Enhanced cortical bone expansion in Lgals3-deficient mice during aging

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INTRODUCTION

Osteoporosis is a growing health concern in the United States due to an aging population.1 During normal bone remodeling, osteoclast precursors from the myeloid lineage are recruited to areas of old or damaged bone primarily by signals from fully mature osteoblasts, known as osteocytes, which sense changes in bone matrix quality or in mechanical stress.2,3 Osteoclast precursors fuse and form a mature osteoclast that is capable of removing damaged tissue via acidic and proteolytic digestion, a process known as bone resorption. This process not only clears out the old bone but also liberates various growth factors from the bone matrix (e.g., Tgfb, Igf1). These growth factors then recruit osteoprogenitors that proliferate and differentiate into osteoblasts that refill the resorption pit with new bone. This cycle of bone resorption and bone formation allows osteoblasts and osteoclasts to regulate each other’s activities, and while these processes are usually balanced,4 aging typically leads to a disproportionate increase in bone resorption and subsequently to osteoporosis.5

Galectin-3 is a chaperone protein which functions in numerous cellular processes intracellularly via protein–protein interactions and extracellularly by binding to specific glycans on glycoproteins.6 These interactions are important for polarized glycoprotein secretion7 and subcellular localization of galectin-3 binding partners.8 Galectin-3 serum levels increase during aging in both mice9 and humans.10 Galectin-3 regulates inflammation11 and Lgals3-deficient mice are protected against fibrosis12–14, suggesting a potential role for galectin-3 in aging related pathologies. Galectin-3 is expressed in bone, but no one has thoroughly addressed whether Lgals3-deficient mice have a skeletal phenotype and whether increased galectin-3 levels during aging play a role in age-related bone loss. Importantly, Lgals3-deficient mice have normal reproduction and life span15 making it unlikely that galectin-3 inhibition would have serious negative health effects.

Galectin-3 is highly expressed by bone cells (osteoblasts, osteoclasts, and chondrocytes).16,17 Previous studies have suggested roles for galectin-3 in osteoblast differentiation18–20 and in the suppression of osteoclast function21 and recruitment.21,22 An early study suggested that adult galectin-3-deficient mice (Lgals3-KO) had normal bone size as determined by standard X-ray analysis.23 However, later studies suggested that there is increased subchondral bone remodeling in Lgals3-KO mice24 and that Lgals3-KO bones have “a higher quantity of trabecular projections in the marrow cavity” of the femoral diaphysis.25 We interpreted the observation of increased trabecular projections as meaning that Lgals3-KO mice may have increased cancellous bone mass due to either increased bone formation and/or reduced bone resorption. The same group that noted the increased trabecular projections25 also suggested that Lgals3-KO femurs were more fragile than wild-type femurs when flushed for bone marrow. Therefore, we hypothesized that Lgals3-KO mice would have increased bone mass, but with reduced bone strength. Because galectin-3 serum levels increase during aging9,10 and initial studies failed to observe a bone phenotype in young adult mice23, we assessed Lgals3-KO mice at multiple time points during aging [12, 24, 36, and 48 weeks (wk)] to determine if the phenotype was age-dependent.

RESULTS

Loss of Lgals3 protected against age-related loss of trabecular bone

To determine if galectin-3 plays a role in trabecular bone mass, we measured trabecular bone parameters in the distal femur and L3 vertebral body by microcomputed tomography (µCT). The results for femurs and L3 vertebrae are displayed in Supplementary Tables 2 and 3, respectively. While Lgals3KO/KO female mice had

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normal trabecular bone measurements in femurs and L3 vertebrae at 12 wk, by 36 wk they had significantly elevated bone mineral density (BMD) and bone volume fraction (BV/TV) in both the femur (+13.3% and +114.8%, respectively; Fig.1) and the L3 vertebral body (+16.7% and +34.7%, respectively). At 36 wk, the increased BV/TV in Lgals3 KO/KO femurs was due to significant contributions from increased trabecular thickness (Tb.Th; +14.5%) and trabecular number (Tb.N; +75%), as well as a significant decrease in trabecular spacing (Tb.Sp; −9.5%). The increase in BV/TV in the vertebral body was primarily due to a significant increase in Tb.Th (+12.8%).

