Fibulin-5, an integrin-binding matricellular protein: Its function in development and disease

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Abstract Interactions between the extracellular matrix (ECM) and cells are critical in embryonic development, tissue homeostasis, physiological remodeling, and tumorigenesis. Matricellular proteins, a group of ECM components, mediate cell-ECM interactions. One such molecule, Fibulin-5 is a 66-kDa glycoprotein secreted by various cell types, including vascular smooth muscle cells (SMCs), fibroblasts, and endothelial cells. Fibulin-5 contributes to the formation of elastic fibers by binding to structural components including tropoelastin and fibrillin-1, and to cross-linking enzymes, aiding elastic fiber assembly. Mice deficient in the fibulin-5 gene (Fbln5) exhibit systemic elastic fiber defects with manifestations of loose skin, tortuous aorta, emphysematous lung and genital prolapse. Although Fbln5 expression is down-regulated after birth, following the completion of elastic fiber formation, expression is reactivated upon tissue injury, affecting diverse cellular functions independent of its elastogenic function. Fibulin-5 contains an evolutionally conserved arginine-glycine-aspartic acid (RGD) motif in the N-terminal region, which mediates binding to a subset of integrins, including α5β1, αvβ3, and αvβ5. Fibulin-5 enhances substrate attachment of endothelial cells, while inhibiting migration and proliferation in a cell type- and context-dependent manner. The antagonistic function of fibulin-5 in angiogenesis has been demonstrated in vitro and in vivo; fibulin-5 may block angiogenesis by inducing the anti-angiogenic molecule thrombospondin-1, by antagonizing VEGF165-mediated signaling, and/or by antagonizing fibronectin-mediated signaling through directly binding and blocking the α5β1 fibronectin receptor. The overall effect of fibulin-5 on tumor growth depends on the balance between the inhibitory property of fibulin-5 on angiogenesis and the direct effect of fibulin-5 on proliferation and migration of tumor cells. However, the effect of tumor-derived versus host microenvironment-derived fibulin-5 remains to be evaluated.

Keywords Angiogenesis · Cutis laxa · Elastic fibers · Fibulin · Fibronectin · Integrin · ROS · Thrombospondin · Tumor

Abbreviations

ECM extracellular matrix
MMP matrix metalloproteinase
LTBP latent TGFβ binding protein
LOXL1 lysyl oxidase-like 1
POP pelvic organ prolapse
RGD arginine-glycine-aspartic acid
ROS reactive oxygen species
TGF-β transforming growth factor-beta
SMC smooth muscle cell
VEGF vascular endothelial growth factor
Fibulin-5: overview

Fibulin-5 was first identified in 1999 by two groups in search of genes involved in phenotypic modulation of vascular smooth muscle cells (SMCs) and in cardiovascular development through subtractive hybridization and signal sequence trap cloning, respectively (Kowal et al. 1999; Nakamura et al. 1999). Fibulin-5 was shown to be a 66-kDa glycoprotein containing six calcium-binding EGF-like (cbEGF) motifs, which are believed to provide stability and facilitate protein interaction. Fibulin-5 also contains a RGD motif involved in the binding to a subset of cell-surface integrins. In the initial reports, the fibulin-5 gene (Fbln5) was shown to be strongly expressed in the embryonic vasculature and neural crest, but was down-regulated in all adult tissues, except in the uterus where active remodeling and angiogenesis takes place. Fbln5 expression, however, was reactivated in injured vessels, including the neointima induced by balloon withdrawal injury, and in atherosclerotic plaques in the mouse model of hypercholesterolemia (Kowal et al. 1999; Nakamura et al. 1999), suggesting a regulatory role in vascular cell function. Fibulin-5 was also identified as a TGF-β-inducible gene in 3T3-L1 fibroblasts and was shown to induce DNA synthesis in a Smad3-dependent manner. In contrast, fibulin-5 inhibited cell proliferation and cyclin A expression in mink lung epithelial cells, suggesting a potential involvement in the control of cell proliferation in a context-dependent manner (Schiemann et al. 2002).

