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Role of laminins in physiological and pathological angiogenesis

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ABSTRACT The interaction of endothelial cells and pericytes with their microenvironment, in particular with the basement membrane, plays a crucial role during vasculogenesis and angiogenesis. In this review, we focus on laminins, a major family of extracellular matrix molecules present in basement membranes. Laminins interact with cell surface receptors to trigger intracellular signalling that shapes cell behaviour. Each laminin exerts a distinct effect on endothelial cells and pericytes which largely depends on the adhesion receptor profile expressed on the cell surface. Moreover, proteolytic cleavage of laminins may affect their role in angiogenesis. We report *in vitro* and *in vivo* data on laminin-111, -411, -511 and -332 and their associated signalling that regulates cell behaviour and angiogenesis under normal and pathological conditions. We also discuss how tissue-specific deletion of laminin genes affects the behaviour of endothelial cells and pericytes and thus angiogenesis. Finally, we examine how coculture systems with defined laminin expression contribute to our understanding of the roles of laminins in normal and pathological vasculogenesis and angiogenesis.

KEY WORDS: *laminin, basement membrane, vasculogenesis, angiogenesis, cancer*

Introduction

The lumen of blood vessels is lined by endothelial cells (EC) that interact with a subendothelial basement membrane (BM) that is composed of a complex of several matrix proteins (Fig. 1; Eble and Niland, 2009), and contains growth factors and proteases. The interaction of EC with this microenvironment, especially with the BM components, plays an essential role during angiogenic processes (Ingberg, 1992). During development, blood vessels are formed by two distinct processes described as vasculogenesis and angiogenesis. Vasculogenesis characterizes the generation of vessels from mesodermally derived angioblasts. Angiogenesis describes the formation of vessels through sprouting from a preexisting plexus (Risau and Flamme, 1995). Both events involve cell migration, proliferation and extracellular matrix (ECM) remodelling. Angiogenesis is also an essential event in tumour growth and metastasis. Similar to physiological angiogenesis, tumour angiogenesis is regulated by BM molecules in particular upon break-down and reconstitution of the vascular BM during tumour progression. Cells within a tumour can promote angiogenesis, e.g. by secretion of soluble and insoluble angiogenic factors as well as by modulating protease activities. In addition to soluble factors such as growth factors and chemokines that can support or inhibit angiogenesis, matrix molecules of the BM also play a decisive role in angiogenesis. For example the interaction of EC with intact collagen IV promote angiogenesis whereas proteolytic fragments of collagen IV and other collagens counteract this process (for reviews see Kalluri, 2003; Davis and Senger, 2005; Hallmann *et al.,* 2005).

In this review we will focus on the role of LM in vasculogenesis and angiogenesis in normal tissue homeostasis and diseases. LM are multifunctional matrix molecules that are widely expressed forming the major scaffold of the BM (Yurchenco and Patton, 2009). LM display organ, site and developmental specificity (Miner and Yurchenco, 2004). They are cross-shaped trimers which are assembled into a triple-stranded coiled-coil structure. Up to 15 distinct

Abbreviations used in this paper: BM, basement membrane; CAM, chick chorioallantoic membrane assay; EC, endothelial cells; ECM, extracellular matrix; EHS, Engelbreth-Holm-Swarm; FGF, fibroblast growth factor; FGFR, fibroblast growth factor receptor; LM, laminin; MMP, matrix metalloproteinase; PDGF-(BB), platelet-derived growth factor-(BB); PDGF-Rβ, platelet-derived growth factor receptor β; PTFE, polytetrafluoroethylene; TGFβ, transforming growth factor β; VE-cadherin, vascular endothelial-cadherin; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor.

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isoforms have been described today and the functional differences of all LM is not well understood (Durbeej, 2010) (Fig. 1). Integrins, dystroglycan, syndecans and Lutheran are cellular receptors for LM (Barczyk *et al.,* 2009; Durbeej, 2010). The globular domains located in the N- and C-terminus of the LM α chains are critical for interactions with cellular receptors which trigger intracellular signalling (Suzuki *et al.,* 2005).

Determining the function of specific LM isoforms by genetic knockout experiments turned out to be difficult. In particular, early lethality was encountered upon knock-out of several α, β and γ chains (Gustafsson and Fässler, 2000; Li *et al.,* 2003; Rozario and DeSimone, 2010). Moreover, partial compensation by other isoforms masks the function of the knocked out isoform (Simon-Assmann *et al.,* 2010). Furthermore, since LM are heterotrimeric molecules, it is difficult to assign a conclusive function to a particular $\alpha\beta\gamma$ combination upon deletion of a single chain by antibody staining. For example an anti-pan-LM antibody, that recognizes either the α1, β1 or γ1 chain, detects 11 distinct isoforms. Yet, specific single chain antibodies had been raised and are now available. They allowed to decipher expression of specific LM isoforms in blood vessels (Sixt *et al.,* 2001; Patarroyo *et al.,* 2002; Hallmann *et al.,* 2005). In most tissues, only LM-411 and LM-511 are found in the endothelial BM (Hallmann *et al.,* 2005). Since only certain LM isoforms are present in vascular BMs, it is assumed that they

