FAST TRACK
Journal of Cellular Biochemistry 107:622–629 (2009)

Journal of Cellular Biochemistry

Transforming Growth Factor-β3-Induced Smad Signaling Regulates Actin Reorganization During Chondrogenesis of Chick Leg Bud Mesenchymal Cells

Dongkyun Kim,[1] Jungsoo Kim,[2†] Shin-Sung Kang,[2] and Eun-Jung Jin[1,3*]

[1] *Department of Biological Sciences, College of Natural Sciences, Wonkwang University, Iksan, Chunbuk 570-749, South Korea*

[2] *Department of Biology, College of Natural Sciences, Kyungpook National University, Daegu 702-701 South Korea*

[3] *Institute of Biotechnology, Wonkwang University, Iksan, Chunbuk 570-749, Korea*

ABSTRACT

Endochondral ossification is characterized by a significant interdependence between cell shape and cytoskeletal organization that accompanies the onset of chondrogenic signaling. However, the mechanisms mediating these interactions have not been well studied. Here, treatment with transforming growth factor (TGF)-β3 at a later stage of chondrogenesis led to activation of Smad-2 signaling and the formation of intense stress fibers, which resulted in suppressing chondrogenic differentiation of leg bud mesenchymal cells. Moreover, specific siRNA knockdown of Smad-2 reduced TGF-β3-induced stress fibers via physical interactions with β-catenin. In conclusion, our results indicate that TGF-β3-induced Smad signaling, in conjunction with β-catenin, is involved in the reorganization of the actin cytoskeleton into a cortical pattern with a concomitant rounding of cells. J. Cell. Biochem. 107: 622–629, 2009. © 2009 Wiley-Liss, Inc.

KEY WORDS: SMAD; β-CATENIN; ACTIN CYTOSKELETON; CHONDROGENIC DIFFERENTIATION

Chondrogenesis is a tightly regulated process in which multipotential mesenchymal cells differentiate into chondrocytes to form cartilage [Hall, 1981; Cancedda et al., 1995; Goldring et al., 2006]. This process is initiated by commitment to the chondrogenic lineage and condensation of cells. This is followed by differentiation into chondrocytes with spatially and temporally regulated expressions of cartilage-specific genes, such as type II collagen, aggrecan and sulfated proteoglycans [Shinomura et al., 1993], to maintain the chondrocyte phenotype [Kamiya et al., 2006]. A dramatic morphological transition in cell shape, from fibroblastoid to round or polygonal morphologies, is a critical regulatory factor for chondrogenesis [Zanetti and Solursh, 1984; Yabu et al., 1991; Benjamin et al., 1994]. In addition, chondrocytes display a primarily cortical organization of actin filaments, whereas undifferentiated mesenchymal cells are characterized by a fibrillar organization [Zhang et al., 2006].

The transforming growth factor (TGF)-β superfamily, comprised of TGF-βs, bone morphogenetic proteins, activins and related proteins, regulates cell function and plays key roles in development, cellular differentiation, apoptosis, and carcinogenesis [Hoodless et al., 1996; Itoh et al., 2000; Massague, 2000; Derynck and Zhang, 2003]. In addition to the BMP/Smad signaling pathway, p38 kinase is also stimulated by BMP-2 [Iwasaki et al., 1999; Kimura et al., 2000]. BMP-2 activates MAP kinase kinase kinases, including TAK1. Subsequently, a MAP kinase kinase kinase induces MKK3 or MKK6 that directly phosphorylates and activates p38 kinase [Raingeaud et al., 1996; Shibuya et al., 1996; Kimura et al., 2000]. The phosphorylated p38 kinase, in turn, regulates gene expressions in the nucleus. In addition, BMP-2 treatment also results in increased protein levels of β-catenin, a known N-cadherin-associated Wnt signal transducer [Haas and Tuan, 2000; Fischer et al., 2002; Jin et al., 2006]. Taken together, these phenomena suggests that functional cross-talk occurs between the BMP-2 and Wnt signaling pathways.

Previously, we demonstrated that an increase of Sox9 protein, resulting from downregulation of β-catenin/Wnt-7a signaling, was mediated by p38 MAPK during BMP-2 induced chondrogenesis

Grant sponsor: Korea Research Foundation (KRF); Grant number: C00731.

†This article was published online on 28 May 2009. An error was subsequently identified. This notice is included in the online and print versions to indicate that both have been corrected 8 June 2009.

*Correspondence to: Dr. Eun-Jung Jin, PhD, Department of Biological Sciences, College of Natural Sciences, Wonkwang University, Iksan, Chunbuk 570-749, South Korea. E-mail: jineunjung@wku.ac.kr

Received 5 January 2009; Accepted 2 April 2009 • DOI 10.1002/jcb.22191 • © 2009 Wiley-Liss, Inc.
Published online 28 May 2009 in Wiley InterScience (www.interscience.wiley.com).

in chick wing bud mesenchymal cells [Jin et al., 2006]. We also demonstrated that TGF-β3 downregulated connexin43. This induced apoptotic cell death via activation of ERK and suppressed PKC-α activation in leg bud mesenchymal cells [Jin et al., 2008]. However, Smad-dependent TGF-β3 signaling during chondrogenic differentiation of chick limb bud mesenchymal cells has not been extensively studied [Hoodless et al., 1996; Heldin et al., 1997; Nakao et al., 1997; Itoh et al., 2000], even though Smad is the major mechanism that mediates the TGF-β3 signaling pathway [Nakao et al., 1997; Miyazono, 2000; Moustakas et al., 2001; Derynck and Zhang, 2003]. Therefore, in the present study, we investigated the functional role of Smad during chondrogenic differentiation using cultured chick leg bud mesenchymal cells. The activation of Smad signaling was found to act as a negative regulator of the morphological transitions of cells at a later stage of chondrogenesis. Furthermore, we found that activation of reorganization of actin cytoskeleton induced by Smad signaling may occur through an interaction with β-catenin.

