

10-Hydroxycamptothecin induces apoptosis in human fibroblasts by regulating miRNA-23b-3p expression

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Abstract. 10-Hydroxycamptothecin (HCPT) effectively controls epidural fibrosis, but the exact underlying mechanisms remain ambiguous. Abnormal microRNA (miR)-23b-3p expression has been detected in various types of fibrotic tissues that are present in different diseases. The aim of the present study was to elucidate the mechanisms through which HCPT induces fibroblast apoptosis. Reverse transcription-quantitative polymerase chain reactions were performed on six traumatic scar samples and matched normal skin samples; traumatic scar formation was revealed to be significantly inversely associated with miR-23b-3p expression. In addition, the miR-23b-3p expression level in human fibroblasts was examined following HCPT treatment. The effects of HCPT and miR-23b-3p on fibroblast apoptosis were assessed using terminal deoxynucleotidyl-transferase-mediated dUTP nick-end labeling, flow cytometry and western blot analysis. The results demonstrated that HCPT treatment notably increased miR-23b-3p expression levels and accelerated fibroblast apoptosis. Therefore, upregulation of miR-23b-3p expression was demonstrated to promote fibroblast apoptosis, consistently with the effects of HCPT. The results of the present study indicated that HCPT may induce fibroblast apoptosis by regulating miR-23b-3p expression.

Introduction

The extensive epidural fibrosis that may occur following lumbar surgery may lead to the development of adverse effects, such as nerve radicular pain or lower back pain (1). This process is associated with a 24% rate of failed back

surgery syndrome (2). Epidural fibrosis is associated with fibroblast hyperplasia and the development of epidural scar tissue. Fibroblasts proliferate at the operative site following stimulation by growth factors and inflammatory cytokines. Local defects of the vertebral plate are repaired by collagen fibers that are produced by these cells. Fibroblasts transform into fibrocytes, and scar tissue replaces the fibrous connective tissue, owing to the production of collagen fibers. The nerve roots in the vertebral canals or dura mater are subsequently constrained by the epidural fibrotic tissue, which may cause restriction of nerve root mobility, nerve root entrapment and dural compression (3).

A number of strategies aiming to prevent epidural fibrosis by inducing fibroblast apoptosis have been introduced and successful outcomes have been reported (4-6). The antitumor agent 10-hydroxycamptothecin (HCPT) has been demonstrated to restrain cell proliferation or induce cell apoptosis; HCPT is a cell cycle-specific agent that mainly acts during DNA synthesis (S phase) (7). HCPT not only restrains the proliferation of several types of tumor cells, but also can inhibit the proliferation of non-cancerous cells (8-10). Although HCPT is known to exhibit antifibrotic properties, the specific underlying mechanisms have not yet been fully elucidated.

MicroRNAs (miRNAs) are short, highly conserved non-coding RNA molecules that regulate gene expression by targeting the 3' untranslated region of target genes during various physiological processes, including cell differentiation, apoptosis and proliferation (11). Each miRNA targets numerous genes; thus, miRNAs serve important roles in physiological processes in several types of cells, including cancer cells (12) and fibroblasts (13). miRNA (miR)-23b is an epidermal differentiation marker and it has several unknown functions in the skin (14). miR-23b belongs to the miR-23b/24/27b cluster, which has been verified to participate in a number of physiological processes, such as cell migration, differentiation and proliferation (15-17). The miR-23b/24/27b cluster serves a cancer-inhibitory role in colorectal, bladder, ovarian and prostate malignancies (18-21), whereas it has been reported to promote breast cancer (22). The aim of the present study was to elucidate the effects of HCPT on fibroblast apoptosis and to determine whether this effect is mediated by the regulation of miR-23b-3p expression.

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Materials and methods

Ethics statement. The present study protocol was approved by the Research Ethics Committee of Northern Jiangsu People's Hospital (Yangzhou, China), and written informed consent was obtained from all the participants for their tissues to be used for the purposes of this research.

