circ_0003170 aggravates human hippocampal neuron injuries by regulating the miR-421/CCL2 axis in cells models of epilepsy

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Abstract. Emerging evidence proposes that circular RNAs (circRNAs) are involved in epileptogenesis. This study aimed to investigate the role and the function mechanism of circ_0003170 in epilepsy models *in vitro*. Epilepsy models were established in human hippocampal neurons treated by magnesium-free (Mg^{2+}-free) solution. The expression of circ_003170, miR-421 and C-C motif chemokine ligand 2 (CCL2) was detected by qRT-PCR. The putative interaction between miR-421 and circ_003170 or CCL2 was validated by dual-luciferase reporter assay and RIP assay. The protein level of CCL2 was detected by Western blot. Cell viability was detected by CCK-8 assay, and cell cycle and apoptosis were monitored by flow cytometry. The content of superoxide dismutase (SOD) and malondialdehyde (MDA) and the activity of caspase-3 were assessed using commercial kits. The results showed that circ_0003170 and CCL2 expression was enhanced, while miR-421 expression was declined in temporal lobe epilepsy serum specimens and Mg^{2+}-free-induced neurons. circ_0003170 knockdown ameliorated Mg^{2+}-free-induced cell cycle arrest, oxidative stress and apoptosis in neurons by enriching miR-421. Further analysis presented that miR-421 overexpression alleviated Mg^{2+}-free-induced cell injuries by depleting CCL2. CCL2 overexpression reversed the effects of circ_0003170 knockdown. Overall, circ_0003170 knockdown ameliorated Mg^{2+}-free-induced human hippocampal neuron injuries by mediating the miR-421/CCL2 axis.

Key words: circ_0003170 — miR-421 — CCL2 — Epilepsy — Hippocampal neuron — Mg^{2+}-free

Introduction

Epilepsy is a chronic neurological disease that affects more than 50 million people worldwide, characterized by recurrent and unexplained attacks (Unehan et al. 2011). Epilepsy is thought to be caused by an abnormal hypersynchronous electrical activity of the neuronal network caused by the imbalance of excitatory and inhibitory neurons in the central nervous system (CNS) (Unterrainer et al. 2004; Guilhoto 2017). Typical clinical features of epilepsy are recurrent seizure disorders and widespread or localized muscle cramps and convulsions (Wan and Yang 2020).

Epileptogenesis is still a challenge to overcome, with molecular, cellular, and structural dynamic changes (Pitkanen et al. 2015). Although accumulating studies have explored the mechanism of epilepsy, the candidate pathogenic factors and mechanisms of epilepsy are still lacking. Exploring the molecular mechanism of genetic regulation in epilepsy helps explain the development of epilepsy.

Non-coding RNAs (ncRNAs), including long ncRNAs (lncRNAs), microRNAs (miRNAs) and circular RNAs (circRNAs), regulate the expression of numerous target genes by several regulatory mechanisms (Jimenez-Mateos and Henshall 2013; Lee et al. 2018). circRNA harbors a covalently closed circular structure and is produced by "back-splicing" by which downstream exon is reversely spliced to upstream exon (Memczak et al. 2013). Expression profiling reveals specific changes to brain circRNA levels in temporal neocortex tissues of temporal lobe epilepsy (TLE) patients (Li et al. 2018), hinting that circRNAs may participate in epilepsy progression. Hippocampus neural network contains a large number of neurons, and its dys-

function or injury plays a crucial role in TLE (Gong et al. 2014). Hippocampal neurons treated by magnesium-free (Mg^{2+}-free) solution were frequently used as TLE *in vitro* models (Gong et al. 2014), which provided available models to explore the function of ncRNAs (Geng et al. 2018). Although the vital role of circRNAs in epilepsy is recognized, few studies have been conducted on circRNAs *in vivo* or *in vitro* models of epilepsy. Here, we focused on circ_0003170 (derived from STK24), which was highly expressed in TLE specimens analyzed by circRNA profiles Li et al. (2018). However, its function and potential functional mechanism in epilepsy are unknown.

It is well known that partial circRNAs target specific miRNAs through miRNA response elements to play competing endogenous RNA (ceRNA) effects (Zhang et al. 2018). Numerous miRNAs were also reported to be implicated in epilepsy pathogenesis, including miR-421. Nevertheless, the role of miR-421 in Mg^{2+}-free-induced hippocampal neurons is deficient, and the associated mechanism of miR-421 linked to circ_0003170 is not unclosed. Moreover, miRNAs modulate gene expression mainly *via* sequence-specific binding sites within the 3' untranslated region (3'UTR) of mRNA transcripts (Jimenez-Mateos and Hendshall 2013). C-C motif chemokine ligand 2 (CCL2) was reported to trigger epileptic seizure by mediating inflammatory responses (Bozzi and Caleo 2016). CCL2 is a target of miR-421, which was verified in this study. However, the interaction between miR-421 and CCL2 is not previously explored, and further mechanism of CCL2 in epilepsy associated with circ_0003170 is worth clarifying.

In the current research, epilepsy *in vitro* models were generated using human hippocampal neurons with the treatment of Mg^{2+}-free. The expression of circ_0003170 was monitored in TLE serum specimens and Mg^{2+}-free-induced neurons, and the function of circ_0003170 on cell viability, cycle, apoptosis and oxidative stress was investigated *in vitro*. In addition, the circ_0003170/miR-421/CCL2 network was assembled to reveal a potential regulatory mechanism of circ_0003170, aiming to reveal the novel pathogenesis of epilepsy through circRNA regulatory network.

Materials and Methods

Serum specimens

A total of 30 TLE patients and 10 normal volunteers were recruited from Panzhihua Municipal Central Hospital and participated in the study. Blood samples from TLE patients and normal subjects were utilized to collect serum specimens by centrifugation. All serum specimens exposed to liquid nitrogen were stored at −80°C conditions. Each

participant had signed written informed consent and approved the use of serum specimens. This study was implemented with the authorization of Panzhihua Municipal Central Hospital.

Experimental cells

Human hippocampal neurons were purchased from Sciencell (catalog: #1540; Carlsbad, CA, USA) and cultured in the recommended medium (Neuronal Medium; catalog: #1521; Sciencell). Human hippocampal neurons were cultured at 37°C conditions with 5% CO_2. After plating for 3 days, the culture medium was replaced every 3 days. The neurons at days 7−21 were used *in vitro* for the following assay.

