

# Increased expression of microRNA-338-3p contributes to production of Dsg3 antibody in pemphigus vulgaris patients

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**Abstract.** Expression of microRNA-338-3p (miR-338-3p) was aberrantly elevated in pemphigus vulgaris (PV), although its role in PV is still unknown. The present study investigated the functional role and possible molecular mechanisms of miR-338-3p in PV. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was performed to detect miR-338-3p expression in peripheral blood mononuclear cells (PBMCs) from patients with PV. Correlation with disease severity and anti-desmoglein 3 antibody (anti-Dsg3) titers were analyzed in patients with PV. The effects of overexpression and knock-down of miR-338-3p expression in PBMCs and effects on Th1 and Th2 cytokines were also examined using ELISA. The luciferase reporter analysis, RT-qPCR and western blot analysis were applied to investigate potential and functional target genes. The data showed that miR-338-3p expression was significantly upregulated in PV and the upregulation of miR-338-3p associated with disease severity and a high anti-Dsg3 antibody titer. Expression of miR-338-3p/mimic in healthy PBMCs significantly downregulated Th1 cytokine (IFN- $\gamma$ ) and upregulated Th2 cytokines (IL-4 and IL-10), whereas knock-down of miR-338-3p expression in PBMCs from patients with

PV induced the reverse effects. Overexpression of miR-338-3p suppressed cell viability. Luciferase reporter, RT-qPCR and western blot assays indicated that TNFR1-associated death domain protein (TRADD) was the direct and functional target of miR-338-3p. Increased expression of miR-338-3p contributed to the production of Dsg3 antibody by inhibiting TRADD expression to induce an imbalance in Th1/Th2 cell functions. Taken together, this study suggests that miR-338-3p may be used as a potential therapeutic target for PV treatment.

## Introduction

Pemphigus vulgaris (PV) is a bullous skin disease mediated by autoantibodies, primarily desmoglein (Dsg) 3 or/and Dsg1 antibodies, and is considered a Th2 cell predominant autoimmune disease. The balance of Th1/Th2 cells in the peripheral blood plays an important role in the PV immunopathogenesis (1). It has been widely demonstrated that levels of Th1 cells and Th1 cytokines (interferon-gamma (IFN- $\gamma$ ), IL-2) are decreased, whereas levels of Th2 cells and Th2-type cytokines (IL-4, IL-10) are significantly increased in the peripheral blood of PV patients (2,3). The mean frequency of Th2 CD4+ T cells significantly elevates in active disease (2). In contrast, Th1 cells show decreased levels in the acute stages of PV (1). Titers of Dsg3-reactive IgG are directly related to the ratio of autoreactive Th1/Th2 cells (1). Altogether, these results suggest that the onset and extent of disease are related to an imbalance of Th1/Th2 cells. However, to date, the exact mechanisms underpinning this phenomenon remain unclear.

Currently, numerous studies have demonstrated that altered expression of miRNAs also play roles in various autoimmune diseases, including multiple sclerosis (4), systemic lupus erythematosus (SLE) (5), rheumatoid arthritis (6), and psoriasis (7). miRNAs are non-coding RNAs and their aberrant expression is involved in various cellular processes, including differentiation, apoptosis and immune response, by suppressing target gene expression. However, it is no report whether miRNAs also play a role in PV. Previously, we demonstrated that there were 124 miRNAs aberrantly expressed in the peripheral blood mononuclear cells (PBMCs) from PV patients after miRNA array analysis (8). miR-338-3p has been listed as one of the most significantly increased miRNAs with more than a 500-fold change between PV

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**Abbreviations:** PV, pemphigus vulgaris; PBMC, peripheral blood mononuclear cell; Th, T helper cells; IFN- $\gamma$ , interferon-gamma; IL, interleukin; TRADD, TNFR1-associated death domain protein; NC, negative control; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; Dsg, desmoglein; miRNA, microRNA; CCK-8, Cell-Counting Kit-8 assay; PVDF, polyvinylidene fluoride; ANOVA, one-way analysis of variance; PAAS, pemphigus area and activity score; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; TRAF2, TNF receptor-associated factor 2; TNFR1, tumor necrosis factor receptor 1

**Key words:** miR-338-3p, pemphigus vulgaris, Dsg3 antibody, Th1/Th2, TRADD

Table I. Polymerase chain reaction primers used in this study.

