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Platelet-Rich Plasma Releasate Promotes Differentiation of Tendon Stem Cells Into Active Tenocytes

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Background: Platelet-rich plasma (PRP) has been used to enhance tendon healing in clinical settings. However, the cellular mechanisms underlying PRP treatment of injured tendons remain unclear. The aim of this study was to determine the effects of PRP, in the form of PRP-clot releasate (PRCR), on tendon stem cells (TSCs), a newly discovered cell population in tendons.

Hypothesis: The PRCR treatment promotes differentiation of TSCs into tenocytes that are activated to proliferate quickly and increase collagen production.

Study Design: Controlled laboratory study.

Methods: After PRCR treatment, cell morphology, expression of stem/progenitor cell marker nucleostemin, and population doubling time were examined. In addition, gene and protein analyses were performed using reverse transcription-polymerase chain reaction, immunocytochemistry, and Western blot to characterize the type of cells that had differentiated after PRCR treatment.

Results: The TSCs without PRCR treatment were small and exhibited an irregular shape, whereas with increasing PRCR dosage, TSCs became large, well spread, and highly elongated with downregulation of nucleostemin expression. The PRCR treatment also markedly enhanced TSC proliferation, tenocyte-related gene and protein expression, and total collagen production, all of which indicated that PRCR treatment induced differentiation of TSCs into activated tenocytes.

Conclusion: The PRCR treatment promotes differentiation of TSCs into active tenocytes exhibiting high proliferation rates and collagen production capability.

Clinical Relevance: The findings of this study suggest that PRP treatment of injured tendons is “safe” as it promotes TSC differentiation into tenocytes rather than nontenocytes, which would compromise the structure and function of healing tendons by formation of nontendinous tissues. Moreover, they suggest that PRP treatment can enhance tendon healing because tenocytes induced to differentiate by PRP are activated to proliferate quickly and produce abundant collagen to repair injured tendons that have lost cells and matrix.

Keywords: platelet-rich plasma (PRP); tendon stem cells; differentiation; tenocytes; collagen

Tendon injuries are a common problem in sports medicine and orthopaedic practice. Healing of injured tendons is slow, especially when the injury is substantial or when rupture with tendon retraction occurs. Tendon healing

also forms scar tissue, which impairs joint function and has inferior mechanical properties that make the healed tendons susceptible to reinjury.^{7,27} Injured tendons undergo a complex healing process that involves several stages, including cell proliferation and matrix production. After these stages is remodeling, in which the healing tendon ultimately regains its mechanical strength. During tendon healing, platelet-derived growth factor (PDGF), epidermal growth factor, transforming growth factor-beta 1 (TGF- β_1), insulinlike growth factor (IGF-I), and certain receptors (eg, TGF- β_1 receptor) are upregulated in tendons.^{8-10,35} This provides a rationale for using autologous platelet-rich plasma (PRP), which contains various types of growth factors, to promote tendon and ligament healing.^{14,31}

Platelets function as a natural reservoir for releasing those growth factors that are essential to the repair of injured tissues. Most of these growth factors, including PDGF, epidermal growth factor, TGF- β_1 , IGF-I, vascular

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endothelial growth factor (VEGF), basic fibroblast growth factor, and hepatocyte growth factor (HGF), are also involved in tendon healing.^{17,23,37}

Previous studies using tenocyte cultures showed that PRP-clot releasate (PRCR) stimulates cell proliferation and total collagen production.^{2,11} Use of scaffolds enriched by growth factor-rich plasma was also shown to increase tendon cell proliferation and deposition of a collagen-rich extracellular matrix.³⁶ All these studies suggest that PRP treatment in vivo may enhance tendon healing by increasing tenocyte number and production of collagen, which, including collagen types I and III, is a major component of the tendon.³⁷

In addition to tenocytes, tendons also contain so-called tendon stem/progenitor cells (TSCs), the newly discovered tendon cell populations in humans, mice, rats, and rabbits.^{5,29,38} Like adult stem cells, TSCs undergo proliferation and differentiation in response to biochemical and biomechanical stimuli.³⁹ Because they are tendon-specific stem cells, TSCs under normal physiological conditions should differentiate into tenocytes to maintain tendon homeostasis and repair tendons when injured. However, the effects of PRP on TSCs are unknown. We reason that when tendons are injured, more tenocytes are needed, and these tenocytes must be activated to produce abundant tendon matrix components, including collagen, to effectively repair injured tendons that have lost both cells and matrix. In addition, it is generally recognized that the effects of PRP in promoting tissue wound healing are due to those growth factors (eg, TGF- β_1 and PDGF) contained in the α -granules of platelets.¹ Once activated, the platelets release the growth factors to repair injured tissues. Therefore, we hypothesized in this study that PRCR, a combination of growth factors released from PRP clots, stimulates TSCs to differentiate into tenocytes that are activated to increase their number and produce a large quantity of collagen. To test this hypothesis, we first derived TSCs from patellar tendons of rabbits and then treated the cells in culture with various concentrations of autologous PRCR. We next determined the types of cells that had differentiated from TSCs after PRCR treatment by examining changes in cell size and shape, proliferation rate, and gene and protein expression. Herein we report the findings of this study.

