12-1-2013

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Anabolic Effect via the Bone Morphogenetic Protein/Smad Pathway

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Objective. Interleukin-10 (IL-10) is a pleiotropic immunoregulatory cytokine with a chondroprotective effect that is elevated in cartilage and synovium in patients with osteoarthritis. However, the role of IL-10 during endochondral bone formation and its mechanism of action have not been elucidated.

Methods. IL-10$^{-/-}$ mice and IL-10–treated tibial organ cultures were used to study loss and gain of IL-10 functions, respectively, during endochondral bone formation. Primary chondrocytes from the long bones of mouse embryos were cultured with and without IL-10. To assess the role of IL-10 in chondrogenic differentiation, we conducted mesenchymal cell micromass cultures.

Results. The lengths of whole skeletons from IL-10$^{-/-}$ mice were similar to those of their wild-type littermates, although their skull diameters were smaller. The tibial growth plates of IL-10$^{-/-}$ mice showed shortening of the proliferating zone. Treatment with IL-10 significantly increased tibial lengths in organ culture. IL-10 also induced chondrocyte proliferation and hypertrophic differentiation in primary chondrocytes in vitro. Mechanistically, IL-10 activated STAT-3 and the Smad1/5/8 and ERK-1/2 MAP kinase pathways and induced the expression of bone morphogenetic protein 2 (BMP-2) and BMP-6 in primary chondrocytes. Furthermore, the blocking of BMP signaling attenuated the IL-10–mediated induction of cyclin D1 and RUNX-2 in primary chondrocytes and suppressed Alcian blue and alkaline phosphatase staining in mesenchymal cell micromass cultures.

Conclusion. These results indicate that IL-10 acts as a stimulator of chondrocyte proliferation and chondrogenic or hypertrophic differentiation via activation of the BMP signaling pathway.

The main pathologic feature of osteoarthritis (OA) is gradual loss of cartilage from exposure to repetitive loading. The initial response of chondrocytes to external mechanical loading is adaptation; that is, they become metabolically active and proliferative (1). However, repetitive stress induces hypertrophic differentiation and, eventually, chondrocyte apoptosis. During this process, the balance between anabolic and catabolic signaling pathways in which SOX9 and RUNX-2, respectively, function as major regulatory transcription factors is critical in determining the fate of chondrocytes (2,3). Several cytokines can affect the expression of SOX9 and RUNX-2 via complex autocrine and paracrine loops and, as a consequence, have an effect on the progression of OA (4,5).

The physiologic and pathologic roles of the main catabolic cytokines, such as interleukin-1β (IL-1β) and tumor necrosis factor α (TNFα), have been well established in chondrocyte biology (4). In contrast, the roles

of anabolic cytokines remain poorly understood, although IL-10 seems to be a strong candidate for detailed study (5,6). In joint tissues, IL-10 and its receptor are typically expressed in chondrocytes and synovial fibroblasts (7,8). Furthermore, levels of IL-10 are significantly elevated in cartilage, synovial tissue, and subchondral bone tissue from OA patients, while serum levels are diminished (9). In addition to mature chondrocytes, mesenchymal stem cells (MSCs) and MSCs differentiated along the chondrogenic lineage express substantial amounts of IL-10 (10). Several in vivo and in vitro studies support the idea that IL-10 works protectively on cartilage tissue (11–16). In one study, subjects in the lowest quartile for the innate ex vivo production of IL-10 upon lipopolysaccharide stimulation had a 3-fold increased risk of having OA as compared to those in the highest quartile (11). In vitro studies have also revealed that IL-10 promotes the production of extracellular matrix proteins of cartilage, such as type II collagen and aggrecan (12–14,16). In addition, IL-10 has been reported to suppress the production of catabolic matrix metalloproteinases (MMPs) as well as the apoptosis of chondrocytes (15,16). Taken together, these results suggest that IL-10 has direct protective effects on cartilage.

These effects of IL-10 on chondrocytes may involve complex interactions with a number of locally acting essential growth factors required for endochondral bone formation, such as Indian hedgehog (IHH), parathyroid hormone–related protein (PTHrP), fibroblast growth factors (FGFs), bone morphogenetic proteins (BMPs), and Wnt proteins. The BMPs, which are members of the transforming growth factor β (TGFβ) superfamily, consist of at least 15 members, and they have been implicated in multiple steps of chondrogenesis (17). During the commitment of mesenchymal cells to the chondrocyte lineage, BMP signals are involved in the maintenance of the NK 3 homeobox 2 (NKX-3.2) and SOX9 autoregulatory loop in response to Sonic hedgehog (SHH) (18). BMP signaling also involves the SOX9-dependent induction of SOX5 and SOX6, which as a SOX trio, play a critical role in chondrogenic differentiation (19). In addition, BMPs promote chondrocyte proliferation, both directly and in an IHH/PTHrP-dependent manner (20,21). Strong evidence for the critical roles played by BMPs in the terminal hypertrophic differentiation of chondrocyte is provided by accelerated differentiation toward hypertrophic chondrocytes in mice with constitutively activated BMP receptor signals (22).

These findings suggest that BMPs promote almost every aspect of chondrogenesis, from commitment to terminal differentiation. With regard to joint homeostasis, there is evidence to show that BMPs play a chondroprotective role in joint cartilage via their anabolic effects (23). In OA tissue, the expression of BMPs 2, 3, 6, and 7 was found to be increased, whereas the expression of BMPs 4 and 5 was decreased (24,25). However, little is known of the regulatory mechanisms that control BMP expression in OA.

The purpose of this study was to investigate the role of IL-10 during endochondral bone formation in order to gain insights into its pathophysiologic or protective functions in OA cartilage. It was found that IL-10 increased chondrogenic and hypertrophic differentiation and chondrocyte proliferation via the BMP pathway. These results suggest that IL-10 is an important upstream regulator of BMP signaling in chondrocyte biology.

MATERIALS AND METHODS

Reagents and antibodies. Recombinant mouse IL-10 was purchased from BioVision, and recombinant human/mouse IHH (C28II) and recombinant human BMP-2 were from R&D Systems. Dorsomorphin and cyclopamine were purchased from Sigma-Aldrich, and recombinant human Noggin was from Joint Protein Central. Rabbit polyclonal antibodies against SOX9, type II collagen, type X collagen, proliferating cell nuclear antigen (PCNA), RUNX-2, and MMP-13 and mouse monoclonal antibody for cyclin D1 were purchased from Abcam. Rabbit polyclonal antibodies against p–STAT-3 (Tyr^{705}), p–STAT-3 (Ser^{727}), p-Smad1/5/8, total Smad1, p-AKT (pTyr^{374}), AKT, p-ERK (pTyr$^{202/204}$), and p-IκB were obtained from Cell Signaling Technology. Rabbit polyclonal antibodies for IHH, IL-10 receptor α-subunit, and ERK-1/2 were obtained from Epitomics, Millipore, and BD Biosciences, respectively, and mouse monoclonal antibody for β-actin was from Sigma-Aldrich.

Animal experiments. Female homozygous IL-10$^{-/-}$ mice on the C57BL/6 background were kindly provided by Professor E. S. Kim (Kyemyung University, Daegu, Korea). IL-10$^{-/-}$ mice were maintained in the Central Animal Facility of the Daegu Fatima Hospital (Daegu, Korea). The IL-10 treatment experiment was performed using pregnant ICR mice obtained from the Hana Breeding Laboratory. Mice were killed on embryonic day 11.5 (E11.5) by dislocation of the cervical spine to use for micromass cultures, on E15.5 for primary chondrocyte cultures and tibial organ cultures, and on E18.5 for histologic analysis. All animal protocols were approved by the Institutional Animal Care and Use Committee of Kyungpook National University School of Medicine.

