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Biglycan knockout mice: New models for musculoskeletal diseases

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Biglycan is a Class I Small Leucine Rich Proteoglycans (SLRP) that is localized on human chromosome Xq28-ter. The conserved nature of its intron-exon structure and protein coding sequence compared to decorin (another Class I SLRP) indicates the two genes may have arisen from gene duplication. Biglycan contains two chondroitin sulfate glycosaminoglycan (GAG) chains attached near its NH_2 terminus making it different from decorin that has only one GAG chain. To determine the functions of biglycan *in vivo,* transgenic mice were developed that were deficient in the production of the protein (knockout). These mice acquire diminished bone mass progressively with age. Double tetracycline-calcein labeling revealed that the biglycan deficient mice are defective in their capacity to form bone. Based on this observation, we tested the hypothesis that the osteoporosis-like phenotype is due to defects in cells critical to the process of bone formation. Our data shows that biglycan deficient mice have diminished capacity to produce marrow stromal cells, the bone cell precursors, and that this deficiency increases with age. The cells also have reduced response to tranforming growth factor-β (TGF-β), reduced collagen synthesis and relatively more apoptosis than cells from normal littermates. In addition, calvaria cells isolated from biglycan deficient mice have reduced expression of late differentiation markers such as bone sialoprotein and osteocalcin and diminished ability to accumulate calcium judged by alizerin red staining. We propose that any one of these defects in osteogenic cells alone, or in combination, could contribute to the osteoporosis observed in the biglycan knockout mice. Other data suggests there is a functional relationship between biglycan and bone morphogenic protein-2/4 (BMP 2/4) action in controlling skeletal cell differentiation. In order to test the hypothesis that functional compensation can occur between SLRPs, we created mice deficient in biglycan and decorin. Decorin deficient mice have normal bone mass while the double biglycan/decorin knockout mice have more severe osteopenia than the single biglycan indicating redundancy in SLRP function in bone tissue. To further determine whether compensation could occur between different classes of SLRPs, mice were generated that are deficient in both biglycan (class I) and fibromodulin, a class II SLRP highly expressed in mineralizing tissue. These doubly deficient mice had an impaired gait, ectopic calcification of tendons and premature osteoarthritis. Transmission electron microscopy analysis showed that like the decorin and biglycan knockouts, they have severely disturbed collagen fibril structures. Biomechanical analysis of the affected tendons showed they were weaker compared to control animals leading to the conclusion that instability of the joints could be the primary cause of all the skeletal defects observed in the fibromodulin/biglycan knockout mice. These studies present important new animal models for musculoskeletal diseases and provide the opportunity to characterize the network of signals that control tissue integrity and function through SLRP activity.
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Introduction

Biglycan was discovered some 20 years ago as a small proteoglycan within the mineral compartment of bone [1]. Amino terminal sequencing showed it was distinct from a closely related proteoglycan now known as decorin. Using peptide antibodies towards the N-terminal biglycan sequence along with a cDNA library constructed from human bone cells, full length cDNA clones were isolated and used to determine the entire coding sequence [2]. The cDNA clones were also instrumental in determining tissue expression profiles, gene map location and intron-exon organization of the biglycan gene. All of this data provided a pivotal foundation for the creation of animal models where the production of the protein was abolished. The goals

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of this review are to (1) summarize the research findings that provided rationale for the creation of genetically engineered animals unable to make biglycan (single and double knockouts) and (2) describe the phenotype of these mice in order to detail the new discoveries that have revealed the *in vivo* biological function of biglycan. We would like to point out that there are several excellent reviews that contain a wealth of information about its function *in vitro* [3,4]. The present review will primarily focus on what we have learned about its function using deficient animal models.

Protein and gene structure

Biglycan is part of the expanding family of proteoglycans now known as small leucine rich proteoglycans (SLRPs) (Table 1). The family has been divided into three classes based on gene structure and protein characteristics [5]. Biglycan (a class I SLRP) contains 10 repeats of an amino acid motif rich in leucine [2]. The leucine rich repeat region makes up almost the entire core protein and is flanked by cysteine clusters whose sequence is characteristic for each class of SLRP [5]. The protein has two glycosaminoglycan (GAG) chains attached to serine 42 (site 1) and serine 47 (site 2) near the amino terminus. The GAG chains are chondroitin sulfate (CS) or dermatan sulfate (DS) depending on the tissue source. The initiation of GAG synthesis begins via transfer of xylose residue to site 1 which influences the subsequent GAG synthesis on site 2 [6]. Biglycan has a hydrophobic signal sequence (pre) used for secretion from the cell followed by a pro-peptide that can be cleaved by bone morphogenetic protein-1 (BMP-1) [7] in a tissue-specific fashion [8]. The function of the pro-peptide is not known at this time but it is speculated that it regulates the positioning of biglycan relative to the cell surface [9].

