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The promotion of bone healing by progranulin, a downstream molecule of BMP-2, through interacting with TNF/TNFR signaling

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Abstract

Endochondral ossification plays a key role in the bone healing process, which requires normal cartilage callus formation. Progranulin (PGRN) growth factor is known to enhance chondrocyte differentiation and endochondral ossification during development, yet whether PGRN also plays a role in bone regeneration remains unknown. In this study we established surgically-induced bone defect and ectopic bone formation models based on genetically-modified mice. Thereafter, the bone healing process of those mice was analyzed through radiological assays including X-ray and micro CT, and morphological analysis including histology and immunohistochemistry. PGRN deficiency delayed bone healing, while recombinant PGRN enhanced bone regeneration. Moreover, PGRN was required for BMP-2 induction of osteoblastogenesis and ectopic bone formation. Furthermore, the role of PGRN in bone repair was mediated, at least in part, through interacting with TNF- α signaling pathway. PGRN-mediated bone formation depends on TNFR2 but not TNFR1, as PGRN promoted bone regeneration in deficiency of TNFR1 but lost such effect in TNFR2 deficient mice. PGRN blocked TNF- α -induced inflammatory osteoclastogenesis and protected BMP-2 mediated ectopic bone formation in TNF α transgenic mice. Collectively, PGRN acts as a critical mediator of the bone healing process by constituting an interplay network with BMP-2 and TNF- α signaling, and this represents a potential molecular target for treatment of fractures, especially under inflammatory conditions.

Keywords

progranulin; bone repair; BMP-2; TNF- α ; endochondral ossification; bone resorption

Introduction

The treatment of large bone defects remains a challenge for the orthopaedic surgeons. Regenerative therapies with the use of mesenchymal stem cells (MSCs), bio-scaffolds and growth factors may provide an alternative to autogenous bone transplantation, callus distraction or the use of allografts. However, there are still a number of patients with critical

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Conflict of interest

We herein declare that we have no conflict of interest.

bone defect that cannot be cured. In the healing process of large bone defects, chondrogenesis and endochondral ossification play a critical role [1]. It is known that defect of cartilage callus formation leads to dramatically impaired bone healing [2]. Besides, some chondrogenic and chondroprotective molecules can also promote bone repair [3–6].

Bone morphogenic proteins (BMPs) are growth factors of the transforming growth factor- β (TGF- β) superfamily, which have proven to be potent agents to induce bone formation [7–9]. Among them, BMP-2 is known to play an important role in bone regeneration and has been applied clinically for fracture healing [9, 10].

TNF- α and its receptors, TNFR1 and TNFR2, are widely investigated for their roles in bone regeneration [11, 12]. It is known that overexpression of TNF- α enhances bone resorption and inhibits osteogenesis induced by BMP-2 [13]. TNFR1 is known to mediate inflammatory function of TNF- α , which also induces inflammatory bone resorption [14]. On the contrary, TNFR2 is reported to be beneficial for anti-inflammatory reaction [15]. In many fracture and bone defect cases, the patients are under local or systemic inflammatory conditions, and the cytokines in particular TNF- α delays the healing process.

Progranulin (PGRN), also known as granulin–epithelin precursor [16], proepithelin [17, 18], acrogranin [19], and GP88/PC cell-derived growth factor [20], is a 593-aa growth factor that is secreted from cells mediated by its signal peptide. PGRN contains 7½ repeats of a cysteine-rich motif (CX5–6CX5CCX8CCX6CCXDX2HCCPX4CX5–6C) in the order, P–G–F–B–A–C–D–E, where A–G are full repeats, and P is the one-half motif [21]. PGRN is digested into 6-kDa GRN peptides by several proteases, including matrix metalloproteinase 9, 12, and 14, elastase, and protease 3 [22–26]. PGRN is known to have multiple functions [27, 28], and plays a critical role in physiological and disease processes such as wound healing [29], tumorigenesis [30], degenerative diseases [31–33] and inflammation [25, 26, 28, 34, 35]. PGRN is a secreted glycoprotein, but it also functions at the cytoplasmic and nuclear levels [36]. We previously reported that PGRN was expressed in chondrocytes and cartilage [37]. Moreover, PGRN acts as a downstream molecule of BMP-2 and activates ERK1/2 as well as AKT signaling pathway, and has been reported to stimulate chondrocyte proliferation [38], differentiation and endochondral ossification during development [39, 40]. Recently, we reported that PGRN antagonized TNF- α signaling through directly binding to TNF receptors which include TNFR1 and TNFR2, and protected inflammatory bone erosion in inflammatory arthritis [27, 28, 35, 41]. Herein we examined the role of PGRN in bone repair and its interplay with BMP-2 and TNF/TNFR signaling through bone healing models based on genetically-modified mice.

1. Materials and methods

2.1 Mice

All animal studies were performed in accordance with institutional guidelines and approval by the Institutional Animal Care and Use Committee of New York University. The generation and genotyping of PGRN deficient (PGRN $^{-/-}$) mice have been described previously [34]. Two-month-old male WT, TNFR1 deficient (TNFR1 $^{-/-}$) mice and TNFR2 deficient (TNFR2 $^{-/-}$) mice from Jackson Laboratories, and 4-month-old human TNF- α transgenic (TNF-Tg) mice from Taconic were used for these experiments.

