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Research article

Fibulin-1 promotes intimal hyperplasia after venous stent implantation through ACE mediated angiotensin II signaling☆

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ABSTRACT

Objective: Stent intimal hyperplasia leads to in stent restenosis and thrombosis. This study determined whether Fibulin-1 activity in smooth muscle cells (SMCs) contributes to stent restenosis or thrombosis.

Methods: Stent implantation was conducted in a pig model. Target vessel samples were stained and analyzed by protein mass spectrometry. Cell experiments and Fibulin-1 SMC specific knockout mice (*Fbln1*^{SMKO}) were used to investigate the mechanism of Fibulin-1 induced SMC proliferation and thrombosis.

Results: SMC proliferation and phenotypic transition are the main pathological changes of intimal hyperplasia in venous stents. Protein mass spectrometry analysis revealed a total of 67 upregulated proteins and 39 downregulated proteins in intimal hyperplasia after stent implantation compared with normal iliac vein tissues. Among them, Fibulin-1 ranked among the top proteins altered. Fibulin-1 overexpressing human SMCs (Fibulin-1-hSMCs) showed increased migration and phenotypic switching from contractile to secretory type and Fibulin-1 inhibition decreased the activity of SMCs. Mechanistically, Fibulin-1-hSMCs displayed increased levels of angiotensin converting enzyme (ACE) expression and angiotensin II signaling. Inhibition of ACE or angiotensin II signaling alleviated the migration of Fibulin-1-hSMCs. Using Fibulin-1 SMC specific knockout mice (*Fbln1*^{SMKO}) and venous thrombosis model, we demonstrated that Fibulin-1 deletion attenuated intimal SMCs proliferation and thrombosis. Further, Fibulin-1 concentration was high in iliac vein compression syndrome (IVCS) patients treated with stent and was an independent predictor of venous insufficiency.

Conclusions: Fibulin-1 promotes SMC proliferation partially through ACE secretion and angiotensin II signaling after stent implantation. Fibulin-1 plays a role in venous insufficiency syndrome, implicating the protein in the detection and treatment of IVCS.

1. Introduction

With the development of endovascular technique, stenting to treat venous stenotic lesions has been used more frequently, especially in patients with iliac vein compression syndrome (IVCS) [1]. Due to the particularity of venous system, stent implantation is

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prone to in-stent restenosis (ISR) or thrombosis [2,3]. Previous studies mainly focused on the changes after arterial stent implantation, and there were few studies on the pathological changes and mechanisms after venous stent implantation.

Fibulin is a family of extracellular matrix (ECM) proteins which are highly expressed in cardiovascular tissues. They exhibit epidermal growth factor (EGF)-like modules followed by calcium-binding-EGF-like modules, all containing a unique Fibulin C-terminal domain [4]. Fibulin-1 is essential during embryonic development as it is required for regulation of migration and survival of cranial neural crest cells [5]. Fibulin-1 is expressed in many organs, often in relation to basement membranes, it is also present as a plasma protein [6]. Fibulin-1 has emerged as a player in the pathogenesis of various chronic injuries [7–9]. In the cardiovascular system, Fibulin-1 is highly expressed within atherosclerotic lesions and areas of thrombosis [6]. A previous study reported that Fibulin-1 contributes to ductus arteriosus (DA) closure by forming an environment favoring directional smooth muscle cells (SMC) migration toward the subendothelial region [10]. Whether Fibulin-1 is involved in venous in-stent restenosis and SMC proliferation is unknown.

Little is understood about the molecular mechanisms of venous in-stent restenosis despite its high incidence. In this study, the molecular mechanism of neointimal in venous stent was explored, and the role of Fibulin-1 in the blood of affected patients was examined by enzyme-linked immunosorbent assay (ELISA). The results indicated that Fibulin-1 promoted the activity of SMCs through angiotensin converting enzyme (ACE)/angiotensin II signaling in ISR. Further, the increased concentration of Fibulin-1 in IVCS patients was correlated with venous insufficiency of the lower limbs, implying a potential value of Fibulin-1 for IVCS.

2. Materials and methods

Fibulin-1 overexpressed SMCs. Primary SMCs (Human Umbilical Vein Smooth Muscle Cells) (HUM-Icell-e007, iCell Bioscience, China) were maintained in primary SMC basal medium (PriMed-iCELL-004, iCell Bioscience, China) containing FBS (PCS-999-010, ATCC, USA). Lenti-viral vectors expressing human Fibulin1-GFP (Lenti-Con335-hFibulin1-GFP) and control con335-GFP (Lenti-Con335-GFP) were purchased from GeneChem Company (China). To overexpress Fibulin1 in SMCs, Lenti-con335-Fibulin1-GFP was added into SMCs at a multiplicity of infection of 10:1 in serum-free medium. The lentivirus containing con335 backbone (Lenti-con335-GFP) was used as a negative control. Fibulin1 expression was observed by positive green fluorescence under a laser confocal microscope (IX-81, Olympus, Japan) and verified by q-PCR and Western blot 72 h after the transduction.

Fibulin-1 knockdown SMCs. Fibulin-1 siRNA was transfected into SMCs using Lipofectamine 3000 (GeneChem, China NM-001996.4) according to the manufacturer's instructions, and backbone con335 was transfected as negative controls. Cells were collected at 48 h after transfection, and the expression of mRNA and protein was confirmed by qPCR and western blotting.

RNA isolation and qPCR. qPCR was carried out using the SYBR Green FastMix Reaction Mixes kit (Roche Switzerland) in a real-time-PCR System (LightCycler 480, Roche, Switzerland). RNA expression was analyzed using the $2^{-\triangle\triangle}$CT methods. Primer sequences are listed in Supplementary Table 1. The operations were performed as previously reported [11].

Protein mass spectrometry analysis Proteins were identified and quantified by secondary mass spectrometry using a Thermo Scientific QEXactive mass spectrometer. All procedures are executed by the capitalbio company.

Immunoblotting. The primary antibodies include, mouse anti-human α-SMA (1:1000, 32575 Abcam USA), mouse anti-human Fibulin1 (1 μg/ml, 211536, Abcam, USA), rabbit anti-human SM22 (1 μg/ml 14106 Abcam USA), rabbit anti-human ACE (1 μg/ml, 254222, Abcam, USA), mouse anti-GAPDH (1:10000,181602, Abcam USA), and mouse anti-β tubulin (1:1000, 2146s, CST, USA). The operations were performed as previously reported [11].

