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1    **Highlights**

2        • Aged female PGRN-KO mice show severe low bone mass compared to WT mice.

3        • PGRN inhibits TNF- $\alpha$ -induced osteoclastogenesis from PGRN-KO mouse spleen

4    cells.

5        • PGRN promotes osteoblastic differentiation by down-regulating ERK1/2 pathway.

# Title

Progranulin plays crucial roles in preserving bone mass by inhibiting TNF- $\alpha$ -induced osteoclastogenesis and promoting osteoblastic differentiation in mice

## Author names

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## **Abstract**

A close correlation between atherosclerosis, inflammation, and osteoporosis has been  
recognized, although the precise mechanism remains unclear. The growth factor  
progranulin (PGRN) is expressed in various cells such as macrophages, leukocytes,  
and chondrocytes. PGRN plays critical roles in a variety of diseases, such as

atherosclerosis and arthritis by inhibiting Tumor Necrosis Factor- $\alpha$  (TNF- $\alpha$ ) signaling.

The purpose of this study was to investigate the effect of PGRN on bone metabolism.

Forty-eight-week old female homozygous PGRN knockout mice (PGRN-KO) (n=8) demonstrated severe low bone mass in the distal femur compared to age- and sex-matched wild type C57BL/6J mice (WT) (n=8) [BV/TV (%): 5.8 vs. 16.6;  $p<0.001$ , trabecular number (1/mm): 1.6 vs. 3.8;  $p<0.001$ ]. *In vitro*, PGRN inhibited TNF- $\alpha$ -induced osteoclastogenesis from spleen cells of PGRN-KO mice (vehicle $\rightarrow$ 5 $\rightarrow$ 50 ng/ml PGRN: 172.3 $\rightarrow$ 138.0 $\rightarrow$ 132.0 number of osteoclasts per six fields; vehicle vs. 50 ng/ml,  $p<0.05$ ). Moreover, PGRN significantly promoted ALP activity (vehicle $\rightarrow$ 5 $\rightarrow$ 50 ng/ml PGRN: 28.1 $\rightarrow$ 36.5 $\rightarrow$ 51.7 ALP/protein units/ $\mu$ g protein; vehicle vs. 50 ng/ml,  $p<0.05$ ), osteoblast-related mRNA (ALP, osteocalcin) expression in a dose-dependent manner and up-regulated osteoblastic differentiation by down-regulating phosphorylation of ERK1/2 in mouse calvarial cells. In conclusion, PGRN may be a promising treatment target for both atherosclerosis and inflammation-related osteoporosis.

#### **Keywords**

Progranulin, osteoclast, osteoblast, TNF- $\alpha$ , bone metabolism, ERK1/2.

## 1. Introduction

Recent reports have demonstrated a close correlation between atherosclerosis, inflammation, and osteoporosis [1-7], and the existence of common factors has been assumed. TNF- $\alpha$  is strongly associated with both atherosclerosis and arthritis [8, 9], and also promotes osteoclastogenesis and inhibits osteoblastogenesis [10, 11]. PGRN is a 593 amino acid autocrine growth factor, which shows protective effects against Alzheimer's disease and wound healing [12, 13]. Recent reports demonstrated that PGRN inhibits TNF- $\alpha$  signaling and plays critical roles in the pathology of atherosclerosis and arthritis [14-16]. Conversely, PGRN enhances endochondral ossification during development and also acts as a critical mediator of the bone healing process modulating BMP-2 and TNF- $\alpha$  signaling [17]. However, another recent report demonstrated that serum PGRN levels were substantially higher in ovariectomized mice than in sham control mice and PGRN strongly induced osteoclastogenesis in the presence of Receptor Activator of Nuclear factor Kappa-B Ligand (RANKL) [18]. Collectively, the physiological role of PGRN in bone metabolism seems controversial. In this study, we demonstrate a difference in bone mass of the distal femurs between homozygous PGRN-KO mice and WT mice and investigate the direct effect of PGRN on TNF- $\alpha$ -induced osteoclastogenesis and osteoblastic differentiation *in vitro*.

