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Phosphoinositide 3-kinase/Akt signaling is essential for prostaglandin E2-induced osteogenic differentiation of rat tendon stem cells ☆

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ABSTRACT

Tissue calcification is a typical histopathological feature of tendinopathy. The osteogenic differentiation of tendon stem cells (TSCs) induced by inflammatory mediators is believed to play a key role in this process. Previous studies showed that the major inflammatory mediator prostaglandin E2 (PGE2) induced osteogenic differentiation of TSCs via bone morphogenetic protein (BMP)-2 production. Using a rat TSC culture model, we showed that PGE2 induced BMP-2 production through up-regulation of BMP-2 mRNA expression. PGE2 activated Akt, but not extracellular-signal-regulated kinase, in TSCs. Increased BMP-2 mRNA expression mediated by PGE2 was prevented by phosphoinositide 3-kinase (PI3K) and Akt inhibitors, but not by a MEK inhibitor. Furthermore, in the presence of exogenous BMP-2, PI3K and Akt inhibitors blocked Runx2 and osteocalcin expression, although BMP-2 did not activate Akt. BMP-2-induced alkaline phosphatase activity and mineralization were also inhibited by PI3K and Akt inhibitors. However, these inhibitors did not block activation of Smad, implying that Akt was involved downstream of Smad. Taken together, these results indicate that the PI3K-Akt signaling cascade is essential for PGE2-induced BMP-2 production and BMP-2-mediated osteogenic differentiation, suggesting that PI3-kinase-Akt signaling contributes to the formation of calcified tissues in tendinopathy.

1. Introduction

Tendons are fibrous bands of connective tissue that connect muscles to bones and transmit muscular forces to the bones, allowing joint motion and subsequent body movement. However, tendons are constantly subjected to large mechanical loading and are thus prone to pathological changes, known as tendinopathy [1]. Tendinopathy is a collective term for tendon disorders involving inflammation and/or degeneration.

Although the precise pathogenic mechanisms of tendinopathy remain unclear, the typical histopathological features include accumulation of lipid cells, mucoid degeneration, tissue calcification, or some combination of these, suggesting that tendons contain cells with multi-differentiation potentials. Tendon stem cells (TSCs) have been identified in humans [2], mice [3], rabbits [4] and rats [5]. These stem cells can differentiate into non-tenocyte lineages such as adipocytes, chondrocytes, and osteocytes under intensive, repetitive mechanical loading [2,6].

Previous studies suggested that prostaglandin E2 (PGE2), a major inflammatory mediator of pain and acute inflammation in injured tendons [7–9], was markedly increased in tendons subjected to repetitive mechanical loading conditions *in vivo* [6]. PGE2 treatment may result in degenerative changes of the tendon, partly by inducing differentiation of TSCs into adipocytes and osteocytes [6]. Moreover, PGE2 treatment of human TSCs (hTSCs) induced the production of bone morphogenetic protein-2 (BMP-2) in culture, and BMP-2 may mediate PGE2-induced osteogenic differentiation of hTSCs [10].

BMPs are a group of polypeptides within the transforming growth factor (TGF)-superfamily, originally named for their ability to promote ectopic bone formation [11]. Among the many BMPs, BMP-2 causes mesenchymal stem cells, and even myogenic cells, to differentiate into osteoblasts [12,13]. Like other members of the TGF-β superfamily, BMPs exert their effects via type I and type II transmembrane serine/threonine kinase receptors [14,15], and activate the intracellular signaling molecules Smad1, Smad5 and Smad8 through their serine phosphorylation [16]. Activated Smads induce the expression of Runx2, the master regulator of osteogenesis [17]. Smad and Runx2 then collaboratively induce other genes in differentiating osteoblasts [18]. However, the molecular mechanisms through which PGE2 induces the production of BMP-2 and

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the subsequent osteogenic differentiation of TSCs remains unknown. PGE2 exerts its downstream effects by signaling through four distinct G-protein-coupled E-prostanoid receptors (EP1–EP4) [19] and then initiates a series of signaling pathways. Although several signaling pathways mediate osteogenic differentiation, a growing literature supports critical roles for the mitogen-activated protein kinase (MAPK) pathway (MEK-ERK) [20–22] and phosphoinositide 3-kinase (PI3K)-Akt signaling [23,24].

