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Biological

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ABSTRACT

Temporomandibular joint osteoarthritis (TMJ OA) is a degenerative disease that affects both cartilage and subchondral bone. We used microarray to identify changes in gene expression levels in the TMJ during early stages of the disease, using an established TMJ OA genetic mouse model deficient in 2 extracellular matrix proteins, biglycan and fibromodulin (*bgn⁻⁰fmod^{-/-}*). Differential gene expression analysis was performed with RNA extracted from 3-week-old *WT* and *bgn⁻⁰fmod^{-/-}* TMJs with an intact cartilage/subchondral bone interface. In total, 22 genes were differentially expressed in *bgn⁻⁰fmod^{-/-}* TMJs, including 5 genes involved in osteoclast activity/differentiation. The number of TRAP-positive cells were three-fold higher in *bgn⁻⁰fmod^{-/-}* TMJs than in *WT*. Quantitative RT-PCR showed up-regulation of *RANKL* and *OPG*, with a 128% increase in *RANKL/OPG* ratio in *bgn⁻⁰fmod^{-/-}* TMJs. Histology and immunohistochemistry revealed tissue disorganization and reduced type I collagen in *bgn⁻⁰fmod^{-/-}* TMJ subchondral bone. Early changes in gene expression and tissue defects in young *bgn⁻⁰fmod^{-/-}* TMJ subchondral bone are likely attributed to increased osteoclast activity. Analysis of these data shows that biglycan and fibromodulin are critical for TMJ subchondral bone integrity and reveal a potential role for TMJ subchondral bone turnover during the initial early stages of TMJ OA disease in this model.

KEY WORDS: temporomandibular disorders, osteoclasts, matrix biology, bone biology osteoarthritis, cartilage.

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Role of Subchondral Bone during Early-stage Experimental TMJ Osteoarthritis

INTRODUCTION

The temporomandibular joint consists of a fibrocartilaginous condyle, disc, synovial membrane, and subchondral bone. Temporomandibular joint osteoarthritis (TMJ OA) is a degenerative disease involving all TMJ tissues and leads to anatomical changes and severe pain (Scrivani *et al.*, 2008). While the contribution of the cartilage to the disease pathology is well-studied, little is known about the role of subchondral bone. Emerging studies have implicated a potential role of subchondral bone during TMJ OA pathology, where sclerosis and increased bone metabolism are involved during cartilage degeneration (Jiao *et al.*, 2011; Lories and Luyten, 2011). Furthermore, the development of new OA therapeutics that target both cartilage and bone highlights the necessity for a better understanding of the role of subchondral bone turnover during OA pathology (Karsdal *et al.*, 2008).

The small leucine-rich repeat proteoglycans (SLRPs) are a family of extracellular matrix proteins highly expressed in both cartilage and bone (Embree *et al.*, 2010). A mouse line doubly deficient in 2 members of the SLRP family, biglycan and fibromodulin (*bgn⁻⁰fmod^{-/-}*), is a well-established genetic model for TMJ OA (Wadhwa *et al.*, 2005a,b; Embree *et al.*, 2010). We have previously reported late-stage osteoarthritic changes in both *bgn⁻⁰fmod^{-/-}* TMJ cartilage and subchondral bone, including age-dependent cartilage degeneration, osteophyte formation, and sclerosis (Wadhwa *et al.*, 2005a,b). One advantage of using an experimental, genetic TMJ OA model is the ability to identify early cellular mechanisms that may predispose the animal to late-onset TMJ OA disease phenotype. Specifically, we have shown that Bgn and Fmod modulate cartilage ECM breakdown at an early age, before overt histological cartilage damage occurred (Embree *et al.*, 2010). The goal of this study was to identify new genes in cartilage and bone involved during early-stage TMJ OA in this genetic model. Based on these data, we aimed to evaluate cellular and tissue defects in the *bgn⁻⁰fmod^{-/-}* TMJ cartilage and subchondral bone.