69

## 70 **2. Materials and methods**

### 71 2.1. Animals

72 Homozygous PGRN-KO mice with a C57BL/6J background were obtained from Riken  
73 BioResource Center (Tsukuba, Japan) [19], and WT mice with a C57BL/6J background  
74 were obtained from Charles River Laboratories (Osaka, Japan). Experiments were  
75 performed using age- and sex-matched PGRN-KO mice and WT mice, which were fed  
76 with normal chow and water from birth to 48 weeks in a temperature- and  
77 humidity-controlled facility with a 12 hour light/dark cycle. Mice were anaesthetized with  
78 an intraperitoneal injection of 5.0 mg/kg butorphanol, 4.0 mg/kg midazolam, and 0.3  
79 mg/kg medetomidine and then sacrificed [20]. All experimental protocols were approved  
80 by the Ethics Review Committee for animal Experimentation of Osaka University,  
81 Graduate School of Medicine.

### 82 2.2 Micro-CT

83 The distal femurs of mice (500  $\mu$ m above the growth plate) were evaluated by  
84 micro-computed tomography (micro-CT) (Rigaku Mechatronics, Tokyo, Japan) and the  
85 results were analyzed using Tri/3D Bon software (Ratoc System Engineering Co., Ltd.,

Tokyo, Japan) for various parameters including total volume (TV), bone volume (BV), BV/TV, trabecular thickness (TbTh), trabecular number (TbN), trabecular space (TbS), cortical volume (Cv), all volume (Av), Cv/Av and mean cortical bone thickness.

### 2.3. Histology

After micro-CT scans, specimens were fixed in 10% neutral-buffered and decalcified for embedding. Histological sections were stained with tartrate resistant acid phosphatase (TRAP) following the manufacturer's protocol (Cosmo bio, Tokyo, Japan). The area of TRAP-positive osteoclasts per unit trabecular surface was counted as previously described [21].

### 2.4. Immunohistochemistry

Sections were incubated with anti-osteocalcin antibody (Takara bio, Shiga, Japan) according to the manufacturer's protocol. The next day, the sections were incubated for 30 minutes with secondary antibody (Vectastain Elite ABC kit Rabbit IgG: Vector Laboratories, Inc., San Diego, CA, USA) and stained with 3, 3'-Diaminobenzidine tetrahydrochloride (DAB) (Dako, Tokyo, Japan).

### 2.5. Serum assay



Serum concentration of osteocalcin (Takara Bio), CTX-1 (CUSABIO, Hubei, China), and TNF- $\alpha$  (R&D Systems, Minneapolis, MN, USA) were measured by ELISA kit according to the manufacturer's protocol.

## 2.6. Cell culture

Mouse spleen-derived cells and mouse bone marrow-derived cells flushed from the femur and tibia were cultured in  $\alpha$ -minimum essential medium ( $\alpha$ -MEM) containing 10% fetal bovine serum (FBS) (Equitech-Bio, Kerrville, TX, USA) and 1% penicillin and streptomycin overnight at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>. Adherent cells were seeded at  $1 \times 10^6$  cells per well in a 12-well plate and then stimulated with 10 ng/ml M-CSF (R&D Systems) and 50 ng/ml RANKL (R&D Systems) as previously described [22]. The following day, cells were stimulated with TNF- $\alpha$  (R&D Systems) (vehicle, 1, 5 or 10 ng/ml) and mouse recombinant PGRN protein (R&D Systems) (vehicle, 5 or 50 ng/ml) simultaneously for 5 days in a 48-well plate.

MC3T3-E1 cells were purchased from Riken Cell Bank (Tsukuba, Japan) and murine primary osteoblasts were isolated from the calvaria of three-day old C57BL/6J mice.

Cells were seeded at  $1 \times 10^5$  cells per well in a 12-well plate or  $5 \times 10^4$  cells per well in a 24-well plate, and treated with mouse recombinant PGRN protein (vehicle, 5, 50 or 100 ng/ml) for 5 days. Media were changed to osteoblast differentiation medium containing

120 50 µg/ml ascorbic acid (Sigma-Aldrich, St. Louis, MO, USA) and 10 mM  
121 β-glycerophosphate (Calbiochem, San Diego, CA, USA) after the cells reached 60-70%  
122 confluence.

