Int. J. Dev. Biol. 55: 511-525
doi: 10.1387/ijdb.103243eo

THE INTERNATIONAL JOURNAL OF DEVELOPMENTAL BIOLOGY
www.intjdevbiol.com

Fibronectin and tenascin-C: accomplices in vascular morphogenesis during development and tumor growth

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ABSTRACT In addition to soluble factors, the extracellular matrix (ECM) also plays a vital role in normal vasculogenesis and in the pathological angiogenesis of many disease states. Here we will review what is known about the role of the ECM molecules fibronectin and tenascin-C in the vasculature and highlight a potential collaborative interplay between these molecules in developmental and tumorigenic angiogenesis. We will address the evolution of these modular proteins, their cellular interactions and how they become assembled into an insoluble matrix that impacts the assembly of other ECM proteins and the bioavailability of pro-angiogenic factors. The role of fibronectin and tenascin-C networks in tumor angiogenesis and metastasis will be described. We will elaborate on lessons learned about their role in vessel function from the functional ablation or the ectopic expression of both molecules. We will also elaborate on potential mechanisms of how fibronectin and tenascin-C affect cell adhesion and signaling that are relevant to angiogenesis.

KEY WORDS: tumor angiogenesis, matrix assembly, fibronectin, tenascin-C

Introduction

Cells and extracellular matrix (ECM) form tissues, and collections of tissues form organs. In the organism different organs act together through blood and lymphatic vessels. Solid tumors resemble organs that are structurally and functionally abnormal. They contain multiple cell types and ECM components and develop through complex interactions between these different components using processes that often resemble those used by developing organs (reviewed in Egeblad *et al.*, 2010). It is long known that tumors need to turn on angiogenesis in order to grow more than a few millimeters in diameter. Tumors have developed several strategies to trigger the so called "angiogenic switch" in order to develop a connection to the hemapoietic and lymphatic vasculature which is believed to be essential for nourishment and oxygenation. Connections to the vasculature also present pathways for motile cancer cells to travel to distant organs to seed metastasis.

In the initial concept of sprouting angiogenesis, largely supported by angiogenesis observed in tumor xenograft experiments, tumors secrete factors that stimulate the process of the outgrowth of new blood vessels from preexisting vessels (Risau, 1997). The development of new vasculature by angiogenesis occurs in two stages. First, a dense, immature, evenly spaced network of new vessels develops by recursive sprouting and fusion of sprouts. Second, the network is remodeled into a hierarchically spaced network by adaptive pruning events and blood flow. How vessel branching is regulated at the molecular level is a matter of debate. Whether the tip cell represents the default state or is actively induced is not clear. At least it is known that VEGFR2, Wnt and notch signaling

Abbreviations used in this paper: αTM, alpha tropomyosin; DKK, dickkopf; E, embryonic day; ECM, extracellular matrix; EDA, extra domain A; EDB, extra domain B; EDNRA (B), endothelin receptor type A (B); ET1, endothelin 1; FAK, focal adhesion kinase; FGF, fibroblast growth factor; Id2, inhibitor of differentiation 2; ILK, integrin-linked kinase; JNK, c-Jun N-terminal kinase; LPA, lysophosphatidic acid; MEK, mitogen-activated protein kinase kinase; MMP, matrix metalloproteinase; MMTV, mouse mammary tumor virus; PDGFR, platelet-derived growth factor receptor; PI3K, phosphoinositide 3-kinase; PLCγ, phospholipase C gamma; RT2, Rip1Tag2, SV40 T antigen induced insulinoma; TFGβ1, transforming growth factor beta 1; VEGF, vascular endothelial growth factor; VM, vasculogenic mimicry.

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Final, author-corrected PDF published online: 12 July 2011.

ISSN: Online 1696-3547, Print 0214-6282

are involved in tip cell organization (Bentley *et al.*, 2008). Apart from sprouting angiogenesis, there are several other mechanisms of tumor vascularization, including intussusceptive angiogenesis, vessel co-option, and recruitment of endothelial progenitor cells. In addition, lymphangiogenesis and vasculogenic mimicry are involved in the formation of the tumor microcirculation (Dome *et al.*, 2007; Hillen and Griffioen, 2007; Kucera *et al.*, 2009).

Much emphasis has been placed on the role of angiogenic cytokines such as vascular endothelial growth factor (VEGF) in endothelial cell biology. However, considerable evidence indicates that the matrix is equally important in vessel homeostasis and remodeling. Its role at the molecular level is still poorly understood. ECM proteins provide instructive signals to cells during development, homeostasis and in disease states. The ECM can regulate cell and tissue behavior by serving as a structural network as well as initiating biochemical signaling cascades in cells through interactions with a number of specialized transmembrane ECM receptors such as integrins (reviewed in Erler and Weaver, 2009). Emerging evidence indicates that distinct ECM molecules act in concert to elicit their biological effects. Thus, deciphering the coordinated action of ECM proteins is key to understanding how the ECM network can support normal vascular function and influence the tumor microenvironment to promote angiogenesis.

Fibronectin is a large multi-domain ECM glycoprotein with a fundamental role in blood vessel morphogenesis during embryonic development and pathological angiogenesis. Since the first investigation of fibronectin distribution by Linder and collaborators in chick embryos (Linder *et al.*, 1975), countless studies have documented its elevated expression at sites of tissue remodeling, organogenesis and in numerous disease states. Whereas fibronectin is strongly expressed around developing blood vessels during embryogenesis (Peters and Hynes, 1996), its expression is barely detectable in the normal adult vasculature (ffrench-Constant and Hynes, 1989; Peters *et al.*, 1996). Re-expression of fibronectin occurs during pathological angiogenesis in various diseases such as cancer, late stage artherosclerosis and in blinding ocular conditions (Astrof and Hynes, 2009; Neri and Bicknell, 2005; Pedretti *et al.*, 2009, 2010; Roy *et al.*, 1996, and references therein).

Fibronectin is commonly classified into two forms, plasma fibronectin (p-fibronectin), a soluble form produced by hepatocytes that circulates in blood at high concentrations, and cellular fibronectin (c-fibronectin) produced in tissues where it is incorporated in a fibilllar matrix. c-Fibronectin differs from p-fibronectin by the presence of additional domains, including the highly conserved fibronectin type III "extra" domains B (EDB) and/or A (EDA), that arise from alternative splicing of the pre-mRNA. (Fig. 1, ffrench-Constant and Hynes, 1989; Peters *et al.*, 1996; White *et al.*, 2008,

