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Original Article

MicroRNA-137 inhibits apoptosis of neuron cells in injured spinal cord by targeting calpain 2

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Abstract: This study is aimed to investigate the therapeutic effect of MicroRNA-137 (miR-137) on the apoptosis of neuron cells in injured spinal cord and further identify the possible mechanism in a rat spinal cord injury (SCI) model. In present study, a total of 30 Sprague-Dawley (SD) rats were assigned randomly to 3 groups: control group, SCI + agomir-NC group and SCI + agomir-137 group (n = 10 per group). Rats of the three groups received injection of agomir-137, agomir-NC and saline at T10 vertebrae respectively, 1 day after which rats of SCI + agomir-137 group and SCI + agomir-NC group were treated with SCI surgery. Both mRNA and the protein levels of miR-137, calpain 2 and cleaved caspase 3 were examined before and after the surgery. Basso-Beattie-Bresnahan scoring was done. Luciferase activity was measured to verify the miR-137 target site in the 3'-UTR of calpain 2 and apoptosis rate was determined by flow cytometry. Overexpression of miR-137 promotes locomotor recovery and inhibits cell apoptosis in SCI rats. Besides, overexpression of miR-137 down-regulated the apoptotic markers calpain 2 and caspase 3 in both gene and protein level. We further predicted a potential target site of miR-137 in the 3'UTR of apoptotic gene calpain 2 and validated that miR-137 can directly target onto the 3'UTR site of calpain 2 and lead to calpain 2 down-regulation at transcriptional level. In conclusion, our results revealed the positive regulation functions of miR-137 in SCI-induced apoptosis by down-regulating calpain 2 and might provide an opportunity for development of novel therapies of spinal cord injury.

Keywords: miR-137, spinal cord injury, apoptosis, calpain 2

Introduction

Spinal cord injury (SCI) is a devastating pathology which associated with life-long disabilities. Reported annual incidence of SCI is about 15-83 per million people worldwide and most of these patients sustain their injury at an average age of 33 [1-3], causing an enormous impact on society and economy globally. The SCI usually evolves through two phases: an unexpected primary injury and a following deteriorative secondary injury [4, 5]. In the secondary injury SCI phases, complications including microvasculature alterations, oxidative damage and biochemical disturbances occur that lead to inflammatory response and cell death, resulting in a worse neurological outcome [6, 7]. Several reports suggested that apoptosis is involved in this secondary SCI progress, causing progressive degeneration of the spinal cord [8-10]. However, till now, the specific cell death mechanism for the secondary injury remains unclear.

In the recent years, substantial studies focused on the relationship between genes expression alteration and the pathogenesis of secondary SCI, aiming to limit the evolution of secondary and develop novel neuroprotective measures [11-14]. Lately, micro RNA-137 (miR-137) was found down-regulated in the injured spinal cords of SCI-treated rats which draw us attention [15]. As miR-137 has been reported plays a positive regulatory role in suppressing neuroblastoma cancer, colorectal cancer and human glioma [16-19]. Here, we hypothesized that miR-137 might also mediate in SCI. By using TargetScan and starBase online platform (www.targetscan.org; http://starbase.sysu.edu.cn/), we found that there might be a potential target site of miR-137 in the 3'UTR of apoptotic gene calpain 2. Yet, the levels and pattern of miR-

137 expression in the spinal cord and how miR-137 functions in apoptosis of neuron cells in injured spinal cord are still unknown and worth studying.

In this study, we further study the functional role of miR-137 in apoptosis of neuron cells in SCI-treated rat model. Both mRNA and protein expression of miRNA-17 and apoptotic markers like calpain 2 and caspase 3 were investigated, and apoptosis of spinal cord neurons were accessed both *in vivo* and *in vitro*. Our results revealed the positive regulation functions of miR-137 in injured spinal cord and might provide an opportunity for development of new therapies for SCI pathology.