MATERIALS AND METHODS

Tissue Culture for TSCs

Nine adult New Zealand White rabbits (6-8 months old, 3.0-4.0 kg) were used in this study. The protocol for collecting tendons and blood from rabbits was approved by the University of Pittsburgh Institutional Animal Care and Use Committee. The TSCs were derived from patellar tendons using a published protocol.³⁸ These TSCs are hence referred to as *patellar TSCs* (PTSCs). The procedures for obtaining PTSCs are briefly described as follows. Patellar tendons were removed by cutting through their bony attachments. The tendon sheath was stripped away and the core portion of the tendons was minced into small

fragments. The fragments were then digested in a solution containing 3 mg/mL of collagenase type I (Worthington Biochemical Corporation, Lakewood, New Jersey) and 4 mg/mL of dispase (StemCell Technologies Inc., Vancouver, BC, Canada) in phosphate-buffered saline (PBS). The resulting cell suspension was centrifuged at 2000 rpm for 15 minutes to obtain a cell pellet. The cell pellet was resuspended in growth medium consisting of Dulbecco's Modified Eagle Medium (DMEM) supplemented with 20% fetal bovine serum (FBS; Atlanta Biologicals, Lawrenceville, Georgia) and 1% penicillin and streptomycin. After diluting the suspension to 10 cells/ μ L, the cells were plated in a 6-well plate for continued cell culture. Approximately 2 weeks later, TSC colonies formed on the culture plates and were subsequently separated from tenocytes by local application of concentrated trypsin (0.25%). The detached PTSCs were transferred to T25 flasks for continued culture in growth medium (ie, DMEM plus 20% FBS). Before PTSCs were used for cell culture experiments in this study, their stem cell identity was confirmed by staining stem cell markers, including Oct-4, SSEA-4, and nucleostemin.³⁸

Preparation of PRP-Clot Releasate

Immediately after rabbits were sacrificed, whole blood was extracted from the hearts using an 18-gauge needle. The blood (7-9 mL) was then mixed with 0.1 M sodium citrate (1 mL) in a centrifuge tube. Blood was centrifuged at 400g for 15 minutes, and then the supernatant, which contained concentrated platelets and is referred to as *PRP*, was collected. The PRP portion composed about 37% of total blood (7-9 mL). To activate platelets, the PRP was treated with 22 mM CaCl₂ at 37°C for 1 hour, followed by centrifugation at 2000g for 10 min. The resulting soluble releasate from the clot preparation contained growth factors released by activated platelets and is referred to as *PRCR*. The PRCR was collected by aspiration and stored at 4°C until use.

During the preparation of PRCR, platelet numbers in 8 samples of PRP and whole blood preparations were measured using an automated cell counter (Nexcelom Bioscience LLC, Lawrence, Massachusetts). Note that PRCR contains a number of concentrated growth factors, including PDGF-AB, TGF- β_1 , epidermal growth factor, VEGF, HGF, and IGF-I.² Considering that TGF- β_1 is known to be a potent inducer of α -smooth muscle actin (α -SMA),¹⁶ which was used as a marker for activated tenocytes in this study, we measured TGF- β_1 concentrations in 5 samples of our PRCR and blood preparations using a TGF- β_1 Single Plex Kit (Millipore, Billerica, Massachusetts; catalog No. TGFB-64K-01).

Cell Culture Experiment

The PTSCs at passage 2 were seeded in a 6-well plate at a density of 8 $\times 10^4$ cells per well and cultured in growth medium (DMEM 20% FBS) for 24 hours before being replaced by growth medium with 10% FBS only (control group) or with 10% FBS plus an additional 2% PRCR (2%

PRCR group) or 10% PRCR (10% PRCR group). The PTSCs were cultured up to 10 days, with medium collected or changed every 3 days. The differentiation of PTSCs was determined by performing the following assays: (1) monitoring changes in cell size and shape during treatment with various concentrations of PRCR using phase contrast microscopy; (2) measuring expression of genes specific to tenocytes, adipocytes, chondrocytes, and osteocytes using reverse transcription-polymerase chain reaction (RT-PCR); (3) staining nucleostemin, a stem cell marker, using immunocytochemistry; (4) measuring expression of α -SMA, a specific marker of myofibroblasts^{16,33}; (5) measuring cellular expression of collagen types I and III, 2 major components of intact and healing tendons by immunocytochemistry and Western blot; and (6) cellular production of total collagen using a Sircol collagen assay kit. In addition, cell numbers were counted using an auto cellometer (Nexcelom Bioscience LLC), and cell proliferation rate was defined by population doubling time (PDT), which is calculated by total culture time divided by the number of generations.

Real-Time qRT-PCR Analysis of Gene Expression

Total RNA was obtained from the cells cultured with various PRCR concentrations for 7 days by extraction using an RNeasy Mini Kit with an on-column DNase I digest (Qiagen, Valencia, California; catalog No. 74104). From total RNA, first-strand cDNA was then synthesized by reverse transcription with SuperScript First-Strand synthesis System for RT-PCR (Invitrogen, Carlsbad, California; catalog No. 11904-018). The following conditions for cDNA synthesis were applied: 65°C for 5 minutes and cooling 1 minute at 4°C, then 42°C for 50 minutes, 72°C for 15 minutes. Rabbit-specific primers were used for collagen types I and III, Tenascin C, *PPAR* γ , *Sox9*, *Runx2*, and glyceraldehyde-3-phosphate dehydrogenase was used for an internal control. The forward and reverse primer sequences were designed according to previous publications.^{13,40} All primers were synthesized by Invitrogen.³⁸

The Qiagen QuantiTect SYBR Green PCR Kit (Qiagen; catalog No. 204143) was used to carry out qRT-PCR. In a 50 μ L PCR reaction mixture, 2 μ L cDNA (total 100 ng RNA) were amplified in a Chromo 4 Detector (MJ Research, Waltham, Massachusetts) with incubation at 94°C for 5 minutes followed by 30 to 40 cycles of a 3-temperature program of 1 minute at 94°C, 30 seconds at 57°C, and 40 seconds at 72°C. The PCR reaction was terminated after a 10-minute extension at 70°C and stored at 4°C until analysis. The products (each 5 μ L) from qRT-PCR were run in a 2% agarose gel in 0.5 Tris-borate-EDTA buffer at 100 V. The separated DNA fragments were analyzed using a gel documentation system (Bio-Rad quantity one 1-D analysis software, Bio-Rad Laboratories, Hercules, California).