To determine whether the increased trabecular bone mass that we observed at 36 wk in Lgals3-deficient females was restricted to the Lgals3-KO allele or the C57BL/6J background, we analyzed bone from mice carrying an independently derived null allele of Lgals3 (Lgals3-Δ; Fig.2) maintained on a mixed genetic background. Results from Lgals3-Δ are presented in Supplementary Table 4. Trabecular parameters were highly congruent with those...
of Lgals3-KO mice, in that 36-wk Lgals3Δ/Δ females had significantly increased femoral trabecular bone BMD and BV/TV (+40% and +95%, respectively) with a 14% increase in Tb.Th. The L3 BMD was also significantly elevated in Lgals3Δ/Δ females (+23%). Also similar to the Lgals3KO/KO mice, we observed no difference in trabecular parameters in Lgals3Δ/Δ males compared to their respective wild-type littermates.

We performed static- and dynamic-histomorphometry on L3 vertebrae of a second cohort of 24-wk Lgals3-KO mice (Table 1). Female Lgals3KO/KO mice had significantly increased number of osteoblasts per unit of bone surface (N.Ob/BS; +48.6%; Fig.1e). N.Ob/BS was also elevated in male Lgals3KO/KO mice (+23%). Also similar to the Lgals3KO/KO mice, we observed no difference in trabecular parameters in Lgals3Δ/Δ males compared to their respective wild-type littermates.

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Lgals3-deficient females showed significantly increased growth plate height at 36 wk (Lgals3KO/−; +25.7%) and 48 wk (Lgals3KO/−; +28%), both presented in Table S2. Altogether, the data suggested that the increased trabecular bone measurements at 36 wk in Lgals3-deficient females were driven by increased osteoblast numbers and possibly a more active growth plate.

Loss of Lgals3 enhanced cortical bone expansion

To determine if increased cortical bone expansion could also contribute to the increased B.Ar observed in 36-wk Lgals3-deficient animals, we measured changes in cortical bone parameters by µCT. These results are shown in Supplementary Table 5. We observed a significant increase in total area (T.Ar) of both male and female Lgals3KO/KO femurs (Fig. 3d, g) starting at 36 wk (+6.5% and +10.7%, respectively). Lgals3KO/KO females also had significantly increased T.Ar at 48 wk (+8.8%). Male Lgals3KO/− mice also had significantly increased T.Ar at 36 wk (+8.8%). In addition, B.Ar was increased at 36 wk which was significant in Lgals3KO/KO females (+9.9%), but failed to reach significance in Lgals3KO/KO males (Fig. 3e, h). The smaller cortical bone mass effect observed in Lgals3-deficient males may be related to a skeletal growth deficit in young Lgals3KO/− (T.Ar; −5.1% and B.Ar; −4.0%) and Lgals3KO/KO (T.Ar; −7.9% and B.Ar; −8.1%) mice at 12 wk (Fig. 3g, h), although these results did not reach statistical significance. Young female Lgals3-deficient mice had mostly normal or slightly elevated cortical bone parameters at 12 wk, which suggested that
galectin-3 may play a role in male bone mass accrual during early development. We also performed µCT on cortical bone from 36-wk Lgals3Δ/A mice and results are shown in Supplementary Table 6. Similar to Lgals3KO/KO mice, Lgals3Δ/A females had significantly increased B.Ar (+11.6%) and increased T.Ar (+9.5%), but the latter did not achieve statistical significance. Unlike the Lgals3KO/KO mice, we did not observe an increase in cortical bone mass in Lgals3Δ males. Because there was an increase in T.Ar, but no change in cortical thickness in Lgals3Δ/A animals, this suggested that the increased B.Ar was primarily due to increased periosteal bone apposition which was balanced by either a reduction in endosteal bone apposition or increased endosteal bone resorption. Consistent with increased periosteal bone formation driving the increase in T.Ar and B.Ar, dynamic histomorphometry of the femoral midshaft of 24-wk mice (Table 2) showed that Lgals3KO/KO females had a significant increase in periosteal bone formation (Ps.BFR/+45.5%; Fig. 3k) that was primarily driven by a significant increase in the periosteal mineral apposition rate (Ps.MAR/+ 34.4%). There was little change in endocortical bone formation parameters in female Lgals3KO/KO mice.

