Excess BMP Signaling in Heterotopic Cartilage Forming in *Prg4*-null TMJ Discs

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Abstract

Heterotopic cartilage develops in certain pathologic conditions, including those affecting the human temporomandibular joint (TMJ), but the underlying molecular mechanisms remain obscure. This is in part due to the fact that a reliable animal model of such TMJ diseases is not available. Here, we show that aberrant chondrocyte differentiation and ectopic cartilage formation occur spontaneously in *proteoglycan 4* (*Prg4*) mutant TMJ discs without further invasive procedure. By 2 mo of age, mutant disc cells displayed chondrocyte transdifferentiation, accompanied by strong expression of cartilage master gene *Sox9* and matrix genes *aggrecan* and *type II collagen*. By 6 mo, heterotopic cartilage had formed in the discs and expressed cartilage hypertrophic markers *Runx2* and *ColX*. The ectopic tissue grew in size over time and exhibited regional mineralization by 12 mo. Bone morphogenetic protein (BMP) signaling was activated with the ectopic chondrogenic cells and chondrocytes, as indicated by phosphorylated Smad 1/5/8 nuclear staining and by elevated expression of *Bmp2*, *Bmpr1b*, *Bmpr2*, and BMP signaling target genes. Likewise, we found that upon treatment with recombinant human BMP 2 in high-density micromass culture, mutant disc cells differentiated into chondrocytes and synthesized cartilage matrix more robustly than control cells. Importantly, a specific kinase inhibitor of BMP receptors drastically attenuated chondrogenesis in recombinant human BMP 2–treated mutant disc cultures. Unexpectedly, we found that *Prg4* was expressed at joint-associated sites, including disc/muscle insertion and muscle/bone interface, and all these structures were abnormal in *Prg4* mutants. Our data indicate that *Prg4* is needed for TMJ disc integrity and function and that its absence leads to ectopic chondrogenesis and cartilage formation in conjunction with abnormal BMP signaling. Our findings imply that the BMP signaling pathway could be a potential therapeutic target for prevention or inhibition of ectopic cartilage formation in TMJ disease.

Keywords: articular disc, temporomandibular joint, TMJ disorder, lubricin, ectopic cartilage, joint lubrication

Introduction

The articular disc is 1) a fibrocartilagenous tissue located between the mandibular condyle and glenoid fossa in the temporal bone, 2) a major component of the temporomandibular joint (TMJ), and 3) essential for joint movement. The disc has important roles in shock absorbance, load distribution, protection of articular cartilage and joint lubrication, and overall TMJ function (Tanaka et al. 2008). Given the multifaceted demands imposed onto the TMJ during mastication, the disc is constantly exposed to considerable biomechanical stress and load. Indeed, anatomic and structural changes in the disc, including disc displacement, perforation, or malformations, significantly increase the risk and incidence of TMJ disorders (Scrivani et al. 2008).

The disc is composed of fibroblasts and chondrocyte-like cells (fibrochondrocytes) and is commonly distinguished into 3 regions along the anteroposterior axis: anterior band, intermediate zone, and posterior band (Molinari et al. 2007). Because fibroblasts predominate, their characteristic type I collagen is more abundant than other collagens, and type II collagen is present in the pericellular matrix of chondrocyte-like cells. Within the disc regions, there is spatial variability in cellular and matrix composition, and changes in these

parameters are observed with age and in TMJ pathologies (Molinari et al. 2007; Lu et al. 2009). For example, calcium content and/or calcification of TMJ discs increases progressively with aging, suggesting that TMJ disc degenerative changes take place as a result of deranged mechanical stress (Jibiki et al. 1999; Takano et al. 1999). In other pathologic conditions, patients with juvenile idiopathic arthritis or chronic TMJ disorders and those undergoing TMJ surgery can display

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ectopic chondrocyte differentiation and cartilage formation within the TMJ's synovial space or soft tissue (Lindqvist et al. 1992; Luz et al. 2003; Ringold et al. 2011). In such conditions, patients have limited TMJ mobility, and their facial development can be compromised (Shibuya et al. 2003; Jensen et al. 2010). Therefore, it is important to define the cellular and molecular mechanisms underlying these conditions and, in particular, heterotopic cartilage formation that could in turn provide insights into prevention or treatment.

