We investigated the effects of bone morphogenetic protein (BMP)-2, a member of the transforming growth factor-β superfamily, on the regulation of the chondrocyte phenotype, and we identified signaling molecules involved in this regulation. BMP-2 triggers three concomitant responses in mouse primary chondrocytes and chondrocytic MC615 cells. First, BMP-2 stimulates expression or synthesis of type II collagen. Second, BMP-2 induces expression of molecular markers characteristic of pre- and hypertrophic chondrocytes, such as Indian hedgehog, parathyroid hormone/parathyroid hormone-related peptide receptor, type X collagen, and alkaline phosphatase. Third, BMP-2 induces osteocalcin expression, a specific trait of osteoblasts. Constitutively active forms of transforming growth factor-β family type I receptors and Smad proteins were overexpressed to address their role in this process. Activin receptor-like kinase (ALK)-1, ALK-2, ALK-3, and ALK-6 were able to reproduce the hypertrophic maturation of chondrocytes induced by BMP-2. In addition, ALK-2 mimicked further the osteoblastic differentiation of chondrocytes induced by BMP-2. In the presence of BMP-2, Smad1, Smad5, and Smad8 potentiated the hypertrophic maturation of chondrocytes, but failed to induce osteocalcin expression. Smad6 and Smad7 impaired chondrocytic differentiation induced by BMP-2. Thus, our results indicate that Smad-mediated pathways are essential for the regulation of the different steps of chondrocyte and osteoblast differentiation and suggest that additional Smad-independent pathways might be activated by ALK-2.

The development of long bones involves first the formation of cartilage primordia, which prefigure the future skeletal elements, and the replacement of the cartilage by bone. The formation of cartilage is initiated by the condensation and differentiation of mesenchymal cells into chondrocytes. This commitment to the skeletal lineage progresses through a switch in gene activation and results in the production of a cartilage matrix, which contains predominantly type II collagen and the proteoglycan aggrecan. During replacement of cartilage by bone through the sequence of events called endochondral ossification, chondrocytes enter a process of maturation, characterized by cellular hypertrophy and onset of type X collagen expression (reviewed in Ref. 1). This is followed by vascular invasion, matrix degradation, and replacement of the cartilage by bone marrow and trabecular bone.

Chondrocyte differentiation and maturation is thus a central cellular aspect of skeletal development, and a molecular understanding of skeletogenesis requires an understanding of how expression of the chondrocytic phenotype is regulated. The fate of the skeletal cells and their precursors is controlled by the extracellular matrix, growth factors, hormones, and cytokines. Bone morphogenetic proteins (BMPs) play a particularly important role in skeletal formation. Although BMPs have been shown to have a broad spectrum of action on proliferation, differentiation, and apoptosis in numerous cellular systems, they are the only members of the TGF-β superfamily that have the ability to stimulate ectopic bone formation by recapitulating all the events occurring during endochondral ossification (2), and to potentiate chondrocyte and osteoblast differentiation in vitro (3–5).

Cytokines of the TGF-β superfamily signal through two types of serine/threonine kinase receptors. The type II receptors are constitutively active kinases, which phosphorylate and activate the type I receptors upon ligand binding (6). Seven type I receptors, originally named activin receptor-like kinases (ALKs), have been cloned in mammals. ALK-1 (TSR-1) and ALK-2 (ActR-I) are structurally highly similar. A recent study has demonstrated that ALK-1 acts as a TGF-β receptor in endothelial cells (7). ALK-2 has been shown to bind activin, BMP-7, and, weakly, BMP-2 (8) and also to mediate Mullerian inhibiting substance signaling (9, 10). ALK-3 (termed also BMP type IA receptor or BMPR-IA) and

1 The abbreviations used are: BMP, bone morphogenetic protein; ALK, activin receptor-like kinase; Co-Smad, common partner Smad; DMEM, Dulbecco’s modified Eagle’s medium; EC, embryonic chondrocyte; FBS, fetal bovine serum; HA, hemagglutinin; I-Smad, inhibitory Smad; m.o.i., multiplicity of infection; PBS, phosphate-buffered saline; PEC, primary embryonic rib chondrocyte; PTHrP, parathyroid hormone/parathyroid hormone-related peptide; R-Smad, receptor-regulated Smad; RT, reverse transcription; TGF-β, transforming growth factor-β.
its close structural and functional homologue ALK-6 (BMPR-IB) bind BMPs. ALK-4 (ActR-IB) and ALK-5 (TβR-1) are the type I receptors for activin and TGF-β, respectively. ALK-7 is structurally similar to the last two receptors, and, although its physiological ligand has not been reported yet, it appears to act as a receptor for nodal proteins and related ligands (11). The type I receptors act downstream from the type II receptors and are the effectors of the signal transduction. When activated, the type I receptors phosphorylate intracellular mediators, the Smad proteins. Eight Smad proteins have been identified in mammals, and these Smads are classified into three groups according to their function (reviewed in Ref. 12): the receptor-regulated Smads (R-Smads), the common mediator Smad (Co-Smad) and the inhibitory Smads (I-Smads). The R-Smads are phosphorylated by the type I receptors upon ligand binding. Smad2 and Smad3 are involved in the TGF-β/activin pathway, whereas Smads 1, 5, and 8 act in response to BMPs. The different phosphorylated R-Smads interact with the single Co-Smad identified in mammals, Smad4. The heteromeric complex then translocates into the nucleus, binds directly or indirectly to the DNA, and is responsible for the transcriptional regulation of target genes. The inhibitory Smads (Smad6 and Smad7) are structurally divergent from the other Smads and antagonize the transcription of the signal.

