Published in final edited form as:
*Matrix Biol*. 2014 July ; 0: 142–149. doi:10.1016/j.matbio.2014.02.004.

# Fibulin-4 and Fibulin-5 in elastogenesis and beyond: insights from mouse and human studies

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## Abstract

The fibulin family of extracellular matrix/matricellular proteins is comprised of long fibulins (fibulins-1, -2, -6) and short fibulins (fibulins-3, -4, -5, -7) and is involved in protein-protein interaction with the components of basement membrane and extracellular matrix proteins. Fibulins-1, -2, -3, -4, and -5 bind the monomeric form of elastin (tropoelastin) in vitro and fibulins-2, -3, -4, and -5 are shown to be involved in various aspects of elastic fiber development in vivo. In particular, fibulins-4 and -5 are critical molecules for elastic fiber assembly and play a non-redundant role during elastic fiber formation. Despite manifestation of systemic elastic fiber defects in all elastogenic tissues, fibulin-5 null (*Fbln5*$^{-/-}$) mice have a normal lifespan. In contrast, fibulin-4 null (*Fbln4*$^{-/-}$) mice die during the perinatal period due to rupture of aortic aneurysms, indicating differential functions of fibulin-4 and fibulin-5 in normal development. In this review, we will update biochemical characterization of fibulin-4 and fibulin-5 and discuss their roles in elastogenesis and outside of elastogenesis based on knowledge obtained from loss-of-function studies in mouse and in human patients with *FBLN4* or *FBLN5* mutations. Finally, we will evaluate therapeutic options for matrix-related diseases.

### Keywords

elastic fibers; integrin; collagen fibers; aortic aneurysm; cutis laxa; ECM

## 1. Introduction

Since the discovery of prototype fibulin-1 as a 100 kDa integrin-binding protein in 1989 (Argraves et al., 1989), the fibulin family has expanded to include seven members over the last 20 years (reviewed in (Argraves et al., 2003, Timpl et al., 2003, Yanagisawa and Davis, 2010)). Fibulins are characterized by the presence of repeated calcium binding EGF-like motifs and a C-terminal fibulin domain. Much progress has been made to determine biochemical properties of fibulins as well as elucidate the biological roles of fibulins using loss-of-function studies in vivo and human genetic and pathological studies. In particular,



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fibulins-3, -4, and -5 have been established as elastogenic short fibulins (as opposed to long fibulins that include fibulins-1, 2- and -6) and shown to play a crucial role in various aspects of elastic fiber development in vivo (reviewed in (Wagenseil and Mecham, 2007, Yanagisawa and Davis, 2010)). Although fibulin-2 was shown to interact with tropoelastin most potently in vitro (Sasaki et al., 1999), inactivation of fibulin-2 in vivo did not cause appreciable phenotypes until animals were bred on a *Fbln5*-null background to unmask the role of fibulin-2 in the formation of internal elastic lamina of the aortic wall (Chapman et al., 2010, Sicot et al., 2008). Fibulin-3, on the other hand, was shown to bind weakly to tropoelastin in vitro; however, a loss of fibulin-3 in mice exhibited a unique defect of elastic fibers in a tissue-restricted manner, i.e. disrupted elastic fibers in fascia connective tissues of the body wall (Kobayashi et al., 2007, McLaughlin et al., 2007).

Fibulins-4 and -5 are thus far the most critical molecules for aiding assembly of elastic fibers. Fibulin-5 exhibits higher binding affinity to tropoelastin compared to fibulin-4 (Choudhury et al., 2009, Kobayashi et al., 2007), and in vitro elastogenic assays using human foreskin fibroblasts showed that addition of fibulin-5 induced robust elastin-positive fibrous structures (Hirai et al., 2007b). We also observed that fibulin-5 potently induced elastic fibers compared to fibulin-4 (unpublished observation). Conversely, the knockdown of either fibulin-4 or fibulin-5 abolished elastin fiber formation in human gingival fibroblasts and dermal fibroblasts (Yamauchi et al., 2010). Fibulin-5 is expressed at much higher levels than fibulin-4 in major elastogenic organs, including the aorta, lung, and skin (Kobayashi et al., 2007). It is interesting, however, that fibulin-4-null (*Fbln4*$^{-/-}$) mice exhibit a remarkably more severe phenotype and die between late gestation and the perinatal period (Horiguchi et al., 2009, Huang et al., 2010, McLaughlin et al., 2006). Fibulin-5-null (*Fbln5*$^{-/-}$) mice, in contrast, live through adulthood with progressively worsening elastic fiber defects that involve all elastogenic tissues (Nakamura et al., 2002, Yanagisawa et al., 2002). These in vivo data indicate that either fibulin-4 and fibulin-5 are involved in different aspects of elastic fiber formation that cannot be compensated for, or that fibulin-4 has additional roles that affect embryonic development. We will review biochemical properties of fibulin-4 and fibulin-5 and discuss how these molecules are involved in elastogenesis, as well as describe differential roles of fibulin-4 and fibulin-5 beyond elastogenic functions.

