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Original Article

Transforming growth factor-β1 promotes early odontoblastic differentiation of dental pulp stem cells via activating AKT, Erk1/2 and p38 MAPK pathways

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KEYWORDS
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Background/purpose: TGF-β1 (Transforming growth factor-β1) plays an important role in the regeneration and repair of pulp-dentin complex. However, the biological function of TGF-β1 on odontoblastic differentiation remains unclear, mainly due to the processes of differentiation were controlled by complex signaling pathways. This study aimed to investigate the signaling pathways involved in regulating the early differentiation of dental pulp stem cells (DPSCs) by TGF-β1 and their functional role.
Materials and methods: DPSCs were treated with 1 ng/mL TGF-β1 and Western blotting was conducted to examine the activation of protein kinase B (AKT), small mothers against decapentaplegic 3 (Smad3), p38 mitogen-activated protein kinase (p38 MAPK), c-Jun N-terminal kinase (JNK) and extracellular signal-regulated kinase 1/2 (Erk1/2). DPSCs were exposed to mineralization medium contained TGF-β1 with/without the specific signaling pathway inhibitors, and early odontogenic differentiation was evaluated by assessing the expression of alkaline phosphatase (ALP), collagen type 1 alpha 1 (COL1A), dentin matrix protein 1 (DMP-1) and runt-related transcription factor 2 (Runx2).

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Results: TGF-β1 stimulated AKT, Smad3, p38 MAPK, Erk1/2 and JNK phosphorylation in DPSCs within 120 min. TGF-β1 enhanced ALP activity and elevated levels of COL1A, DMP-1 and Runx2. LY294002, U0126 and SB203580 attenuated the effect of TGF-β1 on DPSCs, however, the SIS3 and SP600125 treated groups had no significant effect.
Conclusion: TGF-β1 promotes the early stage of odontoblastic differentiation in DPSCs by activating AKT, Erk1/2 and p38 MAPK signaling pathways, but not by Smad3 and JNK.

Introduction

Dental pulp stem cells (DPSCs) are isolated from the dental pulp of permanent teeth and display the same ability to differentiate into multiple cell types as mesenchymal stem cells (MSCs). It is known that DPSCs have the ability to differentiate into odontoblast-like cells after given the appropriate stimulus.[1] Taking into account their functional abilities, DPSCs are an exciting new source for regenerative endodontic treatment (RET).[2,3] Transplantation of DPSCs or stem cells from human exfoliated deciduous teeth (SHED) has achieved regeneration of the pulp and dentin-pulp complex in several studies.[4,5] In addition, a recent study found that human dental pulp with irreversible pulpitis contained putative stem cells,[6] which indicated that the inflamed pulp had the capacity to heal itself. Several clinical trials have suggested that irreversible pulpitis can be treated with vital pulp therapy (VPT) instead of root canal treatment.[7,8] Therefore, more research is needed to understand how to regulate the fate of DPSCs to ensure the RET and VPT work efficiently.

Cell fate can be controlled by growth factors by regulating migration, proliferation, and differentiation.[9,10] A reservoir of growth factors is embedded within the dentin matrix and released upon decayed dentin or root canal irrigation to modulate cellular functions.[9] Furthermore, a selection of effective growth factors can be transplanted into the root canal alone or in combination with stem cells to regenerate the pulpal-dentin complex.[11,12] Hence it is important to note how these molecules regulate cellular events during the regeneration process of dentin-pulp complex.

TGF-β1 (Transforming growth factor-β1) is a member of the TGF-β family, which is a pleiotropic molecule involved in multiple biological processes.[13] Ethylenediamine tetraacetic acid (EDTA) irrigation or mineral trioxide aggregate significantly increased TGF-β1 release from dentin.[14–16] Furthermore, TGF-β1 can be released from the extracellular matrix during pulp repair due to reduced blood flow.[17] But the biological function of TGF-β1 on cell activity remains controversial. Some previous studies indicated that TGF-β1 functioned as a positive regulator in proliferation, migration or differentiation of stem cells from dental.[18–20] However, overexpressing TGF-β1 significantly reduced tooth mineralization and dentin sialophosphoprotein gene expression in transgenic mice.[21] When treated with TGF-β1, the mineralization capacity of DPSCs or stem cells from the apical papilla (SCAPs) was inhibited.[22,23] In general, the function of TGF-β1 depends on treatment, cell types or different stages of dentin formation.

