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Extracellular matrix scaffolding in angiogenesis and capillary homeostasis

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Abstract

The extracellular matrix (ECM) of blood vessels, which is composed of both the vascular basement membrane (BM) and the interstitial ECM is identified as a crucial component of the vasculature. We here focus on the unique molecular composition and scaffolding of the capillary ECM, which provides structural support to blood vessels and regulates properties of endothelial cells and pericytes. The major components of the BM are collagen IV, laminins, heparan sulfate proteoglycans and nidogen and also associated proteins such as collagen XVIII and fibronectin. Their organization and scaffolding in the BM is required for proper capillary morphogenesis and maintenance of vascular homeostasis. The BM also regulates vascular mechanosensing. A better understanding of the mechanical and structural properties of the vascular BM and interstitial ECM therefore opens new perspectives to control physiological and pathological angiogenesis and vascular homeostasis. The overall aim of this review is to explain how ECM scaffolding influences angiogenesis and capillary integrity.

Keywords

Extracellular Matrix ; Basement Membrane ; Vascular Development ; Angiogenesis ; Capillary

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1. Introduction

The extracellular matrix (ECM) is a crucial component of the cellular microenvironment and forms a complex three-dimensional network. The vascular ECM is composed of two different compartments: the basement membrane (BM) and the interstitial ECM. Their respective ratio, composition and architecture differ depending on the nature (artery, vein, capillary) and the surrounding environment of the blood vessel. In the wall of large vessels, the interstitial ECM present in the media and embedding vascular smooth muscle cells displays the structural and mechanical properties that are necessary for supporting shear stress and pressure, i.e. high stiffness and elasticity. This interstitial ECM is thus mainly composed of elastic fibers and fibrillar collagens, collagen I being by far the most abundant protein in the wall of large vessels. It is crucial for establishing and maintaining the mechanical stability of the vascular system, as demonstrated in mice deficient for the $\alpha 1$ chain of collagen I, which die between E12 and E14 due to rupture of major blood vessels [1]. In capillaries, the vascular wall is limited to the BM. This specialized macromolecular assembly of proteins separates the endothelium from the surrounding stromal tissue [2]. It forms a continuous sheet-like structure of 50 to 150 nm thickness that surrounds the basal surface of the endothelial monolayer and ensheathes perivascular cells, which also participate to BM synthesis and organization [3, 4]. Since capillaries lack smooth muscle cells and the associated interstitial ECM, the BM alone provides the structural and mechanical features that support the endothelium and vascular integrity, and allows distribution of nutrients and oxygen. BM also plays a major role in pathologies including tumor angiogenesis [5].

We review here the unique molecular properties of the capillary BM, which are crucial to control plasticity of endothelial cells. BM indeed vary in composition in a temporal and tissue-specific manner [6]. BM composition and structure are modified in many ways to generate specialized and context-specific assemblies [7]. Its core components are laminins 411 and 511, collagen IV, perlecan and nidogen 1 and 2. The vascular BM also contains associated proteins including collagen XVIII, VI and VIII and fibronectin. In addition to the properties of each of these components, their scaffolding in the BM is a key process in vascular development and homeostasis. The capillary microenvironment also plays the role of a reservoir for many growth factors and proteins that regulate vascular biology. The complete vascular surroundings therefore orchestrate the capacity of vascular cells to sense and respond to both mechanical and chemical cues from the environment and is thus a key regulator of vascular homeostasis. The aim of the review is to give an overview of the role of BM components and scaffolding in vascular development and homeostasis.

2. Core components of the vascular basement membrane

The two major components of BM are laminins and collagen IV. Both undergo auto-assembly into macromolecular sheets interconnected by nidogen and perlecan. Laminin network is first assembled through interaction with cell surface receptors and then triggers deposition of nidogen and perlecan, which both participate to cross-linking of the collagen IV network in order to generate the core of the BM.

2.1. Laminin

Laminins are a large family of high molecular weight glycoproteins. They consist in heterotrimers composed of disulfide-linked subunits, the α , β and γ chains, and are named according to the composition of the heterotrimer, i.e. laminin 411 is composed of α 4, β 1 and γ 1. Laminins have a cross-shaped structure where the long arm of the cross (80 nm) is an α -helical coiled-coil formed by all three chains, whereas the short arms (35–50 nm) are composed of one chain each [8] (**Figure 1**). At the C-terminal extremity of the long arm, the α chains contain five laminin G-like (LG) domains that include the major cell-adhesive sites. The homologous short arms are arranged in a unique globular structure, the laminin N-terminal (LN) domain, connected to tandem repeats of laminin type epidermal growth factor-like (LE) domains. The overall structure is interrupted by two to three internal globular domains [9].

Whereas expression of the β and γ chains is ubiquitous, the α chain is restricted to certain tissues and cell types, thus allowing tissue-specific distribution of laminins. In addition, their expression is also modulated during development and in pathological states [8]. They are found underlying the endothelium and surrounding pericytes in the capillary wall. The most abundant laminins in the vascular BM are composed of the α 4 and α 5 chains combined with laminin β 1 and γ 1 chains to form laminins 411 and 511 [10-13]. Laminin α 2 is expressed during development (laminin 211) and also in the mature BM (laminin 221) [14] (**Table 1**).

Laminin interactions with cellular receptors, such as integrins via the globular domain at the C-terminus of its long arm, support assembly of other protein networks in the BM. Indeed, retention of laminin at the cell surface promotes further polymerization of the network, as demonstrated using *in vitro* planar lipid bilayers containing sulfated glycolipids [15]. The N-terminal short arms of the three chains mediate self-assembly of laminin polymers (**Figure 1**). Laminin network undergoes self-assembly at the endothelial cell surface and is considered to initiate BM deposition. The role of laminin scaffolding on vascular development and homeostasis will be further described in chapter 4.1.

2.2. Collagen IV

2.2.1. Synthesis and secretion

Whereas collagen IV is also a heterotrimer, its subunits share quite similar structure with one another. Six distinct collagen IV α chains (from α 1 to α 6 encoded by *COL4A1* to *COL4A6* genes, respectively) are expressed and assemble with a remarkable specificity to form only three distinct heterotrimers (α 1 α 1 α 2, α 3 α 4 α 5 and α 5 α 5 α 6), α 1 α 1 α 2 being the most predominant in all tissues

including vascular BM (**Table 2**). The α chains are approximately 400 nm long and can be separated into three domains: a short N-terminal 7S domain, a long middle triple helical collagenous domain, and a C-terminal globular non-collagenous (NC1) domain (**Figure 2**).

