Animal Study

Reduction of SIRT1 Mediates Monosodium Iodoacetate-Induced Osteoarthritic Pain by Upregulating p53 Expression in Rats

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Background: Clinically, chronic pain is the most common and disabling symptom of osteoarthritis (OA). OA pain is associated with OA lesion of the knee and the plastic changes in the peripheral and central nervous systems. However, the central mechanisms involved at the spinal cord level are not fully understood.

Objectives: The aim of this study was to identify the mechanism underlying the role of spinal cord Sirtuin 1 (SIRT1) in OA pain induced by intraarticular injection of monosodium iodoacetate (MIA) in rats.

Study Design: Controlled animal study.

Methods: MIA was injected intraarticularly into the rat knee joint for the induction of OA. The OA lesion of the knee was assessed by histopathological examination. The mechanical allodynia were measured over 21 days post-injection by von Frey filaments. The messenger RNA and protein levels of SIRT1 and p53 were determined by real-time quantitative polymerase chain reaction and western blotting, respectively. Involvement of SIRT1-mediated p53 expression in the development of MIA-persistent pain was studied using intrathecal (i.t.) injection of the SIRT1-activating molecule resveratrol and intraperitoneal (i.p.) injection of the p53 inhibitor pifithrin-mu (PTF-μ).

Results: MIA induced mechanical allodynia, decreased the expression of SIRT1, and upregulated the expression of p53 in the spinal dorsal horn. Consecutive i.t. injection of resveratrol or i.p. injection of PTF-μ alleviated the MIA-induced mechanical allodynia. Upregulation of dorsal horn SIRT1 expression by i.t. injection of resveratrol also inhibited the increase of dorsal horn p53 induced by MIA. Moreover, silencing of dorsal horn SIRT1 expression by i.t. administration of small interfering RNA SIRT1 into normal rats induced the mechanical allodynia and upregulation of p53 expression in the dorsal horn.

Limitations: More underlying mechanism(s) of the role of p53 signaling pathway in OA pain need to be explored in future research.

Conclusions: These findings suggest that the reduction of dorsal horn SIRT1 mediated upregulation of p53 expression, which plays a critical role in persistent pain induced by OA. The i.t. drug delivery treatments targeting the spinal cord SIRT1/p53 pathway might be novel therapeutic options for OA-induced persistent pain.

Key words: Osteoarthritis, persistent pain, sirtuin 1, p53, spinal dorsal horn

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Osteoarthritis (OA) (1) is one of the most common forms of arthritis worldwide, characterized by chronic pain and substantial functional impairment. Pain is the predominant clinical feature and cannot be completely cured due to its incompletely elucidated pathogenesis. Apart from the

enhanced responsiveness of peripheral nociceptors induced by joint inflammation and structural bone tissue damage (1-3), pain sensitization by means of neuropathic pain mechanisms (4) seems to be present in a large proportion of OA patients. While there are considerable insights into the peripheral pain mechanisms in OA (5,6), there is a poor understanding of the central mechanisms underlying OA pain. Thus, there is an urgent need for the in-depth study of its molecular mechanisms at the level of the spinal cord, in hope of finding better therapy strategies.

Accumulating data indicate that tumor suppressor p53 (7), a sequence-specific transcription activator, regulates the expression of multiple genes that promote the adaptation, survival, or elimination of targeted cells thereby maintaining the genomic stability of an organism. Evidence suggests that the increase of p53 expression produces mitochondrial dysfunction and reactive oxygen species (ROS) accumulation, leading to pain hypersensitivity in diabetic mice (8). In addition, the p53 inhibitor pifithrin-mu (PFT-μ), which mediates cell apoptosis by preventing p53 from binding to Bcl-xL and Bcl-2 at the mitochondrial surface without affecting p53 transactivation activities (9), has been reported to inhibit the early cisplatin-induced increase in mitochondrial p53 (10) in the dorsal root ganglia and peripheral nerves, thereby protecting against chemotherapy-induced peripheral neuropathy. Studies have demonstrated that mitochondrial dysfunction in chondrocytes was also involved in the pathogenesis of OA (11,12) and targeting mitochondrial dysfunction attenuated OA (13). Notably, evidence suggests that mitochondrial superoxide and abnormal mitochondrial distribution in the spinal dorsal horn contribute to neuropathic and inflammatory pain (14). However, whether the expression or inhibition of p53 in the spinal cord contributes to or attenuates OA pain has not been determined.

Silent information regulator 1 (sirtuin 1, SIRT1), a member of the mammalian sirtuin family protein, functions as a conserved nicotinamide adenine dinucleotide-dependent histone deacetylase to participate in the modulation of transcriptional silencing and cell survival (15,16). In addition, SIRT1 knockdown increases p53 acetylation in cortical neuron apoptosis (17), and resveratrol decreases p53 acetylation and promotes hippocampus neuronal survival in Alzheimer's disease and amyotrophic lateral sclerosis models (18). SIRT1 also plays crucial roles in inflammatory and neuropathic pain (19-21). Our previous evidence has demonstrated that decreased SIRT1 in the spinal dorsal horn contributes to the development and maintenance of neuropathic pain via upregulating pain-related inflammatory gene expression (22). Oral administration of the SIRT1 activator resveratrol (23) has been reported to attenuate OA pain in monosodium iodoacetate (MIA)-induced OA model. However, whether SIRT1 mediates p53 expression in the spinal dorsal horn, thereby contributing to OA pain remains largely unknown.

