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Fibulin-4 deficiency results in ascending aortic aneurysms: a potential link between abnormal smooth muscle cell phenotype and aneurysm progression

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Abstract

Rationale—Loss of fibulin-4 during embryogenesis results in perinatal lethality due to aneurysm rupture, and defective elastic fiber assembly has been proposed as an underlying cause for the aneurysm phenotype. However, aneurysms are never seen in mice deficient for elastin, or for fibulin-5, which absence also leads to compromised elastic fibers.

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Novelty and Significance

Fibulin-4 (Fbln4) is a secreted glycoprotein essential for the formation of elastic fibers. *Fbln4*-null mice develop aortic aneurysms and die at birth from aneurysm rupture. Interestingly, however, mice deficient for the components of elastic fibers, such as elastin or fibulin-5, do not develop aneurysms despite severe elastic fiber defects. These observations led us to hypothesize that fibulin-4 may play crucial roles not only in elastogenesis but also for normal development of the aortic wall. By analyzing *Fbln4*-null and smooth muscle cell (SMC)-specific *Fbln4* knockout mice (*Fbln4^{SMKO}*), we showed that fibulin-4 is critical for maintenance of a permissive and stable environment for SMC differentiation. Second, we described focal proliferation of SMCs with marked upregulation of phosphorylated (p)-ERK1/2 levels and disruption of the extracellular matrix (ECM) in aneurysm lesions. The aneurysm involving defective SMCs is remarkably similar to a subset of human thoracic aortic aneurysms in which mutations of SMC-specific contractile genes have been identified to be the cause. This new animal model provides opportunities for pharmacological interventions that target the ERK1/2 MAPK signaling pathway for treatment of thoracic aortic aneurysms. In addition, investigations of the mechanism underlying the altered signaling in *Fbln4^{SMKO}* mice will expand our knowledge on the role of ECM as a modulator of cell signaling.

What is known?

- Fibulin-4 is an elastin binding ECM protein.
- Congenital loss of fibulin-4 results in severe disruption of elastic fibers and aortic aneurysms in mice and humans.

New information contributed by this study?

- Provides evidence that fibulin-4-induced phenotypic alterations of SMCs play a role in aneurysm development.
- Shows that congenital loss of fibulin-4 leads to marked upregulation of phosphorylated (p)-ERK1/2 in SMCs and loss of SMC contractile phenotype in aneurysm lesions.
- Provides a basis for potential ERK1/2 signaling-targeted therapy for ascending aortic aneurysms in humans.

Objective—We sought to determine the mechanism of aneurysm development in the absence of fibulin-4 and establish the role of fibulin-4 in aortic development.

Methods and Results—We generated germline and smooth muscle cell (SMC)-specific deletion of the fibulin-4 gene in mice (*Fbln4*^{GKO} and *Fbln4*^{SMKO}, respectively). *Fbln4*^{GKO} and *Fbln4*^{SMKO} aortic walls fail to fully differentiate, exhibiting reduced expression of SM-specific contractile genes and focal proliferation of SMCs accompanied by degenerative changes of the medial wall. Marked upregulation of ERK1/2 signaling pathway was observed in the aneurysmal wall of *Fbln4*^{GKO} and *Fbln4*^{SMKO} mice and both mutants developed aneurysm predominantly in the ascending thoracic aorta. In vitro, *Fbln4*^{GKO} SMCs exhibit an immature SMC phenotype with a marked reduction of SM-myosin heavy chain and increased proliferative capacity.

Conclusion—The vascular phenotype in *Fbln4* mutant mice is remarkably similar to a subset of human thoracic aortic aneurysms caused by mutations in SMC contractile genes. Our study provides a potential link between the intrinsic properties of SMCs and aneurysm progression in vivo and supports the dual role of fibulin-4 in the formation of elastic fibers as well as terminal differentiation and maturation of SMCs in the aortic wall.

Keywords

Aneurysms; ECM; smooth muscle cells; mouse; elastic fibers

Introduction

Aortic aneurysms are characterized by abnormal enlargements of the aorta caused by global or localized weakness of the vessel wall. Traditionally, aneurysm formation is believed to involve defects in synthesis or assembly of extracellular matrix (ECM) proteins or accelerated degradation of vascular ECM (reviewed in 1, 2). For example, mutations in the fibrillin-1 (*FBN1*) or type III alpha 1 collagen gene (*COL3A1*) genes are responsible for Marfan syndrome and vascular Ehlers-Danlos syndrome^{3, 4}, respectively. Mutations in *Fbn1* profoundly affect the formation of microfibrils that surround an elastin core and are required for proper assembly of elastic fibers⁵. Genetically-engineered mice provide further evidence that the vascular ECM is the critical determinant of aneurysm development, including an allelic series of *Fbn1* mutant mice, and mice homozygous for null alleles of the genes encoding lysyl oxidase, a crosslinking enzyme for elastin and collagen, and ECM proteins such as fibulin-4 (*Fbln4*) and biglycan (*Bgn*)⁶⁻⁹. In addition to the disruption of ECM in the vessel wall, alterations of SMC intracellular contractile proteins have recently been shown to be responsible for subsets of thoracic aneurysms in humans¹⁰⁻¹². Two heterozygous mutations in *MYH11* (SM-MHC) were identified in kindreds presenting with thoracic aortic aneurysms and/or aortic dissections (TAADs) and patent ductus arteriosus. Heterozygous missense mutations in *ACTA2* (SM α -actin) were also found in 14% of inherited TAADs¹². Thus, the pathogenesis of aortic aneurysm formation may involve abnormalities in homeostasis of vascular ECM proteins or abnormal SMC development, or both.

Fibulin-4 belongs to the fibulin family of ECM proteins consisting of seven known members characterized by tandem repeats of calcium-binding epidermal growth factor (cbEGF)-like motifs and a C-terminal fibulin module¹³. In the aorta, fibulin-5 is expressed at one magnitude higher than fibulin-4 and its absence leads to compromised elastic fibers with aggregates of elastin, but does not develop aneurysms.^{14, 15} *Fbln4*-null (*Fbln4*^{-/-}) mice, in contrast, exhibit a more severe phenotype with perinatal lethality due to rupture of aortic aneurysms and marked disruption of elastic fibers⁸. Genetic mutations in the *FBLN4* have also been identified in two human cutis laxa patients, both of which exhibited aortic aneurysms^{16, 17}. Defective elastogenesis was proposed to be an underlying cause of aortic aneurysms in *Fbln4*^{-/-} mice, however, *elastin*-null mice (*Eln*^{-/-}) do not develop aortic aneurysms, rather they exhibit stenosis

of the aorta due to subendothelial proliferation of SMCs¹⁸. It is not clear, therefore, whether the phenotypic differences between *Fbln4*^{-/-} and *Fbln5*^{-/-} mice are due solely to the difference in severity of elastic fiber defects. It is possible that fibulin-4 has additional function(s) that act independent of, or in concert with, elastogenesis in the developing aorta. Recently, hypomorphic *Fbln4*^{R/R} mice were shown to contain increased phosphorylated (p)-Smad2, enhanced production of CTGF and collagen fibers, and increased proliferation of SMCs in the aortic wall¹⁹. However, the precise mechanism of upregulation of TGF- β in *Fbln4*^{R/R} mice or whether it is a primary cause of the vascular phenotype is unclear, especially since SMC-specific ablation of TGF- β receptor type 2 causes aortic aneurysms by down-regulating elastogenic genes²⁰.

