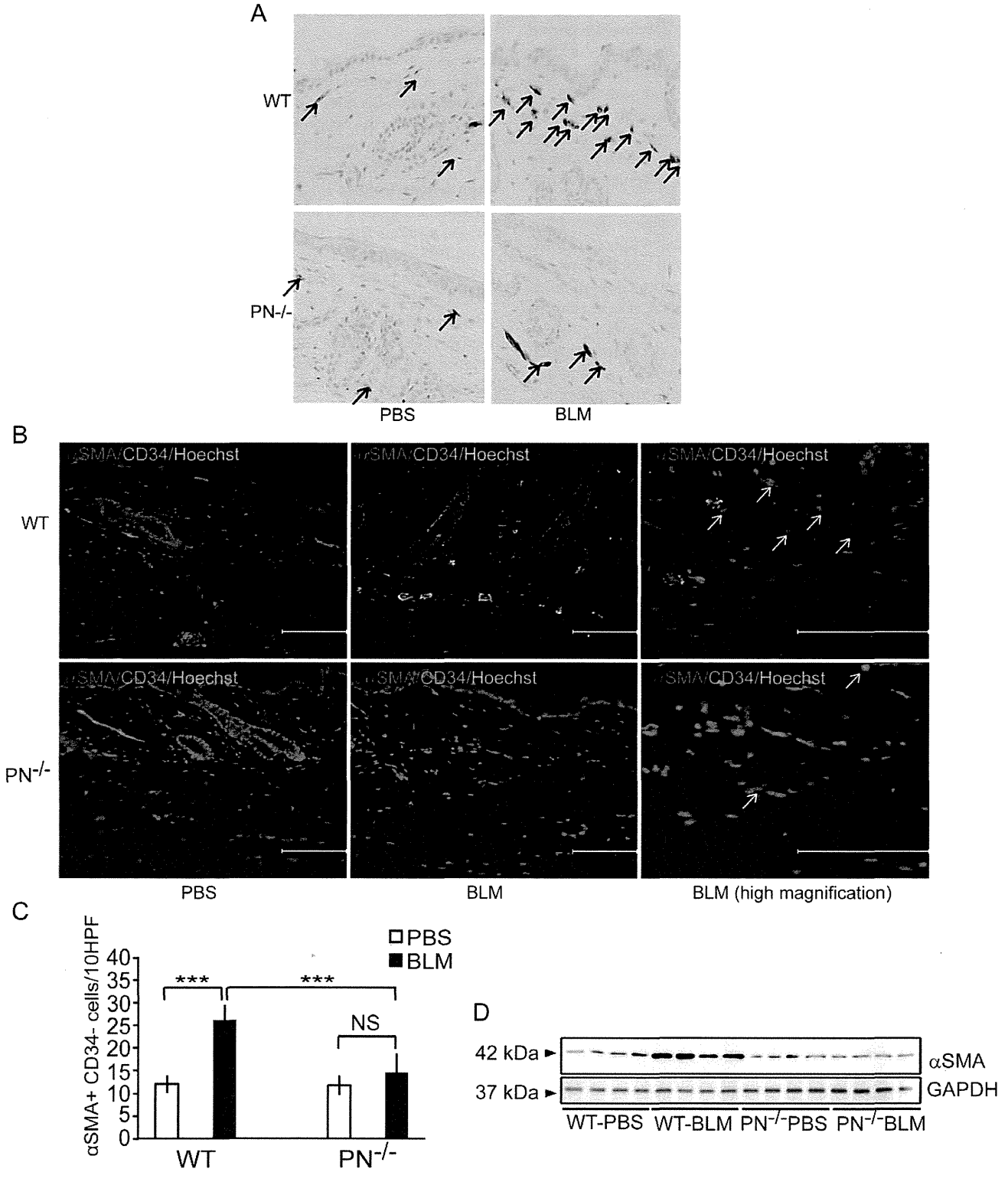

Figure 4. Periostin is required for dermal myofibroblast development in BLM-treated mice *in vivo*. A, Representative skin sections from WT and $PN^{-/-}$ mice, stained by immunohistochemistry with anti-α-SMA antibody (original magnification, ×400). α-SMA-positive myofibroblasts are indicated by arrows. B, Representative skin sections from WT and $PN^{-/-}$ mice, doubly stained by immunofluorescence for anti-α-SMA (red) and anti-CD34 (green). α-SMA^{+} $CD34^{-}$ spindle-shaped myofibroblasts are indicated by arrows. Scale bar = 100 μm. Nucleic staining: Hoechst 33342 (blue). C, The number of myofibroblasts per 10 hyper power microscopic fields is shown in the histogram. D, Western blotting analysis of protein extracted from WT and $PN^{-/-}$ mice skin tissues. For all assays, 10 mice from each group were analyzed. Values in C are shown as the mean ± SD. NS, no significance; ***, $p<0.01$.

doi:10.1371/journal.pone.0041994.g004

$^{-}$ fibroblasts were significantly lower than those in WT fibroblasts ($P<0.01$) (Figure 5A). Western blotting analysis, using protein samples extracted from cultured fibroblasts 24 hrs after TGFβ1 stimulation, confirmed that α-SMA protein levels were strongly induced in WT fibroblasts but not in $PN^{-/-}$ fibroblasts (Figure 5B). In addition, WT fibroblasts stimulated with TGFβ1 for more than 12 hrs could upregulate periostin at the protein levels (Figure S2B). These results raise the possibility that periostin protein induced by TGFβ1 may directly or indirectly mediate α-SMA expression in fibroblasts. Therefore, we next stimulated cultured WT dermal fibroblasts with different concentrations of rmPeriostin alone or in combination with TGFβ1 for two hours. While neither α-SMA transcript expression (Figure 5C) nor α-SMA protein expression (Figure 5D) was increased by rmPeriostin stimulation alone, the α-SMA expression level was synergistically enhanced by the combined stimulation of rmPeriostin with TGFβ1, compared to that with TGFβ1 stimulation alone (Figure 5C and 5D). These results suggest that periostin can enhance α-SMA expression in fibroblasts, not by acting alone but by cooperating with TGFβ1.

Periostin Upregulates Col1α1 Expression via the αv-integrin Mediated Phosphoinositide 3 Kinase (PI3K)/Akt Signaling Pathway *in vitro*

TGFβ1 is also known as a major inducer of collagen synthesis. We therefore investigated Col1α1 transcript levels in WT and $PN^{-/-}$ fibroblasts when they were stimulated with TGFβ1. Similar to the results of α-SMA expression, Col1α1 expression in $PN^{-/-}$ fibroblasts became to be significantly lower than WT fibroblasts after 12 hours of stimulation (Figure 6A). This result suggests that periostin may play a role in the Col1α1 expression.

To elucidate whether periostin directly enhances collagen synthesis, the effects of periostin on Col1α1 expression were also examined in cultured WT dermal fibroblasts. Interestingly, Col1α1 expression was induced two hours after stimulation with rmPeriostin alone in a dose-dependent manner (Figure 6B). In addition, Col1α1 expression level was further enhanced by the combined stimulation of rmPeriostin and TGFβ1, compared to TGFβ1 or rmPeriostin stimulation alone (Figure 6B), indicating the additive effect of rmPeriostin on TGFβ1-induced collagen induction.

Finally, to further clarify the signaling pathway by which periostin regulates Col1α1 expression, receptor neutralizing and kinase inhibition analyses were performed. After identification the optimal concentration of each inhibitor by a series dilution prior to the initiation of experiments, mouse dermal fibroblasts were pre-treated for two hours with or without various inhibitors at the identified concentrations: a neutralizing antibody against the known periostin receptor of αv-integrin (anti-αv integrin Ab), a tyrosine kinase inhibitor (genistein), a PI3K/Akt kinase inhibitor (LY294002), a mitogen-activated protein (MAP) kinase inhibitor (PD98095), an extracellular signal-related kinase (ERK) inhibitor (U0126), a p38 MAP kinase inhibitor (SB203580), or mammalian target of rapamycin (mTOR) inhibitors (temsirolimus and rapamycin). Fibroblasts were then stimulated with rmPeriostin for two hours to measure Col1α1 mRNA levels by real-time quantitative PCR (Figure 6C). Among these pharmacological inhibitors, only the addition of

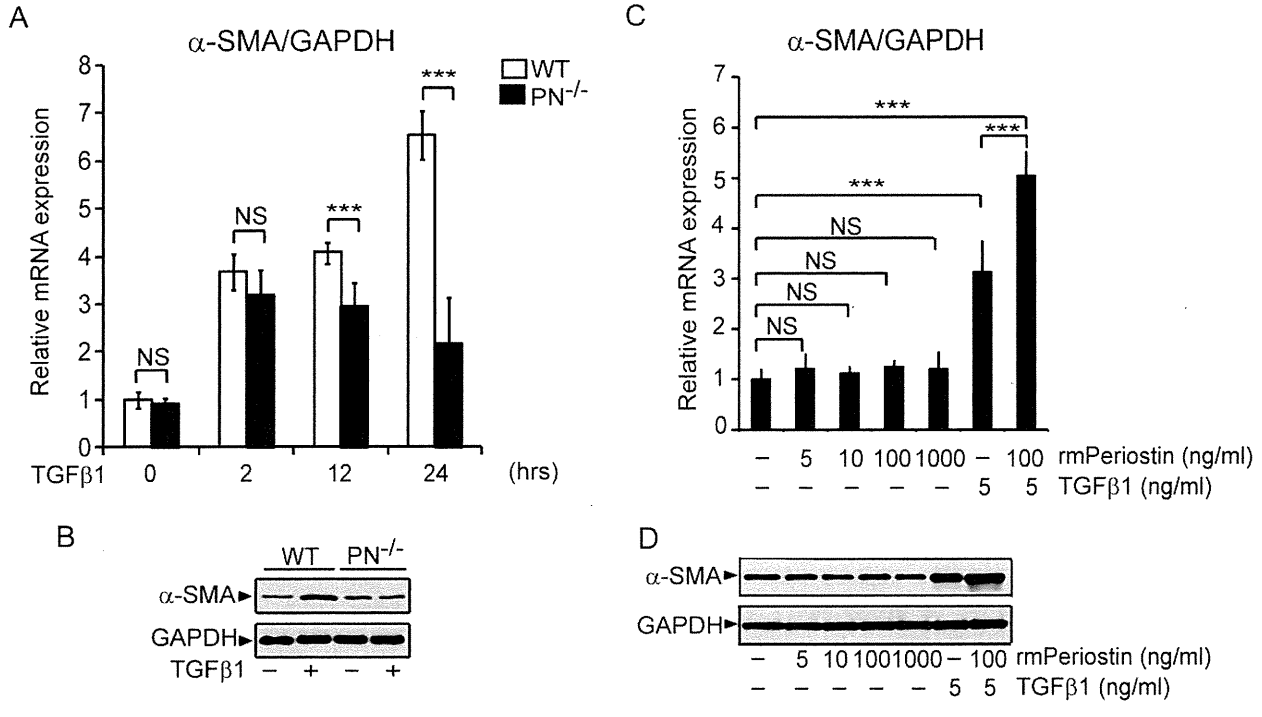

Figure 5. Periostin is required for TGFβ1-induced myofibroblast differentiation *in vitro*. A, Real-time quantitative PCR was performed to determine relative mRNA levels of α-SMA in cultured mouse dermal fibroblasts after TGFβ1 stimulation at the indicated times. B, Western blotting analysis for α-SMA with protein extracted from the indicated mouse dermal fibroblasts after TGFβ1 stimulation. C, Relative mRNA levels of α-SMA in cultured WT mouse dermal fibroblasts after the indicated stimulation. D, Western blotting analysis for α-SMA with protein extracted from WT mouse dermal fibroblasts after the indicated stimulation. Values in A and C were normalized to GAPDH levels and expressed as relative mRNA levels compared with WT mice fibroblasts (A) or WT dermal fibroblasts without stimulation (C). Values in A and C are shown as the mean ± SD. NS, no significance; ***, $p<0.01$.
doi:10.1371/journal.pone.0041994.g005

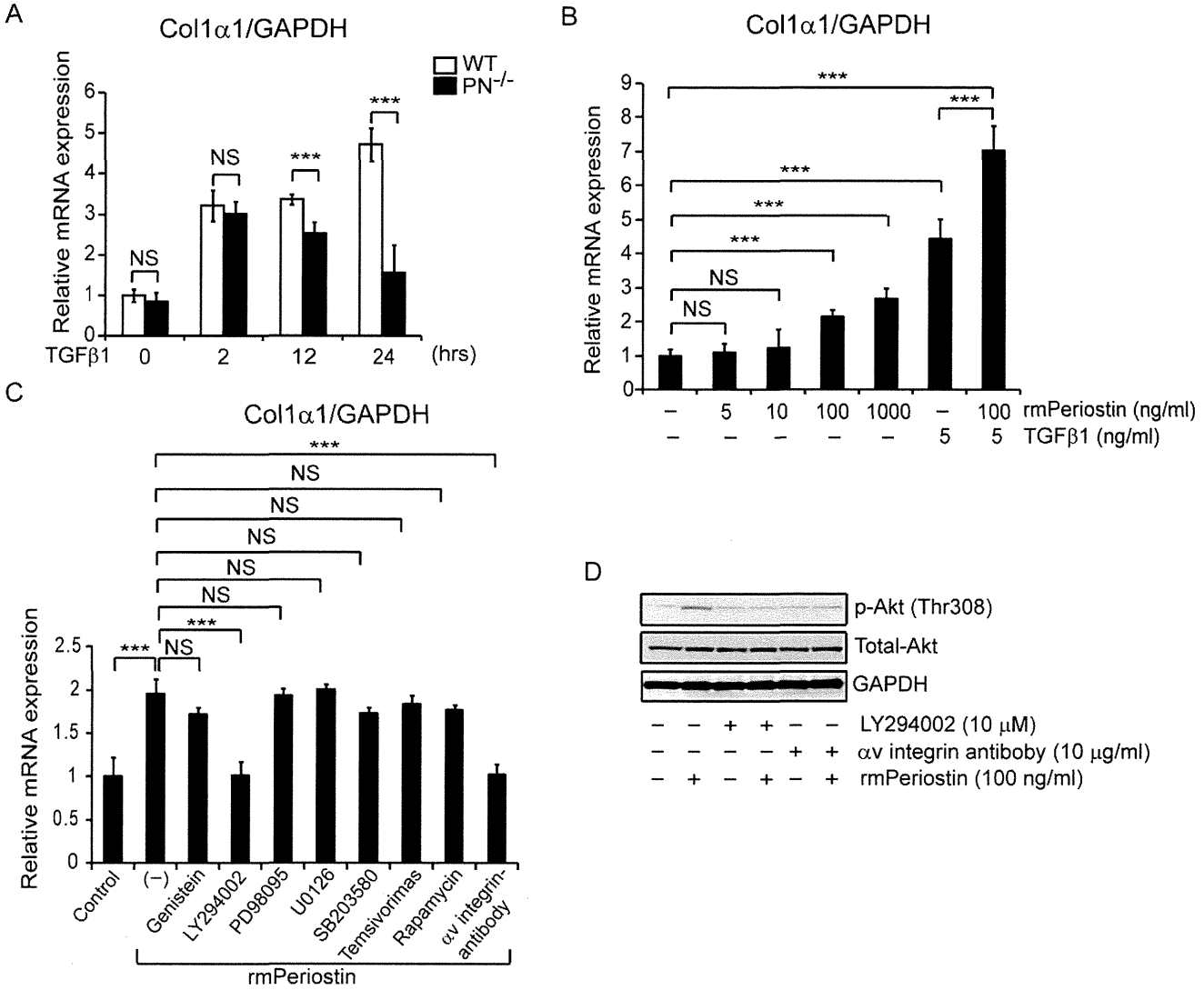

Figure 6. Periostin upregulates the expression of Col1α1 via αv-integrin mediated-PI3K/Akt signaling pathway *in vitro*. A, Real-time quantitative PCR was performed to determine relative mRNA levels of Col1α1 in cultured dermal fibroblasts from WT and $PN^{-/-}$ mice after TGFβ1 stimulation at the indicated times. B, Relative mRNA levels of Col1α1 in WT mouse dermal fibroblasts with the indicated stimulation. C, Relative mRNA levels of Col1α1 in cultured WT mouse dermal fibroblasts treated with rmPeriostin in the presence or absence of the indicated neutralizing antibody or kinase inhibitors. D, Phosphorylation of Akt in cultured WT mouse dermal fibroblasts treated with or without rmPeriostin in the presence or absence of LY294002 or anti-αv neutralizing antibody. Values in A, B, and C were normalized to GAPDH levels and expressed as relative mRNA levels compared with WT mice fibroblasts (A) or WT dermal fibroblasts without stimulation (B and C). NS, no significance; ***, $p<0.01$.
doi:10.1371/journal.pone.0041994.g006

