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# Induction of Lrp5 HBM-causing mutations in Cathepsin-K expressing cells alters bone metabolism

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ABSTRACT

High-bone-mass (HBM)-causing missense mutations in the low density lipoprotein receptor-related protein-5 (Lrp5) are associated with increased osteoanabolic action and protection from disuse- and ovariectomy-induced osteopenia. These mutations (*e.g.*, A214V and G171V) confer resistance to endogenous secreted Lrp5/6 inhibitors, such as sclerostin (SOST) and Dickkopf homolog-1 (DKK1). Cells in the osteoblast lineage are responsive to canonical Wnt stimulation, but recent work has indicated that osteoclasts exhibit both indirect and direct responsiveness to canonical Wnt. Whether Lrp5-HBM receptors, expressed in osteoclasts, might alter osteoclast differentiation, activity, and consequent net bone balance in the skeleton, is not known. To address this, we bred mice harboring heterozygous Lrp5 HBM-causing conditional knock-in alleles to Ctsk-Cre transgenic mice and studied the phenotype using DXA, μCT, histomorphometry, serum assays, and primary cell culture. Mice with HBM alleles induced in Ctsk-expressing cells (TG) exhibited higher bone mass and architectural properties compared to non-transgenic (NTG) counterparts. *In vivo* and *in vitro* measurements of osteoclast activity, population density, and differentiation yielded significant reductions in osteoclast-related parameters in female but not male TG mice. Droplet digital PCR performed on osteocyte enriched cortical bone tubes from TG and NTG mice revealed that ~8–17% of the osteocyte population (depending on sex) underwent recombination of the conditional Lrp5 allele in the presence of Ctsk-Cre. Further, bone formation parameters in the midshaft femur cortex show a small but significant increase in anabolic action on the endocortical but not periosteal surface. These findings suggest that Wnt/Lrp5 signaling in osteoclasts affects osteoclastogenesis and activity in female mice, but also that some of the changes in bone mass in TG mice might be due to Cre expression in the osteocyte population.

## 1. Introduction

Most FDA-approved therapies for osteoporosis target the bone-resorbing activities of osteoclasts [1]. More recently, anabolic treatments have been considered a promising approach to improve bone properties. Approved agents that stimulate anabolic action are focused around the PTH/PTHrP axis, but discoveries regarding anabolic potential of the Wnt signaling pathway have provided other avenues to achieve anabolism in bone [2,3]. Several plasma membrane receptors are involved in the Wnt signaling pathway, including the low density lipoprotein receptor-related protein-5 (LRP5). Numerous reports indicate that LRP5 is a key protein involved in the regulation of bone mass [4]. The importance of LRP5 in skeletal regulation has been addressed using genetically engineered mouse models, all of which consistently show significant changes in bone mass and strength with gain- and loss-of function mutations in the gene [5–9].

Much of the high bone mass (HBM) phenotype described in mice with LRP5 HBM-causing mutations (*e.g.* Lrp5-HBM transgene or knock-in point mutations) has been ascribed to altered anabolic signaling in the osteoblast/osteocyte. Mice with HBM-causing missense mutations

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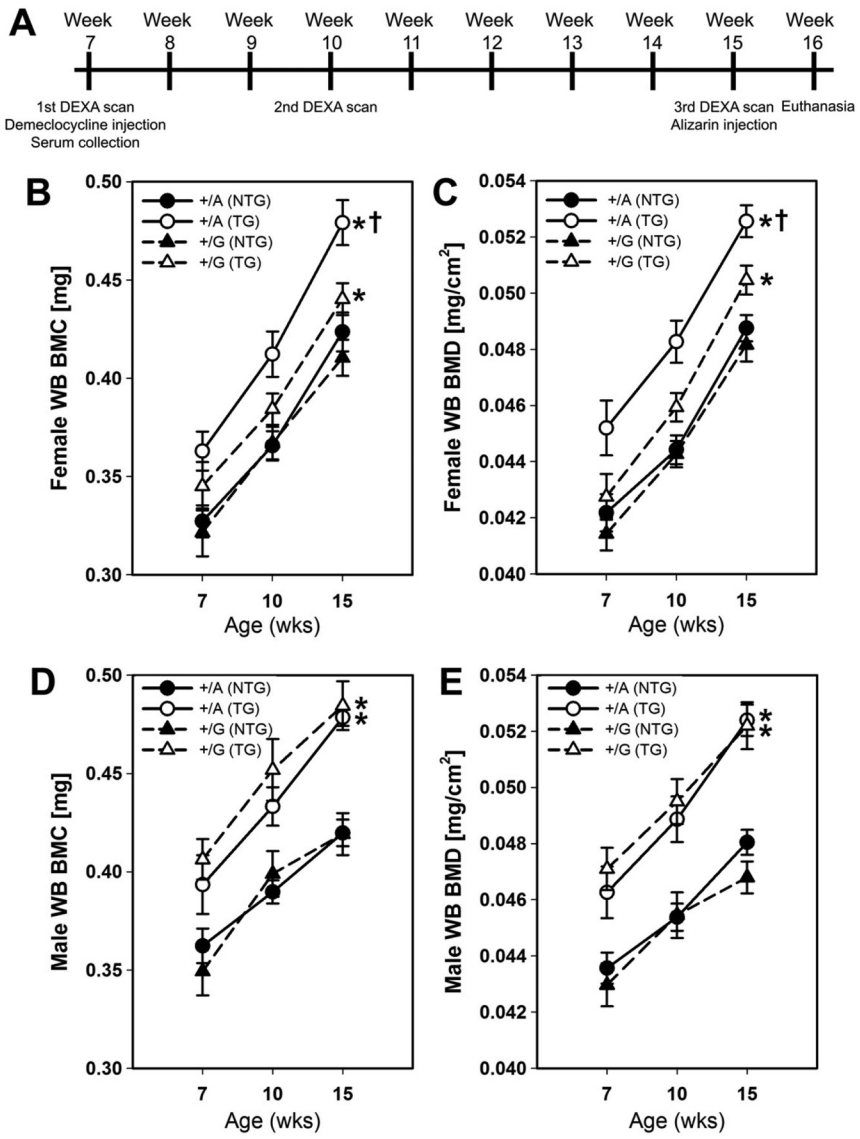


**Fig. 1.** Increased bone DEXA values in mice with activated *Lrp5* HBM alleles in *Ctsk*-Cre expressing cells. (A) Schematic diagram of the experimental design. (B–E) Skeletal mass and density were increased significantly in *Lrp5* HBM conditional mice (+/A or +/G) when the Ctsk-Cre transgene was present (TG, open symbols) compared to non-transgenic mice (NTG, filled symbols). Whole body bone mineral content (WB BMC) and density (WB BMC) were measured by DXA at 7, 10, and 15 weeks using an ROI that encompassed the entire postcranial skeleton of (B and C) female and (D and E) male mice. $^{*}p < 0.05$ compared to Lrp5-matched NTG counterpart, $^{\dagger}p < 0.05$ compared to +/G TG mice. Sample size is $n = 11$/group.

in *Lrp5* provide robust gain-of-function models because LRP5 HBM receptors respond normally to Wnt ligands but exhibit resistance to endogenous inhibitors such as sclerostin (SOST) and Dickkopf homolog-1 (DKK1) [10–13]. Additionally, these mutations enhance responsiveness to mechanical loading and protect the skeleton from the bone-wasting effects of mechanical disuse [14,15].

