Department of Dermatology[1], The Second Affiliated Hospital of Hunan University of Chinese Medicine, Changsha, Hunan Province; Department of Dermatology[2]; Department of Ophthalmology[3], Ruikang Hospital affiliated to Guanxi University of Chinese Medicine, Nanning, Guangxi Province, China

MiR-99a inhibits keratinocyte proliferation by targeting Frizzled-5 (FZD5) / FZD8 through β-catenin signaling in psoriasis

Hui Shen[1,2], Yi Tian[1], Xiaolei Yao[3], Wen Liu[1], Yujin Zhang[1], Zhibo Yang[1,*]

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**Corresponding author: Zhibo Yang, No.233 Caie North Road, Tianxin District, Changsha, Hunan Province, China, 410005.*
dr_yangzhibo@163.com

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Psoriasis, a common chronic skin disorder, is characterized by hyperproliferation and aberrant differentiation of keratinocytes and infiltration of inflammatory cells into the dermis and epidermis. MicroRNAs (miRNAs) are a large family of highly conserved small non-coding RNA which regulates diverse biological process, including cell proliferation, by modulating gene expression at the posttranscriptional level. In the present study, we indicated that miR-99a was specifically downregulated in psoriatic dermatic lesions, and could inhibit HaCaT cells' proliferation; by direct targeting, miR-99a could also regulate the expression of Frizzled-5 (FZD5)/Frizzled-8 (FZD8). In addition, we found that the downstream factor of FZD5/FZD8 signaling, β-catenin, and the downstream target gene of β-catenin, cyclinD1, could be suppressed by miR-99a; the suppressive effect of miR-99a on β-catenin and cyclinD1 could be partially abolished by forced FZD5/FZD8 expression. Taken together, we assume that miR-99a inhibits HaCaT cells' proliferation by targeting FZD5/FZD8 through downstream factors β-catenin and cyclinD1, and provide diagnostic markers and a novel target for psoriasis treatment.

1. Introduction

Psoriasis is a common skin disease affecting 2 – 3 % of the world population. It begins as red, scaly patches on the scalp, elbows, and knees that is associated with systemic inflammation and comorbidities, such as psoriatic arthritis, cardiovascular disease, diabetes, and depression (Boehncke et al. 2011; Gladman et al. 2005; Koo 1996; Neimann et al. 2006; Schon 2005). Abnormal proliferation of human keratinocytes is an important feature of psoriasis, along with local hypoxia and vascular abnormal growth (Albanesi et al. 2007; Tao et al. 2008). However, the precise mechanism of exaggerated proliferation is not yet fully understood.

MicroRNAs (miRNAs) are ~22 nucleotide-long single-stranded noncoding RNAs that can mediate post-transcriptional silencing by binding with partial complementarity to the 3' UTR of the target messenger RNA (mRNA) (Ambros et al. 2003). miRNAs have been shown to participate in many fundamental life processes such as development, differentiation, organogenesis, growth control, and apoptosis (Lui 2016). Accordingly, deregulation of miRNA expression has been shown to contribute to cancer, heart diseases, infectious diseases, inflammatory diseases, and other medical conditions, making them potential targets for medical diagnosis and therapy (Czech 2006). In our previous study, the microarray results showed that 26 miRNAs were upregulated and 13 miRNAs were decreased in psoriasis patients. qRT-PCR validated three upregulated miRNAs (miR-146a, miR-31, miR-192-5p) and two downregulated miRNAs (miR-99a, miR-200c) in peripheral blood mononuclear cells (PBMCs) from psoriasis patients compared with healthy controls (P<0.01) (Yang et al. 2016). In the present study, we focused on the functional role of miR-99a in psoriasis and the underlying mechanism.

The Wnt/β-catenin pathway is best known for its role in embryogenesis and development (Clevers 2006; Logan and Nusse 2004). Some researchers highlight that the Wnt/β-catenin pathway is also involved in the regulation of cancer cell proliferation (Xiao et al. 2016; Zhan et al. 2017). Wnt/β-catenin pathway is triggered by binding of Wnt ligands to Frizzled (FZD) receptor proteins; FZD was essential for Wnt/β-catenin pathway activation (Corrigan et al. 2009). In the present study, by using online tools, we screened out several candidate downstream genes of miR-99a; combined with previous studies, FZD5 and FZD8, which were reported to be related to proliferation, were chosen as the further subjects.

In the present study, we determined the expression of miR-99a in psoriasis tissues; by direct binding to the 3'UTR of FZD5/FZD8, miR-99a suppressed the protein levels of FZD5/FZD8 and the proliferation of the psoriasis cell line, HaCaT. Moreover, the downstream factors of FZD signaling, β-catenin and CyclinD1 were involved in the regulation process. Taken together, these findings provide a novel molecular basis for the potential effect of miR-99a in treatment of psoriasis.

2. Investigations and results

2.1. MiR-99a was specifically downregulated in psoriatic skin lesional tissues

In our previous study, we demonstrated that miR-99a expression is downregulated in peripheral blood mononuclear cells (PBMCs) from psoriasis patients compared with healthy controls. Here, real-time PCR analysis was performed to quantify the expression of miR-99a in psoriatic skin lesional tissues. In 23 paired of psoriasis lesional tissues and normal non-lesional tissues, the expression of miR-99a was significantly downregulated in psoriasis lesional tissues, compared with the normal non-lesional tissues (Fig. 1, P<0.01).

