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Prostaglandin E2 Inhibits BMP Signaling and Delays Chondrocyte Maturation

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Abstract

While cyclooxygenases are important in endochondral bone formation during fracture healing, mechanisms involved in prostaglandin E2 (PGE2) regulation of chondrocyte maturation are incompletely understood. The present study was undertaken to determine if PGE2 effects on chondrocyte differentiation are related to modulation of the BMP signaling pathway. In primary murine sternal chondrocytes, PGE2 differentially regulated genes involved in differentiation. PGE2 induced *type II collagen* and *MMP-13*, had minimal effects on *alkaline phosphatase*, and inhibited the expression of the maturational marker, *type X collagen*. In BMP-2 treated cultures, PGE2 blocked the induction of *type X collagen*. All four EP receptors were expressed in chondrocytes and tended to be inhibited by BMP-2 treatment. RCJ3.1C5.18 chondrocytes transfected with the Protein Kinase A (PKA) responsive reporter, CRE-luciferase, showed luciferase induction following exposure to PGE2, consistent with activation of PKA signaling and the presence of the EP2 and EP4 receptors. Both PGE2 and the PKA agonist, dibutyryl cAMP, blocked the induction of the BMP-responsive reporter, 12XSBE, by BMP-2 in RCJ3.1C5.18 chondrocytes. In contrast, PGE2 increased the ability of TGF- β to activate the TGF- β -responsive reporter, 4XSBE. Finally, PGE2 down regulated BMP-mediated phosphorylation of Smads 1, 5, and 8 in RCJ3.1C5.18 cells and in primary murine sternal chondrocytes. Altogether, the findings show that PGE2 regulates chondrocyte maturation in part by targeting BMP/Smad signaling and suggest an important role for PGE2 in endochondral bone formation.

Introduction

Endochondral ossification occurs during long bone development and in response to injury during the process of bone repair (20;40). During this process, mesenchymal stem cells proliferate, undergo condensation, and begin producing cartilage-specific type II collagen (20). Immature chondrocytes form a template for bone formation. A key event is the transition from a proliferating to a hypertrophic chondrocyte. Pre-hypertrophic chondrocytes exit the cell cycle and express the genes *alkaline phosphatase* and *type X collagen (colX)*, and calcify the matrix (20;46). Subsequently, these cells terminally differentiate, express *metalloproteinase 13 (MMP-13)* and *vascular endothelial growth factor (VEGF)* and undergo apoptosis (30; 46), leaving behind a cartilage matrix that provides a scaffold for vascular invasion and primary bone formation by osteoblasts (9;20;40;41).

The progression of chondrocytes through the process of endochondral ossification is controlled by growth factors and signaling molecules. One signaling molecule implicated in this process,

particularly in bone reparative events, is PGE2. PGE2 is a major metabolite in the cyclooxygenase pathway. PGE2 is synthesized and secreted by growth plate chondrocytes as are all four receptor isoforms, EP1, EP2, EP3, and EP4 (3;7;26;37). Addition of PGE2 to cell cultures increases expression of *Sox9*, type II collagen, aggrecan, and proteoglycan and induces proliferation (3;7;26;28;31). The expression of later maturational markers seen in hypertrophic and terminally differentiated chondrocytes is delayed with continued PGE2 treatment (26). PGE2 dose-dependently inhibits the expression of type X collagen, VEGF, MMP13, and alkaline phosphatase (26;46). Thus, studies to date suggest that PGE2 acts to stabilize the chondrocyte phenotype in a less differentiated state and inhibits the maturation process.

PKA signaling has been implicated in the regulation of chondrocyte maturation (17;18;25;49). PGE2 activates PKA signaling through two of the receptor isoforms, EP2 and EP4. While others have shown that PKA signaling is involved in the induction of type II collagen and proteoglycans, work in our laboratory suggests that PGE2 inhibits chondrocyte maturation through a PKA-dependent mechanism (26;31). Although PGE2 signaling through PKA tends to enhance expression of immature chondrocyte markers and inhibit chondrocyte maturation, the manner in which PGE2 signals are integrated with other modulators of chondrocyte maturation is not known (2;17;18;25).

