Down-regulation of Fibulin-5 is associated with aortic dilation: role of inflammation and epigenetics

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Aims

Destructive remodelling of extracellular matrix (ECM) and inflammation lead to dilation and ultimately abdominal aortic aneurysm (AAA). Fibulin-5 (FBLN5) mediates cell–ECM interactions and elastic fibre assembly and is critical for ECM remodelling. We aimed to characterize FBLN5 regulation in human AAA and analyse the underlying mechanisms.

Methods and results

FBLN5 expression was significantly decreased in human aneurysmatic aortas compared with healthy vessels. Local FBLN5 knockdown promoted aortic dilation and enhanced vascular expression of inflammatory markers in Ang II-infused C57BL/6J mice. Inflammatory stimuli down-regulated FBLN5 expression and transcriptional activity in human aortic vascular smooth muscle cells (VSMC). Further, aortic FBLN5 expression was reduced in LPS-challenged mice. A SOX response element was critical for FBLN5 promoter activity. The SOX9 expression pattern in human AAA parallels that of FBLN5, and like FBLN5, it was reduced in TNFα-stimulated VSMC. Interestingly, SOX9 over-expression prevented the cytokine-mediated reduction of FBLN5 expression and transcription. The inhibition of Class I histone deacetylases (HDACs) by MS-275 or gene silencing attenuated the inflammation-mediated decrease of FBLN5 expression in VSMC and in the vascular wall. Consistently, HDAC inhibition counteracted the reduction of SOX9 expression induced by inflammatory stimuli and prevented the TNFα-mediated decrease in the binding of SOX9 to FBLN5 promoter normalizing FBLN5 expression.

Conclusion

We evidence the deregulation of FBLN5 in human AAA and identify a SOX9/HDAC-dependent mechanism involved in the down-regulation of FBLN5 by inflammation. HDAC inhibitors or pharmacological approaches that aimed to preserve FBLN5 could be useful to prevent the disorganization of ECM induced by inflammation in AAA.

Keywords

Abdominal aortic aneurysm • Fibulin-5 • SOX9 • Histone deacetylases • Vascular smooth muscle cells

1. Introduction

Abdominal aortic aneurysm (AAA) is a common degenerative vascular disease that affects 6–9% of men over the age of 65 years. It is characterized by a progressive dilatation of the abdominal aorta due to an impairment of vascular integrity. Acute AAA rupture, the most feared complication of this pathology, is associated with an 80–90% mortality rate.1 AAA formation involves an inflammatory process of the aortic wall characterized by the up-regulation of proteolytic pathways, increased neovascularization, and exacerbated apoptosis of vascular smooth muscle cells (VSMC), leading to an inexorable and destructive connective tissue remodelling. Currently, therapeutic options are limited to surgical repair of those AAA under a high risk of rupture, and there are no pharmacological strategies hindering AAA progression.2 As a result of the progressive increase in lifespan, AAA incidence has augmented, making the development of therapeutic approaches limiting AAA growth and rupture a challenge that requires a better understanding of the underlying molecular mechanisms.
2. Methods

An expanded Methods section is available in the Supporting information.

2.1 Aneurysm and donor sampling and preservation

Aortas from patients (n = 27) undergoing open repair for AAA (Hospital de la Santa Creu i Sant Pau; HSCSP; Barcelona, Spain), and healthy aortas (n = 15), from multi-organ donors were obtained.\textsuperscript{18} Tissue-conditioned medium from abdominal aortic samples of patients enrolled in the RESAA protocol and from donors was used as described (n = 12).\textsuperscript{19} The research was performed in accordance with the Declaration of Helsinki and approved by the HSCSP Ethics Committee (12/031/1316), a French ethics committee (CPB, Cochin Hospital) and the French Biomedicine Agency (PFS 09-007).

2.2 Animal procedures

Male C57BL/6j mice (2 months old; n = 12; Charles River, Barcelona, Spain) were randomly distributed into two groups: control mice receiving a siRNA random (sIRD; Acell Non-targeting pool; GE Healthcare Dharmacon Inc., Little Chalfont, UK), and mice receiving the Smart pool Acell FBLN5 siRNA (siFBLN5; GE Healthcare Dharmacon Inc.). siRNA delivery was achieved with an atelocollagen formulation (Atelogene Local Use Quick Gelation; Koken Co., Ltd, Tokyo, Japan). Four days after siRNA delivery, mice were infused with Angiotensin II (Ang II; 1000 ng/kg/min, Sigma-Aldrich, St Louis, MO, USA) using osmotic mini-pumps (model 1002, Alzet. DURECT Corporation, CA, USA) for 2 weeks.

