Cell. Mol. Life Sci. 65 (2008) 3494 – 3506
1420-682X/08/213494-13
DOI 10.1007/s00018-008-8342-5

Cellular and Molecular Life Sciences

Research Article

Mediation of Chondrogenic and Osteogenic Differentiation by an Interferon-Inducible p202 Protein

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Received 21 June 2008; received after revision 05 August 2008; accepted 01 September 2008
Online First 15 September 2008

Abstract. p202, an interferon-inducible protein that belongs to an interferon-inducible p200 family, was highly induced in the course of osteogenesis of pluripotent C2C12 cells and the chondrogenesis of C3H10T1/2 cells. Differential expression of p202 is probably due, at least in part, to the transactivation of the p202 gene by Smad transcription factors. Overexpressing p202 inhibited, whereas lowering p202 via a siRNA approach enhanced, chondrocyte differentiation. In contrast, overexpression of p202 enhanced, whereas knockdown of p202 inhibited, osteoblast differentiation. Molecular mechanism studies revealed that p202 and parathyroid hormone-related peptide (PTHrP) formed a positive feedback loop, since (1) overexpressing p202 markedly enhanced whereas knocking down p202 suppressed the expression of PTHrP; and (2) p202 expression was increased in growth plate chondrocytes of PTHrP receptor transgenic mouse embryos; however, its expression was reduced in PTHrP knockout mouse embryos. Taken together, our findings demonstrate that p202 protein is a novel, important mediator of chondrogenic and osteogenic differentiation.

Keywords. p202, interferon, PTHrP, chondrogenesis, osteogenesis.

Introduction

Endochondral bone formation is a tightly regulated developmental process [1, 2]. During bone growth, chondrocyte undergoes proliferation, prehypertrophy, and hypertrophy followed by matrix calcification and chondrocyte apoptosis [3, 4]. Cartilage is eventually replaced by trabecular bone and bone marrow [5]. Multiple signaling pathways and growth factors are involved in endochondral bone formation and resorption [6], and among them bone morphogenetic proteins (BMPs), a subgroup of the transforming growth factor β family (TGF-β), have been known to be a potent inducer of bone formation [7]. BMP-2 was first described for its activity inducing ectopic bone formation [8], and subsequently it was found to play an important role in skeletal development [9, 10], activating differentiation of mesenchymal cells into chondrocytes and osteoblasts *in vivo* [11–13]. BMP-2 has been reported to accelerate differentiation of osteoblasts in monolayer cultures [14] and chondrocytes in micromass cultures [15]. BMP-2 was also observed to enhance hypertrophic chondrocyte differentiation [16] and stimulate expression of genes encoding MMP-13 and type X collagen, which are

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the phenotypic markers of these cells [17]. BMP-2 signals through heteromeric complexes of transmembrane types I and type II serine/threonine kinase receptors that propagate signals to the Smad pathway. Smad proteins mediate BMP-induced signals from the cell surface to the nucleus. Three classes of Smads have been defined: receptor-regulated Smads (R-Smads), common-mediator Smads (Co-Smads), and inhibitory Smads (I-Smads), each of which has a distinct function. R-Smads (Smad1, 5, and 8) are phosphorylated by the type I receptor in response to BMP signals. Once phosphorylated, R-Smads bind to a Co-Smad (Smad4) and enter the nucleus. After translocation into the nucleus, heteromeric Smad complexes regulate transcription of target genes by binding to their consensus DNA sequences, interacting with other transcription factors, and recruiting transcriptional coactivators or corepressors [18, 19]. The interferons (IFNs) are cytokines with antimicrobial, immunomodulatory, and cell growth- and differentiation-regulatory activities [20, 21]. The activities of interferon are mediated by numerous interferon-inducible proteins. Interferons have been found to inhibit the differentiation of osteoclasts by interfering with RANKL-induced expression (RANKL: receptor-activated NF-κB ligand) of c-Fos, an essential transcription factor for the formation of osteoclasts [22, 23]. We previously reported that p204, an interferon-inducible protein that belongs to an interferon-inducible p200 family [24, 25], acts as an important mediator of osteogenesis via associating with Runx2, pRb, and Id helix-loop-helix proteins [6, 26, 27]. Our recent studies demonstrated that p204 also plays a crucial role in chondrocyte hypertrophy [28]. In this study we report the involvement of p202, another well-characterized member of the p200 family, in both osteogenic and chondrogenic differentiation. p202 is an interferon-inducible murine phosphoprotein (52 kDa) and contains two repeats of 200-amino-acids, whose basal levels are detectable in a variety of mouse tissues and cultured cells [25, 29]. p202 slows down cell proliferation [30–34] and modulates apoptosis [35, 36]. p202 negatively regulates cell proliferation in part through the pRb/E2F pathway, since p202 contains the pRb binding motif LxCxE and binds to pRb *in vitro* and *in vivo*[30, 37]. p202-mediated inhibition of cell growth depends on pRb[38]; furthermore, overexpression of E2F-4 partially overcomes p202-mediated inhibition of cell proliferation.

p202 was found to be an important regulator of myogenesis [39]. Expression of p202 inhibits the transcriptional activity of both MyoD and myogenin, and the inhibition by p202 correlates with an interaction of p202 with both proteins, as well as an inhibition of their sequence-specific DNA binding activity. A recent report suggested that p202 regulates hematopoietic cell proliferation and differentiation [40]. Ifi202 (encodes the protein p202) revealed little or no expression in the Lin(-)/c-Kit(+) fraction enriched for immature hematopoietic progenitor cells but higher levels in more differentiated Lin(-)/c-Kit(-) and Lin(+) populations. The highest levels of Ifi202 expression were observed in CD11b (+)/Gr-1(dim) immature granulocytes in the bone marrow. In the peripheral blood, Ifi202 was expressed only in the myeloid lineage, with the highest level of expression seen in CD11b (+)/Gr-1(dim) immature granulocytes. Direct evidence linking p202 to the human autoimmune diseases came from the finding that the interferon-activatable Ifi202 was isolated as a candidate lupus-susceptibility gene in a mouse model of systemic lupus erythematosus (SLE) [41–48], a prototype systemic autoimmune disease, which has potential to involve multiple organ systems. In this study we reveal that p202, a direct target of BMP/Smad signaling, is a novel regulator of both osteogenic and chondrogenic differentiation, and we also elucidate the molecular events involved.

