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Transcriptional Cross-talk between Smad, ERK1/2, and p38 Mitogen-activated Protein Kinase Pathways Regulates Transforming Growth Factor-β-induced Aggrecan Gene Expression in Chondrogenic ATDC5 Cells*

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In chondrogenesis, members of the transforming growth factor-β (TGF-β) superfamily play critical roles by inducing gene expression of cartilage-specific molecules. By using a chondrogenic cell line, ATDC5, we investigated the TGF-β-mediated signaling pathways involved in expression of the aggrecan gene (*Agc*). At confluency, TGF-β induced *Agc* expression within 3 h, and cycloheximide blocked this induction, indicating that *de novo* protein synthesis is essential for this response. At this stage, TGF-β induced rapid, transient phosphorylation of Smad2, extracellular signal-activated kinase 1/2 (ERK1/2), and p38 mitogen-activated protein kinase (MAPK). Inhibition of the Smad pathways by transfection with a dominant negative Smad4 construct significantly reduced TGF-β-induced *Agc* expression, indicating that Smad signaling is essential for this response. Furthermore, an inhibitor of the ERK1/2 pathway, U0126, or inhibitors of the p38 MAPK pathway, SB203580 and SKF86002, repressed TGF-β-induced *Agc* expression in a dose-dependent manner, indicating that ERK1/2 or p38 MAPK activation is also required for TGF-β-induced *Agc* expression in confluent ATDC5 cells. In differentiated ATDC5 cells, persistently high basal levels of ERK1/2 and p38 MAPK phosphorylation correlated with elevated basal *Agc* expression, which was inhibited by incubation with inhibitors of these pathways. Whereas Smad2 was rapidly phosphorylated by TGF-β and involved in the initial activation of *Agc* expression in confluent cells, Smad2 activation was not required for maintaining the high level of *Agc* expression. Taken together, these results suggest an important role for transcriptional cross-talk between Smad and MAPK pathways in expression of early chondrocytic phenotypes and identify important changes in the regulation of *Agc* expression following chondrocyte differentiation.

During development, cartilage serves as a template for most bones. Cartilage formation is initiated with condensation of mesenchymal cells, followed by progression of chondrocyte differentiation toward proliferation, prehypertrophy, and hypertrophy (1, 2). A number of growth factors, such as fibroblast growth factors (3, 4), insulin-like growth factor (5–7), transforming growth factor βs (TGF-βs)[1] (8–10), and bone morphogenetic proteins (BMPs) (4, 11, 12), have been implicated in this differentiation process. During differentiation, chondrocytes secrete extracellular matrix (ECM) molecules characteristic of cartilage, such as type II collagen, aggrecan, and link protein, providing an environment that maintains the chondrocyte phenotype. Thus, chondrocytes are defined both by their morphology and capacity to synthesize these characteristic ECM molecules.

Among the ECM molecules, aggrecan is a major proteoglycan of cartilage (13), and its deposition corresponds well with Alcian blue staining, which is commonly used for identification of cartilage. Although low levels are detected in the heart (14) and brain (15), aggrecan is largely restricted to cartilage, and it contributes to water retention, resistance to deformation, and the gel-like property of the cartilage. Aggrecan-null mice develop perinatal lethal dwarfism with little ECM in cartilage and defective chondrocyte differentiation, indicating critical roles of aggrecan in cartilage development (16). Therefore, aggrecan serves as a good marker for differentiated chondrocytes.

A number of *in vitro* studies have been performed using primary chondrocytes and chondrogenic cell lines, such as CFK 2 (17), ATDC5 (12, 18, 19), C1 (20), C3H10T1/2 (11), and RCJ3.1C5 cells (21, 22), to elucidate the mechanisms of chondrocyte differentiation. Among them, ATDC5 cells are a well characterized chondrogenic cell line derived from mouse teratocarcinoma. In culture, these cells mimic the multistep process of chondrocyte differentiation. After plating, they proliferate until confluency, at which point they undergo growth arrest following contact inhibition. After 4 days, these cells undergo a phenotypic change characterized by the secretion of a variety of ECM molecules, such as type II collagen, aggrecan, and link protein, and by the formation of cartilaginous nodules on the culture plate (12, 18, 19). Recent studies have demonstrated that TGF-β, BMP-2 (12), and growth/differentiation factor-5 (GDF-5, also known as cartilage-derived morphogenetic protein-1, CDMP-1) (23) rapidly induce type II collagen expression in confluent ATDC5 cells, suggesting critical roles of signaling

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[1] The abbreviations used are: TGF-β,transforming growth factor-β; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; BMPs, bone morphogenetic proteins; ECMs, extracellular matrix; JNK, c-Jun N-terminal kinase; DN, dominant negative; PCR, polymerase chain reaction; RT-PCR, reverse transcriptase-PCR; CHX, cycloheximide; GFP, green fluorescent protein.

by the TGF-β superfamily for chondrocyte-specific gene expression.

Receptor complexes of the TGF-β superfamily consist of a ligand-binding type II receptor serine-threonine kinase that, following ligand binding, binds to and transphosphorylates the signal transducing type I serine-threonine kinase (24). These receptors, in turn, activate, through a phosphorylation event, members of a family of downstream signaling intermediates, the Smads (25). Within this family of proteins, the receptor-activated Smads, R-Smads, are the direct substrates for the activated type I serine-threonine kinase and are phosphorylated on a conserved C-terminal -SS*X*S motif. Smad1, Smad5, and Smad8 are phosphorylated following activation of type I bone morphogenetic protein (BMP) receptors, whereas Smad2 and Smad3 are activated by the type I TGF-β and activin receptors (25). The phosphorylated R-Smads then form heteromeric complexes with the common Smad mediator, Smad4, which is then translocated to the nucleus. Once in the nucleus, these Smad complexes function as transcriptional activators, binding to specific *cis*-acting elements in Smad-dependent promoters (26–29), interacting with and recruiting a number of other DNA binding (30–36) and non-DNA binding transcriptional activators (37), inhibitors (38–41), and bridging coactivator proteins CBP and p300 (28, 42). Smad4 is an essential component in many of the Smad-dependent responses (43), serving both to stabilize the Smad-transcription factor complex (44) and to form functional interactions with critical transcriptional adapter proteins including CBP and p300 (45).