Fibroblast culture and treatment. Fibroblasts were acquired from scar tissue resected from patients undergoing reoperation laminectomy in Northern Jiangsu People's Hospital of Yangzhou City in October 2017; patient information is provided in Table I. Under sterile conditions, the epidural scars were dissected into 5x5 mm pieces and dissociated in 0.25% trypsin (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) for 6 min at 37°C, and the cell suspension was centrifuged at 240 x g for 5 min. Cells were maintained in Dulbecco's modified Eagle's Medium (Gibco; Thermo Fisher Scientific, Inc.) with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) and penicillin (100 U/ml)/streptomycin (100 mg/l) (Gibco; Thermo Fisher Scientific, Inc.) at 37°C in a humidified atmosphere of 5% CO₂ and 95% air. Cells in the exponential growth phase were selected for used in all the experiments. The fibroblasts were incubated in Petri dishes of different specifications until they reached 60-80% confluence, and subsequently washed with PBS (pH 7.4) and treated with or without HCPT at 1 µg/ml for 24 h following previous studies (23-25).

Lentiviral infection. Lentiviruses containing the Lv-miR-23b-3p, Lv-anti-miR-23b-3p or their respective control were purchased from Shanghai Genechem Co. Ltd. (Shanghai, China). Lentiviral transfection was performed according to the manufacturer's protocols. Briefly, when fibroblasts reached 60-80% confluence, fibroblasts were incubated for 72 h at 37°C in the lentiviral vector (multiplicity of infection=200) in the presence of Polybrene Infection/Transfection Reagent (2 mg/ml; Gibco; Thermo Fisher Scientific, Inc.). The medium was removed and replaced by newDMEM. Transfected fibroblasts were screened by culturing for 72 h in puromycin (2 mg/ml; Sigma-Aldrich; Merck KGaA, St. Louis, MO, USA). When the screening is completed, it may be regarded as the stable expression of the target gene in the cell. Cells with stable expression were used in the subsequent experiments.

Flow cytometry. Fibroblasts were seeded in 6 cm plates and incubated for 24 h in 5% CO₂ at 37°C. When the fibroblasts reached 60-80% confluence, following treatment under the various experimental conditions aforementioned, adherent and detached fibroblasts (1x10⁶ cells/ml) were centrifuged at 13,000 x g for 5 min at 4°C, resuspended in 500 µl 1X binding buffer and then double-stained with propidium iodide (PI) and Annexin V-allophycocyanin (APC; BD Biosciences, San Jose, CA, USA), according to the manufacturer's protocol. Each sample was detected using FACSDiva Software 6.0 (BD Biosciences). All experiments were performed in triplicate.

Western blot analysis. Total protein was extracted from the fibroblasts when they reached 60-80% confluence and protein

concentrations were measured using a Bicinchoninic Protein Assay kit (Thermo Fisher Scientific, Inc.). A total of 10 µg protein was separated by 10% SDS-PAGE and transferred onto polyvinylidene fluoride membranes. Following the blocking of non-specific binding with 5% non-fat milk dissolved in TBS + 0.05% Tween-20 at room temperature for 2 h, the membranes were incubated with the following antibodies: Anti-cleaved poly [ADP-ribose] polymerase [PARP; 1:1,000; catalog no. 5625; Cell Signaling Technology, Inc. (CST), Danvers, MA, USA], anti-GAPDH (1:1,000; catalog no. 8884; CST), anti-B cell lymphoma 2 (Bcl-2; 1:1,000; catalog no. 4223; CST), anti-Bax (1:1,000; catalog no. 5023; CST) and anti-mothers against decapentaplegic homolog 3 (Smad3; 1:1,000; catalog no. ab40854; Abcam, Cambridge, UK) at 4°C overnight. Following washing with TBST, the membranes were incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit secondary antibodies (1:1,000; catalog no. 7074; CST) and the protein expressions were visualized using the Enhanced Chemiluminescence System (EMD Millipore, Billerica, MA, USA). The bands were quantified by densitometry using Image J 1.46r software (National Institutes of Health, Bethesda, MD, USA). All reactions were performed in triplicate.

RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. Total RNA was isolated from the treated fibroblasts using TRIzol[®] reagent (Invitrogen; Thermo Fisher Scientific, Inc.) following the manufacturer's instructions. The IQ SYBR Green Supermix kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA) was used to determine the expression levels of miR-23b-3p. Briefly, a special looped RT primer was used for each miRNA and the RevertAid First-Strand cDNA Synthesis kit (Fermentas; Thermo Fisher Scientific, Inc.) was used to reverse transcribe 3 µg total RNA, following the manufacturer's protocol. Subsequent amplification was performed using IQ SYBR Green Supermix in a CFX connect Real-Time PCR System (Bio-Rad Laboratories, Inc.). U6 was used as internal reference, U6 forward, 5'-CGGCGGTAGCTTATCAGACTGATG-3' and reverse, 5'-CCAGTCGAGGGTCCGAGGTATT-3'. The primers used were as follows: miR-23b-3p, forward 5'-GCGGCGGATCACATTGCCAGGG-3'; and we use Universal Primer (catalog no. 1046471; Qiagen GmbH, Hilden, Germany) as miR-23b-3p as reverse primer. The 2^{-ΔΔC_q} method was performed to calculate the relative expression (26). All reactions were performed in triplicate.

Terminal deoxynucleotidyl-transferase-mediated dUTP nick-end labeling (TUNEL) staining of fibroblasts. The apoptotic rate of HCPT-treated fibroblasts and Lv-transfected fibroblasts was evaluated using the TUNEL assay (Nanjing KeyGen Biotech Co., Ltd., Nanjing, China), following the protocol recommended by the manufacturer. Following TUNEL and DAPI nuclear staining, images were captured using fluorescence microscopy (Zeiss AG, Oberkochen, Germany). TUNEL-stained fibroblasts were considered to be apoptotic; DAPI staining was performed to count the total number of fibroblasts. All reactions were performed in triplicate.

Statistical analysis. Data are expressed as the mean ± standard deviation. Using GraphPad Prism 6.0 software (GraphPad

Table I. Clinicopathological characteristics of the six patients used in the study.

Patient no.	Sex	Age (years)	Biopsy site ^a	Duration of the lesion (months)	Etiology
1	Male	64	S: Leg NS: Leg	12	Trauma
2	Male	46	S: Leg NS: Leg	12	Trauma
3	Female	65	S: Leg NS: Leg	12	Trauma
4	Female	40	S: Leg NS: Leg	12	Trauma
5	Male	63	S: Leg NS: Leg	12	Trauma
6	Female	21	S: Shoulder NS: Shoulder	12	Trauma

^aThe position of normal skin is at a distance of 5 cm from scar. NS, normal skin tissue; S, scar tissue.

Software, Inc., La Jolla, CA, USA), comparisons between two groups were performed using Student's t-test and comparisons between multiple groups were performed using one-way analysis of variance followed by Tukey's post hoc test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

miR-23b-3p is overexpressed in traumatic scar tissues. The morphological characteristics of fibroblasts obtained from patients identified by phase contrast microscopy (Fig. 1A). Skin tissues were collected from six patients with traumatic scars and the expression levels of miR-23b-3p were examined using RT-qPCR. The results demonstrated that miR-23b-3p expression levels in traumatic scar tissues was significantly lower compared with expression in the normal skin tissues ($P < 0.05$; Fig. 1B). These results indicated that miR-23b-3p is associated with epidermal fibrosis.