Materials and methods

Establishment of SCI model

A total of 30 male Sprague-Dawley rats (Animal Center of Chinese Academy of Sciences, China) were maintained in a specific pathogen free facility under a 12-h dark/light cycle and then assigned randomly to three groups: SCI + agomir-137 group (n = 10), SCI + agomir-NC group (n = 10) and sham control group (n = 10). SCI surgery or sham operation was performed using a modified Allen's method as previously described [20, 21]. Chemically modified agomir were used for miR-137 overexpression in vivo. The day before the SCI surgery, rats of SCI + agomir-137 group, SCI + agomir-NC group and sham control group received injection of agomir-137, agomir-NC and saline at T10 vertebrae, respectively.

Quantitative RT-PCR (QRT-PCR)

Total RNA was extracted from the T10 vertebrae tissues using TRIzol Reagent (Invitrogen, USA) according to the manufacturer's instructions. Purified RNA (500 ng) was reverse transcribed into cDNA using the Prime Script RT (Takara, Janpan). QRT-PCR was performed using Sybr Green (Takara) with the ABI 7300 HT Sequence Detection system (Applied Biosystems), and relative gene expression was calculated via a $2^{-\Delta\Delta Ct}$ method.

Behavioral test

Motor coordination and integration capabilities of rats in each group were evaluated with Basso-Beattie-Bresnahan (BBB) [22, 23] score 28 days after the SCI surgery.

Quantification of cell apoptosis in vivo

DNA fragmentation *in vivo* was detected using TdT mediated dUTP nick end labeling (TUNEL) techniques [24]. Cells isolated from the T10 vertebrae were fixed with paraformaldehyde and then stained by TUNEL Apoptosis Asssy kit (Yeasen biotechnology, China) according to the manufacturer's instructions. Images were taken under a × 200 fluorescence microscope. The bright green labeled dots were defined as TUNEL-positive or positive-apoptosis cells. For quantification of cell apoptosis, numbers of apoptosis cells in each view were counted.

Western blotting assay

Total protein (~50 mg) extracted from the T10 vertebrae tissues was prepared at preconcerted time points. Protein samples (~25 ug) were separated by SDS-PAGE gel subsequently transferred on to PVDF membranes. After blocking, the blots were incubated with primary antibodies at 4°C overnight. Antibodies against calpain 2 (Cell Signaling), cleaved caspase 3 (Cell Signaling), procasapase 3 (Cell Signaling), β-actin (Sigma-Aldrich, USA) and β-actin (Sigma-Aldrich) were used. All the western bands (n = 3) were detected using ECL and also quantified by densitometry.

Luciferase reporter assay

To test the direct binding of miR-137 to the target gene, calpain 2, a luciferase reporter assay was performed as previously described [25, 26]. Briefly, the 3'UTR fragment of calpain 2 was amplified and inserted into pMIR-Ctrl reporter plasmids, and plasmids pMIR-Ctrl, calpain 2-UTR and calpain 2-UTR-mut were constructed. HEK 293T cells were planted into 24-well plates, and cells in each well were co-transfected with miR-24 mimics or negative control, pMIR-Ctrl, calpain 2-UTR and calpain 2-UTR-mut. 24 h after the transfection, luciferase activity of the cells were assayed using the Dual luciferase assay kit (Promega, USA). The data were normalized to Renilla luciferase activities.

Quantification of cell apoptosis in vitro

Spinal cord tissues of neonatal rats were dissected, dissociated by papain and primary spinal cord neurons were seeded into 12 well cluster with serum-free culture medium after differential adherence. Then spinal cord neurons

