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Ecto-5'-nucleotidase (CD73) regulates bone formation and remodeling during intramembranous bone repair in aged mice

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Abstract

Ecto-5'-nucleotidase (CD73) generates adenosine, an osteoblast activator and key regulator of skeletal growth. It is unknown, however, if CD73 regulates osteogenic differentiation during fracture healing in adulthood, and in particular how CD73 activity regulates intramembranous bone repair in the elderly by using a CD73 knock-out (CD73^{-/-}) mouse model. Monocortical tibial defects were created in 46 to 52-week-old wild type (WT) and CD73^{-/-} mice. Injury repair was analyzed at post-operative days 5, 7, 14 and 21 by micro-computed tomography (micro-CT), histomorphometry, proliferating cell nuclear antigen (PCNA) immunostaining, alkaline phosphatase (ALP) and tartrate-resistant acid phosphatase (TRAP) histochemistry. Aged CD73 knock-out mice exhibited delayed bone regeneration and significantly reduced bone matrix deposition during bone healing detected by histomorphometry and micro-CT. Cell proliferation, ALP activity and osteoclast number were reduced in the CD73^{-/-} mice, suggesting a combined defect in bone formation and resorption due the absence of CD73 activity in this model of intramembranous bone repair. Results from this study demonstrate that osteoblast activation through CD73 activity is essential during bone repair in aged mice, and it may present a drugable target for future biomimetic therapeutic approaches that aim at enhancing bone formation in the elderly patients.

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Conflicts of interest

B.N.C. has filed a patent on the use of anti-netrin-1 antibodies for the treatment of bone disease (pending), holds patents 5,932,558; 6,020,321; 6,555,545; and 7,795,427—adenosine A1R and A2BR antagonists to treat fatty liver (pending). B.N.C. is a consultant for Bristol-Myers Squibb, Novartis, Eli Lilly & Co., CanFite Biopharmaceuticals, Cypress Laboratories, Regeneron (Westat, DSMB), Endocyte, Protalex, Allos, Inc., Savient, Gismo Therapeutics, Antares Pharmaceutical, Medivector, King Pharmaceutical, Celizome, Tap Pharmaceuticals, Prometheus Laboratories, Sepracor, Amgen, Combinatorx, Kyowa Hakka, Hoffman-LaRoche, and Avidimer Therapeutics and has stock in CanFite Biopharmaceuticals.

P.L. is a consultant for Ankasa Regenerative Therapeutics, Inc and Zimmer Biomet.

Graphical abstract

Keywords

Fracture repair; aging; ecto-5'-nucleotidase; CD73; adenosine

1. Introduction

The skeleton possesses remarkable self-maintenance and self-healing capacity. As individuals age, however, these important hallmarks of bone homeostasis are disturbed by mechanisms that are not fully understood. In addition to the increased risk of skeletal complications such as bone fragility and greater fracture risk due to osteoporosis (Gibon et al., 2016), the healing potential of fractures in the elderly is also decreased, as previously shown (Leucht et al., 2013; Leucht et al., 2007). This decreased healing capacity of fractures in the elderly may be attributed to a decreased number of osteoprogenitor cells and osteoblasts, as well as reduced expression of osteogenic growth factors, altogether culminating in a decreased bone mass (Kusumbe et al., 2014).

Fracture repair is a complex process that requires the recruitment, differentiation and activity of different cell types which coordinately produce and remodel bone matrix in order to reestablish the anatomy and function of the injured bone. During fracture healing several molecules contribute to a cascade of cell signaling events, and one of the key signaling mechanisms that triggers osteoblast differentiation is through adenosine (Costa et al., 2011). Adenosine is generated through de-phosphorylation of adenine nucleotides by ecto-5'-

