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Original Article

Increased HDAC3 and decreased miRNA-130a expression in PBMCs through recruitment HDAC3 in patients with spinal cord injuries

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Abstract: The study was performed to investigate the molecular mechanism for SCI patients. The interaction between miRNA-130a and HDAC was demonstrated in PBMCs from SCI patients. Increased HDAC3 and decreased miRNA-130a were observed in PBMCs from AS patients. Next, HDAC3 loss-of-function or HAAC3 inhibition promoted the expression of miRNA-130a, and HDAC3 could be recruited to the promoter region of the gene, miRNA-130a, in PBMCs. In addition, linear regression analysis indicated that mRNA expression results were highly negative correlated between HDAC3 and miRNA-130a in PBMCs from SCI patients. Furthermore, miRNA-130a down expression increased the expression of HDAC3 in PBMCs. Loss-of-function of miRNA-130a promoted PBMCs apoptosis, but HDAC3 loss-of-function had no significant effect on the apoptotic cell. In addition, miR-130a overexpression decreased, whereas miR-130a inhibition increased, the expression of TNF-α in PBMCs. Furthermore, HDAC3 loss-of-function or HAAC3 inhibition associated with simultaneous up-regulation the expression of miR-130a and down-regulation the expression of TNF-α in PBMCs. In conclusion, HDAC3 regulated a distinct underlying molecular and pathogenic mechanism of SCI by forming a negative feedback loop with miR-130a and enhanced TNF-1α expression.

Keywords: Spinal cord injury, HDAC3, miRNA-130a, PBMCs

Introduction

Acetylation or deacetylation of the histone proteins are controlled by histone acetyltransferase (HAT) or histone deacetylase (HDAC), respectively [1]. The equilibrium between HAT and HDAC gives insights into the level of gene transcription, including genes coding for inflammatory cytokines [2]. HAT coordinates the recruitment and activation of transcription factors with conformational changes in histones that allow gene promoter exposure. HDAC counteracts HAT activity through the targeting of both histones as well as non-histone signal transduction proteins important in inflammation [3]. Conditional deletion of HDAC1 in T cells leads to enhance airway inflammation and increase Th2 cytokine production [4]. Recent observations that HDAC activity is depressed in rheumatoid arthritis patient synovial tissue have predicted that strategies restoring HDAC function may be therapeutic in this disease as well [3]. Moreover, valproic acid, a histone deacetylase inhibitor, showed a reduction in the development of secondary damage in rat spinal cord trauma with an improvement in the open field test (BBB scale) with rapid recovery [5]. However, research increasingly suggests that HDAC inhibitors may contribute to reduction spinal cord lesions [5-7].

On the contrary, inhibition of HDAC with HDAC inhibitors has been reported to limit production of proinflammatory cytokines, including TNF-α and MMP9 [7, 8], and expression of the sirtuin 1 (SIRT1) gene is under the control of NF-κB, which is activated by TNF-α [9]. Moreover, pharmacological inhibitors of HDAC activity, however, have demonstrated potent therapeutic effects in animal models of spinal cord injury

and other chronic inflammatory diseases [10, 11]. A recent study indicates a clear increase of the HAT/HDAC ratio in ankylosing spondylitis during anti-TNFα herapy, while rituximab increased both HAT and HDAC [12]. An imbalance between HAT and HDAC evaluated in PBMCs or synovial tissue has previously been reported in RA and AS [13, 14].

AS is a chronic inflammation arthritis that affects both axial and peripheral skeletons and soft tissues. Altered microRNA (miRNA) expression has been implicated in the pathogenesis of different forms of spinal cord injury (SCI) [15, 16]. Many studies have demonstrated that altered expression of miRNAs in synovia, peripheral blood mononuclear cells (PBMCs) or T cells from patients with SCI is associated with innate immunity and inflammation [17-19]. A recent study shows that miR-16, miR-221 and let-7i are over-expressed in SCI T cells. In the functional studies, the increased let-7i expression facilitates the T helper type 1 (IFN-γ) immune response in T cells [20]. Bioinformatics analyses are used to identify the potential targets of miR-130a in endothelial progenitor cells [21], hepatitis C virus [22] and cardiomyocytes [23, 24]. To date, the underlying mechanisms for miR-130a regulation PBMCs from patients with SCI are quite limited.

