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Fibulin-5 promotes fibroblast activation through LTBP-4 to improve the pathogenesis of hemorrhoids

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Abstract

Background Hemorrhoid disease is a widespread gastrointestinal ailment caused by the degeneration of supporting tissues within the anal cushions. Despite its high incidence, the pathogenic processes involved remain poorly elucidated.

Objective This study sought to clarify the functions of Fibulin-5 (FBLN5) and latent transforming growth factor beta binding protein 4 (LTBP-4) in activating fibroblasts and their combined impact on hemorrhoid pathogenesis, while exploring their potential as therapeutic targets.

Methods A rat model of hemorrhoid was created by injecting glacial acetic acid into the region surrounding the anus. Fibroblast migration was assessed using a scratch test, while cell viability was assessed with CCK-8. Protein and gene expression levels of FBLN5, LTBP-4, COL1A2, Elastin, and fibronectin were measured by Western blotting and RT-PCR. Immunofluorescence and immunohistochemistry were employed to localize and visualize protein localization and expression. Plasmid transfection experiments assessed the effects of FBLN5 and LTBP-4 overexpression or knockdown on fibroblast behavior.

Results Hemorrhoidal tissues in the experimental group exhibited significant structural alterations, with notable reductions in the expression of FBLN5, LTBP-4, α -SMA, COL1A2, Elastin, and Fibronectin compared to controls. Primary fibroblasts derived from hemorrhoid tissues exhibited reduced migratory ability and downregulated key fibrotic markers. Overexpression of FBLN5 or LTBP-4 restored fibroblasts' viability and migration, while LTBP-4 knockdown attenuated the beneficial effects of FBLN5, highlighting their synergistic role in extracellular matrix remodeling.

Conclusion FBLN5 and LTBP-4 play significant roles in activating fibroblasts and contributing to the pathogenesis of hemorrhoid disease. Targeting these proteins could provide novel therapeutic approaches to enhance the outcomes of hemorrhoid treatment.

Keywords Hemorrhoid disease, Fibulin-5, LTBP-4, Fibroblast activation, Extracellular matrix, Therapeutic targets

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Introduction

In the United States, hemorrhoid disease is the fourth most common outpatient gastrointestinal diagnosis, accounting for around 3.3 million annual clinic visits, with over 10 million self-reported cases each year, affecting 4.4% of the population [1, 2]. The peak incidence occurs between the ages of 45 and 65, and is more common in Caucasians and individuals with higher socioeconomic status [3]. Factors like pregnancy, straining, and weakened supportive tissues elevate intra-abdominal pressure, thereby increasing the risk of symptomatic hemorrhoids [4]. Despite hemorrhoid disease having low morbidity, it significantly affects the quality of life. Management includes both surgical and nonsurgical treatments. Historically attributed to varicose veins, the pathogenesis is now understood as degeneration of the supportive structures in anal cushions, exacerbated by aging, strenuous activities, and prolonged sitting [5].

Fibroblast activation is essential for tissue repair and fibrosis, yet this mechanism appears to be impaired in hemorrhoid disease [6]. Histopathological examinations of hemorrhoidal tissues revealed abnormal venous dilation, vascular thrombosis, and degenerative changes in collagen and fibroelastic tissues [7, 8]. In severe cases, inflammation, mucosal ulceration, and ischemia further underscore the failure of fibroblast-mediated repair. Pathological displacement of anal cushions and venous dilation reflect inadequate fibroblast activation, compromising tissue integrity in hemorrhoidal disease [9, 10].

Recent research has identified Fibulin-5 (FBLN5) and Latent Transforming Growth Factor Beta Binding Protein 4 (LTBP-4) as key players in hemorrhoid pathogenesis [11, 12]. FBLN5, an extracellular matrix (ECM) glycoprotein, is essential for elastogenesis and structural integrity of connective tissues. Altered FBLN5 expression is linked to pathological vascular remodeling and inflammation [13]. Concurrently, LTBP-4 modulates the bioavailability and activation of transforming growth factor-beta (TGF- β), a key cytokine maintaining tissue homeostasis and facilitating repair processes. Dysregulation of LTBP-4 may result in aberrant TGF- β signaling, promoting abnormal angiogenesis and excessive fibrosis within hemorrhoidal tissues [14].

Both FBLN5 and LTBP-4 significantly impact fibroblast activation. FBLN5 regulates fibroblast activity by promoting elastin fiber assembly and cellular adhesion; its deficiency leads to abnormal fibroblast proliferation and matrix deposition [15]. Similarly, LTBP-4 influences the bioavailability and activation of TGF- β , a key cytokine in fibroblast activation. LTBP-4 levels can enhance TGF- β signaling, resulting in heightened fibroblast activity, excessive ECM production, and fibrosis [16, 17]. Together, these proteins orchestrate fibroblast-mediated tissue remodeling processes in hemorrhoid disease.

Importantly, FBLN5 and LTBP-4 interact structurally to facilitate elastic fiber organization. Specifically, the C-terminal domain of FBLN5 interacts with the N-terminal region of LTBP-4, enabling the coordinated deposition of elastin onto fibrillin-rich microfibril scaffolds [18]. Knockdown of LTBP-4 in fibroblasts disrupts elastin and FBLN5 deposition, confirming their cooperative role in ECM assembly [19]. This molecular synergy suggests that FBLN5–LTBP-4 interaction is central to fibroblast function and tissue homeostasis, highlighting a promising therapeutic axis for hemorrhoid intervention.

This study investigated how FBLN5 and LTBP-4 regulate fibroblast activation and jointly influence hemorrhoid disease pathogenesis, with the goal of identifying novel therapeutic targets for improving treatment outcomes.

Methods and materials

Patient recruitment and tissue collection

A total of 30 patients with hemorrhoids were recruited from the First Affiliated Hospital of GZUCM. All participants were ≥ 18 years old and were referred for surgical management of hemorrhoids. Hemorrhoid severity was assessed using the Goligher classification, and patients with grade II–IV disease who provided written informed consent were eligible. Exclusion criteria included: (1) a history of inflammatory bowel disease or malignant gastrointestinal disease within the past 5 years; (2) medical conditions rendering the patient unsuitable for surgery; (3) pregnancy; (4) any condition potentially interfering with clinical evaluation or tissue collection. During surgery, hemorrhoidal tissues were collected from the excised hemorrhoids. For the control group, adjacent non-lesional anal canal tissues were obtained from the same patients at a site proximal to the hemorrhoidal tissue. This paired-sample design minimizes inter-individual variability. All tissue samples were immediately processed for fibroblast isolation or snap-frozen in liquid nitrogen and stored at -80°C for subsequent molecular analysis.

Primary extraction of fibroblasts from hemorrhoidal tissue

Hemorrhoidal tissues were taken from patients with hemorrhoids using aseptic techniques and then sliced into small pieces using sterile scissors. The minced tissues were treated with a solution containing (0.1% collagenase I + 0.25% trypsin-EDTA) at 37°C for 1 h while gently stirring. Subsequent filtration through a $70\text{ }\mu\text{m}$ mesh filter ensured removal of residual tissue debris from the dissociated cell suspension. Subsequently, the mixture was then centrifuged at $300\times g$ for 10 min. The cell pellet was suspended in DMEM containing 10% FBS and antibiotics (Gibco, USA). The cells were cultivated in T25 flasks at 37°C in a humidified atmosphere with 5% CO_2 (Thermo Fisher Scientific, USA). Medium was replaced

every two days until confluence was achieved. Fibroblasts were distinguished based on their elongated, spindle-shaped appearance and verified through immunostaining for fibroblast-specific markers, including vimentin and α -smooth muscle actin (α -SMA).

Establishment of a rat hemorrhoid model

Animal Selection and Feeding: We acquired a group of 14 male Sprague-Dawley (SD) rats from Rigor Bioscience (Guangzhou, China). The rats were 8–10 weeks old and weighed 180 g to 220 g. The animals were kept in separate cages with ventilation following typical laboratory settings, maintaining 22 ± 2 °C and $55 \pm 10\%$ relative humidity. Participants were exposed to a 12-hour alternating pattern of light/dark cycle with ad libitum access to food and water. Before the experiment, all rats were allowed to acclimate to a regular diet for one week to establish consistent physiological conditions.

