Platelet-Rich Plasma Promotes Migration, Proliferation, and the Gene Expression of Scleraxis and Vascular Endothelial Growth Factor in Paratenon-Derived Cells In Vitro

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Background: Platelet-rich plasma (PRP) is a treatment option for tendon injury because of its effective tendon-healing properties. At the early stage of tendon repair, paratenon-derived cells (PDCs) are thought to play a more important role than tendon proper–derived cells (TDCs). However, there has been no study investigating the effects of PRP on PDCs.

Hypothesis: PRP promotes the migration, proliferation, and differentiation of PDCs in vitro.

Study Design: Controlled laboratory study.

Methods: TDCs and PDCs were isolated from the tendon proper and paratenon of rat Achilles tendons and were cultured to the third passage. PRP was prepared from the rats using the double-spin method. Third-passage TDCs and PDCs were cultured in Dulbecco's modified Eagle medium with 2% fetal bovine serum (control group) or 2% fetal bovine serum plus 5% PRP (PRP group), and cell migration, proliferation, and differentiation were evaluated. The relative mRNA expression levels of scleraxis (Scx), tenomodulin (Tnmd), collagen type I alpha 1 (Col1a1), collagen type III alpha 1 (Col3a1), and vascular endothelial growth factor A (VEGF) were examined by quantitative real-time reverse transcription polymerase chain reaction.

Results: The cell migration rate was significantly higher in the PDCs of the PRP group than in the control group (1.4-fold increase; $P = 0.02$). Cell proliferation was significantly higher in the PDCs of the PRP group (2.2-fold increase; $P < 0.01$). In the PDCs, the gene expression levels of Scx, Col1a1, and VEGF were significantly increased by PRP (Scx: 2.0-fold increase, $P = 0.01$; Col1a1: 5.3-fold increase, $P = 0.01$; VEGF: 7.8-fold increase, $P = 0.01$), but the gene expression level of Tnmd, a factor for tendon maturation, was significantly reduced by PRP (0.11-fold decrease; $P = 0.02$).

Conclusion: In vitro PRP promoted migration, proliferation, and tenogenic differentiation with the upregulation of Scx in PDCs. PRP also upregulated the expression of the angiogenic marker VEGF.

Clinical Relevance: Our results suggest that PRP treatment in vitro may enhance the tendon-healing properties of PDCs at the initial stage of tendon repair.

Keywords: platelet-rich plasma (PRP); paratenon-derived cells (PDCs); tendon proper–derived cells (TDCs); migration; proliferation; differentiation

Acute tendon injury is a common musculoskeletal impairment that can occur during athletic activity. The natural healing process of tendon is slow and often results in incomplete repair.[9] Tendon injury remains a persistent clinical challenge, although previous studies have provided extensive knowledge on its mechanical and biological properties.

Platelet-rich plasma (PRP) is an autologous blood product that contains high concentrations of growth factors. PRP promotes human and animal tendon cell migration and proliferation in vitro[7,11,17] and is a treatment option for tendon injury because of its effective tendon-healing potential. However, the underlying mechanisms of PRP treatment for tendon repair have not yet been fully elucidated.

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Tendon repair requires cell migration, proliferation, and differentiation. These processes can be achieved through 2 distinct stem/progenitor cell populations: One is directly derived from the core of the tendon for intrinsic healing (tendon proper–derived cells [TDCs]), and the other is derived from the paratenon for extrinsic healing (paratenon-derived cells [PDCs]).[12] Although both TDCs and PDCs have the potential to repair tendon, recent studies have demonstrated that PDCs play a more important role in the early phase of tendon healing.[10] As such, this stem/progenitor cell population should be the focus in studies of biological strategies for promoting tendon repair with PRP. However, the biological effects of PRP on PDCs have not yet been well characterized.

We hypothesized that PRP promotes the migration, proliferation, and differentiation of PDCs in vitro.

