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Biglycan-Deficient Mice Have Delayed Osteogenesis after Marrow Ablation

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Abstract. Biglycan (*bgn*) is a small proteoglycan in skeletal tissue that binds and regulates collagen and TGF-beta activities. Mice deficient in *bgn* (*bgn*-KO) develop age-dependent osteopenia and have multiple metabolic defects in their bone marrow stromal cells including increased apoptosis, reduced numbers of colony-forming units-fibroblastic (CFU-F) and decreased collagen production. In the present study we tested the hypothesis that bone formation capability in response to a physiological stress is compromised in *bgn* deficiency. We tested this theory using an *in vivo* bone marrow ablation assay. Ablation was performed on 6-week-old wild type (*wt*) and *bgn*-KO mice and bones were analyzed at days 7, 10, and 17 postsurgery. X-ray analysis showed that bone marrow ablation in femora induced vigorous new bone formation within 10 days in both genotypes but appeared greater in the *wt* compared to the *bgn*-KO. In order to quantitate the changes in bone formation in the ablated animals, bone densities of the proximal, midshaft, and distal femora were assessed using peripheral quantitative computed tomography (pQCT). The ratio of cancellous bone density at the midshaft (ablated limb/control limb) was significantly higher in *wt* compared to *bgn*-KO at day 10 postsurgery. *Wt* and *bgn*-KO femora had similar total and cancellous bone densities at days 7 and 17 postsurgery at all three locations indicating that the ablation effects were temporal and limited to the cancellous bone of the mid-shaft region. These data indicate that the absence of *bgn* directly impeded bone formation. Our results support the concept that *bgn* is important in controlling osteogenesis following marrow ablation.

Key words: Biglycan — Marrow ablation — Bone formation — Bone repair

Biglycan (*bgn*) is a small proteoglycan that is enriched in bone and other skeletal connective tissues [1]. It is highly posttranslationally modified, containing two chains of glycosaminoglycans that are either chondroitin or dermatan sulfate, depending on the tissue source (for review see: [2, 3]). The core protein is made almost exclusively of a tandem repeat of a 24 amino acid long motif that is rich in leucine [4]. These leucine-rich repeats (LRR) are flanked on either end by cysteine clusters [4]. *Bgn* is part of a growing family called SLRPs (small leucine-rich proteoglycan) that is presently composed of 13 members [3]. The family is divided into three classes (I, II, and III) based on (1) the amino acid sequence and number of leucine repeats, (2) the sequence and position of the N-terminal cysteine cluster, and (3) the intron-exon character of the gene structure [3]. Biglycan is a class I member and, like the other two members of this class, decorin and asporin, it contains 8 exons and 10 LRR.

The human biglycan gene was first mapped broadly to the X chromosome [5] and then more specifically to Xq27-ter [6] and is highly expressed in bone and cartilage [1]. These two observations led researchers to speculate *BGN* expression could be altered in humans with X chromosome anomalies such as Turners (XO) or Kleinfelters (supernumerary X and Y) syndromes noted for abnormal bone length and density. Indeed, cells derived from patients suffering from such X-chromosome anomalies have *BGN* mRNA and protein levels that have a significantly positive correlation with the number of X (or Y) chromosomes present [7]. This and other data linking *bgn* to TGF-beta [8] and collagen binding [9] together pointed to the concept that *bgn* could have some role in regulating bone formation and integrity.