ELISA Commercially human Fibulin1 ELISA kit (ZellBio GmbH Germany) was used to measure Fibulin1 in the supernatant of cell cultures according to the manufacturer's instructions. The results were listed in Supplemental Table 2. The procedures were carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki). Informed consent was obtained from all subjects.

Pig All animal procedures were approved by the Institutional Animal Care and Use Committee of Soochow University. The 8-to-10-weeks-old Bama pigs (30–35 kg n = 6) were used for iliac vein stent (Wallstent Boston) implanting. Balloon dilatation angioplasty and stent implantation was conducted in pig iliac vein under DSA. Target vessel samples were collected at 2 months after surgery [11]. To avoid staining difficulties caused by the stent, we pulled out the stent after removing vessel samples before staining.

Mice. A mouse specifically expressing Cre recombinase in SMC was purchased from Jackson Laboratories (SM22-cre Bar Harbor, USA ID 017491) and mate with a mouse strain that carries Fibulin-1 conditional alleles (Fibulin-1flox/flox Cyagen Biosciences Inc China ID S–CKO-02367) to obtain the SMC specific Fibulin-1 knockout mice (SM22-cre; Fibulin$^{flox/flox}$ mice Fbln1^{SMKO}). The results were confirmed by immunofluorescence staining (Supplementary Fig. S1). Mice were maintained under Specific Pathogen Free (SPF) raising conditions. Eight-twelve-week-old mice, weighing 22 g–28 g, were used for the experiments. The left iliac vein was injured by FeCl3 to induce thrombosis, then the mice were injected with DIOC6 fluorescent dye through the jugular vein. And the real time images were recorded in real-time by a stereo fluorescence microscope and the size of thrombi were quantified. After 1 weeks, vascular samples were dissected for staining.

Immunostaining. The primary antibodies include, rabbit anti-pig CD31(1:1000, 28364, Abcam, USA), rabbit anti-mouse VWF (1:1000, 6994, Abcam, USA), mouse anti-mouse fibronectin (1:1000, 6328, Abcam, USA), mouse anti-human/mouse α-SMA (1:500, 32575, Abcam, USA), mouse anti-human Fibulin1 (1 μg/ml, 211536, Abcam, USA). The operations were performed as previously reported [11].

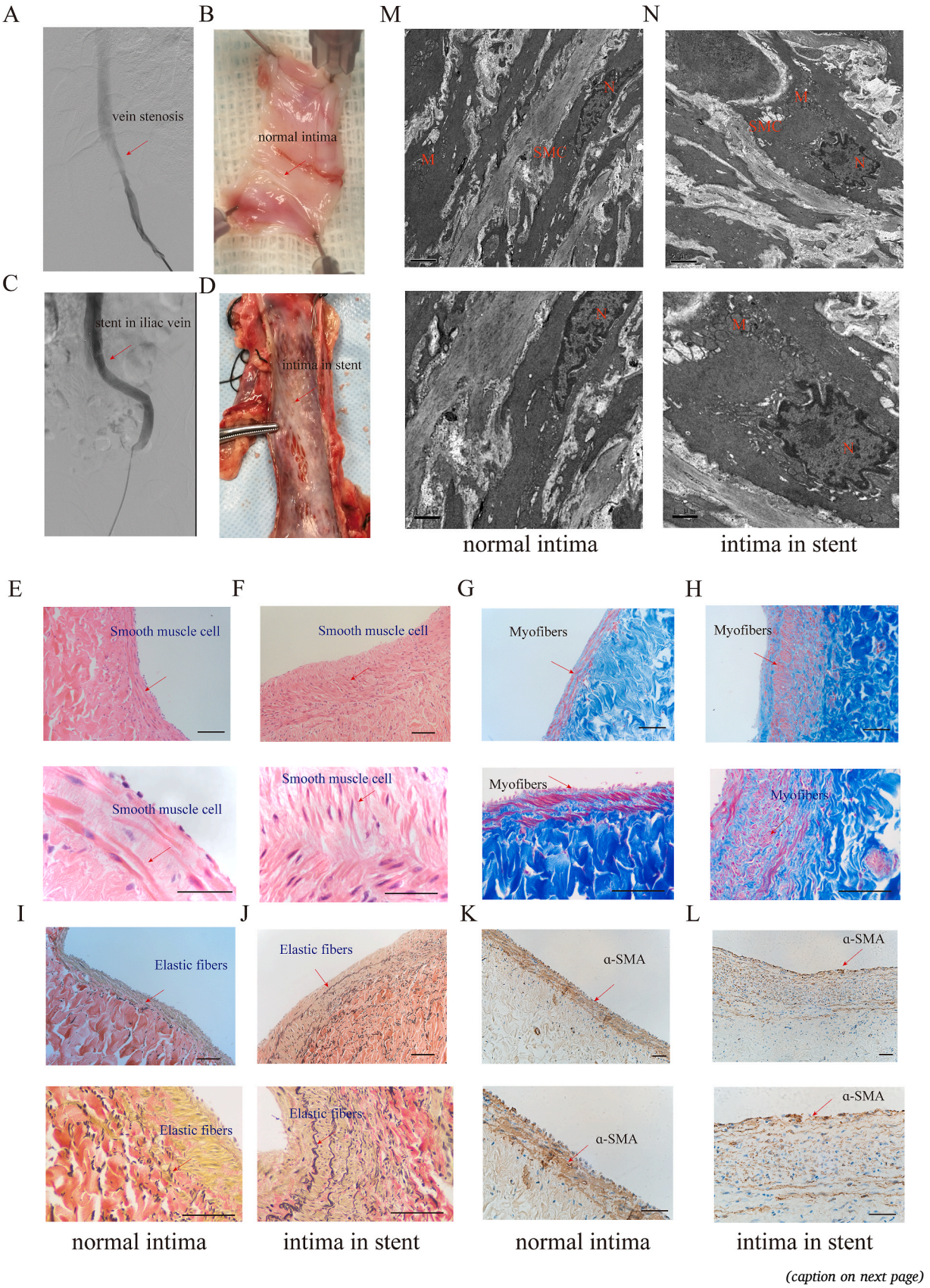

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Fig. 1. SMC proliferation and phenotypic transformation promote intimal hyperplasia in IVCS treated with stent.**A-D**. IVCS treated with stent animal models in pig. **E-F.** Normal and intima in stent were stained by HE 2 months after operation. **G-H** Masson staining the red streak represent Myofibers and blue collagenous fiber. **I-J** EVG staining Stripe of brown represent elastic fibers. **I-J** Immunohistochemical stain of α-SMA Staining with brown represent α-SMA positive signal. Scale Bar = 50 μm. **M-N.** Electron microscopy was used to detect SMCs. Bar = 1 μm N nuclear M mitochondria. The down photo is the enlarged image of the above picture for E-N.