Proteoglycan 4 (Prg4) encodes superficial zone protein/lubricin, megakaryocyte-stimulating factor, and other secreted products (Hui et al. 2012). Lubricin is synthesized by synovocytes and articular cartilage surface cells and is secreted into the joint space (Flannery et al. 1999; Schmidt et al. 2007; Coles et al. 2010). It contains an extensive mucin-like region rich in O-linked oligosaccharides and is thought to be responsible for boundary lubrication by itself or together with other lubricants during joint movement (Swann et al. 1985; Seror et al. 2015). *PRG4* loss-of-function mutations in humans cause camptodactyly-arthritis-coxa-vara-pericarditis syndrome (CACP; Bahabri et al. 1998; Marcelino et al. 1999). The limb joints in CACP patients are characterized by noninflammatory hyperplasia and fibrosis of the synovial membrane (Bahabri et al. 1998). Importantly, altered lubricin levels have been associated with osteoarthritis and rheumatoid arthritis (Elsaid et al. 2008; Ungethuen et al. 2010). We and others recently reported that mice lacking *Prg4* exhibit 1) hyperplasia of articular cartilage of glenoid fossa and condyle, 2) disappearance of superficial cells, 3) abnormal protein deposition, and 4) articular cartilage degradation (Hill et al. 2014; Koyama et al. 2014). *Prg4*-null TMJ discs were also severely deranged, lost their characteristic concave shape, and importantly, exhibited increased chondrocytic cell differentiation by as early as 3 mo (Koyama et al. 2014).

Bone morphogenetic protein (BMP) signaling has well-established roles in endochondral bone formation during skeletal development and growth, and its abnormal activation causes heterotopic cartilage and bone formation (Billings et al. 2008; Huegel et al. 2015; Long and Ornitz 2015). Thus, we hypothesized that BMP signaling could be involved in heterotopic chondrocyte differentiation and cartilage formation in the discs of *Prg4*-null mice. Here we do show that BMP signaling was excessive in heterotopic cartilage forming in *Prg4*-null TMJ discs and that inhibition of BMP signaling blocked the increased responsiveness of mutant disc cells to exogenous recombinant human BMP 2 (rhBMP-2).

Materials and Methods

Mice

Prg4^{-/-} mutant mice were kindly provided by Dr. Matthew Warman (Boston Children's Hospital, Boston, MA, USA; Rhee et al. 2005). Animals were maintained in accordance with the National Institutes of Health's *Guide for Care and Use of Laboratory Animals*, and protocols were approved by the Institutional Animal Care and Use Committee of The Children's Hospital of Philadelphia.

Histologic, Histochemical, Immunohistochemical, Immunocytochemical, and In Situ Hybridization Analyses

Age-matched *Prg4* mutants and wild-type littermates were evaluated at postnatal day 14 (2 wk, $n = 6$), 2 mo ($n = 12$), 4 mo ($n = 6$), 6 mo ($n = 12$), 12 mo ($n = 6$), and 15 mo ($n = 4$) of age. Mice were fixed with 4% paraformaldehyde overnight, decalcified for 2 wk in 10% ethylenediaminetetraacetic acid (EDTA) / 2% paraformaldehyde, dehydrated, and embedded in paraffin. Immunostaining for phosphorylated Smad 1/5/8 (pSmad1/5/8) was carried out with paraffin sections. After deparaffinization, antigen retrieval was performed in citrate buffer (pH 6.0) by boiling for 10 min and then incubating in citrate buffer at room temperature for 10 min. Endogenous peroxidase was blocked by incubation with 3% H₂O₂ for 10 min. Sections were treated with Tween-20 in phosphate-buffered saline 1% bovine serum albumin for blocking and were incubated with a rabbit pSmad1/5/8 antibody (1:100; Cell Signaling, Beverly, MA, USA) overnight at 4 °C. Following rinsing, sections were then incubated with biotinylated goat anti-rabbit secondary antibody, and the signal was visualized with a horseradish peroxidase / DAB detection immunohistochemistry kit according to the manufacturer's instructions (Abcam Plc., Cambridge, UK). Companion serial sections were treated together, except for inclusion of primary antibodies, and served as negative controls.

For in situ hybridization, sections were hybridized with antisense or sense ³⁵S-labeled probes as described in detail previously (Koyama et al. 2007).