We have previously demonstrated that *bgn* is important for bone tissue function *in vivo* by generating *bgn*-deficient mice (*bgn*-KO) and showing that they developed age-dependent osteopenia [10]. The *bgn*-deficient mice acquire age-dependent defects in the ratio of their mineral/matrix FTIR spectrum beginning as early as 3 weeks of age [11]. Further *in vitro* analysis showed that bone marrow stromal cells isolated from *bgn*-KO mice had multiple defects including increased apoptosis, reduced numbers of colony-forming units (CFU-F), and decreased collagen mRNA and protein [12]. These abnormalities, however, only appeared after 3 months of

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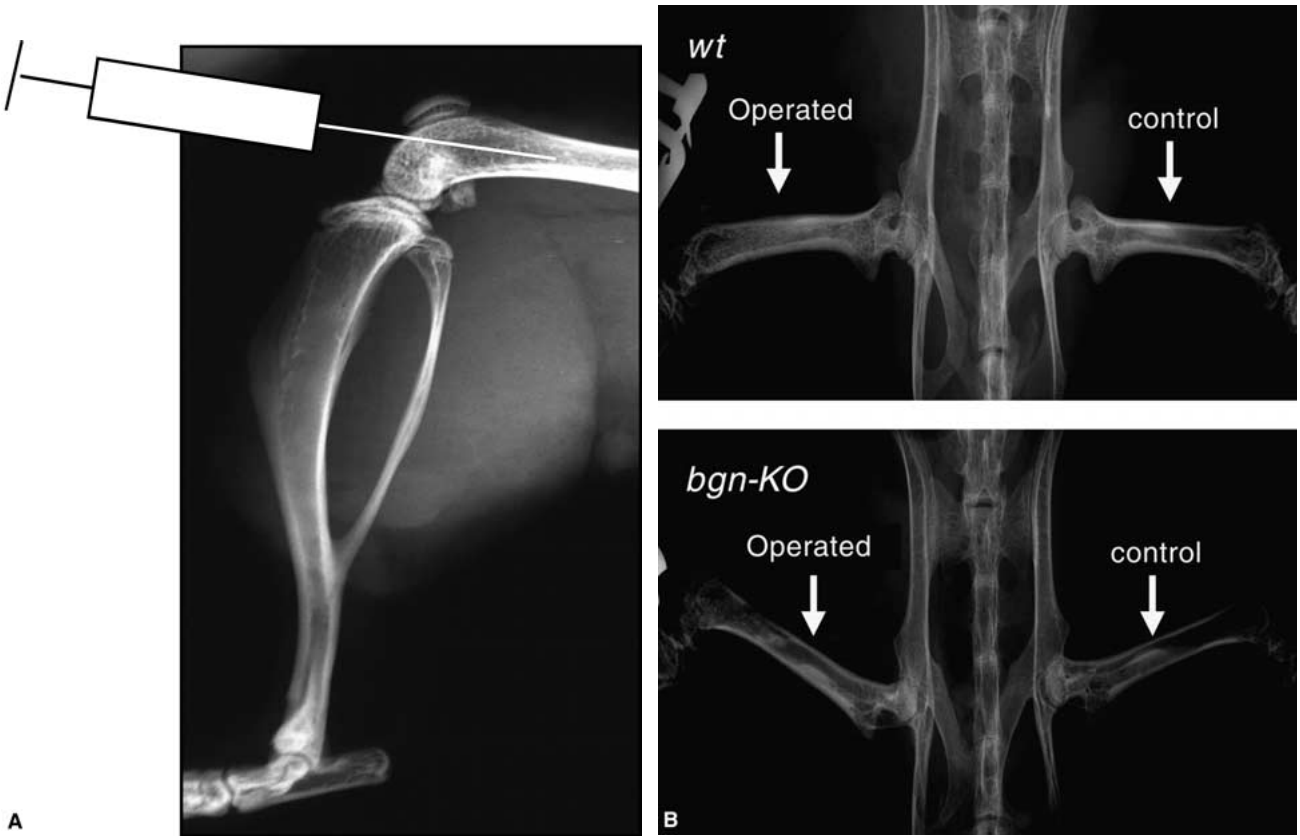

Fig. 1. Diagram illustrating the orientation of the surgical procedure for the ablation (**A**) Faxitron X-rays of control and ablated (operated) femurs from *wt* and *bgn* KO mice (**B**). Increased trabecular mass in the mid-shaft region is evident in the ablated (operated) femur (shown on the left) compared to the control(shown on the right).

age and were most prominent at 6 months [12]. In order to determine whether biglycan has *in vivo* functions at early ages, we wished to test bone formation capability in response to a unique physiological stress in 6-week-old *bgn*-KO mice. Bone marrow ablation was used since it is a highly reproducible way to evaluate *in vivo* bone formation and resorption in a relatively short time span [13–15]. Our data point to the conclusion that *bgn* is a positive regulator of bone formation *in vivo* and can modulate bone tissue structure and integrity at an early age.

