

Biglycan deficiency increases osteoclast differentiation and activity due to defective osteoblasts

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Abstract

Bone mass is maintained by a fine balance between bone formation by osteoblasts and bone resorption by osteoclasts. Although osteoblasts and osteoclasts have different developmental origins, it is generally believed that the differentiation, function, and survival of osteoclasts are regulated by osteogenic cells. We have previously shown that the extracellular matrix protein, biglycan (Bgn), plays an important role in the differentiation of osteoblast precursors. In this paper, we showed that Bgn is involved in regulating osteoclast differentiation through its effect on osteoblasts and their precursors using both in vivo and in vitro experiments. The in vivo osteolysis experiment showed that LPS (lipopolysaccharide)-induced osteolysis occurred more rapidly and extensively in *bgn* deficient mice compared to wild type (*WT*) mice. To further understand the mechanism of action, we determined the effects of Bgn on $1\alpha, 25$ -dihydroxyvitamin D_3 ($1,25$ -(OH) $_2D_3$)-induced osteoclast differentiation and bone resorption in a co-culture of calvariae-derived pre-osteoblasts and osteoclast precursors derived from spleen or bone marrow. Time course and dose response experiments showed that tartrate-resistant acid phosphatase-positive multinuclear cells appeared earlier and more extensively in the co-cultures containing calvarial cells from *bgn* deficient mice than *WT* mice, regardless of the genotype of osteoclast precursors. The osteoblast abnormality that stimulated osteoclast formation appeared to be independent of the differential production of soluble RANKL and OPG and, instead, due to a decrease in osteoblast maturation accompanied by increase in osteoblastic proliferation. In addition to the imbalance between differentiation and proliferation, there was a differential decrease in secretory leukocyte protease inhibitor (*slpi*) in *bgn* deficient osteoblasts treated with $1,25$ -(OH) $_2D_3$. These findings point to a novel molecular factor made by osteoblasts that could potentially be involved in LPS-induced osteolysis.

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Keywords: Biglycan; Proteoglycan; Osteoclast; Osteolysis; ECM

Introduction

Bgn is an extracellular matrix (ECM) small proteoglycan, a part of the family of genes called small leucine rich proteoglycan (SLRPs), and is abundant in skeletal tissues [5,38,40]. Understanding of the role of ECM in controlling skeletal function has made substantial progress by studying human diseases

or mouse models defective in specific ECM components. The Bgn gene is localized to the X (and not Y) chromosome [18] and is differentially transcribed in patients with X-chromosome anomalies including Turners (XO) and Klinefelters Syndrome (XXY) [20,23], diseases characterized by short and long skeletal stature, respectively. To directly test whether there is a connection between the expression of Bgn and skeletal function, deficient mice were generated [41]. Those mice develop an osteoporosis-like phenotype with significant decreases in trabecular bone volume, mineral apposition rate, and bone formation rate by 6 months of age [41]. Further experiments showed that there is an age dependent decrease in the number and differentiation of osteogenic precursors in the bone marrow of

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bgn deficient mice [13], which is theorized to be the cellular basis for the decrease in osteoblast surface and number observed in the mutant mice [41]. When the bone marrow of *bgn* deficient mice was ablated, the mutant mice had a delayed accrual of new bone compared to normal mice providing further proof that Bgn could be important for modulating osteogenesis in vivo [10].

When the number and surface area of osteoclasts were examined in *bgn* deficient mice, there were no significant differences at 3 and 9 months of age. However, the older *bgn* deficient animals showed increased amounts of both osteoclast area and number [41] compared to normal age matched controls. This observation coupled with others studies showing *bgn* deficient osteoblasts had reduced responses to BMP2/4 [12] prompted us to theorize that changes in osteoclast formation and function might be uncovered by applying physiological stress to the *bgn* deficient mice. To test this possibility, normal and mutant mice were subjected to calvarial titanium particle overlay [4,27], a procedure previously shown to induce rapid, transient local bone resorption due to the LPS carried by the particles. The experiments presented here show that animals deficient in Bgn had more induced bone resorption than controls due to increased differentiation of osteoclasts. In vitro studies showed that the increased differentiation of the osteoclasts is due to defects in the proliferation and differentiation of *bgn* deficient osteoblasts and their precursors. This study underscores the importance of the ECM in controlling the relationship and subsequently, the coordinated differentiation and activity between osteoblasts and osteoclasts in maintaining bone tissue integrity.

Materials and methods

Animals

All experiments were performed using *WT*, and *bgn* deficient male mice under an institutionally approved protocol for the use of animals in research (#NIDCR-DIR-04-300). Generation of *bgn* deficient mice has been previously reported [41]. The genotype of the *WT* (*bgn*⁺⁰, *bgn* is on X chromosome) and *bgn* deficient (*bgn*⁻⁰) mice was determined by a PCR-based assay as described previously [14].

Culture medium

The culture medium for calvarial cells was α -Modified Minimum Essential Medium (Life Technologies, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS, Becton Dickinson, Franklin Lakes, NJ, USA), 2 mM glutamine, 100 units/ml penicillin, 100 μ g/ml streptomycin (Biofluids, Rockville, MD, USA), and 55 μ M 2-mercaptoethanol (GIBCO BRL, Gaithersburg, MD). The culture medium for co-culture was phenol red-free Minimum Essential Medium (GIBCO) containing 10% FBS, nonessential amino acids (Mediatech, Herndon, VA), 2 mM L-glutamine, 100 units/ml penicillin, and 100 μ g/ml streptomycin.

