ECM mediates BMP-2 in a model of temporomandibular joint-osteoarthritis

M. Shirakura[1,2], **V. Kram**[1], **J. Robinson**[3], **S. Sikka**[1], **T.M. Kilts**[1], **S. Wadhwa**[3], and **M F. Young**[1]

[1]NIDCR, National Institutes of Health, Bethesda, MD

[2]Hiroshima University, Hiroshima, Japan

[3]Columbia University, NY, NY

Abstract

Temporomandibular joint osteoarthritis (TMJ-OA) is a complex disease that affects both cartilage and subchondral bone. It is accompanied by loss of extracellular matrix (ECM) and may be controlled by Bone Morphogenetic Protein 2 (BMP-2). We analyzed the effect of BMP-2 in both cartilage and subchondral bone in a TMJ-OA animal model that is deficient in Bgn and Fmod ($Bgn^{-/-}Fmod^{-/-}$). Whole mandibles were dissected from 3 week-old wild-type (WT) and $Bgn^{-/-}Fmod^{-/-}$ mice, and incubated with and without 250 μg/ml BMP-2 for 2 days using an explant culture system. Condyle growth was measured by μCT and the expression levels of cartilage and bone related genes were analyzed using real time (RT)-PCR or by immunohistochemistry (IHC) from condyles that contained an intact cartilage/subchondral bone interface. Osteoclast activity was estimated by TRAP staining and by *TRAP, Rankl, AdamTs4* mRNA expression levels. Our results showed that most parameters examined were slightly up-regulated in WT samples treated with BMP-2 and this up-regulation was significantly enhanced in the $Bgn^{-/-}Fmod^{-/-}$ mice. The up-regulation of both catabolic and anabolic agents did not appear to positively affect the overall growth of $Bgn^{-/-}Fmod^{-/-}$ condyles compared to WT controls. In summary, the up-regulation of both anabolic and catabolic genes in the WT and $Bgn^{-/-}Fmod^{-/-}$ TMJs treated with BMP-2 suggests that BMP increases matrix turnover in the condyle and, further, that Bgn and Fmod could have protective roles in regulating this process.

Keywords

biglycan; fibromodulin; mineralization; growth factor; turnover; osteoclast

Introduction

Temporomandibular joint (TMJ) disorders afflict approximately 5–10% of the US population [Liu and Steinkeler 2013]. These disorders encompass a collection of at least 12 clinical phenotypes, one of which is TMJ degenerative joint disease (TMJ-DD) [Schiffman

Corresponding Author: Marian Young, PhD, 9000 Rockville Pike, Building 30 Room 225, MSC 4320, National Institute of Dental and Craniofacial Research, National Institutes of Health, Bethesda, MD 20892, Phone: 301-496-8860, FAX: 301-402-0824, Myoung@dir.nidcr.nih.gov.

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et al. 2014]. Individuals with TMJ-DD experience irreversible damage to the joint complex including degradation and loss of extracellular matrix proteins necessary for joint function. Unfortunately, in most cases, this irreversible damage occurs before symptoms present and the disease is diagnosed [Wang et al. 2015]. Therefore, efforts to identify markers and the molecular mechanisms characteristic of TMJ-DD in the early phases are needed in to detect and to develop treatments that can prevent the irreversible process of the disease.

The TMJ is a unique articulating joint comprised of the mandibular condyle which fits into the mandibular fossa of the temporal bone. The articular disc and the articular cartilage of the condyle are both fibrocartilagenous tissues that cover these two bones and promote articulation. The fibrocartilage of the mandibular condyle is comprised of extracellular matrix proteins and proteoglycans that provide the necessary properties to support the loads on the condyle during mastication and verbal communication. Within this extracellular matrix, there exists small leucine-rich proteoglycans (SLRPs) including biglycan (Bgn) and fibromodulin (Fmod), which play a role in sequestration of important signaling molecules and regulating collagen fibrillogenesis [Ameye and Young 2002]. Specifically, biglycan and fibromodulin modulate TGF super family signaling activity including bone morphogenetic proteins signaling (BMP) [Young et al. 2006, Wadhwa et al. 2004]. BMP signaling has well established roles in chondrogenesis and endochondral bone formation [Long and Ornitz 2013]. Abnormal activation of this signaling causes heterotopic cartilage and bone formation [Huegel et al 2015, Billings et al, 2008]. Specifically, excessive BMP-2 signaling has recently been shown to be involved in heterotopic cartilage formation and the pathogenesis of TMJ-OA [Bechtold et al. 2016, Albilia et al, 2013]. However, the mechanism of excessive BMP-2 signaling on the mandibular condylar cartilage and its implications in TMJ-OA are unknown.

We have previously developed a murine model with accelerated of TMJ-OA in mice double-deficient in biglycan and fibromodulin ($Bgn^{-/-}Fmod^{-/-}$). Using this model, we determined that $Bgn^{-/-}Fmod^{-/-}$ mice develop TMJ-OA at 6–9 months of age whereas WT mice develop TMJ-OA at 12–18 months of age [Wadhwa et al. 2005a, Wadhwa et al, 2005b, Chen et al. 2009]. Consequently, the goal of this study was to examine BMP-2 signaling in $Bgn^{-/-}Fmod^{-/-}$ mice. We hypothesized that BMP-2 treatment would promote over-activation of chondrogenic markers in the absence of Bgn and Fmod compared to WT mice due to dysregulation of the signaling pathway. Greater understanding of the early signaling events that mediate TMJ-OA is critical in the development of diagnostic tools and interventional therapeutics.

