

## Original Article

# Upregulation of P27<sup>Kip1</sup> by mitomycin C induces fibroblast apoptosis and reduces epidural fibrosis

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**Abstract:** Fibroblast excessive proliferation is considered as one of the major reasons of epidural fibrosis after laminectomy. Recent studies have shown that mitomycin C (MMC) could successfully reduce the formation of epidural fibrosis by inducing fibroblasts apoptosis. However, the detailed mechanism was still unclear. Increasing evidence indicated that P27<sup>Kip1</sup> (P27) could result in apoptotic cell death in various cells. In this study, we investigated whether MMC could induce fibroblasts apoptosis and reduce epidural fibrosis by regulating P27. Western blot analysis, Hoechst staining, Flow cytometry, and Cell Counting Kit-8 (CCK-8) assay were used to detect the effect of MMC on fibroblasts apoptosis by regulating P27 expression in vitro. Moreover, histological and immunohistochemical assays were used to evaluate the effect of MMC on reducing epidural fibrosis by regulating P27 expression in rats. The results showed that MMC could induce fibroblasts apoptosis and upregulate P27 expression in vitro. Knockdown of P27 partially attenuated MMC-induced expressions of P27 and cleaved PARP as well as increased the cell viability. MMC could reduce epidural fibrosis in a dose-dependent manner in rats by histological analysis. The expression of P27 was increased by MMC treatment as shown by immunohistochemical analysis. In conclusion, this study demonstrated that MMC could upregulate P27 expression, which subsequently induced fibroblasts apoptosis and reduced epidural fibrosis.

**Keywords:** Mitomycin C, P27<sup>Kip1</sup>, fibroblast apoptosis, epidural fibrosis

## Introduction

Epidural fibrosis is a common complication after laminectomy and results in failed back surgery syndrome, which is mainly characterized by recurrence of radicular nerve pain or lower back pain [1, 2]. Fibroblasts excessive proliferation is one of the main reasons that can produce extracellular matrixes and form the fibrous connective tissues, which subsequently transfer to scar tissues and result in serious epidural fibrosis [3, 4]. A lot of strategies, such as microsurgical technique, material implantation and drug application, have been used to reduce epidural fibrosis and achieved satisfactory results.

Mitomycin C (MMC), a famous antitumor drug, is usually used intravenously to treat various malignant tumors [5, 6]. It has been shown that MMC could prevent the formation of multiple fibrosis by inhibiting fibroblasts activities follow-

ing the ophthalmological, otolaryngologic and orthopedic surgeries [7-9]. Recently, MMC exerts obvious ability in reducing epidural fibrosis after laminectomy and discectomy by inhibiting fibroblasts proliferation or inducing fibroblasts apoptosis, which provides a potential therapy for preventing fibrosis after surgery [10]. However, MMC is an antimetabolic drug that can affect the wound healing process and cause serious complications. The detailed mechanisms associated with the function of MMC on reducing epidural fibrosis need to be further elucidated.

Aberrant genes have been reported to involve in fibroblasts activities that regulate the formation of various fibrosis. P27<sup>Kip1</sup> (CDKN1B, hereafter P27) is a member of cyclin-dependent kinase (CDK) inhibitors that regulate cell cycle progression from late G1 into S [11, 12]. It has been reported that P27 was involved in the regulation of cell proliferation, apoptosis and me-

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tabolism [13-15]. Increasing evidences demonstrated that overexpression of P27 resulted in apoptosis in human carcinoma cells, osteosarcoma cell and melanoma cells [16-18]. Moreover, knockdown of P27 could decrease dasatinib- and paclitaxel-induced apoptosis or reduced apoptosis of bone marrow mesenchymal stem cells from systemic lupus erythematosus [19, 20]. Recently, overexpression of P27 could lead to the apoptosis in human lung fibroblasts and rat fibroblasts [18]. These studies indicated that P27 involved in the regulation of various cell apoptosis.

In this study, we cultured human fibroblasts and established the laminectomy model in rats, and investigated the effect and mechanism of the MMC in inducing fibroblast apoptosis and reducing epidural fibrosis by regulating P27 expression. The study demonstrated that MMC could induce fibroblast apoptosis and reduce epidural fibrosis by upregulating P27 expression.

### Methods

#### *Fibroblast culture and treatment*

Human fibroblasts were obtained from epidural scar tissues of patients who underwent a lysis operation for epidural fibrosis. The fibroblasts were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, CA, USA) supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 100 µg/mL streptomycin (Gibco, CA, USA) in a stable environment with 5% CO<sub>2</sub> at 37°C. The fibroblasts were starved overnight and then subjected to various treatments until the cells were 80-90% confluent. Fibroblasts from three and five were used in all of the experiments.