TABLE 1

ROLE OF LAMININS IN ANGIOGENESIS - EXPERIMENTAL APPROACHES AND BIOLOGICAL RESPONSES

Experimental approach	Biological response	Reference
	Laminin α1 chain	
Purified LM-111		
Collagen assay	Tube formation	Dixelius *et al.* 2004
Embryoid body assay	Angiogenesis	
CAM assay	Angiogenesis	
Overexpression in human cancer cells		
Xenografting in nude mouse	Increased tumour angiogenesis	De Arcangelis *et al.,* 2001
Peptide IKVAV		
Cell adhesion assay	EC attachment	Grant *et al.* 1992 and 1994
In vitro and *in vivo* Matrigel assays	Inhibition of tubulogenesis, inhibition of angiogenesis	Kibbey *et al.* 1992
CAM assay	Angiogenesis	
Zymography	Activation of proteolytic enzymes	Stack *et al.* 1993 Faisal Khan and Falcone, 1997
Peptide A10, A13, A55, A64, A118, A119, A124, A203		
Matrigel assay	Tube inhibition	Malinda *et al.* 1999
Aortic ring assay	Vessel sprouting	
Boyden chamber assay	Migration (A10, A55, A64)	Kuratomi *et al.* 1999
Peptide AG73		
CAM and aortic ring assay	Angiogenesis, vessel sprouting	Mochizuki *et al.* 2007
Immunoblotting	Interaction with Syndecans	Hoffman *et al.* 1998
RGD peptide		
Cell adhesion assay	EC attachment, EC spreading	Aumailley *et al.* 1991
Matrigel assay	Tube inhibition	Grant *et al.* 1989
Zebrafish with mutated Lama1	Delayed EC differentiation Defect in hyaloid vasculature	Semina *et al.* 2006
Mouse with mutated Lama1, and mouse with conditional Lama1 knockout	Defect in vessel formation Defect in hyaloid vasculature, Impaired angiogenesis	Edwards *et al.* 2010
	Laminin β1 chain	
Peptide B7, B49, B77, B97, B160		
Matrigel assay	Tube inhibition	Malinda *et al.* 1999
Aortic ring assay	Vessel sprouting	
Boyden chamber assay	Migration (B160)	
Peptide YIGSR		
Matrigel assay	Tube inhibition	Kubota *et al.*1988, Grant *et al.* 1989
CAM assay	Inhibition of angiogenesis	Sakamoto *et al.* 1991
	Laminin γ1 chain	
Peptide C16, C25, C30, C38, C64, C75, C102		
Matrigel assay	Tube inhibition	Ponce *et al.* 1999
Cell adhesion and aortic ring assay	EC attachment, vessel sprouting	
CAM assay	Angiogenesis (C25, C30, C38, C64)	
	Laminin α4 chain	
Blocking antibodies and recombinant protein		
Cell adhesion assay	EC adhesion, EC migration,	Gonzales *et al.* 2001
Matrigel assay	Tube inhibition	
In vivo Matrigel assay	Stimulation of angiogenesis	Gonzalez *et al.* 2002
Overexpression in EC		
Scratch wound assay	EC spreading, EC migration	Li *et al.* 2006
Collagen assay	Tube formation	
Blocking antibodies		
Proliferation assay	Inhibition of proliferation	DeHahn *et al.* 2004
TUNEL assay	Enhanced apoptosis	
Knockout mouse	Impaired microvessel maturation	Thyboll *et al.* 2002
Xenografting of cancer cells into Lama4 knockout mouse	Increased tumor angiogenesis	Zhou *et al.* 2004
	Laminin α5 chain	
Knockout mouse	Reduced vessel stability	Miner *et al.*1998; Miner, 2008
Recombinant LM-511		
Cell adhesion assay	EC attachment	Doi *et al.* 2002
Migration assay	EC migration	
Purified LM-511/521		
Embryoid body assay	No effect	Dixelius *et al.* 2004

Effects of LM chains on angiogenesis are summarised. Experimental approaches and biological responses of EC such as adhesion, migration and tube formation are presented for each LM as either the full length molecule or specific peptides. Standard procedures such as Boyden chamber assay, CAM assay, aortic ring assay, embryoid body assay, scratch wound assay, collagen assay, TUNEL assay (Cimpean *et al.,* 2011) as well as overexpression and gene ablation were applied to investigate the effect of the various LM on angiogenesis. Note that multiple peptides competed responses of cells toward LM-111 within Matrigel.

are critical for maintenance of vessel stability and/ or integrity. Formation of new vessels during angiogenesis requires degradation of the BM, a process in which EC are exposed to other LM isoforms such as LM-111, that are usually not expressed or weakly expressed in most healthy tissues but are present in the abnormal microenvironment. Most of the data reported in the present review, and summarized in Table 1, are based on *in vitro* and *in vivo* models that were applied to elucidate the angiogenic properties of LM (Cimpean *et al.,* 2011).

In vitro *angiogenesis assays with reconstituted basement membrane proteins*

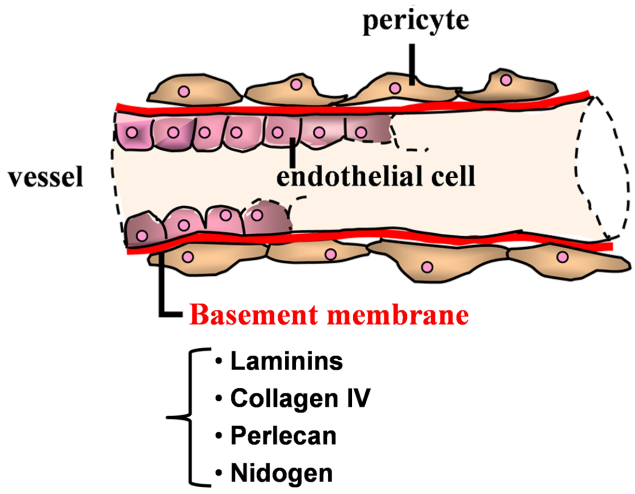
The first LM discovered was isolated from the matrix of the murine EHS tumour (Engelbreth-Holm-Swarm) and is now designated as LM-111 (Timpl *et al.,* 1979). The antibody raised against the EHS LM preparation, formed by α1, β1 and γ1 chains, was considered to exclusively recognize LM-111 which turned out to be wrong. Indeed, several LM isoforms share the α1, β1 or γ chain and therefore can be recognized by the anti-pan laminin antibody. Yet, by using α1 chain-specific antibodies it had been observed that α1-containing LM are not ubiquitously expressed in BM but exhibit a limited distribution. This is in contrast to β1γ1-containing LM forming trimers with α chains other than α1 that are found ubiquitously in BM. LM-111 is expressed during early embryogenesis (Sasaki *et al.,* 2002) where it plays an essential role in developing kidney tubules, lung alveoli and submandibular glands (Ekblom *et al.,* 2003). LM-111 is essential for embryogenesis due to its expression in the extraembryonic Reichert's BM. Thus it is not surprising that Lama1 knockout mice die by embryonic day 7 (Miner *et al.,* 2004; Alpy *et al.,* 2005). Despite a restricted expression in the adult, LM-111 is expressed in polarized tissues such as the mammary gland and the intestine, which suggests an essential role in cellular polarization in tissues with a high turnover and remodelling (Simo *et al.,* 1991, Streuli *et al.,* 1995; Virtanen *et al.,* 2000; Simon-Assmann *et al.,* 2010). But how LM-111 would induce or regulate polarization is unknown.

