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5-Azacytidine Alters TGF-β and BMP Signaling and Induces Maturation in Articular Chondrocytes

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Abstract Maintenance of the articular surface depends on the function of articular chondrocytes (ACs) which produce matrix and are constrained from undergoing the maturation program seen in growth plate chondrocytes. Only during pathologic conditions, such as in osteoarthritis, are maturational constraints lost causing recapitulation of the process that occurs during endochondral ossification. With the aim of establishing a model to identify regulatory mechanisms that suppress AC hypertrophy, we examined the capability of 5-azacytidine (Aza) to have an impact on the maturational program of these cells. Primary ACs do not spontaneously express markers of maturation and are refractory to treatment by factors that normally regulate chondrocyte maturation. However, following exposure to Aza, ACs (i) were induced to express type X collagen (colX), Indian hedgehog, and alkaline phosphatase and (ii) showed altered colX and AP expression in response to bone morphogenetic protein-2 (BMP-2), transforming growth factor-β (TGF-β), and parathyroid hormone-related protein (PTHrP). Since Aza unmasked responsiveness of ACs to BMP-2 and TGF-β, we examined the effect of Aza treatment on signaling via these pathways by assessing the expression of the TGF-β Smads (2 and 3), the BMP-2 Smads (1 and 5), and the Smad2 and 3-degrading ubiquitin E3 ligase Smurf2. Aza-treated ACs displayed less Smad2 and 3 and increased Smad1, 5, and Smurf2 protein and showed a loss of TGF-β signaling on the P3TP-luciferase reporter. Suggesting that Aza-induction of Smurf2 may be responsible for the loss of Smad2 and 3 protein via this pathway, immunoprecipitation and metabolic labeling experiments confirmed that Aza accelerated the ubiquitination and degradation of these targets. Overall, Aza-treated ACs represent a novel model for the study of mechanisms that regulate maturational potential of articular cartilage, with the data suggesting that maturation of these cells may be due to up-regulation of Smad1 and 5 coupled with a Smurf2-dependent degradation of Smad2 and 3 and loss of TGF-β signaling. J. Cell. Biochem. 92: 316–331, 2004.

Key words: articular chondrocyte; TGF-β; BMP; Smad2; Smad3; Smurf2; 5-azacytidine

The principle function of articular cartilage is to provide a low friction load-bearing surface that facilitates free movement of joints. Maintenance of this surface depends on the maturational arrest of chondrocytes before terminal hypertrophic differentiation occurs [Bohme et al., 1995; Serra et al., 1997, 1999; Lotz et al., 1999]. In contrast to endochondral ossification which involves a programmed process of chondrocyte maturation culminating in terminal hypertrophy and mineralization [Wright et al., 1995], articular chondrocytes (ACs) are constrained from completing the maturational program as evidenced by a lack of type X collagen (colX) and alkaline phosphatase expression [Sandell and Aigner, 2001; Sztrolovics et al., 2002]. Also, ACs are not responsive to factors that impact the maturational process, including bone morphogenetic protein-2 (BMP-2), a potent stimulator of chondrocyte maturation [Sailor et al., 1996]. Factors that constrain AC maturation are only relieved under unique circumstances such as in osteoarthritis, where proliferation and an increase in the expression of hypertrophic hallmarks indicates that the

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cells have differentiated into a mature phenotype [von der Mark et al., 1992; Pullig et al., 2000]. Osteoarthritis may thus involve the functional loss of mechanisms that arrest articular cartilage differentiation. These mechanisms are yet to be characterized because ACs do not spontaneously mature in culture, nor do they respond to factors that alter the maturation program.

In order to study the molecular constraints that prevent differentiation of ACs, a novel culture model was developed that utilizes the cytidine analog 5-azacytidine (Aza) to alter the regulation of gene expression. The action of Aza, which is used as an anti-tumor agent in the treatment of childhood leukemia [Leonhardt and Cardoso, 2000], is based on its ability to block methylation of DNA by replacing cytidine bases during replication. Aza un-masks the expression of genes that drive maturation in a number of model systems because an underlying mechanism that constrains the progression of cellular differentiation involves methylation-dependent suppression of genes that drive maturation [Taylor, 1993]. During normal progression through the cell cycle, genes with hypomethylation of cytidine bases in their promoter are actively transcribed while hypermethylated genes, which contain a large population of methylated cytidines (CpG islands), are transcriptionally repressed [Cedar and Razin, 1990; Ng et al., 1999; Leonhardt and Cardoso, 2000; Wade, 2001]. For example, blockade of DNA methylation in C3H10T1/2 mesenchymal cells by Aza un-masks the potential to differentiate into either myoblastic, adipositic, or chondrocytic cell types [Konieczny and Emerson, 1984]. A similar un-masking of maturational potential in ACs with Aza would, for the first time, facilitate the identification of genes that regulate maturational progression. In particular, identification of un-masked genes that can over-ride maturational suppression in ACs may be possible in this novel model system.

Chondrocyte maturation is regulated by a few key growth factors and their associated downstream signaling pathways. One of the most potent regulatory systems in chondrocytic cell types involves signaling via receptors for transforming growth factor-β (TGF-β) and the BMPs [Ballock et al., 1993; Grimsrud et al., 2001]. These receptors exert their effects via a shared constellation of transcription factors called the Smads, the details of which have been reviewed [Miyazono, 2000]. Smads are a family of intracellular proteins that comprise three classes of signaling molecules: receptor-associated Smads (2 and 3 for TGF-β; 1, 5, and 8 for the BMPs), the co-factor Smad4, and the inhibitory Smads (6 and 7) [Heldin et al., 1997; Ishisaki et al., 1999; Massague et al., 2000a; Massague and Chen, 2000b]. The receptor-associated Smads are phosphorylated following receptor activation, form heteromeric complexes with the co-factor Smad4 and translocate to the nucleus where they directly influence gene transcription [Massague et al., 1997]. Besides via Smad6 and 7, negative regulation of TGF-β/BMP signaling is facilitated via the Smad ubiquitination regulatory factors, commonly referred to as Smurf1 and Smurf2. Smurf1 has been found to interact with BMP-activated Smad1 and Smad5, thereby triggering their ubiquitination and degradation [Zhu et al., 1999]. Smurf2 has been shown to also target Smad1 for degradation while possessing additional activity toward Smad2 [Badger et al., 2000; Zhang et al., 2001]. Another target in this signaling cascade is the type I TGF-β receptor, which is targeted for degradation by both Smurf1 and Smurf2 in a Smad7-dependent manner [Kavsak et al., 2000; Ebisawa et al., 2001]. Since cartilage formation and maturation is strongly linked to the balance of TGF-β and BMP signaling, these actions of the Smurfs as negative regulators may be central in the control of the maturational program in chondrocytes.

In this study, we characterized the phenotype of ACs that were treated with Aza to evaluate the potential of this system as an in vitro model of AC maturation. Our findings indicate that treatment of ACs with Aza enables the expression of maturational hallmarks including colX, alkaline phosphatase, and Indian hedgehog (Ihh). Further, Aza-treated cells become responsive to factors that regulate the hypertrophic program, including parathyroid hormone-related protein (PTHrP), TGF-β, and BMP-2. Our findings also suggest that stimulation of maturation following Aza treatment is at least partially dependent on the un-masking of genes that promote BMP signaling, Smad1 and 5 and the ubiquitin E3 ligase that mediates degradation of the TGF-β Smads, Smurf2. We propose that the net result is a shift of regulatory dominance from a suppression of maturation via TGF-β signaling to an acceleration of maturation via BMP-2 signaling. Overall, this model

will facilitate a comprehensive characterization of the role of these signaling pathways in the regulation of AC maturation and will directly impact our understanding of the progression of these cells to hypertrophy, such as in osteoarthritis.