2.1. Migration assay

Smooth muscle cells were seeded in 6-well plates (3×10^5 cells/well) with serum, and a 200-μL pipette tip was used to create a linear scratch at nearly 95% confluence. Images were captured by an inverted microscope at 24 and 48 h, and the migration area (the migration area = the initial scratch value subtract the surplus value) was assessed by Image J and GraphPad Prism software.

2.2. Transwell assay

Corning Transwell chamber in 24-well plates was used to perform cell invasion assays. Matrigel was added to the upper chamber and placed at 37 °C for 30 min, followed by the addition of cells. Basal medium supplemented with 10% FBS was transferred to the lower compartment of the chamber. Images of invasion cells were obtained using an inverted microscope. The invasion assay analysis was performed using Image J software and GraphPad Prism software.

Plasma samples From December 2018 to January 2021, 256 IVCS patients (confirmed by angiograph) treated with stent at the 19 participating centers 20 healthy people were recruited, the blood sample of which were collected to detect Fibulin1 concentration through ELISA.

2.3. Statistical analysis

All statistical analyses were performed using SPSS 22.0 (SPSS, Chicago, IL, USA). Continuous data are presented as means ± standard deviation and categorical data as counts and percentages. Student's t-test and the $\chi 2$ test were employed to compare continuous and categorical data, respectively. All statistical tests performed were two-tailed.

3. Results

SMC proliferation and phenotypic transformation promote intimal hyperplasia after stent implantation.

To investigate changes of venous intima after stenting, we established animal models in pig as previously reported [11]. Intimal hyperplasia after stenting was observed in the animal models (Fig. 1A–D). By HE staining, we confirmed that SMC proliferation was the

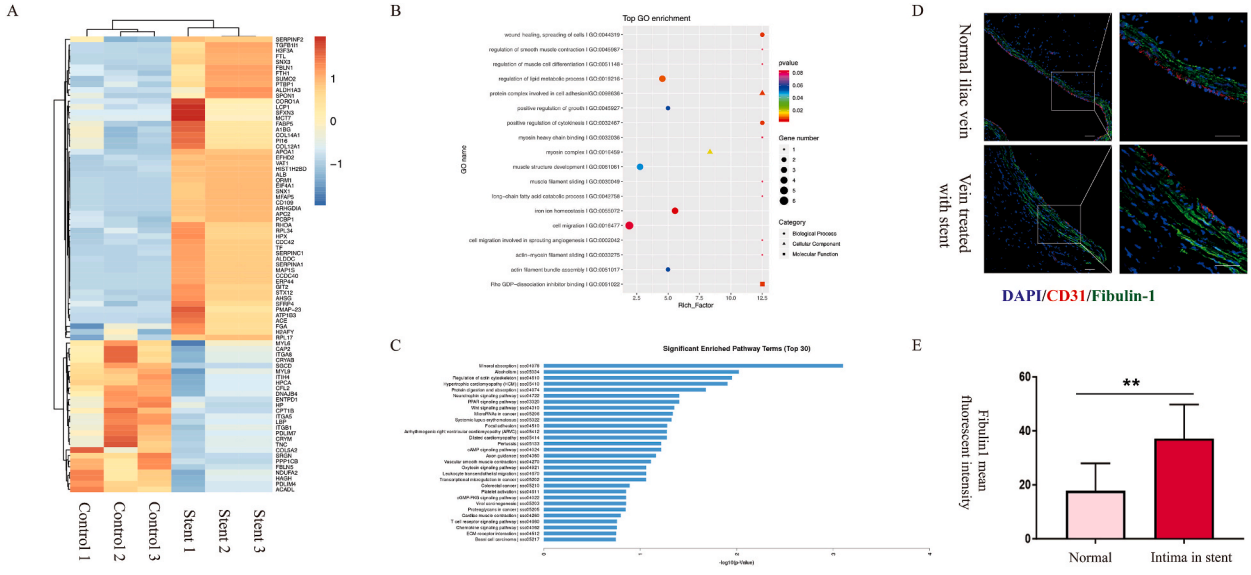

Fig. 2. Fibulin-1 gene expression is augmented in-stent intimal hyperplasia samples. **A.** The top changed protein between normal pig iliac vein intima with in-stent intimal hyperplasia samples. **B**. Gene ontology enrichment analysis of biological processes. **C.** Gene ontology enrichment analysis of KEGG pathway with up-regulated genes. **D.** Normal Iliac vein and iliac vein treated with stent in pig were immunofluorescent stained for CD31 (red), Fibulin1 (green), and DAPI (blue) 2 months after operation. the right column is the enlarged image of the box in the left column Scale Bar = 50 μm. **E.** Quantification of fluorescence intensity of intima is shown. Data are mean ± SEM. Results are representative of 3 independent experiments. $^{**}P < 0.005$.

main pathological characteristic of intimal hyperplasia in stent, followed by SMC migration into the subendothelial region (Fig. 1E and F). Masson and EVG staining further illustrated hyperplasia of the muscle fibers and increased extracellular collagen fiber occurrence (Fig. 1G–J). SMC marker α-SMA was widely expressed though its intensity was significantly weakened in stent intima staining compared with normal iliac vein tissues (Fig. 1K and L). Electron microscopy was used to explore the subcellular structural changes, revealing enlarged nuclear size, reduced chromatin density and increased mitochondria of SMC in hyperplasia intimal compared to control (Fig. 1M and N). These results indicated the phenotypic transition of SMCs from contractile to proliferative in in-stent intimal hyperplasia.

