

MicroRNA-219-5p inhibits wound healing by targeting TMEM98 in keratinocytes under normoxia and hypoxia condition

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Abstract. – **OBJECTIVE:** Wound healing is closely related to the production of inflammatory cytokines, keratinocytes proliferation and migration. This study aims to investigate the role of transmembrane protein 98 (TMEM98) on wound healing and whether miRNA-219-5p could inhibit wound healing by targeting TMEM98 in keratinocytes.

MATERIAL AND METHODS: Hypoxia model was established by CoCl₂ (2000 μmol/L) treatment. TMEM98 protein expression, inflammatory cytokines (IL-6, IL-8, TNF-α) and cell proliferation and migration were detected in hypoxia group.

RESULTS: Overexpression of TMEM98 could significantly reverse the effects caused by hypoxia. MiR-219-5p was markedly increased in hypoxia group and miR-219-5p could downregulate TMEM98 expression by direct binding its 3'-UTR.

CONCLUSIONS: We demonstrated that miR-219-5p could inhibit wound healing by targeting TMEM98.

Key Words:

MicroRNA-219-5p, Keratinocytes, TMEM98, Healing.

Introduction

Hypoxia in the skin tissue is one of the major pathophysiological factors for some chronic diseases¹. Lack of oxygen can cause chronic inflammation of the skin tissue². Therefore, anti-inflammatory treatment is a therapeutic strategy to promote wound healing. The changes in proliferation and migration of keratinocytes are closely related to wound healing. Human skin immortalized keratinocytes (HaCaTs) is a commonly used *in vitro* model for the study of skin-related diseases including wound healing³. Thus, hypoxia-treated HaCaTs was employed in this study.

Human Transmembrane protein 98 (TMEM98) is a newly discovered gene, the function of which was largely unknown. TMEM98 is proved widely

expressed in various tissues including the immune system such as the spleen, thymus, lymph nodes, and bone marrow^{4,5}. It is speculated that TMEM98 may serve as an important immune regulator and plays an important role in the immune system⁶. In addition, TMEM98 was upregulated by the induction of a variety of inflammatory cytokines, indicating that it may be closely related to the occurrence and development of inflammation⁷. Wound healing is a typical immunization processes accompany with low-grade inflammation; however, the expression and role of TMEM98 remain unknown. miRNAs are short, endogenous, noncoding RNAs that have been considered as transcriptional or post-transcriptional regulators of gene expression. Previous evidence⁸ revealed important roles of microRNA-mediated mechanisms in wound healing. During the proliferative phase of wound healing, miRNAs can affect wound healing by regulating the proliferation, migration, and differentiation of keratinocytes⁹. It has been indicated that antagonists of miR-205 could promote the expression of SH2-containing phosphoinositide 5-phosphatase 2 (SHIP2), so that the healing rate of wound scratches of keratinocyte keratinocytes was significantly lower than that of the control group, which played a promoting role in the development and migrations of keratinocytes¹⁰. In addition, in a mouse ischemic wound model, hypoxia-inducible factor-1α induces miR-210 to inhibit the expression of E2F3, thereby affecting the downstream genes B-myb, cyclin A, cdc2, cdc6. The proliferative capacity of formed cells was reduced and wound healing was inhibited^{11,12}. MiR-219 has been reported to regulate cell proliferation and differentiation on pluripotent stem cells¹³. However, the effect of miR-219 on wound healing and underlying mechanisms remains to be clear. We aim to determine the effect of miR-219 and related mechanism in affecting wound healing.

Material and Methods

Reagents

Dulbecco's Modified Eagle's Medium (DMEM) was obtained from Gibco (Rockville, MD, USA). 5-ethynyl-2'-deoxyuridine (EdU) fluorescent probe and enhanced chemiluminescence (ECL) were obtained from Beyotime (Beijing, China). Proliferating cell nuclear antigen (PCNA) and Matrix metalloproteinase-1 (MMP-1) antibody were purchased from Proteintech (Rosemont, IL, USA). Agomir-219-5p was bought from Genepharma (Shanghai, China). Thiazolyl Blue Tetrazolium Bromide (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide, MTT) was from Sigma-Aldrich (St. Louis, MO, USA).

Cell Culture and Treatment

The human HaCaTs were purchased from the Cell Bank of Shanghai Institute for Biological Sciences (Shanghai, China). All the cell lines were cultured in complete high-glucose DMEM (HyClone, South-Logan, MA, USA) in 5% CO₂ in a humidified incubator at 37°C, and the medium was supplemented with 10% FBS (Gibco, Rockville, MD, USA) and 100 U/mL penicillin and 100 µg/mL streptomycin (Invitrogen, Carlsbad, CA, USA) for cell culture.