2.2 Generation of PGRN stable line and purification of recombinant PGRN protein

The method to generate PGRN stable line and purify recombinant PGRN has been described in our previous publication [40]. A total of 293 EBNA cells were cultured in tissue culture dishes in DMEM supplemented with 10% heat-inactivated FCS (Invitrogen) and antibiotics. Cells were cultured for 1 day before transfection using Lipofectamine reagent (Invitrogen),

following the manufacturer's instructions, at a density of 1.5×10^5 cells/30-mm plate. The plasmid pHis/Myc-PGRN was transfected into 293 cells. Two days after transfection, cells were divided into 100mm dishes at a density of 10^5 cells/dish in 10ml of DMEM containing 1000 μ g/ml G418. After 14 days in selective medium (medium changed every 3 days), cells were expanded in DMEM containing 500 μ g/ml G418. To prepare recombinant PGRN protein, we collected medium from the PGRN stable line. For each liter of medium, 30ml of nickel-nitrilotriacetic acid (Ni-NTA)-agarose was added, and the suspension was incubated overnight with agitation at 4°C. After sedimentation, the Ni-NTA-agarose was packed into a 3- x30-cm column. With extensive washing, the bound protein was eluted, and the eluted protein was adjusted to 10ng/ml (0.2% BSA was added to stabilize PGRN for storage). Then 5 μ l of purified proteins was analyzed by 8% SDS-PAGE and visualized by Coomassie Blue staining and Western blotting with polyclonal anti-PGRN antibodies.

2.3 Surgical procedure

All animals were provided with water and food ad libitum throughout the duration of the study. In all in vivo experiments mice were anesthetized with a mixture of xylazine and ketamine (0.15% xylazine and 0.85% ketamine), which was injected intraperitoneally at 1 μ l/g body weight, and operations were performed (table 1).

2.3.1 Segmental femoral bone defect model—The method by which we created the segmental femoral bone defect model in WT and PGRN^{-/-} mice was based on a previously described protocol with modifications [42, 43]. A 1cm skin incision was made overlying the lateral right femur and lateral femur was exposed. Through the same incision, the knee joint was exposed using a medial parapatellar approach, and the patella was dislocated laterally. A 1.5-inch 25-gauge needle was then inserted between the femoral condyles. A tranverse osteotomy was made through the mid-femoral shaft using sharp surgical scissors. Fractures with significant comminution were excluded. Using a 0.75mm drill bit and motorized drill, two holes were then placed in an anteroposterior direction at 2.2mm from the fractured ends. The femur was then stabilized by advancing the needle that was then cut. A fracture gap of 0.5mm was established by inserting a custom 5mm metallic clip into the previously drilled holes. The wound was then irrigated with saline. The muscle and skin were then closed. A dissecting microscope was used throughout the operation.

2.3.2 The femoral drill-hole model—The drill-hole model in the femur was created as described previously [44]. Briefly, unicortical holes of 0.8mm in diameter were generated in WT and PGRN^{-/-} mice through anterior cortices using a 21-gauge needle. Holes were rinsed by injection of saline to remove bone fragments from the cavity. Thereafter, the mice from both genotypes were sacrificed on days 0, 5, 15 and 21 (n=6 for each group) post injury.

2.3.3 Nonunion segmental radial defect model—The method by which we created a nonunion segmental radial defect model was based on a previously described protocol [45]. Briefly, male WT, TNFR1^{-/-} and TNFR2^{-/-} mice were anesthetized in the manner described above. The skin was cut and a 1.5mm long defect was created in the right radius bone. A 1.5mm long collagen sponge (a kind gift of Dr. Gino Bradica, Kensey Nash Corp. Exton, PA) was then implanted into the defect and the skin was sutured. For WT mice, the animals were randomly allocated to the following groups: (i) PBS (n = 6); (ii) recombinant PGRN (4 μ g, n = 6); (iii) recombinant PGRN (8 μ g, n = 6). For TNFR1^{-/-} and TNFR2^{-/-} mice, the animals were randomly allocated to the following groups: (i) PBS (n = 6); (ii) recombinant PGRN (8 μ g, n = 6). All of the reagents were diluted into 10 μ l PBS, and dripped slowly and gently into the collagen sponge to make them distribute well, and all of the

process was performed on ice in the sterile environment. Thereafter, the collagen sponges were implanted into bone defect positions.

2.3.4 BMP-2 induced ectopic bone formation model—The muscle trauma-associated ectopic bone formation model was created as previously described [46]. Briefly, 3 mm × 1 mm × 1 mm collagen discs were prepared by punched out of collagen sponge sheets (a kind gift of Dr. Gino Bradica, Kensey Nash Corp. Exton, PA). Longitudinal skin incisions were made on the medial surface of calf muscles in 2-month-old WT, PGRN^{-/-} and 4-month-old human TNF- α transgenic mice. An intramuscular pocket was created microscopically, and one collagen disc was placed in it; the skin was then closed. WT mice received recombinant BMP-2 (1 μ g, n = 6). For PGRN^{-/-} and TNF- α transgenic mice, the animals were randomly allocated to the following groups: (i) recombinant BMP-2 (1 μ g, n = 6, respectively); (ii) BMP-2+PGRN (1 μ g for each growth factor, n = 6, respectively). All of the reagents were diluted into 5 μ l PBS, and then absorbed into collagen discs slowly and gently. All of this process was performed in sterile environment on ice.

