

Effects of microRNA-338-3p on morphine-induced apoptosis and its underlying mechanisms

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Abstract. The aim of the present study was to investigate the effects of microRNA-338-3p (miR-338-3p) on morphine (MP)-induced apoptosis, and its underlying mechanisms. Freshly-isolated mouse peritoneal macrophages were cultured *in vitro* and treated with MP following transfection with miR-338-3p mimic, inhibitor or controls. miR-338-3p expression levels increased significantly following MP treatment ($P<0.01$). This increase was enhanced following transfection with miR-338-3p mimic ($P<0.05$) and abrogated following transfection with miR-338-3p inhibitor ($P<0.05$). The apoptotic rate increased significantly in groups treated with MP ($P<0.05$); however, this increase was abrogated by transfection with miR-338-3p inhibitor ($P<0.05$). Bioinformatics software predicted that sex determining region Y-box 4 (SOX4) was the target gene of miR-338-3p and this was verified using a dual-luciferase reporter gene system. SOX4 mRNA and protein expression levels decreased significantly following MP treatment ($P<0.05$); however, this decrease was abrogated following transfection with miR-338-3p inhibitor ($P<0.05$). Caspase-3 protein expression levels increased markedly following MP treatment ($P<0.05$); however, this increase was inhibited by transfection with miR-338-3p inhibitor ($P<0.05$). Therefore, decreased expression of miR-338-3p may suppress MP-induced apoptosis, potentially via the upregulation of SOX4 expression and the caspase-3-dependent apoptotic signaling pathway.

Introduction

MicroRNA (miR) is a short-segment (length, 20-23 nt) RNA, which binds to the 3'-untranslated region (UTR) of its target DNA by reverse complement, thus preventing the translation of mRNA or causing its degradation (1-3). Extensively expressed

in mammals, miR regulates various physiological processes, cell functions and signaling pathways via the regulation of gene expression (1-5). Thus far, >2,000 miRs associated with humans have been identified, many of which perform various gene regulatory functions (6,7). However, the precise functions and underlying regulatory mechanisms of miR remain to be fully elucidated. A number of studies have suggested that miR is associated with certain diseases (8-10). Therefore, investigating the function of a specific miR and its underlying mechanisms may improve our understanding of the regulation of signaling pathways by miR and the subsequent effects, including cell differentiation, proliferation and apoptosis. This may contribute to improvement of human health and disease treatment.

Morphine (MP) is commonly used during the treatment of cancer. In addition to easing pain, it has been demonstrated to increase the susceptibility of cancer cells to apoptosis by promoting the expression of apoptosis-associated proteins, which suppresses the proliferation of cancer cells (11-13). However, MP treatment for cancer is complex and is often associated with other factors, including miR (14,15). In addition, as an opioid, MP is addictive and is associated with various side effects (16,17), which may alter miR expression (15,18,19). miR-338-3p is a widely-reported miR that promotes cancer cell death by regulating specific signaling pathways or associated genes during cancer treatment (20,21). Investigating the association between MP and miR-338-3p may contribute to our understanding of the activity of miR-338-3p and the underlying molecular mechanisms of the effects of MP in cancer treatment. In the present study, mouse macrophages were transfected with miR-338-3p and treated with MP to observe the effect on miR-338-3p expression. Furthermore, the interaction between MP and miR-338-3p was investigated and the target gene of miR-338-3p was verified.

Materials and methods

Isolation of mouse peritoneal macrophages. A total of 5 C57BL/6J mice (regardless of gender; weight, 20 ± 2 g), purchased from the laboratory animal center of Xinjiang Medical University (Ürümqi, China), were housed at 21°C in 50% humidity, with a 12 h light/dark cycle, and free access to food and water. A week later, the mice (6-8 weeks old) were sacrificed by cervical dislocation; the whole mouse was soaked in 75% ethanol for 5 min and dried with sterile

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gauze. A total of 5 ml Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) was injected i.p. A total of 5 min later, 3.5-4 ml liquid was withdrawn from the abdominal cavity via syringe and centrifuged at $134 \times g$ for 10 min at 4°C . The supernatant was discarded and the cell pellet resuspended in Hank's balanced salt solution (Beijing Huamaike Biotechnology Co., Ltd, Beijing, China). DMEM containing 10% fetal bovine serum (Biowest, Nuaille, France) and double-antibody [penicillin (100 U/ml) and streptomycin (100 mg/ml)] was added to achieve the correct cell concentration ($1 \times 10^9/\text{l}$), and the cells were cultured in a 25T-flask under 5% CO_2 for 48 h at 37°C . At this stage, certain adherent cells exhibited round or oval morphology when observed under a Zeiss Axio Observer A1 microscope (Carl Zeiss MicroImaging, Inc., Thornwood, NY, USA). DMEM was then exchanged for RPMI-1640 medium (HyClone; GE Healthcare Life Sciences, Logan, UT, USA) containing 10% fetal calf serum (Hyclone; GE Healthcare Life Sciences) and the macrophages were cultured under 5% CO_2 , 37°C and saturated humidity. Culture medium was replaced every day; digestion was performed every 4 days with 0.25% trypsin; when cell confluence reached 90%, the cells were passaged at a ratio of 1:3. The present study was approved by the Experimental Animals Ethics Committee of Linyi Yishui Central Hospital (Linyi, China). All studies performed on the mice were strictly in accordance with the Provisions of Protection and Use of Experimental Animals from the International Association for the Study of Pain (22).