2.2. Screening of the candidate downstream target genes of miR-99a

Commonly, miRNAs exert their functions through imperfect pairing with the 3' untranslated region (UTR) of target mRNAs. To investigate the potential role of miR-99a in psoriasis and the underlying mechanism, online tools including miRWalk, RANDA, Targetscan, miRDB were used to screen out several

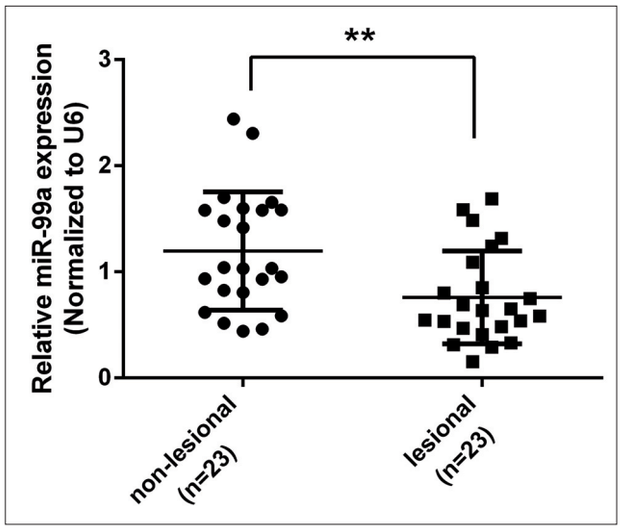

Fig. 1: MiR-99a was specifically down-regulated in psoriasis tissues. The expression of miR-99a in a panel of 23 paired psoriasis lesion tissues and normal non-lesional tissues was determined by real-time PCR assays. The data are presented as mean±SD of three independent experiments. **P<0.01.

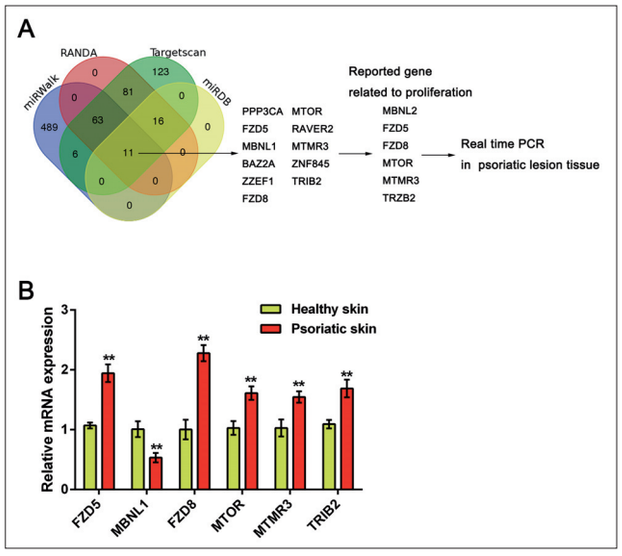

Fig. 2: Screening of the candidate downstream target genes of miR-99a. (A) Online tools including miRWalk, RANDA, Targetscan and miRDB were used to screen out potential candidate downstream genes of miR-99a. (B) The expression levels of six candidate downstream genes of miR-99a: FZD5, MBNL1, FZD8, MTOR, MTMR3 and TRIB2, in psoriasis lesion tissues and normal non-lesional tissues were determined by using real-time PCR assays. The data are presented as mean±SD of three independent experiments. **P<0.01.

candidate downstream genes of miR-99a; combined with previous studies, six candidate genes (FZD5, MBNL1, FZD8, MTOR, MTMR3, TRIB2), which were reported to be related to cell proliferation, were chosen as further sampling object of real-time PCR (Fig. 2A). The mRNA expression levels of the indicated candidate genes were determined in psoriasis lesional tissues and normal non-lesional tissues. Results showed that the expression levels of FZD5 and FZD8 mRNA were the most strongly upregulated in psoriasis lesional tissues compared with the normal non-lesional tissues (Fig. 2B, P<0.01). Given that FZD5 and FZD8 are essential for cell proliferation-related Wnt/β-catenin pathway activation (Corrigan et al. 2009), we then performed a series of functional assays to figure out their detailed role in psoriatic keratinocyte proliferation.

2.3. MiR-99a inhibited HaCaT cells' proliferation through FZD5/FZD8

miR-99a mimics and miR-99a inhibitor was transfected into HaCaT cells to achieve miR-99a overexpression and miR-99a inhibition. The transfection efficiency was verified by real-time PCR assays (Fig. 3A). The protein levels of FZD5 and FZD8 in response to miR-99a overexpression and inhibition were determined by using Western blot assays. Results showed that the protein levels of both FZD5 and FZD8 were significantly downregulated by miR-99a overexpression while upregulated by miR-99a inhibition (Fig. 3B and C). Next, we monitored the cell viability and proliferation of HaCaT cells under the co-transfection of miR-99a mimics and FZD5 or FZD8 overexpression vector. As showed in Fig. 3D, transfection of FZD5 or FZD8 vectors effectively increased the FZD5 or FZD8 expression in HaCaT cells. MTT assays showed that miR-99a significantly suppressed the cell viability of HaCaT cells, while the forced expression of either FZD5 or FZD8 significantly promoted the cell viability; the suppressive effect of miR-99a on HaCaT cells' viability could be partially restored by FZD5 or FZD8 overexpression (Fig. 3E and F). Similar results were observed from BrdU assays: miR-99a significantly suppressed the cell proliferation of HaCaT cells, while the forced expression of either FZD5 or FZD8 significantly promoted the cell proliferation; the suppressive effect of miR-99a on HaCaT cells' proliferation could be partially restored by FZD5 or FZD8 overexpression (Fig. 3G and H). These data suggested that miR-99a inhibits HaCaT cells' proliferation and the protein levels of FZD5 and FZD8; FZD5 and FZD8 were involved in the process of miR-99a inhibiting HaCaT cells' proliferation, respectively.

