Osteoarthritis and Cartilage 23 (2015) 985–995

Osteoarthritis and Cartilage

OARSI OSTEOARTHRITIS RESEARCH SOCIETY INTERNATIONAL

The high affinity ALK1-ligand BMP9 induces a hypertrophy-like state in chondrocytes that is antagonized by TGFβ1

A. van Caam †, E. Blaney Davidson †, A. Garcia de Vinuesa ‡, E. van Geffen †, W. van den Berg †, M.-J. Goumans ‡, P. ten Dijke ‡, P. van der Kraan † *

† *Experimental Rheumatology, Radboud University Medical Center, Nijmegen, The Netherlands*
‡ *Department of Molecular Cell Biology and Cancer Genomics Centre Netherlands, Leiden University Medical Center, Leiden, The Netherlands*

ARTICLE INFO

Article history:
Received 1 September 2014
Accepted 3 February 2015

Keywords:
Chondrocyte
Hypertrophy
BMP-9
TGF-beta1
Smad signaling

SUMMARY

Objective: In osteoarthritic cartilage, expression of the receptor ALK1 correlates with markers of deleterious chondrocyte hypertrophy. Recently, bone morphogenetic protein 9 (BMP9) was identified as a high affinity ligand for ALK1. Therefore, we studied if BMP9 signaling results in expression of hypertrophy markers in chondrocytes. Furthermore, because transforming growth factorß1 (TGFβ1) is a well known anti-hypertrophic factor, the interaction between BMP9 and TGFβ1 signaling was also studied.
Design: Primary chondrocytes were isolated from bovine cartilage and stimulated with BMP9 and/or TGFβ1 to measure intracellular signaling *via* pSmads with the use of Western blot. Expression of Smad-responsive genes or hypertrophy-marker genes was measured using qPCR. To confirm observations on TGFβ/Smad3 responsive genes, a Smad3-dependent $CAGA_{12}$-luc transcriptional reporter assay was performed in the chondrocyte G6 cell line.
Results: In primary chondrocytes, BMP9 potently induced phosphorylation of Smad1/5 and Smad2 to a lesser extent. BMP9-induced Smad1/5 phosphorylation was rapidly (2 h) reflected in gene expression, whereas Smad2 phosphorylation was not. Remarkably, BMP9 and TGFβ1 dose-dependently synergized on Smad2 phosphorylation, and showed an additive effect on expression of Smad3-dependent genes like *bSerpine1* after 24 h. The activation of the TGFβ/Smad3 signaling cascade was confirmed using the $CAGA_{12}$-luc transcriptional reporter. BMP9 selectively induced *bAlpl* and *bColX* expression, which are considered early markers of cellular hypertrophy, but this was potently antagonized by addition of a low dose of TGFβ1.
Conclusions: This study shows that *in vitro* in chondrocytes, BMP9 potently induces pSmad1/5 and a chondrocyte hypertrophy-like state, which is potently blocked by TGFβ1. This observation underlines the importance of TGFβ1 in maintenance of chondrocyte phenotype.

Introduction

Osteoarthritis (OA) is the most common joint disease, characterized by degeneration of articular cartilage. This loss of articular cartilage is the result of an imbalance between matrix synthesis and degradation. Chondrocytes play a crucial role in balancing both processes with their capacity to produce both matrix molecules, like collagens, and catabolic proteolytic enzymes such as Matrix Metalloproteases (MMPs). During OA, a subpopulation of chondrocytes differentiates towards a hypertrophy-like phenotype, characterized by increased production of catabolic enzymes such as MMP13[1–3]. As a consequence, these chondrocytes actively break down their surrounding matrix, resulting in pathology.

Signaling by members of the Transforming Growth Factor β (TGFβ)-superfamily, which include TGF-βs, Activins and Bone morphogenetic proteins (BMPs), regulate matrix synthesis, matrix degradation and cellular differentiation in chondrocytes[4]. The TGFβ-superfamily members signal *via* Activin receptor-Like Kinase (ALK) receptors[5]. There are seven ALKs, which phosphorylate specific sets of receptor-Smads: ALK1/2/3 and 6 phosphorylate Smad1/5 (pSmad1/5), whereas ALK4/5 and 7 phosphorylate Smad2/3 (pSmad2/3). Both pSmad2 and pSmad3, but predominantly

* Address correspondence and reprint requests to: P. van der Kraan, Experimental Rheumatology, Radboud University Medical Center, Geert Grooteplein 28, 6525 GA, Nijmegen, The Netherlands. Tel: 31-24-3616568; Fax: 31-24-3540403.
E-mail address: Peter.vanderkraan@radboudumc.nl (P. van der Kraan).

http://dx.doi.org/10.1016/j.joca.2015.02.007
1063-4584/

pSmad3, have been shown to be essential for chondrogenesis and maintenance of chondrocyte phenotype by potently blocking hypertrophy[6–8], whereas pSmad1 and pSmad5 are required for chondrocyte hypertrophy and terminal differentiation[9–12].

In human osteoarthritic cartilage, ALK1 expression is positively correlated with expression of *MMP13*, the main cartilage degrading enzyme, and of *COL10A1*, a marker of chondrocyte terminal differentiation[13]. Recently, BMP9 was identified as a high affinity ALK1 ligand[14,15]. BMP9 is produced in the liver and is present in human, bovine and murine serum in high amounts of up to 10 ng/ml[16–18], responsible for over 60% of all BMP activity found in serum[15]. In endothelial cells, BMP9 potently induces pSmad1/5 and downstream signaling[14,15]. Furthermore, in mesenchymal stem cells, BMP9 is a potent inducer of chondrocyte hypertrophy, and has been shown to induce osteogenic differentiation[19,20]. Combined, these observations suggest a pro-hypertrophic effect of BMP9 in chondrocytes. However, it has been reported that BMP9 is also able to induce pSmad2 and subsequent downstream effects in endothelial cells[21,22]. Furthermore, a synergistic action of BMP9 with TGFβ has been described in endothelial cells, in which BMP9 enhances TGFβ induced Smad2 phosphorylation and Smad3-mediated transcriptional responses[23].

In view of the opposing functions of pSmad1/5 and pSmad2/3 in chondrocyte differentiation, we wondered what the effect of BMP9 signaling on chondrocytes would be. Therefore, the aim of this study was to investigate BMP9 induced Smad phosphorylation and downstream gene expression, and to unravel if BMP9 signaling results in induction of a hypertrophy-like phenotype in chondrocytes. Furthermore, because of the reported synergy with TGFβ, we also studied the interaction of BMP9 with TGFβ.

We show that in primary chondrocytes, BMP9 indeed induces both phosphorylation of Smad1/5 and Smad2. Additionally, we demonstrate that BMP9 can synergize with TGFβ on Smad2 phosphorylation and expression of certain Smad3-dependent target genes. Furthermore, we report that BMP9 induces a hypertrophy-like state in chondrocyte as characterized by *Alkaline phosphatase* (*Alpl*) and *Collagen 10a1* (*Col10a1*) expression, which is potently counteracted by TGFβ1. Our results underline the idea that pSmad1/5 and pSmad2/3 have opposing roles in regulation of chondrocyte differentiation.