The expression of BMP receptors has been shown to be required for the maintenance of the differentiated phenotype and hypertrophic maturation of chondrocytes (13). However, despite the recent reports on the involvement of TGF-β superfamily receptor and Smad pathways in the osteogenic conversion of pluripotent cells or in the induction of chondrogenic differentiation of teratocarcinoma cells (14–16), similar detailed analysis of the chondrogenic lineage has not yet been fully accomplished, particularly during hypertrophic maturation of chondrocytes. MC615 is an established immortalized clonal mouse chondrocyte cell line that has been shown to express several biochemical markers of the articular cartilage (17–19), and has been used to determine the role of Sox transcription factors in the control of chondrocyte differentiation (20, 21). We previously reported that BMP-2 could trigger hypertrophic maturation of MC615 chondrocytes and their osteoblastic differentiation (22). In the present study, we first extended these observations with a detailed identification of key genes targeted by BMP-2 in MC615 cells and freshly isolated mouse embryonic chondrocytes. We further determined the role of various type I receptors and Smads in chondrocyte phenotype maintenance, hypertrophic maturation, and osteoblastic differentiation.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—The MC615 cell line has been characterized previously (17). For the studies with freshly isolated chondrocytes, embryonic mouse chondrocytes were prepared from the ventral parts of the rib cages of 17.5-day post coitum mice, as described (23). The MC615 cells and rib chondrocytes were maintained in 1:1 high glucose DMEM/Ham’s F-12 containing 10% FBS and supplemented with 2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin (all products from Invitrogen) on Corning dishes, as previously described (22).

For the test assays, MC615 cells and primary embryonic rib chondrocytes were grown for 3 days as above, except that FBS was 1% and BMP-2 (0–100 ng/ml) was added to the medium. Recombinant human BMP-2 was produced and purified by Genetics Institute, Inc. (Cambridge, MA). The culture medium supplemented with BMP-2 was replaced each day.

**Immunofluorescence**—Freshly isolated chondrocytes were grown for 3 days on Lab-tch chamber slides (Nunc) and were fixed with 2.5% paraformaldehyde in PBS (Sigma) for 30 min and rinsed in PBS. After treatment with hyaluronidase (800 units/ml, Sigma type I) for 30 min at 37 °C, intracellular staining was achieved by permeabilizing cells with 0.1% Triton X-100 in PBS for 20 min followed by a wash in PBS. Cultured chondrocytes were incubated for 30 min with 1% bovine serum albumin in PBS and then incubated for 1 h with the 2B1 primary monoclonal antibody specific for type II collagen (24). Cells were then washed with PBS, and Cy3-conjugated secondary antibodies against mouse immunoglobulins (Jackson) were applied for 1 h. Chondrocytes were then washed with PBS and slides were mounted in refro/ PBS (1:1) for their observation by epifluorescence with an Axioplan 2 Zeiss microscope.

**Reverse Transcription-Polymerase Chain Reaction (RT-PCR)**—Total RNAs were extracted from cultured cells and from rib cage of 2-day-old mice with the RNeasy kit (Qiagen), and digested with DNase to remove any contaminating genomic DNA. For RT, a 40-μl reaction contained 1 μg of total RNA, 12.5 ng/μl oligo(dT)12–18 (VN) (V represents A, G, or C, and N represents A, T, G, or C), 500 μM each dNTP, 100 ng/μl bovine serum albumin, 10 mM dithiothreitol, 4 units of RNasin (Promega), and 200 units of SuperScript II RNAse H− (Invitrogen). Reactions were carried out at 42 °C for 50 min, followed by an inactivation of the enzyme at 70 °C for 15 min. The cDNAs were then incubated with 4 units of RNase H (Invitrogen) at 37 °C for 30 min. For PCR amplification, each 20-μl reaction contained 1% agarose-formamide gels and transferred to nylon N-membranes (Amersham Biosciences). Northern hybridization was carried out as previously described (17) with probes labeled with [α-32P]dCTP by random priming (26). Probes included mouse cDNAs encoding α1(I) procollagen (27), α1(I) procollagen (28), and osteocalcin (29).

**LacZ Assay**—The mouse osteocalcin gene 2 (OG2) promoter fused to the luciferase reporter gene (pL316-luc) was generously provided by P. Ducy (30). The β-galactosidase-encoding expression vector, pL51, and the plasmid pGL2-Basic (Promega) were included in all transfections as internal controls. MC615 cells were seeded in six-well tissue culture plates with 1.75×104 cells/well and were incubated for 2 days with 1:1 high glucose DMEM/Ham’s F-12 supplemented with 1% FBS ± BMP-2. Cells were then transfected with 1 μg of pGL2-Basic or 1 μg of pL316-luc in combination with 1 μg of pL316-luc using LipofectAMINE (Invitrogen), according to the manufacturer’s instructions. After 5 h of incubation, cells were washed and incubated for 18 h with 1:1 high glucose DMEM/Ham’s F-12 supplemented with 1% FBS ± BMP-2, without antibiotics. Cells were harvested, rinsed twice in cold PBS, and solubilized in Reporter Lysis buffer (Promega). After clearing by centrifugation, luciferase and β-galactosidase activities in the cell lysates were determined with a luminometer by using Luciferase Assay System (Promega) and Galacto-Light Plus (Tropix) detection kits, respectively.

**Alkaline Phosphatase Activity**—Histochemical analysis of alkaline phosphatase activity was carried out as described (4). Cells were washed with PBS and fixed with 2.5% paraformaldehyde in PBS at 37 °C for 10 min. After washing twice with PBS, cells were incubated for 5 min in a mixture of 0.1 mg/ml naphthyl phosphate (Sigma), 0.5% N,N-dimethylformamide, 2 mM MgCl2, and 0.6 mg/ml fast blue BB salt (Sigma) in 0.1 M Tris-HCl, pH 8.5, at 37 °C, followed by observation using phase-contrast microscopy.

