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Ptprj-as1 mediates inflammatory injury after intracerebral hemorrhage by activating NF-κB pathway

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Abstract. – OBJECTIVE: We aimed at investigating the expression of long non-coding RNA (lncRNA) Ptprj-as1 and the role of lncRNAPtprj-as1 in inflammatory cells after intracerebral hemorrhage and its potential mechanism.

MATERIALS AND METHODS: The rat model of intracerebral hemorrhage was established. Expressions of Ptprj-as1 and inflammatory cytokines in inflammatory cells were detected by quantitative Real-time PCR (qRT-PCR). After BV2 cells were transfected with lentivirus, cell proliferation, migrative ability and apoptosis were detected by cell counting kit-8 (CCK-8) assay, transwell chamber and flow cytometry, respectively. Immunofluorescence was used to explore the ratio of M1 and M2 glial cells. The detection of tumor necrosis factor alpha (TNF-α) expression was performed using enzyme-linked immunosorbent assay (ELISA). Moreover, the expressions of key genes in NF-κB pathway were evaluated using Western blot.

RESULTS: Ptprj-as1 was highly expressed in inflammatory tissues caused by intracerebral hemorrhage (ICH). Overexpressed Ptprj-as1 promoted the migration of BV2 cells and expression levels of inflammatory cytokines such as TNF-α, interleukin-1β (IL-1β), interleukin-6 (IL-6), iNOS and NO. Meanwhile, Ptprj-as1 enhanced the proportion of M1 glial cells, the mechanism of which might be related to the activation of NF-κB pathway.

CONCLUSIONS: Ptprj-as1 activates NF-κB pathway in microglia and promotes the secretion of inflammatory cytokines, which is involved in inflammatory injury caused by intracerebral hemorrhage.

Key Words:
Ptprj-as1, Inflammatory factor, NF-κB pathway, Apoptosis.

Introduction

Intracerebral hemorrhage (ICH) is a serious injury in central nervous system with high morbidity and mortality[1]. Brain damage after ICH is a complex pathophysiological process, including primary injury caused by hematoma expansion and increased intracranial pressure, and secondary injury caused by cytotoxicity and inflammation. Effective control of secondary damage is essential for neuroprotective treatment of ICH. Additionally, inflammation is found to play an important role in the secondary injury induced by ICH[2,3]. After intracerebral hemorrhage, microglia, as an important effector of neuroinflammation, is the first to be activated. After a series of morphological and physiological changes, microglia releases a series of inflammatory mediators and biological active factors such as tumor necrosis factor alpha (TNF-α), interleukin-1β (IL-1β), interleukin-6 (IL-6), IL-8, etc., leading to the occurrence of inflammatory reactions caused by intracerebral hemorrhage, which may further result in secondary injury[4,5]. Therefore, microglia plays a crucial role in the inflammatory response induced by intracerebral hemorrhage.

Long non-coding RNA (lncRNA) accounts for the majority of non-coding RNA. According to the genome-specific affinity of lncRNA with the encoding gene, lncRNA is divided into five categories, including the sense strand, antisense strand, bidirectional, introns and intergenic RNA[6,7]. lncRNA can play a regulatory role in the cell cycle, proliferation, migration, invasion and metabolism through DNA methylation, post-transcriptional regulation, imprinted genes and histone modification, thus affecting expressions of coding genes[8-11]. Abnormally expressed lncRNA is associated with various diseases, which is also closely related to the occurrence and development of inflammation. At present, inflammation-related lncRNA has been continuously found, such as Mirt2 and PlncRNA1[12-14].

Ptprj-as1 is the antisense strand of Ptprj gene with 1006 nucleotides in length. Previous studies have suggested that its expression is regulated by

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lipopolysaccharide (LPS) and is mainly expressed in mouse macrophages. However, the specific function of Ptprj-as1 remains unclear[15]. We aimed at exploring the effect of Ptprj-as1 in microglia after intracerebral hemorrhage and its underlying mechanism.