MIA-induced OA model is known to be of benefit to preclinical or research studies involving inflammation and OA. Therefore, in this study, we established a rat model of OA with MIA and investigated the role of dorsal horn SIRT1 in MIA-induced OA pain by using the physiological, histological, and behavioral analysis in rats. The goal of this study was to test the hypothesis that the dorsal horn SIRT1 inhibition-mediated p53 upregulation (22) contributes to OA pain in rats.

Methods

Animals

Male Sprague-Dawley rats (180-220 g) were purchased from the Experimental Animal Center of Southern Medical University, which is certified by the Guangdong Provincial Bureau of Science. All Sprague-Dawley rats were housed separately under controlled conditions (23 ± 1°C, 55 ± 3% humidity, 12/12-hour light/dark cycles) with food and tap water ad libitum. All rats were randomly assigned to different groups. All experimental protocols were approved by the Jinan University Animal Care and Use Committee (Guangzhou, China) and were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All efforts in this study were made to minimize the number and the suffering of rats used.

Experimental Models

The MIA model of OA was induced as previously described (24). The rats were anesthetized by sodium pentobarbital (50 mg/kg, intraperitoneally [i.p.]). Then, the left joint was shaved, swabbed with 75% ethanol, and MIA (3 mg in 50 μl of sterile saline, Sigma-Aldrich, United States) was injected into the joint space (intraarticular) using a 26.5 G needle. The knee was manually extended and flexed for 30 s to make MIA disperse throughout the joint. The control rats received an equivalent volume of vehicle saline.

Histopathological Examination

The rats were euthanized with sodium pentobarbital (50 mg/kg, i.p.), the left knee joints with an MIA injection were fixed with 10% formalin for 24 hours, decalcified in 10% EDTA in phosphate buffered saline for 8 weeks, and then dehydrated in increasing concentrations of ethanol and embedded in paraffin. Then, each sample was sectioned into 3 μM, followed by staining with Hematoxylin-Eosin (HE) and Safranin O-Fast green. The stained sections were observed under microscopy and statistically graded on a scale of 0-5 by double-blind observation (26), according to the Osteoarthritis Research Society International (OARSI) scoring system (25). The OA severity was determined by using a 0-5 scoring system: 0 for normal cartilage; and 1, 2, 3, 4, and 5 for 5-10%, 11-25%, 26-50%, 51-75%, and > 75%, respectively, of the total projected cartilage area affected by matrix or chondrocyte loss (27).

Drug Treatment and Lumbar Subarachnoid Catheterization

PFT-μ (25 mg/mL in dimethyl sulfoxide (DMSO), Sigma Aldrich, United States) or resveratrol (15 mg/mL in DMSO, Sigma Aldrich, United States) was diluted in sterile saline. PFT-μ was administered i.p. at a dose of 8 mg/kg (10,28) for 8 consecutive days from day 14 to day 21 following MIA treatment. Resveratrol (50 μg/10 μl, 250 μg/10 μl, and 500 μg/10 μl, Sigma-Aldrich, United States) or vehicle saline (10 μl) was administrated intrathecally (i.t.) for 8 consecutive days from day 14 to day 21 following MIA treatment. A small interfering RNA (siRNA) was chemically modified for enhancing stability with methylation and efficiency delivery with cholesterol-conjugation, and it was commercially purchased from Ribobio. The saline was used for siRNA delivery. SIRT1 siRNA (50 μg/10 μl, Ribobio, China) or scrambled siRNA (50 μg/10 μl, Ribobio, China) was i.t. administrated for 10 consecutive days from day 1 to day 10.

The i.t. injection was performed according to the previously described method from our study (22). After each rat was anesthetized with sodium pentobarbital (50 mg/kg, i.p.), a polyethylene i.t. catheter (PE-10, Becton Dickinson, United States) was implanted into the lumbar subarachnoid space of the rat between the fifth and sixth lumbar vertebrae with the catheter tip located near the L5 spinal segmental level. Following i.t. implantation of the catheter, each rat was allowed to recover from surgery for 5 days prior to the subsequent drug treatment, and any rats exhibiting paresis or paralysis of the hind limb were excluded from this study. Ten microliters of 2% lidocaine were injected into each rat to ensure the correct catheter position, as indicated by transient paralysis of the bilateral hind limb.

Behavioral Assessment

The 50% paw withdrawal threshold was tested using von Frey filaments as described previously (29). The rats were placed in a plastic box on a metal mesh and von Frey filaments with different bending forces were presented alternately to the midplantar surface of each rat's hind paw after being acclimated to the mesh. The mechanical allodynia of each rat was assessed by the mechanical withdrawal threshold of its hind paw in response to probing with von Frey filaments for 6 s or until the rat withdrew. A nociceptive response of a rat was defined as a brisk paw withdrawal or a paw flinching after von Frey filament application. Each trial was repeated 2 or 3 times at 2 min intervals. The 50% paw withdrawal threshold was calculated for each rat following a previous validation with the "up-down" calculating method (29). The behavioral tests of rats were conducted by the experimenter who was blinded to all treatments.

