PDGF-BB Enhances the Proliferation of Cells in Human Orbital Fibroblasts by Suppressing PDCD4 Expression Via Up-Regulation of microRNA-21

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Purpose. The aim of this study was to investigate the effect of platelet-derived growth factor (PDGF)-BB on the proliferation of cells and its possible mechanism in human orbital fibroblasts.

Methods. Human orbital fibroblasts were obtained from orbital fat from decompression surgery in patients with thyroid-associated ophthalmopathy (TAO). The cells were treated with PDGF-BB, and the number of cells was counted using an Advanced Detection and Accurate Measurement (ADAM) automatic cell counter. The expression of programmed cell death 4 (PDCD4) was determined by Western blotting. The effect of PDCD4 on cell proliferation was evaluated using PDCD4 small interfering RNA (siRNA)-transfected cells. The level of microRNA-21 (miRNA-21) was measured by quantitative real-time RT-PCR. In addition, the role of miRNA-21 in the proliferation of PDGF-BB-treated cells was assessed by means of anti-miRNA-21 siRNA and resveratrol (*trans*-3,4′,5-trihydroxys-tilbene), an inhibitor of miRNA-21.

Results. PDGF-BB was found to enhance cell proliferation, whereas it inhibited PDCD4 expression in human orbital fibroblasts. Down-regulation of PDCD4 by PDCD4 siRNA transfection significantly increased the number of human orbital fibroblasts. In addition, PDGF-BB increased the level of miRNA-21 in human orbital fibroblasts. Transfection with anti-miRNA-21 and treatment with resveratrol partially restored the expression of PDCD4 and led to a reduction in cell number in PDGF-BB-treated orbital fibroblasts.

Conclusions. PDGF-BB enhances proliferation by suppressing PDCD4 expression by up-regulation of miRNA-21 in human orbital fibroblasts. These results suggest that PDGF-BB stimulates cell proliferation through microRNA-21-mediated PDCD4 down-regulation, leading to the development of TAO.

Keywords: microRNA-21, orbital fibroblasts, PDCD4, PDGF-BB, thyroid-associated ophthahlmopathy

Thyroid-associated ophthalmopathy (TAO) is an autoimmune disease that occurs in 25% to 50% of patients with Graves' disease.[1,2] Exophthalmos, the main clinical feature of TAO, is caused mainly by to swelling of fatty and muscular orbital tissue. These edematous changes in TAO are caused by infiltration of inflammatory cells, accumulation of extracellular matrix, proliferation of fibroblasts, and an increase in the amount of fatty tissue.[3] It has been reported that orbital fibroblasts produce glycosaminoglycan hyaluronan, proliferate excessively, and differentiate into adipocytes in response to stimuli, leading to the expansion of orbital tissue.[1,4,5]

Platelet-derived growth factor (PDGF) is a dimeric protein that plays a prominent role in tissue repair and fibrotic diseases by promoting the proliferation and survival of myofibroblasts.[6] In addition, PDGF-A and PDGF-B chains have been reported to be increased in orbital tissues obtained from patients with TAO.[7,8] Of the three PDGF isoforms, PDGF-BB is the most potent isoform in terms of activating orbital fibroblasts to proliferate and to produce interleukin-6 (IL-6) and hyaluronan, whereas PDGF-AA is the least potent.[8] Thus, PDGF isoforms that contain the PDGF-B chain may contribute to orbital tissue expansion in TAO.

Programmed cell death 4 (PDCD4), a well-known tumor suppressor, has been a potential target for a number of anticancer therapies.[9] Expression of PDCD4 has been reported to be down-regulated in colon cancer cells.[10] It has been reported that over-expression of PDCD4 inhibits 12-*O*-tetraadecanoyl phorbol-13-acetate (TPA)-induced neoplastic transformation of murine epidermal JB6 cells in an in vitro model.[11] In a transgenic mouse model, over-expression of PDCD4 also inhibits 7,12-dimethylbenz(a) anthracene (DMBPA)/TPA-induced skin papilloma formation.[12] In contrast, down-regulation of PDCD4 in transgenic mice has been reported to significantly induce lymphoma development[13] and to promote DMBPA/TPA-induced papilloma formation.[14] Guo et al.[15] reported that knockdown of PDCD4 up-regulates cyclin D1 expression through activation of AKT, subsequently promoting the proliferation of HT29 colon cancer cells. PDCD4 protein expression is down-regulated by phosphatidylinositol-3-kinase (PI3K)-Akt-mammalian target of rapamycin (mTOR)-dependent

