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LncRNA-UC.25 + shRNA Alleviates $P2Y_{14}$ Receptor–Mediated Diabetic Neuropathic Pain via STAT1

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Abstract

Diabetic neuropathic pain (DNP) is a common complication of diabetes, and its complicated pathogenesis, as well as clinical manifestations, has brought great trouble to clinical treatment. The spinal cord is an important part of regulating the occurrence and development of DNP. Spinal microglia can regulate the activity of spinal cord neurons and have a regulatory effect on chronic pain. $P2Y_{12}$ receptor is involved in DNP. $P2Y_{14}$ and $P2Y_{12}$ receptors belong to the Gi subtype of P2Y receptors, but there is no report that the $P2Y_{14}$ receptor is involved in DNP. Closely related to many human diseases, the dysregulation of long noncoding RNA (lncRNA) has the effect of promoting or inhibiting the occurrence and development of diseases. The aim of this research is to investigate the function of the spinal cord $P2Y_{14}$ receptor in type 2 DNP and to understand the function as well as the possible mechanism of lncRNA-UC.25 + (UC.25 +) in rat spinal cord $P2Y_{14}$ receptor–mediated DNP. Our results showed that $P2Y_{14}$ shRNA can reduce the expression of $P2Y_{14}$ in DNP rats, thereby restraining the activation of microglia, decreasing the expression of inflammatory factors and the level of p38 mitogen–activated protein kinase (p38 MAPK) phosphorylation. At the same time, UC.25 + shRNA can downregulate the expression of the $P2Y_{14}$ receptor, reduce the release of inflammatory factors, and diminish the p38 MAPK phosphorylation, indicating that UC.25 + can alleviate spinal cord $P2Y_{14}$ receptor–mediated DNP. The RNA immunoprecipitation result showed that UC.25 + enriched signal transducers and activators of transcription1 (STAT1) and positively regulated its expression. The chromatin immunoprecipitation result indicated that STAT1 combined with the promoter region of the $P2Y_{14}$ receptor and positively regulated the expression of the $P2Y_{14}$ receptor. Therefore, we infer that UC.25 + may alleviate DNP in rats by regulating the expression of the $P2Y_{14}$ receptor in spinal microglia via STAT1.

Keywords Diabetic neuropathic pain · Spinal cord · Microglia · $P2Y_{14}$ receptor · lncRNA

Introduction

Diabetes mellitus (DM) is a universal chronic illness. The rising morbidity and mortality rate have seriously affected the health and quality of life of patients. The latest ninth edition statistics of the International Diabetes Federation in 2019 show that diabetes has become a global epidemic, and the number of patients suffering from diabetes is increasing with the prevalence rate of 9.3% (approximately 463 million) [1, 2]. Diabetic neuropathic pain (DNP) develops from peripheral neuropathy. The main features are hyperalgesia, tactile pain, secondary pain, and spontaneous pain. The pathogenesis and clinical manifestations of DNP are relatively complex, and it is even difficult to relieve pain symptoms in patients by comprehensive treatment measures

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[3]. Therefore, the research on the pathogenesis and prevention of diabetic neuropathic pain is particularly urgent.

The pathogenesis of DNP involves not only the peripheral but the central nervous systems. As one part of the central nervous system, the spinal cord plays significant roles on the regulation of the occurrence and development of DNP [4]. The traditional treatments for pain mainly focus on neurons, and the clinical analgesic effect of a large number of neuron-targeted therapeutic drugs is not quite satisfactory, which boost the exploration of glial cells, a new hot spot in pain research. Research has shown that glial cells, such as microglia and astrocytes, can regulate the activity of neurons in the spinal cord and have a significant regulatory effect on the pathological changes in chronic pain [5]. Glial cell activation is observed in a variety of neuropathic pain models. The activated glial cells release multiple inflammatory factors, which enhance the excitability of neurons and promote the spread of the pain [6]. As immunocompetent cells, like macrophages, glia cells participate in the function of the immune barrier under normal circumstances. When the peripheral or central nervous system suffers from diabetes-related injuries, microglia are rapidly activated and undergo morphological and functional changes [7]. Activated microglia produce and secrete a series of pro-inflammatory factors, which increase sensitivity and affect synaptic transmission, leading to pathological pain. Therefore, the reason why the current pain treatment effect is not ideal may be the incorrect selection of the target, and glial cells may be a more suitable target [8].

As an energy donor for normal cellular metabolism, intracellular adenosine triphosphates (ATPs) play an important biological role. Inflammation as well as tissue damage can cause intracellular ATP release. Besides, the extracellular ATPs can also act as signal molecules and participate in signal transduction by binding to purinergic receptors located on the cell membrane [9]. Purinergic receptors include P1 receptors and P2 receptors. P2 receptors include ligand-gated ion channel type P2X receptors and G protein-coupled P2Y receptors. P2Y receptors include eight subtypes ($P2Y_{1, 2, 4, 6, 11, 12, 13, 14}$). Research has shown that in the rat model of neurogenic neuropathic pain, the expression of $P2Y_{14}$ receptors in spinal microglia is significantly increased, lasting at least 2 weeks. The application of $P2Y_{14}$ receptor antisense oligonucleotide can significantly relieve pain and inhibit the expression of $P2Y_{14}$ receptor, which proves that $P2Y_{14}$ receptor can participate in neuropathic pain [7]. So, it can be speculated that $P2Y_{14}$ receptors may participate in the development of DNP.

LncRNA is a DNA transcription product over 200 nucleotides in length, which can be found in the nucleus or cytoplasm and is stably expressed in tissues and cells. With the development of whole-genome sequencing technology and transcriptome sequencing, lncRNA has also received more attention [10]. LncRNA is not only an intermediary between DNA and protein, but also an important participant in cell function, which can regulate gene expression in a variety of ways from different levels, including epigenetic level, transcription level, and post-transcriptional processing [11]. Recent research has shown that the dysregulation of lncRNA is going hand in hand with numerous human diseases, and has the effect of promoting or inhibiting the development of diseases [12]. The abnormal expression of lncRNA was closely related to DM [13]. Therefore, the expression of individual lncRNA may be suitable for disease diagnosis and prognostic evaluation. LncRNA UC.25 + (UC.25 +) is one of the ultra-conserved orthologous regions, which is 100% identity between rats, mice, and humans [14]. Our microarray result revealed the expression of UC.25 + was raised in the DM rats. However, there is no report on the function of UC.25 + .

In this research, a rat model of DNP was built to explore whether spinal microglia $P2Y_{14}$ receptor is involved in DNP and whether UC.25 + affects the $P2Y_{14}$ receptor–mediated DNP via the speculated mechanism.