Staining of the skeleton. Skeletons from IL-10$^{-/-}$ mice and their wild-type littermates on postnatal day 1 were prepared and stained with Alcian blue and alizarin red as previously described (26). Briefly, skin, muscles, and visceral organs were removed prior to overnight fixation in 95% ethanol at room temperature. Specimens were then incubated overnight in a solution containing 0.015% Alcian blue (Sigma), rinsed

Table 1. Primer sequences used in real-time polymerase chain reaction analyses

Gene	Forward	Reverse
Acan	5′-CCAGCCTACACCCCAGTG-3′	5′-GAGGGTGGGAAGCCATGT-3′
Col2a1	5′-CGGTCCTACGGTGTCAGG-3′	5′-TTATACCTCTGCCCATTCTGC-3′
Col10a1	5′-GCATCTCCCAGCACCAGA-3′	5′-CCATGAACCAGGGTCAAGAA-3′
Opn	5′-GGAGGAAACCAGCCAAGG-3′	5′-TGCCAGAATCAGTCACTTTCAC-3′
PthrP (Pthlh)	5′-GGTTCAGCAGTGGAGTGTCC-3′	5′-CAGACACAGCGCGTTTGA-3′
Ihh	5′-TGCATTGCTCTGTCAAGTCTG-3′	5′-GCTCCCCGTTCTCTAGGC-3′
Ptch1	5′-GGAAGGGGCAAAGCTACAGT-3′	5′-TCCACCGTAAAGGAGGCTTA-3′
Runx2	5′-TAAAGTGACAGTGGACGGTCCC-3′	5′-GCCCTAAATCACTGAGGCGAT-3′
Sox9	5′-CAGCAAGACTCTGGGCAAG-3′	5′-TCCACGAAGGGTCTCTTCTC-3′
Mmp13	5′-GCCAGAACTTCCCAACCAT-3′	5′-TCAGAGCCCAGAATTTTCTCC-3′
Bmp2	5′-CGGACTGCGGTCTCCTAA-3′	5′-GGGGAAGCAGCAACACTAGA-3′
Bmp3	5′-CTCTCCCAAGTCATTTGATGC-3′	5′-GCGTGATTTGATGGTTTCAA-3′
Bmp4	5′-GAGGAGTTTCCATCACGAAGA-3′	5′-GCTCTGCCGAGGAGATCA-3′
Bmp6	5′-ACTGACTAGCGCGCAGGA-3′	5′-TGTGGGGAGAACTCCTTGTC-3′
Bmp7	5′-CGAGACCTTCCAGATCACAGT-3′	5′-CAGCAAGAAGAGGTCCGACT-3′
Gapdh	5′-TGTCCGTCGTGGATCTGAC-3′	5′-CCTGCTTCACCACCTTCTTG-3′

with 95% ethanol for 3 hours, and transferred to 2% KOH for 24 hours. Specimens were then counterstained overnight with 0.005% alizarin red S in 1% KOH, and cleared by placing them in 1% KOH/20% glycerol for 2 days. Samples were then stored in a 1:1 mixture of glycerol and 95% ethanol.

Isolation and culture of mouse primary chondrocytes. Primary chondrocytes were isolated from the humerus, radius, and ulna of E15.5 mice as previously described (27). Isolated bones were equilibrated in α-minimum essential medium (α-MEM)–based organ culture media supplemented with 0.2% bovine serum albumin, 0.25 mM ascorbic acid, 1 mM β-glycerophosphate, 0.25% L-glutamine, and 0.25% penicillin/streptomycin for 24 hours at 37°C in an atmosphere containing 5% CO_2. Specimens were then incubated for 15 minutes at 37°C in 0.25% trypsin–EDTA, with gentle shaking, and digested for 2 hours with 3 mg/ml of collagenase P (Sigma-Aldrich) in complete Dulbecco's modified essential medium (DMEM). Fractioned cells were filtered through a 40-μm nylon mesh (BD Biosciences) and collected by centrifugation. Cell culture was performed in a 3:2 mixture of Ham's F-12 to DMEM containing 10% fetal bovine serum (FBS), and 0.25% L-glutamine. For starvation and treatment, 1% DMEM was used. Primary chondrocytes at passage 0 were used for experiments because of their dedifferentiation capacity (28).

Organ culture and histologic analysis. Tibiae isolated from E15.5 mice were equilibrated for 24 hours at 37°C in α-MEM–based organ culture medium as previously described (29,30). Treatment with recombinant mouse IL-10 (20 ng/ml) was performed 1 day after dissection and carried out for 6 days, with media changes on alternate days. For morphometric analysis, tibial explants were photographed under a dissecting microscope (Nikon). For histologic analyses, tibiae were fixed overnight in 4% buffered paraformaldehyde, embedded in paraffin, cut into 5-μm–thick sections, and stained with Safranin O. For immunohistochemical analyses for SOX9, PCNA, IHH, RUNX-2, and type X collagen α1-chain, sections were incubated overnight at 4°C with primary antibodies (31). Horseradish peroxidase–conjugated secondary antibody (Promega) was used for localization. Sections from at least 3 mice and at least 3 sections per mouse were analyzed, and the numbers of positive cells within a 400-μm^2 rectangle were

counted under a microscope. For all antibodies, mouse IgG at the same concentrations was used as a negative control following the same protocol in the absence of primary antibody.

Micromass culture. Micromass cultures were performed as described previously (32,33). Briefly, limb buds from E11.5 mouse embryos were dissected, and mesenchymal cells were isolated by digestion for 90 minutes in Dispase II (Roche), with gentle shaking. Cells were resuspended in a 3:2 concentration of Ham's F-12 to DMEM containing 10% FBS, 0.25% penicillin/streptomycin, and 0.25% L-glutamine, and plated at a density of 2.5×10^5 cells/10-μl drop. One hour after plating, the wells were flooded with growth medium supplemented with 1 mM β-glycerophosphate and 0.25 mM ascorbic acid. Cells were cultured for up to 12 days in culture medium, which was replaced every 2 days. Cells were differentiated in micromass culture for 3 days to allow chondrogenesis and then treated with IL-10 (20 ng/ml) or Noggin (100 ng/ml). Cells were fixed for 1 hour in 10% formalin and stained overnight with 1% Alcian blue in 0.1N HCl. To quantify Alcian blue incorporation, cultures were incubated overnight with 6M guanidine hydrochloride, and the absorbance was measured at 600 nm. Alkaline phosphatase staining was performed after fixation with citrate–acetone–formaldehyde fixative solution. Fixed cells were incubated with an alkaline dye mixture for 15 minutes at room temperature, washed with phosphate buffered saline, and photographed.

RNA isolation and real-time polymerase chain reaction (PCR) analysis. Total RNA was isolated from primary chondrocyte cultures using TRIzol reagent (Invitrogen), and first-strand complementary DNA was synthesized using Superscript III reverse transcriptase (Invitrogen). Real-time PCR was performed using a LightCycler 480 instrument and Light-Cycler 480 SYBR Green I Master Mix (both from Roche). Real-time PCR oligonucleotide primers for mouse chondrocyte-specific or related genes were designed using Universal Probe Library System software (https://www.roche-applied-science.com). Primers used for real-time PCR are shown in Table 1.

Protein isolation and Western blot analyses. Proteins from primary chondrocyte cultures (n = 3 independent isolations) were extracted using 300 μl of radioimmunoprecipita-
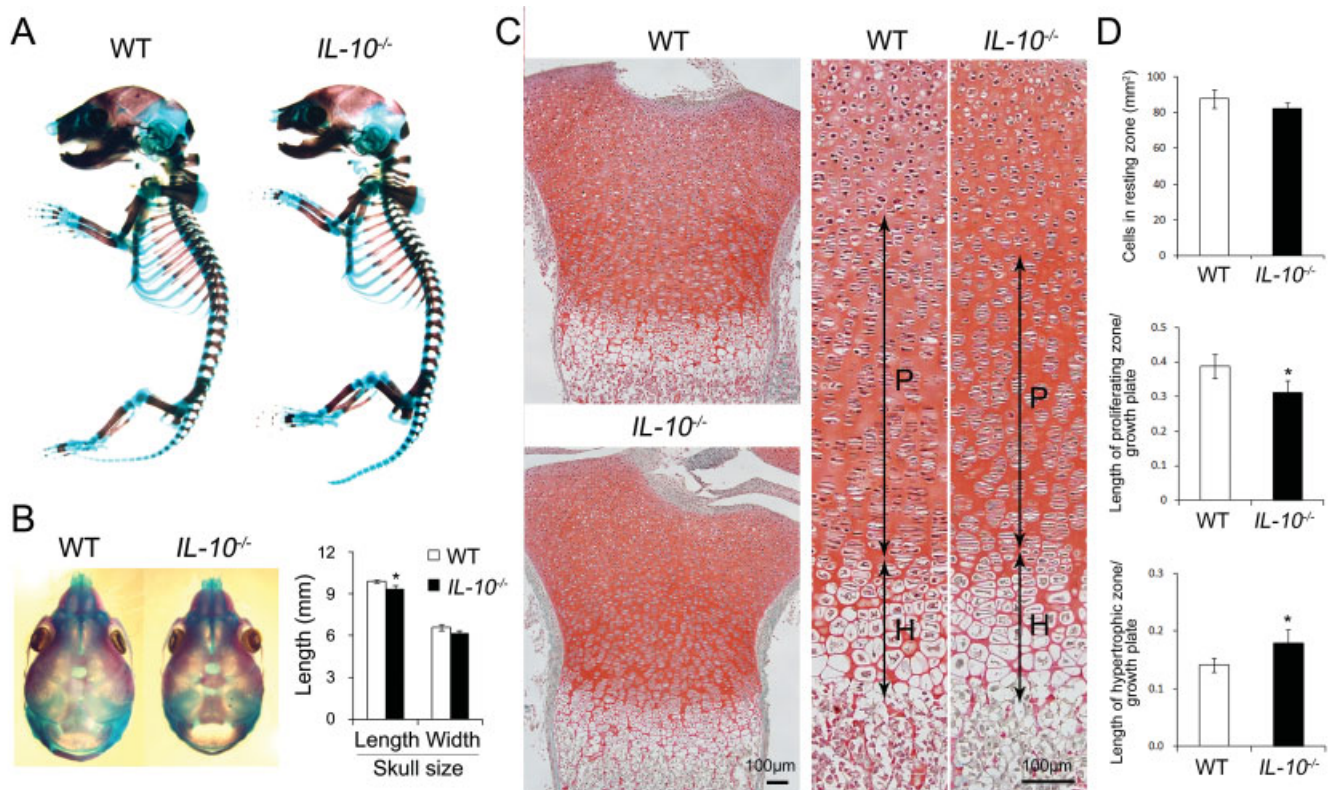
Figure 1. Whole skeletal and growth plate phenotypes of IL-10$^{-/-}$ mice. **A** and **B,** Alizarin red and Alcian blue staining of whole mouse skeletons (**A**) and skulls (**B**) obtained on postnatal day 1 from IL-10$^{-/-}$ mice and their wild-type (WT) littermates. Mineralized bone matrix is stained red; cartilaginous tissue is stained blue. The diameters and widths of the skulls in **B** were measured, and these data are also shown (right). **C,** Safranin O staining of tibial growth plates from wild-type and IL-10$^{-/-}$ mice on embryonic day 18.5, illustrating the resting zone (top area), proliferating zone (**P**), and hypertrophic zone (**H**). **D,** Quantification of cell densities in the resting zone, as well as the relative length of the proliferating and hypertrophic zones versus the total growth plate. IL-10$^{-/-}$ mice showed a shorter proportion of the proliferating zone and a longer proportion of the hypertrophic zone than did their wild-type littermates. Values in **B** and **D** are the mean ± SD of 4 mice per group. ∗ = $P < 0.05$ versus wild-type mice.