Real-time Quantitative PCR

The rats were euthanized with sodium pentobarbital (50 mg/kg, i.p.) at different time points. The rats' L4-L6 spinal cord tissues were immediately removed and the dorsal horn was dissected under a light microscope. Total RNA was extracted from the L4-L6 spinal dorsal horn with TRIzol Reagent (Invitrogen, United States). The RNA was transcribed into complementary DNA (cDNA) using RT Master Mix (Takara, Japan) according to the protocol based on the manufacturer's instructions. TB Green™ Premix Ex Taq™ II (Takara, Japan) and Bio-Rad CFX96 touch Detection System were used for real-time quantitative polymerase chain reaction (PCR). The amount of synthesized cDNA was evaluated by PCR using the following primer sequences: β-actin (rat): forward 5′-AGGGAAATCGTGCGTGACAT-3′, and reverse 5′-GAACCGCTCATTGCCGATAG-3′; SIRT1 (rat): forward 5′-TTGGCACCGATCCTCGAA-3′, and reverse 5′-ACAGAAACCCCAGCTCCA-3′; P53 (rat): forward 5′-GTTCGTGTTTGTGCCTGTCC-3′, and reverse 5′-TGCTCTCTTTGCACTCCCTG-3′. PCR amplification was conducted with an initial denaturing step at 95 °C for 30 s, then 40 cycles at 95 °C for 5 s, and at 60 °C for 30 s. The results reflect the average results from at least 3 technical replicates. The relative messenger RNA

(mRNA) expression ratio in the L4-L6 spinal dorsal horn was quantified by the 2-△△CT method (30).

Western Blotting

The rats were euthanized with sodium pentobarbital (50 mg/kg, i.p.) at different time points. The rats' L4-L6 spinal cord tissues were immediately removed and the dorsal horn was dissected under a light microscope. Then, the rats' L4-L6 spinal dorsal horn tissues were homogenized in the ice-cold Tris solution (15 mmol/l) containing a cocktail of proteinase and phosphatase inhibitors. Next, the lysate tissues of the L4-L6 dorsal horn were prepared. The homogenate was centrifuged at 13,000 g for 15 min at 4 °C, and the supernatant was collected. The total protein was quantified by using the Bradford assay (P0006, Beyotime, China) based on the manufacturer's instructions. Protein lysates (50 μg) were separated by sodium dodecyl-sulfate polyacrylamide gel electrophoresis (10% gels) and subsequently transferred onto a polyvinylidene fluoride membrane (Millipore Corporation, United States) at 100 V for 120 min. The membrane was incubated for 1 h at room temperature with 5% nonfat milk, then incubated overnight at 4 °C with primary antibodies against SIRT1 (1:2000, Abcam, United Kingdom), P53 (1:1000, Abcam, United Kingdom), and GAPDH (1:1000, Santa Cruz Technology, United States). The membrane was washed 3 times with tri-buffered saline Tween and was then incubated with a goat anti-mouse secondary antibody in horseradish peroxidase-conjugated (1:10000) secondary antibody (Beyotime, China) for 1 h at room temperature. Finally, immunoblots in the membrane were developed and visualized by an enhanced chemiluminescence system (BeyoECL Star, China) based on the manufacturer's instructions. Images of the protein bands on the Western blots were measured with a computer-assisted imaging analysis system (NIH ImageJ, United States).

Statistical Analysis

All data were expressed as the mean values ± standard error of mean, and analyzed with SPSS 19.0 (SPSS, United States) and Origin 8.0 (Origin Lab Corporation, United States). For the painful behavior tests, one-way or two-way analysis of variance (ANOVA) with repeated measures followed by a Tukey post-hoc test was carried out. The western blotting and real-time quantitative PCR data were analyzed by one-way ANOVA followed by a Tukey post-hoc test. A value of $P < 0.05$ was considered significant. While no power analysis was performed, the sample size was determined according to our and peers' previous publications in painful behavior and pertinent molecular studies.

Results

Validation of the Rat Model of Osteoarthritic Pain

As articular cartilage degeneration is the main histopathological feature of OA joints, the histopathological changes of articular cartilage and severity of cartilage damage in the MIA-induced OA rats were assessed by HE and Safranin O-Fast green staining. The HE staining revealed that the vehicle control group rats showed normal articular cartilage structures with smooth articular surfaces, normal chondrocytes with columnar orientation, and intact tide marks and subchondral bone (Fig. 1A). The MIA group rats exhibited the severity of surface irregularity and cleft, matrix loss of articular cartilage, degeneration of columnar orientation, degeneration of the tide mark, and the penetration of subchondral bones on days 14 and 21 after injection (Fig. 1A). Safranin O-Fast green staining also indicated moderate saffron staining in OA rats on day 7 following MIA injection (Fig. 1A), and severe cartilage destruction occurred on days 14 and 21 after injection (Fig. 1A). The severity of the articular cartilage degradation was measured using the OARSI grading system, and the combined score of the articular cartilage was significantly increased on days 7, 14, and 21 after MIA injection (Fig. 1B). With the prolongation of post-injection time, cartilage destruction showed a tendency to be aggravated. These results confirmed the successful induction of OA in rats with MIA intraarticular injection. In addition, behavioral tests revealed that the MIA rats showed a significant decrease in paw withdrawal threshold on day 7 following MIA injection, and the decrease persisted to study termination on day 21 (Fig. 1C). These results confirmed the successful induction of OA pain in rats with MIA intraarticular injection.

