Abstract. – OBJECTIVE: The aim of this study was to investigate the molecular mechanism into the keratinocyte migration, which is promoted by Transforming growth factor-β1 (TGF-β1) during wound healing.

MATERIALS AND METHODS: In the present study, we investigated the regulation by TGF-β1 on phosphatase and tensin homolog (PTEN) expression, and microRNA-21 (miR-21) level with real-time quantitative PCR or/and Western blotting, and then examined the regulatory role of miR-21 on the PTEN expression and the mesenchymal transition, with real-time quantitative PCR, western blotting and luciferase reporter assay, and the migration of keratinocytic HaCaT cells with scratch assay.

RESULTS: It was demonstrated that miR-21 was upregulated by TGF-β1 treatment in HaCaT cells; and the upregulated miR-21 targeted the 3’ UTR of PTEN gene and downregulated the PTEN expression, along with the Smad3/4 upregulation. Moreover, the miR-21 manipulation with miR-21 mimics or miR-21 inhibitor not only upregulated or downregulated the miR-21 level, but also associated with the mesenchymal transition and the migration of HaCaT cells via promoting or downregulating the FSP1 and Collagen I and the E-cadherin, and via upregulating or downregulating the migration of HaCaT cells.

CONCLUSIONS: Our results demonstrate that miR-21 mediates the TGF-β1-promoted mesenchymal transition and migration of keratinocytes during skin wound healing via targeting PTEN. This study implies that miR-21 might be an important target to promote the skin wound healing.

Key Words: TGF-β1, PTEN, miR-21, Keratinocyte migration.

Introduction

Cutaneous wound repair is highly orchestrated by multiple molecular, cellular and humoral events, which include coagulation, inflammation, epithelialization, granulation tissue formation, matrix deposition, and tissue remodeling. And the growth factor transforming growth factor-β (TGF-β) involves in various phases of the repair process. In particular, as the essential feature of a healed wound, re-epithelialization is largely influenced by TGF-β. The upregulated TGF-β targets keratinocytes, and promotes the cell migration, whereas inhibits the cell proliferation. Interestingly, the abrogation of TGF-β, of its receptors or its downstream mediators, Smads, has been reported to either inhibit or promote the wound healing. Therefore, to better recognize the role of TGF-β in wound healing process, it is important to identify the downstream effectors of TGF-β signaling during wound healing.

microRNAs (miRNAs) are a group of 18-23 nt-long noncoding RNAs, being capable of inducing posttranscriptional gene regulation by blocking translation or by degrading the target mRNA. Accumulating evidence demonstrates that miRNAs also involve in the multiple steps of wound healing, with specific miRNA profile identified in wound healing. And miRNAs have been suggested to regulate the leukocyte function and the cytokine network, which are necessary for the migration and capillary formation of endothelial cells, and also to control the proliferation and differentiation of wound-specific cells and the production of extracellular matrix composition. Several lines of evidence have shown that miRNAs regulate keratinocyte proliferation and migration. miR-205 promotes epidermal keratinocyte migration through targeting SHIP2. In a mouse model of the ischemic wound, HIF-1α-induced miR-210 attenuates keratinocyte migration by targeting E2F3. Particularly, lots of miRNAs have been identified to regulate the TGF-β...
signaling. miR-24 was upregulated during myoblast differentiation and could be inhibited by TGF-β, Smad3-dependently. The miR-106b-25 and miR-17-92 clusters are emerging as key modulators of TGF-β signaling in gastrointestinal and other tumors, interfering with cell cycle arrest and apoptosis, and miR-224 is confirmed to be involved in TGF-β-mediated mouse granulosa cell proliferation and granulosa cell function by targeting Smad4.

miR-21 is frequently upregulated in human cancers and other diseases, and plays important roles in cell proliferation, apoptosis, epithelial to mesenchymal transition (EMT) and migration through its distinct targets. The regulatory role of miR-21 in cutaneous wound healing has also been recognized, recently. miR-21 overexpression promoted, whereas miR-21 knockdown attenuated the keratinocyte migration and re-epithelialization, suggesting that miR-21 was essential for TGF-β-driven keratinocyte migration. However, the mechanism of miR-21-promoted keratinocyte migration and cutaneous wound healing is still under-determined.

