Published in final edited form as:

Matrix Biol. 2016 ; 52-54: 141–150. doi:10.1016/j.matbio.2016.03.008.

Biglycan potentially regulates angiogenesis during fracture repair by altering expression and function of endostatin

Maja Myren¹, David J. Kirby¹, Megan L. Noonan¹, Azusa Maeda¹, Rick T. Owens², Sylvie Ricard-Blum³, Vardit Kram¹, Tina M. Kilts¹, and Marian F. Young¹

¹Craniofacial and Skeletal Diseases Branch, NIDCR, NIH, Bethesda MD 20892

²Life Cell Corporation, Branchburg, NJ 08876

³University of Lyon, UMR 5246 CNRS - University Lyon 1, ICBMS, 69622 Villeurbanne, France

Abstract

The small proteoglycan biglycan (Bgn) is highly expressed in the organic matrix of bone and plays a role in bone formation. Previous work implicated Bgn in vessel growth during bone healing (1). By infusing barium sulfate (BaSO₄) into *WT* and *Bgn*-deficient mice we discovered the positive effect of Bgn in modulating angiogenesis during fracture healing. Using micro-computed tomography angiography we found significant differences in the vessel size and volume among other parameters. To further understand the mechanistic basis for this, we explored the relationship between Bgn and the anti-angiogenic protein endostatin. Immunohistochemistry (IHC) showed co-localization of Bgn and endostatin in regions of bone formation, with increased endostatin staining in *Bgn*-KO compared to *WT* at 14 days post-fracture. To further elucidate the relationship between Bgn and endostatin, an endothelial cell tube formation assay was used. This study showed that endothelial cells treated with endostatin had significantly decreased vessel length and vessel branches compared to untreated cells, while cells treated with endostatin and Bgn at a 1:1 molar ratio had vessel length and vessel branches comparable to untreated cells. This indicated that Bgn was able to mitigate the inhibitory effect of endostatin on endothelial cell growth. In summary, these results suggest that Bgn is needed for proper blood vessel formation during fracture healing, and one mechanism by which Bgn impacts angiogenesis is through inhibition of endostatin.

Keywords

biglycan; fracture healing; angiogenesis; endostatin

Corresponding Author: Marian Young, Chief, Molecular Biology of Bones and Teeth Section, Craniofacial and Skeletal Diseases Branch, National Institute of Dental and Craniofacial Research, National Institutes of Health, 9000 Rockville Pike, Building 30 Room 211, Bethesda, MD 20892, ; Email: Myoung@dir.nidcr.nih.gov, Tel: 301-496-8860.
Maja Myren, David J. Kirby, (Co-first authors)

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Introduction

The annual incidence of adult fractures has been estimated at 9.1 to 36 per thousand per year (2,3), with 5–10% of fractures complicated by non-union or delayed union (4). Non-union, the failure of a broken bone to heal, causes significant morbidity and is the result of impaired healing. Poor vascularity of the fracture zone disrupts healing and has been identified as a risk factor for non-unions (5). By studying the mechanisms controlling angiogenesis in fracture healing, a framework can be formed which would allow for the development of therapies to improve fracture site vascularity and decrease non-unions.

We previously identified the matrix component, biglycan (Bgn), as a potential regulator of angiogenesis during fracture healing (1). Bgn is a member of the small leucine-rich proteoglycan (SLRP) family (6) and is abundant in mineralized tissue. *Bgn*-deficient mice have defective bone formation and mineralization (7), which may, in part, be caused by changes in the expression (8) and hierarchical structure of other important matrix components of bone such as type I collagen. Indeed, mouse models have shown that the absence of Bgn leads to abnormal collagen fibril shape and character (9–11), which could be one of the foundations for the mineralized tissue defects observed in the *Bgn*-deficient (KO) mice. The cellular and molecular basis for the bone abnormalities found in the absence of Bgn appears to be from defects in osteogenic progenitors that have a reduced ability to undergo osteogenesis *in vitro* (12). Several factors have been implicated in modulating the Bgn-osteogenesis regulatory axis, including TGF-beta (8), BMP-2 (12) and Wnt signaling (13). Recently we found that Bgn has strong binding affinity for the angiogenic factor, VEGF; however, it did not potentiate the effect of VEGF in a human umbilical vein endothelial cell (HUVEC) endothelial vessel forming assay (1). This observation led us to consider that there were yet unidentified factors that work with Bgn to control vessel formation. Our attention was drawn to endostatin, a 20 kDa C-terminal fragment of type XVIII collagen that has potent antiangiogenic activities (14), and appears to regulate angiogenesis in multiple ways (15–18). Previous work using solid phase binding and immunoprecipitation (IP) showed Bgn directly binds to endostatin (15). The goal of this investigation was to determine if Bgn could work through inhibition of endostatin to regulate angiogenesis during fracture healing.

