Progranulin growth factor protects against osteoarthritis through interacting with TNF-α and β-Catenin Signaling

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Materials and Method

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 mice have been described previously[1]. C57/BL6 background age-matched male wild type (WT) mice, TNFR1 deficient (TNFR1-/-) mice and TNFR2 deficient (TNFR2-/-) mice from Jackson Laboratories were used for these experiments.

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

Generation of PGRN stable cell line and purification of recombinant PGRN protein has been reported in our previous study[2]. 293 EBNA cells were cultured in DMEM supplemented with 10% heat-inactivated FCS (Invitrogen) and antibiotics. Cells were cultured at a density of 1.0×10^5/30-mm plate for 1 day before transfection using Lipofectamine reagent (Invitrogen), following the manufacturer's instructions. The plasmid pHis/Myc-PGRN was transfected into 293 cells. Two days after transfection, cells were divided into 100-mm dishes at a density of 10^5 cells per dish in 10 ml of DMEM containing 1000μg/ml G418. After cultured in selective medium for 14 days (medium changed every 3 days), cells were expanded in DMEM containing 500μg/ml G418. To prepare recombinant PGRN protein, we collected medium from the PGRN stable line. For each liter of medium, 30 ml of nickel-nitrilotriacetic acid (Ni-NTA)-agarose was added, and the suspension was incubated overnight with agitation at 4℃. After sedimentation, the Ni-NTA-agarose was packed into a 3× 30-cm column. With extensive washing, the bound protein was eluted, and the eluted protein was adjusted to 10 ng/ml (0.2% BSA was added to stabilize PGRN for storage). Then 5ml of purified proteins was analyzed by 8% SDS-PAGE and visualized by Coomassie Blue staining and Western blotting with polyclonal anti-PGRN antibodies.

Aging-associated and surgically-induced OA models.

For the aging-associated model of OA, WT and PGRN-/- mice were kept up to the age of 6 and 10 months and were followed up for spontaneous development of OA. For surgically-induced DMM (destabilization of medial meniscus) model, surgery was performed in 10-week-old PGRN-/- mice and their corresponding age-matched WT control. Animals were anesthetized with isoflurane. The right knee joint was destabilized by transection of the medial meniscotibial ligament to generate destabilization of the medial meniscus[3]. The left knee joint was sham-operated, in which the joint was prepared using the same approach as that for the right knee joint but without any ligament transection. Seven mice were used per time point in each group. These animals were then killed at 4, 8 or 12 weeks after surgery. Knee joint tissues were processed for histological evaluation. To investigate the therapeutic function of recombinant PGRN, 10-week-old WT mice, TNFR1-/- mice and TNFR2-/- mice (n=6,

respectively) were anesthetized and the medial meniscotibial ligament and ACL (anterior cruciate ligament) of right knee joint were transected, followed by intra-articular injection of 6μg PGRN once a week for 4 weeks. To determine whether the expression of this growth factor was altered in the course of OA progression, we used a surgical OA rat model, with anterior cruciate ligament transection and partial medial meniscectomy as described previously [4].

Micro-CT

Prior to histological processing, paraformaldehyde-fixed knee joints from 6-month old WT and PGRN-/- mice were evaluated with micro-CT using a Scanco vivaCT40 cone-beam scanner (SCANCO Medical, Switzerland) with 55kVp source and 145μAmp current. We scanned the knee joints at a resolution of 10.5μm. The scanned images from each group were evaluated at the same thresholds to allow 3-dimensional structural reconstruction of each sample.

Histological analysis and immunostaining

For the mice of each experimental group, knee joints were fixed in 4% PFA for 3 days and decalcified for 2 weeks in 10% w/v EDTA before dehydrated and embedded in paraffin, and 5μm thick sections were cut. Serial sections were stained with Safranin-O/fast green/iron hematoxylin. For immunohistochemistry of indicated biomarkers, sections were pretreated with 0.1% trypsin for 30 min at 37°C, while for the other matrix protein in the cartilage sections were pretreated with chondroitinase ABC (Sigma-Aldrich, 0.25 U/ml) for 60 min at 37°C and then hyaluronidase (Sigma-Aldrich, 1U/ml) for 60 min at 37°C, followed by protein blocking with 20% normal goat serum and 3% BSA for 30 min at room temperature to reduce nonspecific staining. Hybridoma cells supernatant containing Col X antibody (no dilution, DSHB), MMP13 antibody (1:200 dilution, ab3208, Abcam), Aggrecan neoepitope 374ARGSV polyclonal antibody (1:100 dilution, AB8135, Millipore), anti-PGRN antibody (1:200 dilution, sc-28928, Santa Cruz), anti-ADAMTS-7 serum, anti-ADAMT-12 antibody [5] and affinity-purified monoclonal anti-COMP fragments(1:200)[4] were incubated overnight at 4°C. Detection was performed using the Vectastain Elite ABC kit (Vector, Burlingame, CA), and 0.5 mg/ml 3,3′-diaminobenzidine (DAB) in 50 mM Tris-Cl substrate (Sigma-Aldrich) was used for visualization, and sections were then counterstained with 1% methyl green.