Several studies have characterised the behaviour of cells when placed on natural or reconstructed BM. The most widely used example is Matrigel, a commercialized cell culture matrix, extracted from the EHS tumour. EC cultured on Matrigel cease to proliferate, align and form capillary-like structures (Kubota *et al.,* 1988; Pauly *et al.,* 1992). Furthermore, Matrigel has been demonstrated to support the formation of tubules from EC *in vitro* as well as vascular networks when implanted into mice (Passaniti *et al.,* 1992; Garrido *et al.,* 1995). One major application of Matrigel is to support tumour grafting in murine xenograft experiments (Kleinman and Martin, 2005). Yet, due to several growth factors and other components in Matrigel, conclusions to what molecule the result relates to should be taken with caution (Hughes *et al.,* 2010). As LM-111 is the major glycoprotein found in Matrigel, several studies aimed to define its precise function in regulating angiogenesis. In particular, LM-111 recombinant proteins, proteolytic fragments and synthetic peptides have been generated and were used to identify and characterize functional domains of the molecule.

Pro-angiogenic properties of sequences within the laminin α1 chain

It has been shown that matrix-derived synthetic peptides, corresponding to active sites in proteins, could have biological activities on their own. Using synthetic peptides from the α1 chain, Grant *et al.,* (1992, 1994) had demonstrated that an IKVAV (Ile-Lys-Val-Ala-Val) containing-peptide (Fig. 2) promoted EC migration and invasion into Matrigel and increased angiogenesis in the chick chorioallantoic membrane assay (CAM). The angiogenic activity of this IKVAV-containing peptide was further confirmed in an *in vivo* model when the peptide was co-injected with melanoma cells

A



B

Laminin isoforms

Name	Chain composition
Laminin-111	α1β1γ1
Laminin-121	α1β2γ1
Laminin-211	α2β1γ1
Laminin-213	α2β1γ3
Laminin-221	α2β2γ1
Laminin-311	α3β1γ1
Laminin-321	α3β2γ1
Laminin-332	α3β3γ2
Laminin-411	α4β1γ1
Laminin-421	α4β2γ1
Laminin-423	α4β2γ3
Laminin-511	α5β1γ1
Laminin-521	α5β2γ1
Laminin-522	α5β2γ2
Laminin-523	α5β2γ3

Fig. 1. The basement membrane constituents in vessels and presentation of the laminin family. (A) *In a quiescent capillary a BM separates endothelial cells from supporting cells such as pericytes and the connective tissue. The BM is composed of 4 major types of molecules: LM, collagen type IV, perlecan and nidogen.* **(B)** *The 15 different LM isoforms are composed of α, β and γ chains forming hetrotrimers. Name and chain composition are presented.*

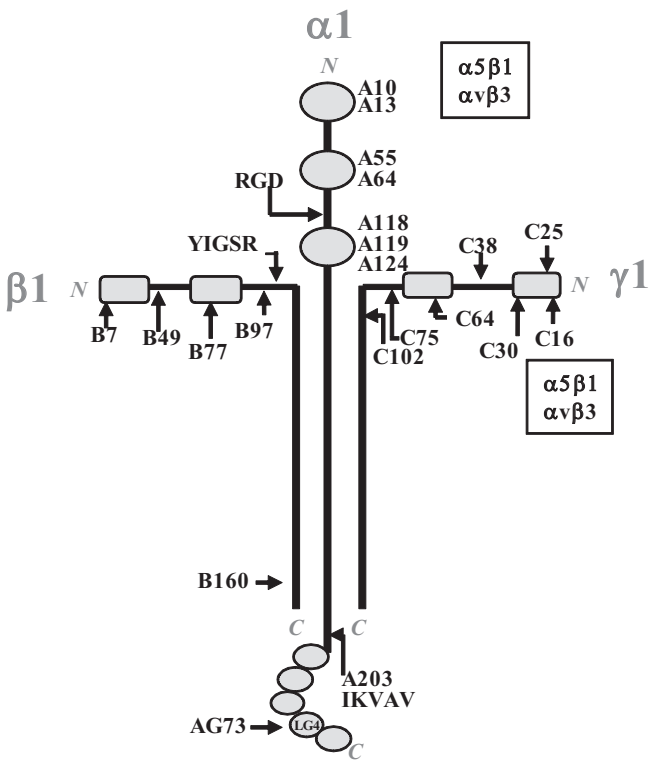


Fig. 2. Schematic model of the LM-111 molecule (α1β1γ1) and its peptide domains active on endothelial cells (EC). *The location of LM-111 synthetic peptides that act on angiogenesis is indicated by arrows. Most of the identified peptides located on the α, β or γ arm of LM (designated respectively by A, B or C letters) are positively regulating EC behaviour. Peptide YIGSR located on the α1 chain inhibits angiogenesis. Peptides A13 or C16 bind to integrin α5β1 and αvβ3 receptors whereas intact LM-111 does not bind to these integrins. Note that B7 peptide is located within the putative signal sequence of the β1 chain. Globular domains of LM-111 are represented in grey. N, amino terminus domain; C, carboxy terminus domain; LG4, LM C-terminal G domain (module 4).*

and Matrigel into nude mice. Under this condition, the peptide enhanced tumour vascularisation as well as tumour growth over that observed with melanoma cells or Matrigel alone (Kibbey *et al.,* 1992). In order to identify additional active sites within the LM molecule, a screen was performed on 405 synthetic partially overlapping peptides that were derived from the α1 chain (Nomizu *et al.* 1995; Malinda *et al.,* 1999). Using the HUVEC tube formation and EC rat aortic ring sprouting assay, eight peptides A10, A13, A55, A64, A118, A119, A124 and A203 were found to be active in angiogenesis (Fig. 2). Of interest is the fact that the α1 chain peptides are predominantly clustered in the first, second and third globular domain (Malinda *et al.,* 1999). The A13 peptide (RQVFQVAYIIIKA), which was the most active in the angiogenesis assays, also promoted melanoma lung colonization (Kuratomi *et al.,* 1999).

The synthetic peptide AG73 derived from the LM α1 chain carboxy-terminal globular domain (G domain, LG4 module) (Fig. 2) turned out to be a very potent stimulator of angiogenesis (Mochizuki *et al.,* 2007). Again, this peptide promoted angiogenesis in the aortic ring and in the CAM assays. Interestingly, peptides with homologous sequences in LM α2, α3, α4 or α5 chain were not as angiogenic as was AG73 (Mochizuki *et al.,* 2007) which suggests a particular role of the intact as well as the proteolysed LM α1 chain in angiogenesis.