MATERIALS AND METHODS

Growth Factors and Molecular Reagents

BMP-2 was a gift from Wyeth, PTHrP was purchased from Bachem AG, and TGF-β1 was purchased from R&D Systems. Wild type and Dominant Negative Smad1, Smad2, and Smad3 were a gift from Dr. R. Derynck [Zhang et al., 1996], and were subcloned into the mammalian expression vector pCMV [Schwarz et al., 1996]. A 520 base pair double stranded cDNA fragment from exon 1 of Ihh was provided by Dr. C.J. Tabin [Vortkamp et al., 1996]. The flag-tagged Smurf2 mammalian expression plasmid was provided by Dr. J. Wrana (University of Toronto). The TGF-β responsive p3TP-Lux reporter construct was a gift from Dr. J. Massague [Wrana et al., 1992]. Expression of mRNAs for colX, Ihh, and GAPDH were analyzed by real time reverse transcription PCR (real time RTPCR) using the following primer sets. ColX: left primer—5′-acatgcatttacaaatatcgttac-3′; right primer—5′-aaaatagtagacgttaccttgactc-3′. Ihh: left primer—5′-ctgctatttgtgtgtgtgt-3′; right primer—5′-gtacaaggctctggtttg-3′. GAPDH: left primer—5′-tatgatgatatcaagagggtagt-3′; right primer—5′-tgtatccaaactcattgtcatac-3′.

Chondrocyte Isolation

Chick articular and growth plate chondrocytes were isolated from the femora and tibiae of 4–6 week old *Gallus domesticus* chickens as previously described [Crabb et al., 1990; Pateder et al., 2000]. After sacrifice with CO_2, the skin and soft tissue surrounding the distal femur and proximal tibia were aseptically removed. Thin slices of the articular cartilage were shaved from the articular surface with a scalpel and were placed in modified F12 medium that was magnesium free, and contained 0.5 mM $CaCl_2$ (F12, Sigma, St. Louis, MO). Following harvest of articular cartilage, the remaining articular surface was removed to expose growth plate tissue which was also harvested with a scalpel and placed in a separate F12 bath. Shavings from articular and growth plate cartilage were processed identically: cartilage shavings were rinsed twice in F12 and then incubated for 30 min in F12 containing 5 mg/ml trypsin (Sigma) at 37°C. The cartilage was rinsed a second time with fresh F12 and digested for 1 h at 37°C with 5 mg/ml hyaluronidase (Sigma, type 1-S) in F12. The cartilage was rinsed again in fresh F12 and was digested overnight at 37°C in F12 containing 5 mg/ml collagenase type IA (Roche, Indianapolis, IN). The cells were then filtered through a 400 μM mesh, centrifuged for 5 min at 600*g*, and washed three times with F12. Articular and growth plate chondrocytes were then re-suspended separately in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Carlsbad, CA) and counted with a hemocytometer.

Cell Culture

Growth plate chondrocytes were plated at a density of 5×10^6 cells/100 mm dish in DMEM containing 5% fetal bovine serum (FBS, Invitrogen). After 24 h in culture, growth plate chondrocytes were harvested for isolation of mRNA.

Chick ACs were cultured in DMEM containing 5% FBS at a density of 1×10^6 cells/100 mm dish or at 0.5×10^6 cells per well of a 6-well plate or per 60 mm dish and were treated as described below. Three hours after plating, half of the cultures were supplemented with 15 μg/ml Aza (Sigma) and both the treated and control groups were incubated for 48 h as previously described [Cheung et al., 2001]. After this treatment, all cultures were rinsed with fresh DMEM and were maintained in DMEM + 5% FBS supplemented with 5 mg/ml L-ascorbic acid (Sigma). Medium was replaced every 48 h. ACs plated in 6-well plates were used for assays involving transient transfection, alkaline phosphatase activity determination, and immunoprecipitation. Cells plated in 100 mm dishes were used for Northern and Western analyses and real-time PCR. Cells plated in 60 mm dishes were used for pulse chase experiments protein degradation studies.

Northern Analysis

RNA extraction was performed from cultures using the RNAeasy kit (Qiagen, Valencia, CA). Ten micrograms aliquots of total RNA were denatured, run on a 1.2% agarose gels containing 17.5% formaldehyde, transferred to a GeneScreen Plus nylon membrane (PerkinElmer Life Sciences, Boston, MA) and UV cross-linked

to the membrane. Analysis of colX expression was performed using a synthetic type X oligonucleotide probe that was end labeled with ^{32}P as previously described [Grimsrud et al., 2001]. All blots were pre-hybridized in QuickHyb solution (Stratagene, La Jolla, CA) for 20 min at 72°C. Probes were added directly to the pre-hybridization solution and then blots were hybridized at 68°C for 1 h. Blots were washed twice for 15 min with 2× SSC + 0.1% SDS, washed once for 30 min with 0.1× SSC + 0.1% SDS, and exposed to X-Omat AR film (Kodak, Rochester, NY).

Western Analysis

After rinsing the cell layer with phosphate-buffered saline (PBS), protein was extracted from AC cultures using Golden lysis buffer containing protease inhibitor cocktail tablets (Roche Molecular Biochemicals, Indianapolis, IN) as previously described [Samuels et al., 1993]. The lysate was centrifuged at 12,000*g* and insoluble material was removed. Protein concentration of the soluble material was determined by using the Coomassie Plus Protein Assay kit (Pierce Chemical, Rockford, IL). One hundred micrograms aliquots of protein extract were separated by SDS–PAGE (10% polyacrylamide) and then transferred to a nitrocellulose membrane (Schleider and Schuell, Keene, NH). The blots were probed overnight at 20°C with the following antibodies: goat-anti-human Smad1, 2, and 3 polyclonals (Santa Cruz, Santa Cruz, CA), goat-anti-mouse Smad5 polyclonal (Santa Cruz), a rabbit-anti-mouse actin monoclonal (Sigma), or a goat-anti-rabbit Smurf2 monoclonal (Upstate Biotechnology, Lake Placid, NY), all at a 1:3,000 dilution. Blots were further incubated for 1 h at 20°C in the presence of horseradish peroxidase-conjugated secondary antibodies against rabbit (Sigma), mouse (BioRad, Hercules, CA) or goat (Santa Cruz), also at a dilution of 1:3,000. The immune complexes were detected using ECL-Plus (Amersham Pharmacia Biotech, Piscataway, NJ) and visualized following exposure to X-OMAT AR film (Kodak).

Transient Transfection

ACs plated in 6-well plates were transiently transfected on day 8 after isolation. All transient transfections were carried out in DMEM using the commercially available reagent Mirrus TransIT-LT1 (Panvera Corporation, Madison, WI) according to the manufacturers instructions. The DNA to transfection reagent ratio used for all experiments was 1:3 (w/w).

Alkaline Phosphatase Activity

Alkaline phosphatase activity in AC cultures was measured using a previously described technique which measures cellular conversion of *p*-nitrophenyl phosphate to *p*-nitrophenol [Grimsrud et al., 2001]. Briefly, the medium was aspirated from the culture wells and the cells were rinsed once with 150 mM NaCl. After aspirating the rinse solution, a reaction buffer containing 0.25 M 2-methyl-2-aminopropanol, 1 mM $MgCl_2$, and 2.5 mg/ml *p*-nitrophenyl phosphate (pH 10.3) was added to the wells. After 30 min at 37°C, the reaction was stopped by the addition of 0.5 ml of 0.3 M Na_3PO_4 (pH 12.3). Alkaline phosphatase activity was determined by measuring the absorbance at 410 nm using a spectrophotometer. A standard curve was constructed by measuring the 410 nm absorbance of several standard solutions of *p*-nitrophenol.

cDNA Synthesis and Real Time RTPCR

Total RNA was extracted from cultures using the RNAeasy kit (Qiagen) and the recovered RNA was treated with RNase-free DNase I (Invitrogen) at 37°C for 30 min. One microgram aliquots of RNA were then reverse transcribed into cDNA using the Advantage RT-for-PCR Kit (Clontech, Palo Alto, CA). Before real-time PCR, the cDNA samples were further treated with RNase (Invitrogen) at 37°C for 20 min and then were diluted five-fold prior to PCR amplification.