proteasome-mediated degradation and/or by microRNA-21 (miRNA-21)-mediated-inhibition of its translation.[16–20] PDCD4 has emerged as a major, functionally significant target of microRNA (miRNA)-21.[16,19] It has been reported that treatment with lipopolysaccharide (LPS) reduces PDCD4 expression and that transfection with antisense oligonucleotides to miRNA-21 or targeted protection of the miRNA-21 site in *Pdcd4* mRNA inhibits LPS-reduced PDCD4 expression in RAW264.7 cells, suggesting that LPS reduces PDCD4 expression through induction of miRNA-21.[16]

Given the above findings, we hypothesized that PDGF-BB may promote proliferation of orbital fibroblasts through miRNA-21-mediated down-regulation of PDCD4 during the development of TAO.

We investigated the effect of PDGF-BB on cell proliferation and its possible mechanism in orbital fibroblasts. We initially assessed the effects of PDGF-BB and PDCD4 on the proliferation of orbital fibroblasts. We also assessed the effect of miRNA-21 on PDCD4 expression and cell proliferation in PDGF-BB-treated orbital fibroblasts. The findings reported herein demonstrate that PDGF-BB stimulates cell proliferation through the miRNA-21-mediated down-regulation of PDCD4, subsequently leading to the development of TAO. This study provides evidence to suggest that the PDGF-BB/miRNA-21/PDCD4 pathway is a promising target for TAO treatment.

Materials and Methods

Reagents and Antibodies

Recombinant human PDGF-BB was purchased from R&D Systems, Inc. (Minneapolis, MN, USA). Resveratrol (*trans*-3,4′,5-trihydroxys-tilbene) was obtained from Sigma-Aldrich Corp. (St. Louis, MO, USA). Human PDGF-BB was dissolved in 4 mM HCl. Resveratrol was dissolved in dimethyl sulfoxide (DMSO). The final vehicle concentration was adjusted to 0.1% (v/v), and the control medium contained the same quantity of vehicle. The antibody against PDCD4 (product ab80590) was obtained from Abcam (Cambridge, UK). Anti-GAPDH antibody (sc-25778) and goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (sc-2301) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell Cultures

Human orbital fibroblasts were obtained from orbital fat obtained during decompression surgery in patients with TAO ($n = 4$). Before the decompression surgery, all patients with TAO had experienced at least 6 months of inactive disease status with a euthyroid condition. Patient characteristics are presented in the Table. Orbital fat explants were chopped into small pieces, attached to plastic culture dishes, and covered with Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, Grand Island, NY, USA) supplemented with 20 mM HEPES (Fisher Scientific, Atlanta, GA, USA), 10% fetal bovine serum (FBS; Gibco), 100 U/mL penicillin, and 100 μg/mL streptomycin (BioWhittaker, Inc., Walkersville, MD, USA). Cultures were maintained at 37°C in a 5% CO_2 humidified incubator until the fibroblasts reached 70% confluence. Nonadherent cells and fat tissues were then removed, and the established fibroblasts were passaged with gentle trypsin/EDTA treatment. Fibroblasts were not used in studies beyond passage 10 from the initial culture. These activities were undertaken after obtaining informed consent from the donors, according to procedures approved by the Institutional Review Board of Seoul St. Mary's Hospital (KC10TISE0743) and the tenets of the Declaration of Helsinki.

