Biology of Extracellular Matrix 12
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# Matrix Pathobiology and Angiogenesis

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# Biology of Extracellular Matrix

Volume 12

Extracellular matrix (ECM) biology, which includes the functional complexities of ECM molecules, is an important area of cell biology. Individual ECM protein components are unique in terms of their structure, composition and function, and each class of ECM macromolecule is designed to interact with other macromolecules to produce the unique physical and signaling properties that support tissue structure and function. ECM ties everything together into a dynamic biomaterial that provides strength and elasticity, interacts with cell-surface receptors, and controls the availability of growth factors. Topics in this series include cellular differentiation, tissue development and tissue remodeling. Each volume provides an in-depth overview of a particular topic, and offers a reliable source of information for post-graduates and researchers alike.

All chapters are systematically reviewed by the series editor and respective volume editor(s).

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# Matrix Pathobiology and Angiogenesis

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# Introduction on the Interplay Between the Extracellular Matrix and Angiogenesis

Angiogenesis is a complex, tightly regulated process during which new blood vessels derive from pre-existing ones through various mechanisms. The most common and best studied mechanism that occurs during pathological angiogenesis is sprouting from pre-existing vessels, which can be facilitated by endothelial progenitor cells that exist in adult organisms. Numerous and diverse factors regulate the steps of sprouting angiogenesis (Senger and Davis 2011; De Palma et al. 2017; Duran et al. 2017), making it difficult to effectively control pathological angiogenesis by simple pharmacological interventions.

Among the various molecules that regulate steps of the angiogenic process are components of the extracellular matrix (ECM), a concept that is being studied since before the explosion of the interest and the knowledge on the role of the numerous growth factors that regulate angiogenesis and are sequestered in the ECM (Lu et al. 2011; Kostourou and Papalazarou 2014; Marchand et al. 2019). The ECM components are produced by both endothelial and mural cells and are nearly 300 proteins grossly categorized in three main groups: (a) structural proteins, such as fibrillar and non-fibrillar collagens, fibronectin, laminin, elastin, and hyaluronan; (b) matricellular proteins, such as thrombospondin, osteopontin, periostin, and osteonectin (also known as Secreted Protein Acidic and Cysteine Rich, SPARC); (c) matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs). Other protease families also remodel ECM and differentially affect various steps of the angiogenic process. The structure and remodeling of the ECM is critical for many developmental processes, differs between tissues and organs, provides unique structure and elasticity, and is involved in tissue homeostasis (Brown 2011; Nerger et al. 2022). A specialized and conserved type of ECM is the basement membrane (BM) that underlies epithelia/endothelia and also regulates cell signaling and tissue growth and function. BM is mainly composed of collagen IV and laminin and also contains nidogen-1 and heparan sulfate proteoglycans, such as perlecan and agrin (Pozzi et al. 2017).

Endothelial cells interact with the various components of the ECM or BM mostly through integrins (Gullberg and Ekblom 1995; Larsen et al. 2006; Davis and Senger

2015; Kadry and Calderwood 2020). Integrins are heterodimeric proteins that consist of an α and a β subunit that are type 1 transmembrane proteins non-covalently associated on the cell surface. Both subunits are composed of a large extracellular domain and a relatively small intracellular region that interacts with a cluster of associated proteins that link the ECM to the cytoskeleton. There are 18 α and 8 β subunits that give rise to 24 different heterodimers in humans and respond to a broad range of ligands, including ECM proteins. Although the role of integrins in angiogenesis has been extensively studied and integrin inhibitors have even reached clinical studies, there is no conclusive evidence on either how integrins control angiogenesis or how we could exploit integrins to develop effective therapeutics for the regulation of angiogenesis (Bachmann et al. 2019; Cox 2021; Bergonzini et al. 2022).

During development, as well as in pathological situations, such as inflammation and cancer, ECM is remodeled to facilitate the changes needed for the altered cellular functions. Numerous enzymes mediate ECM remodeling, among which the MMPs and plasminogen activator/plasmin families, which thus regulate the angiogenic process (Pepper 2001; Montuori and Ragno 2014; Deryugina and Quigley 2015; Niland and Eble 2020). Proteolysis of the matrix is a regulated process and is localized on the cell surface through interaction of the proteases with cell surface receptors, such as the urokinase-type plasminogen activator receptor (uPAR) (Blasi and Sidenius 2010) and integrins, e.g., $\alpha_v\beta_3$ integrin that has been shown to localize MMP2 activity on the surface of activated endothelial cells (Brooks et al. 1996). However, the latter interaction has been questioned and it has been suggested that membrane-type 1-MMP on endothelial cells is important for localized ECM proteolysis (Nisato et al. 2005).

In this book, aspects of the role of major ECM components in the regulation of angiogenesis are presented and discussed, including factors that affect ECM composition and potential therapeutic interventions that regulate angiogenesis through ECM remodeling.

The first chapter deals with fibronectin fibrillogenesis, which depends on the interaction of fibronectin with cells through cell surface integrins that act as fibronectin receptors, such as $\alpha_5\beta_1$ (Leiss et al. 2008). Fibronectin is the sole ECM protein that is also found as a soluble protein in the plasma and is overexpressed during the initial phases of blood vessels development (Papadimitriou et al. 1993). More recently, it has been shown that endothelium-derived but not fibronectin from other sources regulates proper retinal vascular development (Turner et al. 2017). As described in the chapter, fibronectin is involved in numerous phases of the angiogenic process, acting as a structural support, sequestering growth factors, activating growth factor receptors in an integrin-dependent manner, and forming fibrils based on cellular inside-out signaling that is also dependent on the fibronectin binding integrins $\alpha_5\beta_1$ and $\alpha_v\beta_3$. The chapter also discusses the potential involvement of fibronectin in pathological angiogenesis and the effect of fibronectin-derived peptides, such as anastellin, which in contrast to the full-length ECM protein, inhibits angiogenesis. The chapter concludes that although many details of the exact regulatory pathways related to fibronectin fibrillogenesis are still to be uncovered, fibronectin remodeling in the adult is limited at sites of active angiogenesis,

suggesting that it warrants exploitation as a potential therapeutic target in pathologies characterized by enhanced angiogenesis, such as cancer.

The second chapter is focused on the impact of the interplay between fibronectin and matricellular proteins on angiogenesis. Matricellular proteins are heterogeneous non-structural ECM components that do not affect cell adhesion but regulate cell morphology and functions through interaction with numerous cell surface receptors and structural ECM components. Matricellular proteins are classified into seven families: thrombospondins (TSPs), short fibulins, the secreted protein acidic and rich in cysteine (SPARC) family, the centralized coordination network (CCN) family, tenascins, the small integrin-binding ligand N-linked glycoprotein (SIBLING) family, and the Gla protein family. In addition, an increasing number of other proteins have been shown to possess matricellular properties but have not been categorized into one of the families (Murphy-Ullrich and Sage 2014). The chapter focuses on TSPs, tenascin C, SPARC, and CCN1, which have been implicated in the regulation of various cellular functions, such as proliferation, differentiation, migration, and apoptosis. They also regulate angiogenesis through various mechanisms that include and are initiated by the direct interaction of the matricellular proteins with cell surface receptors, such as integrins, growth factor receptors, and Toll-like receptor 4, or they stem from an interplay between the matricellular proteins and structural ECM components, such as fibronectin. The latter is extensively discussed.

Chapter 3 focuses on the role of laminins and their integrin receptors on angiogenesis. Laminins form a group of 16 high molecular weight, cross-shaped heterotrimeric glycoproteins, composed of one α, one β, and one γ polypeptide chains. Different combinations of the α, β, and γ chains (five, four, and three variants, respectively) lead to the 20 laminin isoforms that have been studied in mammals. Laminin is the major constituent of the BM and forms complexes with collagen IV through nidogen (Yao et al. 2017). Laminin isoforms are differentially expressed in a tissue- and developmental stage-specific manner and interact with cells through the laminin-binding integrins $\alpha_3\beta_1$ and $\alpha_6\beta_4$ that mediate the various and sometimes contradictory laminin functions (Yamada and Sekiguchi 2015; Ramovs et al. 2017). Laminins in the vascular BMs are important for proper endothelial cell BM assembly and vascular homeostasis, and contain mostly the α4 and α5 chains; the laminin α2 chain is detected in larger vessels (Yousif et al. 2013). On the other hand, degradation of the BM during angiogenesis exposes the endothelial cells to other laminin isoforms in the surrounding ECM that may also affect endothelial cell activation (Dixelius et al. 2004). The chapter focuses on the endothelial cell expressed laminins 411 and 511 that are responsible for vessel growth and maturation, respectively; however, there are studies that support that laminin 411 may be overexpressed during sprouting angiogenesis to act as an endogenous break against excessive angiogenesis through the Dll4/Notch signaling pathway. Laminins act on endothelial cells through interaction with integrins, some of which are also receptors for other ECM proteins, e.g., collagen; the complexity of their regulation and the difficulty to unfold the tangle is highlighted.

Chapter 4 discusses the importance of collagen homeostasis in the regulation of angiogenesis. Collagens are the most abundant ECM proteins and consist of 28 types that are divided into six groups based on the location, size, and distribution of their

triple-helical domains. Collagens affect angiogenesis directly, through interaction with integrins on endothelial cell membrane, or indirectly, by affecting the availability of growth factors that are sequestered in the ECM. They are also the major source of the so-called matrikines that are released from collagens following cleavage by collagen-degrading enzymes, such as MMPs. The most abundant type of collagen in the ECM is collagen I, which has the major function to maintain tissue integrity and actively participates in the ECM remodeling that favors the angiogenic process (Iruela-Arispe et al. 1991; Kirkpatrick et al. 2007; Feng et al. 2013). Another collagen type that significantly affects angiogenesis is the BM collagen IV, which is secreted by endothelial cells at later stages during vessel formation (Papadimitriou et al. 1993), in line with the suggested involvement of collagen IV in vessel maturation (Bonanno et al. 2000; Bahramsoltani et al. 2014). Collagen IV is also the source of angiogenesis inhibitors/matrikines, such as tumstatin and other statins (Mundel and Kalluri 2007). Endostatin that derives from collagen XVIII (Walia et al. 2015) is also discussed. Finally, the direct and indirect effects of the 24 different MMPs found in humans, secreted and transmembrane, on endothelial cells during the process of new vessel formation, are presented.

Chapter 5 deals with the role of Kruppel-like factor 2 (KLF2) and MMPs on angiogenesis. KLF2 belongs to the family of Kruppel-like transcription factors that consists of 17 members in vertebrates, is expressed in endothelial cells, and has a role in endothelial cell biology and blood vessel homeostasis (Nayak et al. 2011). It is regulated by many factors that also affect angiogenesis, seems to have a role in endothelial cell quiescence, and has been shown to affect various steps of the angiogenic process. The chapter also focuses on the effect of MMPs in regulating inflammatory signaling from chemokines or transcription factors, such as NF-κB and p53, that then affect angiogenesis. It also discusses how KLF2 or MMPs seem to differentially affect common pathways that are activated in angiogenic endothelial cells and finishes by mentioning how MMPs and KLF2 interact with thrombin and clotting pathways and how they may affect atherosclerosis; in both pathologies, there is deranged endothelial cell function and differences in the angiogenic potential of endothelial cells.

Chapter 6 gives an updated overview of natural compounds derived from plants, fungi, algae, bacteria, and several animal species, which inhibit angiogenesis by targeting ECM-remodeling enzymes, such as MMPs, ADAMs (a disintegrin and metalloprotease), ADAMTSs (a disintegrin and metalloproteinase with thrombospondin motifs), (neutrophil) elastase, uPA, heparanase, and cathepsins. ADAMs are transmembrane proteins that modify cell adhesion through binding to integrins. Moreover, as enzymes, they release membrane proteins' ectodomains, leading to fragments with biological activities that may be distinct from the protein of origin. For example, ADAM10 has been shown to cleave vascular endothelial-cadherin, vascular endothelial growth factor (VEGF) receptor 2, collagen IV, the tyrosine kinase hepatocyte growth factor receptor Met and interleukin 6, among others (Reiss and Saftig 2009; Donners et al. 2010). Similarly, the ADAMTS family has been linked to angiogenesis by affecting collagen maturation, among others (Sun et al. 2015). The role of uPA in angiogenesis is further discussed in Chap. 8.

Heparanase, (neutrophil) elastase and cathepsins have also been shown to affect angiogenesis (Shamamian et al. 2001; Ilan et al. 2006; van Hinsbergh et al. 2006; Mans et al. 2012). Heparanase is further discussed in Chap. 9. Several natural products described in the chapter, concomitantly inhibit one or more of the above-mentioned ECM-remodeling enzyme families and downregulate angiogenesis; a few of these compounds are at more advanced stages of potential clinical development.

Chapter 7 focuses on the functions and the pathways activated by endothelial cell adhesion receptors, such as integrins, upon interaction with the corresponding ECM proteins during endothelial cell specification, vascular morphogenesis, and angiogenesis. Adhesome is the highly dynamic network of scaffolding and signaling proteins that link integrins to the cell cytoskeleton and besides integrins, it includes structural/adaptor proteins, such as talin, vinculin, integrin-linked kinase-PINCH-parvin, and paxillin, signaling proteins, such as intracellular kinases and phosphatases, and proteins that regulate the cytoskeleton, such as the Rho GTPase family (Nikolopoulou et al. 2021; Arapatzi et al. 2022). The current state of knowledge regarding the regulation and the role of adhesome components in angiogenesis, both developmental and pathological, is being extensively reviewed.

Chapter 8 deals with the remodeling of the ECM resulting from hypoxia and affecting angiogenesis. Hypoxia is a well-known enhancer of angiogenesis through activation of the hypoxia-inducible transcription factors HIF-1α and HIF-1β and the upregulation of the expression of numerous angiogenic growth factors, such as angiopoietin 2, platelet-derived growth factor B, and VEGF (Semenza 2013; Lv et al. 2016). Hypoxia also affects ECM remodeling by regulating (a) expression of collagens and other ECM molecules; (b) enzymes that are important for the maturation and collagen fibril formation, such as lysyl oxidase (LOX) and LOX-like proteins; (c) collagen and other ECM degrading enzymes, such as MMPs and members of the plasminogen activators/plasmin family; (d) integrins that are the major ECM protein receptors. The chapter highlights the complexity of all the above regulations that has resulted in some potential therapeutic opportunities but also addresses the many unanswered aspects that still require extensive research.

Chapter 9 discusses the role of heparan sulfates (HS) in angiogenesis and cancer. HS are linear polysaccharides composed of a variable number of glucosamine and glucuronic or iduronic acid disaccharide repeats that are modified by epimerization or sulfation. These modifications fine-tune ligand-binding selectivity/affinity and ECM remodeling enzyme activities, thus affecting cell functions. HS exists as covalently attached to core proteins to form HS-proteoglycans (HSPGs), whose functions are mostly mediated by the HS moieties, although some of the functions may also depend on the protein core. HS is abundantly expressed in both the developing and the mature vasculature, but the different HS modifications derived from HS-modifying enzymes, such as sulfatases or heparanases, are those that affect the final balance between a pro- and an anti-angiogenic state (van Wijk et al. 2014; Chiodelli et al. 2015). The chapter summarizes what is known on how HS regulates several steps of the angiogenic cascade and thus affects normal or cancer angiogenesis and highlights the importance of targeting HS to regulate angiogenesis and cancer.

Chapter 10 deals with the role of pericytes in angiogenesis. Although pericytes are heterogeneous in their origin, phenotype, distribution, and function, they cover capillaries and interact with endothelial cells to promote vessel maturation and vascular BM formation. Pericytes have been shown to have an important role in the regulation of angiogenesis at both early and late stages. At early stages, they may activate endothelial cells through production and secretion of VEGFA, thus guiding them to form tubes. At the late stages, they inhibit endothelial cell growth and strengthen the new capillary barriers, contributing to the maturation of the new vessels (Gerhardt and Betsholtz 2003; Payne et al. 2019). Pericyte recruitment during vessel formation correlates with specific induction of fibronectin, nidogen-1, laminin, and perlecan deposition, as well as up-regulation of their integrin receptors expression (Stratman et al. 2009). Brain pericytes also have a significant role in the maintenance of the integrity of the blood brain barrier through secretion of the ECM protein vitronectin that binds to $\alpha_5$ integrin in endothelial cells and suppresses transcytosis through endothelial cells (Ayloo et al. 2022). In pathological settings, pericytes release MMP-9 and contribute to blood brain barrier disruption (Machida et al. 2015) and ECM remodeling during tumor angiogenesis in human breast cancer (Nielsen et al. 1997). Although much is still needed to be elucidated, pericytes are discussed as potential targets in angiogenesis-related tumor therapy.

Finally, Chap. 11 discusses how ECM composition and stiffness regulate lymphatic endothelial cells. Lymphatic endothelial cells are heterogeneous and important for the formation of new lymphatic vessel sprouting from veins through the process of lymphangiogenesis. Like angiogenesis, lymphangiogenesis and lymphatic homeostasis are regulated by the ECM (Lutter and Makinen 2014; Frye et al. 2018), while lymphatic endothelial cells seem to significantly contribute to ECM remodeling (Detry et al. 2012; Becker et al. 2021). Since the lymphatic system seems to be implicated in interstitial fluid balance regulation, immune cell trafficking, edema, and cancer metastasis (Petrova and Koh 2020), elucidation of the ECM-related mechanisms that affect lymphatic development and homeostasis may eventually lead to the discovery of novel therapeutic approaches for lymphatic-related diseases associated with microenvironmental alterations, as discussed.

Conclusively, as highlighted in all chapters, although the regulation of angiogenesis by ECM components or the role of vascular cells in ECM remodeling is highly complicated, it seems that we can isolate some of the discussed mechanisms and exploit them for the development of effective therapeutic approaches for angiogenesis- and lymphangiogenesis-related disorders. Many such approaches are long being used experimentally, and a few have progressed in clinical trials. Better understanding of the interactions/pathways involved will gradually lead to better therapeutics for yet unresolved clinical problems, such as diabetes-related angiogenesis deregulation and some types of cancer.

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# Abbreviations

| | |
|---|---|
| α-SMA | Alpha smooth muscle actin |
| 2-OST | 2-*O*-sulfotransferase |
| 3-OST | 3-*O*-sulfotransferase |
| 4EBP1 | Eukaryotic initiation factor-4E-binding protein-1 |
| 6-OST | 6-*O*-sulfotransferase |
| ACE | Angiotensin-converting enzyme |
| ACE2 | Angiotensin-converting enzyme 2 |
| ADAM | A disintegrin and metalloproteinase |
| ADAMTS | A disintegrin and metalloproteinase with thrombospondin motifs |
| ADME | Absorption, distribution, metabolism, and excretion |
| AFM | Atomic force microscopy |
| AITC | Allyl isothiocyanate |
| ALP | Alkaline phosphatase |
| AMD | Age-related macular degeneration |
| AMPK | Adenosine monophosphate-activated kinase |
| Ang | Angiogenin |
| ANGPT(s) | Angiopoietin(s) |
| ANKRD1 | Ankyrin repeat domain 1 |
| ApoE | Apolipoprotein E |
| ARE | AU-rich elements |
| AT | Antithrombin |
| BBB | Blood–brain barrier |
| BEC(s) | Blood vascular endothelial cell(s) |
| bFGF | Basic fibroblast growth factor |
| BM | Basement membrane |
| BMP | Bone morphogenetic protein |
| BMPR2 | Bone morphogenetic protein receptor type 2 |
| CADASIL | Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy |
| CAM | Chorioallantoic membrane |

| | |
|---|---|
| CaMKIIα | Calcium/calmodulin-activated kinase IIα |
| CBP | CREB binding protein |
| CCBE1 | Collagen and calcium-binding EGF domain 1 |
| CCH | Cas-family C-terminal homology |
| CCL | CC chemokine ligand |
| CCMs | Cerebral cavernous malformations |
| CD | Crohn's disease |
| CD31 | Cluster of Differentiation 31 |
| cFN | Cellular fibronectin |
| CHO | Chinese hamster ovary |
| COL1A2 | Collagen type I alpha 2 chain |
| COL27A1 | Collagen type XXVII alpha 1 chain |
| Col2α1 | Collagen type II alpha 1 chain |
| COL4A1 | Collagen type IV alpha 1 chain |
| COUP-TFII | COUP transcription factor 2 |
| COX2 | Cyclo-oxygenase 2 |
| CRAC | Calcium release-activated calcium |
| CREB | cAMP-response element binding protein |
| CTGF | Connective tissue growth factor |
| CV | Cardinal vein |
| CXCL | CXC chemokine ligand |
| DAG | Diacylglycerol |
| DKK1 | Dickkopf WNT signaling pathway inhibitor 1 |
| Dll4 | Delta-like4 |
| DSS | Dextran sulfate sodium |
| DT | Diphtheria toxin |
| DXR | Doxorubicin |
| EAE | Experimental autoimmune encephalomyelitis |
| EC(s) | Endothelial cell(s) |
| ECM | Extracellular matrix |
| EDA | Extra domain A |
| EDB | Extra domain B |
| EGCG | Epigallocatechin-3-gallate |
| EGF | Epidermal growth factor |
| EGF-L | Epidermal growth factor-like |
| EGFR | EGF receptor |
| eNOS | Endothelial nitric oxide synthase |
| EPC(s) | Endothelial progenitor/precursor cell(s) |
| EPO | Erythropoietin |
| ER | Endoplasmic reticulum |
| ERK | Extracellular signal-regulated kinase |
| ERK5 | Extracellular signal-regulated kinase 5 |
| EXT | Exostosin |
| EXTL3 | Exostosin-like 3 |

| | |
|---|---|
| FACITs | Fibril-associated collagens with interrupted triple helices |
| FAK | Focal adhesion kinase |
| FA(s) | Focal adhesion(s) |
| FB(s) | Fibrillar adhesion(s) |
| FCS | Fetal calf serum |
| FGF(s) | Fibroblast growth factor(s) |
| FGFR(s)1 | FGF receptor(s) |
| FN | Fibronectin |
| FOXC2 | Forkhead box protein C2 |
| FRP | Follistatin-related protein |
| FSS | Fluid shear stress |
| FUD | Functional upstream domain |
| FC(s) | Focal contact(s) |
| GAG(s) | Glycosaminoglycan(s) |
| GalT-I | Galactosyltransferase I |
| GalT-II | Galactosyltransferase II |
| GBM | Glioblastoma |
| GDNF | Glial cell-derived neurotrophic factor |
| GlcA | Glucuronic acid |
| GlcAT-I | Glucuronyl transferase I |
| GlcNAc | N-acetylglucosamine |
| Glut1 | Glucose transporter type 1 |
| GPI | Glycosyl-phosphatidylinositol |
| GSK3β | Glycogen synthase kinase 3 beta |
| HA | Hyaluronic acid |
| HB-EGF | Heparin-binding epidermal growth factor-like growth factor |
| HDAC | Histone deacetylase |
| HDAC4 | Histone deacetylase 4 |
| HDAC5 | Histone deacetylase 5 |
| Hep | Heparin |
| HGF | Hepatocyte growth factor |
| HIF(s) | Hypoxia-inducible factor(s) |
| HIF-α | Hypoxia-inducible factor alpha subunit |
| HIF-β | Hypoxia-inducible factor beta subunit |
| HNE | Human neutrophil elastase |
| hpf | Hours post-fertilization |
| HS | Heparan sulfate |
| HSPG(s) | Heparan-sulfate proteoglycan(s) |
| HUVEC | Human umbilical vein endothelial cell(s) |
| IAP | Integrin-associated protein |
| IBD | Inflammatory bowel disease |
| ICAM | Intercellular adhesion molecule |
| Id1 | Inhibitor of DNA binding 1 |
| IdoA | Iduronic acid |

| | |
|---|---|
| IGFBP | IGF-binding protein |
| IGF | Insulin-like growth factor |
| IL | Interleukin |
| ILK | Integrin-linked kinase |
| IM | Interstitial matrix |
| IP | Immunoprecipitation |
| $IP_3$ | Inositol 1,4,5-trisphosphate |
| JAM(s) | Junctional adhesion molecule(s) |
| JLS | Jugular lymph sac |
| JNK | c-Jun N-terminal kinase |
| KANK | KN motif and ankyrin repeat domain |
| KLF(s) | Kruppel-like factor(s) |
| LAMA3 | Laminin subunit alpha 3 |
| LAMB2 | Laminin subunit beta 2 |
| LD | Leucine-aspartic acid |
| LD | Lymphedema-distichiasis |
| LDL | Low-density lipoprotein |
| LEC(s) | Lymphatic endothelial cells |
| LOX | Lysyl-Oxidase |
| LOXL2 | Lysyl oxidase-like protein 2 |
| LPS | Lipopolysaccharide |
| LRP1 | Low-density lipoprotein receptor 1 |
| LTBP | Latent TGFβ binding protein |
| LV(s) | Lymphatic valve(s) |
| LYVE-1 | Lymphatic vessel endothelial hyaluronan receptor 1 |
| M6P | Mannose-6-phosphate |
| MAPK | Mitogen-activated protein kinase |
| MCAM | Melanoma cell adhesion molecule |
| M-CSF | Macrophage colony-stimulating factor |
| MEF | Myocyte enhancer factor |
| MEF2 | Myocyte enhancer factor 2 |
| MGO | Methylglyoxal |
| miRs | microRNAs |
| MLC | Myosin light chain |
| MLCK | Myosin light chain kinase |
| MLCP | Myosin light chain phosphatase |
| MMP(s) | Matrix metalloproteinase(s) |
| MMRN2 | Multimerin 2 |
| MSC(s) | Mesenchymal stem cell(s) |
| MT1-MMP | Membrane type 1 MMP |
| MT-MMP | Membrane-type MMP |
| mTOR | Mammalian target of rapamycin |
| MYPT1 | Myosin phosphatase targeting 1 |
| NADPH | Reduced nicotinamide adenine dinucleotide phosphate |

| | |
|---|---|
| NC | Non-collagenous |
| NF-κB | Nuclear factor kappa-light-chain-enhancer of activated B cells, or Nuclear factor kappa B |
| NG2 | Neural/glial antigen 2 |
| NO | Nitric Oxide |
| NR2F2 | Nuclear receptor subfamily 2 |
| Nrp1 | Neuropilin 1 |
| NS | N-sulfated |
| OF | Oscillatory flow |
| OSS | Oscillatory shear stress |
| OST | O-sulfotransferase |
| oxLDL | Oxidized low-density lipoprotein |
| P4H | Prolyl 4-hydroxylase |
| P4HA | Prolyl 4-hydroxylase α-subunit |
| P4HA1 | Prolyl 4-hydroxylase subunit alpha 1 subunit |
| P4HA2 | Prolyl 4-hydroxylase subunit alpha 2 subunit |
| P4HB | Prolyl 4-hydroxylase β-subunit |
| PA | Plasminogen Activator |
| PAI | Plasminogen Activator Inhibitor |
| PAPS | 3′-phosphoadenosine-5′-phosphosulfate |
| PAR | Protease-activated receptor |
| PAR1 | Protease-activated receptor 1 |
| PCAF | CBP-associated factor |
| PDGF | Platelet-derived growth factor |
| PDGFR | PDGF receptor |
| PDL1 | Programmed death ligand 1 |
| pFN | Plasma fibronectin |
| PG(s) | Proteoglycan(s) |
| PGE2 | Prostaglandin E2 |
| PGF or PlGF | Placental growth factor |
| PHD(s) | Prolyl hydroxylase(s) |
| PI(4,5)P2 | Phosphatidylinositol-4,5-bisphosphate |
| PI3K | Phosphatidylinositol 3-kinase |
| PIEZO1 | Piezo-type mechanosensitive ion channel component 1 |
| PINCH | Particularly interesting new Cys-His protein |
| $PIP_2$ | Phosphatidylinositol 4,5-bisphosphate |
| $PIP_3$ | Phosphatidylinositol 3,4,5-trisphosphate |
| PITC | Phenyl isothiocyanate |
| PKA | Protein kinase A |
| PKC | Protein kinase C |
| PLC | Phospholipase C |
| PLLV | Peripheral longitudinal lymphatic vessel |
| PLOD | Procollagen-lysine 2-oxyglutarate 5-dioxygenase |
| PL(s) | Lymphatic progenitor(s) |

| | |
|---|---|
| PLVAP | Plasmalemma vesicle-associated protein |
| PMA | Phorbol 12-myristate 13-acetate |
| PPE | Porcine pancreatic elastase |
| PROX1 | Prospero homeobox protein 1 |
| PSS | Pulsatile shear stress |
| PTD | Primordial thoracic duct |
| PTP(s) | Protein tyrosine phosphatase(s) |
| PTPR | PTP receptor |
| pVHL | Von Hippel–Lindau tumor suppressor protein |
| Rap1 | Ras-related protein 1 |
| RECK | Reversion-inducing cysteine-rich protein with Kazal motifs |
| RGS5 | Rregulator of G protein signaling 5 |
| RhoA | Ras homology family member A |
| RPE | Retinal pigment epithelium |
| RTK | Receptor tyrosine kinase |
| S6K | Ribosomal S6 kinase |
| sc-uPA | Single chain urokinase plasminogen activator |
| SD | Substrate domain |
| SDC(s) | Syndecan(s) |
| SDF | Stromal cell-derived factor |
| SFK | Src family kinase |
| SH | Src homology |
| shRNA(s) | Short hairpin RNA(s) |
| SIBLING(s) | Small integrin-binding ligand N-linked glycoprotein(s) |
| SLC39A13 | Solute Carrier family 39 member 13 |
| SMC(s) | Smooth muscle cell(s) |
| SMOC | Secreted modular calcium binding protein |
| SND4 | Syndecan 4 |
| SOCS3 | Suppressor of cytokine signaling 3 |
| SOX18 | SRY-Box transcription factor 18 |
| Sulf(s) | endoglucosamine-6-*O*-sulfatase(s) |
| SUR2 | Sulfonylurea receptor 2 |
| TBX18 | T-Box transcription factor 18 |
| tc-uPA | Two-chain urokinase plasminogen activator |
| TF | Tissue factor |
| TGF | Transforming growth factor |
| Th | T helper cells |
| TIMP(s) | Tissue inhibitor of metalloproteinase(s) |
| TLR4 | Toll-like receptor 4 |
| TM | Thrombomodulin |
| TN | Tenascin |
| TNF | Tumor necrosis factor |
| tPA | Tissue-type plasminogen activator |
| TSAd | T cell- specific adapter |

| | |
|---|---|
| TSP | Thrombospondin |
| TSP1 | Thrombospondin 1 |
| UC | Ulcerative colitis |
| UF | Unidirectional flow |
| uPA | Urokinase plasminogen activator |
| uPAR | uPA receptor |
| VCAM | Vascular cell adhesion molecule |
| VE-cadherin | Vascular endothelial cadherin |
| VEGF | Vascular endothelial growth factor |
| VEGFR | VEGF receptor |
| VE-PTP | Vascular endothelial-protein tyrosine phosphatase |
| VMSC(s) | Vascular smooth muscle cell(s) |
| WWTR1 | WW domain-Containing transcription regulator 1 |
| XylT | Xylosyltransferase I |
| YAP | Yes-associated protein |
| ZO-1 | Zonula occludens 1 |

# Chapter 1
# Fibronectin Fibrillogenesis During Angiogenesis


**Xiangyi Gan, Lariza Ramesh, Nidhi Nair, and Ananthalakshmy Sundararaman**

**Abstract** Vascular basement membrane is a critical regulator of angiogenesis. Fibronectin, a component of the vascular matrix, acts as a scaffold between the endothelial and perivascular cells that guide the assembly of other matrix components like the laminins and collagen IV. Fibronectin is unique in being the only extracellular matrix protein present in both soluble and insoluble forms. Fibronectin is secreted as a dimeric glycoprotein that can self-assemble into a network of fibrils. The process of fibronectin fibrillogenesis is carefully tuned according to extracellular cues to regulate the multistep angiogenic processes. This chapter details the regulators of fibronectin remodelling in endothelial cells and the role of fibronectin secretion and fibrillogenesis in guiding physiological and pathophysiological angiogenesis.

## 1.1 Fibronectins

Fibronectin (FN) is a vertebrate-specific matrix protein. It is secreted as a disulfide-bonded dimer. The dimer is unfolded and assembled into fibrils by a cell-driven process resulting in fibrillogenesis. Our understanding of the role of FN in angiogenesis comes from knockout mouse models that delineate the different functions of FN in developmental and postnatal angiogenesis. The importance of FN in cardiovascular morphogenesis is underscored by the lethal phenotype in knockout mice. *FN* knockout results in embryonic death at embryonic day (E) 8.5 due to vascular and neural tube defects (George et al. 1993). FN is particularly enriched and remodelled at sites of physiological and pathophysiological angiogenesis. FN acts as a guidance cue for cell migration, particularly evident in retinal angiogenesis,

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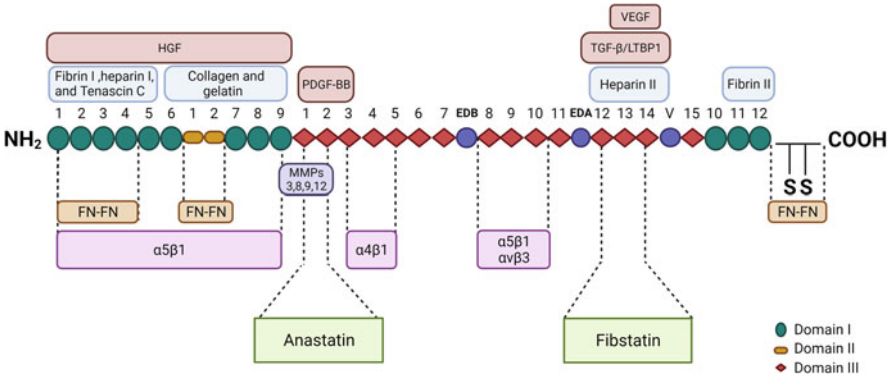


**Fig. 1.1** Fibronectin (FN) and its binding domains. FN is composed of three types of repeating subunits; called FNI, FNII, and FNIII. Some FNs also contain three different alternatively spliced domains—Extra Domain A (EDA), Extra Domain B (EDB), and Variant (V) region. These domains and subunits make up the numerous binding sites for structural proteins, mitogens, and receptors. Multiple fibrillogenesis sites exist across the FN (shown as FN-FN), allowing FN molecules to bind to one another. Growth factor binding sites on FN regulate the availability of mitogens in the ECM. Figure created with Biorender.com

where astrocyte-secreted FN acts as a provisional matrix for endothelial cell (EC) migration. FN signals through integrin receptors, causes actin cytoskeletal reorganisation, and acts as a growth factor reservoir, as detailed in subsequent sections.

FN has three different types of domains (known as FN type I, II, and III domains). There are 12 Type I, 2 Type II, and 14 Type III repeats in FN, as shown in Fig. 1.1. Interestingly, the source of FN determines its domain structure and its eventual function in angiogenesis. The hepatocytes are the primary source of plasma FN (pFN), present in micromolar concentrations in our blood. The soluble pFNs were discovered by fractionating human blood plasma and were initially called "cold-insoluble globulin" (Morrison et al. 1948). The other major form of FN is the multimeric and insoluble component of extracellular matrices derived from cells or cellular FN (cFN). The cFN differs from the pFN in containing the conserved type III 'extra' domains B (EDB) and/or A (EDA).

Since FN is a single complex gene with 45 exons that can be alternatively spliced into different isoforms, different tissues and cells secrete different variants of cFN. Interestingly, 40–60% of extracellular FN in tissues is derived from the remodelling of the soluble pFN (Moretti et al. 2007) and the contribution of EDA containing cFN to pFN is negligible. These observations suggest that in ECs, there is polarised secretion of cFN towards the abluminal side. Experiments with $EDA^{+/+}$ FN hepatocytes in engineered mice reveal a drop in pFN. This is due to the defective secretion of EDA-containing FN by these hepatocytes (Moretti et al. 2007). These together suggest that the EDA domain determines the secretory trajectory of FN in ways we do not yet understand completely. The two forms of FN possibly take different secretory routes out of cells.

### *1.1.1 Cardiovascular Development*

FN is strongly expressed around developing blood vessels during early development, but its expression in normal adult vasculature is barely detectable (Astrof et al. 2007; Peters et al. 1996). Re-expression of FN occurs during pathological angiogenesis, as seen in tumours, ocular diseases, and atherosclerosis. The absence of FN leads to early embryonic lethality (George et al. 1993). EDA/EDB double null embryos also show severe defects in cardiovascular development, including haemorrhages and defective remodelling of the yolk sac vasculature leading to embryonic lethality (Astrof et al. 2007), while the single EDA or EDB individual KO mice are viable and fertile with normal embryonic and adult angiogenesis.

Using quail development as a model, vasculogenic tube assembly was studied, which demonstrated that pericytes induce the expression of integrin receptors on ECs and catalyse the deposition and remodelling of FN among other matrix proteins. By inhibiting the FN matrix assembly using the N-terminal FN peptide fragment, the authors show that collagen IV and to a lesser extent laminin, depend on FN assembly for deposition onto the vascular basement membrane (BM). Indeed, defective pericyte recruitment or lack of FN assembly leads to vessels that are wider in diameter (Stratman et al. 2009). The absence of $\alpha_5$ integrin (a receptor for FN) in mouse embryos also led to distended vessels in the yolk sac, phenocopying the FN null mouse embryos. Together, we see that across model systems, the FN assembly and signalling through its receptors is essential for normal vascular development and a lack of the scaffolding function of remodelled FN might result in increased vessel diameters.

Early postnatal deletion of cFN, specifically in vascular smooth muscle cells (SMCs) following tamoxifen injections from postnatal day (P) 1 to P3, results in no reduction in life span, possibly due to uptake and transfer of pFN that completely rescues the amount of FN deposition at the aortic wall. Postnatal deletion of pFN preferentially from P3 also does not affect the vasculature. However, the mice with a double knock out of cFN in SMCs and pFN showed perinatal lethality at a median age of P11 (Kumra et al. 2018). At P8, these mice (both dKO and single pFN and cFN KOs) also showed reduced deposition of other extracellular matrix (ECM) proteins, like fibrillin1 and fibulin, in the aortic wall (Kumra et al. 2018), demonstrating the role for both cFN and pFN in forming scaffolds in the vessel wall for guiding the deposition and fibrillogenesis of other matrix proteins. Together, we find a robust regulation of FN fibrillogenesis by both the cFN and pFN forms. They are capable of compensating for one another, at least in their postnatal functions, to maintain vascular integrity. Therefore, it is only when both these forms of FN are knocked out in blood vessel walls that we get perinatal lethality.

Further experiments done in vitro, using FN null mouse embryonic fibroblasts provided with both cFN and pFN in equal amounts in the soluble form, revealed that the EDA/B containing cFN acts as a better substrate for fibrillogenesis, forming longer and more mature fibres (Kumra et al. 2018). This also translates to better scaffolding for the deposition of other matrix proteins by cFN compared to pFN

(Kumra et al. 2018). Taken together, although the two isoforms, cFN and pFN are capable of compensating for the loss of one another in vivo, in vitro studies suggest that they differ in their ability to form fibres and guide fibrillogenesis. There are possibly factors influencing fibrillogenesis in vivo that allow pFN to be efficiently transferred across the inner endothelial layer to the vessel walls and bundled in the absence of cFN in SMCs (Kumra et al. 2018).

A tamoxifen-induced complete knockout of FN in 4-week-old mice did not show any vascular phenotype (Yuan et al. 2020). Taken together, except for early post-natal development, it appears that FN has minimal roles to play in adults under physiological conditions. This is in keeping with the highly reduced FN expression in quiescent vessels, suggesting that the role of FN becomes apparent only under conditions of increased physiological or pathophysiological angiogenesis in adults.

### *1.1.2 Evolution*

Unlike other ECM proteins, like laminins and collagens that arose very early in the course of evolution in basal metazoa, FN is a vertebrate-specific matrix protein that arose in conjunction with the endothelium-lined closed blood vascular system (Adams et al. 2015). While integrins themselves and molecules in the integrin signalling pathway, such as paxillin and integrin-linked kinase (ILK), are very ancient, known to occur in fungi and single-celled holozoans, this response pathway was co-opted by FN in vertebrates. Indeed, both FN and endothelium-lined blood vessels are absent in non-vertebrate chordates, like the tunicates and cephalochordate lineages. The appearance of FN may have, therefore, played a crucial role in the evolution of the closed vascular system, which is a distinctive feature of vertebrates.

### *1.1.3 Fibrillogenesis*

Fibrillogenesis of FN is a cell-mediated process (McDonald 1988). Assembly of FN into fibrils begins with the binding of the soluble dimer to cell surface receptors, like the $\alpha_5\beta_1$ integrins. Receptor binding triggers self-association through the N-terminal assembly domain. The self-association of FN and receptor clustering triggers actin cytoskeletal rearrangements through the integrin cytoplasmic tails that promote contractility. The pulling force exerted by actin through the integrin receptor causes conformational changes in FN, exposing more binding sites that trigger several FN proteins to associate and form fibrils (Mao and Schwarzbauer 2005). These are eventually stabilised by non-covalent interactions and converted to insoluble fibrillar FN (Fig. 1.2). Once assembled, FN acts as a scaffold for other ECM proteins, as described previously (Singh et al. 2010).

FN fibrils are initially extended linearly and unidirectionally (Winklbauer and Stoltz 1995), but lateral additions of fibrils across the entire length could also occur

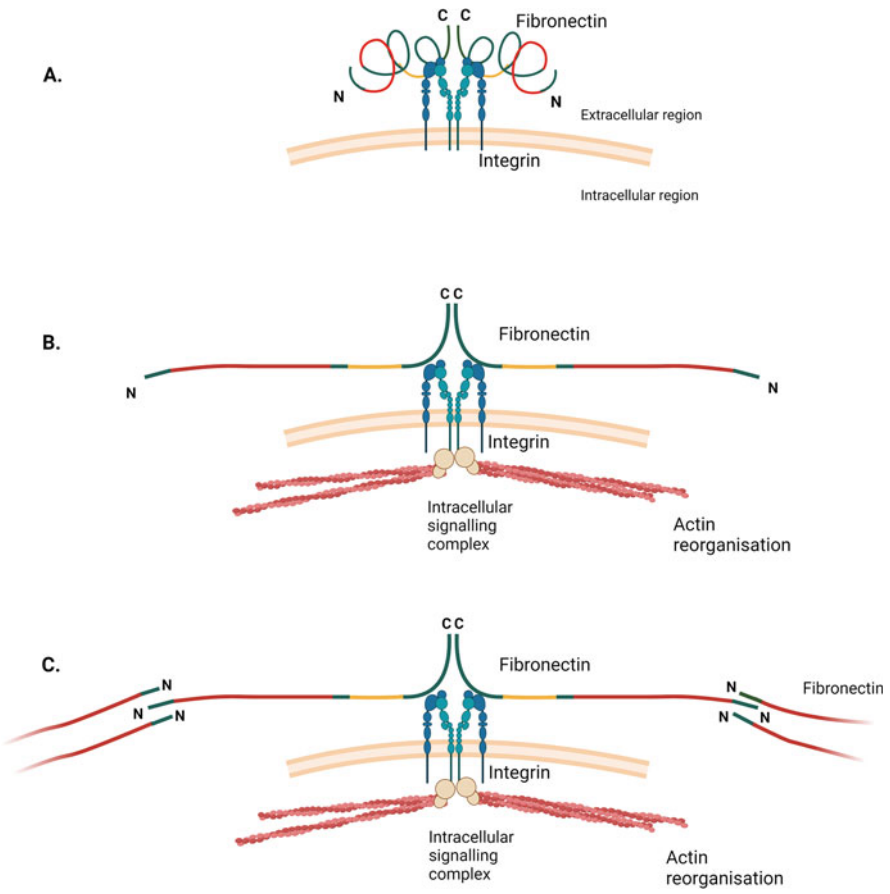


**Fig. 1.2** FN fibrillogenesis. Adapted from (Mao and Schwarzbauer 2005). (**a**) Newly synthesised FN dimers are in a folded state. Upon binding to the FN receptor, for example, the $\alpha_5\beta_1$ integrins, there is a conformational change in FN. FN-binding sites are exposed for fibrillogenesis. (**b**) FN binding to integrins induces actin reorganisation, which aids in unfolding the FN and revealing the sequestrated FN binding sites. (**c**) Fibrillar FN networks are formed through FN-FN interactions. Figure created with Biorender.com

subsequently. Recent studies using time-lapse atomic force microscopy (AFM) have revealed new mechanistic details of fibrillogenesis. In fibroblasts used for this study, FN nanofibrils are formed within 5 min of cell-matrix contact and then extended at 0.25 μm/min rates that are increased threefold by $Mn^{2+}$ mediated increase in integrin adhesion (Gudzenko and Franz 2015).

As a unique ECM protein that exists in both soluble and fibrillar forms, it seems imperative that the process of FN assembly would be carefully coordinated to prevent the inappropriate generation of insoluble fibrils. This would be particularly true for ECs that face a high concentration of soluble plasma FN on their luminal

side. We will subsequently discuss the key regulators of this process in ECs and their role in angiogenesis (Sect. 1.3).

## 1.2 Role of Fibronectin in Angiogenesis

### *1.2.1 Overview of Angiogenic Sprouting*

Angiogenesis describes the process by which new blood vessels are formed from pre-existing vessels, enabling the distribution of oxygen, nutrients, and immune cells throughout the body. There are two main mechanisms for blood vessel formation—the development of a primitive vascular network in the embryos, called vasculogenesis, and the vessel branching from a pre-existing one, termed angiogenesis. During embryonic development, angioblasts, the precursors of EC, are derived from embryonic mesoderm. Upon induction by fibroblast growth factor 2 (FGF2), angioblasts differentiate into ECs and subsequently develop into new blood vessels through proliferation and junctional remodelling (Vailhe et al. 2001; Wilting and Christ 1996). Generation of an ancillary network from the existing blood vessels is essential in physiological conditions, including physical growth, female reproductive cycles, and wound healing. Angiogenesis also plays an important part in several pathologies, such as tumour progression and diabetes (Chung and Ferrara 2011; Otrock et al. 2007).

The process of physiological angiogenesis is tightly regulated in healthy tissues, and FN plays a significant supportive role in this context. In general, ECs in the quiescent blood vessels are wrapped around by the subendothelial BM and supported by vascular SMCs in large vessels or pericytes in microvessels. Hypoxia in tissues triggers the production of vascular endothelial growth factor A (VEGFA) that diffuses to the nearby blood vessel and activates the quiescent ECs. Activated ECs undergo a series of changes in secretory phenotype and morphology. First, the ECs degrade the surrounding ECM and BM by secreting metalloproteinases (MMPs) to invade through the BM. The predominant MMPs are MMP1, MMP2, and MMP9, while their specificity depends on tissue types. The signalling of VEGFA also induces ECs to transform into high-mobility tip cells, which lead the migration of the angiogenic sprout along fibrillar FN tracks guided by the VEGFA gradient. The ECs that follow tip cells in the sprout are named stalk cells. Unlike tip cells, stalk cells are highly proliferative and lack filopodia (Carmeliet and Jain 2011).

The specification of tip cells and stalk cells is a consequence of the VEGFA-Dll4/Notch feedback loop, which restricts the number of tip cells in a sprout. A high level of VEGFA is sensed by VEGFRs on the tip cell as it moves at the very front. Meanwhile, VEGFR sensing also traps VEGFA within the receptor, reducing the amount of VEGFA available to the following stalk cells. FN around the angiogenic sprout also regulates the bioavailability of GFs in the extracellular environment, as discussed later. The activation of VEGFRs in tip cells increases the presentation of DII4 to the adjacent stalk cells, which subsequently activate Notch signalling. Notch

inhibits VEGFR expression and therefore decreases stalk cell response to VEGFA. While the VEGFA-Dll4/Notch feedback loop specifies the behaviour of ECs, Dll4 also promotes cell-cycle progression and junction stability, which ensure vessel structure integrity. As the angiogenic sprout moves towards the hypoxic site guided by the tip cell, it forms a lumen by hollowing and elongation. This process requires the rearrangement of cell-cell junctions and cell polarisation (Potente et al. 2011). Before the newly formed blood vessels become functional, they undergo maturation. This includes increasing ECM stiffness for stronger adhesion, refining the new vascular network by pruning, and recruitment of pericytes, which are mural cells that strengthen blood vessels by adhering to ECs. Pericytes contribute to increased synthesis and deposition of BM proteins (Korn and Augustin 2015).

FN is involved in almost every aspect of the angiogenic process. Its role as a structural component of the ECM, as a regulator of growth factor availability, as well as its functions as a ligand for surface integrin receptors are discussed below.

### *1.2.2 Fibrillar Fibronectin as a Structural Support for Angiogenesis*

In the process of angiogenesis, the elongation of the vessel sprout relies on physical support and guidance from the ECM. By depositing EC-coated beads on different ECM surfaces, it was shown that the activity of angiogenic sprouting was much stronger on fibrillar FN compared to soluble FN-coated surfaces (Mitsi et al. 2015). In vitro models have provided insight into the vessel formation facilitated by FN fibrillogenesis. Tubulogenesis of ECs on three-dimensional matrices was greatly reduced in the presence of several fibrillogenesis-inhibiting peptides. One of them is the functional upstream domain (FUD)—a short peptide that can interact with FN-FN interaction sites, thereby inhibiting the process of fibrillogenesis (Zhou et al. 2008). Similarly, using decellularized matrix isolated from a co-culture of human new-born foreskin fibroblasts and breast cancer MDA-MB 231 cells, it was demonstrated that ECs cultured on fibrillar FN show signs of angiogenic morphogenesis and increase the expression of MMPs. In addition, imaging demonstrates high co-localisation of collagen I, IV, laminin, and tenascin-C with fibrillar FN. These features are aborted when inhibiting fibrillogenesis with pUR4B, a peptide that prevents the binding of soluble FN to the ECM (Tomasini-Johansson et al. 2001; Hielscher et al. 2016). Thus, fibrillar FN efficiently supports vessel lumen formation during angiogenesis.

### 1.2.3 Fibronectin in Growth Factor Regulation

In addition to providing physical support, the presence of FN around the angiogenic sprout serves as a reservoir for growth factors, creating concentration gradients, and enabling potent release when triggered (Sawicka et al. 2015). A typical example is the VEGFA binding to two known domains on FN. VEGFA is a master regulator of angiogenesis, with its expression and secretion largely upregulated under hypoxia. Upon the stimulation of VEGFA, ECs undergo remarkable changes in cell shape and polarity to form tip and stalk cells, as described above. The cell differentiation process, the correct migrating direction, as well as the subsequent cell signalling during elongation, are all achieved by the VEGFA gradient. It is believed that FN regulates the bioavailability and signalling output of VEGFA through direct interactions. Early studies have identified two VEGFA binding sites on FN, one located at the N-terminus of FN (Wijelath et al. 2002), while the other one is located at the C-terminus. By performing solid-phase binding assays on recombinant FN fragments, it was discovered that VEGFA has the highest binding affinity towards the type $III_{13–14}$ region, where the heparin-binding domain is also located (Wijelath et al. 2006). Interestingly, the FN-VEGFA association is greatly enhanced in the presence of heparin. An extended conformation of FN was observed under AFM after administrating heparin treatments (Wijelath et al. 2006), suggesting that heparin-induced unfolding could make more VEGFA sites available on FN. In addition, to determine the possible function of such an interaction between VEGFA and FN, EC migration and proliferation were assessed in the presence of VEGFA or FN or both (Wijelath et al. 2006). The migration and proliferation of ECs increase with VEGFA or FN alone, but the combination of the two manifested much stronger effects. Taken together, FN containing heparin-binding domain can associate with VEGFA and regulate EC behaviour. However, under normal circumstances, the C-terminus of FN exists in a closed conformation, where the binding sites are not available. The presence of heparin exposes the domains on the C-terminal region to which VEGF interacts, resulting in enhanced angiogenesis-inducing activity.

Transforming growth factor beta (TGFβ), unlike VEGFA, exerts both pro-angiogenic and anti-angiogenic effects by activating different protein effectors that belong to the decapentaplegic (Smad) family. TGFβ induces angiogenesis by increasing the expression of VEGFA, while it can also oppose angiogenesis by arresting the cell cycle (Nakagawa et al. 2004). Although it is unclear how exactly TGFβ specifies the two completely opposing signalling cascades, given that TGFβ signalling in ECs requires multiple receptors (Goumans et al. 2009), the receptors may respond to TGFβ at different concentrations. FN stores TGFβ by immobilising the latent TGFβ binding protein (LTBP) on the ECM. Inhibiting fibrillogenesis with the functional upstream domain (FUD) peptide causes LTBP-1 to be lost from the ECM. Thus, fibrillar FN regulates TGFβ availability through LTBP1 sequestration (Zilberberg et al. 2012). The binding affinity is enhanced in the FN with the EDA segment, compared to other splicing forms, such as FN-EDB. An interesting competitive assay was performed by supplying human dermal fibroblasts with soluble

FN fragments, and it was observed that when treating cells with fragments containing EDA, the amount of LTBP-1 present in the ECM was decreased, while more soluble LTBP-1 can be found in the media. The same effect did not occur when supplying cells with EDA-free fragments. This suggests that the soluble EDA fragments compete for LTBP1 binding, thereby reducing the amount bound to the fibrillar ECM. Therefore, in angiogenesis, ECs regulate the downstream signalling cascade of TGFβ by producing multiple isoforms of FN with different binding affinities. By controlling the ECM composition, the bioavailability of TGFβ can be regulated. Other examples include platelet-derived growth factor BB (PDGF-BB), for which FN contains two binding sites. The regulation of PDGF-BB by FN is believed to promote cell survival (Battegay et al. 1994; Lin et al. 2014).

Interestingly, while FN embedded in the ECM can enhance growth factor activities, as discussed above, pFN is also capable of regulating mitogen signalling. For example, hepatocyte growth factor (HGF), a mitogen that stimulates EC proliferation in angiogenesis (Xin et al. 2001; Newman et al. 2013) was found in complex with FN when derived from platelets (Rahman et al. 2005). Similarly to VEGFA, there are two binding sites on FN for HGF, located at either termini of the protein. Both HGF and FN can induce EC sprouting individually (Newman et al. 2013), while the FN/HGF complex can increase migration by sixfold (Rahman et al. 2005). An endogenously derived FN type $III_{12–14}$ fragment—fibstatin—can inhibit FGF2 activities. It is suggested that this FN fragment has a high binding affinity towards FGF2, which could, in turn, inhibit FGF2 interaction with its receptors (Bossard et al. 2004). Taken together, full-length pFN interacts with HGF and promotes its signalling output, while fibstatin binds to FGF2 and reduces its availability. These studies suggest that freely diffusing pFN functions as a chaperone, capable of binding mitogens and regulating their downstream function.

### *1.2.4 Fibronectin and Cell Signalling*

In angiogenesis, the behaviour of ECs relies on the signalling effect of extracellular mitogens and proteins. Extracellular signals induce cellular response mostly by signal transduction through receptors on the plasma membrane. For example, the angiogenic ligand VEGFA induces vascular remodelling mainly through its receptors VEGFR1, 2, and 3. The subsequent conformational and biochemical changes in the receptor initiate downstream signalling in the ECs. FN is a ligand for surface receptor integrins. The major integrin receptors which bind to FN are reported to be $\alpha_5\beta_1$ and $\alpha_v\beta_3$, and FN–integrin complex signalling is critical for cell-matrix adhesion (Danen et al. 2002), cell migration (Zou et al. 2012), proliferation, and survival (Wang and Milner 2006). As an integrin ligand, FN signalling requires direct contact with integrins. Early studies revealed that when human umbilical vein endothelial cells (HUVECs) adhere to FN, intracellular focal adhesion kinase (FAK) and mitogen-activated protein kinase (MAPK) were phosphorylated. However, when the integrin function is inhibited with cytochalasin D, a drug that disrupts actin

cytoskeleton and integrin signalling, the activation of MAPK and FAK were blocked (Short et al. 1998). A similar study on brain capillary ECs showed that adhesion to FN increased $\beta_1$ integrin expression and proliferation through MAPK signalling (Wang and Milner 2006).

In addition to the direct activation of downstream pathways by FN–integrin complex, early studies have also suggested that mitogen signalling requires the presence of FN (Short et al. 1998). FN–integrin complex can couple with multiple receptors, which enhance or suppress their effects in cell signalling. One of the examples of angiogenic signalling is the proximity activation of VEGFR3 by the FN-$\beta_1$ complex. Dermal microvascular endothelial cells and 293/VEGFR-3 cells plated on FN showed high VEGFR3 phosphorylation, a key step in the activation of these receptors. When treated with antibodies against $\beta_1$ integrin, the phosphorylation event induced by FN was greatly reduced. Secondly, using co-immunoprecipitation (IP), it was shown that VEGFR3 association with $\beta_1$ relies on FN stimulation, suggesting the significant role of FN in $\beta_1$-VEGFR3 interaction and signalling (Wang et al. 2001). A similar mechanism in VEGFR2 activation has also been discovered where $\alpha_v\beta_3$ integrin binds VEGFR2 and promotes its phosphorylation (Soldi et al. 1999). TGFβ receptor downstream signalling pathway was also activated by the FN–$\alpha_5\beta_1$ complex, as detailed subsequently (Tian et al. 2012). In addition to mitogen receptors, FN–$\alpha_5\beta_1$ also couples with other integrins. For example, $\alpha_v\beta_3$-mediated EC migration in angiogenesis relies on $\alpha_5\beta_1$ ligation with FN. $\alpha_v\beta_3$ mediates ECs migration on vitronectin, however, the migration is aborted by either inhibiting $\alpha_5\beta_1$ function or cellular FN synthesis. The cross-talk between $\alpha_5\beta_1$ and $\alpha_v\beta_3$ is described in depth under Sect. 1.3.

Although FN is generally pro-angiogenic, several fragments of FN, as well as modified FN, have anti-angiogenic properties. It is worth noting that anastellin, an FN fragment from the type III1-C region, reduces tumour angiogenesis and metastasis in mice. As FN type III segments are involved in fibrillogenesis, anastellin inhibits angiogenesis by occupying the fibrillogenesis sites (Yi and Ruoslahti 2001). Another interesting case is the anti-angiogenic effect of glycated-FN. VEGFA-VEGFR signalling of cell migration was enhanced by FN, however, after incubating FN with methylglyoxal (MGO), which glycated the FN, it no longer activated VEGFR. In addition, one of the downstream effectors of VEGFR, c-Src, was found to be uncoupled from the receptor by glycated FN (Chen et al. 2020).

The FN–integrin complex can activate some receptors without the need for their own ligands. Two receptor tyrosine kinases—fibroblast growth factor receptor-1 (FGFR1) in angiogenesis and the c-Met receptor in tumour metastasis—have been shown to have ligand-independent FN-dependent activation mechanisms. In the case of FGFR1, it was observed that the phosphorylation-dependent activation of FGFR1 occurred when plating human liver ECs on FN without FGF2. Surprisingly, by studying the activation states of the two major downstream effectors of FGFR1, Akt and Erk, an alternative FN-dependent signalling cascade was identified, where FGF2-dependent FGFR1 activation preferentially triggered the Erk pathway, while FN–β1 complex-dependent activation of FGFR1 tends to signal through the Akt pathway (Zou et al. 2012). c-Met, on the other hand, is believed to signal tumour

metastasis induced by HGF. In the absence of HGF, FN–$\alpha_5\beta_1$ was found to activate c-Met and its signalling pathway via FAK and Src. A direct interaction between the FN–$\alpha_5\beta_1$ complex and c-Met was observed in co-immunoprecipitation (Mitra et al. 2011). Other examples include epidermal growth factor receptors, which were also reported to follow a similar mechanism to increase cell proliferation and cell migration in a ligand-independent and FN–integrin-dependent manner (Cabodi et al. 2004).

### *1.2.5 Lumen Formation and Cell Polarity*

At the final stages of angiogenesis, when the angiogenic sprout meets another sprout to anastomose, the ECs stop migrating and form lumen through hollowing. Interestingly, although FN facilitates angiogenesis by promoting cell migration, reducing but not depleting the concentration of FN in vitro, led to a stalling of migration and increased tubulogenesis (Cox et al. 2001). This suggests that the concentration dynamics of FN could directly serve as a cue for tubulogenesis. A matrigel plug assay is commonly used to assess lumen formation in vivo. By implanting matrigel containing FGF2 with or without $\alpha_5\beta_1$ inhibitory antibodies in mice, authors have observed far less lumen formation in the $\alpha_5\beta_1$-inhibiting plug. The newly formed vessels were also scattered across the plug instead of being organised as a network (Kanda et al. 2005). The same effect has been repeated in vitro in another study, where, in tube formation assays, brain capillary ECs showed reduced tubulogenesis in the presence of anti-$\alpha_5\beta_1$ antibodies (Zhou et al. 2008). Therefore, FN is essential in tubulogenesis and vessel stability.

As ECs firmly attach to their neighbouring cells by junctions during migration, the hollowing process first requires the relocation of junction proteins to separate the interface. By delivering negatively charged glycocalyx to the cell–cell contact site, which causes repulsion between the cell surfaces, a gap can be formed at the interface of the sprout, ultimately contracting to form a lumen. Meanwhile, ECM around the angiogenic sprout starts remodelling in order to increase matrix stiffness, as the newly formed lumen is stabilised by actomyosin contractility that requires a rigid ECM for strong cell adhesion (Shamloo and Heilshorn 2010). While glycocalyx is delivered to the apical side of ECs, FN is deposited to the basolateral side, becoming part of the BM. Such polarised apical trafficking of negatively charged protein podocalyxin was found to be RhoJ-directed (Richards et al. 2015). RhoJ-containing vesicles were also reported to be enriched in integrins $\alpha_5\beta_1$, and RhoJ negatively regulates active receptor trafficking and basolateral FN fibrillogenesis, as detailed in Sect. 1.3 (Sundararaman et al. 2020). Together, basolateral FN could dictate the apical polarised trafficking of proteins necessary for lumen formation.

## 1.3 Fibronectin Remodelling

### *1.3.1 Integrin Receptors for Fibronectin*

The role of integrin binding and trafficking in FN remodelling and angiogenesis is explored by many studies. The $\alpha_5\beta_1$ and $\alpha_v\beta_3$ integrins play a crucial role in FN remodelling (Francis et al. 2002). Both integrins cooperate during development to remodel the vasculature and could compensate for the loss of one another (van der Flier et al. 2010). Endothelium-specific $\alpha_5$ KO mice have normal developmental angiogenesis, while endothelial $\alpha_5/\alpha_v$ DKO mice have normal vasculogenesis up to E11.5 but further on developing defects in the remodelling of the large vessels and heart, leading to death at E14.5.

A well-studied model for the role of FN remodelling in the context of angiogenesis is retinal angiogenesis, where astrocytes lay out a provisional matrix for ECs to migrate on. In this context, regulators of astrocyte FN remodelling also need to be considered to understand angiogenesis. In response to reduced oxygen tension, astrocytes secrete VEGFA and FN. Additionally, pro-angiogenic astrocytes strongly express the nuclear receptor Tailless (Tlx). In Tlx null mice, FN matrix formation is impaired due to FN mRNA expression deficits, leading to complete failure of retinal vascularisation (Uemura et al. 2006). Post P7, however, the role of FN secretion and remodelling around vessels shifts to the ECs. The astrocyte-derived FN and its remodelling plays a more crucial role early on, as evidenced by a gene dose-dependent reduction in endothelial migration and vascular plexus formation in the *FN* KO astrocyte model (Stenzel et al. 2011). Interestingly, endothelial postnatal integrin $\alpha_5$ deletion results in normal retinal vasculature (Stenzel et al. 2011). This is in keeping with reduced dependence on FN postnatally after P8 once early postnatal development is accomplished, as detailed previously. FN RGE expression (replacing the RGD domain that binds to integrins) in astrocytes also barely altered EC migration and only altered filopodia alignment. Together, it suggests that the FN RGD domain is not essential for FN assembly (Takahashi et al. 2007). This suggests that alternate integrin binding sites on FN like the iso-DGR motif can compensate and act redundantly with RGD domain for FN assembly (Takahashi et al. 2007).

### *1.3.2 Actin Regulators Involved in Fibronectin Bundling*

Central F-actin fibres in arterial ECs are oriented to extracellular FN in vivo. These fibres end in focal adhesions that dynamically couple the actin cytoskeleton to extracellular FN. The veins, on the other hand, show the presence of actin predominantly at the cell cortex lining the cell boundaries (van Geemen et al. 2014). Thus, the presence of high shear in arteries causes coupling of actin and FN orientations. The role of shear stress in actin cytoskeletal reorganisation and FN remodelling has been studied. As seen previously, cellular contractile forces are transmitted through

integrins like $\alpha_5\beta_1$ to FN, to unfurl the protein and bundle it into fibrils. Since actin cytoskeletal tension is predominantly regulated by the Rho family of GTPases, we will firstly explore Rho family proteins and their ability to cause FN remodelling.

RhoA-ROCK axis is generally appreciated as a key regulator of cytoskeletal tension. RhoA signalling downstream of VEGFA regulates cytoskeletal rearrangement in ECs. However, it does appear that RhoA activity is dispensable for angiogenesis in vivo. Endothelial RhoA deficiency did not perturb embryonic development and induction of RhoA deletion postnatally showed minimal perturbation of retinal angiogenesis. RhoA deletion has minimal effect on angiogenesis, possibly due to compensatory mechanisms we are yet to understand. The lack of phenotype is surprising, given the complete inhibition of myosin light chain and cofilin phosphorylation downstream of RhoA and the lack of RhoB and RhoC upregulation in murine ECs derived from KO mice (Zahra et al. 2019).

Bone morphogenetic protein receptor type 2 (BMPR2) axis is also a negative regulator of FN remodelling. BMPR2 KO ECs display a hyperactivation of the TGFβ signalling pathway leading to increased actomyosin contractility. FN, as well as fibrillin remodelling, was increased in BMPR2 KO ECs, especially at junctions leading to cell stiffening, increased $\beta_1$ integrin activation and endothelial to mesenchymal transition. FN, provided as part of a functionalised polydimethylsiloxane scaffold, was remodelled more efficiently by *BMPR2* KO endothelia at cell junctions. Interestingly, while in wild-type cells soluble rhodamine FN enters vesicles, it remains majorly extracellular in KO ECs and gets remodelled at cell-to-cell contacts (Hiepen et al. 2019). These results raise interesting questions about the differences in FN remodelling for intrinsically secreted FN and extrinsically provided soluble FN.

### *1.3.3 Integrin Trafficking in Fibronectin Remodelling*

As key proteins coupling the contractile forces of the actin cytoskeleton to FN, the integrins $\alpha_5\beta_1$ and $\alpha_v\beta_3$ play critical roles in FN remodelling. The spatial distribution, trafficking and downstream signalling of these two key receptors are differentially regulated. For example, studies in fibroblasts indicate that nascent adhesion plaques called focal contacts (FX) are rich in $\alpha_v\beta_3$. In contrast, the maturation of FX into focal adhesions (FAs) involves the incorporation of $\alpha_5\beta_1$ and the further translocation of FAs centripetally along the base of the cells, leads to the formation of fibrillar adhesions (FBs) that exclude $\alpha_v\beta_3$ and are rich in $\alpha_5\beta_1$. The engagement of $\alpha_5\beta_1$ in FAs and FBs can activate RhoA, while $\alpha_v\beta_3$ in FX and FAs do not generally signal through RhoA (Morgan et al. 2009).

In ECs, using nano-patterned substrates functionalised with either $\alpha_5\beta_1$ or $\alpha_v\beta_3$ selective peptidomimetics, it was demonstrated that $\alpha_v\beta_3$ gets recruited to $\alpha_5\beta_1$ focal adhesions and not vice versa. This recruitment of $\alpha_v\beta_3$ after the initial engagement of $\alpha_5\beta_1$ is essential for effective FA formation and cell spreading. Surprisingly, cell-derived FN was dispensable and FAK was required for the recruitment of $\alpha_v\beta_3$ integrins to $\alpha_5\beta_1$ adhesions (Diaz et al. 2020). This study contrasts with studies in

fibroblasts, where the initial FXs containing $\alpha_v\beta_3$ mature into FAs with $\alpha_5\beta_1$ recruitment. The medial end of these adhesions enriched in $\alpha_5\beta_1$ translocates centripetally, while vitronectin-bound $\alpha_v\beta_3$ serves to anchor the other end, allowing for actomyosin-based contractility to unfurl the FN–$\alpha_5\beta_1$ complex (Zamir et al. 2000). It is plausible that ECs, which primarily depend on $\alpha_5\beta_1$ to adhere to FN (Diaz et al. 2020), utilise a different order of recruitment of integrins to ECM proteins compared to fibroblasts, where $\alpha_v$ integrins are shown to outcompete $\alpha_5\beta_1$ for initial attachment to FN (Bharadwaj et al. 2017).

Vascular injury upregulates $\alpha_5\beta_1$ expression in SMCs, as seen in studies with rat carotid artery balloon injury models. This is accompanied by increased FN fibrillogenesis in situ on the luminal face of the injured artery and absence in uninjured areas, suggesting localised fibril assembly (Pickering et al. 2000).

Neuropilin 1 (Nrp1), a VEGFA co-receptor, is known to promote EC adhesion to FN and fibrillogenesis through $\alpha_5\beta_1$. Specifically, the Nrp1-GIPC PDZ domain containing family, member 1 complex stimulates the internalisation of active $\alpha_5\beta_1$ into Rab5-positive early endosomes and its recycling to newly formed adhesion sites (Valdembri et al. 2009). Indeed, lack of Nrp1 leads to defective development of blood vessels, which does not phenocopy malformations due to lack of VEGFA or semaphorin 3A, suggesting that the Nrp1-dependent regulation of $\alpha_5\beta_1$ is a distinct arm compared to its role as a co-receptor for these ligands. Indeed, Nrp1–$\alpha_5\beta_1$ axis could be a critical modulator of developmental angiogenesis.

Syntaxin 6, an endosome and Golgi-associated target SNARE is seen to play a key role in $\alpha_5\beta_1$ recycling in HUVECs using biochemical recycling assays (Tiwari et al. 2011). Lack of syntaxin 6 leads to increased $\alpha_5\beta_1$ ubiquitinylation and routing to lysosomes. Functionally, this results in impaired EC adhesion and migration on FN. Although this study does not distinguish total versus active $\alpha_5$ regulation by syntaxin 6, active $\beta_1$ was reduced at the plasma membrane upon syntaxin 6 inhibition, which also causes the total heterodimer to relocalise to vesicles (Tiwari et al. 2011).

$\alpha_5\beta_1$ adopts a bent conformation when inactive, and a straight conformation with cytosolic tails pulled apart when active and bound to FN. Conformation-sensitive trafficking of $\alpha_5\beta_1$ has been reported in ECs. Liprin α1, a focal and fibrillar adhesion localised adaptor protein, interacts preferentially with active $\alpha_5\beta_1$ integrins and controls their recycling to regulate basolateral FN secretion and fibrillogenesis (Mana et al. 2016). RhoJ is yet another regulator of $\alpha_5\beta_1$ integrin trafficking in a conformation-specific manner. RhoJ negatively regulates the internalisation of the active $\alpha_5\beta_1$, while having no effect on the inactive bent conformation. RhoJ opposes Cdc42 in regulating subendothelial FN fibrillogenesis through a competition for shared effector proteins like PAK3 (Sundararaman and Mellor 2021; Sundararaman et al. 2020).

Integrin $\alpha_5\beta_1$ is known to co-internalise with endoglin in ECs. TGFβ increases integrin $\alpha_5\beta_1$ levels, as well as their activation in an endoglin-dependent manner (Tian et al. 2012). Interestingly, the increase in the levels of integrin $\alpha_5\beta_1$ is evident within 15 min and independent of proteasomal inhibitor MG132. While authors speculate that TGFβ prevents lysosomal degradation of integrin $\alpha_5\beta_1$, it is generally

known that $\alpha_5\beta_1$ is degraded over the course of several hours and is reported to have a half-life of 24 h (Lobert et al. 2010). Thus, the experimental evidence of increased integrin levels and downstream signalling in response to TGFβ in minutes could be due to a change in receptor trafficking that needs to be studied in further detail. Authors show that endoglin/integrin $\alpha_5\beta_1$ complex is essential for developmental angiogenesis in zebrafish. A mutant of endoglin that cannot interact with $\alpha_5\beta_1$ fails to rescue the phenotypes observed in morpholino-mediated knock down of endogenous endoglin. The cross-talk between the TGFβ/bone morphogenetic protein 9 signalling axis and the FN- integrin $\alpha_5\beta_1$ signalling, both potentiating each other through endoglin, determines the response of the endothelium to TGFβ (Tian et al. 2012).

Taken together, integrin receptors $\alpha_5\beta_1$ and $\alpha_v\beta_3$, which are known to have different trafficking routes in cells (Morgan et al. 2009), can dictate FN secretion and fibrillogenesis. The availability of these receptors at the surface is regulated by proteins governing their trafficking and this adds an additional layer of regulation to the fibrillogenesis process.

## 1.4 Fibronectin in Pathological Angiogenesis

### *1.4.1 Tumour Angiogenesis*

Targeting tumour angiogenesis as a means to prevent tumour growth was an idea initially put forth by Judah Folkman (Folkman 1971). One of the means of achieving it was through the inhibition of FN remodelling by tumour ECs. EDA and EDB containing FN is highly expressed around angiogenic vasculature, including tumour neovessels (Astrof et al. 2004). In fact, recent use of nanobodies against EDB domain of FN for immune-PET studies to image tumours, showed excellent specificity for tumours and metastasis, surpassing conventional 18F-2-fluorodeoxyglucose PET imaging (Jailkhani et al. 2019). Prophylactic vaccination against EDB domain of FN (Huijbers et al. 2010) and therapeutic vaccination against the EDA domain (Femel et al. 2014), were both shown to significantly reduce the tumour burden in mice. CAR-T cells redirected to the EDB-FN also show anti-tumour effects (Wagner et al. 2021). In all these studies, in addition to targeting tumour ECs, cancer cells secreting EDA/EDB splice variants of FN may also be directly targeted.

However, genetic models suggest that a near-complete deletion of FN or cognate $\alpha_5/\alpha_v$ receptors does not affect tumour growth, even in angiogenesis-dependent pancreatic adenocarcinomas, suggesting a very robust mechanism of driving tumour angiogenesis even in the absence of FN, either globally or specifically in ECs (Murphy et al. 2015). This study also reports that the deposition of other BM proteins is also not perturbed upon the inducible knockout of FN. Yet another study reports similar findings using EDA- or EDB-FN null mouse strains (Astrof et al. 2004). This is in keeping with other reports of normal physiology in mice with

FN KO 4 weeks postnatally (Yuan et al. 2020), underscoring the dispensability of FN beyond the embryonic and early postnatal development window. Therefore, the therapeutic utility of targeting FN splice variants in the context of cancer awaits results from robust clinical trials.

CD93 is a transmembrane receptor upregulated in tumour-associated vessels. The CD93–multimerin 2 (MMRN2) complex localises abluminally in the ECs supplying gliomas, in EC filopodia and at the sprout front in the developing retinal vasculature. Interestingly, in cultured cells, this CD93-MMRN2 complex was found to localise with FN fibrils tracing the same fibrillar pattern. MMRN2 is a soluble glycoprotein that multimerises in the ECM. MMRN2 is required for CD93 localisation along FN fibrils. MMRN2 prevents the proteolytic cleavage of CD93. Both proteins, when knocked down, reduce FN fibrillogenesis and it is proposed that the interaction happens along the cell membrane–matrix interface, where they enhance the interaction of fibrillar FN with $\alpha_5\beta_1$ integrins, thereby amplifying downstream signalling (Lugano et al. 2018). Since exogenous supply of conditioned media from control cells rescues fibrillogenesis in CD93-null cells, it is possible that some soluble matrisome component overcomes the deficit in FN remodelling. Although the paper elegantly shows the correlation of CD93-MMRN2 expression with tumour vessel fibrillogenesis (Lugano et al. 2018), an understanding of why the bundling of FN is affected by the loss of this complex or how MMRN2 recruits CD93 to FN fibrils remains unclear. The integrin signals, trafficking, and actin reorganisation that could help us understand how fibrillogenesis is regulated remain to be explored in this context.