Histopathologic and quantificational evaluation of OA.

The proteoglycan content of the articular cartilage was graded on Safranin-O–stained sections using OARSI histology scoring system[6], as shown in Table 1. To determine whether the OA changes in mice were associated with loss of chondrocytes, articular chondrocytes were counted per unit area, and the average diameter of articular chondrocytes was determined following calibration of the microscope at a convenient magnification (100×). The articular cartilage thickness was analyzed by Adobe Photoshop 7.0 (Adobe Systems)[7]. Five random regions of interest were chosen from each joint, and the diameter of all cells within each region of interest was measured. All

3 parameters were determined and averaged in all sections from each mouse, and four mice were analyzed each group. Safranin O staining was performed in 6-month old WT and PGRN-/- mice for osteophyte formation assay between the two genotypes as previously reported[8]. The presence or absence of osteophytes was scored in three histological sections per knee joint. In each section, a value of 1 (osteophyte present) or 0 (no osteophyte) was assigned for medial tibia plateau. The average score of three sections was calculated for medial tibia plateau. A high score (closer to 1.0) indicates a large osteophyte, or more (isolated) small osteophytes.

Sandwich ELISA for COMP

Serum concentration of COMP was analyzed by our new sandwich ELISA[9], using rabbit anti-COMP pAb as a capture antibody, anti-COMP typeIII mAb 2127F5B6 as a detection antibody, both of the antibodies were purified by the Protein A agarose (Invitrogen), and anti-COMP typeIII mAb 2127F5B6 was labeled by HRP using Lightning-Link™ Horseradish Peroxidase Labeling Kit (Innova) as per the manufacturer's protocols. Ninety-six well ELISA plates (Becton, Dickinson and Company) were coated with 50µl /well of purified rabbit anti-COMP pAb diluted with PBS to 2µg/ml, kept on an orbital shaker overnight at 4ºC , then coated wells were completely washed with PBST three times and blocked with 5% BSA(w/v) in PBS for 1.5 h at room temperature. Purified recombinant mouse type III standards (6.25, 12.5, 25, 50, 100ng/ml) and serum samples (1/10 for mouse) diluted with 0.5%BSA in PBST were transferred to blocked wells at 100µl/well, incubated for 2h on a shaker at room temperature. Plates were washed with PBST for three times and 100µl diluted HRP conjugated detection antibody (diluted to 1µg/ml with 0.5% BSA in PBST) were added to each well, incubated for 2h on a shaker at room temperature, washed plates with PBST for six times. Peroxidase substrate TMB solution (eBioscience) was applied to plates at 100µl/well, and development of color was stopped by adding 100µl 2M sulfuric acid to each well, and absorbance at wavelength of 450nm was read. The COMP concentrations in serum were calculated from the linear range of a standard curve. All the samples were assayed in triplicate and repeated three times.

Primary cultures of human chondrocytes

Human cartilage samples were harvested from patients receiving total knee joint replacement surgery for OA at New York University Hospital of Joint Diseases (New York, USA). Informed consent was collected from each patient before surgery and the whole process was approved by Institutional Review Board (IRB#12758). Human articular chondrocytes were isolated from tibial plateau cartilage by enzymatic digestion according to a previously described method[10].

Briefly, Cartilage slices were minced finely and washed several times with 1% PBS. They were then digested in a mixture of: 0.25% collagenase II in DMEM medium with 5% FBS and 0.1% Penn Strep for 12-16 hours in a spinner flask in an incubator at 37 degrees in an atmosphere of 5% CO_2. The cell suspension was used to establish cultures in T175 dishes. Within 2–3 days of harvesting, primary chondrocytes were re-plated at 80% confluence in 6-well plates before being used in the experiments.

