

BMP-2 Mediates PGE₂-Induced Reduction of Proliferation and Osteogenic Differentiation of Human Tendon Stem Cells

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ABSTRACT: Tendon stem cells (TSCs) have been proposed to play a major role in the development of tendinopathy, which refers to pathological changes, such as calcification, in affected tendons. Using a human TSC (hTSC) culture model, this study investigated the effects of PGE₂, an inflammatory mediator present in injured tendons, on hTSC proliferation and differentiation as well as the molecular mediator for such PGE₂-induced effects. We found that PGE₂ treatment of hTSCs decreased cell proliferation and caused osteogenic differentiation of hTSCs in a dose-dependent manner. Also, PGE₂ treatment of hTSCs induced dose-dependent BMP-2 production in culture, and moreover, addition of BMP-2 to hTSC culture decreased cell proliferation and induced hTSC differentiation into osteoblasts. Finally, addition of BMP-2 antibodies to hTSC culture treated with PGE₂ nearly abolished PGE₂ effects on both cell proliferation and osteogenic differentiation. Taken together, the findings of this study showed that BMP-2 mediates PGE₂-induced reduction of proliferation and osteogenic differentiation of hTSCs. We suggest that such a mechanism may be partially responsible for the formation of calcified tissues in tendinopathic tendons seen in clinical settings. © 2011 Orthopaedic Research Society. Published by Wiley Periodicals, Inc. *J Orthop Res*

Keywords: tendinopathy; tendon stem cells; proliferation; differentiation; osteogenesis

Tendons are fibrous bands of connective tissue that connect muscles to bones. Traditionally, tendons are thought to contain mainly tenocytes, or fibroblast-like cells responsible for the maintenance and repair of tendons. However, in recent years, a new type of tendon cells termed tendon stem cells (TSCs) has been identified in humans, mice,^{1,2} rabbits,³ and rats.⁴ TSCs differ from tenocytes in that they possess multi-differentiation potential;³ for example, under appropriate induction conditions, these tendon-specific stem cells can differentiate into osteoblasts, or bone cells, among other non-tenocyte lineages of cells.

The function of tendons is to transmit muscular forces to bone, thus enabling joint movements. During such force transmission, large mechanical loads act on the tendons; consequently, tendons are frequently injured. Tendon injuries are divided into two categories, namely acute tendon injury (or traumatic injury) and chronic tendon injury. The latter is also referred to tendinopathy.⁵ A prevalent tendon disorder in both occupational and athletic settings, tendinopathy is associated with pain, localized tenderness, swelling, and impaired performance. Histopathologically, tendinopathy at later stages (or the degenerative stages⁶) is characterized by variable zones of reduced number of cells^{7,8} and calcific tissues, among other symptoms, in tendon lesions.⁹ While the precise mechanisms for the development of tendinopathy remain elusive, previous studies^{10–12} suggest that prostaglandin E₂ (PGE₂), a major inflammatory mediator of pain and acute inflammation in injured tendons,¹³ may be involved due to its catabolic effects

on tendon cells. PGE₂ treatment of tenocytes, for instance, decreases cell proliferation and cellular production of collagen.¹⁴ In addition, when rabbit TSCs were treated with PGE₂ in vitro, the treatment suppressed cell proliferation and induced TSC differentiation into osteoblasts.¹³ Despite these observations of PGE₂ effects, the molecular mediators responsible for such effects on TSCs were not known. Considering that bone morphogenetic protein-2 (BMP-2) is a bone-inducing differentiation factor¹⁵ that causes mesenchymal stem cells (MSCs) to differentiate into osteoblasts,¹⁶ we hypothesized that PGE₂ induced reduction of TSC proliferation and osteogenic differentiation is mediated through BMP-2. To test this hypothesis, we performed cell culture experiments using human TSCs (hTSCs). We found that BMP-2 mediated the reduction of proliferation and osteogenic differentiation of hTSCs due to PGE₂ treatment. The findings of this study suggest a TSC-based mechanism for the development of calcific tendinopathy.

MATERIALS AND METHODS

hTSC Culture

hTSCs were derived from the patellar tendons of six human donors, ages 26–49 years old. Briefly, using our published protocol,³ a single-cell suspension was obtained from core portions of patellar tendons and cultured in flasks at 37°C with 5% CO₂. After 8–10 days in culture, hTSCs formed colonies on the culture surface of the flask. These colonies were isolated by local treatment with 0.25% trypsin and cultured in growth medium (DMEM plus 20% FBS). hTSCs were expanded once (or passage 1), and cells at passage 1 were used for all cell culture experiments described as follows.