Materials and methods

Chondrocyte culture

Primary bovine chondrocytes were isolated from the metacarpophalangeal joint of cows *post mortem*. Cartilage slices were incubated overnight in 1.5 mg/ml Collagenase B (Roche Diagnostics, Germany) in DMEM/F12 (Gibco, UK) supplied with 50 mg/L gentamicin (Centrafarm Services, the Netherlands) and 100 mg/L pyruvate (Gibco UK) in a ratio of 1 g cartilage per 10 ml medium. Subsequently, chondrocyte suspension was spun down at 1500 rpm for 10 min, washed three times using saline (Aguettant, France) and resuspended in DMEM/F12 containing 10% fetal calf serum (FCS) (Thermo Scientific UK). Chondrocytes were seeded at a density of 1×10^5 cells per cm^2 in 6 wells plates (Greiner Bio-one International, the Netherlands) for protein studies, or in 24 wells plates (Bio-one International, the Netherlands) for mRNA experiments. Cells were cultured for 1 week at 37°C and 5% CO_2 before start of experiments. Subsequently, cells were serum starved and stimulated with recombinant hBMP9, hBMP2, hBMP7 (R&D systems, USA), or $hTGF\beta_1$ (BioLegend, the Netherlands) or a combination for indicated time periods and dosages. Each experiment was conducted at least three times, each time with cells of a different animal. In each experiment, conditions were tested in duplicate.

Detection of pSmads using SDS-PAGE and western blot

Cells were placed on ice and washed twice using ice cold saline. Subsequently, cells were lysed using lysis buffer (Cell signaling, USA) containing protease inhibitor cocktail (complete, Roche Diagnostics, Germany). Cell lysates were sonicated on ice for 10 cycles, of 30 s sonication and 30 s rest, using a Bioruptor (Diagenode, USA). Hereafter, samples were centrifugated at 4°C at 13,500 rpm for 15 min and pellets were discarded. With a BCA-assay (Thermo Scientific, USA), protein concentration was measured. An equal amount of protein was loaded on a 7.5% Bisacrylamide gel, for SDS-PAGE. Next, proteins were transferred to a nitrocellulose membrane using wet transfer (Towbin buffer, 2.5 h 275 mA at 4°C). After overnight incubation at 4°C with 1:1000 polyclonal Rabbit anti P-Smad1/5 (S463/465)/Smad8 (S426/428) (Cell signaling, USA) or P-Smad2 (S465/467) (Cell signaling, USA), membranes were incubated with 1:1500 polyclonal Goat anti Rabbit labeled with Horseradish peroxidase (DAKO, Belgium) for 1 h. Hereafter, enhanced chemiluminescence (ECL) using ECL plus kit (GE Healthcare, UK) was used to visualize the proteins. As loading control β-Actin rabbit mAb (13E5) (Cell signaling) or Gapdh mouse mAb (1G5) (Sigma Aldrich, Germany) was used, both 1:1500.

Detection of gene expression

RNA was isolated using TRI-reagent (Sigma–Aldrich, Germany) by following manufacturer's protocol. After this procedure, RNA was dissolved in ultra pure water and RNA concentration was measured using a NanoDrop photospectrometer (Thermo Scientific, USA). Subsequently, 1 μg of RNA dissolved in 8 μl water was treated with 1 μl DNAse (Life Technologies, USA) for 10 min at room temperature, and incubated at 65°C with 1 μl 25 mM EDTA (Life Technologies, USA) for 10 min. To perform reverse transcriptase (RT) reaction; 1.9 μl ultra pure water, 2.4 μl 10 × DNAse buffer, 2.0 μl 0.1 M DTT, 0.8 μl 25 mM dNTP, 0.4 μg oligo dT primer, 1 μl 200 U/μl M-MLV Reverse transcriptase (all Life Technologies, USA) and 0.5 μl 40 U/μl RNAsin (Promega, the Netherlands) was added, and samples were incubated for 5 min at 25°C, 60 min at 39°C, and 5 min at 95°C using a thermo cycler. Gene expression was measured using 0.5 μM of validated cDNA-specific primers (see Table I) (Biolegio, the Netherlands) in a quantitative real time polymerase chain reaction (qPCR) using SYBR green master mix (Applied Biosystems). The following protocol was used: after 10 min at 95°C, 40 cycles of 15 s 95°C and 1 min 60°C each were run. For calculations of the −ΔCt, two reference genes were used: glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and ribosomal protein S14 (RPS14).

$CAGA_{12}$-luciferase transcriptional reporter assay

The $CAGA_{12}$-luciferase reporter construct produces luciferase in response to Smad3-Smad4 activation[24]. For this assay a human chondrocyte derived cell line (G6) was used. Cells were transduced with an adenovirus containing the $CAGA_{12}$-luciferase construct at a multiplicity of infection of 10. Two days after transduction, cells were serum starved for 8 h, and hereafter stimulated with the reported doses of rhTGFβ1 and rhBMP9 for 16 h. Subsequently, cells were lysed using and luciferase activity was measured after adding Bright glo™ (Promega) on a luminometer according to manufacturer's protocol.

Statistics

All quantitative data are expressed as a mean of multiple repeats ± SD. For every analysis data was checked for normality

Table I
Primer sequences as used in this study

Gene	NCBI reference sequence	Efficiency (%)	Forward sequence (5′ → 3′)	Reverse sequence (5′ → 3′)
bRps14	NM_001077830.2	105	CATCACTGCCCTCCACATCA	TTCCAATCCGCCCAATCTTCA
bGapdh	NM_001034034.2	100	CACCCACGGCAAGTTCAAC	TCTCGCTCCTGGAAGATGGT
bAlk1	NM_001083479.1	99.5	ACAACACAGTGCTGCTCAGACA	TGCTCGTGGTAGTGCGTGAT
bAlk2	NM_176663.3	104.8	CGTTGGAGACAGCACTTTAGCA	AGAGCCGCTTCCCGATGTA
bSerpine1	NM_174137.2	99	CGAGCCAGGCGGACTTC	TGCGACACGTACAGAAACTCTTGA
bSmad7	NM_001192865.1	103	GGGCTTTCAGATTCCCAACTT	CTCCCAGTATGCCACCACG
bTgfb1	NM_001166068.1	107	GGTGGAATACGGCAACAAAATCT	GCTCGGACGTGTTGAAGAAC
bJunb	NM_001075656.1	97.2	CCTTCTACCACGACGACTCA	CCGGGTGCTTTGAGATTTCG
bId1	NM_001097568.2	107	GCTCCGCTCAGCACTCTCAA	GATCGTCCGCTGGAACACA
bCol10a1	NM_174634.1	92	CCATCCAACACCAAGACACAGT	TGCTCTCCTCTCAGTGATACACCTT
bAlpl	NM_176858.2	92	CTTTCCCTCCTGGACCAAATTA	CCTGGGTCCTTGGAGTGGT
bMmp13	NM_174389.2	92	CTTTCCCTCCTGGACCAAATTA	CCTGGGTCCTTGGAGTGGT
bSPP1	NM_174187.2	104.1	ACCCATCTCAGAAGCAGACT	TGGGAGGGTATTTTGTTTGTTGT
bCol2a1	NM_001001135.2	97.2	TGATCGAGTACCGGTCACAGAA	CCATGGGTGCAATGTCAATG

using the Shapiro–Wilk test. One-way analysis of variance (ANOVA) with Tukey's multiple comparison post test was used to determine the significance. The statistical analyses were performed with the SPSS 20.0 (IBM, Chicago, USA) statistical software package.