Materials and Methods

Animals

Female $Bgn^{-/-}Fmod^{-/-}$ mice and their strain matched wild-type counterparts (C57BL/6) were used with approval from the Animal Care and Use Committee, National Institutes of Health (#NIDCR-12-655). The generation and genotyping of the $Bgn^{-/-}Fmod^{-/-}$ have been reported previously [Wadhwa et al. 2005a].

Mandibular ex-vivo organ culture and treatment with BMP-2

Mandibular condyles were harvested from 3-week-old wild-type (WT) or $Bgn^{-/-}$ $Fmod^{-/-}$ mice (for histology) or 5-week-old mice (for mRNA), dissected in half and cultured in 24 well plate in DMEM and Pen/Strep antibiotics supplemented with 100 nmol/L ascorbic acid for 24 hours. The condyles were then incubated with or without 250 ng/ml human carrier-free recombinant BMP-2 (eBioscience). The left mandibular condyle was treated with BMP-2 and the right was treated with vehicle control. After 2 days, the explant cultures were collected and prepared for histology and quantitative real-time PCR (Fig. S1). For each sample, 5 condyles were combined and each experiment was repeated at least three times.

MicroCT

Treated mandibles were fixed and scanned using a μCT 50, Scanco Medical AG, (Bassersdorf, Switzerland). Scans were performed at 70 kV, 85 μA, 300 ms integration time, and at a resolution of 10 μm. After reconstruction, the images were stored in 3D arrays and used to measure the height of the mandible as shown in Figure 1A–B).

Quantitative RT-PCR

Expression levels of cartilage and bone related genes were analyzed by RT-PCR using RNA obtained from condyles that contained both cartilage and subchondral bone. RNA was isolated using Trizol (Invitrogen) and purified with RNeasy. cDNA was generated by reverse transcribing total RNA using iScript from BioRad and subjecting it to real-time PCR using SYBER-mastermix (Applied Biosystem) in a Biorad MyiQ real-time PCR thermocycler. Each cDNA (5–10 ng) was amplified using the primers shown in Table S1. Gene expression was normalized to the housekeeping gene, S29. For each sample, 5 condyles were combined and each experiment was repeated at least three times.

Histology, Staining and Quantification

Paraffin-embedded sections were dewaxed in xylene washes and rehydrated through graded ethanol (100%, 95%, and 75% and water). Tissue sections were stained histochemically with hemotoxylin and eosin (H&E) or Safranin O. For immunohistochemistry, rehydrated sections were enzymatically treated with ABCase and incubated with primary antibodies at 4° C overnight including polyclonal rabbit anti-mouse aggrecan (1:100=5 mg/ml rabbit IgG cat # AB1031, ABCAM), polyclonal rabbit anti-mouse type I collagen (1:2000, rabbit antiserum, LF-68, gift from Dr. Larry Fisher). The broad spectrum immunoperoxidase AEC kit (Picture Plus, Zymed) was subsequently used to detect the immunoreactivity according to the manufacturers' instructions. The sections were counterstained with hematoxylin. Images of the stained sections were captured and the area of positive staining accessed using software from Image Pro. Non-immune immunoglobulins of the same isotype as the primary antibody served as negative controls and showed no positive staining (not shown). Safranin O positive staining in the treated TMJs was also accessed using Image Pro software (S2). Osteoclast formation was estimated by counting the number of tartrate-resistant acid phosphatase (TRAP, 387A Sigma) positive multinucleated cells in 5–7 sections/sample.

Statistical Analysis

Values are presented as the mean ± standard deviation. Statistical significance of differences among means was determined by two-way analysis of variance (ANOVA) with post hoc analysis by the Bonferonni method using SPSS. Statistical significance was defined as $p < 0.05$.

Results

The experimental strategy for examining BMP-2 function in ex-vivo explants is outlined in S1. Briefly, hemi-mandibles were dissected from the heads of WT and $Bgn^{-/-}Fmod^{-/-}$ mice and included the entire condyle with its subchondral bone. Samples from 3 week-old mice were used for histology while 5 week-old mice were used for mRNA extraction. The hemi-mandibles were then cultured in the presence or absence of BMP-2 for 48 hours and processed for μCT, histology, and mRNA isolation for RT-PCR.

Hemi-mandibles from WT and $Bgn^{-/-}Fmod^{-/-}$ incubated with or without BMP-2 were first analyzed by microCT to determine the relative height of the condyle after treatment (Figure 1A). This analysis showed that WT condyles responded with a slight but significant increase in length (Figure 1B), but the $Bgn^{-/-}Fmod^{-/-}$ condyles did not. This finding prompted us to further examine the underlying cell and molecular foundation by histology, immunohistochemistry and quantitative RT-PCR.

