Laminin α1 as a target for the treatment of epidural fibrosis by regulating fibrotic mechanisms

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Abstract. Excessive proliferation and migration of fibroblasts in the lumbar laminectomy area can lead to epidural fibrosis, eventually resulting in failed back surgery syndrome. It has been reported that laminin α1, a significant biofunctional glycoprotein in the extracellular matrix, is involved in several fibrosis-related diseases, such as pulmonary, liver and keloid fibrosis. However, the underlying mechanism of laminin α1 in epidural fibrosis remains unknown. The present study aimed to explore the effect and mechanism of laminin α1 in fibroblast proliferation, apoptosis and migration, and epidural fibrosis. Following the establishment of a laminectomy model, hematoxylin and eosin, Masson's trichrome and immunohistochemical staining were performed to determine the degree of epidural fibrosis, the number of fibroblasts, collagen content and the epidural expression levels of laminin α1, respectively. Furthermore, a stable small interfering RNA system was used to knock down the expression of laminin α1 in fibroblasts. The transfection efficiency was confirmed by reverse transcription-quantitative PCR and immunofluorescence staining. Western blot analysis, scratch wound assay, EdU incorporation assay, flow cytometric analysis and Cell Counting Kit 8 assay were performed to assess the proliferation, apoptosis, migration and viability of fibroblasts, as well as the expression levels of the AKT/mechanistic target of rapamycin (mTOR) signaling-related proteins. *In vivo* experiments revealed that laminin α1 was positively and time-dependently associated with epidural fibrosis. In addition, laminin α1 knockdown attenuated cell proliferation, viability and migration, and promoted apoptosis. Furthermore, the results revealed that the activation of the AKT/mTOR signaling pathway was involved in the aforementioned processes. Overall, the current study illustrated the positive association between laminin α1 and epidural fibrosis, and also verified the effect of laminin α1 on fibroblast proliferation, apoptosis and migration. Furthermore, the results suggested that the AKT/mTOR signaling pathway may serve a significant role in regulating the behavior of laminin α1-induced fibroblasts.

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Introduction

Lumbar laminectomy is considered an effective treatment approach for diseases of the lumbar vertebrae, such as slipped disc, spinal stenosis and intraspinal tumor, which are often accompanied by neuropathic pain and other clinical symptoms (1-3). However, epidural fibrosis, which occurs in >20% of patients undergoing lumbar laminectomy worldwide, remains an intractable clinical complication, that results in failed back surgery syndrome (FBSS), which is characterized by chronic lower back and leg pain, as well as disability (4-6). It has been reported that the excessive proliferation and migration of fibroblasts in the lumbar laminectomy area serve a significant role in the development of epidural fibrosis. The aggregation of fibroblasts can promote the deposition of abundant extracellular matrix (ECM) components, eventually leading to the formation of local fibrosis and lumbosacral adhesive arachnoiditis (7-9). Previous studies have suggested that E8002, a biological antiadhesive membrane, as well as immunosuppressants and cytotoxic drugs could attenuate FBSS by regulating fibroblast proliferation and migration (10-12). However, their controversial clinical safety has limited the further advancement of this treatment strategy. Therefore, further research is required in this field.

Laminins are significant biofunctional glycoproteins of the ECM, which are composed of three polypeptide chains, namely α, β and γ, linked with disulfide bonds. Accumulating evidence has suggested that there are five α chains (α1-α5), four β chains (β1-β4) and three γ chains (γ1-γ3), which are assembled into >16 different laminin molecules (13-16). In terms of function, laminins are associated with several cell

surface receptors, and are involved in the transmission of biochemical signals in the microenvironment inside and outside the cell, in order to regulate cellular biological behaviors, such as proliferation, migration, adhesion and differentiation (17-20). Furthermore, the biological activity of laminins is principally governed by five different α chains (laminin α1, laminin α2, laminin α3, laminin α4 and laminin α5), which exhibit a greater effect compared with β and γ chains (21-23). However, the underlying biological mechanism of laminin α1 in regulating the biological behaviors of fibroblasts remains unknown and requires further investigation. To the best of our knowledge, the present study was the first to investigate the role of laminin α1 in the proliferation, apoptosis and migration of fibroblasts in epidural fibrosis. The present study also investigated the association between the expression of laminin α1 and activation of the classical AKT/mechanistic target of rapamycin (mTOR) signaling pathway. Overall, the results of the current study may provide a novel target for the prevention and treatment of epidural fibrosis.

Materials and methods

Animal preparation and establishment of a laminectomy model. A total of 48 Sprague-Dawley (SD) male rats (weight, 250 g; age, 8 weeks) were purchased from the Hubei Provincial Center for Disease Control and Prevention. SD rats were acclimated for 1 week to the laboratory environment of 25±2˚C and 30-50% humidity, under a 12-h light/dark cycle, and were provided with *ad libitum* access to food and water. A total of 36 SD rats were numbered and randomly divided into the 2-, 3- and 4-weeks groups using the random-number-table function in Excel version 2016 (Microsoft Corporation); the number of weeks indicates when rats were sacrificed post operation. The remaining 12 SD rats were set as the control group, which did not undergo laminectomy, and were directly sacrificed to provide a control epidural area. During the preparation process, all rats were appropriately treated according to the Regulations of Laboratory Animals of Hubei province (24).

To simulate laminectomy in clinical patients, a laminectomy model was established in SD rats by carefully excising the vertebral plate, as previously described (3). Briefly, SD rats were anesthetized with 1% pentobarbital sodium (50 mg/kg) through intraperitoneal injection, and their backs were shaved above the first and second lumbar vertebrae to clearly expose the surgical area. The operative skin was sterilized three times with iodine. Following skin incision, the local soft tissue was separated to expose the vertebral plate. Subsequently, the vertebral plates of the first and second lumbar were carefully excised using a rongeur to expose the spinal cord. After appropriate hemostasis, the wound was sutured using Vicryl Plus antibacterial sutures (Johnson & Johnson). During establishment of the laminectomy model, all procedures were performed in sterile conditions by professional orthopedists. After the surgery, the following signs were considered humane endpoints and if these endpoints were met, the rats were sacrificed by cervical dislocation under anesthesia [1% pentobarbital sodium (50 mg/kg), intraperitoneal injection]: i) Persistent tiredness and failure to clean the fur (rough and dull); ii) weakness, dehydration, decreased food and water intake, urine and stool volume; iii) abnormal physical responses to human touch (including excessive withdrawal, limping, unusual aggression, screaming and abdominal clamping); iv) weight loss of >20% (the maximum percentage of body wight loss was 20% in the present study); v) paralysis, unable to walk normally; vi) abnormal body temperature, pulse and breathing (rates were too high or too low); vii) persistent self-harming behavior, self-harm of the incision site and post-operative area; viii) severe inflammation and infection at the surgical site; ix) abnormal central nervous system reactions (convulsions, shaking, paralysis, head tilting).