To determine the role of fibulin-4 in aneurysm formation, we generated mice with a germline deletion or vascular cell-specific deletion of *Fbln4*. SMC-specific, but not endothelial cell (EC)-specific knockout of *Fbln4*, resulted in the formation of large aneurysms exclusively in the ascending aorta. We found that SMCs failed to fully differentiate in the absence of fibulin-4 in the aorta as evidenced by down-regulation of SMC-specific contractile protein genes and an immature phenotype of *Fbln4*-null SMCs accompanied by focal hyperproliferation of SMCs and degenerative changes affecting the integrity of the vessel wall. These data highlight a novel role for fibulin-4 in the development of SMCs, and together with its role in the formation of elastic fibers, fibulin-4 functions to protect the aortic wall against aneurysm formation in vivo.

Materials and Methods

Mouse

Generation of conditional knockout mice is described in the expanded Materials and Methods sections online.

Mechanical stretch

Two or 4×10^5 cells were plated on 6-well silicon elastomer-bottomed culture plates covalently coated with type I collagen (Flexcell). After allowing cells to adhere to the plates (6-12 h), the media was changed to serum free and incubated for 24 h to synchronize the cell cycle prior to initiation of stretch stimulation. Cells were subjected to unidirectional cyclic strain (0.2 Hz and 20% strain) for 24 h in a humidified incubator with 5% CO₂ at 37°C using FX-4000T Flexcell Tension Plus system (Flexcell International). Cells incubated in static condition served as controls.

Standard protocol was used for immunostaining, Western blotting, qPCR, gelatin zymography, histological and electron microscopic analysis and is available online.

Results

Germline and SMC-specific deletions of *Fbln4* in vivo

To investigate aneurysm development in the absence of fibulin-4, we generated a conditional allele of *Fbln4* using Cre-loxP technique in ES cells (Online Figure IA). *Fbln4*^{neo-loxP} mice were mated with transgenic mice expressing the heat stable *Flp*-recombinase to generate mice homozygous for *Fbln4*^{loxP/loxP} (Online Figures IB, C). *Fbln4*^{neo-loxP} mice were also mated with *CAG-Cre* transgenic mice to delete *Fbln4* in germline, and subsequently homozygous mice for null alleles were generated (termed *Fbln4*^{GKO}). In concordance with the previous reported data⁸, all *Fbln4*^{GKO} mice died in or after parturition due to aneurysm rupture (Online Figure ID) and quantitative RT-PCR (qPCR) confirmed absence of *Fbln4* in the aorta of *Fbln4*^{GKO} mice (Online Figure IE).

Fbln4 is ubiquitously expressed during embryogenesis and is detected in both ECs and SMCs in the aorta (Online Figures IIA, IIB). To determine the cell type responsible for the aneurysm phenotype, we generated EC-specific and SMC-specific knockout mice for *Fbln4* by mating *Fbln4^{loxP/loxP}* mice with transgenic mice expressing *Cre*-recombinase under a control of the *Tie2* promoter and *SM22 α* promoter, respectively^{21, 22}. EC-specific knockout mice (*Fbln4^{loxP/GKO};Tie2-Cre*, termed *Fbln4^{ECKO}*) were healthy and indistinguishable from control littermates. Histological finding of the aorta was normal (Online Figure III). In contrast, SMC-specific knockout mice (*Fbln4^{loxP/GKO};SM22-Cre*, termed *Fbln4^{SMKO}*) began to die spontaneously at approximately 2 months of age despite absence of embryonic or neonatal lethality. Aortae harvested from adult *Fbln4^{SMKO}* mice exhibited large aneurysms exclusively in the ascending aorta (Figure 1A). Aneurysms were observed in *Fbln4^{SMKO}* mice with complete penetrance. The ductus arteriosus was closed and the ligamentous remnant was markedly elongated. qPCR confirmed a significant reduction of *Fbln4* transcripts in the ascending aorta where the aneurysms developed, as well as in the descending thoracic aorta where only elongation was evident (Figure 1B). These data indicate that loss of SMC-derived fibulin-4 is responsible for the aneurysm formation and that fibulin-4 derived from other cell types compensated for aneurysm formation during embryonic life and perinatal lethality in *Fbln4^{SMKO}* mice.

Compromised elastic fibers, increased wall thickness and disarray of SMCs in the *Fbln4^{GKO}* aorta

To better understand the pathological processes of aneurysm formation in the absence of *Fbln4*, we performed a developmental analysis of the ascending aorta in *Fbln4^{GKO}* embryos. Consistent with a previous observation⁸, elastic fibers were clearly detected in the aortic wall of wild-type embryos at E14.5, whereas elastic fibers were decreased in the aorta from *Fbln4^{GKO}* embryos (Online Figures IVb, d). Despite compromised elastic fibers in the *Fbln4^{GKO}* aorta, at this timepoint, the overall morphology of SMCs was indistinguishable from the wild-type (Online Figures IVa, IVc). From E15.5, elastic fibers increased progressively in the wild-type aorta and SMCs became spindle-shaped with elongated nuclei (Online Figures IVe-f, IVi-j). In the *Fbln4^{GKO}* aorta, however, elastic fiber assembly remained defective and SMCs appeared immature with round nuclei (Online Figure IVg-h, IVk-l). Although not reported previously, eosin-positive focal degeneration and marked proliferation of SMCs were observed in aneurysmal lesions of the *Fbln4^{GKO}* aorta at E17.5 (Online Figure IVo) and E18.5 (arrow in Figure 2b). The degenerative area was devoid of elastic fibers and did not stain with Alcian Blue-PAS, indicating no accumulation of polysaccharides or mucins (Figure 2d and Online Figure IVp, data not shown). Only a small numbers of neutrophils and mononuclear cells were detected around degenerative lesions and some infiltrating through the intima (Online Figure VAa). In advanced lesions with dissection, more infiltrates were observed within the vessel wall (Online Figure VAb). No difference in proMMP-2 or MMP-2 levels was observed between wild-type and *Fbln4^{GKO}* descending thoracic aortae (Online Figure VB). Interestingly, this focal degeneration of the aortic wall was not detected in embryos younger than E17.5. Since *Fbln4^{GKO}* mutants die in or after parturition, we further characterized the aortic wall at E18.5. A significant upregulation of p-ERK in ECs and SMCs was observed in the *Fbln4^{GKO}* aorta (Figure 2f) and GRP78, a marker of the endoplasmic reticulum stress response, was upregulated in SMCs surrounding the degenerative area (Figure 2h). Increased apoptotic cells were also observed in both ECs and SMCs in degenerative areas (data not shown) as well as in advanced aneurysmal lesions (Figure 2j). These observations suggest that aneurysm development in *Fbln4^{GKO}* mice involved not only defective elastic fibers but also abnormal SMCs with degenerative medial wall changes.

Defects in terminal differentiation of SMCs in *Fbln4^{GKO}* aorta

Histological abnormalities in SMCs of the *Fbln4^{GKO}* aorta led us to further evaluate the SMC phenotype in *Fbln4^{GKO}* embryos. Specifically, we examined expression of SMC differentiation markers in wild-type and *Fbln4^{GKO}* descending thoracic aortae at E16.5. In the wild-type aorta, SM α -actin and SM-MHC, early and late SMC differentiation markers, respectively, were expressed throughout the aortic wall (Figures 3Aa and 3Ac). In the *Fbln4^{GKO}* aorta, the intensity of both markers was not homogenous along the longitudinal axis of the aorta, particularly for SM-MHC (Figures 3Ab and 3Ad). At E18.5, SM α -actin and SM-MHC levels were increased in both wild-type and mutant aortae. The mutant aorta, however, still exhibited reduced marker expression compared to the wild-type aorta (Figures 3Ai-3Al). To confirm these findings, tissue extracts from the descending thoracic aorta devoid of aneurysm lesions from *Fbln4^{GKO}* embryos were compared with those of wild-type embryos by Western analysis (Figure 3B). Consistent with tissue immunostaining, we observed significant decreases in SM-MHC and α -SM-actin expression. In addition, a marked increase in MAPK signaling pathways, including p-ERK1/2 and p-MEK1/2, was observed in mutant aortae (Figure 3B). Other components of MAPK pathways were only moderately increased in the mutants.