#### *Western blot analysis*

Fibroblasts were collected after receiving various treatments and were lysed in RIPA buffer (Beyotime, Hangzhou, China) according to the manufacturer's instructions. The lysates were centrifuged and the supernatants were collected for western blot analysis. The protein concentrations were quantified using the BCA Protein Assay Kit (Thermo Scientific, Waltham, MA). The proteins were subjected to SDS-polyacrylamide gel electrophoresis and transferred onto PVDF membranes. The protein-

bound membranes were blocked with 5% skimmed milk and incubated with appropriate primary antibodies overnight at 4°C. The primary antibodies used were anti-P27 antibody (R&D system, Minneapolis, USA), anti-Bax, anti-Bcl-2 and anti-cleaved-poly ADP-ribose polymerase (cleaved PARP) antibodies (Cell Signaling Technology, USA). The anti-β-actin antibody, anti-mouse or anti-rabbit IgG were obtained from Santa Cruz Biotechnology (USA). The following day, the membranes were washed and then incubated for 1 h at room temperature with anti-rabbit/mouse secondary antibodies (Santa Cruz). The membranes were exposed using the ECL system (Millipore, Bedford, USA) and immunoreactive protein bands were observed.

#### *RNA preparation and quantitative real-time PCR*

RNA Preparation and Quantitative Real-Time PCR Total RNA and miRNA in treated cells were isolated using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Quantitative real-time PCR was performed using the Applied Biosystems 7300HT machine and Maxima TM SYBR Green/ROX qPCR Master Mix (Fermentas, USA). The primers used are as follows: P27 sense, 5'-TAATTGGGGCTCCGGCTAACT-3', reverse, 5'-TTGCAGGTCGCTTCCTTATTC-3'; GAPDH sense, 5'-TGCACCACCAACTGCTTAGC-3', reverse, 5'-GGCATGGACTGTGGTCATGAG-3'. The PCR reaction was evaluated by melting curve analysis and by checking the PCR products on 2% agarose gels. GAPDH amplification was used as an internal control. The data were processed using 2<sup>-ΔΔCt</sup> methods.

#### *Hoechst 33342 staining*

Hoechst 33342 staining was performed to observe the apoptotic fibroblasts. The fibroblasts were seeded in 6-well plates and were incubated for adherence overnight at 37°C. After treated with different concentrations of MMC for 5 min, each well and the plates were then incubated at 37°C for 24 h. The fibroblastic monolayer was then rinsed with phosphate buffered saline (PBS) and stained with 5 µg/ml Hoechst 33342 for 30 min at 37°C in the dark. Following staining, the cells astrocytes were rinsed 3 times with PBS. The morphological features of apoptosis were observed by fluorescence microscopy.

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## *Detection of apoptosis by flow cytometry*

The fibroblasts were seeded in 6-well plates and were incubated for adherence overnight at 37°C. Different concentrations of MMC were then added to each well and the plates were then incubated at 37°C for 24 h. The fibroblasts were then collected and washed twice with ice-cold PBS. The cells were resuspended in 1× binding buffer at a concentration of 1×10<sup>6</sup> cells/ml and 100 µl cell suspension was incubated with 5 µl Annexin V-FITC and 5 µl propidium iodide (BD Biosciences, USA) for 15 minutes at room temperature in the dark. Before analysis by Beckman Coulter FC500 flow cytometry system 400 µl 1× binding buffer was added in the mixture.

## *Cell transfection and infection*

The lentivirus vectors pLV-P27<sup>kip1</sup>-inhibitor (target sequences: 5'-AAGGTTGCA TACTGAGCCA-AG-3') along with the packaging plasmid mix were purchased from Shanghai GenePharma Co., Ltd. Individual plasmids and the packaging mix were cotransfected into HEK293TN packaging cell lines using a commercial reagent (Invitrogen). The culture supernatant was concentrated using the Lenti-Pac Lentivirus Concentration Solution (Gene-Copoeia, Inc., Guangzhou, China). The control plasmid provided with the lentivirus kit was processed as described above to obtain the control pseudovirus (sequence: 5'-GTCCCGGATACCTAATAAAA-3'). Human fibroblasts were incubated with the lentiviruses in the presence of 2 µg/ml polybrene (Gibco, CA, USA) and were cultured with 2 µg/ml puromycin (Sigma, CA, USA) for at least 96 hours to select stably transfected cells. Stably transfected cells were used for the following subsequent experiments: MMC-treated, CCK-8 assay, Hoechst staining and Western blot.

## *Cell viability test*

Cell Counting Kit-8 (CCK-8) was used to determine cell viability. The human fibroblast cells were cultured in triplicate in 96-well plates. The cell viability was determined according to the manufacturer's instructions. The CCK-8 solution (10 µl) was added to each well and incubated for 2 h at 37°C. The cells that were stained with CCK-8 were considered viable and the data are presented as a percentage compared to control cells.