Results

Vessel quantitation in WT and *Bgn*-deficient mice

Fracture callus vascularity at 7 days post-fracture in wild type (*WT*) and *Bgn*-KO mice was compared using micro-computed tomography (μ CT) angiography. Three-dimensional μ CT rendering of bones 7 days post-fracture using both anterior (Fig. 1B) and mid-coronal (Fig. 1D) views of the whole fractured femurs displayed decreased vascularity in *Bgn*-KO mice compared to the *WT* mice (Fig. 1A,C). When the bone was digitally removed to display only the vascular bed at the fracture site, the anterior (Fig. 1G) and mid-coronal views (Fig. 1H) again showed decreased vasculature in *Bgn*-KO mice compared to *WT* (Fig. 1E,F). Transverse sections at the level of the fracture with and without the bone also demonstrated decreased vasculature in the *Bgn*-KO mice (Fig. 1K, L) compared to *WT* (Fig. 1I,J). The three-dimensional μ CT rendering of 7 days post-fracture callus was quantified and showed a

decreased trend (but not significant with this sample size) in total tissue volume in the *Bgn*-KO compared to *WT* (Fig. 1A). Despite the limited sample size many other features of the vessels were affected. Specifically, there was significantly reduced vessel volume (Fig. 2B), total vessel volume/total volume (Fig. 2C) vessel number (Fig. 2E) and vessel thickness (Fig. 2F) with significantly increased vascular separation (Fig. 2G) in *Bgn*-KO mice compared to *WT* mice. Vessel connectivity (Fig. 2D) exhibited a reduced trend in the *Bgn*-KO but was not significantly different from *WT* mice. Quantitative real-time RT-PCR of mRNA from the fracture callus was performed which showed the expression of *Pecam1* mRNA, a marker of endothelial cells, was significantly decreased in *Bgn*-KO mice compared to *WT* mice 7 days after fracture (Fig. 2H).

Bgn and endostatin expression in healing bone callus

In an effort to identify the mechanism by which Bgn impacts angiogenesis, the interaction between Bgn and angiogenic factors was studied. Endostatin, a strong angiogenesis inhibitor, was previously found to bind Bgn through solid-phase binding assays (15). In the context of this relationship, immunohistochemistry (IHC) of the *WT* mouse fracture callus 7 days post-fracture was performed. Consecutive sections were stained for Bgn and endostatin, which revealed spatially interrelated expression. Specifically, Bgn (Fig. 3A,B) and endostatin (Fig. 3D,E), shown at low (Fig. 3A,D) and high power (Fig. 3B,E), were colocalized around forming woven bone spicules. Endostatin and Bgn expression was most prominent at the endochondral ossification sites where cartilage is transitioning to bone and where vessels are beginning to infiltrate (Fig. 3). No staining was observed in isotype matched negative controls (Fig. 3C,F). Interestingly, immunohistochemistry of *WT* and *Bgn*-KO mouse callus 14 days post-fracture appeared to show increased endostatin expression in *Bgn*-KO mice compared to *WT* mice, visualized at low (Fig. 3G,I) and high power (Fig. 3H,J). The expression of endostatin mRNA in fracture healing was then studied with real time RT-PCR. As predicted, no appreciable *Bgn* mRNA was found in the Bgn-deficient mice (Fig. 3K). Endostatin (*Col18a1*) mRNA levels were also examined and found to be significantly higher in the fracture callus of *Bgn*-KO mice compared to *WT* mice (Fig. 3L) but not until 14 days post fracture.

Bgn and endostatin impact on endothelial cell tubule formation

To explore the functional relationship between Bgn and endostatin, a HUVEC tube formation assay was performed. Endothelial cells were grown in media containing VEGF (as a positive control and induces angiogenesis), suramin (as a negative control that inhibits angiogenesis), endostatin, Bgn, or combinations thereof. After 14 days of growth, cultures were stained for *Pecam1*, the previously mentioned endothelial cell marker. Analysis of the staining showed that cultures treated with endostatin alone (Fig. 4D) had significantly decreased vessel length and branch points (Figure 4 I,J) compared to untreated cells (Fig. 4B,I,J), while cultures treated with endostatin and equimolar or greater amounts of Bgn had significantly increased vessel length and branch points compared to endostatin alone (Fig. 4E–G,I, J). Moreover these cultures had vessel length and branch points equivalent to that of untreated cells.