We investigated the involvement of these pathways in PGE2-induced osteogenic differentiation using cultured rat TSCs, and defined a key role for the PI3-kinase-Akt pathway in PGE2-induced BMP-2 production and BMP-2-mediated osteogenic differentiation.

2. Materials and methods

2.1. Isolation and culture of rat TSCs

Three 8-week-old Sprague–Dawley rats weighing 250–300 g were used. All experiments were approved by the Animal Research Ethics Committee, Third Military Medical University, China. The isolation and culture of rat TSCs were performed as described previously [5]. Briefly, the whole piece of intact flexor tendon was excised from both limbs of each rat following euthanasia. Only the midsubstance tissue was collected and peritendinous connective tissue was removed carefully. The tissues were minced in sterile phosphate-buffered saline (PBS), digested for 2.5 h at 37 °C with type I collagenase (3 mg/ml Sigma–Aldrich, St. Louis, MO, USA), and passed through a 70-mm cell strainer (Becton Dickinson, Franklin Lakes, NJ, USA) to yield a single-cell suspension. The released cells were washed in PBS followed by centrifugation at 300 g for 5 min and resuspended in Dulbecco's modified Eagle's medium (Gibco, Carlsbad, CA, USA) with 10% fetal bovine serum, 100 U/ml penicillin, 100 mg/ml streptomycin, and 2 mM L-glutamine (all from Invitrogen, Carlsbad, CA, USA). The isolated cells were diluted to different cell densities and cultured at 37 °C in 5% CO_2 to form colonies. At day 2 after initial plating, the cells were washed twice with PBS to remove the nonadherent cells. At day 7, they were trypsinized and mixed together as passage 0 cells. Cells from passages 1–3 were used for other experiments. Culture medium with or without PGE2 (Sigma–Aldrich), BMP-2 (Sigma–Aldrich), noggin (R&D Systems Inc., Minneapolis, MN, USA), Akt Inhibitor IV (Santa Cruz, Santa Cruz, CA, USA), U0126 (Cell Signaling, Danvers, MA, USA) and LY294002 (Cell Signaling), was changed every 3 days throughout the experiments.

2.2. Immunofluorescence

Immunofluorescence analysis was performed using rat TSCs with octamer-binding transcription factor 4 (Oct-4), stage-specific embryonic antigen-4 (SSEA-4), and nucleostemin as stem cell markers. The TSCs were fixed with 4% paraformaldehyde in PBS for 30 min and blocked in 2% goat serum for 1 h at room temperature. After washing, the cells were incubated at room temperature with Oct-4 antibody (Abcam, Cambridge, MA, USA), SSEA-4 antibody (Abcam) and nucleostemin antibody (Santa Cruz) for 3 h. After washing the cells with PBS, Cy3-conjugated secondary antibody (Abcam) was applied for 30 min at room temperature. The cells were also counterstained with DAPI (Sigma–Aldrich). The stained cells were examined under a fluorescence microscope and color images of same cells were merged.

2.3. Measurement of BMP-2 levels

Cell-conditioned medium was collected and BMP-2 levels in the culture medium were measured using an enzyme-linked immunosorbent assay kit (R&D Systems Inc.) following the manufacturer's instructions.

2.4. Alizarin red staining

Cells were fixed in 70% ethanol for 1 h and stained with 2% alizarin red solution (pH 4.1–4.5) for 30 min at 20 °C. The stained cells were examined under an inverted microscope. Photomicrographs were obtained using a charge-coupled device (CCD) camera.

2.5. Alkaline phosphatase staining

Cells were washed with PBS, fixed with 70% ethanol for 30 min, and incubated with 500 ml NBT/BCIP solution (Sigma–Aldrich) for 20 min at 20 °C [25]. After three washes with distilled water, images were captured using a CCD camera.