tion assay buffer supplemented with protease and phosphatase inhibitors (Roche Diagnostics) and were microcentrifuged for 20 minutes at 10,000g. Total cell lysates (containing 20–30 μg of protein) were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (Immobilon-P; Millipore). Membranes were then blocked for at least 1 hour at room temperature using 5% skimmed milk, and membrane-bound proteins were probed with primary antibodies against SOX9, type II collagen, cyclin D1, PCNA, RUNX-2, type X collagen, p-STAT-3, p-Smad1/5/8, p-Akt, p-IκB, p–ERK-1/2, and p-p38. Membranes were washed in Tris buffered saline–Tween and incubated for 1 hour at room temperature with horseradish peroxidase–conjugated secondary antibody (Santa Cruz Biotechnology), and proteins were detected using enhanced chemiluminescent detection reagents (GE Healthcare).

MTT assay. Proliferation of primary chondrocytes was determined using an MTT assay. Briefly, E15.5 primary chondrocytes (1×10^4/well) were seeded into 96-well plates in a 3:2 (volume/volume) mixture of Ham's F-12 and DMEM, with or without IL-10 (10 ng/ml), in the presence or absence of BMP-2 (100 ng/ml). After 48 hours of culture, 50 μl of MTT was added to each well, and the plate was incubated for 4 hours at 37°C. Medium was then removed and cells were solubilized with 150 μl of DMSO. Spectrophotometric absorbance was measured at 570 nm.

Statistical analysis. Student's t-test and one-way analysis of variance with Tukey's multiple comparison post-tests were used to determine differences between means. All analyses were conducted using SPSS version 14.0 software. Results are presented as the mean ± SD. P values less than 0.05 were considered significant.

RESULTS

Reduction in the proliferating zone of embryonic growth plates following genetic deletion of IL-10. To determine the role played by IL-10 during endochondral bone formation in vivo, we analyzed the skeletons of
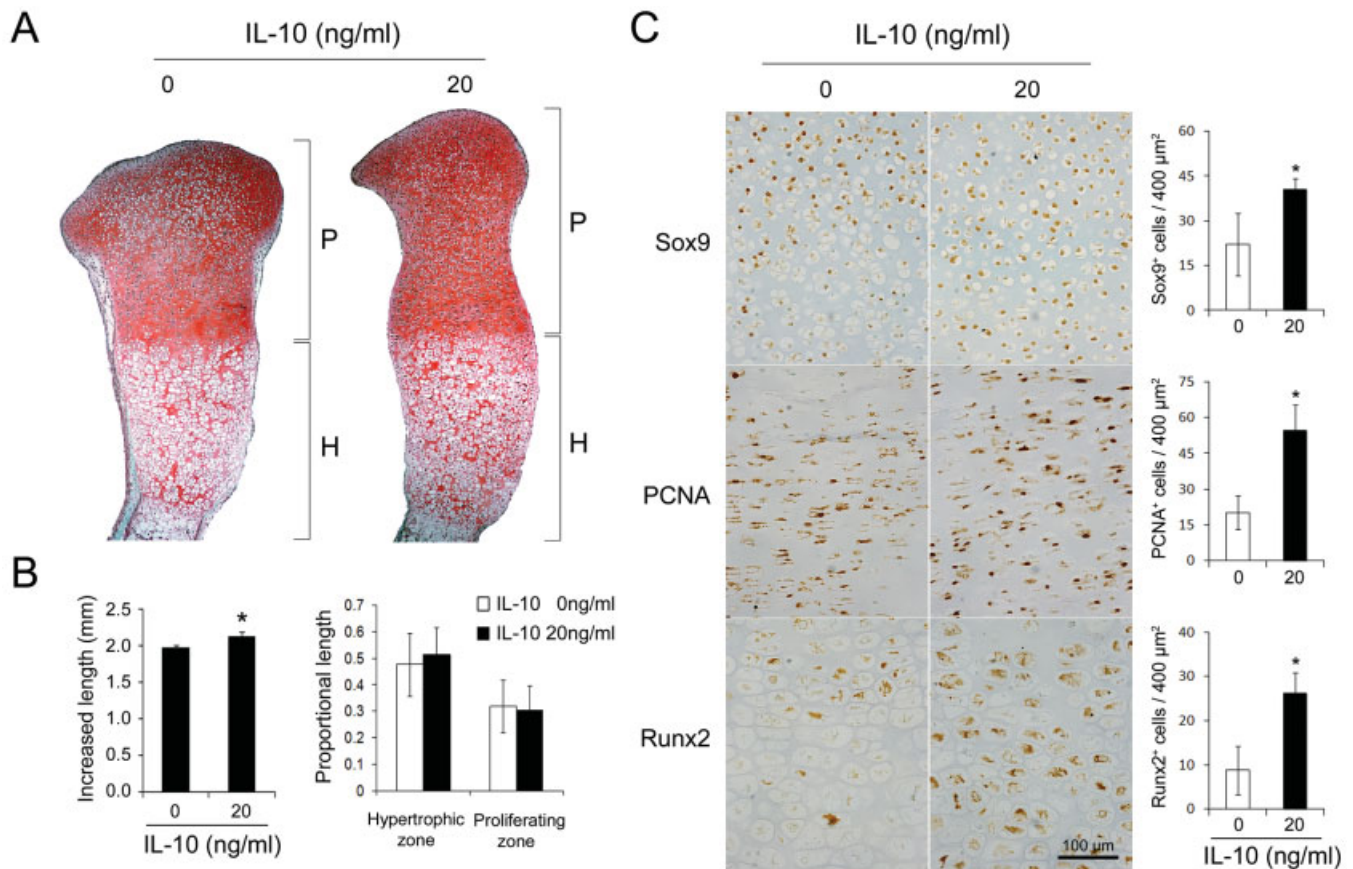
Figure 2. Increased tibial length in organ cultures following interleukin-10 (IL-10) treatment. **A,** Safranin O staining of tibiae in organ culture. Tibiae isolated from wild-type mice on embryonic day 15.5 were cultured for 6 days in the absence (left) and presence (right) of 20 ng/ml of IL-10 and then stained with Safranin O. The proliferating (P) and hypertrophic (H) zones of the tibiae increased in response to IL-10. Original magnification $\times$ 40. **B,** Quantification of the longitudinal growth of tibiae over the culture period (left) and proportions of hypertrophic and proliferating zones with respect to the total growth plate length (right). **C,** Immunohistochemical analysis of tibial organ cultures (left) and quantification of positive cells (right). The numbers of positive cells for SOX9 in the resting zone, proliferating cell nuclear antigen (PCNA) in the proliferating zone, and RUNX-2 in the hypertrophic zone were counted in 5 different 400-μm^2 rectangles. IL-10 treatment increased the numbers of cells positive for SOX9, PCNA, and RUNX-2. Values in **B** and **C** are the mean $\pm$ SD of 4 mice per group. $*$ = $P < 0.05$ versus no IL-10 treatment.