An FN fragment, anastellin inhibits tumour angiogenesis (Yi and Ruoslahti 2001) and interestingly, this inhibitory effect is completely lost in mice with *pFN* KO (Yi et al. 2003). Anastellin is a 76 aa peptide fragment derived from the first type III repeat of FN. In vitro, it polymerises FN. The in vivo results suggest that the anti-angiogenic effects of anastellin depend on its ability to bind to/polymerise FN. We can speculate that the presentation of polymerised FN, or FN of altered conformation on the luminal side, skews endothelial polarity thus affecting angiogenesis potential in the matrigel plug assays used in vivo in this study (Yi et al. 2003). Since *pFN* KO per se is hardly expected to perturb tumour angiogenesis, as seen with global *FN* KO studies, anastellin represents a 'gain of function' perturbation of homeostasis. Since anastellin also triggers polymerisation of fibrinogen, it offers a viable therapy option against tumour angiogenesis. A similar mechanism of adhesion protein binding is also utilised by other angiostatic peptides, like endostatin (Yi et al. 2003).

### *1.4.2 Wound Healing Angiogenesis*

Wound healing is a well-orchestrated process involving inflammation, cell proliferation, migration, matrix deposition, and tissue remodelling. Plasma FN gets incorporated into fibrin clots and mediates haemostasis by regulating platelet function. ECs and fibroblasts invade the clot and synthesise cFN, which is remodelled to form

a scaffold that regulates cell function. The EDA/EDB containing cFN is highly upregulated around neovessels and capillary sprouts in the granulation tissue (Singh et al. 2004). Indeed, excess ECM deposition, of which FN constitutes a major component, leads to fibrosis (To and Midwood 2011). Dysregulated FN assembly could lead to excessive scarring of wounds.

The capillary sprouts that invade the wound granulation tissue at the early stages express the FN receptor $\alpha_v\beta_3$. This expression is transient, as once the wound is completely filled with granulation tissue and invasive angiogenesis is terminated, the microvasculature shows no expression of $\alpha_v\beta_3$ (Clark et al. 1996). A corresponding study in wound healing models for endothelial $\alpha_5\beta_1$ is presently lacking.

In line with previous adult *FN* KO studies, *pFN* KO mice show no difference in skin wound healing and haemostasis compared to WT controls. This could be due to the copious amounts of platelet-derived cFN at the wound site, or other redundant mechanisms to ensure clotting and wound angiogenesis. However, in contrast to skin wounding, brain injury induced by focal ischemia in pFN KO mice led to a 35% larger infarct area (Sakai et al. 2001). It is proposed that the difference possibly lies in two factors; ischaemic brain tissue is unable to synthesise cFN as a compensatory mechanism and ischaemia-induced damage to the blood–brain barrier causes a leak of only plasma and not blood cells, resulting in virtually no FNs in the infarct area in pFN null mice (Sakai et al. 2001).

Hepatic fibrosis is accompanied by excessive ECM deposition and pathological angiogenesis. Recent studies indicate that EDA-FN is positively correlated with the extent of pathological angiogenesis in mouse models and human hepatic tissues. EDA-FN knockdown in mouse livers resulted in reduced neovessel density and fibrosis, as well as reduced VEGFR2 phosphorylation. These results indicate that EDA-FN mediates pathological angiogenesis in hepatic fibrosis (Su et al. 2020). Thus, several studies indicate that FN fibrillogenesis potentiates wound angiogenesis and if excessive, can lead to scarring in certain tissues.

### *1.4.3 Atherosclerosis*

Atherosclerosis describes a pathological process by which a fatty streak builds up underneath the endothelium in arteries. The atheroma protrudes from the vascular wall and destabilises it over time as atherogenesis progresses, which eventually leads to thrombosis or even haemorrhage, due to the rupture of blood vessels in the atheroma. In general, atherosclerotic plaque comprises of lipids deposited by foam cells, increased ECM content deposited by dysfunctional cells, a fibrous cap that stabilises the structure, and the accumulation of SMCs caused by abnormal proliferation. The plaque is relatively stable at this point (Bentzon et al. 2014); however, as lipids continue to build up in the atheroma, it forms a necrotic core inside the plaque where ECM proteins, such as FN and collagens are lacking. Therefore, without the structural support from the ECM, the plaque becomes vulnerable and is prone to rupture (Thim et al. 2008). In addition, an increase in atheroma size leads to

hypoxemia. Hypoxia signals reach the vasa vasorum surrounding the arteries and induce angiogenesis from this network of vessels in the outermost layer of large arteries. The angiogenic sprouts then invade the arterial walls and tissues to reach the plaque centre. The process of angiogenesis in atherosclerosis further destabilises the plaque and it is believed to be one of the major causes of atherothrombosis (Camare et al. 2017).

Despite the complex structure of atherosclerotic plaques and the severe consequence of plaque rupture, at the early stages of atherosclerosis, there are almost no symptoms manifested. However, a huge alteration can be observed at the cellular level. The major cause of atherosclerosis is the uptake of oxidised low-density lipoprotein (oxLDL) by the endothelium and macrophages. In brief, as low-density lipoprotein (LDL), a cholesterol-carrying macromolecule, circulates in the vascular system, it is oxidised by free radicals and turned into oxLDL, which is taken up by macrophages and ECs. Macrophages become lipid-rich foam cells and keep depositing lipids underneath the endothelium, while ECs become dysfunctional and abnormal protein secretory activity is observed. One of the secretory abnormalities of ECs is reported to be polarised FN secretion. Under physiological conditions, ECs deposit FN towards the abluminal side of the cells alongside the BM, while in dysfunctional ECs, it has been reported that FN can be found at the luminal side of the ECs in both in vitro (Shih et al. 1999) and in vivo studies (Rohwedder et al. 2012). FN containing EIIIA region was found to colocalise with surface marker wheat germ agglutinin on the luminal surface of ECs after treatments with oxLDL (Shih et al. 1999). A layer of FN can also be found on the luminal side of the aortic endothelium in atherosclerotic mice (Rohwedder et al. 2012). It is suggested that surface FN-EIIIA induces monocyte adhesion through interaction with integrin $\alpha_5\beta_1$ (Park et al. 2021; Budatha et al. 2021).

In-depth studies in ECs and atherosclerotic mouse models have highlighted the role of FN in inflammation and fibrous cap formation. Alternative splicing of FN in different cell types creates diversity in domain arrangement and functions. FN variants produced from different cell types are suggested to contribute differently to atherosclerosis. For instance, in $ApoE^{-/-}$ mice, a common atherosclerotic mouse model, a specific knockout of EC-derived FN-EDA attenuated the progression of atherosclerotic plaque. A reduction in EC activation, less recruitment of neutrophils and macrophages, and smaller lesion size were observed, all of which suggests that EC-derived EDA-FN plays a key role in the progression of the early stages of atherosclerosis. Interestingly, atherosclerotic mice without SMC-derived FN-EDA showed no effect on plaque development. As atherosclerosis progressed to the late stages, fewer macrophages and collagen were found within the plaque in $FN\text{-}EDA^{SMC\text{-}KO}$ mice (Doddapattar et al. 2020). Therefore, it is suggested that EC-derived FN-EDA contributes to the early stages of atherogenesis (Tan et al. 2004), while SMC-derived FN-EDA functions in the late stages (Doddapattar et al. 2020).

Plasma FN circulating throughout the vascular system contributes to clotting and wound healing upon vessel injuries. Using ApoE-defective mice, with FN gene specifically knocked out in hepatocytes, it was discovered that lack of pFN

decreased the FN deposition in atherosclerosis-prone areas resulting in fewer and smaller plaques. Therefore, pFN increases the atherosclerotic lesion area by accumulating on the vascular wall and inducing inflammatory responses. On the other hand, pFN deficient mice failed to form a fibrous cap. Therefore pFN also helps stabilise the plaque by assisting the formation of a fibrous cap (Rohwedder et al. 2012). Since plaque rupture in humans is the most dreaded aspect of atherosclerosis, this study suggests that blocking FN function using inhibitors may not be the right path forward in atherosclerosis, even if that might reduce plaque area. Together, FN acts as a double-edged sword fuelling atherosclerotic plaque formation, while simultaneously stabilising the plaque through the formation of a fibrous cap.

## 1.5 Summary

FN remodelling is a crucial step in angiogenesis as summarised under both physiological and pathophysiological context in Fig. 1.3. Developmental angiogenesis is tightly regulated by FN. Basolateral fibrillogenesis of FN governs endothelial polarity and vascular morphogenesis. This chapter attempts to synthesise the various factors regulating fibrillogenesis, ranging from FN isoform expression to integrin expression and trafficking, and actin contractility. Several outstanding questions, including the potential use of FN polymerisation inhibitors in treating pathological angiogenesis and atherosclerosis, remain. The mechanisms governing the different secretory routes of FN isoforms based on the presence of EDA/EDB domains remain unanswered. The transcytosis pathways that regulate pFN incorporation basolaterally into fibrils are also not understood even though different tissues incorporate up to 60% of plasma FN into fibrils. The physiological inhibition of apical fibrillogenesis in ECs, which is disrupted in atherosclerosis, also remains to be understood.

Since adult conditional *FN* KO mice are healthy, the significant enrichment of FN remodelling at sites of angiogenesis offers hope that therapeutic targeting of FN fibrillogenesis is likely to be tolerated systemically and might be beneficial in diseases, like cancer and wet age-related macular degeneration (AMD). While EDB-FN-based imaging of tumours with PET is currently under clinical trial, newer ways to impact FN remodelling can emerge with a more thorough understanding of how FN fibrils are weaved by cells.

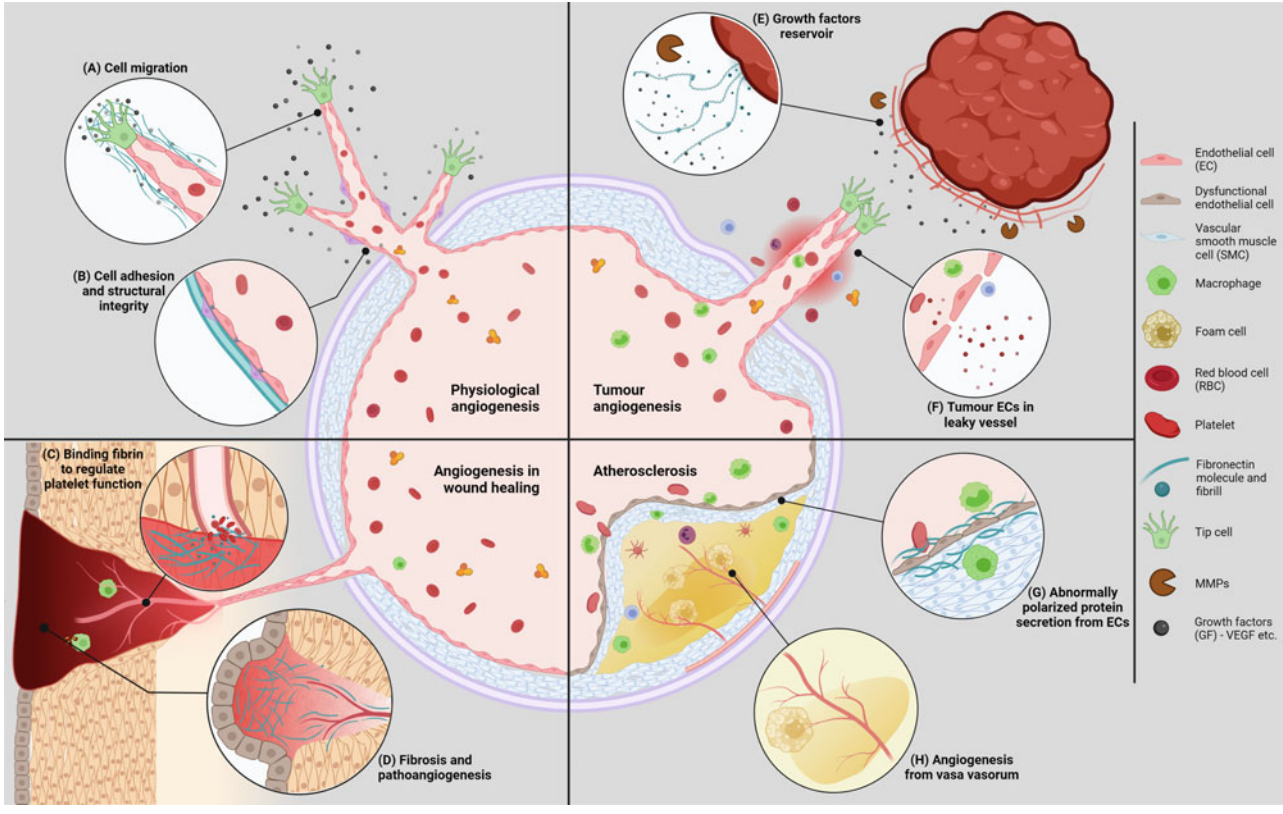


**Fig. 1.3** Angiogenesis in physiological and pathological conditions. (**a**) In physiological angiogenesis, FN is enriched at the sprout front. It functions as a scaffold that promotes cell migration and regulates growth factor availability. (**b**) Strong cell-matrix adhesion ensures vessel integrity. (**c**) Soluble pFN polymerises with fibrin at the injury site and promotes clot formation. (**d**) Excessive deposition of FN by angiogenic ECs leads to scarring. (**e**) MMPs secreted by

the tumour, degrade the ECM network, and release many growth factors, resulting in signalling abnormalities. (**f**) Irregular BM around tumour neovessels. Newly formed vessels are leaky and unstable due to weak junctions and cell-matrix adhesion. (**g**) Dysfunctional ECs produce FN on the apical surface to trigger inflammatory responses. pFN on the luminal side promotes the formation of the fibrous cap. (**h**) Hypoxia signals angiogenesis from the vasa vasorum. The microvessel invasion puts the atherosclerotic plaque at risk of rupture and thrombosis. Figure created with Biorender.com.in

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# Chapter 2
# Functional Interplay Between Fibronectin and Matricellular Proteins in the Control of Endothelial Tubulogenesis


**Verônica Morandi, Laila R. Fernandes, and Aline O. Silva de Barros**

**Abstract** Fibronectin (FN) has stood out as the main component of the so-called provisional matrix, essential to trigger the angiogenic process. FN orchestrates several pathways in endothelial tubulogenesis, through interactions with multiple extracellular matrix molecules, growth factors, and cell receptors, but especially with vascular endothelial growth factor A, integrins, and matricellular proteins. A few iconic matricellular proteins that have been now studied for decades were clearly involved in sprouting angiogenesis and are implicated in the modulation of many of the FN activities during vessel morphogenesis. The original group of matricellular proteins included the families of thrombospondins, tenascins, and SPARC, all involved in the regulation of both normal and pathological angiogenesis. In this review, we discuss what appears to be evidence for the evolution of important cooperation between FN and few iconic matricellular proteins, in the context of endothelial and vascular morphogenesis, in health and disease contexts.

## 2.1 An Evolutive Glimpse: The Origins of Endothelium, Fibronectin, and Matricellular Glycoproteins

According to studies of gene organization, toolbox analysis of protein domains, comparative genomic sequencing, and molecular clock estimates, the advent of the *endothelium* in vertebrates (~540–510 million of years ago, or Mya) is part of a narrow window of time in metazoan evolution (~550–450 Mya), when the emergence of a closed circulatory system, a fibrin-dependent coagulation system, and

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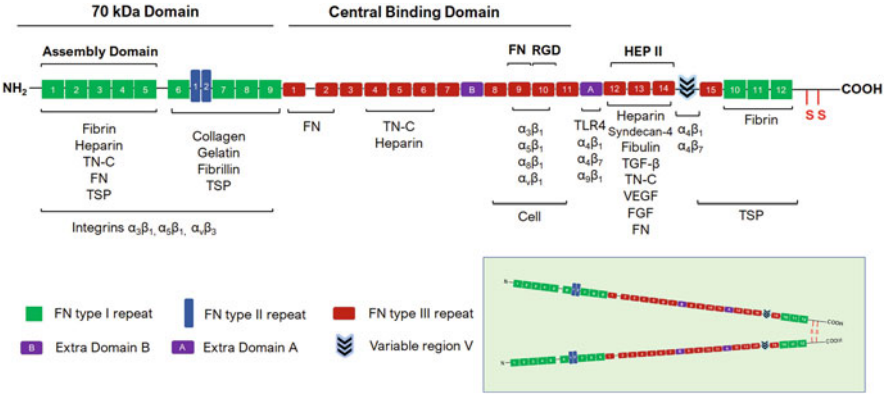


**Fig. 2.1** Schematic representation of the modular structure of human FN monomer and most of its known ligands. FN orchestrates several pathways in endothelial tubulogenesis, through interactions with multiple ECM molecules and growth factors. While the three types of repeated motifs are present in both cellular and plasma FN, extra domains A and B (EDA and EDB), together with motifs of the variable region, can be alternatively spliced, thus generating multiple FN cellular isoforms. Plasma FN does not contain EDA and EDB motifs. The colored box (at the right bottom) represents the dimeric structure of FN. Flexible FN dimers can copolymerize in the process of FN fibrillogenesis, which is crucial for vascular morphogenesis. Details about the functions of the different structural domains of FN can be found in the text. (Adapted from Singh et al. 2010; Van Obberghen-Schilling et al. 2011; Mezzenga and Mitsi 2019)

adaptive immunity were also in course (Monahan-Earley et al. 2013; Anitua et al. 2020). Invertebrates bearing open circulation had already developed short proteolytic-dependent cascades aiming to generate clotting activity for closing wounds—to prevent potentially harmful fluid leakage—and eventually entrapping and killing invader pathogens (Theopold et al. 2004). These early "glue-like" adhesive systems focused on repairing and defending these simpler organisms, did not rely on the thrombin/fibrinogen axis that evolved later in vertebrates (Cerenius and Söderhäll 2011).

The selection of a variated set of molecules able to exhibit cell adhesive properties was also key for the emergence of multicellularity (Rokas 2008; Brunet and King 2017). Among these, the *extracellular matrix (ECM)* molecules played a pivotal role, by contributing to the rising of polarized cell barriers and by controlling other activities known to be essential for the homeostasis of differentiated cell phenotypes, such as the sorting and maintenance of specialized cells in functional units, cell migration, proliferation, and viability (Hynes 2012). Since most ECM proteins were generated by extensive exon/domain shuffling and gene duplication events during their evolution, many module-based ECM proteins sharing either structural and/or functional similarities emerged across the Metazoan kingdom (Hynes and Naba 2012).

*Fibronectin (FN)* genes code large, highly adhesive, and multifunctional glycoprotein dimers of 230- to 250-kDa subunits, connected by a pair of disulfide bonds at their C-termini, generally as a single copy in most of the vertebrate classes (Fig. 2.1). The "true" fibronectins (as opposed to some proteins containing FN-like modules,

found in early-diverging chordates), bearing different FN-type repeated modules, namely FN-I, FN-II and FN-III domains, and portions of the variable or alternatively spliced (V, EDB, EDA and IIICS) domains, have a strongly conserved structure in the entire vertebrate subphylum, including the consensus RGD integrin-binding motif. Phylogenetic estimates vertebrate FNs as one of the most recent ECM proteins, dating to the appearance of jawless fish (~530 Mya) (Hynes 2012; Orend and Tucker 2021), an evolutionary period that closely coincides with the advent of the endothelium and tissue factor-, thrombin-, and fibrinogen-dependent coagulation systems, as mentioned above.

By the time FN genes emerged in vertebrates, a few ECM toolkits existed already since early multicellular metazoans, including structural molecules, such as collagen domains/repeats (~900 Mya), fibrillar collagens (~750 Mya), fibrillins (~580 Mya), and some prototypical basement membrane (BM) components, spread from the Porifera phylum to the Cnidarians (~750–580 Mya) (Hynes 2012). Vertebrate FNs were also preceded by the evolution of some members of the wide and heterogeneous group of *matricellular proteins*, from as early as the Porifera phylum emergence (Mosher and Adams 2012). The term "matricellular," proposed by Paul Bornstein in the mid-1990s, referred to ECM proteins that, rather than constituting structural elements *per se*, do modulate the adhesive activity or organization dynamics of other structural matrix molecules in BMs and connective tissues. Most matricellular proteins exhibit counteradhesive properties, in general toward FN, and modulate the activity of both soluble and matrix-bound growth factors (Bornstein 1995; Mosher and Adams 2012). Some matricellular families are among the oldest proteins in the Metazoan kingdom, dating from invertebrate rising. For example, while thrombospondins (TSPs) and SPARC families were found in Porifera phylum (~760 Mya), fibulins and CCN protein families were proposed as emerging in the interval between the divergence of the sponges and the Cnidaria–Bilateria split (Segade 2010). The tenascin (TN) family emerged more recently, early in the phylum of chordates (urochordates and cephalochordates, ~675 Mya) (Mosher and Adams 2012).

In this review, our focus will be, on the one hand, to discuss the roles played by FN as the main orchestrator of endothelial morphogenesis in the ECM and, on the other hand, to show what appears to be evidence for the evolution of important cooperation between FN and some iconic matricellular proteins, in the context of endothelial and vascular morphogenesis, in health and disease situations.

## 2.2 Steps in Endothelial Tubulogenesis: Major Signaling Pathways

Endothelial tubulogenesis is part of the mechanism of *sprouting angiogenesis*, in which new vascular branches may arise from pre-existing vessels. The heterogeneity of endothelial phenotypes along the same nascent branch has become evident and

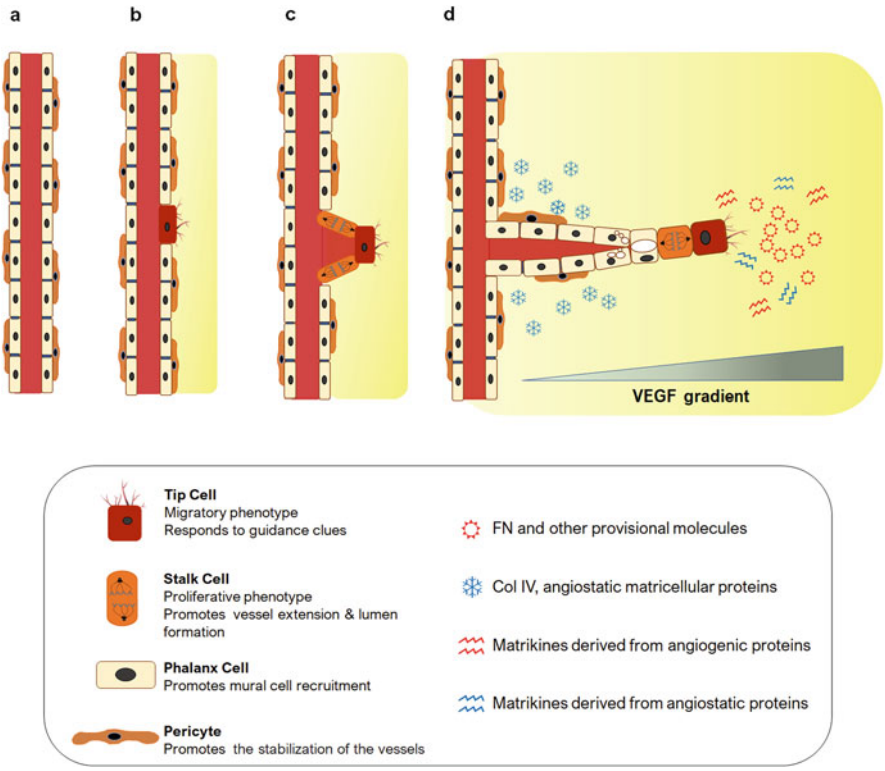


**Fig. 2.2** Sprouting angiogenesis. (**a**) In adult organisms, vessels remain quiescent for long periods, usually measured in years; (**b**) and (**c**) In some pathophysiological situations, endothelial cells can be activated and break the quiescence, starting the sprouting process (see details in the text); different endothelial subpopulations share tasks, while building new functional structures bearing the lumen, as indicated in the figure; (**d**) the angiogenic factor VEGF is the main driver for the differentiation of tip cells, which take the lead and give direction to the whole process. The provisional ECM (e.g., FN) also contributes to potentiate angiogenic responses to growth factors, including endothelial proliferation and migration, in addition to the maintenance of endothelial viability. Matricellular proteins modulate endothelial cell responses to the provisional matrix, including stabilization of newly formed structures, by recruiting pericytes and stimulating the reorganization of an angiostatic BM. Especially in the early phase of sprouting angiogenesis, all ECM molecules involved in the process can be cleaved by proteases relevant to the angiogenic process, generating bioactive fragments that may differ from the roles played by the corresponding intact proteins (More detailed information can be found in Eelen et al. 2020; Okuda and Hogan 2020; Ricard-Blum and Vallet 2016)

better characterized for nearly two decades now (Gerhardt et al. 2003), but many aspects of the role of each endothelial phenotype remain to be fully clarified. Briefly, the process can be summarized in the following main steps (Fig. 2.2) (Eelen et al. 2020):

(a) One or more endothelial cells are activated by molecular cues from the micro-environment (soluble ones or associated with ECM), assuming the role of

leaders or *tip cells*, and giving directionality to the whole morphogenic process. Tip cells do not proliferate, but rather track extracellular signals with their filopodia-rich front, by exhibiting a highly motile behavior.

(b) *Stalk cells* follow the tip cells and are implicated in lumen formation, matrix biosynthesis (or remodeling), and cell proliferation.

(c) A third endothelial phenotype, the *phalanx cells*, mostly remain distant from the main stage of morphogenic action, in a state of quiescence.

(d) New formed branches are then stabilized by the reconstitution of intact BMs and the association of recruited pericytes.

The generation of endothelial tip cells is largely controlled by vascular endothelial growth factor (VEGF) chemotactic gradients. VEGFs can be released from the provisional matrix by local proteases or secreted by activated inflammatory cells, fibroblasts, and platelets that invade the fibrin-rich edema. This culminates in the activation, in tip cells, of the VEGFR2/delta-like canonical Notch ligand 4 (Dll4)-Notch1 axis, which in turn dampens the ability of neighboring endothelial cells to respond to the angiogenic factor, by NOTCH1-mediated downregulation of VEGFR2/3 and upregulation of VEGFR1. This topic has been extensively reviewed (Blanco and Gerhardt 2013; Mack and Iruela-Arispe 2018). Conversely, as soon as VEGF becomes scarce and the provisional matrix is gradually substituted by an organized vascular BM, an intensive pericyte recruitment signaling program is mobilized to ensure vessel stabilization (Davis et al. 2015). Evidence points to the impact of endothelial plasticity in the assumption of these different phenotypic/functional roles, through a permanent competition for the position of endothelial leadership (Jakobsson et al. 2010). The importance of a differential regulation of energy metabolism (or "metabolic signatures") among the three endothelial phenotypes has also been highlighted in recent publications (reviewed in Dumas et al. 2020).

In quiescent vessels, BM molecules—type IV collagen, laminins, perlecan, etc.—set the conditions for vessel stability, while the edema associated with injured vessels ultimately primes some endothelial cells to become more responsive to angiogenic growth factors and to differentiate into motile tip cells. FN is an essential component of the so-called angiogenic "provisional matrix," together with fibrinogen/fibrin, fibrillar collagens, vitronectin, and different proteoglycans (Barker and Engler 2017). In adult organisms, this pro-coagulant/inflammatory microenvironment is essential for building efficient tissue repair responses, including the promotion of angiogenesis (Dvorak 2015; Barker and Engler 2017). Microenvironmental cues are also represented by matricellular proteins, whose expression is usually increased at tissue remodeling sites (Murphy-Ullrich and Sage 2014), and by sequestered angiogenic growth factors. Interaction of matricellular proteins with specific target receptors is largely controlled by their molecular association with ECM molecules, especially FN (Zhu and Clark 2014). In both endothelial quiescence and endothelial sprouting, several members of the integrin family of ECM receptors have been implicated in sensing and transducing environmental cues (Avraamides et al. 2008).

An insightful nomenclature was previously proposed to describe the opposing triggering ("fire") and suppressive ("ice") scenarios governing sprouting angiogenesis (Davis and Senger 2005). The "fire" signaling program initiated by FN/fibrin/fibrinogen/vitronectin or interstitial collagen fibers activates endothelial tip cells, mainly through a few $\beta_1$-integrin receptors, provokes actin cytoskeleton rearrangements involving the activation of Src, focal adhesion kinase (FAK), RhoA → Rho kinase (or ROCK), p38MAK, which leads to the induction of actin stress fibers, and disruption of VE-cadherin- and JAM-mediated cellular junctions (Szymborska and Gerhardt 2018). Additionally, the small GTPase Cdc42 is known to induce the extension of the angiogenic front and filopodia formation in angiogenic tip cells and its positive regulation by the Hippo-YAP/TAZ pathway during tubulogenesis has been recently demonstrated (Sakabe et al. 2017). Importantly, $\alpha_v\beta_3$ integrin plays a pivotal role in sprouting angiogenesis, mainly by keeping endothelial cells viable along the differentiation process (Brooks et al. 1994a, b). The direct interaction between this integrin and VEGFR2 has been shown to increase VEGFR2 phosphorylation, signaling to PI3K and cell migration, when endothelial cells adhere to provisional substrates, such as FN (Soldi et al. 1999).

On the other hand, as part of the "cold" signaling program, in stabilized portions of new branches bearing organized laminin-rich BMs, cAMP-dependent protein kinase A (PKA) acts to inhibit the activity of RhoA/Rho kinase signaling axis (Davis and Senger 2005). Upstream suppression of the RhoA/ROCK pathway is also exerted by a few members of the Pak protein kinase family in cooperation with the scaffold polarity protein Par-3, which probably plays a key role in stabilizing interendothelial junctional contacts (Davis et al. 2015; Mack and Georgiou 2014). In addition, the combined activation of Cdc42/RhoJ/Rac1 also helps to suppress RhoA/ROCK activity in the endothelial cells implicated in lumen formation—a role mostly attributed to the stalk cell subpopulation (Tung et al. 2012). The proteolytic activity of the surface matrix metalloproteinase (MMP), membrane type 1 MMP (MT1-MMP), was shown to be critical to the vacuolization-dependent lumen extension (Sacharidou et al. 2010). In fact, MT1-MMP expression is upregulated in endothelial cells adhering on FN or inside a tridimensional collagen type I-rich microenvironment (Yan et al. 2000; Chun et al. 2004).

## 2.3 FN Roles in the Vascular Compartment and Sprouting Angiogenesis

Mammalian vasculogenesis occurs in an FN-rich environment (Risau and Lemmon 1988). FN-null mouse embryos show lethality at embryonic day E9.5, due to severe vascular defects and other malformations (George et al. 1993). Genetic models in which integrin chains were knocked out, showed phenotypes like those observed in FN-null mice, suggesting that $\alpha_5$ and $\alpha_v$ chains seemed to functionally compensate for the lack of each other, while $\beta_1$ chain was critical for development (Astrof and

Hynes 2009). The high lethality observed in animals derived from global genetic knockout strategies has led to the development of new cell-targeted knockout methods, to trace their outcomes beyond the embryonic stages. Using endothelial-targeted knockout mice for either FN or selected integrin chains known to recognize FN, allowed the confirmation of the essential role of endothelial FN in neonatal vessel morphogenesis. Moreover, when the expression of endothelial $\alpha_5$ and $\alpha_v$ integrin chains are both ablated in combination (but not either alone), failure in remodeling great vessels and the heart caused death at ~E14.5 (van der Flier et al. 2010; Turner et al. 2017).

The biological properties of FN are mediated by a plethora of binding motifs, functional domains, and cell receptors (Fig. 2.1). FN is found in two forms: a plasma FN (pFN), which circulates at high concentrations in blood, constitutively produced solely by hepatocytes; and a set of variants of the so-called cellular isoform (cFN) (Astrof and Hynes 2009). Different isoforms are generated by alternative splicing of the unique vertebrate gene (White et al. 2008). The pFN and cFN isoforms have sites for recognition by integrins (including the RGD consensus motif) and heparin/heparan sulfate proteoglycans, in addition to sites for self-association, and heterotypical interactions with several ligands (see below). cFN is expressed during development and in adult organisms and is largely related to vessel remodeling and angiogenesis. The alternatively spliced EDA and EDB of cFN modules are upregulated around neovessels and capillary sprouts (Cseh et al. 2010; To and Midwood 2011; Hielscher et al. 2016).

Interestingly, pFN, which also exhibits proangiogenic properties (Nicosia et al. 1993) but lacks EDA and EDB modules in its structure, was able to rescue cFN functions in mice bearing early neonatal mural aortic defects, due to the targeted deletion of cFN expression in smooth muscle cells (Kumra et al. 2018). This observation is consistent with the fact that cFN of endothelial origin has not been shown to be essential for the vascularization of tumors in vivo (Murphy et al. 2015), suggesting that FN from other sources (including plasma) could compensate for the lack of endothelial cFN. These fascinating data suggest that circulating pFN may probably subserve a strategic safeguard role in vessel integrity and health, especially in the early phases of tissue repair (To and Midwood 2011).

Mature, stabilized, and insoluble FN fibers, generated through a cell-mediated mechanism (extensively reviewed in Singh et al. 2010) were shown to be essential for the angiogenic properties of FN (Zhou et al. 2008; Chang et al. 2014; Hielscher et al. 2016). Fibers are formed after FN dimerization, through their self-association N-terminal assembly domain. In vitro, it was demonstrated that endothelial cells first engage into tubulogenesis by unfolding soluble FN and depositing a pericellular network of fibrils that serve to support cytoskeletal organization and actomyosin-dependent tension (Zhou et al. 2008). Although FN is recognized by several integrins—namely, $\alpha_4\beta_1$, $\alpha_5\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_5$, and $\alpha_9\beta_1$—the key receptor in FN fibrillogenesis seems to be $\alpha_5\beta_1$, via the RGD adhesion motif and its synergy site (Astrof and Hynes 2009). FN adhesion motifs, placed in proximity by fibrils assembly, cause integrins to form clusters, which participate either in fibrillar or focal adhesions: although both types of adhesion complexes induce signaling

pathways leading to cell contractility and the formation of stress fibers, differences apply depending on their size, positioning along the cell body and the nature of the intracellular scaffold/signaling proteins recruited (Singh et al. 2010). Conversely, disruption of the actin cytoskeleton inhibits fibronectin matrix assembly (Wu et al. 1995), showing that cellular components of adhesion complexes and the polymerized FN fibrils/fibers form a dynamic mechano-transduction unit that operates both outside-in and inside-out, which is constantly submitted to conformational stress, linked to cell-mediated contractility (Friedland et al. 2009). Interestingly, exogenous pFN can be incorporated into pre-existing or newly assembled cFN matrices (Peters et al. 1990).

The organized fibrillar FN matrix can provide structural scaffolding for ligands such as fibrinogen/fibrin, heparin/heparan sulfates, collagens, TNC, TSP1, and several heparin-binding growth factors—such as VEGFs and fibroblast growth factors (FGFs)—and the latent form of the pro-fibrotic transforming growth factor beta1 (TGFβ1) (Sawicka et al. 2015; Zollinger and Smith 2017; Efthymiou et al. 2020). Thus, the circumstantial fluctuation of these ligands in the ECM environment might change the conformation of FN fibrils and therefore, modulate the proangiogenic role of the FN-rich provisional matrices. Accordingly, the flexibility of FN fibers was suggested by the occurrence of cryptic sites on the dimer structure, which can be selectively exposed for cell interaction, depending on the stretching forces generated either by the interaction of FN with other molecules or by ECM proteolysis (Mezzenga and Mitsi 2019).

$VEGFA_{165}$, a variant of the VEGFA isoform with a 165 aa in length can be found associated in large amounts to the ECM (Ferrara 2010). A heparin-binding motif in $VEGFA_{165}$ mediates most of its interactions with the ECM (Park et al. 1993). Matrix-bound VEGFA can elicit strong signaling from its tyrosine kinase VEGFR2 receptor (Chen et al. 2010). The interaction of VEGFA to the high-affinity heparin-binding Hep II domain of FN results in increased VEGFR2-dependent signaling, which then promotes cell proliferation, migration, and ERK activation (Wijelath et al. 2006). In the retinal angiogenesis model, astrocyte-derived FN has been shown to enhance tip endothelial cell migration in a VEGFA-dependent manner (Stenzel et al. 2011). The decreasing gradient of FN from the tip of the neovessel branch toward its rear seems to guarantee adequate concentrations of VEGFA at each stretch of nascent branch, so that VEGFA still provides the survival and proliferative cues to the other endothelial phenotypes (e.g., stalk and phalanx cells), but stimulates a migratory and directional behavior only in the tip cell subpopulation (Stenzel et al. 2011).

Although the RGD-dependent cell binding motif of FN recognized by integrins is considered the primary cell adhesion site on this ECM molecule, it has been shown that the engagement of other domains is necessary for full matrix assembly and focal adhesion organization, leading to cytoskeleton formation. *Syndecan* and *neuropilin* families of adhesion co-receptors were shown to play important roles in the angiogenic process in cooperation with FN-binding integrins, especially with $\alpha_5\beta_1$ integrin. These single-pass transmembrane proteins bearing GAG modifications have been strongly implicated in angiogenesis, through VEGFA, FGF2, and

integrin-dependent pathways (De Rossi and Whiteford 2014; Goel and Mercurio 2012; Ellison et al. 2015; De Rossi et al. 2021).

Syndecan-4 (SND4) is the only of the four syndecan isoforms consistently found in association with focal adhesions (Woods 2001). SND4 is the most ubiquitous member of the family and the major cell surface heparan sulfate proteoglycan (HSPG) expressed by endothelial cells and, like VEGFA, also binds to the Hep II domain of FN (Woods et al. 2000). Once cooperatively engaged with the activated $\alpha_5\beta_1$ integrin cluster, SND4 promotes focal adhesion maturation and potentiates F-actin contractility, because of the activation of RhoG and Rac1 proteins through a PKCα-dependent pathway (Elfenbein et al. 2009). Recruitment of vinculin to F-actin bundles was recently shown to require SND4 expression by endothelial cells (Cavalheiro et al. 2017).

Neuropilins, originally identified as receptors for class-3 semaphorins involved in neuronal guidance, were subsequently found to bind several isoforms of VEGF and to form complexes with VEGFR1 and VEGFR2 (Lampropoulou and Ruhrberg 2014). In endothelial cells, besides its role as a VEGF co-receptor that enhances the binding of $VEGFA_{165}$ to VEGFR2 and $VEGFA_{165}$-mediated chemotaxis (Wang et al. 2003a, b; Lampropoulou and Ruhrberg 2014), neuropilin-1 (NRP1) binds to FN and $\alpha_5\beta_1$, enabling the activation of this integrin. Probably due to $\alpha_5\beta_1$ activation, NRP1 also promotes FN fibrillogenesis (Valdembri et al. 2009) and focal adhesion turnover through direct interaction with filamin A (Seerapu et al. 2013).

## 2.4 Modulation of the Angiogenic Properties of FN by Matricellular Proteins

Matricellular proteins are characterized by their transient expression all along development, tissue injury repair, inflammatory processes, and neoplastic progression (Chiodoni et al. 2010). Families of proteins capable of modulating cell-matrix adhesion have been increasingly tagged as matricellular proteins (Murphy-Ullrich and Sage 2014), although not all among them have been implicated in the morphogenic process that leads to the formation of endothelial tubular, lumen-bearing structures. A few iconic matricellular proteins that have been studied for decades have been clearly implicated in sprouting angiogenesis, and in the modulation of FN activity, as we intend to demonstrate in the following sections. The original group of matricellular proteins included the families of TSPs, TNs, and SPARC, all involved in the regulation of both normal and pathological angiogenesis (Sage and Bornstein 1991).

Firstly, we will focus on TSP1 and TNC, as representatives of two matricellular families exhibiting high molecular weights, whose biological properties have been unequivocally connected to the proangiogenic role of FN in provisional matrices. Then, we will also comment on the role played by CCN1 and SPARC, two glycoproteins that, despite their low molecular weights and much simpler modular

structures, have been recently implicated in crucial aspects of the sprouting angiogenesis process induced by FN-rich provisional matrices.

### 2.4.1 Tenascin-C

TNC is an ECM glycoprotein formed by six monomers (~240 kDa) that are joined at the N-terminal region by disulfide bonds, forming one of the largest known ECM components (>1000 kDa) (Chiquet 2020) (Fig. 2.3). The basic domain structure of each monomer has a TN assembly domain, epidermal growth factor-like (EGF- L) repeats, 8 constant FN type III repeats and 9 FN type III repeats that result from alternative splicing, in addition to a fibrinogen-like C-terminal globular region. Each domain of TNC interacts with other matrix molecules and cell receptors including $\alpha_9\beta_1$, $\alpha_v\beta_3$, and $\alpha_v\beta_6$ integrins, Toll-like receptor 4 (TLR-4) and SND4. These interactions have a wide range of effects on cell adhesion, motility, differentiation, growth control, and ECM production (Midwood et al. 2016). In most adult tissues, despite its extremely low expression, TNC is found in connective tissues, tendons, muscles, under epithelia, in reticular fibers, and in some stem cell niches (Chiquet-Ehrismann et al. 2014; Midwood et al. 2011).

TNC is found in the embryonic ECM participating in the development of the vasculature of various tissues and organs (Tucker 1993), around mobile cells, in sites of epithelial-mesenchyme interactions and branching morphogenesis, and in dense connective tissues, such as those found in bone, cartilage and tendons, as well as in the central nervous system (Akbareian et al. 2013; Sahlberg et al. 2001; Wiese and Faissner 2015; Midwood et al. 2016). For example, in corneal development, neural

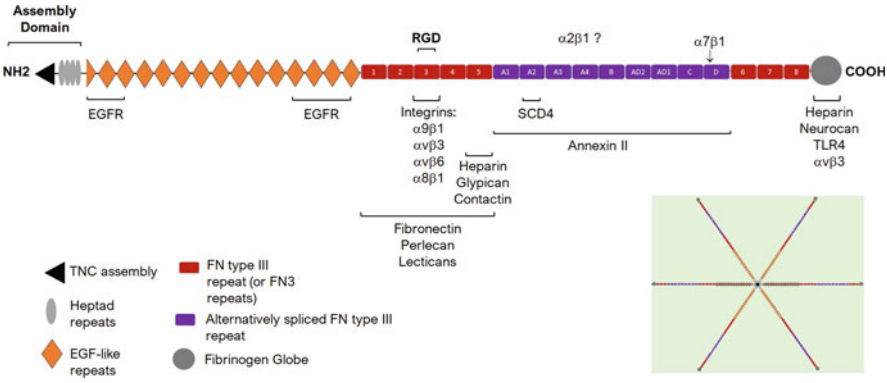


**Fig. 2.3** Domain structure of human TNC. On the top of the figure, the modular organization of a single chain of the molecule, with an emphasis on most of its ligands and identified receptors. Multiple splicing variants can potentially be generated that differ in the FN type III-like repeats, and cleavage of the protein by proteases can generate otherwise cryptic FN-binding motifs. The hexameric structure of TNC is shown on the bottom right. (Adapted from Van Obberghen-Schilling et al. 2011; Midwood et al. 2016)

crest-derived cells destined to become endothelium, migrate strictly along a TNC-rich stroma, suggesting that TNC functions as rails that guide these cells (Kaplony et al. 1991).

Indeed, FN and TNC seem to cooperate during development in the process of branching morphogenesis, not only for the formation of vessels but also for the generation of branched epithelial patterns, such as those found in the lungs and kidneys (To and Midwood 2010). Lung explants from TNC knockout mouse embryos exhibit reduced branching (defective cleft formation and increased terminal lung buttons) and decreased vascularity (Roth-Kleiner et al. 2004). Studies indicate that pulmonary branching morphogenesis and vascularization are dependent on a Wnt/cFN-dependent axis (Van Obberghen-Schilling et al. 2011). Wnt signaling is activated between E10.5 and E12.5 in the developing lung. Later (E13.5), Wnt signaling is greatly reduced by Dickkopf (DKK) 1-3, and this coincides with the induction of the Wnt target gene, FN. Since TNC blocks FN signaling, represses DKK1, and plays a role in pulmonary branching morphogenesis, it was suggested that a tight balance between FN and TNC regulates normal pulmonary branching and vascularization (Warburton et al. 2005). In this scenario, DKK1 repression by TNC would result in Wnt activation and cFN induction, suggesting a division of tasks between FN and TNC proteins for the establishment of a functional vascular network.

TNC was the first matricellular protein shown to compete with FN for binding to SND4. The specific interference of TNC with the SND4-binding site on FN HepII domain inhibited adhesion and proliferation of tumor cell lines (Huang et al. 2001) and led to cell growth arrest in normal fibroblasts (Orend et al. 2003). In both cases, overexpression of SND4 reverted the cell adhesion defect elicited by TNC. Since the inhibition of SND4 by TNC prevented cell spreading, actin stress fiber formation, and activation of FAK and RhoA-mediated signaling pathways (Midwood et al. 2004), the mode of action of the TNC seems to be rooted mainly in the direct antagonism of the adhesive activity of FN.

Using mouse fibroblast cultures and a model for studying epithelial branching morphogenesis (e.g., based on MDCK cell line), To and Midwood (2011) demonstrated that TNC domains exert multistage control in the assembly of the FN matrix. They observed that the TNfn1-8 domains block fibril formation, which may ensure that an adequate amount of fibrillar FN is deposited at the correct time and place, while the reduction of FN in "mature" matrices mediated by the fibrinogen-like globular domain of TNC would act to neutralize any excess activity of FN. It was suggested that, during development and tissue repair, this regulation may be important in orchestrating vascular morphogenesis as well, controlling where tubules arise, how branches are initiated, and how they extend in the right direction.

We have studied the role of glioblastoma (GBM)-derived ECM in endothelial angiogenic behavior (Alves et al. 2011), using primary human umbilical vein endothelial cells. Despite the initial adhesion provided by GBM matrices (up to 2 h), when even vinculin-containing focal adhesions were organized by the adhering cells, a gradual detachment process was triggered and proceeded for over ~18 h. We went further to observe that TN-rich GBM matrices—which are almost devoid of

cFN, positively selected a subpopulation of proliferative, anoikis-resistant endothelial cells (20–50% of the total seeded population). Moreover, surviving cells failed to undergo tubulogenesis in vitro. Adhesion substrata containing TNC:FN ratios aimed to mimic the ratios found in native GBM matrices, recapitulated the tubulogenesis defect, which was partially reversed by neutralizing antibodies raised against the EGF-like domain of TNC. Nevertheless, these antibodies were not able to prevent endothelial *anoikis*, thus confirming that anoikis-resistant and anoikis-sensitive endothelial cells constituted diverse endothelial subpopulations. Although the mechanisms involved in the tubulogenic defect have not been studied, it might not be interpreted as anti-angiogenic role of TNC, since it was shown to be largely reversible, and the anoikis-resistant cells exhibited a proliferative behavior.

Another evidence in favor of the importance of the TNC: FN correlation was the fact that the matrix secreted by primary human dermal fibroblasts, which contained high levels of the two proteins, did not generate any tubulogenic defect in endothelial cells (Alves et al. 2011). This result was surprising to some extent, as TNC content in the fibroblast matrix was ~40-fold more abundant than in GBM-derived matrices, for the same relative amount of FN. Overall, this set of experiments clearly demonstrated the importance of TNC/FN interplay for the fate of tubulogenic differentiation and we suggested that heterogeneity of endothelial cells bearing variable adhesion capabilities toward the TNC-rich/FN-poor GBM matrices may contribute to the abnormal, chaotic vasculature prevalent in GBM. Altered tumor matrices may thus act in cooperation with excessive VEGFA signaling, which was classically proposed by R. Jain (2005) as the main driver for the aberrant vessels often observed in malignant glioma, in support of the ever-evolving concept of vessel "normalization" and its therapeutic applications (Martin et al. 2019).

More recently, the biological relevance of such regulatory pathways was also demonstrated, in a neuroendocrine tumoral context in vivo, in which TNC downregulated Dickkopf WNT Signaling Pathway Inhibitor 1 (DKK1) promoter activity through the blocking of actin stress fiber formation and the activation of Wnt signaling, whereas adhesion to FN-rich/TNC-deprived substrata reversed DKK1 ablation, both in tumor and endothelial cells in vitro (Saupe et al. 2013). Moreover, DKK1 downregulation by TNC could be reversed through enforcement of actin polymerization and stress fiber formation by overexpression of syndesmos, a scaffolding protein that interconnects SND4 and $\alpha_5\beta_1$ integrin, in focal adhesions (Bass and Humphries 2002). Interestingly, DKK1 expression was directly dependent on actin stress fibers that were disrupted by TNC and induced by FN, suggesting a mechano-transducing pathway for the control of gene expression. Therefore, this is further evidence that the excessive predominance of TNC over FN in the tumor microenvironment can have as a major outcome a defective, hyperpermeable vasculature, which is a basic feature of various solid malignant tumors (Martin et al. 2019).

Using various experimental in vitro and in vivo approaches, a recent study has shown that TNC can exert both pro- and anti-angiogenic roles in a GBM-related context (Rupp et al. 2016). The direct contact of endothelial cells with TNC led to a decrease in actin polymerization, resulting in cytoplasmic retention of the

transcriptional coactivator YAP and impairment of cell migration, proliferation, and tubulogenesis. On the other hand, GBM cells primed by TNC substrata started to express a proangiogenic secretome that rescued endothelial cells from the effects of TN-rich matrices. The authors identified Ephrin-B2 as a major proangiogenic effector in the secretomes of various GBM cell lines.

Interestingly, in another work by the same group, immobilized TNC was used as a support on top of which endothelial cells were seeded and cultured to organize a new secreted matrix. Cell-free matrices were then prepared and used to challenge naïve endothelial cells (Radwanska et al. 2017). Endothelial cells growing on this "TNC-primed," mixed-native matrix became more tubulogenic. Despite the significant technical differences that were observed between this work and the study by Alves and co-workers (2011), both investigations reinforce the idea of a cooperation between FN and TNC in TNC-rich microenvironments, in which an appropriate FN: TNC balance would favor endothelial tubulogenesis (Radwanska et al. 2017).

### *2.4.2 Thrombospondin-1*

TSPs form a conserved family of five extracellular, oligomeric, multidomain, calcium-binding glycoproteins (Adams and Lawler 2011), which are synthesized, secreted, and incorporated into the ECM of normal and transformed cells of both mesenchymal and epithelial origin (Adams and Lawler 2011). TSP1 was the first member to be discovered and isolated from human α-platelet granules, since the exposure of platelets to thrombin determines the rapid release of a thrombin-sensitive protein (Baenziger et al. 1972; Lawler et al. 1978). Unlike FN, TSP1 expression in the adult organism is minimal (except for the reservoir found in platelet α-granules). However, usually in situations of injury, neoplasia, and other chronic diseases, there is an increase in its expression (Agah et al. 2002; Hohenstein et al. 2008; Rogers et al. 2012).

The TSP1 gene and protein had their primary structures solved by Lawler and Hynes in 1986, in what was a key step that significantly accelerated our knowledge about this complex matricellular protein, allowing the rapid characterization of functional domains, cell-binding sites and structural motifs for interacting with other molecules (Fig. 2.4). TSP1 is a 450 kDa homotrimer, formed by 150 kDa chains, containing the following structural domains: (1) an N-terminal globular domain, which contains basic consensus motifs for binding to heparin (Heparin-Binding Domain, or HBD) (Elzie and Murphy-Ullrich 2004); (2) a sequence of three different types of repeated motifs, type 1 with homology to properdin (TSRs), type 2 with homology to EGF (EGF-like) and type 3 with highly conserved $Ca^{+2}$ binding domains on all TSPs; and (3) a C-terminal globular (lectin-like) domain, also present in all family members. The TSP family signature domain in the C-terminal half of the molecule comprises the three EGF-like repeats, 13 calcium-binding type 3 (T3) repeats, and the C-terminal lectin-like module arranged in a "bat and ball"-like conformation. In the presence of physiological concentrations of $Ca^{+2}$, type

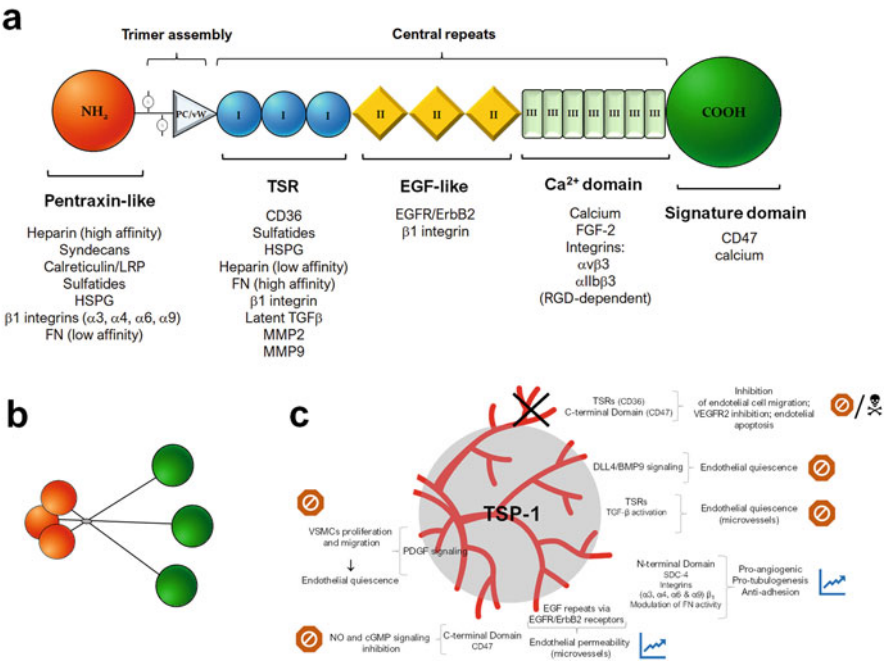


**Fig. 2.4** Structure and function of TSP1 in the vascular context. (**a**) Monomeric structure of human TSP1, with various ligands and receptors involved in their recognition. No evidence for mechanisms of alternative splicing in the TSP family has been described so far. (**b**) TSP1 assembled as a trimer; the position of the oligomerization domain leads to substantial compaction of the three N-terminal domains. The C-terminal globular domain is highly conserved among the different TSPs, being considered the structural "signature" of the family; (c) TSP1, proposed as the first identified physiological inhibitor of angiogenesis, acts mainly as an angiostatic molecule that keeps endothelial cells in a quiescence state, mediated by the TSRs and C-terminal domains. Induction of endothelial apoptosis (dependent on the CD36 receptor) has also been described for TSP1, with possible implications for the process of vascular regression or pruning. The N-terminal domain, with a high affinity for heparin and HSPGs, is proangiogenic and can be readily produced by several proteases, in the angiogenic context

3 repeats involve the C-terminal globular domain (Adams and Lawler 2011). Multiple receptors have been identified in TSP1 recognition: integrins ($\alpha_v\beta_3$, $\alpha_{IIb}\beta_3$, $\alpha_9\beta_1$, $\alpha_6\beta_1$, $\alpha_4\beta_1$, $\alpha_3\beta_1$), the scavenger receptor CD36, syndecans, sulfatides, calreticulin, CD47 (also known as IAP—integrin-associated protein), and low-density lipoprotein receptor 1 (LRP-1) (Morandi et al. 2021).

A considerable bulk of research on the TSP field was focused on the roles of the protein in endothelial/vascular contexts, in the wake of TSP1 characterization as the first physiological inhibitor of angiogenesis, in 1990 (Good et al. 1990). While TSP1 has been described as an adhesive/chemotactic adhesive factor for several malignant cell types in vitro, increased TSP1 levels in solid tumors have been mostly been correlated with better patient prognosis and with lower microvascular densities of experimental tumors in vivo, suggesting that the anti-tumor effect of the protein is

related to TSP1 ability to inhibit angiogenesis (Kazerounian et al. 2008; Kaur et al. 2021). Accordingly, TSP1 expression is down-regulated by oncogenes and upregulated by tumor suppressor genes (Stenina-Adognravi 2014). However, in a few pre-clinical and clinical tumor sets, evidence is quite controversial and the anti-angiogenic activity of TSP1 seems insufficient to stop tumor growth, especially in metastatic contexts of breast, prostate, and colorectal carcinomas, besides melanoma (Bertin et al. 1997; Filleur et al. 2001; Poon et al. 2004; Fontana et al. 2005; Yee et al. 2009; Jayachandran et al. 2014; Borsotti et al. 2015; Sun et al. 2020).

Several mechanisms of inhibition of endothelial functions have been identified for TSP1:

(a) TSP1 inhibits the proliferation and migration of vascular endothelial cells in vitro and inhibits neovascularization in vivo (Tolsma et al. 1993; Jiménez et al. 2000). The induction of endothelial apoptosis was also characterized, mainly related to the interaction of TSR domains with CD36 receptor on endothelial cell surface (Dawson et al. 1997), but the occurrence of apoptosis by TSRs in cells that do not express CD36 has also been reported (Guo et al. 1997).
(b) CD36 engagement by TSP1 via the TSR motifs leads to dephosphorylation of VEGFR2 (Zhang et al. 2009) through the recruitment of the phosphatase SHP1 to sites of CD36 engagement by TSP1 (Chu et al. 2013); CD36-mediated inhibition of VEGFR2 phosphorylation requires $\beta_1$ integrin (Primo et al. 2005) and receptor clustering also includes tetraspanin receptors (Zhang et al. 2009).
(c) TSP1 directly binds to VEGF and prevents its association with the VEGFR2 receptor, either by competing with extracellular VEGF for binding to cell surface proteoglycans or by stimulating VEGF clearance through LRP-1 (Greenaway et al. 2007; Gupta et al. 1999).
(d) TSP1 binds to FGF2, thus sequestering the growth factor and inhibiting its angiogenic activity. The site for FGF2 binding on TSP1 structure seems to be in the type III repeats (Margosio et al. 2008).
(e) Finally, TSP1 has been also implicated in the blockade of nitric oxide (NO) signaling pathways through both CD36 and CD47. The latter recognizes two peptide motifs on the carboxy-terminal signature domain of TSP1 (Gao et al. 1996). The VEGF-dependent activation of NO–cGMP signaling pathway induces endothelial migration and proliferation (Isenberg et al. 2009), thus contributing to the formation of the angiogenic provisional matrix by increasing the microvascular permeability. The interaction of TSP1 with CD47 also inhibits angiogenesis independently of NO, by inhibiting the constitutive direct interaction of CD47 with VEGFR2 (Kaur et al. 2010; Roberts et al. 2012).

Additionally, TSP1 modulates angiogenesis by intertwinement with the TGFβ pathway, although the outcomes from this partnership may depend on quite specific cellular contexts. TGFβ exerts paradoxical roles in angiogenesis: while TGFβ signaling through ALK5 receptor induces Smad2/3 phosphorylation and blocks angiogenesis by inhibiting endothelial proliferation, tube formation, and migration, the TGFβ/ALK1 axis through Smad1/5/8 activation promotes the opposite effect on

these same cell behaviors, in different experimental sets. In the same line, TGFβ has opposing effects on direct tumor growth, being able either to inhibit tumor progression at early neoplastic stages or to promote growth in advanced/metastatic disease (reviewed in Guerrero and McCarty 2017; and in Goumans and Ten Dijke 2018).

TSP1 released from platelet α-granules upon injury or other stimuli associates with latent TGFβ and promotes its activation (Murphy-Ullrich and Poczatek 2000). The amino acid sequence RFK that lies between the second and third TSRs was found to be essential for TSP1-mediated activation of the latent cytokine, in vitro and in vivo. In some tumor contexts, TSP1 seems to efficiently inhibit tumor growth and angiogenesis by activating latent TGFβ (reviewed in Ren et al. 2006). At the microvascular level, it was suggested that latent TGFβ activated by TSP1 might contribute to vessel stability, since physical contacts established by endothelial cells with mural cells (pericytes and VSMCs) stimulate the activation of TGFβ and the growth inhibition of endothelial cells (Antonelli-Orlidge et al. 1989; Sato and Rifkin 1989; RayChaudhury et al. 1994).

However, other tumors seem able to override the anti-angiogenic role of TSP1 in vivo, by growing more efficiently in the presence of TSP1-activated TGFβ (Filleur et al. 2001). TGFβ might contribute to such bypassing mechanism by up-regulating the expression of angiogenic growth factors—e.g., VEGF, FGF2—and provisional proteins, such as FN and fibrillary collagens (Guerrero and McCarty). These actions of TGFβ are further expected to fuel epithelial-to-mesenchymal transition, cancer invasion and, possibly, angiogenesis (Ignotz et al. 1987; Gonzalez and Medici 2014).

The proangiogenic properties of the isolated N-terminal heparin-binding domain of TSP1 (NTSP1) have been independently described by different groups (Taraboletti et al. 2000; Chandrasekaran et al. 2000; Outeiro-Bernstein et al. 2002), with the identification of different mechanisms of action in each case. A 25 kDa NTSP1 has been found to induce angiogenesis by stimulating endothelial invasiveness, through increasing the activity of MMPs 2 and 9 (Taraboletti et al. 2000). Together with two other members of the $\beta_1$ integrin subfamily ($\alpha_4\beta_1$ in venous and $\alpha_6\beta_1$ in microvascular cells), $\alpha_3\beta_1$ integrin has been implicated in the recognition of NTSP1, or of peptide motifs derived from this region (Calzada and Roberts 2005). These interactions promote proangiogenic responses in endothelial cells, both in vitro and in vivo (Chandrasekaran et al. 2000).

Additionally, in a shorter, recombinant 18 kDa NTSP1, TSP HepI and TSP HepII, two GAG-binding motifs comprised in this domain, potently induced endothelial tubulogenesis and PKCα/Akt signaling axis (Nunes et al. 2008). Such responses were effectively associated with the recognition of NTSP1 by SDC4, a cell surface HSPG enriched in focal adhesions (Woods and Couchman 1994). Both TSP HepI and TSP HepII peptides retained most of the proangiogenic capacity of the NTSP1 and were identified as competitors of the FN Hep II for binding to SDC4 (Nunes et al. 2008). TSP Hep I potentialized FGF2 angiogenic differentiation in vivo (Dias et al. 2012).

Long before the unraveling of the proangiogenic activities of the heparin-binding domain of TSP1, NTSP1 was shown to promote the destabilization of fibroblast and

endothelial focal adhesions mediated by FN in endothelial cells (Murphy-Ullrich and Höök 1989). This activity is mediated by calreticulin/LRP-1 receptor complex (Goicoechea et al. 2000), through recognition of TSP HepI, but not of TSP Hep II motif (Murphy-Ullrich et al. 1993). The focal adhesion-labilizing activity of TSP HepI required RhoA inactivation mediated by FAK (Orr et al. 2004). Thus, the roles performed by TSP1 in the angiogenic context are closely related to those played by the FN itself.

Several works have shown that FN and TSP1 physically interact within ECM. TSP1 associates with FN through site-specific interactions. Lahav et al. (1982) showed that the interaction of platelet TSP1 with FN was independent of the presence of calcium and magnesium and involves the 70 kDa chymotryptic fragment of TSP1 that contains the anti-angiogenic motifs (Lawler and Hynes 1986). In the case of endothelial TSP1, an additional site of interaction with FN in the N-terminal heparin-binding domain of TSP1 has been described (Dardik and Lahav 1989, 1999). Intact TSP1 and FN cooperatively support endothelial adhesion (Morandi et al. 1993) and an intact FN fibrillary matrix seems essential for the assembly of both TSP1 and collagen I within the matrix (Sottile and Hocking 2002). Interestingly, hypoxia, which is a major driver of pathological angiogenesis, especially in models of retinal neovascularization, upregulates the expression of both FN and TSP1 (Germain et al. 2010), suggesting a division of tasks between the two proteins in the building of new vessels.

Consistently with this view, a few seminal works showed that, in vitro, TSP1 was part of the surrounding matrix of mature tube-like endothelial structures, while it had been barely expressed during tube formation (Iruela-Arispe et al. 1991a, b). In another investigation, transformed, highly proliferative endothelial cells that did not express TSP1 and generated hemangiomas in vivo were unable to form tubes in vitro (Sheibani and Frazier 1995). But, when these cells were transfected to overexpress TSP1, they "learnt" how to form tubes, demonstrating the importance of TSP1 in maintaining vessel architecture, probably by a combination of mechanisms that ultimately curb serino-protease activity, endothelial proliferation, and favors cell-cell interactions (Sheibani and Frazier 1995). Accordingly, it was recently shown that TSP1 is upregulated by the activation of the Dll4/bone morphogenetic protein 9 axis, through the engagement of TGFβ receptors, leading to endothelial quiescence. FN and collagen IV are also increased, suggesting that TSP1 may participate in bridging the transition between "fire" and "cold" elements of the vascular ECM (Rostama et al. 2015).

The complex role of TSP1 in vascular morphogenesis was studied in vascularization models of retinal development and pathology. In these models, FN from an astrocytic source was considered critical to sprouting angiogenesis (Stenzel et al. 2011). Retinal endothelial cells deficient in TSP1 expression proliferate faster and exhibit enhanced migratory activity (Wang et al. 2003a, b). Oxygen-induced retinopathy (OIR) is a hypoxia-driven angiogenesis model that recapitulates features of diabetic retinopathy and retinopathy of prematurity (Scott and Fruttiger 2010). The overexpression of TSP1 in the lens resulted in an attenuated neovascular response in OIR (Wu et al. 2006) and pericytes isolated from "global" TSP1-knockout mice, had

decreased proliferation capacity, and failed to migrate when challenged with platelet-derived growth factor BB (Scheef et al. 2009). Accordingly, when TSP1 expression was genetically manipulated through cell-targeted knockdown strategies in three different cell types—endothelial cells, pericytes and astrocytes—robust evidence arose that the newly formed retina vasculature after OIR is not protected from full regression, corroborating the concept that TSP1 is essential in the later stages of sprouting angiogenesis, for vessel maturation and stabilization (Sorenson et al. 2021). Hence, the role of intact TSP1 in vascular morphogenesis may be better defined as a pro-quiescence factor, which may eventually contribute to inhibition of angiogenesis or even to vascular regression, depending on the local balance between anti- and pro-survival signals.

TSP1 has been implicated in another eye disorder, the age-related macular degeneration (AMD), which occurs through the accumulation of subretinal debris, exacerbating inflammation and neovascularization, as well as progression to degeneration of photoreceptors and retinal pigment epithelium (RPE) (Housset and Sennlaub 2015). In normal physiological situations, TSP1 is highly expressed by RPE (Miyajima-Uchida et al. 2000; Uno et al. 2006), resident macrophages (Gautier et al. 2013), and retina endothelial cells (Bornstein 2009), but its expression is severely decreased in AMD (Uno et al. 2006). TSP1 plays an important immunosuppressive role, preventing excessive endothelial growth and accumulation of mononuclear phagocytes in the subretinal microenvironment (Masli et al. 2014; Housset and Sennlaub 2015). A correlation between high levels of human high temperature requirement serine protease A1 (HtrA1), and the occurrence of AMD was identified (Lin et al. 2018). The analysis of RPE secretoma samples revealed that TSP1 is a cleavage substrate for HtrA1, which readily releases the proangiogenic NTSP1 domain, thus suggesting that the mechanism responsible for the increase in vascularization in AMD occurs through the elevation of HtrA1 activity, with the increased availability of the proangiogenic domain of TSP1 (Chen et al. 2018). Interestingly, in a pre-clinical model of AMD, it has been demonstrated that a soluble form of the extracellular domain of SND4, a receptor for the NTSP1 (Nunes et al. 2008), inhibits angiogenesis (De Rossi et al. 2021). This new evidence suggests that TSP1 may have its expression and structural integrity tightly regulated in a spatiotemporal manner, strikingly differing in its actions between the early/proteolytic and late/stabilizing phases of endothelial tubulogenesis.

Vascular lesions caused by the absence of an intact BM and defects in the recruitment of mural cells are characteristic of neurovascular anomalies called cerebral cavernous malformations (CCMs), in which the vessels are hyperpermeable (reviewed in Su and Calderwood 2020). Mutations in genes of the CCM family (CCM1, CCM2, and CCM3) are at the origin of most cavernomas. The complex formed by the three CCM proteins is essential for the homeostasis of interendothelial junctions. The hyperactivation of the RhoA/ROCK pathway leading to excessive stress fiber-dependent contractility was found to be crucial for the establishment of the cavernous phenotype. Interestingly, these aberrant phenotypes could be efficiently reversed by both TSP1 (or with the anti-angiogenic TSR motifs) or FN (Lopez-Ramirez et al. 2017; Schwefel et al. 2020), as an additional indication that

both proteins cooperate for the formation and stabilization of new vascular structures.

### 2.4.3 CCN Proteins and SPARC: Little but Fierce

So far in this review, we have discussed the role of high molecular weight matrix proteins FN, TNC, and TSP1, whose diversity of functional domains and large sizes are crucial for the high degree of complexity of their actions in vessel morphogenesis. However, the field of matricellular proteins encompasses proteins that also exhibit modular organizations but with a much smaller size (usually <100 kDa). Nonetheless, protein families like CCNs, SPARC, the group of genetically clustered Small Integrin-Binding Ligand N-linked Glycoproteins (SIBLINGs), and fibulins, among others (Thakur and Mishra 2016), are also proving to be important actors in the control of angiogenesis. Even though most of these exhibit sizes smaller than ~70 kDa, they have functional modules like those found in larger matricellular proteins, including integrin-binding motifs, calcium-sensitive domains, heparin-binding domains, among others. Accordingly, they can bind growth factors and be recognized by several integrins (especially $\alpha_v\beta_3$, but also $\alpha_v\beta_5$, $\alpha_v\beta_1$, $\alpha_4\beta_1$, $\alpha_8\beta_1$, and $\alpha_9\beta_1$, for example) and other adhesion mediators, such as CD44 (Bellahcène et al. 2008). Most of these proteins were discovered in processes related to the development, morphogenesis, and repair occurring in connective tissues, such as bones, joints, cartilages, or were found in association with elastic fibers (e.g., fibulins) and muscles. Evidence is also accumulating that the expression of these proteins is highly modulated in the tumor microenvironment, with drastic consequences for angiogenic responses (Thakur and Mishra 2016).

In the scope of this review, it would be impossible to explore all the evidence currently available about this ever-growing "matricellular world," but we will briefly focus on two other iconic glycoproteins in this heterogenous group, CCN1 and SPARC, on which important contributions to sprouting angiogenesis mechanisms have been made.

### 2.4.4 CCN family

The first member to be described in the CCN family was Cyr61 or CCN1 (see Fig. 2.5a, for an appraisal of its modular structure), a 40–45 kDa glycoprotein that has its expression associated with the development of the skeletal, cardiovascular, and neuronal systems during mouse embryogenesis (Kubota and Takigawa 2007), while in adult life the protein can be upregulated at sites of tissue remodeling, chronic inflammation, and cancer growth (Jun and Lau 2011; Yeger and Perbal 2021).

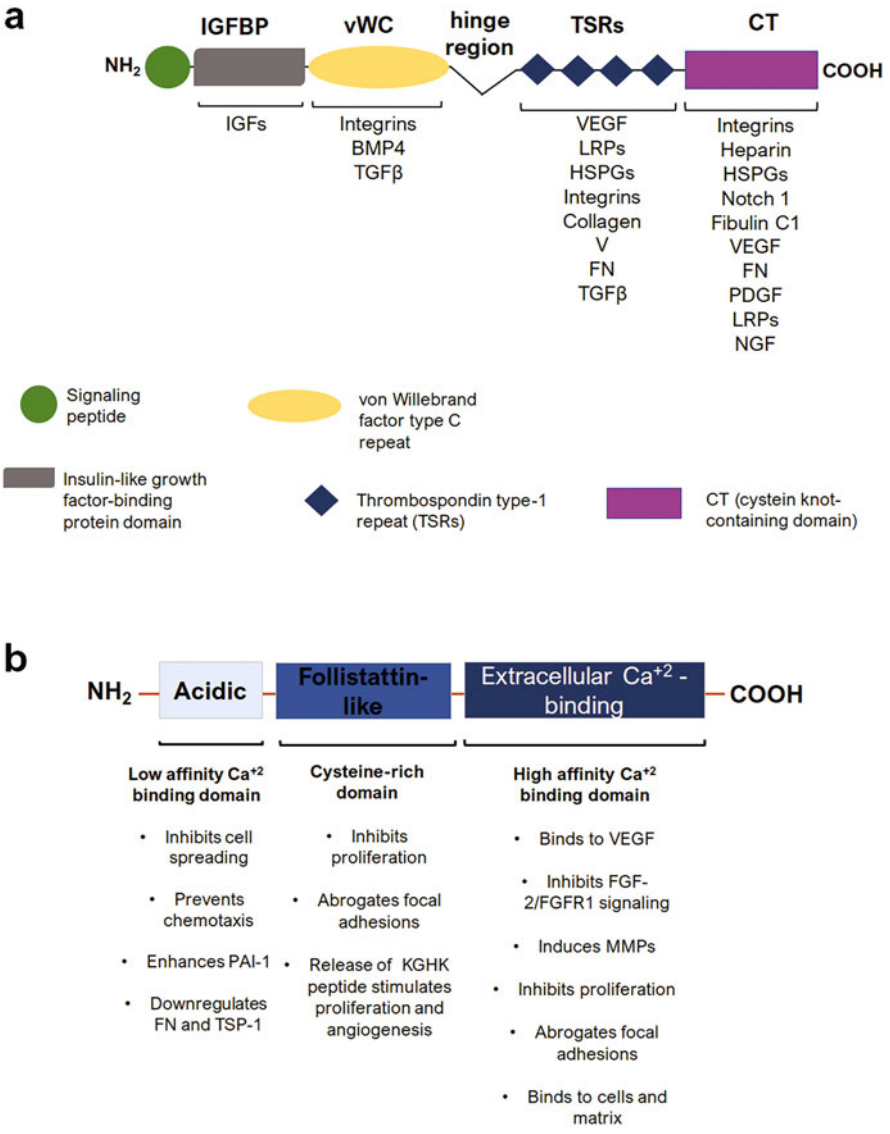


**Fig. 2.5** CCN1 and SPARC modular structures. Despite their low molecular weights and the relatively small number of modular domains, the glycoproteins CCN1 (**a**) and SPARC (**b**) play important roles during angiogenesis. As with TNC and TSP1, these glycoproteins can bind to growth factors and other matrix elements. CCN1 is upregulated by hypoxia and plays a major role in sprouting angiogenesis through interaction with the "master angiogenic" $\alpha_v\beta_3$ integrin in cancer contexts (for details, see the text). However, the lack of receptors that specifically recognize SPARC suggests that throughout the angiogenic process, this protein may act indirectly on other ligand-receptor interactions

The CCN family currently has six identified members, but only the first three discovered (CCN1/CCN2/CCN3) have so far been implicated as positive modulators of angiogenesis (Kubota and Takigawa 2007). In this regard, most of the available data relates to CCN1 and CCN2 isoforms. Like other matricellular proteins, CCNs can be incorporated into the ECM, where they can interact with other ECM components and growth factors. As could be predicted from its importance in the morphogenesis of the cardiovascular system, CCN1 expression is increased by FGF2, TGFβ1, and by hypoxia (Jun and Lau 2011). Although CCN1-3 proteins are recognized by several integrins ($\alpha_6\beta_1$, $\alpha_{IIb}\beta_3$, $\alpha_v\beta_5$, $\alpha_M\beta_2$, and $\alpha_5\beta_1$), $\alpha_v\beta_3$ integrin seems to be the most relevant receptor for angiogenesis modulation, mainly by stimulating endothelial migration (Leu et al. 2002). CCN1-$\alpha_v\beta_3$ interaction is not mediated by RGD motif since it is absent in CCN1 structure. When CCN1 protein was mutated at the site of recognition by $\alpha_v\beta_3$ integrin, its proangiogenic activity was lost (Chen et al. 2004). Besides integrins, HSPG, LRP, and the cation-independent mannose-6-phosphate (M6P) receptor have also been described as receptors for CCN proteins, with potential impact on angiogenesis regulation (Lau 2016).