The biological function of fibulin-5 in vivo, however, was unknown until the generation of Fbln5 knockout mice, in which systemic elastic fiber defects were revealed (Nakamura et al. 2002; Yanagisawa et al. 2002). Fbln5-null mice survive to adulthood but progressively develop severe elastinopathy, including loose skin, tortuous aorta, emphysematous lung, and genital prolapse. These observations establish the first animal model for congenital elastic fiber defects. Fbln5-null mice exhibit an elevated pulse pressure, and Fbln5-null aortic explants show a significant decrease in extensibility compared to the wild-type vessels, demonstrating that compromised elastic fibers lead to stiff vessels with decreased elasticity. Fbln5-null skin and lungs contain only short, disrupted elastic fibers, and no signs of inflammatory infiltrates were observed. In addition, the aorta, lungs, and skin did not show a disruption in collagen fiber formation, confirming that the defects in Fbln5-null mice were confined to the elastic fiber system.

The biological functions of fibulin-5 have been investigated in in vitro studies, and can be segregated into elastogenic and extra-elastogenic functions. However, distinguishing these functions in vivo is challenging due to the fact that insoluble elastin is known to influence cellular behavior (Karnik et al. 2003).

Fibulin-5: an integrin-binding member of the Class II fibulin subfamily

Fibulins are characterized by tandem repeats of calcium-binding EGF (cbEGF)-like motifs and a globular C-terminal fibulin module. There are seven known fibulins (Fig. 1), which can be subdivided into two subfamilies based on their size and domain structure. The Class I fibulin subfamily includes the prototype fibulin-1, fibulin-2, and fibulin-6 (reviewed in Argraves et al. 2003; Timpl et al. 2003). In this subfamily, cbEGF repeats are longer than in Class II fibulins, and there are additional N-terminal domains that are not
shared by Class II fibulins. Fibulin-6 contains the largest N-terminal domain, consisting of a von Willebrand factor domain, more than forty immunoglobulin domains depending on the species, and six thrombospondin type I repeats (Vogel and Hedgecock 2001). Class II fibulins include fibulin-3, fibulin-4, fibulin-5 and fibulin-7. Fibulins-3, -4 and -5 contain six cbEGF domains, the first of which contains a proline-rich insertion sequence, and the sixth domain, which is a divergent type with 8 cysteines. The identity score of the primary amino acid sequence between fibulin-5 and fibulins-3, -4 and -7 in humans is 41%, 49%, and 24%, respectively. Fibulin-7 is the newest member and is rather atypical because of the shorter cbEGF domains and the presence of a sushi domain, which is frequently found in complement proteins, but is absent in fibulins-3, -4, and -5 (de Vega et al. 2007).

Fibulin-5 contains an evolutionally conserved RGD sequence in the first cbEGF motif (Fig. 2). The RGD sequence is present in various matricellular and ECM proteins, including fibronectin, vitronectin, osteopontin and thrombospondins, and is recognized by heteromeric integrin receptors to participate in cellular functions (Davis et al. 2000; Ruoslahti and Pierschbacher 1987). For example, ECM-cell binding is essential for the assembly of fibrous ECM proteins such as fibronectin (Wu et al. 1995), to trigger cellular effects via the cytoplasmic tail of integrin (Legate et al. 2006), and to form cell-surface protease complexes, involving urokinase type plasminogen activator (uPA)/uPA receptor, vitronectin, and activated integrins (Madsen and Sidenius 2008).