Complex signaling pathways may be the determinants of TGF-β1 function. TGF-β1 regulates cell differentiation not only via canonical small mothers against decapentaplegic 2 (Smad2) and Smad3 signaling, but also through noncanonical pathways like mitogen-activated protein kinase (MAPK), and phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT).[24] Therefore, maybe the multifunction of TGF-β1 on cell odontoblastic differentiation is related to the different signaling pathways regulating different downstream cellular activities. However, the regulatory mechanism of TGF-β1 in the differentiation of DPSCs remains to be elucidated, this could be due to the signaling pathways affect specific stages differently.

As a result, in this study, we focused on the function of TGF-β1 on the early differentiation of DPSCs aimed to find out the signaling mechanisms involved.

Materials and methods

Cells isolation and culture

The third molars without diseases were collected from patients for tooth extraction at the Stomatological Hospital of the Air force Medical University (n = 10, aged between 15 and 25 years, no gender requirement). All protocols were approved by the institutional review board. DPSCs were isolated based on the method previously reported.[1] Briefly, pulp tissue was retrieved from the dental pulp cavity and dissociated with 4 mg/mL type I collagenase (Gibco, Grand Island, NY, USA) at 37 °C for 1 h. The reaction was terminated by adding α-minimum essential medium (α-MEM) (Gibco) supplemented with 10% fetal bovine serum (FBS) (Gibco). Then the pulp tissue and cells were transferred into a 6-well plate containing α-MEM supplemented with 20% FBS, 1% penicillin—streptomycin (HyClone, Logan, OH, USA) and cultured at 37 °C in a 5% CO_2 incubator. Cells were harvested at 80% confluence with 0.25% (w/v) trypsin and EDTA (HyClone). Two to five passages were used for this experiment.

Identification of human DPSCs

For the multi-lineage differentiation assay, cells were seeded in 6-well plate. Some of cells were treated with osteo/odontoblast differentiation medium (OM) containing 10% FBS, 1% penicillin—streptomycin, 50 mg/mL ascorbic acid, 10 mM β-glycerophosphate, and 10 nM dexamethasone (Sigma-Aldrich, St Louis, MO, USA) for 2 weeks and the cells were stained with 2% Alizarin Red S (pH 4.2) (Sigma).

The rest were incubated in adipogenic medium containing 10% FBS, 1% penicillin-streptomycin, 0.1 μM dexamethasone, 0.2 mM indomethacin, 0.01 mg/mL insulin, and 0.5 mM IBMX (Sigma) for 4 weeks and stained with 0.5% Oil Red O and hematoxylin (Sigma).

The expression of stem cell associated phenotypic markers were analyzed by flow cytometry. Cells in the third passage (1.0×10^6 cells) were harvested and washed with PBS three times, then incubated with the following specific antibodies: CD29-phycoerythrin (PE), CD34-PE, CD45-PE, CD90-PE, and CD146-PE (1:100; all from BioLegend, San Diego, CA, USA) for 1 h at room temperature. After being washed with PBS the cells were resuspended and analyzed by Becton & Dickinson flow cytometry. The data were assessed with the Mod-Fit 2.0 cell cycle analysis program.

Treatments

To investigate the involvement of signaling pathways, cells were treated with 1 ng/mL TGF-β1 for 0, 15, 30, 60 and 120 min. DPSCs were blocked with specific signaling pathway inhibitors after being serum starved for 24 h as follows: 10 μM LY294002 (PI3K inhibitor), 5 μM SIS3 (Smad3 inhibitor), 10 μM SB203580 (p38 mitogen-activated protein kinase [p38 MAPK] inhibitor), 10 μM SP600125 (c-Jun N-terminal kinase [JNK] inhibitor) and 10 μM U0126 (extracellular signal-regulated kinase 1/2 [Erk1/2] inhibitor) were purchased from Selleck Chemicals (Houston, TX, USA), then incubated in 1 ng/mL TGF-β1 for 15 or 60 min. Cell lysates were collected for Western blot analysis.