Materials and Methods

Cell culture. The micromass culture was performed as described previously [15]. Briefly, trypsinized C3H10T1/2 cells were resuspended in Dulbecco's modified Eagle's medium (DMEM) with 10% FBS at a concentration of 5×10^6 cells/ml, and six drops of 50 µl of cells were placed in a 60-mm tissue culture dish (Becton Dickinson). After incubation for 2 h at 37°C, 3 ml of DMEM containing 10% FBS and 300 ng/ml exogenous recombinant BMP-2 protein were added. The pluripotent murine mesenchymal cell line C2C12 [49] is a well-established cell line for *in vitro* osteoblast differentiation assays. C2C12 cells cultured in 60-mm dishes in DMEM with 20% fetal bovine serum were treated with 300 ng/ml exogenous recombinant BMP-2. The mouse embryonal carcinoma-derived clonal cell line ATDC5 has been widely used as a model of chondrogenesis in the early stages of endochondral bone development [50, 51]. ATDC5 cells were cultured in the maintenance medium consisting of a 1:1 mixture of DMEM and Ham's F-12 medium containing 5% FBS as previously described [52]. Murine osteoblastic MC3T3-E1 cells were cultured at 37 °C in 5% CO_2 atmosphere in α-modified minimal essential medium (α-MEM; GibcoBRL) containing 10% fetal bovine serum (FBS). MC3T3-E1 cells were then treated at 90% confluence with culture medium containing 10 mM β-glycerophosphate, and 50 µg/ml

ascorbic acid to induce to differentiate. Unless otherwise specified, the medium contained 100 U/ml penicillin and 100 μg/ml streptomycin and was replaced approximated every 2~3 days, and cultures were harvested at various time points for different experimental assay.

Plasmid constructs. To generate two mutants of the p202-ST-luc reporter plasmid, the corresponding segments were amplified using PCR with the following primers: 5'-ctattctctggtctgtctcgtgtgggagagctgcca-3' and 5'-tggcagctctcccacacgagacagaccagagaatag-3' for Mut 1, and 5'-ctattctctggtctgtcagacgtgggagagctgcca-3' and 5'-tggcagctctcccacgtctgacagaccagagaatag-3' for Mut 2, and then digested with Dpn I before being transformed into XL-10 gold ultracompetent cells, following the protocol of the Quickchange XL-site-directed mutagenesis kit (Stratagene, La Jolla, CA).

Real-time PCR. To examine the expression of p202 mRNA in the course of chondrogenesis and osteogenesis, total RNA prepared from various time points of micromass cultures of C3H10T1/2 cells and monolayer cultures of C2C12 cells was isolated with the RNAeasy Mini kit (QIAGEN, Alameda, CA), and then reverse-transcribed to cDNA as described in the protocol of the Improm-II Reverse Transcriptase system kit (Promega, Madison, WI). The oligo 5'-ggcaatgtccaaccgtaact-3' (position 221 to 240 of the mouse p202 cDNA) was used as forward primer, and the oligo 5'-taggtccaggagaggcttga-3' (position 345 to 326 of the mouse p202 cDNA). Reactions were performed in a 50 μl SYBR Green PCR reaction volume in a 96-well optical reaction plate formatted in the 7300 Sequence Detection System (ABI PRISM, Applied Biosystems, Foster City, CA) using the following PCR conditions: 40 cycles: 95 °C for 15 s, 60 °C for 1 min. The transcript of GAPDH was employed as an internal control. For each gene, three independent PCR reactions from the same reverse transcription sample were performed. The presence of a single specific PCR product was verified by melting curve analysis and confirmed on an agarose gel.
To examine whether Samd transcription factors or PTHrP regulate p202 expression, total RNA was extracted from C3H10T1/2 or C2C12 cells transfected with Smads expression plasmids, or treated with PTHrP recombinant protein, and real-time PCR was performed as described above.

Immunoblotting analysis. To examine the expression of p202 protein, total cell extracts prepared from different time points of BMP-2-treated C3H10T1/2 or C2C12 cells were mixed with 5x sample buffer (312.5mM Tris-HCl pH 6.8, 5% β-mercaptoethanol, 10% SDS, 0.5% bromphenol blue, 50% glycerol). Proteins were resolved on a 10% SDS-polyacrylamide gel and electroblotted onto a nitrocellulose membrane. After blocking with 10% nonfat dry milk in Tris buffer saline Tween 20 (10 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.5% Tween 20), blots were incubated with rabbit polyclonal anti-p202 antisera (diluted 1:1000) for 1 h. After washing, the secondary antibody (horseradish peroxidase-conjugated antirabbit immunoglobulin, 1:1000 dilution) was added, and bound antibody was visualized using an enhanced chemiluminescence system (Amersham Biosciences, Piscataway, NJ).

Reporter gene assay. To test whether Smads can activate p202-specific reporter genes, either C3H10T1/2 cells or C2C12 cells grown to about 80% confluence in 35 mm culture dishes were transfected with 1 μg of reporter construct (202–LG-luc or 202–ST-luc) [53] along with 1 μg of β-Gal plasmid (internal control) and mammalian expression plasmids encoding Smad1, Smad5, and Smad4 or various combination, as indicated in the Results section's Figure 2. Forty-eight hours after transfection, the cultures were harvested and lysed. Luciferase assays were performed using 20 μl of cell extract and 100 μl of luciferin substrate (Promega). A β-galactosidase assay was performed using a kit (Applied Biosystems/Tropix, Foster City, CA) according to the manufacturer's protocol, and luciferase activity was measured using a Mini-Lum liminometer (Bioscan, Washington, DC).

Generation and selection of p202 siRNA expression constructs. For silencing the expression of p202, three regions of p202 were targeted for small interfering RNA (siRNA) using mammalian expression pSUPER vector (OligoEngine, Seattle, WA) according to the manufacturer's instructions. To generate siRNAs, equimolar amounts of complementary sense and antisense strands were separately mixed, annealed, and slowly cooled to 10 °C in a 50 μl reaction buffer (100 mM NaCl and 50mM HEPES, pH 7.4). The annealed oligonucleotides were inserted into the BglII/HindIII sites of pSUPER vector. The resultant plasmids and control vector pSUPER were transfected into p202 expression plasmid stable transfected Cos-7 and C3H10T1/2 cells using Lipofectamine 2000 reagent (Invitrogen), and the expression of p202 was determined using an RT-PCR assay. The data demonstrated that siRNA corresponding to the coding sequence of the p202 gene (5'-GGCATCCTAGAGATCAATG -3') were able to efficiently reduce the expression of p202.

Alcian blue staining. To assess the extent of chondrogenesis, micromass cultures of C3H10T1/2 cells were transfected with p202 expression plasmid, pSUPER-p202 encoding siRNA of p202, or corresponding control plasmids maintained in Ham's F-12 medium containing 10 % fetal calf serum in the presence of 300 ng/ml recombinant BMP-2 for 14 days. ATDC5 cells transected with p202 expression plasmid, pSUPER-p202 encoding siRNA of p202, or corresponding control plasmids cultured in the maintenance medium consisting of a 1:1mixture of DMEM and Ham's F-12 medium containing 5 % FBS with addition of 10 mg/ml insulin to the medium for 14 days were rinsed with PBS and fixed with methanol for 20 min and stained overnight with 1 % Alcian blue 8GX (Sigma-Aldrich, St. Louis, MO) in 3 % acetic acid.
For quantitation of alcian blue staining, cells cultured in 96-well dishes were stained with Alcian blue (1 % in 3 % acetic acid) for 30 min, washed three times for 2 min in 3 % acetic acid, rinsed once with water, and solubilized in 1 % SDS. The absorbance at 605 nm was determined for triplicate samples.