Materials and Methods

Rat Intracerebral Hemorrhage Model

Blood samples were collected from the right femoral artery by a catheter after rats were anesthetized. Rats were positioned in a stereotactic apparatus, keeping the anterior and posterior fontanelle at the same level. Acranial hole was drilled, followed by the insertion of a 200 μL syringe into the right basal ganglia (coordinates: 0.2 mm anterior, 5.5 mm ventral, 3.5 mm lateral to the anterior fontanelle) using the stereotactic instrument. Autologous whole blood was injected in through the micro-pump at a speed of 100 μL/min. The needle was slowly removed after injection for 5 min and the hole was then sealed with sterile bone wax. All rats in this experiment were purchased from SLAC Laboratory Animal Co. Ltd, (Shanghai, China). This study was approved by the Animal Ethics Committee of Jinan University Animal Center.

Cell Culture and Transfection

The murine BV2 microglial cells and MN9D neuronal cells were cultured at 37°C in Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% penicillin and streptomycin in a 5% CO_2 incubator. BV2 cells were stimulated with 1 g/ml LPS for different time points. For cell differentiation in MN9D, 1 mM sodium butyrate was added in the medium and cells were incubated for 6 consecutive days. For lentivirus infection, cells were seeded into a 12-well plate. LV-Vector and LV-Ptprj-as1 were transfected according to the Hiperfect instructions, respectively. 48 h after the transfection, cells were harvested for further analysis. The lentiviruses used were purchased from Gene Pharma (Shanghai, China).

Total RNA Isolation and Quantitative Real-Time PCR (qRT-PCR)

Total RNA was extracted by TRIzol reagent. Reverse transcription was performed with the Reverse Transcription Kit. Real-time PCR was performed to detect the relevant gene expression. The expression level was calculated by the $2^{-\Delta\Delta Ct}$ method and normalized by the internal control gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Primer sequences were as follows: Ptprj-as1 (F)CCATCTCCCATTGTCCAAAC, (R)TGATTGAAGGACAGCTGGAA; TNF-α (F)CAAGGGACAAGGCTGCCCCG, (R)GCAGGGGCTCTTGACGGCAG; IL-1β (F) CCAAGCCTTATCGGAAATGA, (R)TTTTCACAGGGGAGAAATCG; IL-6 (F)AACGATGATGCACTTGCAGA, (R)CTCTGAAGGACTCTGGCTTTG;iNOS(F)GAGCTTCTACCTCAAGCTATC, (R)CCTGATGTTGCCATTGTTGGT.

Immunofluorescence

2×10^4 BV2 cells were digested and seeded in 24-well plates. Cells were then transfected with LV-Vector or LV-Ptprj-as1, respectively. After that, cells were treated with LPS. Next, cells were fixed with 4% paraformaldehyde followed by blocking with 5% goat serum. Cells were incubated overnight with the primary antibodies. In the next day, cells were incubated with fluorescently labeled secondary antibody and stained with 4',6-diamidino-2-phenylindole (DAPI). The inverted fluorescence microscopy was used to take the images.

Apoptosis

Pre-treated cells were washed with phosphate-buffered saline (PBS) twice, digested with EDTA-free trypsin and gently centrifuged. The supernatant was discarded after centrifugation and cells were resuspended with 500 μL of binding buffer. Next, cells were added with 5 μL of Annexin V-allophycocyanin (APC) and 5 μL of 7-aminoactinomycin D (7-AAD), followed by incubation for 15 min in the dark. The apoptosis rate was analyzed by a FACS flow cytometer.

Nitrite Quantification

The levels of stable metabolite nitrite in BV2 cell culture medium were detected according to the instructions of Colorimetric BioAssay™ kit (Biological, Kibbutz Beit Haemek, Israel). The nitrate standard curve was used to assess the nitric oxide expression in different samples.

