The role and mechanisms of action of SIRT6 in the suppression of postoperative epidural scar formation

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Received July 26, 2015; Accepted January 28, 2016

DOI: 10.3892/ijmm.2016.2522

Abstract. In order to investigate the role which sirtuin-6 (SIRT6) plays in lumbar spinal epidural fibrosis (EF) and scar formation in vitro and in vivo, SIRT6 and transforming growth factor β (TGF-β) protein levels in the lumbar discs of patients who had undergone a laminectomy. The results demonstrated that SIRT6 expression was significantly reduced in the lumbar discs of patients in whom an epidural scar had formed, but the expression pattern of TGF-β2 was much higher. Subsequently, a pcDNA-SIRT6 expression vector was constructed and transfected into the primary fibroblasts isolated from the epidural scar formation. Flow cytometric and MTT analyses indicated that overexpression of SIRT6 suppressed the proliferation of the fibroblasts, and TGF-β2 and interleukin-1α expression, as well as collagen type I (Col I) production. The results of bioinformatics and molecular biological analyses demonstrated that TGF-β2 was a target of microRNA-21 (miR-21) and SIRT6 overexpression suppressed the levels of TGF-β2 through promoting the expression of miR-21. Finally, by injecting the pcDNA-SIRT6 vector, it was possible to observe that SIRT6 suppressed EF and epidural scar formation in vivo. In conclusion, we noted that SIRT6 suppressed EF and epidural scar formation in vitro and in vivo. It was also noted that SIRT6 overexpression suppressed TGF-β2 levels by promoting the expression of miR-21.

Introduction

Epidural fibrosis (EF), which causes spinal epidural adhesion and scarring, is frequently found in the epidural space and contributes greatly to postoperative pain and recurrent lumbar disc herniation after a laminectomy (1,2). Various agents or mechanical barriers for preventing EF have been studied both in animal models and humans, including autologous fat grafts, Adcon-L, polytetrafluoroethylene membranes, and fibrinolytic agents (3). However, it is still difficult to treat patients with established spinal EF.

EF formation is known to be a complicated process, involving hypermorphic proliferation of fibroblasts, the increased expression of inflammatory cytokines, the exorbitant production of collagens and other changes in cellular and molecular components (4,5). After laminectomy, fibrous connective tissues adjacent to the laminar traumas are activated to repair the wound; fibroblast proliferation and inflammatory cytokine release are greatly increased during the repair process (6). As a major cell component involved in this repair, fibroblasts are driven to excessively proliferate and produce collagens (mainly Col I) by the increased growth factors and inflammatory mediators (7).

Transforming growth factor β (TGF-β) is a secreted protein that controls proliferation, differentiation, metastasis and other functions in the majority of types of cells (8). TGF-β has been shown to be involved in the progression of many chronic inflammation-related diseases, including diabetes, multiple fibrosis/sclerosis and also in scar formation (9-12). Of the members of the TGF-β family, TGF-β1 and TGF-β2 are believed to play a central role in the fibrosis and scar formation in tissues (13). During these processes, these were upregulated, and induced the production of multiple growth factors, inflammatory cytokines and collagens (14). A previous study on TGF-β1 and TGF-β2 expression in mice with spinal injury revealed that TGF-β1 was induced at an early stage while TGF-β2 was induced at a later stage (15). Another study demonstrated that expression of TGF-β2 but not TGF-β1 was correlated with the deposition of scar tissue in the spinal lesion (16). These studies revealed that TGF-β2 plays a pivotal role in the recovery of patients with spinal lesions.

Sirtuins (SIRTs) are a highly conserved NAD+-dependent deacetylase family that plays multiple roles in metabolism, cell
apoptosis, cell fate determination and lifespan regulation (17). As an important member of the SIRT family, SIRT6 has been proven to deacetylate many important proteins involved in physiological and pathological processes, and plays a role in DNA repair, metabolism and aging (18). Depletion of SIRT6 has been shown to result in the suppression of cell apoptosis, cellular senescence, DNA damage, and telomere dysfunction in many types of cells (19-21). However, the role and mechanism of SIRT6 in spinal EF have not been studied in depth.