To induce epileptiform cell models, hippocampal neurons cultured *in vitro* at day 6 were exposed to magnesium-free (Mg^{2+}-free) solution containing NaCl (145 mM), KCl (2.5 mM), HEPES (10 mM), $CaCl_2$ (2 mM), glucose (10 mM) and glycine (0.002 mM) for continuing 3 h (Jiang et al. 2010; Dai et al. 2019). Then, treated neurons were re-cultured in Neuronal Medium in standard conditions. Untreated hippocampal neurons for normal culture were used as the control.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Firstly, total RNA was extracted using RNAiso Plus Kit (Takara, Dalian, China) and used for cDNA synthesis using the Real Time One Step RT-PCR Kit (Takara) or using miRNA First Strand Synthesis Kit (Takara) following the protocols. cDNA was then used for qRT-PCR using a TB Green qRT-PCR Kit (Takara) under a CFX96 Touch PCR system (Bio-Rad, Hercules, CA, USA). β-actin or U6 was served as the internal control, and the method of $2^{-\Delta\Delta Ct}$ was used to calculate relative expression. The primer sequences were listed as below: circ_0003170, F: 5'-GGAGTGGGGT-TATTGGGTCA-3' and R: 5'- TGGCCAGGTAGTCA-GACAAG-3'; miR-421, F: 5'-TGCGGATCAACAGACAT-TAATTGGGC-3' and R: 5'-CCAGTGCAGGGTCCGAG-GT-3'; CCL2, F: 5'-AGCAGCAAGTGTCCCAAAGA-3' and R: 5'-TTTGCTTGTCCAGGTGGTCC-3'; β-actin, F: 5'- CCAAGGCCAACCGCGAGAAGATGAC-3' and R: 5'-AGGGTACATGGTGGTGCCGCCAGAC-3'; U6, F: 5'-GGAACGCTTCACGAATTTG-3' and R: 5'-ATTG-GAACGATACAGAGAAGATT-3'.

Bioinformatics analysis

Bioinformatics analysis was performed to predict the potential targets of circ_0003170 and miR-421. In brief, the target miRNAs of circ_0003170 were analyzed by Starbase (http://starbase.sysu.edu.cn/) and Circular RNA interactome

(https://circinteractome.nia.nih.gov/). The target mRNAs of miR-421 was analyzed by Starbase.

Dual-luciferase reporter assay

The targeting sites between miR-421 and circ_0003170 or CCL2 3'UTR were mutated to form mutant sequences of circ_0003170 or CCL2. Then the wild- and mutant-type of circ_0003170 sequence and CCL2 sequence were cloned into pmirGLO reporter plasmid (Promega, Madison, WI, USA), named as circ_0003170-WT, circ_0003170-MUT, CCL2-WT and CCL2-MUT. Human hippocampal neurons were transfected with miR-421 or miR-NC and circ_0003170-WT, circ_0003170-MUT, CCL2-WT or CCL2-MUT using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA). After maintaining for 48 h, the luciferase activity in cells was checked using the Dual-Luciferase Assay System (Promega).

RNA immunoprecipitation (RIP) assay

Magna RIP Kit (Millipore, Bedford, MA, USA) was utilized to conduct RIP assay to validate the interaction between miR-421 and circ_0003170 or CCL2 in line with the directions. Briefly, human hippocampal neurons were lysed, and cell lysate was mixed with RIP buffer with magnetic beads conjugated with Argonaute 2 antibody (anti-Ago2) or Immunoglobulin G antibody (anti-IgG; control). Afterwards, the RNA samples bound to beats were isolated, purified and used for qRT-PCR.

Cell transfection

For functional analysis, circ_0003170 knockdown or overexpression was accomplished using small interference RNA (siRNA) (si-circ_0003170#1, #2 and #3) or pcDNA overexpression vector (circ_0003170) (Genepharma, Shanghai, China), with siRNA random sequence or pcDNA vector as a negative control (si-NC or pcDNA). miR-421 mimic (miR-421) or miR-421 inhibitor (anti-miR-421) was used for miR-421 overexpression or inhibition (Ribobio, Guangzhou, China), with mimic negative control (miR-NC) or inhibitor negative control (anti-miR-NC) as control. CCL2 overexpression was conducted using pcDNA overexpression vector (CCL2) (Genepharma), also with pcDNA vector as control. According to the protocol's procedures, cell transfection was carried out using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA).

RNase R treatment

Total RNA was isolated and exposed to 2 U/μg RNase R (Epicentre, Madison, WI, USA) for 15 min at 37°C. Then, qRT-PCR was performed to check the expression of circ_0003170 and its linear mRNA STK24. The primers used of STK24 were: F, 5'-CCCGGCATGCAGAACCTAAA-3' and R, 5'-CCAAAGGAGCCCTTCCCAAT-3'.

Subcellular distribution

Nuclear RNA and cytoplasmic RNA were isolated using the Cytoplasmic & Nuclear RNA purification kit (Norgen Biotek, Thorold, Canada) according to the guidelines. The isolated RNA was used for qRT-PCR analysis to detect the abundance of circ_0003170 in subcellular parts. GAPDH or U6 was used as the control in cytoplasm and nucleus, respectively.

Cell counting kit-8 (CCK-8) assay

Cells with transfection were plated into 96-well plates at a density of 5000 cells/well. Cells were then incubated for 48 h, and 10 μl CCK-8 reagent was added into each well for another 2 h. The absorbance at 450 nm was monitored using a microplate reader (Bio-Rad) to observe cell viability.

Flow cytometry assay

For cell cycle analysis, after transfection, cells were collected, following by trypsinization, and resuspended in phosphate-buffered saline (PBS). Cells were next fixed in 70% ethanol at 4°C overnight and collected for staining using propidium iodide (PI) staining solution from a Cell cycle analysis kit (Beyotime, Shanghai, China) for 30 min at 37°C in the dark, followed by detection on a flow cytometer (BD Biosciences, San Jose, CA, USA).

For apoptosis analysis, cells were collected and resuspended in PBS. Then, cells were exposed to binding buffer and orderly stained with Annexin V-fluorescein isothiocyanate (FITC) and PI from a Cell apoptosis detection kit (Beyotime) according to the protocol, for 15 min at room temperature in the dark. The apoptotic cells were analyzed using a flow cytometer (BD Biosciences).

Detection of superoxide dismutase (SOD) and malondialdehyde (MDA)

The levels of SOD and MDA were detected using Total SOD Assay Kit (Beyotime) and Lipid Peroxidation MDA Assay Kit (Beyotime), respectively. All experimental procedures were conducted in line with the protocol.

Caspase-3 activity

The activity of caspase-3 was measured using a GreenNuc Caspase-3 Assay Kit (Beyotime) referring to the manufacturer's protocol. The result was monitored to detect optical density (OD) of caspase-3 activity at 405 nm.

Western blot

The expression of CCL2 at the protein level was detected by Western blot. In brief, total protein was extracted, quantified and separated by electrophoresis. The isolated protein was transferred into polyvinylidene fluoride (PVDF) membranes followed by blockage. The membranes were then exposed to the primary antibody against CCL2 (anti-CCL2; sc-32771; Santa Cruz Biotechnology, Santa Cruz, CA, USA) or β-actin (anti-β-actin; sc-47778; Santa Cruz Biotechnology) at 4°C overnight and subsequently exposed to the secondary antibody (sc-516102; Santa Cruz Biotechnology) at 37°C for 1.5 h. The protein plots were finally examined using an enhanced chemiluminescence kit (Beyotime). The original gels containing protein signals are shown in Figure S1 (see Supplementary material).