3.1. *Fibulin-1 gene expression is augmented in stent intima*

We performed a proteome analysis on normal pig iliac vein and in-stent intimal hyperplasia samples. There are 67 genes upregulated and 39 genes downregulated. Among these, molecules associated with proliferation were upregulated in in-stent intimal hyperplasia samples (Fig. 2A). We subsequently performed functional gene ontology (GO) analysis on the relevant up-regulated genes, observing that proteins involved in spreading of cells, regulation of smooth muscle contraction, regulation of lipid metabolic processes, protein complexes involved in cell adhesion, positive regulation of cytokinesis, myosin heavy chain binding, myosin complexes, muscle structure development, muscle filament sliding, long-chain fatty acid catabolic processes, regulation of iron ion homeostasis, cell migration related to sprouting angiogenesis, actin-myosin filament sliding, actin filament bundle assembly and Rho GDP-dissociation inhibitor binding increased in in-stent intimal hyperplasia samples (Fig. 2B). We further investigated the expression and activation of signaling pathways affected in intimal hyperplasia (Fig. 2C). Among those identified, pathways involved in the regulation of the actin cytoskeleton, hypertrophic cardiomyopathy, vascular smooth muscle contraction, cardiac muscle contraction, the Wnt signaling pathway, PI3K-Akt signaling pathway, TGF-beta signaling pathway and angiotensin II signaling pathway all have shown participation in SMC proliferation and phenotypic transformation. Fibulin-1 has been previously implicated across various diseases such as thyroid lesion [12], atherosclerosis [6], and tumor metastasis [9]. Proteome analysis revealed that Fibulin-1 was one of the highest upregulated genes in stent intimal (Fig. 2A), confirmed by Fibulin-1 immunofluorescent staining (Fig. 2D and E).

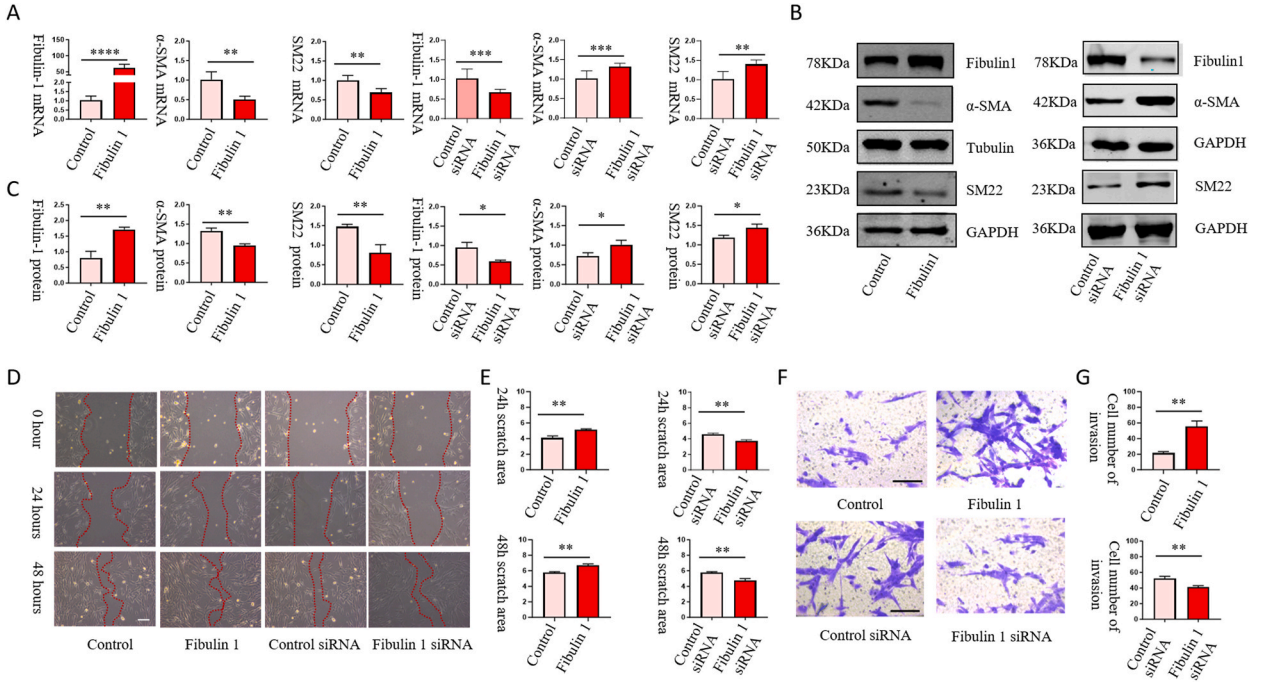

Fig. 3. Fibulin-1 promotes SMC migration and phenotypic transition. **A.** Fibulin1, α-SMA and SM22 mRNA expression in the Lenti-Con335-hFibulin1-GFP -transduced SMCs (Fibulin1) and Fibulin-1 siRNA were analyzed by quantitative polymerase chain reaction (q-PCR) relative to Lenti-Con335-GFP-transduced control SMCs (Control). (n ≥ 3 per group). Data are mean ± SEM., ***$P < 0.001$; ****$P < 0.0001$. **B–C.** Fibulin1, α-SMA and SM22 proteins in Fibulin1 siRNA、Fibulin1-SMCs and Con335(Control) were analyzed by Western blotting normalized to Tubulin or GAPDH and displayed as fold changes relative to Con335-SMCs. Data are mean ± SEM. Results are representative of ≥3 independent experiments. **$P < 0.005$; ***$P < 0.001$; ****$P < 0.0001$. **D-E.** Migration assay showing the effects of fibulin1 on SMCs migration, Scale bar = 100 μm, migration area were analyzed. Results are representative of ≥3 independent experiments. **$P < 0.005$. **F-G** Transwell cell invasion assay provided results similar to those for migration assay, Scale bar = 100 μm, invasion cell number was analyzed. Results are representative of ≥3 independent experiments. **$P < 0.005$.