Induction of the hemorrhoid model: The rats were separated into two groups using random assignment: a control group of 7 rats and a model group of 7 rats. All surgical procedures were performed under anesthesia with 2% sodium pentobarbital (40 mg/kg, intraperitoneally). Following the application of 75% ethanol to the perianal area, a small amount (0.05 mL) of 75% glacial acetic acid was injected into the perianal skin using a syringe with a volume capacity of 0.1 mL. This glacial acetic acid–induced perianal ulceration model has been validated for hemorrhoid research, as it reproduces key pathological features such as inflammation, vascular permeability changes, and alterations in pain- and angiogenesis-related molecules [20]. Although it primarily reflects acute inflammatory injury, it provides a reliable and reproducible system for investigating fibroblast dysfunction, extracellular matrix remodeling, and tissue repair mechanisms relevant to hemorrhoid pathogenesis. Within 24 h, visible perianal ulceration was observed in the model group. The model was sustained for seven days, during which the rats were closely observed for clinical manifestations of hemorrhoids, including edema, inflammation, and bleeding. At the end of the experimental period, all rats were humanely euthanized by inhalation of an overdose of carbon dioxide.

Scratch test

To measure the fibroblast migration, cells were placed in 6-well plates (2×10^5 cells/well) and allowed to grow until they covered approximately 90% confluency. To minimize the influence of cell proliferation on wound closure, cells were serum-starved in DMEM containing 0.5% FBS for 12 h before scratching [21]. A sterile 200 μ L pipette tip was then used to introduce a linear abrasion on the layer of cells. Dislodged cells were removed by rinsing the wells twice using phosphate-buffered

saline (PBS; Sigma-Aldrich, USA). Subsequently, cells were maintained in serum-free DMEM (0% FBS) during the migration period to ensure that wound closure primarily reflected cell migration rather than proliferation. Scratch wound healing was recorded at 0 and 24 h with an Olympus inverted microscope. Each experiment was performed independently three times (biological replicates), with duplicate wells per condition.

Western blotting

Protein Extraction: Hemorrhoidal samples and fibroblast cells were disrupted in RIPA buffer (Beyotime, China), which included substances that prevent the breakdown of proteins by enzymes called proteases and phosphatases (Thermo Fisher Scientific, USA). The lysates were placed on ice for 30 min, then centrifuged at 14,000 rpm for 15 min at 4 °C. The supernatant proteins were collected, and their concentrations were determined using the bicinchoninic acid (BCA) assay.

Electrophoresis and Blotting: A 10% SDS-polyacrylamide gel (Bio-Rad, USA) was used to separate 30 μ g of protein samples, followed by electrophoretic transfer onto PVDF membranes (Millipore, USA). To prevent nonspecific binding, the membranes were incubated with 5% skim milk prepared in TBST (Tris-buffered saline containing 0.1% Tween-20, Sigma-Aldrich, USA) for one hour at ambient temperature. Following protein transfer, the membranes were probed with primary antibodies targeting FBLN5, LTBP-4, FN, COLIA2, Elastin, α -SMA, and GAPDH (loading control) for an extended period at 4 °C. Following the washing step with TBST, HRP-linked secondary antibodies were applied for 1 h at room temperature (detailed antibody information is provided in Table 1). Protein signals were observed by enhanced chemiluminescence (ECL) and measured using densitometry with ImageJ software. Images were first converted to an 8-bit grayscale format. For each protein band, a rectangular region of interest (ROI) was defined using the rectangle selection tool, maintaining consistent dimensions across all samples. An adjacent background region of equal size was also selected for each band. The integrated density of each band was measured, and the background signal was subtracted to obtain the corrected band intensity. The expression level of each target protein was normalized to the corresponding GAPDH loading control by calculating the ratio of target protein intensity to GAPDH intensity. Western blotting was performed using protein lysates from three independent biological replicates, and densitometric quantification was conducted in triplicate for each blot.

Real-time PCR (qRT-PCR)

RNA Preparation and cDNA Conversion: Total RNA from hemorrhoidal tissues and cultured fibroblasts

Table 1 Antibodies used in this study

Target Protein	Host Species	Application	Dilution	Manufacturer	Catalog No.	Validation Method
FBLN5	Rabbit	WB, IF, IHC	1:1000	Abcam (UK)	ab109428 ab66339 ab202977	Single band at ~ 50 kDa in WB; manufacturer-validated
LTBP-4	Rabbit	WB, IF	1:500	Bioss (China) Abcam (UK)	bs-5779R ab222844	Single band at ~ 220 kDa; literature validated
FN	Rabbit	WB	1:1000	Abcam (UK)	ab45688	Single band at ~ 263 kDa; manufacturer-validated
COL1A2	Rabbit	WB	1:1000	Abcam (UK)	ab308455	Expected MW ~ 125 kDa; manufacturer-validated
Elastin	Rabbit	WB	1:1000	Abcam (UK)	ab213720	Band ~ 85 kDa; literature validated
α -SMA	Rabbit	WB, IF, IHC	1:100	Abcam (UK)	ab124964	Single band at ~ 42 kDa; confirmed by literature
GAPDH	Rabbit	WB	1:1000	Abcam (UK)	ab181602	Single band at ~ 36 kDa; widely validated
HRP-linked anti-rabbit IgG)	Goat	WB, IHC	1:1000	Abcam (UK)	ab6721	Manufacturer validated

WB Western blotting, IHC Immunohistochemistry, IF Immunofluorescence

Table 2 mRNA (Human) primer pairs used in recent study

Gene Name (H)	Forward Primer (5'–3')	Reverse Primer (5'–3')
FBLN5	TCGCTATGGTTACTGCCAGCA	TTGGCAAGACCTCCATCGTC
LTBP4	GTGTACACTATGCCACTGGC-CAAC	CACGTGGACGCTCAC-CATAGA
FN1	TGGGCAACTCTGTCAACGAA	GAGCAATGGCACCGAGATA
COL1A2	TCTTGGTCAGTCCTATGCGGATA	CATCGCAGAGAACGGATCCT
ELN	CAGCTAAATACGGTGCTGCTG	AATCCGAAGCCAGGTCTTG
ACTA2	GGGACATCAAGGAGAACT-GTGT	TCTCTGGGCGCGGAAAC
GAPDH	GCACCGTCAAGGCTGAGAAC	TGGTGAAGACGCCAGTGGA

Table 3 mRNA (Rat) primer pairs used in recent study

Gene Name (R)	Forward Primer (5'–3')	Reverse Primer (5'–3')
FBLN5	CGCTGTGACCCAGGATATG	TCGGAGAAGCTGCACTCAT
LTBP4	TGCTGTACTGTGGGAGAAGG	TTCATCCACATCTCTCCGGG
FN1	GTGATCTACGAGGGACAGC	GCTGGTGGTGAAGTCAAAG
COL1A2	CAGAGCACCATTTTCCAAAGCA	GGTACAGAGTCTCTT-GCTTCCT
ELN	TCCCGGTGGAGTCTATTATCCAG	GAAGCATAGGAGAGACCT-GCC
ACTA2	GGATCAGCGCCTTCAGTTCT	AGGGCTAGAAGGGTAGCACA
GAPDH	CCTTCGTGTCTACCCCC	TAGCCAGGATGCCCTTTAG

was extracted using TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions. The RNA concentration and purity of the RNA were evaluated using a NanoDrop spectrophotometer, and integrity was assessed by electrophoresis on a 1% agarose gel. Using 1 μ g of total RNA, first-strand cDNA was synthesized with a reverse transcription kit (Takara, Japan) according to the manufacturer's instructions, employing oligo (dT) primers.

PCR Amplification: qRT-PCR was performed in 20 μ L reactions containing SYBR Green Master Mix, 1 μ L cDNA template, and 0.5 μ M gene-specific primers (FBLN5, LTBP-4, or GAPDH reference) using a commercial kit. Thermal cycling on a real-time PCR instrument was initiated with 95 °C for 10 min, followed by 40 cycles of 95 °C (15 s) and 60 °C (1 min). Post-amplification melting curve analysis confirmed product specificity. Relative expression levels normalized to GAPDH were quantified via the $2^{-\Delta\Delta C_t}$ method. qRT-PCR assays were performed in triplicate for each biological sample, with at least three independent biological replicates. Primer sequences are detailed in Tables 2 and 3.