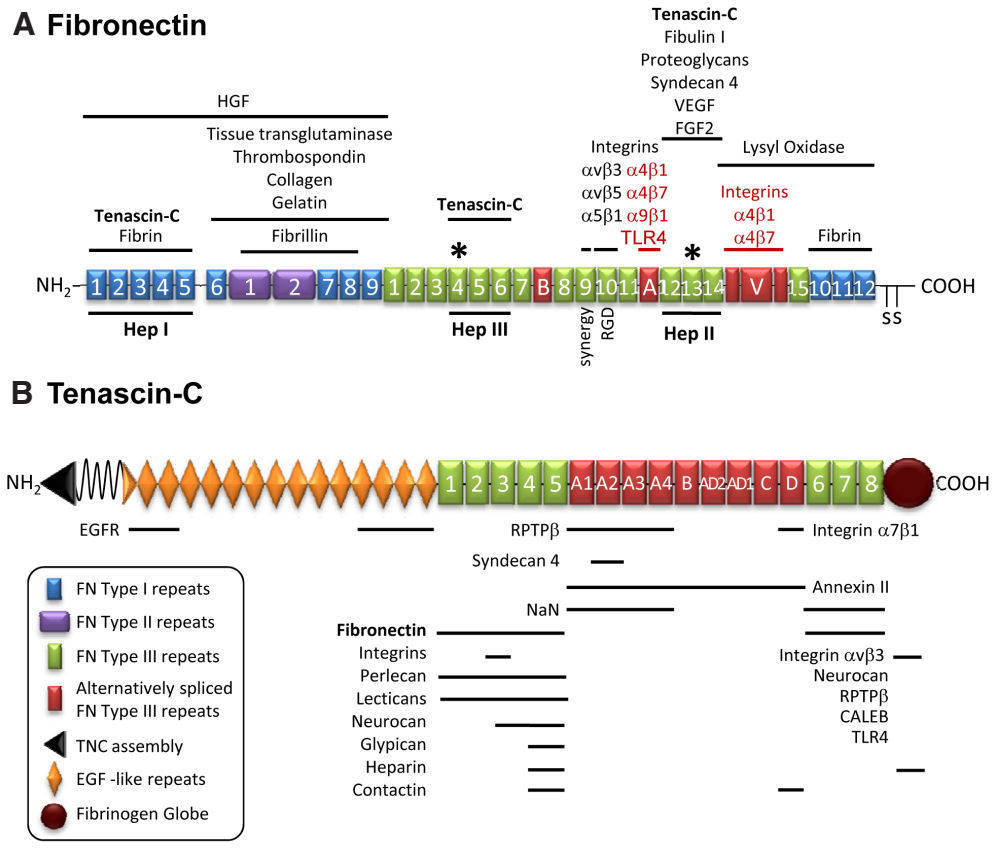

Fig. 1. Domain structure of fibronectin and tenascin-C and potential binding partners. (A) *Fibronectin is a dimeric protein of 240-270KDa, composed of 2 similar or identical monomers joined by a pair of disulfide bonds near the C terminus. Each monomer is organized into type I, type II and type III repeats (FN I-III). Extra Domains B (B) and A (A) correspond to type III repeats. Alternate splicing at a third site (V) gives rise to inserts of variable length (up to 5 in humans) that are nearly always included in c-fibronectin. Heparin-binding domains (Hep) I-III and binding domains for cellular receptors, ECM components, enzymes and growth factors are indicated. Multiple cellular binding sites for fibronectin contribute to angiogenesis (reviewed in Avraamides et al., 2008; Hynes, 2007). Most notably, the tri-peptide motif Arg-Gly-Asp (RGD) located in the 10th FN type III repeat (FN III-10) is the site of binding to α5β1 integrin, as well as αv-based integrins (Leiss et al., 2008; Pankov and Yamada, 2002) including αvβ3 and αvβ5, which are all currently targeted in anti-angiogenic strategies (Desgrosellier and Cheresh, 2010). The asterisks correspond to sites of mutations FN1 identified in patients with glomerulopathy and fibronectin deposits (GFND) (Castelletti et al., 2008). Integrin α5β1 binding to the RGD motif in FN III-10 requires the synergy region in the 9th FN type III repeat (Danen et al., 1995).* **(B)** *Tenascin-C is a modular molecule composed of an oligomerization domain, EGF-L and FN type III (constant and alternate) repeats, and a fibrinogen like domain (see Orend and Chiquet-Ehrismann, 2006). Binding sites for interacting molecules are indicated. CALEB, chicken acidic leucine-rich EGF like domain containing brain protein, EGFR, epidermal growth factor receptor, NaN, sodium channel subunit β 2, RPTPβ, protein tyrosine phosphatase β, TLR4, toll like receptor 4.*

and references therein). Importantly for clinical applications, fibronectin splice variants containing the EDB and EDA domains, often referred to as oncofetal variants, are amongst the most specific markers of angiogenic blood vessels to date (Kaspar *et al.*, 2006). In addition to promoting adhesion and signaling through cell surface receptors, the fibronectin matrix functions as a fibrillar scaffold for the assembly of other matrix proteins. Itprovides a platform for angiogenic signaling by increasing the bioavailability of soluble angiogenic factors, and cooperating with their transmembrane receptors (Hynes, 2007; Hynes, 2009; Miyamoto *et al.*, 1996; Mosher *et al.*, 1980).

Tenascin-C is another large modular ECM protein that exhibits a restricted and low expression in normal tissue. It is the founding member of the tenascin familiy with four members, tenascin-C, -R, -W and tenascin-X (reviewed in Chiquet-Ehrismann and Chiquet, 2003). All tenascins harbor several homologous domains that have been extensively described elsewhere (reviewed in Chiquet-Ehrismann and Chiquet, 2003; Orend, 2005; Orend and Chiquet-Ehrismann, 2006, Fig. 1). Tenascin-C can assemble into hexamers, it can be processed into monomers and it interacts with several cell surface receptors and other matrix molecules (reviewed in Midwood and Orend, 2009, Fig. 1). Its expression is elevated in embryonic tissues and in tissue of several cancers, and high tenascin-C expression has been found to correlate with lymph node metastasis and poor prognosis (reviewed in Midwood and Orend, 2009). Tenascin-C is one of the few genes within the signature of predictive value for lung metastatis in breast cancer patients (Minn *et al.*, 2005). Moreover, a robust expression of tenascin-C is associated with resistance to tamoxifen therapy in patients with estrogen receptor positive breast cancer (Helleman *et al.*, 2008). Tenascin-C plays a yet poorly defined role in enhancing tumor cell proliferation, promoting angiogenesis, invasion and metastasis (Orend and Chiquet-Ehrismann, 2006). Tenascin-C interacts with several ECM molecules and cell surface receptors, thus affecting tissue architecture, tissue resilience and cellular responses relevant in angiogenesis, metastasis and the stem cell niche (reviewed in Midwood and Orend, 2009).

At present, little is known about the interdependence of tenascin-C and fibronectin, yet a functional complicity of these two ECM molecules is strongly supported by their overlapping expression, physical interaction and modulatory role in cell adhesion-dependent processes (reviewed in Midwood and Orend, 2009). Here, we will address when both matrix molecules have evolved and how this may further our knowledge about their potential role in the vasculature of vertebrates in normal tissue homeostasis and in cancer. We will describe how the ablation of fibronectin and tenascin-C affects developmental and pathological angiogenesis. Finally, we will summarize and discuss what is known about fibronectin and tenascin-C in angiogenesis, in pathologies including cancer.

Evolutionary aspects of fibronectin and tenascin-C in the vasculature

The vasculature of vertebrates has a number of anatomical features that sets it apart from the vasculature of other members of the phylum Chordata. Notably, it is composed of a closed system of tubes lined by endothelial cells that are in turn invested by a basement membrane (Fig. 2). The urochordates (also known as tunicates or sea squirts), which are generally believed to be the closest relatives to the vertebrates (e.g., Putnam *et al.*, 2008)), have what appear to be inside-out vessels, at least from a vertebrate's point of view: the lumen of the tunicate heart and major vessels is lined by the basement membrane of myoepithelial cells and not by an endothelium (Davidson, 2007). Cells are occasionally encountered lining the lumen of urochordate vasculature, and though these may represent endothelial-like cells, recent evidence suggests that in at least some colonial tunicates they are Piwi-positive stem cells (Rinkevich *et al.*, 2010). In contrast to the closed system of vertebrates, the circulatory system of the tunicates is open, with the vessels leading from the heart emptying into lacunae where the hemolymph can bathe the organs. The cephalochordates (also known as lancelets or amphioxus), which are most likely a more distantly related invertebrate chordate, also have an open vascular system lined by ECM, but it lacks the epithelial lining of tunicate vessels (Fig. 2). For example, the aorta of amphioxus forms from the basement membranes of the gut endoderm and the ECM of the surrounding mesenchyme (Kucera *et al.*, 2009). This primitive acellular matrix-tube vasculature is widely found in other invertebrates as well (Rupert

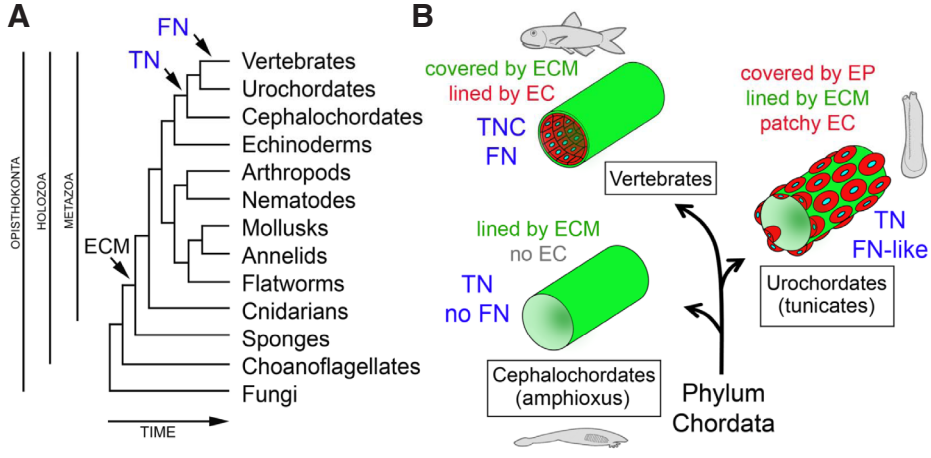

Fig. 2. Evolution of fibronectin and tenascin-C. (A) *Integrins are found in single-celled holozoans and fungi, but extracellular matrix (ECM) first appeared in early metazoans. Tenascins (TN) and fibronectin (FN) evolved much later in basal chordates.* **(B)** *The evolution of blood vessels in the Phylum Chordata. In cephalochordates (also known as lancelets or amphioxus) the circulatory system is open, and blood vessels are matrix tubes without endothelial cells (EC) or surrounding epithelial cells (EP). Cephalochordates have a tenascin gene, but not a fibronectin gene. In urochordates (also known as tunicates or sea squirts) the circulatory system is also open. Blood vessels are lined by extracellular matrix and surrounded by epithelial cells. Occasionally cells are encountered lining the lumen of the vessels, but it is not known if these are endothelial cells. The urochordate Ciona has a tenascin gene and a fibronectin-like gene that lacks key features of vertebrate fibronectin. In vertebrates the circulatory system is closed and lined by endothelial cells; all vertebrates examined have multiple tenascin genes and a highly conserved fibronectin gene.*

and Carle, 1983; reviewed by Kucera and Lammert, 2009), even though invertebrates are perfectly capable of making tubular organs lined with epithelia like the trachea or salivary glands (e.g., see Castillejo-Lopez *et al.*, 2004). Other anatomical features that are unique to the vertebrates include a lymphatic system and thymus (Bajoghli *et al.*, 2009): no comparable systems are found in tunicates or amphioxus.