A further investigation of whether signaling pathways are involved in TGF-β1-induced osteogenic differentiation of dental pulp cells was performed as follows: cells were treated in OM with or without PI3K inhibitors, Smad3 inhibitors and MAPK inhibitors for 7 days. Then we examined the activity of alkaline phosphatase (ALP) or expression of mineral-associated proteins in the samples.

Alkaline phosphatase activity and staining

For ALP activity analysis, cells were lysed with 200 μL/well RIPA lysate (Beyotime, Shanghai, China) on ice. Then ALP activity was determined in the lysate by measuring the release of p-nitrophenol according to the manufacturer's protocol (Beyotime). After being fixed in 4% paraformaldehyde for 30 min and rinsed with phosphate buffer, the cells were stained according to the manufacturer's instructions with an ALP staining kit (Beyotime).

Western blot analysis

Cells were lysed in RIPA buffer with protease inhibition and phosphatase inhibitors (Roche, Mannheim, Germany) on ice and the protein concentration was determined by bicinchoninic acid protein assay (Beyotime). Then proteins (20 μg) were separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (Bio-Rad, Hercules, CA, USA) and transferred onto 0.22 μm polyvinylidene fluoride membrane (EMD Millipore, Billerica, MA, USA). After blocking in QuickBlock Blocking Buffer (Beyotime) for 20 min, the membrane was incubated overnight at 4 °C with primary antibodies as follows: anti-AKT, anti-phosphor-AKT (p-AKT), anti-Erk1/2, anti-p-Erk1/2, anti-p38 MAPK, anti-p-p38 MAPK, anti-Smad3, anti-p-Smad3, anti-JNK, anti-p-JNK, anti-collagen type 1 alpha 1 (COL1A), anti-runt-related transcription factor 2 (Runx2) (1:1000; all purchased from CST, Boston, MA, USA), anti-dentin matrix protein-1 (DMP-1) (1:1000; Novus, Littleton, CO, USA) and anti-glyceraldenhyde-3-phosphate dehydrogenase (GAPDH) (1:10000; Proteintech, Rosemont, IL, USA). The membranes were rinsed and incubated with a diluted 1:6000 concentration of Horseradish peroxidase-conjugated secondary antibody (Yeasen, Shanghai, China). Enhanced chemiluminescence reagents (Millipore) were added and protein bands were captured using the ChemiDoc MP system (Bio-Rad). A gray value (intensity) of each protein band was determined using ImageJ software to enable comparison.

Statistical analyses

Data are presented as mean ± standard deviation (SD) and all experiments were performed at least three independent experiments. GraphPad 8 (GraphPad Software Inc., La Jolla, CA, USA) was used to perform one-way ANOVA or Student's t-tests for comparisons between groups, and asterisks indicate significant differences ($^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

Results

Isolation and characterization of DPSCs

Clone-like growth of primary cells emerged after 5 days culture (Fig. 1A). The cells from clones were spindly in shape (Fig. 1B). DPSCs cultured with OM showed significant mineralized nodules two weeks after culturing (Fig. 1C). Staining of the cells with oil red O revealed the presence of lipid deposits after 4 weeks of adipogenic induction (Fig. 1D). Cell surface marker analysis of DPSCs showed they positively expressed CD29 (99.8%), CD90 (97.2%) and CD146 (64%) and negative for CD34 (1.1%) and CD45 (1.0%) (Fig. 1E).