2.6. Immunoblotting

Cells were scraped and homogenized in lysis buffer (50 mM Tris–HCl, pH 8.0, 1 mM EDTA, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 150 mM NaCl) containing a mixture of proteinase inhibitors (Thermo Fisher Scientific Inc., Rockford, IL, USA). Protein concentrations were measured using a BCA protein assay kit (Thermo Fisher Scientific Inc.). Protein samples (30 μg/lane) were resolved by SDS–polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes. After blocking with 0.1% TBS-Tween containing 5% non-fat milk for 1 h at 20 °C, membranes were incubated sequentially with primary and secondary antibodies. The following primary antibodies were used: anti-Akt, anti-phospho-Akt (Ser473), anti-phospho-Smad1/5/8, anti-Smad (all from Santa Cruz), anti-ERK1/2, anti-phospho-ERK1/2 (all from Abcam). Results were visualized and images captured using a LiCoR Odyssey imager (LI-COR Biosciences, Lincoln, NE, USA). Densitometric analysis was performed using ImageJ software.

2.7. Gene analysis by quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

The expression levels of specific marker genes in differentiated TSCs were determined by qRT-PCR. Total RNA was obtained as described previously [26] and was reverse-transcribed using a Superscript III first-strand synthesis kit (Invitrogen), with oligo (dT) primers in a final volume of 20 μl. qRT-PCR was carried out with an ABI Prism 7900 Sequence Detection System (PE Applied Biosystems, Foster City, CA, USA) using a QuantiTect SYBR Green RT-PCR kit (Qiagen, Hilden, Germany). Rat-specific primers were used for BMP-2, Runx2 and osteocalcin as follows:

5'-TGATCACCTGAACTCCACCAACCA-3' and 5'-AACCCTCCACAACCATGTCC TGAT-3' for BMP-2; 5'-TGATGACACTGCCACCTCTGACTT-3' and 5'-TGGATAGTGCATTCGTGGGTTGGA-3' for Runx2; 5'-AGAACAGACAAGTCCCACACAGCA-3' and 5'-TCCTGCTTGGACATGAAGGCTTTG-3' for osteocalcin. Glyceraldehyde-3-phosphate dehydrogenase was used as an internal control (primers: 5'-TGACT CTACCCACGGCAAGTTCAA-3' and 5'-ACGACATACTCAGCACCAGCATCA-3'). Relative gene expression levels are expressed as $2^{-\triangle\triangle Ct}$ formula, and at least three independent experiments were performed.

2.8. Statistical analysis

Data were expressed as mean ± SD. Multiple comparisons were made using one-way analysis of variance followed by Fisher's tests. A P value < 0.05 was considered to be statistically significant.

3. Results

3.1. BMP-2 mediates PGE2-induced osteogenic differentiation

Rat TSCs exhibited slender fibroblast-like shapes with an irregular distribution (Fig. 1A). The stem cell markers Oct-4, SSEA-4, and nucleostemin were expressed in TSCs, as detected by immunofluorescence (Fig. 1B–D).

After culture of TSCs in PGE2-containing growth medium for 14 days, mineralization of the extracellular matrix and bone-specific alkaline phosphatase activity were significantly increased (Fig. 1E and F), compared to the control group without PGE2 treatment. We investigated the ability of the BMP-2 antagonist noggin to reverse the osteogenesis induced by PGE2. Alkaline phosphatase activity and mineralization were prevented by noggin, supporting the hypothesis that PGE2-induced osteogenesis was mediated by upregulation of BMPs (Fig. 1E and F).

3.2. Inhibition of PI3-kinase-Akt signaling blocks PGE2-induced BMP-2 production

Rat TSCs were incubated with PGE2 (100 ng/ml) for different times, and BMP-2 mRNA expression levels in TSCs and in the culture medium were significantly increased on days 7 and 14 (Fig. 2A and B).

To determine if ERK or PI3K signaling was involved in PGE2-triggered osteoblast development of TSCs, we examined the phosphorylation of Akt and/or ERK. PGE2 treatment activated Akt phosphorylation, but had no obvious effect on ERK phosphorylation in TSCs (Fig. 2C and D). Furthermore, we treated TSCs with the MEK inhibitor U0126, the PI3K inhibitor LY294002, or Akt Inhibitor IV, in the presence of PGE2. LY294002 and Akt inhibitor markedly inhibited Akt phosphorylation and significantly reduced BMP-2 mRNA expression and BMP-2 levels in TSCs (Fig. 2C–F). In contrast, U0126 had no effect on BMP-2 mRNA expression or BMP-2 levels (Fig. 2C–F).