LY294002 (10 μM) and anti-αv integrin Ab (10 μg/ml) abrogated periostin-induced upregulation of Col1α1 expression. In addition, rmPeriostin promptly activated Akt (Thr308) in WT mouse dermal fibroblasts (Figure 6D), implying the direct activation of the PI3K/Akt pathway by rmPeriostin. We also confirmed that the nontoxic concentration of LY294002 and anti-αv integrin Ab efficiently blocked Akt phosphorylation in fibroblasts treated with rmPeriostin (Figure 6D). Thus, periostin appears, at least in part, to directly increase Col1α1 expression in murine scleroderma via the αv integrin-mediated PI3K/Akt pathway.

Discussion

Matricellular proteins are ECM proteins that modulate cell-matrix interactions as well as cellular functions. They are highly expressed in injured and remodeled tissues and during embryonic development, and have been implicated in the pathophysiology of various fibrotic conditions. Like other matricellular proteins, periostin is thought to play a fundamental role in tissue development and remodeling [10,27,35]. Using $PN^{-/-}$ mice, the importance of periostin in various fibrotic conditions has been uncovered. However, it is still unknown whether periostin is involved in scleroderma. Our study is the first to assess the role of periostin in scleroderma.

As expected, we show herein the enhanced expression of periostin in lesional skin from patients with scleroderma and in BLM-induced sclerotic mouse skin, compared with hypertrophic scar, keloid, normal skin and PBS-treated mouse skin. These observations support the notion that periostin is involved in the process of skin fibrosis.

$PN^{-/-}$ mice were used to examine the contribution of periostin in the pathogenesis of scleroderma. The results of histological analysis showed that before the subcutaneous injection of BLM, there were no significant differences in dermal thickness or collagen production between WT and $PN^{-/-}$ mice. However, in the BLM-induced mouse scleroderma model, a reduced sclerotic response was shown in the skin of $PN^{-/-}$ mice, suggesting that periostin is critically involved in the pathogenesis of scleroderma.

The enhanced generation of α-SMA-positive myofibroblasts is determined to be a hallmark of and an essential process for scleroderma [33]. In the present study, BLM-induced myofibroblast formation was distinctly impaired in $PN^{-/-}$ mice. A similar reduction in the development of α-SMA-positive myofibroblasts has been observed previously in $PN^{-/-}$ mice subjected to various pathogenic conditions such as myocardial infarction [17,36], wound healing [37] and tumor engraftment [27]. These observations collectively indicate the important role of periostin in myofibroblast development *in vivo*.

One possible mechanism by which periostin can increase myofibroblast number is the promotion of myofibroblast recruitment through the αv-integrin pathway [17,21]. It is also well known that myofibroblast differentiation is critically regulated by TGFβ1 and TGFβ1-induced matricellular proteins such as CCN2 and fibronectin [4,38,39]. In the present study, myofibroblast differentiation induced by TGFβ1 *in vitro* was attenuated in $PN^{-/-}$ fibroblasts (Figure 5A and 5B), although we found no impairment of cell viabilities in $PN^{-/-}$ fibroblasts during culture (Figure S1 and Text S1). Moreover, this impairment in $PN^{-/-}$ fibroblasts was rescued by the addition of rmPeriostin *in vitro* (Figure S3A). Interestingly, however, we found that periostin stimulation alone did not induce α-SMA expression in WT fibroblasts, but the TGFβ1-induced α-SMA expression could be enhanced in combination with rmPeriostin. Similar to our findings, a previous study showed that periostin is required for embryonic fibroblasts to respond properly to TGFβ1 [40]. Thus, it appears that periostin likely plays a critical role as a co-factor that augments TGFβ1-induced α-SMA expression. This action of periostin is reminiscent of other matricellular proteins such as CCN2 in facilitating TGFβ1 action [38]. Thus, periostin, in cooperation with other TGFβ1-induced matricellular proteins, may provide integrated extracellular signals for a proper TGFβ1 response. In addition, periostin may also augment TGFβ1 activity *via* the activation of latent TGFβ1, as suggested by a previous study on airway epithelial cells [41].

Our findings also suggest that periostin directly contributes to excessive collagen synthesis in scleroderma. Previously, in various disease models utilizing $PN^{-/-}$ mice, reductions in collagen accumulation, similar to our observations, were reported [17,27–29]. However, it is unknown whether periostin directly regulates collagen synthesis. In this study, both $PN^{-/-}$ mice upon bleomycin injection *in vivo* and $PN^{-/-}$ fibroblasts stimulated with TGFβ1 *in vitro* exhibited reduced Col1α1 mRNA production. Furthermore, rmPeriostin induced Col1α1 mRNA expression in dermal fibroblasts *in vitro*. These effects of periostin are presumably direct and mediated *via* the αv-integrin mediated-PI3K/Akt pathway because 1) rmPeriostin can induce a prompt activation of Akt in fibroblasts and 2) Col1α1 induction was abrogated by αv-integrin neutralization or PI3K inhibition. It is known that periostin can bind to several types of integrins (e.g., αvβ3, αvβ5, and αvβ4), which act as receptors that activate downstream signaling pathways including PI3K/Akt [13]. Our findings also raise the intriguing possibility that TGFβ1-induced Col1α1 expression, unlike α-SMA expression, is mediated by the action of periostin. These observations of periostin differ from those obtained using $CCN2^{-/-}$ fibroblasts, in that Col1α1 production normally increases after TGFβ1 stimulation [4]. It is tempting to speculate that Col1α1 production in $CCN2^{-/-}$ fibroblasts might be compensated by the effects of TGFβ1-induced periostin. Thus, we assume that periostin, upon induction by TGFβ1, not only acts as a co-factor of TGFβ1 activity, but also, at least in part, directly mediates part of the TGFβ1 response.

Our time-course experiments *in vitro* revealed that mRNA levels of α-SMA and Col1α1 were similar between WT and $PN^{-/-}$ fibroblasts at the early phase of TGFβ1 stimulation (0 hrs, 2 hrs), but became prominently lower in $PN^{-/-}$ fibroblasts than that in WT fibroblasts after longer incubation with TGFβ1 (12 hrs, 24 hrs) ($P<0.01$) (Figure 5A and 6A). This difference at late phase can be explained by de novo periostin secretion, which is induced by TGFβ1 in WT fibroblasts. Indeed, as reported previously [19], periostin was strongly induced in fibroblasts by TGFβ1 in a dose-dependent manner (Figure S2A). Moreover, the protein synthesis and secretion of periostin was undetectable at 2 hrs but became detectable after 12 hrs of stimulation (Figure S2B). Notably, TGFβ1-induced expression of α-SMA and Col1α1 in $PN^{-/-}$ fibroblasts could be rescued by addition of rmPeriostin to the culture media (Figure S3A and S3B). Upon these results described above, periostin, induced by TGFβ1 in fibroblasts, is likely involved in fibrosis process of scleroderma, at least in part *via* enhancing α-SMA expression and mediating Col1α1 induction in these cells.

The unexpected data we encountered in the present study was that, in $PN^{-/-}$ fibroblasts, TGFβ1-induced α-SMA and Col1α1 mRNA levels were peaked at 2 hrs and slightly declined thereafter (Figure 5A and 6A). Because it is well known that the fibrotic effect of TGFβ1 is regulated by its negative feedback mechanisms, the absence of periostin may render these feedback mechanisms predominant. Furthermore, our preliminary data suggest that the expression of decorin, which is known as a potent inhibitor of TGFβ1/Smad signaling [42], is increased in $PN^{-/-}$ fibroblasts compared to WT cells (data not shown). Thus, periostin may accelerate the fibirotic action of TGFβ1 not only by increasing α-SMA and Col1α1 mRNA expression but also by counteracting against negative feedback signaling of TGFβ1. Further studies are underway to reveal the role of periostin in regulating negative-feedback signaling molecules such as decorin and Smad7 in TGFβ1 signaling.

It should be noted that periostin is reported to have a number of functions that may related to skin fibrosis. Similar to other matricellular proteins like thrombospondin-2 [41] and SPARC (secreted protein acidic and rich in cysteine, also known as osteonectin or BM-40) [42], periostin is known to be involved in collagen assembly [10]. Moreover, we recently reported that rmPeriostin can promote the proliferation of mouse dermal fibroblasts *in vitro* [24], at least in part *via* periostin-PI3K/Akt pathway. Additionally, according to recent evidence [29,43], periostin may also contribute to scleroderma *via* the regulation of the Notch1 signaling pathway, another important pathway in skin sclerosis [44–46].

It is generally known that fibrotic processes in skin are regulated by a complex network of matricellular proteins. Inhibition of just one matricellular protein can often disrupt the balance of this organized network and lead to exacerbation [43] or attenuation [4] [6,44] of skin fibrosis under pathogenic conditions. The present study is the first to show that periostin is one of these pivotal matricellular proteins that accelerates pathologic fibrosis in both BLM-induced skin sclerosis and human scleroderma. Our findings suggest that periostin promotes disease by enhancing myofibroblast differentiation and collagen synthesis via the augmentation

and mediation (at least in part) of TGFβ1 activity. Periostin may also contribute to the pathogenesis of scleroderma via the proliferation and recruitment of myofibroblasts [17,24], enhancement of Notch1 signaling [29,45], and promotion of collagen assembly [10]. Thus, our observations and those of others collectively indicate that periostin is involved in the multiple steps of skin fibrosis and is an attractive target for the treatment of scleroderma.

We hope that our findings will contribute to both a better understanding of scleroderma pathogenesis and the development of novel therapeutic approaches, including the possible inhibition of periostin function, for the treatment of scleroderma.

Materials and Methods

Human Samples

The frozen biopsy tissues and paraffin-embedded tissue sections obtained from lesional skin of well-defined patients with diffuse systemic scleroderma (n = 12; male: female ratio 2:10, mean age 52.4 years [range 24–76 years]), lesional skin of patients with keloid (n = 8; male: female ratio 2:4, mean age 48.5 years [range 21–68 years]), hypertrophic scar (n = 7; male: female ratio 2:5, mean age 50.5 years [range 34–72 years]), and corresponding sites of healthy donors (n = 12; male: female ratio 3:9, mean age 49.2 years [range 26–65 years]) were used in this study. Written informed consent was obtained from all participants prior to study inclusion. The study was approved by the Medical Ethics Committee of Osaka University (Case number 2011-3/17-10193).

Rearing Management of Animals

WT mice (C57BL/6 strain) were obtained from CLEA Japan, Inc. (Osaka, Japan). Periostin gene knockout ($PN^{-/-}$) mice (C57BL/6 strain) were generated as previously described [27]. All animal care and experimentation were performed in accordance with the institutional guidelines of the National Institute of Biomedical Innovation, Osaka, Japan (NIBIO) (Approval No. DS2147R1).

BLM-induced Scleroderma Model and Tissue Sample Preparation

BLM (Nippon Kayaku, Tokyo, Japan) was dissolved in phosphate-buffered saline (PBS) at a concentration of 1 mg/ml and sterilized by filtration. BLM or PBS (100 μl) was injected subcutaneously as described by us previously [46]; one day after the final injection, the skin at the injected site was removed and processed for analysis as previously described [47].

Histopathological Analysis, Assessment of Skin Thickness, and Collagen Synthesis

Paraffin-embedded tissue sections were stained with hematoxylin and eosin (H&E Fisher Scientific), and dermal thickness was calculated as described previously [48]. To assess dermal collagen deposition, semi-quantitative analysis using Masson's trichrome staining, in which collagen fibers are stained blue, was used. Collagen deposition was graded by examining five randomly chosen fields at 100× magnification in a blinded manner using three observers. The grading criteria were as follows: grade 0 = no collagen fibers; grade 1 = few collagen fibers; grade 2 = moderate amount of collagen fibers; and grade 3 = excessive amount of collagen fibers.

Immunohistochemistry and Immunofluorescence Staining

Paraffin sections were prepared as referred to above and then subjected to immunohistochemistry and immunofluorescence staining as described previously [47,49]. The primary antibodies used were rabbit anti-periostin (1:3,000 dilution; Abcam, Cambridge, MA), mouse anti α-smooth muscle actin (α-SMA; 1:3,000 dilution; Sigma-Aldrich, St. Louis, MO) and rat anti-CD34 antibody (1:50 dilution; Abcam, Cambridge, MA), followed by the DAKO LSAP+System-AP (DakoCytomation) and Dako ChemMate Envision kit/HRP(DAB), or followed by the secondary antibody (anti-mouse Alexa Fluor 555, anti-rat Alexa Fluor 488, Invitrogen). The slides were visualized using a light microscope or Keyence Biozero confocal microscope. α-SMA-positive spindle cells (α-SMA^+ cells) or α-SMA-positive and CD34-negative spindle-shaped cells (α-SMA^+ $CD34^-$ cells) were counted in 10 non-contiguous random grids under high-power magnification fields (400×) by confocal microscope. Results are expressed as the mean ± standard deviation (SD) of positive spindle-shaped fibroblasts per field.

Cell Culture

Neonatal murine primary dermal fibroblasts were isolated from the skin of 10-day-old WT mice and cultured as previously described [47]. After 24 hours of serum starvation, dermal fibroblasts were treated with TGFβ1 (2–12 ng/ml) or recombinant mouse periostin (rmPeriostin) (5–1,000 ng/ml) for the indicated periods prior to extraction of RNA and protein extraction. Cells were used at passage three. In each experiment, obtained fibroblasts were examined at the same time and under the same culture conditions (e.g., cell density, passage, and days after plating).