Primary cultures of murine articular chondrocytes

Immature murine articular chondrocytes were isolated by enzymatic digestion of articular cartilage from the hip and knee joints of 6-day-old newborn WT, TNFR1-/- and TNFR2-/- mice, or of 6-month old WT and PGRN-/- mice, as previously described[11-13]. After sacrificing the mouse, we dislocated femoral heads, femoral condyles and tibial plateau. The cartilage samples from one mouse were isolated under the microscope and special attention was paid to avoid subchondral bone[11]. After rinsed in PBS buffer, the cartilage samples were kept in digestion buffer containing 0.25% collagenase II in DMEM medium with 5% FBS and 0.1% Penn Strep for 12-16 hours. After digestion, cells were seeded in 6-well plates (10,000 cells/cm^2) and allowed to grow for 6–7 days in DMEM (1 mg/L glucose) supplemented with 10% FBS and antibiotics.

Cartilage explant cultures

Cartilage explants from human and mouse were cultured as reported in our previous studies[2, 5]. Briefly, cartilage samples were isolated from mouse femoral head and human tibial plateau cartilage. Then the samples were dissected into tiny pieces with the diameter of 1mm and thickness of 1-2mm, then the tiny cartilage pieces were dispensed into tissue-culture flasks with serum free DMEM (containing 25mM HEPES, 2mM glutamine, 100μg/ml streptomycin, 100IU/ml penicillin, 2.5μg/ml gentamicin), supplemented with or without TNF-α(10ng/ml) and PGRN(200ng/ml). After indicated incubating time, the conditioned medium was collected for GAG synthesis analysis.

Real-time RT-PCR

Total RNA was extracted from the articular cartilage or cultured chondrocytes using RNeasy kit (Qiagen), and first-strand cDNA was generated with ImProm-II reverse transcription system (Promega). Real-time PCR was performed with SYBR Green I dye used to monitor DNA synthesis. Data from each sample were normalized to GAPDH. Primers used for real-time RT-PCR were designed to generate products between 100bp and 200bp in length. Oligonucleotides used as the specific primers to amplify mouse genes are as follows: 5′-AATGCTGGTACTCCAAACCC-3′ and 5′-CTGGATCGTTATCCAGCAAACAGC-3′ for Aggrecan; 5′-ACTAGTCATCCAGCAAACAGCCAGG-3′ and 5′-TTGGCTTTGGGAAGAGAC-3′ for Col II; 5’-AACCTATGCCCGTTTCCTCT-3’ and 5’-CCACACATTTCTCCCTCTCC-3’ for AXIN2; 5’-TGATGACACTGCCACCTGTG-3’ and 5’-ACTCTGGCTTTGGGAAGAGC-3’ for RUNX2; 5’-AATCTCACAGCAGCACATCA-3’ and 5’-AAGGTGCTCATGTCCTCATC-3’ for IL-1β; 5’-ACA GGAGGGGTTAAAGCTGC-3’ and 5’-TTGTCTCCAAGGGACCAGG-3’ for iNOS; 5′-ACACCTTGACTGTGGTTACTGCTGA-3′ and 5′-CCTTGTAGCCAGGCCCGTTA-3′ for ALP; 5′-CTTGAAGACCGCCTACAAAC -3′ and 5′-GCTGCTGTGACATCCATAC-3′ for osteocalcin;

5′-GCATTGACGCATCCAAACCC-3′ and 5′-CGTGGTAGGTCCAGCAAACAGTTAC-3′ for ADAMTS-5; 5′-ACTTTGTTGCCAATTCCAGG-3′ and 5′-TTTGAGAACACGGGGAAGAC-3′ for MMP13; 5′-ACCCCAAGGACCTAAAGGAA-3′ and 5′-CCCCAGGATACCCTGTTTTT-3′ for Col X; 5′-AAAATGGCAGTGCGTTTAG-3′ and 5′-TTTGAAGGCAGTCTGTCGTA-3′ for β-catenin; 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.

Western blotting

Total protein extracts from articular cartilage or isolated chondrocytes of each indicated experimental group were collected. 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 (10 mM Tris-HCl, pH 8.0; 150 mM NaCl; and 0.5% Tween 20), blots were incubated with polyclonal anti-ERK1/2, anti-phosphorylated ERK1/2 (diluted 1:1000, Cell Signaling Technology) or anti-β-catenin (diluted 1:1000, Santa Cruz Biotechnology) antibody for 1 h. After washing, the secondary antibody (horseradish peroxidase-conjugated anti-rabbit immunoglobulin; 1:2000 dilution) was added to detect ERK and p-ERK, while the secondary antibody (horseradish peroxidase conjugated anti-goat immunoglobulin; 1:2000 dilution) was added to detect β-catenin, and bound antibody was visualized using an enhanced chemiluminescence system (Amersham Life Science, Arlington Heights, IL, USA).