In accordance with this pro-angiogenic effect of the LM α1 chain, its overexpression by transfection in human colon adenocarcinoma cells increased angiogenesis and growth of the arising tumours and promoted recruitment of stromal cells (De Arcangelis *et al.,* 2001; Fig. 3 panel I). In contrast, mutated versions of Lama1 in zebrafish morphants delayed or reduced EC differentiation, formation of capillaries as well as blood flow in the hyaloid vasculature (Semina *et al.,* 2006). In mice with a recessive mutant Lama1 gene that was generated by chemical mutagenesis, EC development was also delayed. A more severe phenotype was observed in a Lama1 null mouse mutant that derived from Sox2-cre induced deletion of the floxed Lama1 gene (Fig. 3 panel II). These mice were blind and exhibited tortuous vessels with persistence of hyaloid vessels in the retina (Edwards *et al.,* 2010). This observation points at a crucial role of the LM α1 chain in proper retinal blood vessel development.

Pro- and anti-angiogenic properties located within the laminin β1 chain

As for the α1 chain, short synthetic peptides from the β1 chain of LM were screened for their potential effect on EC adhesion in Matrigel, the aortic ring and the Boyden chamber assays (Nomizu *et al.*1995; Malinda *et al.,* 1999). Five peptides of the β1 chain, B49, B77, B97, B160 and B7, were found to be active in angiogenesis (Fig. 2). Yet, another sequence (YIGSR) located in the β1 chain had an opposite biological activity since it inhibited angiogenesis (Fig. 2). Kubota *et al.,* (1988) and Grant *et al.,* (1989) found that a peptide containing the YIGSR sequence blocked the morphological differentiation of EC into capillary-like structures. This peptide also inhibited embryonic angiogenesis in the CAM assay (Sakamoto *et al.,* 1991). Of interest is the fact that this peptide also inhibited experimental metastasis formation of melanoma cells (Iwamoto *et al.,* 1987) suggesting that this peptide may not only block angiogenesis but also other steps during metastasis. Thus this peptide may potentially find application in cancer therapy.

Activation of signalling pathways in response to cell interactions with laminin-111

Binding of angiogenic peptides of the laminin α1 and γ1 chains to αv β3 and α5 β1 integrins

To search for angiogenic sequences 154 overlapping synthetic peptides were generated that span the entire LM γ1 chain (Ponce *et al.,* 1999). The authors identified 7 peptides (C16, C25, C30, C38, C64, C75 and C102) that inhibited Matrigel-induced formation of capillary-like structures of EC by competition. Moreover, these peptides promoted angiogenesis in the aortic ring assay and in the CAM assay (Fig. 2). The most active peptide C16 (KAFDITYVRLK) bound to integrins αvβ3 and α5β1 (Ponce *et al.,* 2001). Unexpectedly, the angiogenic peptide A13 located in the N-terminal globular domain of the LM α1 chain represented a redundant active site found in the C16 peptide (Ponce and Kleinman, 2003). Both peptides A13 and C16 bound to the same integrin and competed with each other in cell adhesion and angiogenesis. Binding of these peptides to integrins αvβ3 and α5β1 was rather surprising since these integrins are not classical LM receptors

(Avraamides *et al.,* 2008; Silva *et al.,* 2008). Since intact LM do not bind to these integrins it is likely that the A13 and C16 sequences represent crytic sites. Whether and how they are released from the intact molecule is not known. Although A13 and C16 bound to integrins, they did not induce classical integrin signalling such as activation of mitogen-activated protein kinase or serine/threonine kinases (Ponce *et al.,* 2001). There is nothing known about what signalling is induced upon ligation of the integrins by these laminin peptides. It is intriguing that the A13 or C16 sequences are found in almost all LM isoforms: A13 in LM-111 and LM-121, and C16 in 11 out of the 15 isoforms. This high conservation may indicate the importance of this sequence for development and angiogenesis.

In cancer, a peculiar mechanism of tumour cell dissemination was described where tumour cells migrated along the external surface of vessels; this mechanism is called extravascular migratory metastasis (Lugassy and Barnhill, 2007). In the CAM assay, the C16 peptide was shown to increase extravascular migration of human GFP labelled melanoma cells along vessels although it was not addressed whether it affected angiogenesis (Lugassy *et al.,* 2009). Because of the putative significance of this LMγ1 sequence in modulating angiogenesis, Ponce *et al.,*2003) had searched for a more potent peptide and discovered C16Y which was five times more active. Intraperitoneal injections of this peptide also inhibited angiogenesis and growth of xenografted breast cancer cells in mice. The minimal active sequence of C16Y was identified as DFKLFAVY and presumably functions as an antagonist for integrins αvβ3 and α5β1 during angiogenesis. Since these integrins are a target in cancer therapy the described LM sequences blocking these integrins have a potential for drug development.

A cryptic RGD domain in the laminin α1 chain

Binding of various integrins to extracellular ligands is a major initial event in cell-matrix interactions. EC and pericytes express a large subset of integrins that include typical LM receptors such as α2β1, α3β1, α6β1, α6β4, α7β1 (Avraamides *et al.,* 2008; Silva *et al.,* 2008). Interestingly, the repertoire of integrins on EC changes during angiogenesis (Silva *et al.,* 2008). Among these are integrins that recognize the well-known cell-adhesive RGD (Arg-Gly-Asp) site in matrix molecules. Yet, RGD sequences are rarely found in laminins. In the mouse α1 chain, a cryptic RGD site was identified and was located in the fragment P1 upon pepsin digestion (Aumailley *et al.,* 1991). Although some conflicting results exist in the literature regarding the implication of this RGD site in regulating cell adhesion of EC to LM, it was shown that the differentiation of EC into tube-like structures required an initial integrin-dependent attachment to an RGD-containing sequence present in LM α1. This initial integrin-mediated interaction was followed by a cell-cell interaction through the YIGSR sequence on the LM β1 chain (Grant *et al.,* 1989; Aumailley *et al.,* 1991). Thus proteolytic release of the RGD sequence from the α1 chain was necessary for cell adhesion. This raises the question about the role of this mechanism in normal tissue where proteolytic activity is low. Yet, this mechanism might be relevant in inflammatory and tumourigenic tissues that exhibit a high proteolytic activity.