## 2. Fibulin-4 and fibulin-5 during elastic fiber assembly

Elastogenesis involves multiple protein-protein interactions that are precisely regulated in a spatiotemporal manner (reviewed in (Wagenseil and Mecham, 2007, Yanagisawa and Davis, 2010)). Both fibulin-4 and fibulin-5 have a modular structure that interacts with various molecules implicated in each step of elastogenesis in vitro (Fig. 1) (Giltay et al., 1999, Horiguchi et al., 2009, Liu et al., 2004). Elastin is first secreted as a monomer, called tropoelastin, from elastogenic cells, including dermal fibroblasts, vascular smooth muscle cells (SMCs), and lung alveolar cells. Tropoelastin is shown to form self-aggregates and undergo phase separation, called coacervation (reviewed in (Yeo et al., 2011)). It is not clear whether coacervation indeed occurs in vivo or if it requires binding of tropoelastin to the cell surface; however, coacervation is suggested to render efficient crosslinking to elastin (Keeley et al., 2002). Fibulin-5 binds full-length tropoelastin, potentiates coacervation efficiency, and lowers coacervation temperature in a dose-dependent manner (Hirai et al.,

2007b, Wachi et al., 2008). Fibulin-4 also binds tropoelastin in a $Ca^{2+}$-dependent manner (McLaughlin et al., 2006). Using elastin-like polypeptides, fibulin-5 and (to a lesser extent) fibulin-4 were shown to inhibit maturation of coacervation without affecting the coacervation temperature (Cirulis et al., 2008). These results suggest that coacervation can be compromised and the maturation phase of coacervation may be dysregulated in the absence of fibulin-5 or fibulin-4. Interestingly, oxidization of tropoelastin has been shown to facilitate coacervation, but decreases binding to fibulin-4 and fibulin-5 and results in markedly reduced cross-linking and formation of elastin-positive aggregates instead of intact elastic fibers (Akhtar et al., 2010). These notions are consistent with the in vivo observation that large elastin aggregates are accumulated in the skin of *Fbln5*$^{-/-}$ mice (Choi et al., 2009).

Microfibrils, which are mainly composed of fibrillin-1 and fibrillin-2, serve as scaffolds for deposition of tropoelastin (Rock et al., 2004, Trask et al., 2000). Fibrillin-1 null mice (*Fbn1*$^{-/-}$) showed a neonatal lethal phenotype due to ascending aortic rupture with disorganized elastic fibers (Carta et al., 2006). Knocking out *Fbn2* on a *Fbn1*-null background further exacerbated the aortic defects with poorly organized elastin and embryonic lethality, suggesting functional redundancy between fibrillin-1 and fibrillin-2 in vivo (Carta et al., 2006). Fibrillin-1 microfibrils are dependent on the fibronectin matrix for deposition, and knockdown of fibronectin completely abolishes the formation of fibrillin-1 microfibrils (Kinsey et al., 2008, Sabatier et al., 2009). Since neither fibulin-4 nor fibulin-5 bind fibronectin in vitro, it is unlikely that these molecules affect initial formation of a microfibrillar scaffold (El-Hallous et al., 2007, Kobayashi et al., 2007).

Fibulin-5 was shown to bind N-terminal fibrillin-1 fragments and co-localize with microfibrils (Freeman et al., 2005, Zheng et al., 2007). It was further demonstrated that fibulin-5 binding to fibrillin-1 markedly potentiates binding between fibrillin-1 fragments and tropoelastin, acting as an adaptor to form a ternary complex (El-Hallous et al., 2007). In other experiments, fibulin-5 was shown to bind tropoelastin or fibrillin-1 without influencing the overall complex formation and it was suggested that fibulin-5 facilitates targeting and deposition of tropoelastin onto microfibrils (Choudhury et al., 2009). Although the precise sequence of events during tropoelastin deposition onto microfibrils is not completely determined, these data collectively suggest that fibulin-5 binds tropoelastin to navigate onto a fibrillin-1 dominant microfibrillar scaffold while simultaneously mediating proper coacervation prior to cross-linking. However, fibulin-4 seems to play relatively a minor role in this process compared to fibulin-5 (Fig. 2).

Cross-linking of elastin is the next critical step for generating insoluble polymerized elastin. This process is mediated by the lysyl oxidase enzyme family, which includes lysyl oxidase (LOX) and lysyl oxidase like (LOXL)-1 (reviewed in (Lucero and Kagan, 2006)). LOX and LOXL-1 are shown to be involved in elastic fiber formation in vivo and *Lox* knockout mice exhibit perinatal lethality due to rupture of aortic aneurysm with failure to cross-link both collagen and elastin (Hornstra et al., 2003, Maki et al., 2005). Interestingly, *Loxl1* knockout mice live into adulthood but exhibit defective elastic fiber remodeling, manifested as development of pelvic organ prolapse following vaginal delivery and progressive pulmonary emphysema (Liu et al., 2004). In vitro binding analysis showed that LOX strongly binds

fibulin-4, whereas LOX binding to fibulin-5 was only observed by solid-phase binding assays but not by BIAcore (Choudhury et al., 2009). Co-immunoprecipitation (Co-IP) assays also confirmed the binding between fibulin-4 and LOX, which was mediated between the N-terminal region of fibulin-4 and propeptide of LOX. It was further shown that fibulin-4 markedly increases tropoelastin binding to LOX (Hirai et al., 2007b). Fibulin-5, in contrast, binds LOXL-1 in vitro through its C-terminal domain and is suggested to tether LOXL-1 onto elastic fibers (Liu et al., 2004) and/or activate LOXL-1 by facilitating cleavage of preproLOXL-1 into an active LOXL-1 (Choi et al., 2009). These findings indicate that the preferential binding between LOX and fibulin-4, and between LOXL-1 and fibulin-5, together facilitates crosslinking of elastic fibers in vivo. The fact that LOX is also essential for collagen crosslinking, along with the striking phenotypic similarity between *Lox*$^{-/-}$ and *Fbln4*$^{-/-}$ mice, raises the question of whether the fibulin-4-LOX interaction may also be biologically relevant outside of elastogenesis. However, LOX activation and localization have not yet been assessed in *Fbln4*$^{-/-}$ mice, and this question remains to be investigated.