On the cellular, physiological and molecular levels the vertebrate circulatory system also has a number of unique features. For example, an adaptive immune system appears to have evolved with jawed fish (e.g., see Bajoghli *et al.*, 2009), and amphioxus and tunicates lack the complex coagulation systems of vertebrates (Doolittle, 2009). Amphioxus and tunicates have many blood cells that appear to share roles with leukocytes, but they do not have erythrocytes (Cima *et al.*, 2001; Huang *et al.*, 2007). The tunicate *Ciona* has both globin and hemocyanin-related genes, but it is unknown if one or both play roles in respiration (Ebner *et al.*, 2003).

Many ECM genes have origins that coincide with the appearance of basal metazoa, including collagens (Exposito *et al.*, 2008), thrombospondins (Bentley and Adams, 2010) and syndecans (Chakravarti and Adams, 2006). Wnt and key players that make up the Wnt signaling pathways are present in sponges but not choanoflagellates, suggesting that they also evolved with the first metazoans (Richards and Degnan, 2009). Even more ancient are the integrins and molecules involved in integrin signaling like paxilin, talin and Integrin-linked Kinase (ILK) (Sebe-Pedros *et al.*, 2010), some of which are in single-celled holozoans as well as fungi. In contrast, tenascins and fibronectin are encoded by relatively new genes, having evolved early in the chordate lineage. Of the two, tenascins appear to have evolved first, as the amphioxus *Branchiostoma* has a gene that encodes a tenascin that closely resembles vertebrate tenascins, but it lacks a fibronectin gene (Tucker and Chiquet-Ehrismann, 2009). Nothing is known about the expression of the amphioxus tenascin, but it is remarkable for having multiple (at least 7) copies of RGD motifs predicted to be exposed to integrin binding. It is interesting to speculate that at least some of the functions of fibronectin in vertebrates may be carried out in amphioxus by this RGD-rich form of tenascin. In contrast, the tunicate *Ciona* has a tenascin gene and a fibronectin-like gene, but the predicted protein encoded by the latter has distinctive features (e.g., it lacks an RGD motif and domains that correspond to EDA and EDB domains) that leads one to suggest that it may play different roles than its highly conserved vertebrate counterpart (Tucker and Chiquet-Ehrismann, 2009). Interestingly, the *Ciona* tenascin gene is expressed in the notochord and muscle cells, and is not associated with the developing heart (Kawashima *et al.*, 2009; Tucker *et al.*, 2006).

What can we learn from the evolution of ECM about the origins of the distinctive anatomical and molecular features of the circulatory system in vertebrates? Only in vertebrates do we see a closed circulatory system with proper endothelial cells, and during vertebrate development cells lining blood vessels, which are likely to be endothelial cells, express both tenascin (Tucker, 1993) and fibronectin (Astrof and Hynes, 2009; ffrench-Constant and Hynes, 1989). As we will see below, both tenascin-C and fibronectin are critical for normal vascularization, so the appearance of these interrelated matrix molecules may have played a role in the evolution of this distinctive feature of vertebrates.

Fibronectin and tenascin-C in loss of function models

Effect of a loss of fibronectin and its receptors on the vasculature

Genetic evidence points to a major role for fibronectin and its receptors in vascular development. Ablation of the fibronectin gene leads to embryonic lethality at embryonic day 9.5 (E9.5) with severe cardiovascular defects and aberrant somitogenesis (George *et al.*, 1993). Interestingly, the severity of the defects was found to vary as a function of the genetic background of the fibronectin-null mice (George *et al.*, 1997). A search for gene modifiers of the heart defects led to the identification of potential candidates on chromosome 4, proposed to affect a migratory process involved in coalescence of the two heart primordia into a single heart tube (Astrof *et al.*, 2007b). The gene encoding tenascin-C is also located on chromosome 4, at a distinct locus. It is intriguing to speculate that tenascin-C could participate in genetic interactions that determine the severity of the phenotype. Integrin α5-null mice lacking the main fibronectin receptor, $\alpha5\beta1$, die at E10.5 with a phenotype similar to fibronectin-null mice (Francis *et al.*, 2002; Yang *et al.*, 1993). A comparable phenotype was observed in mice with an inactivating mutation of the $\alpha5\beta1$-binding motif of fibronectin (RGD to RGE) (Takahashi *et al.*, 2007), attesting to the functional importance of this ligand-receptor pair for vascular morphogenesis. Concerning the role of other fibronectin-binding integrins in vascular development and angiogenesis (reviewed in Hynes, 2007)), $\alpha v\beta3$ and $\alpha v\beta5$ integrins that bind to the RGD sequence in the cell-binding domain of fibronectin have received the most attention. Studies of integrin antagonists indicate that αv integrins promote angiogenesis, while genetic deletion studies indicate that αv integrins are not required for angiogenesis. Although their exact function in angiogenesis has been subject to much debate (see (Astrof and Hynes, 2009; Desgrosellier and Cheresh, 2010; Hodivala-Dilke, 2008), αv-targeting agents are currently being developed or used in the clinic for cancer therapeutics.

Genetic ablation of the fibronectin gene deletes all fibronectin variants (up to 20 in humans). What about the role of the alternately spliced isoforms, and their cellular receptors? Selective ablation of EDB and EDA domains suggests that these domains confer essential functions to fibronectin, as evidenced by the early embryonic death of mice lacking both exons (Astrof *et al.*, 2007a). However, the precise roles of these domains and the molecular events involved have yet to be fully understood, as compensatory mechanisms can rescue mice with single knock outs of either the EDB or EDA variant (recently reviewed in Astrof and Hynes, 2009; White *et al.*, 2008). The EDB domain has been proposed to generate a conformational modification of fibronectin and improve the access to nearby integrin binding domains (see Balza *et al.*, 2009; Bencharit *et al.*, 2007; Carnemolla *et al.*, 1992; Hashimoto-Uoshima *et al.*, 1997; Ventura *et al.*, 2010). This function is consistent with results from isoform-selective knockdown studies in endothelial cells (Cseh *et al.*, 2010).

Whereas no EDB-specific cellular receptor has been identified to date, inclusion of the EDA repeat in c-fibronectin creates new binding sites for $\alpha4\beta1$, $\alpha4\beta7$ and $\alpha9\beta1$ integrins (Kohan *et al.*, 2010; Liao *et al.*, 2002) (Fig. 1). $\alpha4\beta1$ and $\alpha9\beta1$ are structurally similar integrins that can bind to several ECM proteins; in the case of $\alpha9\beta1$ this includes tenascin-C (Humphries *et al.*, 2006; Yokosaki *et al.*, 1998). Genetic and pharmacological studies in mice reveal a

role for these integrins in the lymphatic vasculature. Hence, $\alpha 9\beta 1$ null mice die between 8-12 days after birth from major defects in development of the lymphatic system (Huang *et al.*, 2000). More recently, it was shown that the interaction between integrin $\alpha 9$ and fibronectin containing the EDA domain is required for fibronectin matrix assembly during lymphatic valve morphogenesis (Bazigou *et al.*, 2009). Integrin $\alpha 4$ knock out mice die at E11.5 with cardiac malformations and placental defects. Interestingly, targeted deletion of $\alpha 4$ in lymphatic vessels or pharmacological inhibition of $\alpha 4\beta 1$ was found to suppress growth factor- and tumor-induced lymphangiogenesis and prevent metastatic spread *in vivo*. In this same study, $\alpha 4\beta 1$ and c-fibronectin were identified as markers of proliferative lymphatic endothelium in invasive tumors (Garmy-Susini *et al.*, 2010). In addition to the EDA domain, sequences in the variable (V) region (Fig. 1) can also bind to $\alpha 4\beta 1$ integrin and thus contribute to the observed effects. Collectively, these findings shed light on the role of c-fibronectin variants as fundamental regulators of both blood and lymphatic vessels.

In humans, mutations in the fibronectin gene were identified in patients with glomerulopathy with fibronectin deposits (GFND), an autosomal dominant disease characterized by proteinuria, microscopic hematuria, hypertension, and massive glomerular deposits of non-fibrillar fibronectin in the mesangium and subendothelial space that lead to end-stage renal failure (Castelletti *et al.*, 2008). These mutations affect the heparin binding domains Hep-II and Hep-III. Functional studies showed that mutant recombinant Hep-II fragments display lower binding to endothelial cells and podocytes, compared to wild-type Hep-II, and an impaired ability to induce endothelial cell spreading and cytoskeletal reorganization. Hep-II and -III domains participate in fibronectin assembly in ECM, through complex fibronectin-fibronectin and fibronectin-cell surface proteoglycan interactions (Singh *et al.*, 2010). Interestingly, heparin-binding domains of fibronectin are sites of tenascin-C binding and have been shown to mediate functional interactions between fibronectin and tenascin-C that involve cell surface proteoglycans of the syndecan family, as discussed below.

