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miR-590-3p Alleviates diabetic peripheral neuropathic pain by targeting *RAP1A* and suppressing infiltration by the T cells

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Background: MicroRNAs play a crucial role in diabetic peripheral neuropathic pain (DPNP). *miR-590-3p* is a novel miRNA and involved in multiple diseases. However, the pathological mechanism of *miR-590-3p* in DPNP needs to be elucidated. Materials and methods: The db/db mice and db/m mice were selected to mimic diabetes and control, respectively, for *in vivo* studies. The *miR-590-3p* agomir was injected into db/db mice and pain-related behavioral tests were performed. The interaction of *miR-590-3p* with target gene was confirmed by dual-luciferase reporter assay. The expression of target gene was determined by qRT-PCR and western blot assay. The levels of inflammatory cytokines were measured by enzyme-linked immunosorbent assay (ELISA). Results: *miR-590-3p* was down-regulated in diabetic peripheral neuropathy mice. More importantly, *miR-590-3p* agomir alleviated pain-related behavior, reduced TNF-α, IL-1β and IL-6 concentrations, and inhibited neural infiltration by immune cells in db/db mice. Interestingly, *RAP1A* was predicted to be the target of *miR-590-3p* by Targetscan, and was actually regulated by *miR-590-3p*. Finally, the rescue experiments proved that overexpression of *RAP1A* partially abrogated the suppressive impact of *miR-590-3p* on T cells proliferation and migration. Conclusion: *miR-590-3p* ameliorates DPNP *via* targeting *RAP1A* and inhibiting T cells infiltration, indicating that exogenous *miR-590-3p* may be a potential candidate for clinical treatment of DPNP.

Key words: *miR-590-3p*, diabetic peripheral neuropathic pain, pro-inflammatory cytokines, T cell

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Abbreviations: DPNP, diabetic peripheral neuropathic pain; DRG, dorsal root ganglion; PN, diabetic peripheral neuralgia; ELISA, enzyme-linked immunosorbent assay; IHC, immunohistochemistry; IL-1β, interleukin-1 beta; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TBST, tris buffered saline with Tween; TNF-α, tumor necrosis factor alpha

INTRODUCTION

Diabetes has a high prevalence rate and has become a common chronic disease all over the world. It also induces a complication — diabetic neuropathy, which causes diabetic peripheral neuropathic pain (DPNP) (Davies *et al.,* 2006). Diabetic patients with DPNP experience limb numbness, spontaneous pain, hyperalgesia, or allodynia (Calcutt 2004). Given that diabetic peripheral neuralgia (DPN) is a typical chronic neurogenic pain, it is difficult to treat, affects the physiological functions and quality of life of the patients, and brings a heavy burden to individuals and society (Boulton *et al.,* 2004). The pathogenesis of DPNP is complex, including inflammation, repair process, and gene expression (Vanotti *et al.,* 2007). Although the diverse treatment strategies have advanced, DPNP is still difficult to cure. Therefore, the pathogenesis of DPNP should be further clarified and new treatment strategies should be developed.

MicroRNA (miR), is a non-coding RNA, which generally has a length of 18–22-nucleotides. MiRs can bind to the 3'-UTR of the target gene to modulate the gene expression (Wu *et al.,* 2013). Currently, the expression of miRs has been proved to be closely related to DPN. *miR-146a* influences the severity of DPN through the regulation of inflammation (Feng *et al.,* 2018). Moreover, knockdown of *miR-25* can significantly aggravate diabetic peripheral neuralgia *via* the production of the reactive oxygen species (Zhang *et al.,* 2018). Overexpression of *miR-146a* can suppress hyperglycemia-induced proinflammatory genes and alleviate diabetic peripheral neuralgia (Liu *et al.,* 2017). Moreover, *miR-590-3p* is a novel miRNA involved in multiple diseases. The expression of *miR-590-3p* is reduced in Alzheimer's disease, suggesting that it may be related to neural degradation (Villa *et al.,* 2011). More importantly, *miR-590-3p* is down-regulated in DPN model (Gong *et al.,* 2015). However, the specific role and mechanism of *miR-590-3p* in DPN remain unknown.