Osteoclast function in vivo

A murine calvarial osteolysis model was used to estimate inductive bone resorption as previously described [27]. Briefly, 5 week-old *WT* and *bgn* deficient mice were first anesthetized with isoflurane (2.5–4.5% in O₂). Titanium particles with adherent LPS (5×10^5 in 40 μ l of PBS, Johnson Matthey, Ward Hill, MA) or vehicle (40 μ l PBS) alone were implanted on the surface of parietal bones of each mouse. Mice were sacrificed at 4, 7, and 10 days post-surgery. Calvariae were harvested and processed for histology or radiographs. For radiographs, the parietal bones were imaged using a Faxitron MX-20

Specimen Radiography System (Faxitron X-ray Corp., Wheeling, IL, USA) at an energy of 20 kV for 90 s. The extent of osteolysis in each parietal bone was determined from the X-ray image, in a blinded fashion, by computer assisted histomorphometry using the computer program, imageJ (NIH image, <http://rsb.info.nih.gov/nih-image>). For histology, parietal bone was fixed in freshly prepared 4% paraformaldehyde for 24 h, decalcified with buffered 10% EDTA (pH 8.0) for 1 week, and then processed for standard paraffin embedding and sectioning. The sections were deparaffinized, rehydrated, and stained histochemically for tartrate-resistant acid phosphatase (TRAP) using a Leukocyte Acid Phosphatase kit (Sigma, St. Louis, MO), following procedures recommended by the manufacturer.

Preparation of murine calvarial cells

Neonatal murine calvarial cells were prepared as previously described [12,36]. Briefly, calvariae of 1 to 5-day-old *WT* or *bgn* deficient mice were dissected and washed twice with 4 mM EDTA in PBS in a 37°C water bath with shaking. The calvariae were then digested with 200 U/ml collagenase type II (Worthington Biomedical Corporation, Freehold, NJ) in PBS for 10–15 min each for 5 times. The cells from the last three digestions were collected and served as the starting population of cells that are highly enriched in osteoblastic cells [30]. The cells were washed twice in culture medium, seeded into 150 mm dishes at a density of 4000 cells/cm², and cultured at 37°C until confluent. The cells were detached with trypsin-EDTA (GIBCO), and replated for the experiments as described below.

Preparation of osteoclast precursors

Two types of cells were used as a source of osteoclast precursors, spleen cells and bone marrow cells. Spleen cells were obtained from 6–8 week old *WT* or *bgn* deficient mice. Marrow cells were flushed from the femora and tibiae of mice using phosphate-buffered saline with antibiotics. Red blood cells were lysed in 0.83% ammonium chloride, 10 nM Tris-HCl (pH 7.4). Cells were washed twice with phosphate-buffered saline and resuspended in the culture medium described above.

Osteoclast differentiation assay

Calvarial cells (1×10^4) were plated into a well of 48-well plates. After incubating overnight (5% CO₂, 37°C), nucleated spleen cells (5×10^4) were added and cultured in medium supplemented with 100 nM dexamethasone (Sigma), 100 μ M ascorbic acid 2-phosphate (WAKO, Tokyo, Japan), and the indicated concentrations of 1 α ,25-dihydroxyvitamin D₃ (1,25-(OH)₂D₃, Biomed, Plymouth Meeting, PA, USA). The media were changed every 3 days. On the indicated days, the cells were stained histochemically for TRAP and the number of TRAP-positive multinuclear cells (TRAP⁺ MNCs) containing 3 or more nuclei was determined as previously described [4]. In some assays, spleen cells (2×10^4) were plated into a well of 96-well plates and cultured with α -MEM supplemented 10% FBS and 30 ng/ml M-CSF for 5 days. The cells were then cultured in the presence of both 30 ng/ml M-CSF and 100 ng/ml RANKL for an additional 3–4 days to stimulate the differentiation of osteoclasts. The number of TRAP⁺ MNCs was counted.

Bone resorption assays

Calvarial cells (1×10^4) and bone marrow cells (5×10^5) were plated into a well of a 96-well plate containing bovine bone slices (0.2 mm) in culture medium supplemented with 100 nM dexamethasone, 50 μ g/ml ascorbic acid (Sigma), and 10 nM 1,25-(OH)₂D₃. After 8 days, the cells were fixed with 4% paraformaldehyde in PBS and washed twice with PBS. The bone slices were brushed using a cotton swab, and washed in distilled water, and stained with Mayer's hematoxylin (Bie and Berntsen A/S). Resorbed bone areas were determined using a CastGrid (Microsoft, Olympus, Glostrup, Denmark), workstation incorporating an Olympus 1X70 microscope. Collagen degradation was determined using CrossLaps™ ELISA kit (Nordic Bioscience Diagnostics A/S, Herlev, Denmark) to measure type I collagen fragments released into the medium [19], following procedures recommended by the manufacturer.

Enzyme-linked immunosorbent assay (ELISA) and Western blot analysis

Calvarial cells (1×10^5 /dish) were plated into a 60 mm dish and cultured overnight (5% CO₂, 37°C). The cells were then treated with 10 nM 1,25-(OH)₂D₃ or vehicle for the indicated period of time. The culture medium was collected for measurement of OPG and RANKL concentration using the Quantikine ELISA Kit (R and D Systems, Minneapolis, MN).

Attached cells were washed twice with PBS and lysed with M-PER mammalian protein extraction reagent (Pierce, Rockford, IL) containing a protease inhibitor cocktail (Complete™ Mini, Roche, Indianapolis, IN). The protein content of the lysate was measured using a BCA Protein Assay Kit. Equal amounts of protein were loaded and separated on a 10% NuPAGE™ Bis-Tris gel (Invitrogen, Carlsbad, CA). The resolved proteins were then transferred onto a BA85 nitrocellulose membrane (Schleicher and Schuell, Keene, NH) and blocked for 1 h at room temperature in blocking buffer (NAP™-Blocker, Geno Technology, Inc., St Louis, MO). Rabbit anti-human RANKL antibody (cross-reacts with mouse, 1:500 dilution, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) in tris-buffered saline (TBS) containing 0.05% Tween-20, 3% BSA, and 1% goat serum was incubated with the membrane for 1 h at room temperature. The membrane was washed, and then incubated with a 1:50,000 dilution of goat-anti rabbit IgG conjugated to HRP (Kirkegaard and Perry Laboratories Inc., Gaithersburg, MD) in blocking buffer for 1 h at room temperature. The membrane was then washed and reacted with Super Signal Chemiluminescence HRP substrate (Pierce Chemical Co., Rockford, IL) according to the manufacturer's recommendations, and imaged using Kodak BIOMAX MR film (Kodak, Rochester, NY).

