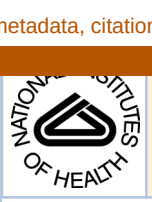



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# Role of Glycosaminoglycans of Biglycan in BMP-2 Signaling

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## Abstract

Recently we have reported that biglycan (BGN) promotes osteoblast differentiation and that this function is due in part to its ability to positively modulate bone morphogenetic protein (BMP) functions. In this study we investigated the role of glycosaminoglycans (GAGs) of BGN in this function using *in vitro* and *in vivo* models. C2C12 myogenic cells were treated or untreated with BMP-2 alone or in combination with glycanated, partially glycanated or de-glycanated BGN, and the effects on BMP signaling and function were assessed by Smad1/5/8 phosphorylation and alkaline phosphatase (ALP) activity. Furthermore, the effect of de-glycanation of BGN on BMP-2 induced osteogenesis was investigated employing a rat mandible defect model. The defects were filled with collagen scaffolds loaded with glycanated, partially glycanated or de-glycanated BGN alone or in combination with a sub-optimal dose of BMP-2 (subBMP). In *in vitro* experiments, BMP signaling and function were the greatest when BMP-2 was combined with de-glycanated BGN among the groups tested. In the rat mandible experiments, μCT analyses revealed that the newly formed bone was significantly increased only when subBMP was combined with de-glycanated BGN. The data indicate that the GAG component of BGN functions as a suppressor for the BGN-assisted BMP function.

### Keywords

biglycan; BMP-2; C2C12 cells; Smad pathway; microcomputed tomography; osteogenesis

## Introduction

Small leucine rich proteoglycans (SLRPs) are a large family of extracellular matrix proteins composed of a core protein that includes 10–20 leucine-rich repeats (LRRs) and covalently bound glycosaminoglycan chains (GAGs) [1,2]. The latter are highly negative-charged polymers of repeating disaccharides. One of the key functions of SLRPs in the extracellular matrix is the modulation of growth factor functions through its core protein or GAG

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component. [3,4]. Biglycan (BGN) is a multifunctional Class I SLRP member playing roles in collagen assembly [5,6], regulation of inflammation by interacting with TLR 2 and 4 receptors [7,8], binding and modulation of transforming growth factor (TGF)-β [3,9] and bone morphogenetic protein (BMP)-2 and -4 functions [10,11]. Previously, we have demonstrated that BGN promotes osteoblast differentiation, accelerates and increases matrix mineralization *in vitro*, and enhances the formation of a highly organized mineralized matrix *in vivo* [12]. These functions could be due to the capability of BGN to bind BMP-2/4 and their receptors [10,11,13]. However, the binding- and/or functional domains of BGN for its positive modulation of BMP functions are unknown. In this study, we investigated the role of GAGs of BGN in the BGN-assisted BMP-2 functions employing both *in vitro* and *in vivo* models.

## Materials and Methods

### Generation of partially glycanated, de-glycanated BGN and non-glycanated recombinant BGN

GAG containing BGN (glycanated) purified from bovine articular cartilage was commercially obtained (Sigma), suspended in distilled water (2 μg/μl) and an aliquot (4 μl) was treated with chondroitinase-ABC (C-ABC) (Seikagaku) overnight at 37°C [14]. Two concentrations of C-ABC, i.e. 0.3U and 0.1U/100 μg of protein were used to generate de-glycanated or partially glycanated BGN. C-ABC alone, C-ABC treated and non-treated BGNs were subjected to 4–12% SDS-PAGE and stained with Coomassie brilliant blue R-250 (CBB, Bio-rad) to confirm the extent of de-glycanation of BGN.

Non-glycanated, glutathione S-transferase (GST)-fused BGN and GST-protein were generated in bacteria as previously described [11]. Briefly, the primer sequences of mouse BGN cDNA corresponding to its mature core protein (amino acids 38-369; Asp38-Lys369) were designed as in Mochida et al., 2006 [11]. The PCR products amplified were then ligated into pGEX4T-1 vector (GE Healthcare), sequenced, and the plasmid harboring the mature BGN cDNA (pGEX4T-1-BGN) was obtained. The pGEX4T-1-BGN or pGEX4T-1 empty vector was transformed into BL21-CodonPlus bacterial strain (Stratagene), and GST-BGN or GST protein was synthesized. Supernatants of the bacterial lysates were incubated with glutathione-sepharose beads (Amersham Biosciences) and the eluted proteins were dialyzed against distilled water and lyophilized. The identity and purity of the proteins generated were confirmed on SDS-PAGE and Western blot (WB) analyses with anti-BGN polyclonal antibody LF-159 (generous gift from Dr. Larry W. Fisher at NIDCR, Bethesda) and anti-GST antibody (Sigma)[11].