In a second experimental approach, C57BL/6j mice were randomly distributed into two groups, one receiving LDS (0.5 mg/kg; i.p.; Sigma-Aldrich) and a control group receiving an equivalent volume of saline.\textsuperscript{20} Animals were sacrificed 24 h after treatment. Alternatively, animals were distributed in four groups: control mice receiving vehicle, LDS-treated mice, LDS + MS-275 animals, which were pre-treated with MS-275 (5 mg/kg/day; via i.p.; SelleckChemicals, Houston, TX, USA) 1 day prior LDS and received a second dose of the drug at the same time than LDS, and MS-275-treated mice. Doses and routes of administration were chosen according to previous studies.\textsuperscript{21} After 24 h, mice were anaesthetised with ketamine (150 mg/kg) and medetomidine (1 mg/kg) and sacrificed by thoracotomy. Aortas were excised, frozen in liquid nitrogen, and stored at –80 °C. All animal procedures were reviewed and approved by the Ethical Committee at the Centro de Investigación Cardiovascular (CSIC-ICCC, Barcelona, Spain; ICCC-055) as stated in Law 5/1995, 21 June, (Generalitat de Catalunya) and follow the Spanish Policy for Animal Protection RD53/2013, which meets the European Union Directive 2010/63/UE.

2.3 Cell culture

Human aortic VSMC were obtained by using a modification of the explant technique and cultured as previously described.\textsuperscript{18,22} Human VSMC were obtained from non-atherosclerotic arteries of hearts removed in transplant surgeries at the Hospital de la Santa Creu i Sant Pau (Barcelona, Spain) and cultured in M199 (Gibco, Carlsbad, CA, USA) supplemented with 20% FCS, 2% human serum, 2 mmol/L L-glutamine, and antibiotics. VSMC from at least four donors were used. Studies were performed with cells between Passages 3 and 6. The research was performed in accordance with the Declaration of Helsinki and approved by the HSCSP Ethics Committee (12/031/1316).

2.4 Real-time PCR

Total RNA was isolated using Ultraspec\textsuperscript{TM} (Biotex, Houston, TX, USA) and was reverse transcribed into cDNA using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Thermo Fisher Scientific Inc., Waltham, MA, USA) in the presence of random hexamers. Quantification of mRNA levels was performed by real-time PCR.

2.5 FBLN5 promoter constructs and site-directed mutagenesis

The pGL3 luciferase reporter vectors pFBLN5-1650, pFBLN5-635, and pFBLN5-329 containing serial fragments of the FBLN5 promoter were used.\textsuperscript{15} The FBLN5 SOX9 sites located at position -304 and -251 were mutated using the QuickChange II Site-directed mutagenesis kit (Agilent Technologies, Inc., Santa Clara, CA, USA).

2.6 Transient transfection and luciferase assays

Lipofectamine\textsuperscript{TM} LTX and Plus\textsuperscript{TM} Reagent (Life Technologies) were used for transient transfection of VSMC as described.\textsuperscript{20}

2.7 siRNA transfection

VSMC were transfected with a pool of Silencer predesigned siRNAs for HDAC1, 2 and 3 (0.4 μg each; ID#573, ID#6493, and ID#16878, respectively; Life Technologies), or Silencer Negative Control siRD (ID#AM4611; Life Technologies) by nucleofection.

2.8 Lentivirus production and VSMC infection

The mouse SOX9 cDNA was obtained from the pWPXL-SOX9 vector (Addgene, Cambridge, MA, USA) and cloned into the pLVX-puro lentivector vector to generate the pLVX/SOX9 plasmid. The empty vector was used as control. Viral particles were obtained according to the Lenti-X\textsuperscript{TM} Lentiviral Expression System (Clontech, Mountain View, CA, USA). Infected VSMC were selected with puromycin during 6 days.