Immunostaining for Detecting Expression of Nucleostemin, α -SMA, and Collagen

Cells were fixed for 20 minutes using 4% paraformaldehyde in PBS. The cells were then washed with 0.1%

Triton-X100 in PBS for 5 minutes, followed by blocking with 3% goat serum for 1 hour. Next, goat antihuman nucleostemin antibody (Neuromics, Edina, Minnesota; catalog No. GT15050;) was applied, followed by application of a Cy3-conjugated donkey antigoat immunoglobulin G (IgG) secondary antibody (Millipore; catalog No. AP180S). Finally, the cells were counterstained with Hoechst 33342 (Sigma-Aldrich, St Louis, Missouri; catalog No. 33270). Similarly, for immunostaining α -SMA and collagen types I and III, the cells were blocked with 2% mouse serum after 4% paraformaldehyde treatment. They were then reacted with mouse monoclonal anti- α -SMA antibody (Sigma-Aldrich) at a dilution of 1:800, rabbit antihuman collagen type I (Rockland Immunochemicals for Research, Gibertsville, Pennsylvania; catalog No. 600-401-103) at a dilution of 1:1000 for 5 hours, or mouse antihuman collagen III (Sigma-Aldrich; catalog No. C7805) at a dilution of 1:500 for 5 hours in PBS solution, followed by application of a Cy3-conjugated donkey anti-rabbit IgG secondary antibody at a dilution of 1:1000 for 2 hours (Millipore; catalog No. AP180S) or Cy3-conjugated goat antimouse IgG secondary antibody at a dilution of 1:1000 for 2 hours. The stained cells were examined on an inverted fluorescent microscope (Nikon eclipse, TE2000-U, Nikon Inc, Melville, New York) and cell images were taken with a charge-coupled device camera using SPOT imaging software (Diagnostic Instruments, Inc, Sterling Heights, Michigan). To determine the change in cellular expression of nucleostemin with increasing PRCR dosage, we randomly sampled 36, 40, and 40 views of cell staining in 3 groups (Control, 2% PRCR, and 10% PRCR), respectively, for a total of approximately 1000 cells each. Then, the percentage of nucleostemin-positive cells was calculated by dividing the number of nucleostemin-positive cells by total number of cells sampled.

Western Blot

The PTSCs were seeded in 6-well plates at a density of 5 10^4 per well and grown in growth medium with or without PRCR treatment for 7 days. The PTSCs were lysed using a mammalian protein extraction reagent cocktail (Pierce, Rockford, Illinois) containing 1.5% protease inhibitors (Sigma-Aldrich). After centrifugation at 12 000 rpm for 10 minutes, the protein concentrations of the supernatants were determined using a BCA Protein Assay (Pierce). Equal amounts of total protein were run on 12% SDS-polyacrylamide gels (Bio-Rad) at a constant voltage of 100 V for 60 minutes. Proteins were blotted to a nitrocellulose membrane using a Semi-Dry transfer module (Bio-Rad) at 200 mA for 90 minutes. The membrane was blocked in a 5% dry milk/TBS-Tween 20 solution for 1 hour at room temperature and then probed for 5 hours with a mouse monoclonal anti- α -SMA antibody (Sigma-Aldrich; catalog No. A2547) at a dilution of 1:1000; rabbit antihuman collagen type I (Rockland; catalog No. 600-401-103) at a dilution of 1:1000 for 5 hours; and mouse antihuman collagen III (Sigma-Aldrich; catalog No. C7805) at a dilution of 1:500 in a 1% dry milk/PBS-Tween

20 solution. Incubation with the primary antibody was followed by a peroxidase-conjugated goat antimouse antibody (Jackson ImmunoResearch Lab, Inc, West Grove, Pennsylvania) or goat antirabbit IgG (Santa Cruz; catalog No. SC-2004) at a dilution of 1:2000 in a 1% dry milk/PBS solution. The targeted protein bands were detected using an ECL (enhanced luminol-based chemiluminescence) detection kit (Amersham Biosciences, Piscataway, New Jersey), followed by exposure of the membrane to x-ray film. Membranes were also reprobbed for rabbit antihuman β -actin (Abcam Inc, Cambridge, Massachusetts; catalog No. ab8227) or rabbit antihuman glyceraldehyde-3-phosphate dehydrogenase (Sigma-Aldrich; catalog No. G8795) to verify equal protein loading in the gels.

Cellular Production of Total Collagen

The PTSCs were seeded in 6-well plates at a density of 5×10^4 per well and grown in growth medium with or without PRCR treatment for 7 days. The assay of collagen production was performed when the cell culture became highly confluent. After the cell-conditioned medium was collected, cells were detached by trypsinization. With use of an auto cellometer (Nexcelom Bioscience LLC), cell numbers were then counted. To measure total soluble collagen in cell-conditioned media, we used a Sircol collagen assay (Biodye Science, Biocolor Ltd, Carrickfergus, Northern Ireland, UK). Briefly, the cell-conditioned medium was mixed with Sircol dye reagent on an orbital shaker for 30 minutes. This solution was then centrifuged to obtain a collagen-dye complex pellet, which was solubilized with an alkali reagent. A microplate reader (Spectra MAX 190, Molecular Devices, Sunnyvale, California) was used to measure absorbance of the samples at a wavelength of 540 nm. A standard curve for calculating collagen concentration was obtained using a manufacturer-supplied acid-soluble type I collagen calibration standard solution. Finally, to compare the PRCR treatment group with the control group, we normalized the amounts of collagen produced by each group with the total cell number per group.

Statistical Analysis

One-way analysis of variance followed by Fisher PLSD (protected least significant difference) for multiple comparisons was used for statistical analysis. Wherever applicable, a 2-tailed Student *t* test was used to compare mean values of 2 groups. A *P* value < .05 was considered to be statistically significant.

