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BMP-2 induces ATF4 phosphorylation in chondrocytes through a COX-2/PGE2 dependent signaling pathway

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ARTICLE INFO

SUMMARY

Objective: Bone morphogenic protein (BMP)-2 is approved for fracture non-union and spine fusion. We aimed to further dissect its downstream signaling events in chondrocytes with the ultimate goal to develop novel therapeutics that can mimic BMP-2 effect but have less complications.
Methods: BMP-2 effect on cyclooxygenase (COX)-2 expression was examined using Real time quantitative PCR (RT-PCR) and Western blot analysis. Genetic approach was used to identify the signaling pathway mediating the BMP-2 effect. Similarly, the pathway transducing the PGE2 effect on ATF4 was investigated. Immunoprecipitation (IP) was performed to assess the complex formation after PGE2 binding.
Results: BMP-2 increased COX-2 expression in primary mouse costosternal chondrocytes (PMCSC). The results from the C9 Tet-off system demonstrated that endogenous BMP-2 also upregulated COX-2 expression. Genetic approaches using PMCSC from ALK2$^{fx/fx}$, ALK3$^{fx/fx}$, ALK6$^{-/-}$, and Smad1$^{fx/fx}$ mice established that BMP-2 regulated *COX-2* through activation of ALK3−Smad1 signaling. PGE-2 EIA showed that BMP-2 increased PGE2 production in PMCSC. ATF4 is a transcription factor that regulates bone formation. While PGE2 did not have significant effect on ATF4 expression, it induced ATF4 phosphorylation. In addition to stimulating COX-2 expression, BMP-2 also induced phosphorylation of ATF4. Using *COX-2* deficient chondrocytes, we demonstrated that the BMP-2 effect on ATF4 was COX-2-dependent. Tibial fracture samples from *COX-2$^{-/-}$* mice showed reduced phospho-ATF4 immunoreactivity compared to wild type (WT) ones. PGE2 mediated ATF4 phosphorylation involved signaling primarily through the EP2 and EP4 receptors and PGE2 induced an EP4-ERK1/2-RSK2 complex formation.
Conclusions: BMP-2 regulates COX-2 expression through ALK3−Smad1 signaling, and PGE2 induces ATF4 phosphorylation via EP4-ERK1/2-RSK2 axis.

Abbreviation: BMP, Bone Morphogenic Protein; COX, Cyclooxygenase; ATF, Activating Transcription Factor; ALK, Activin Receptor-like Kinase; MAPK, Mitogen Activated Protein Kinase; TGF, Transforming Growth Factor; TAK, TGF-β Activated Kinase; PGE, Prostaglandin E; RSK, The 90 kDa Ribosomal S6 Kinase; KO, Knock-out; WT, Wild Type; Hh, Hedgehog; ERK, Extracellular Signal Regulated Kinase; RCS, Rat Chondrosarcoma Cells; PMCSC, Primary Mouse Costosternal Chondrocytes.
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Introduction

Bone morphogenic protein (BMP)-2 regulates cellular function by binding serine/threonine kinase receptors composed of a complex of type I and type II receptors with subsequent phosphorylation/activation of Smad1/5/8. Three different type I BMP receptors (ALKs2, 3, and 6) have been identified[1,2]. Recombinant BMP-2 is FDA approved to stimulate bone formation in spine fusion surgery and for the treatment of non-union of tibial fractures[3,4]. Recent clinical reports have documented adverse events related to the use of BMP-2 that include ectopic bone formation, osteolysis and soft tissue inflammation[5–7]. More clearly defining the downstream

signaling events that are stimulated by BMP-2 may improve safety as well as lead to the development of more selective agents for skeletal diseases.

A classic Smad signaling pathway mediates BMP-2 receptor signaling. Smads 1, 5 and 8 associate with the type I receptors and are phosphorylated and released into the cytoplasm following ligand binding to the receptor. The receptor associated Smads form a complex with Smad4 in the cytoplasm, and then translocate to the nucleus and modulate gene transcription. In addition, BMP receptors also activate the mitogen activated protein kinase (MAPK) signaling pathway. TAK1 is a MAP-kinase-kinase-kinase that associates with the receptor complex and initiates signaling through the MAPK pathways. Recent reports show that TAK1 is an important mediator of BMP-2 effects in chondrocytes[8–10]. We have also shown that the classic Smad and a non-classic TAK1-p38-ATF2 pathways are both involved in maintenance of articular cartilage and have a role in the pathogenesis of osteoarthritis (OA)[11].

In addition to its effect on osteochondral specific transcriptional factors, BMP-2 also increases the expression of cyclooxygenase (COX)-2 in osteoblasts[12]. COX-2 is a critical factor in the skeletal repair process as COX-2 deletion mutant (COX-2$^{-/-}$) mice have delayed fracture union[13]. PGE2 is a major metabolite downstream of COX-2. PGE2 exerts its effect through binding to G-protein coupled cell receptors, EP1, EP2, EP3, and EP4[14]. Our previous study indicates that PGE2 inhibits the differentiation of chicken growth plate chondrocytes[15]. However, the reports from other groups demonstrate a complex interaction of BMP-2 and COX-2. PGE2 can upregulate BMP-2 expression in human mesenchymal stem cells via its EP4 receptor[16,17]. We also previously demonstrate that systemic administration of EP4 agonist accelerates fracture healing in COX-2$^{-/-}$ mice[18]. The findings from other groups have shown that both EP2 and EP4 agonists enhance fracture healing[19,20]. In contrast, our previous study demonstrates that EP1 receptor is a negative regulator of fracture healing[21].