IL-10$^{-/-}$ mice on postnatal day 1. Staining of whole skeletons showed no differences in the extremities or spine of the IL-10$^{-/-}$ mice as compared to their wild-type littermates, based on measurements of bone lengths and mineralized portions (Figure 1A). On the other hand, IL-10$^{-/-}$ mice had smaller skulls and showed less Alcian blue staining in the occipital bones as compared to their wild-type littermates. In addition, the distal phalanges of IL-10$^{-/-}$ mice showed faint Alcian blue staining as compared to their wild-type littermates (Figure 1B). We next examined phenotype differences in growth plates obtained on E18.5 from IL-10$^{-/-}$ mice and their wild-type littermates. Safranin O staining of tibial growth plates revealed a shortened proliferating zone and a corre-

sponding increase in the hypertrophic zone in IL-10$^{-/-}$ mice (Figures 1C and D), with no changes in cell density in the resting zone.

Lower PTHrP expression by IL-10$^{-/-}$ chondrocytes during BMP-2–induced differentiation. Given the histologic results, we sought to determine the expression of messenger RNA (mRNA) for major transcription factors, regulatory proteins, and chondrocytes markers. Primary chondrocytes obtained on E15.5 were cultured for 4 days in insulin–transferrin–selenium and BMP-2–containing media representing proliferating and hypertrophic conditions, respectively. Real-time PCR analysis revealed that chondrocytes from IL-10$^{-/-}$ mice showed significantly less PTHrP expression than did those from
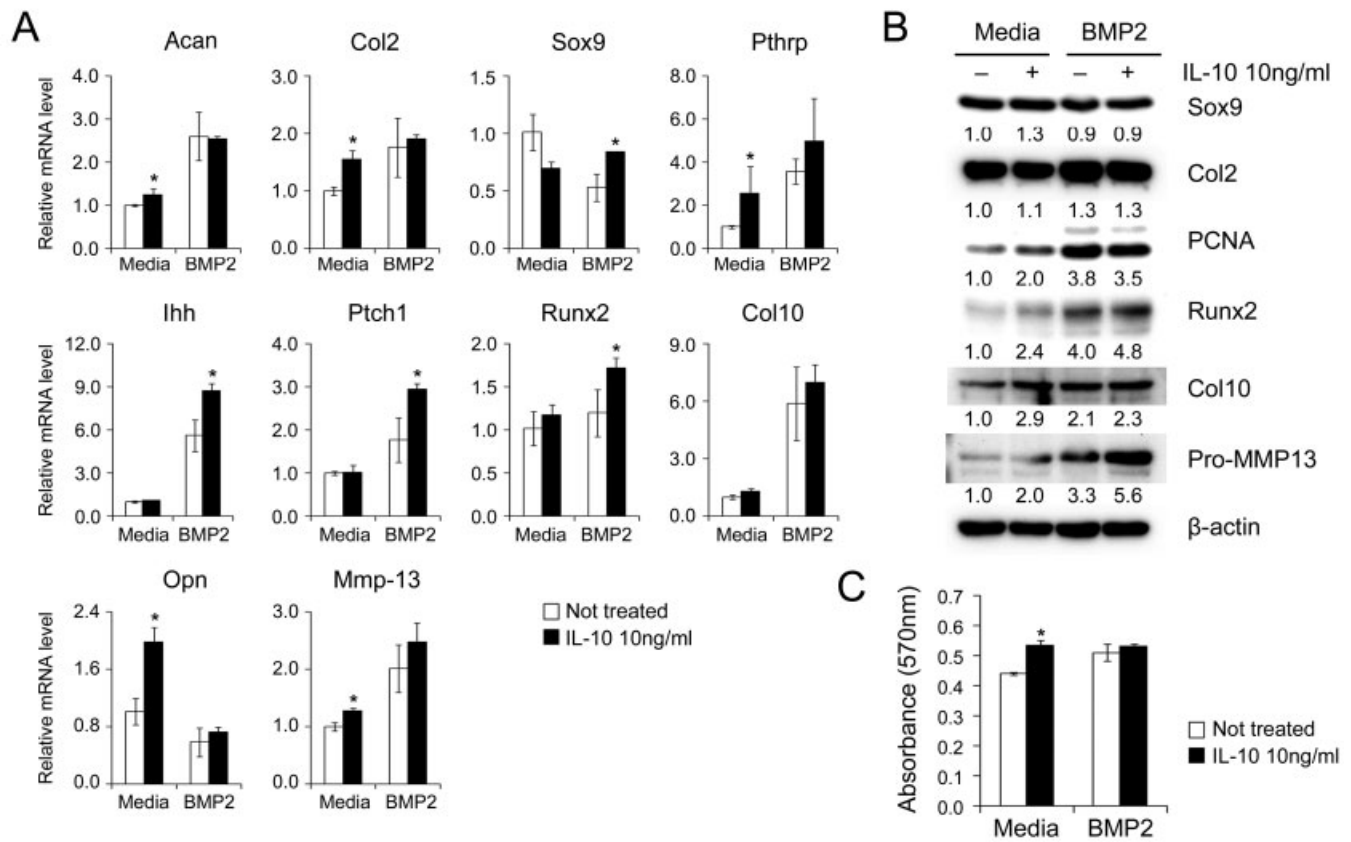
Figure 3. Effects of interleukin-10 (IL-10) on chondrocyte proliferation and hypertrophic differentiation. **A,** Expression of mRNA for chondrogenic and hypertrophic marker genes. Primary chondrocytes obtained on embryonic day 15.5 (E15.5) from wild-type mice were cultured for 4 days in the presence or absence of 10 ng/ml of IL-10 in either chondrogenic media (5 mM β-glycerophosphate and 50 μg/ml of ascorbic acid) or media containing bone morphogenetic protein 2 (BMP-2; 100 ng/ml). RNA was isolated and analyzed by quantitative polymerase chain reaction (qPCR). Results are representative of 3 independent real-time qPCR experiments. Values are the mean ± SD. ∗ = $P < 0.05$ versus no IL-10 treatment. **B,** Western blot analysis of SOX9, type II collagen, proliferating cell nuclear antigen (PCNA), RUNX-2, type X collagen, and pro–matrix metalloproteinase 13 (proMMP-13) proteins in whole lysates of primary chondrocytes cultured for 4 days in the presence or absence of 10 ng/ml of IL-10. β-actin was used as a loading control. Results are representative of 3 independent experiments. **C,** Effect of IL-10 on cell numbers, as determined by MTT assay. Primary chondrocytes obtained on E15.5 were cultured in the presence or absence of IL-10 in media alone or in media containing BMP-2. After 2 days, cells were isolated and counted. Values are the mean ± SD of 5 separate experiments. ∗ = $P < 0.05$ versus no IL-10 treatment.

wild-type littermates under BMP-2–mediated differentiation conditions (data available upon request from the corresponding author).

Increased growth of tibiae in organ cultures following IL-10 treatment. Next, we investigated the gain-of-function effects of IL-10 on chondrocyte proliferation and differentiation using a tibial organ culture system. Tibiae obtained on E15.5 from wild-type mice were cultured for 6 days in the presence or absence of IL-10 (20 ng/ml). Gross analysis showed that IL-10 treatment resulted in a small but significant increase in longitudinal growth. Histologic analysis revealed that IL-10 caused an increase in the length of proliferating

and hypertrophic zones as compared to the untreated controls, but the proportions of both zones with respect to the total growth plate length were similar in tibiae from IL-10–treated mice and untreated controls (Figures 2A and B).