Safranin O staining and DMMB assay of GAG for cartilage explants

Cultured human cartilage explants were fixed in 10 % formalin, bisected in the middle, embedded in paraffin, and cut into 5 mm sections. GAG content was visualized using standard Safranin O staining. Briefly, dewaxed and hydrated sections were stained with 2% hematoxylin for 5 min, 1.0% Safranin O for 30 min, and counterstained with 0.02% Fast Green for 1 min. Stained slides were dehydrated, coverslipped and photographed. Conditioned medium was collected from human and mouse cartilage explant culture, and the presence of GAG released from explants was quantified using dye DMMB (Polysciences, Warrington, PA, USA). Culture medium was pre-treated with 0.5 units/ml of hyaluronidase (Seikagaku, Tokyo, Japan) at 37^{o}C for 3 h to remove exogenous HA which interferes with the DMMB assay. Digests (in duplicate) were mixed with DMMB in 96-well plates and read at 520 nm using SpectraMax 384 Microplate Reader (Molecular Devices, Sunnyvale, CA, USA). The amount of GAG in the conditioned medium was extrapolated using chondroitin-6-sulfate sodium salt from Shark cartilage (Sigma e Aldrich, St. Louis, MO, USA) as a standard. The average values of the duplicates were normalized to the wet weights of cartilage explants. The data were shown as the mean of GAG released into the condition media from three explants (treated in separate wells).

Kockdown of TS7 in chondrocytes

Knockdown of ADAMTS-7 in chondrocyte was performed in a method as we have previously reported[14]. Primary chondrocytes isolated from 6-month old WT and PGRN-/- mice were transfected with pSUPER-ADAMTS7 vector for 48h, using X-tremeGENE HP DNA transfection reagent following the manufacturer's protocol (Roche). Total RNA was isolated from cells, and mRNA levels of MMP13, ADAMTS-5 were quantified through real-time PCR.

Assay for determining the effects of MEK inhibitor on PGRN-mediated chondrocyte anabolism

Primary chondrocytes isolated from OA patients were treated with 200 ng/ml PGRN in the presence or absence of 10 uM MEK inhibitor U0126 (Promega), which has been reported for antagonizing activation of ERK1/2 signaling pathway, for 24h[15]. Thereafter, total RNA was isolated from cells, and mRNA levels for Aggrecan as well as collagen 2 were quantified by real-time PCR.

Luciferase reporter assay for β-catenin

Luciferase reporter gene assay was performed in a method as we have previously reported[16]. Primary chondrocytes isolated from 6-month old WT and PGRN-/- mice were cotransfected with b-catenin/TCF Reporter Plasmid and renilla plasmid in the presence or absence of 200ng/ml PGRN using X-tremeGENE HP DNA transfection reagent following the manufacturer's protocol (Roche). 18 h after transfection, cells were treated with 75 ng/ml Wint3a (R&D), luciferase activity was measured 24h later using Dual-Luciferase® Reporter Assay System according to the manufacturer's instructions (Promega).

Assay for examining the effects of β-Catenin inhibitor on elevated expressions of β-Catenin downstream molecules seen in PGRN-deficient chondrocytes

Primary chondrocytes isolated from 6-month old PGRN-/- mice were treated with 15uM β-Catenin/Tcf Inhibitor, FH535 (Santa Cruz) for 24h as previously reported[17]. Thereafter, total RNA was isolated from cells, and mRNA levels for RunX2 and Axin2 were quantified by real-time PCR.

TUNEL Staining

TUNEL Staining of knee joint articular cartilage from 6-month or 10-month old WT and PGRN-/- mice was performed according to previous reports[18] by using Promega™ DeadEnd™ Colorimetric TUNEL System (Promega) following the manufacturer's protocol.

TABLE 1 Murine osteoarthritis scoring system

Grade	Osteoarthritic damage
0	Normal cartilage without damage
0.5	Loss of Safranin-O staining while no detectable structural change
1	Small fibrillation
2	Vertical damage of cartilage limited to superficial layer
3	Vertical damage, no more than 25% of the cartilage surface
4	Vertical damage, 25-50% of the cartilage surface
5	Vertical damage, 50-75% of the cartilage surface
6	Vertical damage, more than 75% of the cartilage surface