2.9 Chromatin immunoprecipitation assay

Chromatin immunoprecipitations were performed with antibodies (5 μg) against Histone 3 acetyl K18 (ac-H3K18; ab1191, Abcam, Cambridge, UK), pan acetylated Histone H3 (acetyl K9 + K14 + K18 + K23 + K27)
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2.10 Western blot analysis
VSMC were lysed with a buffer containing 10 mM Tris–HCl (pH 7.4), 1% SDS, and 1 mM EDTA and sonicated. SDS–PAGE and electrotransference onto polyvinylidene difluoride membranes (Immobilon, Millipore) were then processed. Proteins were separated on SDS–PAGE and electroblotted onto polyvinylidene difluoride membranes (Immobilon, Millipore). Blots were incubated with antibodies against FBLN5 (ab66339, Abcam). The blot was then treated using an appropriate horseradish peroxidase-labelled secondary antibody. The bands were visualized using an enhanced chemiluminescence detection kit (Dako, Agilent Technologies, Inc.) and the Supersignal West Dura detection system (Life Technologies).

2.11 Statistical analysis
Results are shown as mean ± SEM. Significant differences were established by Student’s t-test or one-way ANOVA for repeated measures, according to the number of groups compared (GraphPad Instat V2.03; GraphPad Software Inc., La Jolla, CA, USA). In the latter case, when significant variations were found, the Student–Newman–Keuls method was applied. To determine association between variables, data were Log_10 transformed to normalize their distribution, and the Pearson Product Moment Correlation method was then used. Differences were considered significant at P < 0.05.

3. Results

3.1 FBLN5 expression and secretion are reduced in human AAA
The expression of FBLN5 was analysed in abdominal aorta samples from both AAA patients and multi-organ donors (controls). Clinical data of patients and donors are shown in Supplementary material online, Table S1. FBLN5 mRNA and protein levels were significantly lower in aneurysmata samples compared with controls (a decrease of 72 and 89%, respectively, (P < 0.0001)) (Figure 1A and B). Western blot analysis evidences the presence of two main bands corresponding to both native and cleaved-FBLN5 forms, as has been previously described in human lung tissue.24 FBLN5 immunostaining was detected in the media of both non-aneurysmata and aneurysmata aortas, although it was lower in AAA samples (Figure 1C). α-Actin staining in consecutive sections indicates that FBLN5 localize in VSMC (see Supplementary material online, Figure S1), in which FBLN5 staining is observed in the extracellular space, cell tips, and cell periphery (Figure 1C), with a similar pattern to that previously reported in endothelial cells.16 Accordingly, in tissue-conditioned medium from media of aneurysmata aortas, FBLN5 protein levels were significantly reduced compared with those from healthy samples (Figure 1D).

3.2 Vascular inhibition of FBLN5 expression by in vivo siRNA delivery promotes aortic dilation
To investigate the potential role of FBLN5 down-regulation in AAA pathophysiology, vascular FBLN5 expression was knocked down in vivo using an atelocollagen formulation that allows an efficient siRNA delivery. FBLN5 siRNA or random siRNA(siRD)/atelocollagen complexes were applied in the supraparenchymal aorta of C57BL/6j mice and, 4 days later, animals were subjected to Ang II infusion for another 14 days. All the animals survived to the end of the study, when the specific aortic region in which the siRNA/atelocollagen complex was applied was analysed. Local atelocollagen-mediated delivery of a siRNA against FBLN5 efficiently reduced abdominal aortic FBLN5 mRNA levels (siRD: 1 ± 0.16 vs. siFBLN5: 0.48 ± 0.075; P < 0.05). Furthermore, FBLN5 silencing induced a significant enhancement in aortic diameter recorded by ultrasonography (Figure 1E and F) and promoted a concomitant increase in the vascular expression of EMR-1 (marker of macrophage infiltration; Figure 1G) and IL-1β (Figure 1H), compared with siRD mice.

3.3 Vascular FBLN5 expression is decreased by inflammatory stimuli
Because inflammation is a key feature of AAA, we tested whether vascular FBLN5 could be regulated by inflammatory stimuli. In human aortic VSMC exposed to TNFα or LPS, we observed a significant down-regulation of FBLN5 mRNA (Figure 2A) and protein levels as determined by western blot and immunocytochemistry (Figure 2B and C). FBLN5 secretion into the cell media was also reduced by inflammatory stimuli (Figure 2B). Afterwards, we assessed whether vascular expression of FBLN5 is sensitive to inflammatory stimuli in vivo. Mice challenged with LPS (0.5 mg/kg, 24 h) exhibited a strong decrease in aortic FBLN5 expression, at both mRNA and protein levels (Figure 2D and E). In these animals, the increased vascular expression of IL6 and IL8 was used as a control of the inflammatory response triggered by LPS (data not shown).20