Effect of tenascin-C knock out on the vasculature

Tenascin-C knock out mouse was generated in two different laboratories (Forsberg *et al.*, 1996; Saga *et al.*, 1992). In both studies the signal peptide and the heptad repeat sequences were disrupted. Saga and colleagues inserted a lacZ-neo construct just in front of the translational initiation codon in exon 2 of the tenascin-C gene, deleting parts of the exon 2 and intron 2 and keeping the regulatory unit of the tenascin-C gene for lacZ expression (Saga *et al.*, 1992). Since expression of a truncated tenascin-C in these mice was detected (Mitrovic and Schachner, 1995), a second independent tenascin-C knock out mouse was generated where a neomycin resistance cassette was inserted into exon 2 leading to two aberrant splice products of tenascin-C in homozygous mice inducing a frameshift and translation stop after 99 and 18 nucleotides (Forsberg *et al.*, 1996). It is now clear that tenascin-C expression is lost in most tissues. Surprisingly, in both cases tenascin-C knock out mice were alive and fertile and exhibited an apparently normal development. The apparently normal development and tissue organization of these mice has been attributed to compensation mechanisms (reviewed in Orend and Chiquet-Ehrismann, 2006). It was noticed that in the subventricular zone of the brain oligodendrocyte precursor cells respond differently to growth factors and proliferate less, but this appears to be compensated by a reduced apoptosis rate later on. Thus the number of oligodendrocytes ends up being similar in the tenascin-C knock out and wildtype mouse (Garcion *et al.*, 2001). Later studies showed that the absence of tenascin-C imposes problems for tissue homeostasis which is particularly evident during wound- or inflammation-associated tissue repair (reviewed in Orend and Chiquet-Ehrismann, 2006). Meanwhile tenascin-C knock out mice present valuable tools for addressing the roles of tenascin-C in development, angiogenesis, inflammation, heart failure and tumorigenesis.

By using a cardiac allograft model it was shown that tenascin-C is a mediator of postnatal cardiac angiogenic mechanisms in mice (Ballard *et al.*, 2006). Upon subdermal transplantation of wild-type cardiac tissue into a syngenic host, a fibrin clot forms around the allograft and both the clot and cardiac tissue become vascularized, resulting in engraftment of viable cardiac tissue. Clot formation is unaffected in tenascin-C-null mice; however, these mice fail to form any vessels and no engraftment of cardiac tissue is observed. In wildtype mice the donor endothelial cells engrafted at sites of tenascin-C expression, suggesting that tenascin-C acts to promote homing and incorporation of endothelial or progenitor cells. Indeed, cultured rat cardiac microvascular endothelial cells adhere to tenascin-C substrata, but spreading and monolayer formation are delayed compared to cells plated on fibronectin or collagen. Futhermore, migration of these cells into a collagen gel is enhanced when cultured on tenascin-C (Ballard *et al.*, 2006). These data support a role for tenascin-C in the early stages of angiogenesis by modulating endothelial cell adhesiveness, and thus promoting migration.

Tenascin-C also seems to play a role in vascularization associated with lung development. The tenascin-C knock out mouse does not show apparent defects in lung anatomy and function, presumably due to unknown compensatory mechanisms. This compensation seems not to apply when the embryonic lung is placed in culture since the lung explants from tenascin-C knock out embryos display reduced branching (defective cleft formation and enlarged terminal lung buds) and decreased vascularization (Roth-Kleiner *et al.*, 2004). Results from another report indicate that lung vascularization and branching morphogenesis are dependent on Wnt and fibronectin signaling. Wnt signaling is turned on between E10.5 and E12.5 in the developing lung. Later (E13.5) Wnt signaling is largely reduced by Dickkopf (DKK) 1–3, and this coincides with induction of the Wnt target gene, fibronectin. Moreover DKK1 and fibronectin are instrumental in promoting lung branching morphogenesis and angiogensis, since recombinant DKK1 and anti-fibronectin antibody both block cleft formation and angiogenesis. DKK1 treatment causes thinner blood vessels, reduced sprouting from existing vessels and impaired formation of large vessels with fewer interconnections (De Langhe *et al.*, 2005). Given that tenascin-C blocks fibronectin signaling, represses DKK1, and plays a role in lung branching morphogenesis, it is possible that a tight balance between fibronectin and tenascin-C regulates normal lung branching and vascularization. In this scenario DKK1 repression by tenascin-C would result in Wnt activation and induction of fibronectin.

The role of tenascin-C in tumorigenesis was also investigated in a mouse model that develops metastasizing mammary gland tumors (due to ectopic expression of the polyoma virus middle T-antigen in the mammary epithelium) in the presence of wildtype

tenascin-C or in a tenascin-C knock out setting (Talts *et al.*, 1999). There was no difference observed in tumor onset, angiogenesis or metastasis between the genotypes, but the authors observed an altered organization of the tumor tissue. In tenascin-C wildtype tumors ECM molecules such as fibronectin, collagen I, nidogen and fibulin-2 were arranged in continuous long tracks whereas they were assembled in shorter matrix patches in the tenascin-C knock out background. These structures might represent matrix channels that were subsequently identified by others in metastasizing melanomas (Kaariainen *et al.*, 2006, see below). In contrast to melanomas, where the tenascin-C matrix channels seem to promote metastasis, other mechanisms might exist to promote metastasis in mammary gland tumors, in the absence of tenascin-C. It was also noted that tenascin-C knock out breast carcinomas are significantly more infiltrated by activated macrophages (Talts *et al.*, 1999). Since M2 macrophages promote tumor metastasis (Mantovani *et al.*, 2008) it is possible that this species is increasingly attracted to the tissue that lacks tenascin-C. Previously it was shown that tenascin-C inhibits T lymphocyte adhesion to fibronectin (Hauzenberger *et al.*, 1999) and activation (Puente Navazo *et al.*, 2001). Thus, it is possible that in the tenascin-C knock out mammary gland carcinomas the inhibitory effect of tenascin-C is absent and this situation allows the attraction of macrophages. The reason why this would only affect a subset of macrophages needs to be addressed in the future.

In Balb/c-nude mice lacking tenascin-C, subcutaneously xenografted human melanoma cells made smaller tumors (Tanaka *et al.*, 2004). In this model blood vessels were visualized by immunofluorescence upon injection of rhodamine-labeled gelatin, which allows selective visualization of perfused vessels that arise through sprouting angiogenesis. Despite a lack of quantitative data, the authors showed in tissue stainings that the arising tumor vasculature is reduced in the absence of host tenascin-C. They link this to reduced VEGFA expression in the tumor tissue. Although the melanoma cells exhibit strong tenascin-C expression, this does not appear to have a significant impact on VEGFA levels in the tumor. These data suggest that tenascin-C made by stromal cells has a major impact on VEGFA expression and that this mechanism potentially accounts for the angiogenesis promoting effect of tenascin-C (Tanaka *et al.*, 2004).

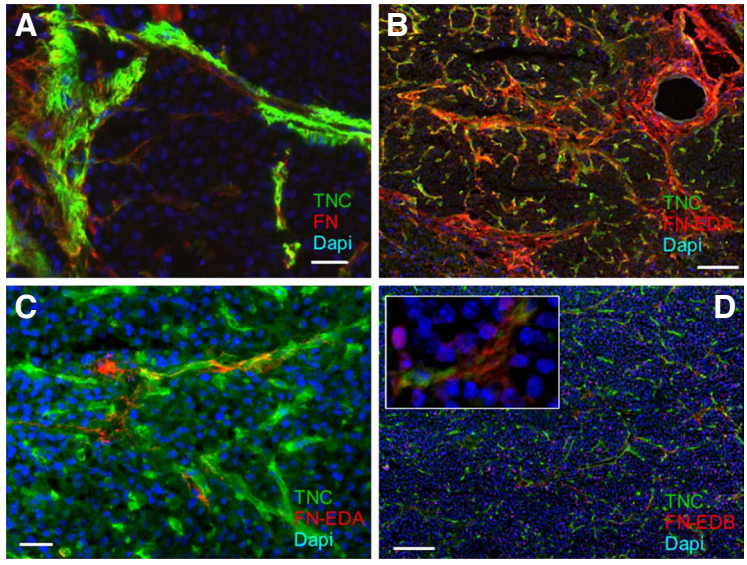

Fig. 3. Colocalization of fibronectin and tenascin-C in the tumor vasculature. (A-D) *Tenascin-C and fibronectin expression in tumors. Coexpression of total fibronectin* **(A)**, *FN-EDA* **(B,C)** *or FN-EDB* **(D)** *and tenascin-C in fibrils of an RT2 tumor (Gasser and Orend, unpublished). The anti FN-EDB antibody was kindly provided by Dr. A. K. Olsson (Uppsala University, Sweden). Scale bar 20 μm (A,C) and 100 μm (B,D). Note the fibrillar organization of both molecules and their close apposition in tube-like structures, presumably representing blood vessels and/or matrix tubes.*

Effects of fibronectin and tenascin-C on the vasculature in tumors and in other pathological tissues

Similar to fibronectin, tenascin-C is only weakly expressed, or undetectable, in the ECM of quiescent vasculature. However, following vessel injury, tenascin-C and fibronectin are highly upregulated. Tenascin-C expression is strongly associated with sites of vascular remodeling during dermal tissue repair (Betz *et al.*, 1993; Fassler *et al.*, 1996; Latijnhouwers *et al.*, 1996; Mackie *et al.*, 1988). Tenascin-C expression is also highly associated with angiogenesis in a wide range of disease states, including diabetes, aortic aneurysm (Castellon *et al.*, 2002; Jallo *et al.*, 1997; Paik *et al.*, 2004), artherosclerosis (Fischer, 2007), ulcerative colitis (Dueck *et al.*, 1999), inflammatory bowel disease (Geboes *et al.*, 2001), Crohn's disease (Riedl *et al.*, 2001), vasculitis (Gindre *et al.*, 1995) and cancer.

