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Laminins in blood vessel development and disease

**-functional aspects in angiogenesis, atherosclerosis,
and muscular dystrophy**

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Faculty of Medicine

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Abstract

Basement membranes are sheet like extracellular matrices that underlie or surround many cell types. They are crucial for key cellular events such as cell migration, proliferation, survival and differentiation. Vital components of all basement membranes are laminins, heterotrimeric combinations of different laminin chain isoforms. The different laminins and their subunits largely have a developmental and tissue specific distribution. To determine the function of laminin isoforms in pathological conditions, three model diseases were used. Special focus was on the vasculature, but also on the most blood vessel specific laminin chain, the laminin $\alpha 4$ chain.

Atherosclerosis is an inflammatory process in large and medium sized arteries. During progression of atherosclerosis the vascular smooth muscle cells (VSMC) in the vessel wall become activated and start to migrate and proliferate. Extracellular matrix molecules are important for this process, but not much was known about VSMC laminin expression in health or disease. Therefore, this was studied in healthy and atherosclerotic vessels. Major laminin chains in VSMC were laminin $\alpha 2$, $\alpha 4$, $\alpha 5$, $\beta 2$ and $\gamma 1$. In plaques, the laminin $\beta 1$ chain was up-regulated and the laminin $\beta 2$ chain down-regulated and in a cell culture system, mimicking the events of VSMC activation, the laminin $\alpha 5$ chain was also seen to be reduced.

Tumour growth and metastasis are both dependent on incorporation of vessels into the tumour. An approach to treat cancers would therefore be to inhibit endothelial cell proliferation and migration. The C-terminal part of the laminin $\alpha 4$ chain, the LG4-5 domain, is proteolytically released. This domain was shown to be retained in the circulation and to reduce endothelial cell migration *in vitro*. Further studies showed that laminin $\alpha 4$ LG4-5 inhibited new blood vessel formation both *ex vivo* as well as *in vivo*. Purified integrin $\alpha 3\beta 1$ was shown to bind laminin $\alpha 4$ LG4-5 and is therefore suggested as a potential receptor for laminin $\alpha 4$ LG4-5.

The extraocular muscles are selectively spared from muscle wasting in some muscular dystrophies, including a mouse model of muscular dystrophy arising from complete absence of laminin $\alpha 2$ chain. This model was used to identify the cause of this exclusion. Immunohistochemical and quantitative PCR studies suggested that extraocular muscles were spared due to high expression of the laminin $\alpha 4$ chain, which could enable a sustained activation of the integrin $\alpha 7\beta 1$.

In conclusion laminin isoform transition and processing of chains appear to occur in several of societies major diseases.

List of papers

This thesis is based on the following papers referred to in the text by their Roman I-III

I. Nyström, A., Sasaki, T., Talts, J.F., and Hultgårdh-Nilsson, A. (2006) Changes in vascular smooth muscle cell laminin expression in the transition from healthy aorta to atherosclerotic plaques.

II. Nyström, A., Yu, H., Ekblom, P., and Talts, J.F. (2006) The natural extracellular matrix cleavage product, laminin α 4LG4-5, is a novel endogenous angiogenesis inhibitor, lamstatin-4. *Submitted*.

III. Nyström, A., Holmblad, J., Pedrosa-Domellöf, F., Sasaki, T., Durbeej, M. (2006) *Extraocular muscle is spared upon complete laminin α 2 deficiency: Comparative expression of laminin and integrin isoforms. *Matrix Biol.* 25(6):382-5.

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Abbreviations	6
Introduction	7
Basement membranes and laminins	7
A historical preface	7
Basement membrane structure	8
Laminins	9
Laminin structure	10
Laminin globular (LG) domain	12
Laminin isoforms	14
Laminin α 1 chain containing laminins	15
Laminin α 2 chain containing laminins	15
Laminin α 3 chain containing laminins	16
Laminin α 4 chain containing laminins	16
Laminin α 5 chain containing laminins	19
Laminin β and γ chains	20
Laminins in disease	20
Laminin receptors	22
Dystroglycan	22
Integrins	23
The vascular system	26
Hierarchy and constituents	26
Vascular development during embryogenesis	27
Vascular remodelling in the adult	28
Blood vessel formation in the adult body	28
Angiogenesis in the adult	29
Angiogenesis inhibitors	30
Tumour angiogenesis	32
Atherosclerosis	33
Specific aims of the study	35
Results and discussion	35
Laminin presence in blood vessels	35
Laminin expression in vascular smooth muscle cells (Paper I)	35
Release of laminin α 4LG4-5 (Paper II)	35
Shifts in laminin expression from healthy to diseased tissue	39
Laminin expression in atherosclerotic plaques (Paper I)	39
Laminin α 4 functionally compensates for absence of laminin α 2 in laminin α 2 deficient extraocular muscle (Paper III)	40
Concluding remarks and future perspectives	43
Populärvetenskaplig sammanfattning	48
Acknowledgements	50

Abbreviations

Ang	Angiopoietin
ApoE	Apolipoprotein E
bHLH	basic helix-loop-helix
BM	basement membrane
CMD	congenital muscular dystrophy
DGC	dystrophin glycoprotein complex
ECM	extracellular matrix
EHS	Engelbreth-Holm-Swarm
EOM	extraocular muscle
EPC	endothelial progenitor cells
ERK	extracellular signal-regulated kinase
FAK	focal adhesion kinase
FGF2	fibroblast growth factor 2
HIF	hypoxia inducible factor
HUVEC	human umbilical vein endothelial cells
Hsp	heat shock protein
IBEC	immortalised brain endothelial micro vessel cells
ILK	integrin linked kinase
LDL	low density lipoprotein
LG	laminin globular
LN	laminin N-terminal
LM	laminin
MAPK	mitogen activated protein kinase
MEK	mitogen-activated protein kinase
MMP	matrix metalloproteases
NC	non-collagenous domain
NO	nitric oxide
PDGF	platelet derived growth factor
PI3K	phosphoinositide 3'-kinase
PKA	protein kinase A
VEGF	vascular endothelial growth factor
VHL	von Hippel Lindau tumor suppressor
VSMC	vascular smooth muscle cell

Introduction

This work concerns aspects of the pathology of the two major causes of death in western societies, cancer and atherosclerosis. A third severe inherited disorder, a congenital muscular dystrophy, is also studied. What connects these apparent widespread and non-converging investigations are the group of molecules, the laminins, which I have pursued studies on. For a multi-cellular organism to function as a whole, cells within that organism have to communicate with each other. Cells constantly send and receive signals, either via soluble factors, direct cell-cell contacts or by the extracellular matrix. The extracellular matrices are specialised to meet different demands. Basement membranes (BM) are extracellular matrices that are in direct contact with cells. Laminins (LM)s are one of the principal building blocks of the BMs. They are a family of so far 16 different heterotrimers and these largely have a developmental and tissue specific distribution. In the first paper I have characterised and studied functional aspects of the differences in laminin expression in healthy and atherosclerotic vessels. The other two works concerns functional aspects of the major vessel specific LM subunit, the LM α 4 chain.

Basement membranes and laminins

A historical preface

The insight that a body consisted of cells and those were the basis of life appeared late in history. Up until the early 19th century, fibres of connective tissues that spontaneously replicated were considered to be the foundation of existence. That such a theory arose is not surprising considering that an adult body consists of about 80 % extracellular proteins and just 20 % cells. Therefore, it was easy for early scientists with crude methods to observe connective tissues, given its name (Bindegewebe) by the Prussian anatomist and physiologist Johannes Müller 1830, as structural parts that provided form, joined parts of the body and built tubing for movement of air and blood. With development of techniques and microscopes carrying better resolution, cells within the connective tissue could be observed, and the cellular theory of life shot off. By the mid 19th century it had become apparent that gelatine extracted from different sources of cells or tissues were of the same substance, this substance was named collagen - Greek for glue producing. Extracellular matrix as a term for the non-cellular part of the connective tissue and other tissues was introduced during the 1930's (Piez, 1997).

In 1842 the English surgeon William Bowman, while investigating and comparing kidneys from different species, described epithelial cells sitting on an extracellular flat structure, which he called a basement membrane (BM) (Bowman, 1842). This was not the first report of BMs, he had actually two years earlier described the existence of BM in muscle, but that structure he named sarcolemma

(Bowman, 1840). More than a century after their initial observation, in the beginning of the 1950's, Krakower and Greenspon performed the first biochemical studies on BMs. These studies done on glomerular BMs revealed that it consisted of collagenous and non-collagenous glycoprotein parts (Kefalides, 1978). With the development of electron microscopy the BM could be seen to appear as three discrete layers, an electron dense layer - the lamina densa - sandwiched between two electron sparse layers, the lamina lucida close to the cells, and the lamina reticularis close to the fibrous connective tissue.

Basement membrane structure

BMs are thin extracellular matrices that underlie all epithelia and endothelia and surround many cell types such as muscle, fat and peripheral nerve cells (Nguyen and Senior, 2006). In general, BMs are thin (30-70 nm). However, the glomerular BM and the lens capsule BM are extraordinarily thick (about 100 nm). This is the reason why many of the initial studies on BMs were performed on these two BMs (Kefalides, 1978). An abnormal thickened BM due to imbalance in the production and degradation are seen in major diseases like diabetes and asthma (Nihlberg et al., 2006; Tsilibary, 2003). In cancers, a loss of the BM between the tumour and connective tissues is often a sign of poor prognosis, this indicates more aggressive and invasive cancers (Patarroyo et al., 2002).

The BMs function as signalling units via interactions with cell surface receptors, they modify signalling by presenting growth factors, and they act as structural elements of tissues. BMs regulate cell migration, proliferation, survival, and differentiation. Furthermore, they provide tissue stability and create compartmentalisation of tissues and cell types (Sasaki et al., 2004; Timpl, 1996). As anticipated when considering the diverse effects of BMs, their molecular compositions are heterogeneous and organ specific. Their major building blocks are various isoforms of laminins (LMs), type IV collagens, nidogens and the heparin sulphate proteoglycan perlecan. These provide the molecular foundation of most BMs. The BMs can in addition, depending on the specific tissue, hold contributions from e.g. the heparin sulphate proteoglycan agrin, fibronectin, fibulins (calcium binding proteins with affinity for many BM proteins), collagen XVIII (which can be considered as a hybrid of collagen and heparin sulphate proteoglycans), collagen XV and collagen XIX (Iozzo, 2005; Yurchenco et al., 2004). BMs are considered to be composed of two self-polymerising meshworks, a collagen type IV and a LM network. These are held together by nidogens and heparin sulphate proteoglycans to stabilise the BM.

There are genetic and molecular biological evidence that formation of an initial LM network attached to the cell surface via receptors is essential for the creation of BMs. This LM network functions as a scaffold to attract other BM components (Li et al., 2003). Many of the studies on the involvement of different molecular components in BM assembly have been done on embryoid bodies.

Embryoid bodies can be used as a culture system mimicking the early events of embryogenesis *in vitro*; here an initial outer endoderm is formed on aggregated embryonic stem cells. The endoderm synthesizes and lays out an extraordinarily thick BM (250-1500 nm), which polarises and induces inner cells adjacent to the BM to form epiblast. *In vivo* the epiblast would make up the proper embryo. Embryoid bodies with reduced synthesis of LMs or deficient in LM γ 1-chain (a component present in all early embryonic LMs) fail to form BMs although type IV collagen and nidogen were produced (Aumailley et al., 2000; Li et al., 2002; Smyth et al., 1999). Disruption of LM network polymerisation by chemical treatment or by addition of LM fragments inhibiting polymerisation cancelled BM formation (Li et al., 2002). However, gene deletion studies indicate that formation of BM as such is not crucial for early development, but rather signalling through polymerised LMs is. Mice deficient in either nidogens, perlecan, or type IV collagens succeed early gestation, but die when the physical stress on specific organs increase (Bader et al., 2005; Costell et al., 1999; Poschl et al., 2004), while absence of embryonic LM components leads to failure to complete early development (Miner et al., 2004; Scheele et al., 2005; Smyth et al., 1998). All these studies indicate that LMs are the foremost cell signaling molecules in embryonic BMs. Extrapolation would also suggest them to have this role also in adult tissues.

Laminins

LMs are large glycosylated heterotrimeric proteins with multi domain structure. They were first isolated 1979 from the murine Engelbreth-Holm-Swarm (EHS) tumour, which produces great amounts of BM proteins. Localisations of LMs are largely but not absolutely confined to the BMs (Colognato and Yurchenco, 2000; Talts et al., 2000). Each LM trimer is composed of an α -, a β -, and a γ -chain. There are eleven characterised LM chains in mammals of which five are α (LM α 1- α 5), three are β (LM β 1-3), and three are γ (LM γ 1-3) chains. A fourth LM β gene exists (Miner and Yurchenco, 2004), but it has not yet been further studied. It is flanking the LM β 1 gene on the human chromosome 7 and shares strong identity with this gene on the proposed mRNA level and moderate similarity on the proposed amino acid level. The adjacent localisation and the similarity of the two LM β genes, suggest that the LM β 4 gene has arisen from duplication of the LM β 1 gene. So far, there are no reports supporting that the LM β 4 gene is transcribed or translated. It is likely that the LM β 4 gene is just a silenced duplication of the LM β 1 kept in the genome. The LM trimers are named after their chain composition with the α chain followed by the β and γ chains, e.g. LM 521 made up of α 5 β 2 γ 1, according to a recent nomenclature (Aumailley et al., 2005). Previously they were largely named after the order of discovery i.e. the first presented LM, LM 111, was named LM-1 etc.

Laminin structure

LMs carry a cross or T-shaped structure depending on the chain content. The N-terminal parts of the chains each make up one of the three short arms. Two α -chains, the LM α 3A chain that is a splice variant of the LM α 3 chain and the LM α 4 chain (Figure 2), have shortened N-terminal parts giving the trimers they participate in more T than cross shaped appearances. The C-terminal parts of the LM β and γ chains and the mid part of the α chain make up an α -helical coiled-coil trimeric long arm, and the most C-terminal region of the α chain is freely exposed to interact with many LM receptors (Tunggal et al., 2000). LM α , β , and γ chains, to a large extent, share a common domain structure. Presence or absence of discrete domains can differ in the chains. LMs are large molecules, this makes it difficult to study the function of different parts of the molecules by use of intact LM trimers. To circumvent this hindrance, discrete LM domains or regions have been recombinantly produced.