The PGE₂ Effect Experiment on Cell Proliferation

hTSCs were seeded in six-well plates at a density of 6 × 10⁴/well and cultured in growth medium with three

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concentrations (1, 10, and 100 ng/ml) of PGE₂. Cells without PGE₂ treatment were used as controls. The medium was changed every 3 days, with addition of fresh PGE₂ each time. The cell proliferation was measured at 3 and 7 days by counting cells using a hemocytometer.

The Measurement of BMP-2 Levels in Culture

In the cell proliferation experiment above, cell-conditioned medium at 7 days was collected, and BMP-2 levels in culture were measured using an ELISA kit (R&D Systems Inc., Minneapolis, MN).

The PGE₂ Effect Experiment on Osteogenic Differentiation of hTSCs

In a separate experiment, hTSCs were seeded in six-well plates at a density of 24×10^4 /well and cultured in growth medium with various concentrations of PGE₂ for 14 days. Alizarin S Red assay³ was used to assess osteogenic differentiation of hTSCs. In addition, immunostaining for osteocalcin expression was also performed to verify osteogenesis of hTSCs by PGE₂. Osteocalcin is a noncollagenous protein produced solely by osteoblasts;¹⁷ hence, it was used as a marker of osteogenesis of hTSCs in this study.

The BMP-2 Effect Experiment on Cell Proliferation and Differentiation

hTSCs were seeded in six-well plates at a density of 9×10^4 /well and cultured in growth medium for 2 days. Then the cells were divided into five groups as follows. Group 1: the control cells without any treatment; group 2: the cells treated with PGE₂ (100 ng/ml); group 3: the cells treated with the same amount of PGE₂ plus a saturated concentration of BMP-2 antibodies (100 ng/ml); group 4: the cells treated with BMP-2 (10 ng/ml), and group 5: the cells treated with the same amount of BMP-2 plus a saturated concentration of BMP-2 antibodies (100 ng/ml).

The cells were grown in culture for up to 15 days. During culture time, media were replaced with new growth media containing fresh PGE₂ and anti-BMP-2 antibodies every 3 days.

The proliferation of hTSCs in each group was assessed by measuring cell population doubling time (PDT),³ and the differentiation of hTSCs in each group was assessed using Alizarin Red S assay for staining calcium deposits and immunostaining for osteocalcin.

Alizarin Red S Assay

Osteogenesis of hTSCs induced by PGE₂ or BMP-2 was evaluated by Alizarin Red S assay. After removing culture medium, cells were fixed in ice-cold 70% ethanol for 1 h. Then the cells were rinsed with distilled water two times, each 5 min, and stained with Alizarin Red S (Millipore, Billerica, MA, Cat. # 2003999) at room temperature for 30 min. The stained cells were examined on the microscope, with images taken by a CCD camera and analyzed by SPOTTM imaging software. The osteoblasts containing mineral deposits would be stained brown by Alizarin Red S assay, which is an established method to assess osteogenesis of stem cells.^{1,3}

Immunostaining of hTSCs and Differentiated Cells

Immunocytochemistry was performed on the stem cell markers, including Oct-4, SSEA-4, and nucleostemin, using the protocol previously described.³ In addition,

immunostaining was performed on differentiated cells to detect the expression of osteocalcin. Briefly, cells were fixed with 4% paraformaldehyde in PBS for 30 min at room temperature and blocked in 2% mouse or goat or rabbit serum for 1 h. Then the cells were incubated at room temperature with mouse anti-human Oct-4 (1:400; Millipore, Cat. P20263) for 5 h, mouse anti-human SSEA-4 (1:350; Invitrogen, Carlsbad, CA, Cat. # 414000) for 5 h, goat anti-human nucleostemin (1:500; Neuromics, Edina, MN, Cat. # GT1505) for 4 h, and rabbit anti-osteocalcin (1:350; Millipore, Cat. # AB10911) for 3 h. After washing the cells with PBS for three times, Cy3-conjugated goat anti-rabbit IgG (1:500; Invitrogen, Cat. # A10521) for Oct-4 and SSEA-4, Cy3-conjugated Donkey anti-goat IgG (1:500; Millipore, Cat. # AP180C) for nucleostemin, and Cy3-conjugated goat anti-rabbit IgG (1:500; Millipore, Cat. # AP132C) for osteocalcin were applied. Negative controls were used during immunostaining by omitting primary antibodies, and at least two independent stainings were performed. The stained cells were examined and color images of cells were obtained on a fluorescence microscope.