The advances in effective therapy for SCI has been limited because the pathological mechanisms causing immune and inflammatory response are not known. Therefore, revealing the molecular mechanism for the SCI is indispensable for developing effective treatment. In this study, PBMCs are chosen to investigate the pathogenesis of SCI through miR-130a via HDAC pathways.

Materials and methods

Serum samples and cell culture

Human serum samples were obtained with written informed consent from The Central Hospital of Taian. The study was approved by the Ethics Committee of The Central Hospital of Taian. 28 serum samples of SCI patients and 28 cases of normal control group were collected between 02/2013 and 6/2014. The peripheral blood mononuclear cells (PBMCs) was obtained from the serum samples, and maintained in RPMI-1640 (Invitrogen, USA) supplemented with 10% FBS (Invitrogen, USA) at 37°C in a humidified incubator (Thermo, USA), 5% CO_2, 95% air atmosphere. The medium was replenished every day.

Quantitative real-time PCR

The PBMCs RNA extraction was performed according to the TRIzol manufacturer's protocol (Invitrogen, Carlsbad, CA, USA). Synthesis of cDNAs was performed by reverse transcription reactions with 2 μg of total RNA using moloney murine leukemia virus reverse transcriptase (Promega, Switzerland) with oligo dT (15) primers (Fermentas) as described by the manufacturer. The first strand cDNAs served as the template for the regular polymerase chain reaction (PCR) performed using a DNA Engine (ABI 9700). The cycling conditions were 2-min polymerase activation at 95°C followed by 40 cycles at 95°C for 15 s and 55°C for 60 s. PCR with the following primers: HDAC3, Forward 5'-TTGCCATTCTGTAATTGCT-3' and Reverse 5'-CTGGCGTCGTCAGTGGG-3'; miRNA-130a, Forward 5'-TTCCGAATGTGTTTTGTCCT-3' and Reverse 5'-CTGCGGTCCTGAGAGGG-3'; β-actin, Forward 5'-AGAGCCGAGGTGATTGCATT-3' and Reverse 5'-GAGCAAAAGCGTACATACATCTC-3'. β-actin as an internal control was used to normalize the data to determine the relative expression of the target genes. The reaction conditions were set according to the kit instructions. After completion of the reaction, the amplification curve and melting curve were analyzed. Gene expression values are represented using the $2^{-\Delta\Delta Ct}$ method.

Western blotting

The PBMCs were homogenized and extracted in NP-40 buffer, followed by 5-10 min boiling and centrifugation to obtain the supernatant. Samples containing 50 μg of protein were separated on 10% SDS-PAGE gel, transferred to PVDF Transfer Membrane (Millipore). After saturation with 5% (w/v) non-fat dry milk in TBS and 0.1% (w/v) Tween 20 (TBST), the membranes were incubated with the following antibodies, HDAC3 at dilutions ranging from 1:500 to 1:2,000 at 4°C over-night. After three washes with TBST, membranes were incubated with secondary immunoglobulins (Igs) conjugated to IRDye 800CW Infrared Dye (LI-COR), including donkey anti-goat IgG and donkey anti-mouse IgG at a dilution of 1:10,000-1:20,000. After 1