Micromass Cell Culture

Micromass cultures were prepared from the disc cells of 2-month-old wild-type and *Prg4* mutant mice described previously (Asai et al. 2014) with some modification. Isolated articular discs were incubated with 2.5 U/mL of Dispase (MP Biomedicals, LLC, Solon, OH, USA) and 600 U/mL (3 mg/mL) of type I collagenase (Worthington Biochemical Corporation, Lakewood, NJ, USA) in Hank's balanced salt solution with Ca²⁺ and Mg²⁺ for 30 min at 37 °C with agitation. The dissociated cells were grown on collagen 1 substrate-coated 6-well culture plates in Dulbecco's Modified Eagle's Medium containing 10% fetal bovine serum and antibiotics. The cultures were passaged 3 to 5 times prior to use in experiments.

Micromass cultures were initiated by spotting 20 µL of the cell suspensions (4×10^5 cells) onto collagen 1 substrate in 24-well tissue culture plates. The cultures were grown for 10 d in Dulbecco's Modified Eagle's Medium containing 10% fetal bovine serum and supplemented with rhBMP-2 (R&D Systems, Minneapolis, MN, USA) at a concentration of 100 to 200 ng/mL and in absence or presence of the BMP signaling inhibitor LDN-193189, which selectively blocks the transcriptional activity of the BMP type 1 receptor kinases (Selleckchem, Houston TX, USA; Cuny et al. 2008) at a concentration of 0.2 or 0.4 µM. Day 10 cultures were fixed and stained with alcian blue (pH 1.0), and the integrated density of alcian blue-stained cultures was semiquantified with ImageJ.

Results

Prg4 Expression in Discs and Heterotopic Chondrogenic Differentiation in Prg4-null Discs

Little is known about possible links between chondrogenic transdifferentiation and *Prg4* expression in TMJ discs, and such information could provide important insights into heterotopic cartilage formation in certain TMJ diseases. To this end, we further examined the expression patterns of *Prg4* expression and the structural and phenotypic changes occurring in *Prg4*-null discs over time. Parasagittal (Fig. 1A) and frontal (Fig. 1C) disc sections were prepared, and the histologically identified disc regions are schematically depicted in Figure 1B and D. Chondrocyte-like cells were assessed by a round shape and *type II collagen* (*Col2a1*) gene expression. In 2-wk- and 2-mo-old discs, flat and compacted fibroblasts predominated in all regions, while few if any chondrocyte-like cells were present in anterior (Fig. 1E, F), posterior (Fig. 1M, N), and intermediate zones (Fig. 1I, J). By 6 mo of age, the chondrocyte-like cells became appreciable in the central portion of anterior/posterior bands (Fig. 1G, O, arrowheads), while fibroblasts lined the disc surfaces (hereafter referred to as *disc-lining cells*). *Prg4* transcripts were present in disc-lining cells as early as at 2 wk of age and continued to be present over time (Fig. 1Q–S, arrow). Interestingly, *Prg4* expression became detectable in chondrocyte-like cells at 6 mo (Fig. 1S, arrowhead). In *Prg4*-null discs, however, chondrocyte-like cells were already appreciable at 2 mo, were present throughout (Fig. 1H, L, P), and increased in number with age (Fig. 1U–W). Importantly, there were almost no detectable disc-lining cells in 2-mo-old mutants (Figs. 1H, L, P), and some unknown material coated the tissue surface. In situ hybridization for *Prg4* with *Prg4*-null sections produced no signals, indicating specificity of signal (Fig. 1T).

Molecular Evaluation of Heterotopic Cartilage Formation in Prg4-null Discs

Next, we characterized in greater detail the process of ectopic chondrogenesis in mutant discs. RNAs isolated from 2-mo-old control and *Prg4*-null discs were processed for quantitative polymerase chain reaction (PCR; Fig. 2A, B). *Sox 9*, a key transcription factor regulating early chondrocyte differentiation, was upregulated in mutant versus wild-type discs, as were the cartilage characteristic genes *aggrecan* and *Col2a1* (Fig. 2A, B). Similar trends were seen with semiquantitative PCR (Fig. 2C). Importantly, *Runx2*, a key transcription factor for chondrocyte hypertrophy and endochondral ossification, was slightly increased in mutants and accompanied the increased expression of *type X collagen* (*ColX*), a marker for chondrocyte hypertrophy. To verify data, we performed in situ hybridization to assess the topographic expression of *Sox-9*, *Col2a1*, and *aggrecan* (Fig. 2E, G, I, K). Safranin O/fast green staining clearly revealed that the medial region of mutant discs contained ectopic cartilage (Fig. 2I [arrowheads], N) composed of chondrocytes expressing *Sox9*, *aggrecan*, *Col2a1*, and *ColX*