Histopathological analysis. At 2, 3 and 4 weeks following the lumbar laminectomy, the SD rats from the three groups were sacrificed. The SD rats in the control group were directly sacrificed after the 1-week acclimation. To evaluate the degree of fibrosis, the spinal tissue from the surgical area was collected and a histopathological analysis was then carried out. Briefly, all of the SD rats in each group were anesthetized with 1% pentobarbital sodium (50 mg/kg) through intraperitoneal injection and cardiac perfusion was performed using 4% paraformaldehyde. When decreased corneal reflexes, abnormal pulse and respiration were observed, cervical dislocation was performed to ensure the rats were euthanized. Subsequently, the postoperative lumbar column with external soft tissues was excised and fixed in 4% paraformaldehyde at room temperature for 1 week. After fixation, the column was immersed in EDTA for 40 days for decalcification. Finally, for histopathological analysis, the column was embedded in paraffin and was then sliced into 4-μm transverse sections. For hematoxylin and eosin (H&E) staining, the sections were first stained with hematoxylin for 5 min and then with eosin for an additional 5 min. For Masson's trichrome staining, the sections were immersed overnight in 50% potassium dichromate, stained with hematoxylin for 3 min and incubated in Ponceau S dye for 5 min. The sections were then washed and incubated with 1% phosphomolybdic acid for 2 min prior staining with aniline blue for 5 min. All of the procedures were performed at room temperature. The tissue sections were finally observed under an optical photographic light microscopy (magnifications, x40 and x200) to ascertain the local fibroblast proliferative level, epidural fibrotic level and the content of collagen in epidural tissues. The results were collected and processed using Image Pro Plus 6.0 software (Media Cybernetics, Inc.).

Immunohistochemical staining. Immunohistochemical staining was performed using the Ready-to-use HP IHC detection kit (cat. no. abs957; Absin Bioscience, Inc.). Briefly, the tissue sections were deparaffinized in xylene at room temperature and rehydrated with a descending alcohol series (100, 85 and 75%). For antigen retrieval, the tissue sections were immersed in sodium citrate at 100˚C for 20 min followed by blocking in 3% hydrogen peroxide (from kit) and 100% FBS (Gibco; Thermo Fisher Scientific, Inc.) for 10 and 15 min, respectively, at room temperature. Subsequently, the sections were first incubated with primary antibodies against laminin α1 (1:200; cat. no. bs-4973R; BIOSS) and α smooth muscle actin (α-SMA; 1:200; cat. no. NBP2-33006; Novus Biologicals, Ltd.) at 4˚C overnight and then with the corresponding secondary antibody provided by the HP IHC detection kit at room temperature for 2 h. The tissue sections were then

stained with DAB reagent and hematoxylin for 2 min each at room temperature. Finally, the sections were observed under an optical photographic light microscope at x200 magnification and the expression levels of laminin α1 and α-SMA were analyzed using Image Pro Plus 6.0 software.

Cell culture. Primary human fibroblasts were purchased from ScienCell Research Laboratories, Inc. (cat. no. #2320), and were cultured in DMEM supplemented with 15% FBS and 1% penicillin and streptomycin (all from Gibco; Thermo Fisher Scientific, Inc.) at 37˚C in an incubator containing 5% CO_2. Fibroblasts at passages 3-8 were collected and divided into three groups: The control group, negative control group and small interfering (si)RNA group for the subsequent experiments.

Transfection with siRNA. The siRNA against laminin α1 (5'-TGCCATAGATGGCACCAATAACT-3') and the corresponding negative control siRNA (5'-GGCTCTAGAAAA GCCTATGC-3') were purchased from Guangzhou RiboBio, Co., Ltd. Human fibroblasts (60% confluence) were transfected with 50 nM siRNA (siRNA against laminin α1 for siRNA group, and negative control siRNA for negative control group) for 48 h at 37˚C with Opti-MEM (Gibco; Thermo Fisher Scientific, Inc.) and Lipofectamine® 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.) to knock down the expression of laminin α1. The cells in the control group were not transfected. All procedures were performed according to the protocol of Guangzhou RiboBio, Co., Ltd. The transfection efficiency was assessed by reverse transcription-quantitative PCR (RT-qPCR) and immunofluorescence staining. The transfected cells were collected for the subsequent experiments within 48 h to guarantee transfection efficiency.

RNA preparation and RT-qPCR. Total RNA was extracted from the transfected cells using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. RNA was then reverse transcribed into cDNA using the FastKing DNA Dispelling RT SuperMix (Tiangen Biotech Co., Ltd.), according to the manufacturer's instructions. Subsequently, qPCR was performed using the SYBR®-Green Master Mix kit (Vazyme Biotech Co., Ltd.). The primer sequences used are shown in Table I. The thermocycling conditions were as follows: Initial denaturation at 95˚C for 5 min; followed by 40 cycles at 95˚C for 10 sec and 60˚C for 30 sec; then, 95˚C for 15 sec, 60˚C for 60 sec and 95˚C for 15 sec. The relative mRNA expression levels were calculated using the $2^{-\Delta\Delta Cq}$ method (25). GAPDH served as the internal reference.

Immunofluorescence staining. The fibroblasts were seeded in 24-well plates containing glass slides until they reached 60% confluence (generally within 24 h). After fixing in 4% paraformaldehyde at room temperature for 15 min, the fibroblasts were immersed in 0.1% Triton X-100 for permeabilization and blocked in 3% BSA (Gibco; Thermo Fisher Scientific, Inc.) for 30 min at room temperature. Subsequently, the cells were incubated with anti-laminin α1 (1:100) at 4˚C overnight and then with Alexa Fluor® 647-conjugated anti-rabbit IgG antibody (1:200; cat. no. ab150075; Abcam) for 1 h at room temperature. The nuclei were stained with Hoechst for 5 min at room temperature and the cells were observed under a Zeiss inverted fluorescence microscope (magnification, x200; Zeiss AG). The fluorescence density of laminin α1 in each group was analyzed using Image Pro Plus 6.0 in five randomly selected fields with the same size.

Table I. Primers for reverse transcription-quantitative PCR.

Gene	Primer sequence, 5'-3'
Laminin α1	F: AGAGGGCTACAAAGTCCAGT
	R: GCTCTAGTCCAATGGCATCC
GAPDH	F: GAAGCTTGTCATCAATGGAAAT
	R: TGATGACCCTTTTGGCTCCC

F, forward; R, reverse.