Discussion

The potential role of Bgn in regulating angiogenesis has previously been examined in gastric cancer (19) and in tumor endothelial cells isolated from human melanoma xenografts (20). In both studies, Bgn appears to play a role in enhancing vessel formation and tumor cell migration; however, the exact mechanism for this was unclear. In our studies on fracture healing, we found *Bgn*-deficient mice have reduced vessel volume, number, thickness and spacing supporting the notion that Bgn has positive angiogenic properties in this unique pathological setting. Considering Bgn can directly bind to endostatin (15), we speculated it could, in some way, interfere with endostatin and regulate its function during bone repair. For the first time, we show Bgn localizes with endostatin at sites of new bone formation, and that Bgn can counteract endostatin's inhibitory function during vessel formation.

Endostatin has potent antiangiogenic activities (14) and appears to regulate angiogenesis in multiple ways. A comprehensive analysis by Abdollahi (21) mapped endostatin's antiangiogenic signaling network by mRNA profiling endostatin treated and showed that endostatin's effects on cell signaling pathways were enormous encompassing over 6635 Unigene clusters. Interestingly some of the mRNA's controlled by endostatin included VEGF a finding recently verified by Zhang et al (16) and integrin alpha V (21) which we also found down regulated in the *Bgn*-KO fracture callus (not shown). In this regard it is important to note that endostatin was shown to inhibit endothelial cell migration (17) possibly by a direct interaction with alpha-5 and alpha-V integrins at the surface of endothelial cells (22). While much is known about the generation and processing of endostatin by proteases (23) the cell source of endostatin particularly during fracture healing is more elusive. Additional studies will be needed to precisely track endostatin expression using genetic "knockin" technology or in-situ hybridization to effectively address this point. Regardless of its origin, in bone endostatin is localized in the basement membranes of periosteal and bone marrow vessels (24). The periosteum is a key source of progenitor cells that upon fracture, expand and migrate to the affected site to play essential roles in the repair process. It is possible that endostatin has multiple roles in bone healing related to periosteal expansion, migration, and subsequent bone repair.

An interesting mechanistic point that remains unanswered is: how does Bgn fit into the endostatin-VEGF-integrin responsive axis? We do not know at this time which of the skeletal cells are important for Bgn's effect on angiogenesis but we presume it might involve the osteoblast a key target for Bgn's activities in bone (12). Clearly more experiments are needed to clarify the molecular interplay and subsequent cascade of events that unfolds during angiogenesis during fracture healing. Another interesting point that has emerged from anti-tumor studies is that endostatin has a biphasic efficacy response (18). In this context, it is tempting to speculate that Bgn could be an upstream "fine-tune" regulator of the many downstream functions that are ascribed to endostatin in pathological situations.

Decorin (Dcn) is a SLRP that is most closely related to Bgn (6), yet in contrast to what we found for Bgn, Dcn has inhibitory effects on angiogenesis (25,26). It is unclear why Dcn and Bgn have what appears to be opposite effects on angiogenesis, but it is likely that tissue context is involved (27). That is to say, it is possible that vessel formation might be

controlled in different ways when angiogenesis is acting as the “enemy” such as in cancer or in pathologies such as diabetic retinopathy (28), compared with situations like fracture healing where angiogenesis is the “friend” (29). Considering the large body of evidence now linking Dcn inhibition of angiogenesis to autophagy (30,31), we can not ignore the fact that Bgn could regulate autophagy during fracture healing. Indeed in this regard it is interesting to note that endostatin induces autophagy in endothelial cells by modulating Beclin 1 and beta-catenin (32). In this context it should be noted that the decorin levels are higher in the callus of Bgn KO mice compared to WT mice (1) potentially adding another level of complexity to the situation. Whatever the case may be, many additional unanswered questions remain. For example, the first stage of fracture repair begins with a robust immune response that is thought to be important to the entire sequence of bone repair (33). Interestingly, Bgn has previously been shown to regulate the immune response in a TLR2/4 dependent fashion in macrophages (32,34). It is possible that in the absence of Bgn, the inflammatory response at early stages of fracture healing could be compromised, subsequently effecting down stream processes such as cartilage formation and vessel infiltration. Experiments are underway to determine the precise sequence of events controlled by Bgn during the entire fracture healing process.

The burden associated with fractures necessitates a thorough understanding of the fracture healing process to optimize treatment. Crucial to this process is the formation of new blood vessels to deliver the components required for the fracture to heal. In this study, Bgn was shown to play a role in the process of angiogenesis, and that effect appears to be partially mediated through endostatin suppression. Although its role in angiogenesis has been shown, questions remain on how Bgn mediates its effect on this process.