Statistical analysis

Data from at least three independent experiments were statistically analyzed using GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA, USA) and finally shown as the mean ± standard deviation (SD). In addition, differences between two groups or among multiple groups were analyzed by Student's t-test or analyses of variance with Tukey *post hoc* test. The correlation of expression levels between two groups was analyzed by Spearman's correlation test. p value ≤ 0.05 was regarded to be statistically significant.

Results

The expression of circ_0003170 was elevated, while the expression of miR-421 was declined in serum specimens from TLE patients and Mg^{2+}-free-induced human hippocampal neurons

QRT-PCR analysis was performed to ensure the expression of circ_0003170 and miR-421 in TLE serum specimens and cell models. Firstly, the formation of circ_0003170 was validated by RT-qPCR, and we found that circ_0003170 could be amplified by divergent primers from cDNA but not gDNA. In contrast, linear transcripts, including GAPDH and STK23 (parental mRNA of circ_0003170), could be amplified by convergent primers from both cDNA and gDNA (Fig. 1A). As shown in Figure 1B and C, the expression of circ_0003170 was significantly increased in serum
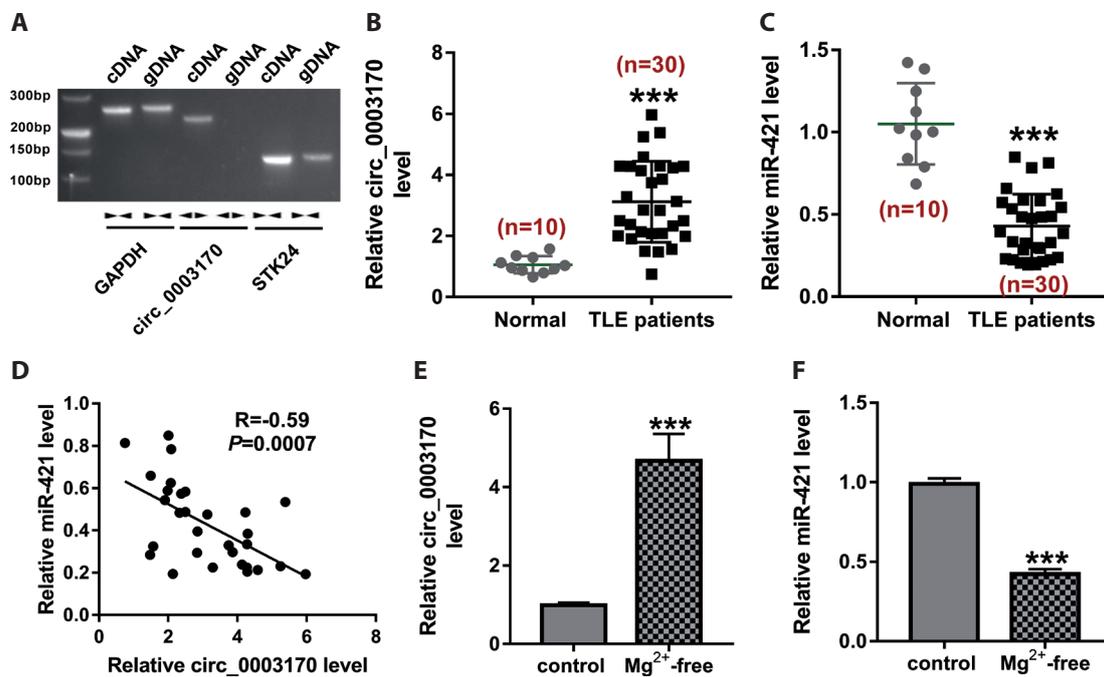

Figure 1. circ_0003170 was upregulated, while miR-421 was downregulated in temporal lobe epilepsy (TLE) serum specimens and Mg^{2+}-free-induced human hippocampal neurons. **A.** The formation of circ_0003170 was identified by divergent primers, using GAPDH and STK24 as linear control. **B, C.** The expression of circ_0003170 and miR-421 in serum specimens from TLE patients and normal subjects was detected using qRT-PCR. **D.** Spearman's correlation analysis revealed the correlation between miR-421 and circ_0003170 expression in TLE serum specimens. **E, F.** The expression of circ_0003170 and miR-421 in Mg^{2+}-free-induced human hippocampal neurons was detected using qRT-PCR. *** $p < 0.001$.

Figure 2. miR-421 was verified as a target of circ_0003170. **A.** The potential miRNAs targeted by circ_0003170 were predicted by Starbase and Circular RNA interactome. **B.** Dual-luciferase reporter assay examined the interaction between circ_0003170 and target miRNAs. **C.** The binding sites between circ_0003170 and miR-421. **D.** The efficiency of miR-421 overexpression was checked using qRT-PCR. **E, F.** The relationship between circ_0003170 and miR-421 was validated by dual-luciferase reporter assay and RIP assay. **G.** The efficiency of circ_0003170 overexpression and downregulation was checked using qRT-PCR. **H.** The expression of miR-421 in cells with circ_0003170 overexpression and downregulation was detected using qRT-PCR. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 3. circ_0003170 knockdown ameliorated Mg²⁺-free-induced injuries of human hippocampal neurons by targeting miR-421. **A.** The stability of circ_0003170 was examined using RNase R. **B.** The distribution of circ_0003170 in the cytoplasm and nucleus was checked using qRT-PCR. Mg²⁺-free-induced human hippocampal neurons were transfected with si-circ_0003170, si-NC, si-circ_0003170+anti-miR-421 or si-circ_0003170+anti-miR-NC. **C.** The expression of miR-421 was measured using QRT-PCR. **D.** Cell viability was monitored using CCK-8 assay. **E, F.** Cell cycle was monitored by flow cytometry assay. **G, H.** The levels of SOD and MDA were investigated using the corresponding detection kit. **I.** The activity of caspase-3 was also checked using a kit. **J, K.** Cell apoptosis was determined using flow cytometry assay. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

specimens from TLE patients compared with that from normal subjects, while the expression pattern of miR-421 was opposite to circ_0003170. Besides, the expression of miR-421 in TLE serum specimens was negatively correlated with the expression of circ_0003170 (Fig. 1D). As expected, circ_0003170 was notably upregulated in Mg^{2+}-free-induced hippocampal neurons compared to control, while miR-421 was downregulated in Mg^{2+}-free-induced hippocampal neurons (Fig. 1E,F). The dysregulation of circ_0003170 and miR-421 hinted that they might be involved in the development of TLE.