Upregulation of p53 Expression in the Dorsal Horn was Involved in MIA-Induced Osteoarthritic Pain

Next, we observed the time course of the expression of p53 mRNA and protein levels in the spinal dorsal horn of the rats with OA to illustrate the function of p53 in OA pain. The results showed that the expression of p53 mRNA and protein levels were upregulated in the spinal dorsal horn of the rats with MIA injection on days 14, 18, and 21 compared with the expression

in vehicle rats (Figs. 2A and 2B). In addition, behavioral results showed that injection of PFT-μ at a dosage of 8 mg/kg i.p. from days 14 to 21 for 8 consecutive days following MIA treatment attenuated the mechanical allodynia induced by MIA (Fig. 2C). Moreover, i.p. injection of PFT-μ inhibited the increase of p53 mRNA and protein levels in the rats' dorsal horn on day 21 following MIA administration (Figs. 2D and 2E). These results demonstrated that p53 upregulation in the dorsal horn contributed to the persistent pain hypersensitivity induced by OA.

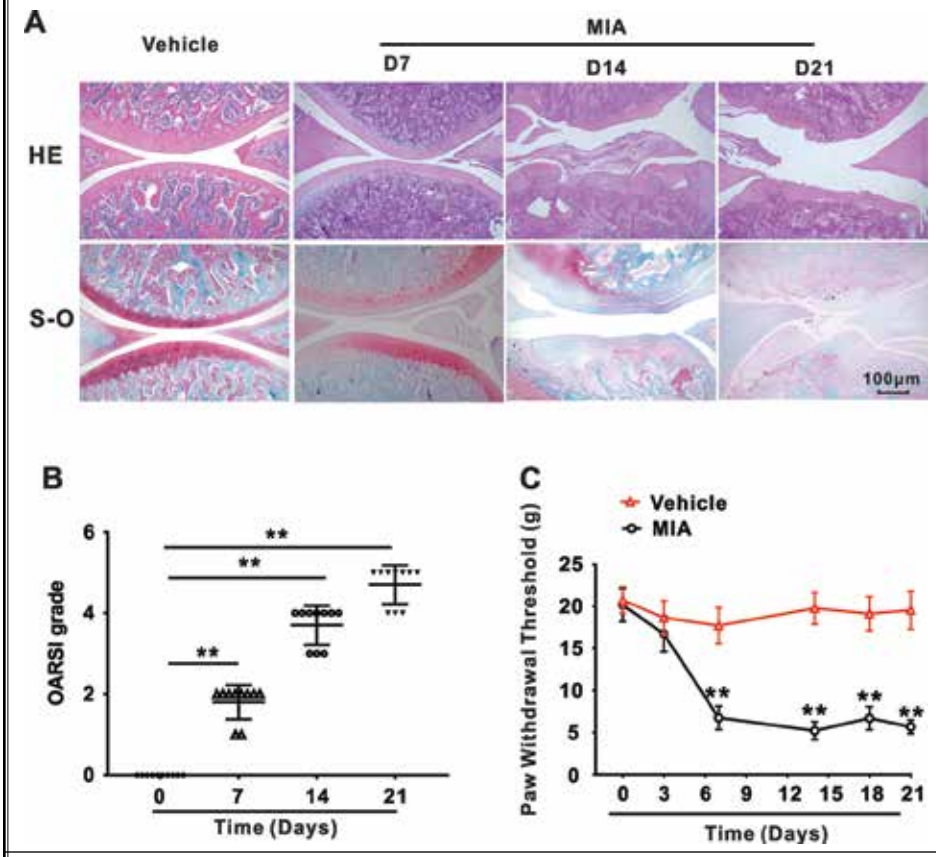

Fig 1. *MIA induces osteoarthritis and mechanical allodynia in rats. (A) MIA induced time-dependent knee articular lesion. Hematoxylin-Eosin (HE) and Safranin O-Fast green (S-O) histologic section images of knee joints in MIA-treated rats and vehicle rats. Scale bar = 100 μm. (B) Histograms show the cartilage lesion on days 7, 14, and 21 after the injection of MIA. The knee joint lesions are graded on a scale of 0–6 using OARSI score, giving a combined score for cartilage structure in MIA-induced osteoarthritic and vehicle rats. n = 10 in each group, **P < 0.01 versus vehicle group. (C) Von Frey filaments are used to measure mechanical allodynia. Intra-articular injections of MIA markedly decrease the mechanical paw withdrawal threshold of rats. n = 12 in each group, **P < 0.01 versus vehicle group.*

Reduction of SIRT1 Expression in the Spinal Dorsal Horn Contributed to MIA-Induced Osteoarthritic Pain