Experimental Procedures

Animal Experiments

Male C57BL/6 (WT) (Taconic, USA) or *Bgn*-KO mice (7) were used in these experiments. Animals were 6–8 weeks old when fractured. The National Institutes of Dental and Craniofacial Research Animal Care and Use Committee approved all experimental procedures (protocol #13-676).

Surgical procedures

Mice were anesthetized by an intraperitoneal injection of 0.01 mL/g 10% ketamine, 5% xylazine and 2 mg/kg acepromazine. Mice were supplemented with 2–3% isoflurane as needed. 0.1 mg/kg buprenorphine was administered subcutaneously before the procedure and postoperatively as needed. The right leg was shaved and scrubbed with a 10% povidone-iodine and alcohol solution to prepare it for surgery. A 5-mm medial parapatellar incision was created. The patella was dislocated to expose the femoral condyle. A hole was burred into the femoral intracondylar notch using a 25-gauge (25G) hypodermic needle. Sometimes holes were enlarged using a 23G needle. A 26G cannula (MP06226, Millpledge Veterinary, USA) was inserted into the burred hole. The needle was retracted and the plastic sheath left in the bone. The fractures were created using a 3-point bending device (1). A 100-g weight was dropped to apply a precise force that would create one single oblique fracture.

Immediately after fracture the leg was X-rayed to confirm the nature of the fracture. The cannula needle was inserted back into the plastic sheath, pulled back 1 mm and the protruding end of the needle was cut off. The cannula needle and sheath were gently pushed back into the hole to provide stabilization. A drop of tissue glue (VetBond™) was used to seal the hole thus preventing the cannula from moving out. The patella was repositioned, and the incision was closed using the same tissue glue. The fractured legs were X-rayed again to document the fracture with a stabilizer inside the femur.

Perfusion of the vascular system and μ CT scanning

At 7 days post-fracture, animals were sedated using 4% isoflurane and subjected to X-ray to assess fracture severity. Only animals with simple transverse fractures were used. After anesthesia, the thoracic area was shaved, an incision made, and the heart exposed. A 25 G butterfly needle was inserted into the left ventricle and a solution of heparinized saline was infused using a peristaltic pump. Next, the right atrium was punctured and the entire vascular system flushed with the heparinized saline solution for 5 minutes, followed by infusion of 10% barium sulfate solution with 1.5 % gelatin for 7 more minutes. The mice were stored at 4°C until the barium sulfate solution had cast, and then the femurs were dissected out for further evaluation. The whole fractured legs were scanned as soon as possible after the harvest with a maximum of twelve hours after harvest to avoid diffusion of barium sulfate in the μ CT system (μ CT 50, Scanco Medical AG, Bassersdorf, Switzerland). Scans were performed at 70 kV, 85 μ A, 300 ms integration time, and at a resolution of 10 μ m. After reconstruction, the images were stored in 3D arrays. The tissues were differentially segmented by a global thresholding procedure (35) requiring two separate threshold inputs to allow for the adjustment of two masked outputs [e.g. bone and vessels]. Morphometric parameters were determined by a direct 3D approach (36). Quantitative parameters were determined in a volume of interest (VOI) that was 1.5mm proximal and distal to the fracture site. Vascular parameters were based on a trabecular bone script and included: total tissue volume (TV), vessel volume (VV), vessel volume/total volume (VV/TV), connectivity, vessel number (Vs.N), vessel thickness (Vs.Th), and vascular spacing (Vs.Sp).

Immunohistochemistry

Femurs from *WT* and *Bgn*-KO mice at 7 and 14 days post-fracture were surgically isolated and fixed for 24 hours in Z-fix (170; Anatech, LTD) at 25°C. Samples were paraffin-embedded and sectioned in a sagittal plane with every 5th slide stained with H&E for orientation. Comparable sections from *WT* and *Bgn*-KO samples were deparaffinized in xylene washes and rehydrated in graded ethanol series into water. For Bgn staining, sections were subjected to chondroitinase ABC (100330-1A; Seikagaku) for 1 hour at 37°C; for endostatin staining, sodium citrate (pH 6.0) for 20 min at 95°C was used. A peroxidase block with 3% H₂O₂ for 20 min at room temperature was then performed. Sections were incubated with primary antibodies at 4°C overnight including polyclonal rabbit anti-mouse Bgn (1:500 dilution, rabbit total serum, LF-106; Dr. Larry Fisher, NIH), anti-human Endostatin (1:100 dilution, rabbit polyclonal antibody; Dr. Sylvie Ricard-Blum, University of Lyon) (37). Isotype-matched antibodies were used as negative controls under the same conditions. The broad-spectrum immunoperoxidase AEC kit (Picture Plus, Zymed) was

subsequently used to detect the immunoactivity according to the manufacturer's instructions. The sections were counterstained with hematoxylin and scanned using a Model CS Aperio Scanscope.