The EP4 receptor has a unique long intracellular domain that can be internalized upon ligand binding and forms complex in cytoplasm with other molecules[22]. For examples, PGE2 can activate ERK1/2 pathway through EP4 and induce ATF4 phosphorylation in cancer cells[23,24]. Previous studies have established that ERK1/2 can phosphorylate and activate RSK-2[25]. ATF4 plays a critical role in skeletal development and maintenance. ATF4 null mutant mice exhibit an osteoporotic phenotype due to a reduced bone formation rate. Enzymatic activity assay revealed that a 90KD ribosomal S6 kinase 2 (RSK2) is a direct upstream kinase able to phosphorylate ATF4. RSK2 mutation has been found in the Coffin–Lowry Syndrome, an X-linked disorder characterized by skeletal anomalies and mental retardation. RSK2 deficient mice display a similar phenotype as ATF4 mutant mice[26,27]. Interestingly, ATF4 also promotes osteoclast differentiation and bone resorption via direct and indirect mechanisms[28,29]. Further, ATF4 may regulate differentiation of osteochondral progenitors in an Ihh dependent mechanism[30,31].

Based on these observations, we hypothesized that BMP-2 regulates ATF4 expression and activity in chondrocytes in a COX-2 dependent manner. Our current study aimed to establish a molecular cascade linking these pathways. Our results show that BMP-2 increases COX-2 expression via a classic ALK3–Smad1 pathway. PGE2 induces ATF4 phosphorylation mainly through EP4 receptor with downstream signaling involving ERK1/2-RSK2 activation.

Materials and methods

Cells

Primary mouse costosternal chondrocytes (PMCSC) were isolated from the mice with different genetic backgrounds as previously described[32,33]. Briefly, the rib cages including sterna were dissected from 3-day-old pups. Soft tissue was removed after serial incubation with pronase (Roche Laboratory, Nutley, NJ, USA, 2 mg/ml in PBS) at 37°C shaker for 45 min and then in collagenase D (Roche, 3 mg/ml in full medium) at 37°C for 60 min. After thorough wash, the rib cages were further digested with collagenase D in a Petri dish for 5 h at 37°C incubator. Then the cells were filtered through 40 μm mesh to remove tissue debris. After centrifuge, the cell pellet was washed and re-suspended with Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS).

The C9 Tet-off cells were developed from mouse mesenchymal C3H10T1/2 cells after transfection with the BMP-2 gene and antibiotic selection with hygromycin. C9 cells are under the control of the inducible Tet-off system, e.g., no BMP-2 over-production in the presence of doxycycline (1 μg/ml, Sigma, St. Louis, MO). After the withdrawal of doxycycline, C9 cells overexpress BMP-2 and secrete a large amount of BMP-2[34].

Rat chondrosarcoma cells (RCS) were used in some experiments including reporter luciferase assay.

In some experiments, cells were pretreated with BMP-2 type I receptor (BMPRI, ALK 2, 3, 6) inhibitor Dorsomorphin (10 nM, Sigma) or a TAK1 inhibitor (5Z)-7-Oxozeaenol (10 nM, EMD Millipore, Billerica, CA) for 1 h followed by other treatments.

Mice

The following genetically modified mice have been used in this study: global knock-out (KO) mice: ALK6$^{-/-}$ mice were from Dr. Karen Lyons[35], EP1$^{-/-}$ mice from Dr. Matthew Breyer[36], EP2$^{-/-}$ mice from Dr. Richard Breyer[37]; conditional gene floxed mice: ALK2$^{fx/fx}$ mice were from Dr. Vesa Kaartinen[38]; ALK3$^{fx/fx}$ from Dr. Yuji Mishina[39], Smad1$^{fx/fx}$ mice from NIH[40]; EP4$^{fx/fx}$ mice from Dr. Matthew Breyer[41], and TAK1$^{fx/fx}$ mice from Dr. Michael Schneider[42].

Luciferase assay

COX-2 gene promoter reporter was purchased from Panomics (Santa Clara, CA) and transient transfection was performed in RCS using Lipofectamine 2000 from Invitrogen (Grand Island, NY). Cell culture medium containing Lipofectamine 2000 was removed after 12 h and BMP-2 (100 ng/ml, R&D Systems, Minneapolis, MN) was added to culture medium for 48 h with vehicle as a control. Reporter luciferase assay was done in triplicate with a luminometer (Opticom 1, Promega, Madison, WI).

Real time quantitative PCR (RT-PCR)

Total RNA was harvested using an RNeasy Mini kit (Qiagen, Valencia, CA). Reverse transcription was done with a SuperScript First Strand Synthesis System (Invitrogen). RT-PCR was performed with QuantiTech SYBR Green PCR kit (Qiagen) in Rotor-Gene Real-Time DNA amplification system (Corbett Research, New South Wales, Australia) using the COX-2 primers: forward: 5'-AGAAGGAAATGGCTGCAGAA-3', reverse: 3'-GCTCGGCTTCCAG-TATTGAG-5'; Atf4 primers: forward: 5'-TCGATGCTCTGTTTC-GAATG-3', reverse: 3'-GGCAACCTGGTCGACTTTTA-5'. The expression levels of the genes of interest (GOI) were analyzed with the software from Qiagen. For each GOI, a standard curve was established and a value of each treatment group was obtained. Similarly, a standard curve was established for β-actin and its value in each group was calculated. RT-PCR was done in quadruplicate and each GOI value was normalized by β-actin values. The mean and SD were generated with software from these normalized values.

EIA for PGE2

Prior to BMP-2 treatment, PMCSC were starved for 24 h in serum-free medium. Then, cells were treated with BMP-2 (100 ng/ml) for 12 h and supernatants were collected. PGE2 concentration was measured using an enzyme immunoassay (EIA) kit from Cayman Chemical (Ann Arbor, MI), and the results were represented as the means values from six wells and standard deviations.