Proliferation of calvarial cells

Proliferation was assessed by BrdU incorporation under conditions that allow visualization of dividing cells. Calvarial cells (6×10^3 cells) were plated into a well of 8-well chamber slides (Nalge Nunc International, Naperville, IL) and incubated overnight. The cells were then labeled with BrdU-labeling reagent (Zymed Laboratories, Inc., Southern San Francisco, CA) for 7 h and fixed in 70% alcohol for 20 min at 4°C. The BrdU-labeled cells were visualized using the BrdU Staining Kit (Zymed Laboratories, Inc., Southern San Francisco, CA) following procedures recommended by the manufacturer.

The proliferation of calvarial cells was also measured by [³H]-thymidine incorporation. Calvarial cells were washed with thymidine-free α-MEM, and then incubated in culture medium containing radioactive labeled [³H]-thymidine (1 μCi/well, Amersham Biosciences, Hørsholm, Denmark). After 24 h, cells were washed 3 times with 0.24% thymidine in PBS and detached with trypsin. The DNA was precipitated by addition of 100 μl 75% TCA. Samples were vortexed for 1 h, incubated at 4°C overnight, and pelleted by centrifugation. The pellet was washed twice with 7.5% TCA and dissolved in 300 μl 0.1 M NaOH before being counted in 2.5 ml scintillation fluid Ecoscint A (National Diagnostics, Atlanta, Georgia, USA) on a LS6500 Multi-purpose Scintillation counter (Beckman, USA).

Differentiation of calvarial cells

Osteoblast differentiation was estimated as described previously [17]. Calvarial cells (1.9×10^4 cells) were plated into a well of 12-well plates (Corning Inc., Corning, NY, USA) and cultured in 0.75 ml culture medium supplemented with 0.01 M β-glycerolphosphate (Sigma) and 50 μg/ml ascorbic acid (Sigma) for 1, 2, and 3 weeks. Medium was changed every 2–3 days. At the indicated time, the cells were fixed with 70% ethanol for 1 h and stained with 40mM alizarin red-S (pH 4.2, Sigma) for 10 min with gentle shaking. To quantify the bound dye, the stain was solubilized with 1 ml 10% (w/v) cetylpyridinium chloride for 15 min while shaking shielded from light. The absorbance of solubilized stain was measured at 561 nm. Cell viability was determined at the end of each culture period using an alamarBlue™ Assay (Trek Diagnostic Systems, Cleveland, Ohio, USA), which is based on the incorporation of a fluorometric/calorimetric growth indicator to detect the metabolic activity, and viability of the cells, following procedures recommended by the manufacturer. Briefly, 10% of the culture medium was replaced with alamarBlue solution and

incubated at 37°C until a color change was observed (approximately 1–2 h). The level of fluorescence in the medium was measured at excitation 561 nm and emission at 595 nm.

Semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR)

Calvarial cells (1×10^5 cells) were plated into a 60 mm dish and cultured in the presence and absence of 10 nM 1,25-(OH)₂D₃. After 3 days, mRNA was isolated using Tri-Reagent (Molecular Research Center, Cincinnati, OH) following the manufacturer's instructions. One microgram total RNA from the sample preparation was reverse transcribed with 50 units of SuperScript II RT using random hexamer primers (In Vitrogen Life Technology, Carlsbad, CA) following the manufacturer's instructions. The primers used to amplify the cDNA were designed with Primer 3 software (<http://www-genome.wi.mit.edu/cgi-bin/primer/primer3.cgi>). The following primers were used: biglycan (*bgn*) forward 5'-acctgtcccctccatctct-3'; reverse 5'-ccgtgtgtgtgtgtgtgtg3', secretory leukocyte protease inhibitor (*slpi*), forward 5'-atgtgagcctgacccac-3'; reverse 5'-gaatgctgagccaaaaggag-3', *gapdh*, forward 5'-gagagccctatccaactc-3'; reverse 5'-gtgggtgcagcgaacttat-3', β-actin, forward 5'-agccatgtacgtageccatcc-3'; reverse 5'-ctctcagctgtgtgtgtgaa-3', bone sialoprotein (*ibsp*), forward 5'-attttgctcagcattttggg-3'; reverse 5'-ctgaagagtactgctccc. PCR was performed using a hot start in the presence of AmpliTaq Gold™ at 94°C for 5 min followed by 35 cycles of a three temperature program of 1 min at 94°C, 20 s at 57°C, and 30 s at 72°C. The PCR reaction was terminated after a 7 min extension at 70°C and the entire reaction was chilled and stored at 4°C until analysis. Five microliters of the PCR product and a 0.25 μg of φX174RF DNA/HaeIII DNA ladder (Gibco BRL) were run in a 10% acrylamide gel in (TBE) buffer at 100 V. The separated DNA fragments were visualized after ethidium bromide staining under a UV light.

Results

Induced osteolysis in normal and *bgn* deficient bone

To assess the potential role of Bgn on osteoclast function, we used a quantitative version of murine calvarial model [27] to determine LPS-induced local bone resorption, termed osteolysis, in normal and *bgn* deficient mice. Titanium particle, used as a carrier for LPS, rapidly induces osteoclast differentiation and osteolysis in this model [4,27]. Titanium particles were implanted on the surface of the parietal bones of each mouse. After 4, 7, and 10 days, calvariae were harvested and subjected to contact X-ray analysis. Osteolysis was visualized as dark pockets on the X-ray image (Fig. 1A). The areas of resorption were quantitated using NIH image. Quantitative results showed that osteolysis occurred more rapidly and extensively in *bgn* deficient mice compared to *WT* mice (Fig. 1A, right panel). There was significantly more bone resorption in *bgn* deficient mice than in *WT* mice at days 7 and 10 (Fig. 1A, right panel). Histology of the parietal bone confirmed that there was increased resorption in the calvaria of *bgn* deficient mice compared to *WT* mice and further, that it was accompanied by increased numbers of TRAP positive cells (Fig. 1B, left panel). When the relative number of osteoclasts in the affected tissue was quantified by cell counting, the increased number of osteoclasts in *bgn* deficient compared to *WT* calvaria was shown to be statistically significant (Fig. 1B, right panel). These results suggested that the increased bone resorption could be primarily due to increased differentiation of osteoclasts.