mRNA extraction and real time RT-PCR

Calluses from fractured *WT* and *Bgn*-KO mouse femurs at 14 days post fracture were surgically isolated, snap-frozen in liquid nitrogen, and stored at -80°C . The calluses were pulverized with a mortar and pestle in liquid nitrogen and total RNA was isolated using TriPure Isolation Reagent (11667165001; Roche). Isolated RNA was treated with RNase-free DNase (18068-015; Invitrogen). cDNA was generated with iScriptTM cDNA Synthesis Kit (170-8891; Bio-Rad). Primers listed in Table 1 were designed with Beacon Designer Software (Bio-Rad) with nucleotide sequences imported from the GeneBank accession numbers indicated. Real-time PCR was performed with primers, iQTM SYBR[®] Green Supermix (170-8886; Bio-Rad) and CFX96TM Real-Time PCR Detection System (Bio-Rad). Target gene expression was normalized to S29 expression.

Endothelial cell tube formation assay

HUVEC cells (ZHA-4000, Cellworks V2a KitTM) were grown according to kit protocol guidelines. Briefly, V2a Seeding Media was exchanged for V2a Growth Media with appropriate control and test components after 48 hours. Untreated and VEGF treated cultures served as positive controls; suramin treated cultures served as negative control (Fig. 4). For test compounds, human recombinant endostatin (E8154; Sigma) and human recombinant Bgn (glycosylated form) (Dr. Rick T. Owens, LifeCell Corporation) were used. Endostatin was added at 2.5×10^{-9} moles/L. Bgn was added at 2.5×10^{-9} moles/L to samples treated with Bgn alone, and to Bgn/endostatin treated samples at 0.5, 1, and 2 moles of Bgn per 1 mole endostatin. Compounds were added to V2a Growth Media and incubated at 4°C for 1 hour with agitation for prior pre-equilibration and to allow binding to occur between Bgn and endostatin. All medias were allowed to pre-equilibrate at 37°C for 30 minutes prior to media exchange. V2a Growth Media was replaced every 48 hours in this manner (with fresh Bgn and endostatin) for 14 days.

Imaging and tube quantification were performed according to kit protocol guidelines. Briefly, whole well images were taken using an Olympus SZ61 microscope at $0.67\times$ magnification, with contrast and lighting set to minimize shadow. Tube quantification and analysis were performed with AngioSys 2.0 Image Analysis Software. A threshold of 129 was chosen to maximize vessel identification and minimize background. AngioSys 2.0 Software prepared and quantified a skeletonized vessel map of each well.

Statistics

The data was presented as means with standard deviations. To determine statistical significance, an unpaired two-tailed Student's *t*-test was utilized with $p < 0.05$ considered significantly different.

Acknowledgments

This research was supported in part by the Intramural program of the NIDCR, NIH, DHHS. We would like to thank, Dr. Rhonda Prisby (University of Delaware) for sharing her expertise in vessel casting *in vivo* and Dr. Neal Fedarko for advice on the use of the HUVEC system. We also wish to thank Ms. Aalia Farukhi for her technical assistance and Ms. Li Li for her help in sectioning femurs for immunohistochemistry.