Materials and Methods

Animal Model

In order to define that the $P2Y_{14}$ receptor participates in DNP, an animal model of DNP was established. Adult male Sprague–Dawley (SD) rats (430,726,220,100,076,156) weighing between 180 and 220 g were purchased from Changsha Tianqin Biotechnology Co., Ltd., Hunan Province. All experimental operations have been reviewed and authorized by the Medical Laboratory Animal Ethics Committee of Nanchang University. Before the rats were sacrificed, 3 ml/kg 10% hydrous chloric acid was injected intraperitoneally for anesthesia to relieve the pain of the rats. Before establishing the model, the rats were fed with normal diet in a clean environment for 1 week to fully adapt to the environment and reduce the influence of environmental differences on this experiment. After 1 week of normal diet, the rats were randomly divided into Control group ($n = 12$) and DNP group ($n = 50$). Rats in the control group were fed with normal diet, while DNP group was fed with high-lipid and high-glucose diet, including 77.8% normal diet, 10% lard, 10% sugar, 2% cholesterol, and 0.2% cholic acid sodium. After a high-lipid and high-sugar diet for a month, the rats in the DNP group were fasted for 8–10 h and injected intraperitoneally with streptozotocin 35 mg/kg. After 7 days, the fasting blood sugar ≥ 7.8 mmol/L and the obvious neuropathic pain symptoms were used as the criteria of the DNP model. The rats in the normal group were left untreated, and the tail vein blood glucose test showed normal. Finally, 36 rats were randomly chosen from the modeled rats and divided into three groups:

DNP group (DNP group), DNP + $P2Y_{14}$ shRNA treatment group (DNP + $P2Y_{14}$ shRNA), and DNP + scramble shRNA as negative control treatment group (DNP + NC shRNA), with 12 rats in each group.

In order to explore the role and possible mechanism of lncRNA-UC.25 + in rat spinal cord $P2Y_{14}$ receptor–mediated DNP, the animal model was established again, and the establishment method was the same as above. The test was divided into four groups: control group (Control), diabetic neuropathologic pain group (DNP group), DNP + lncRNA-UC.25 + shRNA treatment group (DNP + UC.25 + shRNA), and DNP + scramble shRNA treatment group (DNP + NC shRNA). There were 12 rats in each group.

Intrathecal Injection

According to the instructions of the animal transfection kit of Beijing Engreen Biotechnology Co., Ltd., $P2Y_{14}$ shRNA, lncRNA-UC.25 + shRNA and NC shRNA plasmid were transfected into rats by intrathecal injection at a dose of 5 μg. The absorbent cotton soaked in ether was placed in the centrifuge tube, and the mouth and nose of the prone rats were placed in the tube. The needle was inserted vertically along the middle of the L5–L6 spinous process, and there was an obvious sense of breakthrough when entering the subarachnoid space. Wagging the tail was a sign of successful intrathecal injection. Injected the plasmid. The rats were put in an observation cage to wake up until they resumed normal activities. No treatment for control group and DNP group.

Cell Experiment

Select human umbilical vein endothelial cells (HUVEC) for the experiment. The cultural conditions were endothelial cell medium (ECM), 5% fetal bovine serum, 1% penicillin and streptomycin, 1% growth factor, and the cultural environment was 37 °C and 5% CO_2. HUVEC cells were divided into control group and high-glucose group. The glucose concentration in the high-glucose group and the control group were 33.3 mmol/L and 5.5 mmol/L. In order to explore the relationship between UC.25 + and the transcription factor, signal transducer and activator of transcription 1 (STAT1), the cells were set up in the following groups: control group (Control), UC.25 + overexpression group (UC.25 + OE) and vector plasmid group (Vector) and high-glucose cell group (HG), high-glucose plus UC.25 + low-expression group (HG + UC.25 + shRNA), and high-glucose plus scramble shRNA group (HG + NC shRNA). Meanwhile, set up the following groups: control group (Control), STAT1 overexpression group (STAT1 + OE), vector plasmid group (Vector), and high-glucose cell group (HG), high-glucose plus STAT1 low-expression group (HG + STAT1 shRNA), and high-glucose plus scramble shRNA group (HG + NC shRNA) to detect the effect of STAT1 on $P2Y_{14}$ receptor. STAT1 (Gene ID: 6772; NCBI Reference Sequence: NM_007315.3), and UC.25 + were inserted into the pCDNA3.1 plasmid, respectively. The overexpression plasmid (1000 ng/μl) was transfected into cells by FuGENE6.

Measurement of the Thermal Withdrawal Latency and Mechanical Withdrawal Threshold

Each group of rats was placed in a transparent plastic box and allowed to acclimate to the environment for 30 min. The electromechanical pain meter was used for testing, adjusting the relevant parameters of the instrument in advance, and then stimulating the center of the left hind plantar of the rat with a test needle with a force sensor. The intensity is gradually increased until the rat feels pain and lifts the left hind foot. The displayed data at this time is recorded as the rat's mechanical hyperalgesia threshold. Place each group of rats in a transparent plastic box on a horizontal glass table. After the start of the test, turn on the tungsten lamp with a thermal light source and aim at the center of the left hind plantar of the rat to give thermal stimulation. Until the rat feels pain and lifts the left hind foot, the value recorded at this time is the thermal hyperalgesia threshold. Repeat the measurement 6 times for each rat, and take the average of the measurement results.

Quantitative Real-Time PCR

The separated lumbar 4–6 (L4–6) spinal cord is washed with pre-cooled phosphate buffer saline (PBS); it was placed in a homogenizer that had been treated with ribozyme-free water in advance. Collect total RNA with TRIZOL total RNA reagent and use Transgeen Reverse Transcription Kit for RNA reverse transcription. Then, add the complementary DNA (cDNA) template obtained by reverse transcription according to the Promega's Real-time PCR kit, and use the step-one plus real-time fluorescent quantitative PCR instrument for experiment. All primers are from Beijing Shenggong Biological Co., Ltd. Primer sequence is as follows: $P2Y_{14}$ Forward: 5′-GCATTGTGCTCGTATTTGTCG -3′, Reverse: 5′-CTAAACGGCTGGCATAAGAAG-3′; UC.25 + Forward: 5′-TGGTCAAAAGCAAAACAAG-3′, Reverse: 5′-TATGCA AGAAAAGGCAGAG-3′; STAT 1 Forward: 5′-GCCAAA GGAAGCACCAGAGCC-3′, Reverse: GAGCCCACTATC CGAGACACC-3′; Rat β-actin Forward: 5′-GCTCTCTTC CAGCCTTCCTT-3′, Reverse: 5′-CTTCTGCATCCTGTC AGCAA-3′; Homo β-actin Forward: 5′-CAAGAGATGGCC ACGGCTGCT-3′, Reverse: 5′-TCCTTCTGCATCCTGTCG GCA-3′. Real-time quantitative PCR was performed with SYBR® Green Master Mix by an ABI PRISM® 7500 (Applied Biosystems, Inc., Foster City, CA, USA). Individual experiments were performed on each sample and

repeated three times. The quantification of gene expression was calculated by the $2^{-\Delta\Delta Ct}$ method in comparison with respective levels of β-actin mRNA.

Western Blotting

Freshly isolated L4-6 spinal cord tissue was washed in pre-cooled PBS, and put into the radio immunoprecipitation assay (RIPA) lysis solution. The mixture was thoroughly ground and decomposed on ice for 30 min, followed by centrifugation to obtain supernatant. The protein in the supernatant was separated by polyacrylamide gel electrophoresis, and the separated protein was transferred to the polyvinylidene fluoride (PVDF) membrane. The protein-containing PVDF membrane was incubated with 5% nonfat dried milk for 1 h, and then incubated with anti-rabbit $P2Y_{14}$ (1:500, APR026, Alomone Labs), OX42 (1:1000, AB1211, Abcam), interleukin-1 beta (IL-1β) (1:500, AF5103, Affinity), tumor necrosis factor-alpha (TNF-α) (1:500, PB0082, Boster), p38 mitogen-activated protein kinase (p38 MAPK) (1:800, 8690, Cell Signaling Technology), and phosphorylated p38 (p-p38) MAPK (1:800, 4511, Cell Signaling Technology) overnight at 4 °C. After three washes with triethanolamine-buffered saline-tween (TBST), the membranes were incubated in secondary antibody with horseradish peroxidase (1:2000, Beijing Zhongshan Biotechnology Co.) for 1 h. Put the membranes into the gel imaging system, and the enhanced chemiluminescence (ECL) luminescent solution was added to expose the image. The results were recorded and stored. The integrate optical density (IOD) analysis of protein results was analyzed by Image-Pro Plus software, and the expression of target protein in each group was standardized by β-actin.