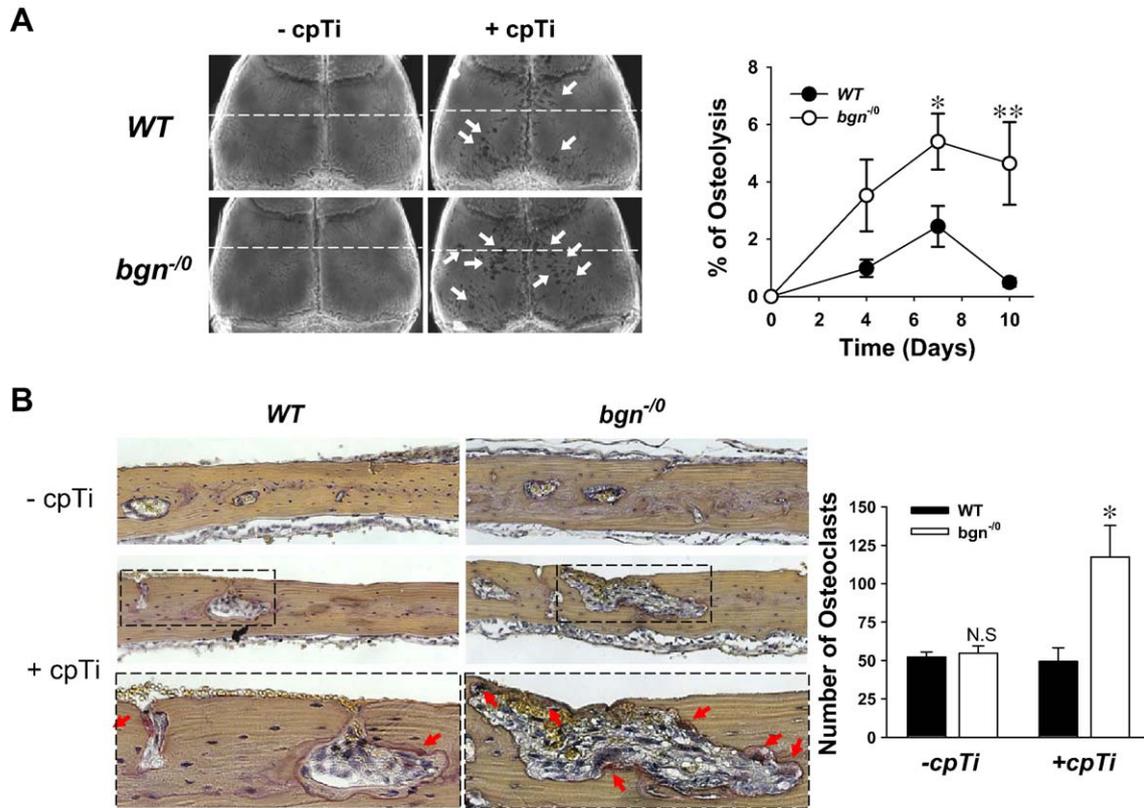


Fig. 1. Titanium-LPS-induced osteolysis. Titanium particles with adherent LPS endotoxin were implanted on the calvariae of *WT* or *bgn* deficient mice. Parietal bones were harvested and processed for histology and X-ray analysis using a Faxitron at the indicated times. The extent of resorption area (arrows) on each parietal bone was determined from the X-ray image, in a blinded fashion, by computer-assisted histomorphometry. (A) Titanium-LPS-induced osteolysis occurred more rapidly and extensively in *bgn* deficient mice (*bgn*^{-/-}, open circles) compared to *WT* mice (solid circles). All groups are $n = 6$. *Denotes $P < 0.05$ and ** denotes $P < 0.02$ compared to *WT* mice at the same time point. (B) Parietal bones were sectioned as indicated by the white dash lines on X-ray image. Histological sections showed the TRAP staining of *WT* and *bgn* deficient calvaria that were treated with titanium particles (+ cpTi) or vehicle (- cpTi) for 5 days. The boxes in the middle panel indicate the regions of osteolysis that are shown magnified in the right panel. Red arrows in the magnified sections point to purple TRAP-positive cells. The numbers of TRAP positive cells (right) on the section of parietal bones were counted under microscopy. All groups are $n = 3$. *Denotes $P < 0.05$ compared to *WT* mice at the same treatment.

The differentiation and activity of osteoclasts regulated by normal and *bgn* deficient osteoblasts and their precursors

To gain insight into the cellular basis for the increased osteolysis observed in the *bgn* deficient mice, we first used an in vitro osteoclast differentiation assay to determine how the absence of *Bgn* affects osteoclast differentiation. Pre-osteoblasts isolated from calvaria were co-cultured with spleen-derived osteoclast precursors in the presence of various concentrations of $1,25\text{-(OH)}_2\text{D}_3$ for different periods of time. Both time course and $1,25\text{-(OH)}_2\text{D}_3$ dose-response studies showed that there were significantly more TRAP⁺ MNCs formed in the co-culture of spleen cells with *bgn* deficient calvarial cells compared to that observed with *WT* calvarial cells (Figs. 2A and B). This increase in the differentiation of osteoclasts was observed only when *bgn* deficient calvarial cells were used in the co-culture (Fig. 2A). The genotype of osteoclast precursors did not affect the number of TRAP⁺ MNCs. To confirm this finding and to use as an alternative to the co-culture of spleen cells with calvarial cells, we measured the differentiation of spleen cells from *WT* and *bgn* deficient mice in the presence of M-CSF and

RANKL. The results showed that there was no significant difference in the number of cells (data not shown) and the number of TRAP⁺ MNCs formed in the culture of spleen cells between *WT* and *bgn* deficient mice (Fig. 2C). Therefore, *bgn* affects the differentiation of osteoclasts through its effect on osteogenic cells.