Alkaline phosphatase and osteocalcin assays. C2C12 or MC3T3-E1 cells transfected with p202 expression plasmid, pSUPER-p202 encoding siRNA of p202, or corresponding control plasmids were cultured in DMEM containing 20 % fetal bovine serum with BMP-2 (300 ng/ml), or α-MEM medium containing 10 mM β-glycerophosphate, and 50 μg/ml ascorbic acid, respectively, for 4 days. Cells were then lysed for measuring alkaline phosphatase (ALP) activity, and medium was used for determining osteocalcin (OCN) production. In brief, the ALP assay mixtures contained 0.1 M 2-amino-2-methyl-1-propanol (Sigma-Aldrich), 1 mM $MgCl_2$, 8 mM p-nitrophenyl phosphate disodium, and cell homogenates. After 30 min incubation at 37°C, the reaction was stopped with 0.1 M NaOH, and the absorbance was read at 405 nm. A standard curve was prepared with p-nitrophenol (Sigma-Aldrich). Each value was normalized to the protein concentration. The amount of OCN secreted into the culture medium was determined by enzyme-linked immunosorbent assay using a mouse osteocalcin assay kit (Biomedical Technologies, Stoughton, MA) per the manufacturer's protocol.

Immunohistochemistry. Sections of postcoital day 18.5 mouse embryos from wild type mice, PTHrP knockout mice [54], or PTHR1 transgenic mice (the constitutively active PTHR1 in Jansen transgenic mice driven by a rat Col2 promoter) [55] were deparaffinized, rehydrated, and placed in 3 % hydrogen peroxidase (dilute with PBS) for 10 min in dark. 20 % goat serum in 3 % BSA was applied for 1 h at room temperature prior to incubation of the primary antibody. Affinity-purified rabbit anti-mouse p202 was diluted 1:100, incubated at 4 °C overnight. For detection, biotinylated anti-goat secondary antibody (Vector ABC Elite kit) was diluted at 1:200 and incubated for I h at 37 °C followed by Vector ABC peroxidase (Vector) at 37 °C for 1 h and developed with DAB (Sigma) for 2 min at room temperature. Sections were counterstained with Methyl Green (Dako).

Data analysis. Two-sample Student's *t*-tests were used to determine significant differences ($p < 0.05$, $p < 0.01$) in levels of gene expression between experimental and control groups.

Results

Differential Expression of p202 in the course of chondrogenesis and osteogenesis. We previously reported that p204 acts as a cofactor of Cbfa1 and plays an important role in regulating chondrocyte and osteoblast differentiation [6, 26–28]. Given that both p202 and p204 belong to the interferon-inducible p200 protein family [30, 56, 57], which shares a conserved 200-amino-acid-long repeat (either a-type or b-type) [29, 58], we sought to determine whether p202 is also involved in chondrocyte and osteoblast differentiation. We first examined the expression of p202 during chondrocyte differentiation using a micromass culture of the pluripotent C3H10T1/2 cell line, a well-established *in vitro* cell model for study chondrogenesis [59]. As shown in Figure 1A, p202 mRNA was steadily induced in the course of chondrogenesis, reached a peak level by day 5 (approximately 18–fold increase), and then was markedly reduced after that. We further tested the protein level of p202 using the same cell model (Fig. 1C). p202 protein was not detectable until day 5 and highly expressed at day 7 in BMP-2-induced chondrocyte differentiation from C3H10T1/2 cells; collagen X, a specific marker for hypertrophic chondrocyte, was also immunopositve at day 7. The discrepancy between protein and mRNA levels of p202 during chondrogenesis suggests that posttranscription regulation, such as translation, mRNA stability, and protein degradation, might also be important in the control of p202 expression in the course of chondrocyte differentiation.
We next examined whether the p202 level changes in the course of osteoblast differentiation. The pluripotent murine mesenchymal cell line C2C12 [49, 60], an established cell line for *in vitro* differentiation assays, was used because it is capable of differentiating into different cell types, including osteoblasts [7, 61–65].

