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Original Article

Hylonome

The effects of substance P on pluripotent tendon cells: an *in vitro* and *in vivo* study

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Abstract

Pluripotent tendon cells (PTCs) play important roles in maintaining metabolic balance and the development of tendinopathy. Recently, substance P (SP) was identified as an important factor in the pathogenesis of tendinopathy. A better understanding of SP's effects on PTCs is essential to gain more insight into tendinopathy. This study aims to investigate the effects of SP on PTCs *in vitro* and *in vivo*. For the *in vitro* study, we quantified exogenous SP production by PTCs and determined the effects of SP on the proliferation and differentiation of PTCs. For the *in vivo* study, we used a rat patella tendon injection model to investigate the biological effects of SP on PTCs and tendons. Our results showed that exogenous SP production by PTCs was 1.80 pg/10^6 cells and SP significantly enhanced the proliferation of PTCs. Reverse transcription polymerase chain reaction (RT-PCR) results showed that SP upregulated the expression of non-tenocyte genes but downregulated the expression of tenocyte-related genes in the differentiation of PTCs. Moreover, Histological examination showed that SP induced disorganizes of tissue and tendinosis-like changes *in vivo*. Taken together, these findings indicate that SP enhanced PTCs' proliferation, induced non-tenocyte differentiation and plays an important role in the development of tendinopathy.

Keywords: Tendons, Stem Cell, Substance P, Patella Tendon, Tendinopathy

Introduction

The role of neuropeptides in tendon pathology has been increasingly discussed and substance P (SP), a peripheral neurochemical mediator, has been identified as an important factor in the pathogenesis of tendinopathy. Recent studies show that tenocytes produce SP, and its receptor, neurokinin-1 receptor (NK-1R), is expressed throughout tendon tissue, especially in patients with tendinopathy[1]. Bi firstly isolated some cells from both human and mouse tendon, which possessed several universal criteria of stem cells in 2007[2] and were then subsequently confirmed to exist in rat and rabbit tendons[3]. We termed these cells as pluripotent tendon cells (PTCs). PTCs differ from tenocytes in that they form colonies and have the ability of self-renew in culture. Additionally, PTCs have multi-differentiation potential, which enables them to differentiate into tenocytes and non-tenocytes[2,3]. PTCs have been confirmed to play an important role in maintaining metabolic balance of tendons[4,5] and in the development of tendinopathy[6,7]. However, whether PTCs, like tenocytes, also produce SP and the characteristic responses of PTCs to SP remain unknown.

Chronically painful Achilles tendon tendinopathy has been verified by histological findings to contain abnormal tissue structure, hypercellularity, and neovascularization[8]. Recent studies show that SP accelerates hypercellularity in tendon tissue in response to Achilles tendon overuse in a tendinopathy model[9]. There is little doubt that PTCs in tendons are responsible for tenogenesis and tenocyte proliferation; however, whether SP enhances the proliferative ability of PTCs is unknown.

Although etiology of tendinopathy is considered to be multifactorial, the underlying mechanisms of tendinopathy are unclear. SP accelerates the development of tendinopathy-like

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changes in the rabbit Achilles tendon, which supports theories of a potential role of SP in tendinosis development[9]. Furthermore, PTCs play an important role in maintaining metabolic balance and repairing injured tendons. PTCs have demonstrated non-tenocyte differentiating potential, including differentiation into adipocytes, chondrocytes, and osteocytes under appropriate conditions[2,3]. To date, there is no information in the literature concerning the effects of SP on PTCs s' differentiation. We hypothesize that SP accelerates tendinopathy changes by inducing aberrant differentiation of PTCs into non-tenocytes.

Therefore, this study was designed to determine the response of PTCs to SP *in vitro*, specifically in terms of proliferation and differentiation. Secondly, we aimed to determine the biological effects of SP on PTCs and tendons *in vivo*. To this end, we experimentally injected SP into rat patella tendons model, and then assessed structural changes of tendons tissues.

Materials and methods

Ethics statement

The ethics committee of Southwest hospital approved all experimental protocols for the use of rats for tendon samples and the tendon injection model used in this study.

Study design

Male Sprague-Dawley rats (n=18; 6-8 month-old) were obtained from the Animal Experiment Centre of the Third Military Medical University. The rats accessed food and water freely, and were housed in a 12-hour light/12-hour dark cycle. Six rats were used for isolating PTCs *in vitro*. Another 12 rats were divided into two groups for the *in vivo* tests. Six rats underwent SP injection in the right patella tendon (10^{-6} mol/kg; Abcam, Hong Kong, People's Republic of China). Another six rats underwent the same volume injection of saline in the right patella tendon as a control. Rats were sacrificed by intramuscular injection of 10 mg/kg ketamine for histology 14 days after injection.