References

1. Berendsen AD, Pinnow EL, Maeda A, Brown AC, McCartney-Francis N, Kram V, Owens RT, Robey PG, Holmbeck K, de Castro LF, Kilts TM, Young MF. Biglycan modulates angiogenesis and bone formation during fracture healing. *Matrix biology : journal of the International Society for Matrix Biology*. 2014; 35:223–231. [PubMed: 24373744]
2. Donaldson LJ, Cook A, Thomson RG. Incidence of fractures in a geographically defined population. *Journal of epidemiology and community health*. 1990; 44:241–245. [PubMed: 2273364]
3. Donaldson LJ, Reckless IP, Scholes S, Mindell JS, Shelton NJ. The epidemiology of fractures in England. *Journal of epidemiology and community health*. 2008; 62:174–180. [PubMed: 18192607]
4. Moucha, CS.; Einhorn, TA. Bone morphogenetic proteins and other growth factors to enhance fracture healing and treatment of nonunions. In: Lieberman, JR.; Friedlaender, GE., editors. *Bone regeneration and repair: Biology and clinical applications*. Totowa, New Jersey: Humana Press; 2005. p. 169
5. Santolini E, West R, Giannoudis PV. Risk factors for long bone fracture non-union: a stratification approach based on the level of the existing scientific evidence. *Injury*. 2015; 46(Suppl 8):S8–S19. [PubMed: 26747924]
6. Iozzo RV, Schaefer L. Proteoglycan form and function: A comprehensive nomenclature of proteoglycans. *Matrix biology : journal of the International Society for Matrix Biology*. 2015; 42:11–55. [PubMed: 25701227]
7. Xu T, Bianco P, Fisher LW, Longenecker G, Smith E, Goldstein S, Bonadio J, Boskey A, Heegaard AM, Sommer B, Satomura K, Dominguez P, Zhao C, Kulkarni AB, Robey PG, Young MF. Targeted disruption of the biglycan gene leads to an osteoporosis-like phenotype in mice. *Nature genetics*. 1998; 20:78–82. [PubMed: 9731537]
8. Chen XD, Shi S, Xu T, Robey PG, Young MF. Age-related osteoporosis in biglycan-deficient mice is related to defects in bone marrow stromal cells. *Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research*. 2002; 17:331–340.
9. Corsi A, Xu T, Chen XD, Boyde A, Liang J, Mankani M, Sommer B, Iozzo RV, Eichstetter I, Robey PG, Bianco P, Young MF. Phenotypic effects of biglycan deficiency are linked to collagen fibril abnormalities, are synergized by decorin deficiency, and mimic Ehlers-Danlos-like changes in bone and other connective tissues. *Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research*. 2002; 17:1180–1189.
10. Ameye L, Aria D, Jepsen K, Oldberg A, Xu T, Young MF. Abnormal collagen fibrils in tendons of biglycan/fibromodulin-deficient mice lead to gait impairment, ectopic ossification, and osteoarthritis. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology*. 2002; 16:673–680. [PubMed: 11978731]
11. Goldberg M, Septier D, Rapoport O, Iozzo RV, Young MF, Ameye LG. Targeted disruption of two small leucine-rich proteoglycans, biglycan and decorin, exerts divergent effects on enamel and dentin formation. *Calcified tissue international*. 2005; 77:297–310. [PubMed: 16283572]
12. Chen XD, Fisher LW, Robey PG, Young MF. The small leucine-rich proteoglycan biglycan modulates BMP-4-induced osteoblast differentiation. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology*. 2004; 18:948–958. [PubMed: 15173106]
13. Berendsen AD, Fisher LW, Kilts TM, Owens RT, Robey PG, Gutkind JS, Young MF. Modulation of canonical Wnt signaling by the extracellular matrix component biglycan. *Proceedings of the National Academy of Sciences of the United States of America*. 2011; 108:17022–17027. [PubMed: 21969569]