Cell Counting kit-8 (CCK-8) Assay

Pre-treated cells were digested and seeded into 96-well plates (1×10^4/well). 24 h later, each well was added with 10 μL of CCK-8 reagent. Cells were gently mixed and continued to incubate for 1-4 h at 37°C. The absorbance (OD value) at 450 nm was detected by a microplate reader.

Enzyme-linked immunosorbent Assay (ELISA)

The supernatants of BV2 cells with different treatments were collected for detecting TNF-α expression according to the instructions of ELISA kit (EBiosciences, San Diego, CA, USA). Each well was blocked with 5% bovine serum for 40 min and washed with phosphate-buffered saline (PBS) for three times. Samples were then added into each well, followed by adding enzyme-labeled antibody. Next, the substrate solution was added to terminate the reaction. The absorbance was detected by a microplate reader within 20 min.

Western Blotting Analysis

The total proteins were extracted by radioimmunoprecipitation assay (RIPA) lysis buffer. Protein concentration was quantified by bicinchoninic acid (BCA) method. Isodose proteins were electrophoresed in 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride (PVDF) membrane. The immunoblots were blocked with 5% non-fat milk and incubated with primary antibodies at 4°C overnight. After that, the immunoblots were washed with Tris-buffered saline-Tween (TBS-T) and incubated with secondary antibody. Protein bands were detected by enhanced chemiluminescence (ECL) method.

Cell Migration Assay

Single cell suspension was prepared at a dose of 2.5×10^5/mL. 200 μL of the cell suspension were added into the upper chamber of the transwell and 500 μL of complete medium were added into the lower chamber. Cells were placed in the incubator overnight. The cells on the upper membrane were gently removed with a cotton swab and cells on the lower membrane were fixed with an appropriate amount of methanol for 15 min. After that, cells were stained with crystal violet for 10 min, washed by running water and naturally dried. 5 fields of views were randomly selected for observation and image taking.

Statistical Analysis

All statistics were analyzed with statistical product and service solutions (SPSS 19.0, Armonk, NY, USA) statistical software. Data in the article were represented as mean ± standard deviation. The Student's *t*-test was applied to compare the differences between two groups. $p<0.05$ was considered statistically significant.

Results

Ptprj-as1 is Highly Expressed in Inflammatory Cells of ICH Model

We detected Ptprj-as1 expression in the perihematoma and contralateral normal brain tissues in rat ICH model. Results showed that Ptprj-as1 expression was significantly elevated in inflammatory tissues around the hematoma (Figure 1A). At the same time, *in vitro* experiment showed that Ptprj-as1 was upregulated after LPS treatment in BV2 cells, which reached to the highest level after 6 h-stimulation (Figure 1B). Next, we overexpressed Ptprj-as1 in BV2 cells by lentivirus to explore the impact of Ptprj-as1 on M1 and M2 polarized microglia (Figure 1C). We found that Ptprj-as1 could significantly activate M1 microglia and inhibit M2 microglia by detecting CD68 and CD206 expressions, M1 and M2 markers, respectively (Figure 1D). These results demonstrated that Ptprj-as1 is highly expressed in inflammatory cells of ICH model.

Ptprj-as1 Promotes Expressions of Inflammatory Cytokines

We next investigated the effect of Ptprj-as1 on cellular functions of microglial cells. CCK-8 assay showed that Ptprj-as1 had no significant effect on the proliferation of microglial cells (Figure 2A). Migration of microglial cells to the inflammatory site is an important part of the immune response. In our research, overexpressed Ptprj-as1 could significantly increase the migration of microglia under LPS stimulation (Figure 2B), suggesting that Ptprj-as1 could promote the immune response. Besides, we detected levels of several inflammatory cytokines and found that Ptprj-as1 did not affect IL-1β expression (Figure 2C), but could significantly increase the mRNA level of IL-6 under LPS stimulation (Figure 2D). Meanwhile, Ptprj-as1 increased TNF-α expression under LPS stimulation not only at the intracellular mRNA level (Figure 2E), but also at extracellular level (Figure 2F). At the same time, Ptprj-as1 could elevate the mRNA expression of iNOS under LPS treatment (Figure 2G) and promote NO production (Figure 2H). To further investigate whether Ptprj-as1 could affect the survival of nerve cells through inflammatory response to microglia, we collected the culture medium of BV2 cells to incubate the well-differentiated MN9D neurons. Our data found that the culture medium collected from Ptprj-as1 overexpression and LPS treatment group could significantly increase the