MATERIALS AND METHODS

CELL CULTURE AND TREATMENTS

Mesenchymal cells derived from the distal tips of Hamburger–Hamilton (HH) stage 22/23 embryo leg buds of fertilized White Leghorn chicken eggs were micromass cultured as previously described [Jin et al., 2006]. Briefly, the cells were suspended at a density of 2×10^7 cells/ml in Ham's F-12 medium containing 10% fetal bovine serum (FBS), 100 IU/ml penicillin, and 100 μg/ml streptomycin (Gibco Invitrogen, Grand Island, NY). The cells were plated in 3 drops (15 μl each) dispensed into 35-mm culture dishes or 19 drops (15 μl each) into 60-mm Corning culture dishes, and incubated for 1 h at 37°C under 5% CO_2 to allow attachment. The cells were maintained in 1 ml of culture medium after 72 h of culture in the absence or presence of 5 ng/ml recombinant Human TGF-β3 (R&D, Minneapolis, MN).

TRANSFECTION OF SMAD-2-SPECIFIC SMALL INTERFERING RNA (siRNA)

The siRNA oligonucleotide (5′-CAGGACAGCTGGATGAGCTTGAG-AA-3′) and the corresponding sense oligonucleotide (′5-TTCT-CAAGCTCATCCAGCTGTCCTG-3′) were obtained from Invitrogen. Transfection of the RNA oligonucleotides (50 nM final RNA concentration) into chick wing bud mesenchymal cells used Fugene 6 (Roche) following the manufacturer's protocol.

ANALYSIS OF CELL DIFFERENTIATION

Chondrogenesis was measured by Alcian blue staining of sulfated cartilage glycosaminoglycans. To demonstrate the deposition of cartilage matrix proteoglycans, representative cultures were collected at day 5 of incubation and stained with 0.5% Alcian blue 8GX, pH 1.0 [Lev and Spicer, 1964; Hassel and Horigan, 1982]. Alcian-blue-bound sulfated glycosaminoglycans were extracted with 6 M guanidine–HCl and quantified by measuring the absorbance of the extracts at 600 nm.

SEMI-QUANTITATIVE RT-PCR

Total RNA was isolated using Trizol (Gibco Invitrogen) at days 1, 3, and 5 of culture. cDNA was synthesized from a 2 μg sample of RNA in 20 μl of master mix for reverse transcription containing 200 U/μl Superscript III (Gibco Invitrogen), 5 mM $MgCl_2$, PCR Buffer II, 1 mM dNTP, 1 U/μl RNase inhibitor, and 2.5 mM oligo dT in DEPC-treated distilled water. The master mix was incubated in a Perkin-Elmer GeneAmp PCR system 9600 (Wellesley, MA) at 42°C for 55 min and 99°C for 5 min. Synthesized cDNAs were subjected to 25 cycles of PCR amplification under the following conditions: 94°C denaturing for 40 s, 55°C annealing for 40 s, and 72°C extension for 40 s. Subsequently, PCR products were electrophoresed on a 2.0% agarose gel. Primers for *Smad2*: 5′-ACACTCATTCCATTCCTGAG-3′, 5′-GAAGGTTTCTCCCACTCTTT-3′, for *Smad3*: 5′-AGAACACTAA-CTTCCCAG-3′, 5′-GGTTTACAGACTGAGCCAAG-3′, for *GAPDH*: 5′-GATGGGTGTCAACCATGAGA AA-3′, 5′-ATCAAAGGTGGAAGA ATGGCTG-3′.

WESTERN BLOT ANALYSIS

Proteins (30 μg) or conditioned media were separated by 10% polyacrylamide gel electrophoresis containing 0.1% SDS and transferred to nitrocellulose membrane (Schleicher and Schuell, Germany). The membranes were incubated for 1 h at room temperature in blocking buffer (20 mM Tris–HCl, 137 mM NaCl, pH 8.0, containing 0.1% Tween and 3% non-fat dry milk), and probed with antibodies against Smad, pSmad, β-catenin (R&D), glycogen synthase kinase (GSK), phosphorylated glycogen synthase kinase (pGSK; Calbiochem, La Jolla, CA), and HSP70 (Stressgene, San Diego, CA). The blots were developed with a peroxidase-conjugated secondary antibody and reacted proteins were visualized using the electrochemiluminescence (ECL) system (Pierce Biotechnology, Inc., Rockford, MN).

ACTIN STAINING AND IMMUNOCYTOCHEMISTRY

Cells grown on cover slips were treated with various chemicals as indicated in the figure legends, washed three times with phosphate-buffered saline (PBS), then fixed and permeabilized as described above. For actin staining, each culture was stained with Alexa488-phalloidin (Molecular Probes, Eugene, OR) prepared in PBS containing 1% (v/v) bovine serum albumin for 1 h at room temperature in a lightproof box. For immunocytochemistry, cultured cells were double stained with antibodies against Smad2 and β-catenin, washed three times with PBS, and incubated with secondary antibody conjugated with either Cy2 (for Smad2 detection) or TRITC (for β-catenin detection). Cultures were then washed three times with water and mounted with Gel/Mount (Biomedia, Foster City, CA). The slides were examined using a confocal microscope (MRC 1024/ES, Bio-Rad Laboratory, CA). Cultures were imaged at scan speed 8 (1.76 μs per voxel, 1 s per section) in 1-μm-thick Z-sections for a total of 4–10 slices.

CELL VIABILITY AND PROLIFERATION ASSAY

Proliferation of mesenchymal cells was determined by direct counting of cells from micromass cultures. Control and treated cultures were maintained for the indicated number of days, cells were detached with trypsin/EDTA solution and counted in triplicate using a hemacytometer.