Results

In primary chondrocytes, BMP9 potently induces pSmad1/5 phosphorylation, and Smad2 phosphorylation to a lesser extent

First, expression of the two receptors BMP9 can use; ALK1 and ALK2[15,25] was measured using qPCR, and detected at 0.11 and 0.23 fold of reference gene value respectively. Subsequently, chondrocytes were stimulated with a dose range of rhBMP9 to investigate its potency in inducing Smad phosphorylation. Concentrations reported[16–18] as physiological (50 pg, 250 pg, 1 ng, and 2.5 ng/ml) as well as excessive (25 ng/ml) were tested. In this range, rhBMP9 dose-dependently induced pSmad1/5 after 1 h, starting from a dose of 50 pg/ml [Fig. 1(A)]. Furthermore, from a dose of 1 ng/ml or higher, BMP9 also induced pSmad2, but not in all donors. Both BMP9-induced Smad1/5 and Smad2 phosphorylation lasted up to at least 2 h after stimulation. Therefore we conclude that in primary chondrocytes, BMP9 can induce phosphorylation of both pSmad1/5 and pSmad2, but that the induction of pSmad2 requires a higher dose of BMP9.

Physiological doses of BMP9 induce expression of bId1, a Smad1/5-dependent gene, but not of Smad3-dependent genes

To investigate if the observed Smad phosphorylation results in gene transcription, mRNA expression of Smad-dependent genes was measured 2, 6 and 24 h after stimulation with rhBMP9 using qPCR [Fig. 1(B)]. The effect of three doses of BMP9 (0.25, 1 and 2.5 ng/ml) with varying induction of Smad phosphorylation, was measured. Expression of *bId1*, marker of pSmad1/5 signaling, was profoundly upregulated ~32 fold (~5 ΔC_t) at all time points compared to unstimulated samples, but with little difference between dosage. *bSerpine1*, a classical Smad3-dependent gene[24,26], was measured to study if BMP9 induced pSmad2 was reflected in gene expression, because in adults not many Smad2-dependent genes have been identified[26,27], and pSmad2 and pSmad3 follow a very similar expression pattern, even when induced TGFβ independently[28,29]. In contrast to *bId1*, expression of *bSerpine1* was not induced by BMP9 at any time point. Two other Smad3-dependent genes; *bTgfb1*[26] and *bAlk5*[30] were also not affected by BMP9. Expression of *bSmad7* and *bJunb*, two genes that have been described as responsive to both pSmad1/5 and pSmad2/3[31,32] were upregulated by BMP9, but remarkably only at 1 ng/ml or higher. In conclusion, BMP9 potently induced expression of Smad1/5-dependent genes, but not Smad3 specific genes.

BMP9 synergizes with TGFβ1 on induction of Smad2 phosphorylation

Because previously a synergistic effect of BMP9 and TGFβ1 on Smad2/3 phosphorylation has been reported[23], we investigated if this synergy also occurs in chondrocytes. To investigate a possible dose-dependency, two doses of rhTGFβ1, 0.1 and 1 ng/ml, and three doses of BMP9, 0.5, 5 and, based on the previously mentioned study[23], 50 ng/ml were used [Fig. 2(A)]. As expected, stimulation of chondrocytes with TGFβ1 led to phosphorylation of Smad2 and Smad1/5. Furthermore, BMP9 strongly induced pSmad1/5 but not pSmad2 in this donor. Most remarkably, BMP9 dose-dependently enhanced TGFβ1-induced Smad2 phosphorylation of both TGFβ1 doses. In addition, inhibition of BMP9-induced pSmad1/5 was observed in the presence of 0.1 ng/ml TGFβ1, but not in the presence of 1 ng/ml TGFβ1. These data suggest that TGFβ1 and BMP9 synergize on the induction of pSmad2 in chondrocytes.

Co-stimulation of chondrocytes with BMP9 and TGFβ1 results in an additive effect on expression of Smad3-dependent genes

To investigate the observed synergy between BMP9 and TGFβ1 further downstream, mRNA levels of Smad3-dependent genes were measured after 2 and 24 h. A low dose of TGFβ1, 0.1 ng/ml, and high dose of BMP9, 50 ng/ml were used, because these two conditions showed profound synergy on pSmad2 in the previous experiment. Two hour after stimulation, both TGFβ1 and BMP9 induced expression of *bId1*, ~2.8-fold (~1.5 ΔC_t) and ~16-fold (~4 ΔC_t) respectively, but no additive, synergistic of inhibitory effect, was observed between both growth factors [Fig. 2(B)]. Expression of the Smad3-dependent genes *bSerpine1*, *bTgfb1*, *bAlk5* was not yet induced by either growth factor alone or in combination. Single stimulation with TGFβ1 or BMP9 both induced bSmad7 expression ~2.5-fold (~1.3 ΔC_t), and co-stimulation increased the expression ~6-fold (~2.5 ΔC_t). Furthermore, *bJunb* levels were only significantly upregulated ~4-fold (~2 ΔC_t) after co-stimulation with TGFβ1 and BMP9.

Twenty-four hour after stimulation, *bId1* expression was still upregulated ~2.8-fold (~1.5 ΔC_t) by BMP9 but no longer by TGFβ1. Again, no additive, synergistic of inhibitory effect was observed on this pSmad1/5 dependent gene [Fig. 2(C)]. In contrast to the 2 h timepoint, Smad3-dependent genes were upregulated after 24 h. Only co-stimulation with TGFβ1 and BMP9 significantly upregulated *bSerpine1* expression 2-fold (~1 ΔC_t), suggesting enhanced pSmad2/3 signaling. Enhanced pSmad2/3 signaling is further