In the present study, we found that SIRT6 expression was significantly reduced the lumbar disc of the patients in whom an epidural scar formed, displaying an opposite expression pattern to TGF-β2. Overexpression of SIRT6 in primary epidural fibroblasts suppressed cell proliferation, TGF-β2 and interleukin-1α (IL-1α) expression, and Col I production. Our results of the bioinformatics and molecular biological analyses revealed that SIRT6 overexpression suppressed TGF-β2 levels through inducing the expression of microRNA-21 (miR-21). Finally, a study of pcDNA-SIRT6 vector injection indicated that SIRT6 could suppress EF and epidural scar formation in vivo.

Patients and methods

Patients and sampling. This study enrolled 96 post-laminectomy patients with lumbar disc herniation, of which 48 patients developed an epidural scar and the other 48 patients did not develop an epidural scar. Their lumbar disc tissues were sampled during the laminectomy, and the removed tissues were collected, labeled and saved at -80°C for future use. This study was approved by the Ethics Committee of Xi’an Red Cross Hospital (Xi’an, China). All participants provided written informed consent.

Cell culture and transfection. Samples of epidural scar tissues were taken, and human primary epidural fibroblasts were isolated from these as described previously (22). The primary fibroblasts were obtained from the epidural scars using the enzymatic digestion method in a sterile superclean bench. A total of 5 mg obtained tissues were cut into small pieces (approximately 1 mm³) and mixed with 3 ml 0.2% collagenase II. The mixture was then transferred to sterile tubes and centrifuged at 500 x g for 10 min. The precipitate was resuspended with 2 ml medium and incubated for 0, 24, 48 and 72 h before adding the MTT reagent to each well at a final concentration of 0.5 mg/ml and incubated at 37°C for 4 h. After medium removal, 500 µl dimethylsulfoxide was added to each well. Viable cells were measured at an absorbance of 550 nm wavelength using a microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

ChIP analysis. The regulatory association between SIRT6 and miR-21 was revealed using ChIP analysis. Cell proliferation was evaluated using an MIT assay (Sigma, St. Louis, MO, USA). The cells were seeded in 12-well culture plates at 5x10⁴ cells/well, and then transfected with the indicated vectors or siRNA. The cells were incubated for 0, 24, 48 and 72 h before adding the MIT reagent to each well at a final concentration of 0.5 mg/ml and incubated at 37°C for 4 h. After medium removal, 500 µl dimethylsulfoxide was added to each well. Viable cells were measured at an absorbance of 550 nm wavelength using a microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Cell proliferation and apoptosis analyses. Cell proliferation was evaluated using an MIT assay (Sigma, St. Louis, MO, USA). The cells were seeded in 12-well culture plates at 5x10⁴ cells/well, and then transfected with the indicated vectors or siRNA. The cells were incubated for 0, 24, 48 and 72 h before adding the MIT reagent to each well at a final concentration of 0.5 mg/ml and incubated at 37°C for 4 h. After medium removal, 500 µl dimethylsulfoxide was added to each well. Viable cells were measured at an absorbance of 550 nm wavelength using a microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

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Western blotting. A total of 50 µg of protein of each sample were separated by 12% SDS-PAGE and electro-transferred to a PVDF membrane (Millipore Corp., Billerica, MA, USA) for western blotting. The following primary antibodies (purchased from Abcam, Cambridge, UK) were used: anti-Col I (1:300; ab34710), anti-SIRT6 (1:300; ab88494), anti-TGF-β2 (1:500; ab66045) and anti-β-actin (1:200; ab8226), which was used as the internal reference. After incubation with the appropriate horseradish peroxidase-conjugated secondary antibody (goat anti-rabbit IgG, 1:3,000, ab7090; Abcam), proteins were detected using chemiluminescence reagent (ECL; Invitrogen Life Technologies) in a ChemiDoc XRS imaging system and analyzed with Quantity One software (both from Bio-Rad Laboratories, Inc.).
for decoding transcription factor binding maps, expression profiles and the transcriptional regulation of non-coding RNAs and protein-coding genes from ChIP-seq data. The targeting association between miR-21 and TGF-β2 was predicted with TargetScan (http://www.targetscan.org/), an online resource.