At the N-terminus of all LM chains but LMs α 3A, α 4 and γ 2 a single globular laminin N-termina (LN)-domain (previously known as domain VI) can be found (Figure 1). These domains are important for LM self-assembly, they interact with each other to create a meshwork. LN domains from α chains also interact with integrin receptors (Colognato and Yurchenco, 2000). The interactions between LN-domains are somewhat calcium dependent as binding calcium ions are thought to provoke conformational changes helping the polymerisation. Previously, there were thought to be many restrictions in which other LN domains a specific LN domain can interact with. These restrictions have been studied with recombinantly expressed fragments and revealed that more interactions are possible than previously figured (Odenthal et al., 2004). The α and β LN-domains show wide binding spectra and can even interact with homologous domains, the exception being the LN β 1 domain. The γ 1 and γ 3 LN-domains show much more restricted binding and never occur in homologous pairs. The ability to form LM meshworks is thought to be essential for BM creation. LMs α 4 and α 3A lack the LN domain and are considered as non-polymerising LMs and are therefore believed unable to create a BM without support from other α -chains. This idea was strengthened by studies on embryoid bodies deficient in LM γ 1. These do not form a BM by themselves, as previously described. The exogenous addition of LMs containing α 1 or α 2 could create a BM, but addition of LMs with α 3A or α 4 could not support the formation of BMs. The idea of LM self-assembly dependence on LN domains is further strengthened in that the dystrophic mice dy2J lacking parts of the α 2 chain LN domain has impaired BM formation. However, antibodies against LM α 3 or α 4 show intensive staining in pericellular regions reminiscent of BM as seen in (Hager et al., 2005) and colocalise with BM components also when they are the only LM α chain expressed e.g. the LM α 4 chain in neonatal vascular BM (Thyboll et al., 2002).

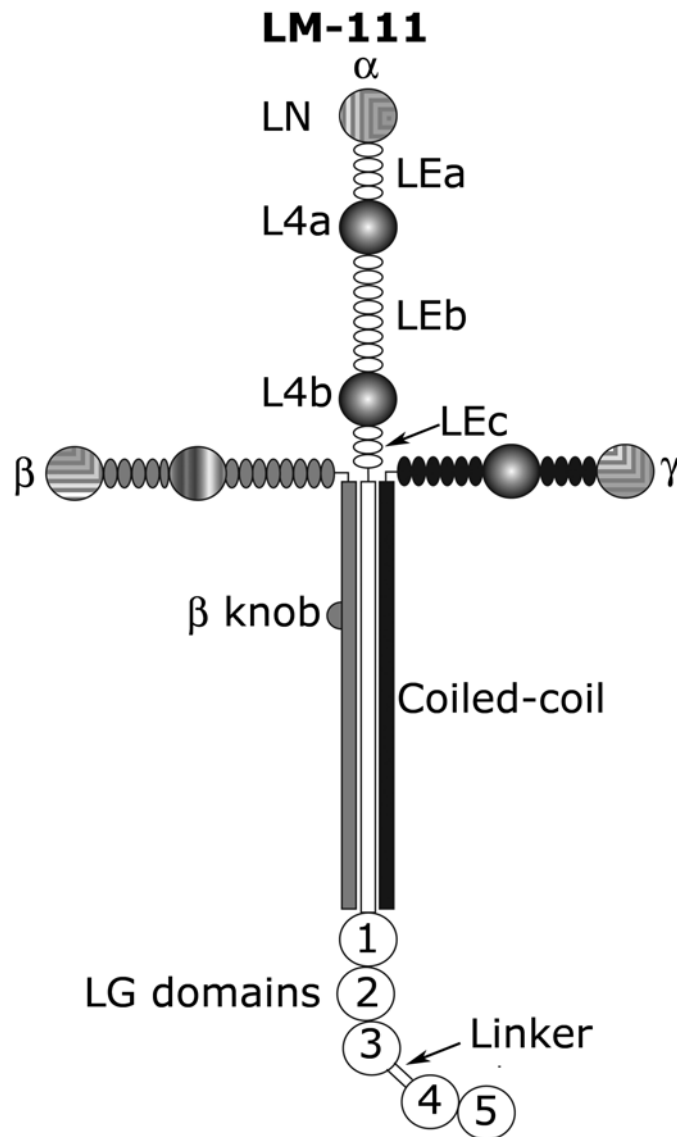


Figure 1. Structural model of the domain structure of laminin 111, abbreviations used are: LN laminin N-terminal, LE laminin epidermal growth factor domain, L4 laminin domain 4, LG laminin globular domain.

In the short arms, C-terminal to the LN domain lies LM epidermal growth factor like repeats abbreviated LE. The first LE-repeat (LEa) is interrupted by a globular domain, which is named L4a. L4a is homologous for the LM $\alpha 1$, $\alpha 2$, and the three γ chains. The L4a domains of LM $\alpha 3B$ and $\alpha 5$ show homology with L4a domains of LM $\beta 1$ and $\beta 2$, although the C-termini differ. Towards the LM chain C-termini lies another

LE repeat (LEb). In this region, for the LM $\gamma 1$ chain, a binding site to nidogen has been mapped (Mayer et al., 1993). After LEb lies another globular domain L4b. In LMs $\alpha 1$, $\alpha 2$, $\alpha 3B$ and $\alpha 5$, this sequence is continued with a third LE stretch (LEc). Apart from the nidogen interaction little is known about the function of L4 globules and LE repeats. After this sequence, the three short arms gather to form the long arm. In this long arm, α -helices from the three chains interact to make up a triple-helical coiled-coil rod domain. In LM β chains the helical structure is shortly interrupted with a loop, called β knob (Aumailley et al., 2005; Tunggal et al., 2000).

Laminin globular (LG) domain

LM α chains carry a tandem of five LM globular (LG1-5) domains at the C-terminus. The LG domain is not unique as such for the LMs. In fact, about 80 different LG domains have been identified in extracellular and transmembrane proteins. However, the organisation with five LG domains in a row without interruptions by other modules is unique (Timpl et al., 2000; Tisi et al., 2000). Between the LG3 and the LG4 domains is a short (23-31 amino acid residues) linear linker region inserted. Therefore, the LG1-3 and LG4-5 domains are sometimes considered as two units. The linker can be proteolytically cut in many α chains. Much of LMs signalling activity is attributed to LG domains. LM α LG1-3s are classically considered to contain integrin binding sites while the LM α LG4-5 for the $\alpha 1$ and $\alpha 2$ chains interact with α -dystroglycan (Sasaki et al., 2004). Both LG1-3 and LG4-5 domains have affinity for heparin. Each LG domain includes 180-210 amino acid residues. This means that the whole stretch of LG domains contain about one third of the amino acid residues in α chains with a full length N-terminus and about half for LM $\alpha 3A$ and $\alpha 4$. Most of the LG domains are N-glycosylated and this can explain the discrepancies in receptor affinity between mammalian and bacterially produced recombinant domains e.g. bacterially produced $\alpha 4$ LG1-2 has strong affinity for integrin $\alpha v\beta 3$ (Gonzalez et al., 2002) while $\alpha 4$ LG1-3 expressed in a mammalian system does not interact with integrin $\alpha v\beta 3$ (Yu and Talts, unpublished observations).

The crystal structure has been solved for LM $\alpha 2$ LG4-5 and from this a model for the complete LG domain tandem has been proposed (Tisi et al., 2000). The domains are spherical with a diameter of about 3.5 nm, made up of 14 β strands that are divided into two β sheets creating an antiparallel β sandwich. The β sandwich is bent giving it a concave and convex side. The C-terminal end of the β sandwich is stabilised by two cysteine residues creating a disulfide bridge. The N- and C-termini of the domain lay in close connection. This is quite rare for repeated modules, which normally have the N- and C-termini at opposite ends (Timpl et al., 2000). This unusual feature has implications on the whole LG domain tandem structure, giving the hypothetical model of the LG1-3 domain a cloverleaf shape. Further, the crystal structure of the $\alpha 2$ LG4-5 tandem revealed that the linker region was well integrated

with these domains forming a disulfide bond with LG5. The path of the linker puts LG5 in closer contact with LG1-3 domain, leaving LG4 at the tip of the α chain.

The crystal structure also revealed a binding site for calcium ions provided by aspartic acid residues at the edge of the β sheets of both the LG4 and the LG5 domains (Tisi et al., 2000). These sites have been implicated in binding to the LM receptor α -dystroglycan. Primarily, the calcium ion and surrounding positively charged amino acid residues in LG4 for both LM α 1 and LM α 2 seem important for this binding (Andac et al., 1999; Tisi et al., 2000; Wizemann et al., 2003). However, for LM α 1 the LG4 domain alone was sufficient for α -dystroglycan binding (Andac et al., 1999; Talts et al., 1999), while α -dystroglycan also required contributions from basic residues in the LG5 domain for LM α 2 interaction (Wizemann et al., 2003). The suggestion that calcium ions are important for dystroglycan binding is further strengthened by the fact that α 4LG4-5 and α 5LG 4-5 both lack the aspartic acid residues necessary for binding calcium and that these LM chains interact only weakly with α -dystroglycan (Talts et al., 2000; Timpl et al., 2000; Yu and Talts, 2003).

Heparin and sulphatide-binding were the first interactions recognised for LG domains (Ott et al., 1982; Taraboletti et al., 1990). These properties enable interaction with heparin sulphate containing receptors such as syndecans or allow sulphate and sugar groups at the cell surface to work as co-receptors for LMs. In the case of sulphatides, this interaction is also suggested to participate in LM network polymerisation (Kalb and Engel, 1991; Li et al., 2005). There is variability in the heparin affinity of LG domains for different LM α chains. However, the LG4 domain has the strongest heparin affinity in all α chains except for the LM α 2 chain where LG4 interacts weakly with heparin and LM α 2LG5 possess substantial heparin binding (Talts et al., 1999; Utani et al., 2001; Yamaguchi et al., 2000; Yu and Talts, 2003). Epitopes and basic amino acid residues interacting with heparin have been mapped by site-directed mutagenesis of LM α 1LG4 to regions close to the calcium-ion binding site also known to be important for dystroglycan binding. The sites important for heparin binding in LM α 1LG4 are also active for the closely related LM α 2LG4 heparin interactions (Wizemann et al., 2003), whereas site-directed mutagenesis studies on LM α 4LG4 revealed a more spread distribution and localisation of the lysine residues responsible for heparin binding (Yamashita et al., 2004). By competition studies for heparin binding with synthetic peptides, the major binding sites for heparin in LM α 3 LG4 and α 5 LG4 were mapped towards the opposite end of the LG domain compared to the LM α 1 LG4 and α 2 LG4 (Nielsen et al., 2000; Utani et al., 2001).

Recombinantly produced LM α LG1-3 domains for LM α 2, α 4, and α 5 are adequate to support integrin mediated cell binding (Talts et al., 1999; Talts et al., 2000; Yu and Talts, 2003). For the LM α 1 chain, the LG1-3 domain may not be sufficient to support cell adhesion as contribution from parts of the rod domain have been reported to be necessary for cell binding (Deutzmann et al., 1990; Sung et al., 1993).

Proteolytic processing can modulate LG domains. The LM $\alpha 2$ chain is cut in the LG3 domain by a furin-like convertase. However, the resulting domain consisting of half of LG 3 to LG5 is not released from the remaining LM $\alpha 2$ but stays non-covalently associated with the chain. This has been observed both for recombinantly produced LG 1-3 domains, as well as for placental preparations of LM $\alpha 2$ containing LMs (Brown et al., 1994; Smirnov et al., 2002; Talts et al., 1998). The cleaved form stimulated acetylcholine receptor clustering on muscle cells, whereas the uncut form did not (Smirnov et al., 2002). LM $\alpha 3$ is processed in the linker region releasing the LG4-5 domain (Marinkovich et al., 1992; Tsubota et al., 2005). The biological function of this cleavage is unknown and studies with the cleavage site mutated have given contradictory results. One study indicated a role in stimulating migration of keratinocytes (Tsubota et al., 2005), whereas another showed no such effect (Baudoin et al., 2005). Mouse LM $\alpha 4$ LG4-5 has three potential sites for proteolytical processing in the linker between LG3 and 4. Two of these sites were shown to be major cleavage sites with minor contributions from the third one (Talts et al., 2000). Human LM $\alpha 4$ LG4-5 has not the exact same sequence, but still potential proteolytical processing has been reported for the human LM $\alpha 4$ chain (Fujiwara et al., 2001; Geberhiwot et al., 1999). The cut $\alpha 4$ LG4-5 is not associated with LM $\alpha 4$ or contained within the vicinity of the BM, but lost from the tissue (Talts et al., 2000). One report show the LM $\alpha 4$ LG4 domain to be present in skin, but did not further deal with if the LG4 domain was associated with the LM $\alpha 4$ chain or not (Matsuura et al., 2004). The LM $\alpha 5$ chain linker region contains sites for furin-type cleavage (Timpl et al., 2000). However, there has not to my knowledge, up till today been any reports showing that such processing occurs. Known is however that LM $\alpha 5$ exists in several different sizes in mouse, but what this is due to needs to be clarified (Miner et al., 1997). Lastly, the LM $\alpha 1$ chain, which was previously thought to be excepted from proteolytical modification (Timpl et al., 2000) was recently suggested to be processed based on immunostainings that detected free LM $\alpha 1$ LG4-5 in the ectoplacental cone (Scheele et al., 2005).

Laminin isoforms

To date 16 different LM trimers have been presented, if the two different forms of LM 332 made up by the splice variants LM $\alpha 3A$ or B are included (Aumailley et al., 2005). The low number of known LMs compared to the hypothetical maximum number of 45 ($5\alpha \cdot 3\beta \cdot 3\gamma$) indicates that there are restrictions in the trimerisation of different LM chains. For example it has been suggested that there are restrictions in the trimerisation of different LM chains. To a large extent the LMs have a developmental and tissue specific distribution (Miner and Yurchenco, 2004). As a majority of the receptor binding activity lies in the LG domains of the α chains, much of LMs functions can be attributed to the α chain. Therefore, it is natural to distinguish different LMs by their α -chain content.