Immunofluorescence

The isolated L4-6 spinal cord was rinsed with pre-cooled PBS and dehydrated overnight in an eppendorf (EP) tube containing 20% sucrose solution. The spinal cord was embedded by optimum cutting temperature (OCT) and sectioned with a frozen microtome. First rinse the sections with PBS, then soak them in 0.3% Triton X-100 PBS solution for 30 min, and incubate with 10% normal goat serum to block non-specific staining. The sections were incubated overnight with anti-rabbit $P2Y_{14}$ receptor (1:200, APR026, Alomone Lab) and anti-mouse OX42 (1:200, AB1211, Abcam). After washing the next day, the sections were incubated with goat anti-rabbit tetramethyl rhodamin isothiocyanate (TRITC) (1:200, Zhongshan Biotech Co.) and goat anti-mouse fluorescein isothiocyanate (FITC) (1:200, Zhongshan Biotech Co.). The images of the dorsal horns of the spinal cord were captured by fluorescence microscopy (Olympus DP72, Japan).

Bioinformatics Technology Predicts the Transcription Factor STAT1 of the $P2Y_{14}$ Receptor

The transcription factors of $P2Y_{14}$ were searched and screened through UCSC, GENECARDS and other databases, and multiple prediction results showed that transcription factor STAT1 might regulate the expression of $P2Y_{14}$ receptor. Finally, the sequence of binding sites with higher STAT1 score was searched through JASPAR website and subsequent validation experiments were carried out.

RNA Immunoprecipitation

The interaction between UC.25 + and STAT1 protein was detected by RNA immunoprecipitation (RIP) experiment. HUVEC cells were cultured and divided into two groups: Control group: co-transfected with two plasmids pcDNA3.0–12 × MS2bs and pcDNA3.0-Flag-2 × MS2; Experimental group: co-transfected with two plasmids pcDNA3.0-UC.25 + -12 × MS2bs and pcDNA3.0-Flag-2 × MS2. After plasmid transfection, the cells were cultured for 48 h. After the cells were cross-linked by UV and lysed, collected the supernatant, added ANTI-FLAG M2 magnetic beads eluted with the eluent I, and turned over overnight. The supernatant was collected as the output group, and the remaining magnetic beads were continuously eluted through eluent I and eluent II. Finally, the obtained supernatant was subjected to a Western blot experiment to detect the protein bound to UC.25 + .

Chromatin Immunoprecipitation

In order to explore the binding of the transcription factor STAT1 to the DNA fragments in the promoter region of the $P2Y_{14}$ receptor, a ChIP assay kit was used, and experiments was performed according to the instructions. HUVEC cells were cross-linked with 1% formaldehyde and chromatin was disrupted by an ultrasonicator (UH-250A, Beijing, China). STAT1 antibody (1:50, 14,994, Cell Signaling Technology) was used to immunoprecipitate cross-linked protein-DNA complexes. After purification, the obtained DNA was amplified by PCR. The PCR primers were designed, and the sequences were Homo STAT1 290 bp, Sense: 5′-TCCAGC AACCTGATGTAA-3′; Antisense: 5′-ACACTTGGGAGT AGAGGG-3′.

Statistical Analysis

The experimental results were analyzed by SPSS 21, and the results of each group were expressed as mean ± SEM. The results of each group were compared by one-way analysis of variance (ANOVA) combined with least significant difference (LSD). Two-way ANOVA was performed to compare

2 points between groups for TWL and MWT. Paired *t*-test was used for comparison between the two groups. $p < 0.05$ was considered to be statistically significant.

Results

P2Y$_{14}$ Receptor Mediates DNP

Determination of MWT and TWL

The MWT and TWL were measured to define the changes of pain behavior. The data indicated that MWT and TWL in DNP group were significantly lower than those in Control group after STZ injection ($p < 0.01$); after intrathecal injection of P2Y$_{14}$ shRNA, compared with the DNP group, the MWT and TWL of DNP + P2Y$_{14}$ shRNA group were obviously raised, indicating that P2Y$_{14}$ shRNA can alleviate pain behavior in DNP rats ($p < 0.01$). There was no significant difference in MWT and TWL between DNP + NC shRNA and DNP group ($p > 0.05$) (Fig. 1).

Expression of P2Y$_{14}$ Receptor and OX42 in Rat Spinal Cord

Compared with the Control group, the expression of P2Y$_{14}$ receptor protein and mRNA in the DNP group were obviously raised ($p < 0.01$); after intrathecal injection of P2Y$_{14}$ shRNA, the expression of P2Y$_{14}$ receptor protein and mRNA in DNP + P2Y$_{14}$ shRNA group were obviously lower than those in DNP group ($p < 0.01$); there was no significant difference between the DNP + NC shRNA group and the DNP group ($p > 0.05$) (Fig. 2A, B). The co-expression of P2Y$_{14}$ receptor and microglial marker OX42 was detected by immunofluorescence in the control group (Fig. 2C). The results indicated that P2Y$_{14}$ receptor was co-localized with OX42, and P2Y$_{14}$ receptor could be expressed in spinal microglia. Compared with Control group, the expression level of OX42 in DNP group was significantly raised ($p < 0.01$); after P2Y$_{14}$ shRNA treatment, the expression level of OX42 was obviously reduced than that in the DNP group ($p < 0.01$). Compared with the DNP group, the expression of OX42 was not obviously and statistically different in the DNP + NC shRNA group ($p > 0.05$) (Fig. 2D).

P2Y$_{14}$ shRNA Downregulated the Expression of TNF-α, IL-1β, and p-p38 Protein in the Spinal Cord

Western blot was used to detect the expression of pro-inflammatory factors and p-p38 protein of each group. The results indicated that the expression levels of TNF-α and IL-1β protein in the DNP group were obviously increased than those in the Control group ($p < 0.01$); compared with the DNP group, after intrathecal injection of P2Y$_{14}$ shRNA, the expression of TNF-α and IL-1β in DNP + P2Y$_{14}$ shRNA group were obviously reduced ($p < 0.01$); there was no significant difference between the DNP + NC shRNA group and the DNP group ($p > 0.05$) (Fig. 3A, B). The expression of p38 of rats was not statistically different in each group ($F = 3.798$, $p = 0.058$). Compared with the Control group, the phosphorylation level of p38 in the DNP group was obviously raised ($p < 0.01$); after intrathecal injection of P2Y$_{14}$ shRNA, the phosphorylation level of p38 was obviously lower than that in the DNP group ($p < 0.01$); the phosphorylation level of p38 was not significantly different between the DNP + NC shRNA group and the DNP group ($p > 0.05$) (Fig. 3C).