Materials and Methods

Normal (wt) and Biglycan (bgn) KO Mice

Normal (*wt*) and biglycan-deficient mice (*bgn* KO) were generated at the NIDCR and genotyped as previously described [12]. The mice used in this experiment were generated by backcrossing the original *bgn*-KO strain (126 B6/C57 mixture) to the C3H strain to a purity greater than 95%. All procedures were carried out following guidelines recommended by the NIDCR Institutional Animal Care and Use Committee with approval under protocol # 01-173. Briefly, tail biopsies were obtained from animals at weaning (21 days) and DNA was extracted using a commercial purification kit (Highpure™ from In-Vitrogen). Purified DNA (10 µl) was used for a PCR-based genotype assay [12]. After analysis of each genotyping reaction *wt* (+/0) and *bgn*-KO (−/0), male mice were identified and housed separately until the time of surgery.

Surgical Procedures

Animals were anesthetized with a combination of ketamine (150 mg/kg), xylazine (9 mg/kg) and acepromazine (5 mg/kg). Prior to surgery, the hair was gently shaved over the knee joint. A 1.0 cm incision was made over the knee joint and the patella tendon was exposed as a landmark. The intracondylar notch above the tendon was opened by inserting a 26-gauge needle (see Fig. 1a) followed by insertion of a dental drill (typically used for root canal procedures, number 10-30 dental files). The contents of the bone marrow cavity were back-flushed by injecting 100 µl of PBS into the femur using a syringe attached to a 26-gauge needle. The skin incision was closed with surgical metallic clips. The right femora of all mice (n = 3) underwent ablation and the left femora was untreated serving as an internal control and indicated as "control".

Radiography

Animals were sacrificed on sequential days as indicated in Figure 3 by CO_2 inhalation and subject to radiography using Kodak X-OMAT TL film and an Faxitron X-ray (model MS-20) at a setting of 30 kv and a 90-second exposure. Femora from the mice were then surgically removed and soft tissue was dissected free from the hard tissue. Bones were stored in 70% ethanol at 4°C until pQCT analysis was performed.