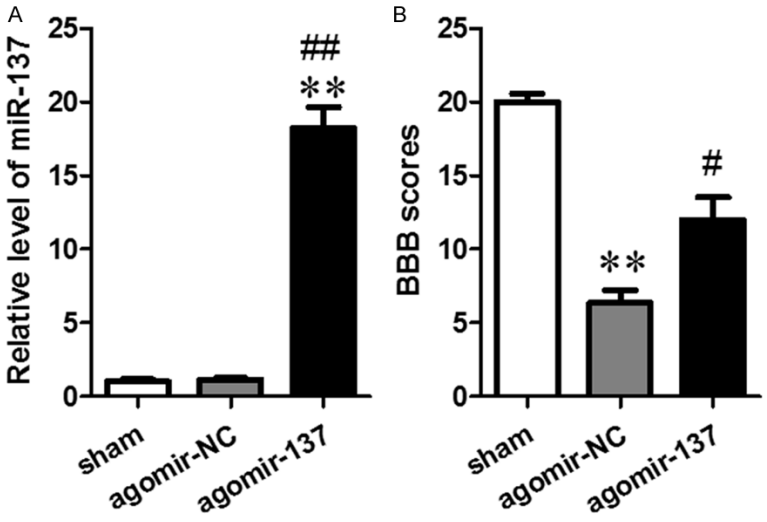

Figure 1. MiR-137 was down-regulated in SCI model. A: QRT-PCR analysis of miR-137 expression in T10 vertebrae tissue of agomir group, agomir-137 group and sham control group rats right before the SCI surgery. B: BBB scoring of SCI + agomir-NC group, SCI + agomir-137 group and sham control group rats 28 days after the SCI surgery. Means ± SD are shown. **P < 0.01 vs. sham control group; #P < 0.05, ##P < 0.01 vs. SCI + agomir-NC group.

were transfected with miR-137 mimics or negative control oligos (NC), and 24 hours later, 1 mM monosodium glutamate (MSG) was added to induce injury. The apoptotic rates of the cell apoptosis was analyzed by FACScan with PI/Annexin V-FITC staining. The cell apoptotic rates of the spinal cord neurons, *in vitro*, were measured using a PI/Annexin V-FITC kit (Beckman Coulter, USA) by FACScan flow cytometer (Beckman Coulter, USA) as the manual description. Spinal cord neurons treated with Entranster™ R4000 and subjected to the same protocol were taken as control.

Results

Overexpression of miR-137 promotes locomotor recovery in SCI rats

To explore a potential role of miR-137 in SCI, we first overexpressed miR-137 via miR-137 agomir in rats. Rats of SCI + agomir-137 group, SCI + agomir-NC group and sham control group received injection of agomir-137, agomir-NC and saline at T10 vertebrae respectively, on the day before the SCI surgery. 24 h later, right before the SCI surgery, RNA expression level of miR-137 in the T10 vertebrae tissue was measured. As expected, samples from SCI + agomir-137 group presented a dramatic rises of miR-137 (**Figure 1A**). We next assessed the locomotion of these three group rats 28 days after the SCI surgery, by BBB scoring. The sham control group rats had scores of 20-21 and were able to walk normally, while those in the SCI + agomir-NC group had scores of about 7, showing obvious deficits in stability (**Figure 1B**). Interestingly, BBB scores of SCI + agomir-137 group was 12-13, significantly higher than for the SCI + agomir-NC group; and rats of SCI + agomir-137 group showed only mild injury. These results indicated that overexpression of miR-137 improves motor functioning and promotes locomotor recovery in rats after SCI.

MiR-137 inhibits apoptosis of neuron cells in SCI rats

We next investigated the effect of miR-137 on cell death *in vivo* by TUNEL staining with T10 vertebrae tissue obtained 7 days after the SCI surgery. We found that, after the injury a high ratio of the neuron cells became TUNEL-positive; whereas with the overexpression of miR-137, samples of SCI + agomir-137 group showed a decrease in the amount of TUNEL-positive cells (**Figure 2A**). We counted the TUNEL-positive cells in three different group, then further confirmed that the number of TUNEL-positive cells was significantly lower in the SCI + agomir-137 group comparing to that in the SCI + agomir-NC group (**Figure 2B**). Besides, the RNA expression levels of miR-137 and apoptotic markers calpain 2 and caspase 3 in T10 vertebrae tissues were examined. 7 days after the SCI surgery, the miR-137 expression level in the SCI + agomir-NC group was significantly lower than that in the sham control group, but the miR-137 expression level in SCI + agomir-137 group was much higher than those in the SCI + agomir-NC group and sham control group (**Figure 2C**). However, the calpain 2 expression level in the SCI + agomir-NC group was higher than that in the sham control group, whereas the calpain 2 expression level in the SCI + agomir-137 group was obviously lower than those in the SCI + agomir-NC group and