Western blot analysis. Total protein was extracted from the fibroblasts using RIPA lysis buffer (Beyotime Institute of Biotechnology) and protein concentration was determined using the BCA kit (Thermo Fisher Scientific, Inc.). Subsequently, 30 μg proteins/lane were separated by SDS-PAGE on 10% gels and transferred onto a PVDF membrane. After blocking in 5% skim milk in TBS with 0.05% Tween-20 for 2 h at room temperature, the membranes were incubated with the primary antibodies at 4˚C overnight and then with the corresponding secondary antibodies (anti-rabbit; 1:2,000; cat. no. #7074; Cell Signaling Technology, Inc.) at room temperature for 2 h. The following primary antibodies were used: Anti-proliferating cell nuclear antigen (PCNA; 1:1,000; cat. no. #13110), anti-cyclin D1 (1:1,000; cat. no. #55506), anti-matrix metalloproteinase (MMP)-2 (1:1,000; cat. no. #40994), anti-MMP-9 (1:1,000; cat. no. #13667), anti-AKT (1:1,000; cat. no. #4685), anti-phosphorylated (p)-AKT (1:1,000; cat. no. #4060), anti-mTOR (1:1,000; cat. no. #2983), anti-p-mTOR (1:1,000; cat. no. #5536), anti-Bax (1:1,000; cat. no. #5023), anti-Bcl-2 (1:1,000; cat. no. #4223) and anti-GAPDH (1:1,000; cat. no. #5174) (all from Cell Signaling Technology, Inc.). GAPDH served as an internal loading protein. The protein expression level were then detected using BeyoECL Moon (cat. no. P0018FS; Beyotime institute of Biotechnology) and the ChemiDoc XRS+ system (Bio-Rad Laboratories, Inc.) according to the manufacturer's protocol. ImageJ version 1.46r software (National Institute of Health) was used to perform densitometric analysis of all protein bands.

Cell viability assay. Cell viability was assessed using the Cell Counting Kit-8 (CCK-8) assay (cat. no. CK04; Dojindo Molecular Technologies, Inc.). Briefly, the prepared fibroblasts from each group were cultured in 96-well plates and then treated with 10 μl CCK-8 reagent for 2 h at 37˚C. Subsequently, optical density (OD) values were measured at a wavelength of 450 nm using a microplate absorbance reader (Elx800; Biotek Instruments, Inc.). Finally, the cell survival rate was calculated according to the formula in the manufacturer's protocol: [(As-Ab)/(Ac-Ab)] x100. As indicates the absorbance of the samples (negative control group or siRNA group), Ac indicates the absorbance of the control group, and Ab indicates the absorbance of wells without cells.

EdU incorporation assay. The EdU incorporation assay was performed to evaluate cellular DNA synthesis using the Click-iT EdU Alexa Fluor 488 Imaging kit (Nanjing KeyGen Biotech Co., Ltd.), according to the manufacturer's protocol. Briefly, fibroblasts from each group were cultured in 24-well plates until they reached 60% confluence (generally within 24 h). Subsequently, cells were incubated in 10 μmol/l EdU working solution at 37˚C for 2 h followed by fixing in 4% paraformaldehyde at room temperature for 30 min and treatment with 0.5% Triton X-100 for 20 min in the dark at room temperature. Cells were then immerged in the click-iT mixture system and cell nuclei were stained with Hoechst at room temperature for 5 min. Finally, the fibroblasts were observed under a Zeiss inverted fluorescence microscope (magnification, x200) to analyze the EdU-positive fluorescent signal. The EdU-positive rate was calculated using ImageJ.

Flow cytometric analysis. Flow cytometry was performed to determine the apoptotic rate (early + late apoptosis) of fibroblasts according to the protocol of the Annexin V-FITC Apoptosis Detection kit (cat. no. C1062L; Beyotime institute of Biotechnology). Briefly, the fibroblasts from each group were cultured in 6-well plates at a density of 10^6 cells/well. When the cells reached 80% confluence, the supernatant was removed. The prepared cells were then collected and stained with Annexin V (5 μl/10^5 cells) and PI (10 μl/10^5 cells) at room temperature for 20 min. After staining, the cells were washed twice with PBS, resuspended in PBS and detected by flow cytometry (BD Accuri C6; BD Biosciences). The data were analyzed using FlowJo 10.8.1 software (FlowJo, LLC).

Scratch wound assay. The fibroblasts from each group were cultured into 6-well plates until they covered the entire bottom of the well (80% confluence) (26,27). Subsequently, a 10-μl pipette tip was used to scratch the bottom of the well. The scraped cells were removed after washing with PBS. Subsequently, the complete culture medium was replaced with DMEM supplemented with 0.1% FBS. The fibroblasts were observed under an optical photographic light microscopy (magnification, x40). The migratory ability of fibroblasts from each group was assessed at 0, 12 and 24 h after scratching. The results were collected and processed using ImageJ software. The wound closure rate was calculated as follows: Wound closure rate=the area of migrated cells in the total scratched area at 0, 12 or 24 h/the total scratched area.

Statistical analysis. All of the data in the present study are presented as the mean ± standard deviation and the statistical analysis was performed by SPSS 19.0 statistical software (IBM Corp.). Each experiment was performed in triplicate. The differences among multiple groups were assessed using one-way ANOVA followed by Tukey's post hoc test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Laminin α1 is positively and time-dependently associated with epidural fibrosis. As shown in Fig. 1, the results of H&E staining and Masson's trichrome staining showed a low level of epidural fibrosis, few fibroblasts (spindle cells indicated with red arrows) and low collagen content in the SD rats of the control group, which illustrated the normal epidural site without laminectomy. By contrast, following the laminectomy, the level of epidural fibrosis, the number of fibroblasts and the collagen content were increased in the 4-weeks group. These changes were also apparent but were more moderate in the 3-weeks group and were even more moderate in the 2-weeks group (Fig. 1A-F). These findings indicated that the level of postoperative epidural fibrosis in the laminectomy area was increased in a time-dependent manner. In addition, some lymphocytes were observed in the epidural area after laminectomy (round cells indicated with green arrows).

To determine whether laminin α1 was involved in the progression of epidural fibrosis, the expression levels of laminin α1 in the three groups were analyzed by immunohistochemical staining. As shown in Fig. 2A, laminin α1 was significantly increased in the 4-weeks group and was moderately increased in the 3-weeks group compared with in the 2-weeks group. To exclude the influence of neovascularization, which commonly occurs during fibrosis formation in local tissues, the expression levels of α-SMA were also detected. The results showed that there was no significant difference in the expression of α-SMA among the three groups (Fig. 2B), thus suggesting that the expression of laminin α1 changed with the progression of epidural fibrosis. Overall, these data suggested that the expression of laminin α1 was positively and time-dependently associated with epidural fibrosis and it could be involved in postoperative FBSS.

siRNA transfection interferes with the expression of laminin α1 in fibroblasts. The results of immunofluorescence staining and RT-qPCR analysis confirmed transfection efficiency (Fig. 3A and B). After transfection, the expression levels of laminin α1 were significantly reduced in the siRNA group compared with in the control group, and a negligible difference in expression was detected between the control and negative control groups.