## 123 2.7. TRAP staining

124 Cells were washed once with PBS and fixed with 10% formalin. TRAP staining was  
125 performed using a TRAP staining kit (Cosmo Bio) according to the manufacturer's  
126 protocol. The total number of TRAP-positive cells with three or more nuclei was counted  
127 in six fields [23].

## 128 2.8. Alkaline phosphatase (ALP) staining, ALP activity assay, and Alizarin red staining

129 Osteoblastic cells were treated with PGRN (vehicle, 5 or 50 ng/ml) and incubated for 5  
130 days. For ALP staining, cells were washed twice with PBS after fixation with 10%  
131 formalin and incubated with ALP substrate solution, 0.1 mg/ml naphthol AS-MX  
132 (Sigma-Aldrich), and 0.6 mg/ml fast violet B salt (Sigma-Aldrich) in 0.1 M Tris-HCl for 30  
133 minutes. For the ALP activity assay, cells were washed twice with PBS and lysed with  
134 Mammalian Protein Extraction Reagent (Pierce, Rockford, IL, USA), and ALP activity  
135 was measured using a Lab Assay ALP activity kit (Wako Pure Chemical Industries, Ltd.,  
136 Osaka, Japan) according to the manufacturer's protocol. ALP protein was quantified

using the Bicinchoninic Acid Protein Assay Kit (Pierce). For Alizarin red staining, cells were washed once with distilled water (DW) after fixation with 10% formalin and stained with alizarin red solution (PG Research Inc., Tokyo, Japan) according to the manufacturer's protocol. Absorbance of the released alizarin red was measured at 415 nm with a microplate reader [24].

## 2.9. Extraction of RNA from bone tissue and cells and first-strand cDNA synthesis

RNA was extracted from the radial bone of PGRN-KO and WT mice according to the QIAzol standard protocol (Qiagen, Düsseldorf, Germany). RNA was extracted from cells in 12-well plate using a RNeasy Mini Kit (Qiagen). First-strand cDNA was reverse-transcribed from total RNA (1 µg) using the SuperScript<sup>III</sup> First-Strand Synthesis system (Life Technologies, Tokyo, Japan).

## 2.10. Quantitative real-time PCR analysis

Real-time PCR was performed using a Step One Plus Real-Time PCR System (Life Technologies) and Fast SYBR Green Master Mix (Life Technologies), in which each cDNA sample was evaluated and expression values were normalized to GAPDH. PCR primers (forward and reverse, respectively) were as follows: *GAPDH*, 5'-AGGTCGGTGTGAACGGATTTG-3' and 5'-TGTAGACCATGTAGTTGAGGTCA-3';

154 *ALP*, 5'-AATCGGAACAACCTGACTGACC-3' and  
155 5'-TCCTTCCACCAGCAAGAAGAA-3'; *Osteocalcin*, 5'-CTCACTCTGCTGGCCCTG-3'  
156 and 5'-CCGTAGATGCGTTTGTAGGC-3'; *TNF- $\alpha$* ,  
157 5'-GGACAGTGACCTGGACTGTGG-3' and 5'-AGTGAATTCGGAAAGCCCATT-3'; *IL-6*,  
158 5'-ACAACCACGGCCTTCCCTACTT-3' and 5'-CACGATTTCCTCCAGAGAACATGTG-3'.

## 159 2.11. Western blotting

160 Cells from mouse calvaria were cultured in 12-well plates and homogenized with 100  $\mu$ l  
161 of RIPA buffer (Pierce), and complete cell lysis was obtained using a sonicator for 7.5  
162 minutes. The lysates were centrifuged at 12,000 rpm for 5 minutes at 4°C and the  
163 supernatants were used for electrophoresis after a protein assay using a BCA assay kit  
164 (Pierce) [25]. Western blotting was performed using the following antibodies purchased  
165 from Cell Signaling Technology (Danvers, MA, USA): phosphate anti-Akt antibody  
166 (Ser473) (1:2000), anti-Akt antibody (pan) (1:1000), phosphate anti-ERK1/2 antibody  
167 (Thr202/Tyr204) (1:2000), anti-ERK1/2 antibody (p44/42) (1:1000), phosphate anti-p38  
168 antibody (Thr180/Tyr182) (1:1000), anti-p38 antibody (1:1000), phosphate  
169 anti-SAPK/JNK antibody (Thr183/Tyr185) (1:1000), anti-SAPK/JNK antibody (1:1000),  
170 phosphate anti- $\beta$ -Catenin antibody (Ser675) (1:1000), anti- $\beta$ -Catenin antibody (1:1000),  
171 and anti- $\beta$ -actin antibody (1:2000).