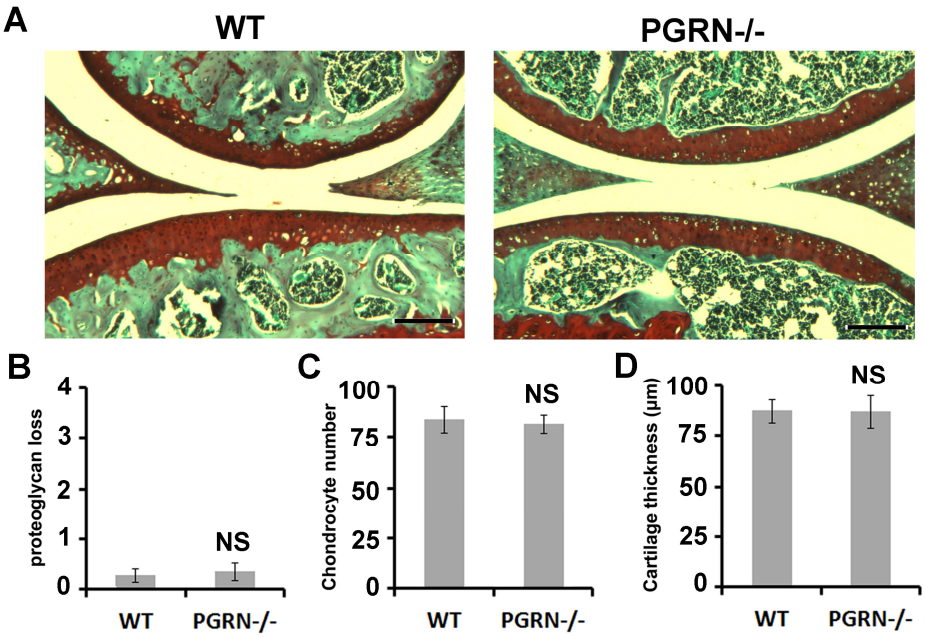

Fig. S1 No significant osteoarthritic phenotype between 3-month old WT and PGRN-/- mice. (**A**) Safranin O staining of knee joint articular cartilage. (**B-D**) Comparison of OA severity between 3-month old WT and PGRN-/- mice, as assessed by Safranin-O staining loss score, chondrocyte number and articular cartilage layer thickness in the tibia. Values are the normalized mean±SEM. Scale bar=100μm. N=6 for each group. NS=No significant difference (P>0.05 versus WT mice).

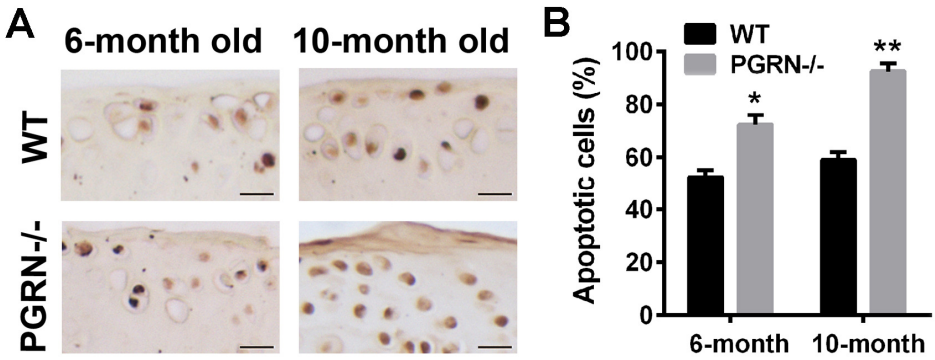

Fig. S2 TUNEL staining of 6-month and 10-month old WT and PGRN-/- mice. (**A**) Cartilage samples were isolated from 6-month and 10-month old WT and PGRN-/- mice and apoptosis of chondrocyte was detected through TUNEL staining assay. (**B**) Statistical analysis of apoptotic cells on the basis of TUNEL staining. Representative sections are shown. Scale bar=25μm. N=6 for each genotype.

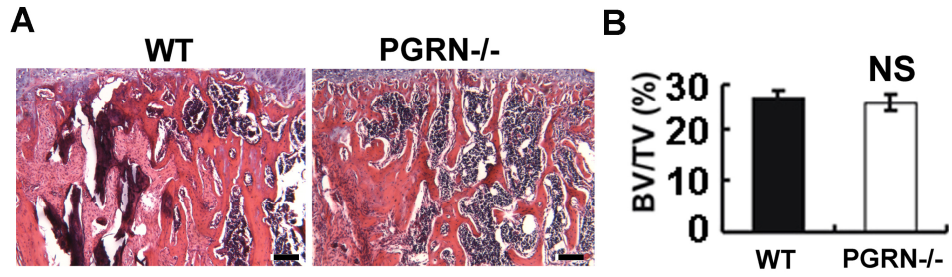

Fig. S3 No significant bone metabolic phenotype between 3-month old WT and PGRN-/- mice. (**A**) HE staining images of bone from proximal tibia in 3-month old WT and PGRN-/- mice. (**B**) BV/TV percentage in WT and PGRN-/- mice, as measured by one morphological assay according to HE staining Values are the normalized mean±SEM. Scale bar=100μm. N=6 for each group. NS=No significant difference (P>0.05 versus WT mice).