Table 1. Small leucine rich proteoglycan classification

	SLRP	*Gene and protein structure, and amino terminal cysteine cluster*
Class I	Asporin	8 exons
	Biglycan	10 LRR
	Decorin	CX_3CXCX_6C
Class II	Fibromodulin	3 exons
	Lumican	10 LRR
	Keratocan	CX_3CXCX_9C
	PRELP	
	Osteoadherin/osteomodulin	
Class III	Epiphican/PG-Lb/DSPG3	7 exons
	Mimican/osteoglycin	6 LRR
	Opticin/oculoglycan	CX_2CXCX_6C
Others	Chondroadherin	3 exons
	Nyctalopin	11 LRR
		CX_3CXCX_8C or CX_3CXCX_6C

Legend: Classes of SLRPs were developed based on gene structure and DNA and protein homology. Column 1 lists the different classes of SLRPs. Column 2 shows the members of the SLRP family belonging to a each class. Column 3 lists the number of exons in the gene, the number of leucine-rich repeats LRR in the protein in each class and the sequence of the amino terminal cysteine cluster (where C represents a cysteine and X any amino acid). (Reprinted with permission from Glycobiology)

The biglycan gene (*bgn*) is divided into 8 exons (the largest being located at the most 3 prime (3′) end). The exon structures have no obvious relationship to the structural domains in the protein; however, they are identical in length and position to the related SLRP, decorin. The complete conservation of intron-exon organization of these two Class I SLRPs indicates they may have arisen from a common ancestor during evolution. Using a combination of somatic cell hybrids [10], and *in situ* hybridization of chromosome spreads [11], biglycan has been localized to a distal region of the X chromosome within the gene dense region Xq28 [11,12].

Observations that biglycan is present in copious amounts in the growth plate of bone, coupled with the fact that the gene resides on the X (but not Y) chromosome, led researchers to postulate that its expression could be altered in patients with X chromosome anomalies such as Turner (X0 and Fragile X) or Klinefelter (supernumerary X and Y). These genetic syndromes are associated with short stature and osteoporosis, and extreme height respectively. When fibroblasts were isolated from patients with Turner Syndrome or with other supernumerary sex chromosome anomalies, *BGN* expression at the mRNA and protein level was significantly correlated with the number of X (and surprisingly Y) chromosomes in the cell. Specifically, *BGN* expression in Turner (X0) and Klinefelter cells (XXY or variants thereof) was ∼50% and 150% respectively compared to age-matched controls. This kind of expression pattern resembles genes that escape inactivation. Most genes on the X chromosome have one copy inactivated during development resulting in the same level of expression in males (XY) and females (XX). However, a small set of genes on the X chromosome escapes inactivation; in this case females (XX) can have twice as many transcripts of the gene compared to males (XY). The correlation of *BGN* to sex chromosome number therefore makes it resemble a gene that escape inactivation. However, *BGN* was found to be X-inactivated [13,14]. In order to explain these contradictory observations, a theory was proposed that there is another gene that regulates *BGN* expression located on both the X and Y chromosomes that escapes inactivation. In this model, this control gene would have less expression in Turner (X0) (50%) and greater expression in supernumerary X and Y abnormalities (>150%). Thus, its altered expression would, hypothetically, result in parallel changes in transcription (and translation) of the *BGN*.