Cellular Immunofluorescence

Fibroblasts were incubated overnight on glass coverslips in 24-well plates at a concentration of 5×10^4 cells/well. Cells underwent fixation with 4% paraformaldehyde for 15 min at room temperature for immobilization, followed by permeabilization using 0.1% Triton X-100 for 10 min to allow the substances to pass through the cell membrane. Subsequently, A 1-hour blocking step using 5% BSA in PBS to prevent nonspecific binding. Primary antibodies targeting FBLN5, α -SMA, and LTBP-4 (diluted in 1% BSA/PBS) were applied, and an extended period at 4°C. After PBS washing, specimens underwent 1-hour dark incubation at room temperature with fluorophore-conjugated secondary antibodies. Nuclei staining was employed with DAPI (4',6-diamidino-2-phenylindole; Beyotime, China). Following DAPI staining, slides were coverslipped in antifade medium, and images were captured with a fluorescence microscope. Immunofluorescence staining was repeated three times using independent fibroblast cultures, and representative images are shown.

Immunohistochemistry

Paraffin-embedded hemorrhoidal tissue slices (5 μ m) underwent deparaffinization in thick xylene to remove the paraffin and were then gradually rehydrated using graded ethanol. The antigen retrieval employed microwave heating in citrate buffer (pH 6.0, 20 min). After cooling to room temperature, endogenous peroxidase activity was suppressed by exposure to a 3% H₂O₂ (10 min). Endogenous binding sites were saturated with 5% BSA (w/v) in PBS, 1 h at 22 °C. Subsequently, the sections were then exposed to primary antibodies targeting FBLN5 and α -SMA and incubated overnight at 4 °C. Following PBS washes, HRP-conjugated secondary antibodies were applied. Chromogenic development used 3,3'-DAB as the chromogen and stained with hematoxylin as a counterstain. Ultimately, sections were dehydrated, clarified using xylene, affixed with coverslips, and imaged using a light microscope.

Hematoxylin and Eosin (H&E) staining

Sections of hemorrhoidal tissue encased in paraffin (5 μ m in thickness) were treated to remove the paraffin and restore hydration following a previously described procedure. The sections were stained with hematoxylin (Beyotime, China) for 5 min, followed by rinsing under flowing tap water. The differentiation process was accomplished by immersing the sections in a solution of 1% acid alcohol for a brief period, then rinsing again in flowing water. Sections were then treated with 0.2% ammonia water to enhance nuclear blue staining and washed thoroughly. Subsequently, after 2-minute eosin staining, tissue processing included graded ethanol dehydration, xylene clearing, and glass mounting. Microstructural changes were characterized by bright-field microscopy.

CCK-8 assay

Fibroblast viability was assessed via Cell Counting Kit-8 (CCK-8) test. Briefly, cells (5,000/well) were placed in 96-well plates and incubated for 24 h. Afterwards, after adding 10 μ L of CCK-8 solution per well, plates were placed in an incubator at 37 °C for 2 h. Absorbance at 450 nm was measured with a microplate reader. Each CCK-8 assay was repeated in triplicate (three independent biological replicates). Viability percentages, calculated by comparison to that of the control wells, are expressed as mean $\pm$ SD ($n = 3$).

Plasmid transfection

Fibroblasts were cultured in 6-well plates (2×10^5 cells/well) and incubated overnight to promote attachment. All transfections were performed according to the manufacturer's instructions. For overexpression experiments, cells were transfected with either a plasmid containing the full-length cDNA for human Fibulin-5 (OE-FBLN5)

or LTBP-4 (OE-LTBP4), or an empty vector plasmid as a negative control (OE-NC). Plasmids were purified using an EndoFree Plasmid Mini Kit (Tiangen, China). Transfection was performed by incubating cells with a mixture of plasmid DNA and Lipofectamine™ 3000 reagent (Invitrogen, USA) in Opti-MEM™ medium. For knockdown experiments, cells were transfected with either a small interfering RNA (siRNA) specifically targeting human LTBP-4 (si-LTBP4) or a non-targeting scrambled siRNA as a negative control (si-NC). siRNA transfection was performed using Lipofectamine™ RNAiMAX reagent (Invitrogen, USA) in Opti-MEM™ medium. Following a 6-hour incubation for both plasmid and siRNA transfections, the medium was exchanged for complete growth media. The efficiency of overexpression or knockdown was assessed 48 h post-transfection via Western blot.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 9.0 (San Diego, CA, USA). Data are expressed as the mean $\pm$ SEM. Comparisons between two groups used an unpaired Student's *t*-test. Multi-group analyses utilized one-way/two-way ANOVA with Tukey's post hoc testing where applicable. A *p*-value < 0.05 was considered statistically significant. Quantification of imaging data was carried out using ImageJ software (NIH, Bethesda, MD, USA). For in vitro experiments, all assays were performed with at least three independent biological replicates (fibroblasts derived from distinct patient samples or passages), each containing three technical replicates. For animal experiments, each group consisted of seven rats ($n = 7$).

Results

Reduced expression of fibroblast activation markers and ECM components in hemorrhoid tissues

Tissue specimens were obtained from patients who were diagnosed with hemorrhoids, with matched non-lesional anal canal tissue serving as controls. To examine the molecular mechanisms responsible for these changes, we examined the protein and gene expression levels of various important markers: FBLN5, LTBP-4, α -smooth muscle actin (α -SMA), COL1A2, Elastin, and fibronectin (FN), which are crucial markers associated with fibroblast activity and ECM remodeling. As shown in Fig. 1A, Western blot demonstrated a clear reduction in the protein expression levels of all six markers in hemorrhoidal tissues relative to controls. Densitometric quantification (Fig. 1B) confirmed statistically significant decreases in protein levels of FBLN5, LTBP-4, FN, COL1A2, Elastin, and α -SMA in the hemorrhoid group ($p < 0.01$). qRT-PCR (Fig. 1C) demonstrated that mRNA levels of these genes were similarly downregulated, corresponding to the protein-level alterations ($p < 0.01$). This indicates a significant

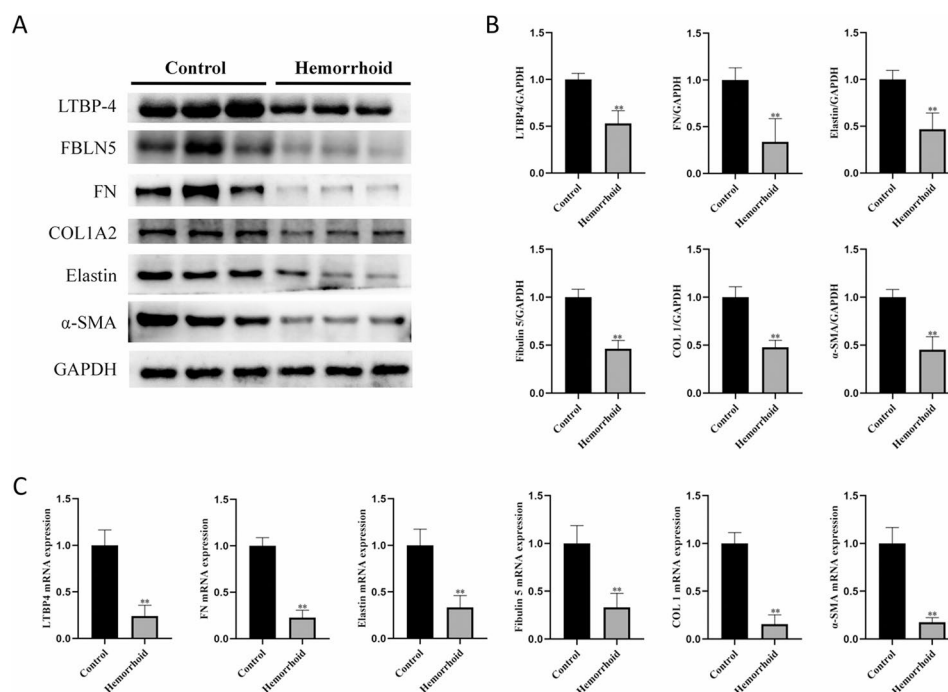


Fig. 1 Reduced expression of FBLN5 and fibroblast activation markers in hemorrhoid tissues. **A** Representative Western blot showing protein expression of LTBP-4, FBLN5, FN, COL1A2, Elastin, and α-SMA in hemorrhoidal versus control tissues. Molecular weight markers are indicated on the right. **B** Densitometric quantification of Western blot bands normalized to GAPDH. **C** qRT-PCR analysis of FBLN5, LTBP-4, α-SMA, COL1A2, Elastin and FN mRNA expression. Data are presented as mean ± SEM from $n = 7$ patients per group (biological replicates), with each sample analyzed in triplicate (technical replicates). Statistical significance was determined by unpaired Student's *t*-test. ** $p < 0.01$ vs. Control group

reduction in the activation of fibroblasts and the creation of an ECM in hemorrhoid tissues. The decrease in the expression of these markers in hemorrhoid tissues suggests that disturbances in FBLN5 may be implicated in the onset of hemorrhoids.