In the present study, we observed the time course of the expression of SIRT1 mRNA and protein levels in the spinal dorsal horn of rats with OA to illustrate the function of SIRT1 in OA pain. The results showed that the expression of SIRT1 mRNA and protein levels were significantly downregulated in the spinal dorsal horn of rats with MIA injection on days 14, 18, and 21 compared with the expression in vehicle rats (Figs. 3A and 3B). In addition, behavioral results showed that i.t. injection of resveratrol, a specific SIRT1 activator, from days 14 to 21 for 8 consecutive days at a dosage of 500 or 250 μg, but not 50 μg attenuated the mechanical allodynia induced by MIA (Fig. 3C). The i.t. injection of resveratrol also prevented the decrease of SIRT1 mRNA and protein levels in the rats' dorsal horn on day 21 following MIA administration (Figs. 3D and 3E). These results demonstrated the critical role of dorsal horn SIRT1 reduction in persistent pain hypersensitivity induced by OA.

p53 Upregulation Induced by SIRT1 Reduction Contributes to MIA-Induced Mechanical Allodynia

Next, we observed the expression of SIRT1 in the spinal dorsal horn and the paw withdrawal threshold of the normal rats following i.t. injection of SIRT1 siRNA from days 1 to 10 for 10 consecutive days. The results showed that SIRT1 siRNA decreased the SIRT1 protein and mRNA levels in the normal rats on day 10 after i.t. injection (Figs. 4A and 4B). Meanwhile, SIRT1 siRNA

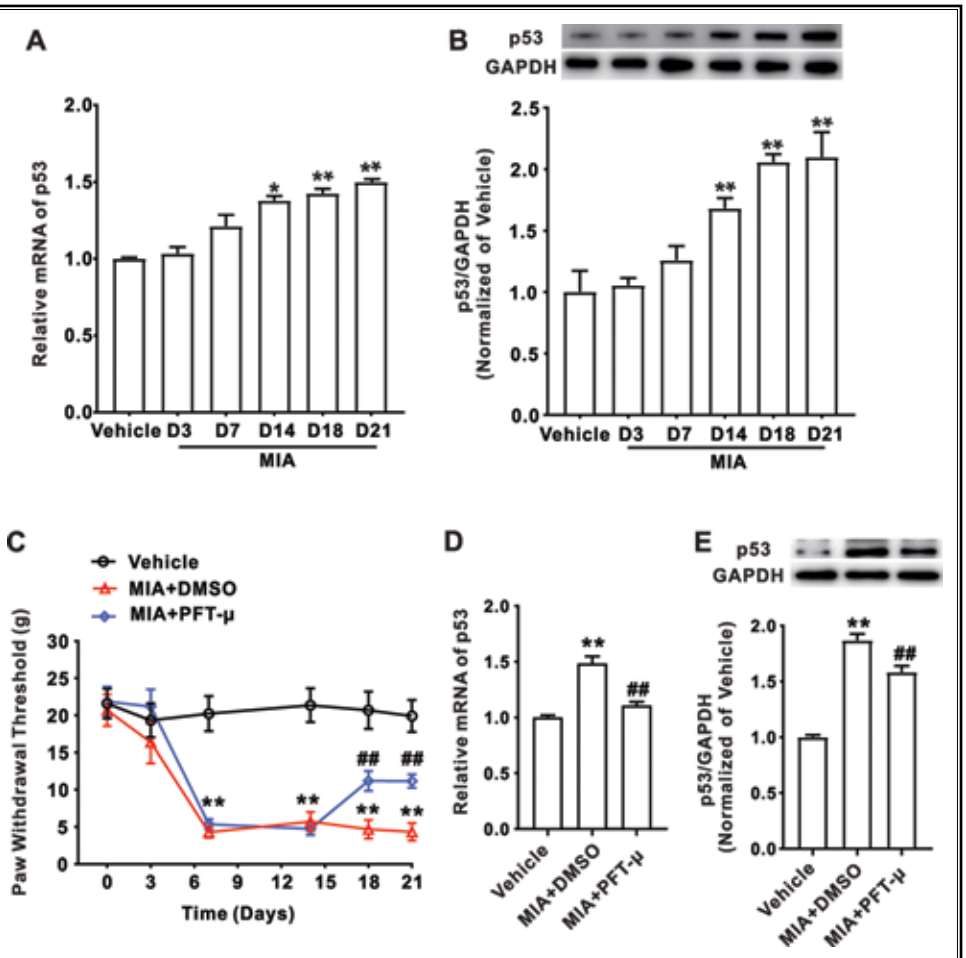

Fig 2. *Upregulation of p53 expression contributes to MIA induced mechanical allodynia. (A) MIA induced time-dependent increase of p53 mRNA expression. RT-PCR analyses results showed the quantification of p53 mRNA expression, normalized to GAPDH expression in the spinal dorsal horn from vehicle and MIA rats. n = 6 in each group, *P < 0.05, and **P < 0.01 versus the vehicle group. (B) MIA induced time-dependent upregulation of p53 protein expression. Protein level of p53 in the spinal dorsal horn of vehicle and MIA rats was detected by western blotting. Values were normalized against GAPDH. n = 3 in each group, **P < 0.01 versus the vehicle group. (C) PFT-μ inhibited the mechanical allodynia induced by MIA. Mechanical hypersensitivity behavioral testing showed that the effect of intraperitoneal administration of PFT-μ at a dosage of 8 mg/kg for 8 consecutive days on the paw withdrawal threshold of the MIA rats. n = 12 in each group, **P < 0.01 versus the vehicle group, ##P < 0.01 versus the corresponding MIA+DMSO group. (D and E) PFT-μ prevented the increase of p53 mRNA and protein expression levels in the spinal dorsal horn of the MIA rats. RT-PCR and western blotting analysis showed the expression of p53 mRNA and protein levels in the dorsal horn on day 21 in the vehicle and MIA rats with an intraperitoneal injection of PFT-μ or DMSO. n = 6 in each group for PCR and n = 3 in each group for western blotting, **P < 0.01 versus the corresponding vehicle group. ##P < 0.01 versus the corresponding MIA+DMSO group.*