Table. Characteristics of Patients With Thyroid-Associated Ophthalmopathy From Whom Orbital Fibroblasts Were Obtained

Characteristic	Patients With TAO, $n = 4$
Mean age, y (range)	42.8 (23–54)
Males/females	2/2
Smoking, yes	1
Graves disease, yes	4
Radioactive iodine therapy	1
Surgery	0
Methimazole therapy	4
Treatment of TAO	4
Surgery	4
Prednisolone therapy	3
Radiation therapy	1
Euthyroid	4
TSH* receptor antibodies	4
CAS*	2 or 3

*CAS, clinical activity score; TSH, thyroid-stimulating hormone.

All patients with TAO had experienced at least 6 months of inactive disease status with a euthyroid hormonal condition.

Cell Proliferation Assay

Human orbital fibroblasts were plated at 2.5×10^4 cells/well in a 24-well plate. After 24 hours, the cells were treated with 50 ng/mL PDGF-BB in DMEM containing 10% FBS or 0.1% bovine serum albumin (BSA) for 24 or 48 hours. Cell numbers were counted using an Advanced Detection and Accurate Measurement (ADAM) automatic cell counter (NanoEnTek, Inc., Seoul, Korea).

PDCD4 siRNA Transfection

For silencing PDCD4, PDCD4-directed small interfering RNA (siRNA) pool (ON-TARGET plus SMARTpool reagent) and control siRNA were purchased from Dharmacon (Lafayette, CO, USA). Cells were transfected with PDCD4 siRNA or control siRNA by electroporation at 1350 V for 35 ms, using a pipette electroporator (MicroPorator-Mini; Digital Biotechnology, Suwon, Kyounggi-do, Korea) according to the manufacturer's instructions. The efficiency of the PDCD4 siRNA transfection was confirmed by Western blot analysis at 48 hours after transfection.

Western Blot Analysis

Treated cells were removed from the incubator at designated times and placed on ice. Cells were then washed three times with ice-cold phosphate-buffered saline. Cells were then lysed for 30 minutes with Radioimmunoprecipitation lysis buffer (50 mM Tris-HCl [pH 7.4], 1% Triton X-100, 150 mM NaCl, 0.1% sodium dodecyl sulfate [SDS], 0.5% sodium deoxycholate, 100 mM phenylmethylsulfonyl fluoride, 1 μg/mL leupeptin, 1 mM Na3VO4, and 1× complete protease inhibitor cocktail [Santa Cruz Biotechnology]). Equal amounts of protein were loaded onto 10% to 15% SDS-polyacrylamide gels, electrophoresed, and transferred to polyvinylidene fluoride membranes (Millipore, Bedford, MA, USA). Membranes were blocked in Tris-buffered saline with 0.05% Tween-20 (TBST) supplemented with 5% powdered milk or 5% BSA and then incubated with a primary antibody against the designated protein. The blot was then washed with TBST and incubated with a horseradish peroxidase-conjugated secondary antibody in TBST plus 5% powdered milk. Bound antibodies were detected with Super Signal Ultra Chemiluminescence reagents (Pierce Biotechnology, Inc., Rockford, IL, USA).

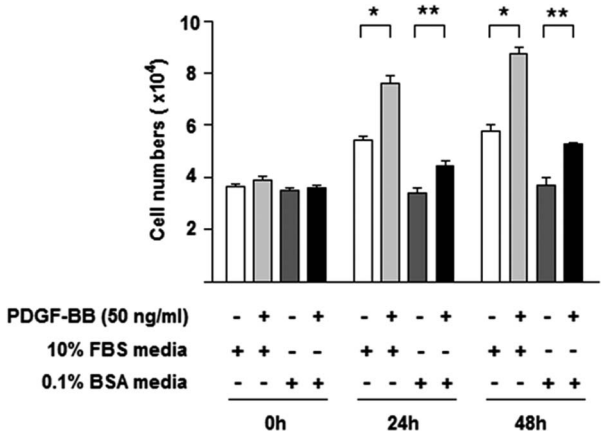

FIGURE 1. PDGF-BB enhances the proliferation of orbital fibroblasts. Orbital fibroblasts were treated with 50 ng/mL PDGF-BB in DMEM containing 10% FBS or 0.1% BSA for 24 or 48 hours. Cell numbers were counted using an ADAM automatic cell counter (NanoEnTek, Inc.). $^{*}P < 0.01$ and $^{**}P < 0.05$ between indicated groups. Similar results were observed in three independent experiments.