Recently, Berndt and collaborators reported the distribution of tenascin-C and fibronectin in several different carcinomas using antibodies specific for different splice variants (Berndt *et al.*, 2010). Both proteins were generally present in the vessel wall, with fibronectin being preferentially localized at the luminal side and tenascin-C at the extraluminal side of the vascular basement membrane. Interestingly, tumour vessels showed a heterogenous positivity for oncofetal fibronectin and tenascin-C variants, with some vessels lacking both proteins, some vessels exclusively positive for fibronectin or tenascin-C, and other vessels surrounded by both matrix proteins. As an example, expression and partial colocalization of c-fibronectin variants and tenascin-C in a murine RT2 insulinoma is shown (Fig. 3). This stratified pattern clearly suggests a temporally and spatially regulated expression of these ECM proteins in the tumor vasculature and may reflect different maturation states of the vessels. In the study by Berndt *et al.*, fibronectin was expressed by endothelial cells and carcinoma associated fibroblasts (CAFs) whereas tenascin-C was abundantly produced by carcinoma cells. It will be important to identify which cell types in various cancer tissues express tenascin-C and fibronectin with different domain structures. Further, it remains to be determined whether tenascin-C and c-fibronectin variants with different domain compositions fulfill distinct roles in tumor angiogenesis.

Some light was shed on these questions by RNA expression analysis in breast cancer tissue. Amongst the more than 500 theoretically possible splice variants only 2 or 3 are usually expressed in cancer tissue, and these differ between cancers of different organs (reviewed in Orend, 2005). In invasive breast cancer, the authors found a prominent expression of a tenascin-C molecule with extra repeats B and D that is derived from the tumor cells, whereas a tenascin-C molecule only expressing the D domain appears to be expressed by carcinoma associated fibroblasts (reviewed in

Guttery *et al.*, 2010).

During tissue neovascularization, endothelial cells undergo pro-angiogenic activation, and assume a migratory morphology (Carmeliet, 2000; Ingber, 2002). Tenascin-C may promote tumor angiogenesis through several mechanisms, such as by serving as a chemoattractant for endothelial cells by initiating endothelial cell differentiation, or by promoting survival and proliferation. *In vitro*, tenascin-C is specifically upregulated by sprouting and cord forming bovine aortic endothelial cells but not by non-sprouting (i.e., resting) cells (Canfield and Schor, 1995; Schenk *et al.*, 1999). This angiogenic phenotype is inhibited when cells are grown in the presence of anti–tenascin-C antibodies, suggesting that the transition from a resting to a sprouting phenotype may be promoted by tenascin-C (Canfield and Schor, 1995). Indeed, soluble tenascin-C reduces focal adhesions in endothelial cells (Chung *et al.*, 1996; Murphy-Ullrich *et al.*, 1991) and enhances endothelial cell migration (Chung *et al.*, 1996). These events appear to involve integrin $\alpha v\beta 3$, FAK and Prx1 amongst other, not yet identified, molecules (reviewed in Orend and Chiquet-Ehrismann, 2006).

Counter-adhesive activities of fibronectin and tenascin-C

Shortly after its discovery in the early 1980s as myotendinous antigen (Chiquet and Fambrough, 1984), as glioma-mesenchymal extracellular matrix molecule (GMEM) (Bourdon *et al.*, 1983) and as neuronal protein janusin J1 (Faissner *et al.*, 1988) it was noted that tenascin-C can bind to fibronectin (Chung *et al.*, 1995; Lightner and Erickson, 1990). Since then, several reports have extended this finding although some controversy exists whether the long and/or the short form of tenascin-C (i.e. including or lacking the alternatively spliced fibronectin type III repeats, respectively) (Chiquet-Ehrismann *et al.*, 1991; Chung *et al.*, 1995; Huang *et al.*, 2001) have different affinities for fibronectin. All three heparin binding domains seem to bind tenascin-C, with Hep-I being cryptic and exhibiting low affinity (Ingham *et al.*, 2004). Tenascin-C binds to the Hep-III domain in fibronectin, but it is not known whether this interaction competes with binding of cell adhesion receptors. Binding of tenascin-C to the fibronectin-Hep-II domain blocks cell spreading (Chiquet-Ehrismann *et al.*, 1991; Huang *et al.*, 2001; Midwood *et al.*, 2004a; Orend *et al.*, 2003) and fibronectin fibrillogenesis (To and Midwood, 2010) through competition with syndecan-4. The Hep-II domain serves as coreceptor for the major fibronectin binding integrin $\alpha 5\beta 1$ (Fig. 4 A,B). The exact binding site in fibronectin has been mapped to the 13th fibronectin type III repeat within the Hep-II domain, and a peptide representing 10 amino acids of the cationic craddle rescued tenascin-C induced cell rounding (Huang *et al.*, 2001; Orend *et al.*, 2003). Activation of syndecan-4 signaling induced upon ectopic expression of syn-

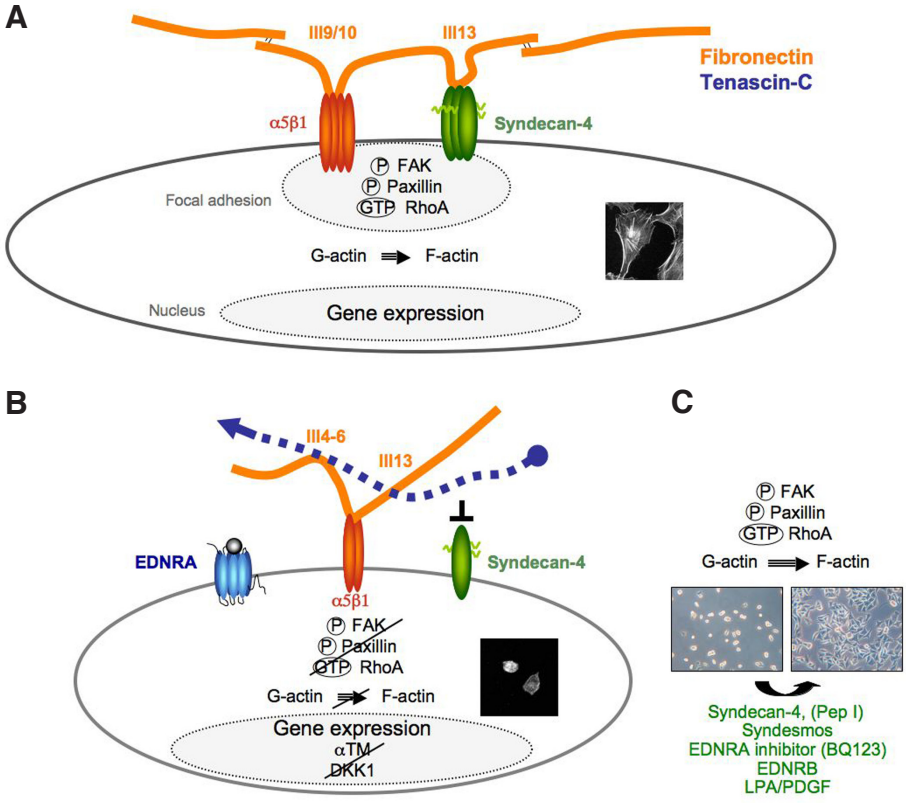

Fig. 4. Inhibition of syndecan-4 by tenascin-C. (A) *Cells activate integrin $\alpha 5\beta 1$ and syndecan-4 upon adhesion to their respective binding sites in fibronectin (FN type III repeats 9 and 10, and 13, respectively). This induces formation of focal adhesions. Crucial steps in cell spreading are phorphorylation of focal adhesion kinase (FAK) and paxillin and GTP loading of RhoA. Consequently, G-actin is polymerized into F-actin and cells spread on fibronectin.* **(B)** *Tenascin-C binds to the 13th FN type III repeat in fibronectin thus competing for cell binding to this domain. This mechanism applies to tumor cells and fibroblasts, but has yet to be addressed in endothelial cells. In the presence of tenascin-C, FAK and paxillin stay unphosphorylated and RhoA remains inactive (Huang et al., 2001, Midwood and Schwarzbauer, 2002). No focal adhesions or actin stress fibers are formed. The rounded cell shape translates into altered gene expression and causes repression of alpha tropomyosin (αTM) and Dickkopf-1 (DKK1) as well as induction of endothelin receptor type A (EDNRA) amongst changes in expression of several other genes (Ruiz et al., 2004).* **(C)** *The rounded cell shape can be reverted on a fibronectin/tenascin-C substratum upon activation of syndecan-4 with a peptide (pep I) that mimics the cationic craddle in syndecan-4 or upon overexpression of syndesmos, a molecule that binds to the cytoplasmic tail of syndecan-4 and provides a molecular bridge in the focal adhesions to proteins binding to integrins (Baciu et al., 2000; Lange et al., 2008; Orend et al., 2003). Inhibition of EDNRA with BQ123 also induces cell spreading (Lange et al., 2007). Combined signaling from platelet derived growth factor (PDGF) and the lysophosphatidic acid (LPA) and by endothelin receptor type B (EDNRB) involving the EGFR induce cell spreading on the mixed fibronectin/tenascin-C substratum. Signaling by these molecules induces focal adhesion and actin stress fiber formation although with a cell shape that is particular for each treatment. Nevertheless, repression of αTM is ablated and tropomyosins 1-3 are expressed to stabilize actin stress fibers (Lange et al., 2007; Lange et al., 2008). Regulation of αTM is crucial for cell spreading on fibronectin since its knock down inhibits cell spreading on fibronectin and interferes with restored cell spreading on the fibronectin/tenascin-C substratum upon treatment with pep I and activation of LPA receptors and PDGF receptors or EDNRB (Lange et al., 2007; Lange et al., 2008).*