Fibulin-5 was shown to mediate binding to human umbilical vein endothelial cells (HUVECs) in a RGD-dependent manner (Nakamura et al. 1999). Further, it was shown that the N-terminal half of fibulin-5 mediates cell attachment via αvβ3, αvβ5 and α9β1 integrins (Nakamura et al. 2002). On the other hand, Lomas et al. found that fibulin-5 mediates attachment and spreading of primary aortic SMCs through binding to the fibronectin receptor α5β1 and α4β1, but not to αvβ3 (Lomas et al. 2007). Although the RGD motif and insertion sequence of fibulin-5 was suggested to be exposed to the cell surface (Albig and Schiemann 2005), direct protein interaction assays revealed that fibulin-5 was only able to bind to αvβ3 after reduction and alkylation, which unmarks the RGD sequence (Kobayashi et al. 2007). Furthermore, truncated fibulin-5, containing the first cbEGF domain alone, did not support binding and spreading of SMCs. Taken together, these results indicate that fibulin-5-integrin interactions may require efficient exposure of the RGD motif and presence of the flankling domains of fibulin-5 (Lomas et al. 2007). Interestingly, fibulin-5 failed to activate downstream signaling after binding to α5β1 and α4β1 integrins. Fibulin-5 antagonized fibronectin-induced stress fiber formation and focal adhesions in SMCs in a dose-dependent manner, suggesting that fibulin-5 acts in a dominant-negative fashion to inhibit fibronectin receptor-mediated signaling.
Repeating cbEGF motifs are present in ECM and transmembrane proteins and are involved in protein-protein interactions (Maurer and Hohenester 1997). Various binding partners for fibulin-5 have been reported, including fibulin-5 itself (Jones et al. 2009; Zheng et al. 2007) (Table 1). Most notably, fibulin-5 binds to several molecules, critical for elastic fiber assembly, which will be discussed later in this review. All class II fibulins except fibulin-7 were shown to bind to tropoelastin (Kobayashi et al. 2007) and are responsible for different aspects of elastic fiber development in vivo (McLaughlin et al. 2006; McLaughlin et al. 2007). Although a direct binding of Ca\(^{2+}\) to the cbEGF domains of fibulin-5 has not been demonstrated, reducing the Ca\(^{2+}\) concentration significantly decreased tropoelastin binding to fibulin-5 in solid-phase binding assays (Wachi et al. 2007; Yanagisawa et al. 2002). Furthermore, a large deletion of the cbEGF domains of fibulin-5 exhibited a significant reduction in tropoelastin binding, suggesting that cbEGF domains play a significant role in tropoelastin-fibulin-5 binding (Zheng et al. 2007). Fibulin-5 also binds to extracellular-type superoxide dismutase (SOD3), which regulates extracellular superoxide anion (O\(_2\)^\(-\)) levels by facilitating the conversion of O\(_2\)^\(-\) to hydrogen peroxide (H\(_2\)O\(_2\)) and protecting the formation of peroxynitrite. Consistent with this observation, Fbln5-null aorta was shown to contain higher amounts of O\(_2\)^\(-\) compared to wild-type controls because of the impaired tethering of SOD3 (Nguyen et al. 2004).

Fibulin-5 was shown to undergo partial proteolytic cleavage at arginine at position 77 in the N-terminal region, creating a truncated form of fibulin-5 (Hirai et al. 2007b). A point mutation of arginine to alanine inhibited proteolytic cleavage of fibulin-5 in vitro. The truncated fibulin-5 increased with aging in the skin of wild-type mice, and showed impaired ability to assemble elastic fibers in in vitro elastogenic assays. The putative protease responsible for the cleavage was suggested to be a serine protease (Hirai et al. 2007b); however, the in vivo significance of this cleavage and its relationship with human skin diseases still needs to be established.