## 2.12. Statistical analysis

All data were expressed as mean  $\pm$  standard deviation (SD). Differences between the groups were assessed by a Mann-Whitney U-test. A probability value of  $<0.05$  was considered to indicate statistical significance.

## 3. Results

### 3.1. Decreased trabecular bone mass in homozygous PGRN-KO mice

Trabecular and cortical bone in the distal femur were assessed by micro-CT (Fig. 1A). The values of BV/TV and TbN in PGRN-KO mice were significantly decreased compared to WT mice, while TbS in PGRN-KO mice was significantly increased compared to WT mice (Fig. 1B). No significant differences were observed in the cortical bone parameters (Cv/Av, cortical bone thickness) between the groups (Fig. 1C). TRAP staining of the distal femur demonstrated a significantly larger number of osteoclasts in PGRN-KO mice compared to WT mice (Fig. 2A, B). On the other hand, immunostaining of osteocalcin revealed that the number of osteocalcin-positive cells was smaller in PGRN-KO mice compared to WT mice (Fig. 2C). Gene expression levels of TNF- $\alpha$  and IL-6 in bone tissue were higher in PGRN-KO mice compared to WT mice as evaluated

by real-time PCR (Fig. 2D). ELISA of serum bone turnover markers revealed that osteocalcin levels were significantly lower and TNF- $\alpha$  levels were significantly higher in PGRN-KO mice compared to WT mice (Fig. 2E).

### 3.2. Effects of PGRN on osteoclastogenesis

The effect of PGRN on osteoclastogenesis *in vitro* was evaluated. Osteoclast differentiation was induced by TNF- $\alpha$  (vehicle, 1, 5 or 10 ng/ml) using spleen cells of PGRN-KO and WT mice. The number of osteoclasts did not change in WT mice, while they were significantly increased in PGRN-KO mice in a dose-dependent manner (Fig. 3A, B). After inducing osteoclast differentiation by adding 10 ng/ml TNF- $\alpha$  to cells of PGRN-KO mice, treatment with PGRN recombinant protein (vehicle, 5, or 50 ng/ml) significantly decreased the number of TRAP-positive cells in a dose-dependent manner (Fig. 3C, D).

### 3.3. Effects of PGRN on osteoblasts

PGRN significantly promoted ALP activity of MC3T3-E1 cells (Fig. 4A) and WT mouse calvarial cells (Fig. 4B). In addition, PGRN significantly promoted osteoblast-related mRNA (ALP, osteocalcin) expression in a dose-dependent manner (Fig. 4C). Consequently, PGRN promoted mineralization of MC3T3-E1 cells as evaluated by

Alizarin red stain (Fig. 4D). Western blotting revealed that PGRN down-regulated the phosphorylation of ERK1/2 and p38 in a dose dependent manner (Fig. 4E).

#### **4. Discussion**

We have previously demonstrated that PGRN plays important roles in atherogenesis by modulation of local and systemic inflammation [26]. Pro-inflammatory cytokines such as TNF- $\alpha$  enhance osteoclastogenesis partially by inducing RANKL from various cells [10, 11]. Previous reports showed that PGRN binds directly to the Tumor Necrosis Factor receptor (TNFR) and disrupts TNF- $\alpha$  signaling [14, 15, 17]. Therefore, we hypothesized that PGRN may play an important role in bone metabolism, especially in inflammatory conditions. In this study, we have demonstrated for the first time that physiological levels of PGRN (35-70 ng/ml) [27] inhibits TNF- $\alpha$ -induced osteoclastogenesis and also promotes osteoblastogenesis. Furthermore, aged homozygous PGRN-KO mice showed an increased number of osteoclasts and severe trabecular bone loss in the distal femur compared to WT mice.