2.4 Soft X-ray assay

X-ray radiographic analysis was performed on fracture samples with both antero-posterior and lateral directions immediately after femoral segmental bone defect operation to ensure the fracture pattern and the position of the fixation needle. The fracture healing quality (analyzed with 5.0-kV for 6.0 s, Faxitron X-ray, Wheeling, IL) was examined 4 weeks and 6 weeks post surgery and 6 mice of each genotype were sacrificed and tested for each time point. The residual gap size was measured by two independent masked investigators by using an electric caliper based on X-ray result.

2.5 Micro CT assay

Prior to histological processing, paraformaldehyde-fixed samples were evaluated with micro CT using a Scanco vivaCT40 cone-beam scanner (SCANCO Medical, Switzerland) with 55kVp source and 145 μ Amp current. We scanned the samples at a resolution of 10.5 μ m. For femoral drill-hole model, we scanned the whole length of the operated femur which included the position of the hole; for radial bone defect model, whole length of the forearm which involved all the gap location; for ectopic bone formation model, whole length of the hind-limb which involved all the ectopic bone. The scanned images from each group were evaluated at the same thresholds to allow 3-dimensional structural reconstruction of each sample. The newly formed bone in each experimental group was analyzed through structural reconstruction.

2.6 Culture of Mesenchymal Stem Cells (MSCs) and C2C12 cells

Primary MSCs were harvested from femora and tibiae of 2-month-old WT and PGRN^{-/-} mice as previously reported [47]. Single cell suspensions from three mice of each genotype were pooled, and then cultured in MPC media (alpha-minimum Eagle's medium supplemented with 10% fetal bovine serum, 2mM glutamine, 25 μ g/mL sodium ascorbate, and 100U/mL /streptomycin). Afterwards, the MSCs were split and stimulated with or without 200ng/ml BMP-2 for indicated time points based on different experiments. C2C12 cells were cultured in DMEM supplemented with 20% fetal bovine serum, 2mM glutamine, 25 μ g/mL sodium ascorbate, and 100U/ml/streptomycin, and treated with recombinant PGRN (200ng/ml) and Noggin (100ng/ml) as indicated.

2.7 Real-time RT-PCR

Total RNA was extracted from cultured MSCs, C2C12 cells and newly formed bone in ectopic bone formation models as indicated using an RNeasy kit (Qiagen, Valencia, CA,

USA). Reverse transcription was performed using a RT-for-PCR kit (Qiagen, Valencia, CA) following the manufacturer's protocol. Reactions were performed in a 20- μ l SYBR Green PCR volume in a 96-well optical reaction plate formatted in the 7300 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Sequence-specific primers in the present study are synthesized as follows: 5'-TGGTGGAGCAGCAAGAGCAA-3' and 5'-CAGTGGACAGTAGACGGAGGAAA-3' for PGRN; 5'-TGATGACACTGCCACCTGTG-3' and 5'-ACTCTGGCTTTGGGAAGAGC-3' for RUNX2; 5'-ACACCTTGACTGTGGTTACTGCTGA-3' and 5'-CTTGTAGCCAGGCCCGTTA-3' for ALP; 5'-CTTGAAGACCGCCTACAAAC-3' and 5'-GCTGCTGTGACATCCATAC-3' for osteocalcin; 5'-TTGCGACCATTGTTAGCCACATA-3' and 5'-TCAGATCCATAGTGAAACCGCAAG-3' for TRAP; 5'-CAGCAGAACGGAGGCATTGA-3' and 5'-CTTTGCCGTGGCGTTATACATACA-3' for Cathepsin K; 5'-CAAGAACCTTAGCTGCCAGAG-3' and 5'-CAAGCACGCGGACAATGTTG-3' for calcitonin receptor; 5'-AGAACATCATCCCTGCATCC-3' and 5'-AGTTGCTGTTGAAGTCGC-3' for GAPDH. The presence of a single specific PCR product was verified by melting curve analysis and for each gene, the experiments were repeated three times.

2.8 Western blotting

Total C2C12 cell extracts from indicated time points were collected and proteins were isolated. Proteins were resolved on a 10% SDS-polyacrylamide gel and electroblotted onto a nitrocellulose membrane. After blocking in 5% nonfat dry milk in Tris buffer-saline-Tween 20 (10mM Tris-HCl, pH 8.0; 150mM NaCl; and 0.5% Tween 20), blots were incubated with anti-phosphorylated ERK1/2 (diluted 1:1000) antibody for 1 h. After washing, the secondary antibody (horseradish peroxidaseconjugated anti-rabbit immunoglobulin; 1:2000 dilution) was added, and bound antibody was visualized using an enhanced chemiluminescence system (Amersham Life Science, Arlington Heights, IL, USA).