Real-time PCR was performed using the Rotor Gene real-time DNA amplification system (Corbett Research, Westborough, MA) according to the manufacturer's instructions. Reactions were performed in a 25 μl volume using a 0.5 μM final concentration of primers of interest. SYBR green PCR master mix (Applied Biosystems, Foster City, CA), which included dNTP's, Taq polymerase, SYBR Green and buffers for PCR amplication, was used to amplify target cDNAs. The PCR protocol included an initial 5 min 94°C denaturation step followed by 35 cycles of 94°C denaturation for 20 s, 45–47°C annealing (primer dependent) for 20 s and 68°C extension for 30 s. Detection of the fluorescent product was carried out at the end of the 68°C extension period. PCR products

generated from each pair of primers were subjected to a melting curve analysis and agarose gel electrophoresis. Detection of the fluorescent product was carried out at 5 s intervals as the temperature ramped up from 50 and 90°C (i.e., prior to each denaturation). The data was analyzed and quantified with the RotorGene analysis software as directed by the manufacturer. Dynamic tube normalization and noise slope correction were used to remove background fluorescence. In order to elucidate a standard curve, we performed real-time PCR analysis on dilutions of GAPDH template DNA ranging from 10^1 to 10^9 copies [Simpson et al., 2000]. The GAPDH template was generated by using chicken GAPDH primers to amplify a PCR product that was purified using Concert Rapid PCR Purification System (Life Technologies, Gaithersburg, MD) and quantified spectrophotometrically.

Metabolic Labeling and Immunoprecipitation

ACs plated in 100 mm dishes (70% confluence) were lifted from the plate with trypsin and split 1:5 into 60 mm dishes and given fresh DMEM + 5% FBS. Twenty four hours after replating, cells were transfected with Smad2 and 3 (flag-tagged) pCMX plasmids of interest. Forty eight hours after transfection, cells were washed twice in PBS and incubated for 1 h at 37°C with 3 ml of cysteine- and methionine-free DMEM (Cellgro, Herndon, VA). Following this, cells were pulsed with 0.25 mCi/ml of ^{35}S-methionine (Amersham) for an additional 1 h at 37°C. The cells were washed twice with normal DMEM and then chased with DMEM + 5%FBS for various periods up to 120 min. The chase was terminated by lysing the cells with RIPA buffer (20 mM Tris, 100 mM NaCl, 0.2% Triton X-100, 0.2% NP40, 0.2% DOC. Flag-tagged Smad2 and Smad3 protein was immunoprecipitated from these extracts using a 1:200 dilution of a mouse anti-flag M2 monoclonal antibody (Sigma) covalently bound to protein A/G sepharose beads (Amersham Pharmacia Biotech). Pelleted beads were washed once in RIPA buffer and bound proteins were eluted in gel-loading buffer and separated by electrophoresis on SDS–10% PAGE gels. Dried gels were exposed to X-OMAT AR film (Kodak) and the relative intensity of the visualized Smad2 and 3 bands was quantitated using NIH image (version 1.6).

For studies aimed at visualizing ubiquitinated forms of Smad2 and 3, the HA-tagged ubiquitin pCMX plasmid was co transfected into ACs along with the Smad2 and 3 plasmids. Forty eight hours post-transfection, immunoprecipitations were performed as described above for the pulse chase experiments and pulled-down proteins were electrophoresed on SDS–10% PAGE gels. Western analyses were performed as described previously using a 1:200 dilution of a mouse-anti-HA monoclonal antibody (Sigma) as a primary antibody and a 1:3,000 dilution of an anti-mouse secondary antibody.

Statistical Analysis

Numerical data are presented as mean ± standard error ($N \geq 3$) and statistical significance between groups was identified using the two-tailed student's *t*-test (*P* values are reported in the figure legends).

RESULTS

Aza Induces Maturation in ACs

In vitro models that support the expression of hypertrophic markers by ACs have not yet been established. To elucidate the molecular mechanisms that drive inappropriate AC maturation during pathologic conditions (such as during osteoarthritis), we developed a novel culture system using the cytidine analog Aza to unmask hypertrophic potential in chick ACs. We initially tested the efficacy of Aza to induce maturation by treating with 15 μg/ml Aza for 5 or 10 days and then evaluating the expression of colX. ColX mRNA expression was increased at both the 5 and 10 day time point as measured by Northern analysis (Fig. 1A), indicating the ability of Aza to induce maturation in chick ACs. This correlated with the robust expression of colX by growth plate chondrocytes, a positive control for this experiment. These findings suggest that chick ACs can be induced to mature beyond their quiescent, matrix-producing, immature state by treatment with Aza.

Using real-time RTPCR and Northern analysis to measure colX gene expression, the maturational effects of PTHrP and TGF-β, two suppressors of chondrocyte maturation, and BMP-2, a stimulator of maturation, were evaluated. As expected, compared to the stimulatory effect of Aza, PTHrP, TGF-β, and BMP-2 did not affect colX mRNA expression in chick ACs (Fig. 1B,C). Additionally, the effect of Aza, PTHrP, TGF-β, and BMP-2 on alkaline phosphatase activity was determined. In agreement

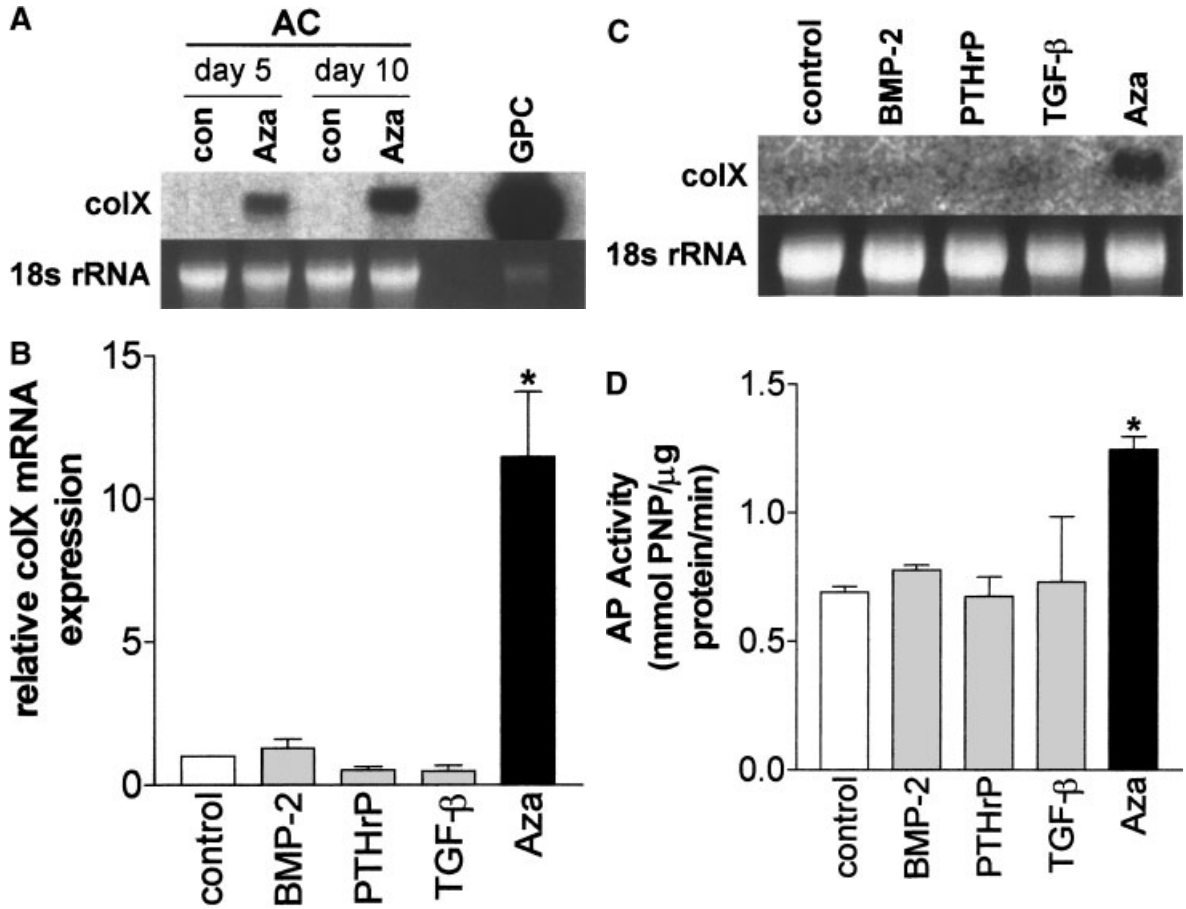
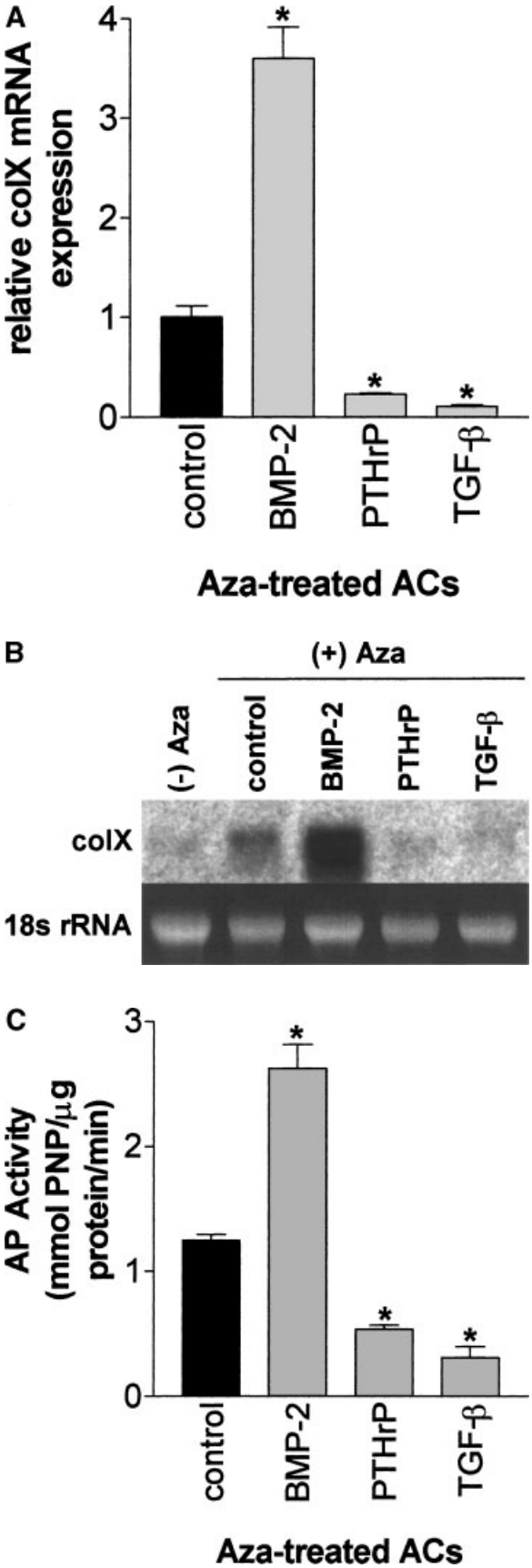