2.4. MiR-99a inhibited FZD5/FZD8 expression by direct binding to the 3'UTR of FZD5/FZD8

To investigate the mechanism by which miR-99a inhibits the expression of FZD5 and FZD8, a wt-FZD5 or FZD8 3'UTR luciferase reporter gene vector, a mut-FZD5 or FZD8 3'UTR luciferase reporter gene vector containing a 6 bp mutation on the predicted binding site of miR-99a in the 3'UTR of FZD5 or FZD8 3'UTR was constructed (Fig. 4A). The indicated vectors were co-transfected into HaCaT cells with miR-99a mimics or miR-99a inhibitor, respectively. The luciferase activity was then determined by using dual luciferase assays. Results showed that the luciferase of wt-FZD5 3'UTR and wt-FZD8 3'UTR vectors was suppressed by miR-99a mimics transfection, while amplified by miR-99a inhibitor transfection; the effect of miR-99a mimics or miR-99a inhibitor could be abolished by the mutations in mut-FZD5 3'UTR and mut-FZD8 3'UTR vectors (Fig. 4B and C). These data indicated that miR-99a could inhibit FZD5 and FZD8 expression through direct binding to the 3'UTR of FZD5 and FZD8, respectively.

2.5. MiR-99a/FZD5/FZD8 regulated HaCaT cells' proliferation through β-catenin signaling

Wnt/β-catenin pathway has been reported to be involved in the regulation of cancer cell proliferation (Xiao et al. 2016; Zhan et al. 2017). Most frizzled receptors are coupled to the β-catenin canonical signaling pathway (Carron et al. 2003; Ring et al. 2014). Here we investigated whether β-catenin and cyclinD1, the downstream factors of FZD5/FZD8, were involved in the regulation of HaCaT cells. MiR-99a mimics was transfected into HaCaT cells with the absence or presence of FZD5/FZD8, and the protein levels of β-catenin and cyclinD1 were determined by using Western blot assays. Results showed that the protein levels of β-catenin and cyclinD1 were reduced by miR-99a, increased by FZD5/FZD8; the suppressive effect of miR-99a on the protein levels of β-catenin and cyclinD1 could be partially restored by forced expression of FZD5/FZD8 (Fig. 5A and B). These data indicated that miR-99a/FZD5/FZD8 regulated HaCaT cells' proliferation through β-catenin signaling.