2.9 ALP activity assay

ALP activity assay was performed as previously described [48]. Briefly, ALP activity was measured colorimetrically using p-nitrophenyl phosphate liquid substrate (MP Biomedicals, Santa Ana, CA, USA). Cells were lysed in RIPA buffer (Santa Cruz Biotechnologies, Santa Cruz, CA, USA), sonicated on ice, and centrifuged at 15,000g for 3 minutes at 4°C to remove cellular debris. Each lysate (50 μ l) was mixed with 150 μ l of phosphate substrate in a 96-well plate. After 30 minutes of incubation at 37°C, the reaction was terminated by adding 50 μ l of 1M NaOH. ALP activity was measured as absorbance at 405nm using a UV-Vis microplate reader (Molecular Devices, Sunnyvale, CA, USA). All samples were quantified in triplicate and further normalized by the concentrations of total cellular proteins (BioRad, Hercules, CA, USA).

2.10 Histology

The specimens from bone defect models and ectopic bone formation models were fixed in 4% paraformaldehyde, decalcified with 10% EDTA, dehydrated in ethanol gradient, cleared with dimethylbenzene, and were then embedded in olefin. At least 4 consecutive 6- μ m sections were obtained from the saggital planes, and stained using hematoxylin and eosin (HE) for routine morphologic analysis, Safranin O staining for cartilage change, Masson Trichrome staining for ossification and TRAP staining for identifying osteoclasts, and images were acquired with a light microscopic system (Olympus IX71, Olympus America Inc., Center Valley, PA).

2.11 Immunohistochemistry

Samples from BMP-2 induced ectopic bone formation models were harvested and fixed in 4% PBS buffered paraformaldehyde at 4°C overnight. After the tissue was dehydrated and embedded in paraffin, 6-μm sections were cut. Thereafter, sections were deparaffinized by xylene immersion, rehydrated by graded ethanol, and treated with 0.1% trypsin for 30 minutes at 37°C. After blocking in 20% goat serum for 60 minutes at room temperature, these sections were incubated with anti-PGRN polyclonal antibody [38] (1:100 dilution; Santa Cruz Biotechnology) at 4°C overnight, followed by incubation with a horseradish peroxidase-conjugated secondary antibody for 60 minutes at room temperature. The signal of PGRN was detected using the Vector Elite ABC Kit (Vectastain; Vector).

2.12 Statistical analysis

The Statistical Package for Social Sciences version 17.0 (SPSS Inc, Chicago, IL) was used for standard statistical analysis including one-way ANOVA and Student's t-test. Statistical significance was achieved when a value of $P < 0.05$.

3 Results

3.1 Impaired bone healing in PGRN-deficient mice

To investigate the role of PGRN in the normal procedure of bone regeneration, a well-established bone defect model was used in WT and PGRN^{-/-} mice as described previously with modifications [49]. First, a 0.5mm segmental defect was made in the femur and the mice of both genotypes were sacrificed 4 weeks and 6 weeks postoperatively, followed by X-ray assay and measurement of residual gap size between the two ends of the bone defect. As shown in Fig. 1A, PGRN^{-/-} mice displayed impaired bone regeneration compared with WT littermates in both time points. Intriguingly, no gap was observed in a large part of WT mice after 6 weeks, while PGRN^{-/-} mice still exhibited a bone defect in situ. Statistical analysis of residual gap size was performed and Fig. 1B revealed that the residual gap size was significantly larger in PGRN^{-/-} mice ($n=6$, $p<0.01$), indicating that deficiency of PGRN caused the delayed bone healing. Secondly, a unicortical drill-hole model was established in WT and PGRN^{-/-} mice [44]. To compare the healing process between WT and PGRN^{-/-} group in a time-dependent manner, mice of both genotypes were sacrificed respectively at day 5, day 10, day 15 or day 21, followed by micro CT and histological analysis. Micro CT assay demonstrated that the hole was covered by new bone in WT mice at day 10, and the bone surface became smooth at day 21, while in PGRN^{-/-} mice the hole was not totally filled even on day 15 (Fig. 1C). As shown in Fig. 1D, statistical analysis of micro CT parameter BV/TV indicated that except day 5, deficiency of PGRN led to significantly impaired new bone quality ($n=6$, $p<0.01$). HE staining of samples from day 10 postoperatively revealed that PGRN^{-/-} mice exhibited dramatically less newly formed bone tissue than WT littermates (Fig. 1E).

3.2 PGRN treatment in bone regeneration

To determine whether treatment with recombinant PGRN can promote bone regeneration, a radial bone defect model was established in WT mice as described previously [45]. Collagen sponges with PBS or different doses of recombinant PGRN were implanted into the bone defect location. Fourteen days later, mice were sacrificed and radius samples were collected and analyzed. As shown in Fig. 2A, there was hardly any new bone formation in PBS control group, while PGRN groups displayed markedly enhanced bone regeneration. Fig. 2B, C, D and E are statistical analysis of parameters based on micro CT, including BV/TV, Tb.Th, Tb.N and Tb.Sp, and PGRN treatment significantly increased the quality of newly formed bone in a dose-dependent manner ($n=6$, $p<0.05$). Endochondral ossification plays a

critical role in bone repair, and various chondroprotective molecules have been proved to enhance the bone healing process in certain microenvironments [3–6]. As shown in Fig. 2F, Safranin-O staining of newly formed bone indicated that treatment with recombinant PGRN led to remarkably more cartilage callus formation during bone regeneration process than in the PBS group, suggesting enhanced endochondral ossification by administration of PGRN. Enhanced bone repair by PGRN was further confirmed with Masson Trichrome staining (Fig. 2G).

