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MiR-20a-3p regulates TGF-β1/Survivin pathway to affect keratinocytes proliferation and apoptosis by targeting SFMBT1 in vitro

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Abstract
Psoriasis is a common immune-mediated chronic inflammatory skin disease characterized by abnormal keratinocyte proliferation, differentiation and apoptosis. However, the exact etiology and pathogenesis are still unclear. Evidence is rapidly accumulating for the role of microRNAs in psoriasis. It has been demonstrated that Interleukin-22 (IL-22) plays vital role in T cell-mediated immune response by interacting with keratinocytes in the pathogenesis of psoriasis. The aim of our study
was to explore the possible functional role of miR-20a-3p in psoriasis and in IL-22 induced keratinocyte proliferation. Here, we found that miR-20a-3p was down-regulated in psoriatic lesions and in HaCaT cells (human keratinocyte cell line) treated by IL-22 stimulation. Functional experiments showed that overexpression of miR-20a-3p in HaCaT cells suppressed proliferation and induced apoptosis while its knockdown promoted cell proliferation and reduces cell apoptosis. Mechanistically, SFMBT1 was identified as the direct target of miR-20a-3p by dual luciferase reporter assay. SFMBT1 knockdown was demonstrated to inhibit cell growth and induced apoptosis, which was consistent with the function of miR-20a-3p upregulation in HaCaT cells. In addition, results of western blot analysis showed that miR-20a-3p upregulation or SFMBT1 knockdown changed the protein expression levels of TGF-β1 and survivin. Our findings suggest that miR-20a-3p play roles through targeting SFMBT1 and TGF-β1/Survivin pathway in HaCaT cells, and loss of miR-20a-3p in psoriasis may contribute to hyperproliferation and aberrant apoptosis of keratinocytes.

**Keywords:** miR-20a-3p; HaCaT cells; psoriasis; SFMBT1; TGF-β1; Survivin

1. Introduction

Psoriasis is a common chronic inflammatory skin disease that affects about 2% to 3% of the worldwide population [1,2]. Psoriatic lesions are characterized by dysfunctional epidermal keratinocytes including hyperproliferation, abnormal cell differentiation and apoptosis [3,4]. MicroRNAs (miRNAs) are small single-stranded noncoding RNAs (18–25nt), which play a key role in cellular process including proliferation, differentiation, apoptosis, invasion and metabolism [5]. They can negatively regulate
gene expression through translational repression by binding to the 3’ untranslated region (UTR) of the target mRNA [6,7]. Recently, an increasing miRNAs abnormal expression was reported in psoriasis, and understanding the role of miRNAs may provide potential insight in the pathogenesis and treatment of psoriasis [8].

Evidence indicates that immune system plays a critical role in the pathogenesis of psoriasis, and numerous inflammatory cytokines (such as IL-17, IL-22 etc) produced by immune cells contribute to the proliferation of keratinocytes [3,5]. Interleukin-22 (IL-22) is mainly produced by Th17 and Th22 cells. Increased levels of IL-22 have been detected in lesional skin and blood of patients with psoriasis [9-11]. IL-22 receptor is exclusively expressed on epithelial cells rather than immune cells and therefore is the uniquely communication between the immune system and keratinocytes [12]. IL-22 could promote keratinocyte proliferation and lead to aberrant cell differentiation in vitro [13]. All evidence suggests that IL-22 plays a pivotal role in the pathogenesis of psoriasis.

Using miRNA microarrays, we identified 116 differentially expressed miRNAs in the psoriasis skin tissues and healthy skin, of which we found that miR-20a-3p was downregulated in psoriatic lesions. In our previous work, we performed a microarray analysis to identify changes in miRNA and mRNA profiles in human keratinocytes exposed to IL-22, which plays a pivotal role in the pathogenesis of psoriasis[14]. Of interest, we found that miR-20a-3p was also decreased in human keratinocytes treated with IL-22 compared with the control group. Therefore, we selected miR-20a-3p to further study and investigated the possible functional role of miR-20a-3p in psoriasis and provided a novel molecular basis for IL-22 induced keratinocyte proliferation.

The effects of miR-20a on the proliferation, migration, and invasion in tumor cells have been studied for a long time [15]. However, the effects of miR-20a-3p was rarely reported in studies. MiR-20a-3p, as miRNA passenger strand, does not accumulate to higher levels than its complementary strand (guide strand) [16]. It was elevated in plasma of HT (Hashimoto’s thyroiditis) patients and related with TgAb level [17]. Aberrant expression of miR-20a-3p was also reported in patients with β-thalassemia and older breast cancer [18,19]. But the exact mechanism need to be further explored. In this study, our results suggest that decreased expression of miR-20a-3p may play a role in the pathogenesis of psoriasis and may be a novel therapeutic target for psoriasis.

