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miR-339-5p inhibits alcohol-induced brain inflammation through regulating NF-κB pathway

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ABSTRACT

Alcohol-induced neuroinflammation is mediated by the innate immunesystem. Pro-inflammatory responses to alcohol are modulated by miRNAs. The miRNA miR-339-5p has previously been found to be upregulated in alcohol-induced neuroinflammation. However, little has been elucidated on the regulatory functions of this miRNA in alcohol-induced neuroinflammation. We investigated the function of miR-339-5p in alcohol exposed brain tissue and isolated microglial cells using *ex vivo* and *in vitro* techniques. Our results show that alcohol induces transcription of miR 339-5p, IL-6, IL-1β and TNF-α in mouse brain tissue and isolated microglial cells by activating NF-κB. Alcohol activation of NF-κB allows for nuclear translocation of the NF-κB subunit p65 and expression of pro-inflammatory mediators. miR-339-5p inhibited expression of these pro-inflammatory factors through the NF-κB pathway by abolishing IKK-β and IKK-ε activity.

1. Introduction

Chronic alcohol consumption is known to increase cytokines and inflammatory mediators in the rat brain, which activates neuroinflammatory-associated signaling pathways and triggers cell death [1]. This inflammatory effect by alcohol is initiated by activation of Toll-like receptors (TLRs) [2]. When TLRs are activated, downstream expression of NF-κB, inflammation-associated molecules and cytokines is induced [3]. The protein complex NF-κB controls DNA transcription and is involved in many cellular responses [4–8]. Activation of NF-κB is associated with the pathogenesis of alcohol-induced brain inflammation [2,9] and stimulates expression of tumor necrosis factor-α (TNF-α), interleukin-1-β (IL-1β) and interleukin-6 (IL-6), which are all increased in alcohol-induced brain inflammation [2,9–11].

MicroRNAs are small non-coding RNAs that regulate gene expression post-transcriptionally by binding to complementary sequences found in the 3′-UTR of target mRNAs, thus degrading or inhibiting translation of the mRNA. MicroRNAs have been implicated in many biological processes including metabolism, cell proliferation, developmental timing, apoptosis, morphogenesis and stress-response [12–16].

In the brain, miRNAs are widely expressed and involved in biological processes such as neuronal differentiation, synapse formation and plasticity and neuronal degeneration [17–20]. Altered miRNA expression has recently been shown to correlate with pathogenesis in Alzheimer's disease and neuroviral infections [21,22]. MiRNAs are also believed to play important roles in neuroadaptive processes related to chronic drug use [23] and may be important in drug and alcohol addiction-related behavior [24,25].

The miRNA miR-339-5p is a tumor suppressor [26], a regulator of the p53 pathway [27], a physiological regulator of β-site amyloid precursor protein cleaving enzyme 1 (BACE1) and is involved in axon guidance [28]. This miRNA is dysregulated in neurodegenerative diseases and has also been found to be altered in murine frontal cortex during alcohol dependency [29–32].

However, the specific role of mir-339-5p in alcohol-induced inflammation has not been characterized. The aim of our study was to examine the regulatory function of miR-339-5p in alcohol-induced brain inflammation. Our data shows that alcohol increases miR-339-5p and pro-inflammatory factors expression in microglial cells and brain tissue and that miR-339-5p inhibits pro-inflammatory factor expression by targeting specific NF-κB activators.

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2. Materials and methods

2.1. Animals

Animal studies were approved by the Institutional Animal Care and Use Committee (IACUC) of Harbin Medical University. Six-week old C57BL/6 mice were randomly divided into two groups: control and alcohol ($n = 9$/group). Alcohol consumption followed a two-bottle choice-drinking in the dark paradigm [33]. For 3 weeks, the animals received 15% alcohol or 100% water from two separate bottles during a 3 h period starting three hours into dark cycle to avoid side preference, and were pair-fed diet with an equal amount of water. Study mice were sacrificed under anesthesia and the brain tissues were excised for further analysis.