Cell surface binding of fibulin-5 to subsets of integrin receptors, including αVβ1, αVβ3, α9β1 and α5β1, have been demonstrated in vitro (Lomas et al., 2007, Nakamura et al., 2002). Since fibulin-5 contains a RGD integrin-binding motif, it was initially hypothesized that fibulin-5 bridges the gap between the cell surface and elastic fibers during the final step of elastic fiber assembly. Solid phase binding assays using immobilized αVβ3 integrin showed that fibulin-5 binding to αVβ3 was weak unless reduction and alkylation was introduced to unmask the RGD site in fibulin-5 (Kobayashi et al., 2007). In addition, mice containing alleles in which RGD was mutated to RGE to disrupt integrin binding showed normal elastic fibers in the aorta, lungs, skin and vaginal wall. This finding suggested that RGD-mediated integrin binding was not required for elastic fiber assembly in vivo (Budatha et al., 2011). Recently, it has been shown that all short fibulins (fibulins-3, -4, and -5) adhere to human fibroblasts and SMCs, possibly mediated by cell surface heparan sulfate in a calcium-dependent manner (Djokic et al., 2013). It is not clear, however, whether fibulin-5 binding to the cell surface is a necessary step for elastogenesis in vivo, or induces specific intracellular signaling pathways.

Several molecules have been reported to interact with fibulin-4 or fibulin-5 and affect the formation of elastic fibers in vitro. Latent TGFβ binding protein-2 (LTBP-2), which belongs to the fibrillin/LTBP family but lacks binding to the latency-associated propeptide-TGFβ complex (small latency complex or SLC), interacts with fibulin-5 and facilitates fibrillin-1 microfibril-dependent elastin deposition (Hirai et al., 2007a). When LTBP-2 is knocked down, elastin preferentially deposits onto fibrillin-2 microfibrils. Another study showed that LTBP-2 binds fibulin-5 and inhibits tropoelastin-fibulin-5 interaction (Sideek et al., 2013). LTBP-1 binds strongly to fibulin-4 and forms a ternary complex involving LTBP-1, fibrillin-1 and fibulin-4 (Massam-Wu et al., 2010). LTBP-4, which can bind the SLC, also binds fibulin-5 and induces linear deposition of fibulin-5 onto microfibrils and promotes elastic fiber assembly (Noda et al., 2013).

In addition to the different biochemical properties of fibulin-4 and fibulin-5, distinct tissue distribution, localization within the tissue, expression levels, and temporal expression patterns may account for the differential roles of fibulin-4 and fibulin-5 in elastogenesis. It is

also important to identify interacting proteins and examine their tissue distribution to understand whether the mechanism of elastogenesis differs among tissues in vivo. Finally, primary structural defects caused by the loss of elastic fibers need to be distinguished from secondary defects resulting from abnormal tissue architecture, such as increased mechanical stress and stress-induced signaling pathways, in order to obtain a full understanding of pathophysiology resulting from the loss of fibulin-4 and fibulin-5 in vivo.

## 3. Functions of fibulin-4 outside of elastogenesis

### 3.1. Fibulin-4 and aortic development

Since *Fbln4*$^{-/-}$ mice die early in life, it has been challenging to identify extra-elastogenic functions of fibulin-4 from in vivo phenotypes. We previously reported that *Fbln4*$^{-/-}$ aortic SMCs were less differentiated as judged by the reduced expression of SMC-specific terminal differentiation markers and persistent expression of embryonic SMC markers (Huang et al., 2010). In addition, *Fbln4*$^{-/-}$ cells exhibited an increase in phosphorylated Histone H3 under serum-starved conditions, mimicking the embryonic SMC phenotype. Interestingly, these immature and proliferative phenotypes are strikingly similar to *Eln*$^{-/-}$ SMCs as well as cells from patients with deletion of the elastin gene (supravalvular aortic stenosis and Williams-Beuren Syndrome (Li et al., 1998, Urban et al., 2002)). Since tropoelastin mRNA levels are comparable between *Fbln4*$^{-/-}$ and wild-type aortas (Horiguchi et al., 2009) and soluble tropoelastin protein levels are even higher in the mutants (Le et al., 2014), it is possible that the absence of fibulin-4 in SMCs may influence elastin-dependent intracellular signaling mediated by a putative elastin receptor (Mochizuki et al., 2002). Alternatively, fibulin-4 deficient SMCs may acquire a hyperproliferative SMC phenotype secondary to the disorganized elastin matrix in the aortic wall. It is also possible that a fibulin-4-containing vascular microenvironment is necessary to support maintenance of a differentiated SMC phenotype in vivo.

