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Staurosporine induces chondrogenesis of chick embryo wing bud mesenchyme in monolayer cultures through canonical and non-canonical TGF-β pathways

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Abstract Staurosporine has been known to induce chondrogenesis in monolayer cultures of mesenchymal cells by dissolving actin stress fibers. The aim of this study was to further elucidate how the alteration of actin filaments by staurosporine induces chondrogenesis. Specifically, we examined whether the transforming growth factor (TGF)-β pathway is implicated. SB505124 strongly suppressed staurosporine-induced chondrogenesis without affecting the drug's action on the actin cytoskeleton. Staurosporine increased the phosphorylation of TGF-β receptor I (TβRI) but had no significant effect on the expression levels of TGF-β1, TGF-β2, TGF-β3, TβRI, TβRII, and TβRIII. Phosphorylation of Smad2 and Smad3 was not increased by staurosporine. However, SB505124 almost completely suppressed the phosphorylation of Smad2 and Smad3. In addition, inhibition of Smad3 blocked staurosporine-induced chondrogenesis. Inhibition of Akt, p38 mitogen-activated protein kinase (MAPK), and c-jun N-terminal kinase (JNK) suppressed chondrogenesis induced by staurosporine. Phosphorylation of Akt, p38 MAPK, and JNK was increased by staurosporine. SB505124 reduced the phosphorylation of Akt and p38 MAPK, while it had no effect on the phosphorylation of JNK. The phosphorylation level of extracellular signal-regulated kinase (ERK) was not significantly affected by staurosporine. In addition, inhibition of ERK with PD98059 alone did not induce chondrogenesis. Taken together, these results suggest that staurosporine induces chondrogenesis through TGF-β pathways including canonical Smads and non-canonical Akt and p38 MAPK signaling.

Keywords Chondrogenesis · Cytoskeleton · Staurosporine · TGF-β

Introduction

Chondrogenesis is a multistep process by which mesenchymal cells differentiate into chondrocytes and then ultimately give rise to skeletal tissues during the formation of limbs (for review, see DeLise *et al.* 2000). During the development of limb buds, mesenchymal cells undergo a shape change from stellate to rounded (Wezeman 1998). Acquisition of a spherical morphology is also observed in micromass culture, which mimics in vivo chondrogenesis (Ahrens *et al.* 1977). The actin cytoskeletal change that accompanies the change in cell shape is also observed in vivo and in vitro. Actin microfilaments are present inside the cell membrane as a predominantly cortical structure in mature bovine articular cartilage (Langelier *et al.* 2000). The three-dimensional culture condition permits chondrogenesis through reorganization of the actin cytoskeleton. When cultured on chitosan film, de-differentiated chondrocytes regain the phenotype of differentiated chondrocytes, with a rounded form and cortical actin (Park *et al.* 2008).

The important role of actin cytoskeleton reorganization in chondrogenesis has been observed by treating mesenchymal cells with chemical compounds that eliminate actin stress fibers. Cytochalasin D, a blocker of actin polymerization, disrupts the actin stress fibers and induces chondrogenesis of limb bud mesenchyme cells cultured at subconfluent densities (Zanetti and Solursh 1984). ML-7 (an inhibitor of myosin light chain kinase) and blebbistatin (an inhibitor of myosin

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II) also remove actin stress fibers and induce chondrogenesis (Kim *et al.* 2012b). Staurosporine, a microbial alkaloid, similarly disrupts the actin stress fibers and induces chondrogenesis of limb bud mesenchyme cells (Kim *et al.* 2012a). Staurosporine also restores the differentiated functionality of de-differentiated chondrocytes (Borge *et al.* 1997; Rottmar *et al.* 2014).

Transforming growth factor betas (TGF-βs) are known as multifunctional growth factors involved in key events in development and tissue repair (Shi and Massague 2003). TGF-β signaling is mediated by two serine/threonine kinase receptors on the cell membrane, type I (TβRI) and type II (TβRII). When a ligand binds, the receptors form a heterodimeric complex. This allows receptor II to phosphorylate the receptor I kinase domain, which then phosphorylates the downstream signaling molecules Smad2/3 leading to forming a heterocomplex with Smad4. The Smad complexes are then translocated into the nucleus to regulate the transcription of target genes (Shi and Massague 2003; Feng and Derynck 2005; Massague *et al.* 2005). TGF-βs have been known to be critical for chondrogenesis. Several studies have reported that TGF-β promotes chondrogenesis (Kulyk *et al.* 1989; Schofield and Wolpert 1990; Leonard *et al.* 1991; Chimal-Monroy and Diaz de Leon 1997; Zhang *et al.* 2004), while others showed that TGF-βs have an inhibitory influence (Chen *et al.* 1991; Tsuiki *et al.* 1996; Seo and Serra 2007; Jin *et al.* 2008). In order to elucidate the molecular mechanism by which alteration in actin cytoskeleton by staurosporine induces chondrogenesis, we employed low-density cultures of chick wing bud mesenchymal cells. We examined whether staurosporine activates a TGF-β receptor. We also determined the role of canonical and non-canonical TGF-β pathways in staurosporine-induced chondrogenesis.