In the present study, we investigated the indispensable role of miR-21 in keratinocyte migration during wound healing. Particularly, we determined the mediation of Phosphatase and tensin homolog (PTEN) in the miR-21-promoted keratinocyte migration during wound healing. Our study implies the key regulatory role of miR-21 in the TGF-β-promoted wound healing.

Materials and Methods

Cells, Reagents and Cell Treatment

Immortalized human keratinocytic HaCaT cell line was obtained from the cell resource center of Chinese Academy of Medical Sciences. HaCaT cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM), which was supplemented with 10% fetal calf serum (FCS) (GIBCO, Rockville, MD, USA) and with antibiotics (100 units/ml penicillin and 100 mg/ml streptomycin, both from Sigma-Aldrich, St. Louis, MO, USA), under 37 °C and 5% CO₂ in humidified air. TGF-β (Sigma-Aldrich, St. Louis, MO, USA) was utilized to treat HaCaT cells with a concentration of 0, 3, 10 or 30 ng/ml for 0, 12 or 24 hours. miR-21 mimics (Sigma-Aldrich, St. Louis, MO, USA) with 25 or 50 nM, miR-21 inhibitor (Qiagen, GmbH, Hilden, Germany) with 50 nM, or miRNA control (GenePharma, Shanghai, China) were transfected with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) into HaCaT cells to regulate the miR-21 level. And the Smad4-specific siRNA or scramble siRNA (Sigma-Aldrich, St. Louis, MO, USA) were transfected with Lipofectamine 2000 to knockdown the Smad3 level. To overexpress PTEN in HaCaT cells, PTEN coding sequence was cloned into eukaryotic pcDNA3.1(+) vector (Invitrogen, Carlsbad, CA, USA); and the PTEN-pcDNA3.1(+) or control CAT-pcDNA3.1(+) vectors was transfected into HaCaT cells to promote the PTEN level. On the other side, SF1670 (Sigma-Aldrich, St. Louis, MO, USA) was utilized to inhibit PTEN with a concentration of 200 nM.

mRNA isolation, miRNA Purification and Real-Time Quantitative PCR

Total cellular mRNA from HaCaT cells were extracted with the TRIzol reagent (Life Technologies, Grand Island, NY, USA) and were added with RNase inhibitor (Takara, Tokyo, Japan). miRNA samples were extracted with the mirVana miRNA Isolation Kit (Ambion, Austin, TX, USA) according to the manufacturer’s manual. The quantitative analysis of PTEN, Smad3, Smad4 or TGF-β1 at mRNA level was performed with the One-step SYBR real-time RT-PCR kit (Takara, Tokyo, Japan) and with the PTEN-, Smad3-, Smad4-, TGF-β1- or β-actin-specific primers (5′-ACA GGC TCC CAG ACA TGA CA-3′ AND 5′-TCA GAG TTT TGT AAT TTG TGT ATG-3′ for PTEN33, 5′-GAG TAG AGA CGC CAG TTC TACC-3′ AND 5′-GGT TGAG ACG CCT CGT CCA-3′ for Smad334, 5′-ATA CGC CTG ACG AGA GTG CAG-3′ AND 5′-GCC CTG TAT TCC GTT TTT GGT-3′ for TGF-β135, 5′-TGT CCA CCT TCC AGC AGA TGT-3′ AND 5′-AGC TCA GTA ACA GTC CGC CTA GA-3′ for β-actin). And the Quantitative assay for the miR-21 level was conducted using the mirVana qRT-PCR miRNA Detection Kit (Ambion, Austin, TX, USA), with U6 small nuclear RNA as internal control. The real-time RT-PCR was performed in Lightcycler 480 II. Each value was normalized to β-actin and expressed as the fold change over control and calculated with the ΔΔCt method36.