MTT Assay

HaCaTs (8×10³/mL) were seeded into each of the 96-well culture plates overnight and kept in a humidified atmosphere of 5% CO₂ and 95% air at 37°C. After 24 h of incubation, the medium was exchanged for DMEM and with or without CoCl₂ (2000 µmol/L) for 24 h to induce hypoxia model. Meanwhile, cells without any treatment were used as a control. Following this, the culture medium was removed, and 5 mg/mL methyl thiazolyl tetrazolium (MTT) were added to each well. The plates were incubated for 4 h at 37°C. The supernatant was carefully removed, the formazan crystals in each well were dissolved in 200 µL of dimethyl sulfoxide (DMSO) for 30 min at 37°C, and optical density at 570 nm was read on a Microplate Reader (Thermo Fisher Scientific, Waltham, MA, USA).

Wound Healing Assay

Cell migration ability was measured by using scratch wound healing assay. Cells were grown to 90% confluence in a six-well plate, a micropipette tip was used to scratch a wound. The cellular

debris was washed with serum-free medium for three times. The wound area was photographed under an Upright Metallurgical microscope (Olympus CX41, Shinjuku, Tokyo, Japan) at 0 and 48 h.

Western Blot

Cells were harvested and protein extracts prepared according to established methods¹⁴. Extracts were separated in sodium dodecyl sulfate-polyacrylamide electrophoresis gels (SDS-PAGE) (8-15%) and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). The membranes were blocked with 5% milk, and then incubated with indicated primary antibodies (1:500) at 4°C overnight. After washing, the membranes were incubated with the appropriate secondary antibodies (1:5000). The membranes were exposed to ECL. The emitted light was captured by a Bio-Rad imaging system (Hercules, CA, USA) with a Chemi HR camera 410 and analyzed with a Gel-Pro Analyzer Version 4.0 (Media Cybernetics, Rockville, MD, USA).

Transfection of Agomir and TMEM98 Overexpression

HaCaTs were transiently transfected using Lipofectamine 2000 (Life Technologies-Invitrogen, Carlsbad, CA, USA) in Opti MEM according to the manufacturer's protocol, as described¹⁵. Agomirs, TMEM98 overexpression plasmid and the corresponding NC were obtained from GenePharma (Shanghai, China). The transfection procedures were performed according to the manufacturer's protocols.

miRNA Expression

Total RNA was extracted using the miRNeasy kit (Qiagen, Hilden, Germany) and 250 ng was reverse transcribed using stem-loop Multiplex primer pools (Applied Biosystems, Foster City, CA, USA). Reverse transcription (RT)-specific primers (forward 5'-AAAAGAATTCCCACTTCCCACTCCAGACATT-3' and reverse 5'-AAAGCGGCCGCCCTCACTTCTCCGTAACCC-3') for rat miR-219-5p were used for all miRNA RT. Quantitative polymerase chain reaction (qPCR) was carried out using a 7500HT Fast Real-time System (Applied Biosystems, Foster City, CA, USA) and TaqMan microRNA assays (Applied Biosystems, Foster City, CA, USA). Endogenous RNA U6 small nuclear 2 (RNU6B) was used for normalization. The relative fold

change in expression of the target gene transcript was determined using the comparative cycle threshold method ($2^{-\Delta\Delta CT}$).

EdU Staining

Cells were harvested and fixed with 70% alcohol overnight at 4°C, and then incubated with the EdU cell proliferation kit (Ribobio, Beijing, China) according to the instructions.

Luciferase Reporter Activity Assay

Plasmids containing the TMEM98 3'-UTR response element (3'-UTR-wt) and the corresponding mutant (3'-UTR-mut) were obtained from Obio Technology Corp., Ltd., (Shanghai, China). Plasmid DNA and the agomir or the NC were conducted 36 h after transfection. The luciferase activity was determined with a Dual-Luciferase Reporter Assay Kit (TransGen, Beijing, China) using a Dual-Light Chemiluminescent Reporter Gene Assay System (Berthold, Oak Ridge, TN, China) and was normalized to the Renilla luciferase activity.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 19.0 software (SPSS Inc., Chicago, IL, USA) were used for data processing. The *t*-test was used for the intergroup comparison, and χ^2 -test was used for enumeration data. Continuous data from multiple groups were analyzed by using one-way ANOVA, with the Tukey's post-hoc test. *p*-values <0.05 were considered statistically significant.