### Alkaline phosphatase activity

The BMP function was assessed by the C2C12 cell assay system previously described [11,15,16]. The mouse C2C12 myoblastic cells were obtained from American Type Culture Collection (ATCC, CRL-1772). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) as described [11,16]. C2C12 cells were untreated or treated with glycanated BGN (4 μg), C-ABC alone, BMP-2 (R&D Systems) (150ng), BMP-2 + glycanated BGN or BMP-2 + C-ABC treated BGN (0.3U/100 μg of protein) (de-glycanated). After 3 days of culture, the cell lysates were collected and subjected to Alkaline phosphatase (ALP) activity in the same manner as described [11,16]. ALP assay was performed in triplicate and the values obtained were subjected to factorial analyses One-Way ANOVA and Tukey at 95% confidence interval using JMP 8.0 software (SAS).

### Smad phosphorylation assay

The effect GAGs of BGN on BMP signaling was assessed by the Smad1/5/8 phosphorylation in C2C12 cells. BGN glycanated, partially glycanated and de-glycanated with BMP-2 (see above) were added to cell medium. Cell extracts were then applied to 4–12% SDS-PAGE followed by WB analyses with anti-phospho-Smad1/5/8 antibody (Cell Signaling) or anti-β actin antibody (Cell Signaling) for normalization.

To assess a possible effect of C-ABC on BMP signaling, two control groups were investigated for Smad phosphorylation: C-ABC + BMP-2 (150ng), and C-ABC + GST-BGN (non-glycanated) + BMP-2.

### Effect of removal of GAGs of BGN in *in vivo* bone formation

The protocol of animal experiments was approved by the Institutional Animal Care and Use Committee (IACUC) at the University of North Carolina at Chapel Hill (IACUC ID: 09-237.0). To investigate craniofacial bone regeneration using BGN and BMP, 5mm-critical-sized defects were generated in mandibles of sixteen Sprague-Dawley male breeder rats weighing ~525g [17–20]. According to Arosarena et al., 100ng of BMP-2 is considered as “sub-optimal” dose of BMP as defects exhibit minimal osteogenesis even after 8 weeks of healing [20]. Thus, this “suboptimal” dose of BMP (subBMP) was used to evaluate the effect of BGN on BMP-induced bone formation. Briefly, all animals were given a pre-operative dose of antibiotic Cefazolin (10mg/kg). Anesthesia was achieved by Ketamine (80 mg/kg)/Xylazine (10 mg/kg). Two cm incisions were made along the inferior border of the hemi-mandibles and the masseter muscle and periosteum detached to expose the ramus. Using a 5mm-diameter trephine (Salvin Dental), a critical-sized defect was placed at the ramus ~3mm above the lower border of the mandible and 2mm distal to the incisor root (Fig 3). The defects were unfilled or filled with a UV-cross-linked collagen sponge (Nitta Gelatin) as a scaffold. Each scaffold precut with a 5mm-diameter tissue punch (Miltex Inc.) was soaked uniformly in 10 μl total solution of phosphate buffer saline containing the experimental protein groups. Control groups included: 1. unfilled, 2. collagen scaffold-filled with glycanated BGN, 3. collagen scaffold-filled with de-glycanated BGN [0.3U/100 μg protein], and 4. C-ABC alone (a total of 4 control groups without any addition of subBMP). Treatment groups received the scaffold loaded with: 5. subBMP alone or combined with 6. glycanated BGN, 7. de-glycanated BGN, or 8. C-ABC (a total of 4 treatment groups). For each group, 2 rats were used. The muscle layer was tightly sutured with 5-0 chromic gut (Ethicon) and the skin with 4-0 polypropylene suture (Ethicon) and the rats were maintained on a diet of soft rat chow (Harlan Taklad) and water for 4 days.