As already mentioned, $\alpha_v\beta_3$ integrin plays a major role during sprouting angiogenesis, mediating pro-survival signaling and potentializing the activation of VEGFR2 in angiogenic vessels. Since active VEGFR2 is concentrated on the surface of endothelial tip cells, it would be expected that the activity of $\alpha_v\beta_3$ would also be concentrated in this endothelial subpopulation. In recent breakthroughs, it was demonstrated that CCN1 is a key regulator of endothelial tip cell fate, in an $\alpha_v\beta_3$-dependent manner, during retinal vascular development (Chintala et al. 2015; Park et al. 2019). CCN1/$\alpha_v\beta_3$ interaction in tip cells has been also coupled to VEGFR2 activation and triggers downstream MAPK/PI3K signaling and the pro-migratory YAP/TAZ pathway, besides also activating RhoA/ROCK pathway and increasing the expression of Dll4, a marker for tip cell specification (Park et al. 2019). In these works, the authors did not address the role of FN in experimental sets in which CCN1 expression was genetically manipulated, but it is known that endothelial-derived cFN actively stimulates angiogenesis in the neonatal retinal angiogenesis model, with the combined involvement of both $\alpha_5$ and $\alpha_v$ integrins, through its EDA and EDB domains (Miller et al. 2017; Turner et al. 2017). It would be exciting to examine whether CCN1 and FN somehow share roles in regulating the tip cell phenotype dynamics through the engagement of $\alpha_v\beta_3$ integrin, both in the normal development of retinal vascularization and in situations of remodeling induced by pathological contexts (hypoxia, diabetes, AMD, etc.).

### *2.4.5 SPARC*

SPARC (secreted protein acidic and rich in cysteine) is the 32 kDa founding member of a family of related proteins that today also comprises hevin/SPL1 (SPARC-like 1) (Hambrock et al. 2003), secreted modular calcium-binding protein (SMOC) 1 and 2 (Vannahme et al. 2002, 2003), SPOCK/testicans 1, 2 and 3 (Schnepp et al. 2005;

Vannahme et al. 1999, Alliel et al. 1993, Charbonnier et al. 1998), and follistatin like protein 1 (fstl1, TSC 36/Flik, follistatin related protein (FRP), TGFβ inducible protein) (Hambrock et al. 2004).

SPARC is a small glycoprotein (32 kDa) that has three types of structural/functional domains (Fig. 2.5b): besides the common central follistatin-like domain and a C-terminal extracellular $Ca^{2+}$-binding EF-hand motif that together constitute the "signature" of the SPARC-related protein family (Bradshaw 2012), SPARC has a highly acidic $NH_2$-terminal domain that interacts with hydroxyapatite and has been implicated in the mineralization of cartilage and bone (Romberg et al. 1985). SPARC-null phenotypes generate several connective tissue defects in dermis, bone, heart, adipose tissue, and periodontal ligament, which contain decreased collagen content, with fibers smaller than normal in size and diameter (Bradshaw et al. 2003a, b; Bradshaw 2009; Trombetta and Bradshaw 2010), indicating that SPARC expression is essential for the normal development. In contrast to other matricellular proteins, however, no specific receptors have been identified in the specific recognition of SPARC by cells, even though the protein was found in association with endothelial cell surface (Murphy-Ullrich et al. 1995). This fact suggests that SPARC may exert its effects through interference with other ligand-receptor interactions rather than through dedicated cell receptors.

In adult life, expression of SPARC is augmented in situations of repair, remodeling, inflammation, and cancer growth (Sage et al. 1989; Murphy-Ullrich and Sage 2014). In cancer, SPARC can either promote or inhibit tumor growth, depending on the cell and tissue types considered (Feng and Tang 2014). Like TSP1 and TNC, SPARC can destabilize focal adhesions and disrupt stress fibers, thus provoking cell rounding in various cell types, including endothelial cells (Murphy-Ullrich et al. 1995; Rivera et al. 2011). The protein has been considered a counter-adhesive and anti-proliferative ECM component. In most in vivo tumor experimental sets, SPARC has been shown to act as an angiogenesis inhibitor (Nagaraju et al. 2014). Besides interfering with focal adhesions, intact SPARC can also inhibit the proliferation and migration of endothelial cells by abrogating endothelial responses to growth factors in vitro, by directly binding to VEGF, or by interfering with FGFR1, the main signaling receptor for FGF2 (Bradshaw 2012). On the other hand, tumor promotion by SPARC has been attributed to its ability to activate MMPs, and to induce cell invasion and migration (Feng and Tang 2014), and tumor-derived SPARC was shown to promote paracellular endothelial permeability, resulting in increased tumor metastasis (Tichet et al. 2015).

Interestingly, SPARC downregulated the expression of both FN and TSP1 in endothelial cells undergoing tubulogenesis in vitro (Lane et al. 1992). This is consistent with the observation that fibroblasts isolated from SPARC-null mice fail to assemble an organized FN matrix (Barker et al. 2005), which may also contribute to the defects observed in fibrillary collagen deposition in these animals. Indeed, FN matrix organization precedes that of collagen and is essential for collagen fiber organization (Sottile and Hocking 2002; Velling et al. 2002). In the angiogenic context, the contribution of SPARC to the organization of provisional ("fire") components, such as fibrillar collagens and FN, may sound paradoxical, in face of

its anti-angiogenic activity described above. Indeed, one of the first roles described for SPARC was the ability to bind not only fibrillar collagen but also basal lamina collagen IV (Mayer et al. 1991), an attribute that places this small glycoprotein in an intersecting scenario between provisional/angiogenic and BM/quiescent vascular matrices. SPARC was shown to contribute to vessel maturation and stability, by promoting the migration of pericytes to nascent vessels (Arnold et al. 2010; Rivera and Brekken 2011) but, upon proteolysis in the early phases of sprouting angiogenesis, SPARC can generate proangiogenic peptides, suggesting that the role of the protein throughout the sprouting process is spatially and temporally regulated by proteolysis (Iruela-Arispe et al. 1995).

## 2.5 Conclusions and Perspectives

In summary, organized FN fibrils in provisional matrices decisively orchestrate endothelial tubulogenesis through a multitasking program that includes potentiating VEGFA-dependent signaling through VEGFR2, inducing MT1-MMP expression and activity, and stimulating endothelial contraction through RhoA/ROCK pathway, leading to the disruption of cell-cell junctional complexes, with the consequent increases in both endothelial permeability and tip cell activation and migration. These concerted actions largely depend on the engagement, by the FN-rich matrix, of diverse receptors from integrin, syndecan, and neuropilin families, and on the partnership of these adhesion receptors with growth factor receptors and matricellular proteins.

On the other hand, the important degree of conservation of matricellular proteins throughout the evolution of metazoans, which suggests that these molecules might have represented an evolutionary advantage in early invertebrates (e.g., in primitive responses to injuries and in organism defense), might also have been essential later, during evolution and adaptation of endothelium-based vascular systems. The repertoire of examples detailed in this review, concerning the role of four major matricellular glycoproteins that associate with the provisional matrix for the control of angiogenesis, is rather consistent with this hypothesis.

The term "anti-adhesive," often used to define the nature of matricellular proteins, was originally to convey the idea of antagonism to the adhesive properties of FN. However, FN and matricellular proteins seem to cooperate in a spatiotemporal manner, rather than to antagonize each other, throughout many of the essential steps of vascular morphogenesis. Specific cell-targeted knockout animal models should continue to generate more readily interpretable data about the role of each cell type in the angiogenic microenvironment. Additionally, the recent advent of new technologies for large-scale analysis of proteomes, especially of those specifically associated with pathological contexts in which angiogenesis plays critical roles, may allow, in the years to come, a better understanding of the physiological relevance of bioactive fragments (matrikines or matricryptins) of ECM proteins, for the angiogenic process.

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# Chapter 3
# Endothelial Cell–Matrix Interactions in Angiogenesis and Vessel Homeostasis: A Focus on Laminins and Their Integrin Receptors


**Susan E. LaFlamme and Hao Xu**

**Abstract** Integrin-mediated interaction of cells with proteins present in the extracellular matrix and basement membranes direct many cellular processes required for both normal and pathological processes. These interactions guide angiogenesis and vessel homeostasis, both in the embryo and the adult. Mouse genetic studies have explored the contribution of individual integrin heterodimers; laminins and their integrin receptors have emerged as important regulators of these processes. This chapter discusses findings from both in vivo studies and organotypic cell culture models that provide insight into the molecular mechanisms by which the laminin-binding integrins $\alpha_3\beta_1$, $\alpha_6\beta_1$, $\alpha_6\beta_4$, and their ligands, laminin-411 and laminin-511, regulate endothelial cell signaling, cell–cell interactions, and gene expression that contribute to the regulation of endothelial cell function in angiogenesis and vessel homeostasis.

## 3.1 Introduction

Angiogenesis is a process that is critical to tissue repair, cancer progression, as well as inflammation, and involves the sprouting of endothelial cells from the preexisting vasculature (Potente et al. 2011). Angiogenic signals, such as vascular endothelial growth factor (VEGF), activate endothelial cells leading to the proteolytic breakdown of the endothelial basement membrane, the loosing of endothelial cell–cell junctions, the dissociation of mural cells, and the sprouting of endothelial cells into surrounding extracellular matrix. Sprouting is guided by endothelial tip cells with

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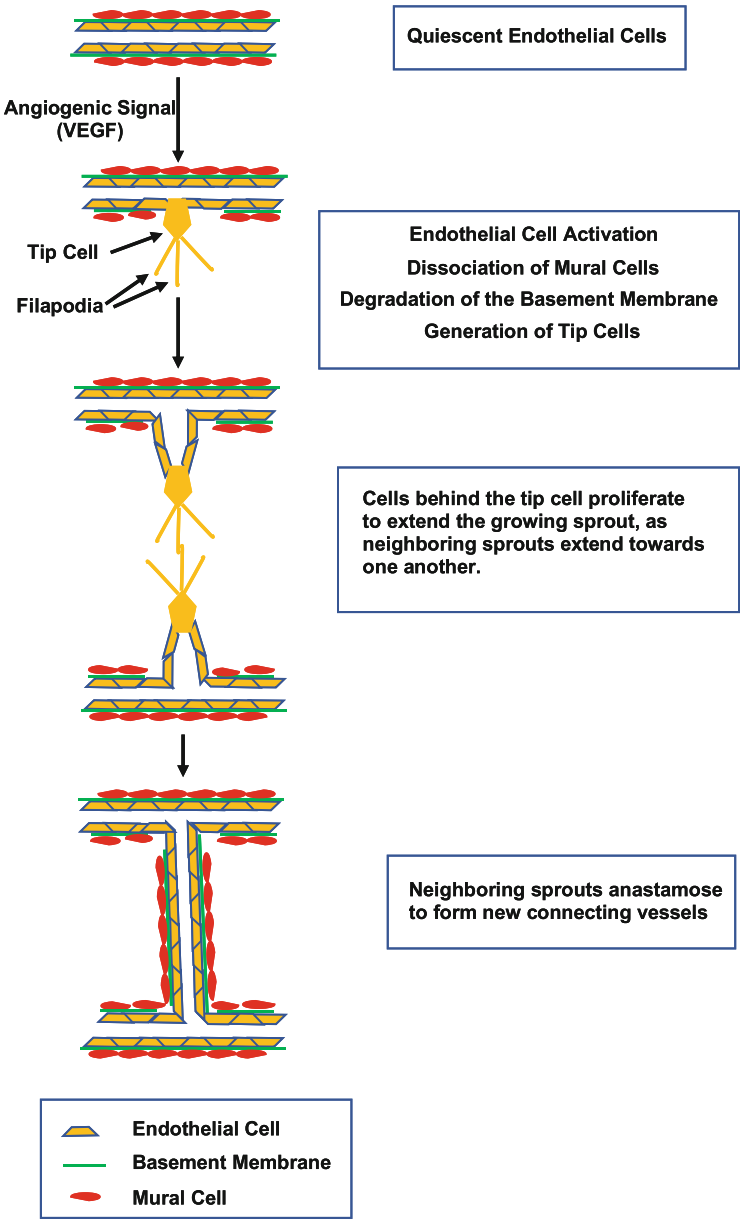


**Fig. 3.1** Angiogenesis. Shown is a schematic representation of the steps involved in angiogenesis

proliferating stalk cells in tow (Potente et al. 2011). Tip cells from neighboring sprouts anastomose to create a network of new vessels that form lumens, assemble new basement membranes, and recruit mural cells (Potente et al. 2011) (Fig. 3.1).

Sprouting endothelial cells can interact with the plasma proteins, fibronectin, vitronectin, or fibrinogen present in the provisional matrix or with collagens present in the interstitial matrix (Senger and Davis 2011; Eming et al. 2007). Endothelial cells can also interact with endothelial-secreted extracellular matrix (ECM) proteins, including fibronectin, and laminins (Hallmann et al. 2005; Avraamides et al. 2008; Turner et al. 2017). Members of the integrin family of adhesion receptors (Hynes 2002a) mediate these interactions that can contribute to the formation and stabilization of endothelial tubes (Hallmann et al. 2005; Turner et al. 2017; Senger and Davis 2011; Xu et al. 2020; Song et al. 2017; Avraamides et al. 2008).

## 3.2 Integrins in Angiogenesis

Integrins are heterodimeric transmembrane proteins that contain one α and one β subunit. Mammals have 18 α subunits and 8 β subunits that combine to form 24 α/β heterodimers (Hynes 2002a). Their extracellular domains engage ECM and basement membrane components, including fibronectin, collagens, and laminins. Integrin intracellular domains interact with the cell's cytoskeleton and signaling networks to regulate many aspects of cell behavior including cell adhesion, migration, proliferation, survival, and invasion, as well as gene expression, which together contribute to complex biological processes, such as tissue morphogenesis and development (Danen and Sonnenberg 2003; Streuli and Akhtar 2009).

Several endothelial integrin heterodimers, including $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_3\beta_1$ $\alpha_5\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_6\beta_1$, and $\alpha_6\beta_4$, are known to regulate angiogenesis, and their individual roles can be context-dependent (Avraamides et al. 2008; Senger and Davis 2011; Van Der Flier et al. 2010; Murphy et al. 2015). Much of what is known about the contribution of integrins has been gleaned from mouse genetic studies. The $\alpha_5\beta_1$, $\alpha_v\beta_3$, and $\alpha_v\beta_5$ integrins all bind to the RGD motif in fibronectin (Hynes 2002a). Both the global deletion of fibronectin (*Fn1*-null) in mice and the global deletion of integrin $\alpha_5$ subunit (*Itga5*-null) in mice are embryonic lethal, with both mutants exhibiting defects in embryonic vascular development (Avraamides et al. 2008). The specific deletion of fibronectin alleles in endothelial cells demonstrated that the endothelial expression of fibronectin is required for retinal developmental angiogenesis (Turner et al. 2017). However, the endothelial-specific deletion of the integrin $\alpha_5$ subunit alleles was not sufficient to alter developmental angiogenesis; however, endothelial-specific deletion of both integrin $\alpha_5$ (*Itga5*) and $\alpha_v$ (*Itgav*) subunit alleles inhibited embryonic vascular remodeling, suggesting that $\alpha_5\beta_1$ and $\alpha_v$ integrins can compensate for one another's endothelial function during the development of the embryonic vasculature (Van Der Flier et al. 2010). Significant effort both at the bench and in the clinic has been concerned with the RGD-binding integrins, $\alpha_5\beta_1$, $\alpha_v\beta_3$, and $\alpha_v\beta_5$; however, drugs targeting these integrins to suppress tumor angiogenesis have met with discouraging results (Hynes 2002b; Desgrosellier and Cheresh 2010; Paolillo et al. 2016). Additionally, the endothelial-specific deletion of the prominent RGD-binding integrins, as well as fibronectin, failed to inhibit tumor angiogenesis

(Murphy et al. 2015). Thus, it seems that other integrins and matrix components contribute to the regulation of angiogenesis.

The $\alpha_1\beta_1$ and $\alpha_2\beta_1$ integrins bind to both interstitial collagens, as well as collagens present in basement membranes (Hynes 2002a). An endothelial-specific deletion of $\alpha_1$ subunit gene (*Itga1*) has not been generated. However, mice with null mutations in the $\alpha_1$ integrin subunit gene, *Itga1-null* animals, are viable and fertile (Gardner et al. 1996), indicating that $\alpha_1\beta_1$ is not required for developmental angiogenesis. However, $\alpha_1\beta_1$ can regulate angiogenesis in the adult. Tumor angiogenesis was suppressed in *Itga1*-null mice, and this was correlated with the increased plasma levels of the metalloproteinases MMP-7 and MMP-9 and defects in endothelial proliferation (Pozzi et al. 2000; Pozzi et al. 2002). However, it is unclear whether endothelial cells are responsible for increased levels of MMPs, as $\alpha_1\beta_1$ is expressed in multiple cell types in addition to microvascular endothelial cells (Belkin et al. 1990; Glukhova et al. 1993; Gullberg et al. 1992; Defilippi et al. 1991; Glukhova et al. 1995; Loeser et al. 1995; Sobel et al. 1998; Senger et al. 1997).

The $\alpha_2\beta_1$ integrin is expressed on microvascular endothelial cells and has been observed on sprouting tips of angiogenic vessels, suggesting a role for $\alpha_2\beta_1$ in angiogenesis (Senger et al. 1997; Enenstein and Kramer 1994). An endothelial-specific deletion of the $\alpha_2$ subunit gene (*Itga2*) has not been generated. Mice lacking both $\alpha_2$ alleles (*Itga2*-null mice) have been made. These mice develop normally and are fertile (Chen et al. 2002; Holtkotter et al. 2002); thus, similar to $\alpha_1\beta_1$, $\alpha_2\beta_1$ is not required for developmental angiogenesis. However, adult *Itga2*-null mice exhibited enhanced wound and tumor angiogenesis, suggesting anti-angiogenic role for $\alpha_2\beta_1$ (Zweers et al. 2007; Grenache et al. 2007; Zhang et al. 2008). Surprisingly, the angiogenic response in *Itga2*-null mice was found to be tumor-dependent. An enhanced angiogenic response was triggered by B16F10 melanoma cells, but not by Lewis lung carcinoma cells (Zhang et al. 2008). VEGFR1 is the receptor for the VEGF family member, placental growth factor (PlGF) and is upregulated in endothelial cells from *Itga2*-null mice. B16F10 melanoma cells secrete much higher levels of PlGF compared to Lewis lung carcinoma cells, which explains the differences in the angiogenic response and underlines the importance of crosstalk between tumor and endothelial cells, as well as the tumor microenvironment in the angiogenic response (Zhang et al. 2008). Interestingly, increased expression of metalloproteinases, including MMP-9, was observed in dermal wound tissue from *Itga2*-null mice compared to control animals. In vitro studies suggested the enhanced protease expression was derived from *Itga2*-null keratinocytes, pointing to this as a potential mechanism for enhanced wound angiogenesis (Grenache et al. 2007). Others have shown the importance of integrin-regulated secretion of paracrine factors from keratinocytes in the crosstalk between keratinocytes and endothelial cells in the regulation of angiogenesis during dermal wound healing (Mitchell et al. 2009). Nonetheless, it is unclear how increases in MMP expression inhibit angiogenesis in the case of *Itga1-null* mice and enhance angiogenesis in the case of *Itga2*-null mice. Possibly the cellular source and localization of increased MMP expression determine the effect on angiogenesis. It is notable that these two collagen receptors have opposing roles in the regulation of angiogenesis, raising the question of

whether they balance one another's function, or whether $\alpha_1\beta_1$ or $\alpha_2\beta_1$ plays a dominant role. Analysis of animals lacking alleles for both the integrin $\alpha_1$ and integrin $\alpha_2$ subunits demonstrated that both wound and tumor angiogenesis were inhibited, indicating that the *Itga1-null* phenotype is dominant (Ghatak et al. 2016). This was further supported by explant aortic ring angiogenesis assays, which showed that sprouting was inhibited in explants from doubly null mice (Ghatak et al. 2016). Although mostly discussed in terms of their ability to bind to collagen, $\alpha_1\beta_1$ or $\alpha_2\beta_1$ can also bind to a subset of laminin isoforms, which will be discussed in more detail below.

The remainder of this chapter focuses on the contribution of endothelial laminins and their integrin receptors in the regulation of normal and pathological angiogenesis, as well as in vessel homeostasis. We discuss results from both mouse genetic models as well as in vitro angiogenesis assays.

## 3.3 Endothelial Laminins

Laminins are heterotrimeric proteins, each containing an α, β, and γ chain, are assembled intracellularly and are secreted as heterotrimers (Yurchenco et al. 1997). Laminins are important contributors to the formation and structure of basement membranes, that are present in epithelial, endothelial, muscle, and neural tissues (Colognato and Yurchenco 2000; Yurchenco 2011). In mammals, there are five distinct α chains, four β chains, and three γ chains, that can associate to form at least 15 laminin isoforms, with distinct tissue distribution (Yurchenco 2011). Endothelial cells express two laminin isoforms: laminin-411, which contains the α4, β1, and γ1 chains, and laminin-511, which contains the α5, β1, and γ1 chains. It is important to note that laminin-411 and laminin-511 were previously named laminin-8 and laminin-10 respectively (Aumailley et al. 2005).

Since laminin-411 and laminin-511 contain the same β and γ chains, their endothelial expression in the vasculature can be monitored by immunostaining with antibodies that recognize the laminin α4 chain or the laminin α5 chain, respectively. In mice, the laminin α4 chain is expressed ubiquitously by endothelial cells and is observed in the vasculature beginning at embryonic day 8.8 (Frieser et al. 1997; Iivanainen et al. 1997; Miner et al. 1997). The endothelial expression of the α5 chain of laminin is first observed a few weeks after birth and is detected primarily on capillaries and venules (Patton et al. 1997; Sorokin et al. 1997). Because laminin-411 and laminin-511 share the same β and γ chains, the effects of deletion of laminin-411 and laminin-511 on endothelial function can be examined by deletion of the α4 (*Lama4*) or α5 (*Lama5*) chain alleles, respectively. To date, an endothelial-specific deletion of *Lama4* gene has not been generated. Analysis of *Lama4*-null mice revealed significant hemorrhaging both during embryonic and neonatal development. This was accompanied by defects in the assembly and structure of endothelial basement membranes. Collagen IV and nidogen are also key components of basement membranes; their expression was lacking in endothelial basement

membranes of developing *Lama4*-null mice. Electron microscopy confirmed the presence of defective basement membranes associated with capillary endothelial cells. Together these data suggest that laminin-411 is required for the proper assembly of the endothelial basement membrane that in turn functions to stabilize the embryonic vasculature (Thyboll et al. 2002). Interesting, *Lama4*-null mice recover and develop into adulthood due to the endothelial expression of laminin-511, which becomes more widely expressed by vascular endothelial cells in mutant mice; thus, laminin-511 can compensate for the loss of laminin-411 (Thyboll et al. 2002; Zhou et al. 2004). Mice null for the laminin α5 chain, die during embryogenesis, before laminin-511 is expressed by endothelial cells (Miner et al. 1998). However, an endothelial-specific deletion of the laminin α5 chain alleles (*Lama5*) has been generated. The vasculature in these mice appears to develop normally with no significant defects in vasculature at homeostasis (Song et al. 2013).

## 3.4 Endothelial Laminin-Binding Integrins

Multiple integrin heterodimers bind laminin isoforms (Hynes 2002a). Endothelial cells express the $\alpha_3\beta_1$, $\alpha_6\beta_1$, and $\alpha_6\beta_4$ laminin-binding integrins, as do many epithelial cells (Avraamides et al. 2008; Hynes 2002a). The α chains of laminin heterotrimers contain the binding sites for integrins, and thus, the identity of the α chain present in a particular laminin isoform determines its integrin binding partners (Yurchenco 2011). Several approaches have been employed to determine whether $\alpha_3\beta_1$, $\alpha_6\beta_1$, and $\alpha_6\beta_4$ can bind to laminin-411 and/or laminin-511. Purified laminin-411 or laminin-511 has been used in cell adhesion assays together with antibodies to inhibit the function of specific integrin subunits. Endothelial cell adhesion to laminin-411 was inhibited by function-blocking antibodies to either the $\alpha_6$ or $\beta_1$ integrin subunit, demonstrating that laminin-411 is a ligand for $\alpha_6\beta_1$ (Kortesmaa et al. 2000). K562 cells do not express any laminin receptors. The ability of K562 cells expressing individual recombinant laminin-binding integrins demonstrated that the $\alpha_3\beta_1$, $\alpha_6\beta_1$, and $\alpha_6\beta_4$ integrins can each function as adhesion receptors for laminin-411 (Kortesmaa et al. 2000; Fujiwara et al. 2001). Endothelial cell adhesion to laminin-511 was inhibited using function-blocking antibodies to integrin $\alpha_3\beta_1$ (Doi et al. 2002). The ability of $\alpha_6\beta_4$ to bind laminin-511 was demonstrated using the murine $\beta_1$ *Itgb1*-null GD25 cells that express only one laminin-binding integrin, $\alpha_6\beta_4$ (Kikkawa et al. 2004). The ability of $\alpha_6\beta_1$ to bind laminin-511 was demonstrated using in vitro binding assays (Nishiuchi et al. 2006), as well as in cell adhesion assay with antibodies to block the function of $\alpha_6$ integrins. Although $\alpha_1\beta_1$ and $\alpha_2\beta_1$ are usually considered collagen-binding integrins, they have also been shown to bind to a subset of laminin isoforms (Humphries et al. 2006, Yurchenco 2011 41). The $\alpha_2\beta_1$ was shown to function in cell adhesion to laminin-411, but not laminin-511 (Stenzel et al. 2011). The ability of $\alpha_1\beta_1$ to bind to these laminin isoforms has not been tested. It is also important to note that the $\alpha_v\beta_3$ integrin was shown to bind a recombinant fragment of the laminin α4 chain (Gonzalez et al. 2002). The significance of the

interaction between $\alpha_v\beta_3$ and the α4 chain of laminin-411 to endothelial cell function is not yet fully appreciated. The laminin α5 chain contains an RGD sequence and the $\alpha_5\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_1$ and $\alpha_v\beta_5$ were shown to bind recombinant fragments of the α5 chain containing the motif (Hallmann et al. 2005; Sasaki and Timpl 2001). Whether these interactions contribute to angiogenesis or vessel homeostasis is yet to be appreciated; however, adhesion to laminin-511 mediated by $\beta_1$ and $\beta_3$ integrins was implicated in maintaining the surface expression of the adherens junction protein, VE-cadherin (Song et al. 2017), as discussed below.

## 3.5 Laminins in Pathological Angiogenesis

As mentioned above, embryonic vessels that lack the expression of laminin-411 are leaky, suggesting that laminin-411 functions to promote vessel stability, at least in the embryo (Thyboll et al. 2002). To determine the role of laminin-411 in new vessel formation in adult animals, the effects of inhibiting the expression of laminin-411 were assessed in cornea angiogenesis assays. Compared with wild-type littermates, *Lama4*-null mice exhibited enhanced endothelial sprouting in response to FGF2 (Thyboll et al. 2002), suggesting that laminin-411 may function as a negative regulator of angiogenesis. Interestingly, in wild-type corneas, a well-organized vasculature developed in response to FGF2, but most of these vessels had regressed by 42 days (Zhou et al. 2004). Vessels that formed in *Lama4*-null corneas were disorganized and dilated, but surprisingly at day 42, these vessels had undergone dramatic remodeling into what appeared as a normal vasculature (Zhou et al. 2004). This correlated with the upregulation of laminin-511 expression, suggesting that laminin-511 promotes vessel maturation. To examine the contribution of laminin-411 to tumor angiogenesis, control mice and mice deficient in the α4 chain of laminin-411 were challenged with subcutaneously implanted Lewis lung carcinoma cells or B16-F10 melanoma cells (Zhou et al. 2004). Tumor growth and angiogenesis were enhanced in mutant compared to control in response to both tumor types. Additionally, greater B16-F10 lung metastases were observed in *Lama4*-null compared to control mice; however, the mechanisms involved have yet to be identified. Although the tumor vasculature in control mice mostly expressed laminin-411, in *Lama4*-null mice the tumor vasculature exhibited strong expression of laminin-511, which presumably compensated for the loss of laminin-411 (Zhou et al. 2004).

In post-natal retinal developmental angiogenesis, the expression of laminin α4 chain RNA localized to extending endothelial sprouts, suggesting a positive role for laminin-411 in regulating endothelial sprouting. *Lama4*-null mice, however, showed enhanced endothelial sprouting, suggesting that the expression of laminin-411 puts the breaks on sprouting (Stenzel et al. 2011). This is consistent with the studies discussed above, which reported enhanced tumor angiogenesis in *Lama4*-null mice.

As mentioned in the Introduction, sprouting is guided by endothelial tip cells, which send out filopodia that direct the growing sprout. VEGF-VEGFR2 signaling in tip cells promotes the secretion of the Notch ligand Dll4 to limit the generation of

additional tip cells nearby (Potente et al. 2011). Dll4-Notch signaling is critical to the regulation of angiogenesis; the loss of Dll4/Notch signaling is known to lead to hyper-sprouting (Eilken and Adams 2010; Phng and Gerhardt 2009). Thus, the loss of laminin-α4 expression phenocopied the defects observed when Dll4/Notch signaling is inhibited. Indeed, Notch signaling was inhibited in *Lama4*-null retinas (Stenzel et al. 2011). Additionally, in vitro adhesion of endothelial cells to laminin-411, but not laminin-511, significantly increased the expression of Dll4 RNA (Stenzel et al. 2011). Antibody inhibition studies and siRNA knockdown experiments indicated that the $\alpha_2\beta_1$ and $\alpha_6\beta_1$ integrins contributed to this regulation (Stenzel et al. 2011). Taken together, these results suggest that $\alpha_2\beta_1$- and $\alpha_6\beta_1$-dependent endothelial cell adhesion to laminin-411 regulates the expression of Dll4 to promote properly regulated endothelial sprouting (Stenzel et al. 2011).

An endothelial-specific deletion of the laminin α5 chain is available (Song et al. 2013). No developmental angiogenesis defects were observed, as expected, since the endothelial expression of laminin α5 chain occurs postnatally. The contribution of laminin-511 to adult or pathological angiogenesis has yet to be explored. However, loss of endothelial laminin-511 decreases endothelial barrier function (Song et al. 2017). Such a phenotype might be permissive to angiogenesis. The contribution of laminin-511 to the regulation of endothelial barrier function is discussed below.

## 3.6 Laminin-Binding Integrins in Pathological Angiogenesis

Mouse genetic models were established to characterize the function of the laminin-binding integrins $\alpha_3\beta_1$, $\alpha_6\beta_1$, and $\alpha_6\beta_4$ during development. Mice null for the $\alpha_3$ (*Itga3*), $\alpha_6$ (*Itga6*), or $\beta_4$ subunit gene (*Itgb4*) died soon after birth with no defects in developmental angiogenesis reported (Kreidberg et al. 1996; Georges-Labouesse et al. 1996; Dowling et al. 1996; da Silva et al. 2010; Germain et al. 2010; Bouvard et al. 2012). To study the role of $\alpha_3\beta_1$ and $\alpha_6$ integrins during angiogenesis in the adult, several labs used conditional endothelial deletion of either the *Itga3*, *Itga6, or Itgb4* gene (Germain et al. 2010; da Silva et al. 2010; Bouvard et al. 2012; Bouvard et al. 2014; Seano et al. 2014; Welser-Alves et al. 2013). To conditionally delete integrin $\alpha_3$ subunit alleles in endothelial cells, Cre recombinase was expressed from the Tie-1 promoter (da Silva et al. 2010). This mutant will be referred as ec-*Itga3*-null. The $\alpha_3\beta_1$ integrin was not expressed by angiogenic vessels in mutant mice; however, $\alpha_3\beta_1$ expression was maintained in quiescent endothelial cells in dermal vasculature of ec-*Itga3*-null mice at levels similar to littermate controls (da Silva et al. 2010). Ec-*Itga3*-null mice exhibited increased angiogenesis in three distinct models. Tumor angiogenesis was analyzed in response to the subcutaneous injection of either B16F0 melanoma cells or CMT19T lung carcinoma cells (da Silva et al. 2010). Both tumor cell types showed enhanced angiogenesis in mutant compared to control mice. Hypoxia-induced retinal angiogenesis was also greater in ec-*Iga3*-null

mice compared to control, as was endothelial sprouting in aortic ring explant angiogenesis assays. These results supported a role for $\alpha_3\beta_1$ as a general negative regulator of angiogenesis in the adult. The mechanism reported for the ec-*Itga3*-null phenotype was surprisingly elaborate. The $\alpha_3\beta_1$ integrin was shown to be a positive regulator of the endothelial expression of VEGF, which led to the inhibition of the VEGFR2 RNA expression (da Silva et al. 2010). Thus, endothelial cells from mutant mice express higher levels of VEGFR2, explaining their increased angiogenic activity (da Silva et al. 2010).

There are conflicting reports as to whether and how $\alpha_6$ integrins ($\alpha_6\beta_1$ and $\alpha_6\beta_4$) regulate adult angiogenesis. The phenotype of targeting the endothelial expression of $\alpha_6$ integrins is dependent upon the promoter used to express Cre recombinase in endothelial cells (Germain et al. 2010; Bouvard et al. 2014). Like the Tie-1-dependent deletion of the integrin $\alpha_3$ subunit genes, the Tie1-Cre-dependent deletion of the integrin $\alpha_6$ subunit alleles (*Itga6*), referred to as ec1-*Itga6*-null, resulted in enhanced tumor angiogenesis (Germain et al. 2010). Subcutaneous injection of either B16F0 melanoma cells or Lewis lung carcinoma cells resulted in larger more vascularized tumors in ec1-*Itga6*-null, compared to those in littermate controls, suggesting that $\alpha_6$ integrins, like $\alpha_3\beta_1$, are negative regulators of angiogenesis. The authors note that this is consistent with the decreased expression of $\alpha_6$ integrins in angiogenic vessels associated with invasive ductal carcinoma as compared to normal breast tissue (Germain et al. 2010). The observed enhanced angiogenesis was not due to the upregulation of other integrins reported to promote angiogenesis, such as the $\alpha_1\beta_1$, $\alpha_3\beta_1$, $\alpha_5\beta_1$, and $\alpha_v\beta_3$ integrins (Germain et al. 2010). Endothelial cells from ec1-*Itga6*-null mice displayed increased surface expression of VEGFR-2 and downstream signaling, which explain the enhanced angiogenesis observed ec1-*Itgα6*-null mice (Germain et al. 2010).

Tie-2 (Tek) driven Cre recombinase was also used to examine the effect of the endothelial deletion of $\alpha_6$ integrins (Bouvard et al. 2012, 2014). This mutant is referred to as ec2-*Itgα6*-null. The angiogenic phenotype of this mutant was first examined following ischemic injury to mouse hind limb (Bouvard et al. 2012). A significant reduction in angiogenesis was observed in mutant animals. It is important to note that in addition to endothelial cells, the Tie-2-lineage includes pericytes and subsets of endothelial progenitors and macrophages (Kisanuki et al. 2001). The decrease in angiogenesis observed in ec2-*Itgα6*-null was accompanied by a reduction in the recruitment of bone marrow-derived endothelial progenitor cells and Tie-2 expressing macrophages to the site of injury. Thus, the phenotype of ec2-*Itgα6*-null could be due to the loss of $\alpha_6$ integrins in multiple cell types (Bouvard et al. 2012). Notably, the loss of expression of $\alpha_6$ integrins did not affect the surface expression of other integrin heterodimers, or surprisingly, the expression of VEGFR2.

Tumor angiogenesis was also examined in ec2-*Itgα6*-null mice. Angiogenesis was inhibited following the subcutaneous injection of B16F10 melanoma cells (Bouvard et al. 2014). This was associated with a decreased recruitment of Tie-2 expressing macrophages to the site of tumor growth. Macrophages significantly contribute to the tumor microenvironment; thus, their loss could affect the

angiogenic response (Coussens and Werb 2002). Notably, pericyte coverage of tumor vessels was similar in wild-type and mutant mice (Bouvard et al. 2014). Although the reduction in macrophage recruitment to the site of tumor formation in ec2-*Itga6*-null mice, may explain the difference in the angiogenic responses in ec2-*Itga6*-null versus ec1-*Itga6*-null mice, it is difficult to understand how the loss of $\alpha_6$ integrins affects VEGFR2 expression in one case and not the other.

A pro-angiogenic role for $\alpha_6\beta_1$ is supported by the finding that the $\alpha_6\beta_1$ integrin localizes invasive structures referred to as podosomes (Seano et al. 2014) These structures are integrin-extracellular matrix contact sites that aid in targeting proteases to sites of invasion (Linder and Aepfelbacher 2003; Gimona et al. 2008). Antibodies or RNAi approaches to inhibit the activity or expression of $\alpha_6$ integrins suppressed VEGF-induced formation of podosomes and the recruitment of the metalloproteinase MT1-MMP (Seano et al. 2014). The importance of $\alpha_6\beta_1$ in the formation of these structures was further supported by the finding that podosome formation was impaired in aortic explants from ec2-*Itga6*- null mice, compared to littermate controls (Seano et al. 2014). A tight balance in integrin-laminin interactions appears critical to the formation of these structures in endothelial cells, as more VEGF-induced podosomes were observed in explants from *Lama4*-null mice compared to control (Seano et al. 2014).

As noted above, the integrin $\alpha_6$ subunit heterodimerizes with either the $\beta_1$ or $\beta_4$ subunit. Thus, the effects of deletion of the $\alpha_6$ subunit could be due to the loss of either $\alpha_6\beta_1$ or $\alpha_6\beta_4$. Since the $\beta_1$ subunit dimerizes with multiple α subunits, distinct roles for endothelial $\alpha_6\beta_1$ and $\alpha_6\beta_4$ have been inferred from the effects of targeted endothelial deletion of the $\beta_4$ integrin subunit gene (*Itgb4*) together with information on the endothelial expression of $\alpha_6\beta_4$ in different vascular beds.

The $\beta_4$ integrin subunit has a very large intracellular domain compared with other β subunit intracellular domains. The membrane-proximal region is known to connect the α6β4 integrin to the intermediate filament cytoskeletal system (te Molder et al. 2021; Mercurio et al. 2001): keratin intermediate filaments in the case of epithelial cells and vimentin intermediate filaments in endothelial cells (te Molder et al. 2021; Homan et al. 1998, 2002). Mice null for the $\beta_4$ integrin subunit gene (*Itgb4*) die after birth due to extensive defects in epithelial tissues thought to be mostly due to the loss of a transmembrane connection between the basement membrane and the keratin intermediate filament system (Dowling et al. 1996; Van Der Neut et al. 1996). The membrane distal region is involved in the ability of $\alpha_6\beta_4$ to initiate intracellular signaling events to control cell behavior (te Molder et al. 2021; Mercurio et al. 2001). The global deletion of only the signaling portion of the $\beta_4$ subunit cytoplasmic domain was reported to inhibit angiogenesis in several types of assays (Nikolopoulos et al. 2004). The loss of the signaling portion of the $\beta_4$ cytoplasmic domain inhibited hypoxia-induced retinal angiogenesis. The subcutaneous injection of a number of different tumor cell lines, including B16F0 melanoma cells and Lewis lung carcinoma cells resulted in significantly decreased tumor angiogenesis in mutant mice compared to wild-type littermate controls (Nikolopoulos et al. 2004). The loss of the signaling portion of the $\beta_4$ cytoplasmic domain correlated with reduced nuclear localization of ERK and NFκB, which are known to become

activated downstream of $\alpha_6\beta_4$ and to promote endothelial sprouting and invasion (Nikolopoulos et al. 2004).

As indicated above, a conditional endothelial deletion mutant has been generated using Tie-2-dependent expression of Cre recombinase (Welser-Alves et al. 2013). This mutant is referred to as ec-*Itgb4*-null. No defects in vascular development were observed and analysis of endothelial expression of $\alpha_6\beta_4$ was reported to be restricted to arterioles (Welser-Alves et al. 2013). However, it is important to note that others reported the expression of $\alpha_6\beta_4$ in angiogenic vessels associated with tumors and dermal wound repair, although the $\alpha_6\beta_4$ expression appeared dynamic in angiogenic endothelial cells in some contexts (Nikolopoulos et al. 2004; Desai et al. 2013). Additionally, single-cell RNA sequencing of vascular cells from the murine lung, found $\beta_4$ mRNA widely expressed in endothelial cells in veins, venules and with higher expression in capillary endothelial cells (He et al. 2018), consistent with the ability of $\alpha_6\beta_4$ to contribute to angiogenesis. Also of note, the endothelial expression pattern of $\alpha_6\beta_4$ is similar to the endothelial expression of the α5 chain of laminin (Patton et al. 1997; Sorokin et al. 1997). The same single-cell RNA sequencing study indicated that arterial endothelial cells of the brain vasculature had relatively higher levels of $\beta_4$ mRNA expression (He et al. 2018). The function of $\alpha_6\beta_4$ in brain endothelial cells was analyzed in a chronic hypoxia model (Welser-Alves et al. 2013). Although wild-type and ec-*Itgb4*-null mice did not exhibit a significant difference in the early angiogenic response to hypoxia, the number of arteriolar size vessels was significantly lower in ec-*Itgb4*-null mice, implicating $\alpha_6\beta_4$ in regulating arteriolar remodeling. Previous work indicated a role for TGF-β signaling in this process (Seki et al. 2003). Consistent with this previous work, the expression of the type-I TGF-β receptor, activin-like kinase 1 (ALK1) and the activation of downstream signaling protein was inhibited in ec-*Itgb4*-null mice, providing a mechanism for defective arterial remodeling in mutant mice (Welser-Alves et al. 2013). Nonetheless, with reports of $\alpha_6\beta_4$ expression in venules, capillaries, and angiogenic vessels, it will be interesting to examine the effect of an endothelial-specific deletion of *Itgb4* employing other models of adult angiogenesis.

## 3.7 Laminin-511 and $\alpha_6\beta_4$ in the Regulation of Endothelial Barrier Function

Previous studies demonstrated that the endothelial expression of $\alpha_6\beta_4$ integrin was upregulated during neuroinflammation (Milner and Campbell 2006). To understand the implication of the upregulation, the effect of the endothelial deletion of $\beta_4$ was examined using ec-*Itgb4*-null mice in experimental autoimmune encephalomyelitis (EAE), which is a mouse model for multiple sclerosis (Welser et al. 2017). Although the timing of the onset of disease was similar in mutant and wild-type mice, the clinical outcome was worse in ec-*Itgb4*-null mice, which exhibited increased leukocyte infiltration and was accompanied by the loss of the tight junction proteins

claudin-5 and ZO-1 from cell–cell junctions (Welser et al. 2017). These studies implicate the endothelial expression of $\alpha_6\beta_4$ in the protection of the blood–brain barrier by regulating the integrity of cell–cell junctions (Welser et al. 2017).

Leucocyte extravasation from the vasculature occurs predominately at postcapillary venule sites with both laminin-411 and laminin-511 expression (Hallmann et al. 2005). In the EAE model of neuroinflammation, T lymphocytes were found to transmigrate at areas of the endothelial basement membrane that contained laminin-411, but low levels of laminin-511 (Sixt et al. 2001; Wu et al. 2009). In *Lama4*-null mice, the expression of laminin-511 is ubiquitously present in endothelial basement membranes. In the EAE model, *Lama4*-null mice exhibited reduced T-cell extravasation across the blood–brain barrier and decreased disease severity, suggesting that laminin-511 promotes endothelial barrier function (Wu et al. 2009 38). Later studies demonstrated that laminin-511 regulates endothelial barrier function by stabilizing the localization of VE-cadherin at endothelial cell–cell junctions, known as adherens junctions, to limit leukocyte extravasation (Song et al. 2017). Endothelial adhesion to laminin-511 by $\beta_1$ and $\beta_3$ integrins regulates the localization of VE-cadherin by promoting the activation of the small GTPase protein RhoA (Song et al. 2017). Taken together these results suggest that multiple laminin-511-binding integrins may cooperate to promote the blood–brain barrier in the brain, with $\beta_1$ and $\beta_3$ integrins functioning to stabilize adherens junctions and $\alpha_6\beta_4$ stabilizing tight junctions. It will be interesting to know whether similar interactions function to limit inflammation in other vascular beds.

## 3.8 Dissecting the Contribution of Laminin-Binding Integrins to Processes Involved in Angiogenesis Using In vitro Angiogenesis Assays

There are several in vitro angiogenesis assays that have been used to identify molecular mechanisms involved in regulating the morphogenesis of endothelial cells into endothelial cords and tubes (Simons et al. 2015). Two organotypic co-culture angiogenesis assays have been particularly useful. These are the "bead sprout" and the "planar co-culture" assays (Nakatsu et al. 2003; Nakatsu and Hughes 2008; Bishop et al. 1999; Donovan et al. 2001; Bajaj et al. 2012; Li et al. 2018). In the bead sprout assay, endothelial cells, usually human umbilical vein endothelial cells, are adhered to gelatin-coated beads and embedded in a fibrin gel (Nakatsu et al. 2003; Nakatsu and Hughes 2008). Endothelial sprouting from beads can easily be observed microscopically by phase contrast or by confocal following immunostaining (Fig. 3.2). Fibrin is present in the provisional matrix of tumors and wounds and provides an adhesive ligand for migration through interactions with RGD-binding integrins (Eming et al. 2007). In the planar co-culture assay, endothelial cells are plated at low density onto dermal fibroblasts, which have grown to confluence (Bishop et al. 1999; Donovan et al. 2001; Bajaj et al. 2012). Endothelial

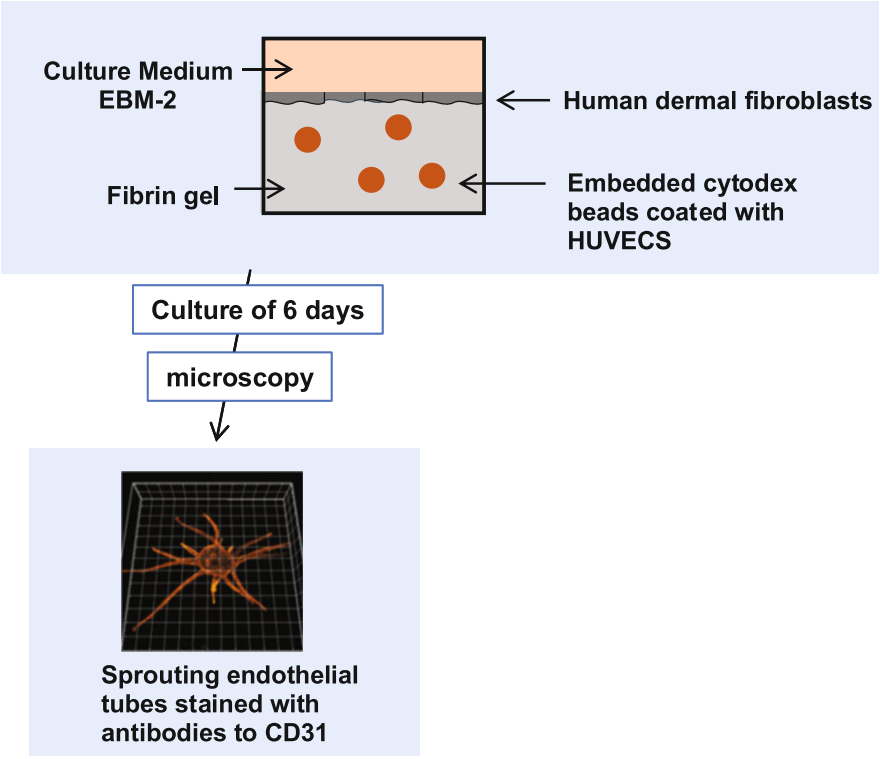


**Fig. 3.2** Bead sprout assay. Shown is a schematic of the bead sprout assay

morphogenesis then occurs in the dense fibroblast-secreted ECM, containing fibronectin and fibrillar collagen, like what is observed in wound and tumor ECM (Eming et al. 2007) (Fig. 3.3).

Endothelial cells secrete collagen IV (Col IV) and laminin-411 and laminin-511 on their basal surfaces, as they form tubes in both co-culture assays (Fig. 3.4 and (Xu et al. 2020)). Nonetheless, without the demonstration that other components of basement membrane are present and without ultrastructure analysis, it is unclear whether a basement membrane is fully assembled in these assays. However, the expression of these laminins in co-culture allowed for the examination of their contribution to endothelial morphogenesis. RNAi-dependent depletion of either the α4 or α5 chains of laminin-411 or laminin-511 inhibited endothelial sprouting and the formation of endothelial tubes in both the bead-sprout and planar co-culture assays, respectively (Xu et al. 2020).

As discussed above, $\alpha_6\beta_1$ and $\alpha_6\beta_4$ bind to both laminin-411 and laminin-511. The same organotypic angiogenesis assays were used to explore the contribution of $\alpha_6$ integrins to endothelial morphogenesis. RNAi-dependent depletion of $\alpha_6$

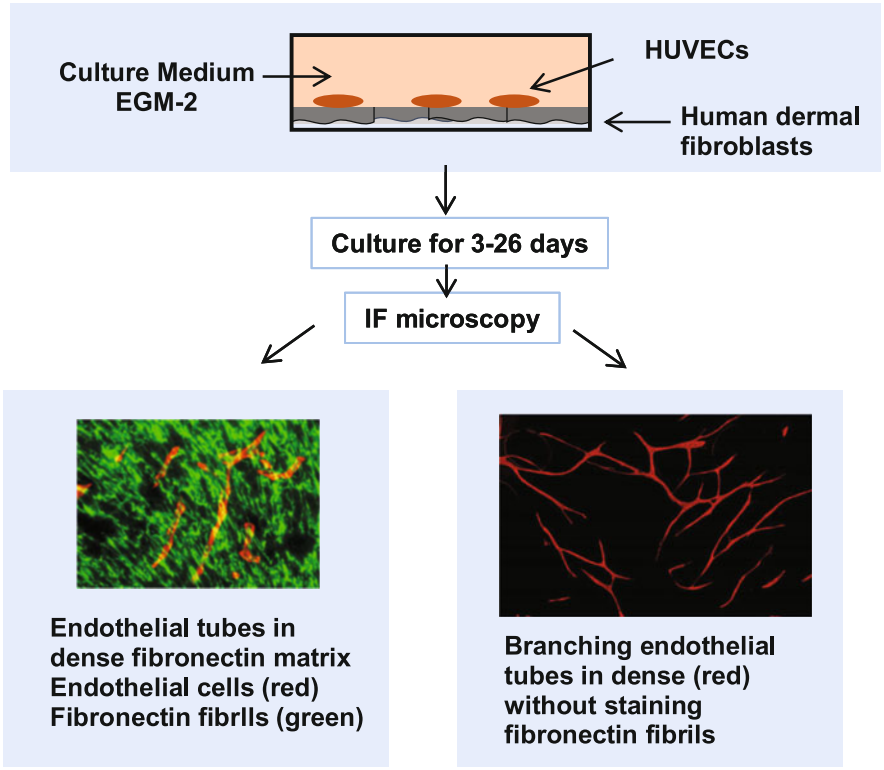


**Fig. 3.3** Planar co-culture assay. Shown is a schematic of the planar co-culture assay

integrins inhibited both endothelial sprouting and tube formation (Xu et al. 2020). The surface expression of the $\alpha_2\beta_1$, $\alpha_5\beta_1$ or $\alpha_v\beta_3$ integrins, previously shown to regulate angiogenesis, was not altered by depletion of $\alpha_6$ integrins. Notably, the expression of $\alpha_3\beta_1$ was significantly increased (Xu et al. 2020), which is interesting as the endothelial-specific depletion of the $\alpha_3$ subunit gene promoted angiogenesis (da Silva et al. 2010). However, depletion of $\alpha_3\beta_1$ did not enhance endothelial sprouting in our co-culture assays, as discussed further below (Xu and Laflamme 2022).

The bead-sprout assay allows the easy isolation of endothelial cells for the analysis of gene expression, as the layer of fibroblasts is easily removed (Li et al. 2018). Several genes have been associated with endothelial sprouting and angiogenesis, including *VEGFR2*, *CXCR4*, *ANGPT2*, *DLL4*, *PDGFB*, *NRP1*, *JAG1*, and *MMP14* (MT1-MMP) (de Smet et al. 2009; del Toro et al. 2010; Strasser et al. 2010). To determine whether endothelial $\alpha_6$ integrins or their laminin substrates regulate the expression of any of these genes in the bead-sprout assay, RNAi technology was employed to deplete endothelial cells of either $\alpha_6$ integrins, laminin-411 or laminin-511 and changes in gene expression were analyzed by qPCR. Defects in endothelial morphogenesis correlated with significant decreases in the expression of the

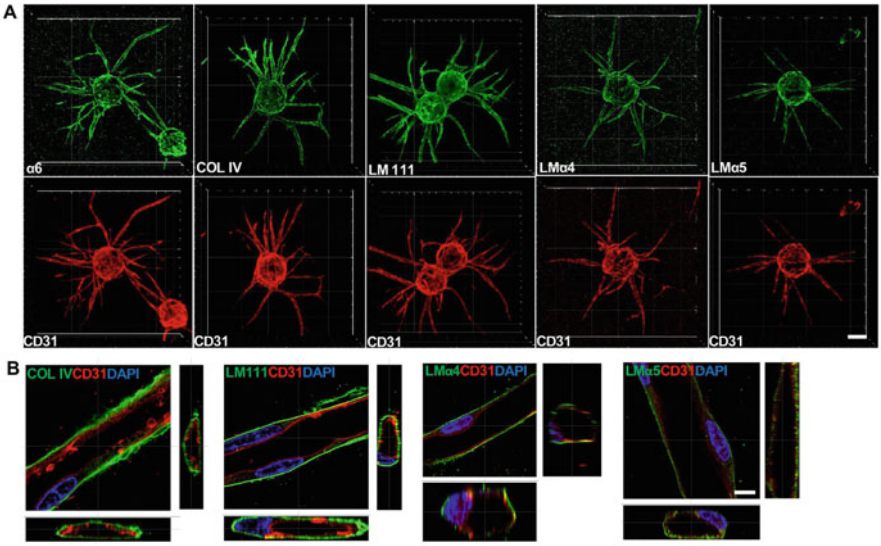


**Fig. 3.4** Laminin-411 and laminin-511 are deposited by growing sprouts in organotypic cultures. (**a**) Confocal images of sprouting endothelial cells stained in green for collagen IV (COLIV), laminin β1 and γ1 chains (LM 111), laminin α4 chain (LM-α4) and laminin α5 chain (LM-α5). CD31 staining is shown in red. Scale bar, 100 μm. (**b**) High magnification confocal images of lumenized sprouts and basement membrane components expressed on the endothelial basal surface. Main images show xy sections, with xz and yz shown below. Nuclei are stained with DAPI. Scale bar, 6 μm (Xu et al. 2020)

chemokine receptor (*CXCR4*) and angiopoietin-2 (*ANGPT2*), as well as α5 chain of laminin-511, L*AMA5* (Xu et al. 2020). A similar decrease in the expression of chemokine *CXCR4* receptor and *ANGPT2* was observed when endothelial cells were depleted of the α5 chain of laminin-511, suggesting that the interaction of $\alpha_6$ integrins with laminin-511 significantly contributes to the regulation of these pro-angiogenic genes (Xu et al. 2020). Importantly, the expression of recombinant *CXCR4* in $\alpha_6$-depleted endothelial cells partially rescued endothelial tube formation (Xu et al. 2020). It will be interesting to test whether the expression of recombinant *ANGPT2* alone or in combination with recombinant CXCR4 is sufficient to restore endothelial morphogenesis in the absence of $\alpha_6$ integrins and whether $\alpha_6$ integrins regulate the expression of these pro-angiogenic genes in vivo. Notably, no overlap was observed in the genes regulated by $\alpha_6$ integrins and laminin-411. Of note, Dll4 was upregulated in endothelial cells depleted of the α4 laminin chain, but this increase did not reach statistical significance (Xu et al. 2020).

To distinguish the contribution of $\alpha_6\beta_4$ in organotypic co-culture assays, RNAi was employed to inhibit the expression of the $\beta_4$ subunit (Xu and Laflamme 2022). The depletion of the $\alpha_6\beta_4$ integrin also inhibited endothelial sprouting. Interestingly, this was accompanied by a decrease in the expression of ANGPT2 mRNA. It is unclear whether $\alpha_6\beta_4$ acts alone or collaborates with $\alpha_6\beta_1$ in this regulation. The

depletion of $\alpha_6\beta_4$, however, did not alter the expression of CXCR4, implicating $\alpha_6\beta_1$ in this regulation (Xu and Laflamme 2022).

As discussed above, in vivo studies implicated the $\alpha_3\beta_1$ integrin as a negative regulator of angiogenesis (da Silva et al. 2010). Also, the expression of the $\alpha_3\beta_1$ integrin was enhanced in endothelial cells depleted of $\alpha_6$ integrins in bead sprout assays (Xu et al. 2020), suggesting a potential inhibitory role in organotypic assays, as well. Surprisingly, RNAi-dependent depletion of $\alpha_3\beta_1$ also inhibited endothelial sprouting in co-culture angiogenesis assays. However, this inhibition was not accompanied by the downregulation of *CXCR4*, *ANGPT2*, or *LAMA5* expression, suggesting that $\alpha_3\beta_1$ regulates sprouting by distinct mechanisms. This is consistent with the inability of $\alpha_6$ integrins to compensate for the loss of $\alpha_3\beta_1$ and vice versa. Notably, the expression of neuropilin-1 (NRP1) mRNA was most significantly decreased in $\alpha_3$-depleted cells (Xu and Laflamme 2022). NRP1 is enriched in endothelial tip cells and functions as a co-receptor for VEGFR2 receptor signaling (Kofler and Simons 2015). Thus, the downregulation of NRP1 expression may be responsible for the inhibition of sprouting observed when endothelial cells are depleted of $\alpha_3\beta_1$. A comparison of gene expression regulated by the $\alpha_3\beta_1$, $\alpha_6\beta_1$, and $\alpha_6\beta_4$ integrins suggested that these laminin-binding integrins regulate distinct but overlapping sets of genes previously identified to be important in angiogenesis (Xu and Laflamme 2022).

The Tie-1-dependent deletion of $\alpha_3$ resulted in increased mRNA expression of VEGFR2 in $\alpha_3\beta_1$-deficient endothelial cells (da Silva et al. 2010). However, depletion of $\alpha_3\beta_1$ in bead sprout assays did not result in increased VEGFR2 expression. In fact, RNAi-dependent depletion of the $\alpha_3$ subunit resulted in a small but significant decrease in VEGFR2 with two of the three siRNA targeting sequences (Xu and Laflamme 2022). The differences in the effects of depleting the endothelial expression of $\alpha_3\beta_1$ in vivo versus in cell culture experiments may be due to the timing when $\alpha_3\beta_1$ expression is lost. As mentioned above, expression of $\alpha_3\beta_1$ in ec-Itga3-null mice appears to occur at some point after the initial response to angiogenic stimuli (da Silva et al. 2010); in contrast, in bead sprout assays, the expression of $\alpha_3\beta_1$ was already inhibited before the onset of the assay. Thus, this in vivo model may not be ideal to examine the role of $\alpha_3\beta_1$ during the initial steps of angiogenesis. Perhaps the use of a promoter that is active in quiescent endothelial cells to drive the inducible expression of Cre recombinase, such as the VE-Cadherin promoter may be better for this purpose.

The stable interaction of endothelial cells with their underlying basement membrane promotes vessel stability. As mentioned above, the endothelial expression of laminin-511 promotes endothelial barrier function (Song et al. 2017). Since $\alpha_6$ integrins, but not $\alpha_3\beta_1$, were shown to regulate the endothelial expression of laminin-511, the effect of deleting $\alpha_6$ integrins or $\alpha_3\beta_1$ was examined in the planar co-culture assay ((Xu et al. 2020) and Xu and LaFlamme, unpublished data). The inducible expression of either an $\alpha_6$, $\alpha_3$, or non-targeting shRNA was used to inhibit integrin expression after the formation of endothelial tubes and to analyze the effect on the maintenance of endothelial tube stability. Indeed, depletion of $\alpha_6$ integrins after the establishment of endothelial tubes resulted in the loss of detectable

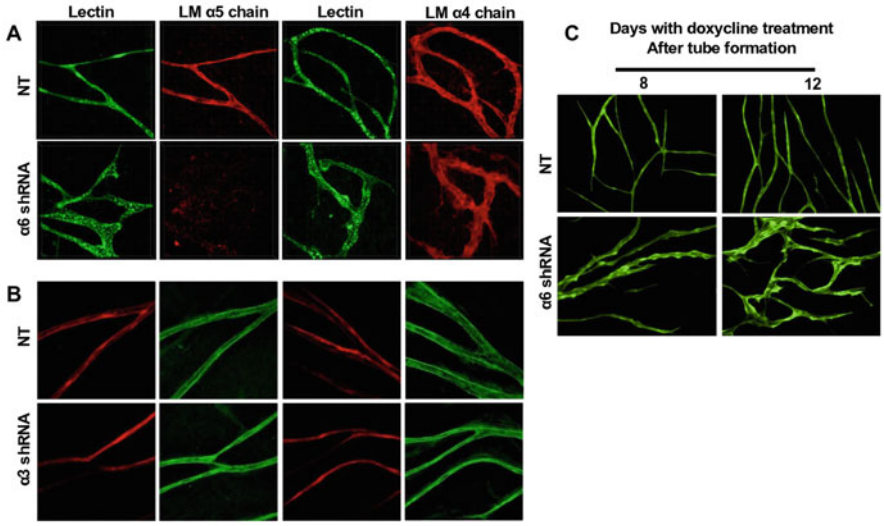


**Fig. 3.5** Laminin-511 expression and tube morphology are regulated by $\alpha_6$ integrins and not by the $\alpha_3\beta_1$ integrin. Planar co-cultures were set up with endothelial cells transduced with lentiviral vectors to direct the doxycycline-inducible expression of $\alpha_6$ (**a**), $\alpha_3$ (**b**) or non-targeting (NT) shRNA, together with fluorescent protein as a readout of shRNA expression. (**a**) After the formation of endothelial tubes, cultures were treated with doxycycline for up to 8 days and imaged. Induction of $\alpha_6$-targeting led to the loss of laminin-511 expression and changes in endothelial tube morphology (Xu et al. 2020). (**b**) This did not occur following the induction of non-targeting or $\alpha_3$-targeting shRNAs. (**c**) Changes in tube morphology are even more pronounced 12 days after induction of $\alpha_6$ shRNA (Xu et al. 2020)

laminin-511 expression, as well as normal tube morphology (Fig. 3.5). Notably, the expression of laminin-411 was maintained as expected (Fig. 3.5 and (Xu et al. 2020)). The depletion of $\alpha_3\beta_1$ integrins did not affect laminin-511 expression or tube morphology (Fig. 3.5). This suggests an important role for the association of $\alpha_6$ integrins and laminin-511 in stabilizing endothelial structures. Unfortunately, the role of $\alpha_6\beta_4$ in the process was not examined in a similar assay, as the endothelial expression of both $\alpha_6\beta_4$ and laminin-511 have been associated with promoting endothelial barrier function in vivo (Welser et al. 2017; Wu et al. 2009).

Laminin-binding integrins likely regulate angiogenesis by multiple mechanisms, as they are known to activate pathways to promote migration and invasion not only in endothelial cells, but in other cellular contexts. Additionally, the laminin-binding integrins, $\alpha_3\beta_1$, $\alpha_6\beta_1$, and $\alpha_6\beta_4$ form complexes with the tetraspanin CD151 (Sterk et al. 2002). *Cd151*-null mice exhibit decreased angiogenesis in multiple in vivo and ex vivo models, including tumor angiogenesis and aortic ring explant assays (Takeda et al. 2007). Additionally, integrin-laminin-dependent activation of multiple signaling pathways was decreased in *Cd151*-null endothelial cells that adhered to laminin (Takeda et al. 2007). In the case of $\alpha_3\beta_1$, CD151 forms a complex with the membrane-anchored matrix metalloproteinase MT1-MMP (*MMP14*) and $\alpha_3\beta_1$ to

promote appropriate proteolysis, and the loss of CD151 results in a dramatic loss of $\alpha_3\beta_1$/MT1-MMP association (Yanez-MO et al. 2008), suggesting an additional mechanism by which $\alpha_3\beta_1$ can contribute to angiogenesis.

## 3.9 Concluding Remarks

Mouse genetic models have provided important insights into mechanisms that both positively and negatively regulate angiogenesis and vessel stability. These studies have highlighted the importance of individual laminins and laminin-binding integrins in limiting angiogenesis and inflammation. Organotypic cell culture models have provided additional insight into the underlying molecular mechanisms. Cross-fertilization of ideas regenerated from in vivo and cell culture experimental approaches are likely to facilitate the identification of new therapeutic targets to limit pathological angiogenesis and inflammation.

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# Chapter 4
# Collagens and Collagen-Degrading Enzymes in the Regulation of Angiogenesis


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**Abstract** The role of extracellular matrix (ECM) proteins in the regulation of angiogenesis has been the focus of numerous studies aiming at understanding the interplay of endothelial cells with their microenvironment. Collagens are the most abundant ECM proteins that regulate angiogenesis through complicated, well-orchestrated mechanisms that are not elucidated yet for all collagen types. It seems that collagens can have both stimulatory and inhibitory effects on angiogenic processes, depending on the type of collagen and the presence and activity of the numerous enzymes that act proteolytically and either free growth factors from the ECM or result in the generation of the inhibitory collagen fragments known as matrikines. In the present chapter, we summarize the basic knowledge on the effect of collagens, collagen-degrading enzymes, and collagen fragments on angiogenesis in vitro and in vivo, highlighting the complexity of the described data and the necessity to develop better models to include the heterogeneity of the collagen-related microenvironment for the regulation of vessel growth.

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## 4.1 Collagens: Introduction

The term "collagen" derives from the synthesis of two Greek words, "colla" ("κόλλα") which stands for glue, and "gen" (comes from "γένεση," genesis) which means the process of giving birth to and was first described as a component of tissues that has the potential to produce glue when boiled (Sorushanova et al. 2019). Collagens represent approximately 30% of the human body's total protein mass. The most recent definition is that collagen is the major extracellular matrix (ECM) molecule that self-amasses into cross-striated fibrils, provides support for cell growth, and is responsible for the mechanical resilience of connective tissues (Sorushanova et al. 2019). The collagen family consists of 28 types, with types I, II, and III comprising 80–90% of the total collagen in the human body. Collagens are the most abundant ECM proteins in vertebrate animals, and their ubiquity throughout the animal kingdom indicates their importance in biological viability (Ricard-Blum 2011).

Collagen types are symbolized by Roman numerals and Greek letters identify the polypeptide chains of each type. Collagens are divided into six groups based on the location, size, and distribution of their triple-helical domains: (a) fibril-forming collagens, (b) fibril-associated collagens with interrupted triple helices (FACITs), (c) network-forming collagens, (d) membrane collagens, (e) multiplexins, and (f) others (Table 4.1) (Bielajew et al. 2020; Sorushanova et al. 2019). All collagens have N and C terminus non-collagenous (NC) domains that have a non-triple-helical structure.

Fibril-forming collagens form triple helices of uninterrupted Gly-X-Y repeats, approximately 300 nm in length, in which X usually represents proline and Y hydroxyproline. Collagen fibrils can be formed by either one collagen type (homotypic fibrils) or by mixtures of different collagen types (heterotypic fibrils). The key feature of the FACITs group is that they do not form fibrils but they associate with fibril surfaces. The triple-helical Gly–X–Y repeats of these collagens are multiply interrupted and by associating with collagen fibrils, they add

**Table 4.1** Collagen groups and their features (Bielajew et al. 2020; Gordon and Hahn 2010; Sorushanova et al. 2019)

| Group name | Features | Collagen types |
|---|---|---|
| Fibril-forming collagens | Long triple-helical domains of uninterrupted Gly-X-Y | I, II, III, V, XI, XXIV, XXVII |
| Fibril-associated collagens with interrupted triple helices (FACITs) | Do not form fibrils but associate with fibril surfaces | IX, XII, XIV, XVI, XIX, XX, XXI, XXII |
| Network-forming collagens | Do not form fibrils—Form repeating patterns | IV, VIII, X |
| Membrane collagens | Span the cell membrane | XIII, XXIII, XXV |
| Multiplexins | Multiple interruptions in the central triple-helical domain | XV, XVIII |
| Others | Do not belong to any of the previous groups | VI, VII, XXVIII |

functionality to them. Among the network-forming collagens, collagen IV is the most important and is the main component of basement membranes (BM). The triple helices of collagen IV form polygonal networks that interact with other ECM components, such as laminins and proteoglycans (Khoshnoodi et al. 2008). The membrane collagens possess transmembrane domains and project their triple-helical domains outward into the extracellular space. Multiplexins include the types XV and XVIII collagen and contain multiple triple-helical domains interrupted by NC sequences that carry glycosaminoglycans (GAGs), forming proteoglycans that are widely expressed and regulate numerous biological processes (Bretaud et al. 2020; Elamaa et al. 2003; Hurskainen et al. 2010; Saied-Santiago and Bülow 2018). Last, the types VI, VII, and XXVIII collagen do not belong to any of the previous groups. Collagen VI is nonfibrillar (Cescon et al. 2015), collagen VII is a component of anchoring fibrils that provide stability to BM (Chung and Uitto 2010) and collagen XXVIII belongs to the class of von Willebrand factor A domain-containing proteins and is present in the peripheral nervous system in all glial cells when myelin is absent (Gebauer et al. 2016). The distribution of the different collagen types is shown in Table 4.2.

## 4.2 Collagens and Angiogenesis

The vascular ECM consists of two distinct parts: BM and interstitial matrix. The BM is mainly composed of collagen IV and minor amounts of other collagens, such as types XVIII or XIX, fibronectin, laminin, proteoglycans, and nidogens. The interstitial matrix primarily consists of cross-linked collagen I and elastin (Sorushanova et al. 2019). The vascular ECM components interact with endothelial cell integrins and thus regulate endothelial cell proliferation, migration, morphogenesis, and survival, all of which are critical for angiogenesis (Davis and Senger 2005).

Collagen I is long known to induce angiogenesis in vitro and in vivo and is being used as a model substrate that stimulates the organization of endothelial cells into tubule-like structures (Auler et al. 2017; Cross et al. 2010; Folkman and Haudenschild 1980; Koh et al. 2008; Montesano et al. 1983; Morgan et al. 2013; Schor et al. 2001; Sweeney et al. 1998; Tetzlaff and Fischer 2018; van Kempen et al. 2008). The receptors for collagen I on endothelial cells are the $\alpha_1\beta_1$ and $\alpha_2\beta_1$ integrins (Davis and Camarillo 1996), with the latter being most important for collagen I signaling based on the observation that inhibition of $\alpha_2\beta_1$ but not $\alpha_1\beta_1$ integrin disrupts tube formation in collagen I gel angiogenesis assays (Li et al. 2022; Sweeney et al. 2003). In the same line, $\alpha_2\beta_1$ integrin has higher affinity and mediates cell binding to the fibrillar collagen I, while $\alpha_1\beta_1$ integrin binds the monomeric form and may affect fibril morphogenesis (Xu et al. 2000). In vivo, the expression of both $\alpha_1\beta_1$ and $\alpha_2\beta_1$ integrins is upregulated by vascular endothelial growth factor A (VEGFA), and their inhibition results in the abolishment of VEGFA-induced angiogenesis (Senger et al. 1997). Stimulation of endothelial cells with collagen I fibrils

**Table 4.2** Distribution of collagens (Banyard et al. 2003; Bielajew et al. 2020; Bretaud et al. 2020; Gebauer et al. 2016; Gonçalves et al. 2019; Gelse et al. 2003; Hjorten et al. 2007; Koch et al. 2003, 2004; McGuire et al. 2014)

| Type | Distribution |
|---|---|
| Fibril-forming collagens | |
| I | Bone, dermis, tendons and ligaments, blood vessel wall, adipose tissue, skeletal muscle, heart valve, cornea |
| II | Cartilage, vitreous body, intervertebral disc |
| III | Skin, blood vessel wall, bone, intervertebral disc, reticular fibers of most tissues |
| V | Lung, cornea, bone, adipose tissue, tendons, ligaments |
| XI | Cartilage, vitreous body |
| XXIV | Cornea, bone |
| XXVII | Bone, cornea, trachea, lung, skin |
| FACITs | |
| IX | Cartilage, vitreous body, cornea |
| XII | Perichondrium, ligaments, tendons |
| XIV | Dermis, tendons, blood vessel wall, placenta, lung, liver |
| XVI | Amnion, hyaline cartilage, skin |
| XIX | Central neurons, BMs |
| XX | Corneal epithelium, embryonic skin, sternal cartilage, tendon |
| XXI | Blood vessel wall |
| XXII | Tissue junctions in the muscle, tendons, heart, articular cartilage, skin |
| Network-forming collagens | |
| IV | Heart valve, blood vessels, BMs |
| VIII | Cornea Descemet's membrane, blood vessel wall |
| X | Hypertrophic cartilage |
| Membrane collagens | |
| XIII | Epidermis, bone, cartilage, intestine, lung, liver |
| XXIII | Heart, brain, prostate |
| XXV | Skeletal muscle |
| Multiplexins | |
| XV | BMs, kidney, pancreas |
| XVIII | BMs, lung, liver |
| Others | |
| VI | Skin, adipose tissue, skeletal muscle, cornea |
| VII | Skin, BMs, oral mucosa, cervix |
| XXVIII | Peripheral nervous system |

results in redistribution and clustering of $\alpha_2\beta_1$ integrin to their apical site, thus inducing angiogenesis (Turner et al. 2020).

One of the first observations that has highlighted the importance of collagen on angiogenesis involved the use of inhibitors of collagen helix formation and cross-linking, which thus prevent collagen deposition and inhibit angiogenesis in vivo (Ingber and Folkman 1988). The notion that angiogenesis is regulated by collagen production has been further supported by observations showing a positive

correlation between collagen production/deposition and new blood vessel formation in vitro and in vivo (Haralabopoulos et al. 1994; Kirkpatrick et al. 2007; Nicosia et al. 1991; Papadimitriou et al. 1993, 1997; van Kempen et al. 2008). The size of the newly formed microvessels in vitro has been shown to be influenced by the amount of collagen in the ECM (Nicosia et al. 1991) and collagen synthesis inhibitors significantly inhibit angiogenesis in various experimental models of physiological or tumor angiogenesis, (Haralabopoulos et al. 1994; Mammoto et al. 2013; Maragoudakis et al. 1993; Turner et al. 2020). In the same line, the decreased angiogenesis observed in polyvinyl alcohol sponges subcutaneously implanted in the dorsa of aged mice has been associated with the decreased synthesis of collagen I, compared with both collagen I synthesis and angiogenesis in the corresponding young animals (Reed et al. 1998). The endogenous angiogenesis inhibitor thrombospondin 1 (TSP1) has been shown to decrease the mRNA levels of collagen I$\alpha_1$, I$\alpha_2$ III$\alpha_1$, and IV$\alpha_1$ polypeptides and the protein levels of collagen I. The absence of TSP1 leads to enhanced angiogenesis, which is reversed by the downregulation of collagen I biosynthesis, supporting the significance and the regulatory role of collagen I for angiogenesis (Zhou et al. 2006).

Besides type I collagen, type III collagen seems to also regulate angiogenesis and be even more effective in enhancing tube formation in cultures of human endothelial cells (Kanzawa et al. 1993), although data are limited.

Type IV collagen is a major component of the blood vessels' BM. It is produced by endothelial cells and has been implicated in the regulation of angiogenesis (Maragoudakis et al. 1988). In the rat aortic ring in vitro model of angiogenesis, when collagen IV was added in gels of type I collagen, newly formed vessels were elongated, and vascular regression was prevented (Bonanno et al. 2000). This is in line with a more recent study showing that collagen I is supportive of endothelial cell migration and collagen IV supports vascular integrity (Turner et al. 2020). Collagen IV enhances endothelial cell proliferation, migration, and tube formation and mediates the angiogenesis-promoting effect of nitric oxide through $\alpha_v\beta_3$ integrin and focal adhesion kinase signaling in lung endothelial cells (Wang and Su 2011). Secretion and subsequent deposition of collagen IV by endothelial cells are strictly regulated and determine the consecutive steps of the angiogenic cascade in vivo and in vitro (Bahramsoltani et al. 2014; Papadimitriou et al. 1993, 1997). Accumulation of collagen IV in the endothelial cell endoplasmic reticulum and disruption of its extracellular secretion has been shown to result in endothelial cell apoptotic death and defective developmental, neonatal, and pathological angiogenesis, further supporting an important implication of collagen IV in endothelial cell survival and angiogenic potential (Chen et al. 2022).