induced the mechanical allodynia in the normal rats on days 7 and 10 after i.t. injection (Fig. 4C). In addition, the p53 proteins and mRNA levels were significantly upregulated in the normal rats' dorsal horn on day 10 following SIRT1 siRNA treatment (Figs. 4D and 4E). Furthermore, upregulation of SIRT1 by i.t. administration of resveratrol significantly inhibited the upregulation of p53 proteins and mRNA in the dorsal horn on day 21 following MIA injection (Figs. 4F and 4G).

Discussion

Our data suggest a new mechanism by which SIRT1 regulates p53 expression in the dorsal horn and subsequently contributes to MIA-induced mechanical allodynia. We found that MIA treatment increased the p53 expression in the spinal dorsal horn, and i.p. application of PTF-μ attenuated MIA-induced mechanical allodynia. In addition, MIA treatment decreased SIRT1 expression in the dorsal horn, and i.t. injection of resveratrol ameliorated MIA-induced p53 upregulation and mechanical allodynia. Moreover, knockdown of SIRT1 using SIRT1 siRNA increased p53 expression and induced mechanical allodynia in the normal rat group. These results demonstrated the critical role of SIRT1 reduction in p53 upregulation in the dorsal horn and pain behavior induced by MIA.

The levels of p53 proteins and mRNA were sig-

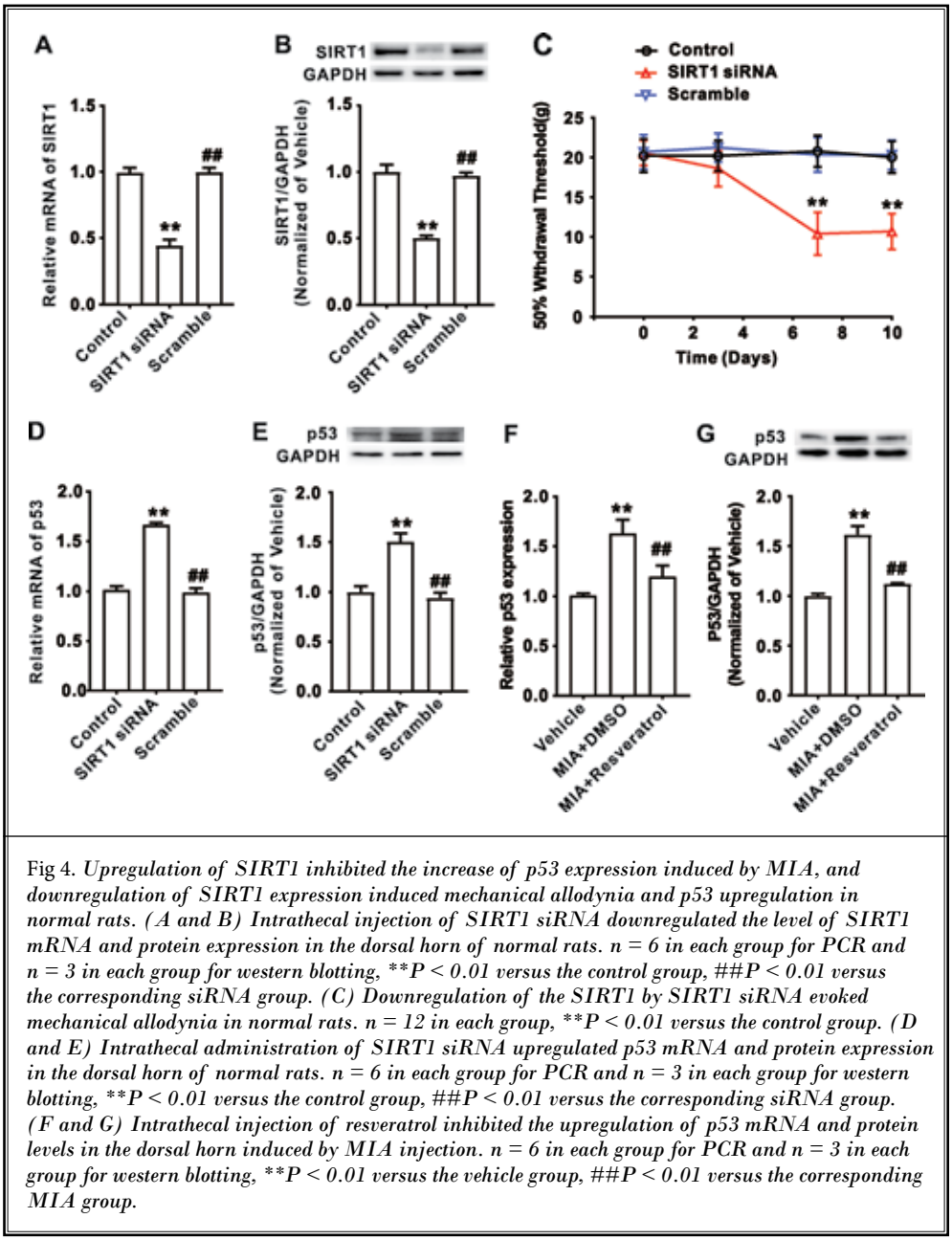

Fig 4. *Upregulation of SIRT1 inhibited the increase of p53 expression induced by MIA, and downregulation of SIRT1 expression induced mechanical allodynia and p53 upregulation in normal rats. (A and B) Intrathecal injection of SIRT1 siRNA downregulated the level of SIRT1 mRNA and protein expression in the dorsal horn of normal rats. n = 6 in each group for PCR and n = 3 in each group for western blotting, **P < 0.01 versus the control group, ##P < 0.01 versus the corresponding siRNA group. (C) Downregulation of the SIRT1 by SIRT1 siRNA evoked mechanical allodynia in normal rats. n = 12 in each group, **P < 0.01 versus the control group. (D and E) Intrathecal administration of SIRT1 siRNA upregulated p53 mRNA and protein expression in the dorsal horn of normal rats. n = 6 in each group for PCR and n = 3 in