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Mocetinostat suppresses epidural fibrosis following laminectomy by inhibiting myofibroblast activation and increasing apoptosis

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Abstract. – OBJECTIVE: To investigate the effect and mechanism of mocetinostat on diminishing epidural fibrosis. Dysregulated wound repair usually occurs after injury or surgery and is featured by excessive scar tissue contributed by fibrosis. Increasing researches demonstrated that histone acetylation, an epigenetic alteration, plays a crucial role in fibrosis. However, the mechanism of the complicated proces[illegible] remains unclear. In the current study, the[illegible] of histone deacetylase (HDAC) inhibito[illegible] tinostat in a rat model of epidural fibros[illegible]s detected, and it was discovered that mocet[illegible] tat suppressed myofibroblast activation and[illegible] creased apoptosis by reducing Akt/GSK3b si[illegible] naling.

PATIENTS AND METHOD[illegible] levels of histone acetylation in th[illegible]ients' [illegible]dural fibroblasts were analyzed[illegible], mRN[illegible] teins obtained from hum[illegible]robla[illegible] TGF-β activation and [illegible]oce[illegible]eatment *in vitro* were used to e[illegible]e the [illegible]ce of mocetinostat on the ac[illegible]ion and s[illegible] of fibroblasts, so as to[illegible]e the related [illegible]anism of mocetinosta[illegible] [illegible]ectomy mode[illegible] was established in r[illegible]o obs[illegible]e therapeutic effect of mocetin[illegible]at on epidur[illegible] tissues.

RESUL[illegible] In this researc[illegible]as found that the inc[illegible]e of HDAC1 in human dura scar was acco[illegible]nied b[illegible]e aggravation of fibrosis. In add[illegible] cell[illegible]y demonstrated that mocetinos[illegible]ib[illegible] fibrobl[illegible]ctivation and accelerated [illegible]is by i[illegible]iting Akt/GSK3b pathway. In th[illegible] mo[illegible] mocetinostat weakened [illegible]yperpl[illegible] collagen deposition and effe[illegible]ely inhib[illegible] the process of epidural fibros[illegible]

[illegible]: The above results indicate th[illegible]nocetin[illegible]at inhibits HDAC1 expression and [illegible]reases the conduction of the AKT/GSK3b [illegible]ay in fibroblasts, leading to myofibroblast ac[illegible]n and apoptosis elevation. Hence, mocetinos[illegible] ameliorates epidural fibrosis.

Key W[illegible][illegible]t, Mocetinostat, [illegible]brosis, HDAC, Epidur[illegible].

[illegible]troduction

Dysreg[illegible]ation of wound repair often leads [illegible] excessive scar tissue proliferation caused by [illegible]. Epidural adhesion is the main patho[illegible]gica[illegible]anifestation of excessive fibrosis following laminectomy, which causes failed back surgery syndrome (FBSS) and seriously affects the outcome of lumbar surgery[2,3]. However, the [illegible]ormation of epidural scar adhesion is triggered by a variety of factors, including inflammatory response post-surgical trauma, excessive proliferation, and migration of fibroblasts, and a large amount of synthesis of extracellular matrix (ECM)[4,5]. Histone deacetylases (HDACs) are enzymes that remove acetyl groups from the amino-terminal lysine residues of histones, causing the densification of chromatin, inhibiting transcription, and reducing gene expression[6,7]. Therefore, HDAC inhibitors (HDACI), as a new class of anti-tumor drugs, have the characteristics of high efficiency and low toxicity, and can inhibit tumor cell proliferation, induce cell cycle arrest, promote cell differentiation or apoptosis after acting on tumor cells[8,9]. Many reports prove that HDAC expression changes during fibrosis after injury and HDACI inhibits fibrosis and prevents multiple insults and defects of the organs[10,11]. HDACI can reduce ischemic myocardial infarction in the body and protect myocardial structures from fibrous hyperplasia *in vivo*[11,12]. In addition, the HDAC inhibitor valproic acid

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(VPA) alleviated Ang II-induced cardiac fibrosis and myocardial pericytes by inhibiting HDAC 4-dependent phosphorylation of ERK[13]. Mocetinostat is a novel type of benzamide HDACI with high oral activity, which is used as a single drug or combined with gemcitabine and docetaxel for the treatment of hematologic malignancies and solid tumors[14,15]. Moreover, mocetinostat attenuates ischemic heart failure and exerts anti-fibrotic effects both *in vitro* and *in vivo*[16,17]. However, the effects of mocetinostat on epidural fibrosis remain to be elucidated. In the present study, it was discovered that mocetinostat inhibited fibroblasts in epidural scar tissues, reduced collagen deposition, and enhanced apoptosis from epidural fibrosis.