RNA Extraction and Quantitative Real-Time RT-PCR

Total RNA was extracted using a mirVana miRNA isolation kit (Ambion, Austin, TX, USA), and quantified using an RNA BR assay and spectrophotometric analysis (Invitrogen, Carlsbad, CA, USA). The relative expression levels of miRNA-21 were measured using a two-step TaqMan assay according to the manufacturer's instructions (Applied Biosystems, Waltham, MA, USA). Quantitative real-time RT-PCR was performed with iQ SYBR Supermix kit (Bio-Rad Laboratories, Hercules, CA, USA) in a Peltier thermal cycler-200 system (MJ Research, Berlin, Germany). RNA input was normalized by human U6 small nuclear RNA.

Anti-miRNA-21 Transfection

The anti-miRNA-21 and anti-miR-negative controls were purchased from Panagene Ltd. (Daejeon, Korea). Orbital fibroblasts were transfected with anti-miRNA-21 and anti-miR-negative control (Panagene Ltd.) using Lipofectamine 2000 transfection reagent (Invitrogen). After 6 hours, culture medium was changed to 10% FBS. At 24 hours after transfection, transfected cells were treated with 50 ng/mL PDGF-BB for the indicated times.

Statistical Analysis

Results are mean ± SD data from at least three separate experiments. Statistical significance was determined by Student's *t*-test for two points or 1-way ANOVA. A P value of <0.01 or <0.05 was considered statistically significant.

RESULTS

PDGF-BB Enhances Proliferation of Orbital Fibroblasts

We initially examined the effect of PDGF-BB on cell proliferation in orbital fibroblasts. As shown in Figure 1, treatment with PDGF-BB induced a significant increase in the number of orbital fibroblasts in DMEM containing 10% FBS or 0.1% bovine serum albumin. These results show that PDGF-BB enhances the proliferation of orbital fibroblasts, irrespective of whether FBS is present or absent. However, the stimulatory effect of PDGF-BB on cell proliferation was more prominent in DMEM containing 10% FBS, than when DMEM containing 0.1% BSA was used. Thus, in the following experiments, orbital fibroblasts were maintained in DMEM containing 10% FBS.

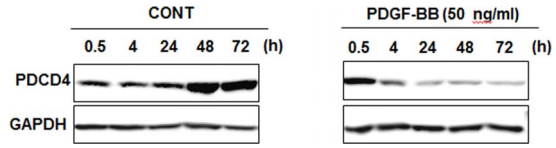

FIGURE 2. PDGF-BB inhibits PDCD4 expression in orbital fibroblasts. Orbital fibroblasts were treated with 50 ng/mL PDGF-BB for 0.5 to 72 hours. PDCD4 expression was determined by Western blot analysis at designated times. Similar results were observed in three independent experiments.

PDGF-BB Enhances Proliferation of Orbital Fibroblasts Via the Down-Regulation of PDCD4

To assess the mechanism responsible for PDGF-BB stimulation of cell proliferation, we initially examined the effect of PDCD4 on proliferation of orbital fibroblasts. Treatment with PDGF-BB decreased the expression of PDCD4 in orbital fibroblasts, whereas the level of PDCD4 was elevated in untreated orbital fibroblasts as the cell culture continued (Fig. 2). In addition, down-regulation of PDCD4 by PDCD4 siRNA transfection increased the number of cells in orbital fibroblasts (Fig. 3). These results suggest that PDGF-BB enhances cell proliferation through down-regulation of PDCD4 in orbital fibroblasts.