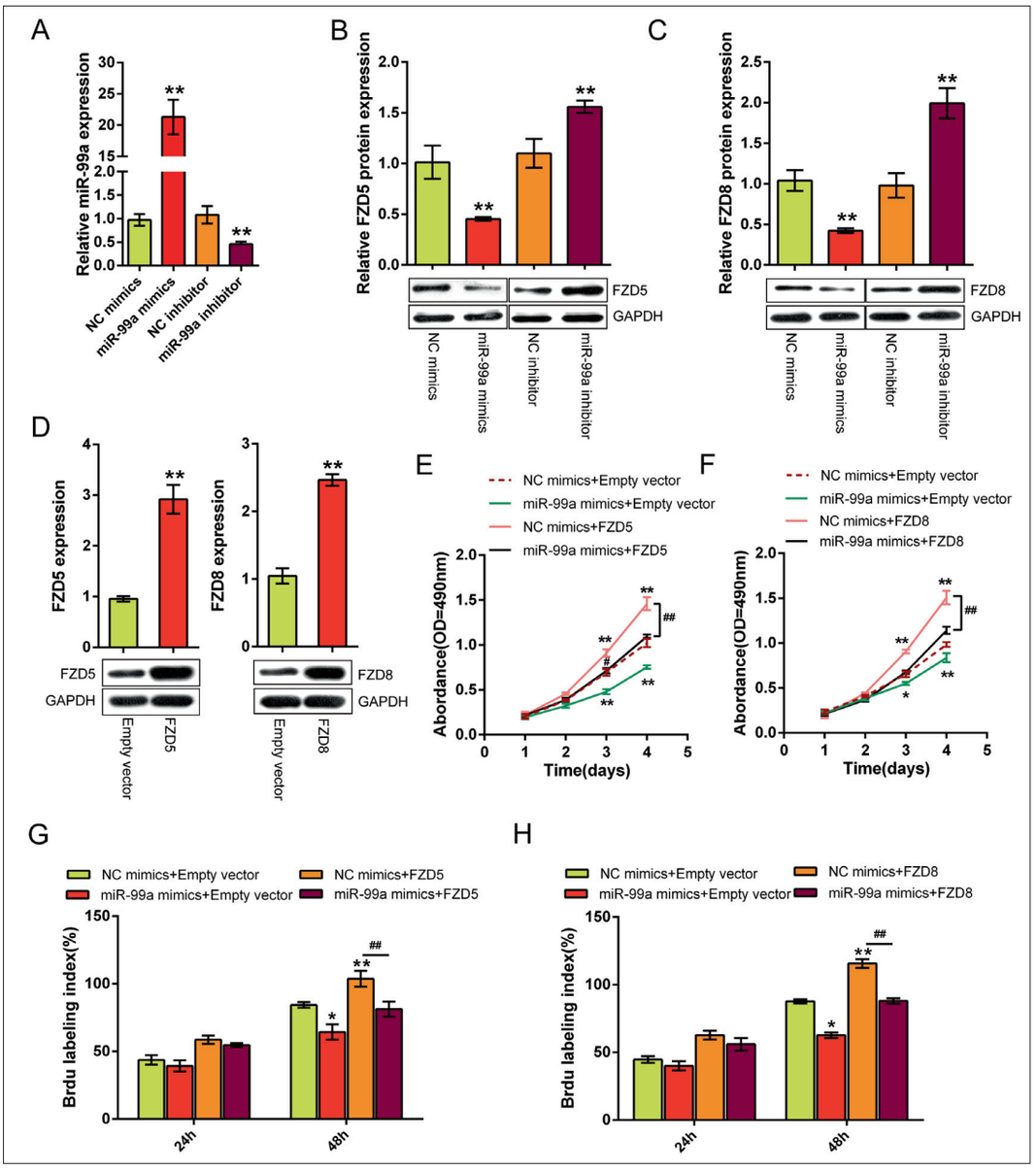

Fig. 3: MiR-99a inhibited HaCaT cells' proliferation through FZD5/FZD8 (A) miR-99a mimics or miR-99a inhibitor was transfected into HaCaT cells to achieve miR-99a overexpression or miR-99a inhibition. The transfection efficiency was verified by using real-time PCR assays. (B) and (C) The protein levels of FZD5 and FZD8 in HaCaT cells in response to miR-99a overexpression or miR-99a inhibition were determined by using Western blot assays. (D) The protein levels of FZD5 and FZD8 in HaCaT cells in response to FZD5 or FZD8 overexpression. (E) and (F) The cell viability of HaCaT cells in response to co-transfection of miR-99a mimics and FZD5/FZD8 was determined by using MTT assays. (G) and (H) The cell proliferation of HaCaT cells in response to co-transfection of miR-99a mimics and FZD5/FZD8 was determined by using BrdU assays. The data are presented as mean±SD of three independent experiments. *P<0.05, **P<0.01, ##P<0.05.

2.6. Expression of FZD5/FZD8 in psoriasis tissues and the correlation between FZD5/FZD8 and miR-99a

We determined the expression levels of FZD5 and FZD8 in 23 paired of psoriatic skin lesional tissues and normal non-lesional tissues by using real-time PCR assays. Results showed that FZD5 and FZD8 expression was up-regulated in psoriasis lesional tissues, compared with normal non-lesional tissues (Fig. 6A and B). The Spearman's rank correlation analysis was performed to analyze the correlation between miR-99a expression and FZD5/FZD8 expression psoriatic skin lesional tissues, respectively. Results showed that miR-99a was inversely correlated with FZD5 (r = - 0.4899, p= 0.0177) and FZD8 (r = -0.5909, p= 0.0030), respectively (Fig. 6C and D).

3. Discussion

Psoriasis is an autoimmune disease with multiple consequences, and keratinocyte abnormality is a major one of its various pathological changes (Chong et al. 2013). During the psoriasis process, keratinocytes often show rapid cell proliferation, delayed differentiation and apoptosis (Harden et al. 2015). Regulation of the pathological changes initiated or perpetuated by keratinocytes may be effective in psoriasis management (Albanesi and Pastore 2010). Aside from the disorder of keratinocytes proliferation, differentiation or apoptosis, miRNA expression disorders have been reported (Sonkoly 2017; Van Gele et al. 2016). MiR-181a has been reported to negatively regulates the proliferation of human epidermal keratinocytes in psoriasis through targeting TLR4 (Feng