Transfection and grouping of mouse macrophages. When cell confluence reached 80-90%, macrophages were transferred to a 24-well plate ($3 \times 10^4/\text{well}$). The cultured cells were divided into 6 groups: i) Blank control group; ii) MP group, in which the macrophages were treated with $10 \mu\text{M}$ MP (Sigma-Aldrich, St. Louis, MO, USA) for 24 h; iii) MP+miR-338-3p mimic group, in which the macrophages were treated with $10 \mu\text{M}$ MP for 24 h following transfection with a miR-338-3p mimic for 24 h; iv) MP+control mimic group, in which the macrophages were treated with $10 \mu\text{M}$ MP for 24 h following transfection with a negative control mimic for 24 h; v) MP+miR-338-3p inhibitor group, in which the macrophages were treated with $10 \mu\text{M}$ MP following transfection with a miR-338-3p inhibitor for 24 h; and vi) MP+control inhibitor group, in which the macrophages were treated with $10 \mu\text{M}$ MP following transfection with a negative control inhibitor for 24 h. miR-338-3p mimic, mimic control, miR-338-3p inhibitor and inhibitor control were purchased from Shanghai GenePharma Co., Ltd., Shanghai, China, and transfection sequences are listed in Table I. Transfection was performed using Lipofectamine[®] 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions, immediately when the confluence of the cells in each group reached 50-60% in each well. Each treatment group was performed in triplicate and the experiment was repeated at least three times with similar results.

miR-338-3p and sex determining region Y-box 4 (SOX4) mRNA expression levels detected by reverse transcription-quantitative polymerase chain reaction (RT-qPCR). TRIzol[®] reagent (Invitrogen; Thermo Fisher Scientific, Inc.)

was used to extract the total RNA and diethylpyrocarbonate (DEPC; $50 \mu\text{l}$; Sigma-Aldrich) was used to inactivate RNases. RNA concentration was determined on a DU-800 Spectrophotometer (Beckman Coulter, Inc., Brea, CA, USA). RNA purity and integrity were controlled in accordance with the width ratio of 28S and 18S RNA bands following formaldehyde modified gel electrophoresis (5 V for 1 h). The width ratio of 28S and 18S ≥ 1.5 and $A_{260 \text{ nm}}/A_{280 \text{ nm}} \geq 1.8$ indicated that RNA had a good completeness and no degradation. The extracted total RNA showed clear and bright 28S and 18S bands; no degradation was observed. The $A_{260 \text{ nm}}/A_{280 \text{ nm}}$ ratio was 2.0, indicating that the extracted total RNA was of high quality. miR-338-3p RT-qPCR was performed with the All-in-One[®] First-Strand cDNA Synthesis kit and the SYBR Green I based All-in-One[®] qPCR Mix (GeneCopoeia, Inc., Rockville, MD, USA) according to the manufacturer's instructions. The reverse transcription reaction contained total RNA with a final concentration of $2 \mu\text{g}$ ($2 \mu\text{l}$), $2.5 \text{ U}/\mu\text{l}$ PolyA polymerase ($1 \mu\text{l}$), reverse transcriptase RTase Mix ($1 \mu\text{l}$), 5X reaction buffer ($5 \mu\text{l}$) and DEPC H_2O ($16 \mu\text{l}$), in a total volume of $25 \mu\text{l}$. The reverse transcription reaction conditions were: 37°C for 60 min and 85°C for 5 min. The qPCR reaction system contained cDNA ($2 \mu\text{l}$), 2X All-in-ONE Q-PCR Mix ($10 \mu\text{l}$), universal PCR primer ($2 \mu\text{l}$), 50X Rox Reference Dye ($0.4 \mu\text{l}$), miR-338-3p or U6 primer ($2 \mu\text{l}$) and DEPC H_2O ($3.6 \mu\text{l}$). qPCR cycling conditions were as follows: Preheating for 10 min at 95°C , degeneration for 10 sec at 95°C , annealing for 20 sec at 60°C and extension for 34 sec at 72°C . A total of 40 cycles were performed. SOX4 RT-qPCR was performed with Prime Script[™] RT reagent kit (Takara Bio, Inc.) and SYBR[®] Green Real time PCR Master Mix (Toyobo Co., Ltd., Osaka, Japan) according to the manufacturer's instructions. The reverse transcription reaction contained 5X Moloney-murine leukemia virus (M-MLV) buffer ($2 \mu\text{l}$), deoxynucleotides (2 mM; $2 \mu\text{l}$), Random 6 mers ($0.5 \mu\text{l}$), RTase M-MLV ($200 \text{ U}/\mu\text{l}$; $0.25 \mu\text{l}$) and RNA ($1 \mu\text{l}$), and DEPC H_2O was added to make up a final volume of $10 \mu\text{l}$. Reaction conditions were as follows: 42°C for 10 min, 95°C for 2 min and 4°C for 10 min. The qPCR reaction system contained 2x SYBR Green ($10 \mu\text{l}$), forward and reverse primers ($0.8 \mu\text{l}$), cDNA template ($1 \mu\text{l}$) and dd H_2O was added to make up a total volume of $20 \mu\text{l}$. U6 and GAPDH served as internal reference genes. All primer sequences are listed in Table II. The relative quantitative expression of the target gene was calculated by the $2^{-\Delta\Delta\text{Cq}}$ method (23). The formula was expressed as follows: $\Delta\Delta\text{Cq} = (\text{Cq}_{\text{experimental target gene}} - \text{Cq}_{\text{experimental reference gene}}) - (\text{Cq}_{\text{control target gene}} - \text{Cq}_{\text{control reference gene}})$.