The assembly of collagen IV trimers is initiated by interactions between the NC1 globular domains of three α chains that lead to formation of a heterotrimeric protomer. Both the 7S and NC1 domains are then responsible for the assembly of collagen IV networks [16]. Two collagen IV protomers associate via their C-terminal NC1 trimers and four protomers are cross-linked at the N-terminal 7S domain. In parallel, lateral associations between these assembled molecules form an elaborate supramolecular scaffold and allow formation of the complex mesh network of collagen IV. Unlike fibrillar collagens, the central collagenous domain of collagen IV is characterized by numerous (21 to 26 depending on the α chain) interruptions of the Gly-X-Y motifs that confer the structural flexibility responsible for its mesh-like network organization in the BM. In addition, the high level of covalent crosslinks provides stiffness to this structural scaffold.

Collagen IV secretion and supramolecular assembly into the BM require a variety of complex intracellular and extracellular molecular interactions. Among these, interactions with intracellular chaperones such as Hsp47 and the chaperone-binding Tango1 are required for proper collagen IV protomer stability and secretion. Hsp47 does not bind to unfolded or misfolded proteins but preferentially recognizes properly folded triple helices of fibril-forming procollagens and collagen IV protomers in the endoplasmic reticulum (ER). This protein may stabilize collagen IV protomers from the ER to the cis-Golgi [17] and is required for proper BM biosynthesis [18]. Moreover, osteonectin also known as secreted protein acidic and rich in cysteine (SPARC) or basement-membrane protein 40 (BM-40) has recently been proposed to act as an extracellular chaperone-like protein for collagen IV. It is proposed to ensure the proper deposition and assembly of collagen IV molecules in the BM, and to prevent premature collagen IV organization at sites of production, enabling diffusion and assembly to more distal sites [19].

2.2.2. Assembly in the BM

In all BMs, the collagen IV network is cross-linked to the laminin network through perlecan and nidogen. Synthesis, deposition and scaffolding of collagen IV networks by endothelial cells have been shown to be a central regulator of blood vessel formation and is mandatory for blood vessel survival and maturation *in vivo* ([20] and chapter 4.2 of the present review). Collagen IV networks display a highly oriented macromolecular organization in the BM. For long, the hypothesis has been that collagen IV form a horizontally organized network through its NC1 and 7S domains that serves as the main scaffold for the BM [21]. However, recent studies on $\alpha 3\alpha 4\alpha 5$ heterotrimers in kidney or retina BM have reported a polarized distribution for collagen IV trimers as the N- and C-terminal-domains are localized to opposite sides of the BM [22]. Indeed, the 7S domain is detected at the stromal side whereas the NC1 domains have been detected as primarily associated to the cell surface. Other recent studies also support this hypothesis [23], suggesting that collagen IV could be organized in such a way that the protein extends throughout the entire width of the vascular BM with the C-terminus pointing out towards the endothelium and the N-terminus towards the interstitial ECM. This highly orientated distribution thus questions not only the horizontal positioning of collagen IV molecules proposed in the current BM model, but also the functional roles of the

macromolecular organization of collagen IV. Besides, it has also been demonstrated that distribution of laminin is very similar to NC1 collagen IV at the cell-BM interface. These collagen IV and laminin networks are tightly connected by aggregated perlecan [24]. In addition to this specific orientation, the chains composing the collagen IV heterotrimers are also specific depending on their cellular expression. For example, in the kidney glomerular BM, collagen IV $\alpha1\alpha1\alpha2$ protomers produced by the endothelial cells form a network closely juxtaposed to the endothelium, whereas the $\alpha3\alpha4\alpha5$ protomers secreted only by podocytes are tightly attached to the $\alpha1\alpha1\alpha2$ network [25]. These distinct compositions of collagen IV protomers may be fundamental to establish and maintain the BM architecture therefore suggesting that organization of the BM plays a critical role in its function, and especially in this example, in the filtration capacity of the kidney.

2.2.3. Cross-linking

In addition to disulfide crosslinks, collagen IV network assembly is catalyzed by specific cross-linking enzymes, including peroxidase and lysyl oxidases (LOXs).

Peroxidase has recently been identified as cross-linking collagen IV through formation of sulfilimine bonds between a methionine sulfur and a lysine nitrogen of the NC1 domains, thus stabilizing the hexameric structure of collagen IV [26] (**Figure 2**). Peroxidase-deficient mice show reduced collagen IV sulfilimine crosslinks and reduced stiffness of the renal tubular BM, thus providing direct evidence for the contribution of collagen IV cross-linking to BM mechanical properties [27]. This however does not result in a vascular phenotype in these mice.

LOXs are secreted copper-containing amine oxidases that catalyze the oxidative deamination of lysines and hydroxylysines in collagens to form semi-aldehydes known as allysines. These highly reactive groups then undergo spontaneous reactions resulting in collagen cross-linking. LOX enzymes thus do not directly cross-link collagens but catalyze the last step required for its cross-linking [28]. They play important roles at different levels of the vascular system. Gene inactivation experiments have demonstrated that whereas LOXL1 only targets elastin [29], LOX cross-links both fibrillar collagen and elastin in the aorta [30, 31], and does not affect collagen IV cross-linking or BM assembly [32]. The inactivation of LOX results in defects of aorta development leading to rupture and perinatal birth. Deletion of *Loxl2* gene in mice promotes perinatal lethality with incomplete penetrance associated to congenital heart defects and distension of hepatic blood vessels [33]. We have shown that LOXL2-depletion in endothelial cells results in defects in collagen IV deposition and cross-linking in the ECM [34]. More recently, cross-linking of the 7S N-terminal domain by LOXL2 was demonstrated [35]. Besides, we demonstrated that LOXL2 regulates angiogenesis *in vivo*, as LOXL2 down-regulation inhibits formation of intersomitic vessels in zebrafish embryos, and *in vitro*, using 3D culture models [34].

2.3. Perlecan and nidogen

Perlecan, a secreted heparan sulfate proteoglycan (HSPG), is another major component of the core endothelial BM. Perlecan has a very large multi-domain core protein (approximately 470 kDa), carrying three long N-terminal heparan sulfate (HS) side chains of 65 kDa each. The core protein, composed of five distinct structural domains, is bound to the laminin network, whereas the HS are bound to the collagen IV network [24] most likely at its 7S and NC1 domains [8]. Perlecan has

the potential to provide a bridge between laminin and the cell surface through its α -DG-binding LG domains [36]. The N-terminal HS side chains also allow perlecan to sequester and present growth factors to their appropriate receptors (see chapter 5). This role is quite important for establishing growth factor gradients required for developmental processes and regulating angiogenesis (reviewed in [37]).

