Abstract

Purpose: To determine the effect of miR-485-5p on keratinocyte proliferation and migration.

Methods: Human primary keratinocytes (HaCaT cells) were treated with different concentrations of transforming growth factor-β1 (TGF-β1). miR-485-5p expression levels were determined using quantitative reverse transcription-polymerase chain reaction (qRT-PCR). MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) and wound healing assays were performed to investigate the regulatory effects of miR-485-5p on cell viability and migration of HaCaT cells. Downstream target gene expression of miR-485-5p was determined using a luciferase activity assay.

Results: In HaCaT cells, miR-485-5p was time- and dose-dependently downregulated by TGF-β1 treatment ($p < 0.05$). Forced expression of miR-485-5p decreased cell viability and migration of HaCaT cells ($p < 0.05$). Knockdown of miR-485-5p enhanced HaCaT cell viability and migration. Integrin subunit alpha-5 (ITGA5) was predicted and verified to be a downstream target of miR-485-5p in HaCaT cells. Overexpression of ITGA5 attenuated the miR-485-5p-induced decrease of HaCaT cell viability and migration ($p < 0.05$).

Conclusion: MiR-485-5p reduces cell proliferation and migration of keratinocytes through the regulation of ITGA5. This mechanism provides a potential therapeutic strategy for skin wound healing.

Keywords: ITGA5, Keratinocyte, Cell migration, MiR-485-5p, Cell proliferation, Wound healing
Therefore, the particular mechanisms of wound healing require further investigation.

MicroRNAs (miRNAs) are important gene regulators that function by targeting the 3' untranslated regions of messenger RNAs (mRNAs). A recent study revealed important roles of miRNAs in the inflammatory, proliferative, and tissue remodeling stages of wound healing [3]. Specific miRNAs either enhance or suppress proliferation and migration of keratinocytes during wound healing. miR-485-5p was recently reported to have a suppressive effect on the migration and proliferation of melanoma cells [4]. However, whether miR-485-5p is implicated in the proliferation and migration of keratinocytes during skin wound healing has not yet been reported. Integrin subunit alpha-5 (ITGA5) functions as a fibronectin or osteopontin binding receptor and participates in cell proliferation and migration signaling [5]. Knockdown of ITGA5 reduces the migration capacity of stem cells [6]. ITGA5 is also involved in miR-205-mediated re-epithelialization during skin wound healing [7]. This study was performed to evaluate the effects of miR-485-5p on proliferation and migration of keratinocytes and to determine whether miR-485-5p binds to ITGA5 to regulate cell proliferation and migration.

EXPERIMENTAL

Cell culture, treatment, and transfection

The human epidermal keratinocyte cell line HaCaT, acquired from American Type Culture Collection (Manassas, VA, USA), was cultured in Dulbecco’s Modified Eagle Medium (DMEM; HyClone, Logan, UT, USA) containing 10% fetal bovine serum (HyClone) at 37°C. For treatment with transforming growth factor-β1 (TGF-β1), HaCaT cells were seeded and incubated with different concentrations of TGF-β1 (2, 5, and 10 ng/mL; Sigma-Aldrich, St. Louis, MO, USA) for 24 h before miR-485-5p expression was measured. HaCaT cells were also incubated with 5 ng/mL TGF-β1 (Sigma-Aldrich) for various times (6, 12, and 24 h) before miR-485-5p expression was measured. For transfection experiments, HaCaT cells were seeded and transfected with miR-485-5p mimics or inhibitors, a negative control (control, NC inh) (Ribobio, Guangzhou, China), pcDNA-ITGA5, or empty pcDNA vector (Invitrogen, Carlsbad, CA, USA) using Lipofectamine 2000 (Invitrogen).