Materials and Methods

Cell culture and reagents. Monolayer cultures of mesenchymal cells were prepared from the wing buds of Hamburger-Hamilton (HH) stage 23/24 chick embryos as described previously (Kim *et al.* 2012a). Briefly, wing bud cells were separated by digestion with 0.1% trypsin and 0.1% collagenase in Ca^{2+}-Mg^{2+}-free Hanks's balanced salt solution for 10 min at 37°C. Cells were pelleted at 350×*g* for 10 min and resuspended with Ham's F12 medium supplemented with 10% fetal bovine serum (FBS) and antibiotics. Cells were filtered through eight layers of lens paper, and the monolayer cultures were plated at 2×10^6 cells/60-mm culture dish. They were allowed to adhere for 1 h in a humidified atmosphere at 37°C with 5% CO_2. The following biochemical agents were administered either alone or together with staurosporine: PD98059 and SP600125 (Enzo Life Sciences, Plymouth, PA); SB505124 (TOCRIS Bioscience, Ellisville, MO); SIS3 and Akt inhibitor IV (Calbiochem, La Jolla, CA); SB203580 (Selleck Chemicals, Houston, TX); and TGF-β1, TGF-β2, and TGF-β3 (R&D, Minneapolis, MN). The micromass culture was performed as described previously (Ahrens *et al.* 1977). Briefly, the concentration of a mesenchymal cell suspension was adjusted to 2×10^7 cells/ml, and a 10-μl drop of this cell suspension was placed in a culture dish. After incubating for 1 h at 37°C and 5% CO_2, F12 medium containing 10% FBS was added to the culture.

Alcian blue staining and quantitation of chondrogenesis. To obtain the photomicrographs, micromass cultures were fixed with Kahle's fixative for 20 min and then stained with 0.5% Alcian blue 8-GX (Sigma, St. Louis, MO) in 0.1 N HCl overnight. For quantitative analysis, cultures were fixed with 2% glacial acetic acid solution in ethanol for 30 min and rehydrated by sequential incubation with 95 and 70% ethanol for 10 min each. Cells were then stained with 0.5% Alcian blue (MP Biomedicals, Illkirch, France) overnight. After washing with 0.1 N HCl three times, the bound dye was extracted with 4 M guanidine HCl overnight at 4°C. The absorption of the extracted dye was measured at 595 nm using a microplate reader.

Immunofluorescence. Wing bud mesenchymal cultures were washed twice with phosphate-buffered saline (PBS) and fixed for 10 min in 4% paraformaldehyde, followed by incubation with 0.1% Triton X-100 in PBS for 4 min to render the cell membranes permeable. After rinsing and blocking with 1% BSA for 1 h, cultures were incubated with anti-type II collagen antibody (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h. Cells were washed three times in PBS and then incubated with Alexa Fluor 555-conjugated donkey anti-goat IgG secondary antibody (Invitrogen, Grand Island, NY) for 1 h. To visualize the polymerized actin, cells were incubated with Alexa Fluor 488 phalloidin (Invitrogen) along with the secondary antibody. The cells were then washed and counterstained with 4′,6′-diamidino-2-phenyindole (DAPI; Vector Laboratories, Burlingame, CA) to identify the nuclei. Images were obtained using a fluorescent microscope (Axiovert 2, Carl Zeiss, Oberkochen, Germany). All incubations were carried out at room temperature in a dark, humid chamber.

Immunoblotting. Cells were washed with ice-cold PBS and lysed with lysis buffer [50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 mM NaF, 1 mM sodium orthovanadate, 0.25% sodium deoxycholate, 1% NP-40, and protease inhibitor cocktail (Sigma)]. After intermittent agitation for 30 min at 4°C, the lysates were centrifuged at 15,000×*g* for 10 min at 4°C. The protein concentration of the supernatant was measured using a BCA protein assay (Pierce, Rockford, IL). Samples containing 30 μg of total

protein were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane. After blocking with 4% nonfat milk in 0.1% Tween 20 in Tris-buffered saline (TBS-T) for 1 h at room temperature, the membranes were probed overnight at 4°C with the indicated primary antibodies diluted in blocking buffer. The primary antibodies were obtained as follows: anti-type II collagen, TGF-β1, p38, pp38, (Santa Cruz Biotechnology), TGF-β2, TGF-β3, TβbRI, TβRII, TβRIII, pSmad2, pSmad3, Akt, pAkt, ERK, pERK, GAPDH (Cell Signaling, Denver, MA), Smad2 (Abcam, Eugene, OR), Smad3 (Pierce), and pJNK (Millipore, Billerica, MA). Membranes were washed with TBS-T three times and incubated with the respective horseradish peroxidase (HRP)-conjugated secondary antibodies (Cell Signaling) for 1 h at room temperature. The blots were developed with the Super Signal West Femto kit (Pierce) and exposed to X-ray film. Some bands were quantified by densitometry using ImageJ software.

Immunoprecipitation. For immunoprecipitation, equal amounts of the protein extracts (300 μg) were pre-cleared for 2 h with protein A agarose (Sigma). The pre-cleared samples were immunoprecipitated with 1 μg of anti-phosphoserine antibody (Millipore) at 4°C overnight. The immune complexes were recovered by adding 30 μl of protein A agarose bead slurry. The beads were blocked with 1% nonfat dry milk in lysis buffer, washed in lysis buffer, and then incubated with the samples for 2 h at 4°C. Immunocomplexes were collected by centrifugation, separated by SDS-PAGE, and blotted onto a nitrocellulose membrane. Immunoblotting was carried out with the anti-TβRI antibody.

RNA isolation and reverse transcription. Total RNA was extracted from the cultures using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. One microgram of total RNA was reverse-transcribed using SuperScript III (Invitrogen). The cDNAs (1 μL) were then subjected to PCR using PCR Master Mix (Takara, Otsu, Shiga, Japan) and primers according to the manufacturer's protocol. The PCR conditions were as follows: denaturation at 95°C for 10 min; 29 cycles each at 95°C for 1 min, annealing temperature for 1 min, and 72°C for 1 min, followed by 72°C for 10 min. The primer sequences and annealing temperatures for each primer are shown in Table 1. The PCR products were analyzed by electrophoresis on 1% agarose gel. The above experiments were repeated at least three times.