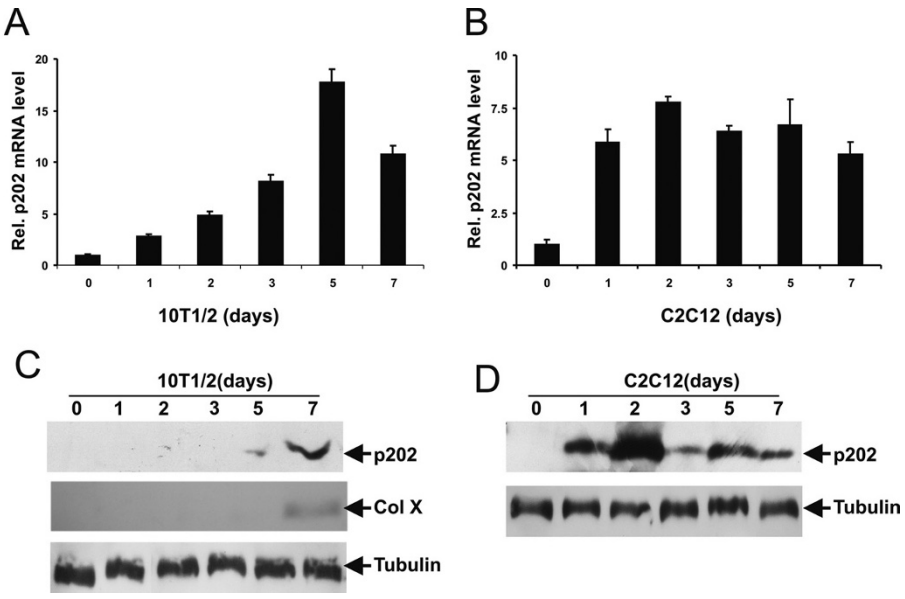

Figure 1. Differential expression of p202 in the course of chondrogenesis and osteogenesis. (*A*) mRNA expression of p202 in the course of chondrogenesis from micromass cultures of C3H10T1/2 cells, assayed by real-time PCR. Total RNA was prepared from micromass cultures of C3H10T1/2 cells in the presence of 300 ng/ml recombinant BMP-2 for various time points, as indicated, and p202 mRNA expression was determined by real-time PCR. Expression of p202 was normalized against GAPDH endogenous control. The normalized values were then calibrated against day 0 control values that were arbitrarily set to 1. Mean values from three independent experiments are shown (error bars indicate standard deviations); *(B)* mRNA expression of p202 in the course BMP-2-induced osteoblastic differentiation of C2C12 cells. Total RNA was prepared from C2C12 cells treated with BMP-2 for various time points, as indicated, and p202 mRNA expression was determined by real-time PCR as in (*A*); (*C*) Expression of p202 protein in chondrogenesis in micromass cultures of C3H10T1/2 cells, assayed by western blotting. Total protein prepared from the same micromass cultures as described in (*A*) was subjected to 10% SDS-PAGE and detected with anti-p202, anticollagen X or antitubulin (used as an internal control), respectively; (*D*) Expression of p202 protein in the course of BMP-2-induced osteoblastic differentiation of C2C12 cells. Cell lysates prepared from C2C12 cells as indicated in (*B*) were assayed by western blotting with either anti-p202 or antitubulin antibodies (used as an internal control).

C2C12 cells cultured in 60 mm dishes in DMEM with 20% fetal bovine serum were treated with 300 ng/ml exogenous recombinant BMP-2. Cultures were harvested at various time points, and real-time PCR (Fig. 1B) and western blot (Fig. 1D) were performed. The results showed that p202 was differentially expressed in the course of osteogenesis: p202 mRNA and protein levels were highly induced as early as day 1 and reached highest level at day 2 following BMP-2 treatment. It remained at a high level for at least 7 days tested. These findings suggest that the gene encoding p202 is an early response gene in the BMP-2-induced signaling for osteoblast differentiation from mesenchymal stem cells.

Smad actives p202-specific report genes. A 1.6-kb segment from the 5'-flanking region of the *Ifi202* gene was found to contain two consensus Smad-binding sites (not shown). This finding prompted us to test for the involvement of Smad-binding elements (SBEs) in the induction of p202 during both chondrocyte and osteoblast differentiation. Two reporter gene plasmids, 202–LG-luc and 202–ST-luc, were used in which segments with SBEs from the 5'-flanking region of *Ifi202*a (–1630 to 59 and –1630 to –1326) were linked to the upstream end of a region encoding luciferase in the pGL3 vector [53] (Fig. 2A). These two p202-specific reporter constructs, together with Smad expression plasmids, were transfected into either C3H10T1/2 or C2C12 cells, and reporter genes activities were measured. As showed in Figure 2B, cotransfection of the reporter plasmids with an expression plasmid encoding Smad1, Samd4, or Smad5 (cDNA constructs kindly provided by Drs. Riko Nishimura and Regis O'Keefe), increased the expression of each of the reporter genes. Although Smad4 alone did not activate the two reporters as much as did Smad 1 and Smad5, it boosted levels of Smad1- and Smad5-activated 202-specific reporter genes. Similar activation of p202-specific reporter constructs by Smad transcription factors was observed in C2C12 cells (Figure 2C). To further determine whether Smad transcription factors activated the endogenous p202 gene, a real-time PCR assay was performed. As shown in Figure 2D, transfection of the expression plasmids encoding Smad1, 4, 5 as well as their combinations into either C3H10T1/2 or C2C12 cells resulted in increased expression of the p202 gene, with higher induction observed in C2C12 cells than in 10T1/2 cells.