Patients and Methods

Patient Tissue Samples

Human epidural scar tissues were obtained from patients undergoing secondary decompression surgery, and the procedure was ap[illegible] by the Hospital Ethics Committee of th[illegible] Hospital, Shanghai Jiaotong University [illegible]ol of Medicine. The informed consent was obt[illegible] from patients or their families before sam[illegible] collection. A total of 25 patients including [illegible] males and 11 females aged 38[illegible]old, with a mean age of 47 years old d[illegible]ed th[illegible]pidural scar tissues. Pending tissu[illegible]ere stor[illegible] liquid nitrogen for later experi[illegible]

Cell Culture and [illegible]g Tre[illegible]t

Fresh epidural [illegible] tissues we[illegible]hed by Dulbecco's Mo[illegible]le's Medium[illegible] Ham's F-12 mediu[illegible]MEM[illegible] Keygen, China) and fragmentiz[illegible]d. The disso[illegible] was performed using 0.2[illegible]rypsin solution[illegible]C for 15 min. Then, t[illegible] mixture was treated with 0.15% type II co[illegible]enase [illegible]°C overnight, and the cell sol[illegible]was t[illegible]ferred to a cell strainer with 100 µ[illegible]zes an[illegible]suspended in DMEM/F12 (The[illegible]Fishe[illegible]ientific, Waltham, MA, [illegible]ontai[illegible] fetal bovine serum (FBS, Gib[illegible]Rockvi[illegible]MD, USA). After that, the cell[illegible]re seeded in 5′5 cm^2 flask and TGF-β (10 n[illegible]Aldrich, St. Louis, MO, USA), [illegible]ibroblasts were stimulated to evoke differ-[illegible]ation. Then, mocetinostat (20 ng/mL Med-C[illegible]xpress, Monmouth Junction, NJ, USA) was [illegible]d to treat cells.

Rats

Eight-week-old Sprague Dawley (S[illegible] tained from the Shanghai Jiaoto[illegible]niver[illegible] School of Medicine Animal Ce[illegible], were bred and maintained at the Shangh[illegible]otong Uni-versity School of Medicine Anim[illegible]ter. This study was approved by the[illegible]imal E[illegible]Com-mittee of Shanghai Jiao[illegible]g University of Medicine Animal C[illegible], and [illegible] experi[illegible] were performed in [illegible]rdanc[illegible]th the Guide for the Care and [illegible]f L[illegible]atory [illegible]mals of the Shangha[illegible]ote[illegible]versity [illegible]ool of Medicine.

Laminec[illegible]d Drug Inje[illegible]on

The [illegible] were [illegible]etized with 10% chloral hydrate (4 mL/kg) to p[illegible]e skin. After disinfec-tion[illegible] incision was [illegible] to separate the fas-c[illegible]yer and the muscle layer, and laminectomy [illegible] performed at T10. After rinsing by normal [illegible]ne, the inci[illegible] was closed and disinfected [illegible] hemostasis [illegible]en, 20 mg/kg was adminis-te[illegible]nce dai[illegible]r 7 days *via* intraperitoneal inje[illegible]

Cell Viability Assay

[illegible]ounting Kit-8 (CCK-8) assay was per-[illegible]rmed to measured fibroblasts viability using CCK-8 Cell Viability/Cytotoxicity Assay Kit (C0009; Beyotime, Shanghai, China) following the manufacturer's protocol. Briefly, fibroblasts were transferred in 96-well plates with the density of 1×10^4 cells/well. Following the treatment with different concentrations of mocetinostat, the absorbance was then measured using a microtiter plate reader (Labsystems Multiskan, Helsinki, Finland) at 570 nm.