3'‑UTR luciferase reporter assay. The 3'‑UTR of TGF-β2 mRNA was amplified by PCR, using primers linked with the XhoI and NotI restriction sites, respectively. Both the primers were designed and synthesized by Genscript Co., Ltd., (Nanjing, China). The PCR products were excised with NotI and XhoI and inserted into the pGL-2 vector (Promega, Madison, WI, USA) at the 3'-end of the Renilla gene CDS. Firefly luciferase activity was used as the internal control. The 3'-UTR dual-luciferase vectors were transfected or co-transfected with the miR-21 mimic or pcDNA-TGF-β2 expression vector into the primary fibroblasts (100% confluence) using X‑tremeGENE 9 DNA transfection reagent (Roche, Basel, Switzerland). The medium was changed 6 h later. Cells were incubated for another 48 h with commercial cell lysis buffer (Merck, Whitehouse Station, NJ, USA). The luciferase activity was measured using a luminometer (Promega) according to the manufacturer's instructions.

Lumbar disc herniation models and injection. In the present study, a Xenopus tropicalis model of lumbar disc herniation was established using the method of caudal vertebral pulposus transplantation, as described previously (23). The animals (aged approximately 12 weeks; purchased from the Laboratory Animal Center, Beijing Institute of Genetics and Development, Chinese Academy of Sciences, Beijing, China) underwent a laminectomy and subsequently those which developed epidural fibrotic scars were selected and used in the following experiments. The X. tropicalis were sacrificed using a dissecting needle inserted into the vertebral foramen. For caudal vertebral pulposus transplantation, the animals were anaesthetized with 40 mg/kg after adaptive feeding for 1 week. We cut the tail vertebrae at the root of the tail (about 1 cm from the anus), and the incision was then sutured. The caudal nucleus pulposus was removed, and we weighed 10 mg of the pulposus with an analytical balance (Pingxuan Scientific Instrument Co., Ltd., Shanghai, China). We then cut open the back of the Xenopus tropicalis, removed the spinous processes and lamina of L4-L6, and gently set the obtained pulposus on the nerve root. Finally, the incisions were sutured. The miR-21 inhibitor (1 µg/g body weight) or pcDNA-SIRT6 (5 µg/g body weight) was locally injected into the animals around the surgical site. Over a 6-week period, the injection was administered once a day. Subsequently, expression of SIRT6 and TGF-β2 was assessed by one-way ANOVA followed by Dunnett's tests. A P-value <0.05 was considered to indicate a statistically significant difference.

Results

Expression of SIRT6 is reduced in the lumbar disc tissues of the patients with an epidural scar. As mentioned in Materials and methods, following laminectomy, the removed lumbar disc tissues were collected, labeled and saved at -80°C. Two years later, an investigation was undertaken to confirm whether each patient had an epidural scar and whether there had been a relapse of lumbar disc herniation. A total of 48 scar-free patients and 48 patients who developed an epidural scar were randomly selected. Expression of SIRT6 and TGF-β2 in their lumbar disc tissues samples previously was detected using western blotting, and the results showed that SIRT6 levels in the patients with epidural scarring were significantly lower than those of scar-free patients (Fig. 1A). By contrast, TGF-β2 expression in the patients who suffered an epidural scar was much higher than that of scar-free patients (Fig. 1B). These results suggested that lumbar disc SIRT6 expression is associated with epidural scar formation and also TGF-β2 expression.

SIRT6 suppresses fibroblast proliferation, TGF-β2 and IL-1α expression, and Col I production. In order to explore the role of SIRT6 in EF and scar formation in vitro, epidural scar samples were taken from the patients, and primary fibroblasts were isolated using the collagenase I digestion method. A pcDNA-SIRT6 expression vector was built and transfected
into the primary cells. After incubation for 72 h, the cells were stained with PI/annexin V followed by flow cytometric analysis. The results showed that the proportion of viable cells was reduced by pcDNA-SIRT6 transfection but there were no marked changes in the proportion of early apoptotic cells among the groups (Fig. 2A). MTT analysis also demonstrated that pcDNA-SIRT6 transfection reduced the viability of the cells (Fig. 2B). The levels of TGF-β2 and Col I proteins, as well as IL-1α mRNA, were determined by western blotting and qPCR, respectively. The data indicated that SIRT6 overexpression caused a significant decrease in TGF-β2 and Col I protein levels (Fig. 2C) and the IL-1α mRNA level (Fig. 2D). These data indicated that SIRT6 suppressed EF, which manifested as suppression in fibroblast proliferation, TGF-β2 and IL-1α expression, as well as Col I production.