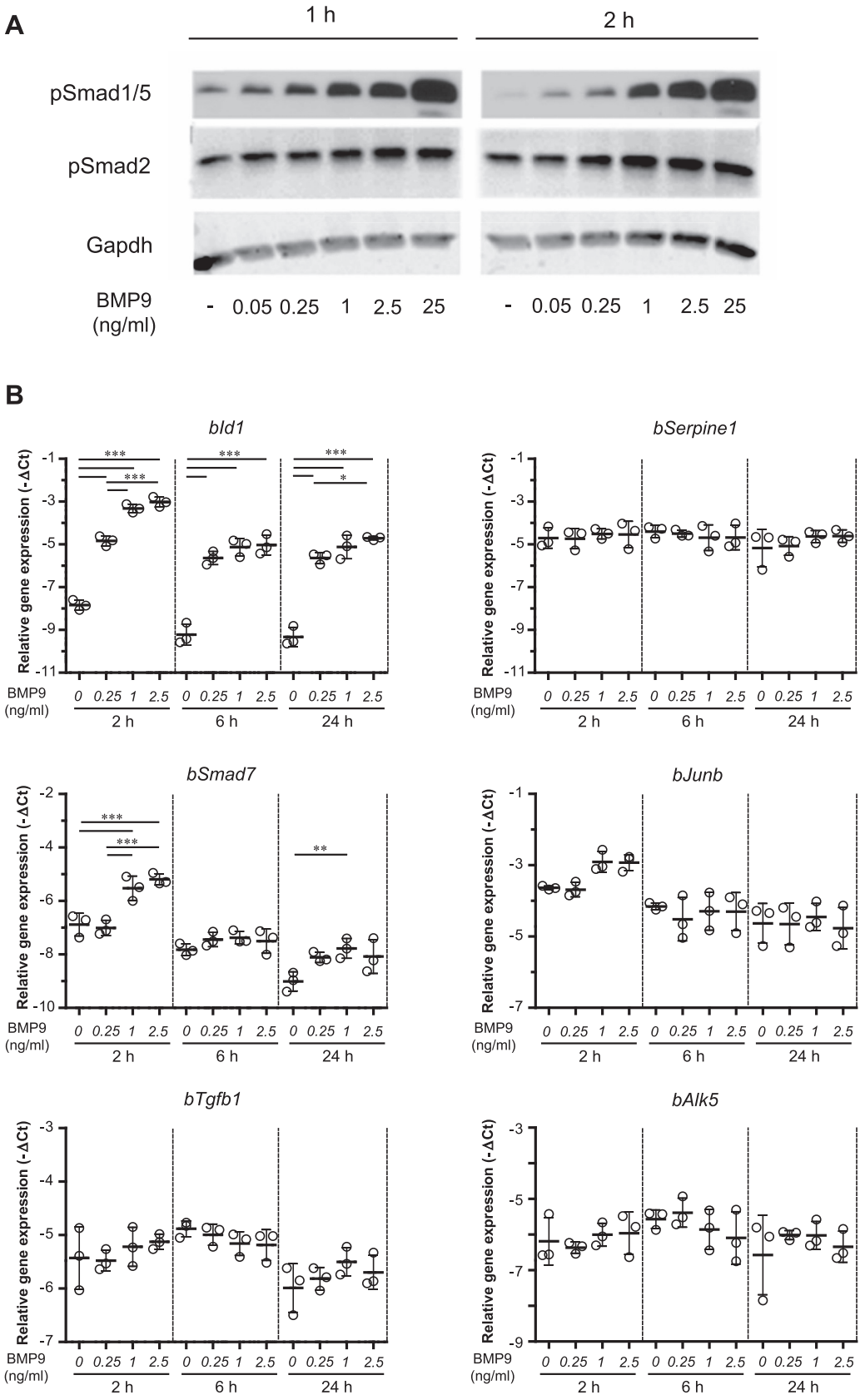


Fig. 1. BMP9 induces Smad phosphorylation and downstream gene expression in primary bovine chondrocytes. **(A)** Primary bovine chondrocytes were stimulated with a dose range of rhBMP9 for 1 and 2 h, and induction of Smad1/5 and Smad2 phosphorylation was analyzed on Western blot using specific antibodies. pSmad1/5 is potently dose-dependently induced by BMP9 from 50 pg/ml, whereas pSmad2 is induced from a dose of 1 ng/ml and higher. (**B**) Chondrocytes of three donors were stimulated in duplo with 0.25, 1 and 2.5 ng/ml BMP9 for 2, 6 and 24 h, to study BMP9-induced gene expression downstream pSmad1/5 and pSmad2/3 using qPCR. BMP9 significantly induced expression of *bId1* at all time points, but not of *bSerpine1*. For Western blot, a donor is shown in which BMP9-stimulation resulted in both Smad1/5 and Smad2 phosphorylation. For qPCR data, average ± S.E.M. was plotted, with each dot representing the average of one donor. Statistics were calculated using one-way ANOVA with a Tukey's post-hoc test ($^{*} = P < 0.05$, $^{**} = P < 0.01$ and $^{***} = P < 0.001$).