Neutralizing and Kinase Inhibition Assays

Cells were grown on 6-well plates; after extensive washing with PBS to remove all sera, cells were serum-starved for 24 hours. Subsequently, the cells were incubated for 2 hours with the neutralizing antibody against αv-integrin (anti-αv-integrin Ab, Biolegend, San Diego, CA) and kinase inhibitors (Cell Signaling Technology, Beverly, MA) at the indicated concentrations: anti-αv-integrin Ab (10 μg/ml), genistein (10 μM), LY294002 (10 μM), PD98095 (50 μM), U0126 (20 μM), SB203580 (25 μM), temsirolimus (10 μM), and rapamycin (500 nM). Cells were then stimulated for 2 hours with 100 ng/ml rmPeriostin in the same media. After stimulation, total RNA was isolated. To analyze protein phosphorylation, cells were collected after five minutes of periostin stimulation. We performed a serial dilution to identify the optimal concentration of each inhibitor prior to the initiation of experiments by MTT assay and western blotting analysis, the nontoxic and effective concentration was used in neutralizing and kinase inhibition assay.

RNA Isolation and Real-time Quantitative Polymerase Chain Reaction (PCR)

Total RNA from mouse skin tissues or cultured fibroblast cell pellets was isolated with RNeasy spin columns (Qiagen, Valencia, CA) following the manufacturer's instructions. The integrity of the RNA was verified by gel electrophoresis. Total RNA (100 ng) was reverse-transcribed into first-strand complementary DNA (cDNA) (QuantiTect Reverse Transcription Kit, Qiagen). The primers used for real-time PCR were as follows: TGFβ1, sense 5′-cgaatgtctgacgtattgaagaaca-3′, antisense 5′-ggagcccgaagcggacta-3′; CCN2/CTGF, sense 5′-caaagcagctgcaaatacca-3′, antisense 5′-gacaggcttggcgattttag-3′; α-SMA, sense 5′-tctctatgctaa-caacgtcctgtca-3′, antisense 5′-ccaccgatccagacagagtactt-3′; collagen type-I alpha 1 (Col1α1), sense 5′-gagccctcgcttccgtactc-3′, antisense 5′-tgttccctactcagccgtctgt-3′; and GAPDH, sense 5′-tgtcatca-

tacttggcaggtttct-3′, antisense 5′-catggccttccgtgttccta-3′. Each reaction was performed in triplicate. Variation within samples was less than 10%. Statistical analysis was performed with the Student's paired *t* test.

Western Blotting Analysis

Proteins from skin samples and cell pellets were extracted, and 5 μg of extracted protein was used for western blotting analysis as described previously [47]. The primary antibodies were used at the following dilutions: anti-α-SMA (Sigma-Aldrich), 1:500; anti-periostin (R&D Systems. Minneapolis, MN), 1:500; anti-periostin (Abcam, Cambridge, MA), 1:1,000; anti-phospho-Akt (Cell Signaling Technology, Beverly, MA), 1:1,000; anti-Total Akt (Cell Signaling Technology), 1:1,000; and anti-GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA), 1:500. We used anti-GAPDH antibody as a loading control.

Statistical Analysis

The data were expressed as the mean ± SD. The Student's two-tailed *t*-test (Microsoft Excel software, Redmond, WA) was used for comparison between two groups. When analysis included more than two groups, one-way analysis of variance was used. P-values less than 0.05 were considered statistically significant.

Supporting Information

Figure S1 TGFβ1 does not affect cell viability of WT and $PN^{-/-}$ dermal fibroblasts. Cell viabilities of WT and $PN^{-/-}$ dermal fibroblasts were assessed by MTT assay after treatment with TGFβ1 (5 ng/ml) for 2–24 hours. Data are shown as mean ± SD. NS, no significance.
(TIF)

Figure S2 Periostin is induced by TGFβ1 in WT dermal fibroblasts in a dose- and time-dependent manner. A, Real-time quantitative PCR was performed to determine relative mRNA levels of periostin in cultured WT dermal fibroblasts at two hours after TGFβ1 treatment at the indicated concentrations. B, Western blotting analysis for periostin with protein extracted from WT dermal fibroblasts or culture supernatants after TGFβ1 treatment at the indicated times. Values in A were normalized to GAPDH levels and expressed as relative mRNA levels compared with WT dermal fibroblasts without TGFβ1 treatment. Values in A are shown as the mean ± SD. NS, no significance; ***, $p<0.01$.
(TIF)

Figure S3 The effects of TGFβ1 in the induction of α-SMA and Col1α1 were recovered by addition of rmPeriostin to cultured $PN^{-/-}$ fibroblasts. Real-time quantitative PCR was performed to determine relative mRNA levels of α-SMA (A) and Col1α1 (B) in cultured dermal fibroblasts at 24 hours after TGFβ1 treatment. Values in A and B were normalized to GAPDH levels and expressed as relative mRNA levels compared with WT dermal fibroblasts without TGFβ1 treatment. Values in A and B are shown as the mean ± SD. NS, no significance; ***, $p<0.01$. (Note: Data of WT and $PN^{-/-}$ group shown here and those presented in Figure 5A and 6A are from the same data set.)
(TIF)

Text S1 Supplementary materials and methods.
(DOC)

Acknowledgments

We acknowledge Laboratory of Animal Models for Human Diseases (National Institute of Biomedical Innovation, Osaka, Japan) for animal care and husbandry, Dr. Kenju Nishida for tissue processing and embedding, Dr. Barry Ripley for evaluation of the manuscript.

Author Contributions

Conceived and designed the experiments: LY SS HM MF AK TN IK. Performed the experiments: LY SS HM MF MT SM YK SK. Analyzed the data: LY SS HM MF MT YK SK. Contributed reagents/materials/analysis tools: AK MT SM YK SK. Wrote the paper: LY SS HM MF.

References

1. Gabrielli A, Avvedimento EV, Krieg T (2009) Scleroderma. N Engl J Med 360: 1989–2003.
2. Leask A, Abraham DJ, Finlay DR, Holmes A, Pennington D, et al. (2002) Dysregulation of transforming growth factor beta signaling in scleroderma: overexpression of endoglin in cutaneous scleroderma fibroblasts. Arthritis Rheum 46: 1857–1865.
3. Leask A (2009) Signaling in fibrosis: targeting the TGF beta, endothelin-1 and CCN2 axis in scleroderma. Front Biosci (Elite Ed) 1: 115–122.
4. Liu S, Shi-wen X, Abraham DJ, Leask A (2011) CCN2 is required for bleomycin-induced skin fibrosis in mice. Arthritis Rheum 63: 239–246.
5. Jun JI, Lau LF (2010) The matricellular protein CCN1 induces fibroblast senescence and restricts fibrosis in cutaneous wound healing. Nat Cell Biol 12: 676–685.
6. Liu S, Kapoor M, Denton CP, Abraham DJ, Leask A (2009) Loss of beta1 integrin in mouse fibroblasts results in resistance to skin scleroderma in a mouse model. Arthritis Rheum 60: 2817–2821.
7. Takeshita S, Kikuno R, Tezuka K, Amann E (1993) Osteoblast-specific factor 2: cloning of a putative bone adhesion protein with homology with the insect protein fasciclin I. Biochem J 294 (Pt 1): 271–278.
8. Kudo A (2011) Periostin in fibrillogenesis for tissue regeneration: periostin actions inside and outside the cell. Cell Mol Life Sci 68: 3201–3207.
9. Kii I, Amizuka N, Minqi L, Kitajima S, Saga Y, et al. (2006) Periostin is an extracellular matrix protein required for eruption of incisors in mice. Biochem Biophys Res Commun 342: 766–772.
10. Norris RA, Damon B, Mironov V, Kasyanov V, Ramamurthi A, et al. (2007) Periostin regulates collagen fibrillogenesis and the biomechanical properties of connective tissues. J Cell Biochem 101: 695–711.
11. Chen CC, Lau LF (2009) Functions and mechanisms of action of CCN matricellular proteins. Int J Biochem Cell Biol 41: 771–783.
12. Gillan L, Matei D, Fishman DA, Gerbin CS, Karlan BY, et al. (2002) Periostin secreted by epithelial ovarian carcinoma is a ligand for alpha(V)beta(3) and alpha(V)beta(5) integrins and promotes cell motility. Cancer Res 62: 5358–5364.
13. Baril P, Gangeswaran R, Mahon PC, Caulee K, Kocher HM, et al. (2007) Periostin promotes invasiveness and resistance of pancreatic cancer cells to hypoxia-induced cell death: role of the beta4 integrin and the PI3k pathway. Oncogene 26: 2082–2094.
14. Ruan K, Bao S, Ouyang G (2009) The multifaceted role of periostin in tumorigenesis. Cell Mol Life Sci 66: 2219–2230.
15. Larsen M, Artym VV, Green JA, Yamada KM (2006) The matrix reorganized: extracellular matrix remodeling and integrin signaling. Curr Opin Cell Biol 18: 463–471.
16. Norris RA, Moreno-Rodriguez R, Hoffman S, Markwald RR (2009) The many facets of the matricelluar protein periostin during cardiac development, remodeling, and pathophysiology. J Cell Commun Signal 3: 275–286.
17. Shimazaki M, Nakamura K, Kii I, Kashima T, Amizuka N, et al. (2008) Periostin is essential for cardiac healing after acute myocardial infarction. J Exp Med 205: 295–303.
18. Tilman G, Mattiussi M, Brasseur F, van Baren N, Decottignies A (2007) Human periostin gene expression in normal tissues, tumors and melanoma: evidences for periostin production by both stromal and melanoma cells. Mol Cancer 6: 80.
19. Horiuchi K, Amizuka N, Takeshita S, Takamatsu H, Katsuura M, et al. (1999) Identification and characterization of a novel protein, periostin, with restricted expression to periosteum and periodontal ligament and increased expression by transforming growth factor beta. J Bone Miner Res 14: 1239–1249.
20. Takayama G, Arima K, Kanaji T, Toda S, Tanaka H, et al. (2006) Periostin: a novel component of subepithelial fibrosis of bronchial asthma downstream of IL-4 and IL-13 signals. J Allergy Clin Immunol 118: 98–104.
21. Kühn B, del Monte F, Hajjar RJ, Chang YS, Lebeche D, et al. (2007) Periostin induces proliferation of differentiated cardiomyocytes and promotes cardiac repair. Nat Med 13: 962–969.
22. Dorn GW (2007) Periostin and myocardial repair, regeneration, and recovery. N Engl J Med 357: 1552–1554.
23. Hamilton DW (2008) Functional role of periostin in development and wound repair: implications for connective tissue disease. J Cell Commun Signal 2: 9–17.

24. Ontsuka K, Kotobuki Y, Shiraishi H, Serada S, Ohta S, et al. (2012) Periostin, a matricellular protein, accelerates cutaneous wound repair by activating dermal fibroblasts. Exp Dermatol 21: 331–336.
25. Zhou HM, Wang J, Elliott C, Wen W, Hamilton DW, et al. (2010) Spatiotemporal expression of periostin during skin development and incisional wound healing: lessons for human fibrotic scar formation. J Cell Commun Signal 4: 99–107.
26. Oku E, Kanaji T, Takata Y, Oshima K, Seki R, et al. (2008) Periostin and bone marrow fibrosis. Int J Hematol 88: 57–63.
27. Shimazaki M, Kudo A (2008) Impaired capsule formation of tumors in periostin-null mice. Biochem Biophys Res Commun 367: 736–742.
28. Nishiyama T, Kii I, Kashima TG, Kikuchi Y, Ohazama A, et al. (2011) Delayed re-epithelialization in periostin-deficient mice during cutaneous wound healing. PLoS One 6: e18410.
29. Tkatchenko TV, Moreno-Rodriguez RA, Conway SJ, Molkentin JD, Markwald RR, et al. (2009) Lack of periostin leads to suppression of Notch1 signaling and calcific aortic valve disease. Physiol Genomics 39: 160–168.
30. Gordon ED, Sidhu SS, Wang ZE, Woodruff PG, Yuan S, et al. (2012) A protective role for periostin and TGF-β in IgE-mediated allergy and airway hyperresponsiveness. Clin Exp Allergy 42: 144–155.
31. Yamamoto T, Takagawa S, Katayama I, Yamazaki K, Hamazaki Y, et al. (1999) Animal model of sclerotic skin. I: Local injections of bleomycin induce sclerotic skin mimicking scleroderma. J Invest Dermatol 112: 456–462.
32. Yamamoto T, Kuroda M, Nishioka K (2000) Animal model of sclerotic skin. III: Histopathological comparison of bleomycin-induced scleroderma in various mice strains. Arch Dermatol Res 292: 535–541.
33. Desmoulière A, Geinoz A, Gabbiani F, Gabbiani G (1993) Transforming growth factor-beta 1 induces alpha-smooth muscle actin expression in granulation tissue myofibroblasts and in quiescent and growing cultured fibroblasts. J Cell Biol 122: 103–111.
34. Border WA, Noble NA (1994) Transforming growth factor beta in tissue fibrosis. N Engl J Med 331: 1286–1292.
35. Norris RA, Borg TK, Butcher JT, Baudino TA, Banerjee I, et al. (2008) Neonatal and adult cardiovascular pathophysiological remodeling and repair: developmental role of periostin. Ann N Y Acad Sci 1123: 30–40.
36. Oka T, Xu J, Kaiser RA, Melendez J, Hambleton M, et al. (2007) Genetic manipulation of periostin expression reveals a role in cardiac hypertrophy and ventricular remodeling. Circ Res 101: 313–321.
37. Elliott CG, Wang J, Guo X, Xu SW, Eastwood M, et al. (2012) Periostin modulates myofibroblast differentiation during full-thickness cutaneous wound repair. J Cell Sci 125: 121–132.
38. Shi-wen X, Stanton LA, Kennedy L, Pala D, Chen Y, et al. (2006) CCN2 is necessary for adhesive responses to transforming growth factor-beta1 in embryonic fibroblasts. J Biol Chem 281: 10715–10726.
39. Lygoe KA, Wall I, Stephens P, Lewis MP (2007) Role of vitronectin and fibronectin receptors in oral mucosal and dermal myofibroblast differentiation. Biol Cell 99: 601–614.
40. Snider P, Hinton RB, Moreno-Rodriguez RA, Wang J, Rogers R, et al. (2008) Periostin is required for maturation and extracellular matrix stabilization of noncardiomyocyte lineages of the heart. Circ Res 102: 752–760.
41. Sidhu SS, Yuan S, Innes AL, Kerr S, Woodruff PG, et al. (2010) Roles of epithelial cell-derived periostin in TGF-beta activation, collagen production, and collagen gel elasticity in asthma. Proc Natl Acad Sci U S A 107: 14170–14175.
42. Yamaguchi Y, Mann DM, Ruoslahti E (1990) Negative regulation of transforming growth factor-beta by the proteoglycan decorin. Nature 346: 281–284.
43. Ikonomidis JS, Hendrick JW, Parkhurst AM, Herron AR, Escobar PG, et al. (2005) Accelerated LV remodeling after myocardial infarction in TIMP-1-deficient mice: effects of exogenous MMP inhibition. Am J Physiol Heart Circ Physiol 288: H149–158.
44. McCann MR, Monemdjou R, Ghassemi-Kakroodi P, Fahmi H, Perez G, et al. (2011) mPGES-1 null mice are resistant to bleomycin-induced skin fibrosis. Arthritis Res Ther 13: R6.
45. Tanabe H, Takayama I, Nishiyama T, Shimazaki M, Kii I, et al. (2010) Periostin associates with Notch1 precursor to maintain Notch1 expression under a stress condition in mouse cells. PLoS One 5: e12234.
46. Kitaba S, Murota H, Terao M, Azukizawa H, Terabe F, et al. (2011) Blockade of Interleukin-6 Receptor Alleviates Disease in Mouse Model of Scleroderma. Am J Pathol.
47. Terao M, Murota H, Kitaba S, Katayama I (2010) Tumor necrosis factor-alpha processing inhibitor-1 inhibits skin fibrosis in a bleomycin-induced murine model of scleroderma. Exp Dermatol 19: 38–43.
48. Murota H, Hamasaki Y, Nakashima T, Yamamoto K, Katayama I, et al. (2003) Disruption of tumor necrosis factor receptor p55 impairs collagen turnover in experimentally induced sclerodermic skin fibroblasts. Arthritis Rheum 48: 1117–1125.
49. Terao M, Ishikawa A, Nakahara S, Kimura A, Kato A, et al. (2011) Enhanced epithelial-mesenchymal transition-like phenotype in N-acetylglucosaminyltransferase V transgenic mouse skin promotes wound healing. J Biol Chem 286: 28303–28311.