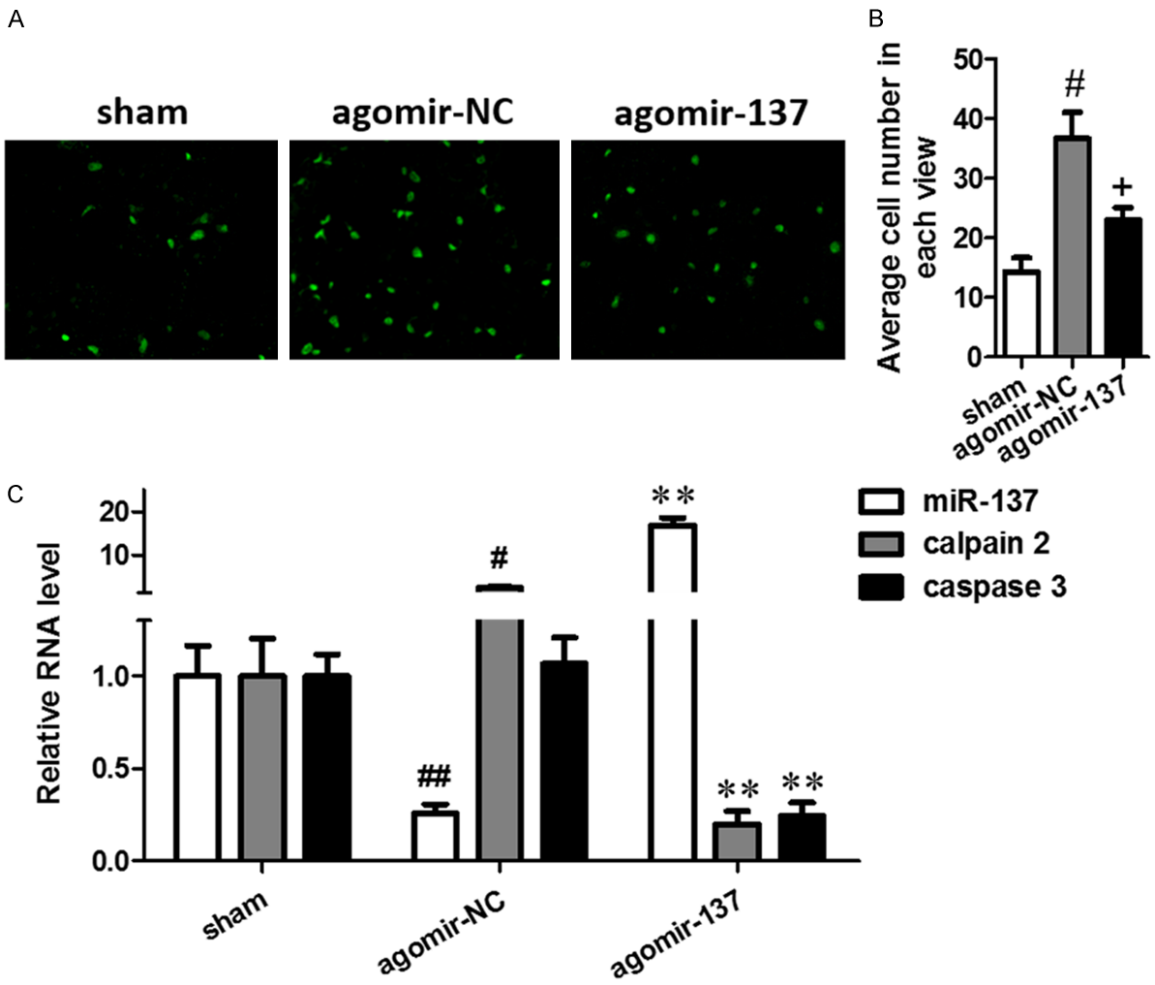

Figure 2. MiR-137 inhibits apoptosis of neuron cells in SCI rats. A: Cell apoptosis in T10 vertebrae tissue measured by TUNEL assay 7 days after the SCI surgery. B: Average number of apoptotic cells in each view was counted (200 ×). C: RT-PCR analysis of miR-137, calpain 2 and caspase 3 expression in T10 vertebrae tissue 7 days after the SCI surgery. Means ± SD are shown. #P < 0.05, ##P < 0.01 vs. sham control group; +P < 0.05 vs. SCI + agomir-NC group; **P < 0.01 vs. SCI + agomir-NC group or sham control group.

sham control group. These results suggested a negative correlation between miR-137 and apoptotic markers calpain 2 and caspase 3 in RNA expression level.