3.3 PGRN-TNFR association in bone repair

TNF- α and its receptors TNFR1 and TNFR2 have been widely studied for their roles in bone healing process [12]. Our recent finding that PGRN binds to TNFRs [35] prompted us to determine whether the role of PGRN in enhancing endochondral ossification was mediated by TNFRs. To do so, we established radial bone defect model in TNFR1 $^{-/-}$ and TNFR2 $^{-/-}$ mice. Afterwards, a collagen sponge with PBS or 8 μ g recombinant PGRN was implanted in the defect location. Fourteen days postoperatively, the mice were sacrificed and radius samples were harvested for micro CT analysis of newly formed bone. For the TNFR1 $^{-/-}$ mice, no new bone formation was detected in the defect of PBS group; however, treatment with PGRN induced dramatically more newly formed bone in the gap (Fig. 3A). The parameters of micro CT including BV/TV, Tb.Th, Tb.N and Tb.Sp were statistically analyzed, and PGRN treatment resulted in significantly higher bone quality than PBS group ($n=6$, $p<0.05$) (Fig. 3B, C, D and E). In contrast, PGRN-mediated bone formation was largely lost in TNFR2 $^{-/-}$ mice models (Fig. 3F). In addition, all the parameters of micro CT images were measured and analyzed in TNFR2 $^{-/-}$ mice with treatment of PBS or recombinant PGRN, and no significant difference was detected ($n=6$, $p<0.05$) (Fig. 3F, G, H and I). Collectively, this set of experiments indicated that PGRN-mediated endochondral ossification during bone healing process relies on TNFR2 but not TNFR1.

3.4 Role of PGRN in BMP-2-mediated bone repair

It is known that BMP-2 is one of the most crucial growth factors in osteoblastogenesis and ossification [9]. We previously reported that PGRN was a downstream molecule of BMP-2 and mediated BMP-2-induced chondrocyte differentiation, and knockdown of PGRN suppressed BMP-2 function in chondrogenesis [40]. We next determined whether PGRN was also involved in BMP-2 induced osteoblastogenesis and bone healing. Firstly, primary mesenchymal stem cells (MSCs) were harvested from bone marrow of WT and PGRN $^{-/-}$ mice, and cultured in the absence or presence of 200ng/ml BMP-2. 48h later, cells were collected, RNA isolated and real time PCR was performed for examining the levels of osteoblastic marker genes, including RUNX2, osteocalcin and alkaline phosphatase (ALP). As shown in Fig. 4A, 4B and 4C, expressions of all three osteoblastic markers following stimulation with BMP-2 were significantly lower in PGRN-deficient MSCs ($p<0.05$). Secondly, primary MSCs from WT and PGRN $^{-/-}$ mice were cultured in presence or absence of BMP-2 for 7 days, followed by relative alkaline phosphatase (ALP) activity assay. As shown in Fig. 4D, there was no significant difference of ALP activity between the two genotypes without stimulation of BMP-2, while deficiency of PGRN resulted in significantly lower ALP activity compared with WT group following BMP-2 stimulation ($p<0.05$). These results indicated that PGRN plays an important role in BMP-2-induced osteoblastogenesis in vitro. Intriguingly, it appears that PGRN-mediated signaling and osteogenic activity does not depend on BMP-2 signaling, as Noggin, a known inhibitor of BMP-2, did not affect PGRN-induced Erk1/2 phosphorylation and RUNX2 expression (Fig. 4E and 4F).

To further reveal the dependence on PGRN of BMP-2 mediated bone regeneration in vivo, an ectopic bone formation model was established in WT and PGRN $^{-/-}$ mice. Briefly, an

intramuscular pocket was created microsurgically in the calf muscle, and collagen sponge carrying BMP-2 with or without PGRN was then implanted. Fourteen days later, the mice were sacrificed and samples were collected for analysis. Ectopic bones induced with BMP-2 in WT mice were harvested and immunohistochemistry for PGRN was performed (Fig. 5A). PGRN was clearly detectable in the ectopic bone induced by BMP-2, indicating that BMP-2 stimulated the expression of PGRN in newly formed bone. In accordance with previous reports [46, 50], implantation of BMP-2 induced ectopic bone formation, however, the volume of BMP-2-induced ectopic bone in PGRN^{-/-} mice was dramatically reduced. Importantly, this reduction seen in PGRN-deficient mice could be largely corrected by recombinant PGRN (Fig. 5B). The parameters of micro CT, including BV/TV, Tb.Th and Tb.N, were analyzed, and results indicated that deficiency of PGRN significantly suppressed new bone formation induced by BMP-2, while recombinant PGRN can partially restore the new bone quality in PGRN^{-/-} mice (n=6, p<0.05) (Fig. 5C, 5D and 5E). In addition, the samples from each group were collected and histology was performed. Ectopic bone formation totally depends on endochondral ossification, in which process cartilage callus formation plays a critical role in final bone volume and quality. As shown in Fig. 5F, Safranin O staining (**upper panels**) indicated the new bone tissue in PGRN^{-/-} mice presented dramatically less positive staining compared with WT mice, while treatment with PGRN largely reestablished the formation of cartilage-like structure during new bone formation. To verify the bone tissue formed, Masson Trichrome staining (**lower panels**) was performed, and consisting with micro CT analysis, PGRN^{-/-} mice exhibited less bone formation, which was rescued by additional use of recombinant PGRN.