METHODS

Animals

This study protocol with the use of rats was approved by the Animal Research Committee of Yokohama City University (protocol number: F-A-15-045). A total of 12 Sprague-Dawley male rats (6-8 weeks old; weight, 191-301 g) (Charles River Laboratories Japan) were used in this study.

Isolation and Characterization of TDCs and PDCs

TDCs and PDCs were isolated from the Achilles tendon proper and paratenon, respectively, as previously described.[14] Rats were euthanized, and the Achilles tendon with paratenon was dissected from each ankle. The tendons with paratenon were first treated with 0.5% type I collagenase (CLS-1; Sigma-Aldrich) and 0.25% trypsin (Gibco) in high-glucose Dulbecco's modified Eagle medium (DMEM; Wako Pure Chemical) for 10 minutes at 37°C. Thereafter, the paratenon was removed from the tendon proper. The remaining tendon tissues were cut into 1-mm^3 pieces and then treated with 3 mg/mL CLS-1 and 4 mg/mL Dispase II (Wako Pure Chemical) for 20 minutes at 37°C. The paratenon and tendon proper were strained in a 70-µm cell strainer, and cells were collected by centrifugation for 10 minutes at 2000 rpm. The cell pellets were resuspended in growth medium consisting of high-glucose DMEM supplemented with 10% fetal bovine serum (FBS; Biowest), 100 U/mL penicillin, and 100 µg/mL streptomycin (Gibco). The cell suspensions were cultured in a 6-well plate at 37°C in a humidified atmosphere of 5% CO_2. Cells from the paratenon were cultured as PDCs, and those from the tendon proper were cultured as TDCs.

Gene expression profiles were investigated to characterize the TDCs and PDCs. Total RNA was isolated from the TDCs and PDCs of the second passage using RLT Lysis Buffer (Qiagen). The RNA was quantified by measuring absorbance at 260 nm, and the quality was assessed by determining the 260/280 nm absorbance ratio. First-strand cDNA synthesis was performed with 1 µg of total RNA in a total volume of 20 µL using an iScript Advanced cDNA Synthesis Kit (Bio-Rad). Quantitative real-time polymerase chain reaction (PCR) was carried out using TaqMan gene expression assays (Applied Biosystems) on a CFX96TM real-time PCR detection system (Bio-Rad) in 20 µL of reaction volume. The expression level of each gene of interest was normalized to that of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The TaqMan gene expression assays used were the following: scleraxis (Scx, Rn01504576_m1), tenomodulin (Tnmd, Rn00574164_m1), sex-determining region Y-box9 (Sox-9, Rn01751070_m1), vascular endothelial growth factor A (VEGF, Rn01511602_m1), and GAPDH (Rn01775763_g1).

The isolated TDCs and PDCs were analyzed for their ability to form tendon constructs in vitro. A 6-well plate was coated with Agarose 28CC (Funakoshi), and a 7 × 24 × 3-mm mold was made. Polyethylene terephthalate artificial ligament (Telos; Aimedic MMT) was placed on both sides of the mold. Type I-A collagen gel matrix (Cellmatrix; Nitta Gelatin) was poured into the mold, and third-passage TDCs and PDCs were seeded onto the collagen gel at a density of 5.0×10^6/mL and cultured in 2 mL of growth medium. The culture medium was changed every 2 to 3 days.

Preparation of PRP

Whole blood was extracted from the hearts of the rats. Nine milliliters of blood were mixed with 1 mL of 0.1 mol/L sodium citrate in a centrifuge tube and centrifuged for 7 minutes at 1700 rpm. The plasma layer and the buffy coat were collected into another centrifuge tube and centrifuged for 5 minutes at 3200 rpm. The top half of the plasma was transferred and retained as platelet-poor plasma (PPP). The platelet pellet was resuspended in the bottom half of the plasma and was retained as the PRP. The number of platelets in the PRP, PPP, and whole blood was determined using an automated cell counter (CDA-1000; Sysmex). The PRP was stored at –80°C until use.