RAP1A (Ras-associated protein 1A) is a member of the Ras-like GTPases family and plays a vital role in the cell-matrix and cadherin-mediated cell-cell contacts (Duchniewicz *et al.,* 2006). In addition, RAP1A regulates T cells *via* augmenting lymphocyte responses and activating integrins (Sebzda *et al.,* 2002). Moreover, inhibition of RAP1A alleviates neuropathic pain (Li *et al.,* 2015; Fang *et al.,* 2019). Also, accumulating evidence has proved that pro-inflammatory T cells migrate into the spinal cord in several pain models, and the immune cell infiltration is involved in diabetic neuropathy (Costigan *et al.,* 2009; Grace *et al.,* 2011; Agarwal *et al.,* 2018). Given all these results, RAP1A might be related to the pathogenesis of DPNP.

The aim of our study was to elucidate the role of *miR-590-3p* in DPNP. We found that *miR-590-3p* was downregulated in db/db mice (type 2 diabetes model). Overexpression of *miR-590-3p* alleviated DPNP in db/db mice by targeting *RAP1A*, providing strong evidence for the role of altered *miR-590-3p*/*RAP1A* axis in the development of DPNP.

MATERIALS AND METHODS

Animals. All animal experiments were performed in accordance with the Guide for the Care and Use of

Laboratory Animals and were approved by the Ethics Committee of the First Affiliated Hospital of Soochow University. The 20-week-old male db/db mice and db/m mice use in this study were purchased from Nanjingjunke (China).

miR-590-3p Agomir and NC agomir (Genepharma, China) were successfully injected into 20-week-old mice according to the manufacturer's protocol. We delivered *miR-590-3p* agomir or NC agomir to db/db mice via tail vein once a week for 4 weeks under anesthesia with isoflurane. Before sacrificing the mice, pain-related behavioral tests were performed. Before the end of the animal experiments, the blood glucose in one drop of tail blood and body weight were determined with a blood glucose monitoring system (Bayer, Germany) and electronic balance (Meitele, Switzerland), respectively. Finally, the dorsal root ganglion (DRG) tissues were extracted, frozen and stored for further experiments.

Isolation of T cells and transfection. pleens from db/m mice were collected and disaggregated, and then the erythrocytes were lysed with Lysing Buffer (BD Biosciences, USA). The T cells were isolated using $CD4^+$ T cell Isolation Kit (Miltenyi Biotec, Germany). Next, the T cells were cultured and stimulated with 0.5 μg/ml anti-CD3e (BD Biosciences, USA) in RPMI-1640 medium (Gibco, USA) containing 10% fetal bovine serum (FBS; Gibco, USA) at 37°C.

The T cells were transfected with pcDNA3 or pcDNA3-*RAP1A* (GenePharma, China) and *miR-590-3p* mimic or NC mimic (GenePharma, China) using Lipofectamine 2000 (Invitrogen, USA). The sequences for NC mimic were: 5'-UUCUCCGAACGUGUCACGU-3' and for *miR-590-3p* mimic: 5'-UAAUUUUAUGUAUAAGCUAGU-3'.

Pain-related behavioral tests. The pain-related behavioral tests were performed as follows: the mice were placed on a hot plate (approx. 55°C), and then the reaction time (latency of the first recoil or struggle of the rear paw) was recorded. To avoid skin damage, we applied a cutoff time of 10 seconds. The shortened withdrawal latency indicated hyperalgesia (Chopra *et al.,* 2010). In addition, each mouse's tail-swinging delay was determined by dipping the distal end of the tail 2–3 cm into a vessel filled with cold water (10±1°C). The data on the duration of the struggle, tail-swinging reaction, or tail-swinging were recorded. The threshold value was set at 15 seconds. A shorter soaking time indicated hyperalgesia which is also attributed to a central mechanism (Kamboj *et al.,* 2010).

