

GuaLou GuiZhi decoction represses LPS-induced BV2 activation via miR-155 induced inflammatory signals

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Abstract: Inflammatory response that occur post-ischemia is a serious problem in the treatment of ischemic brain disease. MicroRNA-155 is a brain-specific or brain-enriched miRNA, which mediates inflammatory reactions in cerebral ischemic tissue by regulating inflammatory signal and the expression level of SOCS1. The present study was aimed to assess the effect of GuaLou GuiZhi Decoction (GLGZD) on miR-155 expression in activated microglia following inflammation and further explore the role of GLGZD on expression of the inflammation-related gene. BV2 cells were used to simulated by LPS to make the inflammatory model. Expression level of miR-155 was detected by Real-Time PCR. BV2 cells after simulated by LPS were then transfected with miR-155 mimic and its negative controls. Cytokines release were measured by corresponding purchased ELISA kits, respectively. Then target protein expression of miR-155 were detected by western blotting assay. After miRNA over expression transfections, expressions of inflammation-related factors, SOCS-1 and SMAD in BV2 cells after activation were measured by Western blot assay. Results showed that in BV2 cells after simulated by LPS, miR-155 was upregulated. The elevated miR-155 expression enhanced the inflammatory cytokine release. miR-155 directly target and negatively regulated SOCS-1 and SMAD-1 expression. Over expression of SOCS-1 and SMAD reduced inflammatory action that was enhanced by miR-155 mimic transfection. miR-155 was positively related with activation of NF- κ B signal pathways via SOCS-1 and SMAD. In conclusion, GuaLou GuiZhi Decoction (GLGZD) might exert its anti-inflammatory action by inhibiting the expression of miR-155, indicating that miR-155 may be used as a treatment target in clinical treatment with GuaLou GuiZhi Decoction (GLGZD) in ischemic brain.

Keywords: Traditional Chinese Medicine, GuaLou GuiZhi Decoction, miR-155, inflammation, stroke.

INTRODUCTION

Stroke is widely organized as the leading cause of human death and disability around the world (Komolafe *et al.*, 2015). Ischemic brain injury were associated with several processes, including excitotoxicity, oxidative stress and inflammation, promoting neuron damage. There are several cytokines and chemokines release in inflammatory responses are involved in the pathophysiology of ischemia, including tumor necrosis factor- α (TNF- α), interleukin-6 (IL-6) and interleukin-1 β (IL-1 β), affected critically in ischemic brain injury (Rong *et al.*, 2013). MicroRNAs (miRNAs) play a central role in gene expression in stimulated microglia and activated NF- κ B signaling and its downstream targets (Wimmer *et al.*, 2018, Veltkamp and Gill, 2016, Li *et al.*, 2019). MiR-155, which is a key and widely reported microRNA in immune action and involved in neuroinflammation, induces the expression of inflammatory cytokines via NF- κ B signaling pathway (Marques-Rocha *et al.*, 2015, Elton *et al.*, 2013). Previous study have confirmed that GuaLou

GuiZhi Decoction (GLGZD), a traditional Chinese medicine for spasticity treatment, exerts potent anti-inflammatory actions by inhibiting expression of inflammation-related genes (Hu *et al.*, 2013, Hu *et al.*, 2013, Hu *et al.*, 2016). However, the functions of GuaLou GuiZhi Decoction (GLGZD) on miR-155 in ischemia-induced cerebral inflammation remain unclear. Thus, we examined the role of GuaLou GuiZhi Decoction (GLGZD) in miR-155 expression and its targeted genes in LPS-activated microglial cells, establishing innovative therapeutic target to regulate inflammatory response which is vital for GuaLou GuiZhi Decoction (GLGZD) therapeutic mechanism, leading to functional recovery after stroke.

MATERIALS AND METHODS

Cell Culture. The murine microglial BV2 cell was obtained from Kaiji Biocology (Nanjing, China) and was cultured as described in previous study (Hu *et al.*, 2015). DMEM cell culture medium (HyClone, South Logan, UT, USA) added with 10% new born calf serum, 100 U/ml-1 penicillium and streptomycin sulfate (Gibco, Carlsbad,

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CA, USA) was employed for cell cultivation at 37°C with 5% CO₂ atmosphere in a humidified incubator.