Protein Extraction and Western Blotting

Whole cellular proteins were extracted with a cell lysis reagent (Sigma-Aldrich, St. Louis, MO, USA) according to the reagent’s manual, and
were quantified with a BCA Protein Assay Reagent Kit (Pierce, Rockford, IL, USA). Then the protein samples were separated via SDSPAGE electrophoresis, and were blotted using rabbit polyclonal antibody against PTEN (Abcam, Cambridge, UK), Smad3/4 (Abcam, Cambridge, UK), TGF-β (Cell Signaling Technology Inc., Danvers, MA, USA), E-cadherin (Pierce, Rockford, IL, USA), FSP1 (Abnova, Walnut, CA, USA), Collagen I (Abcam, Cambridge, UK), or β-actin (Sinobio, Beijing, China). Goat anti-rabbit IgG antibody conjugated to horseradish peroxidase (Pierce, Rockford, IL, USA) and ECL detection systems (Amersham Pharmacia Biotech, Amersham, UK) were used for specific band detection.

**Luciferase Activity Assay**
To investigate the targeting regulation by miR-21 on PTEN expression, we construct a luciferase reporter with the wild 3’-UTR or the mutated 3’-UTR of PTEN. The 3’-UTR or the mutated 3’-UTR sequence was amplified by PCR from human genomic DNA and cloned into the multiple clone sites between Bgl II and Nhe I, downstream from the stop codon of luciferase, of the pGL3 vector. 90%-confluent HaCaT cells which were seeded in 6-well plates were successively transfected with 1 μg of the constructed luciferase report vector for 1 hour, and then with 50 nM miR-21 mimics or control miRNA. 24-hour post transfection, firefly luciferase activity was assayed using luciferase assays (Pierce, Rockford, IL, USA).

**Cell Migration Assay**
To investigate regulatory roles of TGF-β1, miR-21 mimics, miR-21 inhibitor on the wound healing of HaCaT cells, cell migration was examined by the scratch assay. HaCaT cells with 90% confluence in 12-well plates were transected with miR-21 mimics, miR-21 inhibitor or miR-21 control. 6-hour post the transfection, the confluent cells were scratched with CellScrapers (Corning Coster, Cambridge, MA, USA), and were observed for the cell migration across the baseline at 48h post treatment. All the experiments were performed respectively in triplicate.

**Statistical Analysis**
Statistical analysis was performed in GraphPad Prism 6 (GraphPad Software, La Jolla, CA, USA). The significant difference between two groups was analyzed by Student’s t-test. A p-value less than 0.05 was considered statistically significant.

**Results**

**TGF-β1 Downregulates PTEN in Keratinocytic HaCaT cells, Smad-Dependently**
To investigate the regulatory role of TGF-β1 in the wound healing, we treated HaCaT cells with a various concentration of TGF-β1, and then examined the PTEN expression in the HaCaT cells. Figure 1A demonstrated that the TGF-β1 treatment with 10 or 30 ng/ml significantly downregulated the PTEN mRNA level (p < 0.05 or p < 0.01, respectively), and such downregulation was reconfirmed by the luciferase reporter assay with the untranslated region of PTEN (p <0.01 or p < 0.001, respectively; Figure 1B). The protein level of PTEN expression was also examined via the western blot assay (Figure 1C), Figure 1D indicated that the PTEN protein level was also markedly (p < 0.01 or p < 0.001, respectively) downregulated, with a dose-dependence (p < 0.05).