### Fibulin-5 and elastic fiber development

Elastic fibers are composed of an amorphous-appearing elastin core and a peripheral mantle of 10-nm fibrillin-containing microfibrils. In addition to elastin, fibrillin-1, and fibrillin-2, more than 30 microfibril- and elastin-associated proteins, have been identified, including fibulins, microfibril-associated glycoproteins MAGP-1 and MAGP-2, and latent TGF-\(\beta\) binding proteins-1 through -4 (LTBPs) (reviewed in Kielty et al. 2002). Elastogenesis occurs through a series of highly regulated steps that involve secretion of the tropoelastin monomer, self-aggregation of tropoelastin, called coacervation, correct assembly and cross-linking of tropoelastin, and final organization of the insoluble elastin into functional fibers. In the mouse aorta, elastin expression starts during mid-gestation in vascular smooth muscle cells and continues for approximately 1 month after birth (Kelleher et al. 2004). Fbln5 expression coincides with elastin expression in the aorta. During this time, the secreted elastin is cross-linked and organized into insoluble elastin sheets or laminae. Interestingly, microfibril bundles form first in the regions where elastic fibers will develop, and insoluble elastin is never observed in the...
absence of microfibrils. Recently, double knockout mice for the genes encoding fibrillin-1 (Fbn1) and fibrillin-2 (Fbn2) were generated. These mice exhibited a severe disruption of elastic fibers and a marked dysregulation of TGF-β-mediated signals (Carta et al. 2006). The latter event occurs due to the inability to tether proTGF-β onto microfibrils via the interaction between large latency complex and fibrillin-1 (Charbonneau et al. 2004; Isogai et al. 2003; Neptune et al. 2003); however, the elastin core does not seem to be involved in this process. These observations, together with biochemical and ultrastructural studies, have led to the suggestion that an interaction between microfibril proteins and tropoelastin may be critical for proper elastic fiber assembly.

Role of fibulin-5 in elastic fiber assembly

A dose-dependent, direct interaction between fibulin-5 and tropoelastin was demonstrated by solid-phase binding assays, and co-localization of fibulin-5 and elastic fibers was revealed at an electron microscopic level, providing a basis for the molecular function of fibulin-5 (Yanagisawa et al. 2002). Fibulin-5 was shown to bind tropoelastin preferentially, but not polymerized α-elastin, suggesting its role in an early step of elastogenesis (Zheng et al. 2007). Using full-length recombinant fibulin-5, it was shown that fibulin-5 accelerates coacervation (Hirai et al. 2007b; Wachi et al. 2008), and the interaction between fibulin-5 and tropoelastin is enhanced by increased temperature and sodium chloride concentrations, which is consistent with the conditions for the efficient coacervation in vitro (Wachi et al. 2008). Furthermore, Cirulis et al. showed that fibulin-5 limits the maturation of coacervated elastin fragments (Cirulis et al. 2008). Consistent with these data, Choi et al. showed by using electron microscopy that the average size of elastin aggregates was increased in the skin of Fbln5-null mice, compared with wild-type mice (Choi et al. 2009). Taken together, these findings show that fibulin-5 functions at a formation and maturation step of coacervation to 1) control coacervation efficiency, and 2) to regulate the size of self-aggregates to achieve optimal cross-linking of tropoelastin during elastic assembly.

It was also demonstrated that fibulin-5 binds to fibrillin-1, a major component of microfibrils (ElHallous et al. 2007; Freeman et al. 2005). Fibulin-5 co-localizes with microfibrils in elastogenic cells, as well as in non-elastogenic cells, engineered to overexpress tropoelastin and fibrillin-5 (Hirai et al. 2007b; Nonaka et al. 2009; Zheng et al. 2007). Consistent with these observations, a significant alteration of fibulin-5 expression was observed in tight skin mice, in which a Fbn1 mutation caused abnormally tight skin, dermal fibrosis, and impaired elastogenesis (Lemaire et al. 2004). Furthermore, it was shown that knocking down Fbn1 in human skin fibroblasts abrogates the formation of elastic fibers and decreases fibulin-5 immunoreactivity. Binding between LTBP-2 and fibulin-5 was shown to promote the deposition of fibulin-5 onto fibrillin-1 microfibrils (Hirai et al. 2007a). Whether the formation of a fibrillin-1-fibulin-5-tropoelastin ternary complex is necessary to bind microfibrils, or whether

