Cell. Mol. Biol. (ISSN: 1165-158X)

Journal Homepage: www.cellmolbiol.org

Cellular and Molecular Biology

Original Article

Tubastatin alleviates epidural fibrosis via negatively targeting TGFβ/PI3K/Akt pathway

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Article Info

Article history:

Received: December 15, 2023
Accepted: March 12, 2024
Published: April 30, 2024

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Abstract

Epidural fibrosis (EF) is a chronic, progressive and severe disease. Histone deacetylase 6 (HDAC6) regulates biological signals and cell activities by deacetylating lysine residues and participates in TGF-β-induced epithelial-mesenchymal transition (EMT). Nevertheless, the effect and mechanism of HDAC6 in EF remain unclear. To investigate the effect and mechanism of HDAC6 inhibition on repressing epidural fibrosis. HDAC6 expression and α-smooth muscle actin (α-SMA) in normal human tissue and human EF tissue were assessed by quantitative real-time PCR (qRT-PCR) and western blotting. Human fibroblasts were treated with TGF-β ± HDAC6 inhibitors (Tubastatin) and fibrotic markers including collagen I, collagen III, α-SMA and fibronectin were assessed using western blotting. Then TGFβ1 receptor (TGFβ1-R), PI3K and Akt were analyzed using qRT-PCR and western blotting. Rats were undergone laminectomy± Tubastatin (intraperitoneally injection; daily for 7 days) and epidural scar extracellular matrix (ECM) expression was gauged using immunoblots. Increasing HDAC6 expression was associated with α-SMA enrichment. Tubastatin remarkably restrained TGF-β-induced level of collagen and ECM deposition in human fibroblasts, and the discovery was accompanied by decreased PI3K and Akt phosphorylation. Moreover, Tubastatin also inhibited TGF-β-mediated HIF-1α and VEGF expression. In the epidural fibrosis model, we found that Tubastatin weakened scar hyperplasia and collagen deposition, and effectively inhibited the process of epidural fibrosis. These results indicated that Tubastatin inhibited HDAC6 expression and decreased TGF-β/ PI3K/ Akt pathway that promotes collagen and ECM deposition and VEGF release, leading reduction of myofibroblast activation. Hence, Tubastatin ameliorated epidural fibrosis development.

Keywords: Collagen; fibroblast, HDAC6, Tubastatin, Epidural fibrosis.

1. Introduction

Failed back surgery syndrome (FBSS) leads to a diverse series of spinal cord and nerve root disorders following surgery, which is characterized as different levels of pain and numbness resulting in neurologic loss of function and even dysfunction [1,2]. Common etiology of FBSS is epidural fibrosis [3]. Representative pathological feature of epidural fibrosis (EF) is the formation of fibroblast foci, consisting of excessive collagen and ECM deposition and abundant fibroblast activation [4,5]. Myofibroblasts are the critical initiators that synthesize and release a multitude of fibrotic proteins including collagen I, collagen III, α-SMA and fibronectin, etc. [6-8].

Although a variety of types of cells can transform into myofibroblasts, fibroblasts are thought to be the dominant position of myofibroblasts transition [9]. In multiple fibrogenic factors related to the pathogenesis of EF, transforming growth factor (TGF)-β has been suggested to be a major source for myofibroblast accumulation *via* regulating Smad2/3 and PI3K /Akt signaling pathways [10,11]. Clearly, EF remains a huge therapeutic challenge and there is an urgent need to develop effective anti-fibrotic drugs. Histone deacetylases (HDACs) are aimed to remove the acetyl groups from lysine residues in histone and nonhistone proteins [12]. HDACs participate in the composition of inhibiting transcription and regulate diverse cellular processes, such as controlling cell proliferation, inducing cell cycle arrest, and promoting cell differentiation or apoptosis [3,141].

Recent studies suggested that histone deacetylase inhibitors (HDACIs) might also inhibit collagen production in myocardial remodeling [15] and pulmonary fibrosis [16]. Valproic acid alleviated Ang II-induced cardiac fibrosis and myocardial pericytes through inhibiting HDAC 4-dependent phosphorylation of ERK [17]. Phenylbutyrate has been reported to decrease TGF-β-stimulated collagen I mRNA and protein levels in the lung fibroblast [18]. Trichostatin A inhibited collagen production in sclerosing fibroblasts throughout the body and reduced the total amount of collagen deposition in bleomycin-induced skin fibrosis in mice [19].