Vascular SMC-specific *Fbln4* knockout mice (*Fbln4*$^{SMKO}$) and hypomorphic *Fbln4* mice (*Fbln4*$^{R/R}$) were generated, in which *Fbln4* levels in the aorta were reduced to approximately 6–8% and 40%, respectively (Hanada et al., 2007, Horiguchi et al., 2009, Huang et al., 2010). These mice develop ascending aortic aneurysms and tortuous descending aorta with disorganized elastic fibers and accumulation of collagen fibers. It is interesting to note that endothelial cell (EC)-specific knockout *Fbln4* mice do not develop an aneurysmal phenotype; however, EC-SMC double knockout mice exhibit an exacerbated aneurysmal phenotype with the lesion extending over the aortic arch and involving the descending thoracic aorta (Huang et al., 2010), our unpublished observation). In addition, *Fbln4*$^{R/R}$ mice which maintain as much as 40% of *Fbln4* in the aorta still exhibit an aneurysm phenotype similar to *Fbln4*$^{SMKO}$, indicating that fibulin-4 derived from ECs or other cell types may play additional roles in protecting the aortic wall from aneurysm formation during the embryonic and postnatal period.

### 3.2. Fibulin-4 and angiotensin-TGFβ pathway

It was reported that TGFβ-mediated signaling was elevated in the aortas of *Fbln4*$^{R/R}$ mice and in patients carrying mutations in the *FBLN4* gene, as indicated by an increase in

phosphorylated (p-) Smad2/3 levels (Hanada et al., 2007, Renard et al., 2010). We have reported that local angiontensin-converting enzyme (ACE) was increased in the aneurysmal wall, resulting in an increase of mature angiontensin II and p-ERK1/2 in *Fbln4*$^{SMKO}$ mice (Huang et al., 2013). Angiotensin II is a positive regulator of TGFβ signaling by inducing transcription of TGFβ, and *Fbln4*$^{SMKO}$ aortas showed increased p-Smad 2/3, but not Smad1/5/8. Interestingly, ACE levels were comparable between isolated wild-type and *Fbln4*$^{SMKO}$ SMCs, indicating that additional mechanical stress on the vessel wall (i.e. pressure or flow), disruption of cell-matrix connections, or contribution from other cell types may be required for upregulation of ACE in vivo. In both *Fbln4*$^{R/R}$ and *Fbln4*$^{SMKO}$ mouse models, angiotensin II receptor blockade (ARB) effectively prevented the development of aortic aneurysms. It was suggested that the protective effect of ARB was due to reduction of systemic blood pressure in *Fbln4*$^{R/R}$ (Moltzer et al., 2011) and blocking of angiotensin II-mediated signaling in *Fbln4*$^{SMKO}$ (Huang et al., 2013), respectively. It is noteworthy that an ACE inhibitor also effectively prevented aneurysms in *Fbln4*$^{SMKO}$ mice, which shows a distinct difference from a Marfan mouse model (*Fbn1*$^{C1039G/+}$) in which an ARB was more effective than an ACE inhibitor in preventing aortic root aneurysms (Habashi et al., 2011, Habashi et al., 2006). However, it remains unknown whether TGFβ upregulation is a direct consequence of loss of fibulin-4 or a secondary event due to upregulation of angiotensin II in the aortic wall.

Another unanswered question is whether TGFβ plays a causative role in the pathogenesis of aortic aneurysms in *Fbln4*-mutant mice similar to what has been suggested in Marfan syndrome and related diseases (Dietz et al., 1991, Loeys et al., 2005, Neptune et al., 2003). Fibrillin-1 tethers the large latency complex (LLC) comprised of covalently linked LTBP (predominantly LTBP-1) and SLC via direct binding, aiding in sequestration of latent TGFβ in the ECM (Isogai et al., 2003). Immuno-EM studies indicated that fibulin-4 is localized on microfibrils (Kobayashi et al., 2007), and one study suggested that fibulin-4 stabilizes LLC on microfibrils by directly binding to LTBP-1 and fibrillin-1 and forming a ternary complex (Massam-Wu et al., 2010). Conversely, LTBPs, fibulin-2, and fibulin-4 were found to compete for binding to the third EGF-like domain (EGF3) of fibrillin-1 in in vitro binding assays (Ono et al., 2009). These biochemical studies are informative but limited in their ability to directly assess the effect of loss of fibulin-4 on TGFβ bioavailability in vivo. Therefore, additional tools such as a TGFβ reporter mouse will be useful to monitor active TGFβ levels during aneurysm formation. In addition, genetic ablation of TGFβ signaling components or treating *Fbln4*$^{-/-}$ mice with a TGFβ neutralizing antibody should prove useful in addressing the contribution(s) of TGFβ to the pathology of *Fbln4*-mutant mice.

## 4. Non-elastogenic functions of fibulin-5: Protease regulation mediated by the integrin binding domain of fibulin-5

Systemic elastic fiber defects in *Fbln5*$^{-/-}$ mice are already present during early postnatal life, thus the role of fibulin-5 has been suggested to be predominantly developmental. However, deterioration of elastic fibers is observed in *Fbln5*$^{-/-}$ mice as they age, especially in the lung and vaginal tissues, resulting in emphysema and pelvic organ prolapse, respectively (Drewes et al., 2007, Yanagisawa et al., 2002). Pelvic organ prolapse is

characterized by abnormal protrusion of female pelvic organs (i.e. uterus, bladder and vagina) and this phenotype is not observed until later in life in *Fbln5*$^{-/-}$ mice. This observation led to the hypothesis that abnormal elastic fibers alone may not be sufficient to induce pelvic organ prolapse, and suggested additional, non-elastogenic functions of fibulin-5.