Construction of EP4-V5 plasmid

The plasmid was constructed using the Gateway Technology System (Invitrogen). Briefly, mouse EP4 receptor cDNA was produced by PCR with high fidelity polymerase (Invitrogen). A Gateway® entry clone from an *att*B-flanked PCR product was created with a TOPO® cloning reaction. Through an LR reaction, the product was subcloned to the destination vector pcDNA™3.2/V5-DEST. After sequencing, the correct clone was amplified for future study.

Western blotting and immunoprecipitation (IP)

The nonprostanoid EP4 receptor agonist CP-734432 was obtained from Pfizer Global Research and Development (Groton, CT). For *in vitro* studies, it was used at a final concentration of 10^{-6} M. Cells were lysed with golden lysis buffer (20 mM Tris, 137 mM NaCl, 5 mM Na_2EDTA, 10% glycerol, 1% Triton X-100, 1 mM phenyl-methylsulfonyl fluoride, 1 mM aprotinin, 1 mM leupeptin, 1 μM pepstatin A, 10 mM NaF, 1 mM Na_3VO_4, 1 mM EGTA, 1 mM tetrasodium PP1, and 100 μM P-glycerophosphate). Western blot was performed with the invitrogen system. Chemiluminescence was done with West Femto reagent (Thermo Fisher Scientific, Rockford, IL). The following antibodies were used in the experiments: COX-2 (Cayman Chemicals), ATF4 and phospho-ATF4 (Abcam, Cambridge, MA), phospho-ERK1/2 and phospho-RSK-2 (Cell Signaling Technology, Boston, MA). The experiments were usually repeated for three times. After establishing the trends of protein expression, the clear results with less variation were chosen and represented. IP was performed using the Catch and Release Reversible IP System (EMD Millipore). The protein was pulled down with V5 antibody (Invitrogen) and GST antibody (Sigma) was used as antibody control. IP was performed using antibodies against phospho-EKR1/2 and RSK-2.

In vitro gene deletion

PMCSC were isolated and cultured in DMEM containing 10% FBS. After starvation for 24 h in serum-free medium, the cells were infected with adenovirus (multiplicity of infection of 10) encoding either Cre or GFP (Baylor Collage, TX, website: http://www.bcm.edu/vector/instock-av) for 3 days. After recovering in the standard culture medium containing 10% FBS for 2 days, these cells were subjected to different treatments.

Immunohistochemistry (IHC)

The Day 7 fracture tibial callus samples harvested from wild type (WT) and $COX-2^{-/-}$ mice were fixed, decalcified and embedded. Paraffin sections were cut and immunostained using an avidin-biotin peroxidase detection system (Vector Labs, Burlingame, CA). After quenching endogenous peroxidase and blocking non-specific signals, the sections were incubated overnight at 4°C with the primary antibody against phospho-ATF4 (1:100, Abcam). The sections were thoroughly washed and then incubated with a biotinylated secondary antibody (Vector) for 2 h at room temperature. Color reactions were developed with diaminobenzidine (DAB) as substrate (Vector).

Statistical analysis

The PASW software was used in this study. After testing the homogeneity of variances, the log-transformed values were used for analysis of variances (ANOVA). Following analysis of overall group differences on the mean values at 0.05 alpha levels were adjusted using the Turkey correction for multiple comparisons.

Results

BMP-2 increases COX-2 promoter activity and COX-2 protein expression

RCSs were transiently transfected with the *COX-2* promoter construct for 12 h and then treated with BMP-2 for 48 h. Luciferase assay showed that BMP-2 treatment increased *COX-2* promoter activity [Fig. 1(A)]. A time-course study was performed to examine the effect of BMP-2 on COX-2 protein expression in PMCSC. BMP-2 treatment increased COX-2 protein levels at 6 h and the peak effect occurred at 12 h [Fig. 1(B)]. The COX-2 protein levels remained elevated in the BMP-2 treated cultures over the 48 h culture period. Subsequent experiments were designed to determine the signaling pathway through which BMP-2 stimulates COX-2 expression. PMCSC were first incubated with either BMPRI inhibitor or TAK1 inhibitor for 1 h and then treated with BMP-2 for 12 h. Treatment with BMP-2 in control cultures caused approximately a 3-fold upregulation of *COX-2* mRNA expression. This induction in *COX-2* was abolished when the cells were pretreated with the BMPRI inhibitor. In contrast, pre-treatment of the cell cultures with TAK1 inhibitor resulted in a normal induction of *COX-2* expression by BMP-2 [Fig. 1(C)]. These findings suggest that BMP-2 regulates COX-2 expression mainly through the classic Smad signaling pathway. The effect of the endogenous BMP-2 on COX-2 expression was examined using C9 Tet-off cells. These cells have normal levels of BMP-2 expression in the presence of doxycycline, but have activation of a BMP-2 transgene upon the withdrawal of doxycycline and thus over-expression of BMP-2. BMP-2 was increased 48 h after removal of doxycycline compared to C9 cells maintained in doxycycline containing medium. The cultures with over-expression of endogenous BMP-2 also had upregulated COX-2 expression [Fig. 1(D)].