Isolation of PTCs and cell culture

PTCs isolation procedure was carried out as previously described[10]. Briefly, the midsubstance patellar tendons were harvested after the rats were sacrificed. Peritendinous connective tissue was then carefully removed and surrounding adipose tissue and blood were cleaned off using phosphate buffered saline (PBS; pH 7.4). Next, the tendon samples were minced into fine pieces (1 x 1 x 1 mm^3) and 10 mg of tendon tissue was digested with 3 mg type I collagenase (Worthington Biochemical Corp, Lakewood, NJ, USA) and 4 mg dispase (Stem Cell Technologies, Vancouver, BC, Canada) in 1 mL PBS at room temperature for 2 hours. The cell suspension was then centrifuged at 1500 g for 5 minutes. The supernatant was discarded and the cells were resuspended in Dulbecco's Modified Eagle's Medium (DMEM; Shanghai Juncheng Biotechnology Co Ltd, Shanghai, People's Republic of China) supplemented with 20% fetal bovine serum (FBS; Shanghai Juncheng Biotechnology Co Ltd). Cells were seeded in growth medium (DMEM +20% FBS) and cultured separately in 6-well culture plates. The PTCs formed adherent colonies after 9-12 days in culture. We detached cell colonies using trypsin and visually examined the cells under a microscope, and then used a micropipette to collect each individual detached cell colony. Finally, 100 cells from detached colonies were subcultured in culture plate for colony tests and to obtain sufficient PTCs for further studies. A minimum of triplicates of cells from six rats were used for each experimental condition; we used passage numbers one to four for all PTCs *in vitro* tests.

Flow cytometric analysis

Flow cytometric analysis was used to characterize PTCs and to examine stem cell gene expression in PTCs. Briefly, PTCs at passage one were harvested by trypsinization of the T75 flasks, centrifuged at 500 g for 5 min at room temperature, and resuspended in microcentrifuge tubes at a density of 1 x 10^4 cells in 20 µl staining buffer (Becton, Dickinson and Co, Franklin Lakes, NJ, USA). Next, 20 µl of the appropriate primary antibody (mice anti-rat PE conjugated CD90, CD44 or mice anti-rat FITC conjugated CD34, CD31) was added to the cells and incubated at 4°C for 2 hrs. The cells were again washed with PBS and analyzed in the Guava Personal Cytometry system using the protein express software package, analyzing at least 100,000 cells per sample. Antibodies were used according to the manufacturers recommended dilutions.

Immunostaining for stem cell markers

We examined the expression of stem cell markers on PTCs, including nucleostemin (NS), octamer-binding transcription factor 4 (Oct-4), and Nanog. Briefly, PTCs at passage one were seeded into 12-well plates at a density of 30,000 cells/well in 1.5 ml medium and cultured at 20% O2 for 48 hrs. Cells were then fixed in 4% paraformaldehyde for 10 min at room temperature and washed 3 times in PBS. Fixed cells were further treated with 0.5% Triton-X-100 in PBS for 20 min and coated with 2% goat serum/PBS for 30 min. After washing in PBS the cells were incubated with rabbit anti-rat Oct-4 (1:500), rabbit anti-rat Nanog (1:500) or rabbit anti-rat NS (1:500) overnight at 4°C, washed three times in PBS and again incubated with Cy3-conjugated goat anti-rabbit IgG (1:500) for 2 hrs at room temperature. After a final wash in PBS, stained cells were observed under fluorescence microscopy and digital images were recorded. All antibodies were obtained from Santa Cruz Biotechnology Inc (Santa Cruz, CA).

Immunoassay measurement of SP

SP levels produced by PTCs were measured using a commercial reagent (SP: EIA 7451; Bachem Holding AG, Bubendorf, Switzerland). PTCs at passage three were seeded in 25 cm^2 flasks with 5 mL growth medium for 4 days. Next, 1 mL of culture medium was harvested for immunoassay measurement of SP and the number of cells were counted using the cellometer Auto T4 automated cell counter (Nexcelon Bioscience LLC, Lawrence, MA, USA); the assay was performed according to the manufacturer's instructions. The lowest de-

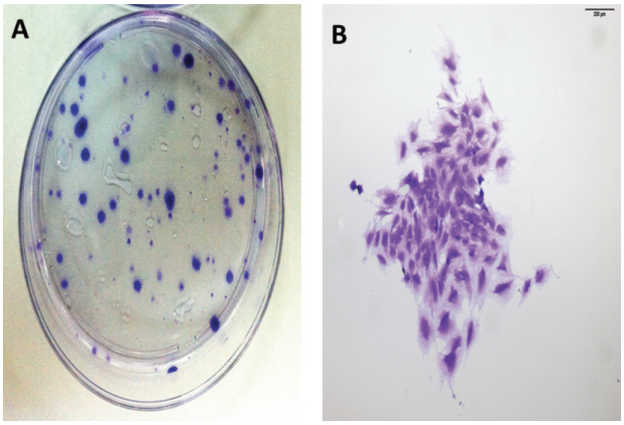

Figure 1. PTCs isolated from rat patella tendons formed colonies. The tendon samples were minced into fine pieces (1 x 1 x 1 mm^3) and 10 mg of tendon tissue was digested with 3 mg type I collagenase and 4 mg dispase in 1 mL PBS at room temperature for 2 hours. The cell suspension was centrifuged at 1500 g for 5 minutes. Next, the supernatant was discarded and the cells were resuspended in growth medium. Colonies formed after 8-10 days of culture; we detached cell colonies using trypsin and visually examined the cells under a microscope, and then used a micropipette to collect each individual detached cell colony. Finally, 100 cells from detached colonies were subcultured in culture plate for colony tests the colonies were staining with crystal violet. N=6. Bar: 100 μm.