In order to test this theory, DNA encoding the *BGN* promoter was isolated and characterized. Various lengths of the promoter were connected to a reporter gene and transfected into cells

isolated from Turner patients, or age matched controls. These studies indicated that a 44 bp region located between −262 to −218 (relative to the start of transcription) was important for differential activation of the *BGN* promoter in cells with supernumerary chromosomes [15]. A combination of DNAse footprinting and mobility shift experiments using nuclear protein isolated from bone cells showed that the transcription factor c-Krox could possibly control the *BGN* expression in skeletal cells. Other studies indicated that the transcription factors SP1 and SP3 may also be important [16]. One intriguing transcription factor called pseudoautosomal homeobox-containing osteogenic gene (PHOG) has been identified and proposed to be a candidate gene for involvement in the short stature of Turner Syndrome [17]. This proposal was based on the fact that it is expressed by osteogenic cells and deleted in 45,X individuals. Because the gene has features that match a putative control factor for biglycan in Turner, it is tempting to suggest either a direct or indirect role in controlling *BGN* promoter activity. On the other hand many environmental factors such as vitamins and hormones regulate *BGN* transcriptional activation in a tissue-specific fashion [4]. Thus, it is clear that much more needs to be done to unravel the mechanisms that control its production in diseases and during development and aging.

Tissue localization

Using a combination of *in situ* hybridization and immunohistochemistry, biglycan and decorin have been localized to many skeletal regions including articular and epiphyseal cartilage, vascular canals, subchondral regions and the periosteum [18]. In non-skeletal tissues, biglycan is localized to specialized cell types such as skeletal myofibers and differentiating keratinocytes. Decorin, in contrast, localizes with all major type I and II collagen-rich tissues and appears to be directly associated with the extracellular matrices [17]. The notable localization of biglycan in a cell surface and "pericellular" arrangement indicates it may have regulatory functions at the cell surface.

Single *bgn* knockout mouse

In order to test the function of biglycan *in vivo*, knockout mice ($bgn^{-/0}$) were made using gene targeting and homologous recombination. The mice are designated as $bgn^{-/0}$ because they are males with no second *bgn* allele on their Y chromosome. These mice were viable and had no profound skeletal patterning abnormalities at birth, as judged by alizarin red and alcian blue staining, two staining methods classically used to label calcium in bone and glycosaminoglycans in cartilage. With age, however, X-ray images showed that long bones had decreased length coupled with decreased mineral density and mass compared to age matched controls. Histological analysis showed that the mice had less trabecular volume and reduced cortical thickness [19]. The thinned cortices in the absence of biglycan may explain why the bones also had reduced biomechanical strength [18]. In this regard it is important to note that both Fourier transformed infrared and back scattered EM analysis showed that the bones from the $bgn^{-/0}$ mice have a reduced mineralization [19,20], although the exact biochemical reason why this occurs is unclear.

Double labeling of the $bgn^{-/0}$ mice using tetracycline and calcein showed that they had less bone formation compared to controls indicating biglycan is, at some level, important for the cells that control bone production [17]. To understand the cellular basis of this control, we examined the quantity and metabolic activity of biglycan-deficient bone marrow stromal stem cells (undifferentiated osteoprogenitor cells). Compared to control mice, the number of stem cells detected by colony forming unit fibroblastic (CFU-F) efficiency assay was significantly diminished by 24 weeks of age [20]. This may be due to the increased programmed cell death as suggested by TUNEL (TdT-mediated dUTP-X nick end labeling used for apoptosis) assay. The response of $bgn^{-/0}$ cells to TGF-β was also lower than in normal cells. In contrast to normal cells, it was found that TGF-β neither increased the size of biglycan-negative colonies nor their synthesis of type I collagen [21]. This data may, at first, appear contradictory to previous *in-vitro* data which showed that biglycan (and other SLRPs including decorin and fibromodulin) bind TGF-β and thereby inhibit its activity [22]. In this regard, we suggest that each tissue responds uniquely to TGF-β depending on the abundance of other influencing proteins accumulated in the tissue. Thus, in some tissues, biglycan could enhance TGF-β function (as in stroma cells) while in other cases (such as in fibrosis) it would inhibit or buffer TGF-β activities.

When more mature osteoblast cells were isolated from the calvaria of newborn mice, we found that $bgn^{-/0}$ cells expressed lower levels of the bone-related genes for osteopontin, bone sialoprotein, and osteocalcin, and furthermore, accumulated less calcium in nodules during the differentiation than control cells. To understand further the molecular basis for the apparent defects in differentiation, we examined the effects of BMP-4 on the production of Cbfa1 by $bgn^{-/0}$ osteoblasts. Our data showed that the $bgn^{-/0}$ cells produced a significantly lower level of Cbfa1, possibly due to decreased BMP-4-induced signaling events [23]. Taken together, we propose that biglycan participates in bone formation at multiple levels by influencing both the number of available osteogenic precursors, as well as their subsequent development, maturation and function. During this process, biglycan may modulate the activity of BMP-4 or other growth factors to ultimately control osteoblast function. The outcome is decreased bone formation and mass with compromised strength and structure.