Loss of Lgals3 reduced bone strength and stiffness. The increased T.Ar and B.Ar in Lgals3KO/KO animals at 36 wk was accompanied by significantly increased measurements of inertia and normal to slightly elevated tissue mineral density (TMD; Fig. 4c, f), which suggested that femurs from these animals were likely to have improved mechanical strength. To test this, 4-point bending was performed on femurs and full results are shown in Table S5. Despite the improved bone geometry, there was not a corresponding increase in whole bone strength (max force) or stiffness (Fig. 4a, d) in Lgals3-deficient females. When max force and stiffness were converted to their tissue level values (max stress and elastic modulus) both Lgals3KO/+ and Lgals3KO/KO female bone tissue withstood significantly less stress at 48 wk (−18.9% and −11.7%) and both had a striking reduction in elastic modulus at 24 wk (−16.4% for both genotypes) and 36 wk (Lgals3KO/+ : −20% and Lgals3KO/KO : −18.3%). Lgals3KO/+ females had significantly reduced elastic modulus at 48 wk (−16.7%), as shown in Fig. 4b.

Lgals3-deficient males trended toward a reduction in both max stress (Table S5) and elastic modulus (Fig. 4e) at 24 and 36 wk, and Lgals3KO/KO males had a significant reduction in whole bone stiffness at 24 wk (−17.4%; Fig. 4d). These results suggest that while the loss of Lgals3 led to increased cortical bone formation and normal mineralization, the quality of the deposited bone was relatively lower in aged Lgals3-deficient mice, particularly in females.

Loss of Lgals3 affected osteoblast, but not osteoclast formation in vitro. We isolated primary calvarial osteoprogenitors from neonatal pups and cultured them with ascorbic acid containing media to stimulate osteoblastogenesis. Calvarial cells isolated from Lgals3KO pups showed enhanced expression of the osteoblast transcription factor, osterix (Osx) and alkaline phosphatase on day 6 (Fig. 5b). Congruently, alkaline phosphatase activity was also increased (Fig. 5a). There were no changes in other osteoblast related genes including Runx2 or osteocalcin (Ocn). These results are consistent with increased osteoblast formation from cultures of Lgals3KO calvarial cells, particularly in the regulation of Osx expression. We also looked at genes involved in osteoblast stimulation of osteoclast differentiation and observed a strong elevation of Opg in Lgals3KO cells on day 6 (Fig. 5b). Opg suppresses osteoclastogenesis by antagonizing Rankl (receptor activator of NF-kB ligand) signaling. We observed no difference in Rankl or macrophage colony stimulating factor (mCSF) expression. However, despite the increased transcription of Opg in calvarial osteoblasts in vitro, we actually observed reduced plasma levels of Opg in Lgals3 KO mice and alterations to the Opg/Rankl ratio (Table 3), suggesting that there is another factor in vivo that disfavors Opg protein levels in Lgals3-KO mice.

Due to a recent paper that suggested that galectin-3 negatively regulates osteoblastogenesis by activating Notch1 signaling, we looked at the expression of a set of Notch pathway genes to see if

**Table 1.** Histomorphometry values from L3 vertebrae of wild-type (Lgals3KO/+), heterozygous (Lgals3KO/KO), and mutant (Lgals3KO/KO) mice at 24 wk