2.2. Culture of primary microglial cells

Mouse primary microglial cells cultures were prepared as previously described [34]. In brief, cerebral cortices from 1-day-old neonatal mice were dissected, stripped of their meninges and vessels after washing in ice-cold PBS, and digested with 0.25% trypsin for 30 min at 37 °C. Trypsin was stopped with an equal volume of RPMI medium (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (Invitrogen), 100 U/mL penicillin and 100 μg/mL of streptomycin (Invitrogen). The tissue suspension was centrifuged, supernatant removed and the pelleted cells made into a single cell suspension in culture medium by repeated pipetting following by passage through a 40 μm cell strainer and then centrifuged at 1250×*g* for 5 min at room temperature. Supernatant was discarded and the pellet was resuspended in 3 ml 70% Percoll and transferred to a 15 ml conical tube. Six ml of 50% Percoll followed by 2 ml of 2% FBS and 0.2% glucose in PBS were layered on top of the 70% Percoll cell suspension and centrifuged at 2400×*g* for 30 min at room temperature. The layer containing enriched microglia was collected from the interface between the 70% and 50% Percoll phases and washed twice with 1 ml of PBS containing 2% FBS and 0.2% glucose and centrifuged at 1250×*g* for 5 min at room temperature. Isolated brain microglia were seeded in growing medium (250,000 cells/ml) and non-adherent cells were removed by washing cells with PBS 1 h after plating. Medium was replaced every 3–4 days, and cultures were used between 12 and 20 days. For alcohol stimulation, cells were incubated with medium containing 50 mM alcohol at 37 °C, 5% CO_2 and harvested 12 h post-treatment.

2.3. RT-PCR

RNA was extracted using TRIzol (Invitrogen) according to the manufacturer's instructions. The TaqMan microRNA Reverse Transcription kit and TaqMan MicroRNA Assay (Applied Biosystems, Foster City, CA) were used to examine levels of mature miR-339-5p. U6 was used as control. cDNA was transcribed from 1 mg of total RNA using Reverse Transcription System (Promega, Madison, WI) in a final volume of 30 μl. SYBR-Green-based real-time quantitative PCR was performed using the iCycler (Bio-Rad Laboratories Inc, Hercules, CA). Comparative threshold cycle (Ct) method was used to calculate expressions relative to control groups. The final results were expressed as fold changes between the sample and the controls corrected with internal control. Sense and antisense primer sequences were: IL-1β sense 5′-AATCTCACAG CAGCACATCAA-3′; IL-1β antisense 5′-AGCCCATACTTTAGGAAG ACA-3′; IL-6 sense 5′-TGGAGTCACAGAAGGAGTGGCTAAG-3′; IL-6 antisense 5′-TCTGACCACAGTGAGGAATGTCCAC-3′. TNF-α sense 5′-CCCCTCAGCAAACCACCAAGT-3′; TNF-α antisense 5′-CTTGG GCAGATTGACCTCAGC-3′; β-actin sense 5′-CCACACCCGCCAC-CAGTTCG-3′; β-actin antisense 5′-CCCATTCCCACCATCACACC-3′.

2.4. ELISA

Brain sections were prepared in ice-cold RIPA buffer containing protease and phosphatase inhibitors (Santa Cruz Biotechnology, Santa Cruz, CA). Protein was dissociated from tissue using an ultrasonic cell disrupter. The sonicated samples were immediately centrifuged at maximum speed for 30 min at 4 °C. The supernatants were removed and stored at −80 °C until ELISA measurement. ELISA kits (Thermo Scientific, Rockford, IL) and Bio-Rad protein assay dye (Bio-Rad Laboratories Inc., Richmond, CA) were used to quantify IL-1β, IL-6 and TNF-α according to manufacturer's instructions.

2.5. miRNAs, plasmid and siRNAs transfection, and luciferase assay

Mouse negative control miRNA, miR-339-5p and miR-339-5p-in were acquired from RiboBio (Guangzhou, China). Control siRNA and siRNA against p65 were purchased from Santa Cruz Biotechnology. Partial wild-type sequences of mouse IKK-β and IKK-ε UTR's or those with a deleted miR-339-5p target site were inserted into the pMIR-Reporter construct (Ambion, Austin). NF-κB luciferase reporter plasmid was purchased from Addgene (Cambridge, MA). The pRL-SV40 vector (Promega) carrying the Renilla luciferase gene was used as an internal control to normalize transfection efficiency. Luciferase values were determined using the Dual-Luciferase Reporter System (Promega). Transfection of miRNAs, siRNAs and miRNA were performed using Lipofectamine™ RNAiMAX (Invitrogen) according to the manufacturer's instruction. In brief, cells were seeded in a 24-well plate in triplicate. miRNA (10 pmol) and plasmid (0.8 μg) were cotransfected into cells of each well. Luciferase activity was measured after 48 h.

2.6. Western blot

Cells or tissues were harvested and lysed with ice-cold lysis buffer (50 mM Tris–HCl, pH 6.8, 32 mM 2-ME, 2% SDS, 10% glycerol). After centrifugation (20,000×*g*, 10 min, 4 °C), proteins were separated by 10% SDS PAGE and transferred to polyvinylidene difluoride membranes (Bio-rad). After blocking with 10% non-fat milk in PBS, membranes were incubated with primary antibodies, followed by HRP-linked secondary antibodies (Santa Cruz). Bound antibodies were detected by SuperSignal West Pico Chemiluminescent Substrate kit (Pierce, Rockford, IL) according to manufacturer's instructions. Anti-IKK-β, anti-IKK-ε, p65 and ant-β-actin antibodies were purchased from Santa Cruz Biotechnologies (Santa Cruz). Protein levels of β-actin were used as loading controls.