miR-421 was identified to be a target of circ_0003170

miR-421 as a target of circ_0003170 was predicted by both Starbase and Circular RNA interctome. Meanwhile, a total of ten miRNAs were simultaneously predicted by two bioinformatics tools (Fig. 2A). Ten miRNA mimics were cotransfected with circ_0003170 luciferase reporter plasmid in human hippocampal neurons, respectively, and the results showed that miR-421 mimic could reduce luciferase activity more effectively (Fig. 2B). Hence, miR-421 was chosen for further analyses. Besides, the target relationship between miR-421 and circ_0003170 was validated by dual-luciferase reporter assay and RIP assay. As shown in Fig. 2C, mutant-type sequence of circ_0003170 was designed according to its wild-type sequence. miR-421 mimic transfection strikingly promoted the expression of miR-421 in human hippocampal neurons (Fig. 2D) and diminished the luciferase activity in human hippocampal neurons transfected with circ_0003170-WT but not circ_0003170-MUT (Fig. 2E). RIP assay displayed that circ_0003170 and miR-421 could be significantly enriched in the RIP group with Anti-Ago2 compared to Anti-IgG (Fig. 2F). For circ_0003170 overexpression and knockdown efficiency analysis, the data showed that circ_0003170 expression was remarkably enhanced in cells transfected with circ_0003170 compared to pcDNA, and circ_0003170 expression was remarkably declined in cells transfected with si-circ_0003170 (#1, #2 and #3), suggesting that overexpression efficiency and knockdown efficiency are available (Fig. 2G). Moreover, the expression of miR-421 was reduced with circ_0003170 overexpression and strengthened with circ_0003170 knockdown (Fig. 2H). Collectively, miR-421 was a target of circ_0003170 and negatively regulated by circ_0003170.

circ_0003170 knockdown ameliorated Mg^{2+}-free-induced injuries of human hippocampal neurons by regulating miR-421

circ_0003170 was stably expressed in human hippocampal neurons due to its significant resistance to the digestion of RNase R relative to corresponding linear mRNA (Fig. 3A).

Subcellular distribution analysis showed that the abundance of circ_0003170 in the cytoplasm was significantly higher than that in the nucleus (Fig. 3B). For functional experiments, partial human hippocampal neurons treated with Mg^{2+}-free were transfected with si-circ_0003170 or si-NC, and partial human hippocampal neurons treated with Mg^{2+}-free were cotransfected with si-circ_0003170+anti-miR-421 or si-circ_0003170+anti-miR-NC. The expression of miR-421 inhibited in Mg^{2+}-free-induced cells was largely recovered in cells with the transfection of si-circ_0003170 but partly reduced in cells with the cotransfection of circ_0003170+anti-miR-421 (Fig. 3C). CCK-8 assay showed lower cell viability in Mg^{2+}-free-induced human hippocampal neurons, and the transfection of si-circ_0003170 notably recovered cell viability, which was lessened by the cotransfection of circ_0003170+anti-miR-421 (Fig. 3D). Flow cytometry cycle assay exhibited that Mg^{2+}-free-induced cell cycle arrest was relieved by the transfection of si-circ0003170 but promoted by the cotransfection of circ_0003170+anti-miR-421 (Fig. 3E,F). Besides, the level of SOD was weakened by Mg^{2+}-free, while the transfection of si-circ_0003170 improved the level of SOD, and the cotransfection of circ_0003170+anti-miR-421 impaired the level of SOD (Fig. 3G). The level of MDA was always opposite to the levels of SOD (Fig. 3H). Caspase-3 was an important indicator of apoptosis, and the activity of caspase-3 was significantly elevated in Mg^{2+}-free-induced human hippocampal neurons. However, circ_0003170 knockdown diminished caspase-3 activity, while combined circ_0003170 knockdown and miR-421 inhibition restored the activity of caspase-3 (Fig. 3I). The result was verified by flow cytometry apoptosis assay that introduced that apoptotic rate enhanced by Mg^{2+}-free was ameliorated by si-circ_0003170 transfection and partly recovered by circ_0003170+anti-miR-421 transfection (Fig. 3J,K). In short, circ_0003170 knockdown mediated miR-421 to ameliorate Mg^{2+}-free-induced human hippocampal neuron injuries.

miR-421 bound to CCL2 3'UTR, thus suppressing the expression of CCL2

We further used Starbase to analyze the potential target mRNAs of miR-421. There were several binding sites between miR-421 and CCL2 3'UTR, and the sequence of CCL2 3'UTR mutated at the miR-421 binding sites was assembled to conduct dual-luciferase reporter assay (Fig. 4A). Luciferase activity of human hippocampal neurons with cotransfection of miR-421 and CCL2-WT was significantly declined, while cotransfection of miR-421 and CCL2-MUT never reduced the luciferase activity in neurons (Fig. 4B). RIP assay also verified the interaction between CCL2 and miR-421 (Fig. 4C). The data from qRT-PCR and Western blot showed an increase of CCL2 expression in TLE serum

specimens and Mg^{2+}-free-induced human hippocampal neurons (Fig. 4D,E). Besides, miR-421 expression was negatively correlated with CCL2 mRNA expression in TLE serum specimens (Fig. 4F). Moreover, miR-421 transfection strikingly promoted the expression of miR-421 but weakened the expression of CCL2, while anti-miR-421 transfection strikingly reduced the expression of miR-421 but reinforced the expression of CCL2 (Fig. 4G,H). In conclusion, CCL2 was a target of miR-421, and the expression patterns of miR-421 and CCL2 were opposite in TLE.

miR-421 restoration ameliorated Mg^{2+}-free-induced injuries of human hippocampal neurons by downregulating CCL2

To monitor the function of miR-421 and CCL2, partial Mg^{2+}-free-induced human hippocampal neurons were transfected with miR-421, miR-NC, miR-421+CCL2 or miR-421+pcDNA. The expression of CCL2 was impaired in Mg^{2+}-free-induced cells transfected with miR-421 but recovered in Mg^{2+}-free-induced cells transfected with miR-421+CCL2 (Fig. 5A). miR-421 transfection promoted cell viability of human hippocampal neurons blocked by Mg^{2+}-free relative to miR-NC, while miR-421+CCL2 cotransfection impaired cell viability relative to miR-421+pcDNA (Fig. 5B). Besides, miR-421 overexpression lessened Mg^{2+}-free-induced cell cycle arrest relative to miR-NC, and miR-421+CCL2 transfection promoted cell cycle arrest relative to miR-421+pcDNA transfection (Fig. 5C). miR-421+CCL2 cotransfection blocked the level of SOD, which was induced by miR-421 transfection alone (Fig. 5D), while miR-421+CCL2 cotransfection strengthened the level of MDA which was inhibited by miR-421 transfection alone in Mg^{2+}-free-induced human hippocampal neurons (Fig. 5E). The activity of caspase-3 was suppressed by miR-421 transfection but recovered by miR-421+CCL2 cotransfection in Mg^{2+}-free-induced human hippocampal neurons (Fig. 5F). The apoptotic rate was also repressed in Mg^{2+}-free-induced cells transfected with miR-421 but improved in cells transfected with miR-421+CCL2 (Fig. 5G). All data suggested that miR-421 restoration lessened Mg^{2+}-free-induced injuries, which was reversed by CCL2 overexpression.