Signalling events triggered by cell adhesion to laminin-111

It is not yet clear how small peptides or the entire LM-111 molecule exhibit a biological response. In the CAM assay, Dixelius *et al.,* (2004) demonstrated that intact LM-111 was as potent as FGF-2

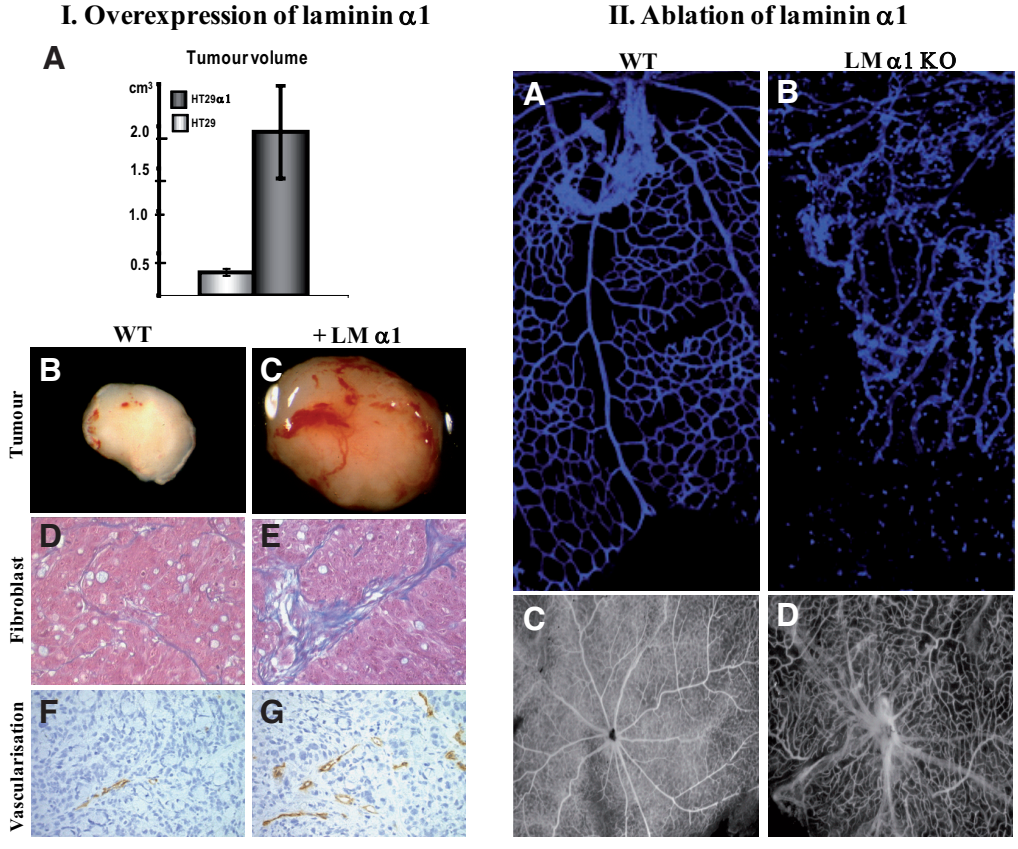


Fig. 3. Documentation of the proangiogenic properties of the LM α1 chain. (Panel I) *Ectopic expression of LM α1 under control of the cytomegalovirus promoter in HT29 colorectal carcinoma cells that do not express endogenously LM α1 in cell culture. Overexpression of LM α1 caused an increased tumour growth* **(A;C** *versus* **B)**, *a strong recruitment of fibroblasts (Masson's Trichrome staining),* **(E,D)** *and enhanced angiogenesis (immunostaining for CD31)* **(G**,**F)** *upon xenografting under the skin of nude mice (De Arcangelis* et al., *2001).* **(Panel II)** *The absence of LM α1 in knockout mice interfered with normal blood vessel development in the retina (Edwards* et al., *2010). Retinas from postnatal day 7 LM α1 knockout mice* **(B)** *exhibited a poor primary retinal plexus in contrast to wildtype mice that showed an organised vasculature with capillaries and small branched vessels* **(A)** *as visualised with* Griffonia simplicifolia isolectin staining. *Abnormal blood vessel organisation is still obvious in the adult stage as visualised by staining for ADPase showing tortuous capillaries and larger vessels extending into the vitreous* **(D,C)**.

to induce angiogenesis, that vascular development in embryoid bodies was stimulated in a synergistic manner by FGF-2 together with LM-111, and that formation of tubular structures was accompanied by increased expression of Jagged-1, a ligand of the Notch receptor family and a marker of endothelial differentiation. They further showed that the combined presence of LM-111 and FGF-2 led to a transient increase of FGFR-1, VEGF-A and VEGFR-2 mRNA levels. Yet, LM-111 failed to activate classical angiogenic growth factor-induced signalling such as phospho-ERK-1/-2 or phospho-Akt. Cell attachment to LM is mediated by cell adhesion receptors such as integrins, syndecans, dystroglycan and Lutheran (Durbeej, 2010). Although studies were not done with EC, one can assume that the pro-angiogenic A13 peptide of the α1 chain likely binds to an integrin containing the β1 subunit since an interaction of A13 with a β1 integrin was found in HT-1080 human fibrosarcoma cells and hepatocytes (Nomizu *et al.,* 1998; Kikkawa *et al.,* 2009). Moreover, the angiogenic AG73 peptide also found in the α1 chain, was shown to interact with members of the transmembrane syndecan family of proteoglycans, syndecan-1 and presumably syndecan-4 (Hoffman *et al.,* 1998; Mochizuki *et al.,* 2007) that are critical regulators of angiogenesis.

Potential role of proteolytic processing of laminin in angiogenesis

Except a role of Notch and VEGF signalling (Jakobsson *et al.,* 2009), little is known how EC are stimulated to form a branch from a parental vessel. The BM itself or BM associated factors and enzymes might play a decisive role in branching of capillaries. Matrix remodelling is a key step in vessel branching and LM-111 might play a role in this process. In particular, the IKVAV-containing peptide in the α1 chain was shown to promote endothelial collagenase IV activity (Grant *et al.,* 1992; Schnaper *et al.,* 1993). This peptide also increased plasminogen activation, resulting in the degradation of LM, as well as of matrix metalloproteinase MMP9 in melanoma cells and macrophages (Stack *et al.,* 1993; Faisal Khan and Falcone, 1997). An increased protease activity could also trigger release of matrix bound growth factors such as VEGF, FGF, PDGF and thus the action of IKVAV would be rather indirect. In contrast to the IKVAV peptide, all other angiogenic peptides derived from the globular domains of the LM α1 chain, including the A13 sequence, did not stimulate MMP activity in a zymogram assay (Malinda *et al.,* 1999).