| Variables compared | Females | | Males | |
|--------------------|---------|-------------------------|---------|-------------------------|
| OV/BV              | Wild type | 0.06 ± 0.01           | Mutant | 0.04 ± 0.01           |
|                   |         | 0.06 ± 0.01           |         | 0.05 ± 0.01           |
| OS/BS              | 0.43 ± 0.03 | 0.54 ± 0.02           | 0.29 ± 0.02 | 0.30 ± 0.05           |
|                   |         | 0.47 ± 0.06           |         | 0.40 ± 0.05           |
| O.Wi/μm           | 2.93 ± 0.14 | 3.11 ± 0.13           | 2.80 ± 0.08 | 2.91 ± 0.20           |
|                   |         | 3.08 ± 0.17           |         | 2.58 ± 0.12           |
| (N.OB/BS)/mm⁻¹     | 48.22 ± 4.52 | 71.66 ± 2.87*          | 43.61 ± 4.15 | 40.55 ± 7.82           |
| (N.OC/BS)/mm⁻¹     | 10.70 ± 1.42 | 9.20 ± 0.98           | 9.42 ± 1.93 | 8.04 ± 1.44           |
| Oc.S/BS            | 0.29 ± 0.03 | 0.26 ± 0.04           | 0.25 ± 0.04 | 0.17 ± 0.03           |
|                   |         | 0.26 ± 0.04           |         | 0.24 ± 0.04           |
| Oc.B/BS            | 0.42 ± 0.04 | 0.46 ± 0.04           | 0.41 ± 0.06 | 0.37 ± 0.01           |
|                   |         | 0.46 ± 0.04           |         | 0.36 ± 0.02           |
| Oc/BV              | 1.03 ± 0.17 | 1.00 ± 0.16           | 1.35 ± 0.13 | 1.47 ± 0.22           |
|                   |         | 0.79 ± 0.09           |         | 1.12 ± 0.12           |
| MAR/(μm-d⁻¹)       | 1.01 ± 0.07 | 1.16 ± 0.13           | 1.06 ± 0.07 | 0.72 ± 0.03           |
|                   |         | 1.16 ± 0.13           |         | 0.79 ± 0.04           |
| Aj.AR/(μm-d⁻¹)     | 1.06 ± 0.19 | 1.12 ± 0.16           | 0.82 ± 0.09 | 0.98 ± 0.10           |
|                   |         | 0.82 ± 0.09           |         | 1.13 ± 0.13           |
| (BFR/BS)/(μm³·μm⁻² per day) | 0.43 ± 0.05 | 0.52 ± 0.05           | 0.42 ± 0.05 | 0.25 ± 0.02           |
|                   |         | 0.52 ± 0.05           |         | 0.28 ± 0.02           |
| (BFR/BS)/% per day | 16.61 ± 1.95 | 19.16 ± 1.57           | 15.64 ± 2.05 | 11.28 ± 1.15           |
|                   |         | 15.64 ± 2.05           |         | 12.94 ± 0.54           |
| Omt/d              | 2.97 ± 0.24 | 2.75 ± 0.29           | 3.02 ± 0.25 | 4.01 ± 0.16           |
|                   |         | 2.91 ± 0.69           |         | 3.58 ± 0.25           |
| Mlt/d              | 3.09 ± 0.62 | 2.91 ± 0.69           | 2.40 ± 0.35 | 5.34 ± 0.42           |
|                   |         | 2.40 ± 0.35           |         | 5.21 ± 0.81           |

*Values are expressed as mean ± S.E.M. (n = 6–9). Holm–Sidak post hoc analysis adjusted P-values compared to wild type; bold values highlight *P < 0.05.
those were conversely downregulated in Lgals3-deficient calvarial cells. This did not appear to be the case as Hes1 and Hey1 were modestly increased (~50% each) in Lgals3-KO cells on day 6 (Fig. 5c). Increased Notch signaling has been observed in T-cells and dendritic cells of Lgals3-KO mice. This suggests that genomic loss of galectin-3 may lead to increased Notch signaling.

Galectin-3 has been shown to positively regulate β-catenin protein levels in multiple cell types, so we were also

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Fig. 3 Increased axial expansion of the femur between 24 and 36 wk in Lgals3-deficient mice. a Femoral region of interest (green). b Representative femoral cortical bone sections of 36-wk mice, by μCT. c Diagrams corresponding to total area (T.Ar), bone area (B.Ar), and marrow area (M.Ar). Average ± S.E.M. at indicated time points for T.Ar (d, g), B.Ar (e, h), and M.Ar (f, i) for females and males, respectively. j Representative calcein labeled sections showing extended region of periosteal double labeling in mutant females. k Scatter plot of periosteal bone formation rates. F, Female; M, Male. Asterisks indicate a significant difference, *$P<0.05$. 

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interested in whether there was evidence of downregulation of Wnt/β-catenin mediated transcription in Lgals3-deicient calvarial osteoblasts. However, we observed no difference in the expression of Axin2 (Fig. 5c), a β-catenin target gene.⁴³ Therefore, we do not believe a decrease in Wnt/β-catenin signaling occurs in Lgals3-KO osteoblasts, which is consistent with published models showing reduced bone mass with reduced β-catenin activity.⁴⁴

We wanted to determine if expression of any other galectin family members might occur in the absence of galectin-3. The most striking change in galectin genes was an ~5.5-fold increase in galectin-12 (Lgals12) expression in Lgals3-KO cells after 6 d in osteogenic media (Fig. 5d). Galectin-12 is primarily expressed by adipocytes ³⁵,³⁶, which prompted us to compare expression of adipocyte-related genes from our calvarial cultures. Indeed, we observed a nearly 4-fold increase in Pparg transcripts from Lgals3KO cells (Fig. 5e). However, we did not see significant changes in other adipocyte-related genes from our gene set.

When bone marrow osteoclast progenitors were stimulated to form osteoclasts in response to media containing macrophage colony stimulating factor (mCSF) and receptor activator of nuclear factor κ-B (Rankl), we observed no difference in the formation of multinucleated tartrate resistant acid phosphatase positive cells (TRAP⁺; Fig. 5f–h). This was consistent with our histomorphometry data where we observed no major changes in osteoclast surface values (Table 1) and suggests that the loss of Lgals3 does not have a major effect on osteoclast formation.