Fig. 1. 5-Azacytidine (Aza) induces a hypertrophic phenotype in articular chondrocytes (ACs). **A**: ACs were treated with 15 μg/ml Aza for 48 h and then were maintained in culture for 5 or 10 days. RNA was extracted with the Qiagen RNAeasy kit and Northern analysis of colX expression in control and Aza-treated cultures was performed. Ethidium bromide staining of the gel prior to transfer was performed to detect the 18s rRNA as a load control. Growth plate chondrocytes (GPC), which are terminally differentiated in culture, provide a positive control for colX expression. **B**: ACs were treated for 48 h with DMEM + 5% FBS (control) or with similar medium supplemented with 15 μg/ml Aza, 50 ng/ml BMP-2, 100 nM PTHrP or 5 ng/ml TGF-β. RNA was extracted as in (A) and colX mRNA levels were determined using real time RTPCR. **C**: ACs were treated as described in (B) and colX mRNA levels were assessed via Northern analysis. The 18s rRNA band was used as a load control. **D**: ACs were treated as described in (B), cell extracts were prepared and alkaline phosphatase activity was determined. The Northern blots shown in (A) and (C) are representative of three separate experiments. Error bars in (B) and (D) represent the standard error of the mean and significance from control is denoted with an asterisk ($P < 0.05$).

with the colX results, Aza stimulated while PTHrP, TGF-β, and BMP-2 were without effect on alkaline phosphatase activity (Fig. 1D). These findings further demonstrate the efficacy of Aza to induce maturational progression in ACs and confirms the concept that key regulators of chondrocyte hypertrophy do not affect these cells.

Besides inducing maturation, Aza was also found to un-mask growth factor responsiveness in chick ACs. Following treatment with 15 μg/ml Aza for 2 days, not only did the cells begin to show colX expression and increased alkaline phosphatase activity, but regulation of these responses was observed. Specifically, PTHrP and TGF-β inhibited while BMP-2 enhanced colX expression (Fig. 2A,B) and alkaline phosphatase activity (Fig. 2C) in Aza-treated cells. Whereas growth factors are typically unable to influence maturation of ACs, these findings suggest that Aza eliminates the silencing of maturational constraints, thus un-masking responsiveness to factors that regulate maturation.

Correlating with the effect of Aza on colX and alkaline phosphatase activity, expression of Ihh, another hallmark of maturation, was significantly up-regulated by Aza in chick ACs. Aza-treated cells displayed a significant induction of Ihh mRNA expression relative to control as determined by Northern (Fig. 3A)



and real-time RTPCR (Fig. 3B). Although not nearly as robust, the Aza effect correlated with basal Ihh expression seen in more mature growth plate chondrocytes (Fig. 3A). As was the case with colX expression and alkaline phosphatase activity, PTHrP and BMP-2 did not induce significant Ihh expression unless the cells were pre-treated with Aza. Following Aza treatment, PTHrP inhibited and BMP-2 stimulated Ihh mRNA relative to the Aza-treated group (Fig. 3B). These findings support our hypothesis that treatment of ACs with Aza alleviates molecular constraints that prevent these cells from achieving terminal maturation.

Effects of Aza on TGF-β/BMP Signaling

Due to the strong dependence of chondrocyte maturation on the balance between TGF-β and BMP signaling, we predicted that ACs treated with Aza express maturational hallmarks due to Aza-induced alterations in Smad signaling. To test determine if this is a plausible idea, we compared the ability of Aza to stimulate colX mRNA expression with gain and loss of Smad function models including over-expression of wild type and dominant negative (Δ) forms of Smad1, 2, and 3. As measured by real time RTPCR, colX induction by Aza was mimicked by Smad1, ΔSmad2, ΔSmad3, and Smurf2, while Smad2 and 3 inhibited expression (Fig. 4). These findings demonstrate that alterations in TGF-β/BMP signaling can indeed impact the maturational potential of ACs, raising the possibility that the mechanism of Aza action may therefore involve alterations in TGF-β/BMP/Smad signaling.

Based on these findings, we hypothesized that ACs treated with Aza express maturational hallmarks due to an Aza-induced shift in signaling dominance from TGF-β to BMP. Specifically, if Aza induces maturation in ACs

Fig. 2. Aza un-masks AC responsiveness to BMP-2, PTHrP, and TGF-β. **A**: ACs were treated with 15 μg/ml Aza for 48 h and then incubated for an additional 48 h in DMEM + 5% FBS (control) or similar medium containing 50 ng/ml BMP-2, 100 nM PTHrP or 5 ng/ml TGF-β. RNA was extracted with the Qiagen RNAeasy kit and colX mRNA levels were determined using real time RTPCR. **B**: ACs were treated as described in (A) and colX mRNA levels were assessed via Northern analysis. The 18s rRNA band was used as a load control. **C**: ACs were treated as described in (A), cell extracts were prepared and alkaline phosphatase activity was determined. The Northern blot shown in (B) is representative of three separate experiments. Error bars in (A) and (C) represent the standard error of the mean and significance from control is denoted with an asterisk ($P < 0.01$).

