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CaMKII inhibition in human primary and pluripotent stem cell-derived chondrocytes modulates effects of TGFβ and BMP through SMAD signaling

B. Saitta † ‡, J. Elphingstone †, S. Limfat †, R. Shkhyan †, D. Evseenko † § *

† Department of Orthopaedic Surgery, Keck School of Medicine of University of Southern California, Los Angeles, CA, 90033, USA
‡ Division of Nephrology and Hypertension, Department of Medicine and USC/UKRO Kidney Research Center, Keck School of Medicine of the University of Southern California, Los Angeles, CA, 90033, USA
§ Stem Cell Research and Regenerative Medicine Keck School of Medicine of University of Southern California, Los Angeles, CA, 90033, USA

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SUMMARY

Objective: Upregulation of calcium/calmodulin-dependent kinase II (CaMKII) is implicated in the pathogenesis of osteoarthritis (OA) and reactivation of articular cartilage hypertrophy. However, direct inhibition of CaMKII unexpectedly augmented symptoms of OA in animal models. The role of CaMKII in OA remains unclear and requires further investigation.
Methods: Analysis of CaMKII expression was performed in normal human and OA articular chondrocytes, and signaling mechanisms were assessed in articular, fetal and Pluripotent Stem Cell (PSC)-derived human chondrocytes using pharmacological (KN93), peptide (AC3-I) and small interfering RNA (siRNA) inhibitors of CaMKII.
Results: Expression levels of phospho-CaMKII (pCaMKII) were significantly and consistently increased in human OA specimens. BMP2/4 activated expression of pCaMKII as well as COLII and COLX in human adult articular chondrocytes, and also increased the levels and nuclear localization of SMADs1/5/8, while TGFβ1 showed minimal or no activation of the chondrogenic program in adult chondrocytes. Targeted blockade of CaMKII with specific siRNAs decreased levels of pSMADs, COLII, COLX and proteoglycans in normal and OA adult articular chondrocytes in the presence of both BMP4 and TGFβ1. Both human fetal and PSC-derived chondrocytes also demonstrated a decrease of chondrogenic differentiation in the presence of small molecule and peptide inhibitors of CaMKII. Furthermore, immunoprecipitation for SMADs1/5/8 or 2/3 followed by western blotting for pCaMKII showed direct interaction between SMADs and pCaMKII in primary chondrocytes.
Conclusion: Current study demonstrates a direct role for CaMKII in TGF-β and BMP-mediated responses in primary and PSC-derived chondrocytes. These findings have direct implications for tissue engineering of cartilage tissue from stem cells and therapeutic management of OA.

Introduction

Alterations in chondrocyte homeostasis and differentiation play a critical role in osteoarthritis (OA). Recent mouse studies demonstrated that activation of the calcium/calmodulin-dependent kinase II (CaMKII) pathway in primary articular chondrocytes induces chondrocyte hypertrophy and triggers OA[1]. Previously, CaMKII signaling was shown to promote hypertrophy in developing chondrocytes, while reduction of CaMKII activity delayed chondrocyte maturation[2]. Additionally, *in vivo* studies have reported that loss of CaMKII function using inhibitors (e.g., small molecule inhibitor KN93 and permeable peptides AIP-II or NtideII) blocked the transition from proliferation to hypertrophy of growth plate chondrocytes, resulting in the blockade of hypertrophic chondrocyte formation[3]. Therefore, an inverse gradient model of CaMKII activity has been proposed: higher in the hypertrophic zone and lower in the proliferative zone(3). Several studies suggested that intracellular Ca^{2+} levels could act as an inducer of CaMKII activity, enhancing chondrocyte maturation[2–5], and that calcium cascades triggered by mechanical stimuli promoted the expression of chondrocyte markers (e.g., Sox9, type II collagen and Aggrecan)

* Address correspondence and reprint requests to: D. Evseenko, Stem Cell Research and Regenerative Medicine, Keck School of Medicine of USC, 1450 Biggy St, NRT 4509. Los Angeles, CA 90033, USA.
E-mail address: evseenko@usc.edu (D. Evseenko).

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during early development[6–9], but the mechanism(s) remains largely unclear. Unlike chondrocytes in the growth plate, articular chondrocytes and human/mouse mesenchymal stromal cells are sensitive to minimal levels of pCaMKII, which have been shown to phosphorylate a variety of downstream signaling targets[10,11], including SMADs[12,13]. Chondrocyte terminal differentiation is stimulated by SMAD1/5/8 and inhibited by the SMAD2/3 pathway. Multiple studies suggest that the fine balance between different SMAD proteins controls phenotypic stability of adult articular chondrocytes. However, this balance can be disrupted in

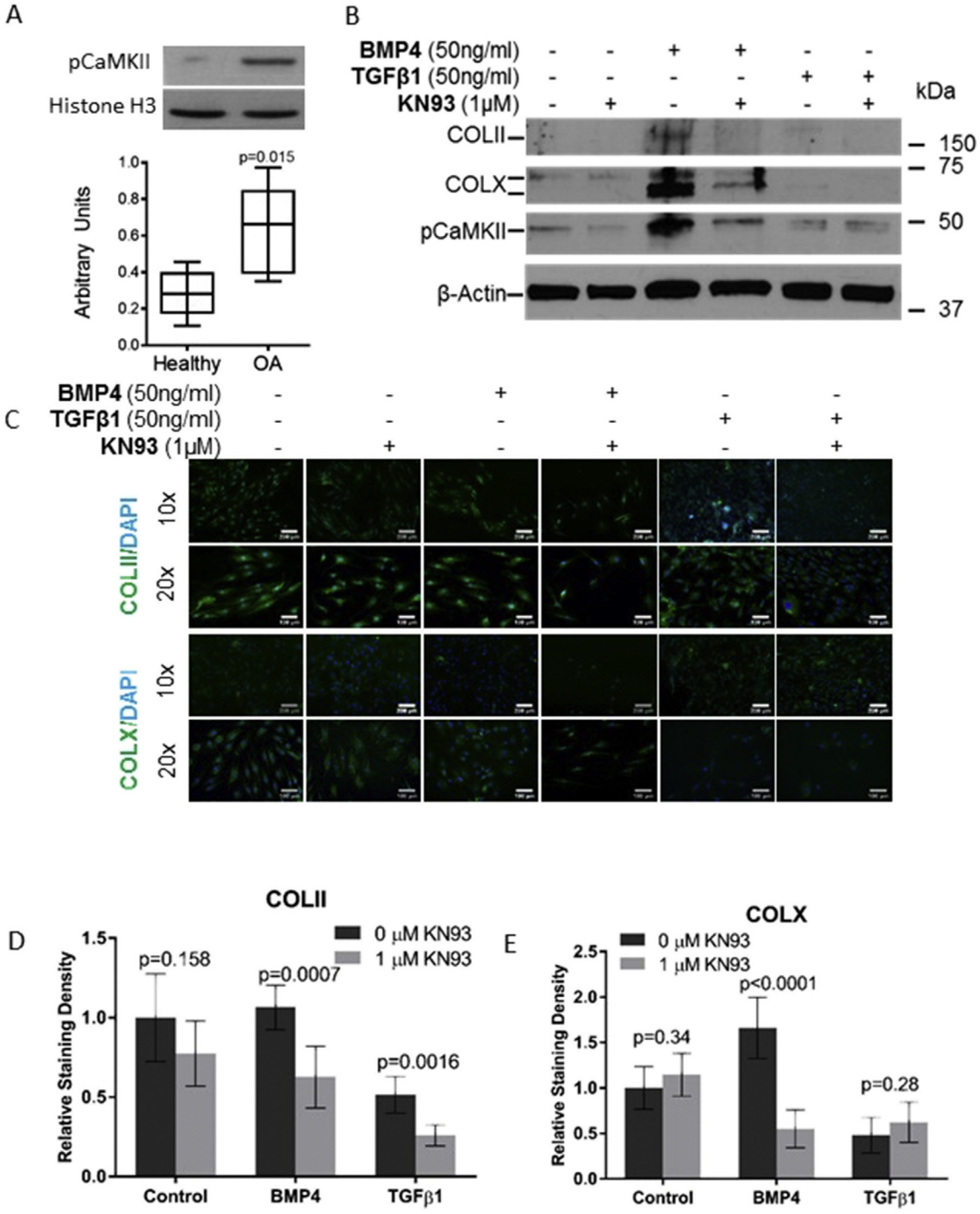

Fig. 1. Levels of phosphorylated CaMKII are upregulated in osteoarthritis specimens and after exposure of human adult articular chondrocytes to BMP4 *in vitro*. A. Western blot assessment of phosphorylated CaMKII in healthy and osteoarthritis cartilage tissue). Histone 3 is a housekeeping gene. Non-cultured cells from biologically different normal ($n = 4$) and OA donors ($n = 4$) were used. Data was analyzed using Student's t test **B.** Western blot (COLII, COLX, α-pCaMKII, β-actin) of HAC monolayers after 7-day treatment with either BMP4, TGFβ1 or combination of the two, with and without KN93 (1 μM). Image shows representative data from 1 of 3 independent experiments (3 healthy donors). **C.** Articular chondrocytes grown in chamber slides as a monolayer were treated for 7 days with BMP4, TGF-β1, or alone, with and without KN93 (1 μM). Immunofluorescent staining for either COLII or COLX (green) with a nuclear DAPI (blue) counterstain at two different magnifications. Scale bars, 100 and 200 μm. Images from all panels show representative data from 1 of 3 independent experiments (3 different donors). **D, E.** Quantification of staining showed decreases in COLII (**D**) and COLX (**E**) in all groups after treatment with KN93 (1 μM). $n = 3$ independent experiments (3 different donors). Data presented as mean ± 95% confidence interval and analyzed using Student's t test.