Although a few studies investigated the effect of HDACIs on visceral fibrosis, the effects of Tubastatin, a selective inhibition of HDAC6, on EF has never been reported. Therefore, we evaluated Tubastatin, which has been promising drug to treat systemic sclerosis. Here, we explored

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Doi: http://dx.doi.org/10.14715/cmb/2024.70.4.36

the anti-fibrosis potential of Tubastatin in human fibroblasts treated with TGF-β, as well as its anti-fibrosis effect after laminectomy *in vivo*.

2. Patients and methods

2.1. Human Samples

Human tissue was obtained from patients undergoing secondary decompression surgery and the procedure was approved by the ethics committee of Zhejiang Provincial People's Hospital. We also acquired informed consent from patients or their families prior to sample collection. A total of 18 patients (mean age, 45; age range, 35–56; 10 males, 8 females) donated their epidural scar tissues. Pending tissues were stored in liquid nitrogen until the continuing study. Fresh epidural scar tissues and surrounding normal tissue were washed by Dulbecco's modified Eagle's medium and Ham's F-12 medium (DMEM/F12, Keygen, Nanjing, China) and fragmentized. Dissociation was performed using 0.25% trypsin solution at 37°C for 15 min. Then, the mixture was waiting for total protein or RNA isolation.

2.2. Cells and drug treatment

Human fibroblasts were purchased from the Chinese Academy of Medical Science (Shanghai, China). All the cells were seeded in 5×5 cm^2 flask and TGF-β (10 ng/mL, Sigma-Aldrich, St. Louis, MO, USA) was stimulated fibroblasts to evoke differentiation for 24 h when cell confluence was 90%. Tubastatin (20 μM MedChemExpress, Monmouth Junction, NJ, USA) was used to treat cells.

2.3. Rats

Eight-week-old Sprague Dawley (SD) rats (male, 200 g - 220 g) obtained from Zhejiang Provincial People's Hospital, were bred and maintained at the Zhejiang Provincial People's Hospital Animal Center. This study was approved by the Animal Ethics Committee of Zhejiang Provincial People's Hospital Animal Center., and all experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals of the Zhejiang Provincial People's Hospital.

2.4. Laminectomy and drug dose

Briefly, rats have conducted skin preparation following anesthesia with 10% chloral hydrate (4 mL/kg). After disinfection, a back incision was made to separate the fascia layer and the muscle layer and laminectomy was performed at T10. After rinsing by normal saline, the incision was closed and disinfected post-hemostasis. Then 20 mg/kg Tubastatin was administered once daily for 7 days via intraperitoneal injection.

2.5. Cell viability assay

NP cell viability was determined by utilizing the cell counting kit 8 (CCK8) assay. cells were seeded in 96-well plates with a density of 1×10^4 cells/well and treated with different concentrations of Tubastatin (0-20 μM) for 24 h. Subsequently, cell viability was determined using a CCK8 Cell Viability/Cytotoxicity Assay Kit (KeyGen, Nanjing, China) following the manufacturer's instructions. Absorbance at 570 nm was then examined with a microtiter plate reader.

