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BMP-2 and TGF-β1 differentially control expression of type II procollagen and α10 and α11 integrins in mouse chondrocytes

Jérôme Gouttenoire[a], **Carole Bougault**[a], **Elisabeth Aubert-Foucher**[a], **Emeline Perrier**[a], **Marie-Claire Ronzière**[a], **Linda Sandell**[b], **Evy Lundgren-Akerlund**[c], and **Frédéric Mallein-Gerin**[a,*]

[a]Institut de Biologie et Chimie des Protéines, CNRS UMR 5086, Université de Lyon, and IFR 128 BioSciences Gerland-Lyon Sud, 7 passage du Vercors, Lyon F-69367, France

[b]Department of Orthopaedic Surgery and Department of Cell Biology, Washington University, St. Louis, USA

[c]Ideon Bioincubator BMC-D10 SE 22184 Lund, Sweden

Abstract

Bone morphogenetic protein (BMP)-2 and transforming growth factor (TGF)-β1 are multifunctional cytokines both proposed as stimulants for cartilage repair. Thus it is crucial to closely examine and compare their effects on the expression of key markers of the chondrocyte phenotype, at the gene and protein level. In this study, the expression of α10 and α11 integrin subunits and the IIA/IIB spliced forms of type II procollagen have been monitored for the first time in parallel in the same in vitro model of mouse chondrocyte dedifferentiation/redifferentiation. We demonstrated that TGF-β1 stimulates the expression of the non-chondrogenic form of type II procollagen, IIA isoform, and of a marker of mesenchymal tissues, i.e. the α11 integrin subunit. On the contrary, BMP-2 stimulates the cartilage-specific form of type II procollagen, IIB isoform, and a specific marker of chondrocytes, i.e. the α10 integrin subunit. Collectively, our results demonstrate that BMP-2 has a better capability than TGF-β1 to stimulate chondrocyte redifferentiation and reveal that the relative expressions of type IIB to type IIA procollagens and α10 to α11 integrin subunits are good markers to define the differentiation state of chondrocytes. In addition, adenoviral expression of Smad6, an inhibitor of BMP canonical Smad signaling, did not affect expression of total type II procollagen or the ratio of type IIA and type IIB isoforms in mouse chondrocytes exposed to BMP-2. This result strongly suggests that signaling pathways other than Smad proteins are involved in the effect of BMP-2 on type II procollagen expression.

Keywords

BMP-2; TGF-β1; Type IIA and IIB procollagens; α10 and α11 Integrins; Chondrocytes; Smad6

Introduction

Type II collagen is the most abundant protein component in cartilage. This collagen is first synthesized as a procollagen molecule consisting of three identical α1(II) chains, containing amino- and carboxy-terminal propeptides. Although type II collagen was first considered to be cartilage specific, findings based upon in situ localization of α1(II) collagen mRNAs (Cheah et al., 1991) and protein (Wood et al., 1991) have revealed that type II collagen

*Corresponding author: Tel.: +33 4 3765 2919; Fax: +33 4 7272 2604; f.mallein-gerin@ibcp.fr (F. Mallein-Gerin).

presents a much wider tissue distribution during mouse embryogenesis. For instance the mouse α1(II) collagen gene (*Col2a1*) was found transiently expressed in tissues such as the notochord, neural retina, tail, tendon, heart, surface ectoderm, calvarial mesenchyme, fetal brain and the sensory epithelium of the inner ear (Cheah et al., 1991). In other species such as chicken and *Xenopus* the non-chondrogenic localization of type II collagen during embryonic development was also revealed (Fitch et al., 1989; Kosher and Solursh, 1989; Mallein-Gerin, 1990; Su et al., 1991), supporting the idea that this collagen could have other functions than cartilage formation during embryogenesis. In fact, type II collagen can be generated under two forms by alternative RNA splicing (Ryan and Sandell, 1990). The type IIA form includes in its amino propeptide a cysteine-rich (CR) domain encoded by exon 2, which is not present in type IIB. Concerning the developmental standpoint mentioned above, it is important to note that type IIA procollagen mRNA represents the major form of *Col2a1* transcript present in embryonic mouse tissues up to 12.5 days in both pre-chondrogenic and non-chondrogenic tissues. After 12.5 days, type IIB mRNA levels increase rapidly and finally exceed that of type IIA mRNAs (Ng et al., 1993). Type IIB mRNA becomes the major *Col2a1* transcript by 14.5 days and is predominantly expressed in mature chondrocytes (Ng et al., 1993; Sandell et al., 1994). In chicken and human, type IIA procollagen was also found to be expressed early in pre-chondrogenic and non-chondrogenic tissues with type IIB procollagen being expressed later in differentiating cartilage (Nah and Upholt, 1991; Sandell et al., 1991; Lui et al., 1995). Thus, the shift from type IIA to type IIB is a sign of chondrocyte differentiation. Once cartilage is formed, the function of exon 2 would be no longer required and the function of the type IIB isoform could be related to maintaining structural integrity of the extracellular matrix, as suggested previously (McAlinden et al., 2008). Moreover, the precise role of type II procollagen in the non-cartilaginous tissues is unclear but could be related to the presence of the CR domain. This CR domain has been shown to interact with transforming growth factor (TGF)-β1 and bone morphogenetic protein (BMP)-2 in vitro (Zhu et al., 1999). These observations led to the proposal that the type IIA isoform functions as a reservoir for (TGF)- β superfamily members required for development of cartilage (Zhu et al., 1999). Furthermore, CR domains are present in a large number of extracellular proteins, several of which appear to be involved in BMP signaling regulation. For instance chordin has four CR domains and prevents the binding of BMP-2 and BMP-4 to their receptors (Piccolo et al., 1996). Chordin is intimately involved in dorsoventral patterning in *Xenopus* (De Robertis and Sasai, 1996; Larraín et al., 2000). Thus, it has been speculated that type IIA, via its CR domain, could have an anti-BMP activity during development. This speculation is supported by the finding that, in vivo, overexpression of full-length type IIA procollagen (but not of a construct mimicking type IIB procollagen) in *Xenopus* embryos, results in dorsalization, a phenotype indicative of BMP inactivation (Larraín et al., 2000). In this case, type IIA procollagen binding to BMP was predicted to prevent BMP–receptor interactions, thereby inactivating the BMP-induced ventralizing activity required for normal *Xenopus* development. Thus, the type IIA procollagen amino-propeptide may play an important role in growth factor regulation during early development. In addition, genes homologous to BMP and chordin, designated *Dpp* (*Decapentaplegic*) and *sog* (*short gastrulation*) have been identified in the fruit fly *Drosophila melanogaster* (Francois et al., 1994; Holley et al., 1995). Phylogenetic, biochemical and in vivo analyses of human, mouse, chicken, *Xenopus* and zebrafish chordin proteins and of *Drosophila* Sog reveal a high degree of conservation and activity for each specific CR domain among the different species. This suggests an evolutionarily conserved function for these domains in the regulation of the BMP signals (Garcia-Abreu et al., 2002). Very little information is available on the mechanisms that control *Col2a1* alternative splicing. The constructions of human and mouse *Col2a1* minigenes allowed the identification of cis-acting RNA elements and trans-acting protein factors that regulate this splicing (Hatano et al., 2002; Nishiyama et al., 2003; McAlinden et al., 2005, 2007). Another recent study on the effects of different serine-arginine (SR) proteins on the