### Table 1 Interacting partners of Fibulin-5

| Interacting proteins | Binding site(s) within Fibulin-5 | Cellular and/or Biological functions | Reference |
|---------------------|---------------------------------|-------------------------------------|-----------|
| Fibulin-5           | ND                              | Unknown                             | Zheng et al. 2007 |
| Tropoelastin        | cbEGF domains                   | Elastic fiber assembly              | Jones et al. 2009 |
|                     | N-terminal cbEGF domains        |                                     | Zheng et al. 2007 |
|                     | C-terminal EB domain           |                                     |           |
| Loxl-1              | a. C-terminal fibulin domain > 1st cbEGF | Elastic fiber assembly               | a. Hirai et al. 2007a |
|                     | b. C-terminal region (245-448)  |                                     | b. Liu et al. 2004 |
| Loxl-2, 4           | C-terminal fibulin domain       | Elastic fiber assembly              | Hirai et al. 2007a |
| Emilin-1            | ND                              | Elastic fiber assembly              | Zanetti et al. 2004 |
| LTBP-2              | 6th cbEGF                       | Elastic fiber assembly              | Hirai et al. 2007b |
| Fibrillin-1         | ND                              | Elastic fiber assembly              | Freeman et al. 2005 |
| SOD3                | C-terminal region (320-448)     | Superoxide scavenge                 | Nguyen et al. 2004 |
| α5β1, α4β1          | N-terminal half containing RGD  | Cell attachment, Antagonize          | Lomas et al. 2007 |
|                     |                                 | fibronectin function                |           |
| ανβ3, αβ5           | N-terminal half containing RGD  | Cell attachment                     | Nakamura et al. 2002 |
| α9β1                | ND                              | Cell attachment                     | Nakamura et al. 2002 |
| Lipoprotein(a)      | C-terminal domain (350-448)     | Unknown                             | Kapetanopoulos et al. 2002 |

ND not determined
fibulin-5-tropoelastin binding alone is sufficient, needs to be determined.

Fibulin-5 also binds cross-linking enzymes, including lysyl oxidase like (Loxl)-1, -2, and -4 (Hirai et al. 2007b; Liu et al. 2004). Overexpression of Fbln5 increased deposition of elastin and the desmosine level of elastin, indicating that fibulin-5 facilitates cross-linking of tropoelastin (Nonaka et al. 2009). Retrovirus- or adenovirus- mediated gene transfer of Fbln5 accelerated deposition of elastic fibers in human skin fibroblasts (Katsuta et al. 2008) and regenerated elastic fibers in the skin of Fbln5-null mice, respectively (Zheng et al. 2007). The deletion mutant of fibulin-5 lacking its N-terminal region, however, was unable to rescue elastic fiber defects both in vitro and in vivo, despite the presence of the elastin-binding domain located within the C-terminal fibulin module (Hirai et al. 2007b; Zheng et al. 2007). Therefore, the C-terminal elastin-binding domain is required but not sufficient for the formation of elastic fibers.

Although the significance of the RGD sequence in elastic fiber assembly was initially suggested, generation of mutant mice expressing fibulin-5 D56E (Fbln5^RGE/RGE^), a mutation known to disrupt the binding of the ECM to RGD-dependent integrins (Yang et al. 2007), developed completely normal elastic fibers (our unpublished observation), suggesting that cell-surface binding of fibulin-5 mediated by integrins is dispensable for the formation of elastic fibers. Taken together, we propose a model in which fibulin-5 binds tropoelastin to regulate the coacervation step and serves as an adaptor to bind the cross-linking enzymes, tropoelastin and microfibrils, to aid in elastic fiber assembly (Fig. 3).

Mutation of fibulin-5 in human elastic fiber disease

Human genetic studies have identified two homozygous missense mutations of FBLN5 (p.S227P) and (p.C217R) in autosomal recessive (AR) cutis laxa families (Claus et al. 2008; Elahi et al. 2006; Loeys et al. 2002). The first mutation was reported in two families of ethnically different groups and the disease manifested as a severe form of cutis laxa with internal organ involvement (AR cutis laxa type I). Biochemically, both substitutions of fibulin-5 (S227P and C217R) were shown to decrease binding to tropoelastin. The S227P mutation further revealed a significant decrease in synthesis and secretion of the mutant protein and impaired association with fibrillin-1 (Hu et al. 2006). A heterozygous tandem duplication of fibulin-5 was also reported in a patient with a mild form of cutis laxa, suggesting that the large mutant protein might act in a dominant negative fashion (Markova et al. 2003).