nucleotidase (CD73). CD73 exists as a cytoplasmic and membrane-bound enzyme. However, in osteoblast, the membrane-bound enzyme is responsible for its function (Mediero and Cronstein, 2013). The adenosine generated by CD73 plays an important tissue-protective role promoting the recruitment of bone marrow mesenchymal stem cells to a variety of wounds, including bone and skin injuries, by activating its receptor A2AR (Katebi et al., 2009). The stimulation of A2AR by topical administration of A2AR specific agonists stimulated bone formation in calvarial defects of young adult mice (Mediero et al., 2015). Previous studies in 13-week-old CD73-knockout (CD73^{-/-}) mice showed a decreased adenosine expression and consequently reduced osteoblast activity, which resulted in mice with osteoporotic phenotype, including reduced trabecular bone volume, decreased trabecular number and thickness and increased trabecular separation. Mice exhibited a lower serum expression of osteocalcin and gene expression of Runx2, alkaline phosphatase, osteocalcin and bone sialoprotein. CD73 deficiency also impairs osteoblast differentiation and mineralization in vitro, however, the importance of CD73 during fracture healing of aged mice was never analyzed (Takedachi et al., 2012).

Improving skeletal regeneration in the aged population is a challenge that remains to be solved. Potential mechanisms that can be targeted to promote fracture healing of mature bone need to be discovered, and osteoblast signaling by adenosine is a promising candidate mechanism to be stimulated in aged bones. The aim of the present study is to investigate the role of CD73-generated adenosine during bone repair of a mono-cortical tibial defect in aged CD73-deficient mice.

2. Materials and methods

2.1. Mice

All procedures were approved by the New York University Committee on Animal Research. Forty-six to fifty-two-week-old, male CD73^{-/-} mice, C57BL/6J background, were purchased from Taconic Biosciences (Hudson, NY, USA). Male wild-type (WT) C57BL/6J mice at the same age range were used as controls. All mice were maintained on a 12-h light/dark cycle with food and water provided ad libitum.

2.2. Surgical procedures

Bone repair by intramembranous ossification was studied using an established monocortical tibial defect model, as previously described (Kim et al., 2007; Minear et al., 2010). After adequate anesthesia and analgesia, the surgical site was clipped and prepped with betadine. A 3mm incision was performed over the anteromedial tibia, the pes anserine insertion was identified and sharply elevated off of the tibia. A 1 mm monocortical defect was drilled with a dental drill (NSK Ultimate XL, Japan) in the location of the pes insertion (approx. 3mm distal from joint line), which allows consistent placement of the injury site. Next, the anterior muscle compartment, which was previously elevated, was then laid over the defect and sutured in place with a 7-0 Vicryl, followed by skin closure. Mice were allowed to ambulate freely post-operatively. Mice were euthanized on post-operative days 5, 7, 14 or 21 (n=5 per time point per group).

2.3. Micro-computed tomography analyses

All injured mice from the study were submitted to micro-CT analysis. Injured tibiae from CD73^{-/-} and WT mice at post-operative days 5, 7, 14 and 21 were carefully dissected and fixed in 4% paraformaldehyde overnight. Specimens were scanned using a high-resolution SkyScan micro-CT system (SkyScan 1172, Kontich, Belgium). Images were acquired at 10µm isotropic resolution using a 10MP digital detector, 10W energy (100kV and 100A), and a 0.5mm aluminum filter with a 9.7µm image voxel size. A fixed global threshold method was used based on the manufacturer's recommendations and preliminary studies, which showed that mineral variation between groups was not high enough to warrant adaptive thresholding. The following parameters were analyzed: total bone volume (BV), total tissue volume (TV), respective mineralized volume fraction (BV/TV), trabecular number (Tb.N), trabecular thickness (Tb.Th) and trabecular spacing (Tb.Sp) following the guidelines described by Buxsein et al. (Buxsein et al., 2010).

The volume of interest (VOI) included a region of the proximal tibia with the defect centered, including 1.1 mm proximal and distal to the injury site in order to capture periosteal callus formation outside of the 1mm defect. The VOI was contoured to capture the entire callus region and total volume (TV) represents the entire callus volume within the above mentioned VOI.

2.4. Structural and compositional bony regenerate strength assessment

The maximum and minimum bending moment of inertia (I_{max} and I_{min} , respectively), as well as the polar moment of inertia (J), where $J = I_{max} + I_{min}$, were measured for the region of the tibial diaphysis containing the defect using the CTan software (Bruker microCT). The volume of interest (VOI) was defined as a region spanning from the most proximal to the most distal extent of the tibial defect in the longitudinal direction (1mm) and therefore included the entire bone injury. Thresholding was performed on the VOI to include all of the volume inside the perimeter of the injured tibia based on a previously described protocol (O'Neill et al., 2012). The I_{max} , I_{min} and J were averaged from all cross-section microCT images in the VOI using the CTan software. I_{max} and I_{min} represent resistance to bending while J indicates resistance to torsion.