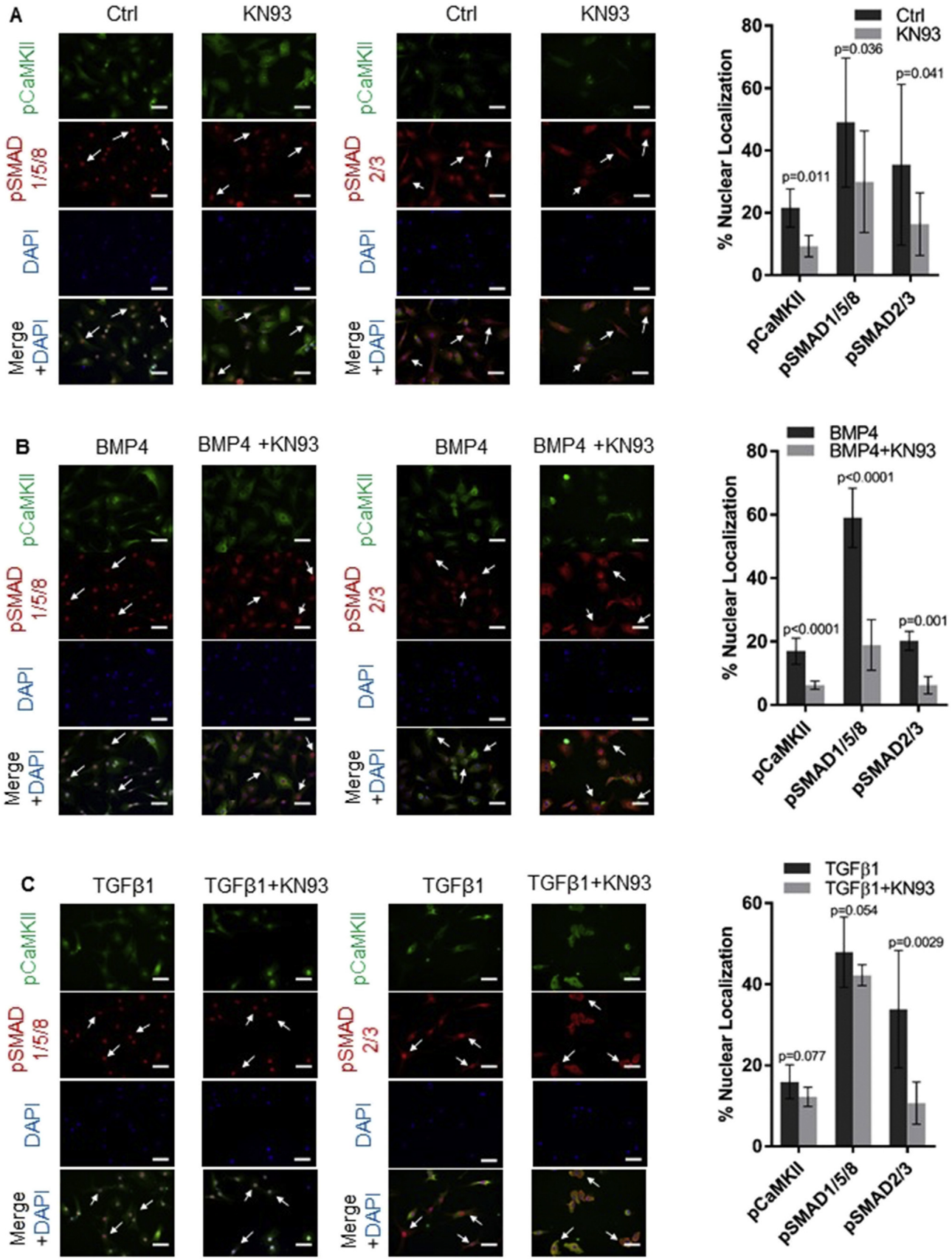

Fig. 2. Inhibition of pCaMKII activity by 1 μM KN93 decreased nuclear localization of pCaMKII, pSMAD1/5/8 and pSMAD 2/3 in human articular chondrocytes. A-C. Human articular chondrocytes were grown in chamber slides and treated for 1 h with BMP4 or TGFβ1, and KN93. The left columns include immunofluorescence staining for pCaMKII (green), pSMAD 1/5/8 (red), and a nuclear DAPI counterstain (blue). The right columns show pSMAD2/3 (red) with the aforementioned stains. The greatest decrease of pSMAD 1/5/8

pathological conditions and multiple signaling pathways have been discussed in this context. A direct link between CaMKII and SMAD signaling has never been studied in chondrocytes. We hypothesized that CaMKII is directly implicated in bone morphogenetic protein (BMP)-mediated activation of SMAD1/5/8 in articular chondrocytes.

Here, we report for the first time that CaMKII levels are upregulated in human OA cartilage; we also show that inhibition of CaMKII results in significant modulation of pSMAD levels and nuclear localization in the presence of BMP2/4 and transforming growth factor beta (TGFβ)1/3. Using primary human articular and fetal chondrocytes, we demonstrated that inhibition of CaMKII in the presence of BMPs decreased the levels of SMAD1/5/8 and reduced expression of cartilage genes, while inhibition of CaMKII in the presence of TGFβ decreased pSMAD2/3 levels as well as chondrogenic gene expression. Moreover, CaMKII inhibition is shown to delay chondrogenic differentiation of human pluripotent stem cells. Our studies also demonstrate direct physical interactions between pSMADs and pCaMKII.

Overall, these findings further dissect the role of CaMKII in regulation of chondrocyte differentiation and offer important knowledge for developing novel strategies for cartilage engineering and therapeutic management of OA.

Methods

Isolation and culturing of primary fetal and adult chondrocytes from human and porcine cartilage tissue

Normal (four donors) and OA adult articular cartilage (two males and two females, 50–70 years old, 2–3 on the Kellgren–Lawrence grading scale for OA) were obtained from the National Disease Research Interchange (NDRI). Fetal specimens (17 weeks post conception) (Novogenix Laboratories) were obtained from Dr April Pyle (UCLA) and cartilage cells were isolated as described previously[14]. Those samples from elective terminations were collected after obtaining informed consent. All donated material was anonymous and carried no personal identifiers. Samples were processed as we previously described[14]. Porcine cartilage (three donors) was obtained from S&S Farms. Legs from 5-month-old Yucatan minipigs were obtained and digested following previously described protocols[16].

Chondrogenic differentiation of human pluripotent stem cells

Human embryonic stem cells (H1 and H9) were purchased from the USC Stem Cell Core (Chang Stem Cell Engineering Facility http://stemengineering.usc.edu/services/). Pluripotent Stem Cell (PSC)-derived cartilage-committed progenitors were generated and re-aggregated in chondrospheres using methods previously developed and described in our lab[14,18] Ferguson *et al*., 2018 (*In Press*). After this initial induction, cells were switched to treatment media, X–VIVO with FGF2 (10 ng/ml) and subjected to treatment with BMP4, TGFβ1, KN93, or a combination of several factors.