To gain more insight into the mechanisms responsible for this modulation, human aortic VSMC were exposed to TNFα and LPS in the presence of inhibitors of two classical pathways involved in vascular inflammation: parthenolide (an NFκB inhibitor) or nordihydroguaiaretic acid (NDGA, an AP-1 inhibitor). However, neither parthenolide nor NDGA or their combination prevented the down-regulation of FBLN5 elicited by inflammatory mediators in human VSMC (see Supplementary material online, Figure S2A and C). The effect of parthenolide on the expression of Chemokine (C-C motif) ligand 20 (CCL20; see Supplementary material online, Figure S2B), a well-known NFκB target,20 and on the nuclear translocation of p65 (see Supplementary material online, Figure S2D) was assessed as positive controls.

3.4 HDAC inhibitors normalized vascular FBLN5 expression altered by inflammatory stimulus
Epigenetics is now emerging as a relevant factor in cardiovascular diseases.4 In fact, the main risk factors for AAA (i.e. cigarette smoking, age, and male gender) have been linked with epigenetic responses. Therefore, we sought to determine whether HDAC inhibition could affect the down-regulation of FBLN5 under pro-inflammatory conditions. Trichostatin A (TSA; an inhibitor of Class I and II HDACs) prevented the reduction of FBLN5 mRNA levels elicited by either TNFα or LPS (Figure 3A). Furthermore, MS-275 (a Class I-specific HDAC inhibitor) attenuated FBLN5 down-regulation with a similar efficiency than TSA, suggesting a major contribution of Class I HDACs (Figure 3A). Similarly, MS-275 also prevented the decrease in FBLN5 protein levels triggered by inflammatory stimuli in VSMC (Figure 3B). As expected, MS-275 induced histone hyperacetylation, increasing the levels of Ac-H3K18 (Figure 3B). More interestingly, inflammatory...
Figure 1  FBLN5 expression and secretion is reduced in human AAA. (A) FBLN5 mRNA levels in AAA (n = 27) and healthy abdominal aorta (Control; n = 15). Data are expressed as mean ± SEM (*P < 0.0001; Mann–Whitney). (B) FBLN5 protein levels were evaluated by western blot in arterial wall lysates from AAA and control samples. Representative immunoblot and graph corresponding to quantification are shown (Control: n = 7; AAA: n = 12). Results are expressed as mean ± SEM (*P < 0.0001; Mann–Whitney). (C) Immunohistochemical analysis of FBLN5 expression in haematoxylin counterstained sections from AAA and healthy aortas. The indicated areas are shown at high magnification (right panel). Bar: 100 μm. (D) FBLN5 protein levels analysed by western blot in tissue-conditioned medium from AAA (n = 12) and control vessels (n = 12). Results are expressed as mean ± SEM (*P < 0.001; Mann–Whitney). (E–H) Atelocollagen-mediated delivery of a siRNA against FBLN5 (siFBLN5) or a random siRNA (siRD) in Ang II-infused C57BL/6J mice for 14 days. (E) Representative high-frequency ultrasound frames of abdominal aortas from each group. (F) Maximal suprarenal abdominal aortic diameter (in mm) measured from ultrasound images at Day 14 post-infusion. EMR-1 (G) and IL-1β (H) mRNA levels were determined by real-time PCR. Results are expressed as mean ± SEM (n = 6; *P < 0.05; Mann–Whitney).
mediators strongly decreased H3K18 acetylation, effect that was counteracted by MS-275 (Figure 3B). The role of Class I HDACs was tested in knockdown experiments. The efficient blockade of HDACs 1, 2, and 3 (see Supplementary material online, Figure S3) significantly prevented the down-regulation of FBLN5 by inflammatory stimuli (Figure 3C). Finally, to investigate whether inflammatory stimuli could affect the occupancy of acetylated histones at the FBLN5 promoter, we performed chromatin immunoprecipitation assay (ChIP) analyses using an antibody against Ac-H3K18 or a Pan ac-H3 antibody. Immunoprecipitated DNA was amplified using primers surrounding FBLN5 transcription start site (proximal FBLN5 promoter region: from -353 to -204). Independently of the antibody used, we observed that TNFα significantly decreased the level of acetylated H3 bound to FBLN5 promoter, while MS-275 prevented this effect (Figure 3D). Interestingly, MS-275 also attenuated the decrease in aortic FBLN5 mRNA levels observed in mice challenged with LPS (Figure 3E), supporting the relevance of this mechanism in vivo.