The temporomandibular joint at 3 weeks of age is composed of a thick fibrocartilage surface that covers a layer of cartilage that can be clearly visualized by staining with safranin O (S2). The cartilage layer lies on top of the subchondral bone that composes the majority of the condyle structure. The area of safranin O positive staining in WT mandibles treated with BMP-2 was unchanged after the 48 h BMP treatment. However, the area of safranin O in condyles from $Bgn^{-/-}Fmod^{-/-}$ mice was significantly increased (Fig. 2A, B). To confirm these observations, we next stained the sections for the expression of aggrecan (Acan) a known marker of differentiated cartilage tissue. This showed that BMP-2 slightly increased the extent of Acan staining in WT tissue, and more so in the $Bgn^{-/-}Fmod^{-/-}$ condyles (Fig. 2C, D). When the relative expression of mRNA for *Agan* was examined with BMP-2 treatment we found that both WT and $Bgn^{-/-}Fmod^{-/-}$ had increased *Agan* levels but that in the $Bgn^{-/-}Fmod^{-/-}$ the BMP-2 response much was more robust that in the WT (Fig. 2E).

To deepen our understanding of the molecular basis for the observed changes that we saw in cartilage tissue morphology, the mRNA expression levels for additional key cartilage genes were determined. This analysis showed that BMP-2 increased the levels of type II collagen (*Col2*, Fig 3A) and type X collagen (*Col10*, Fig3B), *Sox9*, the master gene known to regulate cartilage formation (Fig 3C), and chondrocyte controlling gene *ihh* (Fig 3D) and that the relative level of induction was much greater in the $Bgn^{-/-}Fmod^{-/-}$ condyles. Taken together these data showed that the $Bgn^{-/-}Fmod^{-/-}$ condyles are more sensitive to the effects of BMP-2 causing expansion of the cartilage zone resulting in increased levels of safranin 0, Agan positive tissue as well in the expression of the cartilage related genes *Col2*, *Col10*, *Sox9* and *ihh*.

We next examined the effects of BMP-2 on the proteins and mRNAs that are made by the surrounding fibrocartilage and bone. This analysis showed that the extent of type I collagen (Col1) in the fibrocartilage was slightly increased (but not significantly) in WT condyles treated with BMP-2 and that the $Bgn^{-/-}Fmod^{-/-}$ condyles were much more responsive (Fig 4A). Real-time PCR of mRNA encoding *Col1* mRNA showed a parallel significant increase of type I collagen in the $Bgn^{-/-}Fmod^{-/-}$ condyles treated with BMP-2 compared with similarly treated WT explants (Fig 4B). The master gene, *Runx2*, known to regulate many bone enriched genes including type I collagen was found to be regulated in the same manner as type I collagen Fig 4 C). Osterix (*Osx*), the master regulatory factor operating down stream of *Runx2* showed a different pattern compared to *Runx2* whereby induction by BMP-2 in WT condyles was almost (but not completely) at the same level as the $Bgn^{-/-}Fmod^{-/-}$ condyles (Fig 4D). A down-stream target of Wnt called *Wisp1/Ccn4* that is known to control bone turnover was also examined and showed constitutive elevation in WT vs. $Bgn^{-/-}Fmod^{-/-}$, and that this increased expression was further enhanced by treatment with BMP-2 (Fig 4E).

The enhancement of genes known to regulate bone and cartilage formation led us to question whether skeletal turnover in the $Bgn^{-/-}Fmod^{-/}$ condyles was differentially affected by BMP-2. To test this possibility, WT and $Bgn^{-/-}Fmod^{-/}$ condyles were treated with BMP-2 and stained for tartrate resistant acid phosphatase (TRAP) to identify osteoclasts. This analysis showed that the number of multinucleated TRAP positive cells was not significantly changed in BMP-2 treated WT condyles while in contrast $Bgn^{-/-}Fmod^{-/}$ condyles showed a higher number of TRAP positive cells that was increased with BMP-2 (Figure 5A). Analysis of mRNA for TRAP (*Acp5*) showed a similar outcome where BMP-2 treated $Bgn^{-/-}Fmod^{-/-}$ condyles had much greater expression than BMP-2 treated WT samples (Fig 5 B). Interestingly, untreated $Bgn^{-/-}Fmod^{-/-}$ condyles also had elevated TRAP mRNA expression compared to untreated WT condyles. Analysis of additional catabolic mRNAs *Rankl* (Fig. 5C) and *Adamts4* (Fig 5D) showed a similar pattern to TRAP mRNA where the $bgn^{-/0}fmod^{-/-}$ condyles had constitutively higher levels than WT condyles. BMP-2 treatment of $Bgn^{-/-}Fmod^{-/-}$ condyles further increased *Rankl* and *Adamts* mRNA compared to WT controls.

Discussion

For patients who experience TMJ-OA, irreversible degeneration of the joint often occurs prior to detection or diagnosis. Thus, early detection and diagnosis with biomarkers could lead to early intervention and reduce the incidence of irreversible damage to the TMJ. The goal of this study was to determine whether excessive BMP-2 signaling results in over-activation of chondrogenesis in a $Bgn^{/-}Fmod^{-/-}$ model of TMJ-OA. Results from this study illustrate that BMP-2 treatment potentiated the induction of *Acan, Col2, Col 10*, and *Ihh* in $Bgn^{-/-}Fmod^{-/-}$ mice. Excess BMP-2 signaling has also been implicated in the pathogenesis of accelerated TMJ-OA in Pgr4-KO mice [Bechtold et al. 2016]. In contrast, absence of BMP signaling in transgenic mice with *Bmp1Ra* conditionally deleted in neural crest cells led to failure of articular disc separation from the hypoplastic condyle and under development of the mandibular condylar cartilage [Gu et al. 2014]. Taken together, the results suggest that a threshold of BMP-2 is needed for normal TMJ development.