2. Materials and methods

2.1 Clinical samples

Patients in the study were diagnosed with vulgaris psoriasis histopathological at the department of Dermatology, Qilu Hospital. Recruited patients did not have other coexistent autoimmune disorders, acute or chronic infections, and malignancies. Patients were excluded from our study if they had received systemic treatment, immunosuppressive drugs, or phototherapy during the past 1 months. Samples of normal skin tissue were obtained from plastic surgery of Qilu Hospital. The study was
approved by the Ethics Committee of Shandong University (China), and informed consent was obtained from all subjects included in the study.

2.2 Cell culture
HaCaT cells were cultured in DMEM (Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Sangon Biotech, Shanghai, China), 100 U/ml penicillin and 100µg/ml streptomycin. All cells were incubated at 37°C in a humidified condition supplemented with 5% CO2.

2.3 Cytokine stimulation
HaCaT cells were seeded into a six-well plate (5×10^5 cells/well). When it grew to approximately 70% confluent, the cells were starved in serum-free DMEM for 12h and then stimulated with IL-22 (100ng/ml, proteintech) or TGF-β1(20ng/ml, R&D Systems) in serum-free DMEM. The concentration of IL-22 was all used 100ng/ml in the light of previous experiments [14]. The cells were then harvested for extraction of RNA or protein.

2.4 Cell transfection
MiR-20a-3p mimics or inhibitor and their negative control were designed and synthesized by RiboBio Inc. (Ribobio, Guangzhou, China). The sequences of miR-20a-3p mimics: sense strand: 5’ ACUGCAUUAUGAGCACUUAAAG 3’ and antisense strand: 5’ CUUUAAGUCUCUAUAUGCAGU 3’; miR-20a-3p inhibitor is single-strand: 5’ CUUUAAGUCUCUAUAUGCAGU 3’. The siRNA targeting SFMBT1 was designed and synthesized by Shanghai GenePharma. HaCaT cells were transfected with miR-20a-3p mimics and its negative control at a final concentration of 100nM or miR-20a-3p inhibitor and its negative control at a final concentration of 200nM. The siRNA for SFMBT1 (50nM) and non-targeting siRNA (siNC) (50nM) used as negative control, were transfected into cells using Lipofectamine 2000 Transfection Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. The transfection efficiency was measured by qRT-PCR or western blot analysis.

2.5 Cell proliferation, cell cycle and apoptosis assays
Cell proliferation was measured by a Cell Counting Kit-8 (CCK-8) (Beyotime, Shanghai, China) and 5-Ethynyl-2’- deoxyuridine (EDU) incorporation assay (RiboBio, Guangzhou, China). In CCK-8 assay, the HaCaT cells were seeded in 96-well plates at a density of 1×10^4 cells/well. When grew to 30-50% confluency, the cells were transfected with miRNA or siRNA. After transfecting for 24, 48 and 72h, CCK-8 (10 µl) was added to each well, and then the plates were incubated for 1 h at 37°C. Absorbance was measured at a wavelength of 450 nm. DNA synthesis in proliferating cells was determined by measuring 5-Ethynyl-2’- deoxyuridine (EDU) incorporation. EDU assays were performed at 24h or 48h after transfecting HaCaT cells with miRNA or siRNA in 24-well culture plates. All experimental procedures were according to the manufacturer’s instructions.

For cell cycle, transfections were done in 12-well plates (2 × 10^5 cells per well). At 24h or 48h after transfection, the cells were fixed into ice-cold 75% ethanol at -20 °C overnight. Then, the cells were washed with phosphate buffer saline (PBS), centrifuged, incubated with 0.1% RNAse A for 30 minutes at 37 °C. The cells were
subsequently incubated in 10g/ml propidium iodide (PI) at 4°C for 30 min in the dark and the cell cycle distribution was detected by flow cytometric analysis. The results were analyzed using ModFit software.

For apoptosis analysis, cells were seeded in 12-well plates (2 × 10⁵ cells per well). After transfecting with miRNA or siRNA for 24h or 48h, cells were stained with FITC-Annexin V and propidium iodide (PI) at 4°C away from light and the percentage of early and late apoptotic cells were detected using flow cytometry.

2.6 Quantitative Real-time PCR
Total RNA was extracted from cultured cells with Trizol reagent (TaKaRa Otsu, Shiga, Japan). The qRT-PCR for miRNA were performed using All-in-One™ miRNA qRT-PCR Detection Kits (GeneCopoeia, Inc, USA). The primer of miR-20a-3p and U6 were purchased from Guangzhou Genecopoeia, but the company did not provide the primer sequences. The cDNA of SFMBT1 was synthesized with the PrimeScript™ RT reagent Kit with gDNA Eraser (TaKaRa Otsu, Shiga, Japan) and Real-time PCR was performed using a SYBR Premix Ex TaqTM (Tli RNaseH Plus) (TaKaRa Otsu, Shiga, Japan). The primer sequences for siRNA were listed as follows: SFMBT1, Forward: 5’-GGAGGAAGGCTGAAGCTACG-3’ and Reverse: 5’ GTCTAATGGCTGAAGGGGC-3’; GAPDH, Forward: 5’-AACCTTGGGATTGTGGAAGG-3’, Reverse: 5’-ACACATTGGGGTAGGAACA -3’. U6 and GAPDH were used as the endogenous controls for miR-20a-3p and SFMBT1, respectively. Relative gene expression levels were calculated using 2^−ΔΔCt method.

