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Original Article

Knockdown of miR-125-3p protects against oxidative stress in PC-12 cells by targeting MEF2

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Abstract: Aim: Spinal cord injury (SCI) is a devastating and common neurological disorder which causes local oxidative damage. The study aimed to investigate the underlying role of miR-125-3p mediated by expression of myocyte enhancer factor-2 (MEF2) in oxidative stress induced during spinal cord injury. Methods: Rat derived pheochromocytoma of adrenal medulla cells (PC-12) were cultured in plates and H_2O_2 was used as stimulus. PC-12 cells were transfected with miR-125-3p mimics to facilitate overexpression or suppression (silencing) of miR-125-3p expression, respectively. qRT-PCR was performed for testing the expression levels of miR-125-3p in PC-12 cells. CCK-8 and apoptosis assays were performed to evaluate the cell survival and apoptosis rates of PC-12 cells. ROS and dual luciferase activity assays were also performed. Western blot analysis was also performed to evaluate the levels of expression of different proteins associated with PI3K/AKT and Notch pathways. Results: H_2O_2 induced oxidative damage in PC-12 cells in a concentration dependent manner. H_2O_2 induced apoptosis and increased the ROS levels in PC-12 cells. MiR-125-3p was abnormally expressed in PC-12 cells and reduced oxidative damage in PC-12 cells and vice versa. Expression of miR-125-3p is directly regulated by myocyte enhancer factor 2 (MEF2). Knockdown of miR-125-3p activated PI3K/AKT pathway and suppressed Notch pathway by upregulation of MEF2. Conclusion: These findings suggested that knockdown of miR-125-3p protects PC-12 cells against oxidative stress by targeting MEF2. This might in turn provide novel insights regarding the role of circulating miRNAs especially miR-125-3p in pathogenesis of oxidative damage caused during SCI.

Keywords: MiR-125-3p, oxidative stress, spinal cord injury, myocyte enhancer factor 2

Introduction

Spinal cord injury (SCI) is a highly devastating and common neurological disorder that has profound influences on modern society affecting not only individuals, but also affecting the family members and society physically, psychosocially, and socioeconomically [1, 2]. Therefore, the prevention, treatment, and rehabilitation of SCI have become a major issue in medical field. SCI can cause local oxidative damage; hence, it is very important to study the molecular mechanism of oxidative damage after SCI [2]. Although the etiology and pathogenesis of SCI is still unclear, according to the studies over the last two decades, it has been suggested that there is an increased formation of reactive oxygen species (ROS) and oxidative stress which are considered to be important events associated with SCI. Due to several factors such as high content of polyunsaturated fatty acids, increased oxidative metabolic activity, increased production of reactive oxygen metabolites, and relatively low antioxidant capacity, the neurons, glia and spinal cord in the central nervous system are subjected to oxidative and electrophilic stress [3, 4]. Along with these factors, oxidative stress appears to be a pathological hallmark of secondary phase of SCI and hence, alleviation of oxidative stress can be an effective therapeutic intervention in patients of SCI [2].

MiRNAs are a class of small, non-coding RNAs that enter the RNA interference (RNAi) pathway to regulate the expression of protein-encoding genes at the post-transcriptional level. miRNAs are involved in cell proliferation, apoptosis, and differentiation [5]. miR-125 is considered as one of the most important miRNAs which either suppresses or promotes different varieties of

carcinomas and other diseases [6]. It has been reported that the expression of miR-125-3p was decreased in different cancer cells [7]. Overexpression of miR-125-3p can inhibit cancer cell proliferation, migration, and invasion. In addition, it has also been found that hypoxia upregulates expression of miR-125-3p [7]. Therefore, we assumed that upregulation of miR-125-3p was related to cell stress response. Also, recently few studies have demonstrated that miRNAs belonging to this family are widely expressed in brain tissue [8] and play significant roles in nervous system development. miR-125 have demonstrated to induce the irreversible commitment of human pluripotent stem cells to the neural lineage [9] and glial progenitors to undergo astroglial differentiation [10]. The presence of miR-125 in many neural diseases has been demonstrated previously [11], but its role in the oxidative damage of SCI has not been thoroughly investigated.

