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MicroRNA-17-3p promotes keratinocyte cells growth and metastasis via targeting MYOT and regulating Notch1/NF-κB pathways

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Wound healing is a fundamental biological process to restore skin integrity. The role of microRNAs (miRNAs) during this process remains elusive. Thus, our study aimed to investigate the biological functions and its molecular mechanisms of miR-17-3p in cutaneous wound healing. Human keratinocyte cell line HaCaT was transfected with miR-17-3p mimic, antisense oligonucleotides (ASO)-miR-17-3p and corresponding controls respectively. After transfection, MTT, flow cytometry, qRT-PCR and western blot were performed to analyze cell viability, colony-formation and cell cycle. Next, scratch wound, Transwell and western blot were used to examine cell migration ability. Besides, prediction of miR-17-3p target was performed by TargetScan and microRNA database, dual-luciferase reporter assay, qRT-PCR and western blot. Moreover, the functions of the MYOT on cell proliferation and metastasis were detected by transfection with its expression vector. Signal pathways of Notch1 and NF-κB were performed by qRT-PCR and western blot. Results showed that miR-17-3p overexpression distinctly promoted cell viability, colony formation, arrested cells at G2/M phase, and upregulated cyclin D1, cyclin B1 and CDK2. Simultaneously, miR-17-3p overexpression increased cell migration and downregulated E-cadherin but upregulated vimentin and α-SMA expression. Moreover, MYOT was verified as a target of miR-17-3p, and it remarkably inhibited HaCaT cells proliferation and metastasis. Protein expression levels of cyclin D1, cyclin B1, CDK2, vimentin and α-SMA were downregulated while E-cadherin was upregulated by MYOT. Furthermore, miR-17-3p signally activated Notch1/NF-κB pathways. These data demonstrated that miR-17-3p promoted keratinocyte cells proliferation and metastasis via targeting MYOT and activating Notch1/NF-κB signal pathways in cutaneous wound healing.

1. Introduction

The skin is the largest human organ and plays a natural protective role in the intentional or accidental injury (Chen et al. 2015). Skin wound healing is a complicated process that can be divided into inflammatory reaction, proliferation and maturation of newly formed tissue (Lofrumento et al. 2016). It has been reported that re-epithelialization is a crucial process, which requires proper cell proliferation and migration of keratinocytes at the periphery of the wound healing (Seeger and Paller 2015). Despite the most recent advances in cutaneous wound treatment, up to 50% of wounds remain incurable (Chen et al. 2012). These complex pathogenesis and biological processes of cutaneous wound healing make it challenging to develop effective therapies.

MicroRNAs (miRNAs) are endogenous small non-coding RNA molecules which can regulate gene expression at the post-transcriptional (Hecker et al. 2013). Several studies certified that miRNAs play an important role in various diseases and are associated with biological functions (Hruška 2014). In terms of cutaneous wound healing, it has been proved that miR-31 as a key regulator promoted keratinocyte proliferation and migration during wound healing (Li et al. 2015). Similarly, Yu et al. (2010) demonstrated that miR-205 promoted keratinocyte migration via the lipid phosphatase SHIP2 (Yu et al. 2010). Besides, miR-21, miR-Let-7p, miR-182 and miR-221 also have been found to participate in the regulation of skin biology process (Banerjee and Sen 2011; Wang et al. 2012; Wu et al. 2017).

MiR-17-3p is a member of the miR-17-92 cluster which expresses six miRNA precursors and plays important roles in gene regulation (Shan et al. 2013). Previous studies indicated that miR-17-3p as a suppressive factor, could regulate cell proliferation, metastasis and apoptosis in all kinds of diseases (Velez and Yong 2011). For instance, miR-17-3p inhibited angiogenesis by downregulating FIK-1 in cell growth (Yin and Zhang 2013). In addition, miR-17-3p inhibited cell apoptosis in cardiac fibroblasts by targeting Par4 (Du et al. 2015). However, less is known about how miR-17-3p regulates cell proliferation and migration in cutaneous wound healing. Thus, our study aimed to uncover the roles of miR-17-3p in cell growth and metastasis in cutaneous wound healing. Human keratinocyte cell line HaCaT was transfected with miR-17-3p mimic, antisense oligonucleotides (ASO)-miR-17-3p and corresponding controls. Subsequently, cell viability, cell cycle, colony-formation and migration were assessed. A target of miR-17-3p has been predicated with the help of bioinformatics software, and their targeting regulatory relations were verified *in vitro*. Besides, the correlative signaling pathways were investigated to reveal the potential mechanisms. This study may provide opportunities for a development of miRNA-targeted therapies for cutaneous wound healing.

2. Investigations and results

2.1. MiR-17-3p promotes keratinocyte cells proliferation

To explore the roles of miR-17-3p in cutaneous wound healing, human keratinocyte cells were transfected with miR-17-3p mimic, ASO-miR-17-3p or corresponding controls. After transfection, cell viability, colony formation, cell cycle and cyclin-CDK genes (cyclin B1/D1 and CDK2) were analyzed. MTT assay results showed that cell viability was time-dependently improved by miR-17-3p overexpression, whereas ASO-miR-17-3p resulted

in an opposite effect (Fig. 1A, $P < 0.05$ or $P < 0.01$). Colony formation assay showed similar results. The number of colonies was increased by miR-17-3p overexpression but was reduced by ASO-miR-17-3p (Fig. 1B, $P < 0.05$).