Type VIII collagen, which is synthesized by some but not all types of endothelial cells, seems to be also involved in the regulation of angiogenesis and remodeling of arteries (Sage et al. 1984), and its levels are increased in several different types of cancer that have been associated with angiogenesis (Hansen et al. 2016).

In contrast to the stimulatory effect of the above-described collagens, type VII collagen inhibits angiogenesis in cutaneous squamous cell carcinoma through

binding to $\alpha_2\beta_1$ integrin and suppressing the expression and signaling of transforming growth factor beta (TGFβ) (Martins et al. 2015).

The role of other types of collagens has not been extensively and specifically studied but there is data supporting direct or indirect involvement in the regulation of angiogenesis. For example, mice lacking collagen XV present with collapsed capillaries and degenerating endothelial cells in multiple tissues, suggesting that it may be involved in the survival and proper functioning of endothelial cells during angiogenesis (Eklund et al. 2001). Collagen XIII indirectly enhances bone angiogenesis through binding to $\beta_1$ integrin on osteoblasts and activating the JNK-ERK1/2 pathway that results in enhanced VEGFA expression and increased bone angiogenesis (Koivunen et al. 2019).

## 4.3 Collagen-Degrading Enzymes and Angiogenesis

Matrix metalloproteinases (MMPs) or matrixins are ubiquitously expressed in all organisms and contain $Zn^{2+}$ in their active site. They belong to the metzincin family of zinc-endopeptidases which also include astacins and adamlysins that are both involved in procollagen processing. Up to date, 25 different types of MMPs have been identified in vertebrates (24 of which in humans) and are classified as matrilysins (MMP7, MMP26), collagenases (MMP1, MMP8, MMP13, MMP18), gelatinases (MMP2, MMP9), stromelysins (MMP3, MMP10, MMP11), metalloelastase (MMP12), membrane-tethered MMPs (MMP14, MMP15, MMP16, MMP17, MMP23, MMP24, MMP25) and other MMPs (MMP19, MMP20, MMP21, MMP22, MMP27, MMP28), with selectivity for different types of collagens and other ECM proteins (Table 4.3). The majority of MMPs share a common protein core that consists of a hydrophobic signal peptide at the N-terminus that is responsible for the proMMP latency, a 170 amino acid catalytic domain with the zinc-ion binding site, a 200 amino acid hemopexin C-terminal domain that determines the substrate specificity, and a proline-rich hinge region as linker between the catalytic and the hemopexin domains. In MMP7, MMP23, and MMP26, the hinge region and the hemopexin domain are absent; furthermore, MMP23 incorporates a cysteine-rich domain and an immunoglobulin domain (Jabłońska-Trypuć et al. 2016). Moreover, some MMPs, such as MMP12, may perform functions that do not involve their catalytic activity (Houghton et al. 2009). Almost all MMPs are synthesized as inactive pro-enzymes called zymogens and except for the membrane-tethered MT-MMPs, they are secreted into the extracellular space. MT-MMPs, as well as MMP11, MMP21, and MMP28 belong to the group of furin-activatable MMPs, as between the propeptide and the catalytic domain there is a furin recognition sequence. This sequence is recognized intracellularly by furin-like proteases leading to endoproteolytic cleavage of the propeptide. Thus, MMP11, MMP21, and MMP28 are already in active state when secreted in the extracellular space and MT-MMPs are already activated when they are localized at the plasma membrane (Cui et al. 2017; Fanjul-Fernández et al. 2010; Laronha and

**Table 4.3** Classification of MMPs (Andreuzzi et al. 2017; Bajbouj et al. 2021; Barksby et al. 2006; Bigg et al. 2007; Cui et al. 2017; Han et al. 2016; Itoh 2015; Mimura et al. 2009; Motrescu et al. 2008; Taddese et al. 2010; Uría and López-Otín 2000; Visse and Nagase 2003)

| Enzyme | Position | Collagen substrate | Non-collagen substrate | Angiogenesis effect |
|---|---|---|---|---|
| MMP1 (collagensase-1) | Secreted | Collagens I, II, III, VII, VIII, X | Aggrecan, gelatin, proMMP2, proMMP9, nidogen, perlecan, proteoglycan link protein, serpins, tenascin C, versican | Stimulates angiogenesis directly and indirectly (Agarwal et al. 2010) Generation of restin—inhibition of angiogenesis (Ma et al. 2006) |
| MMP2 (gelatinase-A) | Secreted | Collagens I, II, III, IV, V, VII, X, XI, XIV | Gelatin, fibronectin, elastin, aggrecan, laminin, nidogen, versican, proteoglycan link protein, active MMP9, CCL7, CXCL12, TGFβ, proMMPs (1,2,9,13) | Stimulates angiogenesis in vitro and in vivo (Ohno-Matsui et al. 2003) Generation of angiostatin—inhibition of angiogenesis (Brauer et al. 2011) |
| MMP3 (stromelysin-1) | Secreted | Collagens I, II, III, IV, V, VII IX, X, XI | Gelatin, Aggrecan, decorin, elastin, fibronectin, laminin, nidogen, perlecan, proteoglycan link protein, versican, proMMPs (1,7, 8, 9, 13) and other | Stimulates angiogenesis (Frieling et al. 2020; Jin et al. 2006) Generation of endostatin—inhibition of angiogenesis (Brauer et al. 2011; Heljasvaara et al. 2005) |
| MMP7 (matrilysin-1) | Secreted | Collagens II, III, IV, IX, X, XI | Elastin, gelatin, aggrecan, laminin, syndecan-1, fibronectin, E-cadherin, proMMPs (1,7,8,9,13) and other | Stimulates angiogenesis directly and indirectly (Ito et al. 2009; Zhang et al. 2012) Generation of endostatin and angiostatin—inhibition of angiogenesis (Brauer et al. 2011; Heljasvaara et al. 2005) |
| MMP8 (collagenase-2) | Secreted | Collagens I, II, III, VII, VIII, X | Gelatin, aggrecan, elastin, fibronectin, laminin, nidogen | Stimulates angiogenesis in vitro and in vivo (Fang et al. 2013) |
| MMP9 (gelatinase-B) | Secreted | Collagens I, III, IV, V, VII, X, XI, XIV | Gelatin, elastin, aggrecan, laminin, fibronectin, nidogen, proteoglycan link protein, versican, Multimerin-2, | Stimulates angiogenesis in vitro and in vivo (Andreuzzi et al. 2017; Ardi et al. 2009; Leu et al. 2016; Xu et al. 2001a, b) Generation of |

(continued)

**Table 4.3** (continued)

| Enzyme | Position | Collagen substrate | Non-collagen substrate | Angiogenesis effect |
|---|---|---|---|---|
| | | | proMMP9, proMMP13, and other | tumstatin, endostatin, and angiostatin—inhibition of angiogenesis (Brauer et al. 2011; Hamano et al. 2003; Heljasvaara et al. 2005) |
| MMP10 (stromelysin-2) | Secreted | Collagens III, IV, V, IX, X | Gelatin, aggrecan, elastin, laminin, nidogen, fibrinogen, proMMPs (1,7,8,9,13) | Acts as a pro-angiogenic factor (Heo et al. 2010) |
| MMP11 (stromelysin-3) | Secreted | Collagen VI | Aggrecan, fibronectin, laminin | No effect on angiogenesis has been reported |
| MMP12 (macrophage metalloelastase) | Secreted | Collagen I, III IV | Elastin, laminin, fibronectin | Inhibits angiogenesis in vitro and in vivo (Brauer et al. 2011; D'Alessio et al. 2004; Li et al. 2012) |
| MMP13 (collagenase-3) | Secreted | Collagens I, II, III, IV, IX, X, XIV | Gelatin, aggrecan, fibronectin, tenascin, laminin, perlecan | Stimulates angiogenesis directly and indirectly (Hattori et al. 2009; Kudo et al. 2012)<br>Generation of endostatin—inhibition of angiogenesis (Heljasvaara et al. 2005) |
| MMP14 (MT1-MMP) | Membrane-tethered | Collagen I, II, III | Gelatin, aggrecan, laminin proteoglycan, CD44, E-cadherin, ADAM9, vitronectin, ICAM-1, perlecan, decorin, integrin $\alpha_v\beta_3$, VEGFR1, LYVE-1 fibronectin, syndecan-1, proMMP2, proMMP13, and others | Stimulates angiogenesis directly and indirectly (Alfranca et al. 2008; Basile et al. 2007; Eisenach et al. 2010; Esteban et al. 2020; Han et al. 2016; Hawinkels et al. 2010; Kaimal et al. 2013; Kang et al. 2019; Mimura et al. 2009; Niewiarowska et al. 2011; Sounni et al. 2002; Zhou et al. 2000) |
| MMP15 (MT2-MMP) | Membrane-tethered | Collagen I, IV | Fibronectin, laminin-1, perlecan, fibrin, nidogen, proMMP2, | Stimulates angiogenesis in vitro and in vivo (Chen et al. 2014; Lafleur et al. 2002) |

(continued)

**Table 4.3** (continued)

| Enzyme | Position | Collagen substrate | Non-collagen substrate | Angiogenesis effect |
|---|---|---|---|---|
| | | | proMMP13, tenascin, aggrecan, gelatin, | |
| MMP16 (MT3-MMP) | Membrane-tethered | Collagens I, III | Gelatin, laminin-1, vitronectin, fibronectin, fibrin, proMMP2, proMMP13, and others | |
| MMP17 (MT4-MMP) | Membrane-tethered | – | Gelatin, ADAMTS4 fibrin/fibrinogen, proTNF | Increased tumor vasculogenesis (Chabottaux et al. 2009). |
| MMP18 Xenopus laevis (collagenase-4) | | Collagens I, II, III | Gelatin | No effect on angiogenesis has been reported |
| MMP19 (RASI-1) | Secreted | Collagens I, IV | Gelatin, aggrecan, fibronectin, laminin, nidogen, tenascin | Generation of angiostatin—inhibition of angiogenesis (Brauer et al. 2011) |
| MMP20 (enamelysin) | Secreted | Collagen V | Aggrecan, cartilage oligomeric protein, amelogenin | Generation of endostatin—inhibition of angiogenesis (Heljasvaara et al. 2005) |
| MMP21 | Secreted | – | Gelatin | No effect on angiogenesis has been reported |
| MMP23A | Membrane-tethered | – | – | No effect on angiogenesis has been reported |
| MMP23B | Membrane-tethered | – | – | No effect on angiogenesis has been reported |
| MMP24 (MT5-MMP) | Membrane-tethered | – | Gelatin, proMMP2, pro-MMP13, chondroitin sulfate, dermatin sulfate, fibrin, fibronectin, laminin-1, N-cadherin, and other | No effect on angiogenesis has been reported |
| MMP25 (MT6-MMP) | Membrane-tethered | Collagen IV | Gelatin, fibrinogen/fibrin, vimentin, proMMP2, cystatin C, fibronectin, and other | No effect on angiogenesis has been reported |
| MMP26 (matrilysin-2) | Secreted | Collagen IV | Fibrinogen, gelatin fibronectin, vitronectin, proMMP2, proMMP9 | Increased angiogenesis in MMP-26-transfected tumors (Yang et al. 2012) |
| MMP27 (human | Secreted | – | Gelatin | |

(continued)

**Table 4.3** (continued)

| Enzyme | Position | Collagen substrate | Non-collagen substrate | Angiogenesis effect |
|---|---|---|---|---|
| MMP-22 homolog) | | | | No effect on angiogenesis has been reported |
| MMP28 (epilysin) | Secreted | – | – | No effect on angiogenesis has been reported |

Caldeira 2020; Ra and Parks 2007). To date, six different MT-MMPs have been identified in humans and are classified as MT1-MMP (MMP14), MT2-MMP (MMP15), MT3-MMP (MMP16), MT5-MMP (MMP24), the glycosylphosphatidylinositol (GPI)—anchored MT4-MMP (MMP17) and MT6-MMP (MMP25) (Cui et al. 2017). Among MT-MMPs only MT4-MMP and MT6-MMP lack the ability to activate proMMP2 (Kojima et al. 2000).

Balance in MMP activity is achieved by the inhibitory action of tissue inhibitors of metalloproteinases (TIMPs) and $a_2$-macroglobulin. TIMPs comprise a family of four enzymes, TIMP1, TIMP2, TIMP3, and TIMP4, which form selective stoichiometric complexes (1:1) with active MMPs and inhibit their intrinsic proteolytic activity (Brew and Nagase 2010; Löffek et al. 2011). The structure of TIMPs is composed of an N-terminal domain containing 125 amino acids and a C-terminal domain containing 65 amino acids, each with six conserved cysteine residues forming three conserved disulfide bonds (Laronha et al. 2020). Inhibition of MMPs by TIMPs has been initially considered the main function of these inhibitors. More recent data, however, suggest that TIMPs may also have other functions, including direct signaling in the cells (Eckfeld et al. 2019). For example, TIMP3 inhibits angiogenesis by blocking the binding of VEGFA to VEGF receptor 2 (VEGFR2), independently of its MMP-inhibitory activity (Qi et al. 2003). TIMP2 inhibits VEGFA-induced signaling and migration of endothelial cells by enhancing the expression of the membrane-anchored MMP inhibitor RECK (Oh et al. 2004), which is a key regulator of ECM integrity and angiogenesis (Oh et al. 2001). TIMP2 through its N-terminal domain also interacts with $\alpha_3\beta_1$ integrin and through regulation of tyrosine phosphatase interaction with various receptors, it inhibits VEGFA-stimulated endothelial cell growth in vitro (Seo et al. 2003, 2011).

MMPs have been linked to the regulation of angiogenesis (Deryugina and Quigley 2015), with MMP9 being the first MMP to be correlated with angiogenesis regulation in the growth plate of homozygous mice with a null mutation in the *mmp9* gene (Vu et al. 1998). Inhibition of MMP9 and MMP2 secretion concomitantly with stimulation of TIMP1 secretion by IL-10 in prostate cancer cells have been considered responsible for inhibition of angiogenesis in co-culture with endothelial cells in vitro (Stearns et al. 1999). Inhibition of MMP9 seems to inhibit prostate cancer angiogenesis (London et al. 2003) and stimulation of MMP2 secretion by pituitary tumor transforming gene (Malik and Kakar 2006) or stimulation of MMP9 by late

SV40 factor (Santhekadur et al. 2012) or an inhibitor of DNA binding/differentiation 1 (Id1) in endothelial progenitor cells (Su et al. 2013) stimulate angiogenesis in vitro and in vivo. In the same line, the expression of both MMP2 and MMP9 positively correlated with angiogenesis in colorectal adenocarcinoma (Kim and Kim 1999). MMP genetic or pharmacological inhibition inhibits collagen degradation and endothelial cell differentiation in vitro (Jia et al. 2000; Ohno-Matsui et al. 2003; Pratheeshkumar and Kuttan 2011; Webb et al. 2017) and angiogenesis in vivo (Giraudo et al. 2004; Gondi et al. 2004; Jiang et al. 2021; Kargiotis et al. 2008; Tummalapalli et al. 2007; Wang et al. 2016), supporting the stimulatory effect of MMPs on angiogenesis. Interestingly, retinal angiogenesis was significantly decreased in *Mmp2*$^{-/-}$ compared to the corresponding wild-type mice, while there was no difference in *Mmp9*$^{-/-}$ mice, and this effect was independent of the expression of VEGFA (Ohno-Matsui et al. 2003). Similarly, the vascular density in wound granulation was not affected in *Mmp9*$^{-/-}$ mice but it was significantly lower in *Mmp13*$^{-/-}$ and *Mmp9*$^{-/-}$/ *Mmp13*$^{-/-}$ mice compared to the wild-type mice (Hattori et al. 2009). On the other hand, MMP9 seems to play a crucial role in endothelial progenitor cell mobilization and angiogenesis in experimental critical limb ischemia (Leu et al. 2016). It also cleaves the anti-angiogenic ECM protein multimerin 2 that binds and inhibits VEGFA and other pro-angiogenic molecules, thus promoting angiogenesis, especially in tumors (Andreuzzi et al. 2017). MMP9 secreted from neutrophils can act as a potent inducer of angiogenesis. The reason why neutrophil MMP9 exhibits strong angiogenic potential lies in the fact that human neutrophils do not express TIMPs. Consequently, the secreted MMP9 is free from its endogenous inhibitor (Ardi et al. 2007; Bekes et al. 2011; Deryugina et al. 2014) and potentiates angiogenesis by upregulating the fibroblast growth factor 2 (FGF2)/FGF receptor 2 (FGFR2) pathway (Ardi et al. 2009). Vascular remodeling is also linked to upregulated MMPs in asthma (Bajbouj et al. 2021), especially MMP9 which is upregulated downstream of VEGFA signaling (Lee et al. 2006). One mechanism that has been suggested to contribute to the angiogenesis-promoting effect of MMP9 is that it may proteolytically expose cryptic sites on collagen IV, which then enhance angiogenic properties of human umbilical vein endothelial cells in vitro (Xu et al. 2001a, b), as well as neovascularization and migration of retinal endothelial cells in vivo (Hangai et al. 2002).

Besides MMP2 and MMP9, other MMPs may also affect angiogenesis. MMP1 has been shown to enhance the angiogenic phenotype of endothelial cells by increasing the levels of VEGFR2. This effect seems to be mediated by the MMP1-induced activation of the protease-activated receptor 1 (PAR1), which then activates the nuclear factor-κB (NF-κB) pathway in endothelial cells and leads to increased VEGFR2 and endothelial cell proliferation (Mazor et al. 2013). Activation of PAR1 by MMP1 has been also observed in ovarian cancer cells and leads to increased expression of numerous pro-angiogenic molecules that act in a paracrine manner to cause endothelial cell proliferation, migration, and tube formation in vitro (Agarwal et al. 2010). MMP3 has been suggested to mediate the pro-angiogenic activities of matriptase that enhances tumor growth (Jin et al. 2006) and to be the target of miR-152 that attenuates glioma cell angiogenesis (Zheng et al. 2013). Nanoparticles

that inhibit MMP3 also inhibit angiogenesis (Ma et al. 2015) and MMP3 inhibition inhibits prostate cancer angiogenesis and metastasis (Frieling et al. 2020). A positive effect of MMP7 in angiogenesis has been suggested by studies showing that downregulation of MMP7 abolishes the enhanced angiogenesis observed following secreted protein acidic and rich in cysteine silencing in gastric cancer xenografts (Zhang et al. 2012). In endothelial cells, MMP7 also interacts with and degrades the soluble VEGF receptor 1 (VEGFR1), which acts as a VEGF trap, thus enhancing the interaction of VEGFA with its transmembrane receptors on endothelial cells, stimulating angiogenesis (Ito et al. 2009). Downregulation of MMP8 in human endothelial cells inhibits endothelial cell proliferation, migration, and tube formation in vitro through inhibition of platelet/endothelial cell adhesion molecule-1 expression and decreased nuclear accumulation of β-catenin. Decreased angiogenic potential is also observed in aortic rings, in Matrigel plugs and within the atherosclerotic lesions of *Mmp8*$^{-/-}$/*apoE*$^{-/-}$ compared to *MMP8*$^{+/+}$/*apoE*$^{-/-}$ mice (Fang et al. 2013). VEGFA-stimulated MMP10 secretion in endothelial cells mediates its angiogenic effects in vitro and in vivo (Heo et al. 2010). MMP13 has been suggested to stimulate angiogenesis both directly by acting on endothelial cells, and indirectly by stimulating the secretion of VEGFA by fibroblasts or other cells (Kudo et al. 2012). In contrast to other MMPs, MMP12 seems to have an inhibitory effect on angiogenesis. Endothelial cells have been shown to overexpress MMP12, which cleaves the endothelial urokinase-type plasminogen activator receptor and thus inhibits angiogenesis (D'Alessio et al. 2004). In the same line, lack of MMP12 accelerates revascularization of avascular retina in oxygen-induced retinopathy (Li et al. 2012).

MT1-MMP is the first membrane-type MMP to be discovered and its functions have been extensively studied, especially its role in the activation of proMMP2 (Deryugina et al. 2001). MT1-MMP has low substrate selectivity and can proteolyze a wide variety of ECM components (Itoh 2015), among which collagens I–III (Chun et al. 2004). Although it does not directly affect collagen IV, MT1-MMP cleaves it indirectly, through both the activation (Ohuchi et al. 1997) and the increased expression (Lee et al. 2013) of MMP2. MT1-MMP is expressed in endothelial cells and numerous observations support its involvement in angiogenesis. First, MT1-MMP-deficient mice exhibit defective angiogenesis (Zhou et al. 2000). It has also been shown to have a significant role in endothelial cell invasion of collagen I gels (Aplin et al. 2009; Nisato et al. 2005), and to mediate the pro-angiogenic effects of the endothelial mechanosensor Piezo1 (Kang et al. 2019). MT1-MMP expression is higher in the sprouting endothelium tip cells compared to the stalk cells and decreases when the vessels mature, maybe due to the presence of mural cells (Yana et al. 2007). MT1-MMP interacts with VEGFR1, and its enzymatic activity is necessary for VEGFA-induced endothelial cell activation in vitro and corneal angiogenesis in vivo (Han et al. 2016). It also promotes tumor angiogenesis through proteolytic cleavage of semaphorin 4D in cancer cells into its soluble form that then activates neighboring endothelial cells (Basile et al. 2007). Proteolytic cleavage of corneal decorin has been also suggested as a mechanism for the pro-angiogenic role of MT1-MMP in the cornea (Mimura et al. 2009). MT1-MMP cleaves TSP1, leading

to the release of a TSP1 C-terminal fragment that binds to $\alpha_v\beta_3$ integrin and induces nitric oxide production, vasodilation, and intussusceptive angiogenesis in inflammatory colitis (Esteban et al. 2020). The membrane clustering of MT1-MMP is promoted by prostaglandin E2 (PGE2) to cleave latent TGFβ and release its active form which mediates the pro-angiogenic effects of PGE2 (Alfranca et al. 2008). One of the mechanisms through which lumican inhibits angiogenesis has been suggested to be the inhibition of MT1-MMP in endothelial cells (Niewiarowska et al. 2011). A monoclonal antibody against MT1-MMP has been shown to suppress angiogenesis stimulated by ovarian cancer cells in vitro and in vivo (Kaimal et al. 2013). In breast cancer cells, MT1-MMP not only regulates VEGFR2 cell surface localization but also forms a complex with VEGFR2 and Src that is dependent on the MT1-MMP hemopexin domain and independent of its catalytic activity. Downstream of the MT1-MMP/VEGFR2/Src complex, Akt and mTOR are activated leading to increased VEGFA transcription (Eisenach et al. 2010). In the same cancer cells, overexpression of MT1-MMP, but not MMP2, up-regulates VEGFA expression, enhances in vitro invasiveness through Matrigel-coated filters, and stimulates the in vivo development of highly vascularized tumors in nude mice (Sounni et al. 2002).

Although the pro-angiogenic role of MT1-MMP is supported by numerous data as those described above, it seems that MT1-MMP is not universally required for angiogenesis, possibly depending on the nature of the angiogenic stimulus (Genís et al. 2006). In the same line, proteolytic cleavage of endoglin, a transforming growth factor beta (TGFβ) coreceptor, at a site close to its transmembrane domain by MT1-MMP releases soluble endoglin that negatively regulates angiogenesis in vitro (Hawinkels et al. 2010).

Regarding the role of other MT-MMPs in angiogenesis, the data are deficient and there is a literature gap that needs to be filled. In tumor sections from patients with non-small cell lung cancer, higher expression in MT2-MMP is associated with highly vascularized tumors (Chen et al. 2014) and MT2-MMP appears to be also involved in the vasculogenesis of human umbilical vein endothelial cells in vitro (Lafleur et al. 2002). LINC00482 has been shown to contribute to angiogenesis stimulation by recruiting FOXA1 and promoting MT2-MMP expression in bladder cancer; overexpression of MT2-MMP abrogates the suppressed tumor-associated angiogenesis observed following silencing of LINC00482 (Wang et al. 2020), supporting the pro-angiogenic effect of MT2-MMP. Although MT4-MMP lacks collagenolytic activity, it can affect angiogenesis, especially in tumors. In mice bearing tumors from cancer cells that overexpress MT4-MMP, tumors were highly vascularized with the vessels displaying distorted architecture. At the same time, a decrease in supportive cells, such as pericytes, renders the vessels in the environment of the tumor prone to intravasation (Chabottaux et al. 2009).

## 4.4 Collagen-Derived Anti-angiogenic Peptides

Several extracellular proteins and glycosaminoglycans (GAGs) are enzymatically cleaved, resulting in the release of fragments with biological activities that differ from those of the full-length molecules. Matrikines are called those fragments derived from proteins of the ECM, which have a different biological function than their parent molecule. The most known collagen-derived matrikines are fragments of the NC1 domains of α chains from type IV and XVIII collagens that have an inhibiting role in functions of endothelial cells in vitro and angiogenesis in vivo (Mutgan et al. 2020). Collagen IV-derived matrikines are arresten ($\alpha_1$), canstatin ($\alpha_2$), tumstatin ($\alpha_3$), and tetrastatin ($\alpha_4$). There are also some references for NC1 fragments from $\alpha_5$ and $\alpha_6$ chains, pentastatin and hexastatin, respectively (Colorado et al. 2000; Kamphaus et al. 2000). Collagen XVIII matrikine is called endostatin, derived from the $\alpha_1$(XVIII) chain (O'Reilly et al. 1997).

Arresten was first mentioned in 2000, as a 26-kDa fragment from the NC1 domain of the $\alpha_1$ chain of collagen IV that inhibits the proliferation, migration, and tube formation and induces apoptosis of human endothelial cells. The inhibitory effects of arresten seem to be mediated by binding to $\alpha_1\beta_1$ integrin and inhibiting hypoxia-induced expression of hypoxia-inducible factor 1a and VEGFA, as well as the MAPK signaling pathway in endothelial cells (Mundel and Kalluri 2007; Nyberg et al. 2008). Due to its anti-angiogenic properties, arresten has a limiting role in tumor growth, (Aikio et al. 2012; Lv and Zheng 2012; Nyberg et al. 2008). It has been shown that p53 protein directly activates transcription of the *col4α1* gene and increases the release of arresten in the ECM through an MMP-dependent mechanism (Assadian et al. 2012).

Canstatin is a 24 kDa fragment derived from NC1 domain of collagen IV $\alpha_2$ chain that inhibits endothelial cell proliferation, migration, and tube formation in vitro (He et al. 2003, 2004) and induces apoptosis (Kamphaus et al. 2000). The inhibitory effects of canstatin in human endothelial cells seem to be mediated by its inhibitory effect on the phosphorylation of numerous signaling mediators, such as Akt, focal adhesion kinase (FAK), mammalian target of rapamycin (mTOR), eukaryotic initiation factor-4E-binding protein-1 (4EBP1) and ribosomal S6 kinase (S6K). Apoptosis induction is in line with its stimulatory effect on Fas ligand expression, activation of procaspase 8 and 9 cleavage, and reduction of mitochondrial membrane potential (Panka and Mier 2003). $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins act as receptors for canstatin and mediate the canstatin-induced mitochondrial apoptotic mechanism through procaspase-9 cleavage in both endothelial and tumor cells (Magnon et al. 2005). Both arresten and canstatin, as well as a canstatin-derived peptide also bind to the capillary Morphogenesis Gene 2 protein, which is a transmembrane, integrin-like receptor that also plays a role in angiogenic processes (Finnell et al. 2020). Due to its anti-angiogenic properties, canstatin has anti-tumor effects (Kamphaus et al. 2000). Both N- and C-terminal domains of canstatin also have anti-angiogenic and anti-tumor properties (He et al. 2003; He et al. 2004). Recombinant canstatin decreases the expression of angiopoietin-1 (Ang1) in hypoxia-treated colon carcinoma cells

and inhibits the Ang1-induced endothelial cell proliferation, migration, and tube formation, as well as the expression of Tie-2. In vivo, recombinant canstatin decreases the growth and vascularity of colon cancer by decreasing the expression of Ang1 and Tie-2 (Hwang-Bo et al. 2012).

Tumstatin is the best studied anti-angiogenic fragment derived from the NC1 domain of collagen IV $\alpha_3$ chain following cleavage by MMP9 (Hamano et al. 2003; Hamano and Kalluri 2005). Tumstatin is present in the circulation and acts through binding to $\alpha_v\beta_3$ integrin (Colorado et al. 2000; Hamano et al. 2003; Sudhakar et al. 2003), inhibiting the proliferation and tube formation of endothelial cells from different species and induces apoptosis of the proliferating endothelial cells (Hamano and Kalluri 2005; Maeshima et al. 2000b, 2001, 2002). It also inhibits tumor growth in vivo (Hamano et al. 2003). Tumstatin possesses an N-terminal (amino acids 54-132) domain with anti-angiogenic activity and a C-terminal (amino acids 185-203) domain with anti-tumor cell activity that both bind to $\alpha_v\beta_3$ integrin through two distinct RGD-independent binding sites (Maeshima et al. 2000a). Tumstatin has been suggested to function as an endothelial cell-specific inhibitor of protein synthesis through interaction with $\alpha_v\beta_3$ integrin and inhibition of FAK, phosphatidylinositol 3-kinase (PI3K), Akt, mTOR, and 4EBP1 (Maeshima et al. 2002). Tumstatin expression is downregulated in cancer and may be associated with poor outcomes (Hamano and Kalluri 2005; Luo et al. 2012; Xu et al. 2010). In glioma cells, tumstatin also attenuates the effect of IL-17 that is secreted by Th17 cells on their stemness maintenance (Yu et al. 2021).

Tetrastatin is the NC1 domain of the $\alpha_4$ chain of collagen IV, with well-reported anti-cancer effects in vitro and in vivo (Brassart-Pasco et al. 2012; Lambert et al. 2018; Monboisse et al. 2014). Overexpression of tetrastatin or exogenous administration of recombinant tetrastatin in melanoma cancer cells decreases their cell proliferation and invasion capabilities, possibly due to decreased MT1-MMP activation. The suggested tetrastatin receptor is $\alpha_v\beta_3$ integrin (Brassart-Pasco et al. 2012; Lambert et al. 2018). Although tetrastatin showed no anti-angiogenic effect in the chicken embryo chorioallantoic membrane (CAM) angiogenesis model (Petitclerc et al. 2000), shorter synthetic peptides from tetrastatin exerted anti-angiogenic activity by suppressing endothelial cell proliferation and migration in vitro (Karagiannis and Popel 2007). One such peptide is a 13 amino acid sequence of tetrastatin (QS-13), which exerts anti-angiogenic properties, in contrast to tetrastatin which has no effect. Administration of QS-13 suppresses vascularization of Matrigel plugs in vivo and endothelial cell migration and tube formation in vitro, with no effect on cell proliferation. The potential receptor through which QS-13 mediates its anti-angiogenic effects is $\alpha_5\beta_1$ integrin (Vautrin-Glabik et al. 2020).

Pentastatin and hexastatin are less studied but for both there are some references related to their anti-angiogenic and anti-tumor effects (Koskimaki et al. 2009, 2010; Mundel et al. 2008; Petitclerc et al. 2000). Hexastatin also counteracts the activation of endothelial cells caused by an elastin-derived pro-angiogenic peptide (Gunda et al. 2013).

Endostatin (20 kDa) is released from the NC1 domain of collagen XVIII $\alpha_1$ chain by MMPs, elastase, and cathepsins. Endostatin partially inhibits endothelial cell

proliferation and efficiently arrests the growth of several experimental tumors (Heljasvaara et al. 2005; O'Reilly et al. 1997). It also plays a crucial role in the regression of vitreous blood vessels in the eye (Fukai et al. 2002) and suppresses angiogenesis in the CAM assay (Dixelius et al. 2000). Endostatin has been implicated in the induction of autophagy in endothelial cells by upregulating beclin-1 expression (Nguyen et al. 2009). There are many receptors that have been suggested as endostatin receptors, such as $\alpha_5\beta_1$, $\alpha_v\beta_3$, and $\alpha_v\beta_5$ integrins (Rehn et al. 2001; Sudhakar et al. 2003; Wickström et al. 2002), caveolin-1 (Wickström et al. 2002), cell surface nucleolin (Shi et al. 2007), and VEGFR2 (Kim et al. 2002). Decreased levels of endostatin are detected in wound lysates of mice that lack MT1-MMP expression in epidermis, as well as in cultured keratinocytes. In wounds of these mice, angiogenesis is significantly increased (Zigrino et al. 2012). Endostatin has a potent anti-cancer activity in many different tumors (Folkman 2006) that has been attributed to its ability to decrease VEGFR expression (Hajitou et al. 2002) or to inhibition of the FGF2-activated MAPK signaling pathway (Xiao et al. 2015). The estrogen receptor antagonist tamoxifen increases the activity of MMP9 and the generation of endostatin in estrogen-dependent breast and ovarian cancer cells, thus decreasing their proliferation rate. Through the same mechanism, it also significantly decreases tumor growth and vascularization (Bendrik et al. 2010). Based on its anti-tumor effect on several types of cancer, a structurally modified synthetic recombinant human endostatin (Endostar) has been examined for integration in various cancer treatment guidelines (Hu et al. 2022; Li et al. 2018; Zhan et al. 2022; Zhang et al. 2022a, b), although its large-scale production is facing challenges (Mohajeri et al. 2017).

Restin is a C-terminal fragment of the NC1 domain of collagen XV and has a similar 3D structure and approximately 60% sequence homology with endostatin, features that lead to similar physiological in vitro and in vivo effects (Sasaki et al. 2000). It serves as an anti-angiogenic peptide that inhibits endothelial cell migration in vitro and tumor growth (Ramchandran et al. 1999) and results from MMP1 proteolytic activity (Ma et al. 2006).

Vastatin derives from the $\alpha_1$ chain of type VIII collagen and was first described to inhibit the proliferation and migration of bovine aortic endothelial cells (Xu et al. 2001a, b). Vastatin is expressed in normal liver tissue, but its liver expression is lost in most hepatocellular carcinoma patients and has been used as an anti-angiogenic therapy and a potential biomarker for hepatocellular carcinoma (Shen et al. 2016) and colorectal cancer (Willumsen et al. 2019). Due to its anti-angiogenic properties, vastatin may also have a significant potential therapeutic benefit for glioblastoma (Li et al. 2017).

Lastly, plasmin releases an anti-tumor peptide from the NC1 domain of collagen XIX (Oudart et al. 2015). This fragment was initially shown to inhibit melanoma cell migration and invasion following binding to $\alpha_v\beta_3$ integrin, inhibition of the FAK/PI3K/Akt/mTOR pathway, and activation of GSK3β (Oudart et al. 2016). It also inhibits VEGFA-induced endothelial cell activation through interaction with $\alpha_v\beta_3$ and $\alpha_1\beta_5$ integrins (Oudart et al. 2021).

## 4.5 Non-collagen-Derived Angiostatic Peptides Because of MMPs' Proteolytic Activity

Angiostatin is a 38 kD plasminogen fragment following cleavage by several proteases, including plasmin, elastase, cathepsin D, MMP2, MMP3, MMP7, MMP9, MMP12, and MMP19 (Brauer et al. 2011). Angiostatin selectively inhibits endothelial cell proliferation, is a potent endogenous angiogenesis inhibitor, and seems to be responsible for the inhibition of metastases by the primary tumors (Jung et al. 2003). Angiostatin has been found to suppress the expression of MMP2 and MT1-MMP in lung microvascular endothelial cells, thus inhibiting their migration (Radziwon-Balicka et al. 2013). The NG2 proteoglycan that is expressed on immature but not mature capillary vessels, binds, and internalizes angiostatin, thus enhancing angiogenesis and glioma progression (Chekenya et al. 2002). Inhibition of NO synthase has been shown to enhance MMP2 and MMP9 activities and increase angiostatin levels, which may be responsible for the decreased coronary angiogenesis observed when NO production is compromised due to endothelial dysfunction (Matsunaga et al. 2002). Angiostatin presents in different forms that differ in the number of cringles they contain. All angiostatin molecules, however, increase the expression of p53 protein, affect the mRNA levels of 189 genes related to growth, apoptosis, migration, and inflammation, enhance FasL-mediated signaling pathways, and decrease activation of Akt following binding to Fas, $\alpha_v\beta_3$ integrin, or ATP synthase (Chen et al. 2006). Another mechanism through which angiostatin may inhibit angiogenesis is the inhibition of the hepatocyte growth factor/c-Met signaling pathway and the decreased c-met phosphorylation (Brauer et al. 2011). In vivo studies have shown that the inhibitory effect of angiostatin is mediated by interleukin-12, suggesting that the immune modulating effect is required for effective angiogenesis inhibition by angiostatin (Albini et al. 2009). Angiostatin levels are increased in diabetic rats, in line with the impaired angiogenesis observed following focal ischemia (Zhu et al. 2010) and its expression is decreased by dietary linoleic acid that enhances tumor growth in an animal model (Nishioka et al. 2011). The use of angiostatin in tissue-engineered constructs for fibrocartilage regeneration has resulted in suppressed angiogenesis when implanted in vivo, which may prove to be beneficial for preventing endochondral ossification (Helgeland et al. 2020).

## 4.6 Concluding Remarks

The extensive direct and indirect (fragments upon proteolytic cleavage) roles of collagen members on angiogenesis are an autonomous scientific field that can be only summarized in a book chapter, mentioning the best-characterized effects of specific, well-known, individual members of the collagen family, which have rendered them as important potential targets in the fields of oncology and angiogenesis (Fig. 4.1). Similarly, matrix metalloproteases, the main collagen degrading

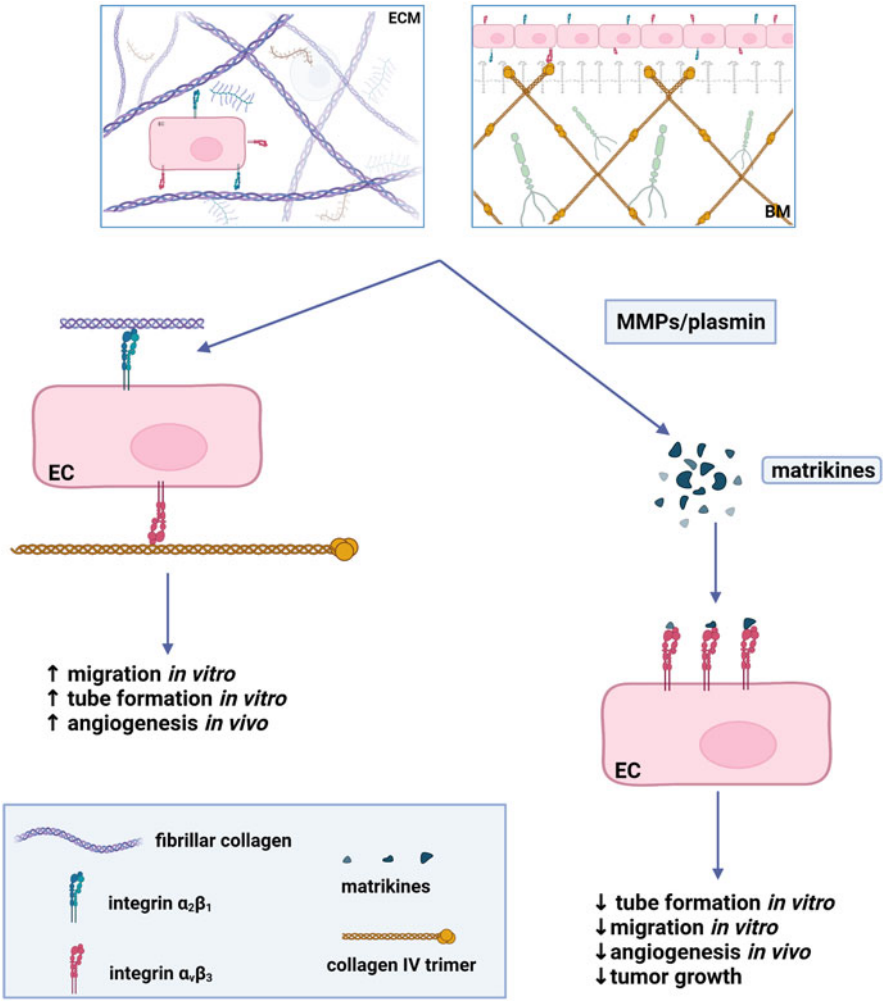


**Fig. 4.1** Schematic representation of the known collagen-related effects on endothelial cells and angiogenesis. Fibrillar collagen I and network-forming collagen IV interact with $\alpha_2\beta_1$ and $\alpha_v\beta_3$ integrins, respectively, and enhance angiogenic functions of endothelial cells. Other collagens, such as type III and VIII also stimulate angiogenesis, while type VII collagen has an inhibitory effect; however, the receptors and signaling pathways are unknown. On the other hand, collagen fragments (matrikines) derived from degradation by numerous MMPs interact mostly with $\alpha_v\beta_3$ integrin and have anti-angiogenic and anti-cancer activity. Other integrins that have been involved in the effect of matrikines are $\alpha_1\beta_1$, $\alpha_v\beta_5$, and $\alpha_5\beta_1$, while VEGFR2, cell surface nucleolin, and caveolin-1 are also shown to act as receptors for endostatin (for more details see text). Created with BioRender.com

enzymes, is a family of numerous proteolytic enzymes with complicated mechanisms of acting on endothelial and other cells and the ECM, thus affecting angiogenesis directly or indirectly, positively, or negatively (Fig. 4.2). The complexity

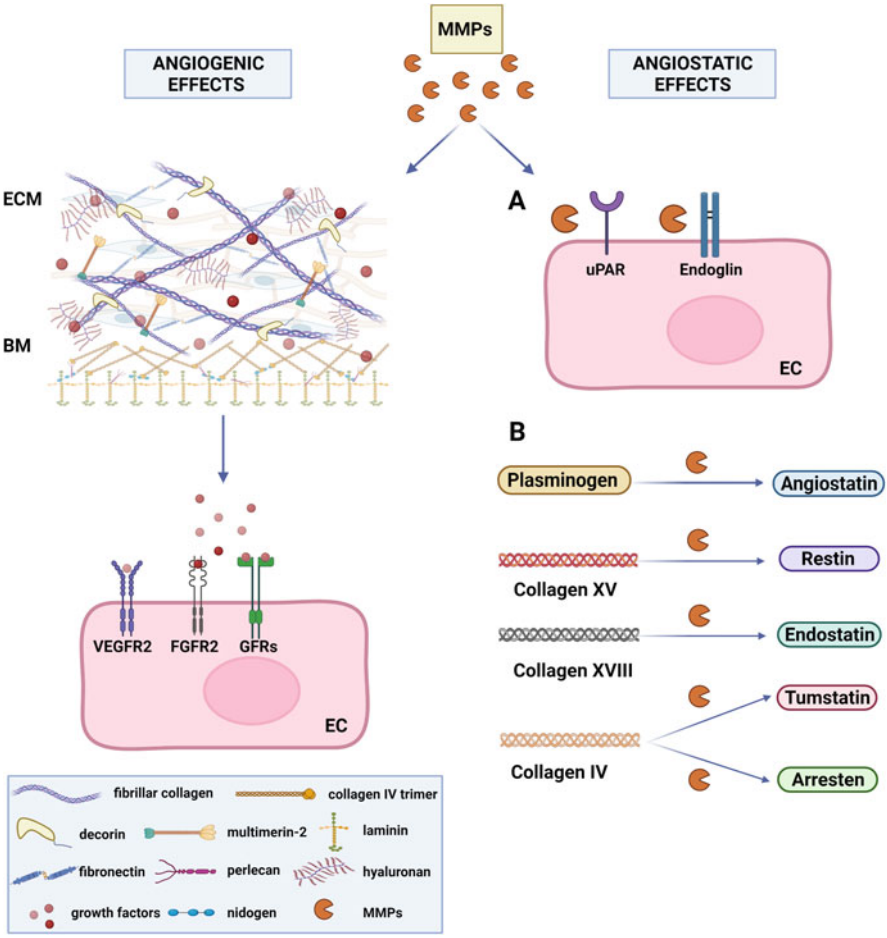


**Fig. 4.2** Schematic representation of MMPs implication in angiogenesis. MMPs exert a bivalent role in regulating angiogenesis. On the one hand (left), MMPs induce angiogenesis, which is mainly attributed to their ability to degrade ECM components. A reservoir of growth factors is embedded in the ECM and can be released upon proteolytic degradation. The secreted growth factors potentiate angiogenic response bound to their receptors on the adjacent endothelial cells. MMPs can also cleave anti-angiogenic components of ECM, such as multimerin-2 and decorin, thus further upregulating angiogenesis. On the other hand (right), MMPs can directly suppress angiogenesis though cleavage of endoglin and uPAR on endothelial cells (**a**) or indirectly, through the release of matrikines that arise from collagens (IV, XV, XVIII) and plasminogen breakdown (**b**). Created with BioRender.com

and the multifaceted role of the different collagen members and collagen-driven products have been and remain the focus of extensive scientific research. Further studies on the effect of the not well-characterized members of this family, as well as the elegant balance or interaction among them, are areas worth investing and should be considered in future preclinical and clinical studies.

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# Chapter 5
# Kruppel-Like Factor 2 and Matrix Metalloproteinases in the Context of Vasculature


**Sarah Anderson, Derek Barthels, and Hiranmoy Das**

**Abstract** Matrix metalloproteinases (MMPs) and Kruppel-like Factor 2 (KLF2) are proteins present in endothelial cells that play opposing roles in these cells in response to angiogenic and inflammatory stimuli. MMPs are endoproteases that play a large part in the degradation of various components of the extracellular matrix (ECM) and tissue remodeling. They are essential mediators of inflammation, angiogenesis, and vascular remodeling. KLF2 is a transcription factor in endothelial cells that helps maintain homeostasis and signals the endothelial cell to enter a state of quiescence. It is induced by unidirectional flow and is anti-inflammatory, anti-thrombotic, and anti-angiogenic, and promotes vasodilation. The goal of this chapter is to provide insight into this complex system by focusing on one cell type, one transcription factor, and one protease family. Herein, we review the functions of these proteins, especially in endothelial cells, and their roles in various processes, including angiogenesis, atherosclerosis, and clotting.

## 5.1 Kruppel-like Factor-2

Kruppel-like Factors (KLFs) are a family of transcription factors expressed in a wide variety of cell types. There are currently 17 known transcription factors that fall into the KLF family. KLF2 is a transcription factor expressed in tissues such as the lungs, spleen, heart, thymus, adipose tissue, skeletal muscle, and vascular endothelium. KLF2 is required for growth and development, as homozygous KLF2 knockout mice do not survive in utero (Turpaev 2020; Gimbrone Jr. and Garcia-Cardena 2013). Studies have shown that KLF2 plays an important role in regulating monocyte differentiation into osteoclasts and mesenchymal stem cell differentiation into osteoblasts. KLF2 inhibits osteoclastic differentiation by inhibiting autophagy. In mesenchymal stem cells, KLF2 modulates autophagy and is in turn modulated by

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autophagy during osteoblastic differentiation. These findings are of particular importance for autoimmune diseases, such as rheumatoid arthritis (Das et al. 2006, 2012, 2019a, b; Laha et al. 2019; Maity et al. 2020; Rolph et al. 2018).

In endothelial cells, KLF2 helps maintain homeostasis and signals for the endothelial cell to enter a state of quiescence. This results in decreased cell turnover and a low proliferation rate. KLF2 expression also causes reduced vessel permeability and represses inflammatory responses to tumor necrosis factor (TNF)-α and interleukin (IL)1β (Nakajima and Mochizuki 2017; Novodvorsky and Chico 2014). It does this by inducing endothelial nitric oxide synthase (eNOS) and thrombomodulin (TM) and preventing the expression of cellular adhesion molecules. These cellular adhesion molecules are critical for immune cells to pass from the blood vessel into the surrounding tissue (Nakajima and Mochizuki 2017; Jha and Das 2017; Hiroi et al. 2009). KLF2 expression is regulated by the transcription factor myocyte enhancer factor-2 (MEF2) and histone deacetylase 5 (HDAC5) (Turpaev 2020; Kumar et al. 2011; Wang et al. 2010). MEF2, which increases KLF2 expression, is induced by extracellular signal-regulated kinase 5 (ERK5) and inhibited by Ras homolog family member A (RhoA) and nuclear factor-kappa B (NF-κB). KLF2 transcription activation is associated with its binding to histone acetyltransferases CREB binding protein (CBP), p300, and p300/CBP-associated factor (PCAF) (Turpaev 2020; SenBanerjee et al. 2004). KLF2 can be induced by pharmacological, as well as pathophysiological factors (Das et al. 2019a; SenBanerjee et al. 2004; Niedzielski et al. 2020; Chiu et al. 2020).

### *5.1.1 Pathways Regulating the Induction of KLF2*

As mentioned earlier, several proteins are responsible for inducing the level of KLF2. Human umbilical vein endothelial cells (HUVEC) exposed to metformin exhibited increased KLF2 expression due to adenosine monophosphate-activated kinase (AMPK)-mediated phosphorylation of HDAC5 at serine 498 (Tian et al. 2019). When phosphorylated, HDAC5 moves from the nucleus to the cytosol. This causes the DNA to unwind from the histone and allows MEF2 access to the KLF2 genetic sequence, thereby promoting greater expression of KLF2. This is supported by a recent study demonstrating that when EA.hy926 cells were exposed to betulinic acid, there was a significant increase in KLF2 and its downstream product, eNOS. The effects observed were mediated by the kinases AMPK and calcium/calmodulin-activated kinase IIα (CaMKIIα), which phosphorylate HDAC5, ERK5, and MEF2C. This phosphorylation resulted in lower nuclear HDAC5 levels and increased phosphorylation of MEF2C by ERK5, which promotes the transcription of KLF2 (Gimbrone and Garcia-Cardena 2013; Lee et al. 2020). Another study shows a similar pathway that was used to induce KLF2 expression by stimulation with steady laminar flow. Steady laminar flow leads to the phosphorylation of HDAC5. This phosphorylation is dependent on the calcium/calmodulin pathway. Additionally, this study showed that flow-mediated phosphorylation and nuclear export of HDAC5

induce the dissociation of HDAC5 and MEF2C. This frees MEF2C to perform transcriptional activity on target promoters, such as KLF2 (Wang et al. 2010; Lee et al. 2012).

### 5.1.2 *Pathways Reducing the Levels of KLF2*

The nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway is a principal inhibitor of KLF2 expression. The NF-κB pathway has a variety of proteins that interact with KLF2 expression and activity, such as cRel, IκB, p50, and p65. p65 binds to the KLF2 gene and recruits histone deacetylase (HDAC) 4 and HDAC5. This reduces KLF2 expression by inhibiting MEF2 transcriptional activity (Fig. 5.1) (Turpaev 2020; Nakajima and Mochizuki 2017). One study demonstrated that the IKK-β protein, which phosphorylates Iκβα to release NF-κB, is a key protein in modulating KLF2's transcription of TM. They showed that IKK-β, independent of p65, inhibited KLF2 genetic expression. This reduced KLF2 expression is key to decreased TM protein expression (Pathak et al. 2014). RhoA, a GTPase protein, has

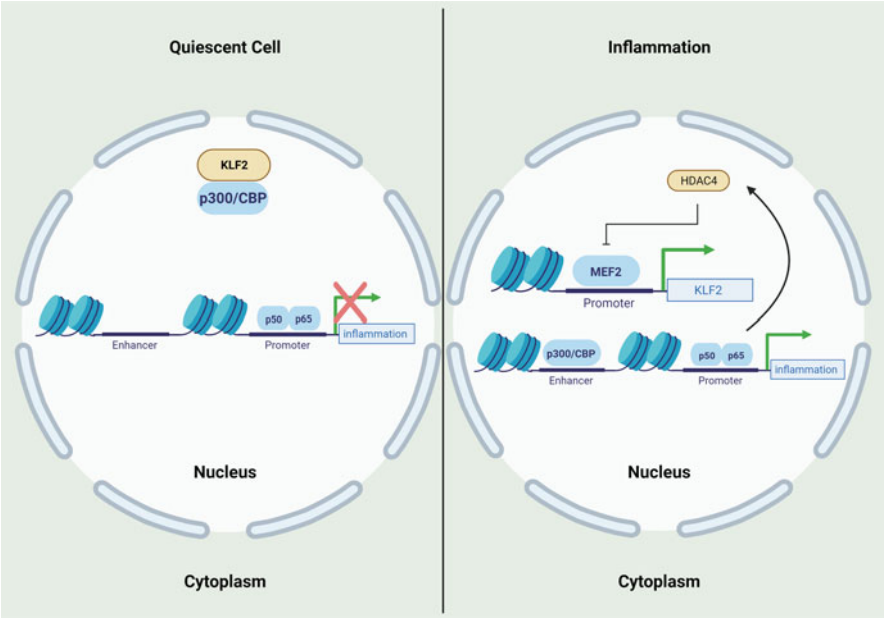


**Fig. 5.1** Crosstalk between KLF2 and the inflammatory mediator NF-κB. In quiescent cells, KLF2 is induced by the binding of MEF2 to the promoter region of the KLF2 gene. This binding is inhibited by HDAC4, which deacetylates the region around the gene, preventing its transcription. HDAC4 is induced through the transcriptional activators p300/CBP, p50, and p65. However, the activity of these inflammatory activators can also be regulated by KLF2. KLF2 binds the p300/CBP complex, preventing the transcription of inflammatory genes. *Created with Biorender*

also been shown to inhibit KLF2 expression by blocking MEF2 activity (Turpaev 2020).

The transcription factor p53 is another molecule that regulates KLF2 expression. P53 is activated during times of stress and is responsible for initiating apoptosis. Kumar et al. demonstrated that p53 inhibits KLF2 expression at the transcriptional level. P53 inhibited KLF2 promoter activity, indicating a role for p53 in suppressing KLF2 transcription. They showed that p53-dependent repression of KLF2 was not carried out through the NF-κB pathway, but rather that p53 represses KLF2 by blocking the promoter site for KLF2, but not on the MEF2 binding site. Instead, p53 binds to a novel 27 bp sequence in the KLF2 promoter site to repress KLF2 expression (Kumar et al. 2011). This data was used to inform the work of Wu et al. who showed that in primary human brain endothelial cells, the accumulation of amyloid-beta peptide induced expression of p53, which inhibited KLF2 and therefore led to downregulation of occludin. This study demonstrates how the interaction between p53 and KLF2 is important in Alzheimer's disease (Wu et al. 2013).

## 5.2 Matrix Metalloproteinases

Matrix metalloproteinases (MMPs) are zinc-dependent endoproteases that play a large role in the degradation of various components of the extracellular matrix (ECM) and tissue remodeling and are absolutely critical for the allostasis of various tissues in the body (Serra 2020; Wang and Khalil 2018). MMPs can promote cell proliferation and differentiation and have been thought to promote angiogenesis, as well as apoptosis and tissue repair (Rohani and Parks 2015). MMPs play important roles in normal biological functions, such as pregnancy and wound regeneration, but are also implicated in pathological conditions like vascular complications, cancer progression, and neurodegenerative disorders (Cabral-Pacheco et al. 2020). There are 28 different members in the MMP family in vertebrates; 23 of them are expressed in humans and 14 of those are expressed in the vasculature (Agren and Auf dem Keller 2020; Cui et al. 2017). MMPs are further classified based on which substrate they interact with: collagenases, gelatinases, stromelysins, matrilysins, and membrane-type (MT) MMPs (Laronha et al. 2020). Although a large number of cell types secrete MMPs, the most prominent sources are dermal fibroblasts, leukocytes, and endothelial cells (Montero et al. 2006; Shin et al. 2019; Larochelle et al. 2011). The MMPs produced by these cells are either secreted or anchored to the cell's plasma membrane. MMPs are primarily localized in connective tissues because that is where many of their primary targets are, but they have also been found in various cell types in the vasculature, indicating that they may serve other physiological roles (Cui et al. 2017). The specificity of MMPs for these functions is essential because certain targets of MMPs, such as collagens I, II, and III, are resistant to proteolysis from more generalized proteinases and need more targeted activity to carry out normal physiological processes (Nissinen and Kahari 2014). There are several ways that activation of MMPs is regulated. For example, MMPs

can be regulated through their genetic expression and tissue compartmentalization (Parks et al. 2004). Additionally, MMPs are typically expressed as zymogens with pro-peptide domains that need to be removed to allow for binding and cleaving of substrates, providing a third method of regulation (Rempe et al. 2016).

In addition to these mechanisms for activation, the functions of MMPs are balanced with endogenous inhibitors, called tissue inhibitors of metalloproteinases (TIMPs). Four classes of TIMPs have been identified thus far and are labeled TIMP1, TIMP2, TIMP3, and TIMP4 (Raeeszadeh-Sarmazdeh et al. 2020). Each TIMP variant differs in its expression in certain tissues and has a distinct affinity profile for which MMPs it inhibits. But, while each TIMP has its own affinities for certain MMPs, there is overlap between each affinity profile (Arpino et al. 2015). The balance of MMPs with TIMPs is vital to the proper regulation of the ECM. Overexpression of MMPs or reduced expression of TIMPs can lead to excessive breakdown of the ECM and cause the nearly complete loss of regulatory capacity of the ECM (Moore and Crocker 2012). Conversely, the overabundance of TIMPs and blanket inhibition of MMPs prevents the remodeling of the ECM that is essential for survival, which underlines the reason why each TIMP has specific binding affinities rather than inhibiting all MMPs (Cabral-Pacheco et al. 2020).

### *5.2.1 MMPs in Regulating Inflammatory Signaling*

While MMPs are primarily thought of as tools for the regulation of the ECM, they also play roles in inflammatory signaling (Franco et al. 2017). The inflammatory mediator TNF activates neutrophils and causes elastase to be released, which activates MMPs. MMPs, in turn, activate latent transforming growth factor-β (TGF-β) released from macrophages to recruit additional neutrophils (Nathan 2002). Some treatments using MMP inhibitors reinforce the association of MMPs with inflammation, such as in the treatment of periodontal disease, where MMP inhibition also decreases inflammation (Checchi et al. 2020). MMPs are also thought to exacerbate tissue destruction in arthritis (Bedoui et al. 2019). Some functions of MMPs in inflammatory signaling are better understood, such as in the case of epithelial wound healing. MMP1 is essential in the closure of wounds, as it alters the $\alpha_2\beta_1$-integrin to a lower affinity integrin. This process allows for the increased migration of cells that carry out the wound healing process as they can more easily attach and detach from different regions of the wound bed (Parks et al. 2004).

Other MMPs can influence inflammatory signaling more directly, by influencing the binding activity of certain chemokines (Van den Steen et al. 2000). For example, MMP2 cleaves four amino acids from the (N)-terminal of CC-chemokine ligand (CCL) 7, turning it into an antagonist for the factor's target receptors. Similar processes happen for the chemokine targets of MMP1, 3, 13, and 14, but the interaction of MMP9 with IL8 increases its chemotactic reactivity. Additionally, MMP13 activates TNF-α in a murine model of inflammatory bowel disease and MMP8 increases the activity of CXC-chemokine ligand (CXCL) 5, which increases

the chemotactic attraction of neutrophils (Nissinen and Kahari 2014; Parks et al. 2004). This shows that MMPs are not exclusively pro-inflammatory factors, but may simply act as another regulator of inflammatory function.

Imbalances in the inflammatory regulation by MMPs can have dire consequences. While the monitory nature of the innate immune system is a significant contributor to the prevention of cancer in the body, chronic inflammation can trigger the progression of a number of cancer variants, including both colon and breast cancers (Coussens and Werb 2002). For example, some malignant tumors secrete TNF-α which can be activated by MMP activity. This, in turn, activates the NF-κB pathway that induces inflammation-driven cancer proliferation (Nissinen and Kahari 2014). MMPs also contribute to immune suppression by cancer cells. MMPs can activate TGF-β in its active form to promote an anti-inflammatory environment and can also cleave IL2Rα on the surface of T cells to prevent T cell proliferation, which is a process carried out by MMP9 in cervical cancer. The activity of MMP11 also decreases the effectiveness of natural killer cells on their cancer targets (Nissinen and Kahari 2014; Sheu et al. 2001). Some MMPs also interact with the protein kinase C (PKC) and p53 pathways, which are responsible for monitoring DNA damage to cells and initiating certain cell pathways, such as the cell cycle, DNA repair, and apoptosis. This, of course, has vital consequences in the development and progression of cancer cells, as they manipulate p53 to ensure their survival (Meyer et al. 2005). MMP9 typically works to break down apoptotic signaling molecules, while activating cell survival factors. The transcription of the MMP9 gene is inhibited by p53 activity, ensuring increased expression of apoptotic factors in cells damaged beyond repair (Liu et al. 2006). For this reason, increased expression of MMP9 is associated with poor prognosis in cancer patients. This increased expression has also been linked to increases in angiogenesis and cancer metastasis (Meyer et al. 2005).

### *5.2.2 MMPs and Endothelial Cells*

The extravasation of immune cells seen in the inflammatory environment of cutaneous wounds is facilitated by the increased permeability of intercellular junctions between endothelial cells (Govindaraju et al. 2019). MMPs are primarily responsible for carrying out this operation. MMPs 2 and 9 cleave the tight junction protein occludin and MMP13 breaks down ZO-1. These processes occur in multiple tissues after injuries, such as in regions near a cutaneous wound or at the blood-brain barrier after a stroke and are even seen in conditions such as epilepsy (Nissinen and Kahari 2014; Rempe et al. 2018).

MMPs are contained in secretory vesicles of endothelial cells so that they can be quickly released, and act as pro-angiogenic factors (Taraboletti et al. 2002). These MMPs are essential for migration and tube formation by endothelial cells. For example, MMP7 promotes the proliferation of endothelial cells and triggers the release of MMPs-2 and -3 (Huo et al. 2002). MMPs play other roles in this process,

such as the breaking of cell-to-cell adhesions. Additionally, the proteolytic activity of MMPs reveals binding sites for $\alpha_v\beta_3$ integrin, which promotes angiogenesis. These sites are available for binding in both angiogenic and cancerous vessels but are covered when vessels are quiescent (Rundhaug 2005).

MMPs that assist in angiogenesis can be produced from multiple sources, including inflammatory cells and cancer cells, as mentioned above, and from endothelial cells themselves. Endothelial cells can be induced to express MMP1 by vascular endothelial growth factor (VEGF). Additionally, endothelial cells constitutively secrete MMP2, but it must be activated. This activation can be achieved in a variety of ways, including endothelial cell exposure to TNFα. TNFα upregulates MT1-MMP, which then activates MMP2 (Rundhaug 2005). MMP2 can also be induced by clotting factors in the aftermath of an injury. Additionally, MMPs can contribute to the release of additional angiogenic molecules. Some factors, such as VEGF, basic fibroblast growth factor (bFGF), and TGF-β, are contained in the ECM and released when the ECM is broken down by MMP activity (Rundhaug 2005).

## 5.3 KLF2 in Endothelial Cells

In endothelial cells, KLF2 is a transcription factor that targets proteins that decrease coagulation and fibrinolysis, increase vascular tone, and promote barrier protection. KLF2 inhibits the expression of adhesion molecules, making the endothelium anti-inflammatory. It also inhibits the expression of proteins that reverse the effects of the proteins described above.

It is well established that eNOS is a downstream product of KLF2. This enzyme is required for the synthesis of nitric oxide (NO), which causes smooth muscle cells to relax, resulting in vasodilation. Vasodilation allows more blood to accumulate in the blood vessel, which is essential in situations such as exercise. Impaired vasodilation contributes to high blood pressure and is a sign of endothelial dysfunction, which puts people at risk for cardiovascular disease (Jha and Das 2017; Heiss et al. 2015).

Another protein downstream of KLF2 is angiotensin-converting enzyme 2 (ACE2). This protein is particularly well-known for cleaving angiotensin II into angiotensins (1–7), which induce anti-inflammatory and anti-oxidant effects (El-Hashim et al. 2012). One study demonstrated that pulsatile shear stress (PSS) upregulates ACE2 partially through the AMPKα2—KLF2 pathway. ACE2 induced by PSS increased eNOS expression and helped reduce endothelial cell proliferation. These results were not seen in oscillatory shear stress (OSS), which is important regarding atherosclerosis. OSS only induced a short-term ACE2 expression that was weaker compared to cells exposed to PSS (Song et al. 2020).

KLF2 also helps maintain endothelial cell membrane integrity. Another study found that KLF2 hemizygous deficient mice showed increased vascular leakage when treated with mustard oil compared to control mice. This occurs because KLF2 promotes transcription of the tight junction protein occludin. Additionally, KLF2 inhibited the contractile action of endothelial cells by inhibiting the phosphorylation

of the myosin light chain (Lin et al. 2010). A separate study found that levels of KLF2 expression determined infarct volume and damage in a mouse model of stroke. This was found to be related to levels of occludin in the endothelial cells (Shi et al. 2013).

KLF2 and MEF2 inhibit NF-κB activation, function, and expression of adhesion molecules. KLF2 binds to CBP/p300, a coactivator required for optimal NF-κB activity in the nucleus. By competing with NF-κB for binding to CBP/p300, KLF2 reduces NF-κB transcriptional activity. This results in a decrease in proteins such as vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), and E-selectin, which are adhesion molecules for immune cells. Furthermore, KLF2 inhibits nuclear translocation of ATF2, one of the heterodimeric components of AP-1, in response to laminar shear stress. AP-1 is a transcription factor that has similar actions as NF-κB in that it promotes inflammation and has increased expression under OSS (Nakajima and Mochizuki 2017; Niu et al. 2019; Atkins and Jain 2007).

## 5.4 Factors that Induce Angiogenesis in Endothelial Cells

Endothelial cells typically have a longer lifespan than most cells in the body and form extremely stable connections with the ECM. These interactions change, however, with the initiation of angiogenesis. To form new vessels, endothelial cells must be able to dissolve their current connections with the ECM, migrate into the ECM, and then form new connections with the ECM to vascularize tissues. To accomplish this, endothelial cells must be able to sense the ECM and how it surrounds them. Integrins are the most common receptors on endothelial cells that perform this function. While there are many types of integrins, $\alpha_v\beta_3$ has been most associated with angiogenesis. The expression of $\alpha_v\beta_3$ is induced when endothelial cells are exposed to growth factors, such as VEGF, and is also induced in hypoxic conditions, which is consistent with angiogenic mechanisms (Iivanainen et al. 2003). However, experiments that inhibit the function or expression of $\alpha_v\beta_3$ have shown that angiogenesis can still occur without these subunits, indicating that there is likely some redundancy in integrin functions. However, experiments that knock out genes for $\beta_1$ integrins resulted in neonatal mortality and high levels of vascular abnormalities. This is likely because the $\beta_1$ subunit makes up part of the dimers that form fibronectin, collagen, and laminin receptors on endothelial cells (Iivanainen et al. 2003). In addition to integrins, endothelial cells express heparan-sulfate proteoglycans (HSPGs) to help regulate both the availability and reactivity of growth factors that can induce or inhibit angiogenesis (Pozzi et al. 2017).

When all the factors above combine and pro-angiogenic signals outweigh anti-angiogenic signals, neovascularization takes place (Fig. 5.2). Fibrinogen will then create a sheath that penetrates the ECM, allowing endothelial cells and endothelial cell progenitors to migrate and proliferate into a space that will become a new blood vessel (Iivanainen et al. 2003; Moccia et al. 2019). The intracellular signaling

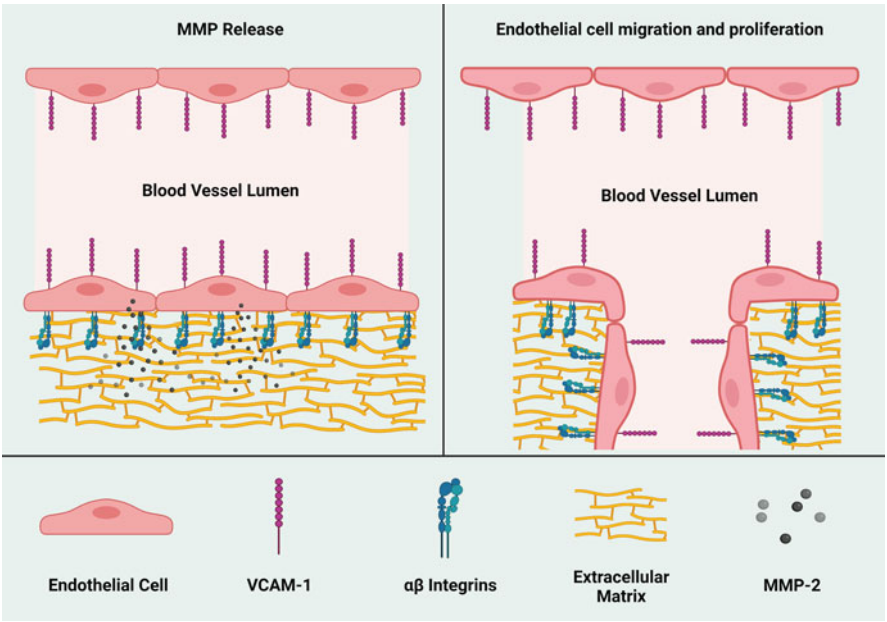


**Fig. 5.2** MMP-mediated initiation of angiogenesis. Endothelial cells form tight bonds with both other endothelial cells, as well as with the ECM. For endothelial cells and their progenitors to penetrate the ECM, these bonds must be broken down. To accomplish this, endothelial cells release pro-MMP2, which is then activated by a complex made up of MT1-MPP and TIMP2. Activated MMP2 breaks down tight junctions between endothelial cells and the ECM outside of the vessel. Integrins on the surface of endothelial cells sense where the new boundaries of the ECM are, and the endothelial cells use these signals as indicators of where to bind and form new vessels, as they migrate and proliferate into the newly vacant space in the ECM. *Created with Biorender*

pathways in endothelial cells that drive the angiogenic processes are complex, but they are mainly driven by the increase in the cell's intracellular $Ca^{2+}$ concentration (Moccia et al. 2019). This rise in intracellular $Ca^{2+}$ concentration is usually mediated through the binding of pro-angiogenic factors to their receptors, as seen with VEGF. When VEGF binds to its receptor tyrosine kinase (RTK), it recruits phospholipase C (PLC), which acts as a catalyst to cleave phosphatidylinositol 4,5-bisphosphate ($PIP_2$) into inositol-1,4,5-trisphosphate ($IP_3$) and diacylglycerol (DAG) (Moccia et al. 2019). This signaling pathway works in conjunction with calmodulin and ends with the localization of NF-κB in the nucleus, triggering both proliferation and tube formation by endothelial cells.

For angiogenesis to progress, endothelial cells and their progenitors must have a space in which to move to produce new blood vessels. While there are many cells capable of releasing MMPs that break down the ECM to help clear this space, endothelial cells can release MMPs directly to the target site. The most likely candidate released from endothelial cells that can most quickly affect endothelial tight junctions is MMP2, which is constitutively expressed by endothelial cells,

rather than MMP9, which must be induced by treatments with cytokines. Upon its release to the cell membrane, MMP2 requires multiple factors to activate it. A combination of TIMP2 and membrane type 1 (MT1)-MMP react to change the membrane-bound pro-MMP2 to MMP2 (Alexander and Elrod 2002). This may be part of the reason that MT1-MMP is upregulated in migrating endothelial cells, and when the activity of MT1-MMP is blocked with monoclonal antibodies, endothelial migration is stopped in vitro (Galvez et al. 2001).

Additionally, the binding of lymphocytes to VCAM-1 induces nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. This process is normally needed for VCAM-1-dependent lymphocyte migration. This process also activates concentrations of hydrogen peroxide produced by NADPH oxidase (Deem and Cook-Mills 2004). Another study investigated the role of microparticles, small secreted vesicles released via exocytotic budding, in the activation of MMP2. Microparticles have gained interest in terms of clinical relevance, as the overabundance of these particles increases clotting complications, while scarcity of microparticles can lead to excessive bleeding. Factors secreted with these particles have been shown to activate both MMP2 and MT1-MMP and may initiate processes to degrade the ECM surrounding the endothelial cells (Lozito and Tuan 2012).

There is increasing evidence that KLF2 both promotes quiescence in adult endothelial cells and is important for neovascularization. In endothelial progenitor cells, KLF2 is essential for angiogenesis. The protein erythropoietin (EPO), which is well-known for its importance in red blood cell production but has demonstrated a range of other effects, increases KLF2 expression in these cells through AMPK. A recent study showed that knockdown of KLF2 abrogated the angiogenesis induced by EPO. The protein VEGF was at least partially responsible for triggering angiogenic processes in the endothelial progenitor cells. VEGF was downregulated when KLF2 was suppressed, demonstrating that it is a target protein of KLF2 in endothelial progenitor cells (Wang et al. 2017; Chen et al. 2019). Another study showed that flow-induced KLF2 induces the expression of an endothelial-specific microRNA during zebrafish embryogenesis. This microRNA, mir-126, inhibits spred1 and pik3r2, which are proteins that inhibit VEGF signaling. This allows for VEGF-mediated angiogenesis during embryogenesis (Nicoli et al. 2010).

There is conflicting evidence when it comes to KLF2 and VEGF. One study showed VEGF and VEGFR2 are target proteins of KLF2 in HUVECs when exposed to shear stress. Constitutive VEGF—VEGFR2 expression decreases apoptosis compared to static cells, making it another mechanism by which KLF2 promotes quiescence (dela Paz et al. 2012). In contrast, a second study showed that KLF2 inhibited VEGFR2 expression, preventing angiogenesis (Bhattacharya et al. 2005). Finally, a third study showed that angiopoietin-1 inhibited VEGF-mediated VCAM-1 expression on endothelial cells through KLF2 (Sako et al. 2009). Therefore, KLF2's influence on the VEGF-VEGFR2 pathway is still unclear. However, each study showed that KLF2 was promoting quiescence in the endothelial cells.

## 5.5 Interaction of MMPs and KLF2 with Thrombin and Clotting Pathways

Thrombin is a central part of the clotting pathway and is instrumental in wound healing and other hemostatic processes. In addition to its hemostatic utility, however, thrombin also regulates various aspects of vascular remodeling. The non-hemostatic functions are typically induced by the binding of thrombin to protease-activated receptors (PARs). The family of PARs is made up of four members, PAR1 through PAR4, which differ in both their affinity for thrombin and their expression levels in various cells (Posma et al. 2016). One example of this activity is the binding of thrombin to PAR1. The binding triggers the induction of G protein pathways, such as the PKC and Src pathways that induce mitogen-activated protein kinase (MAPK) and NF-κB. This activation, in turn, induces the expression of pro-MMP9 which, when activated, induces cell migration (Fig. 5.3) (Yang et al. 2018). Additionally, there is evidence that the binding of thrombin to heparan sulfates decreases the rate at which activated MMP2 is broken down (Koo et al. 2010). This mechanism is believed to facilitate the migration and invasion of tissues

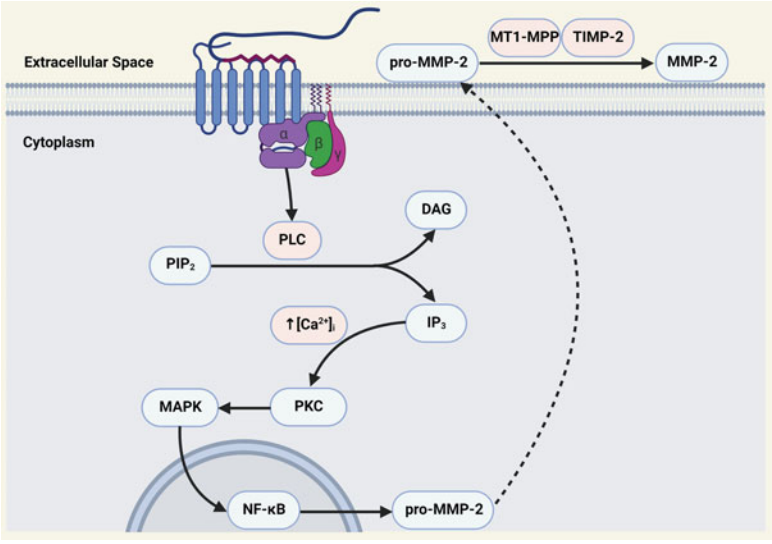


**Fig. 5.3** Activation of MMP2 by PAR-1 Activation via thrombin. Upon the binding of thrombin to the G-protein coupled PAR-1 receptor, phospholipase C (PLC) is induced and cleaves phosphatidylinositol 4,5-bisphosphate ($PIP_2$) into diacylglycerol (DAG) and inositol triphosphate ($IP_3$). This causes an increase in intracellular calcium levels, activating protein kinase C (PKC), which phosphorylates mitogen-activated protein kinase (MAPK). This leads to the induction of NF-κB and the production of pro-MMP2, which is released from the cell and activated to MMP2 by a complex made up of MT1-MMP and TIMP2, allowing it to perform its angiogenic roles. *Created with Biorender*

by smooth muscle cells, and the pathological activation of MMP2 is commonly associated with cancer metastasis (Alcantara and Dass 2013).