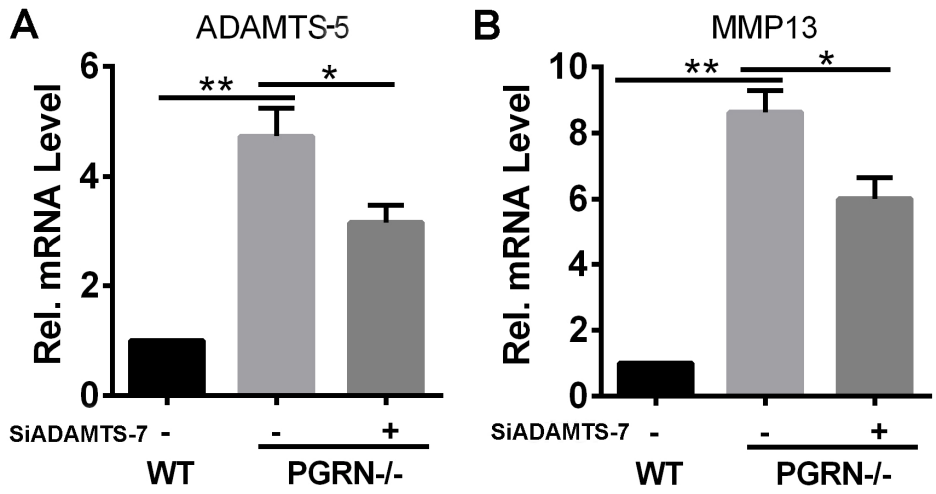

Fig. S4 Knockdown of ADAMTS-7 attenuated the levels of ADAMTS-5 (A) and MMP13 (B) in PGRN-/- chondrocyte. Primary chondrocytes isolated from 6-month old WT and PGRN-/- mice and indicated experiments were performed. Values are the normalized mean±SEM of at least 3 independent experiments. *, $P < 0.05$, **, $P<0.01$.

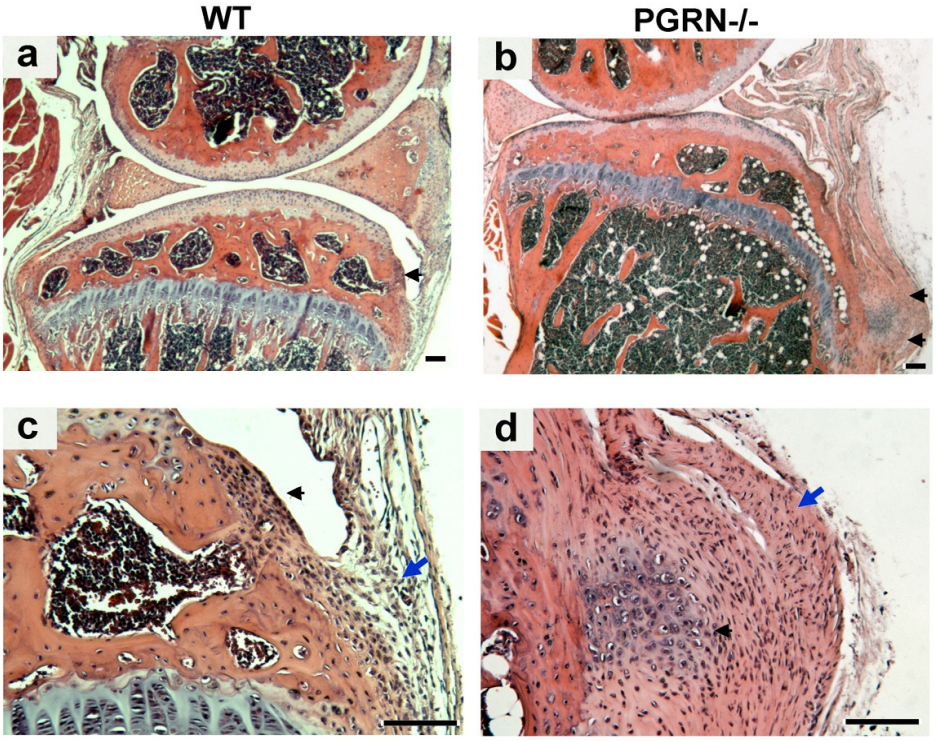

Fig. S 5 Phenotypes of synovium and osteophyte in DMM models established in WT and PGRN-/- mice at 4 weeks. (**a, b**) HE staining of knee joints samples from DMM models established in WT and PGRN-/- mice. Black arrows indicate osteophyte formation. (**c, d**) Magnified field of osteophyte and synovium in WT and PGRN-/- groups. Black arrows indicate osteophyte formation, and blue arrows indicate synovium. Representative sections were shown. Scale bar=100μm. N=7 for each group.