qRT-PCR assay. The total RNA from DRG tissues or T cells was extracted using TRIzol reagent (Takara, Japan), and cDNA was synthesized using PrimeScript™ RT Master Mix (Takara, Japan). The expression of the target gene was evaluated using SYBR Green PCR Master Mix (Takara, Japan) based on $2^{-\Delta\Delta Ct}$ method. U6 or β-actin was used as an endogenous control for the normalization. The primer sequences were as follows: *miR-590-3p* forward: 5'-AAAGATTCCAAGAAGCTAAGGGTG-3' and reverse: 5'-CCTAACTGGTTTCCTGTGCCTA-3'; U6 gene forward: 5'-GCTTCGGCAGCACATATACTAAAAT-3' and U6 gene reverse: 5'-CGCTTCACGAATTTGCGTGTCAT-3'; *RAP1A* forward: 5'-TGTCTCACTGCACCTTCA-3' and *RAP1A* reverse: 5'-GACTTCCCAACGCCTCCT-3'; β-actin gene forward: 5'-TCACCCACACTGTGCCCATCTACGA-3' and β-actin gene reverse: 5'-CAGCGGAACCGCTCATTGCCAATGG-3'.

Western blot. The cells or DRG tissue were lysed with RIPA lysis buffer (Beyotime, China) to isolate the proteins, and the protein concentration was determined using a BCA kit (Beyotime, China). The equal amount of protein was separated by SDS-PAGE and transferred onto a PVDF membrane. Then the membranes were washed with TBST (Tris Buffered Saline with Tween) buffer three times and incubated with anti-RAP1A antibody (1:800; Abcam, UK) or anti-β-actin antibody (1:800; Abcam, UK) overnight at 4°C. After washed with TBST buffer for three times, the membranes were then incubated with Goat Anti-Rabbit IgG H&L (HRP) (1:8000; Abcam, UK) or Goat Anti-Mouse IgG H&L (HRP) (1:8000; Abcam, UK) for 2 h. Finally, an electrochemiluminescence kit (Beyotime, China) was used to measure the corresponding protein expression levels.

Luciferase reporter assay. The 3'-UTR of *RAP1A* and the potential target sequences of *miR-590-3p* were predicted by Targetscan (http://www.targetscan.org) and inserted into pGL3 plasmids (Promega, USA). When isolated T cells grew to about 80% confluence, they were co-transfected with pGL3-*RAP1A*-WT vectors (*RAP1A* WT) or pGL3-*RAP1A*-MUT vectors (*RAP1A* MUT) along with *miR-590-3p* mimic or NC mimic (GenePharma, China) using Lipofectamine 2000 (Invitrogen, USA). After 24 hours, the luciferase activity was evaluated.

ELISA assay. The levels of IL-1β, TNF-α and IL-6 in DRG tissues were evaluated by ELISA. After the preparation of the samples, the concentration of IL-6, IL-1β and TNF-α in the sample supernatant was determined with IL-6, IL-1β and TNF-α ELISA kit (Elabscience, China), respectively. Each measurement was repeated three times.

Immunohistochemistry (IHC). The collected DRG tissues were subjected to IHC staining. After fixation with 10% paraformaldehyde, the samples were sliced horizontally, blocked with goat serum (Jackson ImmunoResearch, USA), and probed with anti-CD4 antibody (1:200; Abcam, UK). DAB and hematoxylin were used to stain the tissue slices. RAP1A staining was visualized using a light microscope (Olympus, Japan).

Cell Counting Kit-8 (CCK-8) assay. For CCK-8 assay, after transfection, T cells were seeded into 96-well plates at the density of 3×10^3 cells/well. After cultured for 48 h, the cell proliferation was detected using Cell Counting Kit-8 (CCK-8) assay (Beyotime, China). Finally, the absorbance at the 450 nm was determined.

Transwell assay. After transfection, T cells were seeded onto the upper chamber of a Transwell plate (filters diameter: 6.5 mm, pore size: 5 μm; Corning, USA). The lower chamber was filled with medium containing sphingosine-1-phosphate (S1P; Sigma, USA). After 4 hours of incubation, the cells in the lower chamber were harvested and counted under a light microscope (Olympus, Japan).

Data statistics. The data in this study were analyzed with SPSS 20.0 (SPSS, USA) and presented as mean and Standard Deviation (S.D.). The Student's *t*-test or one-way analysis of variance was performed for comparison between two groups or more than two groups, respectively. $P<0.05$ was considered to indicate a significant difference.