**DISCUSSION**

The identification of genes or proteins that can support gains in bone mass is important for the development of novel therapeutic
agents to prevent bone loss due to aging or disease. While there exist efficacious and affordable therapies for preventing bone loss (e.g., sex-hormone replacement or bisphosphonates), the currently available bone anabolic agents, teriparatide and abaloparatide, as well as the anti-sclerostin antibodies that may reach market in the near future, are biologics and therefore carry significantly high price tags. Their cost combined with their limited duration of effectiveness leaves room for the identification of other anabolic targets, particularly those that are targetable by a less expensive small-molecule inhibitor. We believe that galectin-3, which has bone anabolic properties, is a suitable target.

Galectin-3 has been purported to associate in various protein complexes in both glycan-dependent (e.g., Itgβ1, Muc1, and InsR) and glycan-independent (e.g., KRas, spliceosome components, and Bcl-2) interactions. Most galectin-3 interactions can be disrupted by targeting the glycan binding domain with small molecules known as glycomimetics, a class of drugs whose structures resemble glycans. This makes it more likely galectin-3 inhibitors could be synthesized in a cost-effective way.

Fig. 5 Increased osteoblastogenesis and normal osteoclastogenesis observed following differentiation of Lgals3-deficient bone cells in vitro. Calvarial osteoblasts from 2 animals per genotype were cultured for 6 d in osteogenic conditions.  

(a) Differentiation was analyzed by alkaline phosphatase (ALP) staining (Abs = 405 nm) relative to DNA content (Hoechst 33342; 350 nm, 461 nm) and normalized to wild-type ratios. In identical cultures, gene expression was assessed by qPCR (b–e). The results represent the mean ± S.E.M. and are representative of duplicate experiments. Bone marrow myeloid cells from 1 animal per sex per genotype were differentiated into osteoclasts for 5 d in osteoclastic conditions. Representative images of osteoclast cultures. Ostoclast counts for male (g) and female (h) osteoclast differentiation experiments.
Lgals3-KO mice were grossly normal, Lgals3-KO femurs underwent a period of cortical bone expansion between 24 and 36 wk due to enhanced periosteal bone formation. The cortical bone expansion was stronger in Lgals3-KO females, and those mice also showed protection against age-related trabecular bone loss.

We do not yet have a mechanism identified for the age-dependent enhancement of bone mass and cortical expansion in Lgals3-KO mice, but we suspect the phenotype may be dependent upon the age-related boost in osteoclast activity that occurs in C57BL/6 mice around the time of the onset of the bone phenotype we observed in Lgals3-KO mice.8 A possible mechanism for the age-dependent bone phenotype could be that Lgals3-KO osteoblasts have increased sensitivity to one or more of the growth factors (e.g., Igf1) that are released upon the age-related boost in osteoclast activity in high bone turnover.8

Materials and Methods

Animal care
All portions of this study were approved by the Institutional Animal Care and Use Committee at the Van Andel Research Institute (VAR; Grand Rapids, MI, USA). Any animals that showed signs of suffering or distress (e.g., dermatitis, abnormal behavior, injury, etc.) were humanely killed. Mice were monitored every 2–3 d by VARI vivarium staff members trained to identify adverse health events in mice. If mice began showing early signs of an adverse outcome, the cage was marked and monitored daily to determine if the condition advanced or resolved. All mice were housed in climate-controlled conditions (25°C, 55% humidity, and 12 h of light alternating with 12 h of darkness) and fed standard LabDiet Rodent Chow 5010 (Purina Mills, Gray Summit, MO).

Table 3. Plasma Opg and Rankl concentrations in wild-type (Lgals3+/+), heterozygous (Lgals3KO/+), and mutant (Lgals3KO/KO) mice