2.7. Immunofluorescent staining

Microglial cells were seeded in 4-well chamber slides in triplicate and then transfected with miRNA. After 48 h, cells were fixed with freshly prepared 4% paraformaldehyde for 15 min and permeabilized with 0.5% Triton X-100 for 15 min. After 1 h incubation with 10% normal goat serum, cells were incubated with rabbit anti-p65 (1:100, Santa Cruz). After washing with PBS, a FITC-conjugated goat anti-rabbit secondary antibody (1:1000, Invitrogen) was used to probe the primary antibody for 1 h. Thereafter, the cells were mounted with DAPI containing mount media and scanned under an Olympus fluorescence microscope. Cells probed with only a secondary antibody were used as negative controls.

2.8. Statistical analysis

Data was expressed as mean ± SEM from at least three separate experiments. Differences between groups were analyzed using one-way ANOVA. $P < 0.05$ was considered statistically significant.

3. Results

3.1. miR-339-5p and pro-inflammatory cytokines are increased in microglial cells and mouse brain treated with alcohol

From published literature, we found that miR-339-5p is overexpressed in mouse alcohol-induced brain injury model [32]. However, it has not been investigated whether and how miR-339-5p regulates alcohol-induced brain inflammation. To explore this, we first isolated mouse primary microglial cells from new born mice and treated the cells with alcohol (50 mM) for 12 h, then performed RT-PCR to analyzed miR-339-5p expression. We found that expression of miR-339-5p was 50 times higher in alcohol treated cells compared to control (Fig. 1A). Furthermore, we fed mice with 15% alcohol for three weeks to investigate whether alcohol can induce miR-339-5p *in vivo*. RT-PCR of brain tissues showed almost 70 times increase of miR-339-5p in alcohol-treated mouse brain compared to control group (Fig. 1B). To analyze whether pro-inflammatory mediators are activated by alcohol, we performed RT-PCR and detected that IL-1β, IL-6 and TNF-α were stimulated by alcohol (Fig. 1C). This result was confirmed using ELISA for IL-1β, IL-6 and TNF-α in mouse brain tissues (Fig. 1D). Taken together, miR-339-5p and pro-inflammatory cytokines are increased in alcohol-induced microglial cells and brain tissues.

3.2. miR-339-5p inhibits alcohol-stimulated NF-κB signaling

To investigate if the increase of miR-339-5p expression is promotive or protective in alcohol-induced inflammation, we transfected miRNA negative control, miR-339-5p mimics and miR-339-5p inhibitor (miR-339-5p-in) into microglial cells, then detected the expression of IL-1β, IL-6 and TNF-α by RT-PCR. As shown in Fig. 2A, IL-1β, IL-6 and TNF-α expression was significantly decreased 4–5-fold by miR-339-5p, but increased more than 3-fold by miR-339-5p-in in alcohol treated microglial cells. Since IL-1β, IL-6 and TNF-α are the downstream genes of NF-κB signaling pathway, we then examined the miR-339-5p regulation of NF-κB activity in the presence or absence of alcohol treatment using luciferase assay. We found that in the absence of alcohol, neither miR-339-5p nor miR-339-5p-in affected NF-κB activity. Alcohol treatment activated NF-κB signaling which could be inhibited by miR-339-5p and stimulated by miR-339-5p-in (Fig. 2B). Further, we used western blot to detect nuclear p65. As shown in Fig. 2C, alcohol promoted p65 nuclear translocation, and miR-339-5p, but not miR-339-5p-in, inhibited alcohol-induced p65 translocation. To confirm this result, fluorescence staining for p65 in microglial cells was performed, and it was observed that more p65 translocated into cell nuclei after alcohol treatment, and that this process was inhibited by miR-339-5p (Fig. 2D). In summary, alcohol induces nuclear translocation of p65 and miR-339-5p inhibits this translocation, thereby inhibiting alcohol-induced NF-κB signaling and expression of pro-inflammatory factors.