While most of the published reports on Wnt action in bone have focused on the anabolic effects in osteoblasts, or on the anabolism-relaying effects in osteocytes, much less is known about Wnt signaling in osteoclasts. Osteoclasts are modulated by Wnt proteins, secreted by neighboring osteoblasts and osteocytes [16]. Beyond indirect effects on the osteoclast (*e.g.*, Wnt-modulated RANKL/OPG production by osteocytes/osteoblasts), more recent data suggest that Wnt stimulation can have direct effects on osteoclasts [17]. The most thoroughly characterized mechanism for direct osteoclast stimulation by Wnt is the Wnt5a/Ror2 pathway, which activates one of the non-canonical arms of intracellular Wnt signaling [18]. However, osteoclasts also express the molecular machinery to transduce canonical Wnt signaling (*e.g.*, LRP5/6, FZDs, β-catenin, GSK3β) [19], and deletion of LRP5/6 in early osteoclasts results in increased resorption and low bone mass, whereas treatment of osteoclast cultures with the canonical ligand Wnt3a induces β-catenin nuclear translocation and suppresses osteoclast differentiation [17]. In light of the accumulating evidence that canonical Wnt signaling might have a significant role in the osteoclast, we hypothesized that osteoclast-selective (*i.e.*, *Ctsk*-Cre-mediated) expression of LRP5 receptors harboring the HBM-causing G171V or A214V mutations would exhibit increased bone mass *via* Wnt-mediated inhibition of osteoclasts.

In this communication, we sought to investigate whether expression of LRP5 gain-of-function mutations (G171V, A214V) in osteoclasts would alter bone homeostasis *in vivo* and osteoclastogenesis *in vitro*. Mice harboring heterozygous *Lrp5* HBM-causing conditional knock-in alleles were bred with Ctsk-Cre transgenic mice. We evaluated *in vivo* actions of both *Lrp5* mutant alleles individually, expressed in osteoclasts, on bone mass and degradation. We also explored the *in vitro* effects of the mutations on osteoclast differentiation and activity, using bone marrow hematopoietic stem cells isolated from the mutant mice. Here, we report that *Lrp5* HBM-causing knock-in alleles in *Ctsk*-expressing cells significantly increase overall bone mass and reduce resorption in female mice, but have more mild effects in male mice.

## 2. Materials and methods

### 2.1. Experimental mice

Mice with *Lrp5* conditional knockin alleles for HBM-causing

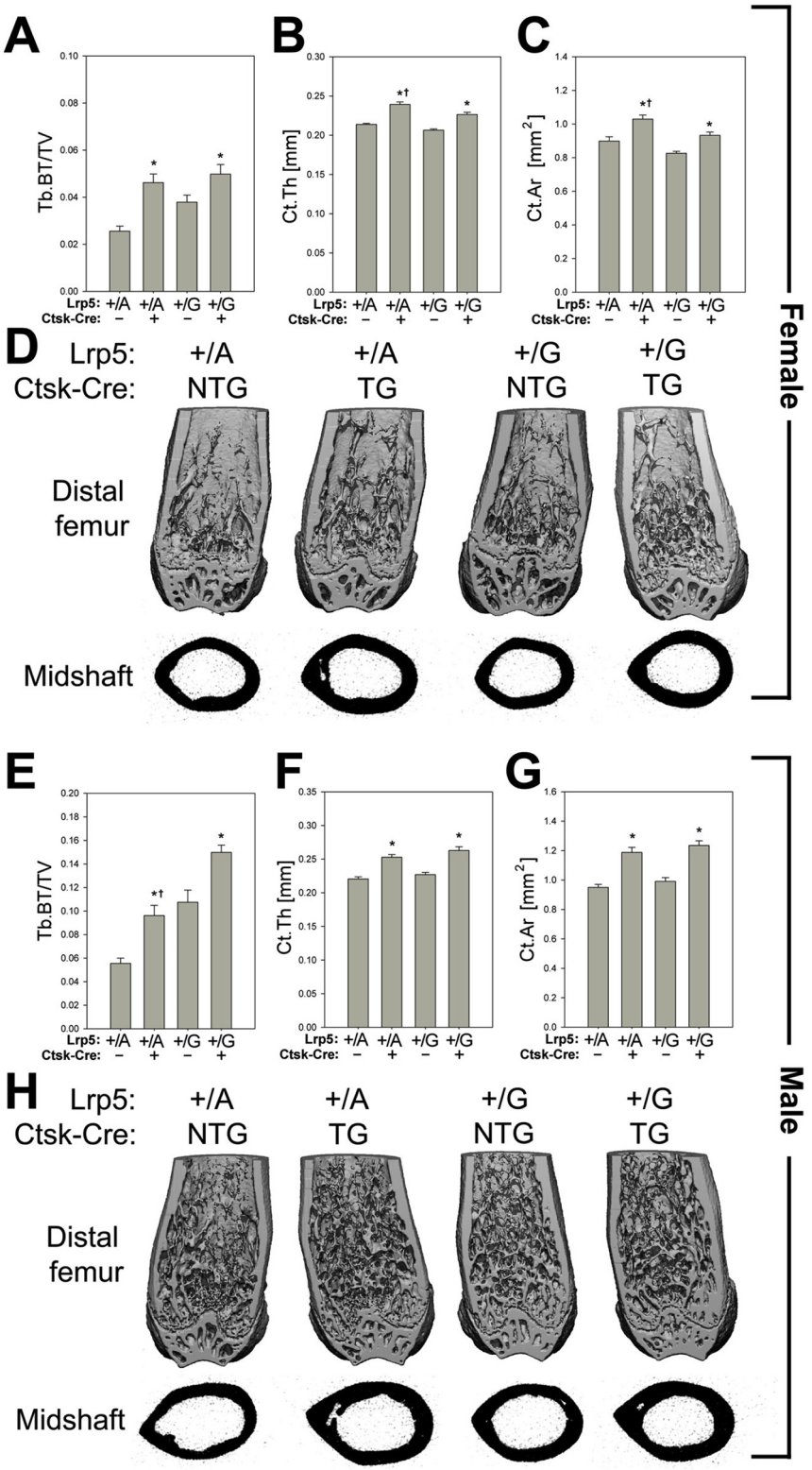


**Fig. 2.** Increased bone μCT values in mice with activated *Lrp5* HBM alleles in *Ctsk*-Cre expressing cells. (A) Trabecular bone volume fraction (Tb.BV/TV), (B) cortical thickness (Ct.Th), and (C) cortical area (Ct.Ar) were measured in the femora of 16-week-old female Lrp5 heterozygous +/A and +/G mice, with (+) or without (−) the Ctsk-Cre transgene (*$p < 0.05$ compared to each NTG counterpart, +$p < 0.05$ compared to +/A TG mice). (D) Representative μCT reconstructions of the distal femur of all female experimental groups. (E–G) Same parameters measured in femora from 16-week-old male mice. (*$p < 0.05$ compared to each NTG counterpart, †$p < 0.05$ compared to +/G TG mice). (F) Representative μCT reconstructions of the distal femur of all male experimental groups. Sample size in $n = 11$/group.