decan-4 (but not of syndecan-1 or –2) rescued tenascin-C-inhibited cell spreading on fibronectin (Huang *et al.*, 2001; Midwood *et al.*, 2004b; Orend *et al.*, 2003) as did overexpression of syndesmos, a molecule that binds to the cytoplasmic tail of syndecan-4 and triggers downstream signaling (Lange *et al.*, 2008, Fig. 4C). The major binding site for fibronectin-Hep-II may reside in the 6-8 fibronectin type III repeats of tenascin-C as was deduced from antibody blocking experiments (Chiquet-Ehrismann *et al.*, 1988; Riou *et al.*, 1990). This is now supported by a recent report demonstrating that meprinβ-cleaved tenascin-C loses its anti-adhesive properties in a fibronectin context (Ambort *et al.*, 2010). The authors showed that meprinβ cleaves within the 7th fibronectin type III repeat of tenascin-C thus destroying this interaction site, and that meprinβ-cleaved tenascin-C does not interfere with cell spreading on fibronectin (Ambort *et al.*, 2010). Inhibition of syndecan-4 by tenascin-C prevents focal adhesion formation, blocks activation of FAK and paxillin and has a strong negative impact on expression and protein stability of RhoA and tropomyosin 1–3 (Lange *et al.*, 2007; Lange *et al.*, 2008), and Rho activation (Midwood *et al.*, 2006). The effect on the cytoskeleton appears to be instrumental in cell rounding by tenascin-C since ectopic expression of tropomyosin-1, an actin stress fiber stabilizing molecule with tumor suppressor activity, restores cell spreading which is linked to FAK and paxillin phosphorylation (Lange *et al.*, 2007).

Binding of tenascin-C to the Hep-II domain of fibronectin can also have implications for angiogenic growth factor signaling. Indeed, it has been shown using molecular and biochemical approaches that several growth factors (up to 25, including VEGF, HGF, FGF-2, PDGF-BB and TGF-β1) bind to this domain (see (Hynes, 2009; Martino and Hubbell, 2010). Moreover, fibronectin-Hep-II-bound growth factors are even more potent than their un-bound counterparts in triggering capillary morphogenesis of endothelial cells in fibrin gels (Martino and Hubbell, 2010). Although not shown in endothelial cells, cellular responses to tenascin-C can be modulated by growth factors. Thus, in fibroblasts and tumor cells growth factor signaling can override the necessity of syndecan-4 in fibronectin-induced cell spreading. Combined signaling from LPA and PDGF-BB (but not from each factor alone) restores cell spreading on a fibronectin/tenascin-C substratum even in cells that lack syndecan-4 (knock out) in a PI3K- and MEK-dependent manner, and this is linked to restored high expression of tropomyosins 1-3 and RhoA (Lange *et al.*, 2008, Fig. 4C). Again, high levels of tropomyosin 1-3 are essential since sh-mediated knock down of the tropomyosins 1, 2 and 3 counteracts LPA/PDGF-BB-induced cell spreading on the fibronectin/tenascin-C substratum (Lange *et al.*, 2008).

Adhesion to a fibronectin/tenascin-C substratum also has long term consequences as revealed by RNA profiling (Ruiz *et al.*, 2004). In particular, 12h after plating, endothelin receptor type A (EDNRA) is induced 5-fold, and, signaling through this receptor maintains cell rounding by tenascin-C since it is blocked by a specific EDNRA inhibitor. EDNRA associated cell rounding occurs in a MEK-dependent manner and EDNRA inhibition causes cell spreading with activation of FAK and restoration of tropomyosin and RhoA levels (Fig. 4 B,C). These studies also reveal that, depending on the receptors present on the membrane, interactions with tenascin-C can be interpreted very differently. In particular, in contrast to EDNRA signaling that induces tenascin-C cell rounding, activation of EDNRB restores cell spreading through a different pathway that does not involve MEK but does involve EGFR, PLCγ, PI3K and JNK (Lange *et al.*, 2007, Fig. 4C). Thus, whether cells respond to a fibronectin/tenascin-C matrix by rounding or spreading appears to be highly regulated and may have an impact on cell function, tissue stiffness and vessel diameter. Both endothelin receptors play an important role in modulating blood pressure and are linked to high blood pressure in heart disease and artherosclerosis (Nguyen *et al.*, 2010). Since tenascin-C is expressed in diseased heart tissue and artherosclerosis, it remains to be determined whether tenascin-C-associated EDNRA signaling plays a role in blood pressure regulation that has an impact in heart diseases.

Tenascin-C potentially plays a role in EDNRA-associated events involving angiogenesis, e.g., in ovarian cancer progression. Primary and metastatic ovarian cancer cells not only overexpress tenascin-C (Wilson *et al.*, 1999; Wilson *et al.*, 1996) but also EDNRA and its ligand endothelin-1 (ET1) (Rosano *et al.*, 2001). EDNRA signaling contributes to tumor angiogenesis presumably through stabilization of HIF-1α, induction of VEGFA (Grimshaw, 2007) and β-arrestin-linked Wnt signaling (Rosano *et al.*, 2001).

A tenascin-C – EDNRA axis might also be relevant in tumor cell migration and tumor lymphangiogenesis (Cueni *et al.*, 2010). Tumors derived from xenografted breast adenocarcinoma cells that ectopically express the orphan receptor podoplanin induce lymphatic vessels, whereas this was not observed in tumors of control cells with low or no podoplanin expression. RNA profiling of microdissected areas of the invading tumor front revealed overexpression of tenascin-C, ET1 and the ERM member villin. Previously it was shown that binding of the ERM family member ezrin to the cytoplasmic tail of podoplanin induces filopodia which was linked to collective tumor cell migration (Wicki *et al.*, 2006). These results suggest that ET1 signaling may induce migration on a tenascin-C substratum by podoplanin through its link to the actin cytoskeleton.

In addition to EDNRA, Wnt signaling is also induced in glioblastoma cells on a fibronectin/tenascin-C substratum. In particular, DKK1 is repressed, β-catenin stabilized and Wnt targets such as Id2 are induced (Ruiz *et al.*, 2004). This observation could be relevant in glioblastomas where a high expression of Id2 and tenascin-C correlated with malignancy (Ruiz *et al.*, 2004). Given that Wnt signaling is instrumental in angiogenesis, by triggering endothelial cell proliferation and sprouting (reviewed in Franco *et al.*, 2009), it remains to be determined whether tenascin-C promotes angiogenesis through Wnt signaling. Since EDNRA and Wnt signaling are linked through β-arrestin (Rosano *et al.*, 2009) and through ET1-induced DKK1 repression (Clines *et al.*, 2007) it is possible that tenascin-C enhances this cross-talk by activating both pathways.

Organization of fibronectin and tenascin-C into matrices

Fibronectin assembly and angiogenesis

Fibrillar organization is a key feature of the ECM. Many of the functions of fibronectin depend not only on its linear sequence but on the 3-dimensional structure of the protein and its assembly into a functional fibrillar matrix (see Mao and Schwarzbauer, 2005). Due to its compact conformation, fibronectin does not form fibrils in solution. Rather, fibril assembly is a cell-driven process in which α5β1 integrin plays a major role (recently reviewed in Singh *et al.*, 2010) and shown diagrammatically in Fig. 5). Importantly, soluble fibronectin selectively binds to α5β1 integrin, and not other RGD-binding

integrins (Huveneers *et al.*, 2008). Hence, bloodborne p-fibronectin in quiescent vessels is segregated from α5β1 integrins, located on the abluminal surface of endothelial cells. Studies to elucidate the mechanisms of fibronectin fibrillogenesis in endothelial cells have revealed a determinant role for ILK in this process (Vouret-Craviari *et al.*, 2004). ILK, an integrin beta subunit adaptor, regulates actin dynamics and fibronectin fibrillogenesis by recruiting actin-binding regulatory proteins such as α-parvins and tensin (Legate *et al.*, 2006; Stanchi *et al.*, 2009) involved in generating acto-myosin contractility for fibril growth. More recently, loss of function studies have revealed that fibronectin fibrillogenesis in endothelial cells is a cell autonomous process, wherein basally directed secretion of autocrine fibronectin is tightly coupled to fibronectin assembly and cadherin-based junction formation (Cseh *et al.*, 2010). These results highlight the importance of spatial and temporal regulation of c-fibronectin expression and they support a model in which the induction of cellular c-fibronectin expression by angiogenic factors triggers the deposition of a perivascular fibrillar matrix.

