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

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Appendix

Immunocytochemistry

Immunocytochemistry was performed for the detection of phosphorylated Smad 1/5/8 nuclear translocation. Disc cells from wild-type mice at 2 mo of age were plated onto 3 to 4 coverslips placed in 6-well culture plates at a density of approximately 30,000 cells per well and cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum for 12 h. Cultures were serum depleted (Dulbecco's modified Eagle's medium + 0.1% fetal bovine serum) for 12 h and then stimulated with recombinant human bone morphogenetic protein 2 at a concentration of 100 ng/mL for 10, 30, and 60 min. Cultures were fixed with 2% paraformaldehyde for 20 min, permeabilized in 0.2% Triton X-100 for 10 min, and then blocked with Tween-20 in phosphate-buffered saline / 1% bovine serum albumin. The cultures were incubated with a rabbit phosphorylated Smad 1/5/8 primary antibody (1:100; Cell Signaling, Beverly, MA, USA) for 12 h at 4 °C. The cultures were then incubated with an Alexa Fluor 488–conjugated goat anti-rabbit polyclonal secondary antibody (1:200; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) with rhodamine phalloidin (1:50; Life Technologies, Grand Island, NY, USA) for 1 h at room temperature. The coverslips were mounted with the Vectashield HardSet antifade mounting medium containing DAPI for nuclear counterstain (Vector Laboratories, Inc., Burlingame, CA, USA).

Quantitative and Semiquantitative PCR Analyses

Total RNA was extracted from articular discs from *Prg4* mutants and age-matched wild-type littermates at 2 and 6 mos of age and the day 10 micromass cultures by TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocols, and yields were quantified with a NanoDrop spectrophotometer (Life Technologies). Briefly, 2 μg of total RNA was reverse transcribed with the SuperScript III First-Strand Synthesis System (Life Technologies). Quantitative real-time polymerase chain reaction (PCR) was carried out with the SYBR Green PCR Master Mix (Qiagen, Valencia, CA, USA) and Applied Biosystems 7500 (Life Technologies) according to manufacturers' protocol. *Gapdh* was used as an endogenous control, and relative expression was calculated with the ΔΔCt method. Primer information is presented in the Appendix Table. Semiquantitative PCR was performed to detect the relative abundance of expressed genes in the same cDNA samples prepared for real-time PCR.

In Situ Hybridization

cDNA clones used as templates for probes included: *Sox9* (nt. 116-856; NM_011448), collagen II (*Col II*; nt. 1095-1344; X57982), collagen X (nt. 1302-1816; NM009925), *Bmp2* (nt. 952-1299; XM_006498619), *Prg4* (nt. 41-2646; AB034730), and aggrecan (*Acan*; nt. 880-1733; NM_007424).

Statistical Analysis

Data were validated by 2-tailed Student's *t* tests. The threshold for significance for all tests was set as $P < 0.05$.