Smad proteins have been widely conceived to transduce signals from TGF-β and to mediate the regulation by TGF-β on cell proliferation, differentiation and death through the activation of receptor serine/threonine kinases. To investigate the role of Smad proteins in the TGF-β-downregulated PTEN, we also determined the expression of Smad3 and Smad4 in the TGF-β-treated HaCaT cells. As indicated in Figure 2A, the mRNA levels of both Smad3 and Smad4 were markedly upregulated by the TGF-β1 treatment with 10 or 30 ng/ml (p < 0.01 or p < 0.001, respectively). And the upregulation in protein level of both Smads was also recognized with western blotting assay (Figure 2B), protein levels of Smad3 and Smad4 were significantly higher in TGF-β-treated HaCaT cells with 10 or 30 ng/ml (p < 0.05 or p < 0.01, Figure 2C). To investigate the role of Smad signaling in the TGF-β-downregulated PTEN, we then knocked down the Smad4 with Smad4-specific siRNA. It was demonstrated in Figure 2D that the Smad4 expression was significantly knocked down by the transfection with 25 or 50 nM siRNA-Smad4, compared to the scramble siRNA. Moreover, the siRNA-Smad4 transfection could markedly ameliorate the TGF-β1-induced...
PTEN reduction (\(p < 0.05\) or \(p < 0.01\); Figure 2E and 2F). Therefore, we confirmed that TGF-\(\beta_1\) downregulated PTEN level via Smad proteins in the HaCaT cells.

**TGFB1-Promoted miR-21 Inhibits the PTEN Translation**

miR-21 is conceived to play a critical role in TGF-\(\beta\) pathway. It is reported that TGF-\(\beta\) upregulates mature miR-21 by promoting the transformation of primary transcripts of miR-21 (pri-miR-21) into precursor miR-21 (pre-miR-21) through the interaction of Smads and Drosha\(^{38,39}\). To examine the miR-21 induction in HaCaT cells by TGF-\(\beta\), we then quantified the miR-21 level in the HaCaT cells subject to 10 ng/ml TGF-\(\beta\). As shown in Figure 3A, the miR-21 level was significantly promoted in the TGF-\(\beta\)-treated HaCaT cells at 12 or 24-hour post treatment (\(p < 0.01\) or \(p < 0.001\), respectively). Then we manipulated the miR-21 level with miR-21 mimics transfection, and Figure 3B indicated that the transfection with 25 or 50 nM miR-21 mimics markedly promoted the miR-21 level, with a fold change of more than 7 or 12 (\(p < 0.001\) respectively). And the miR-21 level rose quickly from 1-hour post transfection, and peaked at 4-hour post-transfection, with a significant time-dependence (Figure 3C).

To confirm the regulation by miR-21 on the PTEN expression, we then evaluated the influence by the miR-21 mimics transfection on the luciferase-based reporter, with wild or mutated 3’ untranslated region (UTR) of PTEN gene. It was demonstrated in Figure 3D that the transfection with 50 ng/ml miR-21 mimics, rather than miRNA control, markedly inhibited the luciferase activity of the reporter with the wild, rather than the mutated, 3’ UTR of PTEN gene (\(p < 0.01\)). To reconfirm the PTEN downregulation by miR-21 in HaCaT cells, we then determined the PTEN expression in both mRNA and protein levels in the miR-21-transfected HaCaT cells. Figure 3E indicated that there was a significant reduction of PTEN mRNA level in the miR-21 group, compared to the miRNA control group, whereas there was no such significant difference in TGF-\(\beta\) mRNA between the two groups. Figure 3F and 3G demonstrated that not only both SF1670 and TGF-\(\beta_1\) markedly reduced the PTEN protein level (\(p < 0.001\) or \(p < 0.05\)), but also the miR-21 transfection (50 nM) led to such PTEN reduction significantly (\(p < 0.001\)). Thus, we confirmed the PTEN downregulation by miR-21 and TGF-\(\beta_1\) in HaCaT cells.
Role of miR-21 in the TGF-β-Induced Mesenchymal Transition of HaCaT Cells

The mesenchymal transition is promoted by TGF-β in keratinocytes during the skin wound healing. And we then investigated the regulation by miR-21 on the TGF-β-induced mesenchymal transition of HaCaT cells. Figure 4A and 4B indicated that the transfection with 50 nM miR-21 mimics significantly reduced the expression of E-Cadherin ($p < 0.01$), whereas markedly promoted the levels of fibroblast-specific protein-1 (FSP-1) and Collagen I ($p < 0.01$ or $p < 0.05$). And the miR-21 transfection aggravated the TGF-β-induced mesenchymal transi-
microRNA-21 mediates the TGF-β1-induced migration of keratinocytes via targeting PTEN