| Variables compared | Females | Males |
|--------------------|---------|-------|
|                    | 24 wk   | 36 wk | 24 wk | 36 wk |
| Opg/(pg·mL⁻¹)      |         |       |       |       |
| Wild type          | 2 662.7 ± 213.0 | 2 552.5 ± 155.2 | 2 320.6 ± 137.1 | 2 751.5 ± 293.8 |
| Heterozygote       | 2 388.1 ± 189.5 | 2 216.4 ± 246.1 | 2 215.3 ± 180.1 | 2 153.9 ± 198.0* |
| Mutant             | 2 302.0 ± 70.9 | 2 205.6 ± 166.5 | 1 744.9 ± 87.4* | 1 900.9 ± 89.9** |
| Rankl/(pg·mL⁻¹)    |         |       |       |       |
| Wild type          | 153.4 ± 12.4 | 152.1 ± 14.3 | 161.7 ± 16.6 | 188.4 ± 17.5 |
| Heterozygote       | 151.7 ± 10.9 | 175.4 ± 20.7 | 140.5 ± 8.1 | 169.9 ± 9.7 |
| Mutant             | 145.8 ± 10.1 | 188.9 ± 14.9 | 124.5 ± 3.9 | 174.9 ± 7.9 |
| Opg/Rankl          |         |       |       |       |
| Wild type          | 17.9 ± 1.7 | 17.9 ± 2.5 | 15.2 ± 1.5 | 14.7 ± 1.1 |
| Heterozygote       | 16.4 ± 1.9 | 13.2 ± 1.6* | 16.3 ± 2.2 | 13.0 ± 1.6 |
| Mutant             | 16.4 ± 1.3 | 12.3 ± 1.2* | 14.0 ± 0.6 | 11.1 ± 0.9 |

**Values are expressed as mean ± S.E.M. (n = 6–10); Holm–Šidák post-hoc analysis adjusted P-values compared to wild type; bold values highlight *P < 0.05, **P < 0.01.
antibody,\(^5^9\) (gift from John Wang, MSU, East Lansing, MI) which recognizes an epitope that is N-terminal of the region encoded by exon 4.\(^4^0\) No protein product corresponding to full-length or truncated galectin-3 was detected from lung lysates of Lgals3\(^-/-\) mice. The additional benefits of using Lgals3 DNA is that they avoid potential transcriptional effects on neighboring genes by a neomycin resistance cassette;\(^5^7\) also, the genomic deletion is downstream from the galectin-3 internal gene, Galg, which utilizes a promoter in intron 2 and encodes a small protein via an alternative open reading frame of exon 3 of Lgals3.\(^6^2,^6^3\) Figure 2 shows the null alleles utilized in this study.

Genomic DNA was isolated from tail clips at weaning and necropsy by proteinase K digestion and ethanol precipitation. Genotypes were assigned by allele specific PCR. The sequences of primers (synthesized by Integrated DNA Technologies, San Diego, CA) were as follows: The forward primer common to both the Lgals3-wild-type and Lgals3-KO alleles corresponded to intron 1 of Lgals3 (5'-GAG TGA AAT TGC CCA TGA AC-3'), a reverse primer specific for the Lgals3 wild-type allele corresponded to intron 2 of Lgals3 (5'-GAG GAG GAG CTA AGG GAA AG-3'), and a reverse primer specific for the Lgals3-KO allele corresponded to the Neo cassette that replaced the region encompassing exons 2–4 (5'-TGC CCT TCT TGA CGA GTT CT-3'). To identify mice carrying the Lgals3-\(\Delta\) allele we used\(^6^4\) a forward primer common to both the Lgals3-wild-type and Lgals3-\(\Delta\) allele corresponding to intron 3 upstream from the 5' \(loxp\) site (5'-GAT CAG AGC AAG AGA TGG GAG-3'), a reverse primer specific for the Lgals3-wild-type allele corresponding to intron 3 downstream from the 5' \(loxp\) site (5'-GAC TCA TGA CAT ACA GCA CGA CTG-3'), and a reverse primer to detect the Lgals3-\(\Delta\) allele following Cre-mediated excision of the floxed exon 4 that is located in intron 4, downstream from the 3' \(loxp\) site (5'-CCT CAT CTG AAG GCT GCT ATC-3'). The PCR reaction included a hot start at 94 °C for 1.5 min followed by 32 amplification cycles of 94 °C for 30 s, 55 °C for 30 s, 68 °C for 1.5 min + 3 s/cycle. These cycles were followed by 68 °C for 5 min. Then the amplified PCR products were analyzed by 2% agarose gel electrophoresis. For the Lgals3-KO reaction (primers 1+2+3), wild-type mice (Lgals3\(^+/-\)) yielded a single 220-bp band, homozygous mutant mice (Lgals3\(^-/-\)) yielded a single 150-bp band, and heterozygotes (Lgals3\(^-/-\)) both had a 220-bp and a 150-bp band. For the Lgals3-\(\Delta\) reaction (primers 4+5+6), wild-type mice (Lgals3\(^-/-\)) yielded a single 267-bp band, homozygous mutant mice (Lgals3\(^-/-\)) yielded a single 501-bp band, and heterozygotes (Lgals3\(^-/-\)) had both a 267-bp and a 501-bp band.