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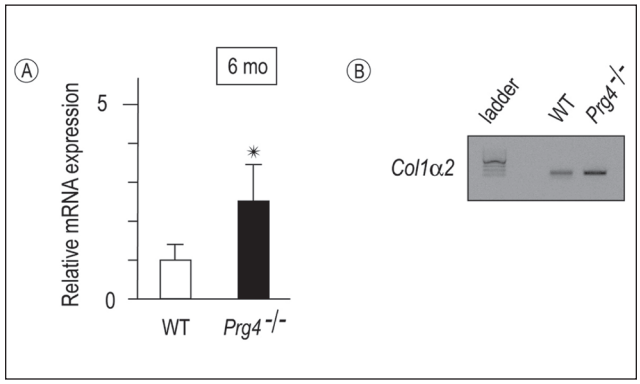
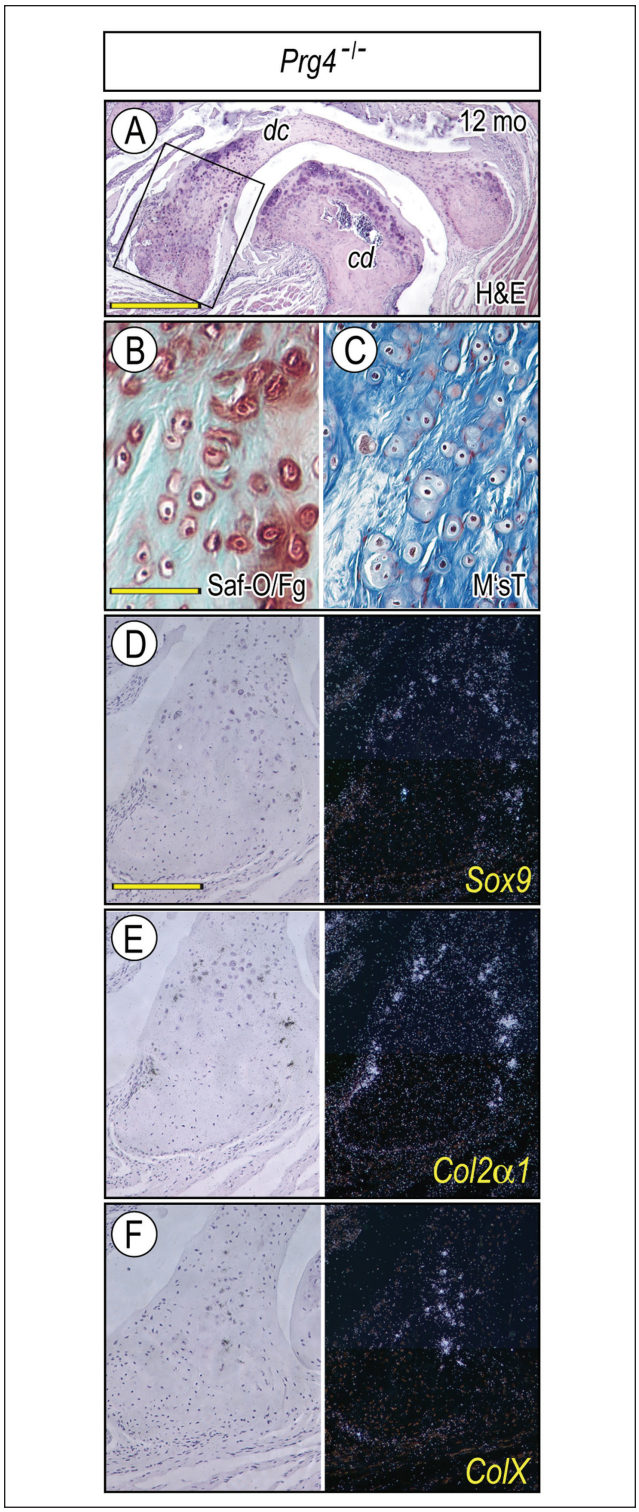
Appendix Table. Primer Information.

Gene	Accession No.	Forward Sequence (5′-3′)	Reverse Sequence (5′-3′)	Size, bp
Acan	NM_007424	GGAGCGAGTCCAACTCTTCA	CGCTCAGTGAGTTGTCATGG	120
Bmp2	NM_007553	TCTTCCGGGAACAGATACAGG	TCTCCTCTAAATGGGCCACTT	249
Bmp4	NM_007554	TCCATCACGAAGAACATC	TAGTCGTGTGATGAGGTG	239
Col2α1	NM_031163	CTACGGTGTCAGGGCCAG	GTGTCACACACACAGATGCG	116
Runx2	NM_001146038	CCGTGGCCTTCAAGGTTGT	TTCATAACAGCGGAGGCATTT	118
Sox9	NM_0011448	GAGCTCAGCAAGACTCTGGG	CGGGGCTGGTACTTGTAATC	13
ColX	NM_009925	ACCAGGAATGCCTTGTTCTC	CCTGGTTCATGGGATGTTTT	120
Bmpr1a	NM_009758	GGCAATGACTTTCACCTGCT	GGTCAAAGCTGTTCGGAGAA	249
Bmpr1b	NM_007560	TCCAGAGCTTCGTAAGAGCA	ATTTGGCGCTGAGCTATGAC	239
Acvr1	NM_ 001110204	GAGGCCCTCACACACACAC	TGCTAATGATGATGGCTTTCC	116
Bmpr2	NM_007561	CACACAGCCGTTCTTGATTC	AGGGATGACTTCCTCGCTG	118
Gapdh	NM_008084	ATCTTGGGCTACACTGAGGA	CAGGAAATGAGCTTGACAAAGT	122
Col1a2	NM_007743	AGCAGGTCCTTGGAAACCTT	AAGGAGTTTCATCTGGCCCT	101