HCPT upregulates miR-23b-3p expression in human fibroblasts. To determine the effects of HCPT on miR-23b-3p expression, human fibroblasts were treated with or without 1 $\mu\text{g/ml}$ HCPT for 24 h and miR-23b-3p expression levels were determined by RT-qPCR. The results revealed that miR-23b-3p expression in the HCPT-treated fibroblasts was significantly higher compared with the expression levels in the untreated control group ($P < 0.05$; Fig. 2). These data suggested that HCPT may upregulate miR-23b-3p expression in human fibroblasts.

miR-23b-3p promotes fibroblast apoptosis. To determine the role of miR-23b-3p in fibroblast apoptosis, human fibroblasts were successfully transfected with Lv-miR-23b-3p, Lv-anti-miR-23b-3p or their respective controls (Fig. 3A). Cell apoptosis was measured using TUNEL and flow cytometry. TUNEL staining data revealed that miR-23b-3p overexpression induced a significant increase in the number

of TUNEL-staining cells compared with untreated and Scramble control-transfected cells, indicating an increase in human fibroblast apoptosis. However, miR-23b-3p inhibition induced a decrease in the number of TUNEL-staining cells compared with untreated and Negative control-transfected cells, which exhibited an decrease in human fibroblast apoptosis. The apoptotic rate of the Negative control group and the Scramble control group was significantly increased compared with the control group, and it was hypothesized that the apoptosis was caused by the transfection virus operation methods, reagents and viral vectors (Fig. 3B). Similar results were observed from flow cytometric analysis, which demonstrated that miR-23b-3p upregulation resulted in a significantly higher cell apoptosis compare with Scramble control, while miR-23b-3p inhibition reduced cell apoptosis rate compared with Negative control (Fig. 3C).

Western blot analysis revealed that miR-23b-3p promoted fibroblast apoptosis, while inhibition of miR-23b-3p exerted the opposite effect. It was also observed that Smad3 and Bcl-2 decreased significantly in the overexpression group compared with the Scramble control group, while Smad3, Bcl-2 was significantly upregulated in the knockout group compared with Negative control (Fig. 3D). The apoptotic proteins PARP and Bax demonstrated the opposite; PARP and Bax increased significantly in the miR-23b-3p upregulation group compared with Scramble control and PARP and Bax decreased significantly in miR-23b-3p inhibition group compared with Negative control. These results indicated that miR-23b-3p upregulation may induce fibroblast apoptosis.

HCPT induces fibroblast apoptosis by upregulating miR-23b-3p. To verify the role of miR-23b-3p in HCPT-induced fibroblast apoptosis, human fibroblasts were transfected with Lv-miR-23b-3p and Lv-anti-miR-23b-3p, followed by co-treatment with HCPT. TUNEL assays revealed that miR-23b-3p increased HCPT-induced fibroblast apoptosis (Fig. 4A). Flow cytometric analysis confirmed the TUNEL assay results; HCPT treatment increased the

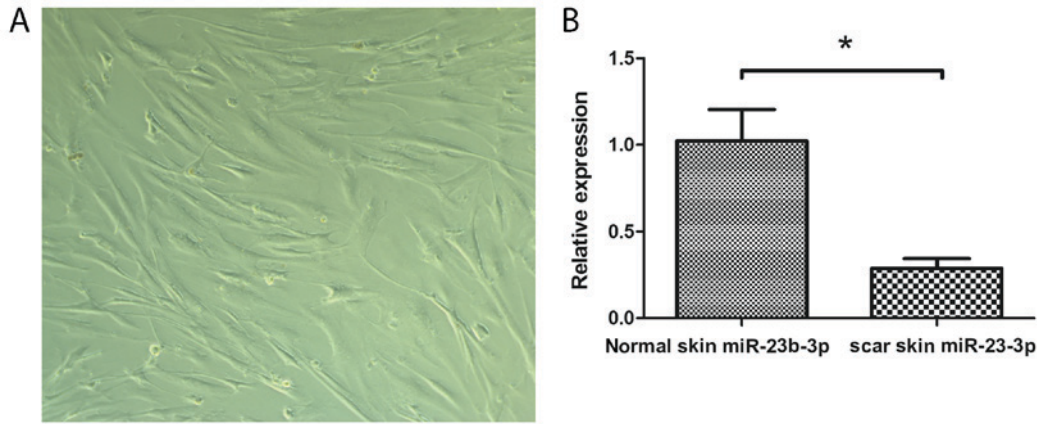


Figure 1. Culture and identification of fibroblasts isolated from humans and miR-23b-3p expression in specimens. (A) Morphology of fibroblasts derived from scar tissues; magnification, x100. (B) Expression levels of miR-23b-3p in traumatic scars and normal skin as determined by reverse transcription-quantitative polymerase chain reaction; * $P < 0.05$. miR, microRNA.