miR-338-3p	RT primer	5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTTCGCACTGGATACGACCAACAA-3'
	Forward	5'-GTCAGTTCCAGCATCAGTGATT-3'
	Reverse	5'-GTGCAGGGTCCGAGGT-3'
TRADD	Forward	5'-GGACCCTGAAACTCCACTTG-3'
	Reverse	5'-GATGAAGTCCAGGACACCAA-3'

miR, microRNA; TRADD, TNFR1-associated death domain protein.

patients and healthy control samples. As reported, miR-338-3p was considered as a tumor-suppressor and showed to play a role in various diseases, including nasopharyngeal (9), non-small cell lung (10) and hepatocellular carcinoma (11), gastric cancer (12) and breast cancer (13), and esophageal squamous cell carcinoma (14). miR-338-3p also contributed to formation of basolateral polarity in epithelial cells (15) as well as differentiation of odontoblasts (16) and oligodendrocytes (17). In addition, decreased expression of miR-338-3p was shown to increase expression of innate and adaptive immune proteins in celiac disease (18). Thus, miR-338-3p can not only regulate differentiation and apoptosis, but also possess immunomodulatory functions. In PV, however, the impact of the increased expression of miR-338-3p remains to be elucidated. In this study, we investigated the role of miR-338-3p in PV and the immune response.

## Materials and methods

**Patients and peripheral blood samples.** This study was approved by Research Center Ethics Committee, Nanfang Hospital, The Southern Medical University (Guangzhou, China) and the informed consent was obtained from all participants. PV patients were recruited from Nanfang Hospital and only new-onset patients or recurrent patients without immunosuppressant and steroid hormone treatment for at least three months were included in this study. Peripheral blood samples were collected using EDTA anticoagulant tubes when patients were admitted to our hospital. PBMCs from freshly drawn blood of patients and healthy volunteers were purified by gradient centrifugation with Ficoll-Paque Plus, and collected for RNA isolation or transfection experiments.

**RNA isolation, cDNA synthesis and reverse transcription-quantitative polymerase chain reaction (RT-qPCR).** RNA was isolated from PBMCs using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and reversely transcribed into cDNA using All-in-One First-Strand cDNA Synthesis kit (GeneCopoeia Inc., Germantown, MD, USA). qRT-PCR was performed with primers specific for miR-338-3p or TNFR1-associated death domain protein (TRADD) (Table I). 18srRNA and U6 gene regions were used as controls. The qPCR was run on an ABI PRISM® 7500 Sequence Detection System using SYBR-Green PCR Master Mix (Toyobo Co., Ltd., Osaka, Japan). The  $\Delta\Delta C_t$  method was used to normalize transcripts to 18srRNA and U6 and to calibrate the fold changes.

**Transfection of miRNA into PBMCs.** Oligonucleotides of miR-338-3p mimics, miR-338-3p inhibitors, and negative control (NC or inhibitor NC) were synthesized by RiboBio (Shanghai, China). For their transfection, purified PBMCs were suspended in Opti-MEM (Invitrogen) and then transfected with 50 nM of miR-338-3p mimics, miR-338-3p inhibitors or negative control oligonucleotides using Lipofectamine™ RNAiMAX (Invitrogen) for 4 h and after that, the medium was replaced with RPMI-1640 (HyClone, Logan, UT, USA). Transfected cells were harvested at different time points for analysis. Each transfection experiment was carried out in triplicate.

**ELISA detection of cytokine levels.** The blood samples were centrifuged for 5 min at 2,000 rpm, and the serum was saved for anti-Dsg3 antibody detection using MESACUP Desmoglein TEST 'Dsg3' (Medical and Biological Laboratories, Nagano-ken, Japan). In addition, 72 h after gene transfection, PBMC culture medium was collected for detection of T-type cytokines and levels of IFN- $\gamma$ , IL-4 and IL-10 were also measured by ELISA according to the instructions of manufacture of human ELISA Kits (RayBiotech, Norcross GA, USA).

**Cell viability CCK-8 assay.** Transfected cells were seeded into 96-well plates at  $1 \times 10^4$  cells/well and grown for 0, 24, 48 or 72 h, respectively. At the end of each experiment, 10  $\mu$ l of CCK-8 solution (dilution 1:10; CCK-8; Beyotime Institute of Biotechnology, Shanghai, China) was added and incubated for 4 h. The optical density of each well was then measured at the wavelength of 450 nm (OD=450).