Statistical Analysis

Unless otherwise indicated, data were expressed in mean \pm SD. For statistical data analysis, one-way ANOVA was used, followed by Fisher's PLSD test for multiple comparisons. A *p*-value <0.05 was considered to be significantly different.

RESULTS

Human TSCs (hTSCs) exhibited a cobblestone-like shape in confluent conditions (Fig. 1A), in contrast to highly elongated fibroblast-phenotype of tenocytes (not shown). Immunostaining also showed that hTSCs expressed stem cell markers Oct-4, SSEA-4, and nucleostemin (Fig. 1B–D), thus verifying their stem cell identity.

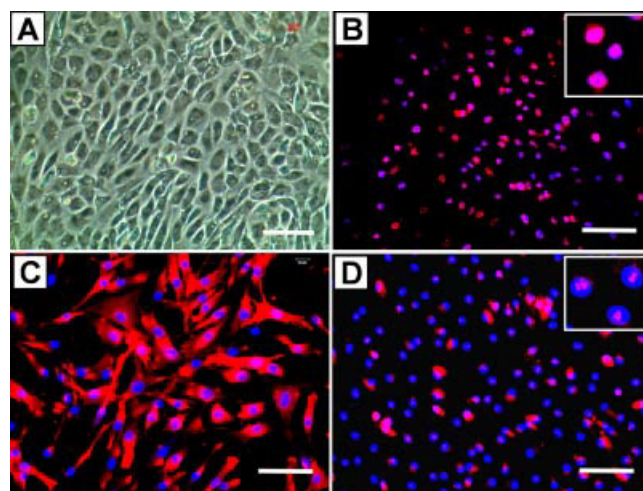


Figure 1. The identity of human tendon stem cells (hTSCs) was verified. Specifically, hTSCs exhibited a cobblestone-like shape when grown to confluence (A). However, human tenocytes were highly elongated in shape (not shown). Moreover, hTSCs in culture expressed stem cell markers Oct-4 (B, pink dots in the inset), SSEA-4 (C, red), and nucleostemin (D, pink dots in the inset). Bar: 100 μ m.

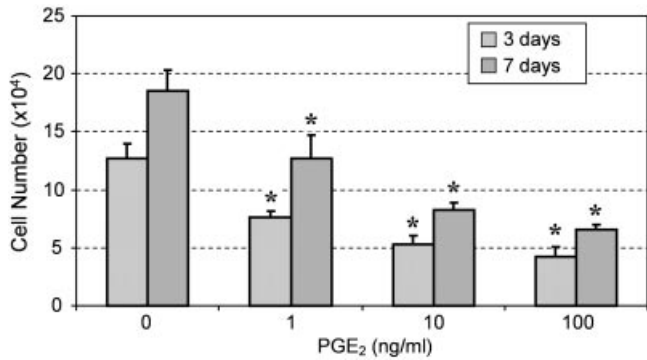


Figure 2. PGE₂ treatment of hTSCs decreased cell proliferation at both time points (3 and 7 days) in a dose-dependent manner (**p* < 0.05, with respect to control and to each other).

After hTSCs were cultured in PGE₂-containing growth medium for 3 and 7 days, cell proliferation decreased at both time points in a dose-dependent manner (Fig. 2). Moreover, PGE₂ treatment induced osteogenic differentiation of hTSCs, as evidenced by the accumulation of calcium deposits (Fig. 3A–D) and the expression of osteocalcin (Fig. 3E–H), a marker for osteoblasts. The PGE₂ effects on osteogenic differentiation of hTSCs also appeared to be dose dependent.

In addition, PGE₂ treatment of hTSCs induced production of BMP-2 in the medium, with increasing levels dependent on PGE₂ dose (Fig. 4). Addition of PGE₂ (100 ng/ml) or BMP-2 (10 ng/ml) to hTSC culture decreased cell proliferation, but each inhibitory effect was abolished by co-addition of BMP-2 antibodies, as cell proliferation returned to control levels (Fig. 5). Furthermore, hTSCs treated with BMP-2 (10 ng/ml) underwent osteogenic differentiation (Fig. 6A and B), as shown by staining of calcium deposits and osteocalcin; however, addition of BMP-2 antibodies to hTSC culture treated with PGE₂ (100 ng/

ml) largely blocked hTSC osteogenesis as well (Fig. 6C and D).