3.5 Inflammatory mediators decrease FBLN5 expression in VSMC through a transcriptional mechanism sensitive to HDAC inhibition

These results prompted us to investigate the molecular mechanisms underlying FBLN5 inhibition by inflammatory stimuli and its modulation by HDAC inhibition. Transcription blockade with actinomycin D abolished LPS- and TNFα-induced inhibition of FBLN5 expression in VSMC (Figure 4A), while a time-course mRNA decay analysis evidenced that the turnover of FBLN5 mRNA was unaffected by these effectors (Figure 4B). Consistent with this, transient transfection of VSMC with a FBLN5 promoter-driven luciferase reporter construct evidenced that both TNFα and LPS down-regulated FBLN5 transcriptional activity (Figure 4C), while MS-275 abolished their effect. Thus, FBLN5 is down-regulated by inflammatory stimuli through transcriptional mechanisms involving a promoter region targeted by HDAC inhibitors.
Figure 3  HDAC inhibitors normalize vascular FBLN5 expression altered by inflammatory stimuli. (A) Human VSMCs were exposed to LPS (100 ng/mL) or TNFα (TNF, 50 ng/mL) in the presence of MS-275 (2 μM) or trichostatin-A (TSA, 1 μM) during 24 h. FBLN5 mRNA levels were analysed by real-time PCR. Results are expressed as mean ± SEM (at least n = 9; *P < 0.0001; #P < 0.01; ANOVA). (B) FBLN5 and acetylated Histone H3 (Ac-H3K18) protein levels in VSMC treated as described in (A). The graphs corresponding to immunoblot densitometric analysis are shown in the lower panel (n = 4; *P < 0.0001; #P < 0.001; ANOVA). (C) VSMC were transfected with a siRNA pool against HDACs 1, 2, and 3 (siHDAC) or a control siRNA (siRandom) and exposed to TNFα. FBLN5 mRNA levels are expressed as mean ± SEM (n = 9; *P < 0.0001; #P < 0.001; ANOVA). (D) Association of acetylated Histone H3 with the human FBLN5 promoter analysed by ChIP with Ac-H3K18 (upper panel) or pan acetylated Histone H3 (Pan-ac-H3; lower panel) antibodies. Data were normalized to the total input DNA and represented as mean ± SEM (n = 4; *P < 0.05; ANOVA). (E) FBLN5 mRNA levels determined in mouse aorta from LPS-challenged animals (0.5 mg/kg; i.p; 24 h). Half of the animals were pre-treated with MS-275 (5 mg/kg; i.p.) 24 h before LPS administration. Control animals received saline. A group exclusively treated with MS-275 was also included. Results are represented as mean ± SEM (at least n = 6; *P < 0.001; #P < 0.05; Kruskal–Wallis).
3.6 SOX9 is down-regulated in VSMC exposed to inflammatory stimuli and in human AAA

Serial deletion analysis of FBLN5 promoter enclosed the responsiveness to inflammatory stimuli into the proximal 329 bp region (Figure 5A). In silico analysis of this region identified two putative cis-acting elements (at -304 and -251) highly homologous to the heptameric SOX (Sry-related high-mobility group box) consensus motif (A/T A/T CAA A/T G) and conserved among species (Figure 5B, upper panel). Site-directed mutagenesis of the -304 motif significantly decreased FBLN5 transcriptional activity, while mutagenesis of the proximal site had a negligible effect (Figure 5B, lower panel).

Among members of SOX family, SOX9 has been reported to participate in the control of genes involved in ECM synthesis/deposition.25,26 Both LPS and TNFα reduced SOX9 expression in VSMC (Figure 6A), and time-course experiments showed that the decline in SOX9 expression (mRNA and protein levels) induced by TNFα preceded FBLN5 down-regulation (Figure 6B and C). Interestingly, SOX9 mRNA levels were down-regulated in aneurysmatic aortic samples (Figure 6D) and significantly correlated with those of FBLN5 in these human tissues \( r = 0.6843; p < 0.0001; \) Figure 6E). Further, immunohistochemical analysis revealed reduced SOX9 staining in AAA samples compared with donor aortas (Figure 6F). SOX9 expression was detected in areas of α-actin staining (see Supplementary material online, Figure S4). These results are consistent with a potential regulation of vascular FBLN5 by SOX9 under inflammatory conditions.

