Hederagenin promotes *SIRT6* to attenuate epidural scar formation by aggravating PRMT1 deacetylation

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Aims

The formation of a postoperative epidural scar induced by epidural fibrosis is the main reason for recurrence of lumbar disc herniation after laminectomy. Hederagenin (HE) has been found to be widely present in various medicinal plants and has various pharmacological functions. This study aimed to investigate the effect and regulatory mechanism of HE on epidural scar formation.

Methods

Transforming growth factor beta 1 (TGF-β1)-stimulated epidural scar fibroblasts were used as an in vitro cell model. Based on that, HE treatment was carried out along with sirtuin-6 (*SIRT6)* silence or protein arginine N-methyltransferase 1 (*PRMT1*) overexpression. The interaction between SIRT6 and PRMT1 was evaluated by pulldown and co-immunoprecipitation (CoIP) assays. Then, cell proliferation, apoptosis, and fibrosis were measured by Cell Counting Kit (CCK)-8, flow cytometry, and western blotting. Moreover, the effects of receptor activator of nuclear factor-κB ligand (RANKL) supplementation and endoplasmic reticulum (ER) stress were also evaluated by supplementing recombinant protein and specific inhibitor or activator.

Results

HE depressed cell proliferation and fibrosis, while inducing apoptosis of epidural fibroblasts. Meanwhile, HE promoted *SIRT6* expression which suppressed PRMT1 acetylation and protein stability. Additionally, HE induced ER stress and upregulated RANKL in epidural fibroblasts via mediating *SIRT6*/*PRMT1* axis.

Conclusion

Generally, the therapeutic role of HE treatment on epidural scar formation was exerted by regulating *SIRT6*/*PRMT1* axis-mediated ER stress and RANKL pathway. This study provides evidence of a novel therapeutic measure for epidural scar formation.

Article focus

- The effect and regulatory mechanism of hederagenin (HE) on epidural scar formation.

Key messages

- HE depressed transforming growth factor beta 1 (TGF-β1)-induced excessive fibroblast proliferation; HE regulated protein arginine N-methyltransferase 1 (PRMT1) expression by mediating sirtuin-6 (SIRT6) deacetylation, which attenuated epidural fibrosis; receptor activator of nuclear factor-κB ligand (RANKL) was involved in the effect of HE on epidural fibrosis.

Strengths and limitations

- This study provides evidence of a novel therapeutic measure for epidural scar formation.

Bone & Joint Research

- The clinical value of HE in therapying epidural scar formation should be clearly illustrated by rigorous in vivo explorations.

Introduction

Laminectomy is a common treatment for spinal stenosis patients.[1] Unfortunately, there is often a recurrence of the disease postoperatively due to the epidural scar formation. Excessive proliferation of fibroblasts is a crucial factor that contributes to epidural fibrosis and leads to epidural scar formation, secondary to laminectomy.[2,3] The financial and pain burden for treating this secondary disease is high due to its difficult reoperation and intractable complications.[4-6] Therefore, there is an urgent need to discover an effective therapeutic strategy or drug for epidural fibrosis.

Hederagenin (HE), an active compound derived from *Hedera Helix* (common ivy), is reported to exert anti-depressive, anti-cancer, and anti-inflammation effects when used in medicine.[7,8] HE is isolated from different species of traditional medicine plant sources.[8] Emerging studies also indicate the participation of HE in regulating cellular processes. Rodríguez-Hernández et al[9] illustrated HE as an effective potent cytotoxic agent by exerting an antiproliferative function. Elsewhere, HE treatment also inhibited excessive fibroblast proliferation in bleomycin-induced pulmonary fibrosis.[10] However, the exact role and potential molecular mechanism of HE in epidural fibrosis pathogenesis have yet to be elucidated.

As a lysine deacetylase from the nicotinamide adenine dinucleotide (NAD^+)-dependent class III conserved family, sirtuin-6 (*SIRT6*) possesses the ability to deacetylate proteins by post-translational modification.[11] Liu et al[12] highlighted the nucleus-located regulation of *SIRT6* in various cell processes, including cell apoptosis, DNA repair, transcription, ageing, and inflammation. In addition to its regulatory role in chronic kidney disease, cell-senescent atherosclerosis, and cancer, *SIRT6* was shown to modulate the pathological fibrosis in the liver, cardiovascular system, and kidneys.[13,14] A recent study also indicated that *SIRT6* depletion promoted bone resorption and cartilage destruction in osteoblasts.[15] However, there is a lack of evidence for the way in which *SIRT6* effects epidural fibrosis after laminectomy.

According to the previous findings by Fan et al,[16] *SIRT6* was abnormally altered in epidural fibrosis tissues and participated in its development. Therefore, we inferred that HE could attenuate the excessive fibrosis of epidural fibroblasts and epidural fibrosis, and that *SIRT6* participates in this process. In this study, we established a commonly used in vitro cell model of epidural fibrosis,[17] by treating fibroblasts from human epidural scar tissue with transforming growth factor beta 1 (TGF-β1). Based on this, the effect of HE was evaluated, as well as the role of *SIRT6* during epidural fibrosis progression.

Methods

Primary fibroblast isolation and culture

We collected human epidural scar tissues from seven lumbar disc herniation patients who had undergone a laminectomy and developed epidural scars. Any patients with a history of any kind of operative site-located drug treatment, substance abuse, extreme keloid formation, tumour, hypertrophic scarring, or laminectomy along with fusion were excluded. Written informed consent was supplied by the seven patients who were included in the study. The Ethics Committee approved our study, in line with the Declaration of Helsinki.[18] The scar tissues of patients were sampled during the laminectomy and immediately stored at -80°C.

To isolate fibroblasts, the scar tissues from different patients were mixed, and 75% alcohol was used to soak the tissue mixture (5 mg); trypsin-ethylenediaminetetraacetic acid (EDTA) was used for the digestion of fragmented tissue pieces (1 mm^3) at 37°C (15 mins) in sterile superclean benches. Then, the mixture was centrifugated at 1,300 rpm (5 mins), with fetal bovine serum (FBS) supplemented in order to terminate the digestion from tissue to cell in a sterile tube. After three-times centrifugation and resuspension, the isolated fibroblast cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) which contained 100 μg/ml penicillin and streptomycin, as well as 10% FBS. A humidified incubator (95% O_2 and 5% CO_2) at 37°C was used to incubate fibroblasts. After 4-6 passage cultivation, the cells were collected for subsequent experiments.