Rat hemorrhoid tissues exhibit histopathological remodeling with suppressed fibroblast activation markers

The anal wound tissue of the rat hemorrhoid model was subjected to H&E staining to examine the histological differences between the model and control groups. As shown in Fig. 2A, acetic acid-treated tissues exhibited prominent inflammatory infiltration, disorganized cellular arrangement, and glandular hyperplasia in both the epidermal and dermal layers. Additionally, vascular dilation and thickening of vessel walls were observed, with the possible presence of histopathologic features such as venous stasis associated with pathogenic mechanisms.

Additional molecular analysis was performed to measure the protein and gene expression levels of FBLN5, LTBP-4, α-SMA, Elastin, COL1A2, and FN. The results indicated a significant decrease in the expression levels of these proteins and genes in the hemorrhoid model group compared to those in the control group ($p < 0.01$) (Fig. 2B–D). Immunohistochemistry further assessed the expression of FBLN5 and α-SMA in tissue samples (Fig. 2E–F). Immunohistochemical analysis confirmed

the Western blotting and qPCR results, demonstrating reduced staining of FBLN5 and α-SMA in the model tissues versus control tissues. This finding provides additional evidence that fibroblast activation and synthesis of the ECM are decreased in the hemorrhoid model, and that abnormalities in the pathways involving these proteins may have a crucial impact on the formation of hemorrhoids.

Hemorrhoid-Derived fibroblasts exhibit impaired proliferation, migration, and ECM marker expression

Fibroblasts were isolated and derived from clinical hemorrhoid specimens and compared with those obtained from adjacent non-hemorrhoidal tissues. Using the CCK-8 assay, we observed a significant decline in fibroblast activity from hemorrhoid samples ($p < 0.01$) (Fig. 3A). The scratch test was utilized to assess the migratory capability of fibroblasts, which revealed significantly reduced migration in fibroblasts from hemorrhoid patients relative to controls (Fig. 3B).

The protein and gene expression levels of FBLN5, LTBP-4, α-SMA, COL1A2, Elastin, and FN were measured in fibroblasts from both normal control and hemorrhoid tissues using Western blotting and qPCR. Expression of these markers was significantly reduced in fibroblasts derived from hemorrhoid tissues ($p < 0.05$) (Fig. 3C–E). Immunocytochemical staining further

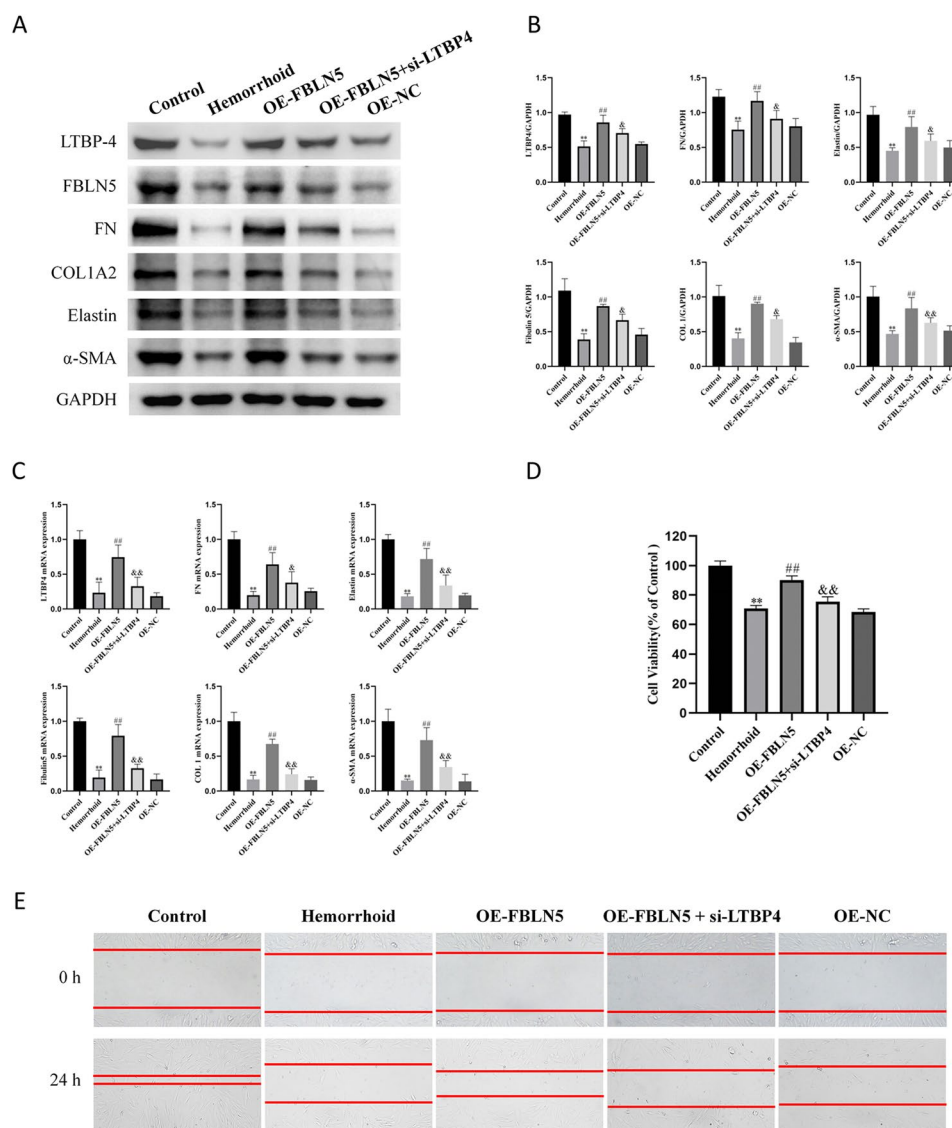


Fig. 2 Analysis of tissue pathology and reduced fibroblast marker expression in the hemorrhoid model. **A** H&E staining showing inflammatory infiltration, disorganized epidermal/dermal architecture, and vascular dilation in hemorrhoid tissues versus controls (100×). These images reflect typical findings observed in multiple subjects from each group ($n=5$). **B** qRT-PCR analysis of FBLN5, LTBP-4, α-SMA, COL1A2, Elastin, and FN mRNA expression levels normalized to GAPDH. **C** Representative Western blot images showing protein expression of LTBP-4, FBLN5, FN, COL1A2, Elastin, and α-SMA in acetic acid-treated versus control rat perianal tissues. **D** Quantification of Western blot band intensities normalized to GAPDH. **E, F** Immunohistochemical staining confirms decreased FBLN5 and α-SMA protein levels (200×). Images are representative of several individuals from each group ($n=5$). Data are presented as mean $\pm$ SEM ($n=3$, biological replicates), with each sample analyzed in triplicate for qRT-PCR and Western blot (technical replicates). Statistical significance was determined by unpaired Student's *t*-test. ** $p < 0.01$ versus Control group

confirmed these findings by demonstrating reduced staining intensity for FBLN5 and α-SMA proteins in hemorrhoid-derived fibroblasts (Fig. 3F). Overall, these data indicate that fibroblasts from hemorrhoidal tissues exhibit impaired activation, reduced ECM production, and migratory capability. Such functional impairments may hinder tissue repair and compromise structural integrity, thereby contributing to the pathogenesis of hemorrhoid disease.