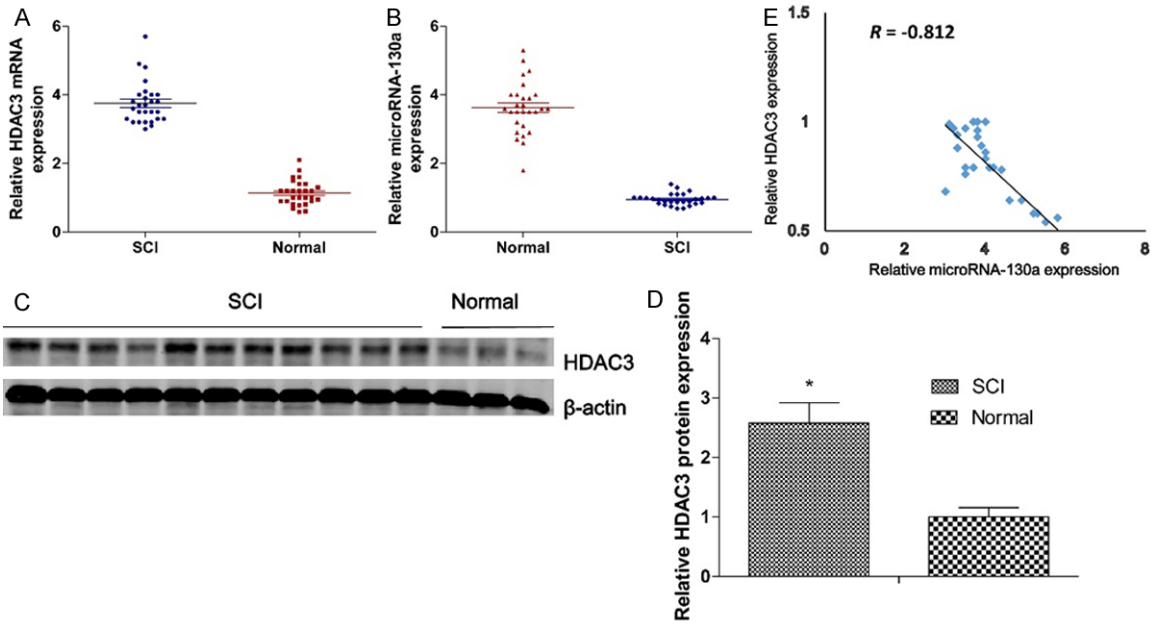

Figure 1. The expression of HDAC3 and miRNA-130a in PBMCs from SCI patients. The mRNA (A) and protein (C) expression of HDAC3 are respectively measured by Quantitative real-time PCR and western blotting in PBMCs from SCI patients. Densitometric quantification for western blotting (D). miRNA-130a expression is measured by Quantitative real-time PCR (B). Correlation between HDAC3 and miRNA-130a (E). Values are expressed as mean ± SEM, n = 3 in each group. $R \leq -0.8$ means highly negative correlation between cervical scrapings and tumor tissue PAX1 methylation levels.

hour incubation at 37°C, membranes were washed three times with TBST. Blots were visualized by the Odyssey Infrared Imaging System (LI-COR Biotechnology). Signals were densitometrically assessed (Odyssey Application Software version 3.0) and normalized to the β-actin signals to correct for unequal loading using the mouse monoclonal anti-β-actin antibody (Bioworld Technology, USA).

Chromatin immunoprecipitation (ChIP)

PBMCs were cross-linked with 0.5% formaldehyde for 10 min at room temperature. Cross-linking was stopped by adding 125 mM glycine. Cells were solubilized in a buffer containing 10 mM Tris-HCl (pH 8.0), 1% Triton X-100, 1% sodium deoxycholate, 1 mM PMSF and PIC for 10 min at 4 uC. Pellets obtained by centrifugation at 10000 g for 5 min were suspended in RIPA buffer and sonicated using a Bioruptor Sonicator (Diagenode, Belgium) to shear chromatin into 500 bp fragments. Sonicated chromatin was subjected to immunoprecipitation using ChIP-grade agarose beads with protein G (Cell Signaling), blocked with 1% bovine albumin and 1% salmon sperm DNA.

RNA interference

The small interfering (si) RNA or scramble siRNA was obtained from Dharmacon (Lafayette, USA). The small interfering with the following primers: HDAC3, Forward 5'-CACUCUGAGUGGGACAAGCUCUUCA-3' and Reverse 5'-UGAAGAGCUUGUCCCACUCAGAGUG-3'; miRNA-130a, Forward 5'-GCUAUCAGUCCACUGUGCUUGUGGU-3' and Reverse 5'-ACCACAAGCACAGUGGACUGAUAGC-3', scramble, Forward 5'-CACGAGUGGGUAACACUCGUCUUCA-3' and Reverse 5'-UGAAGACGAGUGUUACCCACUCGUG-3'. The siRNA oligonucleotides (at a final concentration of 100 nM) were transfected into human umbilical vein endothelial cells using Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's instructions.