Cell treatment. Once cell growth reached 70%–80% confluence, and then treated with LPS (1µg/mL) and different concentrations of GLGZD. LPS was obtained from Sigma-Aldrich (St. Louis, MO, USA). Water extract from GLGZD was prepared and the working concentrations of GLGZD was made following previous description (Hu *et al.*, 2013). Cells were firstly cultured with serum-free for 4 h followed by LPS stimulation.

Cell transfection of miR-155 mimic and GLGZD treatment. The miR-155 mimic were purchased from Thermo Fisher Scientific (Invitrogen, Carlsbad, CA, USA). BV2 were seeded in six-well plates at 3x10⁵ cell/mL, and cells reached 50%-80% confluence for miRNA transfection studies. Transfection reagent Lipofectamine RNAiMAX 0.25% (Invitrogen, Carlsbad, CA) was utilized for transfection of miR-155 mimic according with the manufactures' protocol. BV2 cells were transfected with 30 nM miR-155 mimic and the corresponding negative controls (NC) prior to LPS stimulation. After 3h of transfection, LPS and GLGZD were added to the cell culture at 1µg/mL and 100ng/mL. To this end, cells were harvested after 24 hours for subsequent analyses. Experiments were performed in triplicate.

ELISA Assays. Cell culture supernatants were harvested and measured the cytokine concentration of pro-inflammatory mediators TNF-α, IL-6, TGF-β, IL-10 by enzyme-linked immunoabsorbent assay (ELISA) (R&D Systems), then compared them to miRNA-transfected cells.

RNA isolation and qPCR. All cells were collected and then the total cell RNAs were extracted after treatment. Efficacy of miR-155 overexpression and their target genes SOCS-1 and SMAD-1 were measured using qPCR after transfection. Total RNA was extracted from cell lines using RNA Isolation Kit (9108, TaKaRa Bio Inc.) according to the manufactures' instruction. After the reverse transcription of RNA into cDNA, the RNA concentration were quantified using the NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific). MiRNA-specific primers were designed and synthesized as following: the forward primer 5'-CAGTTTGTGGAACGGTGCTG-3' and the reverse primer 5'-TGGTAGGGTCATCGGGTTCT-3'. The following primers for SOCS-1 and SMAD-1 were used: the SOCS-1 forward primer 5'-GCTATAATCCTGGACTTCTG-3' and the reverse primer 5'-GAGGAAGGCTGTGAACATGA-3', the SMAD-1 forward primer 5'-TGGTGACTCTCGGGAAC-3' and the reverse primer 5'-ATCGGCTTGAAGTCTT-3', with β-actin (the forward primer 5'-TGTGTCCGTCGTGGATCTGA-3' and the reverse primer 5'-TTGCTGTTGAAGTCGAGGAG-3'), as an internal reference. Expression of

all mRNA were assessed by Real-Time qPCR using SYBR[®] Premix Ex Taq[™] (DRR420A, TaKaRa Bio Inc.). qPCR analysis for PCR amplification experiments were performed as following: initial denaturation at 95°C for 5 minutes, after reacted for 45 cycles, which including denaturation at 95°C for 30 seconds, annealing at 60°C for 13 seconds, and extension at 72°C for 30 seconds. The amplification system was as following: 12.5 µL 2× SYBR Premix Ex Taq, 0.5 µL PCR primers (10mM), and 2 µL cDNA template. mRNA expression by each sample was performed in triplicate. The qRT-PCR results of the target genes expression were quantified by the comparative Ct (2^{-ΔΔCt}) method.

Western Blot Analysis. Cell proteins were extracted by RIPA complemented with protease-inhibitors at 4°C for 15 minutes. Protein concentration of cell lysates was measured using BCA Protein Assay kit. 50µg of protein from each sample was loaded into and purified on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE, 10%). They were transferred on the PVDF membrane and then immersed in 5% bovine serum albumin (BSA) to block the non-specific protein for 1 hour. The membranes were immunoblotted at 4°C overnight with the the primary antibodies: SOCS-1 (1:1000; 3950, Cell signaling, Boston, MA, USA), SMAD-1 (1:1000; 9743, Cell signaling, Boston, MA, USA), β-actin antibody (1:500; 3700, Cell signaling, Boston, MA, USA). After incubation, the blots were washed with TBST for three times, followed by being incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (7074, Anti-rabbit IgG, 7076, Anti-mouse IgG, Cell signaling, Boston, MA, USA) for 1 hour at room temperature. Proteins were determined by ECL detection reagent (P0018, Beyotime Biotechnology) and ChemiDoc instrument (Bio-Rad). The intensity of each band was measured using Bio-Image Analysis System (Bio-Rad, Hercules, CA, USA).