FBLN5 rescues fibroblast function and ECM marker expression in hemorrhoid

Fibroblasts derived from hemorrhoid tissues were transfected with a plasmid to overexpress FBLN5. The cells were divided into four groups: control group, hemorrhoid group, hemorrhoid fibroblasts transfected with FBLN5 overexpression plasmid (OE-FBLN5), and hemorrhoid fibroblasts transfected with empty vector (OE-NC). The efficacy of FBLN5 overexpression was verified using Western blotting and qPCR (see the FBLN5 expression, $p < 0.01$), showing significantly elevated FBLN5

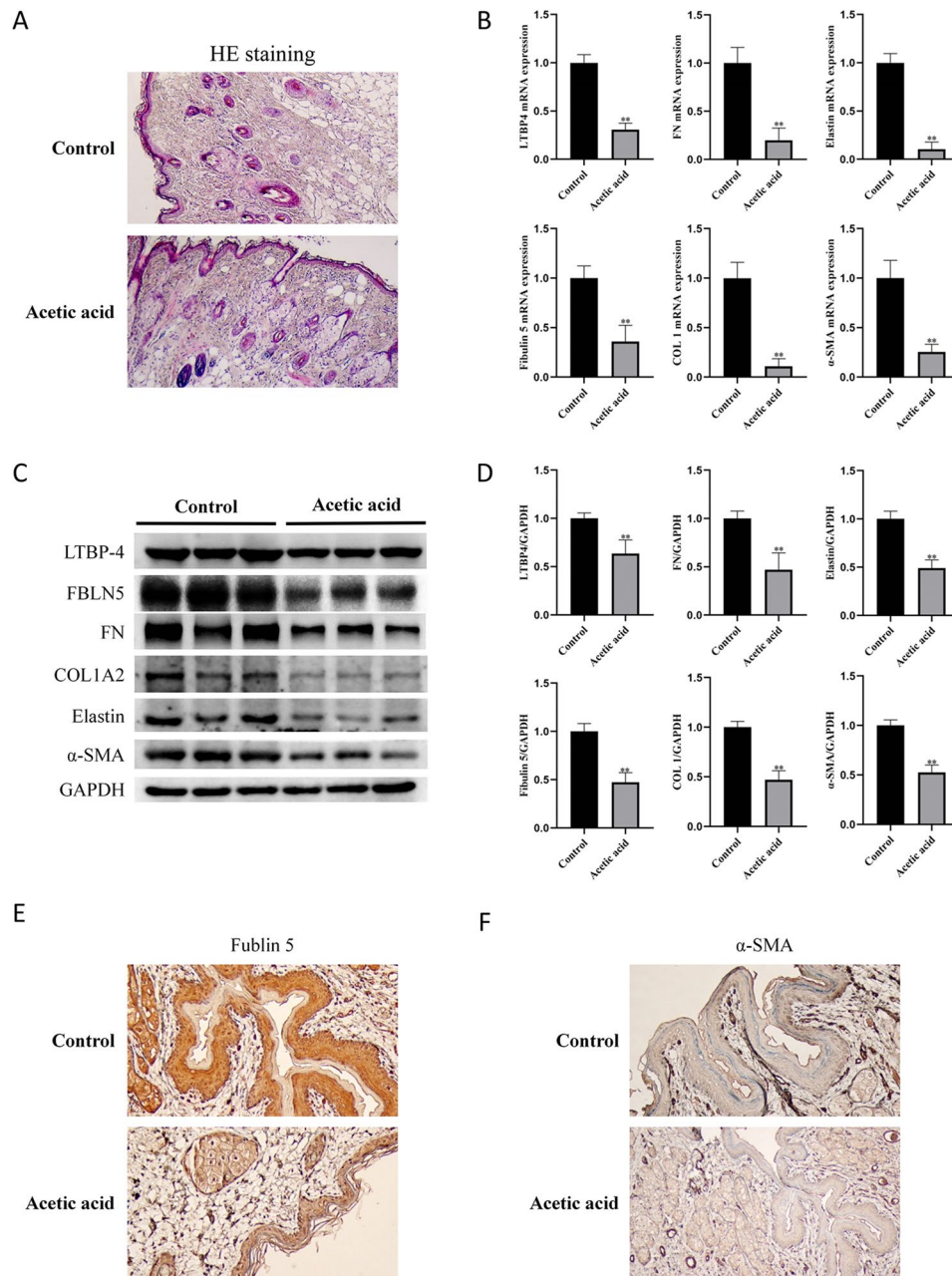


Fig. 3 Dysfunctional fibroblasts from hemorrhoid tissues show reduced viability and marker expression. **(A)** CCK-8 assay showing decreased cell viability in hemorrhoid fibroblasts. **(B)** Scratch assay demonstrating impaired migratory capacity in hemorrhoid fibroblasts (100 $\times$). Images are representative of several individuals from each group ($n = 3$). **(C-E)** Western blot **(C, D)** and qPCR **(E)** quantification of FBLN5, LTBP-4, α -SMA, COL1A2, Elastin, and FN expression showing decreased gene and protein expression in hemorrhoid fibroblasts. **F** Immunocytochemical staining confirms reduced FBLN5 and α -SMA protein levels (400 $\times$). Data are presented as mean $\pm$ SEM from at least three independent experiments using cells from different donors (biological replicates). Statistical significance was determined by an unpaired Student's t-test. * $p < 0.05$, ** $p < 0.01$ versus Control group

expression in OE-FBLN5 cells relative to OE-NC. Next, the protein and gene expression levels of FBLN5, LTBP-4, α -SMA, COL1A2, Elastin, and FN were evaluated in fibroblasts from all four groups using Western blotting and qPCR ($p < 0.05$). As shown in Fig. 4A-C, protein and gene levels were markedly lower in hemorrhoid samples relative to controls. Conversely, FBLN5 overexpression

reinstated their expression. Subsequently, an immunocytochemistry assay further validated the results that FBLN5 overexpression enhanced the expression of FBLN5 and α -SMA (Fig. 4D). Functional assays showed that FBLN5 overexpression significantly improved fibroblast viability and migratory ability, as determined by the