Laminin α 1 chain containing laminins

LMs containing the α 1 chain shown to exist are LM 111 and 121 (Aumailley et al., 2005). The LM 111 was the first LM identified (Chung et al., 1979; Timpl et al., 1979), it is the most biochemically analysed and studied LM. This is due to that the EHS tumour has a high production of this LM, which has made LM 111 easy to obtain. However, LM α 1 is also the most restrictively expressed α -chain in the adult and can primarily be considered as an embryonic LM (Ekblom et al., 2003). LM α 1 is expressed early after fertilisation already at the 16-cell stage (Hogan, 1994). The α 1 chain is crucial for early embryonic progression, with complete deficiency leading to failure before gastrulation in mice. This is possibly due to defects caused by absence of the extra embryonic BM present in rodents, the Reichert's membrane, as the embryonic BM was present in the LM α 1 deficient mice due to compensation by LM α 5. The embryonic BM formed appeared to be functional as the embryonic ectoderm was organised and had cavitated (Alpy et al., 2005; Miner et al., 2004). Further, it was recently shown that extra embryonic expression of LM α 1 was sufficient for development of otherwise LM α 1 depleted mice. These mice were viable and macroscopically normal (Ogawa et al., 2005). However, signalling by the LM α 1LG4-5 domain (known as E3) also seems to be important for embryonic development. Absence of this domain leads to failure of the embryonic ectoderm to polarise and cavitate, vital steps for early development. During organ development LM α 1 is involved in epithelial morphogenesis for most tissues (Ekblom et al., 2003). In the adult, LM α 1 expression is rare and mainly found in some epithelial BMs, e.g. the proximal tubules of the kidney, ovary, testis and in the parenchymal BM, a second BM lining brain blood vessels (Falk et al., 1999; Sixt et al., 2001). Not much has been reported regarding LM 121. It has been found in human placenta (Champlaud et al., 2000).

Laminin α 2 chain containing laminins

LM α 2 is the major α chain of skeletal and heart muscle BMs. It has been shown to exist in the trimers LM 211, 221 and 213. Contributions of both LM 211 and 221 are seen in mature muscle BMs, with LM 221 being enriched in mature neuromuscular junctions (Patton, 2000). As for the LM 121 isoform, LM 213 has been found in placental LM preparations. Genetic and immunohistochemical analysis also suggest that the LM 213 is present in the testicular BM (Hager et al., 2005).

LM α 2 is mainly expressed in BMs of mesodermally derived cells or tissues with large mesodermal contributions. Apart from muscles, LM α 2 is also highly present in e.g. peripheral nerves, testis, thyroid and adrenal glands (Sasaki et al., 2002a). During mouse development the expression begins after embryonic day 11 (Sasaki et al., 2002a). LM α 2 is similar to the α 1 chain, with a 72% identical nucleotide sequence and 45% identical amino acid sequence. The similarity between

the $\alpha 1$ and $\alpha 2$ chains is further revealed by the fact that the LM $\alpha 1$ chain can functionally compensate for the absence of LM $\alpha 2$ *in vivo* (Gawlik et al., 2004).

Laminin $\alpha 3$ chain containing laminins

Splicing of the LM α chains occur in at least the LM $\alpha 2$, $\alpha 3$, and $\alpha 4$ chains. LM $\alpha 3$ is expressed in two splice variants as discussed earlier, the LM $\alpha 3A$ with a truncated N-terminal or the $\alpha 3B$ with an extended N-terminal. There has also been an intermediate length transcript of LM $\alpha 3$ reported (Miner et al., 1997). Both the A and B forms can be extensively proteolytically processed in the N-terminal as well as in the C-terminal ends (Aumailley et al., 2003). LM $\alpha 3$ is primarily expressed in stratified epithelium in the trimers LM 332 and LM 311 (Ekblom et al., 2003). They participate in formation of anchoring filaments binding to hemidesmosomes, attaching the epidermis to the dermis. The $\gamma 2$ chain has been thought to be unique for LM 332, although there are indications that it participates in an additional trimer, the partially described LM 522 (Aumailley et al., 2005) and from studies in nerve there are indications that LM 121 or 122 and LM 212 or 222 are also possible. All chains of LM 332 can be extensively proteolytically processed, but the biological effects of processing are unknown (Aumailley et al., 2003). Apart from the skin, LM $\alpha 3$ is also highly expressed in intestine and lung (Miner et al., 1997). In lung, LM 311 complexed with nidogen and perlecan interacts with dystroglycan to sense mechanical force (Jones et al., 2005). The third identified LM $\alpha 3$ containing trimer, LM 321, was identified from human amnion and has subsequently been shown to be expressed in epithelial structures of teeth (Champlaud et al., 1996; Oksonen et al., 2001)

Increased LM-332 expression, or more frequently great LM $\gamma 2$ syntheses alone have been reported as prognostic markers for aggressive carcinomas and their invasion (Katayama and Sekiguchi, 2004). LM 332 is principally an epithelial LM and carcinomas arise from epithelial cells. Carcinoma cells lack hemidesmosomes. This deficiency enables them to migrate, and their migration is in addition supported by excessive production and deposition of LM 332 (Katayama and Sekiguchi, 2004). Further, there are reports that the cleaved off LM $\gamma 2$ short arm stimulates migration as a soluble factor (Miyazaki, 2006).

Laminin $\alpha 4$ chain containing laminins

In the mouse embryo LM $\alpha 4$ is first expressed at around day 7 and expression peaks in late gestation at day 15-17. Initial mRNA expression studies detected LM $\alpha 4$ in organs and tissues mainly derived from cells of mesenchymal origin. High expression was seen in heart, lung, skeletal muscle and in fat cells (Iivanainen et al., 1997; Iivanainen et al., 1995; Niimi et al., 1997) and in expression studies of several LMs on multiple tissues, the LM $\alpha 4$ chain along with the LM $\alpha 5$ chain were reported to have the widest expression pattern (Miner et al., 1997). In capillary BMs of skeletal muscle, LM $\alpha 4$ is introduced at embryonic day 11 (Iivanainen et al., 1997). LM 411 is

highly expressed by endothelial cells (Frieser et al., 1997; Sixt et al., 2001) and can be found in or in the vicinity of the BM of all blood vessels (Zhou et al., 2004a). This can account for the wide expression pattern observed. Immunofluorescent staining for LM $\alpha 4$ gives BM like staining around capillaries (Nystrom et al., 2006). Given that LM $\alpha 4$ lacks the LN domain important for LM network formation (Figure 2), it is believed that LM $\alpha 4$ containing trimers cannot participate in BM formation as previously discussed (Colognato and Yurchenco, 2000). This notion is strengthened by transmission electron microscopy immunogold stainings that failed to detect localisation of the LM $\alpha 4$ chain within endothelial BMs of muscle capillaries. Instead it was found in interstitial tissues (Talts et al., 2000). However, the absence of other BM components in neonatal LM $\alpha 4$ null mouse capillary BM and the ruptured capillary BM in these mice (Thyboll et al., 2002) indicate that LM $\alpha 4$ is present in and important for formation of most vascular BMs. More studies need to be performed to clarify the apparent discrepancies.

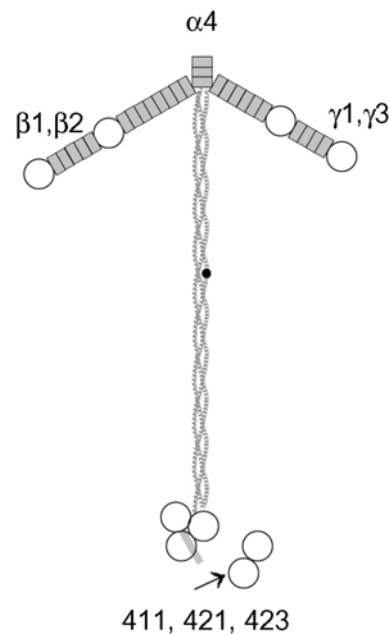


Figure 2. A hypothetical model of a LM $\alpha 4$ trimer showing the short N-terminal and the release of the LG4-5 domain.

Interestingly, LM $\alpha 4$ shows a developmentally regulated expression pattern. Replacement of LM $\alpha 4$ with other LM α chains during maturation of some tissues, as well as introduction of other LM α chains into BMs holding only LM $\alpha 4$ containing LMs, occurs. In developing embryonic skeletal muscles, LM $\alpha 4$ is expressed in the skeletal muscle BM. As the muscle matures this expression is lost and remains only at the neuromuscular junctions and in capillaries (Ringelmann et al., 1999). For intestinal smooth muscle cells, the LM $\alpha 4$ chain is up-regulated during differentiation and kept at a high level after maturation. The LM $\alpha 4$ chain is the major and in many

cases only LM α chain in late embryonic and neonatal vascular BMs (Hallmann et al., 2005). LM α 5, which is the other major vascular α chain, is at the time of birth only expressed in a few large vessels. A high LM α 4 content has been suggested to lead to formation of BMs that have looser structure due to the shortened N-terminus. This could result in a BM easier for blood cells to penetrate (Hallmann et al., 2005). It was shown that T-cells passed over capillary BMs containing LM α 4, while they did not go through BMs containing a mixture of LM α 4 and α 5 in an experimentally induced cell infiltration of the brain (Sixt et al., 2001). LM α 5 is introduced in capillary and venule BMs 3-4 weeks after birth in mice (Hallmann et al., 2005; Sorokin et al., 1997). This introduction could lead to stabilisation of the capillary bed.

Mice lacking the α 4 chain have been created. They were viable and fertile (Patton et al., 2001; Thyboll et al., 2002). However, these mice showed an increase in perinatal lethality, where about 20 % died within the first two days after birth. The mice exhibited haemorrhages at the head, neck, back, and hind limbs. There were also minor bleeding around heart and meninges. Larger vessels were unaffected and the bleedings were limited to the smaller vessels and capillaries. The excessive haemorrhages also led to anaemia. Bleedings were detected already as early as embryonic day 11, when LM α 4 makes its first appearance in the capillaries. When the mice had reached one week of age the vascular phenotype healed out. This could be explained by the introduction of LM α 5 in the capillary BMs (Thyboll et al., 2002). Normally, LM α 5 starts being expressed in these BMs at 3-4 weeks after birth. However, subsequent studies of LM α 4 null mice revealed a premature introduction of the LM α 5 chain, which was present already after the first week (Zhou et al., 2004a). Further, these mice exhibited more extensive and disorganised new blood vessel growth in an *in vivo* experimental model. They also showed increased experimental tumour growth, vascularisation, and metastasis. A later study has shown that LM α 4 deficient mice have partially malfunctioning hearts, which can cause pre-mature death. The defect is due to impaired capillary patterning leading to hypoxia. It is likely that other organs also have this vascular disorganisation, but since the heart is the most oxygen-demanding organ in the body it is in this organ the defect makes itself marked (Wang et al., 2006). All of the presented imperfections of the LM α 4 null mice could be explained by the particular importance of LM α 4 chain in regulating vascular formation.

In mature muscle, the LM α 4 chain is present at the neuromuscular junctions as previously mentioned. At this site it occurs in the LM 421 trimer. The LM α 4 deficient mice also displayed mild neuromuscular defects in that some dragged their hind limbs when moving or slipped in a beam-walking test (Patton et al., 2001). Synapses in these mice were present and properly formed, but there were subtle morphological defects in the neuromuscular junctions (Patton et al., 2001). An additional neural imperfection was that the axons were not always properly myelinated (Wallquist et al., 2005). A third LM α 4 trimer, LM 423, has been reported from studies on retinal matrix (Libby et al., 2000).

Two mRNA splice variants of the LM $\alpha 4$ chain exist. They differ in a seven amino acid insertion toward the N-terminal end and show differences in supporting adhesion of glial tumour cells (Hayashi et al., 2002).

The LM $\alpha 4$ chain is up-regulated in vasculature of human gliomas, a tumour of glial cells (Ljubimova et al., 2001). Expression of LM $\alpha 4$ was also correlated with the prognosis of the disease; more aggressive tumours had a higher LM $\alpha 4$ content. Moreover, there was a switch in the $\alpha 4$ trimer content related to the malignancy of the tumour. Benign tumours expressed the LM 421 trimer, while more malignant tumours expressed LM 411 (Ljubimova et al., 2001). Antisense RNA directed against LM $\alpha 4$ or LM $\beta 1$ have been able to reduce tumour vascularisation in rats (Fujita et al., 2006). An up-regulation of LM $\alpha 4$ has also been described for benign enlargement of the prostate (Luo et al., 2002).

Many blood cells such as thrombocytes, monocytes, T-cells, and neutrophils have been reported to synthesize LM $\alpha 4$ (Geberhiwot et al., 2001; Geberhiwot et al., 1999; Pedraza et al., 2000). However, in the studies on monocytes, expression of LM $\alpha 4$ was very low, as mRNA could only be detected by nested PCR and the protein only visualized in immunoblots after immunoprecipitation (Pedraza et al., 2000). In neutrophils, LM $\alpha 4$ promoted cell migration and protected these cells from apoptosis (Wondimu et al., 2004). Induced infiltration of neutrophils was reduced in mice lacking LM $\alpha 4$ (Wondimu et al., 2004).

Laminin $\alpha 5$ chain containing laminins

The LM $\alpha 5$ chain is the most widely expressed LM α chain in the body (Ekblom et al., 2003). It is the largest LM α chain and this α chain is to some extent more similar to the partially identified amino acid sequence of the only known α chain in hydra than the LM $\alpha 1$ or $\alpha 2$ chains are. This indicates that LM $\alpha 5$, among the mammalian α chains, is most similar to the ancestral LM gene (Zhang et al., 2002). By comparing the two LM α genes in drosophila with the mammalian α chain genes it is also evident that a duplication of the ancestral LM α chain occurred early in metazoan evolution. This separated the gene giving rise to LM $\alpha 1$ and $\alpha 2$ chains from the gene giving rise to LM $\alpha 5$ and later to the LM $\alpha 3$ and $\alpha 4$ chains (Miner et al., 1995; Miner and Yurchenco, 2004).

LM $\alpha 5$ is expressed early in development and the embryonic BMs are rich in this LM (Miner et al., 2004). This chain by itself is not exclusively important for early embryonic development as mice deficient in LM $\alpha 5$ survive early development and die between embryonic days 13.5 and 16.5. The null mice exhibit failure of neural tube closure, placental defects, shortened limbs and failure to separate digits. During mid embryonic development, LM $\alpha 5$ expression is reduced and only sustained in subsets of BMs, to be up-regulated in late development and after birth (Miner et al., 1998). This pattern of LM $\alpha 5$ regulation is not uniform for all organs. Embryonic skeletal muscles have a high LM $\alpha 5$ content. This is strongly reduced after birth, leaving LM $\alpha 5$ chain mainly in the neuromuscular junctions in mature muscles (Miner

and Yurchenco, 2004; Ringelmann et al., 1999). The opposite is seen for blood vessel maturation as earlier stated.