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

The total RNA of fibroblasts or scar tissue were isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) abided by the manufacturer's protocol. The complementary deoxyribose nucleic acid (cDNA) synthesis was conducted using the PrimeScript™ RT Master Mix (Applied Biosystems, Foster City, CA, USA). Then, HDAC 1, collagen I, Akt, GSK3b, caspase 3/8, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were detected using the SYBR PremixEx TaqII kit (RR820A, TaKaRa, Otsu, Shiga, Japan). The primers are listed as follows: HDAC 1: Forward, 5′-CGCATGACTCATAAT-3′, Reverse, 5′-GCTGTGGTACTTGGTCATCT-3′;

Collagen I: Forward, 5′-CATCAAGGTCTTCTG-CGACA-3′, Reverse, 5′-CTTGGGGTTCTTGCT-GATGT-3′; Caspase3: Forward, 5′-GCCATCGT-GGCTAAACAGGTA-3′, Reverse, 5′- GTTG-GTGTTCATCCGCTTGC-3′; Caspase8: For-ward, 5′-CTGGAAGATGGTCGTACCCTG-3′, Reverse, 5′-GGTCTTGCCAGTGAGTGTCT-3′; Akt: Forward, 5′-ACCGTGTGACCATGAAC-GAG-3′, Reverse, 5′-GGTCGTGGGTCTG-GATGAG-3′; GSK3b: Forward, 5′-ATGGCAG-CAAGGTAACCACAG-3′, Reverse, 5′-TCTCG-GTTCTTAAATCGCTTGTC-3′; Bax-2: Forward, 5′-CTGACAGTTTTCTGACGG-3′, Reverse, 5′-TCAGCCACTTCCAGA-3′; Bcl-2: Forward, 5′-GCTACCGTCGTGACTTCGC-3′, Reverse, 5′-CCCCACCGAACTCAAAGAAGG-3′; GAP-DH: Forward, 5′-GCAAGTTCAACGGCACAG, Reverse, 5′-GCCAGTAGACTCCACGACCAT. The $2^{-\Delta\Delta Ct}$ method was used to calculate the rela-tive mRNA levels.

Western Blotting Analysis

Scar tissue or fibroblasts were treated using a Total Protein Extraction Kit (KeyGEN, Nanjing, China) with phosphatase and protease i tors. Following violent oscillation and l perature centrifugation, the protein con tion was measured with the bicinchoninic (BCA) protein assay kit (Thermo Fisher Sc tific, Waltham, MA, USA) and b anced. Se arated in 10% sodium dode te-poly-acrylamide gel electrophor (SDS GE) gel and transferred to a pol ylidene luoride (PVDF) membranes (M e, B USA), the proteins w b h a Quick Block Solution (Epi e, Chi d incubated overnight at low perature wit primary antibodies (ant l (Abcam, C bridge, MA, USA, 1 0), ax-2 (Abcam, Cam-bridge, MA USA, 1:100 ti-Bcl-2 (Abcam, Cambrid MA, USA, 1:10 ti-caspase3/8 (Abcam ambridge, MA, USA, 1:1000), an-ti-col n I (M pore, Billerica, MA, USA, 1:1 anti gen I (Abcam, Cambridge, MA, 00), a -SMA (Abcam, Cam-bridge, USA 1000), anti-fibronectin n, Ca MA, USA, 1:500), and anti PDH (C ignaling Technology, Dan-vers A, USA, 1:2000)). Washed by Tris-Buff-er en (TBST) and incubated with secondary antibody (Abcam, Cambridge, USA, 1:2000) at room temperature, the p were visualized and using the enhanced chem minescence system.

Flow Cytometry Analysis

Fibroblasts apoptosis degree was m ing Apoptosis Detection Kit (KeyG Nanji China). Following the manufact s protocol, Annexin V-FITC and propidiu de (PI) were stained with fibroblasts for 30 mi Then, the cells were sorted and ana d usin ores cence-activated cell sort low cytom Biosciences, San Jose, USA).

Statistical Ana sis

Data were ressed an standard deviation. differences the two groups w d using the dent's *t*-test. Compar bet ultiple groups was done using One-way AN st followed by post-hoc east Signific fference). $p < 0.05$ s sted that the difference was statistically ificant.

Results

HDAC was Differentially Expressed in Human Epidural Scar

o determine whether there are differ-ces the expression of HDAC 1 in the epidural scar tissue and HDAC 1 impacts on the fibrosis process, human epidural scar tissues were used to measure the proteins and RNAs of HDAC and collagen I, the marker of pro-fibrosis. It was found that collagen I protein expression in severe fibrosis tissues was significantly increased com-pared with those with mild fibrosis, which was accompanied by increased HDAC 1 expression (Figure 1A). RNA analysis consistently revealed marked increases in collagen I and HDAC levels in the severe group compared to the mild group (Figure 1B and 1C). The results indicated that the increase of HDAC 1 expression was accompanied by the exacerbation of progress and degree of fibrosis.

