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p38 Mitogen-activated protein kinase affects Transforming growth factor-β/Smad signaling in human dental pulp cells

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Abstract

Transforming Growth Factor-β (TGF-β) plays an essential role in differentiation of dental pulp cells into odontoblasts during reparative dentine formation. However, the mechanism by which TGF-β stimulates dental repair remains rather obscure. Human dental pulp cells were used as an *in vitro* model in the present work. We showed that TGF-β signaled through mitogen-activated protein kinases (MAPKs), such as ERK1/2 and p38, along with Smad pathway. Distinct pathways exerted different time response. SB203580, a specific p38 MAPK inhibitor, reduced phosphorylation of Smad3, while it slightly enhanced phosphorylation of Smad2. Increased phosphorylation of ERK1/2 and p38 confirmed that SB203580 did not block activation of TGF-β receptors. In addition, the inhibition of ERK1/2 activity with MEK1/2 inhibitor U0126 increased TGF-β mediated phosphorylation of Smad3. Our results suggest that p38 affects the phosphorylation of Smad2 and Smad3 differentially during TGF-β signaling in human dental pulp cells and ERK1/2 might be involved in the process. (Mol Cell Biochem **291:** 49–54, 2006)

Key words: transforming growth factor-β, Smad, p38, ERK1/2, dental pulp cells

Introduction

Dental pulp contains a heterogeneous cell population including fibroblast-like cells, vascular cells, pericytes, neural cells, histiocytes/macrophages, dendritic cells, lymphocytes and mast cells etc. Most of the cells are fibroblast-like cells, which are responsible for the formation and turnover of a complex non-mineralized extracellular matrix [1]. Thus dental pulp fibroblast-like cells, termed dental pulp cells, can serve as an *in vitro* model to determine the effects of growth factors implicated in dental repair.

Transforming Growth Factor-β (TGF-β) is an important mediator of extracellular matrix biosynthesis and is involved in many aspects of the regulation of cell growth, differentiation and function [2]. During reparative dentine formation, TGF-β1 is up-regulated at the injury site and acts as a signaling molecule directing tissue regeneration. TGF-β1 plays a vital role in differentiation of dental pulp cells into odontoblasts [3–5]. Studies over the past several years have elucidated how TGF-β initiates its response. TGF-β signals from its receptor to nucleus through receptor-regulated Smads (R-Smads): Smad2 and Smad3 [6]. However, the mechanism by which TGF-β stimulates dental repair remains to be documented.

Members of the mitogen-activated protein kinase (MAPK) family, extracellular signal-regulated kinase (ERK), c-Jun amino-terminal kinase (JNK), and p38 kinase are central elements, which transduce the signals generated by growth factors and stressing agents [7]. Activation of p38 kinase in response to cytokines and environmental stresses is associated

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with the expression of alkaline phosphatase (ALP) during osteoblastic cell differentiation [8, 9]. Our previous study has demonstrated that p38 MAPK pathway might act downstream of TGF-β signaling and that inhibition of p38 MAPK with its inhibitor SB203580 reduces ALP activity in human dental pulp cells [10]. Recent studies also revealed that Smad3 promotes ALP activity and mineralization of osteoblasts [11, 12]. In addition, p38 MAPK has been shown to interact with Smad pathway. p38 MAPK mediates phosphorylation of Smad3 in rat myofibroblasts [13–15]. Therefore, it is possible that p38 MAPK might interact with Smad pathway through the phosphorylation process in human dental pulp cells. Our aim of this work was to clarify the possible implications of p38 MAPK in mediating Smad2 and Smad3 pathway during TGF-β signaling.