To explore the molecular actions of IL-10 in endochondral bone growth, we performed immunohistochemistry for major transcription factors and the proliferation marker PCNA in tibial organ cultures. Immunohistochemical analysis revealed that IL-10 significantly increased the number of chondrocytes expressing SOX9 and PCNA in resting and proliferating layers of the growth plate. RUNX-2 was also significantly
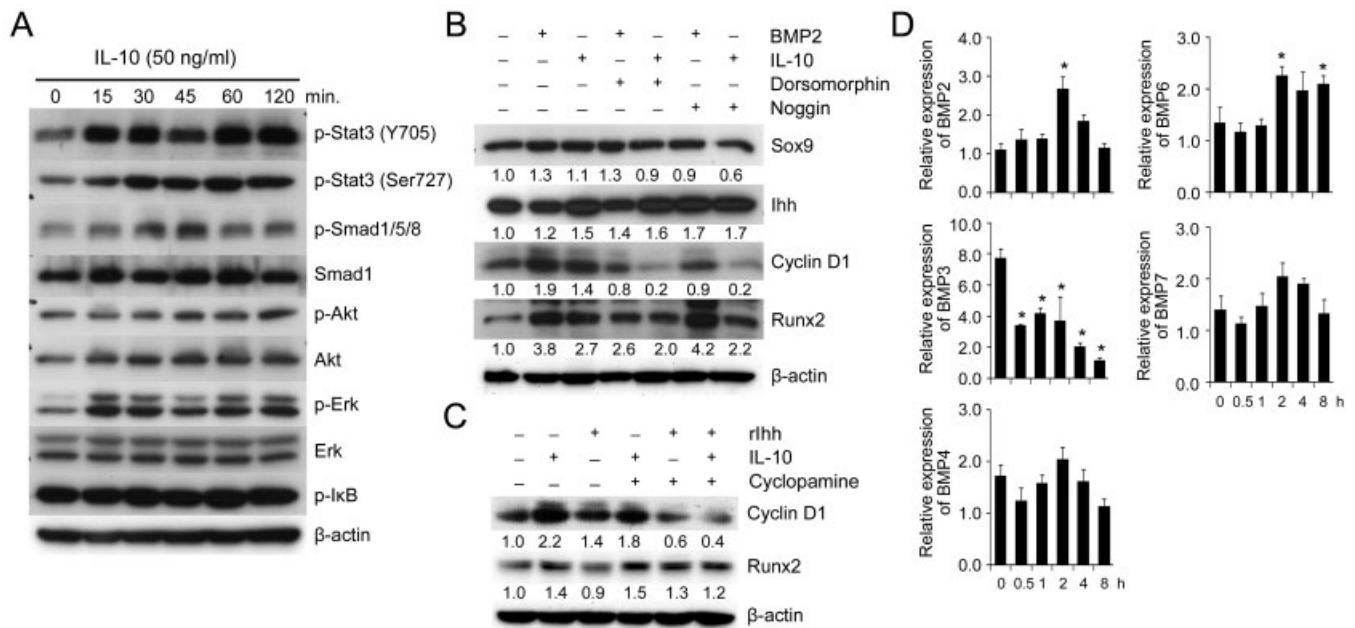
Figure 4. Interleukin-10 (IL-10)–mediated chondrocyte signaling and interaction with the bone morphogenetic protein (BMP) and Indian hedgehog (IHH) pathways. **A,** Signaling mechanisms involved in the effects of IL-10 on chondrocytes. Serum-starved primary chondrocytes obtained from wild-type mice on embryonic day 15.5 (E15.5) were stimulated for the indicated times with 50 ng/ml of IL-10, and cell lysates were analyzed by Western blotting. IL-10 induced the phosphorylation of STAT-3, ERK-1/2, and Smad1/5/8, but not Akt or IκB. **B,** Interaction of IL-10 signaling with BMP/Smad signaling. Primary chondrocytes were cultured with BMP-2 (100 ng/ml) or IL-10 (10 ng/ml) in the presence of the receptor or ligand BMP-2 antagonists dorsomorphin (2 μM) or Noggin (150 ng/ml), respectively. After 4 days, cells were harvested, and protein expression was examined by Western blotting. **C,** Partial dependence of IL-10–mediated chondrocyte proliferation on IHH signaling. Primary chondrocytes were cultured for 4 days with recombinant IHH (rIHH; 100 ng/ml) or IL-10 (10 ng/ml) in the presence or absence of cyclopamine (5 μM), an inhibitor of smoothened. Results shown for all Western blot experiments are representative of 3 experiments. β-actin was used as a loading control in all Western blot experiments. **D,** Time course of the expression of BMPs 2, 3, 4, 6, and 7 in response to IL-10 treatment. Real-time polymerase chain reaction analysis was performed on RNA extracted from primary chondrocytes treated with 10 ng/ml of IL-10 for the indicated times. Values are the mean ± SD. ∗ = $P < 0.05$ versus baseline, by analysis of variance with Tukey's post hoc test.

increased by IL-10 in the hypertrophic layer of growth plate compared to untreated controls (Figure 2C). The control group showed positive staining for IHH only in the prehypertrophic zone, whereas the IL-10–treated group showed more widespread expression from the prehypertrophic to the hypertrophic layer (data available upon request from the corresponding author). We also examined the expression of the IL-10 receptor, which was abundant from the resting to the prehypertrophic layer, with gradual attenuation in the hypertrophic layer of the growth plate. A similar expression pattern of the IL-10 receptor α subunit was verified during the differentiation of ATDC5 chondroprogenitor cells as well. Treatment with IL-10 increased the expression of IL-10 receptor in the hypertrophic layer (data available upon request from the corresponding author).

IL-10–induced chondrocyte proliferation and hypertrophic differentiation in primary chondrocytes. We then studied the effect of IL-10 on mRNA and protein expression in primary chondrocytes. Primary chondrocytes obtained on E15.5 from wild-type mice were cultured for 4 days with or without IL-10 in medium alone or in the presence of BMP-2. Real-time PCR analyses revealed that IL-10 treatment increased the levels of mRNA for both chondrogenic and hypertrophic marker genes, including aggrecan, type II collagen, osteopontin, and MMP-13. In addition, PTHrP mRNA was up-regulated by IL-10. Under BMP-2–mediated differentiation conditions, IL-10 significantly increased the mRNA expression of SOX9, IHH, patched 1, and RUNX-2 (Figure 3A). However, other chondrogenic and hypertrophic marker genes were unaffected by IL-10 in the presence of BMP-2. At the protein level,
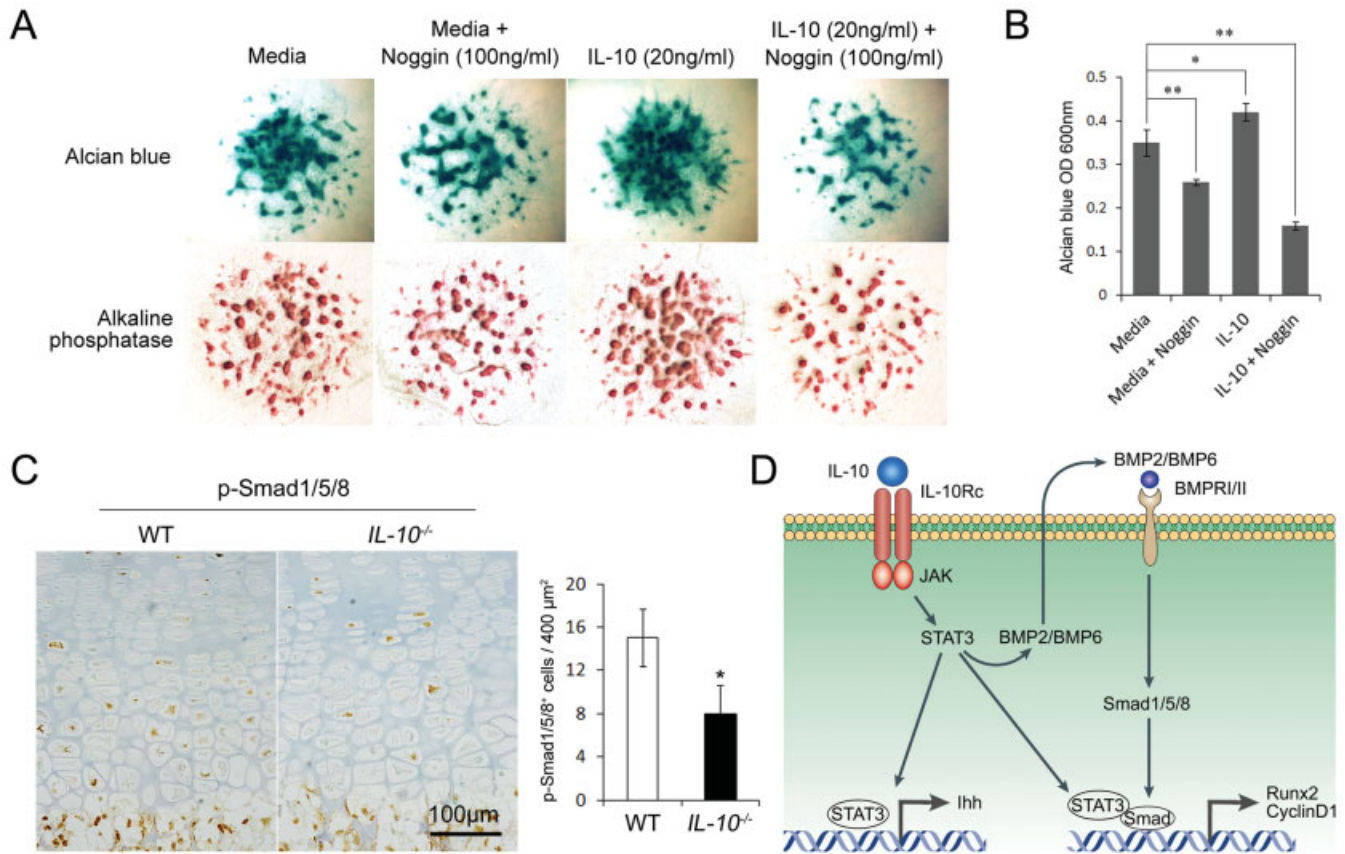
Figure 5. Dependence of interleukin-10 (IL-10)–mediated chondrogenic and hypertrophic differentiation on bone morphogenetic protein (BMP) signaling. **A,** Attenuation of IL-10–mediated chondrocyte differentiation by Noggin. Mesenchymal cells obtained on embryonic day 11.5 (E11.5) from normal mouse limb buds were plated at 2.5×10^5 cells/10-μl drop and cultured in the absence (media control) or presence of Noggin (100 ng/ml), IL-10 (20 ng/ml), or both. After 12 days, micromass cultures were stained with Alcian blue to visualize the degree of chondrogenic differentiation or with alkaline phosphatase to visualize hypertrophic maturation. Original magnification $\times$ 2.5. **B,** Quantification of Alcian blue binding to sulfated glycosaminoglycans in the extracellular matrix following cell culture as in **A**. Values are the mean $\pm$ SD of triplicate experiments. $* = P < 0.05$; $** = P < 0.01$. **C,** Immunohistochemical analysis of p-Smad1/5/8 expression in the prehypertrophic and hypertrophic layers of tibia obtained on E18.5 from IL-10$^{-/-}$ mice and their wild-type (WT) littermates (left). The numbers of p-Smad1/5/8–positive cells in 400-μm^2 rectangles were also quantified (right). Values are the mean $\pm$ SD. $* = P < 0.05$ versus wild-type mice. **D,** Schematic representation of the mechanistic insights into cross-talk between IL-10/STAT-3 and BMP signaling deduced from this study of the IL-10–mediated up-regulation of chondrocyte proliferation and differentiation. IL-10 signaling induces the expression of BMP-2 and BMP-6. Subsequently, BMP/Smad signaling participates in the IL-10–mediated induction of RUNX-2, SOX9, and cyclin D1. Indian hedgehog (IHH) signaling, which contributes to IL-10–mediated cyclin D1 induction, is independent of BMP signaling. IL-10Rc = IL-10 receptor; BMPRI/II = BMP receptor type I/II.