alternative splicing of pre-mRNAs generated from the endogenous mouse *Col2a1* gene has identified the translocation liposarcoma protein-associated SR protein-1 (TASR-1) as a factor regulating this splicing (Matsushita et al., 2007). However, less information is available regarding upstream regulators of this splicing event. We previously showed by using reverse transcription-polymerase chain reaction (RT-PCR) that BMP-2 favors expression of type IIB, whereas TGF-β1 potentiates the expression of type IIA induced by sub-culture (Valcourt et al., 2003). Considering that re-expression of type IIA procollagen has been reported in human osteoarthritic cartilage (Aigner et al., 1999) and that both BMP-2 and TGF-β1 are widely proposed as candidates for cartilage cell therapy (Oshin and Stewart, 2007; Blaney Davidson et al., 2007), we feel it is very important to closely compare the effects of each growth factor on the expression of the chondrocytic phenotype. In the present study, we first extended our previous observations by a more precise characterization of key markers targeted by BMP-2 and TGF-β1 in mouse embryonic chondrocytes, at the gene or protein level. We paid particular attention to the synthesis of the IIA procollagen form and of the α10 and α11 subunits of the collagen-binding integrins α10β1 and α11β1. α10 Integrin is the main collagen-binding integrin on chondrocytes in cartilage (Camper et al., 1998) whereas α11 integrin is more characteristic of mesenchymal tissues (Zhang et al., 2002). In addition, it has been shown recently that chondrocytic differentiation of mesenchymal stem cells is associated with an increase in α10 expression and a decrease in α11 expression (Varas et al., 2007). Thus α10 and α11 are good markers to evaluate the state of the chondrocytic phenotype. Our results demonstrate that BMP-2 primarily stimulates expression of a well-differentiated chondrocytic phenotype whereas TGF-β1 stimulates a pre-chondrocytic phenotype. We also found that overexpression of Smad6, an inhibitory Smad of BMP signaling, does not significantly affect expression of type II procollagen and the ratio of type IIA and type IIB forms in mouse chondrocytes exposed to BMP-2.

Materials and methods

Antibodies

For collagen II, a monoclonal antibody (2B1; Thermo Fisher Scientific Inc, France; dilution 1:100) recognizing an epitope located in the triple helix of type II collagen (Mayne et al., 1994) was used for immunofluorescence (IF) and polyclonal rabbit antibodies to mature collagen II (Novotec, Lyon, France; ref. 20251; dilution 1:2000) for immunoblotting (IB). Detection of collagen IIA was done with a rabbit antiserum against recombinant type IIA-GST which only recognizes the exon 2 domain of type IIA procollagen (Oganesian et al., 1997) at 1:1000 or 1:2000 dilutions for IF or IB, respectively. Polyclonal antibodies to collagen I (ref. 20151; dilution 1:100 for IF or 1:2000 for IB) and to aggrecan (ref. 24421; dilution 1:100) were from Novotec. Monoclonal antibody against α10 integrin (dilution 1:100 for IF or 1:2000 for IB) and polyclonal antibodies against α11 integrin (dilution 1:800 for IF or 1:2000 for IB) were from Cartela AB, Lund, Sweden. Other antibodies were as follows: anti-Phospho-Smad1/5/8 (#9511, 1:1000) and HRP-conjugated anti-rabbit (#7074, 1:2000), Cell Signaling Technology, Danvers, MA; anti-Flag M2 (F3165, 4 mg/ml) and anti-actin (A5060, 1:800), Sigma-Aldrich, St Louis, MO; Cy2- or Cy3-conjugated secondary antibodies (dilutions 1:100 or 1:200, respectively), Jackson ImmunoResearch Laboratories, West Grove, PA; anti-mouse (170–6520) or anti-rabbit (170–6518) IgG-AP conjugates and anti-mouse IgG-HRP conjugates (170–6516), Bio-Rad, Richmond, CA, all used at a 1:5000 dilution.