2.6. Quantitative real-time -polymerase chain reaction (qRT-PCR)

Total RNA of fibroblasts or scar tissue was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) abided by the manufacturer's protocol. cDNA synthesis was conducted using the PrimeScript™ RT Master Mix (Applied Biosystems, Foster City, CA, USA). Then human HDAC6, human collagen I, rat collagen I, rat α-SMA, rat fibronectin and GAPDH were detected using the SYBR PremixEx TaqII kit (RR820A, TaKaRa, Tokyo, Japan). The primers were listed as follows and the $2^{-\Delta\Delta Ct}$ method was used to calculate the relative mRNA levels. Human HDAC6: Forward, 5'-CAACTGAGACCGTGGAGAG-3', Reverse, 5'-CCTGTGCGAGACTGTAGC-3; Human collagen I: Forward, 5′-CATCAAGGTCTTCTGCGACA-3′, Reverse, 5′-CTTGGGGTTCTTGCTGATGT-3′; Rat collagen I: Forward, 5′- GCCAAGAAGACATCCCTGAAG-3′, Reverse, 5′-CACAAGGAACAGAACAGAACAG-3′; Rat α-SMA: Forward, 5′- GGCTCTGGGCTCTGTAAGG -3', Reverse, 5'-CTCTTG CTCTGGGCTTCATC-3'; Rat fibronectin: Forward, 5'-ACAACCCCTACAAACGGC-CA-3', Reverse, 5'-TAGTCAATGCCCGGCTCCAG-3; glyceraldheyde 3-phosphate dehydrogenase (GAPDH): Forward, 5′-GCAAGTTCAACGGCACAG, Reverse, 5′-GCCAGTAGACTCCACGACCAT.

2.7. Western blotting analysis

Scar tissue or fibroblasts were treated using a Total Protein Extraction Kit (KeyGEN, Nanjing, China) with phosphatase and protease inhibitors. Following violent oscillation and low-temperature centrifugation, protein concentration was measured with the bicinchoninic acid (BCA) Protein Assay Kit (Thermo Fisher, Waltham, MA, USA) and balanced. Separated in10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA, USA), the protein was blocked with a Quick Block Solution (Epi-Zyme, Shanghai, China) and incubated overnight at low temperature with the primary antibodies (anti-HDAC 6 (1:3000; Upstate Biotechnology, Lake Placid, NY, USA), anti-acetylated a-tubulin (1:2500; Upstate Biotechnology, Lake Placid, NY, USA), anti-a-tubulin (1:1500; Upstate Biotechnology, Lake Placid, NY, USA), anti-collagen I (Millipore, Billerica, MA, USA, 1:1000), anti-α-SMA (Abcam, Cambridge, MA, USA, 1:1000), anti-fibronectin (Abcam, Cambridge, MA, USA, 1:500), anti-Akt (Abcam, Cambridge, MA, USA, 1:1000), anti- p-Akt (Abcam, Cambridge, MA, USA, 1:1000), anti-PI3K (Abcam, Cambridge, MA, USA, 1:1000), anti-p-PI3K (Abcam, Cambridge, MA, USA, 1:1000), anti-HIF-1α (Abcam, Cambridge, MA, USA, 1:1000), anti-VEGF (Abcam, Cambridge, MA, USA, 1:1000) and anti-GAPDH (Cell Signaling Technology, Danvers, MA, USA, 1:2000)). Washed by tris buffered saline-tween (TBST) and incubated with the secondary antibody (Abcam, Cambridge, MA, USA, 1:2000) at room temperature, protein was visualized and using the enhanced chemiluminescence (ECL) system.

2.8. Statistical analysis

Data are normality displayed by the means ± standard deviations. Data compared between the two groups were analyzed using Student's t-test. Comparison between multiple groups was done using One-way ANOVA test fol-

lowed by Post Hoc Test (Least Significant Difference). Statistical analysis was performed using Statistical Product and Service Solutions (SPSS) 18.0 (SPSS Inc., Chicago, IL, USA). P-values <0.05 were considered statistically significant.

3. Results

3.1. Difference of HDAC6 expression in normal human tissue and human epidural scar

Firstly, we extracted normal tissue and epidural scar into RNA and protein, which conducted qRT-PCR and immunoblot respectively. It was found that HDAC6 RNA expression in normal tissue was lower than that in epidural scar (Figure 1A). Moreover, immunoblot consistently exhibited HDAC6 protein content was an increased level in epidural scar compared with that in normal tissue. Besides, we also detected acetylation of α-tubulin to reflect HDAC6 activity, showing that acetylated α-tubulin was reduced in epidural scar tissue and acetylated α-tubulin/α-tubulin ratio was consistently decreased compared with the normal tissue (Figure 1B-1D). Therefore, HDAC6 expression was increased both in RNA and protein levels, and acetylated α-tubulin and acetylated α-tubulin/α-tubulin ratio were decreased in human epidural fibrosis.