Although the Smad pathway is widely represented in most of the cell types and tissues studied, additional pathways may be activated following treatment with TGF-β in specific contexts. For example, activation of Ras, extracellular signal-regulated kinase 1/2 (ERK1/2), and c-Jun N-terminal kinase (JNK) by TGF-β signaling has been reported in primary intestinal epithelial cells and some breast cancer cell lines (46, 47), whereas activation of protein kinase A contributes to TGF-β-signaling responses in murine mesangial cells (48). In addition, TGF-β-activated kinase-1 (TAK1), a member of the MEKK family and activator of JNK and p38 MAPK pathways (14, 49), is rapidly activated by TGF-β in certain cell systems, notably in murine C2C12 myocytes (50).

The relative contribution of these different pathways in chondrocytic responses to TGF-β is poorly understood. The aim of our study is to define the contribution of specific TGF-β-dependent signaling pathways involved in the regulation of chondrocyte differentiation. In particular, we have focused on the regulation of aggrecan gene (*Agc*) expression as a unique marker gene that is characteristic of the chondrocyte phenotype. In this paper, we demonstrate that TGF-β rapidly induces *Agc* expression in ATDC5 cells and that this response requires TGF-β-induced activation of both R-Smad2/4 complexes as well as p38 MAPK and ERK1/2 pathways. In contrast, following differentiation, high levels of *Agc* expression are maintained without activation of R-Smad2 but are associated with, and require, persistently high basal activation of p38 MAPK and ERK1/2 pathways. These findings suggest that there are distinct transcriptional cross-talk mechanisms regulating *Agc* expression in differentiating chondrocytes, and they provide new insights into the regulatory mechanisms defining chondrocytic phenotypes.

MATERIALS AND METHODS

Cell Culture—ATDC5 cells (18) were grown in Dulbecco's modified Eagle's medium/F-12 (Life Technologies, Inc.) containing 5% fetal bovine serum (HyClone) and 10 μg/ml insulin, 10 μg/ml transferrin, and 10 μg selenium (ITS) (Biofluids, Inc) at 37 °C under 5% CO_2. Cells were maintained at 20–80% confluency, replacing media every other day. For inhibition of protein synthesis, a concentration of 5 μg/ml cycloheximide (CHX, Sigma), which blocks 95% of protein synthesis without cell toxicity, was added to culture media at the indicated times.

Transfection and Luciferase Assay—Cells at 50–60% confluency were transfected with DNA constructs using FuGene™ 6 (Roche Molecular Biochemicals). Transfection efficiency was monitored using a constitutively expressed GFP expression vector under the control of the EF1 promoter, pCEFL-GFP (a gift from J. S. Gutkind), confirming that ~65% of the transfected cells expressed GFP under fluorescence microscopy. For dominant inhibitory experiments of the Smad pathway, cells were cotransfected with the following: 0.25 μg of p3TP-Lux, which is a TGF-β-responsive reporter containing three 12-*O*-tetradecanoylphorbol-13-acetate-responsive elements and a small TGF-β-responsive element from the plasminogen activator inhibitor 1 promoter (51); 1.7 μg of a dominant negative (DN−) Smad4 construct, Smad4-(Δ275–322) (45); or empty vector pcDNA3 (Invitrogen); and 0.1 μg of pRLSV40 (Promega), a *Renilla* luciferase expression vector driven by SV40 promoter, was used as an internal control vector. Twenty four hours after transfection, when cells reached confluency, the culture medium was replaced with Dulbecco's modified Eagle's medium/F-12 containing ITS and 0.2% fetal bovine serum. After 12 h, cells were treated with 10 ng/ml TGF-β_2 (R & D Systems) for 16 h and were lysed. Luciferase activity of cell lysate was assayed with the Dual-Luciferase reporter assay system (Promega) using a microtiter plate luminometer (Dynex). For inhibition studies, U0126 (Promega), SB203580, and SKF86002 (Calbiochem) were used at the concentrations indicated and were added 1 h before TGF-β (10 ng/ml) treatment. U0126 is a specific inhibitor of an upstream molecule, MEK1/2, that activates ERK1/2 (52). In preliminary experiments, a concentration of 10 μM showed optimal inhibition of ERK1/2 phosphorylation. SB203580 (53) inhibits p38 MAPK activity but not its phosphorylation. SKF86002 (54, 55) is an inhibitor of MAPK-kinase 6 (MKK6), an activator of p38 MAPK. Concentrations of 20–50 μM showed optimal inhibition of p38 MAPK activity without cytotoxicity. Because of the relatively short half-life of SB203580 and SKF86002, these compounds were added to the media every 4 h during the course of the assay. Gal4-Smad experiments were performed as reported elsewhere (45). Briefly, cells were similarly transfected with 1 μg of p147-Gal4-Smad2 or -Smad4 (266–552), coupled with 1 μg of Gal4-Luc (56) and 0.1 μg of pRLSV40 (Promega). Gal4-VP-16 was also used as an independent transactivator for a control. After 24 h, inhibitors were added under serum-starved conditions. After 1 h, cells were treated with TGF-β. Cells were collected after 16 h, and cell lysate was similarly subjected to the Dual-Luciferase reporter assay system (Promega).

Immunoblot Analysis for Phosphorylation and Activation of Smad and MAPK—Phosphorylation of ERK1/2, JNK, and p38 MAPK was determined with corresponding PhosphoPlus Antibody™ kits (New England Biolabs), respectively. Briefly, a pair of immunoblot analyses was performed with phosphospecific antibodies or antibodies to detect the proteins themselves. For Smad2 phosphorylation, anti-phospho-Smad2 (Upstate Biotechnology, Inc.), which detects the C-terminal phosphorylated form of Smad2, and anti-total Smad2 (Zymed Laboratories Inc.) were used. For ERK1/2 and Smad2 phosphorylation, cells were starved for 16 h to decrease basal phosphorylation levels. After treatment with TGF-β for the time indicated, cells were washed with ice-cold phosphate-buffered saline and collected, and cell lysate was subjected to a SDS-polyacrylamide gel electrophoresis under a reducing condition, followed by immunoblotting. For the inhibition studies, optimized concentrations of U0126, SB203580, and SKF86002 were added 1 h prior to TGF-β treatment. An inhibitor of protein kinase A, H-89, was used at concentrations of 10 μM as a negative control. ERK1/2 assay was carried out using p44/42 MAP kinase assay kit (New England Biolabs) according to the manufacturer's protocol. For p38 MAPK activity, p38 MAP kinase assay kit (New England Biolabs) was used according to the manufacturer's protocol.