2.7 Western blot analysis
HaCaT cells were extracted using RIPA lysis buffer with 1% PMSF and phosphatase inhibitor. Then total protein was quantified using BCA kit (Beyotime, Shanghai, China) and equal amount of protein was separated on SDS-PAGE gels. Separated protein bands were transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were then blocked with 5% fat-free milk for 1h, followed by against appropriate primary antibodies SFMBT1 (1:2000; Abcam), Cyclin D1(1:1000; Abcam), Cdk4 (1:1000; Abcam), Cdk2 (1:1000; Abcam), p21clip1(1:2000; Abcam), Phospho S811-Rb (1:1000; Abcam), TGF-β1(1:400; Abcam), Phospho-Smad3 (S423/S425) (1:1000; abways), Phospho-Smad2(S250) (1:1000; abways), Survivin (1:5000; Abcam) overnight at 4°C. The bands were subsequently incubated with HRP-conjugated secondary antibody (1:2000) at room temperature for 1h. Protein bands were visualized using ECL substrates on Amersham Imager 600 (General Electric Company, USA) and quantified by Image-Pro Plus 6.0. β-actin (1:2000, proteintech) was used as an endogenous control for normalization.

2.8 Dual-luciferase reporter assay
The DNA sequences of SFMBT1 3’-UTR were amplified and inserted into pmiR-RB-REPORTTM vectors (RiboBio, Guangzhou, China) to generate a wild-type (WT) SFMBT1 3’-UTR luciferase vector. The mutated (MUT) SFMBT1 3’-UTR luciferase vector, was generated using site-directed mutagenesis based on the WT vector. HEK293 cells were seeded into 48-well plates (4 × 10⁴ cells per well) and then co-transfected with miRNAs (50nM) and luciferase reporter plasmid ((RiboBio,
100ng) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). After 48 hours of transfection, the firefly and renilla luciferase activities were measured using the Luc-Pair Duo-Luciferase Assay Kit 2.0 (Genecopoeia, Guangzhou, China). The results of firefly luciferase reporter were used for normalization.

2.9 Immunohistochemistry analysis
Paraffin-embedded tissue sections (20 of psoriasis and 8 of normal) were cut into 5μm thick and were treated with 3% hydrogen peroxide to block endogenous peroxidase activity. Then the sections were incubated with normal goat serum for 20min, and subsequently incubated with antibodies against SFMBT1 (1:1000, HPA036153; Sigma, USA) and survivin (1:200, ab76424; Abcam, USA) at 4°C for overnight. Finally, the sections were treated with biotinylated goat anti-rabbit secondary antibodies and were analyzed using an inverted microscope.

For the evaluation of immunostaining, we only analyzed the entire epidermis. Any cells with nuclear staining positive was considered immunoreactivity for both survivin and SFMBT1. Immunostaining was defined as “high” expression if the immunoreactivity was observed in 25% or more of the cells in the whole epidermal layers, while tissue with lower percentages of immunoreactive cells (< 25%) were deemed to have “low” or "no" expression.

2.10 Statistical analysis
The results were presented as mean ± SD. The differences between groups were evaluated using two-tailed student t-test. The results of immunohistochemistry were analyzed using Fisher’s Exact Test. SPSS 20.0 (St Louis, MO, USA) was used for the statistical analysis and P< 0.05 was considered statistically significant.

3. Results

3.1 Expression of miR-20a-3p in psoriasis tissues and in HaCaT cells treated by IL-22 stimulation
Recently, a microarray analysis was conducted to detect a possible distinct miRNA expression in the psoriasis tissues (n=4) compared to healthy controls (n=4), and 116 differentially expressed miRNAs were identified. Of those, we found that miR-20a-3p was downregulated in psoriasis lesional skin (fold change of 2.35, P<0.001) (Supplementary Table S1), and then the results of microarrays were confirmed by qRT-PCR. MiR-20a-3p was shown 2.73-fold downregulation in skin lesions from psoriasis (n=6) compared with healthy controls (n=6) (Fig. 1A). In our previous work, total RNA from IL-22 treated HaCaT cells and the control were extracted and run the microarrays to study the molecular mechanism of IL-22 in the pathogenesis of psoriasis. We found 20 miRNAs were differentially expressed compared with the control (Using threshold criteria of fold changes ≥ 2 and P-values < 0.05). Among them, 5 miRNAs were down-regulated and 15 were up-regulated (Table 1) [14]. Interestingly, miR-20a-3p was also downregulated in HaCaT cells treated by IL-22 stimulation (fold change of 3.28, P<0.001), and then the results of microarrays were confirmed. MiR-20a-3p was shown 2.08-fold downregulated in HaCaT cells when stimulated with IL-22 using qRT-PCR (Fig. 1B).
Table 1. MiRNAs differentially expressed in HaCaT cells treated by IL-22 compared with the control (fold changes ≥ 2 and P-values < 0.05)