BMP-2 regulates COX-2 expression in a classic ALK3-Smad pathway

PMCSC were isolated from $Alk2^{fx/fx}$, $Alk3^{fx/fx}$, and $Alk6^{-/-}$ mice. The floxed chondrocytes were infected with Ad-GFP or Ad-Cre for 3 days to induce Cre-mediated gene recombination. Then, PMCSC were treated with BMP-2 for 48 h and protein samples were harvested. Western blotting analysis showed that the $Alk2^{fx/fx}$ PMCSC infected with Ad-GFP were responsive to BMP-2 and had increased COX-2 protein expression in BMP-2 treated cultures. Deletion of the *Alk2* gene in Ad-Cre treated cultures resulted in a slight increase in the basal level of COX-2 protein, and the cells continued to respond to BMP-2 with an increase in COX-2 expression [Fig. 2(A)]. *Alk3* deficiency resulted in essentially a complete loss of the BMP-2 response of COX-2 induction [Fig. 2(B)]. Similar to the observations in *Alk2* deficient mice, The $Alk6^{-/-}$ PMCSC had an elevated basal level of COX-2 and continued to respond to BMP-2 with induction of COX-2 expression [Fig. 2(C)]. Using a similar approach, we have confirmed that regulation of COX-2 by BMP-2 is mediated through BMPR/Smad signaling pathway. PMCSC isolated from $Smad1^{fx/fx}$ and $Tak1^{fx/fx}$ mice were infected with either Ad-GFP or
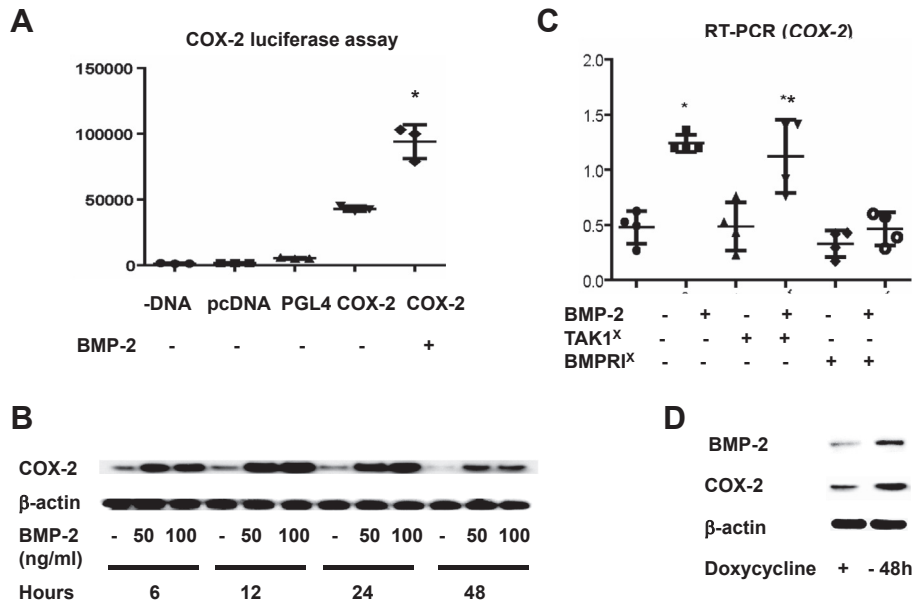
Fig. 1. Luciferase assay was performed in triplicate after transient transfection of human *COX-2* gene promoter reporter construct to RCS. BMP-2 treatment for 48 h resulted in approximately 2-fold increase of *COX-2* luciferase activity (**A**). Western blotting analysis demonstrated that BMP-2 mediated COX-2 protein upregulation occurred at 6 h and peaked at 12 h after treatment (**B**). PMCSC were pretreated with BMPRI or TAK1 inhibitor and followed by a BMP-2 treatment for 12 h. RT-PCR was performed in quadruplicate and the results showed that TAK1 inhibitor did not have significant effect on the BMP-2 induced *COX-2* expression. In contrast, BMPR1 inhibitor almost completely abrogated BMP-2 effect on *COX-2* mRNA expression (**C**). TAK1^x: TAK1 inhibitor, BMPRI^x inhibitor, *significant difference between lane 1 and 2 ($P = 0.01$), **significant difference between lane 3 and 4 ($P = 0.03$). The effect of endogenous BMP-2 on COX-2 expression was investigated in Tet-off C9 cells. Western blotting analysis showed that 48 h after removal of doxycycline, BMP-2 expression was upregulated with a subsequent increase in COX-2 protein expression (**D**).
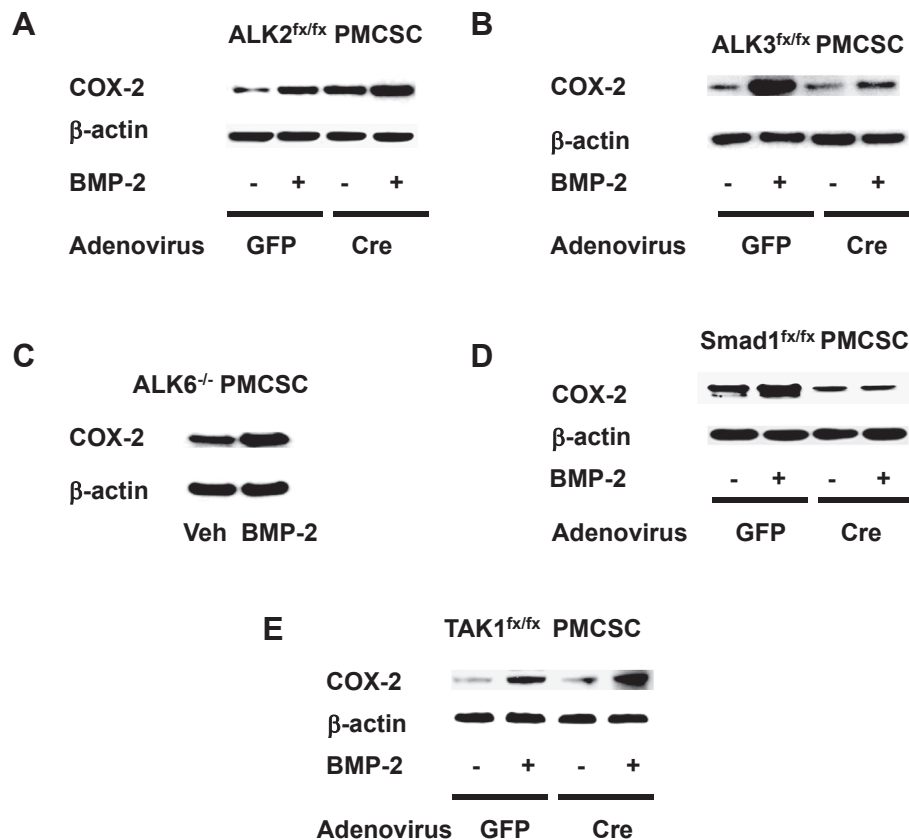