mutations A214V and G171V have been described previously [5]. These alleles, *Lrp5*$^{AN}$ and *Lrp5*$^{GN}$, respectively) are activated by Cre-recombination. Male mice with conditional *Lrp5* alleles were crossed to hemizygous female *Ctsk*-Cre transgenic (TG) mice, which is expressed in osteoclasts [20]. Offspring with the following genotypes were studied TG; +/A (mice with the Lrp5$^{AN}$ allele and the *Ctsk*-Cre transgene), NTG; +/A (mice with the Lrp5$^{AN}$ allele but not the *Ctsk*-Cre transgene), TG; +/G (mice with Lrp5$^{GN}$ allele and the *Ctsk*-Cre transgene), and NTG; +/G (mice with the Lrp5$^{GN}$ allele but not the *Ctsk*-Cre transgene). The genetic background of all mice was a mixture of 129S1/SvIMJ and C57Bl/6J. Offspring were same sex–housed in cages of 3 to 5 (independent of *Ctsk*-Cre genotype) and given standard mouse chow [Harlan Teklad 2018SX; 1% Ca; 0.65% P; vitamin D3 (2.1 IU/g)] and water *ad libitum*. The *Lrp5* alleles and the *Ctsk*–Cre transgene were genotyped using standard PCR on genomic DNA from ear notches. All animal procedures were performed in accordance with relevant federal

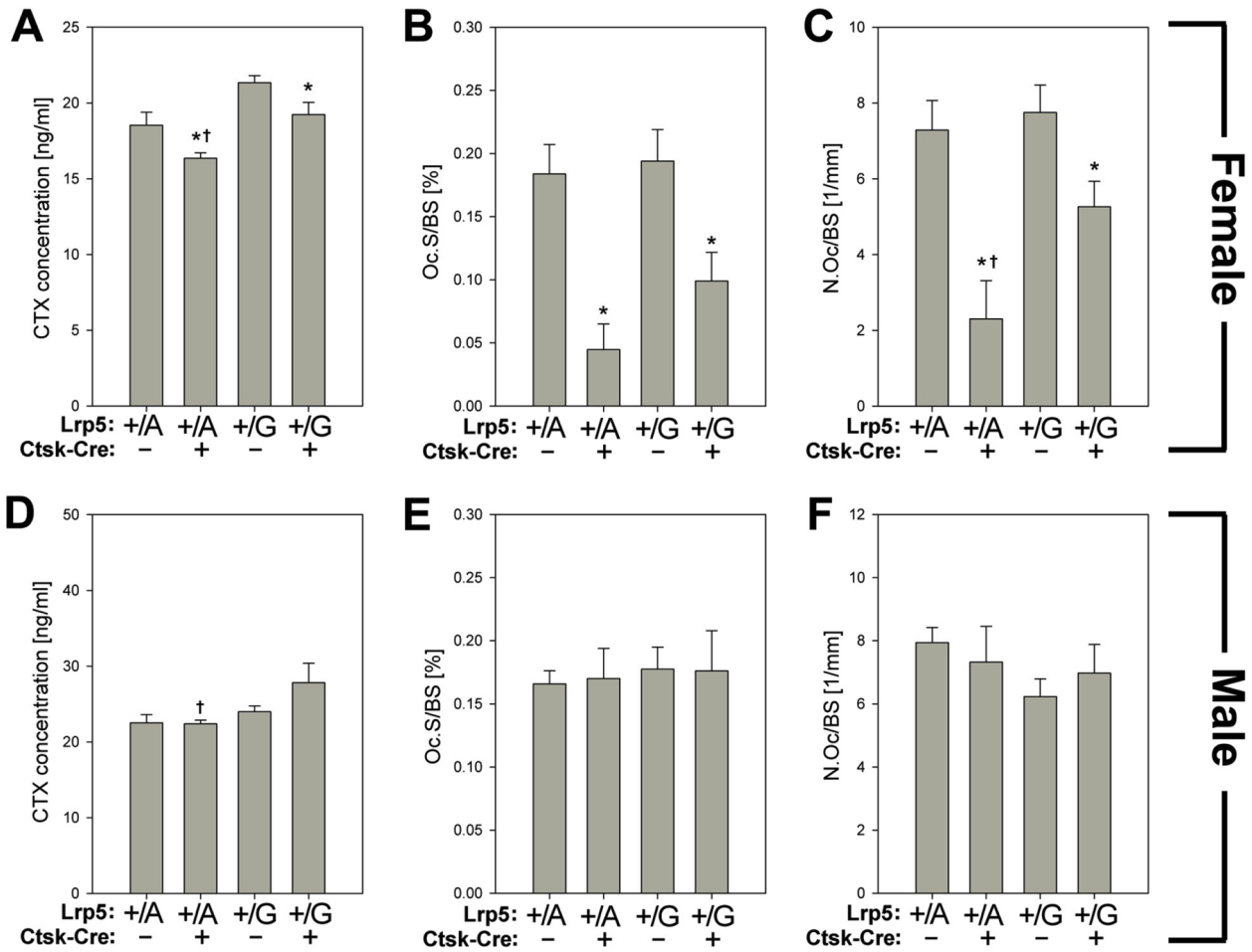


**Fig. 3.** Reduced bone resorption in female, but not male, mice with activated *Lrp5* HBM alleles in *Ctsk*-Cre expressing cells. The resorption marker carboxyl-terminal collagen crosslinks (CTx) was measured from serum collected from (A) female and (D) male Lrp5 heterozygous +/A and +/G mice, with (+) or without (−) the Ctsk-Cre transgene at 7 weeks of age. Osteoclast surface per bone surface (Oc.S/BS; panels B and E) and number of osteoclast per bone surface (N.Oc/BS; panels C and F) were measured in the distal femoral metaphysis in sections from 16-week-old mice by counting tartrate-resistant acid phosphatase (TRAP)-positive cells over bone surface. *$p < 0.05$ compared to each Cre-negative counterpart, †$p < 0.05$ compared to +/G Cre-positive mice. Sample size in $n = 11$/group.

guidelines and conformed to the Guide for the Care and Use of Laboratory Animals (8th Edition). The animal facility at Indiana University is an AAALAC-accredited facility.

### 2.2. Dual-energy X-ray absorptiometry (DEXA)

Whole-body DEXA scans were collected on isoflurane-anesthetized mice using a PIXImus II (GE Lunar, Madison, WI) densitometer. All experimental mice were scanned at 7, 10, and 15 weeks of age as indicated in Fig. 1A. From the whole body scans, areal bone mineral density (BMD) and bone mineral content (BMC) were calculated for the entire postcranial skeleton using the Lunar ROI tools.