Treatments and transfection

HE reagent (> 98%) was obtained from Medchemexpress (China) and used to treat the isolated fibroblast at different concentrations (0, 1, 5, 10, 20, 40, 80, and 100 μM) and different times (0, 6, 12, 24, 48, and 72 hrs) for the evaluation of HE treatment dose and time. Similarly, for TGF-β1-cell model construction, the fibroblasts were treated with 0, 2, 5, 10, and 20 ng/ml for 0, 6, 12, 24, and 48 hours, respectively.

At first, cells were transfected with short hairpin RNA (shRNA) or overexpression vectors based on pc-DNA 3.1 vector to silence or overexpress target genes. The synthesis of sh-SIRT6s and pc-protein arginine methlytransferase 1 (PRMT1) was provided by GenePharma (China). For transfection, cells seeded in 12-well plates were incubated with shRNAs (50 nM) or pc-DNA vectors (0.5 μg) for 48 hours by using a Lipofectamine 3000 kit (Thermo Fisher Scientific, USA) in line with the manufacturer's protocol. Then, 4-PBA, tunicamycin (TM, 2 μg/ml), denosumab (DE, 100 ng/ml), or recombinant receptor activator of nuclear factor-κB ligand (r-RANKL, 100 ng/ml) was used to treat the fibroblast cells for 24 hours. After that, TGF-β1 induction was performed followed by HE treatment.

Cell viability and cell death

To evaluate the cell viability, a CCK-8 Detection kit (Dojindo Molecular Technology, Japan) was used. Briefly, the cultured cells (3×10^3 cells/well) were planted into the holes of 96-well plates. After 48 hours' incubation, the system was supplemented with CCK-8 solution (10 μl). The final optical density at 450 nm was measured following another two-hour incubation.

An annexin V/PI staining cell death detection kit (BD Biosciences, USA) was used for cell apoptosis evaluation. After treatment, cells were washed with ice-cold phosphate-buffered saline (PBS) three times and resuspended to 1×10^6 cells/ml concentration in binding buffer. Then, annexin V labelled with fluorescein isothiocyanate (V-FITC) and propidium iodide (PI) were added to incubate for staining (15 mins, 25°C) before flow cytometry analysis.

Real-time quantitative polymerase chain reaction

The expressions of target genes were initially investigated by treating specimens using TRIzol reagent (Invitrogen, China) for extracting total RNA. After spectrophotometrically detecting the concentration, RNA samples (1 μg) were incubated with Primescript Reverse Transcriptase (Tiangen, China) to synthesize complementary DNA (cDNA). Subsequently, SYBR Master Mix and a real-time polymerase chain reaction (PCR) system (Applied Biosystems, USA) were used to perform the amplification. The final quantification of data was processed based on the $2^{-\Delta\Delta Ct}$ method with glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) as the endogenous reference gene.

Western blotting

As part of the routine protocol, fibroblast proteins were initially extracted by treating the cells with ice-cold radioimmunoprecipitation assay (RIPA) buffer (Beyotime, China). A bicinchoninic acid (BCA) protein assay kit (Invitrogen) was used to determine protein concentration. After the separation of protein (20 μg) through sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and the transference onto polyvinylidene fluoride (PVDF) membrane, the protein was blocked with Tris-buffered saline (TBS) (supplemented with 5% bovine serum albumin (BSA)) for one hour at room temperature. The incubation of primary antibodies (anti-ki67 ab15580, anti-caspase-3 ab2302, anti-fibronectin ab2413, anti-collagen I ab233080, anti-CTGF ab6992, anti-SIRT6 ab88494, anti-PRMT1 ab73246, anti-GRP78 ab21685, anti-CHOP ab11419, anti-p-PERK ab192591, anti-β-actin ab8227 (Abcam); anti-α-SMA BN42131R (BioDee, China); and p-eIF1α BS4787 (Bioworld, USA)) were conducted overnight at 4°C. Finally, peroxidase-conjugated secondary antibody was used for a one-hour incubation at room temperature, and enhanced chemiluminescence was performed to visualize target protein blots on an Image Reader (Fujifilm, Japan).

Pulldown assay

First, the cells were incubated with IP lysis buffer (Beyotime) supplemented with protease inhibitor (Solarbio, China), and protein A plus G agarose (Solarbio) was used to purify the lysates. Then, anti-immunoglobulin G (anti-IgG), anti-SIRT6, and anti-PRMT1 cross-linked beads were used to pull down target protein complex (overnight antibody incubation at 4°C; beads immunoprecipitation for four hours at 4°C). After washing with IP lysis buffer (Beyotime) three times, the beads were incubated using 4× Laemmli sample buffer (Bio-Rad, USA) and protein pulldown complex was immunoblotted using anti-SIRT6 and anti-PRMT1 after being boiled and separated by SDS-PAGE. Input and IgG pulldown protein complexes were set as controls in this study.

Co-immunoprecipitation

A universal magnetic co-immunoprecipitation (Co-IP) kit purchased from Active Motif Technology (USA) was used to investigate the interaction of SIRT6 and PRMT1, as well as acetyl-lysine. First, the antibodies of SIRT6, PRMT1, and acetyl-lysine were supplemented into total protein samples (1 mg) for a four-hour incubation at 4°C. Then, the mixture was incubated with the magnetic beads (1 hr, 4°C) followed by four times' wash and resuspension on a magnetic stand using loading buffer. The final pellets were immunoblotted by anti-SIRT6 (ab88494), anti-PRMT1 (ab73246), anti-histone (ab1791), and anti-acetyl-lysine (ab22550).

Statistical analysis

This study used Tukey's test and independent-samples *t*-test to analyze statistically significant differences between data (presented as mean and standard error of the mean (SEM)) from different groups. Post-hoc tests were applied for pairwise comparisons. A p-value less than 0.05 was considered statistically significant. The experiments were conducted individually at least three times. GraphPad Prism 10.1.2 software (GraphPad Software, USA) provided the analysis platform.