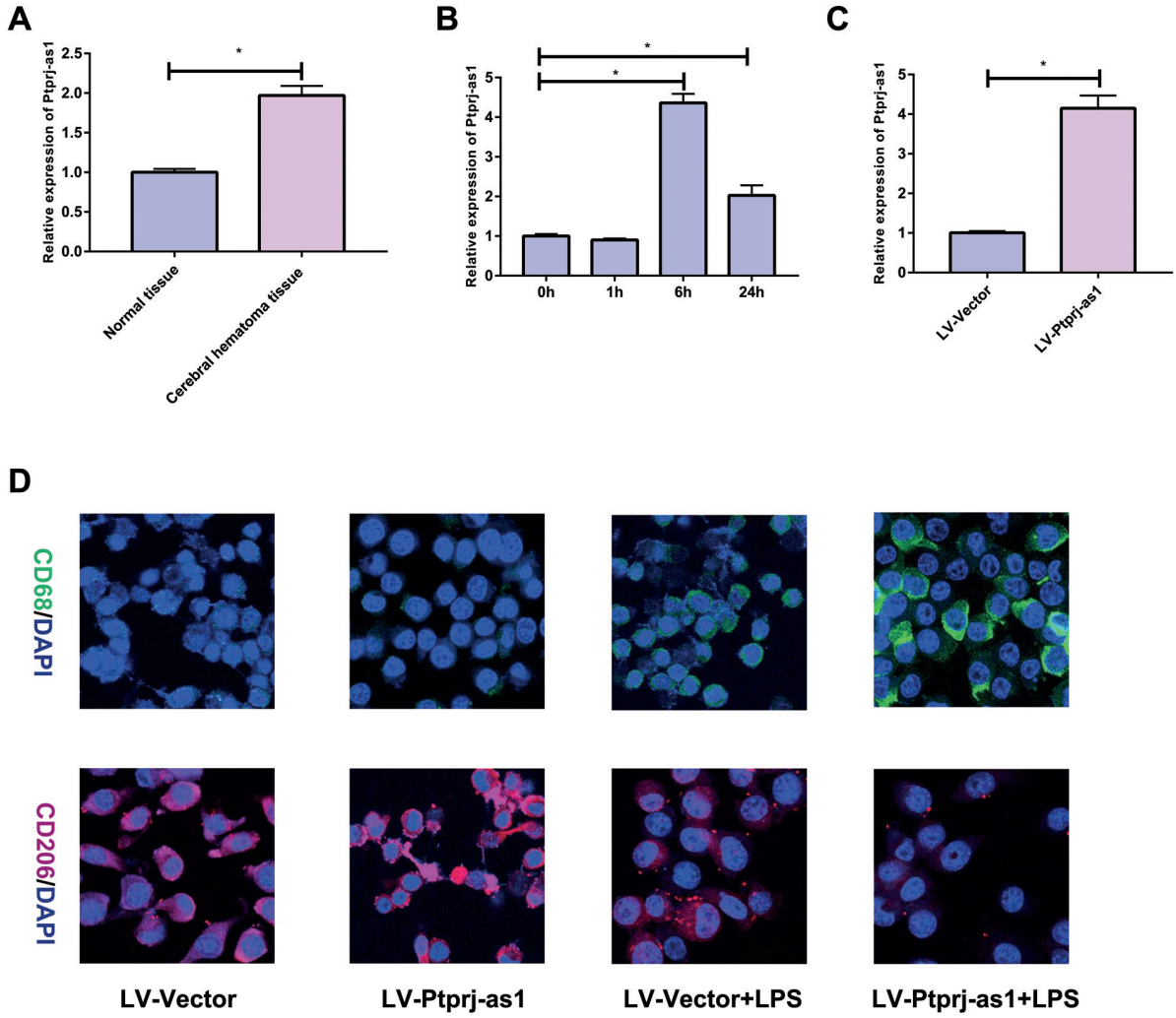

Figure 1. Ptprj-as1 is highly expressed in inflammatory cells of ICH model. ***A,*** Ptprj-as1 expression was detected in normal brain tissue and hematoma tissue in intracerebral hemorrhage rat model; ***B,*** Expression of Ptprj-as1 in BV2 cells stimulated by LPS at different time points; ***C,*** The expression of Ptprj-as1 was detected after lentivirus infection of LV-Vector and LV-Ptprj-as1; ***D,*** The ratio of M1 and M2 was detected after Ptprj-as1overexpression under normal conditions and LPS stimulation.

apoptotic rate of MN9D cells than that of the single LPS treatment group (Figure 2I). These above results indicated that Ptprj-as1 could participate in the inflammatory damage of nerve cells after ICH through regulating microglial inflammation.

Ptprj-as1 Activates the NF-κB Pathway

Both NF-κB and MAPK are important inflammatory response pathways. We found that NF-κB pathway was significantly activated after LPS treatment. Besides, Ptprj-as1 overexpression resulted in a decreased IκB expression and increased p-p65 expression (Figure 3). These results suggested that NF-κB pathway was further activated. However, there was no difference in the p38 expression between LPS + Ptprj-as1 group and LPS group (Figure 3). The above results suggested that Ptprj-as1 may participate in the inflammatory response by activating NF-κB pathway.

Discussion

Intracerebral hemorrhage (ICH) is one of the most common intractable diseases in neurosurgery, which is characterized by high morbidity, mortality and disability. ICH mainly occurs in elderly male people with low incomes. In Western countries, such as the United States, UK and Australia, ICH accounts for 8-15% of total stroke

patients[16]. One month after the onset of cerebral hemorrhage, the mortality rate is up to 40%, and the one-year mortality rate is about 54%. ICH incidence was 5-9/100,000 in people aged from 35-54 years, and 176/100,000 in those aged from 75-94 years. In all age groups, male ICH incidence is higher than that of females[17]. Therefore, it is of great importance to study mechanism of the secondary injury and functional repair after ICH, which could improve life quality of ICH patients and reduce its mortality and morbidity.

Microglia is the inherent non-neuronal cell in the brain. Activated microglial cells experience morphological and functional changes, including enlargement and deep staining, proliferation, migration and phagocytosis. Meanwhile, expressions of pro-inflammatory proteins are elevated[18]. Due to the strong morphological and functional plasticity, microglial cells can be divided into the M1 and M2 type according to their different functional status by peripheral T cell immunophenotyping. M1-type microglia could participate in the secondary brain