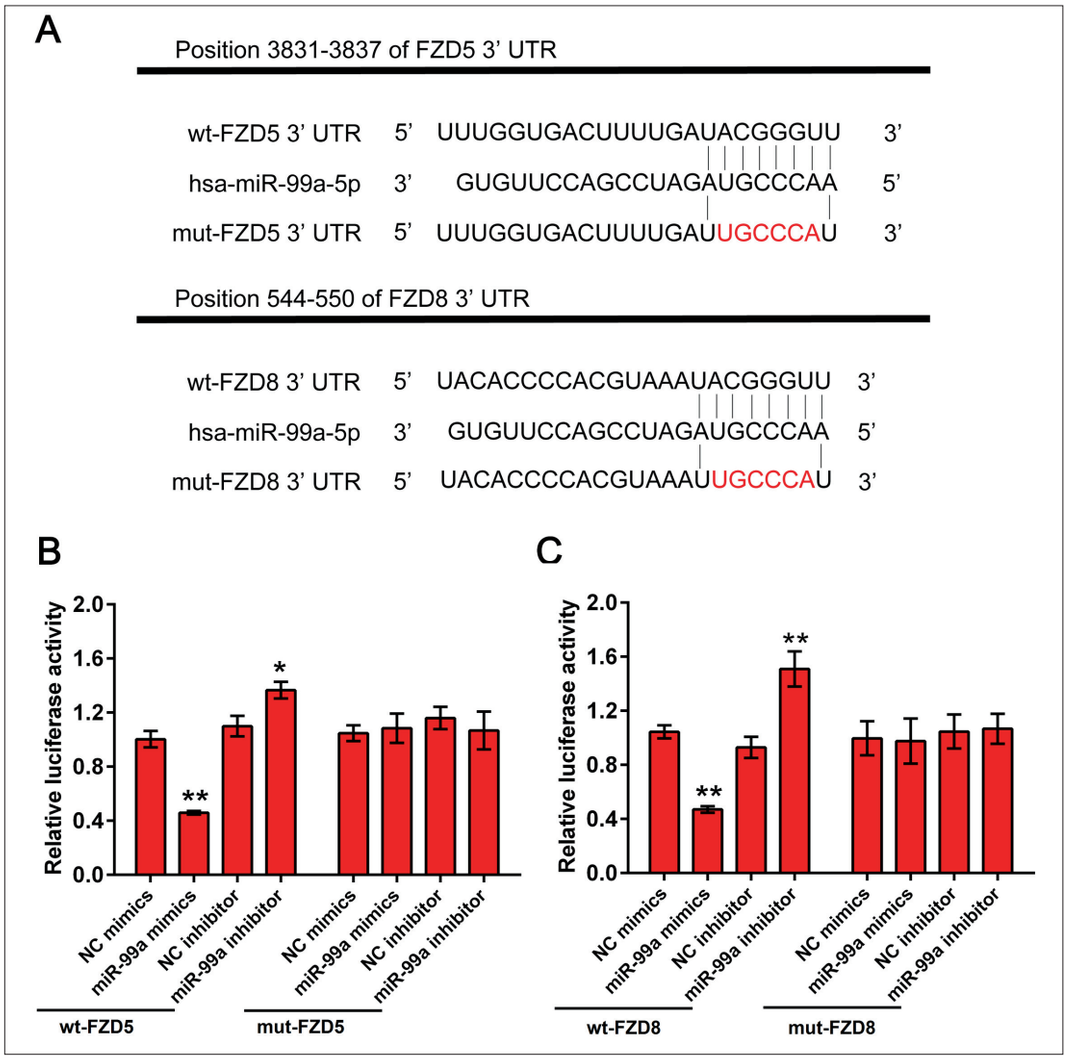

Fig. 4: MiR-99a inhibited FZD5/FZD8 expression by direct binding to the 3'UTR of FZD5/FZD8 (A) A wt-FZD5 or FZD8 3'UTR luciferase reporter gene vector, a mut-FZD5 or FZD8 3'UTR luciferase reporter gene vector containing a 6 bp mutation on the predicted binding site of miR-99a in the 3'UTR of FZD5 or FZD8 3'UTR was constructed. (B) The indicated vectors were co-transfected into HaCaT cells with miR-99a mimics or miR-99a inhibitor, respectively. The luciferase activity was then determined by using Dual luciferase assays. The data are presented as mean±SD of three independent experiments. *P<0.05, **P<0.01.

et al. 2017). In the present study, we observed that the expression of miR-99a in psoriasis lesional tissues was significantly downregulated compared in normal non-lesional tissues, which was consistent with our previous study showing that miR-99a expression is downregulated in peripheral blood mononuclear cells (PBMCs) from psoriasis patients compared with healthy controls (Yang et al. 2016).

To date, more than 250 miRNAs have been reported as aberrantly expressed in psoriasis tissue, the majority of which are found in peripheral blood or involved psoriatic skin, but only small subsets of these dysregulated miRNAs in psoriasis have confirmed mRNA targets with established biological functions in the skin (Hawkes et al. 2016). In the present study, we used online tools to screen out several candidates downstream target genes of miR-99a. Among six candidate downstream genes, the expression levels of FZD5/FZD8 showed to be most strongly up-regulated in psoriasis lesional tissues was significantly downregulated compared to normal non-lesional tissues. We also determined the protein levels of FZD5 and FZD8 in response to miR-99a overexpression and miR-99a inhibition in HaCaT cells; as shown by Western blot assays, both FZD5 and FZD8 could be inversely regulated by miR-99a. FZD5/FZD8 belong to a family of G protein-coupled receptor proteins (Malbon 2004) that serves as receptors in the Wnt signaling pathway and other signaling pathways, including Wnt/β-catenin pathway, Wnt/calcium pathway, and planar cell polarity (PCP) pathway (Huang and Klein 2004). *Frizzled* genes are essential for embryonic development, tissue and cell polarity, formation of neural synapses, and the regulation of proliferation, and many other processes in developing and adult organisms (Huang and Klein 2004), as well as tumor progression (Ueno et al. 2013). In the present study, we investigated the detailed role of miR-99a and FZD5/FZD8 in regulating HaCaT cells' proliferation. Ectopic miR-99a expression significantly suppressed HaCaT cells' viability and proliferation, while forced FZD5 or FZD8 expression promoted HaCaT cells' vitality and proliferation; the suppressive effect of miR-99a on HaCaT cells' proliferation could be partially abolished by forced FZD5 or FZD8 expression.