The Effect and Mechanism of lncRNA-UC.25 + on P2Y$_{14}$ Receptor–Mediated DNP

Determination of MWT and TWL

In order to evaluate the pain behavior of rats, the MWT and TWL were measured for each group. After successfully modeling, these data indicated that the TWL and MWT in the DNP group were obviously lower than those in the Control group ($p < 0.01$); after intrathecal injection of UC.25 + shRNA, the TWL and MWT of the DNP + UC.25 + shRNA group were obviously higher than those in the DNP group ($p < 0.01$); there were no significant

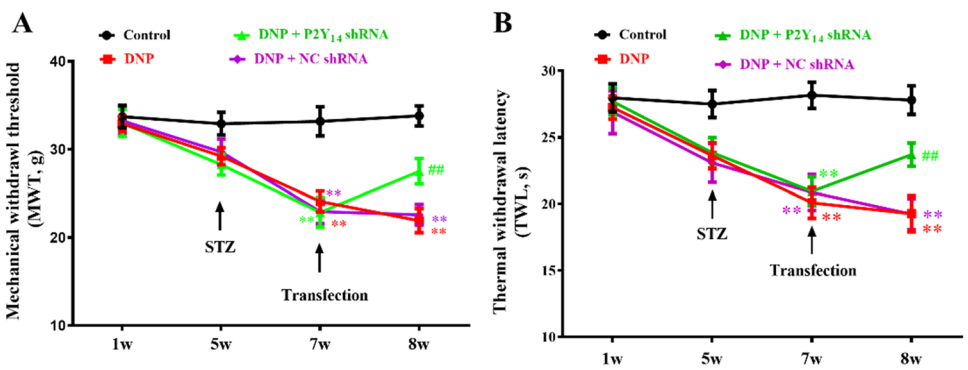

Fig. 1 P2Y$_{14}$ shRNA increased the MWT (**A**) and the TWL (**B**) of DNP rats. The results are presented as the mean ± SEM. $n = 12$ rats per group. $^{**}p < 0.01$ vs. Control group; $^{\#\#}p < 0.01$ vs. DNP group. All data were statistically analyzed by two-way ANOVA

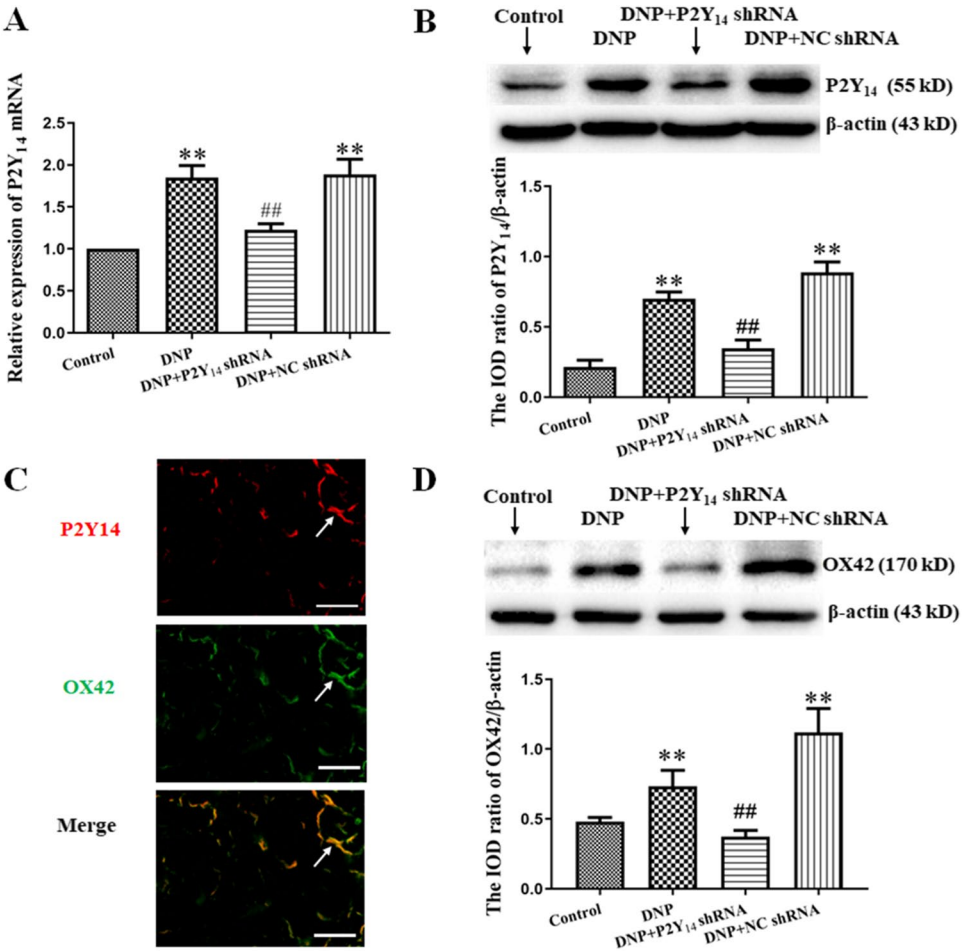

Fig. 2 The expression of $P2Y_{14}$ receptor and OX42 in the spinal cord. **A** Relative expression of $P2Y_{14}$ mRNA. **B** The expression levels of $P2Y_{14}$ proteins and the bar histograms of $P2Y_{14}$ proteins analysis results. **C** The co-expression of $P2Y_{14}$ receptor and OX42 in the spinal cord was detected by immunofluorescence double labeling. The red signal means that $P2Y_{14}$ is stained with TRITC. The green signal means that OX42 is stained with FITC. The yellow signal means the $P2Y_{14}$ and OX42 double staining. Scale bar, 50 μm. **D** The expression of OX42 is detected by Western blot. The results are expressed as the mean ± SEM. $n = 12$ in each group. $^{**}p < 0.01$ vs. Control group; $^{\#\#}p < 0.01$ vs. DNP group. All data were statistically analyzed by one-way ANOVA

difference in TWL and MWT between the DNP group and the DNP + NC shRNA group ($p > 0.05$). These results indicated that UC.25 + shRNA can reduce hyperalgesia threshold in DNP group rats (Fig. 4).

Expression of UC.25 +, $P2Y_{14}$ Receptor, and OX42

Compared with Control group, the expressions of UC.25 +, $P2Y_{14}$ receptor, and OX42 in DNP group were obviously raised ($p < 0.01$); after intrathecal injection of UC.25 + shRNA, the expressions of UC.25 +, $P2Y_{14}$ receptor, and OX42 in DNP + UC.25 + shRNA group were obviously decreased than those in the DNP group ($p < 0.01$); there was no significant difference in the expression of UC.25 +, $P2Y_{14}$ receptor, and OX42 between DNP + NC shRNA group and DNP group ($p > 0.05$) (Fig. 5A, B, C, and D).