RESULTS

We found that the platelet number in our PRP preparations was 1.44×10^8 \pm 0.84×10^8 /mL (mean \pm SD, *n* = 8). With respect to the baseline platelet number in whole rabbit blood, the concentration ratio was 3.25 ± 0.73 (mean \pm SD, *n* = 8) (Figure 1A). In addition, we found that TGF- β_1 levels in activated and nonactivated PRCR were 4.49 \pm 0.41 and 0.97 \pm 0.32 ng/mL, respectively. In contrast, TGF- β_1 level

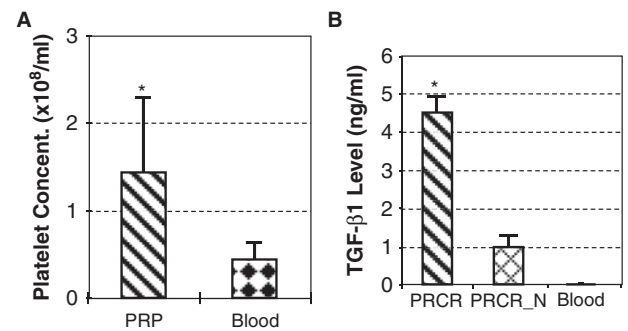


Figure 1. A, quantification of the concentration of platelets in platelet-rich plasma (PRP) preparations. The number of platelets in PRP was significantly increased compared with whole blood (**P* < .05; *n* = 8). B, the concentration of transforming growth factor beta-1 (TGF- β) in PRP-clot releasate (PRCR) preparations was found to be significantly increased compared with nonactivated PRCR (PRCR_N) (**P* < .05; *n* = 5). TGF- β_1 levels in blood samples, either with or without addition of CaCl₂, were less than 41 pg/mL.

in blood was less than 41 pg/mL for all 5 samples measured (Figure 1B).

We then went on examining the effects of PRCR on PTSCs. We noted that in control culture conditions, PTSCs were small and exhibited an irregular shape (Figure 2A). However, after growing in culture containing PRCR with an increasing concentration from 2% to 10%, PTSCs markedly increased in cell size, were well spread, and became more elongated in shape (Figure 2 B and C), suggesting that PRCR treatment induced PTSC differentiation into fibroblastlike cells. To confirm that PTSCs had undergone differentiation, we examined nucleostemin expression of the PRCR-treated cells. We found that under control culture conditions, most PTSCs expressed nucleostemin; with 2% PRCR treatment, fewer cells expressed nucleostemin; while under 10% PRCR treatment, only a small number of cells expressed nucleostemin (Figure 2 D-F). The semiquantitative analysis found that 76.1% of cells in control culture were nucleostemin-positive, whereas the number was decreased to 53.1% for 2% PRCR treatment group and further decreased to 6.4% for the 10% PRCR treatment group. There was a statistically significant difference in the percentage of nucleostemin positive cells among the 3 groups (Figure 2G). Furthermore, PRCR-treated cells increased their proliferation rate in a PRCR dose-dependent manner, as shown by decreased PDT values compared with nontreated control cells (Figure 3).

To determine the types of cells present after TSC differentiation as a result of PRCR treatment, we performed gene analysis on PRCR-treated cells. We found that those genes related to tenocytes (collagen type I and III, and tenascin C) were highly expressed, whereas those nontenocyte-related genes, *PPAR γ* , *SOX-9*, and *Runx2*, were either not expressed at all or minimally expressed (Figure 4). *PPAR γ* , *SOX-9*, and *Runx2* are essential transcription factors for adipogenesis, chondrogenesis, and osteogenesis of stem cells, and hence they are established markers for adipocytes,