3.5 PGRN-TNF- α interaction in BMP-2-induced bone formation

It has been reported that TNF- α impaired BMP-2-induced ectopic bone formation in mice, and treatment with anti-TNF- α agents maintained bone mass and protected against bone loss caused by TNF- α [13]. To demonstrate the relationship between PGRN and TNF- α in BMP-2 mediated bone formation, an ectopic bone formation model was created in the calf muscle of WT and human TNF- α transgenic (TNF-Tg) mice. Thereafter, a collagen sponge with BMP-2 alone or BMP-2 plus PGRN was implanted. Fourteen days postoperatively, the mice were sacrificed and samples were collected and analyzed. As shown in Fig. 6A, micro CT assay indicated that new bone formation induced by BMP-2 in TNF-Tg mice was markedly impaired with smaller volume and more porous structure, while this was partially rescued by use of PGRN together with BMP-2. To further reveal whether the difference was due to osteoclastogenesis, TRAP staining was performed in samples from each group. Osteoclast distribution in WT mice was scattered and moderate in newly formed bone, which was required for normal bone remodeling. However, TNF-Tg mice exhibited much condensed and stronger TRAP signal at the border of newly formed bone, which implied the erosion of bone in these mice. In contrast, when BMP-2 plus PGRN was implanted in TNF-Tg mice, the distribution and signal of osteoclast was rescued (Fig. 1A). In addition, the ectopic bones from each group were collected, RNA was isolated and real time PCR was performed for markers of osteoclasts including TRAP and Cathepsin K. Fig. 6B and 6C indicated that both markers were in significantly higher in samples of TNF-Tg mice than WT littermates, while use of PGRN largely abolished the elevation of these genes (p<0.05).

4. Discussion

Bone healing requires organization of several different processes, including hematoma, cartilage callus formation, calcification, ossification and bone remodeling [44]. It is possible that PGRN plays a role in multiple steps through interacting with different molecules. Formation of cartilaginous callus during fracture healing requires the recruitment of progenitor cells and their differentiation into chondrocytes and osteoblasts [1, 51–53].

Failing to form a proper cartilaginous callus results in impaired fracture healing [54, 55]. Identifying the involved growth factors and understanding the molecular mechanisms of progenitor cell differentiation into chondrocytes is important for developing therapeutic approaches to prevent delayed healing and rescue non-unions. It is well-established that BMP-2 plays a dominant role in new bone formation during bone healing and skeletal development [7–9]. Recently, it was reported that deficiency of BMP-2 in the chondrocyte impairs the bone healing process, which suggests that BMP-2 signaling in the chondrocyte plays a role in cartilage callus formation during bone regeneration [2]. It has been known that PGRN promotes chondrocyte proliferation [38]. Our previous studies revealed that PGRN was a BMP-2 inducible molecule in chondrocyte differentiation, and enhanced chondrogenesis as well as cartilage repair [40]. Moreover, PGRN plays a crucial role in endochondral ossification during development [39]. In the current study we created bone defect models in WT and PGRN^{-/-} mice, and found that deficiency of PGRN remarkably delayed bone regeneration (Fig. 1). Interestingly, BMP-2-induced cartilage callus in our ectopic bone formation model was markedly inhibited in PGRN^{-/-} mice, while additional treatment of recombinant PGRN reversed this impairment (Fig. 5F). Furthermore, recombinant PGRN enhanced bone regeneration, and Safranin O staining indicated that PGRN promoted cartilage callus formation during the healing process (Fig. 2F). Taken together, the literature and present data imply that PGRN is a potential regulator for cartilage callus formation in bone regeneration process. In addition, mesenchymal stem cells from PGRN^{-/-} mice displayed impaired stimulation of osteoblastic marker genes following BMP-2 treatment (Fig. 4), suggesting that PGRN may also directly participate in BMP-2 induced osteoblastogenesis. In addition, it appears that PGRN functions as a downstream mediator of BMP2 signaling and stimulates osteogenic process in BMP2-independent manner, as noggin, a well-accepted antagonist of BMP signaling pathway [56, 57], did not affect PGRN-stimulated Erk1/2 activation and Runx2 induction (Fig. 4).