Materials and methods

Materials

Human TGF-β1 was purchased from R&D Systems (Minneapolis, MN). For kinase inhibition experiments, SB203580 and U0126 were from Alexis and Calbiochem (San Diego, CA), respectively. Both inhibitors were first dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO) and then diluted into the culture medium. Rabbit monoclonal anti-phospho-Smad2 (Ser465/467) (138D4), rabbit polyclonal anti-phospho-Smad3 (Ser433/435), rabbit polyclonal anti-Smad2/3, rabbit monoclonal anti-phospho-p38 kinase (Thr180/Tyr182), rabbit monoclonal anti-phospho-ERK1/2 kinase (Thr202/Tyr204), rabbit polyclonal anti-ERK1/2 kinase were purchased from Cell Signaling Technology (Beverly, MA). Rabbit polyclonal anti-p38 was from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell culture

Normal human impacted third molars were collected from adults (22–30 years of age) with informed consent at West China Stomatology Hospital of Sichuan University. Human dental pulp cells were isolated and cultured as previously described [10]. Briefly, pulp tissue was minced with scalpels and digested in a solution of 3 mg/mL collagenase type I (Sigma-Aldrich, St. Louis, MO) for 50 min, at 37 °C. Enzymatically released cells were cultured in α-modified minimum essential medium (α-MEM; Gibco-BRL, Grand Island, NY) supplemented with 10% fetal calf serum (FCS; Hyclone, South Logan, UT), 2 mM l-glutamine, 100 units/mL penicillin and 100 μg/mL streptomycin. Confluent cells were detached with 0.20% trypsin and 0.02% EDTA. Cell cultures between the fourth and sixth passages were used.

TGF-β treatment

Cells were plated into 6-well plates or 35 mm dishes (Corning, NY) in α-MEM containing 10% FCS. Subconfluent cells were grown in serum-free medium for 24 h followed by treatment with 10 ng/mL TGF-β1 for 5, 15, 45 and 135 min. Where indicated, the cells were treated with SB203580 or U0126 at the different concentration for 1 h before the treatment of TGF-β1 for 45 min.

Protein extraction and western blot analysis

Cells total protein were extracted with cell lysis buffer (containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.25% Na-deoxycholate, 1 mM EDTA, and 1 mM NaF, 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulphonylfluoride, 1 mM activated Na3VO4, 1 μg/mL aprotinin, 1 μg/mL leupeptin, and 1 μg/mL pepstatin) on ice for 15 min. After centrifugation at 14,000$\times$g for 15 min, supernatants were collected and protein concentration was determined using the bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL). Twenty micrograms total protein extracts were separated by 10% SDS-PAGE under reducing conditions and electroblotted onto polyvinylidene difluoride membrane (PVDF; Roche. Diagnostic, Germany). Membranes were blocked in 5% nonfat dry milk in TBS-T (10 mM Tris, pH 8.0, 150 mM NaCl, and 0.1% Tween-20) and probed with the indicated primary antibodies. After incubation with horseradish peroxidase–conjugated secondary antibodies, signal was visualized using enhanced chemiluminescence reagents (ECL; Pierce, Rockford, IL).

In order to examine expression of additional proteins on the Western blots, antibodies were removed by incubating blots in stripping buffer (62.5 mM Tris, pH 6.8, 2% SDS, and 0.1 M β-mercaptoethanol) for 30 min at 50 °C, blocked, and incubated with the next antibody. Blots were initially incubated with phospho-specific antibodies (phospho-Smad2, phospho-Smad3, phospho-ERK1/2 and phospho-p38 kinase antibodies), followed by incubation with their respective antibodies against total proteins. Band intensity was quantified using Quantity One software (Bio-Rad). Data were representative of three independent experiments.

Results

TGF-β activated Smad, ERK1/2 and p38 MAPK pathways

Previous studies indicated that TGF-β stimulates MAPKs, such as ERK1/2 and p38 in several kinds of cells [12, 16]. We have also shown that TGF-β induces nuclear translocalization of Smad2/3 and p38 [10]. Therefore, we investigated whether

TGF-β would phosphorylate these signaling proteins in dental pulp cells with Western Blot using phospho-specific antibodies (p-Smad2, Smad3, p38, and ERK1/2 kinase antibodies). TGF-β1 (10 ng/mL) treatment induced phosphorylation of Smad2, Smad3, p38, and ERK1/2 (Fig. 1). Phosphorylation of both Smad2 and Smad3 was observed as early as 15 min after treatment and peaking by 45 min (Fig. 1A and B), whereas phosphorylation of either p38 or ERK1/2 kinase had different time responses (Fig. 1C and D). These results indicated that TGF-β can activate numerous other pathways including the p38 and ERK1/2 MAPK pathway along with Smad signaling.