Summary

Several sequences within the LM α1-, β1- and γ1 chains regulate angiogenesis. Some of them such as the RGD and IKVAV sites are presumably cryptic in the intact LM-111 molecule and need to be unmasked by proteases to be active. These sequences promote early steps in angiogenesis such as cell migration, formation of differentiated spindle-shaped cells, production of proteases, loss of contact inhibition, and expression of matrix molecules that facilitate cell attachment to the ECM (Grant *et al.,* 1994). Another class of pro-angiogenic peptides located in the N-terminal domain of the LM α1 chain, such as the A13 peptide, is also active in context of the intact molecule and may be accessible upon a conformational change. The reason for the large number of pro-angiogenic peptides in LM-111 is not clear. Depending on the surrounding microenvironment and proteolytic activities, alternative peptides may be generated from the LM α1 chain which may modulate angiogenesis by different mechanisms. Finally, synthetic peptides of LM-111 could be used to improve our understanding of the biological functions of this laminin in angiogenesis and may provide the basis for drug development to regulate angiogenesis in pathological situations.

The laminin α4 chain regulates endothelial cell behaviour and angiogenesis

The LM α4 chain is a component of two laminins, LM-411 (α4β1γ1) and LM-421 (α4β2γ1) and is widely distributed in vascular endothelial BMs of several tissues. Since the β2, β3, γ2 and γ3 chains have not been found in capillaries, LM-411 is presumably the only LM trimer in the capillary BMs of most embryonic and neonatal tissues (Hallmann *et al.,* 2005). The widespread and exclusive expression of the α4 chain in BM of developing microvessels suggests a role in angiogenic processes. Indeed, it was shown that the LM α4 subunit has a strong impact on EC behaviour (Gonzales *et al.,* 2001; Gonzalez *et al.,* 2002). In addition it was demonstrated that the G domain of LM α4 is a specific high affinity ligand for αvβ3 and α3β1 integrins. These two integrins together with the α6β1 integrin cooperatively mediate EC interactions with α4 containing LM by promoting cell adhesion and migration (Gonzalez *et al.,* 2002). Similarly, the overexpression of the LM α4 chain in human dermal microvascular EC promoted cell spreading and migration in a scratch wound assay and accelerated angiogenic tube formation in collagen gel overlay assays (Li *et al.,* 2006). By using a panel of dominant-negative mutants of Rho family GTPases, Fujiwara *et al.,* (2004) showed the importance of Rac activation in α6β1 integrin-mediated EC adhesion and migration on LM-411. Other *in vitro* studies suggested that the α4 LM chain can directly regulate cell proliferation and inhibit apoptosis. In particular an antibody directed against the G domain was able to trigger a mitochondrial-dependent programmed cell death pathway in EC (DeHahn *et al.,* 2004) which suggests that cell binding to the LM α4 chain is essential for survival. The authors further showed that the anti-apoptotic signal emanating from the interaction with the α4 LM chain was transduced via integrin α3β1 and/or α6β1.

Studies of LM-α4 deficient mice revealed that this chain is not required for blood vessel formation; yet, Lama4 knock-out mice displayed certain vascular abnormalities. In particular they exhibited an unstable and weakened capillary BM. Rupturing of the microvascular wall led to mild transient haemorrhages in the skin of newborn mice. Within the first week after birth, however, the vascular phenotype of Lama4 deficient mice disappeared most likely due to compensation by LM-511 which was largely deposited under these conditions (Thyboll *et al.,* 2002). In adult Lama4 null corneas, the formation of new blood vessels was dramatically affected. In response to FGF-2 an extensive bleeding and disorganisation of the microvessel growth was observed in Lama4 deficient corneas (Thyboll *et al.,* 2002; Zhou *et al.,* 2004). In contrast to most microvessels where the α4 chain is produced by EC, in the kidney LM α4 is expressed by specialized pericytes, the mesangial cells. In Lama4 null mice the lack of the LM α4 chain caused a progressive renal lesion which was presumably due to deregulated PDGF-Rβ signalling. In consequence pericyte recruitment and vessel lining was reduced (Abrass *et al.,* 2010).

Comparing the expression between normal brain tissue and gliomas using a gene array approach combined with qRT-PCR and immunohistochemical staining revealed that the Lama4 gene was

consistently overexpressed in the tumorigenic blood vessels as opposed to low levels found in normal brain tissue (Ljubimova *et al.,* 2001). Moreover, in gliomas, LM-411 potentially plays a role in tumour regrowth since a high LM-411 expression correlated with a more rapid recurrence, but the underlying mechanism is unknown. Data suggest that during tumour progression a switch from β2-containing LM to LM with a β1 chain occurs. Indeed, normal blood vessels of brain and breast tissue expressed LM-421 which was in contrast to vessels in high grade gliomas and breast carcinoma that expressed LM-411 (Fujita *et al.,* 2005; Ljubimova *et al.,* 2006).

Yet, opposing data on the role of LM α4 on tumour angiogenesis were obtained by Zhou *et al.,* (2004). To determine the role of LM-411 in tumour growth and angiogenesis, they xenografted lung carcinoma cells under the skin of a Lama4 knockout mouse. Under these conditions the absence of host LM α4 accelerated tumour growth in comparison to control mice expressing the Lama4 gene. This was accompanied by an increased tumour vascularisation in the Lama4 knockout mice. These surprising data could be due to compensation by other LM such as α5 or α1 which may be induced in the absence of LM α4. An expression analysis of other α chains in tumours of Lama4 knockout mice may clarify this issue.

Potential role of laminin α5 during angiogenesis

LM α5 expression is developmentally regulated in vessels. In the mouse embryo, the α5 chain is only detected in large vessels; its expression in capillaries becomes evident only at 3-4 weeks after birth (Sorokin *et al.,* 1997). Data on the influence of α5-containing LM on angiogenesis are still fragmentary and not conclusive. Targeted inactivation of the Lama5 gene encoding the LM α5 chain resulted in disturbed placental vessel development (Miner *et al.,* 1998). As the Lama5 knockout embryo died at day 17, no data on later stages are available. But in the very early Lama5 knockout embryo it appears that the lack of this LM chain affects vessel stability and / or maturation (Miner, 2008). Using recombinant human LM-511 as substratum, Doi *et al.,* (2002) showed that human vein EC adhered and migrated on this LM molecule in an integrin α2β1- and α3β1-mediated manner. Quantitative cell adhesion assays revealed that cells bound to LM-511 upon ligation of β1 and αvβ3 integrins which acted in concert with Lutheran, another α5 specific transmembrane adhesion receptor (Vainionpää *et al.,* 2006). Yet, α5-containing LM seems to be unable to stimulate angiogenesis in the embryoid body model when ES cells were cultured together with purified LM. This is in contrast to LM-111 that had strongly induced angiogenesis (Dixelius *et al.,* 2004).