3.2. HDAC6 alteration in human fibroblasts treated with TGF-β

To verify whether HDAC6 is involved in TGF-β activating fibroblast differentiation, we next investigated changes in HDAC6 levels and deacetylation activity in activated TGF-β fibroblasts. Interestingly, we found that TGF-β increased HDAC6 RNA levels in fibroblasts (Figure 2A), and the increase in HDAC6 protein was consistent with variations in RNA level. In addition, α-tubulin acetylation showed a decreasing trend in fibroblasts following TGF induction (Figure 2B-2D), indicating that HDAC6 participated in TGF-β-mediated fibroblasts activation through regulating acetylation.

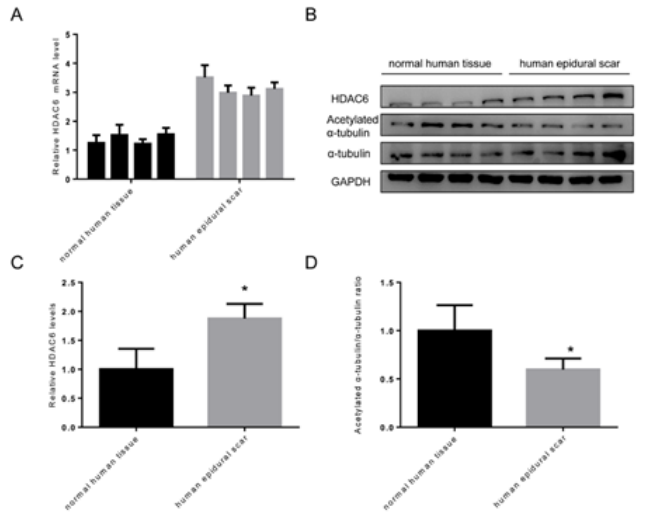

Fig. 1. Difference of HDAC6 expression in normal human tissue and human epidural scar. (A) Representative level of HDAC6 mRNA in the normal human tissue and human epidural scar. (B) Representative protein level of HDAC6, acetylated α-tubulin, and α-tubulin in the normal human tissue and human epidural scar. (C) Quantitative analysis of HDAC6 protein expression. (D) Quantitative analysis of acetylated α-tubulin/ α-tubulin ratio. "*" means *vs.* normal human tissue with statistical significance.

3.3. Tubastatin suppresses TGF-β-induced ECM deposition in human fibroblasts

We further investigated whether HDAC6 inhibition affected TGF-β-mediated ECM production of fibroblasts. Hence we pretreated fibroblasts with Tubastatin and then stimulated cell differentiation with TGF-β. Firstly, we observed whether Tubastatin treatment with different gradients affected cell viability, and found that the gradient of Tubastatin concentration from 5 to 20 μM did not affect the biological viability of fibroblasts (Figure 3A). Subsequently, fibroblasts were treated with 5-20 μM Tubastatin and activated to determine collagen I RNA level in the

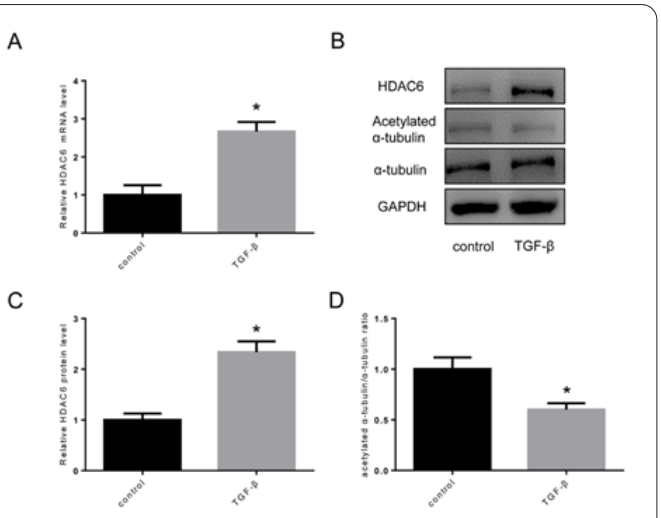

Fig. 2. HDAC6 alteration in human fibroblasts treated with TGF-β. (A) Representative RNA level of HDAC6 in the control and TGF-β group. (B) Representative western blotting of HDAC6, acetylated α-tubulin, and α-tubulin in the the control and TGF-β group. (C) Quantitative analysis of HDAC6 protein expression. (D) Quantitative analysis of acetylated α-tubulin/ α-tubulin ratio. "*" means *vs.* control group with statistical significance.