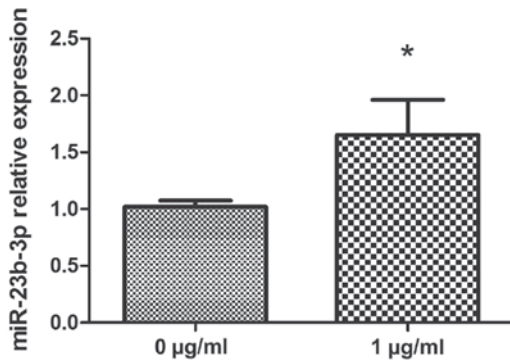


Figure 2. HCPT treatment increases the expression of miR-23b-3p in fibroblasts. Reverse transcription-quantitative polymerase chain reaction analysis of the relative miR-23b-3p expression levels in fibroblasts following treatment with or without of HCPT for 24 h. * $P < 0.05$. HCPT, 10-hydroxycamptothecin; miR, microRNA.

fibroblast apoptotic rate compare to the control group, and transfection with Lv-anti-miR-23b-3p partially reversed the increased apoptosis caused by HCPT, whereas transfection with Lv-miR-23b-3p significantly increased cell apoptosis caused by HCPT (Fig. 4B). Consistent with these apoptosis data, treatment with HCPT alone or with Lv-miR-23b-3p co-transfection increased the expression of apoptosis-related proteins cleaved-PARP and BAX, and decreased the expression of Bcl-2, compared with the Control group (Fig. 4C). Transfecting Lv-anti-miR-23b-3p into the fibroblasts partially attenuated the HCPT-induced increased expression of these proteins. These data indicated that upregulation of miR-23b-3p expression by HCPT treatment increases human fibroblast apoptosis.

Discussion

Unsatisfactory outcomes following back surgery, including persistent lower back pain, radiculopathy and even disability, is often attributed to extensive epidural fibrosis (27,28). The development and progression of epidural fibrosis are affected by several factors, such as postoperative chronic inflammation, lumbar instability and the degree of hemostasis during

surgery (29,30), which likely promote the proliferation of fibroblasts and ultimately leads to epidural fibrosis (31). Thus, research has been focused on inducing fibroblast apoptosis as a measure of preventing epidural fibrosis. Owing to the target-specific DNA-damaging ability of HCPT, this agent has achieved remarkable results in the inhibition of the proliferation of a number of tumor cells, and has been used in the treatment of several types of malignant tumors (32,33). However, as an antitumor agent, HCPT does not only inhibit fibroblast proliferation, but also may exert an inhibitory effect on the proliferation of other types of cells.

In our previous studies, fibroblasts treated with 1 µg/ml HCPT for 24 h exhibited typical morphological changes in chromatin condensation and apoptosis in the nucleus, and a significant increase in cell apoptotic rate (23,24). A previous study (25) indicated that fibroblasts exposed to HCPT at a concentration of 1 µg/ml for 12 h did not cause significant cytotoxicity, but the effects of exposure became significant after 24 and 48 h, and cell growth was arrested in S phase. At the same time, treating fibroblasts with various concentrations of HCPT for 24 h. Active-caspase 3 and cleaved PARP, which are markers of apoptosis, can only begin to detected in 1 µg/ml of HCPT. This indicates that under this condition, HCPT successfully inhibited fibroblast proliferation by S phase arrest and promotion of apoptosis. Therefore, 24 h and 1 µg/ml was used as the processing time and processing concentration.