Concerning osteoclastogenesis, a recent report demonstrated that PGRN-KO mice showed a higher number of osteoclasts and lower bone mineral density (BMD) in

223 vertebra compared to WT mice, but no *in vitro* data were shown [28]. However, another  
224 recent report showed hyper-physiological levels of PRGN (500 ng/ml) promotes  
225 multinucleated osteoclast formation and bone resorption from mouse bone marrow cells  
226 when stimulated with M-CSF (30 ng/ml) and RANKL (100 ng/ml) [18]. In this study, we  
227 demonstrate that physiological levels of PGRN inhibited osteoclastogenesis of  
228 PGRN-KO mouse spleen cells induced by TNF- $\alpha$ . However, even hyper-physiological  
229 levels of PRGN (200 ng/ml) showed no significant effects on osteoclastogenesis of WT  
230 mice spleen cells induced by TNF- $\alpha$  or of WT mouse bone marrow cells induced by  
231 M-CSF and RANKL (data not shown). Taken together, a depletion of physiological  
232 levels of PGRN may lead to elevated serum and local TNF- $\alpha$  levels, which may promote  
233 osteoclastogenesis and consequent bone loss in aged mice.

234 Concerning osteoblastogenesis, a previous report demonstrated that PGRN was  
235 required for BMP-2-induced osteoblastogenesis *in vitro*, although they only showed the  
236 effect of recombinant PGRN protein on Runx2 gene expression of C2C12 cells [17]. In  
237 this study, PGRN-KO mice showed significantly lower levels of serum osteocalcin  
238 compared to WT mice. In addition, physiological levels of PGRN down-regulated the  
239 phosphorylation of ERK1/2 which signaling inhibition leads to osteoblast differentiation  
240 [29], and consequently promoted ALP activity, osteoblast-related gene expression



241 (ALP, osteocalcin), and mineralization of mouse calvarial cells and MC3T3-E1 cells.

242 These results clearly demonstrate that physiological levels of PGRN are effective in

243 promoting osteoblastogenesis.

244 In conclusion, PGRN may be one of the crucial factors to maintain bone mass,

245 especially in aged mice, where it may play interactive roles in both inhibiting

246 TNF- $\alpha$ -induced osteoclastogenesis and promoting osteoblastogenesis by

247 down-regulating phosphorylation of ERK1/2.

248

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253

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355

## Figure Legends

Fig. 1. (A) Micro-CT images of the distal femur from forty-eight-week old WT and PGRN-KO mice. (B) Quantitation of trabecular bone parameters (BV/TV, TbN, TbTh and TbS). (C) Quantitation of cortical bone parameters (Cv/Av and Mean cortical bone thickness). Data are the mean  $\pm$  SD for 8 mice of each group. \*\*\* $p < 0.001$  WT vs. PGRN-KO mice.

Fig. 2. (A) TRAP staining in the distal femur of WT and PGRN-KO mice (200 $\times$ ). (B) The number of TRAP-positive cells per unit trabecular surface (200 $\times$ ). \*\*\* $p < 0.001$  WT vs. PGRN-KO mice. (C) Immunostaining of osteocalcin in the distal femur of WT and PGRN-KO mice (200 $\times$ ). (D) Gene expression of TNF- $\alpha$  and IL-6 in bone tissue of WT and PGRN-KO mice assessed by real-time PCR. (E) Serum levels of osteocalcin, CTX1 and TNF- $\alpha$  of WT and PGRN-KO mice assessed by ELISA. \*\* $p < 0.01$ , \* $p < 0.05$  WT vs. PGRN-KO mice. All data are expressed as the mean  $\pm$  SD.

Fig. 3. (A) (B) Induction of osteoclasts from spleen cells of WT and PGRN-KO mice under TNF- $\alpha$  stimuli assayed by TRAP-staining. \* $p < 0.05$  WT vs. PGRN-KO mice,



373 †p<0.05 vs. vehicle (200×). (C) (D) Induction of osteoclasts from spleen cells of  
374 PGRN-KO mice under TNF-α stimuli and treatment with PGRN assayed by  
375 TRAP-staining \*p<0.05 vs. vehicle (200×). All data are expressed as the mean ± SD.