Myocyte enhancer factor 2 (MEF2) is a family of transcription factors which include different isoforms and are highly expressed in developing neurons during early dendritic maturation and synapse formation [12-14]. These transcription factors play important roles in several key intracellular pathways, including neuronal survival, and apoptosis [15-23]. It is also involved in the neuronal differentiation by the survival of newly formed neurons [16, 18, 22, 23] and regulate the functions of excitatory synapses in hippocampal and cerebellar granule neurons [24-26]. Few studies have suggested that MEF2 down-regulation was associated with death of the cortical neurons [20, 27]. Hence, this study aims to explore the role of miR-125-3p in oxidative damage of PC-12 cells, induced by H_2O_2 exposure.

Materials and methods

Cell culture and treatment

The PC-12 (pheochromocytoma of the rat adrenal medulla) cells were purchased from Kunming Institute of Zoology (Kunming, China) and used throughout the study. The cells were seeded onto flasks at a density of 1×10^4 cells/ml in DMEM with 10% (v/v) fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin. It was maintained at 37°C in a humidified incubator containing 5% CO_2. Culture medium was changed every other day. For the H_2O_2 treatment, the cells were plated in cell culture multi-well plates (Thermo Scientific, Nunc™, Denmark) at a density of 5×10^4 cells/m for 24 h. The cells were treated with fresh medium with different concentrations of H_2O_2 for 24 h to construct the injury model. The control group was treated with the same medium without H_2O_2.

MiRNA transfection

MiR-125-3p mimic, si-miR-125-3p and the NC controls were synthesized by GenePharma Co. (Shanghai, China). Cell transfections were conducted using Lipofectamine 3000 reagent (Invitrogen) following the manufacturer's protocol.

qRT-PCR

Total RNA was extracted from cells and tissues using Trizol reagent (Life Technologies Corporation, Carlsbad, CA, USA) according to the manufacturer's instructions. The Taqman MicroRNA Reverse Transcription Kit and Taqman Universal Master Mix II with the TaqMan MicroRNA Assay of miR-125-3p and U6 (Applied Biosystems, Foster City, CA, USA) were used for testing the expression levels of miR-125-3p in cells.

CCK-8 assay

Cells were seeded in 96-well plates with 5000 cells/well, cell proliferation was assessed by a Cell Counting Kit-8 (CCK-8, Dojindo Molecular Technologies, Gaithersburg, MD). Briefly, after stimulation, the CCK-8 solution was added to the culture medium, and the cultures were incubated for 1 h at 37°C in humidified 95% air and 5% CO_2. The absorbance was measured at 450 nm using a Microplate Reader (Bio-Rad, Hercules, CA).

Apoptosis assay

Flow cytometry analysis was performed to identify and quantify the apoptotic cells by using Annexin V-FITC/PI apoptosis detection kit (Beijing Biosea Biotechnology, Beijing, China). The PC-12 cells (100,000 cells/well) were seeded in 6 well-plates. Treated cells were washed twice with cold PBS and resuspended in buffer. The adherent and floating cells were combined and treated according to the manufacturer's instruction and measured with flow cytometer