Results

Staurosporine activates transforming growth factor beta receptor I. The administration of staurosporine to the limb bud mesenchymal cells resulted in the complete loss of actin stress fibers and the induction of type II collagen expression, a chondrocyte marker, as determined by immunostaining and western blot (Fig. 1*a*, *b*). This is consistent with a previous report by Kim *et al.* (2012a).

To determine whether TGF-β signaling is involved in staurosporine-induced chondrogenesis, the effect of SB505124, an inhibitor of TβRI (DaCosta *et al.* 2004), on the TGF-β signaling was evaluated. SB505124 almost completely blocked chondrogenesis induced by staurosporine (Fig. 1*a*, *c*). Although SB505124 did slightly alter cell shape, it did not block the dissolution of actin stress fibers by staurosporine. These results indicate that TGF-β signaling is implicated in the chondrogenic process following dissolution of actin stress fibers by staurosporine.

To further investigate the involvement of TGF-β signaling in staurosporine-induced chondrogenesis, gene and protein expression levels of TGF-β1, TGF-β2, and TGF-β3; TβRI; TβRII; and TβRIII were determined by RT-PCR and Western blot analysis, respectively. RT-PCR showed a decrease in the transcript of TGF-β1, TGF-β3, and TβRIII and an increase in the transcript of TβRII by staurosporine administration, whereas the transcript levels of TGF-β2 and TβRI remained unchanged (Fig. 2*a*). Western blot analysis showed generally similar patterns of transcript level, except for an increase in TGF-β3 and TβRIII (Fig. 2*b*). Next, we analyzed the possible involvement of TβRI in staurosporine-induced chondrogenesis. As shown in Fig. 2*c*, treatment with staurosporine increased phosphorylation of TβRI. Our findings indicate that activation of TβRI is implicated in staurosporine-induced chondrogenesis.

TGF-β alone is not enough to induce chondrogenesis of wing bud mesenchymal cells in monolayer culture. In order to examine whether TGF-β is able to induce chondrogenesis as does staurosporine, TGF-β1, TGF-β2, or TGF-β3 was administered to the cultures. As shown in Fig. 3*a*, none of them induced type II collagen production in single mesenchymal cells. Immunofluorescence microscopy using anti-type II collagen confirmed these results (data not shown). TGF-β1 has been known to stimulate chondrogenic differentiation in micromass culture conditions, in which cells aggregate and spontaneously differentiate into chondrocytes (Bang *et al.* 2000; Jin *et al.* 2006). Therefore, the micromass culture system was employed to examine whether the TGF-βs used in this study function normally. TGF-β1, TGF-β2, or TGF-β3 was administered to the micromass cultures for 3 days, and cultures were stained with Alcian blue to assess chondrogenic differentiation. Alcian blue staining revealed that all of the TGF-βs significantly enhanced chondrogenesis (Fig. 3*b*). Western blot analysis using the anti-type II collagen antibody confirmed these results (Fig. 3*c*). The present results imply that TGF-β promotes chondrogenesis under micromass

Table 1 Primers for RT-PCR

Gene	Forward primer 5′–3′	Reverse primer 5′–3′	Temperature (°C)
TGFβ1	GCCCTGGATACCAACTACTGC	GCTGCACTTGCAGGAACGCCAC	52
TGFβ2	GGAGCCTGAAGCAAGATTTGC	TGCCAATGTAGTAGAGGATGGTGAG	55
TGFβ3	AGTGGCTGTCCTTCGATGTC	TAGGCTCATGGACCCATTTC	52
TβRI	GCAGTGCTTCGCCTCCCTGA	CAGGAATGGTCCAATAAATC	52
TβRII	CTGTGCAGCTGTATGGAGAC	ATCATCCAACATAATAGCAC	55
TβRIII	TGTCCTCAATCTCAGAAATC	CACGTGGGAGGAAAAACTTG	52
GAPDH	AGTCATCCCTGAGCTGAATG	ACCATCAAGTCCACAACACG	55

culture conditions but is unable to induce chondrogenesis in single-cell culture conditions.

Smad canonical Smad signaling pathways are necessary for staurosporine-induced chondrogenesis. In order to understand the signaling pathways by which staurosporine induces chondrogenesis, we first sought to discover whether there is an association with the Smad signaling pathway. Since the canonical TGF-β signaling pathway involves activation of Smads via phosphorylation of Smad2 and/or Smads3, we sought to determine whether staurosporine affects this phosphorylation. Cells were cultured in the presence of staurosporine for 2 days, and the phosphorylation of Smads was examined. Staurosporine inhibited the phosphorylation of Smad2 but had little effect on the phosphorylation of Smad3 (Fig. 4*a*). Co-treatment of SB505124 with staurosporine almost completely abolished the phosphorylation of Smad2 and Smad3 (Fig. 4*b*). Administration of SIS3, a Smad3 inhibitor (Jinnin *et al.* 2006), suppressed the expression of type II collagen that was induced by staurosporine. These results indicate that the Smad pathway is required for staurosporine-induced chondrogenesis.