Activation of p38 MAPK interacted with Smad2 and Smad3 signaling differentially

Recently, inhibition of p38 MAPK was shown to have an adverse effect on nuclear translocation of Smad [10, 13]. We thereby initiated experiments to examine the interaction between Smad and p38 MAPK with the specific p38 MAPK inhibitor SB203580. As shown in Fig. 2A, phosphorylation of Smad3 was reduced by SB203580 dose-dependently, while phosphorylation of Smad2 was slightly induced by SB203580 (Fig. 2B). Meanwhile, SB203580 did not inhibit the phosphorylation of p38, which is regulated by upstream MAPK kinases activation [7], confirming that SB203580 did not block activation of TGF-β receptors in human dental pulp cells (Fig. 2C). Interestingly, SB203580 induced the phosphorylation of ERK1/2 in cells treated with TGF-β (Fig. 2D). We next used MEK1/2 inhibitor U0126 to investigate the possible regulative roles of ERK1/2 in process of Smad2 and Smad3 phosphorylation. U0126 acts by inhibiting the kinase activity of MAPK kinases MEK1/2 thus preventing the activation of ERK1/2 [17]. As shown in Fig. 3, U0126 enhanced TGF-β mediated phosphorylation of Smad3 (Fig. 3A), whilst it did not affect the phosphorylation of Smad2 (Fig. 3B). These results taken together indicate that phosphorylation of p38 may regulate the phosphorylation of Smad2 and Smad3 differentially with or without the phosphorylation of ERK1/2 involved in.

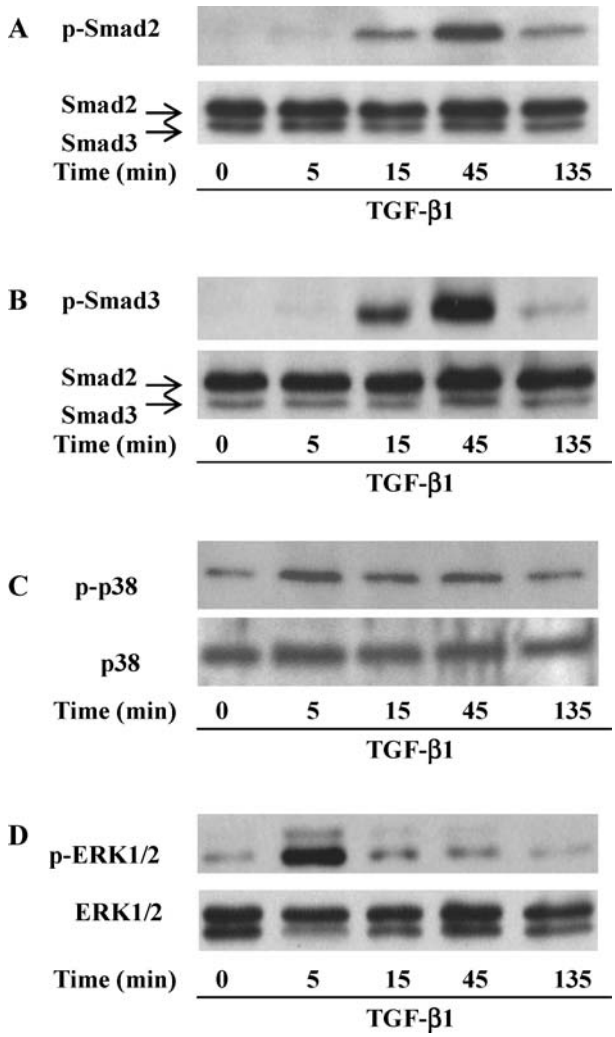

Fig. 1. TGF-β activates Smad, ERK1/2 and p38 MAPK pathways. Subconfluent human dental pulp cells were grown in serum-free medium for 24 h followed by treatment with 10 ng/mL TGF-β1 in serum-free α-MEM for 0, 5, 15, 45, 135 min. Total protein extracts (20 μg/lane) were subjected to Western blot analysis. Blots were incubated with different antibodies. Three independent experiments were performed. The representative blots show the expression of phosphorylated and total Smad3 (A), phosphorylated and total Smad2 (B), phosphorylated and total p38 (C), and phosphorylated and total ERK1/2 (D).