Perlecan can act as a regulator of cell adhesion, proliferation and growth factor signaling [38]. The C-terminal domain, responsible for oligomeric self-assembly and integrin-binding activity, is crucial for developmental and tumor angiogenesis. This domain, named endorepellin, can be processed by various proteases, and displays anti-angiogenic activities [39, 40]. In zebrafish embryo, knocking down perlecan expression using morpholinos leads to defects in sprouting of primary intersegmental vessels, showing the importance of perlecan in vascular morphogenesis. The phenotype is partially rescued by microinjection of human perlecan protein, or endorepellin [41]. Half of the perlecan-deficient mice die early in development, at E10.5, when expression of the Perlecan gene is normally initiated [42]. At E10–11, many perlecan-deficient embryos show signs of cardiac failure characterized by intrapericardial hemorrhages, weak heartbeats, or even cardiac arrest. Mutants that survive show abnormal BM formation.

Agrin is another secreted HSPG that interacts with core components of the BM. It directly binds the laminin γ 1 chain via its N-terminal domain [8]. The agrin bridge has been shown to strengthen laminin anchorage both *in vitro* and *in vivo* [43, 44]. In the BM, agrin was recently shown to have a polarized distribution with the C-terminus pointing to the podocyte or endothelial cell surface, and the N-terminus to the center of the glomerular BM [25]. The functions of agrin are regulated by alternative splicing of its LG3 domain [45]. The non-neural splice variant of agrin is responsible for its contribution to BM formation and stability, whereas the neural splice variant plays an important role in the formation of neuromuscular junctions. Agrin might also play a role in the blood-brain-barrier as it accumulates in the BM of cerebral microvessels [46] and contributes to the localization of β -catenin, VE-Cadherin and ZO-1 at the endothelial adherens junctions [47].

Two nidogen proteins, nidogen-1 and -2 (entactin-1 and -2, respectively) are encoded by two different genes but share the same structure. Like perlecan, nidogens act as linkers of the core components of BM. They contain binding sites in the G3 domain for the short arm of the γ 1 laminin subunit and in the G2 domain for collagen IV and perlecan [48-50]. Both isoforms are ubiquitous BM components. However, nidogen-1 is predominant in the vascular BM in adult. Only deletion of both nidogen genes results in perinatal lethality possibly due to cardiovascular and/or respiratory failure, whereas individual knockouts are viable and fertile [51]. Locally restricted bleeding within the heart wall has been reported in mutant embryos. This bleeding seems to be associated to leakage of the microvasculature and absence of capillary BM. Therefore, nidogens do not seem essential for BM formation but may play a role in maintenance of capillary integrity.

3. Extracellular matrix proteins associated to the vascular basement membrane

Besides these core components, many associated proteins also play a role in maintaining the BM functions and in connecting the BM to the interstitial ECM. We will present here collagens associated to the BM and fibronectin, which constitutes a functional bridge between the endothelial cell surface and the surrounding microenvironment.

3.1. Collagens

3.1.1. Collagen XVIII

Collagen XVIII is a ubiquitous BM collagen, reaching high levels in the endothelial BM. It is a high molecular weight protein (approximately 300 kDa) that possesses structural properties of both collagen and proteoglycans, with several glycosaminoglycan chains consisting mainly in HS chains. Collagen XVIII is classified under the group of multiplexins due to the presence of 11 non-collagenous interruptions alternating with 10 collagenous repeats within the central triple helix, conferring high flexibility to the protein. Collagen XV, the other multiplexin collagen specific for BM, is mainly involved in cardiovascular development [52]. Homotrimerization of collagen XVIII is triggered by assembly of the NC1 domains. As collagen IV, collagen XVIII displays a very polarized orientation in epidermal and endothelial BMs, the C-terminal domain being orientated towards the cell surface, whereas the N-terminus resides at the BM-interstitial ECM interface [53]. This polarization suggests an anchoring role for collagen XVIII between cells and interstitial ECM. Co-localization of the C-terminal domain of collagen XVIII with perlecan and laminin in the BM is consistent with such polarization [54]. Collagen XVIII binding to the BM could be mediated by HS chains [55]. Collagen XVIII-deficient mice show thickened epidermal BM, suggesting an important structural role for collagen XVIII in maintaining BM physical integrity [56].

Three different tissue-specific isoforms, with core proteins ranging from 135 to 178 kDa, that differ in their N-terminal non-collagenous sequence, have been identified. The shortest variant is found in most vascular BMs. Collagen XVIII became a focus of interest when an 18 kDa C-terminal tryptic peptide, named endostatin, turned out to have anti-angiogenic and tumor-suppressing activities [57]. *In vitro* and *in vivo* anti-angiogenic effects of endostatin have been further confirmed [53]. Several ocular abnormalities including abnormal outgrowth of retinal vessels [58] and reduced susceptibility to high oxygen-induced retinal neovascularization were reported in collagen XVIII-deficient mice [59].

3.1.2. Collagen VIII

Collagen VIII was initially termed endothelial collagen due to its high expression in the endothelial BM [60]. Collagen VIII is a trimeric non-fibrillar short-chain collagen expressed as homotrimers of $\alpha 1$ or $\alpha 2$ chains of 74 and 67 kDa, respectively. They are very similar and share an identical domain structure consisting of a short non-collagenous domain 2 (NC2), followed by a collagen triple helix domain and a C-terminal non-collagenous domain 1 (NC1).

While the function of collagen VIII is uncertain, the importance of this collagen in the vasculature has been particularly reported in maintaining the smooth muscle cell phenotype. It may provide a substratum for a variety of cells and facilitate migration of endothelial cells in angiogenesis, smooth muscle cells in intimal invasion and myofibroblasts in fibrotic conditions [61]. Other studies suggest that the close association of collagen VIII to the BM may improve its resistance to compression, and that it would be synthesized during capillary sprouting [62].

