2$/year)	1.10 (0.80)	1.16 (0.22)	1.36 (0.11)	1.23 (0.21)

$N =$ represents the number of animals studied. Mean values (±SD) are shown. Significant differences (*, **, ‡, and ‡‡) are noted between mice with specific genotypes. Note that femur trabecular thickness (Tb·Th) and polar moment of inertia (pMOI) are significantly increased in OI + HBM compared to OI mice. Also note that OI + HBM mice have increased vertebral trabecular number (Tb. N) and Tb·Th compared to OI mice. No significant differences were detected between male OI + HBM and OI mice for quantitative histomorphometry measures of periosteal (Ps) and endochondrial (Ec) mineralizing surface/bone surface (MS/BS), mineral apposition rate (MAR) and bone formation rate (BFR). * $p < 0.05$ compared to WT, ** $p < 0.01$ compared to WT, ‡ $p < 0.05$ compared to OI, ‡‡ $p < 0.01$ compared to OI.

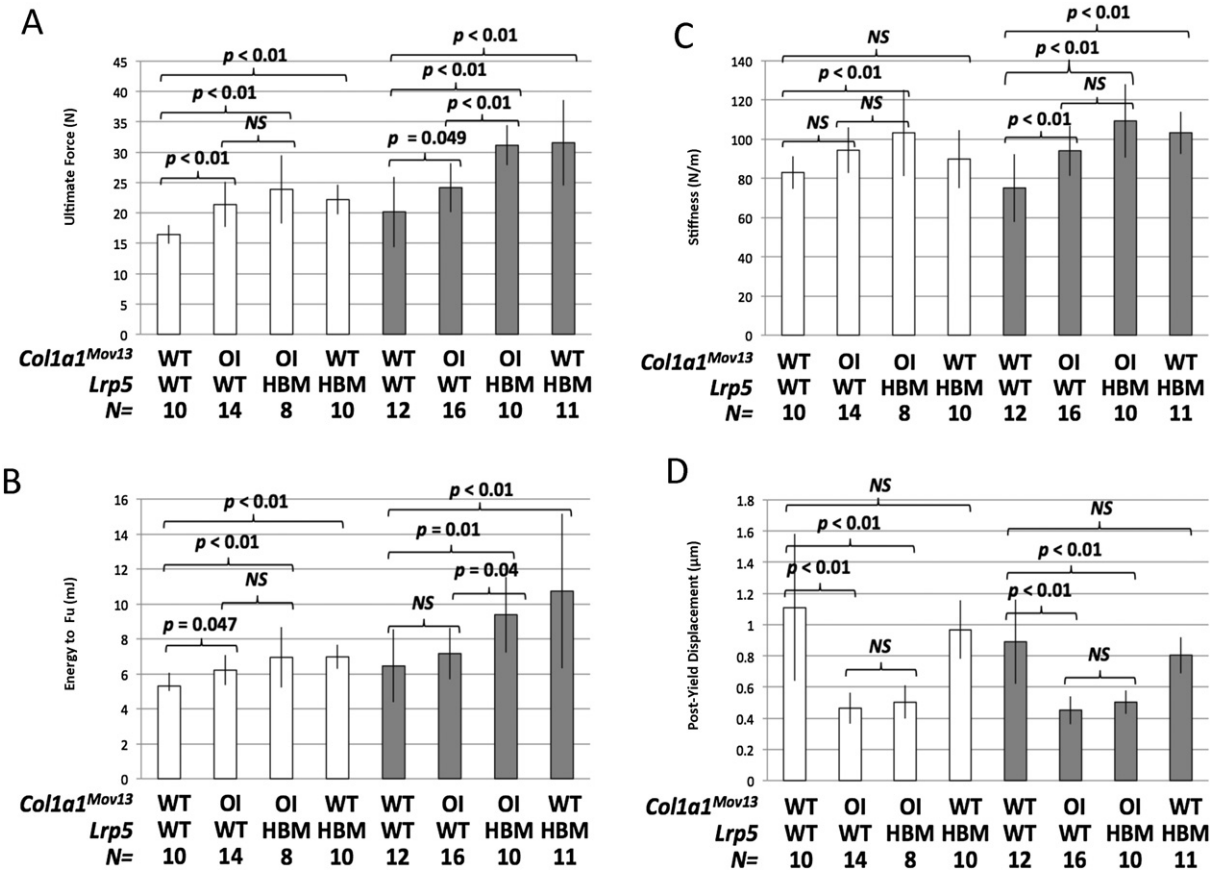

Fig. 3. An *Lrp5* HBM allele increases bone strength but not post-yield displacement in male mice with a *Mov13* OI allele. Graphs depicting mean (±SD) measures of Ultimate Force (A), Energy to Ultimate Force (B), Stiffness (C) and Post-Displacement Yield (D) in female (open bars) and male (shaded bars) 8-week-old WT, OI, OI + HBM, and HBM mice. Genotypes, WT or heterozygous mutant (OI or HBM, with respect to *Col1a1 and Lrp5*), are indicated, as is the number (*N*) of animals with each genotype that were studied. Brackets indicate comparisons and *p* values between mice with the indicated genotypes. *NS* – not significant.