Migration Assay

Cell migration was assessed using the Oris Cell Migration Assay Kit (Platypus Technologies). Third-passage TDCs and PDCs were seeded into Oris 96-well Tissue Culture Treated Plates with Oris Cell Seeding Stoppers at a density of 5×10^5/mL and cultured in 100 µL of growth medium for 24 hours. After the initial culture, the stoppers were removed, and the medium was changed to DMEM with 2% FBS (control group) or DMEM with 2% FBS plus 5% PRP (PRP group). Twenty-four hours after the medium was changed, the cell migration area was evaluated by using a Diff-Quick Stain Kit (Sysmex). The cell migration rate was calculated by dividing the cell migration area by the area of the stopper.

Proliferation Assay

Third-passage TDCs and PDCs were seeded into 96-well plates at a density of 2.5×10^4/mL and cultured in 100 µL of growth medium for 2.5 hours. After the initial culture, the medium was changed to DMEM with 2% FBS (control group) or DMEM with

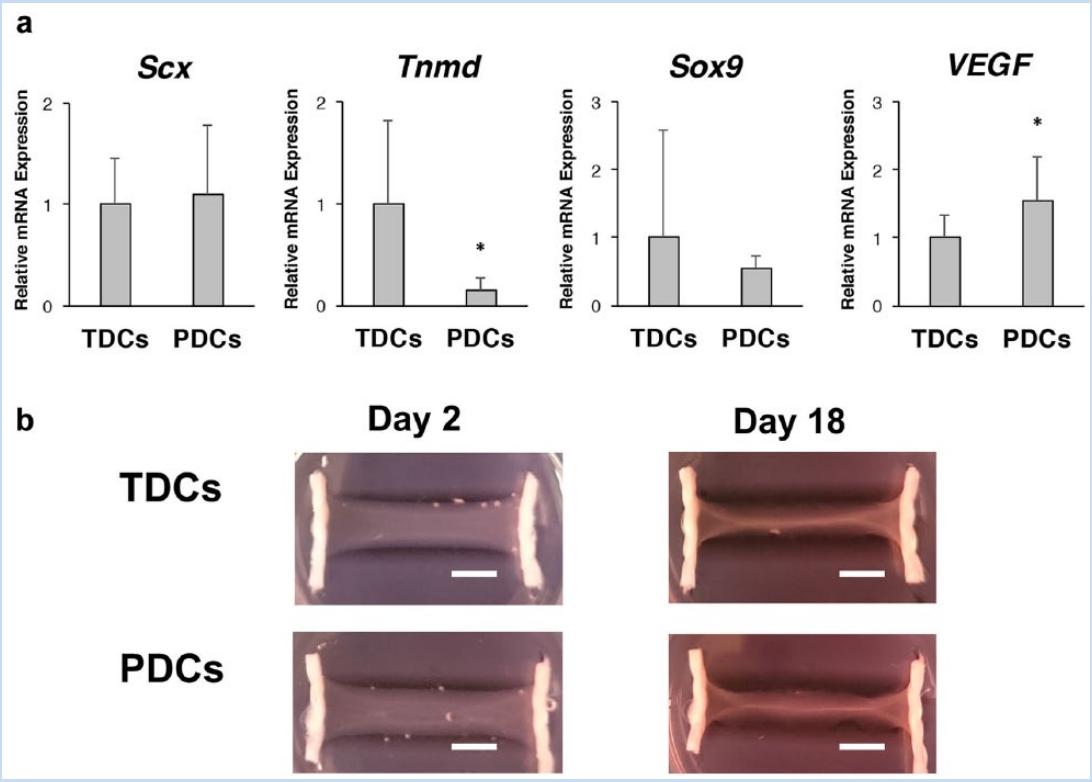

Figure 1. (a) Relative mRNA expression levels in tendon proper–derived cells (TDCs) and paratenon-derived cells (PDCs). n = 6, *$P < 0.05$. (b) In vitro tendon constructs on day 2 and day 18. White scale bar = 5 mm. Scx, scleraxis; Sox9, sex-determining region Y-box9; Tnmd, tenomodulin; VEGF, vascular endothelial growth factor A.