3.3. PI3K-Akt signaling is involved in BMP-2-induced osteogenic differentiation

To determine if PI3K-Akt signaling was needed for BMP-2-induced osteogenic differentiation, BMP-2 (200 ng/ml) was added to the TSC culture medium. As shown in Fig. 3A, alkaline phosphatase activity and mineralization were increased slightly at day 3, and then increased progressively to reach a significant level by day 14.

BMP-2 activated downstream signaling pathways, as indicated by the increased serine phosphorylation of Smad1, Smad5 and Smad8 in protein extracts (Fig. 3B). However, unlike PGE2, BMP-2 did not activate the phosphorylation of Akt (Fig. 3B). LY294002 and Akt inhibitor did not inhibit BMP2-activated Smad phosphorylation, but significantly down-regulated the mRNA expression levels of Runx2 and osteocalcin, while U0126 had no effect (Fig. 3B and C). The down-regulation of alkaline phosphatase activity and mineralization by LY294002 and Akt inhibitor with no change in cell viability also indicated that PI3K-Akt signaling was

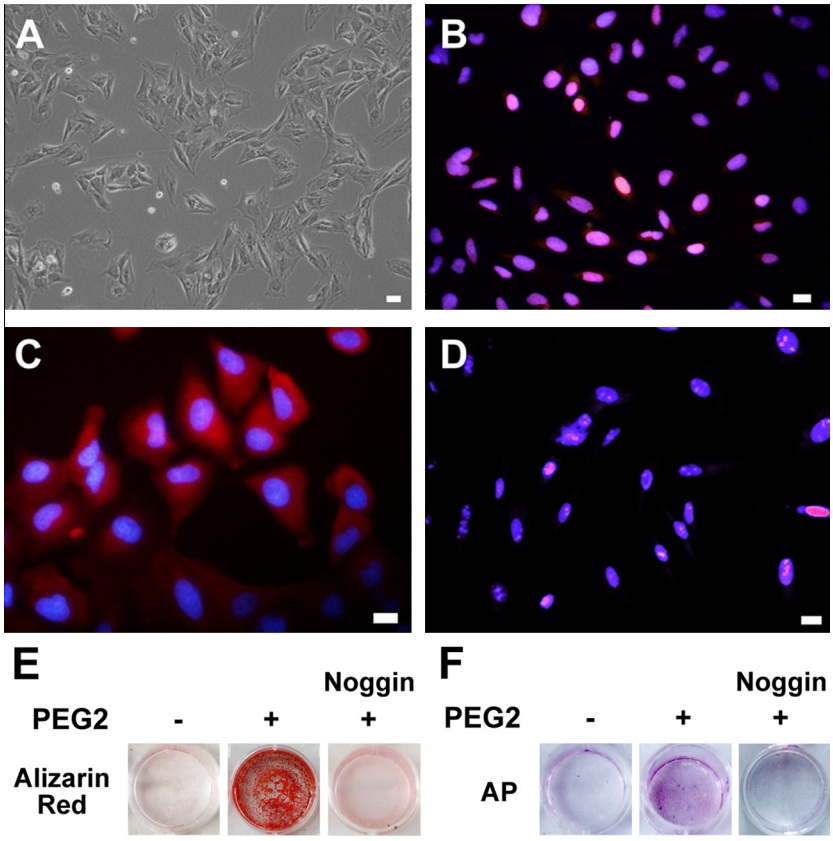

Fig. 1. Identity of rat tendon stem cells (TSCs) and PGE2-induced osteogenic differentiation. TSCs in culture exhibited fibroblast-like shapes (A) and expressed the stem cell markers Oct-4 (B, pink dots), SSEA-4 (C, red), and nucleostemin (D, pink dots). Bar: 10 μm. TSCs were incubated in medium containing PGE2 (100 ng/ml) with or without noggin (BMP-2 antagonist, 300 ng/ml) for 14 days. Osteogenic differentiation was evaluated by Alizarin red staining (E) and alkaline phosphatase (AP) staining (F). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