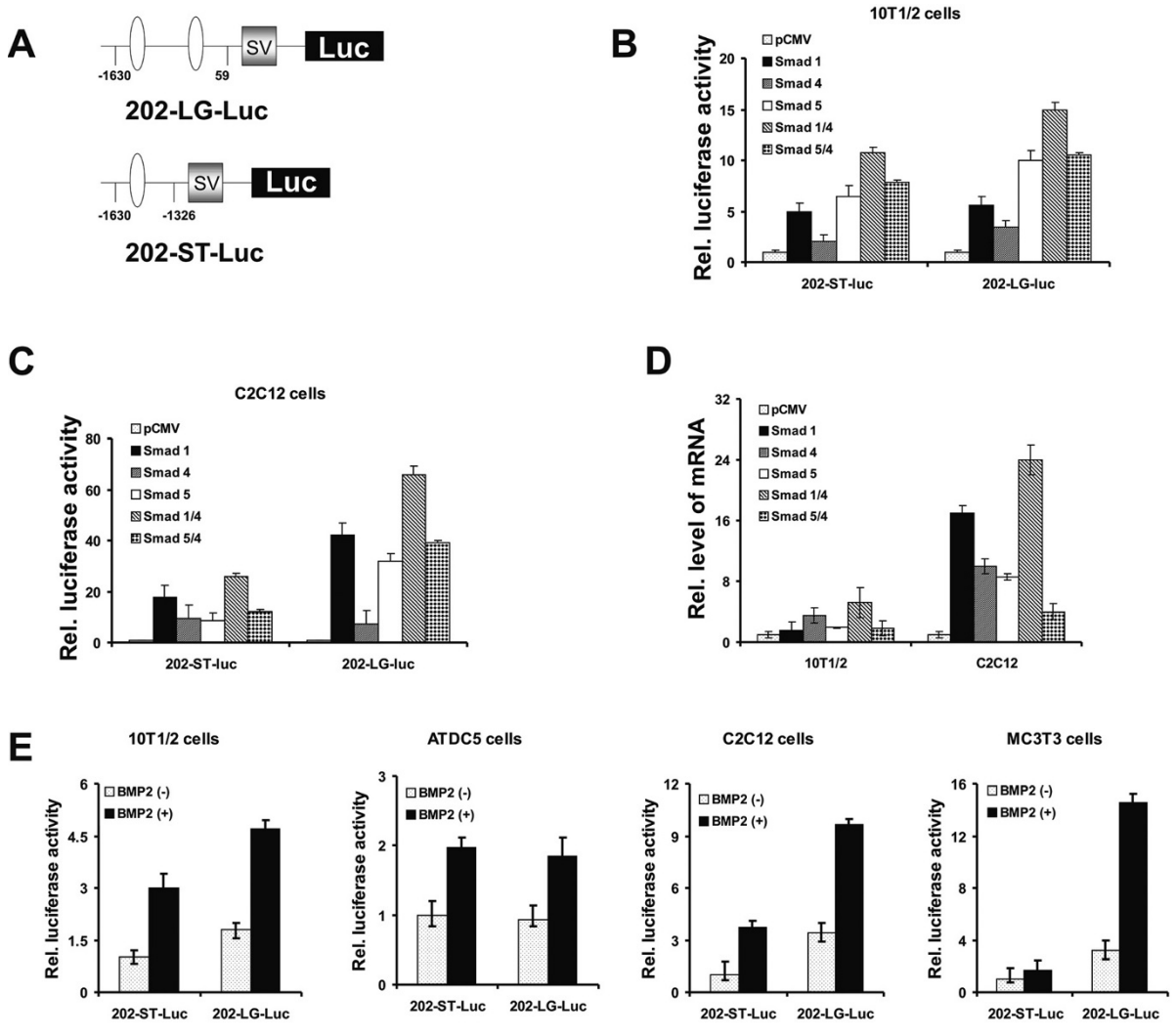

Figure 2. Smad transcription factors activate p202 gene expression. (*A*) Schematic structures of two p202-specific reporter genes. The indicated segments from the 5'-flanking region of the p202 gene were linked to an SV40 promoter ("*SV*") and a DNA segment encoding luciferase ("*Luc*"). Ovals indicate SBEs (Smad-binding site). (*B, C)* Smad activates p202-specific reporter genes (reporter gene assays): Smads can drive the expression of p202-specific reporter genes in C3H10T1/2 (*B*) cells and C2C12 (*C*) cells. The indicated reporter gene was transfected into either 10T1/2 or C2C12 cells together with the indicated Smad expression plasmids (i.e., Smad1, Smad4, Smad5), as well as a β-Gal internal control plasmid. The luciferase activities were normalized to the β-galactosidase activities. The normalized values were then calibrated against the control values that were arbitrarily set to 1. (*D*) Smads activate expression of p202 gene in both C3H10T1/2 and C2C12 cells, assayed by real-time PCR. The indicated Smad expression plasmids were transfected into either C3H10T1/2 or C2C12 cells, total mRNA was extracted, and real-time PCR performed. Expression of p202 was normalized against GAPDH endogenous control. The normalized values were then calibrated against the control values that were arbitrarily set to 1. Means from three independent experiments are shown (error bars indicate standard deviations). (*E*) BMP-2 treatment enhances the expression of p202-specific reporter genes. The indicated reporter genes and a β-Gal internal control plasmid were transfected into either 10T1/2, ATDC5, C2C12, or MC3T3-E1 cells in the presence or absence of 300 ng/ml BMP-2 for 48 h, and the cells were processed as described in (*B*).

Because p202 levels increased substantially in the course of both BMP-2-induced chondrogenesis and BMP-2-induced osteoblastic differentiation (Fig. 1), the effects of BMP-2 on the activity of the two p202-specific reporter genes bearing the 1.6 and 0.25 segments from the 5'-flanking region of the p202 gene (Fig. 2A) were also tested in C3H10T1/2, ATDC5 chondroprogenitors, C2C12, and preosteoblastic MC3T3-E1 cells. As revealed in Figure 2E, exposure of all these transfected cells to BMP-2 clearly increased the expression of both the p202-specific reporter genes tested.

As Smad is known to activate the expression of various genes, including other transcription factors in chondrocytes and osteoblasts, the activation of the p202 gene by Smad observed above (Fig. 2B-D) might be due to the Smad-activatable transcription factor(s). To determine whether the increase in the expression

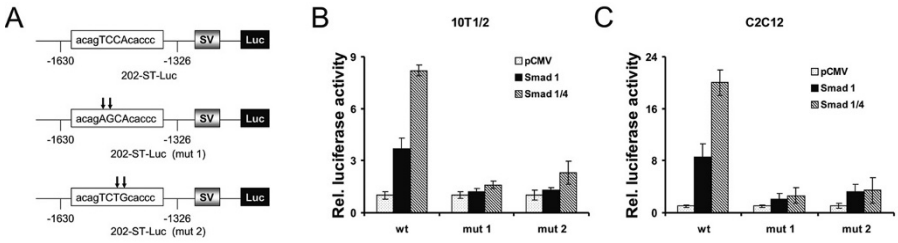

Figure 3. Smad-mediated transactivation of p202 gene depends on direct association of Smad and p202 promoter. (*A*) Diagrams show the alternations in the binding sites in the p202-ST-Luc report gene. Mutant nucleotides are indicated by arrows. (*B*) Smad-dependent transactivation of p202 gene was dramatically reduced when Smad binding site was mutated. The wild-type or mutant reporter gene specified and the β-Gal internal control plasmid were transfected into C2C12 cells together with either control or Smad1 and/or Smad 4 expression plasmid, and the same procedure as described above was followed.

of 202-ST-luc reporter genes in C3H10T1/2 cells and C2C12 cells was directly dependent on the Smad-specific sequences, the Smad-specific binding element in pGL3-202-ST-luc was altered by replacing core nucleotides from the sequence (Fig. 3A). The replacement of the four TCCA with either AGCA or TCTG resulted in a strong decrease in the responsiveness to Smad of the expression of the reporter (Fig. 3B-C). These data suggested that the Smad-dependent increase in the expression of the reporter genes depends on the direct association of Smad and p202 promoter.

p202 inhibits chondrogenesis whereas it enhances osteogenesis. To gain an understanding of the role of p202 in chondrogenesis and osteogenesis, we examined the effect of either overexpression or knockdown of p202 on chondrocyte differentiation from micromass cultures of C3H10T1/2 cells and osteoblast differentiation from C2C12 cells. p202 expression plasmid or pSuper-p202 encoding siRNA were used to overexpress p202 or inhibit its expression, respectively. As revealed in Figure 4A, transfection with pSUPER-p202 in C3H10T1/2 cells resulted in almost a 90% decrease in p202 expression, assayed by western blotting. Micromass cultures of C3H10T1/2 cells transfected with either p202 expression plasmid or p202 siRNA were cultured in the presence of BMP-2, and chondrocyte differentiation was monitored by examining the expression of types II and X collagen, two specific markers for chondrocytes. At the various time points indicated, total RNA was collected and real-time assay was performed. As showed in Figure 4B, peak level of collagen II (approximately 60% reduction) was observed 3 days later in the p202 overexpression group than in the control group; however, there was an approximate 40% increase in collagen II expression when p202 was knocked down via its siRNA. In addition, overexpression or knockdown of p202 led to 60% inhibition or a 2-fold increase in collagen X, a specific marker for hypertrophic chondrocytes, at day 10 after BMP-2 induction (Fig. 4C). In addition, expression of Sox9, Cbfa1, and MMP-13 were also reduced when p202 was overexpressed, whereas their levels, specially that of Cbfa1, were markedly induced when p202 was knocked down (Fig. 4D). Alcian blue staining was also performed using either micromass 10T1/2 cultured in 300 ng/ml BMP-2 or ATDC5 cells cultured in differentiation medium with the addition of 10 mg/ml insulin for 14 days. As showed in Figure 4E, overexpression of p202 in both cell lines gave much weaker staining, while knockdown of p202 appeared to be stronger compared to the control. If we quantify this difference, a greater than 50% reduction or approximately a 40% increase were found in conjunction with overexpression or knockdown of p202 (Fig. 4F). Collectively, these results clearly indicated that p202 is a novel negative regulator of chondrogenesis.