**Infection with Reombinant Adenovirus**—Different recombinant adenoviruses containing cDNAs for hemagglutinin (HA)-tagged type I receptors, FLAG-tagged Smads, and β-galactosidase were constructed, amplified, and titrated as described (15, 31). For infection with the
adenoviruses, MC615 cells and embryonic rib chondrocytes (after one passage) were seeded in six-well tissue culture plates (with 1.75 × 10^5 MC615 cells/well and 2 × 10^5 rib chondrocytes/well) and maintained for 24 h in standard medium supplemented with 10% FBS. The medium was replaced with fresh medium containing 1% FBS 2 h before the infection. Infection with recombinant adenoviruses was performed at a m.o.i. ranging between 100 and 500 plaque-forming units/cell. Infected cells were cultured for 3 days.

**Immunoblotting**—Cells infected with adenoviruses were washed twice with PBS and solubilized in a buffer containing 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% (w/v) Triton X-100, and protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, 0.1 μg/ml leupeptin, and 0.1 μg/ml pepstatin A). Lysates were cleared by centrifugation and subjected to SDS-polyacrylamide gel electrophoresis. The proteins were electrotransferred to nitrocellulose membranes (PROTRAN BA83, Schleicher & Schuell), immunoblotted with anti-HA 3F10 antibody (Roche Molecular Biochemicals) or anti-FLAG M2 antibody (Sigma), and visualized using the COVALIGHT chemiluminescence detection system (CovalAb, Oullins, France) with horseradish peroxidase-labeled secondary antibodies.

| Gene          | Primers                                      | Strand | Product size (bp) | T (°C) | PCR cycles | Reference   |
|---------------|----------------------------------------------|--------|-------------------|--------|------------|-------------|
| Col2a1        | GCCTCGCGGTGAGCCATGATC CTCCATCTGCGGAGGTT     | +      | 472               | 60     | 30         | 22          |
|               | -                                            |        | 268               |        |            |             |
| Aggrecan      | CCAGTGGCAGTTGCGGATAGG TGTTCTGCCGAGAAGGGT    | +      | 271               | 55     | 30         | 5           |
| Sox9          | TGCGAGACAGTGACCAGCCAGCATG TCTTTTCGTCGAGGCG | +      | 136               | 35     | 35         | 5           |
| Ihh           | CATGACCGGAGCTGAGG AGCTCTGAGGTGCTG          | -      | 470               | 60     | 40         | U85610      |
| Col10a1       | GCAACTAAGGCGTTCAATG GAGGCCTAGGAATCTGAG     | -      | 596               | 37     | 35         | M18443      |
| Alkaline      | GCCGAAGCAGAAGAAGCC GAGGAGGCTCCAGGCG       | +      | 450               | 57     | 35         |             |
| phosphatase   | -                                            |        | 59                |        |            |             |
| PTH/PTHrP      | TTCTGGTGTCACATGACCG TCTGAGGCAAGGCG         | +      | 579               | 59     | 40         | X78936      |
| Receptor      | GCCACTGCTGCGGCTCTGC                         | -      | 293               | 65     | 40         | AF010284    |
| Chfa1         | GAGCCGGCGCCAGCAACGAGCAGCTGA                | -      | 371               | 57     | 38         | X04142      |
| Osteocalcin   | CAAGTCCACACGAGACCTT GAGCAGCTGGAAGGTT        | +      | 653               | 57     | 35         | 45          |
| Bone sialo    | GAGCGACGCAGTGGCAGAAAGGACC GCTCTGACGCTG    | +      | 293               | 65     | 40         | AF010284    |
| protein       | -                                            |        | 371               |        |            |             |
| Smad1         | GTATGCAGATTGGCTAGTGG GAGGAGGATGATGGTAC   | +      | 486               | 47     | 30         | NM_005900   |
| Smad2         | GTAGACATGCTGGCAGGCAACAGGAGAAGGAGTG       | +      | 490               | 50     | 35         | U60530      |
| Smad3         | CTGACAAAGCCAGCAATGGC GCTGTGGCAGCTGCTG    | +      | 417               | 57     | 35         | AB008192    |
| Smad4         | CTGCCGAGCTGAGGCAACACAC TACCTGACGCTG    | +      | 438               | 57     | 37         | NM_008540   |
| Smad5         | CTGCACATTCCACATGGGCT CTATGGTCAATACATTCAAC | +      | 564               | 49     | 35         | U77638      |
| Smad6         | GACCAGTAAGCCAAGAGAGAGAGAATCTGAGTGGG      | +      | 628               | 57     | 37         | AF010133    |
| Smad7         | CAGATTTCCAACTTTCTCTTG TGGTGGAGTACAGCTC   | +      | 533               | 57     | 35         | AF015260    |
| Smad8         | CACCTAGGCTGCCGTCTCTCCA GCTGCTGCTGACTCAGC | +      | 429               | 49     | 40         | AF175408    |
| GAPDH         | ATCTGCGGCCACCAAGAACGATGAGGTTCTACCTTGT    | +      | 443               | 57     | 25         | 22          |
Fig. 1. Effect of BMP-2 on chondrogenic and osteogenic expression of MC615 cells and PEC. A, RT-PCR analysis of mRNA expression of proc-II collagen, aggrecan, and Sox9. MC615 cells and PEC were grown for 3 days in presence of 1% FBS (0) or in the presence of 1% FBS with 50 or 100 ng/ml BMP-2 as indicated, or in presence of 10% FBS. The type IIA and IIB forms of type II procollagen mRNA were amplified in the same PCR reaction. Total RNAs extracted from rib cartilage (ribs) of 2-day-old mice were used as a positive control for RT-PCR. B, Northern blot analysis of mRNA expression of type II and type X procollagen, and osteocalcin. MC615 cells were grown for 3 days in presence of 1% FBS (0) or in the presence of 1% FBS with 50 or 100 ng/ml BMP-2 as indicated, and total RNAs were extracted as described under “Experimental Procedures.” Each lane was loaded with 20 μg of RNA. After transfer, the membrane was hybridized sequentially with the probes corresponding to the gene products indicated on the right. Bottom, ethidium bromide staining of the agarose gel shows the integrity and equivalent amounts of RNAs, with the positions of 28 and 18 S ribosomal RNAs. C, BMP-2 stimulates type II collagen synthesis in PEC. Cells were grown for 3 days in presence of 1% FBS (0) or in the presence of 1% FBS with 50 or 100 ng/ml BMP-2 as indicated, and immunostained using an anti-type II collagen antibody.