2% FBS plus 5% PRP (PRP group). Cell counts were evaluated on days 0, 1, 2, and 3 using Cell Counting Kit-8 (CCK-8; Dojindo). A 10-µL solution of CCK-8 was added to the cells and incubated at 37°C for 1.5 hours. Subsequently, the absorbance of each well was measured by using a microplate reader at 450 nm. The difference in absorbance between the experimental wells and the control wells containing medium only was calculated.

Gene Expression Analysis Using Real-Time Reverse Transcription PCR

Third-passage TDCs and PDCs were seeded into 10-cm dishes at a density of 5×10^4/mL and cultured in 10 mL of growth medium for 4 days. After the initial culture, the medium was changed to DMEM with 2% FBS (control group) or DMEM with 2% FBS plus 5% PRP (PRP group). Total RNA was isolated 3 days after the medium was changed. The experiment was performed using the same protocol that was used for the characterization assay. The TaqMan gene expression assays used were the following: Scx (Rn01504576_m1), Tnmd (Rn00574164_m1), collagen type I alpha 1 (Col1a1, Rn01463848_m1), collagen type III alpha 1 (Rn01437681_m1), VEGF (Rn01511602_m1), and GAPDH (Rn01775763_g1).

Statistical Analysis

All experiments were performed at least 4 independent times. All data are presented as the mean ± SEM. The analysis was performed using JMP Pro software version 12.0 (SAS Institute). The Mann-Whitney *U* test was used to test for significant differences in the characterization of TDCs and PDCs. A paired *t* test was used to test for significant differences among the test groups in the other experiments. An adjusted *P* value <0.05 was considered to be statistically significant.

RESULTS

Characterization of Isolated TDCs and PDCs

TDCs and PDCs were isolated from the Achilles tendon of 6- to 7-week-old male Sprague-Dawley rats, and the second passage of the cells was characterized using real-time reverse transcription PCR. The gene expression levels of Scx and Sox-9 were not significantly different between the TDCs and PDCs. In contrast, the gene expression level of Tnmd was significantly higher in TDCs than in PDCs ($P = 0.02$; n = 6), and the gene expression level of VEGF was significantly lower in TDCs than in PDCs ($P = 0.02$; n = 6) (Figure 1a). These results suggest that both TDCs and PDCs express Scx, a marker of the progenitor