Disrupted wall structure and alterations of SMC gene expression in *Fbln4^{GKO}* aorta

It has been shown that the aorta lacking elastin exhibits increased SMC proliferation and changes in SMC organization¹⁸. We therefore examined aortic ultrastructure at the electron microscopic level in E18.5 embryos. In the wild-type aorta, near continuous elastic laminae were observed throughout the aortic wall, with those closer to the lumen being more completely formed at this time (Figures 4Aa, 4Ac). SMCs maintained close contacts with neighboring cells and developing elastic fibers. In contrast, discontinuous aggregates of elastin were observed in the *Fbln4^{GKO}* aorta (Figure 4Ab, arrow). Higher magnification showed loose cell-cell associations and very few cell-elastin contacts with increased pericellular spaces in the *Fbln4^{GKO}* aorta, exhibiting an immature vessel wall (Figure 4Ad).

To examine if the absence of fibulin-4 affected SMC-specific gene expression in the aortic wall, we performed qPCR analysis using E18.5 descending thoracic aorta from wild-type and *Fbln4^{GKO}* mice. SMC-specific genes, including *Acta2* and *Myh11*, were downregulated in the mutant aorta (Figure 4B), consistent with the data obtained from Western blot and immunostaining analyses (Figure 3). Interestingly, transcription factors involved in SMC differentiation, including *Myocd* (myocardin), *Mkl1* (myocardin-like 1/MAL/MRTF-A) and *Mkl2* (myocardin-like 2/MRTF-B) were also downregulated. Because the lack of intact elastic fibers has been shown to affect SMC phenotype in the *Eln^{-/-}* aorta, and recombinant tropoelastin induced actin polymerization without changing actin expression in *Eln^{-/-}* SMCs²³, the immature phenotypes of *Fbln4^{GKO}* SMCs may be secondary to the loss of intact elastic fibers. To examine this possibility, we evaluated the expression profile of SMC marker genes in *Eln^{-/-}* aortae. Transcripts for *Myocd*, *Mkl1*, *Mkl2*, *Myh11* or *Acta2* were not downregulated in *Eln^{-/-}* aortae (Online Figure VI). Rather, *Myocd*, *Myh11* and *Acta2* were upregulated compared to wild-type aortae. Although we cannot completely distinguish a direct or elastic fiber-mediated role of fibulin-4 on differentiation of SMCs in the current experimental system, the distinct transcriptional profile of the *Fbln4^{GKO}* aorta suggest a unique and/or additional role of fibulin-4 in development of the aortic wall.

Loss of fibulin-4 results in an immature phenotype in *Fbln4^{GKO}* SMCs

To further investigate the effect of loss of fibulin-4 on SMC phenotype, we isolated SMCs from the aorta of wild-type or *Fbln4^{GKO}* embryos between E17.5 and E18.5. Morphological analysis with phalloidin staining revealed that stress fibers were decreased and cortical actin staining more prominent in *Fbln4^{GKO}* SMCs compared to wild-type SMCs (Figure 5A). SM-

MHC protein content was markedly reduced in *Fbln4^{GKO}* SMCs by Western analysis (Figure 5Ac). Separation of SM1 (204 kDa) and SM2 (200 kDa) by SDS-PAGE revealed that loss of SM-MHC was predominantly due to loss of SM1, the predominant myosin heavy chain in the developed aorta. SM α -actin was highly expressed in SMCs and we did not detect any appreciable difference in SM α -actin content between the genotypes by Western blot analysis (data not shown). Next, we examined the capacity of *Fbln4^{GKO}* cells to proliferate in the absence of serum, a characteristic feature of embryonic-type SMCs²⁴. Wild-type and *Fbln4^{GKO}* SMCs were allowed to adhere to the culture dish and the medium was changed to a serum-free medium and grown for 24h. Immunostaining with anti-phosphorylated histone H3 (PH3), a mitosis marker, showed a significant increase in mitotic cells in *Fbln4^{GKO}* SMCs (Figures 5Bb, 5Bd), whereas positive cells were virtually absent in wild-type SMCs (Figures 5Ba, 5Bc). Proliferation assays performed following stimulation with 20% FBS also exhibited a marked increase in the proliferation of *Fbln4^{GKO}* cells (Figure 5C).

Compromised contractile forces resulting from the mutations of genes encoding for SMC-specific contractile proteins or cardiac sarcomeric proteins are proposed to cause familial thoracic aortic aneurysms and familial hypertrophic or dilated cardiomyopathy, respectively^{25, 26}. Therefore, it is possible that *Fbln4*-null SMCs with reduced expression of SMC contractile proteins may exhibit an abnormal response to mechanical strain compared to wild-type SMCs. To examine this possibility, we performed in vitro stretch assays. Wild-type and *Fbln4^{GKO}* SMCs were plated on a type I collagen pre-coated elastomer well and subjected to cyclic stretch (0.2 Hz, 20% strain) for 24 h without serum. Cells incubated in a static condition served as controls. Changes in SM-MHC and activation of downstream signaling pathways were evaluated by Western analysis. As Figure 5Da shows, wild-type SMCs maintained SM-MHC levels after the stretch stimulation, however, *Fbln4^{GKO}* cells showed a marked decrease in SM-MHC levels in both static and stretch conditions compared to wild-type cells ($p < 0.005$). Although a trend indicating a decrease in SM-MHC expression was observed after stretch stimulation in mutant cells by densitometric analysis, no statistical significance was obtained ($p = 0.056$). Cyclic stretch showed a moderate increase in p-ERK in *Fbln4^{GKO}* SMCs compared to wild-type cells (Figure 5Db), however, statistical significance was not obtained under the given condition ($p = 0.088$, Figure 5Dc). These data suggest that absence of fibulin-4 results in an immature SMC phenotype with compromised level of SM-MHC and altered proliferative response.

Differentiation defect of SMCs and marked upregulation of p-ERK1/2 in the *Fbln4^{SMKO}* aorta

Finally, we asked if postnatal aneurysms in the *Fbln4^{SMKO}* mice share the same underlying pathology as those of *Fbln4^{GKO}* mice. Histological analysis of *Fbln4^{SMKO}* ascending aorta at P90 revealed focal lesions with a thickened aortic wall, which primarily due to an increase in medial SMC layers (Figures 6Aa, 6Ad). Disarray of SMCs was particularly prominent in the outer two-thirds of the aortic wall in which disorganized elastic fibers were also prominent (Figures 6Ab, 6Ae). Inflammatory infiltrates, including neutrophils and macrophages, were not prominently seen in *Fbln4^{SMKO}* aortae. An overall increase of collagen fibers was also detected in the mutant aorta (Figures 6Ac, 6Af). Surprisingly, in contrast to the dramatic changes seen in the ascending aorta, the descending thoracic aorta appeared normal without hyperproliferation of SMCs (Online Figure VIIId). Elastic laminae were more organized compared to those in the ascending aorta, although discontinuities were more frequently observed in mutants compared to the wild-type aorta (Online Figure VIIe). Masson-Trichrome staining revealed significant increases in collagen fibers in the mutant descending thoracic aorta (Online Figure VIIf).