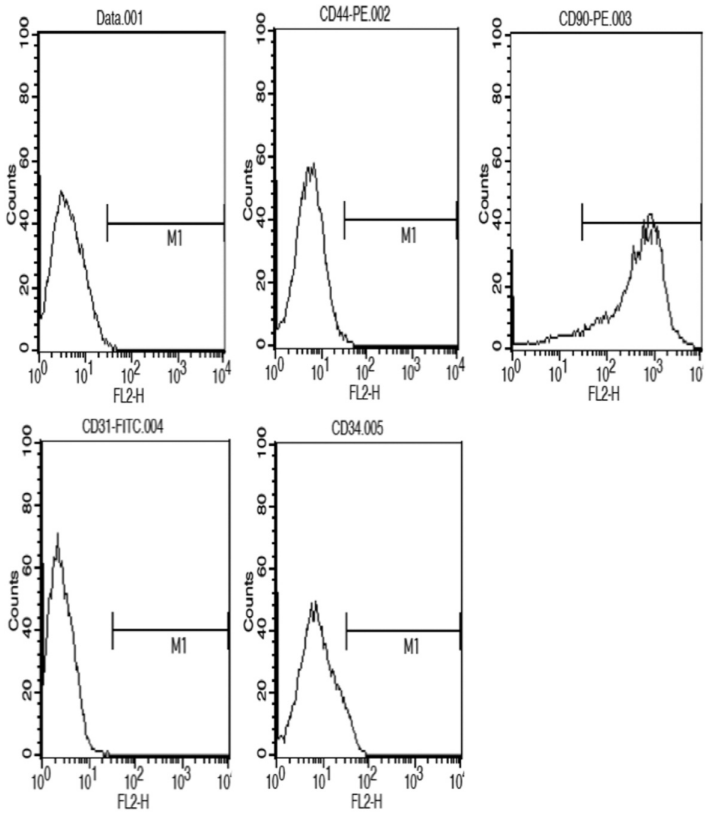

Figure 2. Flow cytometry analysis. Briefly, PTCs at passage one were harvested by trypsinization of the T75 flasks, centrifuged at 500 g for 5 min at room temperature, and resuspended in microcentrifuge tubes at a density of 1 x 10^4 cells in 20 μl staining buffer. Next, 20 μl of the appropriate primary antibody (mice anti-rat PE conjugated CD90, CD44 or mice anti-rat FITC conjugated CD34, CD31) was added to the cells and incubated at 4°C for 2 hrs. The cells were again washed with PBS and analyzed in the Guava Personal Cytometry system using the protein express software package, analyzing at least 100,000 cells per sample. Results showed that PTCs were negative for CD34 (cell surface marker for hematopoietic cells), CD44 (cell surface marker for MSCs) and CD31 (cell surface marker for endothelial cells). However, these cells were positive for CD90 (cell surface marker for fibroblasts). N=3.

tectable concentration was 2 pg/50 mL sample. The tracer was biotinylated SP, and SP cross-reaction was 100%. Triplicates of cells from six rats were used.

PTCs proliferation experiment

To measure proliferation, PTCs (passage three) were seeded in 6-well plates at a density of 5 x 10^4/well and cultured in growth medium with or without 1 nM SP for 72 hours; the number of cells were counted at 24, 48, and 72 hours using the cellometer (Nexcelom Bioscience LLC). We used the cell counting Kit-8 test (CCK-8; Beyotime Institute of Biotechnology, Jiangsu, People's Republic of China) to also assess proliferation. For this test, PTCs were seeded onto 96-well culture plates at a density of 3,000 cells/well in 100 µL of medium with or without 1 nM SP and maintained in regular 20% O_2 culture conditions. Next, 10 µL of CCK-8 solution was added to each well of the plate after seeding for 24 hours and incubation for another 2 hours, then the absorbance was measured at 450 nm using a microplate reader (SpectraMax Plus384 Absorbance Microplate Reader; Molecular Devices, LLC, Sunnyvale, CA, USA).