RESULTS

BMP-2 Maintains or Stimulates Chondrogenic Expression—The type II procollagen gene (Col2a1) can be expressed in two forms by differential splicing of the primary gene transcript. The two mRNAs either include (type IIA) or exclude (type IIB) exon 2, which encodes the major portion of the amino-propeptide (32). MC615 cells weakly expressed both forms when cultured in medium containing 1 and 10% FBS, as revealed by RT-PCR analysis (Fig. 1A). The treatment with BMP-2 stimulated expression of both transcripts in MC615 cells, type IIA representing the predominant form. The most pronounced stimulation was shown with 50 ng/ml BMP-2 (Fig. 1A), and Northern blotting analysis with a probe recognizing both IIA and IIB forms of type II collagen mRNA confirmed this dose-dependent stimulation (Fig. 1B). Sox9 is a transcription factor expressed in the chondrogenic lineage during skeletogenesis (33) and involved in the regulation of the expression of Col2a1 and aggrecan genes (34–36). RT-PCR analysis showed that the basal level of aggrecan and Sox9 expression was very low in MC615 cells grown in 1% FBS and was stimulated in the presence of BMP-2 (Fig. 1A). Total RNAs were also extracted from rib cage of 2-day-old mice. This tissue undergoes endochondral ossification and therefore was used as a positive control for RT-PCR analysis of gene expression characteristic of chondrocytes and osteoblasts. The type IIB form of Col2a1 was the only form detected in rib cage (Fig. 1A) as expected, because type IIB mRNA represents an expression of the differentiated chondrocyte phenotype whereas type IIA mRNA represents the major form in both non-chondrogenic and pre-chondrogenic cells (37). In parallel, expressions of aggrecan and Sox9 were barely detectable in this tissue (Fig. 1A) and this should be related to the fact that aggrecan and Sox9 are much less expressed than Col2a1 in cartilage, and that endochondral ossification is well advanced in rib cage of 2-day-old mice. This is further supported by the clear detection of numerous gene expressions associated with hypertrophic chondrocytes and osteoblasts in rib cage (see Fig. 2).

The chondrocytic cell line MC615 was obtained by immortalization of embryonic mouse chondrocytes with the large T oncogene of the simian virus 40 (17). To ascertain that large T expression did not interfere with the response of MC615 cells to BMP-2, we monitored the same experiments using freshly isolated embryonic mouse chondrocytes. Primary embryonic chondrocytes (PEC) expressed type IIB mRNA but barely expressed type IIA mRNA, as judged by RT-PCR analysis (Fig. 1A). In comparison with MC615 cells, higher levels of aggrecan and Sox9 were expressed in PEC, and no sign of BMP-2 effect was observed on these expressions (Fig. 1A). A slight increase in Col2a1 expression was observed in PEC treated with 50 ng/ml BMP-2 (Fig. 1A). Consistent with this expression pattern, BMP-2 treatment led to an increase in type II collagen synthesis in PEC, as indicated by the higher number of cells positively stained for type II collagen when compared with cells grown in the presence of 1% FBS only (Fig. 1C). These results together indicate that BMP-2 stimulates chondrogenic expression in MC615 cells and PEC.

BMP-2 Induces Hypertrophic Maturation and Osteoblastic Differentiation of Chondrocytes—During endochondral ossification, chondrocytes undergo hypertrophy. During this process of maturation, cells start to synthesize type X procollagen, a characteristic marker of hypertrophic chondrocytes (38), and alkaline phosphatase. The rate and extent of endochondral bone formation is tightly regulated by Indian hedgehog (Ihh) and parathyroid hormone-related peptide (PTHrP), two molecules acting coordinately as a negative feedback loop on chondrocyte hypertrophy (39–41). The Ihh gene is expressed in prehypertrophic chondrocytes and although PTHrP itself is secreted far away from the expression domain of Ihh, the gene coding its receptor (PTH/PTHrP receptor) is expressed in the prehypertrophic cartilage zone, as well as in osteoblasts (42). BMP-2 induced expression of type X collagen gene (Col10a1), Ihh, and PTH/PTHrP receptor in MC615 cells. This effect was dose-dependent, as revealed by Northern blotting (Fig. 1B) and/or RT-PCR analyses (Fig. 2).

During bone formation, osteoblast differentiation is marked by the sequential activation of many genes encoding characteristic bone matrix proteins like alkaline phosphatase, type I collagen, bone sialoprotein, osteopontin, and osteocalcin (43). Osteocalcin is exclusively synthesized by mature osteoblasts (44) and therefore represents a bone-specific marker. Cbfa1 is a transcription factor that has been shown to regulate the expression of all the major genes expressed by osteoblasts, such as the genes coding for osteocalcin (30, 45), alkaline phosphatase (46), the α1 and α2 chains of the type I procollagen (47), and osteopontin (48). Our RT-PCR analysis indicated that cbfa1 expression in MC615 cells was not modulated by BMP-2 (Fig. 2). However, osteocalcin expression in MC615 cells was induced by BMP-2 in a dose-dependent manner, as shown by Northern blotting (Fig. 1B) and RT-PCR (Fig. 2) analyses. In
parallel, PCR assays showed that alkaline phosphatase and bone sialoprotein expressions were stimulated in response to BMP-2 (Fig. 2). We further tested whether the increase in the steady-state level of osteocalcin mRNA observed in MC615 cells treated with BMP-2 reflected an increase at the transcriptional level. MC615 were treated for 3 days with 100 ng/ml BMP-2, and transcriptional activation was measured by assessing luciferase expression from a 1,316-bp promoter segment of the mouse osteocalcin 2 gene (OG2). This promoter segment has been shown to confer the same regulation and expression pattern in vivo as the endogenous promoter (49). In DNA transfection experiments, BMP-2 stimulated the transcription from this promoter (Fig. 3A).