TGF-β1 caused the activation of AKT, Smad3 and MAPK pathways in DPSCs

To explore which pathways in DPSCs can be activated by TGF-β1, we evaluated the phosphorylated protein expression levels of critical members. The results showed that TGF-β1 upregulated levels of phosphorylated AKT, p38 MAPK, Smad3, JNK and Erk1/2, which indicated that the AKT, Smad3 and MAPK pathways were activated by TGF-β1 in DPSCs (Fig. 2A). Compared with the TGF-β1 treated group, Western blot analysis indicated the inhibitors blocked the corresponding pathways efficiently (Fig. 2B).

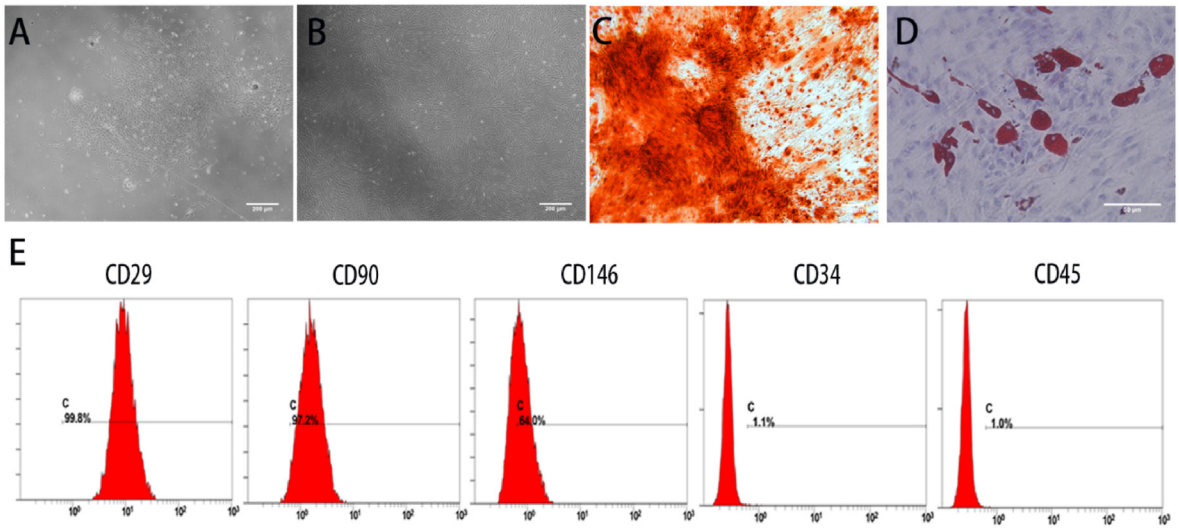

Figure 1 Characterization of DPSCs. (A) Primary DPSCs cultures on day 5 (40×). (B) DPSCs at passage 3 exhibit a spindle-shaped morphology (40×). (C) Calcified nodules stained with Alizarin red S (50×). D: Oil red O staining for lipid droplets (200×). E: Flow cytometry showed that cells were positive for CD29, CD90, and CD146, but negative for CD34 and CD45. Scale bars are shown. DPSCs, dental pulp stem cells.

Involvement of the AKT, p38 MAPK and Erk1/2 signaling pathways in TGF-β1 induced early odontoblastic differentiation of DPSCs

ALP staining was more intense and ALP activity was statistically significantly higher in TGF-β1 treated group (Fig. 3A). Consistent with ALP activity, levels of COL1A, DMP-1 and Runx2 were increased obviously when DPSCs were cultured with 1 ng/mL TGF-β1 (Fig. 3B). After incubated in different medium for 7 days, compared with TGF-treated group, the LY294002, U0126 and SB203580 attenuated ALP staining and activity induced by 1 ng/mL TGF-β1, while the SP600125 and SIS3 exerted minimal effect (Fig. 3A). Consistently, the protein levels of COL1A, DMP-1 and Runx2 were reduced significantly when cotreated with LY294002, U0126 and SB203580, but no significant differences presented in SIS3 and SP600125 treated groups (Fig. 3B).