Additionally, cell cycle was detected by flow cytometry. The percentage of cell population showed a slight increase at G2/M phase in miR-17-3p overexpression compared with its control (Fig. 1C). Furthermore, qRT-PCR and western blot showed that both the mRNA and protein levels of cyclin B1, cyclin D1 and CDK2 were upregulated by miR-17-3p overexpression, while were downregulated by ASO-miR-17-3p (Fig. 1D and 1E, $P < 0.05$ or $P < 0.01$). In summary, these results indicated that miR-17-3p promoted cell proliferation in cutaneous wound healing.

2.2. MiR-17-3p enhances keratinocyte cells metastasis

To investigate whether miR-17-3p has any effect on cell metastasis, scratch wound and Transwell assays were performed. Scratch wound assay showed that the relative migration distances between wound edges of miR-17-3p overexpression groups were markedly wider than its control in HaCaT cell line from 24 h post-scratch, but ASO-miR-17-3p showed contrary results (Fig. 2A, $P < 0.05$ or $P < 0.01$). In addition, the migration cell numbers were obviously increased by miR-17-3p overexpression, while decreased by ASO-miR-17-3p (Fig. 2B, $P < 0.05$). Besides, western blot results in Fig. 2C showed that, miR-17-3p overexpression signally suppressed E-cadherin expression level, but promoted vimentin