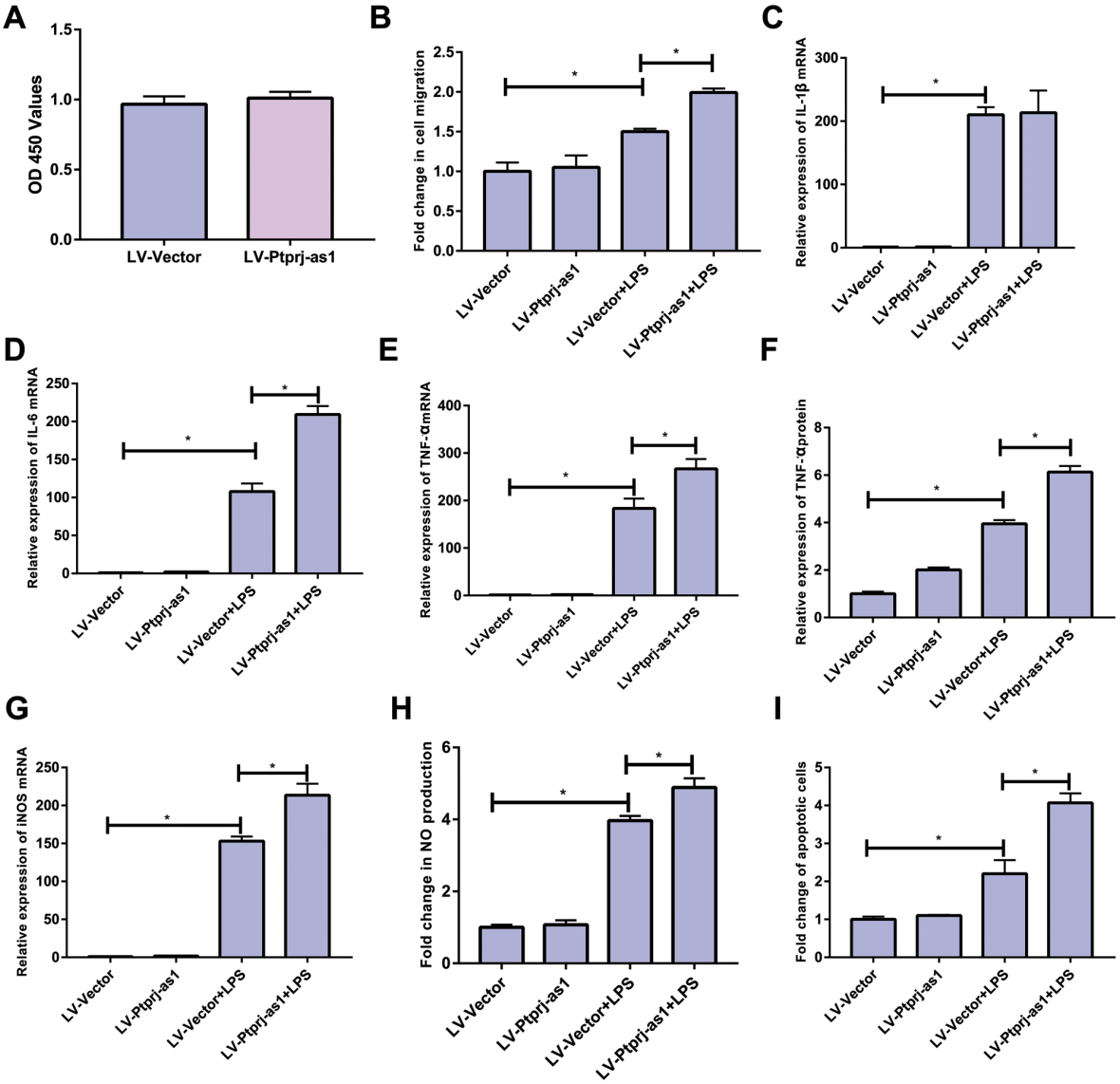

Figure 2. Ptprj-as1 promotes the expression of inflammatory cytokines. ***A,*** The effect of Ptprj-as1on cell proliferation was analyzed by CCK8 assay; ***B,*** The effect of Ptprj-as1 on cell apoptosis was analyzed by flow cytometry under normal conditions and under LPS stimulation; ***C-E,*** The levels of IL-1β, IL-6 and TNF-α were examined by Real-time PCR. ***F,*** TNF-α protein level was detected by Western blot in BV2 cells under normal conditions and under stimulation of LPS; ***G,*** The mRNA level of iNOSwas detected by Real-time PCR; ***H,*** NO production was evaluated by Nitrite quantification. ***I,*** Effect of Ptprj-as1on MN9D neuronal apoptosis under normal conditions and under the stimulation of LPS was analyzed by flow cytometry.