**Identification of miRNA targets.** The target genes of miR-338-3p were predicted using three microRNA target databases (MiRanda, PITA and TargetScan). The selected target genes were first validated by dual-luciferase assay and then confirmed using qRT-PCR and western blot assays.

**Dual-luciferase assay.** HEK293 cells were cultured in 24-well plates for a dual-luciferase reporter assay. Briefly, cells were co-transfected with 50 nM of miRNA or miRNA NC and 500 ng of wild or mutant type reporter plasmid (psi-CHECK2) using Lipofectamine 2000 reagent (Invitrogen). Forty-eight hours later, cells were harvested and lysed to measure Firefly and Renilla luciferase activities using the Dual-Glo Luciferase Reporter Assay Kit (Promega, Madison, WI, USA).

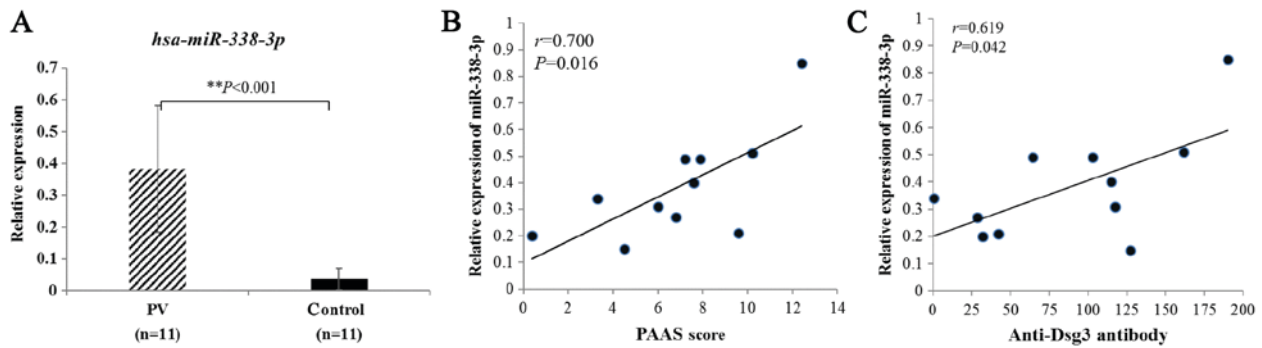


Figure 1. Association of elevated miR-338-3p levels with PV severity. (A) RT-qPCR. Levels of miR-338-3p in PBMCs from PV patients and controls (n=11 each) were detected by RT-qPCR. miR-338-3p was significantly elevated in patients with PV compared to healthy controls. (B) Relative expression of miR-338-3p was positively correlated with PAAS score of patients, analyzed by Person method. (C) Titers of anti-Dsg3 antibodies in serum were detected by ELISA. Relative expression of miR-338-3p was also positively associated with the titers of anti-Dsg3 antibodies of patients.

Table II. Baseline demographics and clinical characteristic of PV patients and healthy volunteers.

Characteristic	Patients with PV (n=11)	Healthy volunteers (n=11)	P-value
Sex			0.68
Female	5 (45.5%)	4 (36.4%)	
Male	6 (54.5%)	7 (63.6%)	
Age, years			0.90
Mean $\pm$ SD	44.27 $\pm$ 12.65	43.64 $\pm$ 11.35	
Median (range)	42 (26-64)	45 (28-65)	
Disease stages			
Initial stage	7 (63.6%)	-	
Recurrent stage	4 (36.4%)	-	
Anti-Dsg3 antibodies, mean $\pm$ SD, median (range)	87.48 $\pm$ 57.07, 103 (0.63-170.3)	-	
PAAS, median (range), IQR			
Cutaneous score	3.6 (0.4-12.4), (1.2-5.95)	-	
Mucus membrane score	6 (0-9), (0-6)	-	
Total score <sup>a</sup>	7.2 (0.4-12.4), (5.25-8.25)	-	

IQR (25th-75th percentiles). <sup>a</sup>Total score is sum of cutaneous score and mucus membrane score. PV, pemphigus vulgaris; PAAS, pemphigus area and activity score; IQR, interquartile range.

**Western blotting.** Cells were harvested and lysed for western blot analysis after 48 gene transfection. Total crude proteins were extracted from PBMC lysates and separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) electrophoresis. Proteins were then electrophoretically transferred on to polyvinylidene fluoride (PVDF) membranes and the membranes were incubated with the rabbit monoclonal antibody against human TRADD at a dilution of 1:1,000 (Abcam, Cambridge, UK) at 4°C overnight followed by horseradish peroxidase-conjugated secondary Goat Anti-Rabbit IgG (1:5,000; Southern Biotech, Birmingham, AL, USA) for 1 h at the room temperature. The immuno-complexes were detected by chemiluminescence. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a protein-loading control.