## *Laminectomy models and topical application of drug*

The study was approved by the Animal Research Committee of Clinical Medical College of Yangzhou University and the animals were received care in compliance with the principles of International Laboratory Animal Care. Twenty-four healthy male Sprague Dawley rats (200~250 g) were randomly divided into four groups (six rats per group), 0.5 mg/ml MMC group, 0.2 mg/ml MMC group, 0.1 mg/ml MMC group and control group (saline). The spinous process and vertebral plate of L1 were removed after anesthesia by intraperitoneal injection of ketamine. The dura mater was exposed and the laminectomy of L1 was performed. After satisfactory hemostasis of laminectomy defects, cotton pads soaked with different concentrations of MMC or saline were topically applied to the laminectomy defects for 5 min. The muscles and skin were subsequently sewed in layers. The rats were housed in cages individually and had access to chow and water.

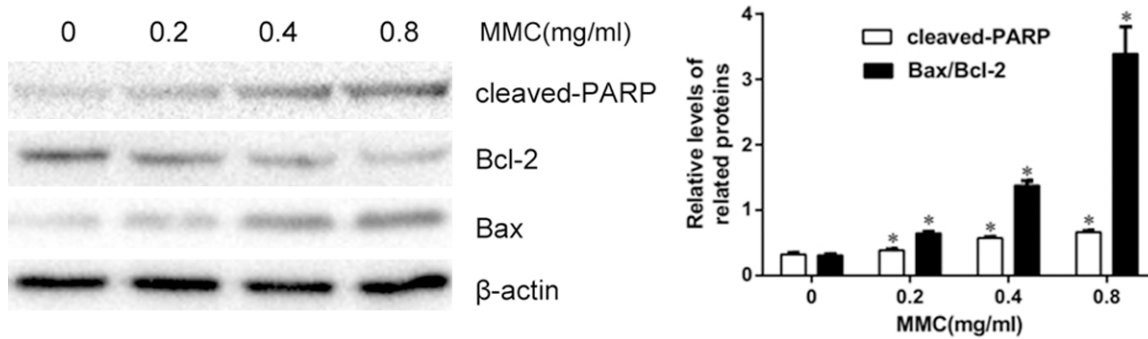
## *Histological analysis*

The rats were selected from each group for histological analysis at four weeks postoperatively. The rats were anesthetized by an overdose of urethane and underwent intracardial perfusion with 4% paraformaldehyde. The entire L1 spinal column was removed en bloc and immersed in 10% buffered formalin. The specimens were embedded in paraffin after decalcification by ethylenediamine tetraacetic acid (EDTA) and glycerol solution. Successive transverse sections were made through the L1 vertebra from the top to the bottom. The odd sections were stained with hematoxylin-eosin and the epidural fibrosis was evaluated by light microscopy using ×40 magnification.

## *Immunohistochemical staining*

After sections were made through the L1 vertebra, the even sections were deparaffinized and rehydrated through gradient ethanol solutions. The sections were incubated in citrate buffer to activate the antigenicity and exposed to 3% H<sub>2</sub>O<sub>2</sub> to block endogenous peroxidase. All subsequent incubations were performed in a humidified chamber at 37°C unless otherwise stated. The sections were blocked in common goat serum solution, followed by incubation

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**Figure 1.** MMC could increase the expression of apoptotic proteins. Western blot analysis showed that MMC increased the expression of cleaved PARP and Bax, and decreased the expression of Bcl-2 in a dose-dependent manner. The ratio of Bax/Bcl-2 was significantly increased by MMC treatment. The histogram in this panel represents the mean  $\pm$  S.E.M of three independent experiments. \* $P < 0.05$  versus control group (0 mg/ml).

overnight with rabbit polyclonal anti-P27 at 4°C. The sections were then washed in phosphate-buffered saline and incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG antibody. The fibroblasts in sections were visualized by using 3,3'-diaminobenzidine for 5 min at room temperature, and nuclei were counterstained with hematoxylin for 3 min. The sections were analyzed under the light microscope with  $\times 400$  magnification. The expression level of P27 was quantified by mean OD value.

### Statistical analysis

Statistical analysis was performed using SPSS software (version 19.0). The data from the experiments are presented as the means  $\pm$  S.E.M. The differences among treatment groups were analyzed using Student's t-test or one-way ANOVA followed by the Student-Newman-Keuls (SNK) test.  $P < 0.05$  was considered statistically significance.