Pigment Cell Melanoma Res. **25**; 219–230

ORIGINAL ARTICLE

Dysregulation of melanocyte function by Th17-related cytokines: significance of Th17 cell infiltration in autoimmune vitiligo vulgaris

Yorihisa Kotobuki[1,2,*], Atsushi Tanemura[1,*], Lingli Yang[1,2], Saori Itoi[1], Mari Wataya-Kaneda[1], Hiroyuki Murota[1], Minoru Fujimoto[2], Satoshi Serada[2], Tetsuji Naka[2] and Ichiro Katayama[1]

1 Department of Dermatology Integrated Medicine, Osaka University Graduate School of Medicine, Osaka, Japan **2** Laboratory for Immune Signal, National Institute of Biomedical Innovation

CORRESPONDENCE Atsushi Tanemura, e-mail: tanemura@derma.med.osaka-u.ac.jp
*These authors contributed equally to this work.

KEYWORDS vitiligo/Th17 cell/Th17-related cytokines/melanocyte/interaction with skin-resident cells

PUBLICATION DATA Received 19 July 2011, revised and accepted for publication 30 November 2011, published online 3 December 2011

doi: 10.1111/j.1755-148X.2011.00945.x

Summary

The aim of this study was to determine whether CD4$^+$IL-17A$^+$Th17 cells infiltrate vitiligo skin and to investigate whether the proinflammatory cytokines related to Th17 cell influence melanocyte enzymatic activity and cell fate. An immunohistochemical analysis showed Th17 cell infiltration in 21 of 23 vitiligo skin samples in addition to CD8$^+$ cells on the reticular dermis. An in vitro analysis showed that the expression of MITF and downstream genes was downregulated in melanocytes by treatment with interleukin (IL)-17A, IL-1β, IL-6, and tumor necrosis factor (TNF)-α. Treatment with these cytokines also induced morphological shrinking in melanocytes, resulting in decreased melanin production. In terms of local cytokine network in the skin, IL-17A dramatically induced IL-1β, IL-6, and TNF-α production in skin-resident cells such as keratinocytes and fibroblasts. Our results provide evidence of the influence of a complex Th17 cell-related cytokine environment in local depigmentation in addition to CD8$^+$ cell-mediated melanocyte destruction in autoimmune vitiligo.

Introduction

In the epidermis, the epidermal melanin unit is reliant on the close interaction between a melanocyte and the associated pool of keratinocytes, and several inflammatory cytokines affect melanocyte migration, proliferation, and differentiation. Therefore, the local skin microenvironment generated by the skin-resident cells may be considered a crucial milieu for the normal life and functions of epidermal melanocytes (Chalraborty and Pawelek, 1993).

Vitiligo, a representative depigmented skin disorder associated with melanocyte destruction, affects an estimated 1% of the world's population (Howitz et al., 1977). Although the cellular immunoresponse, mainly of CD8$^+$ cytotoxic T cells, to the melanocyte-specific proteins MART-1, tyrosinase (TYR), and TRPs-1 and -2 has been shown to destroy functional melanocytes in

Significance

Here we show that not only cytotoxic T cells, which have been thought to play a major role in autoimmune vitiligo, but also infiltration of Th17 cells may play a role in vitiligo skin. In fact, we find that in vitro, a network of Th17 cell-related cytokines directly affect melanocyte activity and function, including downregulation of melanin production and shrinkage of melanocytes. These observations may shed light on the functional significance of TH17 cells in autoimmune vitiligo.

autoimmune vitiligo, this does not provide a full explanation for the etiology of vitiligo (Norris et al., 1994; Ogg et al., 1998; Okamoto et al., 1998; Ongenae et al., 2003). In addition to the autoimmune mechanism, recent reports have shown that there is a significant increase in the expression of inflammatory cytokines in affected skin compared with unaffected skin, and several investigators have proposed that the influence of local cytokines may be related to the induction and maintenance of vitiligo (Basak et al., 2009; Moretti et al., 2002, 2009; Ratsep et al., 2008). Although the representative cytokines increased in vitiligo skin have been reported to include interleukin (IL)-2, tumor necrosis factor (TNF)-α, and interferon (IFN)-γ (Caixia et al., 1999), there is no direct evidence of their function in the melanocyte destruction observed in vitiligo.

Upon induction by transforming growth factor (TGF)-b and IL-6, a subset of $CD4^+$ helper T cells develops as Th17 cells (Diveu et al., 2008). IL-17A is a cystine-linked homodimeric proinflammatory cytokine produced by Th17 cells, which form a distinct subset of the $CD4^+$ T-cell lineage. IL-17A stimulates the production of IL-1β, TNF-α, and IL-6 (Kolls and Linden, 2004; Liang et al., 2006). In the past decade, Th17 cells have been identified in autoimmune skin inflammatory disorders such as psoriasis and atopic dermatitis (Asarch et al., 2008; Fitch et al., 2009). A recent study showed a positive correlation between serum IL-17 levels and the extent of the depigmentation patch area in vitiligo, thus suggesting that Th17 cells, rather than regulatory T cells, are involved in vitiligo (Basak et al., 2009). Another study demonstrated elevated IL-17 levels in lesional skin and serum of patients with vitiligo compared with those of controls (Bassiouny and Shaker, 2011). These results indicated the importance of the secreted cytokine environment surrounding vitiliginous melanocytes in terms of vitligo etiology. In the present study, we investigated whether Th17 cells infiltrate vitiligo skin as in cases of psoriasis and whether the proinflammatory cytokines produced by Th17 cells, keratinocytes, and fibroblasts are altered in vitiligous lesions in a series of non-segmental vitiligo patients. The Th17-related cytokines tested included IL-17A and IL-22, in addition to IL-1β and IL-6, which have been reported to inhibit melanocyte activity (Kamaraju et al., 2002; Kholmanskikh et al., 2010).

MITF-M (microphthalmia-associated transcription factor-M) is a master transcription factor regulating melanocyte fate and melanogenic activity; it is distinctly expressed in melanocytes and mast cells (Levy et al., 2006). MITF expression and phosphorylation are important for the regulation of melanogenesis and melanocyte survival because the target genes of MITF encode the apoptosis regulator protein, B-cell lymphoma 2 (Bcl-2), in addition to melanogenic enzymes, tyrosinase, tyrosinase-related protein-1 (TRP-1), and dopachrome tautomerase (DCT), which are indispensable for maintaining melanocyte function (Levy et al., 2006). Because of the reduction in active melanocytes expressing these proteins in the vitiligo epidermis, the dysregulation of MITF expression has to be resolved to effectively treat vitiligo. In addition, the mRNA levels of *MITF* and *BCL2* were decreased in the lesional skin compared with the non-lesional skin of vitiligo patients (Kingo et al., 2008). The expression levels of IL-6 and TNF-α were also significantly higher in the lesional skin, indicating that in vitiligo lesions, there is increased expression of cytokines that are paracrine inhibitors of melanocytes (Moretti et al., 2002, 2009). These cytokines are produced mainly by keratinocytes, so it is possible that these cells may be abnormal in vitiligo. In addition, the expression of cytokines was unchanged in healthy skin compared with non-lesional skin, suggesting that the change observed in vitiligo lesional skin is possibly related to, or contributes to, depigmentation. Therefore, it is conceivable that there is a previously unrecognized mechanism involved in the regulation of the pigmentation-hypopigmentation balance in addition to a cytotoxic effect by $CD8^+$ T cell.

In this study, we examined the direct effect of Th17-related cytokines on MITF expression to determine the effects on the resulting cytokine involvement on the regulation of critical melanocyte behavior. We discuss the significance of Th17 cell infiltration in autoimmune vitiligo skin and propose a functional involvement of Th17 cell-related proinflammatory cytokines in vitiligo.

Results

Vitiligo skin develops in association with Th17 cell infiltration

Approval for this study was obtained from the Institutional Review Board of the Osaka University Hospital. To investigate whether Th17 cells infiltrate vitiligo skin, we performed immunostaining for IL-17A and CD4 using specific antibodies. Th17 cells were defined as the cells expressing both markers after exclusion of gamma delta T cells. Twenty-three vitiligo patients were enrolled in this study (see Table 1 for details) and were divided into 17 generalized, four localized, and two seg-

Table 1. Patients' characteristics and the infiltration status of Th17 cells

Age	27–81			
Gender				
Female	13			
Male	10			
Disease duration (yr)	0.1–26			
Mean	6.2			
	(n)	>50/field	<50/field	Not detected
Th17 cell infiltration				
Generalized type	17	11	6	0
Segmental type	4	2	0	2
Localized type	2	2	0	0
Total	23	15	6	2

mental types. The ages of the enrolled patients ranged from 27 to 81 yr, and the subjects included 13 women and 10 men. As a representative case, we show a 79-yr-old man who had experienced enlarging symmetrical depigmented macules on the whole body including face starting 2 yr previously who was positive for antithyroid antibody in the blood test (Figure 1).

Biopsy specimens were obtained from the leading edge of lesional skin on the left upper arm and were processed for the designated immunostaining. The immunohistochemical analysis revealed significant infiltration of IL-17A$^+$CD4$^+$ cells, that is, Th17 cells, mainly on the reticular dermis and perivascular region (Figure 1A). IL-17A expression was confirmed by RT-PCR using vitiligo tissue RNA. Psoriasis skin, a representative skin disease with Th17 cell infiltration, was loaded as a positive control for RT-PCR (Figure 1A). CD8$^+$ T cells were also observed, mainly below the epidermis, whereas Foxp3$^+$ cells and CD20$^+$ B cells had only faintly infiltrated (Figure 1B). Melan-A positive melanocytes were not observed in the vitiligo epidermis with inflammatory cell infiltration, whereas they were frequently located in the non-lesional skin (Figure 1B lower right panel and Inbox, respectively).

We observed a significant number of Th17 cells in 21 of 23 of the patient skin samples, and more than 50 double-positive cells per high power field were observed in 15 patients, while there was sparse infiltration in normal skin. Th17 cells were not detected in the two cases of localized type. Psoriatic skin was used as a positive control for this staining and showed the involvement as dense infiltration through the epidermis and upper dermis of pathogenic inflammatory cells whose localization was different from that in vitiligo (Figure 1C). Although we suspected that early onset and generalized type vitiligo had more opportu-

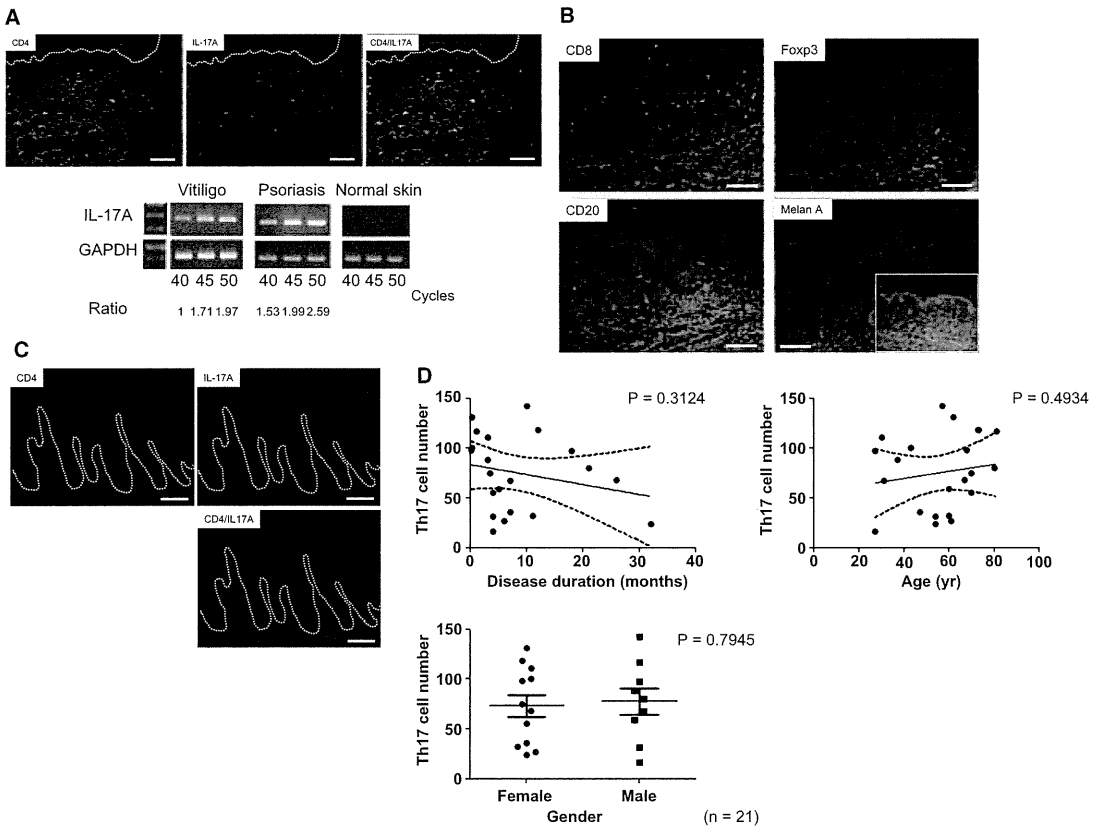

Figure 1. Photographic features of a representative generalized vitiligo patient and the immunohistochemical analysis for infiltrating cells in vitiligo skin. Multiple- and symmetrical-depigmented macules were present on the face and upper arm. A spindle-shaped skin specimen was obtained from the leading edge of an upper arm lesion. Immunostaining for CD4 and IL-17A in the vitiligo skin lesion indicated the significant infiltration of Th17 cells (yellow) positive for both CD4 (green) and IL-17A (red) mainly on reticular dermis and perivascular region. RT-PCR confirmed the same level of IL-17A expression in the vitiligo skin as in psoriasis skin (A). CD8-positive cytotoxic T lymphocytes (CTLs) (red, upper left) infiltrated the upper dermis and epidermis, whereas Foxp3 and CD20 positive cells (upper right and lower left) were only faintly detected. Melan-A-positive cells, highly differentiated melanocytes, were present in the normal region (lower right, small window), whereas they were absent in the vitiligo epidermis (lower right) (B). Psoriatic skin showed Th17 cell infiltration in the papillary dermis in addition to the epidermis (C). All images are original magnification ×40 for vitiligo and ×100 for psoriatic skin. The white bar indicates 100 μm. (D) The mean Th17 cell number present in vitiligo skin was counted on three independent fields, and the correlation with disease parameters such as disease duration, age, and gender was evaluated (n = 16).

nity to be infiltrated by Th17 cells, there was no significant correlation between the number of infiltrating Th17 cells and the clinical type, or with disease parameters such as disease duration, age, or gender in 21 patients with Th17 cell infiltration (Figure 1E).