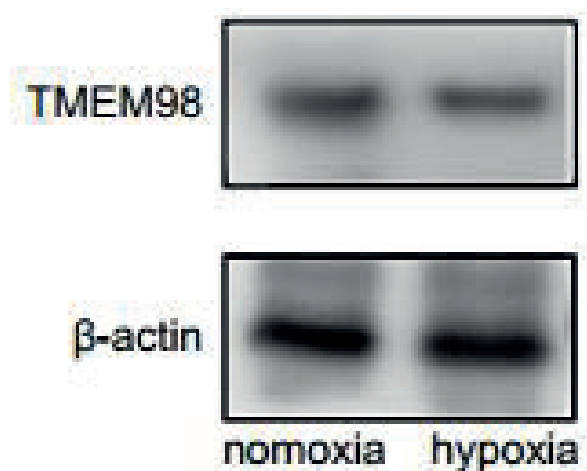


Figure 1. TMEM98 protein expression (n=6).

Results

TMEM98 Protein Expression in Keratinocytes Under Normoxia and Hypoxia Condition

TMEM98 protein expression level was determined by Western blot. As shown in Figure 1, in hypoxia group, the TMEM98 protein expression level was significantly decreased compared with normoxia group.

TMEM98 Was Involved in Inhibiting Inflammatory Response

TMEM98 was overexpressed in normoxia or hypoxia condition. We found that, in hypoxia group, the level of TNF- α , IL-6 and IL-8 were significantly increased; however, the levels of TNF- α , IL-6 and IL-8 were markedly decreased by TMEM98 overexpression (Figure 2).

Effect of TMEM98 in Cell Proliferation

Cell proliferation is an important step in wound healing. In this study, MTT assay and ki67 protein expression were employed to evaluate cell proliferation. As shown in MTT assay, cell viability rate was decreased in hypoxia group compared with that of normoxia group, and TMEM98 overexpression significantly reversed the decreased cell viability rate under hypoxia condition (Figure 3A). Accordingly, ki67 protein expression was reduced in hypoxia group but increased by TMEM98 overexpression (Figure 3B). Above results were also validated by EdU staining (Figure 3C).

Effect of TMEM98 on Cell Migration

Effect of TMEM98 on cell migration was measured by detecting MMP-1 expression and with wound healing assay. Hypoxia treatment significantly reduced MMP-1 expression but TMEM98 overexpression markedly elevated MMP-1 expression (Figure 4A). In wound healing assay, TMEM98 overexpression also promoted the inhibited migration rates in hypoxia group (Figure 4B).

In the current view, mRNAs can negatively regulate gene expression by binding the 3'-UTR of a specific miRNA, causing its degradation or translational repression^{16,17}. We hypothesize that TMEM98 may regulated by miRNAs. The miRNA target prediction programs miRanda (www.microrna.org/), TargetScan (www.targetscan.org/), and PicTar (<http://pictar.mdc-berlin.de/>) were used to identify miRNAs that target the TMEM98 3' untranslated region (3'UTR). MiR-

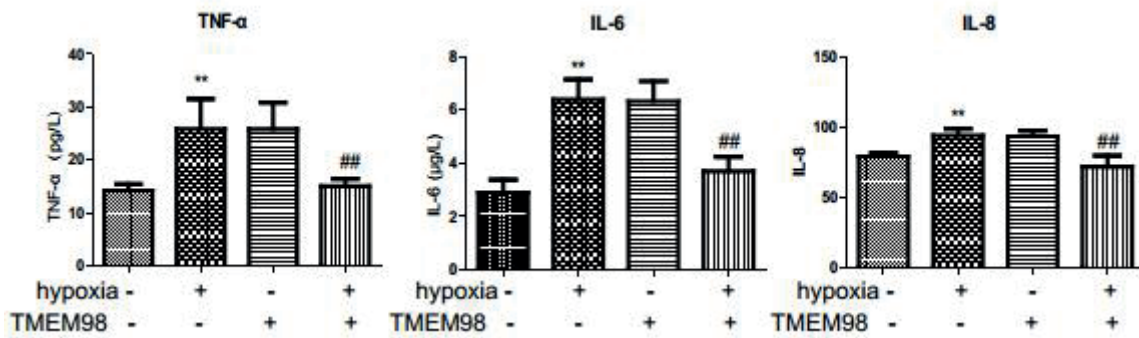


Figure 2. TNF- α , IL-6 and IL-8 levels. The results are the mean \pm SD (n=10), ** p < 0.05 vs. Control group, ## p < 0.05 vs. hypoxia group.