Nuclear Translocation Analysis—Confluent cells were serum-starved for 16 h, pretreated with U0126 (10 μM) or SB203580 (20 μM) for 1 h, and treated with TGF-β (10 ng/ml) for 1 h. Then cells were fixed with 4% paraformaldehyde, permeabilized in 0.2% Triton X-100, and incubated with an anti-Smad2/3 antibody (Transduction Laboratories) overnight at 4 °C, followed by incubation with Alexa Fluor™ 488 (Molecular Probes) secondary antibody. Cells were then visualized with immunofluorescence microscopy (Nikon). The percentage of cells with Smad2/3 staining in the nucleus was determined by observing 100 cells.

Quantitative RT-PC—For analysis of *Agc* transcription, cells were cultured for 12 h in a culture medium containing 0.2% fetal bovine serum and were treated with TGF-β at a concentration of 10 ng/ml for 12 h, unless otherwise indicated. Poly(A) RNA was prepared from cells using the Micro-Fast Track™ kit (Invitrogen). This mRNA (200–600 ng) was reverse-transcribed to generate cDNA using the Superscript

Preamplification™ System (Life Technologies, Inc.). For semiquantitative analysis of *Agc* expression, PCR was performed using a set of primers, 5′-TGGAGCATGCTAGAACCCTCG-3′ and 5′-GCGACAAGAAGACACCATGTG-3′. After confirmation of the *Agc* transcript, real time quantitative PCR was performed using the TaqMan™ 7700 (PE Applied Biosystems). Sequences for a probe and a set of primers were chosen by the Primer Express™ program as follows: 5′-CCCTGGGCAGCGTGATCCTCAC-3′ for a probe, 5′-CTGCCCTTGCCCCGTA-3′ for a forward primer, and 5′-GACAGGTCAAAGATGGGCTTTG-3′ for a reverse primer. The probe was labeled with fluorescent reporter dyes 6-carboxyfluorescein and 6-carboxy-*N,N,N′,N′*-tetramethylrhodamine at 5′ and 3′ ends, respectively. As an internal control, a set of primers and a probe of rodent GAPDH labeled with VIC™ (PE Applied Biosystems) were used according to the manufacturer's protocol.

RESULTS

Expression of Aggrecan Gene (Agc) Is Rapidly Induced by TGF-β in ATDC5 Cells—In confluent ATDC5 cells, RT-PCR analysis revealed induction of *Agc* transcription as early as 3 h after the treatment with TGF-β (Fig. 1*A*). These data were confirmed by direct measurement of the transcript levels of *Agc* by a real time quantitative RT-PCR assay. By using this assay, the fold induction of *Agc* mRNA by TGF-β was 4.1 ± 0.38, 10.22 ± 2.75, 22.3 ± 9.1, and 59.6 ± 0.5 for 3, 6, 12, and 24 h, respectively, where 0 h was 1.0 ± 0.03 (mean ± S.D., $n = 3$, Fig. 1*B*). Without TGF-β, *Agc* expression after 24 h was 5.35 ± 0.41 that of 0 h.

Next, we used CHX to examine whether protein synthesis was required for *Agc* induction. Pretreatment with CHX 30 min before TGF-β addition completely blocked transcription of *Agc*. When CHX was added 2 h after TGF-β treatment, *Agc* transcription was observed (Fig. 1*C*). These results indicate that protein synthesis initiated within 2 h of treatment with TGF-β is required for *Agc* expression and suggest that this expression requires induction of an immediate early response gene (IEG) to a TGF-β-dependent signal.

The Smad Pathway Mediates Induction of Agc Expression—To determine the nature of this signaling response, we examined a number of pathways previously shown to be activated by TGF-β in different cell systems. First, we examined if Smads are involved in *Agc* expression. As shown in Fig. 2*A*, using phospho-Smad2-specific antibodies, Smad2 was rapidly phosphorylated within 5 min of treatment with TGF-β and persisted for 4 h. We next examined the functional significance of this Smad pathway activated by TGF-β in regulating *Agc* expression by using a dominant negative (DN−) Smad4 construct, Smad4-(Δ275–322), which has previously been shown to inhibit TGF-β-dependent transcriptional responses in a number of different cell types and reporter systems (45).[2] When the cells reached confluency after 24 h of transfection, cells were starved for 12 h and treated with TGF-β for an additional 12 h. The expression of p3TP-lux reporter gene was inhibited in a dose-dependent manner, with a maximal inhibition of 65%, confirming down-regulation of the Smad pathway by DN-Smad4 construct (Fig. 2*B*). Under the same conditions, this dominant negative construct inhibited TGF-β-mediated *Agc* expression as determined by real time RT-PCR, by ~65% (Fig. 2*C*). In parallel experiments, transfection efficiency of 65 ± 10% (mean ± S.D.) was obtained using the GFP expression construct, suggesting that the DN-Smad4 construct strongly inhibits TGF-β signaling in the transfected cells. These results indicate involvement of the Smad pathway in *Agc* expression.

Both ERK1/2 and p38 MAPK Pathways Are Required for TGF-β-induced Agc Gene Expression—We next examined the effects of TGF-β on activation of ERK1/2, SAPK/JNK, and p38 MAPK in confluent ATDC5 cells. TGF-β increased phosphorylation of ERK1/2 5–10 times that of the basal level. The increased phosphorylation appeared as early as 5 min, reached the peak at 15–30 min, and was sustained until 1 h after TGF-β treatment (Fig. 3*A*). Correlation between phosphorylation and activation of ERK1/2 was confirmed by an ERK1/2 assay system using Elk1 as a substrate (Fig. 3*A, lower panel,* at *15 min*). Immunoblot analysis using anti-phospho-p38 showed negligible basal levels of phospho-p38 MAPK, and TGF-β dramatically phosphorylated p38 MAPK, although compared with ERK1/2 phosphorylation, phosphorylation of p38 MAPK was delayed (Fig. 3*B*). JNK was not phosphorylated or activated by TGF-β, indicating that this pathway is not involved in TGF-β signaling in these cells (data not shown).