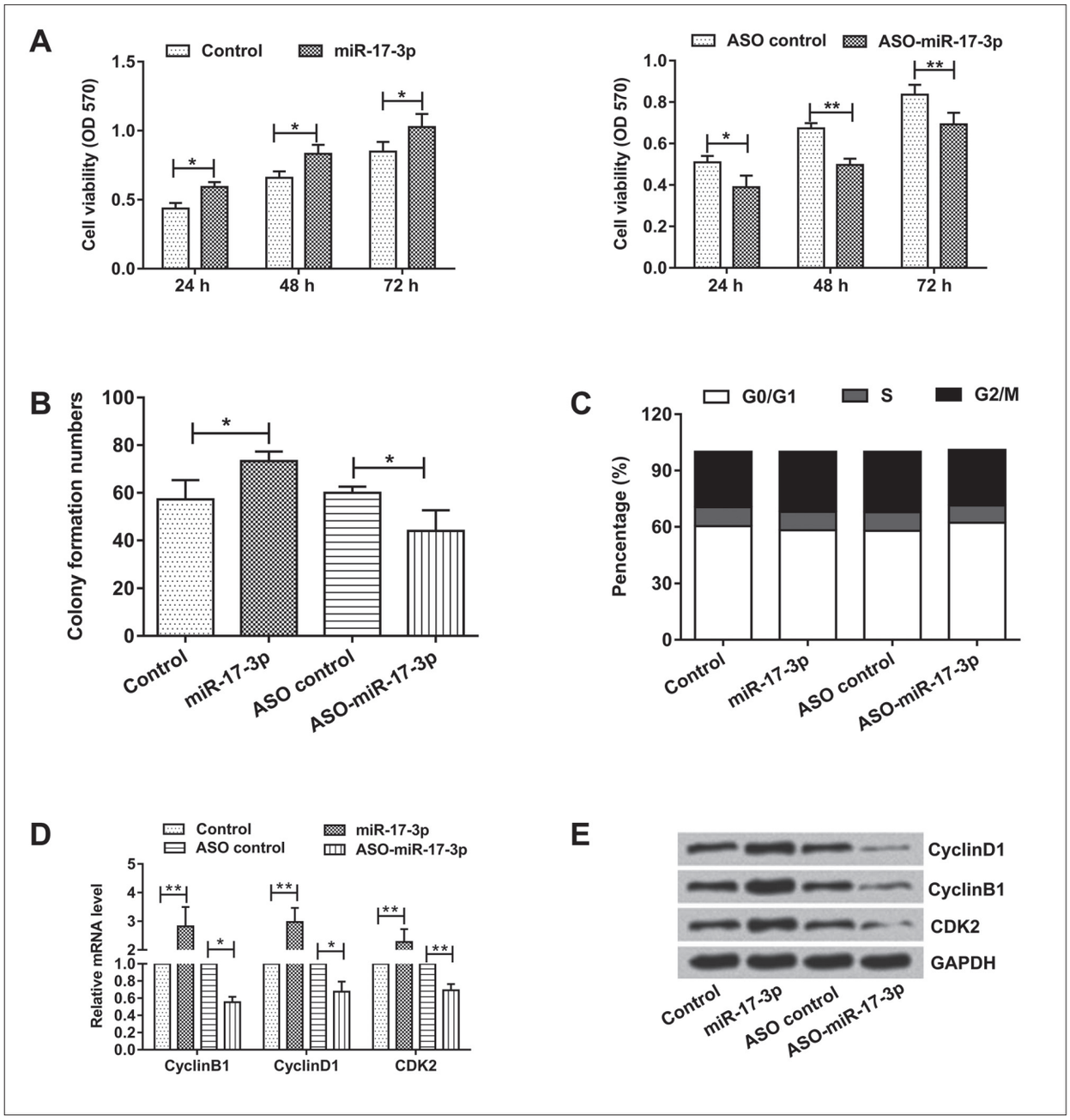

Fig. 1: MiR-17-3p promoted keratinocyte cells proliferation. Keratinocyte cell line HaCaT was transfected with miR-17-3p mimic, ASO-miR-17-3p and corresponding controls. After 48 h of transfection, (A) Cell viability was detected by MTT; (2) Colony formation capacity conducted by colony formation assay; (C) Cell cycle was examined by flow cytometry; (D and E) The mRNA and protein levels of cyclin D1, cyclin B1 and CDK2 were detected by qRT-PCR and western blot. MiR-17-3p: microRNA-17-3p; ASO: antisense oligonucleotides; MTT: 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide; CDK2: cyclin-dependent kinase 2; qRT-PCR: quantitative real time polymerase chain reaction; * $P < 0.05$, ** $P < 0.01$.

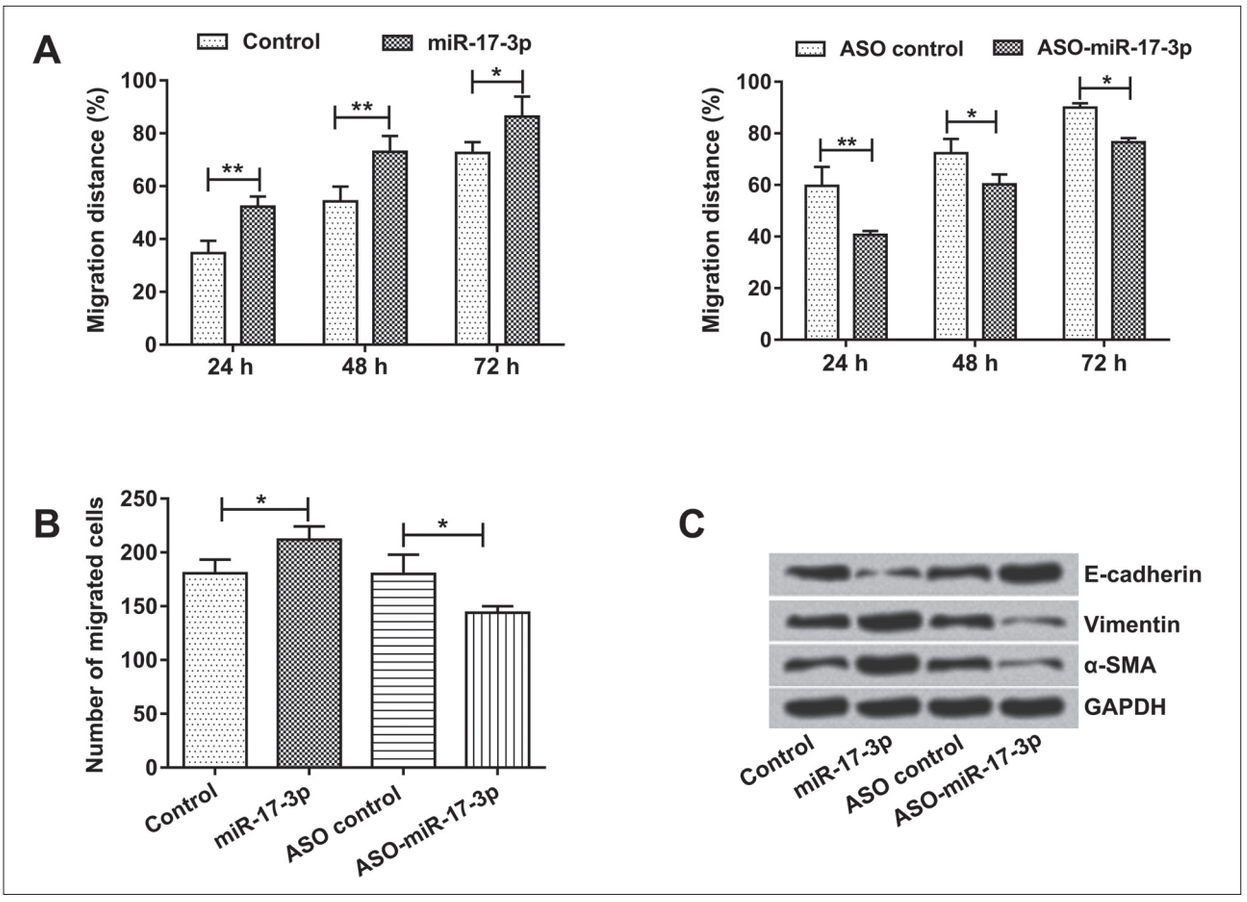

Fig. 2: MiR-17-3p promoted keratinocyte cells metastasis. (A) Migration distance was evaluated by scratch wound assay; (B) Cell migration was detected by Transwell assay; (C) The protein levels of E-cadherin, vimentin and α-SMA were assessed by western blot. MiR-17-3p: microRNA-17-3p; α-SMA: alpha smooth muscle actin. * $P < 0.05$, ** $P < 0.01$.

and α-SMA expressions. Overall, miR-17-3p promoted cell metastasis in cutaneous wound healing.