Results

HE depressed TGF-β1-induced excessive fibroblast proliferation

As presented in Figures 1a and 1b, HE at concentrations higher than 5 μM ($p = 0.005$) and durations longer than 12 hours ($p = 0.006$) significantly reduced cell viability in both dose- and time- dependent manners, with 40 μM ($p = 0.005$) as the half-inhibitory concentration and 48 hours ($p = 0.008$) the time of fibroblast cell viability under which cell status was considered normal, and the impact on subsequent indicator testing was relatively small. Subsequently, the results of modelling showed that TGF-β1 dramatically enhanced the cell viability of fibroblast (concentration, $p = 0.006$; time, $p = 0.007$) (Figures 1c and 1d). HE treatment inhibited cell proliferation (non-TGF-β1, $p = 0.009$; TGF-β1, $p = 0.020$) and induced cell apoptosis (non-TGF-β1, $p = 0.021$; TGF-β1, $p = 0.009$), along with decreased expression of ki67 and increased cleaved-caspase-3 expression, regardless of whether fibroblasts were stimulated by TGF-β1 (Figures 1e to 1g). In addition, HE treatment dramatically depressed the fibrosis process induced by TGF-β1 in epidural fibroblasts, presenting as the downregulated protein expressions of alpha-smooth muscle actin (α-SMA), fibronectin, collagen I, and connective tissue growth factor (CTGF) (Figure 1g). Taken together, HE inhibited TGF-β1-induced excessive proliferation and fibrotic process of scarring fibroblasts, and induced fibroblast cell apoptosis.

HE upregulated *SIRT6* to depress cell survival of scarring fibroblasts

To illustrate the mechanism of HE in epidural scarring, the role of *SIRT6* exerted during the disease progression was investigated. *SIRT6* was found to be significantly downregulated in TGF-β1-induced fibroblasts ($p = 0.009$); conversely, HE treatment increased (non-TGF-β1, $p = 0.006$; TGF-β1, $p = 0.009$) *SIRT6* expression in a dose-dependent manner in fibroblasts with or without TGF-β1 stimulation (Figures 2a and 2b). The transfection of SIRT6 shRNAs depressed ($p = 0.028$) the elevated *SIRT6* expression induced by HE in TGF-β1-stimulated fibroblasts, with sh-SIRT6b presenting the most effective depression ($p = 0.007$) (Figures 2c and 2d). Then, HE suppressed cell proliferation ($p = 0.009$) of TGF-β1-stimulated fibroblasts and induced cell apoptosis ($p = 0.007$); however, the silence of *SIRT6* greatly reversed these effects of HE (proliferation, $p = 0.006$; apoptosis, $p = 0.008$) (Figures 2e and 2f). In addition, the fibrosis progression in TGF-β1-stimulated fibroblasts was inhibited by HE, presented as the

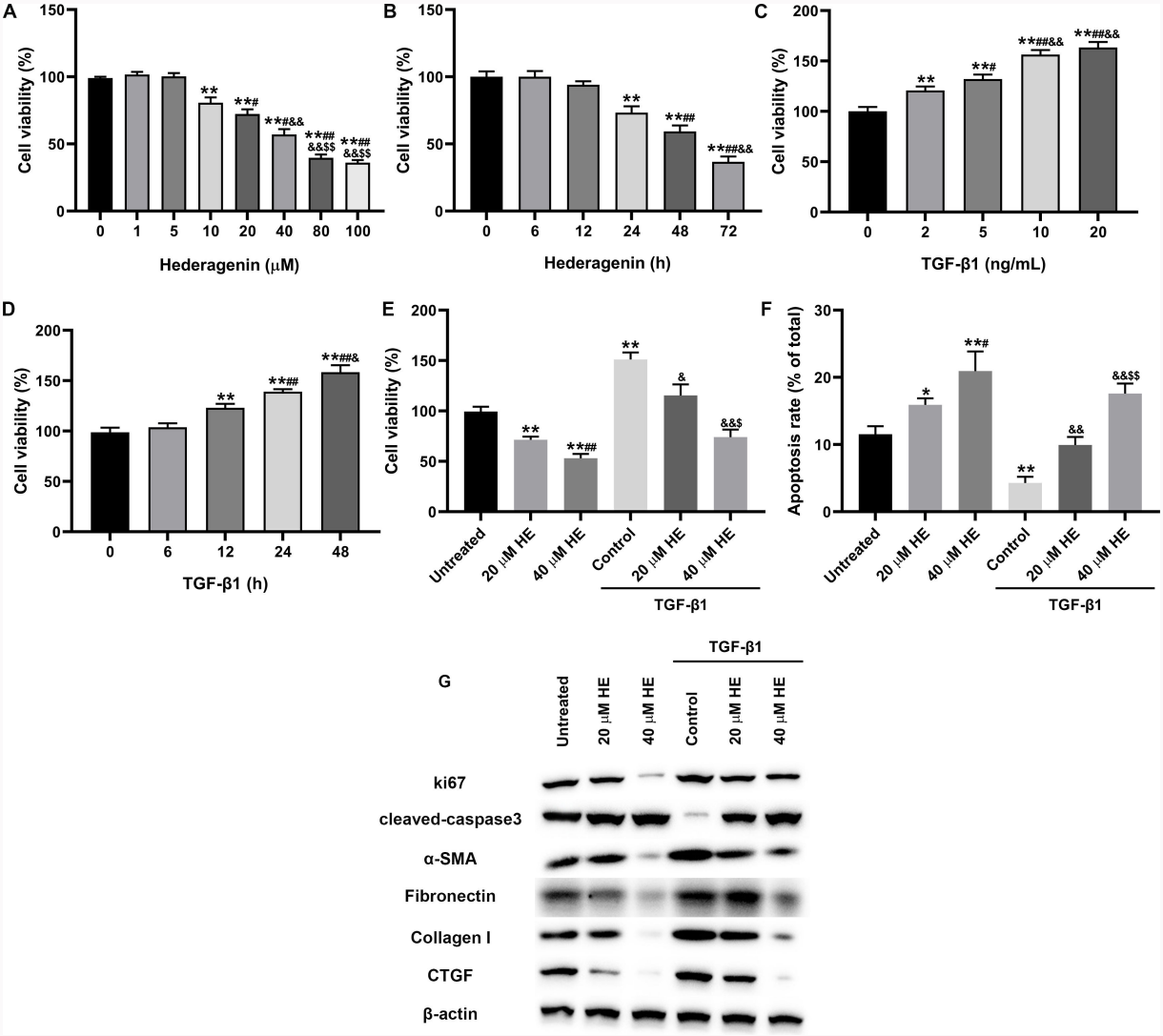

Fig. 1

The effect of hederagenin (HE) on transforming growth factor beta 1 (TGF-β1)-stimulated fibrosis in epidural fibroblasts. a) to e) Cell Counting Kit (CCK)-8 method was performed to evaluate cell viability. f) Apoptosis rate of fibroblasts was measured using flow cytometry. g) The expressions of target proteins were evaluated by western blotting. *p < 0.05; **p < 0.01; #p < 0.05; ##p < 0.01; &p < 0.05; &&p < 0.01; $p < 0.05; $$p < 0.01. Data are presented as mean (standard error of the mean) derived from at least three experiments and analyzed by post-hoc test. α-SMA, alpha smooth muscle actin; CTGF, connective growth tissue factor.

depressed protein expressions of α-SMA, fibronectin, collagen I, and CTGF; but sh-SIRT6b promoted the fibrotic process again (Figure 2g).