Akt and p38 MAPK are downstream targets of TGF-β signaling during staurosporine-induced chondrogenesis. TGF-β has been described to utilize a multitude of intracellular signaling pathways in addition to the Smads in order to regulate a wide array of cellular functions (Zhang 2009). These non-canonical, non-Smad pathways include various branches of the mitogen-activated protein (MAP) kinase and phosphatidylinositol-3-kinase/Akt pathways. In order to determine which downstream kinase in the TGF-β pathway was affected during staurosporine-induced chondrogenesis, staurosporine was administered alone or together with SB505124 to mesenchymal cells in a monolayer culture, and the phosphorylation of MAP kinases and Akt was examined. Administration of staurosporine increased the phosphorylation level of Akt, while SB505124 reduced phosphorylation (Fig. 5*a*, *b*). Inhibition of Akt activity with Akt Inhibitor IV blocked staurosporine-induced chondrogenesis (Fig. 5*b*). In addition, the phosphorylation of p38 mitogen-activated protein kinase (MAPK) was increased by staurosporine and decreased by SB505124 (Fig. 5*c*, *d*). Inhibition of p38 activity by SB203580 suppressed staurosporine-induced chondrogenesis (Fig. 5*d*). These findings suggest that Akt and p38 MAPK act downstream of the TGF-β receptor, which is activated by staurosporine.

Phosphorylation of extracellular signaling-regulated kinase (ERK) was slightly increased by staurosporine and SB505124 (Fig. 6*a*, *b*). PD98059, a mitogen-activated protein kinase kinase (MEK) inhibitor, blocked the phosphorylation of ERK but did not induce chondrogenesis (Fig. 6*b*). These

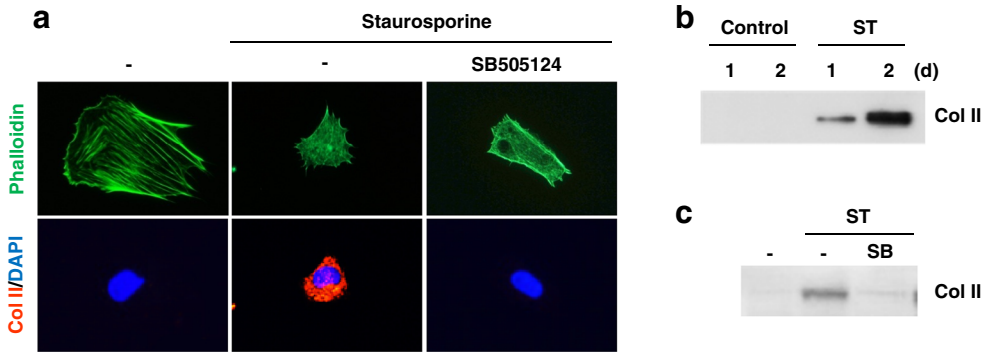

Figure 1 SB505124 blocks chondrogenesis induced by staurosporine. Chick wing bud mesenchymal cells were treated with staurosporine (5×10^{-8} M) in the presence or absence of SB505124 (5 μM) for 2 d (*a*, *c*) or 1 or 2 d (*b*). (*a*) Cells were stained for type II collagen (*red*) with F-actin (phalloidin, *green*) and nuclear (DAPI, *blue*) staining (×100). (*b*, *c*) Cell extracts were prepared on the indicated days, and the blots were probed with anti-type II collagen antibody (Col II). The images are epresentatives from three or more independent experiments.