To examine if the differentiation defect of SMCs is also present in the *Fbln4^{SMKO}* aorta, qPCR analysis was performed using ascending and descending thoracic aortae from *Fbln4^{SMKO}* and

control mice at P90. Transcripts for SMC contractile genes were significantly downregulated in the diseased ascending aorta as well as in the descending aorta where no aneurysms were observed (Figure 6B). In addition, *Myocd* was downregulated in both ascending and descending thoracic aortae. Immunostaining of the ascending aorta at P25 with anti-SMemb, a marker of embryonic SMCs²⁷, showed faint staining in the wild-type aorta, whereas persistent expression was detected in the mutant aorta (Figures 6Ca and 6Cc). In contrast, immunostaining with anti-SM-MHC detected strong expression in P90 wild-type ascending aorta, whereas a marked reduction of SM-MHC was observed in the mutant aorta (Figures 6Cb and 6Cd). The reduction of SM-MHC levels was further confirmed by Western blot analysis using ascending aortae harvested from P25 and P90 *Fbln4^{SMKO}* mice (Figure 6D). At both time points, before or after aneurysm development, reduction of SM-MHC was observed in the mutant aorta

Finally, we examined signaling pathways responsible for the proliferative changes of the aneurysmal wall. Upregulation of p-ERK1/2 was observed in the *Fbln4^{SMKO}* aorta at P25 and P90 by Western blot analysis (Figure 7A). Immunostaining of the P90 ascending aorta revealed strong upregulation of p-ERK1/2 in the mutant SMCs compared to control vessels (Figure 7B). Phosphorylated p38 level was moderately increased in the mutant aorta (data not shown). Levels of p-Smad2/3, p-Smad1/5/8, and CTGF, a downstream target of TGF- β , were comparable between control and *Fbln4^{SMKO}* aortae examined at P25 and 3-months of age (Online Figure VIII Aa, B). In addition, we did not detect any difference in p-Smad2/3 levels between wild-type and *Fbln4^{GKO}* aortae by Western blot or immunostaining (Online Figure VIII Ab, data not shown). Taken together, these observations in the *Fbln4^{GKO}* and *Fbln4^{SMKO}* aortae suggested that 1) alteration of SMC-specific genes was not simply a consequence of aneurysm formation in the mutant aorta, and 2) the ERK1/2 pathway played a pivotal role in the focal proliferative response of abnormal SMCs in the aneurysmal wall.

Discussion

Potential contribution of abnormal SMCs to aneurysm development in *Fbln4* mutant mice

Deletion of fibulin-4 in a global or SMC-specific manner resulted in a differentiation defect of SMCs with marked down-regulation of SMC contractile genes. Contractile forces in SMCs are generated by cyclic interactions of myosin heavy chain and actin filaments induced by MLCK-mediated phosphorylation of myosin light chains (Reviewed in 28-29). The mutations in components of the contractile apparatus in SMC such as *ACTA2* and *MYH11* lead to a focal proliferation of SMCs with disarray, disruption of elastic fibers, and eventual loss of SMCs¹⁰⁻¹². It is also shown that patients with *ACTA2* disease haplotype exhibit reduced aortic compliance and increased pulse velocity, altering aortic stiffness¹⁰. Although aortic aneurysms were not observed in genetic ablation of the SM2 isoform of SM-MHC in mice, the mutants did develop an enlarged thin bladder and hydronephrosis and died within 4 weeks of age³⁰. A concomitant reduction of SM1 was observed in the *SM2^{-/-}* bladder and an appropriate ratio of SM1 to SM2 was shown to be critical for normal contractility of SMCs. Absence of *Acta2* also exhibited a significant decrease in vascular contractility and impaired maintenance of blood pressure and blood flow³¹. Thus, quality and quantity of contractile proteins significantly affect contractile functions of SMCs and eventually affect mechanical properties of the aortic wall. Taken together, we suggest that *Fbln4* mutant SMCs with reduced expression of contractile proteins and highly synthetic phenotype, together with the abnormal ECM, alter vessel wall stability and results in maladaptation of the vessel wall, providing a potential link to an aneurysm phenotype (Figure 7C).

Fibulin-4 provides a permissive environment for differentiation and maintenance of SMCs

SMC differentiation is largely governed by the myocardin family of transcriptional co-activators (reviewed in 32). Myocardin, Mkl1 and Mkl2 serve as a potent co-factor of SRF and

upregulate a variety of SMC contractile genes 33-36. The reduced expression of *Myocd*, *Mkl1*, and *Mkl2* in the *Fbln4^{GKO}* mutant aorta indicates a potential mechanism of extracellular regulation of SMC differentiation genes by fibulin-4. Fibulin-4 is located in microfibril bundles which tether elastic fibers to SMCs via integrin-mediated binding at regions of the cell membrane occupied by intracellular membrane-associated dense plaques, anchoring sites for actin filaments 37, 38. Thus, loss of fibulin-4 may disrupt the interaction between elastic fibers and SMCs, leading to the alterations in actin cytoskeleton organization. The actin dynamics between filamentous (F) and globular (G) actin are known to regulate the activity of Mkl1: G-actin binds to cytoplasmic Mkl1 and inhibits translocation of Mkl1 to the nucleus thereby disrupting the interaction between Mkl1 and SRF and downregulating the transcription of SMC contractile genes 39. Our observation that actin filaments were less prominent in *Fbln4^{GKO}* SMCs compared to wild-type cells is consistent with this hypothesis. A similar disruption of the elastin-SMC connection was reported in a mouse deficient for the polycystic kidney disease 1 (*Pkd1*) gene, which developed descending aortic aneurysms with hyperproliferative SMCs 40. Ultrastructural studies of *Fbn1^{C1039G/+}* aortae also revealed disruption between SMC and elastic fiber connections, leading to activation of SMCs 41. Taken together, fibulin-4 is critical for the maintenance of the permissive and stable environment for SMC differentiation.

Elastic fibers and protection of aortic aneurysms

Our present study, together with other reports, confirmed that a loss of fibulin-4 profoundly affects formation of elastic fibers 8, 19. Various experimental manipulations that disrupt elastic fibers have successfully recapitulated pathological changes of aortic aneurysms in rodents 42, 43. Genetic or pharmacological stabilization of elastin or inhibition of its degradation was shown to attenuate development of abdominal aortic aneurysms 44-46. Although elastic fibers or insoluble elastin have been shown to suppress proliferation and migration of SMCs 23, a causal relationship between disrupted elastic fibers and aneurysm formation during development has not been fully established. Interestingly, mice deficient in the fibrillin-1 gene (*Fbn1^{mgN/mgN}*) that die from aneurysm rupture within two weeks of age, exhibit maturation defects of the aortic wall and persistent expression of embryonic SMC markers 47. Thus, components of elastic fibers affect SMC phenotypes directly or indirectly during development, a time when SMCs have a high degree of plasticity.