Proinflammatory cytokines associated with Th17 cells influence in melanin activity

Because a significant number of Th17 cells were found in most of the vitiligo skin samples examined in this study, we hypothesized that there was a possible role for Th17 cell-related cytokines in melanocyte activity. Previous reports have shown that several cytokines downregulated tyrosinase activity through the activation of designated intracellular signaling pathways (Englaro et al., 1999; Kamaraju et al., 2002; Kholmanskikh et al., 2010). We therefore decided to examine the effects of IL-1β, IL-6, IL-17A, and IL-22, which are important cytokines induced by Th17 cell differentiation and maintenance, on melanocyte development and activity. MITF, a pivotal transcription factor related to melanocyte function and survival, expression and translocation was at first examined by immunocytochemistry (Figure 2A, C). Whereas there was apparent translocation of the MITF protein to the nucleus in untreated cultured melanocytes (Figure 2A), the MITF expression was decreased in the nucleus of the melanocytes treated with 10 ng/ml of IL-1β or IL-17A (Figure 2B, C), suggesting that there was a reduction in MITF-related signaling in melanocytes following cytokine treatment. In contrast, IL-22 treatment had no effect on melanocytes (data not shown), so we decided not to include IL-22 in the further experiments.

Next, we examined the expression of cytokine receptors by RT-PCR to confirm the ligand-to-receptor correspondence in melanocytes. Cultured human melanocytes were found to express IL-1R1, IL-6R, and IL-17RA without the addition of cytokines, whereas treatment with 10 ng/ml of their corresponding

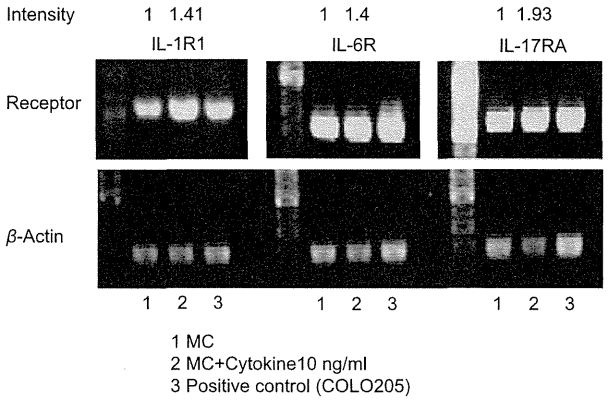

Figure 3. The expression of cytokine receptors in human melanocytes. IL-1R1, IL-6R, and IL-17RA mRNA were expressed in human melanocytes and were upregulated following treatment with their corresponding cytokines. COLO205 cells (colon cancer cell line) were used as a positive control. β-actin was used as a housekeeping gene.

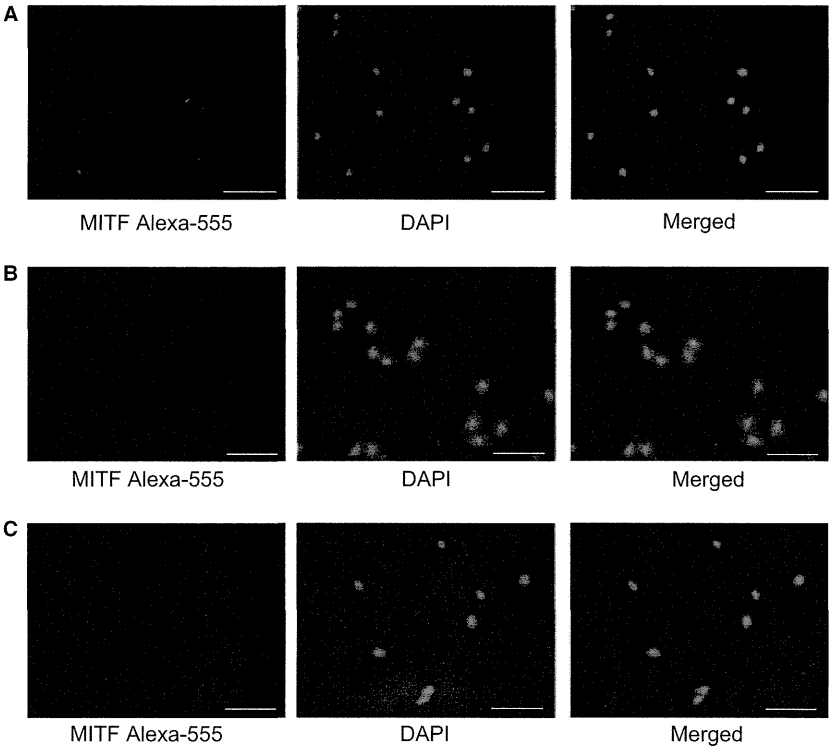

Figure 2. Immunocytochemical staining for MITF in human melanocytes. MITF was expressed mainly in the nuclei of untreated cells (A). In contrast, MITF expression was decreased after treatment with recombinant IL-1β (B) and IL-17A (C). The white bar indicates 50 μm.

cytokines increased receptor expression (Figure 3). COLO205 cells, the human colon cancer cell line expressing these receptors endogenously, were loaded in parallel with cultured melanocytes as a positive control.

To investigate the direct effects of Th17 cell-related cytokines on melanocytes in vitro, we examined the mRNA expression of melanogenic and melanocyte survival molecules after treatment of human melanocytes with recombinant human cytokines (Figure 4). The expression of *MITF*, which encodes a master transcription factor that regulates melanocyte function; of *TYR*, *TRP-1*, and *DCT*, which encode enzymes involved in melanin synthesis; and of *BCL2*, which encodes an anti-apoptotic protein, was measured by quantitative PCR. MITF expression was found to be significantly decreased in a dose-dependent manner after treatment with IL-1β, IL-6, and TNF-α (Figure 4A). The *MITF* transcription level decreased to <50% after treatment with 1 ng/ml of TNF-α. MITF was downregulated by 10 ng/ml IL-17A. In terms of the expression of its downstream enzymes, IL-1β significantly downregulated the genes, but only at a concentration of 10 ng/ml, whereas basic FGF upregulated their expression. IL-6 downregulated *TYRP1* and *DCT*, but there was no significant decrease in *TYR*. A 10 ng/ml concentration of IL-17A was needed to induce their significant downregulation. On the other hand, TNF-α significantly suppressed the expression of

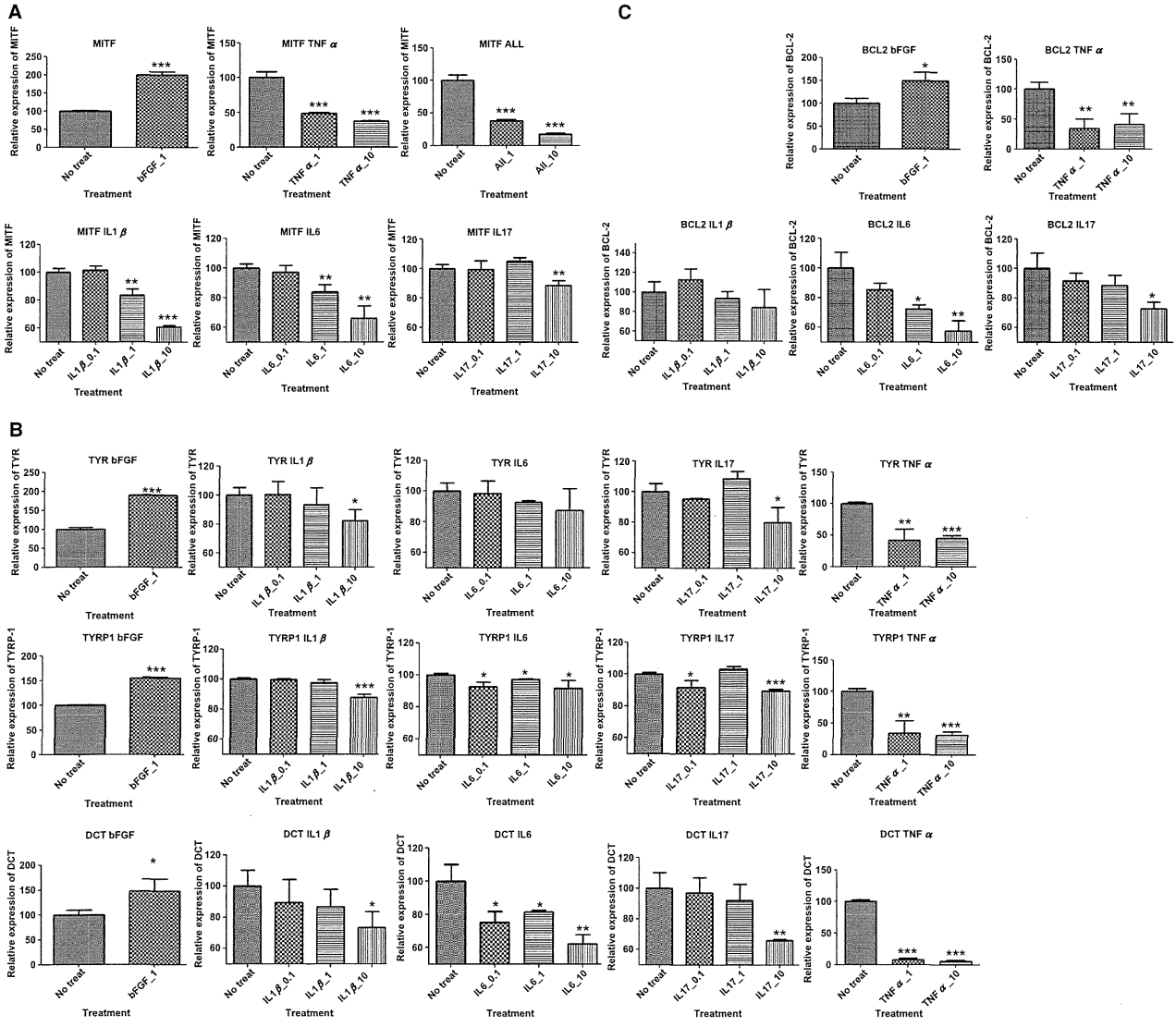

Figure 4. The quantitative analysis of the mRNA expression of MITF and genes encoding melanogenic enzymes. Human melanocytes were incubated with recombinant cytokines for 4 h at concentrations of 0.1, 1, or 10 ng/ml in the culture medium. The mRNA expression levels of MITF (A), genes encoding melanogenic enzymes (B), and B-cell lymphoma 2 (Bcl-2) (C) were measured by qRT-PCR. *P < 0.05; **P < 0.01; ***P < 0.001 compared with the expression level in untreated control cells.

these genes and *BCL2*, even at the concentration of 1 ng/ml, suggesting that TNF-α likely had the strongest suppressive effect on gene expression. BCL2 expression was decreased following treatment with IL-1β, IL-6, and IL-17A in a dose-dependent manner. Overall, there was a tendency for molecules related to melanocyte function to be downregulated following treatment with exogeneous cytokines (Figure 4B, C).

To determine the direct effects of Th17 cells on melanocytes, we performed coculture of the Th17 cells induced from peripheral blood mononuclear cells by an in vitro protocol with and without TGF-β treatment (Wilson et al., 2007) with melanocytes and real-time PCR. Th17-polarized cells without TGF-β decreased the expression of *MITF* and its downstream melanogenic molecules more than Th2-polarized cells did (Figure S1).

Furthermore, melanin production was measured after continuous treatment with exogenous cytokines including IL-1β, IL-6, IL-17A, and TNF-α. The percentage of melanin production was significantly lower in melanocytes treated with 1 and/or 10 ng/ml of exogenous cytokines than in untreated cells. In contrast, no reduction in total protein was observed after the addition of any of the cytokines (Figure 5). Because these cytokines are critical for the maintenance and development of Th17 cells from naïve $CD4^+$ T cells, we suggest that the presence of a specific local cytokine environment might be indispensable for Th17 cell recruitment and activation in vitiligo lesions, thereby indicating that they contribute significantly to depigmentation in addition to CTL (cytotoxic T cell) activation.

Production of cytokines by skin-resident cells

We have shown infiltration of Th17 cells in vitiligo skin and have demonstrated the inhibitory effects of Th17 cell-related cytokines on melanocyte function. As the cytokines examined in this study are produced not only by inflammatory cells but also by the surrounding cells, such as keratinocytes and fibroblasts, the source of the cytokine production was examined. We treated normal human epidermal keratinocyte (NHEK) and normal human dermal fibroblast (NHDF) cells with recombinant IL-17A and measured IL-1β, IL-6, and TNF-α production (Figure 6A, B). IL-17A exponentially increased the pro-

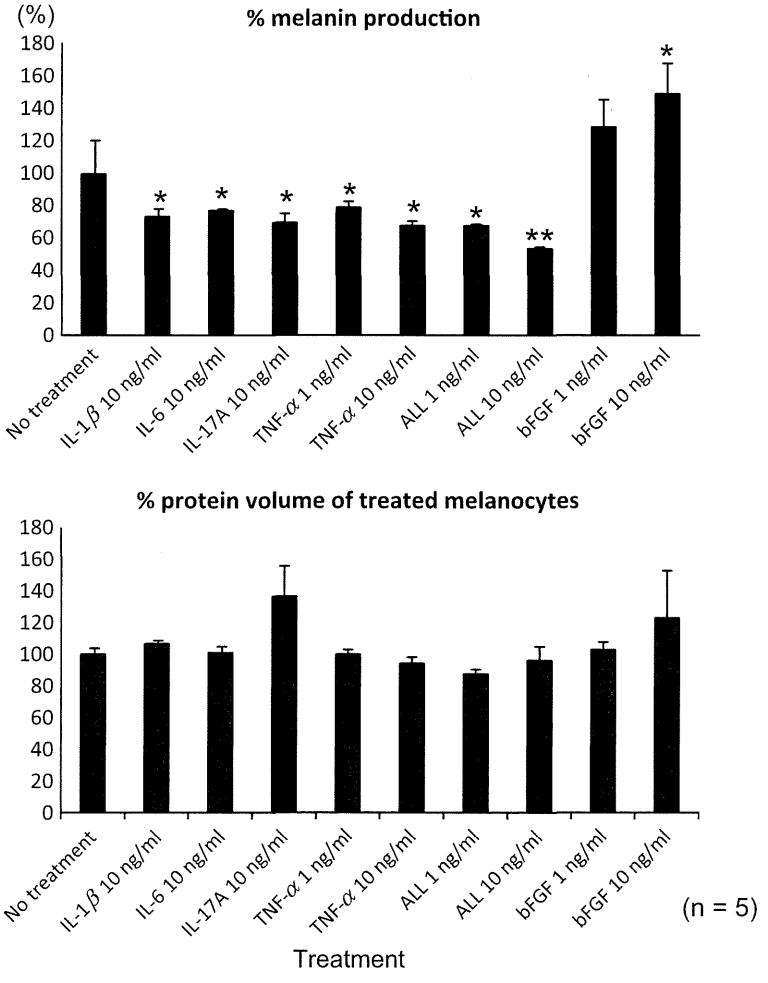

Figure 5. There is a decrease in melanin production after treatment with cytokines. Human melanocytes were incubated with 1 ng and/or 10 ng/ml of recombinant cytokines for 5 days in the culture medium (n = 5). Recombinant cytokines were added everyday. Cultured melanocytes were treated with 1 N NaOH and processed for absorbance at 450 nm to quantify the melanin volume. The protein volume of the cell extracts was measured to demonstrate whether the cytokines exerted the reduction of whole cell protein. *P < 0.05; **P < 0.01; ***P < 0.001 compared with the expression level of the untreated controls.