Multi-lineage differentiation potential test

The differentiation capacity of PTCs *in vitro* was examined by testing their ability to undergo chondrogenesis, adipogenesis, and osteogenesis. PTCs (passage four) were seeded on 6-well plates at a density of 1 x 10^6 cells/well in basic growth medium. Once the cultured cells were confluent, growth medium was replaced by chondrogenic, adipogenic, and osteogenic induction medium (Shanghai Juncheng Biotechnology Co Ltd) with or without 1 nM of SP. PTCs were grown in the aforementioned three types of media for 21 days followed by Safranin O, Oil red O, or Alizarin red S assay as described previously[11]. A semi-quantification method was implemented in which ten random images were obtained with a camera attached to a Nikon eclipse microscope (Nikon Instruments Inc, Melville, NY, USA). In each image, the positively stained cells were identified manually and analyzed using SPOT™ imaging software (Diagnostic Instruments Inc, Sterling Heights, MI, USA). Next, the percentage of positively stained cells in each image was calculated by averaging values.

Gene expression analysis

To characterize the response of PTCs to SP, we determined the expression of tenocyte-related and non-tenocyte related genes using qRT-PCR. PTCs at passage three were seed in T25 flasks in basic growth medium with or without 1 nM SP for 72 hours. Total RNA was extracted from PTCs using the RNeasy Mini Kit (Qiagen, Limburg, Netherlands). Tenocyte-related genes and non-tenocyte related genes including collagen (Col) types I, tenomodulin, Col II, transcription factor Sox 9, osteogenic transcription factor runt-related transcription factor 2 (Runx2), Osteocalcin, and peroxisome proliferator-activated receptor y (PPARγ) were examined. Glyceraldehyde-3-phosphate dehydrogenase was used as an internal control. Forward and reverse primers for all genes were designed based on previously published sequences[6] and were synthesized by Life Technologies (Carlsbad, CA, USA). A minimum of three replicates were performed for each gene and each experimental condition.

Histology

Twelve rats underwent SP or saline injection in the right patella tendon. Rats were sacrificed 14 days after SP injection. Patellar tendons from both control and SP injection groups were harvested, quickly snap frozen in tissue freezing medium and stored at -80°C. Using a cryostat, these frozen tendon tissues were sectioned at a thickness of 6 mm and subjected to hematoxylin and eosin (H&E) or Masson staining. Three sections from each rat were analyzed.

Statistical analysis

The Statistical Package for Social Sciences for Windows, version 21.0 (SPSS Inc, Chicago, IL, USA) was used for the statistical analyses. All data are presented as mean ± standard deviation (SD). Independent t-test and Shapiro-Wilk test were used for statistical analysis. Differences between two groups were considered significant when the p-value was less than 0.05.

Results

Verification of rat PTCs

First, PTCs were identified by clonogenicity and flow cytometry of cell surface markers. The cells isolated from rat patella tendons formed colonies after 8-10 days in culture (Figure 1). Flow cytometry results showed that these cells were negative for CD34, CD45, CD44, and CD31; however, these sells were positive for CD90 (Figure 2). The results of immunostaining showed that PTCs were positively stained by the stem cell markers of NS, Oct-4, and Nanog (Figure 3).

SP significantly enhanced the proliferation of PTCs

We found that PTCs produce SP at a rate of 1.80±0.27 pg/10^6 cells. We also tested the effect of SP on the proliferation capability of PTCs by performing PDT and CCK assays. The results showed that SP significantly enhanced PTCS proliferation. The PDT of PTCs cultured with or without 1 nM SP was 25.4±0.91 hours and 29.1±1.59 hours, respectively (P=0.007; Figure 3A). Consistent with the PDT results, significant increases in OD values in PTCs cultured with 1 nM SP were observed on the CCK test; the OD values of PTCs cultured with or without SP were 1.81±0.14 and 1.15±0.10, respectively (P=0.000; Figure 3B).

SP induced differentiation of PTCs into non-tenocytes in vitro

The multi-differentiation potential toward osteogenesis, adipogenesis, and chondrogenesis in PTCs cultured with or without SP group was also determined. qRT-PCR results showed that non-tenocyte related genes (Sox9, PPARγ, Runx2, and Osteocalcin) were significantly upregulated in PTCs cultured with SP. However, the expression of Col I was significantly