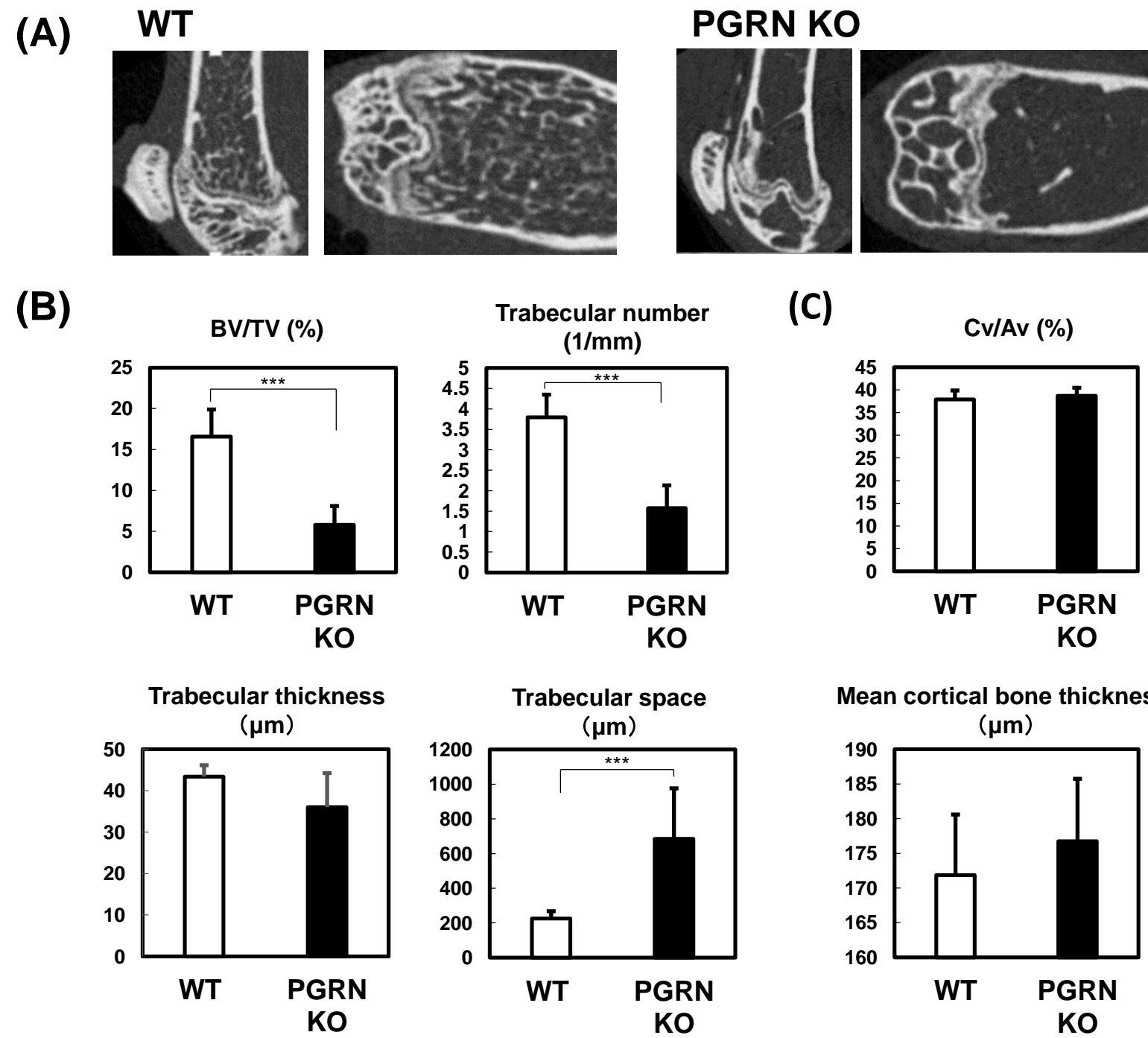
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377 Fig. 4. (A) ALP activity in MC3T3-E1 cells treated with PGRN. \*p<0.05 vs. vehicle. (B)  
378 ALP activity in mouse calvarial cells treated with PGRN. \*p<0.05 vs. vehicle. (C) ALP  
379 and osteocalcin gene expression change in mouse calvarial cells treated with PGRN.  
380 \*p<0.05 vs. vehicle. (D) Mineralization of MC3T3-E1 cells treated with PGRN assayed  
381 by Alizarin red staining. \*p<0.05 vs. vehicle. (E) Western blotting analysis of the  
382 phosphorylation of osteoblast differentiation-related signals in mouse calvarial cells. All  
383 data are expressed as the mean ± SD.