3.2. Fibulin-1 promotes SMC migration and phenotypic transition

To investigate the direct effect of Fibulin-1 on SMCs, we generated a Fibulin1-over-expressing human SMC line (Fibulin1-SMC) by transducing lentiviral vectors expressing human Fibulin1 with a GFP (green fluorescent protein) tag (Lenti-hFibulin1-GFP) into SMCs. SMCs transduced with Lenti-con335-GFP (Con335-SMC) were used as control cells and a Fibulin1-knockdown SMC line (Fibulin-1 siRNA SMC) by transducing lentiviral vectors Fibulin-1 RNAi. Fibulin1 expression in the cell line was determined by q-PCR and confirmed by Western blotting (Fig. 3A–C). We next explored the role of Fibulin1 in the SMC phenotype transition. Compared with con335-SMCs, Fibulin1-SMCs presented a significantly lower level of α-SMA and SM22 mRNA and protein expression and the expression of α-SMA and SM22 increased after Fibulin-1 inhibition (Fig. 3A–C). As the activity of secretory SMCs was increased compared with contractile type SMCs, we detected the migration and invasion ability of SMCs through wound healing assay and transwell assay. The results indicated that Fibulin1 overexpression enhances the ability of SMCs to migrate and invade surrounding tissue, which was reduced in Fibulin-1 siRNA-treated SMCs. (Fig. 3D–G), confirming its role in the induction of the SMC transition from contractile to secretory type.

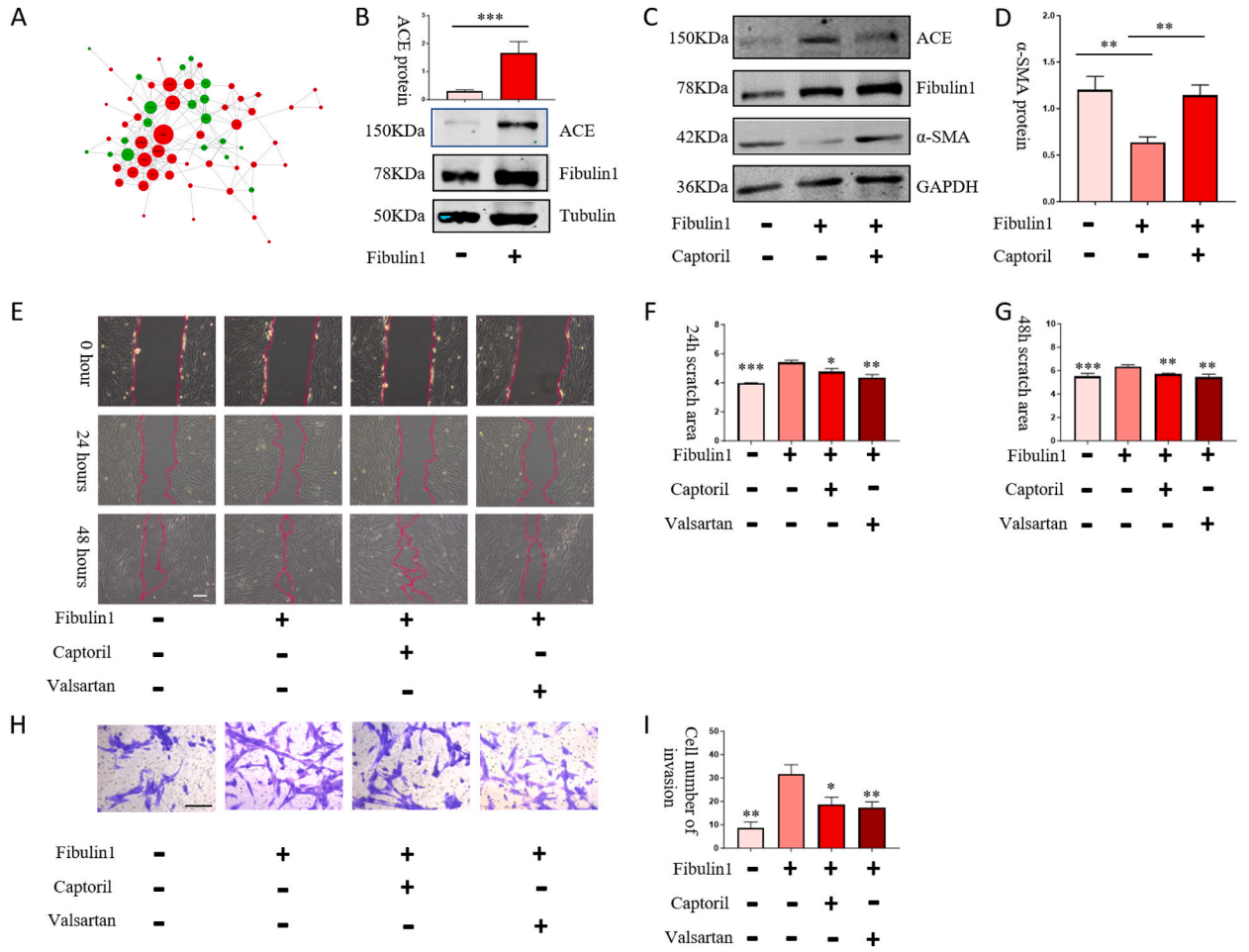

Fig. 4. ACE mediates Fibulin-1 function in SMC regulation via angiotensin II signaling. **A.** Protein interaction analysis of molecular interaction through proteome analysis on normal pig iliac vein intima and stenosis-affected iliac vein intima. **B.** ACE proteins in Fibulin1-SMCs were analyzed by Western blotting normalized to Tubulin and displayed as fold changes relative to Con335-SMCs. Data are mean ± SEM. Results are representative of ≥3 independent experiments $^{***}P < 0.001$. **C-D.** ACE and α-SMA proteins in Fibulin1-SMCs were analyzed by Western blotting normalized to GAPDH and displayed as fold changes relative to Con335-SMCs and Fibulin1-SMCs added with Captoril. Data are mean ± SEM. Results are representative of ≥3 independent experiments $^{**}P < 0.005$. **E-G.** Migration assay assay showing the effects of captopril and Valsartan on Fibulin1-SMCs migration, Scale bar = 100 μm, migration area were analyzed. Results are representative of ≥3 independent experiments. $^{*}P < 0.05$; $^{**}P < 0.005$; $^{***}P < 0.001$. **H–I.** Transwell cell invasion assay provided results similar to those for migration assay, Scale bar = 100 μm, invasion cell number was analyzed. Results are representative of ≥3 independent experiments. $^{*}P < 0.05$; $^{**}P < 0.005$.