3.3. miR-339-5p directly targets IKK-β and IKK-ε

To explore the mechanism by which miR-339-5p inhibits NF-κB signaling, we investigated potential miR-339-5p targets in microglial cells. Using three publicly available algorithms (RNAhybrid, miRDB and PICTAR4), we identified five proteins (Bcl6, Kif11, IKK-β, PFTK1 and IKK-ε) as potential targets of miR-339-5p (Fig. 3A). Subsequently, a luciferase reporter assay using pMIR-Reporter plasmid carrying IKK-β and IKK-ε 3′UTR confirmed that IKK-β and IKK-ε are targets of miR-339-5p (Fig. 3B). To confirm that miR-339-5p directly targets IKK-β and IKK-ε, we constructed plasmids with IKK-β and IKK-ε 3′-UTR with six common nucleotides of miR-339-5p target sequences mutated (IKK-β and IKK-ε 3′-UTR-mut, Fig. 3C) and performed the luciferase assay. The results show that miR-339-5p significantly inhibited luciferase activity in wild type IKK-β and IKK-ε 3'UTR constructs, but not in mutant IKK-β and IKK-ε 3'UTR (Fig. 3D). Accordingly, the protein level of IKK-β and IKK-ε were also dramatically reduced after miR-339-5p overexpression and elevated after the inhibition of miR-339-5p in alcohol-induced microglial cells (Fig. 3E). These

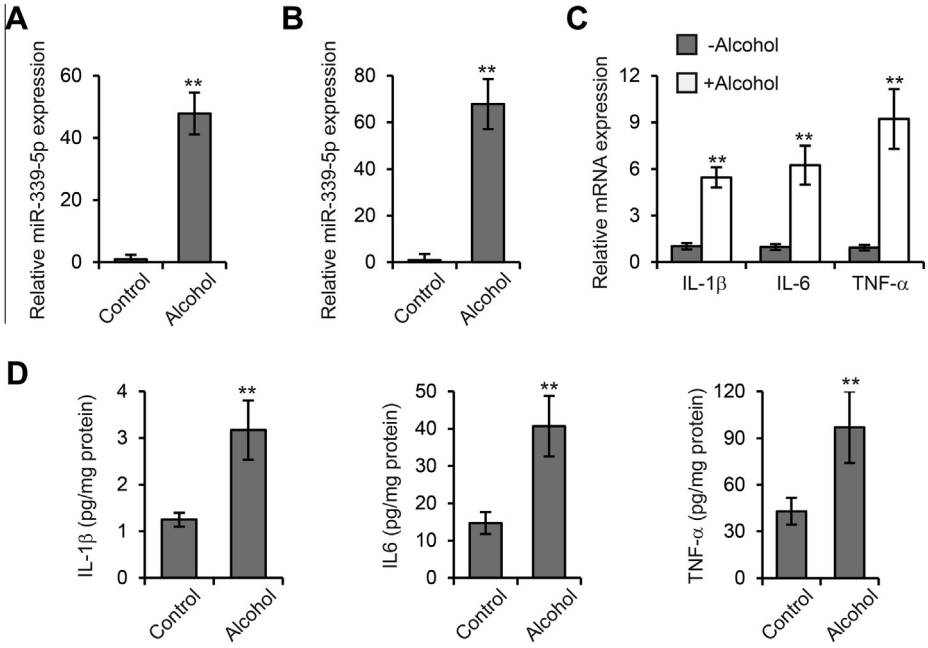

Fig. 1. miR-339-5p and pro-inflammatory cytokines were increased in alcohol-induced microglial cells and brain tissue. (A) miR-339-5p expression in mouse microglial cells treated with alcohol (50 mM) for 12 h. (B) miR-339-5p expression in mice fed with 15% alcohol for 3 weeks. (C) RT-PCR analysis of IL-1β, IL-6 and TNF-α expression in microglial cells treated with alcohol for 12 h. (D) ELISA assay of IL-1β, IL-6 and TNF-α in mouse brain tissues. $^{**}P < 0.01$ compared with the control group. Data expressed as mean ± SEM of three independent experiments.