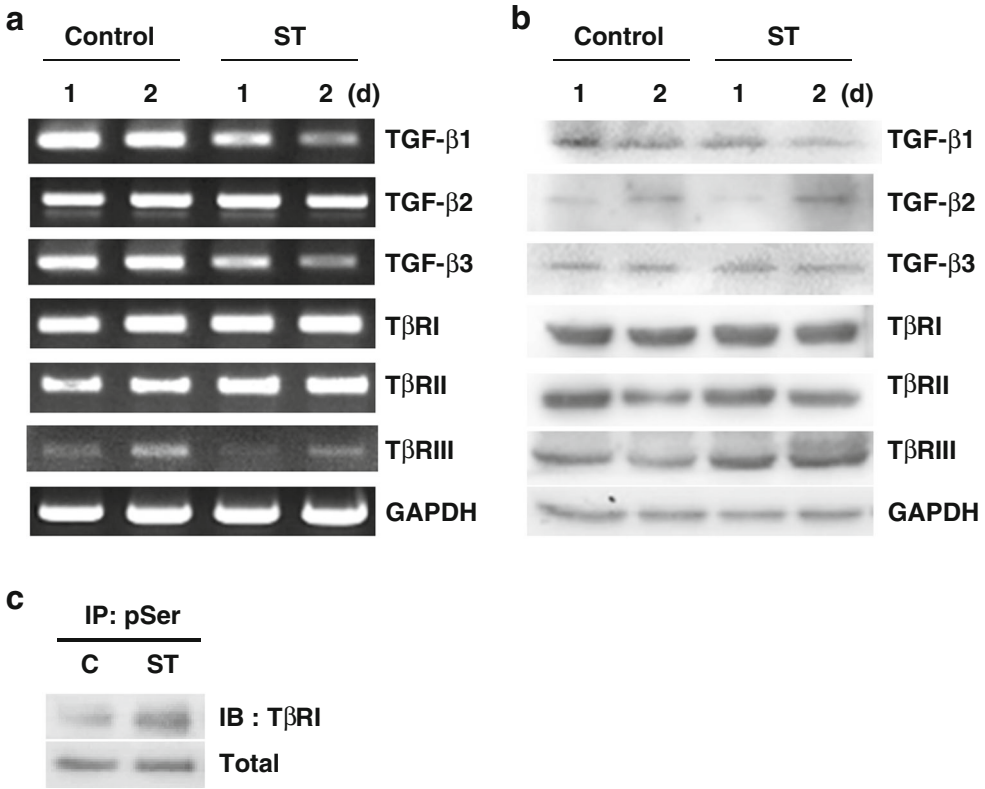

Figure 2 Staurosporine increased the phosphorylation of TβRI. Mesenchymal cells were cultured in the presence or absence of staurosporine (ST) for 1 or 2 d. (*a*) RNA was extracted from mesenchymal cells at the indicated time points and subjected to RT-PCR to detect the mRNA of TGF-β1, 2, and 3 and TβRI, II, and III. GAPDH was used as a loading control. (*b*) Proteins were extracted from mesenchymal cells at the indicated time points and subjected to Western blot analysis. GAPDH was used as a loading control. (*c*) Cells were cultured in the presence or absence of staurosporine for 2 d. Cell lysates were immunoprecipitated with anti-phosphoserine antibody (pSer) and immunoblotted with anti-TβRI antibody. Total protein levels of TβRI are also shown. The images are representatives from three or more independent experiments.

results suggest that ERK is not implicated in staurosporine-induced chondrogenesis. Phosphorylation of c-Jun NH(2)-terminal kinase (JNK) was increased by staurosporine but was not affected by SB505124 (Fig. 6*c*, *d*). SP600125, a JNK inhibitor, decreased the phosphorylation of JNK and suppressed staurosporine-induced chondrogenesis, indicating that JNK is involved in chondrogenesis induced by staurosporine but is not downstream of the TGF-β signaling pathway activated by staurosporine.

Discussion

Because cells in a micromass culture progress through chondrogenesis in multiple steps and the steps are not clearly separated from one another, it is difficult to investigate the role of each step separately (e.g., reorganization of the cytoskeleton). However, using the monolayer culture system, the role that the cytoskeleton plays during chondrogenesis induced by cytoskeletal alteration can be investigated. Early studies focused on staurosporine's ability to induce chondrogenesis (Borge *et al.* 1997; Lee *et al.* 2003; Hoben and Athanasiou 2008). More recently, the possible regulatory mechanisms of actin filament disruption during staurosporine-induced chondrogenesis have been of interest (Kim *et al.* 2012a). The report by Rottmar *et al.* (2014) that PI3K, PKC, and MAPKs are implicated in staurosporine-induced re-differentiation was the first study investigating the signaling pathways associated with staurosporine-induced chondrogenesis in a low-density culture. To further elucidate the mechanisms of action in staurosporine-induced chondrogenesis, the present study investigated the possible role of TGF-β and its downstream pathways.

We observed that staurosporine disrupted actin stress fibers and induced chondrogenic differentiation of mesenchymal cells. SB505124 almost completely blocked this chondrogenesis without affecting cell morphology or the F-actin staining pattern. These results imply that staurosporine uses the TGF-β pathway not for disrupting actin filaments, but rather for inducing chondrogenesis after filament disruption. Interestingly, staurosporine did not significantly affect the expression of either the mRNA or protein of TGF-βs and TβRs (Fig. 2). However, the phosphorylation of TβRI was increased by staurosporine treatment. These results indicate that

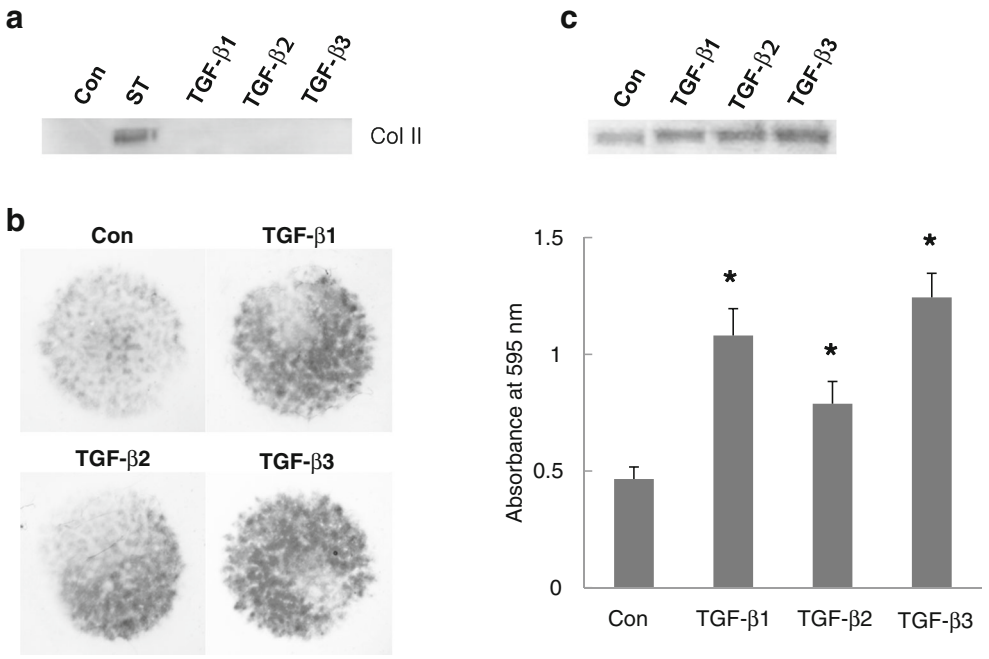

Figure 3 Effect of TGF-βs on the chondrogenic differentiation of mesenchymal cells in micromass culture or monolayer culture. (*a*) Cells in monolayer culture were treated with TGF-β1 (5 nM), TGF-β2 (5 nM), or TGF-β3 (5 nM) for 2 d, and the cell lysates were probed for type II collagen (Col II). (*b*, *c*) Chicken wing bud mesenchymal cells grown in high-density micromass culture were treated with TGF-β1 (5 nM), TGF-β2 (5 nM), or TGF-β3 (5 nM) for 3 d. (*b*) Cultures were fixed and stained for proteoglycan accumulation using Alcian blue (*left panel*). After staining, the dye was extracted with guanidine HCl and the optical density was measured at 595 nm (*right panel*). The data are presented as the mean±SD (n=3). Statically significant differences compared with control cells (*p<0.01). (*c*) Cell lysates were subjected to Western blot analysis with anti-type II collagen antibody.

staurosporine induces chondrogenesis by activating TβRI. Just how staurosporine activates TβRI remains to be determined.