Cell culture

Embryonic mouse chondrocytes were isolated from the ventral parts of the rib cages of 17.5-days post coitum mice, as described (Lefebvre et al., 1994). These chondrocytes were

seeded on 90-mm Corning dishes with 1×10^6 cells/dish and cells at this stage were designated P0. Chondrocytes were cultured with 1:1 Dulbecco's modified Eagle's medium/F-12 with GlutaMAX-I containing 10% fetal bovine serum (FBS) and supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin (all products from Invitrogen), at 37 °C in 5% CO_2. Confluent cells were detached using 0.05% trypsin-EDTA, seeded in 6-well culture dishes and designated P1. For treatment with growth factors, 1.5×10^5 cells/well were grown for 3 days in medium containing 1% FBS supplemented or not with 50 ng/ml BMP-2 or 5 ng/ml TGF-β1. Recombinant human BMP-2 was purchased from Wyeth or R&D Systems and recombinant human TGF-β1 was purchased from R&D Systems. The culture medium was replaced and supplemented with fresh BMP-2 or TGF-β1 every day.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNAs were extracted and reverse-transcribed as previously described (Valcourt et al., 2003). RT-PCR with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers was performed to ascertain that an equivalent amount of cDNA was synthesized from the different samples. The conditions for the PCR amplifications have been previously reported (Valcourt et al., 1999, 2003). Specific primers were designed from sequences available in the data banks (Table 1). Following an initial denaturation step of 2 min at 95 °C, samples were amplified for 20–35 cycles of 30 s at 95 °C, 30–45 s at optimal temperature (Table 1) and 30 s at 72 °C, followed by a final extension step of 5 min at 72 °C. The RT-PCR products were separated by electrophoresis on 2% agarose gels and stained with ethidium bromide. Photographs of gels of RT-PCR experiments using cells obtained from four independent dissections were scanned (Epson perfection 1640SU) and analyzed with Image Quant software (GE Healthcare).

Immunofluorescence

P0, P1 and P2 chondrocytes were seeded on glass coverslips in 6-well culture dishes with 4×10^5 cells/well (P0) or 1.5×10^5 cells/well (P1, P2). For one set of experiments, P0, P1 and P2 chondrocytes were grown for 3 days in the presence of 10% FBS. For another set of experiments, P1 were grown for 3 days in the presence of 1% FBS supplemented or not with 50 ng/ml BMP-2 or 5 ng/ml TGF-β1. The cell cultures were then fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS, Sigma) for 30 min at 4 °C and rinsed in PBS. After treatment with hyaluronidase (800 units/ml, Sigma type I) for 30 min at 37 °C, cells were permeabilized with 0.1% Triton X-100 in PBS for 20 min at room temperature followed by washes in PBS. To visualize collagens, chondrocytes were first incubated for 15 min with 1% bovine serum albumin in PBS then incubated for 1 h with the indicated primary antibodies. To visualize integrins, cells were incubated for 30 min with 0.5% casein in PBS and then overnight with antibodies to α10 or α11 subunits. Cells were then washed with PBS and incubated for 1 h with Cy3- or Cy2-conjugated anti-mouse or anti-rabbit secondary antibodies in PBS containing 0.25 μg/ml Hoechst (Fluka) to visualize nuclei. After a last wash in PBS, the coverslips were mounted on microscope slides in glycerol/PBS (1:1) for their observation by epifluorescence with a Zeiss Axioplan 2 microscope equipped with a CoolSNAP Fx camera (Roper Scientific). Image acquisition and overlay were achieved with Metaview software (Universal Imaging). For each condition, stained cells were counted by using ImageJ software. Four independent fields were selected under the microscope to determine the average score count. The results were expressed as percent of stained cells to total cell number that was determined as number of Hoechst-positive cells counted.

Infection with recombinant adenoviruses

Recombinant adenoviruses containing cDNAs for Flag-tagged Smad6 or β-galactosidase were constructed, amplified, and titrated as previously described (Saitoh et al., 1998; Fujii et

al., 1999). For infection with the adenoviruses, P1 chondrocytes were seeded in 6-well culture plates (2×10^5 cells/well) and maintained for 24 h in standard medium supplemented with 10% FBS. The medium was replaced with medium containing 1% FBS 2 h before the infection. Infection with recombinant adenoviruses was performed at a multiplicity of infection (m.o.i.) ranging between 300 and 500 plaque-forming units/cell for 24 h. Infected cells were cultured for 3 days in fresh medium containing 1% FBS supplemented or not with 50 ng/ml BMP-2.

Immunoblotting

Cells were washed three times with PBS and scraped into Laemmli sample buffer. After boiling for 5 min in the presence of 2-mercaptoethanol, equivalent amounts of proteins were separated on 10% or 4–12% polyacrylamide gradient minigels and transfered to PVDF membranes (Millipore). The membranes were probed with the primary antibodies, washed and incubated with HRP- or alkaline phosphatase-conjugated anti-mouse or anti-rabbit IgG. After multiple washes, bound antibodies were detected on X-ray films using a Bio-Rad Immun-star chemiluminescent substrate. The membranes were sequentially re-probed with the indicated antibodies after stripping (Re-Blot Plus Strong, Chemicon).

Results and discussion

Synthesis of the type IIA form of procollagen is a sign of dedifferentiation for embryonic mouse chondrocytes expanded in monolayer