Mocetinostat Increased Fibroblast Apoptosis In Vitro

To investigate the effect of mocetinostat con-centration on fibroblast viability, fibroblasts were treated with mocetinostat at different concentrations (5 ng/mL-50 ng/mL), and vi-ability was detected *via* CCK-8 assay, whose results revealed that mocetinostat administra-tion reduced fibroblast viability resulting from increasing concentrations (Figure 2A). There-

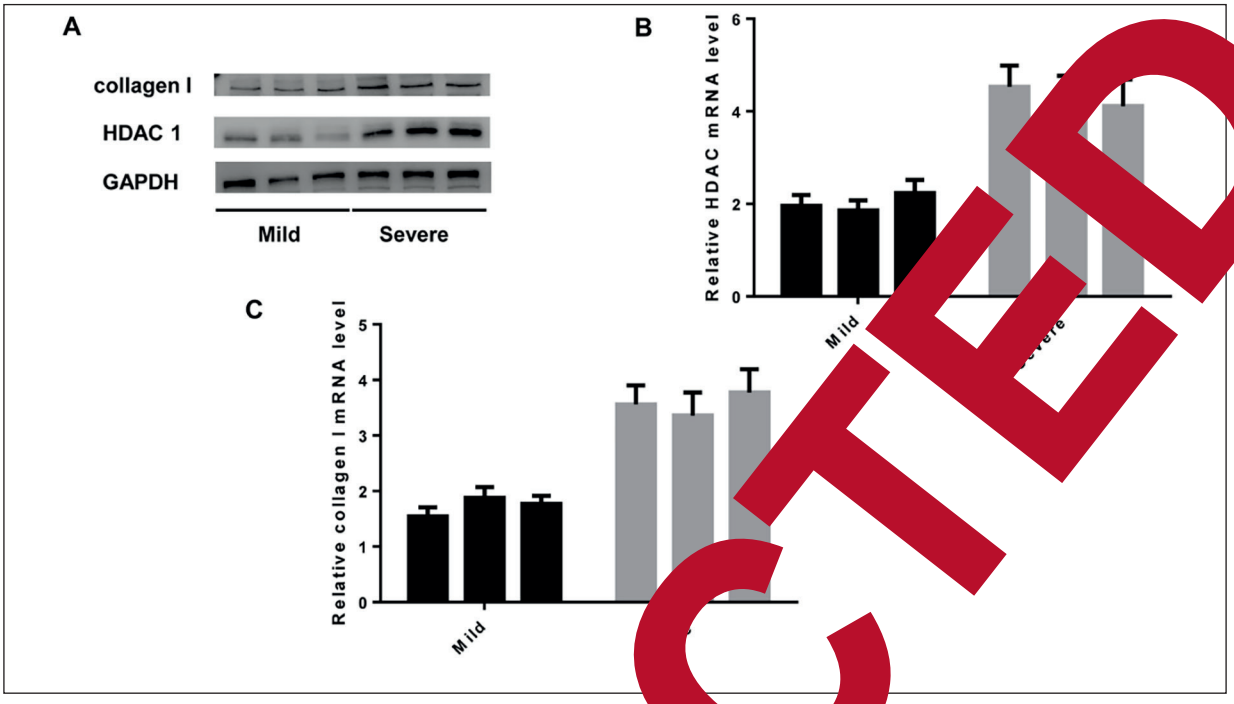


Figure 1. HDAC is differentially expressed in human epidural sca[illegible]pres[illegible]e Western blotting of collagen I and HDAC in the Mild group and the Severe group. **B,** Representative mR[illegible]n of HDAC in the Mild group and the Severe group. **C,** Representative mRNA level of HD[illegible] the Mild group and Severe group.

fore, 20 ng/mL was selected as the treat[illegible]t concentration of cells under the condition maintaining most cell viability. In addition, cell cytometry manifested th[illegible]poptosis level of fibroblasts in mo[illegible]ostat[illegible]eatment was significantly increas[illegible]ompare[illegible]ith that in the control group an[illegible]F-β g[illegible] 2B). Meanwhile, the [illegible]NA[illegible]in expression levels of apopt[illegible]related[illegible]Caspase3/8 were remarkably [illegible]eased (Figu[illegible]nd 2D). Therefore, it ca[illegible]luded that m[illegible]inostat could increas[illegible]aspa[illegible]xpression to induce fibroblast a[illegible]optosis.