3.3. ACE mediates Fibulin-1 function in SMC regulation via angiotensin II signaling

Upregulation of Angiotensin converting enzyme (ACE) is associated with various pathological conditions, including hypertension, atherosclerosis and diabetic nephropathy [13]. Protein interaction analysis demonstrated that Fibulin1 interacts with ACE (Fig. 4A), particularly, that ACE is upregulated in Fibulin1-overexpressing SMCs (Fig. 4B). We therefore proposed that Fibulin-1 induces the SMC transition via activation of ACE. To further test this hypothesis, we inhibited ACE activity through the use of the ACE inhibitor captopril in Fibulin-1-overexpressing SMCs. The findings demonstrated that inhibition of ACE attenuated the inhibitory effect of Fibulin1 on α-SMA (Fig. 4C and D). Moreover, inhibition of ACE reduced the migration and invasion ability of Fibulin-overexpressing SMCs (Fig. 4E–I). Pathway enrichment further showed that the Angiotensin II (AngII) signaling pathway, activated by ACE, is enriched in Fibulin-overexpressing SMCs. Subsequent inhibition of AngII signaling by valsartan in Fibulin-overexpressing SMCs similarly demonstrated attenuated SMC activity induced by Fibulin-1 over-expression (Fig. 4E- I).(See. Fig. 6)

3.4. Fibulin-1 knockout reduced intima hyperplasia and thrombosis in a mouse model

ISR in the venous system is related to local thrombosis. To investigate the effects of Fibulin-1 on intimal hyperplasia in vivo, we established a Fibulin-1 SMC specific knockout mice (*Fbln1*^{SMKO}) line treated to thrombosis model. Compared with WT mice, the intima hyperplasia of the iliac vein was reduced in *Fbln1*^{SMKO} mice (Fig. 5A and B). Similarly, the proliferation activity of SMCs was normalized in the *Fbln1*^{SMKO} mice (Fig. 5C and D). Meanwhile we found that compared with the WT mice, *Fbln1*^{SMKO} mice had a significant reduction of thrombi in injury venous (Fig. 5E- I) (Supplemental Table 3).

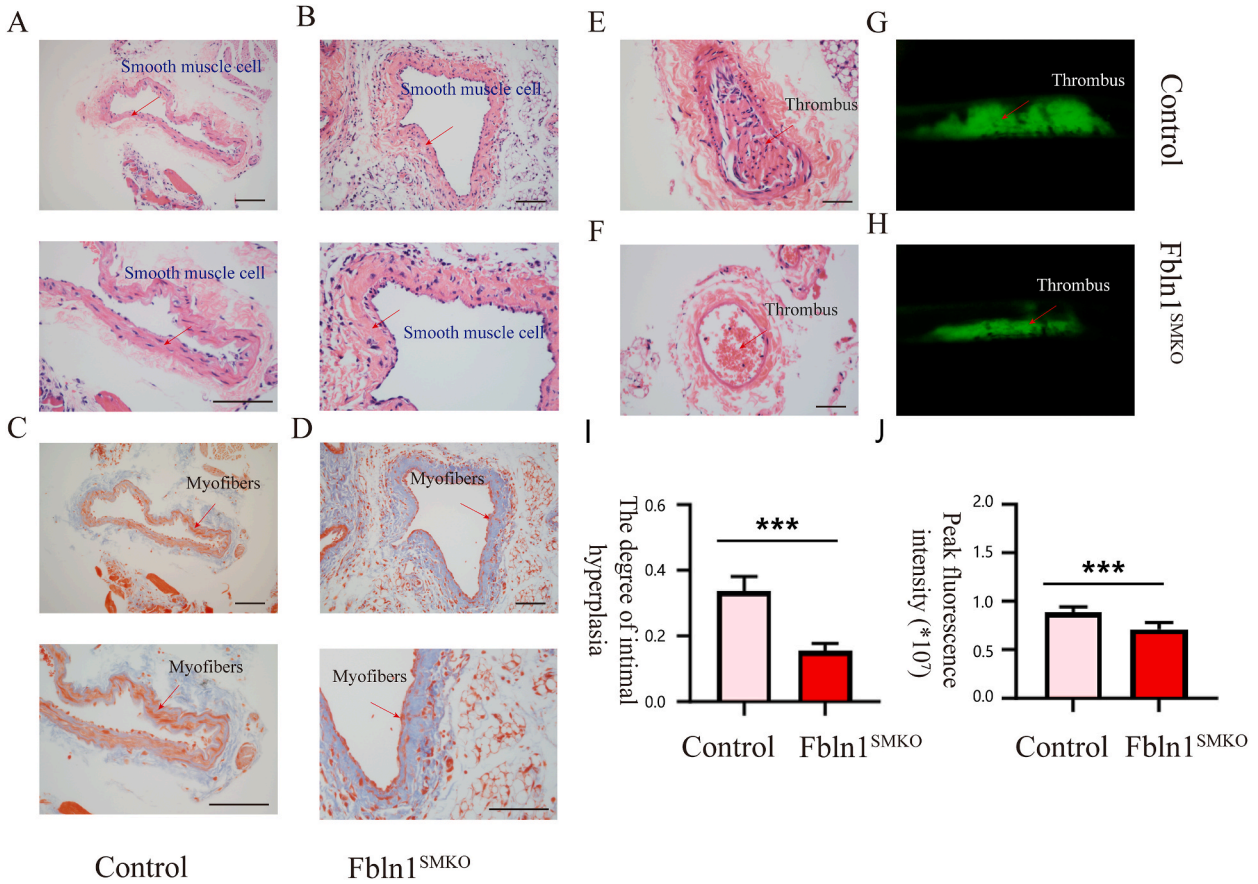

Fig. 5. Fibulin-1 knockout and ACE inhibitor reduced intimal hyperplasia and thrombosis in a mouse model of thrombus.A-B. Iliac vein of thrombosis model from Fibulin1$^{+/+}$, Fbln1^{SMKO} were stained by HE 1 week after operation. **C-D** Masson staining the red streak represent Myofibers and blue collagenous fiber. **E-F** Thrombus in injury vein by HE staining. **G-H** Real time images were recorded in real-time by a stereo fluorescence microscope The green signal represents the thrombus. **I.** Statistic analysis of the degree of intimal hyperplasia. Use Qwin image analysis software. Intimal area (IA) = area surrounded by internal elastic lamina (AIEL) subtract lumen area (LA). Medial area (MA) = area surrounded by external elastic lamina (AEEL) subtract IA. The degree of intimal hyperplasia = IA/MA. **J.** Statistic analysis of the peak Fluorescence indicate the thrombus size n = 10 ***$P < 0.001$. Scale Bar = 50 μm. Down photo is the enlarged image of the above picture for A-D.