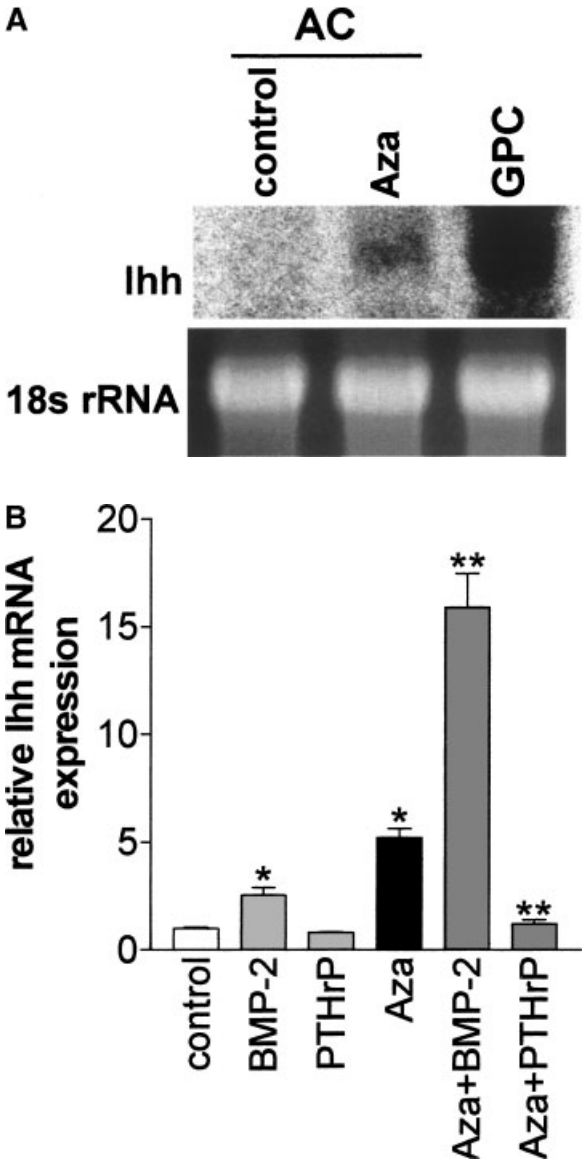


Fig. 3. Aza stimulates Ihh expression in ACs. **A**: ACs (AC) were treated with 15 μg/ml Aza for 48 h and then were maintained in culture for 8 days. RNA was extracted with the Qiagen RNAeasy kit and Northern analysis of Ihh expression in control and Aza-treated cultures was performed. The 18s rRNA was used as a load control. Growth plate chondrocytes, which are terminally differentiated in culture, provide a positive control for Ihh expression. **B**: ACs were treated for 48 h with DMEM + 5% FBS (control) or with similar medium supplemented with 15 μg/ml Aza, 50 ng/ml BMP-2, 100 nM PTHrP or with 15 μg/ml Aza in combination with 5 ng/ml BMP-2 or 100 nM PTHrP. RNA was extracted as in (A) and Ihh mRNA levels were determined using real time RTPCR. The Northern blot shown in (A) is representative of three separate experiments. Error bars in (B) represent the standard error of the mean; significance from control is denoted with an asterisk ($P < 0.01$) and significance from the Aza-treated group is denoted with a double asterisk ($P < 0.01$).

by altering Smad signaling, expression and function of the various Smads should be a target of Aza action. Suggesting this to be the case, Western analyses of Aza-treated ACs showed a distinct pattern of protein expression, with the TGF-β Smads (2 and 3) showing reduced expression and the BMP Smads (1 and 5) showing significantly increased expression (Fig. 5A). Also, Smurf2 protein levels were increased by Aza, suggesting that besides inducing BMP signaling via up-regulation of Smad1 and 5 protein, Aza may induce loss of TGF-β signaling due to increased degradation of Smad2 and 3.

If Aza induces loss of TGF-β signaling by enhancing degradation of Smad2 and 3, it should mimic the action of Smurf2 by blocking TGF-β-induced signaling on the P3TP-luciferase promoter/reporter. Consistent with this idea, in P3TP-luciferase transfected ACs, Aza not only reduced basal signaling, but it caused a greater than 50% reduction in TGF-β-induced promoter activation (Fig. 5B). This was comparable to the loss of signaling under both basal and TGF-β-stimulated conditions in cells transfected with the human Smurf2 expression plasmid (Fig. 5B). This finding suggests that Aza-induced degradation of Smad2 and 3 is sufficient to inhibit TGF-β signaling and raises the possibility that Aza facilitates maturational progression in ACs by alleviating the suppressive effect of TGF-β on these cells.

Since Smad2 and 3 protein levels were reduced in Aza-treated chick ACs, we proceeded to confirm that the reduced protein levels were a result of enhanced degradation that may be Smurf2-dependent. To test this possibility, Western analyses and a pulse-chase method were employed to measure ubiquitination and half life of Smad2 and 3 protein in Aza-treated cells. In the first experiment, control and Aza-treated cells were co-transfected with HA-tagged ubiquitin and either flag-tagged Smad2 or Smad3. Flag-tagged Smad2 and 3 proteins were pulled down from cell extracts and Western analyses were performed using an HA antibody to detect ubiquitin conjugated Smad targets. Consistent with the idea that Aza-induced Smurf2 function leads to enhanced degradation of Smad targets, Aza enhanced the ubiquitination of both Smad2 and 3 targets relative to the control group (Fig. 6A). In the second experiment, control and Aza-treated cultures were transfected with flag-tagged Smad2 or 3 and the half-life of these proteins was determined via a

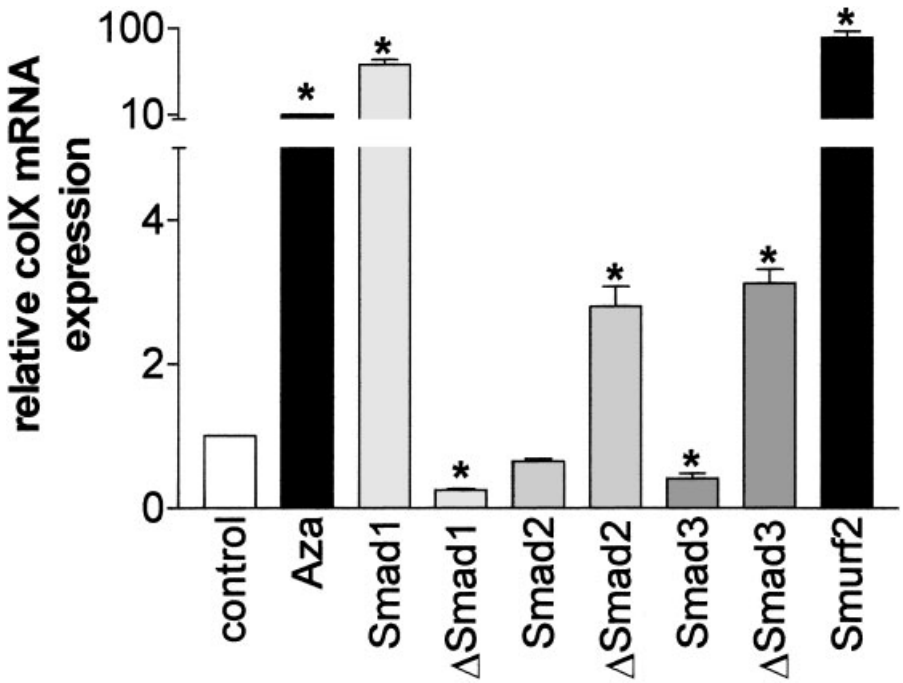


Fig. 4. Over-expression of Smad1, ΔSmad2, ΔSmad3, and Smurf2 mimics Aza stimulation of colX in ACs. ACs were transiently transfected with empty vector or with plasmids encoding Smad1, DSmad1, Smad2, DSmad2, Smad3, DSmad3 or Smurf2. Empty vector-transfected cells were either un-treated (control) or were pre-treated with 15 μg/ml Aza for 48 h prior to transfection (Aza). Forty eight hours post-transfection, RNA was extracted with the Qiagen RNAeasy kit and real time RTPCR analysis for colX was performed. Error bars represent the standard error of the mean and significance from control is denoted with an asterisk ($P < 0.01$).

metabolic labeling approach. Following pulse-chase with ^{35}S-methionine, flag-tagged Smad2 or 3 were pulled down from cell extracts, run on SDS–PAGE gels and analyzed by autoradiography. Compared to control, Aza-treated ACs showed more rapid degradation of Smad2 and 3 as evidenced by the shorter half life of both proteins relative to control cultures (Fig. 5B,C). Overall, these findings provide direct evidence that besides up-regulating Smad1 and 5, Aza induces expression of Smurf2 protein (Fig. 4A) and a subsequently increased rate of Smad2 and 3 degradation.