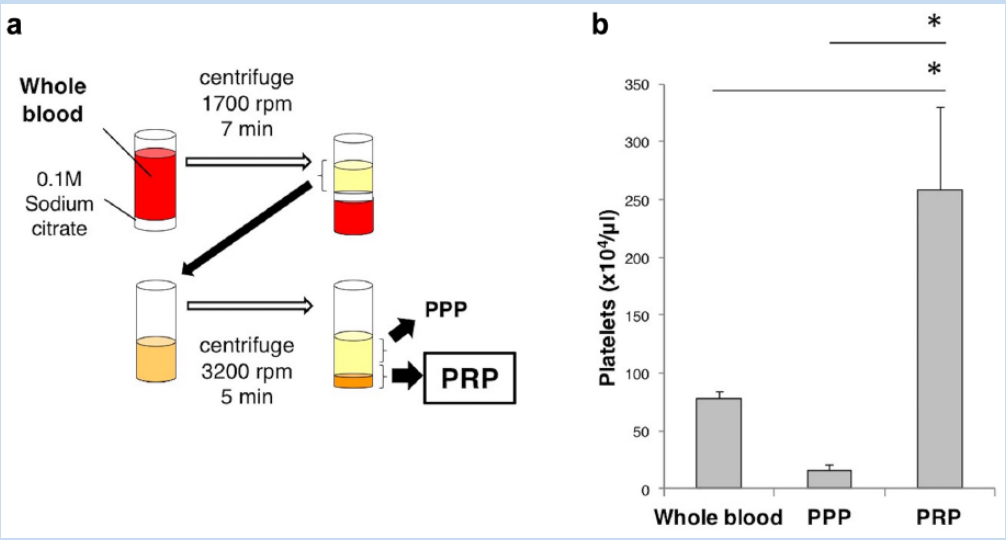

Figure 2. (a) Preparation method of rat platelet-rich plasma (PRP). (b) The PRP contained 3.3 and 16.4 times more platelets than whole blood and the platelet-poor plasma (PPP), respectively. n = 6, $*P < 0.05$.

populations of tendons and tenocytes, whereas the gene expression of Tnmd, a marker of tendon maturation, and VEGF, a marker of angiogenesis, differ between the TDCs and PDCs. In the in vitro tendon construct assay, tendon-like structures were observed in both TDCs and PDCs on day 18 (Figure 1b).

Characterization of Prepared PRP

PRP and PPP were prepared from fresh whole blood from rats (Figure 2a), and the number of platelets was determined. The PRP contained 3.3 and 16.4 times more platelets than whole blood ($P < 0.01$; n = 6) and the PPP ($P < 0.01$; n = 6), respectively (Figure 2b). These results confirmed that the PRP contained a sufficiently high amount of platelets, as previously described.[5] This PRP was used for the following experiments.

Migration Assay

The PRP group demonstrated a significantly higher migration rate than the control group in both TDCs (1.3-fold increase; $P = 0.02$; n = 4) and PDCs (1.4-fold increase; $P = 0.02$; n = 4) (Figure 3). These results suggested that PRP promoted the migration of PDCs as well as TDCs.

Proliferation Assay

The PRP group showed significantly greater cell proliferation than the control group in both TDCs (2.8-fold increase; $P < 0.01$; n = 4) and PDCs (2.2-fold increase; $P < 0.01$; n = 4) on day 3 (Figure 4). These results suggested that PRP promoted the proliferation of PDCs as well as TDCs.

Gene Expression Analysis Using Real-Time Reverse Transcription PCR

PRP significantly increased the gene expression level of Scx in the PDCs (2.0-fold increase; $P = 0.01$), but not in the TDCs (Figure 5a). The gene expression level of Tnmd was significantly reduced by PRP in both the TDCs and PDCs (TDCs: 0.06-fold decrease; $P = 0.04$; PDCs: 0.11-fold decrease; $P = 0.02$) (Figure 5b). The gene expression level of Col1a1 was significantly increased by PRP in both the TDCs and PDCs (TDCs: 5.5-fold increase; $P = 0.02$; PDCs: 5.3-fold increase; $P = 0.01$) (Figure 5c). There was no significant difference in the gene expression level of Col3a1 in both the TDCs and PDCs (Figure 5d). The gene expression level of VEGF was significantly increased by PRP in both the TDCs and PDCs (TDCs: 5.6-fold increase; $P = 0.03$; PDCs: 7.8-fold increase; $P = 0.01$) (Figure 5e). These results suggested that PRP promoted the gene expression of Scx in PDCs as well as the gene expression of Col1a1 and VEGF in TDCs and PDCs but that it inhibited the gene expression of Tnmd in TDCs and PDCs.

DISCUSSION

Understanding of the mechanisms of tendon repair, including the magnitude of the contribution of different cell types to the healing process, is important for developing efficient treatment strategies. A recent study using a mouse Achilles tendon injury model demonstrated that the progenitor cell subpopulation localized in the paratenon was induced to express Scx, migrated to the wound site, and produced extracellular matrix to fill the defect at the early stage of healing. In addition, the resident Scx-expressing tenocytes also contributed to the healing process, but only at the later stages after the progenitor cells had made their contribution.[15] To promote tendon repair from an early stage, treatments should be focused on PDCs rather than the TDCs.