MATERIALS & METHODS

Animals

Male *bgn⁻⁰fmod^{-/-}* mice and their strain-matched *WT* counterparts (C57BL/6-129) were used with approval from the ACUC, National Institutes of Health (NIDCR-DIR-#07-414), and generated as previously reported (Embree *et al.*, 2010).

Figure 1. Microarray analysis. **(A)** TMJ condylar cartilage (arrow) was dissected (black dashed lines) with intact subchondral bone interface from WT and *bgn^{-/-}fmod^{-/-}* mice. RNA was isolated for microarray and quantitative RT-PCR analysis. **(B)** Hierarchical clustering is shown for expression patterns of genes differentially expressed between WT and *bgn^{-/-}fmod^{-/-}* samples. Colorimetric scaling of standardized expression values (Z-standardization) is indicated at the bottom. **(C)** Quantitative RT-PCR for *Cartpt*, *Ptpv*, and *Scl4a1* with RNA extracted from 3-week-old WT (open bars) and *bgn^{-/-}fmod^{-/-}* (black bars) TMJs with an intact cartilage/subchondral bone interface. Gene expression levels were normalized to housekeeping gene *s29*. Graph shown is representative of 3 biological replicates. **p* < 0.01, *bgn^{-/-}fmod^{-/-}* vs. WT.

RNA Isolation and Microarray Analysis

TMJ condyles with an intact cartilage/subchondral bone interface were dissected from 3-week-old WT and *bgn^{-/-}fmod^{-/-}* mice (Fig. 1A, black arrow). Tissue from 3 animals was pooled to create single samples, and 3 independent WT and *bgn^{-/-}fmod^{-/-}* pooled samples were prepared for analysis. RNA was isolated by Trizol (Invitrogen,

Carlsbad, CA, USA), purified with RNeasy Mini Kit (Qiagen, Valencia, CA, USA), and treated with RNase-freeTM DNase (Ambion, Foster City, CA, USA). Synthesis of cDNA, *in vitro* transcription of biotin-labeled cRNA targets, and fragmentation of target cRNAs were performed following established Affymetrix[®] procedures (Allen *et al.*, 2009) at the MUSC Proteomics Facility (Charleston, SC, USA). Fragmented cRNA samples were hybridized to Affymetrix[®] Mouse Genome 430 2.0 microarrays in accordance with manufacturer protocols. Data were normalized by the Bioconductor gcRMA algorithm (Sean and Meltzer, 2007) implemented with the ArrayQuest Web tool (Argraves *et al.*, 2005) and imported into dChip software (Li and Wong, 2001) for comparative analyses. Genes differentially expressed between WT and *bgn^{-/-}fmod^{-/-}* samples were defined by the criteria: (1) 'present' detection scores in ≥ 2 samples of either group; (2) fold change > 2; and (3) *p* < 0.05 (unpaired Student's *t* test). False-discovery rate, estimated by permutation of sample groupings, approximated 19.4%.

Histology and Immunohistochemistry

Paraffin-embedded sections from 3-week-old WT and *bgn^{-/-}fmod^{-/-}* TMJ condyles were treated as previously described (Embree *et al.*, 2010). For tissue orientation, every 5th slide was stained with hematoxylin and eosin (H&E) or tartrate-resistant acid phosphatase (TRAP; Sigma 387A; Sigma, St. Louis, MO, USA). To quantify the percentage of TRAP-positive cells, we counted TRAP-positive cells and total cell number at the cartilage/subchondral bone interface from comparable tissue sections from 3 WT and 3 *bgn^{-/-}fmod^{-/-}* mice. Three serial sections within fixed areas were counted *per* mouse.