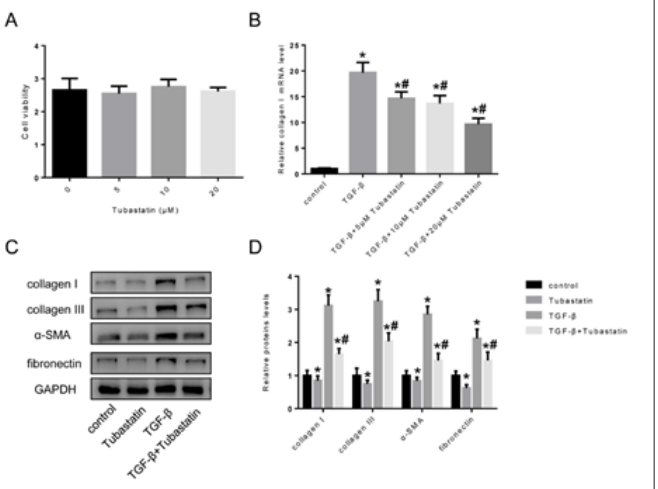

Fig. 3. Tubastatin suppresses TGF-β-induced ECM deposition in human fibroblasts. (A) Cell viability of fibroblasts in the 0, 5, 10 and 20 μM Tubastatin treatment. (B) Representative RNA level of collagen I in the control, TGF-β and TGF-β+5 μM Tubastatin, TGF-β+10 μM Tubastatin and TGF-β+20 μM Tubastatin group. (C) Representative western blotting of collagen I, collagen III, α-SMA and fibronectin in the control, Tubastatin, TGF-β and TGF-β+Tubastatin group. (D) Quantitative analysis of collagen I, collagen III, α-SMA and fibronectin expression. "*" means vs. control group with statistical significance. "#" means *vs.* TGF-β group with statistical significance.

cells. It was found that Tubastatin at different concentrations inhibited collagen I RNA expression, and 20 μM Tubastatin had the most significant inhibitory effect (Figure 3B). Then we selected 20 μM Tubastatin to pretreat fibroblasts and activate them with TGF-β, and then detected the expression of ECM. Western blotting showed that protein expression of α-SMA, collagen I, III and fibronectin decreased in fibroblasts treated with 20 μM Tubastatin, and TGF-β activation had the most significant inhibitory effect on ECM expression (Figure 3C, 3D). These data suggest that Tubastatin restrains TGF-β-induced ECM generation in fibroblasts.

3.4. Tubastatin inhibits fibroblast activation through down-regualting phosphorylation of Akt and VEGF expression

To identify the specific regulatory mechanism of the inhibition of Tubastatin to mediate fibroblast activation, we then examined the levels of PI3K and Akt phosphorylation in cells and found that Tubastatin inhibited the expression of phosphorylated PI3K and Akt, especially in TGF-β stimulated cells. However, Tubastatin did not affect the total levels of PI3K and Akt, suggesting that Tubastatin regulates fibroblast activation through the TGF-β-PI3K-Akt pathway (Figure 4A, 4B). Furthermore, we continued to measure hypoxia-inducible factor-1α (HIF-1α) level, a downstream effector of Akt pathway. HIF-1α has been shown to regulate ECM expression in the course of fibrosis. Our results showed that Tubastatin inhibited secondary HIF-1α protein expression. Consistently, Tubastatin suppressed TGF-β-induced vascular endothelial growth factor (VEGF), a known HIF-1α target, protein expression (Figure 4C, 4D). Therefore, their results indicated that Tubastatin represses ECM expression through down-regulating Akt pathway and inhibiting HIF-1α and VEGF levels.