<table>
<thead>
<tr>
<th>miRNA</th>
<th>Fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Downregulated</strong></td>
<td></td>
</tr>
<tr>
<td>hsa-miR-20a-3p</td>
<td>3.28</td>
</tr>
<tr>
<td>hsa-miR-664a-5p</td>
<td>2.37</td>
</tr>
<tr>
<td>hsa-miR-3131</td>
<td>2.25</td>
</tr>
<tr>
<td>hsa-miR-378e</td>
<td>2.10</td>
</tr>
<tr>
<td>hsa-miR-4498</td>
<td>2.09</td>
</tr>
<tr>
<td><strong>Upregulated</strong></td>
<td></td>
</tr>
<tr>
<td>hsa-let-7d-3p</td>
<td>5.83</td>
</tr>
<tr>
<td>hsa-miR-122-5p</td>
<td>2.97</td>
</tr>
<tr>
<td>hsa-miR-509-3p</td>
<td>2.77</td>
</tr>
<tr>
<td>hsa-miR-548a-3p</td>
<td>2.72</td>
</tr>
<tr>
<td>hsa-miR-766-3p</td>
<td>2.55</td>
</tr>
<tr>
<td>hsa-miR-744-3p</td>
<td>2.50</td>
</tr>
<tr>
<td>hsa-miR-940</td>
<td>2.45</td>
</tr>
<tr>
<td>hsa-miR-1228-3p</td>
<td>2.44</td>
</tr>
<tr>
<td>hsa-miR-1208</td>
<td>2.41</td>
</tr>
<tr>
<td>hsa-miR-1825</td>
<td>2.33</td>
</tr>
<tr>
<td>hsa-miR-3201</td>
<td>2.18</td>
</tr>
<tr>
<td>hsa-miR-4274</td>
<td>2.17</td>
</tr>
<tr>
<td>hsa-miR-4461</td>
<td>2.10</td>
</tr>
<tr>
<td>hsa-miR-4695-3p</td>
<td>2.04</td>
</tr>
<tr>
<td>hsa-miR-371b-5p</td>
<td>2.03</td>
</tr>
</tbody>
</table>

Fig. 1 Expression level of miR-20a-3p in psoriasis tissues and in HaCaT cells treated by IL-22 stimulation using qRT-PCR. (A) Comparisons of miR-20a-3p expression in the psoriatic skin (n = 6) and healthy control skin (n = 6). (B) Comparisons of miR-20a-3p expression in HaCaT cells treated by IL-22 stimulation and the control group. *P < 0.05
3.2 MiR-20a-3p inhibits proliferation in HaCaT cells

MiR-20a-3p mimics or inhibitor was transiently transfected into HaCaT cells to increase or decrease expression of the miRNA. The expression of miR-20a-3p was found to be increased in miR-20a-3p mimics group compared to NC group (P<0.01) (Supplementary Fig. S1) after transfection. A CCK8 assay and 5-Ethynyl-2’-deoxyuridine (EDU) incorporation assay were used for cell proliferation. The results of CCK8 assay showed that upregulation of miR-20a-3p suppressed proliferation while knockdown of miR-20a-3p had the opposite effect in HaCaT cells (Fig. 2A and B). For EDU assay, the EdU-positive rate (percentage of cells that underwent cell division) was significantly reduced by miR-20a-3p mimics (P<0.05). Conversely, inhibition of endogenous miR-20a-3p increased the EdU-positive rate compared with the control group (P<0.05). (Fig. 2C and D)

Flow-cytometric analysis showed that the proportion of cells at G1 phase increased (P<0.01) while that of cells at S phase decreased (P<0.05) after upregulation of miR-20a-3p in HaCaT cells (Fig. 2E and F). In contrast, knockdown of miR-20a-3p resulted in a reduced G1 phase and increased S phase, the differences were statistically significant (P<0.05) (Fig. 2G and H). These results suggested that miR-20a-3p could result in cell cycle arrest (cells arrested in G1 phase) and suppressed G1/S transition. Corresponding, at the protein level, HaCaT cells transfected with miR-20a-3p mimics or inhibitor led to a significant change in the expression of cell cycle control protein, which affect G1 progression and G1/S transition (such as CDK2, CDK4, Cyclin D1, P21cip1, and phospho S811-Rb) (Fig. 3C, E and F).
Fig. 2 Effect of miR-20a-3p on proliferation and cell cycle distribution in HaCaT cells. (A) The proliferation curves of HaCaT cells after transfection with miR-20a-3p mimics and negative control at 24, 48, and 72 h by CCK-8 assay. (B) The proliferation curves of HaCaT cells after transfection with miR-20a-3p inhibitor and negative control at 24, 48, and 72 h by CCK-8 assay. (C) EdU incorporation assay of HaCaT cells that were treated with miR-20a-3p mimics, inhibitor, or negative control. (E and F) Cell cycle distribution for miR-20a-3p mimics and negative control by flow-cytometric analysis. (G and H) Cell cycle distribution for miR-20a-3p inhibitor and negative control by flow-cytometric analysis. *P < 0.05, **P < 0.01.
3.3 MiR-20a-3p induces cell apoptosis