The mandibular condylar cartilage is unique in that it is derived from the periosteum and undergoes endochondral ossification [1]. In other growth plates, it is believed that there are a finite number of progenitor cells and once these cells are depleted, growth stops [Hinton et al. 2015]. A similar mechanism may occur in the TMJ. For example, in mice the mandibular condylar cartilage rapidly undergoes endochondral ossification until 2 months of age [Ghassemi et al. 2016]. Afterwards, the mandibular condylar cartilage becomes senescent and phenotypically resembles other articular cartilages [Levne et al. 1985]. Therefore, in the absence of Bgn and Fmod, excessive BMP-2 signaling may cause accelerated progenitor cell depletion resulting in accelerated age-related TMJ degeneration. Future studies will focus on delivering BMP-2 inhibitors to young (growing) and adult (non-growing) mice to examine if they can inhibit age related TMJ-OA in the $Bgn^{-/-}Fmod^{-/-}$ mice in order to further test this hypothesis.

It is well known that aggrecan is a major proteoglycan in cartilage and that it has important roles in regulating cartilage integrity. In the present study, we focused on Bgn and Fmod because we found they are both abundantly expressed and localized in the articular cartilage of the mandibular condyle [Wadhwa et al. 2001]. Interestingly in the $Bgn^{-/-}Fmod^{-/-}$ condyles the expression of aggrecan is first increased at 3 weeks of age, and then, at 5 weeks of age decreased with further reduction at 24 weeks of age compared to WT condyles. We believe this is due, in part, to overactive TGF-beta production leading to an up-regulation proteolytic enzymes that degrade aggrecan including ADAMTS4 and ADAMTS5 [Embree et al. 2010]. In this context, we cannot exclude the possibility that some of the effects we see in the BMP-2 responsiveness in the $Bgn^{-/-}Fmod^{-/-}$ mice could, in fact, be from alterations in aggrecan levels and function.

In our previous study we found that TGF-ß signaling was elevated in $Bgn^{-/-}Fmod^{-/-}$ mice. Our results with BMP-2 are similar; however, in this study, we found that BMP-2 also caused an increase in *Rankl* [Embree et al. 2010]. Increased *Rankl* in the $Bgn^{-/-}Fmod^{-/-}$ mice was confirmed by our previous published microarray data [Embree et al. 2011], suggesting that osteoclastogenesis may be specific for BMP-2 signaling pathway rather than the TGF-ß signaling pathway.

Bgn and Fmod have a highly ordered structure that is composed of a core protein with extensive post-translational modifications including the addition of chontroitin/dematan sulafate (Bgn) or keratin sulfate glycosaminoglycan (Fmod) glycosaminoglycan chains. Currently it is not known what region of these small proteoglycans is important for the functions we show they have in the present study. An important next step will be to test the role of the core protein compared to the proteoglycan form (with GAG chains) to see what part of these SLRP molecules is biologically active. In this regard, it's interesting to note that three different families with point mutations in the core protein of in biglycan have bone shortening suggesting the overall importance of the core protein in biglycan function (Cho et al, Am J Hum Genet 2016 98(6):1243-8). Clearly more experiments are needed to fully understand SLRPs mechanism of action requiring a comprehensive and systematic test all the parts of Bgn and Fmod's structure either by gain of function or "knockin" genetic manipulation.

Considering how many SLRPs populate skeletal tissues it is likely there is some overlap in their functions including in the ability to bind and regulate growth factors such as the TGF-beta family members. When we examined either decorin or Fmod single deficient mice we did not see any major bone phenotype so did not pursue the single decorin KO or fmod KO further in this context. With regards to cartilage, the single Bgn KO and Fmod KO both display mild osteoarthritis and because Fmod and Bgn are so highly co-expressed in the cartilage of the condyle we suspected they had overlapping and possible compensatory functions there. By making mice deficient in both Fmod and Bgn we subsequently uncovered new roles for these SLRPS that were masked by a compensation effect. This was evident in the $Bgn^{-/-}Fmod^{-/-}$ which had much earlier and severe OA compared to the single bgn and fmod deficient mice [Ameye et al. 2002]. Because of the nature of the genetic model used we cannot tell whether Bgn or Fmod dominates in the functions we discovered namely SLRP control of BMP-2 induced bone and cartilage turnover.

As mentioned earlier Bgn and Fmod are members of the small leucine-rich repeat proteoglycans. Members of this family have been linked to OA by their action in mediating extracellular collagen organization, mediating TGF-ß signaling and/or regulating subchondral bone turnover (for review see [Ni et al. 2014]). Therefore, decreased expression of Bgn and Fmod may be a biomarker for the early stages of TMJ-OA and/or potentially indicate a higher probability of developing TMJ-OA.