MiRNAs exert their functions through binding to the 3'UTR of target mRNA genes and subsequently disrupt translation or triggers mRNA degradation (Hawkes et al., 2016). We demonstrated that miR-99a suppresses the protein levels of FZD5 and FZD8, and the proliferation of HaCaT cells, while the suppressive effect of miR-99a on HaCaT cells' proliferation could be partially abolished by forced FZD5 or FZD8 expression; here we performed

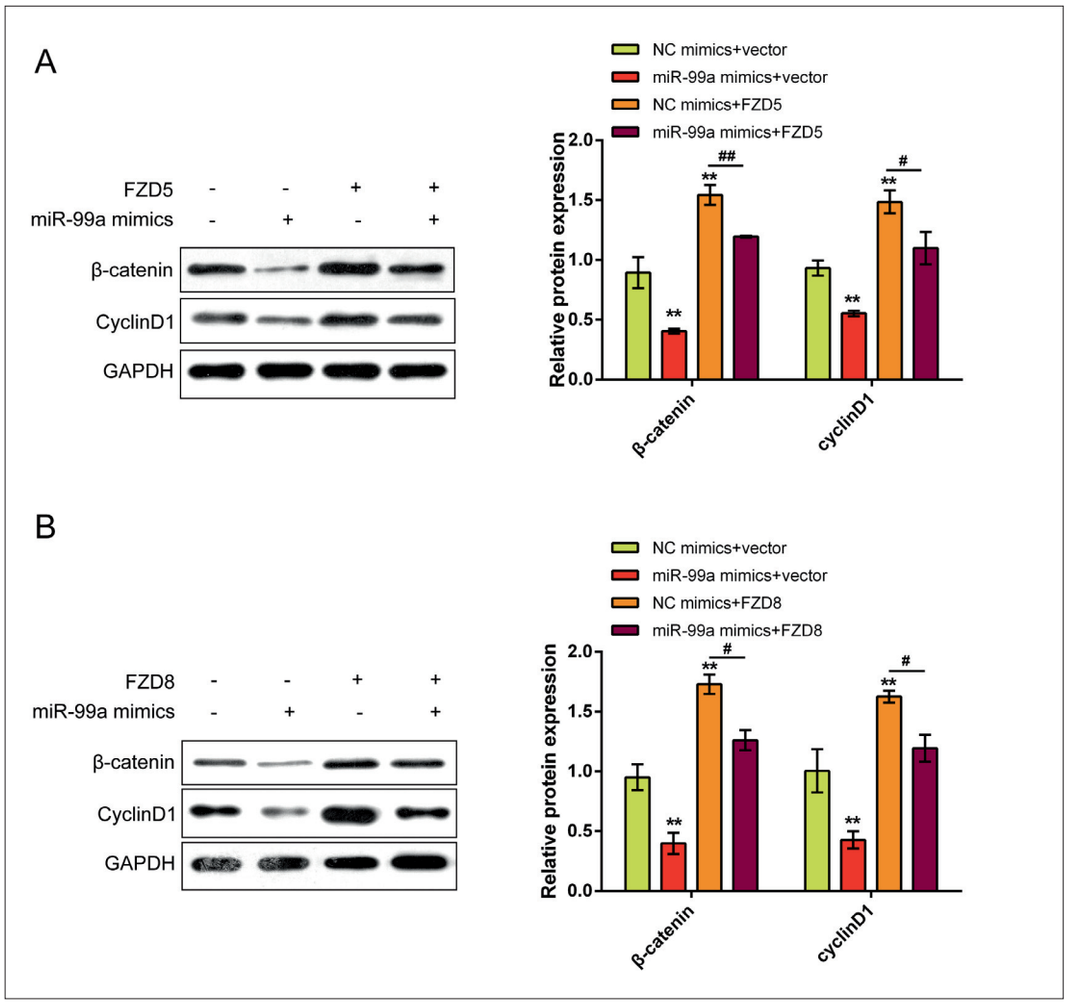

Fig. 5: MiR-99a/FZD5/FZD8 regulated HaCaT cells' proliferation through β-catenin signaling (A) and (B) The protein levels of β-catenin and cyclinD1 in miR-99a mimics- or NC mimics-transfected HaCaT cells with the absence or presence of FZD5/FZD8 were determined by using Western blot assays. The data are presented as mean±SD of three independent experiments. $^{**}P<0.01$, $\#P<0.05$, $\#\#P<0.01$.

a mechanistic analysis to validate whether miR-99a regulates FZD5 or FZD8 expression and HaCaT cells' proliferation through direct binding to the 3'UTR of FZD5 and FZD8, respectively. By performing luciferase assays, we revealed that miR-99a could directly bind to the predicted binding site in the 3'UTR of FZD5 and FZD8, respectively.

As we have mentioned, FZD5 and FZD8 are essential receptors in the Wnt/β-catenin pathway (Huang and Klein 2004). Here we further investigated whether β-catenin and its downstream target gene, cyclinD1, were involved in HaCaT cell proliferation regulation. In HaCaT cells, the protein levels of β-catenin and cyclinD1 were significantly reduced after ectopic expression of miR-99a, increased after forced FZD5 or FZD8 expression; the suppressive effect of miR-99a on the protein levels of β-catenin and cyclinD1 could be partially abolished by forced FZD5 or FZD8 expression. These data suggested that miR-99a/FZD5/FZD8 might regulate HaCaT cell proliferation through downstream β-catenin signaling. In the present study, we provide evidence that the expression of miR-99a is significantly downregulated in psoriasis tissues, and are specifically related to FZD5 and FZD8, respectively, during the psoriasis process. Besides, miR-99a inhibits FZD5 and FZD8 expression by direct binding to the 3'UTR of FZD5 and FZD8 to suppress HaCaT cells' proliferation. Moreover, miR-99a/FZD5/FZD8 might regulate HaCaT cell proliferation through downstream β-catenin signaling. Taken together, we demonstrated that miR-99a/FZD5/FZD8 may serve as useful diagnostic markers and novel targets for treatment strategies of psoriasis.