circ_0003170 sponged miR-421 to upregulate CCL2

The expression of circ_0003170 was positively correlated with CCL2 mRNA expression in TLE serum specimens
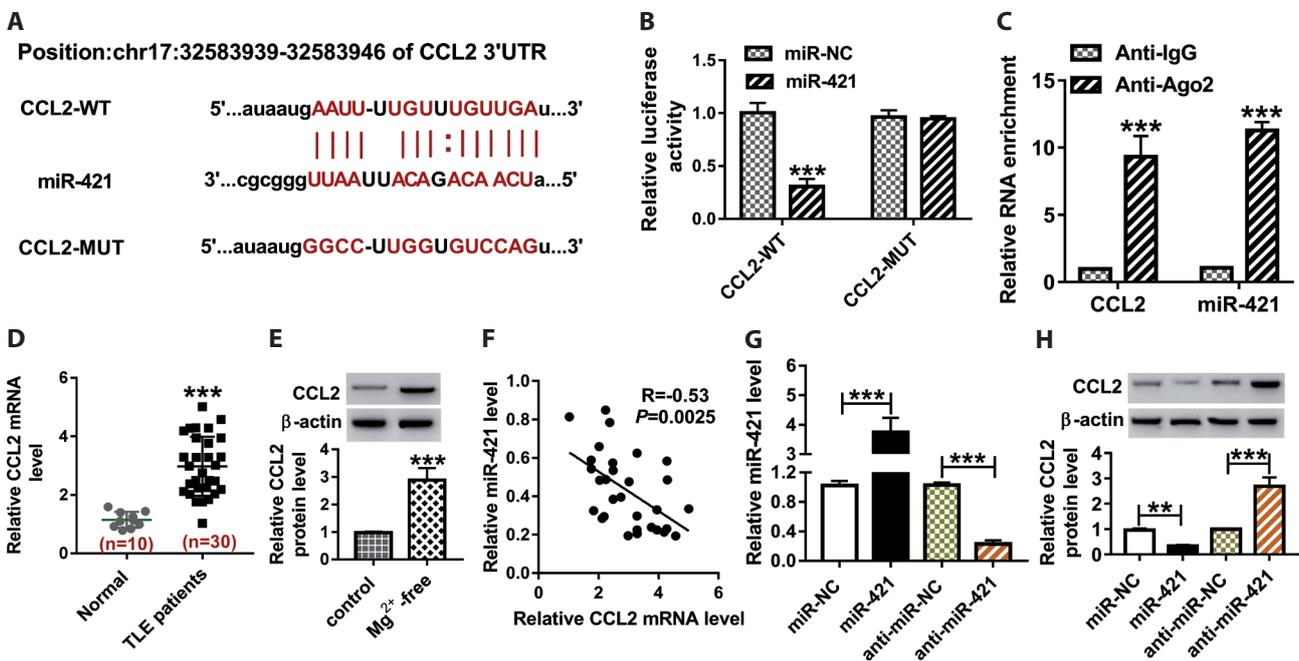
Figure 4. CCL2 was a target of miR-421. **A.** The binding sites between miR-421 and CCL2 3'UTR were analyzed by Starbase. **B, C.** The interaction between miR-421 and CCL2 was verified by dual-luciferase reporter assay and RIP assay. **D.** The expression of CCL2 in serum specimens from temporal lobe epilepsy (TLE) patients or normal subjects was measured using qRT-PCR. **E.** The expression of CCL2 in Mg^{2+}-free-induced human hippocampal neurons was measured using Western blot. **F.** The correlation between miR-421 expression and CCL2 expression in TLE serum was analyzed using Spearman's correlation analysis. **G.** The efficiency of miR-421 overexpression or inhibition was examined using qRT-PCR. **H.** The expression of CCL2 in cells with miR-421 overexpression or inhibition was detected using Western blot. ** $p < 0.01$, *** $p < 0.001$.
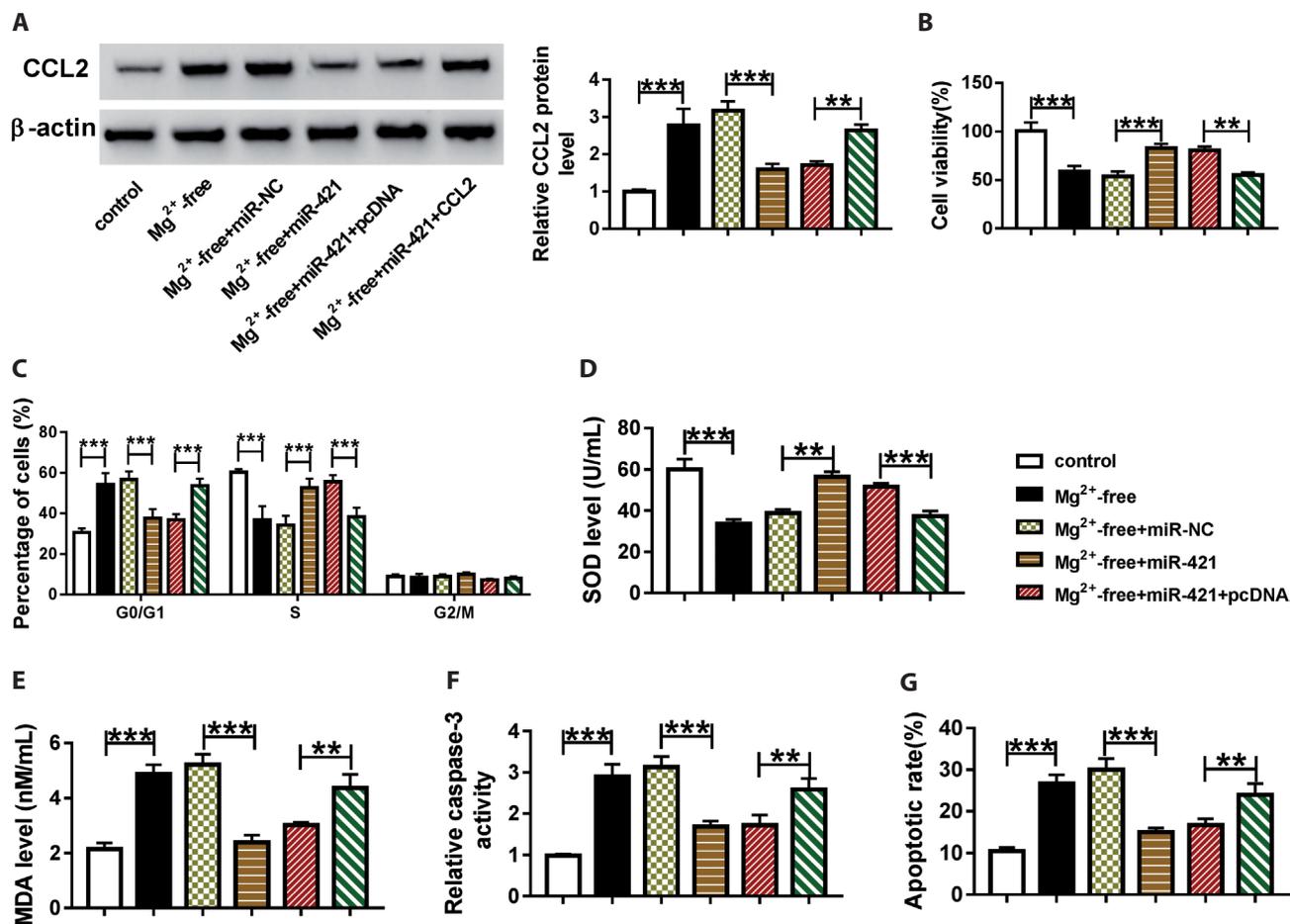
**Figure 5.** miR-421 restoration ameliorated Mg^{2+}-free-induced injuries of human hippocampal neurons by degrading CCL2. Mg^{2+}-free-induced human hippocampal neurons were transfected with miR-421, miR-NC, miR-421+CCL2 or miR-421+pcDNA. **A.** The expression of CCL2 in these cells was detected by Western blot. **B.** Cell viability was assessed using CCK-8 assay. **C.** Cell cycle was monitored by flow cytometry assay. **D–F.** The levels of SOD and MDA and the activity of caspase-3 were checked using the corresponding kits. **G.** Cell apoptosis was checked using flow cytometry assay. ** $p < 0.01$, *** $p < 0.001$.