Transfection With Anti-miRNA-21 Partially Restores PDCD4 Protein Level and Leads to Reduction in Cell Number in PDGF-BB-Treated Orbital Fibroblasts

We then examined the issue of whether miRNA-21 was involved in the PDGF-BB-reduced expression of PDCD4 in orbital fibroblasts. We measured the level of miRNA-21 in PDGF-BB-treated orbital fibroblasts by quantitative real-time RT-PCR. Treatment with PDGF-BB caused a significant increase in miRNA-21 expression in orbital fibroblasts (Fig. 4). In addition, transfection with anti-miRNA-21 partially restored the level of PDCD4 protein (Fig. 5A) and suppressed cell proliferation (Fig. 5B) in PDGF-BB-treated orbital fibroblasts. These results suggest that PDGF-BB enhances proliferation by suppressing PDCD4 expression through upregulation of miRNA-21 in human orbital fibroblasts. However, in non-treated control cells, transfection with anti-miRNA-21 resulted in a significant increase in the number of orbital fibroblasts at 48 hours after the PDGF-BB treatment (Fig. 5B).

Resveratrol Partially Restores PDCD4 Expression and Leads to Reduction in Cell Numbers in PDGF-BB-Treated Orbital Fibroblasts

Consistent with the results of a transfection experiment using anti-miRNA-21 (Fig. 5), treatment with resveratrol, an inhibitor of miRNA-21 expression, reduced the PDGF-BB-induced expression of miRNA-21 in orbital fibroblasts (Fig. 6). In addition, treatment with resveratrol partially restored the level of PDCD4 protein (Fig. 7A) and suppressed cell proliferation (Fig. 7B) in PDGF-BB-treated orbital fibroblasts. However, in non-treated orbital fibroblasts, treatment with resveratrol significantly increased the number of orbital fibroblasts at 48 hours after treatment (Fig. 7B).

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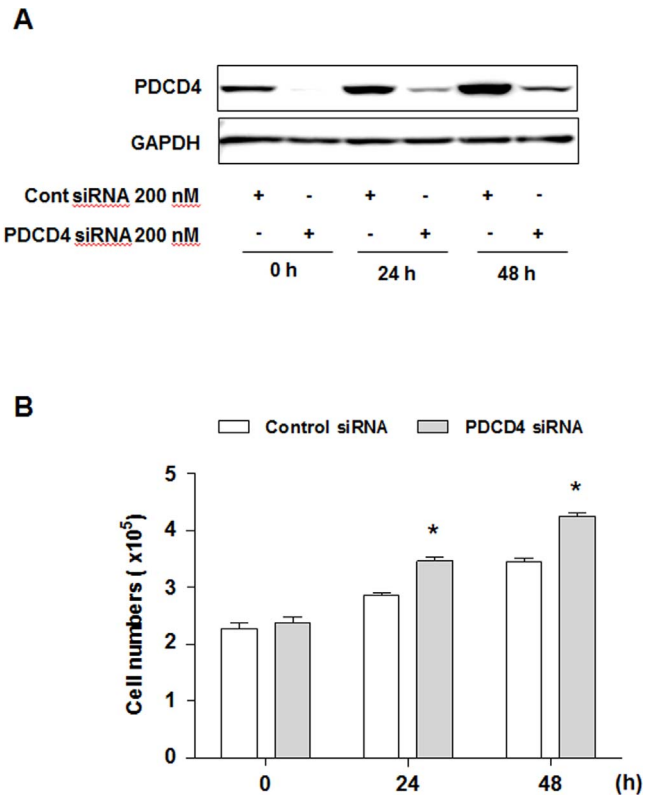

FIGURE 3. Down-regulation of PDCD4 enhances proliferation of orbital fibroblasts. Cells were transfected with 200 nM PDCD4 siRNA or control siRNA. At 48 hours after transfection, the transfected cells were treated with 50 ng/mL PDGF-BB for 24 or 48 hours. The effect of PDCD4 siRNA transfection was confirmed by Western blot analysis (**A**), and cell numbers were counted using an ADAM automatic cell counter (NanoEnTek, Inc.) (**B**). $^{*}P < 0.01$ compared with control siRNA-transfected cells, as calculated by 1-way ANOVA. Similar results were observed in three independent experiments.