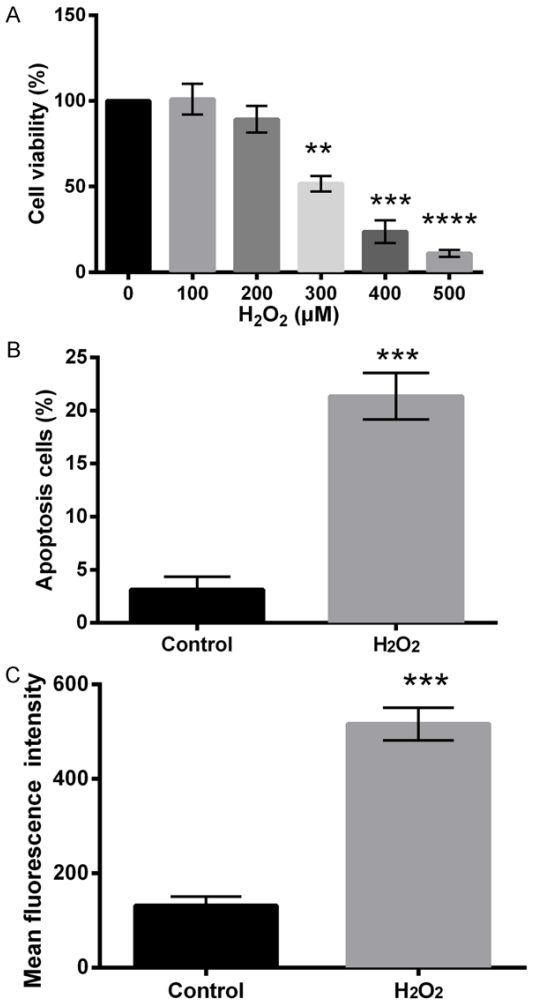

Figure 1. H_2O_2 induced oxidative damage in PC-12 cells. A. PC-12 cells were treated with increasing concentrations of H_2O_2, the cell viability was reduced by H_2O_2 in a concentration dependent manner; B. H_2O_2-induced PC-12 cell apoptosis; C. H_2O_2 increased the level of ROS in PC-12 cells. $^{**}P<0.01$, $^{***}P<0.001$, $^{****}P<0.0001$. ROS, reactive oxygen species.

(Beckman Coulter, USA) to differentiate apoptotic cells (Annexin-V positive and PI-negative) from necrotic cells (Annexin-V and PI-positive).

ROS assay

ROS was measured by flow cytometry using 2,7-dichlorofluorescein diacetate (DCFH-DA) (Nanjing Jiancheng, Nanjing, China). The cells were seeded into a 6-well plate, after treatment, which were washed twice with PBS and co-incubated with serum-free culture medium containing 10 μM DCFH-DA (20 min, 37°C, in dark). Subsequently, the cells were washed with PBS, a trypsin digestion method was used for sample collection. All samples were centrifuged and the supernatants were removed. The cells were resuspended to 500 μl PBS and the fluorescent intensities were measured using a flow cytometer (488 nm excitation, 521 nm emission).

Dual luciferase activity assay

The 3'UTR target site was generated by PCR and the luciferase reporter constructs with the MEF2 3'UTR carrying a putative miR-125-3p-binding site into pMiR-report vector were amplified by PCR. Cells were co-transfected with the reporter construct, control vector and miR-125-3p or scramble using Lipofectamine 3000 (Life Technologies, USA). Reporter assays were done using the dual-luciferase assay system (Promega) following to the manufacturer's information.

Western blot

The protein used for western blotting was extracted using RIA lysis buffer (Beyotime Biotechnology, Shanghai, China) supplemented with protease inhibitors (Roche, Guangzhou, China). The proteins were quantified using the BCA™ Protein Assay Kit (Pierce, Appleton, WI, USA). The western blot system was established using a Bio-Rad Bis-Tris Gel system according to the manufacturer's instructions. GAPDH antibody was purchased from Sigma. Primary antibodies were prepared in 5% blocking buffer at a dilution of 1:1,000. Primary antibody was incubated with the membrane at 4°C overnight, followed by wash and incubation with secondary antibody marked by horseradish peroxidase for 1 h at room temperature. After rinsing, the Polyvinylidene Difluoride (PVDF) membrane carried blots and antibodies were transferred into the Bio-Rad ChemiDoc™ XRS system, and then 200 μl Immobilon Western Chemiluminescent HRP Substrate (Millipore, MA, USA) was added to cover the membrane surface. The signals were captured and the intensity of the bands was quantified using Image Lab™ Software (Bio-Rad, Shanghai, China).

Statistical analysis

All experiments were repeated three times. The results of different experiments are presented as mean ± SD (standard deviation). Statistical analyses were performed using SPSS 19.0 sta-

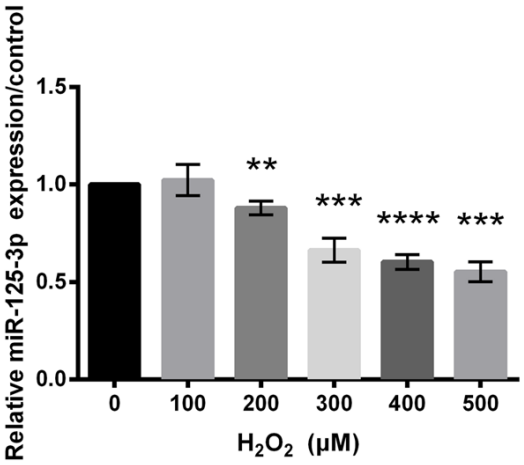

Figure 2. H_2O_2 decreased the expression of miR-125-3p. $^{**}P<0.01$, $^{***}P<0.001$, $^{****}P<0.0001$.