RESULTS

miR-590-3p was down-regulated in diabetic peripheral neuropathy mice

Compared to db/m mice, the blood glucose in db/db mice was markedly elevated, suggesting the suc-

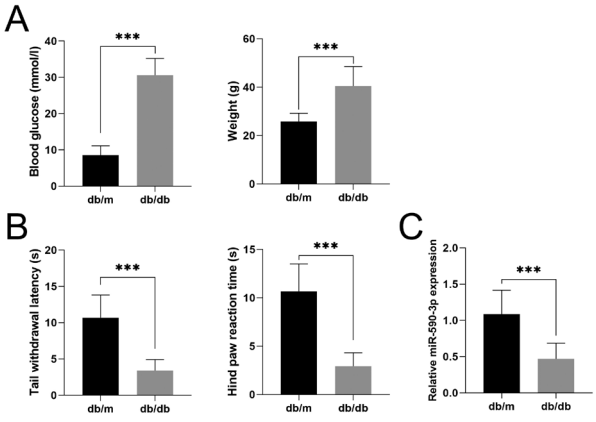

Figure 1. *miR-590-3p* was down-regulated in diabetic peripheral neuropathy mice
The 20-week-old db/db mice (diabetes) and db/m mice (control) were studied. (**A**) Blood glucose and weight were measured. (**B**) The tail immersion and hot plate tests were performed. (**C**) The expression of *miR-590-3p* was determined by qRT-PCR in DRG tissue. Ten mice per group. ***p<0.001.

cessful establishment of diabetes models (Fig. 1A). Besides, compared to db/m mice, the body weight was higher in db/db mice. For pain-related behavior test, the hind paw reaction time and tail withdrawal latency were lower in db/db mice compared to those in db/m mice, suggesting that db/db mice were in hyperalgesia and suffered from diabetic neuropathy pain (Fig. 1B). Interestingly, compared to db/m mice, the db/db mice exhibited a reduction of *miR-590-3p* levels in DRG tissue (Fig. 1C). These results confirmed that diabetic peripheral neuropathy mice models were successfully established and *miR-590-3p* was down-regulated in diabetic peripheral neuropathy mice.

miR-590-3p alleviated peripheral neuropathic pain in diabetic mice

To further clarify how *miR-590-3p* alleviates peripheral neuropathic pain in diabetic mice, the 20-week-old db/db mice were injected with *miR-590-3p* agomir or NC agomir (10 mg/kg) weekly for 4 weeks. Compared to db/m mice, *miR-590-3p* level in db/db mice was significantly declined, while *miR-590-3p* agomir markedly promoted *miR-590-3p* expression compared to NC agomir (Fig. 2A). Moreover, blood glucose and body weight were significantly elevated in db/db mice, whereas *miR-590-3p* agomir did not influence blood glucose and body weight (Fig.2B). As for pain-related behavior of mice, the hind paw reaction time and tail withdrawal latency were increased by *miR-590-3p* agomir, indicating that *miR-590-3p* agomir alleviated diabetic neuropathy pain (Fig. 2C). Thus, these findings proved that *miR-590-3p* decreased peripheral neuropathic pain *in vivo*.

miR-590-3p inhibited the production of pro-inflammatory mediators and neural infiltration by the immune cells

An excessive amount of blood glucose stimulates the production of pro-inflammatory cytokines (including IL-6, IL-1β and TNF-α) and directs the cells toward inflammation (Rains & Jain 2011). When compared to the control, the levels of IL-6, IL-1β, and TNF-α in db/db mice were increased, however, they were significantly reduced by *miR-590-3p* agomir (Fig. 3A). Accumulating evidence has shown a significant neural infiltration by the immune cells in DPNP patients (Younger *et al.,* 1996; Alexandraki *et al.,* 2006). The infiltration by the immune cells was determined by IHC staining of CD4. The expression of CD4 in DRG tissue of db/db mice was elevated (Fig. 3B). After the injection of *miR-590-3p* agomir into db/db mice, the expression of CD4 was reduced (Fig. 3B). These findings proved that exogenous *miR-590-3p* inhibited the production of pro-inflammatory mediators and neural infiltration by immune cells in db/db mice.