### 2.3. Micro-computed tomography (μCT)

The right femur was extracted at sacrifice (16 weeks of age) and fixed in 4% PBF for 2 days, then transferred into 70% ethanol. A 2.6-mm span of the distal femoral metaphysis was scanned on a desktop μCT (μCT 20; Scanco Medical AG) at 13-μm resolution using 70-kV peak tube potential and 151-ms integration time to measure trabecular three-dimensional morphometric properties as previously described [21]. Standard trabecular bone parameters (BV/TV, Tb.N, Tb.Th) were calculated from each reconstructed stack through the metaphysis. Cortical thickness (Ct. Th) and area (Ct.Ar) were obtained from 20 slices reconstructed through the midshaft femur at 9-μm resolution.

### 2.4. Measurements of serum bone resorption markers and osteoclast enumeration in mice

To measure levels of the serum resorption marker carboxy-terminal collagen cross-links (CTx), blood samples were collected from each mouse at 7 weeks of age, allowed to clot for 30 to 60 min, and then centrifuged to separate and collect serum. CTx was measured from the serum using a commercially available plate assay (IDS Ratlaps EIA, Gaithersburg, MD) following the manufacturer's instructions. Serum samples from 10 mice per group were analyzed. Additionally, after conducting μCT measurements on the right femur, we processed those tissues for plastic-embedded thin sectioning and Trap-staining as described previously [22]. Osteoclast number per unit bone surface (Oc.N/BS, #/mm) and osteoclast surface per unit bone surface (Oc.S/BS, %) were measured in metaphyseal cancellous bone.

### 2.5. Osteoclast culture and osteoclastogenic assays

Bone marrow-derived macrophages (BMMs) were prepared as previously described [23]. Briefly, bone marrow was isolated from 6-week old mice heterozygous for the *Lrp5* G171V conditional allele (G171V), with or without the *Ctsk*-Cre transgene. After culturing for 24 h in α-MEM containing 10% FBS, non-adherent cells were collected and cultured in α-MEM containing 10% FBS with M-CSF and RANKL (PeproTech, NJ, USA) For osteoclastogenesis assays, cells were plated at

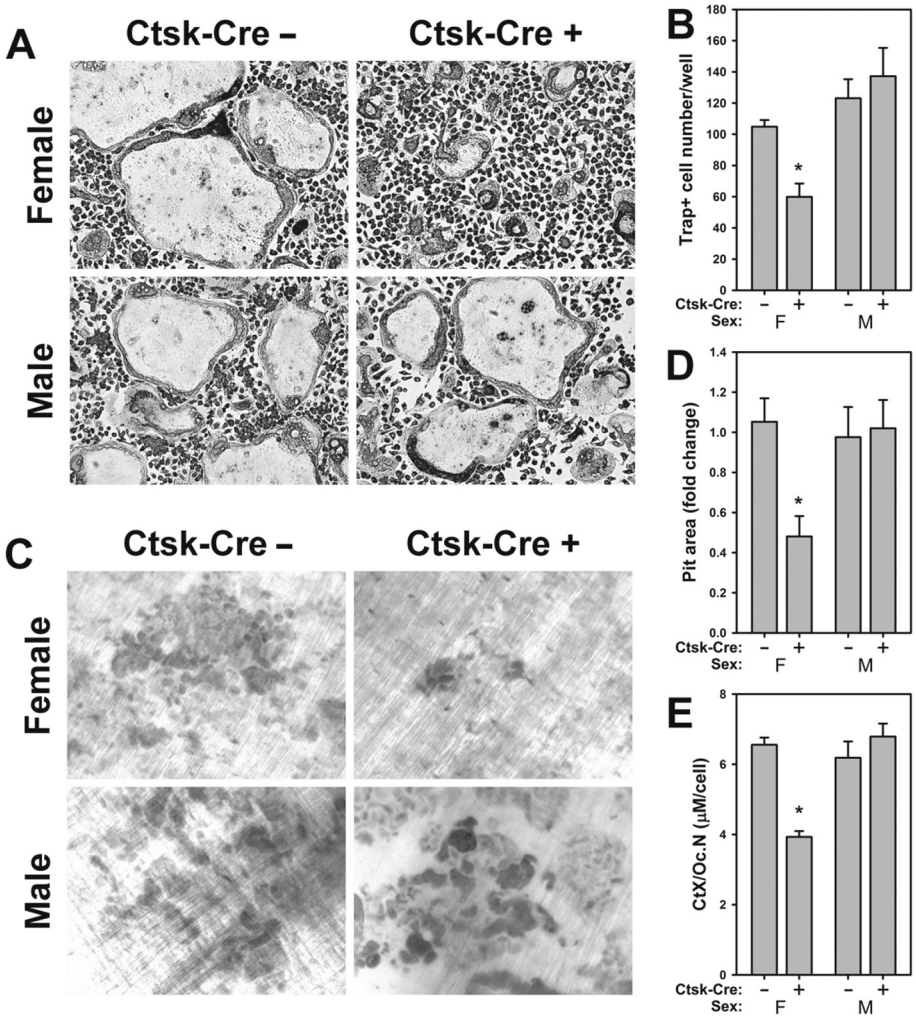


**Fig. 4.** Reduced *in vitro* osteoclastic differentiation and bone resorption in female, but not male, mice with activated *Lrp5* HBM alleles in *Ctsk*-Cre expressing cells. Photomicrographs (A) and graph (B) depicting TRAP stained OCs differentiated from bone marrow, Photomicrograph (C) and graph (D) depicting TRAP stained resorption pit assays, and (E) graph depicting. ELISA-based quantitation of C-terminal telopeptide (CTx) released into the media after 3 days of culture, standardized by osteoclast number (Oc.N). *$p < 0.05$ compared to NTG counterpart. Sample size in $n = 3$/group.

$6 \times 10^4$ cells/well in a 96 well plate and cultured for 4–5 days until the appearance of multinucleated osteoclasts. The osteoclasts were then fixed in 4% paraformaldehyde for 10 min and then stained for TRAP with 0.1 M acetate solution (pH 5.0) containing 6.7 mM sodium tartrate, 0.12 mg/ml naphthol AS-MX phosphate, and 0.07 mg/ml fast red violet. After TRAP staining, the numbers of red-stained TRAP-positive cells that had more than three nuclei and a red cytosol were counted under a light microscope. The average was calculated from 5 wells of 96-well plates. This counting was repeated five times. To determine the bone resorption activity of osteoclasts, multinucleated osteoclasts cultured in the presence of RANKL and M-CSF for 4–5 days were treated with 0.025% trypsin-EDTA for 3–5 min (with light mechanical scraping) to release the cells. The detached mature osteoclasts were washed in growth media, and an identical number of cells were reseeded onto sterilized cortical bone slices (approx. 200 osteoclasts per slice in 96 well plates; IDS, Ltd., Boldon, UK). After 3 days of culture on bone slices, the conditioned media was collected for biochemical evaluation of osteoclast resorption activity (CTx ELISA, IDS Inc., Gaithersburg, MD). The bone slices were TRAP stained for osteoclast enumeration, and the osteoclast counts were used to normalize the CTx data (CTx/OC number). To stain osteoclast resorption pits, the TRAP-stained cells on bone slices were first removed using mechanical agitation, and the slices were incubated with 20 μg/ml of peroxidase-conjugated wheat germ agglutinin for 45 min, followed by the staining of the pits with chromogen 3,3′-diaminobenzidine (Sigma-Aldrich, St Louis, MO). To quantify the resorption on each bone slice, the resorbed pits were identified and measured for area using ImageJ.