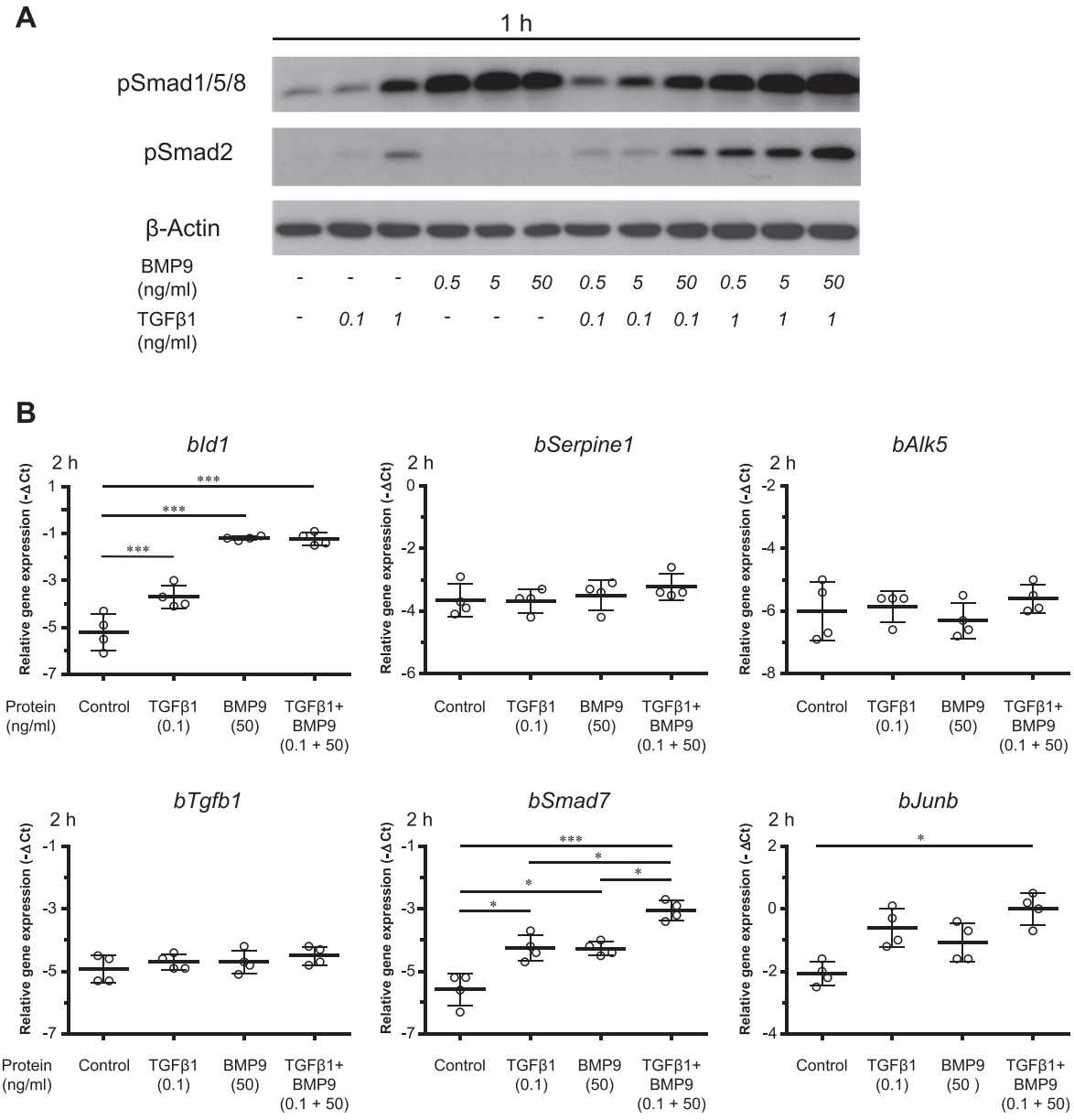
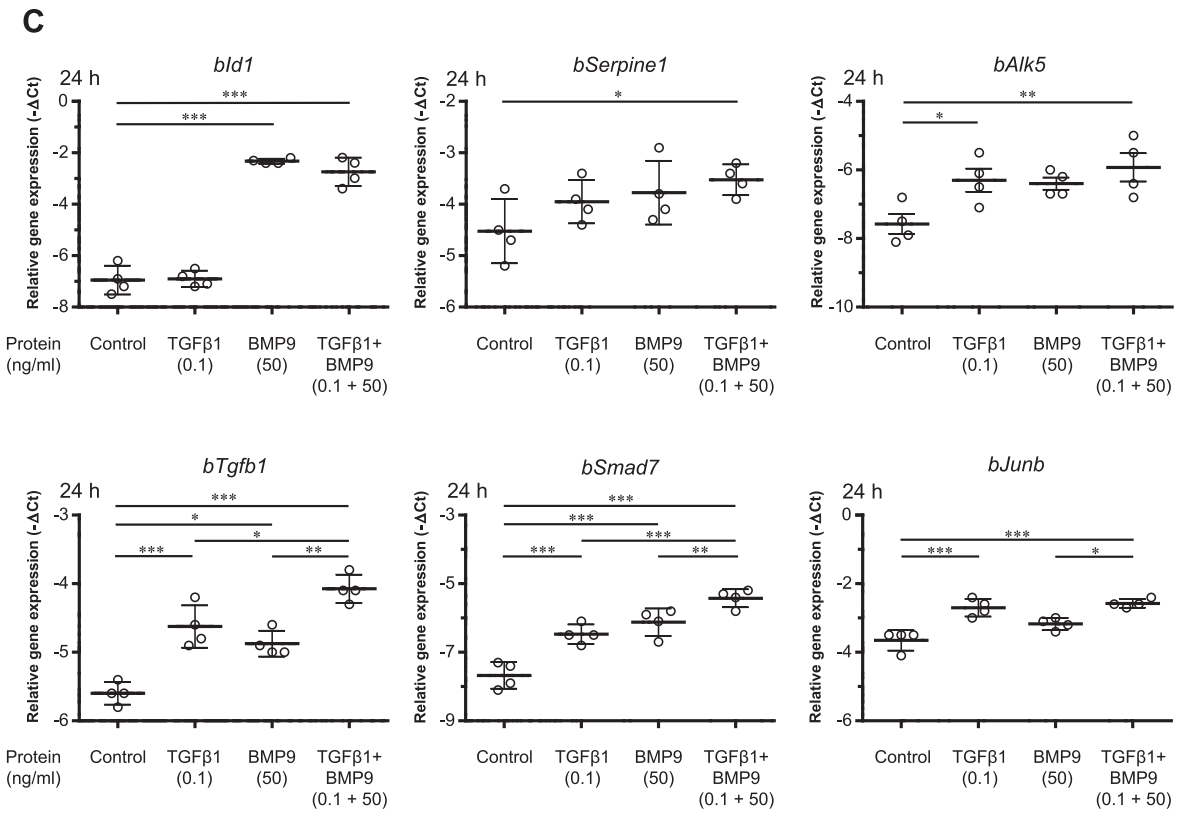


Fig. 2. BMP9 synergizes with TGFβ1 on pSmad2 induction resulting in enhanced expression of pSmad2/3 responsive genes. (**A**) Chondrocytes were treated for 1 h with either 0.1 or 1 ng/ml of rhTGFβ1, 0.5, 5 or 50 ng/ml of rhBMP9 or a combination of both growth factors and subsequently pSmad2 and pSmad1/5 were visualized on Western blot using specific antibodies. An inhibitory effect of 0.1 ng/ml on BMP9-induced pSmad1/5 was observed. Furthermore, a synergistic effect of BMP9 on TGFβ1 induced pSmad2 was observed, as signal intensity was increased for co-stimulated samples compared to samples only stimulated with TGFβ1. (**B**) and (**C**) Co-stimulation of chondrocytes with BMP9 and TGFβ1 was also investigated on gene expression of Smad-dependent genes 2 h (B) or 24 h (C) after stimulation, by stimulating four donors in duplo. At 2 h after stimulation, no effect on *bSerpine1* expression could be detected. *bSmad7* and *bJunB* expression was upregulated more strongly in samples treated with both growth factors compared to single treatment, however not in a synergistic manner. At 24 h after stimulation, combination treatment induced more *bSerpine1*, *bTgfb1* and *bSmad7* expression compared to single treatment. (**D**) To study if co stimulation of chondrocytes with BMP9 and TGFβ1 results in enhanced Smad responsive biologic activity, the chondrocyte G6 cell line was transduced with the pSmad3-responsive CAGA12-luc construct, and treated with either 1 ng/ml TGFβ1, 5 or 50 ng/ml BMP9 and a combination of both for 16 h. Both growth factors synergized, as co-stimulation enhanced luciferase production significantly compared to TGFβ1 stimulation alone. For Western blot, a representative donor is shown. For qPCR data, average ± S.E.M. was plotted with each dot representing the average of one donor. Statistics were calculated using one-way ANOVA with a Tukey's post-hoc test (* = $P < 0.05$, ** = $P < 0.01$ and *** = $P < 0.001$).

indicated by *bTgfb1* expression levels, as co-stimulation induced significantly higher expression of this gene compared to single stimulations; ~3-fold (~1.6 ΔC_t) vs ~2-fold (~1 ΔC_t) respectively. Similar to 2 h stimulation, an additive effect of TGFβ1 and BMP9 was observed on *bSmad7*; both TGFβ1 and BMP9 induced expression ~2.5-fold (~1.3 ΔC_t), but co-stimulation induced expression 4.6-fold (~2.2 ΔC_t). Therefore, these data show that co-stimulation with TGFβ1 and BMP9 results in an additive effect on pSmad2/3-responsive gene expression, but not on pSmad1/5-responsive genes.