The detection of TMJ-OA usually occurs after there is irreversible damage to the joint. Therefore, efforts to detect and prevent and the disease are needed. In our model, there is excessive BMP-2 signaling. Therefore, monitoring the expression of BMP-2, Bgn and Fmod may be beneficial in the early detection of TMJ-OA. Furthermore, manipulating the expression of these proteins may be beneficial in preventing the progression of the disease process.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Abbreviations used in this paper

ECM	extracellular matrix
TMJ-OA	temporomandibular joint osteoarthritis
Bgn	biglycan
Fmod	fibromodulin
WT	wild-type

| **SLRPs** | small leucine-rich proteoglycans |
| **BMP-2** | Bone Morphogenetic Protein 2 |

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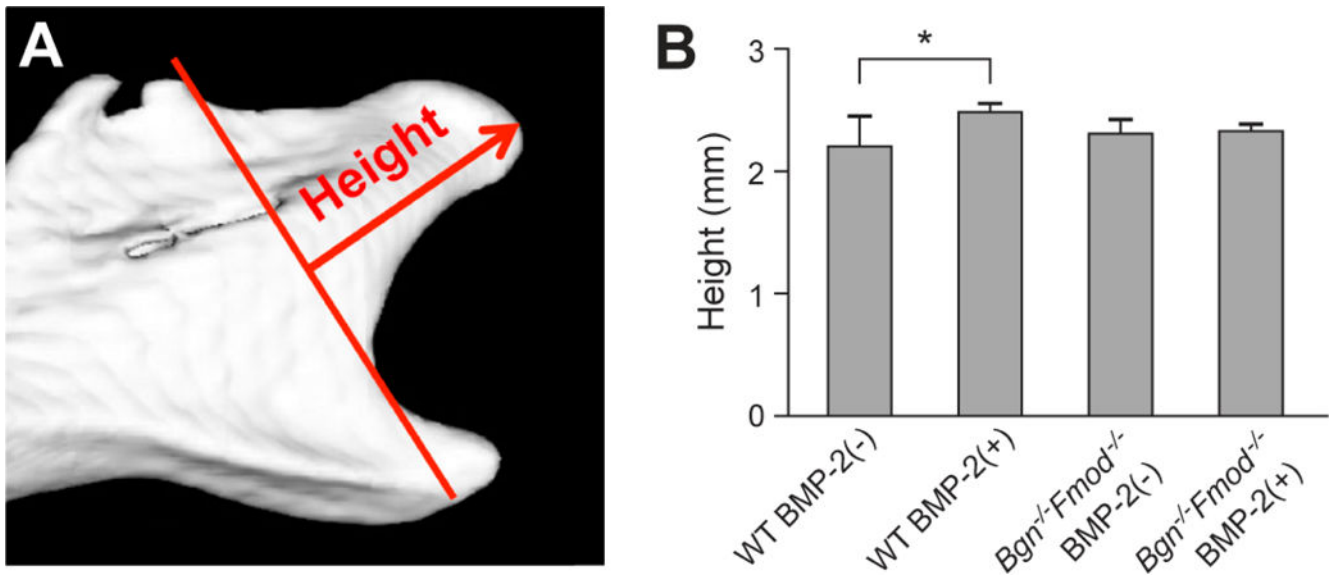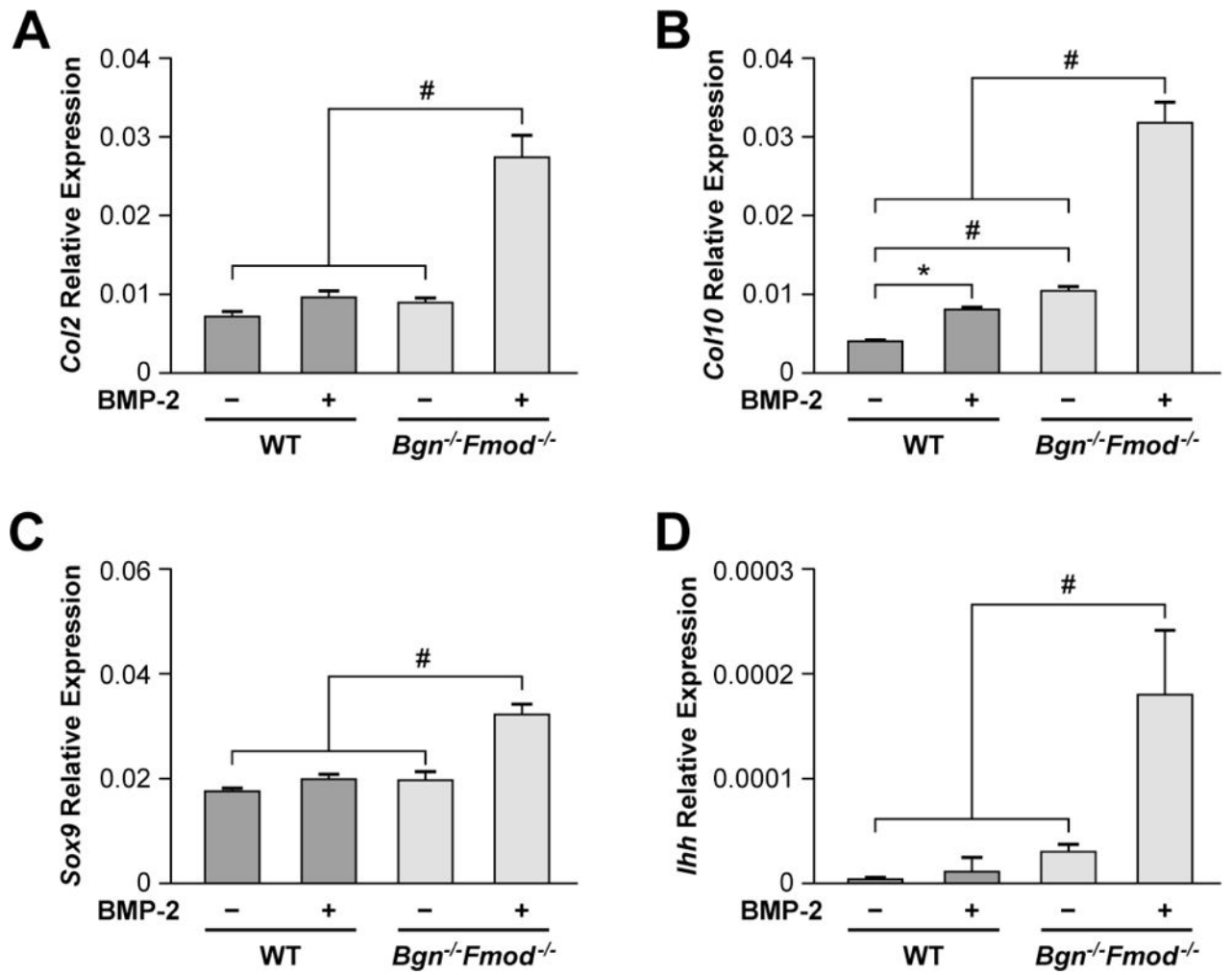
Figure 1.
A) Representative microCT image of a 3 week-old mouse condyles after explant culture indicating the growth regions that were measured. B) Height of the condyles in WT or $Bgn^{-/-}Fmod^{-/-}$ tissue with our without BMP-treatment.
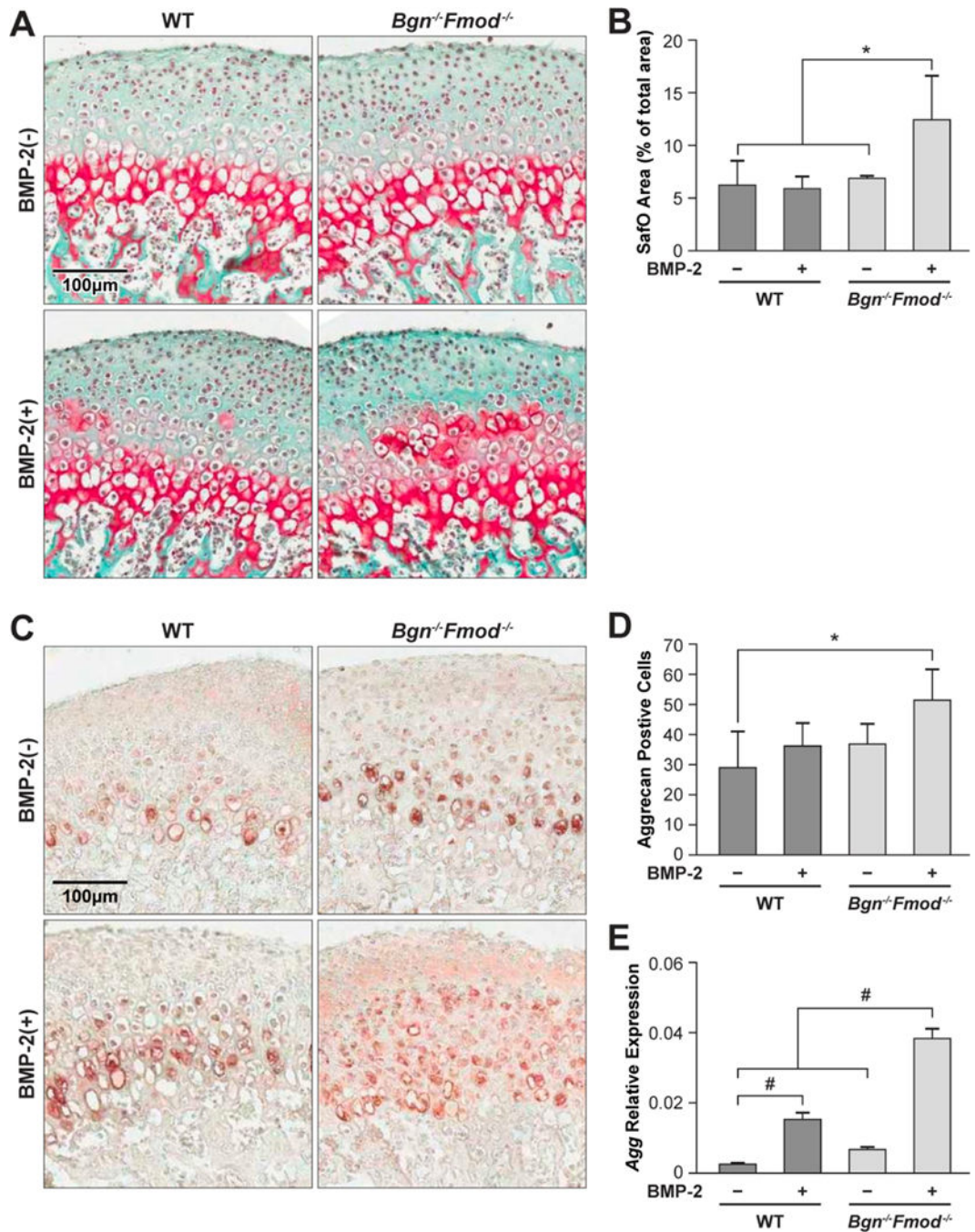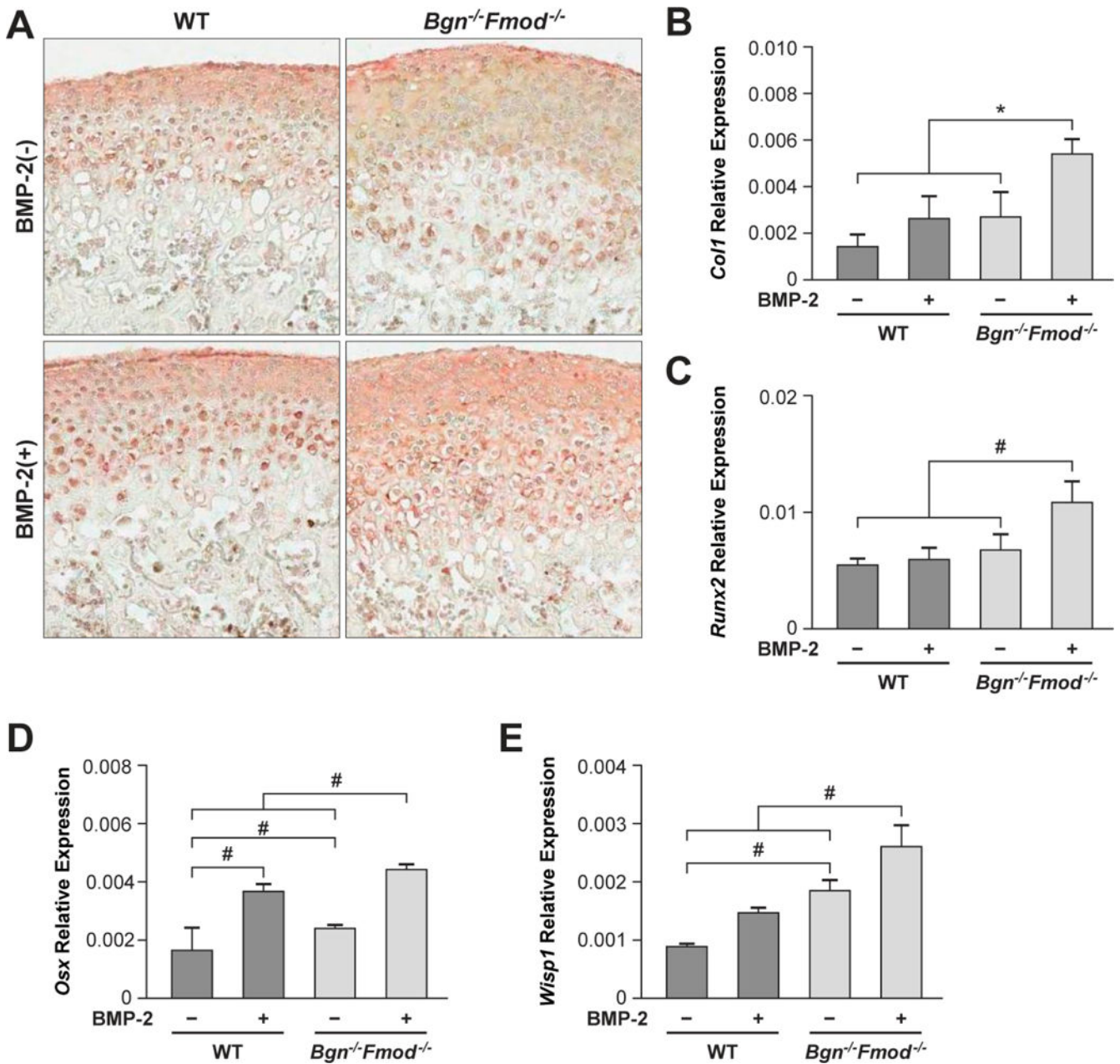
Figure 2.
A) Safranin-O staining of WT (left panels) and *Bgn*$^{-/-}$*Fmod*$^{-/-}$ condyles (right panels) treated without (top panel) or with (bottom panels) BMP-2. B) Histomorphometic quantitation of safranin-O positive