Statistical analysis

The data from these experiments were reported as mean ± standard errors of mean (SEM) for each group. All statistical analyses were performed by using PRISM version 4.0 (GraphPad). Inter-group differences were analyzed by one-way ANOVA, and followed by

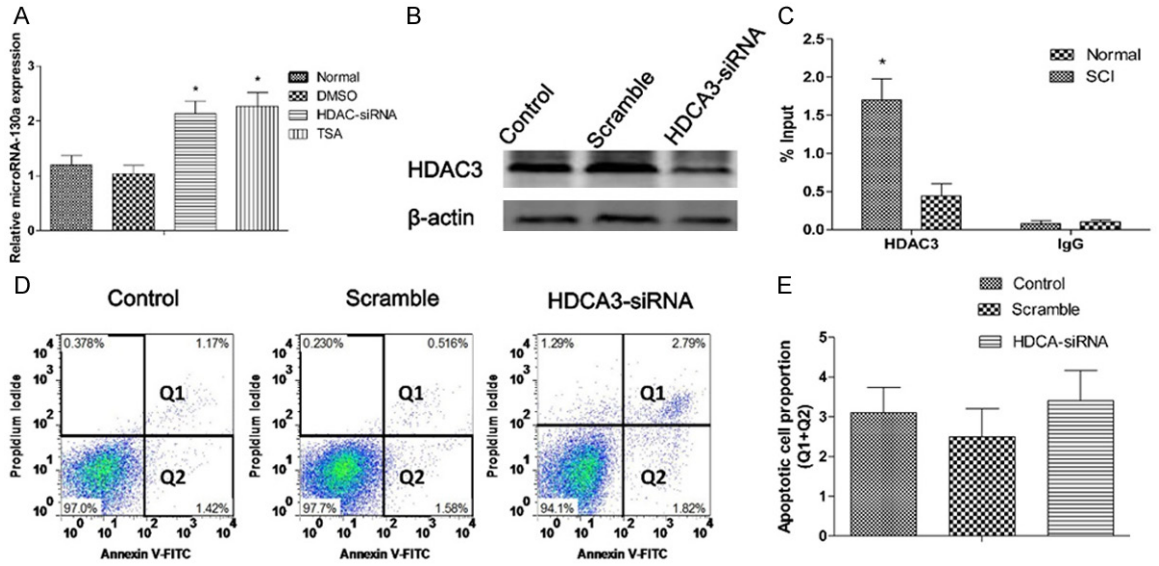

Figure 2. Regulation of miRNA-130a by HDAC3. The PBMCs are treated with vehicle solvent, DMSO, HDAC-siRNA and trichostatin A (TSA), and the expression of miRNA-130a is measured by Quantitative real-time PCR (A). The protein expression of HDAC3 is measured by western blotting in PBMCs from SCI patients (B). ChIP assay is performed using HDAC3 antibody to detect binding at the miRNA-130a promoter region. Percentage input was calculated with $2^{(Ct\ [1\%\ of\ input]-Ct\ [ChIP])}$ (C). The apoptotic cells was detected by flow cytometric analysis of annexin V/PI double staining (D) and the percentage of apoptotic cells (E). Values are expressed as mean ± SEM, n = 3 in each group.

Tukey's multiple comparison test as a post test to compare the group means if overall $P < 0.05$. Differences with *P* value of < 0.05 were considered statistically significant.

Results

The expression of HDAC3 and miRNA-130a in PBMCs from SCI patients

Recent studies have shown that PBMCs nuclear extracts decreased the levels of HAT while HDAC tended to increase in SCI patients [12]. To consistent with this, our result also showed increased HDAC3 mRNA (superior to 3 folds) and protein expression in PBMCs of SCI patients (**Figure 1A**, **1C** and **1D**). We then investigated the possible mechanisms that the interaction between miRNA and SCI. We performed a hierarchical cluster analysis of the differentially expressed miRNAs in the PBMCs from AS patients. The results showed that the down-regulation of miRNA-130a was associated with SCI, the expression of which was at the lowest levels in all miRNAs (**Figure 1B**). Interestingly, when the correlation between HDAC3 and miRNA-130a was analyzed as a continuous variable, we did find a highly negative correlation between HDAC3 and miRNA-130a in the PBMCs from SCI patients (**Figure 1C**).