3.1.3. Collagen VI

Collagen VI is ubiquitously expressed and has been studied in a wide range of tissues including the vessel wall. Though not considered as a BM associated collagen *per se*, collagen VI is a BM-anchoring protein with specific biosynthetic process and structural organization. Filaments of collagen VI are composed of heterotrimers of α chains ranging from approximately 100 to 350 kDa encoded by six different genes (from *COL6A1* to *COL6A6*). They mostly organize into a heterotrimer composed of $\alpha 1\alpha 2\alpha 3$. The $\alpha 4$, $\alpha 5$ and $\alpha 6$ chains share a structure similar to the $\alpha 3$ chain and have been proposed to replace the $\alpha 3$ chain under some pathological conditions [63]. Supra-molecular assembly of collagen VI is driven by a multi-step process and leads to the formation of a characteristic and distinctive network of beaded microfilaments in the ECM. However, in contrast to other types of collagens, collagen VI undergoes an intracellular assembly into large aggregates before being secreted [64]. The three α chains assemble to form a triple-helix monomer that joins into disulfide-bonded antiparallel dimers, which then align to form large tetramers also stabilized by disulfide bonds. These tetramers are finally secreted in the extracellular space and associate together through non-covalent bonds to form the beaded microfilaments [65]. Collagen VI forms an anchoring meshwork that connects the interstitial matrix to the vascular BM, thus bridging cells to the surrounding environment and organizing the three-dimensional tissue architecture of the vasculature through direct interaction with collagen IV [66]. Interactions with other fibrillar collagens, including collagen I, have also been reported [67]. Collagen VI and fibronectin have been shown to interact together [66, 68]. Depletion of collagen VI leads to an increase in both fibronectin expression and extracellular deposition, which is then rearranged into long and parallel fibrils. Exogenous collagen VI restores the basal level of fibronectin deposition, suggesting that collagen VI is a regulator of fibronectin fibrillogenesis [69].

3.2. Fibronectin

3.2.1. Expression and fibrillogenesis

Fibronectin (FN) is a ubiquitously expressed BM-associated protein. In the vessel wall, it is incorporated between endothelial and perivascular cells. It is a dimeric glycoprotein of high molecular weight, composed of three types of structural repeats (I, II, III). The N-terminal part contains a self-assembly domain that allows for individual fibronectin dimers to assemble in a process termed fibrillogenesis, resulting in a three-dimensional network. Different isoforms of fibronectin are generated by alternative splicing: 1) the soluble form, or plasma form (p-FN) produced by hepatocytes that circulates at high concentration in blood, and 2) the cellular form (c-FN) produced in tissues, that contains additional domains called EDA and EDB, and is incorporated in the ECM [70]. Fibronectin plays a crucial role in cell adhesion, growth, migration, and differentiation. It is essential to wound healing, embryo development and blood vessel morphogenesis. Fibronectin is a unique ECM component because it is detected both close to the cell surface, strongly associated to the endothelial BM but also more distantly, associated to the interstitial ECM. Whereas *in vitro* experiments demonstrate that c-FN is required for autocrine activity on cell-cell and cell-ECM interactions [71] recent *in vivo* loss of function experiments proposed that p-FN could play a critical role during vascular development [72].

3.2.2. Assembly in the extracellular matrix

Fibronectin fibrillogenesis is a non-spontaneous process, mediated by cells via integrin binding to the RGD motif. Fibronectin binding to integrins induces their clustering. These integrin clusters locally provide very high concentrations of fibronectin at the cell surface, which may promote fibronectin-fibronectin interactions and fibrillogenesis. Other matrix proteins have been involved in fibronectin fibrillogenesis such as ECM proteoglycans [73]. Transglutaminase-2 (TG2), a calcium-dependent enzyme, stabilizes the ECM through its cross-linking activity. Furthermore, TG2 interacts with β 1 integrin and fibronectin. This ternary complex promotes fibronectin deposition and pericellular network of fibrils [74].

Direct interaction of fibronectin with type IV collagen has been known for long [75]. A recent study further reported that fibronectin fibrillogenesis is required for type IV collagen deposition [76]. Disruption of fibronectin network affects type IV collagen and laminin deposition in the BM of human trabecular meshwork cells [77]. In a similar manner, interactions between type I collagen and fibronectin suggest that fibronectin acts as a scaffold that may participate to the alignment of collagen fibrils. Fibronectin fibers may thus organize and orient cells so that collagen fibers are aligned and parallel, thus constituting a functional bridge between the endothelial cell surface and the surrounding ECM.

4. Basement membrane scaffolding and function in vascular development and homeostasis

This chapter will focus on the functional role of BM core components in capillary formation and vascular homeostasis described in mouse models and human pathologies.

4.1. Laminins

Among the three main laminin isoforms expressed in vascular BMs, we will here focus on laminins 411 and 511 that are the two major isoforms found in vascular BMs. We will not review laminin α 2, present in the mature vessels and vascular smooth muscle BMs [78] but predominantly expressed in striated muscles and peripheral nerves and for which the KO mouse models are reviewed in [79].

4.1.1. Laminin 411

Laminin α 4 expression begins at E8.5 and is not restricted to a special type of vessel. The role of laminin α 4 has been studied during vascularization of the mouse retina. It regulates tip and stalk cell number and vascular density by regulating the endothelial delta-like 4 (Dll4)/Notch pathways [80]. Laminin α 4 is mainly expressed at the growing vascular front in the postnatal retina, and its expression is mostly concentrated at the leading tip cells. It is hypothesized that laminin α 4 could induce Dll4 expression specifically in tip cells through a β 1 integrin-mediated mechanism. The phenotype of the laminin α 4 knockout mouse shows a central role for the laminin α 4 chain in microvessel growth, resulting in bleeding and hemorrhages of capillaries, leaving large vessels unaffected [81]. The hemorrhages are associated with abnormalities of the BM, i.e. reduced deposition of collagen IV, nidogen, and laminin α 1 chain. These defects in vascular BM are observed in newborn but not adult null mice [81], suggesting compensation at later stages. Consistent with this

observation, laminin $\alpha 4$ knockout mice that survive beyond the perinatal period have a lifespan comparable to the wild-type controls. Mice lacking laminin $\alpha 4$ show excessive filopodia activity and tip cell formation in the retina. This phenotype is close to the phenotype observed when Notch is inhibited *in vivo*, leading to aberrant sprouting angiogenesis and branching [80].

4.1.2. Laminin 511

Laminin $\alpha 5$ is the other major laminin chain strongly expressed in the endothelial BM. Laminin $\alpha 5$ expression begins when the heart starts beating (E10) and blood circulation is initiated, mainly in arteries. It is expressed in the BM of microvessels at much later stages, and may be associated with maturation of the endothelium or its tissue/organ-specific characteristics [82, 83]. Laminin $\alpha 5$ is not expressed in fenestrated endothelium of endocrine glands and peritubular capillaries of the kidney [14] suggesting a correlation between absence of laminin $\alpha 5$ and fenestration formation. In postcapillary and larger venules, distribution of laminin $\alpha 5$ is patchy, resulting in BM regions containing only laminin $\alpha 4$ or both laminin $\alpha 4$ and $\alpha 5$ [14]. The exact role of this endothelial laminin in vascular homeostasis has not been fully reported. Deletion of laminin $\alpha 5$ in mouse models results in multiple defects and embryonic lethality at E10-E11 due to placental defects, failure of dorsal aorta closure and structural abnormalities and leakiness of the yolk sac vessels [84]. Kidney defects characterized by abnormal glomerular BM and glomerulogenesis are observed in these Lama5 null embryos [85].