**TGF-β2 is a target of miR-21, and SIRT6 promotes the expression of miR-21 in primary fibroblasts.** Subsequently, the mechanism of SIRT6 suppression of EF was investigated. Bioinformatics analysis using TargetScan revealed that there was a complete match between the miR-21 seed sequence and the 3'UTR of TGF-β2 mRNA, and the match was highly conserved among species (Fig. 3A and B). In order to validate the target relationship, the full length of the 3'UTR of TGF-β2 mRNA was cloned into pGL3-basic luciferase vector (Fig. 3C). After transfection of pGL3-TGF-β2 or pGL3-control vector into the fibroblasts followed by transfection with pcDNA-SIRT6 or pcDNA-vector, luciferase activities were quantitatively measured.

Figure 1. Expression of sirtuin-6 (SIRT6) was lower in patients who developed epidural scars after laminectomy. (A) Comparison of SIRT6 protein levels between patients who developed epidural scars and those who did not. (B) Comparison of transforming growth factor β (TGF-β) protein levels between patients who developed epidural scars and those who did not. Following laminectomy, 48 scar-free patients and 48 patients who developed an epidural scar were randomly selected. Expression of SIRT6 and TGF-β2 in the lumbar disc tissues samples was detected using western blotting. Control, non-scarred patients.

Figure 2. Overexpression of sirtuin-6 (SIRT6) suppressed epidural fibrosis in vitro. (A) Cell apoptosis detected with propidium iodide/annexin V dual staining after pcDNA-SIRT6 transfection for 72 h. (B) Cell viability detected with MTT method after pcDNA-SIRT6 transfection for 72 h. (C) Expression of SIRT6, transforming growth factor β (TGF-β) and collagen type I (Col I) proteins detected with western blotting after pcDNA-SIRT6 transfection for 72 h. (D) Expression of interleukin (IL)-1α mRNA detected via qPCR. *P<0.05, **P<0.01 vs. the control. Control, untreated fibroblasts.
mRNA was inserted into a pGL3 vector and a luciferase reporter gene assay was performed. The results showed that the miR-21 mimic significantly decreased the fluorescence intensity, while the decrease was rescued by pcDNA-TGF-β2 transfection (Fig. 3C). In primary fibroblasts, miR‑21 mimic transfection caused a marked reduction in TGF-β2 and Col I protein expression (Fig. 3D). Subsequently, the relationship between miR‑21 and SIRT6 was predicted using ChIPBase. The results revealed that SIRT6 was likely to be a transcription factor of miR-21 (Fig. 4A). In addition, the overexpression of SIRT6 increased the expression of pri-, pre- and mature miR-21 transcripts (Fig. 4B). These data demonstrated that SIRT6 negatively regulated TGF-β2 expression and fibrosis by promoting miR-21 expression.

SIRT6 suppresses EF and scar formation after laminectomy in the Xenopus tropicalis model of lumbar disc herniation. The role of SIRT6 in the suppression of EF and scar formation was investigated in vivo. A Xenopus tropicalis model of lumbar disc herniation was established with caudal vertebral pulposus transplantation. The pcDNA-SIRT6 vector or miR-21 inhibitor were locally injected into the models around the laminectomy site every day. After 6 weeks, the Xenopus models were sacrificed and their epidural scar tissues were removed carefully. The mass of the scars was examined using an electronic analytical balance. The results showed that pcDNA-SIRT6 injection significantly reduced the mass of the total scar and also Col I (Fig. 5A). The rate of Col I/total scar was also reduced by pcDNA-SIRT6 injection (Fig. 5C). By contrast, injection with the miR-21 inhibitor increased the total mass of the scar, Col I and the proportion of the Col I/total scar (Fig. 5A-C). Masson's staining analysis showed that the fibers in the pcDNA-SIRT6-injected group were more scattered and slightly stained (Fig. 6A), suggesting a lesser degree of fibrosis. Moreover, the number of vimentin positive (vimentin+) cells in the pcDNA-SIRT6 injection group was much smaller than the control (Fig. 6B). By contrast, injection of the miR-21 inhibitor exerted the opposite effect to pcDNA-SIRT6 injection (Fig. 6A and B). These data indicate that SIRT6 had a suppressive effect on EF and scar formation in vivo.