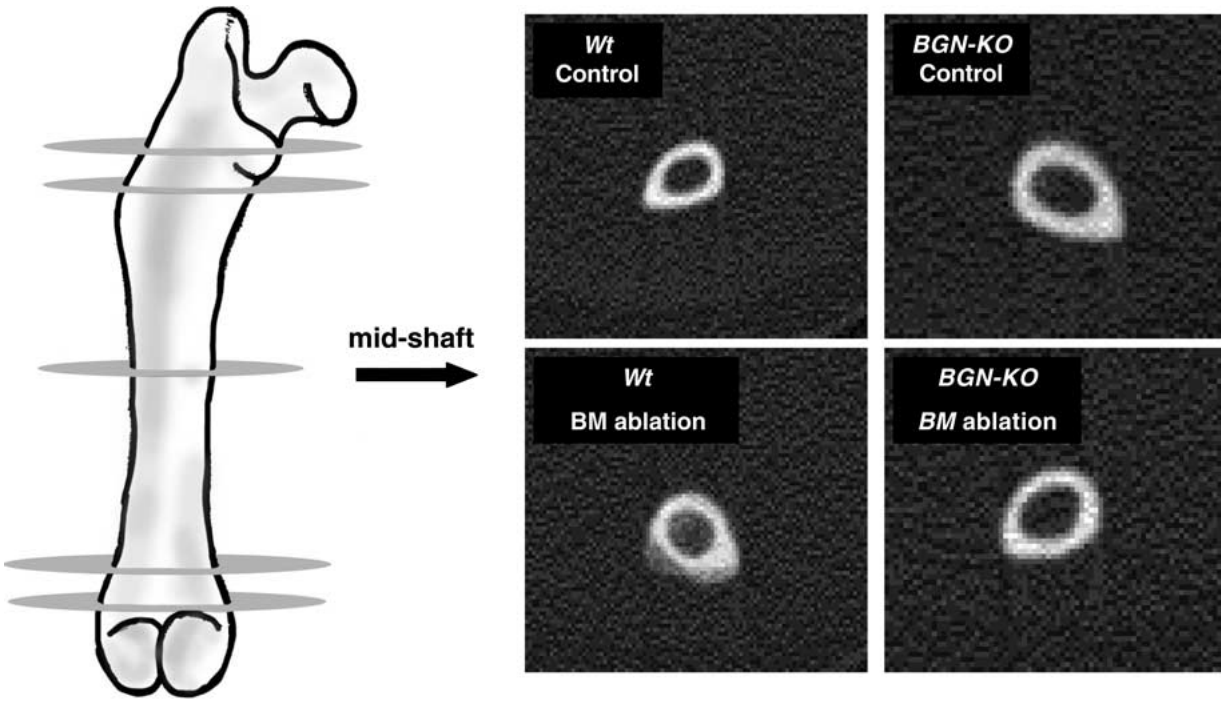

Fig. 2. Diagram showing the sections of the femur used for pQCT analysis. The arrow indicates the region of the mid-shaft shown in the representative pQCT images shown on the right side of the figure which are control and ablated (BM = bone marrow) femurs from *wt* and *bgn* KO mice at 10 days postsurgery. *Wt* control: cancellous/BMD = 167 mg/cm^3, *wt:* BM ablation = 376 mg/cm^3, *bgn* control: cancellous/BMD = 181 mg/cm^3, *bgn* = KO BM ablation: cancellous/BMD = 213 mg/cm^3.

Peripheral Quantitative Computed Tomography (pQCT)

Bone mineral density (BMD) of the femora was assessed using the Stratec XCT Research-M pQCT (Norland Corporation, Fort Atkinson, WI). Machine calibration was performed using a hydroxyapatite standard cone phantom to ensure measurement precision across multiple scanning days. Excised bones were placed in a 70% ethanol-filled vial and a two-dimensional view was obtained of the entire bone for landmark detection. Multiple transverse slices (0.50 mm thickness) were scanned at the proximal metaphysis (2 slices located at 2.25 and 2.75 mm distal to the proximal plateau), mid-diaphysis (1 slice located at 50% total bone length) and distal metaphysis (2 slices located at 2.25 and 2.75 mm proximal to the distal plateau) (see Fig. 1b). For all scans, a CT speed of 2.5 mm/sec and a scanning resolution of $0.07 \times 0.07 \times 0.50$ mm were used. Using standard Stratec software (Version 5.40B), values of total bone mineral content (BMC), total cortical and cancellous BMD, and total bone and marrow areas were obtained for all slices. The two slices obtained at both the proximal and distal metaphyses were averaged to get a mean value for each region. Reproducibility of these measurements was determined from 5 repeat scans of 3 bones. Resulting coefficients of variation (CV) for metaphyseal regions were as follows: total BMD ± 0.70%, cancellous (trabecular) BMD ± 2.26%, total area ± 1.83%, and marrow area ± 0.61%. CVs for mid-diaphyseal cortical BMD, total area, and marrow area were 0.34%, 1 .07%, and 0.97%, respectively.