STATISTICAL ANALYSIS

All values are presented as the mean ± standard error of the mean (SEM). The data obtained from at least three experiments. Multiple comparisons were done using the SPSS 16.0 software (SPSS Inc., Chicago, IL, USA). A *P* value < 0.05 was considered as significant statistically.

RESULTS

GLGZD down-regulated expression of miR-155 in BV2 cells following LPS stimulation. To identify the role of GLGZD in the regulation of miR-155 expression, miR-155 expression was examined in microglial cells treated with different concentrations of LPS and GLGZD. The qRT-PCR assay suggested that miR-155 expression level was significantly down-regulated by GLGZD treatment in LPS-induced BV2 cells compared with the control

untreated cells (fig. 1a). Simultaneously, to confirm that GLGZD affects the expression of miR-155, we infected BV2 cells with a mimic miR-155. The transfection experiment indicated that miR-155 mimic transfection dramatically enhanced the miR155 expression in LPS-stimulated microglials compared to that observed in unstimulated cells, however, GLGZD reduced LPS-induced upregulation of miR155 over expression (fig. 1b).

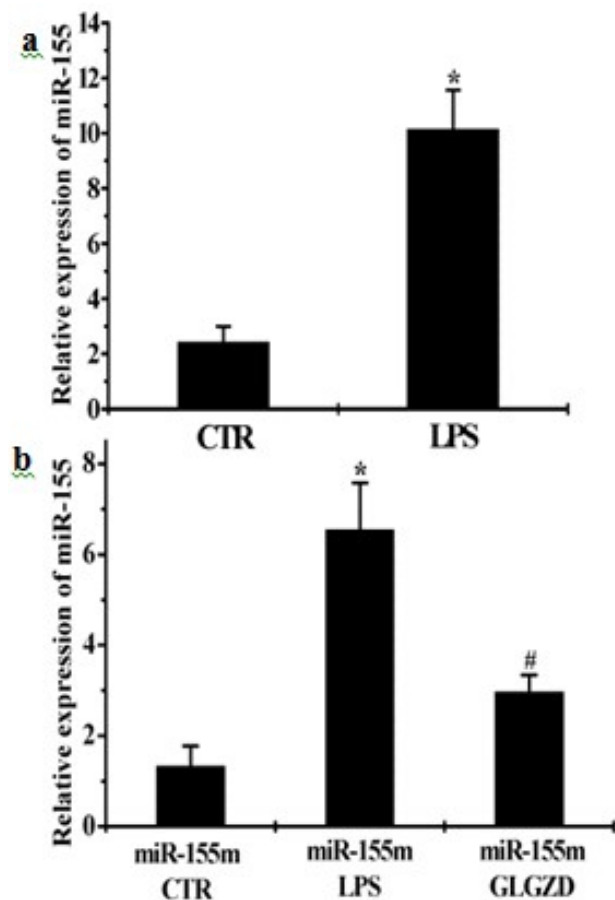


Fig. 1: The expression of miR155 are significantly reduced by GLGZD in transfected and untransfected microglial cells. The transcriptional levels of miR155 were confirmed by real-time RT-PCR (1a-b). Results are representative of three independent experiments (n=3, mean \pm SEM, * p <0.05 compared to unstimulated cells, # p <0.05 compared to LPS-stimulated cells).

GLGZD Mediates LPS-Induced miR155 targeted SOCS-1 and SMAD-1 Regulation. Previous research has shown that the miR-155 were upregulated, whereas its target genes including SOCS-1 and SMAD-1, were downregulated after LPS stimulation. Our studies revealed that the mRNA and protein expression of SOCS-1 and SMAD-1, which evaluated in triplicate by RT-PCR and Western-blotting analysis respectively, decreased in the LPS-stimulated cells compared with those in the control group, yet remarkably increased after GLGZD treatment (fig. 2a-b). It means that GLGZD may enhance

miR155 target expression levels to regulate inflammation.