HE regulated *PRMT1* expression via mediating SIRT6 deacetylation

Emerging studies have demonstrated the crucial role of *PRMT1* in various fibrosis diseases.[19–21] We intended to further explore the potential mechanism of HE attenuating epidural scarring by focusing on *PRMT1*. As our results show, *PRMT1* was dramatically upregulated (p = 0.009) in TGF-β1-treated cells, a process which was depressed by HE (p = 0.005); the silence of *SIRT6* elevated (p = 0.007) *PRMT1* expression again (Figures 3a and 3b). Both in the pulldown protein complex of PRMT1 and SIRT6 antibodies, SIRT6 and PRMT1 specific proteins were detected, while the IgG group presented no target protein expression, which suggested an interaction between SIRT6 and PRMT1 (Figure 3c). Moreover, we investigated whether the deacetylation of SIRT6 was involved in the regulation of SIRT6 expression mediated by HE and PRMT1. As shown in Figure 3d, TGF-β1 substantially increased the enrichment of PRMT1 in acetyl-lysine immunoprecipitated protein complex and acetyl-histone expression, which were depressed by HE treatment, and the silence of *SIRT6* reversed the effect of HE. These findings indicate that HE impacted the acetylation of PRMT1 by mediating *SIRT6*. Subsequently, the deacetylation region, H133Y, on SIRT6 was mutated to verify whether the interaction between SIRT6 and PRMT1 was acting through this region. Results showed that SIRT6 mutated group showed no PRMT1 blot in SIRT6-immunoprecipitated complex and no SIRT6 blots in PRMT1- and acetyl-PRMT1-immunoprecipitated complexes (Figure 3e). Moreover, the mutation of SIRT6 increased (p = 0.009) PRMT1 expression to a certain extent and notably inhibited (p = 0.251) the depression (p = 0.008) of HE on PRMT1 (Figure 3f). Overall, we confirmed that HE not only promoted *SIRT6* expression, but also elevated its deacetylation effect to depress PRMT1.

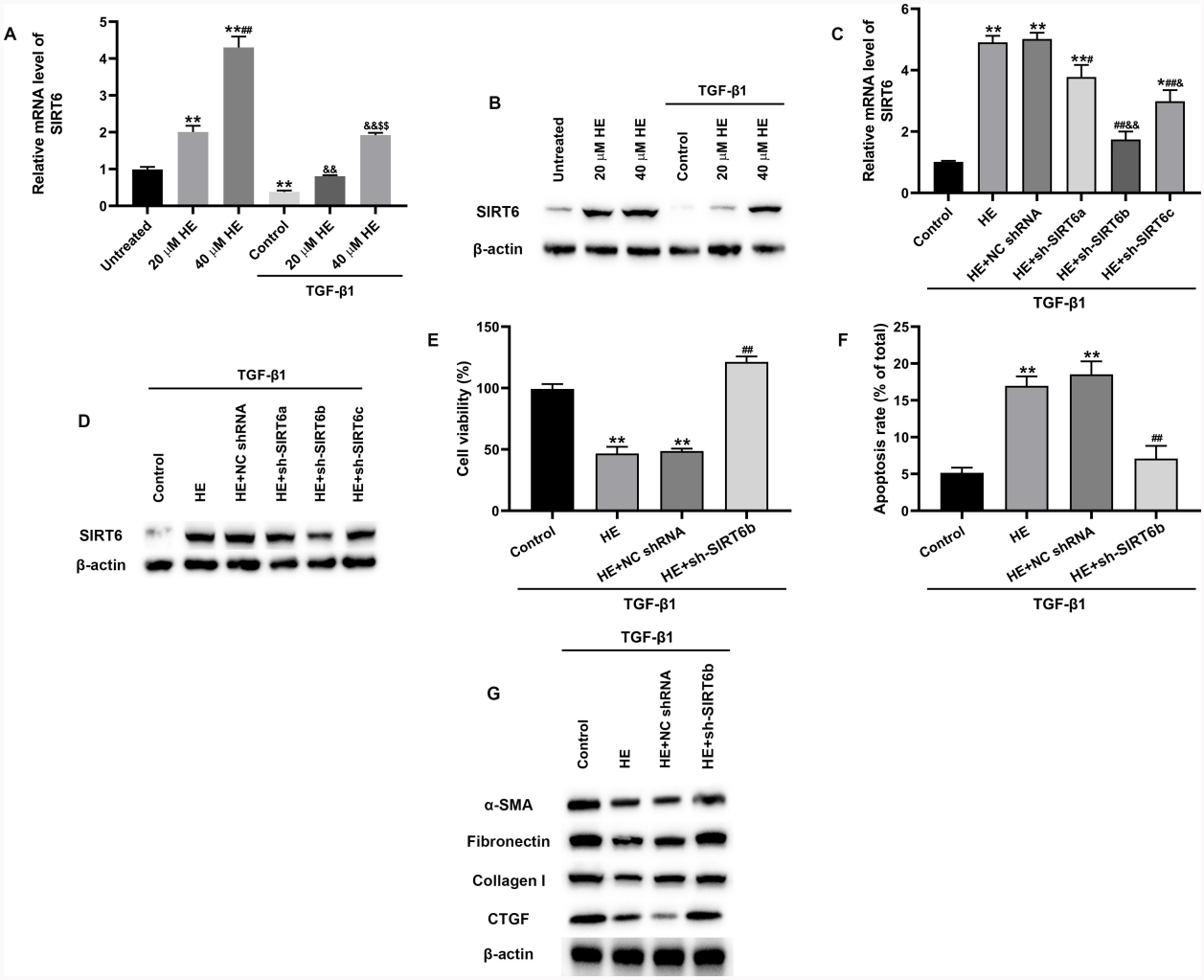

Fig. 2

The role of *SIRT6* during epidural fibrosis progression in vitro. a) and c) The expression of *SIRT6* messenger RNA (mRNA) was measured using real-time quantitative polymerase chain reaction (RT-qPCR). b), d), and g) Western blotting showing the protein levels of SIRT6 and fibrotic proteins. e) Cell Counting Kit-8 assay was used to evaluate cell viability. f) Cell apoptosis was determined using a Cell Death Annexin V/PI kit (BD Biosciences, USA). The results obtained from triple experiments are analyzed by post-hoc test and shown as mean (standard error of the mean). $^{**}p < 0.01$; $^{\#}p < 0.05$; $^{\#\#}p < 0.01$; $^{\&}p < 0.05$; $^{\&\&}p < 0.01$; $^{\$\$}p < 0.01$. α-SMA, alpha smooth muscle actin; CTGF, connective tissue growth factor; HE, hederagenin; NC shRNA, negative control short hairpin RNA; TGF-β1, transforming growth factor beta 1.