When embryonic mouse chondrocytes were examined by immunofluorescence 72 h after isolation and seeding at subconfluence, they all reacted with a monoclonal antibody (2B1) recognizing the triple helix of type II collagen or with a polyclonal antibody that has been raised against aggrecan (Fig. 1). These observations attested the purity and well-differentiated state of the cell population. More precisely, double immunostaining with 2B1 and antiserum to type IIA procollagen amino-propeptide revealed that only a few P0 chondrocytes (3% of total cell number) synthesize the IIA form of type II procollagen (Fig. 1). On parallel coverslips, very few cells (2%) revealed staining for type I procollagen (Fig. 1), a marker of dedifferentiated chondrocytes (von der Mark et al., 1977). When cells were subsequently seeded at P1 and P2, cells progressively spread, became fibroblastic and the amount of cells revealing staining for the triple helix of collagen II or aggrecan decreased (from 97% at P0 to 38% at P2 for collagen II; from 96% at P0 to 29% at P2 for aggrecan) whereas the amount of cells positively stained for the type IIA form or for type I procollagen increased (from 3% at P0 to 71% at P2 for type IIA collagen; from 2% at P0 to 79% at P2 for collagen I) (Fig. 1) showing that type IIA procollagen synthesis accompanies chondrocyte dedifferentiation triggered by subculture. We already showed that type IIA procollagen is expressed at the gene level in embryonic mouse or adult human chondrocytes after their seeding on culture dishes (Valcourt et al., 2003; Hautier et al., 2008), however, this is the first report demonstrating that chondrocytes start to synthesize the corresponding protein when they dedifferentiate in culture. Our observation also underlines asynchronism in the switch to the type IIA form among the cell population. Chondrocyte dedifferentiation in monolayer culture is a process known for a long time but the underlying mechanisms have just started to be unraveled. We previously showed that chondrocyte dedifferentiation is correlated with the organization of actin into stress fibers (Mallein-Gerin et al., 1991). A very recent study has demonstrated that generation of these stress fibers is induced by the GTPase RhoA (Kumar and Lassar, 2009). In addition, RhoA signaling and actin polymerization have been shown to block the function of Sox9 (Kumar and Lassar, 2009). Sox9 is a transcription factor essential for activation of the *Col2a1* gene promoter (Lefebvre et al., 1997) and interestingly, it was found that Sox9 and Sox6, another Sox transcription factor important for chondrogenesis, can regulate pre-mRNA splicing (Ohe et al., 2002).

Thus, it is tempting to speculate that production of the type IIA form of procollagen during chondrocyte dedifferentiation in culture results, at least in part, from a decrease in Sox9 activity under the control of actin polymerization and RhoA signaling.

Type IIB procollagen and integrin subunit α10 are increased by BMP-2 whereas type IIA procollagen and integrin subunit α11 are increased by TGF-β1 in dedifferentiating chondrocytes

Since we previously showed that alternative splicing of type II procollagen pre-mRNA in embryonic mouse chondrocytes is oppositely regulated by BMP-2 and TGF-β1 (Valcourt et al., 2003), we further compared their effect on type IIA procollagen synthesis by using the same cellular model and immunofluorescence analysis. When dedifferentiated P1 chondrocytes were cultured with BMP-2 or TGF-β1, the percentage of cells stained for total type II procollagen was higher than in the control cultures (72% in control cultures, 86% with BMP-2 and 96% with TGF-β1; Fig. 2). Furthermore, most cells stimulated by TGF-β1 were positive for type IIA procollagen (75% positive cells) whereas a much smaller percentage of cells was positive for this collagen isoform when the cells were cultured in the presence of 1% FBS alone (34% positive cells) or supplemented with BMP-2 (25% positive cells) (Fig. 2). This result correlates for the first time the TGF-β1 stimulation of the expression of the type IIA transcript reported before (Valcourt et al., 2003) with the synthesis of type IIA procollagen in dedifferentiated mouse chondrocytes. In accordance with this immunofluorescence study, Western-blotting analysis of P1 chondrocytes confirmed that both BMP-2 and TGF-β1 are able to stimulate total type II procollagen synthesis during a 3-day culture period (Fig. 3). In addition, the differential effect of BMP-2 and TGF-β1 on type IIA procollagen synthesis increased with time in culture and became evident at day 3, with a much higher level of type IIA in the presence of TGF-β1 (Fig. 3). Although there is no direct demonstration of type IIB procollagen synthesis in our experiments since there is no antibody specific for type IIB procollagen, our results also strongly suggest that synthesis of type II procollagen observed in P1 chondrocytes stimulated by BMP-2 corresponds mainly to the type IIB form. In summary, these findings support and complement at the protein level our previous study demonstrating that expression of type IIA is favored by TGF-β1 whereas type IIB is favored by BMP-2 (Valcourt et al., 2003) (see also Fig. 4).

We next extended our studies with a more detailed identification of the effects of BMP-2 and TGF-β1 on key markers involved in chondrogenesis. As expected, expression of the genes encoding type II procollagen and Sox9 decreased from P0 to P1 (20% decrease in 10% FBS) (Fig. 4). When P1 chondrocytes were cultured for 3 days in the presence of 1% FBS supplemented or not with BMP-2 or TGF-β1, the levels of gene expression for type II procollagen and Sox9 appeared to be the highest with BMP-2 when compared to 1% FBS or TGF-β1 (with 145% increase of IIB form with BMP-2 and 180% increase of IIA form with TGF-β1 when compared to 1% FBS) (Fig. 4). Interestingly, our RT-PCR analysis revealed that the gene encoding the α10 integrin subunit (*α10*), robustly expressed in freshly isolated and P0 chondrocytes (Fig. 4), was down-regulated in P1 chondrocytes on day 0 (60% decrease in 10% FBS), then was stimulated by BMP-2 and inhibited by TGF-β1 on day 3 when compared to the 1% FBS control cultures (115% increase with BMP-2 and 55% decrease with TGF-β1). In parallel, expression of the gene encoding the α11 integrin subunit (*α11*) was weakly detectable in freshly isolated and P0 chondrocytes then was clearly up regulated after passage (P1, D0). On day 3, *α11* expression was increased by 25% with TGF-β1 when compared to 1% FBS- or BMP-2-treated cultures (Fig. 4). We further assessed the effects of BMP-2 and TGF-β1 on the regulation of these integrin subunits by Western-blotting analysis: α10 was faintly detectable in chondrocytes 48 h after their seeding but was not detected thereafter (Fig. 5A). In parallel, α11 was weakly detected by

immunoblotting in chondrocytes 48 h after their seeding and in P1 chondrocytes on day 0 (Fig. 5A). The level of α11 subunit then increased in P1 chondrocytes cultured 3 days in the presence of 1% FBS alone or supplemented with TGF-β1 whereas it remained relatively stable in P1 chondrocytes treated with BMP-2 (Fig. 5A). Interestingly, the same responsiveness to BMP-2 and TGF-β1 was observed for type I procollagen synthesis (Fig. 5A), indicating that BMP-2, but not TGF-β 1, opposes chondrocyte dedifferentiation.