2.3. MYOT is a direct target of miR-17-3p

It is generally understood that miRNAs regulate their targets via binding to their 3'UTR (Niniomany et al. 2013). To find the target gene which participates in regulating cell motility triggered by miR-17-3p, bioinformatics software of TargetScan (http://www.targetscan.org/) and microRNA database (http://www.microrna.org/) were used. As demonstrated in Fig. 3A, MYOT 3'UTR (nucleotides 221-228) was highly conserved for miR-17-3p. Moreover, dual-luciferase reporter assay revealed that miR-17-3p overexpression significantly decreased the luciferase activity of reporter genes which contains 3'UTR-Wt of MYOT, but did not suppress that of the reporter fused to the Mut version (Fig. 3B, $P < 0.01$). In addition, MYOT mRNA and protein expression levels in transfected cells of miR-17-3p mimic or ASO-miR-17-3p were detected by qRT-PCR and western blot. Results showed that both the mRNA and protein levels of MYOT were downregulated by miR-17-3p overexpression (Fig. 3C and 3D, $P < 0.05$ or $P < 0.01$). Taken together, MYOT was a direct target of miR-17-3p and was negatively regulated by miR-17-3p.

2.4. MYOT inhibits keratinocyte cells proliferation and metastasis

To investigate if MYOT has similar functions as miR-17-3p in cutaneous wound healing, a RNA-based approach was used. Results showed that MYOT notably suppressed cell viability and also decreased the colony formation numbers (Fig. 4A and 4B, $P < 0.05$ or $P < 0.01$). Western blot result displayed in Fig. 4C, MYOT consistently reduced the cyclin D1, cyclin B1 and CDK2 expressions. Furthermore, MYOT remarkably inhibited cell migration distance and decreased migrated cells numbers (Fig. 4D and 4E). As well as, MYOT promoted E-cadherin expression level but inhibited vimentin and α-SMA expressions (Fig. 4F). In sum, MYOT could inhibit keratinocyte cells proliferation and metastasis.

2.5. MiR-17-3p activates Notch1/NF-kB pathways

It was demonstrated that Notch1 and NF-κB signaling pathways could regulate the expression of miRNAs (Jing et al. 2015). However, whether Notch1 and NF-κB signaling pathways are involved in the regulation of miR-17-3p-mediated biofunction is still unclear. qRT-PCR and western blot results illustrated that miR-17-3p overexpression significantly upregulated Notch1, NICD, IκB-α and p65 expressions ($P < 0.05$ or $P < 0.01$). Conversely, miR-17-3p suppression downregulated these four factors (Fig. 5A-5D). In a word, our results demonstrated that Notch1 and NF-κB pathways were activated by miR-17-3p in cutaneous wound healing.

3. Discussion

In this study, we first demonstrated the effects of miR-17-3p on cutaneous wound healing. These results showed that miR-17-3p overexpression promoted cell proliferation, colony formation, migration and induced G2/M arrest. Several critical cyclin-CDK genes (cyclin D1, cyclin B1 and CDK2) were upregulated by miR-17-3p overexpression. And the epithelial mesenchymal