IL-10 increased the expression of PCNA, RUNX-2, and type X collagen in the presence of media, but in the presence of BMP-2. ProMMP-13, the marker for hypertrophic differentiation, was increased by IL-10 in the presence of media and in the presence of BMP-2. The chondrogenic markers SOX9 and type II collagen were not affected by IL-10 at the protein level (Figure 3B).

To investigate the role played by IL-10 in chondrocyte proliferation, we conducted an MTT assay on primary chondrocytes obtained on E15.5. We have shown previously that MTT values correspond to chondrocyte numbers (34). After 2 days of culture in the current study, the presence of IL-10 significantly increased the numbers of chondrocytes. However, the effect of IL-10 on chondrocyte numbers was lost in the presence of BMP-2 (Figure 3C).

IL-10–mediated chondrocyte signaling and interactions with the BMP and IHH pathways. We next examined the signaling mechanisms involved in the effects of IL-10 on chondrocytes. Serum-starved primary chondrocytes on E15.5 from wild-type mice were stim-

ulated with 50 ng/ml of IL-10, and as expected, phosphorylation at both Tyr^{705} and Ser^{727} of STAT-3 was increased. In addition, Smad1/5/8 and ERK-1/2 MAP kinase phosphorylation was also activated by IL-10 (Figure 4A). Other signaling pathways, including Akt and IκB, were not activated by IL-10. Based on the observed activation of Smad signaling, we further studied the interaction of BMP and IL-10–induced signaling. Accordingly, primary chondrocytes were cultured for 4 days with BMP-2 or IL-10 in the presence of dorsomorphin or Noggin, which antagonize BMP signaling at the receptor and ligand levels, respectively. The induction of cyclin D1 by IL-10 or BMP-2 was dramatically attenuated by dorsomorphin and Noggin. However, the expression of IHH was not suppressed by BMP antagonists (Figure 4B), and induction of RUNX-2 was blocked more efficiently by dorsomorphin. To unravel the role of IHH in IL-10 function, cells were treated with cyclopamine, a smoothened inhibitor, to inhibit IHH signaling. Cyclopamine weakly attenuated cyclin D1 induction by IL-10, but did not affect the induction of RUNX-2 by IL-10 (Figure 4C).

To investigate the temporal regulation of the expression of BMPs by IL-10, we performed real-time PCR analysis on mRNA extracted from primary chondrocytes that had been treated with IL-10 (10 ng/ml). The expression of mRNA for BMP-2 and BMP-6 was significantly elevated by 2–3-fold after 2 hours of IL-10 treatment. However, BMP-3 expression was profoundly suppressed in IL-10–treated primary chondrocytes, and IL-10 did not influence the expression of mRNA for BMP-4 or BMP-7 (Figure 4D).

Increased chondrogenic and hypertrophic differentiation via BMP signaling following treatment with IL-10. To determine whether IL-10–mediated chondrocyte differentiation is dependent on BMP signaling, we conducted mesenchymal micromass cultures. Mouse limb bud mesenchymal cells obtained on E11.5 were seeded at a density of 2.5×10^5 cells/10-μl drop and cultured with IL-10 (20 ng/ml), either alone or with Noggin (100 ng/ml). After 12 days, we examined cartilaginous matrix production and cell differentiation by using Alcian blue and alkaline phosphatase staining. As expected, mesenchymal cells treated with IL-10 showed prominent induction of cartilage matrix production and differentiation as compared to medium-treated controls by Alcian blue and alkaline phosphatase staining. Treatment with Noggin potently suppressed IL-10–mediated cartilage matrix production and differentiation (Figures 5A and B), indicating that IL-10–mediated chondrogenic and hypertrophic differentiation is largely dependent on BMP signaling. We then examined the intensities of BMP signaling in the growth plates of IL-10$^{-/-}$ mice and wild-type littermates. We found that IL-10$^{-/-}$ mice expressed lower levels of phosphorylated Smad1/5/8, which was consistent with the reduced level of BMP signaling we observed (Figure 5C).

DISCUSSION

Although previous in vivo and in vitro studies have raised the possibility that IL-10 has a protective effect on chondrocytes, the mechanisms of IL-10 effects on chondrocytes have been unclear (11–16). In this study, we demonstrated that IL-10 promotes chondrogenic differentiation, proliferation, and hypertrophic differentiation of chondrocytes. In addition, the blocking of BMP signaling attenuated IL-10–induced induction of RUNX-2 and cyclin D1 expression, suggesting that the role of IL-10 in endochondral bone growth is largely dependent on BMP/Smad signaling. IL-10 is known to be constitutively produced in chondrocytes, and its mRNA expression is higher in fetal or OA chondrocytes as compared to healthy adult chondrocytes (7). Thus, our findings suggest that IL-10 as upstream activator of the BMP/Smad cascade may play a significant role in skeletal development and OA. These findings reveal that the IL-10–mediated protective effect on chondrocytes is due to its anabolic effect via the BMP/Smad pathway, in addition to its antiinflammatory effects.

We showed that IL-10 increased the formation of alkaline phosphatase–positive nodules in micromass culture and that this effect was abrogated by Noggin, which indicates that IL-10 induces chondrocyte hypertrophy via BMP/Smad signaling in vitro. This result, however, seems to be in conflict with the chondroprotective effect of IL-10 (11–16), since chondrocyte hypertrophy is associated with OA. On the other hand, mice lacking BMP receptor type IA expression in articular chondrocytes show articular cartilage erosion after birth that resembles OA in humans (35). Considering that BMP signaling promotes almost every aspect of endochondral bone formation resulting from chondrocyte proliferation, differentiation, and terminal hypertrophy (36), this genetic study suggests that BMP signaling has an overall beneficial effect on the maintenance of postnatal articular cartilage. Our findings indicate that IL-10, signaling through BMP/Smad, may also play an important role in protection against chondrocyte damage, despite its role in chondrocyte hypertrophy in vitro.