Immunohistochemistry was performed as previously described (Embree *et al.*, 2010). Tissue sections were enzymatically treated with ABCase and incubated with primary antibodies and isotype-matched negative controls, including polyclonal rabbit anti-mouse Bgn (1:500 dilution, rabbit total serum, LF-106; Dr. Larry Fisher, NIH), polyclonal rabbit anti-mouse Fmod (1:500 dilution, rabbit total serum, LF-149; Dr. Larry Fisher, NIH), and polyclonal rabbit anti-human type I collagen (1:2000 dilution, rabbit antiserum, LF-68; Dr. Larry Fisher, NIH).

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Table. Differential Gene Expression by *bgn*^{+/0} *fmod*^{-/-} vs. WT in TMJ Condyle with Intact Cartilage/Subchondral Bone Interface

Gene	Gene Symbol	Wild-type (mean)	<i>bgn</i> ^{+/0} <i>fmod</i> ^{-/-} (mean)	Fold Change	p-value	Relative Function
Fibromodulin	Fmod	5793	50	-117.0	0.0166	Negative Control
Fibromodulin	Fmod	2353	68	-34.5	0.0037	Negative Control
Fibromodulin	Fmod	2210	91	-24.4	0.0041	Negative Control
Fibromodulin	Fmod	2241	95	-23.8	0.0052	Negative Control
Biglycan	Bgn	3421	150	-22.6	0.0000	Negative Control
Biglycan	Bgn	4270	187	-22.6	0.0000	Negative Control
Fibromodulin	Fmod	2195	103	-21.4	0.0038	Negative Control
Biglycan	Bgn	6165	385	-16.0	0.0002	Negative Control
Fibromodulin	Fmod	1361	124	-10.9	0.0118	Negative Control
Procollagen, type IX, alpha 3	Col9a3	1105	300	-3.7	0.0430	Mutations result in multiple epiphyseal dysplasia and OA (Jackson <i>et al.</i> , 2010)
Hyaluronan and proteoglycan link protein 1	Hapln1	1783	501	-3.5	0.0381	Polymorphism is associated with spinal disc degeneration (Urano <i>et al.</i> , 2010)
Procollagen, type II, alpha 1	Col2a1	4390	1296	-3.4	0.0389	Col2a1 ^{-/-} mice develop TMJ OA (Rintala <i>et al.</i> , 1997)
Procollagen, type IX, alpha 1	Col9a1	2778	861	-3.2	0.0167	Col9a1 ^{-/-} mice develop knee and TMJ OA (Hu <i>et al.</i> , 2006)
Matrilin 3	Matn3	1783	592	-3.0	0.0419	Matn3 ^{-/-} mice develop knee OA (van der Weyden <i>et al.</i> , 2006)
RIKEN cDNA 3110079O15 gene	LOC 344564	534	189	-2.8	0.0431	
Mitochondrial ribosomal protein L30	Mrpl30	70	28	-2.5	0.0476	Energy metabolism (Pagliarini <i>et al.</i> , 2008)
*CART prepropeptide	Cartpt	498	229	-2.2	0.0172	Inhibits bone resorption by modulating Rankl expression (Eleftheriou <i>et al.</i> , 2005)
*Secreted frizzled- related sequence protein 1	Sfrp1	372	765	2.0	0.0402	Expressed in osteoblasts and inhibits osteoclast formation (Hausler <i>et al.</i> , 2004)
Profilin 1	Pfn1	326	671	2.1	0.0073	Required for abscission during late cytokinesis of chondrocytes (Bottcher <i>et al.</i> , 2009)
Beta-site APP cleaving enzyme 1	Bace1	370	798	2.1	0.0006	Proteolytic cleavage of the amyloid precursor protein (Vassar <i>et al.</i> , 1999)
Ribosomal protein S19	Rps19	161	347	2.1	0.0102	Anti-inflammatory (Filip <i>et al.</i> , 2009)
Angiopoietin-like 7	Angptl7	165	367	2.2	0.0225	Support expansion hematopoietic stem cells (Zhang <i>et al.</i> , 2006)
*Arylsulfatase K	Arsk	185	434	2.4	0.0049	Required for rapid inhibition of bone resorption by calcitonin (Baron <i>et al.</i> , 1990)
RIKEN cDNA 1110020P15 gene		77	189	2.5	0.0288	
Transcribed locus		108	265	2.5	0.0038	
*Protein tyrosine phosphatase, receptor type V	Ptprv	263	798	3.0	0.0059	Bone metabolism (Lee <i>et al.</i> , 2007)
Tetraspanin 33	Tspan33	241	809	3.4	0.0390	Cell adhesion and motility (Lazo, 2007)
AE binding protein 1	Aebp1	109	419	3.8	0.0066	Macrophage cholesterol homeostasis and inflammation (Majdalawieh <i>et al.</i> , 2006)
*Solute carrier family 4 (anion exchanger), member 1	Slc4a1	168	719	4.3	0.0423	Mediates osteoclast differentiation/function (Wu <i>et al.</i> , 2008)
Erythroid-associated factor	Ahsp	168	867	5.2	0.0455	Erythropoiesis and cancer (Hardee <i>et al.</i> , 2006)
RIKEN cDNA 4833416E15Rik	4833416E15Rik	132	923	7.1	0.0005	