When expression of genes characteristic of prehypertrophic chondrocytes (Ihh, PTH/PTHrP receptor), hypertrophic chondrocytes (Col10a1), and/or osteoblasts (alkaline phosphatase, bone sialoprotein, cbfa1, and osteocalcin) was examined by RT-PCR in PEC treated with BMP-2, we found similar profiles of expression as with MC615 cells (Fig. 2). The responsiveness of MC615 cells and PEC to BMP-2 was further compared at the protein level. Histochemical analysis of alkaline phosphatase activity showed a few positive cells in the control cultures. The number of positive cells increased in a dose-dependent manner when MC615 cells or PEC were treated with BMP-2 (Fig. 3B), which is in accordance with the mRNA-based data (Fig. 2).

Identification of Critical Type I Receptors Mediating Chondrocyte Differentiation — The type II receptors activate the type I receptors with the phosphorylation of serine residues in a glycine- and serine-rich domain (GS domain). The mutation of the penultimate threonine or glutamine residue in the GS domain into an acidic residue such as aspartic acid leads to constitutive activation of the type I receptor kinase. Thus, these mutated receptors transduce signals without ligand binding or type II receptors activation (50–53).

We analyzed the role of type I receptors in the maturation and induction of osteoblastic differentiation of chondrocytes. To obtain high transfection efficiency, we infected MC615 cells and embryonic rib chondrocytes (passed once and termed EC) with recombinant adenoviruses carrying cDNAs coding for constitutively active forms of type I receptors. More than 90% of cells infected with lacZ-containing adenoviruses displayed a β-galactosidase activity (data not shown). The induction of maturation and osteoblastic differentiation by constitutively active forms of type I receptors was first monitored by analysis of alkaline phosphatase activity. Histochemical analysis revealed that the number of positively stained MC615 cells and EC was significantly higher in cultures overexpressing ALK-1, ALK-2, ALK-3, and ALK-6, in comparison with cultures overexpressing LacZ, ALK-4, or ALK-5 (Fig. 4A). More precisely, expression of ALK-2 led to the highest number of positively stained MC615 cells and EC, whereas expression of ALK-6 showed the mildest effect.

Differentiation of chondrocytes by type I receptors was further examined by analyzing gene expressions characteristic of
hypertrophic chondrocytes and osteoblasts. We carried out RT-PCR experiments with RNAs isolated from MC615 cells and embryonic rib chondrocytes infected with the adenoviruses. ALK-1, ALK-2, and ALK-3 stimulated Col10a1 expression in MC615 cells and EC (Fig. 4B). A slight increase in Col10a1 expression was noted in EC overexpressing ALK-6. However, ALK-5 had no effect on Col10a1 expression, whereas ALK-4 decreased the steady-state level of Col10a1 mRNA, in both MC615 cells and EC (Fig. 4B). Similar results were obtained when alkaline phosphatase expression was examined. Interestingly, the most pronounced up-regulation of Col10a1 and alkaline phosphatase expression was observed with the active ALK-2 receptor, in MC615 cells and EC (Fig. 4B). Moreover, induction of osteocalcin expression was observed only in MC615 cells and EC overexpressing ALK-2 (Fig. 4B). Similarly, RT-PCR analysis showed that PTH/PTHR receptor mRNA was present in MC615 cells and EC only after overexpression of the active ALK-2 receptor (data not shown). The synthesis of HA-tagged type I receptors was confirmed by immunoblotting using anti-HA antibody (Fig. 4B).

TGF-β Receptor ALK-5 Opposes Maturation and Osteoblastic Differentiation of Chondrocytes Induced by BMP-2 or ALK-2—Because TGF-β has been shown to inhibit expression of alkaline phosphatase and osteocalcin in osteoblasts (54, 55), we further investigated whether TGF-β receptor ALK-5 could antagonize BMP-2 or ALK-2 to induce hypertrophic maturation and osteoblastic differentiation of chondrocytes. For this purpose, MC615 cells were infected with ALK-5-containing adenoviruses before treatment with BMP-2, or were coinfected with ALK-2- and ALK-5-containing adenoviruses. As shown in Fig. 5 (A and B), induction of alkaline phosphatase activity triggered by BMP-2 or by overexpression of ALK-2 was markedly reduced by overexpression of ALK-5. In addition, RT-PCR analysis clearly showed that overexpression of ALK-5 inhibits Col10a1, alkaline phosphatase, and osteocalcin expression induced by BMP-2 treatment or overexpression of ALK-2 (Fig. 5C). It is also expected that activin receptor ALK-4 would oppose maturation and osteoblastic differentiation of chondrocytes induced by BMP-2 or ALK-2, similar to the responses observed for ALK-5. This is strongly supported by the fact that ALK-4, like ALK-5, down-regulates Col10a1 and alkaline phosphatase expression in chondrocytes (Fig. 4B).

Smads 1, 5, and 8 Favor Hypertrophic Maturation of Chondrocytes—We first analyzed by RT-PCR the expression of R-Smads and Smad4 in MC615 cells cultured in the absence or presence of 50 or 100 ng/ml BMP-2. As shown in Fig. 6A, Smads 1–5 were constitutively expressed in MC615 cells and their level of expression was not modulated by BMP-2. In contrast, the basal level of Smad8 expression was very low in these cells and treatment with BMP-2 increased the steady-state level of Smad8 mRNA (Fig. 6A). The up-regulation of Smad8 gene expression was also noted by RT-PCR analysis when MC615 cells were infected with adenoviruses carrying cDNAs coding for ALK-1, ALK-2, ALK-3, and, to a lesser extent, ALK-6 (Fig. 6B).