Overexpression of miR-137 reduces calpain 2 expression at transcriptional level in SCI rats

Consist with gene expression results, western blots showed that the protein levels of apoptotic markers calpain 2 as well as cleaved caspase 3 and procasapase 3 in T10 vertebrae tissue were also down-regulated in the SCI + agomir-137 group, comparing to those in the SCI + agomir-NC group (**Figure 3A**). In the bar graph in **Figure 3B**, we can clearly tell a sharp rise in the protein level of calpain 2 and cleaved caspase 3 after the SCI surgery. However, with overexpression of miR-137 in the SCI + agomir-137 group, we observed a significant reduction in all the three apoptotic markers including calpain 2, cleaved caspase 3 and procasapase 3. Western blotting results showed a negative correlation between miR-137 and these findings raise the possibility that calpain 2 might be a direct target of miR-137 in cell apoptosis in SCI rats.

Prediction and validation of calpain 2 as the target of miR-137

Using two publicly available online algorithms platform (TargetScan and starBase), miR-137 was identified as a candidate miRNA that could

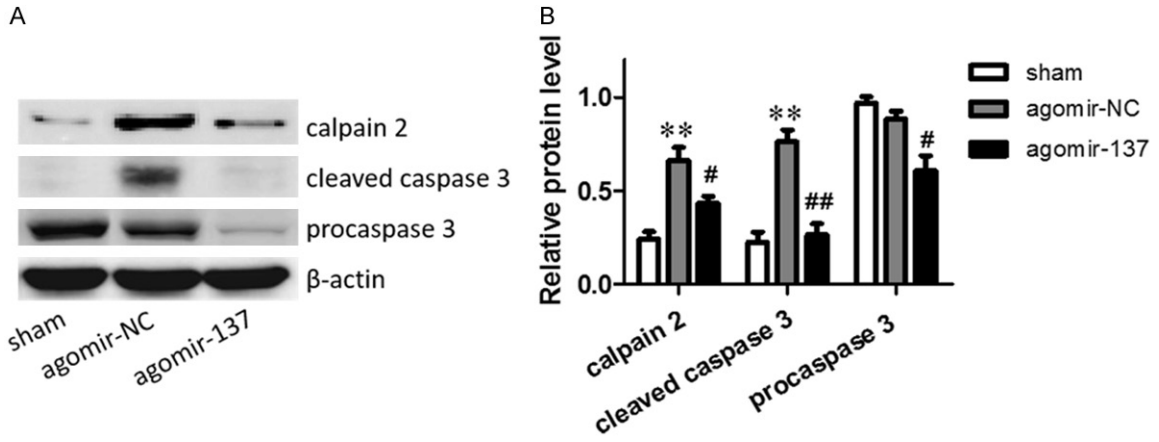

Figure 3. Overexpression of miR-137 reduces calpain 2 expression at transcriptional level in SCI rats. A: Western analysis of calpain 2, cleaved caspase 3 and procasapase 3 expression in T10 vertebrae tissue 7 days after the SCI surgery (n = 3). β-actin was used as a loading control. B: The optical density analysis of calpain 2, cleaved caspase 3 and procasapase 3 protein. **P < 0.01 vs. sham control group; #P < 0.05, ##P < 0.01 vs. SCI + agomir-NC group.

target the 3'UTR of calpain 2. Hypothesized duplexes formed by the interactions between 3'UTR binding site of calpain 2 and miR-137 were shown (**Figure 4A**). Luciferase reporter assay was performed, and results showed that the firefly luciferase reporter activity is significantly reduced in calpain 2-UTR vector compared with calpain 2-UTR-mut (**Figure 4B**), proving that the negative regulatory effect of miR-137 on calpain 2 expression was directly mediated through the binding of miR-137 to the predicted site in the 3'UTR of the calpain 2 mRNA. We further evaluated the anti-apoptotic role of miR-137 *in vitro.* Primary spinal cord neurons were transfected with miR-137 mimics or negative control oligos (NC), and 24 hours later, 1 mM monosodium glutamate (MSG) was added to induce injury, and then the amount of apoptotic cell was quantified with Annexin V-FITC/PI double-labeled flow cytometry. Compared with the controls, with overexpressed miR-137, percentage of viable cells (Annexin V negative and PI negative) increased and the percentage of apoptotic cells (Annexin V positive and PI negative) decreased significantly (**Figure 4C** and **4D**). Similarly to the *in vivo* situation, the *in vitro* protein levels of apoptotic makers calpain 2 as well as cleaved caspase 3 and procasapase 3 in spinal cord neurons transfected with miR-137 mimics were all down-regulated as well (**Figure 4E** and **4F**). Taken together, our findings demonstrated that miR-137 can directly target onto the 3'UTR site of calpain 2 and lead to calpain 2 down-regulation at transcriptional level, playing a prosurvival or anti-apoptotic role in SCI.