each group for western blotting, **P < 0.01 versus the control group, ##P < 0.01 versus the corresponding siRNA group. (F and G) Intrathecal injection of resveratrol inhibited the upregulation of p53 mRNA and protein levels in the dorsal horn induced by MIA injection. n = 6 in each group for PCR and n = 3 in each group for western blotting, **P < 0.01 versus the vehicle group, ##P < 0.01 versus the corresponding MIA group.*

nificantly increased in the spinal dorsal horn of MIA rats, and the i.p. injection of a p53 inhibitor, PFT-μ, inhibited the increase of dorsal horn p53 expression and MIA-induced persistent pain hypersensitivity, indicating the critical roles for dorsal horn p53 in the development and maintenance of OA pain. The tumor suppressor molecule p53, as a transcriptional factor, is a marker of irreversible injury in postmitotic cells of the central nervous system (31). It also has been reported to play critical roles in neuronal death and neuropathic pain (32,33). Based on accumulating evidence, knockout or inhibition of p53 may exert neuroprotection (34) and analgesic effects (28,33). The p53-mediated mitochondrial dysfunction is known to be responsible for diabetic neuropathic pain, and inhibition of p53 prevents the reductions in mitochondrial numbers and accumulation of mitochondrial ROS (8). So, it is possible that mitochondrial dysfunction induced by p53 may contribute to OA pain. This theory is consistent with the previous evidence that the systemic administration of p53 inhibitors also prevents diabetes-, paclitaxel-, and cisplatin-induced pain hypersensitivity (8,10,28,33).

Our data also showed that the levels of the SIRT1 proteins and mRNA were significantly downregulated in the dorsal horn of the rats with OA on days 7, 14, 18, and 21 after MIA injection, which was consistent with the results of the pain behavior assessment. Moreover, activation of SIRT1 by i.t. injection of resveratrol inhibited the mechanical allodynia induced by MIA. These results suggest that the reduction of SIRT1 contributes to MIA-induced mechanical allodynia. Similar findings have also been reported by previous studies in