We next assessed involvement of Smad4 and R-Smads in the maturation of chondrocytes, by monitoring alkaline phosphatase activity after infection of MC615 cells and EC with adenoviruses carrying cDNAs coding for these Smads. Overexpression of Smad4 alone or with Smads 1, 2, 3, 5, or 8 did not modify alkaline phosphatase activity as observed by histochemical analysis (data not shown). Therefore, we examined the effects of Smad4 and R-Smads on alkaline phosphatase activity in MC615 cells treated with 25 ng/ml BMP-2 for 3 days after adenovirus infection (Fig. 7A). As expected, the number of positive cells increased slightly when the cells were treated with 25 ng/ml BMP-2. When expressed alone, Smad4 or each of the R-Smads had no additive effect on the alkaline phosphatase activity induced by BMP-2 (data not shown). Smad1, Smad8, and, to a lesser extent, Smad5, in cooperation with Smad4, increased the number of alkaline phosphatase-positive cells in the presence of BMP-2 (Fig. 7A). However, coexpression of Smad2/Smad4 or Smad3/Smad4 had no cooperative effect on alkaline phosphatase activity. Similar patterns of alkaline phosphatase activity were obtained with EC coexpressing Smad4 and R-Smads in the presence of 50 ng/ml BMP-2 (data not shown).

To characterize more precisely the cellular phenotype induced by overexpression of R-Smads and Smad4, we analyzed Col10a1, alkaline phosphatase, and osteocalcin expression by RT-PCR in MC615 cells infected with the adenoviruses and treated with 25 ng/ml BMP-2. At this low concentration,
BMP-2 alone or BMP-2 in combination with Smad4 showed no effect on these expressions (Fig. 7B). Smad1, Smad8, and, to a lesser extent, Smad5, increased Col10a1 and alkaline phosphatase expression upon BMP-2 treatment. In contrast, Smad2 and Smad3 seemed to down-regulate these expressions. Furthermore, overexpression of R-Smads and Smad4 did not seem to stimulate osteocalcin expression (Fig. 7B). Immunoblotting using anti-FLAG antibody attested that robust expression levels of FLAG-tagged Smads were detected in infected MC615 cells (Fig. 7B). It should be noted that overexpression of R-Smads and Smad4 in the presence of 50 ng/ml BMP-2 remained inefficient to stimulate osteocalcin expression (data not shown).

Inhibitory Smads Inhibit Chondrogenic Expression and Block Maturation and Osteoblastic Differentiation of Chondrocytes Induced by BMP-2—Gene expression of I-Smads was ex-
Smad6 expression was not detected, whereas Smad7 was weakly expressed in MC615 cells cultured with 1% FBS. Addition of BMP-2 up-regulated these expressions in a dose-dependent manner.

We next tested whether I-Smads were involved in the phe-
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nototypic modulation of chondrocytes induced by BMP-2. MC615 cells were infected with adenoviruses carrying cDNAs coding for I-Smads and then treated with 50 ng/ml BMP-2. The expression of characteristic chondrocytic and osteoblastic markers was analyzed by RT-PCR (Fig. 8B). Smad6 and Smad7 impaired the stimulation of Col2a1, aggrecan, and Sox9 expression induced by BMP-2. More precisely, Smad6 and Smad7 decreased aggrecan and Sox9 expression to a lower level than in control cultures with 1% FBS, and Smad7 showed the same effect on Col2a1 expression. In addition, Smad6 and Smad7 opposed the effect of BMP-2 on maturation of chondrocytes and on their osteoblastic differentiation: induction of Col10a1, alkaline phosphatase, bone sialoprotein, and osteocalcin expression by BMP-2 was reduced or abolished by Smad6 or Smad7 (Fig. 8B). Comparable levels of FLAG-tagged Smad6 and Smad7 proteins were detected by immunoblotting using anti-FLAG antibody (Fig. 8B).

In agreement with these RT-PCR results, the blocking effect of I-Smads on the maturation of chondrocytes induced by BMP-2 was also demonstrated at the protein level. MC615 cells were infected with the adenoviruses carrying cDNA coding for Smad6 or Smad7 and treated with 50 ng/ml BMP-2. Histological analysis showed that Smad6 and Smad7 inhibited the increase in alkaline phosphatase activity induced by BMP-2 (Fig. 8C). Moreover, the number of positively stained cells in the presence of each I-Smad was lower than in LacZ-infected cells cultured without BMP-2 (Fig. 8C). Similar patterns of alkaline phosphatase activity were obtained when identical infection experiments were performed with EC (data not shown).