Heterozygous missense variations in FBLN5 were reported to be associated with age-related macular degeneration (ARMD), a common cause of progressive vision loss (Lotery et al. 2006; Stone et al. 2004). Fibulin-5 is normally localized in Bruch’s membrane and the choriocapillaris of the retina, where elastic fibers are present. Fibulin-5 was observed in the pathological basal deposits beneath the retinal pigment epithelium in eyes affected by age-related macular degeneration (Mullins et al. 2007). Some mutations of fibulin-5 (G412E, G267S, J169T, and Q124P) were shown to cause decreased secretion of the mutant protein into the media (Lotery et al. 2006), suggesting compromised elastic fiber formation as the underlying mechanism. However, the causal relationship between heterozygous missense mutations in fibulin-5 and ARMD needs to be further investigated.

Alteration of fibulin-5 in pathological conditions involving elastic fibers

Fbln5-null mice were shown to develop pelvic organ prolapse and have served as one of the first animal models for this condition (Drewes et al. 2007). By 6 months of age, 92% of Fbln5-null females developed vaginal prolapse with severe disruptions in elastic fiber formation. Biomechanical studies using vagina tissue from pregnant and non-pregnant females from wild-type and Fbln5-null mice revealed that the Fbln5-null vagina with prolapse was similar to that of pregnant wild-type females, exhibiting a significant decrease in vaginal stiffness (Rahn et al. 2008). Subsequently, it was shown in the uterosacral ligaments of patients with pelvic organ prolapse (POP) that expression of fibulin-5 was significantly decreased (Jung et al. 2009). Additionally, fibulin-5 mRNA was decreased in para-urethral biopsies obtained from POP women (Soderberg et al. 2009), underscoring defective elastic fibers as an underlying cause of POP.

Another organ affected by the absence of fibulin-5 is the lung, in which the defect is manifested as an emphysematous lung that progressively worsens after birth. Expression of fibulin-5 is observed during embryogenesis and continues after birth to complete the development of the lung. Interestingly, FGF18 expression, which peaks after birth, correlates with the expression of tropoelastin in vivo, and supports elastogenesis by inducing proliferation of lung fibroblasts, as well as by expression of elastogenic genes, including Fbln5 and Lox (Chailley-Heu et al. 2005). Fbln5^expression was confirmed in rat lung interstitial fibroblasts, and was markedly increased after treatment with TGF-β or following elastase-induced lung injury (Kuang et al. 2003; Kuang et al. 2006). On the other hand, treatment of lung interstitial fibroblasts with interleukin -1β completely abolished the expression of Fbln5 (Kuang et al. 2003), demonstrating the regulation of Fbln5 by distinct sets of cytokines.
Effect of fibulin-5 on vascular cells

The function of fibulin-5 in adult blood vessels was suggested by the strong upregulation of \textit{Fbln5} in the neointima after balloon withdrawal injury or carotid artery ligation, and in activated endothelial cells of atherosclerotic plaques (Kowal et al. 1999; Spencer et al. 2005). \textit{Fbln5-null} SMCs displayed enhanced proliferation and migration in response to serum and PDGF. This enhancement was inhibited by over-expression of \textit{Fbln5} (Spencer et al. 2005). Forced expression of \textit{Fbln5} in primary human endothelial cells improved cell attachment against continuous shear stress and enhanced cell retention on artificial grafts subjected to pulsatile flow. However, the proliferation of endothelial cells was decreased in \textit{Fbln5}-overexpressing cells compared to control cells (Preis et al. 2006). Human primary SMCs plated on recombinant fibulin-5 exhibited PDGF receptors with undetectable phosphorylation, and poorly phosphorylated EGF receptors, compared to SMCs plated on fibronectin (Lomas et al. 2007). Interestingly, the presence of integrin β1-activating antibody in SMCs, plated on fibulin-5, restored phosphorylation of PDGF α and β receptors. Together with the observation that fibulin-5 binds fibronectin receptors (α5β1 and α4β1) but fails to activate downstream signaling, these data suggest a possibility that fibulin-5 may regulate vascular cell behavior by antagonizing fibronectin-mediated signaling. However, further investigation is required to establish the in vivo relevance of the fibulin-5-integrin binding during vessel development and pathological insults.