As stated above, thrombin induces the NF-κB pathway in endothelial cells. This results in reducing the expression of KLF2. One study investigated what the effects of inducing expression of KLF2 were when stimulated with thrombin. They discovered that these cells inhibit the expression of tissue factors, reduce cytokine secretion, reduce chemokine secretion, and decrease pro-coagulant proteins. KLF2 also decreased the expression of MMP1, 2, and 9 and increased the expression of TIMP1 and 2. This was done through the inhibition of the thrombin receptor PAR1 promoter activity by KLF2. As this receptor had decreased expression on the endothelial cells in the study, the NF-κB pathway that is activated by thrombin was never induced (Lin et al. 2006).

Additionally, KLF2 expression inhibits thrombin expression and induces TM expression. Thrombin, when bound to TM, switches from a pro-coagulant to an anticoagulant state. The complex causes accelerated activation of protein C (APC). APC proteolyzes the factors Va and VIIIa, which are important coagulant proteins for thrombin generation. Thrombin-TM is unable to cleave fibrinogen, activate factor V or factor XIII, or cleave PAR-1 and PAR-3 on endothelial cells or platelets, all of which are key for ending the clotting cascade (Loghmani and Conway 2018). This makes KLF2 an important anticoagulant transcription factor.

## 5.6 Role of MMPs and KLF2 in Atherosclerosis

Atherosclerosis is a disease in arterial blood vessels where deposits of fatty plaques lead to the narrowing of the blood vessel. Both the narrowing of the blood vessel and the buildup of the plaques are dangerous. Atherosclerotic plaques begin to form when macrophages in the intimal layer of an artery engulf oxidized low-density lipoprotein (LDL) cholesterol and become foam cells. These foam cells continue to grow and impede blood flow by pushing the endothelium toward the center of the lumen, decreasing the blood vessel diameter (Fig. 5.4) (Vacek et al. 2015).

This process leads to the introduction of inflammatory signaling and the activation of MMPs (Wolf and Ley 2019; Olejarz et al. 2020). MMP involvement in the onset of atherosclerosis has been investigated using several animal models. As is common with MMPs, each plays a different role from the others. MMP9, for example, seems to prevent the development of atherosclerotic plaques, while MMP2 contributes to their development (Vacek et al. 2015). The upregulation of inflammatory molecules such as TNFα and IL1, combined with stimuli from growth factors, like platelet-derived growth factor (PDGF) and fibroblast growth factor 2 (FGF2) also triggers the release of MMP1 and MMP3 from vascular smooth muscle cells (VMSCs) (Newby 2005).

The macrophages that contribute to the induction of atherosclerosis express MMPs as well. Adhesion to the ECM induces expression of MMP9, while the presence of inflammatory signaling molecules, PDGF, and macrophage

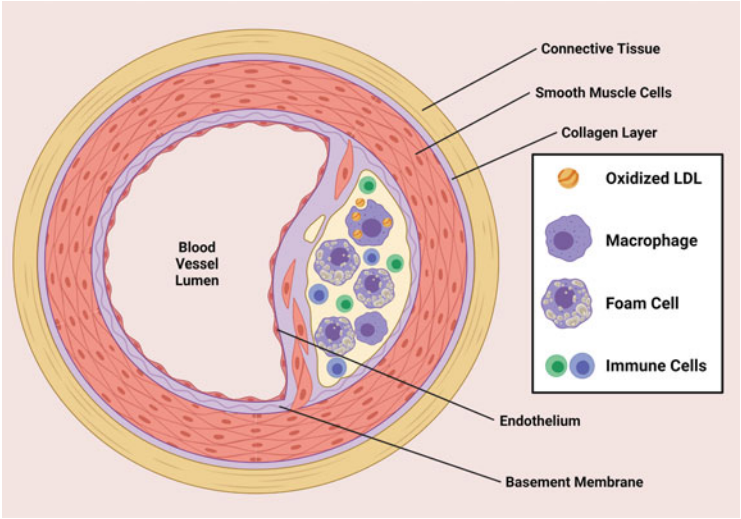


**Fig. 5.4** Formation of atherosclerotic plaques. Atherosclerosis begins to form when macrophages in the intimal layer of an artery begin to engulf oxidized LDL, resulting in the formation of foam cells. These foam cells continue to increase in size until they begin to push on the endothelial lining of the blood vessel, progressively obstructing the vessel more completely as the condition progresses. The development of atherosclerosis is more common in vessels with oscillatory blood flow, such as branches in the vasculature or in tight turns of vessels. The formation of atherosclerosis is combatted by the activity of KLF2 in endothelial and immune cells, however, once initiated, the environment around the atherosclerotic legion becomes inflamed, leading to the upregulated activity of MMPs. *Created with Biorender*

colony-stimulating factor (M-CSF) upregulates the expression of MT1-MMP and MMP16 (Newby 2005). These factors could also induce increased expression of MMP12, which encourages further infiltration by peripheral immune cells, causing increased inflammation in surrounding tissues. Other cells that contribute to MMP production in atherosclerotic plaques are T cells, mast cells, and fibroblasts (Oviedo-Orta et al. 2008; Levick et al. 2011; Evrard et al. 2016). These processes are not solely responsible for atherosclerosis, but animal models indicate that in mammals such as rabbits, the MMPs discussed above are not upregulated in normal macrophages (Newby 2005).

Branching points and arterial bends in blood vessels are more likely to develop atherosclerosis. This is due to the different stimuli caused by oscillatory flow (OF) and unidirectional flow (UF). Shear stress is the frictional force between flowing blood and endothelial cells and is especially important in the development of atherosclerosis (Nakajima and Mochizuki 2017; Zhuang et al. 2019). KLF2 is upregulated by UF but decreased by OF. UF occurs when the blood is flowing in one direction with high laminar shear stress. Cells that are exposed to UF maintain endothelial cell quiescence and alignment to the direction of flow, which helps maintain endothelial homeostasis. OF occurs at branching points and sharply curved areas in arteries. In these locations, the blood flow swirls, creating a non-uniform

flow pattern that generates low shear stress. Lee et al. showed that OF induced the expression of HDAC 5 leading to decreased expression of KLF2 (Lee et al. 2012). Factors such as OF, high blood pressure, and high cholesterol lead to endothelial dysfunction and the buildup of plaques (Gimbrone and Garcia-Cardena 2013; Niu et al. 2019; Zhuang et al. 2019).

KLF2 helps protect against atherosclerosis through endothelial cells, as well as lymphocytes. This was demonstrated by experiments that induced KLF2 deficiencies in two different atherosclerosis mouse models, resulting in increases in atherosclerotic lesion areas. Specifically, in $KLF2^{+/-}$; $ApoE^{-/-}$ mice, this increase was attributed to enhanced lipid uptake in macrophages. Similarly, myeloid cell-specific knockout of *KLF2* also increases the development of atherosclerosis in $LDLR^{-/-}$ mice by increasing the content of neutrophils and macrophages as well as the levels of nitroxidative stress, providing a direct link between KLF2 and atheroprotection (Niu et al. 2019).

## 5.7 Conclusion

The vascular system is well-known for its importance in transporting oxygen and carbon dioxide throughout the body. However, it is also critical for transporting nutrients, allowing immune cells to travel to injured areas, and promoting wound healing. The goal of this chapter is to provide insight into this complex system by focusing on one cell type, one transcription factor, and one protease family. Endothelial cells play a leading role in determining biological functions in blood vessels. They help determine whether immune cells pass through, whether the vessel contracts or expands, and where angiogenesis should occur. KLF2 and MMPs are just two "tools" that these cells use to perform the various functions that are required in the vasculature.

There are many situations in which KLF2 and MMPs are induced. It is important to understand that biological systems are carrying out multiple complex operations at one time. If the stimuli for NF-κB are strong enough that MMPs are induced to promote inflammation or angiogenesis, then that is what happens. If the signal for quiescence, mediated in part by KLF2, is stronger than the stimuli for NF-κB, then KLF2 will inhibit NF-κB and produce proteins that reduce inflammation and promote quiescence.

This chapter explored three different scenarios in which endothelial cells play central roles: angiogenesis, atherosclerosis, and clotting, and examined the different roles that MMPs and KLF2 play in these situations. By focusing on these two proteins that carry out different roles, a better understanding of the complexity and variety of signals that affect endothelial cells and their responses can be gained, and more targeted treatments for vasculature dysregulation can be investigated.

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# Chapter 6
# Extracellular Matrix Remodeling Enzymes as Targets for Natural Antiangiogenic Compounds


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**Abstract** Angiogenesis, or new blood vessels formation, comprises a series of tight regulated and coordinated steps guided by the balance between proangiogenic and antiangiogenic signals. Although physiological angiogenesis occurs in some context, such as embryogenesis or wound healing, it is a very restricted process in adults. A deregulated angiogenesis is associated to several pathologies such as cancer, arthritis, or age-related macular degeneration. For this purpose, plenty of compounds from natural sources have been studied and their antiangiogenic

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potential has been demonstrated. Most of them are isolated from plants, such as the major catechin found in green tea, epigallocatechin-3-gallate (EGCG), or the isoflavonoid genistein. Nevertheless, compounds with antiangiogenic potential can be found in several species of animals, fungi, algae, or bacteria, for instance, aeroplysinin-1, AD0157, carrageenan derivates, and rapamycin, respectively. The extracellular matrix (ECM) remodeling plays a key role in the formation of new blood vessels. The degradation of the ECM components not only provides a structural scaffold for the nascent vessels, but it is also strongly involved in endothelial cell signaling, promoting or inhibiting this complex process. In this regard, targeting the ECM components entails an interesting therapeutic strategy for the treatment of angiogenesis-related diseases. This book chapter is an updated overview of natural compounds with an antiangiogenic effect with the capability to target one or more ECM components.

## 6.1 The Extracellular Matrix and Its Role in Angiogenesis

The extracellular matrix (ECM) term refers to the non-cellular component present within all tissues and organs which provides physical, adhesive, and mechanical support for the cells. The ECM participates in crucial biochemical and biophysical processes for tissue morphogenesis, differentiation, homeostasis, and storage and release of bioactive molecules (Theocharis et al. 2019). Although each tissue has an ECM with a unique topology and composition, which is defined during tissue development, the ECM is generally composed of laminin, nidogens, type IV collagen, and heparan sulfate proteoglycans (HSPG), that separate cells from the interstitial matrix (Walker et al. 2018). Extracellular/pericellular space, a complex molecular network created by interactions of matrix components and interactions with non-matrix molecules establish the dialogue between the cellular components and their microenvironment, further defining cell behavior and function (Muncie and Weaver 2018). In fact, the crosstalk between the ECM components and the proteases, growth factors, cytokines, and chemokines that bind to the ECM plays a pivotal role in physiological but also in pathological processes, ultimately orchestrating complex signaling cascades and the cell response to extracellular stimuli. It is worth mentioning that the ECM is a dynamic structure, since its composition and structural organization change during embryogenesis, adult physiological conditions, and pathological contexts, such as cancer, inflammation, and wound healing (Yamada et al. 2019; Walma and Yamada 2020; Winkler et al. 2020). These variations include the presence of specific proteins typical of embryogenesis or associated with tissue remodeling.

One of the physiological processes that take place in the ECM environment is angiogenesis or the blood vessel growth from the existing vasculature. The new blood vessel is composed of two interacting cell types, endothelial cells, which form the inner lining of the vessel wall, and perivascular cells, also known as pericytes, vascular smooth muscle cells or mural cells that envelop the surface of the vascular

tube (Carmeliet and Jain 2011). It has been largely described how in the angiogenic process the ECM is not a mere physical scaffolding for the endothelial cells but regulates each step of the cascade. The multiple stages that make up angiogenesis are finely coordinated and occur in a sequential and interdependent manner. For the formation of new blood vessels through the process of angiogenesis, proangiogenic factors signal to the quiescent endothelial cells that conform the capillaries, promoting the activated state in these cells and the differentiation into stalk or proliferating cells and tip or migrating cells. This activation includes a cascade of events that promotes the detachment of mural cells from the vessel, the degradation of the underlying basement membrane, the migration and proliferation of endothelial cells, and the formation of an immature capillary structure (Vaeyens et al. 2020). Once the new capillary is formed, its remodeling takes place, characterized by the recruitment of mural cells and the deposition of a new basement membrane to complete vessel maturation. In all these events, endothelial cells interact with the matrix producing proteases necessary for the ECM degradation and produce molecules for the formation of the new basement membrane (Ma et al. 2020) (Fig 6.1).

In a physiological scenario, the above-mentioned steps of angiogenesis are finely coordinated by a delicate balance between proangiogenic and antiangiogenic signals, with the ECM network being a key player in the maintenance of this balance. In fact, a plethora of angiogenesis regulatory factors, both stimulators and inhibitors, are either matrix molecules or fragments derived from the ECM. The number of endogenous inhibitors of angiogenesis that are produced by the ECM protease-mediated cleavage is continuously growing. Some of them, such as endostatin, vastatin, and restin, among others, are being explored for the design of antiangiogenic therapies for neoplastic and non-neoplastic angiogenesis-related diseases (Ma et al. 2020).

Finally, the mechanical properties of the ECM, determined by its composition, can profoundly affect cell behavior (Marchand et al. 2019). The ECM stiffness and spatial dimensionality establish tensile forces that act through the tubulin and the actin cytoskeleton providing traction, directionality, and orientation for the migrating cells in the nascent vessel (Marchand et al. 2019). Thus, stimulated endothelial cells directly influence the physico-mechanical properties of the surrounding ECM to support vessel formation through changes in protein synthesis, polymerization, and fibril formation (Nia et al. 2020; Vaeyens et al. 2020). Figure 6.1 summarizes the angiogenic process and different compounds targeting more than one ECM enzymes.

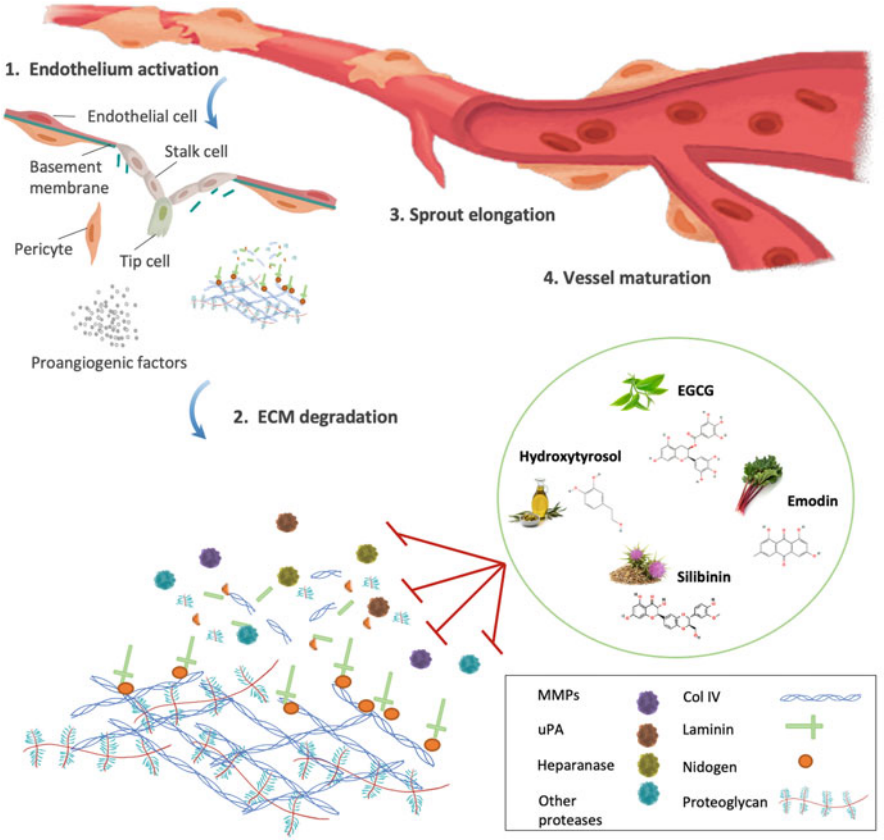


**Fig. 6.1** The angiogenic process and families of compounds targeting ECM enzymes. The scheme illustrates the four main steps of the angiogenic process: (*1*) Endothelium activation, characterized by the binding of proangiogenic factors to their respective receptors on endothelial cells and the subsequent differentiation into tip and stalk cells, parallel to the mural cell detachment; (*2*) ECM degradation, in which the activated endothelium releases proteolytic enzymes to degrade the basement membrane and the surrounding ECM. Several natural compounds have a pleiotropic effect and target different ECM enzymes, resulting in the inhibition of the angiogenic process. Some examples are EGCG, emodin, hydroxytyrosol, or silibinin, which can simultaneously inhibit various ECM proteases; (*3*) sprout elongation, when the activated endothelial cells proliferate and migrate across the ECM; and (*4*) vessel maturation, final step in which the elongation stops. Finally, basement membrane is restored, and mural cells are recruited giving rise to a mature vessel

## 6.2 Pharmacological Targeting of ECM by Natural Compounds in Pathological Angiogenesis

Judah Folkman's studies promoted a fundamental change in the concept of angiogenesis, which went from being understood as a physiological process mainly associated with embryonic development and wound healing, to being considered a

potent pharmacological target with great possibilities for the treatment of diseases that present impaired regulation of the process (Quesada et al. 2010; Ribatti 2021). The basis for antiangiogenic therapy in cancer and other angiogenesis-dependent diseases was then established. However, the apparent simplicity of the model of activation and inactivation of the "angiogenic switch" as a pharmacological strategy against the onset and development of certain diseases, is in contrast with the complexity of the process itself (Quesada et al. 2007). Precisely, this inherent complexity is the basis of most of the observed complications related to the use of antiangiogenic therapies, since different drawbacks, such as the development of compensatory mechanisms, redundancy in the activation pathways, and multiplicity in the effectors, have been described in relation to the use of angiogenic inhibitors in clinics and disease models (Medina et al. 2007; Jászai and Schmidt 2019). For example, because of these events, the initial optimism about the VEGF-targeting therapy in the treatment of cancer was rapidly discarded (Vasudev and Reynolds 2014). Currently, the pharmacological modulation of angiogenesis is mostly conceived as a multi-target strategy, and research in this field is focused on the identification of new targets for the pharmacological modulation of this process and the finding and evaluation of potential antiangiogenic drugs with high effectiveness for the treatment of angiogenesis-dependent diseases, either as monotherapy or in a combinatory approach (Quesada et al. 2006; Lopes-Coelho et al. 2021).

In the search of new potential modulators of angiogenesis, bioactive compounds derived from natural products, including terrestrial and marine plants, fungi, microorganisms, sponges, and even foods (fruits, vegetables, spices, seeds, oils...), are gaining increasing attention as drug candidates or lead structures for drug design, due to their unusual chemical features. In fact, the use of therapeutic compounds derived from plants for the inhibition of pathological angiogenesis may render a series of advantages, such as general availability, low price of products, multiple pharmacological activities, and a good profile of absorption, distribution, metabolism, and excretion (ADME) and tolerability (Khalid et al. 2016; Lu et al. 2016; Ribeiro et al. 2018). In line with the concept of multi-target strategy in the therapeutic intervention of angiogenesis, the ECM represents a complex network of molecules that could be potential pharmacological antiangiogenic targets. Among these molecules, those enzymes that participate in the remodeling of the ECM, revised in the next section, have received a special interest in biomedical and pharmacological research (Su et al. 2016; Liu and Khalil 2017; Fields 2019; Mohan et al. 2019).

Table 6.1 includes an updated compilation of natural antiangiogenic compounds that have been reported to target different enzymes involved in ECM remodeling. For many compounds included in Table 6.1, a pleiotropic function has been described, acting on different ECM remodeling enzymes (see also Fig. 6.1). This is the case of compounds such as curcumin, hydroxytyrosol, genistein, luteolin, or epigallocatechin-3-gallate (EGCG). It is also worth mentioning that many of these compounds show other bioactivities, exhibiting antioxidative and anti-inflammatory potential.

**Table 6.1** Natural antiangiogenic compounds that target ECM enzymes

| Natural product | Source | Kingdom | Molecular Target | References |
|---|---|---|---|---|
| (+)-Catechin | Several plants | Plants | Elastase<br>Collagenase | Wittenauer et al. (2015) |
| 12-O-(2′E,4′E-decadienoyl)-4-hydroxyphorbol-13-acetyl | *Daphne genkwa* | Plants | MMP3 | Wang et al. (2018) |
| 20(R)-ginsenoside Rg3 | *Panax ginseng* (root) | Plants | MMP2<br>MMP9 | Yue et al. (2006) |
| 3-Hydroxyflavone | Several plants | Plants | MMP1<br>MT1-MMP<br>MMP2<br>TIMP1<br>TIMP2<br>uPA<br>PAI1 | Kim (2003) |
| 3′-Hydroxygenkwain | *Daphne genkwa* | Plants | MMP3 | Zhou et al. (2021) |
| 4-Methylumbelliferone | Apiaceae family | Plants | MMP2 | García-Vilas et al. (2013) |
| 7-Epiclusianone | *Garcinia brasiliensis* | Plants | Cathepsin B and G | Murata et al. (2010) |
| Acacetin | *Robinia pseudoacacia* and *Turnera diffusa* | Plants | MMP2 | Bhat et al. (2013) |
| Acidic mucopolysaccharide | *Holothuria leucospilota* | Animals | MMP2<br>MMP9 | Zhang et al. (2009) |
| AD0157 | *Paraconiothyrium sp.* | Fungi | MMP2 | García-Caballero et al. (2014) |
| Aeroplysinin-1 | *Aplysina aerophoba* (and other species of Verongida order) | Animals | MMP2<br>uPA<br>PAI1 | Martínez-Poveda et al. (2013)<br>Rodríguez-Nieto et al. (2002a) |
| AITC (Allyl isothiocyanate) | *Brassica nigra, Lepidium sativum, Wasabia japonica, Raphanus sativus,* and *sinapis species* | Plants | TIMP1 | Thejass and Kuttan (2007a) |
| Alitretinoin | Several foods of animal origin | Animals | MMP2 | Kvestad et al. (2014) |
| Aloe-emodin | *Aloe vera* and others | Plants | MMP2<br>uPA | Cárdenas et al. (2006) |
| Andrographolide | *Andrographis paniculata* | Plants | MMP2<br>MMP9 | Pratheeshkumar and Kuttan (2011) |
| Apigenin | *Matricaria recutita, Daphne genkwa,* and *Chamaemelum nobile* | Plants | MMP1<br>MT1-MMP<br>MMP2<br>MMP3 | Lamy et al. (2012)<br>Kim (2003)<br>Zhou et al. (2021) |

(continued)

**Table 6.1** (continued)

| Natural product | Source | Kingdom | Molecular Target | References |
|---|---|---|---|---|
| | | | TIMP1<br>TIMP2<br>uPA<br>PAI1 | |
| Apigenin-7-O-β-D-glucoside | *Daphne genkwa* | Plants | MMP3 | Zhou et al. (2021) |
| Aplidine (Dehydrodidemnin B) | *Aplidium albicans* | Animals | MMP2<br>MMP9 | Taraboletti et al. (2004) |
| Arctigenin | *Arctium lappa* (seed) | Plants | MMP2<br>MMP9<br>Heparanase | Lou et al. (2017) |
| Baicalein | *Scutellaria baicalensis* | Plants | MMP2<br>Elastase | Liu et al. (2003)<br>Sartor et al. (2002) |
| Baicalin | *Scutellaria baicalensis* | Plants | MMP2 | Liu et al. (2003) |
| Bestatin | *Streptomyces olivoreticuli* | Monera | Heparanase<br>MMP9 | Hossain et al. (2016) |
| BITC (Benzyl isothiocyanate) | Brassicaceae family | Plants | MMP2 | Boreddy et al. (2011) |
| C3G (Cyanidin-3-O-glucoside) | Several plants and food | Plants | MMP2 | Anwar et al. (2014) |
| Carnosic acid | *Rosmarinus officinalis* (leaves) | Plants | MMP2 | López-Jiménez et al. (2013) |
| Carnosol | *Rosmarinus officinalis* (leaves) | Plants | MMP2 | López-Jiménez et al. (2013) |
| κ and λ-carragenaans | Red algae | Protist | Heparanase | Niu et al. (2015)<br>Ishai-Michaeli et al. (1990) |
| Celastrol | *Tripterygium wilfordii* | Plants | MMP1<br>MMP9 | Li et al. (2012) |
| Chymostatin | *Streptomyces sp* | Monera | Cathepsins A, B, D, K | Vidal-Albalat and González (2016)<br>Umezawa et al. (1970) |
| Cryptotanshinone | *Salvia miltiorrhiza* | Plants | MMP2<br>MMP9<br>TIMP1<br>TIMP2 | Zhang et al. (2018) |
| CS5931 | *Ciona savignyi* | Animals | MMP2<br>MMP9 | Liu et al. (2014) |
| Cucurbitacin B | Cucurbitaceae family | Plants | MMP2<br>MMP9 | Sinha et al. (2016) |

(continued)

**Table 6.1** (continued)

| Natural product | Source | Kingdom | Molecular Target | References |
|---|---|---|---|---|
| Curcumin | *Curcuma longa* (rhizome) | Plants | MMP2<br>MMP9<br>MT1-MMP<br>TIMP2<br>uPA<br>Elastase | Yodkeeree et al. (2009)<br>Thaloor et al. (1998)<br>Liu et al. (2003)<br>Sartor et al. (2002) |
| DADS (Diallyl disulfide) | *Allium sativum* | Plants | MMP2<br>MMP9<br>TIMP1 | Meyer et al. (2004)<br>Thejass and Kuttan (2007b) |
| Damnacanthal | *Morinda citrifolia* (root) | Plants | MMP1<br>MMP2<br>TIMP1<br>TIMP4<br>uPA<br>PAI1 | García-Vilas et al. (2017a) |
| Danshensu | *Radix Salviae miltiorrhizae* | Plants | MMP2<br>MMP9 | Zhang et al. (2010a) |
| Daphnetin | *Changbai daphne* | Plants | MMP2 | Kumar et al. (2016) |
| Daphnodorin G | *Daphne genkwa* | Plants | MMP3 | Zhou et al. (2021) |
| DATS (Diallyl trisulfide) | *Allium sativum* | Plants | MMP2<br>MMP7<br>MMP9<br>uPA | Lai et al. (2015) |
| Defibrotide | Porcine mucosal DNA | Animals | Heparanase | Barash et al. (2018) |
| Demethoxycurcumin | *Curcuma aromatica* (rhizome) | Plants | MMP9 | Kim et al. (2002) |
| Dihydroartemisinin | *Artemisia annua* | Plants | MMP9 | Wang et al. (2011) |
| ECAP (Epicatechin 3-O-β-D-allopyranoside) | *Davallia bilabiata* | Plants | MMP2<br>TIMP2<br>MMP14 | Liu et al. (2017) |
| EGCG (Epigallocatechin-3-gallate) | *Camellia sinensis* (leaves) | Plants | MMP2<br>MMP9<br>MT1-MMP<br>uPA<br>Elastase<br>Collagenase<br>PAI1 | Oku et al. (2003)<br>Yamakawa et al. (2004)<br>Cale et al. (2010)<br>Jankun et al. (1997)<br>Madhan et al. (2007)<br>Wittenauer et al. (2015)<br>Donà et al. (2003)<br>Sartor et al. (2002) |
| Elaiophylin | | Monera | | Lim et al. (2018) |

(continued)

**Table 6.1** (continued)

| Natural product | Source | Kingdom | Molecular Target | References |
|---|---|---|---|---|
| | *Streptomyces melanosporus* 17JA11 | | MMP2<br>MMP9 | |
| Ellagic acid | *Phyllanthus urinaria* | Plants | MMP2<br>MMP9 | Wang et al. (2012)<br>Huang et al. (2011b)<br>Huang et al. (2011a) |
| Embelin | *Embelia ribes* (fruit) | Plants | MMP2<br>MMP9<br>PAI1 | Lin et al. (2013)<br>Huang et al. (2014) |
| Emodin | *Rheum palmatum* (root and rhizome) | Plants | MMP2<br>MMP9<br>uPA<br>Trypsin | Kwak et al. (2006)<br>Jedinák et al. (2006a)<br>Shi and Zhou (2018) |
| Esculetin | *Artemisia scoparia* | Plants | MMP2 | Park et al. (2016) |
| Farnesiferol C | *Ferula assafoetida* | Plants | MMP2 | Lee et al. (2010) |
| Ferulic acid | *Cimicifuga heracleifolia*, *Angelica sinensis*, and *Lignsticum chuangxiong* | Plants | MMP2<br>MMP9 | Yang et al. (2015) |
| Formononetin | *Astragalus membranaceus* (dried root) | Plants | MMP2<br>MMP9 | Wu et al. (2015) |
| Gallic acid | *Toona sinensis* (leaf) | Plants | MMP2<br>MMP9 | Hseu et al. (2011) |
| Genistein | Soybeans | Plants | MMP1<br>MMP2<br>MMP9<br>MT1-MMP<br>TIMP1<br>TIMP2<br>uPA<br>PAI1 | Yu et al. (2012)<br>Kim (2003)<br>Fajardo et al. (1999) |
| Genkwanin | *Daphne genkwa* | Plants | MMP3 | Zhou et al. (2021) |
| Ginsenoside Compound K | *Panax ginseng* | Plants | MMP2<br>MMP9 | Shin et al. (2014) |
| Halofuginone | *Dichroa febrifuga* | Plants | MMP2 | Elkin et al. (2000) |
| Harmine | *Peganum harmala* (seed) | Plants | MMP2<br>MMP9<br>TIMP1 | Hamsa and Kuttan (2010) |
| HBA (4-hydroxybenzyl alcohol) | *Gastrodia elata* | Plants | MMP9 | Laschke et al. (2011) |

(continued)

**Table 6.1** (continued)

| Natural product | Source | Kingdom | Molecular Target | References |
|---|---|---|---|---|
| Homocysteine | Methionine | Animals<br>Plants<br>Fungi<br>Monera<br>Protist | MMP2<br>uPA<br>PAI1 | Rodríguez-Nieto et al. (2002b)<br>Midorikawa et al. (2000) |
| Hydrangenol | *Hydrangea macrophylla* | Plants | MMP2 | Gho et al. (2019) |
| Hydroxygenkwanin-5-O-β-D-glucoside | *Daphne genkwa* | Plants | MMP3 | Zhou et al. (2021) |
| Hydroxytyrosol | *Olea europaea* (virgin olive oil) | Plants | MMP1<br>MMP2<br>MMP9<br>TIMP1<br>TIMP2<br>TIMP4<br>uPA<br>uPAR | García-Vilas et al. (2017b)<br>Scoditti et al. (2012)<br>Fortes et al. (2012) |
| Hyperforin | *Hypericum perforatum* | Plants | MMP2<br>uPA | Martínez-Poveda et al. (2005b) |
| Hypericin | *Hypericum sp* | Plants | MT1-MMP<br>uPA<br>Trypsin | Jedinák et al. (2006a)<br>Lavie et al. (2005)<br>Martínez-Poveda et al. (2005a) |
| Hyperoside | Hypericum and Crataegus genera | Plants | MMP2<br>MMP7<br>uPA | Jedinák et al. (2006a)<br>Wu et al. (2020)<br>Liu et al. (2016) |
| Imperatorin | *Angelica dahurica* and *Angelica archangelica* | Plants | MMP2 | Li et al. (2021b) |
| Ipobscurine-A | *Ipomoea obscura* | Plants | MMP2<br>MMP9<br>TIMP1 | Hamsa and Kuttan (2011) |
| Isoliquiritigenin | *Glycyrrhiza uralensis* (root) | Plants | MMP2<br>MT1-MMP<br>TIMP2 | Kang et al. (2010)<br>Wang et al. (2013) |
| Kahweol | *Coffea arabica* (coffee bean) | Plants | MMP2<br>uPA | Cárdenas et al. (2011) |
| Leupeptin | *Streptomyces exfoliatus* | Monera | Cathepsins A, B, D, K | Baici and Gyger-Marazzi (1982)<br>Vidal-Albalat and González (2016) |
| Lupeol | Several plants | Plants | MMP2<br>MMP9<br>Elastase | Mitaine-Offer et al. (2002)<br>Vijay Avin et al. (2014) |

(continued)

**Table 6.1** (continued)

| Natural product | Source | Kingdom | Molecular Target | References |
|---|---|---|---|---|
| Luteolin | Several plants | Plants | MMP2<br>MMP9<br>ADAMTS-4/5<br>Elastase | Pratheeshkumar et al. (2012)<br>Lamy et al. (2012)<br>Moncada-Pazos et al. (2011)<br>Siedle et al. (2007) |
| Luteolin-5-O-β-D-glucopyranoside | *Daphne genkwa* | Plants | MMP3 | Zhou et al. (2021) |
| Lycopene | *Solanum lycopersicum* | Plants | MMP2<br>TIMP2<br>uPA | Chen et al. (2012) |
| Melatonin | Several species | Plants<br>Animals<br>Monera<br>Fungi<br>Protist | MMP2<br>MMP9 | Yan et al. (2021) |
| Nobiletin | *Citrus depressa* and *Citrus reticulata* (fruit) | Plants | MMP2<br>uPA | Kunimasa et al. (2010) |
| Novobiocin | *Streptomyces niveus* | Monera | MMP2 | Yang et al. (2003) |
| Oleanolic acid | Several plants | Plants | Elastase | Kim et al. (2009) |
| Oleuropein | *Olea europaea* (virgin olive oil) | Plants | MMP9 | Scoditti et al. (2012) |
| Panduratin A | *Boesenbergia rotunda* | Plants | MMP2 | Lai et al. (2012) |
| PG (Piceatannol-3-O-β-D-glucopyranoside) | *Rheum undulatum* (rhizome) | Plants<br>Fungi | MMP9 | Kim and Ma (2019) |
| Phloretin | Several plants | Plants | MMP2<br>MMP3<br>Cathepsin S<br>Elastase | Leu et al. (2006)<br>Hsiao et al. (2019) |
| Picroside II | *Picrorhiza kurroa* | Plants | MMP9 | Lou et al. (2019) |
| Piperlongumine | *Piper longum* (fruit) | Plants | MMP2<br>MMP9 | Kumar and Agnihotri (2021) |
| PITC (phenyl isothiocyanate) | *Brassica nigra, Lepidium sativum, Wasabia japonica, Raphanus sativus,* and *sinapis species* | Plants | TIMP1 | Thejass and Kuttan (2007a) |
| Protocatechuic acid | *Pleurotus tuber-regium* and *Agrocybe aegerita* (sclerotium) | Fungi | MMP2 | Hu et al. (2018) |
| Punarnavine | *Boerhaavia diffusa* | Plants | MMP2<br>MMP9<br>TIMP2 | Saraswati et al. (2013a) |

(continued)

**Table 6.1** (continued)

| Natural product | Source | Kingdom | Molecular Target | References |
|---|---|---|---|---|
| Quercetin | Several plants | Plants | MMP2<br>MMP9<br>Trypsin<br>Thrombin<br>uPA<br>Elastase | Tan et al. (2003)<br>Scoditti et al. (2012)<br>Pilátová et al. (2010)<br>Jedinák et al. (2006a)<br>Kanashiro et al. (2007)<br>Sartor et al. (2002) |
| Rapamycin | *Streptomyces hygroscopicus* | Monera | ADAM10 | Zhang et al. (2010b) |
| Resveratrol | Several plants | Plants | MMP2<br>MMP9 | Zhang et al. (2010b)<br>Scoditti et al. (2012)<br>Cao et al. (2005) |
| Rhaponticin | *Rheum undulatum* | Plants | MMP9 | Kim and Ma (2018) |
| Rhein | *Rheum palmatum, Cassia tora, Polygonum multiflorum,* and *Aloe barbadensis* | Plants | Trypsin<br>uPA | Jedinák et al. (2006a) |
| Salicin | Genus *Salix* (willow bark) | Plants | Thrombin | Jedinák et al. (2006a) |
| Silibinin | *Silybum marianum* (fruits) | Plants | MMP2<br>uPA<br>Trypsin<br>Thrombin | Singh et al. (2005)<br>Jedinák et al. (2006a)<br>Jiang et al. (2000) |
| Sulforaphane | Brassicaceae family | Plants | MMP2<br>TIMP2 | Bertl et al. (2006) |
| Sulodexide | Porcine intestinal mucosa | Animals | Heparanase<br>MMP9 | Masola et al. (2012) |
| Syringic acid | *Pleurotus tuber-regium* and *Agrocybe aegerita* (sclerotium) | Fungi | MMP2 | Hu et al. (2018) |
| Tannic acid | Several plants | Plants | PAI1 | Cale et al. (2010) |
| Tanshinone IIA | *Salvia miltiorrhiza* (dried root and rhizome) | Plants | MMP2<br>TIMP2 | Tsai et al. (2011) |
| Theaflavin-3,3′-digallate | *Camellia sinensis* (black tea) | Plants | PAI1 | Jankun (2011) |
| Theaflavin-3′-gallate | *Camellia sinensis* (black tea) | Plants | PAI1 | Jankun (2011) |
| TICMs (Trypsin inhibitor from extract of *C. melo* seeds) | *Cucumis melo* (seeds) | Plants | MMP2<br>MMP9 | Rasouli et al. (2017) |

(continued)

**Table 6.1** (continued)

| Natural product | Source | Kingdom | Molecular Target | References |
|---|---|---|---|---|
| Toluquinol | *Penicillium sp.* | Fungi | MMP2 | García-Caballero et al. (2013) |
| Triptolide | *Tripterygium wilfordii* | Plants | ADAM10 | Soundararajan et al. (2009) |
| Tylophorine | *Tylophora indica* | Plants | MMP2<br>MMP9 | Saraswati et al. (2013b) |
| Ursolic acid | Several plants | Plants | MMP2<br>MMP9<br>TIMP1<br>uPA<br>Cathepsin B<br>Elastase | Kanjoormana and Kuttan (2010)<br>Cárdenas et al. (2004)<br>Ying et al. (1991)<br>Jedinák et al. (2006b)<br>Mitaine-Offer et al. (2002) |
| Zerumbone | *Zingiber zerumbet* (rhizome) | Plants | MMP9 | Samad et al. (2018) |
| β-Carotene | Several plants and food | Plants | MMP2<br>MMP9<br>TIMP1<br>TIMP2 | Guruvayoorappan and Kuttan (2007) |
| γ-Tocotrienol | Several plants and food | Plants<br>Animals | MMP9 | Li et al. (2011) |

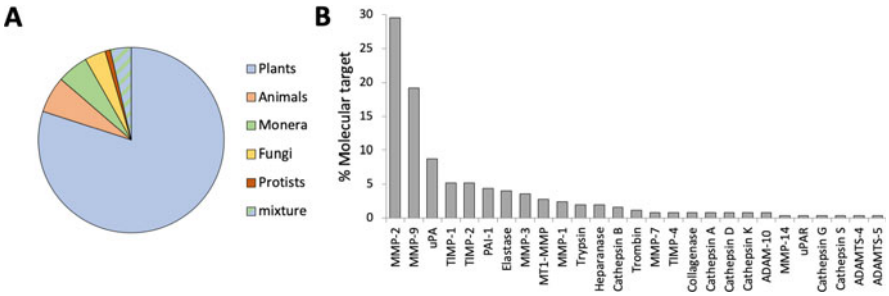


**Fig. 6.2** Distribution of natural antiangiogenic compounds according to kingdoms and ECM targets. (**a**) Distribution of natural antiangiogenic compounds into the different kingdoms. Mixture category includes all those compounds that are present in more than one kingdom. (**b**) Graph shows molecular target distribution for the final 110 natural compounds; 27 different enzymes are involved in the ECM remodeling

In addition, Fig. 6.2 shows the distribution of the compounds listed in Table 6.1 into the different kingdoms (Fig. 6.2a) and the ECM molecular targets of these compounds (Fig. 6.2b).

## 6.3 Matrix Remodeling Enzymes as Therapeutic Targets in Angiogenesis

The main players involved in the ECM remodeling are presented in this section, with a focus on how they can be potential therapeutic targets in modulating angiogenesis. Matrix metalloproteinases (MMPs) are members of an enzyme family of calcium-dependent endopeptidases that require a zinc ion for their catalytic activity. This family targets molecules including growth factors, matrix components, cytokines, adhesion, and signaling molecules (Quintero-Fabián et al. 2019). MMPs are synthesized and secreted as inactive latent enzymes (zymogens) that can be activated after their cleavage by diverse proteases, including other MMPs. MMPs are active at neutral pH catalyzing the normal turnover of various ECM macromolecules. The MMPs can be classified into six different groups: gelatinases, collagenases, matrilysins, stromelysins, membrane-bound MMPs (MT-MMPs), and other MMPs according to their substrate specificity and domain homology (Cui et al. 2017). MT-MMPs are located on the plasma membrane by a transmembrane domain or by a glycosyl phosphatidyl inositol (GPI) anchor, being active once furin convertases recognize a specific motif on their core protein. MMPs function is controlled by other proteases, or by binding with matrix components, and inhibited by MMP tissue inhibitors, named as TIMPs (Cui et al. 2017).

Although MMPs are the main ECM remodeling enzymes studied as therapeutic targets in pathological angiogenesis, there are plenty of compounds isolated from natural sources with the capability to inhibit additional ECM remodeling enzymes, such as those which belong to the PA (plasminogen activator) system, heparanase, or cathepsins, and whose antiangiogenic activity had been demonstrated.

ADAMs (a disintegrin and metalloproteinases) are families of zinc-dependent transmembrane proteases that contain disintegrin, cysteine-rich, and epidermal growth factor (EGF) domains. ADAMTSs (a disintegrin and metalloproteinase with thrombospondin motifs) are proteases secreted in the ECM which contain the thrombospondin (TSP) type-1 motifs instead of EGF motifs (Zhong and Khalil 2019). In the MMP and ADAMTS families, there are more than 20 members and their role in angiogenesis has been largely studied in the context of cancer (Sun et al. 2015; Quintero-Fabián et al. 2019).

Elastases are serine proteases produced by the pancreas, which participate in the breakdown of elastin, a protein that offers elasticity to connective tissue. Elastases catalyze the cleavage of carboxyl groups present in small hydrophobic amino acids, such as glycine, valine, and alanine. Around eight genes encode elastase or elastase-like enzymes, four of which are classified as chymotrypsin-like (Heinz 2020).

Other crucial proteases involved in the ECM remodeling during angiogenesis are the serine proteases urokinase plasminogen activator (uPA) and the tissue-type plasminogen activator (tPA), which participate in the activation of plasminogen to plasmin (Gonias and Zampieri 2020). Plasminogen activators/plasmin cascade can target different matrix elements and molecules and activate MMPs (Breuss and Uhrin 2012; Gonias and Zampieri 2020). Granzymes are serine proteases present

in the ECM that, being produced by immune and non-immune cells, can modulate the cell–matrix interactions by degradation of matrix and junctional proteins, as well as by interacting with cell surface receptors (Velotti et al. 2020). Recently, the role of some members of this family has been described in the modulation of angiogenesis (Wroblewski et al. 2017; Li et al. 2021a, 2021b).

Cysteine cathepsins, although present in the intracellular endolysosomal compartments, also exert their functions in the ECM by degrading numerous matrix and adhesion molecules and activating pro-uPA and MMPs. Some cathepsins are secreted in the ECM and can function in slight acidic conditions, prevalent in diseases including cancer and osteoarthritis (Vizovišek et al. 2019; Vidak et al. 2019).

Aside from proteases, glycosaminoglycan (GAG) degrading enzymes are also implicated in the remodeling of ECM, modifying the proteoglycan (PG) structure and the heparan sulfate (HS) size (Smock and Meijers 2018). Six hyaluronic acid (HA)-degrading enzymes (hyaluronidases 1–4 (HYALs-1, -2, -3, and-4), HYALP, and PH20) have been described in humans (Dogné and Flamion 2020). Some of them are active in acidic or/and in neutral pH and they degrade high molecular weight HA to smaller bioactive fragments in the ECM (Dogné and Flamion 2020). They trigger biological events, such as angiogenesis, inflammation, and cancer progression (Piperigkou et al. 2021). Another GAG-degrading enzyme with pronounced functions in ECM remodeling is heparanase (Masola et al. 2018). Heparanase is an endoglucuronidase that cleaves HS chains, releasing bioactive HS fragments of 4–7 kDa. It is overexpressed in some diseases and involved in the control of processes such as cancer cell growth, metastasis, and angiogenesis (Masola et al. 2018), by activation of VEGF and FGF pathways through HS cleavage and subsequent release of these proangiogenic factors (Jayatilleke and Hulett 2020).

In the last years, numerous studies have shown the important role of these proteolytic enzymes in the modulation of angiogenesis, and their potential as pharmacological targets for the therapeutic inhibition of these processes in angiogenesis-dependent diseases, such as cancer. Despite the initial efforts to develop successful pharmacological approaches focused on the inhibition of ECM enzymes, the clinical trials performed in this line have revealed important side effects derived from the blockage of these enzymes, such as the musculoskeletal syndrome observed with nearly all the inhibitors of MMPs tested (Fingleton 2008). After the first discouragement, the better understanding of the roles of the different members of these families of ECM enzymes in angiogenesis and related diseases has led to reopen the door to this pharmacological strategy (Winer et al. 2018). Therefore, the discovery and development of new inhibitors, highly selective and potent, that avoid the broad-spectrum targeting of different members of the same family of enzymes, may open new avenues for the search of pharmacological alternatives in the treatment of angiogenesis-dependent pathologies. In this context, natural bioactive compounds represent a very attractive source of new putative drugs targeting ECM-related enzymes, and bibliography about their potential activity in these targets is summarized in this chapter.

## 6.4 Natural Antiangiogenic Compounds Targeting MMPs

The role of MMPs in angiogenesis and other disease-related processes promoted the search for chemical inhibitors of these enzymes, aiming at developing new pharmacological strategies. As mentioned above, although the first inhibitors tested in the clinic failed in the attempt to use them as therapeutic drugs, due to the broad-spectrum inhibition and the consequent side effects, the interest in the discovery of more selective and potent drug candidates is increasing year to year. The identification of new compounds (natural and synthetic) that target MMPs is still a hot spot in pharmacological research (Lenci et al. 2021).

After analyzing the current literature regarding the natural compounds exhibiting antiangiogenic activity, the conclusion is that many of them have been described to affect MMPs production, secretion, or activity. Due to the important role of MMPs in angiogenesis, in many cases the inhibition of these key ECM remodeling family of enzymes is included as a part of the mechanism of action of the compounds capable to interfere with the angiogenic process. It is important to remark that MMP activities can be inhibited by TIMPs, which bind to the highly conserved zinc binding site of active MMPs at molar equivalence (Saraswati et al. 2013a). The balance between MMPs and TIMPs plays a key role in angiogenesis regulation and is critical for the eventual ECM remodeling (Nagase et al. 2006; Quintero-Fabián et al. 2019).

Curcumin, isolated from the rhizome of *Curcuma longa* plant, is a powerful angiogenesis inhibitor and it has important inhibitory properties in tumor initiation and growth. Human umbilical vein endothelial cells (HUVEC) treated with curcumin had decreased ability to form tubes, although migration and attachment were not affected in the Matrigel assay. Importantly, upon curcumin treatment, HUVEC exhibit a decrease in the gelatinolytic activities of secreted 53-kDa and 72-kDa MMPs, an effect that may be due to acting at the transcriptional and post-transcriptional level (Thaloor et al. 1998).

Mediterranean diet is an immense source of natural products, many of them with antiangiogenic and antiinflammatory properties. For example, hydroxytyrosol is one of the active compounds of the virgin olive oil that affects a wide spectrum of proteins involved in ECM remodeling, such as MMPs. It has been reported that endothelial cells treated with hydroxytyrosol have decreased MMP1 and MMP2 expression, and increased TIMP1, TIMP2, and TIMP4 levels (García-Vilas et al. 2017b). On the other hand, a reduced angiogenic response has been observed in endothelial cells incubated with some of the Mediterranean diet polyphenolic compounds, due to the inhibition of phorbol 12-myristate 13-acetate (PMA)-induced cyclooxygenase 2 (COX2) protein expression and prostanoid production, as well as MMP9 protein release and gelatinolytic activity (Scoditti et al. 2012).

One of the most important isoflavones is the genistein, which decreases MMP2 and MMP9 secretion and activities induced by VEGF stimulation. In addition, exposure to genistein decreases activation of JNK and p38 induced by VEGF. This kind of mechanism constitutes a common pathway for many compounds to

exert their antiangiogenic activity and their cancer protective function (Yu et al. 2012). Interestingly, genistein blocks the VEGF/bFGF-stimulated TIMP1 increased and TIMP2 decreased expression levels (Kim 2003).

Luteolin is a flavonoid found in many plants (medicinal herbs and some fruits), which suppresses VEGF-induced phosphorylation of VEGF receptor 2 and many downstream targets such as ERK, mTOR, MMP2, and MMP9, among many others (Pratheeshkumar et al. 2012). Other effects observed in cells treated with luteolin are the modulation of interleukin 6-receptor-alpha (IL6Rα) expression levels, the MMP2 secretion, and the expression of suppressor of cytokine signaling 3 (SOCS3). These effects trigger a reduction in endothelial cell proliferation and migration (Lamy et al. 2012).

Panduratin A, a chalcone isolated from *Boesenbergia rotunda*, suppresses MMP2 secretion and F-actin stress fiber formation in endothelial cell, affecting cell migration (Lai et al. 2012). Punarnavine shows antiangiogenic effects in vitro and in vivo (inhibits endothelial cell migration, invasion, and capillary structure formation, suppresses peritoneal angiogenesis in an Ehrlich ascites carcinoma tumor model, and decreases the neovascularization in the sponge implant assay). Additionally, it increases TIMP2 expression in HUVEC (Saraswati et al. 2013a). Other natural compounds, like allyl isothiocyanate (AITC) and phenyl isothiocyanate (PITC), display an in vitro antiangiogenic activity in HUVEC by enhancing the production of TIMP1 (Thejass and Kuttan 2007a).

## 6.5 Natural Antiangiogenic Compounds Targeting PA System

As indicated before, the PA system participates in the conversion of plasminogen to plasmin, which is an active proteolytic enzyme involved in the fibrinolytic process. This conversion is carried out by uPA or tPA, although there are some other players in this enzymatic system, such as uPA receptor (uPAR), plasminogen activator inhibitors (PAI-1/2), and plasmin inhibitors ($\alpha_2$-antiplasmin or $\alpha_2$-AP and $\alpha_2$-macroglobulin or $\alpha_2$-MG). The PA system does not only regulate angiogenesis through the proteolytic degradation of ECM components, but also by interfering with intracellular signaling and modulating gene transcription (Stepanova et al. 2016); therefore, it is assumed as a suitable antiangiogenic target. A compilation of natural antiangiogenic compounds that target the PA system proteins is included in Table 6.1.

The potential of natural compounds as inhibitors of uPA, together with the identification of their mechanism of action, is very well studied. Regarding the uPA-targeting compounds, the polyphenol EGCG should be pointed out, a flavonoid which is the most abundant catechin in green tea and is recognized as the main ingredient responsible for the green tea health benefits (Nagle et al. 2006). EGCG has been described as an antiangiogenic compound both in vitro and in vivo (Cao and Cao 1999; Xu et al. 2011), mostly in the context of vascular tumors, as a

chemopreventive or adjuvant setting (Jung and Ellis 2002; Fassina et al. 2004). Interestingly, this compound has been shown to inhibit uPA activity by direct binding to the enzyme, consequently interfering with its ability to recognize its substrates (Jankun et al. 1997). EGCG also inhibits PAI1 with an $IC_{50}$ of 0.091 μM (Cale et al. 2010).

Silibinin (silybin) is a flavonoid produced by the herb *Silybum marianum* (milk thistle), representing the major component of the plant extract silymarin. The interest in this compound arose from its hepatoprotective effects and its potential antineoplastic activity (Wing Ying Cheung et al. 2010). Regarding uPA targeting, silibinin shows a potent in vitro uPA inhibitory activity, with an $IC_{50}$ of 21 μM (Jedinák et al. 2006a). In the same way, hyperoside, another flavonoid found in traditional medicinal plants of the genera *Hypericum* and *Crataegus*, exhibiting antiangiogenic potential, also has the capability to directly inhibit uPA ($IC_{50}$ 8.3 μM) (Jedinák et al. 2006a; Liu et al. 2016; Wu et al. 2020). Other flavonoids, such as salicin or quercetin, are thrombin and uPA inhibitors, and have been described to exert antiangiogenic effects in vivo and in vitro (Kong et al. 2014; Zhao et al. 2014; Song et al. 2017).

Not only polyphenols, but also other chemicals found in plants, present both PA system and angiogenesis inhibitor effects. This is the case of hypericin, a naphthodianthrone found in *Hypericum* species, which can inhibit uPA activity (Jedinák et al. 2006a) and shows antiangiogenic activity in vitro and in vivo in dark conditions (Lavie et al. 2005; Martínez-Poveda et al. 2005a), or some anthraquinones present in herbs used in traditional medicine, such as rhein or emodin. Rhein (4, 5-dihydroxyanthraquinone-2-carboxylic acid) is extensively found in medicinal herbs and its potential role as hepatoprotective, nephroprotective, anti-inflammatory, antioxidant, anticancer, and antimicrobial compound has been suggested (Zhou et al. 2015). The antiangiogenic effect of rhein has been described in vitro and in vivo (Fernand et al. 2011; He et al. 2011) and this compound also targets uPA, directly inhibiting its activity in vitro (Jedinák et al. 2006a). In the same way, emodin has been used in traditional Chinese medicine as antibacterial or anti-inflammatory and has demonstrated a great antiangiogenic potential in the context of tumor angiogenesis (Kwak et al. 2006; Lin et al. 2015; Qu et al. 2015; Shi and Zhou 2018; Zou et al. 2020).

The mentioned compounds have been described as direct inhibitors of uPA enzymatic activity, but additionally, several natural antiangiogenic compounds are able to produce an indirect inhibition of these enzymes, for example, by decreasing their expression or by preventing their release from the cell. This is the case of curcumin, a polyphenolic compound extracted from *Curcuma longa* (turmeric), described as an inhibitor of angiogenesis (Bhandarkar and Arbiser 2007), which inhibits uPA secretion in casein zymographies of tumoral HT1080 cells, without directly affecting uPA enzymatic activity (Yodkeeree et al. 2009). Hydroxytyrosol, an antiangiogenic phenolic compound present in olive oil, decreases the uPA expression, whereas increases the uPAR expression levels in endothelial cells, which has been proposed to contribute to its mechanism of action in angiogenesis inhibition (García-Vilas et al. 2017b). In the same way, aeroplysinin-1, a secondary

metabolite isolated from a marine sponge, shows antiangiogenic effects with a decrease in uPA and an increase in PAI-1 activity in plasminogen zymographies (Rodríguez-Nieto et al. 2002a). Other examples are damnacanthal, a noni anthraquinone characterized as antiangiogenic, which decreases the uPA/PAI-1 ratio in endothelial cells (García-Vilas et al. 2017a), or aloe-emodin, a hydroxyanthraquinone from *Aloe vera* and other plants, that decreases uPA production in endothelial and tumor cells (Cárdenas et al. 2006).

Belonging to the PA system, PAIs are involved in the inhibition of plasminogen activation by uPA. Although uPA and PAI1 have opposite effects regarding plasminogen activation, both are known to stimulate angiogenesis, depending on the context (Isogai et al. 2001; Devy et al. 2002; Stepanova et al. 2016; Song et al. 2019). Among the natural compounds described to inhibit PAI1, several of them show antiangiogenic activity. The polyphenol tannic acid is produced by a wide range of edible plants, so its consumption is highly frequent around the world. Among its therapeutic potential, activities against oxidation, allergy, inflammation, cancer, dysentery, different infections (parasites, bacteria, virus), etc., have been described (Sharma et al. 2019). In addition, this compound has been shown to inhibit angiogenesis in vitro (Chen et al. 2003), and to strongly inhibit PAI1 activity with an $IC_{50}$ of 0.7 nM (Cale et al. 2010).

Other polyphenols, the black tea theaflavins theaflavin-3′-digallate and theaflavin-3,3′-digallate, have been described to inhibit angiogenesis (Gao et al. 2016a, b). Both theaflavins can inhibit the PAI activity, with theaflavin-3′-digallate having a more potent activity than the theaflavin-3,3′-digallate (Jankun 2011). Furthermore, the hydroxybenzoquinone embelin, isolated from *Embelia ribes* fruit (vidanga), inhibits PAI1 activity at low concentrations ($IC_{50}$ 1.62 μM) and has antiangiogenic potential (Lin et al. 2013; Coutelle et al. 2014; Narayanaswamy et al. 2014; Rouch et al. 2015).

## 6.6 Natural Antiangiogenic Compounds Targeting Heparanase

Heparanase is an ECM remodeling enzyme that represents an interesting target in antiangiogenic therapy. The substrates of heparanase are HSPGs that are involved in self-assembly and integrity of both basement membrane and ECM (Elkin et al. 2001) structures, which must be degraded to allow endothelial cell migration and growth factor availability during angiogenesis. Some natural antiangiogenic compounds that directly target the heparanase enzymatic activity, such as defibrotide and sulodexide, are included in Table 6.1. Defibrotide is a mixture of single-stranded oligodeoxyribonucleotides isolated from porcine mucosal DNA, with recognized antiangiogenic activity in vitro and in vivo (Koehl et al. 2007). The direct inhibition of heparanase by this compound has been reported in an in vitro model of multiple myeloma (Mitsiades et al. 2009; Mohan et al. 2019). Sulodexide is a highly purified

mixture of GAGs isolated from porcine intestinal mucosa, which directly targets heparanase enzymatic activity (Masola et al. 2012) and shows antiangiogenic potential in vitro (Niderla-Bielińska et al. 2019).

Beside the natural inhibitors of heparanase mentioned above, which show the capability of directly inhibiting the enzymatic activity of this enzyme, other natural compounds can produce an indirect inhibition of heparanase. For example, bestatin is a compound that inhibits heparanase expression and angiogenesis in a diabetic retinopathy model (Hossain et al. 2016). Furthermore, carrageenans are polysulfated polygalactans obtained from red algae that can inhibit the release of heparin-binding growth factors and are potent inhibitors of endothelial and cancer cell proliferation. Nevertheless, these molecules can be toxic or proinflammatory due to their high molecular weight; to overcome this, low molecular weight depolymerized variants have been produced, which have shown a great antiangiogenic activity (Chen et al. 2007; Yao et al. 2014; Niu et al. 2015; Poupard et al. 2017; Mohan et al. 2019).

## 6.7 Natural Antiangiogenic Compounds Targeting Cathepsins

Cathepsins are proteases involved in both physiological and pathological angiogenesis and, although they are normally found into cells—inside endosomes and lysosomes—, some of them are at the extracellular space. Among them, cathepsins B, K, L, S are involved in angiogenesis (Vidak et al. 2019), representing potential targets of natural compounds that could be candidates as antiangiogenic drugs. Natural antiangiogenic compounds targeting cathepsins are included in Table 6.1.

The peptidyl aldehydes leupeptin and chymostatin, isolated from *Streptomyces exfoliatus* and *Streptomyces sp.* respectively, have been described as cathepsins A, B, D, and K inhibitors (Vidal-Albalat and González 2016). These natural molecules show antiangiogenic potential, since leupeptin interferes with bFGF-induced angiogenesis in guinea pig cornea (Tamada et al. 2000) and chymostatin inhibits angiogenesis in a hamster sponge angiogenesis model (Muramatsu et al. 2000).

Additionally, 7-epiclusianone, a prenylated benzophenone found in *Garcinia brasiliensis* (bacupari) and used in traditional Brazilian medicine for inflammation (Santa-Cecília et al. 2011), is a cathepsin B inhibitor (Vidal-Albalat and González 2016) that has shown antiangiogenic potential in vitro (Taylor et al. 2019). Finally, ursolic acid is a known natural angiogenesis inhibitor (Cárdenas et al. 2004; Kanjoormana and Kuttan 2010) found in many plants, fruits, and herbs, and is also able to inhibit cathepsins B, L, and D (Jedinák et al. 2006b; Vidal-Albalat and González 2016).

## 6.8 Natural Antiangiogenic Compounds Targeting ADAMs and ADAMTSs

Like the proteases mentioned above, some natural compounds characterized as antiangiogenic are also known to inhibit some ADAM and ADAMTS members (Table 6.1).

Focusing in ADAMs family, ADAM8, ADAM9, ADAM10, ADAM15, and ADAM17 are members that are especially involved in angiogenesis (Donners et al. 2010; van der Vorst et al. 2012). The natural compounds rapamycin (sirolimus), produced by the soil bacterium *Streptomyces hygroscopicus*, and triptolide, isolated from the medicinal plant *Tripterygium wilfordii* Hook F, exhibit antiangiogenic potential in vivo and in vitro (He et al. 2010; Wang et al. 2019) and have been described to indirectly inhibit ADAM10 by decreasing its expression in SweAPP N2a cells and leukemia cells, respectively (Wetzel et al. 2017). The natural compound luteolin, a common flavonoid produced by several plants (López-Lázaro 2009), inhibits angiogenesis in vivo and in vitro (Bagli et al. 2004) and controversially, it has also been reported to inhibit ADAMTS 4/5 activity (Moncada-Pazos et al. 2011) which have shown to have an antiangiogenic role (Kumar et al. 2012).

## 6.9 Natural Antiangiogenic Compounds Targeting Elastases

Human neutrophil elastase (HNE) produces endothelial cell damage, promotes tumor cell intravasation (Grechowa et al. 2017; Deryugina et al. 2020), and is capable to degrade angiogenic factors, such as VEGF and basic fibroblast growth factor (bFGF) (Ai et al. 2007). Despite this apparent antiangiogenic role of HNE, there are some natural antiangiogenic compounds that have also shown an inhibitory effect on HNE, such as the previously mentioned EGCG, which inhibits neutrophil-dependent angiogenesis in vivo (Donà et al. 2003) and strongly inhibits HNE enzymatic activity in A459 cells (Xiaokaiti et al. 2015). The above-mentioned luteolin, the flavonoid compound found in bee pollen and propolis, phloretin (the major polyphenol compound in apples), and the triterpene compound lupeol have also been described to inhibit HNE and angiogenesis (Bagli et al. 2004; Siedle et al. 2007; Lin et al. 2008; Nema et al. 2013; Vijay Avin et al. 2014; Kangsamaksin et al. 2017; Hsiao et al. 2019).

Furthermore, there are natural antiangiogenic compounds which have been reported to inhibit other elastases, such as the polyphenolic compound (+)-catechin (present in tea) or the triterpenoid oleanoic acid (present in olive oil), which inhibit porcine pancreatic elastase (PPE) activity (Guruvayoorappan and Kuttan 2008; Kim et al. 2009; Wittenauer et al. 2015; Li et al. 2016; Yee et al. 2017). The pentacyclic triterpenoid ursolic acid, present in many plants such as *Rosmarinus officinalis*

(rosemary), inhibits leukocyte elastase (Ying et al. 1991), and all of them inhibit angiogenesis (Cárdenas et al. 2004; Kanjoormana and Kuttan 2010).

## 6.10 Conclusions

The aims of this chapter have been to highlight the important role of the ECM in the vascular remodeling and to revise the current literature to classify the natural compounds that exhibit an antiangiogenic activity and affect the production, secretion, or activity of ECM enzymes. The ECM is a rich source of growth factors and enzymes that control and influence vascular morphogenesis and homeostasis by the regulation of its composition. In the last years, a plethora of ECM molecules have emerged as promising targets for the treatment of several human diseases, such as cancer, fibrosis, and inflammation, among others. Interestingly, some of the natural antiangiogenic compounds mentioned in this chapter are currently in clinical trials for the evaluation of their possible therapeutic role in different pathological context related to aberrant angiogenesis, mainly in cancer (https://clinicaltrials.gov/). This is the case of EGCG, which is being studied for the improvement of endothelial function in cardiovascular diseases and several types of cancer, and silibinin or quercetin, which are being tested for the treatment of different cancers. In the context of angiogenesis-dependent ocular pathologies, hydroxytyrosol is being investigated for the treatment of wet macular degeneration, and atarin is in clinical trials for diabetic retinopathy. Of note, much of the compounds compiled in the chapter based on their antiangiogenic potential and their inhibitory activity on ECM-degrading enzymes have been long used (as a part of extracts or herb infusions) in traditional medicine for the treatment of different pathologies, as is the case of luteolin (Lin et al. 2008), and even some of them are approved for their use in certain clinical settings, as is the case of rapamycin, defibrotide, or (+)-catechin.

Although promising, the identification of ECM-based inhibitors of angiogenesis used for clinical applications is still limited and many efforts are needed to unravel their therapeutic potential. Hence, a better understanding on the ECM proteins, their ligands and the molecular interactions between the matrix molecules will help researchers and clinicians to identify new biomarkers and antiangiogenic strategies, further developing novel ECM-based strategies for the treatment of pathological conditions. In this context, nature represents a rich source of new antiangiogenic molecules with the potential to inhibit ECM-degrading enzymes, representing this activity a feasible part of their mechanism of action in the modulation of angiogenesis. The complexity of the angiogenesis process and the better results achieved by multi-target pharmacological strategies, point to the great interest of these natural compounds, which in most of the cases show multiple molecular targets and pleiotropic bioactivities. Future research directions toward the optimization of the existing tools used in the characterization of natural antiangiogenic compounds interfering with the ECM-degrading enzymes, will offer a valuable benefit in the design of novel ECM-based strategies in the antiangiogenic therapy.

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# Chapter 7
# Cell–Extracellular Matrix Adhesions in Vascular Endothelium

**Sophia Valaris and Vassiliki Kostourou**

**Abstract** The vascular system is a conduit network of blood vessels, which supplies tissues with the necessary oxygen and nutrients for survival, coordinates mechanical forces, enables multi-organ communication by circulating hormones and biochemical cues, and provides a gateway for immune surveillance. Genetic deletion of essential components of blood vessel formation leads to embryonic lethality, which emphasises the importance of this system. Blood vessels consist of (1) endothelial cells (ECs) that line the inner surface of the vessel and create the lumen, (2) basement membrane composed of extracellular matrix (ECM) proteins that interact with and support ECs, and (3) mural cells (pericytes and smooth muscle cells) that surround the outer side of the vessel and provide structural support. All vascular cells—ECs and mural cells—, attach to each other and to the basement membrane. These adhesions maintain the organisation of the vascular network and the structural integrity of blood vessels. The cell–ECM adhesions are strictly controlled in time and space, crosstalk with signalling cascades to maintain vascular homeostasis and function, and their remodelling is a prerequisite for new blood vessel development. In this chapter, we focus on the developmental processes of angiogenesis, through the lens of the ECs and ECM interaction. We will present the recent developments in EC specification, highlighting the importance of cell–ECM adhesions in the different steps of vascular morphogenesis and discuss the role of key cell–ECM adhesion proteins in angiogenesis.

## 7.1 Organisation of the Vascular System

Studies of the murine embryonic development, retinal vascularisation, and tumour angiogenesis have been instrumental in understanding how angiogenesis is regulated, the importance of both biochemical and biomechanical cues in the

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development and maintenance of the vascular system and the essential role of key cell–ECM adhesion proteins in the angiogenic process (Stahl et al. 2010; Nikolopoulou et al. 2021; Arapatzi et al. 2022).

The vascular system forms a hierarchical network of blood vessels and lymphatics, consisting of arteries, veins, and capillaries. Each vessel network is specialised for its functional purpose. For example, arteries are supported by specialised extracellular matrix (ECM) and layers of vascular smooth muscle cells which regulate the vascular tone, whereas veins are thinner with fewer mural cells and have valves in their lumen, controlling the direction of the blood flow. In smaller vessels, such as arterioles and post-capillary venules, another type of mural cells, called pericytes, wrap around endothelial cells (ECs), particularly at cell–cell junctions and branch points (Armulik et al. 2010). The simpler thin vessel structures of capillaries, lacking pericytes, allow for the efficient exchange and supply of nutrients and oxygen to tissue. The lymphatic system is another functionally and structurally similar organ, comprising a network of blind-end, thin-walled endothelial tubes. Lymphatics are responsible for fluid balance and lipid metabolism by draining and collecting excess interstitial fluid and proteins and returning them back into the blood circulation via the thoracic duct. Another important function of the lymphatic system is the regulation of the immune responses attained by immune cells trafficking via specialised immune organs, such as the lymph nodes and spleen.

Although ECs form the lumen in all blood vessels, depending on the vessel type, they display distinct cell signalling signatures; for example, arterial ECs highly express Notch pathway components, whereas vein ECs have low expression. The Notch signalling pathway controls the Eph/Ephrin family expression, leading to arterial ECs expressing high levels of Ephrin-B2 ligand, while venous ECs with repressed Notch signalling express Eph-B4 receptor (Potente et al. 2011). Noteworthily, besides the biochemical cues, the formation of the hierarchical network of arteries, veins, and capillaries is dependent on the mechanical stimulation generated by the haemodynamic flow of perfusion (Karthik et al. 2018).

Similarly, the composition of the vascular basement membrane (BM) does not remain constant. The BM of blood vessels consist of three main categories of ECM proteins: (1) Glycosaminoglycans (GAGs): large polysaccharides that mostly present in the proteoglycans, (2) Fibrous proteins, such as collagen and elastin, that regulate the stiffness and elasticity of the ECM, and (3) Adhesive glycoproteins (laminin, fibronectin, tenascin, nidogen), which interact with cells via specific membrane receptors and connect with proteoglycans or collagen fibres to form a functional ECM network. The ECM comes together through a process of "self-assembly", whereas continuous remodelling of ECM occurs through a balance of synthesis, deposition, degradation, and modification of the different components. Both tissue resident cells and the vascular cells, ECs and mural cells, contribute to ECM organisation via secretion of ECM components and modifiers (Kusuma et al. 2012). A more embryonic-like composition of ECM, rich in GAGs, fibronectin, and proteoglycans, promotes EC migration and new blood vessel formation, while a collagen- and laminin-rich ECM, in adults, stabilises blood vessels and maintains their quiescent phenotype (Senger and Davis 2011; Kostourou and Papalazarou 2014).

## 7.2 Tissue-Specific Endothelial Cells

Early studies using electron microscopy had described morphological differences in the vascular beds of several organs, indicating the existence of heterogeneity in ECs (Simionescu et al. 1975, 1976). Advances in transcriptomic and proteomic studies and the use of single cell approaches have illuminated the heterogeneity of ECs and their organ-/tissue-specific phenotypes, even though ECs originate from the same progenitor cells during development, called angioblasts, derived from the extraembryonic mesoderm of the yolk sac (Fig. 7.1a–d) (Aird 2007; Kalucka et al. 2020; Díaz del Moral et al. 2020; Gifre-Renom et al. 2022). This heterogeneity is presumably to achieve the distinct physiological needs of each tissue, but ECs are also

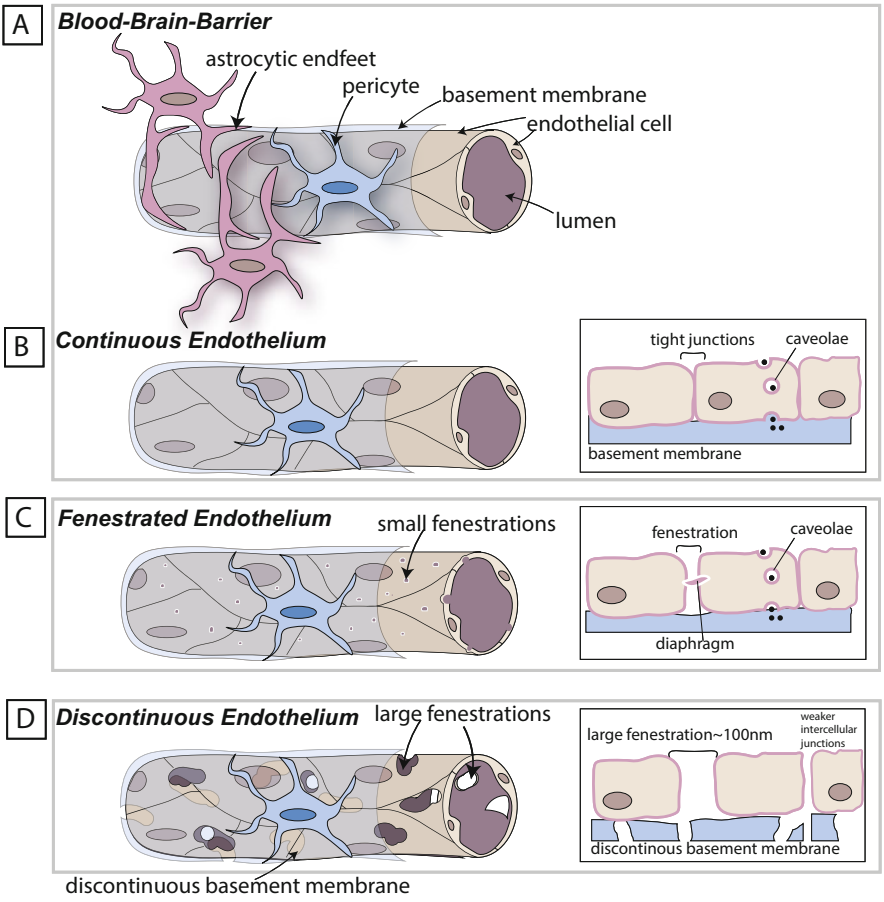


**Fig. 7.1** Endothelium heterogeneity. Schematic of vascular endothelium heterogeneity. (**a**) Blood–brain barrier vasculature, (**b**) Continuous endothelium, (**c**) Fenestrated endothelium, and (**d**) Discontinuous endothelium

dynamic and highly adaptive to changes in their microenvironment, dictated by biochemical and biomechanical cues.

Tissue-specific endothelial subtypes can be broadly categorised into three main phenotypes: discontinuous, fenestrated, and non-fenestrated endothelium (Aird 2007; Gifre-Renom et al. 2022).

The *discontinuous endothelium* is found in sinusoids of the bone marrow, spleen, and liver, where permeability is high (Fig. 7.1d). These endothelia have (1) large fenestrae; transcellular pores extending the length of the cells at ~100–200 nm in diameter, (2) loose cell–cell junctions, and (3) their BM is minimal or partly developed, resulting in high permeability. The highly penetrable characteristics of discontinuous sinusoidal endothelium are ideal for immune surveillance and scavenger function against pathogens or toxins, recycling proteins and lipids/reabsorption of nutrients and releasing haematopoietic cells into the circulation (Shetty et al. 2018; Gifre-Renom et al. 2022). In agreement with this, single-cell RNA sequencing studies showed that liver and spleen ECs have upregulated expression of scavenging and immune regulation transcripts and molecules coming from the intestine (Kalucka et al. 2020). Furthermore, unique to the liver is the use of clathrin-coated pits, which mediate endocytosis of many substances, as part of their effective scavenger functions (Shetty et al. 2018).

The *fenestrated endothelium* is found in blood vessels of kidney, endocrine glands, gastric and intestinal mucosa enabling rapid exchange of molecules between circulation and tissue (Fig. 7.1c). Fenestrated endothelia have (1) transcellular fenestrae; pores of ~70 nm in diameter. Most fenestrae possess thin non-membranous diaphragms across their opening, which act as molecular filters, (2) tight cell–cell junctions, and (3) a normal BM and pericyte coverage. These structural characteristics allow selective permeability by size exclusion in the infiltrated fluid. For example, in the kidney, the glomerular endothelium serves as a "sieve" barrier with its main function being the transportation and reabsorption of components, which are enabled by its diaphragms and a glycocalyx BM (Verma and Molitoris 2015). Similarly, ECs in the intestine act as a gut–vascular barrier, which controls the access of antigens and macromolecules translocating the gut lumen across the blood ECs for nutrient uptake (Spadoni et al. 2015). The cell–cell junctions in enteric ECs consist of tight junctions, formed with claudins, occludins, zonula occludens-1 (ZO-1), cingulins, and adherens junctions, composed by vascular endothelial (VE)-cadherin, junctional adhesion molecules (JAMs), α- and β-catenin, etc.