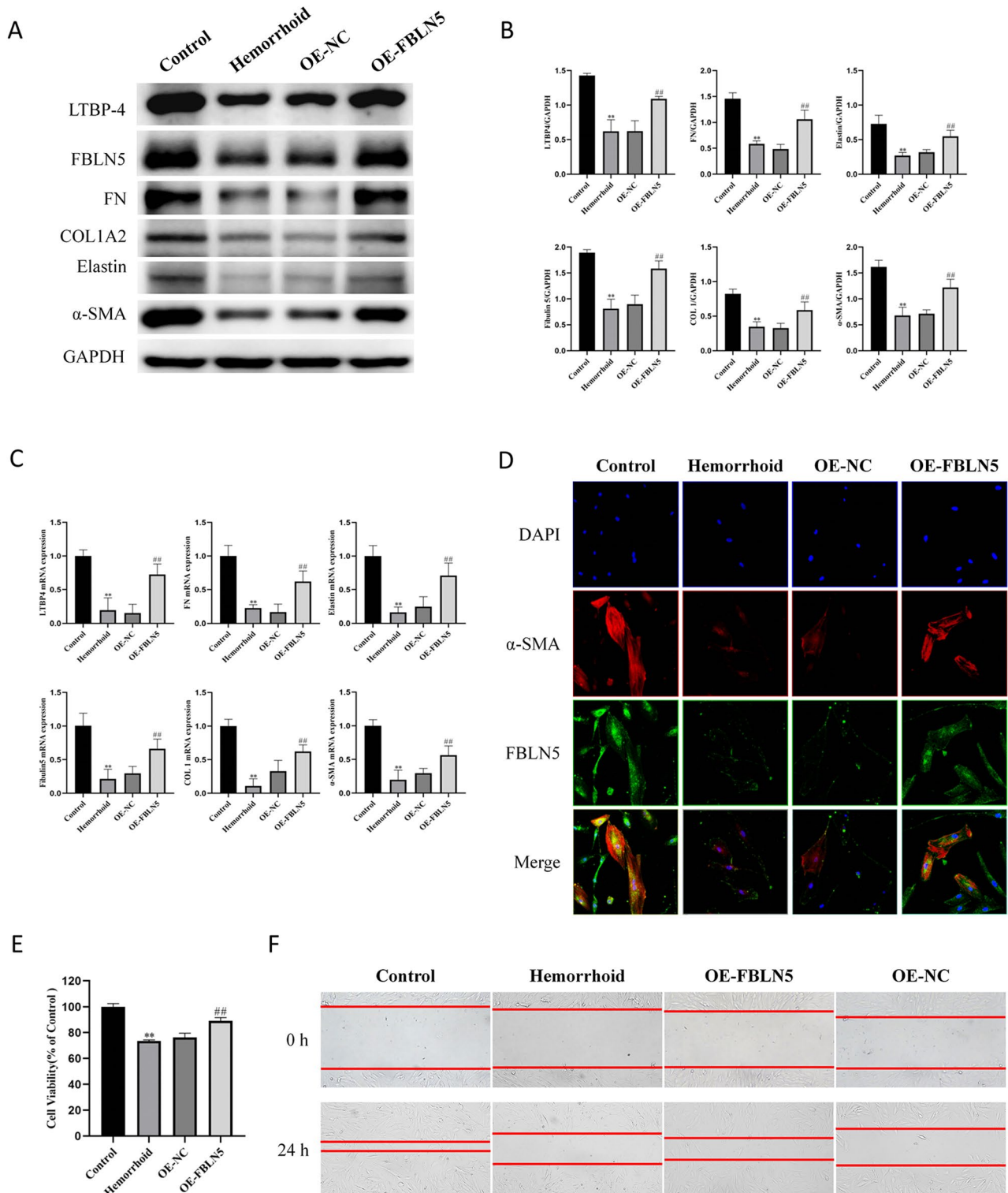


Fig. 4 Rescue of fibroblast function and ECM markers by FBLN5 overexpression. **A–C** Western blot (**A**, **B**) and qPCR (**C**) validation of FBLN5 overexpression efficacy and associated upregulation of LTBP-4, α-SMA, COL1A2, Elastin, and FN. **D** Immunocytochemical staining confirms enhanced FBLN5 and α-SMA expression in the OE-FBLN5 group (400×). Representative images were obtained from three individuals per group ($n = 3$). **E**, **F** CCK-8 (**E**) and scratch assay (**F**) demonstrating restored cell viability and migration in the OE-FBLN5 group versus the Control group (100×). Data are presented as mean $\pm$ SEM from three independent experiments. Statistical significance was determined by one-way ANOVA with Tukey's post-hoc test. ** $p < 0.01$ versus Control group; ## $p < 0.01$ versus OE-NC group

CCK-8 assay ($p < 0.01$) and scratch wound healing test, respectively (Fig. 4E-F).

These data demonstrate that higher FBLN5 expression in fibroblasts supports improved viability and migration, which may help alleviate some of the functional deficiencies observed in fibroblasts from individuals with hemorrhoids. This highlights its potential as a therapeutic target for restoring fibroblast function and the development of hemorrhoids.

LTBP-4 reverses Hemorrhoid-Associated fibroblast dysfunction

To explore how LTBP-4 regulates fibroblast dysfunction associated with hemorrhoids, fibroblasts isolated from hemorrhoid patients were transfected to overexpress LTBP-4. The cells were allocated to four distinct groups: Control group, hemorrhoid group, LTBP-4 overexpression group (OE-LTBP-4), and empty vector control group (OE-NC). The effective amplification of the LTBP-4 protein was verified using Western blotting and qPCR (see the LTBP-4 expression, $p < 0.01$). Fibroblasts from hemorrhoid tissues showed markedly lower FBLN5, LTBP-4, α -SMA, COL1A2, Elastin, and FN expression at both protein and gene levels relative to controls ($p < 0.01$) (Fig. 5A–C). However, overexpression of LTBP-4 in the OE-LTBP4 group markedly restored the expression of these ECM and activation markers ($p < 0.01$). This finding was further confirmed by immunostaining that LTBP-4 overexpression increased the expression of LTBP-4 and α -SMA (Fig. 5D). These results suggest that increasing the expression of LTBP-4 can reverse the decrease in protein levels observed in fibroblasts from patients with hemorrhoids. Functionally, the patterns observed in terms of cell survival ($p < 0.01$) and migratory ability were consistent with the levels of protein expression (Fig. 5E-F). The group that overexpressed LTBP-4 showed enhanced cell survival and migration in comparison to the hemorrhoid group, consistent with the molecular data.

The results indicate that increased expression of LTBP-4 improves the functionality of fibroblasts by enhancing their ability to survive and move, which may help restore impaired fibroblast function in individuals with hemorrhoids. Highlighting its potential as a therapeutic target for modifying fibroblast function and ameliorating hemorrhoid pathogenesis.

LTBP-4 mediates FBLN5-Dependent ECM restoration and functional recovery in hemorrhoid fibroblasts

To confirm LTBP-4 mediates the effects of FBLN5 in hemorrhoid pathogenesis, fibroblasts isolated from hemorrhoid tissues underwent genetic modification to induce FBLN5 overexpression. The cells were divided into five groups: control group, hemorrhoid group, OE-FBLN5

group, OE-FBLN5 + siRNA-LTBP-4 group, and OE-NC group. Western blotting verified the effective suppression of LTBP-4 in the siRNA group (Figure S1). Further investigations were conducted to examine the protein and gene expression levels of FBLN5, LTBP-4, α -SMA, COL1A2, Elastin, and FN in fibroblast samples from the five groups. As depicted in Fig. 6A–C, restoring FBLN5 expression led to a significant recovery of marker levels in hemorrhoidal fibroblasts, which had been markedly downregulated compared with controls ($p < 0.01$). Nevertheless, when LTBP-4 was simultaneously silenced, these restorative effects were largely abrogated, reverting expression levels to those observed in the hemorrhoid group ($p < 0.05$).

Functional assays showed consistent trends: FBLN5 overexpression enhanced fibroblast viability ($p < 0.01$) and migration (Fig. 6D-E), whereas LTBP-4 knock-down reversed these improvements ($p < 0.01$). Together, these results indicate that LTBP-4 facilitates the positive impacts of FBLN5 overexpression on fibroblast function and has the potential to improve hemorrhoid pathology. The interplay between FBLN5 and LTBP-4 appears critical in hemorrhoid pathophysiology, highlighting it as a candidate for targeted treatment.

Discussion

Aging-related degradation of the extracellular matrix (ECM) has been proposed as a key contributor to hemorrhoidal disease. FBLN5, a structural ECM glycoprotein, collaborates with LTBP-4 to regulate ECM remodeling and maintain tissue integrity. The objective of our research was to investigate the essential roles of FBLN5 and LTBP-4 in fibroblast activation and ECM reconstruction, thereby elucidating their involvement in the pathogenesis of hemorrhoids. Our study revealed a significant reduction in FBLN5 and LTBP-4 expression in hemorrhoidal tissues, correlating with decreased fibroblast activity and ECM. These molecular changes were consistent with the typical histological characteristics observed in hemorrhoids, such as aberrant dilatation of veins, formation of blood clots in blood vessels, and degeneration of collagen. The decreased expression of FBLN5 and LTBP-4 indicates a weakened fibroblast-mediated healing mechanism in hemorrhoid illness [22–24].

Hemorrhoids exhibit degenerative connective tissue changes, including deterioration of collagen and elastic fibers, which are fundamental to the structural integrity of the anal cushion [25, 26]. Lohsiriwat et al. comprehensively reviewed the pathophysiology of hemorrhoids and highlighted that several enzymes and mediators involved in the degradation of supporting tissues in the anal cushions have been implicated in disease progression [27]. Among these, matrix metalloproteinases (MMPs),