The IHH/PTHrP and BMP pathways promote chondrocyte proliferation during endochondral bone

formation (20). When one of these pathways is blocked, chondrocyte proliferation is significantly attenuated, which suggests that IHH–PTHrP and BMP signaling are necessary for chondrocyte proliferation (20). In addition, several lines of evidence indicate that the IHH/PTHrP and BMP systems interact closely (20,37–39). Cartilage-specific knockout of Smad1/5, the main signaling molecules in the canonical BMP pathway, display nearly completely suppressed IHH expression in hypertrophic chondrocytes (38). In contrast, the overexpression of IHH in cartilage was shown to increase the expression of BMPs in the perichondrium and in proliferating chondrocytes (20,39), whereas IHH^{−/−} mice showed reduced expression of BMPs (37). These results indicate the existence of a positive feedback loop between BMPs and the IHH/PTHrP pathway during chondrocyte proliferation.

The present study showed that IL-10 promoted chondrocyte proliferation in vivo and in vitro and that this promotion was accompanied by an increase in the expression of BMPs and IHH. In addition, BMP antagonist or hedgehog receptor smoothened antagonist reduced the IL-10–mediated expression of cyclin D1, which is a critical requirement for chondrocyte proliferation (40). In contrast, the increased expression of IHH by IL-10 was not attenuated by BMP antagonists, suggesting that the IL-10/STAT-3 signaling cascade directly controls the expression of IHH. Based on these findings, it would appear that the IL-10/STAT-3 signaling cascade plays a crucial role in the regulation of chondrocyte proliferation. However, chondrocyte proliferation is also controlled by additional signaling pathways, including FGFs and Wnt. Further investigations are necessary to elucidate the precise role of IL-10 in chondrocyte proliferation.

BMP/Smad signaling controls many critical steps in chondrocyte differentiation. The activity and signaling outcomes of this pathway are influenced by many intracellular and extracellular signals (41). Our observations show that IL-10 increased the levels of BMP-2 and BMP-6 transcripts in primary chondrocytes, but decreased the levels of mRNA for BMP-3, which is consistent with the combinatorial nature of the action of BMPs. The expression of BMP-2, an indispensable growth factor that is required throughout endochondral bone formation, is under complex control mechanisms, including hedgehog-Gli, Wnt/β-catenin, PTH/CREB, NF-κB, and estrogen receptor α signaling pathways (42). Our results suggest that IL-10 receptor and its downstream signaling cascade through JAK/STAT-3 contribute to the enhanced expression of BMP-2.

It has been reported that leukemia inhibitory factor–mediated STAT-3 activation induces BMP-2 expression and leads to the activation of Smad1/5/8 during astrocyte differentiation (43). These findings, together with our results, suggest that the JAK/STAT-3 signaling cascade plays an important role in the expression of BMP-2 under inflammatory conditions. Furthermore, we found that together with BMP-2, IL-10 induced the production of BMP-6, which is known to interact functionally with BMP-2 during bone formation (44,45). Evidence from previously reported immunohistochemical analyses revealed that BMP-2 and BMP-6 are coexpressed in hypertrophic cartilage (44,45). Functionally, the loss of BMP-6 on a background of BMP-2 haploinsufficiency causes moderate growth retardation and a delay in endochondral bone formation (45), while the deletion of BMP-6 alone showed no significant change in embryonic skeletal phenotype, except for delayed ossification of the sternum (44). Moreover, an earlier study showed that BMP-2/6 heterodimers are 5–10-fold more potent than BMP-2 homodimers at inducing cartilage and bone production in vivo (46). We found that IL-10 increased the expression of BMP-2 and BMP-6 simultaneously, which may allow them to cooperatively regulate endochondral bone formation.

Smad1/5/8 is the major mediator of canonical BMP signaling and plays a role in the regulation of chondrocyte proliferation and differentiation (41). Our results showed that IL-10 stimulation increased the phosphorylation of the C-terminal Ser[463/465] of Smad1/5/8 in an immediate manner in primary chondrocytes (Figure 4A). Considering that the expression of mRNA for BMP-2 and BMP-6 increased ~2 hours after IL-10 treatment (Figure 4D), this immediate activation of Smad1/5/8 suggests cross-talk with other signaling pathways downstream of the IL-10 receptor. It is well known that the activation of Smad1/5/8 is relatively specific to BMP receptor signaling (41,47). However, recent evidence showed that other ligand/receptor systems can directly activate Smad1/5/8 (48,49). In smooth muscle cells, serotonin (5-hydroxytryptamine [5-HT]) and its receptor 5-HT$_{1B/1D}$ directly phosphorylated Smad 1/5/8, in a Rho/Rho kinase signaling–dependent manner (48). Stromal cell–derived factor 1 and its receptor CXCR4 were also reported to directly activate Smad1/5/8 signaling (49). The present study is the first to suggest IL-10–mediated direct activation of Smad1/5/8. Additional work is required to address the mechanism of IL-10–induced immediate Smad1/5/8 phosphorylation and its biologic meaning.

In summary, the present study shows that IL-10

promotes chondrocyte differentiation and proliferation in primary chondrocytes. As depicted in Figure 5D, the findings of this study provide mechanistic insights into cross-talk between IL-10/STAT-3 and BMP signaling. While our studies were largely focused on embryonic and early postnatal growth plate cartilage, there are numerous connections between cartilage development and OA (50). Thus, the mechanisms identified here may also be partly responsible for IL-10–mediated chondroprotective effects in OA.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Han had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Jung, Kim, Han.
Acquisition of data. Jung, Kim, Park, Lee.
Analysis and interpretation of data. Jung, Kim, Choi, Beier, Han.