3.7 SOX9 is up-regulated by HDAC inhibitors preventing the down-regulation of FBLN5 induced by inflammatory stimuli in human VSMC

Lentiviral transduction of VSMC with a vector that over-expresses SOX9 (pLVX/SOX9; see Supplementary material online) significantly increased FBLN5 mRNA levels (Figure 7A) and FBLN5 promoter activity (Figure 7B), preventing, in both cases, the inhibitory effects of TNFα. Because SOX9 is regulated by HDAC inhibitors in non-vascular cells,27 – 29 we hypothesized that the up-regulation of SOX9 by HDAC inhibitors could underlie the modulation of FBLN5 expression by these drugs in VSMC. Accordingly, MS-275 increased the expression of SOX9 and prevented the down-regulation of this transcription factor evoked by TNFα (Figure 7C and D). Likewise, Class I HDACs knockdown abolished the decrease in SOX9 mRNA levels induced by this cytokine in VSMC (Figure 7E). Finally, we carried out ChIP assays to assess the role of SOX9 and HDACs in the regulation of FBLN5 transcription in vitro (Figure 7F). As observed, TNFα significantly attenuated the interaction of SOX9 with FBLN5 promoter. Interestingly, the inhibition of HDACs by MS-275 increased the recruitment of SOX9 to FBLN5 promoter, thereby counteracting the TNFα-mediated decrease in the association of SOX9 to this region. These results suggest that the modulation of SOX9 expression by HDAC inhibitors is critical to prevent the down-regulation of FBLN5 under pro-inflammatory conditions.

Figure 4  Inflammatory mediators decrease FBLN5 transcriptional activity. (A) FBLN5 mRNA levels evaluated in VSMC exposed to inflammatory stimuli (LPS: 100 ng/mL or TNFα: 50 ng/mL) for 24 h in the presence or absence of actinomycin-D (Act D; 1.6 μM), a transcriptional inhibitor. Data are expressed as mean ± SEM (n = 6; 4p < 0.0001; 6p < 0.001; ANOVA). (B) Effect of inflammatory stimuli on FBLN5 mRNA stability in cells stimulated for 24 h. Then Act D was added (t = 0), and mRNA levels were assessed at different times by real-time PCR. Data are expressed in percentage of mRNA relative to t = 0 for each experimental condition and are the mean ± SEM of n = 6 experiments. (C) Human VSMC transfected with the pFBLN5-635 luciferase construct were pre-treated with MS-275 (2 μM) and exposed to inflammatory stimuli (LPS: 100 ng/mL or TNFα: 50 ng/mL, 24 h). Both Firefly and Renilla luciferase activities were determined. Data (normalised by Renilla luciferase activity) are expressed as mean ± SEM (at least n = 5; 4p < 0.05; 6p < 0.01; ANOVA).

Figure 5  The proximal FBLN5 promoter is an SOX9 target. (A) A schematic representation of the human FBLN5 proximal promoter showing two putative SOX binding sites. (B) A genomic Southern blot using DNA from pulmonary artery was hybridized with a 1.8-kb insert of the FBLN5 promoter region (BamHI-HindIII fragment), and with 5′ and 3′ FBLN5-specific primers (F and R, respectively). (C) Firefly luciferase activity, which reflects FBLN5 promoter activity, was assessed in VSMC exposed to inflammatory stimuli and in human AAA. (D) Immunohistochemical analysis revealed reduced SOX9 staining in AAA samples compared with donor aortas. (E) A representative example of ChIP assay for SOX9 binding to the FBLN5 promoter region in VSMC exposed to inflammatory stimuli. (F) A representative example of ChIP assay for SOX9 binding to the FBLN5 promoter region in human AAA. (G) A schematic representation of the human FBLN5 proximal promoter showing two putative SOX binding sites.
repair of those aneurysms under a high risk of rupture is the only management option for this disease. In search of new therapeutic targets in AAA, we focused our interest on FBLN5, an elastogenic protein essential for proper elastic fibre assembly and vasculogenesis, which plays a critical role in tissue development, remodelling, and repair. Abnormal FBLN5 expression has been related to pathological processes such as chronic obstructive pulmonary disease, cutis laxa, macular degeneration, pelvic prolapse, and tumour progression. The present study evidences for the first time that FBLN5 expression is decreased in aneurysmatic samples, associates this down-regulation with the inflammatory component of the disease, and demonstrates the key role of epigenetics in the underlying mechanisms.