Discussion

As an important member of the TGF-β family, TGF-β1 has been noted to be the master transcription factor of tissue fibrosis (24). The role of TGF-β2 in fibrosis has caught the attention of researchers: previous studies have indicated that TGF-β2 acted synergistically with TGF-β1 in the fibrotic pathway in certain types of cells and even regulated fibrosis‑related biological processes, including spinal fibrosis (16,25,26). In the present study, we found that TGF-β2 was markedly upregulated in patients who developed epidural scars, and we noted a novel SIRT6/miR‑21 pathway and the inhibition of EF and scar formation upon SIRT6 vector transfection.

The suppression of cell apoptosis and cellular senescence means that SIRT6 acts as a critical regulator in growth inhibition in many types of normal and cancerous cells; it has previously been noted that SIRT6 plays a protective role in the progression of many chronic illnesses including obesity, tumorigenesis, fatty liver, diabetes and cardiac hypertrophy (27,28). Previous studies have revealed that SIRT6 also plays a suppressive role in liver and cardiac inflammation or fibrosis (27,29). However, the effect of SIRT6 on spinal EF had not received so much attention. In the present study, we noted a decreased expression of SIRT6 in patients who developed epidural scars after laminectomy. Our in vitro SIRT6 overexpression study indicated that SIRT6 suppressed epidural fibrotic scar formation, as was clear from our examination of fibroblast proliferation, TGF-β2
and IL-1α expression, and Col I production. To the best of our knowledge, this is the first report on the role of SIRT6 and its regulation of spinal EF and scar formation.

The action of SIRT6 in terms of regulating expression or activity of other genes is largely dependent on its deacetylase activity. For instance, SIRT6 overexpression has been shown to induce apoptosis through p53 and p73 activation in many cancer cells (19). However, deacetylating proteins at a post-translational level was not the only functional mechanism of SIRT6. A recent study showed that SIRT6 promoted FoxO1 nuclear exclusion and had a significant effect on gluconeogenesis (30). In the present study, our ChIPBase analysis and detection of

Figure 4. Sirtuin-6 (SIRT6) was a promoter of microRNA-21 (miR-21) in human primary epidural fibroblasts. (A) The transcription factor (TF)-miRNAs network integrated by online platform ChIPBase. (B) Overexpression of SIRT6 promoted expression of pri-, pre- and mature miR-21 transcripts. Human primary epidural fibroblasts were isolated and seeded in 6-well plates. On reaching 80% confluence, 1 µg pcDNA-SIRT6 was transfected into the fibroblasts with Lipofectamine® 3000. After transfection for 72 h, expression of miR-21 transcripts was detected using qPCR. *P<0.05 vs. the control (untransfected cells).
miR-21 transcripts after SIRT6 overexpression showed that SIRT6 is likely a transcription factor for miR-21. We suggest that this is a novel mechanism for SIRT6 regulation of other genes. Moreover, the targeting relationship between miR-21 and TGF-β2 is also a novel finding of this study.

Animal models of lumbar disc herniation are an important tool for the exploration of treatment methods and prognosis evaluation. The animal model used in this study was established using the method of caudal nucleus pulposus transplantation (31), which is generally accepted in research on lumbar disc herniation. Although it would have been optimal to establish the model using animals which are closer to humans, considering both the animal experimental cost and the conservation of the TGF-β2 gene, we finally determined to use Xenopus tropicalis. This is more convenient for obtaining epidural fibrotic scar tissue.

In conclusion, we examined the role and mechanisms of SIRT6 in suppressing postoperative epidural scar formation. We showed that SIRT6 promoted the expression of miR-21 and then suppressed TGF-β2 expression in a targeted manner.
References