### Microcomputed tomography

At 2 weeks post-surgery, all animals were sacrificed, the mandibles were removed, fixed with 10% formalin for 3 days, and μCT scanning (Scanco Medical μCT 40) was performed. The X-ray parameters were 70kVp at 114uA with a 200ms integration time. Image matrix size was 2048 × 2048 with acquired 2000 projections over a 360 degree rotation. A tube of 20.5mm diameter which allows a pixel size of 10 μm was used. Acquisitions were made using a conebeam geometry and a Feldkamp filtered backprojection reconstruction algorithm used to create the reconstructions. All samples were positioned and scanned in a standard manner using an airtight cylindrical sample holder filled with PBS. For the analyses of the acquired images, the CTAn analyser software (Skyscan) was used. The region of interest was selected in all images through a standardized drawing of the area within the defect borders. The region was first positioned in the middle of the surgical defect and it was then extended to all slices of the data set. A new drawing was done every 20 images. Quantitative morphometric analysis of the mineralized tissue inside the defects was

carried out on voxels that corresponded to bone. After tomographic acquisitions, 3D images were reconstructed through direct volume rendering from the series of 2D projections.

## Results

### GAG removal enhances BGN-assisted BMP-2 function

For the removal of GAGs, glycanated BGN was treated with several concentrations of C-ABC and the release of the core protein was examined by SDS-PAGE. At the concentration of 0.3U/100 μg of protein (denoted as ++), the core protein (~45 kDa) was efficiently released (Fig 1A, lane 2) (also see below), which is consistent with the report by Matsuno et al., 2007[14]. The effects of glycanated and de-glycanated BGN on BMP-2 function were evaluated by ALP activity (Fig 1B). No treatment (lane 1) or treatment with BGN (lane 2) or C-ABC alone (lane 3) did not exert significant ALP activity. The BMP-2 induced ALP activity (lane 4) was significantly increased with the addition of glycanated BGN (lane 5), but the increase was further enhanced with de-glycanated BGN (lane 6) ($p<0.05$).

### The levels of GAGs of BGN affect the enhancement of BGN-assisted BMP-2 signaling

Figure 2A shows BGN core protein band when it was untreated (lane 1) or treated with 0.3U/100 μg (++) (lane 2) or 0.1U/100 μg of C-ABC (+) (lane 3). As demonstrated, C-ABC treatment at a concentration of 0.3U/100 μg (++) was sufficient to generate a distinct BGN core band at ~45kDa (lane 2) (de-glycanated). Use of higher concentrations of C-ABC did not change the level of core protein indicating that the BGN is likely fully de-glycanated with this concentration of C-ABC (data not shown). With a concentration of 0.1U/100 μg (+) the BGN core appeared as a smear band with a slightly higher molecular weight indicating that the GAGs were not fully removed (i.e. partially glycanated).

Fig 2B shows the effects of de-glycanation of BGN on BMP-2 signaling assessed by Smad1/5/8 phosphorylation. Without treatment (lane 1) or treated with BGN alone (lane 3) C2C12 cells did not induce Smad phosphorylation. When glycanated BGN was added to BMP-2 (lane 2), the signaling level was increased when compared to that of BMP-2 alone (lane 4). This positive effect was further enhanced with de-glycanated BGN (lane 5), but the enhancement was decreased with partially glycanated BGN (lane 6). C-ABC alone did not affect the BMP-2 signaling (lane 7). The results indicate that though all BGN species exerted positive effects on BMP-2 signaling, the enhancement levels were inversely correlated with the level of GAGs. The positive effect of non-glycanated BGN was further confirmed by the use of recombinant GST-BGN (Fig 2C). The GST-BGN alone did not induce the BMP-2 signaling (lanes 1 and 3). The BMP-2 induced signaling (lane 2) was enhanced with the addition of GST-BGN (lane 4). Addition of C-ABC to (GST-BGN + BMP-2) did not increase the (GST-BGN + BMP-2) induced signaling (lane 5) confirming the lack of C-ABC effect on BMP-2 signaling (also see Fig 2B, lane 7). GST protein alone showed no effect as we previously reported [11].