Differential gene expression $\pm$ 2.0-fold and $p < 0.05$ (unpaired *t* test) for *bgn*^{+/0} *fmod*^{-/-} vs. WT. False-discovery rate for this analysis approximates 19.4%. Mean indicates average normalized fluorescence measurement for 3 replicates. Asterisks denote genes related to osteoclast activity/function and bone metabolism.

Figure 2. Increased osteoclast activity in the *bgn*^{-/-}*fmod*^{-/-} TMJ subchondral bone interface. (A,B) Low magnification and (C,D) high magnification of TRAP staining in the WT and *bgn*^{-/-}*fmod*^{-/-} TMJ cartilage/subchondral bone interface. Orange line indicates the TMJ cartilage/subchondral bone interface and divides TMJ into condylar cartilage (CC) and subchondral bone (SB). Green dashed lines show areas of high magnification. Arrows indicate TRAP-positive cells localized along the TMJ cartilage/subchondral bone interface. Bar = 50 μ m. (E) The number of TRAP-positive cells at the TMJ cartilage/subchondral bone interface/fixed area tissue. Graph shown is representative of a total of 3 mice, with comparable tissue sections with a total of 3 serial sections *per* mouse. **P* = 0.001, *bgn*^{-/-}*fmod*^{-/-} vs. WT. (F,G) Quantitative RT-PCR for *RANKL* and *OPG* with RNA from WT (open bars) and *bgn*^{-/-}*fmod*^{-/-} TMJs (black bars) in an intact TMJ cartilage/subchondral bone interface. Gene expression levels were normalized to housekeeping gene *S29*. Graph shown is representative of at least 3 independent experiments. **p* < 0.01, *bgn*^{-/-}*fmod*^{-/-} vs. WT.

Quantitative RT-PCR

The cDNA was obtained by reverse-transcription of total RNA with TaqManTM reverse-transcription reagents (Applied Biosystems, Foster City, CA, USA). Primers used for the RT-PCR were designed with Beacon Designer Software (BioRad, Hercules, CA, USA) (Appendix Table 1). Real-time PCR was performed as previously described (Embree *et al.*,

2010). Gene expression levels were normalized to the housekeeping gene *s29*.

Statistical Analysis

Data were collected from a minimum of 3 independent experiments. Statistical analyses were conducted with Student's *t* test. Any *p* values < 0.05 were considered statistically significant.