DISCUSSION

The key effector functions of activated orbital fibroblasts in TAO include cytokine production, hyaluronan production, and cell proliferation. van Steensel et al.[8] previously reported that PDGF-BB most potently stimulated these processes by orbital fibroblasts, whereas PDGF-AA was the least effective, and that these differences in stimulatory capacity between the various

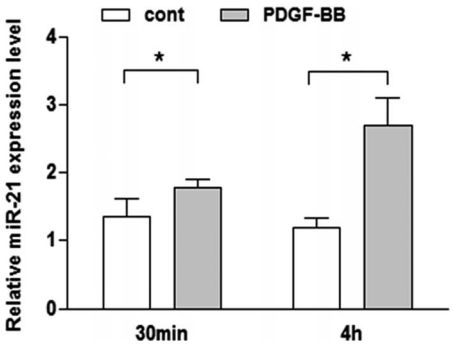

FIGURE 4. PDGF-BB enhances miRNA-21 expression in orbital fibroblasts. Orbital fibroblasts were treated with 50 ng/mL PDGF-BB for 0.5 or 4 hours. miRNA-21 expression was determined by quantitative real-time RT-PCR. $^{*}P < 0.01$ between the indicated groups. Similar results were observed in three independent experiments.

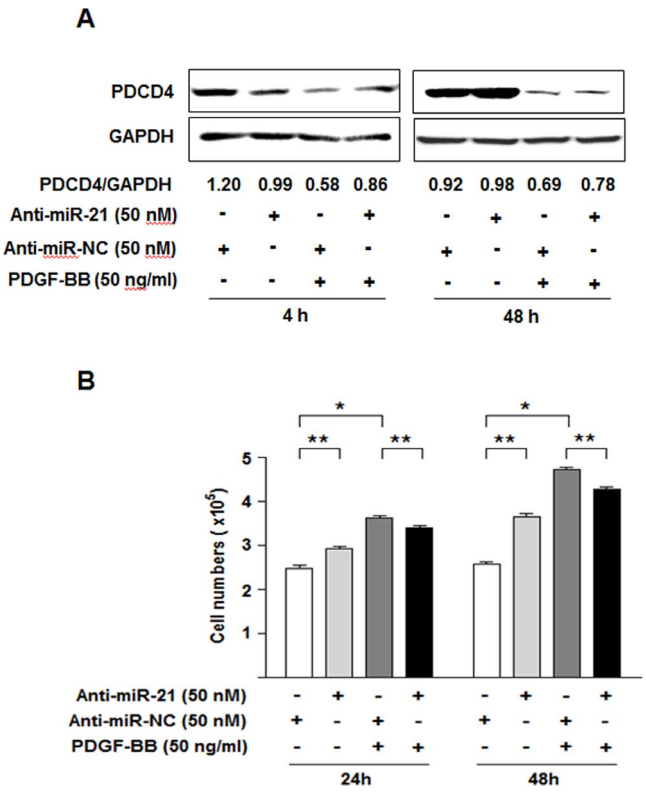

FIGURE 5. Transfection with anti-miRNA-21 partially restored PDCD4 expression and led to a reduction of cell numbers in PDGF-BB-treated orbital fibroblasts. Cells were transfected with 50 nM anti-miRNA-21 or anti-miR-negative control (NC). At 24 hours after transfection, the transfected cells were treated with 50 ng/mL PDGF-BB for the indicated times. PDCD4 expression was determined by Western blot analysis. PDCD4 protein level was quantified densitometrically and normalized to that of GAPDH (**A**). Cell numbers were counted using an ADAM automatic cell counter (NanoEnTek, Inc.) (**B**). $^{*}P < 0.01$ and $^{**}P < 0.05$ between the indicated groups. Similar results were observed in three independent experiments.

PDGF isoforms could be related to differences in PDGF receptor expression.[21] It has been reported that PDGF-BB stimulates secretion of IL-8 and chemokine (C-C-motif) ligand (CCL)2, CCL5, and CCL by orbital fibroblasts.[22] The findings reported herein show that PDGF-BB is capable of enhancing the proliferation of orbital fibroblasts.