DISCUSSION

To determine how PGE₂ treatment of hTSCs affects cell proliferation and differentiation and whether BMP-2 mediates such PGE₂-induced effects, we first derived hTSCs and verified their stemness from their cobblestone shape in confluent conditions and their expression of Oct-4, SSEA-4, and nucleostemin, three TSC markers.³ We then performed a hTSC culture experiment and showed that PGE₂ treatment of hTSCs decreased cell proliferation and induced osteogenic differentiation, which was consistent with our previous findings on rabbit TSCs.¹³ We further showed that BMP-2 was produced in PGE₂ treated hTSC cultures and that BMP-2 treatment of hTSCs also resulted in reduced cell proliferation and osteogenic differentiation, similar to the results of PGE₂ treatment. When BMP-2 antibodies were added to BMP-2 or PGE₂-containing hTSC cultures, however, cell proliferation went back to control levels, and osteogenic differentiation was largely abolished. Taken together, the results indicate that PGE₂ treatment of hTSCs suppressed cell proliferation and induced osteogenic differentiation and that the two cellular events were mediated by BMP-2 produced in culture due to PGE₂ treatment.

PGE₂ is a prostaglandin belonging to the eicosanoid family that plays a prominent role in inflammatory and immune responses as well as various other tissue responses.^{18,19} It is also known that PGE₂ is produced in human, rabbit, and mouse tendons.^{13,20,21} Taken together with the findings of this study, we believe that PGE₂, acting as a local hormone in tendons, may contribute to the development of tendinopathy through two parallel, cellular mechanisms: (a) decreasing the number of TSCs, and (b) inducing osteogenic differentiation of TSCs. Mediated by BMP-2, both cellular events may lead to reduction in TSCs available for

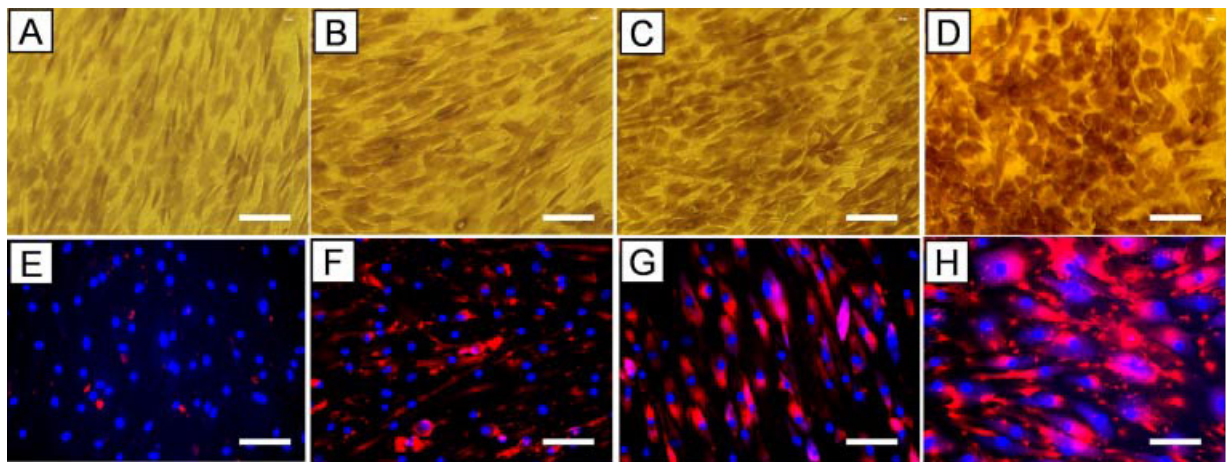


Figure 3. PGE₂ treatment induced osteogenic differentiation of hTSCs. (A and E) Controls (i.e., cells without PGE₂ treatment); (B and F) PGE₂ treatment at a low dose (1 ng/ml); (C and G) PGE₂ treatment at a medium dose (10 ng/ml); and (D and H) PGE₂ treatment at a high dose (100 ng/ml). With increased PGE₂ dosage, more calcium deposits (A–D) were stained, and more extensive expression of osteocalcin were detected (E–H, red). Bar: 100 μm.