**Figure 3.** miR-21 mediates the PTEN downregulation by TGF-β1. **A,** TGF-β1 promoted the miR-21 level in HaCaT cells at 12 or 24 hour post treatment (H.P.T.); **B,** miR-21 mimics transfection promoted the miR-21 level in HaCaT cells, with negative miRNA as control; **C,** Time-dependence of miR-21 promotion by the miR-21 mimics transfection; **D,** miR-21 targeted the 3’ UTR of PTEN and inhibited the luciferase activity of the reporter with PTEN’s 3’ UTR; **E,** miR-21 mimics transfection downregulated the mRNA level of PTEN, rather than of TGF-β; **F,** and **G,** Western blot analysis of PTEN level in the HaCaT cells, which were treated with 200 nM SF1670 (PTEN inhibitor), 10 ng/ml TGF-β1 or were transfected with 50 nM miR-21 mimics or control miRNA. 3’ UTR (WT)/(mt): Reporter plasmid with the wild type or mutant type 3’ UTR of PTEN. All experiments were independently performed in triplicate. *p < 0.05, **p < 0.01, ***p < 0.001, or ns: no significance.
Figure 4. A, miR-21 regulates the TGF-β1-induced mesenchymal transition. A, and B, miR-21 mimics transfection downregulated E-cadherin, whereas upregulated FSP1 and Collagen I. C, miR-21 aggravated the TGF-β1-induced E-cadherin downregulation and the upregulation of FSP1 and Collagen I; D, miR-21 inhibitor downregulated the miR-21 level in HaCaT cells; E, Time-dependence of miR-21 downregulation by the miR-21 inhibitor transfection; F, and G, Western blot analysis of E-cadherin, FSP1 and Collagen I in the HaCaT cells, which were treated with 10 ng/ml TGF-β1 and were transfected with 50 nM miR-21 inhibitor or control miRNA. Each value was averaged for results from independent experiments. *p < 0.05, **p < 0.01, or ***p < 0.001.
tion, via deteriorating the E-Cadherin reduction and the promotion of FSP-1 and Collagen I in HaCaT cells, which were treated with 10 ng/ml TGF-β (p < 0.05, p < 0.01 or p < 0.001; Figure 4C). To reconfirm the regulatory role of miR-21 in the TGF-β-induced mesenchymal transition, we then transfected miR-21 inhibitor into the HaCaT cells with or without TGF-β treatment and re-evaluated the expression of above-mentioned molecules. Results demonstrated that miR-21 inhibitor downregulated the miR-21 level with a concentration of 25 or 50 nM (p < 0.01 respectively; Figure 4D), and the downregulation peaked at 4-hour post transfection (Figure 4E), also with a significant time-dependence. Moreover, 50 nM miR-21 inhibitor could reverse the E-cadherin reduction and the FSP1 and Collagen I upregulation (p < 0.05 or p < 0.01; Figure 4F and 4G). Therefore, miR-21 promoted the TGF-β-induced mesenchymal transition of HaCaT cells.

Role of miR-21 and PTEN in the TGF-β1-Induced Migration of HaCaT cells

Migration of keratinocytes in wounded skin and of epithelial cells in the damaged cornea is regulated and is controlled by transforming growth factor-beta (TGF-β). To determine the role of miR-21 and PTEN in the TGF-β1-induced migration of HaCaT cells, the wound healing assay of HaCaT cells which were subject to TGF-β1 treatment and miR-21 mimics (or miR-21 inhibitor). It was shown in Figure 5A that there were more migratory cells across the baseline in the miR-21 mimics-transfected HaCaT cells, in the presence of 10 ng/ml TGF-β1, than in the miRNA control-transfected cells, which were also subject to 10 ng/ml TGF-β1 (p < 0.05; Figure 5A and 5B). On the other side, the miR-21 inhibitor transfection significantly inhibited the TGF-β1-promoted HaCaT cell migration (p < 0.01; Figure 5A and 5B).