(Fig. 2I, K; Appendix Fig. 2). Heterotopic cartilage also developed in the glenoid fossa, and the medial synovial membrane appeared elongated (Fig. 2N, double arrowhead and arrow, respectively). Alizarin red staining revealed regional mineralization in the ectopic cartilage (Fig. 2N, O, arrowheads).

Aberrant Canonical BMP Signaling Is Associated with Heterotopic Cartilage Formation

We next sought to clarify mechanisms underlying ectopic cartilage formation in mutant discs. BMP signaling is well known for its positive and active roles in chondrogenesis and endochondral ossification in normal and pathologic conditions (Billings et al. 2008; Long and Ornitz 2015). To determine whether ectopic BMP signaling was involved in ectopic cartilage formation in *Prg4*-null discs, we examined expression of BMP and their receptors in 2-mo- and 6-mo-old control and mutant discs (Fig. 3A, B). PCR showed that *Bmp2*, *Bmpr1b*, and *Bmpr2* were all upregulated in 6-mo-old mutants relative to respective controls (Fig. 3B, C). The BMP signaling downstream mediator inhibitor of differentiation 1 (*Id-1*) was also upregulated in mutants, confirming increased Bmp signaling (Fig. 3B, C). In situ hybridization showed that *Bmp2* transcripts were particularly clear in chondrocyte-like cells surrounding the presumptive cartilaginous nodules (Fig. 3E). pSmad1/5/8 immunostaining was quantified as fraction of nuclear-stained chondrocyte-like cells in anterior and intermediate zones, and significant differences were observed between control and *Prg4*-null discs (Fig. 3F, G, I, J; Appendix Fig. 3A). Increased activation of BMP signaling in mutant discs was also evident by Western blotting (Appendix Fig. 3B). Sections in which primary pSmad1/5/8 antibody was omitted displayed no signal, validating specificity of immunostaining data (Fig. 3H, K).

Inhibition of BMP Signaling Blocks Chondrogenesis in Micromass Culture of Prg4-null Disc Cells

The data above indicate that increased BMP signaling is associated with heterotopic chondrogenesis and cartilage formation in mutant discs. Thus, we asked how mutant disc cells would behave in culture with regard to chondrogenic differentiation and whether they would respond to a BMP antagonist. Disc cells were first isolated from control 2-mo-old mice, reared in culture, treated with rhBMP2, and monitored for pSmad1/5/8 nuclear translocation for 10, 30, and 60 min. Immunocytochemistry showed a significant and rapid increase of pSmad1/5/8 nuclear translocation in rhBMP-2-treated as compared with untreated cells (Fig. 4A, B). To analyze chondrogenesis, the cells were seeded in high-density micromass culture (20×10^6 cells/mL) and treated with rhBMP2 (0 to 200 ng/mL). Staining with alcian blue on day 10 showed that exogenous rhBMP2 had induced chondrogenesis in wild-type cell cultures (Fig. 4C, D). As expected, disc cells from *Prg4* mutant mice in companion micromass cultures appeared to undergo more exuberant

Figure 1. Temporospatial *Prg4* expression in wild-type discs and aberrant differentiation of chondrocyte-like cells in *Prg4*^{-/-} discs. Diagram (B, D) illustrating respective disc portions from parasagittal (A) and frontal sections (C) of temporomandibular joints (TMJs). TMJs from mice were analyzed by histology: 2-wk-old (E, I, M, Q), 2-mo-old (F, J, N, R), and 6-mo-old (G, K, O, S) control and 2-mo-old *Prg4*^{-/-} (H, L, P, T). In situ hybridization with isotope-labeled riboprobes for *Prg4* expression (Q–T). Note that *Prg4* is expressed in disc-lining cells (arrows) and chondrocyte-like cells (arrowheads). Chondrocyte-like cells were counted in anterior (Ant) band, intermediate (Int) zone, and posterior (Post) band in distinct TMJ sections from control and *Prg4*-null mice at ages of 2, 6, and 12 mo (U–W; cell number is present per approximately 100 cells in anterior and posterior bands and approximately 50 cells in intermediate zone). Areas were randomly selected from 5 to 8 sections per sample ($n = 3$ for mouse/each group; $*P < 0.05$, $**P < 0.01$) and presented as average $\pm$ SD. Scale bars: 250 μ m in A, also for C, T; 65 μ m in E, also for F–S. ab, anterior band; cd, condyle; dc, disc; gf, glenoid fossa; iz, intermediate zone; la, lateral part; lpm, lateral pterygoid muscle; me, medial part; pb, posterior band; WT, wild type.