3.5. Tubastatin improves epidural fibrosis degree in rat model

We investigated the effect of Tubastatin in murine model of laminectomy. We examined ECM content in rat epidural scar tissue, finding that collagen I, α-SMA and fibronectin protein levels were remarkably decreased following Tubastatin injection (Figure 5A). Moreover, the RNA level displayed a consistent reduction in collagen I, α-SMA and fibronectin treated with Tubastatin (Figure 5B). So these results lead us to conclude that Tubastatin improves epidural fibrosis in rat models.

4. Discussion

Proliferative fibrosis usually occurs in tissue repair, however, excessive fiber stimulation leads to a great crowd of collagen generation, resulting in accumulated scar tissue and affecting tissue healing. Fibroblasts, as a classic fibrogenic cell group, take a critical role in the secretion of ECM and the regulation of the degree of fibrosis scar formation. Epidural fibrosis is associated with differentiation and activation in fibroblasts, including myofibroblasts transition, cell proliferation, and ECM accumulation. Epidural fibrosis developing into severe situations leads to compression of the spinal nervous system, triggering severe neurological dysfunction and disorder. For the complicated mechanism of epidural fibrosis, ECM deposition and myofibroblast proliferation are widely accepted as the specific features during fibrosis procedure.

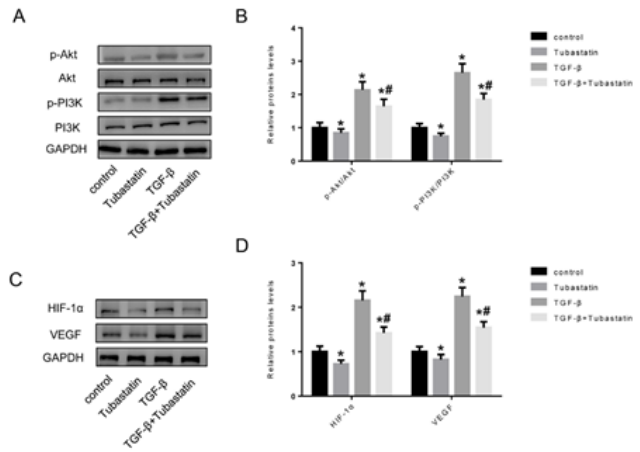

Fig. 4. Tubastatin inhibits fibroblast activation through down-regulating phosphorylation of Akt and VEGF expression. (A) The representative protein level of p-Akt, Akt, p-PI3K and PI3K in the control, Tubastatin, TGF-β and TGF-β+Tubastatin group. (B) Quantitative analysis of p-Akt/Akt and p-PI3K/ PI3K ratio. (C) The representative protein level of HIF-1α and VEGF in the control, Tubastatin, TGF-β and TGF-β+Tubastatin group. (D) Quantitative analysis of HIF-1α and VEGF expression. "*" means *vs.* control group with statistical significance. "#" means *vs.* TGF-β group with statistical significance.

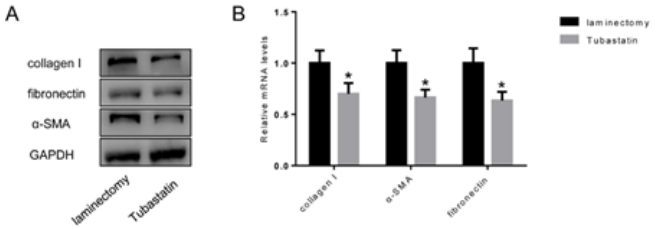

Fig. 4. Tubastatin improves epidural fibrosis degree in rat model. (A) Representative protein level of collagen I, fibronectin and α-SMA in the laminectomy and Tubastatin group. (B) Representative RNA level of collagen I, fibronectin and α-SMA in the laminectomy and Tubastatin group. "*" means *vs.* laminectomy group with statistical significance.