(Fig. 6A). Moreover, the protein level of CCL2 was also significantly promoted in human hippocampal neurons transfected with circ_0003170 but decreased in cells transfected with circ_0003170+miR-421 (Fig. 6B). On the contrary, the expression level of CCL2 was lessened in cells transfected with si-circ_0003170 but recovered in cells transfected with si-circ_0003170+anti-miR-421 (Fig. 6C). The data implied that circ_0003170 regulated the expression of CCL2 by targeting miR-421.

CCL2 overexpression rescued the effects of circ_0003170 knockdown

Mg^{2+}-free-induced human hippocampal neurons were transfected with si-circ_0003170 alone or si-circ_0003170+CCL2. The protein level of CCL2 was decreased in Mg^{2+}-free-

induced human hippocampal neurons transfected with si-circ_0003170 but restored in cells transfected with si-circ_0003170+CCL2 (Fig. 7A). Cotransfection of si-circ_0003170+CCL2 diminished cell viability, which was promoted by si-circ_0003170 transfection alone in Mg^{2+}-free-induced human hippocampal neurons (Fig. 7B). Cotransfection of si-circ_0003170+CCL2 induced cell cycle arrest, which was lightened by si-circ_0003170 transfection alone (Fig. 7C). Besides, circ_0003170 knockdown-promoted SOD level and circ_0003170 knockdown-suppressed MDA level were overturned by combined CCL2 overexpression (Fig. 7D,E). In addition, caspase-3 activity and apoptotic rate were largely blocked in cells transfected with si-circ_0003170 while were heightened in cells cotransfected with si-circ_0003170+CCL2 (Fig. 7F,G). All data indicated that circ_0003170 knockdown-blocked human hippocampal
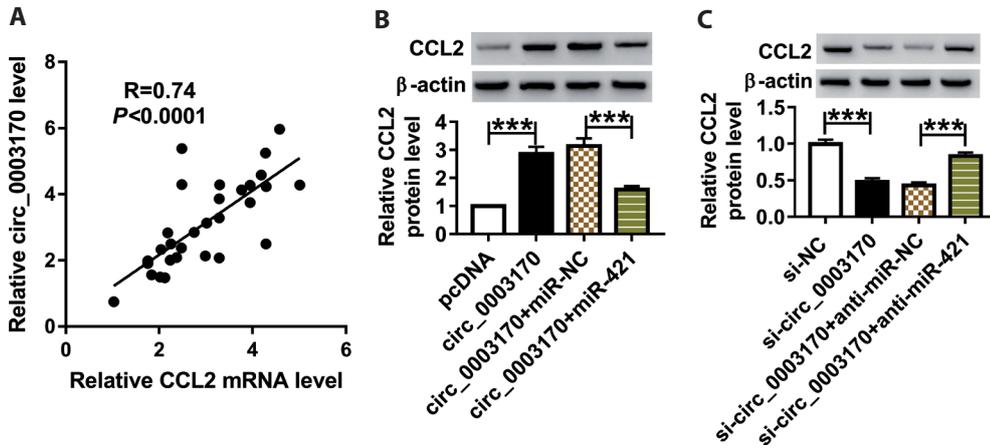
Figure 6. circ_0003170 activated the expression of CCL2 by targeting miR-421. **A.** The correlation between circ_0003170 expression and CCL2 expression in TLE serum was ascertained by Spearman's correlation analysis. **B.** The expression of CCL2 in human hippocampal neurons transfected with circ_0003170, pcDNA, circ_0003170+miR-421 or circ_0003170+miR-NC was detected by Western blot. **C.** The expression of CCL2 in human hippocampal neurons transfected with si-circ_0003170, si-NC, si-circ_0003170+anti-miR-421 or si-circ_0003170+anti-miR-NC was detected by Western blot. *** $p < 0.001$.
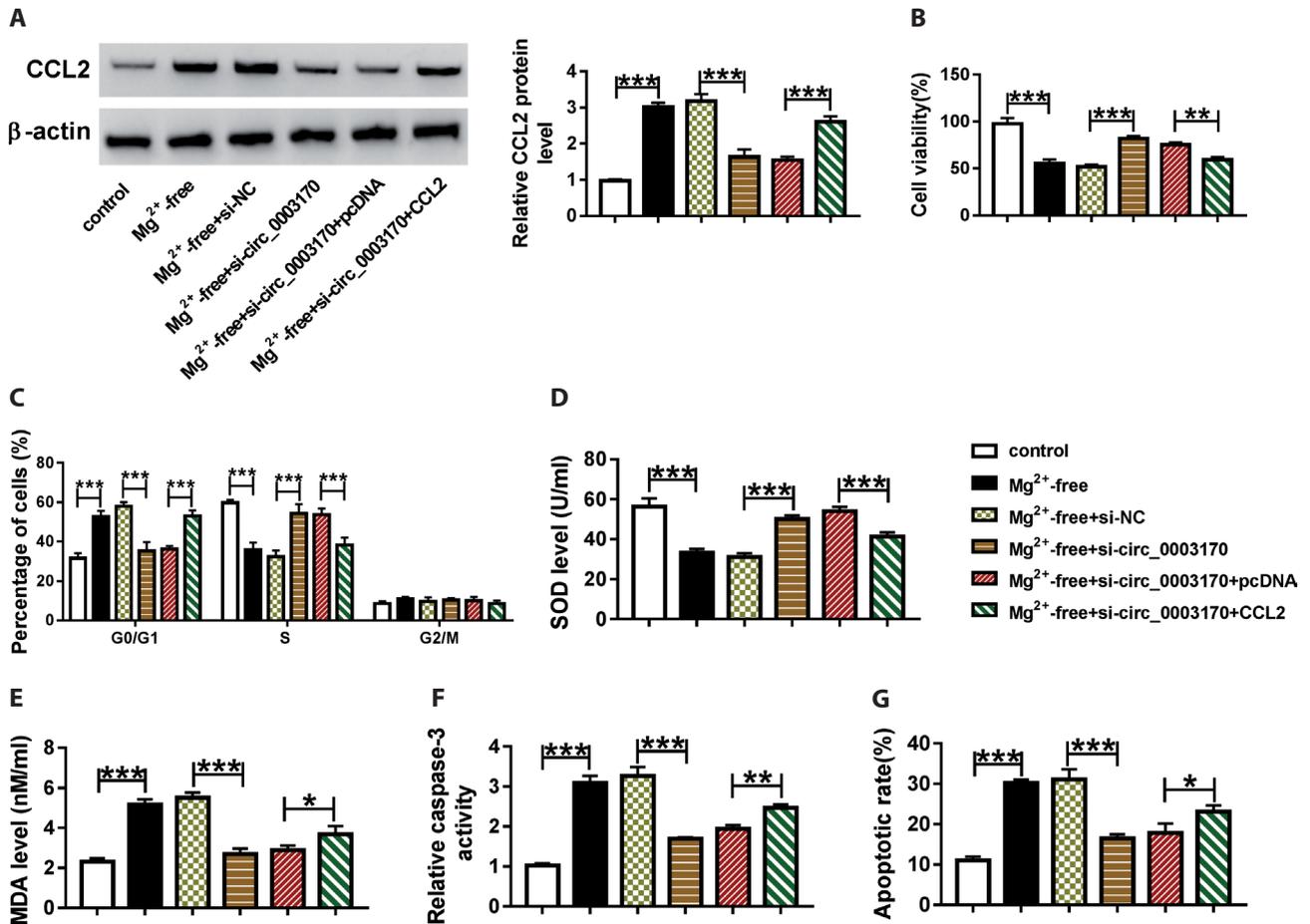
Figure 7. CCL2 overexpression rescued the effects of circ_0003170 knockdown in Mg^{2+}-free-induced human hippocampal neurons. Mg^{2+}-free-induced human hippocampal neurons were transfected with si-circ_0003170, si-NC, si-circ_0003170+CCL2 or si-circ_0003170+pcDNA. **A.** The expression of CCL2 was detected by Western blot. **B.** Cell viability was checked by CCK-8 assay. **C.** Cell cycle was monitored using flow cytometry assay. **D–F.** The levels of SOD and MDA and the activity of caspase-3 were determined by the corresponding kit. **G.** Cell apoptosis was evaluated by flow cytometry. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