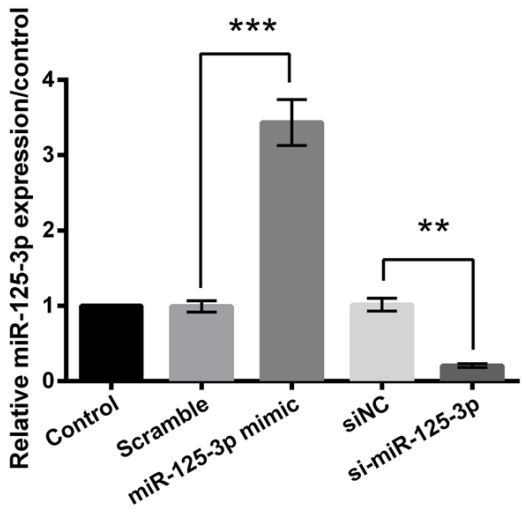

Figure 3. Abnormal expression of miR-125-3p in PC-12 cells. $^{**}P<0.01$, $^{***}P<0.001$.

tistical software. *P*-values were calculated using one-way analysis of variance (ANOVA). *P*-value of <0.05 was considered to be statistically significant.

Results

H_2O_2 induced oxidative damage in PC-12 cells

In the study, we cultured PC-12 cells in 6-well-plates or 96-well-plates and treated with increasing concentrations of H_2O_2 (100, 200, 300, 400 and 500 μM). Cell viability was assessed by CCK-8 assay. Our results showed that H_2O_2 at the concentration of 300 μM significantly induced viability of PC-12 cells ($P<0.01$). These results suggested that different concentrations of H_2O_2 reduced viability of PC-12 cells in a concentration-dependent manner and H_2O_2 at increasing concentrations (300, 400 and 500 μM) significantly reduced cell viability (**Figure 1A**). 300 μM was selected in the forthcoming analyses. Apoptosis assay results revealed that higher percentage of apoptotic cells was observed in the H_2O_2 treated group compared to the control ($P<0.001$, **Figure 1B**). Similarly, ROS assay showed an increase in mean fluorescence intensity (ROS levels) in the H_2O_2 treated PC-12 cells compared to that in control group of PC-12 cells ($P<0.001$, **Figure 1C**). These findings suggested that H_2O_2 at 300 μM induced significant oxidative damage in PC-12 cells ($P<0.001$).

H_2O_2 decreased the expression of miR-125-3p

PC-12 cells after treatment with H_2O_2 showed a decreased expression of miR-125-3p (**Figure 2**).

Abnormal expression of miR-125-3p in PC-12 cells

PC-12 cells were transfected with different miRNAs and then were divided into different groups according to the expression levels of miR-125-3p; the different groups were mimics, scramble (negative control), control, siNC, si-miR-125-3p. Results showed a significant increase ($P<0.001$) in the expression level of miR-125-3p in miR-125-3p mimic group of cells compared to that in the control group. Also, showed a significant decrease ($P<0.01$) in the mRNA expression level of si-miR-125-3p compared to the control group of cells. This suggested that miR-125-3p was abnormally expressed in PC-12 cells (**Figure 3**).