BMP signaling has been shown to be a potent inducer of chondrocyte maturation. (12;19;42). BMP stimulates chondrocyte maturation and the expression of *colX* in numerous cell culture systems, including primary murine sternal chondrocytes (8;27;48). BMPs 2, 4, 6, and 7 are all expressed in the growth plate or adjacent perichondrium and are implicated in the commitment of chondrocytes to hypertrophy in the growth plate (14;29;34;39). BMP binding to a type II serine/threonine kinase receptor (BMPRII) results in association with type I BMP receptors (BMPRI, BMPRII, and the type I activin receptor) and leads to phosphorylation of Smads 1, 5, and 8, which are specific for the BMP receptors (14;38). Once activated, these R-Smads are released from the receptor and associate with Smad 4 in the cytoplasm. The complex translocates to the nucleus where it binds to DNA and influences gene transcription in association with other transcriptional coactivators (1). Expression levels of Smads 1 and 5 increase in late proliferating and early hypertrophic chondrocytes in association with the initiation of chondrocyte differentiation (27;34;38;39).

In the present study we investigate the hypothesis that PGE2 inhibits chondrocyte maturation and targets the BMP/Smad signaling pathway. The findings provide important new insights regarding the mechanisms through which PGE2 regulates chondrocyte differentiation.

Materials and Methods

Chondrocyte cell cultures

Primary murine sternal chondrocytes were isolated and cultured from 3 day old neonatal C57/B6 mice as previously described (23). The anterior rib cage and sternum were harvested *en bloc*, washed with sterile Hank's Buffered Salt Solution (HBSS, Invitrogen, Carlsbad, CA), and then digested with Pronase (Roche Applied Science, Indianapolis, IN) dissolved in HBSS (2 mg/ml) in a 37 °C water bath with continuous shaking for 45 minutes. This was followed by incubation in a solution of collagenase D (3 mg/ml dissolved in serum-free Dulbecco's modified Eagle's medium; Roche Applied Science) for 90 min at 37 °C. The soft tissue debris was removed and the remaining sterna and costosternal junctions were further digested in fresh collagenase D solution in Petri dishes in a 37 °C incubator for 4.5 hours with intermittent shaking. This step allows remnant fibroblasts to attach to the Petri dish while the chondrocytes remain afloat in the medium. The digestion solution was filtered through a 40 µm cell strainer (BD Biosciences, Franklin Lakes, NJ) to remove residual bone fragments. The solution was

centrifuged, and the cells resuspended in Dulbecco's modified Eagle's medium with high glucose, GlutaMax-1, without sodium pyruvate (Invitrogen), 5% fetal bovine serum (FBS), 1% penicillin/streptomycin. The cells were counted and plated at the appropriate density. For all experiments, growth factors were added directly to the individual culture wells. Prostaglandin E2 (PGE2), dibutyrylcyclic AMP, and ascorbic acid were purchased from Sigma (St. Louis, MO). Bone morphogenetic protein 2 (BMP-2) was purchased from Peprotech (Rocky Hill, NJ).

For maturation experiments primary chondrocytes were washed twice with sterile PBS twelve to sixteen hours after plating and fresh 5% DMEM was added containing 50 µg/ml ascorbic acid. Growth factors or control medium were added directly to individual wells at this time. Media containing fresh ascorbic acid and growth factors were added every two to three days until the cell cultures were harvested. For protein extraction primary chondrocytes were placed in control medium overnight and then treated with various growth factors for 4 hours.

RCJ3.1C5.18 chondrocytes (C5.18 cells) were maintained in α -minimal essential medium containing 10% FBS. C5.18 cells were used in transfection and Western blot experiments and were plated in 12-well plates at a density of 35,000 cells per well.