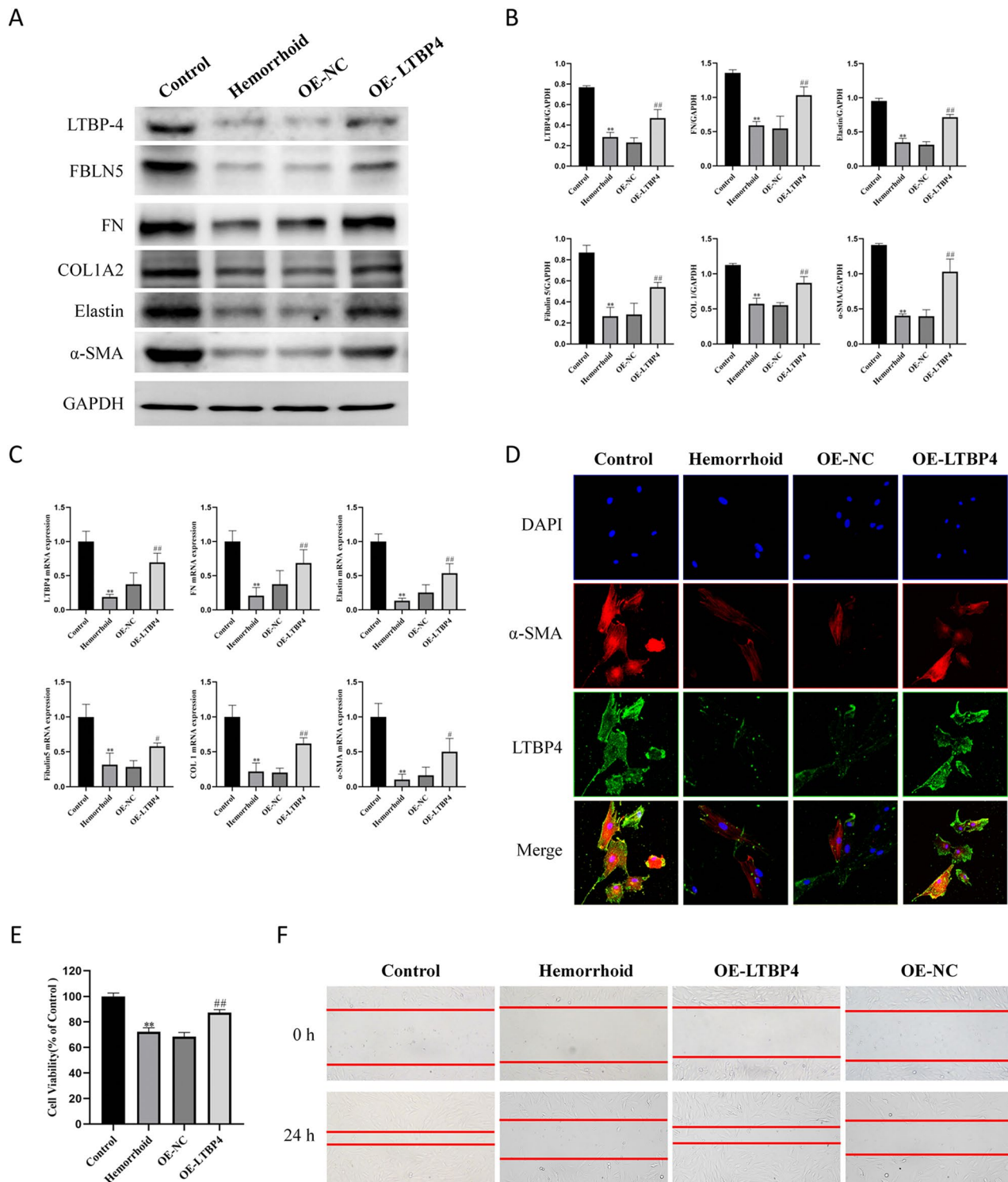


Fig. 5 Functional recovery of hemorrhoid fibroblasts by LTBP-4 overexpression. **A–C** Western blotting (**A**, **B**) and qPCR (**C**) validation of LTBP-4 overexpression efficacy and associated upregulation of FBLN5, α -SMA, COL1A2, Elastin, and FN. **D** Immunocytochemical staining confirms enhanced LTBP-4 and α -SMA expression in the OE-LTBP-4 group (400 $\times$). Representative images were obtained from three individuals per group ($n=3$). **E**, **F** CCK-8 (**E**) and scratch assay (**F**) demonstrating improved cell viability and migration in the OE-LTBP-4 group compared to the Control group (100 $\times$). Data are presented as mean $\pm$ SEM from three independent experiments. Statistical significance was determined by one-way ANOVA with Tukey's post-hoc test. ** $p < 0.01$ versus Control group; # $p < 0.05$, ## $p < 0.01$ versus OE-NC group

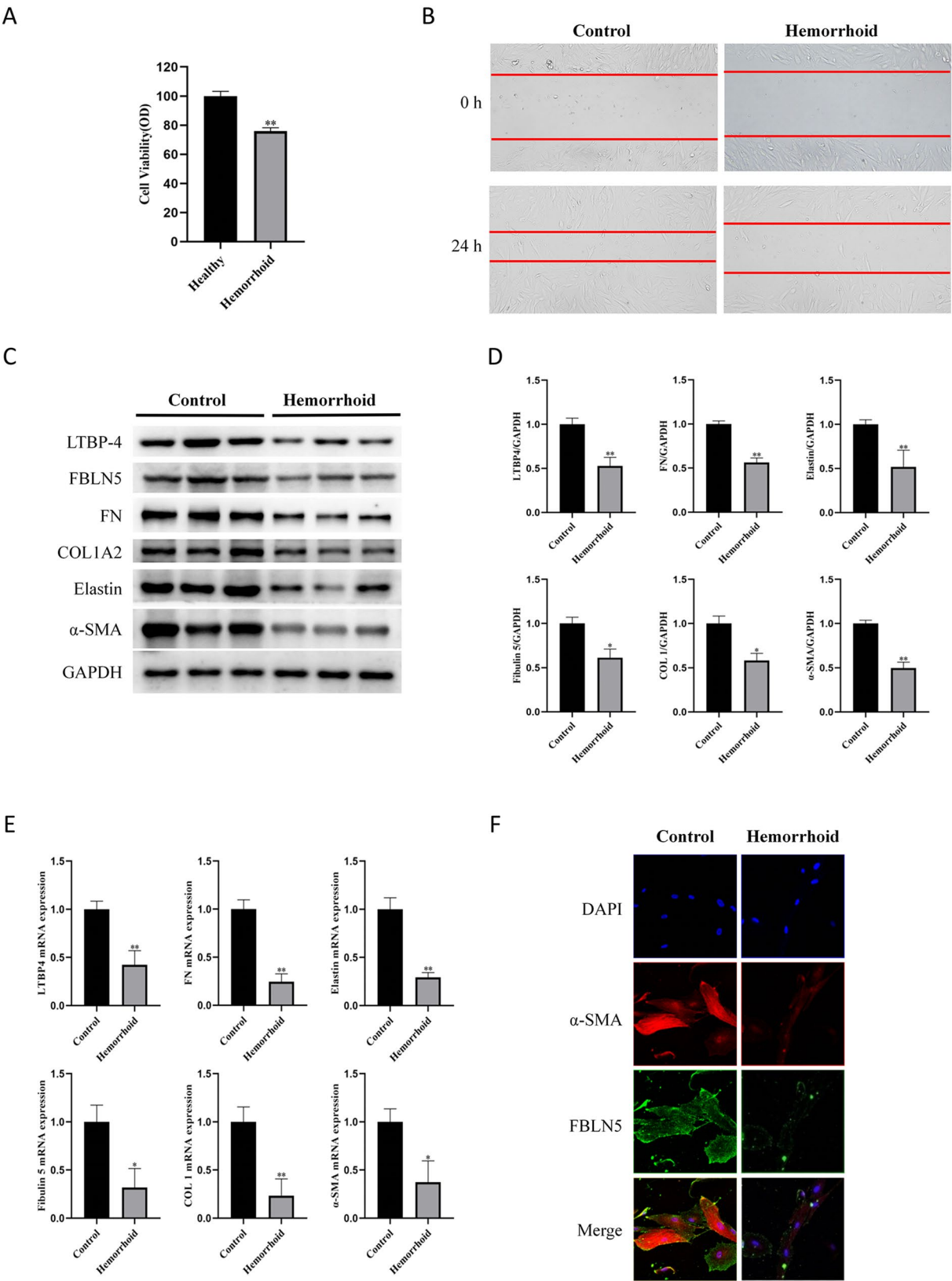


Fig. 6 (See legend on next page.)

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Fig. 6 LTBP-4 is required for FBLN5-induced fibroblast functional recovery. **A–C** Western blot (**A, B**) and qPCR (**C**) analysis showing LTBP-4 knockdown abolishes FBLN5-induced upregulation of α -SMA, COL1A2, Elastin, and FN. **D, E** CCK-8 (**D**) and scratch assay (**E**) demonstrating LTBP-4-dependent rescue of cell viability and migration (100 $\times$). Data are presented as mean $\pm$ SEM from three independent experiments. Statistical significance was determined by two-way ANOVA with Sidak's multiple comparisons test. ** $p < 0.01$ versus Control group; ### $p < 0.01$ versus OE-NC group; & $p < 0.05$, && $p < 0.01$ versus OE-FBLN5 group

particularly MMP-7 and MMP-9, have been detected at elevated levels in hemorrhoidal tissues compared to normal anal cushions, reflecting abnormal ECM degradation activity [28]. Margetis (2019) further demonstrated that the relaxed and hypertrophied connective tissue loses its capacity to support the vascular network, resulting in passive congestion of sinusoids [29]. These alterations suggest that the ECM dynamic imbalance may be a key mechanism in hemorrhoid pathogenesis. Our study revealed dysfunction of fibroblast activation and remodeling of the ECM in patients with hemorrhoid illness. These findings support the idea pivotal role of fibroblast-related proteins in the development of hemorrhoids [24, 30, 31].