Design of cumate-inducible lentivector construct expressing the autocamtide peptide (AC3-I) in hPSCs (H1 ESC)

A cumate (Cu)-inducible lentivector construct with the autocamtide peptide was inserted before the enhanced green fluorescent protein (EGFP) reporter under the control of the CMV5 promoter, creating a cumate-inducible system (CMV + CuO)[15]. The construct was custom-prepared by System Biosciences (https://www.systembio.com/). Next, 293T cells were cultured on a 0.01% L-Lysine (Sigma)-coated 10 cm dish in DMEM 10% FBS with P/S/A media as characterized above and incubated in 5% CO_2 at 37°C for 1 day. Cells were then infected with the lentivirus construct. On the second day the cells were lipofected using Turbo DNAfectin 3000 (Lamda Biotech) following manufacturer protocols.

small interfering RNA (siRNA) transfection

Primary chondrocytes were grown to confluency in a T-75 flask, then ~200,000 cells were plated into a 6-well plate and within 24 h, transfection with either a scrambled siRNA or CaMK2α siRNA (Origene) was completed following manufacturer protocols for Lipofectamine (Invitrogen) for 2 days in OPTI-MEM (Thermofisher).

Immunoprecipitation

Prior to treatment, human fetal and adult pig chondrocytes were grown to confluency in T-75 flasks as described in Supplementary methods. Cells were then treated for 3 days and collected in 50 μl radioimmunoprecipitation assay (RIPA) with phosphatase Inhibitors (ThermoFisher). Immunoprecipitation using Protein G Agarose was completed following manufacturer's protocols (ThermoFisher). Cell lysates were incubated with Protein G Agarose (Pierce) and anti-SMAD1/5/8 or 2/3 antibody (Cell Signaling Technology) at 4°C overnight. The immune complexes were sedimented, washed and separated by SDS-PAGE (see below) and further analyzed by western blot using anti-pCamMK2 antibodies (Abcam).

Detailed description of routine methods including *Quantitative Real-Time Polymerase Chain Reaction (qPCR), western Blot analysis, Immunocytochemistry, Histology Sample Preparation Alcian Blue Staining and Immunocytochemistry Quantification* are described in Supplementary Methods section.

Statistics/analysis

Data was analyzed using Microsoft Excel, statistical tests: Student *t* test for two group comparison and one-way ANOVA with ad hoc Tukey tests were conducted using GraphPad Prism7. Data is presented with 95% C.I. error bars.

Results

pCaMKII levels are upregulated in human osteoarthritic cartilage

Studies in mice have shown that disruption of CaMKII signaling leads to the development of degenerative joint disease resembling human OA. However, no studies to date assessed expression of active forms of CaMKII in human OA cartilage. To address this question, we directly compared the levels of phosphorylated active CaMKII (pCaMKII) in normal and osteoarthritic chondrocytes from patients with moderate OA. The OA chondrocytes show significantly upregulated levels of pCaMKII [Fig. 1(A)].

was observed in the BMP4 treatment group (**B**) and in pSMAD 2/3 in the TGFβ1 group (**C**). The graphs display quantification of % nuclear localization, calculated in ImageJ by measuring fluorescent intensity within a single cell and within the nucleus only as delineated by the DAPI stain. All conditions display decreased % nuclear localization after treatment with KN93. Scale bars, 100 μm. Representative positive cells denoted by white arrow. Images from all panels show representative data from 1 of 3 independent experiments. Graphs show quantification from independent experiments ($n = 3$), data presented as mean ± 95% confidence interval and analyzed using Student's *t* test.

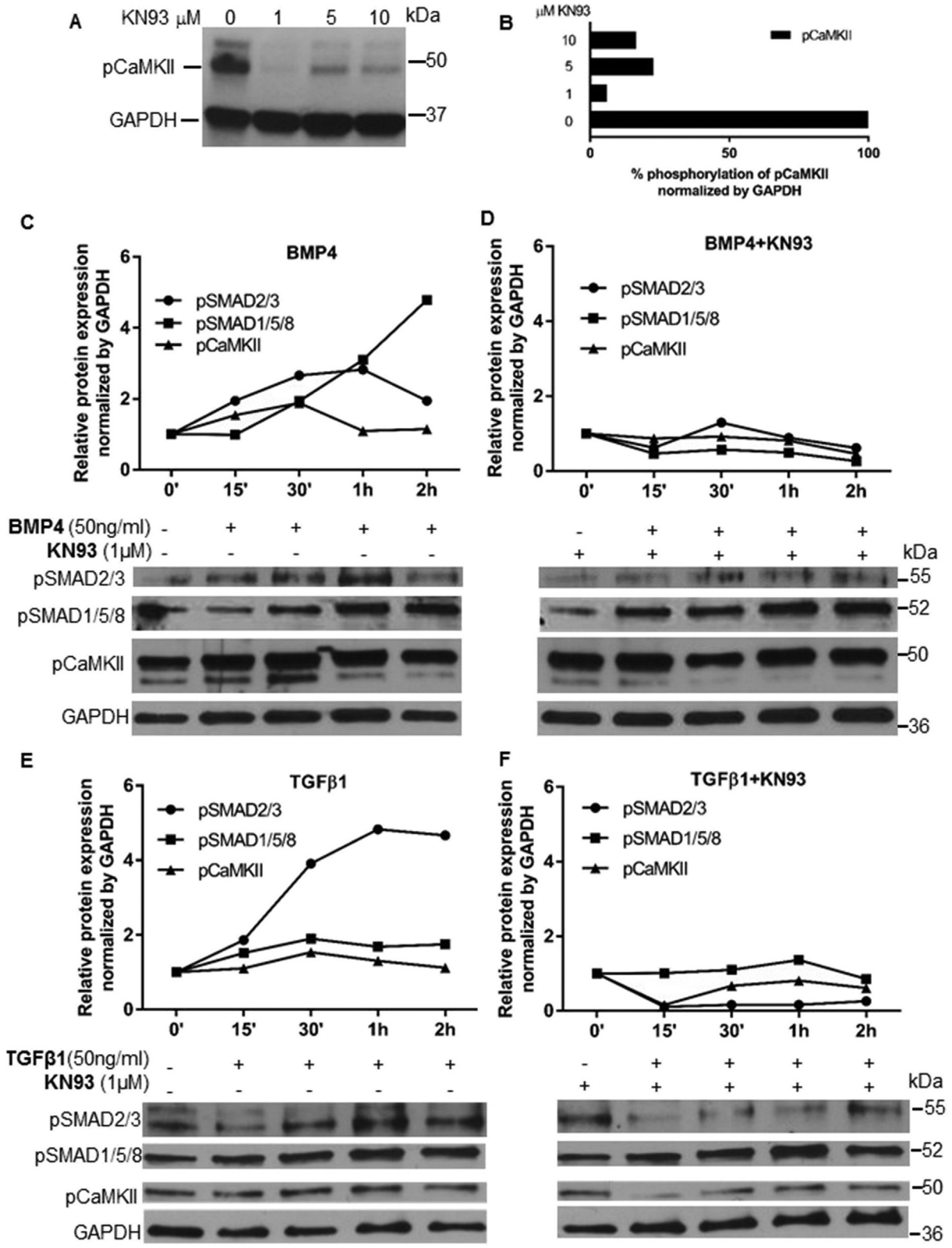

Fig. 3. Inhibition of pCaMKII activity by KN93 decreases phosphorylation of pSMAD 1/5/8 and pSMAD 2/3 during a time course treatment of HFCs with BMP4 and TGFβ1. Total proteins were isolated from human fetal chondrocyte monolayers treated with 0, 1, 5, 10 μM KN93 and western blotting was performed with an antibody against pCaMKII T286. **B.** Quantitative analysis of western blots showed a strong decrease (~80%) of pCaMKII when treated with KN93. **C, D.** Time course of human fetal chondrocytes treated for 0–2 h with BMP4 displayed increased phosphorylation of pCaMKII, pSMAD 1/5/8, and pSMAD 2/3, with a gradual increase observed in pSMAD1/5/8 at 1 and 2 h (**C**), that was abrogated with the addition of KN93 (**D**). **E, F.** Time course of human fetal chondrocytes treated for 0–2 h with TGFβ1 showed increased phosphorylation of pCaMKII, pSMAD 1/5/8, and pSMAD 2/3, with a stronger increase observed in pSMAD2/3 at 30 min, 1 and 2 h (**E**), that was abrogated with the addition of KN93 (**F**). Western blots (**C-F**) were performed using antibodies for pSMAD1/5/8 (Ser463/465), pSMAD2/3 (Ser423/425) and pCaMKII (Thr286). Equal loading of protein was confirmed by monoclonal antibodies against GAPDH. Images from all panels show representative data from 1 of 3 independent experiments (3 different donors). Data are presented as mean ± 95% confidence interval and analyzed using Student's *t* test.