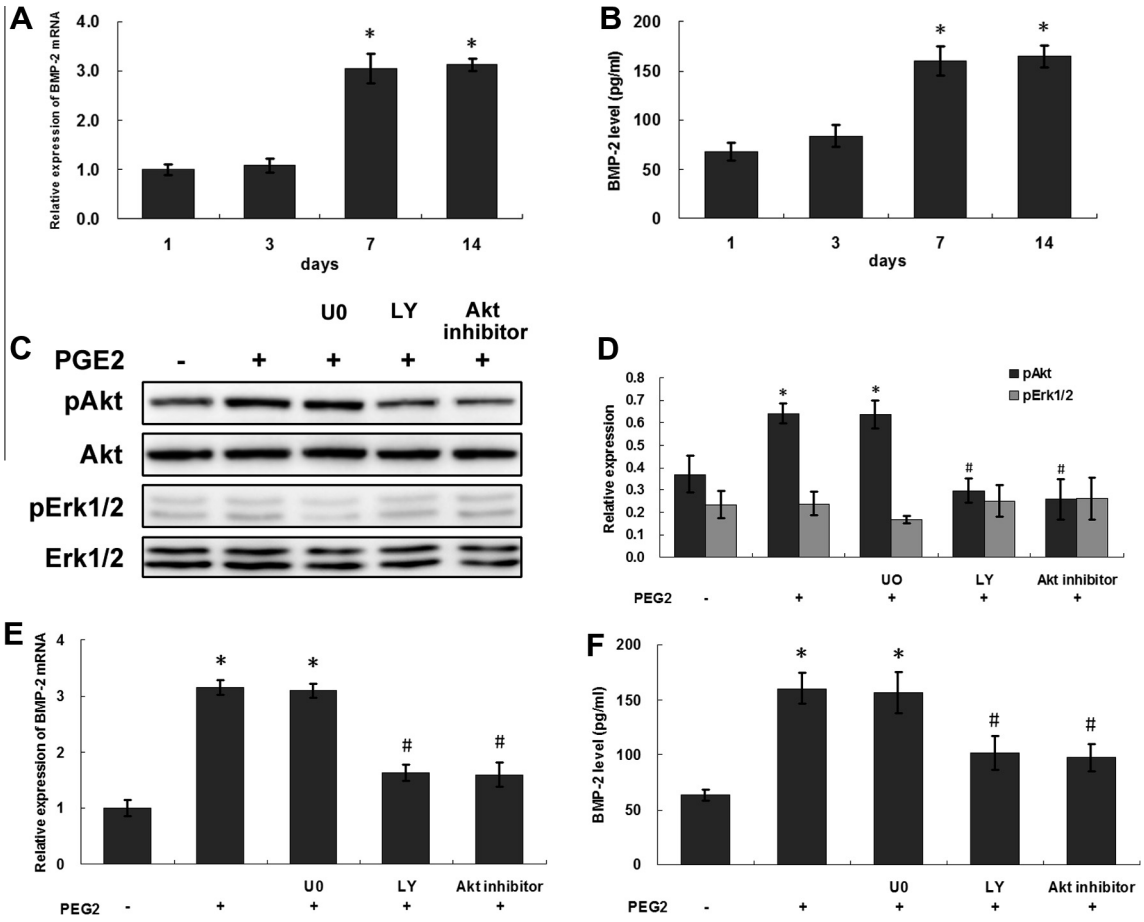

Fig. 2. PI3K/Akt signaling, but not MAPK signaling, is involved in PGE2-induced BMP-2 production. (A) BMP-2 mRNA expression in rat TSCs was detected by qRT-PCR after incubation with PGE2 (100 ng/ml) for the indicated number of days. (B) BMP-2 levels in the culture medium after incubation with PGE2 (100 ng/ml) for the indicated number of days. TSCs were then incubated in medium containing PGE2 (100 ng/ml) for 7 days with or without the MEK inhibitor U0126 (U0, 10 μM), PI3K inhibitor LY294002 (LY, 20 μM) or Akt Inhibitor IV (Akt inhibitor, 10 μM), as described in the Materials and Methods. (C) Immunoblots of whole-cell protein lysates for Akt phosphorylated at Ser473 (pAkt), total Akt, tyrosine- and serine-phosphorylated ERK1 and ERK2 (pERK1/2), and total ERK (ERK1/2). (D) Densitometric analysis of band intensities of pAkt and pERK1/2 was performed and values were normalized using total Akt and total ERK as loading controls. (E) BMP-2 mRNA expression levels in rat TSCs. (F) BMP-2 levels in medium. $^{*}P < 0.05$ with respect to TSCs without PGE2, $^{\#}P < 0.05$ with respect to TSCs with PGE2.

required for BMP-2-induced osteogenic differentiation (Fig. 3D and E).