Laminin α1 regulates the proliferation, apoptosis and migration of fibroblasts. The biological role of laminin α1 in fibroblast proliferation, apoptosis and migration was subsequently assessed. Compared with in the control group, the EdU-positive rate of the siRNA group was decreased following laminin α1 knockdown (Fig. 4A), and the expression levels of the proliferation-related proteins PCNA and cyclin D1 were also significantly reduced; however, there was no difference between the control group and negative control group (Fig. 4B). These results suggested that laminin α1 could regulate the proliferation of fibroblasts.

The results of flow cytometric analysis demonstrated that compared with in the control group, the apoptotic rate of fibroblasts in the siRNA group was significantly increased following laminin α1 knockdown (Fig. 4C). Consistently, western blot analysis of the apoptosis-related proteins Bax and Bcl-2 also indicated a similar tendency; after laminin α1 was knocked down, the expression levels of Bax were increased, whereas those of Bcl-2 were decreased (Fig. 4B). Furthermore, the results of the CCK-8 assay demonstrated that cell viability was decreased in the siRNA group compared

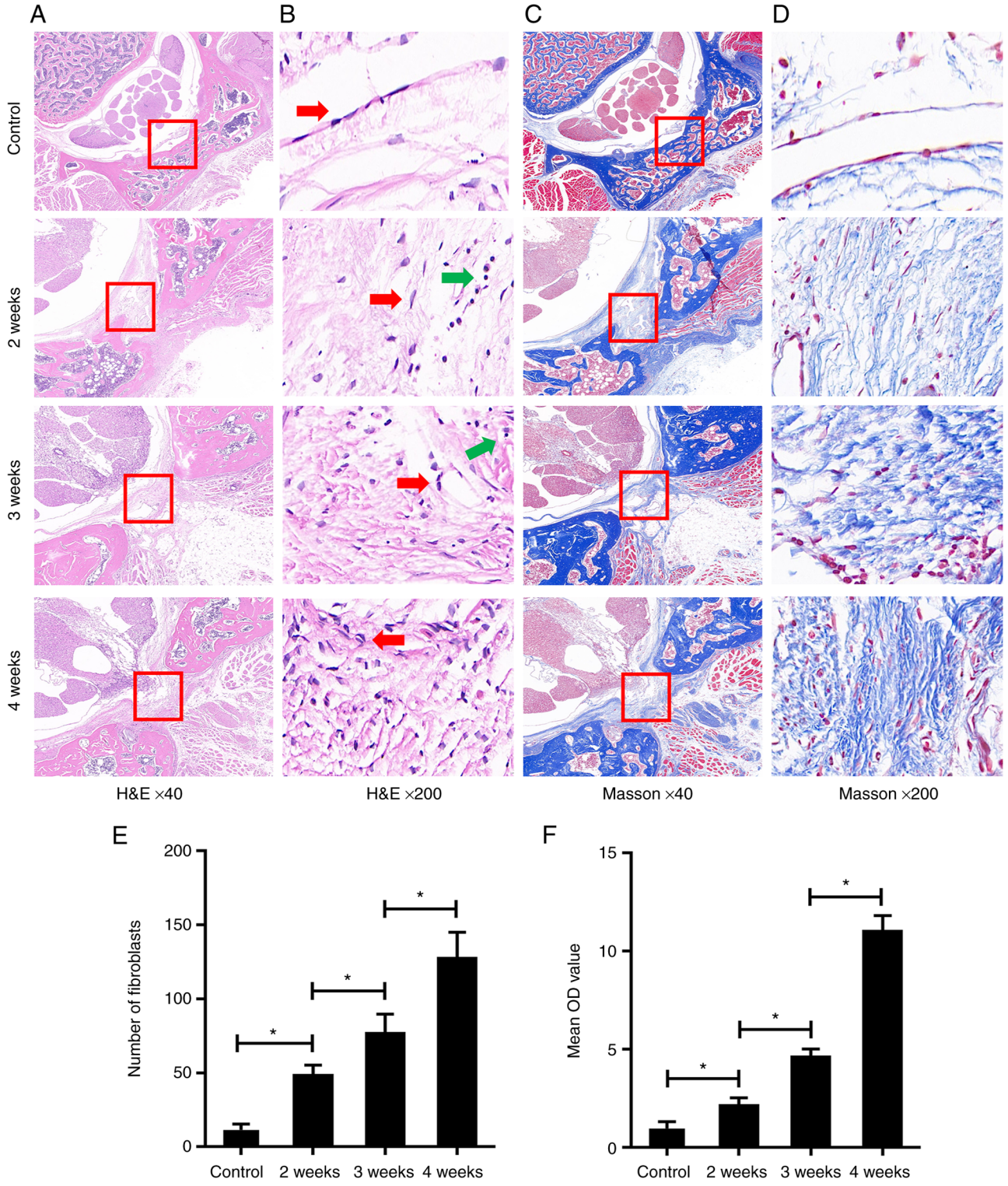

Figure 1. Postoperative epidural fibrosis was increased in the laminectomy area in a time-dependent manner. (A) As determined by H&E staining, compared with the control group, excessive epidural fibrosis (red rectangle) with thick adherence to the spinal dura was observed in the three operated groups; this increased in a time-dependent manner. Magnification, x40. (B) As determined by H&E staining, compared with the control group, the epidural fibroblasts (red arrows) in the three operated groups were observed increasing in a time-dependent manner. Lymphocytes are shown with green arrows. Magnification, x200. (C and D) As determined by Masson's trichrome staining, compared with the control group, epidural collagen (red rectangle) in the three operated groups was observed synthesizing in a time-dependent manner. Magnification, (C) x40 and (D) x200. (E) Semi-quantification of H&E staining in (B); the number of fibroblasts in each group are presented. (F) Semi-quantification of Masson's trichrome staining in (D); the contents of epidural collagen in each group are presented as the mean OD value. Data are shown as the mean ± standard deviation. *P<0.05. H&E, hematoxylin and eosin; OD, optical density.

with in the control group (Fig. 4D). These findings indicated that laminin α1 could regulate the proliferation, apoptosis and viability of fibroblasts.