As a tumor suppressor, PDCD4 inhibits tumorigenesis, tumor progression, and metastasis.[9,12] PDCD4 is ubiquitously expressed in normal tissues, but its expression is absent or suppressed in various cancer cells, including lung, breast, colon, brain, and prostate cancers.[9–11] It has been reported that PDCD4 not only inhibits the translation of growth-regulated genes through interaction with the eukaryotic initiation factors (eIF)4A and eIF4G but also controls transcription factors.[23,24] In addition, the knockdown of PDCD4 promotes cell proliferation and up-regulates cyclin D1 expression in colon cancer cells.[15] Thus, we hypothesized that the effect of PDGF-BB on cell proliferation is related to the down-regulation of PDCD4. Consistent with the previous reports, our results also showed that treatment with PDGF-BB inhibited PDCD4 expression (Fig. 2) and the down-regulation of PDCD4 enhanced cell proliferation (Fig. 3) in orbital fibroblasts.

The level of PDCD4 protein is post-translationally down-regulated by PI3K-Akt-mTOR-dependent proteasome-mediated degradation during inflammation[17,18] and tumor promotion.[20] In addition, PDCD4 translation is also inhibited by miRNA-21.[19]

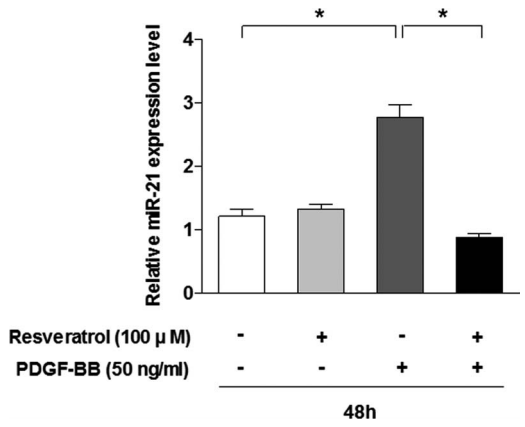

FIGURE 6. Resveratrol reduces miRNA-21 expression in PDGF-BB-treated orbital fibroblasts. Cells were left untreated or were pre-treated with 100 μM Resveratrol for 1 hour, followed by treatment with 50 ng/mL PDGF-BB for 48 hours. miRNA-21 expression was determined by quantitative real-time RT-PCR. $^{*}P < 0.01$ between the indicated groups. Similar results were observed in three independent experiments.

Based on bioinformatic analysis, PDCD4 appears to contain an miRNA-21 binding site, and miRNA-21 has been shown to target the *PDCD4* gene in several types of cancer cells.[25,26] Asangani et al.[26] reported that the level of miRNA-21 was inversely correlated with the level of PDCD4 in colon cancer cells. In that report, the authors reported that anti-miRNA-21-transfected RKO cells produced increased levels of the PDCD4 protein, whereas the over-expression of miRNA-21 significantly reduced the level of PDCD4 in Colo206f cells. miRNA-21 has been reported to be increased in certain types of cancers [24,27,28] and in inflammatory diseases.[29–31] The above-described findings also indicate that the effect of PDGF-BB on cell proliferation is associated with the miRNA-21-mediated down-regulation of PDCD4. Our results show that treatment with PDGF-BB increased the level of miRNA-21 in human orbital fibroblasts (Fig. 4) and transfection with anti-miRNA-21 (Fig. 5) partially restored the expression of PDCD4, thus leading to a reduction in cell numbers in PDGF-BB treated orbital fibroblasts. However, the effect of PDGF-BB on the level of miRNA-21 varies depending on the cell type. Costa et al.[32] reported that over-expression of PDGF-B or a prolonged exposure resulted in decreased miRNA-21 levels and that siRNA-mediated PDGF-B silencing led to increased levels of miRNA-21 in U87 human glioblastoma cells.