The LM $\alpha 5$ chain is along with the LM $\alpha 4$ chain, as previously discussed, the major endothelial LM. Recombinant LM 511 sustains endothelial cell migration better than LM 411 (Doi et al., 2002). This fact together with subsequent studies on tumour growth and blood vessel development in mice lacking the LM $\alpha 4$ chain (these mice have an increased expression of LM $\alpha 5$ in endothelial BM) has led to the notion that LM $\alpha 5$ is more pro angiogenic than LM $\alpha 4$ (Zhou et al., 2004a). This idea is somewhat contradicted by the fact that LM $\alpha 5$ expression is coordinated with vessel maturation.

LM $\alpha 5$ is present in two major forms, LM 511 and 521. LM 521 is, just as LM 421, present in more static structures as e.g. the neuromuscular junction and mature glomerular BMs. LM 523 is as LM 423 present in the retinal matrix (Libby et al., 2000) and a 522 form has also been suggested to exist but has not otherwise been well characterised (Aumailley et al., 2005).

Laminin β and γ chains

The LM $\gamma 1$ chain is along with the LM $\beta 1$ chain the most widely expressed LM chain in the body (Sasaki et al., 2002b; Tunggal et al., 2000). These are the only β and γ chains expressed before gastrulation and deficiency in either of these leads to very early embryonic lethality at embryonic day 5.5 due to failure to form a BM (Miner et al., 2004; Smyth et al., 1999). The LM $\beta 2$ chain was initially found in synapses. It has a wide pattern of expression and can be found in many organs such as kidney glomerular BMs, thymus, heart, lung and vascular smooth muscle cells etc (Glukhova et al., 1993; Ljubimova et al., 2001; Sasaki et al., 2002b). Both LM $\beta 3$ and $\gamma 2$ are primarily incorporated into LM 332, which is important for creating anchoring filaments for keratinocytes (Aumailley et al., 2003). The LM $\gamma 3$ chain is the most recently identified LM chain (Iivanainen et al., 1999; Koch et al., 1999). It was first reported to be present at non BM sites (Koch et al., 1999), but subsequent studies have shown association with the BM (Gersdorff et al., 2005; Hager et al., 2005). LM $\gamma 3$ is expressed in testis, kidney, some sites in the brain, regions in the head, skin BMs, and hair follicles. Although expressed in several organs, the amounts it is present in is minute compared to the LM $\gamma 1$ chain (Gersdorff et al., 2005).

Laminins in disease

Imperfections in the LM chains can lead to severe congenital diseases. Null-mutations for LM $\alpha 5$, $\beta 1$ and $\gamma 1$ chains are embryonic lethal in mouse and therefore it is likely to assume that this is also the case in humans. LM $\alpha 1$ deficiency is also fatal in mice, however this seems to be due to failure of the rodent specific Reichert's membrane (Miner et al., 2004). This makes it difficult to speculate whether lack of the LM $\alpha 1$ chain also cause failure in human development. The LM $\alpha 4$ chain deficient mice show a relatively mild and partially transient phenotype. This gene has not yet been linked

to any human diseases, although there has been a suggestion made that impairment of the LM α 4 chain could account for cases of sudden deaths due to heart failure (Wang et al., 2006). LM γ 3 null mice have not yet been created and no human disease has been connected to malfunction of this gene. The γ 3 chains low level of expression and its similarity to the γ 1 chain suggest that a severe phenotype of γ 3 null mice is unlikely.

Partial or complete deficiency of the LM α 2 chain leads to inherent muscle wasting called congenital muscular dystrophy (CMD) type 1A. From calculations on data from an Italian study on the incidence of all CMDs, the estimated incidence in Europe for CMD type 1A is about 1.4 per 100 000 live births (Miyagoe-Suzuki et al., 2000; Mostacciuolo et al., 1996). Clinical signs of LM α 2 deficient muscular dystrophy are seen with an early onset. The symptoms vary with complete or partial deficiency. Infants can show severe hypotonia and can have problems nursing due to weak sucking. The muscle strength improves slowly after birth and some patients are able to sit upright at 2-3 years of age. The disease can also affect neural functions and patients can suffer from spasms and epilepsy. Eventually the disease is fatal, with death occurring within the first two decades for most patients (McGowan and Marinkovich, 2000; Voit, 1998). There are spontaneously arisen mouse models with partial LM α 2 deficiency. The *dy* mice that produce lower levels of LM α 2 and the *dy*^{2J} mice that express LM α 2 with a shortened N-terminus ((Miyagoe-Suzuki et al., 2000). These mice show less severe symptoms than the created *dy*^{3K} LM α 2 null mice that die within the first 5 weeks after birth (Miyagoe et al., 1997).

Deficiency in either of the chains in LM 332, important for anchoring the epidermis to the dermis, leads to the severe skin blistering disease junctional epidermolysis bullosa (JEB). Most mutations have been linked to the LM β 3 gene. The fatality of the disease depends on how dysfunctional the LM chains are. In the most severe form of JEB 40 % of the patients die within a year (McGowan and Marinkovich, 2000). LM α 3 knock out mice show features similar to severe JEB with lethal skin blistering (Ryan et al., 1999).

A third human syndrome could recently be linked to LM deficiency, the Pierson syndrome. This syndrome is a congenital nephrotic syndrome described in the 1960's, which is characterised by proteinuria and swelling of the body. Along with malfunctioning kidneys, patients suffering from the Pierson syndrome also have a fixed narrowing of the pupils. Mutations in the Pierson syndrome were mapped to the LM β 2 gene (Zenker et al., 2004). LM β 2 null mice display a very similar phenotype. As they were created before the discovery of the link to the Pierson syndrome, they helped in the identification of LM β 2 as the affected gene (Noakes et al., 1995; Zenker et al., 2004).

Laminin receptors

LMs have been shown to interact with at least four different types of cellular anchored receptors. These are the integrins, dystroglycan, syndecans, and the Lutheran blood group receptor (Sasaki et al., 2004). In addition, LMs interact with heparin sulphate proteoglycans and sulfatides. Currently integrins and dystroglycan are considered as the major LM receptors.

Dystroglycan

The dystrophin glycoprotein complex (DGC) is a multiprotein complex important for muscle stability. It connects the ECM with the actin cytoskeleton (Barresi and Campbell, 2006). Dystroglycan was first isolated from skeletal muscles as a protein of the DGC (reviewed in Barresi and Campbell, 2006), and it has subsequently been found in many other tissues (Durbeej et al., 1998). Mice completely deficient in dystroglycan die early during development due to a defect extra-embryonic BM (Williamson et al., 1997) and mutations in many of the DGC components lead to muscular dystrophy (Barresi and Campbell, 2006).

Dystroglycan is composed of an extracellular α unit and a transmembrane β unit. These are coded from the same mRNA and by enzymatic processing of the propeptide separated to two non-covalently attached units. A β unit of 43 kDa and an α unit of variable size (between 120-200 kDa) due to heavy glycosylation (Weir et al., 2006). α -Dystroglycan can bind to many extracellular proteins such as agrin, perlecan, and LMs via their LG-domains. Interestingly, the affinity of α -dystroglycan to different ligands is tightly regulated by α -dystroglycan glycosylation. For example, the glycosylation pattern of α -dystroglycan in different organs seems to be important for LM interaction, as $\alpha 5$ containing LMs could bind to brain dystroglycan but showed weaker or no binding for muscle dystroglycan (McDearmon et al., 2006). Moreover, when the enzymes of the glycosylation machinery are defective, dystrophies and neural defects appear (Barresi and Campbell, 2006). One such important and recently recognised component is the putative glycosyltransferase LARGE (Kanagawa et al., 2004).

LMs are thought to interact with negatively charged sugar chains on α -dystroglycan in a lectin-like manner through a calcium ion and basic residues of the LG4 and LG5 domains (Tisi et al., 2000). Primarily, LM $\alpha 1$ and $\alpha 2$ containing LMs interact with strong affinity to dystroglycan, but the other LM α chains do not. This is due to structural differences between $\alpha 1$ and $\alpha 2$ chains and $\alpha 3$, $\alpha 4$, and $\alpha 5$ chains LG 4-5 domains. These LG domains seem to have no calcium ion binding sites. That LMs have different affinities towards α -dystroglycan has been shown by solid phase interactions of both recombinant LG domains and full-length LM trimers with immobilised DG (Talts et al., 1999; Talts et al., 2000; Yu and Talts, 2003).

In addition to working as a part of the stabilising DGC complex, β -dystroglycan can associate with the adaptor protein Grb-2. Binding of LM $\alpha 1$ LG4-5 to dystroglycan has been reported to initiate Rac signalling via Grb-2 (Zhou et al.,

2006). Rac1 belongs to the Rho-family of GTPases which in various ways are important for directing cell migration and polarisation (Fukata et al., 2003). Signalling via dystroglycan, initiated by LM binding, has also been suggested to modulate integrin mediated LM signalling. In one study, integrins stimulated by LM binding activated phosphorylation of extracellular signal-regulated kinase (ERK), a mitogen activated protein kinase involved in cell growth and differentiation. Occupation of dystroglycan by recombinant LG4-5 domains suppressed this LM initiated ERK phosphorylation (Ferletta et al., 2003).

Integrins

Integrins are a family of glycosylated heterodimeric cell surface receptors that mediate cell-cell, cell-ECM and cell-pathogen interactions. They recognise a versatile set of ligands and are multi-functional, regulating such cellular events as migration, death, and differentiation. They consist of an α and a β subunit that are non covalently linked. There are 18 α and 8 β subunits reported in mammals, but the human genome appears to carry six additional α and one additional β subunit. So far, 24 different dimers have been described (Brakebusch and Fassler, 2005; van der Flier and Sonnenberg, 2001). For ligand binding, association with divalent cations is crucial (Bazzoni and Hemler, 1998). Signals over integrins can be transmitted in two directions, from the outside of the cell to inside or from the inside to the outside. Integrins can be found in three different conformations, an inactive, an active, and a third intermediate conformation (Shimaoka et al., 2003). In the inactive conformation, the integrins possess weak ligand affinity, whereas in the opened active position they have a strong attraction for the ligands, which enables ligand mediated signalling into the cell. The ability to intracellularly regulate the conformation of the extracellular parts of the integrins permits control of integrin activity from the inside of cells. A separation of the cytoplasmic tails of the integrin subunits leads to an open active conformation. While a close association of the cytoplasmic tails are found in the closed inactive form (Springer and Wang, 2004).

Integrins have a large extracellular part, but only a rather short intracellular part, for most integrin chains composed of around 50 amino acids. An exception is the $\beta 4$ subunit that has a 1000 amino acid cytoplasmic stretch (Hynes, 2002). The cytoplasmic tails lack intrinsic enzymatic activities (Hynes, 2002). Therefore, they are by themselves non-signalling and have to attach to adaptor proteins or affect other signalling proteins in order to exert intracellular effects. Integrins can connect the ECM with the cytoskeleton, either by direct binding to F-actin or by interacting with cytoskeletal associated proteins such as talin and filamin. Another way for them to act is by linking directly to signalling proteins like integrin linked kinase (ILK) or focal adhesion kinase (FAK). Moreover, they can interact with adaptor proteins to affect signalling. In addition, integrins associate with calcium binding proteins. Affecting the calcium concentration is a mean to modulate signalling. Integrins also interact with membrane bound proteins like different growth factor receptors, ion channels

and tetraspanins (van der Flier and Sonnenberg, 2001). Tetraspanins are a family of ubiquitously expressed small proteins spanning the membrane four times, thereof their names. They interact with a wide variety of membrane associated proteins and are important for adjusting a broad spectra of cellular activities (Sachs et al., 2006).

The ways that integrins act on cell signalling are truly multifaceted. By activating parallel pathways to the ones used by growth factor receptors they fine-tune the resulting phosphorylation of proteins downstream in the signalling pathway (Chen et al., 1996) Integrins can also increase signalling of growth factor receptors by creating complexes that puts kinases in close contact with their substrates (Geiger et al., 2001). An additional way is by anchoring the ECM to the intracellular actin cytoskeleton and thereby creating tension in the cell. The tensile force a cell experience can regulate gene expression and has been shown to be important for determining the fate of undifferentiated cells (Engler et al., 2006). Integrins can upon adhesion act by clustering several receptor tyrosine kinases and transactivate these (Wang et al., 2001). By organising the surrounding ECM, integrins can regulate the activity of receptor tyrosine kinases, as growth factors can interact with the sugars on proteoglycans. A regulation of the architecture of the surrounding matrix can thereby cause regulation of growth factor presentation to their receptors (Danen and Sonnenberg, 2003). Integrins acts as receptors for, or by signalling affect the protein expression of matrix metalloproteases (MMPs). MMPs are enzymes that degrade components of the ECM, and can therefore modulate the environment surrounding cells and release growth factors bound to the ECM (Iyer et al., 2005; Stefanidakis and Koivunen, 2006).

The integrins can be divided into families according to their β subunit. The largest family is the $\beta 1$ integrins, which comprises 12 different integrin dimers, all binding ECM molecules (Brakebusch and Fassler, 2005). LMs bind mainly to $\beta 1$ integrins, but integrin $\alpha 6\beta 4$ and $\alpha v\beta 3$ have also been shown to be LM receptors. Different LMs have different affinities for different integrins. The LM α chain LG domains provide integrin binding sites with contribution from the most C-terminal rod domain of the LM β and γ chains. This contribution has in some cases been shown to be vital for integrin mediated cell binding (Deutzmann et al., 1990). Some reports have indicated that short arms of LM $\beta 1$ and $\gamma 1$ possess integrin binding activity. However, in these studies synthetic peptides of parts of these regions were used to map the binding and are therefore not very conclusive (Nomizu et al., 1997; Underwood et al., 1995). Most integrin binding in LMs has been mapped to the LM α chain's LG1-3 domains (Sasaki et al., 2004), but as previously stated some binding takes place in the short arms. Integrin $\alpha 1\beta 1$ and $\alpha 2\beta 1$ can bind to the N-termini of LMs containing the $\alpha 1$ or $\alpha 2$ chain (Miner et al., 2004). These contributions to cell binding might be minor (Ekblom et al., 2003). It is likely that LM $\alpha 5$ chain containing LMs also can bind integrin $\alpha 2\beta 1$ as antibodies to some extent blocked recombinantly produced LM 511 cell binding (Doi et al., 2002). LM $\alpha 5$ can via its arginine-glycine-aspartic acid amino acid (RGD) sequence in the L4b domain interact with integrin

$\alpha\beta 3$, as shown using recombinant domains and full length LM 511/521 (Genersch et al., 2003; Sasaki and Timpl, 2001). Integrin $\alpha 9\beta 1$ was reported to bind LM 111 but the region for this binding has not been further mapped (Forsberg et al., 1994).