STATISTICAL ANALYSIS

Results of cell-adhesion experiments were pooled from five replicate samples derived from three independent experiments and expressed as means ± SEM. One-way analysis of variance (ANOVA) with Tukey post hoc comparisons of groups was used to test for significant effects. Differences were considered significant at $P < 0.05$.

RESULTS

Pleiotropic members of the TGF-β superfamily are involved with de novo endochondral bone formation. These factors are soluble signals for tissue morphogenesis during sculpting of the multicellular mineralized structures of the bone marrow organ [Ripamonti et al., 2000; Ripamonti, 2004]. To analyze the TGF-β3-specific signaling, HH stage 22/23 chondroblasts were isolated from chick leg buds and cultured at a density of 2×10^7 cells/ml.

As chondrogenesis progressed, exposing leg bud mesenchymal cells to TGF-β3 resulted in increased and sustained levels of unphosphorylated Smad-2 and -3, pGSK-3β, and β-catenin. These levels decreased at a later stage of chondrogenesis (i.e., day 4 in the control culture; Fig. 1A). These findings suggested that downregulation of Smad and Wnt-related signaling might be required at a later stage of chondrogenesis.

Next, we examined the effects of Smad-2 during the later culture periods. When cells were exposed to 5 ng/ml of TGF-β at 3–4 days of culture, the level of Alcian blue staining was reduced in the micromass culture, which indicated that chondrogenic differentiation had been suppressed. Inhibition of glycosaminoglycan production by TGF-β3 was confirmed quantitatively by dye extraction and measurement of the absorbance. Suppressed chondrogenic differentiation by TGF-β3 was stimulated by Smad-2 knockdown (Fig. 1B).

Chondrogenic differentiation of mesenchymal cells is regulated at various stages, including proliferation of chondroblast competent cells, precartilage condensation, and formation of cartilage nodules [Quarto et al., 1997; DeLise et al., 2000; Knudson and Knudson, 2001]. Therefore, we examined the effects of siRNA-mediated *smad-2* knockdown on the growth of mesenchymal cells using its specific siRNA. Cell numbers were assessed at days 3, 4, and 5 after transfection. Knockdown of *smad-2* had little or no effect on the proliferation of chondroblast competent cells (Fig. 1C). These findings suggest that the positive role of Smad-2 on chondrogenic differentiation of leg bud mesenchymal cells occurred independently of cell proliferation and/or cellular condensation.

It has previously been shown that reorganization of the actin cytoskeleton into a cortical pattern with concomitant rounding of cells and fewer stress fibers, occurred after 4 days of culture [Zakany et al., 2001]. And our laboratory (2007) also showed that treatment with cytochalasin D (CD) at later period of culture to disrupt polymerization of actin cytoskeleton, caused inhibition of chondrogenic differentiation indicating that microtubule polymerization is vital for chondrogenesis. Therefore, we evaluated the blockage of Smad signaling to determine if it was associated with rearrangement of the actin cytoskeleton. Since chondrogenesis of limb mesenchymal cells are cell-density dependent, physical pressure from multilayered culture (micromass culture) would be simply induced actin reorganization. To investigate this, chondroblasts cultured in a density-dependent manner (monolayer vs. micromass) were transfected with Smad-2-specific siRNA in the absence or presence of 5 ng/ml of TGF-β at 3 days of culture. The levels of actin stress fibers were markedly intensified after TGF-β3 treatment when compared to the control culture, pointing to an involvement of the actin cytoskeleton with Smad signaling. However, knockdown of Smad-2 at day 3 of culture resulted in decreased levels of TGF-β3-induced stress fibers as well as a significant increase of cell rounding compared to control cultures both for monolayer- and micromass-cultured chondroblasts at 4 days of culture (Fig. 2). These finding indicate that the physical pressure from increased cell density may not cause actin cytoskeleton and downregulation of Smad signaling supports the establishment of chondrocyte-specific cell shape and actin reorganization.

Knockdown of Smad-2 decreased the TGF-β3-induced levels of N-cadherin and β-catenin at days 3 and 4 of culture (Fig. 3A). Many studies have indicated that physical interactions between Smad and β-catenin occur in a variety of cells [Nishita et al., 2000; Warner et al., 2005]. Therefore, we examined the physical interactions and distributions of Smad2 and β-catenin. Immunoprecipitation using whole lysates of HH stage 23 chick embryos with Smad2 antibody demonstrated that β-catenin protein co-precipitated with Smad2 (Fig. 3B). Next, non-immune IgG was used, instead of anti-Smad2 antibody as a proper control to demonstrate that the interaction was specific and not simply due to binding of the agarose beads. Immunocytochemistry using specific antibodies showed overlapping distributions of Smad2 and β-catenin (Fig. 3C). Actin stress fibers were markedly intensified along with the accumulation of β-catenin by GSK-3β inhibitor VI, which is a specific inhibitor of GSK-3β activity (Fig. 3D).

Previously, we demonstrated that overexpression of β-catenin in primary mesenchymal cells caused a marked inhibition of cellular condensation and chondrogenesis [Jin et al., 2006]. These results suggested that a decreased level of β-catenin was required for precartilage condensation and additional chondrogenic differentiation. The results shown here suggest that TGF-β3-induced β-catenin, via upregulation of Smad signaling, acts as a negative regulator for reorganization of the actin cytoskeleton, as well as inhibition of precartilage condensation.

DISCUSSION

Regulation of actin reorganization and contractility allows cells to control their shape, movement, division and secretion, which are vital processes known to be coordinated by the actions of several signal transduction pathways [Lelkes et al., 1986; Keller and Niggli, 1995; Wang and Newman, 2003].