Recent publications indicate that endothelial laminin $\alpha 5$ is essential to the shear stress response [86]. *In vitro* and *in vivo* analyses suggest that laminin $\alpha 5$ binding to $\beta 1$ integrin stabilizes VE-cadherin at adherens junctions, thus increasing the strength of cell-cell adhesion, which is essential to shear stress response and vessel permeability [86]. There is however at this point only little evidence that laminins may be involved in such mechanosensing functions. Other publications support the fact that the RGD-binding integrins, $\alpha 5\beta 1$ or $\alpha \nu\beta 3$ could play a role in shear-induced intracellular signaling in cultured endothelial cells [87]. Moreover, using a conditional knockout mouse, it was demonstrated that loss of pericyte-produced laminin $\gamma 1$ is involved in the maintenance of the blood brain barrier integrity [88].

4.2. Collagen IV

Double deletion of Col4a1 and Col4a2 results in complete loss of collagen IV in the vascular BM and mouse embryo death at E10.5-11.5 [20]. Embryos display BM structural deficiency associated with dilated, fragile blood vessels and hemorrhages. Brain vascular beds display reduced capillary density and perturbed endothelial protrusions in the neural layers, whereas development of the large vessels is not affected by the deletion [20]. This demonstrates that collagen IV is not critically important for the formation of embryonic BM but is crucial for its structural integrity and function at later stages in development, especially upon mechanical demand after initiation of blood flow. In agreement with these results, we showed that down-regulating COL4A1 in endothelial cells affects capillary formation in a 3D angiogenesis model in fibrin gels [34].

Mutations in collagen IV are associated with defects in capillary formation and vessel integrity in human pathologies [89-91]. In the past decade, COL4A1 and COL4A2 mutations have

been identified as a cause of multi-system disorder for which penetrance and severity of constituent phenotypes can greatly vary. *COL4A1* mutations were found to cause porencephaly. This disease is characterized by cerebral cavities that communicate with the ventricles. Porencephaly is commonly associated with seizures, and mental retardation. It also causes pre- and perinatal hemorrhages, and recurrent intracerebral hemorrhages in young and old patients. The intracerebral hemorrhages can appear spontaneously or can be triggered by specific events. Porencephaly is often associated with systemic small-vessel disease, as mutations in *COL4A1* compromises the BM and weakens the vessels [90, 92-94]. More than 100 mutations identified in patients were analyzed, showing that glycine substitutions within the triple helical domain are the most common type of mutations in patients. Glycine is often replaced by a charged amino acid. The position rather than the nature of the mutation is crucial for disease severity [95]. Other mutations responsible for hereditary angiopathy with nephropathy, aneurysms, and muscle cramps (HANAC) syndrome have been identified in the integrin binding region of the collagen domain (G498V, G519R and G528E). Patients present arteriolar tortuosity in the retina, brain arterial aneurysms and microvessel defects. The vascular BM is interrupted and thinner [91]. Mutations in *COL4A1* and *COL4A2* genes are also associated with systemic small vessel disease and various symptoms especially affecting the eyes, muscles, kidney and brain [96]. The mutations lead to structural instability of all BMs [97]. Perturbations of the matrisome of cerebral blood vessels is a possible proximate cause in familial small vessel disease [98].

A recent study reported the analysis of the BM in human retinal microaneurysms [99]. Interestingly, microaneurysms presented BM thickening associated with increased expression of the core BM proteins (collagen IV, laminin, perlecan and nidogen) along with ECM remodeling enzymes. Indeed, small microaneurysms expressed increased expression of cross-linking enzymes of the LOX family (LOXL2 and LOXL4), whereas large microaneurysms expressed more matrix-metalloprotease MMP9 and plasminogen activator inhibitor-1. Thickening of the BM by accumulation of collagen IV probably produced by recruited pericytes was thus considered as a compensatory mechanism that would strengthen the vascular wall in the early phase of microaneurysm development [99]. Besides, LOXL2 had already been reported as a susceptibility gene to intracranial aneurysms [100] and further exome sequencing analyses have identified mutations in this gene related to familial intracranial aneurysm [101]. These data confirm the hypothesis of an important role for BM cross-linking by LOXL2 in this pathology.

4.3. Fibronectin

Numerous studies showed that fibronectin and its receptors play a major role in embryogenesis and vascular development [102]. Deletion of the Fibronectin gene in mouse leads to embryo lethality at E8.5, with severe cardiovascular defects including heart malformations and aberrant distribution of blood vessels in the yolk sac [103]. During mouse retina vascularization, endothelial tip cells are guided by pre-existing fibronectin deposited by astrocytes but also synthesize and assemble fibronectin *de novo* [34], a crucial step in the angiogenic process [104]. Accordingly, *in vitro* studies have also demonstrated that growing capillaries deposit their own fibronectin [105] whose assembly regulates and is required for capillary morphogenesis [106].

Whereas many effects of fibronectin are mediated by interaction with its integrin receptors,

fibronectin also regulates angiogenesis in an integrin-independent manner, as shown by mutating the heparin-binding domains (HEP-II and HEP-III). Several mutations were identified in patients suffering from glomerulopathy with excessive fibronectin deposition but also in patients with proteinuria, hypertension and massive glomerular deposits of the p-FN, leading to renal failure [107]. The HEP-II and -III domains play a major role in regulating fibronectin assembly into organized fibrils in the ECM, but also keeps p-FN in a compact soluble form thus preventing its deposition in the ECM. It was suggested that these mutations impair the control of the assembly of fibronectin into fibrils but also the balance between soluble and insoluble FN, leading to abnormal incorporation of the non-fibrillar p-FN. These mutants display lower binding to heparin but also to endothelial cells and podocytes. In endothelial cells, this impairs spreading and cytoskeleton organization [107].