Integrin affinity towards different receptors can sometimes be modulated by expression of different extracellular splice variants. For LM binding integrins, this occurs for the $\alpha 7$ subunit. It has two alternatively spliced extracellular variants, the X1 and X2 (Ziober et al., 1993). These are developmentally regulated with the X1 form being expressed in developing muscle and the X2 form being the major variant expressed in mature muscle (Mayer, 2003). Integrin $\alpha 7X1\beta 1$ has a stronger affinity towards LM $\alpha 4$ and $\alpha 5$ containing trimers than the X2 form that only interact weakly with these LMs. For LM 111 the affinity towards the two splice variants is reversed, with strong affinity towards the X2 form, but weak interaction with the X1 form (Nishiuchi et al., 2006; von der Mark et al., 2002). Another major LM binding integrin α subunit, integrin $\alpha 6$, also exist in two alternative extracellular splice variants. These do not differ in affinity for LM 111 (Delwel et al., 1995), but no comparisons with other LMs have been made. Many integrin subunits have splice variants of their cytoplasmic domains, which can contribute to differences in signalling as seen for the integrin $\alpha 6A$ and B variants. Phosphorylation of ERK was seen after stimulation with LM 511/521 in cells expressing both $\alpha 6A$ and $\alpha 6B$, while there was no activation in cells containing just the $\alpha 6B$ variant (Ferletta et al., 2003). Apart from integrin $\alpha 6$, two other major LM binding integrins, $\alpha 3$ and $\alpha 7$, exist in intracellular splice variants. This is also the case for the integrin $\beta 1$ subunit, which has four different splice variants, A-D. The expression of integrin $\beta 1$ cytoplasmic splice variants also shows a developmental pattern in skeletal muscle, with the $\beta 1A$ being present in embryonic and $\beta 1D$ in mature muscles (Bouvard et al., 2001).

The integrin $\alpha 6\beta 1$ is the most promiscuous LM binding receptor. It binds to LMs containing all five α chains although with differences in affinity as seen in studies using purified or recombinant integrins and full length LMs (Nishiuchi et al., 2003; Nishiuchi et al., 2006). Integrin $\alpha 7\beta 1$ shows variability in LM binding, depending on the extracellular splice variant, but both variants interact weakly with LM $\alpha 3$ containing LMs (Nishiuchi et al., 2006). Integrins $\alpha 3\beta 1$ and $\alpha 6\beta 4$ show the largest variability in LM binding among the major LM binding integrins. Integrin $\alpha 3\beta 1$ binds $\alpha 2$, $\alpha 3$, and $\alpha 5$ containing LMs, while it does not seem to interact at all with LM 111 (Delwel et al., 1994; Nishiuchi et al., 2006). Binding of $\alpha 3\beta 1$ to $\alpha 4$ containing LMs is somewhat unclear. Recombinantly produced LM-411 has been reported to bind integrin $\alpha 3\beta 1$ (Fujiwara et al., 2001), but also not to bind this integrin (Kortessmaa et al., 2000). Subsequent analyses of the LM 411 reported to bind $\alpha 3\beta 1$ concluded that this binding was probably due to a contamination in the purified protein (Fujiwara et al., 2004). That $\alpha 3\beta 1$ does not bind LM $\alpha 4$ is further supported by studies on purified and recombinant $\alpha 3\beta 1$ (Nishiuchi et al., 2003; Nishiuchi et al., 2006). The abilities of different LMs to recognise $\alpha 6\beta 4$ is not quite clear. LM 332 and 511 were shown to bind recombinant integrin $\alpha 6\beta 4$, while all other tested LMs failed

to bind (Nishiuchi et al., 2006). Recombinant LM 411 has been suggested to interact with $\alpha 6\beta 4$ as judged from complete inhibition of cell binding to LM 411 with anti integrin $\alpha 6$ antibodies, but incomplete inhibition with anti integrin $\beta 1$ antibodies (Kortessmaa et al., 2000)

The vascular system

Hierarchy and constituents

Circulation of blood is essential for sustaining life in higher metazoans; it provides cells with oxygen and nutrients and relieves them from waste. The vascular system is made up of two hierarchical trees, an arterial and a venous, with larger vessels, arteries and veins, branching in to smaller, arterioles and venules which in turn branches into the smallest vessels, capillaries. Capillaries connect the two vascular trees, to complete the circulation. All vessel lumens are lined with a monolayer of endothelial cells that can be continuous, discontinuous or fenestrated depending on the organ. These cells are resting on an underlying vascular BM. Mural cells cover the outsides of the vessel in variable complexity depending on the nature of the vessel. Mural cells are vessel supporting cells that can either be vascular smooth muscle cells (VSMC) or pericytes, which are spindle shaped supporting cells (Jain, 2003). Arteries and arterioles, that are highly pressurised vessels, are lined with several layers of contractile VSMC separated with elastic fibres that contribute vessel tone and lumen size by contraction or relaxation. These vessels have three histologically discrete layers, an intima made up of endothelium and the vascular BM, a media composed of VSMC and elastic fibres and an outer layer, the adventitia. The adventitia is a connective tissue cover consisting of fibroblasts, nerves, and small blood vessels embedded in ECM. Veins and venules that work under low pressure are dressed in a thin coat of VSMC. Capillaries are associated with a patchy layer of pericytes.

If the vessels take on the fate of becoming arteries or veins during development has been thought to depend on pressure. This idea stems from the initial observations of Thoma in 1893 who, when studying chick embryos, noted that arteries having a greater flow increased in calibre, while those with a low flow decreased in calibre. Based on this initial report it was postulated that hemodynamic forces of the blood flow influenced outcome of arteries or veins (Eichmann et al., 2005). Engrafting experiments from multiple species where veins transplanted to arterial sites adapted the structure of arteries strengthened and retained the hypothesis. Much later, absence of animal models with either early artery or vein specific defects also strengthened the hypothesis. However, mutation studies on e.g. the basic helix-loop-helix (bHLH) transcription factor gridlock in zebrafish indicate that determination of arteries and veins occur before the heart starts pumping (Zhong et al., 2000). Recent engraftment assays of vessels on quail embryos have revealed that their vessels retain the plasticity to become arteries or veins until late in development. Although

endothelial cells early in development showed artery or vein identities, these cells were possible to convert by engrafting experiments until fairly late in development. To sustain the endothelial identity in the later designated vessels the vessel wall was needed (Moyon et al., 2001). Taken together this indicates that the initial set up of artery and vein identities could be regulated by various molecular signals but the vessels remain somewhat adjustable to adapt to pressure.

Vascular development during embryogenesis

The cardiovascular system is the first organ system to develop and function in the embryo (Moore, 1989). Vascular development takes place when the size of the embryo exceeds the limit for diffusion of oxygen and nutrients alone. For human development this occurs during the third week (Moore, 1989) and for mice around embryonic day seven (Daniel and Abrahamson, 2000).

Superficially the initiation of vascular construction in the embryo involves mesodermal stem cells called hemangioblasts and growth factors. Hemangioblasts are derived from the paraxial- and lateral-plate mesoderm, they give rise to both hematopoietic progenitor cells as well as endothelial progenitor cells called angioblasts. For extra-embryonic and some larger embryonic vessels like the dorsal aorta, aggregates of hemangioblasts separates to form endothelia that surround centrally located hematopoietic cells (Shalaby et al., 1997). In the embryo, angioblasts gather at discrete locations, proliferate, differentiate, and aggregate to form vascular plexa (Conway et al., 2001). Later the vascular plexa become linked by major endothelial extensions from the heart (Nguyen and D'Amore, 2001). This de novo formation of blood vessels is referred to as vasculogenesis. Activation of the hemangioblasts to aggregate and further development of the vasculature is dependent on correct growth factor signalling. The vascular endothelial growth factor A (VEGF-A) and its receptors VEGF receptor 1 (formally known as Flt-1), VEGF receptor 2 (formally known as Flk-1) are particularly important for early establishment of vessels. Loss of a single *vegfa* allele leads to embryonic and extra-embryonic vascular defects, with most steps of early vascular development impaired (Carmeliet et al., 1996; Ferrara et al., 1996). Mice deficient of VEGF receptor 1 or VEGF receptor 2 die during midgestation with impaired endothelial organisation and development (Fong et al., 1995; Shalaby et al., 1997; Shalaby et al., 1995).

Angiogenesis is defined as the formation of blood vessels from pre-existing ones. This term dates back to the late 18th century from the British surgeon John Hunter that detected blood vessel formation when studying reindeer antlers, and named this process angiogenesis. Angiogenesis is important for completion of the circular system in the embryo after the major vessels have been laid out by vasculogenesis. Angiogenesis can occur by two different mechanisms, intussusception or sprouting (Conway et al., 2001). Intussusceptional angiogenesis take place when the vessel lumen is separated longitudinally by insertion of interstitial tissues. During sprouting angiogenesis endothelial cells become activated, they migrate out from an

existing vessel, and proliferate to form new vessels and capillaries. These capillaries can then fuse with other sprouts to complete the circulation. For this to progress in an orderly fashion, lateral inhibition between vessels via receptors of e.g the Notch family and ligands such as Jagged and Delta probably occurs (Davis et al., 2002).

In order for the vessels to be stabilised, recruitment of supporting mural cells, VSMC in larger vessels, or pericytes in capillaries, are needed (Jain, 2003; Nguyen and D'Amore, 2001). These mural cells provide endothelial cells with factors needed for survival and stabilisation after retraction of pro-angiogenic signals such as VEGF. The exact nature of the factors that these cells provide is unclear but they are known to express a variety of vasoactive peptides, growth factors and cytokines. Further, there are speculations on that the perivascular cells synthesis ECM components vital for endothelial cell survival that the endothelial cells cannot produce themselves (Conway et al., 2001; Davis and Senger, 2005). Perivascular cells are recruited by signals from the activated endothelium, e.g. the endothelial cells secrete platelet derived growth factor B (PDGFB) in response to VEGF, mural cells unique in their expression of the PDGF-receptor β respond to the growth factor and are recruited. A way to stabilise the endothelial-mural cell interactions is by Angiopoietin-1 (Ang1) - Tie2 interaction. Mural cells synthesize and release Ang1, endothelial cells carry the tyrosine kinase receptor Tie2, a receptor for Ang1. Activation of Tie2 by Ang1 enhances endothelial survival (Jain, 2003).

The origin of the surrounding mural cells is not definite but can stem from different sources. Cells in the surrounding mesenchyme activated by the sprouting endothelium can take on the fate of VSMC or endothelial progenitor cells can commit to become mural cells (pericytes) if they are stimulated by PDGFB. If the endothelial progenitor cells instead are stimulated by VEGF, they will, the former stimuli inducing them to become endothelial cells, the latter committing them to pericytes. It is also possible that the VSMC, myofibroblasts and fibroblasts have a common progenitor cell (Jain, 2003).

Vascular remodelling in the adult

In adults, most vasculature is dormant with only 0.01 % of the endothelium undergoing division, important exceptions are in the female monthly reproductive cycle and during wound healing (Jain, 2003). Deregulation of blood vessel growth is important for many major pathological conditions. In cancers and diabetes, an excessive blood vessel formation brings complications to the disease, whereas in ischemia, after strokes or myocardial infarctions, a limited recruitment of vessels causes problems. It is therefore of great importance to be able to regulate blood vessel formation both negatively and positively.

Blood vessel formation in the adult body

Formation and remodelling of blood vessels in the adult has been thought to depend on angiogenesis. Angiogenesis occurs via endothelial sprouting from capillaries

(Carmeliet and Jain, 2000). This prevailing idea has been challenged by the finding of circulating stem cells, so called endothelial progenitor cells (EPC) that can incorporate into newly formed capillaries (Asahara et al., 1997), indicating that vasculogenesis also takes place in the adult. However, the nature and the actual contribution of these cells to vessel formation in the body are dubious, since vast differences are seen for the relative contribution of these cells to the vasculature between different studies. The differences reported on the contribution of EPC to the endothelium of new vessels could be due to inherited differences between organs or to differences in the isolation and to the extent of manipulation in culture of EPC in the assays prior to injection into mice (Rajantie et al., 2004; Ziegelhoeffer et al., 2004). Recent reports indicate that circulating stem cells can participate in angiogenesis by, instead of acting as endothelial cells and constructing new vessels, operating as supporting cells providing the activated endothelium in vessel sprouts with factors to enhance and sustain proliferation (Grunewald et al., 2006; Rajantie et al., 2004; Ziegelhoeffer et al., 2004).

Angiogenesis in the adult

A complex network of pro- and anti-angiogenic factors tightly regulates angiogenesis. Local hypoxia in tissue is important for activation of the endothelium. Many factors involved in angiogenesis are up-regulated under low oxygen pressure for example VEGF, VEGF-receptors, angiopoietin-2 (Ang-2), and nitric oxide (NO)-synthase. Some genes have hypoxia responsive elements in their promotor regions to which hypoxia inducible factor (HIF) complexes bind. HIF-1 α is rapidly degraded during normal oxygen levels, this occurs through binding to the von Hippel Lindau tumor suppressor that targets the protein for degradation. HIF-1 β is constitutively expressed. Under hypoxia the von Hippel Lindau tumor suppressor is down-regulated leaving the HIF-1 α free to gather with the HIF-1 β and HIF-2 α to a complex. This complex binds to the hypoxia responsive elements, to initiate transcription of for example VEGF and VEGFR1 (Conway et al., 2001).

An increment of the NO concentration leads to dilation of vessels, which is one of the earliest steps during angiogenesis. Moreover, NO, just like hypoxia, increases VEGF transcription. VEGF enhances the permeability of the vessels through redistribution of the cell-cell adhesion molecules important for sustaining a tight endothelium. Further, Ang-2 (an antagonist of Ang-1) up-regulated during low oxygen pressure competes with Ang-1 for binding to Tie-2. This leads to a loosening of the endothelial-pericyte interaction. The loosened interaction between endothelial cells themselves and their interaction with pericytes, enables both of these cell types to migrate. MMPs are activated during angiogenesis. These enzymes degrade the surrounding BM, which eases migration, but also releases pro-angiogenic growth factors sequestered in the ECM like VEGF and fibroblast growth factor 2 (FGF2) (Conway et al., 2001). As an example, genetically modified mice carrying perlecan that is deficient in growth factor binding heparan sulphate side-chains show reduced

angiogenesis (Zhou et al., 2004b). The newly formed vessels are stabilised by recruitment of pericytes and deposition of an endothelial BM (Davis and Senger, 2005). It has been proposed that several cell layers in leading edges of sprouts lack BM (Kalebic et al., 1983), but some studies in embryonic tissues (Ekblom et al., 1991) and tumours (Baluk et al., 2003) suggest an early deposition of BM components.