REFERENCES

1. Rolauffs B, Williams JM, Aurich M, Grodzinsky AJ, Kuettner KE, Cole AA. Proliferative remodeling of the spatial organization of human superficial chondrocytes distant from focal early osteoarthritis. Arthritis Rheum 2010;62:489–98.
2. Kamekura S, Kawasaki Y, Hoshi K, Shimoaka T, Chikuda H, Maruyama Z, et al. Contribution of runt-related transcription factor 2 to the pathogenesis of osteoarthritis in mice after induction of knee joint instability. Arthritis Rheum 2006;54:2462–70.
3. Salminen H, Vuorio E, Saamanen AM. Expression of Sox9 and type IIA procollagen during attempted repair of articular cartilage damage in a transgenic mouse model of osteoarthritis. Arthritis Rheum 2001;44:947–55.
4. Kapoor M, Martel-Pelletier J, Lajeunesse D, Pelletier JP, Fahmi H. Role of proinflammatory cytokines in the pathophysiology of osteoarthritis. Nat Rev Rheumatol 2011;7:33–42.
5. Helmark IC, Mikkelsen UR, Borglum J, Rothe A, Petersen MC, Andersen O, et al. Exercise increases interleukin-10 levels both intraarticularly and peri-synovially in patients with knee osteoarthritis: a randomized controlled trial. Arthritis Res Ther 2010;12:R126.
6. Roman-Blas JA, Stokes DG, Jimenez SA. Modulation of TGF-β signaling by proinflammatory cytokines in articular chondrocytes. Osteoarthritis Cartilage 2007;15:1367–77.
7. Iannone F, De Bari C, Dell'Accio F, Covelli M, Cantatore FP, Patella V, et al. Interleukin-10 and interleukin-10 receptor in human osteoarthritic and healthy chondrocytes. Clin Exp Rheumatol 2001;19:139–45.
8. Ritchlin C, Haas-Smith SA. Expression of interleukin 10 mRNA and protein by synovial fibroblastoid cells. J Rheumatol 2001;28:698–705.
9. Hulejova H, Baresova V, Klezl Z, Polanska M, Adam M, Senolt L. Increased level of cytokines and matrix metalloproteinases in osteoarthritic subchondral bone. Cytokine 2007;38:151–6.
10. Zheng ZH, Li XY, Ding J, Jia JF, Zhu P. Allogeneic mesenchymal stem cell and mesenchymal stem cell-differentiated chondrocyte suppress the responses of type II collagen-reactive T cells in rheumatoid arthritis. Rheumatology (Oxford) 2008;47:22–30.
11. Riyazi N, Slagboom E, de Craen AJ, Meulenbelt I, Houwing-Duistermaat JJ, Kroon HM, et al. Association of the risk of osteoarthritis with high innate production of interleukin-1β and low innate production of interleukin-10 ex vivo, upon lipopolysaccharide stimulation. Arthritis Rheum 2005;52:1443–50.
12. Jansen NW, Roosendaal G, Hooiveld MJ, Bijlsma JW, van Roon JA, Theobald M, et al. Interleukin-10 protects against blood-induced joint damage. Br J Haematol 2008;142:953–61.
13. Muller RD, John T, Kohl B, Oberholzer A, Gust T, Hostmann A, et al. IL-10 overexpression differentially affects cartilage matrix gene expression in response to TNF-α in human articular chondrocytes in vitro. Cytokine 2008;44:377–85.
14. Van Roon JA, van Roy JL, Gmelig-Meyling FH, Lafeber FP, Bijlsma JW. Prevention and reversal of cartilage degradation in rheumatoid arthritis by interleukin-10 and interleukin-4. Arthritis Rheum 1996;39:829–35.
15. Wang Y, Lou S. Direct protective effect of interleukin-10 on articular chondrocytes in vitro. Chin Med J (Engl) 2001;114:723–5.
16. John T, Muller RD, Oberholzer A, Zreiqat H, Kohl B, Ertel W, et al. Interleukin-10 modulates pro-apoptotic effects of TNF-α in human articular chondrocytes in vitro. Cytokine 2007;40:226–34.
17. Umulis D, O'Connor MB, Blair SS. The extracellular regulation of bone morphogenetic protein signaling. Development 2009;136:3715–28.
18. Zeng L, Kempf H, Murtaugh LC, Sato ME, Lassar AB. Shh establishes an Nkx3.2/Sox9 autoregulatory loop that is maintained by BMP signals to induce somatic chondrogenesis. Genes Dev 2002;16:1990–2005.
19. Chimal-Monroy J, Rodriguez-Leon J, Montero JA, Ganan Y, Macias D, Merino R, et al. Analysis of the molecular cascade responsible for mesodermal limb chondrogenesis: Sox genes and BMP signaling. Dev Biol 2003;257:292–301.
20. Minina E, Wenzel HM, Kreschel C, Karp S, Gaffield W, McMahon AP, et al. BMP and IHH/PTHrP signaling interact to coordinate chondrocyte proliferation and differentiation. Development 2001;128:4523–34.
21. Minina E, Kreschel C, Naski MC, Ornitz DM, Vortkamp A. Interaction of FGF, Ihh/Pthlh, and BMP signaling integrates chondrocyte proliferation and hypertrophic differentiation. Dev Cell 2002;3:439–49.
22. Kobayashi T, Lyons KM, McMahon AP, Kronenberg HM. BMP signaling stimulates cellular differentiation at multiple steps during cartilage development. Proc Natl Acad Sci U S A 2005;102:18023–7.
23. Lories RJ, Luyten FP. Bone morphogenetic protein signaling and arthritis. Cytokine Growth Factor Rev 2009;20:467–73.
24. Zoricic S, Maric I, Bobinac D, Vukicevic S. Expression of bone morphogenetic proteins and cartilage-derived morphogenetic proteins during osteophyte formation in humans. J Anat 2003;202:269–77.
25. Bramlage CP, Haupl T, Kaps C, Ungethum U, Krenn V, Pruss A, et al. Decrease in expression of bone morphogenetic proteins 4 and 5 in synovial tissue of patients with osteoarthritis and rheumatoid arthritis. Arthritis Res Ther 2006;8:R58.
26. McLeod MJ. Differential staining of cartilage and bone in whole mouse fetuses by Alcian blue and alizarin red S. Teratology 1980;22:299–301.
27. Woods A, Beier F. RhoA/ROCK signaling regulates chondrogenesis in a context-dependent manner. J Biol Chem 2006;281:13134–40.
28. Gosset M, Berenbaum F, Thirion S, Jacques C. Primary culture and phenotyping of murine chondrocytes. Nat Protoc 2008;3:1253–60.
29. Agoston H, Khan S, James CG, Gillespie JR, Serra R, Stanton LA, et al. C-type natriuretic peptide regulates endochondral bone growth through p38 MAP kinase-dependent and -independent pathways. BMC Dev Biol 2007;7:18.
30. Ulici V, Hoenselaar KD, Gillespie JR, Beier F. The PI3K pathway

regulates endochondral bone growth through control of hypertrophic chondrocyte differentiation. BMC Dev Biol 2008;8:40.

31. Wang G, Woods A, Agoston H, Ulici V, Glogauer M, Beier F. Genetic ablation of Rac1 in cartilage results in chondrodysplasia. Dev Biol 2007;306:612–23.

32. Woods A, Wang G, Beier F. RhoA/ROCK signaling regulates Sox9 expression and actin organization during chondrogenesis. J Biol Chem 2005;280:11626–34.

33. Stanton LA, Sabari S, Sampaio AV, Underhill TM, Beier F. p38 MAP kinase signalling is required for hypertrophic chondrocyte differentiation. Biochem J 2004;378:53–62.

34. Wang G, Woods A, Sabari S, Pagnotta L, Stanton LA, Beier F. RhoA/ROCK signaling suppresses hypertrophic chondrocyte differentiation. J Biol Chem 2004;279:13205–14.

35. Rountree RB, Schoor M, Chen H, Marks ME, Harley V, Mishina Y, et al. BMP receptor signaling is required for postnatal maintenance of articular cartilage. PLoS Biol 2004;2:e355.

36. Yoon BS, Lyons KM. Multiple functions of BMPs in chondrogenesis. J Cell Biochem 2004;93:93–103.

37. Lenton K, James AW, Manu A, Brugmann SA, Birker D, Nelson ER, et al. Indian hedgehog positively regulates calvarial ossification and modulates bone morphogenetic protein signaling. Genesis 2011;49:784–96.

38. Retting KN, Song B, Yoon BS, Lyons KM. BMP canonical Smad signaling through Smad1 and Smad5 is required for endochondral bone formation. Development 2009;136:1093–104.

39. Pathi S, Rutenberg JB, Johnson RL, Vortkamp A. Interaction of Ihh and BMP/Noggin signaling during cartilage differentiation. Dev Biol 1999;209:239–53.

40. Beier F, Ali Z, Mok D, Taylor AC, Leask T, Albanese C, et al. TGFβ and PTHrP control chondrocyte proliferation by activating cyclin D1 expression. Mol Biol Cell 2001;12:3852–63.

41. Shi Y, Massague J. Mechanisms of TGF-β signaling from cell membrane to the nucleus. Cell 2003;113:685–700.

42. Zhang R, Edwards JR, Ko SY, Dong S, Liu H, Oyajobi BO, et al. Transcriptional regulation of BMP2 expression by the PTH-CREB signaling pathway in osteoblasts. PLoS One 2011;6:e20780.

43. Fukuda S, Abematsu M, Mori H, Yanagisawa M, Kagawa T, Nakashima K, et al. Potentiation of astrogliogenesis by STAT3-mediated activation of bone morphogenetic protein-Smad signaling in neural stem cells. Mol Cell Biol 2007;27:4931–7.

44. Solloway MJ, Dudley AT, Bikoff EK, Lyons KM, Hogan BL, Robertson EJ. Mice lacking Bmp6 function. Dev Genet 1998;22: 321–39.

45. Kugimiya F, Kawaguchi H, Kamekura S, Chikuda H, Ohba S, Yano F, et al. Involvement of endogenous bone morphogenetic protein (BMP) 2 and BMP6 in bone formation. J Biol Chem 2005;280:35704–12.

46. Israel DI, Nove J, Kerns KM, Kaufman RJ, Rosen V, Cox KA, et al. Heterodimeric bone morphogenetic proteins show enhanced activity in vitro and in vivo. Growth Factors 1996;13:291–300.

47. Guo X, Wang XF. Signaling cross-talk between TGF-β/BMP and other pathways. Cell Res 2009;19:71–88.

48. Liu Y, Ren W, Warburton R, Toksoz D, Fanburg BL. Serotonin induces Rho/ROCK-dependent activation of Smads 1/5/8 in pulmonary artery smooth muscle cells. FASEB J 2009;23:2299–306.

49. Zhu W, Boachie-Adjei O, Rawlins BA, Frenkel B, Boskey AL, Ivashkiv LB, et al. A novel regulatory role for stromal-derived factor-1 signaling in bone morphogenic protein-2 osteogenic differentiation of mesenchymal C2C12 cells. J Biol Chem 2007;282: 18676–85.

50. Pitsillides AA, Beier F. Cartilage biology in osteoarthritis—lessons from developmental biology [published erratum appears in Nat Rev Rheumatol 2013;9:64]. Nat Rev Rheumatol 2011;7:654–63.