## Results

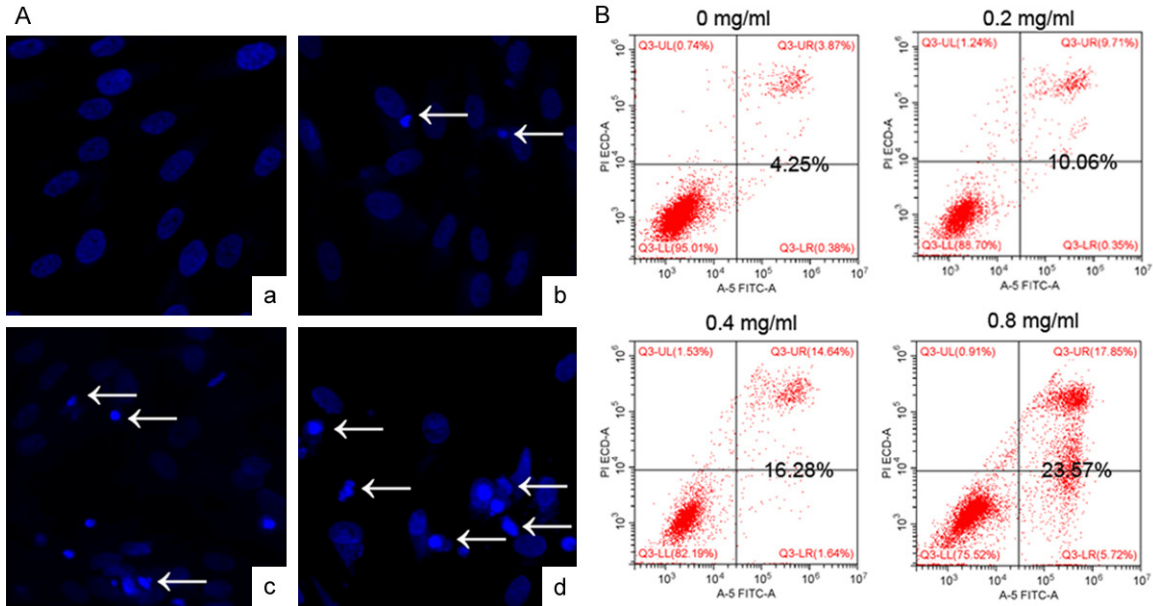
### MMC induces human fibroblasts apoptosis

To determine the effect of MMC on human fibroblasts apoptosis, the fibroblasts were treated with different concentrations MMC (0, 0.2 mg/ml, 0.4 mg/ml, 0.8 mg/ml) MMC for 5 min and subsequently cultured for 24 h. As shown in **Figure 1**, western blot analysis demonstrated that MMC increased the expressions of cleaved PARP and Bax, and decreased the expression of Bcl-2. The ratio of Bax/Bcl-2 was also significantly increased by MMC treatment. Moreover, morphological examinations were

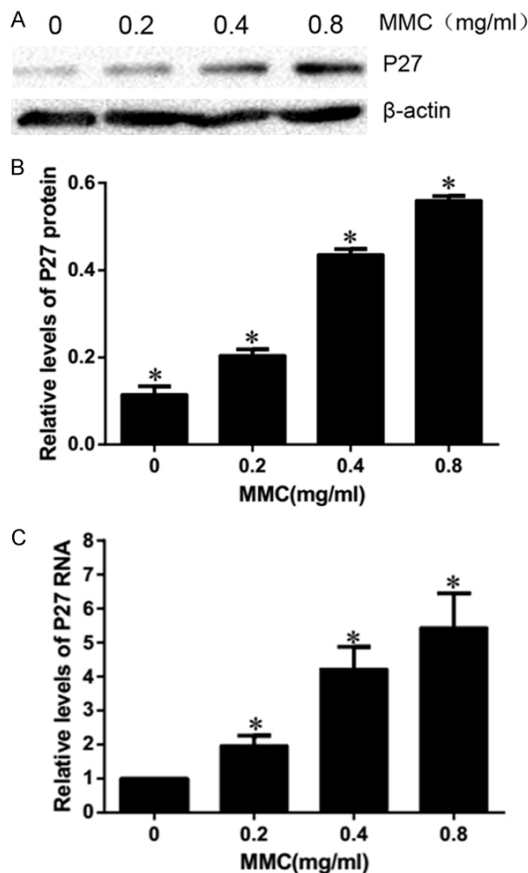
performed using Hoechst 33342 staining to validate the apoptotic status of human fibroblasts after treated with MMC. As shown in **Figure 2A**, normal fibroblasts exhibited intact nuclei and adqulis chromatin in Hoechst staining. However, the significant characteristic of cell apoptosis such as chromatin agglutination and nuclear fragmentation were observed after treatment with MMC by the fluorescence microscopy. As the MMC concentration increased, the extent of fibroblast apoptosis gradually increased. As shown in **Figure 2B**, Annexin V-FITC and propidium iodide double staining result demonstrated MMC could promote fibroblast apoptosis. The results of Western blot analysis, Hoechst staining and Annexin V-FITC and propidium iodide double staining demonstrated that MMC could induce human fibroblast apoptosis and showed a dose-dependent manner.