Discussion

It has been long established that biological molecule like growth factors have potential contributions to reparative/regenerative events.[9] The growth factors function as signaling molecules that modulate cellular activity by mediating intracellular communication, including migration, proliferation, and differentiation of cells.[10] TGF-β1 is a ubiquitous multifunctional growth factor that embeds itself in the dentin matrix or be stored as a latent complex in the pulp's extracellular matrix. It can be released in some cases and influence cellular events during the pulp regeneration or repair process.[25,26] But the role of TGF-β1 in regulating cell differentiation and dentinogenesis is still controversial. The reason may be the signaling pathways differentially affect specific stages.[24]

A previous study pointed out that, the levels of TGFβ receptor I (TβRI) and TGFβ receptor II (TβRII) increased at early odontoblast differentiation and another research found that Biodentine™ prompted early reparative dentine formation may be related to modulation of pulp cell TGF-β1 secretion.[27,28] So we hypothesized that TGF-β1 enhanced the early differentiation of DPSCs. Various markers were used to evaluate odontogenic differentiation of DPSCs, such as ALP, Runx2, COL1A and DMP-1 are well characterized indicator of early stages of odontoblastic differentiation. In this study, we showed that 1 ng/mL TGF-β1 increased ALP levels and expression of Runx2, COL1A and DMP-1, which indicated TGF-β1 enhanced the early stage of differentiation.

TGF-β1 triggers signaling by binding to TβRI and TβRII of receptor serine/threonine kinases.[29] The two pairs of TβRI and TβRII may signal as independent units, for instance, TGF-β can activate the p38 MAPK by promoting the binding of tumor necrosis factor receptor-associated factor 6 (TRAF6) to TβRI, leading to ubiquitination of MAP-kinase.[30] TGF-β efficiently activated Erk1/2 when TβRII levels are high in dermal cells, whereas lower expression inhibits Erk1/2 activation.[31] It has been reported TβRI and TβRII were expressed in odontoblasts and pulp cells,[32] so that the levels of their expression and downstream signals may lead to different outcomes of the effects of TGF-β1 on cell differentiation. The present study reported that 1 ng/mL TGF-β1 can active the MAPK, AKT, and Smad3 pathway in DPSCs. The findings are similar to those in previous studies that found that TGF-β1 can active MAPK and Smad2/3 signaling,[23,33] however it is the first time to find out that TGF-β1 can induce AKT activation in this study. Nonetheless, the function of those pathways has not been well studied in previous studies.

TGF-β1 induces phosphorylation of Smad2/3 by TβRI, and the phosphorylated complexes and co-Smad translocate into the nucleus, where they regulate the transcription of target genes. Our previous studies have shown that Smad3 pathway involved in the inhibitory effects of TGF-β1 on late differentiation of SCAPs or odontoblast cell

