Introduction: The mechanism by which Prostaglandin E2 (PGE2) delays chondrocyte maturation in vitro is unknown. Bone morphogenic proteins (BMPs) are potent inducers of all stages of endochondral ossification. Using a primary murine chondrocyte maturational model, we show PGE2 can inhibit the induction of the hypertrophic chondrocyte marker type X collagen gene expression by BMP-2. PGE2 treatment also diminished BMP signaling and target gene transcription as evidenced by decreases in both the protein levels of phosphorylated forms of the BMP-Smads and the activity of the BMP-specific reporter 12XSBE. This is the first evidence to our knowledge that PGE2 inhibits chondrocyte maturation by altering BMP-signaling.

Materials and Methods: Cell Culture: The anterior rib cage and sternum of 2-day old c57/bl6 mouse pups were harvested as described previously (1). Doses of 1μM Prostaglandin E2 (PGE2), 0.5mM dibutyryl cyclic AMP, 50μg/ml ascorbic acid (Sigma), and 100ng/ml bone morphogenetic protein 2, BMP-2, (gift from Wyeth) were used.

Real-time PCR: Total RNA was extracted, synthesized into cDNA, and real-time PCR was performed using primer sequences and machine settings as described previously (1,2). The relative levels of mRNA for a specific gene were normalized to β-actin levels.

Protein Extraction: For experiments in which protein was extracted, sixteen hours after plating the cells were washed twice with PBS. At this time 0.5% DMEM was added. Fifteen hours later growth factors were added directly to the cell culture wells. Cell lysis and immuno-blots were performed as described previously (2), except 20μg protein was used per sample. Phospho-Smad1 (Ser 463/465)/ Smad5 (Ser 463/465) /Smad8 (Ser426/428) polyclonal antibody (Cell Signaling Technology), and mouse monoclonal β-actin antibody (Sigma) were used. Horseradish peroxidase-conjugated secondary antibodies (Bio-Rad) were used.

Transfections and Luciferase Assay: For transient transfection experiments the chondrocyte cell line RCJ3.1C5.18 (C5.18) was cultured in α-minimal essential medium containing 10% FBS in 12-well plates at a density of 35,000 cells per well. Twenty-four hours later the cells were transfected with Lipofectamine LTX reagent (Invitrogen) at a LipofectamineLTX, PLUS reagent, and DNA ratio of 2.5μl:1μl:1μg. SV40-Revilla luciferase construct was cotransfected with either the 12XSBE or pGL4 firefly reporters to standardize results for transfection efficiency. Dual Luciferase Assay Reporter System (Promega) and a luminometer (Opticom 1) were used to determine luciferase activity in the cell lysate forty-eight hours post-transfection.

Results: In our model, murine chondrocytes underwent chondrocyte hypertrophy with BMP-2 treatment and time in culture as determined by gene expression of type X collagen. This induction of type X collagen by BMP-2 was dramatically diminished with concomitant PGE2 treatment (Fig. 1).

To confirm PGE2 treatment can decrease BMP-2-target-gene transcription, the C5.18 cell line was transfected with the 12XSBE reporter. As seen in Figure 2, PGE2 treatment reduces the induction of 12XSBE activity by BMP-2, as does dbcAMP.

In an attempt to elucidate a potential mechanism by which PGE2 treatment inhibits gene transcription induced by BMP-2, the effect of PGE2 treatment on the induction of activated forms of the BMP-specific Smads was next examined.

While PGE2 treatment alone had no effect on the protein level of the phosphorylated forms of Smads1/5/8, when co-treated with BMP-2, PGE2 reduced this induction by BMP-2 (Fig. 3).

Discussion: We have shown Prostaglandin E2 is able to reduce the activation of the BMP-Smad signaling pathway, BMP-target gene transcription, and the induction of type X collagen by BMP-2. Chondrocyte proliferation and hypertrophy in the growth plate during endochondral ossification are highly regulated processes in which the orchestration of several signaling molecules results in proper bone formation. Our results strongly suggest signaling crosstalk between the prostanglandin and bone morphogenetic protein pathways regulates type X collagen gene expression and chondrocyte hypertrophy, potentially through a PKA-dependent mechanism.