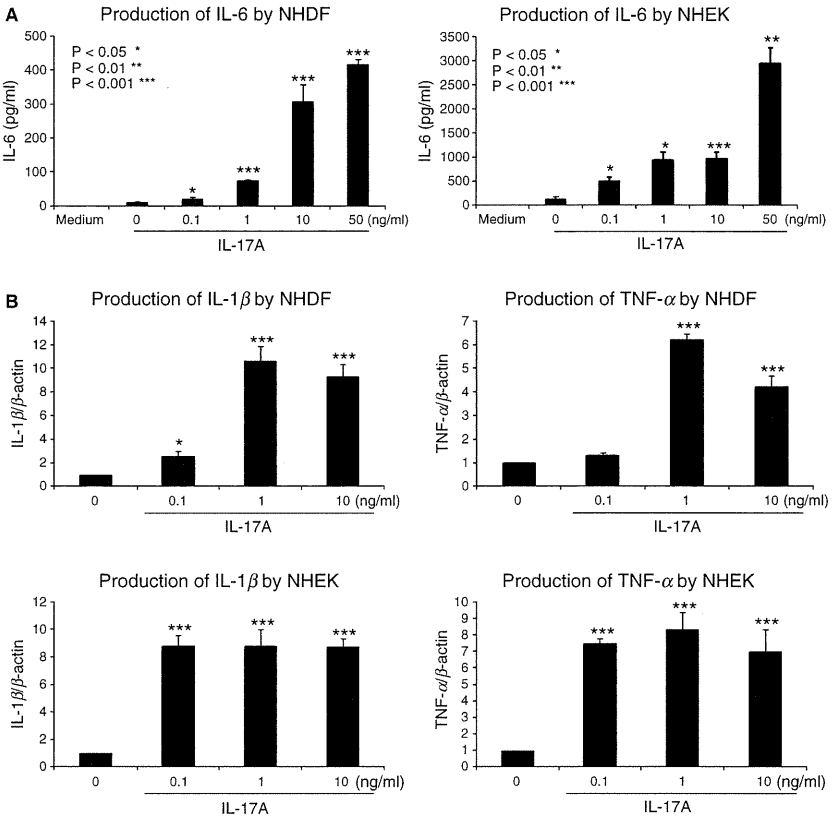

Figure 6. IL-17A induces the release of other Th17 cell-related cytokines from dermal fibroblasts and keratinocytes. (A) Human dermal fibroblasts and keratinocytes were incubated with recombinant IL-17A for 1 day at concentrations of 0.1, 1, 10, and 50 ng/ml in the culture medium, and the IL-6 secreted in the medium was measured by an ELISA. (B) After cells were incubated as in (A), the IL-1β and TNFα mRNA expression levels were measured by RT-PCR. β-actin was used as a housekeeping gene. *P < 0.05; **P < 0.01; ***P < 0.001 compared with the expression level of the untreated controls.

duction of these cytokines in a dose-dependent manner using both of these cell lines.

Cytokine-induced melanocyte dysfunction

Finally, we examined whether proinflammatory cytokines could directly induce melanocyte apoptosis and/or destruction in vitro. The cultured melanocytes were incubated with 1 and 10 ng/ml of recombinant IL-1β, IL-6, IL-17A, TNF-α, or all of the factors for 5 days, and then the melanocytes were observed microscopically under polarized light (Figure 7A). The cells were obviously aggregated and varied in shape after treatment with both the single cytokines and the cytokine cocktail, whereas the untreated cells and those treated with bFGF (basic fibroblast growth factor) grew with a spindle-shaped morphology. Staurosporine, a chemical that induces apoptosis by activating caspase-3, increased the number of round-shaped apoptotic melanocytes. TNF-α induced the greatest extent of melanocyte destruction compared with the other cytokines.

Next, melanocyte apoptosis was assessed by measuring caspase-3 activity after continuous treatment with 10 ng/ml of each of the individual cytokines and the cytokine cocktail. Staurosporine led to an increase in caspase-3 activity (Figure 7B), whereas there was no induction of caspase-3 activity following treatment with any of the cytokines. These results indicate that there appears to be direct inhibition of melanocyte activity by cytokines, rather than induction of cell apoptosis.

Discussion

In the present study, we identified a significant number of Th17 cells that had infiltrated vitiligo skin, and demonstrated the inhibitory effect of Th17 cell-related proinflammatory cytokines on melanocyte activity. We therefore hypothesize that the functional Th17 cell involvement in the initiation of psoriasis and atopic dermatitis may also play an important role in the pathogenesis of vitiligo. Although the precise pathogenic mechanisms underlying the induction of depigmentation in an immunological manner (Ongenae et al., 2003) still remain unknown, non-segmental vitiligo has been thought to be an autoimmune disease because of the high frequency of associated Hashimoto's thyroid disease (Daneshpazhooh et al., 2006; Hegedus et al., 1994; Schallreuter et al., 1994a), type I diabetes (Gould et al., 1985), collagen diseases with antinuclear antibodies (Mihailova et al., 1999), etc. Pathogenic antibodies were also detected in approximately 50% of vitiligo patients (Cui et al., 1992; Ruiz-Arguelles et al., 2007). With respect to the cellular immune condition, the infiltration of cytotoxic T cells targeting melanocyte-specific antigens in vitiligo lesions has been thought to play a critical role in hypopigmentation (Lang et al., 2001; Norris et al., 1994; Ogg et al., 1998). Recent reports have also suggested that there is the local environment of proinflammatory cytokines such as IL-1, IL-6, and TNF-α also contributes to the inhibition of melano-

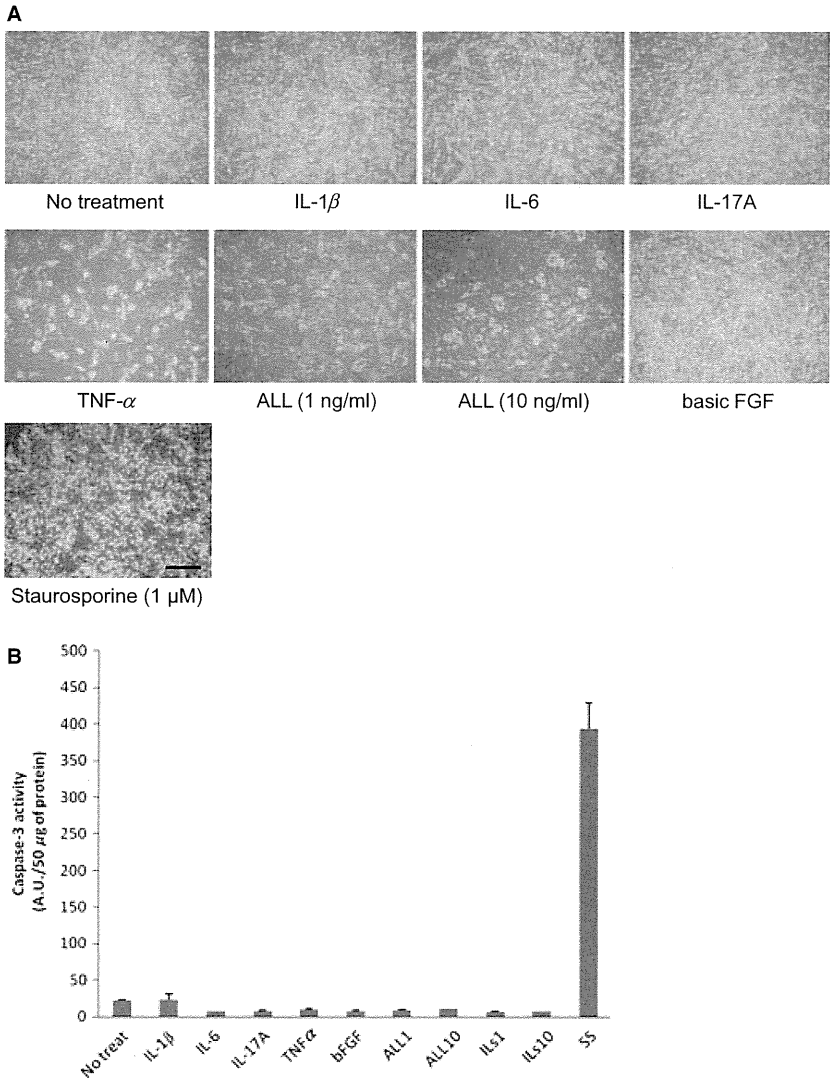

Figure 7. Proinflammatory cytokines induce melanocyte cell destruction, but not apoptosis. (A) Human melanocytes were incubated with recombinant proinflammatory cytokines, including IL-1β, IL-6, IL-17A, and TNF-α continuously for 5 days at concentrations of 1 or 10 ng/ml in the culture medium. These cytokines were used either alone or in combination. Staurosporine was used as a positive control for cell apoptosis. The photographs were taken by a polarized microscope. The bar indicates 50 μm. (B) The absorbance at 450 nm was measured to determine the caspase-3 activity of the cells treated with cytokines. ILs indicates treatment together with IL-1β, IL-6 and IL-17A.

genesis and melanocyte survival (Moretti et al., 2002, 2009).

Direct regulation of melangenic factors by cytokines

The tyrosinase mRNA levels are generally correlated with tyrosinase activity (Ando et al., 1995). In our study, cytokine treatment decreased the mRNA levels of MITF, a transcription factor implicated in regulating melanogenic and antiapoptotic genes, and decreased the expression of genes encoding melanogenic enzymes such as tyrosinase, TYRP-1, and DCT (TYRP-2) in a dose-dependent manner. These results indicate that proinflammatory cytokines can play a pivotal role in the regulation of melanocyte fate through the downregulation of gene expression.

There have been several reports providing evidence that melanocyte functions are regulated by cytokines through several cellular signaling pathways (Kamaraju et al., 2002; Kholmanskikh et al., 2010). For example, IL-1β and 1α were found to direct the downregulation of MITF-M expression through the NF-κB and JNK pathways in two different melanoma cell lines (Kholmanskikh et al., 2010). IL-6/IL-6R signaling silenced the MITF promoter activity and this was mediated by Pax3 downregulation (Kamaraju et al., 2002). IL-6 is a pleiotropic cytokine involved in a variety of inflammatory responses. With regard to the relationship to Th17 cells, IL-6 is essential for induction of Th17 cell development and maintenance (Diveu et al., 2008). As the proinflammatory cytokines involved in Th17 cell fate include IL-1β and TGF-β in addition to IL-6, we examined the expression and activity of some of these cytokines in melanocytes.

Although there is no doubt that cellular and antibody-mediated immune reactions are related to melanocyte destruction, our data suggest that Th17 cells and skin-resident cells, particularly epidermal keratinocytes and dermal fibroblasts, might orchestrate a response that inhibits the stability of melanocytes in some vitiligo skin

through the production of proinflammatory cytokines. In addition, there might be an initial trigger attracting Th17 cells to vitiligo (or pre-vitiligo) skin.

A recent study using several skin samples showed greater numbers of Th17 cells, especially on the leading edge of vitiligo skin (Wang et al., 2011). In the present study, we confirm the presence of Th17 cell infiltration in vitiligo skin and suggest that there was a pathogenic function not only because of cytotoxic T cells but also because of Th17 cells and Th17 cell-related cytokines. Although we expected that there would be more infiltration of Th17 cells in the generalized type and progressive vitiligo compared with other clinical types, there was no significant correlation between the Th17 cell number and the clinical type and disease duration. It is possible that the small sample number, biopsy site, and preceding treatments, including the use of topical steroids, may have affected the status of inflammatory cell infiltration.

We observed that Th17 cells diffusely infiltrated the upper dermis, whereas $CD8^+$ cells were present beneath the basal membrane of the epidermis. In psoriatic skin, Th17 cells mainly infiltrate into the papillary dermis and epidermis. We therefore speculated that Th17 cells might be able to act on melanocytes by producing cytokines, rather than exerting a direct effect on the cells. To address this point, we stimulated dermal fibroblasts and keratinocytes using a characteristic pro-Th17 cytokine, IL-17A. IL-17A robustly upregulated the production of IL-1β and TNF-α by these skin-resident cells, suggesting the presence of mutual cytokine signaling between skin-resident cells and accumulating inflammatory cells. The melanocytes themselves can also synthesize IL-1α and β (Swope et al., 1994).

Previous studies have shown that cytokines associated with skin inflammation, such as IL-1β, IL-6, and TNF-α, inhibited melanin production in vitro (Englaro et al., 1999; Kamaraju et al., 2002; Kholmanskikh et al., 2010). We found that there were significant changes in the expression of epidermal cytokines in vitiligo lesions, where no melanocytes are present, compared with perilesional, non-lesional and healthy skin, where melanocytes are normally present. Therefore, it is conceivable that the cytokines derived from infiltrating cells, as well as the lesional epidermis, would be implicated in depigmented skin disorders. In the present study, treatment with a physiologically relevant concentration of IL-17A, in addition to IL-1β and IL-6, could directly regulate the expression of MITF and downstream molecules, and subsequently melanin synthesis, in human melanocytes.