Fibulin-5 and angiogenesis

The effect of fibulin-5 on angiogenesis was first described using mouse brain microvascular MB114 endothelial cells, in which \textit{Fbln5} overexpression or incubation with recombinant fibulin-5 inhibited sprouting, proliferation, and invasion in matrigel (Albig and Schiemann 2004). Upregulation of thrombospondin-1 expression, as well as antagonization of VEGF \textsubscript{165} -mediated signaling, including activation of P38 MAPK and ERK1/2, were suggested to be the underlying causes of the inhibitory effect of fibulin-5. Fibulin-5 was also shown to reduce migration of human microvascular HMEC-1 endothelial cells toward fibronectin, and overexpression of \textit{FBLN5} in MB114 cells down-regulated MMP-2 expression, as well as enzymatic activity during tubulogenesis on collagen gels (Albig et al. 2006). Matrigel implantation experiments in the skin of wild-type mice showed that matrigel containing high or low doses of fibulin-5 stimulated the invasion of bFGF-induced fibroblasts, but inhibited vessel formation and angiogenesis, independent of RGD-integrin interactions. Consistent with these observations, \textit{Fbln5-null} mice exhibited increased angiogenesis after physiological wound healing and PVA sponge implantation, without an effect on fibroblast invasion (Sullivan et al. 2007; Zheng et al. 2006).

Although exogenous and endogenous fibulin-5 was shown to antagonize angiogenesis, the exact mechanism behind this effect has remained elusive. We recently observed that fibulin-5 exerts its effect on endothelial cells and angiogenesis by controlling integrin-induced production of reactive oxygen species (ROS), which have pro-angiogenic properties (Schlueterman et al. 2009). ROS, including O\textsubscript{2} and H\textsubscript{2}O\textsubscript{2}, are highly reactive molecules produced commonly as by products of aerobic respiration and the mitochondrial transport chain. ROS were originally identified as host defense molecules produced by neutrophils via NAD(P)H oxidases, and have a critical function in eliciting biological processes required for the initiation of angiogenesis (Wu 2006). By functioning as signaling molecules, ROS have been shown to activate pathways such as proliferation, cell adhesion, motility and invasion in endothelial cells (Ushio-Fukai 2007).

Endothelial cells, treated with H\textsubscript{2}O\textsubscript{2}, produced higher levels of VEGF, thereby increasing their proliferation and invasion in matrigel (Albig and Schiemann 2004). Upregulation of thrombospondin-1 expression, as well as antagonization of VEGF \textsubscript{165} -mediated signaling, including activation of P38 MAPK and ERK1/2, were suggested to be the underlying causes of the inhibitory effect of fibulin-5. Fibulin-5 was also shown to reduce migration of human microvascular HMEC-1 endothelial cells toward fibronectin, and overexpression of \textit{FBLN5} in MB114 cells down-regulated MMP-2 expression, as well as enzymatic activity during tubulogenesis on collagen gels (Albig et al. 2006). Matrigel implantation experiments in the skin of wild-type mice showed that matrigel containing high or low doses of fibulin-5 stimulated the invasion of bFGF-induced fibroblasts, but inhibited vessel formation and angiogenesis, independent of RGD-integrin interactions. Consistent with these observations, \textit{Fbln5-null} mice exhibited increased angiogenesis after physiological wound healing and PVA sponge implantation, without an effect on fibroblast invasion (Sullivan et al. 2007; Zheng et al. 2006).