### De-glycanated BGN enhances BMP-2 induced osteogenesis

Figure 3 shows the representative 2D and 3D μCT images obtained from similar anatomical areas including the defects (indicated by dotted line) at the 2 week post-surgery. The graph at the bottom shows the bone volume (BV) of newly formed bone (NFB) in the defects. The NFB in the subBMP group (lane 1) was similar to those of control groups (i.e. empty, collagen scaffold alone or filled with glycanated or de-glycanated BGN, or C-ABC alone) (data not shown). When subBMP was combined with glycanated BGN (lane 2) or C-ABC (lane 3), the newly formed bone was unchanged or slightly lower when compared to the group of subBMP alone. However, when de-glycanated BGN was combined with subBMP, the NFB volume was markedly increased (lane 4).

## Discussion

BGN was originally found in the mineral-associated compartment of bone suggestive of its role in mineralization [21]. In addition, it has been reported that BGN is highly expressed in the areas of growth of skeletal tissues and the cell surface of both chondroblasts and osteoblasts, suggesting its role in modulating the cellular activity [22]. The study reported by Chen et al., 2004 [10] was the first to propose a mechanism by which BGN could facilitate osteoblast functions. Independent of these studies, we have reported that BGN levels were "positively" correlated with the timing, extent and quality of matrix mineralization [12]. Also, Chen et al. and we further reported that BGN suppression resulted in poor response of osteoblastic cells to BMP [10,12]. Furthermore, we demonstrated that BMP-induced ALP activity in C2C12 cells was synergistically increased with the exogenous addition of GST-BGN and that BGN bound to BMP-2 and its type I receptor, ALK6, and possibly other receptors [11].

In the present study, we explored the potential role of GAG component of BGN on the BGN-assisted BMP function. Our study demonstrated that de-glycanation of BGN with C-ABC treatment significantly enhances the BGN-assisted BMP-2 signaling and function. The partial removal of GAGs obtained with a low concentration of C-ABC (0.1U/100 μg) showed that the enhancement was indeed less potent compared to that of fully de-glycanated BGN.

C-ABC alone did not potentiate BMP-2 signaling. In fact, an addition of C-ABC to GST-BGN rather tended to decrease the BMP-2 signaling (Fig 2C). C-ABC could cleave the GAGs of other proteoglycans present at the myoblastic cell surface and/or in the extracellular matrix in the cultures, which may affect BMP-2 signaling. Indeed, myoblasts are known to synthesize chondroitin sulfate (CS)/dermatan sulfate (DS) proteoglycans such as decorin and versican [23,24]. Thus, higher doses of C-ABC (up to 4.0U/100 μg) were also tested in combination with BMP-2, however, this caused even a further decrease in BMP-2 signaling (data not shown). This suggests that the positive effect of de-glycanation on BMP function is specific to BGN.

The mechanism by which the GAG component suppresses the BGN assisted BMP-2 function is not clear at this point. However, it is conceivable that the highly negative charged GAGs can sequester the basic protein, BMP-2, leading to reduced interaction between BGN core protein and BMP-2. During the preparation of this manuscript, a study was published on the effect of BGN on BMP-2 canonical and non-canonical pathways in MC3T3-E1 cells [25]. The authors found that glycanated BGN is able to increase the phosphorylation of ERK in addition to Smad 1/5/8. We have also investigated the effect of glycanated and de-glycanated BGN on ERK phosphorylation and found no effect in C2C12 cells (data not shown).

The *in vitro* ALP and Smad phosphorylation data (Fig 1B and 2B, respectively) unequivocally demonstrated that de-glycanation of BGN greatly increases the BGN-assisted BMP-2 function and signaling. A positive effect of de-glycanation of BGN on BMP-induced osteogenesis was also observed in the *in vivo* study, while glycanated BGN had no effect. For the results from *in vivo* study, a few possibilities should be considered. First, the glycanated BGN used in this study was purified from articular cartilage resulting as a protein with a mixture of CS and DS-GAGs (Sigma). The presence of those GAGs and the ratio of CS to DS in this sample could influence significantly BGN function as these disaccharides possess different affinity to calcium and hydroxyapatite (HAP)[26,27]. Also, due to the increased structural flexibility, DS could function as an inhibitor of mineralization [28]. Second, is that BGN with GAGs may favor alternative interactions rather than with

BMPs and/or its receptors. It has been reported that BGN highly interacts with macrophages stimulating secretion of pro-inflammatory molecules [7,8], which could impair proper osteogenesis. Third, the release kinetics of glycanated vs. de-glycanated BGN from the collagen scaffold could be different. BGN has been found to weakly interact with collagen type I via both core and GAGs [29]. However, if GAGs are present, the interaction is mainly through the disaccharides with a lower dissociation constant ($K_d$), i.e., stronger binding, than through core [30–32]. Thus, longer retention of glycanated BGN in the scaffold could have negatively affected the release of BGN to assist on BMP function. Further studies investigating the kinetics of (glycanated, de-glycanated) BGN release are warranted. However, it is clear that the removal of GAGs from BGN facilitates the BMP-2 induced osteogenesis *in vivo*, which is consistent with the *in vitro* data.