Discussion

Dental pulp cells express TGF-β receptors I and II [18], which contain Ser/Thr-specific protein kinase activity. MAPK belongs to the subfamily of the Ser/Thr-specific protein kinases [19]. In our present study, TGF-β activates p38 and ERK1/2 MAPK in addition to Smad pathway in dental pulp cells, which coincides with the previous observations in other cell lines [12, 13, 15, 16]. Different time response works for distinct pathway (Fig. 1). These results implied that Smad and non-Smad TGF-β signals might mediate different biological effects. Moustakas and Heldin classified non-Smad signal modes of action into three distinct but interrelated signaling mechanisms: *(1) non-Smad signaling pathways that directly modify Smad function; (2) non-Smad proteins whose function is directly modulated by Smads and which transmit signals to other pathways; and (3) non-Smad proteins that directly interact with or become phosphorylated by TGF-β receptors and do not necessarily affect the function of Smads* [20]. Our observations suggest that the stimulation of dental pulp cells

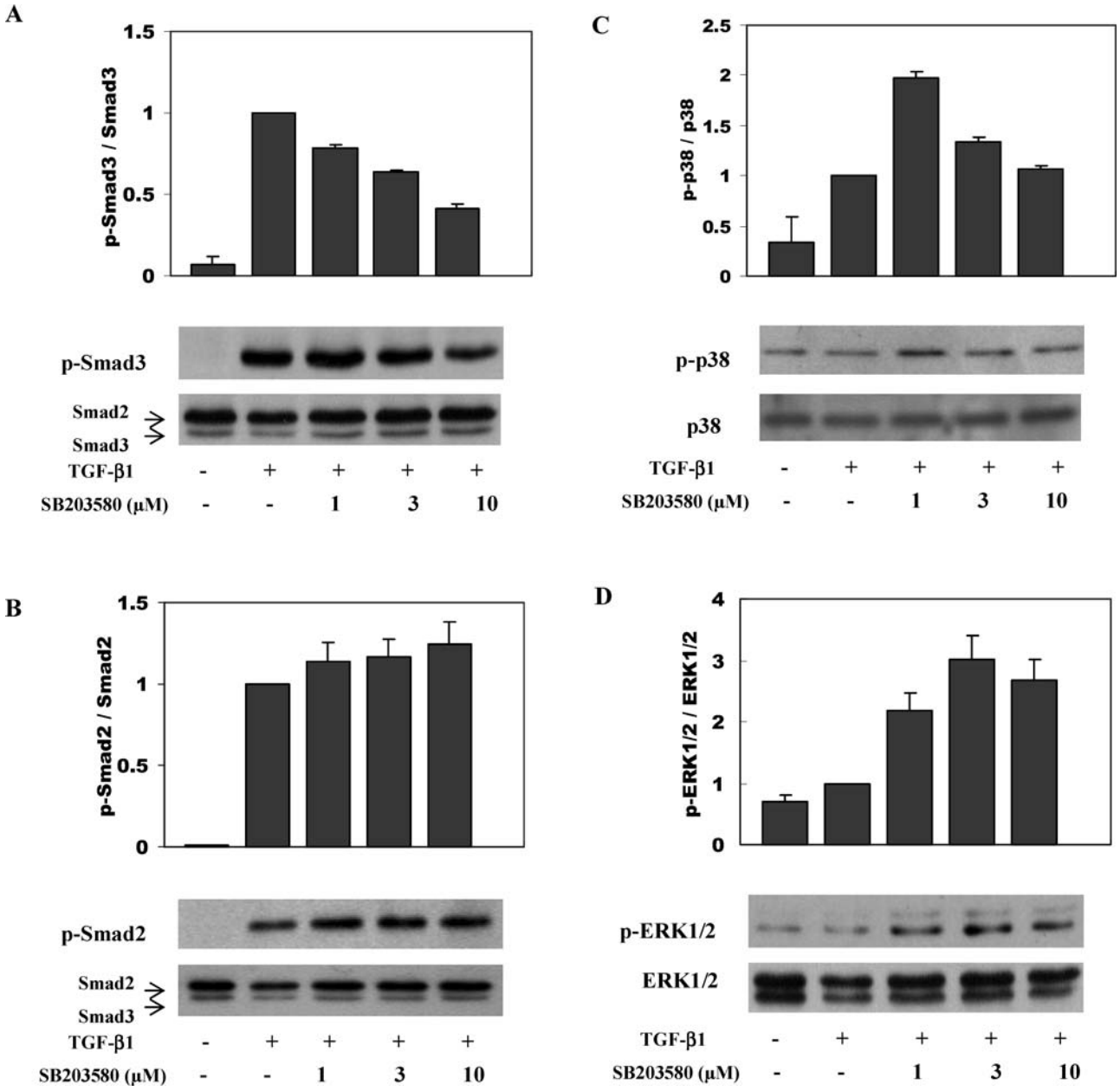

Fig. 2. Activation of p38 MAPK interacts with Smad2 and Smad3 signaling differentially. Serum starved cells were treated with SB203580 for 1 h before the treatment of 10 ng/mL TGF-β1 for 45 min. Total protein extracts (20 μg/lane) were subjected to Western blot analysis as described in Materials and methods. Data are representative of three independent experiments. Top: Graphic analyses of immunoblots showing the ratio of phosphorylated to total Smad3 (A), phosphorylated to total Smad2 (B), phosphorylated to total p38 (C), and phosphorylated to total ERK1/2 (D). Band intensity was quantified using Quantity One software (Bio-Rad). The ratio of the phosphorylated protein to total protein from cells treated with TGF-β1 and without SB203580 is assigned a value of 1.

by TGF-β involves the coordinate activation of several pathways that have specific and distinct roles in the regulation of cellular processes. Elucidation of the contribution of these signals seems essential for our understanding of the physiological roles of TGF-β.

We used the extensively utilized p38 MAPK inhibitor SB203580. This pyridinyl imidazole inhibitor specifically binds to the ATP-binding site of p38, which abrogates its kinase activity. It blocks a number of processes in cells without inhibiting the activity of ERK1/2, suggesting that the effects of this inhibitor are largely attributable to inhibition of p38, in spite of its ability to interact with Raf [21]. We found that treatment of SB203580 does not inhibit the phosphorylation of p38 (Fig. 2C). Although these results contrast