Our group documented that both MMP-9 and MMP-2 were upregulated in vaginal tissues of adult *Fbln5*$^{-/-}$ mice and MMP-9 activity was detected after puberty and preceded the onset of pelvic organ prolapse (Budatha et al., 2011). Since MMP-9 is not elevated in other elastogenic tissues, such as the aorta and skin, it is likely that fibulin-5 suppresses protease activity in tissues that undergo continuous remodeling of elastic fibers. Indeed, using primary vaginal stromal cells, *Fbln5*$^{-/-}$ cells exhibited higher MMP-9 activity in response to fibronectin and this increase was inhibited by recombinant fibulin-5. Interestingly, *Fbln5*$^{RGE/RGE}$ cells, which produce fibulin-5 containing a mutation in the RGD motif that disrupts integrin binding, also exhibited high MMP-9 in response to fibronectin. In both cases, MMP-9 activity was inhibited by a β1 integrin blocking antibody, indicating that fibulin-5 inhibits fibronectin-induced β1 integrin-mediated elevation of MMP-9. This is consistent with an earlier report in which fibulin-5 was shown to bind α5β1 fibronectin receptor and blocked fibronectin-mediated activation of β1 integrin, including stress fiber formation and focal adhesions, and this inhibition was reversed by adding β1 integrin-activating antibody (Lomas et al., 2007).

The biological significance of fibulin-5 binding to β1 integrin has also been reported in mouse pancreas tumor studies. In *Fbln5*$^{-/-}$ mice, pancreatic tumor growth and tumor angiogenesis was suppressed compared with wild-type mice and this was due to an increase in the level of reactive oxygen species (ROS), which resulted in elevated DNA damage and apoptosis of tumor endothelial cells. The ROS production was shown to be dependent on β1 integrin, and treatment with an antioxidant was sufficient to downregulate ROS levels and restore tumor growth in *Fbln5*-mutant mice (Schluterman et al., 2010). In a separate study using lung cancer cell lines, fibulin-5 was shown to suppress MMP-7 expression and activity in an RGD-dependent manner, and this suppression was correlated with increased p-ERK levels (Yue et al., 2009). Interestingly, fibulin-5 was shown to be hypermethylated and the expression was downregulated in primary tumors and lung cancer cell lines, contributing to an increase in cancer cell invasion (Yue et al., 2009). More recently, fibulin-5 was shown to be required for urokinase-type plasminogen activator (uPA)-mediated cell migration (Kapustin et al., 2012). Interaction between uPA and fibulin-5 resulted in generation of plasmin, which cleaves the N-terminal cbEGF-like domain of fibulin-5 containing the integrin-binding motif and disrupts fibulin-5-β1 integrin binding, releasing the inhibitory effects of fibulin-5 on β1 integrin-mediated cell migration (Kapustin et al., 2012). Taken together, these studies point to the role of fibulin-5 in regulating protease activity by interacting with β1 integrin as an endogenous competitive ligand. It seems that fibulin-5 modulates the microenvironment and cellular functions by acting as a negative regulator in a context-dependent manner.

## 5. Fibulins-4 and -5 in human diseases

Mutations in both *FBLN4* and *FBLN5* have been identified in patients with cutis laxa (OMIM 219100, OMIM 614434, OMIM 614437). Consistent with data from knockout mouse models, *FBLN4* mutations lead to a broader, more severe range of phenotypes than *FBLN5* mutations, and the phenotypic abnormalities observed in patients lend further support for the role of these proteins in elastic fiber assembly as well as provide insight for future research directions. The majority of currently identified *FBLN4* mutations occur in the cbEGF-like domains (Al-Hassnan et al., 2012, Erickson et al., 2011, Iascone et al., 2012, Kappanayil et al., 2012, Sawyer et al., 2013), and are predicted to either disrupt disulfide bond formation and proper folding of the domain or disrupt calcium binding (Dasouki et al., 2007, Hucthagowder et al., 2006, Renard et al., 2010). Additionally, one report showed decreased secretion of fibulin-4 into the ECM along with alterations in TGFβ1 signaling in *FBLN4* mutant skin fibroblasts and tissues, consistent with observations in *Fbln4* deficient mice (Renard et al., 2010).

The most prominent and consistent defects resulting from *FBLN4* mutations are ascending aortic aneurysms, arterial tortuosity along with other arterial defects, and cutis laxa, and are consistent with phenotypes observed in *Fbln4*$^{-/-}$ mice (Hoyer et al., 2009, Sawyer et al., 2013). Multiple case studies found respiratory problems including diaphragm abnormalities, which are consistent with diaphragm herniation and rupture found in *Fbln4* deficient mice (Erickson et al., 2011, Horiguchi et al., 2009, Hucthagowder et al., 2006, Iascone et al., 2012). Additional defects identified in multiple patients with *FBLN4* mutations include bradycardia, joint laxity, hypotonia, and craniofacial dysmorphism including features similar to Loeys-Dietz (Loeys et al., 2005) or Arterial Tortuosity Syndrome (Coucke et al., 2006). Multiple *FBLN4* patients were also reported to have skeletal abnormalities including bone fractures, rib/long bone defects, or decreased bone mineral density (Dasouki et al., 2007, Erickson et al., 2011, Hoyer et al., 2009, Hucthagowder et al., 2006, Sawyer et al., 2013), but these findings have not been further investigated. Conditional knockouts of fibulin-4 in skeletal tissues will help clarify which skeletal abnormalities specifically result from fibulin-4 loss, along with their associated mechanisms. Overall, although *FBLN4* mutations are relatively rare, the diverse array of defects has led some to suggest the possibility of as-yet unidentified roles for fibulin-4, and studies are currently underway to address these issues.