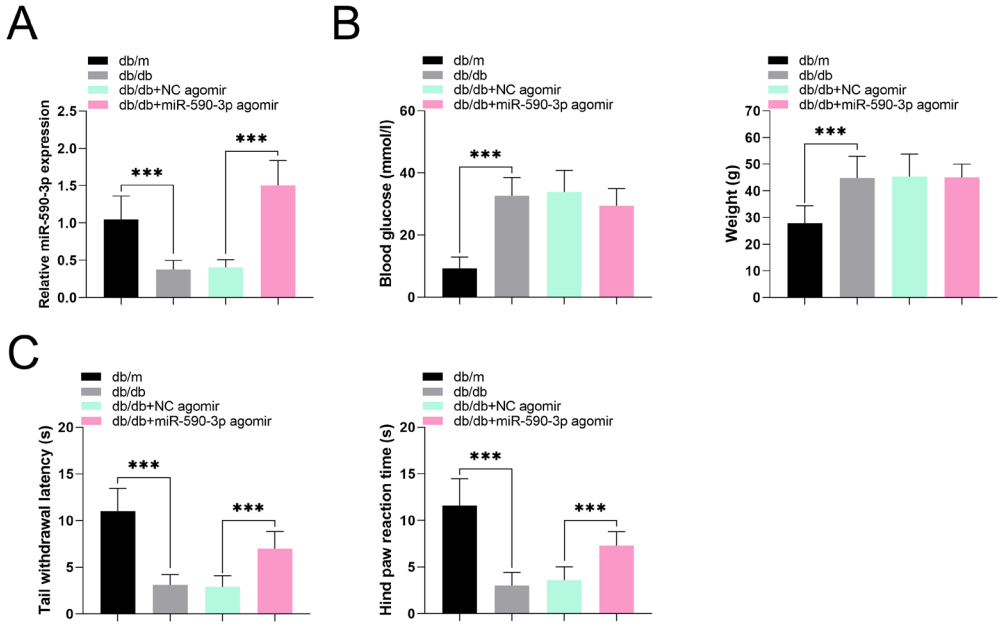

Figure 2. *miR-590-3p* alleviated peripheral neuropathic pain in diabetic mice
The 20-week-old db/db mice were injected with *miR-590-3p agomir* or NC agomir (10 mg/kg) weekly for 4 weeks. (**A**) The expression of *miR-590-3p* in treated mice was detected by qRT-PCR. (**B**) Blood glucose and weight were measured. (**C**) The tail immersion and hot plate tests were performed. Ten mice per group. **p<0.01, ***p<0.001.

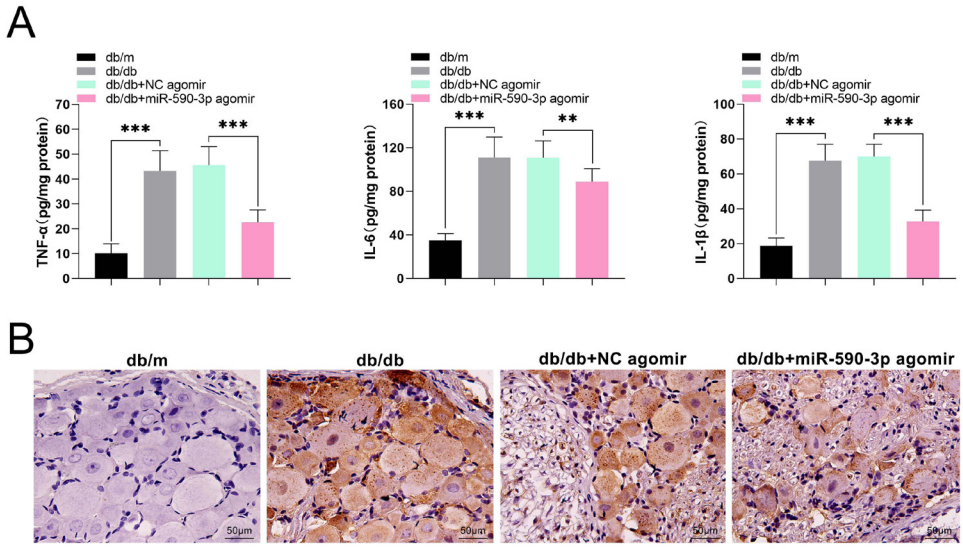

Figure 3. *miR-590-3p* **inhibited the production of pro-inflammatory mediators and neural infiltration by the immune cells**
(**A**) The pro-inflammatory cytokines (such as TNF-α, IL-6, and IL-1β) in differently treated mice were measured by ELISA assay. (**B**) The expression of CD4 in DRG tissues of differently treated mice was detected by IHC, bar=100 μm. **p<0.01, ***p<0.001.