Rapid reorganization of the actin cytoskeleton is one of the primary cellular responses to many extracellular signals. TGF-β induces a rapid reorganization of the actin cytoskeleton in epithelial cells, whereas a prolonged incubation with TGF-β results in the

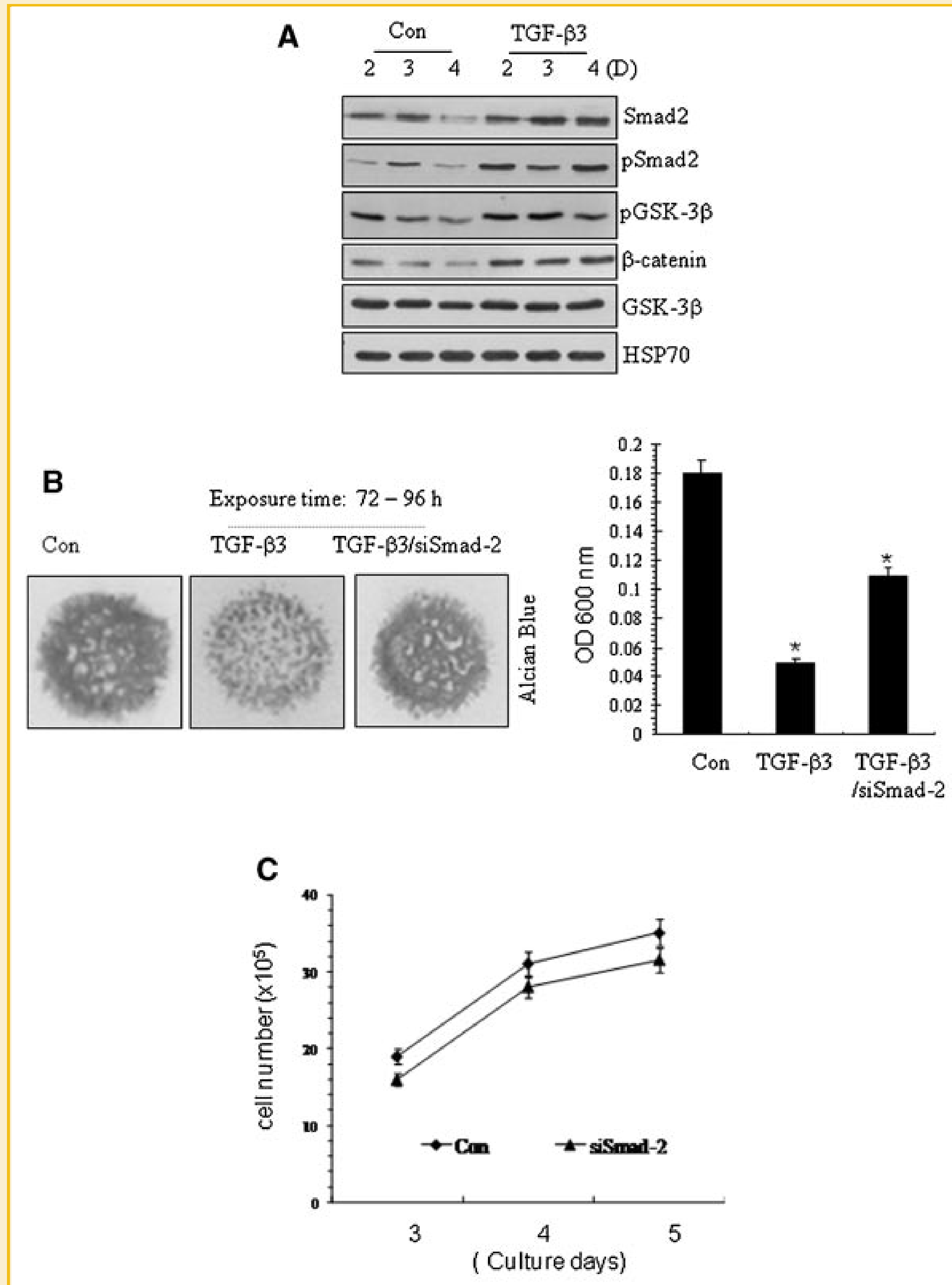

Fig. 1. Mesenchymal competent cells were prepared as described in Materials and Methods Section. A: Changes in the mRNA and protein levels of (p)Smad2, -3, (p)GSK-3β, and β-catenin during chondrogenesis were analyzed. B: Cells were transfected with Smad-2-specific siRNA in the absence or presence of 5 ng/ml of TGF-β and then stained with PNA on day 3 of culture and Alcian blue on day 5 of culture (right panel). Quantification of chondrogenesis was done by measuring the absorbance of bound Alcian blue at 600 nm (left panel). C: The number of viable cells in the control culture was the determined at the indicated days. The results shown are representative of at least four independent experiments. *Statistically significant differences as compared with the control cells ($P < 0.01$).

formation of stress fibers [Bakin et al., 2002; Edlund et al., 2002] with activation of the Rho family of GTPase, Rac, CDC42, and RhoA by regulating organization of actin filaments [Jaffe and Hall, 2002] through TGF-b-mediated signaling including Smads, mitogen-activated protein kinases (MAPKs), Rho kinases, and Akt/PKB [Derynck and Zhang, 2003; Roberts and Wakefield, 2003].

Despite the current understanding of the Smad pathways, the mechanisms by which Smad signals modulate cell shape and the actin cytoskeleton have not yet completely elucidated. Furthermore, the results of studies conducted to evaluate these mechanisms have produced conflicting results. Indeed, some studies have shown that Smad pathway is critical for such responses [Bakin et al., 2002; Tsai et al., 2007], while others have shown that non-Smad signaling effectors, such as Rho GTPases, phospholipid 3′-kinase, or p38 mitogen-activated protein kinase, play more important roles for in these responses [Takenawa and Itoh, 2001;