Fig. 2. Western blot analysis revealed that the BMP-2 mediated COX-2 induction was partially preserved in *Alk2$^{fx/fx}$* PMCSC infected with Ad-Cre (**A**). In contrast, *In vitro* deletion of *Alk3* by infecting *Alk3$^{fx/fx}$* PMCSC with Ad-Cre resulted in a significant loss of BMP-2 responsiveness regarding COX-2 expression (**B**). The ALK6$^{-/-}$ PMCSC showed a similar response to BMP-2 as WT cells regarding COX-2 protein induction (**C**). *In vitro* deletion of *Smad1* in PMCSC significantly abrogated the BMP-2 effect on COX-2 expression (**D**). In contrast, deletion of Tak1 by infecting the *Tak1$^{fx/fx}$* PMCSC with Ad-Cre had a minimal effect on the BMP-2 mediated COX-2 expression (**E**). Veh: vehicle control.

Ad-Cre. *In vitro* deletion of the *Smad1* gene resulted in a loss of COX-2 induction by BMP-2 [Fig. 2(D)]. In contrast, deletion of the *Tak1* gene did not alter the stimulatory effect of BMP-2 on COX-2 expression [Fig. 2(E)]. These findings suggest that BMP-2 regulates COX-2 expression primarily through the classic ALK3–Smad1 signaling pathway.

BMP-2 increases PGE-2 production

PGE-2 EIA revealed that BMP-2 treatment resulted in an enhanced production of PGE2 in mouse articular chondrocytes. PGE2 concentration was measured 12 h after treatment and represented as the mean and standard deviation (BMP-2 treatment: 487.08 ± 57.99; vehicle control: 211.07 ± 31.16).

BMP-2 induces ATF4 phosphorylation through COX-2/PGE2 signaling

As PGE2, the major metabolite of COX-2, has been shown to stimulate ATF4 phosphorylation in prostate cancer cells[24], we subsequently conducted experiments to determine whether PGE2 regulates ATF4 phosphorylation in chondrocytes. Treatment of PMCSC with PGE2 did not result in changes in *Atf4* mRNA expression (data not shown). However, PGE2 strongly stimulated ATF4 phosphorylation after 2 and 4 h of treatment [Fig. 3(A)]. The Tet-off C9 cells were used to examine if endogenous BMP-2 regulates ATF4 phosphorylation. Doxycycline was removed from culture medium and Western blotting analysis was performed with the antibodies against phospho-ATF4, total ATF4, and BMP-2. Over-expression of BMP-2 was detected 48 h after the withdrawal of doxycycline, at which point increased expression of ATF4 protein and enhanced ATF4 phosphorylation was observed [Fig. 3(B)]. To determine if BMP-2 regulates ATF4 phosphorylation in a COX-2 dependent

mechanism, PMCSC were isolated from WT and $COX-2^{-/-}$ mice. While BMP-2 induced ATF4 phosphorylation in WT PMCSC, the induction of ATF4 phosphorylation was absent in $COX-2^{-/-}$ PMCSC [Fig. 3(C)]. Consistent with these findings, callus samples from tibia fracture of $COX-2^{-/-}$ mice harvested at Day 7 post-surgery showed much weaker immunostaining to phospho-ATF4 compared to the staining present in WT mice [Fig. 3(D)].

PGE2 mediates ATF4 phosphorylation through EP4 receptor

As COX-2–PGE2 mediated signaling is necessary for ATF4 phosphorylation, experiments were performed to examine the relative importance of the EP receptors in the PMCSC population. PMCSC were isolated from $EP1^{-/-}$, $EP2^{-/-}$ and $EP4^{fx/fx}$ mice. PMCSC from $EP4^{fx/fx}$ mice underwent Cre recombination *in vitro* following infection with Ad-Cre. Western blot analysis was performed using cell lysates extracted from cultures with 2-h treatment with PGE2. PGE2 stimulated ATF4 phosphorylation in $EP1^{-/-}$ PMCSC [Fig. 4(A)], indicating that the EP1 receptor is not involved in the phosphorylation ATF4. $EP2^{-/-}$ PMCSC had an elevated basal level of phospho-ATF4. In contrast to $EP1^{-/-}$ PMCSC, no increase in ATF4 phosphorylation occurred following PGE2 treatment in $EP2^{-/-}$ PMCSC cultures [Fig. 4(B)]. PMCSC isolated from $EP4^{fx/fx}$ mice were infected with Ad-Cre or Ad-GFP. Deletion of the EP4 gene reduced the basal level of phospho-ATF4 and blocked the induction of ATF4 phosphorylation by PGE2 [Fig. 4(C)].