219-5p was considered to be a potential miRNA candidate that regulates TMEM98. Then, its expression level was determined by Real-time PCR. As shown in Figure 5, miR-219-5p was significantly increased in hypoxia group compared that in control group (p <0.05).

MiR-219-5p Might Inhibit Wound Healing by Targeting TMEM98

Based on above reasons, we suggest that miR-219-5p might inhibit wound healing by targeting

TMEM98. Thus, luciferase reporter activity assay was employed to detect whether TMEM98 could be regulated by miR-219-5p. Luciferase fusion constructs containing either the wild type or mutated TMEM98 3'-UTR were transfected into HaCaT cells (Figure 6). Then, these cells were co-transfected with agomir-219-5p and the corresponding control. Agomir-219-5p clearly suppressed the luciferase activity of the wild-type reporter. However, this repression was not observed for the mutated TMEM98 3'-UTR. Collectively,

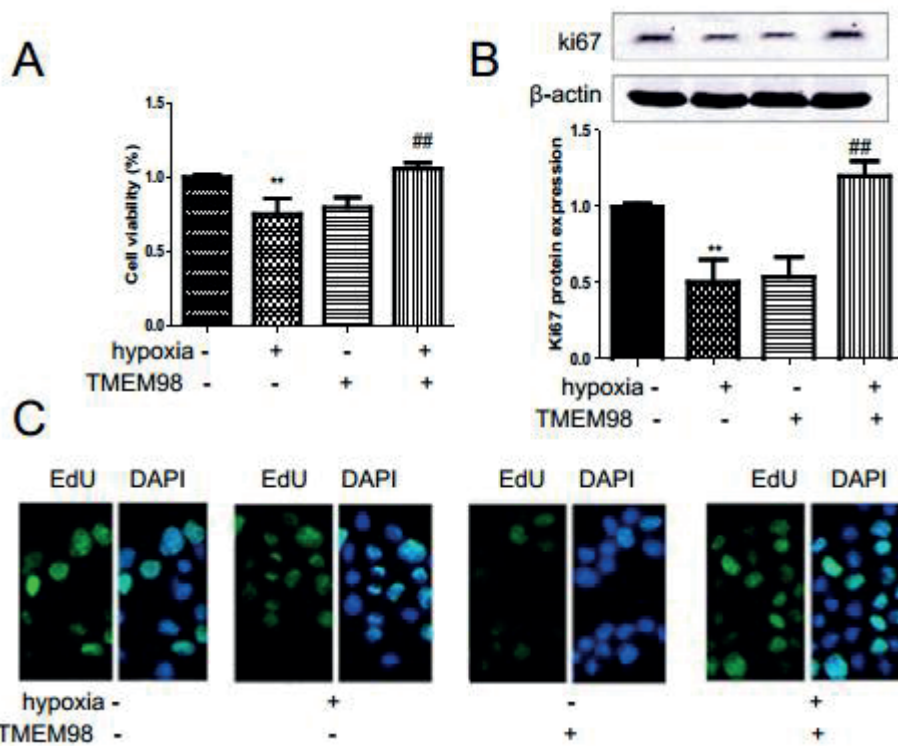


Figure 3. Effect of TMEM98 in cell proliferation. (A) MTT assay. (B) ki67 protein expression. (C) EdU staining. The results are the mean \pm SD (n=10), ** p < 0.05 vs. Control group, ## p < 0.05 vs. hypoxia group.

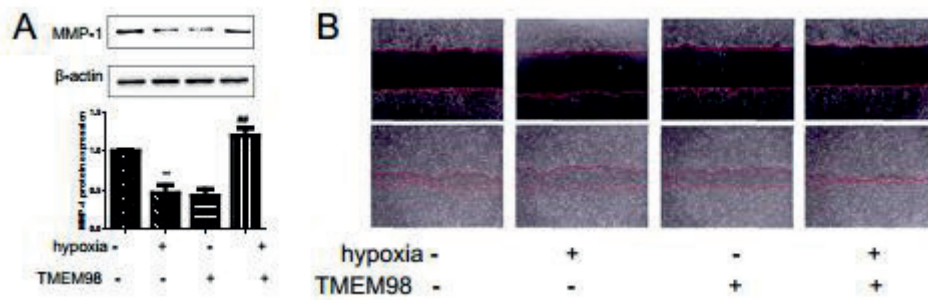


Figure 4. Effect of TMEM98 in cell migration. (A) MMP-1 expression. (B) Wound healing assay. The results are the mean±SD (n=10), ***p*< 0.05 vs. Control group, ##*p*< 0.05 vs. hypoxia group.

these data indicate that miR-219-5p can indeed regulate TMEM98 expression in HaCaT cells.