Inhibi[illegible] of HDAC Reduces Fibroblast Acti[illegible]on In[illegible]o

[illegible]rmo[illegible]whet[illegible] the inhibition of HDA[illegible] imp[illegible]on the activation of TGF-β [illegible]e in[illegible]roblasts was explored. [illegible]ing [illegible] mocetinostat treatment, fibr[illegible]sts were[illegible]rvested to extract proteins and[illegible]As. ECM was measured using Western bl[illegible]was found that after TGF-β acti-[illegible]d fibroblasts, the protein levels of collagen [illegible]ollagen III, fibronectin, and α-SMA were s[illegible]antly increased compared with the control g[illegible]up. However, collagen I, collagen III, [illegible]SM[illegible] and fibronectin were all remarkably downregulated *via* mocetinostat administration (Figure 3A). Besides, it was discovered that the RNA and protein levels of HDAC were decreased after mocetinostat treatment, and the expressions of Akt and GSK3b were also evidently inhibited (Figure 3B). The above results indicate that mocetinostat reduces the Akt/GSK3b signaling pathway by inhibiting HDAC 1 expression, leading to decreased activation of fibroblasts.

Mocetinostat Attenuated Epidural Fibrosis in Rats After Laminectomy

Rat epidural scar in laminectomy group and mocetinostat group at a week and post laminectomy was detected, and collagen deposition and the expression of apoptosis-related factors were measured. The results demonstrated that mocetinostat administration notably decreased the generation of collagen I, fibronectin, and α-SMA in epidural scar following laminectomy (Figure 4A), and the expressions of Bax-2 and Caspase 3/8 were increased, while the expression of Bcl-2 was decreased at one week after laminectomy (Figure 4B and 4C). Besides, the expression levels of HDAC 1 and Akt/GSK3b was examined at a

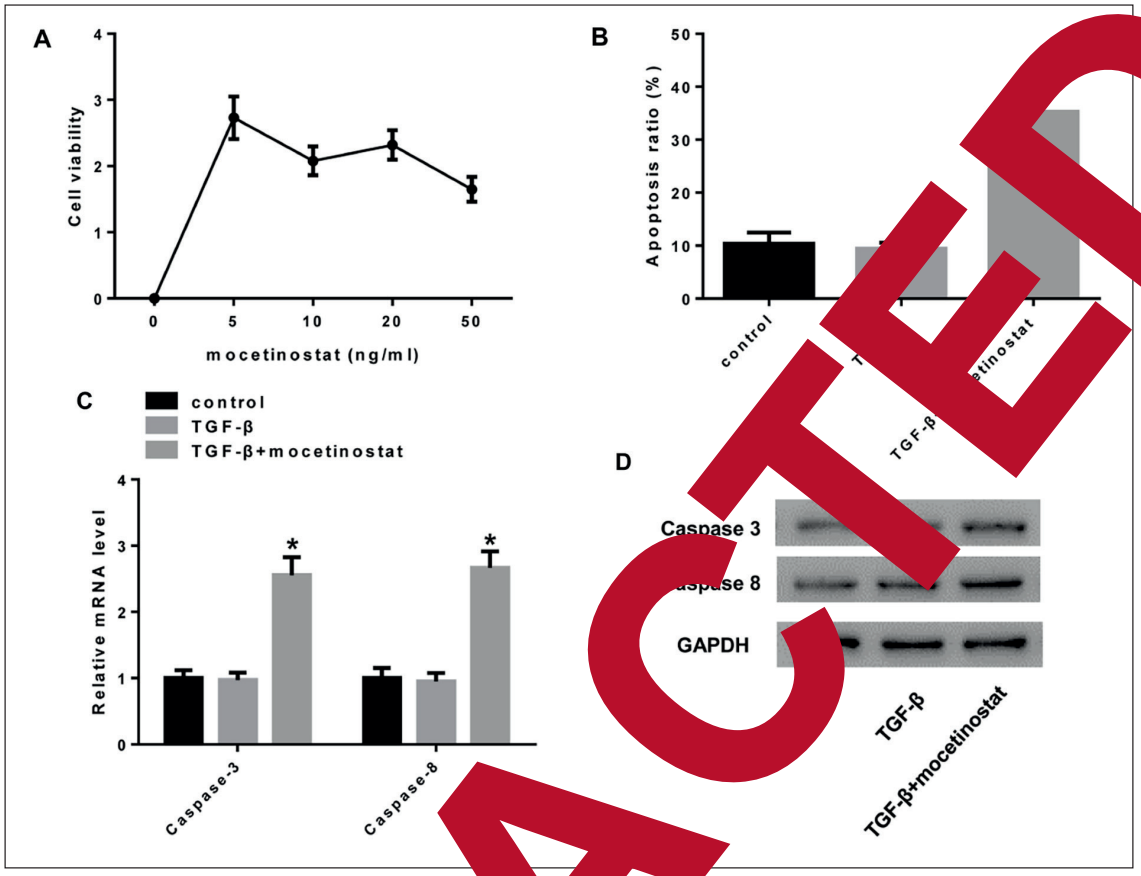
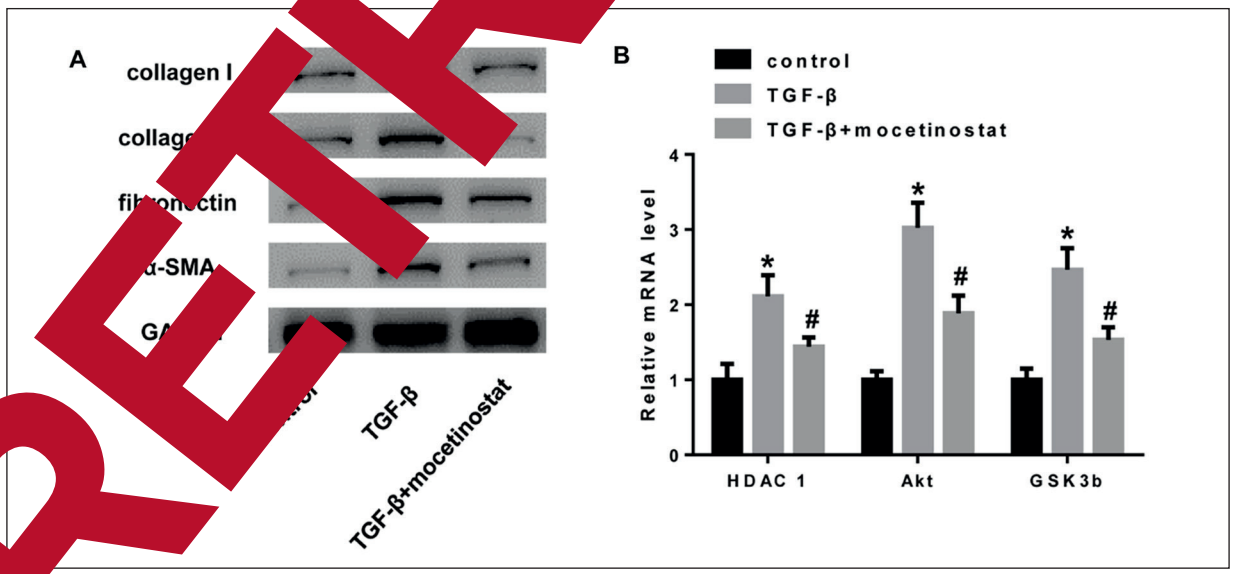