Synergy between BMP9 and TGFβ1 on CAGA$_{12}$-luc activity

To further confirm the synergy between BMP9 and TGFβ1 on Smad2/3 phosphorylation, we made use of a Smad3/Smad4-reporter construct: CAGA$_{12}$-luc. Due to low transfection rates we were not able to use primary cells and used the human chondrocyte-like G6 cell line. Stimulation with 1 ng/ml TGFβ1 strongly induced luciferase activity ~7-fold, whereas BMP9 in doses of 5 and 50 ng/ml did not [Fig. 2(D)]. Although co-stimulation of TGFβ1 with 5 ng/ml BMP9 only shows a trend towards enhanced



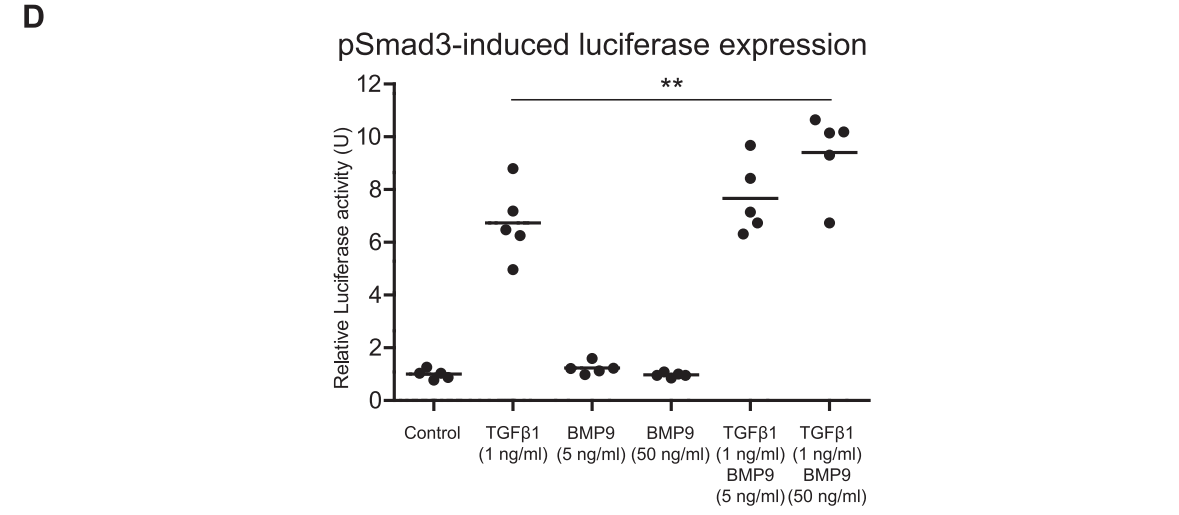


Fig. 2. (*continued*).

pSmad3 signaling, co-stimulation with 50 ng/ml BMP9 strikingly and significantly enhanced luciferase activity up to ~10-fold, displaying clear synergy between TGFβ1 and BMP9, and therefore this dose was used in our next experiments.

A high dose of BMP2 and BMP7 does not synergize with TGFβ1 on pSmad2 phosphorylation

Now that the synergy between BMP9 and TGFβ1 on pSmad2/3 was confirmed in chondrocytes, we investigated if this is a common BMP characteristic. We compared BMP9 to BMP2 and BMP7 due to their importance in chondrocyte biology[33], and their use of the same type II receptors but different type I receptors as BMP9[15,34]. Because the synergy between BMP9 and TGFβ1 is best observed at a high dose of BMP9, we chose to use a high dose (250 ng/ml) of BMP2 and BMP7 as well. Both BMPs induced pSmad1/5 comparable to BMP9 (Fig. 3), but in contrast to BMP9, both BMPs lacked a synergistic interaction with TGFβ1 on pSmad2 phosphorylation. This indicates that the synergy with TGFβ1 on pSmad2 is a unique characteristic of BMP9.

High dose of BMP9 induces expression of chondrocyte hypertrophy marker genes which is blocked by TGFβ1

Overexpression of BMP9's constitutively active receptor ALK1 induces expression of chondrocyte hypertrophy markers[13]. Therefore, we analyzed the effect of BMP9 signaling on expression of

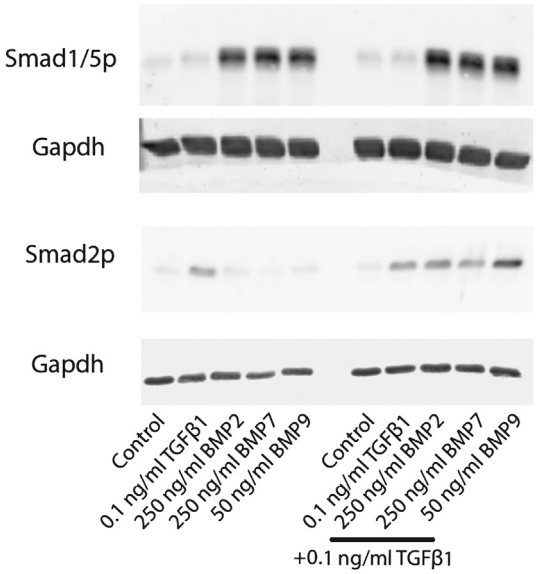


Fig. 3. BMP2 and BMP7 do not synergize with TGFβ1 on pSmad2. Chondrocytes were treated for 1 h with 250 ng/ml of BMP2 or 250 ng/ml BMP7, 50 ng/ml BMP9 and 0.1 ng/ml TGFβ, and pSmad1/5 and pSmad2 induction was analyzed on Western blot using specific antibodies. Although both BMPs induced similar pSmad1/5 compared to BMP9, BMP2 and BMP7, unlike BMP9, did not synergize with TGFβ1 on induction of Smad2 phosphorylation. This Western blot depicts a representative experiment.

hypertrophy markers after 1 week. Considering that pSmad3 is a potent blocker of chondrocyte hypertrophy, co-stimulation with TGFβ1 was included (Fig. 4). After 1 week, *bId1* expression was highly upregulated ~49-fold (~5.6 ΔC_t) by 25 ng/ml BMP9. Remarkably, co-stimulation with 0.1 ng/ml TGFβ1 significantly lowered this induction to ~21-fold (~4.4 ΔC_t). Of the four markers of chondrocyte hypertrophy that were measured, expression of *bMMP13* or *bSPP1* was not significantly regulated by BMP9, TGFβ1, or the combination of both. In contrast, expression of both *bAlpl* and *bCol10a1*, was highly upregulated by 25 ng/ml BMP9; ~10.6-fold (~3.4 ΔC_t) and ~4.6-fold (~2.2 ΔC_t) respectively. Strikingly, 0.1 ng/ml TGFβ1 could block BMP9-induced expression of these hypertrophy markers. Notably, matrix mineralization could not yet be observed by either alcian blue or alizarin red staining in our experiments. However, also after addition of two known inducers of matrix mineralization; β-glycerophosphate and ascorbic acid, no mineralization could be observed either. Apart from the inhibition of hypertrophy marker genes, co-stimulation also significantly induced *bCol2a1* expression ~2.1-fold (~1.1 ΔC_t), showing a beneficial effect of simultaneous stimulation with TGFβ1 and BMP9 on matrix production. Based upon these results we conclude that BMP9 induces the expression of certain hypertrophy-like marker genes, which is counteracted by a low dose of TGFβ1.

Discussion

Increasing evidence suggests pivotal, but opposing, roles for pSmad2/3 and pSmad1/5 in chondrocyte differentiation, with a hypertrophy-inducing role for the latter[35–37]. We investigated *in vitro* in primary chondrocytes the effects of BMP9, a potent pSmad1/5 inducing ligand that circulates in blood. We report that, in primary chondrocytes, BMP9 induces pSmad1/5 and expression of the chondrocyte hypertrophy markers *bCol10a1* and *bAlpl*, but that this effect is potently counteracted by TGFβ1. Possibly, this inhibition is facilitated by the synergy we observed between BMP9 and TGFβ1 on Smad2/3 phosphorylation, a synergy which is unique for BMP9 compared to BMP2 or BMP7.