RESULTS

Differential Gene Expression in the *bgn*^{-/-}*fmod*^{-/-} TMJ Cartilage/Subchondral Bone Interface

To identify potential new genes involved during early stages of TMJ OA in the *bgn*^{-/-}*fmod*^{-/-} genetic mouse model (Wadhwa *et al.*, 2005a,b; Embree *et al.*, 2010), we used a microarray analysis approach. Microarray analysis detected differential expression of 22 genes in *bgn*^{-/-}*fmod*^{-/-} vs. WT samples (Fig. 1B). Eight genes were down-regulated in *bgn*^{-/-}*fmod*^{-/-} samples (Table) and included genes coding for extracellular matrix proteins involved in cartilage degeneration, such as procollagen, type IX, alpha 3 (Jackson *et al.*, 2010), hyaluronan and proteoglycan link protein 1 (Urano *et al.*, 2010), procollagen, type II, alpha 1 (Rintala *et al.*, 1997), procollagen, type IX, alpha 1 (Hu *et al.*, 2006), and matrilin 3 (van der Weyden *et al.*, 2006). Mitochondrial ribosomal protein L30, a gene related to mitochondrial energy metabolism, was also down-regulated (Pagliarini *et al.*, 2008). Interestingly, CART prepropeptide, a peptide of CART that functions to inhibit osteoclasts and bone resorption through the modulation of *RANKL* expression (Eleftheriou *et al.*, 2005), was also decreased in *bgn*^{-/-}*fmod*^{-/-} samples, suggesting that bone metabolism may be changed in the *bgn*^{-/-}*fmod*^{-/-} mice. The 14 genes up-regulated in *bgn*^{-/-}*fmod*^{-/-} samples included 3 additional genes associated with osteoclast differentiation and/or function [secreted frizzled-related sequence protein 1 (Hausler *et al.*, 2004), arylsulfatase K (Baron *et al.*, 1990), solute carrier family 4, member 1 (Wu *et al.*, 2008)], and 1 gene associated with bone metabolism [protein tyrosine phosphatase, receptor type V (Lee *et al.*, 2007)] (Table, asterisks). Other genes up-regulated included those related to: cytokinesis of chondrocytes [profilin 1 (Bottcher *et al.*, 2009)], beta secretase cleavage [beta-site APP cleaving enzyme 1

(Vassar *et al.*, 1999)], stimulation of hematopoietic stem cells [angiopoietin-like 7 (Zhang *et al.*, 2006)], inflammation and fat metabolism [ribosomal protein S19 (Filip *et al.*, 2009)], AE binding protein 1 (Majdalawieh *et al.*, 2006), erythropoiesis and blood cancer [erythroid-associated factor (Hardee *et al.*, 2006)], and cell adhesion/motility [tetraspanin 33 (Lazo, 2007)]. Our microarray analysis demonstrated a relatively high false-discovery rate (FDR = 19.4%), indicating the probability that some false-positives were detected.

Therefore, to confirm the findings from our microarray analysis, we tested 3 responding genes by quantitative RT-PCR (Fig. 1C). RT-PCR confirmed that CART prepropeptide (*Cartpt*) was down-regulated, whereas protein tyrosine phosphatase, receptor type V (*Ptprv*), and solute carrier family 4 (anion exchanger), member 1 (*Slc4a1*), were up-regulated in *bgn*⁻⁰*fmod*^{-/-} samples.

Increased Osteoclast Activity in the *bgn*⁻⁰*fmod*^{-/-} TMJ Cartilage/Subchondral Bone Interface