Knockdown of miR-125-3p reduced oxidative damage in PC-12 cells, and overexpression of miR-125-3p showed opposite results

Cell viability was measured in PC-12 cells after treatment with H_2O_2 and after miR-125-3p transfection. Results showed significant decrease in the cell viability in the H_2O_2+ miR-125-3p mimic group compared to scramble ($P<0.05$), but H_2O_2+ si-miR-125-3p showed a significant increase compared to the control group ($P<0.05$). These results suggested that

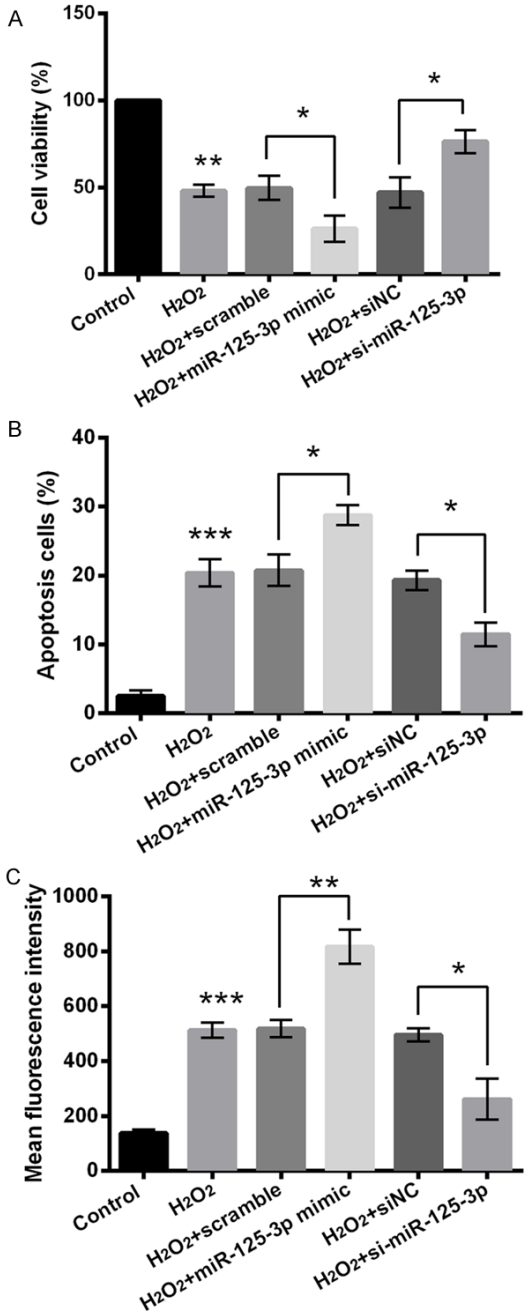

Figure 4. Knockdown of miR-125-3p reduces oxidative damage in PC-12 cells. *P<0.05, **P<0.01, ***P<0.001.

knockdown of miR-125-3p reduced PC-12 cells viability, while overexpression showed opposite results (**Figure 4A**, P<0.05).

Cell apoptosis results showed increased percentage of apoptic cell (P<0.05) in the H_2O_2+ miR-125-3p mimic group compared to scramble, but H_2O_2+ si-miR-125-3p showed a significant decrease compared to the control group (P<0.05, **Figure 4B**). Treatment with H_2O_2 caused nuclear condensation, which is an indicator of apoptosis. ROS assay results were similar to the apoptosis assay results (**Figure 4C**, P<0.05). These results suggested that knockdown of miR-125-3p reduced oxidative damage in PC-12 cells, while overexpression of miR-125-3p showed opposite results.