area/total area. C) Immunohistochemistry for aggrecan (Acan) in WT (left panels) and *Bgn*$^{-/-}$*Fmod*$^{-/-}$ condyles (right panels) treated without (top panel) or with (bottom panels) BMP-2 D) histomorphic evaluation of Agan positive cells. E) Relative expression of *Acan* mRNA in WT vs. *Bgn*$^{-/-}$*Fmod*$^{-/-}$ with or without BMP-2. $*p<0.05$, $\#p<0.01$.
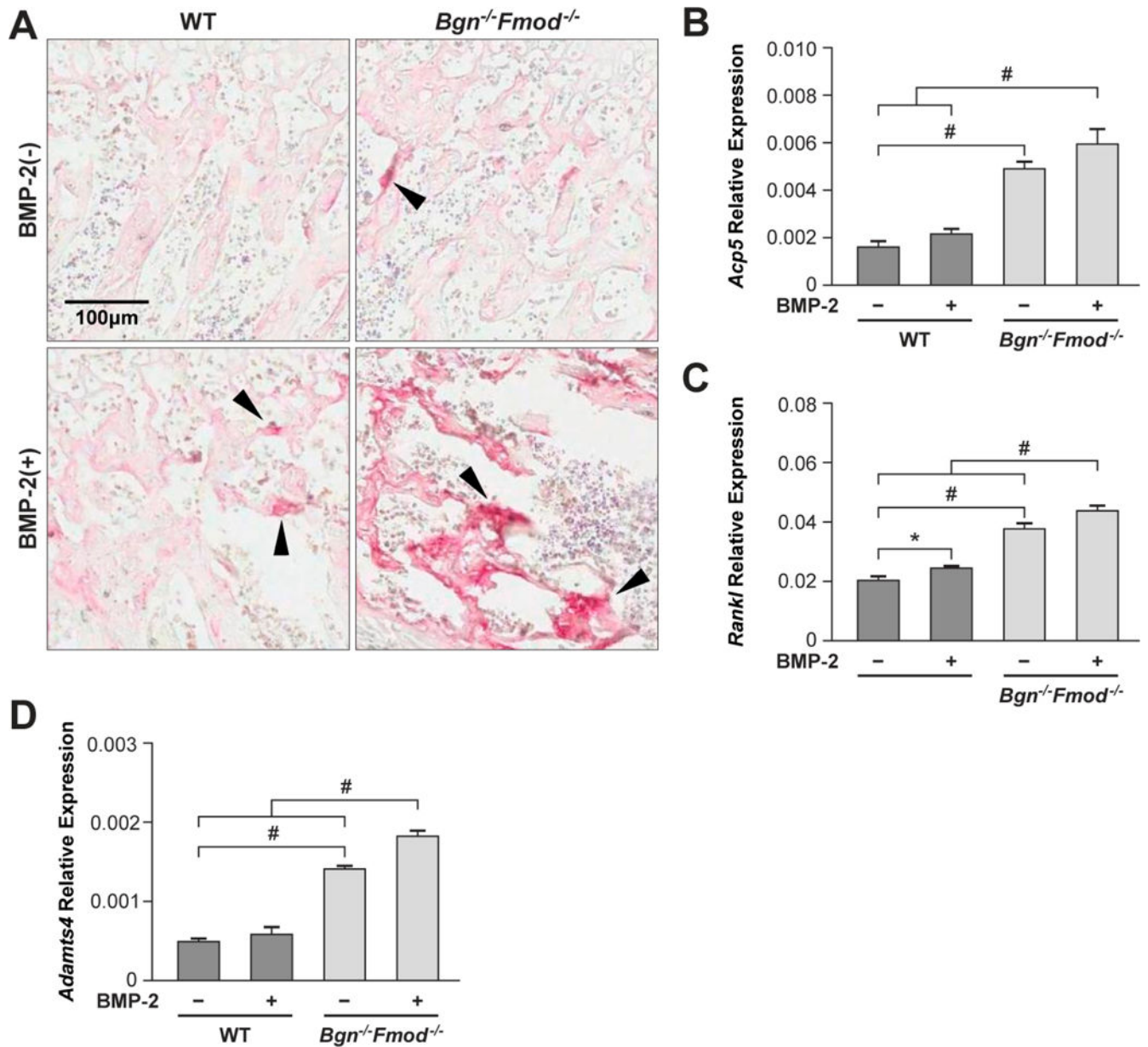
Figure 3.
Real-time PCR of cartilage expressed genes in WT and $Bgn^{-/-}Fmod^{-/-}$ treated without or with BMP-2. A) Type II collagen (*Col2*), B) Type X collagen (*Col10*) C) *Sox9*, D) *ihh*. #*p*<0.01.

Figure 4.
Immunohistochemistry (IHC) and real-time PCR of genes expressed in fibrocartilage and bone. A) IHC of type I collagen in WT (left panels) and *bgn* $^{-/-}$, *fmod* $^{-/-}$ deficient condyles (right panels) treated without (top panel) or with (bottom panels) BMP-2. B) Real-time PCR of type I collagen (*Col1*), C) *Runx2* D) osterix (*Osx*) and E) *Wisp1* mRNA in WT and *Bgn* $^{-/-}$*Fmod* $^{-/-}$ condyles treated with or without BMP-2. *$p<0.05$, #$p<0.01$

Figure 5.
Assessment of bone resorption A) Tartrate Resistant Acid Phosphatase (TRAP) staining of osteoclasts in WT (left panels) and $Bgn^{-/-}Fmod^{-/-}$ deficient condyles (right panels) treated without (top panel) or with (bottom panels) BMP-2. Real-time PCR to measure levels of B) TRAP (*Acp5*) C) *Rankl* D) and AdamTS4 mRNA.