### 2.6. Gene expression of osteoclast-selective transcripts using quantitative PCR

Non-adherent bone marrow cells were prepared as described above and cultured with M-CSF alone to form macrophages (50 ng/ml M-CSF for 3 days) or with M-CSF plus RANKL to form pre-osteoclasts (20 ng/ml M-CSF plus 80 ng/ml RANKL for 3 days) and mature osteoclasts (20 ng/ml M-CSF plus 80 ng/ml RANKL for 5 days). The formation of pre-osteoclasts and mature osteoclasts was confirmed based on their morphology. RNA was isolated from the cell preparations using Qiagen RNeasy kits, and the cDNAs were synthesized from 1 μg of RNA using SuperScript synthesis system (Invitrogen). Quantitative RT-PCR was performed on an ABI 7900HT Real time PCR system (Applied Biosystems) using SYBR Green PCR Master Mix assay (Applied Biosystems) for the following transcripts: *Oscar*, *Dcstamp*, *Ctsk*, *Clcn7*, and *Atp6ap1*. Primer sequences are listed in Table S1. The amplification reaction was performed for 40 cycles with denaturation at 95 °C for 10 min, followed by annealing at 95 °C for 15 s and extension and detection at 60 °C for 1 min. Gene expression was quantitated using the $2^{-\Delta\Delta CT}$ method and normalized to transcripts for the housekeeper

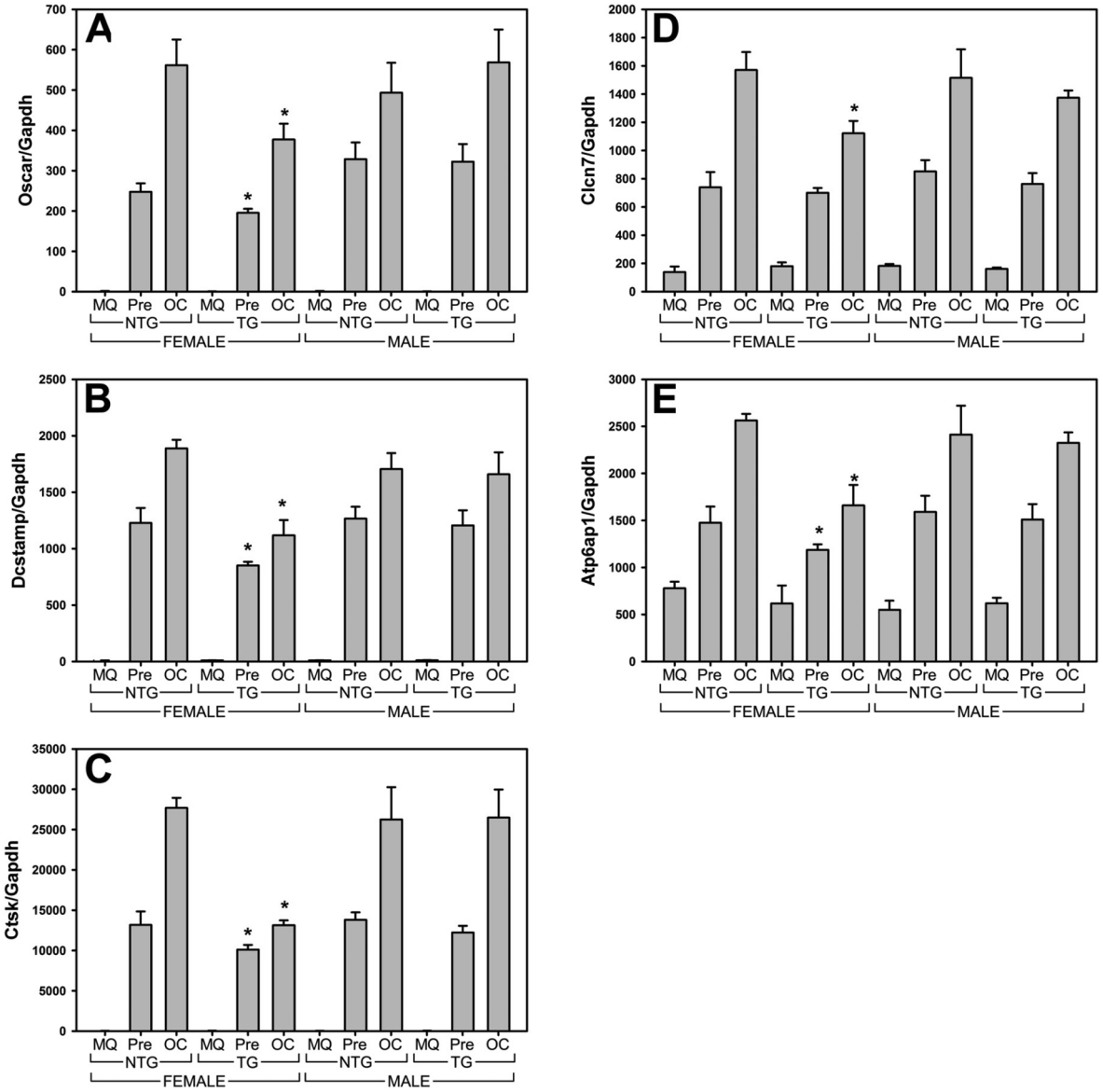


**Fig. 5.** *In vitro* assessment of transcripts associated with osteoclastogenesis and maturity in macrophages (MQ; day 0 of culture from BMMs), preosteoclasts (day 2 of culture in osteoclast-differentiating conditions), and mature osteoclasts (day 4 in osteoclast-differentiating conditions). Quantitative PCR was used to measure expression of (A) osteoclast associated, immunoglobulin-like receptor (*Oscar*), (B) Dendrocyte expressed seven transmembrane protein (*Dcstamp*), (C) Cathepsin-K (*Ctsk*), (D) Chloride voltage-gated channel 7 (*Clcn7*), and (E) V-type proton ATPase (*Atp6ap1*). Expression levels for each gene were normalized to *Gapdh* expression. $^*p < 0.05$ compared to NTG counterpart. Sample size in $n = 3$/group.

GAPDH.