Given that the microarray analysis revealed changes in 4 genes related to osteoclast activity/function and 1 gene associated with bone metabolism in *bgn*⁻⁰*fmod*^{-/-} samples (Table, asterisks), we speculated that osteoclast activity was altered in *bgn*⁻⁰*fmod*^{-/-} TMJ subchondral bone. We examined osteoclast activity in *WT* and *bgn*⁻⁰*fmod*^{-/-} by TRAP staining and quantitative RT-PCR for *RANKL* and *OPG*, 2 genes important for osteoclast activity. In comparison with *WT* TMJ (Fig. 2C, arrows), there was an increase in TRAP-positive regions along the cartilage/subchondral bone interface (Fig. 2, orange line) in *bgn*⁻⁰*fmod*^{-/-} TMJ (Fig. 2D, arrows). Quantification of TRAP-positive cells confirmed that there was a three-fold increase in *bgn*⁻⁰*fmod*^{-/-} TRAP-positive cells (Fig. 2E). Quantitative RT-PCR, with RNA isolated from *WT* and *bgn*⁻⁰*fmod*^{-/-} TMJs, demonstrated a significant increase in *RANKL* (Fig. 2F) and *OPG* (Fig. 2G) gene expression levels in *bgn*⁻⁰*fmod*^{-/-}, with relatively greater up-regulation of *RANKL* (~4.5-fold increase) compared with *OPG* (~3.5-fold increase). Analysis of these data resulted in a 128% increase in *RANKL/OPG* ratio in *bgn*⁻⁰*fmod*^{-/-} and suggested that osteoclast

Figure 3. Defects in TMJ subchondral bone in 3-week-old *bgn*⁻⁰*fmod*^{-/-} mice. (A,B) 20x magnification of H&E staining in 3-week-old *WT* and *bgn*⁻⁰*fmod*^{-/-} TMJs showing a condylar cartilage (CC) region and subchondral bone (SB) regions. Orange dashed box indicates a subchondral bone (SB) region that is shown in images below in higher magnification. Bar = 100 μ m. (C,D) 40x magnification of H&E staining in 3-week-old *WT* and *bgn*⁻⁰*fmod*^{-/-} TMJ subchondral bone (SB) regions, indicated in orange dashed box. Bar = 50 μ m. (E,F) Type I collagen immunostaining in 3-week-old *WT* and *bgn*⁻⁰*fmod*^{-/-} TMJ subchondral bone. Bar = 50 μ m. (G,H) Biglycan (Bgn) and fibromodulin (Fmod) immunostainings in TMJ subchondral bone in 3-week-old *WT* mice. Bar = 50 μ m.

activity was increased in *bgn*⁻⁰*fmod*^{-/-} TMJ subchondral bone. However, differential expression of *RANKL* and *OPG* was not detected in microarray studies (Appendix Table 2). This was likely due to the statistical limitations of the microarray analysis and the increased specificity of quantitative RT-PCR.

Identification of TMJ Subchondral Bone Defects in the *bgn⁻⁰fmod⁻⁰* TMJ

We proposed that increased osteoclast activity would cause phenotypic defects in *bgn⁻⁰fmod⁻⁰* TMJ subchondral bone. We examined the subchondral region from 3-week-old *bgn⁻⁰fmod⁻⁰* TMJ compared with *WT* controls by histology (Figs. 3A-D) and immunohistochemistry (Figs. 3E-F). H&E staining of *WT* TMJ (Fig. 3A) showed distinct bone marrow cavities formed, which were surrounded by trabecular bone tissue (Fig. 3C; orange dashed lines indicate higher magnification). However, H&E stains of *bgn⁻⁰fmod⁻⁰* TMJ (Fig. 3B) revealed significant tissue disorganization and sparse bone tissue present in comparable regions (Fig. 3D; orange dashed lines indicate higher magnification). To confirm the presence of subchondral bone defects in the *bgn⁻⁰fmod⁻⁰*, we examined type I collagen expression immunohistochemistry. Type I collagen was localized within *WT* TMJ subchondral bone (Fig. 3E, red), and a clear decrease was apparent in the *bgn⁻⁰fmod⁻⁰* TMJ (Fig. 3F, red), confirming that less type I collagen-rich bone was present. Interestingly, while type I collagen protein expression was decreased in *bgn⁻⁰fmod⁻⁰* TMJs, type I collagen mRNA was not detected, as differentially expressed by microarray analysis (Appendix Table 2) or by quantitative RT-PCR (Appendix Fig.), suggesting that type I collagen production is unchanged. Taken together, our histological findings suggested that the absence of *Bgn* and *Fmod* led to less bone in the TMJ subchondral bone region in mice. Immunohistochemistry showed that both *Bgn* (Fig. 3G, red, arrows) and *Fmod* (Fig. 3H, red, arrows) were both highly expressed and co-localized in *WT* TMJ at 3 wks in the bone regions shown to be affected by their absence.