In contrast to *FBLN4* mutations, patients with *FBLN5* mutations presented mainly with cutis laxa and showed disruption of elastic fibers in skin and aorta without evidence of aortic aneurysms, consistent with data obtained from *Fbln5*$^{-/-}$ mice. Two mutations in *FBLN5* (S227P and C217R) were separately identified in multiple cases of cutis laxa (Callewaert et al., 2013, Claus et al., 2008, Elahi et al., 2006, Hu et al., 2006, Loeys et al., 2002). Both mutations are found in highly conserved residues in the fourth cbEGF-like domain and are predicted to lead to protein misfolding based on functional studies showing decreased secretion and matrix deposition. Additionally, these mutants showed decreased affinity for tropoelastin (Hu et al., 2006). Jones et al. used multiple biophysical techniques to further demonstrate that these two mutations introduce changes in fibulin-5 that are likely to be

pathogenic, including increased dimerization of S227P, and a likely reduction in protein stability of C217R mutants (Jones et al., 2010).

In addition to cutis laxa, several reports have been published of *FBLN5* variants associated with age-related macular degeneration (AMD) (Jones et al., 2010, Lotery et al., 2006, Stone et al., 2004). Expression of fibulin-5 mutants in COS-7 cells showed four out of nine mutants associated with AMD led to decreased fibulin-5 secretion (Lotery et al., 2006). Interestingly, five heterozygous *FBLN5* mutations were reported in patients with Charcot-Marie-Tooth disease, three of which were novel and two of which were previously identified as variants in AMD patients (Auer-Grumbach et al., 2011, Safka Brozkova et al., 2013). Although the mutations segregated with the disease, functional studies were not performed, and the reported phenotypes were complex and variable. Therefore, further study is needed to identify the specific pathologic mechanisms involved.

## 6. New therapeutic approaches toward matrix-related diseases

Several promising strategies have been proposed to prevent or ameliorate progression of matrix-related defects in animal mouse models. Pharmacological intervention using ARB was first demonstrated in *Fbn1*$^{C1039G/+}$ mice based on the increased bioavailability of TGFβ and improvement of the lung emphysematous phenotype by anti-TGFβ neutralizing antibodies (Neptune et al., 2003). The rationale for selecting an ARB to treat aneurysms in the *Fbn1*$^{C1039G/+}$ mouse was to downregulate TGFβ levels by blocking its upstream regulator, angiotensin II (Habashi et al., 2006). This strategy solidified the notion that disruption of fibrillin-1 not only affects structural integrity but also alters intracellular signaling, which shed light on a novel role of ECM in tissue development and homeostasis. Our study and others also support the effectiveness of ARB in prevention of aneurysms in *Fbln4* mutant mice, but the mechanism of action seems to be different from that of the Marfan mouse model as discussed in the previous section.

A new strategy to target microRNAs (miRs), particularly miR-29, was demonstrated in treatment of angiontensin II-induced aneurysms, elastase-induced abdominal aneurysm, and aneurysms in a Marfan mouse model (Boon et al., 2011, Maegdefessel et al., 2012, Merk et al., 2012). MicroRNAs are well-conserved small noncoding RNAs that regulate protein expression by degrading target mRNA or inhibiting translation (reviewed in (Small and Olson, 2011)). MicroRNA-29 is consisted of three family members (miR-29a, miR-29b, and miR-29c). MicroRNA-29b in particular is increased in mouse and human aneurysms, including *Fbn1*$^{C1039G/+}$, *Fbln4*$^{R/R}$, and angiotensin II-treated mice, and patients with bicuspid or tricuspid aortic valves with thoracic aortic aneurysms. MicroRNA-29b regulates a variety of genes encoding ECM proteins, including elastin, fibrillin-1, collagen type 1 α chain (Col1a1), and Col5a1 (Boon et al., 2011). Others reported that miR-29b also regulates anti-apoptotic genes such as Bcl-2 and Mcl-1 (Mott et al., 2007). Administration of locked nucleic acid-mediated antisense oligonucleotide for miR-29b was shown to prevent the increase in aortic diameter and upregulate target mRNA levels in the aortas of angiotensin II-treated mice and *Fbn1*$^{C1039G/+}$ mice (Merk et al., 2012). These data indicate that deterioration of ECM by suppressing ECM genes and anti-apoptotic genes is a critical aspect of aneurysm formation in vivo. In a separate experiment using an elastase-induced aortic

aneurysm model, miR-29b was shown to be effective in restoration of target gene expression and stabilization of the vessel wall by increasing fibrosis and decreasing MMP-9 levels (Maegdefessel et al., 2012). Interestingly, it was shown that losartan effectively reduced the expression of miR29-b, resulting in a decrease of caspase-3 and caspase-9, and an increase of Bcl-2, Mcl-2, Eln and Col3a1. Taken together, the combination of anti-miR-29b and losartan may work synergistically to prevent aneurysms in vivo.