Figure 2. Mocetinostat increases fibroblasts apoptosi… **A,** Cell viability alteration in 5 ng/mL, 10 ng/mL, 20 ng/mL and 50 ng/mL mocetinostat, respectively. **B,** Cell ap… ratio in the control, TGF-β, and TGF-β+mocetinostat group. **C,** Representative RNA levels of C… nd Caspas… the control, TGF-β, and TGF-β+mocetinostat group. **D,** Representative Western blotting of … Caspase-8 … ntrol, TGF-β, and TGF-β+mocetinostat group.



…3. Inhibition of HDAC reduces fibroblast activation *in vitro*. **A,** Representative Western blotting of collagen I, collagen … …ctin, and α-SMA in control, TGF-β, and TGF-β+mocetinostat group. **B,** Representative RNA levels of HDAC, Akt, and G… …b in control, TGF--β, and TGF-β+mocetinostat group.

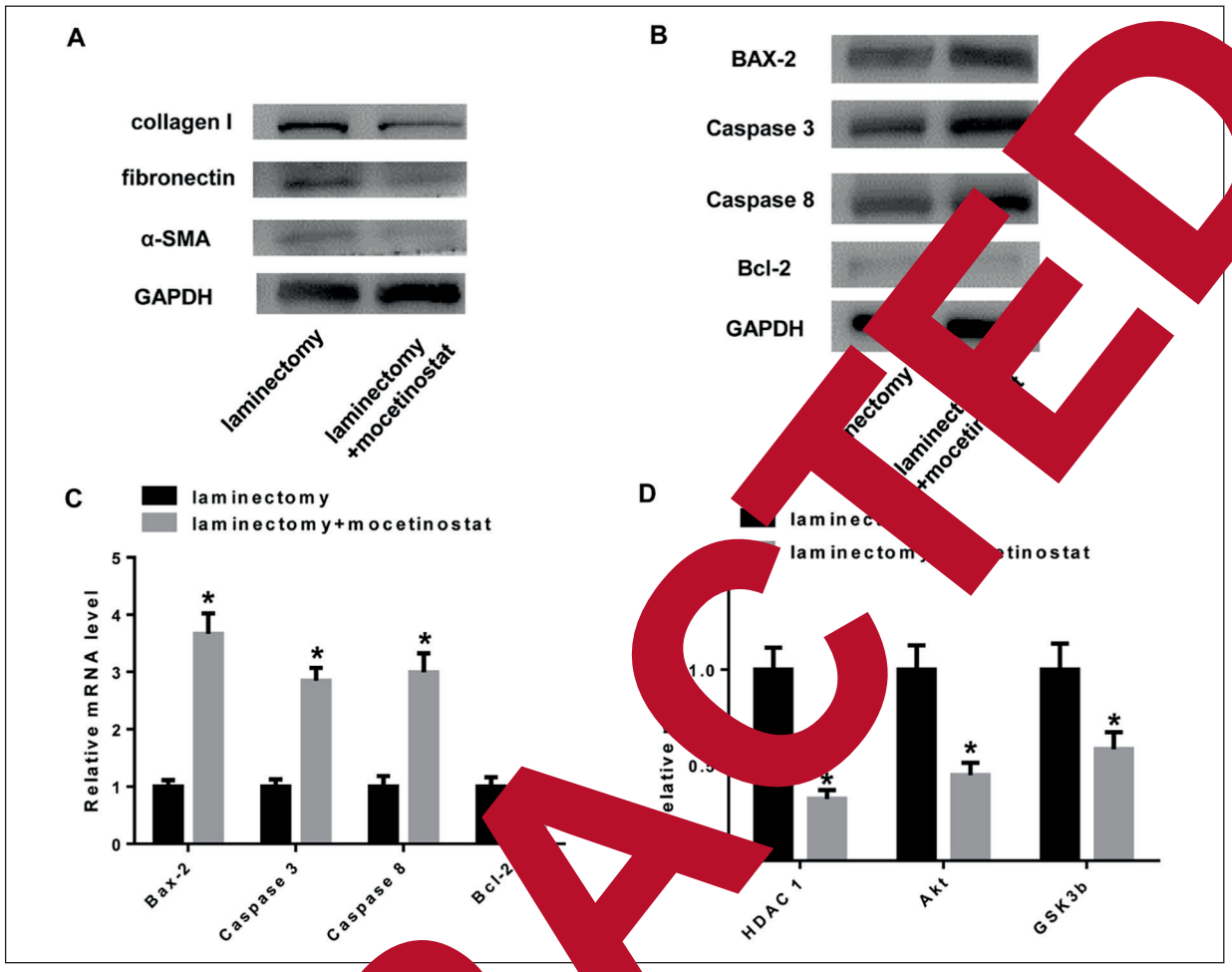


Figure 4. Mocetinostat attenuat dural sis in rats wing laminectomy. **A,** Representative Western blotting of collagen I, fibronectin, and α-SM the lam omy and lam nectomy+mocetinostat group at one week following surgery. **B,** Representative Western blo f Bax-2 se-8, and Bcl-2 in laminectomy and laminectomy+mocetinostat group at one week following su NAs of Bax-2, Caspase-3, Caspase-8, and Bcl-2 in laminectomy and laminectomy+moceti t gr week following surgery. **D,** Representative mRNAs of HDAC, Akt, and GSK3b in laminectomy and la tomy+moc t group at one week following surgery.

week after laminectomy, iting that moceti-nostat sig antly downreg he expression levels DAC 1 and Akt/GSK3b (Figure 4D), whic rther onstrated that mocetinostat am ted ural fib is by inhibiting the Akt/G way.

scussion

rplasia usually occurs in wound r, but excessive fiber activation triggers a amount of collagen deposition, leading to e ve scar tissue, and affecting healing[18,19]. Fibr st plays an important role in the secretion of extracellular matrix and the regulation of the stability of fibrosis scar[20]. Epidural fibrosis is associated with changes in fibroblasts, including activation and differentiation of fibroblasts, proliferation, and apoptosis, as well as accumulation of extracellular matrix. Severe epidural fibrosis can cause compression of the spinal cord and nerve root tissue, resulting in severe neurological dysfunction and disorder. For the complicated mechanism of epidural fibrosis, ECM increase and cell proliferation are widely accepted as the specific features during its process.