Dual-luciferase reporter gene detection. The target gene of *mus musculus* (mmu)-miR-338-3p was predicted by TargetScan (version 7.1; targetscan.org), PicTar (pictar.mdc-berlin.de/) and microRNA.org (August 2010 release; microRNA.org/microRNA/home.do) online bioinformatics prediction software. The 3'-UTR of the SOX4 gene contained sequences that regulated various sites; amplification was performed according to the manufacturer's instructions of the SYBR[®] Green Real Time PCR Master Mix (Toyobo Co., Ltd., Japan). The primer sequences were as follows: Forward, GAGCTCCTCCGCCTTCTTTCTAC and reverse, CTCGAGCACGTCTTCTCA TTTACACC. The reaction conditions were 42°C for 10 min,

95°C for 2 min and 4°C for 10 min. The reaction system contained 2x SYBR Green (10 μ l), primers (0.8 μ l), cDNA (1.0 μ l) and ddH₂O was added to make up a total volume of 20 μ l. The PCR amplified SOX4 3'UTR was cloned into the pmirGLO luciferase miR target expression vector (Promega Corporation, Madison, WI, USA). Site-directed mutagenesis of the miR-338-3p binding site in the 3'-UTR of SOX4 were predicted using the previously mentioned bioinformatics software. The primer sequences were as follows: Forward, TGG ACGACTTTAAAAACAATTCAG and reverse, CAGATT TGAGTTGCGTTTGAATC. The primers were synthesized by Sangon Biotech Co., Ltd., Shanghai, China). The pRL-TK vector (Takara Bio, Inc.) expressing *Renilla* luciferase served as an internal reference for transfection efficiency. miR-338-3p mimic or miR-338-3p mimic control was co-transfected with the luciferase vector containing the wild-type or mutated SOX4 3'-UTR, and the negative control was added directly to macrophages. Dual-luciferase reporter gene detection was performed according to the manufacturer's instructions (24).

Apoptosis detection by annexin V/propidium iodide (PI) double-staining. The apoptotic rate was determined by Annexin V/PI double-staining (Nanjing KeyGen Biotech Co., Ltd., Nanjing, China). Macrophages were digested with pancreatin and 1-5x10⁵ macrophages were transferred to an Eppendorf tube and washed twice with 1 ml phosphate-buffered saline, followed by centrifugation at 134 x g for 10 min at 4°C. Cells were resuspended in binding buffer (500 μ l; Promega Corporation). A total of 5 μ l Annexin V-fluorescein isothiocyanate (FITC) was added to the solution, followed by 5 μ l PI. Cells were incubated for 5-15 min in the dark at room temperature; assessment by flow cytometry was performed within 1 h of the conclusion of the incubation. The green fluorescence of Annexin V-FITC was examined by the FL1 channel and the red fluorescence of PI was examined by the FL2 channel. The excitation wavelength was 488 nm, and FITC and PI fluorescence were examined through filters with wavelengths of 515 and >560 nm, respectively. The lower left quadrant (Q4) of the scatter diagram exhibited healthy living cells (FITC⁻/PI⁻); the lower right quadrant (Q3) contained early stage apoptotic cells (FITC⁺/PI⁻); and the upper right quadrant (Q2) contained necrotic and late stage apoptotic cells (FITC⁺/PI⁺). Apoptotic rate=early stage (Q3) percentage+late stage (Q2) percentage.

SOX4 and caspase-3 protein expression levels detected by western blotting. Following lysis of macrophages with protein lysis buffer [50 mM Tris/HCl, 150 mM NaCl, 50 mM NaF, 1 mM Na₂P₂O₇·10 H₂O, 0.1% deoxycorticosterone, 1.0% Nonidet P40, 50 μ l of Na₃VO₄ and Halt Protease Inhibitor Cocktail (Pierce; Thermo Fisher Scientific, Inc.)], protein concentration was measured by the bicinchoninic method (25). Protein (50 μ g) was loaded onto a 10% SDS-PAGE gel for electrophoresis at 200 V for 1 h. Protein was transferred onto polyvinylidene difluoride membranes. Membranes were blocked with 5% skim milk and probed with primary rabbit anti-SOX4 (1:500; ab80261; Abcam, Cambridge, MA, USA), anti-caspase-3 (1:500; ab4051; Abcam) and anti- β -actin (1:1,000; ab8227, Abcam) overnight on a shaker at 4°C. The membranes were then washed in Tris-buffered saline three

Table I. Transfection sequences of miR-338-3p mimic, inhibitor and controls.

Group	Sequence
miR-338-3p mimic	F, 5'-UCCAGCAUCAGUGAUUU UGUUG-3' R, 3'-UUAGGUCGUAGUCACUA AAACA-5'
Mimic control	F, 5'-UUCUCCGAACGUGUCA CGUTT-3' R, 3'-TTAAGAGGCUUGCACA GUGCA-5'
miR-338-3p inhibitor	F, 5'-CAACAAAAUCACUGAUG CUGGA-3'
Inhibitor control	R, 5'-UUGUAAGUUGCGACAG CCACUCA-3'

miR, microRNA; F, forward; R, reverse.

Table II. Primer sequences.