To our surprise, p202 appears to be a positive mediator of osteogenesis, since overexpressing p202 increased the expressions of osteoblast marker genes, including ALP and OCN, in BMP-2-triggered osteoblast differentiation from C2C12 cells; and knockdown of p202 expression using the siRNA approach effectively inhibited ALP and OCN expression in both C2C12 cells (Fig. 5A) and MC3T3-E1 cells (Fig. 5B). Furthermore, overexpression of p202 also reduced ALP activity and OCN production, whereas knockdown of p202 resulted in an increase in both ALP activity and OCN production in both C2C12 and MC3T3-E1 cells (Figs. 5C-F).

p202 is the downstream molecule of PTHrP signaling. Since p202 negatively regulates chondrocyte differentiation, we next sought to determine whether p202 mediated the expression of the signaling molecules Indian hedgehog (IHH) and parathyroid hormone-related peptide (PTHrP), which established a negative feedback loop that regulates the pace of chondrocyte hypertrophy. For this purpose, micromass cultures of C3H10T1/2 cells transfected with either p202 or pSUPER-p202 were treated with BMP-2 for 7 days

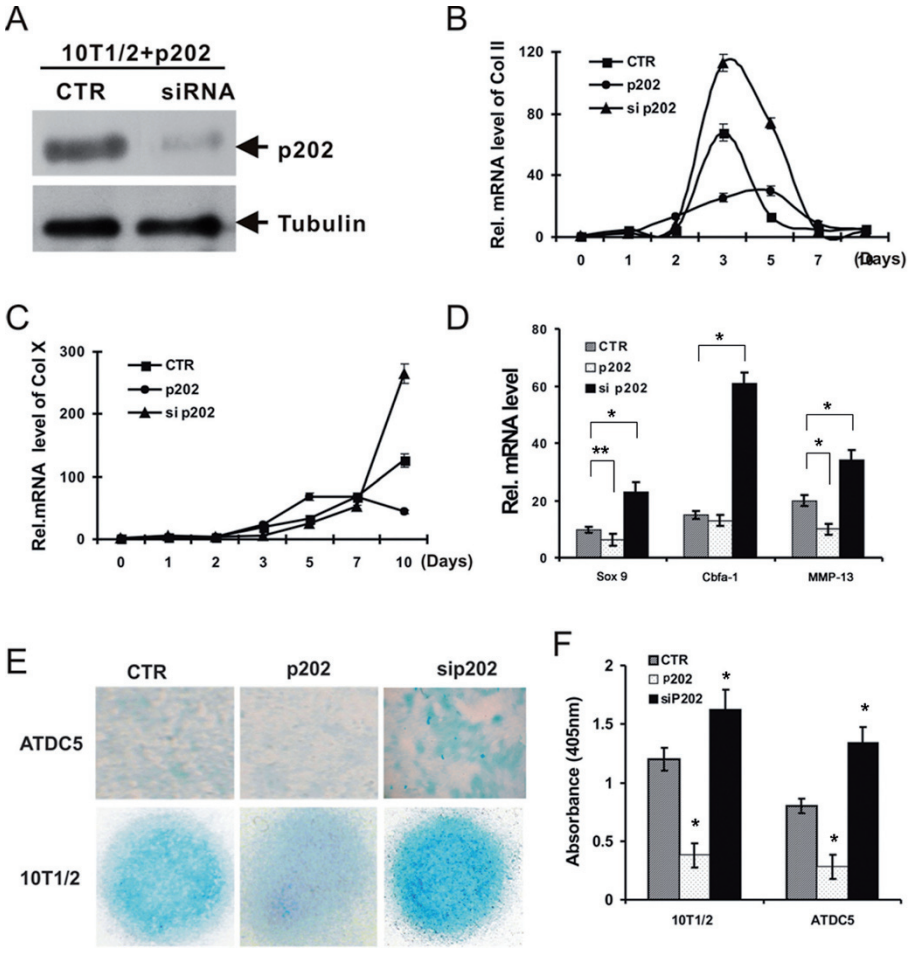

Figure 4. p202 regulates chondrogenesis. (*A*) siRNA against p202 mRNA efficiently inhibit expression of p202. C3H10T1/2 cells were cotransfected with p202 expression plasmid and either a siRNA-p202 expression plasmid pSUPER-p202 (siRNA) or control plasmid pSUPER-CTR encoding a scrambled sequence (CTR), as indicated, for 2 days, and the level of p202 in the cell lysates was visualized by western blotting with anti-p202 antibody; tubulin was used as an internal control; (*B, C)* overexpression of p202 inhibits, while knockdown of p202 increases, chondrogenesis in C3H10T1/2 cells. Transcript levels of collagen II (*B*) and collagen X (*C*) were detected by real-time PCR. Their expressions were normalized against the GAPDH control. The normalized values were then calibrated against the day 0 control values that were arbitrarily set to 1. Means from three independent experiments are shown (error bars indicate standard deviations); (*D*) Effects of altered expression of p202 on Sox9, Cbfa1, and MMP-13 in chondrogenesis of C3H10T1/2 cells, assayed by real-time PCR. *, $P < 0.01$; **, $P < 0.05$. *(E)* Whole-mount Alcian blue histochemistry. Staining was performed on the micromass cultures of transfected C3H10T1/2 cells, as indicated, treated with 300 ng/ml recombinant BMP-2, or transfected ATDC5 cells, as indicated, cultured with 10 mg/ml insulin for 14 days. (*F*) Quantity analysis of Alcian blue staining after 14 days in culture as indicated in *(E).* Cells cultured in 96-well dishes were stained with Alcian blue (1 % in 3 % acetic acid) and solubilized in 1 % SDS. The absorbance at 605 nm was determined for triplicate samples. *, $P < 0.01$.

and real-time PCR performed. The results in Figure 6A demonstrated that overexpression of p202 remarkably increased, whereas knockdown of p202 suppressed, the expression of PTHrP; furthermore, overexpression of p202 inhibited, whereas knockdown of p202 stimulated, the expression of IHH.

We next determined whether PTHrP induced p202 expression. As the data in Figure 6B show, this was the case, and this induction was dose-dependent. Thus, p202 and PTHrP form a positive feedback regulatory loop in the course of chondrogenesis. Furthermore, to determine whether p202 was the target molecule of PTHrP signaling, an immunohistochemistry staining was performed using growth plates of day-18.5 embryos from PTHrP-null or PTHR1 transgenic mice in which constitute active PTHR1 is derived by collagen II promoter. As shown in Figure 7, p202 demonstrated clear expression in the growth plate chondrocytes, with higher expression in prehypertrophic chondrocytes in wild-type mice, which resembled the expression pattern of PTHrP [66–68]. Intriguingly, the expression of p202 was markedly elevated in the growth plate chondrocytes in the PTHrP receptor transgenic mice, whereas it was reduced in the growth plates from PTHrP knockout mice, indicating that *in vivo* expression of p202 depends, at least in part, on PTHrP signaling.