2.5. Histology and immunohistochemistry

After micro-CT scanning, the tibiae were decalcified in 19% ethylenediaminetetraacetic acid (EDTA) for approximately 3 weeks at 4 °C. Decalcified samples were dehydrated in a graded ethanol series, embedded into paraffin, and cut into 10-µm thick sections. Aniline blue staining, which labels osteoid matrix, was used to detect osseous tissues as previously described (Kim et al., 2007). Histochemistry for alkaline phosphatase (ALP) and tartrate-resistant acid phosphatase (TRAP) were performed with BM-purple substrate (Roche, Indianapolis, IN) and Leucocyte Acid Phosphatase kit (Sigma, St. Louis, MO), respectively, according to manufacturers' instructions. Proliferating cell nuclear antigen (PCNA) immunohistochemistry was performed to assess cell proliferation at the injury site by using the anti-PCNA antibody (PCNA D3H8O XP rabbit, Cell Signaling Technologies, Danvers, MA) which was detected by streptavidin-conjugated horseradish peroxidase (HRP) and diaminobenzidine (DAB). followed by 4',6-diamidino-2-phenylindole (DAPI) nuclear stain

(Sigma). DAPI was used to count the total number of cells in the region of interest to serve as the denominator for the quantification of PCNA-positive cells. The stained sections were examined and photographed using a Leica digital imaging system.

2.6. Histomorphometry

The assessment of the bone volume formed within the tibial defects was measured as described previously (Kim et al., 2007). Briefly, the tissue sections were photographed using a 5× objective, encompassing the entire defect, and the digital images were analyzed using Adobe Photoshop CC 2015 software. We selected a rectangular region of interest (ROI) centered around the injury site. Every image corresponded to 10⁶ pixels and included the entire defect site. The aniline blue-positive pixels were selected by using the magic wand tool set to a color tolerance of 60. Cortical surfaces, or bone fragments resulting from the drill injury, were manually deselected. The total number of aniline blue-positive pixels for each section was averaged for each experimental group and the differences were calculated based on these averages (Kim et al., 2007).

Quantification of PCNA-positive cells was done using NIH Image J software. All cells with positive nuclear labeling were considered PCNA-positive. Images were obtained using a 20× objective and the total number of counted PCNA-positive cells was divided by the total number of cells detected by DAPI staining. The number PCNA-stained cells/total cells were also analyzed near the tibial cortices in the periosteal area and within the injury.

We quantified ALP staining on sections photographed at 5× magnification. The digital images were analyzed with Adobe Photoshop CC2015 software. The region of interest was selected in a similar fashion as described above for the assessment of bone volume, i.e., a rectangular selection box corresponding to 10⁶ pixels was centered around the injury site, including the entire defect. The BM purple-positive pixels were selected by using the magic wand tool with the same parameters previously described. The total number of BM purple-positive pixels for each analyzed image was averaged for each experimental group, and the differences were determined based on the averages.

The quantification of TRAP-positive osteoclasts and measurement of the trabecular perimeter was performed using ImageJ. Images were obtained using a 40× objective. At least 3 sections from each bone defect, 70 µm or higher distance between sections, were analyzed. Cells labeled for TRAP containing 3 or more nuclei were considered osteoclasts and the number of counted osteoclasts was divided by the measured trabecular surface length. The osteoclasts attached to the trabecular surfaces were considered activated and the percentage of activated osteoclasts in relation to the total number of osteoclasts was calculated.

2.7. Statistical analysis

Prism 7 (GraphPad Software, Inc., La Jolla, CA) was used for statistical analysis. A Student's t test with Holm-Sidak correction was used to quantify differences described in this study. Error bars represent standard deviation. An asterisk symbol (*) indicates a p value of less than 0.05.