UC.25 + shRNA Downregulated the Expression of TNF-α、IL-1β, and p-p38 Protein in the Spinal Cord

The expression levels of inflammatory factors and p-p38 protein were detected. Those data showed that the protein expressions of TNF-α and IL-1β in DNP group were obviously higher than those in Control group ($p < 0.01$); after treatment with UC.25 + shRNA, the expression levels of TNF-α and IL-1β in DNP + UC.25 + shRNA group were obviously lower than those in DNP group ($p < 0.01$); there was no significant difference in the expression levels of IL-1β and TNF-α between the DNP + NC shRNA group and the DNP group ($p > 0.05$) (Fig. 6A, B). There was no significant difference in the protein expression of p38 MAPK among the groups ($F(3, 8) = 3.622$, $p = 0.065$); the expression of p-p38 MAPK in DNP group was obviously higher than that in Control group ($p < 0.01$); after UC.25 + shRNA treatment, the expression of p-p38 MAPK in DNP + UC.25 + shRNA group was obviously lower than that in DNP group ($p < 0.01$); there was no significant difference in the expression of p-p38 MAPK between DNP + NC shRNA group and DNP group ($p > 0.05$) (Fig. 6C).

Determine the Interaction Between lncRNA and STAT1 and Between STAT1 and $P2Y_{14}$ Receptor

RIP results indicated that compared with the Control group, the enrichment of STAT1 protein in the

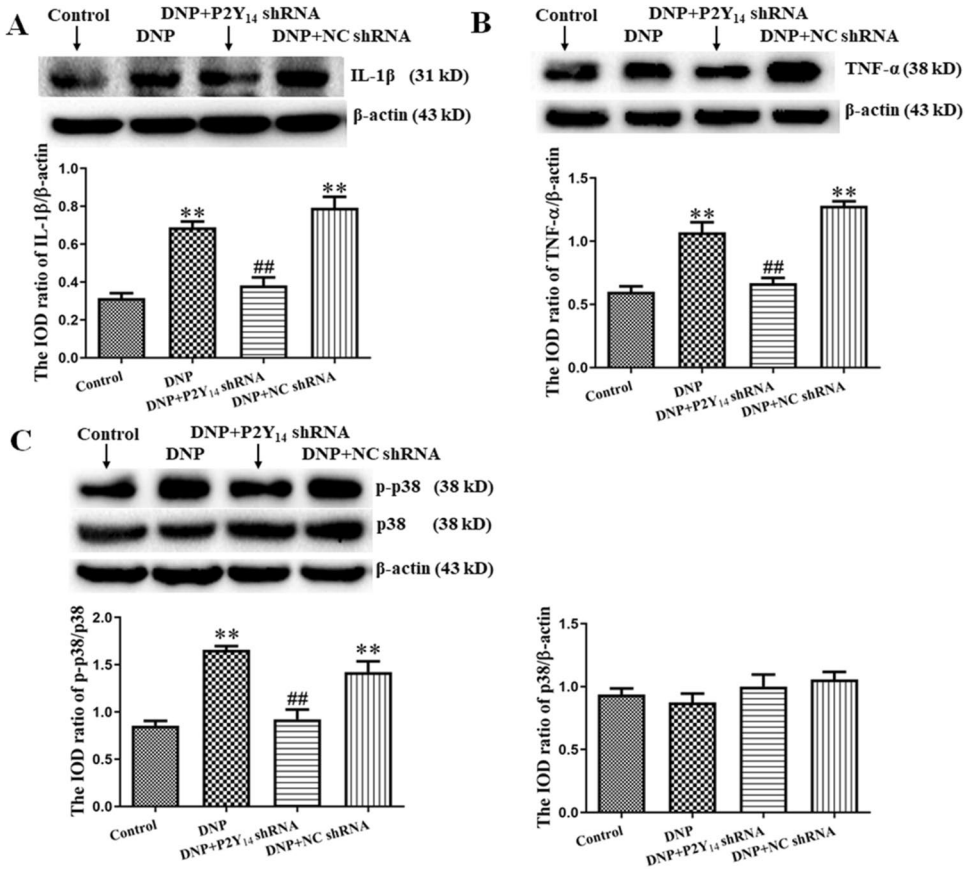

Fig. 3 $P2Y_{14}$ shRNA down-regulated the expression levels of TNF-α, IL-1β, and p-p38 protein in the spinal cord. **A** Expression of IL-1β. **B** Expression of TNF-α. **C** The expression levels of p38 and p-p38. The results are expressed as the mean ± SEM using one-way ANOVA. $n = 12$ rats per group. $^{**}p < 0.01$ vs. Control group; $^{\#\#}p < 0.01$ vs. DNP group. All data were statistically analyzed by one-way ANOVA

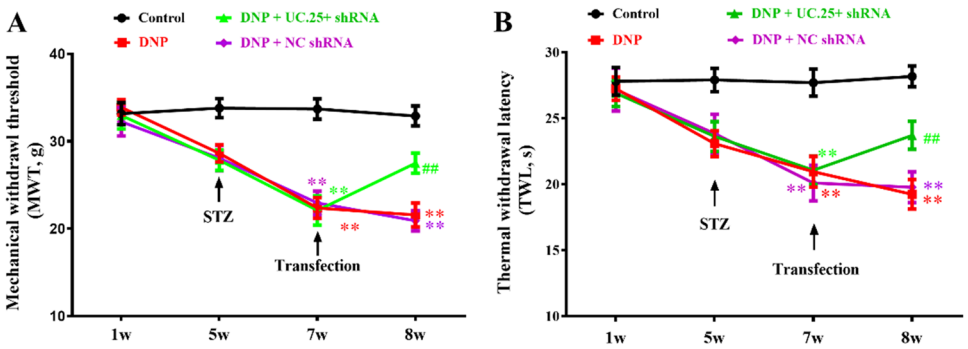

Fig. 4 Effects of UC.25 + shRNA on the MWT (**A**) and the TWL (**B**) of DNP rats. The results are presented as the mean ± SEM. $n = 12$ rats per group. $^{**}p < 0.01$ vs. Control group; $^{\#\#}p < 0.01$ vs. DNP group. All data were statistically analyzed by two-way ANOVA

experimental group was obviously increased ($p < 0.01$), indicating that UC.25 + could bind to STAT1, and there was obvious interaction between them. Flag-Tag (DYKDDDDK) as an internal reference (Fig. 7A). ChIP results indicated that the Input group containing the total DNA of the sample had obvious expression; compared with the Control group, the STAT1 group was specifically expressed at 290 bp, indicating that STAT1 can specifically bind to the promoter of $P2Y_{14}$ gene, and there is an obvious interaction between them (Fig. 7B).

Regulation of UC.25 + on STAT1

HUVEC cells were cultured in high-glucose and normal environments respectively, and UC.25 + was overexpressed in normal cells and low-expressed in high-glucose cells. The effect of UC.25 + on STAT1 was detected by real-time quantitative PCR and Western blot. Results showed that UC.25 + was successfully overexpressed in HUVEC cells. Compared with Control group, the expression of UC.25 + in UC.25 + OE group transfected with