5. The extracellular matrix: a reservoir for growth factors and matricellular proteins

Numerous growth factors, including VEGFs, IGFs, FGFs, TGF- β s, and HGF, have been found to associate with ECM proteins or with HS chains and play a crucial role in vascular development and homeostasis. Some of them are necessary for sprouting angiogenesis, whereas others are tuning this process. We will here review the implication of VEGF and PDGF family of growth factors in vascular homeostasis.

5.1. The VEGF family and its receptors

Vascular endothelial growth factors (VEGFs) are crucial regulators of vasculogenesis and angiogenesis and are essential for vascular homeostasis. The VEGF family comprises several distinct proteins, we here focus on VEGF-A due to its main role in angiogenesis and vascular integrity as well as its predominant association with the vascular BM. Alternative splicing of the VEGF-A pre-mRNA produces several isoforms, each with different affinities for ECM and different biological activity. The predominant form, VEGF₁₆₅, binds to the ECM and to the cell surface by its heparin binding sites. VEGF₁₆₅ binds to the tyrosine kinase receptors VEGFR-1 and VEGFR-2 by diffusion or by presentation through co-receptors such as HSPGs. Binding causes dimerization of receptors, changes in the conformation, and phosphorylation of tyrosine residues that triggers activation of downstream signal transducers and signaling pathways that regulate angiogenesis [108].

ECM-associated HSPGs also bind to VEGF₁₆₅ and potentiate VEGFR-2 signaling [109]. VEGF₁₆₅ signaling in endothelial cells is also fully supported by HS expressed in trans by adjacent perivascular smooth muscle cells [110]. Transactivation of VEGFR-2 leads to prolonged and enhanced signal transduction due to HS-dependent trapping of the active VEGFR-2 signaling complex and constitutes a crosstalk mechanism between adjacent cells. Regulation of VEGF₁₆₅ interaction with HSPG by other HSPG-binding proteins can also modulate VEGFR-2 signaling. Indeed, inactivation of tg2 in mouse resulted in increased vascularization in the retina together as a result of increased phosphorylation of VEGFR-2 at Tyr951 and its downstream targets Src and Akt [105].

Fibronectin also regulates angiogenesis through interactions with growth factors, including TGF- β [111] and VEGF [104]. Indeed, fibronectin binds to VEGF and thus regulates migration and proliferation of endothelial cells. In mouse retina, fibronectin binding to VEGF₁₆₅ secreted by astrocytes promotes the directional migration of tip cells. The role of fibronectin as a “VEGF-A organizer” and as a migration ligand for endothelial cells during sprouting angiogenesis has been well

described [104]. Astrocytes are the major source of cellular fibronectin during retinal angiogenesis and its deletion reduced endothelial migration during vascular plexus morphogenesis. This effect could be recapitulated by selectively inhibiting VEGF-A binding to fibronectin via injection of blocking peptides. A similar phenotype was reported upon double deletion of fibronectin and HSPGs in the retina, suggesting that both components of the ECM synergize to bind to VEGF-A and support migration and orientation of tip cells. Altogether, these data indicate that fibronectin participates to the vascular branching pattern in the retina via binding to VEGF-A.

5.2. The PDGF family and its receptors

Platelet-derived growth factor (PDGF) is a potent mitogen for cells of mesenchymal origin, including fibroblasts, smooth muscle cells and glial cells. It regulates cell growth and division. PDGF plays a significant role in angiogenesis. In both mouse and human, the PDGF signaling network consists of four ligands (PDGF-A, PDGF-B, PDGF-C and PDGF-D) and two tyrosine kinase receptors (PDGFR- α and PDGFR- β). Secreted PDGF acts both in an autocrine and paracrine manner. It regulates angiogenesis through pericyte recruitment and stabilization of neovessels. PDGF-B, released from endothelial tip cells at the migration front, recruits mural cells that express PDGFR- β and especially pericytes [3]. Knockouts of *Pdgfb* and *Pdgfrb* in mouse lead to similar phenotypes of perinatal death due to vascular dysfunction caused by mural cell deficiency [112], leading to vascular leakage and abnormal junctions [113, 114]. These defects induced a compensatory mechanism of up-regulation of VEGF-A, which in turn promotes further vascular leakage and hemorrhage [115].

PDGF-B binds to heparin and HSPGs in the ECM via its C-terminal retention motif that limits the range of action of PDGF-B necessary for adhesion of pericytes to the vessel wall. In mice, deletion of this motif perturbs pericyte coverage in microvessels thus showing that PDGF-B-oriented presentation at the endothelial cell surface is crucial for pericyte recruitment [116]. HSPGs actually constitute a PDGF storage compartment at the cell surface and in the ECM, and these interactions regulate spatial distribution and bioavailability of PDGF at sites of pericyte recruitment such as angiogenic sprouts.

5.3. Matricellular proteins

5.3.1. Angiopoietins

Angiopoietins are part of a family of vascular growth factors that play a role in embryonic and postnatal angiogenesis, as well as in stabilization and maturation of blood vessels. Angiopoietins are involved in regulating vascular permeability, vasodilation, and vasoconstriction. Among them, Angiopoietin-1 (Ang-1) interacts with the ECM, whereas Ang-2 is more diffusible [117]. These interactions provide different local concentrations and are thought to account in part for the differences in the biological effect of the angiopoietins. Indeed, matrix-bound Ang-1 induces cell adhesion, motility and Tie2 activation in cell-matrix contacts, eventually translocated to the trailing edge in migrating endothelial cells. In contrast, Ang1-induced Tie2 translocation to cell-cell contacts triggers the formation of homotypic Tie2-Tie2 trans-associated complexes in contacting cells. Distinct signalling proteins are therefore activated by Tie2 in the cell-matrix and cell-cell contacts, where Ang2 inhibits Ang1-induced Tie2 activation [118]. Also, Ang-3 is tethered on the cell surface via HSPGs [119]. As for angiopoietin like-proteins, angiopoietin like-4 (ANGPTL4), a key regulator of

lipoprotein lipase activity thereby impacting processing and distribution of triglycerides and cholesterol, is also secreted by endothelial cells in hypoxia [120] and interacts with HSPGs [121]. It regulates developmental and pathological retinal vascularization by limiting cell adhesion, cell junction organization, pericyte coverage [122]. ANGPTL4 also plays a role in vessel integrity, limiting vascular permeability in ischemic pathologies [122-124].