In the present study, cells were transfected with Lv-miR-23b-3p, Lv-anti-miR-23b-3p or their respective controls for 72 h, and cell apoptosis was examined by staining with PI and APC Annexin V. miR-23b-3p overexpression caused an increase in the percentage of total apoptotic fibroblasts. Previous studies have reported that the upregulation of miR-23b-3p in airway smooth muscle cells, hypoxia-induced cardiomyocytes and heat-denatured fibroblasts induces cell apoptosis (34-37), which strongly suggested that miR-23b-3p may be a key factor in the apoptotic process. In the present study, the expression of miR-23b-3p was lower in traumatic scar samples compared with normal skin, and the use of HCPT increased the expression of miR-23b-3p compared with normal fibroblasts. These data suggested that HCPT

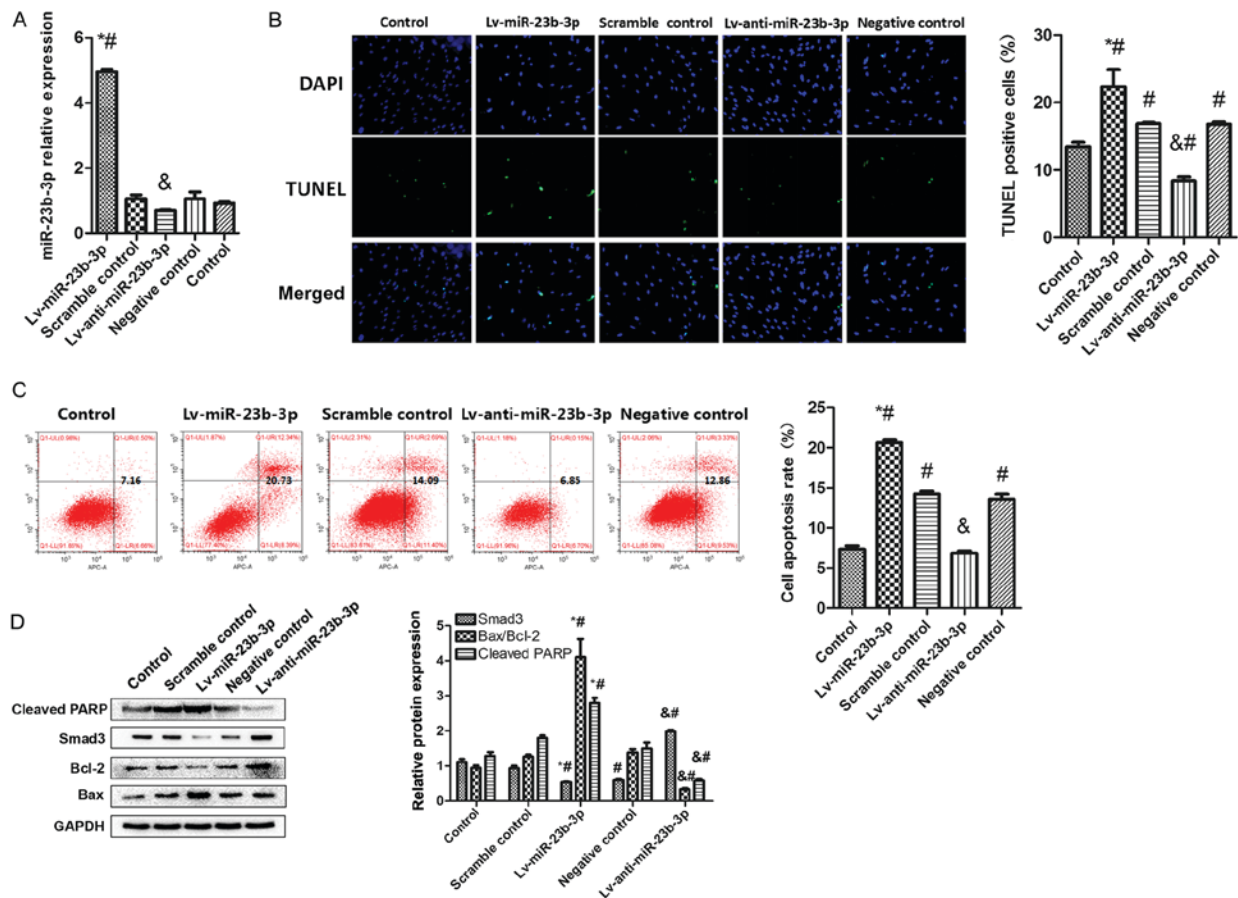


Figure 3. miR-23b-3p induces fibroblast apoptosis. (A-D) Fibroblasts were transfected with Lv-miR-23b-3p, Lv-anti-miR-23b-3p or their respective controls; an untransfected Control was also used. (A) Reverse transcription-quantitative polymerase chain reaction was used to determine the relative miR-23b-3p expression levels in fibroblasts at 72 h post-transfection. (B) TUNEL staining for the detection of apoptosis in fibroblasts. (C) Flow cytometry measurement of cell apoptosis and quantification following transfection of fibroblasts. (D) Western blot analysis of apoptosis-related protein expression levels of cleaved PARP, Smad3, Bcl-2 and Bax in the transfected fibroblasts. * $P < 0.05$ vs. Scramble; # $P < 0.05$ vs. Control; & $P < 0.05$ vs. Negative control. Bcl-2, B cell lymphoma 2; Lv, lentivirus; miR, microRNA; Smad3, mothers against decapentaplegic homolog 3; TUNEL, terminal deoxynucleotidyl-transferase-mediated dUTP nick-end labeling.