Plasma isolation and enzyme-linked immunosorbent assays (ELISA)

Immediately following euthanasia, \(0.5\) mL of whole blood was collected by heart puncture and transferred to a microcentrifuge tube containing 5 \(\mu\)L of 0.5 mol·L\(^{-1}\) ethylenediaminetetraacetic acid (EDTA) pH 8.0. To separate plasma, tubes were centrifuged at 6\,000 \(\times\) g for 6 min. The isolated plasma was stored at \(-80^\circ\)C. Samples were thawed on ice and used for ELISA to look at changes in Rankl (receptor activator of nuclear factor kappa B ligand) and osteoprotegerin (Opg) using Quantikine ELISA kits on plasma from 24- and 36-week-old Lgals3-KO mice. ELISAs were carried out according to the included protocols (R&D Systems, Minneapolis, MN). Plasma galectin-3 from male and female wild-type and heterozygous mice was measured using a DuoSet ELISA Development System kit (D1197; R&D Systems, Minneapolis, MN). Wells were coated with 100 \(\mu\)L of a 2 \(\mu\)g·mL\(^{-1}\) dilution of the galectin-3 capture antibody and only 1 \(\mu\)L of plasma was required per well.

Micro-computed tomography (\(\mu\)CT)

After euthanasia, right lower limbs and spines were defleshed and fixed in 10% neutral buffered formalin (NBF) for 72 h, rinsed with sterile distilled water, and stored in 70% ethanol at 4°C. Whole femurs and L3 vertebrae were imaged using a desktop SkyScan 1172 microCT imaging system (SkyScan, Kontich, Germany). Scans for the Lgals3-KO aging portion of this study were acquired at 50 kV using a 13.3 \(\mu\)m voxel size. For scans for the Lgals3-\(\Delta\) portion of this study were acquired at 50 kV using a 5.98 \(\mu\)m voxel size. The femoral trabecular volume of interest in the Lgals3-KO aging study encompassed regions from 0.25–2.75 mm from the distal growth plate. The femoral trabecular volume of interest in the Lgals3-\(\Delta\) portion of this study encompassed regions from 0.25–1.75 mm from the distal growth plate. For all portions of this study the analyses of trabeculae within the body of L3 vertebrae, a 1.5 mm volume centered on the midpoint was used. For all portions of this study, cortical measurements were obtained from a 0.6 mm segment that was 45% of the distance proximal of the length of the diaphysis from the growth plate (diaphysis length = distance of femoral head-distance of growth plate). Tissue mineral density and bone mineral density values were obtained using a standard regression line generated by converting the attenuation coefficients to mineral density from scans of hydroxyapatite standards with known densities (0.25 and 0.75 g·cm\(^{-3}\)).

Mechanical testing

Following euthanasia, left femurs were defleshed, wrapped in phosphate buffered saline (PBS) pH7.2 soaked gauze, and stored at \(-20^\circ\)C. In order to calculate tissue level mechanical parameters we needed to obtain cortical diameter and inertial measurements by \(\mu\)CT. To accomplish this, femurs were thawed at room temperature for 30 min in PBS pH 7.2, analyzed by \(\mu\)CT, scans were acquired at 50 kV using a 13.3 \(\mu\)m voxel size. Cortical regions of interest were determined as previously described for the right femurs. After scanning, the femurs were returned to \(-20^\circ\)C storage. For mechanical testing, the femurs were thawed a second time and allowed to equilibrate to room temperature for 2 h in PBS pH 7.2. Following equilibration, a standard four-point bend testing procedure was performed using a TestResources 570L axial-torsional screw-driven testing system (TestResources, Shakoee, MN) with displacement rate of 0.005 mm·s\(^{-1}\). The distances between the lower and upper supports were 7.3 and 3.5 mm, respectively. The supports had radii of curvature of 0.5 mm at each point of contact with the femur. Displacement was applied by the upper supports in the anterior-posterior direction such that the anterior of the femur was in compression and the posterior was in tension. Force and displacement were directly measured from the load cell and crosshead, respectively. Tissue level mechanical parameters (max stress and elastic modulus) were calculated as described for four-point bending where max stress = (max force \(\times a/c/2(I_{\text{min}}))\) (3L-4a); where a = the distance between an upper and lower support beam, L is the distance between the lower support beams, \(I_{\text{min}}\) is the minimum calculated value of inertia, and c is the radius of the bone. \(I_{\text{min}}\) and c were obtained by \(\mu\)CT.