Figure 2. Increased expression of chondrocyte markers in *Prg4*^{-/-} discs. Histograms depicting the relative expression of Sox9, aggrecan (*Acan*), and *Col2a1* in *Prg4*^{-/-} and control discs at 2 mo (A) and 6 mo (B) of age and presented as average $\pm$ SD ($n = 3$ for mouse/each group, * $P < 0.05$, ** $P < 0.01$). Semiquantitative reverse transcription polymerase chain reaction reveals increased expression of early (*Sox9*, *Acan*, and *Col2a1*) and maturing chondrocyte (*Runx2* and *ColX*) markers in *Prg4*^{-/-} discs as compared with control littermates at age of 6 mo (C). Frontal sections prepared from TMJs and discs from 6-mo-old (D–G), 12-mo-old (H–K), and 15-mo-old (L–O) control (D, F, H, J, L, M) and *Prg4*^{-/-} (E, G, I, K, N, O) mice were processed for in situ hybridization with isotope-labeled riboprobes for *Sox9* (D, E, arrowhead), *Col2a1* (F, G, J, K, arrowhead), and *Acan* (H, I). Mutant discs were delineated by green dashed lines (E, G). Sections were also stained with safranin O (Saf-O) / fast green (Fg; H, I, L, N) and alizarin red S (M, O) and evaluate proteoglycans and/or mineralization in discs, respectively. Safranin O–stained cartilage (I, bright field, arrowheads) was composed of *Col2a1*- and *Acan*-expressing chondrocytes (I, K, dark field, arrowheads). Alizarin red S–stained mineralized tissues were detected in the mesial and lateral portions of the mutant discs (O, arrowheads). Note also the massive safranin O–stained cartilage developed in the mutant glenoid fossa as compared with control (L, N, double arrowhead, respectively) and the elongated medial synovial membrane in mutants (L, N, arrow). Scale bars: 1.2 mm in D, also for E–K, M, O; 2.5 mm in L, also for N. cd, condyle; dc, disc; gf, glenoid fossa; H&E, hematoxylin and eosin; WT, wild type.

Figure 3. Increased bone morphogenetic protein (BMP) signaling in *Prg4*^{-/-} discs. Histograms depicting the relative expression of *Bmp2*, *Bmpr1b*, and *Bmpr2* in *Prg4*^{-/-} and control discs at age of 2 mo (A) and 6 mo (B) and presented as average $\pm$ SD ($n = 3$ for mouse/each group, * $P < 0.05$, ** $P < 0.01$). Semiquantitative reverse transcription polymerase chain reaction showing that increased expression of *Bmp2*, *Bmp* type I and type II receptors, and *Id-1* in discs from *Prg4*^{-/-} mice as compared with wild type (WT) at age of 6 mo (C). TMJ discs from 4-mo-old control (D, F, I) and *Prg4*^{-/-} (E, G, H, J, K) mice were analyzed by in situ hybridization with isotope-labeled riboprobes for *Bmp2* (D, E) or by immunohistochemistry with phosphorylated Smad 1/5/8 (pSmad1/5/8) antibodies. Sections not treated with primary antibodies served as control (H, K). Note increased *Bmp2* expression (E; arrowheads) and pSmad1/5/8-nuclear translocation (G, J; arrowheads) are detected in chondrocyte-like cells in the anterior band (Ant. Band) and the intermediate zone (Int. Zone) in *Prg4*^{-/-} discs. Scale bars: 90 μ m in D, also for E; 55 μ m in F, also for G–K.