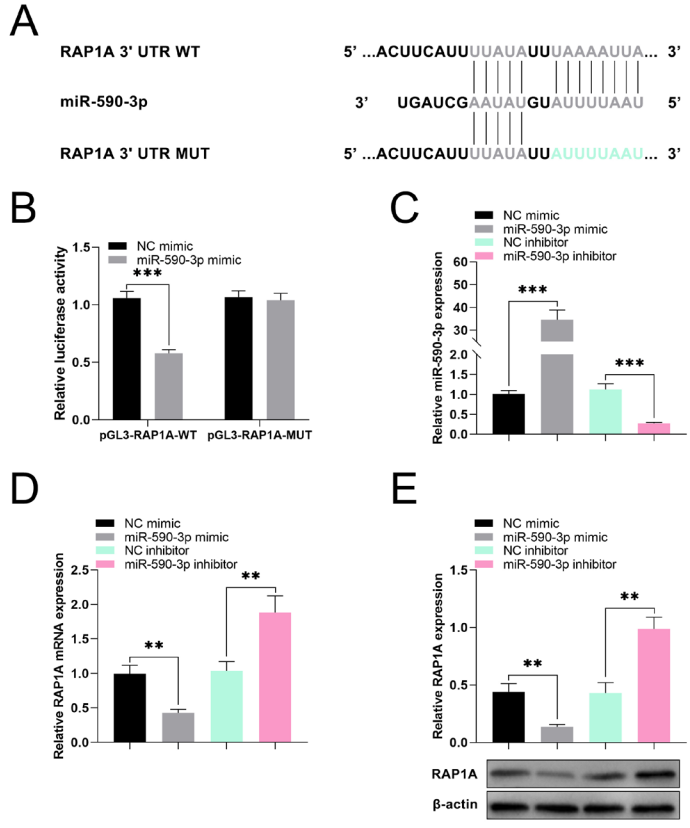

Figure 4. ***RAP1A*** **was a direct target of** ***miR-590-3p***
(**A**) The potential *miR-590-3p* binding sites in the 3'-UTR of *RAP1A*. (**B**) Relative luciferase activity of the T cells transfected with *miR-590-3p* mimic or NC mimic plus the *RAP1A*-WT or *RAP1A*-MUT luciferase reporter gene. (**C**) The efficiency of *miR-590-3p* mimic or *miR-590-3p* inhibitor was judged by qRT-PCR assay. (**D**) The mRNA level of *RAP1A* was determined by qRT-PCR assay. (**E**) The protein level of RAP1A was determined by western blot. Every experiment was repeated three times. **p<0.01, ***p<0.001.

RAP1A was a direct target of *miR-590-3p*

Next, the potential target of *miR-590-3p* was identified. RAP1A, a vital protein, was predicted to be a validated target of *miR-590-3p* by Targetscan. As illustrated in Fig. 4A, *miR-590-3p* binds to a conserved site of *RAP1A* 3'-UTR. The luciferase activity assay confirmed that *miR-590-3p* mimic markedly decreased the luciferase activity of *RAP1A* 3'-UTR WT, but not *RAP1A* 3'-UTR MUT (Fig. 4B). Moreover, *miR-590-3p* mimic or *miR-590-3p* inhibitor significantly increased or decreased *miR-590-3p* expression in T cells (Fig. 4C), respectively. Interestingly,