To further confirm our results, we also used bone marrow cells as a source of osteoclast precursors to co-culture with calvarial cells. Normal or *bgn* deficient calvarial cells combined with normal or *bgn* deficient bone marrow cells were plated on a slice of bovine bone and cultured for 8 days. Consistent with our results using spleen cells as a source of osteoclast precursors, there was significantly more TRAP⁺ MNCs formed in the co-cultures with *bgn* deficient calvarial cells compared to the cultures with *WT* calvarial cells regardless of the genotype of the bone marrow cells (data not shown). Quantitative histomorphometry demonstrated that there was a significantly higher percentage of resorption area on the bone slices when calvarial cells from *bgn* deficient mice were used in the culture (Fig. 3A). Similarly, the level of collagen cross-links, which is another indicator of relative bone resorption, was increased in the culture

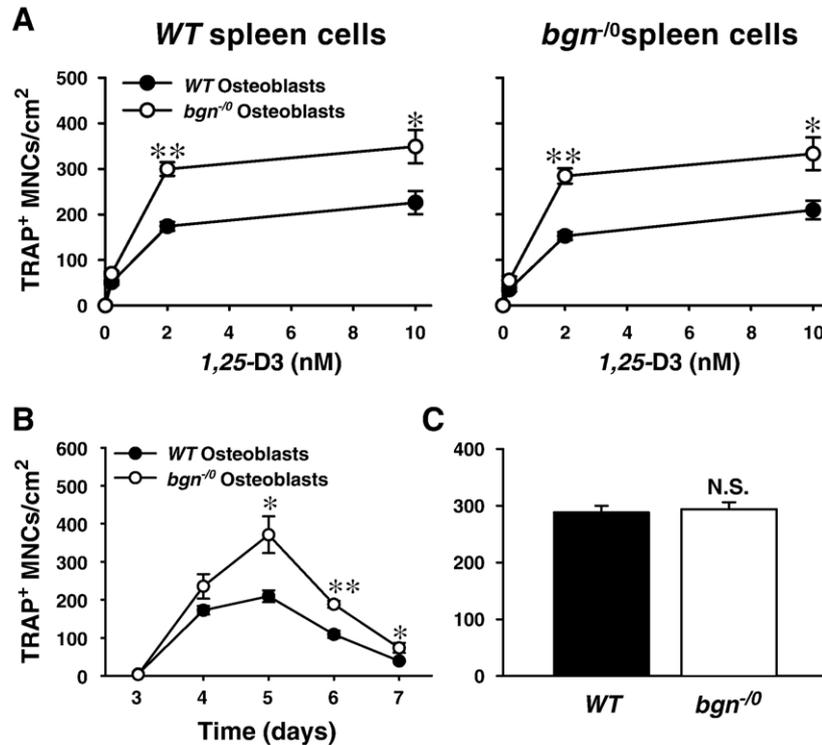


Fig. 2. Osteoclast differentiation in vitro. (A) The number of TRAP⁺ MNCs was assessed after 5 days in the co-cultures of spleen derived osteoclast precursors from wild type (left) or *bgn* deficient mice (right) and calvarial derived osteoblastic cells from WT (solid circles) or *bgn* deficient mice (open circles) in the presence of various concentrations of 1,25-(OH)₂D₃. All groups are *n* = 6. * Denotes *P* < 0.02 and ** denotes *P* < 0.0001 comparing to WT calvarial cells at the same concentration. There is no significant difference between WT and *bgn* deficient spleen cells at all the concentrations. (B) The number of TRAP⁺ MNCs formed in the co-cultures of WT spleen with primary calvarial cells from WT (solid circles) or *bgn* deficient mice (open circles) in the presence of 10 nM 1,25-(OH)₂D₃. All groups are *n* = 6. *Denotes *P* < 0.02 and ** denotes *P* < 0.0001 comparing WT calvarial cells at the same time. (C) The number of TRAP⁺ MNCs was formed in the culture of spleen cells derived from wild type (solid bar) and *bgn* deficient (open bar) mice cultured in the presence of M-CSF and RANKL. All groups are *n* = 6. N.S. denotes no significant difference compared to WT spleen cells.

containing calvarial cells from *bgn* deficient mice (Fig. 3B). These data showed that the ability of calvarial cells to induce differentiation and function of osteoclasts was increased in the absence of Bgn. Taken together, we concluded that the cellular basis for increased osteolysis in the *bgn* deficient mice is likely due to intrinsic defects in osteoblasts and their precursors.

The expression of regulators of osteoclast differentiation in WT and *bgn* deficient calvarial cells

In order to further determine the molecular basis for the osteoblast directed changes in the differentiation and function of osteoclasts in *bgn* deficient osteoblasts and their precursors, we examined the expression of RANKL and OPG in the