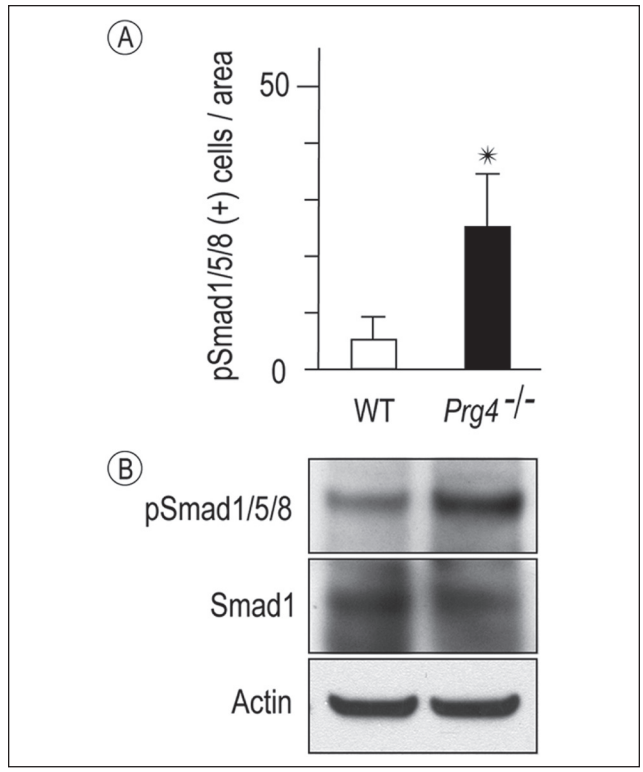
bp, base pairs.



Appendix Figure 1. Comparative expression levels of *type I collagen* (*Col1a2*) in temporomandibular joint discs. (**A**) Histograms depicting the relative expression of *Col1a2* in *Prg4*$^{-/-}$ and control discs at 6 mo of age and presented as average ± SD (n = 3 for mouse/each group, *P < 0.05). (**B**) Semiquantitative reverse transcription polymerase chain reaction. WT, wild type.



Appendix Figure 2. Characterization of ectopic cartilage in *Prg4*$^{-/-}$ discs. Frontal sections prepared from temporomandibular joints and discs from 12-mo-old *Prg4*$^{-/-}$ mice were processed for hematoxylin and eosin (H&E; **A**), safranin O/fast green (Saf-O/Fg; **B**), and Masson's trichrome staining (M'sT; **C**) and in situ hybridization with isotope-labeled riboprobes for *Sox9* (**D**), *Col2a1* (**E**), and *Col X* (**F**). In situ hybridization data corresponding to the boxed area in panel A are shown in panels D–F. Note that the cartilaginous tissue is characterized by nonhomogenous interterritorial and pericellular safranin O–stained proteoglycan matrix and by a collagen-rich matrix stained with Masson's trichrome. Note also that *Sox9*- and *Col2a1*-expressing chondrocytes are present in the peripheral area of the developing cartilaginous tissue and that *ColX*-expressing hypertrophying chondrocytes are located in the central region. Scale bars: 450 µm in A; 35 µm in B, also for C; 250 µm in D, also for E, F. cd, condyle; dc, disc.



Appendix Figure 3. Increased bone morphogenetic protein signaling in *Prg4*$^{-/-}$ discs. (**A**) Parasagittal sections prepared from temporomandibular joints and discs from 4-mo-old *Prg4*$^{-/-}$ and control mice were processed for detection of phosphorylated Smad 1/5/8 (pSmad1/5/8). pSmad1/5/8-positive cells were counted in 3 to 4 randomly selected sections (approximately 100 to 120 cells/area) from each sample (n = 3 for each mouse/each group) and are presented as average number ± SD. P < 0.05 was considered as statistically significant (*P < 0.01). (**B**) Western blot analysis of disc extracts from adult *Prg4*$^{-/-}$ and age-matched control mice, with antibodies of pSmad1/5/8, total Smad1, and actin. Note the increased levels of activated pSmad1/5/8 in *Prg4*$^{-/-}$ discs versus control discs. Disk tissues were lysed and homogenized in ice-cold RIPA buffer with protease and phosphatase inhibitors. Proteins were denatured by SDS sample buffer and electrophoresed on 4% to 15% SDS-bis-Tris gels (20 µg per lane) and transferred to PVDF membranes (Invitrogen). Membranes were blocked by 3% bovine serum albumin in phosphate-buffered saline with 0.1% Tween 20 and then incubated overnight at 4 °C with dilutions of antibodies against pSmad1/5/8 (13820, 1:1,000; Cell Signaling Technology, Danvers, MA, USA), Smad1 (9743, 1:1000; Cell Signaling Technology), or actin (2066, 1:2,000; Sigma-Aldrich, St. Louis, MO, USA). Horseradish peroxidase–conjugated donkey anti-rabbit antibodies were used as the secondary antibodies (NA934V, 1:2,000; GE Healthcare, Little Chalfont, UK). The SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific, Waltham, MA, USA) was used to detect antigen-antibody complexes. The membranes were reprobed with antiactin antibodies as a loading control. WT, wild type.