The flow cytometric results demonstrated that the percentage of early and late apoptotic cells increased after overexpression of miR-20a-3p compared with the control group (early apoptotic rate: 1.79 ± 0.23 vs 1.20 ± 0.10, p<0.05; late apoptotic rate: 22.9 ± 2.07 vs 14.17 ± 2.97, p<0.05) (Fig. 3A and B). Conversely, in the miR-20a-3p knockdown group, early and late apoptotic decreased compared with the control group (early apoptotic rate: 0.67 ± 0.05 vs 0.94 ± 0.11, p<0.05; late apoptotic rate: 6.64 ± 0.76 vs 10.81 ± 0.48, p<0.01) (Fig. 3A and B). The results indicated that miR-20a-3p induced cell apoptosis in HaCaT cells. The expression of apoptosis-related proteins was detected by western blot analysis. We found that upregulation of miR-20a-3p decreased the expression of survivin, whereas knockdown of miR-20a-3p increased the expression of survivin in HaCaT cells (Fig. 3D, E and F). Furthermore, results of immunohistochemistry indicated that 85% of psoriasis patients have high expression of survivin, while that in healthy people was only 12.5% (p<0.05) (Fig. 4B). The staining was mainly seen in basal layer of epidermis (Fig. 4A). However, there were no significant change in the proteins expression of bcl-2 and bax when miR-20a-3p mimics or inhibitor transfected into HaCaT cells (data not shown).

Fig. 3 Effect of miR-20a-3p on apoptosis and the protein expression in HaCaT cells transfected with miR-20a-3p mimic, inhibitor or their negative control. (A and B) Apoptosis rate of HaCaT cells by Flow cytometric analysis of Annexin V/PI staining. (C) Western blot analyses of the expression of cell cycle proteins related to G1/S transition (pRb, Cyclin D1, CDK4, CDK2 and p21). (D) Western blot analyses of the expression of apoptosis-related protein survivin and TGF-β/smads signaling pathway. (E and F) The quantification of protein expression following western blotting assays was exhibited. *P < 0.05, **P < 0.01, ***P<0.001
Fig. 4 Expression of survivin and SFMBT1 in healthy and psoriatic skin. Immunohistochemical staining (A) and analysis (B) of survivin and SFMBT1. (original magnification ×100).

Immunohistochemical staining was performed on healthy (n=8) and psoriatic skin (n=20), and the difference was assessed using Fisher’s Exact Test between the two groups. “+/-” denote “low” or "no" expression and ++/+++ denote “high” expression. *P < 0.05

3.4 SFMBT1 is a direct target of miR-20a-3p

The potential targets of miR-20a-3p were predicted using bioinformatics softwares TargetScan and miRanda binding the results of mRNA microarrays. We got 10 potential target genes (Fig. 5A). Among the candidates, protein levels of SFMBT1 was found to be decreased following miR-20a-3p mimics and increased after transfection with miR-20a-3p inhibitor in HaCaT cells (Fig. 5B and C). In addition, results of immunohistochemistry indicated that 65% of psoriasis patients have high expression of SFMBT1, while that in healthy people was only 12.5% (p < 0.05) (nuclear staining of epidermis) (Fig. 4A and B). The results suggested that SFMBT1 may play roles in the pathogenesis of psoriasis. To further examine whether miR-20a-3p directly targets SFMBT1, we performed dual-luciferase reporter assay. The WT 3’ UTR or the mutant 3’ UTR target sequences of SFMBT1 were cloned into the luciferase reporter vectors (Fig. 5D). Results showed that co-transfection with miR-20a-3p mimics and pmiR-RB-Report™-SFMBT1-WT led to an obvious reduction in luciferase activity when compared to the miR-NC control group (P < 0.01). However, no statistically significant alteration in luciferase activity was observed in the presence of the mutated 3’-UTR site. (Fig. 5E)
SFMBT1 is a direct target of miR-20a-3p. (A) Predicted target genes of miR-20a-3p using bioinformatics softwares TargetScan and miRanda binding the results of mRNA microarrays. (B and C) The expression level of SFMBT1 protein was examined following transfection with miR-20a-3p mimic, inhibitor or their negative control in HaCaT cells. (D) The wild-type and mutated-type miR-20a-3p binding sites in the SFMBT1 3’-UTR. (E) The relative luciferase activity of SFMBT1 wild type or mutant 3’UTR in HEK293 cells following transfection with the miR-20a-3p mimic or negative control. *P < 0.05, **P < 0.01