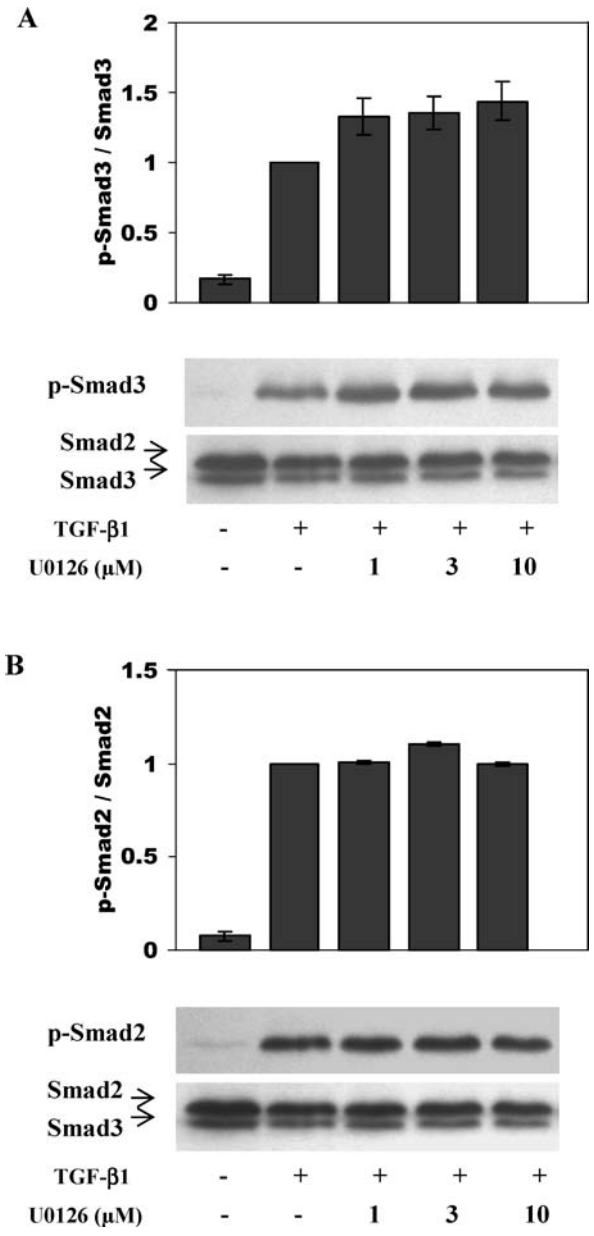

Fig. 3. Inhibition of ERK1/2 activity increases Smad3 phosphorylation. Serum starved cells were treated with U0126 for 1 h before the treatment of 10 ng/mL TGF-β1 for 45 min. Total protein extracts (20 μg/lane) were subjected to Western blot analysis. Data are representative of three independent experiments. Top: Graphic analyses of immunoblots showing the ratio of phosphorylated to total Smad3 (A), phosphorylated to total Smad2 (B). Band intensity was quantified using Quantity One software (Bio-Rad). The ratio of the phosphorylated protein to total protein from cells treated with TGF-β1 and without U0126 is assigned a value of 1.

with previous work in which SB203580 was used to investigate the interaction between p38 MAPK and BMP-2 signaling pathways in human trabecular bone-derived osteoblasts [14], Verma *et al*. also observed the same result using 10 μM SB203580 [22]. Similarly, induction of phosphorylation of ERK1/2 was shown following treatment of SB203580 before exposure of TGF-β (Fig. 2D), further confirming that the effects of SB203580 result in selective blocking of the p38 kinase domain and no inhibition of the TGF-β receptor activation.

Our data suggest that p38 MAPK interacts with Smad2 and Smad3 pathway differentially. Inhibition of p38 MAPK with SB203580 reduced the phosphorylation of Smad3 (Fig. 2A). The result is consistent with the information that SB203580 at 1–10 μM decreased the TGF-β-induced phosphorylation of Smad3 in rat myofibroblasts [13]. In addition, other p38 MAPK inhibitors SB202190 and PD169316 at 10 μM also displayed the same inhibitory effect on Smad3 in rat myofibroblasts and breast cancer cells [13, 23]. However, inhibition of p38 MAPK demonstrated different potency on phosphorylation of Smad2. PD169316 at 10 μM approximately reduced phosphorylation of Smad2 by 90% in ovarian carcinoma cells [15], whereas it was reported that phosphorylation of Smad2 was not affected by SB202190 or SB203580 treatment in chondrogenic cells [24]. Interestingly, in our work, inhibition of p38 MAPK with SB203580 at 1$\sim$10 μM slightly induced the phosphorylation of Smad2 dose-dependently (Fig. 2B). Our results suggest that p38 MAPK has a bilateral effect on TGF-β/Smad cascades in human dental pulp cells. It can also be proposed that kinases other than p38 can enhance the Smad2 phosphorylation by the treatment of SB203580; or else there could be other independent mechanisms involved in the phosphorylation process of R-Smads.

Furthermore, previous study reported that ERK1/2 and p38 MAPK oppositely regulate each other's function during L6E9 skeletal muscle differentiation [25]. In support of this, the activation of ERK1/2 by TGF-β negatively regulates Smad3-induced ALP activity [12]. As was mentioned above, SB203580 promotes TGF-β-induced phosphorylation of ERK1/2. Thus ERK1/2 might be implicated in SB203580 reducing phosphorylation of Smad3. We later revealed that inhibition of ERK1/2 activity results in increases of phosphorylation of Smad3 (Fig. 3A). It has also been shown that the endogenous ratio of Smad2 to Smad3 influences the cytostatic function of Smad3 [26]. Therefore, it can be hypothesized that Smad2 and Smad3 play different roles with the combined regulation of p38 and ERK1/2.

In summary, the present study indicates that p38 MAPK functions differentially during Smad2 and Smad3 activation in response to TGF-β stimulation. We propose that the administration of p38 and ERK1/2 MAPK activities with inhibitors might be of potential value in regulating TGF-β effects on differentiation of dental pulp cells further providing a novel therapeutic strategy for the stimulation of reparative dentine formation after dental injury.

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