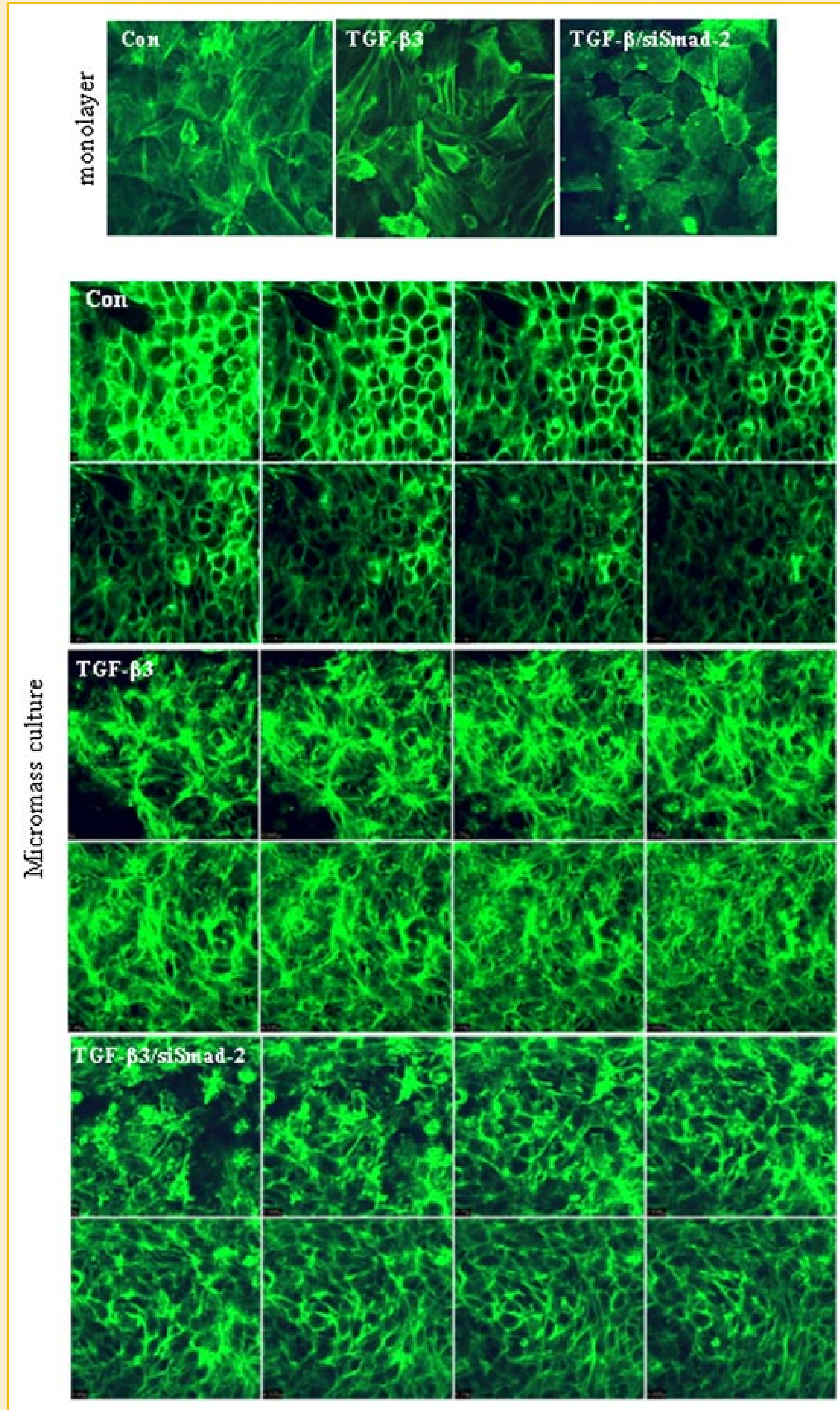

Fig. 2. Downregulation of the Smad pathway blocked TGF-β3-induced chondro-inhibition via modulation of an actin cytoskeleton. Mesenchymal cells were cultured as a monolayer or at a density of 2×10^7 cells/ml and transfected with Smad-2-specific siRNA in the absence or presence of 5 ng/ml of TGF-β at 3 days of culture. Cells were immunostained for F-actin with Alexa488-phalloidin at 4 days of culture. Results shown are representative of at least four independent experiments. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Bettache et al., 2003; Maddox and Burridge, 2003; Hirano et al., 2005]. Ectopically expressed Smad7 inhibited TGF-β1-induced actin cytoskeleton reorganization and RhoA-GTP levels in the Swiss 3T3 system [Vardouli et al., 2005] whereas Smad7 positively modulates the activity of small GTPases Cdc42 and RhoA in prostate carcinoma cells [Edlund et al., 2002]. These studies suggest that