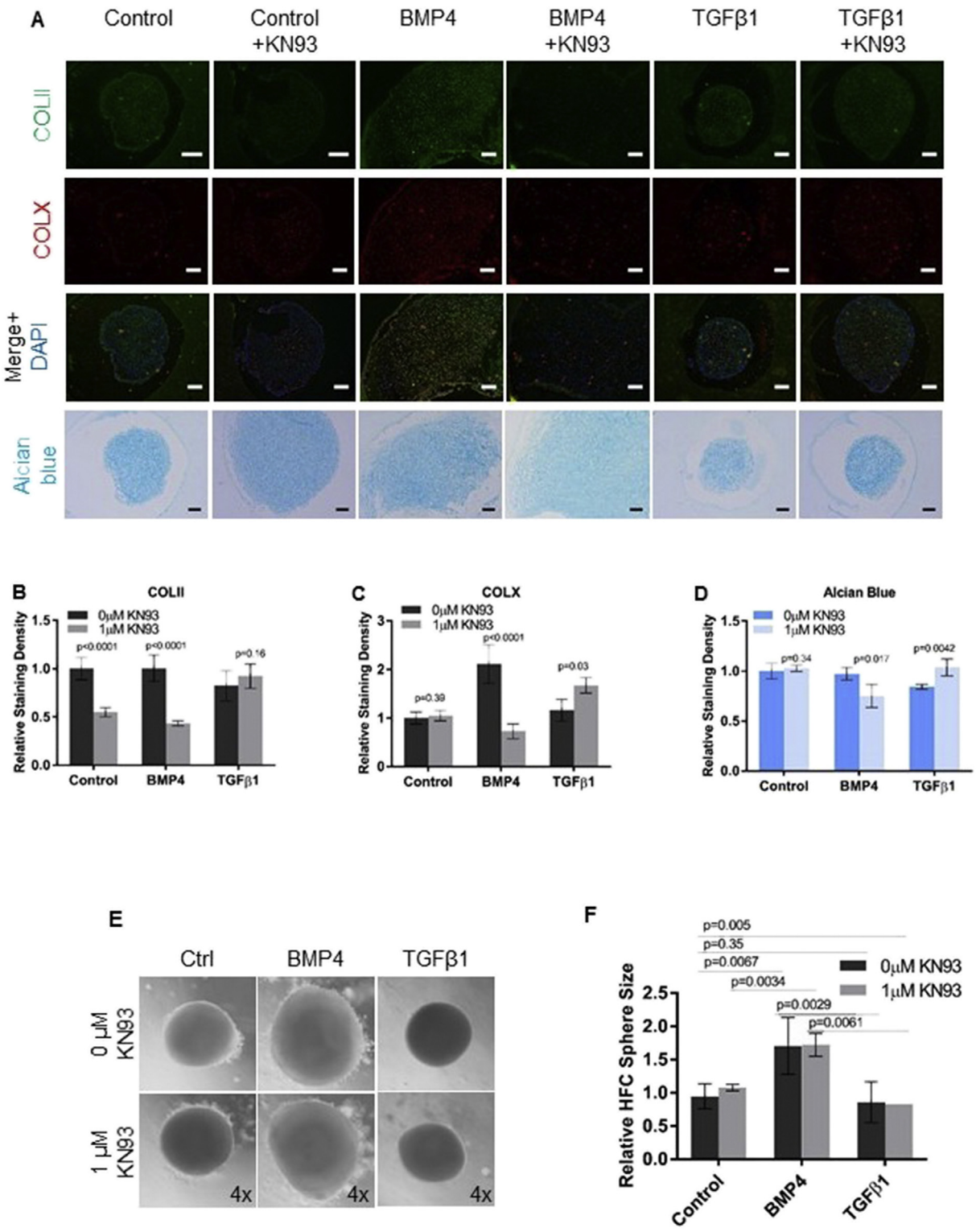

Fig. 4. Human fetal chondrocytes with inhibited CaMKII demonstrate decreased expression of COLII, COLX and Alcian blue staining with BMP4 but not TGFβ1 treatment. **A.** Human fetal chondrocytes were treated for 7 days and then stained with antibodies for COLII (green) or COLX (red) and an Alcian blue stain for proteoglycans. Scale bars, 100 μm. **B.** Quantification of IHC stain for COLII demonstrates statistically significant decreases in expression in control and in BMP4 treated fetal chondrocytes after inhibition by KN93 (1 μM). **C.** Quantification of IHC stain for COLX indicated a statistically significant decrease in expression in BMP4-treated fetal chondrocytes and a statistically significant increase in expression in TGFβ1-treated fetal chondrocytes after inhibition with KN93 (1 μM). **D.** Quantification of Alcian blue stain for proteoglycans showed a statistically significant decrease in expression in BMP4 treated fetal chondrocytes and a statistically significant increase in expression in TGFβ1 treated fetal chondrocytes after addition of KN93 (1 μM). **E.** Representative images of fetal chondrocytes taken prior to fixation and sectioning. **F.** Quantification of fetal cell aggregates showed statistically significant increases in size when treated with BMP4 but not with TGFβ1, compared to controls (analyzed using Student's *t* test). Images from all panels show representative data from 1 of 3 independent experiments. Graphs show quantification from independent experiments (3 different donors), presented as mean ± 95% confidence interval.

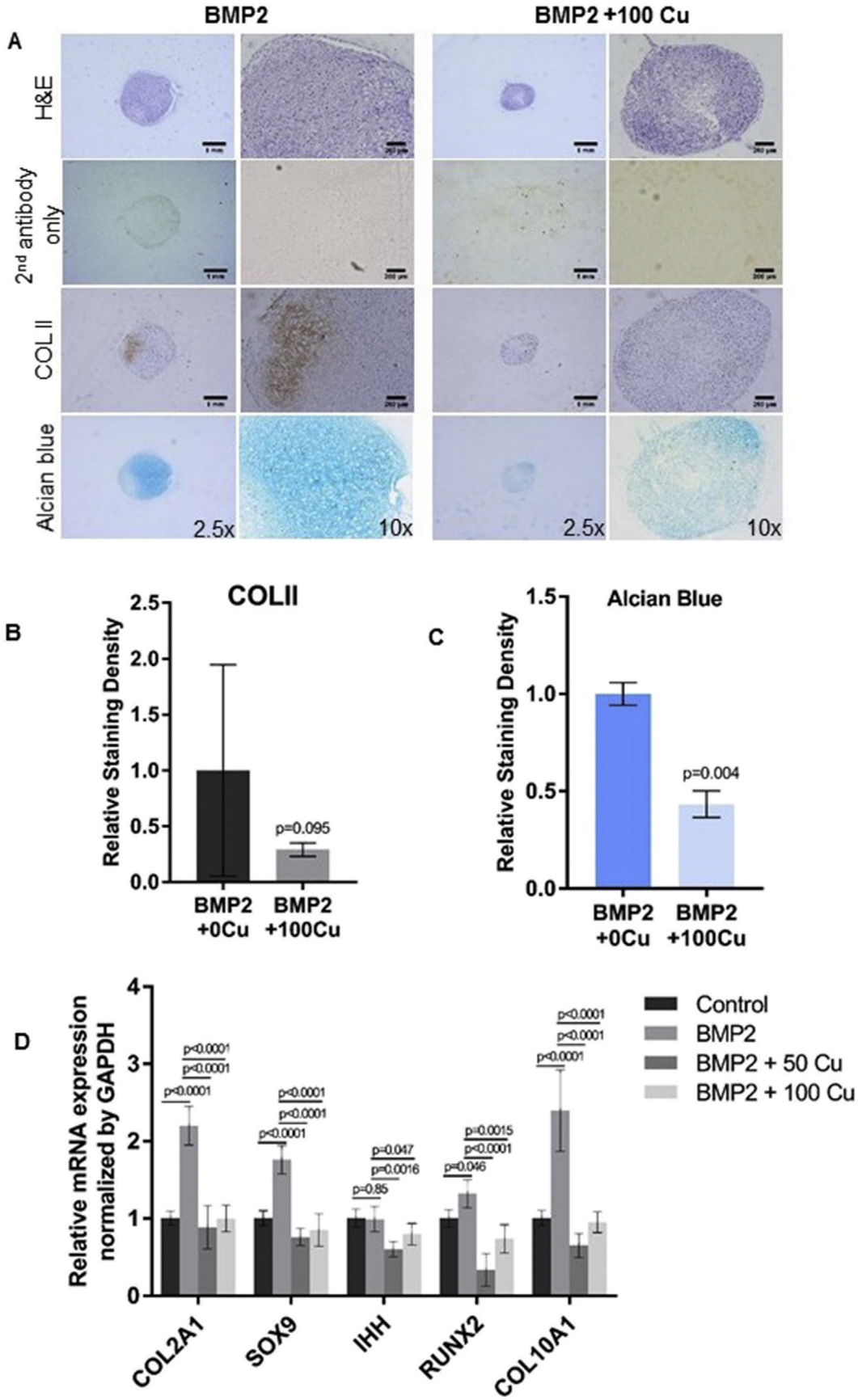

Fig. 5. Immunohistochemistry (IHC) and qPCR of PDCs treated with BMP2 show decreased expression of chondrocyte differentiation markers after Cu treatment. A. IHC and Alcian blue staining of PDCs treated for 7 days with BMP2 (left panels) and BMP2 with Cu (100 μg/ml) induction (right panels) seen at two different magnifications, 2.5× and 10×. First row: H&E staining. Second row: secondary antibody alone. Third row: COLII staining. Fourth row: Alcian blue staining. Upon Cu induction, strong downregulation of COLII and