The *non-fenestrated or continuous endothelium* is found in tissues with low permeability, such as the heart, lung microvessels, large arterial and venous vessels, and the brain (Fig. 7.1b). It is characterised by (1) tight intercellular junctions formed by tight and adherens junctions, (2) lack of any type of fenestrae, and (3) stable BM and pericyte coverage. Communication across the endothelium occurs with caveolae and vesiculo-vacuolar organelles, which are found in higher concentration in most capillaries of non-fenestrated endothelium. In the heart, the low permeability of the non-fenestrated endothelia is necessary for the function of the organ. ECs line each chamber of the heart and the microvascular beds of the heart tissue itself, which all have distinct transcriptomic profiles. This heterogeneity is determined by

mechanical cues, including tissue stiffness governed by the ECM composition and blood flow rate, as well as biochemical cues derived from the neighbouring cells, such as cardiomyocytes (Gifre-Renom et al. 2022). Cardiac EC specialisation has led to their ability to uptake and provide specific nutrients for the efficient functioning of the heart. For example, heart contractions and ion homeostasis are high energy consuming processes and 90% of cardiac ATP is derived from mitochondrial fatty acid oxidation. Therefore, cardiac ECs express specific receptors CD36 and FABP4 to facilitate fatty acid uptake from the circulation (Lother et al. 2018).

A unique structure of the vascular system is the *blood–brain barrier (BBB)* (Fig. 7.1a). In the brain, the vascular network is highly specialised and forms a physical barrier to the circulation with greater selection for permeability compared to the periphery. The brain endothelium exhibits tight inter-cellular junctions and highly selective transporter system, with a size exclusion of 500 Daltons. The continuous non-fenestrated endothelium has few caveolae and high expression of tight junction proteins, among them proteins belonging to occludin and claudin family. The brain ECs express selective membrane transporter proteins such as Glut1 (glucose transporter type 1), due to glucose being the main nutrition source of the brain (Lee and Klip 2012). The structural composition of a BBB vessel consists of a continuous BM, pericytes, and astrocytes, particularly their foot processes (Atkins et al. 2011; Bernardo-Castro et al. 2020). Interestingly, within the brain, there is also heterogenicity of ECs. Recent single-cell transcriptomic analysis identified a subcluster of choroid plexus capillary ECs expressing plasmalemma vesicle associated protein (*Plvap*), a gene that is involved in the formation of fenestrae in endothelia (Kalucka et al. 2020).

The *lymphatic endothelium* is defined as sinusoidal and discontinuous endothelium due to their high permeability. Lymphatic capillaries are lined with lymphatic ECs without pericytes and with minimal BM. Lymphatic ECs possess fenestrae with diaphragms contributing to size-selection of transferred molecules and loosely organised cell–cell junctions (Jalkanen and Salmi 2020).

## 7.3 Vasculogenesis

During embryonic development, vessels are formed de novo by the assembly of mesoderm-derived endothelial precursor cells (angioblasts) that drive the process of *vasculogenesis*, leading to the assembly of primitive vessels, such as the primary capillary plexus in the early embryo. This structure is one of the first systems to develop in the embryo (Hou et al. 2022).

The initial process of vasculogenesis relies on the proliferation, migration, and differentiation of the angioblasts and endothelial precursor cells (EPCs) to the site of neovascularisation, guided by chemokines and growth factors. A critical step of the vasculogenesis process is the deposition and assembly of the ECM of the vessels, due to its importance in maintaining the function and integrity of blood vessels. In embryos, EC and pericyte interaction induces perivascular deposition of fibronectin,

a critical component in the ECM assembly in development, during the formation of vascular tubes (Senger and Davis 2011; Kostourou and Papalazarou 2014).

Once the primitive vasculature has been established, it usually resembles a honeycomb meshwork. Vessels undergo a process of pruning and remodelling to form an intricate architecture that facilitates efficient blood flow to the surrounding tissue. The biomechanical signals play a fundamental role in vascular remodelling. The perfusion of new vessels induces mechanical stimulation through the haemodynamic blood flow, the cyclic stretch generated by the pulsative nature of the blood pressure. These mechanical stimulations lead to the specification of the vessels into arteries and veins (Fig. 7.2a) (Karthik et al. 2018). These primitive vascular networks eventually specialise their vessel structures to form vessels, such as the dorsal aorta and cardinal vein (Risau 1997).

In a homeostatic, mature blood vessel, ECs are quiescent and rarely form new branches. However, ECs retain high plasticity to sense and respond to angiogenic signals given any change to the tissue environment (Potente et al. 2011).

In adults, the process of vascularisation is mediated by the incorporation of EPCs derived from the bone marrow or tissue-resident EPCs, to newly formed blood vessels (Asahara et al. 1997; Liu and Velazquez 2008). The clonogenic and proliferative potential of EPCs supports vessel development. During wound healing, EPCs are stimulated to proliferate and move from the bone marrow towards the tissue site by chemokines and growth factors signals to pave the way for new blood vessels formation (Liu and Velazquez 2008). Likewise, EPCs recruitment has been described in adult post ischaemic injury during new capillary formation (Asahara et al. 1997). Following ischaemic stroke, it has been shown that bone marrow derived EPCs enter the periphery and the site of injury for vascular regeneration (Paczkowska et al. 2013). Tissue-resident EPCs also contribute to the regenerative capacity of vasculature in the postnatal period (Yu et al. 2016). For example, using the CD157+ expressing EPC marker, it has been shown that tissue-resident EPCs exist in large arterioles and veins, and upon liver-induced injury proliferate to repopulate the liver vasculature (Wakabayashi et al. 2018). Finally, the tumour microenvironment can also promote the recruitment of EPCs from tissue resident and haematopoietic origins, inducing neovessel formation in tumours to increase oxygen and nutrient supply (Bussolati et al. 2011).

## 7.4 Angiogenesis

The textbook definition of angiogenesis is the formation of new vessels from pre-established ones, but the term of angiogenesis is generally used for all processes of vascular network formation including sprouting, enlargement, pruning, and remodelling of blood vessels. These processes take place during embryonic blood vessel formation to re-organise and expand the primitive/immature vasculature into a functional blood vessel network.

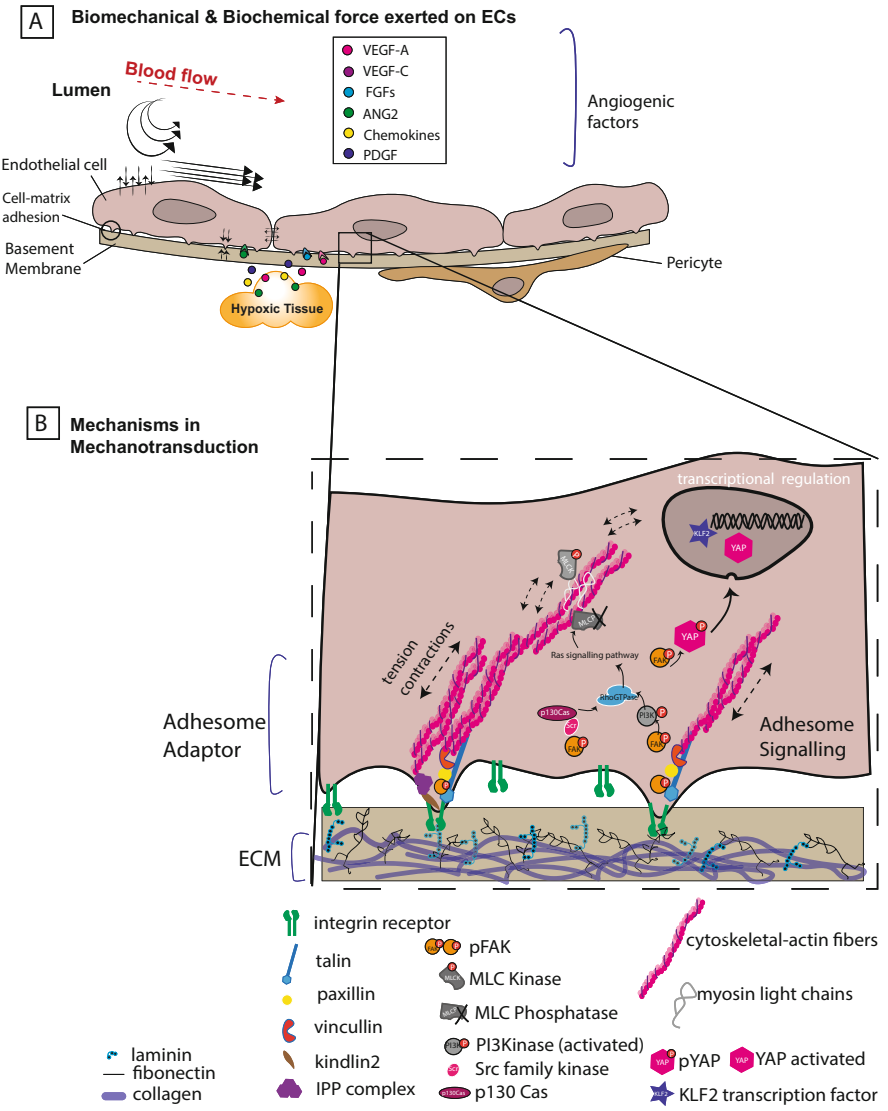


**Fig. 7.2** Extracellular cues and intracellular mechanotransduction and signalling cascades in ECs. (**a**) Schematic of biomechanical and biochemical signals exerted on ECs in the vasculature. Biomechanical signals include shear stress, cyclical and pulsative forces from blood flow, tension generated by cell–cell adhesion and cell–ECM adhesion. Biochemical cues include angiogenic factors, such as VEGF, FGF, ANGPT2 (Ang2), chemokines, and PDGF, released from hypoxic tissue adjacent to blood vessel. (**b**) Molecular components of a cell–ECM adhesion site. Integrin-ECM binding induces actin cytoskeletal rearrangement, adhesion mechanotransduction and signalling by scaffolding proteins and mechanotransducers, such as talin, paxillin, vinculin, kindlin2, and the IPP complex, and adhesome signalling molecules, such as phosphorylated FAK, MLCK, MLCP, PI3Kinase, SFK, YAP, KLF2 transcription factor

The growth of the vascular network mainly occurs by two mechanisms: (1) angiogenic sprouting and (2) intussusception.

### 7.4.1 Sprouting Angiogenesis

Sprouting angiogenesis is the process of developing new branches from pre-existing vessels. This is the most commonly occurring angiogenic process and is strictly controlled in space and time. This multistep process can be divided into the following phases: (1) Endothelial activation, (2) Tip cell selection, (3) Sprout elongation, (4) Lumen formation, (5) Vessel perfusion and patterning, and (6) Vessel maturation.

*Endothelial activation* is induced in ECs of a pre-existing vessel proximal to tissue with increased oxygen or nutrient demand. The hypoxic conditions and/or the release of angiogenic growth factors drives quiescent ECs to become activated. Some angiogenic factors include vascular endothelial growth factor A (VEGF-A) and C (VEGF-C), fibroblast growth factors (FGFs), angiopoietin 2 (ANGPT2), chemokines, and platelet-derived growth factor (PDGF) (Fig. 7.2a). The activation of ECs and other tissue stromal cells starts to produce matrix metalloproteases (MMPs), which remodel the ECM/vascular BM. This allows ECs to remodel their intercellular junctions and ECM-adhesions, proliferate and migrate towards hypoxic tissue and angiogenic stimulus.

*Tip cell selection* is the next stage of the process. During the migration of ECs, one EC is selected as being the leading cell to guide vessel sprout and is defined as the tip cell. The tip cell selection is regulated through the cross-talk between VEGF and Delta-like4 (Dll4)/Notch pathways (Kofler et al. 2011; Lugano et al. 2020). During this process, tip cells are driving the migration and orientation of the new sprouting vessel by using actin-driven elongated membrane protrusions, called filopodia, and newly formed cell–ECM adhesions; these structures allow tip cells to receive and translate microenvironment cues, such as the VEGF-A gradient and the remodelled ECM (Gerhardt et al. 2003; Itoh 2015).

*Sprout elongation*: In response to VEGF, tip cells produce Dll4 ligand, which acts as a negative feedback signal to neighbouring ECs, induces higher levels of Notch signalling, and suppresses additional tip cell formation. This pathway promotes stalk cell phenotype in neighbouring ECs, stimulates their proliferation, and enables sprout elongation (Potente et al. 2011; Kofler et al. 2011; Lugano et al. 2020). Stalk cells establish adherens junctions, which connect neighbouring ECs and produce BM components for structural vessel support.

*Lumen formation* is generated by the rearrangement of cell contacts, polarity, and shape via 3 main mechanisms: (1) budding, (2) cord hollowing, and (3) cell hollowing. Budding results from the expansion of a pre-existing vessel lumen to the neighbouring EC. In cord, hollowing ECs reorganise the contact surface between cells and produce multiple small or large vesicles that fuse at their apical membrane in order to produce an extracellular lumen (Geudens and Gerhardt 2011). The cell

hollowing mechanism is established by intracellular vacuole fusion within the cytoplasm to produce an intracellular lumen that subsequently fuses to the lumen from adjacent cells, to form a continuous and blood filled channel (Betz et al. 2016). The successful completion of these mechanisms requires the efficient remodelling of cell adhesions and cytoskeleton.

*Vessel perfusion* of newly formed blood vessels is achieved by sprout *anastomosis*. This process is accomplished via tip cell fusion from two different sprouts (head-to-head anastomosis) or via one tip cell fusion with a functional blood vessel (head-to-side anastomosis). In some cases, such as the retina neovascularisation, this process is chaperoned by tissue-specific macrophages, enabling the tip cell connection. Following perfusion, newly formed vessels undergo a process of *remodelling* and pruning, which is largely dependent on biomechanical stimuli of perfusion, particularly in segments of the vessels that exhibit low or variable blood flow (Fonseca et al. 2020). In a model of midbrain vascular development in zebrafish, Chen et al. found that pruning was triggered by blood flow changes. Interestingly, they also found that ECs of the vessels undergoing pruning migrate and integrate into unpruned vessel segments, in a process of reconstruction (Chen et al. 2012). However, in murine models of hyaloid vessels in the eye, regression of blood vessels was achieved by macrophages inducing apoptosis in ECs (Lobov et al. 2005; Rao et al. 2007). Although the mechanism of vessel pruning is still under investigation, it is well-established that the mechanical forces induced by the flow and blood pressure in the new lumen stimulate the production of the shear stress response transcription factor Kruppel-like factor 2 (KLF2). KLF2 is believed to act as conductor for vessel remodelling and maintenance in ECs, as low expression of KLF2 leads to vascular regression (Lee et al. 2006; Fonseca et al. 2020).

*Vessel maturation*, stabilisation, and quiescence are the final state of sprouting angiogenesis, where the new vessel becomes a stable part of the functional network. This is dependent on the deposition of ECM proteins in the BM, the recruitment of mural cells, and the establishment of a quiescent state for ECs. ECs secrete growth factors, such as PDGF, epidermal growth factor (EGF), transforming growth factor β (TGFβ), and angiopoietins, which induce mural cell proliferation, migration to the perivascular site and association with ECs. Together, mural cells and ECs begin enriching the ECM with collagens and laminins, creating a stable BM.

ECs enter a quiescent "phalanx" phenotype as a response to Notch signalling, which reduces migration and proliferation. But even in this quiescent state, ECs remain adaptable, detecting and responding to mechanical and biochemical signals by converting them into cellular responses that involve gene expression and protein synthesis (Vandekeere et al. 2015; Charbonier et al. 2019). The formation of perfused new vessels ensures the supply of oxygen and nutrients, switching off the angiogenic factors and hypoxic conditions and establishing a homeostatic quiescent phenotype.

### 7.4.2 *Intussusceptive Angiogenesis*

An alternative mechanism of angiogenesis is intussusceptive angiogenesis, which occurs by splitting of a blood vessel into two new ones via cellular reorganisation. Large vessels can be remodelled to give rise to smaller capillaries. This process is faster and energetically more conservative than sprouting, because it occurs irrespectively of proliferation (Djonov et al. 2003). The intussusceptive angiogenesis develops in response to growth factors or shear stress on vessel walls caused by increased haemodynamic flow (Djonov et al. 2003; Li et al. 2021). ECs lining opposing capillary walls make a protrusion into the vessel lumen creating a zone of contact and a transluminal tissue pillar (Karthik et al. 2018). This endothelial bilayer is perforated, intercellular contacts are reorganised and change the adhesion properties between ECs. This temporarily increases vessel wall permeability, which allows the entrance of growth factors and pericytes. The mural cells produce the ECM foundation of the new vessel wall resulting in the splitting of the vessel. The molecular mechanisms and the conditions by which intussusception occurs are still under investigation.

Central to all processes of blood vessel development are the re-organisation of cell adhesions and the integration of biochemical and biomechanical cues to coordinate EC function.

## 7.5 Cell–ECM Adhesion

Cell–ECM adhesions are the sites where the cell is physically attached to ECM and where the integrin-mediated signalling and mechanotransduction—the process of sensing and translating mechanical stimuli to intracellular signals—occurs (Charbonier et al. 2019; Nikolopoulou et al. 2021) (Fig. 7.2). Cell–ECM adhesion sites can vary greatly in size (0.2–10 μm), protein composition and thus, in their dynamic stability and mechanotransducing capability. The network of scaffolding and signalling proteins associated with cell–ECM adhesions is collectively called the "*adhesome*" (Whittaker et al. 2006; Zaidel-Bar et al. 2007). The molecular composition of adhesome differs in space, time, and tissue, but they include over 232 members. The adhesome proteins can be categorised as (1) integrin receptors, (2) mechanotransducer and adaptor molecules, (3) signalling molecules, and (4) cytoskeletal regulating molecules (Avraamides et al. 2008). Several lines of research in cells and model organisms have established the essential role of many adhesome components in cytoskeletal rearrangements and cell migration, cell proliferation, differentiation, and gene expression (Zaidel-Bar et al. 2007; De Pascalis and Etienne-Manneville 2017).

Below, we will discuss in depth how adhesome components sense and respond to microenvironmental changes and transmit biochemical and mechanical cues to orchestrate endothelial function, focusing on integrins, mechanotransducers, and

adaptor proteins, and signalling adhesome components (Fig. 7.2b). We will present evidence from in vivo, cellular studies for the endothelial dependent function of these components in angiogenic responses.

### 7.5.1 Integrin Receptors

Integrins are heterodimers composed of an α and a β subunit, and they form 24 unique combinations of α and β pairs. Integrin receptor activation, the switching from a low to a high binding affinity to ECM can be induced by external mechanical forces and intracellular mechanical (actomyosin contractions) or biochemical stimulation. Because their cytoplasmic tail does not inherently have enzymatic activity, their signal transduction occurs through recruiting and clustering of adhesome adaptor and signalling proteins, to link them to the cell cytoskeleton and other intracellular signal transduction pathways.

ECs express a diverse repertoire of integrin receptors, including receptors binding to collagen ($\alpha_1\beta_1$, $\alpha_2\beta_1$), fibronectin ($\alpha_5\beta_1$, $\alpha_4\beta_1$, $\alpha_V\beta_3$), vitronectin ($\alpha_V\beta_3$, $\alpha_V\beta_5$), and laminin ($\alpha_3\beta_1$, $\alpha_6\beta_1$) (Silva et al. 2008). Besides, binding ECM components in the BM of blood vessels, integrins also interact with some angiogenic factors, such as VEGF-A (Avraamides et al. 2008; Li et al. 2016) and intercellular adhesion molecules, such as intercellular adhesion molecule 1 (ICAM1) and vascular cell adhesion protein (VCAM) (Hynes 2002).

Genetic ablation of integrin $\beta_1$, the most common subunit of integrin dimers in the ECs, leads to embryonic lethality at embryonic day (E)9.5-E10.5, with severe vascular defects (Tanjore et al. 2008; Lei et al. 2008; Carlson et al. 2008). These results establish an indispensable role of integrins in blood vessel formation. However, the genetic ablation of integrin α subunits has generated mixed results for the role of different integrin pairs on angiogenesis and vasculogenesis. For example, although total deletion of integrin $\alpha_5$ subunit results in embryonic lethality due to angiogenic defects (Yang et al. 1993; Francis et al. 2002), specific EC knock out of integrin $\alpha_5$ alone does not have any obvious developmental effects on angiogenesis. Endothelial knockout of integrin $\alpha_5$ in combination with integrin $\alpha_v$ subunits has normal angiogenesis until E11.5 and then displays extensive defects in great vessel remodelling and heart, resulting in death by E14.5 (Van Der Flier et al. 2010). In agreement with this, in experimental autoimmune encephalomyelitis (EAE), a mouse model for multiple sclerosis, endothelial $\alpha_5$ knockout mice showed faster progression of disease, which correlated to accelerated vascular disruption and reduced vascular density (Kant et al. 2019).

In pathological tumour angiogenesis, both pro-angiogenic and anti-angiogenic activities have been attributed to integrins. Antagonists of integrin $\alpha_4$ subunit (Garmy-Susini et al. 2005) or a combination of $\alpha_1$ and $\alpha_2$ blocking antibodies markedly inhibited VEGF-driven angiogenesis (Senger et al. 1997) and tumour growth. In contrast to this, endothelial-specific deletion of $\alpha_3$ or $\alpha_6$ integrin subunits

enhanced tumour growth and angiogenesis (Da Silva et al. 2010; Germain et al. 2010).

Expression of $\alpha_V\beta_3$ and $\alpha_V\beta_5$ integrins is upregulated in the angiogenic endothelium and integrin $\alpha_v\beta_3$ inhibitors have been shown to decrease tumour angiogenesis and cancer growth (Foubert and Varner 2012). In striking contrast, global deletion of $\beta_3$ integrin enhanced pathological angiogenesis (Reynolds et al. 2002) and low concentration of $\alpha_v\beta_3$ inhibitors stimulated tumour growth (Reynolds et al. 2009). These results were consolidated by a study showing that acute EC-specific $\beta_3$ integrin depletion reduced initial tumour growth and angiogenesis but did not affect established tumours (Steri et al. 2014).

Taken together, these studies indicate a context-specific role of integrins in angiogenesis.

## *7.5.2 Adhesome Scaffolds and Mechanotransducers*

### 7.5.2.1 Talin

Talin is a key player in adhesome network because it connects integrin receptors with the actin cytoskeleton. The structure of talin consists of an N-terminal globular head domain connected via a linker to a C-terminal flexible rod domain. The talin-Head domain contains a FERM domain that allows the binding to the cytoplasmic tail of β-integrin subunit, to induce integrin activation. This FERM domain can also interact with adhesome signalling molecules, such as the small GTPase Ras-related protein 1 (Rap1), focal adhesion kinase (FAK), and phosphatidylinositol-4,5-bisphosphate ($PI(4,5)P_2$), which contribute to integrin activation, cell adhesion, and plasma membrane recruitment of talin. The talin-Rod domain has multiple binding sites for adaptor and signalling adhesome proteins, including vinculin, actin, paxillin and the kidney or KN motif and ankyrin repeat domain-containing (KANK) family of proteins (Rahikainen et al. 2019). It has been proposed that talin remains in a folded autoinhibited state and switches to the active confirmation, assessed by cryoelectron microscopy (Dedden et al. 2019). Deletion of the talin in fibroblasts affects the formation of cell–matrix adhesion and initial cell spreading (Zhang et al. 2008). Cellular and biophysical studies have shown that talin can sense forces and respond by unfolding the rod domain and revealing binding sites for interactions with the adhesome members (Goult et al. 2018). However, proof for an in vivo mechanosensing role in blood vessels is still missing. Although talin has two isoforms (Talin 1 and 2), only talin 1 is expressed in ECs and most haematopoietic cells (Monkley et al. 2011). Endothelial-specific deletion of talin1 is embryonic lethal due to major defects in angiogenesis (Monkley et al. 2011). Furthermore, siRNA knockdown of talin1 in human umbilical vein endothelial cells (HUVEC) decreased the formation of cell–ECM adhesions (Elliott et al. 2010). Postnatal deletion of talin1 in ECs also reduced retinal vascularisation due to impaired sprouting and caused lethality by postnatal day 8 due to widespread haemorrhaging

(Pulous et al. 2019, 2021). Using an EC-specific talin1-mutated mouse, which selectively lacks the ability to activate integrins, Pulous et al. found that disruption of talin1-dependent integrin activation reduces retinal and tumour angiogenesis (Pulous et al. 2021). These data suggest that talin regulates blood vessel formation by activating integrins.

#### 7.5.2.2 Vinculin

Several lines of evidence suggest that vinculin is a key adhesome mechanotransducer that couples, transmits, and regulates mechanical forces between the cytoskeleton and the integrin adhesion receptors. Vinculin exists in an autoinhibitory conformation between its N-terminal head and C-terminal tail domains, and can be activated by mechanical stimulation, which induces changes in the protein conformation. In the open conformation, vinculin's head domain binds to talin, and the C-terminus region binds to F-actin. The vinculin–talin complex drives various adhesome and cytoskeletal proteins coupling, such as α-actinin and paxillin. Studies expressing vinculin and talin mutant constructs in vinculin-deficient cells or talin1/2 knockout cell lines showed that vinculin is not required for cell–ECM adhesion formation but plays a role in their stability. It has been proposed that vinculin acts as a molecular clutch to reinforce the link between talin and actomyosin (Carisey et al. 2013; Atherton et al. 2015). Indeed, vinculin's presence time at cell–matrix adhesion sites correlates with the applied force and the stiffness of the substrate in mouse embryonic fibroblasts (Zhou et al. 2017).

Knockdown of vinculin in human arterial ECs enhanced barrier disruption and increased EC permeability, due to the reduced actin cytoskeleton coupling at cell–ECM adhesion and adherens junctions (Birukova et al. 2016). Further studies have shown that association of vinculin with α-catenin at adherens junctions regulates the force-dependent remodelling of endothelial cell–cell adhesions (Huveneers et al. 2012). Recently, vinculin was shown to regulate angiogenic responses. In a mouse retinal model of angiogenesis, EC-specific knockout of vinculin decreased the radial expansion, vessel density, and increased the vessel regression profile (Carvalho et al. 2019). Using an in vitro wound scratch assay, knockdown of vinculin in HUVEC impaired collective cell polarity and migration. This phenotype correlated with decreased endothelial polarity at sprouting vessels in vivo, indicating that vinculin knockout mice had defective angiogenesis (Carvalho et al. 2019).

#### 7.5.2.3 Paxillin

Paxillin is an adaptor protein recruited at the newly formed cell–ECM adhesions upon integrin activation but is also required for the disassembly of adhesion sites for migration. The recruitment of paxillin to the adhesion sites is mediated by the LIM domains, while its N-terminus LD (leucine-aspartic acid) motif mediates its signalling capacity. It contains multiple protein-binding sites for adhesome docking and

directly interacts with FAK, vinculin, and the actin-binding protein actopaxin, thereby regulating the actin cytoskeleton. Expression of mutant paxillin in fibroblast and HeLa cell lines impaired cell–ECM attachment and mobility (Nikolopoulos and Turner 2000). Similarly, paxillin deletion in embryonic stem cells delayed cell spreading to fibronectin and laminin and reduced phosphorylation of FAK (Wade et al. 2002). Furthermore, studies in cell lines using tagged paxillin constructs have shown that paxillin can translocate to the nucleus, in response to cytoskeletal mechanical cues to stimulate cell proliferation (Dong et al. 2009).

In blood vessels, paxillin can regulate cell migration and endothelial permeability. In lung ECs, phosphorylation of paxillin has been shown to regulate EC permeability via Rac signalling (Birukova et al. 2009). Moreover, paxillin regulates directional cell migration in ECs via the neuropilin2 (NRP2) axis. In retinal angiogenesis murine model, paxillin knockdown increased EC migration and invasiveness, leading to increased sprout formation of the vascular network (German et al. 2014).

#### 7.5.2.4 Zyxin

Zyxin is another mechanosensor component of the adhesome with a LIM domain that concentrates at the cell–matrix adhesion sites. Zyxin forms complexes with the cytoskeletal microfilament protein α-actinin and Ena/VASP, which regulate F-actin polymerisation (Martino et al. 2018). Its presence at cell–ECM adhesions is dependent on the mechanical forces exerted on the cells. When cells are grown on a soft substrate and mechanical load is low, zyxin is released from cell–ECM adhesions. Cell stretching restores zyxin to adhesion sites (Uemura et al. 2011; Hoffman et al. 2012). Zyxin is also able to translocate to the nucleus and regulate gene transcription in response to mechanical stimuli, like paxillin (Babu et al. 2012).

Consistent with a role in mechanotransduction, in human primary ECs, zyxin is also able to translocate to the nucleus, in response to stretch mechanical stimuli, and regulate gene transcription (Babu et al. 2012). Using DNA microarray pathway analysis, it was shown that zyxin could regulate apoptosis and chemokine expression and release (Wójtowicz et al. 2010). Congruently, EC-specific zyxin deletion impaired re-endothelialisation and vascular repair, in an injured carotid artery murine model (Kang et al. 2021).

#### 7.5.2.5 P130Cas (CAS-Crk Associated Substrate)

P130Cas is an adaptor protein, characterised as a mechanosensor and promoter of adhesion stability (Martino et al. 2018). It contains a Src homology 3 (SH3) domain, Cas-family C-terminal Homology (CCH) domains, and a substrate domain (SD) for Src kinase. Integrin activation and Src interactions localise p130Cas to cell–ECM adhesions, where it binds to FAK and vinculin (Donato et al. 2010). Stretch induced

phosphorylation of p130Cas leads to recruitment of other signalling molecules, such as Rap1 and ERK pathway, enhancing the cell adhesion (Sawada et al. 2006).

In ECs, p130Cas was shown to regulate the assembly of cell–ECM adhesions in response to angiogenic factors, such as VEGF and Ephrin-B2 (Nagashima et al. 2002; Avraham et al. 2003). In agreement with this, proteomic analysis of ECs showed that VEGF stimulation enriches p130Cas and other interacting adhesome proteins at cell–ECM adhesions to regulate EC migration and in vitro angiogenesis (Evans et al. 2017). In a cancer study, higher expression of p130cas was found to contribute to endothelial resistance to anti-VEFG antibody therapy, whereas blockage of p130cas reduced adaptive resistance to anti-VEGF treatment. Moreover, EC-specific p130cas knockout mice had decreased tumour growth and neovascularisation and increased survival in an anti-VEGF resistant tumour model, indicating an important role of p130Cas in pathological angiogenesis (Wen et al. 2022).

#### 7.5.2.6 Integrin Linked Kinase (ILK)–PINCH–Parvin (IPP) Complex

Central component of cell–ECM adhesions is the tripartite complex, known as IPP complex, consisting of the Integrin-Linked Kinase (ILK), the Particularly Interesting New Cys–His Protein (PINCH) and parvin proteins. The IPP complex facilitates the connection to the actin cytoskeleton and affects cell attachment to the ECM. Recent studies in Drosophila have indicated an essential role of IPP in mechanosensing and reinforcing cell–ECM adhesions (Vakaloglou et al. 2016).

ILK is the key molecule required for the localisation of the IPP to the cell–ECM adhesions (Zervas et al. 2001; Brakebusch and Fässler 2003). It was discovered in a yeast-two-hybrid system as a direct integrin interactor, with a phosphoinositide 3-kinase-dependent serine/threonine kinase activity (Hannigan et al. 1996). Genetic analysis, however, in Drosophila and later in mice, established that ILK is not a kinase (Zervas et al. 2001; Lange et al. 2009) and interacts with integrins indirectly via kindlins (Karaköse et al. 2010). Nonetheless, ILK is an essential mediator of integrin signalling and cytoskeletal organisation, both in Drosophila and mammalian organisms (Sakai et al. 2003; Zervas et al. 2011).

EC-specific deletion of ILK leads to placental insufficient vascularisation and no viable offspring, because of enhanced EC apoptosis (Friedrich et al. 2004). ILK knockdown in human ECs impaired EC spreading, actin organisation and cell migration, without affecting cell survival (Vouret-Craviari et al. 2004). Deletion of ILK in postnatal retina vasculature decreased angiogenic sprouting and EC proliferation (Park et al. 2019). Furthermore, endothelial depletion of ILK increased vascular permeability by interfering with the wnt pathway (Park et al. 2019). Taken together, these studies highlight an important role of ILK in vascular development. In cancer, ILK kinase inhibitors were reported to reduce tumour angiogenesis and growth (Tan et al. 2004). Given that ILK is not a kinase, further studies are required to elucidate the role of ILK in pathological angiogenesis.

Less is known for the function of PINCH, which interacts with the ankyrin repeats of ILK in the tripartite complex. There are two PINCH proteins, PINCH1 and 2, with broad and overlapping cell expression. PINCH1 ablation in mice is embryonically lethal by E5.5 (Liang et al. 2005). In contrast to PINCH1 null mice, PINCH2 deficient mice are viable with no overt abnormalities (Stanchi et al. 2005). Cell spreading and adhesion are defective in PINCH1/2 deleted fibroblasts and can be rescued by PINCH2 expression (Stanchi et al. 2005), indicating overlapping function of these two proteins. The role of PINCH in vascular morphogenesis is currently unexplored.

Parvin binds to the pseudokinase domain of ILK and interacts with other adhesome components, such as paxillin and α-actinin. Also, it contains an actin-binding domain enabling the association with the actin cytoskeleton (Qin and Wu 2012). There are three parvin genes, expressing α-, β-, and γ-parvin, with established roles in cell shape, adhesion, and migration (Legate et al. 2006). Deletion of *α-parvin* in ECs leads to multiple vascular defects, including decreased angiogenic sprouting and vascular density, and increased EC permeability (Fraccaroli et al. 2015). EC-specific depletion of both *α-* and *β-parvin* genes results in embryonic lethality by E11.5 due to vascular abnormalities, such as reduced vascular branching and increased vessel diameter, due to impaired endothelial apical–basal polarity (Pitter et al. 2018). In a brain tumour mouse model, EC-specific knockout of *α-parvin* reduced vessel density and complexity of tumour vasculature, leading to reduced tumour volume and increased tumour apoptosis (Onetti et al. 2022).

### *7.5.3 Adhesome Signalling*

Cell–matrix adhesion relies on both mechanosensors and signalling molecules to integrate mechanical and biochemical signals and control cell behaviour. All adhesion complexes undergo extensive phosphorylation of adhesome components, which ultimately generate transcriptional responses. These phosphorylation events are under the control of kinases and phosphatases, which collectively comprise the adhesome signalling module. Often, increased phosphorylation correlates with cell–ECM adhesion formation and disassembly activity.

#### 7.5.3.1 Focal Adhesion Kinase (FAK)

One of the first adhesome components recruited to the developing adhesome complex is FAK. FAK exists in an auto-inhibition form; activation by integrins and growth factor receptors initiates the disruption of the interaction between the FERM and kinase domains of FAK. This allows the ATP binding on FAK kinase domain and the autophosphorylation on tyrosine 397 which, in turn, induces Src interaction and promotes the subsequent phosphorylation of other tyrosine and serine FAK residues (Roy-Luzarraga and Hodivala-Dilke 2016). FAK is at the cross-roads of

integrin-mediated and growth factor receptor signalling and has been shown to regulate cell shape and actin organisation, adhesion, migration, proliferation, and survival. Besides biochemical signals, mechanical stimuli can also activate FAK (Ngu et al. 2010). FAK activation is involved in the nuclear translocation and activation of Yes-associated protein (YAP), a transcriptional regulator of mechanotransduction (Kim and Gumbiner 2015).

Shear stress and cyclic stretch in vitro induce site-specific phosphorylation of FAK, small GTPase activation and barrier regulation in human pulmonary ECs (Shikata et al. 2005). Constitutive deletion of FAK from ECs led to embryonic lethality due to deficient angiogenesis and haemorrhaging in late embryonic development. These defects were caused by reduced EC proliferation, migration, and barrier formation (Shen et al. 2005; Braren et al. 2006). Postnatal endothelial-specific deletion of FAK decreased angiogenic sprouting in the developing retina vasculature due to decreased EC migration and increased EC apoptosis (Tavora et al. 2010). Several studies have delineated the role of endothelial FAK in tumour growth and angiogenesis (Nikolopoulou et al. 2021). Interestingly, complete deletion of endothelial FAK impaired tumour development and neovessel formation, tumour vessel leakage and metastasis, whereas reduced FAK activity using suboptimal doses of FAK inhibitors or FAK heterozygous mice increased tumour growth and angiogenesis (Tavora et al. 2010; Chen et al. 2012; Kostourou et al. 2013; Jean et al. 2014). The generation of endothelial-specific FAK mutant mice carrying a kinase dead, non-phosphorylatable, or phospho-mimetic tyrosine residues revealed differential requirements for FAK signalling in angiogenesis (Tavora et al. 2014). Using these mice, it was shown that the kinase activity of FAK regulates EC migration and adherens junction permeability but does not affect EC survival or proliferation (Zhao et al. 2010; Lim et al. 2010). In cancer, endothelial FAK kinase activity regulates vascular permeability and metastasis (Jean et al. 2014; Alexopoulou et al. 2017). Similarly, phosphorylation of tyrosine Y397 is important for regulating remodelling of newly formed vessels, as mice with endothelial-specific expression of the autophosphorylation mutant tyrosine-397 (FAK-Y397F) exhibited defective angiogenesis and vessel density (Corsi et al. 2009; Lim et al. 2010). In a tumour setting, endothelial defective FAK tyrosine-397 phosphorylation decreased pathological angiogenesis and tumour growth (Pedrosa et al. 2019), whereas a phosphor-mimetic FAK tyrosine-397E increased endothelial barrier formation and reduced tumour vascular leakage (Alexopoulou et al. 2017). Taken together, these studies open new avenues for therapeutic explorations in pathological angiogenesis and cancer.

#### 7.5.3.2 Phosphatidylinositol 3 Kinases (PI3Ks)

PI3K is a key molecule in the initiation of signal transduction pathways following extracellular cell surface receptor activation. It initiates multiple intracellular signalling pathways that regulate cellular functions, such as proliferation, migration, and transcription. The PI3K family contains eight catalytic subtypes, which are categorised into three classes, with class I PI3K, particularly PI3Kα isoform, being

key for endothelial function (Graupera and Potente 2013). Activation of PI3K leads to the formation of PIP3 [phosphatidylinositol 3,4,5-trisphosphate], which is a second messenger to phosphoinositide-dependent protein kinase-1 (PDK1) that controls the downstream activation of protein kinase B (PKB)/AKT signalling (Zhao et al. 2021).

The endothelial deletion of p110α, a catalytic subunit of class I PI3K, PI3Kα isoform, leads to embryonic lethality at mid-gestation due to defects in angiogenic sprouting and vascular remodelling (Graupera et al. 2008). Similarly, endothelial-specific deletion of p85 regulatory subunit of PI3K decreased blood vessel development and tumour growth (Yuan et al. 2008). The catalytic subunit, p110α, is critical to the regulation of VEGF-induced endothelial migration through the small GTPase RhoA. Impaired signalling by PI3Kα inhibits EC rearrangement during vessel sprouting and failed anastomosis of newly developed vessels (Angulo-Urarte et al. 2018). These defects in endothelial-specific p110α knockout mice are caused by increased actomyosin contractility due to increased phosphorylation of myosin phosphatase targeting-1 (MYPT1) and thus, decreased activity of myosin light chain phosphatase (MLCP) resulting in increased phosphorylation and activity of myosin light chain (MLC) and enhanced actomyosin contractility. In turn, the enhanced actomyosin contractility destabilises cell–cell junctions in the developing retina vasculature (Angulo-Urarte et al. 2018). Inhibition of p110α isoform of PI3Kα also reduced tumour growth by promoting the formation of a dysfunctional vasculature (Soler et al. 2013). Moreover, endothelial activation of PI3K by mutations in the *PIK3CA*, the gene encoding the p110α, promotes hyperproliferation in ECs and impairs pericyte coverage. Interestingly, the presence of activating *PIK3CA* mutations is found in venous malformation in patients (Castillo et al. 2016). Taken together, these data demonstrate a regulatory role of PI3K in vessel development.

#### 7.5.3.3 Myosin Light Chain Kinase/Phosphatase

Myosin light chain kinases (MLCK) are a family of protein kinases that phosphorylate the regulatory myosin light chain 2 (MLC-2), which induces ATPase driven actomyosin contraction. Specifically, phosphorylation of MLC changes the myosin tertiary structure, allowing contractile movement against actin. Opposing MLCK, myosin light chain phosphatase (MLCP) dephosphorylates MLC, leading to decreased tension and relaxing the cytoskeleton (Shen et al. 2010). Optimal control of contractions arises from a balance between MLCK and MLCP activity. MLCK-mediated MLC phosphorylation and actomyosin contractility are important in muscle contractions, cell migration, and endo/exocytic processing (Shen et al. 2010). In bovine ECs, MLC phosphorylation by increased endothelial MLCK activity caused the translocation of P-MLC to polymerised actin and EC contraction (Garcia et al. 1997). MLC is known to decrease cell–cell adhesions and regulate endothelial vascular leakage induced by permeability factors, such as thrombin (Hirano and Hirano and Hirano 2016). Additional in vivo studies are required to delineate the function of MLCK and MLCP in developmental and pathological angiogenesis.

#### 7.5.3.4 Src Family Kinase

Src family kinases (SFK) are comprised of nine structurally similar molecules with conserved peptide domains, termed Src homology (SH) domains. The SH1 is the enzymatic domain, SH2/3 facilitate intermolecular interactions to form protein complexes, and SH4 is responsible for membrane association (Kim et al. 2009). SFK activity is regulated by both growth factor and integrin-mediated signalling, and activated SFKs regulate many intracellular signal transduction pathways (Frame and Roskoski 2017; Koudelková et al. 2021).

Genetic ablation of all three SFKs (Src, Yes, Fyn) leads to embryonic lethality at E9.5 in mice due to defective vasculogenesis (Eliceiri et al. 1999). Additionally, Src was shown to function downstream of VEGF signalling to promote EC survival and angiogenesis (Eliceiri et al. 1999). Apart from a critical component in angiogenic factor signalling, Src has a role in the maintenance of vascular integrity, by regulating cell–cell adhesion junctions. Activation of Src by VEGF receptor 2 (VEGFR2) phosphorylation in a T cell-specific adaptor (TSAd)-dependent manner stabilised EC junctions and decreased vascular permeability in vivo (Sun et al. 2012). Further studies showed that TSAd can regulate the activity of Src at adherens junctions, hence enabling phosphorylation of VE-cadherin and regulation of endothelial junctions and angiogenic sprouting (Gordon et al. 2016), as well as interactions with VEGFR2/3 and PECAM (Sun et al. 2012; Gordon et al. 2016). In ECs, expression of a dominant negative Src or Src inhibitors disrupted adherens junctions and decreased EC migration and capillary morphogenesis (Liu and Senger 2004). In cancer, inhibition of Src kinase activity reduced tumour vascular permeability and EC proliferation and survival, resulting in decreased tumour growth and metastasis (Criscuoli et al. 2005; Ischenko et al. 2007). Recently, Src was shown to play a role in sprouting angiogenesis in the murine retina. Endothelial-specific deletion of Src reduced actin filopodia protrusions and cell–ECM adhesions, resulting in significantly decreased angiogenic sprouting in murine retina (Schimmel et al. 2020). Interestingly, endothelial cell–cell junctions were not affected by loss of c-Src in the retina vasculature (Schimmel et al. 2020). These findings contradict the previously reported role of Src in regulating vascular permeability. Other in vitro studies, however, had indicated that SFK could promote tube formation and inhibit regression of ECs in 3D matrices, by regulating Rho and cdc42 signalling (Im and Kazlauskas 2007; Koh et al. 2009). Additional experiments could shed light into the context dependent role of Src signalling in vessel development.

#### 7.5.3.5 Protein Tyrosine Phosphatases

Protein Tyrosine Phosphatases (PTPs) are a set of transmembrane and cytoplasmic enzymes that catalyse the hydrolysis of phosphate bonds. Classical PTPs are part of a family of cysteine-based phosphatase that share a core $CX_5R$ motif, and can be in soluble form or as a receptor (Young et al. 2021). PTPs are important catalysts for

the phosphorylation of core adhesome complex structures for the mechanotransduction cascades; therefore, tyrosine phosphorylation modifications are concentrated at the cell–matrix adhesion sites (Young et al. 2021). Given the excellent reviews on phosphatases (Hale et al. 2017; Senis and Barr 2018; Young et al. 2021), we will present evidence highlighting the important role of transmembrane PTP receptors in vascular development, focusing only on in vivo studies.

Vascular endothelial-protein tyrosine phosphatase (VE-PTP), also known as PTP receptor B (PTPRB), is expressed in ECs, especially in arteries and arterioles. Deletion of VE-PTP is embryonic lethal as a result of angiogenic defects, including the failure to remodel the initial vascular plexus into large veins and branched networks (Bäumer et al. 2006; Dominguez et al. 2007). VE-PTP regulates the activation of VEGFR2 by dephosphorylation, in stalk cells during angiogenesis. This dephosphorylation of VEGFR2 by VE-PTP inhibits EC proliferation and enables cell polarity and lumen formation (Hayashi et al. 2013). Besides VEGFR2 activity, VE-PTP regulates Tie2 internalisation and EC proliferation in the developing vasculature (Winderlich et al. 2009). Furthermore, VE-PTP responds to shear stress by rapidly accumulating at the downstream edge of the cell relative to the direction of flow. Its re-distribution is triggered by integrin receptor adhesion to ECM (Mantilidewi et al. 2014). Inhibition of VE-PTP resulted in attenuated cell elongation (shape change) in the direction of flow-induced shear stress in vitro (Mantilidewi et al. 2014). Additionally, VE-PTP has a role in cell–cell adhesion, where it initiates the assembly of mechanosensory complex with VE-cadherin promoting cell–cell junctions (Nottebaum et al. 2008).

PTP receptor F (PTPRF) or LAR is reported to localise to cell–ECM adhesion sites in fibroblasts (Sarhan et al. 2016). However, in ECs, shear stress can initiate the assembly of a mechanosensory complex consisting of PTPRF, VE-cadherin, and TRIO. This complex stimulates Rac signalling, thereby regulating Rho GTPase network and junction assembly to promote barrier function (Polacheck et al. 2017; Young et al. 2021).

PTP-PEST or PTPN12 is a cytoplasmic PTP, which is ubiquitously expressed. Genetic ablation of PTP-PEST results in embryonic lethality with fewer but larger blood vessels (Sirois et al. 2006). Endothelial-specific deletion of PTP-PEST also leads to embryonic lethality due to defects in vascular development. Loss of PTP-PEST in ECs increased tyrosine phosphorylation of several adhesome components, including the cytoskeletal adaptor proteins Cas, paxillin, and Pyk2, indicating that PTP-PEST regulates integrin-mediated adhesion. Indeed, PTP-PEST deficient ECs displayed reduced cell attachment to ECM and decreased cell migration (Souza et al. 2012).

The density-enhanced phosphatase (DEP-1, CD148, RPTPJ) has also been involved in regulating VE-cadherin activity and cell–cell junctional stability (Spring et al. 2012, 2014; Fournier et al. 2016). Besides EC permeability, DEP-1 regulates EC invasion and proliferation, promoting capillary formation in vitro (Brunner et al. 2011; Spring et al. 2012). Expression of a mutant DEP-1, which lacks the phosphatase domain, caused embryonic lethality with enlarged vessels and defective vascular remodelling, resulting in reduced vessel branching and density (Takahashi et al.

2003). Although complete deletion of DEP-1 did not affect embryonic angiogenesis, in a tumour setting, it decreased angiogenesis and vascular permeability (Trapasso et al. 2006; Zhu et al. 2008; Fournier et al. 2016).

## 7.6 Concluding Remarks

The vascular system performs a range of vital functions in the mammalian organism. These diverse roles are achieved by a striking heterogeneity in the identity and morphology of ECs that compose blood vessels. Mechanistically, ECs respond to various biochemical signals (growth factors and cytokines) and biomechanical cues (forces generated from blood flow and the ECM), to drive vascular homeostasis and angiogenesis. Central role in the integration of these signals has the cell–ECM adhesions and the adhesome, the dynamic network of proteins formed around integrins. Accumulating in vivo evidence highlights the significance of scaffolding, signalling, and mechanosensor proteins that comprise the integrin adhesome, in blood vessel morphogenesis and function. Future studies will unravel the essential adhesome molecular composition for angiogenesis and vascular homeostasis and hence, pave the way for innovative therapeutic interventions.

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# Chapter 8
# Hypoxia: A Potent Regulator of Angiogenesis Through Extracellular Matrix Remodelling


**Katerina Karavasili and Pieter Koolwijk**

**Abstract** Angiogenesis, sprouting of new vessels from pre-existing ones, occurs throughout life in both health (physiological angiogenesis) and disease (pathological angiogenesis). The process of angiogenesis is regulated by a delicate balance of pro- and anti-angiogenic stimuli including cell–cell interaction by endothelial cells and bystander cells, production of growth factors and their inhibitors, and the modulation of the extracellular matrix (ECM). One of the driving forces of angiogenesis is the shortage of oxygen (hypoxia) occurring in the tissues. Hypoxia regulates the production of many angiogenic growth factors but also stimulates cells to express proteins of the matrix and enzymes that modify the matrix. Here, we describe the effect of hypoxia on the modification of the ECM components. We particularly focus on the synthesis and modification of collagens, the major ECM molecules that dictate the physical and biochemical properties of the ECM. Finally, we discuss further clinical interest that might be hopeful in aberrant angiogenesis or/and hypoxic conditions that characterize many diseases (e.g. diabetes, cancer).

## 8.1 Introduction

New blood vessel formation includes (a) vasculogenesis that refers to de novo formation of new vessels; (b) arteriogenesis, during which pre-existing vessels enlarge and mature, e.g., the remodelling of arterioles or collaterals into large high flow vessels; and (c) angiogenesis during which new capillaries derive from pre-existing vessels (Semenza 2007). Angiogenesis occurs throughout life in both health (physiological angiogenesis) and disease (pathological angiogenesis). During development, angiogenesis is required for the normal formation and function of a

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living organism. In the adult, physiological angiogenesis is limited and can be only enhanced during wound healing and the menstrual cycle. Pathological angiogenesis, on the other hand, is a major contributor to diseases such as cancer (Hanahan and Folkman 1996; Lugano et al. 2020; Zuazo-Gaztelu and Casanovas 2018), diabetic retinopathy (Capitão and Soares 2016; Patel et al. 2005), age-related macular degeneration (Yamamoto-Rodríguez et al. 2020), rheumatoid arthritis (Elshabrawy et al. 2015), endometriosis and psoriasis (Folkman 2006; Heidenreich et al. 2009).

In the blood vessel wall, three major cell types can be distinguished. A monolayer of endothelial cells is found towards the luminal surface of the vessels and sits on a basal lamina (basement membrane, BM), which is a thin and flexible sheet of extracellular matrix (ECM). In capillaries, scattered pericytes are wrapped around the BM, providing growth factors and cytokines that regulate functions of blood vessels and contribute to their stabilization. The wall of larger blood vessels, such as veins and arteries, are thicker and besides the BM, also contain one or more layers of smooth muscle cells and connective tissue (Ho-Tin-Noé and Michel 2011; Caporali et al. 2017; Marchand et al. 2019; Méndez-Barbero et al. 2021).

The first step in angiogenesis is the signal that comes from injury or disease characterized by a reduced oxygen level, often called hypoxia. Hypoxia upregulates the expression of several genes, including angiogenic growth factors, such as vascular endothelial growth factor A (VEGFA) that is relatively selective for endothelial cells. These angiogenic growth factors activate endothelial cells via binding to specific receptors on their cell surface. Initially, the pre-existing blood vessels vasodilate due to the production of nitric oxide (NO) and become hyperpermeable. In addition, the endothelial cells start to produce new molecules, including enzymes that degrade the BM, e.g. matrix metalloproteinases (MMPs) and members of the plasmin system. Partial degradation of the surrounding matrix facilitates the migration towards the stimulus (diseased tissue, tumour) and the proliferation of endothelial cells. Finally, the newly formed vessel tubes are stabilized by the synthesis of new BM, by recruiting supporting cells, such as pericytes and smooth muscle cells, and blood flow begins (Fong 2008; Senger and Davis 2011; Fraisl 2013; Schito 2019).

Under physiological conditions, the dynamic process of angiogenesis is strictly regulated by the co-ordinated function of numerous angiogenic stimulators and inhibitors that include growth factors, proteases and protease inhibitors, cytokines, and chemokines. This balance is disturbed in pathological conditions that are characterized by low $pO_2$ and/or low pH, such as hypoglycaemia, mechanical stress, injury, inflammation, and cancer. In such cases, the effect of the stimulators exceeds that of the inhibitors and there is a dramatic increase in endothelial cell activity, a phenomenon known as “the angiogenic switch”.

Hypoxia is one of the major drivers of angiogenesis. When oxygen levels drop in inflamed tissues or tumours, either by an inadequate blood supply or an increased oxygen demand, a transcriptional response to hypoxia ensues. Among other transcriptional pathways that may also be activated, the major transcription pathway involved is that of the hypoxia-inducible factors (HIFs). HIFs have been shown to bind to a cis-acting hypoxia-response element in numerous genes that encode

angiogenesis regulators, such as VEGFA, angiopoietin-2 (ANGPT2), platelet-derived growth factor beta (PDGFB), and fibroblast growth factor 2 (FGF2), and many others (Hickey and Simon 2006; Hirota and Semenza 2006; Gilkes et al. 2014).

In the present review, we describe the effect of hypoxia on the modification of angiogenesis-related ECM components, focusing on the synthesis and modification of collagens, the major matrix molecules that dictate the physical and biochemical properties of the ECM. Finally, we discuss further clinical interest that might be hopeful in aberrant angiogenesis or/and hypoxic conditions that characterize many diseases (e.g. diabetes, cancer).

### *8.1.1 Hypoxia Signalling Pathways*

Hypoxia-inducible factors (HIFs) are heterodimeric transcription factors that consist of an oxygen-regulated HIF-α (HIF1α, HIF2α, and HIF3α) subunit and a constitutively expressed HIF-β subunit (Wang et al. 1995; Wang and Semenza 1995). In normoxia, hydroxylation of two proline residues (at positions 402 and 564) and acetylation of a lysine residue of HIF-α promote its interaction with the von Hippel-Lindau (pVHL) ubiquitin E3 ligase complex (Masson et al. 2001) that ubiquitinates HIF-α, which is then degraded in the proteasome (Fig. 8.1). Prolyl hydroxylation of HIF-α is catalysed by HIF prolyl hydroxylase domain-containing protein 1 (PHD1), PHD2, and PHD3, in a reaction that is dependent on oxygen, $Fe^{2+}$, and α-ketoglutarate (Epstein et al. 2001; Kaelin and Ratcliffe 2008). Hydroxylation of an asparagine residue of HIF-α can also take place and inhibits its transcriptional activity (Lando et al. 2002). Under low oxygen conditions, HIF-α hydroxylation, ubiquitination and degradation is inhibited (Semenza 2012), and thereby a HIF-α stabilization and accumulation occurs in the cell (Fig. 8.1).

The mechanisms through which hypoxia regulates angiogenesis are not entirely elucidated. It is well known that hypoxia, via HIF-α activation, rapidly induces VEGFA mRNA expression in all types of cells, and is the major mechanism through which hypoxia induces angiogenesis. However, hypoxia activates both HIF1α and HIF2α that although have several overlapping functions, also have distinct target genes and functions, adding to the complicated transcriptional response of endothelial cells to hypoxia (Nauta et al. 2017). Overall, HIF1α seems to induce the formation of tortuous and leaky vascular structures that are not adequately perfused, while HIF2α that is abundantly expressed in endothelial cells seems to induce the stabilization of new vessels (Skuli et al. 2012; Gong et al. 2015; Nauta et al. 2016).

Hypoxia is traditionally classified into acute and chronic that may differentially affect gene expression (Bayer et al. 2011). Acute hypoxia is mainly caused by temporary, local disturbances in tissue perfusion (Bayer et al. 2011) and has been shown to enhance the expression of the urokinase-type plasminogen activator receptor (uPAR), which—in part—explains the initial increased angiogenic response of endothelial cells, as demonstrated by the increased formation of

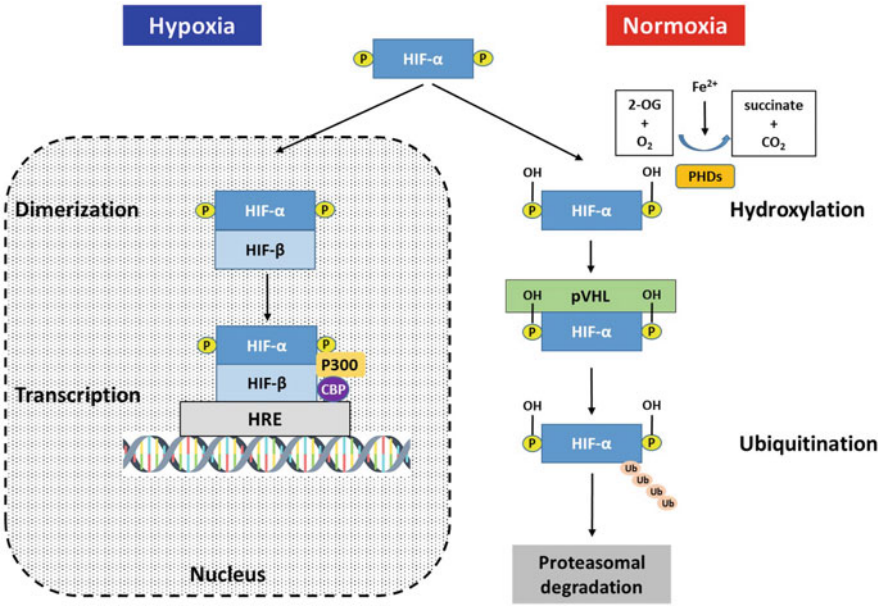


**Fig. 8.1** Oxygen-dependent regulation of HIF-α stabilization and transactivation. In normoxia (right), hydroxylated HIF-α proteins bind to the E3 ubiquitin ligase VHL complex (pVHL), leading to its degradation by the proteasome. In hypoxia (left), the activity of PHDs is reduced due to lack of $O_2$, leading to HIF-α stabilization. CBP and p300 are co-activators that are required for transcriptional activation

capillary-like tubular structures in 3D fibrin matrices (Kroon et al. 2000). Chronic hypoxia lasts relatively long and can stop cell proliferation in the oxygen-depleted regions (Bayer et al. 2011; Saxena and Jolly 2019). It has been suggested that HIF1α predominates in acute hypoxia, whereas HIF2α is mostly responsible for responses to chronic hypoxia (Henze and Acker 2010), such as a more aggressive tumour phenotype through activation of genes such as MMP9, plasminogen activator inhibitor 1, or a 3.5-fold induction of the *Vegfa* gene (Weigand et al. 2012). On the other hand, HIF2α target genes that inhibit endothelial sprouting, such as peroxisome proliferator-activated receptor γ and membrane metalloendopeptidase, have also been found up-regulated during prolonged hypoxia in vitro (Nauta et al. 2017), supporting a shift towards a less aggressive phenotype.

### *8.1.2 The Structure and Function of the ECM*

The ECM is a highly dynamic network that besides its structural role, it also regulates cellular functions dependent on its rigidity/elasticity and its composition. ECM continuously undergoes remodelling that is co-ordinated by the balance

between production of new matrix components and degradation by matrix-degrading enzymes. Deregulation of such balance and changes in ECM composition associates with the development of several pathological conditions (Bonnans et al. 2014; Kastana et al. 2019; Mongiat et al. 2019; Yanagisawa and Yokoyama 2021; Kretschmer et al. 2021; Haller and Dudley 2022; Miller and Sewell-Loftin 2022).

The ECM varies between species and between tissues and is composed of structural biomolecules such as collagens, laminins, elastin, fibronectin, glycosaminoglycans (GAGs: hyaluronate, keratan sulphate, chondroitin sulphate, dermatan sulphate, and heparan sulphate), and proteoglycans. Most of these ECM molecules are synthesized and secreted locally by resident cells, among which endothelial cells. In addition to ECM structural components, there are other non-structural proteins called "matricellular" proteins that function as adaptors and modulators of cell–matrix interactions to guide ECM synthesis (Bonnans et al. 2014; Mongiat et al. 2019; Gopinath et al. 2022).

## 8.2 Hypoxia-Induced Changes in the ECM

The effect of hypoxia on ECM remodelling is long known and follows the effect of hypoxia on the transcription of numerous genes and on cell functions, such as cell proliferation and migration. For example, hypoxia seems to affect fibroblast proliferation to a great degree, so that fibrosis is considered an important outcome of tissue hypoxia (Darby and Hewitson 2016; Xiong and Liu 2017; Valle-Tenney et al. 2020; Foglia et al. 2021; Romero and Aquino-Gálvez 2021). Hypoxia also stimulates vascular smooth muscle cell proliferation and collagen deposition, thus contributing to pulmonary vascular remodelling (Jeffery and Morrell 2002) mediated by endogenous transforming growth factor beta (TGFβ) (Chen et al. 2006) and reversed by ECM remodelling inhibition (Jeffery and Morrell 2002). Remodelling of the ECM following hypoxia has also been considered, at least partly, responsible for the cardiac dysfunction induced by sleep apnoea (Farré et al. 2018), tumour progression and metastasis (Labrousse-Arias et al. 2017), the stemness and differentiation potential of cancer stem cells involved in vasculogenic mimicry (Wei et al. 2021), and for angiogenesis (Germain et al. 2010; Rodriguez et al. 2021).

### *8.2.1 Hypoxia Effect on Collagen Gene Expression*

Collagens, as major and abundantly expressed ECM proteins, provide tensile strength, regulate cell adhesion, support chemotaxis and migration, and direct tissue development. Among the numerous types of collagens, the main types of fibrillar collagens I, II, III, V, XI, and XXIV are found in connective tissues, whereas the networking collagens IV, VIII, and X are predominately found in the BM of epithelial and endothelial cells (Ricard-Blum 2011).

Numerous in vitro and in vivo studies have shown an increased rate of collagen synthesis under hypoxic conditions by fibroblasts (Norman et al. 2000; Liu et al. 2019; Kang et al. 2020), renal epithelial cells (Basu et al. 2011; Rozen-Zvi et al. 2013), and in hepatic stellate cells (Corpechot et al. 2002), contributing to tissue fibrosis, while at the same time it also stimulates angiogenesis (Corpechot et al. 2002). In most of the published data, it is not clarified whether hypoxia has a direct effect on collagen transcription, while there are some studies showing that it is a downstream effect mediated by other factors, such as connective tissue growth factor (CTGF) that promotes collagen I synthesis (Hong et al. 2006). Collagen I is known to positively regulate retinal endothelial cell angiogenic properties and seems to mediate hypoxia-induced retinopathy in zebrafish through $\alpha_2$ integrin (Liu et al. 2022).

In contrast to a positive effect of hypoxia on collagen synthesis, there are numerous studies in other types of cells that show a negative regulation of collagen I by hypoxia. Rabbit aortic smooth muscle cells under hypoxia produce less collagen, despite the increased synthesis of GAGs and hyaluronic acid (Pietilä and Jaakkola 1984). In human articular chondrocytes, HIF1α reduces *Col1a1* gene transcription through the transcription factor Sp3 (Duval et al. 2016), verified by the decreased collagen synthesis observed in mouse growth plate chondrocytes (Stegen et al. 2019). In porcine aortic endothelial cells, hypoxia has been shown to cause a significant decrease in collagen synthesis (Levene et al. 1982) and to induce endothelial-to-mesenchymal transition as inferred by the increased expression of vimentin and α-smooth muscle actin and the decreased expression of VE-cadherin and CD31 (Liu et al. 2019).

Collagen XV has been recently shown to be positively regulated by hypoxia through HIF1α in human mesenchymal stromal cells and may be implicated in their osteogenic potential (Lambertini et al. 2018). In kidney cells, hypoxia suppresses collagen IV $\alpha_2$ expression through HIF1α (Sanaei-Ardekani et al. 2021). In both cases, it is unclear whether such effects occur in endothelial cells and/or if they may have an impact on angiogenesis.

### *8.2.2 Hypoxia Effect on Intracellular Collagen-Modifying Enzymes*

Collagen mRNA is translated to procollagen that undergoes post-translational modifications within the endoplasmic reticulum (ER). Collagen proline hydroxylation is mediated by prolyl 4-hydroxylase α-subunit (P4HA). Three isoforms of P4HA have been identified, namely P4HA1, P4HA2, and P4HA3. All α-subunit isoforms form A2B2 tetramers with the prolyl 4-hydroxylase β-subunit (P4HB) and generate the P4H1, P4H2, and P4H3 holoenzymes, respectively. P4Hs modify proline to 4-hydroxyproline, and this step is essential for the thermal stability of the collagen triple helix (Rappu et al. 2019). Non-prolyl hydroxylated procollagen α-chains are

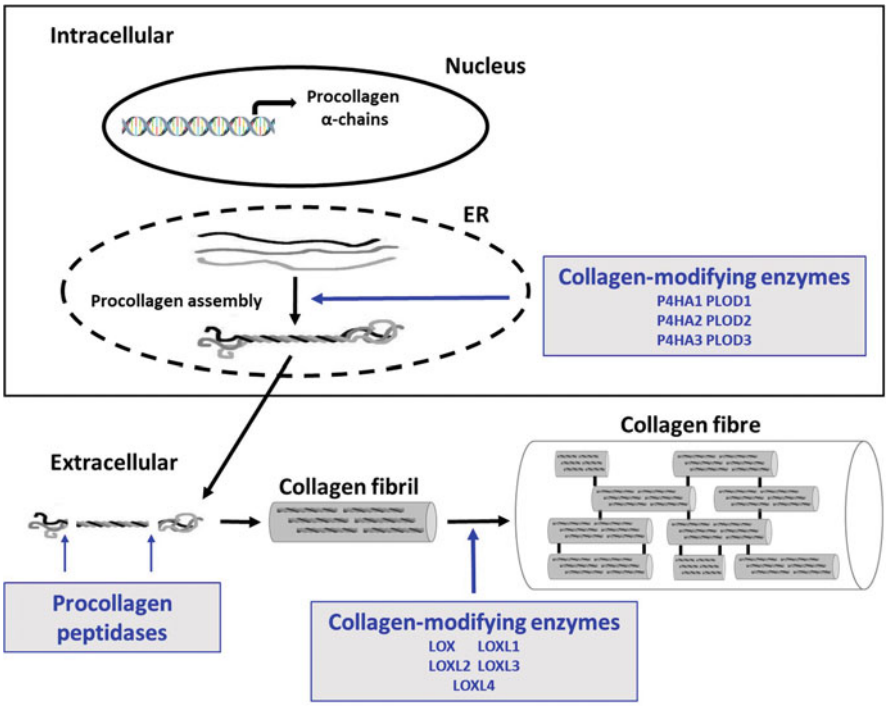


**Fig. 8.2** Biosynthesis of fibrillar collagens. The intracellular steps involve the synthesis of procollagen polypeptides and the modification of these molecules in the ER and the Golgi apparatus by P4HA and PLOD enzymes. In the extracellular space, the non-helical termini are cleaved, and the mature collagen proteins form a collagen fibril. Collagen fibre formation is initiated by collagen crosslinking, catalysed by LOX family members

improperly folded and degraded, leading to decreased collagen deposition (Yamauchi and Sricholpech 2012). Collagen lysine hydroxylation is mediated by the three procollagen-lysine 2-oxyglutarate 5-dioxygenase (PLOD1, PLOD2, and PLOD3) enzymes. Hydroxylated lysine residues contribute to increased stability of the collagen cross-links, leading to increased tissue stiffness (van der Slot et al. 2004) (Fig. 8.2).

Hypoxia increases the expression of P4HA1, P4HA2, PLOD1, and/or PLOD2 in many cell types, e.g., fibroblasts (Gilkes et al. 2013; Rosell-García et al. 2019; Morimoto et al. 2021), chondrocytes (Grimmer et al. 2006), cytotrophoblasts (Highet et al. 2015), human gingival fibroblasts and human periodontal ligament cells (Morimoto et al. 2021), hepatic stellate cells (Copple et al. 2011), sarcoma cells (Eisinger-Mathason et al. 2013), and endothelial cells (Becker et al. 2021 and Table 8.1). This may lead to an increased hydroxylation of collagen and stabilization and stiffening of the collagen matrix. Abrogating the expression of HIF1α or P4HA1 and P4HA2 has been shown to reduce collagen deposition from fibroblasts in vitro (Gilkes et al. 2013). Similarly, a HIF1 pathway inhibitor has been shown to inhibit

**Table 8.1** Expression of collagens and collagen-modifying genes in hypoxia-cultured human microvascular endothelial cells

| Gene | N-fold average | St dev | *t*-test | Mean expression |
|---|---|---|---|---|
| COL1A2 | 5.60 | 3.55 | 0.05 | 2.26 |
| COL4A1 | 1.23 | 0.16 | 0.05 | 457.84 |
| COL27A1 | 1.31 | 0.12 | 0.00 | 18.46 |
| FKBP14 | 0.71 | 0.15 | 0.03 | 13.61 |
| LAMA3 | 0.59 | 0.14 | 0.01 | 3.15 |
| LAMB2 | 1.14 | 0.09 | 0.01 | 98.20 |
| LOXL2 | 1.45 | 0.25 | 0.01 | 264.35 |
| P4HA1 | 2.08 | 0.34 | 0.00 | 57.73 |
| P4HA2 | 1.32 | 0.06 | 0.00 | 91.02 |
| PLOD1 | 1.51 | 0.36 | 0.01 | 183.68 |
| PLOD2 | 1.92 | 0.36 | 0.00 | 175.94 |
| PLOD3 | 1.30 | 0.08 | 0.00 | 90.13 |
| SLC39A13 | 1.09 | 0.07 | 0.04 | 59.65 |

Remark by Pieter Koolwijk

hypoxia-induced P4HA1 and P4HA2, decrease prolyl hydroxylation, and induce proteolytic cleavage of collagen VI, which is the main collagen produced in uveal melanoma, thus inhibiting uveal melanoma cell invasion (Kaluz et al. 2021). Hypoxia can also up-regulate P4H1 at the level of translation, independently of HIF1α, as has been shown in human fibroblasts (Fähling et al. 2006). It has been also suggested that HIF1α but not HIF2α mediates the stimulatory effect of hypoxia on P4H enzymes (Aro et al. 2012; Bentovim et al. 2012).

Collagen hydroxylation by P4H enzymes has been shown to regulate angiogenesis. Compounds that inhibit proline hydroxylation and maturation of collagen in endothelial cells have been shown to inhibit angiogenesis in vitro (Clement et al. 2002). In the same line, up-regulation of P4HA2 by p53 results in inhibition of angiogenesis (Teodoro et al. 2006). On the other hand, such inhibitors have been shown to enhance angiogenesis (Warnecke et al. 2003; Zhu et al. 2019) but the stimulatory effects seem to be attributed to non-collagen related effects of such inhibitors that may also inhibit the HIF PHDs or to a direct effect of P4H enzymes on HIF-α stability (Xiong et al. 2018).

Five different human microvascular endothelial cell isolations were cultured for 14 days at normoxic (20% of oxygen) or hypoxic (1% of oxygen) conditions and genome-wide sequencing was performed on a Illumina platform. For details, see Nauta et al. (2016, 2017).

### 8.2.3 Hypoxia Effect on Extracellular Collagen-Modifying Enzymes

Once secreted extracellularly and following cleavage of the two non-helical termini by proteinases, fibrillogenesis is initiated. During this process, specific lysine and hydroxylysine residues in the N- and C-telopeptides are oxidatively deaminated by lysyl oxidase (LOX), a step necessary for the formation of covalent intra- and intermolecular cross-links (Yamauchi and Sricholpech 2012) (Fig. 8.2).

The expression of LOXs is induced by hypoxia in tumour cells (Wang et al. 2018; Calvo-Anguiano et al. 2018), in fibroblasts (van Vlimmeren et al. 2010), in adipocytes (Anvari and Bellas 2021), and in endothelial cells (Guadall et al. 2011; Becker et al. 2021; Table 8.1). LOX-cross linked collagen activates endothelial cells in vitro and angiogenesis in vivo and correlates with the number of blood vessels in colorectal and breast cancer (Baker et al. 2013), in oral squamous cell carcinoma (Shih et al. 2013), and in hepatocellular carcinoma (Yang et al. 2019). LOX expression has been found higher in tumour endothelial cells compared to normal endothelial cells and to regulate endothelial cell migration and tube formation (Osawa et al. 2013; Shi et al. 2018). In favour of a positive effect of LOX in the regulation of angiogenesis, it has been shown that in synovial membranes, the expression of LOX positively associates with the microvascular density (Wang et al. 2017). More recently, it was shown that an interplay between LOXs and VEGFA/TGFβ is essential for the maturation of blood vessels (Grunwald et al. 2021). Lysyl oxidase-like protein-2 (LOXL2) in endothelial cells has also been identified as a hypoxia target that is involved in the regulation of angiogenesis (Bignon et al. 2011) through its effect on collagen IV scaffolding in the BM of new blood vessels (Bignon et al. 2011), but also on the deposition of other ECM components, such as fibronectin (Umana-Diaz et al. 2020). It has been shown that LOXL2 modulates endothelial-to-mesenchymal transition and thus activates angiogenic functions of endothelial cells (de Jong et al. 2019), a mechanism that has also been described for hypoxia (Liu et al. 2019). It is also of interest that LOXL2 has been found on the exterior of endothelial cell-derived exosomes and is significantly up-regulated in exosomes derived from hypoxic endothelial cells (de Jong et al. 2016). Finally, LOXL2 has been shown to stabilize HIF1α from PHD-dependent hydroxylation, supporting the existence of a positive feedback loop that enhances the effects of hypoxia (Li et al. 2021).

### 8.2.4 Hypoxia Effect on ECM Remodelling Enzymes

Proteolytic cleavage of the ECM is part of its remodelling and is important for regulating its composition and structure, as well as for releasing biologically active molecules. During angiogenesis, endothelial cells produce numerous enzymes that cleave ECM proteins to support various critical steps of the process: (a) BM

breakdown at the initiation of angiogenesis to favour endothelial cell mobility; (b) invasion into collagen I or fibrin matrices; and (c) new lumen formation. Besides degradation of endothelial BM to allow for endothelial cell migration and new lumen formation, proteases also liberate and/or modify pro- and anti-angiogenic factors that are stored in the ECM, contribute to ectodomain shedding of growth factor receptors that are thus activated, cleave and liberate cytokines from membrane-bound precursors, and generate ECM protein fragments that inhibit or activate angiogenesis (Davis et al. 2002; Gonias et al. 2000; Selvarajan et al. 2001; Davis and Bayless 2003; van Hinsbergh and Koolwijk 2008).

MMPs are a major group of enzymes involved in ECM degradation. They are produced either as soluble or as cell membrane-anchored proteinases and have wide substrate specificities towards many ECM proteins (Laronha and Caldeira 2020). Hypoxia has been shown to increase the expression and activity of both MMP2 and MMP9 in pulmonary arterial endothelial cells in vitro and pulmonary artery endothelium in vivo (Liu et al. 2018). Hypoxia has been also shown to stimulate MMP9 expression in brain endothelial cells but not pericytes (Boroujerdi et al. 2015). MMP9 has been implicated in the hypoxia-induced blood–brain barrier disruption and its inhibition has been suggested as a potential basis for therapeutic strategies to treat brain oedema (Bauer et al. 2010). Interestingly, MMP9 seems to not be essential for hypoxic-induced cerebral angiogenesis, but it affects the post-hypoxic vascular pruning following degradation of laminin and claudin-5 (Boroujerdi et al. 2015). Hypoxic pre-treatment of bone marrow mesenchymal stem cells has led to enhanced MMP9 expression levels, among others, and after being transplanted into rats with diabetic lower limb ischaemia, these cells significantly improved angiogenesis (Liu et al. 2015). In monkey choroid-retinal endothelial cells, hypoxia has been shown to primarily induce MMP2 activity (Ottino et al. 2004) and enhancement of MMP2 by hypoxia in endothelial cells has been linked to their enhanced migration and apoptosis but not tube formation; at the same time, hypoxia decreased membrane type 1 MMP (MT1-MMP) and tissue inhibitor of MMP 2 (TIMP2) mRNA and protein levels, suggesting an MT1-MMP-independent MMP2 activation (Ben-Yosef et al. 2005). The decrease of MT1-MMP and TIMP2 by hypoxia has been observed in both short (6 h) and prolong (24 h) hypoxia, while MMP2 has been found up-regulated only at prolonged hypoxia (Ben-Yosef et al. 2002). As has been shown for collagen, the effect of hypoxia on MMPs may be mediated by CTGF that increases the expression of MMPs and decreases the expression of TIMPs by vascular endothelial cells (Kondo et al. 2002). The effect of hypoxia on MMP2 expression and neovascularization in retinas exposed to hypoxia has also been shown to be mediated by ANGPT2 (Feng et al. 2009).