neuron injuries induced by Mg^{2+}-free, including cell viability inhibition, cell cycle arrest, oxidative stress and apoptosis promotion, were improved by CCL2 overexpression.

Discussion

The properties of circRNAs make them promising biomarkers for CNS diseases. For example, the expression of circRNAs in CNS is abundant (Rybak-Wolf et al. 2015), and circRNAs are more stably expressed in CNS (Chen and Shuman 2016). Thus, identifying the expression alteration and functional role of circRNAs in CNS diseases, such as epilepsy, may provide novel diagnostic tools and therapeutic targets. Regrettably, circRNAs are poorly investigated in epilepsy. A previous study introduced that circ_0067835 was strikingly downregulated in plasma and tissues of TLE patients, and overexpression of circ_0067835 suppressed refractory epilepsy progression *via* activating the expression of FOXO3a by targeting miR-155 (Gong et al. 2018). Besides, high-throughput sequencing technology screened numerous differently expressed circRNAs in TLE specimens, which provided several epilepsy-specific circRNAs (Gong et al. 2018; Li et al. 2018). Here, to further understand the pathomechanism of epilepsy, we functionally investigated a poorly studied circRNA, circ_0003170, which was identified to be abundantly expressed in TLE samples (Li et al. 2018). Consistent with the data from the previous study, our results showed that circ_0003170 was aberrantly upregulated in TLE serum specimens and cell models. Yuan et al. utilized Mg^{2+}-free-induced epilepsy cell model to investigate the function of miR-132 on epileptogenesis (Yuan et al. 2016), and Geng et al. (2018) also used Mg^{2+}-free physiological solution to induce epileptiform activity and explored the function of lncRNA UCA1 in this cell model. Similarly, in Mg^{2+}-free-induced human hippocampal neurons, we noted that knockdown of circ_0003170 ameliorated Mg^{2+}-free-induced cell cycle arrest, cell apoptosis and oxidative stress of human hippocampal neurons, thus protecting the normal functions of hippocampal neurons. Mechanically, we validated that miR-421 was a target of circ_0003170.

Previous research explored the role of miR-421 in epilepsy mice model and unclosed that miR-421 expression was weakened in mice model, and miR-421 enrichment blocked apoptosis and autophagy and promoted proliferation of mouse hippocampal neurons (Wen et al. 2018). In line with the data, our results discovered that the expression of miR-421 was prominently declined in TLE serum specimens and Mg^{2+}-free-induced hippocampal neurons. In function, miR-421 restoration relieved Mg^{2+}-free-induced neuron cell cycle arrest, cell apoptosis and oxidative stress, and miR-421 inhibition counteracted the effects of circ_0003170 knockdown. These data suggested that miR-421 prevented the progression of epilepsy.