Other tissues besides bone are also affected in the $bgn^{-/0}$ mice. Light microscope evaluation of tooth tissues showed that the molars from one-day-old mice had multiple defects compared to control animals. Specifically, the teeth had broader, more porous dentin, thicker enamel and a mantle dentin mineralization near the dentino-enamel junction that was diminished

and more heterogeneous than controls. Transmission EM analysis showed that the collagen fibrils were altered, a possible explanation for the observed mineralization defects [24]. Muscles from the $bgn^{-/0}$ were also affected, a defect presumed to be related to the absence of biglycan for binding to one of the members of the muscle plasma membrane, alpha dystroglycin [25]. Considering that biglycan is expressed in numerous tissues including the heart, kidney, cornea, brain and lung, it is likely that $bgn^{-/-}$ mice may also have defects in some of these other tissues as well.

Double knockout mice reveal compensation and synergistic functions among SLRPs

Biglycan and decorin are highly homologous and co-expressed in various tissues such as skin and bone. When proteins were extracted from the $bgn^{-/0}$ tissues, decorin was shown to be in higher quantity than in wild type animals (unpublished observations). This data suggests that the two related class I SLRPs could share common functions and, possibly, compensate for each other's functions when one of them is missing. To test this hypothesis, a $dcn^{-/-}$ mouse previously generated in the laboratory of R. Iozzo [26] was mated to the $bgn^{-/0}$ mouse and double knockouts were created. While lower than expected numbers of $bgn^{-/0}\,dcn^{-/-}$ were born, they were surprisingly viable and free of patterning defects though notably smaller than their littermates. Close inspection of the double knockout $bgn^{-/0}dcn^{-/-}$ mice showed profound defects in the tissues where decorin and biglycan are co-expressed including skin and bone.

Skin tissue in $bgn^{-/0}dcn^{-/-}$ mice was thinner and much more fragile than either single $bgn^{-/0}$ or *dcn* animals (see accompanying article by R. Iozzo for details on the $dcn^{-/-}$). Electron microscope examination showed that the structure of collagen fibrils was profoundly disturbed in all knockouts in a tissue-specific fashion. That is to say, the fibrils were irregular in shape and had mean diameters that were smaller or bigger than controls depending on the tissue source (Table 2 and for review see [5, 27]).

$bgn^{-/0}dcn^{-/-}$ mice exhibited osteopenia (low bone mass) earlier and more severely than the $bgn^{-/0}$ while the *dcn* appeared unaffected [20]. These data suggest that the highly homologous biglycan can compensate for the absence of decorin and possibly rescue the phenotype of the *dcn* (making bone normal even in the absence of decorin). In order to investigate the overlapping functions and cellular nature of the combined $bgn^{-/0-}$/*dcn* defect, we carried out a series of experiments using cells isolated from this new, unique SLRP double knockout model. *In vitro* studies showed that while the proliferation rate of bone marrow stroma cells was higher, the number of cells estimated by the colony forming efficiency (CFU-F) assay was decreased compared to wild type, possibly due to increased apoptosis. This decrease was greater and occurred earlier compared to the $bgn^{-/0}$ animals [21]. In addition, an *in vivo* transplantation bone marrow stem cell assay showed that bone formation is lower from $bgn^{-/0}$ and $dcn^{-/-}$ cells than wildtype cells. However there was no decrease in the expression of the osteoblast markers, such as, cbfa-1, osteopontin or BSP indicating that there is no defect in the normal osteoblast differentiation pathway. The decreased bone formation in the $bgn^{-/0}$ and $dcn^{-/-}$ mice could be due to increased apoptosis of osteogenic precursors, which leads to insufficient numbers of mature osteoblasts [28]. In our working model, we theorize that the high levels of biglycan and decorin normally made by bone marrow stromal cells inhibit uncontrolled proliferation, apoptosis and, subsequently, maintain the proper amount of mature osteoblasts. Current experiments are designed to test this hypothesis and, if it is confirmed, to determine its molecular basis both in and outside the cell.