may induce human fibroblast apoptosis by upregulating miR-23b-3p expression. Transfection of fibroblasts with a lentivirus to inhibit miR-23b-3p expression resulted in enhanced fibroblast proliferation, whereas upregulation of miR-23b-3p resulted in ectopic apoptosis. Furthermore, transfection of Lv-miR-23b-3p enhanced the cytostatic effect of HCPT, and transfection of Lv-anti-miR-23b-3p reduced the apoptotic effect of HCPT. These data suggest that decreasing miR-23b-3p expression may suppress cell apoptosis. A previous study demonstrated that downregulation of miR-23b increased Smad3 protein expression levels, which facilitated the proliferation of heat-denatured fibroblasts (34). Furthermore, a number of other studies have suggested that Smad3 is the target gene of miR-23b-3p in rat fibroblasts and other cells (34-39); thus, it was hypothesized that miR-23b-3p may induce human fibroblast apoptosis by targeting Smad3. The exact effects of miR-23b-3p on fibroblast apoptosis requires further investigation. Results from the present study indicated that the apoptosis of fibroblasts depends on the upregulation of miR-23b-3p. In conclusion, the results of the present study demonstrated that HCPT induces fibroblast apoptosis by regulating miR-23b-3p expression. These data raise the possibility of using miR-23b-3p as a novel therapeutic intervention to prevent epidural fibrosis.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

LZ conceived and designed the experiments, performed the experiments, wrote the paper. YS analyzed the data. LY prepared figures and tables. JW contributed reagents, materials