Besides a direct effect of hypoxia on MMPs in endothelial cells, hypoxia can also regulate MMP expression and activity in other cells that subsequently affect tissue angiogenesis. For example, hypoxia in rheumatoid arthritis fibroblasts has been shown to up-regulate MMPs 2, 8, and 9, as well as MT1-MMP, but to have no effect on TIMPs 1 and 2 and to decrease MMP13. Conditioned medium of these cells stimulated angiogenesis in vitro (Akhavani et al. 2009). In human intervertebral disc cells, hypoxia has been shown to significantly increase MMPs 1 and 3 and decrease

TIMPs 1 and 2, thus enhancing the angiogenic ability of intervertebral disc cells during inflammatory reactions in vitro (Kwon et al. 2017). In human cancer cells, MT4-MMP has been shown to be up-regulated by hypoxia or overexpression of HIF1α through activation of the transcription factor SLUG (Huang et al. 2009), and such up-regulation has been linked to invasiveness, metastasis, and angiogenesis (Huang et al. 2009; Host et al. 2012). In glioblastoma cell lines, hypoxia has been shown to significantly enhance MMPs 2 and 9, as well as collagen I. Interestingly, expression levels of angiostatins, MMP-dependent proteolytic products of plasminogen/plasmin, were also increased by hypoxia, despite the pro-angiogenic phenotype of these cells (Emara and Allalunis-Turner 2014). On the other hand, plasma levels of MMP2 and angiostatin, but not plasminogen/plasmin or MMP9, were found significantly decreased in a swine model of neonatal hypoxia compared to the normoxic group (Emara et al. 2007). Angiostatin has been shown to decrease MMP2 expression in human microvascular endothelial cells exposed to hypoxia, thus inhibiting endothelial cell migration (Radziwon-Balicka et al. 2013). Besides angiostatin, the anti-angiogenic cleavage product of collagen XVIII, endostatin, has been also found increased in extracts from tissues exposed to hypoxia and the elevated amounts of endostatin within the aortic wall of mice exposed to hypobaric hypoxia might contribute to the hypoxia-induced development of pulmonary hypertension due to decreased angiogenesis (Paddenberg et al. 2006).

Another enzyme family that is important in ECM remodelling is that of the serine proteases. The plasminogen activators, urokinase-type (uPA) and tissue-type (tPA), target plasminogen to generate plasmin, an enzyme that degrades many ECM proteins e.g., fibrin, fibronectin, and laminin (Engelse et al. 2004). The uPAR on many cells, including endothelial cells, not only binds uPA and thereby localizes its activity, but can also activate intracellular signalling pathways to coordinate ECM proteolysis (Fig. 8.3). Since uPAR lacks transmembrane and intracellular domains, transmembrane co-receptors, such as integrins, are required for its signalling (Smith and Marshall 2010).

Hypoxia through HIF1α has been shown to up-regulate the expression of both uPA and u-PAR in cancer cells, thus enhancing the proteolytic activity at the invasive front (Krishnamachary et al. 2003; Sullivan and Graham 2007) and promoting the epithelial-to-mesenchymal transition (Gupta et al. 2011), thus favouring invasiveness and metastasis. uPAR expression is also enhanced by hypoxia in endothelial cells and has been linked to enhanced angiogenesis in vitro (Graham et al. 1998; Kroon et al. 2000; Kroon et al. 2001; Choi et al. 2008). Hypoxia has been also shown to increase tPA and plasmin in human retinal microvascular endothelial cells (Valapala et al. 2011) through HIF1α (Huang et al. 2011). PAI1 mRNA and protein levels are also enhanced by hypoxia in bovine aortic endothelial cells (Uchiyama et al. 2000). On the other hand, prolonged hypoxia has decreased the production of uPA without affecting PAI1 (Nauta et al. 2016).

Besides the above-mentioned main families of proteolytic enzymes, several other proteases that play roles during the modulation of the matrix may be affected by hypoxia. For example, hypoxia stimulates the pro-angiogenic heparanase secretion in human retinal microvascular endothelial cells (Hu et al. 2012). Heparanase

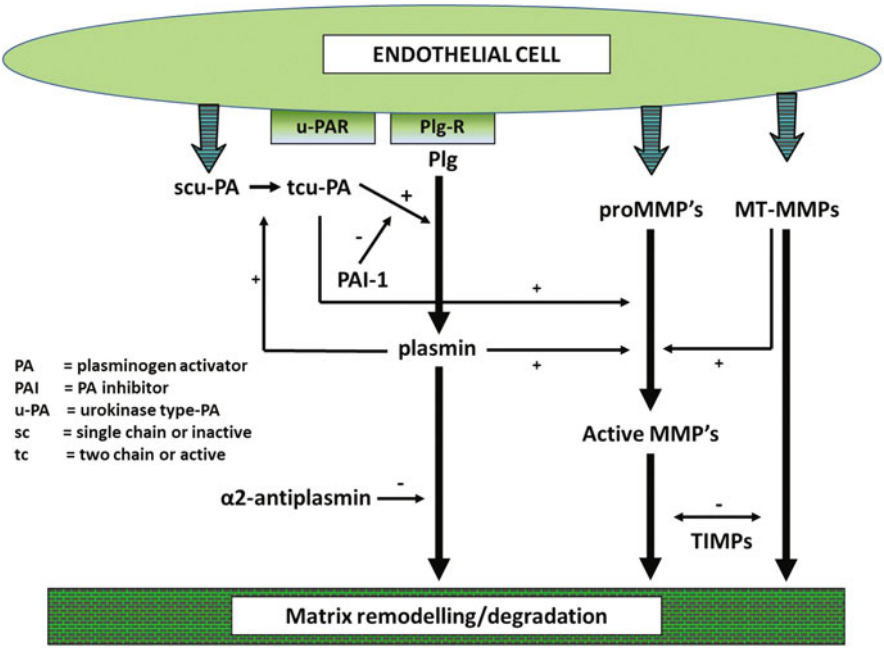


**Fig. 8.3** The uPAR is an important regulator of ECM proteolysis. uPAR regulates the activity of the plasminogen activation system, an extracellular proteolytic cascade, by binding the serine protease uPA and its zymogen form, scu-PA. Plasminogen-derived plasmin cleaves and activates MMPs. Both plasmin and MMPs degrade many ECM components leading to proteolytic fragments with pro- or anti-angiogenic activities and activate or liberate growth factors that are sequestered in the ECM

specifically cleaves cell surface and ECM heparan sulphates at intra-chain sites and its mRNA levels have been correlated with enhanced angiogenesis in the adult rat hippocampus following repeated hypoxia exposures (Navarro et al. 2008). Some members of the ADAM (a disintegrin and metalloproteinase) protein family have also been shown to be up-regulated by hypoxia in other than endothelial cells. Examples include increased expression of ADAM17 following hypoxia treatment of various cancer cell lines (Rzymski et al. 2012). ADAM17 seems to be involved in hypoxia-induced CTGF expression in human lung fibroblasts and may thus play a crucial role in the development of lung fibrosis (Chen et al. 2017). In human glioblastoma specimens, HIF1α expression has been found strongly correlated with endothelial cell markers and ADAM10 expression, implying a potential effect of hypoxia on ADAM10 expression (Musumeci et al. 2015). Hypoxia also enhances ADAM8 expression and activation in human pancreatic cells (Gao et al. 2019).

### 8.2.5 *Hypoxia Effect on Integrins*

Integrins on endothelial cells serve as receptors for various ECM molecules, including collagens or collagen fragments, and regulate angiogenesis. Hypoxia has been shown to enhance expression of $\alpha_v\beta_3$ but not $\alpha_v\beta_5$ integrin in human umbilical vein endothelial cells (Walton et al. 2000; Ben-Yosef et al. 2005). In human microvascular endothelial cells, hypoxia enhances expression of $\alpha_v$, $\beta_1$, $\beta_3$, and $\beta_5$ but not $\alpha_5$ integrins (Befani and Liakos 2017). Hypoxia also increases $\alpha_v\beta_3$ integrin expression in other types of cells, such as melanoma cells (Cowden Dahl et al. 2005) and in myocardium early after infarction (Kalinowski et al. 2008). In human glioblastoma cells, hypoxia recruits $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins to the cell membrane, required for the activation of HIF1α through focal adhesion kinase and for angiogenesis stimulation (Skuli et al. 2009). In the developing mouse central nervous system, hypoxia enhances expression of both fibronectin and its receptor $\alpha_5\beta_1$ integrin localized on brain capillaries (Milner et al. 2008) and this has been correlated with brain endothelial cell proliferation (Li et al. 2012) or spinal cord vessel formation (Halder et al. 2018b) in response to hypoxia. On the other hand, HIF1α has been shown to decrease $\alpha_5$ integrin subunit expression in human gastric cancer cells, thus regulating anoikis and metastasis (Rohwer et al. 2008). Chronic mild hypoxia in the central nervous system enhances vascular integrity by increasing the expression of laminins 111 and 411 and the laminin receptor $\alpha_6\beta_1$ integrin on endothelial cells, without affecting $\alpha_1\beta_1$ integrin (Halder et al. 2018a).

## 8.3 Summary and Future Perspectives

The ECM is important for diverse physiological and pathological processes and is altered in many disease states and following hypoxia. As summarized above, hypoxia enhances ECM stiffness to facilitate endothelial cell proliferation and migration; however, a hypoxia-induced increase of ECM stiffness above a certain level inhibits the formation of new blood vessels and induces fibrosis. Similarly, hypoxia affects the expression and activation of various proteases to remodel ECM, but the physiological consequences of such effects depend on the microenvironment of cells/tissues and the final balance between the pro- and anti-angiogenic molecules produced.

One concern in the interpretation of the data related to the effect of hypoxia on the ECM is the inherent complexity related to the variations in the duration and levels of hypoxia and its categorization as acute or chronic in all the in vitro and in vivo experimental models. It has been shown that in various in vitro studies, acute hypoxia lasts between 0.5 and 72 h, while chronic hypoxia between 4 h to several weeks (Bayer and Vaupel 2012) and it remains unclear whether and how any of such settings resemble pathologies in vivo. Besides chronic and acute, cycling hypoxia (also called intermittent hypoxia) is of interest since it also appears in tumours and

needs to be mimicked in vitro and in vivo (Saxena and Jolly 2019). In all cases, a strategy to control the effects of hypoxia would be the regulation of the expression and activity of HIFs, thus affecting ECM remodelling and angiogenesis (Gilkes et al. 2014; LaGory and Giaccia 2016; Lee et al. 2022).

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# Chapter 9
# Leading Roles of Heparan Sulfate in Angiogenesis and Cancer

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**Abstract** Heparan sulfate (HS) is a member of the broad family of linear heteropolysaccharides known as glycosaminoglycans (GAGs). Except for hyaluronic acid, GAGs are present in tissues as proteoglycans. HS proteoglycans (HSPGs) are found at the cell surface and in the extracellular matrix, where they interact with a plethora of ligands. HSPGs play important roles in cancer initiation and progression, interacting with numerous signaling pathways that affect proliferation, adhesion, invasion, and angiogenesis. Here, we describe the structural characteristics, biosynthesis, post-translational modifications, and degradation of the HS chains. We also summarize the role of HS in cell transformation and angiogenesis. This chapter should help researchers understand the multitude of mechanisms through which HS affects cancer and angiogenesis and inspire the discovery of new therapeutic approaches targeting HS-dependent pathways.

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## 9.1 Heparan Sulfate and Heparin

Heparan sulfate (HS) and Heparin (Hep) are members of a broad family of linear heteropolysaccharides known as glycosaminoglycans (GAGs). Except for hyaluronic acid, GAGs are present in tissues as proteoglycans (PGs), where the polysaccharide chains are O-linked to a protein backbone (Jackson et al. 1991; Kjellen and Lindahl 1991). HSPGs are ubiquitously found on the cell surface and in the extracellular matrix (ECM) of both vertebrate and invertebrate species, whereas Hep is found primarily in cytoplasmic granules of mast cells of some animal species (Medeiros et al. 2000).

HS and Hep share structural similarities. They are mainly composed of repeating disaccharide units of 1 to 4 linked α-D-glucosamine that can be either N-acetylated or N-sulfated, and uronate, either β-D-glucuronate or α-L-iduronate. Moreover, sulfate groups can be present at C2 or C6 of the uronic acid residues and less commonly, at C3 of the glucosamine residues. HS disaccharides are predominantly composed of N-acetylated or N-sulfated α-D-glucosamine and β-D-glucuronate (40–60%), whereas Hep disaccharides consist mainly of α-D-glucosamine N,6 sulfate and α-L-iduronate 2-*O*-sulfate (70–80%) (Fig. 9.1). Consequently, Hep is more sulfated and more charged than HS, containing 2.7 sulfate groups/disaccharide versus 1 sulfate group/disaccharide, respectively (Dietrich 1968; Dietrich et al. 1998; Nader et al. 2004; Meneghetti et al. 2015).

HS disaccharides are distributed along the polymer, creating domains that contain unmodified N-acetylated glucosamine residues at the non-reducing region (NA domains), linked to N-acetylated and N-sulfated glucosamines in the internal hybrid regions (NA/NS domain), and domains containing exclusively N-sulfated, disulfated, and trisulfated glucosamines at the reducing terminal of the polymer (NS domain) (Tersariol et al. 1994). The selective modifications confer the formation of different charge domains within the HS chain, resulting in wide structural variability and favoring interactions with a large number of proteins (Dietrich et al. 1983; Nader et al. 1999) through conserved basic amino acid sequences (Rudd et al. 2017). Therefore, differences in disaccharide composition and substitution pattern and domain structure, along with distinct glycosidic linkages' geometry and iduronate and glucuronate conformation, are essential for the discrimination

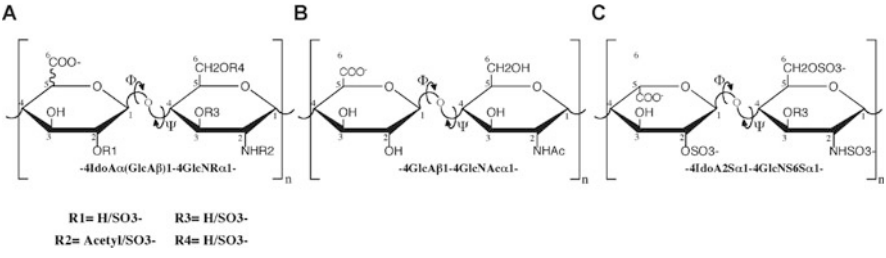


**Fig. 9.1** HS and Hep disaccharide. (**a**) Substitution patterns of HS and Hep, (**b**) HS major disaccharide, and (**c**) Hep major disaccharide (Meneghetti et al. 2015)

of HS and Hep chains. Finally, the molecular weight of HS is usually much higher than Hep, having a maximum around 50 kDa, while Hep has an average of 20 kDa. Also it has been shown that HS chains can adopt more bending and flexibility in solution (Khan et al. 2013).

### *9.1.1 Heparan Sulfate Proteoglycans*

HSPGs are widely expressed in all animal tissues and have great structural diversity. They can be composed of more than one type of GAGs, and each GAG can be associated with different proteins, resulting in numerous different PGs. HSPGs are divided into three groups based on their cellular localization: in the ECM (including interstitial matrix and basement membrane), on the cell surface (transmembrane PGs and PGs linked to GPI anchors), and in cytoplasmic granules (Iozzo and Schaefer 2015).

In general, most PGs found in the ECM are mainly composed of chondroitin and dermatan sulfate chains. The major HSPGs found in the ECM of some tissues are perlecan, agrin, and collagen XVIII (Schaefer and Schaefer 2010). On the other hand, HSPGs are the main PGs on the cell surface. The transmembrane syndecans (SDCs) and glypicans that are linked to the plasma membrane by a glycosylphosphatidyl inositol (GPI) anchor are the two main families of cell surface HSPGs. Apart from them, betaglycan and CD44 can also bear HS chains (Couchman 2010). Lastly, serglycin, a unique intracellular PG, is composed of Hep chains and is located within granules and secretory vesicles cells (Nader et al. 1989; Korpetinou et al. 2014).

Although the biological functions of HSPGs are mainly due to the GAG chains that interact with a variety of proteins, their core protein determines their localization, in addition to contributing to the binding of growth factors, signal transduction as well as the temporal regulation of GAG chains (Annaval et al. 2020). PGs found in the ECM play an essential role in the maintenance of the matrix architecture and integrity, affecting many physiological and pathological processes (Burgess et al. 2000; Farach-Carson and Carson 2007). Cell membrane SDCs are widely involved in the regulation of focal adhesion and cytoskeleton rearrangement (Cavalheiro et al. 2017) and interact with other membrane receptors (Dews and Mackenzie 2007). Glypicans, on the other hand, are involved in the modulation of signaling pathways related to development and morphogenesis, besides being involved in tumor progression events (Iozzo and Schaefer 2015). The function of cell surface HSPGs can be altered through shedding mechanisms performed by proteases or phospholipases, as in the case of glypicans (Traister et al. 2008; Manon-Jensen et al. 2010). Finally, serglycin, found in secretory vesicles, plays an important role in packaging, storage, and activation of proteases and many cytokines and growth factors present in these intracellular granules (Douaiher et al. 2014; Korpetinou et al. 2014).

### 9.1.2 Biosynthesis of HS

HS and Hep chains are synthesized in a non-template driven process, mainly in the Golgi apparatus, involving many enzymes capable of fine-tuning of the structures. Although the biosynthesis can be conceptually divided into polymerization and modification (sulfation and epimerization) steps, these processes occur concomitantly in a dependent way (Dietrich et al. 1988). They also occur quickly, since the complete sulfate substitution in GAG chains is achieved within less than 1 min (Hook et al. 1975). The full polymerization and modification processes are carried out through the catalysis of nucleotide sugars (UDP-sugar) and the nucleotide sulfate donor 3′-phosphoadenosine-5′-phosphosulfate (PAPS) (Esko and Lindahl 2001).

Firstly, the synthesis of HS is initiated through the assembly of the linker region, composed of D-glucuronic acid β (1→3) D-galactose β (1→3) D–galactose β (1→4) D–xylose, on serine residues in a protein backbone. This linker region is stabilized by covalent bond between xylose residue to specific serine groups, belonging to Ser-Gly/Ala motif, consensus sequence for xylosylation, flanked by one more acidic and hydrophobic amino acids (Esko and Zhang 1996). This process is catalyzed by xylosyltransferase I (XylT), galactosyltransferase I (GalT-I), galactosyltransferase II (GalT-II), and glucuronyltransferase I (GlcAT-I) enzymes, which will add specific monosaccharide residues (xylose, galactose I, galactose II, and glucuronic acid, respectively) to the non-reducing end of the growing chains (Fransson et al. 1985). Furthermore, the linker region can also bear some substitution patterns, such as phosphorylation at C2 of xylose, sulfation at C4/C6 of galactose residues, and epimerization at C5 of glucuronic acid, which can regulate further biosynthetic steps and can influence PG secretion (Prydz and Dalen 2000). Although the biosynthesis occurs mainly in the lumen of the Golgi apparatus, the synthesis of the linker region, specifically the xylosylation step, takes place in the earlier secretory pathway and can occur in the endoplasmic reticulum or even in the Cis-Golgi apparatus (Vertel et al. 1993).

After the synthesis of the linker region, the addition of the fifth monosaccharide residue—N-acetylglucosamine (GlcNAc)—to the non-reducing end growing chains, plays an essential role in determining the assembly of HS/Hep chains. This addition is carried out by α-N-acetylglucosaminyltransferase I (EXTL3), an exostosin (EXT)-like enzyme (Fritz et al. 1997). Subsequently, HS polymerase enzymes (EXT1 and EXT2), bifunctional enzymes that display β-glucuronyltransferase and α-N-acetylglucosaminyltransferase activities, catalyze polymerization of HS/Hep chains by alternating addition of β-D-glucuronic acid and α-D-glucosamine N-acetylated residues to the non-reducing end of the chain. EXT1 and EXT2 act as a heterodimeric complex and show higher glucosyltransferase activity than EXT1 or EXT2 alone (Senay et al. 2000).

Following this, a series of enzymatic reactions, carried out by N-deacetylase/N-sulfotransferase (NDST), C5-epimerase, and sulfotransferases, result in complex substitution patterns of HS chains. NDST is a bifunctional enzyme that removes N-acetyl group from specific GlcNAc residues and replaces them with sulfate

groups. Rarely, deacetylated glucosamine residues are kept as free amino group (Nadanaka et al. 2014). Commonly, N-sulfation reaction is attached to N-deacetylation; however, these reactions can be dissociated and different NDST proteins can act on the same glucosamine residue. Nonetheless, the N-deacetylation step is a prerequisite and rate-limiting step for determining the degree of N-sulfation that occurs non-uniformly in the chain (Bengtsson et al. 2003; Gesteira et al. 2013). NDST enzyme has 4 isoforms that have different protein expression levels in each tissue and distinct rates of N-acetylation/N-sulfotransferase activity (Grobe et al. 2002). NDST1 is a critical enzyme responsible for the formation of N-sulfated domain in HS chains (Gesteira et al. 2011a, b; Dou et al. 2015).

Posteriorly, the polymer is then modified by C5-Epimerase, which catalyzes the conversion of glucuronic acid (GlcA) to iduronic acid (IdoA) at C5. This enzyme is specific for HS and Hep chains, not acting on other GAG chains (Crawford et al. 2001; Sugahara and Kitagawa 2002). Furthermore, the process of epimerization of glucuronic acid results in a change in the conformation of the monosaccharide residue from β to α (β-D-glucuronic acid to α-L-iduronic acid). The epimerization reaction is irreversible (Hagner-Mcwhirter et al. 2000). Unlike GlcA and GlcNS residues that are in the $^4C_1$ conformation, IdoA ring can adopt various conformations ($^1C_4$, $^4C_1$, and $^2S_0$) (Mulloy and Forster 2000) and consequently, the sulfates in IdoA residue can be presented in different arrangements, increasing the structural diversity and electrostatic properties of HS/Hep chains (Liu and Pedersen 2007).

In addition to these substitutions, the polymer can be further modified by sulfotransferases that will add sulfate groups at C2 (2-*O*-sulfotransferase, 2-OST) of uronic acid residues, at C6 (6-*O*-sulfotransferase, 6-OST), and at C3 (3-*O*-sulfotransferase, 3-OST) of D-glucosamine residues. 2-OST recognizes both GlcA and IdoA residues as substrates, but has higher affinity for IdoA residues (Rong et al. 2000). GlcA2S/IdoA2S rates can considerably vary during embryonic development and depending on the tissue and the NS domain length (Lindahl et al. 1995; Safaiyan et al. 2000). 6-OST acts preferentially on GlcNS residues and all three isoforms in vertebrates are capable of adding sulfate groups to both GlcNS and GlcNAc residues (Peterson et al. 2009); however, the isoform 1 is the main enzyme responsible for the 6-*O*-sulfation of HS in many tissues (Habuchi et al. 2007). Finally, 3-OST transfers the sulfate group from PAPS to the C3 of the glucosamine residues of HS/Hep chains. 3-OSTs are the biggest family of sulfotransferases among all HS sulfotransferases. In vertebrates, there are seven isoforms divided into two subgroups, gD-type or AT-type. The isoforms 2, 3A, 3B, 4 and 6, which have more than 80% sulfotransferase domain homology, form the group often referred to "gD-type," since all of these enzymes can generate binding sites for the gD glycoprotein of the herpes simplex virus type I (Lawrence et al. 2007). The isoforms 1 and 5, which share 71% sulfotransferase domain similarity, can generate binding sites for antithrombin (AT) and are therefore designated as "AT-type" (Xia et al. 2002; Thacker et al. 2014).

The presence of HS modifying enzymes in organisms is strongly related to the emergence of multicellularity and tissue organization (Medeiros et al. 2000; Bishop et al. 2007) and an evident correlation between the complexity of the organism and

the number of isoforms of HS biosynthesis enzymes seems to support this notion. For example, *C. elegans*, which is a primitive organism, has only one isoform for each of the five HS modifying enzymes, while humans have four isoforms for NDST, one C5-epimerase, one HS2ST (gene for 2-OST), three isoforms for HS6ST (gene for 6-OST), and seven isoforms for HS3ST (gene for 3-OST) (Ori et al. 2011).

#### 9.1.2.1 Regulation of Biosynthesis of HS

Although the mechanisms for the polymerization and modification of HS chains are similar in many organisms, the regulation of the biosynthetic process is not yet fully comprehended (Li and Kusche-Gullberg 2016). Some studies have shown that HS modifying enzymes can form multi-complexes and act collectively, such as EXT1/EXT2 (Senay et al. 2000; Busse et al. 2007), EXT2/NDST1 (Presto et al. 2008), XylT/GalT-1 (Schwartz and Dorfman 1975), C5-Epimerase/2-OST (Pinhal et al. 2001; Qin et al. 2015), and C5-Epimerase/6-OST (Qin et al. 2015) and the modification reactions can be carried out in a hierarchical order (Lindahl 1977). Nonetheless, in order to understand other HS patterns found in nature that are not fully explained by the classical HS biosynthesis route (Gesteira et al. 2011a, b; Rudd and Yates 2012), some studies have also shown the role of other factors in the regulation of HS biosynthesis. For example, localization of EXT1/EXT2 (Chang et al. 2013) and other HS modifying enzymes (Meneghetti et al. 2021) in distinct Golgi cisternae that can modulate the synthesis of HS. Distinct isoforms of 3-OST can also display different subcellular localization (Delos et al. 2018) and can act in different biosynthetic steps in a non-hierarchical way (Meneghetti et al. 2017; Wang et al. 2017). NDST2 can also act in distinct biosynthetic steps (Raman et al. 2011) and different binding orientations of the polysaccharides can regulate the activity of this HS modifying enzyme (Xu et al. 2017; Wander et al. 2021). Finally, it has also been shown that the ZNF263 transcription factor regulates the expression of specific HS biosynthetic enzymes (Weiss et al. 2020) and that availability of PAPS and UDP-sugar is also an essential factor for the regulation of HS biosynthesis (Toma et al. 1996a, b; Dick et al. 2012).

#### 9.1.2.2 Post-translational Modifications in HS Structure

Following HS biosynthesis and PGs targeting, HS chains can be further modified by heparanase and extracellular 6-*O*-endosulfatase (Sulfs) enzymes.

Heparanase is a mammalian endo-β-glucuronidase that specifically cleaves the glycosidic bond between GlcA and GlcNS, modifying the ECM architecture and integrity and releasing bioactive HS oligosaccharides and HS ligands, which are critical for homeostasis of physiological processes and pathological conditions. In addition, heparanase can also participate in the degradation of HSPGs in lysosomes, generating additional non-reducing extremities in HS chains (Vlodavsky et al. 2018).

The mobilization of pro-angiogenic growth factors linked to HS chains in the ECM, basement membrane, and vascular endothelium can be favored by the release of heparanase, thus contributing to tumor cell proliferation and metastasis, as well as angiogenesis (Vlodavsky and Friedmann 2001). The generation of HS oligosaccharides by heparanase favors the interaction of such oligosaccharides with growth factors, allowed the formation of ternary complexes between fibroblast growth factor (FGF)-1 or FGF2 with FGF receptor-1 (FGFR-1), or facilitating the activity of HS as a co-receptor for other growth factors, such as vascular endothelial growth factor (VEGF). Consequently, heparanase inhibition has been suggested as an attractive target for development of an anti-angiogenic and antitumor therapy. On the other hand, in pancreatic neuroendocrine tumors in mice, it has been shown that high heparanase levels positively affect peritumoral lymphangiogenesis in vivo and promote tumor invasion, while heparanase deletion leads to increased angiogenesis and pericyte coverage (Hunter et al. 2014), highlighting the complexity of the involved pathways.

Tissue factor (TF) is crucial in the coagulation cascade and is expressed by pericytes. Heparanase has been shown to participate in the coagulation cascade as a cofactor of tissue factor activity and in patients with lung cancer, heparanase protein levels and activity have been found enhanced compared to control, without changes in TF activity. The heparanase procoagulant activity was inversely correlated to mean patient survival (Nadir et al. 2014). Since many tumors have been identified as having a prothrombotic state (Lip et al. 2002), the procoagulant activity of heparinase may be another mechanism to explain its tumor-promoting effects.

The Sulfs are HS endoglucosamine-6-*O*-sulfatases that were firstly identified in quail embryo (QSuf1) by Emerson and colleagues (Dhoot et al. 2001). Sulfatase-1 (Sulf-1) and Sulfatase-2 (Sulf-2) belong to the Sulfs family that includes 17 members (Diez-Roux and Ballabio 2005), which act in the extracellular compartment but also display endosulfatase activity (Morimoto-Tomita et al. 2002). Sulf-1 and Sulf-2 selectively remove 6-*O*-sulfate groups from glucosamine residues, mainly in domains containing the trisulfated disaccharide IdoA2S-GlcNS6S. In addition, in vitro, the 6-*O*-sulfo groups from disulfated UA-GlcNS6S units have also been shown to be hydrolyzed by Sulf-2. Sulfation at positions other than the 6-*O*-sulfate could not be removed by the enzyme (Pempe et al. 2012; Seffouh et al. 2019). The Sulfs have been shown to be functionally redundant (Morimoto-Tomita et al. 2002; Ai et al. 2003). Regulation of the presence of 6-*O*-sulfation in HS chains consequently modulates the interaction of countless proteins and signaling molecules with HS (Dhoot et al. 2001; Vicente et al. 2015). Glucosamine-6-sulfate modification is required for the high-affinity interaction of HS/Hep chains with several protein ligands, including $VEGFA_{165}$, FGF1 and FGF2, CXCL12, and CCL21, highlighting the potentially important role of HS 6-OSTs and Sulfs in angiogenesis modulation (Chen et al. 2005; Uchimura et al. 2006; Rosen and Lemjabbar-Alaoui 2010; Van Wijk and Van Kuppevelt 2014; Kargozar et al. 2020). Sulfs have been recently associated to angiogenesis promotion and enhancement of VEGFA bioavailability for ischemic heart repair (Korf-Klingebiel et al. 2019).

The Wnt signaling pathway is the best described mechanism for the role of Sulfs on the cell surface (Dhoot et al. 2001; Ai et al. 2003; Nawroth et al. 2007; Tang and Rosen 2009). According to this model, the action of Sulfs weakens the association of Wnt ligands (Wnt1, Wnt3, Wnt3a, Wnt4, and Wnt11) with HSPGs on the cell surface, which allows the Wnt ligands to activate the Frizzled receptor signal transduction (Ai et al. 2003). The Sulfs also promote other signaling pathways, such as those of bone morphogenic protein (BMP) (Otsuki et al. 2010) and glial cell-derived neurotrophic factor (GDNF) (Langsdorf et al. 2011), while inhibiting others, such as FGF2 (Wang et al. 2004) and transforming growth factor beta (TGFβ) (Yue et al. 2008).

Aside from their numerous physiological functions, Sulfs have also been involved in several pathologies, particularly in cancer. Sulfs dysregulation in cancer is extremely complex and has been shown to relate to altering several signaling pathways, gene expression, and resistance to chemotherapeutic drugs (Moussay et al. 2010). Despite having the same substrate and redundant functions, Sulf-1 and Sulf-2 have been described to differentially affect cancer development. Sulf-1 has been reported to have a suppressive role in several cancers, such as hepatocellular carcinoma, myeloma, head and neck, breast, and pancreatic cancer. Sulf-2 seems to have a pro-oncogenic role in hepatocellular, pancreatic, breast, and lung cancer (Vivès et al. 2014). These discrepancies regarding the functions of Sulfs in cancer can be explained by the lack of in vivo studies, the differential expression of Sulf-1 and Sulf-2 in different tissues and cells, and the presence of different HSPGs on the cell surface, which could alter different signaling pathways in each cell type (Vivès et al. 2014).

### *9.1.3 Degradation of Heparan Sulfate*

HS chains are degraded in a highly organized fashion through a hierarchical sequence of enzymatic events, involving a number of exo- and endo-glycosidases and sulfatases, in lysosomes (Freeman and Hopwood 1992). The lysosomal degradation of HS chains occurs at the non-reducing end of the sugar chain and involves three glycosidases, five sulfatases, and one acetyltransferase. In the end of the catabolic process, monosaccharide and inorganic sulfate are produced and recycled for later use by the cell (Kowalewski et al. 2012).

HSPGs are internalized from the cell surface and trafficked through the endocytic pathway, where the protein backbones are initially subjected to proteolytic cleavage to release the HS chains. Afterwards, further non-reducing extremities in HS chains are created by heparinase, and the oligosaccharides generated undergo a stepwise exo-degradative process (Dorfman and Matalon 1976). Overall, nine different enzymatic activities are required for the complete degradation of HS chains. The three specific glycosidases, α-L-iduronidase, β-D-glucuronidase, and α-N-acetylglucosaminidase, are responsible for hydrolysis of the glycosidic bond of specific sugar residues (iduronate, glucuronate, or N-acetylglucosamine,

respectively) present at the non-reducing end, degrading HS oligosaccharides to monosaccharides. Five sulfatases (iduronate-2-sulfatase, glucuronate-2-sulfatase, N-sulfoglucosamine-3-sulfatase, N-acetylglucosamine-6-sulfatase, and sulfamidase) catalyze the removal of the sulfate groups at C2 from iduronate-2-sulfate or glucoronate-2-sulfate and at C3, C6 or amino group from glucosamine residues, respectively. Lastly, α-glucosamide N-acetyltransferase catalyzes the acetylation of the amino group from glucosamine residues generated by the action of sulfamidase, so that they can be cleaved by α-N-acetylglucosaminidase (Toma et al. 1996a, b). Deficiencies in these lysosomal enzymes result in lysosomal storage disorders, known as the mucopolysaccharidoses and are caused by storage of HS oligosaccharides in lysosomes (Meikle et al. 2005).

## 9.2 Heparan Sulfate and Cell Transformation

Changes in the expression of HSPGs have been found in tumor cells, suggesting a potential involvement in cancer. During cancer progression, some events are important to trigger the metastasis process, such as ECM remodeling for survival in the absence of cell–ECM interaction, in a process denominated *anoikis* resistance. Anoikis is a programmed cell death induced upon cell detachment from ECM, behaving as a critical mechanism in inhibiting adherent-independent cell growth and attachment to an inappropriate matrix, thus preventing colonization of distant organs. Anoikis resistance occurs when detached cells circumvent death signaling pathways, enabling survival of cells as a result of several biochemical and molecular alterations. These changes are the hallmark of invasiveness, metastasis, therapy resistance, and relapse of cancer cells (Simpson et al. 2008; Adeshakin et al. 2021). Our previous work has shown that anoikis-resistant endothelial cells exhibit an increase in HS and chondroitin sulfate levels, as well as enhanced expression of SDC4 and heparanase (Carneiro et al. 2014; Onyeisi et al. 2020a). HS mediates cell–cell and cell–ECM communication, leading to different pathological and physiological effects, including embryonic development, cell growth and differentiation, inflammatory responses, and microbial infection (Li and Kusche-Gullberg 2016; Teixeira et al. 2020).

The cell surface SDCs can trigger signaling leading to cell adhesion and spreading, either by exposing binding sites on fibronectin for $\beta_1$ integrin engagement, or by modulating the activation state of the $\beta_1$ integrin. SDC4 is an important regulator of cell adhesion and contributes to tumor cell migration and proliferation, acting as a classical HS co-receptor for soluble ligands, such as growth factors and chemokines, but also via interactions of its protein moiety with growth factor receptors and integrins (Couchman and Woods 1999; Lopes et al. 2006a, b; Onyeisi et al. 2020b, 2021a, b).

Studies indicate a correlation between the cell cycle and the synthesis of HS. Porcionatto and collaborators showed that fetal calf serum (FCS) and phorbol 12-myristate-13-acetate specifically stimulate the synthesis of HSPGs in endothelial

cells (Porcionatto et al. 1998). As mentioned above, SDC4 expression is enhanced in anoikis-resistant endothelial cells (Carneiro et al. 2014) *and* SDC4 gene silencing in anoikis-resistant endothelial cells causes cell cycle arrest at G0/G1, decreasing the number of cells in the S phase. It also decreases the invasive and angiogenic properties of anoikis-resistant endothelial cells and increases their adhesion to collagen and laminin. Besides that, SDC4 gene silenced cells are more FCS-dependent to synthesize HS than anoikis-resistant parental cells (Onyeisi et al. 2020a). In the same line, addition of SDC4 to synchronized cultures of a rat hepatoma cell line just after the first mitosis resulted in an immediate arrest of the cell cycle in G1 (Fedarko et al. 1989). Anoikis-resistant endothelial cells treated with trastuzumab show enhanced adhesion to fibronectin and a decreased proliferation, invasion, and angiogenesis capacity, concomitantly with decreased expression of SDC4, perlecan, and HS that are considered important for its inhibitory effects (Onyeisi et al. 2019). In endothelial cells overexpressing high levels of the cellular ras oncogene isolated from the EJ human bladder carcinoma, SDC4 synthesis is up-regulated but due to the down-regulation of HS modification enzymes, there are changes in the HS structure of SDC4 that are in line with the enhanced cell cycle progression (Lopes et al. 2006a, b).

Using both wild-type Chinese hamster ovary cells (CHO-K1) and a mutant cell line (CHO-745), which is deficient in the synthesis of PGs due to lack of activity of xylosyl transferase, Franco and collaborators demonstrated different adhesive profiles between the two cell lines. However, a decrease in the amount of GAGs does not inhibit the proliferation of mutant CHO-745 cells when compared to the wild-type CHO-K1, since both CHO-K1 and CHO-745 cells take 8 h to enter the S phase (Franco et al. 2001). O'Donnell and Shukla demonstrated that CHO-745 cells have a higher level of Rho GTPase activity compared with CHO-K1 cells, concluding that HS expression may regulate Rho GTPase activity leading to the differences seen in cell–cell fusion (O'Donnell and Shukla 2009). Recent studies have shown that sodium butyrate, a histone deacetylase inhibitor that modulates gene expression, particularly transgene expression in CHO cells, can increase HS trisulfated disaccharides and decrease *N*-sulfated disaccharides in CHO cells (Lee et al. 2016). The synthesis of HSPGs was also characterized in CHO-DG44 cells, which are cells derived from the original CHO-K1 cells by several rounds of mutagenesis that deleted both copies of the dihydrofolate reductase genes. CHO DG44 cells have a greater amount of membrane-bound HSPGs and of enzymes such as C5-epimerase and 2-OST during cell growth (Lee et al. 2016).

The action of HS as a modulator of cell proliferation, adhesion, and migration is linked to its capacity to bind to and act as co-receptor for growth factors, such as FGFs, VEGFs, platelet-derived growth factors (PDGFs), heparin-binding epidermal growth factor-like growth factor (HB-EGF), and hepatocyte growth factor (HGF) (Porcionatto et al. 1998; Lopes et al. 2006a, b; Lee et al. 2012; Li and Kusche-Gullberg 2016) (Fig. 9.2).

FGF signaling can promote cancer development by affecting a range of major downstream biological processes (Turner and Grose 2010). Several studies have demonstrated the existence of aberrant FGF signaling in the pathogenesis of multiple

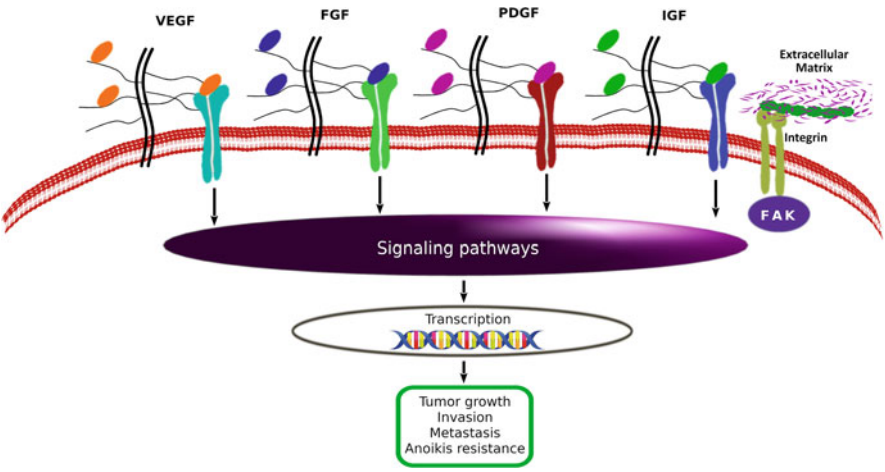


**Fig. 9.2** HS can modulate tumor growth, invasion, metastasis, and anoikis resistance by acting as co-receptor for growth factors. VEGF (vascular endothelial growth factor); FGF (fibroblast growth factor); PDGF (platelet-derived growth factor); and IGF (insulin-like growth factor) (Porcionatto et al. 1998; Lopes et al. 2006a, b; Li and Kusche-Gullberg 2016)

types of cancer. Although FGFs can bind to FGFRs with high affinity, this interaction and the subsequent signaling events are amplified by the presence of HS chains (Elfenbein and Simons 2013; Meneghetti et al. 2015). Different members of the FGF family share binding sites on the HS chain, and their affinities for HS-related oligosaccharides generally correlate with the overall degree of saccharide sulfation. In addition, relatively nonspecific charge interaction may also prevail in the formation of FGF–HS–FGFR complexes (García-García and Anderson 2003; Turner and Grose 2010). The HS undersulfation enhances FGFR1 activation, ultimately resulting in premature senescence through the p53-p21 signaling pathway (Jung et al. 2016). Li and collaborators have demonstrated that hypoxia induces alterations in HS composition on the endothelial cell surface, that enhances cell responsiveness to FGF2. This effect is mediated by an increase in total cell surface HS chains, as well as by an increase in the number of HS-FGF2 binding sites (Turner and Grose 2010).

PDGF signaling is essential for processes involving cell motility and differentiation. The association of PDGF with ECM appears to be dependent on HS (Smith et al. 2009). The long form of PDGF-A includes a positively charged carboxyl-terminal retention motif that can interact with the ECM and HSPGs (Kurup et al. 2006; Smith et al. 2009; Mochizuki et al. 2020). Interestingly, gene expression of the PDGF-receptor subtypes in human embryonic lung fibroblasts is up-regulated by HS (Malmström and Westergren-Thorsson 1998).

Insulin-like growth factors (IGFs) play an important role in regulating growth and development in normal human tissues, by promoting cellular proliferation and differentiation. It has been shown that the biological activity of IGF1 is modified

by IGF-binding proteins (IGFBPs) (Simpson et al. 2017). HS can regulate synthesis of IGF1 (Arai et al. 1994; Park et al. 2018), as well as interaction with IGFBP-2 (Arai et al. 1996). In addition, the reduction in sulfation of HS could result in a decrease in the HS–IGFBP-2 interaction and consequently, in a lower IGF1 activity, constituting a short negative local regulatory loop (Thiébot et al. 1997).

## 9.3 Heparan Sulfate and Angiogenesis

Angiogenesis comprises the formation of new vessels during embryonic development and also in adult life (Herbert and Stainier 2011). In the absence of pro-angiogenic stimuli, endothelial cells remain in a state of quiescence. However, in the presence of high levels of angiogenic factors, initially, there is detachment of pericytes and degradation of the basement membrane, initiating the activation phase of endothelial cells. In this step, the "tip cell" is selected. In short, the cell that has the greatest contact with VEGF is selected as the tip cell. The tip cell starts to present filopodia and migratory cell characteristics, promoting the degradation of the ECM to invade the perivascular space. The tip cell migration is guided by the gradient of angiogenic factors and stimulates neighboring cells to become "stalk cells." The stalk cells have a high proliferative capacity and maintain contact with the pre-existing vessel, while forming the new blood vessel (Herbert and Stainier 2011; Betz et al. 2016). However, the cell proliferation process is not the only way to form a new vessel and maintain contact with the pre-existing vessel. The stem cells also elongate, and the end of the new blood vessel formation process occurs through the anastomosis, which comprises the fusion of two blood vessels (Herbert and Stainier 2011; Betz et al. 2016). Regulation of angiogenesis is essential to maintain homeostasis (Gacche and Meshram 2013).

Among the angiogenic factors that participate in neovascularization, we can highlight VEGF, hypoxia-inducible factor (HIF), placental growth factor (PGF or PlGF), FGF, HGF, Angiopoietin 1 (ANGPT1), PDGF, tumor necrosis factor (TNF), interleukins (IL), epidermal growth factor (EGF), IGF, angiogenin (Ang), stromal cell-derived factor (SDF), and TGF (Hanahan and Folkman 1996; Ferrara and Kerbel 2005; Gacche and Meshram 2013; Van Wijk and Van Kuppevelt 2014).

HSPGs promote the stabilization of various receptors with their respective ligands and serve as a depository for angiogenic factors, which will be released after cleavage of HS chains by the action of the heparanase enzyme or breakdown of the protein skeleton by the action of proteases. The cleavage of HS chains generates a concentration gradient that directs angiogenesis (Sasisekharan et al. 2002; Bishop et al. 2007; Dreyfuss et al. 2009; Fuster and Wang 2010; Sarrazin et al. 2011; Van Wijk and Van Kuppevelt 2014).

In mice, the knockout of the enzyme EXT1 that polymerizes the HS chains causes total inhibition of HS synthesis. This knockout is lethal, and mice die at an early stage of development. These studies showed increased vascular permeability and disorganization of blood vessels, highlighting the importance of HS in the molecular

mechanisms involved in the formation of new blood vessels (Goel and Mercurio 2013).

Neovascularization is a fundamental feature of ischemic retinal diseases and the wet form of age-related macular degeneration (AMD), one of the main causes of today's severe vision loss. VEGF inhibitors, such as brolucizumab, aflibercept, and ranibizumab, have transformed the treatment of AMD and have become the standard treatment. However, many patients do not respond adequately or experience decreased treatment efficacy after repeated administrations (Mitchell et al. 2018; Ricci et al. 2020). Therefore, there is a need to discover and identify new long-acting VEGF inhibitors. The binding to HSPGs has been suggested as a strategy to promote intraocular retention of anti-VEGF, thus leading to fewer intravitreal injections. Among a series of variants of the VEGF receptor 1 (VEGFR1), the variants with the greatest capacity to bind to Hep/HS have been shown to have longer duration of action and higher efficacy in animal models of intraocular neovascularization (Xin et al. 2021). Another approach is to design new synthetic GAG analogs or discover natural GAG-like compounds endowed with angiostatic properties (Dreyfuss et al. 2009). Sulfated oligosaccharides, structural mimics of HS or Hep, have also been discussed as potential drug candidates (Dreyfuss et al. 2010).

In cancer, the formation of neovascularization facilitates the appearance of tumor metastases and supplies the tumor mass with nutrients and oxygen, allowing intense cell proliferation. In cases where angiogenesis is insufficient, ischemia may occur. In metastasis, tumor cells detach from the tumor mass and the ECM, and the transition from the epithelial to the mesenchymal phenotype is taking place. Such tumor cells invade the tissue and may enter the neighboring blood vessels. Tumor cells survive in the bloodstream and invade another organ by migrating through the intercellular junctions of endothelial cells. When tumor cells adhere to the new tissue, they switch back to an epithelial phenotype and proliferate. In colonies of tumor cells that present dimensions greater than 1 $mm^3$, there is a need for neovascularization to occur. Thus, angiogenesis is stimulated. The new vessels formed will oxygenate the metastatic tumor mass, thus allowing the intense proliferation of the metastatic colony (Valastyan and Weinberg 2011; Bielenberg and Zetter 2015).

Vascularization is functionally abnormal in the tumor environment, and the formed vessels leak due to defective endothelium, characterized by openings and holes between cells. One explanation is the high concentration of VEGFA and the alteration in the ECM of the tumor microenvironment (Zanotelli and Reinhart-King 2018). In 1971, it was hypothesized that angiogenesis could be a good target for tumor treatment. Since then, several therapeutic alternatives have been investigated and in 2004, the US Food and Drug Administration approved the first anti-VEGFA monoclonal antibody, called bevacizumab, to treat colorectal cancer. Since then, anti-angiogenic therapy has been shown to inhibit tumor progression and increase patient survival temporarily. Furthermore, in conjunction with chemotherapy treatments, the anti-angiogenic treatment proved to be more efficient with a significant increase in survival (Ferrara and Kerbel 2005; Dreyfuss et al. 2009; Van Wijk and Van Kuppevelt 2014). In addition to bevacizumab, there are other therapies related to angiogenesis. For example, aflibercept is a recombinant protein related to

VEGFR1 and VEGFR2, cetuximab is an anti-EGFR monoclonal antibody, sorafenib is a small molecule VEGFR tyrosine kinase inhibitor, gefitinib and erlotinib are EGFR tyrosine kinase inhibitors, to name a few (Li et al. 2018).

VEGF can bind to three different classes of tyrosine kinase receptors. Each VEGF variant (VEGFA, VEGFB, VEGFC, VEGFD, and PlGF) has specificity in that it binds to one or more VEGFRs (Goel and Mercurio 2013). VEGFA can bind to both VEGFR1 and VEGFR2, with VEGFR2 being the most important modulator of angiogenesis. When VEGFA binds to VEGFR2, it activates the signaling pathway that involves phospholipase C (PLC) and mitogen-activated protein kinase (MAPK), triggering the proliferation of endothelial cells. On the other hand, the migration of endothelial cells occurs mainly through the activation of c-Src and focal adhesion kinase (FAK) (Sasisekharan et al. 2002; Bishop et al. 2007; Fuster and Wang 2010; Goel and Mercurio 2013; Nassar et al. 2021). VEGFA also interacts with co-receptors, such as HSPGs that can modulate VEGFA signaling (Ferrara et al. 2003; Sarrazin et al. 2011).

It is noteworthy that VEGFA undergoes alternative splicing, with the smallest variant, $VEGFA_{121}$ lacking the ability to bind to HS, whereas the larger isoforms $VEGFA_{165}$ and $VEGFA_{189}$ can bind to HS (Ferrara et al. 2003; Goel and Mercurio 2013; Van Wijk and Van Kuppevelt 2014). The binding of $VEGFA_{165}$ to HS depends on the 6-*O*-sulfation domains of glucosamine (Robinson et al. 2006; Uchimura et al. 2006). Decreased expression of 6-OST isoforms in endothelial cells compromises $VEGFA_{165}$-induced endothelial tube formation in vitro, suggesting that sulfation of endothelial HS is significant for the regulation of VEGFA angiogenic activities (Ferreras et al. 2012). Furthermore, both VEGFR1 and VEGFR2 have a binding domain with HS. Therefore, HS generally forms a trimer that stabilizes the complexes formed between VEGFA and the respective receptors (Van Wijk and Van Kuppevelt 2014).

In addition to stabilizing the receptors with their respective ligands, HSPGs also serve as a depot for angiogenic factors, which will be released after HSPG cleavage by the action of heparanase or proteases, in addition to the HS forming a concentration gradient, directing angiogenesis (Sasisekharan et al. 2002; Bishop et al. 2007; Dreyfuss et al. 2009; Fuster and Wang 2010; Sarrazin et al. 2011; Van Wijk and Van Kuppevelt 2014). SDC1 is a HSPG present on the cell surface. A study with co-culture of human triple negative breast cancer cells and human umbilical vein endothelial cells (HUVEC) showed a lower capacity of capillary formation by HUVEC after silencing of SDC1 in breast cancer cells, due to decreased amounts of VEGFA and TF in the secretome of the SDC1-silenced breast cancer cells (Nassar et al. 2021).

Other growth factors also act as mediators of angiogenesis. Activation of the EGF receptor (EGFR) pathway increases the production of angiogenic molecules in a great variety of tumor cells and EGFR tumorigenicity might partially be mediated by VEGFA up-regulation and promotion of angiogenesis (Petit et al. 1997). EGF stimulation of glioma cells has been shown to consistently increase the production of VEGFA by these cells. The conditioned medium of the stimulated glioma cells induces activation of HUVEC, which could be inhibited by an anti-VEGFA

antibody (Goldman et al. 1993). EGF has also been shown to enhance VEGFR mRNA in several gastric cancer cell lines (Akagi et al. 2003).

The role played by the FGF family in neovascularization is complex. In vivo, endothelial cells of quiescent vessels do not express VEGF; nonetheless, several works have indicated that the FGF system requires activation of the VEGF system for its angiogenic activity based on the observation that upon stimulation with FGF2, the endothelium of newly forming capillaries produces VEGF (Seghezzi et al. 1998). Similarly, VEGF expression has been found increased after FGF stimulation of stromal cells and cardiomyocytes (Claffey et al. 2001; Tsunoda et al. 2007). A HS-binding peptide that has high affinity for N-sulfated heparin has been shown to inhibit the proliferation and viability of HUVEC by disrupting the interaction between HS and FGF2. This peptide was also able to inhibit angiogenesis in in vitro, ex vivo, and in vivo assays, as well as to decrease tumor growth obtained from patient-derived xenografts, supporting the importance of HS for tumor growth by modulating angiogenesis (Melo et al. 2021). Decreased expression of 6-OST isoforms in endothelial cells compromises FGF2-induced endothelial tube formation in vitro and angiogenesis in vivo. Interestingly, the affinity of FGF2 for HS with reduced 6-*O*-sulfation is similar compared to HS with control sulfation; however, activation and subsequent internalization of FGFR1 is decreased suggesting that sulfation of endothelial HS is significant for the regulation of FGF2 angiogenic signaling through FGFR1 (Ferreras et al. 2012).

Previous studies have demonstrated the ability of perlecan to modulate FGF2 activity. Perlecan is a HSPG expressed in basement membranes and cartilage, is a key component of the vascular ECM, and is commonly associated with events that occur during the metastatic cascade (Whitelock et al. 2008; Ishijima et al. 2012; Elgundi et al. 2020). FGF2 bound to perlecan HS chains can be released very efficiently by stromelysin, collagenase, and plasmin, which degrade the perlecan core protein, suggesting that perlecan can act as a store of FGFs (Jiang and Couchman 2003). In addition to FGF2 signaling, perlecan interacts with many other pro-angiogenic and anti-angiogenic regulators, such as TGFβ and HGF, TNFα, Ang, IL-8, ANGPTs, and VEGFA (Eliceiri and Cheresh 2001; Jiang and Couchman 2003; Melrose 2020).

FGF2 has also been shown to stimulate the mRNA synthesis of HGF in vascular smooth muscle cells and fibroblasts (Onimaru et al. 2002) and upregulate monocyte chemoattractant protein-1 expression in endothelial cells (Wempe et al. 1997) in vitro. FGF2 also stimulates PDGFR expression in vascular smooth muscle cells, which is important to mural cell recruitment and vascular maturation (Murakami and Simons 2008). All these observations are in line with the notion that the FGF pathway controls the angiogenesis process in concert and synergistically with other growth factor systems (Murakami and Simons 2008).

PDGF overactivity has been related to atherosclerosis, fibrotic diseases, and cancer (Hellberg et al. 2010), potentially playing a relevant role in angiogenesis. PDGF controls the growth of mesenchymal cells, such as fibroblasts, smooth muscle cells, and glial cells (Marchand et al. 2019). PDGF-B synthesized and released from endothelial tip cells recruits mural cells, mainly pericytes, that express its receptor

PDGFR-β to blood microvessels, resulting in proliferation and migration of pericytes along the growing vessel. This process is vital to vessel maturation (Lindblom et al. 2003; Armulik et al. 2005). Endothelial PDGF-B displays a C-terminal protein motif that is required for proper retention of the growth factor within the endothelial pericellular space and interaction with its PDGFR receptor on pericytes (Lindblom et al. 2003; Armulik et al. 2005). Deletion of these retention motifs by gene targeting in mice resulted in defective investment of the microvessel wall with pericytes (Lindblom et al. 2003). Absence of these retention motifs also increases secretion of PDGF-B in the culture medium (Larochelle et al. 1991). All these observations are in line with the data showing that HSPGs bind to PDGF-B through the basic retention motifs, and this interaction is important for proper localization of PDGF-B to the vicinity of the growing vessel (Abramsson et al. 2007).

In tumor tissues, PDGF-BB and FGF2 are highly expressed (Westermark and Heldin 1993; Nguyen et al. 1994; Nissen et al. 2007) and may crosstalk. According to Nissen et al., FGF2 induces up-regulation of PDGFRs expression in endothelial cells that become hyper-responsive to PDGF-BB. PDGF-BB, on the other hand, induces FGFR-1 expression in vascular smooth muscle cells that become more sensitive to FGF2. stimulation The consequence of this complex crosstalk is an uncontrolled neovascularization in cancer, with malformed vasculature (Nissen et al. 2007).

### *9.3.1 Heparan Sulfate and Thrombus Formation*

HS synthesis in cultured rabbit aortic endothelial cells is stimulated by Hep and is rich in iduronic acid (Nader et al. 1987). Such endothelial HS inhibits clot formation by interfering with the conversion of prothrombin to thromboplastin-stimulated thrombin (Colburn and Buonassisi 1982). Such studies suggest that the in vivo antithrombotic activity of Hep may be related, at least in part, to the increased production of this particular HS by endothelial cells (Pinhal et al. 1994). However, endothelial cell HS has been shown to possess both pro- and anti-angiogenic activity (Fuster et al. 2007).

## 9.4 Concluding Remarks

Cancer is a multifactorial disease, and its genesis and progression are extremely complex. The biggest problem in the anticancer drug development effort is the acquisition of multidrug resistance and relapse (Kumar et al. 2017). Tumors produce several molecules that facilitate their proliferation, invasion, and maintenance, among which HSPGs that can act as co-receptors of growth factors and ECM proteins or increase the affinity of ligands for their specific receptors. Changes in

the expression of HSPGs have been found in numerous tumor cells, indicating their involvement in cancer (Blackhall et al. 2001; Belting 2014).

Anticancer drugs can regulate the HS activity in different ways: some directly interact with GAG chains, and others can act on enzymes that regulate HSPGs levels. Other drugs act directly on HSPGs' core protein and can affect a specific type of HSPG. According to literature data, the drugs targeting HSPGs can be divided into four main groups: natural products, monoclonal antibodies, antitumor antibiotics, and mimetic peptides (Onyeisi et al. 2020b, 2021b). HS mimetics have been demonstrated as an important strategy to regulate the tumor biological behavior via a multi-target mechanism of action based on their effects on tumor cells and various components of the tumor microenvironment (Lanzi and Cassinelli 2018). In addition, a recent study demonstrated that targeting HS might serve as a potential strategy for nanoparticle-mediated therapy against desmoplastic stroma-associated neoplasms (Kuo et al. 2020).

Heparanase and Sulfs that act on post-translational modifications of HS are also involved with tumor progression, angiogenesis, and resistance mechanisms to specific antineoplastic treatments. Therefore, several studies have investigated the use of heparanase and Sulfs inhibitors as alternative therapeutic targets for cancer treatment (Lewis et al. 2008; Dredge et al. 2010; Dredge et al. 2011; Ritchie et al. 2011; Liu et al. 2014; Vivès et al. 2014; Jia and Ma 2016; Lanzi et al. 2017; Barash et al. 2018). One of the strategies to regulate the expression of heparanase is based on the post-transcriptional modulation of this enzyme. In this sense, microRNAs (miRs) and short hairpin RNAs (shRNAs) are known to promote the decreased synthesis of this enzyme (Bartel 2004). There are reports in the literature showing that miR-1258 negatively modulates heparanase expression in gastric and breast cancer (Shi et al. 2017; Tang et al. 2013), miR-239-3p and miR-429 in gastric cancer (Sheng et al. 2018; Zhou et al. 2019), and miR-30 in melanoma (Liu et al. 2013).

The fact that HS is ubiquitous in all cells in our body demonstrates its vital importance. Targeting HSPGs and enzymes involved in post-translational modifications of the HS chains is a promising anticancer strategy.

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# Chapter 10
# The Role of Pericytes in Tumor Angiogenesis


**Domenico Ribatti and Antonio Giovanni Solimando**

**Abstract** Pericytes are branched cells surrounded by the basement membrane (BM) of capillaries and post-capillary venules. Pericytes are involved in the control of endothelial cell proliferation, migration, and stabilization. Conversely, endothelial cells boost the expansion and activation of pericyte precursors. The balance amid endothelial cells and pericytes is vastly organized by a sequence of mechanisms active in an autocrine and/or paracrine fashion. In this chapter, we uncover the molecular aspects of the pericyte activating factors secreted by endothelial cells, such as platelet-derived growth factor B (PDGF-B), vascular endothelial growth factor A (VEGFA), Notch and ephrins signaling. We also discuss the multifaceted role of pericytes from different standpoints of angiogenesis, including a potential interplay related to remodeling of the BM, highlighting the potential role of these cells as targets in tumor therapy.

## 10.1 Introduction

Pericytes are located within the basement membrane (BM) of capillary and post-capillary venules and are generally considered to be cells that stabilize the vessel wall, controlling endothelial cell proliferation and thereby the growth of new capillaries. Additionally, they are believed to participate in the regulation of microvascular blood flow via a contractile mechanism. Numerous molecular biomarkers

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**Table 10.1** Pericyte molecular markers

| |
|---|
| Alpha smooth muscle actin (αSMA) |
| Non-muscle myosin |
| Tropomyosin |
| Desmin |
| Nestin |
| Platelet-derived growth factor receptor-β (PDGFR-β) |
| Aminopeptidase A |
| Aminopeptidase N (CD 13) |
| Sulfatide or nerve/glial antigen-2 (NG2) proteoglycan |
| Regulator G protein signaling 5 (RGS5) |
| Sulfonylurea receptor 2 (SUR2) |
| Alkaline phosphatase (ALP) |
| Vimentin |
| Melanoma cell adhesion molecule (NCAM/CD146) |
| Prominin 1 (CD133) |
| Endosialin |
| T-box transcription factor 18 (TBX18) |

classify pericytes (Table 10.1) (Hellström et al. 1999; Ohlsson et al. 1999; Morikawa et al. 2002). In this context, a consensus about the phenotypic identity of pericytes has not to be reached. For example, pericytes on normal capillaries express desmin but not alpha smooth muscle actin (α-SMA), whereas smooth muscle cells on arterioles and pericytes on venules are immunoreactive for both (Nehls et al. 1992; Morikawa et al. 2002). Pericytes wrap around very small vessels, particularly capillaries, playing important roles in endothelial homeostasis (Bergers and Song 2005). In the past, pericytes were said to maintain low growth of blood capillaries. Thus, it has been tempting to manipulate pericytes to halt vessel growth in pathological conditions (Baffert et al. 2006). Nevertheless, several actors on the scene, such as angiopoietins (Angpts), platelet-derived growth factor-B (PDGF-B), transforming growth factor-beta (TGFβ), vascular endothelial growth factor A (VEGFA), and signaling pathways, including Notch and ephrins, control the homeostasis between pericytes and endothelial cell (von Tell et al. 2006; Hughes 2008).

The endothelium PDGF-B shedding is involved in a crosstalk with PDGF receptor beta (PDGFR-β) on pericytes (Betsholtz 2004). The PDGF-B knock-out in pre-clinical model has been shown to disrupt the attraction of PDBFR-β-positive progenitors of pericytes towards the vascular sprouting, thus leading to vascular instability and regression (Levéen et al. 1994; Benjamin et al. 1998; Lindblom et al. 2003).

VEGF receptor 2 (VEGFR2) binding by VEGFA halts PDGFR-β signaling, through the induction of a VEGFR2/PDGFR-β complex (Greenberg et al. 2008), while prompting proliferation and migration of pericytes under hypoxia (Yamagishi et al. 1999). Moreover, VEGFA indirectly stimulates pericyte recruitment via endothelial cell production of nitric oxide (NO), which promotes mural precursor cell migration in vitro and pericyte recruitment to tumor vessels in vivo (Kashiwagi

et al. 2005). Pericytes co-cultured with endothelial cells produce VEGFA that acts as a survival and/or stabilizing factor for endothelial cells (Darland et al. 2003).

Mesenchymal cells co-cultured with endothelial cells or treated with TGFβ1 express smooth muscle cell markers, indicating differentiation of precursor cells into pericytes or smooth muscle cells (Darland and D'Amore 2001). In animal models, the lack of endoglin, a TGFβ1 co-receptor expressed on endothelial cell surface, results in defective vascular development that is not due to defective vasculogenesis but seems to correlate with a decreased association of endothelial cells with smooth muscle cells and pericytes (Li et al. 1999). Mice deficient in TGFβ1 are embryonically lethal due to vascular malformations but for many years, it has not been clear whether TGFβ has a positive or negative effect on angiogenesis. It seems that its effect depends on the TGFβ receptor. Activation of the endothelial cell-specific activin receptor-like kinase (ALK) 1 receptor via Smad1/5 transcription factor stimulates endothelial cell proliferation and migration. On the other hand, activation of the broadly expressed ALK5 via Smad2/3 inhibits endothelial cell functions. Endoglin may act as a negative regulator of TGFβ/ALK5 signaling by recruiting and/or activating Smad1/5 (Lebrin et al. 2005). Interestingly, ALK5 activation induces expression of fibronectin, collagen I $\alpha_1$, and plasminogen activator inhibitor type 1 (Laping et al. 2002; Van Geest et al. 2010; Dhahri et al. 2017) and down-regulates $\beta_5$ integrin in endothelial cells (Ota et al. 2002). The effect of TGFβ on the expression of extracellular matrix (ECM) proteins in both endothelial cells and pericytes may fit with a role of TGFβ in retinal capillary BM thickening in pre-clinical diabetic retinopathy (Van Geest et al. 2010) but it is unclear how it may affect angiogenesis. Murine embryonal germinal matrix microvessels, in which pericytes lack Alk5 expression, have decreased levels of tissue inhibitor of matrix metalloproteinase 3 (TIMP-3) and increased perivascular matrix metalloproteinase (MMP) activity, decreased BM collagen, decreased endothelial cell proliferation and pericyte coverage and abnormal dilation (Dave et al. 2018).

Angpts have been implicated in pericyte recruitment. Angpt1 but not Angpt2 or their receptors Tie-1 and Tie-2, are expressed in pericytes, while endothelial cells express Angpt2, Tie-1, and Tie-2, but not Angpt1. Lack of expression of Angpt1 by endothelial cells and its expression by mural vascular cells has also been confirmed in vivo. Angpt1 synthesized by pericytes and smooth muscle cells interact with endothelial Tie-2 and this may contribute to the interactions between endothelial cells and pericytes that result in blood vessel maturation (Sundberg et al. 2002). Mice deficient for either Angpt1 or Tie-2 die during embryonic development with vascular defects, like those observed for PDGF-B deficient mice (Jones et al. 2001). In the latter, recombinant Angpt1 restores the vascular structure and permeability in the growing retinal vasculature (Uemura et al. 2002) and counteracts VEGF-induced endothelial leakiness (Thurston et al. 1999). Besides endothelial cells, Tie-2 has been found to be expressed by a distinct hematopoietic lineage of bone marrow-derived pro-angiogenic cells that seem to be recruited to spontaneous and orthotopic tumors and promote angiogenesis in a paracrine manner. Tie-2 seems to be also expressed by some mesenchymal precursors that differentiate into tumor pericytes (De Palma et al. 2005). Since neither Angpt1 nor Angpt2 bind to Tie-1, it is

considered to be an orphan receptor. Global Tie-1 deletion in mice leads to late embryonic lethality, while conditional endothelial-specific Tie-1 deletion during primary tumor growth results in a decreased number of tumor microvessels, with increased coverage by mural cells and better perfusion (La Porta et al. 2018). Angpt2 expressed by endothelial cells located at the leading edge of proliferating vessels acts as a destabilizing factor through Tie-2 antagonism (Maisonpierre et al. 1997). VEGF boosts the Angpt2 expression in endothelial cells, leading to the dissociation of pericytes from vessels (Zhang et al. 2003). In the same line, overexpression of Angpt2 has been shown to decrease pericyte coverage of tumor vessels, thus destabilizing them and suppressing tumor growth (Cao et al. 2007). Besides tumors, Angpt2 overexpression leads to dense vascular networks with reduced pericyte coverage in the retina (Feng et al. 2007). Overexpression of Angpt2 in glioma cells has been shown to result in MMP2 activation that results in enhanced angiogenesis and invasiveness and results from binding of Angpt2 to $\alpha_v\beta_1$ integrin (Hu et al. 2003, 2006).

The NG2 proteoglycan is expressed by immature pericytes throughout early vasculogenesis. NG2 interacts with galectin-3 and $\alpha_3\beta_1$ integrin on endothelial cell surface and supports endothelial cell motility and early angiogenesis (Fukushi et al. 2004).

The Notch-3 receptor is highly expressed in pericytes, and the disruption of Notch-3 signaling in *Notch-3*$^{-/-}$ mutant mice results in enlarged vessels due to the lack of pericytes (Rafalowska et al. 2004). Patients suffering from CADASIL (Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy) syndrome, a pathology associated with mutations of Notch-3, have vessels that lack pericytes (Louvi et al. 2006). Knockdown by small interfering RNA revealed that Notch-3 signaling is necessary for endothelial-dependent mural cell differentiation, and Notch-3 contributes to the pro-angiogenic capability of mural cells co-cultured with endothelial cells (Liu et al. 2009). Ephrin-B is also a critical mediator of assembly of pericytes and endothelial cells (Salvucci et al. 2009).

Perivascular pericytes can give rise to mesenchymal stem cells (MSC) that may be differentiated towards osteogenic, adipogenic and chondrogenic cell lineages and thus, may be useful in regenerative medicine (Crisan et al. 2012). Their potential pluripotency seems to be also essential for their protective and remodeling effects in the brain neurovascular unit (ElAli et al. 2014). In the same line, during nephrogenesis, interstitial pericytes are the cells that differentiate into myofibroblasts during fibrosis, a mechanism that may be exploited for therapeutic strategies for fibrotic kidney disease (Humphreys et al. 2010). Osteogenic differentiation of pericytes has been also observed in vitro, accompanied by changes in the ECM proteins laminin, collagen X, tenascin (Schor et al. 1991) and matrix Gla protein (Canfield et al. 2000). Rat heart pericytes produce collagens I, IV and VI, and fibronectin in vitro, and may significantly contribute to the formation of the myocardial capillary matrix (He and Spiro 1995). During blood vessel sprout formation, endothelial cells and pericytes co-assemble and co-contribute to the deposition of the vascular BM components, such as collagen IV, laminin, nidogen, and perlecan,

between the two cell types. In this process, $\alpha_1\beta_1$, $\alpha_3\beta_1$, $\alpha_5\beta_1$, and $\alpha_6\beta_1$ integrins seem to play a key role (Davis et al. 2015). Pericytes also express MMP2 and MMP9 that may play a role in the degradation of the ECM during tumor angiogenesis (Nielsen et al. 1997) or regulate the endothelial barrier integrity of the blood-brain barrier (BBB) (Thanabalasundaram et al. 2010; Takata et al. 2011; Machida et al. 2015; Underly et al. 2017). It was recently shown that pericytes produce and secrete vitronectin, which regulates the integrity of the BBB by interacting with its receptor $\alpha_5$ integrin on brain endothelial cells (Ayloo et al. 2022).

## 10.2 The Role of Pericytes in Angiogenesis

Pericytes are among the first cells to invade newly vascularized tissues, located at the growing front of the endothelial sprouts and guiding newly formed vessels (Nehls et al. 1992). Individual pericytes can be found at the tips of angiogenic sprouts in the corpus luteum and in tumors (Amselgruber et al. 1999; Morikawa et al. 2002; Gerhardt and Betsholtz 2003). Endothelial tube formation is followed by investment of pericytes, which are recruited by differentiation from surrounding mesenchymal precursors or by migration from the mural wall of the adjacent vessel (Gerhardt and Betsholtz 2003). Pericytes suppress endothelial growth and migration (Orlidge and D'Amore 1987; Sato and Rifkin 1989), and there is a striking coincidence of pericyte investment, microvessel stabilization (Bergers and Song 2005; von Tell et al. 2006) and capillary resistance to regression in vivo (Benjamin et al. 1998). Fibronectin, laminin, and integrin expression is actively regulated by endothelial cell–pericyte interactions (Stratman et al. 2009), and these cell communications are crucial in shaping the BM homeostasis.

Pericytes can also exert tissue invasion when endothelial cells are lacking and can form tubes enabling the subsequent penetration of endothelial cells (Ozerdem and Stallcup 2003). Bone marrow-derived $CD11b^+$ and $CD45^+$ hematopoietic progenitor cells expressing the pericyte marker NG2 are located in close proximity to blood vessels in a subcutaneous B16-F1 melanoma model (Rajantie et al. 2004). Bone marrow-derived PDGFR-$\beta^+$/Sca-$1^+$ progenitor pericytes have been demonstrated in a mouse model of pancreatic islet tumorigenesis to differentiate into mature pericytes expressing the NG2 and α-SMA markers (Song et al. 2005). In the growing microvessels in human fetal telencephalon, endothelial cells are preceded and guided by migrating pericytes (Virgintino et al. 2007).

## 10.3 Pericytes, Tumor Growth, and Metastasis

Pericytes cover 73–92% of endothelial sprouts in different murine tumor types, and are located near blood vessels at the growing front of tumors, where angiogenesis is highly operative despite significant morphological alterations (Schlingemann et al.

1990; Wesseling et al. 1995; Morikawa et al. 2002). Pericyte recruitment is significantly more pronounced in colon and breast cancers compared to renal cell carcinoma and central nervous system malignancies (Eberhard et al. 2000). Increased pericyte coverage in melanoma and renal cell carcinoma has been correlated with more aggressive clinic-pathological characteristics, resistance to therapy and unfavorable clinical outcome (Gee et al. 2003). Lack of pericytes is a deficiency that participates in cancer vessel abnormalities (Gerhardt and Semb 2008) and a partial dissociation of pericytes contributes to increased tumor vascular permeability (Hobbs et al. 1998; Hashizume et al. 2000). Of note, based on nestin expression, it has been possible to discriminate between type-2 (nestin-positive) and type-1 (nestin-negative) pericytes; the latter are not recruited during tumor angiogenesis (Ribeiro and Okamoto 2015).

Tumor pericytes are loosely associated with endothelial cells, have abnormal shape, extend cytoplasmic processes away from the vessel wall, and have extra layers of loosely fitting BM (Eberhard et al. 2000). An aberrant PDGF signaling can explain the above-mentioned alterations. Mice genetically deficient in PDGF-B or its receptors have blood vessels with loose pericyte attachment, irregular vessel caliber, luminal projections on endothelial cells, and are hemorrhagic. Similar abnormalities occur in many tumor vessels. Cancer cells actively attract pericytes via PDGF-B in pre-clinical animal models (Guo et al. 2003). Alternatively, genetic abolition of PDGFR-β expressed by embryonic pericytes decreased their recruitment (Abramsson et al. 2003). Inhibition of the expression of endothelial differentiation gene-1 in animal models of Lewis lung carcinoma implanted in mice, strongly reduced pericyte coverage (Chae et al. 2004). Angpt1 orchestrates cancer development, fueling neo-angiogenesis, likely due to increased pericyte infiltration in rats (Machein et al. 2004). Alternatively, in a colon cancer model, overexpression of Angpt1 led to smaller tumors with fewer blood vessels and greater pericyte coverage, decreased vascular permeability and reduced hepatic metastasis (Ahmad et al. 2001). There is a great variability in endothelium coverage by pericytes across different cancer types. Islet carcinomas have dense pericyte coverage, while glioblastomas have a reduced number of pericytes (Bergers and Song 2005).

Neuroblastoma makes no exception, with pericytes ensuing vasculogenesis in a MMP9-dependent manner (Chantrain et al. 2004). In addition, overexpression of TIMP-3, results in decreased pericyte recruitment in neuroblastoma and melanoma models (Spurbeck et al. 2002). Due to MMP expression by tumoral pericytes, it is tempting to envision a pivotal role on ECM degradation (Nielsen et al. 1997).