The present study demonstrated that PRP promoted migration, proliferation, and collagen synthesis in PDCs as well as TDCs; this was consistent with previous reports on the effects of PRP

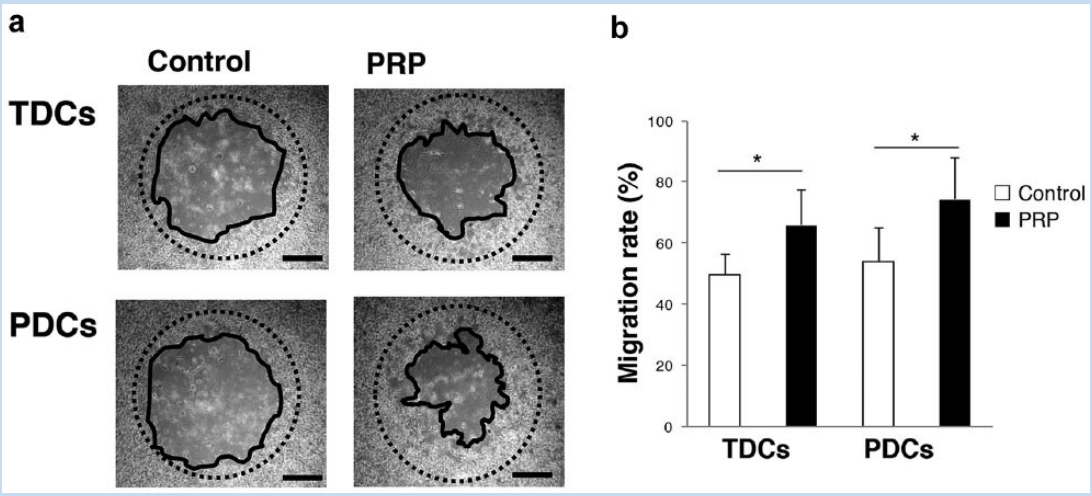

Figure 3. Effects of PRP on the migration of cultured TDCs and PDCs. (a) Photomicrographs of cell migration. Black scale bar = 1 mm. (b) The migration rate was calculated as the area surrounded by a solid line (in figure a) divided by the area surrounded by the dotted circle × 100. n = 4, *$P < 0.05$ versus the control. PDCs, paratenon-derived cells; PRP, platelet-rich plasma; TDCs, tendon proper–derived cells.