**DISCUSSION**

BMP-2 Favors Chondrogenic Expression and Promotes Hypertrophic Maturation and Osteoblastic Differentiation of Chondrocytes—We previously reported that BMP-2 could induce Col10a1 and osteocalcin expression in MC615 cells (22). In the present study, we first asked whether SV40 large T expression interferes with the biological activities of BMP-2 on these immortalized chondrocytes. Our RT-PCR analysis indicated that chondrogenic expression was stronger in PEC than in MC615 cells, as attested by the expression of Col2a1 restricted to the IIB form and by the higher levels of aggrecan and Sox9 expression in PEC. Because the chondrocyte is a cell known to de-differentiate easily in monolayer, these differences should be attributed to a slight de-differentiation of MC615 cells routinely cultured on plastic, not to SV40 large T expression. Furthermore, we found that BMP-2 was able to trigger three concomitant and similar responses in both cell types. First, BMP-2 stimulates the expression of the cartilage phenotype, as shown by the stimulation of Col2a1, aggrecan, and Sox9 expression in MC615 cells, and by the stimulation of Col2a1 expression and type II collagen synthesis in PEC. Second, BMP-2 induces the maturation of chondrocytes into pre- and hypertrophic chondrocytes as revealed by Ihh, PTH/PTHrP receptor, and Col10a1 expression in MC615 cells and in PEC. Third, expression of alkaline phosphatase and bone sialoprotein as well as alkaline phosphatase activity were stimulated by BMP-2 in both cell types. These latter expressions are classically described in the literature as signs of osteoblastic differentiation, but hypertrophic chondrocytes also express alkaline phosphatase and bone sialoprotein (56, 57). The induction of osteoblastic differentiation by BMP-2 was then unequivocally demonstrated by the induction of the steady-state levels of osteocalcin mRNAs in immortalized and freshly isolated chondrocytes and by the stimulation of transcriptional activity of the OC2 promoter in MC615 cells. Taken together, these results indicate that SV40 large T expression does not interfere with the biological activities of BMP-2 on specific genes and, thus, that the MC615 cell line provides a suitable system to examine the molecular mechanisms underlying BMP-2 signaling in chondrocytes. Our results are also in line with previous reports showing that BMP-2 induces chondrogenesis and/or osteogenesis in pluripotent mesenchymal precursor cell lines (4, 58–60) and in teratocarcinoma cells (61), and induces hypertrophic maturation of chondrocytes (62). Here, we present evidence for the first time that BMP-2 induces chondrocytes to transdifferentiate into osteoblastic cells.

Overexpression of ALK-2 Reproduces BMP-2-induced Differentiation of Chondrocytes into Osteoblasts—Specificity of the interaction of type I receptors with R-Smads is determined by
FIG. 7. Smads 1, 5, and 8 potentiate hypertrophic maturation of MC615 cells. A, histochemical analysis of alkaline phosphatase activity in MC615 cells showing the effects of Smads in presence of 25 ng/ml BMP-2. Cells were infected with adenoviruses carrying cDNA coding for Smad4 alone or in combination with adenoviruses carrying cDNAs coding for R-Smads, as indicated. Adenovirus carrying β-galactosidase-coding cDNA was used as a control (LacZ). The m.o.i. are shown in parentheses. Three days after infection, the cells were stained for alkaline phosphatase activity and observed by phase-contrast microscopy. B, top, RT-PCR analysis of type X collagen, alkaline phosphatase, and osteocalcin mRNA expression in MC615 cells infected with recombinant adenoviruses carrying cDNAs coding for β-galactosidase or Smads, under the conditions described in A. Cells were treated with or without 25 ng/ml BMP-2, as indicated. Bottom, the synthesis of Smads was confirmed by anti-FLAG immunoblotting.
FIG. 8. I-Smads (Smad6 and Smad7) inhibit chondrogenic expression and osteoblastic differentiation of MC615 cells induced by BMP-2. A, RT-PCR analysis of Smad6 and Smad7 expression in MC615 cells grown for 3 days in presence of 1% FBS (0) or in the presence of 1% FBS with 50 or 100 ng/ml BMP-2, or in the presence of 10% FBS, as indicated. Total RNAs extracted from 2-day-old mouse rib cartilage (ribs) were used as a positive control for RT-PCR. B, top, MC615 cells infected with adenoviruses carrying Smad6- or Smad7-coding cDNA (m.o.i. of 300) were treated with 50 ng/ml BMP-2. Three days after infection, total RNAs were extracted for RT-PCR analysis of gene expression of markers characteristic of chondrocytes (type II collagen, aggrecan, Sox9), hypertrophic chondrocytes (type X collagen, alkaline phosphatase, bone sialo-protein), and osteoblasts (osteocalcin). Adenovirus with β-galactosidase-coding cDNA (m.o.i. of 500) was used as a control. Bottom, the synthesis of I-Smads was confirmed by anti-FLAG immunoblotting. C, histochemical analysis of alkaline phosphatase activity in MC615 cells infected with adenoviruses carrying cDNAs coding for β-galactosidase, Smad6, or Smad7 and cultured in the absence or presence of 50 ng/ml BMP-2. The m.o.i. are shown in parentheses. Three days after infection, the cells were stained for alkaline phosphatase activity and observed by phase-contrast microscopy.
a short region in the serine/threonine kinase domain, termed the L45 loop (63). The L45 loop of type I receptors interacts with the C-terminal Mad homology 2 domain of R-Smads (64–66). These L45 loop regions can be classified in three subgroups; ALK-4, ALK-5, and ALK-7 have an identical L45 loop amino acid sequence, as have ALK-3 and ALK-6, and there is only one amino acid difference between the L45 loops of ALK-1 and ALK-2 (67). Although the L45 loops of ALK-1 and ALK-2 are the most divergent from those of the other type I receptors, ALK-1 and ALK-2 transduce signals similar to that of ALK-3 and ALK-6 (66, 68, 69). Indeed, our study shows that the constitutively active forms of type I receptors of the ALK-1 group (ALK-1 and ALK-2) and those of the BMP group (ALK-3 and ALK-6) induced a similar pattern of Col10a1 expression and a similar pattern of gene expression and activity for alkaline phosphatase. This latter observation is consistent with the findings of Fuji et al. (15), who reported that overexpression of active ALK-1, ALK-2, ALK-3 and ALK-6 induce alkaline phosphatase activity in myoblastic C2C12 cells. In addition, our results clearly show that ALK-2 allows chondrocytes to undergo further differentiation into osteoblastic cells, as attested by osteocalcin expression and by the highest alkaline phosphatase activity observed in MC615 cells and EC producing active ALK-2. Thus, our results indicate that overexpression of active ALK-2 in chondrocytes mimics the osteoblastic differentiation of chondrocytes induced by BMP-2.