HE promoted ER stress by *SIRT6* upregulation

Currently, ER stress is regarded as an effective signal that regulates cell death during the epidural fibrosis process.[22] We next investigated the alteration of ER stress in epidural fibroblasts under HE treatment. First, HE promoted the expressions of ER stress markers, GRP78, C/EBP homologous protein (CHOP), phosphorylated protein kinase R-like endoplasmic reticulum kinase (p-PERK), and p-eIF1α, which were depressed by TGF-β1. Silencing *SIRT6*, overexpressing *PRMT1*, and treatment with 4-phenylbutyrate (4-PBA), an inhibitor of ER stress, significantly reversed HE effects; the silence of *PRMT1* and the activator of ER stress, TM, counteracted the effect of *SIRT6* silence; additionally, TM reversed the inhibitory effect of *PRMT1* overexpression on ER stress (Figure 4a). Moreover, depressing ER stress ($p = 0.006$) and overexpressing *PRMT1* ($p = 0.023$) both alleviated HE-induced cell death ($p = 0.009$) and slowed down the decline of cell viability ($p = 0.007$) and progression of fibrosis. TM notably reversed ($p = 0.005$) the effects of *SIRT6* knockdown ($p = 0.005$) and *PRMT1* overexpression ($p = 0.005$) on cell survival and fibrosis of TGF-β1-stimulated fibroblast cells under HE treatment (Figures 4b to 4d). It follows that HE mediated epidural fibrosis progression by inducing ER stress in fibroblasts by impacting the *SIRT6*/*PRMT1* axis.

RANKL was involved in the effect of HE on epidural fibrosis

Evidence has shown that RANKL participated in the fibrosis process and ER stress.[23,24] We next investigated whether RANKL contributed to the effect of HE on epidural fibrosis. First, the expression of RANKL was found to be upregulated in TGF-β1-stimulated fibroblasts and was depressed again by HE treatment, while *SIRT6* knockdown, *PRMT1* overexpression, and 4-PBA treatment reversed the effect of HE on RANKL expression (Figure 5a). In addition, exogenous supplementation of RANKL depressed the effect of HE on cell viability ($p = 0.016$), apoptosis ($p = 0.006$), and fibrosis process in TGF-β1-stimulated fibroblasts; DE, an inhibitor of RANKL, significantly counteracted the promotive effects of sh-SIRT6b ($p = 0.009$), pc-PRMT1 ($p = 0.07$), and 4-PBA ($p = 0.010$) on cell viability and fibrosis, and their inhibitions on cell apoptosis (sh-SIRT6b,

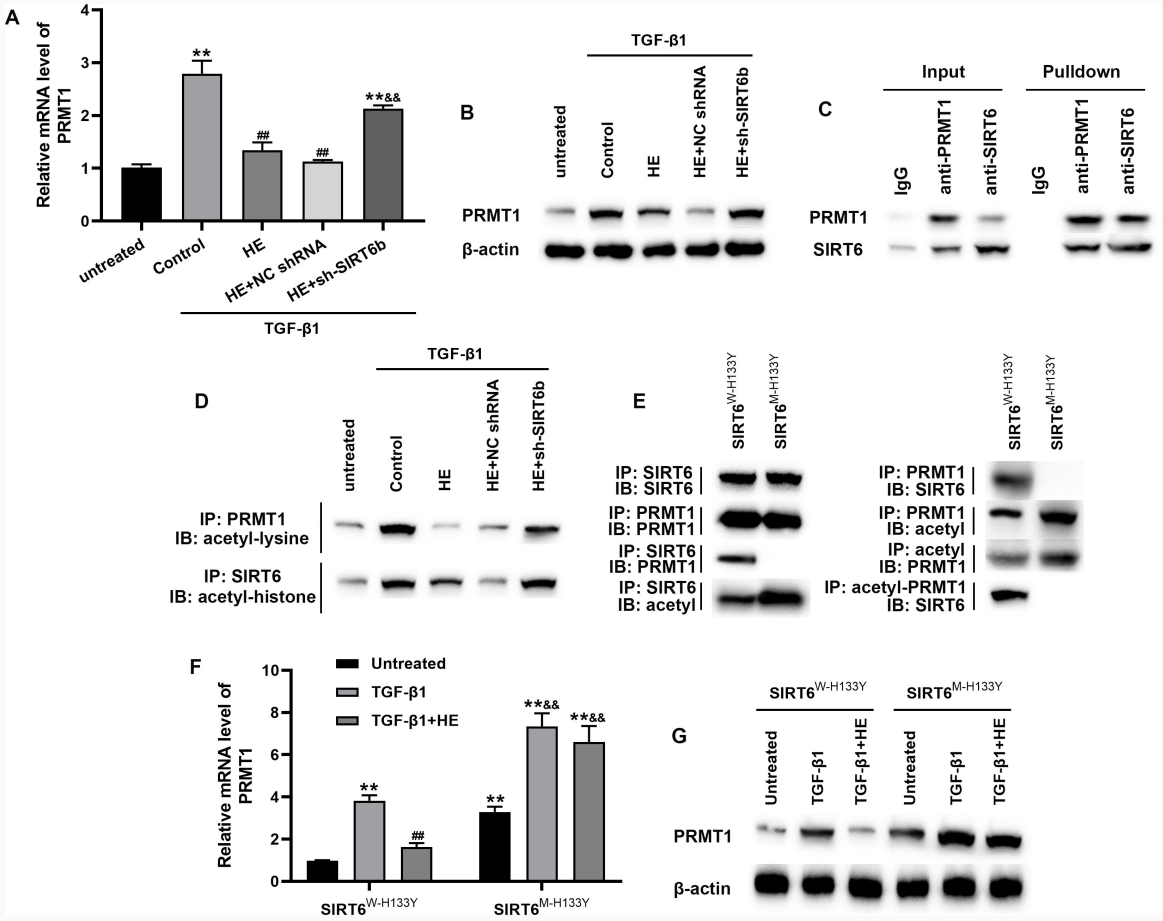

Fig. 3

The interaction and association between SIRT6 and PRMT1. a), b), f), and g) The expressions of *PRMT1* at messenger RNA (mRNA) and protein levels were measured using real-time quantitative polymerase chain reaction and western blotting. c) Pulldown assay was used to evaluate the interaction between SIRT6 and PRMT1. d) and e) Co-immunoprecipitation (Co-IP) determined the interactions between wild-type and mutant type of SIRT6 with PRMT1, acetyl-PRMT1, acetyl-lysine, and acetyl-histone. Experiments were conducted at least three times. The final data were analyzed by post-hoc test and shown as mean (standard error of the mean). $^{**}p < 0.01$; $^{\#\#}p < 0.01$; $^{\&\&}p < 0.01$. NC shRNA, negative control short hairpin RNA; TGF-β1, transforming growth factor beta 1.