One example of how transient c-fibronectin expression participates in a "pro-angiogenic switch" comes from elegant studies on vascular patterning in the developing retinal vasculature (Gerhardt *et al.*, 2003; Jiang *et al.*, 1994; Uemura *et al.*, 2006). During this process, blood vessels use the existing astrocyte network as a template, and fibronectin is the principal component of the astrocyte-derived extracellular scaffold. Upon contact with the growing blood vessels, fibronectin expression becomes dramatically down regulated in the astrocytes and turned on in the endothelial cells that deposit fibronectin matrices. It should be interesting to examine the expression and localization of tenascin-C in this model.

Once assembled, fibronectin fibrils provide a scaffold for the assembly of a growing list of matrix proteins, including fibrillar collagens, thrombospondin-1, fibulin-1, fibrinogen, fibrillins and tenascin-C (reviewed in Dallas *et al.*, 2006). Further, fibronectin interactions can impact higher order fibrils and matrix rigidity by bringing together cross-linking enzymes such as tissue transglutaminase (Mosher *et al.*, 1980) and lysyl oxidase (Fogelgren *et al.*, 2005) as well as their activators (e.g., Bone Morphogenetic Protein-1) and substrates (e.g., procollagen, biglycan and chordin, Huang *et al.*, 2009).

Fibronectin and tenascin-C co-assemble into a matrix

Apart from the α5β1 integrin, other fibronectin-binding integrins have been reported to promote fibrillar assembly, albeit less efficiently (see Leiss *et al.*, 2008). Fibronectin matrix is also regulated by molecules that affect integrin expression or function, including transmembrane molecules (e.g., syndecans 2 and 4, the receptor for urokinase-type plasminogen activator, CD98hc, VEGFR1 and neuropillin), intracellular proteins (e.g., the tumor suppressor von Hippel-Lindau protein) or extracellular components (e.g., extracellular Alix ALG-2-interacting protein X). With regard to functional interactions between fibronectin and tenascin-C that impact matrix formation, they are likely to involve effects on the fibronectin matrix mediated by syndecans 2 and 4, and regulation of intracellular signaling events that accompany fibronectin binding to integrins, as mentioned above. Proteoglycans, such as decorin and periostin, known to modulate fibronectin matrix assembly (Kii *et al.*, 2010; Kinsella *et al.*, 2000) are essential for matrix incorporation of tenascin-C (Chung and Erickson, 1997; Kii *et al.*, 2010). It is known that tenascin-C can bind to purified fibronectin and co-localize with fibronectin fibrils on the surface of cultured cells (e.g., Ramos *et al.*, 1998). A recent study in fibroblasts involving the use of recombinant tenascin-C domains demonstrated an inhibitory effect of tenascin-C domain III 1-8 (fibronectin type III domains 1-8) on the formation of an insoluble fibronectin matrix, whereas the full length protein was without effect (To and Midwood, 2010). These data

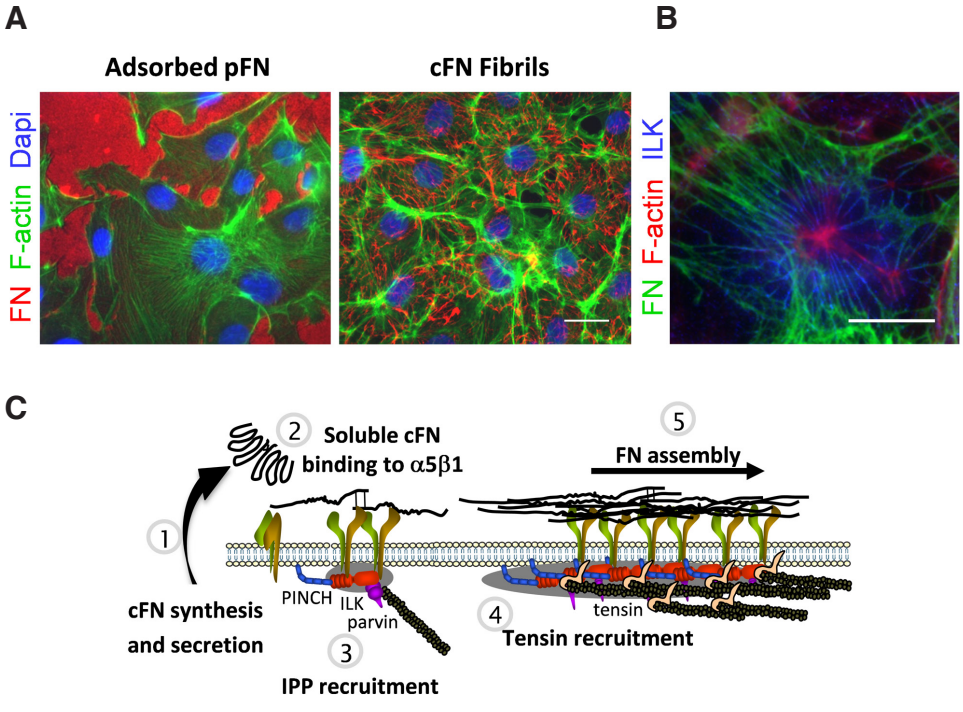

Fig. 5. Fibrillar organization of c-fibronection in endothelial cells and mechanisms of assembly. **(A,B)** *Immunostaining of fibronectin in bovine aortic endothelial cells plated on adsorbed p-fibronectin (left) or non-coated (right) coverslips. Upon adsorption, fibronectin undergoes conformational changes (stretch-induced "activation") that modify its interaction with other proteins (e.g. cellular receptors, ECM components) and affect its biological activity. Note the increase in stress fiber formation in cells plated on a dense carpet of p-fibronectin. Scale bars 20μm.* **(C)** *Sequence of events involved in fibronectin assembly. Work from numerous laboratories have contributed to the understanding of fibrillogenesis that can be summarized as follows (for a detailed review see Singh et al., 2010. Binding of secreted c-fibronectin to inactive α5β1 integrins (bent conformation) leads to integrin activation, clustering, and recruitment of integrin effectors, such as ILK, PINCH, parvin (IPP complex, (Legate et al., 2006)) and tensin that mediate cytoskeletal linkage and actin crosslinking. Acto-myosin-generated contractility "streches" the integrin-bound fibronectin, thereby exposing cryptic self-assembly sites and fibrillogenesis proceeds as integrins translocate along paths of growing fibrils.*

suggest that conformational changes may expose fibronectin-tenascin-C interaction sites that are important for regulation of matrix assembly *in vivo*.

Tenascin-C and fibronectin in tumor matrix tubes

In breast cancer tissue tenascin-C causes remodeling of the matrix. This effect, apparent at first glance by disruption of the basement membrane, can be seen in tumors and in cultured mammary epithelial cells. Using a 3-dimensional model of breast tumor cells, Taraseviciute and colleagues showed that tenascin-C interferes with basement membrane assembly in a c-Met dependent manner. In presence of tenascin-C the mammary epithelial cells proliferate and fill the acini lumen (Taraseviciute, 2009). In cancer tissue, the expression of tenascin-C is frequently not homogenous. Rather, it accumulates in matrix tracks as seen in the tissue of malignant melanomas (Kaariainen *et al.*, 2006), breast (Degen *et al.*, 2007) and colorectal carcinomas (Degen *et al.*, 2008). In the case of melanomas, the combined high expression of tenascin-C and fibronectin, amongst other factors, was found to discriminate between metastatic and non-metastatic malignancies. The switch to an invasive phenotype was associated with the presence of tenascin-C and fibronectin, colocalized with laminin and procollagen in tubular channels containing tumor cells, but not blood endothelial or lymph endothelial cells (Kaariainen *et al.*, 2006). In another study gene profiling revealed a panel of ECM molecules, including tenascin-C, several laminins, and collagens that were highly expressed in metastatic breast cancers from MMTV-VEGF/c-myc transgenic mice, whereas their expression was largely reduced or undetectable in non-metastatic c-myc-induced tumors (Calvo *et al.*, 2008). These studies suggest that a combined high expression of distinct ECM molecules, including tenascin-C and fibronectin, and their assembly in matrix channels, is somehow linked to metastasis.