Angiogenesis inhibitors

Many endogenous negative regulators of angiogenesis exist in the body, e.g. angiostatin, TIMP-2, maspin, fibulin-5, troponin 1 and interferons α , β , and γ (Folkman, 2006). In addition, several other substances have been described as angiogenesis inhibitors and both lists are still rapidly growing

The vascular BMs are structurally and functionally important components of all blood vessels. They make up the barrel of the vessels and are dressed by endothelial cells on the lumen side and pericytes on the outside (Kalluri, 2003). Like all BMs, the vascular BM is important for regulating many key cellular events such as endothelial cell survival, migration, and proliferation (Hallmann et al., 2005). An intriguing feature of vascular BMs is that components of these contain cryptic sites that are exposed after proteolytic processing and possess anti-angiogenic effects (Kalluri, 2003). These fragments are released and can be detected in the circulation. Here I will go through the, in my eyes, three most well-characterised angiogenesis inhibitors derived from components of the vascular BM, i.e. endostatin, tumstatin, and endorepellin.

Collagen XVIII is a heparan sulphate containing non-fibrillar collagen, organised as a homotrimer consisting of three α_1 chains (Ortega and Werb, 2002). It is a constituent of certain epithelial and vascular BMs, with the highest mRNA expression found in liver, kidney, and lung (Marneros and Olsen, 2001). Its most C-terminal part, the globular non-collagenous domain 1 (NC1) is proteolytically released *in vivo*. This processing occurs in the hinge region between the NC1-domain and a triple helical region. Several enzymes have been shown to contribute to this cleavage, e.g. pancreate elastase-like enzyme, cathepsin L and MMP-7 (Bix and Iozzo, 2005). In a quest for powerful angiogenesis inhibitors, endothelial cells stimulated with FGF2 to proliferate were treated with conditioned medium from a murine hemangioendothelioma cell line. The addition of this conditioned medium significantly inhibited cell proliferation. A strongly heparin binding fraction of the conditioned media was subsequently shown to contain the anti-proliferative substance. This substance was further identified as a 20 kDa sized fragment identical to the NC1 domain of collagen XVIII and named endostatin (O'Reilly et al., 1997). Murine endostatin inhibits both endothelial cell proliferation and migration (Sudhakar et al., 2003), whereas human endostatin only affects cell migration, but has no effect on *in vitro* cell proliferation of human endothelial cells (Rehn et al., 2001). Human endostatin exerts its effect on endothelial cells by binding to the fibronectin receptor

integrin $\alpha_5\beta_1$. This leads to an inhibition of a FAK – mitogen or extracellular signal regulated kinase kinase (MEK) - ERK dependent pathway that destabilises the actin cytoskeleton and focal adhesion to cause arrest of migration (Bix and Iozzo, 2005; Sudhakar et al., 2003). Elegant substantiation that endostatin indeed acts as an endogenous inhibitor of blood vessel growth is available from patient based studies. The normal circulating level of endostatin in serum is about 20 ng/ml in healthy individuals. Patients with Down syndrome carry a trisomy of chromosome 21. These patients rarely develop solid tumours, although the incidence of other cancers such as leukaemia, gonadal and soft tissue cancers are higher in persons affected with this syndrome. Collagen XVIII is encoded on chromosome 21, so patients with Down syndrome have an extra copy of the collagen XVIII gene. Naturally, the levels of endostatin are higher (about 40 ng/ml) in these patients (Zorick et al., 2001). In addition, experimental tumours grow slower in mice over-expressing endostatin than in normal mice (Sund et al., 2005). For expansion of tumours blood vessel recruitment is necessary, as discussed in the next section. Taken together, these results strongly indicate that a higher concentration of circulating endostatin inhibits neo-vascularisation and offers protection against certain forms of cancers.

Type IV collagens are the most abundant constituents of BMs (Kalluri, 2003). They are heterotrimers consisting of six different α -chains making up at least three different trimers, $[\alpha_1(\text{IV})]_2[\alpha_2(\text{IV})]$, $[\alpha_3(\text{IV})]_2[\alpha_4(\text{IV})]$, and $[\alpha_5(\text{IV})]_2[\alpha_6(\text{IV})]$ (Bix and Iozzo, 2005). The α_1 and α_2 chains are the most widely expressed, while α_3 , α_4 , α_5 , and α_6 chains are mainly expressed in the kidney (Marnaros and Olsen, 2001). This domain is important for collagen IV network polymerisation (Ortega and Werb, 2002). Also for these collagens, the NC1 domains can be proteolytically released and act as angiogenesis inhibitors. The free NC1 domains from different α -chains have been assigned different names: α_1 NC1 is called arresten (Colorado et al., 2000), α_2 NC1 canstatin (Kamphaus et al., 2000), and α_3 NC1 tumstatin (Maeshima et al., 2000). The α_6 NC1 domain is also anti-angiogenic but was not given a new name in the report showing this (Petitclerc et al., 2000). Most work on the angiogenesis inhibitory properties of the collagen IV NC1 domains have been done on tumstatin. Tumstatin is released by MMP-2, MMP-3, MMP-9, and MMP-13. It is a 28 kDa fragment and share only 14 % homology in the amino acid sequence with endostatin (Bix and Iozzo, 2005). The circulating level in mice is about 336 ng/ml (Hamano et al., 2003). Tumstatin binds to integrin $\alpha_v\beta_3$ and $\alpha_v\beta_5$ in an RGD dependent manner, induces apoptosis, and inhibits endothelial cell proliferation (Sudhakar et al., 2003). Human tumstatin affects endothelial cell proliferation via integrin $\alpha_v\beta_3$ binding. This interaction leads to inhibition of protein translation via inhibition of the FAK – phosphoinositide 3'-kinase (PI3K) pathway (Sudhakar et al., 2003). A recent report suggested that tumstatin also interacts with integrin $\alpha_3\beta_1$ and that this interaction can transdominantly regulate the activity of integrin $\alpha_v\beta_3$ (Borza et al., 2006).

Perlecan is a ubiquitously expressed BM heparan sulphate proteoglycan. Mice deficient in perlecan die between embryonic day of development 10.5 or just after

birth and present myocardial defects, imperfections of the great arteries, and severe cephalic and cartilage abnormalities (Arikawa-Hirasawa et al., 1999; Costell et al., 2002; Costell et al., 1999). The perlecan protein core is composed of five distinct domains, all with sequence homology to domains of other proteins. At the C-terminus lies domain V, an 85 kD region consisting of three LG-domains separated by four EGF-like repeats. This domain has anti-angiogenic effects in *in vitro* and *in vivo* angiogenesis assays and has therefore been named endorepellin (Mongiat et al., 2003). It exerts its inhibitory effect on endothelial cells via integrin $\alpha_2\beta_1$. Endorepellin activation of this integrin increases the cAMP concentration. This triggers protein kinase A leading to a phosphorylation of FAK, which in turn causes transient phosphorylation of p38 mitogen activated protein kinase and heat shock protein 27 with the end results of destabilisation of actin stress fibers and focal adhesion contacts in endothelial cells (Bix et al., 2004). This destabilisation affects migration, adhesion and the morphological changes of endothelial cells occurring during angiogenesis. Most of the anti-angiogenic activities of endorepellin are attributed to the C-terminal LG domain LG3. The LG3 domain is cleaved off and released *in vivo* as a 25 kDa fragment by BMP-1/Tolloid like metalloproteases (Gonzalez et al., 2005). The LG3 have been found the urine of patients suffering from kidney failure (Oda et al., 1996), which indicates that this released domain is retained in the circulation.

Tumour angiogenesis

As the size of a tumour reaches the limit for oxygen diffusion, it needs to incorporate blood vessels for continued growth. For this recruitment local hypoxia, as in tissues, is one of the major driving forces. Growing tumours attract immune cells, fibroblasts and other stromal cells. These cells in turn produce pro-angiogenic factors such as VEGF and FGF-2 that further drives the angiogenesis (Kalluri, 2003). Tumour vessels are not structurally normal. They are disorganised, with no distinction of arteries and veins. They show irregular lumen diameter and the vessels have excessive branching and shunts (Carmeliet and Jain, 2000). This is believed to be due to an imbalance in angiogenic regulatory signals and also due to a pericyte deficiency of the vessels (Carmeliet and Jain, 2000). The structural impairments makes the tumour vasculature leaky and functionally abnormal, which in turn leads to a non-satisfactory circulation that creates regions of hypoxia within the tumour inducing renewed production of angiogenic factors leading to even more angiogenesis (Carmeliet and Jain, 2000; Ruoslahti, 2002). There are reports of six alternative ways in which a tumour can become vascularised (Dome et al., 2005). The way tumour vascularisation occurs depends on the type of cancer and the tissue it grows in. First, blood vessels can arise by sprouting angiogenesis, secondly by intussusceptive microvascular growth, and thirdly by vasculogenesis. These three ways of neovascularisation were discussed in previous sections. The fourth way a tumour can obtain its blood supply by is by vessel co-option, this occurs in blood vessel rich organs such as lungs. Here, the growing tumour initially uses the tissues own vessels and does not by itself provoke

angiogenesis. As the tumour grows the blood supply from the co-opted vessels is not sufficient. Then, the tumour goes through a massive apoptosis leaving tumour cells with a large angiogenic response. These cells can then initiate a second wave of blood vessel recruitment into the tumour (Holash et al., 1999). A fifth possibility is by glomeruloid angiogenesis, this occurs for example in glioblastoma multiforme. Here, the proliferating endothelial cells first form renal glomeruli-like structures called glomeruloid bodies, these endothelial lumps then devolve by apoptosis and reorganises into micro vessels (Sundberg et al., 2001). The last mechanism of tumour vascularisation that has been reported is vasculogenic mimicry, which involves tumour cells that imitate endothelial cells and line hollows in the tumour (Carmeliet and Jain, 2000).

Incorporation of vessels enables the tumour both to grow and to metastasize. Since normal endothelia are quiescent and the tumour vasculature frequently consists of the bodies own non-transformed but activated endothelial cells, an attractive approach to treat cancers would be to inhibit endothelial cell proliferation and migration into tumours.

Atherosclerosis

Circulation of blood is key for life and impairment of blood flow substantially threatens organ function and even existence. Atherosclerosis constitutes one such threat and complications from the disease are the most common cause of death in western societies (Glass and Witztum, 2001). The pathology of atherosclerosis is complex and involves blood borne cells and factors as well as activation of cells in the vessel wall. It is considered as an inflammation of the arterial vessel wall due to accumulation of LDL particles in the intima (Hansson and Libby, 2006). A high LDL concentration injures the endothelium and underlying smooth muscle cells. LDL particles trapped in the vessel wall are progressively oxidised. The oxidised LDL-particles stimulate activation of adhesion molecules on the endothelium that in turn recruit macrophages and T-cells. Macrophages phagocytise the oxidised LDL particles. In the macrophages cholesterol in the LDL-particles is further oxidised and cannot be metabolised. This leads to an accumulation of lipids in the macrophages giving them a cloudy appearance in histological sections. Such cloudy cells are described as foam cells (Ross, 1999). Intimal VSMC may also take up lipids to contribute to the foam cell formation in plaques (Glass and Witztum, 2001).

As a second step in the atherosclerotic process VSMC become activated by various cytokines and growth factors. They lose their contractility, start to proliferate, and migrate from the tunica media into the intima, where they synthesise and deposit great amounts of ECM proteins. Together, these three cell types - macrophages, T-cells, and VSMC - along with ECM-proteins, cell debris, and lipids are the major constituents of atherosclerotic plaques and also what contributes to plaque growth. For smaller plaques the vessel wall can compensate by gradual dilation to keep the size of the lumen intact. However, as the plaque increases in size this compensation

becomes impossible and the vessel lumen narrows (Ross, 1999). This leads to poor blood supply and complications, as seen for angina pectoris where the lumens of coronary arteries are narrowed. Layers of VSMC surrounding the lesion can stabilise a plaque. This cover, called a fibrotic cap, is cell dense compared to the rest of the plaque that is rich in lipids and necrotic (Newby and Zaltsman, 1999). A thick fibrotic cap is considered as a good sign in atherosclerosis as this cap is thought to make the plaque more stable and less prone to rupture. Activated macrophages and lymphocytes produce hydrolytic enzymes that destabilize the plaque. When a plaque ruptures, it releases debris and a thrombotic lipid core that activates blood platelets. This causes thrombosis that can occlude the vessel leading to myocardial infarctions or stroke.

Apolipoproteins are lipid-binding proteins that transport dietary and endogenously synthesized lipids through the bloodstream. Apolipoprotein E (ApoE) associates with lipoprotein complexes and is important for the cholesterol metabolism. Mice deficient in ApoE develop plaques and the formation of these can be provoked by feeding the mice a fat rich diet. These genetically modified mice are a good model for studying events and aspects of atherosclerosis (Daugherty, 2002).

Specific aims of the study

The specific aims of this study were:

- To characterise the expression of LMs in aortic VSMC in healthy and atherosclerotic aortas (Paper I).
- To determine a rationale for the known release of the LG4-5 domain from the LM α 4 in the endothelium (Paper II).
- To investigate whether LM α 2 deficient extraocular muscle (EOM) was spared from muscle wasting as seen for the EOM in other muscular dystrophies (Paper III).

Results and discussion

Laminin presence in blood vessels

Laminin expression in vascular smooth muscle cells (Paper I)

Discrete VSMC in the medial layer of the vessel wall are surrounded by a BM. However, little is known about the LM expression in this cell type. There are some histological studies on LMs in the aortic tunica media on human and rat vessels from the early and mid 90's (Glukhova et al., 1993; Walker-Caprioglio et al., 1995). These studies were performed when many members of the LM family were still unknown. We therefore executed a comprehensive study on the expression of all eleven LM chains in sections of mouse aortas using well-characterised antibodies.

The major LM α chains expressed in VSMC were the LM α 2, α 4, and α 5 chains. High levels of LM α 2 was expected, as it is a major LM α -chain in skeletal and cardiac muscle and smooth muscle of the intestine (Ekblom et al., 2003; Lefebvre et al., 1999). Both LM α 1 and LM α 3 were present at low levels. LM β 2 was the major β chain in aortic VSMC, a finding which concurs with what has previously been reported (Glukhova et al., 1993). LM β 1 expression was mainly localised to the intima and adventitia indicating that adventitial fibroblasts and endothelial cells express this β chain, whereas LM β 3 was weakly expressed. Of the LM γ chains, LM γ 1 gave the strongest staining, suggesting that the major LM trimers in VSMC BM are LM 221 and 421. The LM γ 2 chain was moderately expressed; this pointed to a low occurrence of LM 332 in mouse aortic VSMC as previously reported for rat aortic VSMC (Kingsley et al., 2002).