To localize α10 and α11 integrin expression at the cellular level, P0 and P1 chondrocytes were allowed to spread on glass coverslips for 3 days in the presence of 10% FBS, then were examined by fluorescence microscopy (Fig. 5B). The distribution of α10 and α11 was consistent with a presence of these integrin subunits at focal contacts, as generally described for integrins. In addition, our observations showed the presence of α10 integrin subunits only in P0 chondrocytes whereas α11 integrin subunits were detected only in P1 chondrocytes (Fig. 5B).

The data presented here at the gene and protein level complement previous reports indicating that α10 is a marker of the differentiation state of chondrocytes (Camper et al., 1998, 2001) and reveal that α11 integrin subunit and procollagen I expression are correlated during chondrocyte dedifferentiation. They also reveal that integrin subunits α10 and α11, like type IIA and IIB procollagens, are counter-regulated during chondrocyte dedifferentiation and also by BMP-2 and TGF-β1 once chondrocytes are dedifferentiated. Regarding chondrogenesis, our results are in line with a recent study showing that expression of *α10* increases while expression of *α11* decreases during in vitro chondrogenesis of adult human mesenchymal stem cells isolated from bone marrow (Varas et al., 2007). Furthermore, it is interesting to note that, by using the same source of cells, an independent report has shown that early chondrocytic differentiation is accompanied by a transition in the expression of immature collagen type IIA to the mature collagen IIB (Murdoch et al., 2007).

Overexpression of Smad6 does not affect expression of type II procollagen and the ratio of type IIA and type IIB forms in mouse chondrocytes exposed to BMP-2

We investigated the involvement of the Smad pathway in the effect of BMP-2 on type II procollagen synthesis and expression, with attention given to expression of the type IIA and type IIB forms. We used Smad6, an inhibitor of the canonical BMP Smad signaling through the R-Smads 1, 5 and 8 (for a review, see for example (Massague et al., 2005)). Smad6 blocks Smads 1, 5 and 8 phosphorylation by associating with BMP type I receptors (Imamura et al., 1997) or by sequestration of phosphorylated Smad1 from the common-partner Smad4 (Hata et al., 1998). P1 chondrocytes were infected with adenoviruses carrying a cDNA coding for flagged-Smad6 and the corresponding synthesis was confirmed by immunoblotting before treatment (Fig. 6A) and after 3 days (data not shown) of BMP-2 treatment. The activity of Smad6 was demonstrated by its ability to inhibit basal and BMP-2-stimulated phosphorylation of Smad1/5/8 (Fig. 6B). When total type II procollagen synthesis was examined, there was no effect of Smad6 overexpression in the chondrocytes stimulated or not by BMP-2 (Fig. 6C). In addition, when the relative ratio of type IIA and type IIB procollagen transcripts was examined by RT-PCR, the expected effect of BMP-2 in favor of IIB was not significantly modulated by Smad6 overexpression (Fig. 6D). Globally, these results indicate that Smad6 overexpression does not affect expression of the major cartilage collagen when mouse chondrocytes are exposed to BMP-2. Our results are in concordance with a previous report showing that Smad6 overexpression in cartilage in transgenic mice inhibits Smad1/5/8 phosphorylation in chondrocytes without affecting the early steps of cartilage development as judged by type II collagen or aggrecan expression, but delays the late step of endochondral ossification (Horiki et al., 2004). Moreover, in the same study, when embryonic metatarsal cartilage from these Smad6 transgenic mice was

organ cultured in the absence or presence of BMP-2, proliferative cartilage in transgenic rudiments expanded like that of normal mice. Nevertheless, formation of the hypertrophic center was limited, indicating that Smad6 overexpression inhibited chondrocyte hypertrophy induced by BMP-2 but spared early cartilage development (Horiki et al., 2004). These in vivo analyses and our results in vitro are therefore in agreement and collectively suggest that Smad6 overexpression does not block BMP-2 signaling leading to type II procollagen expression in mouse chondrocytes. This strongly suggests that signaling pathways others than the Smad pathway could be involved in the effect of BMP-2 on type II procollagen expression. In support of this view, it has been reported that BMP-2 can activate p38 mitogen-activated protein kinase (Iwasaki et al., 1999).

Concluding remarks

We present here the first study where the expression of α10 and α11 integrin subunits and the spliced forms of type II procollagen have been monitored in parallel at the protein and mRNA levels in the same model of chondrocyte dedifferentiation/redifferentiation. Our results demonstrate that the relative expressions of type IIB to type IIA procollagen and α10 to α11 integrin subunits are good markers to define the differentiation state of chondrocytes and that BMP-2 has more capacity than TGF-β1 to stimulate chondrocyte redifferentiation. In the same vein we recently showed that BMP-2 stimulates chondrogenic expression in human nasal chondrocytes expanded in monolayer, by favoring the type IIB procollagen transcript (Hautier et al., 2008). Finally our results also strongly suggest that signaling pathways other than Smad proteins could be involved in the effect of BMP-2 on type II procollagen expression. Thus, a major challenge for the future will be to decipher the distinct signaling pathways, both Smad-dependent and -independent (in combination or not) that mediate the regulation of chondrocyte phenotype by BMPs.