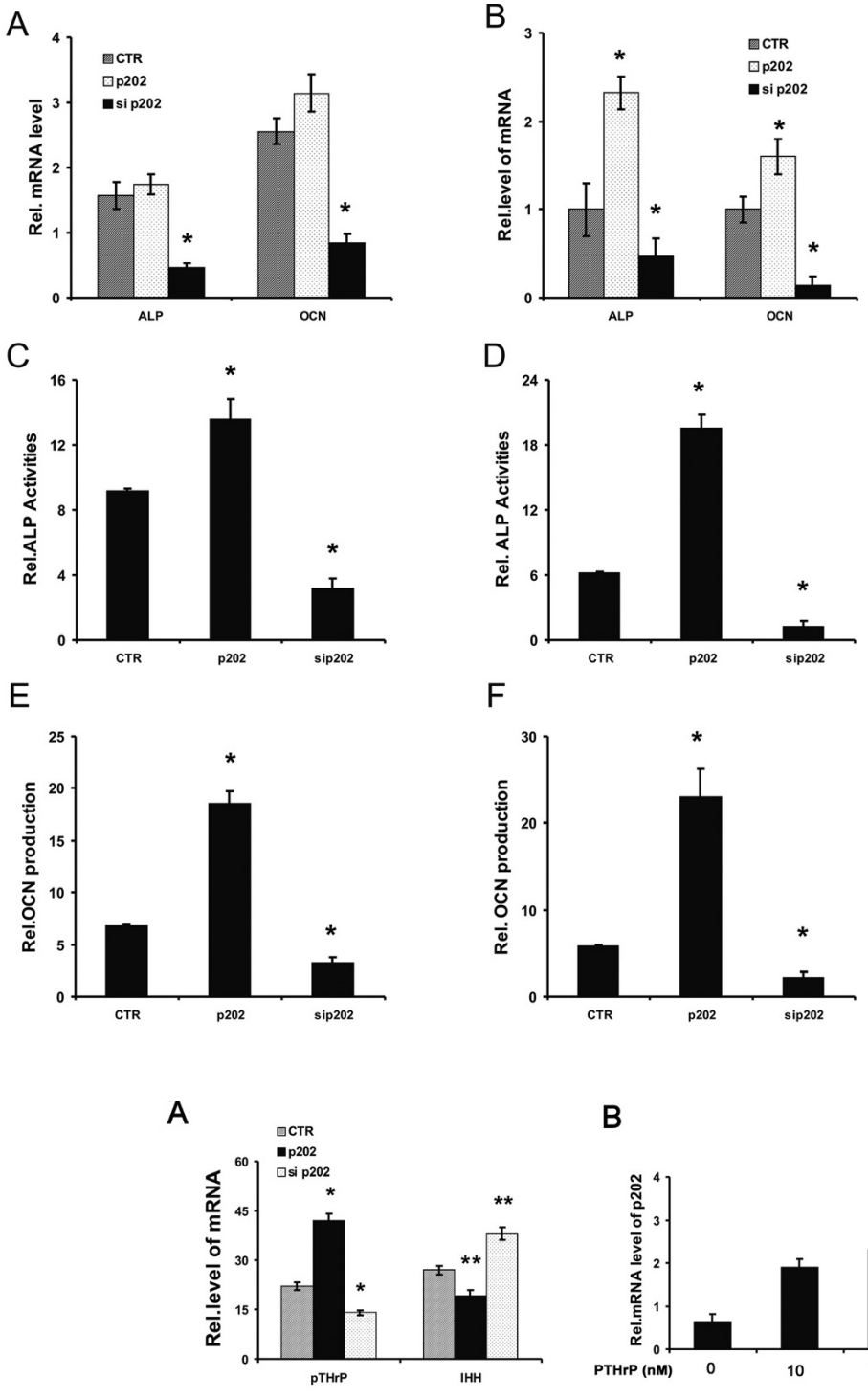

Figure 5. p202 regulates osteogenesis of C2C12 cells. (*A, B*) Repression of p202 dramatically inhibits ALP and OCN expression in osteogenesis of C2C12 cells (*A*) and MC3T3-E1 cells (*B*). Transcript levels of alkaline phosphatase (ALP) and osteocalcin (OCN) were detected by real-time PCR and analyzed as described before. (*C, D*) Overexpression of p202 enhances, while knockdown of p202 inhibits, BMP-2-induced ALP activity. Both C2C12 *(C)* and MC3T3-E1 *(D)* cells were cultured in the presence or absences of 300 ng/ml recombinant BMP-2 for 72 h, and the cell lysates were used for determining ALP activity. (*E, F*) Overexpression of p202 enhances, while knockdown of p202 inhibits, BMP-2-induced OCN production. Cultures were treated as described in (*C, D*) and the medium collected for measuring OCN production. Data are means of triplicate samples (error bars indicate standard deviations). *, $P < 0.01$.

Figure 6. p202 and PTHrP constitute a positive feedback regulation loop. (*A*) Altered expression of p202 affects the expression of PTHrP/IHH in chondrocyte differentiation. Transcript levels of PTHrP and IHH were detected by real-time PCR analysis of RNA isolated from micromass cultures of C3H10T1/2 cells transfected with either an expression plasmid encoding p202 or pSUPER-p202 in the presence of 300 ng/ml of BMP-2 for 7 days. Expression of the target gene was normalized against the GAPDH endogenous control. The normalized values were then calibrated against the day 0 control values that were arbitrarily set to 1. Means from three independent experiments are shown (error bars indicate standard deviations). *, $P < 0.01$; **, $P < 0.05$. (*B*) Dose-dependent induction of p202 expression by PTHrP in chondrocyte differentiation in micromass cultures of C3H10T1/2 cells. Micromass cultures of C3H10T1/2 cells were cultured in the presence of 300 ng/ml BMP-2 for 5 days, various doses of PTHrP, as indicated, were added to the medium, and cells were continued in culture for 48 hr before the total RNA was extracted and real-time PCR performed. Expression of the p202 gene was normalized against the GAPDH endogenous control. The normalized values were then calibrated against the control values that were arbitrarily set to 1. Means from three independent experiments are shown (error bars indicate standard deviations).