TGF-β has been shown to promote chondrogenesis by increasing the aggregation of mesenchymal cells under micromass culture conditions (Iwasaki *et al.* 1993; Chimal-Monroy and Diaz de Leon 1997; Johnstone *et al.* 1998). Consistent with these studies, TGF-β1, TGF-β2, and TGF-β3 were observed to promote cell aggregation and chondrogenesis of mesenchymal cells under micromass culture conditions (Fig. 3). However, none of the TGF-βs used in this study was able to induce chondrogenesis of mesenchymal cells in monolayer culture. The cells treated with TGF-β exhibited a fibroblastic morphology and contained stress fibers similar to the control cells (data not shown). Given that the reorganization of actin filaments is critical for chondrogenesis, failure of chondrogenesis induction by TGF-β attributes to the absence of cytoskeletal change. Our findings suggest that activation of the TGF-β pathway alone is not sufficient to induce chondrogenesis in a monolayer culture, although it is required for staurosporine-induced chondrogenesis.

The Smad pathway has been known to be crucial for chondrogenesis. Smad2 is activated by TGF-β1, leading to the chondrogenic differentiation of mesenchymal cells (Re'em *et al.* 2012). Overexpression of Smad3 strongly induces the chondrogenesis of mesenchymal cells (Furumatsu *et al.* 2005). Activity of Smad2/3 is also important for the continuation of collagen II deposition (Hellingman *et al.* 2011). Consistent with previous observations, we found that Smad signaling is required for chondrogenesis induced by staurosporine. SB505124 almost completely blocked the phosphorylation of Smad2 and Smad3 and resulted in inhibition of chondrogenesis induced by staurosporine. Inhibition of Smad3 by SIS3 also blocked staurosporine-induced chondrogenesis. However, phosphorylation of Smad2 or Smad3 was not upregulated by staurosporine. At this point, it is not clear why phosphorylation of Smad2 and Smad3 was not increased by staurosporine which activated TβRI. After phosphorylated Smad2 and Smad3 mediate their transcription activity, they are dephosphorylated by phosphatase including protein phosphatase, Mg^{2+}/Mn^{2+} dependent, 1A (PPM1A) which is activated by PKCδ (Lin *et al.* 2007; Lee *et al.* 2013). Because staurosporine inhibits PKC isoforms including PKCδ (Seynaeve *et al.* 1994), we speculate that staurosporine has the opposite effect on the phosphorylation of Smad2 or Smad3. In other words, staurosporine stimulates phosphorylation of Smad2 and Smad3 by activating TβRI and dephosphorylates them by inhibiting PKCδ. Consequently, there is no increase in the phosphorylation of Smad2 and Smad3 by staurosporine. However, the exact mechanisms regulating phosphorylation of Smad2 and 3 by staurosporine remain to be elucidated.