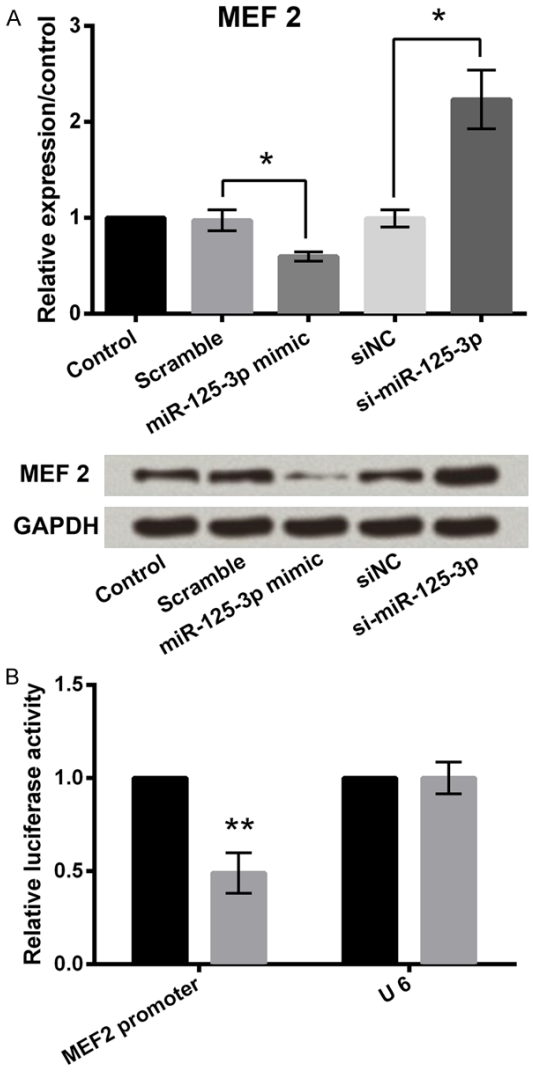

Figure 5. MEF2 is a target of miR-125-3p. *P<0.05, **P<0.01. MEF2: monocyte enhancer factor-2.

MEF2 is a direct target of miR-125-3p

We next sought to identify direct target of miR-125-3p involved in the oxidative damage of SCI. qRT-PCR and western blot analysis were performed to identify the target. Results showed that MEF2 targeted miR-125-3p (**Figure**

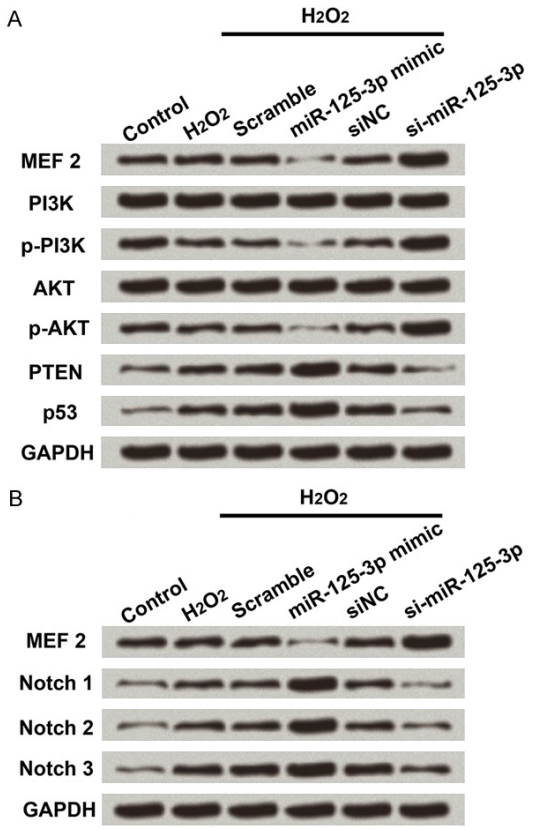

Figure 6. Knockdown of miR-125-3p activated PI3K/AKT pathway and inactivated Notch pathway by upregulation of MEF2. A. Knockdown of miR-125-3p activated PI3K/AKT pathway by upregulation of MEF2; B. Knockdown of miR-125-3p inactivated Notch pathway by upregulation of MEF2. MEF2: monocyte enhancer factor-2, PI3K: phosphoinositide 3-kinase.

5A) as miR-125-3p negatively regulated the expression of MEF2. Next, a dual luciferase reporter assay was performed which showed a significant decrease ($P<0.05$) in the luciferase activity by 50% when cotransfected with miR-125-3p, whereas no significant reduction in luciferase activity was observed when the cells were cotransfected with miR-NC (U 6) (**Figure 5B**).