Discussion

The secondary SCI often carries a poor prognosis, and with its unclear underlying mechanisms, it remains challenging for us in developing effective treatments for SCI. Spinal cord neurons in the secondary SCI phase undergo apoptosis, resulting in progressive degeneration, loss of the tissue in the spinal cord and permanent neurological disability [9, 27]. Certain studies indicated that the success of SCI treatment depends on blocking the secondary SCI progression, and therefore inhibit the neuronal apoptosis may be a therapeutic strategy of SCI [28-30]. MiRNAs might be attractive candidates for the therapeutic strategy as they mediate gene expression at the posttranscriptional level and lead to translational inhibition or sometimes degradation [15, 31]. Previous studies suggested that miRNAs up-regulated post SCI (such as miR-145, miR-214 and miR206 ect.) might present pro-apoptotic effects, whereas miRNAs down-regulated post SCI (such as miR-124, miR-235-3p and miR-137 ect.) are more likely to be anti-apoptotic [15, 32]. Nonetheless, the primordial mechanism of miRNAs on SCI have not yet been well understood.

In this study, for the first time, we identified the functional role of miR-137 in the SCI system. Our data showed that overexpressing miR-137 effectively promotes locomotor recovery in SCI rats. This recovery was associated with the inhibition of neuron apoptosis *in vivo.* We then