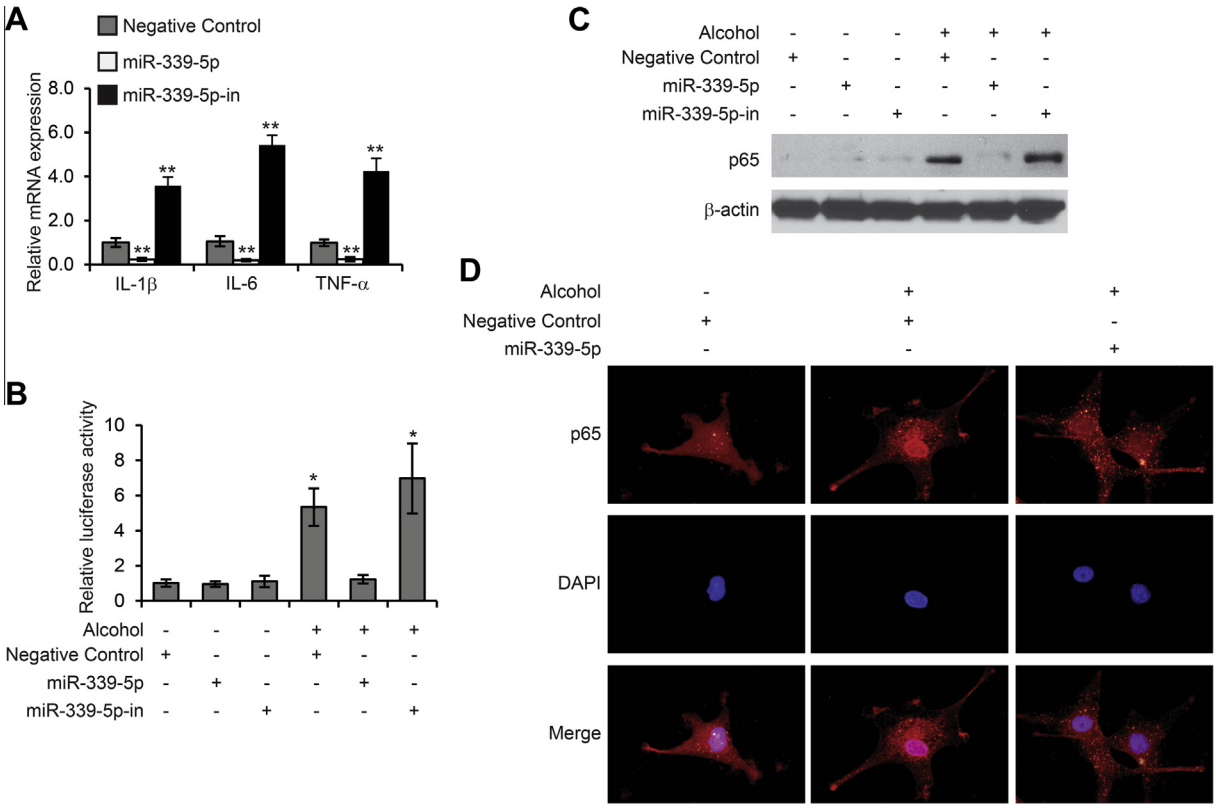

Fig. 2. miR-339-5p inhibits alcohol-induced NF-κB signaling and proinflammatory cytokine expression. (A) RT-PCR analysis of IL-1β, IL-6 and TNF-α expression. Microglial cells were transfected with negative control or miR-339-5p mimics for 48 h, then treated with alcohol. (B) Luciferase assay of microglial cells transfected with negative control or miR-339-5p mimics and NF-κB luciferase reporter plasmids, then treated with or without alcohol. (C) Primary microglial cells were transfected with negative control, miR-339-5p or miR-339-5p-in mimics for 48 h, then cells were treated with or without alcohol, followed by western blot for nuclear p65 expression. (D) Fluorescent staining of p65 (red) in alcohol-treated microglial cells. Nuclei were stained blue by DAPI. $^{*}P < 0.05$ compared with the cells transfected with negative control in the absence of alcohol. $^{**}P < 0.01$ compared with the cells transfected with negative control. Data expressed as mean ± SEM of three independent experiments. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

results demonstrate that IKK-β and IKK-ε are direct targets of miR-339-5p in primary microglial cells.

3.4. miR-339-5p regulates alcohol induced-inflammation through NF-κB pathway

Next we investigated whether miR-339-5p inhibits alcohol-induced inflammatory factor expression through NF-κB pathway. We knocked down p65 in microglial cells using siRNA (Fig. 4A). Our data shows that the expression of IL-1β, IL-6 and TNF-α were down regulated when p65 was knocked down, and that alcohol, miR-339-5p or miR-339-5p-in cannot further stimulate NF-κB signaling (Fig. 4B–D). Taken together, our results suggested that alcohol induces microglial cell inflammation through NF-κB signaling and miR-339-5p inhibits alcohol-induced pro-inflammatory factor expression by targeting the NF-κB pathway (Fig. 4E).

4. Discussion

Alcohol is known to interact with TLR4 [11], which activates the NF-kB pathway, inducing of pro-inflammatory factors expression and inflammation [2]. Pascual et al. [35] demonstrated that ethanol can activate glial cell TLR4, and that mice lacking TLR4 expression are protected against ethanol-induced glial activation, inflammatory factor induction and apoptosis. Downstream of TLR stimulation, NF-κB is activated, causing expression of pro-inflammatory factors.