During the psoriasis process, keratinocyte abnormality is a major but not the only one of various pathological changes (Chong et al. 2013). Hence, despite the reduced keratinocyte proliferation by miR-99a, the potential effect of miR-99a on keratinocyte differentiation and apoptosis might be synergistic in psoriasis treatment, and further studies on these areas are warranted.

4. Experimental

4.1. Clinical specimens, cell lines and transfection

With the approval of the Ethic Committee of The Second Affiliated Hospital of Hunan University of Chinese Medicine, we collected 23 paired psoriatic skin lesional tissues and normal non-lesional tissues from December 2014 to June 2016. All psoriasis patients were diagnosed by dermatologists according to diagnostic standard for psoriasis. All cases signed informed consent. All the tissue samples were snap-frozen and stored at -80 °C in liquid nitrogen.

HaCaT, a spontaneously transformed aneuploid immortal keratinocyte cell line, was obtained from the American Type Culture Collection (ATCC, USA), cultured in 10% fetal bovine serum (Gibco, USA) supplemented RPMI-1640 medium (Invitrogen, USA) at 37 °C with 5% v/v CO_2.

MiR-99a mimics or miR-99a inhibitor (Genepharma, China) was transfected into indicated target cells to achieve miR-99a overexpression or miR-99a inhibition by using Lipofectamine 2000 (Invitrogen). FZD5 and FZD8 pcDNA-vector was used to

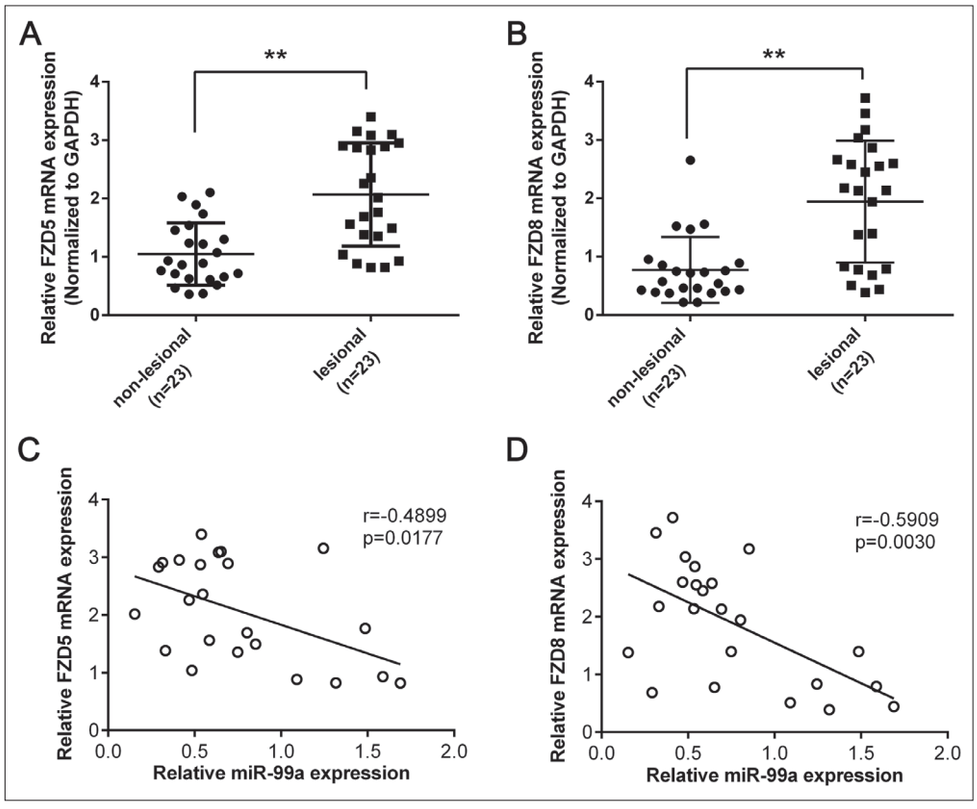

Fig. 6: Expression of FZD5/FZD8 in psoriatic skin lesional tissues and the correlation between FZD5/FZD8 and miR-99a (A) and (B) The expression levels of FZD5 and FZD8 in a panel of 23 paired psoriasis lesion tissues and normal non-lesional tissues was determined by using real-time PCR assays. The data are presented as mean±SD of three independent experiments. $^{**}P<0.01$. (C) and (D) The correlation between miR-99a and FZD5, between miR-99a and FZD8 in psoriatic skin lesional tissues was analyzed by using the Spearman's rank correlation analysis.

achieve ectopic FZD5 and FZD8 expression via transient transfection according to the manufacturer's instructions (GeneCopoecia, China).