Currently, there are 18 human HDAC subtypes. Based on the sequence homology with

yeast, the 18 HDACs were divided into four classes (I, II, III and IV). A total of 11 HDACs in class I, II, and IV are Zn^{2+}-dependent protein but 7 subtypes in class III belong to Sirt 1-7 family. Class I including HDAC1, 2, 3, and 8, showing highly homologous to yeast RPD3 protein, distributes within the nucleus, and mainly takes inhibited function of gene transcription. Class II HDACs (HDAC4, 5, 6, 7, 9, and 10), homologous to yeast Hda1 protein, distributes in cytoplasm, while shuttling between the nucleus and cytoplasm. However, class III HDACs are NAD^{+}-dependent protein deacetylases regulating various cellular processes, such as survival, aging, stress reaction, and metabolism with ADP ribose transferase jointly. Only HDAC 11 is listed in Class IV, characterized as the sequence homology with class I and II enzyme catalytic core region but less similarity[21-26]. HDAC dysfunction is linked to a variety of diseases, including cancer, diabetes, and cardiac hypertrophy[27]. HDACIs are molecules binding to HDAC to interfere with/block its function. HDACIs regulate gene expression and apoptosis through acetylati of histone. A total of six HDACIs have b proved by the Food and Drug Adminis on (FDA) for the treatment of multiple hemato tumors and a few solid tumors. HDACIs into three main categories: (1) ydroxam acids; (2) benzamides; (3) cy des. Lyu et al[28] have demonstrated t DAC deemed as antifibrotic drugs in c ac and monary fibrosis. So, it was sugg that i activity of HDACs y pro-fibrotic signaling loops dir . More he effect of fibroblast apopto as explored HDACI. Mocetinostat, AC class I i itor, is effective in t eati f malignant tumors, and it is f nd to atten ngiotensin II-induced ca fibroblast mig and proliferation b ferentially regulating MMP9, IL-18, and K exp ons.

In the t of different levels of HDAC both M nd Severe group, it is evidently pidu car samples with much fibrosi higher HDAC expression and ower co en I expression. Regarding the oblast cells culture, CCK-8 assay was ar re the optimized concentration stimulation of the mocetinostat. The re- indicated that mocetinostat had an ability te ote the apoptosis of fibroblasts with an o mized concentration. TGF-β has been widely used to mimic the pathophysiology of fibroblast activation *in vitro*. Therefo fibrosis model was constructed t sev targets corresponding to the fib sia and cel apoptosis. It was exhibited th levels of fibrosis markers, such as collagen gen III, fibronectin, and α-SMA ificall ased with the TGF-β stimuli pared with trol one. However, it to a ersion the administration o cetino As expected, the anti-fibrosis e f tinosta the epidural fibrosis pe the in ion of ECM generat and pr to f fibroblasts. Rece idence has s that Akt/GSK3b is l pro-fibrosis thway in the progres ibro tivation, and mocetinostat is an efficient in of it. In this study, it was ed that Akt/ b pathway exerted p rosis function and mocetinostat treatment d induce Akt/GSK3b pathway inhibition to viate fibrosis cess.

Moreover, fib last proliferation is another fa in epidu brosis. Thus, promoting the appr ptosis of fibroblasts may be a potential way to alleviate epidural fibrosis. At the origination of this research, it was observed tinostat could influence fibroblast viability. To analyze whether mocetinostat has the capacity to enhance cell apoptosis, Caspase 3 and Caspase 8, two key factors in the regulation of apoptosis, were analyzed. Previous studies have shown that Caspase 3 and Caspase 8 can participate in apoptosis by regulating oxidative stress and inflammatory response. The findings of this research also strongly supported that Caspase 3 and Caspase 8 are activated by the decreased expression of HDAC. Therefore, mocetinostat is a promising inhibitor that suppresses the influence of Caspase 3/8 to increase the fibroblasts apoptosis in epidural fibrosis.

Conclusions

In summary, it is of great significant to seek a molecule that functions by inhibiting ECM accumulation and elevating cell apoptosis *via* inhibition of HDAC to attenuate epidural fibrosis. The role of mocetinostat treatment on the fibrosis model established by the fibroblasts was systematically evaluated. In a word, the above results reveal that mocetinostat may be a useful method to treat the epidural fibrosis.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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