Real-time PCR

Total RNA was extracted from cultures using Trizol (Invitrogen, Carlsbad, CA). First-Strand Synthesis System for RT-PCR (Invitrogen) was used to synthesize cDNA from 1.5 µg total RNA according to the oligo(dT) version of the protocol. Real-time PCR was performed using the Rotor-Gene real-time DNA amplification system (Corbett Research, South Wales, Australia) and the fluorescent dye SYBR Green I to monitor DNA synthesis (SYBR Green PCR Master Mix, Applied Biosystems, Foster City, CA). The following cycle parameters were used for all experiments: 20s at 94°C, 30s at 60°C, and 30s at 72°C for a total of 45 cycles. The relative level of mRNA for a specific gene was normalized to β -actin levels. Table 1 shows the sequences for all primer sets used in these experiments.

Alkaline Phosphatase Activity

Alkaline phosphatase activity was measured following a previously described protocol (13). Culture medium was aspirated from chondrocytes cultured in 24-well plates. The plates were rinsed with 150 mM NaCl, and 1 ml of reaction buffer containing 0.25 M 2-methyl-2-amino propanol, 1 mM magnesium chloride, and 2.5 mg/ml of *p*-nitrophenyl phosphate (Sigma) at pH 10.3 was added to the wells at 37°C. The reaction was stopped after 30 minutes by the addition of 0.5 ml of 0.3 M Na₃PO₄ (pH 12.3). Alkaline phosphatase activity was determined by measuring absorbance of light at 410 nm and comparing the experimental samples with standard solutions of *p*-nitrophenol and an appropriate blank. Alkaline phosphatase was normalized for protein concentration using BCA Protein Assay Reagent (Pierce, Rockford, IL), as measured by spectrophotometry (562 nm) and compared to standard protein concentrations.

Western Blot

Cell cultures were treated with lysis buffer containing protease inhibitors (Protease Inhibitor Cocktail Set III, Calbiochem, San Diego, CA) and 1mM sodium orthovanadate. Protein concentration was determined using Coomassie Plus protein assay kit (Pierce, Rockford, IL). The protein extracts (20µg) were separated using 10% SDS-PAGE. After transfer to a polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA) and blocking with 5% milk, the blots were probed with the following antibodies overnight at 4°C: phospho-Smad1 (Ser 463/465)/Smad5 (Ser 463/465)/Smad8 (Ser426/428) polyclonal antibody (Cell Signaling Technology, Danvers, MA), or mouse monoclonal β -actin antibody (Sigma, St. Louis, MO)

Horseradish peroxidase-conjugated secondary antibodies (Bio-Rad) were then applied to the membrane and incubated for 30 minutes at room temperature. The immune complexes were detected using SuperSignal West Femto maximum sensitivity substrate or SuperSignal West Pico chemiluminescent substrate (Pierce). For multiple detection of different antibodies in the same membrane, antibody stripping was performed with ReBlot Plus strong antibody stripping solution (Chemicon International, Inc., Temecula, CA).

Transfection and Luciferase Assay

Transient transfection experiments were performed using the chondrocyte cell line RCJ3.1C5.18 (C5.18) cultured in α -minimal essential medium containing 10% FBS in 12-well plates at a density of 35,000 cells per well. These cells maintain a chondrocyte phenotype and are readily transfected and thus are very useful for experiments assessing intracellular signaling events (5;16). Twenty-four hours after culture the cells were transfected with Lipofectamine LTX reagent (Invitrogen) at a Lipofectamine LTX, PLUS reagent, and DNA at a ratio of 2.5 μ l:1 μ l:1 μ g. The following luciferase constructs from Stratagene (La Jolla, CA) were used: pCRE-luc, pFC-PKA, or pCIS. pCRE contains 4 copies of the consensus CRE binding sequence driven by a CMV promoter. pFC-PKA contains the catalytic subunit of PKA driven by a CMV promoter and was used as a positive control for CRE luciferase activation. The empty vector pCIS is the negative control for the pCRE plasmid. The 12XSBE luciferase and the 4XSBE luciferase reporters were used and previously described and are specific for BMP/Smad and TGF- β /Smad signaling respectively (33). A total of 0.1 μ g SV40-*Renilla* luciferase construct was co-transfected with the above firefly reporters (1 μ g) to standardize results for transfection efficiency. Forty-eight hours post-transfection, cells were lysed. Dual Luciferase Assay Reporter System (Promega), and a luminometer (Opticom 1) were used to determine luciferase activity in the cell lysate.