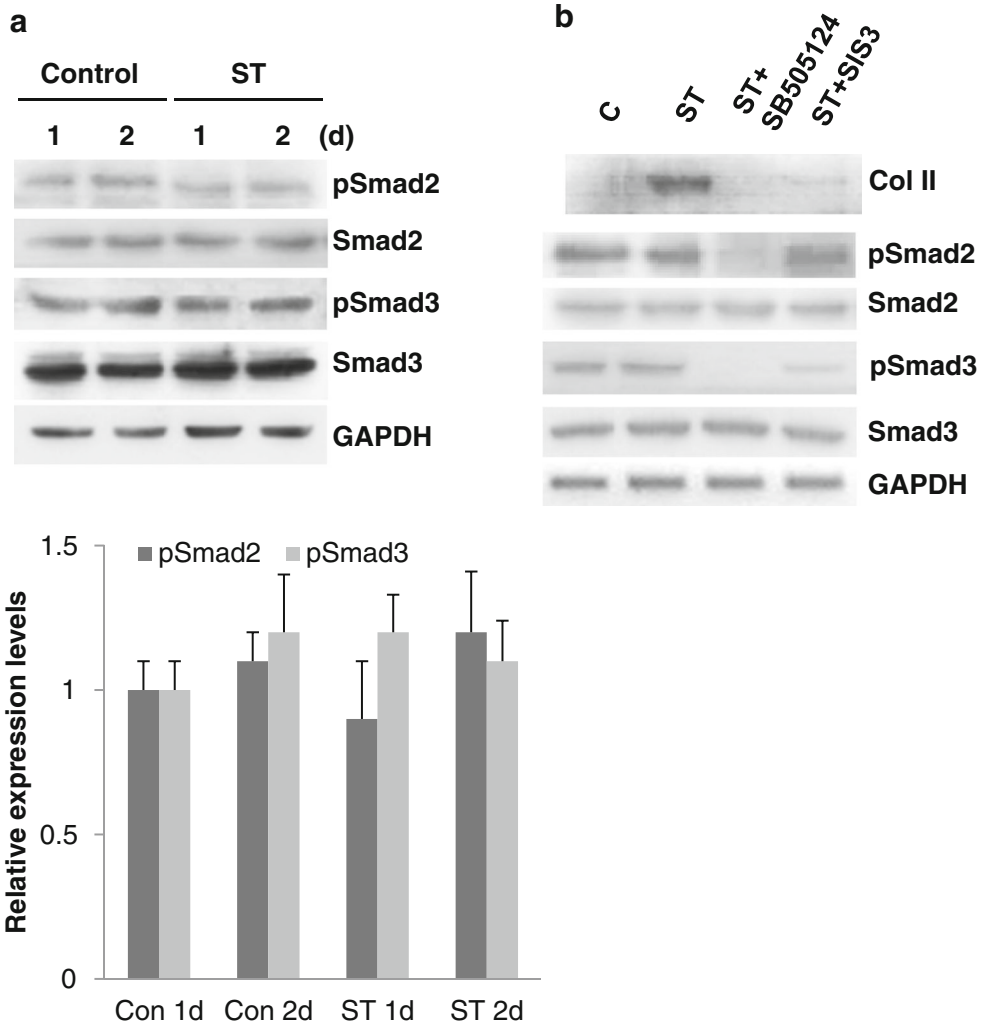

Figure 4 Smad signaling is required for staurosporine-induced chondrogenesis. (*a*) Mesenchymal cells were cultured in the presence or absence of staurosporine (ST) for 1 or 2 d. The phosphorylated forms of Smad2 and Smad3 were analyzed by Western blot (*upper panel*). The expressions of pSmad2 and pSmad3 were quantified and normalized to Smad2 and Smad3, respectively (*lower panel*). The data are presented as the mean± SD (n=3). (*b*) Cells were treated with ST, ST + SB505124, or ST + 10 μM SIS3 for 2 d, and the phosphorylated forms of Smad2 and Smad3 were analyzed by Western blot. GAPDH was used as a loading control. The immunoblots are representatives from three or more independent experiments.

Besides the canonical Smad-mediated TGF-β signaling pathway, TGF-β regulates cellular or physiological processes through non-canonical pathways by activating other signaling pathways, including PI3K/Akt and MAP kinases, that are independent of the Smad proteins (Zhang 2009). The role of MAP kinases in chondrogenesis has been extensively investigated (Bobick and Kulyk 2008). We sought to elucidate the downstream targets of TGF-β signaling in response to the staurosporine alteration of the actin cytoskeleton by investigating four kinases, including Akt, p38 MAPK, JNK, and ERK. As mentioned before, PI3K and p38 MAPK pathways positively regulate chondrogenesis in staurosporine-induced re-differentiation (Rottmar *et al.* 2014). Consistent with these results, the current study showed that inhibition of Akt and p38 MAPK with their respective specific inhibitors blocked staurosporine-induced chondrogenesis (Fig. 5). In addition, we also showed that inhibition of JNK suppressed staurosporine-induced chondrogenesis (Fig. 6), indicating that Akt, p38 MAPK, and JNK are implicated in the process. However, not all the kinases examined in this study are associated with the TGF-β signaling pathway. While SB505124 attenuated the phosphorylation of Akt and p38 MAPK, it did not affect the phosphorylation of JNK. These results suggest that Akt and p38 MAPK are downstream of TGF-β signaling. The current study also suggests that JNK is implicated in chondrogenesis induced by staurosporine but is not downstream of the TGF-β signaling pathway after actin cytoskeletal alteration by staurosporine.

ERK has been known to be a negative regulator of chondrogenesis. Phosphorylation of ERK decreases as chondrogenesis proceeds (Oh *et al.* 2000). Inhibition of ERK with PD98059 promotes the chondrogenesis of mesenchymal cells in a micromass culture (Oh *et al.* 2000) and enhances staurosporine-induced re-differentiation (Rottmar *et al.* 2014). In the present study, phosphorylation of ERK was not significantly affected by staurosporine and was even slightly increased (Fig. 6*a*). Furthermore, inhibition of ERK with PD98059 alone failed to induce chondrogenesis, although it did almost completely abolish the phosphorylation of ERK (Fig. 6*b*). These results suggest that release from ERK