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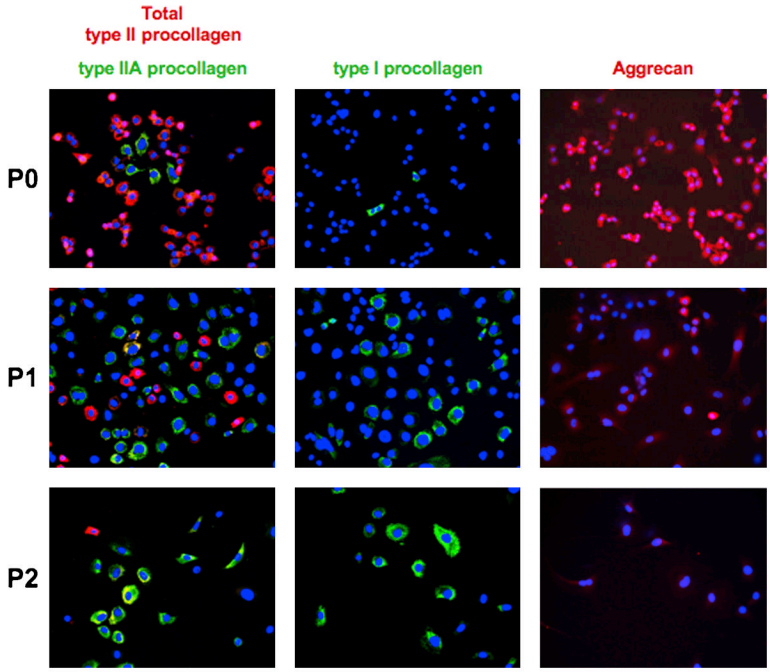

Fig. 1.

Synthesis of type IIA procollagen, like type I procollagen, is a sign of dedifferentiation in mouse chondrocytes expanded in monolayer. Embryonic rib cage chondrocytes were serially cultured in monolayer in culture medium supplemented with 10% FBS. At each passage, cells were fixed 72 h after their seeding. On the panel shown on the left, cells were double-stained for the triple helix part of collagen II with Cy3-conjugated secondary antibodies (red) and for type IIA procollagen with Cy2-conjugated secondary antibodies (green). Note that with passaging, cells spread and synthesis of type II procollagen progressively switches from the IIB to the type IIA form, with cells synthesizing both forms (merged double-staining in yellow). On the panel shown in the middle, cells were stained for type I procollagen with Cy2-conjugated secondary antibodies (green). On the panel shown on the right, cells were stained for aggrecan with Cy2-conjugated secondary antibody (red). Nuclei were stained with Hoechst dye (blue) (×16).

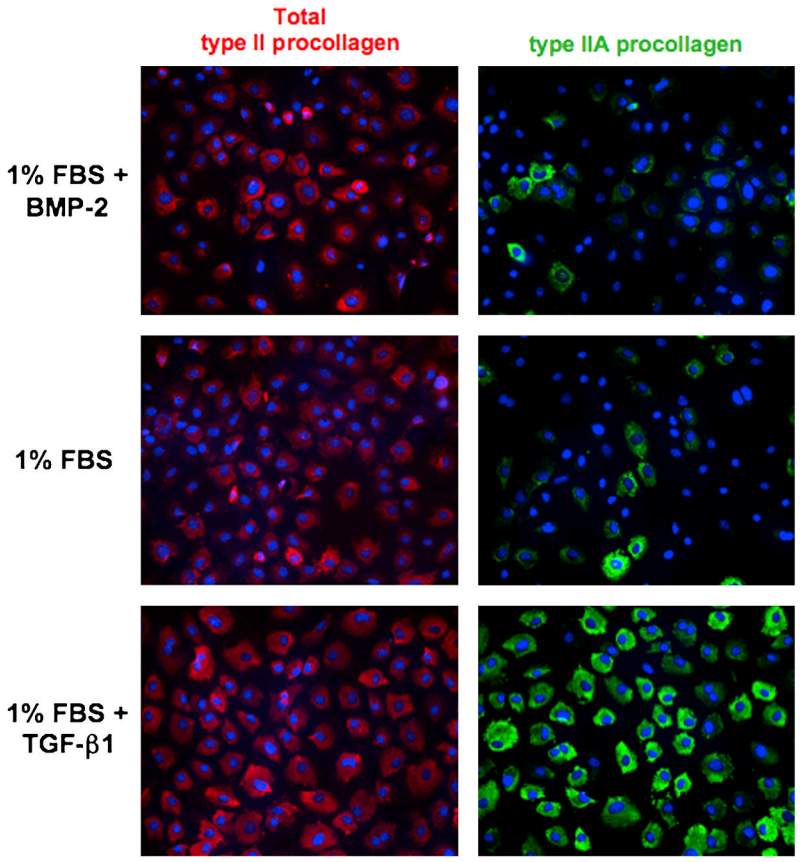

Fig. 2.

Both BMP-2 and TGF-β1 stimulate type II procollagen synthesis in dedifferentiated chondrocytes but only TGF-β1 favors synthesis of the type IIA form. P1 chondrocytes were cultured for 3 days in the presence of 1% FBS alone or supplemented with 50 ng/ml BMP-2 or 5 ng/ml TGF-β1 as indicated. Cells were stained for the triple helix part of collagen II with Cy2-conjugated secondary antibodies (red) and for type IIA procollagen with Cy3-conjugated secondary antibodies (green). Nuclei are stained with Hoechst dye (blue). While a significant amount of cells cultured with 1% FBS are visualized by the presence of their nuclei only, most cells cultured in the presence of BMP-2 or TGF-β1 are stained for total type II procollagen, as observed on the left. In parallel cultures, cells treated with TGF-β1 show intense staining for type IIA procollagen compared with cells cultured with 1% FBS or 1% FBS + 50 ng/ml BMP-2, as observed on the right (×16).