3.5 SFMBT1 knockdown suppresses cell proliferation and induces cell apoptosis

To verify the effects of SFMBT1 on proliferation and apoptosis in HaCaT cells, we knocked down endogenous SFMBT1 expression by small interfering RNA (siRNA). The relative expression of SFMBT1 mRNA and protein in HaCaT cells was significantly decreased after 24 and 48 hours transfection (Supplementary Fig. S2). Results of CCK8 assay indicated that knockdown of SFMBT1 inhibited cell proliferation (Fig. 6A). For EDU assay, the SFMBT1-specific siRNA had less EdU-positive cells at 48 hours after transfection compared with the control group (P<0.05) (Fig. 6B and C). Flow-cytometric analysis showed that SFMBT1 knockdown increased the proportion of cells at G1 phase (P<0.05) and decreased that of S phase (P<0.01) (Fig. 6D and E) in HaCaT cells. In addition, results demonstrated that the percentage of early and late apoptotic cells increased compared with the control group after knockdown of SFMBT1 (early apoptotic rate: 1.45 ± 0.12 vs 0.54 ± 0.02, p<0.001; late apoptotic rate: 11.27 ± 1.25 vs 4.16 ± 1.54, p<0.01) (Fig. 6F and G).

Corresponding, cell cycle proteins were changed and inhibitor of apoptosis protein survivin decreased when the SFMBT1-specific siRNA transfected into HaCaT cells (Fig. 6H, I and J). The data presented above indicated that the effects of SFMBT1 knockdown were similarly to the effect seen following miR-20a-3p
upregulation. This suggests that the effects of miR-20a-3p on proliferation and apoptosis in HaCaT cell are at least partially mediated through SFMBT1.

![Graph showing effect of SFMBT1 knockdown on cell proliferation](image1)

**Fig. 6** Effect of SFMBT1 knockdown on cell proliferation, cell cycle and cell apoptosis in HaCaT cells. (A) The effect of SFMBT1 knockdown on cell proliferation by CCK8 assay. (B and C) EdU incorporation assay of HaCaT cells after knockdown of SFMBT1. (D and E) Cell cycle distribution of HaCaT cells after knockdown of SFMBT1 by flow-cytometric analysis. (F and G) Apoptosis rate of HaCaT cells after knockdown of SFMBT1 by Flow cytometric analysis of Annexin V/PI staining. (H) The expression of cell cycle proteins related to G1/S transition (pRb, Cyclin D, CDK4, CDK2 and p21) by western blot analyses. (I) The expression of apoptosis-related protein survivin and TGF-β/smads signaling pathway by western blot analyses. (J) The quantification of protein expression following western blotting assays was exhibited. *P < 0.05, **P < 0.01, ***P < 0.001

### 3.6 TGF-β1/Survivin signalling axis is affected by miR-20a-3p and SFMBT1 in HaCaT cells

We found that overexpression of miR-20a-3p promoted the protein expression levels of TGF-β1 and P-smad2/3 but suppressed that of survivin (Fig. 3D and E). Similar results were reported after knockdown of SFMBT1 in HaCaT cells (Fig. 6I and J). Conversely, miR-20a-3p knockdown had the opposite results with the protein expression levels of TGF-β1, P-smad2/3 and survivin in HaCaT cells (Fig. 3D and F).

Then, we found that the expression of survivin was reduced in HaCaT cells after TGF-β1 stimulation for 24h (Fig. 7A and B). In addition, a significant change in the
expression of cell cycle control protein (such as CDK2, CDK4, Cyclin D1, P21cip1, and phospho S811-Rb) was also found (Fig. 7A and B).

Fig. 7 The protein expression in HaCaT cells with TGF-β1 and the role of miR-20a-3p in IL-22 induced keratinocyte proliferation. (A and B) The expression of proteins in HaCaT cells treated with TGF-β1 by western blot analyses. (C) The cell proliferation of HaCaT cells under IL-22 stimulation was determined using CCK8 assays, with the presence or absence of miR-20a-3p mimics (*IL-22 vs Control, IL-22 + miR-20a-3p mimics vs IL-22). (D and E) Western blot analyses of the expression of SFMBT1 in HaCaT cells treated by IL-22 stimulation. *P < 0.05, **P < 0.01

3.7 MiR-20a-3p inhibits IL-22 induced keratinocyte proliferation

IL-22 is a cytokine that was shown to play a vital role in the pathogenesis of psoriasis [10]. MiR-20a-3p was down-regulated in psoriatic lesions and in HaCaT cells when stimulated with IL-22, which suggested the potential role of miR-20a-3p in the pathogenesis of IL-22 induced psoriasis. In the present study, we found that IL-22 could promote cell proliferation, whereas miR-20a-3p overexpression attenuated the promotive effect of IL-22 on proliferation in HaCaT cells (Fig. 7C). Further, we monitored that IL-22 stimulation increased the protein levels of SFMBT1 in HaCaT cells (Fig. 7D and E). Data above suggest that reduction of miR-20a-3p may play important roles in IL-22 induced keratinocyte proliferation through upregulating SFMBT1.