Signaling pathways involved in proliferation of SMCs in the *Fbln4* mutant aorta

Hyperproliferation of SMCs is a critical component involved in the alteration of the *Fbln4* mutant aorta and is predominantly mediated by the ERK1/2 signaling. A cue for the activation of ERK1/2 may be provided by mechanical stimuli such as increased hemodynamic force and changes in aortic ECM components, paracrine factors or by undefined mechanisms. Cyclic stretch activates MAPK pathway (both ERK and p38) and increases import of nuclear protein as well as transcription of nuclear pore complex 48. Although the causal relationship between cyclic strain and ERK1/2 phosphorylation was not established in *Fbln4^{GKO}* SMCs using an in vitro system, the observation that aneurysms developed exclusively in the ascending aorta, the region of highest systolic pressure, in *Fbln4^{SMKO}* mice suggests that mechanical strain may influence SMC function in vivo. Angiotensin II activates the ERK pathway and is also a known mediator of the stretch response in SMCs through local production of angiotensinogen or local conversion of angiotensin I to angiotensin II 49. AT1 receptor blockade inhibits gene transcription and proliferation of SMCs induced by stretch stimulation as well as the hypertrophic response of cardiac myocytes induced by pressure overload 50-52. Upregulation of IGF-1 and phosphorylation of IGF-receptor is also induced by cyclic stretch and lead to the proliferation of venous SMCs 53, 54. Interestingly, a high level of angiotensin II and IGF-1 was observed in primary SMC cultures prepared from a patient with a *MYH11* mutation, suggesting the possible involvement of these soluble factors in the proliferative response of SMCs 11. It is also possible that TGF- β activates ERK1/2 pathways by a non-Smad dependent mechanism

⁵⁵. Thus, *Fbln4* mutant mice give us the opportunity to test pharmacological interventions to block specific signaling pathway and attenuate aneurysm formation in vivo.

Limitation of this study

Our current study revealed a critical role for fibulin-4 on differentiation of SMCs and protection from aneurysm development. Since fibulin-4 is a secreted protein, our conditional knockout phenotypes may be partially masked by fibulin-4 supplied by adjacent tissues or remaining production of fibulin-4 within the aortic wall. Fibulin-4 plays a critical role in elastic fiber assembly, therefore, the temporal and causal relationship between defective elastic fibers and alteration of SMC phenotype in *Fbln4*-null aorta remain to be further investigated. In addition, potential cellular functions of fibulin-4 such as the effect on apoptosis or collagen fiber formation, may be explored.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Non-standard Abbreviations and Acronyms

CTGF	connective tissue growth factor
CTL	control
ECM	extracellular matrix
ECKO	endothelial cell-specific knockout
Eln	elastin
ERK	extracellular signal –regulated kinase
Fbln4	fibulin-4
Fbln5	fibulin-5
IGF	insulin growth factor
JNK	Janus kinase
MEK	mitogen-activated protein kinase kinase and extracellular signal-regulated kinase kinase
p38	p38 MAP kinase

SMC	smooth muscle cell
SMKO	smooth muscle cell-specific knockout
SM-MHC	smooth muscle myosin heavy chain
TAAD	thoracic aortic aneurysms and/or aortic dissection
TGF- β	transforming growth factor-beta
BMP	bone morphogenetic protein

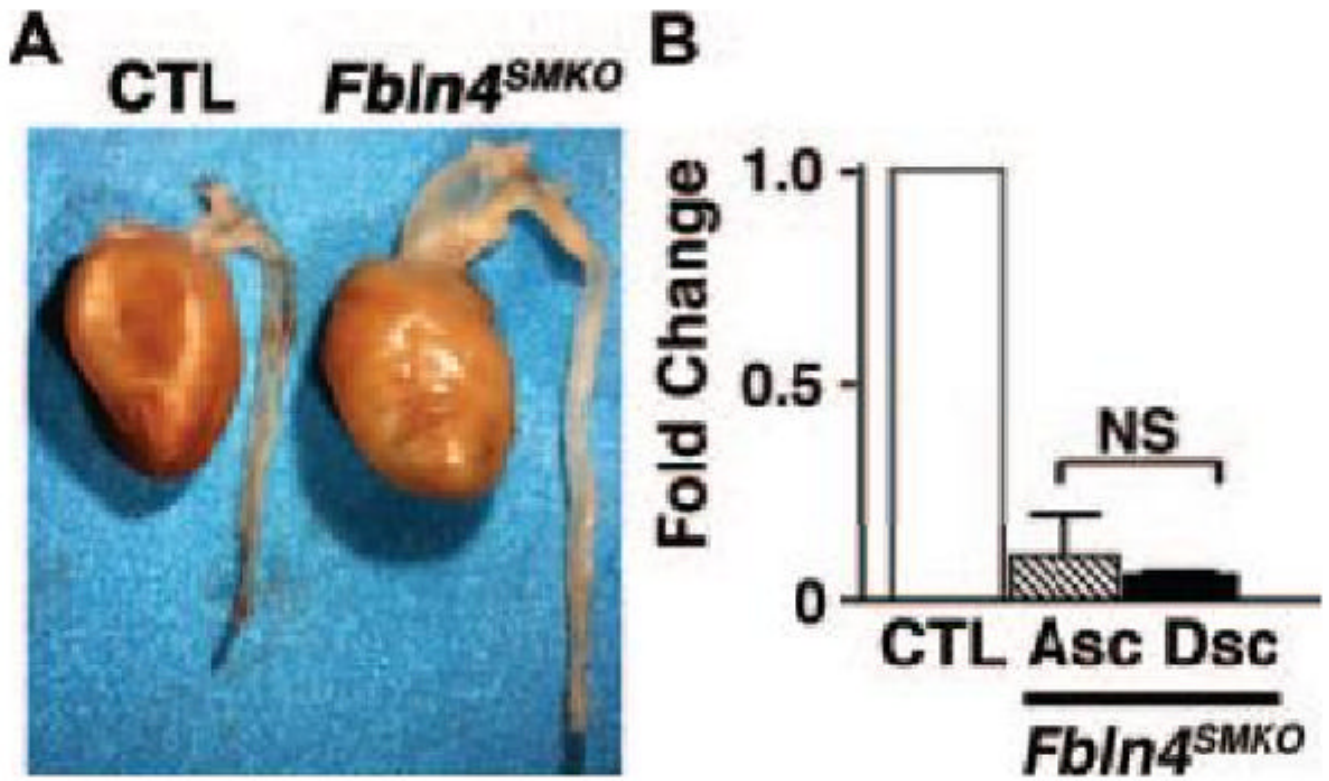


Figure 1.

Aneurysms and tortuous aorta in $Fbln4^{SMKO}$ mice. **A.** Ascending thoracic aortic aneurysm in $Fbln4^{SMKO}$ mouse at P90. **B.** qPCR analysis of $Fbln4$ transcripts in the ascending (Asc) and descending (Dsc) thoracic aortae of $Fbln4^{SMKO}$ mice compared with corresponding controls (CTLs). Aortae from 4 different sibling pairs of CTL and $Fbln4^{SMKO}$ were analyzed in which CTL was considered as 1. Data represent means \pm SD. NS, not significant.