Knockdown of miR-125-3p activated PI3K/AKT pathway and inactivated notch pathway by upregulation of MEF2

Western blot was performed to investigate the expressions of different pathways involved in the regulation of oxidative damage during SCI by miR-125-3p. These results suggested that the cells of si-miR-125-3a in presence of H_2O_2 showed increased expressions of the MEF2 and different proteins associated with PI3K/AKT pathway like p-PI3K, and p-AKT compared to those expressed by miR-125-3a mimic group of cell in presence of H_2O_2. Thus it can be concluded that suppression of miR-125-3p promotes expression of MEF2, which in turn promotes PI3K/AKT pathway (**Figure 6A**). **Figure 6B** suggests that si-miR-125-3p group of cells shows decreased expressions of proteins associated with Notch pathway (notch 1, 2, and 3) while expression of MEF2 was increased compared to miR-125-3p mimic group of cells. It can be concluded that miR-125-3p activates Notch pathway by suppressing MEF2. This suggested that knockdown of miR-125-3p activated PI3K/AKT pathway and inactivated Notch pathway by upregulation of MEF2.

Discussion

In the present study, we explored the effects of miR-125-3p on H_2O_2-induced oxidative damage in PC-12 cells. The results showed that the expression of miR-125-3p was decreased by administration of H_2O_2 in PC-12 cells. Moreover, we found that downregulation of miR-125-3p could alleviate the H_2O_2-induced oxidative damage in PC-12 cells, while overexpression of miR-125-3p aggravated the injury. Furthermore, the results showed that miR-125-3p negatively regulated the expression of MEF2, a direct target of miR-125-3p. In addition, we found that knockdown of miR-125-3p activated PI3K/AKT pathway and inactivated Notch pathway by upregulation of MEF2. Till date there were no published studies demonstrating the role of miR-125-3p in the oxidative damage of SCI.

Several papers have described the importance of miR-125 family in cancer development [6]. Previous studies in both rodents and humans demonstrated abundant expression of miR-125-3p in central nervous system with respect to peripheral tissues, with a higher expression in neurons and oligodendrocytes. Expression studies during brain development showed very high levels of miR-125a-3p, mainly in the neural precursors [7]. According to the recent literature, a single miRNA can regulate hundreds of transcripts, thus having a very broad array of functional consequences [28]. miR-125a-3p acts as a modulator in the differentiation of oligodendrocytes which provide new findings about the complex regulation of myelination processes [7].

According to recent oncology reports, cardiovascular research and infectious disease study the potential diagnostic and prognostic significance of miRNAs, passively leaked or actively released from cells into the biological fluids including circulating blood and cerebrospinal fluid [29] have been established, these miRNAs either contribute to disease pathogenesis or reflect response to treatment. miRNAs were secreted by different neurons, but also by oligodendrocytes and other CNS cells into the extracellular space packaged in exosomes or microvesicles [29, 30]. Recent evidence supports that the levels of miRNAs are altered in bodily fluids in Parkinson's and Alzheimer's Disease [31]. This suggests that their release not only reflects activation of immune cells, but also the indicate underlying neurodegenerative process.

Our study results showed that the expression of miR-125-3p was decreased by administration of H_2O_2 in PC-12 cells. Moreover, we found that downregulation of miR-125-3p could alleviate the H_2O_2-induced oxidative damage in PC-12 cells, while overexpression of miR-125-3p aggravated the injury. Furthermore, miR-125-3p is also regulated by an activity-dependant transcription factor, MEF 2, which negatively regulates the number of excitatory synapses in mature hippocampal neurons [32]. Similar results were observed with miR-134-5p which directly targets MEF2 [33].

Several signaling pathways such as PI3K/AKT as well as Notch pathways were involved in the underlying process of oxidative damage during SCI [33]. PI3K/Akt is a positive signaling pathway, which directly and/or indirectly downregulated by miR-1, miR-133, miR-206 or miR-125b, and upregulated by miR-23a or miR-486 [33]. While Notch3 is initially induced during differentiation even though activated Notch3 normally inhibits differentiation. Notch 3 and MEF 2 are involved in a mutually antagonistic network that is dependent on the actions of the microRNAs and acts as a bi-stable switch. In one position the switch is antagonistic to differentiation, whereas in the opposite position it promotes differentiation [34].

In conclusion, our study results indicate that knockdown of miR-125-3p could protect against oxidative stress in PC-12 cells by improving MEF2 expression following exposure to H_2O_2 by activating PI3K/AKT pathway and inactivating NOTCH pathway. These findings also might provide novel therapeutic targets for treatment of oxidative damage associated with SCI.

Disclosure of conflict of interest

None.

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