4. Discussion

It is well known that psoriasis is a chronic inflammatory skin disorder characterized by aberrant keratinocyte proliferation, immune cells infiltrate and proinflammatory cytokines release [1,7]. The exact pathogenetic mechanisms of psoriasis are largely unknown [20]. Recently, genetic and epigenetic anomalies, particularly genetic regulation by aberrant expressed miRNAs, are indicated to be causative elements in psoriasis [1,21]. An increasing miRNAs abnormal expression was reported in
psoriasis. In this study, data show that miR-20a-3p is down-regulated in psoriatic lesional skin.

It was previously reported that miR-20a play a pivotal role in numerous cancers [22]. In prostate cancer, miR-20a serves as an oncogene and promoted prostate cancer cells invasion and migration [22,23]; While in other types of cancers, such as breast cancers, miR-20a is down-regulated and plays a tumor suppressor role [24]. However, the function of miR-20a-3p has been barely reported. MiR-20a-3p (Previous IDs: miR-20a*) is another mature microRNA product produced by the precursor miR-20a stem loop structure. Traditionally researchers thought that only one mature microRNA could be produced (guide strand) and the other strand (passenger strand) is often considered to be degraded and therefore non-functional [25,26]. Later it became clear that sometimes the passenger strand does not degrade and could act as miRNA [27]. A growing evidence suggests the passenger strand can play functional roles in normal cellular behavior. Pink et al. first found that, the passenger strand miR-21-3p plays a role in mediating cisplatin resistance in ovarian cancer cells [25]. In this study, we found that overexpression of miR-20a-3p in keratinocytes could suppress cell proliferation and induce cell apoptosis. This makes the research very interesting.

Mechanistically, SFMBT1 was identified as a target gene of miR-20a-3p. SFMBT1 belongs to the malignant brain tumor (MBT) domain-containing protein family, which contributes to multiple cellular processes, including cell proliferation and maintenance of the characteristics of stem cells [28-30]. Study conducted by Lin S et al suggested a critical role of SFMBT1 in regulating skeletal myogenesis, likely via promoting muscle stem cell proliferation and preventing premature differentiation [29]. It is reported that human SFMBT localized to the nucleus where it strongly associates with chromatin by binding the N-terminal tail of histone H3 [31]. In this study, 13 cases out of 20 (65%) showed high expression of SFMBT1 in psoriasis patients (nuclear staining of epidermis), while that in healthy people was only 12.5% (p < 0.05). In vitro, our data showed that SFMBT1 knockdown could inhibit cells proliferation and induced apoptosis, which was consistent with the function of miR-20a-3p upregulation in HaCaT cells. The data above suggested that the effects of miR-20a-3p on HaCaT proliferation and apoptosis are at least partially mediated through SFMBT1.

Deregulation of TGF-β signalling has been reported in psoriasis [3]. TGF-β is a multifunctional growth factor which regulates cell growth and differentiation [32]. It initially activates TGF-β RI and propagates the signal intracellularly by phosphorylating SMAD2 and SMAD3 and finally regulates cellular processes [3,33]. TGF-β induces apoptosis in several cell types including hepatocytes and hepatomas [34,35], and is recognized as an important negative regulator of keratinocyte proliferation [36]. In vivo, TGF-β1 knockout mice exhibit hyperproliferation of the epidermis, while overexpression of TGF-β1 in transgenic mice result in an inhibition of skin development [3]. However, several investigators indicate TGF-β1 levels are higher in patients with psoriasis than controls [37,38]. Litvinov IV et.al observed a marked downregulation of the TGF-β RI and RII proteins in the psoriatic skin [3]. Man J et al thought that the decreased expression of TGFβ receptor I in psoriatic
epidermis made the TGFβ/SMAD pathway inactive and led to cell proliferation [39]. It is difficult to determine if increased TGFβ plays a causal role in psoriasis, or it is simply a consequence of psoriasis pathogenesis [40,41].

TGF-β1 inhibits cell proliferation by suppressing G1/S transition in many cell types [42]. TGF-β1 could regulate the levels and activities of cyclins-cyclin-dependent kinase (CDK) complexes and cyclin-dependent kinase inhibitors (p21WAF1/Cip1, p27kip1, and p15) [42]. Detailed functional mechanism is shown in Fig. 8A. Cyclin D is the first cyclin produced during the cell cycle, which binds to CDK4 and forms the active cyclin D–CDK4 complex. The cyclin D–CDK4 complex phosphorylates and inactivates the Rb. Additionally, Rb is partly phosphorylated and inactivated by cyclin E–CDK2 complex. Subsequently, E2F dissociates from the E2F/DP1/Rb complex and the activation of E2F results in the expression of various genes which promotes cell progression from G1 to S phase [34]. In this study, we prove that cell cycle control protein CDK2, CDK4, cyclin D1 and P–Rb were decreased, while p21Cip1 protein was increased in HaCaT cells after TGF-β1 stimulation for 24h. In addition, survivin was downregulation in HaCaT cells when TGF-β1 stimulated. Survivin is known to bind to CDK4 leading to the activation of CDK2/cyclin-E and the subsequent phosphorylation of ribosomes, which can accelerate transformation at the G1/S stage [43-46].