Next, we used a number of known specific inhibitors of the MAPK pathways to determine the functional significance of TGF-β-dependent p38 MAPK and ERK1/2 activation on *Agc* expression. To inhibit the ERK1/2 pathway, we used U0126, a specific inhibitor of MEK1/2, which is an upstream molecule that activates ERK1/2 (52). For the p38 MAPK pathway, SB203580 (53) and SKF86002 (54, 55) were used. These inhibit activity of p38 for its substrate and that of MAPK-kinase-6 (MKK6) for p38, respectively. U0126 (1.0 and 10 μM) inhibited phosphorylation of ERK1/2 (Fig. 4*A, lower panels*) in a dose-dependent manner. Both SB203580 (2.0 and 20 μM) and SKF86002 (2.0 and 20 μM) inhibited p38 activity (Fig. 4, *B* and

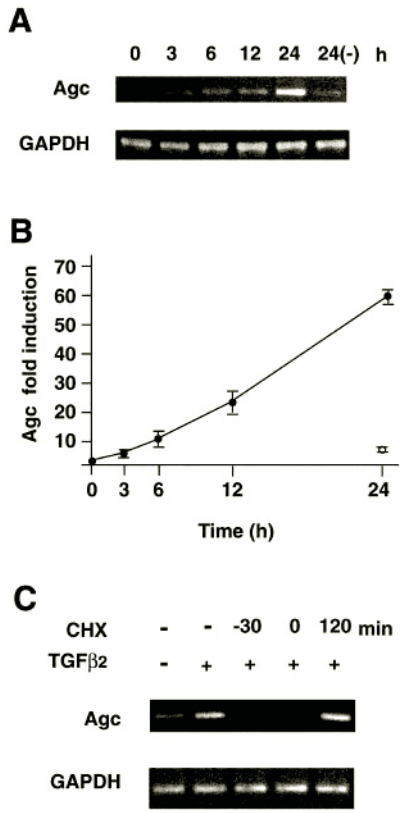

FIG. 1. **Induction of aggrecan gene (*Agc*) expression by TGF-β.** *A,* RT-PCR analysis for *Agc* expression. Electrophoresis gel of RT-PCR products showing *Agc* transcript and GAPDH as control. *B,* quantitation of *Agc* transcript using a real time RT-PCR. TGF-β-induced *Agc* expression (*closed circle*) is observed for 3 h. *Agc* expression without treatment for 24 h is also shown (*open circle*). The fold induction of *Agc* mRNA by TGF-β is 4.1 ± 0.38, 10.22 ± 2.75, 22.3 ± 9.1, and 59.6 ± 0.5 for 3, 6, 12, and 24 h, respectively, and 0 h is 1.0 ± 0.03. Data represent mean ± S.D. ($n = 3$). *C,* electrophoresis gel showing inhibition of *Agc* expression by cycloheximide (*CHX*) treatment, coupled with GAPDH expression as control. RT-PCR products of *Agc* after TGF-β treatment for 6 h are shown. TGF-β-induced *Agc* expression is blocked by CHX treatment. Addition of CHX 120 min after TGF-β treatment does not inhibit *Agc* expression. Two independent experiments showed the same results.

[2] M. P. de Caestecker, unpublished data.

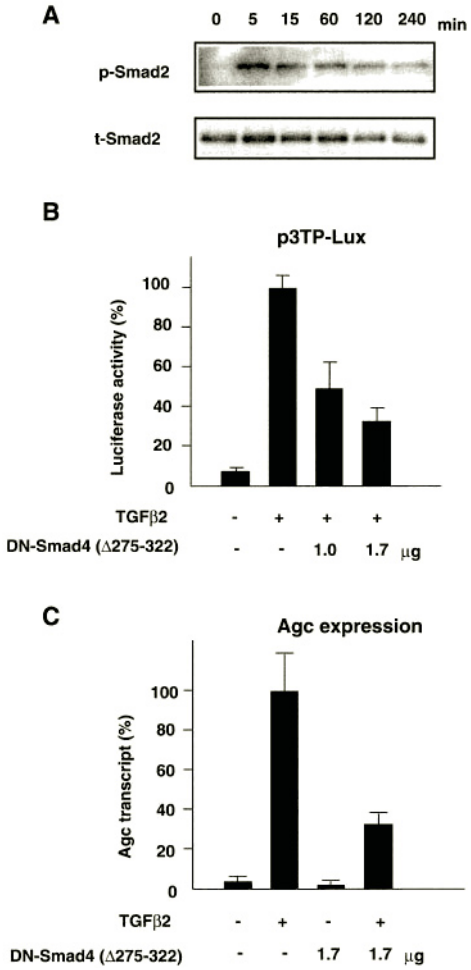

FIG. 2. **Decreased *Agc* expression by inhibition of the Smad pathway with a dominant negative DN-Smad4 construct.** *A,* immunoblot showing phosphorylation of Smad2. Cells were treated with TGF-β for the time indicated. Phospho- and total Smad2 are shown in *upper* and *lower panels*, respectively. *B,* inhibition of p3TP-Lux Smad reporter gene expression by a dominant negative DN-Smad4 construct. Cells were transfected with 1 μg of a dominant negative Smad4 construct, Smad4-(Δ275–322), 1 μg of a reporter p3TP-Lux, and 0.1 μg pRLSV40 as an internal control. After 24 h, cells were treated with TGF-β (10 ng/ml) and were subjected to luciferase assay as described under "Materials and Methods." *C,* inhibition of *Agc* expression. Under the same conditions as in *B*, *Agc* expression was measured by real time quantitative RT-PCR analysis as indicated under "Materials and Methods." Note similar inhibition patterns in both p3TP-Lux and *Agc* expression by transfection of the DN-Smad4 construct. Two additional independent experiments showed similar results.

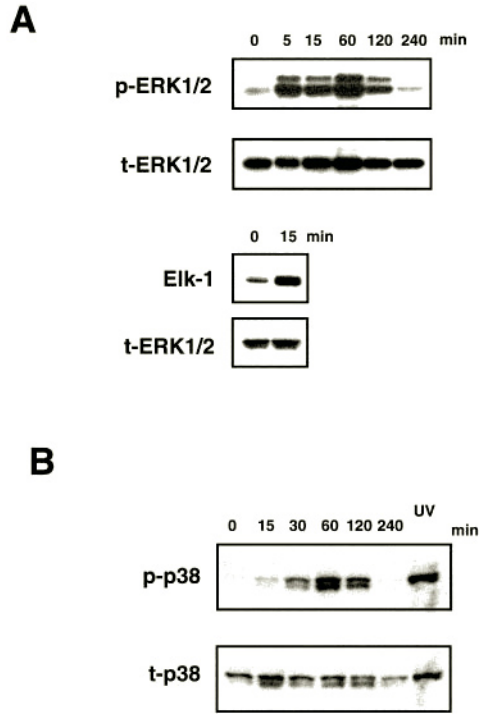

FIG. 3. **Phosphorylation and activation of ERK1/2 (*A*) and p38 MAPK (*B*).** Confluent ATDC5 cells were treated with TGF-β (10 ng/ml). At the indicated times, cells were collected, and phosphorylation of either ERK1/2 or p38 MAPK was analyzed. *A,* phosphorylation and activation of ERK1/2 by TGF-β treatment. The *1st* and the *2nd panels* show immunoblot using anti-phospho-ERK1/2 and anti-ERK1/2 molecules themselves, respectively. The *3rd* and *4th panels* show activity of ERK1/2 using Elk-1 as substrate and control immunoblot using anti-total ERK1/2 of the cell lysate, respectively. Note the correlation of phosphorylation and activation of ERK1/2. *B,* phosphorylation of p38 MAPK by TGF-β treatment. Immunoblot analysis was performed using specific antibodies for phospho-p38 and total p38. *Upper* and *lower panels* indicate patterns of phospho- and total p38 MAPK in transfected cells, detected by their specific antibodies, respectively.