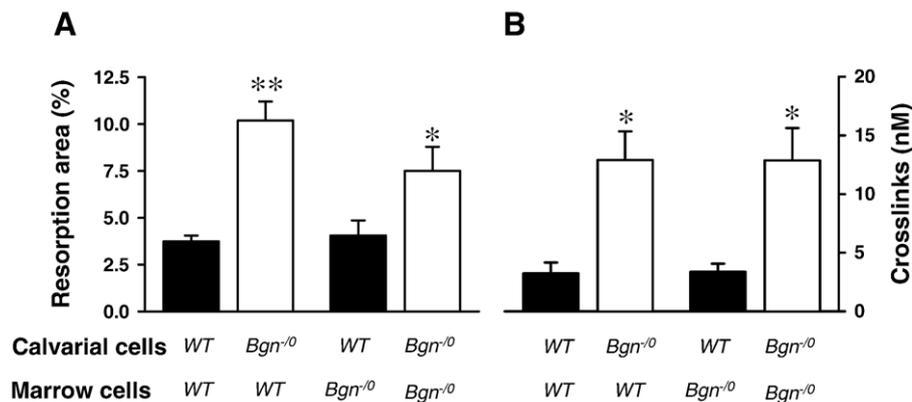


Fig. 3. Bone resorption in vitro. The primary calvarial cells from either WT (solid bars) or *bgn* mice (open bars) were co-cultured on bovine bone slices with bone marrow cells from either WT or *bgn* mice in the presence of 10 nM 1,25-(OH)₂D₃. (A) Bone resorption is expressed at the percentage of resorbing area over total area of bone slices. (B) The concentration (nM) of collagen cross-links (crosslaps) in the culture medium was measured by ELISA. All groups are *n* = 4. *Denotes *P* < 0.06 and ** denotes *P* < 0.01 comparing *bgn* deficient osteoblasts to WT osteoblasts. There are no significant differences between WT and *bgn* deficient bone marrow cells.

presence and absence of 1,25-(OH)₂D₃. RANKL is produced by both osteoblasts and their precursors and is known to be an essential stimulator of the differentiation, activity, and survival of osteoclasts [25]. Western blotting showed that 1,25-(OH)₂D₃ strongly induced the expression of RANKL by calvarial cells. However, there was no significant difference in expression of RANKL between normal and *bgn* deficient calvarial cells (Fig. 4A). Thus, the increased induction of osteoclasts by calvarial cells is not due to a differential increase in the expression of RANKL. OPG, the decoy protein of RANKL, is also produced by mature and immature osteoblastic cells and is an essential inhibitor of osteoclast differentiation, activity, and survival. The differentiation, activity, and survival of osteoclasts and its precursors are dependent on the ratio of RANKL and OPG. *Bgn* might affect the induction of osteoclast differentiation and function by affecting the expression of OPG. To test this, we determined the ratio of RANKL and OPG in the culture medium. 1,25-(OH)₂D₃ strongly stimulated the expression of RANKL and inhibited the expression of OPG in both *WT* and *bgn* deficient calvarial cell cultures. However, when the ratio of RANKL/OPG was plotted over the time course, the maximal levels which were detected at 4 and 5 days after induction (Fig. 4B) were not significantly different between *WT* and *bgn* deficient calvarial cells. Further, RT-PCR analysis showed that M-CSF mRNA was highly expressed in the cultured cells but not differently between normal and *bgn* deficient cells (data not shown). TNF- α mRNA was undetectable in any of the samples analyzed (data not shown).

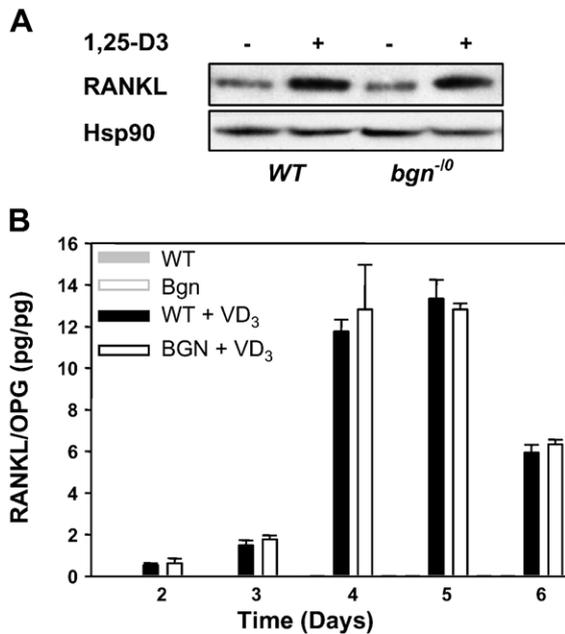


Fig. 4. Expression of RANKL and OPG. (A) The expression of RANKL protein in *WT* and *bgn* deficient osteoblastic cells in the presence and absence of 10nM 1,25-(OH)₂D₃ was analyzed using Western blot analysis. Hsp90 expression level was used as an internal control. (B) The protein levels of RANKL and OPG in the culture media of calvarial cells in the absence (grey/light grey bars) or the presence (black/white bars) of 10 nM 1,25-(OH)₂D₃ were collected at the indicated time and analyzed using ELISA. All groups are $n = 3$.

Decreased differentiation and increased proliferation in *bgn* deficient calvarial cells

Because *bgn* deficient calvarial cells did not have substantial differences in RANK or OPG expression, we turned to more global features of this cell population to try to understand how they may affect osteoclast differentiation and function. It has been shown that the ability of the osteoblast precursors to stimulate osteoclast differentiation and activity is reduced during their maturation to mature osteoblasts [2,15,37]. We, therefore, compared the proliferation and differentiation levels in normal and *bgn* osteoblasts. β -glycerolphosphate and ascorbic acid were used to induce the differentiation of calvarial cells. The in vitro differentiation capacity of calvarial cells was estimated by measuring the accumulation of calcium over a period of 3 weeks (Fig. 5A). This analysis confirmed and extended our previous findings that the ability of osteoblasts from *bgn* deficient mice to differentiate is decreased. The data presented here show differences in calcium accumulation in *bgn* deficient calvarial cells after 2 weeks of culture was significantly lower compared to age matched *WT* controls and, further, showed that this difference became progressively enhanced with additional time in culture. In addition to a delay in calcium accumulation, the *bgn* deficient osteoblasts have altered expression of osteogenic markers. This was shown by isolating mRNA from normal and *bgn* deficient calvarial cells cultured in the presence and absence of 1,25-(OH)₂D₃. *Bgn* deficient osteoblasts expressed constitutively less bone sialoprotein (BSP) mRNA compared to *WT* cells confirming our theory that osteoblast differentiation is impaired in the mutant cells (Fig. 5B). BSP mRNA was also dramatically decreased by treatment with 1,25-(OH)₂D₃ (Fig. 5B) further implying that this decrease in BSP production could somehow be related to the increased osteoclastogenesis. When the differentiation of osteoblasts is impaired, in order to compensate, the proliferation of osteoblast precursors could be increased, which may lead to an increase in the production of the regulators of osteoclast differentiation and activity. To test this hypothesis, we determined the proliferation levels of pre-osteoblasts and more mature osteogenic cells. The proliferation of pre-osteoblasts was first determined by BrdU incorporation in freshly isolated calvarial cells. The results showed that the percentage of dividing cells in the *bgn* deficient cultures was increased compared *WT* cells in early stages of culture (Fig. 5C). [3H]-thymidine incorporation was next used to measure the relatively more mature osteogenic cells after the calvarial cells were induced with osteogenic conditions for 3 weeks. *Bgn* deficient cells showed a trend towards increased proliferation at week 2 that was statistically significant by week 3 (Fig. 5D). Taken together, these data indicate that there is an intrinsic defect in *bgn* deficient osteogenic cells causing them to have increased proliferation and delayed maturation and an altered response to 1,25-(OH)₂D₃ compared to normal cells.