DISCUSSION

Articular cartilage and cartilages that undergo mineralization (e.g., growth plate) are comprised of distinct chondrocytic cell types that are biochemically/genetically distinguishable even though they originate from a common chondroprogenitor cell. For example, ACs, which can be termed 'maturationally arrested,' primarily act to maintain the extracellular matrix by expressing collagen types II, VI, IX, and XI and aggrecan [Buckwalter and Mankin, 1997]. While these cells are metabolically active regarding matrix turnover, they do not proliferate significantly. Comparatively, mineralizing cartilage is composed of chondrocytes that surpass this maturational stage as evidenced by accelerated proliferation, increased cell volume and expression of other phenotypic markers including colX and alkaline phosphatase [Buckwalter and Mankin, 1997], BMP-6 [Grimsrud et al., 1999; Anderson et al., 2000], PTHrP and its receptor [Amizuka et al., 1994; Lee et al., 1995], matrix metalloproteinase-1, 3, 9 and 13 [Kawashima-Ohya et al., 1998; D'Angelo et al., 2000], and Ihh [Lanske et al., 1996; Vortkamp et al., 1996]. Also in contrast to ACs, terminally mature cells ultimately apoptose [Horton et al., 1998]. Only under pathological situations, such as in osteoarthritis, do ACs overcome maturational arrest to present hypertrophic phenotypes [von der Mark et al., 1992; Pullig et al., 2000; Sandell and Aigner, 2001]. In vitro work to examine the mechanisms that constrain AC maturation is limited by the fact that these cells do not spontaneously mature in culture

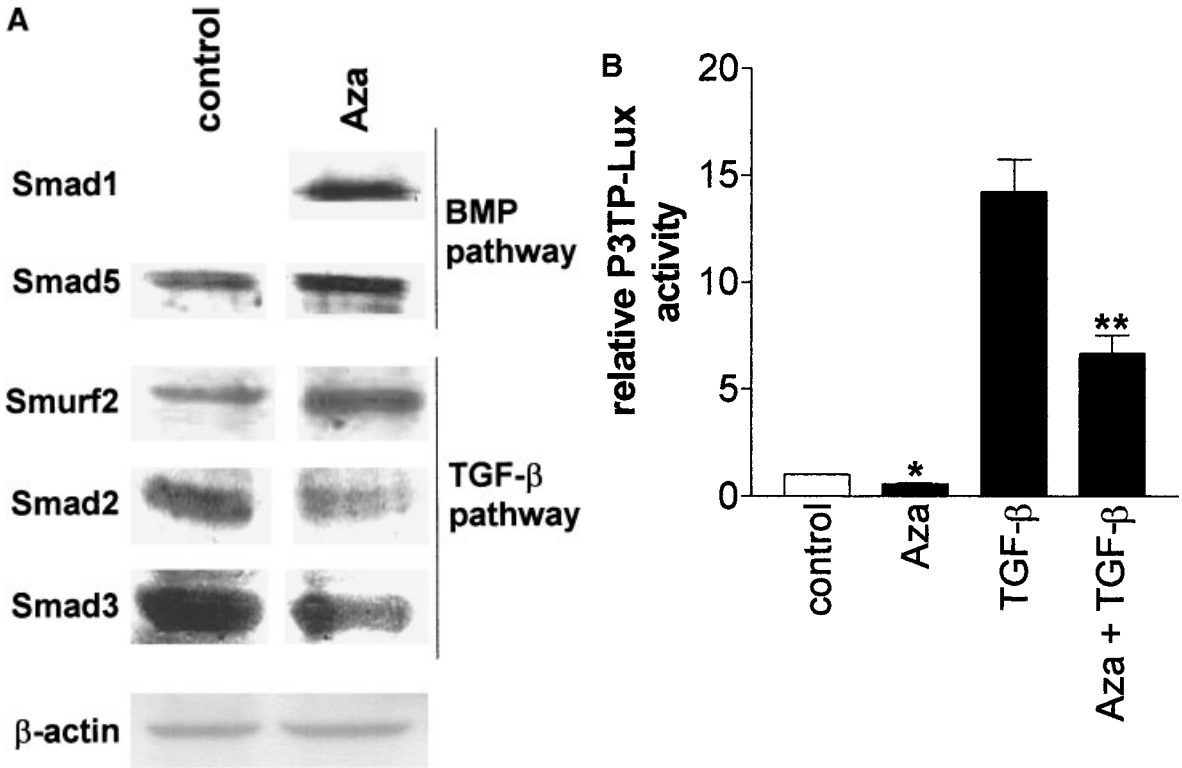


Fig. 5. Smad and Smurf2 protein expression ± Aza and inhibition of basal P3TP-Luc activity by Aza and Smurf2. **A**: ACs were treated with 15 μg/ml Aza for 48 h and then were maintained in culture for 8 days. Protein extracts were prepared from cultures and analyzed via Western analysis for Smad1, 2, 3, 5, and Smurf 2 protein expression in control and Aza-treated cultures. β-actin was used as a loading control. Northern blots shown in (A) and Western blots shown in (**B**) depict representative findings from three separate experiments. B: ACs transfected with the TGF-β-responsive P3TP-Luc promoter/reporter were either transfected with Smurf2, treated with 15 μg/ml Aza for 48 h, or were left untreated (control). Cultures were then treated with and without 5 ng/ml TGF-β for 24 h. Cellular extracts were prepared and luciferase activities were determined for each group as described in ''Materials and Methods.'' Error bars represent the standard error of the mean and significance from control is denoted with an asterisk ($P < 0.01$).

(as mineralizing chondrocytes do) and do not respond to factors that drive maturation. Without a model system to study the mechanisms responsible for suppression of AC maturation, little progress has been made to understand the underlying molecular constraints that are lost during inappropriate maturation of these cells.

The main goal of the study described in this report was to establish a culture model that would facilitate the study of mechanisms that suppress or drive AC maturation. A previously established method to induce commitment/maturation of pluripotent cells involves treatment with Aza. Aza, which replaces cytidine bases in genomic DNA during replication, cannot be methylated by DNA methyltransferases and thus perturbs the methylation pattern of cytidines (CpG islands) present in various target gene promoters. Under normal conditions, repression of hypermethylated genes primarily occurs via transcription factor competition with methyl-CpG binding proteins for binding to regulatory sites in the DNA [Ng et al., 1999; Wade, 2001]. Thus, removing the methylated CpG targets via Aza treatment facilitates transcription factor access to previously occluded sites in the DNA, relieving transcriptional repression.

Since preservation of the normal pattern of methylation is crucial for appropriate mammalian development [Leonhardt and Cardoso, 2000], Aza represents a potent modulator of the developmental process. As such, Aza has been useful as a mechanistic probe of differentiation in several primary culture systems including mouse embryo fibroblasts [Constantinides et al., 1978], chinese hamster embryo fibroblasts [Sager and Kovac, 1982], erythroleukemia cells [Creusot et al., 1982], and in the clonal lines C3H10T1/2 [Konieczny and Emerson, 1984], and HL60

[Christman et al., 1983]. Directly pertinent to the present study, Aza also has been shown to induce differentiation of bovine growth plate chondrocytes to a more mature phenotype as evidenced by increased cell size and increased expression of PTHrP and its receptor, colX, and alkaline phosphatase [Cheung et al., 2001]. Collectively, these reports establish the utility of Aza as a stimulator of maturation and represent the basis of our approach to induce maturation in ACs.