DISCUSSION

The *bgn⁻⁰fmod⁻⁰* mouse is an established TMJ OA genetic model demonstrating a chronological sequence of cellular/histological evidence that signifies TMJ OA pathology (Wadhwa *et al.*, 2005a,b; Embree *et al.*, 2010). We took advantage of this genetic model to identify early changes in gene expression levels that may predispose the animal to the late-onset TMJ OA phenotype. Using microarray analysis, we discovered 22 genes differentially expressed in young *bgn⁻⁰fmod⁻⁰* TMJs that could potentially be involved in disease initiation. Genes that correspond to other ECM proteins involved in cartilage degeneration in other genetic mouse models were also down-regulated in the *bgn⁻⁰fmod⁻⁰* samples, including procollagen, type IX, alpha 3 (Jackson *et al.*, 2010), procollagen, type II, alpha 1 (Rintala *et al.*, 1997), procollagen, type IX, alpha 1 (Hu *et al.*, 2006), and matrilin 3 (van der Weyden *et al.*, 2006). Analysis of these data based on genetic mouse models highlights the possibility that, in humans, the loss/mutation of a single ECM-related gene may initiate pathology or increase susceptibility to TMJ disease. However, the factors that account for the majority of TMJ OA cases in humans, which occur with a high predilection in women during childbearing ages, remain unresolved (Scrivani *et al.*, 2008). TMJ OA genetic mouse models with ECM-related mutations show pathology in aged males (Rintala *et al.*, 1997; Wadhwa

et al., 2005a,b; Hu *et al.*, 2006), thus raising doubt as to whether existing genetic mouse models exhibit TMJ disease analogous to the human condition. It is possible that changes in sex-related hormones may act upstream to contribute to the abnormal expression patterns of ECM-related proteins (Hellio Le Graverand *et al.*, 2000; Talwar *et al.*, 2006; Salgado *et al.*, 2009). Therefore, for a more comparable model of TMJ OA to be developed, it would be important to determine the effects of estrogen levels on TMJ disease in females, using *bgn⁻⁰fmod⁻⁰* mice or other genetic mouse models of TMJ OA.

The TMJ has unique properties compared with other articular joints like the knee, because it is made of fibrocartilage and acts both as an articular joint cartilage and as a site for endochondral ossification (Shen and Darendeliler, 2005). Consequently, the cellular events within the TMJ cartilage/subchondral bone interface are more likely to play a greater role in tissue homeostasis and disease in the TMJ than in the knee joint. TMJ OA is a degenerative disease of the entire joint, including the subchondral bone (Lories and Luyten, 2011). Phenotypic changes in the subchondral bone in osteoarthritic tissue from both animals and humans have been well-documented, including altered trabecular bone structure, sclerosis, and increased bone resorption/formation (Lories and Luyten, 2011). Our microarray analysis revealed that at least 5 genes are changed in *bgn⁻⁰fmod⁻⁰* samples that are related to osteoclast function/differentiation and bone turnover, including CART prepropeptide (Elefteriou *et al.*, 2005), secreted frizzled-related sequence protein 1 (Hausler *et al.*, 2004), arylsulfatase K (Baron *et al.*, 1990), solute carrier family 4, member 1 (Wu *et al.*, 2008), and protein tyrosine phosphatase, receptor type V (Lee *et al.*, 2007). More specifically, CART prepropeptide inhibits bone resorption by modulating *RANKL* expression (Elefteriou *et al.*, 2005). Similar to *bgn⁻⁰fmod⁻⁰* mice, mice deficient in CART also have low trabecular bone mass (Elefteriou *et al.*, 2005). Furthermore, secreted frizzled-related sequence protein 1 is expressed by osteoblasts and inhibits osteoclast formation, suggesting that bone turnover may be higher in the *bgn⁻⁰fmod⁻⁰* TMJ subchondral bone (Hausler *et al.*, 2004). In addition, solute carrier family 4, member 1 (*Slc4a1*), a bicarbonate/chloride exchanger closely related to *Slc4a2*, is also up-regulated in *bgn⁻⁰fmod⁻⁰* samples. This latter protein is a critical mediator of both osteoclast differentiation and function, where *Slc4a2⁻⁰* osteoclasts are unable to resorb mineralized tissue and fail to express TRAP activity (Wu *et al.*, 2008). Taken together, these microarray findings suggested that osteoclast activity and/or bone turnover may be increased in the *bgn⁻⁰fmod⁻⁰* TMJ subchondral bone.