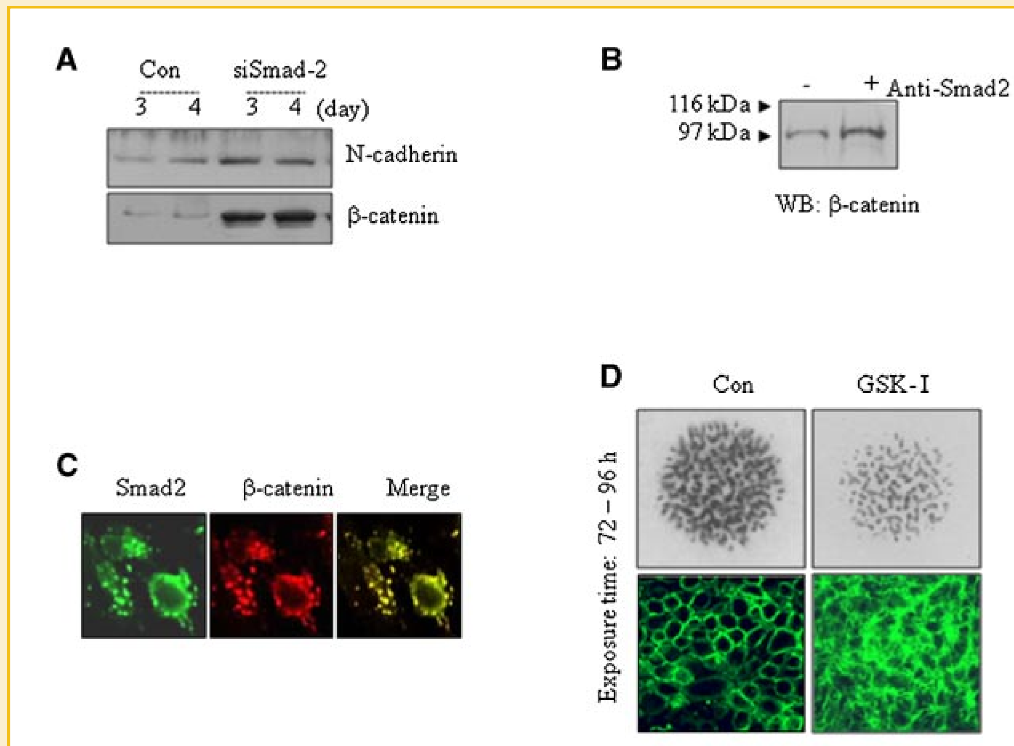

Fig. 3. The Smad pathway affects the actin cytoskeleton in conjunction with β-catenin. A: Cells were transfected with Smad-2-specific siRNA in the absence or presence of 5 ng/ml of TGF-β and analyzed for changes of protein levels for β-catenin and N-cadherin. B: Embryo lysates from HH stage 23 chick immunoprecipitated with anti-Smad2 antibody. Immunoprecipitates were subjected to Western blot analysis with anti-β-catenin antibody. Non-immune IgG (ideally) was used as a negative control. C: Immunocytochemistry using anti-Smad2 and anti-β-catenin antibodies at 1 day of culture. D: Mesenchymal cells were cultured at a density of 2×10^7 cells/ml with or without 10 μM GSK-3β inhibitor VI (GSK-I) at 3-day culture for 24 h. Cells were stained with Alcian blue at day 5 of culture (upper panel) and immunostained for F-actin with Alexa488-phalloidin at 4 days of culture (lower panel). Results shown are representative of at least four independent experiments. *Statistically significant differences compared with control cells ($P < 0.01$). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Smad signaling on actin cytoskeleton reorganization may be due to cell type dependent.

In the present study, we addressed the Smad signaling pathway in the chick leg bud mesenchymal cells that affects reorganization of the actin cytoskeleton. We found that this reorganization occurs independently of cell proliferation and/or cellular condensation Additionally, we found that blocking Smad signaling increased cell rounding, a typical dramatic characteristic of differentiated chondrocytes. Furthermore, the coordination and organization of the cell shape and chondrocyte phenotype by actin was inhibited by activation Smad by TGF-β3.

Several studies have shown that α- and β-catenin interact with the actin cytoskeleton [Akiyama and Kawasaki, 2006; Barth et al., 2006]. Catenin binds to and bundles actin filaments [Rimm et al., 1995; Pokutta et al., 2002], and interacts with actin binding proteins, including vinculin [Watabe-Uchida et al., 1998; Weiss et al., 1998], α-actinin [Knudsen et al., 1995; Hazan et al., 1997], ZO-1 [Itoh et al., 1997], spectrin [Pradhan et al., 2001], Ajuba [Marie et al., 2003], afadin [Pokutta et al., 2002], and formin [Kobielak et al., 2004]. Nishita et al. [2000] identified an endogenous β-catenin/Smad4 complex, capable of forming a Wnt-dependent complex in NIH3T3 and L929 cells. In this study, we also observed physical interactions between Smad2 and β-catenin, which suggested the convergence of these two signaling pathways. This interaction-induced Wnt-signaling triggered a dense fibrillar actin reorganization in chick leg bud mesenchymal cells, which was overcome by downregulation of the Smad pathway.

In the present study, we investigated the functional role of Smad during the late stages of chondrogenic differentiation using cultured chick leg bud mesenchymal cells. Smad was found to be a negative regulator of the morphological transition of the actin cytoskeleton in a conjunction with the β-catenin signaling pathway.

ACKNOWLEDGMENTS

This work was supported by Korea Research Foundation (KRF) by the Korean Government (C00731).

REFERENCES

Akiyama T, Kawasaki Y. 2006. Wnt signaling and the actin cytoskeleton. Oncogene 25(57):7538–7544.

Bakin AV, Rinehart C, Tomlinson AK, Arteaga CL. 2002. p38 mitogen-activated protein kinase is required for TGF-beta-mediated fibroblastic transdifferentiation and cell migration. J Cell Sci 115(Pt 15):3193–3206.

Barth AI, Nathke IS, Nelson WJ. 2006. Cadherins, catenins and APC protein: Interplay between cytoskeletal complexes and signaling pathways. Curr Opin Cell Biol 9(5):683–690.

Benjamin M, Archer CW, Ralphs JR. 1994. Cytoskeleton of cartilage cells. Microsc Res Tech 28(5):372–377.

Bettache N, Baisamy L, Baghdiguian S, Payrastre B, Mangeat P, Bienvenue A. 2003. Mechanical constraint imposed on plasma membrane through transverse phospholipid imbalance induces reversible actin polymerization via phosphoinositide 3-kinase activation. J Cell Sci 116(Pt 11):2277–2284.

Cancedda R, Descalzi Cancedda F, Castagnola P. 1995. Chondrocyte differentiation. Int Rev Cytol 159:265–358.

DeLise AM, Fischer L, Tuan RS. 2000. Cellular interactions and signaling in cartilage development. Osteoarthritis Cartilage 8(5):309–334.

Derynck R, Zhang YE. 2003. Smad-dependent and Smad-independent pathways in TGF-beta family signaling. Nature 425(6958):577–584.

Edlund S, Landstrom M, Heldin C-H, Aspenström P. 2002. Transforming growth factor-beta-induced mobilization of actin cytoskeleton requires signaling by small GTPases Cdc42 and RhoA. Mol Biol Cell 13:902–914.