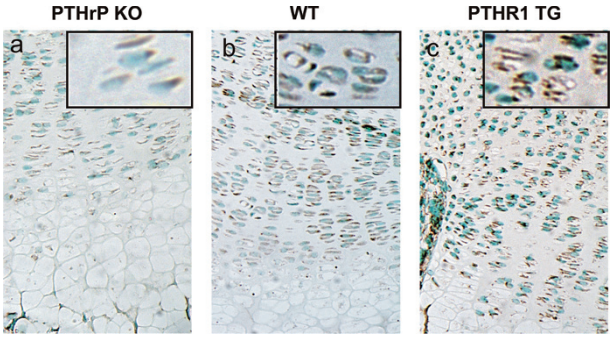

Figure 7. Expression of p202 in growth plate chondrocytes is PTHrP-dependent (immunohistochemistry). Microphotograph of section in growth plate of day-18.5 embryos of PTHrP knockout (*a*, PTHrP KO), wild-type (*b*, WT), and PTHR1 transgenic (*c*, PTHR1 TG) mice. Sections were stained with anti-p202 antibody (brown) and counterstained with methyl green (green). Immunostaining reveals positive nuclear and cytoplasmic staining in proliferating and prehypertrophic chondrocytes (see insets). One representative image from four embryos in each group is shown.

Discussion

Interferons are cytokines with multiple biological functions, and their actions are exerted via numerous interferon-inducible proteins [69–71]. Interferons have been found to inhibit the differentiation of osteoclasts by interfering with the RANKL-induced expression of c-Fos, an essential transcription factor for the formation of osteoclasts [22, 23]. We previously reported that p204, a member of an interferon-inducible p200 family [24, 25], acted as a transcriptional coactivator of Cbfa1 and therefore enhanced osteoblast differentiation [6]. We also demonstrated that pRb is an essential linker between p204 and Cbfa1, thereby increasing its activity [26]. p204, pRb, Cbfa1, and Ids form a protein interaction network and act in concert in regulating the differentiation of pluripotent C2C12 to osteoblasts [27]. The current study elucidates the expression, regulation, and function of p202, another member of the p200 family, during both osteogenic and chondrogenic differentiation. The p202 level demonstrated highest expression at 2 days and remained high level thereafter in the BMP-2 induced osteogenesis of pluripotent C2C12 cells (Fig. 1D). This suggests that the gene encoding p202 is an early response gene in osteoblast differentiation. In contrast, in the course of chondrocyte differentiation of C3H10T1/2 cells, p202 mRNA was found to be induced at a later stage of chondrogenesis (Fig. 1A), and p202 protein was not detectable until day 5 and reached its highest level at day 7 when collagen X (a specific marker for hypertrophic chondrocytes) was also detectable (Fig. 1C). This indicates that p202 expression is prehypertrophic- and hypertrophic chondrocyte-specific. Differential expression of p202 is probably due, at least in part, to the transactivation of p202 gene by Smad transcription factors, including regulatory Smad 1, Smad 5, and Co-Smad 4, based on the findings that (1) Smads activated p202-specific reporter genes bearing Smad-binding sites in both C2C12 and C3H10T1/2 cells; and (2) Smad induced the expression of endogenous p202 mRNA, as assayed by real-time PCR (Fig. 2).

Our recent report showed that p204 positively regulates chondrocyte differentiation via affecting Runx2 and PTHrP activity [72]; however, p202 functions as a negative regulator of chondrogenesis, because overexpression of p202 inhibited, whereas knockdown of p202 enhanced, BMP-2 induced chondrocyte differentiation of pluripotent C3H10T1/2 cells, as assayed by the expressions of collagen II and collagen X, two marker genes of chondrocyte differentiation (Fig. 4). Similar to the case of p204 which was found to positively regulate osteogenesis [6], p202 was also shown for the first time to be a positive mediator in the course of osteogenic differentiation, as demonstrated by the findings that overexpression of p202 increased, whereas knockdown of p202 decreased the levels of alkaline phosphatase and osteocalcin, two specific markers for osteogenesis (Fig. 5). It appears that the major effects are observed when endogenous p202 is downregulated by siRNA rather than when it is overexpressed (Figs. 3, 4), suggesting that p202 is an endogenous protein with relatively rapid turnover.

Multiple signaling pathways are involved in endochondral ossification in epiphyseal growth plate [5]. Among them, PTHrP and IHH coordinately regulate the rate of chondrocyte differentiation through a feedback loop [3, 73, 74]. Several lines of evidences indicated that PTHrP negatively regulated endochondral bone formation. PTHrP prevents chondrocyte hypertrophy in the growth plate and maintains a pool of cells above the hypertrophic zone in a proliferative condition [73]. PTHrP stimulates proliferation and inhibits hypertrophy and thereby controls the transition from proliferation to differentiation. In embryonic mice lacking PTHrP, chondrocytes stop proliferating prematurely, with accelerated differentiation [75]. Mice lacking either PTHrP [54, 76] or the parathyroid hormone (PTH)/PTHrP receptor [77, 78] also have accelerated chondrocyte differentiation and impaired skeletal growth, exhibiting shortened zones of proliferative chondrocytes and premature hypertrophic differentiation. In contrast, animals overexpressing PTHrP in chondrocytes show delayed hypertrophic differentiation [79]. Chondrocyte-specific expression of a constitutively active PTH/PTHrP receptor also delays the conversion of proliferative to hypertrophic chondrocytes [55]. In mouse models both *PTHrP* and *PTH/PTHrP* receptor knockout

mice display advanced endochondral bone formation [54, 77, 78]. Our studies demonstrated that (1) overexpressing p202 enhanced whereas knocking down p202 suppressed the expression of PTHrP (Fig. 6A); (2) PTHrP induced p202 expression in the course of chondrogenesis *in vitro* (Fig. 6B); and (3) p202 expression strictly depends on PTHrP in the growth plate chondrocytes *in vivo* (Fig. 7). These findings suggest that there exists a positive feedback regulatory loop between p202 and PTHrP signaling in the course of chondrogenesis. In addition, these findings also provide a functional link between interferon and PTHrP signaling in regulating skeletogenesis. Sox family transcription factors, including Sox5 and Sox6, are known to play a critical role in controlling chondrogenesis; however, their expression was not particularly affected by p202 (not shown), suggesting that p202-mediated chondrocyte differentiation might function specifically through affecting PTHrP signaling. Whether the regulatory circuit between p202 and PTHrP, and whether such other factors as Cbfa1, pRb, and Ids, which have been shown to be important for p204 action in osteogenesis [6, 26, 27], are involved in the p202-regulated osteoblast differentiation remain to be determined.

Acknowledgments. We thank Drs. H. M. Kronenberg and T. Kobayashi for providing PTHrP knockout and PTHR1 transgenic mice; Drs. H. Wang, P. Lengyel, R. Nishimura and R. O'Keefe for cDNA constructs. We also thank reviewers for their comments; Williams S. Green for critical reading and editing. This work was aided by NIH research grants AR050620, AR052022, AR053210 and AG029388 and a grant from Arthritis National Research Foundation.

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