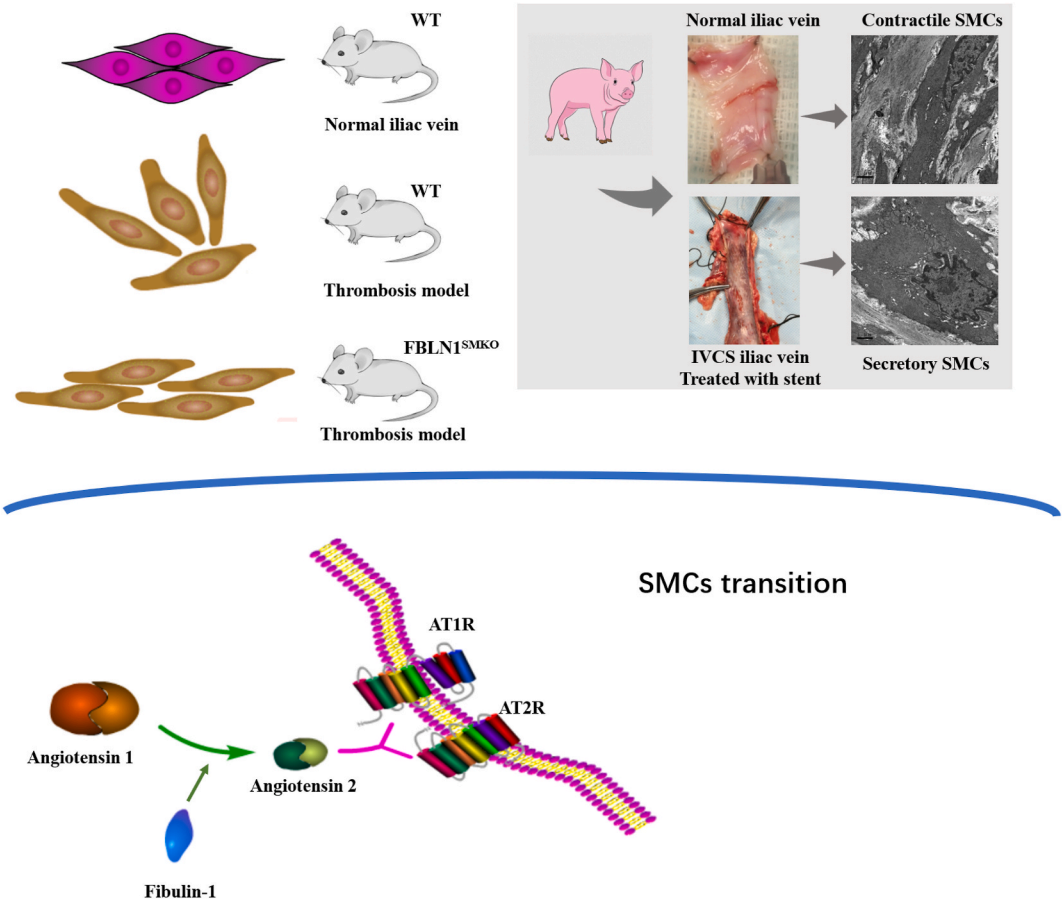

Fig. 6. Fibulin1 is upregulated in in-stent intimal hyperplasia samples, and activation of ACE and angiotensin II signaling, leading SMCs migration and phenotypic transition, and thus inducing vein intimal hyperplasia.**A.** Carotid artery from WT control were immunofluorescence stained for SMA (green Alexa fluor 568), Fibulin-1 (white Alexa fluor 647) and DAPI (blue). **B.** Carotid artery from WT control were immunofluorescence stained for CD31 (red Alexa fluor 488), Fibulin-1 (white Alexa fluor 647) and DAPI (blue). **C.** Carotid artery from WT control were immunofluorescence stained for Fibulin-1 (white Alexa fluor 647) and DAPI (blue) Green arrow represents Fibulin-1 expression in smooth muscle cells, red arrows represent fibulin expression in endothelial cells. **D.** Carotid artery from WT control were immunofluorescence stained for IgG control and DAPI (blue). **E.** Fbln1^{SMKO} mice were immunofluorescence stained for Fibulin-1 (white Alexa fluor 647) and DAPI (blue). Red arrows represent fibulin expression in endothelial cells. **F.** Fibulin-1 intensity of SMC is shown. Data are mean ± SEM, ****$p < 0.0001$. Bar = 50 μm.

3.5. Serum Fibulin-1 is associated with IVCS and chronic venous insufficiency (CVI)

Fibulin-1 is a plasma soluble protein and plasma Fibulin-1 has displayed potential as a biomarker for cardiovascular diseases. For example, the concentration of plasma Fibulin-1 was shown to be associated with arterial stiffness, blood pressure, and glycemic status in a cohort of 305 patients with T2DM [7]. We detected Fibulin-1 plasma concentration using the ELISA assay, demonstrating that plasma concentration of Fibulin-1 is higher in IVCS patients treated with stent compare to healthy people (IVCS vs healthy 8.56 vs 5.11 $p = 0.0006$) (Supplementary Fig. S2, Supplementary Table 2). Revised Venous clinical severity score (r-VCSS) was used to evaluate venous function. The concentration of Fibulin-1 was positively correlated($p = 0.023$)with and independently influenced r-VCSS (Tables 1 and 2).

4. Discussion

In this study, we identified a novel role of Fibulin-1 in the SMC activation preceding ISR. ACE and AngII signaling were shown to participate in the regulation of Fibulin1 in SMCs, indicating that Fibulin-1 may be developed as a regulatory molecule for ISR in venous.

Iliofemoral venous stenting has become the standard of care in the management of chronic iliofemoral venous obstruction (CIVO) [14]. Of the patients who have undergone iliofemoral venous stenting, 5%–20% will require reintervention, mainly for ISR or thrombosis, or a combination of both [2,3,15]. In-stent restenosis in veins appears to differ from that described in the arterial stented literature, by an apparent increase in the prevalence of thrombus (both organizing and old thrombus) noted at all time periods. There

Table 1
Risk factors for r-VCSS in NIVCs.