Cell viability and migration

Two days after transfection, the culture medium was removed and HaCaT cells in each well were treated with 20 µL MTT solution (Sigma-Aldrich). Four hours later, the medium was removed and dimethyl sulfoxide (DMSO) was added to each well. Absorbance at 490 nm was measured 4 h later using an Epoch Microplate Reader (BioTek, Winooski, VT, USA). For the migration assay, HaCaT cells were incubated with 10 µg/mL mitomycin C (Sigma-Aldrich) and then scratched with a plastic pipette tip. Twenty-four hours later, the cultures were viewed under a microscope (Olympus, Tokyo, Japan), and the distances between both sides of the scratch were measured.

Luciferase reporter

Luciferase reporter vectors (Promega, Madison, WI, USA) containing wildtype or mutant 3'UTRs of ITGA5 were constructed and named pGL3-ITGA5-WT and pGL3-ITGA5-MUT, respectively. HaCaT cells were co-transfected with pGL3 vectors, pRL-TK, miR-485-5p mimics, or control. The luciferase activities were measured 24 h later using a Luciferase Assay System (Promega).

Quantitative Reverse Transcription-PCR (qRT-PCR)

Extracted RNA from HaCaT cells was reverse-transcribed into cDNA. A SYBR Green PCR kit (TransGen, Beijing, China) was used to perform qRT-PCR analyses with the primers shown in Table 1.

Table 1: Primer sequences

|  | Sense | Antisense |
|---|---|---|
| **MiR-485-5p** | 5'-AGAGGCTGGCAGATGAATTC-3' | 5'-GCTGTCAACGATACGCTACCTA-3' |
| **U6** | 5'-CTCGCTTCGCGCTCGTTCACG-3' | 5'-AACAGCTTCACGCTAAATTTGCGT-3' |

Western blotting

Protein concentrations of extracted cell lysates from HaCaT cells were measured using a bicinchoninic acid kit (Wuhan Boster Biological Technology, Hubei, China). Total protein (30 µg) was loaded into wells of 10% acrylamide gels. Proteins were separated by SDS-PAGE. After electro-transferring to polyvinylidene-fluoride membranes (Bio-Rad, Hercules, CA, USA), the membranes were blocked in 5% bovine serum albumin and immunoblotted with the following primary antibodies: ITGA5 (1:1500; Abcam, Cambridge, UK) and GAPDH (1:3000; Abcam). Washed membranes were incubated with
horseradish peroxidase-conjugated secondary antibody (1:5000; Abcam). Gray values of the membranes were determined using Pierce ECL Western Blotting Substrate (Pierce Biotechnology, Waltham, MA, USA).

Statistical analysis

Data are reported as mean ± standard deviation and were analyzed using SPSS 21.0 software (IBM-SPSS, Chicago, IL, USA). Student’s t tests were applied to evaluate statistical differences between groups; \( p < 0.05 \) was considered statistically significant.

RESULTS

TGF-β1 treatment suppresses miR-485-5p expression in keratinocytes

The human epidermal keratinocyte cell line HaCaT was treated with TGF-β1 to determine the regulatory role of TGF-β1 on miR-485-5p. qRT-PCR analysis indicated that miR-485-5p expression was decreased in TGF-β1-stimulated HaCaT cells in dose- and time-dependent manners (Figure 1 A and B), suggesting that TGF-β1 treatment suppressed miR-485-5p expression in keratinocytes.

miR-485-5p decreases cell viability and migration of keratinocytes

To determine the effect of miR-485-5p on keratinocytes, HaCaT cells were transfected with miR-485-5p mimics or inhibitor. High expression of miR-485-5p (\( p < 0.01 \)) following transfection with miR-485-5p mimics and low expression of miR-485-5p (\( p < 0.01 \)) following transfection with a miR-485-5p inhibitor indicated high transfection efficiency (Figure 2 A). MiR-485-5p reduced the viability of HaCaT cells, while transfection with a miR-485-5p inhibitor increased cell viability (Figure 2 B), suggesting an anti-proliferative effect of miR-485-5p on keratinocytes. Moreover, cell migration was inhibited by transfection with miR-485-5p mimics and was promoted by transfection with an inhibitor (Figure 3), further suggesting an anti-invasive effect of miR-485-5p on keratinocytes.