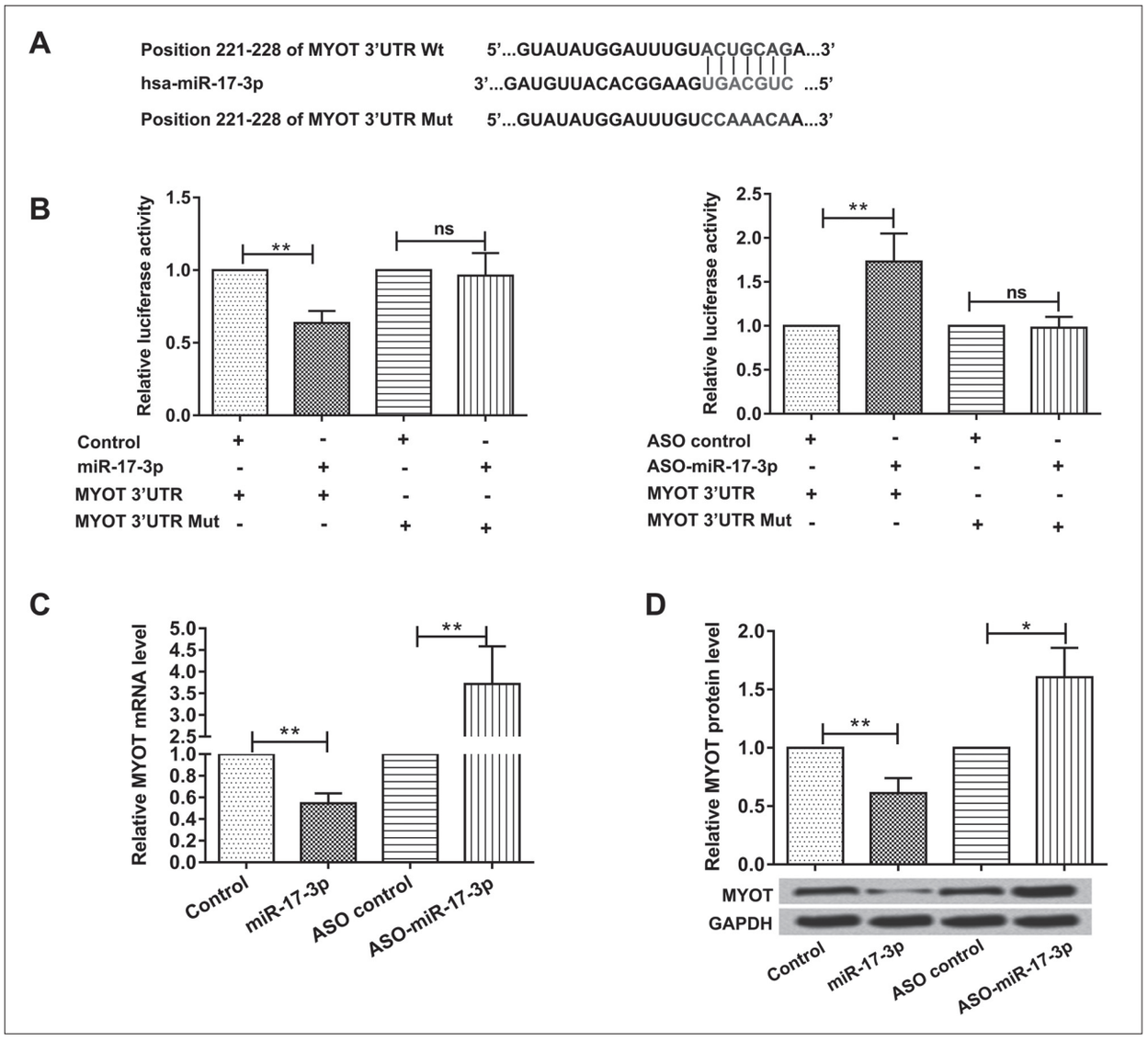

Fig. 3: MYOT was a direct target of miR-17-3p. (A) MYOT was predicated as a target of miR-17-3p by using TargetScan and microRNA database; (B) Dual-Luciferase reporter assay was executed to verify MYOT was a direct target of miR-17-3p; (C and D) The mRNA and protein expression levels of MYOT were detected by qRT-PCR and western blot. MYOT: myotilin; miR-17-3p: microRNA-17-3p; qRT-PCR: quantitative real time polymerase chain reaction. $^{*}P < 0.05$, $^{**}P < 0.01$.

transition (EMT)-associated gene E-cadherin was downregulated but vimentin and α-SMA were upregulated by miR-17-3p overexpression. Most importantly, we found MYOT was a direct target of miR-17-3p and it inhibited cell proliferation and metastasis. Besides, miR-17-3p remarkably activated Notch1/NF-κB pathways.

As a new type of regulatory factor, miRNA has been widely studied in recent years. MiRNAs play an important role in cell proliferation, differentiation, migration, invasion and regeneration (Tanaka et al. 2013). The role of miR-17-3p in many diseases has received the attention from many research groups (Xie 2014). In this regard, miR-17-3p has been reported to promote cell proliferation in multiple myeloma (Shunye et al. 2015). In addition, miR-17-3p enhanced glycolysis pathway by inhibiting mitochondrial metabolism which have been reported in prostate cancer cell (Velez and Yong 2011). However, the role and mechanism of miR-17-3p in cutaneous wound healing remain unclear. Our study first proved that miR-17-3p overexpression promoted cell viability, colony formation and migration.

Current studies suggested that cell cycle and EMT are closely related to cell proliferation and metastasis (Tao et al. 2016). cyclin-CDK genes (cyclin D1, cyclin B1 and CDK2) and EMT-related genes (E-cadherin, vimentin and α-SMA) are key mediators in these biological processes (Cebon et al. 2012; Xu et al. 2017). In our study, the levels of cyclin D1, cyclin B1, CDK2, vimentin and α-SMA were consistently upregulated by miR-17-3p overexpression. These results further indicated that miR-17-3p dramatically promoted cell proliferation and migration via regulating cell cycle and EMT process.

MYOT is a structural protein that induces the formation of actin bundles *in vitro* (Shalaby et al. 2009). A previous study demonstrated that MYOT play a vital role in skeletal muscle, cardiac muscle and peripheral nerves (Nandelstadh et al. 2009). Moreovers, several mutations MYOT have been identified in myofibrillar myopathy (MFM), spheroid body myopathy (SBM) and limb girdle muscular dystrophy (LGMD). However, it is not yet known the effects of MYOT on cell biological processes in wound healing. Our study confirmed that MYOT inhibited cell viability, colony-formation, migration. Meanwhile, cyclin-CDK genes and EMT-associated genes were also downregulated by MYOT. In sum, MYOT might act as a suppressor gene inhibited cell proliferation and metastasis. Whereas, the underlying mechanism of pathology still need further investigation.