14. Walia A, Yang JF, Huang YH, Rosenblatt MI, Chang JH, Azar DT. Endostatin's emerging roles in angiogenesis, lymphangiogenesis, disease, and clinical applications. *Biochimica et biophysica acta*. 2015; 1850:2422–2438. [PubMed: 26367079]
15. Zeng X, Chen J, Miller YI, Javaherian K, Moulton KS. Endostatin binds biglycan and LDL and interferes with LDL retention to the subendothelial matrix during atherosclerosis. *Journal of lipid research*. 2005; 46:1849–1859. [PubMed: 15995169]
16. Zhang L, Ge W, Hu K, Zhang Y, Li C, Xu X, He D, Zhao Z, Zhang J, Jie F, Chen Y, Zheng Y. Endostar down-regulates HIF-1 and VEGF expression and enhances the radioresponse to human lung adenocarcinoma cancer cells. *Molecular biology reports*. 2012; 39:89–95. [PubMed: 21567206]
17. Dixelius J, Cross MJ, Matsumoto T, Claesson-Welsh L. Endostatin action and intracellular signaling: beta-catenin as a potential target? *Cancer letters*. 2003; 196:1–12. [PubMed: 12860282]
18. Parris GE. A Hypothesis Concerning the Biphasic Dose-response of Tumors to Angiostatin and Endostatin. Dose-response : a publication of International Hormesis Society. 2015; 13
19. Hu L, Duan YT, Li JF, Su LP, Yan M, Zhu ZG, Liu BY, Yang QM. Biglycan enhances gastric cancer invasion by activating FAK signaling pathway. *Oncotarget*. 2014; 5:1885–1896. [PubMed: 24681892]
20. Yamamoto K, Ohga N, Hida Y, Maishi N, Kawamoto T, Kitayama K, Akiyama K, Osawa T, Kondoh M, Matsuda K, Onodera Y, Fujie M, Kaga K, Hirano S, Shinohara N, Shindoh M, Hida K. Biglycan is a specific marker and an autocrine angiogenic factor of tumour endothelial cells. *British journal of cancer*. 2012; 106:1214–1223. [PubMed: 22374465]
21. Abdollahi A, Hahnfeldt P, Maercker C, Grone HJ, Debus J, Ansorge W, Folkman J, Hlatky L, Huber PE. Endostatin's antiangiogenic signaling network. *Molecular cell*. 2004; 13:649–663. [PubMed: 15023336]
22. Rehn M, Veikkola T, Kukk-Valdre E, Nakamura H, Ilmonen M, Lombardo C, Pihlajaniemi T, Alitalo K, Vuori K. Interaction of endostatin with integrins implicated in angiogenesis. *Proceedings of the National Academy of Sciences of the United States of America*. 2001; 98:1024–1029. [PubMed: 11158588]
23. Ferreras M, Felbor U, Lenhard T, Olsen BR, Delaisse J. Generation and degradation of human endostatin proteins by various proteinases. *FEBS letters*. 2000; 486:247–251. [PubMed: 11119712]
24. Sipola A, Seppinen L, Pihlajaniemi T, Tuukkanen J. Endostatin affects osteoblast behavior in vitro, but collagen XVIII/endostatin is not essential for skeletal development in vivo. *Calcified tissue international*. 2009; 85:412–420. [PubMed: 19763371]
25. Neill T, Schaefer L, Iozzo RV. Decorin as a multivalent therapeutic agent against cancer. *Advanced drug delivery reviews*. 2015
26. Neill T, Schaefer L, Iozzo RV. Decoding the Matrix: Instructive Roles of Proteoglycan Receptors. *Biochemistry*. 2015; 54:4583–4598. [PubMed: 26177309]
27. Iozzo RV, Schaefer L. Proteoglycans in health and disease: novel regulatory signaling mechanisms evoked by the small leucine-rich proteoglycans. *The FEBS journal*. 2010; 277:3864–3875. [PubMed: 20840584]
28. Behl T, Kotwani A. Possible role of endostatin in the antiangiogenic therapy of diabetic retinopathy. *Life sciences*. 2015; 135:131–137. [PubMed: 26141993]
29. Yuasa M, Mignemi NA, Barnett JV, Cates JM, Nyman JS, Okawa A, Yoshii T, Schwartz HS, Stutz CM, Schoenecker JG. The temporal and spatial development of vascularity in a healing displaced fracture. *Bone*. 2014; 67:208–221. [PubMed: 25016962]
30. Goyal A, Neill T, Owens RT, Schaefer L, Iozzo RV. Decorin activates AMPK, an energy sensor kinase, to induce autophagy in endothelial cells. *Matrix biology : journal of the International Society for Matrix Biology*. 2014; 34:46–54. [PubMed: 24472739]
31. Poluzzi C, Iozzo RV, Schaefer L. Endostatin and endorepellin: A common route of action for similar angiostatic cancer avengers. *Advanced drug delivery reviews*. 2015
32. Moreth K, Frey H, Hubo M, Zeng-Brouwers J, Nastase MV, Hsieh LT, Heceni R, Pfeilschifter J, Iozzo RV, Schaefer L. Biglycan-triggered TLR-2- and TLR-4-signaling exacerbates the

- pathophysiology of ischemic acute kidney injury. *Matrix biology : journal of the International Society for Matrix Biology*. 2014; 35:143–151. [PubMed: 24480070]
33. Claes L, Recknagel S, Ignatius A. Fracture healing under healthy and inflammatory conditions. *Nature reviews. Rheumatology*. 2012; 8:133–143. [PubMed: 22293759]
34. Schaefer L, Babelova A, Kiss E, Hausser HJ, Baliova M, Krzyzankova M, Marsche G, Young MF, Mihalik D, Gotte M, Malle E, Schaefer RM, Grone HJ. The matrix component biglycan is proinflammatory and signals through Toll-like receptors 4 and 2 in macrophages. *The Journal of clinical investigation*. 2005; 115:2223–2233. [PubMed: 16025156]
35. Muller R, Koller B, Hildebrand T, Laib A, Gianolini S, Ruegsegger P. Resolution dependency of microstructural properties of cancellous bone based on three-dimensional mu-tomography. *Technology and health care : official journal of the European Society for Engineering and Medicine*. 1996; 4:113–119. [PubMed: 8773313]
36. Hildebrand T, Laib A, Muller R, Dequeker J, Ruegsegger P. Direct three-dimensional morphometric analysis of human cancellous bone: microstructural data from spine, femur, iliac crest, and calcaneus. *Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research*. 1999; 14:1167–1174.
37. Faye C, Inforzato A, Bignon M, Hartmann DJ, Muller L, Ballut L, Olsen BR, Day AJ, Ricard-Blum S. Transglutaminase-2: a new endostatin partner in the extracellular matrix of endothelial cells. *The Biochemical journal*. 2010; 427:467–475. [PubMed: 20156196]

Highlights

- Biglycan deficient mice have reduced angiogenesis during fracture healing
- Biglycan co-localizes with endostatin at sites of new bone formation
- Endostatin mRNA and protein are up-regulated in the Bgn-deficient callus
- Bgn counteracts the anti-angiogenic effects of endostatin in vessel growth

Fig. 1.