In conclusion, these *in vitro* and *in vivo* results strongly indicate that the BGN core protein is responsible for its function in promoting the BMP-2 functions and that the GAG component could function as a suppressor in this process. BGN core protein could be used in clinical settings to enhance BMP-2 induced bone formation after the optimal conditions *in vivo* are established. The rat mandible defect model described is feasible and appears to be appropriate for these future studies.

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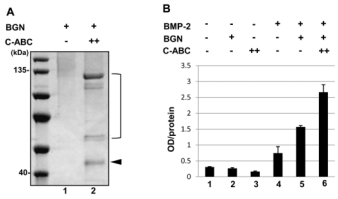


**Figure 1.**

Generation of de-glycanated BGN and its effect on BMP-2 function. (A) SDS-PAGE of BGN without (lane 1) and with (lane 2) chondroitinase ABC (C-ABC) treatment. A bracket indicates C-ABC. An arrow head indicates the core protein of BGN. (B) ALP activity in C2C12 cells. Note that BMP-2 induced ALP activity is significantly increased by the addition of glycanated BGN (lane 5) but the effect is further enhanced by de-glycanation of BGN by C-ABC treatment (lane 6) ($p<0.05$). ++ of C-ABC: 0.3U/100 μg, + for BGN and BMP-2 are 4 μg and 150ng, respectively.

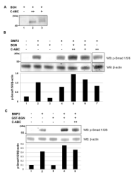

**Figure 2.**

Effects of partially- and fully de-glycanated BGN on BMP-2 signaling. + of C-ABC: 0.1U/100 μg of protein and ++: 0.3U/100 μg of protein. + for BGN and BMP-2 are 4 μg and 150ng, respectively. (A) BGN (glycanated) (lane 1), BGN treated with chondroitinase-ABC (C-ABC) at a concentration of ++ (lane 2) or + (lane 3). Note that only partial de-glycanation was achieved with the latter treatment. (B) Effects of glycanated, partially- (C-ABC +) and fully (++) de-glycanated BGN on BMP-2 signaling measured by Smad1/5/8 phosphorylation. BMP-2 induced signaling (lane 4) was increased by glycanated BGN (lane 2), and it was further enhanced by de-glycanation of BGN with C-ABC (lanes 5–6). Note that the fully de-glycanated BGN (lane 5) shows higher signaling compared to that of partially glycanated (lane 6). C-ABC alone with BMP (no BGN) did not enhance BMP signaling (lane 7). (C) Effect of C-ABC treatment on GST-BGN-enhanced BMP-2 signaling. C-ABC did not increase the effect of GST-BGN (lane 5). GST-BGN: glutathione S-transferase fused BGN.

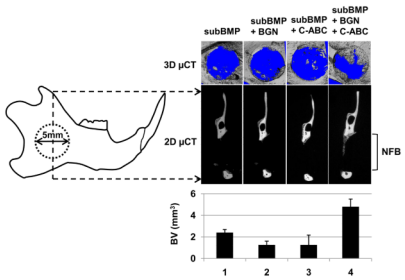


**Figure 3.**

µCT images corresponding to the rat mandible 5mm-surgical defects at 2 weeks post-surgery. subBMP: sub-optimal BMP-2 (100ng), BGN:4 µg. (A) Schematic representation of rat mandible and defect location on the left. On the right, top panel shows 3D µCT and lower panel 2D µCT views of the defects treated with subBMP dose without (lane 1) or in combination with BGN (glycanated) (lane 2), C-ABC (lane 3) or BGN/C-ABC (de-glycanated)(lane 4). Below, the median (represented by columns) and range (error bars) of bone volume (BV) of the newly formed bone (NFB) of 2 defects per group of treatment 2 weeks post-surgery.