The modeling of patients' SMCs has been accomplished by generating induced pluripotent stem (iPS) combined with in vitro differentiation into SMCs using fibroblasts from patients with supravalvular aortic stenosis (SVAS). SVAS is caused by deletion in the elastin gene (ELN) and is characterized by hyperproliferation of SMCs and narrowing of the ascending aorta (Ge et al., 2012, Kinnear et al., 2013). A mouse model of SVAS was established by deleting the *Eln* gene in vivo, and primary *Eln*-null SMCs recapitulate human disease phenotypes (Li et al., 1998). The established iPS-derived SMCs showed decreased SMC markers and increased migration and proliferation, which were reversed by addition of elastin or elastin binding protein ligand 2, or by driving RhoA activity. It is interesting that the abnormal phenotypes of SMCs within the aortic wall can be reproduced in vitro, indicating that the effect of loss of elastin does not only affect vessel integrity but also induces cell-autonomous effects that can be potentially corrected by pharmacological interventions. Since primary *Fbln4*-null SMCs exhibit hyperproliferation and reduction of SMC marker proteins in vitro, it may be useful to establish stable iPS-derived SMCs for characterizing abnormal SMC phenotype and identifying intracellular signaling pathways underlying the disease. Furthermore, iPS-derived SMCs may be useful for engineering of synthetic vessels to examine mechanical properties of the diseased vessels (Hibino et al., 2012).

## 7. Concluding remarks

Considerable progress has been made in the last decade in determining the biochemical properties of fibulins and their biological functions during development and disease progression. Furthermore, it is clear that fibulins-4 and -5 possess extra-elastogenic functions that have yet to be discovered. It is therefore critical to distinguish the effects caused by a primary loss of proteins from a structural consequence of the loss of protein to understand the overall functions in vivo. Moreover, information on the effect of overexpression of these proteins on tissue homeostasis as well as the possibility of one or more fibulins serving as biomarkers for pathological conditions may be important. Finally, the idea that each fibulin has a specific cell surface receptor is an interesting avenue to pursue to understand matrix-cell interactions in various in vivo settings.

## Acknowledgments

The work was supported by grants from National Institutes of Health (R01HL106305 and R01HD06482401 to HY) and an NIH institutional Training in Cardiovascular Research grant (5T32HL007360-34 to CLP). HY is an Established Investigator of the American Heart Association.

## Abbreviation

| | |
|---|---|
| **TGFβ** | transforming growth factor beta |
| **cb-EGF** | calcium binding epidermal growth factor |

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## Highlights

- Fibulins-4 and -5 bind elastin and are essential for development of elastic fibers.
- Mutations in *FBLN4* or *FBLN5* gene cause autosomal recessive cutis laxa.
- Smooth muscle cell-derived fibulin-4 is crucial for preventing ascending aortic aneurysm.
- Fibulin-5 regulates protease activities in an integrin dependent manner.
- Anti-miR29-b therapy may be useful for subsets of ECM-related diseases.