3. Results

3.1. CD73^{-/-} mice exhibit decreased callus size after intramembranous bone repair

Microcomputed tomography (micro-CT) analysis showed no difference in bone volume and mineralized volume fraction (BV/TV) at the injury site between WT and CD73^{-/-} mice at post-operative days (POD) 5 and 7 (Fig. 1A,C,E,G,Q,R). Histomorphometry of aniline blue-stained sections, however, revealed significantly lower osteoid deposition within the defect at POD5 in the CD73^{-/-} group, indicating that at this early time point, osteoid was present, but not calcified enough to be detected by micro-CT (Fig. 1B,D,S). At POD 14 and 21, bone volume and mineralized volume fraction, detected with micro-CT, was significantly lower in CD73^{-/-} compared to controls (Fig. 1I,K,M,O,Q,R), which was confirmed by the measurement of the bone volume deposited within the injury site using histology and histomorphometry (Fig. 1J,L,N,P,S). At day 21, the bony regenerate in the knockout defect sites revealed a very thin cortex and a small number of thin trabeculae (Fig 1P), while sections from WT mice presented more abundant and thicker trabeculae underneath a thick layer of cortical bone (Fig. 1N). As a sign of completed remodeling, trabecular volume within the marrow cavity decreased, leading to an overall decrease in BV/TV at 21 days in all groups. Trabecular thickness, number and separation did not reveal any significant differences between the control and knock out animals (data not shown). Mechanical properties were calculated from the micro-CT data set and revealed that the maximum and minimum bending moment of inertia (I_{max} and I_{min} respectively) (Fig. 1T,U,V), as well as the polar moment of inertia (J) showed a tendency to be lower in the CD73^{-/-} at POD14 and 21, indicating a weaker bony regenerate.

3.2. CD73^{-/-} affects cell proliferation in the injury site

PCNA immunostaining was performed at the early time points of tibial healing to detect a potential direct effect of CD73 deficiency and resulting adenosine depletion on cell proliferation during the repair process. The number of PCNA-positive, proliferating cells at POD5 was significantly higher in the injury site of CD73^{-/-} mice compared to the WT counterparts (Fig. 2A,B,E–G). At POD7 the opposite was noted; WT mice showed abundant PCNA-positive cells in the periosteum and within the injury while proliferating cells were scarce in the injury site of CD73-deficient mice (Fig. 2C–G).

3.3. CD73^{-/-} mice exhibit reduced ALP activity during intramembranous bone healing

Alkaline phosphatase histochemistry was utilized to detect osteoblast activity within the bone injury of WT and CD73^{-/-} mice. At POD7, ALP activity in WT mice was present in the periosteum and entire injury site, while being reduced to a thin region bridging the cortical gap in CD73 knockout animals (Fig. 3A,B). At POD14, ALP activity was noted in a larger region encompassing the marrow cavity of both WT and knockout animals (Fig. 3C,D). After 21 days, there was residual ALP activity in the WT injury, while the injury site of the CD73 knockout animals barely showed any osteoblast activity (Fig. 3E). Histomorphometric analysis of the spatial extent of ALP staining, and therefore osteoblast activity, revealed a significantly decreased activity level in the knockout animals at POD 7 and 21, while the difference at POD 14 did not reach statistical difference (Fig. 3G).

3.4. CD73^{-/-} leads to decreased osteoclast activity during the remodeling phase of intramembranous bone healing

The bone resorptive activity of osteoclasts within the tibial defects was detected by TRAP histochemistry. The first signs of bone remodeling during the tibial injury repair were detected at POD7 (Fig. 4A,B). At this time point, the detected number of TRAP-positive osteoclasts per mm of trabecular surface within the injury site was similar in both groups (Fig. 4G). However, osteoclasts exist in two stages, quiescent and activated. Activated osteoclasts are intimately attached to a bony surface. Sub-analysis of the injury sites at day 7 revealed that despite being equal in total osteoclast number, the injuries of CD73^{-/-} mice showed a significantly smaller number of activated osteoclasts (Fig. 4H). No significant differences in the osteoclast count between both groups were noticed at POD14 (Fig. 4C,D,G,H). At POD21, however, a significantly decreased number of total osteoclasts was observed in CD73^{-/-} bone injuries, without a difference in activated osteoclasts. (Fig. 4E,F,G).