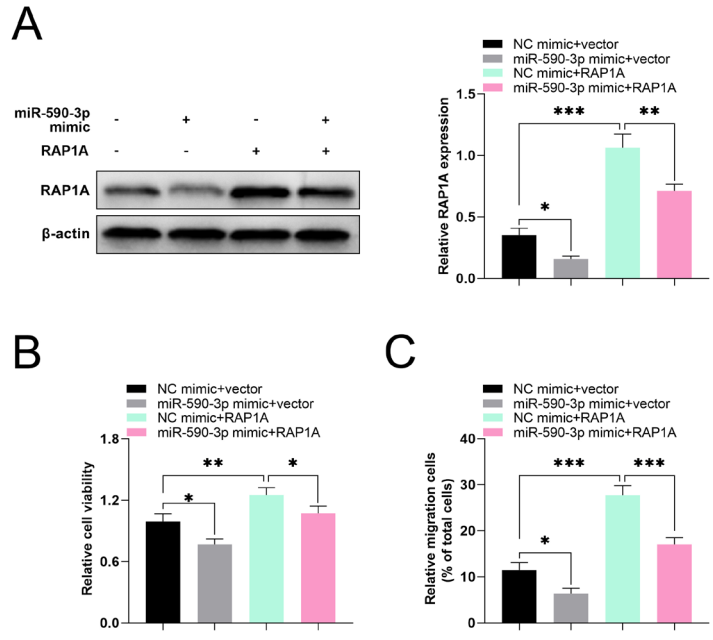

Figure 5. *miR-590-3p* inhibited T cells proliferation and migration *via* targeting *RAP1A*
The RAP1A3-overexpression vector or the corresponding empty vector and NC mimic or *miR-590-3p* mimic were co-transfected into T cells. (**A**) The expression of RAP1A was detected by western blot. (**B**) The cell proliferation was determined using the CCK-8 kit. (**C**) Four hours after transfection, the cell migration ability was evaluated by Transwell assay. Every experiment was repeated three times. *p<0.05, **p<0.01, ***p<0.001.

qRT-PCR and western blot assay confirmed that *miR-590-3p* mimic lowered both mRNA and protein levels of RAP1A in T cells, however, *miR-590-3p* inhibitor elevated both mRNA and protein levels of RAP1A (Fig. 4D–E). These findings suggested that *miR-590-3p* regulated the expression of RAP1A via targeting *RAP1A*.

miR-590-3p inhibited proliferation and migration of T cells *via* targeting RAP1A

To clarify whether *miR-590-3p* affected the production of pro-inflammatory mediators and neural immune cells infiltration in a RAP1A-dependent manner, the isolated T cells were co-transfected with *RAP1A3*-overexpression vector or the corresponding empty vector and NC mimic or *miR-590-3p* mimic. Western blot verified that *miR-590-3p* mimic partially reduced the effect of RAP1A overexpression (Fig. 5A). More importantly, CCK-8 assay indicated that *miR-590-3p* mimic markedly reduced cell viability, whereas this effect was reversed by overexpression of RAP1A (Fig. 5B). Furthermore, Transwell assay results showed that *miR-590-3p* mimic reduced cell migration ability, and the effect was reversed by overexpression of RAP1A (Fig. 5C). With these results, we concluded that overexpression of RAP1A partially abrogated the suppressive impact of *miR-590-3p* on T cells proliferation and migration.

DISCUSSION

In the current study, the diabetic peripheral neuropathy mice models were successfully established and we found that *miR-590-3p* was down-regulated in diabetic peripheral neuropathy mice. Moreover, *miR-590-3p* agomir reduced the production of pro-inflammatory mediators and neural infiltration by the immune cells in db/db mice. Interestingly, *RAP1A* was predicted to be the direct target of miR-590-3p by Targetscan, and *miR-590-3p* regulated the expression of *RAP1A*. Finally, the rescue experiments proved that overexpression of RAP1A partially rescued the suppressive effects of *miR-590-3p* on T cells proliferation and migration. Together, these findings suggested that exogenous *miR-590-3p* may be beneficial to the clinical treatment of DPNP.

The hyperglycemic state may induce the development of DPN, which induces oxidant and inflammatory mediators, thereby leading to deleterious effects in tissues (Anitha *et al.,* 2006; Martyn *et al.,* 2008). Although DPN has been studied, the molecular mechanism underlying inflammatory mediators has not been fully explored (Feldman *et al.,* 2017). Accumulating evidence has confirmed that miRNAs are involved in DPN (Zhu & Leung 2015). For instance, *miR-146a* and *miR-155* regulate inflammation in diabetic peripheral neuropathy (Feng *et al.,* 2018; Chen *et al.,* 2019). Meanwhile, *hsa-miR-590-3p* was identified as an LDHA-suppressing microRNA and can significantly improve glucose metabolism in T2D mice model (Chen *et al.,* 2015). Moreover, a recent study demonstrated that *miR-590-3p* is down-regulated in DR cells (Chen *et al.,* 2018). Similarly, the expression of *miR-590-3p* was found to be decreased in db/db mice, which provided strong evidence for its vital role in diabetes *in vivo*. Furthermore, *miR-590-3p* agomir alleviated DPNP in db/db mice. Nevertheless, the mechanism of how *miR-590-3p* alleviates DPNP remains unknown.