Putative involvement of proinflammatory cytokines in vitiligo

Based on these experimental results, we propose the putative involvement of proinflammatory cytokines in the pathogenesis of vitiligo (Figure 8). Previous studies have shown that the perforin produced from CD8-positive cytotoxic T cells (Lang et al., 2001; Norris et al., 1994; Ogg et al., 1998), antimelanocyte antibodies (Baharav et al., 1996; Cui et al., 1992; Ruiz-Arguelles et al., 2007), and reactive oxygen species were related to the injury of melanocytes and were triggers for vitiligo (Schallreuter et al., 1994b). In the present study, we found a significant infiltration of Th17 cells in vitiligo skin, and demonstrated that Th17-related cytokines such as IL-1β, IL-6, and IL-17A directly or indirectly regulated melanin production and the expression of melanogenic and antiapoptotic molecules. The presence of a cytokine network and the secretion of IL-17A from Th17 cells may therefore represent a new mechanism underlying the pathogenesis of vitiligo concerning the downregulation of melanocyte activity. Indeed, the activation of the innate immune system may lead to the accumulation of Th17 cells in the vitiligo lesion as they do in psoriasis. In fact, the IL-1β released from lesional keratinocytes and melanocytes (Moretti et al., 2002, 2009; Swope et al., 1994) may act as the first inducer of the differentiation of naïve helper T cells into Th17 cells in vitiligo lesions. Thereafter, antimicrobial peptides derived from the lesional epidermis might induce the production of IL-17A by Th17 cells (Infante-Duarte et al., 2000).

Although psoriasis is one of representative skin disorders characterized by pathogenic Th17 cell infiltration, the phenotypic change in this disorder is not akin to that in vitiligo vulgaris. Because the final targets of IL-17A in

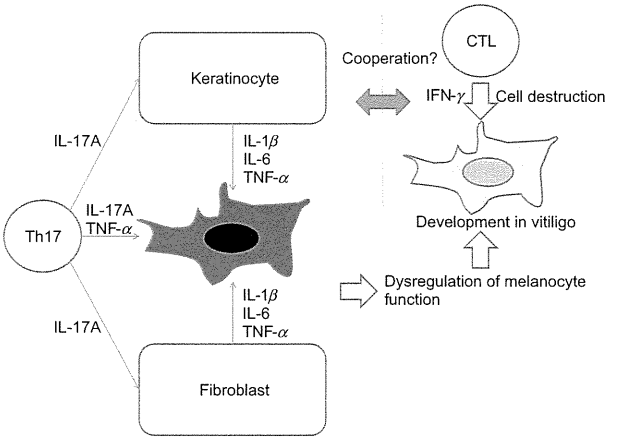

Figure 8. The proposed cues provided by Th17 cells and Th17 cell-related cytokines during the pathogenesis of autoimmune vitiligo. Previous known pathogenic mechanisms of melanocyte destruction in vitiligo include the presence of cytotoxic T cells attacking the melanocytes, local oxidative stress, and downregulation of melanogenesis-inducing factors in the vitiligo epidermis. The newly proposed phenomenon is that an imbalance in the local cytokine network is involved in the downregulation of melanocyte activity found in the present study. The IL-17A secreted from the Th17 cells in vitiligo skin can trigger the production of inhibitory cytokines from dermal fibroblasts and keratinocytes. IL-1β, IL-6, and TNF-α as well as IL17-A also repress melanocyte activity and induce melanocyte destruction.

vitiligo vulgaris and psoriasis are different, that is, melanocyte dysfunction in vitiligo vulgaris and abnormal keratinocyte turnover in psoriasis, Th17 cells may augment vitiliginous skin lesion formation in cooperation with skin-resident cells such as dermal fibroblasts and keratinocytes, as described previously. Microbial lipopeptides may then induce the cell polarization to Th17 cells, producing IL-17 and TNF-α as a result of the stimulation of the innate immune system in vitiligo (Infante-Duarte et al., 2000). Tip dendritic cell (tipCD)-like cells might also be involved in vitiligo formation, as previous reports demonstrated that the number of αDCs was increased in vitiligo vulgaris lesions or there was a unique distribution pattern of Langerhans cells present in such lesions (Mishima et al., 1972). Moreover, recent reports suggest that an altered innate immune response is observed in autoimmune vitiligo in concert with frequent *NALP1* gene mutations (Jin et al., 2007, 2010). An unrecognized micro-organism might stimulate the attending inflammatory cells through antimicrobial peptides and sequentially trigger vitiligo vulgaris. These issues should be clarified in further experiments.

Methods

Cell lines

HeMnMP, a moderately pigmented human melanocyte cell line, was obtained from Cascade Biologics and cultured in Medium 254 with human melanocyte growth supplement (Gibco Inc., Tokyo, Japan), and maintained at 37°C with 5% CO_2 in a humidified incubator. The cells were used for this study by the 5th passage to ensure melanin production. Dermal fibroblasts and epidermal keratinocytes were purchased from TAKARA BIO Inc., (Shiga, Japan) and maintained in DMEM containing 10% FCS and Medium 154 (Gibco Inc.), respectively.

Reagents

Human recombinant cytokines were purchased from Cell Signaling Technology (Tokyo, Japan) and synthetic melanin was from Sigma-Aldrich, Japan. The antibodies used for this study were as follows: anti-MITF mouse monoclonal Ab (D5) from Abcam (San Francisco, CA, USA), horseradish peroxidase (HRP)-conjugated anti-rabbit or mouse IgG from Cell Signaling Technology, anti-CD4 mouse monoclonal antibody from Novocastra Reagents (Tokyo, Japan), anti-CD8, -Foxp3, -CD20 and -Melan A mouse monoclonal antibodies from Dako (Tokyo, Japan), anti-IL17A goat monoclonal antibody from R&D (Minneapolis, MN, USA), Alexa Fluor 488-conjugated anti-mouse IgG and Alexa Fluor 555-conjugated anti-goat IgG from Invitrogen (Tokyo, Japan).

Tissue specimens

Approval for the use of human skin tissue samples was obtained from the local Ethical Committee of Osaka University Hospital and written informed consent was received from each patient after appropriate explanation of this study. Spindle-shaped skin biopsy specimens on the leading edge of vitiligo lesions were taken from 23 vitiligo patients. Twenty-three skin specimens were fixed in buffered 10% formalin and embedded in paraffin and processed for an immunohistochemical analysis as described below. Non-lesional skin from the matched vitiligo patients and normal skin from normal donors were processed as well.

RNA isolation and PCR assay for cytokine and melanocyte markers expression

Total RNA was extracted from HeMnMP cells using the TRIZOL reagent according to the manufacturer's instructions. Reverse transcription (RT) reactions were performed with Moloney murine leukemia virus reverse transcriptase (Promega, Tokyo, Japan) with oligo (dT) primers. For RT reaction of tissue RNA, total RNA was extracted from frozen vilitigo skin tissue using the Sepasol-RNA I reagent (NACALAI TESQUE, INC., Kyoto, Japan) according to the manufacturer's instructions. Genomic DNA contamination was removed by DNase I (TAKARA BIO INC.). The qRT assay was performed using an ABI prism 7900HT Sequence Detection System (Applied Biosystems, Carlsbad, CA, USA) according to the manufacturer's specifications. Briefly, the reaction mixture totaling 10 μl for each qRT consisted of 1 μl of cDNA generated from 250 ng of total RNA, 0.5 μM of Taqman probe labeled with FAM, the master mix for melanogenic markers and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), or the Power SYBR green PCR master mix for cytokines. The mixture was processed by a two-step PCR method with an initial heating at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s, and annealing and extension at 60°C for 60 s for all genes. The obtained PCR amplification curves were analyzed using SDS software program, version 2.1 (Applied Biosystems). GAPDH was used as a control housekeeping gene, and the relative mRNA copy numbers were obtained as the ratio of the mRNA copies of each gene/copies of GAPDH. Each assay was performed at least three times. The specific probe and primers sequences used for this study were as follows:

MITF-M: 5′-AGCTCACAGCGTGTATTTTTCCCAC-3′
TYR: 5′-TCTCCTCTTGGCAGATTGTCTGTAG-3′
TRP-1: 5′-CTTTGTAACAGCACCGAGGATGGGC-3′
DCT: 5′-TGCAAGTGCACAGGAAACTTTGCCG-3′
BCL2: 5′-AACGGAGGCTGGGATGCCTTTGTGG-3′
GAPDH: 5′-GGGCGCCTGGTCACCAGGGCTGCTT-3′
IL-1β: Forward 5′- TGCACGCTCCGGGACTCACA-3′
IL-1 β: Reverse 5′- CGCCTTTGGTCCCTCCCAGG-3′
TNF-α: Forward 5′- CCCCTGACAAGCTGCCAGGC-3′
TNF-α: Reverse 5′- CAGCTCCACGCCATTGGCCA-3′

Reverse transcriptase PCR (RT-PCR) for IL-17A and cytokine receptor expression

To confirm IL-17A expression in vitiligo tissue and determine the expression levels of the cytokines and their receptors, we performed RT reactions with the above-mentioned procedure and PCR with an initial heating at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s, and annealing and extension at appropriate temperatures for 60 s for all genes. Samples were then processed for electrophoresis. The following primer sets were used:

IL-17A: Forward 5′- ACAAACTCATCCATCCCCAG-3′
IL-17A: Reverse 5′- GTGAGGTGGATCGGTTGTAG-3′
IL-1R1: Forward 5′-CCCGTTGCAGGAGACGGAGG-3′
IL-1R1: Reverse 5′-CCACCCAGCCAGCTGAAGCC-3′
IL-1R2: Forward 5′-CTTTAAAGCTGCTTCTGCCACGTG-3′
IL-1R2: Reverse 5′-CATTGCCCGTCCACCACAGCA-3′
IL-6R: Forward 5′-GAGTTCGGGCAAGGCGAGTGG-3′
IL-6R: Reverse 5′-AGGCTCCCTCCAGCAACCAGGAA-3′
IL-17RA: Forward 5′-AAGCCTCAGAACGTTCGTTCGCT-3′
IL-17RA: Reverse 5′-TTGGGCAGGTGGTGAACGGT-3′

Melanin content assay

Melanin production was determined as described previously (Virador et al., 1999). Briefly, 2 days after the plating of 1×10^5 melanocytes into a 6-well culture dish, we performed 5 days of

sequential treatment with 1–10 ng/ml of recombinant cytokines. To determine the melanin content, the pellets of treated cells were dissolved in 200 μl of 1 N NaOH for 30 min, and the concentrations of melanin were calculated by measuring the absorbance at 450 nm. Synthetic melanin was used to generate a standard curve. The melanin content was expressed as nanograms of melanin per microgram of total protein, and the ratio was compared among the samples.

Immunostaining

Vitiliginous skin specimens were processed after receiving written informed consent from vitiligo patients (n = 23). Paraffin-embedded archival tissues were deparaffinized with absolute xylene and dehydrated in a sequential ethanol dilution series. The deparaffinized sections were boiled in an oil bath for 15 min in 10 mM Tris-1 mM EDTA buffer (pH 9.0) for antigen retrieval. The slides were blocked by the Protein Block Serum-Free solution (Dako) for 15 min and incubated with an anti-IL-17A goat monoclonal Ab (×200) at 4°C overnight. After being washed with TBS (pH 7.6), the slides were incubated with Alexa Fluor 555-conjugated anti-goat IgG Ab (×200) and then incubated with anti-CD4 mouse monoclonal Ab (×25) at RT for 1 h, followed by incubation with Alexa Fluor 488-conjugated anti-mouse IgG Ab (×200). The following primary antibodies were used to assess the expression of melanocyte markers and the melanosomal protein MART1: anti-CD8 mouse monoclonal Ab (×25), anti-CD20 mouse monoclonal Ab (×25), anti-Foxp3 mouse monoclonal Ab (×50), and anti-Melan A (recognizing the MART-1 antigen) mouse monoclonal Ab (×50). These antibodies were provided by DAKO Inc.

For the immunocytochemistry analyses, the HeMnMP cells cultured in two-well Lab-Tek chamber slides (Nunc, Tokyo, Japan) were incubated with an anti-MITF mouse monoclonal antibody (×25) at 4°C overnight, followed by incubation with Alexa Fluor 555-conjugated anti-mouse IgG (×500) as the secondary antibody. The mouse isotype IgG was used as a negative control for staining. Nuclei were counterstained after DAPI staining (×1000).

Quantitative analysis of proinflammatory cytokines after the treatment with Th17-related cytokines

To assess the cell–cell interactions between the cells in the skin occurring as a result of paracrine cytokine production, the concentrations of proinflammatory cytokines such as IL-6 and IL-1β in the culture supernatant from dermal fibroblasts was measured 24 h after treatment with recombinant IL-17A using an ELISA kit from R&D.

Apoptosis assay

We determined the cleaved caspase-3 activity following treatment with recombinant cytokines using an apoptosis detection kit (R&D Systems). Briefly, cultured melanocytes were treated with cytokines (1 or 10 ng/ml) for 5 days. The culture medium was not changed until cell extraction, and cytokines were added in the culture medium every day. Thereafter, in addition to observation of melanocyte morphology under a polarized microscope, the melanocytes were processed for measurement of cleaved caspase-3 activity according to manufacturer's instructions.

Statistical analysis

The unpaired *t*-test was used for the analysis of differences in gene and protein expression. The results are shown as the means + SD. A value of $P < 0.05$ (two-tailed) was considered significant. All statistical analyses were performed using the PRISM software program, version 5 (GraphPad Software Inc., La Jolla, CA, USA).

Acknowledgements

We thank Kenju Nishida, Eriko Nobuyoshi, and Tomoka Matsumura for their expert technical assistance. This work was supported in part by a grant from the Ministry of Education, Culture, Sports, Science and Technology of Japan and a grant from the Ministry of Health, Labor and Welfare.

References

Ando, H., Itoh, A., Mishima, Y., and Ichihashi, M. (1995). Correlation between the number of melanosomes, tyrosinase mRNA levels, and tyrosinase activity in cultured murine melanoma cells in response to various melanogenesis regulatory agents. J. Cell. Physiol. *163*, 608–614.

Asarch, A., Barak, O., Loo, D.S., and Gottlieb, A.B. (2008). Th17 cells: a new therapeutic target in inflammatory dermatoses. J. Dermatolog. Treat. *19*, 318–326.

Baharav, E., Merimski, O., Shoenfeld, Y., Zigelman, R., Gilbrud, B., Yecheskel, G., Youinou, P., and Fishman, P. (1996). Tyrosinase as an autoantigen in patients with vitiligo. Clin. Exp. Immunol. *105*, 84–88.

Basak, P.Y., Adiloglu, A.K., Ceyhan, A.M., Tas, T., and Akkaya, V.B. (2009). The role of helper and regulatory T cells in the pathogenesis of vitiligo. J. Am. Acad. Dermatol. *60*, 256–260.

Bassiouny, D.A., and Shaker, O. (2011). Role of interleukin-17 in the pathogenesis of vitiligo. Clin. Exp. Dermatol. *36*, 292–297.

Caixia, T., Hongwen, F., and Xiran, L. (1999). Levels of soluble interleukin-2 receptor in the sera and skin tissue fluids of patients with vitiligo. J. Dermatol. Sci. *21*, 59–62.

Chalraborty, A., and Pawelek, J. (1993). MSH receptors in immortalized human epidermal keratinocytes: a potential mechanism for coordinate regulation of the epidermal-melanin unit. J. Cell. Physiol. *157*, 344–350.