BMP4, but not TGFβ1 increases phosphorylation of CaMKII, expression of COLII and COLX and this response is abrogated by KN93 in human adult articular chondrocytes

It has been shown by our group and by others that cultured human adult articular chondrocytes express relatively low levels of cartilage markers such as COLII due to partial de-differentiation even after only 1–2 passages. Treatment of cultured human adult articular chondrocytes with BMP4, induced strong increases in the expression of COLII, COLX in addition to CaMKII phosphorylation [Fig. 1(B) and (C)]. These effects were abolished after treatment with the CaMKII inhibitor, KN93 (1 μM), for 7 days. The results of the western blot assay were corroborated by immunofluorescence staining [Fig. 1(D)]. In contrast, when human adult articular chondrocytes were treated with TGFβ1, both western blotting and immunofluorescence showed either no change or a decline in expression of these markers [Fig. 1(D)–(E)]. Overall, these findings indicate a strong correlation between CaMKII activity and the maintenance of the chondrogenic program in BMP-treated adult articular chondrocytes. To verify specificity of KN93, primary chondrocytes were treated with KN92, an inactive analog of KN93, and showed no changes in cartilage markers relative to control (Supp. Fig. 3).

KN93 inhibition of activated CaMKII reduces nuclear localization of pSMADs in human primary adult articular chondrocytes treated with BMP4 or TGFβ1

TGFβs canonically transduce signals through SMAD2/3, while BMPs transduce signals through SMAD1/5/8[17]. The effects of SMADs in chondrogenesis are well established by multiple studies[17]. Based on our previous findings, we predicted that CaMKII is involved in the regulation of SMAD phosphorylation by BMP and TGFβ signaling. Using immunofluorescence staining, we showed that inhibition of CaMKII with KN93 treatment reduced nuclear localization of activated, pSMADs in human adult articular chondrocytes [Fig. 2(A)]. Nuclear localization of SMADs indicates activation of signaling by these transcription factors[17]. When treated with BMP4, human adult articular chondrocytes exhibited an increase in pSMAD1/5/8 nuclear localization that was abolished when KN93 was added; additionally, in BMP-treated cells pSMAD2/3 was found to have decreased nuclear localization compared to controls. This was further reduced with KN93 [Fig. 2(B)]. As expected, the addition of TGFβ1 showed an increase in pSMAD2/3 nuclear localization [Fig. 2(C)]. This effect was abrogated by KN93, while pSMAD1/5/8 was not affected in these TGFβ1 treated samples when compared to controls.

Pharmacological inhibition of CaMKII with KN93 in human fetal primary chondrocytes results in opposite outcomes in the presence of BMPs and TGF-beta, respectively

We next tested differentiation of human fetal chondrocytes after CaMKII inhibition by 1, 5, and 10 μM KN93, and found that a concentration of 1 μM was the most efficient inhibitor of CaMKII phosphorylation [Fig. 3(A), (B)]. We next carried out a time course (0, 15′, 30′, 1 h, 2 h) assessment of cells treated with BMP4 [Fig. 3(C) and (D)] or with TGFβ1 [Fig. 3(E) and (F)] with and without KN93. BMP4 treatment increased SMAD2/3 and CaMKII phosphorylation in the first 30′ but decreased after 2 h, while SMAD1/5/8 phosphorylation continued after 2 h [Fig. 3(C)]. Phosphorylation for all SMADs and CaMKII were completely eliminated with the addition of KN93 [Fig. 3(D)]. With TGFβ1 treatment, CaMKII and SMAD1/5/8 phosphorylation slightly increased over 2 h, while here was a major increase of SMAD2/3 phosphorylation at 30′ that started to plateau by 2 h [Fig. 3(E)]. After the addition of KN93, there was an immediate downregulation of CaMKII, SMADs 1/5/8 and SMAD 2/3 phosphorylation [Fig. 3(F)]. Inhibition of CaMKII in the presence of BMP4 showed a decrease of cartilage markers COLII and COLX. In contrast, terminal differentiation of fetal chondrocytes treated with both TGFβ1 and KN93 was higher than in controls treated with TGFβ1 only [Fig. 4A–F].

Thus, analysis of cartilage markers in either BMP or TGFβ-treated fetal chondrocytes showed the same context-specific effects of pCaMKII inhibition on the maintenance of cartilage phenotype as described above for adult articular chondrocytes. These data suggest that described mechanisms of CaMKII-mediated regulation of SMADs are dissimilar at different stages of human cartilage ontogeny.

Inhibition of CaMKII phosphorylation by AC3-I downregulates CaMKII gene expression in human pluripotent stem cell-derived chondrocytes

After dissecting the modulatory effects of CaMKII in primary cartilage cells, we tested how modulation of this kinase affects differentiation of pluripotent stem cell (PSC)-derived chondrocytes (PDC). First, we generated and validated a cumate (Cu)-inducible lentiviral construct with the Autocamtide Peptide inhibitor (AC3-I) as shown [Supp. Fig. 1(A)]. AC3-I mimics the autoinhibitory region of the CaMKII regulatory domain (residues 278–290) by competitively binding to the catalytic site and blocking phosphorylation of Thr 286[19]. To determine if the AC3-I/EGFP construct was functional during chrondrogenic differentiation of human PSC to chondrocytes, we first generated PSC-derived cartilage-committed progenitors and re-aggregated them in chondrospheres using methods previously developed and described in our lab[14,18] Ferguson *et al*., 2018, in press) [Supp. Fig. 2(A) and (B)]. PDCs represent a three-dimensional system for differentiating highly purified skeletal progenitors into fully functional chondrocytes[14,18] Ferguson *et al*., 2018, in press). Cell viability within a given chondrosphere was consistently >75% [Supp. Fig. 2(D) and (E)]. Next, we assessed the progression of differentiation when CaMKII activity was inhibited by AC3-I, expression of which was induced by the addition of Cu. As expected, Cu itself had no effects on gene expression by PSC-derived chondrocytes (data not shown). We therefore examined the effects of BMP2 in conjunction with the modulation of CaMKII activity during *maturation* of PDCs (Fig. 5). We found strong inhibition of COLII expression when PDCs were treated for 7 days with Cu [100 μg/ml; Fig. 5(A)], inducing the expression of AC3-I (optimized in Supp. Fig. 1). Similar to our findings in primary cells, we also observed a decrease in chondrosphere size (~60%) [Fig. 5(A)] and IHC staining for COLII (~40%) [Fig. 5(B)] and a significant reduction (~60%) of Alcian blue staining

Alcian blue staining was observed. An overall decrease in sphere size was observed between the two conditions in columns one and three. Due to the decrease in size, the entire section can be seen in the BMP2 and Cu treated group while only a portion can be seen in the BMP2 only group. Scale bars, 1 mm and 200 μm. **B.** Quantification of sphere sizes after treatment show a roughly 60% decrease in sphere size with Cu treatment. **C.** Quantification of IHC stain for COLII expression shows an ~40% decrease in expression with Cu induction. Data was analyzed using Student's *t* test. **D.** Quantification of Alcian blue staining indicates a statistically significant decrease in proteoglycans as seen at 10× magnification **E.** Analysis of qPCR of PDCs for 7 days shows increased expression of chondrocyte differentiation marker genes (*COL2A1*, *SOX9*, *IHH*, *RUNX2*, and *COL10A1*) with BMP2, and statistically significant downregulation of expression with Cu (50 and 100 μg/ml), normalized by GAPDH. Bars represent data averaged from experiments performed in triplicate. *Y*-axis values indicate fold difference. Images from all panels show representative data from 1 of 3 independent experiments. Graphs show quantification from independent experiments (3 different donors), data presented as mean ± 95% confidence interval and analyzed using one-way ANOVA followed by Tukey test.

for sulfated proteoglycans [Fig. 5(C)] after Cu induction of PDCs. Next, we performed qPCR analyses on the same aggregates to determine if additional genes were also affected by the above treatment. We found, as expected, an upregulation of *COL2A1, SOX9, RUNX2* and *COL10A1* with BMP2, which was abolished by AC3-I induction with Cu (50 and 100 μg/ml), showing statistically significant downregulation of these genes [Fig. 5(D)]. These results show that BMP-driven differentiation was abrogated at the mRNA and protein levels for COLII and for proteoglycans by the activation of the AC3-I inhibitor, suggesting a role for CaMKII during chondrogenic differentiation of PSCs.