Statistical Analysis

Statistical comparisons were made between the groups using either two-way ANOVA with Bonferroni correction or one-way ANOVA with Dunnett's post-test as appropriate. *p* values of < 0.05 were considered significant and are denoted in each of the figures.

Results

PGE2 differentially regulates expression of chondrocyte genes

To test the hypothesis that PGE2 delays maturation in primary murine sternal chondrocyte cultures, cells were treated with or without PGE2 (1 μ M) in the presence or absence of BMP-2 (100 ng/ml) for 2, 5, and 8 days (Figure 1). BMP-2 induced *col2a1*, *MMP-13*, *alkaline phosphatase*, and *colX* expression, with the largest effects observed on *colX* and *alkaline phosphatase*. PGE2 showed gene dependent effects (Figure 1). Alone, PGE2 increased *col2a1* and *MMP-13*, but inhibited *colX* and had minimal effect on *alkaline phosphatase* expression. PGE2 interactions with BMP-2 were similarly gene dependent and varied with time (Figure 1). In 8 day cultures where maximal effects occurred, PGE2 and BMP-2 were additive for *col2a1* induction. In contrast, PGE2 inhibited BMP-2-mediated *colX* induction and had no effect on BMP-2 mediated *alkaline phosphatase* induction. Finally the induction of *MMP-13* by PGE2 was reduced by BMP-2. Alkaline phosphatase activity in the cultures treated with vehicle, BMP-2 and/or PGE2 paralleled the changes in gene expression, showing a correlation between gene expression and functional protein levels (Figure 1E). Altogether, the findings suggest an interaction of the PGE2 and BMP signaling pathways, and suggest that PGE2 effects are greater on the genes expressed later in the differentiation process, *colX* and *MMP-13*, compared to genes expressed earlier, *col2a1* and *alkaline phosphatase* (46).

The relative expressions of the various isoforms of the EP receptor were examined in primary murine sternal chondrocytes after 2 days in culture (Figure 2A). While all receptor isoforms were expressed, the expression of *EP1* was higher than the expression levels of *EP2* (68-fold), *EP3* (21-fold), and *EP4* (3.5-fold), (Figure 2A). Thus, the genes for the *EP1* and *EP4* receptor isoforms are expressed at much higher levels than the *EP2* and *EP3* receptor isoforms.

The regulation of EP receptor expression was examined in primary sternal chondrocyte cultures treated with BMP-2 (100 ng/ml) and PGE2 (1 μ M) alone and in combination for 2, 5, and 8 days (Figures 2B-E). In control cultures, *EP1* receptor expression decreased over time (Figure 2B). PGE2 did not alter the expression of the *EP1* receptor and did not alter the additional suppression of expression observed in BMP-2 treated cultures. The basal expression of the *EP2* receptor remained constant over time but was increased by PGE2 after 5 and 8 days of treatment (Figure 2C). BMP-2 increased the expression of the *EP2* receptor at 2 and 5 days, but had no effect at 8 days and did not show interaction with PGE2 effects. Compared to two day cultures, basal expression of both the *EP3* and *EP4* receptors was increased after 5 and 8 days in culture (Figures 2D and 2E). BMP-2 markedly reduced expression of the *EP3* receptor at all time points, while an inhibitory effect was observed in PGE2 treated cultures after 8 days (Figure 2D). Similarly, *EP4* receptor expression was also reduced by BMP-2 at 5 and 8 days and by PGE2 after 8 days of treatment (Figure 2E). In all cases minimal regulatory interaction was observed between BMP-2 and PGE2 on the various EP receptor expressions. The findings establish that the EP receptors isoforms are differentially expressed and have variable expression patterns over time. The regulation of EP receptor expression following treatment with BMP-2 and PGE2 further suggests that PGE2 is important in regulation of chondrocyte phenotype.