Histomorphometry and histology

Fourteen and 4 d before euthanasia, Lgals3-KO mice were administered calcine via intraperitoneal injection (20 mg·kg\(^{-1}\)). Right femurs and spines were dissected at euthanasia and fixed in 10% NBF. Bones were embedded in methyl methacrylate using dibutyl phthalate as the plasticizer (Sigma Aldrich, St. Louis, MO) and sectioned with a tungsten carbide profile D microtome blade (Dorn & Hart, Loxley, AL) to 5 \(\mu\)m thickness using a rotary microtome by the VARI histology core. One section per mouse was analyzed to measure single and double fluorescent labels on cancellous bone in coronal sections of the L3 vertebral body and on cortical bone in transverse sections of the femoral midshaft (Bioquant Osteo 2014, Bioquant Image Analysis Corporation, Nashville, TN). Goldner’s Trichrome staining of coronal sections of L3 vertebral bodies was used to get measurements of osteoid, osteoblasts, and osteoclasts. The region of analysis for L3 vertebral bodies encompassed approximately 150–200 \(\mu\)m² of tissue area.
with boundaries set 250 µm from the growth plates and cortical wall.

Following µCT, right femurs were decalcified in 10% EDTA for 2 weeks prior to paraffin embedding and sectioning. Five-micrometer sagittal sections were cut and fixed to glass microscope slides prior to staining with hematoxylin and eosin. Stains were imaged with a Nikon Eclipse 55i microscope equipped with a Nikon Digital Sight camera (Nikon Instruments, Melville, NY). For measurement of growth plate height, 10 equidistant spans of the growth plate of the distal femur were measured using ImageJ software (US National Institutes of Health, Bethesda, MD, USA) and averaged.

Primary cell isolation and differentiation

Osteoblast progenitors were isolated from calvariae of newborn Lgals3-KO mice by serial digestion with 12.4 mg·mL\(^{-1}\) collagenase type I (Worthington Biochemical, Lakewood NJ) in Hanks buffered saline solution (HBSS). Calvariae were placed in 10 mL of collagenase solution for 15 min at 37 °C with shaking at 90 r·min\(^{-1}\). The collagenase solution was decanted and replaced with fresh collagenase and the digestion was repeated. This was performed a total of five rounds; only supernatants from first two rounds were discarded. Supernatants 3–5, containing osteoblasts and osteoprogenitors, were pooled. Cells were cultured in alpha-minimum essential medium (a-MEM; Gibco, Life Technologies Corporation, Grand Island, NY) with 10% heat-inactivated fetal bovine serum (FBS; Hyclone, GE Healthcare, Logan, UT) and 1% penicillin/streptomycin (Gibco, Life Technologies Corporation, Grand Island, NY). Cultures were maintained at 37 °C in a fully humidified atmosphere of 5% CO\(_2\) in air. After 3 d, primary calvarial osteoblast cultures were allowed to expand and subconfluent cells were passed into 96-well plates at 10,000 cells per well. After 2 d, the media was changed to osteogenic media (a-MEM containing 10% FBS, 1% penicillin/streptomycin, 50 µg·mL\(^{-1}\) ascorbic acid, and 2.16 mg/mL β-glycerophosphosphate). RNA was collected in Trizol (Invitrogen, Carlsbad, CA) on day 6 to assess changes in gene expression. Alkaline phosphatase activity was assessed using 1-Step (Invitrogen, Carlsbad, CA) on day 6 to assess changes in gene expression. Galectin primer sets were purchased from Origene (Rockville, MD). All other primer sets were synthesized by IDT (San Diego, CA). Galectin sequences can be found in Supplementary Table 1.

Statistical analyses

For the aging study, differences between Lgals3 wild-type (+/+), Lgals3-deficient (KO/+ and KO/KO) mice were determined using two-way ANOVAs within age groups (sex, genotype). For the galectin-3 plasma ELISA, differences between Lgals3 wild-type (+/+ and Lgals3-heterozygous (KO/+)) mice were determined using two-way ANOVAs within sex (age, genotype). The Holm-Sidak method was used in post hoc analyses to identify significant differences (α = 0.05). All statistics and graphs were generated using GraphPad Prism 6 (GraphPad Software Inc, La Jolla, CA).

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