Statistical Analysis

The results were expressed as the mean ± standard deviation (M ± SD) which were calculated from 3 independent ablations. Statistical analysis between experimental groups was performed by one-way ANOVA with InStat 2.01. Differences of $P < 0.05$ were considered significant.

Results

Radiographic analysis from wildtype (*wt*) mice showed that bone marrow ablation induced vigorous new bone formation in the femoral marrow cavity within 10 days. Visual inspection of X-rays from *bgn*-KO mice indicated a less vigorous bone formation in response to the ablation procedure (Fig. 1b) compared to *wt*-animals. Non-ablated contralateral (control) femora appeared to be unaffected in both *wt* and *bgn*-KO mice (Fig. 1b). In order to quantitate the changes noted by X-ray in the experimental and control bones of the *wt* and *bgn*-deficient mice, total cancellous BMD was assessed by pQCT.

The results of pQCT demonstrated that the *wt* and *bgn*-KO mice had similar total bone densities (cortical and cancellous) at day 7 and day 17 postsurgery at all sites scanned (see Fig. 2 for orientation of the sites scanned and Table 1 for BMD). However, at day 10 post-surgery the ablated limb/control limb ratio of cancellous BMD at the midshaft (Fig. 2 and Table 2) was significantly higher in *wt* (2.1 ± 0.32) compared to *bgn*-KO (1.3 ± 0.21) (Fig. 3). No genotype differences in the ratio of ablation/control in total or cancellous

Table 1. Total bone density measured from mid-shaft of femur

	Day 7		Day 10		Day 17	
Experiments	WT	KO	WT	KO	WT	KO
Ablation	793 ± 135	782+138	927 ± 35	838 ± 71	984 ± 118	969 ± 181
Sham	855 ± 174	798 ± 129	850 + 41	826 ± 78	851 ± 154	860 ± 161
Ratio (Ablation/Sham)	0.93 ± 0.26	0.98 ± 0.24	1.09 ± 0.10	1.01 ± 0.13	1.16 ± 0.21	1.13 ± 0.26

No significant differences were observed at any time point for any genotype

Table 2. Cancellous bone density measured from middle shaft of femur

	Day 7		Day 10		Day 17	
Experiments	WT	KO	WT	KO	WT	KO
Ablation	151 ± 31	165 ± 8	346 ± 35	251 ± 40	383 ± 71	391 ± 178
Sham	181 ± 34	176 ± 23	169 ± 8	182 ± 3	158 ± 12	177 ± 23
Ratio (Ablation/Sham)	0.83 ± 0.27	0.94 ± 0.14	2.05 ± 0.11*	1.30 ± 0.21*	2.42 ± 0.59	2.21 ± 0.47

*$P < 0.01$ comparing wild type (WT) with biglycan-deficient mice (KO), n = 3

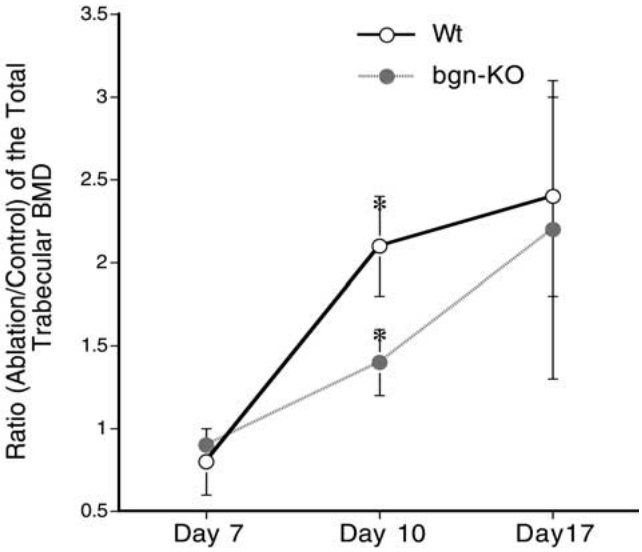

Fig. 3. Comparison of trabecular BMD induced by marrow ablation in *wt* and *bgn* KO mice. In each animal, the right femur was used for the marrow ablation while the left was untreated and served as an internal control. The data were presented as a ratio of cancellous BMD in ablated femur vs. that in the control femur. * $P < 0.01$ between *wt* and *bgn* KO samples, n = 3. Open circle with solid line = *wt*, dotted line with solid circle = *bgn* KO.