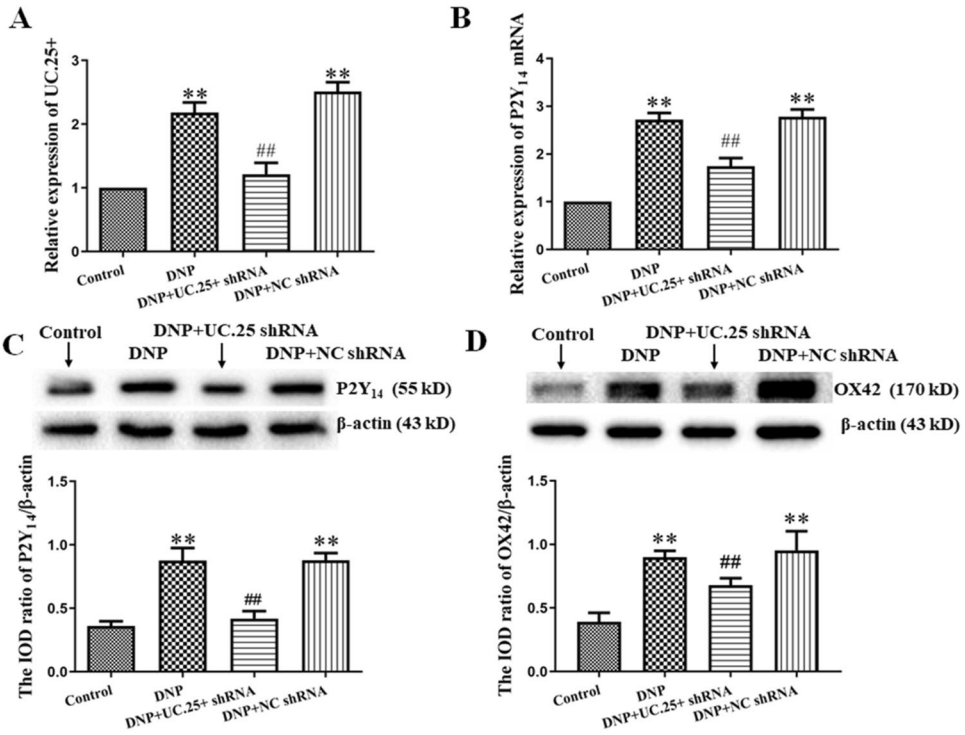

Fig. 5 The expression of UC.25 + , $P2Y_{14}$ receptor, and OX42. **A** Relative expression of UC.25 + . **B** Relative expression of $P2Y_{14}$ mRNA. **C** The expression levels of $P2Y_{14}$ proteins. **D** The expression of OX42 The results are expressed as the mean ± SEM.n = 12 in each group. $^{**}p < 0.01$ vs. Control group; $^{\#\#}p < 0.01$ vs. DNP group. All data were statistically analyzed by one-way ANOVA

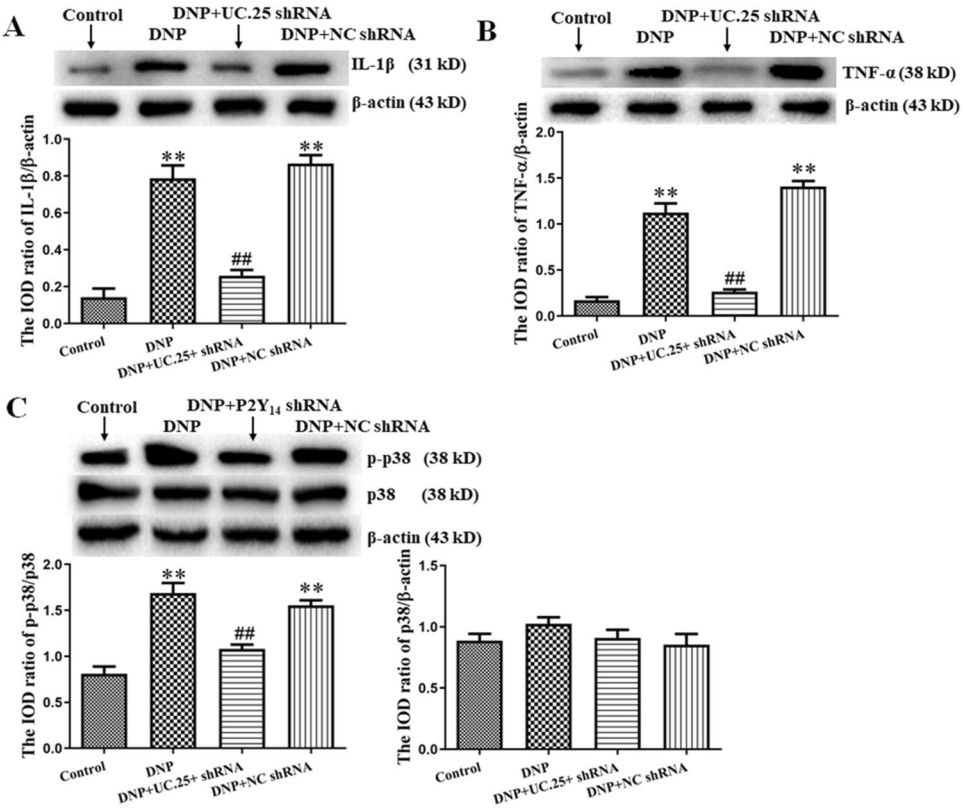

Fig. 6 UC.25 + shRNA downregulated the expression of TNF-α, IL-1β, and p-p38 protein in the spinal cord. **A** Expression of IL-1β. **B** Expression of TNF-α. **C** The expression levels of p38 and p-p38. The results are presented as the mean ± SEM. $n = 12$ rats per group. $^{**}p < 0.01$ vs. Control group; $^{\#\#}p < 0.01$ vs. DNP group. All data were statistically analyzed by one-way ANOVA

UC.25 + overexpression plasmid was obviously increased ($p < 0.01$) (Fig. 8A). Meanwhile, the expression of STAT1 protein was obviously increased ($p < 0.01$) (Fig. 8B); UC.25 + was successfully low-expressed in high-glucose HUVEC cells. Compared with HG group, the expression of UC.25 + in HG + UC.25 + shRNA group transfected with

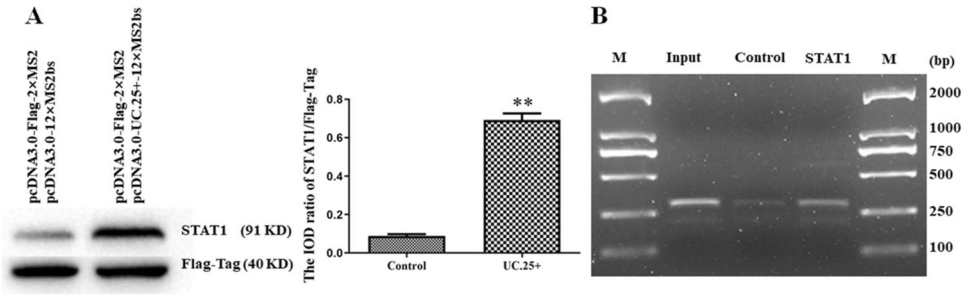

Fig. 7 Determination of interaction. **A**The interaction between UC.25 + and STAT1 protein. The results are expressed as the mean ± SEM of three independent experiments. $^{**}p < 0.01$ vs. Control group. **B** The interaction between STAT1 protein and $P2Y_{14}$ receptor. M: DNA marker; Iuput: total DNA; Control: no antibody; STAT1: STAT1 antibody. Paired *t*-test was used for statistics