### 2.7. Droplet digital PCR assay for genomic recombination of the conditional Lrp5-HBM alleles

Droplet digital PCR (ddPCR) was performed as previously described [24]. Briefly, epiphyseal ends of bone were removed from cleaned long bones and the bone marrow removed by extensive washing with PBS. DNA was extracted from bone pieces using the DNeasy Blood and Tissue Kit (Qiagen) and ~3 ng of cortical bone DNA was used in subsequent PCR reactions. Supermix for Probes mastermix (BioRad, Hercules, CA) was used following the manufacturer's recommendations. PCR was performed using Eppendorf EP gradient S machines, nanodroplets were created using an automatic droplet generator, amplimer containing droplets were counted with a QX200 sample reader, and data were analyzed using Quantasoft software (all instrumentation from BioRad). The primer pairs and probes described below were purchased from IDT (Coralville, IA) and were used to amplify and quantify the number conditional and recombined alleles. At least 200 amplimer-containing droplets per animal were created in order to measure Cre-mediated recombination. PCR primers, P26-AGTACTGGCTGGCACAGA, P27-CAGGCTGCCCTTGCAGAT, and P28-GTCAGTTTCATAGCCTGA were combined and used in a single PCR reaction to generate a 320-bp amplimer for the conditional allele and a 400-bp amplimer for the recombined allele. The conditional allele was detected using a 5HEX/CCGCAAGCTCTAGAGTCAGCTTCTGAT/3IABkFQ probe, while the recombined allele was detected using a 56-FAM/CGGAATTTAGAGGAT CCCCGGGTACC/3IABkFQ probe. PCR parameters were run as follows: 95 °C for 10 min; 94 °C for 30 s, 57 °C for 60 s, 72C for 30 s, for 40 cycles at 20% ramp; 98 °C for 10 min, and then 12 °C indefinitely.

### 2.8. Quantitative histomorphometry

Demeclocycline (60 mg/kg IP) and calcein (12 mg/kg IP) were

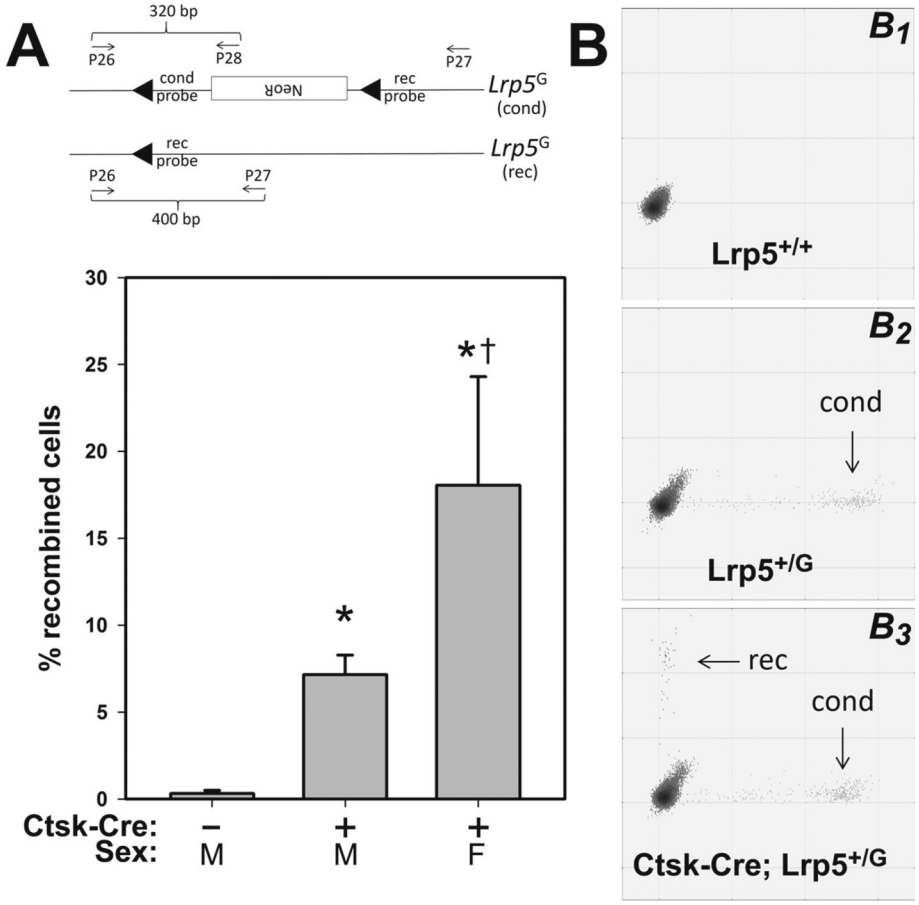


**Fig. 6.** *Ctsk*-Cre is active in bone cells other than osteoclasts. (A) Schematic depicting a portion of the Lrp5 HBM conditional alleles. The ddPCR assay was designed distinguish non-recombined (neo-containing) and recombined (neo-excised) alleles. (B) Scatterplots depicting ddPCR results using cortical bone DNA from $Lrp5^{+/+}$ mice ($B_1$, which contain only empty droplets in the bottom left quadrant), $Lrp5^{+/GN}$ mice without the Ctsk-Cre TG ($B_2$, which also contains droplets with non-recombined alleles ["cond"] in the right lower quadrant), and $Lrp5^{+/GN}$ mice with the *Ctsk*-Cre TG ($B_3$, which now also contains recombined alleles ["rec"] in the left upper quadrant. Graph depicting mean percentages of recombined alleles ( ± 1SD) in male +/G, male TG; +/G, and female TG;+/G mice. $*p < 0.05$ compared to NTG samples, $\dagger p < 0.05$ compared to the TG;+/G male samples. Sample size is $n = 3$/group, and each DNA sample was assayed in triplicate.

injected at 7 and 15 weeks of age, respectively. After collecting left femurs at sacrifice (17 weeks of age), the 4% PBF-fixed femurs were dehydrated in graded ethanols, cleared in xylene, and embedded in methylmethacrylate (MMA). Thick sections were cut from the midshaft using a diamond-embedded wafering saw. Sections were ground and polished to ~30 μm, mounted and coverslipped, then digitally imaged on a fluorescent microscope. Periosteal and endocortical bone formation parameters were calculated by measuring the extent of unlabeled perimeter (nL.Pm), single-labeled perimeter (sL.Pm), double-labeled perimeter (dL.Pm), and the area between the double labeling with Image-Pro software (MediaCybernetics Inc., Gaithersburg, MD). The derived histomorphometric parameters mineralizing surface over bone surface (MS/BS), mineral apposition rate (MAR), and bone formation rate (BFR/BS) were calculated using standard procedures described elsewhere [25].

### 2.9. Statistical methods

Statistical analyses were computed using two-way ANOVA, with *Ctsk*-Cre genotype, TG(transgenic) or non- NTG (non-transgenic), and *Lrp5* genotype, +/A or +/G, as main effects. Significant main effects were followed up with all-pairwise post-hoc comparisons using Fishers protected LSD tests. Significance was taken at $p < 0.05$. All data are presented as mean ± SEM.

## 3. Results

### 3.1. Increased bone mass in mice with activated Lrp5 HBM alleles in Ctsk-Cre expressing cells

To determine changes in bone mineral content and density among mice with *Ctsk*-Cre-driven activation of *Lrp5* HBM alleles, we collected serial whole body DEXA scans from all experimental mice (Fig. 1). *Ctsk*-Cre TG mice with either *Lrp5* HBM allele had increased BMD (5–7% in female, 9–12% in male) and BMC (7–13% in female, 12–15% in male) compared to NTG mice, regardless of sex (Fig. 1). There was no difference in BMC and BMD in male TG mice with the +/A or +/G allele, whereas female TG;+/A mice had significantly higher BMC and BMD than TG;+/G mice (Fig. 1).