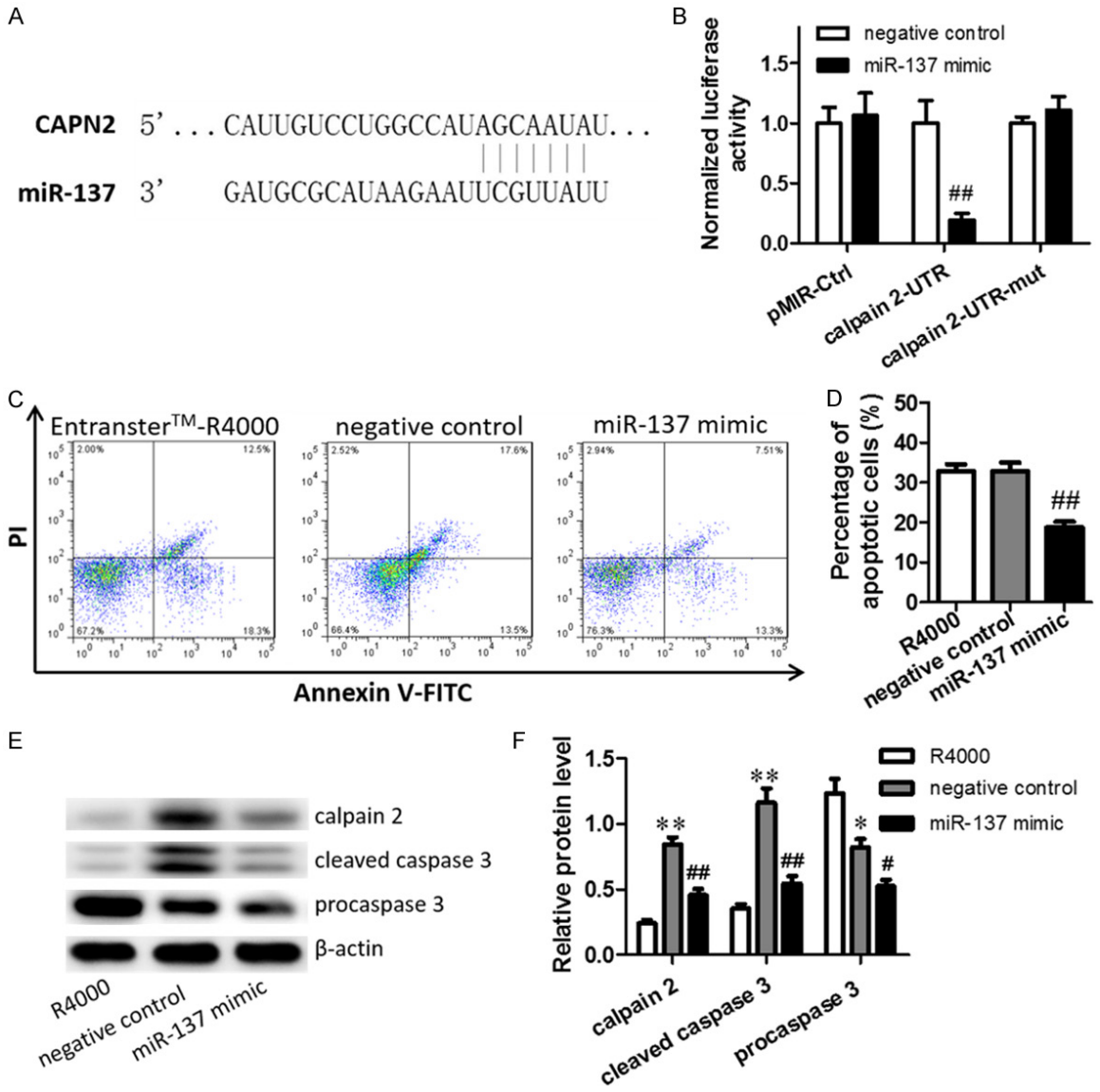

Figure 4. Prediction and validation of calpain 2 as the target of miR-137. A: The predicted structure of the base-paired hybrid between 3'UTR binding site of calpain 2 and miR-137. B: The luciferase activity of reporter plasmids pMIR-Ctrl, calpain 2-UTR and calpain 2-UTR-mut in spinal cord neurons. The data were normalized to Renilla luciferase activities. C: Cell apoptosis analyzed by FACScan with PI/Annexin V-FITC staining. D: Percentage of apoptotic cells, taking Entranster™ R4000-treated spinal cord neurons as control. E: Western analysis of calpain 2, cleaved caspase 3 and procasapase 3 expression in spinal cord neurons transfected with miR-137 mimics, NC oligos or treated with Entranster™ R4000. β-actin was used as a loading control. F: The optical density analysis of calpain 2, cleaved caspase 3 and procasapase 3 protein. *P < 0.05, **P < 0.01 vs. Entranster™-R4000 group; #P < 0.05, ##P < 0.01 vs. negative control group.

identified several apopotic markers calpain 2 and caspase 3 were in negative correlation with miR-137 in SCI rats. Calpain 2 and caspase 3 are proteases that could degrade the cytoskeletal proteins, leading to cellular degradation and death [33-35]. Accordingly, these proteases will be notably activated after SCI in spinal cord neurons [36]. Our findings showed that overexpression of miR-137 significantly reduce these proteases at transcriptional level in SCI rats. With bioinformatic analysis, we predicted a miR-137 target site on 3'UTR site of calpain 2, and mechanistically, we successfully validated hypothesis through luciferase assay and confirmed that miR-137 inhibit SCI-induced apoptosis by down-regulating calpain 2.

The protective efficacy of miR-137 has previously been demonstrated *in vitro* and *in vivo* in cardiac diseases by inhibiting cardiomyocyte apoptosis and suppressing various cancers [37, 38]. In this study, we extend our understanding of the positive regulation functions of miR-137 in SCI system that it inhibits SCI-induced apoptosis by targeting on calpain 2. Our results might provide an opportunity to ameliorate secondary SCI and develop novel therapies of spinal cord injury. Nonetheless, whether miR-137 will function to affect the other aspects of SCI such as the endogenous repair system or whether there are some other targets of miR-137 in SCI, remain further validation.

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Disclosure of conflict of interest

None.

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