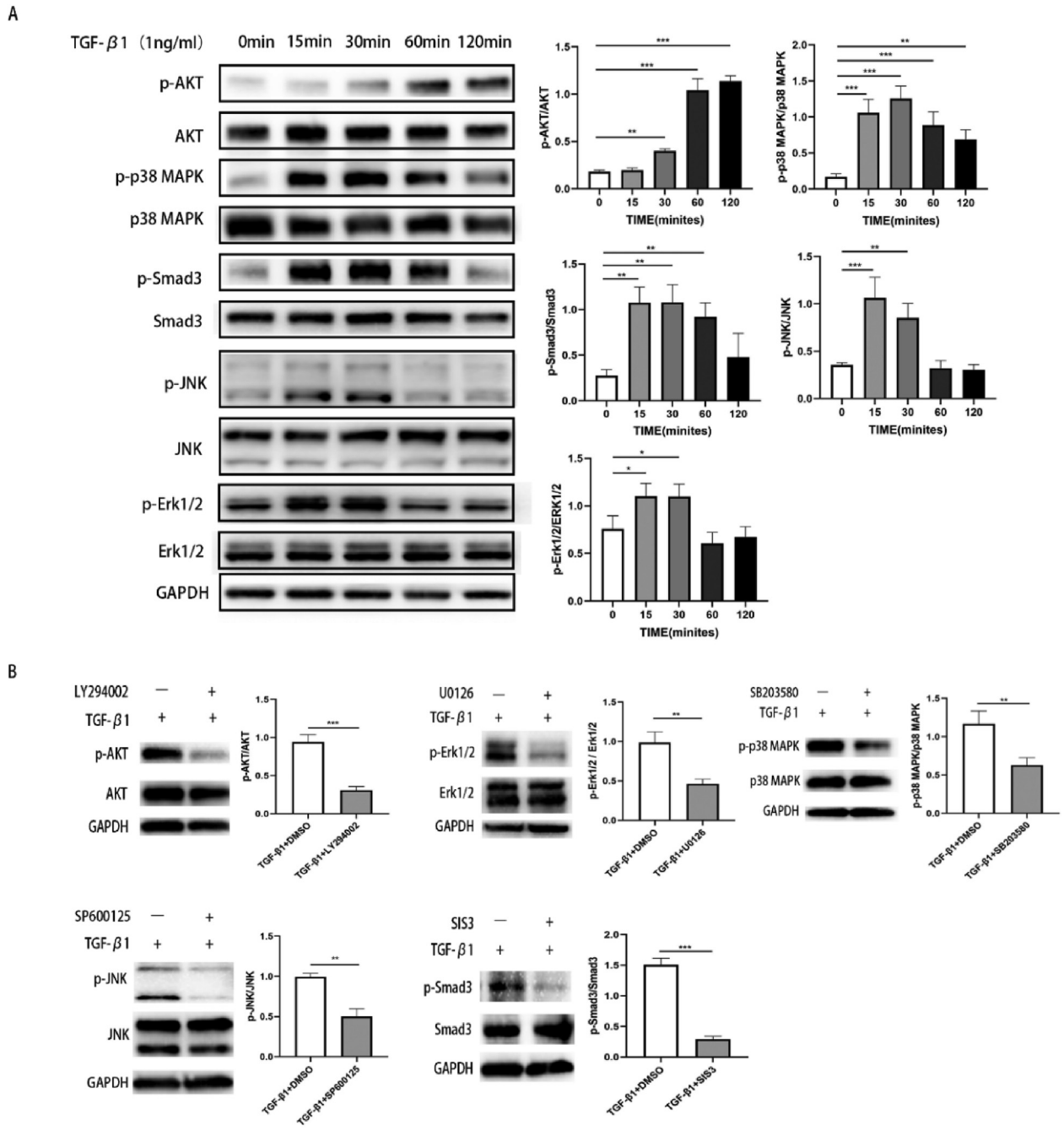

Figure 2 TGF-β1 actives AKT, Smad3 and MAPK pathways in DPSCs. (A) Western blot analysis of p-AKT, AKT, p-p38 MAPK, p38 MAPK, p-Smad3, Smad3, p-JNK, JNK, p-Erk and Erk from DPSCs treated with 1 ng/mL TGF-β1 in a time course. (B) Western blot analysis of p-AKT, p-p38, p-Smad3, p-JNK, p-Erk in DPSCs after treatment with pathways inhibitor respectively for 1 h. Error bars = means ± standard deviation (SD), n = 3, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. AKT, protein kinase B; DPSCs, dental pulp stem cells; Erk 1/2, extracellular signal-regulated kinase 1/2; JNK, c-Jun N-terminal kinase and; p38 MAPK, p38 mitogen-activated protein kinase; Smad3, small mothers against decapentaplegic 3; TGF-β1, Transforming growth factor-β1.

line,[22,34] and another study reported that TGF-β1 reduced Runx-2 and ALP expression via the ALK5/Smad2/3 pathway in dental pulp cells (DPCs).[23] This study concluded that TGF-β1 did not promote early differentiation of DPSCs via Smad3 signaling.