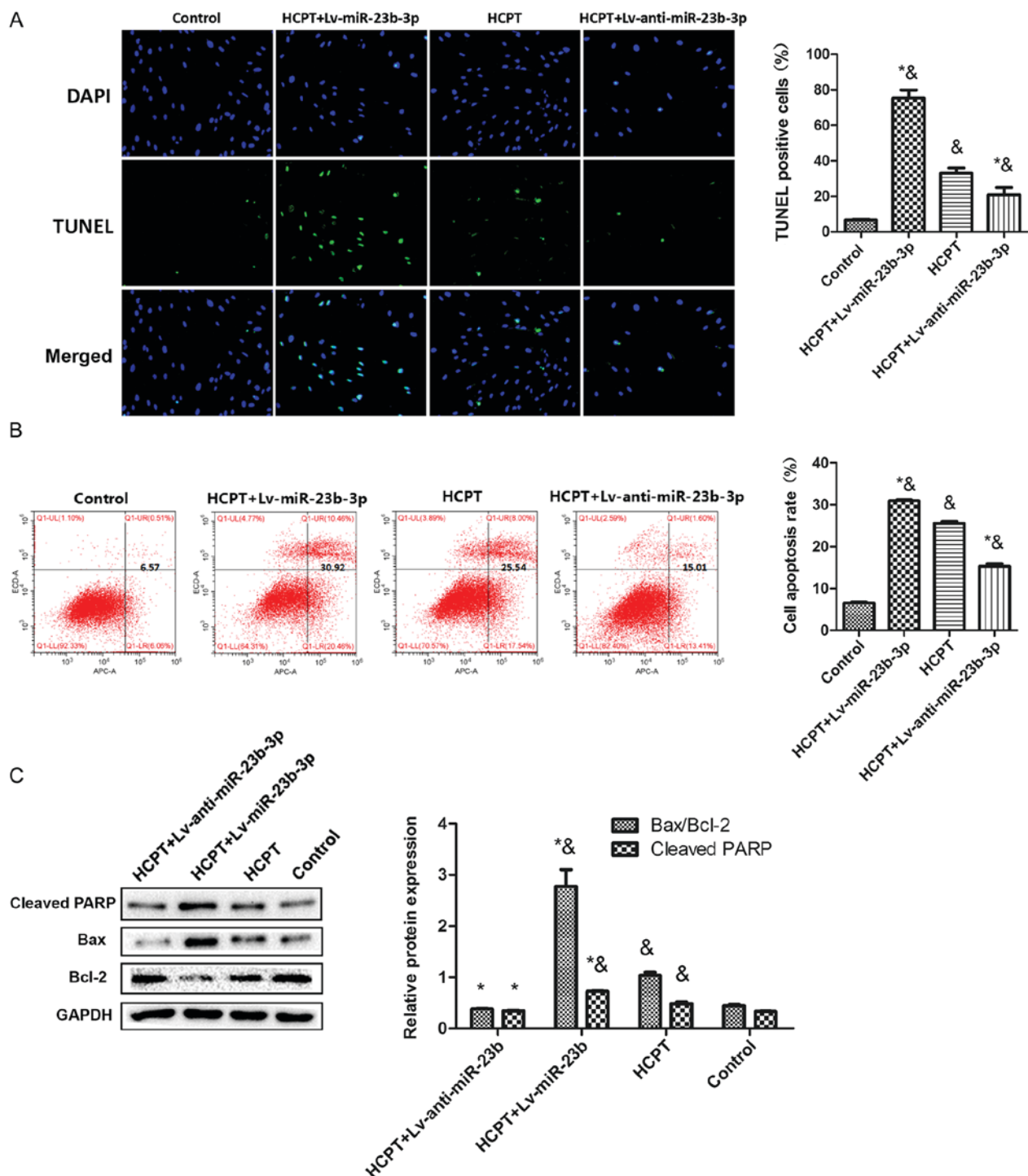


Figure 4. HCPT induces fibroblast apoptosis by upregulating miR-23b-3p expression. Fibroblasts were transfected with Lv-miR-23b-3p or Lv-anti-miR-23b-3p followed by HCPT treatment. (A) TUNEL staining was used to detect apoptosis in transfected and HCPT treated fibroblasts. (B) Flow cytometry measurement of cell apoptosis and quantification. (C) Western blot analysis of apoptosis-related protein expression levels of cleaved PARP, Bax and Bcl-2. ^{*}P<0.05 vs. HCPT; [&]P<0.05 vs. Control. Bcl-2, B cell lymphoma 2; HCPT, 10-hydroxycamptothecin; Lv, lentivirus; miR, microRNA; TUNEL, terminal deoxynucleotidyl-transferase-mediated dUTP nick-end labeling.

and analysis tools, reviewed drafts of the paper and collected tissue samples. XL performed the experiments.

Ethics approval and consent to participate

The present study protocol was approved by the Research Ethics Committee of Northern Jiangsu People's Hospital (Yangzhou,

China), and written informed consent was obtained from all the participants for their tissues to be used for the purposes of this research.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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