p = 0.006; pc-PRMT1, p = 0.009; 4-PBA, p = 0.007) in HE-treated fibroblasts under TGF-β1 stimulation (Figures 5b to 5d). These findings indicated that the HE-induced *SIRT6/PRMT1* axis increased RANKL expression in TGF-β1-stimulated fibroblasts, and that RANKL inhibition hindered the improved effects of HE on fibroblasts, suggesting that RANKL is a downstream molecular target during HE alleviation of fibrosis.

Discussion

The excessive fibrosis of epidural space often presents in patients who have undergone laminectomy, with a worse postoperative outcome.[25] The main cause of epidural fibrosis formation is attributed to the aggravated fibroblast proliferation that results from the accumulated secretion of profibrotic cytokines, for example TGF-β1, induced by inflammatory cell intrusion at the surgical site.[26] Hence, resolving the abnormal proliferation of fibroblasts would be an effective way to prevent epidural fibrosis. Our study provides basic evidence for a new therapeutic substance, HE, which was previously considered to be a pure, natural, plant-extracted and readily available monomer compound with higher pharmacological activities both in vitro and in vivo than other drugs.[27] Moreover, our findings demonstrate that HE attenuated the injury and excessive fibrosis induced by TGF-β1 in epidural fibroblasts. This may offer a novel method of combating post-laminectomy fibrosis.

Plenty of chemical substances isolated from drugs have been discovered to possess antifibrotic effects on epidural fibrosis. Metformin delayed the process of post-epidural fibrosis with decreased collagen and fibronectin in a mouse model of laminectomy.[28] Yang et al[29] indicated that a histone deacetylase inhibitor – suberoylanilide hydroxamic acid – depressed the activation of fibroblasts induced by TGF-β administration in an epidural fibrosis model. Additionally, quercetin prevented epidural fibrosis by inducing autophagy to mediate the cellular functions of fibroblasts.[30] In our study, HE was found to depress cell survival and fibrosis stimulated by TGF-β1, as well as induce apoptosis in fibroblasts to block the formation of epidural scars. HE is a bioactive natural compound that has a therapeutic effect on a variety of diseases. Evidence points to remarkable benefits of HE in the treatment of cardiovascular diseases, cancer progression, bone loss, inflammatory injury, and neurodegenerative disorders.[7,8,31,32] The anti-fibrosis effect of HE has also been uncovered; consistent with our findings, Jia et al[33] demonstrated that HE notably improved renal tubular cell fibrosis in a

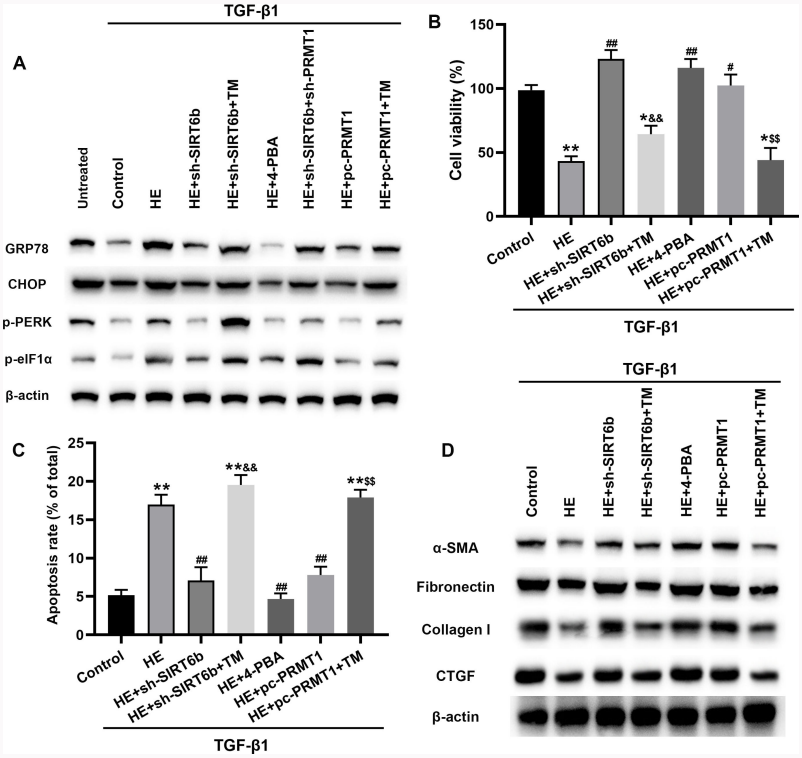

Fig. 4

Endoplasmic reticulum (ER) stress was involved in hederagenin (HE)-induced epidural fibrosis therapy. a) and d) The activation of ER stress and expressions of fibrotic proteins were measured using western blotting assay. b) Cell Counting Kit-8 assay was used to evaluate the cell viability. c) Cell apoptosis was determined by flow cytometry. The statistical results were based on at least three experimental repeats analyzed by post-hoc test and are presented as mean (standard error of the mean). *p < 0.05; **p < 0.01; #p < 0.05; ##p < 0.01; &&p < 0.01; $$p < 0.01. 4-PBA, 4-phenylbutyrate; α-SMA, alpha smooth muscle actin; CHOP, C/EBP homologous protein; CTGF, connective growth tissue factor; PRMT1, protein arginine N-methyltransferase 1; SIRT6, sirtuin-6; TGF-β1, transforming growth factor beta 1; TM, tunicamycin.

chronic kidney disease rat model. Another study also showed the beneficial effect of HE on bleomycin-induced pulmonary fibrosis by reducing collagen deposition and fibrotic dysfunction in rats.[10] In a study of type 2 diabetes, HE treatment presented cardiac-protective effects by alleviating myocardial hypertrophy and fibrosis-induced cardiac remodelling.[34] This evidence emphasizes the anti-fibrotic efficiency of HE.