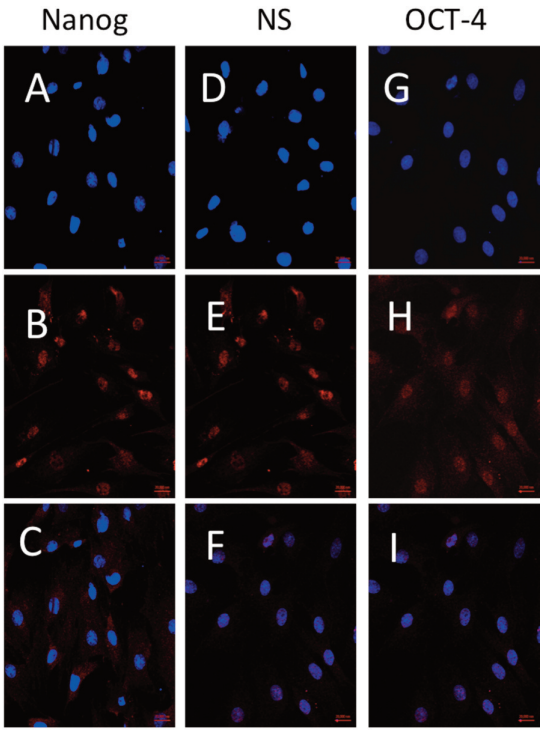

Figure 3. Immunostaining of stem cell markers on PTCs. We examined the expression of stem cell markers on PTCs, including NS, Oct-4, and Nanog. Briefly, PTCs at passage one were seeded into 12-well plates at a density of 30,000 cells/well in 1.5 ml medium and cultured at 20% O2 for 48 hrs. Cells were then fixed in 4% paraformaldehyde for 10 min at room temperature and washed 3 times in PBS. Fixed cells were further treated with 0.5% Triton-X-100 in PBS for 20 min and coated with 2% goat serum/PBS for 30 min. After washing in PBS the cells were incubated with rabbit anti-rat Oct-4 (1:500), rabbit anti-rat Nanog (1:500) or rabbit anti-rat NS (1:500) overnight at 4°C, washed three times in PBS and again incubated with Cy3-conjugated goat anti-rabbit IgG (1:500) for 2 hrs at room temperature. After a final wash in PBS, stained cells were observed under fluorescence microscopy and digital images were recorded. The results of immunostaining showed that PTCs were positively stained by the stem cell markers of NS, Oct-4, and Nanog.

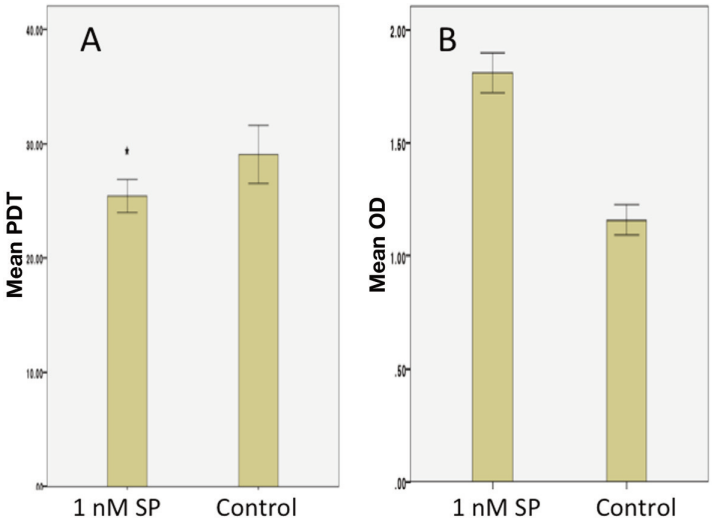

Figure 4. The effects of SP on PTCs proliferation. The effects of SP on the proliferation capability of PTCs were tested by performing PDT and CCK assays. For PDT test, PTCs at passage three were seeded on a 6-well plate at a density of 5 x 10^4/well and cultured in growth medium with or without 1 nM SP for 72 hours; then the cells were counted at 24, 48, 72 hours using a cellometer. Results showed that SP significantly enhanced the proliferation of PTCs from patellar tendons (*P<0.05). For CCK test, PTCs at passage three were seeded on a 96-well plate and cultured to determine proliferation potential. Next, 10 μL of CCK-8 solution was added to each well after seeding for 24 hours and incubated for another 2 hours, then the absorbance was measured at 450 nm using a microplate reader. Results showed that the mean OD value in PTCs cultured with 1nM SP was significantly higher than the control group (P<0.05).

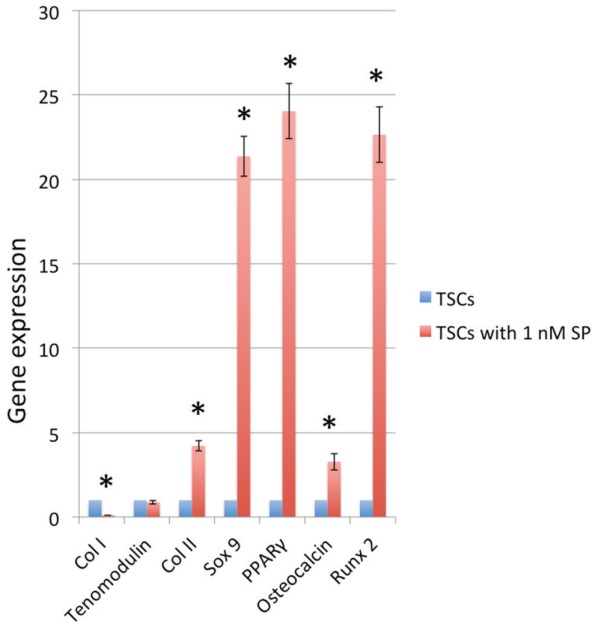

Figure 5. The expression of tenocyte and non-tenocyte related genes in PTCs isolated from rat patellar tendons in response to 1nM SP. Template for qRT-PCR were cDNA transcribed from total RNA extracted from rat PTCs cultured with or without 1 nM SP. GAPDH was used as an internal control. Expression of tenocyte related marker and non-tenocyte related genes were examined. As shown, SP increased the expression of non-tenocyte related genes (Sox9, PPARγ, Runx2, and Osteocascin) but decreased tenocyte related genes (Col I; $P<0.05$ with respect to the corresponding controls). Mean±SD of three samples is shown. Asterisks represent statistical significance at the level of $P<0.05$ when compared to the respective cage controls.