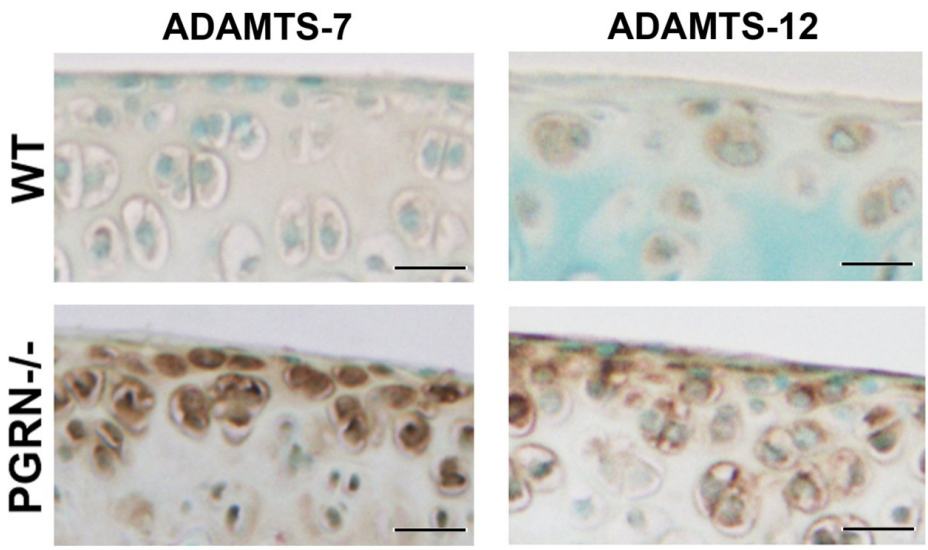

Fig. S6 Upregulated expression of ADAMTS-7 and ADAMTS-12 in PGRN-/- mice. Expression of ADAMTS-7 and ADAMTS-12 in WT and PGRN-/- mice 8 weeks following establishment of DMM model, as measured by immunohistochemistry. Tibial cartilage sections were stained with anti-ADAMTS-7 serum and anti-ADAMTS-12 antibody (brown). Methyl green staining was used as counterstain. Representative sections are shown. Scale bar=50μm