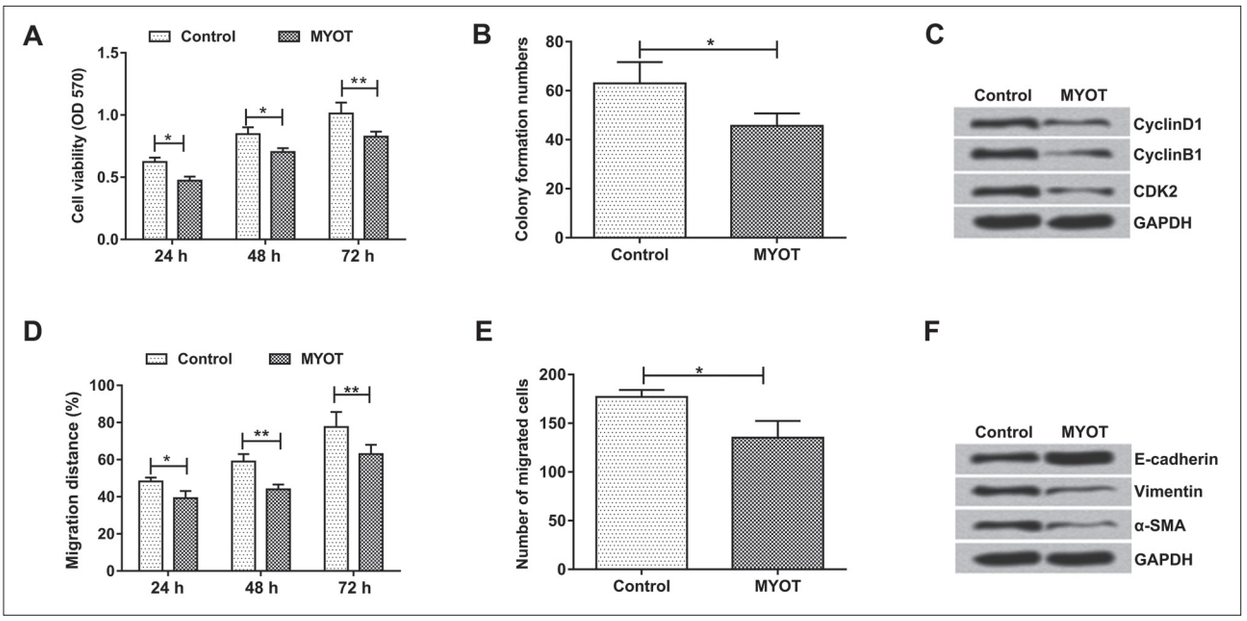

Fig. 4: MYOT inhibited keratinocyte cells proliferation and metastasis. HaCaT cells were transfected with MYOT expression vector or its control. (A) Cell viability was analyzed by MTT; (B) Colony formation capacity was performed by colony formation assay; (C) The protein levels of cyclin D1, cyclin B1 and CDK2 were measured by western blot; (D) Migration distance was evaluated by scratch wound assay; (E) Cell migration was detected by Transwell assay; (F) The protein levels of E-cadherin, vimentin and α-SMA were assessed by western blot. MYOT: myotilin; MTT: 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyltetrazolium bromide; CDK2: cyclin-dependent kinase 2; α-SMA: alpha smooth muscle actin. * $P < 0.05$, ** $P < 0.01$.