4. Discussion

Adenosine signaling is a key mechanism of osteoblast differentiation and activation during skeletal development and bone homeostasis in adult mice (Takedachi et al., 2012). The present study aimed to investigate whether this signaling mechanism is essential for intramembranous bone healing in aged mice. We employed a tibial bone healing model in aged CD73-knockout mice, consisting of a 1-mm monocortical tibial defect (Kim et al., 2007). This bone regeneration model was chosen for its unique property of healing exclusively through intramembranous ossification. Therefore, the monocortical tibial defect model allows for a detailed study of direct bone repair by osteoblasts, without the confounding presence of a cartilaginous callus as seen during endochondral fracture repair.

Cell proliferation within the cambial layer of the periosteum is one of the early events of fracture healing, and precede bone matrix deposition. We observed intense cell proliferation of periosteal cells in the aged CD73-deficient mice at day 5, however at day 7, when proliferative activity in this model is usually at its maximum capacity, the aged CD73-deficient mice showed a significantly lower number of PCNA-positive cells. Adenosine levels are highly increased during early events of fracture repair (Leung et al., 1989), and the generation of adenosine by the enzyme CD73 is essential for signaling the proliferation and differentiation of osteoprogenitor cells (Costa et al., 2011). Our findings show that CD73 activity is not essential for initiation of cell proliferation, but seems to be required for maintenance of cell proliferation, as the aged CD73-deficient mice exhibit a sharp drop-off in cell proliferation at day 7.

In this study, CD73-deficiency resulted in a significant reduction of bone matrix deposition as shown by micro-CT analysis and confirmed by histomorphometry of aniline blue-stained paraffin sections. During the later stages of tibial defect healing the CD73^{-/-} mice showed weaker staining for ALP activity which confirms a decrease in osteogenesis in CD73^{-/-} mice. Decreased bone matrix deposition after fracture leads to overall decreased mechanical strength. We utilized moment of inertia (MOI) measurement from micro-CT data to assess whether the regenerate in the KO animal is weaker. However, the calculation of the MOI

only showed a trend to a lower resistance to bending and torsion of the bony regenerates. Our findings align well with previous studies, such as Takedachi et al, who showed that CD73^{-/-} mice exhibit a phenotype that is characterized by osteopenia in young adult mice, likely resulting from osteoblast deficiency and activity (Takedachi et al., 2012).

Bone remodeling represent the final stage of fracture healing. When we analyzed injuries for their remodeling potential, we noted a decrease in the number of activated osteoclasts within the injury site of CD73-knockout mice. Because osteoblastic bone formation and osteoclastic bone resorption are tightly regulated, we can assume that due to the decreased osteoblastic activity in CD73^{-/-} mice, osteoclastic resorption is also decreased due to the lack of positive feedback from the osteoblastic lineage. This finding points towards an important role of CD73 and adenosine production in promoting remodeling of the primary bone that is laid down in the defect site and later replaced by a mature cortex, which restores the anatomy and function of the injured skeletal element. The osteoporotic phenotype of CD73^{-/-} mice previously described (Takedachi et al., 2012) pointed to the reduced osteoblast activity during skeletal development, but how the CD73 deficiency affects the remodeling process during bone repair is still unknown. CD73 is expressed by bone marrow cells and monocyte-macrophages (Dwyer et al., 2007), and CD73^{-/-} murine bone marrow-derived macrophages have reduced osteoclastogenic differentiation *in vitro* (He et al., 2013). Our findings point towards a possible role of CD73 activity and adenosine signaling on the coupling between osteoblasts and osteoclasts since the deficiency of CD73 activity in knockout mice resulted in decreased ALP activity and decreased activation of TRAP-positive osteoclasts.

Limitations of this study include the lack of biomechanical testing of the uninjured aged CD73 deficient skeleton, as this could potentially reveal an even more profound osteoporotic phenotype compared to the phenotype described by (Takedachi et al., 2012). Also, our current study only describes the effect of CD73 deficiency on intramembranous ossification. The majority of fractures encountered heal through endochondral ossification, therefore it is essential to investigate the effect of CD73 deficiency during endochondral ossification. Future studies will focus on this mechanism and will likely result in a more comprehensive understanding of the effect of CD73 deficiency on the aging skeleton.