Release of laminin α 4LG4-5 (Paper II)

LM α 4 and α 5 are the major LM α chains expressed in the vascular BMs (reported multiple times, for review see Hallmann et al., 2005 and again shown in paper III), with LM α 4 being the principal α chain in late embryonic and neo-natal vasculature. As the vessels mature, this α chain is replaced by LM α 5 (Gu et al., 1999; Sorokin et al., 1997). The most C-terminal part of the LM α 4 chain, the LG4-5 domain, is

proteolytically cleaved off and released *in vivo* from endothelial BMs (Talts et al., 2000). However, it was not known whether this fragment was kept in the circulation. We therefore performed immunoblotting on mouse serum with antibodies against the α 4LG4-5 domain. The released α 4LG4-5 fragment was found to be present in the circulation at a concentration of about 3.7 nM.

Many vascular BM components, and especially cleavage products of these present in the circulation, can modulate angiogenesis (Kalluri, 2003). This knowledge, along with the LM α 4 null mice transient vascular phenotype (Thyboll et al., 2002) and the interesting change in expression levels of LM α 4 during maturation of vessels, provided a good rationale to investigate the LM α 4LG4-5 fragment's effect on endothelial cell behaviour.

Endothelial cells seeded on Matrigel, a reconstituted BM extract from the EHS tumour, spontaneously form tube-like structures. The ability of endothelial cells to structure tubes depends on survival, migration, proliferation, and for true tubes cell differentiation. As angiogenic modulators affect one or several of these parameters, a good first screen to identify novel agents with such effects is to seed endothelial cells on Matrigel in the presence of the proposed substance. However, it is not an optimal screening system since endothelial cells do not divide on Matrigel (Pauly et al., 1992) and not all tubes formed by cells seeded on this substrate are true tubes. We found that recombinant LM α 1LG4-5, α 2LG4-5, α 4LG4-5, and α 5LG4-5 all strongly reduced immortalised brain endothelial micro vessel cells' (IBEC) ability to form tubes on Matrigel.

To dissect what parameters the LM α LG4-5 domains affected in the tube forming process, we looked at the effect of all LM α LG4-5 fragments on cell migration, cell proliferation, and cell survival separately. IBEC did not migrate well in direct migrations assays. We therefore conducted a scratch assay on a monolayer of endothelial cells grown on gelatine. In this system the LM α 4LG4-5 was unique in its ability to delay the fill of the cell denuded scratch area. Even though the cells are grown on gelatine, they most likely do not use this substrate for migration since these cells synthesize and deposit many ECM proteins. Endothelial cells are known to migrate well on endothelial LMs (Doi et al., 2002; Fujiwara et al., 2004) and β 1 containing integrins are important for endothelial cell migration (Short et al., 2005). Therefore, we also tested functional blocking antibodies against integrins α 6 or β 1 (the dimer is the most frequent LM binding integrin (Nishiuchi et al., 2006)) in this system. Both antibodies inhibited wound closure. Further, none of the LG4-5 domains affected cell proliferation or cell survival in our assays. We concluded that LM α 4LG4-5 repressed cell migration. This prompted us to study the effects of the LM α 4LG4-5 fragment in more complex angiogenesis assays.

Micro vessel sprouts can be induced to grow out from rat aortic segments cultured in a matrix-containing environment by addition of pro-angiogenic growth factors such as VEGF and FGF-2. An anti-angiogenic agent will reduce the number of outgrowths. This so called rat aortic ring assay is closely simulating an *in vivo*

situation as it contains not only endothelial cells but also surrounding non-endothelial cells like VSMC and fibroblasts (Auerbach et al., 2003). LM α 4LG4-5 strongly reduced the number of micro vessel sprouts from rat aortic rings supplemented with FGF-2, and this was the only tested LM α 4LG4-5 fragment that significantly affected the angiogenic response in this system (Figure 3).

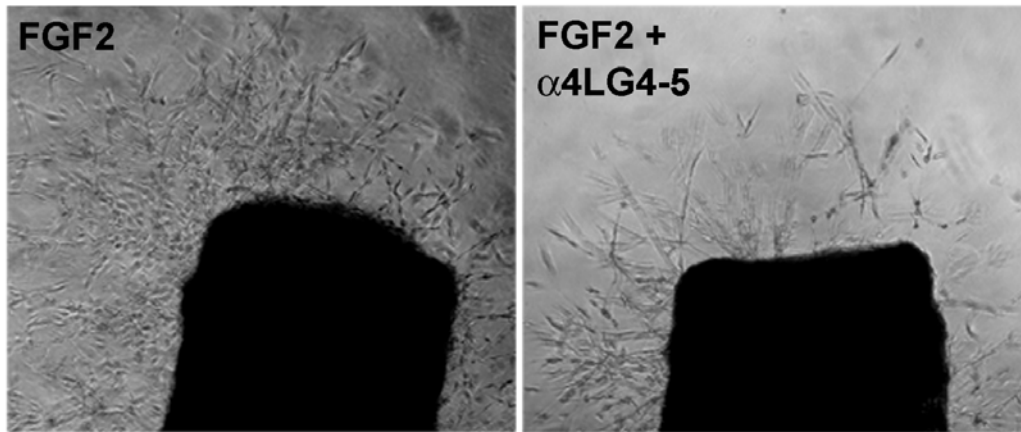


Figure 3. LM α 4LG4-5 inhibits angiogenesis *ex vivo*. Segments of rat aortas were cultured in Matrigel supplemented with FGF2 with or without addition of LM α 4LG4-5. LM α 4LG4-5 strongly reduced the number of micro vessel outgrowths.

To continue to study the effect of LM α 4LG4-5 on blood vessel formation *in vivo*, we chose a Matrigel plug assay as this is an easy method to perform. Liquid Matrigel congeals swiftly to a solid plug at body temperature when injected subcutaneously into mice. The plug formed is avascular. If complemented with a pro-angiogenic factor, it supports extensive blood vessel ingrowth (Norrby, 2006). Due to the high protein content of Matrigel, there is an osmotic pressure towards the plug. This leads to minimal leakage of substances contained within the plug. When this assay system is used for analysis of potentially anti-angiogenic agents, the substance under investigation is mixed with Matrigel supplemented with an angiogenic factor such as FGF-2 and injected subcutaneously into mice. The plugs are removed after one to three weeks and the angiogenic response is quantified either by assessing the number of formed vessels in sections from the plug, or by measuring the haemoglobin content of the plug (Auerbach et al., 2003). A drawback of this assay is that the angiogenic response varies with the position and shape the plugs adopt. This means that to generate trustworthy data the assay needs to be performed in quite a few replicates (Norrby, 2006). In this *in vivo* assay, LM α 4LG4-5 reduced both the haemoglobin content of the plugs as well as the number of recruited cells. Further, this fragment lowered the number of branching points of the vessels formed. No other tested LM α 4LG4-5 domain had any effect on the angiogenic response in the plugs. Collectively the *in vitro*, *ex vivo*, and *in vivo* assays pointed to a role of LM α 4LG4-5 as an endogenous angiogenesis inhibitor.

LM α 4LG4-5 supported cell adhesion for two endothelial cell types, IBEC and primary human umbilical vein endothelial cells (HUVEC) as well as for the non-endothelial human fibrosarcoma cell line HT-1080. But only IBEC and HUVEC adopted spread morphologies on the substrate. To identify what kinds of receptors were used for adhesion by the tested endothelial cells, we tried to block adhesion either by adding heparin to deplete heparan sulphate dependent binding (used by receptors such as syndecans and glypicans) or by adding EDTA to cancel metal-ion dependent binding (used by integrins and dystroglycan). Heparin did not affect IBEC binding and only partially (33%) reduced HUVEC binding. Addition of EDTA totally cancelled cell binding for both tested cell types. Since α 4LG4-5 binds immobilised chicken dystroglycan poorly (Talts et al., 2000) this pointed to an integrin dependent binding. Therefore, functional blocking antibodies against integrins α 3 and α 6, known to form dimers with the β 1 subunit and to bind LM 411 (Fujiwara et al., 2001; Kortessmaa et al., 2000), were used to block cell adhesion. These antibodies reduced cell binding to a variable extent (15 to 35 %) when added singly. When applied jointly the reduction was slightly more efficient (40-55%). Antibodies against integrin β 1 decreased cell binding with (60-70 %). Integrin α v β 3 was reported to bind bacterially produced LM α 4LG1-2 (Gonzalez et al., 2002). We therefore included antibodies directed against this integrin in our study. Addition of an integrin α v β 3 blocking antibody slightly reduced cell attachment for HUVEC, but did not significantly affect IBEC binding. Inactivation of one integrin can change the activities for other integrins (Hodivala-Dilke et al., 1998), a process called transmodulation. We therefore used purified integrins α 3 β 1 and α v β 3 (we could not use α 6 β 1 as it was not commercially available) to directly determine interactions with LM α 4LG4-5. We found that LM α 4LG4-5 bound integrin α 6 β 1 with a dissociation constant of 45 nM. However, the fragment did not interact with α v β 3. With the support of these data it is possible to speculate that integrin α 3 β 1 and α 6 β 1 can act as receptors for the released LM α 4LG4-5 fragment. This is in line with that many other angiogenesis inhibitors exert their activities through integrin signalling.

Cell adhesion of HT-1080 cells to LM 411 can be almost completely blocked using antibodies against the LG1-3 domain, but an antibody against the LG4-5 domain had no effect (Talts et al., 2000). Immunoblotting of purified LM-411 with an antibody directed against LG4-5 showed the LM α 4 chain to be partially intact, indicating that absence of the LG4-5 domain did not account for the lack of inhibition of cell binding with the anti-LG4-5 antibody (Talts et al., 2000). These earlier results along with our new results showing that integrin α 3 β 1 directly interacts with LM α 4LG4-5, suggests that the integrin binding sites of the α 4LG4-5 domain is covered in the intact LM α 4 chain and revealed in the released α 4LG4-5 fragment. Further, LM 411 promotes endothelial cell migration (Doi et al., 2002; Fujiwara et al., 2004; Li et al., 2006) and can be considered as pro-angiogenic. Irregularities in the capillary density and the increased vessel sprouting seen in micro pocket corneal assays of LM α 4 deficient animals points to a role for LM α 4 in stabilising

angiogenesis (Thyboll et al., 2002; Wang et al., 2006). An obvious assumption with our new findings included, is that the intact LM 411 is promoting angiogenesis by supporting endothelial migration, proliferation, and survival (DeHahn et al., 2004) while the released α 4LG4-5 is anti-angiogenic. Taken together, this creates a mechanism that could regulate blood vessel formation. This mechanism is also applicable to other molecules needed for angiogenesis (perlecan) and blood vessel BM integrity, i.e. collagen IV, collagen XVIII, and perlecan. Their C-terminal domains are just as LM α 4's cleaved off to become the angiostatic fragments endostatin, tumstatin and endorepellin (Bix and Iozzo, 2004)

Shifts in laminin expression from healthy to diseased tissue

Laminin expression in atherosclerotic plaques (Paper I)

During progression of atherosclerotic plaques, VSMC become activated and lose their contractile phenotype. The cells start to synthesize large amounts of ECM proteins, proliferate, and migrate into the intima where they participate in the formation of a neo-intima. There are reports suggesting that activated VSMC have lost their BM and LM expression and that these losses are crucial for the activation of VSMC (Hedin et al., 1999; Thyberg et al., 1997). An antibody reacting with LM α 1, LM β 1, and LM γ 1 demonstrated strong immunoreactivity in the fibrotic areas of plaques indicating presence of at least one of these chains (Cuff et al., 2001). Still, as for healthy aortas, there are limited amounts of data available on the expression of the discrete LM chains in aortic plaques. Glukhova et al. studied human atherosclerotic plaques and found an increased expression of LM β 1 chain and a reduced expression of the LM α 5 and LM β 2 chain compared to healthy tunica media (Glukhova et al., 1993). However, there are no data on the expression of other LM chains. Therefore, we stained sections of aortas from the apoE-null mice and wild-type C57 Bl6 mice, with well characterised antibodies against all eleven LM-chains.

To a large extent the LM expression in atherosclerotic plaques correlated with the LM pattern found in the normal aorta, with one major exception. LM β 2 was strongly down-regulated with a concurrent increase of LM β 1. Major LM α chains were as for healthy aortas α 2, α 4 and α 5 and LM γ chains had the same expression pattern as in normal vessels.

In addition to immigrated VSMC, atherosclerotic plaques also contain macrophages and T-cells. Both these cell types produce LM, mainly LM-411(Geberhiwot et al., 2001; Pedraza et al., 2000). Therefore, the hypothesis that deposition of LM from these cells could contribute to the changes seen in LM expression was tested. Plaques from apoE-null mice, which are rich in macrophages (Daugherty, 2002) were double stained with a macrophage marker and with a LM α 4 antibody. By confocal microscopy we could not detect LM α 4 rich areas in vicinity to cells positive for the macrophage marker.

In order to determine if the phenotypic alteration of contractile VSMC to non-contractile synthetic VSMC affected the LM expression, we used the well-established technique of culturing isolated rat VSMC (Thyberg and Blomgren, 1990). Within the first days of culture, rat VSMC lose their contractility and de-differentiate into an actively ECM synthesizing and proliferative phenotype. This *in vitro* model of VSMC phenotypic modulation mimics the de-differentiation seen *in vivo* during atherogenesis. By using this method we could analyse the LM expression by PCR and detect changes that cannot be easily observed by immunohistochemistry. Furthermore, data on the switches in the LM β chain isotype expression had already been observed in this system (Walker-Caprioglio et al., 1995).

As seen by immunohistochemistry and reported earlier, we detected a down-regulation of LM β 2 in VSMC in culture, while LM β 1 was up-regulated (Walker-Caprioglio et al., 1995). The change in LM β 1 expression occurred already at 2-3 days. Furthermore, the LM α 5 chain was significantly reduced in secondary cultures as compared with freshly isolated VSMC. Other LM chains (α 2, α 4, γ 1 and γ 2) showed no significant changes in transcription level after culture. LMs α 1, α 3, β 3 and γ 3 were weakly expressed or absent in both freshly isolated VSMC and cells in secondary culture.