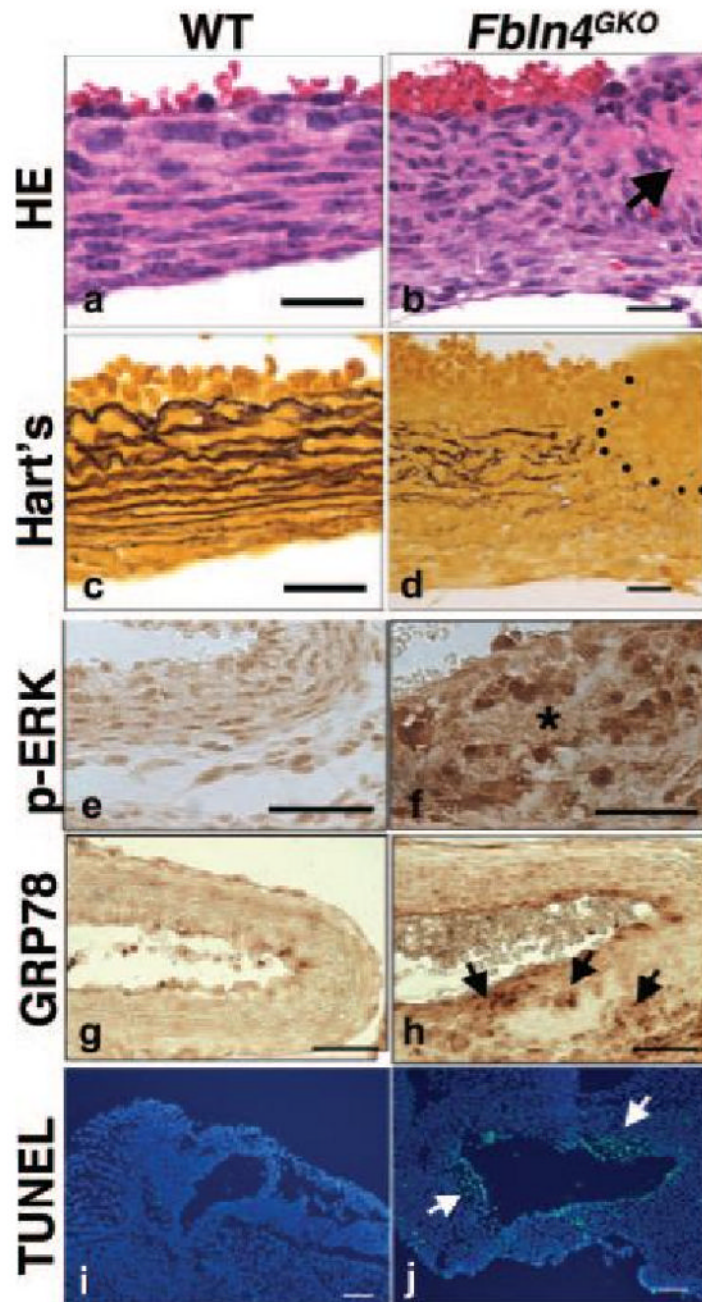


Figure 2. Various pathological changes associated with aneurysms in the *Fbln4^{GKO}* aorta at E18.5. **(a-d)** Representative photos of HE and Hart's staining of transverse sections of the ascending aorta from wild-type (a, c) and *Fbln4^{GKO}* (b, d) embryos (n=5 per genotype). Note focal degeneration of the medial wall in the mutant (arrow in b) devoid of elastic fibers (d). Bars indicate. **(e-j)** Characterization of aneurysmal lesions (n=3 per genotype). (e, f) p-ERK1/2 levels are increased in the *Fbln4^{GKO}* aorta (f) compared to wild-type (e). Asterisk in f indicates degeneration of the medial wall. (g, h) Accumulation of GRP78-positive cells around hyaline degeneration in the *Fbln4^{GKO}* aorta (arrows in h). (i, j) TUNEL staining shows numerous apoptotic cells within

the aneurysmal wall, including the endothelial cells in the *Fbln4^{GKO}* aorta (j). Bars in a-d indicate 20 μm , e-h indicate 40 μm and i-j indicate 100 μm .

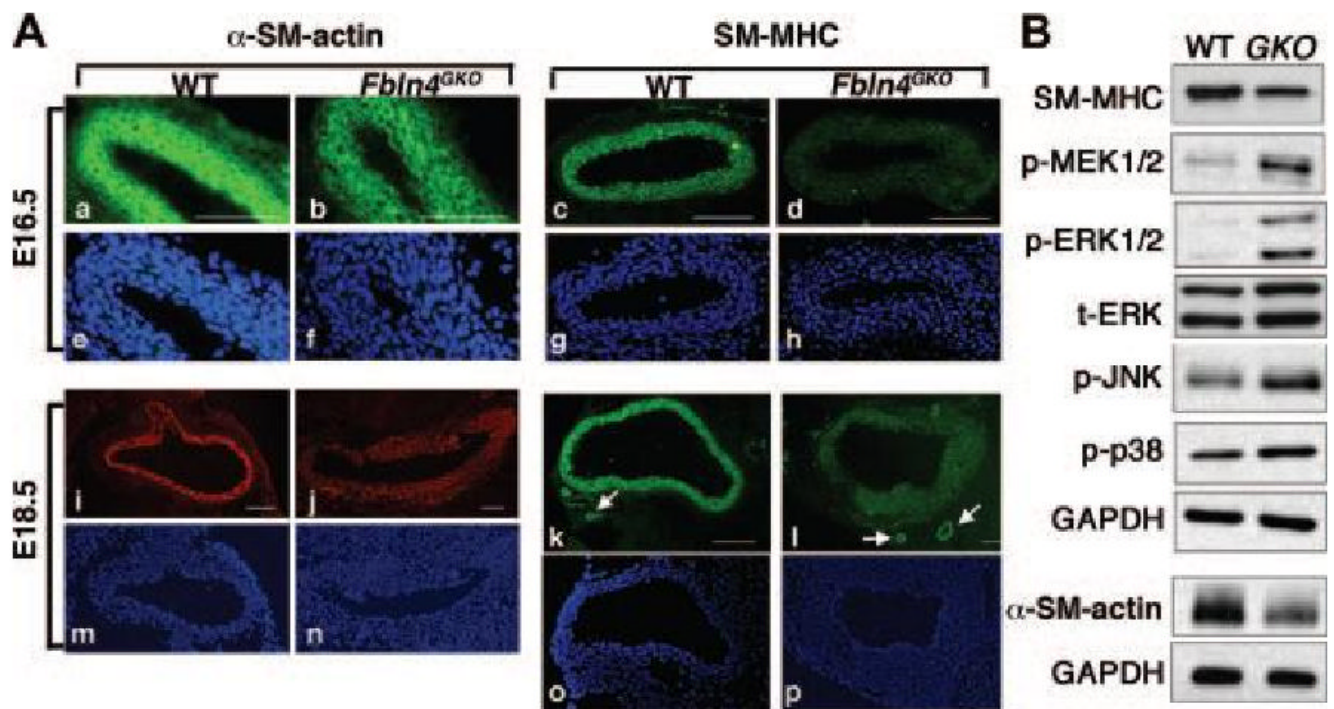


Figure 3.

SMCs exhibit terminal differentiation defects in *Fbln4^{GKO}* aorta. **A.** Representative transverse sections of the descending aorta from wild-type (n=3) and *Fbln4^{GKO}* (n=3) embryos immunostained with SM α -actin and SM-MHC at E16.5 and E18.5. Corresponding DAPI staining is shown. At E16.5, when aneurysm formation is not evident, reduced SM α -actin and SM-MHC staining is already observed in the mutant (b, d) compared with the wild-type aorta (a, c). At E18.5, strong staining for SM α -actin and SM-MHC is observed in the wild-type aorta (i, k) whereas expression of both proteins is reduced in the mutants (j, l). Note comparative expression of SM-MHC in small vessels near the aorta in the wild-type and mutant (arrows in k and l). Bars indicate 100 μ m. **B.** Representative Western blots of descending thoracic aorta extracts from E18.5 wild-type (n=3) and *Fbln4^{GKO}* (n=3) embryos. For α -SM-actin, the experiments were done 2 times using 4 aortae per genotype. SM-MHC is reduced and stress-mediated kinase pathways p-ERK1/2 and p-MEK1/2 are increased in the *Fbln4^{GKO}* aorta. p-JNK and p-p38 are moderately increased.