BMD were observed in regions distal to the ablation (data not shown).

Discussion

Evidence is mounting that biglycan has important roles in mineralized tissue structure and function. Immunohistochemistry using mono-specific antisera against biglycan demonstrates it is expressed in numerous skeletal sites including bone, cartilage and dentin [1, 16, 17]. Cells from patients with X-chromosomal anomalies have altered expression of *BGN* and corresponding abnormalities in bone tissue length and mass [7]. Specifically, *BGN* expression is increased in cells with abnormally high numbers of sex chromosomes (e.g., Klinefelter's syndrome, 47, XXY, 48, XXYY, 47, XXX, 49, XXXXY) and decreased in cells from patients with Turners syndrome (45, X) [7]. Klinefelter's and Turners syndromes are characterized by tall and short stature, respectively, suggesting there could be a link between *BGN* expression and bone growth. Proof of this theory comes from *bgn*-deficient mice which acquire early-onset osteoporosis due to a lower "peak bone mass" and have shorter long bones compared to normal littermates [10]. Structural analyses of the bones of affected animals demonstrate a reduced osteoblast number, suggesting a decrease in bone formation [10]. Indeed, double tetracycline-calcein labeling showed a decreased mineral apposition rate (MAR) and bone formation rate in *bgn*-KO mice compared to *wt*-animals [10] confirming that *bgn* may be a positive regulator bone formation *in vivo*.

In addition to a decrease in the rate of bone formation during normal aging, the quality of the bone appears to be compromised in the absence of *bgn*. FTIR analysis of the tibia from 3- and 6-month-old normal and *bgn*-KO mice reveals diminished phosphate (mineral)/amide (protein) profiles in both the cortex and trabeculae of the *bgn*-KO mice [10]. Back scattered EM analysis also demonstrates that there is a relative decrease in mineralization in the *bgn*-KO long bones [18].

Transmission electron microscopy analysis of osteoid illustrates that the diameters of collagen fibrils are increased and irregularly shaped in the absence of *bgn* compared to normal mice, an alteration that could either be the cause or the effect of defective mineralization [18]. In this regard, it is tempting to speculate that changes in collagen structure and mineralization could be the mechanistic basis for the observation that bones from the *bgn*-deficient mice have decreased biomechanical strength [10]. Specifically, *bgn*-deficient bones show a decrease in load to failure and a decrease in several pre-yield parameters as represented by energy to yield that is in the elastic region of the stress/strain curve [10]. Changes in collagen fibril integrity in the absence of *bgn* are not limited to long bones. Dentin isolated from newborn *bgn*-KO mice has smaller fibrils in the predentin and larger fibrils in the central and distal regions compared to age-matched *wt* controls [19, 20]. The dentinal layer is also expanded and more porous with the mantel dentin near the dentino-enamel junction being more heterogeneous and less mineralized in the *bgn* KO mice compared to *wt* mice. These data all imply a positive function for *bgn* in regulating bone and tooth formation, mineralization and maturation.

Bone marrow ablation is a technique used to elicit massive amounts of new bone formation followed by bone resorption in a rapid and predictable time frame. This is accomplished by physically removing the bone marrow contents by aspiration, or chemically by the injection of drugs such as colchicine. The latter method has been the source of considerable controversy [21–23] due to its variable success. The reasons for this variation are not clear but may be related to the time of application, or to animal species or other genetic differences in the experimental system. After ablation, specific mRNAs are expressed in a typical sequence in the osteogenic phase. These include the matrix proteins bone sialoprotein [24], osteopontin and osteocalcin [25], enzymes such as cyclooxygenase [26], and the growth factors IGF-I [27] and BMP [28], pointing to the fact they may be important for the formation process. Altered production and composition of matrix vesicles is also observed in tibial bones after marrow ablation [13]. Despite these extensive descriptive analyses, the nature of the factors that regulate this formation phase and their exact mechanism of action is not understood.