Neuropoietic cytokines (such as TNF-α, IL-6, and IL-1β) are imperative to keep homeostasis of peripheral neurons (Skundric & Lisak 2003). Previous reports demonstrated that IL-1β, TNF-α and IL-6 levels are elevated in diabetic patients and animals compared to the controls (Abdel Aziz *et al.,* 2001; Lee *et al.,* 2013; Cox *et al.,* 2017). In this study, we demonstrated that *miR-590-3p* agomir reduced the IL-1β, TNF-α and IL-6 concentrations, and alleviated DPN pain-related behavior in db/db mice, indicating that *miR-590-3p* suppressed DPNP via an anti-inflammatory function. More importantly, diabetes-induced inflammation occurs with inflammatory infiltrations (Thaisetthawatkul *et al.,* 2018). Regarding this,

we demonstrated that *miR-590-3p* inhibited neural infiltration by T cells. Besides, under hyperglycemia, TNF-α production aggravates in neural tissues, thereby inducing nerve damage, and finally resulting in the development of diabetic polyneuropathy (Satoh *et al.,* 2003). Many central nervous system paradigms proved that IL-6 induces axonal regeneration, which may be related to immune cells proliferation and migration (Leibinger *et al.,* 2013; Carmel *et al.,* 2015). Thus, we suspected that *miR-590-3p* affects the T cells migration and proliferation, which needed more experiment in the next step.

RAP1A is confirmed to be a target of a series of miRNAs and is involved in cell migration and proliferation in non-small cell lung cancer, breast cancer, cervical cancer and prostate cancer (Xiang *et al.,* 2015; Zhang *et al.,* 2018; Cao 2019; Lu *et al.,* 2020). In this study, *RAP1A* was proved to be a direct target of *miR-590-3p*. As we suspected, *miR-590-3p* inhibited T cells proliferation and migration in DPNP, which was reversed by over-expression of RAP1A. These findings suggested that *miR-590-3p* inhibited T cells proliferation and migration via targeting *RAP1A* in DPNP, which was consistent with the previous research. A recent study pointed out that *lncMIR205HG* acts as a natural decoy for *miR-590-3p* and leads to the process of head and neck squamous cell carcinoma (Di Agostino *et al.,* 2018). Besides, RAP1A contributes to cell migration via regulating the MAPK/ERK pathway (Fujita *et al.,* 2005; Zhang *et al.,* 2018). Given all these reports, we hypothesized that *lncMIR205HG* may target *miR-590-3p* and activate the MAPK/ERK pathway in DPNP. However, this hypothesis needs more experiments to confirm it in the future.

In summary, our data demonstrated for the first time that miR-590-3p inhibited the production of pro-inflammatory mediators and neural infiltration by T cells in DPNP. Moreover, additional *in vitro* studies indicated that *miR-590-3p* inhibited T cells proliferation and migration by targeting *RAP1A*. Our findings provide a novel insight into the molecular mechanism and molecular basis of treating DPN with exogenous *miR-590-3p*. Although the treatment strategy of exogenous *miR-590-3p* can be implemented, the interaction network of the *miR-590-3p* in diabetes remains to be elucidated.

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Not applicable.

Competing interests

The authors state that there are no conflicts of interest to disclose.

Ethics approval

All animal experiments were in accordance with the Guide for the Care and Use of Laboratory Animals and were approved by the Ethics Committee of the First Affiliated Hospital of Soochow University.

Statement of Informed Consent

Not applicable.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

Yihua Wu and Ye Gu designed the study, supervised the data collection, analyzed the data, Bimin Shi interpreted the data and prepared the manuscript for publication, supervised the data collection, analyzed the data and reviewed the draft of the manuscript. All authors have read and approved the manuscript.

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