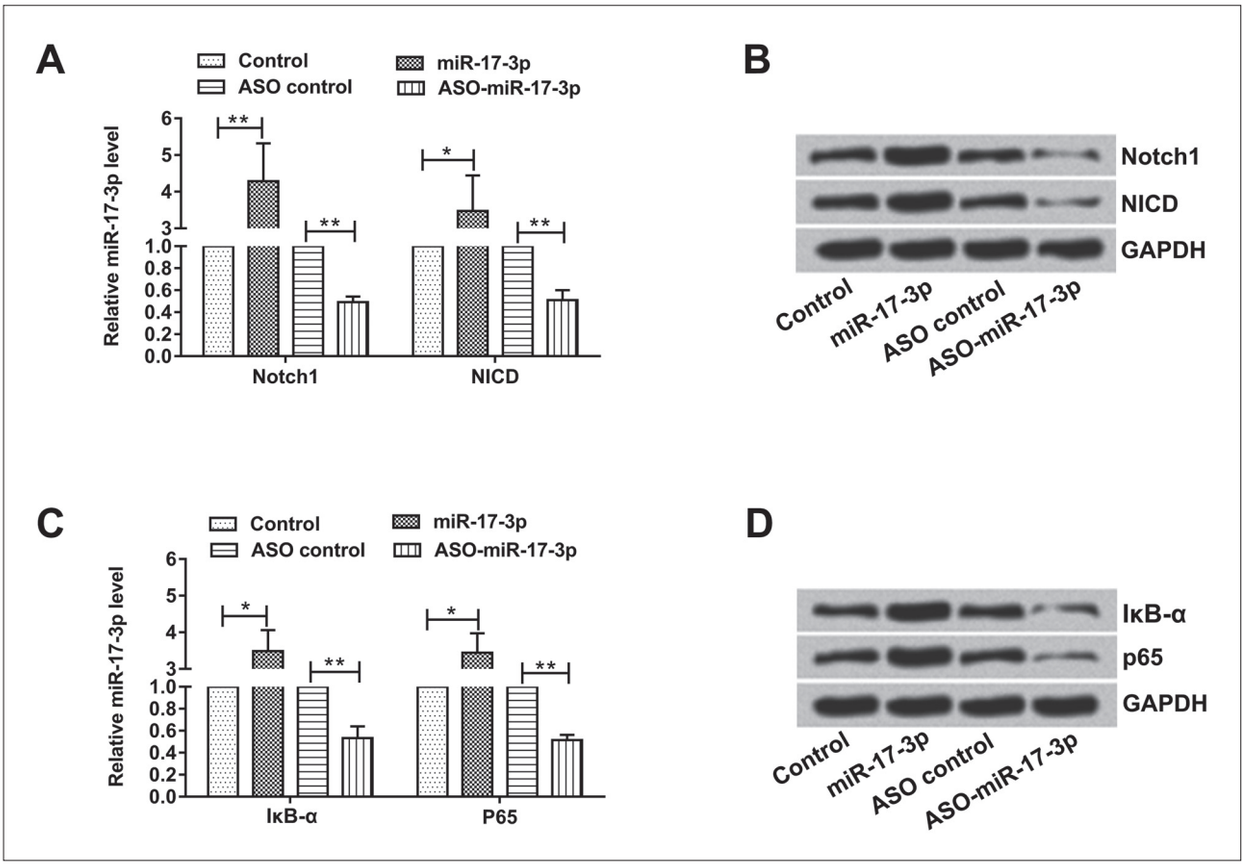

Fig. 5: MiR-17-3p activated Notch1 and NF-κB pathways. HaCaT cells were transfected with miR-17-3p mimic, ASO-miR-17-3p and corresponding controls. Then (A and B) the mRNA and protein levels of Notch1 and NICD were determined by qRT-PCR and western blot; (C and D) the mRNA and protein levels of IκB-α and p65 were detected by qRT-PCR and western blot. MiR-17-3p: microRNA-17-3p; ASO: antisense oligonucleotides; qRT-PCR: quantitative real time polymerase chain reaction. * $P < 0.05$, ** $P < 0.01$.

There are suggestions that miRNAs could regulate Notch1 and NF-κB signaling pathways thereby affecting cell proliferation and migration on several diseases (Zheng 2012). Pang et al. (2010) reported that miR-34a suppressed cell invasion through down-regulation of Notch1 in cervical carcinoma. Kumar et al. (2014) demonstrated that miR-223 expression was regulated by Notch and NF-κB signaling pathways in T-cell acute lymphoblastic leukemia. In terms of cutaneous wound healing, it has been proven that Notch activation extended the ability to promote cell proliferation, differentiation and migration of keratinocytes (Yan et al. 2015). NF-κB activation occurs in the region of the wound edge during keratinocyte wound healing (Na et al. 2016). Compared with previous studies, our study displayed that miR-17-3p overexpression significantly upregulated Notch1, NICD, IκB-α and p65 expressions, indicating that miR-17-3p could promote cell proliferation and migration via activating Notch1 and NF-κB pathways. In conclusion, our results demonstrated that miR-17-3p promoted cell proliferation and migration via targeting MYOT and activating Notch1 and NF-κB signaling pathways. These results will provide a potential therapeutic intervention for the treatment of cutaneous wounds. Nevertheless, further research is needed to clarify the mechanism and expand the application domain of miR-17-3p in therapeutics.

4. Experimental

4.1. Cell culture and transfection

Human keratinocyte cell line (HaCaT) was purchased from the American Type Culture Collection (ATCC, Manassas, VA). All cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco, Grand Island, NY) supplemented with 1% non-essential amino acid (NEAA, Gibco) and 10% fetal bovine serum (FBS, Gibco) at 37 °C with 5% CO_2.

HaCaT cells were transfected with miR-17-3p mimic, ASO-miR-17-3p and their corresponding controls. These plasmids were synthesized by Genepharma (Shanghai, China). These cells were seeded in 6-well plates and cultured overnight at 37 °C in 5% CO_2. Cell transfections were performed by using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions for 48 h. Moreover, the complimentary DNA (cDNA) of myotilin (MYOT) was clone into pcDNA3.1 vector (Sangon Biotech, Shanghai, China) for overexpression of MYOT. The empty pcDNA-3.1 plasmid was used as a negative control.

4.2. Cell viability assay

Cell viability was measured using the 3-(4,5-dimethyl-2-thiazolyl)-2, 5-diphenyltetrazolium bromide (MTT) assay as described previously (Arseculeratne et al. 2007). In brief, 48 h after transfection, cells were seeded in 96-well plates with a density of 5 × 10^3 cells per well. After incubation at 37 °C for 24, 48 and 72 h, 10 µl of 0.5 mg/ml MTT (Sigma-Aldrich, St. Louis, Mo, USA) were added into each well and incubated for another 4 h. Then, 150 ml dimethyl sulfoxide (DMSO; Sigma-Aldrich) was added to dissolve formazan. After incubation for a further 10 min at 37 °C, absorbance at 570 nm for each sample was measured by a microplate reader (Bio-rad, Hercules, CA, USA).