4. Discussion

Chronic mechanical loading of tendons is considered to be the major cause of tendon inflammation and degeneration [27], which are involved in the development of tendinopathy [6,28]. Tissue calcification is a typical histopathological feature of tendinopathy, and the osteogenic differentiation of TSCs induced by inflammatory mediators may play a key role in this process [6,10]. As a major inflammatory mediator, PGE2 is present in tendons subjected to repetitive mechanical loading conditions *in vivo*, and results in the differentiation of TSCs into adipocytes and osteocytes [6]. Which lineage TSCs differentiate into may be related to the microenvironment and downstream signaling pathways. BMP-2 plays an important mediating role in PGE2-induced osteogenic differentiation of TSCs [10]. The results of this study indicate that the PI3K-Akt pathway is also necessary for this differentiation.

This study showed that PGE2 could activate Akt and that both PI3K and Akt inhibitors could inhibit PGE2-induced BMP-2 production, while blocking the MEK-ERK signaling pathway had no effect. The regulation of cell functions by PGE2 involves EP receptor-dependent signaling pathways. Binding of PGE2 to each of the EP receptor subtypes conveys signals to downstream effectors via different pathways [29]. Activation of the PI3K-AKT pathway occurs mainly via the EP2 receptor [29,30], implying that this receptor is enriched in rat TSCs and responsible for mediating PGE2-induced BMP-2 production. BMP-2 is a known osteogenic growth factor with a central role in bone development and osteoblast differentiation [31]. Its expression is regulated by multiple factors, including itself [32], although the molecular mechanisms responsible remain unclear. The current study found that PGE2 induced BMP-2 mRNA expression through the PI3K-Akt pathway. However, Akt cannot promote mRNA expression directly, and transactivation of BMP-2 mRNA may thus be mediated by the NF-κB transcription factor [33–35].

Considering the mediating role of BMP-2, we investigated the need for Akt-regulated signaling in BMP-2-induced osteogenic differentiation. Akt signaling pathways were required for induction of Runx2 gene expression, but not for phosphorylation of Smad1, 5, or 8. These observations are supported by previous studies, which also suggested that Akt signaling was required for BMP-2-induced osteogenic differentiation [24,36].

In summary, the results of this study indicate that the PI3K-Akt signaling pathway is involved in PGE2-induced osteogenic differentiation of rat TSCs. Furthermore, PGE2 activates Akt through PI3K, and then induces BMP-2 mRNA expression. BMP-2 subsequently activates Smad phosphorylation, inducing Runx2 and osteocalcin expression and osteogenic differentiation (Fig. 4). These