KN93 reduces BMP2/BMP4-induced upregulation of cartilage markers and CaMKII genes in human pluripotent stem cell-derived chondrocytes

We next used pharmacological inhibition of CaMKII by KN93 to further confirm the effects of the AC3-I construct on PDC differentiation. Addition of 1 μM KN93 to PDCs treated with BMP2 [Fig. 6(A)] or with BMP4 for 7 days [Fig. 6(B)] decreased COLII expression [40–50%; Fig. 6(C)], confirming the data observed with the AC3-I inhibitor [Fig. 5(A)]. We also found a moderate decrease in COLX (30–40%) expression [Fig. 6(D)]. However, Alcian blue staining for proteoglycans showed minimal to no difference compared with controls [Fig. 6(E)] in the samples treated with KN93. These results were in contrast with the observed strong decrease in Alcian blue staining in chondrospheres inhibited by AC3-I [Fig. 5(A)]. This showed that the two inhibitors could act in different ways since they bind to different regulatory segments of CaMKII[19]. Next, we performed qPCR analyses on the same KN93-treated PDCs to determine if additional chondrogenic differentiation markers were also affected. We found, as expected, an upregulation of *COL2A1, SOX9, IHH, RUNX2, COL10A1, and ACAN* in the presence of BMP2 or BMP4. This upregulation was reduced by the addition of KN93 (1 μM) as shown in Fig. 6(F). ACAN expression, however, was not regulated by KN93, which correlates with the Alcian blue staining results [Fig. 6(E)]. The results of ICC and qPCR experiments confirm that KN93 also robustly reduces COLII and moderately reduces COLX protein expression, showing that it affects the induction of chondrogenic and CaMKII gene expression observed in BMP2 and BMP4-treated PDCs in a manner similar to AC3-I inhibition. However, proteoglycan expression was not decreased as seen with the AC3-I inhibitor, suggesting a different mechanism of action by KN93 on the CaMKII regulatory segment. In addition, we analyzed other chondrocyte progenitor markers (*SOX5, SOX6),* terminal differentiation markers (*MMP13*) and link proteins (*COMP, MATN1, HAPLN1*) and found significant decreases only for *SOX5 and MMP13* in the control group treated with KN93 [Supp. Fig. 4(A)] but not BMP4-treated group. Thus, not all cartilage genes are equally downregulated in response to pCaMK2 inhibition with KN93 [Fig. 6(F)].

KN93 strongly inhibits chondrocyte markers in BMP-treated but not TGF-β-induced human pluripotent stem cell-derived chondrocytes

Our previous studies showed that BMPs drive terminal hypertrophic differentiation of PSC-derived chondrocytes while TGFβ ligands delay terminal differentiation[14,20]. We validated these effects first and showed that BMP4 stimulation when compared to TGFβ3, and TGFβ1 (data not shown) resulted in an increase in the relative expression of the cartilage markers *COL2A1, IHH, RUNX2* and *COL10A1* in PDCs. However, with KN93, there was a strong inhibition of all cartilage markers in BMP4-treated, but not for TGFβ3-treated groups [Fig. 6(G)]. Expression levels of the genes encoding link proteins (*COMP, MATN1, HAPLN1*) was not changed after the exposure TGFβ3 or BMP4 combined with KN93 inhibition [Supp. Fig. 4(B)].

CaMK2α-specific siRNA inhibits CaMK2α, COL2A1, and COL10A1 expression in control, BMP4, and TGFβ1 treated human primary chondrocytes

We have demonstrated that both pharmacological (Figs. 1, 4 and 6) and peptide blockade (Fig. 5) of pCaMKIIα activity decreases expression of cartilage markers in adult (Figs. 1 and 2), fetal (Figs. 3 and 4), and PDCs (Figs. 5 and 6). To confirm our data and better understand the molecular mechanism of this regulation, we then used a different approach by transfecting a 26-bp oligomer siRNA specific for *CaMK2α* mRNA [Fig. 7(A)]. Expression of both *COL2A1* [Fig. 7(B) and (E)] and *COL10A1* [Fig. 7(C) and (F)] was significantly decreased following *CaMK2α* inhibition in BMP4 and TGF β1-treated normal (B, C) and OA chondrocytes (E, F). Fetal chondrocytes had no change in *COL2A1* [Fig. 7(H)] while there was a statistically significant decrease in *COL10A1* expression in the BMP4 treated group [Fig. 7(I)] after knock-down of *CaMK2α*. Thus, our results showed that knockdown of *CaMK2α* with siRNA [Fig. 7(A)] leads to decreased *COL2A1* and *COL10A1* mRNA expression in primary chondrocytes treated with BMP4 and TGFβ1, confirming our prior results obtained using pharmacological inhibitors of pCaMKII (Figs. 1–6).

BMP4 and TGFβ1 trigger direct binding of pCaMKIIα to pSMADs in human fetal and adult chondrocytes

We predicted that an active form of CaMK2 can directly interact with SMADs in chondrocytes. To test this hypotheses we next conducted an immunoprecipitation assay using antibodies against SMAD 2/3 or 1/5/8 followed by the subsequent western blotting with antibodies against pCaMKII. Human fetal chondrocytes treated with BMP4 and TGFβ1 showed increased binding of pCaMKII to both pSMAD1/5/8 and pSMAD2/3, with more prominent effects observed in BMP4 treated group compared to control group, which showed minimal levels of interaction [Fig. 8(A)]. Tested adult chondrocytes had a stronger band for pCaMKIIα in the control group [Fig. 8(B)] indicating some interaction without stimulation with exogenously added BMPs or TGF-beta. Adult chondrocytes also displayed an increase in interaction with the addition of growth factors, though TGFβ1 had a greater effect [Fig. 8(B)]. In control and BMP4 treated adult chondrocytes, pCaMKII had greater interaction with pSMAD1/5/8, while in TGFβ1 group, the pCaMKII interaction with pSMAD2/3 increased and was about equal to pSMAD1/5/8 [Fig. 8(B)]. In conclusion, our studies showed, for the first time, that pCaMK2 can directly interact with SMADs in chondrocytes and this binding can be significantly increased after stimulation of cells with BMP4 and TGFβ1.

Discussion

This study aimed to understand the role of CaMKII in regulating SMAD signaling in chondrocytes. This is a significant question because both CaMKII and SMADs were previously implicated in the pathogenesis of cartilage degeneration and OA. Both of these regulatory mechanisms are also known to be critical for the control of terminal differentiation of chondrocytes during development. However, the direct mechanistic link between CaMKII and SMAD signaling has never been established previously in chondrocytes. We also attempted to study the role of CaMKII in human pluripotent stem cell-derived chondrocytes to advance understanding of how to engineer phenotypically stable articular chondrocytes resistant to terminal differentiation for therapeutic purposes. In