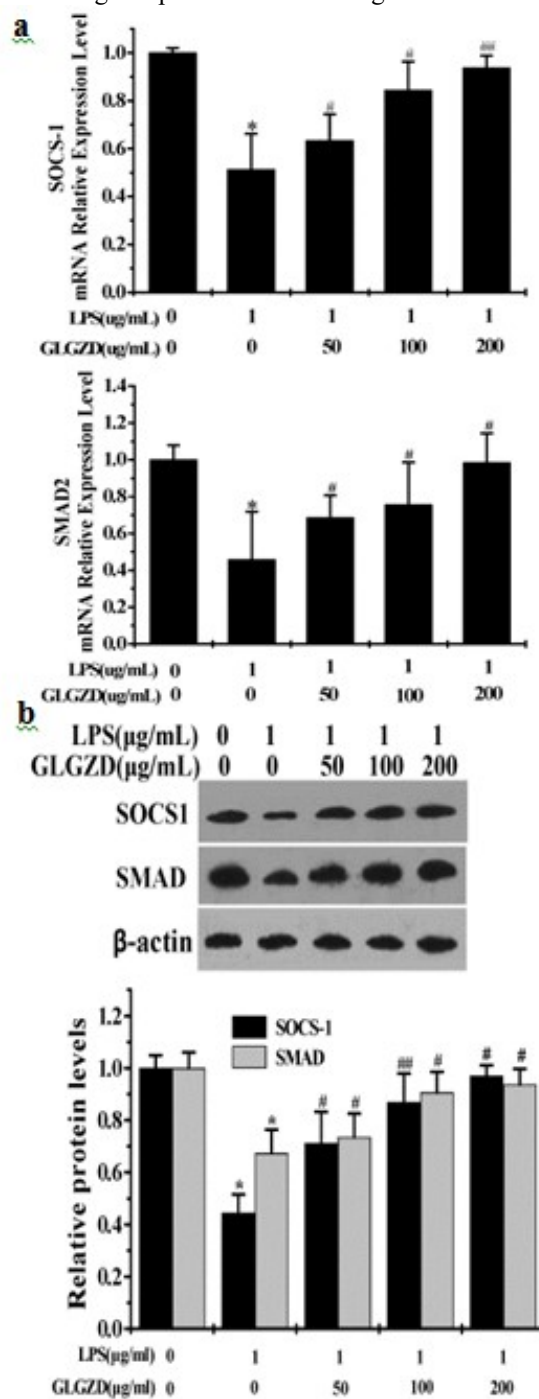


Fig. 2: Expression of the corresponding target genes of miR-155 was affected by GLGZD in LPS-stimulated microglials. The mRNA and protein levels of SOCS1 and SMAD were measured by RT-PCR (A) and western-blotting assay (B) respectively. (n=3, mean \pm SEM, * p <0.05 compared to unstimulated cells; # p <0.05, ### p <0.01 compared to LPS-stimulated cells. Results are representative of three independent experiments.)

Effects of GLGZD on the expression miR-155 and its targeting SOCS-1 and SMAD-1 following transfected by

miR-155 mimic in LPS-induced cells. The BV2 cells were transfected with miR-155 mimic, miR-155 expression significantly upregulated after LPS stimulation as described above. We further detected the expression of SOCS-1 and SMAD-1 after transfection of miR-155 mimic in activated-BV2 cells, the results showed that GLGZD still restored the levels of SOCS-1 and SMAD-1 which suppressed by miR-155 in microglial cells in response to LPS (fig. 3).