From a mechanistic perspective, HE ameliorated tissue fibrosis by regulating a series of target genes. By inducing the depression of NF-kappa B transcription, the downstream profibrosis proteins were downregulated by HE, thereby protecting a diabetic heart from myocardial fibrosis.[34] Evidence has also demonstrated that interferon-stimulated gene 15 (*ISG15*), a promoter gene of fibrosis, was knocked down in HE-intervened renal tubular cells, which contributed to the improvement in chronic kidney disease.[33] Our study first illustrated the crucial role of *SIRT6* in attenuating epidural fibrosis and scar formation. Previous studies established the regulation of *SIRT6* in fibrosis; overexpressing *SIRT6* was shown to inhibit renal interstitial fibrosis, during which the abnormal renal tubular epithelial cell function was improved.[14] Qin et al[35] indicated that upregulated *SIRT6*, induced by Specnuezhenide, depressed inflammatory responses in hepatic stellate cells and hepatocytes, thereby mitigating the pathological process of hepatic fibrosis. The suppressive effect of *SIRT6* on epidural scar formation has been validated in the work by Fan et al.[16] Furthermore, our in-depth investigation pointed out that HE not only stimulated *SIRT6* expression, but also promoted the deacetylation effect of SIRT6 on PRMT1 to decrease PRMT1 protein stability and expression. Acetylation is widely known as a kind of post-translational histone modification which increases the accessibility of gene promoters and enhancers, thereby promoting gene transcription and expression, and is involved in various cellular processes such as signal transduction, DNA repair, and protein metabolism.[36] SIRT6 belongs to the conserved protein family that possesses NAD^+-dependent deacetylase ability.[37] Decreasing target gene expression via deacetylation is regarded as the primary way in which *SIRT6* participates in mechanism regulation. The accumulation of SIRT6 induced by palmitate substantially deacetylated ACSL5, a contributor for lipid gathering, attenuating non-alcoholic fatty liver disease progression in a high-fat diet mice model.[38] Luo et al[39] discovered the physical interaction between SIRT6 and hypoxia-inducible factor 1-alpha (HIF1α), and that increased SIRT6 expression deacetylated HIF1α and promoted HIF1α degradation under capsaicin treatment in a chronic kidney disease mouse model. In a study involving liver fibrosis, *SIRT6* knockdown notably inactivated hepatic stellate cells, a central driver of fibrosis, by deacetylating Smad2, a profibrogenic transcription factor.[40] Moreover, *SIRT6* has proven to be an effective target for therapying liver fibrosis.[41] Based on this evidence, we inferred that *SIRT6* would be a potential clinical target for epidural scar formation treatment.

PRMT1 is a major member of the protein arginine methyltransferase family. Due to its ability to regulate protein positive charge and hydrophilicity by arginine methylation,

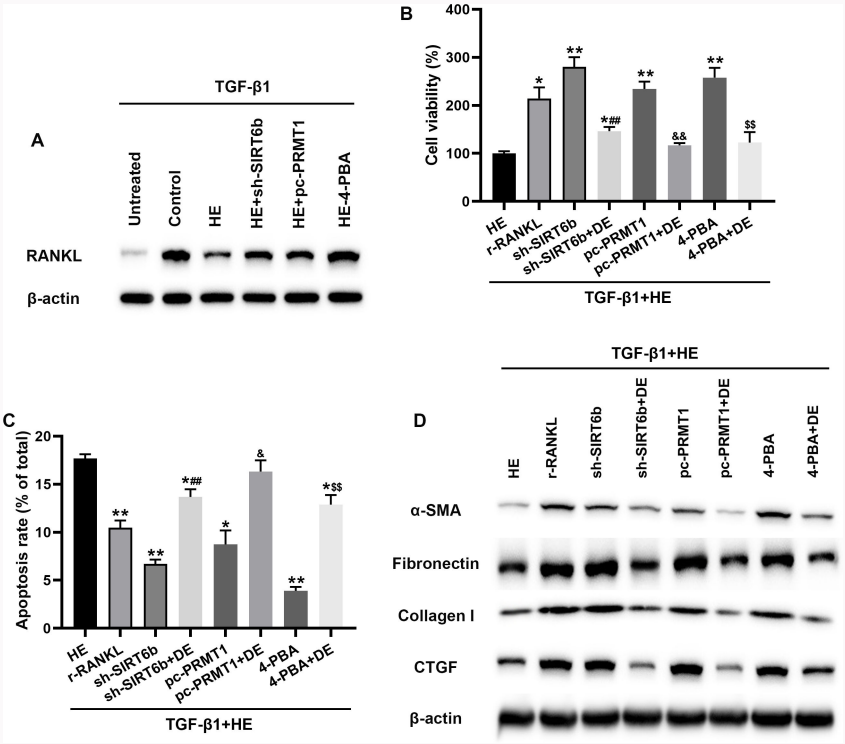

Fig. 5

Receptor activator of nuclear factor-κB ligand (RANKL) plays an important role in HE-induced epidural fibrosis therapy. a) and d) The expressions of RANKL and fibrotic proteins were measured using western blotting. b) Cell Counting Kit-8 method evaluated the viability of fibroblast cells. c) Flow cytometry was used to determine the cell apoptosis rate. Data were based on three experimental repeats analyzed by post-hoc test and are shown as mean (standard error of the mean). *p < 0.05; **p < 0.01; ##p < 0.01; &p < 0.05; &&p < 0.01; $$p < 0.01. 4-PBA, 4-phenylbutyrate; α-SMA, alpha smooth muscle actin; CTGF, connective growth tissue factor; DE, denosumab; PRMT1, protein arginine N-methyltransferase 1; SIRT6, sirtuin-6; TGF-β1, transforming growth factor beta 1.