TNF- α signaling, including TNF- α and TNFR1/2, has been extensively studied for its roles in bone regeneration [11, 12]. Previous studies revealed that TNFR2^{-/-} mice displayed reduced ability for bone regeneration, while TNFR1 was generally believed to play a detrimental role in healing process [14]. Recently, it was found that TNF- α treatment reduced TNFR2 expression in the implanted mesenchymal stem cells and suppressed bone regeneration [15]. In the current study, we used TNFR1^{-/-} and TNFR2^{-/-} mice, and observed that PGRN protein maintained its function in promoting bone regeneration in mice with deficiency of TNFR1, while this therapeutic effect was abolished in TNFR2^{-/-} mice. This implies the role of PGRN in bone regeneration may rely on binding with TNFR2 but not TNFR1. TNF- α is a key inflammatory cytokine, and is well known for inducing inflammatory bone resorption in autoimmune diseases such as rheumatoid arthritis [58]. In many diseases such as diabetes and hyperlipidemia, there are high levels of inflammatory cytokines, in particular TNF- α , which delay bone regeneration through exaggerating osteoclastogenesis [59, 60]. The finding that an anti-TNF drug promoted ectopic bone formation induced by BMP-2 as well as the knowledge that PGRN antagonized TNF- α function [13, 35], prompted us to analyze BMP-2-induced ectopic bone formation in TNF- α transgenic mice. The result revealed that in TNF-Tg mice, osteoclastogenesis was greatly elevated in BMP-2-induced new bone, and the bone quality was impaired. Intriguingly, the additional delivery of PGRN largely repressed the elevated osteoclastogenesis and significantly restored the new bone mass in overexpression of TNF- α . Various studies have suggested that TNF- α -mediated osteoclastogenesis is primarily through TNFR1 [61–63]. Taken together with our previous finding that PGRN antagonizes TNF- α by competitively binding to TNFRs [35], it is possible that PGRN binds to TNFR1 in bone regeneration, which in turn inhibits the function of TNF- α in osteoclastogenesis and protects the newly formed bone mass from erosion in TNF-Tg mice.

To date, the critical bone defects are still a challenge for surgeons, especially when patients are under local or systemic inflammatory conditions. Based on this study and our previous reports [35], PGRN has its own potential advantage in regenerative therapy and tissue engineering, because PGRN not only contributes as a growth factor which promotes bone regeneration, but can also antagonize the dominant inflammatory cytokine TNF- α and protect the newly formed bone structure induced by other growth factors such as BMP-2 during regeneration.

5. Conclusions

The data presented in this study identify PGRN as a regulator in bone regeneration through its association with BMP-2 and TNF/TNFR signaling, which may provide us with a potential target for bone regeneration in clinic. Moreover, the fact that recombinant PGRN protein can promote bone healing and enhance BMP-2 function in the presence of high level of TNF- α suggests that PGRN may be a particular promising biologic in bone repair for patients with systemic inflammatory conditions such as rheumatoid arthritis, or for patients with severe local inflammatory reaction of fracture.

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Fig. 1. Deficiency of PGRN led to impaired bone healing

(A) Bone healing of WT and PGRN^{-/-} mice at 4-week and 6-week time points (n=6 for each time point of both genotypes) in femoral segmental bone defect model, assayed by X-ray. Red arrows indicate the location of bone defect. Scale bar, 1cm. (B) Residual gap size at 4-week and 6-week time points following operation based on radiology results in panel A. ** p<0.01 and *** p<0.005 vs. WT group. At 4 weeks and 6 weeks after operation, X-ray radiographic analysis was performed on fracture samples with both antero-posterior and lateral directions, and average residual gap size between the two ends of bone defect in each sample was measured based on X-ray result, followed by statistical analysis. (C and D)

Impaired bone healing of PGRN^{-/-} mice in femoral drill-hole model, assayed by micro CT. A drill-hole model was created in WT and PGRN^{-/-} mice, followed by micro CT analysis at various time points, and BV/TV of newly formed bone was compared between WT and PGRN^{-/-} mice. Scale bar, 1cm. ** $p < 0.01$ and *** $p < 0.005$ vs. WT group. (F) PGRN^{-/-} mice displayed less callus formation than WT littermates at day 10 post drill-hole operation, assayed by H&E staining. Scale bar, 1mm.

Fig. 2. Recombinant PGRN enhanced bone regeneration in bone defect model

(A) Recombinant PGRN dramatically enhanced bone regeneration in radial bone defect model, assayed by micro CT. 14 days after operation, samples from PBS (n=6), 4µg PGRN (n=6) and 8µg PGRN (n=6) treatment groups were collected and micro CT was performed. (B, C, D and E) Parameters of newly formed bone quality including BV/TV, Tb.Th, Tb.N and Tb.Sp were significantly improved in treatment of PGRN, assayed by micro CT. Samples were collected and analyzed as shown in panel (A). (F) Safranin O staining of newly formed cartilage structure in the radial bone defect model following treatment of PBS or 8µg PGRN. (G) Masson Trichrome staining of newly formed bone in the radial bone defect model following treatment of PBS or 8µg PGRN. The values are the mean±SD of 3 independent experiments. *p<0.05, ** p<0.01 and *** p<0.005 vs. PBS treatment group. Scale bars, 100µm.