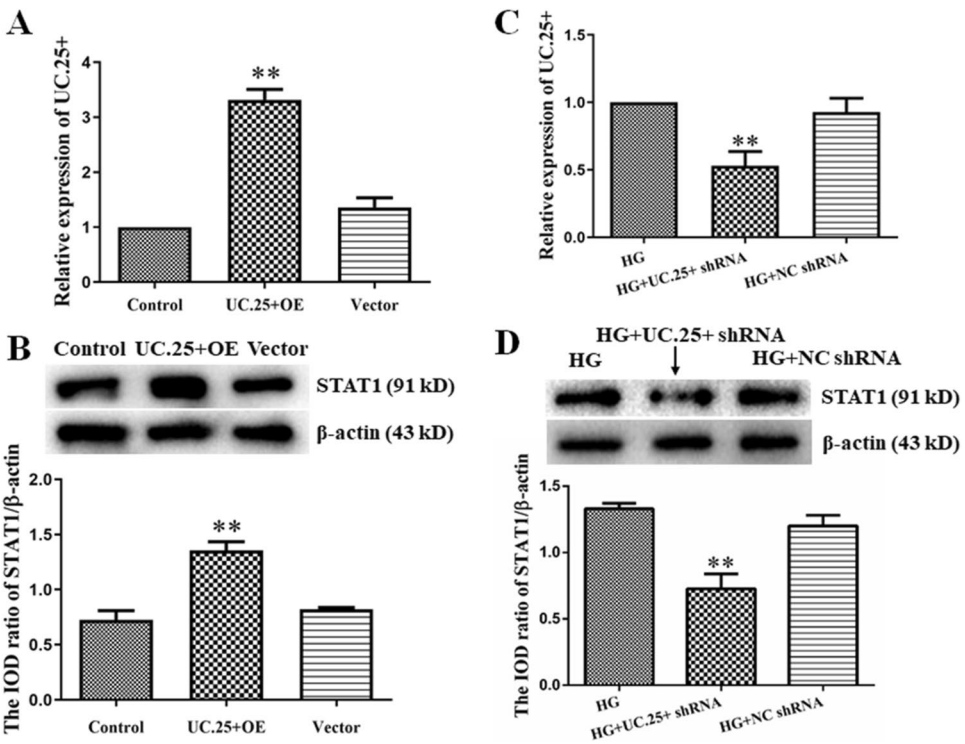

Fig. 8 The expression of STAT1 in HUVEC after UC.25 + overexpression and silence. **A**, **C** Relative expression of UC.25 + . **B**, **D** Expression of STAT1 proteins. The results are displayed as mean ± SEM of three independent experiments. $^{**}p < 0.01$ vs. Control group (or HG group). All data were statistically analyzed by one-way ANOVA

UC.25 + low-expression plasmid was obviously decreased ($p < 0.01$) (Fig. 8C), and the expression of STAT1 protein was also obviously decreased ($p < 0.01$) (Fig. 8D), indicating that UC.25 + can positively regulate the expression of STAT1 in HUVEC cells.

Regulation of STAT1 on $P2Y_{14}$ Receptor

To clarify the regulatory effect of STAT1 on $P2Y_{14}$ receptor, HUVEC cells were cultured again in normal and high-glucose environments. STAT1 was overexpressed in normal cells and low-expressed in high-glucose cells. The effect of STAT1 on $P2Y_{14}$ receptor was detected by real-time quantitative PCR and Western blot. The results showed that STAT1 was successfully overexpressed in HUVEC cells. Compared with Control group, the expression of STAT1 in STAT1 OE group transfected with STAT1 overexpression plasmid was obviously increased ($p < 0.01$) (Fig. 9A), and the expression of $P2Y_{14}$ protein was also obviously increased ($p < 0.01$) (Fig. 9B). STAT1 was successfully low-expressed in high glucose HUVEC cells. Compared with the HG group, the expression of STAT1 in the HG + STAT1 shRNA group transfected with the STAT1 low-expression plasmid was obviously decreased ($p < 0.01$) (Fig. 9C). At the same time, the expression level of $P2Y_{14}$ protein was obviously decreased ($p < 0.01$) (Fig. 9D), suggesting that the transcription factor STAT1 can positively regulate the expression of $P2Y_{14}$ receptor in HUVEC cells.

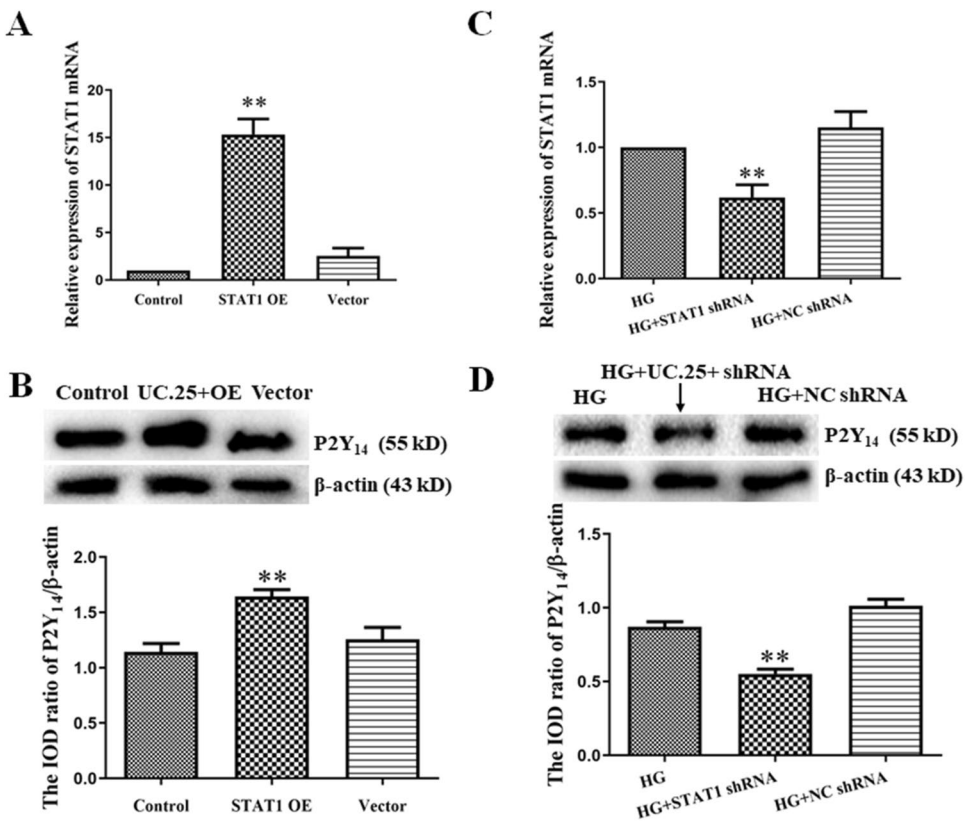

Fig. 9 The expression of $P2Y_{14}$ receptor in HUVEC after STAT1 overexpression and silence. **A**, **C** Relative expression of STAT1 mRNA. **B**, **D** Expression of $P2Y_{14}$ proteins. The results are displayed as mean ± SEM of three independent experiments. $^{**}p < 0.01$ vs. Control group (or HG group). All data were statistically analyzed by one-way ANOVA

Discussion

Diabetes can bring about various injuries in the body thanks to its complications, which include DNP caused by peripheral neuropathy. The symptoms of DNP are obvious, including hyperalgesia, touch-induced pain, spontaneous pain, and hyperalgesia. Most of them can develop into chronic pain, lasting for several years [15]. Thus, it is essential to study the pathogenesis and pretreatment of DNP. In this study, $P2Y_{14}$ shRNA and UC.25 + shRNA treatment increased the mechanical hyperalgesia and thermal hyperalgesia thresholds of rats, indicating that $P2Y_{14}$ shRNA and UC.25 + shRNA can alleviate the pain behavior of DNP rats.