The vascular integrity can be lost due to a lack of pericyte coverage, enabling cancer cells to transit into the circulatory system, thereby boosting the metastatic potential. Experimental interruption of pericyte coverage has triggered enhanced metastasis in the Rip1-Tag2 pancreatic islet tumor model (Xian et al. 2006). A lack of α-SMA-positive pericyte coverage of malignant neovessels has been associated with hematogenous dissemination and correlated with poor prognosis in colorectal cancer (Yonenaga et al. 2005), lung and brain cancer models, pinpointing potential therapeutic windows related to phospholipase A2 targeting (Linkous et al. 2010; Jridi et al. 2017). Pericyte coverage halted cancer cells' invasive phenotype in

prostate cancer models (Welén et al. 2009). Indeed, neo-angiogenesis represents a critical event for malignant progression (Krebs et al. 2020). Pericyte depletion results in hypoxia-associated epithelial-to-mesenchymal transition and metastasis in breast and kidney cancers (Sun et al. 2009; Cooke et al. 2012), two archetypic conditions addicted to neo-vessels with key implications for novel immune targeting strategies (Boudreau and Myers 2003; Argentiero et al. 2020; Shadbad et al. 2021).

According to the "seed and soil" hypothesis, pericytes actively prime a metastatic-prone niche, shaping the cancer milieu of lung malignancies (Murgai et al. 2017). Pericyte-specific deletion of the transcription Kruppel-like factor 4 (KLF4) inhibits pericyte expansion in the lung, decreasing pulmonary metastasis without affecting primary tumor growth (Murgai et al. 2017).

## 10.4 Pericyte Role in Hypoxia, Metabolism, and Tumor Progression

Both solid and hematological cancers are characterized by a stressful microenvironment in terms of access to molecular oxygen (Wang et al. 2012; Solimando et al. 2020b, 2021), a state known as hypoxia. Nonetheless, hypoxia is almost always coupled with decreased availability of nutrients, as observed in many domains within solid tumors leading to ischemia (Sullivan et al. 2019; Marquardt et al. 2021), due to a size extending beyond the natural diffusion limits of either molecular oxygen or blood-born nutrients, such as glucose, glutamine, and lipids. This phenomenon impacts many aspects of disease progression, including the formation of blood vessels, which are needed to sustain tumor growth, but also allow tumor cell dissemination during metastasis at visceral (Eyles et al. 2010) and skeletal (Argentiero et al. 2019; Antonio et al. 2020) sites. Remarkably, the switch from the small avascular tumor to the large more vascularized tumor can take several decades, as inferred from studies performed on several malignancies, such as pancreatic cancer (Maitra and Hruban 2008). Nevertheless, once the tumor obtains blood vessels, it can continue to grow and ultimately metastasize. Tumor blood vessels differ from their normal counterparts by several key features, lacking the typical arterial capillary-venule hierarchy, loosing associations with pericytes, thus being highly fenestrated. They can be torturous and exhibit excessive branching, abruptly resulting in opening and closed structures, with the result of chaotic blood flow patterns and very poor perfusion of the tissue (Weinberg 2007). These dysfunctional blood vessels do not properly fulfill their purpose and the result is a domain where cells receive limited oxygen, glucose, glutamine and other nutrients and respond by rapidly turning to anaerobic metabolism (Nakazawa et al. 2016), inhibiting the mTOR pathway (Saxton and Sabatini 2017; Lamanuzzi et al. 2018), engaging autophagy (Daskalaki et al. 2018; Di Lernia et al. 2020) and the unfolded protein response, along with distress responses (Nakazawa et al. 2016). Hypoxia and 2-oxoglutarate oxygenases regulate DNA methylation, histone methylation, and

collagen modification, among other important biological processes (Ploumakis and Coleman 2015). Pericyte depletion in a mouse model of breast cancer has been associated with increased hypoxia, enhanced epithelial-to-mesenchymal transition and activation of the Met receptor, suggesting that pericytes may act as suppressors against cancer growth and metastasis (Cooke et al. 2012).

BBB pericytes have been shown to respond to hypoxia by migrating away from capillaries, without any apparent effect on endothelial cells (Gonul et al. 2002) and inhibition of pericyte hypoxia-inducible factor-1α signaling seems to be responsible for BBB integrity by safeguarding pericyte survival (Tsao et al. 2021; Baumann et al. 2022). Interestingly, hypoxia-induced BBB dysfunction has recently been shown to be associated with increased fibronectin fibrillogenesis (Jamieson et al. 2022).

## 10.5 Pericytes as Putative Targets in the Therapy of Tumors

Pericytes may represent an Achille's heel and a druggable target in combination with anti-angiogenesis in cancer therapy.

Inhibition of PDGFR-β signaling did not cause significant regression of endothelial cells in tumors (Abramsson et al. 2003). In the same line, the PDGFR inhibitor imatinib did not significantly block tumor angiogenesis when employed alone; however, it boosted the effect of VEGF inhibitors (Bergers et al. 2003). Murphy et al. (2010) generated a series of selective type II inhibitors of PDGFR-β and B-RAF for targeting pericyte recruitment and endothelial survival, respectively, and demonstrated that dual inhibition of both PDGFR-β and B-RAF exerted synergistic anti-angiogenic activity in both zebrafish and murine and translational models of angiogenesis (Sennino et al. 2007; Gnoni et al. 2019).

VEGF inhibitors induce pericytes to become closely associated with surviving tumor vessels in Lewis lung carcinomas, RIP-Tag2 tumors and other tumor models (Willett et al. 2004; Tong et al. 2004; Inai et al. 2004). Anti-angiogenic treatment directed against endothelial cells using VEGF inhibitors induces the regression of tumor vessels without removing pericytes (Morikawa et al. 2002), and decreases tumor size (Baluk et al. 2005), leading to vessel normalization, characterized by increased pericyte coverage, tumor perfusion and chemotherapeutic sensitivity (Jain 2005). In the mouse models of spontaneous RIP-Tag2 tumors or implanted Lewis's lung carcinomas, anti-VEGF treatments significantly decreased tumor vasculature, leaving behind empty sleeves of BM and pericytes. Removal of VEGF inhibition caused rapid tumor re-vascularization through the pericyte-BM scaffold, and this process was not inhibited by MMPs or collagen IV inhibitors (Mancuso et al. 2006).

Pre-clinical mouse models corroborated the effectiveness of anti-PDGF-B/PDGFBR-β-directed therapy, aiming to decrease the pericyte coverage and enhance the response rate in the RIP1-Tag2 animal model (Bergers et al. 2003). AG-013737

or VEGF-Trap is effective in RIP1-Tag-2 cancers and Lewis lung malignancies, leading to cell–cell association between surviving pericytes and endothelial cells (Inai et al. 2004). Treatment of RIP1-Tag2 tumors with anti-PDGFR-β antibody reduces pericytes, increases endothelial cell apoptosis but does not seem to reduce tumor vascular density (Song et al. 2005). Tumor shrinking has also been obtained by receptor tyrosine kinase inhibition: SU6668, modulating PDGFR-β signaling, induces a loss of anchoring properties and decreases RIP1-TAG2 and xenotransplanted tumor growth (Shaheen et al. 2001; Reinmuth et al. 2001). Treatment with a selective DNA aptamer AX102 that blocks the action of PDGF-B led to progressive reduction of pericytes in Lewis lung carcinomas (Sennino et al. 2007). Combined targeting of pericytes and tumor endothelial cells with a mixture of a peptide ligand of aminopeptidase A, discovered by phage display technology for delivery of liposomal doxorubicin (DXR) to perivascular tumor cells, and aminopeptidase N-targeted (to tumor endothelial cells) liposomal DXR, boosts anti-cancer efficacy of liposomal chemotherapy in human neuroblastoma-bearing mice (Loi et al. 2010). Impaired angiogenesis and pericyte coverage have also been obtained by olfactomedin-like-3 targeting (Miljkovic-Licina et al. 2012).

## 10.6 Tumor Immunomodulatory Activity of Pericytes

Pericytes are also involved in vascular immunosurveillance (Stark et al. 2018). Pericytes overexpress adhesion molecules involved in the control of immune cell trafficking through the vasculature, including ICAM1 and VCAM1 (Guijarro-Muñoz et al. 2014). Treatment of brain pericytes with tumor necrosis factor alpha (TNFα), interleukin-1 beta (IL1β), or lipopolysaccharide (LPS), stimulates secretion of IL8 and MMP2, facilitating chemo attraction and transmigration of neutrophils (Pieper et al. 2013). Deletion of the *Rgs5* gene encoding a regulatory G-protein signaling expressed by pericytes, induces changes in the vasculature and enhances CD8-positive T cell infiltration in tumors (Hamzah et al. 2008). In murine pericyte co-cultures with tumor fragments, RGS5 and programmed death-ligand 1 (PDL1) are up-regulated (Bose et al. 2013). Pericytes have a direct role in tumor immune escape by inhibiting CD4-positive T cell activation and by promoting T cell anergy, like the endothelial counterpart in several solid and hematological malignancies (Bose et al. 2013; Rao et al. 2020; Solimando et al. 2020a; Ribatti et al. 2021) (Fig. 10.1). Pericytes isolated from human malignant gliomas inhibit proliferation of cytotoxic T cells (Ochs et al. 2013).

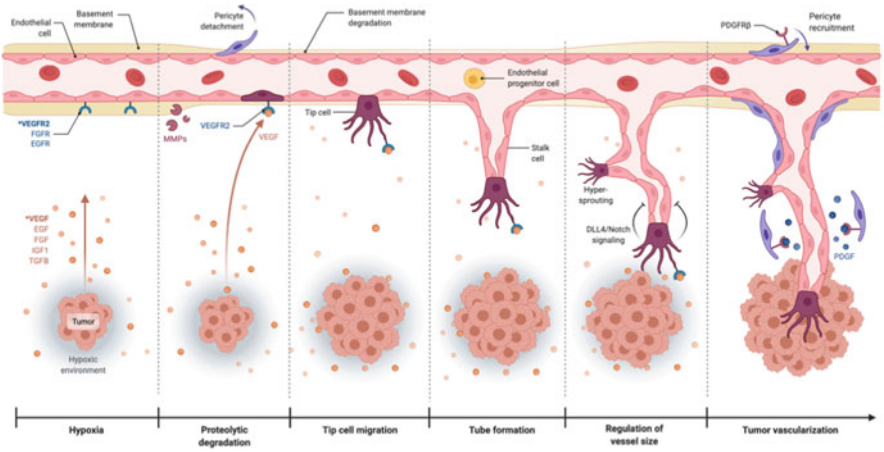


**Fig. 10.1** Tumor vascularization: endothelial cells and pericytes as putative targets in the therapy of malignant progression, across the spatial and temporal cancer heterogeneity. Pericytes support physiological vessel stability. The loss of pericyte growth factor (PDGF-B, secreted by endothelial cells) and its receptor (located on pericytes) may explain at a molecular level why sometimes the support of pericytes fails. A lower amount of pericytes surrounding the tumor-associated endothelium has been correlated with greater instability and permeability of blood vessels. Conversely, the dynamic expression of PDGFR-β receptor sustains tumor vascularization during pericyte recruitment upon massive malignant neovascularization. See text for more details

## 10.7 Concluding Remarks

A stable capillary microvasculature may represent an important prerequisite for preventing tumor cell dissemination. The future use of molecules interfering with the endothelial cell/pericyte unit will also bathe home of interest in tissue engineering, as well as the development of multi-tissue organs. Further studies are needed to highlight aspects of pericyte molecular biology and physiology.

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# Chapter 11
# Lymphatic Mechanoregulation in Development and Disease


**Sandra A. Hemkemeyer, Claudia Carlantoni, and Maike Frye**

**Abstract** Endothelial cells form the innermost layer of lymphatic and blood vessels and continuously interact with their luminal and tissue microenvironment. These interactions confer extracellular mechanical information, such as fluid shear stress, cellular stretch, and matrix stiffness, on the endothelium and are subsequently translated into intracellular biological responses. The impact of changes in fluid shear stress has been extensively studied in both lymphatic and blood endothelial cells. Recent studies suggest that the tissue microenvironment, which is established by the extracellular matrix, endothelial-associated mural cells, and the surrounding tissue, also fundamentally controls vascular development and disease.

In contrast to blood vessels, molecular mechanisms of lymphatic mechanoregulation via the tissue microenvironment are poorly understood. This review briefly compares what is known about the lymphatic and blood endothelial tissue microenvironment. We will further discuss how changes of the tissue microenvironment regulate lymphatic development and could contribute to dysregulation of lymphatic endothelial cells in disease. We aim to point out that a comprehensive analysis of tissue-regulated mechanisms could improve our understanding of lymphatic development and homeostasis and may eventually lead to the discovery of novel therapeutic approaches for lymphatic diseases associated with changes of the lymphatic-proximal microenvironment.

## 11.1 Introduction

In vertebrates, two complementary vessel networks form the vascular system and achieve organ homeostasis: the blood and the lymphatic vasculature. The *blood vasculature* is a circulatory system that transports oxygenated blood from the heart to peripheral tissues via arteries, while veins return oxygen-poor blood to the heart. In a hierarchical tree-like system, blood passes from large arteries to smaller

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arterioles and infiltrates tissues and organs through an extensive network of capillaries. The exchange of oxygen, delivery and recycling of nutrients and waste between blood and the tissue are achieved at the capillary level, which subsequently drains into venules and large veins. For re-oxygenation, blood is transported via the pulmonary artery to the lungs. In contrast, the *lymphatic vasculature* is a blind-ended, unidirectional vessel network that developed evolutionary to transport dietary fats from the intestine to the liver (Kampmeier 1969). Later, the lymphatic system acquired additional functions to drain excessive interstitial fluid and to transport immune cells to lymph nodes for immune surveillance (Stritt et al. 2021).

Endothelial cells (ECs) are essential vessel components and line the inner vessel wall of both vessel networks to form a dynamic barrier between the circulating blood or lymph on their luminal side and the surrounding tissue on their abluminal side. Through their specialized, button-like cell–cell contacts (button-like junctions) (Baluk et al. 2007), lymphatic capillaries (also referred to as initial lymphatics) collect fluid, macromolecules and cells from the interstitial tissues. The lymph is then transported via pre-collecting lymphatic vessels to the larger collecting lymphatic vessels and returns to the bloodstream via lymphovenous valves at the intersection of the jugular and subclavian veins (Jeltsch et al. 2003; Tammela and Alitalo 2010; Geng et al. 2016). Lymphatic collecting vessels are equipped with luminal lymphatic valves (LVs), which support pumping and ensure unidirectional lymphatic transport without reflux (Potente and Mäkinen 2017). In contrast to the lymphatic capillaries, the lymphatic collecting vessels form continuous zipper-like cell–cell contacts and are therefore characterized by a reduced absorption of fluid, macromolecules, and cells from their interstitial environment (Baluk et al. 2007).

Due to their unique functions, each vessel subtype is exposed to a variety of mechanical forces. These vessel subtypes are therefore equipped of specialized EC subtypes with unique properties and genetic profiles that enable them to fulfill their specific function (Potente and Mäkinen 2017). Not only does each vessel subtype exhibit a unique endothelial genetic profile, but endothelial properties also differ in a tissue-specific manner. As an example, specialized lymphatic ECs (LECs) are found in the Schlemm's canal vessels of the eyes (Petrova and Koh 2020), in the ascending vasa recta of the kidney (Kenig-Kozlovsky et al. 2018), and in meningeal lymphatic vessels in the brain (Aspelund et al. 2015; Louveau et al. 2015).

In addition, blood and lymphatic vessels are surrounded by unique tissue microenvironments with different mechanical and structural properties. ECs can be supported by various *extracellular matrix (ECM)* components, as well as by several *mural cell types*, like pericytes or smooth muscle cells (SMCs) (Gordon et al. 2020). The ECM is an essential part of the tissue environment and forms a complex three-dimensional scaffold consisting of the basement membrane (BM), which is mainly formed by the ECs themselves, and the interstitial matrix (IM), which fills the interstitial space between surrounding cells.

ECs recognize and respond to mechanical impacts, such as shear, stretch, and ECM stiffness, through their cell–cell contacts and cell–ECM adhesions, and translate physical stimuli into biological responses, in a process referred to as *mechanotransduction*. Shear mode and amplitude of fluid flow, as well as

composition and mechanical properties of the ECM, differ across the vascular tree, in development and disease.

While the impact of changes in fluid shear stress (FSS) is well studied in blood ECs (BECs) and LECs (reviewed in Baeyens et al. 2016; Bálint and Jakus 2021; Campinho et al. 2020), in this chapter we will focus on *changes of the lymphatic endothelial tissue microenvironment*. We will briefly compare what is known about LEC and BEC tissue microenvironments and further discuss how tissue microenvironment alterations could contribute to (dys)regulation of LECs in development and disease.

## 11.2 The Tissue Microenvironment of Endothelial Cells

### *11.2.1 The Endothelial Basement Membrane and Extracellular Matrix Components*

During cell migration and sprouting in embryonic and postnatal (lymph)-angiogenesis, but also in pathological (lymph)angiogenic processes, the ECM surrounds individual ECs or EC clusters in three dimensions (3D). After vascular remodeling and maturation, ECs form the inner layer of lymphatic or blood vessels and adhere to the surrounding abluminal ECM environment in two dimensions (2D).

In the *established blood vasculature*, the blood vessel BM is mainly composed of laminin 411 (Thyboll et al. 2002; Stenzel et al. 2011) and 511 (Di Russo et al. 2017; Hallmann et al. 2005), collagen IV (Pöschl et al. 2004), fibronectin (Zhou et al. 2008; Van Obberghen-Schilling et al. 2011), and many other molecules, such as the proteoglycans perlecan (Zoeller et al. 2008; Lord et al. 2014; Douglass et al. 2015), agrin (Barber and Lieth 1997; Steiner et al. 2014), and nidogens (Bader et al. 2005). Besides collagen IV, additional collagens, such as collagens XVIII (Marneros et al. 2004), VIII (Sage and Iruela-Arispe 1990) and VI (Kuo et al. 1997; Groulx et al. 2011) have been identified to be part of the blood vessel BM. Genetic deletion or point mutations of genes encoding for these ECM components lead to severe blood vascular dysfunction (George et al. 1993; Thyboll et al. 2002; Van Obberghen-Schilling et al. 2011) and are often associated with embryonic or perinatal lethality (Costell et al. 1999; Pöschl et al. 2004; Bader et al. 2005; Coles et al. 2006). These findings support a pivotal function of BM components in blood vascular development. Additionally, it is not only important that ECM components are correctly expressed and assembled in the extracellular space, they also need to be remodeled and/or degraded to allow for functional blood vessel morphogenesis and homeostasis. This is achieved by matrix remodeling enzymes, such as matrix metalloproteinases (MMPs), reviewed in Sounni et al. (2011).

Although many studies have been performed focusing on the importance and contribution of the BM in the blood vasculature, less is known about the BM composition of the *established lymphatic vasculature*. Lymphatic vessel BM has

been shown to be composed of laminin 421 (Saito et al. 2009), collagen IV (Lutter et al. 2012), fibronectin (Ou et al. 2010; Podgrabinska et al. 2002), hyaluronan (El-Chemaly et al. 2009), and Emilin1 (Danussi et al. 2008). For example, the elastic microfibril-associated protein emilin1 is a component of the anchoring filaments in lymphatic vessels. *Emilin1* knockout mice display hyperplastic and disorganized lymphatic vessels with impaired drainage function, reduced number of anchoring filaments and dysfunctional cell-cell junctions (Danussi et al. 2008).

In contrast to the repertoire of remodeling MMPs expressed by BECs, reelin was initially considered a major lymphatic remodeling enzyme, which degrades fibronectin and laminin components (Lutter et al. 2012; Samama and Boehm 2005). However, important roles for MMPs during physiological and pathological lymphangiogenesis have also been discovered. For example, MMP14 has been shown to control lymphangiogenesis through regulation of lymphatic vessel endothelial hyaluronan receptor-1 (LYVE-1) and vascular endothelial growth factor C (VEGFC) in mice (Wong et al. 2016). MMP2 blockage affects lymphatic tube formation in cell culture spheroids and ex vivo lymphatic ring assays (Ingvarsen et al. 2013). Similarly, inhibition of MMP2 and MMP9 has been associated with lower expression of VEGFC and vascular endothelial growth factor receptor 3 (VEGFR3) followed by impaired corneal lymphangiogenesis (Du et al. 2017). Taken together, these studies highlight a more complex contribution of MMPs in lymphatic vessel growth than previously thought.

To mediate adhesion to the tissue microenvironment, ECs are associated to the ECM through so-called focal adhesions, which are comprised of ECM binding proteins known as integrins (Gordon et al. 2020; Stupack and Cheresh 2004). Integrins bind to their specific ECM ligands and are intracellularly coupled to the cytoskeleton through actin binding proteins, including the mechanosensors vinculin and talin (Bays and Demali 2017; Yan and Schwartz 2018), to sense extracellular mechanical information and transduce them into the cell (Gordon et al. 2020). For example, fibronectin, which is not only an integral component of the lymphatic BM but also of the LV matrix, binds to endothelial integrin $\alpha_9$ (Bazigou et al. 2009). EC-specific deletion of *Itgα9* or mice lacking the EIIIA domain of fibronectin show disorganized fibronectin network in the valve matrix resulting in dysplastic LV leaflets and retrograde lymph flow (Bazigou et al. 2009). Consequently, mechanosensing and mechanotransduction not only depend on ECM ligand availability, but also on the proper endothelial integrin repertoire.

Besides regulating adhesion and providing structural support for the endothelium, ECM components participate in the regulation of additional signaling pathways involved in blood vessel development and homeostasis through sequestration of chemokines or enhancement of chemokine activation. For example, VEGF signaling (Jakobsson et al. 2006; Stenzel et al. 2011; Cecchi et al. 2012), transforming growth factor β (TGFβ) signaling (Fontana et al. 2005), platelet-derived growth factor (PDGF) signaling (Lindblom et al. 2003), and angiopoietin signaling (Xu and Yu 2001; Xu et al. 2004; Chomel et al. 2009) have been identified to be regulated via the ECM. Similar to what has been shown for BECs, ECM components influence lymphatic signaling. Through ligation of integrin $\alpha_5ß_1$ and fibronectin, the

transactivation and phosphorylation of VEGFR3 are enhanced (Zhang et al. 2005). The collagen and calcium-binding EGF domain 1 (CCBE1) protein, which is expressed by mesenchymal cells close to the nascent lymphatics (Facucho-Oliveira et al. 2011), activates the cleavage of VEGFC to its active form, allowing for lymphatic sprouting (Jeltsch et al. 2014; Bos et al. 2011; Hogan et al. 2009).

Within the blood vasculature, relative amounts of ECM components have been shown to correlate with variable stiffness of the EC microenvironment. Arteries are stiffer (50–150 kPa, Kohn et al. 2015) than veins (3–50 kPa, Xue et al. 2017) because they need to withstand high blood pressure. In an ageing-related arterial stiffening mouse model, collagen I and III depositions have been shown to increase the incremental stiffness value of the arterial walls, indicating arterial stiffening in old mice (Fleenor et al. 2010).

*In the developing embryo*, collagen I is the predominant collagen type of the ECM and correlates with tissue stiffness (Majkut et al. 2013; Chen et al. 2012). Interestingly, analysis of collagen I deposition in embryonic day (E) 11 mouse embryos revealed substantial differences in collagen I support of nascent blood vascular versus lymphatic structures (Frye et al. 2018). Arteries and blood capillaries exhibited the highest collagen I density, while the cardinal vein (CV) and the migrating LECs showed moderate or low collagen I deposition, respectively (Fig. 11.1a, b). Decreasing collagen I density also correlated with decreasing local tissue stiffness experienced by ECs, suggesting that already during early vascular development the density of collagen I reflects an essential hierarchy of ECM stiffness required to fulfill different vascular functions.

However, it is important to note that the BM underlying most ECs is only a thin ECM layer (30–500 nm) (Liliensiek et al. 2009). Interestingly, cells can "feel" up to several micrometers deep into a soft substrate. Consistently, induction of EC network formation on compliant substrate (0.4 kPa) was prevented on very thin compliant substrates (<20 mm) as ECs sensed the stiffness of the underlying coverslip (Davidson et al. 2019). These findings suggest that the absolute in vivo stiffness of the ECM microenvironment experienced by ECs might rather be generated by several different surrounding tissue structures, including the ECM and, presumably, several layers of adjacent cells.

### *11.2.2 Mural Cell Support of the Endothelium*

The specific tissue microenvironment of ECs is established not only by various ECM components, discussed in the previous paragraph, but also by surrounding cells. These cells can align the endothelium and provide a passive stiffness scaffold or are closely associated and actively regulating the endothelium, like mural cells.

Mural cells can be distinguished in pericytes and SMCs mostly depending on their morphology and location, although a rigorous distinction is not always possible. Pericytes are usually solitarily associated with small caliber vessels, but their relative frequency to ECs is highly variable (between 1:100 in skeletal muscle to 1:1

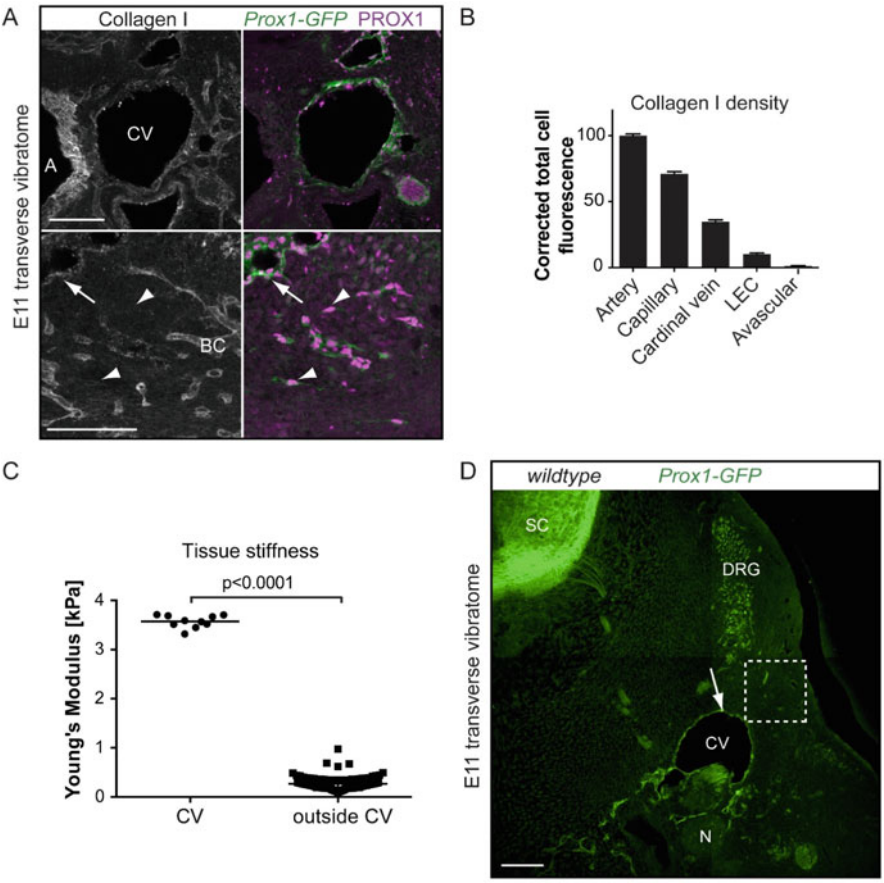


**Fig. 11.1 Changes in matrix stiffness regulate early lymphatic development**. (**a**) Immunofluorescence of transverse vibratome sections of E11 *Prox1-GFP* embryos using antibodies against PROX1 (magenta), GFP (green, Prox1 reporter), and collagen I (single channel images). Note, aorta (A) and blood capillaries (BC) show higher collagen I level compared to the CV (arrow) and migrating LECs (arrowheads). (**b**) Quantification of collagen I density in the respective vessel types and avascular tissue in E11 embryos. Data represent mean integrated density values of corrected total cell fluorescence ± s.e.m. (unpaired Student's t-test) quantified from $n = 10$ images taken from two embryos. (**c**) Ex vivo atomic force microscopy (AFM) measurements on transverse vibratome sections (image on the right) of E11 *Prox1-GFP* embryos. Young's Modulus (kPa) is a measure for the actual tissue stiffness. Horizontal lines represent mean ($n = 10$ measurements from one embryo (CV) and $n = 114$ measurements from three embryos (outside CV)). p value, unpaired Student's t-test. Measurements were done on the dorsal side of the CV (arrow) and the area of LEC migration (boxed area, outside CV). Prox1-GFP$^+$ spinal cord (SC), dorsal root ganglion (DRG), and nerve (N) were used for orientation. Scale bars: 100 μm. Modified from Frye M, et al. Nat Commun. 2018;9(1):1511 und licensed under a Creative Commons Attribution 4.0 International License (https://creativecommons.org/licenses/by/4.0/)

in the retina) (Armulik et al. 2005). Instead, SMCs can form multiple concentric layers around large caliber vessel types and can contract in order to facilitate the movement of fluids (Armulik et al. 2005; Gaengel et al. 2009). Both cell types derive from various cellular sources and through the combined action of hemodynamic forces and signaling induction, are recruited soon after the blood flow and circulation has been initiated (Shen and Mccloskey 2017). A well-studied signaling pathway is the platelet-derived growth factor subunit B (PDGF-B)/PDGF receptor (PDGFR) axis. Binding of PDGF-B, expressed by ECs, to the PDGFR, expressed on mural cells, activates mural cell proliferation and initiates their migration toward the vessels (Shen and Mccloskey 2017; Armulik et al. 2005). Global knockout mice for *Pdgfb* have been associated with microvascular aneurisms, loss of pericyte coverage, and embryonic lethality (Lindahl et al. 1997), while a more recent EC-specific knockout model exhibited increased retinal leakage in adult mice (Park et al. 2017).

In the lymphatic vasculature, mural cell recruitment starts from E 17.5 and proceeds after birth (Norrmén et al. 2009). PDGF signaling has been shown to be regulated downstream of forkhead box protein C2 (FOXC2) transcription factor (Petrova et al. 2004). Global knockout mice for *Foxc2* display upregulated *Pdgfb* expression, resulting in increased recruitment of pericyte/SMCs to the dermal lymphatic capillaries at E17.5, whereas control littermates did not show mural cell coverage of these vessels (Petrova et al. 2004). These studies highlight an essential role of mural cell recruitment to stabilize blood and lymphatic vessels during embryonic development and in vessel maintenance.

In the established vasculature, mural cell coverage and specific deposition of ECM components can distinguish different vessel subtypes by morphology and function. In the blood vasculature, capillaries are surrounded by pericytes and a continuous BM, although organ specific differences are possible; for example, in the liver sinusoids that exhibit discontinuous BM coverage (Potente and Mäkinen 2017). Large caliber vessels are instead surrounded by a continuous BM and an elastic lamina, consisting of numerous layers of SMCs, to withstand higher blood pressure (Potente and Mäkinen 2017). In the lymphatic vasculature, the evident morphological differences between initial and collecting lymphatics resemble the function of these two lymphatic vessel subtypes. Through a highly permeable barrier, which is devoid of a mural cell layer and a continuous BM, lymphatic capillaries drain fluids, macromolecules, and cells from the tissue. In contrast, collecting lymphatics transport the lymph back to the blood circulation and are therefore equipped with a continuous BM and SMCs that contract to allow the movement of the lymph (reviewed in Chen et al. 2014; Stritt et al. 2021).

Taken together, ECM composition and mural cell support can provide first hints about the local stiffness environment of ECs and their exposure to stretch. In the future, it will be necessary to analyze local in vivo stiffness experienced by ECs in more detail, to better understand the relationship between the different microenvironmental factors (ECM composition, mural cell support, and absolute tissue stiffness) and how the combined action of these factors could render ECs more susceptible to dysfunction. Particularly, the heterogeneity of the tissue

microenvironment between BECs and LECs might point toward the idea that selected mechanoregulatory processes and their threshold to malfunction may be essentially different in LECs. In the next paragraph, we will highlight recent findings on lymphatic mechanoregulation during development.

## 11.3 Mechanoregulation of Lymphatic Development

Changes of the microenvironment have been shown to fundamentally regulate cellular processes during development. For example, ECM stiffness regulates the differentiation of multipotent mesenchymal stem cells. Stiff matrices mimicking bone were found to be osteogenic, while soft matrices mimicking brain were neurogenic (Engler et al. 2006). Methylcellulose hydrogels, mimicking external constraints in the bone marrow, positively influenced megakaryocyte differentiation and proplatelet formation (Aguilar et al. 2016). Besides regulating cell fate and behavior at the single cell level, ECM stiffness can regulate tissue morphogenesis (Majkut et al. 2013; Poh et al. 2014).

Interestingly, fate decision of endothelial lineages is also dependent on the mechanical environment of the stem cell niche of vascular progenitor cells, as endothelial lineages prefer softer substrates (10 kilopascal (kPa)) and SMC lineages stiffer substrates (plastic, gigapascal (GPa) range) (Wong et al. 2019). Differences in ECM stiffness experienced by endothelial progenitor cells (EPCs) have been furthermore suggested to regulate arterial-venous differentiation in vitro (Xue et al. 2017). In contrast to EPCs that have been cultured on venous substrate stiffness (7 kPa), EPCs cultured on arterial substrate stiffness (128 kPa) showed an increase in expression of the arterial marker EphrinB2 (Zhang et al. 2005; Xue et al. 2017).

The *development of the mammalian lymphatic system* is initiated in the CV through trans-differentiation of a subpopulation of venous ECs around E9.5. These lymphatic endothelial progenitors express the transcription factor Prospero homeobox protein 1 (PROX1) (Yang et al. 2012; Wigle and Oliver 1999), which is activated through the SRY-Box transcription factor 18 (SOX18) (François et al. 2008; Yang et al. 2012; Srinivasan et al. 2007) and nuclear receptor subfamily 2 (NR2F2, also known as COUP-TFII) (Srinivasan et al. 2010).

Around E10.5, $PROX1^+$ LEC progenitors, expressing VEGFR3, start to delaminate from the CV and intersomitic vessels and migrate dorsolaterally toward a gradient of VEGFC to form the first lymphatic structures, the dorsal peripheral longitudinal lymphatic vessel (PLLV), and the ventral primordial thoracic duct (pTD) (commonly referred to as jugular lymph sacs (JLS)) (Yang et al. 2012; François et al. 2012; Hägerling et al. 2013).

The earliest evidence of lymphatic mechanoregulation via the tissue microenvironment has been demonstrated around E11, when lymphatic endothelial progenitors experience a decrease in ECM stiffness upon delamination from the CV (0.2 kPa outside the CV versus 4 kPa inside the CV) (Fig. 11.1c, d (Frye et al. 2018)). This decrease in matrix stiffness induces a GATA2-dependent transcriptional program,

which is required to form the first lymphatic vessels. Transcriptome analysis showed that LECs grown on a soft matrix (0.2 kPa vs. 25 kPa) exhibit increased GATA2 expression and a GATA2-dependent upregulation of genes involved in cell migration and lymphangiogenesis, including VEGFR3. Analysis of endothelial-specific *Gata2* deletion in mice demonstrated a cell-autonomous function of GATA2 in regulating LEC responsiveness to VEGFC, thereby controlling LEC migration and sprouting (Fig. 11.2). The study further compared the mechanosensitive transcriptional programs activated in LECs in response to different mechanical stimuli, such as increased matrix stiffness and oscillatory flow, and revealed that they appear remarkably different (Frye et al. 2018). Additionally, in contrast to increased GATA2 expression in LECs upon exposure to a soft matrix, GATA2 expression in BECs has been reported to increase in response to increased mechanical stimulus, such as matrix stiffening and induction of oscillatory flow (Mammoto et al. 2009; Kazenwadel et al. 2015). It has been described in several cell types, including ECs, that GATA2 interacts with other transcriptional regulators, including Etv2 (Shi et al. 2014) and Lmo2 (Coma et al. 2013), to form multimeric transcription complexes. An interesting question would be whether oscillatory flow- and soft matrix-induced differences in GATA2-mediated regulation of target genes and cellular responses can be explained by formation of different transcriptional complexes in different EC types.

In parallel to increased responsiveness to VEGFC that ensures efficient dorsolateral migration, proliferation is downregulated in LECs that experience a softer microenvironment. For example, when LECs were cultured on softer 2D substrates (0.2 kPa vs. 25 kPa), these cells also show a reduced proliferation parallel to an induction of VEGFR3 expression (Frye et al. 2018). Similarly, proliferation of BECs is reduced on soft 2D substrates but induced on rigid 2D substrates. Subconfluent human umbilical vein ECs (HUVECs) which were cultured on stiffer substrates (10 kPa vs. 1 kPa) increase vascular endothelial growth factor receptor 2 (VEGFR2) internalization and thus VEGFR2 activation (Lavalley et al. 2017). This phenomenon of reciprocal control of cell proliferation and migration has been described for other cell types (De Donatis et al. 2010). In the context of lymphatic development, it could be speculated that LECs are firstly subjected to an activation of their motility to populate the area of the JLS and secondly, once they have arrested migration, they induce a strong proliferative response to successfully expand the JLS.

A strong induction of proliferation indeed is achieved from E11.5 onward during lymphatic development, once JLS have formed and vessel expansion is induced (Planas-PAZ et al. 2012). Initially, an increase in interstitial pressure between E11.5 and E12 results in swelling of the interstitium, which leads to a stretching of the tissue and the ECM surrounding the JLS (Planas-PAZ et al. 2012), demonstrating again the importance of lymphatic mechanoregulation via the abluminal tissue microenvironment (Fig. 11.2). Stretching of LECs is evident by an elongation of the LECs between E11.5 and E12 and results in activation of VEGFR3 signaling and a transient strong increase in LEC proliferation, which was shown to depend on $\beta_1$ integrins (Planas-PAZ et al. 2012). Conversely, when interstitial fluid pressure decreases between E12.0 and E12.5, possibly due to efficient fluid drainage via

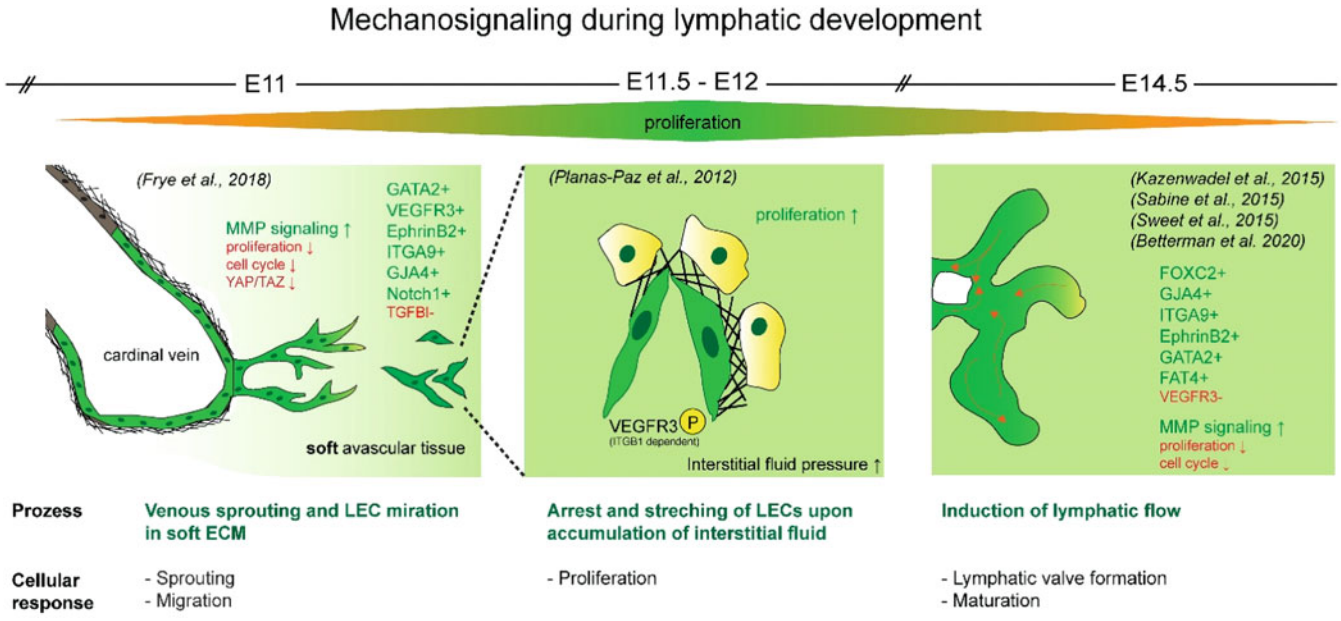


**Fig. 11.2 Mechanosignaling during lymphatic development.** Schematic of mechanoregulatory processes during lymphatic development. Initially, venous-derived LECs migrate out of the CV and are exposed to soft matrix, which induces GATA2-dependent increase in VEGFR3 expression and LEC migration. Next, LECs arrest and initiate proliferation in response to increased stretch, which is caused by an increase in interstitial fluid pressure. Last, initiation of lymphatic flow is necessary to induce lymphatic valve formation and maturation. Top regulated genes and signaling pathways (red, up; green, down) are indicated

the expanded lymphatic vasculature, the proliferation rate of LECs declines. In contrast to BECs, normal elongation of LECs in vitro is around 4%, whereas an increased elongation of 8% already leads to transcriptional induction of inflammatory cytokines, fibrotic markers, and lymphangiogenesis (Wang et al. 2017a). This could suggest that similar to EC type specific FSS set points (Baeyens et al. 2015), LECs have a lower stretch set point.

An indirect contribution of the immediate lymphatic microenvironment has also been identified during early lymphatic development. The CCBE1 protein is expressed by mesenchymal cells close to the nascent lymphatics (Facucho-Oliveira et al. 2011). CCBE1 does not have lymphangiogenic activity on its own, however it has been shown to activate a disintegrin and metalloproteinase with thrombospondin motifs 3 (ADAMTS3), which in turn cleaves VEGFC to its active form to allow for a proper VEGFC gradient and lymphatic sprouting, both in mouse (Jeltsch et al. 2014; Bos et al. 2011) and in zebrafish (Hogan et al. 2009; Wang et al. 2020).

Upon vessel expansion around E13.5, LECs of the pTD and the PLLV start to sprout toward the viscera and the superficial lymphatic plexus, respectively (Jafree et al. 2021). At the same time, non-venous LECs arise and start to coalesce to contribute to lymphatic vessel formation. In addition to the classical venous origin, it has been shown that LECs can also derive from other cell types, such as dermal blood capillary plexus (Pichol-Thievend et al. 2018), hemogenic endothelium (Stanczuk et al. 2015; Klotz et al. 2015), non-endothelial second heart field (Maruyama et al. 2019; Lioux et al. 2020), and paraxial mesoderm (Stone and Stainier 2019). These LECs appear as independent cell clusters that later fuse and incorporate into the nearby forming lymphatic vessels. This process is referred to as lymphvasculogenesis due to its similarity to the vasculogenesis process, where BECs differentiate from single precursors and then coalesce (Martinez-Corral et al. 2015). It seems likely that these additional LEC sources initially experience a different tissue microenvironment compared to the CV microenvironment with moderate stiffness (4 kPa). However, the potential impact of a differential microenvironment on non-venous derived LEC function has not been addressed yet and would be interesting to investigate in future studies.

Approximately at E14.5, an additional mechanical stimulus, the lymph FSS, is exerted on the LECs of the nascent lymphatic network. The onset of the intraluminal lymphatic flow has been shown to fundamentally contribute to the development of LVs (Fig. 11.2). LVs are predominantly formed at lymphatic branches, where the laminar flow pattern of the lymph is disturbed. At these valve initiation sites, oscillatory flow upregulates GATA2 that in turn maintains high PROX1 expression and transcriptionally activates FOXC2 transcription factor (Kazenwadel et al. 2015; Sabine et al. 2012). This transcriptional change activates the calcineurin/NFATC1 axis, connexin 37, and integrin α9 expression leading to valvulogenesis (Danussi et al. 2013; Sabine et al. 2012; Bazigou et al. 2009; Norrmén et al. 2009). Recently, an additional target of GATA2, the atypical cadherin FAT4, has been identified to control LEC polarity in response to flow and is required for lymphatic vessel morphogenesis, including valve formation (Betterman et al. 2020). By using mice that are deficient for the platelet-specific receptor C-type lectin-like receptor

2 (CLEC2), a complete block of lymphatic flow can be achieved as blood backfills the lymphatic network (Sweet et al. 2015). Similar to *Foxc2*-deficient vessels, *Clec2*-deficient lymphatic vessels are premature and excessive and fail to initiate valvulogenesis. Expression of PROX1, FOXC2, and VEGFR3 remains high in LECs of the mature valve but is downregulated in the mature lymphatic collecting vessels after LV formation (Norrmén et al. 2009). Presumably, disturbed flow patterns downstream of the LV maintain those expression patterns.

During postnatal lymphatic development, through secretion of PDGF-B, SMCs are recruited to lymphatic collecting vessels but not lymphatic capillaries (Sabine et al. 2012; Wang et al. 2017b). Another important phenomenon of collecting vessel maturation is the reduction of their diameter starting at E16.5 throughout postnatal stages (Norrmén et al. 2009). However, a potential direct impact of mechanical forces, such as matrix stiffening induced by mural cells or LEC constriction via the tissue microenvironment, has not been studied yet.

As development proceeds, collecting lymphatics and initial lymphatics remodel and mature. At the beginning of lymphatic development, all LECs are connected to each other through continuous zipper-like junctions (Yao et al. 2012). Remodeling of lymphatic capillary junctions to discontinuous button-like junctions increased from only 6% at E17.5 to 35% at birth, 50% at postnatal day (P)7 and 90% at P28 (Yao et al. 2012). This may be caused by mechanical forces generated through transmural lymph flow, which has been shown to induce delocalization and downregulation of vascular endothelial cadherin (VE-cadherin) and PECAM-1 in in vitro experiments (Miteva et al. 2010).

In addition to the mouse model system, *lymphatic development* has been extensively studied in *zebrafish*. Similar to mammals, in zebrafish LECs are distinguished from the rest of the endothelium by PROX1 expression and lymphangiogenesis is highly dependent on VEGFC/VEGFR3 signaling (Yaniv et al. 2006; Van Impel et al. 2014; Dunworth et al. 2014; Küchler et al. 2006; Le Guen et al. 2014; Shin et al. 2016). However, in contrast to mouse lymphatic development, a bipotent progenitor cell division underlies LEC specification in the CV of the zebrafish trunk (Koltowska et al. 2015; Nicenboim et al. 2015). From around 36 hours post-fertilization (hpf), sprouting of LEC progenitors and venous ECs occurs simultaneously, in a process called secondary sprouting (Yaniv et al. 2006; Küchler et al. 2006). About half of the sprouts form venous intersegmental vessels, while the remaining become parachordal lymphatic progenitors (PLs) in the horizontal myoseptum at around 52 hpf (Hogan et al. 2009). Subsequently, the PLs migrate ventrally and dorsally from the myoseptum to form the thoracic duct and the dorsal longitudinal lymphatic vessel, respectively (Yaniv et al. 2006). The network is then completed with the connection of these two main trunk lymphatic vessels through lymphatic intersegmental vessels. As in mouse, other non-venous sources of LECs have been discovered in zebrafish, such as the ventral aorta lymphangioblasts that contribute to facial lymphatics (Eng et al. 2019).

Formation of the proper lymphatic vascular network is highly dependent on the precise regulation of cell proliferation, which is achieved by the mitogenic VEGFC/VEGFR3/ERK signaling. Downstream of VEGFC/VEGFR3/ERK signaling, the

RNA-helicase DDX21 ensures for proper RNA biogenesis and cell cycle progression (Koltowska et al. 2021). Furthermore, similar to the process of LEC progenitor delamination from the CV in mice, during secondary sprouting in zebrafish proliferation is decreased (Jerafi-Vider et al. 2021). The decrease in proliferation was shown to be regulated via a VEGFC/VEGFR3/ERK-controlled cell cycle arrest. However, if the LEC microenvironment also guides cell cycle dynamics in zebrafish remains to be uncovered.

The microenvironment also plays an important role to guide LEC migration in zebrafish. Multiple cellular sources have been shown to guide migrating LECs and secrete guiding cues, such as chemokines and growth factors; these include intersegmental arteries, neurons, and fibroblasts (Bussmann and Raz 2015; Cha et al. 2012; Wang et al. 2020). In addition, LECs migrate alongside notochord sheath cells, which have been found to secrete localized type II collagen (Col2α1) to support PL migration through cell-ECM guidance (Chaudhury et al. 2020). Defects in Col2α1 secretion result in impaired migration of PLs. Similarly, the ECM protein polydom (also called Svep1), a ligand for integrin $\alpha_9\beta_1$, is expressed by mesenchymal cells in intimate proximity of remodeling venous ECs and LECs in zebrafish and mice (Morooka et al. 2017; Karpanen et al. 2017). Zebrafish *polydom/svep1* mutants exhibit a decrease in secondary sprouting, which leads to an increased number of intersegmental arteries. Consequently, a reduced number of PLs in horizontal myoseptum fails to migrate dorsally or ventrally and from the TD (Karpanen et al. 2017). These studies underline the importance to investigate the microenvironment of the developing lymphatic system. Whether, in addition to the local presence of selected ECM proteins, specific mechanical forces, such as matrix stiffness or stretch, are also involved in zebrafish, remains to be studied.

Mechanosensing of FSS-induced extracellular mechanical information has been extensively studied. FSS-induced mechanosensory mechanisms that regulate gene expression and cellular function include the regulation of ion channels and endothelial junctional protein complexes (Bálint and Jakus 2021). For example, the Piezo-type mechanosensitive ion channel component 1 (PIEZO1) has been identified to mediate mechanotransduction in the development and maintenance of the LVs (Nonomura et al. 2018; Choi et al. 2019). Furthermore, the calcium release-activated calcium modulator 1 (ORAI1), a pore subunit of the calcium release-activated calcium (CRAC) channel, is activated upon FSS and mediates $Ca^{2+}$-influx in LECs (Choi et al. 2017b) and induces Kruppel like factor 2 (KLF2) and KLF4 upregulation in LECs to promote VEGFC expression (Choi et al. 2017a).

Once extracellular mechanical information of the tissue microenvironment has been sensed through integrins and the FA complexes in BECs, the intracellular signals are propagated to the actin cytoskeleton. For example, with increasing matrix stiffness (3 kPa, 12 kPa, and 1.5 MegoPa), actin cytoskeleton remodeling becomes more organized, with an increasing amount of actin stress fibers (Jannatbabaei et al. 2019). The actin cytoskeleton is connected to VE-cadherin via its intracellularly associated proteins β- and α-catenin (Wessel et al. 2014). Tension or strain-induced actin remodeling tightly controls assembly and disassembly of VE-cadherin-based junctions (Oldenburg and De Rooij 2014). Additionally, cytoskeletal pulling at the

VE-cadherin complex also recruits the tension sensor protein vinculin via α-catenin to reinforce endothelial junctions (Huveneers et al. 2012; Daneshjou et al. 2015).

In contrast to BECs, our knowledge of stiffness-induced mechanosensory mechanisms in LECs is limited. Besides a direct effect on endothelial junction stability, cytoskeletal mechanotransduction can result in structural modification of membrane-bound or cytoplasmic proteins and their subsequent shuttling to the nucleus. For example, an important class of nuclear shuttling proteins consists of Yes-associated protein (YAP) and WW Domain-Containing Transcription Regulator Protein 1 (WWTR1/TAZ), which are downstream effectors of the Hippo pathway (Zhong et al. 2018). YAP/TAZ are shuttled to the nucleus in lymphatic ECs grown on stiff substrates (25 kPa vs. 0.2 kPa) and induce their target genes connective tissue growth factor *(CTGF)* and ankyrin repeat domain 1 (*ANKRD1)* (Frye et al. 2018). YAP/TAZ function has been extensively studied in the development of the blood (Neto et al. 2018; Sivaraj et al. 2020) and lymphatic vasculature (Cho et al. 2019; Cha et al. 2020; Grimm et al. 2019). Precisely how alterations in ECM stiffness might regulate those processes in LECs remains to be investigated.

Taken together, lymphatic development is guided via luminal (e.g. FSS) and abluminal mechanical forces, such as ECM composition, mural cell support, tissue stiffness and stretch capacity. Besides FSS-induced mechanotransduction, it is now highly relevant to investigate stiffness- and stretch-modulated LEC signaling pathways (including the identification of specific sensors and transducers), not only during lymphatic development but also during the maintenance of the established lymphatic system, as those pathways are likely to be dysregulated in a variety of disease conditions.

## 11.4 Mechano-Dysregulation of Lymphatic Endothelial Cells in Disease

Tissue remodeling and growth require ECM remodeling; however, aberrant ECM alterations have been associated with a variety of diseases, e.g. central nervous system (CNS) injury (Gaudet and Popovich 2014), tumor development and metastasis (Girigoswami et al. 2021; Nicolas-Boluda et al. 2021), lymphedema (Kistenev et al. 2019) or inflammatory bowel disease (Petrey and De La Motte 2017; Gordon et al. 2014). Uncontrolled remodeling of the ECM may lead to either an excessive degradation of ECM (Zhen and Cao 2014) or abnormal deposition and ECM stiffening (Frantz et al. 2010).

Comprehensive analyses of local stiffness changes experienced by LECs in diseased tissues have not been addressed. The temporal sequence of stiffness changes and LEC dysfunction and their interplay during disease progression are not understood. Here, we review selected diseases that are associated with lymphatic dysfunction and aim to point out possible hints that matrix alterations and

modulation of ECM stiffness do not only correlate with lymphatic dysfunction but might mutually define each other.

### *11.4.1 Lymphedema*

Lymphedema is a chronic disease that can occur anywhere in the body, including extremities, face, thorax, and different body cavities. Lymphedema can be inherited (primary lymphedema), affecting 1 in 100,000 Americans (Smeltzer et al. 1985; Sleigh and Manna 2021), or caused by obstruction and injury of the lymphatic system (secondary lymphedema). Secondary lymphedema has a much higher incidence of 1 in 1000 people in developed countries, mainly due to malignant cancer treatment through lymph node (LN) dissection (Azhar et al. 2020). However, it is very likely that the incidence is underreported, especially in lower income countries (Torgbenu et al. 2020).

Several mutations in human genes have been identified to cause primary lymphedema, including *GATA2, SOX18, FOXC2, FLT4, PTPN14, PIEZO1* and *ITGA9*, affecting mainly LEC specification and lymphatic development (Oliver et al. 2020). For example, mutations in *FOXC2* cause Lymphedema-distichiasis (LD) syndrome with lymphatic vessels appearing normal but showing impaired lymphatic drainage due to valve dysfunction (Brice et al. 2002, Petrova et al. 2004, Brice et al. 2002). *Foxc2*-deficient mouse embryos and LD patients also display an ectopic mural cell and BM coverage (Petrova et al. 2004). Mutations in *FLT4* cause congenital bilateral lower limb lymphedema (Nonne-Milroy disease Karkkainen et al. 2004, Gordon et al. 2013) and loss-of-function mutations in *GATA2* lead to impaired development and maintenance of lymphovenous and lymphatic valves (Emberger Syndrome, Ostergaard et al. 2011, Kazenwadel et al. 2012).

Secondary lymphedema can additionally be caused by infection with the nematode *Wuchereria bancrofti*. The adult worm can obstruct lymphatic vessels and lymphatic transport, when lodging in the lymphatic system, and triggers inflammatory responses of the host (reviewed in Bennuru and Nutman 2009), which causes more than 60 million patients with lymphatic filariasis worldwide and about 25% of those patients suffering from lymphedema (Ramaiah and Ottesen 2014). A high prevalence of secondary lymphedema in the USA is also related to malignancy and tumor therapy. It has been described to accompany treatment routines of lymphoma, melanoma, urologic cancers and receives special attention for occurring after surgical and radiation therapy for breast cancer in women. The incidence of lymphedema after mastectomy ranges between 24% and 49%. Other studies report 4% to 28%, probably due to different measurement techniques and criteria (reviewed in Warren et al. 2007, Ly et al. 2017).

Due to ongoing fibrotic processes, it is a commonly accepted idea that lymphedema is associated with tissue stiffening, although lymphatic-proximal stiffness analysis has not been performed. Thus, it remains unclear how changes in ECM stiffness may affect LEC function and disease progression.

To study lymphedema in mice, several experimental lymphedema models, such as the mouse tail surgery model, the popliteal LN dissection and the related axillary LN dissection model, or an inducible transgenic lymphatic ablation model have been described (Ly et al. 2017). The mouse tail surgery model is the most commonly used lymphedema model. After carefully removing the 3–5 mm skin, the superficial and deep lymphatic vessels are ligated, while blood vessels are left intact. The resulting inflammation, lymphatic fluid stasis and vessel dilation, fibrosis and adipose deposition are mimicking the human post-surgical lymphedema. The observed edema increased interstitial pressure and fibrosis formation suggests stiffening of the tissue (Kashiwagi et al. 2011). Recently an advanced tail surgery model has been developed. One lymphatic collector is maintained intact, allowing the study of functional changes during disease progression in the intact vessel, while the common lymphedema phenotype can be observed in the disrupted lymphatic collector (Weiler et al. 2019).

In lymphedema, mainly collagen fibers are excessively deposited in both dermis and subcutaneous tissue (Gardenier et al. 2016). However, not much is known about the spatial organization of the collagen fiber network, although work by Wu et al. showed progressively less compacted and disorganized collagen, due to excessive fluid separating the collagen in the mouse tail model (Wu et al. 2011). Similarly, the development of limb lymphedema in patients with lymphadenectomy is convoyed by a thickening of the BM and an increase in collagen fibers (Mihara et al. 2012). The collagen increase was also confirmed in patients with stage II lymphedema by visualization of collagen fibers using second harmonic generation (Kistenev et al. 2019). More recently, a transgenic mouse model has been developed: a tamoxifen-inducible Cre-loxP system that expresses the human diphtheria toxin (DT) receptor under the control of the LEC-specific *Flt4* promoter. Injection of DT into any of the limbs results in a local ablation of lymphatics (Gardenier et al. 2016), resulting in a histological representation of the human disease by displaying comparable radiographical and clinical symptoms, including progressive dermal fibrosis and deposition of subcutaneous fibroadipose tissue. The latter has also been reported in the mouse tail surgery model, with an increase in fat thickness and subcutaneous fat deposition (Aschen et al. 2012). This is in agreement with several patient studies reporting that tissue swelling in lymphedema, can be caused through fluid stasis and fat deposition; for example, in lymphedemic limbs of breast cancer patients (Schaverien et al. 2018; Azhar et al. 2020).

Changes in ECM deposition are accompanied by another classic hallmark of lymphedema: chronic inflammation of the dermis and its underlying tissue. Lymphatic fluid stasis leads to an inflammatory response triggered by $CD4^+$ T cell infiltration into the surrounding tissue. Almost 70% of all inflammatory cells in lymphedema are $CD4^+$ T cells and their infiltration positively correlates with disease severity (Dayan et al. 2018; Ly et al. 2017). Interestingly, mice lacking all types of T cells or the $CD4^+$ subpopulation T cells fail to develop lymphedema in the tail surgery model. The infiltrating $CD4^+$ cells show a bias toward a T helper (Th) 2 response (Wynn 2008). Th2 cells are the main drivers of the inflammatory response by secreting pro-inflammatory/pro-fibrotic cytokines IL4 and IL13. Together with

upregulation of TGFβ1 signaling, this leads to fibrosis formation, which ultimately impairs lymphatic function (Dayan et al. 2018; Ly et al. 2017). Additionally, T cell-derived cytokines IL4, IL13, interferon gamma and TGFβ1 directly exhibit anti-lymphangiogenic function by decreasing LEC proliferation, migration, survival and responsiveness to VEGFC in human dermal LECs and in a mouse model of suture-induced corneal neovascularization (Savetsky et al. 2015). Besides $CD4^+$ T cells, other immune cells are also involved. Depletion of macrophages, for instance, led to increased $CD4^+$ T cell infiltration and Th2 differentiation and thus causing similar phenotypes with increased fibrosis and impaired lymphatic functions (Ghanta et al. 2015).

It can be speculated that, like chronic inflammation, severe changes in ECM deposition and stiffness are likely to directly contribute to lymphatic dysfunction and dysfunctional lymphangiogenesis or even prevent lymphatic re-growth in lymphedema. In agreement with this, in vitro experiments using human LECs demonstrate that decreasing matrix stiffness primes lymphatic tube formation, while increasing matrix prevented it (Alderfer et al. 2021). In human patients, stiffening of lymphedema tissue is seldomly assessed in a quantitative and objective way. The subjective view of the patients and medical staff (referring to tightening and stiffness) is the most common assessment (see, for example, Pekyavaş et al. 2014). The most common method for evaluating lymphedema severity is the circumference of the limb or volume determination, despite the knowledge that for some patients the affected limb softens due to treatment, but the circumference is not altered. Softening would indicate an improvement of disease (discussed and reviewed in Dayan et al. 2018, Hara and Mihara 2018). Additionally, macroscale indentation techniques, such as elastography and tonometry, have been employed to study tissue stiffness in lymphedema (Nowak and Kaczmarek 2018; Hara and Mihara 2018), but proper local stiffness values or parameters of early lymphedema stages are lacking. Although several studies try to identify risk parameters for lymphedema formation, like body mass index, age, and therapy approach, for example in women who underwent mastectomy for breast cancer treatment (Basta et al. 2017), it remains understudied whether stiffness increase, sensed by LECs, may serve as an early indicator of disease formation prior to clinical symptom development.

For secondary lymphedema, a potential LEC mechano-dysregulation has been proposed: due to surgical removal of a cancerous lymph node, the lymph flow between afferent (upstream) and efferent regions (downstream of the truncated LN) is disrupted. The lymph transport from the afferent region is abolished and leads to lymph accumulation in the lymphatic vessel, causing vessel dilation and a reduction in lymph drainage. It has been hypothesized that, similarly to the embryonic development, an increase in intestinal fluid and the swelling of the ECM is sensed by the LECs, resulting in an increase in $\beta_1$ integrin signaling, phosphorylation of VEGFR3, and elevated LEC proliferation with hyperplasia and further dilation of the lymphatic vessel (Planas-Paz and Lammert 2014).

Functional lymphatic drainage requires correctly organized LEC cell–cell junctions. It has been widely reported that LEC junctions are altered during inflammation. Mature button-like junctions can reversibly transform to zipper-like junctions in

inflammation (Yao et al. 2010). Zipper-like junctions reduce permeability and fluid uptake from the interstitial space is limited. This may be accompanied by reduced lymphatic flow (Zhou et al. 2010; Huggenberger et al. 2010; Cromer et al. 2015), although an initial increased flow has been reported in acute inflammation (Zhou et al. 2010). Both infections and inflammation are implicated in the development of secondary lymphedema (Yuan et al. 2019). Although little is known about how exactly the lymphatic system is altered in structure and function in lymphedema patients, Zhang et al. propose that LEC junction zippering may play a role in fluid retention and tissue swelling (Zhang et al. 2020). The authors suggest that the promotion of button formation could improve lymphatic drainage and subsequently reduce lymphedema. How changes of the tissue microenvironment could contribute to junctional alterations and how this might be addressed therapeutically needs to be further explored.

To date, lymphedema treatment is mainly limited to conservative therapies, such as manual drainage through physiotherapy and compression garments. Alternative treatments offer low-level laser therapy, stem cell therapy, and VEGFC treatment (Dayan et al. 2018; Oliver et al. 2020). However, clinical studies revealed that targeting these key regulators may increase the risk of metastasis and tumor recurrence in cancer patients (Skobe et al. 2001; Baker et al. 2010; Dayan et al. 2018).

Conclusively, identification and characterization of alternative lymphatic signaling pathways that are regulated via the tissue microenvironment could offer new possibilities to modulate and normalize lymphatic behavior in lymphedema disease conditions. At the same time, a comprehensive pre-symptomatic and symptomatic determination of ECM alterations in patients susceptible to lymphedema, such as cancer patients, could increase prevention of lymphedema development or improve treatment strategies.

### *11.4.2 Inflammatory Bowel Disease*

Inflammatory bowel disease (IBD) is an umbrella term for multifactorial disorders of the digestive tract, leading to inflammation. Development and course of these diseases are not only determined via genetic susceptibility and immune dysregulation, but also via the microbial flora and environmental factors (reviewed in Lee and Chang 2021). Crohn's Disease (CD) and ulcerative colitis (UC) are two frequently observed types of IBD. While CD can potentially affect any area of the gastrointestinal tract and frequently causes transmural inflammation (all layers of the intestinal mucosa), UC predominantly affects the colon, and patients present with superficial ulcerations of the intestinal mucosa and submucosa (Eichele and Kharbanda 2017).

To study IBD-like diseases in mice, several mouse models have been developed. Genetically induced IBD can be observed in IL10 deficient mice that spontaneously develop colitis (Schwager and Detmar 2019), probably due to lack of the anti-inflammatory properties of IL10 (Kühn et al. 1993; Spencer et al. 2002), and in

TNFΔARE mice that develop ileitis as a consequence of deleted tumor necrosis factor (TNF) AU-rich elements (ARE) and dysregulated TNF biosynthesis (Kontoyiannis et al. 1999; Rehal and Von Der Weid 2017). Furthermore, murine IBD can be induced by administration of dextran sulfate sodium (DSS), at a concentration of 1–5% in the drinking water (Okayasu et al. 1990). DSS administration subsequently leads to a damage of the intestinal epithelium, thus compromising its barrier function so that luminal bacteria and associated antigens can enter the underlying tissue and release pro-inflammatory factors (Perše and Cerar 2012; Wirtz et al. 2007; Okayasu et al. 1990; Kiesler et al. 2015). If given for seven days, DSS induces an acute inflammation from which animals can recover, while for a chronic disease development, repeated administration cycles are required (Okayasu et al. 1990).

Although most studies have been conducted to investigate the contribution of immune cells, the breakdown of the epithelial barrier (Chidlow et al. 2007; Wei et al. 2020; Stürzl et al. 2021), or the role of the blood vasculature and VEGFA in IBD (Chidlow et al. 2011; Scaldaferri et al. 2009), it is important to note that the disease is also characterized by lymphatic vessel dilation, dysfunctional lymphangiogenesis and increased mesenteric lymphatic vessel leakage (Rehal et al. 2017), phenotypes also described for lymphedema.

Consistently, lymphatic vessel density is increased along with VEGFC expression in inflamed colons of IBD patients (D'Alessio et al. 2014). Furthermore, the study showed that in two murine disease models (DSS and IL10 deficient mice) systemic delivery of VEGFC can reduce disease severity by inducting proliferation to increase lymphatic vessel density. Consequently, lymphatic drainage was partly rescued with immune cells being mobilized from the inflamed intestine to the draining LNs. Together these findings suggest that functional lymphangiogenesis might be an important process for the resolution of intestinal inflammation (D'Alessio et al. 2014). In contrast to that, although DSS concentrations and animal age varied, another study showed that overexpression of VEGFC in older DSS treated mice led to a significant increase in clinical disease index, inflammatory edema, increased lymphatic vessel density and size, suggesting that at different IBD stages, lymphangiogenic processes may have pleiotropic effects (Wang et al. 2016). Similarly, dilated and leaking lymphatic vessels were observed in the ileal mucosa of TNFΔARE mice (Rehal et al. 2017).

BEC-derived MMPs have been shown to play fundamental roles in IBD (O'Shea and Smith 2014). Absence of the blood endothelial derived protease MT1-MMP from ECs impedes colitis progression, which is accompanied by limited deterioration of vascular perfusions and retained well-structured collagen fibers surrounding the colonic crypts (Esteban et al. 2020). Similarly, MT1-MMP expression and activity is elevated in ECs grown on stiffer glycan cross-linked ribose-collagen I substrates (0.5 kPa) compared to softer, more compliant collagen substrates (0.18 kPa) (Bordeleau et al. 2017). The angiogenic sprouting of EC spheroids was reduced in stiffer compared to the softer matrices. It can be speculated that similar signaling pathways might be involved in lymphatic dysregulation in IBD. This

would be in line with observations by D'Alessio et al. (2014) that lower density of lymphatic vessels might be linked to an increased risk of the recurrence of CD.

The lymphatic abnormalities in IBD are accompanied by changes in the ECM, which affect the tissue stiffness (as reported for lymphedema). A study in UC patients measuring the colonic tissue stiffness using a microelastomer, revealed a sixfold stiffness increase of unfixed UC strictures (16.7 kPa) in comparison to unaffected margins of the resected bowel (Johnson et al. 2013). The latter showed no difference compared to healthy intestine with 2.6 kPa and 2.9 kPa, respectively. Using a multi-scale indentation system, another study in seven CD and three UC patients conformingly observed an increase of steady-state modulus in inflamed tissue compared to unaffected areas, both for the colon (0.698 ± 0.463 kPa vs. 1.143 ± 0.488 kPa) and for the ileum (0.641 ± 0.342 kPa vs. 0.991 ± 0.379 kPa) (Stewart et al. 2018). Furthermore, they report an increase in *COL1A1* and *MMP-1* but no alterations of collagen IV and fibronectin content in inflamed tissue compared to unaffected areas. Notably, the study also highlights that colon stiffness (measured as effective total modulus) observed in the mouse, is two-fold higher than in unaffected human biopsies (8.493 ± 5.365 kPa versus 3.985 ± 2.656 kPa, respectively).

Interestingly, a softening of diseased colon tissue was observed in acute DSS at day (d)10 (6 kPa) and in ill IL10 deficient mice (3 kPa) compared to healthy wild-type mice, pre-symptomatic DSS at d4, and healthy IL10 deficient animals (11 kPa) (Shimshoni et al. 2021). This study employed AFM-based microscale stiffness analysis to determine local stiffness values. A softening of diseased colon tissue was further supported by deterioration of collagen, with regional ECM degradation and deposition, as well as a heterogenous ultrastructure (Shimshoni et al. 2021). For the first time, the authors further identify collagen XVIII and fibrillin 1 as biomarkers for a pre-symptomatic state, which still is void of clinical symptoms like body weight loss and endoscopic or histological phenotypes.

More in-depth research is needed to elucidate whether pathological ECM stiffness changes are fundamentally different in murine IBD-like disease compared to human IBD. Macroscale techniques like elastography (Maksuti et al. 2016) or tonography (Nowak and Kaczmarek 2018) to study tissue stiffness in lymphedemic and inflamed tissue analyze the general tissue stiffness or the entire vessel structure. However, they do not take into account cell-scale differences within the tissue, like changes of the EC-proximal tissue microenvironment. Microscale techniques like AFM (Frye et al. 2018) or 4D displacement microscopy (Vaeyens et al. 2020) could be better suited to answer these questions.

### *11.4.3 Tumor Microenvironment and Tumor Metastasis*

Besides lymphedema and IBD, primary tumor development and tumor metastasis are known as lymphatic-associated processes (Oliver et al. 2020). The tumor tissue microenvironment is highly complex. Not only is it composed of different, often

dysregulated, cell types, such as ECs, fibroblasts, pericytes, or immune cells (reviewed in Labani-Motlagh et al. 2020), but the tumor microenvironment is often characterized by ECM stiffening (Trédan et al. 2007). For example, cancer cells can regulate collagen synthesis and in turn collagen may alter cancer cell behavior through integrin signaling (Levental et al. 2009). This observation is however not limited to collagen, but can be extended to fibronectin, laminin, and other ECM proteins (Baghban et al. 2020).

Solid tumors often induce the expansion of the surrounding lymphatic network, with matured lymphatic vessels being restricted to the tumor margin. As a consequence, the intestinal fluid accumulates in the tumor leading to an increase in interstitial pressure (Padera et al. 2016). Moreover, tumors are characterized by a remodeled ECM, including but not limited to collagen deposition and cross-linking (Northey et al. 2017) and stiffening, which strongly correlated with cancer progression and metastasis (reviewed in Emon et al. 2018).

For example, Wei and colleagues showed a positive correlation between an increase in collagen IV expression and human colorectal cancer progression (Wei et al. 2017). Another study, using HUVECs and bovine aortic ECs in spheroid assays showed that tumor angiogenesis (outgrowth, invasion, and vessel branching) is connected to an increased collagen I matrix cross-linking, which is linked to increased matrix stiffness, as assessed by measuring equilibrium compressive modulus. They were able to show that MT1-MMP activity is upregulated in stiffer collagen I matrix (100 mM ribose) compared to softer (0 nM ribose) matrix. The authors further showed that the increased matrix stiffness resulted in an impaired barrier function and mis-localized VE-Cadherin employing tunable polyacrylamide-based hydrogels to mimic stiff (10 kPa) and soft (0.2 kPa) environments (Bordeleau et al. 2017).

These stiffness changes can be aligned with studies that measured the stiffness of tumor tissue. For example, fibrotic colorectal cancer tissue had a median stiffness of 7.51 kPa, compared with about 0.936 kPa for healthy tissue when measured using a macroscale indentation device (Kawano et al. 2015). For mammary cancer (Levental et al. 2009), an almost ten-fold stiffer elastic modulus of around ~2 kPa has been reported compared to healthy tissue (~0.2 kPa), using unconfined compression analysis. How the alterations in stiffness may regulate or prevent (lymph)-angiogenesis is not fully understood, although the increase in interstitial fluid pressure is likely to cause compression of vessels, causing poor perfusion and resulting in hypoxia (Stylianopoulos et al. 2013). Additionally, it has been shown that BECs cultured within a stiffer 3D matrix sprout less compared to BECs cultured in softer 3D matrix (Trappmann et al. 2017), implicating that an unphysiologically stiff matrix could present a physical barrier for growing vessels.

LNs are the most common sites of tumor metastases and are crucial predictors of the cancer prognosis for the patient. The presence of tumor cells in the LN either reflects the cancer probability to metastasize, with the disease within the LN being inconsequential, or reflects the ability of the cancer cells in the lymph node to leave and spread the disease (Padera et al. 2016). Induction of VEGFC-mediated intratumor lymphangiogenesis at the location of the primary tumor can enable

metastatic breast cancer cells to enter the lymphatic vessels, allowing for increased metastasis in LNs and lung (Skobe et al. 2001). However, additional data have recently emerged. Using VEGFR3-blocking antibodies in mouse melanoma models, it has been shown that active VEGFC signaling enables a better immunotherapy response by recruiting naïve T cells through CCL21 induction, which are then locally activated (Fankhauser et al. 2017). In a glioblastoma mouse model, ectopic VEGFC expression led to an enhanced priming of $CD8^+$ T cells in the cervical LNs and migration of these T cells into the tumor, resulting in a rapid clearance of glioblastoma tumor (Song et al. 2020).

Taken together, lymphatic vessels play a crucial role not only in tumor development and metastasis, but also in tumor regression. It is likely that they respond to tumor ECM stiffness changes, and stiffening may prevent functional lymphatics to enter the tumor tissue. In parallel to the frequently discussed concept of blood vessel normalization (to facilitate chemotherapy) versus blood vessel regression (to starve the tumor) (Augustin and Koh 2022), similar questions should be applied to tumor lymphatic vessels. In addition to modulation of well-studied (lymph)angiogenic signaling pathways, normalization of the tumor microenvironment and stiffness (for example via "ECM softening") and, in particular, modulation of ECM stiffness-regulated lymphatic EC signaling pathways, might present a promising approach to tackle persisting hurdles in tumor therapy.

## 11.5 Summary

In contrast to blood endothelial mechanoregulation, the importance of lymphatic endothelial mechanoregulation via the tissue microenvironment has only recently been discovered. ECM composition and signaling capacity, as well as mural cell support of BECs, have been extensively studied. Identification of mechanoregulatory processes during lymphatic development and in vitro studies on LEC regulation via the matrix environment indicate that similar molecular mechanisms are likely to regulate LEC dysfunction in diseases associated with ECM alterations. Therefore, it appears particularly important now to extend our analysis with novel in vivo approaches of local, LEC-proximal tissue stiffness and stretch measurements to obtain a more holistic perspective. We believe it is necessary to analyze tissue stiffness and stretch capacity experienced by ECs, to understand (1) which microenvironmental factors (ECM composition, mural cell support, and absolute tissue stiffness) are key modulators of absolute tissue changes sensed by ECs, (2) how these factors mutually define each other, and (3) to identify thresholds of the combined action of these factors that render ECs more susceptible to dysfunction.

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