It is possible that there is a balance between LM α4 and LM α5 chains since both are expressed in blood vessels. In the Lama4 knockout situation, compensation by the α5 chain presumably rendered mice viable. In contrast, Lama5 knockout mice are embryonic lethal despite local compensation in certain organs by other LM (Miner *et al.,* 1998; Bolcato-Bellemin *et al.,* 2003). No data are available regarding the vessel BM composition in the Lama5 mutants. In Lama4 knockout mice an increased expression of LM α5 was found that presumably stabilized the vessels. But these vessels were far from normal since they were dilated and fragile which may explain why mice developed cardiomyopathy with increased age (Thyboll *et al.,* 2002; Wang *et al.,* 2006b). A well controlled balance between LM α4 and LM α5 chain expression in the endothelial BM was demonstrated in an experimental autoimmune encephalomyelitis model (Wu *et al.,* 2009). The authors showed that focal extravasation of leukocytes via integrin α6β1 was due to the patchy distribution of LM α5 in the BM. Transmigration of leukocytes occurred preferentially at sites that expressed high α4 containing LM but low amounts of α5 LM showing for the first time an instructive role of endothelial BM on T lymphocyte migration.

To identify adhesive sequences within LM α5, 113 overlapping synthetic peptides of the globular domains had been tested in cell attachment assays (Hibino *et al.,* 2004). Four peptides (A5G27, A5G73, A5G81, A5G101) were found to inhibit FGF-induced angiogenesis in the CAM assay. One of these peptides, A5G27, was able to bind the CD44 receptor that is present on EC and inhibited angiogenesis in a dominant-negative manner. It is interesting to note that CD44 is expressed in the vasculature of tumours as a result of an exposure to angiogenic factors such as bFGF and VEGF (Griffioen *et al.,* 1997). Whether this peptide is generated *in vivo* or whether the A5G27 sequence is binding to CD44 in context of the intact LM molecule is not known. This information would be useful for tumour angiogenesis intervention strategies.

Laminin-332 in matrix channels and the vasculature

LM-332, composed of an α3, β3 and γ2 subunit, is typically expressed in the BM of epithelial tissues such as skin, stomach, lung, breast and intestine. In stratified epithelia such as the skin, LM-332 is detected in type I hemidesmosomes, adhesion stuctures containing α6β4 integrin, cytokeratins and several hemidesmosomal proteins (de Pereda *et al.,* 2009). Because LM-332 plays a key role in controlling migration of cells (Giannelli and Antonaci, 2001), it is possible that it plays a similar role in mesodermal tissues. The γ2 chain, found in LM-332 and LM-522, is expressed in the intestinal smooth muscle coat (Orian-Rousseau *et al.,* 1996) and at low levels in the tunica media of aorta and pulmonary arteries (Kingsley *et al.,* 2002a). Interestingly these vascular smooth muscle cells responded to soluble growth factors such as PDGF and TGF-β1 by increasing their endogenous levels of LM-332 while reducing adhesion to this substratum (Kingsley *et al.,* 2002a). PDGF-BB enhanced cell migration of vascular smooth muscle cells on LM-332 via an ERK1/2 signalling pathway (Kingsley *et al.,* 2002b). The importance of the mesenchyme-derived cells in producing and secreting LM-332 is further supported by interspecies tissue recombination experiments using endodermal and mesenchymal anlagen of mouse and chicken, respectively. Species-specific antibodies were used to show that the LM γ2 chain was deposited by the mesenchymal cells only (Orian-Rousseau *et al.,* 1996). These data suggest that LM γ2-containing LM might be important for mesodermal tissue identity.

The available literature regarding the function of LM-332 in vascular biology is limited. Ahesion of cells to LM-322 appears to promote migration through integrin α3β1 and to facilitate hemidesomosome formation through integrin α6β4 (Litjens *et al.,* 2006). Kikkawa *et al.,* (1996) showed that EC bound to this isoform (formerly called ladsin) via integrin α3β1 and this interaction increased cell migration. In contrast, Homan *et al.,* (1998) demonstrated that a LM-332 rich matrix in concert with integrin α6β4 and vimentin promoted the assembly of stable hemidesmosome-like adhesion structures in EC. This knowledge is already applied in bioengineering of biomedical implants (Kidd and Williams, 2004; Kidd *et al.,* 2005). It was shown that LM-332 enriched polytetrafluoroethylene

(PTFE) scaffolds (a porous polymer that permits vessel ingrowth through the pores of the material) increased tissue vascularisation. More importantly, the authors had shown that LM-332 accelerated neovascularisation upon implantation of the PTFE scaffolds into the mouse (Kidd *et al.,* 2005).

In highly aggressive melanomas, cells form a matrix network that appears to be connected to the vasculature and that has been coined "vasculogenic mimicry" (Maniotis *et al.,* 1999) or tubular channel formation (Kaariainen *et al.,* 2006). These conducting structures are rich in LM γ2 containing laminins but are not lined by EC thus potentially recapitulating embryonic vasculogenesis (Hendrix *et al.,* 2003). These channels may play a role in transporting plasma and blood and potentially serve as short distance route for tumour cells (Kaariainen *et al.*, 2006). Interestingly, a high level of similarity between the vasculogenic mimicry of tumours and the ancestral vascular tube formation -found in the invertebrate blood vessels- was noted (Kucera *et al.,* 2009). Similar to the invertebrate blood vessels, LM-containing ECM lined these vessels within the tumour. Upregulation of the *lamc2* gene (encoding the γ2 chain) and of other genes implicated in angiogenesis and vasculogenesis such as cadherin 15, ephrin receptor A2 and several MMPs was found in aggressive melanoma cells (Seftor *et al.,* 2001; Hendrix *et al.,* 2003). An important role of γ2 LM in tube formation was demonstrated upon reducing expression of this isoform by an antisense oligonucleotide knockout strategy in aggressive melanoma cells. Downregulation of the LM γ2 chain resulted in the inability to form vasculogenic-like networks in 3D-cultures (Seftor *et al.,* 2001). A similar matrix-based tube formation had been described in various carcinomas (Hendrix *et al.,* 2003; Kucera and Lammert, 2009). The potential role of LM γ2 in tumour angiogenesis is unknown. It is interesting to note that cathepsin S cleaved the γ2 chain giving rise to pro-migratory fragments (Giannelli and Antonaci, 2001). Deficiency of cathepsin S in the RIP1-Tag2 model of pancreatic islet carcinogenesis impaired angiogenesis (Wang *et al.,* 2006a).