**Statistical analysis.** SPSS 21.0 software was used for all statistical analyses. The data were presented as means  $\pm$  standard deviation (SD). Group comparisons were analyzed by Student's t-test or a one-way analysis of variance (ANOVA) followed by LSD test. Pearson method was used to analyze the correlation between miR-338-3p and PAAS or anti-Dsg3 antibody. A value of  $P < 0.05$  was considered statistically significant.

## Results

**Association of elevated miR-338-3p expression with PV severity.** In this study, we first analyzed level of miR-338-3p expression in PV vs. control sera using RT-qPCR. The results showed that the average level of miR-338-3p relative expression from PV patients was substantially higher than that from healthy controls (Fig. 1A).

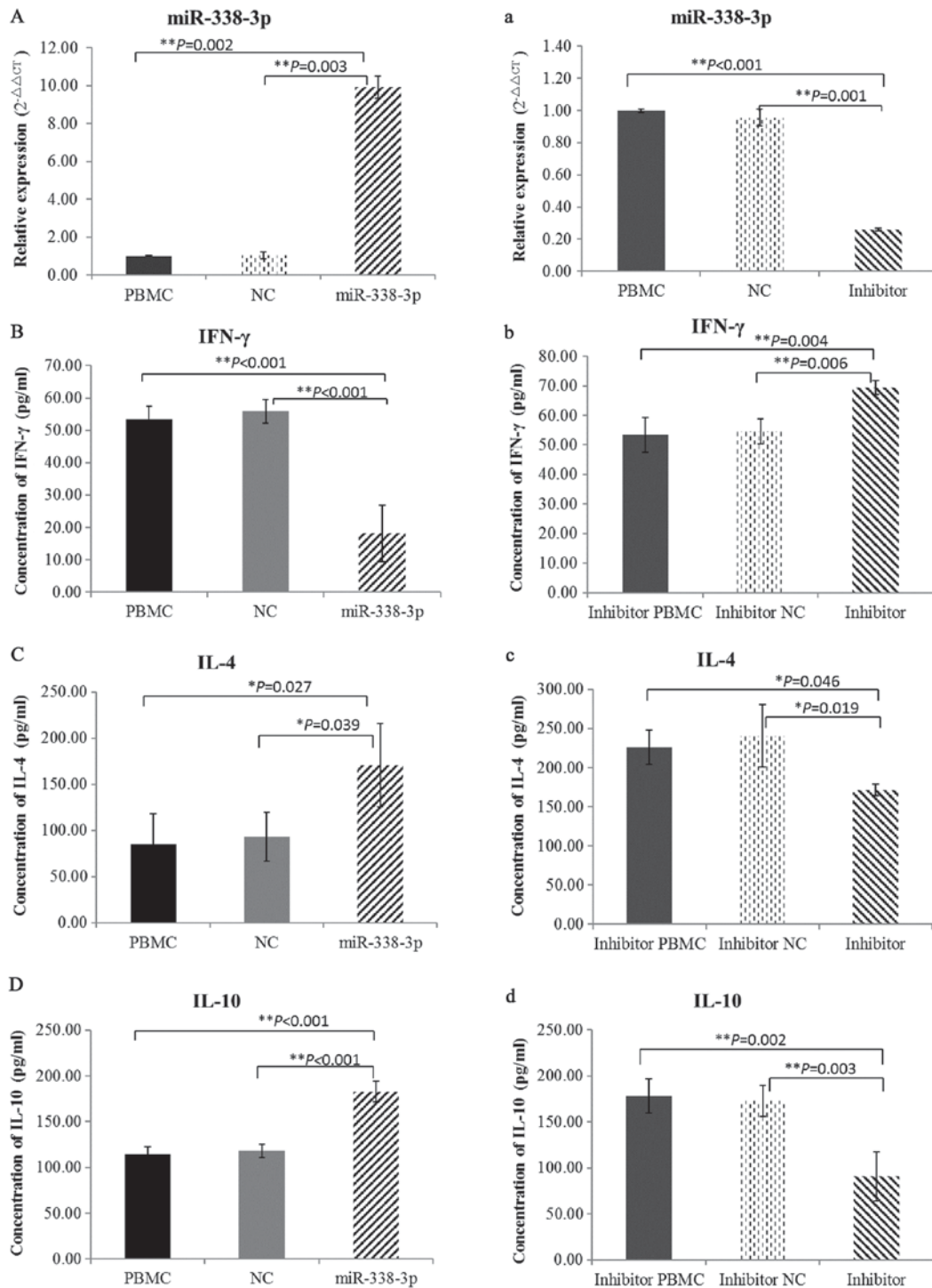


Figure 2. ELISA analyses of T-type cytokines in different groups. PMBCs collected from healthy volunteers or PV patients were cultured *in vitro* for miR-338-3p/mimic or miR-338-3p/inhibitor transfection. Expression of miR-338-3p was detected using RT-qPCR (A-a). When miR-338-3p was overexpressed, the concentration of Th1-type cytokines (IFN- $\gamma$ ) were significantly downregulated in cultural cell supernatant (B), and the concentration of Th2-type cytokines (IL-4 and IL-10) were significant elevated (C and D). However, the opposing findings were also indicated, when miR-338-3p was inhibited (b-d). \* $P<0.05$  and \*\* $P<0.01$ .

We also found that miR-338-3p levels positively correlated with Pemphigus Area and Activity Score (PAAS) (Fig. 1B) and anti-Dsg3 antibody titers (Fig. 1C). Seven initial cases and four recurrent cases were included and no significance was found with respect to sex and age (Table II).

*Effect of miR-338-3p on regulation of Th1/Th2 cell functional balance in cultured cells.* To ensure whether miR-338-3p was

successfully upregulated or downregulated in cultured cells, the expression of miR-338-3p was detected by qRT-PCR after transfection with miR-338-3p mimics, miR-338-3p inhibitor or negative control oligonucleotides (Fig. 2A and a).