Consistent with our observations, miRNA-339-5p was previously found to be upregulated in mouse alcohol-induced brain damage [32]. We also found that alcohol significantly increased the expression of the pro-inflammatory cytokines IL-1β, IL-6 and TNF-α in microglial cells and brain tissue, which is also consistent with previous reports [2,11].

Further, we found that alcohol-stimulated NF-κB signaling is mediated by nuclear translocation of the NF-κB subunit p65, and that overexpression of miR-339-5p inhibits p65 translocation which further inhibits expression of IL-1β, IL-6 and TNF-α. This shows that miR-339-5p directly affects ability of p65 to be released from its inhibitor IκBα. Other miRNAs, including miR-155 and miR-146, have also been reported to be upregulated by ethanol. Similarly to miR-339-5p, these microRNAs play roles in alcohol-induced brain-inflammation. Alcohol induced miR-155 regulates TNF-α and MCP1 expression in a TLR4/NF-κB dependant manner [36]. miR-146 also affects NF-κB activity through the negative feedback loops involved in downregulating IL-1 receptor associated kinase 1 and TNF receptor-associated factor 6 [37].

Our data shows that miR-339-5p inhibits alcohol-stimulated NF-κB signaling in microglial cells by targeting IKK-β and IKK-ε. The transcription factor NF-κB is found in the cytoplasm in its inactive form, bound to the inhibitor IκBα, which masks the nuclear localization signals of NF-κB proteins and keeps them sequester in the cytoplasm. The IKK complex consists of IKK-β, IKK-α and IKK-γ, and activates NF-κB signaling by phosphorylating IκBα, causing release of the p65/p50 dimer of NF-κB, which then translocates to the nucleus and initiates IL-1β, IL-6 and TNF-α transcription.