Role of laminin during basement membrane assembly in angiogenesis

An important step in angiogenesis is the formation of an endothelial lumen giving rise to a tube. On their basal surface EC are first in contact with the BM which maintain the tube structure. This BM and in particular LM appear to be instrumental in forming capillary-like structures (Kubota *et al.,* 1988) by potentially serving as guiding cue for pericytes that will extend cellular processes to contacting EC (von Tell *et al.,* 2006). The recruitment of pericytes to EC-lined tubes will stabilize the vessels and lead to neosynthesis and assembly of BM molecules (Davis *et al.,* 2007) which had been nicely demonstrated in a coculture assay (Stratman *et al.,* 2009). By using species-specific primers in heterospecific cocultures (human EC with bovine pericytes) it was shown that certain LM chains were induced which did not occur if each cell type was cultured alone (Stratman *et al.,* 2009). In particular the α4, α5, β2 and γ1 chains were induced in the coculture setting which points at a possible role of the associated LM, most importantly LM-511 and LM-521 in vascular BM assembly (Miner and Yurchenco, 2004). Coincident with these events, upregulation of integrins α5β1, α3β1, α6β1 and α1β1 occurred in EC-pericyte cocultures that may strengthen the physical interaction between cells and their substratum (Stratman *et al.,* 2009). These findings confirm the essential role of heterotypic cell-cell interactions in controlling BM matrix assembly, a phenomenon not restricted to the vascular BM (Simon-Assmann *et al.,* 2010). These data also emphasise the active participation of pericytes in vessel stabilisation since they are able to produce multiple LM isoforms and type IV collagen (Jeon *et al.,* 1996). The tight interaction between pericytes and EC is presumably further stabilized by cell-cell contacts through integrins and the vascular cell adhesion molecule 1 (Desgrosselier and Cheresh, 2010). Importantly, abnormal interactions of pericytes with EC or the complete lack of pericytes play a role in the pathogenesis of some diseases such as cancer and diabetes (Davis *et al.,* 2007; Huang *et al.,* 2010).

Another example of an important role of BM molecules in the vasculature had been described in the endocrine pancreas (Nikolova *et al.,* 2006). The authors showed that EC provided pancreatic β-cells with a vascular BM which was essential for proliferation and insulin secretion. First, β-cells secreted VEGF-A which attracted EC and triggered formation of a LM-rich BM coat around the capillaries. Subsequently, integrin mediated β-cell interactions with LM promoted insulin expression and β-cell proliferation. In summary it appears that a coordinated binding of cells to LM and other BM components serves as prerequisite for a stable interaction between EC and pericytes that would allow vessel maturation as well as insulin secretion of the endocrine pancreas.

Conclusion and future perspectives

Angiogenesis is a complex process that involves many participants. Amongst them the LM within the BM not only provide a structural basis but also trigger cellular signalling, thus supporting or inhibiting vessel formation. These opposing effects of LM on angiogenesis do reside in their structural configuration that differs if LM are linked to other BM molecules or to cell adhesion receptors. Also cell responses are different if the LM are present as intact molecules or as peptides. The use of recombinant LM proteins or peptides allowed to decipher cell binding sites on individual LM chains. This approach did not take into account glycosylation and the native folding found in the entire molecule, and thus might have missed some interactions. Nevertheless, the fact that some of these sequences were cryptic points to the notion that proteolysis within the tissue is important to generate active or inactive sites. Proteolytic processing of LM appears to be particularly important in tumour angiogenesis.

Since BM are critically involved in blood vessel formation in normal and pathological tissue homeostasis their components are currently an area of intensive research for identification of novel anti-cancer therapies and controlled angiogenesis in tissue repair. One difficulty to encounter is that vascular BMs in distinct tissues and organs exhibit different compositions. In contrast to most organs that express LM-411 and LM-511, blood vessels in the central nervous system express LM-111 and LM-211 (Ljubimova *et al.,* 2001). Under pathological conditions such as in tumour vessels other LM isoforms are expressed than in normal tissue. In particular α2, α3 and β2 containing LM are frequently found in tumour microvessels while they are usually not expressed in healthy tissues (Patarroyo *et al.,* 2002). In addition to LM, also other molecules of the BM are critically involved in regulating vessel homeostasis. Type IV collagen is one such molecule that directly interacts with LM which may affect cell responses to LM. Moreover, in contrast to full length type IV

collagen that supports and promotes angiogenesis, peptides of the N-terminal domain of collagen type IV, known as arrestin, canstatin and tumstatin, are potent inhibitors of angiogenesis (Kalluri, 2003). Similar ambiguous properties, supporting angiogenesis as intact molecule but inhibiting angiogenesis when presented as peptides may also apply to LM. Yet, the underlying mechanisms need to be investigated in more detail.

In the future, a combination of complete or conditional deletion of LM-subunit genes in EC and pericytes will further improve our understanding of the particular function of LM in developmental and pathological angiogenesis. In this respect transgenic mice expressing Cre recombinase by cell type specific promoters such as the EC specific Tie-1, Tie-2, VE-cadherin promoters or the pericyte specific PDGF-Rβ promoter will be useful to remove floxed LM genes.

Finally, the knowledge about the function of LM in the BM of the vasculature can also be used for tissue engineering and tissue repair. Cells derived from host organs are hampered to assemble into a functional tissue without a proper scaffold of a BM and connective tissue. In mice Matrigel presents a scaffold that supports growth of grafted cells (Kleinman and Martin, 2005). But for tissue engineering in humans the mouse-derived Matrigel cannot be used since it would raise an antigenic response. Therefore artificial biomimetic matrices have to be designed to support BM assembly and cell attachment that would provide signals for cell growth and differentiation (Lutolf and Hubbell, 2005). One step in direction of tissue engineering had been done by Nakamura *et al.,* (2008 and 2009). The authors had developped biomimetic supports with collagen-binding activities that promoted the formation of a vascular network to provide artificial tissue substitutes as an alternative for donor tissue and organs.

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