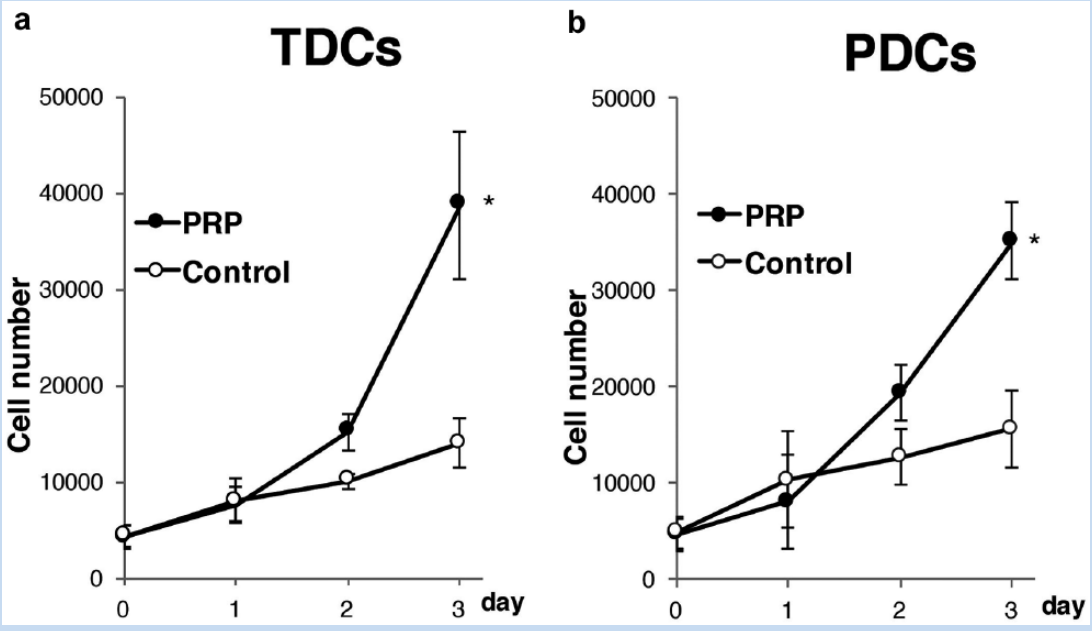

Figure 4. Effects of PRP on the proliferation of (a) cultured TDCs and (b) PDCs. n = 4, *$P < 0.05$ versus the control. PDCs, paratenon-derived cells; PRP, platelet-rich plasma; TDCs, tendon proper–derived cells.

on TDCs.[17] The present study also demonstrated that PRP upregulated the expression of Scx in the PDCs. Previous studies have reported a significantly higher expression level of Scx in TDCs than in PDCs.[4,14] Unlike these previous reports, no difference in Scx level was found between the 2 cell populations when grown in medium with a high concentration of FBS (10%) in the present study. However, PDCs showed a higher level of Scx expression in response to PRP than the TDCs when grown in medium with a low concentration of FBS (2%). These results suggest that the PDCs require growth-promoting factors to be able to induce tenogenic differentiation, and that PRP could compensate for this insufficiency. PRP may have therapeutic potential for enhancing initial tendon repair with PDCs at the early stage.