Gene	Sequence
miR-338-3p	F, 5'-TGCGGTCCAGCATCAGTGAT-3' R, 5'-CCAGTGCAGGGTCCGAGGT-3'
U6	F, 5'-TGCGGGTGCTCGCTTCGGCAGC-3' R, 5'-CCAGTGCAGGGTCCGAGGT-3'
SOX4	F, 5'-GTGAGCGAGATGATCTCGGG-3' R, 5'-CAGGTTGGAGATGCTGGACTC-3'
GAPDH	F, 5'-AGAAGGCTGGGGCTCATTTG-3' R, 5'-AGGGGCCATCCACAGTCTTC-3'

miR, microRNA; SOX4, sex determining region Y-box 4; F, forward; R, reverse.

times. The membranes were subsequently incubated with the secondary goat anti-rabbit horseradish peroxidase-conjugated antibody (1:2,000; ab6721; Abcam) at room temperature for 1 h and visualized with Enhanced Chemiluminescence Plus reagent (GE Healthcare Life Sciences, Chalfont, UK). The protein bands were scanned by a GeneGenius automated gel imaging system (Syngene, Frederick, MD, USA) and analyzed with GeneTools software (version 4.0; Syngene).

Statistical analysis. The data was analyzed with SPSS software version 19.0 (IBM SPSS, Armonk, NY, USA), using the mean \pm standard deviation to denote the measurement data, on which normality tests were conducted. The comparison between two groups or among multiple groups was performed using independent sample *t*-test or one-way analysis of variance (uniformity test of error variance was performed prior to analysis), respectively. The comparison between any two means was conducted using the least significant difference *t*-test. *P*<0.05 was considered to indicated a statistically significant difference.

Results

Variations in miR-338-3p expression levels in MP-treated macrophages. Following treatment with MP, miR-338-3p expression levels in the MP group ($P<0.001$), MP+mimic control group ($P<0.001$), MP+inhibitor control group ($P<0.001$), MP+miR-338-3p mimic group ($P<0.001$) and MP+miR-338-3p inhibitor group ($P=0.006$) increased significantly compared with the blank (untreated) group (Table III; Fig. 1). No significant differences were observed in miR-338-3p expression levels between the MP group and the MP+mimic control and MP+inhibitor control groups ($P=0.521$ and 0.936 , respectively). In addition, no significant differences were observed in miR-338-3p expression levels between the MP+mimic control group and MP+inhibitor control group ($P=0.574$). Compared with the MP+mimic control group, the miR-338-3p expression level in the MP+miR-338-3p mimic group increased significantly ($P<0.001$); compared with the MP+inhibitor control group, the miR-338-3p expression level in the MP+miR-338-3p inhibitor group decreased significantly ($P=0.003$).

Apoptotic rate in MP-treated macrophages. The apoptotic rate in the blank group was $7.70\pm1.83\%$; however, the rate the MP group ($P<0.001$), MP+mimic control group ($P<0.001$), MP+inhibitor control group ($P<0.001$), MP+miR-338-3p mimics group ($P<0.001$) and MP+miR-338-3p inhibitors group ($P=0.013$) increased significantly (Fig. 2). The apoptosis rates of the MP+mimic control group ($23.95\pm3.15\%$) and MP+inhibitor control group ($24.05\pm2.90\%$) were not significantly different from that of the MP group ($23.28\pm2.43\%$; $P=0.720$ and 0.679 , respectively). No significant differences were observed in apoptotic rate between the MP+mimic control group and MP+inhibitor control group ($P=0.956$). Compared with the MP+mimic control group, the apoptotic rate of the MP+miR-338-3p mimic group increased significantly ($35.40\pm5.22\%$; $P<0.001$); compared with the MP+inhibitor control group, the apoptotic rate of the MP+miR-338-3p inhibitor group decreased significantly ($13.01\pm1.02\%$; $P<0.001$).

SOX4 was verified as the target gene of miR-338-3p using a dual-luciferase reporter gene system. Online bioinformatics prediction software TargetScan, PicTar and microRNA.org predicted that the 3'-UTR in the SOX4 gene mRNA contained a locus matching the mmu-miR-338-3p mature sequence, indicating that SOX4 may be the target gene of miR-338-3p (Fig. 3A). A dual-luciferase reporter gene system was adopted to further verify whether SOX4 was the target gene on which miR-338-3p directly acted. The luciferase signal of miR-338-3p mimic and pmirGLO-SOX4-3'-UTR co-transfection group decreased by 50% compared with the other groups (miR-338-3p mimic control+WT-SOX4, miR-338-3p mimic+Mut-SOX4 and miR-338-3p mimic control+Mut-SOX4 groups; $P=0.003$, 0.002 and 0.003 , respectively). However, when the mutated control pmirGLO-SOX4 mut-3'-UTR was co-transfected, the luciferase signal did not decrease and no significant differences occurred among groups regardless of the expression of miR-338-3p mimic or mimic control (miR-338-3p mimic control+WT-SOX4 group vs. miR-338-3p mimic+Mut-SOX4 group, $P=0.887$; miR-338-3p mimic control+WT-SOX4 group

Table III. Relative miR-338-3p expression levels detected by reverse transcription-quantitative polymerase chain reaction.