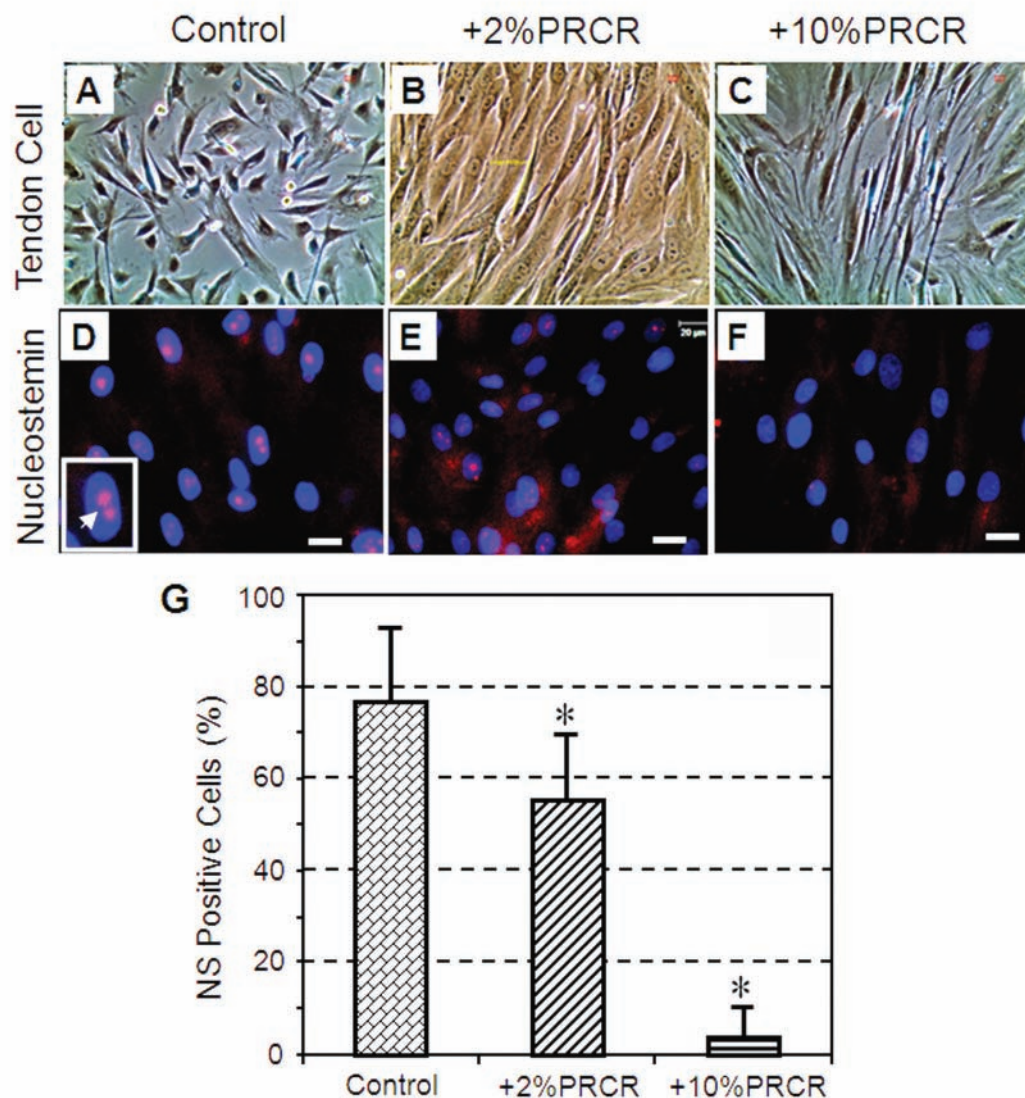


Figure 2. The effect of platelet-rich plasma-clot releasate (PRCR) treatment on tendon stem/progenitor cells (TSCs). A, TSCs in culture medium consisting of Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum (Control); B, TSCs in culture medium with addition of 2% PRCR (2%PRCR); and C, TSCs in culture medium with addition of 10% PRCR (10%PRCR). As seen, with increasing PRCR dosage, TSCs changed from an irregular shape to a well-spread and highly elongated shape. The cell size also markedly increased. D-F, expression of nucleostemin by TSCs in control culture, with 2% PRCR and 10% PRCR treatments, respectively. Inset in D shows an enlarged view of expressed nucleostemin in pink (arrow). With increasing PRCR dosage, fewer cells expressed nucleostemin, indicating that TSCs had undergone differentiation. G, the percentage of nucleostemin (NS)-positive cells. There was a significant decrease in the number of NS-positive cells with increasing PRCR dosage ($*P < .01$). Bar, 20 μm .

chondrocytes, and osteocytes, respectively. Taken together, these results demonstrate that tenocyte differentiation from PTSCs had occurred after PRCR treatment.

We then went on to determine whether the differentiated tenocytes were activated by measuring α -SMA expression, collagen types I and III expression, and total collagen production. We found that α -SMA protein expression was markedly increased after PRCR treatment, as shown by immunostaining (Figure 5 A-C) and Western blot (Figure

5D). Moreover, PRCR treatment increased the expression levels of collagen types I and III, as evidenced by both immunostaining (Figures 6 A and B and 7 A and B) and Western blot results (Figures 6C and 7C). The effects of PRCR treatment on α -SMA and collagen types I and III expression increased with increasing PRCR dosage (Figures 6C and 7C). Finally, we found that total collagen production by these activated tenocytes was markedly increased compared with control cells under the same

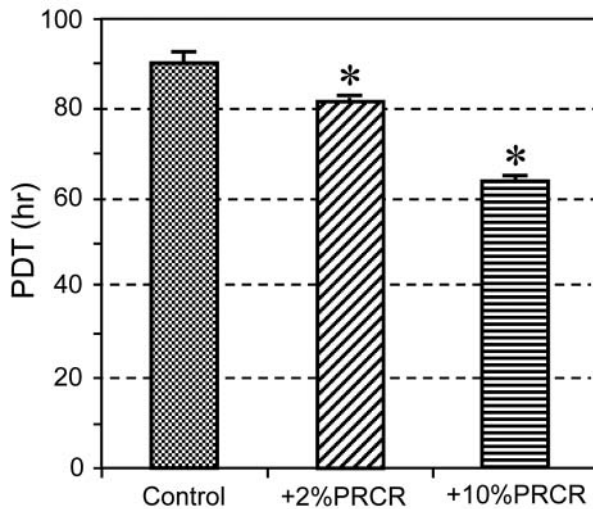


Figure 3. The effect of platelet-rich plasma-clot releasate (PRCR) treatment on cell proliferation. With increasing PRCR dosage from 0% (ie, control culture) to 2% to 10%, cellular population doubling time decreased, indicating that PRCR treatment stimulated tendon stem/progenitor cells to enhance proliferation rate in a dose-dependent manner (* $P < .05$).

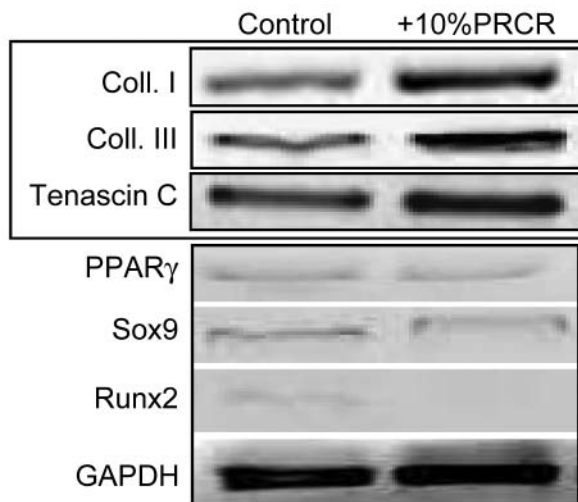


Figure 4. Analysis of gene expression in platelet-rich plasma-clot releasate (PRCR)-treated cells. The PRCR treatment elevated expression levels of those genes related to tenocytes (collagen types I and III, and tenascin C), but not those genes specific to adipocytes ($PPAR\gamma$), chondrocytes (SOX-9), and osteocytes ($Runx2$).

culture conditions except without PRCR treatment (Figure 8).