PGE2 activates PKA signaling in chondrocytes

To confirm that PGE2 treatment regulates PKA signaling in chondrocytes, a pathway implicated in the inhibition of chondrocyte maturation (2;17;18;25), the C5.18 chondrocyte cell line was transfected with the Cre-luciferase reporter and then treated with PGE2 (1 μ M) or vehicle (Figure 3). PGE2 induced reporter activity six-fold, consistent with induction of PKA signaling by PGE2. Control cultures co-transfected with constitutively active PKA had approximately a 15-fold induction of Cre-luciferase reporter activity (Figure 3).

PGE2 inhibits BMP-2 signaling

To determine if the suppressive effect of PGE2 on chondrocyte maturation might involve modulation of the BMP/Smad signaling pathway, experiments were performed to examine the effect of PGE2 on activation of the BMP/Smad-responsive 12XSBE reporter in C5.18 chondrocytes (Figure 4A). PGE2 alone did not alter basal activation of the 12XSBE reporter while BMP-2 treatment resulted in a 10-fold stimulation of reporter activity. The addition of PGE2 to BMP-2 treated cultures resulted in a 20% decrease ($p < 0.05$) in reporter induction by BMP-2 (Figure 4A). To determine if the cAMP/PKA pathway might be involved in this effect, C5.18 cell cultures were also treated with dibutyryl cAMP (0.5 mM). Similar to PGE2, dibutyryl cAMP did not alter basal 12XSBE reporter activity, but significantly suppressed (43%) the ability of BMP-2 to activate the promoter (Figure 4A). The findings show that PGE2 suppresses the ability of BMP-2 to stimulate BMP/Smad signaling and suggest involvement of the PKA signaling pathway.

Since BMP/Smad and TGF- β /Smad signaling have been shown to have a reciprocal relationship, experiments were performed to determine if PGE2 is associated with an increase in TGF- β /Smad signaling (Figure 4B) (6; 24; 48). Addition of TGF- β (5 ng/ml) to C5.18 cell cultures resulted in a 27% stimulation ($p < 0.05$) of the TGF- β /Smad responsive reporter, 4XSBE-luciferase. Addition of PGE2 alone significantly induced TGF- β reporter activity

(31%) and significantly enhanced the stimulatory effect of TGF- β on the reporter (58% compared to control; Figure 4B). Consistent with the reciprocal relationship between BMP and TGF- β signaling, BMP-2 significantly suppressed basal 4XSBE-luciferase activity and inhibited activation of the reporter by TGF- β (Figure 4B). Altogether these findings are further evidence of a modulatory effect of PGE2 on BMP/Smad signaling.

In order to directly determine if PGE2 alters BMP/Smad signaling, Western blot was performed to examine BMP-2-mediated stimulation of Smad 1, 5, and 8 phosphorylation levels in the presence and absence of PGE2 (Figure 5). C5.18 cells (Figure 5A) and primary murine sternal chondrocytes (Figure 5B) were treated with control medium or with medium containing BMP-2 (100 ng/ml) and/or PGE2 (1 μ M). Under basal conditions or following PGE2 treatment for 4 hours, Smads 1, 5, and 8 phosphorylation was absent. In contrast, BMP-2 treatment stimulated a high level of Smads 1, 5, and 8 phosphorylation. When added together, PGE2 reduced BMP-2 mediated phosphorylation of Smads 1, 5, and 8. The effect was present in both the C5.18 cell line and in primary sternal chondrocytes, although inhibition was greater in the primary chondrocytes (Figure 5). These data suggest that PGE2 impairs BMP signaling by inhibiting the activation of Smads 1, 5, and 8.

Discussion

These findings show that PGE2 inhibits chondrocyte maturation and establish the Smad signaling pathway as a target for these events. Although there are multiple potential mechanisms through which PGE2 could suppress maturation, several lines of evidence support suppression of the BMP/Smad signaling pathway as an operant mechanism. First, in the presence of PGE2, the induction of the BMP/Smad responsive 12XSBE reporter was suppressed. Second, PGE2 suppressed BMP-2 mediated induction of Smads 1, 5, and 8 phosphorylation. This latter effect was observed in both C5.18 chondrocytes as well as in primary murine sternal chondrocytes. Finally, PGE2 increased TGF- β activation of the TGF- β /Smad responsive reporter, 4XSBE. This is consistent with the known reciprocal interaction between BMP/Smad and TGF- β /Smad signaling in which suppression of one signaling pathway is associated with elevation of the other (6;24;48). Thus experiments in the current study provide novel mechanistic information regarding integration of PGE2 and BMP signaling during chondrocyte maturation.