Further mechanism analysis disclosed that miR-421 directly bound to CCL2. CCL2 was acknowledged as a pro-epileptogenic factor (Korotkov et al. 2020), and numerous studies documented that CCL2 enhanced epileptic effects by mediating partial or systemic inflammation (Cerri et al. 2016). Besides, CCL2 was also reported to be a target of miR-206, and enforced expression of CCL2 abolished the effects of miR-206 and recovered epilepsy-induced brain injuries (Wu et al. 2019). Here, we reported that CCL2 was a target of miR-421, and the abundance of CCL2 was aberrantly enhanced in TLE serum specimens and Mg^{2+}-free-induced neurons. Functionally, CCL2 overexpression restored Mg^{2+}-free-induced neuron cell cycle arrest, cell apoptosis and oxidative stress, which were impaired by miR-421 enrichment. These functions of CCL2 were accordant with the previous findings. More importantly, circ_0003170 could indirectly regulate the expression of CCL2 by targeting miR-421. Functional analysis noticed that the blocked cell cycle arrest, cell apoptosis and oxidative stress caused by circ_0003170 knockdown were rescued by CCL2 overexpression, supporting the view that CCL2 was a pro-epileptogenic factor.

Conclusion

Taken together, the present study monitored that the expression of circ_0003170 and CCL2 was increased while the expression of miR-421 was decreased in TLE serum specimens and Mg^{2+}-free-induced human hippocampal neurons. circ_0003170 triggered neuron injuries, including cell cycle arrest, apoptosis and oxidative stress, partly by regulating the miR-421/CCL2 axis. Our data supported that circ_0003170 participated in the development of epilepsy and provided new vision into the molecular mechanism of epilepsy progression.

Conflict of interest. The authors declare that they have no financial conflicts of interest.

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circ_0003170 aggravates human hippocampal neuron injuries by regulating the miR-421/CCL2 axis in cells models of epilepsy

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Supplementary Figures
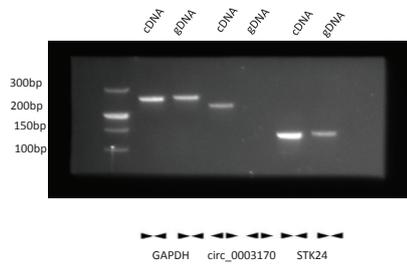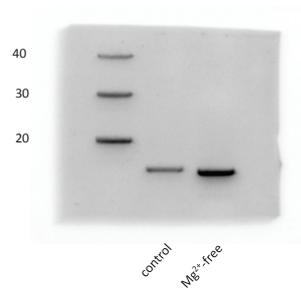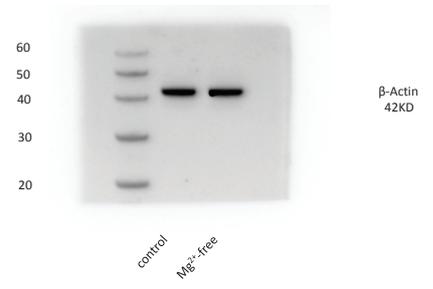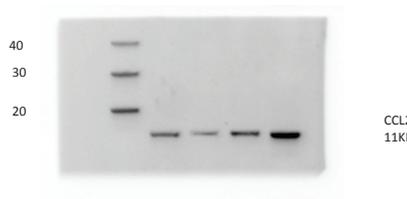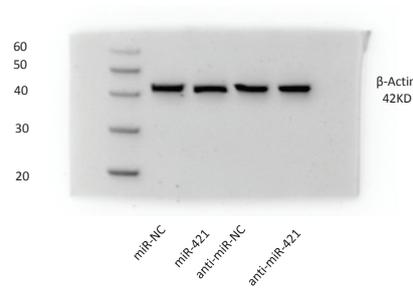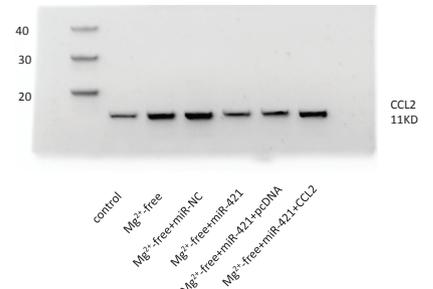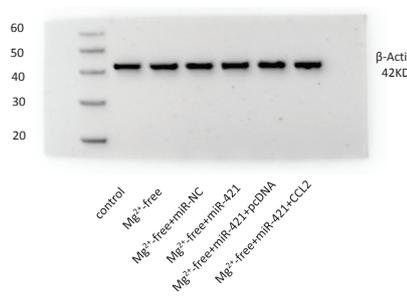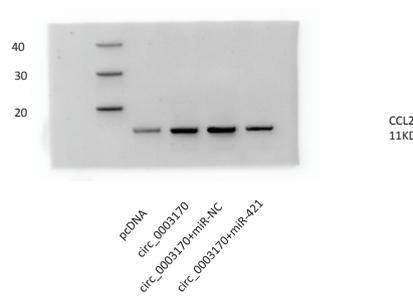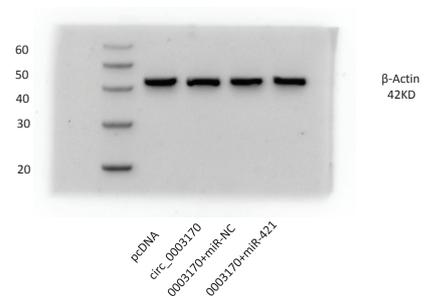
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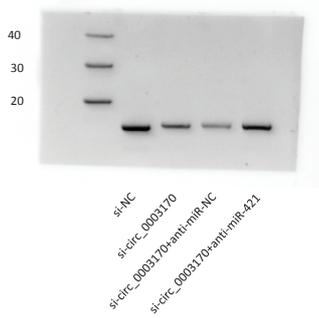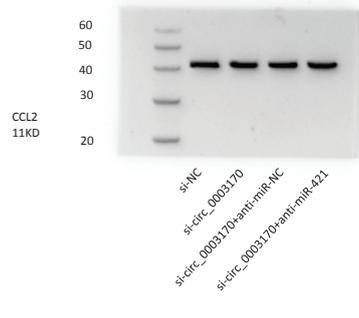
Figure 6C

Figure 7A
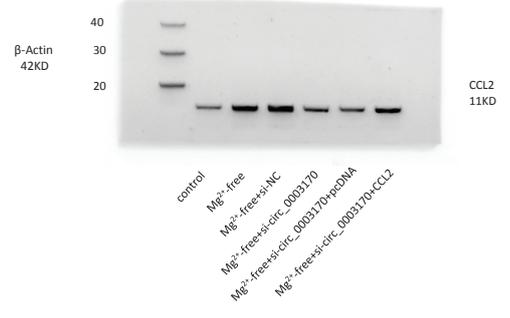
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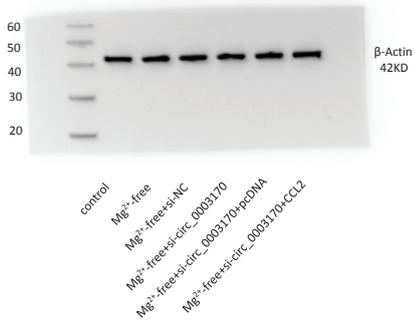
Figure S1. Original Western blots of investigated proteins (blots corresponds to the numbering of figures in the main text).