Previous reports have characterized BMP9 as a potent inducer of pSmad1/5 in a variety of endothelial cell lines[21,22]. We were able to show that also in primary chondrocytes, BMP9 potently induces pSmad1/5. Surprisingly, in some donors, we not only observed Smad1/5 but also Smad2 phosphorylation after stimulation with BMP9. In some, but not all, endothelial cell lines, BMP9-induced pSmad2 has been reported as well[21,22]. BMP9-induced pSmad2 is remarkable, as both type I receptors that BMP9 can bind; ALK1 and ALK2, are not known to directly phosphorylate Smad2. Therefore, how BMP9 induces pSmad2 is yet unclear.

After phosphorylation, Smad1/5 and Smad2/3 form complexes with Smad4, the common-Smad, and translocate to the nucleus where these complexes bind DNA and regulate gene transcription[5]. When analyzing BMP9-induced gene expression in primary chondrocytes, we observed rapid and robust up regulation of the pSmad1/5-dependent gene *bId1*, but not of pSmad3-dependent genes like *bSerpine1*. This lack of Smad3-dependent gene expression is apparently in contradiction with our observations on Western blot, which suggest pSmad2/3 signaling. However, in both endothelial cells and chondrocytes, inhibition of pSmad3 signaling by ALK1 signaling has previously been reported. In both cell types, over-expression of constitutively active ALK1 reduced pSmad3-dependent transcriptional activity, while not affecting Smad phosphorylation itself[38–40]. Possibly, the rapid induction of Smad7 we found in this study can explain the observed lack of pSmad2/3-responsive gene expression. Smad7 is an inhibitor of receptor-Smad signaling (inhibitory-Smad) with a multitude of actions, including the ability to prevent binding of the pSmad2/3-Smad4 complex to DNA[41,42]. Expression of this inhibitory-Smad was rapidly induced ~3.2-fold within 2 h of BMP9 stimulation. Additionally, in chondrocytes, adenoviral over-expression of Smad7 has previously been reported to inhibit *mSerpine1* transcription[43]. Therefore, in primary chondrocytes, Smad7 might be an important regulator of the crosstalk between the BMP9-induced pSmad1/5 and pSmad2/3 signaling pathways.

The crosstalk between pSmad1/5 and pSmad2/3 signaling controls chondrocyte differentiation (reviewed in[37]). Via an interaction with the Runx2, a transcription factor regarded as the key regulator of endochondral ossification, pSmad1 and 5 can directly enhance terminal differentiation by inducing expression of genes like *Col10a1* and *Mmp13*[35,44,45]. Prolonged stimulation with BMP9 indeed induced expression of the early hypertrophy marker genes *bCol10a1* and *bAlpl*, but increased *bMmp13* expression was not observed. However, *bMmp13* expression mainly occurs in late stage hypertrophic chondrocytes[46], a cellular state most likely not achieved in our experimental setting, also indicated by the lack of mineralization in our experiments. Compared to pSmad1/5, pSmad2/3 has an opposite effect on Runx2 activity; it silences Runx2-responsive genes via recruitment of histone deacetylases[36]. In our experiments, BMP9-induced expression of hypertrophy markers was indeed antagonized by a low dose of TGFβ1, a well known inducer of Smad2 and 3 phosphorylation. Possibly, this antagonism is facilitated by the synergy we observed between BMP9 and TGFβ1 on Smad2/3 phosphorylation. In addition to Smad signaling, Smad-independent BMP-signaling *via* e.g., ERK and TAK1 can play a role in chondrocyte hypertrophy[47,48]. A limitation of our study is that we did not investigate these pathways. However, the Smad-dependent and Smad-independent pathways are closely interwoven by regulation of the former by the latter[49], and we think that Smad signaling is the key effector in BMP-induced chondrocyte hypertrophy. Nonetheless, additional experiments are needed to reveal whether this actually holds true.

In cartilage, the inhibition of BMP9 induced hypertrophy is possibly an important physiological role of TGFβ1. TGFβ1 itself is produced by chondrocytes, and present in cartilage in high amounts[50]. Although we were unable to detect BMP9 mRNA in