4.2. Quantitative real time PCR (qPCR) for miRNA and mRNA detection

Trizol reagent (Invitrogen) was used for total RNA extraction following the manufacturer's instructions. As previous described, total RNA (20 ng) was converted into cDNA using the miScriptRT kit (Qiagen, German). qRT-PCR was performed using a miScript SYBR Green PCR kit (Qiagen, Germany) on the ABI 7900HT Real-time PCR Systerm (Applied Biosysterm, USA) following the $2^{-\Delta\Delta Ct}$ method. The expression of miRNAs or mRNA was normalized using the RNU6 and GAPDH as endogenous control, respectively. The samples were amplified in triplicate and each experiment was repeated three times. Primers for qPCR were as follows (Table).

Table: Primers for qPCR

Primer	Sequence
MiR-99a-F	CGAACCCGTAGATCCGATCTT
MiR-99a-R	GTGCAGGGTCCGAGGT
RNU6-F	CTCGCTTCGGCAGCACA
RNU6-R	AACGCTTCACGAATTTGCGT
FZD5-F	CATGCCCAACCAGTTCAACC
FZD5-R	CGGCGAGCATTGGATCTCC
FZD8-F	ATCGGCTACAACTACACCTACA
FZD8-R	GTACATGCTGCACAGGAAGAA
MBNL1-F	GCTGTTAGTGTCACACCAATTCG
MBNL1-R	AGGCGATTACTCGTCCATTTTC
MTOR-F	ATGCTTGGAACCGGACCTG
MTOR-R	TCTTGACTCATCTCTCGGAGTT
MTMR3-F	GACTGAACAACGCAATCCGAC
MTMR3-R	CCTTGAAGTTACATGCTCCCC
TRIB2-F	ATGAACATACACAGGTCTACCCC
TRIB2-R	GGGCTGAAACTCTGGCTGG

4.3. Western blotting

RIPA buffer (Cell-Signaling Tech., US) was used to lyse the cells. The protein levels of FZD5, FZD8, β-catenin and cyclinD1 in HaCaT cells were detected by immunoblotting. Cells were lysed cultured, or transfected in 1% PMSF supplemented RIPA buffer. Protein were loaded onto SDS-PAGE minigel, and then transferred onto PVDF membrane. The blots were probed with 1:1000 diluted rabbit polyclonal FZD5, FZD8, β-catenin and CyclinD1 antibody (Abcam, USA) at 4 °C overnight, and incubated with HRP-conjugated secondary antibody (1:5000). Signals were visualized using ECL Substrates (Millipore, USA). The protein expression was normalized to endogenous GAPDH.

4.4. Luciferase activity

A wt-FZD5 3'UTR luciferase reporter gene vector, a wt-FZD8 3'UTR vector, a mut-FZD5 3'UTR luciferase reporter gene vector containing a 6 bp mutation on the predicted binding site of miR-99a in the 3'UTR of FZD5, and a mut-FZD8 3'UTR luciferase reporter gene vector containing a 6 bp mutation on the predicted binding site of miR-99a in the 3'UTR of FZD8 was obtained from Yrbio Co, ltd (Changsha, China). HaCaT cells were cultured overnight after being seeded into a 24-well plate, co-transfected with the indicated vectors and miR-99a mimics or miR-99a inhibitor. 48 h after transfection, Dual Luciferase Reporter Assay System (Promega, USA) was used to determine the luciferase activity.

4.5. MTT assay

A modified MTT assay was used to evaluate cell viability. After seeding 2×10^3 transfected cells/well into 96-well culture plates we assessed the viability of HaCaT cells at five time points (on day 1, 2, 3, 4 and 5). In brief, quantification of mitochondrial dehydrogenase activity was achieved through the enzymatic conversion of MTT [3-(4,5-dimethyldiazol-2-yl)-2,5- diphenyltetrazolium bromide; Sigma-Aldrich, MO, USA] to a colored formazan product. MTT (10 µl, 10 mg/ml) was added to the cells, incubated for 4 h, and we terminated the reaction by removal of the supernatant and addition of 100 µl DMSO to dissolve the formazan product. After 0.5 h, the optical density (OD) of each well was measured at 490 nm using a plate reader (ELx808 Bio-Tek Instruments, ST, USA).

4.6. BrdU incorporation assay

By measuring 5-bromo-2-deoxy-uridine (BrdU) incorporation, the DNA synthesis in proliferating cells was determined. BrdU assays were conducted at 24 h and 48 h after HaCaT cells were transfected with the indicated miRNA mimics or other vectors. Cells were seeded in 96-well culture plates at a density of 2×10^3 cells/well, cultured for 24 h or 48 h, then incubated with a final concentration of 10 µM

BrdU (BD Pharmingen, San Diego, CA, USA) for 2 h. When the incubation period ended, the medium was removed, the cells were fixed for 30 min at RT, incubated with peroxidase-coupled anti-BrdU-antibody (Sigma-Aldrich) for 60 min at RT, washed three times with PBS, incubated with peroxidase substrate (tetramethylbenzidine) for 30 min, and the 470 nm absorbance values were measured for each well. Background BrdU immunofluorescence was determined in cells not exposed to BrdU but stained with the BrdU antibody.

4.7. Statistical analysis

Data from three independent experiments were presented as mean±SD, processed using SPSS 17.0 statistical software (SPSS, USA). Paired Student's t-test was used to compare the expression of miR-99a and FZD5/FZD8 in glioma tissues and normal tissues. *P* values of <0.05 were considered statistically significant.

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Conflicts of interest: None declared.

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