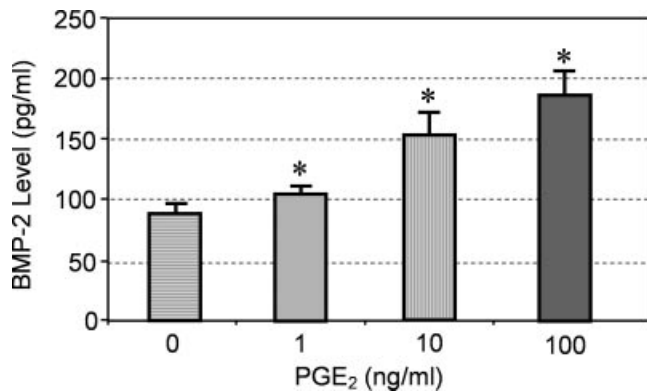


Figure 4. PGE₂ treatment of hTSCs increased BMP-2 production in culture in an apparent dose-dependent manner (**p* < 0.05, with respect to control and to each other).

tendon repair and calcification often seen in tendons at later stages of tendinopathy.⁹

BMP-2 is a known osteogenic growth factor and has been demonstrated to induce bone formation in vivo; for example, implantation of BMP-2 in a collagen sponge induces new bone formation, and this approach is widely used for the treatment of bony defects, delayed union, and non-union.²² In vitro, BMP-2 causes a dose-dependent decrease in proliferation of human fetal osteoblastic cells (hFOB 1.19)²³ and induces osteogenic differentiation of BMSCs.¹⁶ The cellular events caused BMP-2 are likely two separated but coordinated processes.²⁴ In this study, when hTSCs were treated with BMP-2, they also differentiated to osteoblasts, as indicated by the presence of abundant calcium deposits and the expression of osteocalcin. Therefore, our finding that BMP-2 treatment of hTSCs leads to reduced cell proliferation and

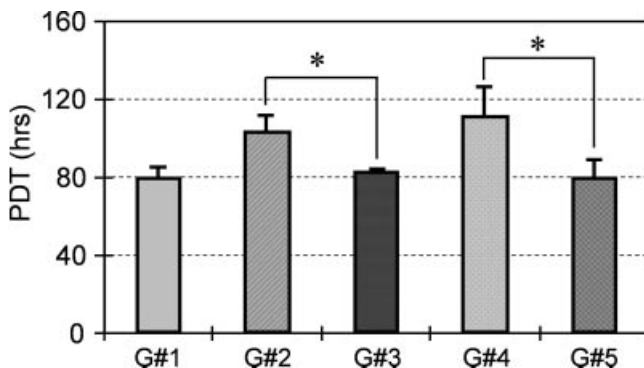


Figure 5. BMP-2 treatment mediates reduction in proliferation of PGE₂-treated hTSCs. Cell proliferation was assessed by measuring the population doubling time (PDT) of cells in culture. Note that G#1 is the control cells without any treatment, G#2 is the cells treated with PGE₂ (100 ng/ml); G#3 is the cells treated with the same amount of PGE₂ plus a saturated concentration of BMP-2 antibodies (100 ng/ml); G#4 is the cells treated with BMP-2 (10 ng/ml), and G#5 is the cells treated with the same amount of BMP-2 plus a saturated concentration of BMP-2 antibodies (100 ng/ml). As indicated, G#3 was significantly different from G#2, so was G#5 with respect to G#4 (**p* < 0.05). Also, note that G#2 and G#4 were significantly different from G#1 (the control group), respectively. However, there was no significant difference between G#2 and G#4.

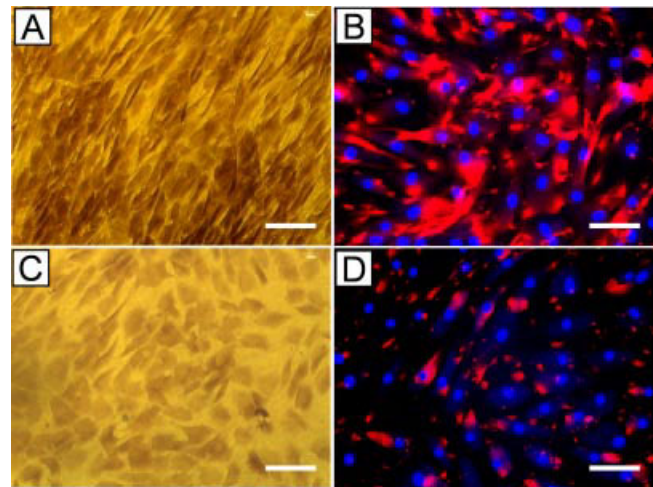


Figure 6. BMP-2 mediated osteogenic differentiation of PGE₂-treated hTSCs. When treated with BMP-2, hTSCs underwent osteogenic differentiation, as evidenced by the accumulation of abundant calcium deposits (A, compared to Fig. 3D) and extensive osteocalcin expression (B, red). Addition of BMP-2 antibodies to hTSC culture treated with PGE₂ (100 ng/ml) markedly decreased the extent of osteogenic differentiation, as indicated by light staining of calcium (C, compared to Fig. 3A) and osteocalcin (D, compared to Fig. 3E). Bar: 100 μm.

osteogenic differentiation is consistent with the known osteogenic functions of BMP-2 as well as its effect on osteoblastic cells.