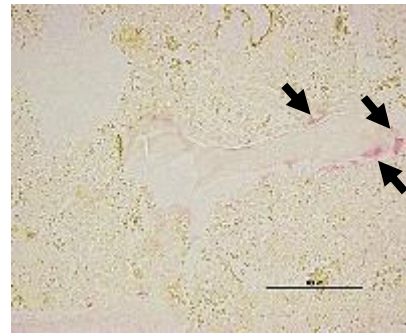
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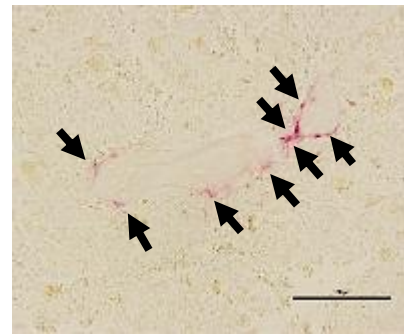
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(A)



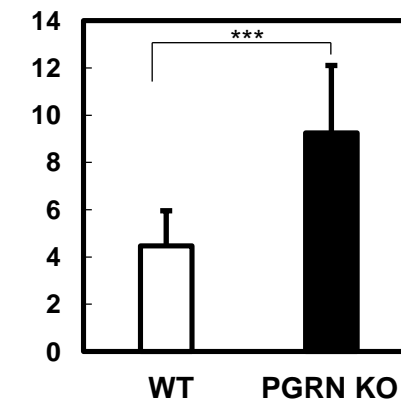
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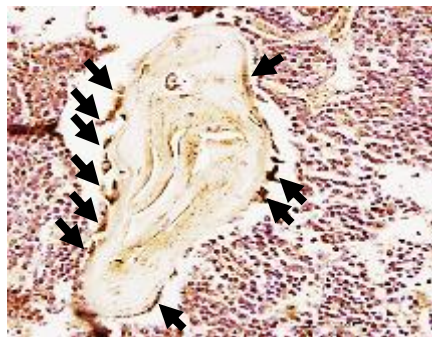
PGRN KO

(B)

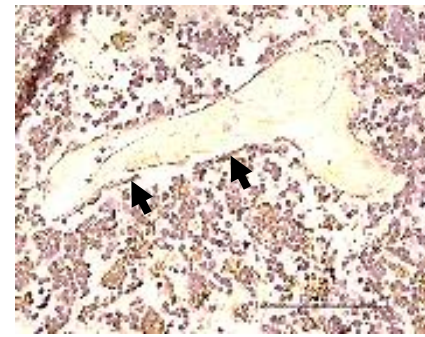
TRAP positive cells / Bone surface (mm)



(C)

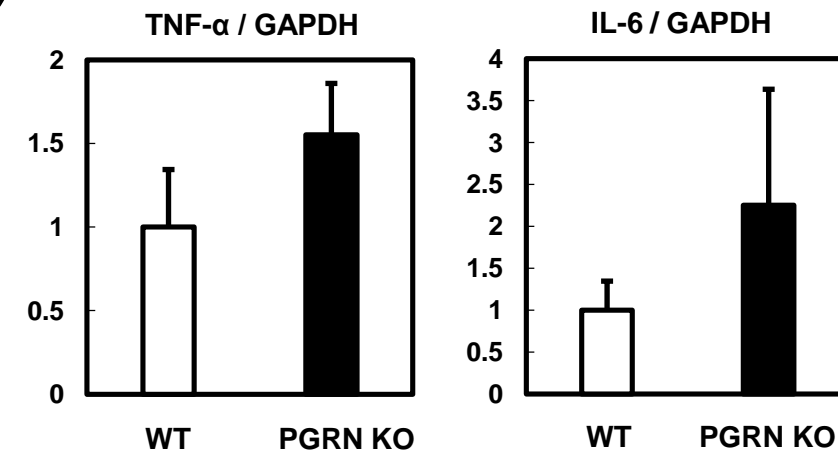


WT



PGRN KO

(D)



(E)