Group	Relative expression of miR-338-3p
Blank	1.00±0.00
MP	1.55±0.07 ^a
MP+mimic control	1.60±0.08 ^a
MP+inhibitor control	1.56±0.10 ^a
MP+miR-338-3p mimic	1.98±0.16 ^{a-c}
MP+miR-338-3p inhibitor	1.26±0.10 ^{a,b,d,e}

^a $P<0.05$ vs. blank; ^b $P<0.05$ vs. MP; ^c $P<0.05$ vs. MP+mimic control; ^d $P<0.05$ vs. MP+inhibitor control; ^e $P<0.05$ vs. MP+miR-338-3p mimic. Analysis of variance results: F-value, 35.03; P-value, <0.001. MP, morphine; miR, microRNA.

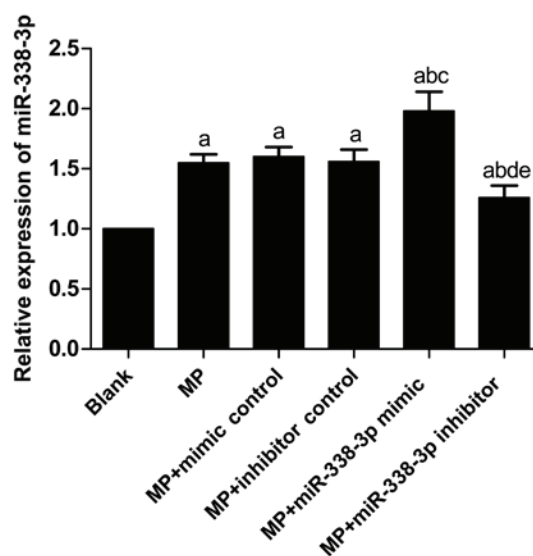


Figure 1. miR-338-3p expression levels were detected by reverse transcription-quantitative polymerase chain reaction. miR-338-3p expression levels increased in macrophages following MP treatment. This increase was enhanced by transfection of macrophages with a miR-338-3p mimic and abrogated by transfection with a miR-338-3p inhibitor. ^a $P<0.05$ vs. blank; ^b $P<0.05$ vs. MP; ^c $P<0.05$ vs. MP+mimic control; ^d $P<0.05$ vs. MP+inhibitor control; ^e $P<0.05$ vs. MP+miR-338-3p mimic. MP, morphine; miR, microRNA.

vs. miR-338-3p mimic control+Mut-SOX4 group, $P=0.810$; miR-338-3p mimic+Mut-SOX4 group vs. miR-338-3p mimic control+Mut-SOX4 group, $P=0.692$) (Fig. 3B). The result demonstrated that SOX4 expression was inhibited following miR-338-3p binding to the appropriate site in the 3'-UTR of SOX4. SOX4 may therefore be the target gene that miR-338-3p directly regulates.

Effects of miR-338-3p on SOX4 mRNA and protein expression levels in MP-treated macrophages. SOX4 mRNA (Table IV; Fig. 4) and protein (Table IV; Fig. 5) expression levels decreased significantly in the MP group ($P<0.001$), MP+mimic control group ($P<0.001$), MP+inhibitor control group ($P<0.001$), MP+miR-338-3p mimics group ($P<0.001$) and MP+miR-338-3p inhibitors group ($P=0.040$ and $P<0.001$,