4.3. Colony formation assay

Three hundred cells of logarithmic phase were planted into 12-well plates and transient transfection for 48 h. Then cells were cultured for 14 days, and the culture medium should be replaced with fresh medium every 3-4 days. Subsequently, the cells were stained with 400 µl of 0.1% crystal violet (Merck, Darmstadt, Germany) for 15-30 min, and colonies including more than 50 cells were counted. The images were captured using an inverted microscope (Olympus, Tokyo, Japan). Three replicate wells were included for each group. All experiments were performed independently at least three times (Borowicz et al. 2014).

4.4. Cell cycle analysis

Cell cycle analysis was performed by using the Cell Cycle and Apoptosis Analysis Kit (Beyotime, Shanghai, China) according to the manufacturer's instructions. In brief, HaCaT cells were harvested after 48 h of transfection with miR-17-3p mimic, ASO-miR-17-3p and their corresponding controls. Next, these cells were washed twice with phosphate-buffered saline (PBS) and fixed in 70% ethanol at 4 °C overnight. Subsequently, cells were re-suspended in 500 µl of PBS containing 0.2 mg/ml RNAse A and 50 µg/ml PI for 30 min in the dark at room temperature. The percentages of cells occupying the different phases (G0/G1, S, and G2/M) of the cell cycle were counted and compared using FACScan flow cytometer (Becton Dickinson, San Jose, USA).

4.5. Scratch wound assay

Scratch wound assay was performed to analyze the migration ability of miR-17-3p-transfected cells. Briefly, HaCaT cells were seeded in 6-well plate and cultured to upon 80-90% confluent. A scratch wound was made by a 10 µl pipette tip on the monolayer, then the cells were washed three times with PBS and added serum-free medium for the incubation of 24 h. Image analysis was measured by Image-Pro Plus software version 6.0 (Media Cybernetics, Bethesda, MD). The scratch wound closure percent was calculated as (the scratch area before incubation – the scratch area after incubation) / (the scratch area before incubation) × 100%.

4.6. Cell migration assay

The cell migration assay of HaCaT cells *in vitro* was performed by the Transwell system with 8-µm pore size (Millipore, Darmstadt, Germany). Cells were transfected with miR-17-3p mimic, ASO-miR-17-3p and corresponding controls. After transfection, 5 × 10^4 cells suspension was added to the upper chamber with serum-free media. The lower chamber was filled with complete medium. After 12 h of incubation, cells were fixed with 4% methanol (NIST, USA) for 30 min. Adhered cells to the upper surface of the insert were removed, and those on the under surface were stained with 0.2% crystal violet (Sigma-Aldrich) for 20 min. The images were captured using inverted microscope (Olympus).

4.7. Luciferase reporter assays

Luciferase reporter plasmids containing 3'untranslated region (3'UTR) of MYTO and empty luciferase vectors were obtained from Promega (Fitchburg, WI, USA). These vectors were co-transfected with 10 nM miR-17-3p mimic or its control into cells using Lipofectamine 2000 (Invitrogen). Luciferase activity was analyzed using Dual-Luciferase Reporter Assay System (Berthold Centro, USA) after 48 h post-transfection following the manufacturer's instructions.

4.8. Quantitative real time polymerase chain reaction (qRT-PCR)

Total RNA was collected from human keratinocyte cells by using TRIzol reagent (Invitrogen). First strand cDNA was used for reverse transcription with mRNA Selective PCR kit (Takara). qRT-PCR was performed by a SYBR Green assay with Applied Biosystems 7500 Fast Real-time PCR system. All primers were synthesized by GenePharma (Shanghai, China). Expression values were normalized to GAPDH expression. The data were calculated with the $2^{-\Delta\Delta Ct}$ method.

4.9. Western blot

Protein from the transfected cells was lysed in Radio Immunoprecipitation Assay (RIPA) lysis buffer (Beyotime) and the protein concentration was measured using the Bradford method (Carlsson et al. 2011). A total of 25 µg protein samples were separated by 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then were transferred to a nitrocellulose membrane (BioRad, Hercules, CA, USA). The membranes were blocked by 2.5% non-fat milk for 1 h at 37 °C, and then incubated with primary antibodies of cyclinB1 (ab32053), cyclinD1 (ab134175), cyclin-dependent kinase 2 (CDK2; ab6433), E-cadherin (ab1416), vimentin (ab92547), alpha smooth muscle actin (α-SMA; ab5694), MYOT (ab68915) and GAPDH (ab8245) (Abcam, Cambridge, UK) at 4 °C overnight. After incubating with horseradish peroxidase-conjugated secondary antibody (Boster Corporation, Wuhan, Hubei, China) for 1 h at room temperature, protein bands were visualized using an enhanced chemiluminescence detection system (Amersham, Little Chalfont, UK).

4.10. Statistical analysis

Statistical significance was determined using *t*-test and one-way analysis of variance (ANOVA) by GraphPad Prism 5.0 software (GraphPad software, Inc., La Jolla, Calif). Mean differences were considered statistically significant if $P < 0.05$. Data were shown as mean±standard errors of the mean (SEM). All experiments were repeated at least three times in triplicate.

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