Similar to the other Aza-induced differentiation models that have been reported, findings presented in this report demonstrate that Aza-treated ACs display a phenotype consistent with maturational progression. Not only does Aza induce the expression of hypertrophic markers including colX, Ihh, and alkaline phosphatase, but it renders the cells responsive to regulation by TGF-β, PTHrP, and BMP-2. This Aza-induced responsiveness to growth factors recapitulates the situation in other established hypertrophic chondrocyte models from the chick growth plate [Ionescu et al., 2001] and sternum [Grimsrud et al., 2001; Pateder et al., 2001]. The induction of matura-

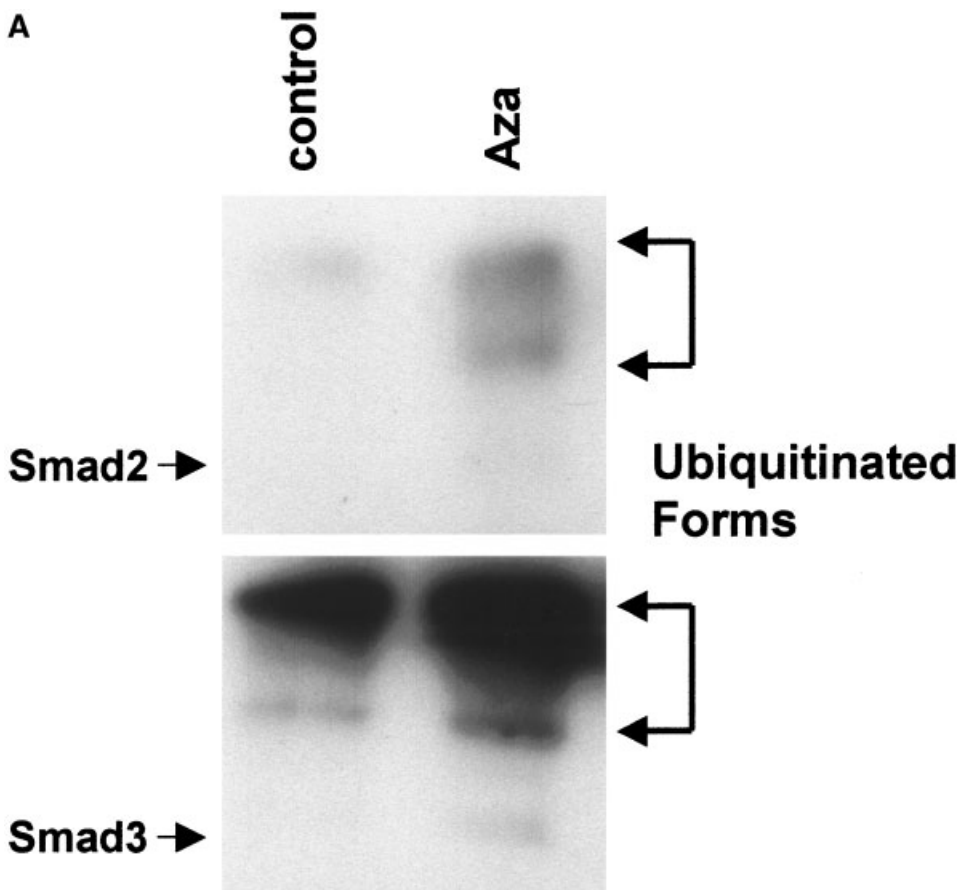


Fig. 6. Aza stimulates ubiquitination and rate of degradation of Smad2 and 3. **A**: ACs that were treated with or without 15 μg/ml Aza for 48 h (Aza) were co-transfected with HA-tagged ubiquitin and either flag-tagged Smad2 or flag-tagged Smad3. Forty eight hours post-transfection, cells were exposed to 50 nM MG-132 for 6 h, protein extracts were prepared and ubiquitinated Smad2 and 3 were pulled down with an anti-flag monoclonal antibody covalently bound to sepharose beads. Pulled down complexes were run on polyacrylamide gels and transferred to nitrocellulose. Western analyses were performed using an anti-HA monoclonal antibody to detect ubiquitinated forms. **B**: ACs that were either treated with 15 μg/ml Aza for 48 h (Aza) or that were left un-treated (control) were transfected with either flag-tagged Smad2. Forty eight hours post-transfection, cells were bathed in methionine-free DMEM for 1 h, were metabolically labeled for 1 h with ^{35}S-methionine and were chased with normal medium (DMEM) for varying amounts of time. Radiolabeled Smad2 was pulled from cell extracts as above using flag antibodies conjugated to sepharose beads pulled down proteins were run on polyacrylamide gels for autoradiographic determination of protein levels. NIH image (version 1.6) was used to determine the optical density of bands in the autoradiographs to permit comparison of protein level at each time point post-chase. **C**: Identical experiment as in (B) was performed, except cells were transfected with flag-tagged Smad3 to facilitate detection of Smad3 degradation.

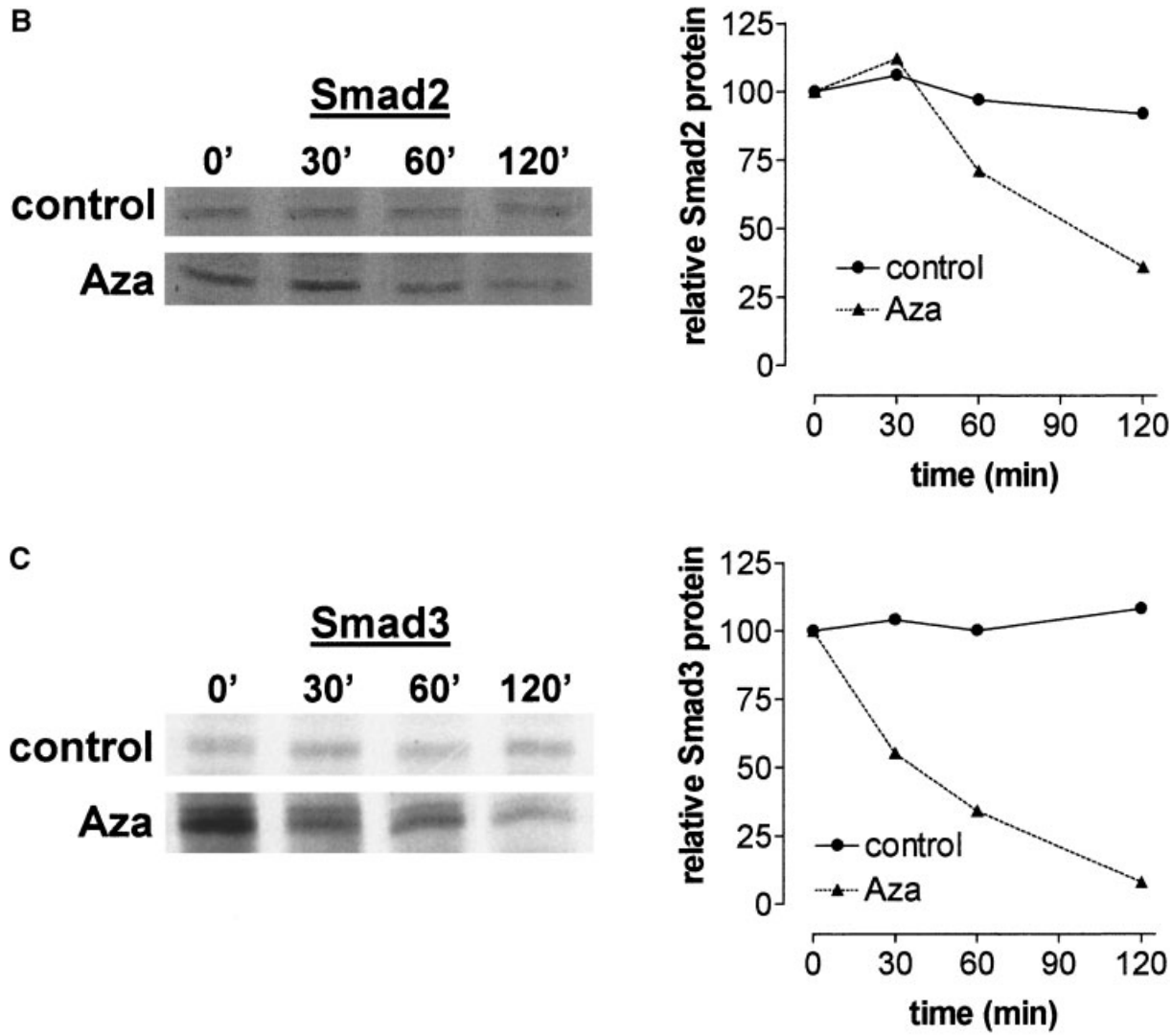


Fig. 6. (*Continued*)

tional hallmarks by Aza is unique in the AC system since, as mentioned, these cells are maturationally arrested both in vivo and in vitro and only rarely can be induced to mature except under pathological circumstances. In fact, stimulation of hypertrophic potential in ACs in vitro has only been demonstrated following infection of cells with a retroviral vector encoding a temperature sensitive mutant of the simian virus 40 large tumor antigen [Oyajobi et al., 1998] or following thyroid hormone treatment [Rosenthal and Henry, 1999]. The strength of maturational induction in these models was not as robust as the Aza effect based on lower level activation of colX and alkaline phosphatase expression. Also, the ability of these treatments to facilitate responsiveness to factors such as BMP-2 was not tested in these studies. Thus, the induction of maturation by Aza in ACs represents a novel in vitro model of AC maturation that we propose will be useful as a tool to examine mechanisms that constrain maturation of these cells.