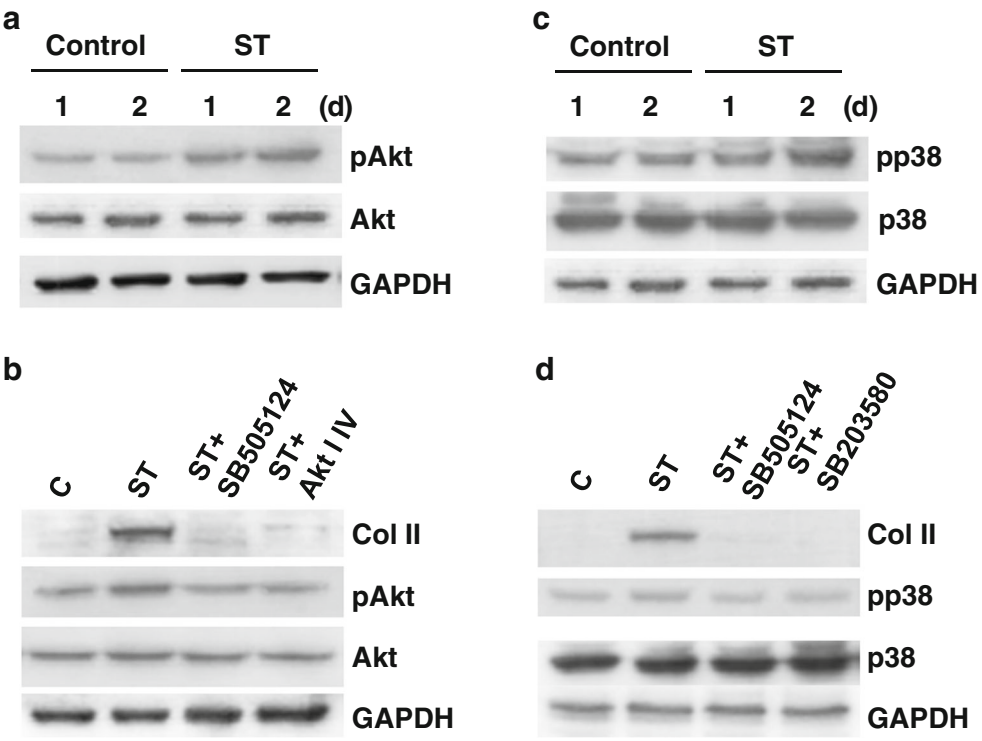

Figure 5 Akt and p38 MAPK function as downstream of TGF-β during staurosporine-induced chondrogenesis. (*a*, *c*) Mesenchymal cells were cultured in the absence or presence of staurosporine for 1 or 2 d, and the cell lysates were subjected to Western blot analysis with pAkt, Akt, pp38, and p38 antibodies. (*b*, *d*) Mesenchymal cells were treated with staurosporine alone or with SB505124, 1 μM Akt inhibitor IV, and 10 μM SB203580 for 2 d. Cell lysates were subjected to Western blot analysis with type II collagen (Col II), pAkt, Akt, pp38, and p38 antibodies. GAPDH was used as a loading control. The immunoblots are representatives from three or more independent experiments.

inhibition is not enough to induce chondrogenesis in a monolayer culture and that staurosporine does not employ ERK during its induction of chondrogenesis.

In summary, our study demonstrates that alteration of actin filaments by staurosporine induces chondrogenesis of mesenchymal cells under monolayer culture conditions through TGF-β pathways including canonical Smads and non-canonical Akt and p38 MAPK signaling. The present study also shows that ERK is not involved in staurosporine-induced chondrogenesis and that JNK activity is regulated by

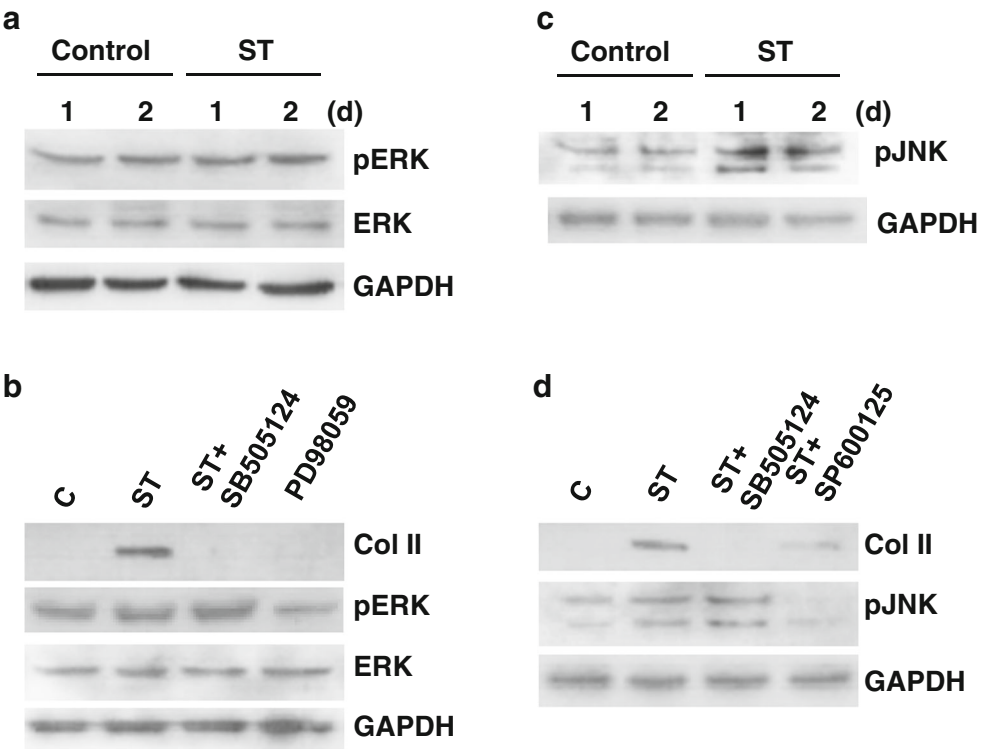

Figure 6 Effect of staurosporine and SB505124 on the phosphorylation of ERK and JNK. (*a*, *c*) Mesenchymal cells were cultured in the absence or presence of staurosporine for 1 or 2 d, and the cell lysates were subjected to Western blot analysis with pERK, ERK, and pJNK antibodies. (*b*, *d*) Mesenchymal cells were treated with staurosporine alone or with SB505124 and 10 μM SP600125 for 2 d. Cells were also treated with 10 μM PD98059 for 2 d. Cell lysates were subjected to Western blot analysis with type II collagen (Col II), pERK, ERK, and pJNK antibodies. GAPDH was used as a loading control. The immunoblots are representatives from three or more independent experiments.

staurosporine but not the TGF-β pathway, suggesting that staurosporine uses multiple pathways to induce chondrogenesis. Staurosporine is known to not only inhibit many protein serine-threonine kinases and tyrosine kinases (Nakano and Omura 2009) but also disrupt actin stress fibers. Therefore, it may be inferred that the mechanisms of action of staurosporine on chondrogenesis are complicated. More extensive and thorough studies are needed to elucidate these mechanisms.

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Conflict of interest Authors declare that they have no conflict of interest.

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