$P2Y_{14}$ receptor can perform specific physiological functions by modulating different signal transduction pathways in cells [16]. Studies have confirmed that in neuropathic pain models, the expression of $P2Y_{14}$ receptors was obviously increased, and the application of $P2Y_{14}$ antisense locked nucleotides can relieve the pain [7]. The upregulation of $P2Y_{12}$ receptors in satellite glial cells of dorsal root ganglion can be increase mechanical or thermal hyperalgesia in diabetic rats, thereby mediating the DNP process [17]. The activation of $P2Y_{13}$ receptors in spinal microglia can promote the transmission of pain in diabetic rats, and the application of $P2Y_{13}$ receptor–specific antagonist MRS2211 can significantly relieve pain. $P2Y_{12}$, $P2Y_{13}$, and $P2Y_{14}$ receptors belong to Gi/o-coupled receptors with strong sequence homologies, and it has been proved that $P2Y_{12}$ and $P2Y_{13}$ receptors exist in spinal microglia [18]. Therefore, we speculated that the $P2Y_{14}$ receptor in spinal cord may participate in the development of DNP. The data showed that the expression of $P2Y_{14}$ receptor protein and mRNA in the DNP group was remarkably increased than that in the Control group. $P2Y_{14}$ shRNA treatment could reduce the expression of $P2Y_{14}$ receptor. Combined with the results of rat pain behavior, it indicated that the abnormal pain in rats was related to the upregulation of $P2Y_{14}$ receptor. Inhibiting the expression of $P2Y_{14}$ receptor could alleviate the pain behavior of rats, implying that $P2Y_{14}$ receptor may participate in the DNP process. At the same time, UC.25 + shRNA treatment could reduce the expression level of $P2Y_{14}$ receptor and alleviate the pain behavior of DNP rats, indicating that UC.25 + may affect the transmission of pain in rats through $P2Y_{14}$ receptor.

Microglia are extremely sensitive to the change in the surrounding environment and can respond quickly. When the nerves are injured or noxiously stimulated, microglia are rapidly activated, releasing a large number of cytokines to act on neurons and alter the synaptic effects [19]. Furthermore, it is found that inhibiting the activation of microglia can effectively relieve the pain [20]. OX42 (CD11b monoclonal antibody) is a marker for identifying the activation of microglia [21]. Immunofluorescence showed that OX42 and $P2Y_{14}$ receptors were co-expressed in microglia, indicating that

$P2Y_{14}$ receptors can be expressed in microglia. Western blot result showed that the expression of OX42 was increased obviously during DNP, indicating that microglia were activated in diabetic rats after nerve injury. $P2Y_{14}$ shRNA treatment reduced the OX42 expression. The activation of microglia is accompanied by the upregulation of $P2Y_{14}$ receptors. The inhibition of $P2Y_{14}$ receptors diminishes the activation of microglia and reduces the transmission of pain. UC.25 + shRNA treatment reduced the expression of OX42 and $P2Y_{14}$ receptor, suggesting that UC.25 + may decrease the activation of microglia by inhibiting the expression of $P2Y_{14}$ receptor, thereby alleviating DNP.

Diabetes is a low-grade systemic inflammatory state [22]. When peripheral nerves are injured, the secretion of pro-inflammatory cytokines increases. The upregulated cytokines will further enhance inflammatory signals and promote the development of pathological pain [23]. The activation of microglia can also promote the secretion of inflammatory factors. This study found that the expression levels of IL-1β and TNFα in the DNP group were obviously higher than those in the control group, indicating that DNP rats released more inflammatory factors and produced a stronger inflammatory response; $P2Y_{14}$ shRNA treatment can downregulate the elevated expression of inflammatory factors induced by DNP, indicating that the $P2Y_{14}$ receptor is related to the release of inflammatory factors. UC.25 + shRNA suppressed the $P2Y_{14}$ expression, suggesting that UC.25 + may reduce the inflammatory response by inhibiting the $P2Y_{14}$ receptor.

Mitogen-activated protein kinases (MAPKs) participate in cell signal transduction. Studies have shown that during inflammatory pain, the activation of p38 MAPK can promote the synthesis and release of inflammatory factors, and p38 MAPK inhibitors can relieve pain; activation of the $P2Y_{12}$ receptor in spinal microglia can over-release inflammatory factors and activate the p38 MAPK pathway, and participate in maintenance of various types of pain [18]. Our data indicated that the phosphorylation of p38 MAPK was obviously raised during DNP, and $P2Y_{14}$ shRNA or UC.25 + shRNA treatment inhibited its phosphorylation, indicating that downregulated expression of $P2Y_{14}$ receptor or UC.25 + could relieve the phosphorylation of p38 MAPK.

Recent studies have verified that lncRNAs can participate in the development of diseases [24]. The dysregulation of lncRNAs may be associated with neuropathic pain [25]. Our results indicated that UC.25 + shRNA inhibited the expression of $P2Y_{14}$ receptor, alleviated the DNP process.

In order to further investigate the potential mechanism of UC.25 + regulating the $P2Y_{14}$ receptor, the screening results through multiple databases showed that the $P2Y_{14}$ promoter region contained the response element of STAT1, suggesting that the transcription factor STAT1 may participate in the regulation of $P2Y_{14}$ receptor expression. In this study, the RIP results showed that UC.25 + can enrich STAT1, indicating that there is an interaction between UC.25 + and STAT1. The overexpression or low-expression of UC.25 + in HUVEC cells respectively increased or decreased the expression of STAT1, suggesting that UC.25 + could positively regulate the expression of STAT1. In addition, ChIP results showed that STAT1 could specifically bind to the response element of $P2Y_{14}$ gene promoter. Similarly, overexpression or low-expression STAT1 in HUVEC cells enhanced or reduced the expression of $P2Y_{14}$, implying that STAT1 could positively regulate the expression of $P2Y_{14}$ receptor. In summary, UC.25 + can enrich STAT1 and positively regulate the expression of STAT1. As a transcription factor of the $P2Y_{14}$ receptor, STAT1 positively regulates the expression of the $P2Y_{14}$ receptor, thereby promoting the occurrence and development of $P2Y_{14}$ receptor–mediated DNP.

Author Contribution Research project conception: Guilin Li, Shangdong Liang. Execution of experimental procedures: Baoguo Wu, Congfa Zhou, Zehao Xiao, Gan Tang. Data analysis: Hongmin Guo, Zihui Hu, Qixing Hu, Hao Peng, Lingzhi Pi, Zhihua Zhang, Miaomiao Wang, Taotao Peng, Jiaqi Huang. Interpretation of results: Baoguo Wu, Congfa Zhou, Hongmin Guo, Zihui Hu. Draft of the manuscript: Baoguo Wu, Gan Tang, Shangdong Liang, Guilin Li. All authors reviewed and approved the final version of the manuscript.

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Data Availability All data generated or analyzed during this study are available from the corresponding author on reasonable request.

Code Availability Not applicable.

Declarations

Ethics Approval Animal experiments were approved by the Institutional Animal Investigation Committee of the Nanchang University and were performed in accordance with the Guidelines for Animal Experiments at Nanchang University.

Consent to Participate No human subjects were involved in this research, so consent to participate is not relevant.

Consent for Publication All authors have approved this manuscript and consented to its submission for publication.

Conflict of Interest The authors declare no competing interests.

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