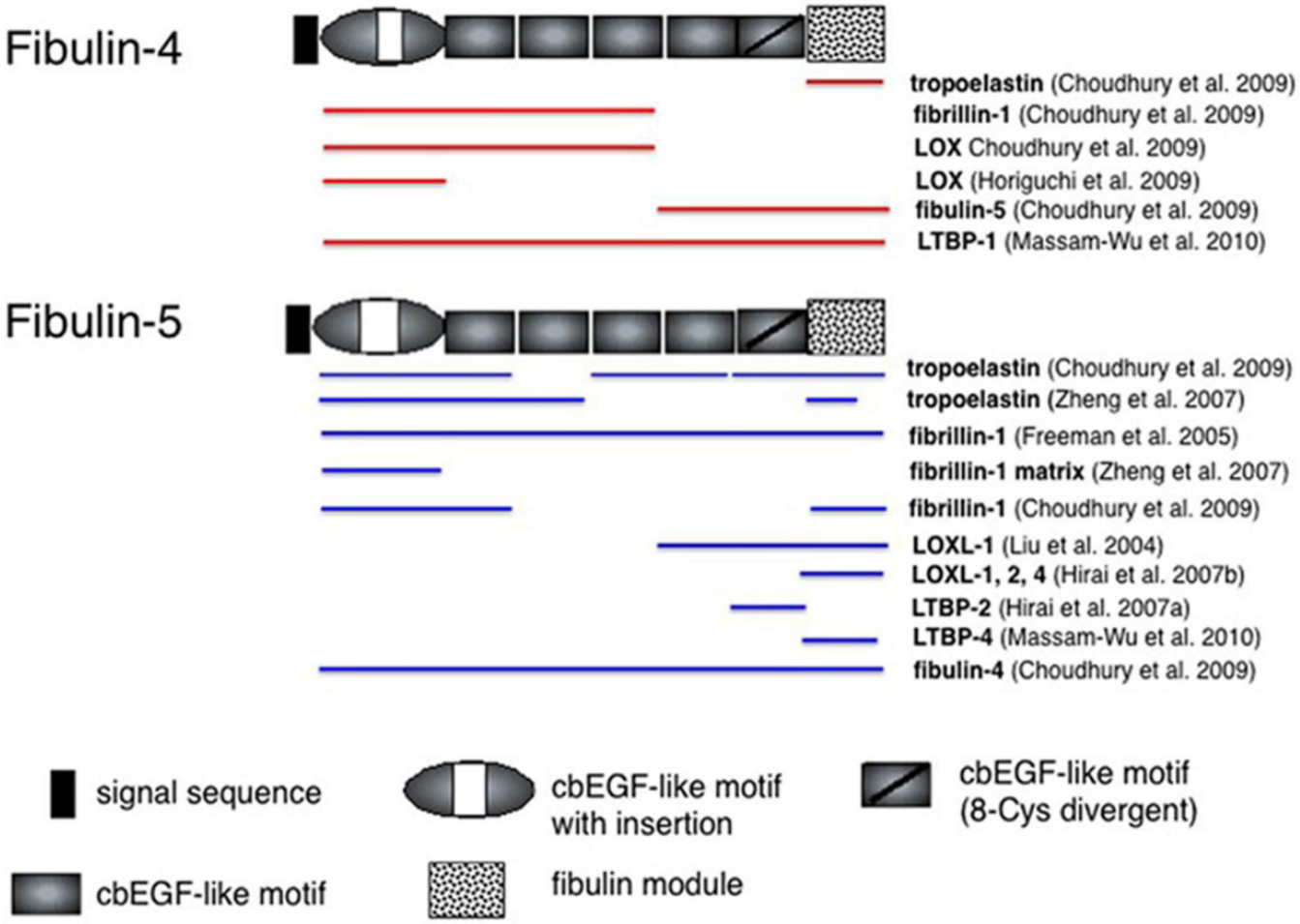


**Fig. 1. Schematic presentation of fibulins-4 and -5**

Fibulin-4 and fibulin-5 are shown with known interacting proteins involved in elastic fiber assembly. Red and blue lines indicate interacting domain(s) for fibulin-4 and fibulin-5, respectively, determined by solid-phase binding assays, BIAcore, or Co-IP. The line encompassing an entire sequence indicates that the binding domain(s) have not been determined.

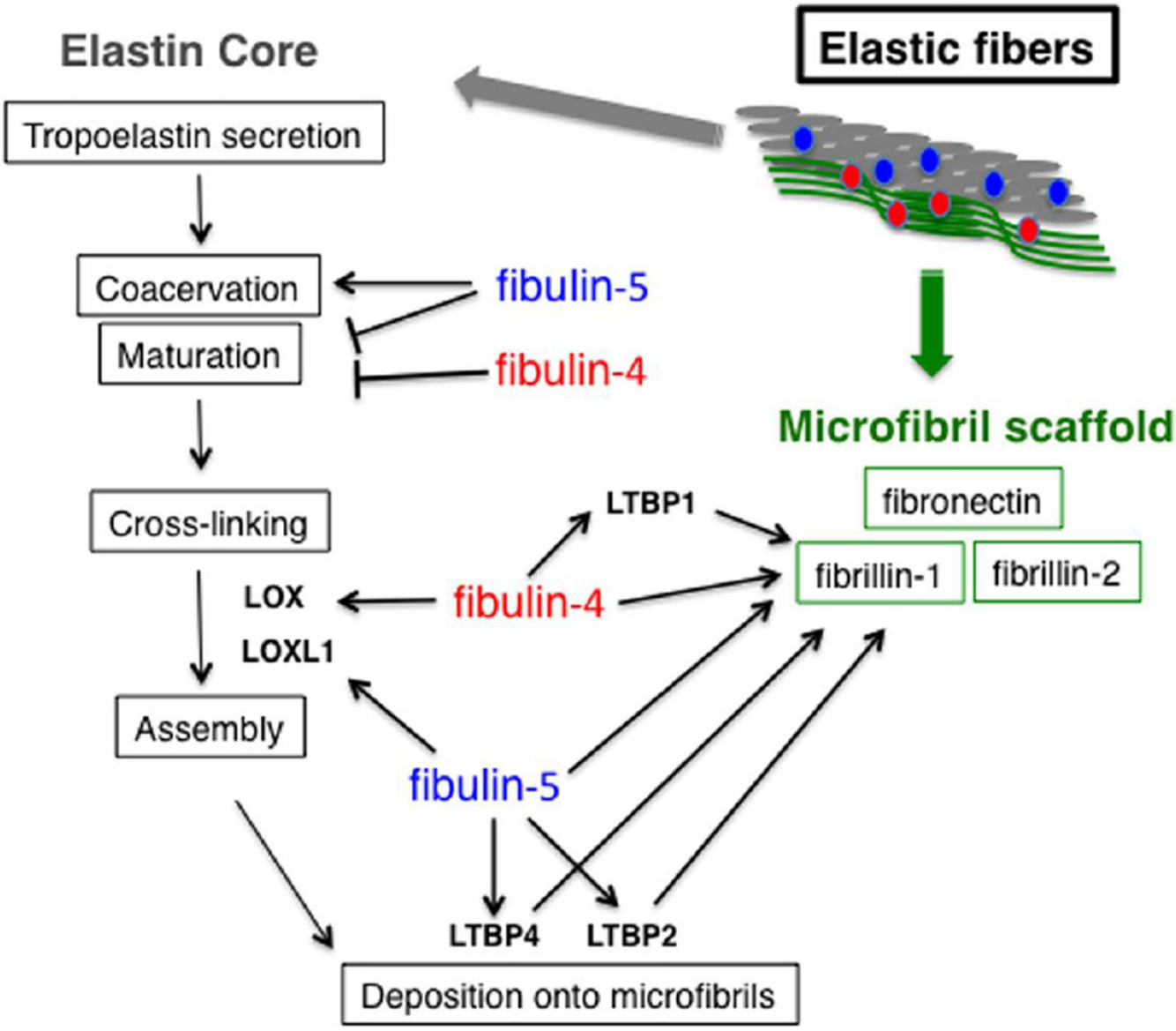


**Fig. 2. Proposed functions of fibulins-4 and -5 during elastogenesis**

Elastic fibers are divided into elastin core (gray) and microfibril scaffold (green). Each step of elastogenesis is shown on the left and molecular interactions among components of elastic fibers and potential functions of fibulins are indicated by arrows.