DISCUSSION

Application of PRP has been reported to enhance healing of injured tissues,²¹ including tendons and ligaments.^{14,31} In

addition, cell culture studies have also shown that PRCR treatment increases tenocyte proliferation and collagen production^{2,11}; however, the effects of PRCR treatment on the newly discovered TSCs are unknown. Like other adult stem cells, TSCs play a major role in repair of injured tendons by proliferation and differentiation in vivo. Using a cell culture model, we found that PRCR treatment induced TSC differentiation into tenocytes, as evidenced by changes in cell size and shape toward a fibroblastlike phenotype and upregulation of those genes related to tenocytes, but not those genes specific to adipocytes, chondrocytes, and osteocytes. The extent of differentiation of TSC populations into tenocytes was PRCR concentration-dependent, as the number of cells expressing nucleostemin appeared to decrease with increasing PRCR concentration. Moreover, the PDT decreased with increasing PRCR concentration, indicating that PRCR treatment increased the cell proliferation rate. Finally, these newly produced tenocytes were activated by PRCR treatment because these tenocytes markedly increased expression of α -SMA, collagen types I and III, and total collagen production compared with control cells without PRCR treatment.

Taken together, these findings suggest that use of PRP for treatment of injured tendons in vivo is "safe," as PRCR-treated TSCs differentiate into tenocytes. It is significant that they do not differentiate into nontenocytes (adipocytes, chondrocytes, and osteocytes), which would produce fatty, mucoid, and calcified tissues often seen in chronically injured tendons (tendinopathy) and thus be detrimental to the structure and function of healed tendons.¹⁹ Moreover, the findings of this study suggest that use of PRP to promote tendon healing in adults is likely effective because these differentiated tenocytes became activated to increase cell number and produce abundant collagen, both of which are necessary to enhance healing of injured tendons that have lost cells and matrix.

Several in vitro studies have investigated the effect of PRP on various types of connective tissue cells. Anitua et al² showed that in addition to increased cellular synthesis of VEGF and HGF, treatment of human tendon cells with PRCR increases cell proliferation. Therefore, our finding of increased cell proliferation after PRCR treatment of PTSCs is consistent with their study. One difference between the 2 studies, however, is that our study used TSCs derived from rabbit patellar tendons, whereas their study used a mixture of tendon cell populations derived from human semitendinosus tendons. In another cell culture study, de Mos et al¹¹ demonstrated that PRCR stimulates proliferation of human tenocytes and total collagen production by the cells. The results of our study are in agreement with their findings. There are notable differences between the 2 studies though: (1) We used rabbit TSCs, whereas they used human tenocytes, which were likely mixed tendon cell populations as TSCs were not isolated in their study; and (2) our study did not separate PRP from platelet-poor plasma, and their study did.

In addition to in vitro studies, numerous in vivo studies have investigated the efficacy of PRP treatment on healing of injured tissues, including tendons^{12,31} and ligaments.^{14,25,26} Although these studies generally showed

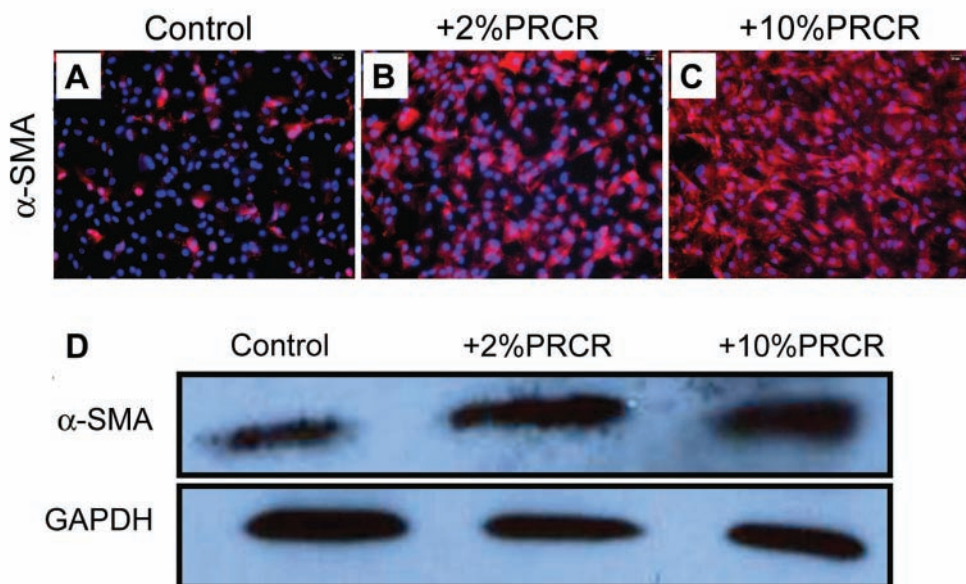


Figure 5. The α -smooth muscle actin (α -SMA) expression of platelet-rich plasma-clot releasate (PRCR)-treated tendon stem/progenitor cells by immunocytochemistry (A, B, and C) and Western blot (D). With increasing PRCR dosage, the extent of α -SMA expression also increased.