TGF-β Receptor ALK-5 Inhibits Maturation and Osteoblastic Differentiation of Chondrocytes Mediated by BMP-2 or ALK-2—TGF-β inhibits osteocalcin expression in various osteoblastic cells (70–72). Here, we have provided evidence that TGF-β receptor ALK-5 antagonizes hypertrophic maturation and osteoblastic differentiation of cartilage cells induced by BMP-2 or ALK-2. This result again strongly suggests that ALK-2 plays a key role in mediating BMP-2 signaling in chondrocytes, particularly in regard of their osteoblastic differentiation. Moreover, our finding is consistent with the results of Spinella-Jaegle et al. (73), who reported that BMP-2 and TGF-β exert opposite effects on osteoblast differentiation. Altogether, these data indicate that TGF-β signaling antagonizes the BMP-2 signaling cascade involved in chondrogenic and osteogenic differentiation. Furthermore, because our data suggest that activin receptor ALK-4 functions like ALK-5, it is highly plausible that activin signaling also antagonizes BMP-2 function in chondrocytes. Further studies are needed to identify the molecular components of these cross-talks.

Smads 1, 5, and 8 Potentiate Hypertrophic Maturation of Chondrocytes—BMPs and constitutively active BMP type I receptors have been shown to transmit intracellular signals by Smad proteins. In our assays, the expressions of genes typical of hypertrophic chondrocytes that were induced by BMP-2 or constitutively active ALK-1, ALK-2, ALK-3, and ALK-6 are probably mediated mainly via a Smad-signaling pathway. The genes coding the five R-Smads were constitutively expressed in MC615 cells, with a weak expression of Smad8. It has been reported that Smad1 and Smad5 are both involved in the intracellular BMP-induced signals, which inhibit myogenic differentiation and induce osteoblastic differentiation, as assessed by alkaline phosphatase activity (15, 74, 75). However, the specific functional role of Smad8, which displays a high homology with Smad1 and Smad5, is not clear. Smad8 has been shown to be phosphorylated by ALK-2, ALK-3, and ALK-6 and thus acts as a downstream mediator for these receptors (53, 76). Moreover, dominant-negative Smad8 was shown to inhibit the increase in alkaline phosphatase activity induced by BMP-2 in mesenchymal C3H10T1/2 cells and C2C12 myoblastic cells, indicating that Smad8 might play a role in this induction (53). Recently, Aoki et al. (16) reported that BMP-4 induces alkaline phosphatase activity equally well in the presence of Smad1, Smad5, and Smad8 in C2C12 cells. However, BMP-6 induces alkaline phosphatase activity in the same C2C12 cells, through the activation of Smad1 and Smad5, but not Smad8 (14). In our study, BMP-2 or constitutively active ALK-1, ALK-2, ALK-3, and ALK-6 induced at the same time Smad8, Col10a1, and alkaline phosphatase expression, as well as alkaline phosphatase activity, suggesting that Smad8 might be involved in the hypertrophic maturation of chondrocytes. Indeed, when chondrocytes were coinfected with Smad4 and each of the R-Smads in the presence of low concentration (25 ng/ml) of BMP-2, Col10a1 expression and alkaline phosphatase activity were clearly stimulated by Smad1 and Smad8 and to a lesser extent by Smad5, in cooperation with Smad4. These latter results first suggest that Smads were physiologically active in our experiments and further indicate that Smad1, Smad5, and Smad8 are major signaling molecules for the hypertrophic maturation of chondrocytes induced by BMP-2. Interestingly, Smad2 and Smad3 showed an antagonistic activity to BMP-2, and this finding is consistent with the role of these Smads in TGF-β/activin signaling and the antagonistic effect of ALK-5 on BMP-2 or ALK-2 function demonstrated in the present study. Furthermore, osteocalcin expression was not upregulated by coexpression of Smad4 and each of the R-Smads. This observation raises the possibility that additional pathways, which are BMP-2- and/or ALK-2-dependent, might be required for osteocalcin expression, a unique trait specific of the osteoblast phenotype. In support of this hypothesis, the p38 mitogen-activated protein kinase and extracellular signal-regulated kinase pathways have recently been shown to control BMP-2-induced osteocalcin expression in myoblastic C2C12 cells (77).

Inhibitory Smads Impair Chondrogenic Expression, Hypertrophic Maturation, and Osteoblastic Differentiation Induced by BMP-2—Smad6 and Smad7 inhibit the effects of R-Smads by competing for binding to activated type I receptors and thus preventing the phosphorylation of R-Smads (78–80). Smad6 can also compete with Smad4 for binding to activated Smad1 (81). In the present study, we showed that Smad6 and Smad7 expressions in MC615 chondrocytes were clearly induced by BMP-2. Induction of I-Smads at the mRNA level by members of the TGF-β superfamily has been reported in other studies. For instance, Smad6 expression is induced by the effects of BMP-activated Smad1/5 on the Smad6 promoter (82), and cooperation of Smads with AP-1 and Sp1 transcription factors is necessary for basal Smad7 promoter activity and induction by TGF-β (83).

Previously, Smad6 and Smad7 have been shown to block spontaneous chondrogenic differentiation of teratocarcinoma ATDC5 cells and to inhibit alkaline phosphatase activity induced by BMP-6 or active ALK-3 and ALK-6 in myoblastic C2C12 cells (15). Our data reveal that Smad6 and Smad7 inhibit the expression of genes specific for cartilage, hypertrophic cartilage, and bone. These results imply that Smad-mediated pathways regulate chondrogenic expression and osteoblastic differentiation of chondrocytes. In conclusion, adenovirus vectors were chosen for transfer and expression of genes to chondrocytes because of the demonstrated efficiency of these vectors in transferring genes to many cell types, including chondrocytes (84). The study presented here is the first systematic analysis of receptor and Smad signaling specificity in primary and established chondrocytes, whereas previous studies have primarily focused on pluripotent cell types that are capable of differentiating to chondrocytes or osteoblasts. We showed that hypertrophic maturation
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Functions of Transforming Growth Factor-β Family Type I Receptors and Smad Proteins in the Hypertrophic Maturation and Osteoblastic Differentiation of Chondrocytes

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