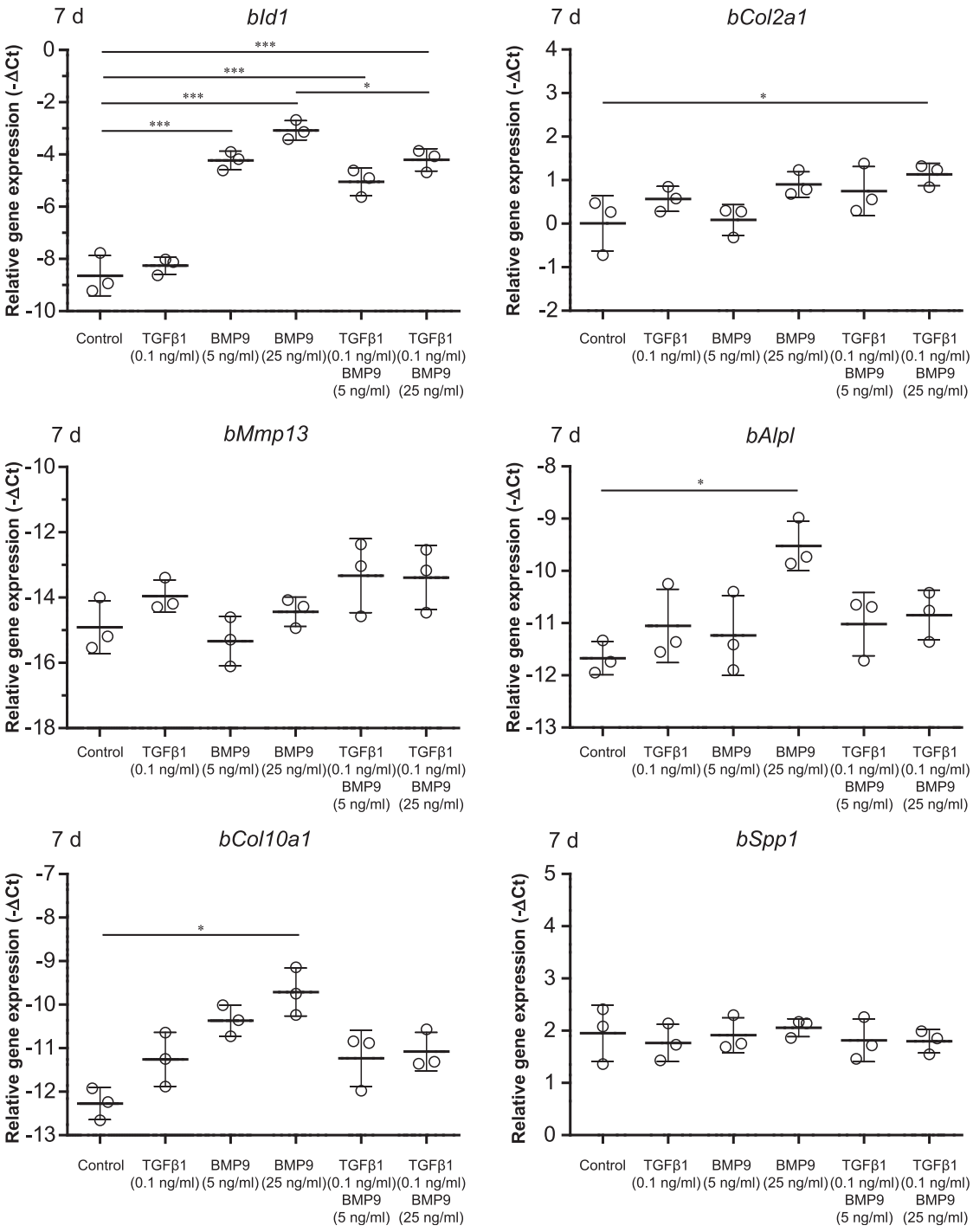


Fig. 4. BMP9 induces expression of early hypertrophy-like marker genes, which is potently blocked by a low dose of TGFβ1. Chondrocytes of three donors were treated in duplo with 0.1 ng/ml of rhTGFβ1, 5 or 50 ng/ml of rhBMP9 or a combination of both growth factors for 7 days, and gene expression of markers of chondrocyte hypertrophy and terminal differentiation were measured using qPCR. Stimulation medium was refreshed every 3 days. After 7 days of treatment, expression of both *Col10a1* and *bAlpl* was significantly increased in samples stimulated with 50 ng/ml rhBMP9, but not in samples treated with 50 ng/ml rhBMP9 and 0.1 ng/ml TGFβ1, indicating an inhibitory effect of TGFβ1. This inhibitory effect of TGFβ1 on BMP9 induced signaling is reflected in *bId1* expression, as addition of TGFβ1 inhibited BMP9 induced *bId1* expression significantly. For qPCR data, average ± S.E.M. was plotted with each dot representing the average of one donor. Statistics were calculated using one-way ANOVA with a Tukey's post-hoc test.

chondrocytes, BMP9 is most likely readily available in cartilage *in vivo*. The liver produces large amounts of BMP9, and BMP9 circulates in levels as high as ~10 ng/ml in bovine and ~12 ng/ml in human serum and plasma[16–18]. Furthermore, BMP9 is responsible for over 60% of all BMP activity in serum, indicating BMP9's relative abundance[15]. Because synovial fluid partly consists of plasma filtrate[51], it is likely that chondrocytes are constantly exposed to BMP9 via synovial fluid, but this still has to be confirmed. Considering that BMP9 signaling can induce hypertrophy, inhibition of this signal would be of crucial importance for the maintenance of articular cartilage, and therefore an important role of TGFβ1. Additional *in vivo* experiments are needed to test this hypothesis. In

a pilot study, we tried to investigate to ability of BMP9 to induce cartilage hypertrophy *in vivo* by intra-articular injection of BMP9, but severe osteophyte formation, resulting in disrupted joint homeostasis, in response to BMP9 made it very difficult to draw conclusions on BMP9's direct effect on cartilage.

BMP9 levels are not known to correlate with OA, but its serum level doesn't decrease during aging in mice[18]. We think that this stable level of BMP9 becomes a problem for cartilage during aging, in view of age-related loss of TGFβ signaling[11,13]. From cartilage-specific TGFβ-receptor type 2 knockout mice it is known that mice lacking TGFβ-signaling develop increased cartilage degradation and expression of hypertrophy markers[52]. It would be very interesting to see if BMP9 is the driving force behind this process by crossing these mice with BMP9 knockout mice.

How BMP9 and TGFβ1 can synergize on pSmad2/3 phosphorylation and downstream gene expression is yet unknown. However, the synergy between both growth factors can be observed rapidly, which suggests a mechanism upstream in the signaling pathway. TGFβ-superfamily signaling requires formation of a hetero-tetrameric receptor complex containing two type II and two type I receptors, in which receptor-Smads are phosphorylated by the type I receptors[5]. TGFβ1 can bind two type I receptors, ALK1 and ALK5, and form complexes containing both. Furthermore, complex formation between ALK1 and ALK5 induces both pSmad1/5 and pSmad2/3[39]. Putatively, a receptor-complex containing both ALK1 and ALK5 can phosphorylate less Smad2/3 compared to a complex containing ALK5 twice because ALK1 cannot phosphorylate pSmad2/3. Using radiolabeled ligands, it has been shown that BMP9 has a far greater affinity for ALK1 than TGFβ1[15]. Possibly, BMP9 affects TGFβ1-induced receptor complex formation by sequestering ALK1, forcing TGFβ1 to signal via receptor complexes containing solely ALK5, thus resulting in more pSmad2. This idea is supported by the observation that two other BMPs, BMP2 and BMP7, do not synergize with TGFβ1 on pSmad2 even at a very high dosage. These BMPs share the induction of pSmad1/5 and the use of type II receptors like BMPR2 with BMP9, but do not use ALK1, making it more likely that ALK1 is involved in the synergy between BMP9 and TGFβ1. Moreover, the synergy between TGFβ1 and BMP9 is most obvious at high levels of BMP9, which would sequester ALK1 more efficiently.

In summary, this study shows that in chondrocytes, the ALK1-ligand BMP9 induces pSmad1/5 and downstream gene expression, leading within a week to a hypertrophy-like state in chondrocytes characterized by *bAlpl* and *bColX* expression. However, we also show that this can potently be inhibited by addition of a low dose of TGFβ1. Possibly, the observed synergy between BMP9 and TGFβ1 on Smad2 phosphorylation plays a role in this inhibitory effect. Although we only investigated this interaction *in vitro*, we propose that the interaction of BMP9 with TGFβ1 underlines the importance of TGFβ1 in maintenance of chondrocyte phenotype and furthermore that our observations support the idea that pSmad1/5 and pSmad2/3 have opposing roles in regulation of chondrocyte differentiation.

Author contributions

Conception and design: Arjan van Caam, Esmeralda Blaney Davidson, Peter van der Kraan.

Collection and assembly of data: Arjan van Caam, Ellen van Geffen.

Analysis and interpretation of data: Arjan van Caam, Esmeralda Blaney Davidson, Amaya Garcia de Vinuesa, Ellen van Geffen, Marie-José Goumans, Peter ten Dijke, Peter van der Kraan.

Drafting of the manuscript: Arjan van Caam, Esmeralda Blaney Davidson, Peter van der Kraan.

Critical revision: Arjan van Caam, Esmeralda Blaney Davidson, Amaya Garcia de Vinuesa, Ellen van Geffen, Wim van den Berg, Marie-José Goumans, Peter ten Dijke, Peter van der Kraan.

Final approval of the article: Arjan van Caam, Esmeralda Blaney Davidson, Amaya Garcia de Vinuesa, Ellen van Geffen, Wim van den Berg, Marie-José Goumans, Peter ten Dijke, Peter van der Kraan.

Funding

This study was supported by a grant from: The Netherlands Organisation for Scientific Research (NWO, ZonMW) for AvC, AGdV, MJG, PtD, and the Dutch Arthritis Association (Reumafonds) for EBD, EvG and PvdK.

Conflict of interest

The authors have no conflict of interest.

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