C, lower panels). Whereas TGF-β induced *Agc* expression to 12.4 ± 3.4-fold, U0126 at 1.0 and 10 μM attenuated the fold induction to 6.13 ± 0.38- and 1.41 ± 0.06-fold, respectively (Fig. 4*A*). Similarly, both SB203580 and SKF86002 attenuated TGF-β-induced *Agc* expression in a dose-dependent manner. Fold induction by TGF-β was 5.1 ± 0.03 and 1.31 ± 0.10 following treatment with SB203580 at 2.0 and 20 μM and 6.55 ± 0.54 and 2.93 ± 0.13 following treatment with SKF86002 at 2.0 and 20 μM, respectively (Fig. 4, *B* and *C*). To ensure that not all protein kinase inhibitors inhibit TGF-β-mediated induction of *Agc* expression, an inhibitor of protein kinase A, H-89, was used as a negative control. H-89 did not show inhibitory activity for TGF-β-induced *Agc* expression (data not shown). These results clearly demonstrate that both ERK1/2 and p38 pathways are required for *Agc* expression.

Cross-talk of the Smad Pathway with p38 MAPK and ERK1/2 Pathways—We demonstrated that both the Smad and the p38 MAPK and ERK1/2 pathways are activated by TGF-β in confluent ATDC5 cells and that each of these pathways is essential for TGF-β-dependent induction of *Agc* expression. Next, we asked whether these pathways act independently or involve some level of intracellular cross-talk. Activation of MAPK pathways has been shown both to activate and induce nuclear translocation of Smad2 (57, 58) or, in other contexts, to inhibit TGF-β-dependent nuclear translocation of Smad2 and -3 (59). We therefore tested whether specific inhibitors of ERK1/2 and p38 MAPK pathways inhibit TGF-β-dependent nuclear translocation of the Smad proteins in ATDC5 cells. Cells were treated with the specific inhibitors and, after 1 h of incubation with TGF-β, the number of Smad2 positive nuclei was counted under fluorescence microscopy. Neither U0126 (10 μM) nor SB203580 (20 μM) inhibited TGF-β-dependent nuclear translocation of Smad 2/3 (Fig. 5*A*), indicating that these MAPK pathways are not inducing nuclear translocation of Smad2/3. Furthermore, immunoblotting with anti-phospho-Smad2 antibody, which recognizes specific C-terminal phosphoserine residues of Smad2, demonstrated that TGF-β increased C-terminal phosphorylation of Smad2, but these phosphorylation events were not inhibited by U0126 or SB203580 (Fig. 5*B*). These findings suggest that TGF-β-dependent activation of p38 and ERK1/2 MAPK pathways does not influence activation of R-Smads by TGF-β and indicate that under the experimental conditions of these studies neither U0126 nor SB203580 affects the ability of TGF-β receptors to phosphorylate downstream R-Smad substrates.

To determine whether these MAPK pathways are affecting

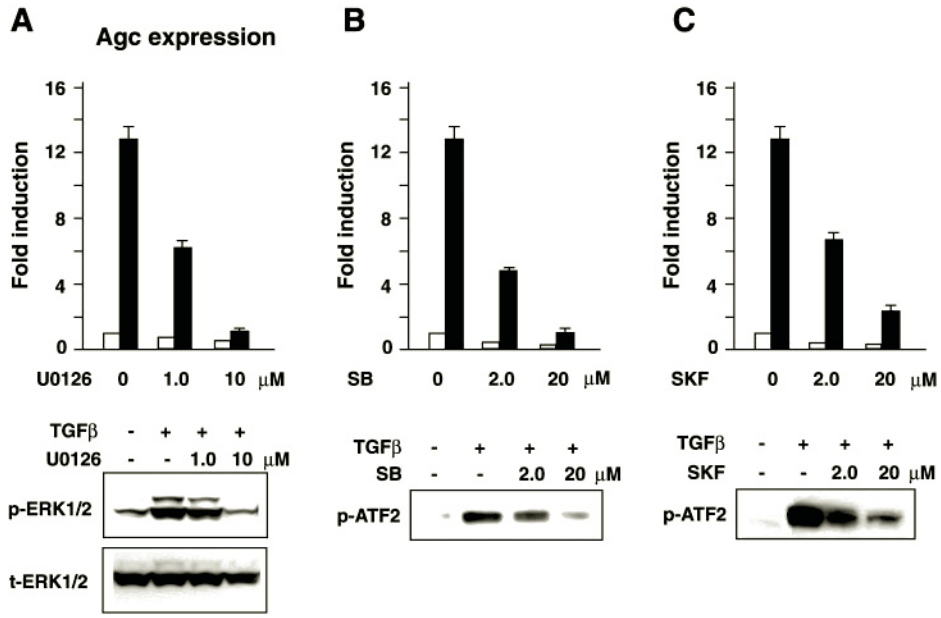

FIG. 4. **Inhibition of *Agc* expression by specific inhibitors of ERK1/2 and p38 MAPK pathways.** Confluent ATDC5 cells were starved for ~16 h. Then U0126, SB203580 (*SB*), or SKF86002 (*SKF*) was added at a concentration indicated. After 1 h, cells were treated with 10 ng/ml TGF-β for 12 h; mRNA was obtained; and real time quantitative RT-PCR analysis was performed. To confirm inhibitory effects of each pathway, immunoblot analysis for ERK1/2 phosphorylation (*lower panel*) and p38 MAPK assay using ATF-2 as a substrate (*B* and *C*, *lower panels*) were performed as described under "Materials and Methods." *Agc* transcription in TGF-β-treated (*solid bar*) is indicated as fold induction that nontreated (*open bar*), where 1.0 equals basal transcription. Whereas TGF-β induces *Agc* expression to 12.4 ± 3.4-fold, U0126 at 1.0 and 10 μM inhibits the fold induction to 6.13 ± 0.38- and 1.41 ± 0.06-fold, respectively (*A*). Both SB and SKF inhibit TGF-β-induced *Agc* expression: at 2.0 and 20 μM, SB inhibits to 5.1 ± 0.03 and 1.31 ± 0.10 and SFK to 6.55 ± 0.54 and 2.93 ± 0.13, respectively (*B* and *C*). The data represent mean ± S.D. ($n = 3$). Similar results were obtained in an additional independent experiment.