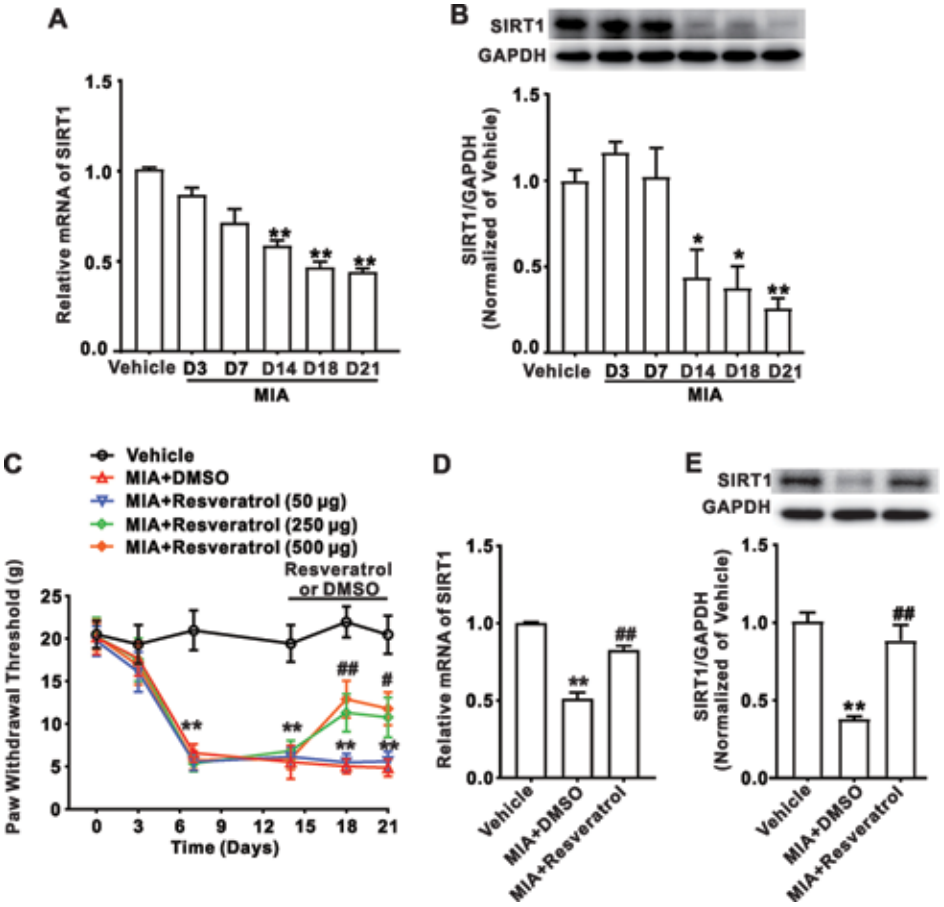

Fig 3. *SIRT1 contributes to MIA induced mechanical allodynia. (A) MIA induced time-dependent decrease of SIRT1 mRNA expression. RT-PCR analyses results showed the quantification of SIRT1 mRNA expression, normalized to GAPDH expression in the spinal dorsal horn from vehicle and MIA rats. n = 6 in each group, **P < 0.01 versus the vehicle group. (B) MIA induced time-dependent downregulation of SIRT1 protein expression. Protein levels of SIRT1 in the spinal dorsal horn of vehicle and MIA rats were detected by western blotting. Values were normalized against GAPDH. n = 3 in each group, *P < 0.05, and **P < 0.01 versus the vehicle group. (C) Resveratrol inhibited the mechanical allodynia induced by MIA in a dose-dependent manner. Mechanical hypersensitivity behavioral testing showed that the effect of intrathecal administration of resveratrol at a dosage of 500 μg/10 μl, 250 μg/10 μl, or 50 μg/10 μl (for 8 consecutive days) on the paw withdrawal threshold of MIA rats. n = 12 in each group, **P < 0.01 versus the vehicle group, #P < 0.05, and ##P < 0.01 versus the corresponding MIA + DMSO group. (D and E) Resveratrol prevented the decrease of SIRT1 mRNA and protein expression levels in the spinal dorsal horn of MIA rats. RT-PCR and western blotting analysis showed the expression of SIRT1 mRNA and protein levels in the dorsal horn of vehicle and MIA rats following intrathecal injection of resveratrol or DMSO. n = 6 in each group for PCR and n = 3 in each group for western blotting, **P < 0.01 versus the corresponding vehicle group, ##P < 0.01 versus the corresponding MIA+DMSO group.*

that SIRT1 expression in the spinal cord was decreased in bortezomib or a chronic constriction injury of the sciatic nerve-induced neuropathic pain, and resveratrol attenuated the neuropathic pain (19,22). Our results are further supported by a study in which oral administration of resveratrol attenuated OA pain in a rat model (23). This study confirmed that silencing of SIRT1 expression in the spinal dorsal horn by SIRT1 siRNA induced mechanical allodynia in the normal rats. SIRT1 originally functioned as a potential tumor promoter, since it negatively regulates the tumor suppressor p53 and other tumor suppressors (35). Previous research has demonstrated that p53 is indeed a SIRT1 target (36) and that SIRT1 deacetylates p53 to modulate p53-dependent functions in neuronal survival (17). Studies also indicated that SIRT1 reduction increased expressions of mGluR1/5 in the spinal dorsal horn via upregulating H3 acetylation levels at Grm1/5 promoter regions, and was involved in the neuropathic pain in type 2 diabetic rats (37). Notably, the present study showed that decreased SIRT1 in the spinal dorsal horn was also involved in upregulation of dorsal horn p53 expression and the persistent pain hypersensitivity induced by OA, as evidenced by our findings that upregulation of dorsal horn SIRT1 expression with resveratrol injection i.t. attenuated the MIA-induced persistent pain hypersensitivity via inhib-

iting the increase of p53 expression. Moreover, similar to the behavioral changes, the levels of p53 proteins and mRNA were increased in the dorsal horn of SIRT1 siRNA-treated rats compared with vehicle rats.

Conclusions

In conclusion, these data suggest that SIRT1 reduction contributes to MIA-induced OA pain via upregulating p53 expression in the dorsal horn of rats. Further research is still warranted to explore and better understand the mechanisms in human OA. Taken together, these findings suggest that SIRT1 inhibition-mediated p53 upregulation in the dorsal horn contributes to pain hypersensitivity induced by OA.

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Author Contributions

Ling-Jun Xu, Cui-Cui Liu, and Xiao-Ping Wang conceived the project, and designed the experiments. Ling-Jun Xu, Cui-Cui Liu, Lu-Miao Chen, and Gui-Hao Wu carried out all experiments. Ling-Jun Xu, Cui-Cui Liu, and Lu-Miao Chen analyzed the data and prepared the figures. Cui-Cui Liu and Xiao-Ping Wang supervised the overall experiment. Ling-Jun Xu and Xiao-Ping Wang revised the manuscript. All authors have read and approved the final manuscript.

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