Fig. 3. PGRN stimulation of bone regeneration was mediated by TNFR2 but not TNFR1
(A) Local delivery of PGRN enhanced bone regeneration in radial bone defect model of TNFR1^{-/-} mice, assayed by micro CT. A radial bone defect model was created in TNFR1^{-/-} mice and a collagen sponge carrying PBS (n=6) or recombinant PGRN (n=6) was implanted into the defect location. Fourteen days later, samples were taken from both treatment groups and micro CT analysis was performed. **(B, C, D and E)** PGRN significantly promoted bone regeneration when TNFR1 was deficient, assayed by micro CT. Surgical process was the same as panel A and parameters for newly formed bone including BV/TV, Tb.Th, Tb.N and Tb.Sp were analyzed through micro CT. **(F)** Deficiency of TNFR2 almost abolished PGRN induced bone regeneration in the radial bone regeneration model, assayed by micro CT. Micro CT analysis of samples from TNFR2^{-/-} mice was performed. Surgical process was the same as panel A. **(G, H, I and J)** PGRN treatment did not significantly enhance bone regeneration in TNFR2^{-/-} mice. The values are the mean±SD of 3 independent experiments. *p<0.05, ** p<0.01 and *** p<0.005 vs. PBS treatment group.

Fig. 4. PGRN was required for BMP-2-induced osteoblastogenesis in vitro

(A, B and C) Deficiency of PGRN impaired induction of osteoblastic markers by BMP-2, assayed by real-time PCR. Mesenchymal stem cells from WT and PGRN^{-/-} mice were cultured in presence of 200ng/ml BMP-2 for 48h, and real-time PCR was performed for RUNX2, Osteocalcin and ALP. (D) Deficiency of PGRN impaired BMP-2-induced ALP activity, detected by ALP activity assay. Mesenchymal stem cells from WT and PGRN^{-/-} mice were cultured in absence or presence of 200ng/ml BMP-2 for 7 days, and ALP activity assay was performed. (E) Noggin did not affect PGRN-induced phosphorylation of ERK1/2, assayed by Western blotting. (F) Noggin did not inhibit PGRN-stimulated Runx-2 expression, as measured by real-time PCR. The C2C12 cells were cultured in presence of 200ng/ml PGRN and 100ng/ml Noggin as indicated for 24 hours, followed by real-time PCR. The values are the mean±SD of 3 independent experiments. *p<0.05, ** p<0.01 and *** p<0.005 vs. control group.

Fig. 5. Loss of PGRN inhibited new bone formation in a BMP-2-mediated ectopic bone formation model

(A) PGRN was expressed in the newly formed ectopic bone induced by BMP-2, assayed by immunohistochemistry. Ectopic bone samples from WT mice (n=5) were collected and stained with anti-PGRN antibody (brown), then counterstained with methyl green (green), and a representative picture is shown. (B) PGRN^{-/-} mice displayed impaired new bone formation in an ectopic bone formation model induced by BMP-2, while implantation of PGRN plus BMP-2 largely restored the newly formed bone mass, assayed by micro CT. (C, D and E) Deficiency of PGRN led to significantly lower quality of new bone, which was largely reestablished by additional treatment of recombinant PGRN. (F) PGRN^{-/-} mice

displayed impaired endochondral bone formation in newly formed ectopic bone compared with WT mice, while additional implantation of PGRN in PGRN^{-/-} mice rescued this impairment, assayed by Safranin O (**upper panels**) and Masson Trichrome staining (**lower panels**). The values are the mean $\pm$ SD of 3 independent experiments. * $p<0.05$, ** $p<0.01$ and *** $p<0.005$ vs. WT group. Scale bars, 100 μ m.

Fig. 6. PGRN protected against TNF- α -induced new bone absorption in BMP-2-mediated ectopic bone formation

(A) Overexpression of TNF- α dramatically suppressed newly formed ectopic bone induced by BMP-2 alone, while combined implantation of BMP-2 and PGRN partially abolished this inhibitory effect of TNF- α , assayed by micro CT (**upper panels**). Osteoclasts scattered in the newly formed bone of WT mice, but condensed and distributed at the boarder between surrounding soft tissue and newly formed bone in overexpression of TNF- α , while additional treatment of PGRN rescued the distribution pattern of osteoclast in new bone, assayed by TRAP staining (**lower panels**). M, muscle; F, fibrous tissue; B, newly formed bone. (**B** and **C**) Levels of osteoclast markers including TRAP and Cathepsin K were significantly elevated in BMP-2 induced ectopic bone tissue of TNF- α transgenic mice, while additional use of PGRN dramatically abolished this elevation, assayed by real-time PCR. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.005$ vs. WT group. Scale bars, 100 μ m.

Table 1

Animal Models used

Name	Genotypes of mice	Objective
Femoral segmental bone defect model	WT mice and PGRN ^{-/-} mice	To verify that deficiency of PGRN impairs bone healing.
Femoral drill-hole bone defect model	WT mice and PGRN ^{-/-} mice	To verify that deficiency of PGRN impairs bone healing.
Nonunion segmental radial bone defect model	WT, TNFR1 ^{-/-} and TNFR2 ^{-/-} mice	To clarify the role of PGRN in promoting bone regeneration and the receptor involved in this process.
BMP-2-induced ectopic bone formation model	WT, PGRN ^{-/-} and TNF-Tg mice	To clarify that PGRN maintains BMP-2 induced bone formation and suppresses TNF- α mediated bone loss.