### MMC upregulates P27 expression in human fibroblasts

To determine the effect of MMC on P27 expression in human fibroblasts, human fibroblasts were treated with different concentration of MMC for 5 min and were then subsequently cultured for 24 h. As shown in **Figure 3**, Western blot analysis showed that MMC could upregulate P27 expression. The P27 expression gradually increased with the increase of time. Moreover, the result of qRT-PCR analysis also showed that MMC could upregulate P27 mRNA expression in a dose-dependent manner. Therefore, the results indicated that MMC could upregulate P27 expression in human fibroblasts.



**Figure 2.** MMC induced fibroblasts apoptosis. Cell treated by 0 mg/ml, 0.2 mg/ml, 0.4 mg/ml and 0.8 mg/ml MMC. A. The morphological features of fibroblast apoptosis were observed by fluorescence microscopy. Arrows were showing condensed or fragmented nuclei (a. 0 mg/ml; b. 0.2 mg/ml; c. 0.4 mg/ml; d. 0.8 mg/ml). The magnification was 400. B. The apoptosis rates were analyzed by Flow cytometry after Annexin V/Propidium Iodide Double Staining. The fibroblasts apoptosis rates increased with the increase of MMC concentration.

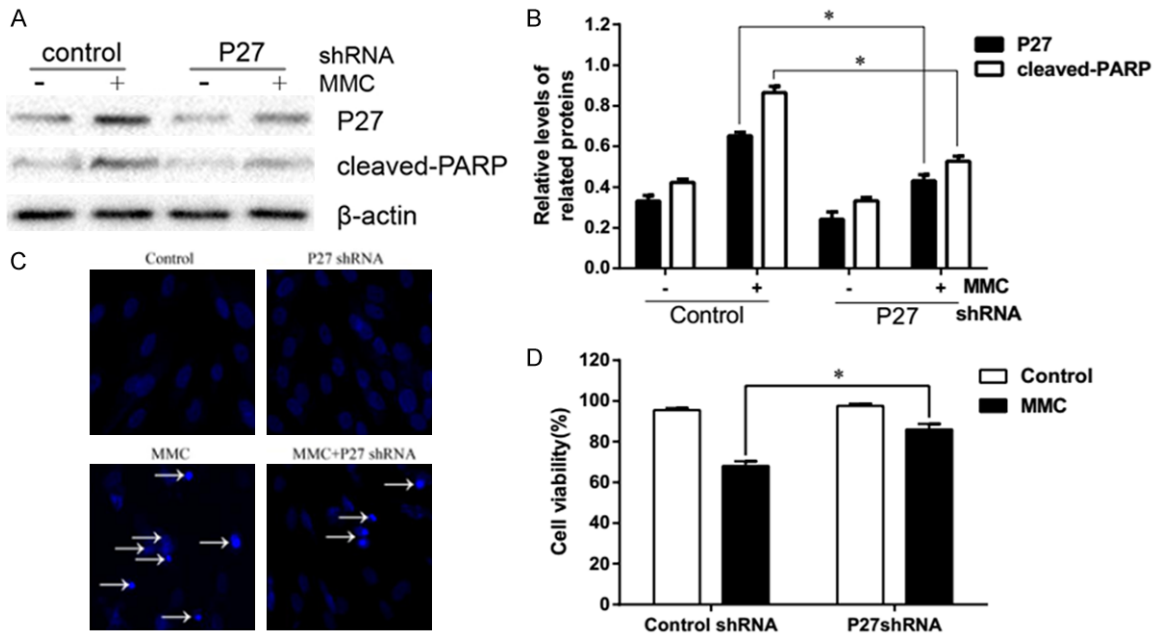


**Figure 3.** Effect of MMC on P27 expression. A. Western-blot showed that MMC could increase in P27 expression in a dose-dependent manner. B. The histogram in this panel represents the mean  $\pm$  S.E.M of three independent experiments. \* $P < 0.05$  versus control group (0 mg/ml). C. qRT-PCR showed that MMC could increase the expression of P27 mRNA. \* $P < 0.05$  versus control group (0 mg/ml).