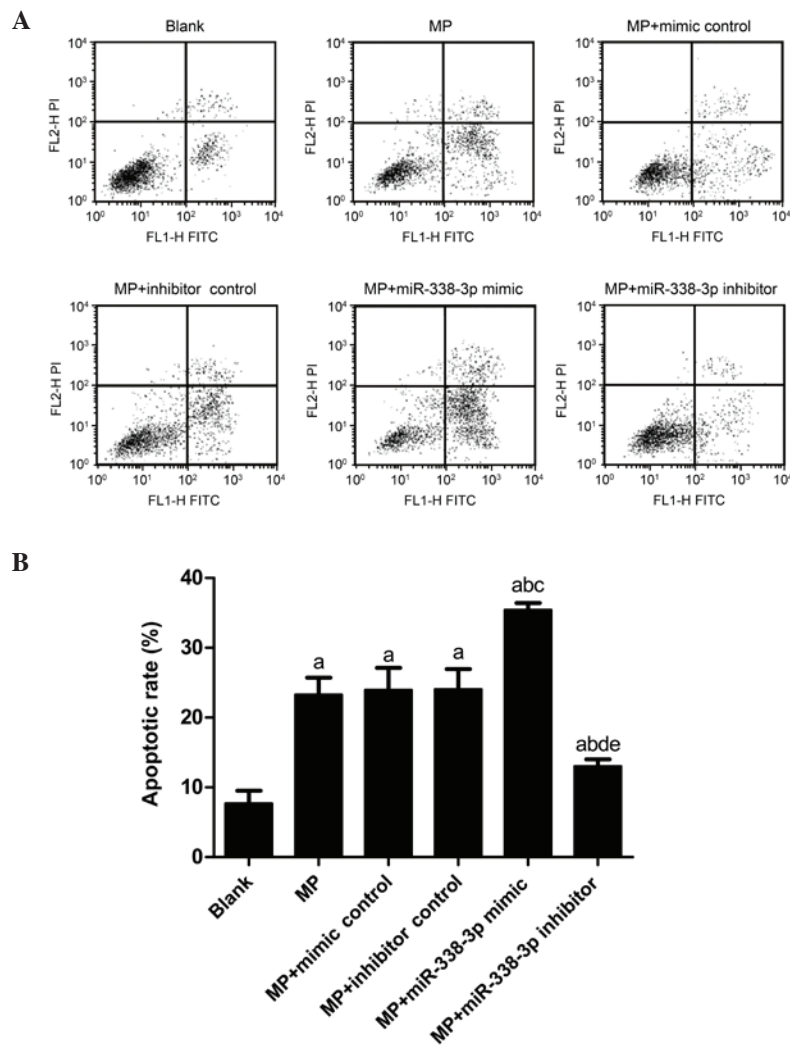


Figure 2. Apoptosis detected by flow cytometry. (A) Apoptosis was examined in macrophages following MP treatment, using Annexin V and PI. (B) Apoptosis increased following MP treatment. This increase was enhanced by transfection of macrophages with a miR-338-3p mimic and abrogated by transfection with a miR-338-3p inhibitor. * $P < 0.05$ vs. blank; ^a $P < 0.05$ vs. MP; ^b $P < 0.05$ vs. MP+mimic control; ^c $P < 0.05$ vs. MP+inhibitor control; ^d $P < 0.05$ vs. MP+miR-338-3p mimic. MP, morphine; miR, microRNA; PI, propidium iodide; FITC, fluorescein isothiocyanate.

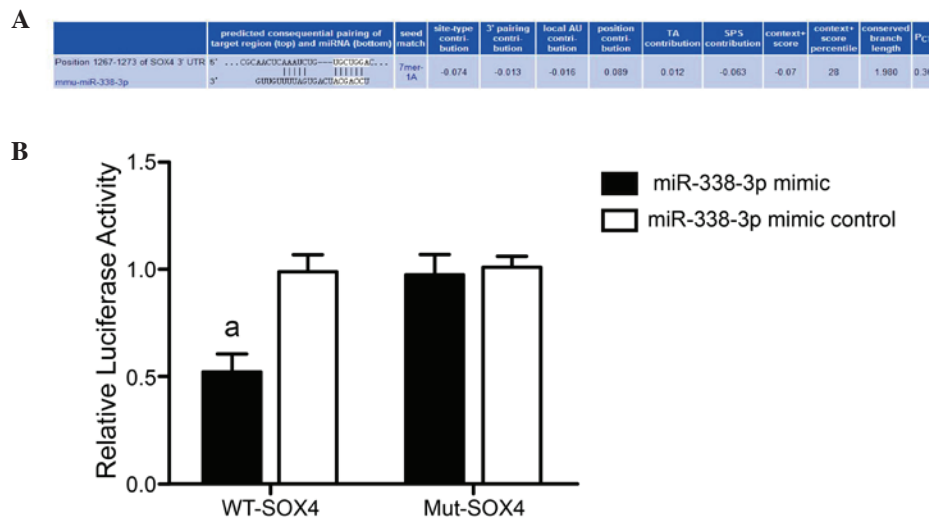


Figure 3. Dual-luciferase reporter gene assessment results. (A) The online bioinformatics prediction software TargetScan predicted that the 3'-UTR in SOX4 mRNA contained a locus that may bind mmu-miR-338-3p. (B) A dual-luciferase reporter gene system was adopted to verify whether SOX4 was the target gene on which miR-338-3p directly acted. Co-transfection of macrophages with the 3'-UTR of SOX4 and a miR-338-3p mimic decreased luciferase activity, while using a miR-338-3p mimic control or a mutated 3'-UTR had no effect. SOX4, sex determining region Y-box 4; UTR, untranslated region; miR, microRNA; mmu, *mus musculus*; WT, wild type; Mut, mutated. * $P < 0.05$ compared with the other three groups.

Table IV. Relative mRNA and protein expression levels of SOX4 detected by reverse transcription-quantitative polymerase chain reaction and western blotting.

Group	Relative SOX4 mRNA expression	Relative SOX4 protein expression
Blank	1.00±0.00	1.00±0.00
MP	0.60±0.12 ^a	0.42±0.10 ^a
MP+mimic control	0.56±0.14 ^a	0.39±0.12 ^a
MP+inhibitor control	0.52±0.09 ^a	0.38±0.08 ^a
MP+miR-338-3p mimic	0.32±0.07 ^{a,b}	0.18±0.05 ^{a-c}
MP+miR-338-3p inhibitor	0.81±0.13 ^{a,c,d,e}	0.68±0.11 ^{a,b,d,e}

^aP<0.05 vs. blank; ^bP<0.05 vs. MP; ^cP<0.05 vs. MP+mimic control; ^dP<0.05 vs. MP+inhibitor control; ^eP<0.05 vs. MP+miR-338-3p mimic. ANOVA results (SOX4 mRNA expression): F-value, 15.95; P-value, <0.001. ANOVA results (SOX4 protein expression): F-value, 33.09; P-value, <0.001. MP, morphine; miR, microRNA; SOX4, sex determining region Y-box 4; ANOVA, analysis of variance.