To examine the levels of T lymphocyte cytokines, we detected IFN- $\gamma$ , IL-4 and IL-10 by ELISA. miR-338-3p overexpression in cultured PBMCs from healthy individuals lead to significantly decreased levels of IFN- $\gamma$  (Fig. 2B)



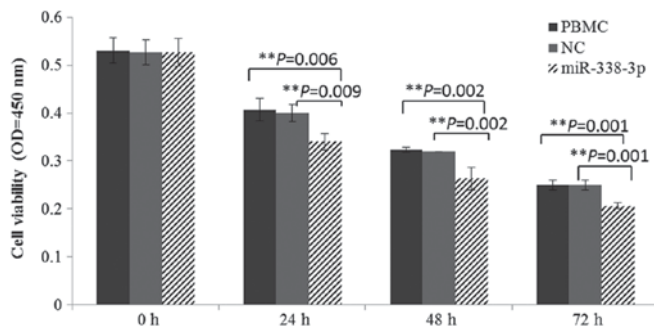


Figure 3. miR-338-3p suppression of cell viability. After transfection, cultured PBMCs were distributed to 96-well plates ( $1 \times 10^4$  cells/well) and collected on 0, 24, 48 or 72 h for CCK-8 kit detection. Cell viability was significantly decreased in miR-338-3p group at 24, 48 and 72 h, respectively.

and the Th2 cytokines, IL-4 and IL-10, were markedly increased (Fig. 2C and D). However, when miR-338-3p was inhibited in cultured PBMCs from patients, opposing findings were observed (Fig. 2b-d). Taken together, these results

suggested that miR-338-3p could regulate the balance of Th1/Th2 cells in PV patients.

**Effect of miR-338-3p on survival of the cultured cells.** To the best of our knowledge, increased expression of miR-338-3p suppresses proliferation and differentiation in tumorigenesis. During *in vitro* culture, cell activity was markedly decreased after miR-338-3p mimic transfection. Cultured cells, with a significantly lower viability in miR-338-3p group, were also confirmed by CCK-8 analysis (Fig. 3). In summary, the overexpression of miR-338-3p could suppress the survival of PBMCs.

**Identification of TRADD as a direct and functional target of miR-338-3p.** Target genes of miR-338-3p were predicted through three databases (MiRanda, PITA and TargetScan), and TRADD, which regulates cell proliferation and apoptosis, was identified as a putative target. Mutation and wild type 3'UTR of TRADD were conducted in Dual-Glo luciferase reporter assay and the results of co-transfected of wt/mut 3'UTR with miRNAs demonstrated that miR-338-3p directly targeted TRADD (Fig. 4A and B).