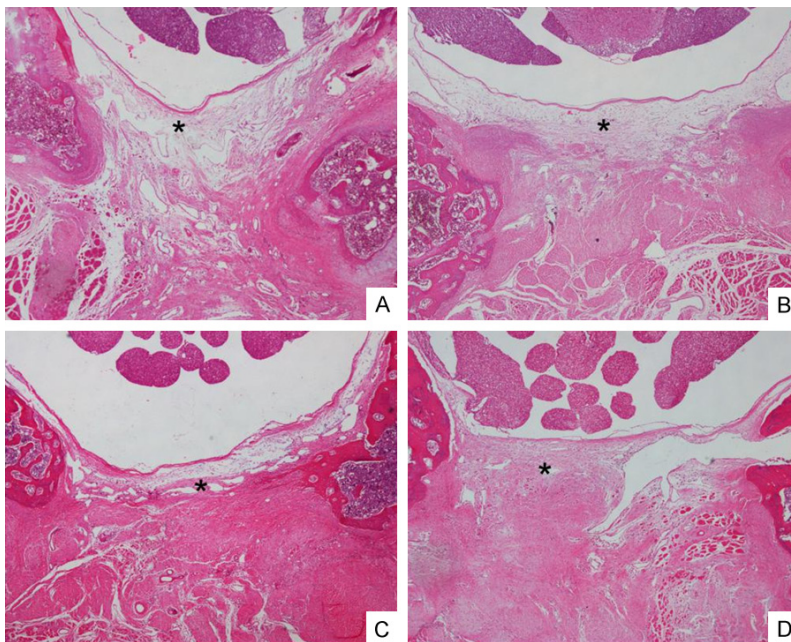
*The effect of P27 on fibroblast apoptosis*

To determine the exact effect of P27 on fibroblast apoptosis, lentiviral-mediated shRNAs were used to knockdown the P27 gene in fibroblast cells. After successfully knockdown of P27, fibroblasts were treated with 0.4 mg/ml MMC and subsequently cultured for 24 h. As shown in **Figure 4**, western blot analysis showed that P27 was decreased after knockdown of P27 as well as the decreased level of cleaved PARP in P27-knockdown cells. The increased expression of P27 and cleaved PARP, which occurred after MMC treatment, was partially attenuated by P27 knockdown. Moreover, the result of Hoechst staining and CCK-8 assay showed that the increased apoptotic cell nuclei and decreased cell viability, which occurred after MMC treatment, was also partially attenuated.

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**Figure 4.** The effect of P27 on fibroblast apoptosis. A, B. Western-blot analysis was used to detect the expressions of P27 and the apoptotic marker (cleaved PARP) after knockdown of P27 in fibroblasts treated with or without MMC.  $\beta$ -actin was used as a loading control. C. Hoechst staining was applied to detect the effect of P27 in fibroblasts treated with or without MMC. The magnification was 400. D. Cell viability was assessed with the CCK-8 assay. \* $P < 0.05$  versus that of the MMC treated group.



**Figure 5.** Photomicrographs of the epidural adhesion issues in the laminectomy sites application with MMC of 0.5 mg/ml (A), 0.2 mg/ml (B), 0.1 mg/ml (C) and saline (D). Loose scar tissues in 0.5 mg/ml MMC group (A). Moderate scar adhesion in 0.2 mg/ml MMC group and 0.1 mg/ml MMC (B, C). Dense scar tissue in control group (D). The sections were stained with hematoxylin-eosin with the magnifications of 40. "\*" represents scar tissue.

tive role in promoting fibroblast apoptosis.

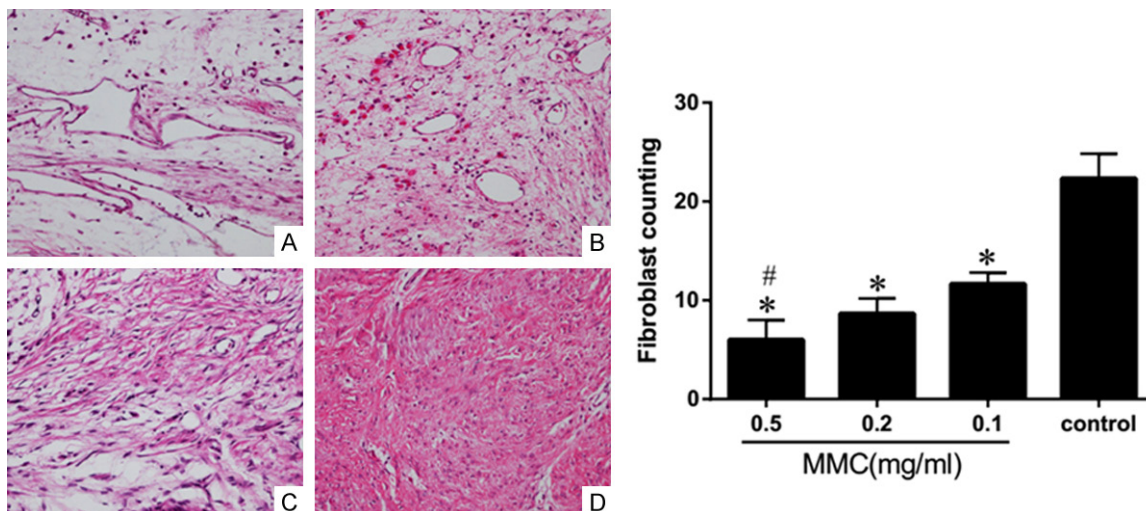
### *MMC reduces epidural fibrosis in rats*