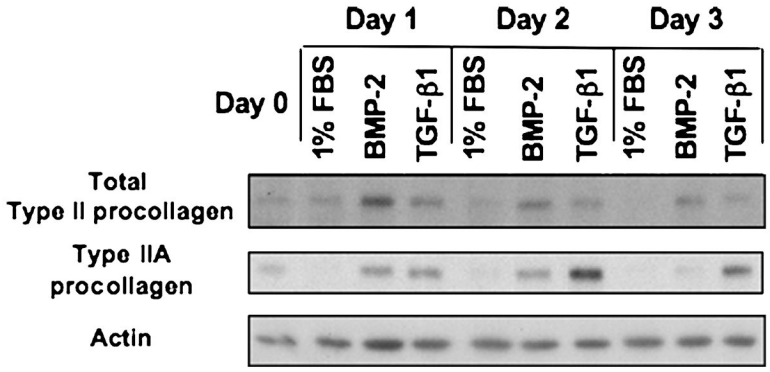

Fig. 3.

The differential effect of BMP-2 and TGF-β1 on type II procollagen isoforms synthesis is dependent on the duration of treatment with the growth factors. P1 chondrocytes were cultured for 18 h in the presence of 10% FBS (day 0), then cultured for 1, 2 or 3 days in the presence of 1% FBS alone or supplemented with 50 ng/ml BMP-2 or 5 ng/ml TGF-β1. Western-blotting analysis of synthesis of global type II or type IIA procollagen in chondrocytes cultured for 1, 2, or 3 days reveals that both BMP-2 and TGF-β1 stimulate total type II procollagen synthesis in dedifferentiated chondrocytes. BMP-2 favors the IIB form whereas TGF-β1 stimulates the type IIA form of procollagen II.

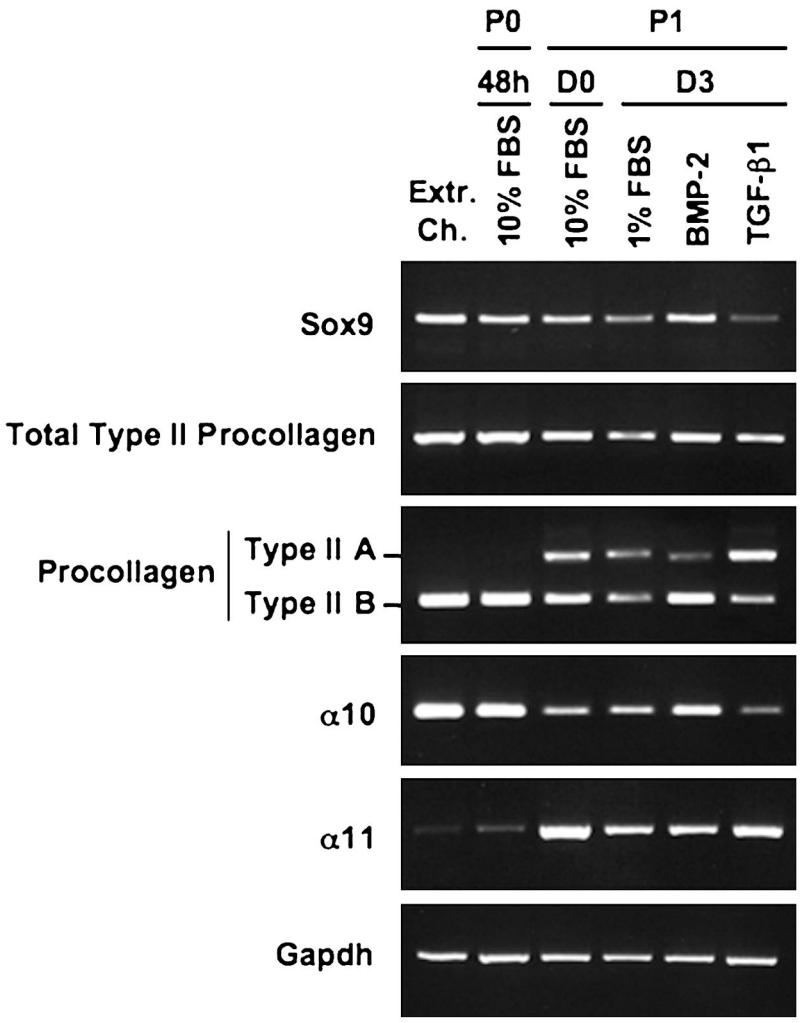

Fig. 4.

RT-PCR analysis of expression of selected genes in mouse chondrocytes in response to treatment with BMP-2 and TGF-β1. RT-PCR analysis was performed with chondrocytes freshly extracted from rib cages (Extr. Ch.), or cultured as P0 chondrocytes for 48 h in the presence of 10% FBS, cultured as P1 chondrocytes for 18 h in the presence of 10% FBS (D0) then for 3 days in the presence of 1% FBS alone or supplemented with BMP-2 or TGF-β1 (D3). The corresponding gene products are indicated on the left.

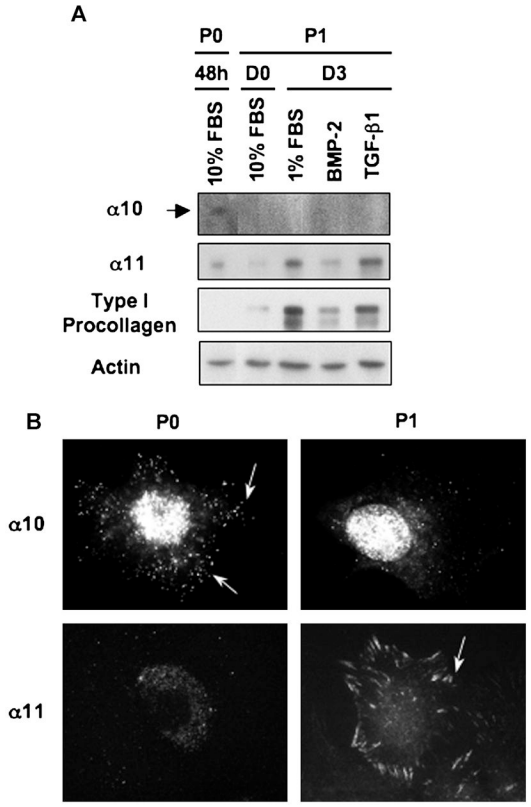

Fig. 5.