Analysis of the distal femur by μCT indicated that male and female TG;+/A and TG;+/G mice had significantly higher trabecular BV/TV, cortical thickness, and cortical area than their NTG littermates (Fig. 2). Vertebral bone mass did not differ between NTG and TG mice for either sex (Fig. S1).

### 3.2. Serum markers of bone resorption and histological measures of osteoclast prevalence are reduced in female but not male mice with Lrp5 mutations induced in Ctsk-Cre-expressing cells

To evaluate osteoclastic activity in these mice, we measured CTx concentration from serum collected at week 7, and we measured the number of, and surface occupied by, osteoclasts in the distal femur metaphysis after sacrifice (Fig. 3). Female TG;+/A and TG;+/V mice had reduced serum CTx concentrations (by 10–13%) compared to their NTG littermates, whereas CTx concentrations did not differ in male TG *versus* NTG mice. Histomorphometric measurements of osteoclast parameters in 16 wk. old mice were consistent with the serum CTx concentration results; female TG;+/A and TG;+/G mice had significantly fewer osteoclasts in the distal femur, reaching a 13–16% decrease in OCL surface and a 32–70% reduction in OCL number, compared to their respective NTG littermates. We did not detect any significant differences in osteoclast histological parameters among male

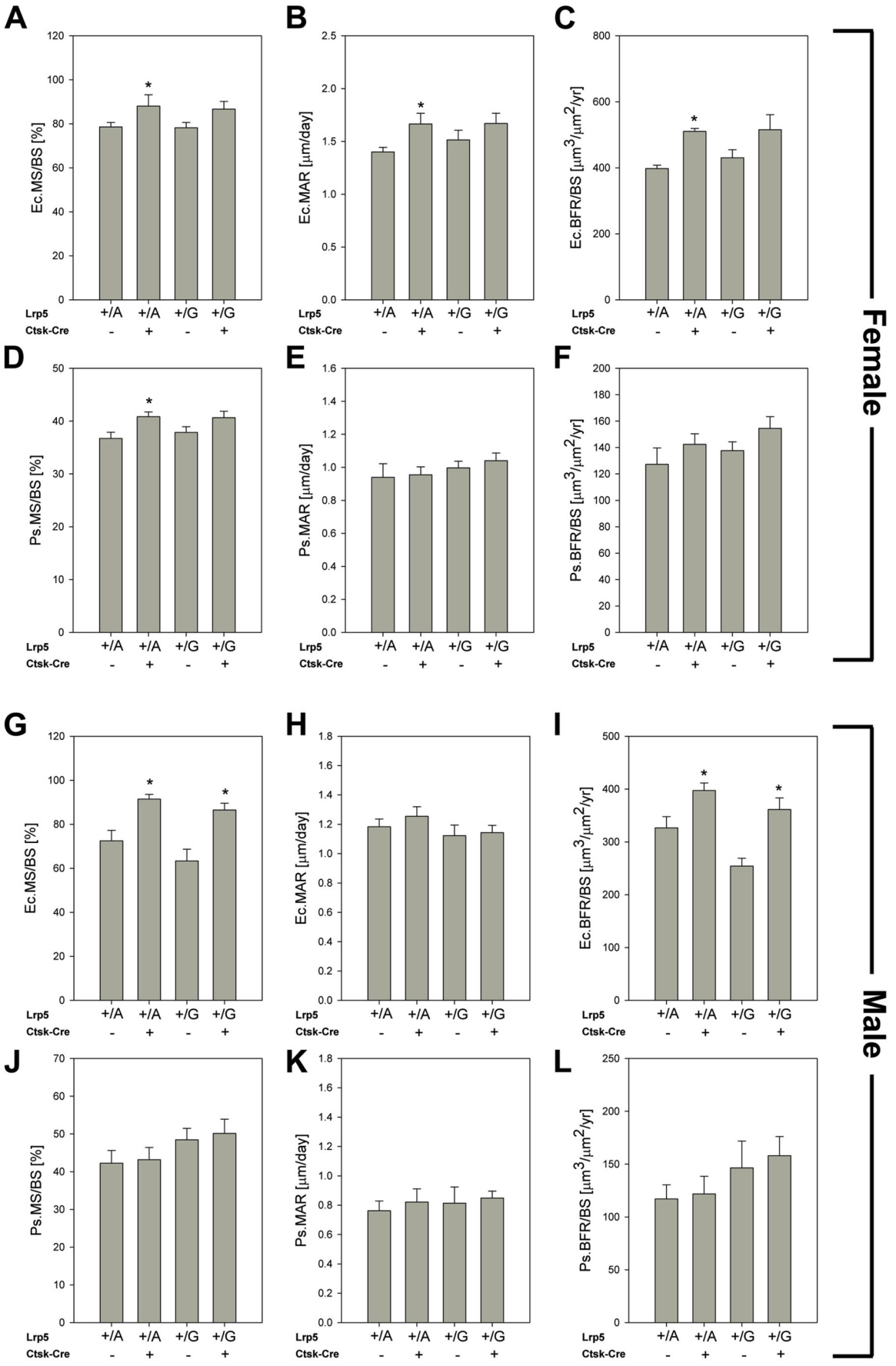


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**Fig. 7.** Increased endocortical, but not periosteal, bone formation in mice with activated *Lrp5* HBM alleles in *Ctsk*-Cre expressing cells. Bone formation parameters were measured in the femoral midshaft from 16-week-old (A–F) female and (G–L) male mice by mineralizing surface per bone surface (MS/BS), mineral apposition rate (MAR), and bone formation rate per unit bone surface (BFR/BS). Endocortical (A–C, G–I) and periosteal (D–F, J–L) were analyzed separately. Both female and male TG mice gained new bones on endocortical region compared to each counterpart but the bone gain in TG;+/G female mice was not significant compared to NTG;+/G female mice. Periosteal BFR/BS in females and males was not statistically noticeable. *$p < 0.05$ compared to each NTG counterpart. Sample size in $n = 11$/group.

mice.

### 3.3. Osteoclast differentiation and activity are reduced in marrow cultures derived from female and not male TG;+/G mice

To determine whether changes in osteoclast-associated measures seen *in vivo* reflect cell autonomous effects of the active Lrp5 allele, we studied osteoclast differentiation and function *in vitro*. Bone marrow-derived hematopoietic stem cells from +/G mice, with or without the *Ctsk*-Cre transgene, were used as a source of OC progenitors, and were induced to differentiate using M-CSF and RANKL. Consistent with what was observed *in vivo*, TRAP staining indicated 45% fewer OCs differentiated *in vitro* from bone marrow progenitors recovered female TG;+/G compared to NTG;+/G mice. Also consistent with the sex specific *in vivo* data, no difference in *in vitro* OC differentiation was observed between male TG;+/G and NTG mice (Fig. 4A–B). The observed sex-related difference in osteoclast formation was not due to initial seeding density, as the same outcome was observed over three different seeding densities (Fig. S1). Additionally, to measure bone resorbing activity of osteoclasts, we re-plated identical numbers of osteoclast-like cells onto bovine cortical bone slices and measured the resorbed ("pit") area after staining. Bone slices containing TG;+/G osteoclasts from female mice had 60% lower pit area than NTG littermate females, while male-derived preparations showed no detectable difference between groups (Fig. 4C–D). Removal of osteoclasts for re-plating onto the bone slices was not associated with a significant loss of, or damage to, cells from female or male mice (Fig. S3). The amount of CTx released into the culture media was also suppressed in female but not male TG mice (Fig. 4E). Expression of osteoclast-selective genes was reduced in female but not male transgenic mice, compared to respective NTG controls (Fig. 5).