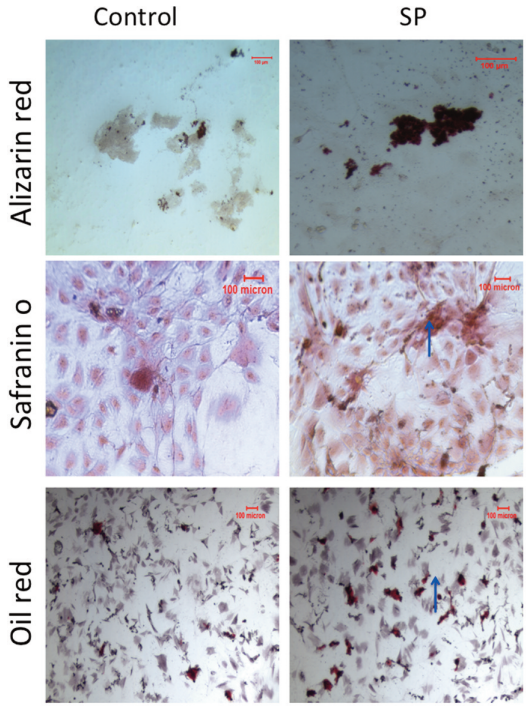

Figure 6. The differentiation capacity of PTCs in response to SP. PTCs were seeded on 6-well plates at a density of 1 x 10^6 cells/well in adipogenic, chondrogenic, or osteogenic induction medium with or without 1 nM SP. PTCs were cultured for 21 days followed by Oil red O assay for adipogenesis, Safranin O assay for chondrogenesis, and Alizarin red S assay for osteogenesis. Results showed that PTCs cultured with 1 nM SP underwent increased adipogenesis, chondrogenesis, and osteogenesis compared to PTCs with control medium. Blue arrows indicate positive cells.

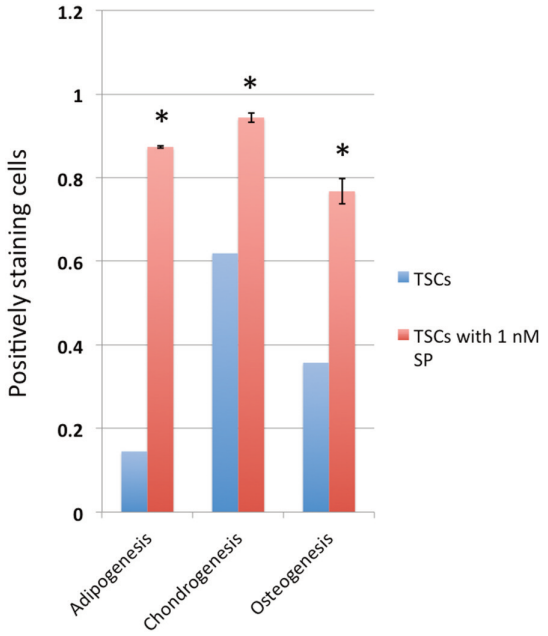

Figure 7. Semi-quantitative results of the differentiation capacity in PTCs. Positively stained cells were also counted to calculate percentage staining. Results showed that PTCs cultured with 1nM SP had a significant increase in the adipogenesis, chondrogenesis, and osteogenesis compared with the control group ($P<0.05$).