Aging remains one of the most challenging obstacles of society. With aging, the skeleton weakens, resulting in a significantly increased incidence of fractures, often resulting from low-energy falls. Orthopaedic surgeons are faced with the sequelae of osteoporosis, and any drug or treatment that may decrease this burden would result in a significant improvement of lifestyle and comfort for our aging generations (Gibon et al., 2016). There is a growing demand for development and optimization of therapies that promote faster and more efficient fracture healing, and understanding the main regulators of fracture healing is crucial in such a search for a novel treatment. Recent findings revealed that targeting adenosine receptors is a promising bone adjuvant therapy (Mediero et al., 2016). Based on previous reports that showed that adenosine generated by CD73 is necessary for the healing of injuries in different organs (Valls et al., 2009);(Feoktistov et al., 2009), we hypothesized that the regulation of CD73 could present a possible mechanism that is responsible for the decline in bone repair in the elderly (Leucht et al., 2013). By using a knockout mouse

model, we identified CD73 activity as essential for osteoblast signaling during intramembranous bone healing of aged mice, which regulates bone formation and subsequent remodeling.

5. Conclusions

Our findings provide evidence that osteoblast stimulation by adenosine signaling is a potential mechanism for the improvement of fracture repair outcomes in the elderly. Further research should explore CD73 and adenosine pathway agonists as a potential mechanism to stimulate bone formation during fracture repair in the elderly population.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- CD73 knock-out significantly delayed bone regeneration in aged mice
- CD73 knock-out results in perturbed cell proliferation and alkaline phosphatase activity in aged mice
- Activation of CD73 may serve as a potential mechanism to improve bone regeneration in the elderly population

Fig. 1. CD73 deficiency impairs intramembranous bone repair

To understand how CD73 contributes to bone healing in aged mice, we used a knockout model in which 1-mm monocortical defects were created in the tibiae and analyzed at different stages of the repair process. At POD 5, micro-CT analysis showed similar bone volume (BV) and mineralized volume fraction (BV/TV) in control and CD73^{-/-} (A,C,Q,R). Histomorphometric analysis revealed a significantly reduced bone volume within the defect in CD73^{-/-} (B,D,S). At POD7, no significant differences in BV and BV/TV were detected by micro-CT and histomorphometry (E,G,Q,R,S), but the aniline-blue stained sections showed a delayed union of the cortices by trabecular bone in the CD73^{-/-} injuries (F,H). At POD 14 and 21 a significantly smaller bony callus was detected in CD73^{-/-} injuries by both micro-CT and histomorphometry analysis (I,K,M,O,Q,R,S). Scale bar = 500 μ m; * $p < 0.05$; c, cortical bone; mu, muscle; m, marrow.

Fig. 2. Altered cell proliferation in CD73^{-/-}

Cell proliferation during the early stages of the tibial defect healing was detected by immunostaining for PCNA. Two separate regions of interest were analyzed: a region within the periosteum adjacent to the cortical bone, and a region in the center of the tibial defect. CD73^{-/-} KO injuries exhibited an increase in proliferative activity at day 5 (A,B, E-F), while WT mice showed a later increase in proliferation at day 7. (C,D, E-F). Scale bar = 50 μ m, * $p < 0.004$; c, cortical bone; p, periosteum.

Fig. 3. ALP activity is decreased in CD73^{-/-} during later stages of bone repair

ALP histochemistry was used to detect bone matrix mineralization during different stages of bone repair. At POD 7 and 14 the intensity of ALP staining was similar within the injuries of both WT and CD73^{-/-} mice (A–D). At POD 21 ALP activity was detected around the bone trabeculae in WT defects, while a very weak signal was seen in the injuries of CD73^{-/-} mice (E,F). Scale bar = 500 μ m, * p < 0.01; c, cortical bone; mu, muscle; m, marrow.

Fig. 4. CD73 deficiency leads to a decreased osteoclast number

TRAP histochemistry was employed to detect osteoclasts in the injury site. The onset of osteoclast recruitment and activation occurs at day 7 in WT and CD73^{-/-} (A,B,G); the number of activated osteoclasts attached to bone was significantly reduced in CD73^{-/-} (H). The number of osteoclasts was similar between both groups at POD 14 (C,D,G,H). At POD 21, the number of osteoclasts detected in CD73^{-/-} was significantly reduced compared to WT (E,F,G). Scale bar = 500 μ m, * $p < 0.05$; c, cortical bone; m, marrow.