The combined bone and skin defects in the $bgn^{-/0}\,dcn^{-/-}$ mice mimic a rare genetic disorder (OIM#130070) that has defective galactosyltransferase I activity [29]. The outcome of this deficiency is the production of small proteoglycans devoid of GAG chains. The similarity of this human disease to the double knockout that is unable to make both decorin and biglycan implies that the GAG chains may have important functions in bone and skin tissue [29].

Biglycan (a class I SLRP) and fibromodulin (a class II SLRP) are co-expressed in cartilage and bone. To determine whether SLRPs of different classes, with greater differences in structure than SLRPs from the same class, could also compensate for each others function, we created mice deficient in both *bgn* and *fm*. The mice were viable with no apparent defects in patterning or development. At one month of age the $bgn^{-/0}\,fm^{-/-}$ mice developed an impaired gait characterized initially by joint laxity, followed by a decrease in the flexibility of the knee and ankle joints. X-ray inspection showed that $bgn^{-/0}$ and $fm^{-/-}$ single and double knockouts had ectopic ossification within many tendons with the ectopic ossification being more abundant in the double knockout. The fact that the defects in absence of both biglycan and fibromodulin were more severe than the added defects of each single knockout defects added together implies synergistic functions for the two SLRPs on tendon ossification. At this point, the exact nature of the synergy is not clear and could be either by direct interaction of

Table 2. Structural defects in $bgn^{-/-}/dcn^{-/-}$ mice

	Skin	*Bone*
$bgn^{+/0}$	Thin dermis Larger irregular fibrils	Lower mass Larger irregular fibrils
$dcn^{-/-}$	Thin dermis Larger irregular fibrils	Normal mass Smaller fibrils
$bgn^{+/0}\,dcn^{-/-}$	Loose disorganized dermis Larger highly irregular fibrils	Severe osteopenia Larger, more irregular fibrils than $bgn^{+/0}$

SLRPs with each other, or with associating factors. Based on structural homology one could imagine that when one SLRP becomes diminished, a different (but similar) SLRP could substitute (for binding) and lead to a sequence of abnormalities in the distribution and abundance of other matrix proteins. Further experimentation will be required to fully understand this "domino" effect and its functional consequences.

Both $bgn^{-/0}$ and $fm^{-/-}$ mice also developed premature osteoarthritis with an earlier and more severe occurrence in the double knockout mice. Based on the nature of the phenotype (i.e. impaired gait, ectopic ossification, and pre-mature osteoarthritis), we theorized that the underlying cause was due to weak, biomechanically compromised tendons. Our mechanistic model is supported by the observations that the affected tendons have reduced stiffness (a measure of their biomechanical strength) [30]. The molecular basis of the biomechanical tendon defect may be caused from a disturbance in collagen fibril organization which ultimately, lead to the formation of ectopic bone. Transmission EM analysis of the tendons showed that the mean diameter and range of the collagen fibrils in deficient tendons was smaller that in controls [29]. All of these defects were further magnified by subjecting the mice to a forced running regime of 30 minutes per day for 30 days. A further characterization of the molecular changes that accompany cartilage breakdown in the $bgn^{-/0}\,fm^{-/-}$ transient mice showed they mimic human osteoarthritis as well, having increased levels of cartilage oligomeric matrix protein, accompanying premature loss of decorin and Type II collagen in the articular cartilage [31].

In summary, SLRP knockouts have provided important new information about their roles in cell and tissue function. The characterization of these models is far from complete and will require input from many disciplines including the areas of signaling, gene activation, proteomics and bioinformatics, using cells and tissues from the SLRP knockout mice. Other avenues yet to be explored are the relationships of the SLRPs to other non-SLRP proteins using multiple knockout or gain-of-function strategies. In order to reveal cooperative functions during aging, it is likely that tissue-specific and temporal-specific SLRP knockouts will need to be generated. In order to test the role of glycosylation in SLRP function we will need to create "knock-in" mice lacking one or more of the amino acids that are essential for GAG attachment. Greater knowledge about both common and rare human diseases models (i.e. osteoporosis, osteoarthritis, Ehlers-Danlos) is already beginning to emerge with the available models. Additional spontaneous (or induced) abnormalities will likely be discovered as the mice are characterized in greater detail.

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