chondrogenesis in response to exogenous rhBMP-2 (Fig. 4E, F). Note that chondrogenesis in control and mutant cell cultures was low in the absence of rhBMP2. To determine whether chondrogenesis in control and mutant cultures could

be counteracted with a BMP signaling inhibitor, cultures were treated continuously with 0.2 μ M and 0.4 μ M LDN-193189. Alcian blue staining (Fig. 4G, H) and expression analysis of cartilage transcription factors and chondrogenic markers

Figure 4. Chondrogenic potential of *Prg4*^{-/-} disc cells stimulated by recombinant human bone morphogenetic protein 2 (rhBMP-2) and attenuated by bone morphogenetic protein (BMP) inhibitor. Disc cells from 2-mo-old wild-type (WT) mice were plated on coverslips and grown in serum-starved Dulbecco's modified Eagle's medium for 12 h. Cultures were then stimulated with rhBMP2 for 30 min, fixed, and incubated with a rabbit phosphorylated Smad 1/5/8 (pSmad1/5/8) primary antibody followed by anti-rabbit Alexa Fluor 488 (green), with rhodamine phalloidin for staining the actin cytoskeleton in cells (red; **A**, upper panel). Culture without rhBMP2 treatment were served as controls (**A**, lower panel). DAPI stains were used to detect nuclei (blue). Images of pSmad1/5/8 (green) or DAPI (blue) were merged with rhodamine phalloidin-stained cytoskeletal images (red). Disc cells with pSmad1/5/8 in the nucleus were counted in randomly selected 6 to 8 areas (approximately 15 to 20 cells/area; $n = 5$, $**P < 0.01$) and presented as average $\pm$ SD (**B**). Day 10 micromass cultures of WT disc cells stimulated with rhBMP-2 (100 or 200 ng/ml) exhibited increased alcian blue staining compared with control cultures (**C**, upper panel; $n = 4$). Micromass cultures were also counterstained with hematoxylin (Htx) to reveal the presence of cells in cultures (**C**, lower panel). Integrated density of alcian blue-stained cultures was measured (**D**; $n = 4$, $*P < 0.05$). Day 10 micromass cultures of *Prg4*^{-/-} disc cells stimulated with rhBMP-2 (100 ng/ml; **E**, upper panel) revealed increased alcian blue staining compared to WT disc cells (**E**, lower panel). Htx counterstaining (**E**, lower panel). Integrated density of alcian blue-stained cultures were measured (**F**; $n = 4$, $*P < 0.05$). Micromass cultures of *Prg4*^{-/-} disc cells were treated with rhBMP-2 (100 ng/ml) alone or rhBMP-2 (100 ng/ml) / LDN-193189 (BMP inhibitor) at indicated concentration (**G**). Alcian blue staining (**G**, upper panel) and Htx counterstaining (**G**, lower panel). Integrated density of alcian blue-stained cultures was measured (**H**; $n = 4$, $*P < 0.05$). Histograms depicting relative expression of chondrocyte makers, *Sox5*, *Sox6*, *Sox9*, and BMP target were decreased in BMP inhibitor-treated cultures as compared with controls (**I**; $n = 3$, $*P < 0.05$, $**P < 0.01$). Semiquantitative reverse transcription polymerase chain reaction revealed decreased expression of chondrocyte markers, *Bmp2*, *Bmp* receptors, and *Runx2* in *Prg4*^{-/-} discs as compared with controls (**J**). Graphs depict mean $\pm$ SD. Control data were set as 1 (**D**, **F**, **H**, **I**).

showed that the antagonist did in fact reduce chondrogenesis (Fig. 4I). The data were further affirmed by semiquantitative PCR, revealing that *Bmp2*, *Bmpr1b*, *Bmpr2*, and *Runx2* were all decreased (Fig. 4J).

Defective Structures at Nonarticulation Sites in *Prg4*-null TMJs

We further investigated the relationship between *Prg4* expression and abnormalities of TMJ soft tissues in mutant mice, with particular attention to the tissue-tissue interface where *Prg4* action may be required. We found that *Prg4* transcripts were also detected in TMJ-associated tissues, including disc-terygoid muscle interface (Fig. 5A) and pterygoid muscle/tendon-condylar neck insertion site (Fig. 5B). In good correlation, *Prg4*-null TMJs showed characteristic defective structures in those sites; in particular, the disc fibers lost a smooth insertion into the muscles (Figs. 5D, arrowheads), and ectopic cartilage had formed in the muscle/tendon insertion site into the condyle (Fig. 5F, arrowheads).