BMP-2 has been found in injured tendons, and it has been suggested to be responsible for the calcification of tendinous tissues in vivo.²⁵ In addition, mechanical loading of so called tendon-derived stem cells increases BMP-2 expression.²⁶ However, because PGE₂ treatment of hTSCs leads to BMP-2 production (Fig. 4), and mechanical loading of tendons and tenocytes increases PGE₂ production,^{10,12,27} we suspect that PGE₂ acts as an upstream molecule of BMP-2 that suppresses hTSC proliferation while inducing osteogenic differentiation. This argument is also supported by the previous finding that treatment of human MSCs (hMSCs) with NS-398, which blocks cellular production of PGE₂, among others, by inhibiting COX-2,²⁸ down-regulates BMP-2 expression.²⁹

In this study, PGE₂ at all three doses (1, 10, 100 ng/ml, or 2.84, 28.4, 284 nM) was shown to decrease hTSC proliferation. A previous study³⁰ showed that while PGE₂ at 25 and 250 nM decreased proliferation of hMSCs, as measured by enhanced ³H-thymidine incorporation, at low concentrations (<25 nM), PGE₂ slightly increased hMSC proliferation. Therefore, Kleiveland's study suggests that PGE₂ at lower concentrations than those used in his study could increase instead of decrease hTSC proliferation. While this possibility is definitely worthy of further investigation, there are two important differences between this study and our study. These include: (a) hMSCs versus hTSCs. Another previous study showed that the two types of stem cells differ from each other in terms of

gene profiles;¹ and (b) hMSCs in serum starvation conditions vs. hTSCs in cultures with 20% FBS. Stem cells in culture are metabolically active.^{1,3} Therefore, there is a possibility that such serum starvation may change the stemness of stem cells. Finally, MSCs may respond differently to PGE₂ treatment than hTSCs, as a previous study showed that addition of PGE₂ to rat MSC cultures increased cell number in a concentration-dependent manner.³¹ One cautionary note is, however, that the stemness of MSCs in this study and Kleiveland's study were not characterized.

It is now known that tendon tissues produce PGE₂ in response to mechanical loading placed on tendons.^{13,32} So the question is: are there any mechanisms in tendons that can minimize the catabolic effects on TSCs? While future studies are obviously required to look into this important question, we speculate that like tendon fibroblasts, leukotriene B₄ may be able to counter-balance the negative effects induced by PGE₂ on TSCs.³³

Now a few comments on the limitations of this study are in place. First, this was a cell culture study that did not take into account the effects of mechanical loading on hTSCs. However, since tendons are subject to large mechanical loading in vivo, future studies should investigate the possible synergistic effects of PGE₂ and mechanical loading on TSCs. Second, in addition to BMP-2, there are other BMPs,¹⁵ such as BMP-4 and BMP-7, that could also play a role in mediating PGE₂ effects on TSC proliferation and osteogenic differentiation. A previous study showed that BMP-4 and BMP-7 were expressed by chondrocyte-like cells in a rat injury model,³⁴ suggesting that during tendon injury, these BMPs could also mediate nontenocyte differentiation of TSCs (e.g., chondrogenesis). Third, the influence of tenocytes on TSCs in terms of their response to PGE₂ treatment was not examined. Future investigation should be done to determine the interactions between the two types of tendon cells. Fourth, in our TSC culture model, exogenous instead of endogenous PGE₂ was used to assess its effects on TSCs. There might be differences between the two sources of PGE₂ in their effects on TSCs. Finally, the molecular signaling pathways of BMP-2 that lead to osteogenic differentiation of TSCs in this study are not known and remain to be determined in future studies. It is likely, however, that p38 kinase and Smad1/5 could be involved in BMP-2 signaling.³⁵

In summary, this study showed that PGE₂ treatment of hTSCs decreases cell proliferation and induces osteogenic differentiation and that these effects are mediated through BMP-2 produced in response to PGE₂ treatment. We suggest that such BMP-2 mediated effects on hTSCs may contribute to the formation of calcified tissues in tendinopathic tendons.

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