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migration (Chua et al. 1998). It has also been shown that ROS, produced in response to hypoxic conditions, can facilitate capillary tube formation in human microvascular endothelial cells, and this process was inhibited by treatment with an anti-oxidant (Lelkes et al. 1998). ROS have also been shown to stimulate angiogenesis in vivo. For example, neovascularization in response to hindlimb ischemia was significantly impaired in mice lacking gp91phox, a critical component of NADPH oxidases (Tojo et al. 2005). However, the production of ROS must be tightly regulated because a large excess of ROS can be detrimental to the remodeling process and result in endothelial cell death (Touyz and Schiffrin 2004). Since α5β1 integrin serves as the primary fibronectin receptor and stimulates ROS production (Chiarugi et al. 2003), and fibulin-5 binding to α5β1 leads to inhibition of fibronectin-mediated downstream signaling (Lomas et al. 2007), it is plausible that fibulin-5 antagonizes endothelial cell proliferation and migration by controlling ROS levels through binding to α5β1 integrin. Interestingly, ROS production increases in response to vascular injury and atherosclerotic changes as part of the inflammatory response to aid in endothelial cell stimulation (Cai and Harrison 2000). Therefore, it is possible that the increase in fibulin-5 expression observed following trauma to the vasculature could be used as a mechanism to control ROS production during such events.

**Fibulin-5 in tumorigenesis**

Given the varying effects of fibulin-5 on different cell populations, it is no surprise that the effect of fibulin-5 on tumor growth is complex and appears to be largely context-dependent. For example, MCA 102 fibrosarcoma cells, stably expressing Fbn5 and subcutaneously injected into isogenic wild-type mice, produced significantly reduced tumor growth, despite the fact that stimulation with fibulin-5 increased the invasiveness of MCA 102 cells into a synthetic basement membrane in vitro (Albig et al. 2006). When Fbn5-overexpressing HT1080 fibrosarcoma cells were injected into BALB/c SCID mice, tumor growth was inhibited and tumor blood vessel formation was significantly decreased compared to the mice injected with control HT1080 cells (Xie et al. 2008). In stark contrast, overexpression of Fbn5 in 4T1 breast cancer cells increased invasiveness by inducing TGF-β-stimulated epithelial-mesenchymal transitions and MMP expression. These alterations led to increased tumor growth in vivo when these cells were implanted into normal wild-type mice. (Lee et al. 2008).

Examination of FBLN5 mRNA levels in various human tumors from kidney, breast, prostate, lung and gastrointestinal organs, revealed that FBLN5 expression was down-regulated in 62% of tumors in comparison to control normal tissues (Schiemann et al. 2002). However, the analysis of FBLN5 expression in human tumors has been limited and has largely centered on whole tumor tissue analysis, allowing for no distinction between tumor cell-derived and host-derived fibulin-5. Given the differential effects of fibulin-5 on cells of epithelial origin and on cells of mesenchymal origin (Schiemann et al. 2002), the overall effect of fibulin-5 on tumor development must be carefully evaluated in human tumor specimens.

**Conclusions and future directions**

Fibulin-5 is a matricellular protein, contributing to the structural development of elastogenic tissues, as well as mediating various cellular functions required for the maintenance of tissue homeostasis. The discovery of fibulin-5 has provided a new insight into the regulated steps of elastic fiber assembly and has given us an opportunity to explore therapeutic implications, including prevention of elastic fiber-degenerative conditions, regeneration of damaged elastic fibers, and development of efficient artificial blood vessels. The availability of genetically engineered mice that enables us to delete Fbln5 after the completion of elastic fibers will allow us to distinguish between the elastic fiber-dependent or -independent effect of fibulin-5. It also remains to be investigated how Class II fibulins are involved in elastic fiber development in vivo, and what are the redundant and specific roles among these fibulins. A diverse cellular effect of fibulin-5, in a cell-type specific and context-dependent manner, suggests that there may be multiple fibulin-5-interacting proteins at the cell surface, including integrins. It remains to be determined how fibulin-5 mediates or antagonizes cellular signaling at the cell surface, and whether modulation of fibulin-5 has the potential for the therapy of angiogenesis-dependent pathologies.

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Fibulin-5 in development and disease

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