5.3.2. CCN proteins

Other ECM-associated integrin- and heparin-binding proteins belong to the CCN family and are functionally involved in the development of the vascular system. CCN is an acronym that refers to the initials of the first three family members namely cysteine-rich 61 (CYR61) for CCN1, connective tissue growth factor (CTGF) for CCN2 and nephroblastoma overexpressed (NOV) for CCN3. There are three other members of this family [125]. They all share structural characteristics but are all functionally different. By far, CCN1 and CCN2 display more important roles in vascular development and diseases [126]. They provide the functional bridge between structural macromolecules and growth factors, cytokines, proteases, and other related proteins [127]. Structurally, they are arranged as multimodular molecules composed of four cysteine-rich motifs.

CCN1 and CCN2 are critical regulators of endothelial cell differentiation, mural cell recruitment and BM formation during embryonic vascular development. Once secreted, CCN1 controls adhesion, proliferation and survival through interaction with integrins. CCN1 signaling often requires HSPGs as co-receptors. Mice lacking CCN1 phenocopied $\alpha 4$ and βV integrin-deficiency, leading to similar vascular defects [128]. CCN1 can also bind ECM proteins such as fibronectin or vitronectin and thus potentiates their activation of αV integrins [129]. As for CCN1, the role of CCN2 is context-dependent and is determined by the bioavailability of membrane receptors and/or presence of interacting molecules in the microenvironment. Through direct binding to $\alpha V\beta 3$ integrin, CCN2 promotes endothelial cell adhesion, migration, and tube formation [130, 131]. CCN2 also regulates gene expression of growth factors and ECM components [132, 133].

6. Concluding remarks

This review describes the specific composition of the vascular BM and highlights the functional roles of this spatially-orientated structure (**Figure 3**). In addition to its proteolytic regulation, increasing number of studies demonstrate that regulation of the assembly of the ECM and its components is a decisive process in vascular morphogenesis. Some ECM remodeling enzymes play a key role, by allowing scaffolding and organization of protein components.

The ECM is a reservoir for growth factors that influence angiogenesis by controlling specific signaling pathways. Regulation of their bioavailability directly impacts on vascular morphogenesis and homeostasis. These combined processes are key events of the dynamic regulation that leads to a specifically and functionally orientated structure, as discussed earlier for the collagen IV and XVIII polarization in the vascular BM.

The ECM is a crucial regulator of the cell microenvironment and tissue organization. Cells assemble soluble proteins into insoluble fibrils embedded in a hydrophilic polysaccharide-based ECM with unique mechanical properties and chemical composition thus generating the cues that will

regulate cell-cell interactions and morphogenesis. Cellular traction forces are indeed considered as effectors activated by mechanosensing in order to regulate matrix remodeling [134]. Cells thus both sense and influence ECM mechanics to maintain tissue homeostasis. Works developed over the last 15 years have demonstrated the crucial role of ECM-mediated mechanotransduction as a major regulator of morphogenetic processes including angiogenesis. Fluid shear stress has been considered as the main source of mechanical signals regulating vascular function for long and is still under deep investigation [135, 136]. It is now also established that substrate mechanical properties can also influence sprouting and vascular network formation. Endothelial cells establish perfused vessels in any tissue but cartilage, thus encountering a huge diversity of microenvironments. Such context pushes forward the role of the vascular BM as the stable structural feature that will allow vascular interactions with- and functions in- any tissue. The mechanisms that stand behind these relationships however still require deep investigation. Overall, a better understanding of the mechanical and structural properties of the vascular BM and interstitial ECM undoubtedly opens new perspectives to control physiological and pathological angiogenesis and vascular homeostasis.

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Table 1. Laminin isoforms in vascular BM

Chains	Expression	Heterotrimers	Vascular specificity	References
$\alpha 4$	endothelial and mural cells	411	all blood vessels	[14]
$\beta 1$	ubiquitous			
$\gamma 1$	ubiquitous			
$\alpha 5$	endothelial and mural cells	511	capillaries	[14]
$\beta 1$	ubiquitous			
$\gamma 1$	ubiquitous			
$\alpha 2$	mural cells	211	blood brain barrier vessels	[3, 137]
$\beta 1$	ubiquitous			
$\gamma 1$	ubiquitous			
$\alpha 2$	mural cells	221	mature blood vessels	[14, 138]
$\beta 2$	widely expressed			
$\gamma 1$	ubiquitous			

Table 2. Collagen IV heterotrimers in BMs

Heterotrimers	Expression	Vascular orientation	References
$\alpha 1\alpha 1\alpha 2$	all vascular BMs	closely juxtaposed to the endothelium	[16, 25]
$\alpha 3\alpha 4\alpha 5$	glomerular, eye, lung, testis BMs	distant from the endothelium	[25]
$\alpha 5\alpha 5\alpha 6$	kidney, skin, smooth muscle BMs	-	[16]

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Figure legends

Figure 1. Organization of laminins in the vascular basement membrane

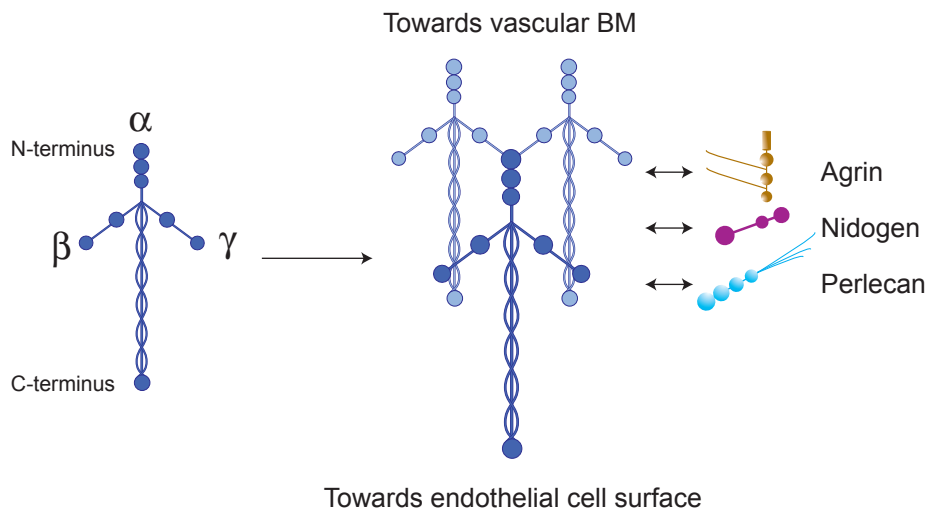
The laminin network is constituted by the assembly of the α , β and γ chains heterotrimer where the C-terminal long arms are orientated towards the cell surface and the N-terminal short arms towards the collagen IV scaffold. The main interacting ECM components are indicated.