EP4, ERK1/2 and RSK2 form a protein complex

ATF4 is a member of the cAMP response element binding proteins (CREB) and is phosphorylated by protein kinase A (PKA) signaling. EP2 and EP4 both are known activators of the PKA signaling pathway. In addition, ATF4 phosphorylation is mediated
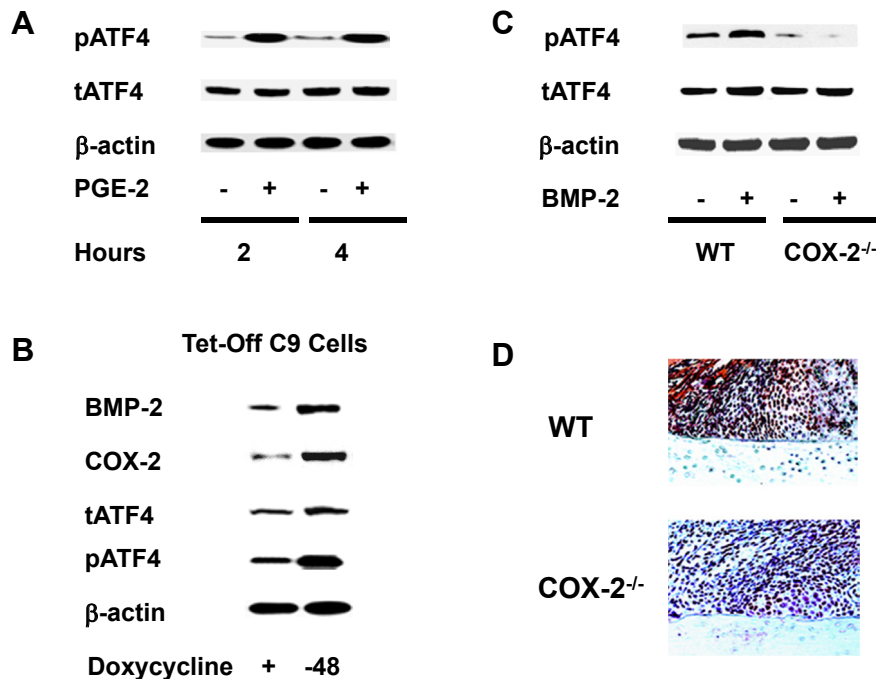
Fig. 3. PMCSC isolated from WT mice were treated with PGE2 (10^{-6} M) for 2 or 4 h and Western blotting analysis showed that PGE2 induced ATF4 phosphorylation (**A**). The effect of endogenous BMP-2 on ATF4 phosphorylation was examined in the Tef-off C9 cells. The results indicated that BMP-2 was overexpressed 48 h after doxycycline withdrawal with a slight increase of total ATF4 protein. ATF4 phosphorylation then became evident (**B**). PMCSC from both WT and $COX-2^{-/-}$ mice were treated with BMP-2 and Western blotting analysis was performed with an antibody for phospho-ATF4. While BMP-2 induced ATF4 phosphorylation in WT PMCSC, its effect was largely lost in $COX-2^{-/-}$ PMCSC (**C**). Day 7 fracture callus samples from $COX-2^{-/-}$ mice showed a much weaker immunoreactivity to phospho-ATF4 than that in WT samples (**D**).
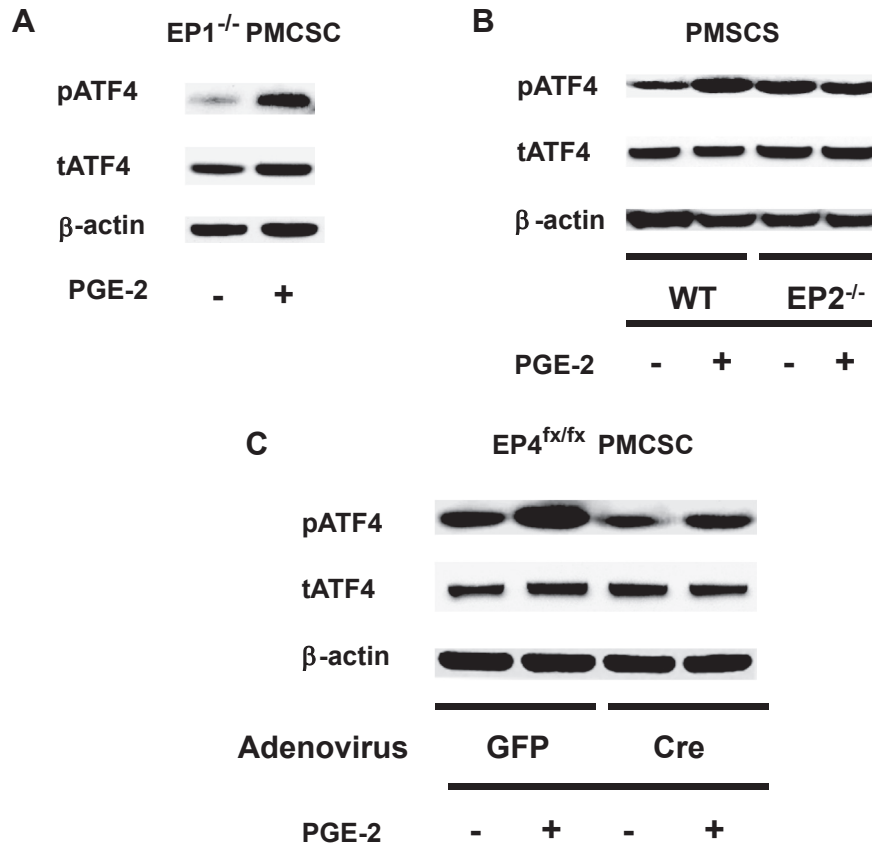
Fig. 4. The PGE2 effect on the phosphorylation of ATF4 was well-reserved in the _EP1$^{-/-}$_ PMCSC (**A**). Although the basal level of phospho-ATF4 was slightly higher in the _EP2$^{-/-}$_ PMCSC, such cells lost their responsiveness to the PGE2 regarding ATF4 phosphorylation (**B**). _In vitro_ deletion of EP4 receptor by infecting _EP4$^{fx/fx}$_ PMCSC with Ad-Cre significantly abrogated the PGE2 effect on ATF4 phosphorylation (**C**).