Regulation of miRNA-130a by HDAC3

The deregulation of miRNAs has been frequently identified in immunopathogenesis and has been linked to SCI [20, 25]. The expression of miRNA-15a/miRNA-16-1 is repressed through recruitment of HDAC3 in mantle cell and other non-Hodgkin B-cell lymphomas [25]. Based on these data, expression of miRNA-130a in PBMCs from SCI patients was chosen for further identification by HDAC3 loss-of-function. Increased miRNA-130a expression was observed in HDAC3 small interference RNA group or HDAC3 inhibitor (TSA) group (**Figure 2A**), and the protein expression of HDAC3 was inhibited by HDAC3 small interference RNA (**Figure 2B**). Moreover, ChIP assay was performed using HDAC3 antibody to detect binding at the miRNA-130a promoter. This approach revealed that HDAC3 could bind to DLEU2 promoters in PBMCs, the percentage input was significantly increased in PBMCs from SCI patients (**Figure 2C**). These findings suggest that HDAC3-mediated histone hypo-acetylation contributes to regulate the expression of miRNA-130a. We next investigated whether HDAC3 loss-of-function induces cell death through an apoptotic mechanism. Annexin V-PI double-labeling was used for the detection of PS externalization, a hallmark of early phase of apopto-

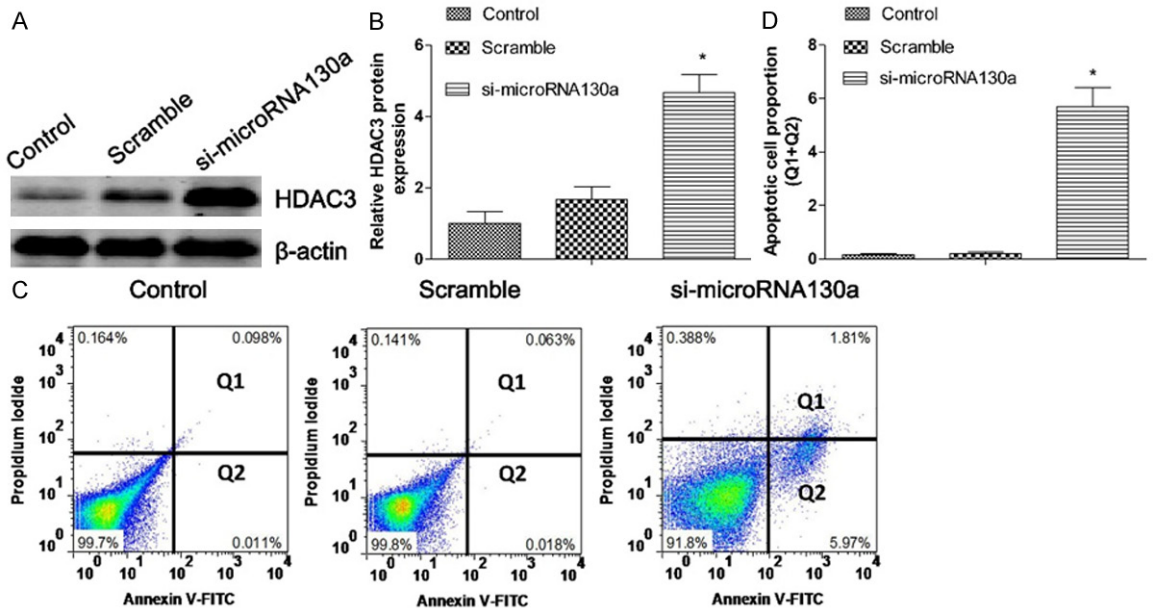

Figure 3. Regulation of PBMCs apoptosis via miRNA-130a. The protein expression of HDAC3 is measured by western blotting in PBMCs from SCI patients (A) and densitometric quantification for western blotting (B). The apoptotic cells was detected by flow cytometric analysis of annexin V/PI double staining (C) and the percentage of apoptotic cells (D). Values are expressed as mean ± SEM, n = 3 in each group.

sis. The results showed that the proportion of the apoptotic cells had no significantly gained as compared to un-treated group (**Figure 2D** and **2E**).

Regulation of PBMCs apoptosis via miRNA-130a

In this study, we did find a highly negative correlation between HDAC3 and miRNA-130a in the PBMCs from SCI patients. Increased HDAC3 expression was observed in miRNA-130a small interference RNA group (**Figure 3A** and **3B**). We next investigated whether miRNA-130a loss-of-function induces cell death through an apoptotic mechanism. Annexin V-PI double-labeling was used for the detection of PS externalization, a hallmark of early phase of apoptosis. The results showed that the proportion of the apoptotic cells had gained as compared to untreated group (**Figure 3C** and **3D**). Therefore, our data suggest that up-regulation the expression of HDAC3 via miRNA-130a loss-of-function was involved in PBMCs apoptosis.