the transcriptional activity of the Smad complex, we used a heterologous transcription assay with Gal4-Smad fusion proteins. This system defines a specific Smad-dependent transcriptional response that bypasses confounding factors associated with different response elements in a variety of other TGF-β-responsive reporters. For these experiments, we cotransfected cells with Smad2 or a transcriptionally active N-terminal truncation of Smad4, Smad4-(266–552), fused to the DNA-binding domain of Gal4 (45), together with pGal4-Luc, a luciferase reporter gene under the control of six Gal4-responsive elements and a minimal TATA-containing promoter. This reporter construct minimized effects of inhibitors on the basal luciferase activity. As shown in Fig. 6, increasing concentrations of U0126, SB203580, and SKF86002 inhibited TGF-β-induced transcriptional activity of both Gal4-Smad2 and Gal4-Smad4 systems. Whereas U0126 (10 μM) and SB203580 (20 μM) inhibited basal Gal4-Smad2 activation by 30 and 25%, TGF-β-induced activation was inhibited by 65 and 70%, respectively. Furthermore, SKF86002 had little effect on the basal levels, indicating that the suppression of ligand-induced reporter activity by these inhibitors is specific. We used Gal4-VP16 as an independent transactivator to evaluate the specific effect of these inhibitors. Transfection of Gal4-VP16 induced activity of the reporter luciferase gene about 95-fold. Addition of U0126 or SB203580 resulted in no significant reduction of transactivation by Gal4-VP16 (data not shown). These results indicate that these inhibitors specifically block the p38 MAPK and ERK1/2 cascades. Taken together, these results indicate that TGF-β-induced activation of p38 MAPK or ERK1/2 is essential for transcriptional activation of Smad2 and Smad4 and that this interaction is necessary for maximal activation of a specific Smad-dependent transcriptional response in ATDC5 cells.

Agc Expression No Longer Requires TGF-β following Differentiation—To determine whether this pattern of the Smad and MAPK pathways was restricted to confluent ATDC5 cells or was a common feature at all stages in chondrocyte differentiation, we performed a time course analysis on their response to TGF-β at various stages of differentiation. First, we examined levels of *Agc* expression, using real time quantitative RT-PCR. As shown in Table I, basal *Agc* expression of nontreated ATDC5 cells increased during differentiation. *Agc* transcript levels increased to 87.5 ± 6.5 and 114.0 ± 1.9, at day 7 and 14 after confluency, respectively, where the level at confluency is 1.0 ± 0.02, confirming chondrocyte differentiation in this time course experiment. Although TGF-β up-regulated *Agc* expression during differentiation, the fold induction by TGF-β became attenuated to 1.5–2.4-fold at day 14, as compared with ~12-fold induction in confluent cells.

Next, we examined phosphorylation patterns of Smad2, ERK1/2, and p38 MAPK, mediated by TGF-β following differentiation in ATDC5 cells. TGF-β dramatically phosphorylated Smad2 at confluency, day 7, and day 14, indicating that receptors for TGF-β and their phosphorylation of Smad2 remain unaffected through differentiation (Fig. 7). Furthermore, low levels of Smad2 phosphorylation in the absence of TGF-β indicate that there is no significant autocrine TGF-β activity in these cells. In contrast, the response of p38 MAPK and ERK1/2 to TGF-β was reversed during differentiation. At day 7, the phosphorylation level of ERK1/2 was unaffected by TGF-β, whereas by day 14, TGF-β decreased the high basal phosphorylation of ERK1/2 and p38 MAPK. Phosphorylation levels of both ERK1/2 and p38 MAPK correlate with elevated basal *Agc* expression in differentiated ATDC5 cells, suggesting that activation of both ERK1/2 and p38 MAPK may be required for continuous *Agc* expression in differentiated cells. Therefore, we examined the effect of inhibitors on *Agc* expression in differentiated ATDC5 cells. As shown in Fig. 8, both U0126 (10 μM) and SB203580 (20 μM) down-regulated *Agc* expression in differentiated cells. These data demonstrate the role of activated ERK1/2 and p38 MAPK for *Agc* expression and together indicate that in differentiated, as opposed to confluent, ATDC5

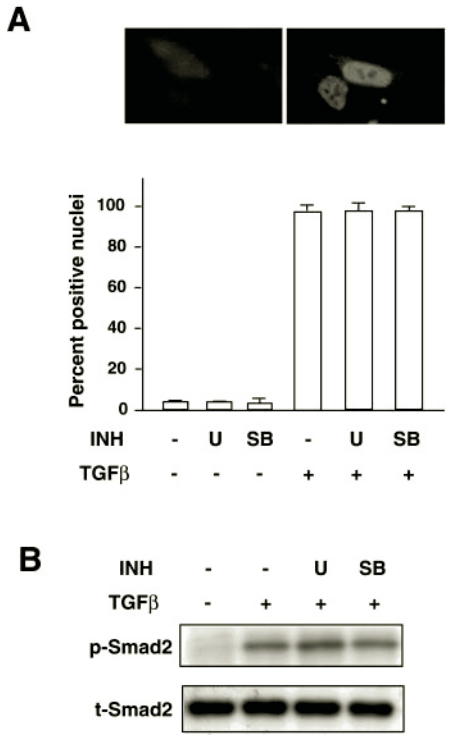

FIG. 5. **Nuclear translocation and phosphorylation of Smad2.** *A,* nuclear translocation of Smad2. *Upper panel* shows nuclear localization of Smad2 in TGF-β-treated ATDC5 cells. Smad2 is observed predominantly in nuclei at 30 min after TGF-β treatment (*right*), whereas cells without treatment contain Smad2 primarily in cytoplasm (*left*). *Bottom panel* shows percentage of nuclei positive for Smad2 after treatment with U126 (*U*) or SB203580 (*SB*) and TGF-β (mean ± S.D., $n = 3$). No significant effects of the inhibitors on nuclear translocation were observed. *B,* phosphorylation of Smad2 after treatment with U126 (*U*) or SB203580 (*SB*) followed by TGF-β. TGF-β treatment of ATDC5 cells increased Smad2 phosphorylation. The inhibitors did not significantly inhibit Smad2 phosphorylation. Similar results were obtained in two additional independent experiments.