The surgery was well tolerated by all animals without any sign of wound infection, neurological deficit and cerebrospinal leak. The recovery of all rats was uneventful after the operations. Six rats in each group were selected for the histological analysis (Figure 5). The results showed that extensive and dense epidural scar tissue that adhered to the dura matter was found in the laminectomy defects in control group. In 0.1 mg/ml MMC group or 0.2 mg/ml MMC group, moderate epidural scar tissue with a decreased density of fibroblasts were found in the laminectomy defects compared with those of control group. However, loose or little epidural scar tis-

ated by P27 knockdown. Thus, these above results demonstrated that P27 played a posi-

tion

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**Figure 6.** Histological analysis of fibroblast density in epidural scar tissue after treated with MMC of 0.5 mg/ml (A), 0.2 mg/ml (B), 0.1 mg/ml (C) and saline (control, D). The number of fibroblasts in the 0.5 mg/ml MMC group (A) was less than those of the 0.2 mg/ml group (B), 0.1 mg/ml group (C) and control group (D). The sections were stained with hematoxylin-eosin with the magnifications of 400. The effect of MMC on fibroblast counting in epidural scar tissue in each group. Fibroblast number was expressed as the number per counting area. \* $P < 0.05$ , compare with control group. # $P < 0.05$ , compare with 0.2 mg/ml group.

sue without significant adhesion was observed in the laminectomy defects in 0.5 mg/ml MMC group. These results demonstrated that MMC could reduce epidural fibrosis and showed in a concentration manner.

### *Effect of MMC on fibroblast density*

The fibroblast number in the epidural scar tissue of each group is shown in **Figure 6**. The fibroblast number in 0.5 mg/ml MMC group was less than those of 0.2 mg/ml MMC group, 0.1 mg/ml MMC group and control group ( $P < 0.05$ ). The fibroblast number in 0.2 mg/ml MMC group was also less than those of control group ( $P < 0.05$ ). Besides, the fibroblast number in 0.1 mg/ml MMC group was also less than that of control group ( $P < 0.05$ ). The results indicated that MMC could inhibit fibroblasts growth in epidural scar tissue.

### *MMC upregulates P27 expression in epidural scar tissue in rats*

Representative sections of P27 expression in epidural fibrotic tissue of each group are shown in **Figure 7**, a large number of fibroblasts but significantly low level of P27 expression in the epidural scar tissue in control group. Moderate number of fibroblasts was found and P27 expression was increased in the epidural scar

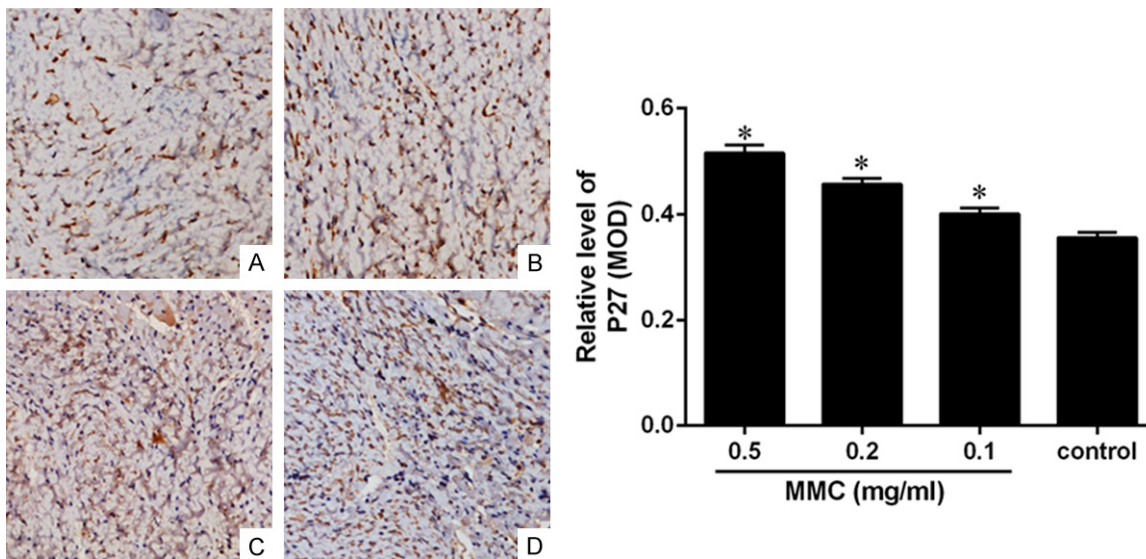
tissue in 0.1 mg/ml MMC group. Lower number of fibroblasts and more P27 expression were found in most of the fibroblasts in epidural scar tissue of 0.2 mg/ml MMC group compared with those in 0.1 mg/ml MMC and control group. Importantly, the lowest number of fibroblasts and the highest P27 expression were found in fibroblasts in epidural scar tissue in 0.5 mg/ml MMC group compared with those of other groups.