Regulation of TNF-α1 by HDAC3 via miRNA-130a

MiRNA-130a overexpression decreased, whereas miR-130a inhibition increased, the mRNA and protein expression of TNF-α in PBMCs (**Figure 4A** and **4B**). Furthermore, HDAC3 loss-of-function or HDAC3 inhibition associated with simultaneous up-regulation the expression of miR-130a and down-regulation the mRNA and protein expression of TNF-α in PBMCs (**Figure 4C** and **4D**). These observations suggested that HDAC3 plays an important role in the pathological courses of SCI by regulating miR-130a expression, via its target TNF-α.

Discussion

Among the numerous HDACs, histone deacetylase-3 (HDAC3) is widely expressed and conserved in a wide range of species [26]. HDAC3 regulates the JNK pathway [27], NF-κB activity [28], MAPK activation [29] and RCAN1 nuclear translocation [30]. In the present study we made several important observations. First, increased HDAC3 and decreased miRNA-130a were observed in PBMCs from SCI patients. Next, HDAC3 loss-of-function or HAAC3 inhibition promoted the expression of miRNA-130a, and HDAC3 could be recruited to the promoter region of the gene, miRNA-130a, in SCI patients. It was reasonable that HDAC3 regulated a distinct underlying molecular and pathogenic mechanism of SCI by forming a negative feedback loop with miR-130a. In addition, miR-130a