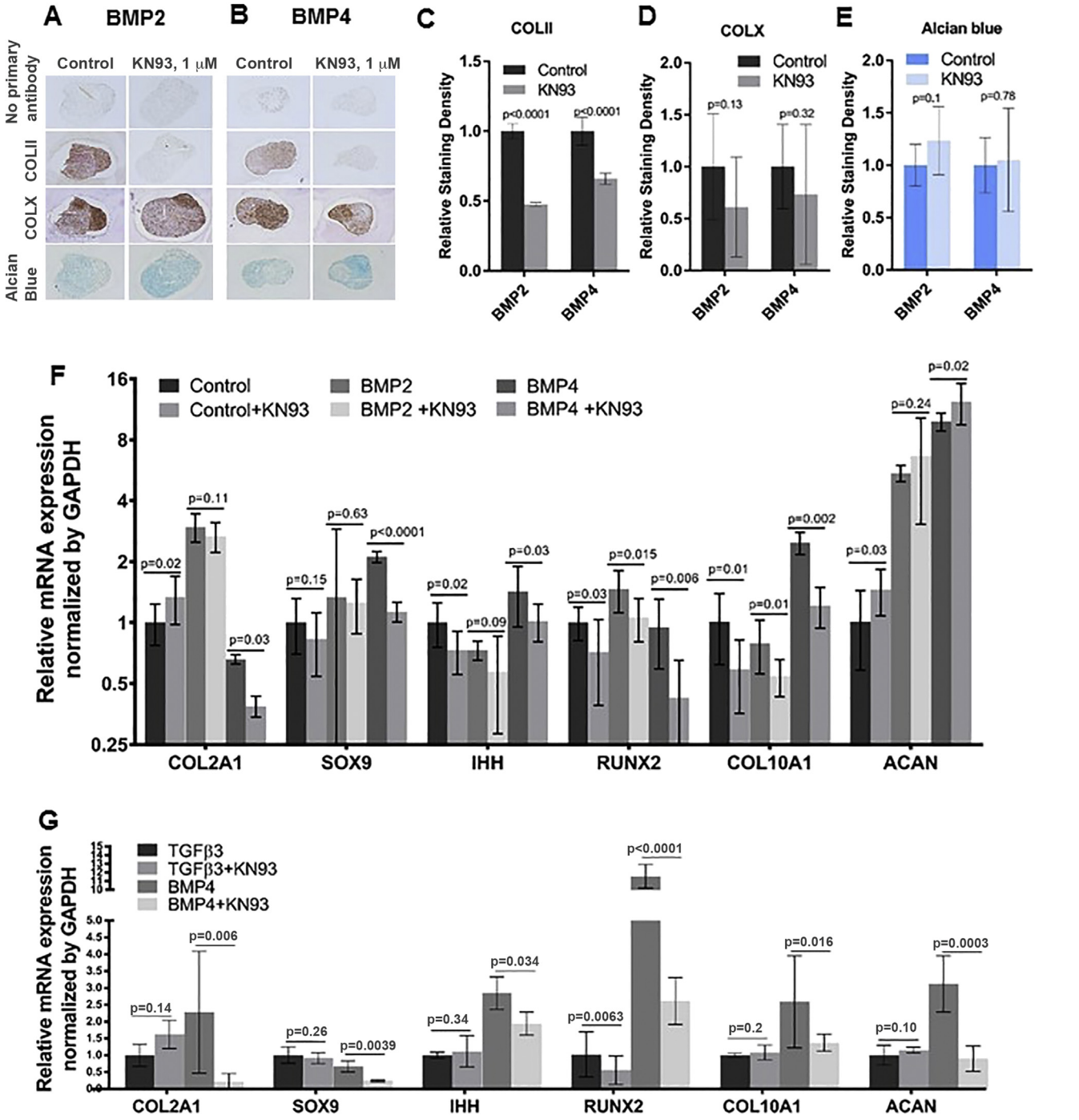

Fig. 6. Inhibition of CaMKII by KN93 decreases expression of chondrocyte differentiation markers in BMP2 or BMP4 treated PDCs. **A, B.** IHC and Alcian blue staining of PDCs treated for 7 days with 50 ng/ml of BMP2 (**A**) or BMP4 (**B**) alone (left columns) and with KN93 (1 μM, right columns). With KN93 treatment, there was a strong decrease in COLII staining (second rows) and moderate decrease in COLX staining (third rows) for both BMP2 and BMP4 treated groups. There was stronger Alcian blue staining (fourth rows) observed after KN93 treatment compared to control. **C.** Quantification of IHC stain for COLII expression showed a decrease in expression (40–50%) with KN93. **D.** Quantification of IHC stain for COLX expression showed a decrease in expression (30–40%) with KN93. **E.** Quantification of Alcian blue stain showed an increase in expression (10–20%) with KN93. **F.** qPCR analysis of PDCs treated for 7 days with BMP2 or BMP4 showed increased expression of chondrocyte differentiation (*COL2A1*, *SOX9*, *IHH, RUNX2*, *COL10A1, and ACAN*) markers with BMPs that was reduced by KN93, with the exception of ACAN. mRNA expression values were relative to controls and normalized by GAPDH. Bars represent data averaged from experiments performed in triplicate. *Y*-axis values indicate fold difference. **G.** qPCR analysis of PDCs treated for 7 days with BMP4 showed significant downregulation of chondrogenic genes (*COL2A1, SOX9, IHH, RUNX2, COL10A1*) when treated with KN93 (1 μM), but not in TGFβ3 treated groups. Values shown were relative to TGFβ3 treated groups, and normalized by GAPDH. Bars represent data averaged from experiments performed in triplicate. *Y*-axis values indicate fold difference. Images from all panels show representative data from 1 of 3 independent experiments. Graphs show quantification from independent experiments, data presented as mean ± 95% confidence interval and analyzed using one-way ANOVA followed by Tukey test.

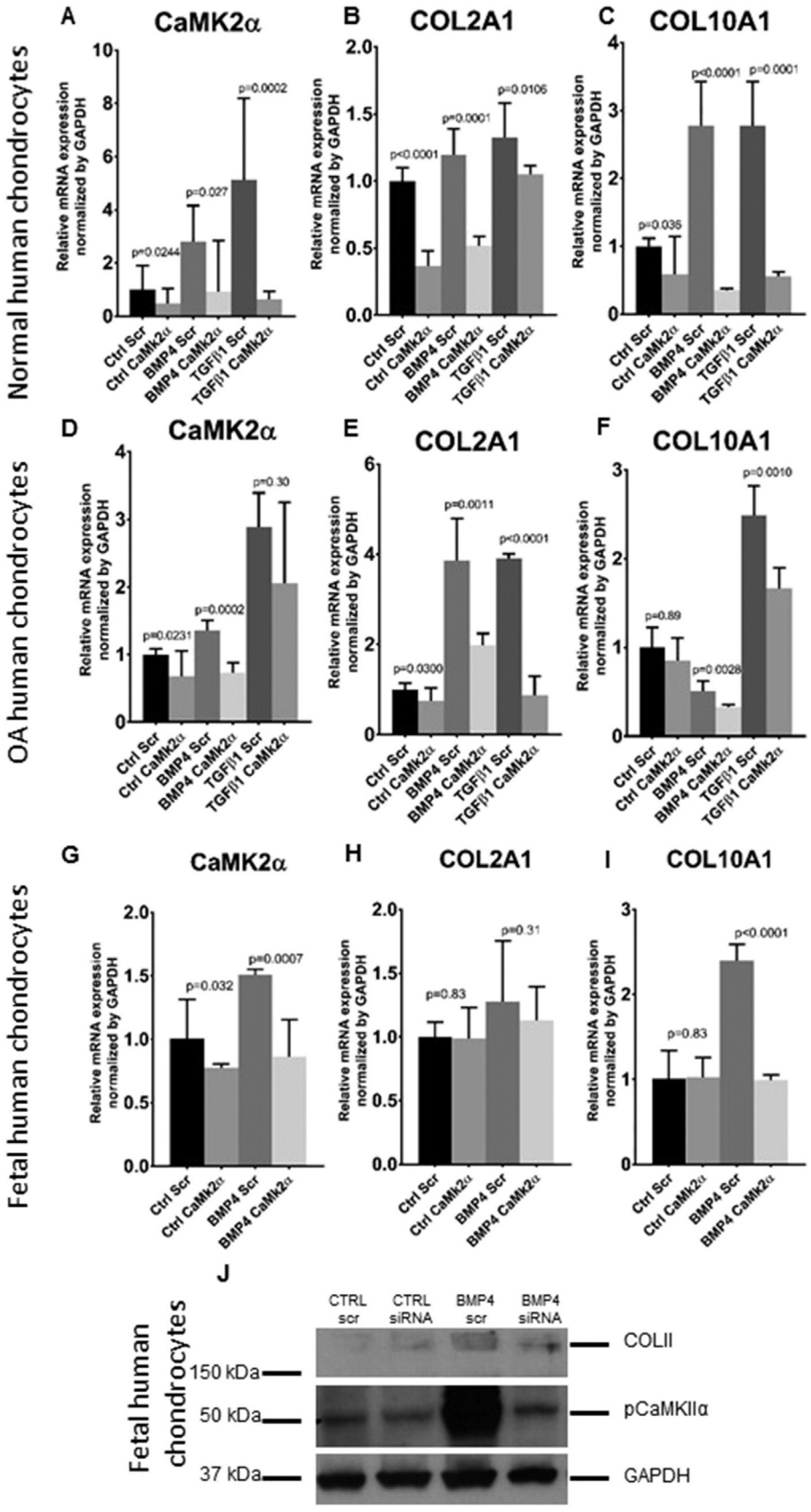

Fig. 7. *CaMK2α* knock-down by siRNA significantly decreases *COL2A1* and *COL10A1* expression in human adult normal, adult OA and in fetal chondrocytes. Effects of *CaMK2α* siRNA on gene expression in human normal (A–C), OA (D–F) and human fetal chondrocytes (G–I) transfected with scrambled (scr) or *CaMK2α*-specific siRNA (CaMK2α). Chondrocytes qPCR expression analysis of the *CaMK2α*, *COL2A1* and *COL10A1* mRNA expression was carried out 72 h after cell transfection and treatments with either BMP4 or TGFβ1. Bars represent data averaged from experiments performed in triplicate. Representative experiments are shown ($n = 3$). *Y*-axis values indicate fold differences, data represented as mean ± 95% confidence interval and analyzed using one-way ANOVA followed by Tukey test.