Risk factors	Univariable odds ratio (95% CI)	Univariable *P* value
Age	0.019 (0.006–0.119)	0.03
Gender	0.058 (0.356–0.119)	0.0001
BMI	0.009 (−0.041–0.269)	0.15
Stenosis degree	0.012 (−0.089–0.006)	0.088
Diameter of stenosis iliac vein	0.005(-0.124–0.445)	0.267
WBC	0.003(-0.216–0.510)	0.43
RBC	0.014(-0.07–2.202)	0.07
PLT	0.014(-0.02–0.0006)	0.07
hemoglobin	0.021(0.006–0.082)	0.02
ALT	0.0008(-0.025–0.04)	0.65
AST	0.0009(-0.061–0.037)	0.64
Creatinine	0.0003(-0.038–0.029)	0.79
glucose	1.28e(-0.413–0.437)	0.96
Direct bilirubin	0.005(-0.134–0.491)	0.26
Total bilirubin	0.011(-0.018–0.188)	0.1
APTT	0.025(-0.241–0.028)	0.01
TT	0.008(-0.281–0.049)	0.17
INR	0.002(-3.07–6.004)	0.52
PT	0.002(-0.25–0.525)	0.48
temperature	0.018(-3.816–0.139)	0.035
Heart rate	0.005(-0.026–0.088)	0.28
MAP	0.0002(-0.059–0.046)	0.82
Fibulin1	0.022 (0.005–0.069)	0.023

The factors of clinical index influenced the r-VCSS by univariate correlation analysis were listed.
P-value was <0.05 for all variables.
Normal laboratory values: WBC: 3.5–9.5 × 10^9/L, RBC: 3.8–5.1 × 10^12/L, PLT: 125–350 × 10^9/L, Hemoglobin: 115–150 g/L, ALT: 5–40 U/L, AST: 8–40 U/L, creatinine: 44–106 μmol/L, Glucose: 3.9–6.1 mmol/L, Direct bilirubin: 1.7–6.8 μmol/L, Total bilirubin: 5.0–20.5 μmol/L, APTT: 25.0–31.3 s, PT: 10–15.0 s, INR: 0.90–1.15 s, FIB: 2–4 g/L.
R- VCSS revised Venous clinical severity score, WBC white blood cell, RBC red blood cell, PTL: platelet, ALT alanine transaminase, AST aspartate aminotransferase, APTT activated partial thromboplastin time, PT prothrombin time, INR international normalized ratio, FIB: fibrous protein, MAP: mean arterial pressure.

Table 2
Risk factors for r-VCSS in NIVCs.

Risk factors	Multivariable odds ratio	Standard error	*P* value
Age	0.060	0.027	0.03
Gender	−1.927	0.535	0.001
Fibulin1	0.207	0.074	0.006
APTT	0.119	0.051	0.022

Multivariate analysis showed that Age, Gender, Fibulin-1 and APTT were risk factors for r-VCSS.
P-value was <0.05 for all variables.
R–VCSS revised Venous clinical severity score, APTT activated partial thromboplastin time.

are two possibilities of evolving vein in-stent stenosis: migration of mesenchymal cells from the vein wall through the stent wire gaps and elaborating within the stent lumen, and/or initial thrombus formation, which is later organized by ingrowing inflammatory cells and mesenchymal cells. Episodic thromboses next to diffuse intimal thickening also may be occurring. Early ISR likely has a predominance of fresh thrombus and late ISR, a predominance of neointima, smooth muscle cells, fibrosis, and so forth. Through animal model, we found that hyperplasia of SMCs is one of the main pathological changes in venous ISR. Although the molecular mechanisms of ISR in venous remain unclear.

Our pathological protein spectrum data revealed the upregulation of Fibulin1, ACE and angiotensin II signaling molecules in intimal hyperplasia. Fibulin-1 has been shown to interact with integrins and the extracellular matrix proteoglycan versican, pointing to a role in cell adhesion, mobility and matrix remodeling [16,17]. The role of Fibulin-1 in venous diseases has not been reported. Although low in healthy veins, Fibulin-1 expressed at high levels in stent intimal hyperplasia. The high incidence of venous thrombosis in IVCS may be an induction factor. Strong extracellular presence of Fibulin-1 was detected by immunostaining in regions containing fresh thrombi [6], which suggests that some factors in thrombosis may induce high expression of Fibulin-1. Stenting destroys the intima lending the formation of local micro thrombosis in stent mesh which can lead to ISR. Our results showed that fibulin-1 knockout reduced thrombosis and intimal hyperplasia in venous injury site.

The treatment of IVCS remains suboptimal, normally IVCS patients with stenosis greater than 50% and CEAP (clinical feature, etiology, anatomic distribution and pathophysiology) greater than grade 3 were treated. Additionally, there remains a lack of physiological indicators to guide the treatment of IVCS. Our results demonstrated that Fibulin-1 is related to CVI, and may therefore provide

a reference for IVCS treatment.

Some of the limitations of the present study include the preliminary nature of exploration of the molecular mechanisms guiding Fibulin-1 regulation of ACE and AngII signaling. Further studies are needed to explore how Fibulin-1 mediates ACE/AngII signaling and the mechanisms of interaction between thrombosis and Fibulin-1. Second, as captopril and valsartan only partially alleviate the increased migration and invasion with Fibulin-1 overexpression, there may be potential non-ACE/angiotensin II mechanisms by which fibulin-1 increase SMC migration, and we will explore in the future. Third, due to the lack of follow-up data, the prognostic value of Fibulin-1 in IVCS treatment remains unclear, though a follow-up at 5 years is currently being planned to evaluate the prognostic value of Fibulin-1 more definitively.

In summary, our results indicate that Fibulin-1 upregulates ACE and activates AngII signaling, leading to the SMC activation that begets intimal hyperplasia after stent implantation. Plasma concentration of Fibulin-1 was also associated with venous insufficiency.

Ethics approval and consent to participate

This program was approved by the Ethics Committee of Soochow University (ECSU-201800069) and obtains informed consent of the people for using they clinic data.

Consent for publication

All authors are consent for publication the paper in Journal of Heliyon.

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Availability of data and material

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CRediT authorship contribution statement

Yuning Gao: Project administration, Methodology, Investigation. **Tianshi Chen:** Validation, Supervision, Formal analysis, Data curation. **Lei Hong:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e27626.

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