injury following ICH. It also leads to neurological deficits and neuronal apoptosis by triggering pre-inflammatory mediators and releasing metabolic toxic products. In contrast, M2-type microglia could down-regulate inflammatory cytokines and delay the effect of cell injury. Additionally, microglia could enhance the scavenging ability of nerve necrosis tissue and actively participate in nerve regeneration[19]. We found that Ptprj-as1 could activate M1 microglia but inhibit M2 microglia cells under inflammatory stimuli, suggesting that it might promote neuroinflammation through activating M1 polarization.

The activation of NF-κB in microglial/macrophages after ICH could up-regulate expressions of pro-inflammatory cytokines and further lead to brain damage[20], which occurs several minutes after intracerebral hemorrhage and persists for weeks[21]. Erythrocytes and plasma may activate NF-κB via a series of signaling pathways, such as free radicals, cytokines and glutamate receptors[22]. Activated NF-|κB enhances the destruction of the blood-brain barrier and cerebral edema after ICH by upregulating expressions of downstream genes TNF-α and IL-1β[23,24]. Consistent with animal experiments, serum level of TNF-a correlates with the degree of cerebral edema around the hematoma in ICH patients. Besides, early inhibition of microglial activation could improve brain injury after ICH[25]. However, long-term inhibition may impair the beneficial effects of microglia. Hematoma induced by ICH could trigger microglial/macrophage activation, which accelerates the clearance of blood and necrotic tissue, thus providing a better tissue repair environment[26]. Therefore, balance of microglial activation provides a new target for controlling inflammatory response of cerebral hemorrhage. We found that Ptprj-as1 significantly activated the NF-κB pathway in microglia under inflammatory environment and promoted the production of IL-6, TNF-α, iNOS and NO. Further studies are urgently needed to keep the microglial cells in a steady state.

Conclusions

We showed that Ptprj-as1 was involved in the inflammatory injury after cerebral hemorrhage by promoting the secretion of inflammatory cytokines and inhibiting the activity of neurons via NF-κB pathway.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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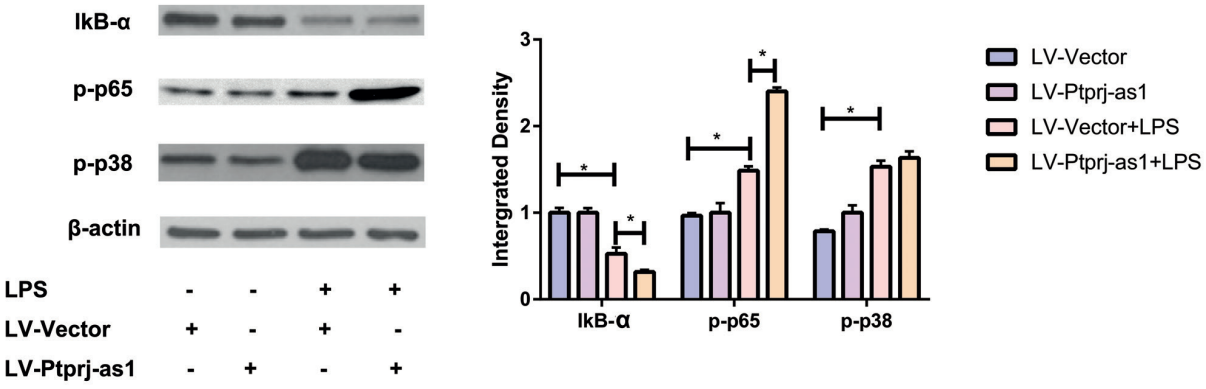

Figure 3. Ptprj-as1 activates the NF-κB pathway. The protein levels of IκB, phosphorylation of p65 and p38 were detected by Western blot under normal conditions and stimulation of LPS.

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