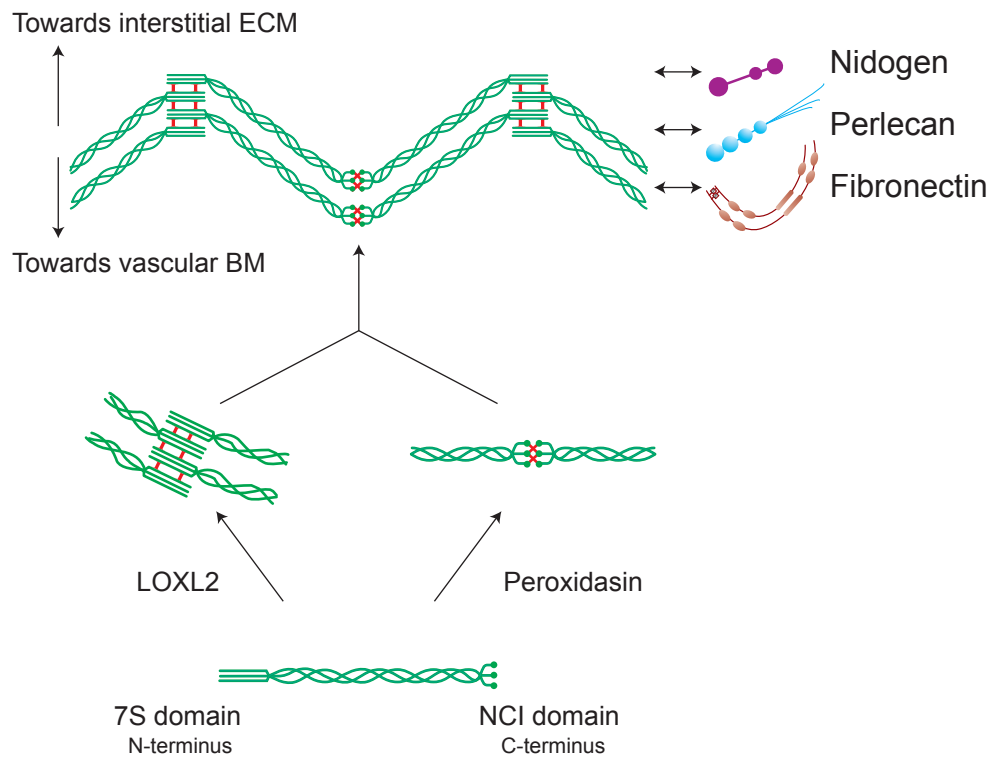
Figure 2. Organization of collagen IV in the vascular basement membrane

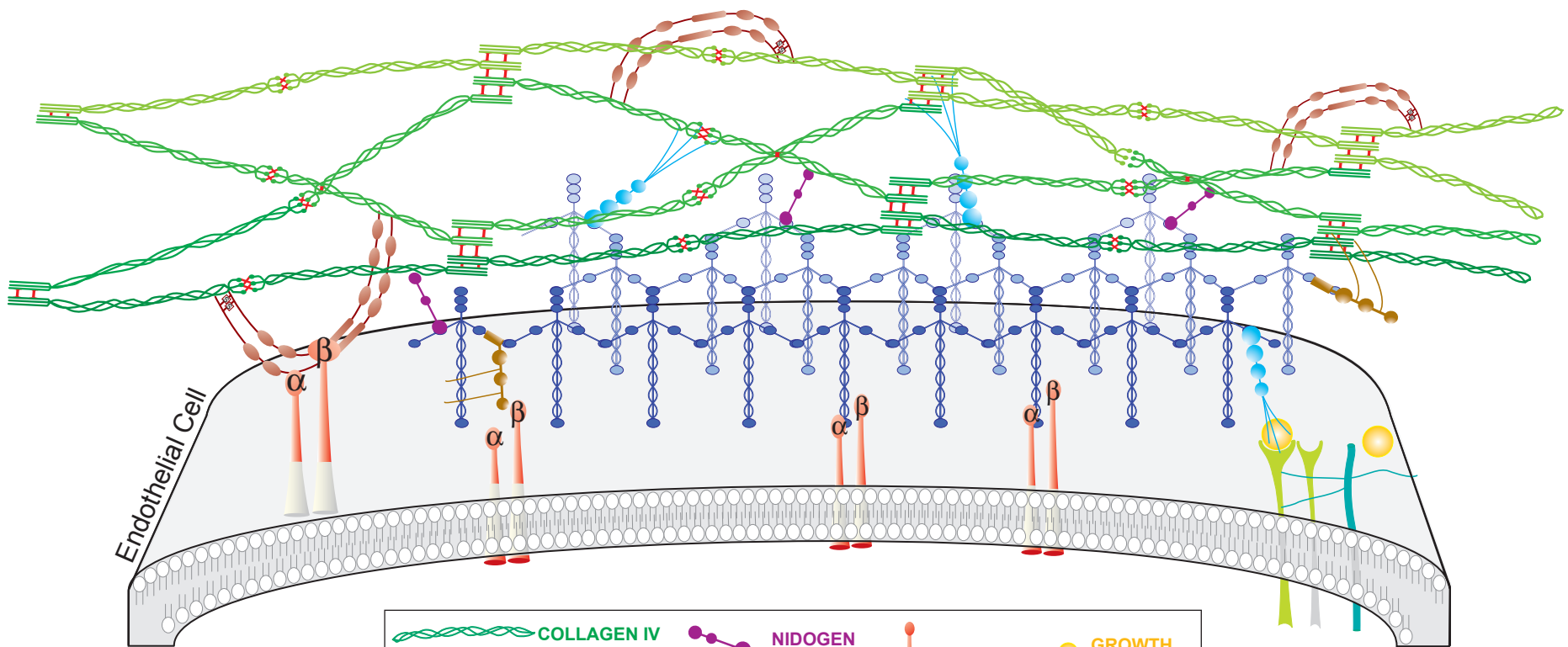
The collagen IV mesh network is constituted by scaffolding of the α chain heterotrimers. The NC1 domains cross-linked by the peroxidase enzyme are orientated towards the laminin network. The 7S domains cross-linked after deamination of lysines by LOXL2 are orientated towards the interstitial ECM. 7S and NCI crosslinks are indicated in red. The main interacting ECM components are indicated.

Figure 3. Polarized basement membrane in capillary wall

The laminin network interacts with the cell surface directly through the integrins or indirectly through agrin and perlecan. The collagen scaffold interacts with the laminin network through perlecan and nidogen. 7S and NCI crosslinks are indicated in red. Fibronectin is present on the cell surface and interstitial sides of the basement membrane.







Endothelial Cell