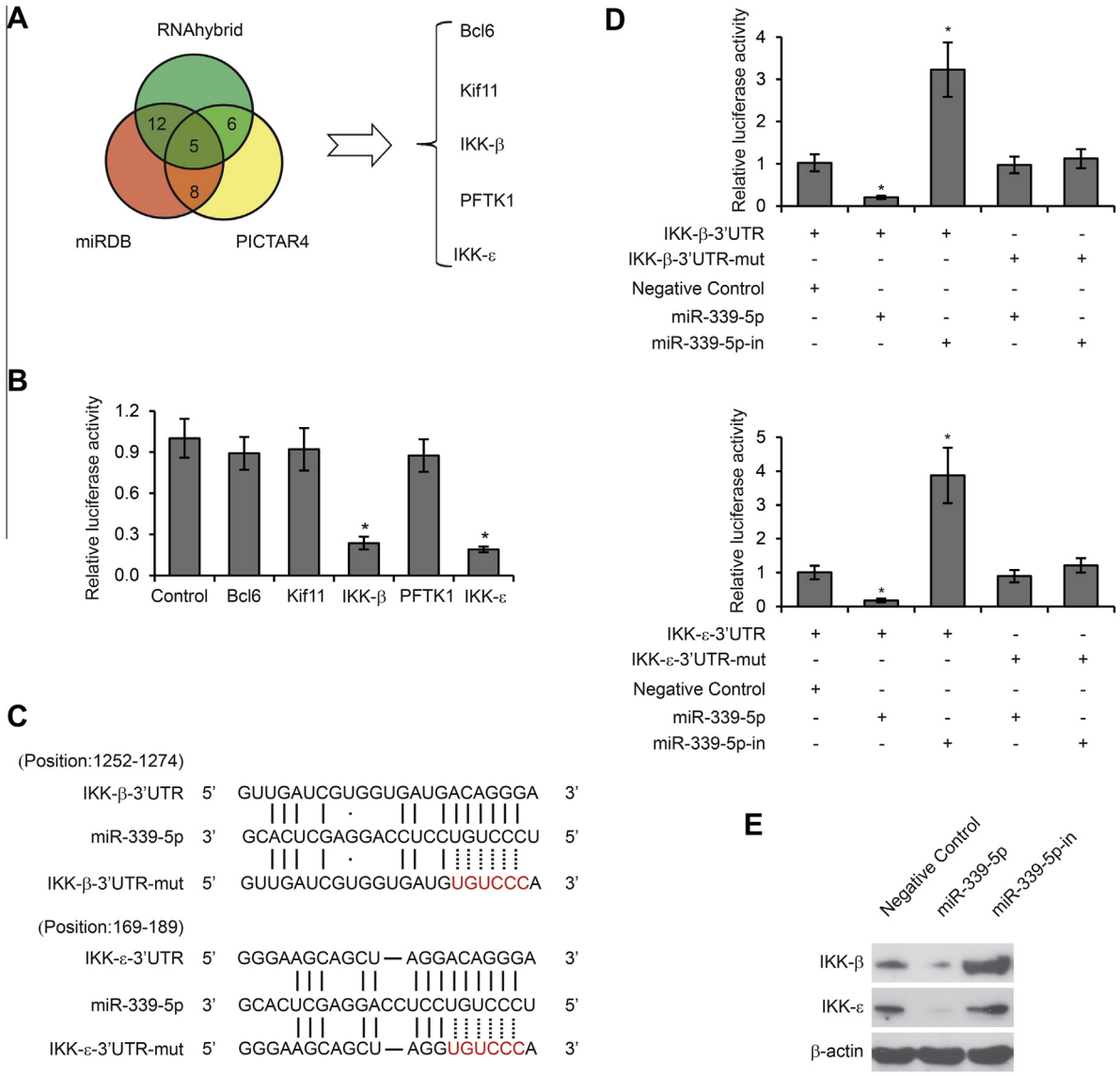

Fig. 3. miR-339-5p directly targets IKK-β and IKK-ε. (A) Five miR-339-5p target genes were predicted by three publicly available algorithms. (B) Luciferase activity of five predicted luciferase reporter constructs (pMIR-Bcl-6-3′UTR, pMIR-Kif11-3′UTR, pMIR-IKK-β-3′UTR, pMIR-PFTK1-3′UTR and pMIR-IKK-ε-3′UTR) co-transfected with miR-339-5p compared to pMIR-reporter luciferase plasmid and miR-339-5p. (C) Predicted miR-339-5p target sequences in the wild-type IKK-β and IKK-ε 3′UTR. Six common nucleotides (red) of IKK-β and IKK-ε 3′UTR were mutated to prevent the broad binding with miR-339-5p. (D) Luciferase activity in microglial cells transfected with pMIR-IKK-β-3′UTR + miR-339-5p, pMIR-IKK-β-3′UTR + miR-339-5p-in, pMIR-IKK-β-3′UTR-mut + miR-339-5p or pMIR-IKK-β-3′UTR-mut + miR-339-5p-in, compared to cells transfected with pMIR-IKK-β-3′UTR + negative control (top); luciferase activity in microglial cells transfected with pMIR-IKK-ε-3′UTR + miR-339-5p, pMIR-IKK-ε-3′UTR + miR-339-5p-in, pMIR-IKK-ε-3′UTR-mut + miR-339-5p or pMIR-IKK-ε-3′UTR-mut + miR-339-5p-in, as compare to cells transfected with pMIR-IKK-ε-3′UTR + negative control (bottom). (E) Western blot of IKK-β and IKK-ε levels in mouse primary microglial cells transfected with negative control, miR-339-5p or miR-339-5p-in. $^{*}P < 0.05$ compared with the control group. Data expressed as mean ± SEM of three independent experiments. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

IKK-β is a serine-kinase which is induced by several different stimuli including inflammation-cytokines, bacterial and viral products and damaged DNA. This kinase also phosphorylates the IKK-related kinase IKK-ε, which may inhibit overexpression of inflammatory mediators by negatively regulating IKKs [38–40]. IKK-β is required for TNF-α induction of NF-κB [41].

IKK-ε is a serine/threonine kinase with critical involvement in regulation of IFN-β and IFN-inducible genes as well as the balance between type I and type II IFN responses and protects from DNA damage-induced cell death [42–44]. IKK-ε is part of the PMA-Inducible IκB kinase complex and can degrade IκBα in response to PMA or T-cell receptor activation [41].

We observed that alcohol induces miR-339-5p and pro-inflammatory cytokine expression in microglial cells and brain tissue. Others found that the expression of several miRNAs, including miR-339-5p, was altered in the rat medial prefrontal cortex after a period of alcohol dependence [45]. Further, we observed that increased expression of miR-339-5p inhibited the NF-κB signaling and the expression of pro-inflammatory factors. To our knowledge, this is the first study to show that miR-339-5p inhibits NF-κB signaling by inhibiting IKK-β and IKK-ε, thereby limiting pro-inflammatory factor expression.