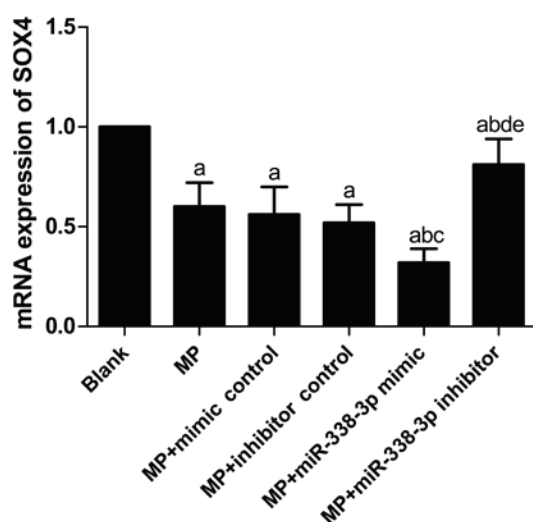


Figure 4. SOX4 mRNA expression levels detected by reverse transcription-quantitative polymerase chain reaction. SOX4 mRNA expression levels in macrophages decreased following MP treatment. mRNA expression levels were additionally decreased in macrophages transfected with a miR-338-3p mimic and the decrease was reversed by transfection with a miR-338-3p inhibitor. ^aP<0.05 vs. blank; ^bP<0.05 vs. MP; ^cP<0.05 vs. MP+mimic control; ^dP<0.05 vs. MP+inhibitor control; ^eP<0.05 vs. MP+miR-338-3p mimic. MP, morphine; miR, microRNA; SOX4, sex determining region Y-box 4.

for mRNA and protein expression, respectively) compared with the blank group. SOX4 mRNA and protein expression levels showed no significant differences between the MP group and the MP+mimic control and MP+inhibitor control groups (mRNA expression, $P=0.616$ and 0.361 , respectively; protein expression, $P=0.681$ and 0.584 , respectively). Compared with the MP+mimic control group, SOX4 mRNA and protein expression levels in the MP+miR-338-3p mimic group decreased significantly (mRNA expression, $P=0.016$; protein expression, $P=0.005$); compared with the MP+inhibitor control group, SOX4 mRNA and protein expression levels in the MP+miR-338-3p inhibitor group increased significantly (mRNA expression, $P=0.011$; protein expression, $P=0.001$).

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Table V. Relative protein expression levels of caspase-3 detected by western blotting.

Group	Relative caspase-3 protein expression
Blank	0.99±0.12
MP	2.51±0.31 ^a
MP+mimic control	2.49±0.35 ^a
MP+inhibitor control	2.50±0.25 ^a
MP+miR-338-3p mimic	4.55±0.27 ^{a-c}
MP+miR-338-3p inhibitor	1.90±0.11 ^{a,b,d,e}

^aP<0.05 vs. blank; ^bP<0.05 vs. MP; ^cP<0.05 vs. MP+mimic control; ^dP<0.05 vs. MP+inhibitor control; ^eP<0.05 vs. MP+miR-338-3p mimic. Analysis of variance results: F-value, 66.99; P-value, <0.001. MP, morphine; miR, microRNA.

macrophages. Caspase-3 protein expression levels (Table V; Fig. 6) in the MP, MP+inhibitor control and MP+mimic control groups increased significantly compared with the blank group ($P<0.001$). No significant differences were observed between the MP group and the MP+mimic control and MP+inhibitor control groups ($P=0.921$ and 0.962 , respectively), as well as between the MP+mimic control group and MP+inhibitor control group ($P=0.962$). Compared with the blank group, caspase-3 expression levels in the MP+miR-338-3p inhibitor and MP+miR-338-3p mimic groups increased significantly ($P<0.001$ and $P=0.001$, respectively). Compared with the MP+mimics control group, caspase-3 protein expression levels in the MP+miR-338-3p mimic group increased significantly ($P<0.001$); compared with the MP+inhibitor control group, caspase-3 protein expression levels in the MP+miR-338-3p inhibitor group decreased significantly ($P=0.012$).

Discussion

Previous studies have demonstrated that MP promotes apoptosis (11,12). miR-338-3p has been revealed to suppress

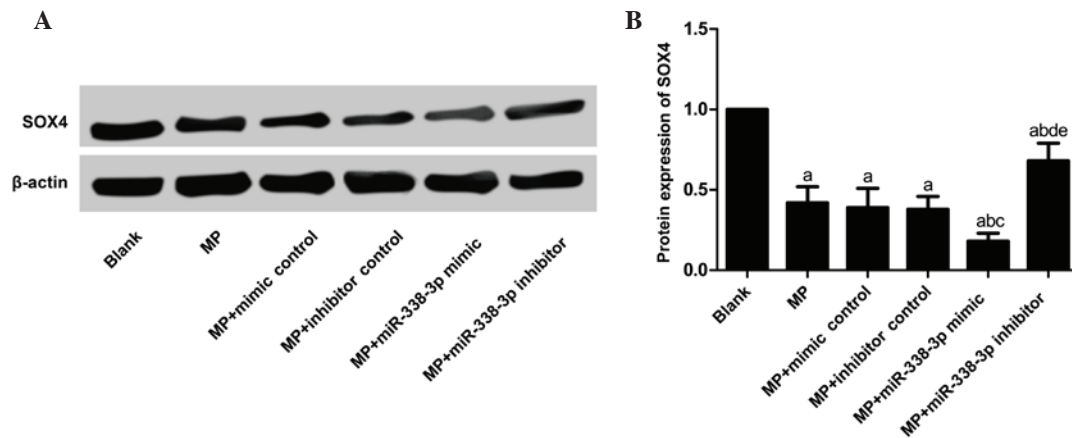


Figure 5. SOX4 protein expression levels detected by western blotting. (A) Western blots of SOX4 and β -actin. (B) Quantification of western blots demonstrated that SOX4 protein expression levels in macrophages decreased following MP treatment. Protein expression levels were additionally decreased in macrophages transfected with a miR-338-3p mimic and the decrease was reversed by transfection with a miR-338-3p inhibitor. ^aP<0.05 vs. blank; ^bP<0.05 vs. MP; ^cP<0.05 vs. MP+mimic control; ^dP<0.05 vs. MP+inhibitor control; ^eP<0.05 vs. MP+miR-338-3p mimic. MP, morphine; miR, microRNA; SOX4, sex determining region Y-box 4.