AKT signaling pathway is reportedly involved in differentiation of MSCs. In this study, LY294002 inhibited the ALP staining and the levels of COL1A, DMP-1 and Runx2 induced by TGF-β1. The result is consistent with previous results in MC3T3-E1 cells, which indicated that TGF-β1 enhanced osteoblast differentiation by activating Akt in the early but not the late phases of differentiation.[35]

MAPK family is comprised of three main subfamilies: p38 MAPK, Erk1/2 and JNK. The MAPK pathway has been shown

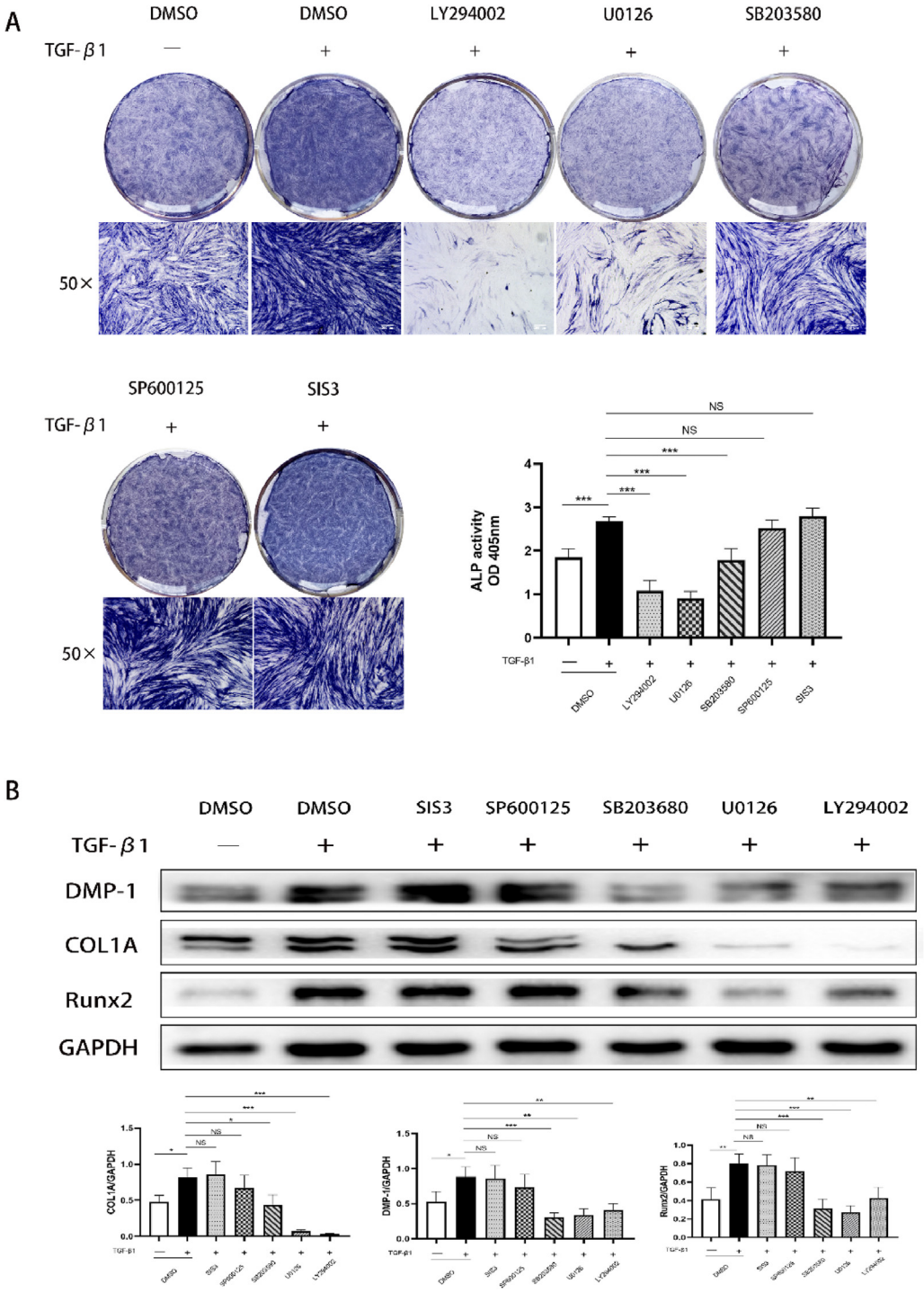

Figure 3 Involvement of p38 MAPK, Erk1/2 and AKT signaling in the TGF-β1-induced early odontoblastic differentiation of DPSCs. (A) ALP activities and ALP staining in the control group, TGF-β1 treated groups with or without pathways inhibitors at day 7(50×). (B) The protein expressions of DMP-1, COL1A, and Runx2 in different groups at day 7. Scale bars are shown. Error bars = means ± standard deviation (SD), n = 3. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. NS $P > 0.5$. COL1A, collagen type 1 alpha 1; DPSCs, dental pulp stem cells; DMP-1, dentin matrix protein 1; Runx2, runt-related transcription factor 2; TGF-β1, Transforming growth factor-β1.

to be involved in dentinogenesis, the inflammation of dental pulp and differentiation of DPSCs.[36] However, studies about whether MAPK is involved in the effect of TGF-β1 on DPSCs differentiation are limited. Also, the previous research had detected TGF-β1 can activate the MAPK pathway in DPCs or DPSCs,[17,33] but only Erk1/2 was confirmed not being the main signaling pathway by which TGF-β1 inhibits ALP activity in DPCs.[23] The current study

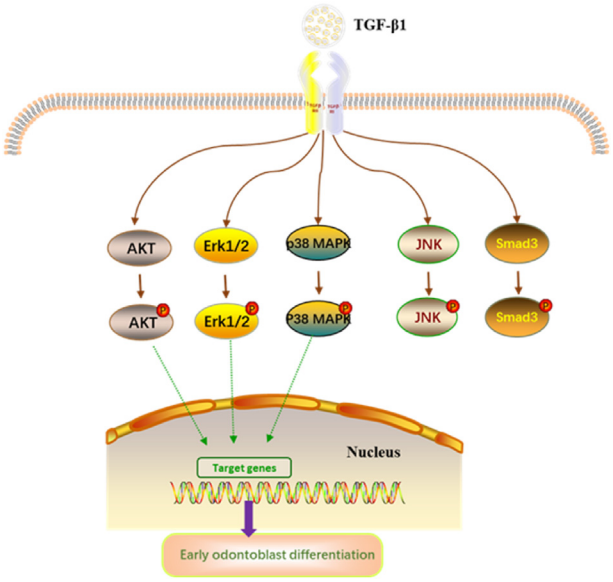