In summary, we found that alcohol induces transcription of miR-339-5p, IL-6, IL-1β and TNF-α in mouse brain tissue and

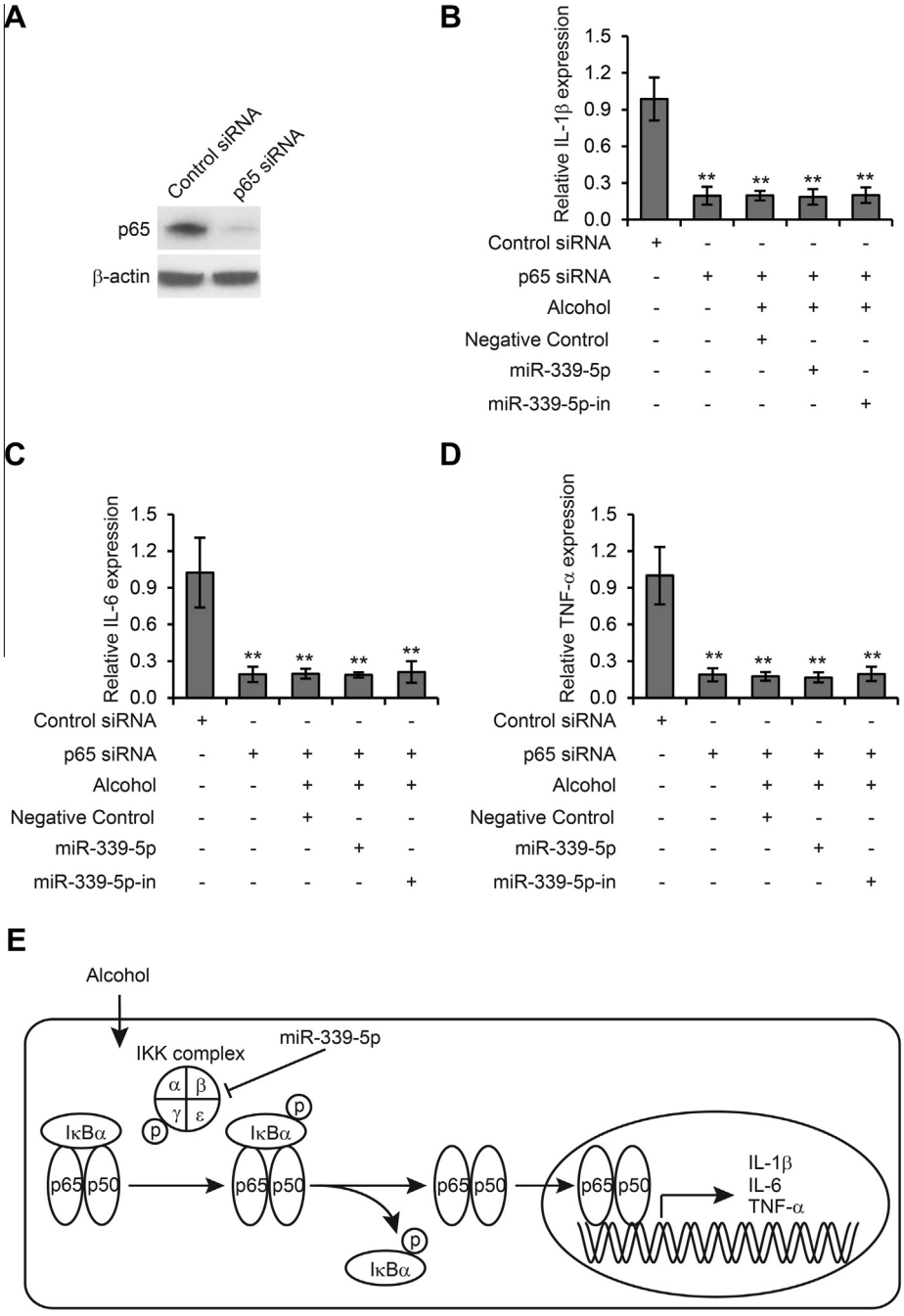

Fig. 4. miR-339-5p and alcohol affect inflammation through NF-κB signaling pathway. (A) Mouse primary microglial cells were transfected with control siRNA or p65 siRNA, followed by western blot for p65 levels. Microglial cells were separately transfected with negative control, miR-339-5p or miR-339-5p-in in p65 knocked down cells. After 48 h, the cells were treated with alcohol for 12 h, followed by RT-PCR assay for IL-1β (B), IL-6 (C) and TNF-α (D) expression. $^{**}P < 0.01$ compared with the control siRNA transfected group. Data expressed as mean ± SEM of three independent experiments. (E) A diagram of alcohol, miR-339-5p and NF-κB signaling pathway.

isolated microglial cells. Alcohol was found to induce cytokine transcription by activating NF-κB nuclear translocation of p65. miR-339-5p inhibited expression of these factors through the NF-κB pathway, by abolishing IKK-β and IKK-ε activity. Thus, miR-339-5p is a negative regulator of alcohol-associated NF-κB-induced pro-inflammatory factor synthesis in the brain.

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