To further investigate the role of PTEN in the TGF-β1-promoted HaCaT cell migration, we then overexpressed PTEN with a eukaryotic expression vector, pcDNA3.1(+), and then re-evaluated the migration of the PTEN-overexpressed HaCaT cells subject to TGF-β1. Figure 5C indicated that the transfection with PTEN-pcDNA3.1(+) significantly upregulated the PTEN level in HaCaT cells (p < 0.001). Moreover, the PTEN-pcDNA3.1(+) transfection markedly inhibited the TGF-β1-induced HaCaT cell migration (Figure 5D and 5E).

Discussion

It is of great importance for the identity of signaling pathways that regulate both cell migration and mesenchymal transition during the wound healing. Here we show that PTEN is involved in the TGF-β1-induced cell migration and mesenchymal transition of keratinocytes during wound healing. Results demonstrated that the PTEN expression in both mRNA and protein levels was significantly downregulated by TGF-β in keratinocytic HaCaT cells. And the downregulation was Smad4-dependent; siRNA targeting Smad4 could markedly reduce the Smad3 level and reverse the PTEN level. miR-21 has been recently recognized as a biomarker in various types of diseases. It has also been confirmed to mediate the TGF-β signaling; mature miR-21 is upregulated through the interaction of Smads and Drosha. And the present study confirmed the miR-21 upregulation by TGF-β treatment in HaCaT cells. And what's more, the miR-21 could target the 3’ UTR of PTEN and mediate the TGF-β-induced PTEN downregulation in HaCaT cells.

The mesenchymal transition was promoted by TGF-β in skin wound healing. And the present study confirmed a positive regulatory role of miR-21 in the mesenchymal transition of HaCaT cells. miR-21 significantly reduced E-Cadherin expression, whereas markedly promoted the levels of fibroblast-specific protein-1 (FSP-1) and Collagen I. Whereas the miR-21 inhibitor could reverse the E-Cadherin reduction and the FSP1 and Collagen I upregulation. The regulatory role of miR-21 was also confirmed in the TGF-β1-induced migration of HaCaT cells. The transfection with miR-21 mimics aggravated, whereas the miR-21 inhibitor inhibited the TGF-β1-promoted HaCaT cell migration. Moreover, the TGF-β1-promoted HaCaT cell migration was markedly inhibited by PTEN overexpression.

The present study firstly recognized the promotion of miR-21 to the TGF-β1-induced mesenchymal transition and migration of HaCaT cells. And such promotion is via downregulating PTEN, which is the target of miR-21. It was shown in Figure 6, the TGF-β/Smad signaling has been widely conceived, and the Smad3 has been confirmed to upregulate the maturing of miRNAs. The present study confirmed the upregulation of matured miR-21 by the TGF-β1-induced Smad3 in HaCaT cells. Therefore, our study has emphasized the important role of Smad signaling in the miRNA processing.
Moreover, we confirmed the key regulatory role of miR-21 in the PTEN level in TGF-β1-treated HaCaT cells, and the downregulation of PTEN by miR-21 significantly influenced the mesenchymal transition and migration of HaCaT cells, which were subject to TGF-β1. Thus, this study highlighted the mediation by miR-21 of TGF-β1-induced mesenchymal transition and
microRNA-21 mediates the TGF-β1-induced migration of keratinocytes via targeting PTEN

migration of keratinocytes via downregulating PTEN. However, there several details need to be further investigation in such mediation, it is not clear about the exact mechanism of the inhibition to EMT and migration by PTEN, and we have known litter about whether there should be other mechanisms of miR-21 in the regulation to the TGF-β1-induced EMT and migration of HaCaT cells.

Conclusions

The present study confirms that miR-21 mediates the TGF-β1-induced mesenchymal transition in and migration of HaCaT cells via targeting the 3' UTR of PTEN gene and thus downregulating PTEN expression. Thus, this work implies that miR-21 might be an important molecular marker to predict the healing of wounded skin or to promote the process of skin wound healing.

Conflict of Interest

The Authors declare that there are no conflicts of interest.

References

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