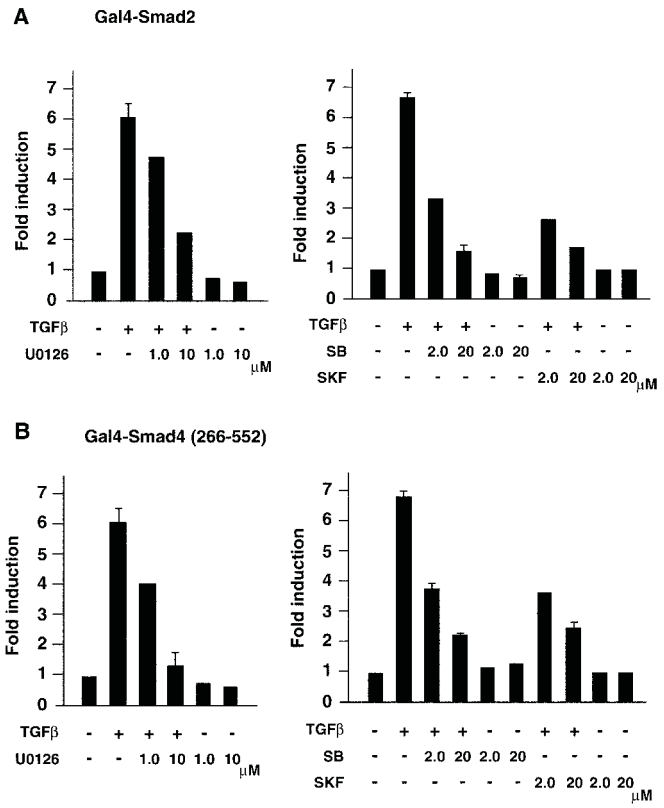

FIG. 6. **Interaction of Smad2/4 proteins with molecules downstream of ERK1/2 and p38 MAPK pathways.** Cells were cotransfected with Gal4-Luc reporter construct, Gal4-Smad2 or Gal4-Smad4-(266–552) expression construct, and pRLSV40 as a control. After 24 h, inhibitors were added under a serum-starved condition. After 1 h, cells were treated with TGF-β. Cells were collected after 16 h, and cell lysate was similarly subjected to the Dual-Luciferase reporter assay system. Luciferase activity is expressed as mean ± S.D. ($n = 3$).

TABLE I
Levels of Agc transcripts in ATDC5 cells

Relative levels of *Agc* transcription were normalized by the levels of GAPDH.

	Confluent	Day 7	Day 14
TGF-β −	1.0 ± 0.02	87.5 ± 6.5	114.0 ± 1.9
TGF-β +	12.5 ± 3.4	210.4 ± 6.1	166.0 ± 15.4

cells, *Agc* expression is maintained without activation of the Smad pathway.

DISCUSSION

In these studies, we have demonstrated that both Smad2/4 and the p38 MAPK and ERK1/2 pathways are rapidly and transiently activated following treatment with TGF-β in a mouse chondrogenic cell line, ATDC5, and that the activation of all three of these pathways is required for the transcriptional activation of *Agc* expression. Furthermore, we show that the TGF-β-induced transcriptional activation of a specific Smad2/4-dependent response element is dependent on the cooperative activation of p38 MAPK and ERK1/2 pathways in these cells. This suggests that there may be an important level of transcriptional cross-talk involving an interaction between these Smad proteins and components downstream of p38 MAPK and ERK1/2 in these cells. Previous studies have shown evidence that TGF-β treatment of ATDC5 cells enhances the expression of chondrocyte differentiation markers, including type II collagen (19), and is associated with activation of p38 MAPK and ERK1/2 pathways (23). Furthermore, it has recently been shown that overexpression of the inhibitory Smads 6 and 7 can block spontaneous differentiation of ATDC5 cells (60), indicating that Smad signaling is also required for this process of cellular differentiation. Our data are consistent with these observations, but they also now define the mechanism of cross-talk between the MAPK and Smad signaling pathways. Furthermore, the fact that p38 MAPK and ERK1/2 activation shows overlapping via distinct kinetics following treatment with TGF-β and that both pathways are required for maximal TGF-β-induced activation of *Agc* expression suggests that there may be some cooperative interaction or overlap of substrates downstream of these pathways that is involved in regulating *Agc* expression. It is still unclear what components downstream of these MAPK pathways are involved in regulating this interaction.

Contrasting with these findings in undifferentiated cells, we now show that differentiated ATDC5 cells demonstrate a distinct pattern of responses following stimulation with TGF-β. Although there is still rapid induction of Smad2 phosphorylation following treatment with TGF-β in these cells, indicating that there is an intact TGF-β receptor/Smad signaling pathway, the persistently high basal phosphorylation of p38 MAPK and ERK1/2 is actually inhibited following treatment with TGF-β. Furthermore, unlike the confluent ATDC5 cells, the differentiated cells have a high basal level of *Agc* expression which requires p38 MAPK and ERK1/2 activation without activation of the Smad2, indicating that *Agc* expression has escaped regulation by TGF-β-activated Smads. The role of Smad2/4 proteins in this response is less clear, as these do not

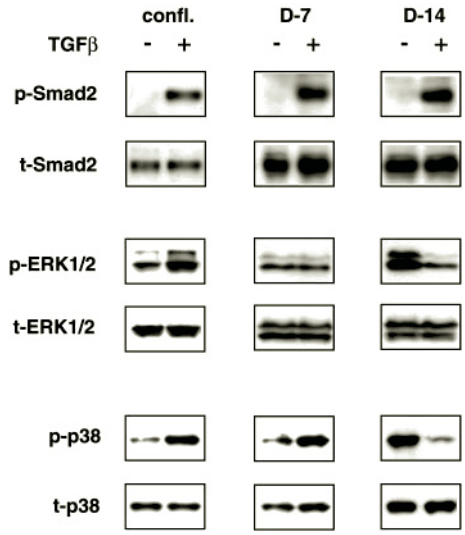

FIG. 7. **TGF-β-induced phosphorylation patterns of Smad2, ERK1/2, and p38 MAPK at different stages (confluency (*confl.*), day 7, and day 14 after confluency, as indicated in the figure) of ATDC5 cells.** Phosphorylation patterns after TGF-β treatment of 15 min for Smad2 and ERK1/2 and that of 1 h for p38 MAPK are shown.