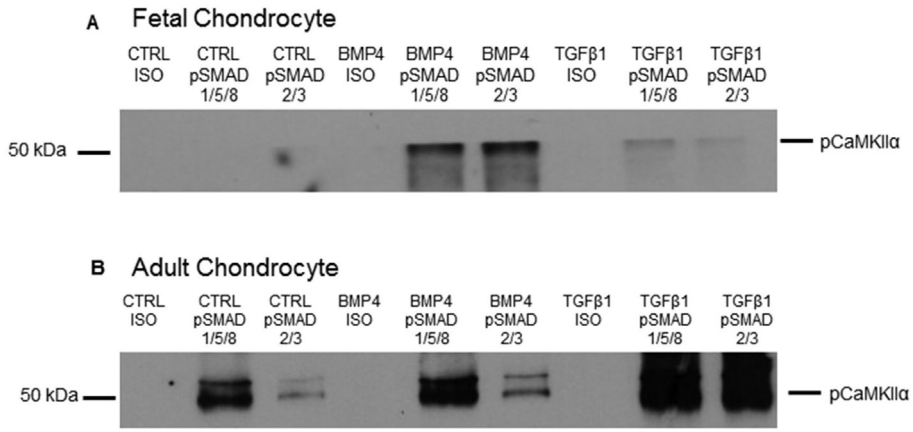

Fig. 8. Immunoprecipitation assay of fetal and adult chondrocytes treated with BMP4 and TGFβ1 using pSMADs and analyzed by Western blot for pCaMKIIα indicates strong physical interactions between SMADs and CaMKII. Western blotting for pCaMK2 expression after immunoprecipitation of fetal (A) and adult (B) chondrocyte lysates with antibodies against pSMAD2/3 or pSMAD1/5/8. Strong interaction between pCaMKIIα and both pSMADs was observed in fetal (A) chondrocytes after exposure to BMP4, while only a moderate increase in signal was observed in TGFβ1-treated cells relative to control. An strong increase in interaction between pCaMKIIα and pSMAD1/5/8 was detected in adult cells treated with BMP4 (B), while activation of pCaMK2 binding to pSMAD2/3 was less prominent. TGFβ1-treated adult chondrocytes (B) had stronger binding between the kinase and both SMADs compared to BMP4 treated groups (representative image shown, $n = 3$).

this study, we hypothesized that CaMKII is directly implicated in BMP- and TGFβ-mediated activation of SMAD1/5/8 in adult articular chondrocytes. We also predicted that CaMKII signaling controls terminal differentiation of PSC-derived chondrocytes and drives their transition to hypertrophic cells, which are not suitable for articular cartilage repair. We are reporting here, for the first time, that active CaMKII is upregulated in human OA cartilage and that activation of CaMKII in human articular chondrocytes can be induced by BMPs. We further show that inhibition of CaMKII activity with KN93 significantly decreases the expression of the *COL2A1* gene and COLII protein and also *COL10A1*/COLX in primary human articular, human fetal and PSC-derived chondrocytes in the presence of BMPs. We also report that inhibition of CaMKII in adult chondrocytes exposed to TGFβ1 results in further suppression of the chondrogenic program, while in immature primary fetal and PSC-derived chondrocytes, inhibition of CaMKII in the presence of TGFβ ligands enhances chondrogenic differentiation. Based on previously published studies[17,21], we speculate that differences in response reflect significant differences in TGFβ and CaMKII signaling between adult and immature chondrocytes.

Upregulation of CaMKII has recently been implicated in the pathogenesis of osteoarthritis (OA)[1] in mice. It has also been shown in mouse models of OA that pharmacological blockade of CaMKII with KN93 causes more severe cartilage damage relative to controls[22]. These results were not expected by the authors who aimed, based on their developmental studies, to limit OA by inhibiting effects of CaMKII signaling on hypertrophy of articular chondrocytes. Our *in vitro* data, using primary and PSC-derived chondrocytes, demonstrated that inhibition of CaMKII activity results in a strong decrease of not only hypertrophic (COLX), but all chondrogenic differentiation markers including SOX9, COLII and proteoglycan expression. We measured intracellular collagen protein expression that does not take into account the secreted portion, which could be a possible limitation of this analysis. However, both type II and type X collagens are homotrimeric molecules and are each regulated by one gene encoding the alpha1 chains specific for each of these collagens, making it less likely that there is a major impact on the data as noted. Moreover, we have previously found that analyzing the intracellular amount of type II collagen as detected by immunofluorescence does adequately reflect the portion secreted in the medium[20].

We also found that inhibition of CaMKII along with exposure to TGF-β is dissimilar in immature fetal and adult cells. Those stage-specific effects and the distinct responses of chondrocytes to CaMKII inhibition in the presence of either BMPs or TGFβ may explain the *in vivo* findings previously reported[22]. These findings support our hypothesis that CaMKII activity plays a crucial role not only in the regulation of the hypertrophic program (marked by e.g., COLX) as reported[3], but also during earlier stages of chondrocyte differentiation. Targeted inhibition of *CaMK2α* at the transcription level with siRNA yields similar results in human normal and OA chondrocytes. This further reinforces the specific effect of KN93 and AC3-I on pCaMKII inhibition.

SMADs promote chondrogenesis at every phase of cartilage development, including pre-chondrocyte, articular, proliferative, and hypertrophic stages[23]. The exact molecular mechanism of CaMKII-dependent modulation of BMP and TGFβ signaling in chondrocytes has only begun to be elucidated. Our study, along with others[12,13], demonstrated that CaMKII activation can phosphorylate SMAD2/3 and SMAD1/5/8 directly. However, only SMAD1/5/8 has been shown to receive activating phosphorylation events, while SMAD2/3 is inactivated by CaMKII. The short-term (15′, 30′, 1 h, 2 h) and long-term (7 days) treatments demonstrated how changes in the rapid phosphorylation of CaMKII and SMADs could also result in the regulation of expression and processing of target proteins (e.g., collagen type II and type X levels). We found that changes after short-term treatments (Figs. 2 and 3) were also maintained in the long term-treatments (Figs. 1, 4–7), allowing us to determine significant changes in collagen gene and protein expression even for collagen proteins with longer turnover times[17]. It is possible that CaMKII can elicit effects on SMAD signaling through filamin. Alli *et al.* demonstrated that CaMKII can phosphorylate, and thereby inactivate and release filamin from the cytoskeleton[24]. Filamin is one of several proteins that negatively regulates TGFβ and BMP signaling by anchoring SMADs to the cytoskeleton near the plasma membrane[25]. Recently, it has been reported that knocking out filamin B expression results in constitutive activation of TGF and BMP signaling in murine intervertebral discs, which markedly increased chondrocyte proliferation and hypertrophy[26]. Lastly, CaMKII has been documented to phosphorylate and inhibit nuclear activity of class II histone deacetylases (HDAC)[27], which negatively regulate BMP and TGFB target gene

expression[28,29]. Vega *et al.* demonstrated that knocking out HDAC4 results in premature ossification and chondrocyte hypertrophy through RUNX2 derepression. Conversely, the group showed that HDAC4 overexpression blocks chondrocyte differentiation and endochondral bone formation[30]. CaMKII signaling is very complex and could potentially contribute to SMAD-mediated chondrogenic gene expression through a variety of molecular interactions. The role of CaMKII in these signaling pathways should be further investigated to provide a deeper insight into chondrocyte development and the etiology of OA.

In conclusion, our work fills a gap in understanding the complex signaling of CaMKII during cartilage development, chondrocyte differentiation and OA. This knowledge will help to refine strategies for engineering functional articular chondrocytes from stem cells and can also provide an insight into the mechanisms of cartilage pathology. An understanding of these processes is essential for developing novel therapeutic approaches for patients with degenerative joint disease.

Author contributions

Conception and design: BS and DE; Analysis and interpretation of the data: BS, SL, JE, RS and DE; Drafting of the article: BS and DE; Critical revision of the article for important intellectual content: DE, BS; Final approval of the article: DE; Collection and assembly of data: SL, JE, BS and RS.

Conflict of interest

The authors declare no financial support or any other benefits from commercial sources received for the work reported on in the manuscript.

Role of the funding source

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Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.joca.2018.08.017.

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