HDACs participate in multiple physiological and pathological procedures. HDACs changes are linked to varieties of diseases, such as cancer [20], cardiac hypertrophy [21] and diabetes [22]. HDACIs are molecules that bind with HDACs to interfere its effect. HDACIs regulate gene expression and metabolism through acetylation of histone. Trichostatin A has been reported to restore Fas-mediated apoptosis in both of IPF lung fibroblasts and bleomycin-induced fibrosis mice [23]. Previous studies have demonstrated that Tubacin inhibited myofibroblast differentiation in isoproterenol-induced cardiac fibrosis rats through elevating RASSF1A expression [24]. Tubastatin, as another HDAC6 inhibitor, is effective in the treatment of idiopathic pulmonary fibrosis through inhibiting Akt pathway. So we hypothesize that tubastatin may decrease pro-fibrotic signaling loops and inhibit the effect of VEGF. From the result of the different levels of HDAC6 and acetylated α-tubulin in both normal human tissue and human epidural scar, it is evidently that normal human tissue has lower HDAC6 and higher acetylated α-tubulin expression, but HDAC6 and acetylated α-tubulin in human epidural scar were reversed their expression. Regarding the human fibroblast cell culture, TGF-β was widely used to mimic the

pathophysiology of fibroblast activation *in vitro*. Tubastatin treatment to fibroblast is used to explore the influence of myofibroblast transdifferentiation *via* TGF-β induction. The result indicated that Tubastatin could interfere with the activation of myofibroblast with collagen I, fibronectin and α-SMA decrease. Inhibiting fibroblast differentiation can inhibit fibroplasia through down-regulating Akt pathway, Moreover, we utilized Tubastatin of different concentrations to test several targets corresponding to the cell viability. It was exhibited that the concentration-independent Tubastatin did not affect fibroblast viability and the HIF-1α and VEGF decreased with SAHA treatment compared with the control one and TGF-β treatment. As we anticipated, the anti-fibrosis effect of Tubastatin on epidural fibrosis depended on repression of PI3K/Akt pathway and inhibition of HIF-1α. Recent evidences have suggested that Tubastatin increased lung fibroblast apoptosis in idiopathic pulmonary fibrosis. In our study, we confirmed again that Tubastatin exerted TGF-β pathway restraining function and could reduce TGF-β-induced fibrosis process.

Moreover, to verify Tubastatin therapeutic effect of epidural fibrosis *in vivo*, we used Tubastatin to treat rats following laminectomy. At 7 days post laminectomy, we wondered whether Tubastatin has the capacity to decrease myofibroblast activation and ECM deposition. Hence we analyzed ECM expression in epidural scar including α-SMA, collagen I and fibronectin. The result demonstrated that Tubastatin could influence ECM protein expression and RNA level. Previous studies have shown that α-SMA, collagen I and fibronectin can participate in fibrosis induced by TGF-β regulating. Our findings also lead to strong support for the view that ECM is reduced by the decreased expression of HDAC6 following TGF-β stimuli. Hopefully, Tubastatin is a promising inhibitor that suppresses the influence of ECM to alleviate fibroplasia in epidural fibrosis.

To summary, it would be nice to find a drug that works by inhibiting ECM accumulation *via* inhibition of HDAC6 and Akt pathway to attenuate epidural fibrosis. We systematically evaluated the role of Tubastatin treatment on the fibrosis model both *in vivo* and *in vitro*. Together, these results revealed a knowledge facilitating investigations that Tubastatin may become a useful measure to ameliorate epidural fibrosis.

5. Conclusions

These results indicated that Tubastatin inhibited HDAC6 expression and decreased TGF-β/ PI3K/ Akt pathway that promotes collagen and ECM deposition and VEGF release, leading reduction of myofibroblast activation. Hence, Tubastatin ameliorated epidural fibrosis development.

Conflict of interest

The authors declared no conflict of interest.

Consent for publications

The author read and approved the final manuscript for publication.

Ethics approval and consent to participate

This study was approved by the ethics committee of Zhejiang Provincial People's Hospital.

This study was approved by the Animal Ethics Committee of Zhejiang Provincial People's Hospital Animal Center.

Informed consent

Signed written informed consent were obtained from the patients and/or guardians.

Availability of data and material

The data that support the findings of this study are available from the corresponding author upon reasonable request

Authors' contributions

Lijiang Tao and Yunlin Ge designed the study and performed the experiments, Lijiang Tao and Liangbang Wu collected the data, Yunlin Ge and Liangbang Wu analyzed the data, and Lijiang Tao and Yunlin Ge prepared the manuscript. All authors read and approved the final manuscript.

Funding

None

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