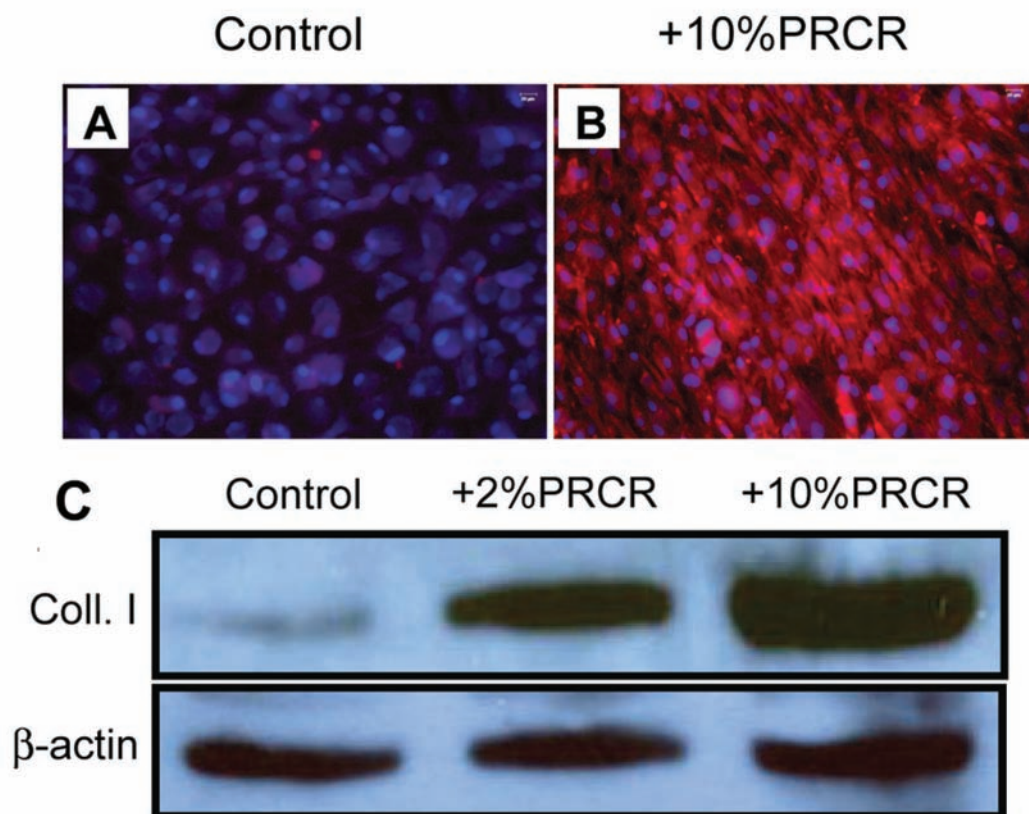


Figure 6. The effect of platelet-rich plasma-clot releasate (PRCR) treatment on collagen type I as shown by immunocytochemistry (A and B) and Western blot (C). The PRCR treatment enhanced collagen type I expression in a dose-dependent manner.

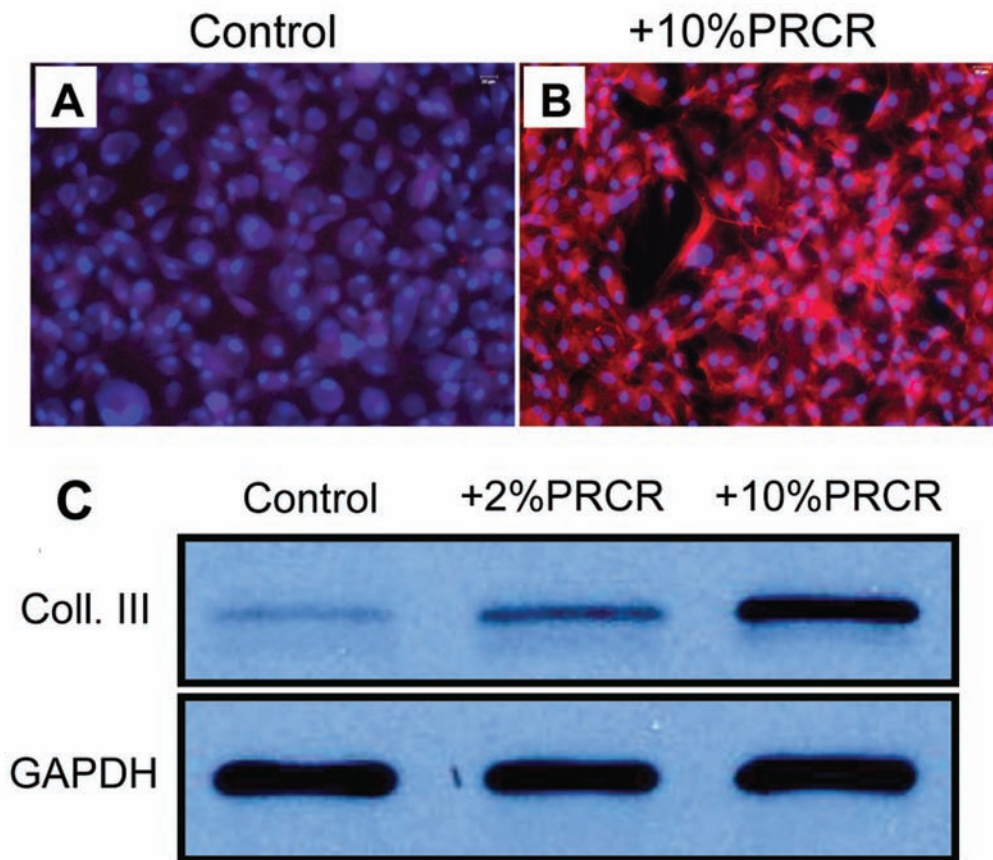


Figure 7. The effect of platelet-rich plasma-clot releasate (PRCR) treatment on collagen type III expression determined by immunocytochemistry (A and B) and Western blot (C). Similar to collagen type I, PRCR treatment induced high levels of collagen type III expression, which is PRCR dose-dependent.