Discussion

Wound healing is closely related to the production of inflammatory cytokines¹⁸. In general, wound healing by keratinocytes involves three stages: the inflammation process, tissue formation, and tissue remodeling¹⁸. Inflammatory cytokines such as interleukin (IL)-6, IL8, and TNF-α are produced during the inflammation process¹⁹. In tissue formation, and tissue remodeling steps, cell proliferation and migration play an important role. Recently, TMEM98 is supposed to be a potential target on anti-tumor⁵. The facilitating effects of TMEM98 on non-small cell lung cancer invasion and migration have been reported⁶. Moreover, TMEM98 was identified to inhibit inflammatory response⁷. Matrix metalloproteinases (MMPs), especially MMP-1 is involved in cell migration during wound healing. The ability of keratinocytes to express and synthesize MMP-1 is closely related to the re-epithelization of wounds. In our study, the reduction

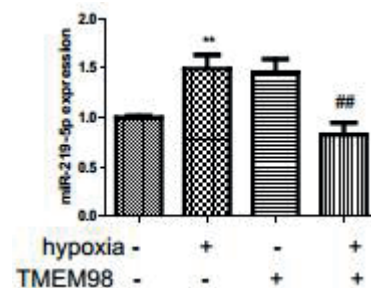


Figure 5. miR-219-5p expression level. The results are the mean±SD (n=10), ***p*< 0.05 vs. Control group, ##*p*< 0.05 vs. hypoxia group.

of TMEM98 protein accompanies with lack of migration and proliferation was demonstrated, and TMEM98 overexpression significantly reversed these effects. The above results indicated the important role of TMEM98 on wound healing.

Database analysis of altered miRNAs was mainly involved in MAPK and Wnt signaling pathways, which affected wound healing^{20,21}. Another group of researchers analyzed differences in expression of miRNAs on the 7th day in both groups and found that the expression of miR-125b-5p, miR-497, and miR-31 were

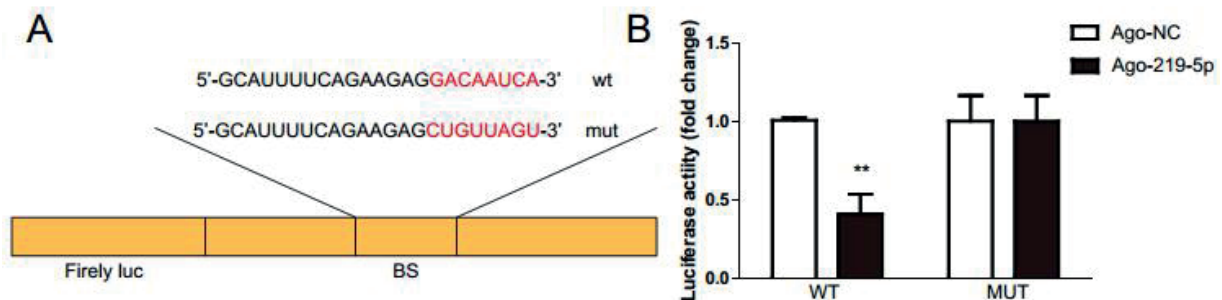


Figure 6. microRNA-219-5p might inhibit wound healing by targeting TMEM98. (A) Vector construction. (B) Luciferase activity assay. The results are the mean±SD (n=10), ***p*< 0.05 vs. Control group.

decreased in diabetic wounds²². In the previous study, it was found that, in the streptozotocin (STZ)-induced diabetic rat model, Dicer's mRNA and protein levels were decreased at the 7th and 12th day after the wound compared to the normal group, and Dicer was transfected in a concentration gradient in HaCaT cells. The results of siRNA showed that wound healing was retarded as the inhibitory intensity of Dicer increased, indicating that certain miRNAs are important for wound healing²³. However, in our work, overexpression of miR-219-5p in hypoxia group indicates a potential role of miR-219-5p in wound healing, which offers alternative for the treatment of wound healing²⁴.

Conclusions

We demonstrated an important role of TMEM98 on inhibiting inflammatory response, promoting cell proliferation and migration. MiR-219-5p impedes wound healing by targeting TMEM98, which provides new insights for the future wound therapy.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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