Survivin, the most important member of IAP (inhibitor of apoptosis protein) family, has been implicated in regulation of cell cycle and protection from apoptosis in many cancers [47,48]. The protein exhibits low or undetectable expression in most adult tissues but is increased in psoriasis and multiple tumor types [49]. In this study, the high expression of survivin has been detected in 85% of psoriasis lesions (n=20) and 12.5% of normal control specimens (n=8). In a study of immunostaining by Bowen et al., survivin expression has been reported in psoriasis (88% of cases), and no survivin expression was detected in normal skin [49]. It is known that keratinocytes develop resistance to apoptosis in psoriasis [50]. However, the mechanism by which survivin inhibits apoptosis remains unclear.

Several studies have shown that TGF-β1 regulates the expression of survivin in cancers [51-55]. Yang JY et al found that TGF-β1 could downregulate survivin expression through a mechanism that is dependent on Smads 2 and 3 [52]. Song k et al found that the expression of survivin was elevated by suppressing TGF-β1 signaling, and lead to the resistance to apoptosis in prostate epithelial cells [53]. But the study conducted by Lee J et al suggested that TGF-β1 led to the upregulation of survivin in ARPE-19 cells and included migration and invasion of local epithelial cells. However, they also found that when survivin was depleted, TGF-β1 induced cell cycle arrest and apoptosis with reduction of Rb phosphorylation [34]. Our data showed that survivin was downregulation in HaCaT cells when TGF-β1 stimulated. We also found that miR-20a-3p mimics or knockdown of SFMBT1 led to the upregulation of TGF-β1 and survivin downregulation. Increased TGF-β1 could suppress G1/S transition and induced cell apoptosis through downregulation of survivin.
To sum up, overexpression of miR-20a-3p could lead to downregulation of SFMBT1 at post-transcriptional level in vitro, which promoted the protein expression levels of TGF-β1 and P-smad2/3 but suppressed that of survivin. Increased TGF-β1 could induce cell cycle arrest and apoptosis through downregulation of survivin. The possible mechanism of miR-20a-3p on proliferation and apoptosis in HaCaT cells was shown in Fig. 8B.

**Fig. 8** Schematic model depicting network effect. (A) The regulatory mechanism of TGF-β1 on cell cycle. (B) The possible regulatory mechanism of miR-20a-3p in HaCaT cells by targeting SFMBT1 and modulating the TGF-β/Survivin signalling pathway. Note that arrows denote activation and bars denote inhibition.

Recent evidence indicates that immune system plays a critical role in psoriasis. The Th17 network, including IL-23, IL-17 and IL-22, plays a critical role in the pathogenesis of psoriasis [11]. IL-22, as the uniquely communication between the immune system and keratinocytes, is increased in serum and skin lesions from psoriatic patients. Studies have shown that IL-22 promotes keratinocyte proliferation, induces keratinocyte migration and downregulates the expression of genes associated with keratinocyte differentiation in vitro [56]. In this study, we found that miR-20a-3p was down-regulated in HaCaT cells treated by IL-22 stimulation, and overexpression of miR-20a-3p could inhibit IL-22 induced keratinocyte proliferation.

In conclusion, our findings showed that miR-20a-3p regulates keratinocytes proliferation and apoptosis by targeting SFMBT1 and TGF-β1/Survivin pathway in vitro, and decreased expression of miR-20a-3p may play a role in the pathogenesis of psoriasis.
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Li RH and Sun Q designed the experiments; Li RH and Zhao XT performed the research; Qiao M and Yan JJ provided the psoriasis patients’ samples; Wang XY helped in Western blotting; Zhong H and Yu XJ contributed essential reagents; Li RH and Qiao M analysed the data and wrote the paper. All authors read the final version of this manuscript and gave their approval for the submitted.

Conflict of interest

The authors declare no conflict of interest.

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**Highlights**

- MiR-20a-3p was down-regulated in psoriatic lesions and loss of miR-20a-3p may play a role in the pathogenesis of psoriasis.
- MiR-20a-3p regulates keratinocytes proliferation and apoptosis by targeting SFMBT1 and TGF-β1/Survivin pathway in vitro.
- MiR-20a-3p was down-regulated in keratinocyte when stimulated with IL-22 and overexpression of miR-20a-3p could inhibit IL-22 induced keratinocyte proliferation.
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