Cui, J., Harning, R., Henn, M., and Bystryn, J.C. (1992). Identification of pigment cell antigens defined by vitiligo antibodies. J. Invest. Dermatol. *98*, 162–165.

Daneshpazhooh, M., Mostofizadeh, G.M., Behjati, J., Akhyani, M., and Robati, R.M. (2006). Anti-thyroid peroxidase antibody and vitiligo: a controlled study. BMC Dermatol. *6*, 3.

Diveu, C., Mcgeachy, M.J., and Cua, D.J. (2008). Cytokines that regulate autoimmunity. Curr. Opin. Immunol. *20*, 663–668.

Englaro, W., Bahadoran, P., Bertolotto, C., Busca, R., Derijard, B., Livolsi, A., Peyron, J.F., Ortonne, J.P., and Ballotti, R. (1999). Tumor necrosis factor alpha-mediated inhibition of melanogenesis is dependent on nuclear factor kappa B activation. Oncogene *18*, 1553–1559.

Fitch, E.L., Rizzo, H.L., Kurtz, S.E., Wegmann, K.W., Gao, W., Benson, J.M., Hinrichs, D.J., and Blauvelt, A. (2009). Inflammatory skin disease in K5.hTGF-beta1 transgenic mice is not dependent on the IL-23/Th17 inflammatory pathway. J. Invest. Dermatol. *129*, 2443–2450.

Gould, I.M., Gray, R.S., Urbaniak, S.J., Elton, R.A., and Duncan, L.J. (1985). Vitiligo in diabetes mellitus. Br. J. Dermatol. *113*, 153–155.

Hegedus, L., Heidenheim, M., Gervil, M., Hjalgrim, H., and Hoier-Madsen, M. (1994). High frequency of thyroid dysfunction in patients with vitiligo. Acta Derm. Venereol. *74*, 120–123.

Howitz, J., Brodthagen, H., Schwartz, M., and Thomsen, K. (1977). Prevalence of vitiligo. Epidemiological survey on the Isle of Bornholm, Denmark. Arch. Dermatol. *113*, 47–52.

Infante-Duarte, C., Horton, H.F., Byrne, M.C., and Kamradt, T. (2000). Microbial lipopeptides induce the production of IL-17 in Th cells. J. Immunol. *165*, 6107–6115.

Jin, Y., Mailloux, C.M., Gowan, K., Riccardi, S.L., Laberge, G., Bennett, D.C., Fain, P.R., and Spritz, R.A. (2007). NALP1 in vitiligo-associated multiple autoimmune disease. N. Engl. J. Med. *356*, 1216–1225.

Jin, Y., Riccardi, S.L., Gowan, K., Fain, P.R., and Spritz, R.A. (2010). Fine-mapping of vitiligo susceptibility loci on chromosomes 7 and 9 and interactions with NLRP1 (NALP1). J. Invest. Dermatol. *130*, 774–783.

Kamaraju, A.K., Bertolotto, C., Chebath, J., and Revel, M. (2002). Pax3 down-regulation and shut-off of melanogenesis in melanoma B16/F10.9 by interleukin-6 receptor signaling. J. Biol. Chem. *277*, 15132–15141.

Kholmanskikh, O., Van Baren, N., Brasseur, F., Ottaviani, S., Vanacker, J., Arts, N., Van Der Bruggen, P., Coulie, P., and De Plaen, E. (2010). Interleukins 1alpha and 1beta secreted by some melanoma cell lines strongly reduce expression of MITF-M and melanocyte differentiation antigens. Int. J. Cancer *127*, 1625–1636.

Kingo, K., Aunin, E., Karelson, M., Ratsep, R., Silm, H., Vasar, E., and Koks, S. (2008). Expressional changes in the intracellular melanogenesis pathways and their possible role in the pathogenesis of vitiligo. J. Dermatol. Sci. *52*, 39–46.

Kolls, J.K., and Linden, A. (2004). Interleukin-17 family members and inflammation. Immunity *21*, 467–476.

Lang, K.S., Caroli, C.C., Muhm, A., Wernet, D., Moris, A., Schittek, B., Knauss-Scherwitz, E., Stevanovic, S., Rammensee, H.G., and Garbe, C. (2001). HLA-A2 restricted, melanocyte-specific CD8(+) T lymphocytes detected in vitiligo patients are related to disease activity and are predominantly directed against MelanA/MART1. J. Invest. Dermatol. *116*, 891–897.

Levy, C., Khaled, M., and Fisher, D.E. (2006). MITF: master regulator of melanocyte development and melanoma oncogene. Trends Mol. Med. *12*, 406–414.

Liang, S.C., Tan, X.Y., Luxenberg, D.P., Karim, R., Dunussi-Joannopoulos, K., Collins, M., and Fouser, L.A. (2006). Interleukin (IL)-22 and IL-17 are coexpressed by Th17 cells and cooperatively enhance expression of antimicrobial peptides. J. Exp. Med. *203*, 2271–2279.

Mihailova, D., Grigorova, R., Vassileva, B., Mladenova, G., Ivanova, N., Stephanov, S., Lissitchky, K., and Dimova, E. (1999). Autoimmune thyroid disorders in juvenile chronic arthritis and systemic lupus erythematosus. Adv. Exp. Med. Biol. *455*, 55–60.

Mishima, Y., Kawasaki, H., and Pinkus, H. (1972). Dendritic cell dynamics in progressive depigmentations. Distinctive cytokinetics of -dendritic cells revealed by electron microscopy. Arch. Dermatol. Forsch. *243*, 67–87.

Moretti, S., Spallanzani, A., Amato, L., Hautmann, G., Gallerani, I., Fabiani, M., and Fabbri, P. (2002). New insights into the pathogenesis of vitiligo: imbalance of epidermal cytokines at sites of lesions. Pigment Cell Res. *15*, 87–92.

Moretti, S., Fabbri, P., Baroni, G., Berti, S., Bani, D., Berti, E., Nassini, R., Lotti, T., and Massi, D. (2009). Keratinocyte dysfunction in vitiligo epidermis: cytokine microenvironment and correlation to keratinocyte apoptosis. Histol. Histopathol. *24*, 849–857.

Norris, D.A., Horikawa, T., and Morelli, J.G. (1994). Melanocyte destruction and repopulation in vitiligo. Pigment Cell Res. *7*, 193–203.

Ogg, G.S., Rod Dunbar, P., Romero, P., Chen, J.L., and Cerundolo, V. (1998). High frequency of skin-homing melanocyte-specific cytotoxic T lymphocytes in autoimmune vitiligo. J. Exp. Med. *188*, 1203–1208.

Okamoto, T., Irie, R.F., Fujii, S., Huang, S.K., Nizze, A.J., Morton, D.L., and Hoon, D.S. (1998). Anti-tyrosinase-related protein-2 immune response in vitiligo patients and melanoma patients receiving active-specific immunotherapy. J. Invest. Dermatol. *111*, 1034–1039.

Ongenae, K., Van Geel, N., and Naeyaert, J.M. (2003). Evidence for an autoimmune pathogenesis of vitiligo. Pigment Cell Res. *16*, 90–100.

Ratsep, R., Kingo, K., Karelson, M., Reimann, E., Raud, K., Silm, H., Vasar, E., and Koks, S. (2008). Gene expression study of IL10 family genes in vitiligo skin biopsies, peripheral blood mononuclear cells and sera. Br. J. Dermatol. *159*, 1275–1281.

Ruiz-Arguelles, A., Brito, G.J., Reyes-Izquierdo, P., Perez-Romano, B., and Sanchez-Sosa, S. (2007). Apoptosis of melanocytes in vitiligo results from antibody penetration. J. Autoimmun. *29*, 281–286.

Schallreuter, K.U., Lemke, R., Brandt, O., Schwartz, R., Westhofen, M., Montz, R., and Berger, J. (1994a). Vitiligo and other diseases: coexistence or true association? Hamburg study on 321 patients. Dermatology *188*, 269–275.

Schallreuter, K.U., Wood, J.M., Pittelkow, M.R., Gutlich, M., Lemke, K.R., Rodl, W., Swanson, N.N., Hitzemann, K., and Ziegler, I. (1994b). Regulation of melanin biosynthesis in the human epidermis by tetrahydrobiopterin. Science *263*, 1444–1446.

Swope, V.B., Sauder, D.N., Mckenzie, R.C., Sramkoski, R.M., Krug, K.A., Babcock, G.F., Nordlund, J.J., and Abdel-Malek, Z.A. (1994). Synthesis of interleukin-1 alpha and beta by normal human melanocytes. J. Invest. Dermatol. *102*, 749–753.

Virador, V.M., Kobayashi, N., Matsunaga, J., and Hearing, V.J. (1999). A standardized protocol for assessing regulators of pigmentation. Anal. Biochem. *270*, 207–219.

Wang, C.Q., Cruz-Inigo, A.E., Fuentes-Duculan, J., Moussai, D., Gulati, N., Sullivan-Whalen, M., Gilleaudeau, P., Cohen, J.A., and Krueger, J.G. (2011). Th17 cells and activated dendritic cells are increased in vitiligo lesions. PLoS ONE *6*, e18907.

Wilson, N.J., Boniface, K., Chan, J.R. et al. (2007). Development, cytokine profile and function of human interleukin 17-producing helper T cells. Nat. Immunol. *8*, 950–957.

Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Coculture of helper T cells and melanocytes, and measurement of the expression of melanogenic markers.

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.

	E X D	1 4 5 4	B	Dispatch: 3.2.12	Journal: EXD	CE: Deepika K
	Journal Name	Manuscript No.		Author Received:	No. of pages: 6	PE: Sathya Kala

DOI:10.1111/j.1600-0625.2012.01454.x
www.blackwellpublishing.com/EXD

Original Article

Periostin, a matricellular protein, accelerates cutaneous wound repair by activating dermal fibroblasts

Kanako Ontsuka[1*], Yorihisa Kotobuki[2,3*], Hiroshi Shiraishi[1], Satoshi Serada[3], Shoichiro Ohta[4], Atsushi Tanemura[2], Lingli Yang[2,3], Minoru Fujimoto[3], Kazuhiko Arima[1], Shoichi Suzuki[1], Hiroyuki Murota[2], Shuji Toda[5], Akira Kudo[6], Simon J. Conway[7], Yutaka Narisawa[8], Ichiro Katayama[2], Kenji Izuhara[1] and Tetsuji Naka[3]

[1]Division of Medical Biochemistry, Department of Biomolecular Sciences, Saga Medical School, Saga, Japan; [2]Department of Dermatology, Osaka University Graduate School of Medicine, Suita, Japan; [3]Laboratory for Immune Signal, National Institute of Biochemical Innovation, Ibaraki, Japan; [4]Department of Laboratory Medicine, Saga Medical School, Saga, Japan; [5]Department of Pathology and Biodefense, Saga Medical School, Saga, Japan; [6]Department of Biological Information, Tokyo Institute of Technology, Yokohama, Japan; [7]Program in Developmental Biology and Neonatal Medicine, Herman B. Wells Center for Pediatric Research, Indiana University School of Medicine, Indianapolis, IN, USA; [8]Department of Dermatology, Saga Medical School, Saga, Japan

Correspondence: Kenji Izuhara, Division of Medical Biochemistry, Department of Biomolecular Sciences, Saga Medical School, 5-1-1, Nabeshima, Saga 849-8501, Japan, Tel.: +81-952-34-2261, Fax: +81-952-34-2058, e-mail: kizuhara@cc.saga-u.ac.jp

**These two authors contributed equally to this work.*

Abstract: Cutaneous wound repair is a highly ordered and well-coordinated process involving various cell lineages and many molecular effectors. Cell–matrix interactions through integrin molecules provide key signals important for wound repair. Periostin is a matricellular protein that may provide signals important during tissue development and remodelling by interacting with several integrin molecules, via the phosphatidylinositol 3-kinase/Akt and MAP kinase pathways. In this study, we examined the role of periostin in the process of cutaneous wound repair using periostin-deficient mice and by analysing the effects of periostin on dermal fibroblasts. We first determined the expression profile and localization of periostin in a well-characterized wound repair model mice. Periostin was robustly deposited in the granulation tissues beneath the extended epidermal wound edges and at the dermal–epidermal junctions in wounded mice. Moreover, periostin-deficient mice exhibited delayed *in vivo* wound repair, which could be improved by direct administration of exogenous periostin. *In vitro* analyses revealed that loss of periostin impaired proliferation and migration of dermal fibroblasts, but exogenous supplementation or enforced periostin expression enhanced their proliferation. Combined, these results demonstrate that periostin accelerates the process of cutaneous wound repair by activating fibroblasts.

Key words: fibroblast – matrix – mice – periostinintegrin – wound repair

Accepted for publication 18 January 2012

Introduction

Cutaneous wound repair is a physiological function that is well ordered and highly coordinated (1–4). The process of repair is divided into three phases: inflammation, new tissue formation and remodelling. In the inflammatory phase beginning with haemostasis, first neutrophils and later macrophages are recruited to the wound site. These infiltrated macrophages not only exert their phagocytic activities but also accelerate re-epithelialization and granulation tissue formation. During new tissue formation, the process of re-epithelialization occurs via extension of wedge-shaped keratinocyte lineage. Fibroblasts and macrophages subsequently form the granulation tissues, assisting in the key process of re-epithelialization. Finally, during the remodelling phase, most of the endothelial cells, macrophages and myofibroblasts undergo apoptosis, leaving a scar containing a few cells with an extensive extracellular matrix (ECM) deposition dominated by collagens.

In the new tissue forming phase, fibroblasts produce mainly collagens and other ECM components such as glycosaminoglycans and proteoglycans, contributing to the formation of granulation tissues, with the provisional fibrin-based matrix eventually being replaced (1,5–7). In addition, fibroblasts secrete various growth factors – fibroblast growth factor-2 (FGF-2)/basic FGF, FGF-7/keratinocyte growth factor, FGF-10, epidermal growth factor and transforming growth factor-β (TGF-β) – which can all affect the process of keratinocyte re-epithelialization (8). TGF-β1 can also drive differentiation of some fibroblasts into transformed myofibroblasts that express α-smooth muscle actin (α-SMA) (9), which are able to contract to draw the wound edges together (1–4). This combination of growth factor receptor–mediated signals and integrin-mediated signals are thought to result in growth, migration, survival, spreading and ECM production responses within the wound fibroblasts (10–12). However, the underlying mechanism of fibroblast activation during cutaneous wound repair has not yet been fully understood.

Periostin is an ECM protein belonging to the fasciclin family (13,14) and is a newly characterized matricellular protein whose main functions are thought to be modulation of cell–matrix interactions and cell functions rather than playing a direct structural role (15,16). Periostin is known to interact with several integrin molecules, specifically $\alpha_v\beta_3$ or $\alpha_v\beta_5$ on cell surfaces, activating the phosphatidylinositol 3-kinase (PI3K)/Akt and MAP kinase pathways during tissue development and remodelling (13,14). We and others have demonstrated the presence of periostin in fibrotic areas in various pathological conditions: bronchial