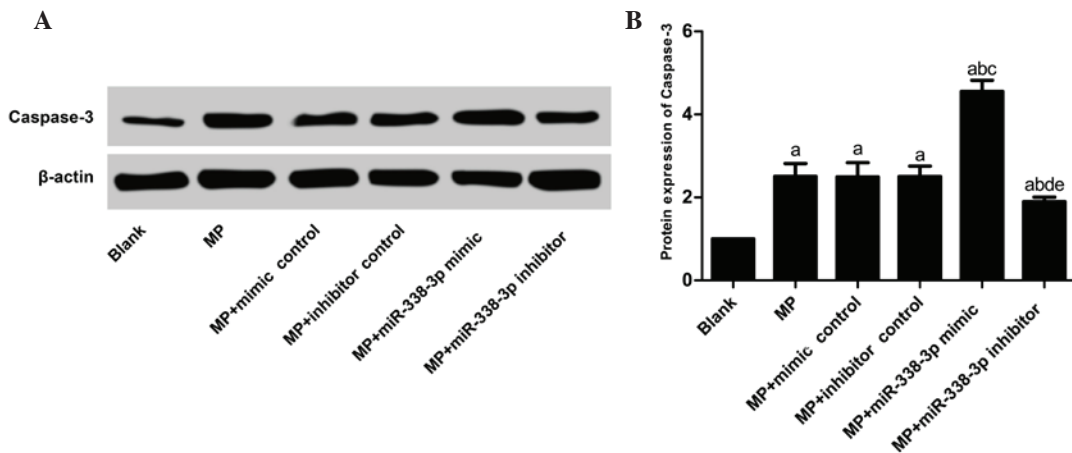


Figure 6. Caspase-3 protein expression levels detected by western blotting. (A) Western blots of caspase-3 and β -actin. (B) Quantification of western blots demonstrated that caspase-3 protein expression levels in macrophages increased following MP treatment. This increase was enhanced by transfection of macrophages with a miR-338-3p mimic and inhibited by transfection with a miR-338-3p inhibitor. ^aP<0.05 vs. blank; ^bP<0.05 vs. MP; ^cP<0.05 vs. MP+mimic control; ^dP<0.05 vs. MP+inhibitor control; ^eP<0.05 vs. MP+miR-338-3p mimic. MP, morphine; miR, microRNA.

signaling pathways, including p38, mitogen-activated protein kinase and AKT, which additionally suppress the proliferation and migration of cancer cells (20). Therefore, MP may promote apoptosis by regulating the expression of miR-338-3p. The aims of the present study were to investigate the association between MP treatment and the expression levels of miR-338-3p, define the underlying mechanism of the effects of miR-338-3p, and investigate whether miR-338-3p is a potential target molecule for the diagnosis and treatment of cancer.

In the present study, MP significantly increased the expression levels of miR-338-3p and enhanced apoptosis. The apoptosis rate decreased significantly when miR-338-3p was inhibited. Previous studies have demonstrated that miR-338-3p promotes apoptosis of cancer cells (26,27). In the present study, it was demonstrated that miR-338-3p may target the SOX4 gene and decrease SOX4 mRNA and protein expression levels. According to a previous study, SOX4 expression levels were significantly upregulated during cancer (28), which

may inhibit apoptosis (29,30). SOX4 suppresses apoptosis by inhibiting the expression of caspase-3, which may result in the proliferation of cancer cells (31). miR-338-3p may suppress the overexpression of SOX4 and prevent the decrease in caspase-3 expression. Therefore, following MP treatment, the increased expression levels of miR-338-3p would decrease the expression levels of SOX4, resulting in an increase in caspase-3 expression levels and the promotion of apoptosis. In the present study, the suppression of SOX4 expression by miR-338-3p was prevented by miR-338-3p inhibitors, resulting in a significant decrease in protein expression levels of caspase-3. This resulted in a decrease in the apoptosis rate. Consistent with previous studies, MP promoted apoptosis in the present study (11-13). Thus, upregulated expression of miR-338-3p may be a primary mediator of the therapeutic effect exerted by MP.

On the other hand, a previous study observed that partial miR expression was easily modulated under the influence of MP (32); in addition, numerous miRs have been demonstrated

to exhibit increased expression during addiction (31,33,34). Therefore, the upregulated expression of miR-338-3p following MP-treatment may be associated with the effects of addiction. The detailed underlying mechanism of this effect remains to be elucidated. However, the side effects associated with MP should not be ignored, as overuse of MP may result in abnormal apoptosis of cells, particularly nerve cells (13,35). Strategies to increase miR-338-3p expression in the absence of MP may be of potential benefit in cancer treatment (21).

In conclusion, the results of the present study demonstrate that MP treatment upregulated the expression levels of miR-338-3p and enhanced its suppression of SOX4 expression, which resulted in increased expression of caspase-3 and increased apoptosis. The apoptosis rate decreased when the expression of miR-338-3p was inhibited. miR-338-3p may be important in MP treatment of cancer, as a mediator of MP-induced apoptosis. The upregulation of miR-338-3p expression provides a potential strategy for the treatment of cancer.

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