Regarding mechanisms that constrain or drive maturation, the hypertrophic program in chondrocytes is tightly regulated by a few key growth factors and their associated downstream signaling pathways. It has been established that of these pathways, one of the most potent regulatory systems in chondrocytic cell types involves signaling via receptors for TGF-β and BMP [Ballock et al., 1993; Grimsrud et al., 2001]. This system exerts its effects via a shared constellation of transcription factors called the

Smads, the details of which have been reviewed [Miyazono, 2000]. Specifically, activation of the TGF-β receptor leads to the secondary activation of Smad2 and Smad3, which then associate with Smad4, translocate into the nucleus and target TGF-β responsive genes. Conversely, when the BMP receptor is turned on, Smad1 and Smad5 act via a similar association with Smad4, translocation into the nucleus and targeting of BMP-responsive genes. Another layer of complexity involves desensitization of Smad signaling, which is partially due to the targeted degradation of Smad proteins. The degradative process occurs via the action of the ubiquitin E3 ligases Smurf1 and Smurf2, which target Smad1/5 and Smad2/3 respectively for proteosomal degradation [Bonni et al., 2001; Zhang et al., 2001]. We predict that the balance of TGF-β signaling with BMP signaling dictates the fate of the maturational program in chondrocytes, with suppression of maturation resulting from TGF-β and Smad2/3 dominance versus acceleration of maturation resulting from BMP and Smad1/5 dominance. Based on this, we hypothesized that the maturation-inducing capability of Aza was at least partially caused by a shift from TGF-β to BMP signaling dominance that results from the un-masking of genes participating in TGF-β/BMP signaling.

Confirming that a shift in signaling dominance from TGF-β to BMP is sufficient to induce AC maturation, over-expression of Smad1, Smad5, ΔSmad2, or ΔSmad3 were each capable of mimicking the effect of Aza-induced maturation as evidenced by induction of colX expression. These findings are in general agreement with previous reports which showed localization of Smad1 and 5 expression in hypertrophic growth plate chondrocytes [Sakou et al., 1999] and induction of maturation by constitutive BMP receptor activation in chondrogenic ATDC5 cells [Fujii et al., 1999]. Furthermore, the inhibition of colX expression by Smad2 and 3 over-expression that we observed was consistent with the previous finding that TGF-β signaling inhibits maturation in cultured growth plate chondrocytes [O'Keefe et al., 1988; Ballock et al., 1993; Ferguson et al., 2000]. The ability to mimic Aza-induced maturation by losing TGF-β receptor Smad function or by gaining BMP receptor Smad function implicate these signaling pathways as possible targets of Aza action. These findings suggest that TGF-β signaling dominance in ACs may represent a critical maturational constraint.

If Aza induces maturation in ACs by causing a shift toward BMP signaling, two possible mechanisms that could facilitate the shift include (i) an un-masking of Smad1 and 5 and (ii) a blockade of Smad2 and 3. Our findings suggest that both of these scenarios play out following Aza treatment. Western analyses of Smad protein levels in Aza-treated ACs confirmed that Smad1 and 5 are up-regulated and Smad2 and 3 are down-regulated. Furthermore, down-regulation of Smad2 and 3 appears to be the result of Aza-induced Smurf2 expression. Confirming that this Aza-induced up-regulation of Smurf2 was functionally relevant, (i) Aza mimicked the inhibitory effect of Smurf2 over-expression on the P3TP-luciferase promoter/reporter and (ii) induction of Smurf2 by Aza caused an increased rate of Smad2 and 3 ubiquitination/degradation. This apparent degradative function of Smurf2 in Aza-treated ACs is consistent with its role as an E3 ubiquitin ligase [Bonni et al., 2001; Zhang et al., 2001]. These findings represent the first evidence indicating that Aza can alter TGF-β/BMP signaling. They also suggest that Aza un-masks maturation in ACs at least in part by inducing signaling through Smad1 and 5 and by a Smurf2-dependent degradation of Smad2 and 3. This type of shift in signaling dominance is sufficient to induce maturation of ACs, raising the possibility that a similar shift in signaling dominance may also occur when maturation of these cells progresses inappropriately, such as in osteoarthritis.

It should be noted that although our data strongly implicate Smad1 and 5 and Smurf2 up-regulation as an underlying mechanism of Aza-induced progression of maturation in ACs, there are other regulatory pathways that could also be participating in this Aza effect. For example, suppression of maturation by TGF-β could also be alleviated by an Aza-induced up-regulation of Ski and/or Sno. These two oncoproteins inhibit transcriptional activation of TGF-β target genes by accompanying Smad2/Smad4 and/or Smad3/Smad4 to their consensus binding sites in the DNA [Liu et al., 2001]. Ski/Sno participation in the transcriptional complex ultimately leads to recruitment of histone deacetylase which induces transcriptional repression. Conversely, Aza treatment could directly activate chondrocyte differentiation

by up-regulating p38 MAP kinase signaling, another important pathway in the induction of chondrocyte differentiation [Thomas et al., 2002]. In fact, the selective p38 MAPK inhibitor SB242235 has been shown to reduce cartilage degradation in animal models of osteoarthritis, suggesting that these compounds could be used as anti-arthritic drugs in humans [Badger et al., 2000]. Overall, un-masking of other important pathways that are involved with regulation of AC hypertrophy may facilitate the Aza-effect in these cells, implicating their dis-regulation as contributing factors in the loss of maturational suppression.

Since it is hypothesized that osteoarthritis arises as a result of inappropriate maturation of ACs [von der Mark et al., 1992; Pullig et al., 2000; Sandell and Aigner, 2001; Sztrolovics et al., 2002], use of Aza-treated cells as an in vitro model will facilitate our major long term aim of identifying candidate causative mechanisms that are capable of abrogating maturational arrest in these cells. Our findings that a shift from TGF-β to BMP dominance can lead to maturation suggest that functional TGF-β signaling is a requirement for ACs to preserve their matrix-maintaining, immature phenotype. This idea is supported by other findings that show emergence of a phenotype that resembles osteoarthritis in the Smad3 knockout mouse [Yang et al., 2001] and in a transgenic model over-expressing a truncated, kinase-defective form of the TGF-β type II receptor [Serra et al., 1997]. Based on our results, it is possible that the manifestation of osteoarthritis can arise from loss of DNA methyltransferase function that in essence mimics the effect of Aza in our culture model (i.e., loss of methylation and unmasking BMP dominance). However, we hypothesize that a more plausible model pivots a progressive loss of TGF-β signaling caused by up-regulation of Smurf2. Although there is no evidence for this to date, we hypothesize that the alteration of TGF-β signaling molecules (Smurf2 in particular) may result from injury or synovitis that causes inflammatory cytokine release that may subsequently impact gene expression.

In conclusion, we have established a novel model to study AC regulation of maturation and have begun to identify that a shift in signaling dominance from TGF-β to BMP is sufficient to induce maturation/hypertrophy of these cells. The transcription factors that regulate the balance between these two signaling pathways are candidate participants in the etiology of osteoarthritis, begging further characterization of their involvement in the disease process.

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