PKA signaling has been shown to inhibit chondrocyte maturation (17). Cell culture studies have established PKA signaling as a key mechanism through which PTHrP suppresses chondrocyte maturation (18;25;49). The stimulatory G protein, G_{α_s} , transduces signals from activated receptors to adenylyl cyclases to generate cAMP and stimulate PKA signaling (2; 21;33). *In vivo* experiments show that G_{α_s} knockout mice with deficient PKA signaling have accelerated hypertrophic differentiation of growth plate chondrocytes similar to the parathyroid hormone/parathyroid hormone related peptide (PTH/PTHrP) receptor knockout mice (2;21).

Two of the PGE2 receptors, EP2 and EP4 are G_{α_s} coupled receptors that activate cAMP/PKA signaling (33). Prior work in our laboratory in chick growth plate chondrocytes showed that PGE2 inhibits expression of the type X collagen gene through a PKA dependent mechanism (26). In the current experiments, we show additional effects downstream of PKA that target BMP/Smad signaling activation. Experiments show that both PGE2 and dibutyryl cAMP, which activates PKA signaling, suppress BMP-2-induced 12XSBE luciferase activity in C5.18 cells. Furthermore, we demonstrate in both C5.18 cells and primary murine chondrocyte cultures that PGE2 inhibits the ability of BMP-2 to induce phosphorylation of Smads 1, 5, and 8. These additional experiments establish modulation of BMP/Smad signaling as a further mechanism involved in the regulation of chondrocytes by PGE2 and extend our previous observations.

The EP receptors were differentially expressed in primary sternal chondrocyte cultures. The *EP1* receptor, which activates PKC signaling, was the most highly expressed under basal conditions, but expression declined over time (7). *EP4* receptors also had a relatively high level of expression, while *EP2* and *EP3* receptors had much lower levels of expression. *EP2* expression remained at a very low level over time. In contrast, the basal expression of the *EP4* receptor was approximately 3-fold higher in 5 and 8 day cultures. BMP-2 inhibited *EP4* expression at 5 and 8 days. Although the current experiments do not directly address the role of EP receptor expression in chondrocyte maturation, the observation that BMP-2 treatment inhibited expression of *EP4* receptors suggests that suppression of PGE2/PKA signaling may be one of the molecular events that allows the induction of maturation by BMP-2. In turn, it is apparent that PGE2 modulates responsiveness to BMP-2 signaling and is an important regulator of endochondral ossification.

The effects of PGE2 treatment on primary murine sternal chondrocyte maturation was gene specific. PGE2 treatment had minimal effect on *alkaline phosphatase*, induced *MMP-13* and *col2a1*, and inhibited *colX* gene expressions. PGE2 suppressed the induction of *colX* by BMP-2, but had no effect on the induction of alkaline phosphatase gene expression or activity by BMP-2. Both BMP-2 and PGE2 have been shown to activate numerous downstream signaling pathways (32). BMP-2 activates Smad and MAP kinase signaling, and interacts with numerous other signaling pathways (32). PGE-2 has been shown to activate PKA, PKC, ERK, and beta-catenin signaling and similarly interacts with other signaling pathways (33). The variable regulation of chondrocyte genes by PGE2 and BMP-2 is likely related to the specificity of the promoters of the various genes. Prior examination of the type X collagen promoter has shown that it is directly induced by activation of BMP/Smad signaling (22). Although the experiments did not directly investigate regulation of the type X collagen promoter, it is likely that the suppression of *colX* expression by PGE, at least in part, involves a BMP/Smad mediated mechanism.