characterizes tissue mineral content and is directly related to ash weight, in either cortical or trabecular bone (Table 2). There were significant differences in carbonate-to-phosphate peak area ratio, collagen cross-link intensity ratio and acid phosphate substitution intensity ratio in trabecular bone suggesting the *Lrp5* HBM mutation alters the mineral lattice, crystal structure and collagen cross-links, at least in trabecular bone (Table 2).

4. Discussion/conclusions

4.1. The *Col1a1*$^{+/Mov13}$ mouse models several features of human OI type I

Although adaptive changes in the shape of the long bones of growing *Col1a1*$^{+/Mov13}$ mice enable the bones to achieve comparable bone strength compared to WT mice, the *Col1a1*$^{+/Mov13}$ mice still incorporate

Table 2
Femur cortical and trabecular bone FTIRI data from male OI and OI + HBM mice.

FTIRI data			
	OI ($N = 6$)	OI + HBM ($N = 6$)	*p*-Value
Cortical bone			
Mineral-to-matrix	8.2 (7.8–8.6)	7.9 (7.5–8.3)	0.256
Carbonate-to-phosphate	0.0047 (0.0045–0.0049)	0.0048 (0.0046–0.0051)	0.520
Collagen cross-link intensity	4.4 (4.0–4.7)	4.1 (3.8–4.4)	0.520
Mineral crystallinity intensity	1.22 (1.20–1.23)	1.24 (1.21–1.27)	0.179
Acid phosphate substitution intensity	0.49 (0.48–0.51)	0.46 (0.43–0.49)	0.069
Trabecular bone			
Mineral-to-matrix	7.2 (6.9–7.5)	6.8 (6.4–7.3)	0.233
Carbonate-to-phosphate	0.0041 (0.0038–0.0043)	0.0045 (0.0043–0.0046)	0.009*
Collagen cross-link intensity	4.6 (4.2–5.0)	4.1 (3.8–4.4)	0.034*
Mineral crystallinity intensity	1.11 (0.90–1.31)	1.24 (1.22–1.27)	0.199
Acid phosphate substitution intensity	0.58 (0.54–0.62)	0.52 (0.50–0.54)	0.011*

$N =$ represents the number of mice studied. Mean values (95% confidence interval) are shown. Significant differences between mice were only observed in the trabecular bone compartment, and did not involve the mineral to matrix ratio.
* Indicates significance of $p < 0.05$.

less type I collagen in their mineralized tissue, have vertebra with lower trabecular BV/TV, and have long bones with lower post-yield deformation in 3-point (this study) and 4-point bending assays [18,26]. We observed that all mice with OI and an *Lrp5* HBM allele had significant increases in femoral and trabecular BV/TV compared to mice with OI alone, and that male OI + HBM mice had significantly increased bone strength. However, the increase in bone strength we observed appears solely related to increased bone mass and/or adaptive changes in bone geometry, not bone quality since the post-yield displacement in OI + HBM mice was not affected. These data are consistent with studies examining the effects of enhanced Wnt signaling in other mouse models of OI, which revealed primarily increased bone formation rather than improved bone quality [13,14].

4.1.1. An Lrp5 HBM allele does not improve bone quality in mice with a Col1a1^{Mov13} allele

There was no significant difference in the mineral-to-matrix ratio of bones from mice with OI compared to those with both OI and an *Lrp5* HBM allele by FTIRI, supporting the lack of improvement in bone quality seen by three-point bending. This poor bone quality is similar to what is seen in bones treated with bisphosphonates, the current standard of care for OI. However, bisphosphonates are anti-catabolic and thus do not induce bone formation [27]. The anabolic nature of enhanced Wnt signaling suggests these therapies could be particularly useful in patients with limited mobility who are unable to accrue bone mass through weight bearing.

4.2. Enhacing Wnt signaling may improve outcomes in patients with OI type I

Type I OI accounts for ~ 2/3 of all reported cases of OI [2,3]. Vertebral compression fractures are a common complication in patients with OI type 1 [3]. The significant increase in trabecular bone BV/TV we observed in the OI + HBM mice could mean that fewer vertebral compression fractures will occur in patients with OI type 1 treated with agents that enhance Wnt signaling. Therefore, our data support the concept of enhancing Wnt signaling to increase bone mass and bone strength in patients with OI type 1. Further benefits in patients with OI will likely be achieved when strategies for improving bone quality, in addition to bone quantity, become clinically feasible.

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