by ERK1/2—RSK2 signaling. We performed further experiments to determine whether EP4 activates ERK1/2 signaling in PMCSC. Treatment of PGE2 for 30 min resulted in a robust stimulation of ERK1/2 phosphorylation, and EP4 agonist, less a lesser extent, also induced ERK1/2 phosphorylation [Fig. 5(A)]. We then constructed a plasmid consisting of EP4 receptor fused to the V5 tag and transfected this plasmid into RCS cells. The cells were treated with PGE2 for 1 h and the cell lysates were subjected to IP using either GST or V5 antibody, followed by immunoblotting with antibodies against phospho-ERK1/2 and phospho-RSK2. Our results suggested that EP4, phospho-ERK1/2 and phospho-RSK2 formed a protein—protein complex [Fig. 5(B)], consistent with the regulation of ATF4 phosphorylation. Such complex formation was not detectible in the lysates from untreated cells.

Discussion

Previous studies have established that both BMP-2 and COX-2 gene expressions are important signals in bone regeneration (4,13). The current study shows that COX-2 expression in PMCSC is regulated by BMP-2 signaling. Pharmacologic and genetic approaches were subsequently used to define the signaling pathways involved in the induction of COX-2 by BMP-2, and to examine subsequent signaling events downstream of COX-2 and its major metabolite PGE2. The experiments establish that BMP-2 induces COX-2 through a pathway that involves ALK3/Smad1 signaling with a subsequent increased PGE2 production. We further establish that induction of ATF4 phosphorylation by BMP-2 is dependent on COX-2 expression and is mediated by a PGE2
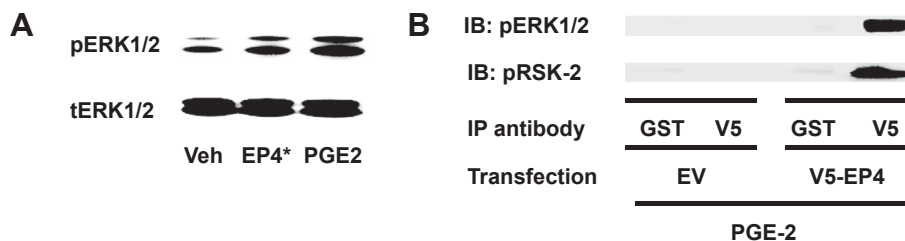

Fig. 5. The lysates from the PMCSC treated with PGE2 or EP4 agonist were subjected to Western blotting analysis with antibodies for total and phospho-ERK1/2. Both PGE2 and EP4 agonist induced the phosphorylation of ERK1/2 (**A**). RCS cells were transfected with either V5-tagged EP4 receptor (V5-EP4) or the emptor vector (EV) and lysates were immunoprecipitated with the antibodies for either V5 or GST. Western blotting was performed with the antibodies against phospho-ERK1/2 and phospho-RSK-2. The result suggested that upon PGE2 treatment, EP4 formed the complex with both phospho-ERK1/2 and phospho-RSK2 (**B**). Such complex formation was not observed in untreated cells. EP4*: EP4 agonist, Veh: vehicle control, EV: empty vector.

through EP2/EP4 receptor signaling events. ATF4 is a key transcription factor in chondrocytes. Since one of the mechanisms for ATF4 phosphorylation involves RSK2/ERK1/2 signaling, we examined the potential for EP4 to activate this pathway in chondrocytes. We demonstrated that PGE2 and an EP4 agonist stimulate ERK1/2 phosphorylation. Furthermore, the IP experiments established that the EP4 receptor co-precipitates with RSK2 and ERK1/2. Altogether the experiments show that BMP-2 stimulates important regulatory pathways in chondrocytes through the induction of COX-2 and subsequent downstream signaling events.

Both BMP-2 and COX-2/PGE2 are critical factors in the early responses to bone injury and are required for normal bone regeneration (4,13). BMP-2 has been shown to be expressed in early fracture repair in mesenchyme and in immature chondrocytes. Because global deletion of BMP-2 results in early embryonic death, the effects of BMP-2 deletion in bone regeneration has been examined in mice with constitutive gene deletion using a Prx1-Cre that results in loss of gene expression in limb mesenchyme derived cell populations. Fractures in mice with constitutive deletion of BMP-2 have marked inhibition of fracture healing with reduced proliferation of periosteal cells along the bone surface, minimal accumulation of callus tissues, and delayed differentiation of cartilage and bone[43]. In contrast, deletion of BMP-4 has minimal effects[44], suggesting that BMP-2 is particularly important in bone regeneration and that other factors do not compensate for absence of BMP-2.

The COX-2 gene is also expressed early in fractures and its expression corresponds primarily with the endochondral phase of bone repair. Peak COX-2 expression has been observed at the time when an immature cartilage callus has developed repair. *In situ* hybridization of murine fracture callus has shown expression of COX-2 primarily in chondro-progenitors that co-express col2a1 and in immature chondrocytes, with reduced expression occurring as chondrocytes mature. Prior work in our laboratory and by others has shown that COX-2 is critical for the early events in fracture healing mature[13,45,46]. The fracture healing phenotype observed with deletion of COX-2 is similar, although not as severe, as that observed with conditional deletion of the BMP-2. COX-2 gene deletion results in reduced proliferation of the periosteal cell population, decreased accumulation of fracture callus, and delayed differentiation of bone and cartilage. The similarities, but less severe phenotype observed in COX-2 compared to BMP-2 gene deletion suggests that these signals may be linked. The current findings demonstrate that BMP-2 regulates the expression of COX-2 in chondrocytes and that COX-2 is involved in the activation of important signals in cartilage.