Fischer L, Boland G, Tuan RS. 2002. Wnt signaling during BMP-2 stimulation of mesenchymal chondrogenesis. J Cell Biochem 84(4):816–831.

Goldring MB, Tsuchimochi K, Ijiri K. 2006. The control of chondrogenesis. J Cell Biochem 97(1):33–44.

Haas AR, Tuan RS. 2000. Murine C3H10T1/2 multipotential cells as an in vitro model of mesenchymal chondrogenesis. Methods Mol Biol 137:383–389.

Hall BK. 1981. Intracellular and extracellular control of the differentiation of cartilage and bone. Histochem J 13(4):599–614.

Hassel JR, Horigan EA. 1982. Chondrogenesis: A model developmental system for measuring teratogenic potential of compounds. Teratog Carcinog Mutagen 2:325–331.

Hazan RB, Kang L, Roe S, Borgen PI, Rimm DL. 1997. Vinculin is associated with the E-cadherin adhesion complex. J Biol Chem 272(51):32448–32453.

Heldin CH, Miyazono K, Ten Dijke P. 1997. TGF-beta signaling from cell membrane to nucleus through SMAD proteins. Nature 390(6659):465–471.

Hirano S, Sun X, DeGuzman CA, Ransom RF, McLeish KR, Smoyer WE, Shelden EA, Welsh MJ, Benndorf R. 2005. p38 MAPK/HSP25 signaling mediates cadmium-induced contraction of mesangial cells and renal glomeruli. Am J Physiol Renal Physiol 288(6):F1133–F1143.

Hoodless PA, Haerry T, Abdollah S, Stapleton M, O'Connor MB, Attisano L, Wrana JL. 1996. MADR1, a MAD-related protein that functions in BMP2 signaling pathways. Cell 85(4):489–500.

Itoh M, Nagafuchi A, Moroi S, Tsukita S. 1997. Involvement of ZO-1 in cadherin-based cell adhesion through its direct binding to alpha catenin and actin filaments. J Cell Biol 138(1):181–192.

Itoh S, Itoh F, Goumans MJ, Ten Dijke P. 2000. Signaling of transforming growth factor-beta family members through Smad proteins. Eur J Biochem 267(24):6954–6967.

Iwasaki S, Iguchi M, Watanabe K, Hoshino R, Tsujimoto M, Kohno M. 1999. Specific activation of the p38 mitogen-activated protein kinase signaling pathway and induction of neurite outgrowth in PC12 cells by bone morphogenetic protein-2. J Biol Chem 274(37):26503–26510.

Jaffe AB, Hall A. 2002. Rho GTPases in transformation and metastasis. Adv Cancer Res 84:57–80.

Jin EJ, Lee SY, Choi YA, Jung JC, Bang OS, Kang SS. 2006. BMP-2-enhanced chondrogenesis involves p38 MAPK-mediated down-regulation of Wnt-7a pathway. Mol Cells 22(3):353–359.

Jin EJ, Lee SY, Jung JC, Bang OS, Kang SS. 2008. TGF-beta3 inhibits chondrogenesis of cultured chick leg bud mesenchymal cells via down-regulation of connexin 43 and integrin beta4. J Cell Physiol 214(2):345–353.

Kamiya N, Watanabe H, Habuchi H, Takagi H, Shinomura T, Shimizu K, Kimata K. 2006. Versican/PG-M regulates chondrogenesis as an extracellular matrix molecule crucial for mesenchymal condensation. Biol Chem 281(4): 2390–2400.

Keller H, Niggli V. 1995. Effects of cytochalasin D on shape and fluid pinocytosis in human neutrophils as related to cytoskeletal changes (actin, alpha-actinin and microtubules). Eur J Cell Biol 66(2):157–164.

Kimura N, Matsuo R, Shibuya H, Nakashima K, Taga T. 2000. BMP2-induced apoptosis is mediated by activation of the TAK1-p38 kinase pathway that is negatively regulated by Smad6. J Biol Chem 275(23):17647–17652.

Knudsen KA, Soler AP, Johnson KR, Wheelock MJ. 1995. Interaction of alpha-actinin with the cadherin/catenin cell-cell adhesion complex via alpha-catenin. J Cell Biol 130(1):67–77.

Knudson CB, Knudson W. 2001. Cartilage proteoglycans. Semin Cell Dev Biol 12(2):69–78.

Kobielak A, Pasolli HA, Fuchs E. 2004. Mammalian formin-1 participates in adherens junctions and polymerization of linear actin cables. Nat Cell Biol 6(1):21–30.

Lelkes PI, Friedman JE, Rosenheck K, Oplatka A. 1986. Destabilization of actin filaments as a requirement for the secretion of catecholamines from permeabilized chromaffin cells. FEBS Lett 208(2):357–363.

Lev R, Spicer SS. 1964. Specific staining of sulfate groups with alcian blue at low pH. J Histochem Cyctochem 12:309.

Maddox AS, Burridge K. 2003. RhoA is required for cortical retraction and rigidity during mitotic cell rounding. J Cell Biol 160(2):255–265.

Marie H, Pratt SJ, Betson M, Epple H, Kittler JT, Meek L, Moss SJ, Troyanovsky S, Attwell D, Longmore GD, Braga VM. 2003. The LIM protein Ajuba is recruited to cadherin-dependent cell junctions through an association with alpha-catenin. J Biol Chem 278(2):1220–1228.

Massague J. 2000. How cells read TGF-beta signals. Nat Rev Mol Cell Biol 1(3):169–178.

Miyazono K. 2000. TGF-beta signaling by Smad proteins. Cytokine Growth Factor Rev 11(1–2):15–22.

Moustakas A, Souchelnytskyi S, Heldin CH. 2001. Smad regulation in TGF-beta signal transduction. J Cell Sci 114(24):4359–4369.