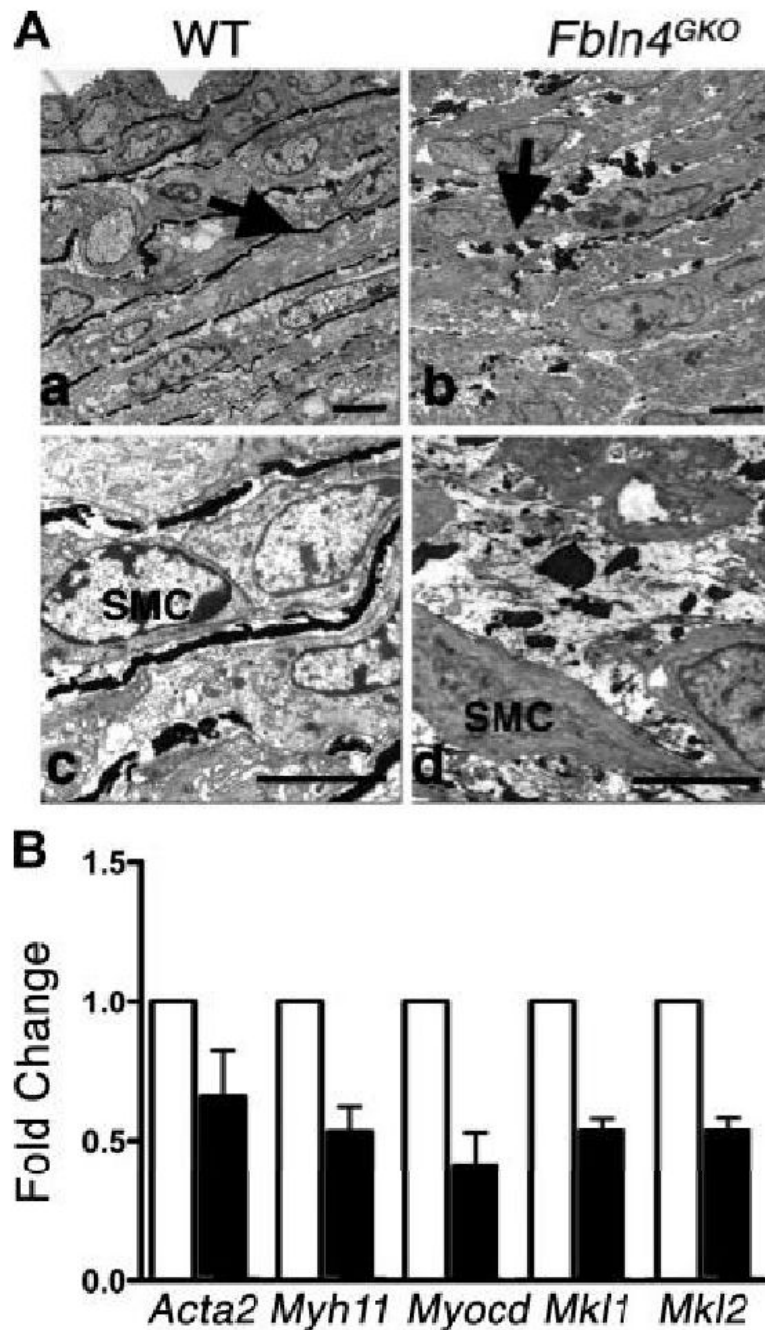


Figure 4. Disorganized elastic laminae and altered expression of SMC marker genes in *Fbln4^{GKO}* aortae. **A.** Electron micrographs of E18.5 aortae from wild-type and *Fbln4^{GKO}* embryos. Elastic fibers (arrow in a) are being formed the wild-type aorta. In the *Fbln4^{GKO}* aorta, numerous elastin globules are seen in the mutant vessel (arrow in b). At higher magnification, disarray of SMCs and an increased pericellular ECM is observed (d). Bars indicate 5 μ m. **B.** qPCR analysis of SMC-specific genes in the descending thoracic aortae devoid of aneurysm lesions from E18.5 wild-type (designated as 1, white column) and *Fbln4^{GKO}* embryos (black column). N=4 using 12 embryos per genotype. Bars indicate means \pm SD.

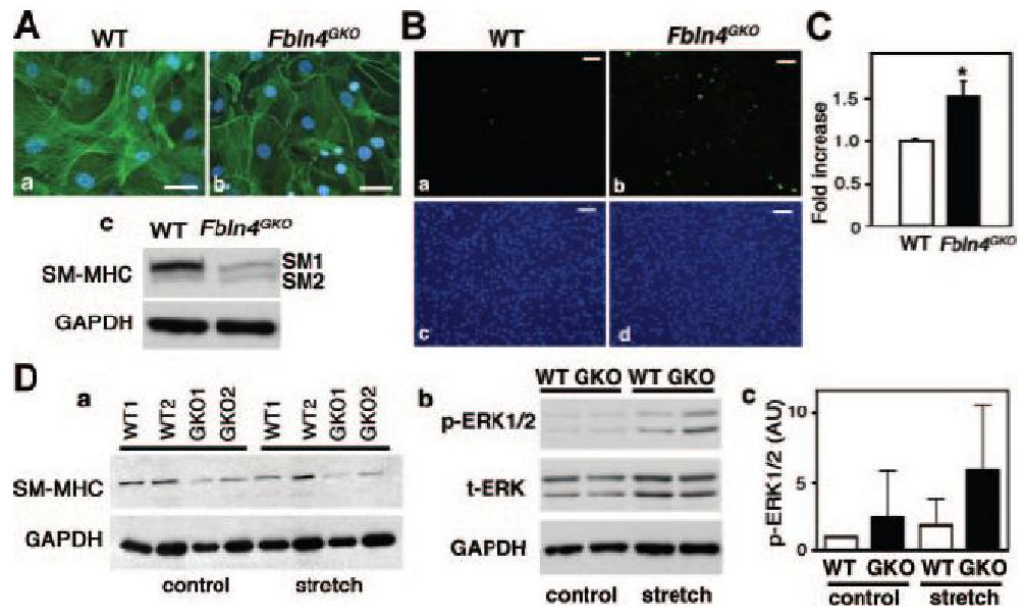


Figure 5. *Fbln4^{GKO}* SMCs display an embryonic phenotype. **A.** Actin fibers detected by fluorescein-conjugated phalloidin in wild-type (a) and *Fbln4^{GKO}* (b) SMCs. Note an overall decrease in actin fibers in *Fbln4^{GKO}* SMCs. Five captured images were analyzed for each of the three independent experiments. Bars indicate 50 μ m. (c) Representative Western blots showing a marked reduction of SM-MHC protein in *Fbln4^{GKO}* SMCs. **B.** Immunostaining of wild-type (a, c) and *Fbln4^{GKO}* SMCs (b, d) grown in serum-free media with anti-PH3 antibody (a, b). The experiment was done twice in duplicates. A significant increase in the number of proliferating cells was observed in *Fbln4^{GKO}* SMCs. Bars indicate 100 μ m. **C.** Proliferation assays. The experiment was done twice in triplicate. Bars indicate means \pm SD. **D. a.** Cyclic stretch assays. Two $\times 10^5$ cells (group 1) or 4 $\times 10^5$ cells (group 2) of wild-type and *Fbln4^{GKO}* SMCs were subjected to stretch stimulation followed by Western blot analysis with anti-SM-MHC and anti-GAPDH. The experiments were done five times. **b.** Representative Western blot showing p-ERK1/2 and t-ERK1/2 levels upon cyclic stretch stimulation in wild-type and *Fbln4^{GKO}* SMCs. **c.** Densitometric analysis of p-ERK1/2 levels normalized to t-ERK1/2 and GAPDH. Bars are means \pm SD (n=5). No statistical significant difference is observed in p-ERK levels.