Cyclooxygenase and PGE2 have been shown to be critical for bone repair (10;35;36;45). The expression of COX-2 occurs during early fracture healing, in the chondrogenesis and proliferative stage of endochondral bone formation (11). However, COX-2 levels return towards basal levels during the onset of chondrocyte maturation (11). Mice lacking expression of COX-2 have delayed fracture healing (35;45). Cartilage effects include decreased chondrogenesis and delayed endochondral bone formation (35;45). In addition, there are also important effects on the osteoblast population. Prior work in our laboratory has shown that osteoblast differentiation of bone marrow stem cells is deficient in the absence of COX-2 and that this can be compensated for by the addition of PGE2 to bone marrow stem cell cultures (45). Others have demonstrated that addition to PGE2 to the femoral periosteal surface in mice stimulates bone formation in an *EP4* dependent manner (43). Thus, PGE2 has a critical role in endochondral bone repair, supporting the importance of approaches designed to define the mechanisms through which PGE2 regulates chondrocyte differentiation.

Prior studies have established that the balance between TGF- β and BMP signaling determines the rate of chondrocyte hypertrophy, and include gain and loss of function approaches (6;23). Addition of TGF- β to BMP-treated cultures inhibits chondrocyte maturation (15). Over-expression of the TGF- β signaling molecule, Smad2, inhibited induction of the type X collagen promoter by BMP-2 in chick chondrocytes (22). In contrast, loss of TGF- β signaling results in accelerated chondrocyte maturation (23). Previously we showed that retinoic acid induces chondrocyte differentiation in chicken caudal sternal chondrocytes by inducing the expression of Smad 1 and Smad5 and thereby enhancing responsiveness to BMP signaling (27). In contrast, the current findings show that PGE2 inhibits chondrocyte maturation and acts to suppress both BMP-mediated signaling and phosphorylation of Smads 1, 5, and 8 following BMP-2 treatment.

While the present work establishes inhibition of BMP/Smad signaling as a mechanism of PGE2 activity in chondrocytes, the manner in which PGE2 mediates these effects is unknown. Potential additional targets involve regulation of the BMP receptors and other upstream targets including the BMP binding proteins chordin and noggin that antagonize BMP signaling events (4;44). Similarly regulators downstream of the BMP receptors, such as the E3 ubiquitin ligase Smurf 1, reduce BMP responsiveness and down regulate intracellular phospho-Smad levels (47). Future work will be focused on further defining the mechanisms involved the down regulation BMP signaling by PGE2.

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Figure 1. PGE₂ regulates chondrocyte gene expression and interacts with BMP-2 in a gene specific manner

Primary murine sternal chondrocytes were isolated and plated in monolayer culture. Sixteen hours later, the cultures were treated with either vehicle, 1 μ M PGE₂, 100 ng/ml BMP-2, or PGE₂ and BMP-2 in combination. Total RNA was isolated from the cultures after 2, 5, or 8 days of culture and real time PCR was performed as described in Methods (A–D). The gene expression levels of *type II collagen (col2a1)* (A), *matrix-metalloproteinase-13 (MMP-13)* (B), *type X collagen (colX)* (C), and *alkaline phosphatase (AP)* (D) were normalized to β -actin. The relative expression levels were based upon comparison to the level of gene expression observed in the two day vehicle treated control cultures. Alkaline phosphatase activity was determined

in the cultures by measuring the conversion of *p*-nitrophenyl phosphate to *p*-nitrophenol as described in Methods (E). Asterisk denotes $p \leq 0.05$ with comparisons made to the control value at each respective day.

Figure 2. The EP receptors are expressed in chondrocytes and regulated by BMP-2 and PGE₂
 Primary murine sternal chondrocytes were isolated and plated in monolayer culture. Sixteen hours after plating, chondrocyte cultures were treated with either vehicle, 1 μ M PGE₂, 100 ng/ml BMP-2, or PGE₂ and BMP-2 in combination. Total RNA was isolated from the cultures after 2, 5, or 8 days of culture and real time PCR was performed as described in Methods. Real-time PCR analysis was performed using primers specific for the *EP 1, 2, 3, and 4 receptors* and expression levels were normalized to β -actin. The relative expressions of the various EP receptors were measured in 2 day vehicle cultures (A). The gene expression of the EP1 (B), EP2 (C), EP3 (D), and EP4 (E) normalized to the 2-day vehicle control level was determined after 2, 5, and 8 days in culture. Asterisk denotes $p \leq 0.05$ with comparisons made to the control value at each respective day.