Classic BMP signaling starts with the ligand binding to the type II receptor with subsequent recruitment and phosphorylation of the type I receptors (ALK2, 3, 6). The activated type I BMP receptor phosphorylates the receptor associated Smad3 (Smad1, 5, 8). In this study, we took advantage of the genetically modified mice to establish the pathway through which BMP-2 regulates COX-2 expression. We first demonstrate that the classic ALK3–Smad1 pathway is the major transducer of the BMP-2 effect on COX-2 expression. In addition to classic Smad pathway, many non-Smad pathways are activated following BMP-2 treatment. We focused on the TAK1/MAPK pathway because this pathway has been studied intensively and mounting evidence suggests a pivotal role of this pathway in chondrocyte differentiation. Our recent publication shows an interdependent relationship between TGF-β-TβRI-Smad3 signaling and TAK1–MAPK pathway[11]. The report from Dr. Glimcher's group suggests an essential role of TAK1 in mediating BMP-2 signaling in chondrocytes[9]. We thus focused on TAK1/MAPK

signaling in the BMP-2 mediated COX-2 expression in chondrocytes, and compared the effect of this non-Smad pathway to the classic Smad pathway. Our results for the first time establish that classic ALK3-Smad1 pathway plays a major role in the BMP-2 effect on COX-2.

PGE2 is a major end product of COX-2 and it takes effect through its cell surface receptors from EP1 to EP4. While EP1 is a negative regulator of fracture healing[21], both EP2 and EP4 signaling accelerate skeletal repair[19,20]. These two receptors belong to the G-protein coupled receptor family able to activate the cAMP/PKA signaling. In addition to EP2/4–PKA signaling, other pathways may also mediate PGE2 effect. EP4 is unique because it has a long intracellular domain. Upon ligand binding, EP4 can be internalized to cytoplasm, where it binds to and interacts with different molecules transmitting different PGE2 effect. Previous studies have demonstrated that PGE2 activates ERK1/2 MAPK pathway through EP4, and ERK1/2 can phosphorylate RSK2. In cancer cells, PGE2 induces ATF4 phosphorylation. RSK2 is a direct upstream kinase for ATF4 phosphorylation, and its mutation in human results in insufficient ATF4 phosphorylation, which causes the Coffin–Lowry Syndrome characterized by mental retardation and skeletal anomalies. Not surprisingly, RSK2 mutant mice exhibit a bone loss phenotype.

Based on these observations, we postulated that modulation of RSK2 activity may partially mimic BMP-2 effect on cartilage and bone. Our current study aimed to establish a molecular reaction cascade linking COX-2 upregulation, PGE2 over-production, and ATF4 phosphorylation. Our observations made in genetically engineered mice suggest that both EP2 and EP4 receptors are involved in the PGE2 mediated ATF4 phosphorylation. We focused on EP4 because it has a unique molecular structure and its interaction with other molecules in cytoplasm may transduce different PGE2 effect. Our IP experiment indicates a complex formation of EP4-pERK1/2-pRSK2. Such complex was not detectible in the absence of PGE2. Based on the observations from others and our own, we hypothesized that PGE2 may induce ATF4 phosphorylation via the EP2/4-ERK1/2-RSK2 axis. Noteworthy, different phosphorylation sites in ATF4 molecule may have different effect on ATF4 activity/function. Thus, more studies are needed to generate more detailed information of ATF4 phosphorylation.

Although our current study did not focus on chondrocyte differentiation through these molecular cascades, accumulating data suggest that ATF4 work either alone or together with other transcription factors to regulate differentiation of osteochondral progenitor cells. Recent findings suggest that ATF4 takes its effect through Ihh, an important molecule driving chondrocyte differentiation[30,31]. Prior work in our laboratory showed reduced expression of *Ihh* in fracture callus of *COX-2*$^{-/-}$ mice. Treatment of these mice with an EP4 agonist markedly increased Ihh expression[18]. In this study, we demonstrate reduced expression of phospho-ATF4 in fracture callus of *COX-2*$^{-/-}$ mice. Together, these observations further support a signaling cascade linking BMP-2-COX-2-PGE2-EP4-RSK2-ATF4, with possible involvement of Ihh. Our future work will focus on the relationship between the phosphorylation status of ATF4 and some skeletal diseases.

Conclusions

BMP-2 upregulates COX-2 expression via the classic Smad signaling pathway. In addition, BMP-2 regulates ATF4 phosphorylation in a COX-2 dependent manner. Further, BMP-2 increases PGE2 production and PGE2 induces ATF4 phosphorylation primarily through EP2/4 receptor with the downstream signaling events involving the activation of ERK1/2 and RSK2.

Author contribution

TFL: the conception and design, acquisition, analysis and interpretation of data, manuscript preparation, and final approval of the version to be submitted. KY: the study design, acquisition and analysis of data. GY: the acquisition, analysis and interpretation of data. TS: the acquisition, analysis and interpretation of data. TM: the acquisition, analysis and interpretation of data. JHJ: the acquisition, analysis and interpretation of data, manuscript preparation. WS: the conception and design, and critical revision of the manuscript. XZ: The conception and design. GX: preparation and critical revision of the manuscript. YTK: the conception and design, and critical revision of the manuscript.

DC: the conception and design, critical revision of the manuscript. RJO: obtaining funding, the conception and design, interpretation of data, manuscript preparation, and final approval of the version to be submitted.

Conflict of interest

No author in this study has received financial support of any form. None of them has a potential of conflict of interest.

Role of funding source

The sponsor had not involved in any process of the current study, e.g., the study design, the collection, analysis, and interpretation of data, manuscript preparation and journal submission.

Acknowledgments

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