### **Discussion**

Increasing studies have shown that MMC had satisfactory effect on preventing the formation of postoperative fibrosis in many surgeries. For example, topical applied various concentration of MMC can reduce epidural fibrosis by inhibiting fibroblasts activities after laminectomy and discectomy [21, 22]. However, MMC can inhibit multiple cells proliferation around the laminectomy sites, which may interferes with the natural wound healing, result in injury of nerve root and disturbance of wound healing [2, 23]. Therefore, the detailed mechanism by which MMC reduces epidural fibrosis should be elucidated.

Multiple parameters have been used to determine the effect of MMC on reducing epidural fibrosis by regulating fibroblast activities. In the

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**Figure 7.** Immunohistochemistry analysis of P27 expression in epidural scar tissue treated with 0.5 mg/ml MMC (A), 0.2 mg/ml MMC (B), 0.1 mg/ml MMC (C) and saline (D). The results of P27 expression were expressed as mean OD and are shown in the histogram. \* $P < 0.05$ , compared with the fibroblast number in control group.

rat model, histological analysis showed that MMC could inhibit fibroblasts proliferation and reduce the epidural fibrotic tissues, indicating that MMC reduced epidural fibrosis. Moreover, MMC increased the expressions of apoptotic proteins such cleaved PARP and Bax in human fibroblast cell lines by western blot analysis, suggesting that MMC could induce fibroblasts apoptosis. Take together, these results demonstrated that MMC could reduce epidural fibrosis by inducing fibroblasts apoptosis.

Previous reports have indicated that P27 could regulate the progression of cell cycle through its interaction with cyclin E-cdk2 complexes [24, 25]. Many studies have shown that P27 was involved in the regulation of various cell apoptosis. For example, Xu reported that matrine could inhibit osteosarcoma cells proliferation and induces apoptosis by regulating cell cycle and apoptosis regulators, such as P27 and Bax [26]. Indovina has reported that SRC inhibition can induce malignant mesothelioma cells apoptosis and P27 silencing was able to suppress the apoptosis [27]. Importantly, overexpression of P27 could result in apoptotic cell death in HeLa cells and ectopic expression of Bcl-2 can protect cells from apoptosis mediated by P27 overexpression [18]. Recently, it has been reported that overexpression of P27 could inhibit Tenon's fibroblasts proliferation and result in the reduction

of the scar formation [28, 29]. Moreover, Wang reported that overexpression of P27 leads to apoptotic cell death in human lung fibroblasts and rat fibroblasts line, indicating that P27 participate in the regulation of fibroblasts apoptosis [18].

In our study, we found that downregulation of P27 expression in fibroblasts using lentiviral knockdown could decrease the expression of cleaved PARP. The upregulation of P27 and cleaved PARP, which occurs after MMC treatment, were partially attenuated by P27 knockdown. These results indicated that P27 played a positive role in promoting fibroblasts apoptosis. Moreover, MMC could significantly upregulate P27 expression in human fibroblasts by western blot analysis and real time PCR detection. Therefore, we concluded that MMC could induce fibroblast apoptosis by upregulating the expression of P27. In rat models, the expression level of P27 was quantified by mean OD value. Mean OD refers to average optical density which has a positive correlation with expression level of P27. That is to say, high level expression leads to high mean od value and vice versa. In this study, we found that MMC could reduce epidural fibrosis and upregulate P27 expression by histological and immunohistochemistry analysis. Previous studies and our studies have demonstrated that P27 was an apoptotic promoter for fibroblasts cell lines.



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Considering the effect of P27 on cell apoptosis, our study indicated that MMC could induce human fibroblast apoptosis by upregulating P27 expression, which may be the main mechanism by which of MMC reduced epidural fibrosis.

However, epidural fibrosis is a complicated process and fibroblasts proliferation is one of the factors that are involved in the development of fibrosis. In the process, many cells and signal pathways such as various inflammatory cells and factors, are involved in the fibroblasts proliferation and apoptosis after MMC treatment. In present study, we only investigated the effect of P27 on fibroblast apoptosis; further investigation about the definite signaling pathways should be elucidated in the future. In summary, the study demonstrated that MMC could induce fibroblast apoptosis and reduce epidural fibrosis by upregulating P27 expression. Considering the role of P27 in fibroblasts apoptosis, it can provide an ideal therapeutic target for preventing postoperative epidural fibrosis after laminectomy.

## Acknowledgements

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## Disclosure of conflict of interest

None.

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