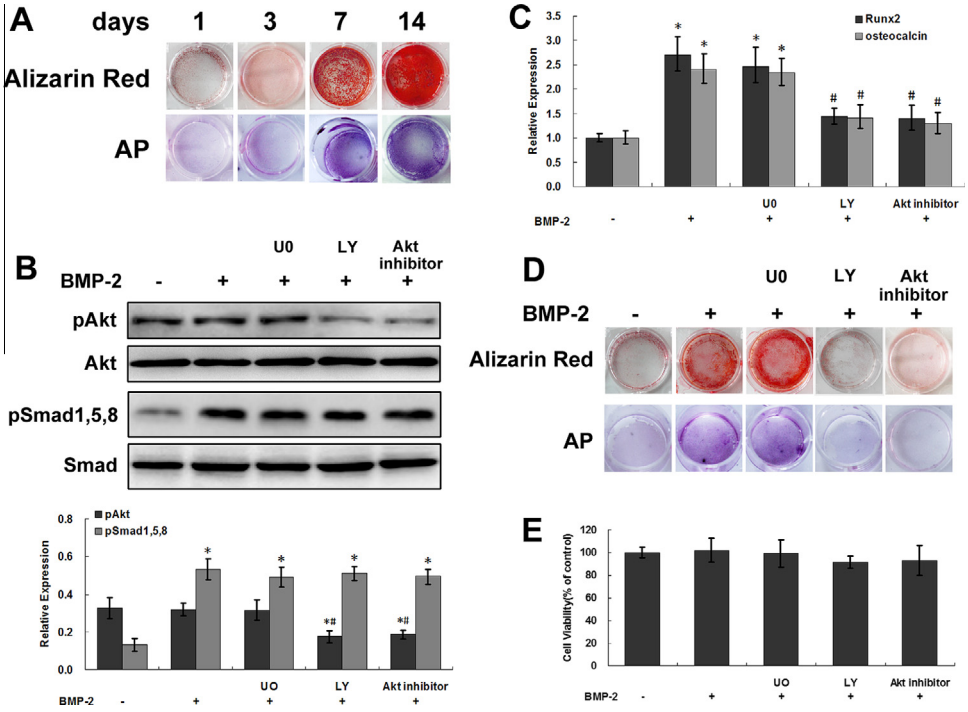

Fig. 3. PI3K and Akt inhibitors block BMP-2-induced osteoblast differentiation. Rat TSCs were incubated in medium containing BMP-2 (200 ng/ml) for the indicated number of days. Osteogenic differentiation was evaluated by Alizarin red staining and alkaline phosphatase (AP) staining (A). TSCs were then incubated in medium containing BMP-2 (200 ng/ml) for 7 days with or without the MEK inhibitor U0126 (U0, 10 μM), PI3K inhibitor LY294002 (LY, 20 μM) or Akt Inhibitor IV (Akt inhibitor, 10 μM). (B) Immunoblots of whole-cell protein lysates for Akt phosphorylated at Ser473 (pAkt), total Akt, tyrosine and pSmad1, 5, and 8, and total Smads. Densitometric analysis of band intensities of pAkt and pSmad1, 5, and 8 was performed and values were normalized using total Akt or total Smads as loading controls. (C) qRT-PCR assays showing expression of osteoblast-specific genes Runx2 and osteocalcin. (D) Osteogenic differentiation was evaluated by Alizarin red staining and alkaline phosphatase (AP) staining. (E) Cell viability was determined by MTT assay. $^{*}P < 0.05$ with respect to TSCs without BMP-2, $^{\#}P < 0.05$ with respect to TSCs with BMP-2. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

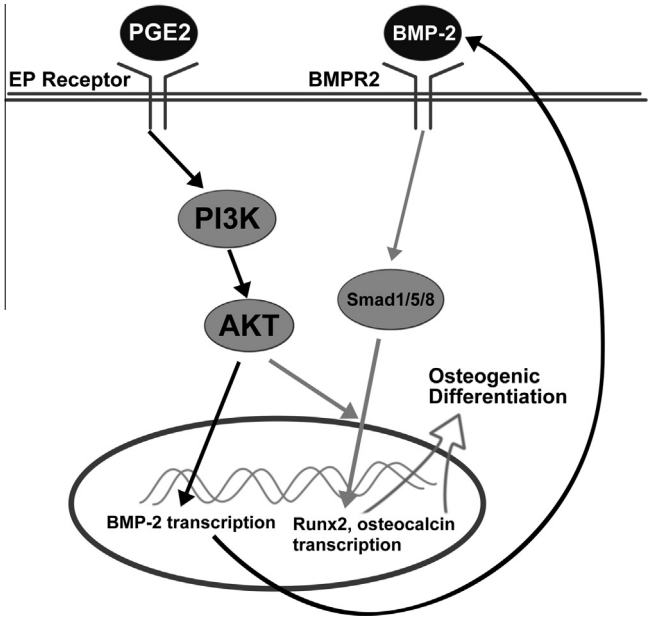

Fig. 4. Model for PI3K/Akt signaling in PGE2-induced osteogenic differentiation of rat TSCs. PGE2 accumulates in tendons subjected to repetitive mechanical loading conditions. Through the PGE2 receptor, PGE2 activates Akt, which in turn up-regulates the expression of BMP-2. Increased BMP-2 levels activate Smad, which subsequently promotes the transcription of Runx2 and osteocalcin in PI3K/Akt signaling, resulting in osteogenic differentiation.

results suggest that the PI3K-Akt signaling pathway may contribute to the formation of calcified tissues in tendinopathic tendons, indicating a potential therapeutic role for PI3K and Akt inhibitors.

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