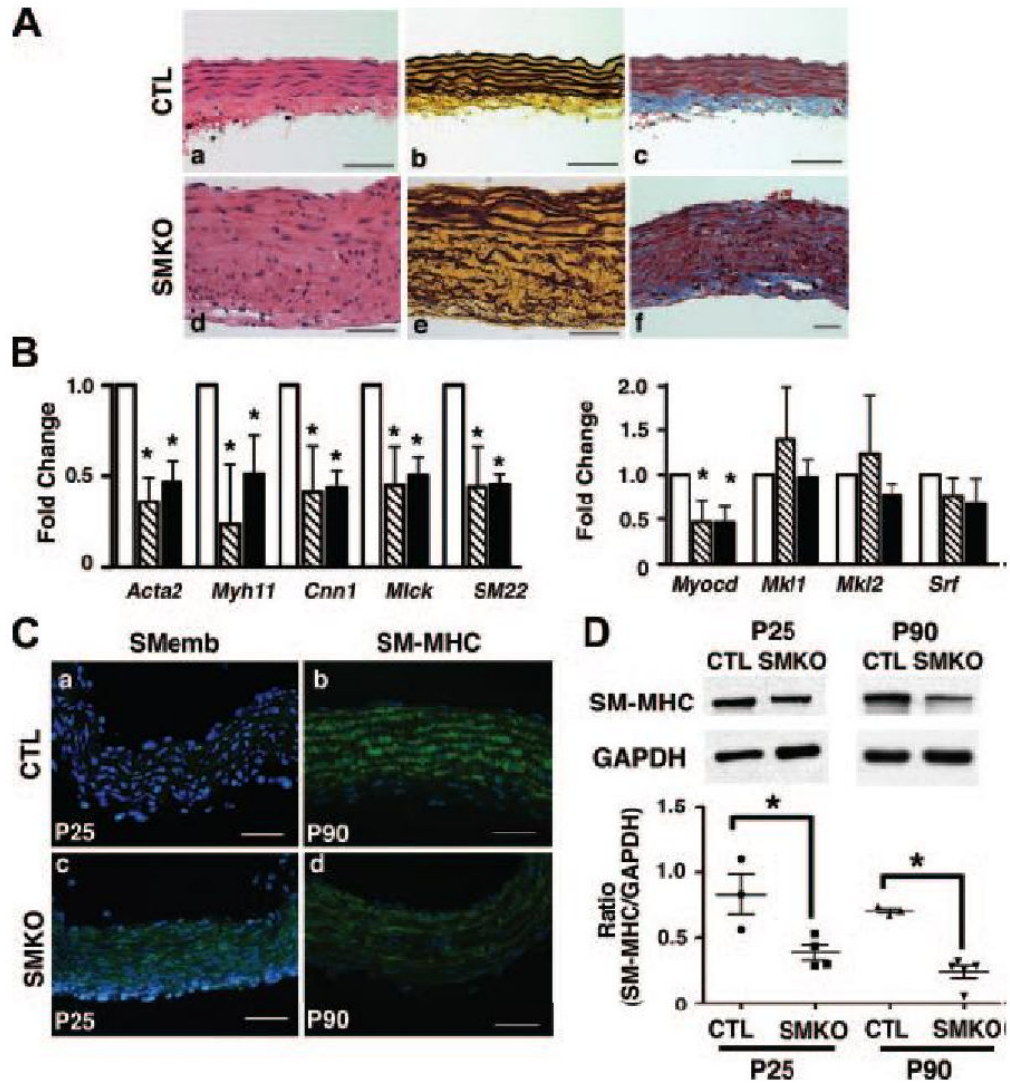


Figure 6. SMC-specific deletion of *Fbln4* recapitulates the aortic phenotype in *Fbln4*-null embryos. **A.** Histological evaluation of focal proliferative lesions in the *Fbln4*^{SMKO} aorta at P90. Representative photos of transverse sections of ascending aorta from control (a-c) and *Fbln4*^{SMKO} (d-f) mice stained with HE (a, d), Hart's (b, e) or Masson-Trichrome (c, f). **B.** qPCR analysis of SMC specific genes in ascending (stripe column) and descending (black column) thoracic aortae from *Fbln4*^{SMKO} mice and corresponding controls (white column). Four sibling pairs were used. Bars indicate means \pm SD. **C.** Representative immunostaining of the ascending aorta from control (a, b) and *Fbln4*^{SMKO} (c, d) mice stained with anti-SMem (P25) and SM-MHC (P90) antibodies. Note strong expression of SMemb and reduced expression of SM-MHC in the mutant aorta. **D.** (upper panel) Representative Western blot of the ascending aorta before (P25) and after (P90) the formation of aneurysms. (lower graph) A ratio between SM-MHC to GAPDH in P25 and P90 ascending aortae is determined densitometrically and plotted. Each data point represents a measurement from an ascending aorta at P25 or P90.

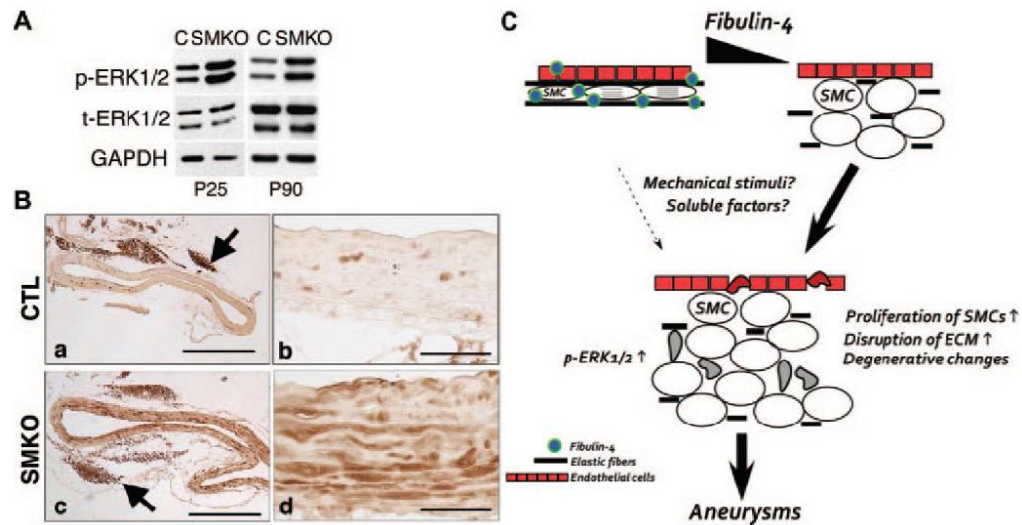


Figure 7.

Increased ERK1/2 signaling in the *Fbln4*^{SMKO} aorta. **A.** Representative Western blot of ascending aortae from control and *Fbln4*^{SMKO} mice at P25 and P90. An increase in p-ERK1/2 is already evident at P25. **B.** Representative immunostaining of P90 ascending aorta from control (a, b) and *Fbln4*^{SMKO} (c, d) mice stained with p-ERK1/2. Increased level of p-ERK1/2 is observed in SMCs of the *Fbln4*^{SMKO} aorta. Note strong signal in perivascular adipose tissue from both control and the mutants (arrow in a and c). Bars in (a, c) indicate 500 μm and in (b, d) indicate 50 μm. **C.** A model illustrating pathological changes in *Fbln4* mutant aorta. Absence of fibulin-4 in the aortic wall affects formation of elastic fibers and terminal differentiation of SMCs. *Fbln4*-null SMCs exhibit an increase in ERK1/2 phosphorylation, possibly mediated by external factors, including mechanical stimuli or soluble factors. Proliferation of SMCs, apoptosis of vascular cells (illustrated by deformed cells) and further destruction of vascular ECM by secondary inflammatory cell response, together contribute to the aneurysm development.