In order to identify novel genes that were differentially regulated in *bgn* deficient calvarial cells that might explain

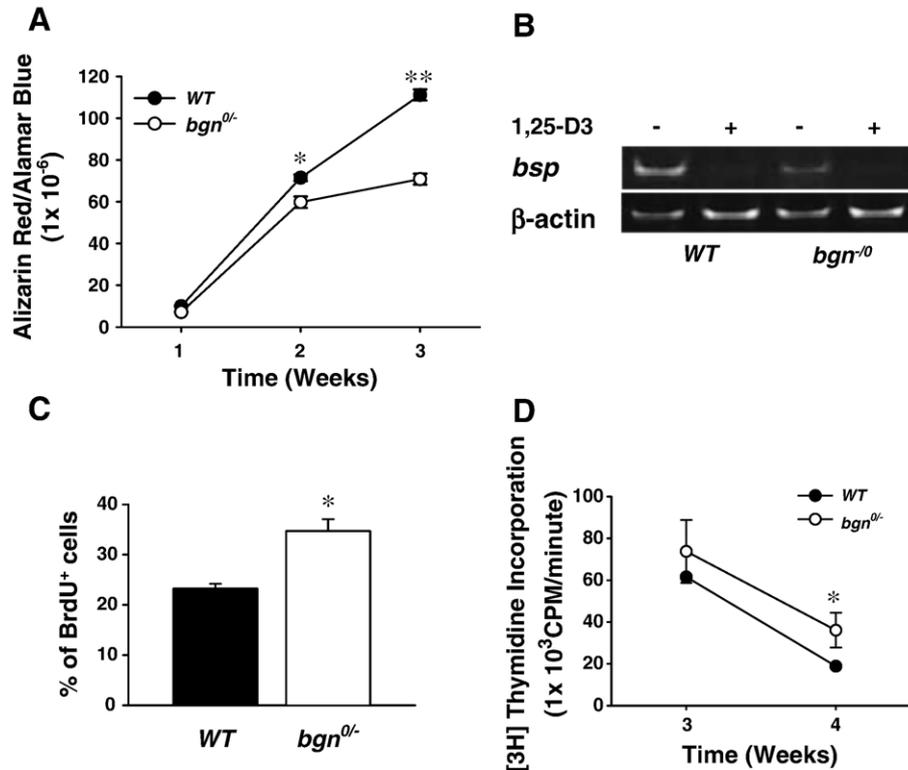


Fig. 5. Differentiation and proliferation of osteoblastic cells. (A) Differentiation capacity was determined using calvarial cells isolated from *WT* (solid circles) or *bgn* deficient (open circles) mice cultured in the presence of 0.01 M β -glycerolphosphate and 50 μ g/ml ascorbic acid. The ratio of alizarin red/amar blue was calculated after 1, 2, and 3 weeks of culture. (B) Semi-quantitative RT-PCR assay showing the relative mRNA levels of bone sialoprotein (BSP) in *WT* or *bgn* deficient calvarial cells treated with or without 10 nM 1,25-(OH)₂D₃ for 72 h. β -actin mRNA was used as an internal control. (C) The proliferation of BMSCs was determined by BrdU incorporation. The primary calvarial cells were labeled with BrdU for 7 h. The percentage of BrdU positive cells was quantified by scoring all of the cells in randomly chosen field (\times 200) on chamber slides containing *WT* (black bar) or *bgn* deficient (open bar) calvarial cells. The results are the average of 3 fields of each sample. (D) The proliferation of calvarial cells was determined by [³H]-thymidine incorporation. [³H]-thymidine (1 μ Ci/ml) was added to *bgn* deficient and *WT* cultures for 24 h prior to harvesting. All groups are $n = 3$. *Denotes $P < 0.01$ and ** denotes $P < 0.001$ compared to *WT* calvarial cells at the same time points.

the molecular mechanisms causing increased osteolysis, the expression of several genes was examined by RT-PCR in normal and mutant cells treated with or without 1,25-(OH)₂D₃. When calvarial cells were treated with 1,25-(OH)₂D₃, the expression of the LPS sensitive gene, secretory leukocyte protease inhibitor (slpi), significantly decreased (Fig. 6). Interestingly, the sensitivity of slpi to 1,25-(OH)₂D₃ was greater in the *bgn* deficient cells compared to *WT* controls (Fig. 6). These data point to a possible role for slpi that might link *bgn* deficiency with 1,25-(OH)₂D₃-induced osteoclastogenesis.

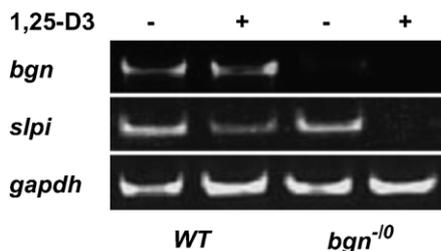


Fig. 6. Expression of secretory leukocyte protease inhibitor. The expression of *bgn* and *slpi* in *WT* and *bgn* deficient calvarial cells was quantified by RT-PCR after treatment with and without 10 nM 1,25-(OH)₂D₃ for 72 h. *Bgn* and Glyceraldehyde dehydrogenase (*gapdh*) mRNAs were used as internal controls.