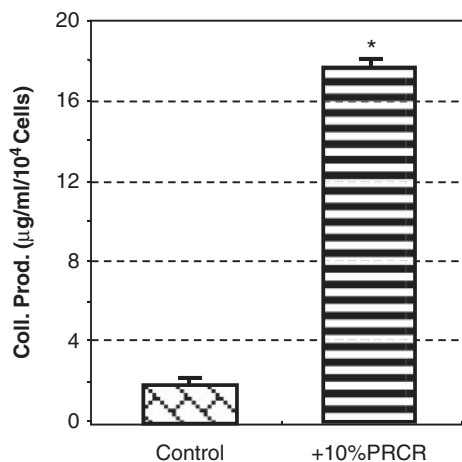


Figure 8. The effect of platelet-rich plasma-clot releasate (PRCR) treatment on total collagen production. The PRCR treatment (10% PRCR) markedly increased total collagen production by activated tenocytes resulting from PRCR-induced tendon stem/progenitor cell differentiation (* $P < .01$).

positive effects on injured tissues in terms of functional recovery and enhanced structural properties,²¹ a notable limitation of these types of studies is that the cellular mechanisms that are responsible for the positive outcomes are not clear. On the basis of the findings of this study, we suggest that TSCs and ligament stem cells³² play a major role in enhanced tissue healing because of PRP treatment in these studies. It should be noted, however, that unlike in vitro usage, in vivo PRP application for repair of injured tissues often requires “bioscaffolds” for effective delivery of PRP to cells to elicit the biological effects of treatment. Two commonly used bioscaffolds for embedding PRP are fibrin and collagen gels, which, when implanted in vivo, enable PRP to gradually release its contents, or growth factors, and thereby elicit cellular events such as proliferation and differentiation. Without such scaffolds, PRP could be dissipated quickly in injured tissues, which may be one reason that a previous study found no apparent treatment effects on chronic Achilles tendinopathy of human subjects by one-time PRP injection.¹² Murray et al²⁴ showed that PRP alone without use of scaffolding materials does not enhance suture repair of the injured ACLs in skeletally immature animals.

As shown in our recent study,³⁸ rabbit TSCs possess multidifferentiation potential, as the stem cells can differentiate into adipocytes, chondrocytes, and osteocytes in vitro and form cartilage and bonelike tissues in vivo in addition to tendonlike tissue. The question then is, why do PRCR-treated TSCs differentiate into tenocytes but not other types of cells? The precise molecular mechanisms responsible for PRCR-induced, tenocyte-specific differentiation in this study have yet to be worked out and are expected to be complicated as PRCR contains many growth factors that have complex actions on cells. For example, PDGF, IGF-I, and TGF- β_1 from PRP enhance cell proliferation, migration, and cellular synthesis of matrix proteins (eg, collagen),^{1,3} whereas VEGF, HGF, and basic fibroblast growth factor exert chemotactic and mitogenic effects on endothelial cells, thus promoting angiogenesis by these cells, which is essential for tissue healing.^{15,30} However, although general mechanisms of PRCR-induced TSC differentiation are complicated, the tenocyte-specific differentiation observed in this study may be related to culture conditions used. These cells were grown in culture media consisting of DMEM and 10% FBS. Under such culture conditions, most of these cells still expressed nucleostemin, a nucleolar protein that is expressed in stem cells and progenitor cells.^{4,20,34} Terminally differentiated cells, such as tenocytes, do not express nucleostemin.³⁸ In this study, we found that in addition to expressing nucleostemin, TSCs in control medium (DMEM plus 10% FBS) also expressed a baseline level of those genes related to tenocytes (eg, collagen type I and tenascin C), a finding consistent with that by Bi et al.⁵ The results suggest that under control culture conditions, these TSCs were exposed to the "tenogenic" environment, meaning that they became progenitor cells that were committed to the tenocyte differentiation pathway. Therefore, PRCR treatment may have just pushed those already committed progenitor cells down a tenocyte lineage. It is worth noting that buffered PRP induces chondrogenic differentiation of human mesenchymal stem cells,²² suggesting that individual types of stem cells vary in their differentiation response to PRP treatment.

This study shows that PRCR treatment not only induced tenocyte differentiation but also increased the number of tenocytes that were activated, as evidenced by marked upregulation of α -SMA. It is known that when fibroblasts are activated, they express α -SMA and are termed *myofibroblasts*.¹⁶ Tenocytes exhibit a fibroblastlike phenotype; hence, α -SMA is used as a marker of activated tenocytes, or myofibroblasts.³³ Because of their high proliferation rates and ability to produce abundant collagen, these activated tenocytes are expected to promote healing of injured tendons in vivo. Nevertheless, myofibroblasts are also responsible for fibrosis, characterized by excessive production of matrix, including collagen.⁶ Therefore, appropriate regulation of their number and/or activities may be necessary for optimal tendon healing. A previous study showed that use of PRP with collagen scaffolds to treat injured ligaments led to ligament hypertrophy.¹⁴

This study has a few limitations that should be taken into consideration when interpreting the results. First, TSCs used in this study were obtained from normal

tendons of adult rabbits. Although it seems reasonable to extrapolate the data to repair of injured tendons in young adults, it may not be appropriate for aging adults. It is known that depletion of stem cell reserves and/or diminished stem cell function occurs with aging.^{18,28} Hence, PRP treatment may not work well on aging adults based on the results of the PRCR treatment effects in this study. Second, we used a cell culture model to assess PRCR effects on TSCs without inclusion of mechanical loading. Future studies should investigate the interaction between PRCR treatment and mechanical loading, an inherent part of the tendon environment in vivo. Mechanical loading has been shown to regulate TSC proliferation and differentiation in a loading-magnitude dependent manner.³⁹ Therefore, mechanical loading is an important factor to be considered when one looks into the effects of PRP treatment in vitro and in vivo. Second, the influence of existing tenocytes on TSC differentiation and proliferation under PRP treatment was not investigated. There may be additive or synergistic effects by existing tenocytes and TSCs in response to PRP treatment, and future studies should look into these effects.

In conclusion, this study showed that PRCR treatment induces differentiation of TSCs from adult rabbits into tenocytes that are activated in that they increase proliferation rates and produce abundant collagen in vitro. Thus, our data show for the first time that PRCR treatment is likely safe for treating injured tendons clinically in terms of its ability to direct TSC differentiation into tenocytes instead of nontenocyte lineages, which would form nontendinous tissues and consequently compromise the structure and function of healed tendons. Moreover, PRCR induces an anabolic effect on TSCs in terms of increasing number of activated tenocytes and producing abundant collagen to promote healing of injured tendons in adults.

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