Nakao A, Imamura T, Souchelnytskyi S, Kawabata M, Ishisaki A, Oeda E, Tamaki K, Hanai J, Heldin CH, Miyazono K, Ten Dijke P. 1997. TGF-beta receptor-mediated signaling through Smad2, Smad3 and Smad4. EMBO J 16(17):5353–5362.

Nishita M, Hashimoto MK, Ogata S, Laurent MN, Ueno N, Shibuya H, Cho KW. 2000. Interaction between Wnt and TGF-beta signaling pathways during formation of Spemann's organizer. Nature 403(6771):781–785.

Pokutta S, Drees F, Takai Y, Nelson WJ, Weis WI. 2002. Biochemical and structural definition of the l-afadin- and actin-binding sites of alpha-catenin. J Biol Chem 277(21):18868–18874.

Pradhan D, Lombardo CR, Roe S, Rimm DL, Morrow JS. 2001. alpha -Catenin binds directly to spectrin and facilitates spectrin-membrane assembly in vivo. J Biol Chem 276(6):4175–4181.

Quarto R, Campanile G, Cancedda R, Dozin B. 1997. Modulation of commitment, proliferation, and differentiation of chondrogenic cells in defined culture medium. Endocrinology 138(11):4966–4976.

Raingeaud J, Whitmarsh AJ, Barrett T, Dérijard B, Davis RJ. 1996. MKK3- and MKK6-regulated gene expression is mediated by the p38 mitogen-activated protein kinase signal transduction pathway. Mol Cell Biol 16(3):1247–1255.

Rimm DL, Koslov ER, Kebriaei P, Cianci CD, Morrow JS. 1995. Alpha 1(E)-catenin is an actin-binding and -bundling protein mediating the attachment of F-actin to the membrane adhesion complex. Proc Natl Acad Sci USA 92(19):8813–8817.

Ripamonti U. 2004. Soluble, insoluble and geometric signals sculpt the architecture of mineralized tissues. J Cell Mol Med 8(2):169–180.

Ripamonti U, Crooks J, Matsaba T, Tasker J. 2000. Induction of endochondral bone formation by recombinant human transforming growth factor-beta2 in the baboon (Papioursinus). Growth Factors 17(4):269–285.

Roberts AB, Wakefield LM. 2003. The two faces of transforming growth factor β in carcinogenesis. Proc Natl Acad Sci USA 100:8621–8623.

Shibuya H, Moriguchi T, Kuroyanagi N, Yamaguchi K, Gotoh Y, Irie K, Kano T, Shirakabe K, Muro Y, Matsumoto K, Nishida E, Hagiwara M. 1996. A novel kinase cascade mediated by mitogen-activated protein kinase kinase 6 and MKK3. J Biol Chem 271(23):13675–13679.

Shinomura T, Nishida Y, Ito K, Kimata K. 1993. cDNA cloning of PG-M, a large chondroitin sulfate proteoglycan expressed during chondrogenesis in chick limb buds. Alternative spliced multiforms of PG-M and their relationships to versican. J Biol Chem 268(19):14461–14469.

Takenawa T, Itoh T. 2001. Phosphoinositides, key molecules for regulation of actin cytoskeletal organization and membrane traffic from the plasma membrane. Biochim Biophys Acta 1533(3):190–206.

Tsai TT, Guttapalli A, Oguz E, Chen LH, Vaccaro AR, Albert TJ, Shapiro IM, Risbud MV. 2007. Fibroblast growth factor-2 maintains the differentiation potential of nucleus pulposus cells in vitro: Implications for cell-based transplantation therapy. Spine 32(5):495–502.

Vardouli L, Moustakas A, Stournaras C. 2005. LIM-kinase 2 and Cofilin Phosphorylation Mediate Actin Cytoskeleton Reorganization Induced by Transforming Growth Factor-β. J Bio Chem 280(12):11448–11457.

Wang Z, Newman WH. 2003. Smooth muscle cell migration stimulated by interleukin 6 is associated with cytoskeletal reorganization. J Surg Res 111(2):261–266.

Warner DR, Greene RM, Pisano MM. 2005. Cross-talk between the TGF-beta and Wnt signaling pathways in murine embryonic maxillary mesenchymal cells. FEBS Lett 579(17):3539–3546.

Watabe-Uchida M, Uchida N, Imamura Y, Nagafuchi A, Fujimoto K, Uemura T, Vermeulen S, van Roy F, Adamson ED, Takeichi M. 1998. alpha-Catenin-vinculin interaction functions to organize the apical junctional complex in epithelial cells. J Cell Biol 142(3):847–857.

Weiss EE, Kroemker M, Rudiger AH, Jockusch BM, Rudiger M. 1998. Weiss Vinculin is part of the cadherin-catenin junctional complex: Complex formation between alpha-catenin and vinculin. J Cell Biol 141(3):755–764.

Yabu M, Takaoka K, Hashimoto J, Fujita H. 1991. Ultramicroscopic aspects of the conversion of fibroblasts to chondrocytes in the mouse dorsal subfascia induced by bone morphogenetic protein (BMP). Arch Histol Cytol 54(1): 95–102.

Zakany R, Bako E, Felszeghy S, Hollo K, Balazs M, Bardos H, Gergely P, Modis L. 2001. Okadaic acid-induced inhibition of protein phosphatase 2A enhances chondrogenesis in chicken limb bud micromass cell cultures. Anat Embryol (Berl) 203(1):23–34.

Zanetti NC, Solursh M. 1984. Induction of chondrogenesis in limb mesenchymal cultures by disruption of the actin cytoskeleton. J Cell Biol 99(1 Pt 1): 115–123.

Zhang Z, Messana J, Hwang NS, Elisseeff JH. 2006. Reorganization of actin filaments enhances chondrogenic differentiation of cells derived from murine embryonic stem cells. Biochem Biophys Res Commun 348(2):421–427.