Discussion

Many studies suggest that the osteoblasts and osteoclasts have an intimate, symbiotic relationship [31]. Considering the fact that *bgn* deficient mice have decreased osteoblast differentiation and bone formation [2,15,37], we suspected that osteoclast differentiation and function could also be affected in the absence of Bgn. We further predicted that pathological induction of bone resorption would uncover the effects of osteoblasts on osteoclasts in the *bgn* deficient mice. To test this theory, we induced local bone resorption using titanium particles containing LPS. Our data showed that *bgn* deficient mice had progressively more osteolysis compared to normal controls throughout the 10-day periods of stimulation by LPS. These observations confirmed our hypothesis that Bgn could influence osteoclast differentiation and activity through osteoblasts and their precursors under certain stress-induced conditions.

In order to determine the cellular basis for increased osteolysis in the absence of Bgn, a series of co-culture experiments were performed. These experiments revealed that both osteoclast differentiation and function were increased when the origin of the pre-osteoblast was from *bgn* deficient mice regardless of the genotype of osteoclast precursors. These data suggest that Bgn modulates osteoclast differentiation and

activities through osteoblasts and their precursors. Two potent cytokines, RANKL and OPG, both of which are made by osteoblasts and pre-osteoblasts, were not differentially regulated in the cells from the *bgn* deficient mice. In this regard, it is interesting to note that serum from patients afflicted with Turners Syndrome (XO) [6] did not show altered levels of RANKL or OPG compared to age matched controls. Previously, we showed that *Bgn* mRNA levels are decreased in cells derived from Turners patients [20]. We conclude, therefore, that *Bgn* expression may not parallel OPG/RANKL levels and may, in some cases, operate independently of this regulatory control system.

In order to examine how more global features of the mutant osteogenic cells could be affected, we assessed the relative proliferation and differentiation capacities of osteogenic cells in normal and *bgn* deficient mice. Our data pointed to the conclusion that *bgn* deficient cells have an imbalance in both proliferation and differentiation, either of which could be responsible for the increase observed in osteoclast differentiation and function in the mutant mice. Precedent for this concept is supported by previous studies suggesting a link between the level of osteoblast maturation and its ability to support osteoclastogenesis [2,15,37]. Moreover, studies using transgenic mice showed that *Cbfa1/RUNX2* over-expression in osteoblasts caused a delay in osteogenic maturity and a parallel increase in cortical osteoclast invasion [29] and activity [21] providing further evidence that there may be a link between osteoblast maturity and osteoclast formation. Increased proliferation and impaired maturation of osteoblast precursors in *bgn* deficient mice might support formation of more osteoclasts and increase the activity of existing osteoclasts, resulting in increased bone resorption. The decreased bone formation due to impaired differentiation of osteoblasts coupled with excess of bone resorption relative to bone formation rates might explain the continuous bone loss observed in *bgn* knockout mice with aging.

Other matrix proteins have also been implicated in osteoclast function but, for the most part, appear to have positive roles. For example, in contrast to *bgn* deficient mice, osteopontin deficient mice have decreased osteoclast function in vitro [33] and in vivo [1,26]. Mice deficient in osteonectin have decreased osteoclast number and function [16], indicating that, like osteopontin, osteonectin has a positive role in regulating osteoclast activities. The role of other ECM proteins such as BAG-75 [34], thrombospondin [7], and the ADAM-12 associated FLRG gene [3] appears to have inhibitory functions on osteoclasts when tested in vitro but further study will be needed to determine whether these functions also occur in vivo. It is possible that the matrix proteins made by osteoblasts such as osteopontin and bone sialoprotein regulate osteoclast functions directly such as by binding to cell surface receptors [28], or indirectly by altering the matrix micro-environment and controlling the distribution, balance, or accessibility of critical growth factors or nutrients.

In an attempt to uncover novel factors that might link *Bgn* to LPS-induced osteolysis, a comprehensive analysis of mRNA levels in normal and *bgn* deficient osteoblasts was

performed [11]. One gene known as *slpi* was significantly down-regulated in *bgn* deficient cells and examined further in the present study. Our analysis showed that *slpi* mRNA production was sensitive to 1,25-(OH)₂D₃ treatment and, further, that *bgn* deficient osteoblasts were more affected than control *WT* samples. This is the first report we know of that shows that *slpi* is a target for 1,25-(OH)₂D₃ regulation in osteogenic cells. This observation is interesting for several reasons. First, gain and loss of function experimental approaches show that *slpi* reduces inflammation by working through TNF- α and NF- κ B [22,24,39] both of which are known to be important to osteoclast function. Secondly, *slpi* is increased with estrogen treatment [9], decreased with advanced age [35], and inactivated after exposure to cigarette smoke [8]. Advanced aging, estrogen depletion, and cigarette smoking are all risk factors in osteoporosis [32], a disease characterized by abnormal osteoclast activity. Clearly more experiments will be needed to determine what role if any *slpi* has in regulating osteoclast function. Nevertheless, the data in the present study provide a foundation to further explore the potential relationship between *slpi*, LPS-induced osteolysis, and its potential modulation by *Bgn*.

In summary, we show, for the first time, a new function for the ECM protein, *Bgn*. *bgn* deficient mice subject to calvarial titanium particle-LPS overlay have increased osteolysis due to an increase in osteoclast formation, and that it is dependent on the mutant osteoblasts and their precursors. The mechanism of action appears to be from an imbalance of proliferation and differentiation of osteogenic cells when *Bgn* is absent. Defective osteoblastic function could cause numerous abnormalities in the ECM microenvironment including abnormal expression of BSP and *Slpi*, factors that could influence osteoblast–osteoclast coupling during bone turnover.

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