Western-blot analysis of integrin and type I procollagen expression of mouse chondrocytes in response to BMP-2 or TGF-β1 treatment. (A) Western-blot analysis of chondrocytes cultured as P0 chondrocytes for 48 h in the presence of 10% FBS, cultured as P1 chondrocytes for 18 h in the presence of 10% FBS (D0) then for 3 days in the presence of 1% FBS alone or supplemented with BMP-2 or TGF-β1 (D3). As shown here, protein levels of type I procollagen and α11 integrin subunit are highly correlated in chondrocytes. (B) Immunolocalization of the α10 and α11 integrins subunits in chondrocytes adhered to glass coverslips. The arrows indicate clustering of α10 and α11 subunits likely corresponding to focal contacts. Note that α10 is detected in differentiated (P0) chondrocytes and α11 in dedifferentiated (P1) chondrocytes (×100).

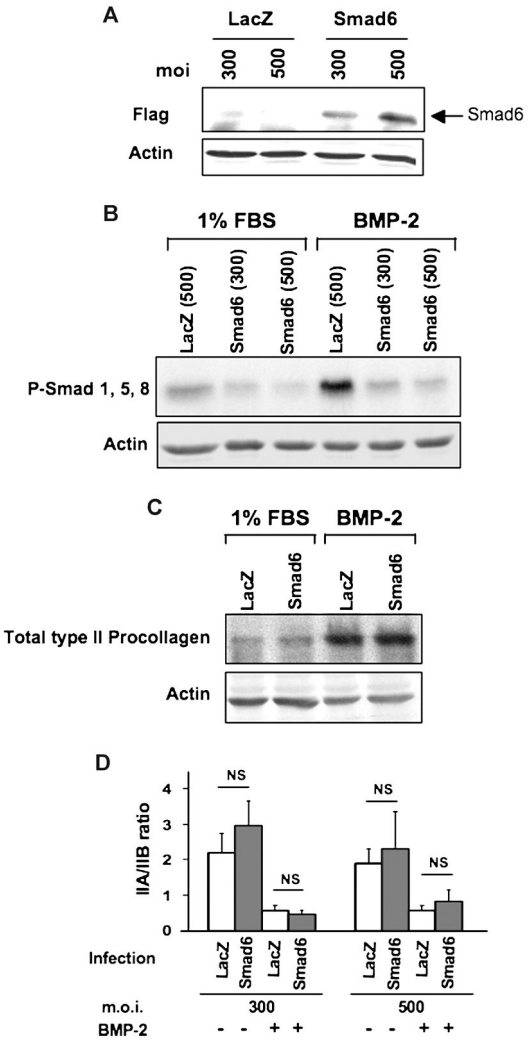

Fig. 6.

Adenoviral overexpression of Smad6 does not influence expression of type II procollagen and the ratio of type IIA and type IIB forms in mouse chondrocytes exposed to BMP-2. P1 chondrocytes were infected with adenoviruses carrying a β-galactosidase-cDNA as a control or a Smad6-coding cDNA (m.o.i. of 300 or/and 500). (A) Twenty-four h following infection, Western-blotting using anti-FLAG antibody confirms the synthesis of Smad6. (B–D) Infected P1 chondrocytes were cultured in the presence of 1% FBS alone or supplemented with 50 ng/ml BMP-2. (B) After 1 h, Western-blotting shows that the basal and BMP-2-stimulated level of phosphorylation of Smad1/5/8 is inhibited by Smad6. (C) After 3 days of culture, Western-blotting shows that overexpression of Smad6 (m.o.i. 500) does not modulate the type II procollagen synthesis stimulated by BMP-2. (D) In parallel, expression of the type IIA and type IIB procollagen spliced forms was analyzed by RT-PCR. The type IIA/IIB ratio was quantified on the scans of photographs of agarose gels using Image Quant software. Results obtained from four independent experiments were expressed as means ± SEM. Statistical analyses were performed using Student's t-test ($p < 0.05$ was considered significant; NS: not significant). The BMP-2 effect on the IIA/IIB ratio is not affected by overexpression of Smad6.

Table 1

Oligonucleotide primers used for the PCR analyses.

Gene	Primers (5′ to 3′)	Strand	Product size (bp)	Annealing temperature (°C)	PCR cycles	Reference
Sox9	TGGCAGACCAGTACCCGCATCT TCTTTCTTGTGCTGCACGCGC	+ −	136	55	35	(Kramer et al., 2000)
Col2a1	gcctcgcggtgagccatgatc ctccatctctgccacggggt GGTTTGGAGAGACCATGAAC TGGGTTCGCAATGGATTGTG	+ − + −	IIA: 472 IIB: 268 Total: 463	60 55	30 20	(Valcourt et al., 1999)
α10	CTGCTGAGGCTGGTTCACA GCTCCAACTTCTCCTCACTTTCT	+ −	303	65	35	NM_001081053
α11	CCGCCTTCCTCTGCTTCATACCCAT GCCGCCTCTCCTCGTTCACACACTC	+ −	654	62	30	NM_176922
Gapdh	atcactgccacccagaagac atgaggtccaccaccctgtt	+ −	443	57	25	(Valcourt et al., 1999)