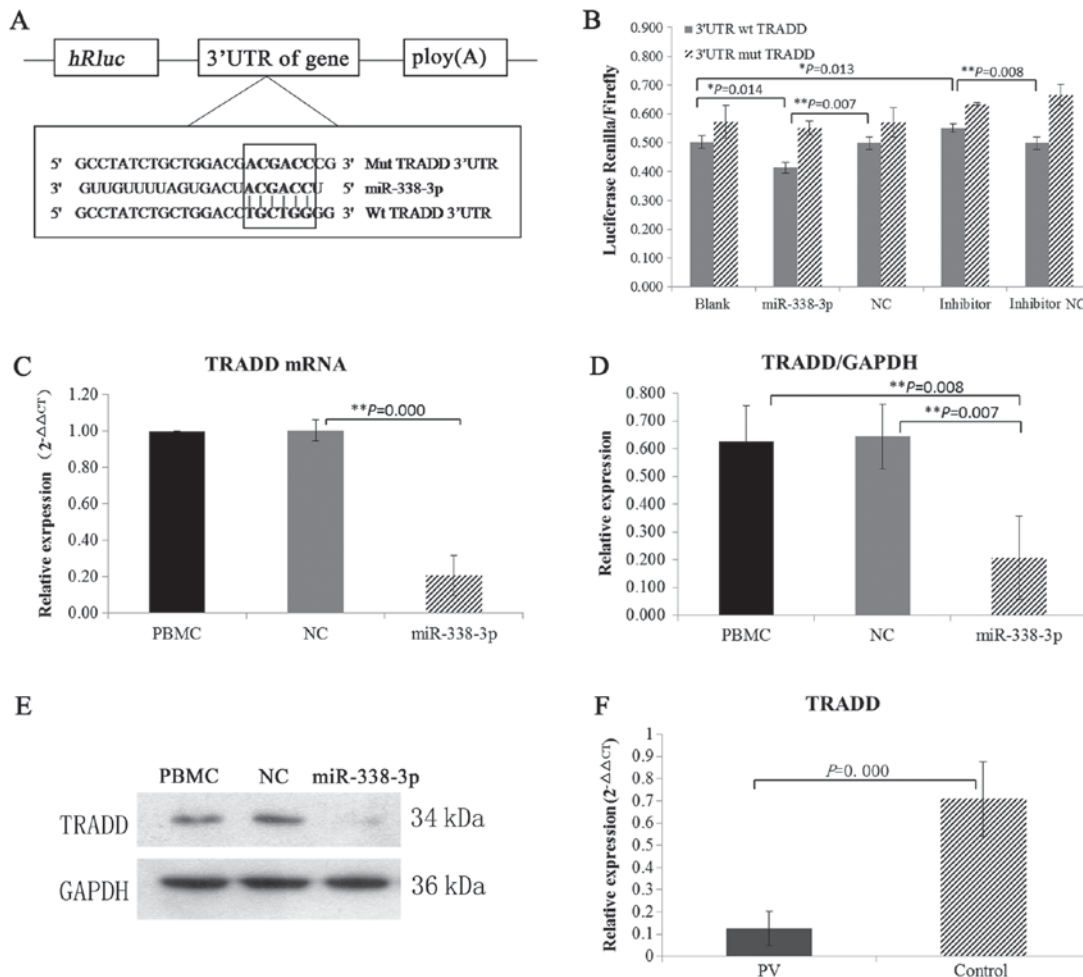


Figure 4. Downregulation of TRADD expression by miR-338-3p overexpression. The diagram showed that the sequences of wt 3'UTR and mutated 3'UTR of TRADD cDNA were contained in reporter constructs (A). Co-transfection of wt/mut 3'UTR with miRNAs in luciferase reporter assays confirmed that miR-338-3p directly targeted TRADD gene (B). The mRNA expression of TRADD in the PBMCs transfected with miR-338-3p mimics *in vitro* was significantly decreased (C). The protein expression of TRADD normalized to GAPDH was also markedly decreased (D and E). The downregulated expression of TRADD can be similarly observed in PBMCs from patients with PV in which the expression of miR-338-3p was significantly increased (F). \* $P < 0.05$  and \*\* $P < 0.01$ .