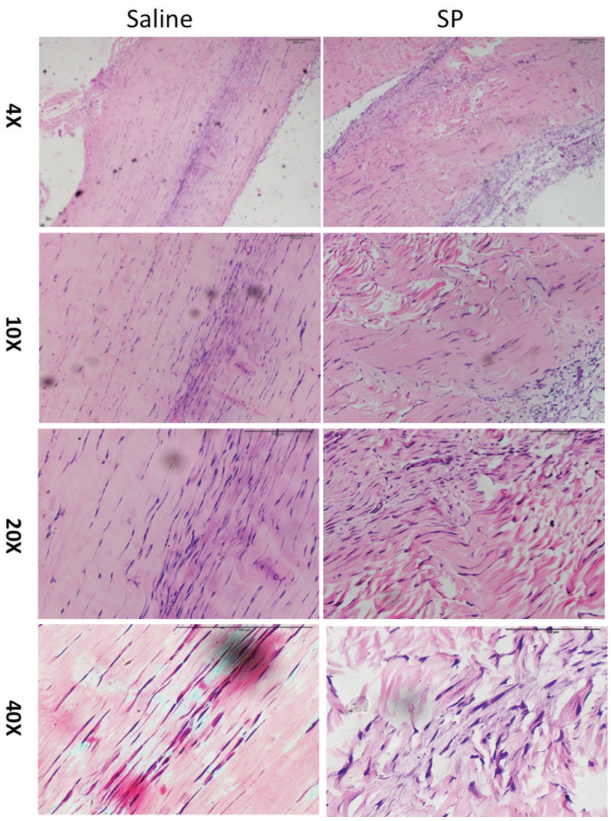

Figure 8. HE staining of patella tendons tissue. Twelve rats underwent SP or saline injection in the right patella tendon. Rats were sacrificed 14 days after SP injection. Patellar tendons from both control and SP injection groups were harvested, quickly snap frozen in tissue freezing medium and stored at -80°C. Using a cryostat, these frozen tendon tissues were sectioned at a thickness of 6 mm and subjected to HE staining. Patella tendons exhibited tendinosis-like changes after SP injection. After HE staining, results showed that the alignment of collagen fibers was disorganized and more cells appeared in the injection location 2 weeks after SP injection compared to with the saline injection.

decreased following culturing with SP, and no significant difference was seen in tenomodulin expression (Figure 4). These results were further demonstrated by multi-lineage differentiation staining (Figure 5). Significant increases were observed in the numbers of cells that stained positive for chondrogenesis, adipogenesis, and osteogenesis following culture with SP (Figure 6).

SP injection induced tendinosis-like tissue changes

We also performed *in vivo* experiments to examine the effects of SP injection on PTCs and patella tendons; patella tendons appeared to undergo tendinosis-like changes after SP injection. HE staining showed that the alignment of collagen fibers was disorganized and more cells appeared at the injection site 2 weeks after SP injection compared with saline injection (Figure 7). These results were corroborated by Masson staining, which showed significant disorganization and dis-alignment of the collagen fibers at 2th weeks postoperatively in the SP group compared to the saline control group (Figure 8).

Discussion

A recent study showed that tenocytes produce SP[1] and that SP accelerates the development of tendinosis-like changes in rabbit Achilles tendons[9]. Furthermore, SP plays a potential role in a tendinosis development[12,13]. However, PTCs also play a major role in maintaining metabolic balance and repairing injured tendons[3,14]. Therefore, it is essential to define the effects of SP on PTCs in order to gain a better understanding of tendinopathy. In our *in vitro* study, results showed that PTCs produced SP and SP significantly enhanced the proliferation of PTCs. PTCs promoted the differentiation of PTCs into non-tenocytes in the induction media. Our *in vivo* experiments showed that SP injections led to tendinosis-like tissue changes. Taken together, these results suggest that a critical role for SP in maintaining metabolic balance and inducing tendinopathy.

Bi[2] firstly isolated tendon stem/progenitor cells (TSPCs), which possessed several universal criteria of stem cells. De Mos et al[15] showed that tendon-derived fibroblasts (TDFs) possessed mesenchymal stem-cell-like characteristics. However, many stem cell-related markers expressed by TSPCs were not specific[2,16]; moreover, the markers expressed by tendon stem

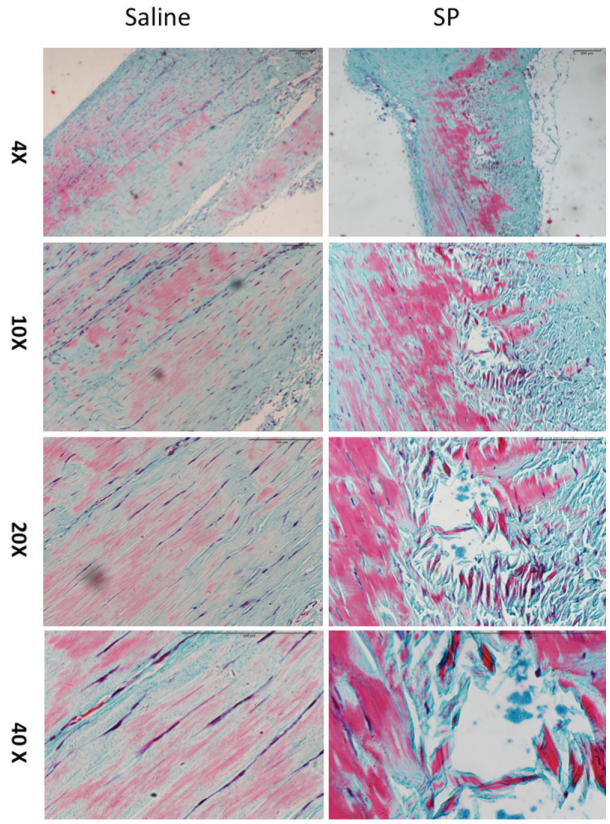

Figure 9. Masson staining of patella tendons tissue. Twelve rats underwent SP or saline injection in the right patella tendon. Rats were sacrificed 14 days after SP injection. Patellar tendons from both control and SP injection groups were harvested, quickly snap frozen in tissue freezing medium and stored at -80°C. Using a cryostat, these frozen tendon tissues were sectioned at a thickness of 6 mm and subjected to Masson staining. Patella tendons exhibited tendinosis-like changes after SP injection. After Masson staining, results showed that the alignment of collagen fibers was disorganized and more cells appeared in the injection location 2 weeks after SP injection compared to with the saline injection.