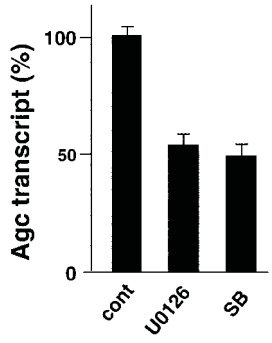

FIG. 8. **Inhibition of *Agc* expression in differentiated ATDC5 cells (day 14 after confluency) by specific inhibitors of ERK1/2 and p38 MAPK pathways.** Differentiated cells were serum-starved for 16 h and then U0126 or SB203580 (*SB*) was added to the culture for 1 h. The cells were then treated with TGF-β for 12 h. mRNA was prepared, and real time quantitative PCR analysis was performed. Both U0126 (10 μM) and SB203580 (*SB*, 20 μM) inhibit *Agc* transcript levels by ~50%. Similar results were obtained in two independent experiments.

seem to be essential to maintain the high basal levels of *Agc* expression seen in differentiated ATDC5 cells. Although TGF-β treatment does augment basal *Agc* expression, albeit to a lesser extent even in these cells, the functional requirement for the Smad2/4 protein complex appears to be critically dependent on the flux of p38 MAPK and ERK1/2 activation. In the presence of persistently high levels of p38 MAPK and ERK1/2 activation in the differentiated cells, activation of Smad2/4 is redundant, whereas at low transient levels of p38 MAPK and ERK1/2 activation in the confluent cells, the Smad2/4 pathway interaction is essential.

In differentiated chondrocytes, other members of the TGF-β superfamily may also participate in high levels of *Agc* expression. GDF5 and BMP-2 are induced in differentiating ATDC5 cells.[3] GDF5 has been shown to activate p38 MAPK and ERK1/2 in ATDC5 cells, and treatment with either of GDF5 or BMP-2 promotes chondrocyte differentiation (23). Constitutive up-regulation of these proteins could therefore account for the enhanced basal MAPK activation and subsequent *Agc* expression following differentiation in ATDC5 cells.

We have also shown that TGF-β-induced transcriptional activation of Smad2/4 in confluent ATDC5 cells is absolutely dependent on the activation of ERK1/2 and p38 MAPK pathways by TGF-β in heterologous transcriptional response assays. Although this is an artificial system that may not reflect the true interaction between these pathways in the context of a physiological TGF-β-dependent response, taken together with our observations on *Agc* expression, it does suggest that additional levels of cross-talk between these pathways may be involved in regulating these responses. One explanation for this cross-talk is that components of these pathways interact directly in the transcriptional complex. For example, although we do not know which specific intermediates are substrates of the p38 MAPK and ERK1/2 pathways in confluent ATDC5 cells, we do know that Smad4 is able to interact with a variety of different transcription factors, including ATF2, which is a downstream substrate of p38 MAPK (50). An alternative, although not exclusive, explanation for the cross-talk between Smad2/4 and MAPK pathways is that the activated MAPKs are themselves directly activating the Smad2/4 complex in these cells. This may result from the phosphorylation of specific MAPK sites in Smad2 if this phosphorylation event is required for its transcriptional activity and/or its ability to form heteromeric complexes with Smad4. It is notable that although we did not detect phospho-Smad2 in differentiated cells in the presence of a high basal levels of MAPK activity, it is possible that the phospho-Smad2-specific antibody, raised against a C-terminal phosphoserine peptide, could not recognize these phosphorylated residues in other domains of the molecule. This interpretation is compatible with our earlier findings that hepatocyte growth factor- and epidermal growth factor-induced activation of the ERK1/2 pathway phosphorylates Smad2 outside of the C-terminal phosphoreceptor sites and yet enhances the transcriptional activity of Smad2/4 (57). Furthermore, other groups studying the role of JNK/SAPK (58, 61) have shown that transient activation of these MAPK pathways can include phosphorylation and transcriptional activation of Smad2 and Smad3. These findings, along with our own, contrast with the observation that Ras transformation, which is associated with persistently high levels of MAPK activation, inhibits TGF-β-dependent activation of Smad2 and Smad3 (59). This suggests that the overall effects of these MAPK pathways on the transcriptional activation of these Smad proteins may be fundamentally dependent on both the level and persistence of MAPK pathway activation in a particular cell type.

Finally, our studies using cycloheximide to inhibit *de novo* protein synthesis also help to define the levels of transcriptional intermediates regulated by Smad2/4, p38 MAPK, and ERK1/2 pathways in defining *Agc* expression. We showed that concomitant treatment of the confluent ATDC5 cells with cycloheximide inhibited TGF-β-induced *Agc* expression, indicating that this response requires *de novo* protein synthesis and is not a direct signaling event. However, a subsequent time course of treatment with cycloheximide showed that the induction of *Agc* expression by TGF-β was not blocked if cycloheximide treatment was delayed for 2 h after treatment with TGF-β. The relatively short time course of this effect suggests that TGF-β signaling directly induces the transcription of an unidentified, and possibly cartilage-specific, immediate early response gene, which may act directly on the *Agc* promoter to activate gene expression. Sox9 and scleraxis are implicated for *Agc* expression in cartilage-derived cell line TC6 (62) and in osteoblastic osteosarcoma cell line ROS17/2.8 (63), respec-

[3] H. Watanabe, unpublished data.

tively. However, little expression of Sox9 is observed in both undifferentiated and differentiated ATDC5 cells. TGF-β also does not induce Sox9 expression in ATDC5 cells.[4] Scleraxis is expressed in undifferentiated ATDC5 cells, and its expression level is not changed with TGF-β treatment.[3] Thus, Sox9 and Scleraxis do not appear to be involved in TGF-β-mediated induction of aggrecan expression in ATDC5 cells.

Taken together, these data indicate that there are distinct transcriptional cross-talk mechanisms between p38 MAPK, ERK1/2, and Smad signaling pathways that are involved in regulating *Agc* expression at different stages of chondrocyte differentiation, and these data provide new insights into the regulatory mechanisms defining chondrocytic phenotypes. Further work will be necessary to define the precise nature of the cartilage-specific transcriptional intermediates regulating *Agc* expression.

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