*miR-338-3p directly targets TRADD in PV.* The changes of TRADD protein expression were detected when miR-338-3p was overexpressed *in vitro*. The levels of TRADD mRNA and protein were found to be significantly lower in the miR-338-3p group compared with PBMC group and control group (Fig. 4C-E). We also examined the expression of TRADD *in vivo*. We found that mRNA expression of TRADD was decreased in PV patients when miR-338-3p was overexpressed (Fig. 4F). Altogether, these findings suggested that miR-338-3p directly targets TRADD in PV.

## Discussion

PV is an intractable autoimmune bullous disease, and the mechanism of the main pathogenic antibody Dsg3, remains elusive. As mentioned earlier, miR-338-3p has been previously suggested to play a role in the pathogenesis of PV. Firstly, increased miR-338-3p expression in PV compared to controls was demonstrated (Fig. 1A). In addition, the expression of miR-338-3p showed a significant positive correlation with PAAS scores (Fig. 1B) and anti-Dsg3 antibody titers (Fig. 1C), which are known to be positively associated with disease extent in PV. Hence, we speculated that miR-338-3p may play a role in the synthesis of Dsg3 antibody.

To study the functions of miR-338-3p in the production of Dsg3 antibody, the experiments of miR-338-3p overexpressed in healthy PBMCs and inhibited in PV PBMCs were conducted, respectively. Results showed that increased expression of miR-338-3p downregulated IFN- $\gamma$  production, and upregulated IL-4 and IL-10 (Fig. 2b-d). However, opposing findings were demonstrated when miR-338-3p was inhibited in PV PBMCs (Fig. 2b-d). These results imply that altered expression of miR-338-3p may be a trigger factor in the imbalance of Th1/Th2 cells in autoimmunity. As previously demonstrated, IFN- $\gamma$  insufficiency and over secretion of IL-4 and IL-10, usually representing the altered proportion of Th1 and Th2 respectively, has been confirmed in peripheral blood of patients with PV (1-3). The balance of Th1/Th2 cells in an immune response plays a pivotal regulative role in the production of Dsg3 antibody, though it is directly secreted by B cells (19). Taken together, elevated expression of miR-338-3p contributes to the production of Dsg3 antibody by mediating an abnormal balance of Th1/Th2 cells in PV.

miRNA-338-3p is known to be a suppressor in tumor cell proliferation. In miR-338-3p overexpression experiments, the declined cultured cell activity was observed in miR-338-3p group (Fig. 3), suggesting that increased expression of miR-338-3p suppressed *in vitro* survival. The imbalance of Th1/Th2 cells in PV may result from an unbalanced inhibition by elevated miR-338-3p between Th1 and Th2 cells. However, miR-338-3p cannot directly mediate cell proliferation or apoptosis. Thus, the potential and functional target genes of miR-338-3p were further predicted through three databases (MiRanda, PITA and TargetScan), and TRADD was identified as a putative target (Fig. 4A and b). TRADD was initially identified as an adaptor molecule, transducing the signal downstream of tumor necrosis factor receptor 1 (TNFR1) that induces either apoptosis or proliferation (20). The death domain of TRADD can recruit FADD or RIP, interacting with TRAF2, leading to apoptosis or the activation

of NF- $\kappa$ B pathway protecting against cell death. TRADD is the key transduction molecule for apoptosis or proliferation, but may not be required for the induction of TNF-induced apoptosis (21). It may play a protective role against apoptosis in TRAIL/TRAIL-R signaling (22). In addition, TRADD contributes to the formation of the membrane survival TRADD-RIP1-TRAF2 complex I leading to proliferation. As a result, insufficiency of TRADD induces apoptosis but not proliferation (23). In miR-338-3p overexpression *in vitro* expression, we observed that the mRNA and protein expression of TRADD was significantly downregulated in response to miR-338-3p overexpression (Fig. 4C-E). In addition, mRNA expression of TRADD was also downregulated in PV patients *in vivo* (Fig. 4F). These results imply that increased miR-338-3p regulates the imbalance of Th1/Th2 cells by directly suppressing the function of TRADD.

In conclusion, we found that miR-338-3p was significantly elevated in PV patients and positively correlated with disease severity. Increased expression of miR-338-3p contributed to the production of Dsg3 antibody by inhibiting the expression of TRADD to induce an imbalance of Th1/Th2 cells. Taken together, we, for the first time, revealed the novel mechanism of miR-338-3p to further the understanding of the pathogenesis of PV.

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## Competing interests

The authors declare that they have no competing interests.

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