Figure 3. PGE2 activates PKA signaling in chondrocytes

C5.18 cells were transfected with 1 μ g pCRE-luc and 0.1 μ g SV40 renilla luciferase 24 h after plating and co-transfected with either control vector (left and middle bars) or pFC-PKA (right bar). Twenty-four hours later, cultures were treated with control medium (left and right bars) or 1 μ M PGE2 (middle bar). Forty-eight hours post-transfection, luciferase activity was measured, and normalized using SV40 renilla luciferase activity. Asterisk denotes $p \leq 0.05$ with comparisons made to the untreated control (left bar).

Figure 4. PGE₂ suppresses BMP reporter activation and stimulates TGF-β reporter activation
 Twenty-four hours after plating, C5.18 cells were co-transfected with 1 μg of 12XSBE-luc in the presence of 0.1 μg SV40 renilla luciferase (A). After an additional twenty-four hours, cells were treated with 100 ng/ml BMP-2 or control medium in the presence or absence of 1 μM PGE₂ or 0.5 mM db-cAMP. Forty-eight hours post-transfection, luciferase activity was measured. (B) Twenty-four hours after plating, C5.18 cells were co-transfected with 1 μg of 4XSBE-luc in the presence of 0.1 μg SV40 renilla luciferase. After an additional 24 hours, the cells were treated with 5 ng/ml TGF-β or control medium in the presence or absence of both PGE₂ (1 μM) or BMP-2 (100ng/ml). The experiments were done in triplicate, and values were

normalized using SV40 renilla luciferase activity. Asterisk denotes significance at a level of $p \leq 0.05$ with comparisons made to the control value or to data points indicated by the bars.

A. C5.18 (Chondrocyte Cell Line)

B. Primary Murine Sternal Chondrocytes

Figure 5. PGE2 suppresses Smad 1, 5, and 8 phosphorylation following BMP-2 treatment

C5.18 chondrocytes (A) or primary murine sternal chondrocytes (B) were plated in monolayer culture for twenty-four hours. The cells were then serum-starved for fifteen hours. Cells were then treated with vehicle control medium or with 100 ng/ml BMP-2 in the presence or absence of 1 μ M PGE2 in serum-reduced conditions. After four hours of treatment, total protein from whole cell lysates was collected and Western blot performed. Phospho-Smad1/5/8 antibody was used to detect the activated forms of these Smads. The membranes were stripped and re-probed for β -actin antibody for loading control.

Table 1

Primer sequences for real-time RT-PCR.

Gene	Forward Primer Sequence (5' → 3')	Reverse Primer Sequence (5' → 3')
β-actin	TGT TAC CAA CTG GGA CGA CA	CTG GGT CAT C TT TTC ACG GT
col2a1	ACT GGT AAG TGG GGC AAG AC	CCA CAC CAA ATT CCT GTT CA
colX	ACC CCA AGG ACC TAA AGG AA	CCC CAG GAT ACC CTG TTT TT
Alk Phos	CCC ATA CAG CAG AGC ACT CA	CCT GAG CCA GTA AGA CCG AG
MMP13	TTT GAG AAC ACG GGG AAG AC	ACT TTG TTG CCA ATT CCA GG
EP1	TAC ATG GGA TGC TCG AAA CA	TTT TAA GCC CGT GTG GGT AG
EP2	ATG CTC CTG CTG CTT ATC GT	TAA TGG CCA GGA GAA TGA GG
EP3	GGA TCA TGT GTG TGC TGT CC	AAC TGG AGA CAG CG T TTG CT
EP4	CCA TCG CCA CAT ACA TGA AG	TGC ATA GAT GGC GAA GAG TG