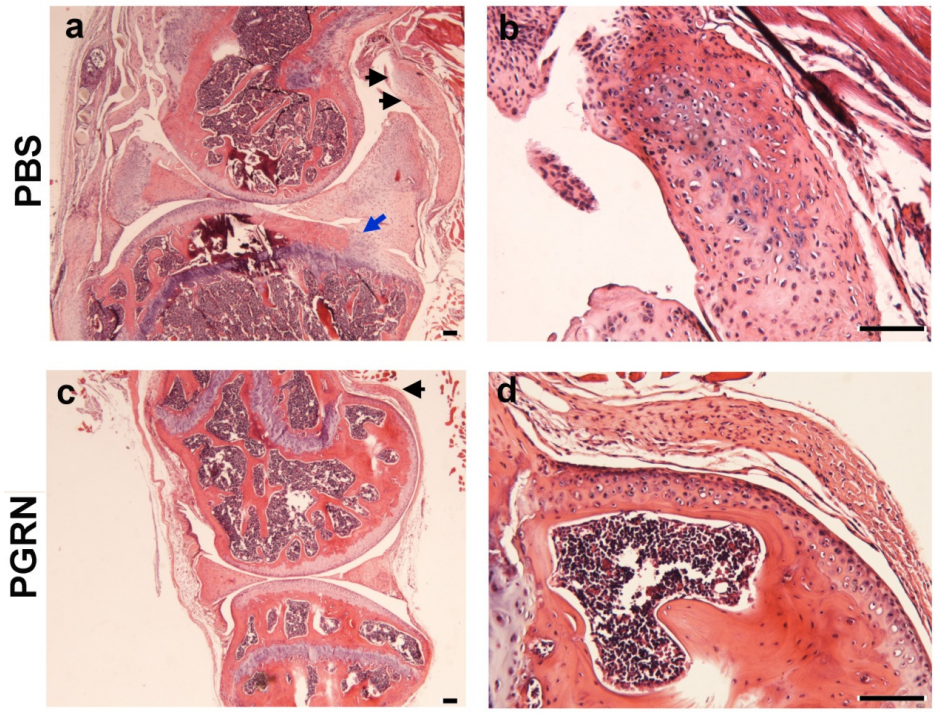

Fig. S7 Phenotypes of synovium and osteophyte in ACLT models at 4 weeks treated with PBS and recombinant PGRN protein. (**a, c**) HE staining of knee joints samples from ACLT models treated with PBS and recombinant PGRN protein. Black arrows indicate synovium, and blue arrow indicates osteophyte formation in PBS treated ACLT model. (**b, d**) Magnified field of synovium in PBS and PGRN treated groups. **N=6 for each group** and representative sections were shown. Scale bar=100μm.

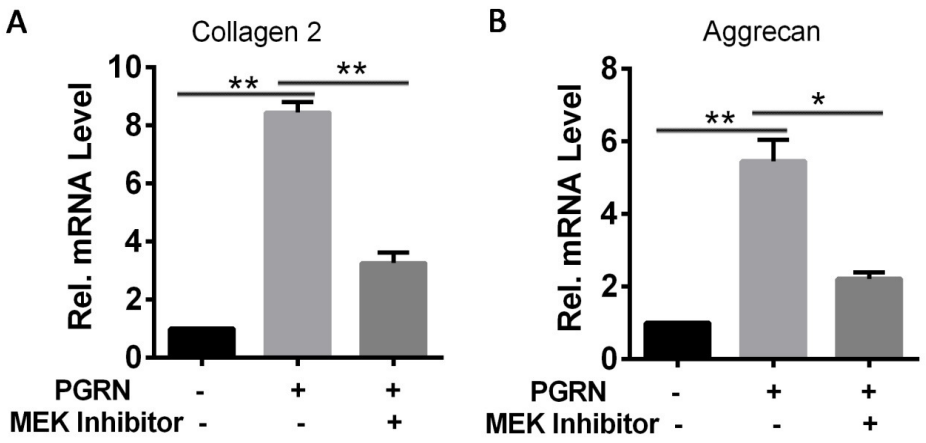

Fig. S8 ERK1/2 signaling is required for PGRN mediated anabolism in human chondrocyte. (**A**) MEK inhibitor largely abolished the PGRN-mediated stimulation of Collagen 2. (**B**) MEK inhibitor significantly impaired the PGRN-mediated stimulation of Aggrecan. Primary chondrocytes isolated from OA patients were treated with 200 ng/ml PGRN in the presence or absence of 10 uM MEK inhibitor U0126 for 24h, as assayed by real time PCR. Values are the normalized mean±SEM of at least 3 independent experiments. *, $P < 0.05$, **, $P<0.01$.

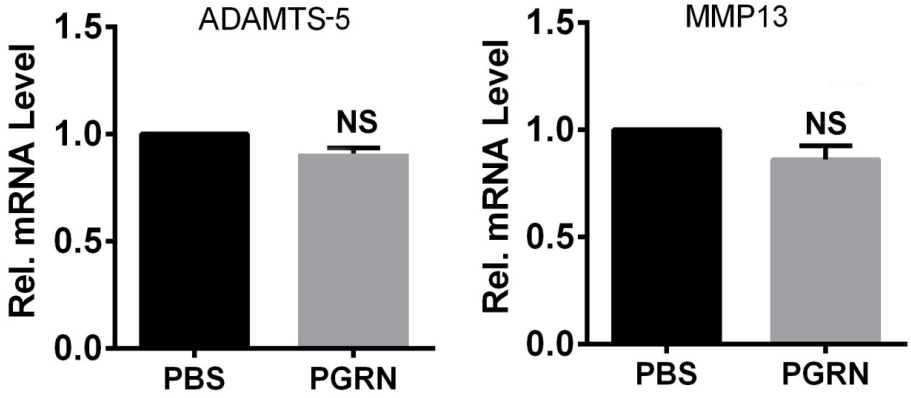

Fig. S9 Effect of PGRN on the expressions of ADAMTS-5 (A) and MMP-13 (B) in human chondrocyte. Primary chondrocytes isolated from OA patients were treated with PBS or 200 ng/ml PGRN for 24h, then total RNA were collected and mRNA levels of ADAMTS-5 and MMP-13 were assayed using real time PCR. Values are the normalized mean±SEM of at least 3 independent experiments. NS=No significant difference (P>0.05).

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