Bgn-KO mice display reduced callus vascularity 7 days post-fracture A-L: Three-dimensional rendering by micro-CT with barium sulfate angiography of typical callus vasculature in *WT* (left) and *Bgn*-KO (right) mice. A,B: an anterior view of the entire fractured femur (gray) with vasculature (red) and C,D: a mid-coronal section of fractured femur with vasculature. E,G: an anterior view of the callus region and F,H: a mid-coronal view of the isolated callus showing the vasculature without bone. I,K: a transverse section of vasculature in the callus region with bone and J,L: the same region without bone. Size bars are 1 mm.

Fig. 2.

Bgn-KO mice display reduced vessel architecture and reduced *Pecam1* expression 7 days post-fracture. A–G: Micro-CT analysis with barium angiography performed on *WT* and *Bgn*-KO mouse femurs for A: total tissue volume, B: vessel volume, C: relative vessel volume (the ratio of the total vessel or volume/total volume or VV/TV), D: connectivity, E: vessel number, F: vessel thickness and G: vascular spacing (*WT*: n=5, *Bgn*-KO: n=5). H: Quantitative PCR of *Pecam1* mRNA expression relative to *S29* mRNA at 7 days post-fracture (*WT*: n=3, *Bgn*-KO: n=3). * $p < 0.05$ *WT* vs. *Bgn*-KO, ** $p < 0.01$ *WT* vs. *Bgn*-KO.

Fig. 3.

Bgn and endostatin expression were elevated in spatially interrelated regions of the callus 7 days post-fracture with increased endostatin expression in *Bgn*-KO mice 14 days post-fracture. A–F: Immunohistochemistry of A,B: Bgn and D,E: endostatin expression at 7 days post-fracture in *WT* mice with A,D: low and B,E: high power. C: and F: Negative controls for Bgn and endostatin antibody staining. Arrow heads point to regions of positive staining. G–J: Immunohistochemistry of endostatin expression at 14 days post-fracture in G,I: *WT* and H,J: *Bgn*-KO mice at low and high power. Arrow heads point to regions of positive

staining. Size bars are shown. K–L: Quantitative PCR of K: *Bgn* and L: *Col18a1* mRNA expression relative to *S29* mRNA 14 days post-fracture. *Bgn* (*WT*: n=3, *Bgn*-KO: n=3), *Col18a1* (*WT*: n=4, *Bgn*-KO: n=4). * $p<0.05$ *WT* vs. *Bgn*-KO, ** $p<0.01$ *WT* vs. *Bgn*-KO.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Fig. 4. HUVEC cultures stained with anti-CD31 antibody at 14 days of growth. Cells treated with endostatin had decreased vessel length and vessel branching, while cells treated with endostatin and Bgn in equimolar amounts had growth comparable to untreated cells. Untreated cells served as a positive control; suramin, an inhibitor of angiogenesis, treated cells served as a negative control. Representative photos of cultures treated with (A) VEGF, (B) untreated, (C) suramin, (D) endostatin, (E) one mole endostatin to 0.5 moles Bgn, (F) equimolar endostatin to Bgn, (G) one mole endostatin to 2 moles Bgn, and (H) Bgn alone.

Quantitation of vessel length (I) and branch points (J) normalized to plate area. n=3 for each experimental variable. * $p<0.05$, ** $p<0.01$.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Table 1

Primers used for real-time RT-PCR

Gene	GeneBank accession no.	Primer Sequence
<i>S29</i>	NM_009093	F 5'-GGAGTCACCCACGGAGTTCG-3' R 5'-GGAAGCAGCTGGCGGCACATG-3'
<i>Bgn</i>	BC052857	F 5'- AGACAAACCGACAGCCTGACAAC-3' R 5'-GCCAGCAGCAAGGTGAGTAGC-3'
<i>Pecam1</i>	LO6039	F 5'- TCCAACAGAGCCAGCAGTATGAGG-3' F 5'- TCCAATGACAACCAACGCAATGAG-3'
<i>Col18a1</i>	NM_009929	F 5'-ACCAGTTTGGTCTTGCTGCT-3' R 5'-TTCTCAGGTAGGGGGTCTCC-3'

F – Forward/Sense; R – Reverse/Antisense