The focus of our study was to determine whether *bgn* could be one of the factors that play a role in this unique bone activation process. Based on the controversial nature of colchicine-induced ablation and to avoid confounding systemic effects on bone tissue formation, we chose to induce osteogenesis *in vivo* using the physical aspiration method. This method resulted in the induction of bone accrual within 1 week that appeared to reach a plateau at 10 days postsurgery. Our data show that *bgn*-deficient mice have delayed or temporarily attenuated cancellous osteoid and bone deposition compared to normal mice indicating that biglycan could indeed be one of the factors that regulates formation in this model of bone induction *in vivo*.

The use of pQCT to study the response to marrow ablation has numerous advantages. In addition to having excellent spatial resolution (70 μm) and the ability to scan specifically focused locations (0.50 mm slice thickness), pQCT allows the separation of the cortical shell from the marrow/cancellous compartment. This latter point is vital to the current study, as traditional densitometry methods (e.g. DXA), which can only measure whole bone properties, may not detect subtle changes in marrow cavity density due to the overriding contribution of cortical bone to overall density. Despite its powerful ability to differentiate bone compartments, however, there is a "partial volume" effect, where voxels on the cortical/marrow compartment interface are partially filled with cortical bone and marrow/cancellous bone. Depending on which compartment these voxels are assigned to, the density of that compartment may be artificially increased or decreased. To minimize this effect in the current study, the same compartment separation threshold was used on all sections, thus any error is assumed to be equal in all groups.

There are a number of possibilities that could explain *bgn*'s role in osteogenesis following marrow ablation. Diminished numbers of stem cells (CFU-F) that make less collagen mRNA and protein are found in the *bgn*-KO mice compared to wild-type mice [12]. The *bgn-deficient* stem cells are also less responsive to TGF-beta and form smaller colonies with fewer cells compared to controls [12]. The TGF-beta induction of collagen mRNA is also diminished in the *bgn*-deficient stromal cells [12]. Based on observations that *bgn* is localized in a pericellular fashion around skeletal cells [1] and that it can bind to TGF-beta [8], we speculate that the diminished response could be due to improper positioning of the growth factor at the cell surface. This could in turn lead to poor activation of the TGF-receptor and its downstream targets. Experiments are underway to test this hypothesis and extend it to include other important TGF-beta family members such as the BMPs. Because the decreased number of CFU-F and increased apoptosis in stromal cells from the biglycan KO mice is not evident until 24 weeks of age, it is likely that the primary basis for the defect in osteogenesis in the young animal is not related to altered stem cell number or apoptosis.

A more likely explanation for the delayed osteogenesis in the *bgn*-KO bone is that the matrix composition is altered in the absence of *bgn*. For example, if collagen protein levels are depleted in the *bgn*-KO mouse, this could lead to changes in other collagen-binding proteins and ultimately affect the function of collagen as a substrate for mineral deposition. Collagen changes resulting from *bgn* depletion could also have direct effects on

bone cells. Suzawa et al. [29] have recently shown that collagen-dependent osteoblast differentiation is a complex process involving the stimulation of Smad1 transcription using Ras-ERK mediated pathways. Along these lines, one could imagine that depletion of a single matrix protein such as *bgn* could lead to multiple and possibly amplified defective pathways, resulting in a sequence of events ultimately leading to compromised osteogenic capacity. The challenge for the future will be to identify the many steps that may be altered in *bgn* deficiency to understand the nature of other related factors and how they interact to control the bone mineralization process.

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