The effect of laminin α1 on the migration of fibroblasts was also assessed. It is widely accepted that several members of the MMP family are involved in the degradation of

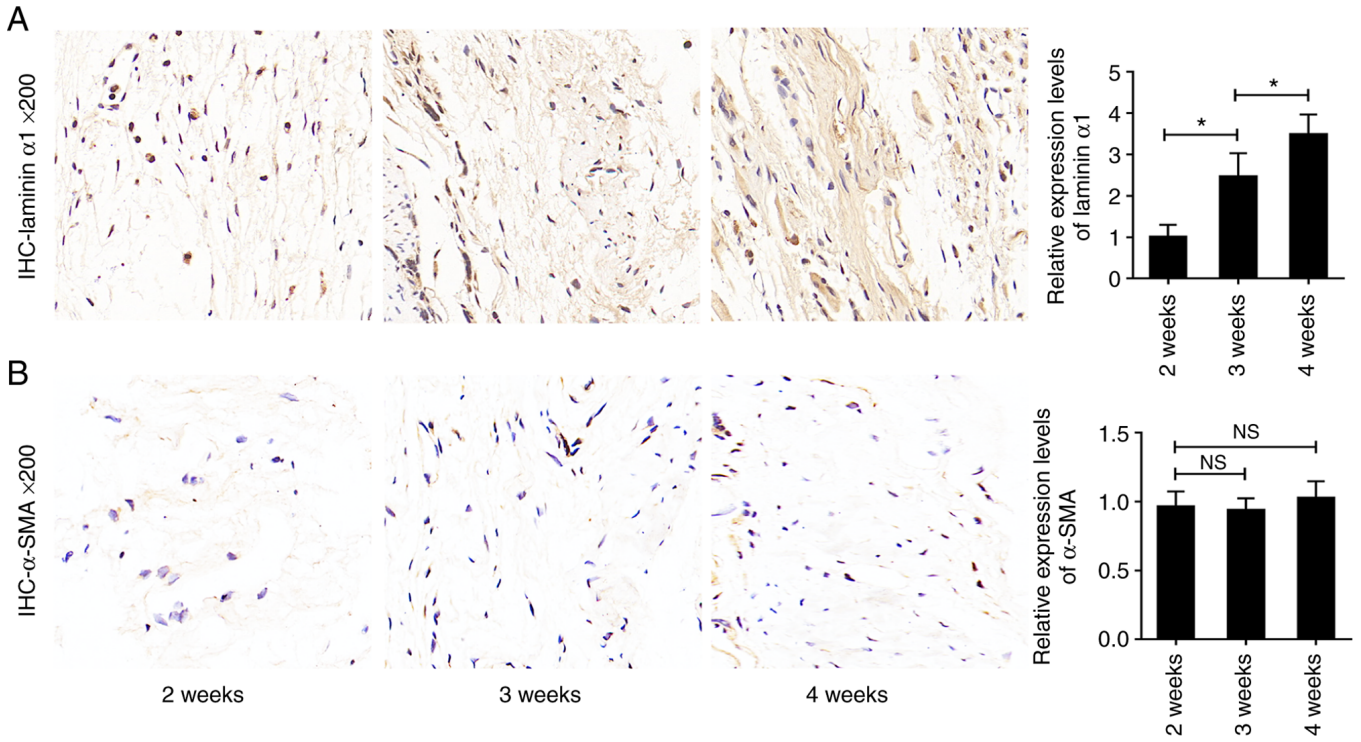

Figure 2. Laminin α1 expression is positively and time dependently increased in response to laminectomy. (A) Immunohistochemical staining of laminin α1 in epidural fibrosis tissues, the expression of laminin α1 increased in a time-dependent manner. Results are shown as the mean OD value. Magnification, x200. (B) Immunohistochemical staining of α-SMA in epidural fibrosis tissues; there was no significant difference among the three groups. Results are shown as the mean OD value. Magnification, x200. Data are presented as the mean ± standard deviation. *P<0.05. α-SMA, α smooth muscle actin; IHC, immunohistochemistry; NS, not significant; OD, optical density.