TGF- β , a key cytokine in tissue repair and fibrosis, promotes fibroblast proliferation and differentiation into α -SMA-positive myofibroblasts, enhancing fibronectin production and collagen deposition. Dysregulated TGF- β expression is central to fibrosis pathogenesis. Notably, LTBP-4 modulates the secretion of latent TGF- β as well as the deposition of ECM, thereby modulating TGF- β /SMAD signaling. Previous studies showed that LTBP-4 knockdown suppresses this pathway, leading to reduced collagen expression [32]. Regarding the molecular mechanism, Noda et al. demonstrated through direct protein-protein interaction studies that LTBP-4 physically binds to fibulin-5, and this interaction is essential for the incorporation of elastin-fibulin-5 complexes into microfibril bundles during elastic fiber assembly [19]. Dabovic et al. further elucidated that LTBP-4 regulates the rate of incorporation of elastin-fibulin-5 complexes into the microfibril bundles, and that elastin-fibulin-5 aggregates accumulate in the absence of LTBP-4, leading to defective elastogenesis [33]. Therefore, the mechanism by which LTBP-4 mediates FBLN5 function involves both direct protein-protein interaction for elastic fiber assembly and indirect regulation through TGF- β /SMAD signaling for collagen synthesis. In addition, LTBP-4 can bind directly to FN. Willis et al. reported that an imbalance in the collagen-to-protein ratio compromises connective tissue stability, potentially contributing to the development of hemorrhoids [34]. Consistent with these findings, our study indicates that decreased expression levels of α -SMA, COL1A2, and fibronectin in hemorrhoidal tissues result in compromised fibroblast activation and ECM remodeling. This finding aligns with prior studies suggesting that fibroblast activation plays a vital role in tissue healing and fibrosis. Histopathological analysis

further supported these molecular changes, revealing abnormally dilated veins, intravascular thrombosis, deterioration of collagen and fibroelastic tissues. This finding emphasizes the importance of fibroblast dysfunction in the development of hemorrhoids [23, 35, 36].

Vascular abnormalities are also central to hemorrhoid pathology. Aigner et al. emphasized the vascular characteristics of hemorrhoids, explicitly addressing the imbalances in arterial and venous blood flow that led to dilatation of the hemorrhoidal plexus and the degradation of connective tissue. Our research confirmed that the key proteins FBLN5 and LTBP-4, which are essential for maintaining vascular and connective tissue integrity, are significantly downregulated in hemorrhoidal tissues [37]. Our study corroborates this vascular-connective interaction by showing that FBLN5 and LTBP-4—key proteins for vascular and ECM stability—are significantly reduced in hemorrhoidal tissues. In vitro upregulation of these proteins restored fibroblast survival and migration, underscoring their therapeutic potential. Similarly, Azeemuddin et al. and Tripathi et al. demonstrated that herbal treatments alleviated hemorrhoid symptoms, reduced inflammation and vasodilation, and enhanced tissue repair, supporting the notion that improving fibroblast activity and ECM integrity is central to disease recovery [36, 38, 39].

These findings collectively suggest that the FBLN5–LTBP-4–TGF- β axis is a promising therapeutic target. Modulation of TGF- β signaling—through receptor agonists, monoclonal antibodies, or ligand traps currently under clinical evaluation for fibrotic diseases—could potentially restore ECM synthesis and tissue integrity in hemorrhoidal tissues [40]. In addition, strategies promoting elastic fiber assembly, such as exogenous or gene-based FBLN5 supplementation, may reinforce ECM resilience [41]. Given the high recurrence rates of the disease and its resistance to current treatments, developing intervention strategies based on these proteins holds substantial clinical significance. Our results indicate that modulating FBLN5/LTBP-4-mediated fibroblast activation and ECM synthesis offers valuable perspectives on the pathophysiology of hemorrhoids and offers a theoretical basis for targeted therapies [42, 43].

Nevertheless, this study has several limitations. First, the glacial acetic acid-induced rat model, although effective in replicating key pathological features such as inflammation, vascular dilation, and ECM disruption, primarily represents an acute inflammatory injury rather

than the chronic degenerative process typical of human hemorrhoids [44, 45]. Therefore, our model primarily reflects the acute exacerbation phase rather than the chronic baseline state. Future studies employing genetic models (FBLN5/LTBP-4 knockout), long-term venous hypertension, or aging models are needed to mimic chronic remodeling and fibroblast senescence better. Second, while our sample sizes ($n = 7$ for animal studies) are consistent with similar mechanistic studies and yielded large, statistically significant effect sizes for our primary outcomes, a formal a priori power analysis was not conducted. Consequently, some secondary comparisons may have been underpowered, and these findings should be interpreted as exploratory. Future studies with larger cohorts, guided by formal power calculations, are warranted to confirm these secondary findings. Finally, the FBLN5–LTBP-4 axis, while central to our findings, likely interacts with other pathogenic mechanisms. Elevated inflammatory cytokines (IL-1 β , IL-6, TNF- α), dysregulated VEGF signaling, and mechanical or hemodynamic stresses may further impair fibroblast function and ECM integrity [46, 47]. Age-related changes and genetic polymorphisms in collagen or MMP genes could also influence disease susceptibility [48]. Integrating these factors with the FBLN5–LTBP-4 pathway will be essential to develop a more comprehensive understanding of hemorrhoid pathophysiology and guide the design of more effective therapeutic strategies.

Valuable insights into the functions of FBLN5 and LTBP-4 in hemorrhoid pathogenesis are provided by this study, yet extensive research remains essential to completely unravel the molecular basis of these effects. Moreover, it is crucial to investigate the therapeutic capacity of explicitly targeting these proteins through clinical trials to evaluate their effectiveness and safety.

Conclusion

This study emphasizes the vital roles of FBLN5 and LTBP-4 in hemorrhoid development. We observed a significant downregulation of these proteins in hemorrhoidal tissues, which was associated with reduced fibroblast activation and compromised ECM integrity. These molecular alterations align with key histological features of hemorrhoids, including aberrant expansion of veins and degradation of collagen.

Restoring FBLN5 and LTBP-4 expression in fibroblasts generated from hemorrhoidal tissues increased cell viability, migration, and ECM formation. Moreover, the reversal of FBLN5's beneficial effects by LTBP-4 knock-down further emphasizes their interdependent roles in fibroblast-driven tissue repair.

Collectively, this work advances understanding of the molecular mechanisms involved in hemorrhoids and identifies FBLN5 and LTBP-4 as promising therapeutic

targets. Augmenting their expression or functionality could represent a novel opportunity to enhance tissue remodeling and clinical outcomes for patients. Future research should focus on developing targeted therapies and conducting clinical trials to examine both the effectiveness and safety of modulating FBLN5 and LTBP-4 in hemorrhoid management.

Glossary

FBLN5	Fibulin 5
LTBP-4	Latent transforming growth factor-beta binding protein 4
FN	Fibronectin
α -SMA	α -Smooth muscle actin
SD	Sprague-Dawley
CCK-8	Cell counting kit-8
ECL	Electro chemiluminescent
PVDF	Polyvinylidene difluoride
ECM	Extracellular matrix

Supplementary Information

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Supplementary Material 1.

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Clinical register number

Not Applicable.

Authors' contributions

We declare that all the listed authors have participated actively in the study and all meet the requirements of authorship. YCZ and FS contributed to the study design and concepts. YCZ and KHX prepared and edited the paper. YNW, DQL, RJX and MCL performed the clinical studies and experimental studies. KHX contributed to the data analysis and statistical analysis. FS provided laboratory space and funding, and supervised the research. All authors reviewed and approved the final manuscript.

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Data availability

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

Declarations

Ethics approval and consent to participate

This study was approved by the Ethics Committee of Guangzhou University of Chinese Medicine (No. K-2021-094), in accordance with the Declaration of Helsinki. All participants provided written informed consent. Animal experiments adhered to the Guidelines for the Care and Use of Laboratory

Animals and were approved by the Animal Ethics Committee of Ruige Biotechnology (No. 20211228-021).

Consent for publication

Not Applicable.

Competing interests

The authors declare no competing interests.

Conflict of interest

The authors declare that they have no conflict of interest.

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