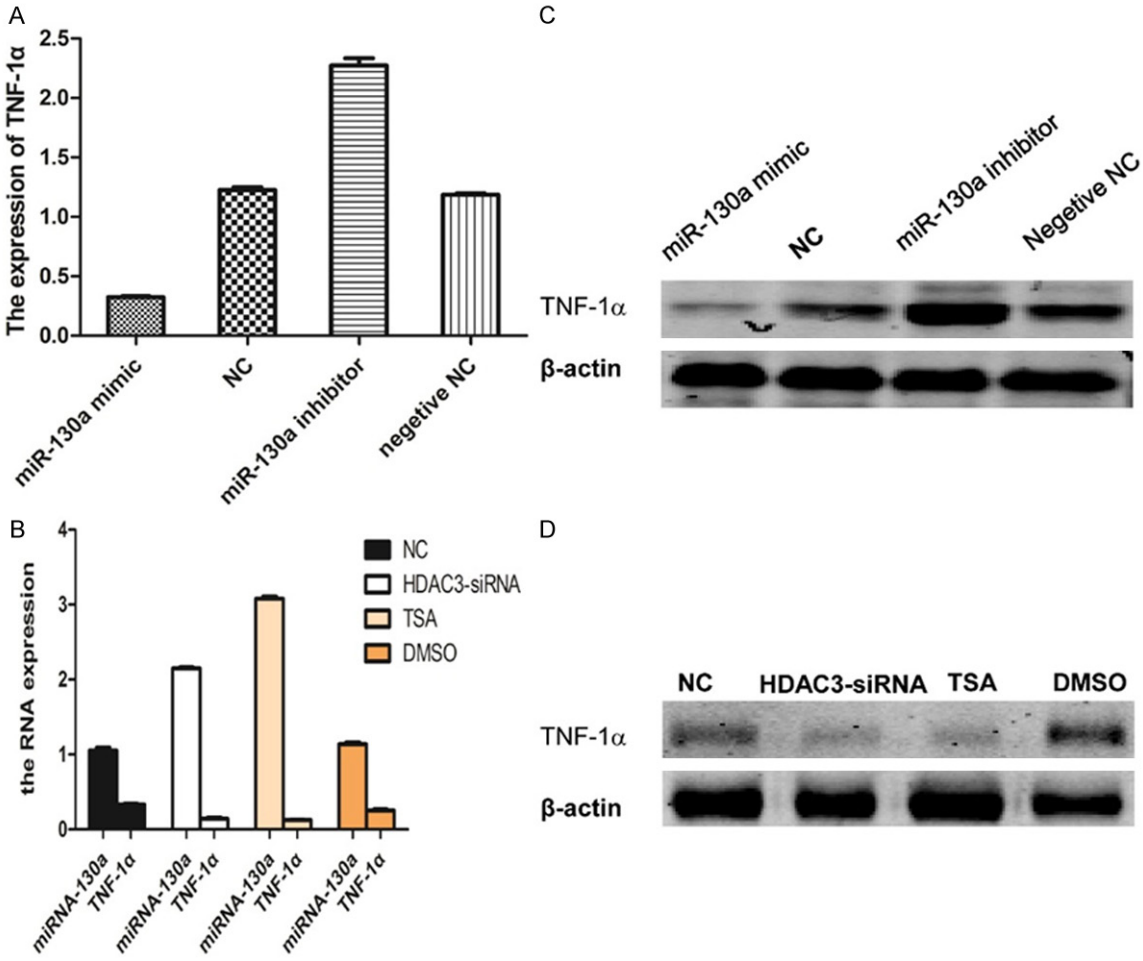

Figure 4. Regulation of TNF-α1 by HDAC3 via miRNA-130a. The mRNA (A) and protein (B) of TNF-1α are respectively measured by Quantitative real-time PCR and western blotting in PBMCs with miRNA-130a loss-of-function or gain-of-function. The mRNA expression of TNF-1α and miRNA-130a are measured by Quantitative real-time PCR in PBMCs with HDAC3 loss-of-function or inhibition (C). The protein expression of TNF-1α is measured by western in PBMCs with HDAC3 loss-of-function or inhibition (D). Values are expressed as mean ± SEM, n = 3 in each group.

overexpression decreased, whereas miR-130a inhibition increased, the expression of TNF-α in PBMCs. Furthermore, HDAC3 loss-of-function or HAAC3 inhibition associated with simultaneous up-regulation the expression of miR-130a and down-regulation the expression of TNF-α in PBMCs. Together, these observations suggested that HDAC3 plays an important role in the pathological courses of SCI by regulating miR-130a expression, via its target TNF-α.

Recent reports have shown that miRNA-130a is required for normal immune function. miRNA-130a significantly inhibits HCV replication in both HCV replicon and J6-/JFH1-infected cells. Over expression of miR-130a upregulates the expression of type I IFN (IFN-α/IFN-β), ISG15, USP18 and MxA, which are involved in innate immune response and decreased expression of miR-122, a well-defined miRNA promoting HCV production [22]. miRNAs are found potentially differential expression in the T cells of SCI patients may alter expression of the downstream target molecules that may contribute to the pathogenesis of SCI [20]. In our study, miRNA-130a inhibition could increase the expression of TNF-α, and miRNA-130a gain-of-function could decrease the expression of TNF-α. These results indicated that TNF-α might be a candidate target of miRNA-130a, as it appeared to play an important role in regulating the pathological courses of SCI.

SCI therapy has been revolutionized by increasing knowledge of the pathogenesis of the disease, involving dysfunction and over secretion

of multiple proinflammatory molecules, in particular TNF-α [20, 31]. Currently, among five biological agents used in SCI therapies, anti-TNF-α monoclonal antibody, adalimumab (ADA), TNF receptor fusion protein, etanercept (ETA), and anti-TNF-α monoclonal antibody, infliximab (INF). These agents have been largely demonstrated to be effective at reducing disease activity and controlling joint damage and various aspects of the diseases and reasonably safe in SCI [31-33]. Clinical studies indicate that SCI patients respond to treatment. With TNF-α inhibitors, HAT activity increases considerably in patients with SCI (+198%), and a clear increase of the HAT/HDAC ratio in SCI patients during anti-TNF-α therapy. With HDAC inhibitor, trichostatin A (TSA), induces a decline in HDAC (-43.6%) [12]. In our study, inhibition the function of HDAC3 with TSA seem to exert direct regulations on the expression of miRNA-130a and indirect regulations on the expression of TNF-α. We provided HDAC3/miRNA-130a/TNF-α axis as a novel regulator of the response to SCI.

In conclusion, our results identified that HDAC3 played an important role in the pathological courses of SCI by regulating miR-130a expression, via its target TNF-α, suggested that such an inflammatory mediator which was usually elevated in PBMCs might at least in part contribute to increased morbidity of SCI.

Disclosure of conflict of interest

None.

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