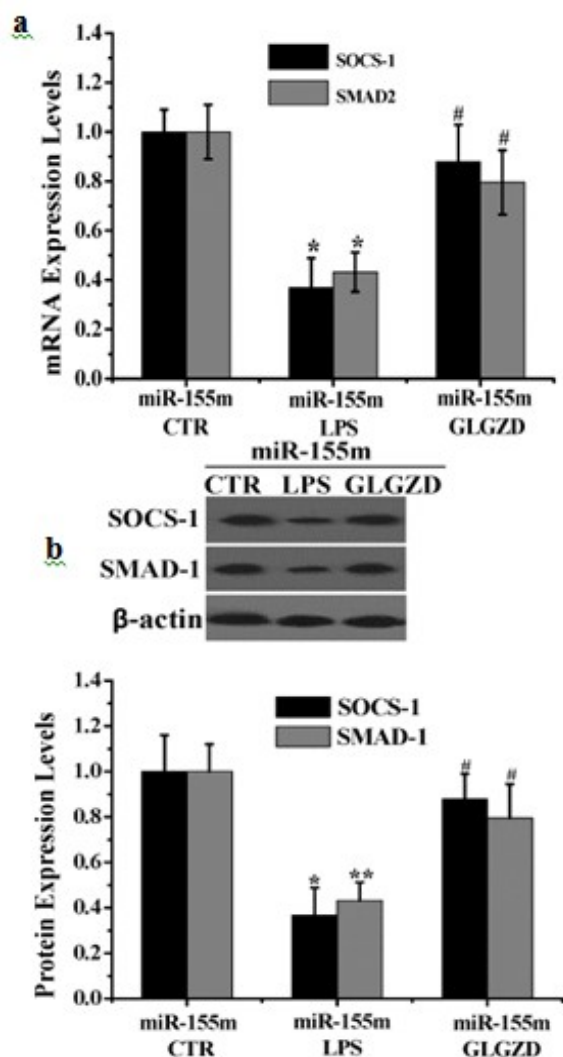


Fig. 3: The expression of SOCS1 and SMAD was affected by GLGZD in the presence and absence of miR155 mimic after LPS stimulation in microglial cells. The results were confirmed by real-time RT-PCR(3a) and western-blotting experiment(3b)(n=3, mean±SEM, * $p < 0.05$, ** $p < 0.01$ compared to unstimulated cells; # $p < 0.05$ compared to LPS-stimulated cells). Results are representative of three independent experiments.

GLGZD Regulated the cytokine expression After miR-155 mimic-transfected Microglial Activation. Post-stroke neuroinflammation and microglial activation play

important roles in the pathogenesis of stroke(Olivera Rajkovic, *et al.*, 2018). To measure the microglial activation, we evaluated the levels of the pro- or anti-inflammatory cytokines in the LPS-stimulated cells transfected by miR-155 mimic. Comparison with the transfected cells, those transfected with miR-155 mimic exhibited significant upregulation of inflammatory cytokines. The results indicated that GLGZD significantly suppressed the elevation of the production of pro-inflammatory mediators (TNF α and IL6), meanwhile, GLGZD cloud also significantly increased the amounts of anti-inflammatory cytokines (IL10 and TGF β) significantly, as demonstrated in fig. 4.

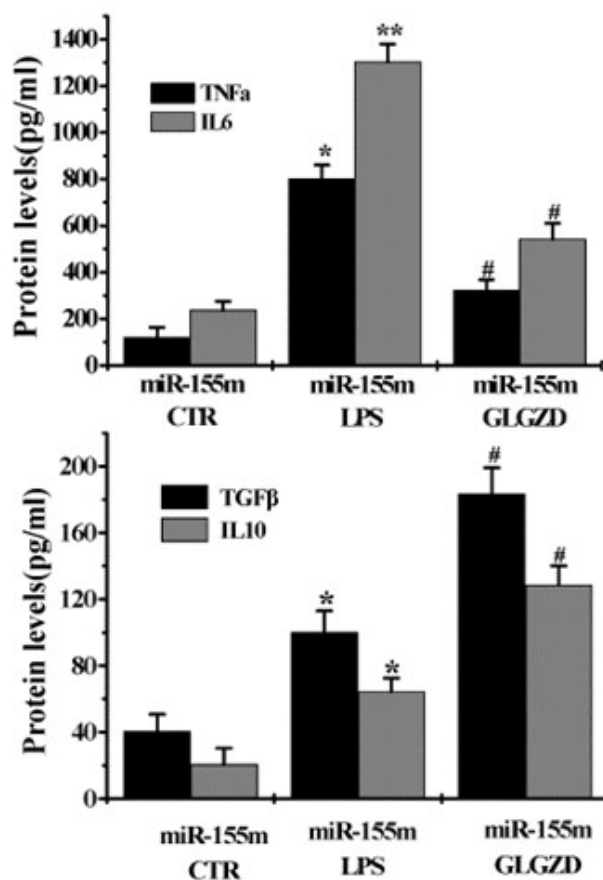


Fig. 4: The influence of GLGZD on the induction of cytokine production in untransfected and miR155-transfected microglial cells. Cytokine production regulated by treatment with GLGZD was determined by ELISA analysis. Results are representative of three independent experiments (n=3, mean ± SEM, * $p < 0.05$, ** $p < 0.01$ compared to unstimulated cells and # $p < 0.05$ compared to LPS-stimulated cells).