To explore osteoclast activity in the *bgn⁻⁰fmod⁻⁰* mice, we examined TRAP stainings in the TMJ subchondral bone and also OPG and *RANKL* by quantitative RT-PCR. In agreement with our microarray findings, we discovered increased TRAP staining specifically localized along the *bgn⁻⁰fmod⁻⁰* TMJ cartilage/subchondral bone interface, suggesting active bone turnover in this region. Similarly, we discovered a 128% increase in *OPG/RANKL* gene expression ratio in *bgn⁻⁰fmod⁻⁰* samples, which could cause increased TMJ subchondral bone turnover in *bgn⁻⁰fmod⁻⁰* mice. In support of our findings, previous studies have demonstrated that non-TMJ OA joints also display

increased subchondral bone turnover (Goker *et al.*, 2000). Moreover, increased osteoclast activity in *bgn⁰fmod^{-/-}* mice was also coupled with increased chondrogenesis in 3-week-old mice (Embree *et al.*, 2010). This concept raises the possibility that crosstalk between the cartilage and subchondral bone interface during younger ages is critical for maintaining tissue homeostasis. The disruption of bone and cartilage metabolism in younger *bgn⁰fmod^{-/-}* mice could disrupt the overall TMJ tissue homeostasis and predispose the mice to the late-onset TMJ OA phenotype previously reported in the older *bgn⁰fmod^{-/-}* mice, including osteophyte formation, TMJ subchondral bone sclerosis, and cartilage degeneration (Wadhwa *et al.*, 2005a,b).

We further show that a possible consequence of high bone turnover is defective trabecular bone structure formed in *bgn⁰fmod^{-/-}* TMJ subchondral region, as evidenced by histology and type I collagen immunostaining. Given that the functional integrity of the cartilage can depend on the mechanical properties of the underlying bone (Bailey *et al.*, 2004), it is possible that the disorganized and defective TMJ subchondral bone in *bgn⁰fmod^{-/-}* mice contributes significantly to TMJ OA pathology. Interestingly, while type I collagen protein expression was decreased in *bgn⁰fmod^{-/-}* TMJs, type I collagen mRNA was unchanged. We speculate that the production of type I collagen mRNA is not changed, and that other things such as translational control of type I collagen mRNA or ECM resorption cause less type I collagen protein expression in *bgn⁰fmod^{-/-}* TMJs. Given that TRAP-positive cells and *RANKL/OPG* gene expression levels are significantly higher in *bgn⁰fmod^{-/-}* TMJs, it is likely that there is increased resorption and, therefore, less type I collagen protein expression. Taken together, we show for the first time that *Bgn* and *Fmod* are critical for the integrity of TMJ subchondral bone and reveal a potential role for subchondral bone turnover during early stages of TMJ OA in this mouse model.

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