PRMTs are attributed to gene translation, protein trafficking, DNA damage, and cell signalling.[42] One recent study confirmed the crucial role of *PRMT1* in the development of fibrotic diseases. Depressing *PRMT1* could be an effective way to alleviate myocardial injury and fibroblast fibrosis during myocardial infarction progression.[20] Moreover, profibrotic signals in liver fibrosis mice have been shown to be inhibited by hepatic stellate cell-specific *PRMT1* knockdown.[21] Zakrzewicz et al[43] discovered that increased *PRMT1* expression in idiopathic pulmonary fibrosis lungs and the silencing of *PRMT1* diminished the proliferation and profibrotic effect of fibroblast cells. Similarly, our research demonstrated that *PRMT1* was downregulated by HE-induced *SIRT6* upregulation, and that overexpressing *PRMT1* reversed cell viability and fibrosis responses of epidural fibroblasts. Furthermore, recent studies have illustrated the regulatory mechanism of *PRMT1* in fibrotic diseases. Downregulated *PRMT1* exerted an inhibitory effect on profibrotic mediator and hepatic stellate cell activations which contribute to liver fibrosis.[21] The research of Wang et al[44] demonstrated that *PRMT1* interference was indicated as an effective way to weaken high glucose-induced fibrotic progression in renal tubular epithelial cells via *TGF-β/SMAD3* inhibition. HE-induced *SIRT6* activation was crucial for alleviating epidural scar formation and fibrosis through the mechanism regulated by *PRMT1*. These findings may provide support for *PRMT1* as a valuable therapy target in fibrotic disease, and indicate the clinical potential of HE in treating epidural scar formation.

Inflammatory response is considered to be a crucial process during the development of epidural scar formation.[45] The abnormal activation of NF-κB signalling was found to be a great contributor to the unfavourable progression of various inflammatory diseases.[46] Interestingly, various studies demonstrated that upregulating *SIRT6* would lead to the depression of NF-κB signalling, thereby alleviating the inflammatory ageing of skin fibroblasts induced by UVA.[47] Yan et al[21] discovered the inhibitory effect of *PRMT1* knockout on the inflammatory activation of NF-κB, which attenuated the progression of liver fibrosis. Moreover, in another study, HE exerted its therapeutic effect on acute lung injury by suppressing NF-κB signalling.[48] Our findings indicated that HE alleviated epidural fibrosis by promoting *SIRT6* to inhibit the aberrant upregulation of *PRMT1*, which was consistent with the aforementioned evidence. Furthermore, the crucial role of inflammatory NF-κB signalling could be inferred to emerge in HE-mediated improvement in epidural scar formation. However, this requires further investigation.

As a core organelle, ER is pivotal for the synthesis, modification, folding, and secretion of transmembrane proteins. The disturbance of ER protein-folding ability contributes to an ER stress state under which the function, fate, and survival of cells are ultimately changed.[49] In recent years, more and more studies have demonstrated the pivotal role of ER stress in fibrotic disorders. Bleomycin induced pulmonary fibrosis and activated ER stress, while inhibiting the activation of ER stress weakened the proliferation

of fibroblasts and improved lung function.[50] Zhang et al[51] indicated that ER stress was restrained by *STING* knockout in the cardiac tissues of cardiac hypertrophy mice, leading to a reduction in fibrosis and inflammation in cardiomyocytes. However, although our research also found that ER stress was activated in TGF-β1-induced scar fibroblasts, HE treatment further intensified the activation of ER stress, leading to cell death, inhibition of cell fibrosis, and attenuation of scar formation progression. Similar to our findings, Delbrel et al[52] demonstrated that the overexpression of *HIF-1α* resulted in alveolar epithelial cell apoptosis during pulmonary fibrosis development by triggering unfolded protein response pathway and ER stress. As for the functional duality of ER stress in the progression of fibrosis disease, according to the existing evidence, we inferred that this may be due to the following reasons: first, ER stress has different modes of action in different cell types; second, although ER stress is a self-protection mechanism of cells, excessive ER stress can cause irreversible damage to cells and even promote apoptosis; finally, ER stress is an induction pathway of cell apoptosis, and activation of this pathway can inhibit cell survival. Supporting this point, evidence has indicated that ER stress presents both cell survival improvement by activating adaptive response, and the acceleration of cell death by inducing apoptosis signals in tumour cells.[53] Moreover, Siwecka et al[54] demonstrated that the dual role of ER stress may be the result of the status of IRE1α, which is considered a positive regulator of cell survival, but its interaction with RNases domain and subsequent TNF receptor-associated factor 2 could stimulate pro-apoptotic mediator activation under stress. However, the specific mechanism of ER stress in HE-improved epidural fibrosis progression needs to be studied further.

We also verified that the suppression on RANKL was pivotal for the HE treatment of epidural scar formation. Supplementing RANKL dramatically offsets the inhibitory effect of HE on cell survival and profibrotic signals in epidural fibroblasts. RANKL is a crucial mediator responsible for osteoclastogenesis. More and more studies indicate that RANKL is widely expressed and functions in various aspects, such as immune response, mammary gland development, and tissue fibrosis.[55] Osteoprotegerin is mainly a decoy mediator which impacts the interaction between RANKL and RANK, and has a crucial link with fibrogenesis.[56] It is known that RANKL is involved in the process of fibrosis. In fact, early research had already discovered that the elevated RANKL/osteoprotegerin ratio was proved to be a contributor for myocardial fibrosis.[57] Ju et al[58] indicated that a RANKL-targeted peptide inhibited the profibrotic M2 activation of macrophages to depress lung fibroblast differentiation, and had a preventive effect on pulmonary fibrosis mice. More importantly, the content of RANKL in the serum of nonalcoholic fatty liver disease patients with fibrosis progression was shown to be reduced.[59] This evidence supports our findings.

Nevertheless, there are limitations to our study. Further investigations should be conducted into the clinical application of HE on epidural scar formation. According to current research on HE, it possesses a good gastrointestinal absorption rate with a short eliminating half-life, which makes HE an effective agent in depressing a wide range of pathogenic processes.[60] Recent studies have also indicated that HE presented safe and valid effects during the therapy of various pathological diseases in vivo.[33,61] The present focus of scientists is to improve the bioavailability and pharmacological activity of HE.[62] The current evidence supports HE's potential as a clinical medication. However, the pharmacology, pharmacokinetics, toxicology, and pharmacy of HE on alleviating epidural scar formation in clinic should be clearly demonstrated by rigorous in vivo studies.

Taken together, our study illustrates the therapeutic role of HE treatment on epidural scar formation. HE regulates *SIRT6/PRMT1* axis-mediated ER stress to depress the aggravated proliferation and fibrosis of epidural fibroblasts by blocking the RANKL pathway (Supplementary Figure a). Our findings present a basis for the novel therapeutic strategy of epidural scar formation.

Supplementary material

Figure showing the effects and mechanism of hederagenin on epidural scar formation.

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Data sharing

The data used to support the findings of this study are available from the corresponding author upon request.

Ethical review statement

This study was approved by the Ethics Committee of Honghui Hospital. Informed consents were provided by every patient in accordance with the Declaration of Helsinki (NO.202406018).

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