cells *in vivo* might be altered as a consequence of *in vitro* cell culture. Therefore, it is difficult to characterize these cells by cell markers. In the present study, we termed these cells as PTCs. PTCs possessed the ability of colony forming and multi-differentiate. PTCs were negative for CD34 (cell surface marker for hematopoietic cells), CD44 (cell surface marker for mesenchymal stem cells), and CD31 (cell surface marker for endothelial cells) and were positive for CD90 (cell surface marker for fibroblasts). These results verify the absence of MSCs, hematopoietic cells, endothelial cells, and fibroblasts. De Mos et al[15] showed that tendon fibroblasts or tenocytes may have trans-differentiation potential. Mienaltowski et al[17] reported that different stem/progenitor cell populations existed within distinct niches at the tendon proper and peritenon. Lui[18] verified that TDSCs and BMSCs might be distinct cell types. However, the possibility that TDSCs and BMSCs were derived from a common ancestor that gradually assumed tissue-specific phenotypes under the influence of local niche could not be excluded. In together, PTCs are heterogeneous and its detailed characteristics remained to be studied in future investigation.

Classically, SP was known to be involved in a number of important functions, such as in sensory neurons. In this study, we observed that PTCs produced SP, which has never previously been shown. In our *in vitro* study, the expression of tenocyte- related genes decreased with SP treatment. Because SP is rapidly degraded by neutral endopeptidases and SP appeared under both normal physiological conditions and tendinopathy tendons[1,9], we assumed that local SP levels in the tendon are mostly determined by the production of PTCs and tenocytes together. Recent study showed that the production of SP increased with tendinopathy[13]; therefore, there is a possible autocrine positive feedback loop that might contribute to the production of SP in tendinosis tendon tissue acting though PTCs[19]. In conclusion, these finding indicated that PTCs played an important roles in the production of SP.

It is now widely accepted that the underlying tissue changes in tendinopathy constitute hypercellularity, collagen discontinuity, and aberrant differentiation[20]. In this study, we showed that SP significantly enhanced PTCs' proliferation *in vitro*. Our *in vivo* results further supported these results, as there were more cells in the injection site 2 weeks after SP injection. These results were consistent with previous studies showing that SP accelerated hypercellularity in tendon tissue[9,21,22]. However, it is hard to identify that the enhanced proliferation of tenocytes are from PTCs or migrated MSCs. On one hand, we still do not know too much about the niche and special cell markers of PTCs. On the other hand, though MSCs has shown their engraftment in several tissues, particularly after injury[23,24]; another study reported that autologous adipose-derived MSCs labeled with nanocrystals were treated with the intralesional implants in the superficial digital flexor tendon. Results showed that labeled cells were present peripheral bloodstream and intralesional implants, but not superficial digital flexor tendon in the contralateral limb[25]. Chemokine receptors and their ligands and adhesion molecules play an important role in tissue-specific homing of MSCs[23], we speculate it may be a potential main direction of study. Taken together, we conclude that SP played a critical role in increasing hypercellularity. The mechanisms underlying proliferation of PTCs remain to be clarified and need more intensive study.

In addition, we investigated the effects of SP on differentiation of PTCs *in vitro*. Our *in vitro* experiments showed that SP enhanced non-tenogenic differentiation and decreased tenogenic differentiation. Based on these *in vitro* and *in vivo* results, it is reasonable to assume that SP can lead to tendinopathy though aberrant differentiation of PTCs. Previous reports showed that the loss of collagen organization was considered one of the effects of SP as it enhanced collagen remodeling and MMP-3 expression[26]. Furthermore, it has been shown that expression of SP increased in collagenase-induced tendon injury models[27] and in rotator cuff diseases[28]. Taken together, it can be concluded that SP induces aberrant differen-

tiation of PTCs, which leads to the tendinopathy-like tissue changes seen in the rat patella tendons.

The limitations of this study were that we only treated PTCs with one concentration (1 nM) and one *in vivo* dose (10^{-6} mol/kg) of SP. However, this does not devalue our conclusion that SP injections in the patella tendon induced tendinopathy because of the obvious pathological changes *in vivo* and enhanced expression of non-tenocyte genes *in vitro*. Further studies are needed to identify whether the effects of SP on PTCs and tendons are dose-dependent.

In summary, this study was designed to define the effects of SP on PTCs using *in vivo* and *in vitro* models. The findings reported here show that PTCs produce SP and that SP enhances PTCS proliferation. Furthermore, the presence of SP may be detrimental, as it directs differentiation of PTCs into non-tenocytes both *in vitro*.

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