Figure 4 Schematic illustration showing the molecular mechanism of TGF-β1 on early odontoblastic differentiation of DPSCs. AKT, protein kinase B; DPSCs, dental pulp stem cells; Erk 1/2, extracellular signal-regulated kinase 1/2; JNK, c-Jun N-terminal kinase and; p38 MAPK, p38 mitogen-activated protein kinase; Smad3, small mothers against decapentaplegic 3; TGF-β1, Transforming growth factor-β1.

suggested that activation of Erk1/2 and p38 MAPK pathways contributed to TGF-β1-induced early differentiation of DPSCs, but not JNK pathway. A previous study suggested that JNK was not involved in the early odontoblastic differentiation but required for the late stage differentiation of odontoblasts induced by bone morphogenetic proteins-2.[37] Combined with the results of the current study, we hypothesize that the JNK signaling pathway does not contribute to the early stage differentiation of DPSCs regulated by TGF-β1.

In conclusion, we showed that the TGF-β1 promoted the early phases of differentiation in DPSCs by activating AKT, Erk1/2 and p38 MAPK signaling pathways, but not Smad3 and JNK in this research (Fig. 4). TGF-β1 can trigger different signaling ligands and corresponding antagonists depending on the cellular environment.[38] Further research is needed to illustrate the exactly mechanisms paradoxes, such as how the nature and intensity of the signal in the nucleus or the crosstalk between the signaling pathways determine the expression of downstream target genes.

Declaration of competing interest

The authors declare no potential conflicts of interest with respect to the authorship and/or publication of this article.

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