Resveratrol is a polyphenolic antioxidant found in peanuts, grapes, and red wine.[33,34] It has been reported to have antitumor effects by inhibiting cell proliferation and inducing apoptosis in various cancer cells, including prostate carcinoma DU 145 cells and acute lymphoblastic leukemia cells.[35–37] Resveratrol has been reported to modulate the level of miRNA-21 in SW480 cells.[38] Sheth et al.[39] also reported that treatment with resveratrol increased the level of PDCD4 through suppression of the Akt/miRNA-21 pathway, leading to a reduction in cell growth in prostate cancer. Our results also showed that treatment with resveratrol restored the level of PDCD4 and inhibited cell proliferation in PDGF-BB-treated orbital fibroblasts (Fig. 6). However, the effects of transfection with anti-miRNA-21 and resveratrol treatment on cell proliferation were different between in PDGF-BB-treated and in non-treated orbital fibroblasts. In non-treated orbital fibroblasts, transfection with anti-miRNA-21 and treatment with resveratrol resulted in a slight increase in cell numbers, although they did not significantly affect the level of PDCD4 protein (Figs. 5 and 6). The reason for such a discrepancy is presently unclear but

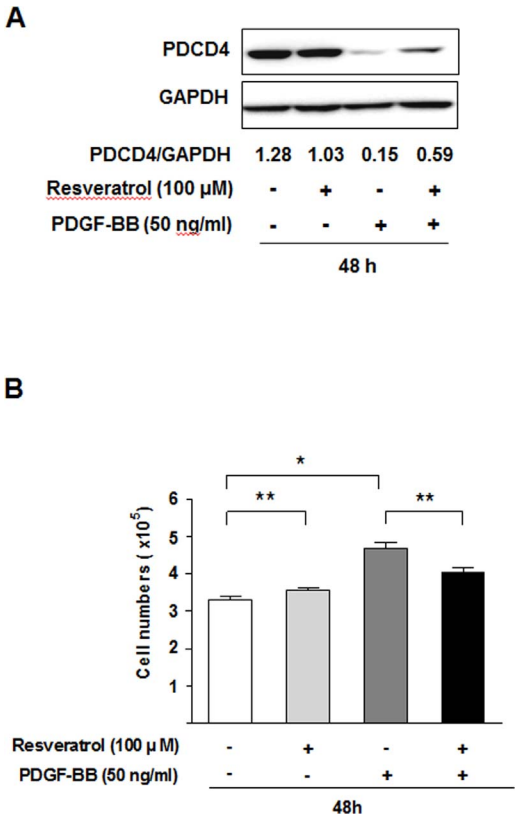

FIGURE 7. Resveratrol partially restored PDCD4 expression, leading to a reduction in cell numbers in PDGF-BB-treated orbital fibroblasts. Cells were left untreated or were pre-treated with 100 μM Resveratrol for 1 hour, followed by treatment with 50 ng/mL PDGF-BB for 48 hours. PDCD4 expression was determined by Western blot analysis. PDCD4 protein level was quantified densitometrically and was normalized to that of GAPDH (**A**). Cell numbers were counted using ADAM automatic cell counter (NanoEnTek, Inc.) (**B**). $^{*}P < 0.01$ and $^{**}P < 0.05$ between the indicated groups. Similar results were observed in three independent experiments.

could be due to a different level of miRNA-21, a target of anti-miRNA-21, and resveratrol, between PDGF-BB-treated and untreated orbital fibroblasts. The inhibitory effect of anti-miRNA-21 and resveratrol on miRNA-21 may be more prominent when their target, miRNA-21, is present in relatively high concentrations, as shown in PDGF-BB-treated cells, whereas their effects may result in a different pattern when their target does not interact sufficiently, as shown in non-treated orbital fibroblasts. However, further studies will clearly be needed to define the difference between them.

In conclusion, the findings presented in the current study suggest that PDGF-BB stimulates cell proliferation through miRNA-21-mediated down-regulation of PDCD4, leading to development of TAO. These findings also show that the PDGF-BB/miRNA-21/PDCD4 pathway may be a reasonable target of therapeutic intervention in the case of TAO. In addition, to clarify the role of PDGF-BB/miRNA-21/PDCD4 pathway in the pathophysiology of TAO further, more detailed studies are clearly needed, including measurement of the levels of PDGF-BB, miRNA-21, and PDCD4 in orbital tissues from patients with TAO.

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