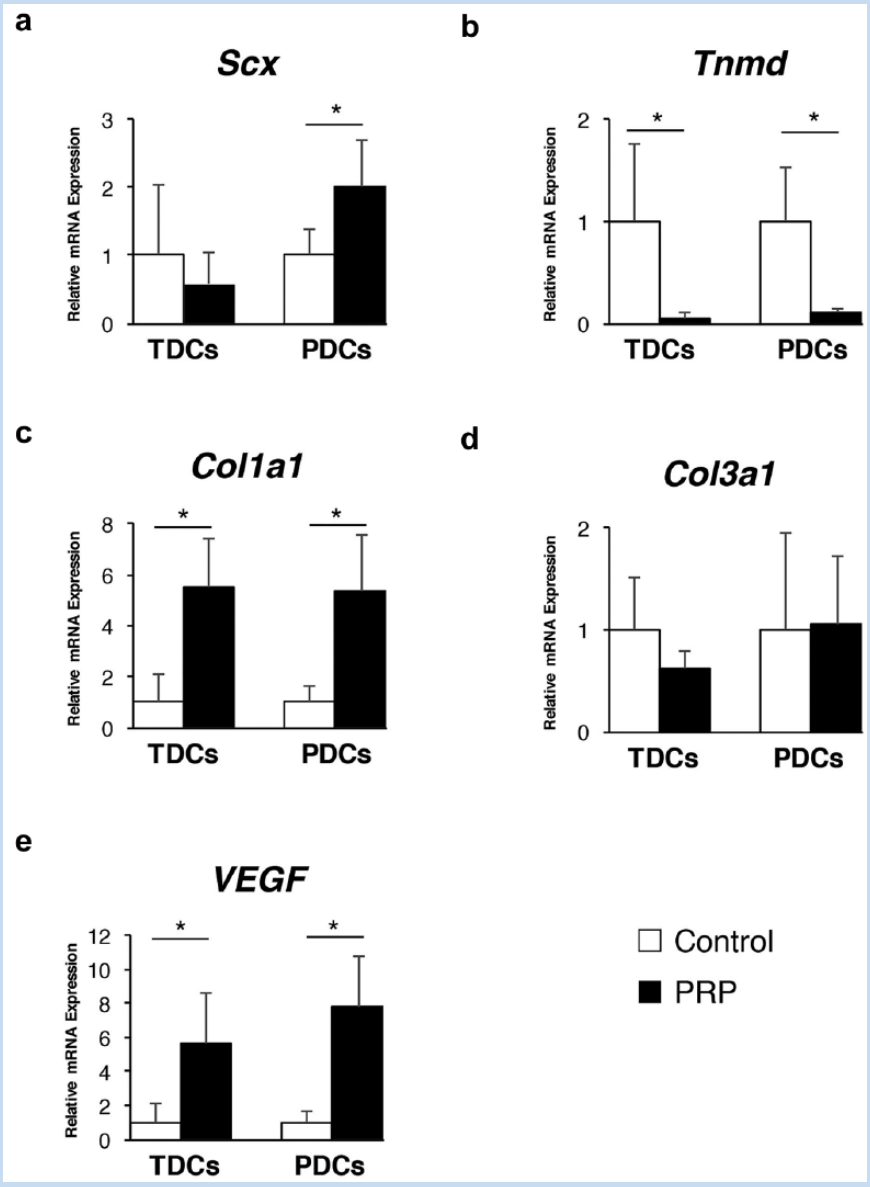

Figure 5. Effects of PRP on the differentiation of cultured TDCs and PDCs. Relative mRNA expression levels of (a) scleraxis (Scx), (b) tenomodulin (Tnmd), (c) collagen type I alpha 1 (Col1a1), (d) collagen type III alpha 1 (Col3a1), and (e) vascular endothelial growth factor A (VEGF) after 3 days. The mRNA expression levels in TDCs and PDCs were individually normalized to that of GAPDH. n = 4, $*P < 0.05$ versus the control. GAPDH, glyceraldehyde 3-phosphate dehydrogenase; PDCs, paratenon-derived cells; PRP, platelet-rich plasma; TDCs, tendon proper–derived cells.

Our results also suggest that PRP inhibits the expression of Tnmd and upregulates the expression of markers for angiogenesis. Tendons are poorly vascularized, and 99% of intrinsic tendon-derived cells are negative for CD34, an endothelial cell marker.[6] Tnmd is a protein predominantly expressed in tendons, and it has anti-angiogenic properties.[16] Tendon stem/progenitor cells require Tnmd at the maturation step of the tenogenic differentiation cascade.[8] Thus, PRP may have conflicting effects in terms of the final maturation in tendon repair.

Tendon repair occurs via overlapping processes, including inflammatory, reparative, and remodeling phases.[9] Treatment strategies may differ for each step. Several studies have demonstrated the temporal accumulation of VEGF mRNA at the repair site during the early stage of tendon repair.[2,3] As PDCs are the main contributor at the early stage of tendon repair,[15] PRP-induced upregulation of both Scx and VEGF in PDCs appears to be a reasonable treatment strategy for promoting the initial healing process. In the rabbit Achilles tendon transection model, a single PRP injection promoted angiogenesis for 2 weeks

and reduced the number of newly formed vessels thereafter, suggesting that the healing process was shortened.[13] When acute tendon rupture is treated with PRP in clinical practice, it should be done with a single injection.[1] The timing of the PRP injection appears to be important, and treatment should be provided as early as possible after injury to accelerate the healing process.

This study has limitations. First, the TDCs and PDCs used in this study were neither established cell lines nor were they sorted. Second, tensile force is required for tendon cell maturation, but we only performed an in vitro study and did not account for tensile force.

This study provides the first insights into the effects of PRP on PDCs in vitro. PRP promoted migration, proliferation, and tenogenic differentiation with the upregulation of Scx in PDCs. PRP also upregulated the expression of the angiogenic marker VEGF. These results suggest that treatment with PRP may enhance the healing properties of PDCs at the initial stage of tendon repair.

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