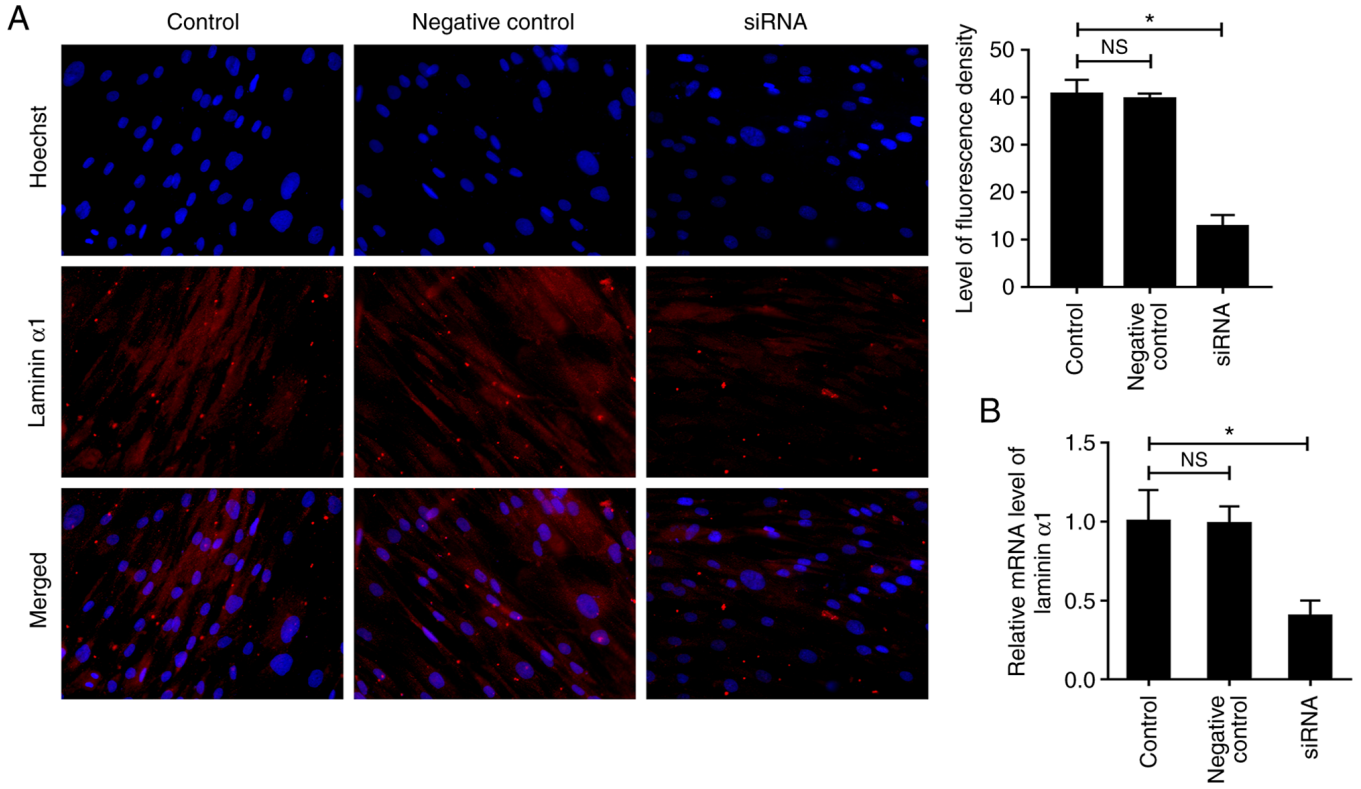

Figure 3. Transfection efficiency of laminin α1 siRNA determined by immunofluorescence staining and RT-qPCR. (A) Expression levels of laminin α1 were measured by immunofluorescence staining among the three groups; the fluorescence density was significantly decreased in the siRNA group. No statistical significance was observed between the control and negative control groups. Magnification, x200. (B) Results of RT-qPCR; the mRNA expression levels of laminin α1 were downregulated in the siRNA group. GAPDH was used as the control. Data are presented as the mean ± standard deviation. *P<0.05. NS, not significant; siRNA, small interfering RNA; RT-qPCR, reverse transcription-quantitative PCR.

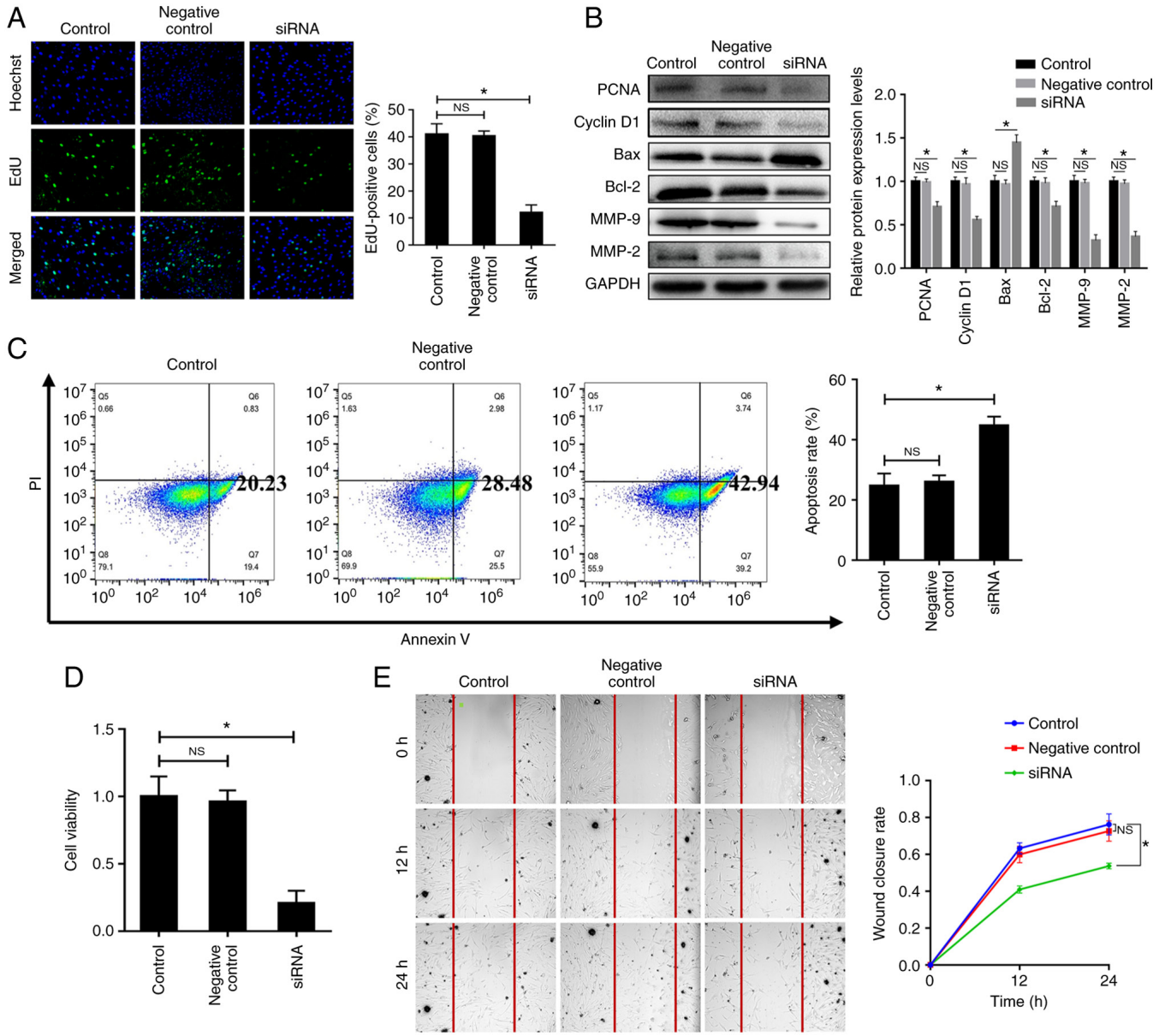

Figure 4. Laminin α1 regulates the proliferation, apoptosis and migration of fibroblasts, and affects their viability. (A) Fibroblast proliferation was detected using the EdU incorporation assay. The proliferative rate was decreased after laminin α1 was knocked down. Magnification, x200. (B) Protein expression levels of PCNA, cyclin D1, Bax, Bcl-2, MMP-2 and MMP-9 were detected by western blotting in each group. (C) Fibroblast apoptosis was detected by Annexin V-FITC/PI double labeling and the apoptotic rates are presented. (D) Fibroblast viability was detected using the Cell Counting Kit-8 assay. (E) Fibroblast migration was detected using the scratch wound assay (magnification, x40). Data are presented as the mean ± standard deviation. *P<0.05. MMP, matrix metalloproteinase; NS, not significant; PCNA, proliferating cell nuclear antigen; siRNA, small interfering RNA.

ECM-related proteins to promote cell migration (28). In addition, previous studies have indicated that MMP-2 and MMP-9 (two classical MMPs) could promote the proliferation, activation and migration of fibroblasts in an inflammatory site, thus resulting in local fibrosis (29-31). As shown in Fig. 4B, the results of western blotting revealed that after laminin α1 was knocked down, the expression levels of MMP-2 and MMP-9 in the siRNA group were downregulated compared with in the control group. These changes suggested that migration may be inhibited in response to laminin α1 knockdown. Moreover, the results of a scratch wound assay demonstrated that after laminin α1 knockdown the migration of fibroblasts was significantly reduced compared with in the control group, whereas there was no obvious difference between the control group and negative control group (Fig. 4E). These results indicated that laminin α1 could regulate cellular migration.