DISCUSSION

Ischemic stroke was known as a major challenge to public health. Inflammatory reaction following cerebral ischemia are crucial for the progression in ischemic damage

(Gairolla, *et al.*, 2017, Iadecola and Anrather, 2011). Post-stroke molecules released from injured tissue and necrotic cells activate Toll-like receptors (TLRs) to release pro-inflammatory cytokines, these cytokines cause post-ischemic inflammation, which is known as a key driving pathogenesis and subsequently leading to exacerbated brain injury (Esenwa *et al.*, 2016, Zhou, *et al.*, 2019, Dimitrova, *et al.*, 2018) in ischemic stroke. Therefore, pharmacological inhibition of inflammatory events may be one of the most promising method contributing to stroke therapy.

MicroRNAs (miRNAs), which are approximately 19 to 24 nucleotides, are small noncoding RNA molecules and regulate gene expression. It is now clear that miRNA are a major class of gene expression regulators involved in several biologic functions such as inflammation (Yu *et al.*, 2017, Khoshnam, *et al.*, 2017). The inflammation-related genes expression can be modulated by microRNAs (miRNAs, miRs) at the post-transcriptional level. Recent study have reported that miR-155 is a critical inflammation-associated noncoding RNA regulating multiple specific target genes, which have the important immunomodulatory effect (Marques-Rocha, *et al.*, 2015). It is one of the significantly upregulated miRNAs in the brain. MiR-155, a brain-enriched miRNA, has been documented to mediate LPS-Induced SOCS1 and SMAD Regulation. It means that SOCS-1 and SMAD-1 are direct target of miR-155, increasing miR-155 expression in microglial cell lines led to a decreased expression level of SOCS-1 and SMAD-1, which negatively regulate the inflammatory cytokines related with neuroinflammation following post-ischemic stroke (Elton, *et al.*, 2013, Pal, *et al.*, 2016).

Given the critical role of miR-155 in the progression of post-ischemic neuroinflammation, the regulation of GLGZD on miR-155 and the mechanism of GLGZD treatment in brain damage will provide novel insights into ischemic stroke therapy. Our previous studies demonstrate that GLGZD reduces the expression of inflammation-related genes by suppressing the phosphorylation and degradation of I κ B- α , inhibiting the activation of NF- κ B and restraining the binding of active NF- κ B (Hu, *et al.*, 2016, Hu, *et al.*, 2015). In the present study, the fold changes of miR-155 expression were detected in the real-time PCR, the result demonstrated that miR-155 were upregulated, whereas its target genes were down regulated after LPS stimulation, finally regulated the inflammatory cytokine secretion. It means that miR-155 expression is inversely correlated with SOCS-1 and SMAD-1 expression. After transfected by miR155 mimic, the miR155 and its target genes levels were consistent with the results obtained from the primary result. In addition, we found that GLGZD ultimately down regulated the inflammatory-associated cytokines expression of TNF α and IL6, up regulated TGF β and IL10.

CONCLUSION

Taken together, our study aimed to evaluate the effects of GLGZD on miR-155 and underlying mechanism in a inflammatory cell model. These results implicate that miR-155 were affected after GLGZD treatment, that is GLGZD decreased expression levels of miR-155 and enhanced its targeted genes expression levels, which subsequently regulates inflammatory cytokines. Preventing miR-155 expression resulted in impaired LPS-induced inflammatory responses. After transfection with miR-155 mimic, GLGZD has been shown to have similar anti-inflammatory effect in vitro. It suggested that miR-155 might be a promising predictive target of GLGZD therapy.

ACKNOWLEDGMENTS

This study was supported by Natural Science Foundation of Fujian Province (2017J01849), Science and technology platform construction project of Fujian science and Technology Department (2015Y2001), New Century Outstanding Talent Support Program of Fujian Universities (2017).

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