By real-time PCR, western blot, and immunohistochemistry, we confirmed the significant reduction of FBLN5 levels in human aneurysmatic aortic tissues. Further, FBLN5 levels were also significantly reduced in tissue-conditioned medium from human AAA. The depletion of VSMC in the media layer characteristic of human AAA could explain the decrease in FBLN5 expression observed in aneurysmatic samples. However, our immunohistochemistry data showed low expression of FBLN5 in AAA even in VSMC-rich areas. Furthermore, both studies in VSMC in culture and in vivo approaches in LPS-challenged mice, support that inflammation plays a critical role in the inhibition of FBLN5 in the vasculature.

FBLN5 deficiency in mice triggers an acute elastinopathy that results in severe vascular abnormalities characterized by an elongated...
Figure 6  SOX9 is down-regulated by inflammatory stimuli in AAA and VSMC. (A) Human VSMC were exposed to inflammatory stimuli (LPS: 100 ng/mL or TNFα: 50 ng/mL; 24 h), and SOX9 mRNA levels were analysed. Results are expressed as mean ± SEM (n = 6; *P < 0.001; ANOVA). (B and C) Time-course analysis of FBLN5 (empty circles) and SOX9 (filled circles) mRNA (B; n = 6) and protein levels (C, n = 4) in VSMC stimulated with TNFα. The graph showed in the lower panel corresponds to the densitometric analysis of immunoblots. Data are expressed as mean ± SEM (*P < 0.01; ANOVA). (D) SOX9 mRNA evaluated in abdominal aortas from aneurysmatic patients (n = 17) and healthy donors (Control; n = 15). Results are expressed as mean ± SEM, (*P < 0.01; Mann–Whitney). (E) Positive correlation between FBLN5 and SOX9 mRNA levels in patients and donors (r = 0.6843, P < 0.0001; Pearson Product Moment Correlation). (F) Immunohistochemical analysis of SOX9 expression in haematoxylin counterstained sections from human AAA and healthy aortas (Bar: 100 μm).

and tortuous aorta. Although aortic elastic laminae were disrupted in FBLN5 knockout animals, they show no signs of spontaneous aneurysm or aortic dissection.9,10 Our results, however, support the pathophysiological role of FBLN5 in aneurysm development, since the partial vascular FBLN5 silencing triggers an aortic dilation and increases the local inflammatory response elicited by Ang II in
Figure 7 The up-regulation of SOX9 by MS-275 prevents the TNFα-mediated inhibition of FBLN5 in human VSMC. (A) Human VSMC were transduced with pLVX-SOX9 (SOX9; filled circles) or the corresponding empty vector (V; empty circles) and stimulated or not with TNFα (50 ng/mL, 24 h). FBLN5 mRNA levels were assessed by real-time PCR and expressed as mean ± SEM (n = 6; *P < 0.001; ANOVA). (B) VSMC were co-transfected with the pFBLN5-635 luciferase construct and the pLVX-SOX9 (SOX9; filled circles) or the corresponding empty vector (V; empty circles) and stimulated with TNFα (50 ng/mL, 24 h). Luciferase activity (normalized by Renilla) is expressed as mean ± SEM (n = 5; *P < 0.05; #P < 0.0001; ANOVA). (C and D) Cells were treated with TNFα in the presence or absence of MS-275 (2 µM). SOX9 mRNA (C; at least n = 6) and protein levels (D; n = 4) were analysed. The graph (lower panel) corresponds to the densitometric analysis of immunoblots. Results are expressed as mean ± SEM (*P < 0.03; #P < 0.0001; ANOVA). (E) VSMC were transfected with a pool of specific siRNAs against HDACs 1, 2, and 3 (siHDAC) or with a control siRNA (siRandom) and exposed to TNFα (50 ng/mL; black bars) for 24 h. SOX9 mRNA levels are expressed as mean ± SEM (n = 9; *P < 0.001; ANOVA). (F) Human VSMC were cultured as described in (C), and ChIP assays were performed using a SOX9 specific antibody or a non-specific rabbit IgG. SOX9 recruitment to FBLN5 promoter was quantified by real-time PCR using FBLN5 promoter specific primers (upper panel). Data, normalized to the total input DNA, are represented as mean ± SEM (at least n = 4). (*P < 0.05; #P < 0.005; ANOVA). Lower panel: agarose gel electrophoresis of PCR products. Equal input DNA and control IgG immunoprecipitations are shown.
adult C57BL/6J mice, a strain little susceptible to Ang II-induced aneurysms. The present study shows that vascular FBLN5 is epigenetically regulated by histone acetylation. To our knowledge, this is the first study reporting the modulation of FBLN5 expression by HDACs. In human VSMC, the selective inhibition of Class I HDACs (by MS-275 and gene silencing) abolished the inflammation-induced down-regulation of FBLN5. This effect was corroborated in vivo, in LPS-challenged mice treated with MS-275, further supporting that histone acetylation may control FBLN5 transcription. In VSMC, inflammatory stimuli reduced global levels of histone H3 acetylation, as has been reported in non-vascular cells,34,35 and MS-275 counteracted this effect. In fact, this drug efficiently modulated the occupancy of acetylated histones at the proximal promoter of FBLN5. Since decreased promoter acetylation has been related to transcriptional repression,36 these findings pointed to a role of epigenetics in the down-regulation of FBLN5 by inflammatory stimuli. Although in dendritic and granulose cells inflammatory stimuli were able to alter HDAC expression,37,38 our data in VSMC do not support a similar effect on Class I HDACs. Therefore, the specific mechanisms involved in the regulation of histone acetylation by pro-inflammatory stimuli in VSMC are probably cell type dependent.