Laminin α1 is involved in activation of the AKT/mTOR signaling pathway. The AKT/mTOR signaling pathway serves an important role in the regulation of diverse cellular functions, including proliferation, differentiation, apoptosis and migration (32,33). In previous studies, it was demonstrated that AKT/mTOR signaling could regulate fibroblast behaviors, such as proliferation, apoptosis and autophagy (9,34). To explore the potential interaction between laminin α1 and AKT/mTOR signaling, the expression levels of the AKT/mTOR signaling-related proteins (p-AKT, AKT, p-mTOR and mTOR) were detected. The results of western blotting showed that

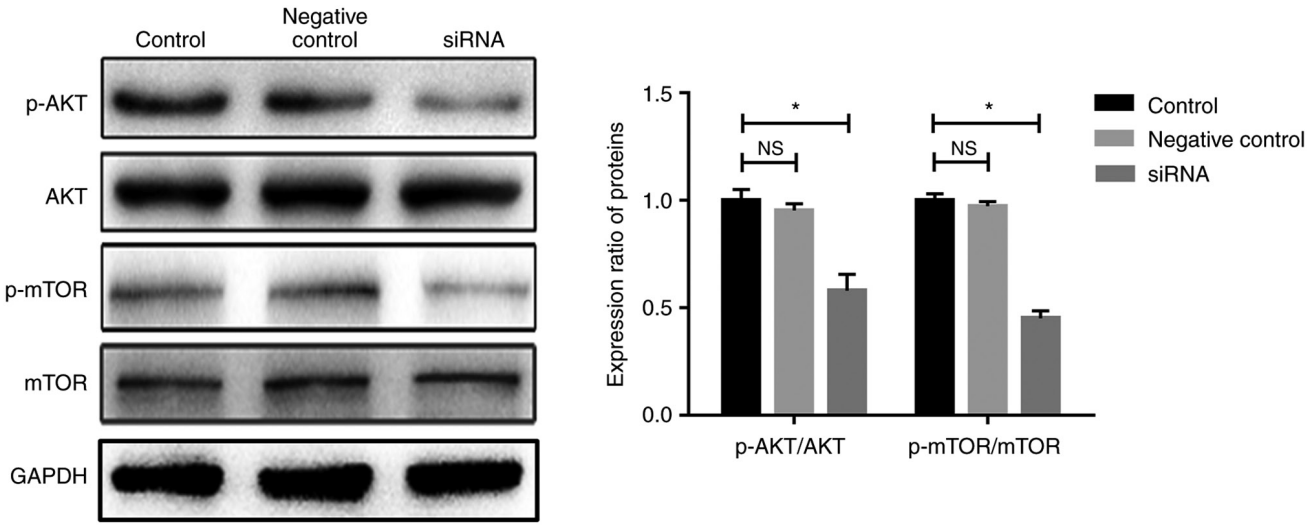

Figure 5. Laminin α1 interferes with activation of the AKT/mTOR signaling pathway. Protein expression levels of p-AKT, AKT, mTOR and p-mTOR were detected by western blotting in each group. Data are presented as the mean ± standard deviation. *P<0.05. mTOR, mechanistic target of rapamycin; NS, not significant; p-, phosphorylated; siRNA, small interfering RNA.

following laminin α1 knockdown, the expression ratios of p-AKT/AKT and p-mTOR/mTOR were significantly reduced compared with in the control group (Fig. 5). These findings suggested that activation of the AKT/mTOR signaling pathway was prevented after laminin α1 was knocked down.

Discussion

Lumbar laminectomy is often accompanied by an increased incidence of FBSS and unavoidable symptoms, including chronic lower back and leg pain (5). Although the clinical complications are apparent to orthopedists, the underlying mechanisms of epidural fibrosis have not been thoroughly explored. Therefore, it is important to clearly study the cellular and molecular mechanisms, which could be beneficial for the improved understanding of the appearance and development of epidural fibrosis. An in-depth understanding of the physiological mechanism is essential for the development of effective methods to prevent and treat fibrosis. The current dominant view is that fibroblasts have a critical role in the progression of epidural fibrosis (3,12). After lumbar laminectomy, local resident fibroblasts are recruited to the operative region and start to deposit abundant ECM proteins, including collagens and laminins (35). The ECM serves an important role in cell construction, and regulates the cell biological behavior by interacting with specific plasma membrane receptors in the extracellular microenvironment. The ECM also affects the physiological structure and characteristics of the cell basement membrane (36,37). The abnormal parameters and functions of ECM can lead to a variety of diseases, including abnormal blood filtration, adipogenesis and fibrosis, muscle, vessel and skin dyspoiesis, as well as tumorigenesis (38,39). As a type of important biofunctional protein in the ECM, laminins can determine the properties of basement membranes and serve a crucial role in several cellular behaviors (20). Across the five types of laminin α chains, laminin α1 has been demonstrated to have a wide expression in human tissues, including skin, retina, neural stem cells and testes, and may have an important role in the biological behaviors of cells (40-43). Furthermore, increasing studies have indicated that laminin α1 is upregulated in fibrotic diseases, such as pulmonary fibrosis, liver fibrosis and keloid fibrosis (44-46).

Based on the aforementioned evidence, the present study first hypothesized that laminin α1 may be involved in the formation of epidural fibrosis. According to the results of histopathological analysis in the present study, after laminectomy, the level of epidural fibrosis increased with the passage of postoperative time compared with in the control group. Combined with the results of immunohistochemical staining, the present study demonstrated that the expression of laminin α1 was positively and time-dependently associated with epidural fibrosis following lumbar laminectomy. Furthermore, the expression levels of α-SMA were detected to exclude the influence of neovascularization, which ultimately confirmed the original hypothesis. Moreover, the results of histopathological analysis indicated that several lymphocytes were present in epidural fibrosis. Lymphocytes are involved in the early stage of inflammation, and can produce inflammatory factors and profibrotic molecules to promote inflammatory chemotaxis and fibrosis (47,48). The presence of lymphocytes indicated the development of inflammation at the postoperative site. This finding was consistent with previous studies, which demonstrated that following lumbar laminectomy, inflammation and fibrosis occurred in the local area and finally led to clinical FBSS (7-9). Therefore, it was further hypothesized that laminin α1 may be related to fibroblast proliferation, apoptosis and migration, which are considered the predominant pathophysiological hallmarks of epidural fibrosis. A stable siRNA-transfected system was thus established. Following laminin α1 knockdown, the viability, proliferation, apoptosis and migration of fibroblasts were detected using CCK-8 assay, scratch wound assay, western blot analysis, EdU incorporation assay and flow cytometry. Notably, the results revealed that the levels of cellular viability and proliferation were significantly decreased in response to laminin α1 knockdown. Furthermore, the expression levels of proliferation-related proteins, such as

PCNA and cyclin D1, were also downregulated. The expression levels of apoptosis-associated proteins, Bax and Bcl-2, and the results of flow cytometry all demonstrated that apoptosis was promoted following laminin α1 knockdown. These data indicated that the laminin α1 could interfere with and regulate the proliferation and apoptosis of fibroblasts.