Discussion

Here, we report that heterotopic cartilage develops spontaneously in *Prg4*-null discs without additional insult or invasive surgical intervention. The expression levels of *Bmp2*, *BMP type I* and *type II receptors*, and *Id-1* were all significantly and concurrently increased in mutant discs, indicative of abnormal activation of BMP signaling. Consistent with this notion, mutant discs displayed an increased number of chondrocytic cells and chondrocytes concomitant with pSmad1/5/8-nuclear staining. In good correlation, we found that mutant disc cells in micromass cultures were more responsive to rhBMP2 treatment than control cells were, as indicated by cartilage matrix accumulation. Importantly, a BMP signaling inhibitor attenuated chondrogenesis in control and mutant cultures. Taken together, the data indicate that the mutant disc cell population is more prone than control cells to undergo chondrogenic cell differentiation in conjunction with enhanced BMP signaling. The data also suggest that the *Prg4*-null mice are a useful model for the study of TMJ heterotopic ossification and that

by absence of *Prg4* action and involvement of abnormal BMP signaling. (G) Model of increased chondrogenesis and subsequent cartilage formation in *Prg4* mutant discs. TMJ appears to develop normally, and the disc separates the joint space into the upper and lower joint cavities. With further development, the disc displays a concaved shape; the disc-lining cells cover the surface of the disc; and a few chondrogenic cells and chondrocytes differentiate in the anterior and posterior bands in wild-type disc (with *Prg4* action). In sharp contrast, in *Prg4* mutant mice (without *Prg4* action), the disc becomes much thicker, and this is accompanied by increased chondrocyte number and ectopic cartilage formation throughout the disc. In addition, the lateral pterygoid muscle loses its smooth integration into the disc. The increased chondrogenesis and subsequent cartilage formation throughout the disc are accompanied by activation of Bmp signaling and increased expression of *Sox9* and *Runx2*. Scale bars: 120 μ m in A, also for E, F; 60 μ m in B, also for C, D. cd, condyle; dc, disc; lpm, lateral pterygoid muscle; po, periosteum.

Figure 5. *Prg4* expression at nonarticulation sites and tissue derangement in *Prg4*^{-/-} mice. Parasagittal sections of TMJs from 12-mo (A, B) and 15-mo (C–F) control (A, B, C, E) and *Prg4*^{-/-} (D, F) mice were processed for histology and in situ hybridization with isotope-labeled riboprobes for *Prg4* expression (A, B). Sections were stained with safranin O/fast green (Saf-O/Fg; B), Masson's trichrome (M'sT; C, D), and hematoxylin and eosin (H&E; E, F). Note that *Prg4* is expressed at disc/lateral pterygoid muscle junction (A, arrowheads) and muscle/tendon insertion site into the mandibular condyle (B). Note also the disrupted muscle insertion into the articular disc in mutant (D, arrowheads) as compared with control (C, arrowheads) and ectopic cartilage formation at muscle/tendon insertion site into the mandibular condyle in mutant (F, arrowheads). (G) Model of heterotopic ossification in TMJs caused

Prg4 is normally needed for postnatal TMJ disc integrity and function.

Our findings clearly show that wild-type TMJ discs dynamically undergo remodeling with age, as observed in discs in humans and other animals (Minarelli and Liberti 1997; Berkovitz and Pacy 2000). Chondrocyte-like cells preferentially differentiate in anterior and posterior bands, likely representing an adaptive response of disc cells to the demands of TMJ function. The results correlate well with analysis of biomechanical properties that were found to change significantly in terms of compressive modulus in those sites with age (Wang et al. 2014). Interestingly, we find that these chondrocyte-like cells/chondrocytes in discs of aged mice start to express *Prg4*, indicating that the interface between these cells may need lubricating action for proper TMJ function. Prior studies have shown an increased coefficient of friction in loaded joints from *Prg4*-null mice (Jay et al. 2007; Drewniak et al. 2012). Therefore, abnormal friction could be one of major culprits for aberrant chondrocyte differentiation and subsequent cartilaginous tissue formation in discs as well as glenoid fossae and condyles. Given our finding from this and previous work (Shibukawa et al. 2007; Koyama et al. 2014), we propose the following abnormal modeling process in the TMJs of *Prg4*-mutants (Fig. 5G). The disc primordium first becomes apparent once the superior and inferior articular cavities are created by early postnatal life. Soon after, the mutant disc cells show increased cell proliferation throughout the disc, leading to disc hypertrophy. In conjunction with the deformation of articular cartilage of glenoid fossa and condyle, the disc could be subjected to abnormal mechanical stimuli, making the cells more prone to differentiate into chondrocytes. In such processes, aberrant BMP signaling could be activated and would induce heterotopic cartilage and bone formation.