Transient transfection assays demonstrated the involvement of transcriptional mechanisms in the modulation of FBLN5 by inflammatory stimuli and HDAC inhibitors. Our studies delimit this regulation into the proximal promoter of FBLN5 (329 bp long), which contains critical elements for FBLN5 expression, as we and others have reported.16,39 In this region, we identified two putative SOX elements conserved between primates and mice. In fact, mutation of the -304 motif significantly decreased basal FBLN5 transcriptional activity, suggesting the role of SOX transcription factors in the regulation of its expression.

The SOX family of transcription factors comprises a versatile group of proteins involved in embryonic development and human diseases that play a wide range of functional roles, including the control of cell proliferation and differentiation.36,40,41 In the last years, a great effort has been made to identify target genes of the SOX family.26 Our investigations identify FBLN5 as a SOX9-target gene in human VSMC. SOX9 regulates multiple ECM proteins and is critical for chondrogenesis.25,26 Interestingly, we found that SOX9 expression decreases in human aneurysmatic samples and correlates with FBLN5 expression. Since FBLN5 protects the vessel wall structure against injury17 and SOX9 is required for the normal deposition of ECM proteins,42 the parallel regulation of SOX9 and FBLN5 in AAA is consistent with a role for both proteins in the impaired ECM remodelling in AAA. Furthermore, the down-regulation of FBLN5 and SOX9 has been related to increased apoptosis in vascular cells and mesenchymal cells, respectively.16,25,43 However, further research will be required to clarify whether the inhibition of SOX9 could participate in the pathogenesis of AAA. We observed that SOX9 expression was inhibited by inflammatory mediators in VSMC, in agreement with previous data in other cell types.44,45 The temporal expression pattern of SOX9 in VSMC treated with TNFα (preceding the down-regulation of FBLN5), and the ability of SOX9 over-expression to overcome the inhibitory effect of inflammatory stimuli on FBLN5, supported a role for this transcription factor in the regulation of FBLN5 gene expression, which we subsequently confirmed by transient transfection studies and ChIP assays.

HDAC inhibitors induced SOX9 expression and abrogated the down-regulation of both SOX9 and FBLN5 triggered by inflammatory stimuli. Further, in cells treated with TNFα, MS-275 prevented the decrease in the binding of SOX9 to FBLN5 promoter. Taken together, these results support that HDAC inhibitors modulate FBLN5 expression by enhancing the recruitment of SOX9 to its promoter. Interestingly, we have recently reported the up-regulation of specific HDACs in human AAA and that HDAC inhibitors limit aneurysm progression in a preclinical model, suggesting the therapeutic interest of HDAC inhibition in AAA.46

Overall, our data provide novel insights into the mechanisms underlying the destructive ECM remodelling associated with aortic dilation which could culminate in AAA. These results point to a pathophysiological role of FBLN5 down-regulation in AAA, its close relationship with the inflammatory component of this disease, and the ability of HDAC inhibitors to normalize vascular FBLN5 expression through a SOX9-dependent mechanism. Our data suggest that pharmacological approaches aimed to preserve FBLN5 could be useful to prevent the disorganization of ECM induced by inflammation in AAA.

Supplementary material
Supplementary material is available at Cardiovascular Research online.

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