The existence of vessel-like structures that are distinct from blood and lymphatic vessels has been known for a long time and was described as vasculogenic mimicry (VM) (Hendrix *et al.*, 2003). VM is characterized by the large absence of endothelial cells and by staining with PAS (Periodic Acid Schiff reagent), which identifies proteoglycans with glycosaminoglycan residues present in the ECM without further information on the molecular nature of these proteoglycans. It is likely that the matrix tubes that contain tenascin-C and fibronectin described in melanomas (Kaariainen *et al.*, 2006) are part of the PAS-positive structures that are characteristic of VM. In a large number of different cancers including melanoma, uveal melanomas, colorectal carcinoma, ovarian carcinoma (summarized in Kucera and Lammert, 2009) and astrocytoma (El Hallani *et al.*, 2010), VM is frequently associated with metastasis and bad prognosis. It is claimed that several forms of VM exist, which are classified according to the degree and nature of cells that associate with the ECM: no endothelial cells, patchy distribution of endothelial cells, tumor cells only, or a combination of both cell types. By comparing *in vitro* cultures of cancer cells that do or do not exhibit VM *in vivo*, several VM-associated molecules have been identified. These include γ2 chain containing laminins, several metalloproteases (MMPs 1, 2, 9 and 14), Cox2, PI3K, EphA2, nodal and pigment epithelial derived factor, amongst a list of growing candidates (reviewed in Dome *et al.*, 2007; Paulis *et al.*, 2010). Eventually, cells are found associated with matrix structures: melanoma cells, and erythrocytes in tenascin-C matrix channels (Kaariainen *et al.*, 2006), and macrophages along collagen-rich tracks found in so called co-opted vessels (Pollard, 2008). Although matrix structures had been identified that appear to be different, more work is needed to clarify whether they are potentially part of the same matrix network.

Currently there is little known about the functional significance of these matrix structures in respect to tumor angiogenesis and progression. However, there is experimental evidence that cancer cells can use the tenascin-C-containing matrix tubes to disseminate. In a coculture experiment of fibroblasts together with squamous carcinoma cells, the fibroblasts scouted their way through collagen-enriched matrigel by degrading the ECM at the front. Tubes were left behind that were filled with fibronectin and tenascin-C. The squamous carcinoma cells invaded the matrigel by using these matrix tubes (Gaggioli *et al.*, 2007). It was previously shown that carcinoma-associated fibroblasts or TGFβ1 treated fibroblasts (differentiating into myofibroblasts) secrete tenascin-C into the collagen gels preparing a path for colorectal carcinoma cells to invade in a c-MET- and EGFR-dependent manner, involving activation of Rac and inhibition of RhoA (De Wever *et al.*, 2004). They proved a crucial role of tenascin-C in these events since invasion was inhibited with an anti-tenascin-C antibody. Whether fibronectin plays a role in these structures is unknown, but likely.

Evolutionary development of endothelium-lined blood vessels in vertebrates seems to have followed laminin-based matrix tracks in invertebrates (Kucera *et al.*, 2009) (Fig. 2). In amphioxus, laminin-filled tubes are laid down as a scaffold in which a hole is drilled by cells of unknown origin to generate a coelom that allows blood to circulate (Kucera *et al.*, 2009). As mentioned above the tenascin-C gene is present in amphioxus and it remains to be seen whether tenascin-C is part of this matrix circulation network. In co-cultures of endothelial cells with a macrophage cell line, where the endothelial cells deposit laminin, macrophages create a coelom-like cavity by partially digesting and clearing the ECM to generate space. Thus, it is possible that this ancient vessel program is turned on in tumors to establish the observed matrix-based networks. More information about the composition of the matrix blood vessels in chordata and in human cancers is necessary to elucidate this possibility further. The observed tenascin-C matrix tubes in melanomas and other cancers may offer a route for dissemination of tumor and other cells through their continuum with blood vessels. They also potentially provide a scaffold to support growth of blood vessels. This possibility is interesting, considering that anti-angiogenic therapeutic approaches, despite efficient killing of the endothelial cells, fail in the long-run, and even promote tumor progression and earlier tumor metastasis (Paez-Ribes *et al.*, 2009; Stockmann *et al.*, 2008). Tenascin-C matrix tubes would not be affected by anti-angiogenic drugs. Indeed, Fusenig and coworkers (Vosseler *et al.*, 2005) demonstrated that expression of tenascin-C in xenografts of squamous carcinoma cells is unchanged upon elimination of the endothelial cells with a VEGFA-targeting antibody.

Matrix tubes containing tenascin-C are also present in a normal setting in mammals in so called reticular fibers of secondary lymphoid tissues such as lymph nodes, thymus and spleen (Lokmic *et al.*, 2008). They combine characteristics of basement membranes and fibrillar matrices, resulting in scaffolds that are strong and flexible, and in certain organs, such as the spleen and the thymus, form conduit networks for rapid fluid transport and cells (Lokmic *et al.*, 2008). In the thymus, the conduits exhibit a collagen core, a laminin wrapping and an outer lining of tenascin-C (Drumea-Mirancea *et*

al., 2006). Fibronectin is also part of the reticular fiber network (Sobocinski *et al.*, 2010). Whether tenascin-C and or fibronectin are required for the formation and function of the conduits is not known.

We speculate that a program may exist for the establishment of structured matrix that is potentially turned on inappropriately in cancer resulting in the described tenascin-C/fibronectin-containing matrix tubes. It is also tempting to consider that tubular matrix structures containing fibronectin and tenascin-C had developed once during evolution and were potentially further developed to fulfill other needs such as a transport system for maturing macrophages in reticular fibers, as scaffold for endothelial cell lined vessels and as an instructive matrix for branching morphogenesis. It will be important to understand how these matrix networks are created, and which signals induce their emergence.

Lysyl oxidase, transglutaminase and other enzymes modifying fibronectin may be relevant in modulating interactions of fibronectin with tenascin-C and other matrix molecules. It is likely that monomeric rather than bulky hexameric tenascin-C is part of a dense tubular matrix network. Monomeric tenascin-C can be generated upon cleavage by several proteases that are abundant and active in cancer tissue. In particular, separation of the N-terminal part from the remainder of the molecule seems to result in the release of monomeric tenascin-C from the hexamer (Mackie, 1997). The N-terminal oligomerizing part of tenascin-C can be cleaved off by meprinα and β (Ambort *et al.*, 2010), pepsin (Chiquet *et al.*, 1991), trypsin (Fischer *et al.*, 1995) and MMP7 (Siri *et al.*, 1995).

It will be necessary to determine what exact role tenascin-C plays in the matrix tubes in cancer. Recently, it was found that cancers are able to trick the immune system by using a chemokine signaling program that would mischievously tell the body that the tumor is a lymphoid tissue (Shields *et al.*, 2010) and thus trigger tumor evasion. Given that lymphocytes use the reticular fiber system to translocate within the lymphoid tissue and that tenascin-C and fibronectin are structural components of the reticular fibers it will be interesting to see whether these matrix molecules play a role in immune evasion in reticular fibers and in cancer. Assuming that ancient programs developed in evolution and are potentially involved in the creation of tubular matrix structures in mammals, it is intriguing to speculate that laminins and integrins may play an initial role followed by tenascin-C, fibronectin and other ECM molecules that were developed later during evolution.

Potential applications and outlook

Together, these data demonstrate that tenascin-C and fibronectin are key players in tumor angiogenesis and metastasis, and they represent attractive anti-cancer targets. Drugs targeting tenascin-C and c-fibronectin, or interactions with their cellular receptors are currently being developed, or have already reached clinical trials (reviewed in Desgrosellier and Cheresh, 2010; Midwood and Orend, 2009; Pedretti *et al.*, 2009; Schliemann and Neri, 2010). Novel approaches involving immunization against the EDB domain of c-fibronectin also may provide interesting alternative strategies for interfering with tumor angiogenesis and cancer growth (Huijbers *et al.*, 2010). To optimize potential treatments, several questions related to the biology of these relatively new (evolutionarily speaking) ECM proteins remain to be further addressed. Many secrets appear to be hidden in their topographical organization, some of which may be revealed by comparing cancer tissue with embryonic tissue, and the ECM of different types of cancer. Beyond circumstantial evidence, do these molecules collaborate or counteract each other in induction of pro-angiogenic signaling and blood vessel remodeling? Does this occur through a receptor-mediated mechanism or indirectly e.g. by modulating signaling of proangiogenic growth factors? What are the mechano-regulatory mechanisms involved? How is their spatial regulation (different cells) and temporal regulation in tumors controlled? Do both molecules serve as chemoattractants for endothelial cells or their precursors? How are they involved in the recruitment of mural cells or cells of the hematopoetic system? Finally, do fibronectin and tenascin-C-containing matrix channels support regrowth of vessels in residual tumor tissue upon an anti-angiogenic therapy? Finding answers to these questions will require the complicity of many researchers. The answers should clarify the function of ECM in the evolution and development of vasculature, and should lead to the discovery of more effective therapies for fighting tumor growth and metastasis.

Acknowledgements

We apologize to all authors whose work we did not have space to mention. The laboratories of EVOS and GO are financially supported by INSERM, the Agence National de la Recherche, the Association pour la Recherche sur le Cancer and the Institut National du Cancer. EVOS was additionally supported by the University of Nice-Sophia Antipolis, CNRS and the Centre A. Lacassagne; and GO by Oncosuisse, the University Hospital Hautepierre and the University Strasbourg. I.G. was supported by Inserm Region Alsace. Anti-FN-EDB antibody was generously provided by A.K. Olsson, Uppsala University, Sweden.

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