We utilized high-density micromass cultures to analyze the chondrogenic potentials of cells isolated from the fibrocartilaginous disc of TMJs (Hayes and Ralphs 2011). We show here that cells isolated from wild-type discs were able to undergo chondrogenesis but that cells isolated from *Prg4*-mutant discs displayed higher chondrogenic potential. Interestingly, proteoglycan accumulation in wild-type cultures was relatively low but increased substantially after rhBMP-2 treatment (Fig. 4C, D). The data indicate that disc cells are able to retain their chondrogenic potentials during culture expansion and can undergo chondrogenesis in response to BMP signaling. The more exuberant chondrogenesis that we observe in mutant cell cultures in response to exogenous rhBMP-2 (Fig. 4E, F) may reflect the possibility that mutant cultures contain a more numerous population of chondroprogenitor and/or chondrocytic cells.

Our data clearly indicate that a characteristic BMP ligand-receptor signaling axis operates in the *Prg4*-mutant discs. We find in mutant TMJ discs at 6 mo of age that expression levels of *Bmp2*, *Bmpr1b*, and *Bmpr2* are about 3-fold higher than controls. Previous studies have shown that BMP type I receptors possess distinct biological functions during skeletal development. Intriguingly, expression of dominant-negative *Bmpr1b*

displayed more severe skeletal defects when compared with *Bmpr1a*, implying that *Bmpr1b*-mediated BMP signaling is essential for early chondrogenesis (Chen et al. 1998). In agreement with previous studies, our results show that blocking BMP signaling can markedly decrease the expression of *Bmpr1b* and *Bmp2*, resulting in decreased proteoglycan synthesis and accumulation and decreased chondrocyte differentiation. Our findings imply that the BMP signaling pathway could be a potential therapeutic target for prevention and/or treatment for ectopic chondrocyte differentiation and/or cartilage formation in affected TMJ discs.

Our histomorphometric observations and gene expression analyses clearly indicate that mutant discs in juvenile mice contain more chondrogenic cells than those in age-matched controls. In our mouse model, however, ectopic cartilage formation appears to be slower than that seen in discs subjected to surgical intervention (Embree et al. 2015). Therefore, our model may represent a chronic pathologic process of heterotopic ossification in discs. Note that there are no apparent inflammatory signs in synovial joints in CACP patients (Rhee et al. 2005). It is therefore intriguing to ask if there are any links between the acceleration of heterotopic cartilage formation and inflammatory response in synovium and other TMJ sites accompanied by surgical intervention or joint injuries.

Finally, we show that *Prg4* is expressed in nonarticulation sites of TMJ associated tissues: disc/lateral pterygoid muscle (LPM) interface and condylar neck/LPM insertion site. The LPM is a muscle with 2 separate heads, attaches to the antero-medial surface of the TMJ disc and the condylar neck, and coordinates the movement of TMJ components (Juniper 1981). Clinical studies have pointed out relationships among muscle hypertrophy/atrophy, damaged discs and TMJ internal derangement (Dergin et al. 2012; Imanimoghaddam et al. 2013). Our data indicate that loss of *Prg4* could alter the associations of LPM/TMJ disc and LPM/condyle and could compromise TMJ function. Ectopic cartilage also develops in the muscle/bone insertion site. Taken together, our data lead to the intriguing possibility that *Prg4* has previously unsuspected roles in such joint-associated tissues and sites. Further investigations will be necessary to clarify this and other possibilities.

Author Contributions

T.E. Bechtold, C. Saunders, C. Mundy, H. Um, R.S. Decker, I. Salhab, N. Kurio, P.C. Billings, contributed to data analysis, drafted the manuscript; M. Pacifici, H.D. Nah, E. Koyama, contributed to conception and design, critically revised the manuscript. All authors gave final approval and agree to be accountable for all aspects of the work.

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