miR-485-5p targets ITGA5 in keratinocytes

ITGA5 was predicted to contain a binding site for miR-485-5p (Figure 4 A). A luciferase reporter assay was performed to investigate whether miR-485-5p directly targets ITGA5. The results showed that ITGA5-WT luciferase activity was decreased by transfection with miR-485-5p mimics when compared to the control (Figure 4 B), while luciferase activity of ITGA5-MUT was not affected by miR-485-5p (Figure 4 B). This suggests that miR-485-5p directly binds the 3'UTR of ITGA5. Moreover, over-expression of miR-485-5p decreased expression of ITGA5 and knockdown of miR-485-5p increased ITGA5 expression (Figure 4 C).

miR-485-5p decreases cell viability and migration of keratinocytes through regulation of ITGA5

To determine the effect of the miR-485-5p/ITGA5 axis on keratinocyte proliferation and migration, HaCaT cells were co-transfected with miR-485-5p mimics and/or pcDNA-ITGA5. Data from western blot analysis demonstrate that ITGA5 over-expression reversed the suppressive effect of miR-485-5p on ITGA5 protein expression (Figure 5 A). ITGA5 attenuated the miR-485-5p-induced decrease in viability (Figure 5 B) and
migration (Figure 5 C) of HaCaT cells, suggesting that miR-485-5p decreases cell viability and migration of keratinocytes through regulation of ITGA5.

DISCUSSION

The proliferative stage of wound healing, including rebuilding the extracellular matrix, re-epithelialization, and angiogenesis, is crucial for effective healing. Proliferation and migration of keratinocytes contribute to reestablishing coverage of the wound site and are important for rebuilding tissue integrity [8]. Application of Y27632, a promoter of human keratinocyte proliferation and migration, enhances cutaneous wound closure [9]. Because miR-485-5p exerts anti-proliferative and anti-invasive effects on tumor cells, the effect of miR-485-5p on keratinocyte proliferation and migration was investigated in this study.

Additional studies are needed to fully understand the role of miR-485-5p in wound healing. To determine the correlation between miR-485-5p expression and its biological function in skin wound healing, MTT and wound healing assays were performed. miR-485-5p suppressed the proliferation and migration of keratinocytes, while knockdown of miR-485-5p promoted their proliferation and migration.

Keratinocytes, which have mitotic ability in wound margins, initiate proliferation at the end of the inflammatory phase and then migrate to close the wounds [13]. Therefore, down-regulation of miR-485-5p in TGF-β1-treated keratinocytes may contribute to the re-epithelialization process in the proliferative phase of wound healing. Moreover, miR-132 and miR-21 promote keratinocyte migration and growth in an anti-inflammatory manner [14], suggesting that miRNAs may be involved in the regulation of all phases of wound healing. miR-485-5p regulates secretion of inflammatory factors [15], but it remains unknown whether miR-485-5p is involved in the inflammatory phase or other phases of wound healing.

ITGA5, which is related to angiogenesis, is up-regulated in the early healing response and is essential for keratinocyte migration [16]. In this study, ITGA5 was validated as a target of miR-485-5p in keratinocytes. Knockdown of miR-92a demonstrated a healing-supportive effect during re-epithelialization of wound healing [16], and inhibition of miR-205 promoted re-epithelialization through targeting ITGA5 [7]. The present study has demonstrated that over-expression of ITGA5 counteracts the suppressive effects of miR-485-5p on cell proliferation and migration of keratinocytes.

CONCLUSION

This study has demonstrated a close involvement of miR-485-5p in the regulation of keratinocyte migration and proliferation during wound healing. Down-regulation of miR-485-5p...
Yuan et al led to proliferation and migration of keratinocytes via up-regulation of ITGA5. These results provide a potential therapeutic target for chronic wounds.

DECLARATIONS

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Keyu Yuan designed the study, supervised data collection, and analyzed the data. Yi Sun interpreted the data and prepared the manuscript for publication. Yu Ji supervised data collection, analyzed the data, and reviewed the draft of the manuscript. All authors have read and approved the manuscript.

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