### 3.4. The Ctsk-Cre transgene recombines conditional Lrp5-HBM alleles in bone cells other than osteoclasts, with greater "off-target" recombination occurring in females rather than males

Although *Ctsk*-Cre is commonly used to induce Cre-mediated recombination in osteoclasts, it may also be active in other skeletal cell types. These "off-target" effects could contribute to the increased bone mass observed in TG;+/A and TG;+/G mice. Because osteocytes express *Ctsk*, particularly under resorption-inducing conditions (26), and since we previously reported that activation of conditional HBM alleles in osteocytes was sufficient to increase bone mass [5], we determined whether the *Ctsk*-Cre transgene was active in cortical bone which is enriched for osteocytes. We extracted DNA from femoral cortical bone of TG;+/G and NTG mice and used ddPCR to measure the percentage of *Lrp5* conditional alleles in this tissue that had recombined. The rate of recombination in NTG mice did not exceed the background level for this assay (< 1%). In contrast, male TG;+/G mice had ~8% of their cortical bone *Lrp5* alleles recombined; the rate of cortical bone Lrp5 recombination in female TG;+/G mice was even higher (Fig. 6).

In addition to observing off-target expression in osteocytes, we also noted that the *Ctsk*-Cre TG is active during male gametogenesis. If a sire has the *Ctsk*-Cre TG and a floxed allele, the allele will be inherited recombined in the offspring. This is why all animals in this study inherited the floxed allele from the sire and the *Ctsk*-Cre TG from the dam.

### 3.5. Midshaft femur bone formation parameters are increased on the endocortical but not periosteal surface in mice with Lrp5-HBM mutations induced in Ctsk-Cre-expressing cells

Having found evidence for *Ctsk*-Cre induced Lrp5 HBM activation in osteocytes, we performed dynamic histomorphometry to assess whether some of the increased bone mass in TG;+/A and TG;+/G mice might be due to increased bone formation. Endocortical bone formation (MS/BS and BRF/BS) was significantly increased in male and female TG;+/A mice compared to NTG controls and increased in male, but not female, TG;+/G mice compared to controls (Fig. 7). Presence of the TG had no effect on periosteal bone formation.

## 4. Discussion

The importance LRP5 signaling to bone anabolism is well known. Mice with global *Lrp5* HBM mutations and mice with conditional activation of the *Lrp5* HBM allele in osteocytes/osteoblasts, had significantly increased bone formation compared to controls [5,14]. Transgenic mice overexpressing a human LRP5 G171V cDNA in osteoblasts ($^{2.3kb}$Col1α1–Lrp5.G171V) also had significantly increased bone formation [15,27]. LRP5 also appears to have a role in preventing bone catabolism. We reported that *Lrp5* HBM mice were protected from the bone catabolic effects of disuse and estrogen deficiency (OVX) [14]. Unknown, was whether the HBM mice were protected from catabolism because of anti-osteoclastogenic signals that originated from osteocytes/osteoblasts, from a cell-autonomous role for LRP5 during osteoclast differentiation/function, or both. Experiments in the present communication were designed to address a cell-autonomous role for LRP5 in osteoclasts.

We observed that *Ctsk*-Cre-driven recombination of conditional *Lrp5* HBM alleles led to increased bone mass and architectural properties in male and female mice (Figs. 1 and 2), which could be due to increased anabolism or decreased catabolism. *In vivo* evidence of decreased catabolism was observed in female, but not male, mice (Fig. 3). Since osteocytes/osteoblasts help regulate osteoclast activity by expressing RANKL and OPG, we performed *in vitro* osteoclast differentiation and activity assays to determine if the LRP5 effects were cell autonomous. We observed significant reductions in the number of osteoclasts that could be differentiated bone marrow precursors *in vitro* from in female (Fig. 4), but not male, TG;+/G mice, and decreased resorptive ability of isolated TG;+/G osteoclasts *ex vivo* (Figs. 4 and 5). Taken together, these data are consistent with LRP5 having a cell-autonomous role in osteoclast function; this role appears sex-specific.

Because bone mass was increased in male TG;+/A and TG;+/G mice, despite there being no apparent effect on osteoclast differentiation or resorption, we sought another reason these mice developed increased bone mass. We looked for "off target" recombination of the *Lrp5* HBM allele in osteocytes, for which pro-anabolic roles of LRP5 are known. Osteocytes express Ctsk, albeit at much lower levels than osteoclasts [26]. We therefore asked whether *Ctsk*-Cre is also expressed in osteocytes. Droplet digital PCR indicated that Ctsk-Cre was active in ~8 to 17% of osteocytes, with females having greater rates of cortical bone recombination than mice. This low, but appreciable, percentage of recombined cells could account for the 2 to 4% increased bone volume fraction and the elevated endocortical bone formation rate seen in TG;+/A and TG;+/G mice (Fig. 7). Puzzling, however, is that only endocortical bone formation increased, since global HBM and osteocyte/

osteoblast specific activation of HBM alleles increased endocortical and periosteal bone formation [5]. Therefore, we cannot preclude the possibility that increased endocortical bone formation in *Ctsk*-Cre TG mice is the consequence of recombined osteoclasts releasing pro-anabolic coupling factors that affect osteoblasts. The results we obtained using *Ctsk*-Cre to activate Lrp5 HBM alleles are not inconsistent with the results obtained by Weivoda et al. [17], who used *Ctsk*-Cre to inactivate Lrp5; they found no effect on bone mass. However, these investigators did show that bone mass was reduced when Lrp5 was inactivated using a different and earlier-acting osteoclast Cre driver (Rank-Cre) [17].

In conclusion, our results support the hypothesis that there is a cell-autonomous role for LRP5 in osteoclasts. In female mice, *Ctsk*-Cre mediated activation of Lrp5 HBM alleles reduced osteoclast differentiation and bone resorbing activity *in vivo* and *in vitro*. Ctsk-Cre mediated activation of Lrp5 HBM alleles also significantly increased bone mass in male mice, however the mechanism is less certain. It is possible that osteoclasts with active Lrps5 HBM alleles released pro-anabolic factors that affected osteoblasts. Alternatively, “off target” Ctsk-Cre activity in a small percentage of osteocytes may have been sufficient to promote increased endocortical bone formation. Additional experiments are needed to address these two possibilities.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bone.2018.10.007.

## Disclosure statement

The authors have nothing to disclose.

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