MMPs are closely related to fibrosis and can regulate a range of biological processes in the immune system and tissue repair. A previous study demonstrated that several MMPs could turn over ECM components, and influence cellular proliferation, survival, gene expression and inflammation to impact migration and fibrosis (49). Another study revealed that the two classical MMPs, MMP-2 (gelatinase A) and MMP-9 (gelatinase B), could degrade type IV collagen and gelatin substrates, which were highly associated with cellular migration and invasion (50). Hence, the decreased expression of MMP-2 and MMP-9 detected in response to laminin α1 knockdown in the present study suggested that migration may be inhibited. The reason for this could be attributed to the limited degradation of collagen and gelatin. Combined with the results of the scratch wound assay, it was suggested that the migratory ability of fibroblasts could be suppressed after laminin α1 knockdown. It has been reported that laminin α1 serves a critical role in the proliferation, migration and invasion of esophageal squamous cells, which indicated a potential prognostic and therapeutic significance for laminin α1 in esophageal squamous cell carcinoma (51). Previous studies have also indicated that laminin α1 is closely related to the melanoma (52), pulmonary fibrosis (44), colorectal carcinoma (53), cerebellar hypoplasia (54) and vitreoretinal disease (55). The present findings also indicated that laminin α1 could interfere with and regulate the proliferation, apoptosis and migration of fibroblasts. As the functioning cells of epidural fibrosis, the decreased migration and proliferation, and enhanced apoptosis of fibroblasts may further relieve epidural fibrosis during the postoperative period of lumbar laminectomy, and laminin α1 could be an effective therapeutic target in the treatment of epidural fibrosis.

During the regulation of cellular behavior, laminins can interact with cell surface receptors and lead to a series of changes in intracellular signaling pathways (17). In previous studies, the AKT/mTOR signaling pathway has been demonstrated to have an important role in the regulation of cellular behaviors, such as proliferation, differentiation, apoptosis and migration (32,33). To further confirm the underlying molecular mechanism of laminin α1 knockdown, the activation status of the AKT/mTOR signaling pathway was detected. In response to laminin α1 knockdown, the AKT/mTOR signaling pathway was significantly suppressed. These results suggested that laminin α1 could affect activation of the AKT/mTOR signaling pathway. A previous study reported that the AKT/mTOR signaling pathway promoted the cellular activities of fibroblasts; notably, after intervening with LY294002 (an inhibitor of the AKT/mTOR signaling pathway), cellular viability and proliferation were reduced (3). Previous studies have also confirmed the inhibitory effect of AKT/mTOR signaling inactivation on fibroblast migration; notably, applying Morin (an anti-arthritis compound) has been shown to reduce the migration of fibroblasts in rheumatoid arthritis (56) and Danlou tablet (a Chinese medicine used to treat cardiovascular diseases) has been reported to restrain the migration of vascular adventitial fibroblasts in atherosclerosis (57). Furthermore, Lu *et al* revealed that an antibody against programmed cell death ligand 1 could inhibit fibroblast proliferation, migration and ECM deposition in pulmonary fibrosis through regulating the AKT/mTOR signaling pathway (58). In combination with the changes in the AKT/mTOR signaling pathway detected following laminin α1 knockdown, it may be hypothesized that laminin α1 could regulate the proliferation, apoptosis and migration of fibroblasts, partially via regulating the AKT/mTOR signaling pathway.

The findings of the present study provided novel insights into the treatment of epidural fibrosis and FBSS; however, due to limitations associated with technology, time and finance, there are some deficiencies that should be overcome to further strengthen the conclusion of the present study. For example, the following experiments may provide a better understanding of the relationships and mechanisms among laminin α1, the AKT/mTOR signaling pathway, epidural fibrosis and fibroblast behaviors: The establishment of a laminin α1 knockdown animal model; more cellular staining experiments; a Transwell migration assay; and a possible reversal experiment (activating AKT/mTOR in fibroblasts in which laminin α1 was knocked down and reconfirming the cellular behavior). Furthermore, previous studies have used fibroblasts derived from the epidural postoperative site to study the mechanisms of epidural fibrosis (59,60); however, due to the difficulty of extracting primary fibroblasts, a high cell contamination rate and their low cell viability, there are limits to their application. Scars and fibrosis after injury are caused by tissue repair induced by the proliferation of surrounding fibroblasts, and the physiological mechanisms and cellular functions of fibroblasts around the dura may be similar to those around the derma. Therefore, in the present study, commercially available human dermal fibroblasts [classic fibroblasts usually applied in the study of fibrosis (61)] were purchased as an alternative, as they have better cell viability and more stable cell passages. The results of the present study could provide a novel idea in the clinical treatment of postoperative epidural fibrosis. In addition, previous studies have selected L929 (mouse epithelial fibroblasts) and NIH-3T3 (mouse embryo fibroblasts) cells to study epidural fibrosis (62,63). However, validation of the results demonstrated in the present study in other fibroblast cell lines or the fibroblasts extracted from the epidural site would still be valuable. In the future, these could drive the directions for further research.

In conclusion, the present study first illustrated the positive association between laminin α1and epidural fibrosis, and confirmed the regulatory effect of laminin α1 on the proliferation, apoptosis and migration of fibroblasts. Furthermore, a possible mechanism was revealed; the regulatory effect of laminin α1 on proliferation, apoptosis and migration of fibroblasts may be realized via the AKT/mTOR signaling pathway.

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Availability of data and materials

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

Authors' contributions

PL, DZ and GH designed the study and performed most of the experiments together. MX, YF, LL and JZ established the animal models, helped to perform the experiments, and contributed to the reagents, materials, data analysis and figure preparation. MX and ZY guided and modified the whole study, interpretated the data, and reviewed and amended the manuscript. PL and MX confirmed the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The purchase and application of human primary fibroblasts in the present study were reviewed and approved by the Medical Ethics Committee of Union Hospital, Tongji Medical College, Huazhong University of Science and Technology [(2021) IEC (approval no. 554)], and the animal experiment was reviewed and approved by the Institutional Animal Care and Use Committee of Huazhong University of Science and Technology [(2021) IACUC (approval no. 2955)].

Patient consent for publication

Not applicable.

Competing interests

The authors declared that they have no competing interests.

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