Previous studies have shown that the de-differentiation process of VSMC in culture can be delayed by growth on LM but not on fibronectin. VSMC held on LM to a higher degree produced and organised smooth muscle α -actin than cells on a self produced matrix or fibronectin (Thyberg and Hultgardh-Nilsson, 1994). However, in these studies however, LM 111 was used. Our and others data (Glukhova et al., 1993) suggest that LM α 5 and LM β 2 could potentially be important for keeping the VSMC in a contractile and quiet phenotype. Hence, we analysed the effect of different LM preparations on VSMC ability to organise smooth muscle α -actin. Cells were grown on fibronectin, LM 111, LM 511/521, and a placental LM preparation containing several LMs (LM 211/221 and substantial amounts of the α 4 chain), for 1 and 3 days. Staining for smooth muscle α -actin in VSMC was stronger on all LM extractions compared to fibronectin. No visible differences were seen in the number of cells positive for smooth muscle α -actin between the different LM extractions. These results do not exclude that the LM β 2-chain could be more efficient than the LM β 1-chain in organising the contractile cytoskeleton of VSMC, since the LM β 2 content in the preparations used is fairly low. A purer β 2-chain containing LM preparation would be needed to further explore the contributions of the LM β chains to cytoskeletal organisation.

Laminin α 4 functionally compensates for absence of laminin α 2 in laminin α 2 deficient extraocular muscle (Paper III)

Although deficiencies of some LM α chains manifest in severe clinical symptoms such as congenital muscular dystrophy type1A and junctional epidermolysis bullosa, LM α chains can compensate for each other *in vivo* under some circumstances, both

artificially and naturally. Transgenically expressed LM α 1 significantly reduced muscular dystrophy and ameliorated the peripheral nerve imperfection of LM α 2 deficient mice (Gawlik et al., 2004; Gawlik et al., 2006a). LM α 2 and LM α 4 chains could partially compensate for each other's deficiency in peripheral nerves (Yang et al., 2005). The extraocular muscles (EOMs) are selectively spared from muscle wasting in many types of muscular dystrophies (Andrade et al., 2000). One such disease is Duchenne's muscular dystrophy, where functional impairment of dystrophin leads to muscle degeneration. Protection of EOMs from muscle wasting has in mice lacking dystrophin been attributed to unique intrinsic properties of this muscle group rather than adaptation. These intrinsic properties most likely involve a high and unique expression of various ECM components (Porter et al., 2003). Further, the EOMs have a distinct embryonic origin different from other skeletal muscles in mammals and show a protein profile related to developing and immature muscles (Andrade et al., 2000). In agreement with the resistance of EOMs in various muscular dystrophies, mice that express low levels of LM α 2 develop dystrophies of the limbs, but the EOMs are selectively spared (Porter and Karathanasis, 1998; Ringelmann et al., 1999). We wanted to investigate if the EOMs in mice that completely lack LM α 2 (dy^{3K}/dy^{3K}) also were protected from dystrophy.

Haematoxylin and eosin staining of cryo-sections from dy^{3K}/dy^{3K} EOMs, in contrast to dy^{3K}/dy^{3K} quadriceps, revealed no histopathological signs of muscle wasting such as fibrosis or centrally located nuclei. Tenascin-C is a molecular marker of fibrosis in muscle. In healthy mature muscles tenascin-C is restricted to myotendinous junctions, whereas in dystrophic muscles it is up-regulated in areas of fibrosis (Ringelmann et al., 1999). No areas positive for tenascin-C staining were seen in sections from dy^{3K}/dy^{3K} EOMs. This strongly indicated that the EOMs were spared also in the complete absence of LM α 2. From studies on human EOMs it has been proposed that differences in the LM composition of muscle BMs between limb muscles and EOM could partly explain the selective rescue of EOM (Kjellgren et al., 2004). Hence, we studied the expression of all LMs in EOM in both wild type and dy^{3K}/dy^{3K} mice. For this purpose we used antibodies against all eleven LM chains. As in limb muscles, EOM BMs were rich in LM α 2. Interestingly, LM α 4 was also highly expressed. This is in sharp contrast to limb muscle BMs where LM α 4 is lowly expressed, if at all. LM α 5 showed minor staining in EOM BMs as in limb muscle BMs. The major changes in LM α 2 chain deficient EOM were that LM α 4 stained stronger and that LM β 2 was absent. By quantitative PCR we saw that WT EOM contained twice as much LM α 4 mRNA as WT limb muscle. In both LM α 2 deficient limb muscle and EOM LM α 4 mRNA expression were 2.5 fold-increased compared to WT. We found the rich presence of LM α 4 in EOM BMs particularly interesting as this could possibly explain the sparing of EOMs upon depletion of LM α 2.

Two LM receptors are ubiquitously found in mature skeletal muscle, integrin α 7 β 1 and α -dystroglycan. In developing muscle the LM binding integrin α 6 β 1 is additionally present, but it is down-regulated in early postnatal stages as the muscles

mature (Mayer, 2003). LM 411 is found in developing and neo-natal skeletal muscle BMs and is strongly reduced in adult skeletal muscle BMs (Ringelmann et al., 1999). This LM is recognised by integrins $\alpha 6\beta 1$, and $\alpha 7X2\beta 1$ (Nishiuchi et al., 2006; von der Mark et al., 2002) and possibly by $\alpha 3\beta 1$ (Fujiwara et al., 2001), but it interacts weakly with α -dystroglycan (Talts et al., 2000). The retained expression of LM $\alpha 4$ in adult EOM is in line with the preservation in EOM of protein isoforms unique for muscle development. This led us to speculate that sustained presence of integrin $\alpha 6\beta 1$ in EOM BM and its interaction with LM 411 could explain sparing of EOM in congenital muscle dystrophy. However, staining for integrin $\alpha 6$ revealed localisation to capillary BMs rather than to muscle BMs. EOM is a highly vascularised tissue and the capillary density in EOM is more than double that of limb muscle (Kjellgren et al., 2004). A possibility was that the large number of vessels in EOM could account for the LM $\alpha 4$ staining seen in EOM. Since integrin $\alpha 6$ stained capillaries, we made use of this as vessel marker. Double staining of EOM with LM $\alpha 4$ and integrin $\alpha 6$ revealed expression of LM $\alpha 4$ in the muscle BMs as well as in capillary BMs.

As integrin $\alpha 6$ was not highly expressed in the EOM BMs, other possible candidates for muscle cell binding to LM $\alpha 4$ and subsequent rescue of EOM during LM $\alpha 2$ deficiency had to be considered. Integrin $\alpha 7$ has two extracellular splice variants X1 and X2 (Ziober et al., 1993). These variants show, in dimers with integrin $\beta 1$, selective affinity towards different LMs. Both forms recognise LM 211 with similar affinity, whereas the X1 variant but not X2 binds LM 411 and 511 (von der Mark et al., 2002). Interestingly, integrins $\alpha 7$ X1 and X2 have a developmental expression pattern in skeletal muscles, where both forms are present during development and the X1 variant down-regulated in mature muscle (Mayer, 2003) and up-regulated after injury in regenerating muscle (Kaariainen et al., 2002). The high expression level of LM $\alpha 4$ in EOM BMs pointed to the possibility that the integrin $\alpha 7X1$ subunit could be expressed and via its interaction with LM $\alpha 4$ partially be responsible for the rescue of EOM during LM $\alpha 2$ deficiency. PCR analyses of the $\alpha 7X1$ and $\alpha 7X2$ in EOM showed that the X1 form was expressed while X2 could not be detected. In the dy^{3K}/dy^{3K} mouse, integrin $\alpha 7$ expression is greatly reduced at the skeletal muscle sarcolemma. This is despite an increased transcription and protein synthesis of the integrin subunits $\alpha 7$ and $\beta 1D$, the muscle specific integrin $\beta 1$ isoform (Gawlik et al., 2006b). We stained EOM from WT and dy^{3K}/dy^{3K} mice with anti-integrin $\alpha 7$ antibodies and detected similar staining intensities, indicating that integrin $\alpha 7$ was localised with equal efficiency to the sarcolemma in dy^{3K}/dy^{3K} and WT EOM. Further, quantitative PCR showed similar levels of $\alpha 7$ and $\beta 1D$ in LM $\alpha 2$ deficient and WT EOM. Transcription of dystroglycan mRNA, which is increased in LM $\alpha 2$ deficient skeletal muscles (Gawlik et al., 2006b) was however not normal in dy^{3K}/dy^{3K} EOM. This is most likely due to that LM $\alpha 4$ interacts weakly with dystroglycan and thus is unable to down-regulate the dystroglycan expression to normal levels.

EOMs have been claimed to be saved from muscle wasting in muscular dystrophies by their inherent properties (Andrade et al., 2000). Indeed, a high expression of LM $\alpha 4$ and sustained synthesis of the integrin $\alpha 7 X 1$ variant after muscle maturation support this notion. The constitutive properties of the EOMs enable integrin $\alpha 7 X 1 \beta 1 D$ retention at the cell surface and normalisation in the expression of this integrin after loss of LM $\alpha 2$. This normalisation is possibly caused by occupancy of the receptor from the ligand LM $\alpha 4$. The continuation of integrin $\alpha 7 X 1 \beta 1 D$ activity in LM $\alpha 2$ deficient mice by functional compensation from LM $\alpha 4$ may not be crucial for protecting EOM from wasting, as this muscle group is not dystrophic in a mouse model lacking integrin $\alpha 7$ (Porter et al., 2003). However, our results obtained from using the EOM as a model system, strongly suggest that an induced high LM $\alpha 4$ expression could rescue other skeletal muscles from muscle dystrophy introduced by LM $\alpha 2$ deficiency. A potential answer to why other skeletal muscles are not spared in the absence of LM $\alpha 2$, even though they do produce $\alpha 7 X 1$ can be found in the level of LM $\alpha 4$ these muscles synthesize. Normal mature limb muscles have very low LM $\alpha 4$ expression. Even if this is increased in the absence of LM $\alpha 2$, the amount of LM $\alpha 4$ these muscles produce is still very low in comparison to EOM.

Concluding remarks and future perspectives

As most cells in the body are either surrounded by, or sitting on a BM and LMs are vital parts of all BMs it follows that LMs are involved in many aspects of both normal physiology as well as pathological conditions. In this thesis studies on different functional aspects of LMs are presented and the thesis is therefore seemingly spread. Nevertheless, the papers are quite closely connected in that they either deal with LM functions in blood vessels or the major vessel specific LM chain, the LM $\alpha 4$ chain. All three studies have laid out a foundation of knowledge from where many interesting and important studies should be continued.

The proteolytically released fragment LM $\alpha 4 LG 4-5$ was identified as an angiogenesis inhibitor and it possibly exerts its actions via integrins $\alpha 3 \beta 1$ or $\alpha 6 \beta 1$ (paper II). In future studies it would be interesting to study if the effects of this fragment is unique for endothelial cells, as many other angiogenesis inhibitors are known to specifically affect endothelial cells. Hints that this may be so are given by the specificity in spreading on this fragment displayed by endothelial cells (paper II). However, it is not fully elucidated if LM $\alpha 4 LG 4-5$ acts as a general inhibitor of migration. Therefore, migration studies with other cell types are needed. Indications from paper I where LM $\alpha 4 LG 4-5$ did not affect VSMC secondary culture VSMC migration over self-conditioned matrices could vaguely hint at specific effects on endothelial cells. However, aortic VSMC do not express integrin $\alpha 6$ (Moiseeva, 2001) so this could also point to that the integrin $\alpha 6 \beta 1$ is a functional receptor for LM $\alpha 4 LG 4-5$. This leads into a second future *in vitro* study. That would be to determine if LM $\alpha 4 LG 4-5$ affects cell migration via $\alpha 3 \beta 1$, $\alpha 6 \beta 1$, both or none of these. The ways to test this would vary depending on the outcome of the first study. If LM $\alpha 4 LG 4-5$

acts as a general inhibitor of cell migration GD25 cells lacking the $\beta 1$ integrin could possibly be used for an initial study and further investigations using cells depleted of integrin $\alpha 3$ or $\alpha 6$ would follow. Such cells could be obtained either by isolating embryonic fibroblasts from $\alpha 3$ and $\alpha 6$ null mice embryos, as these animals die neonatally, or by knock-down studies in established cell lines using si-RNA. If the effect of LM $\alpha 4$ LG4-5 would prove to be endothelial specific, the most straightforward way to test involvement in migration of the two proposed integrin receptors would be to transfect IBEC with si-RNA directed against $\alpha 3$ and $\alpha 6$ and perform migration studies with LM $\alpha 4$ LG4-5.

There are strong therapeutic interests for using angiogenesis inhibitors in treatments of cancer and other diseases. Some identified proteolytically released anti-angiogenic fragments of vascular BMs are, or have been, in clinical trials (Kalluri, 2003). Therefore, it would be obvious to test if and how systemic injections of LM $\alpha 4$ LG4-5 could affect growth of engrafted tumours in mice. It would also be of interest to determine the normal circulating levels of LM $\alpha 4$ LG4-5 in humans and in patients with various types of cancers in order to see if levels are elevated in patients, and to evaluate if higher circulating concentrations of LM $\alpha 4$ LG4-5 give protection or better prognosis for the disease.

We have not investigated what signalling pathways are activated by LM $\alpha 4$ LG4-5. A study clarifying this is essential to pursue. One way in which such a study could be carried out, is to compare the activation or phosphorylation by immunoblotting and immunostaining of certain signalling cascade proteins known to be involved in migration and LM signalling e.g. FAK, Rho family GTPases, MEK-ERK, and PI3 Kinase-AKT pathways, in cells with the potential receptor ($\alpha 3\beta 1$ or $\alpha 6\beta 1$) for LM $\alpha 4$ LG4-5 present or absent. These studies would be performed on cells treated or not treated with this fragment in different doses. Ideally, the cells should be grown on a self-conditioned matrix. However, in cells with changed integrin expression the synthesis and composition of the matrix should be carefully checked. Since absence of integrin $\beta 1$ in embryoid bodies leads to of LM 111 synthesis (Aumailley et al., 2000). Instead of using LM $\alpha 4$ LG4-5 as a therapeutic agent, the studies could lead to the use of substances for anti-cancer therapies that inhibit angiogenesis by specifically hitting targets in the LM $\alpha 4$ LG4-5 signalling pathway. To continue this train of thought, it would be valuable to compare the signalling pathways of different angiogenesis inhibitors affecting the same processes in endothelial cells e.g. migration and proliferation, to find common features in the genes that they affect. By such studies hopefully new targets to hit or direct therapeutic agents for anti-angiogenesis treatments could be found.

To further scrutinise the released LM $\alpha 4$ LG4-5 fragments effect on blood vessel formation during development and in disease, a few transgenic animal models can be proposed. First it would be valuable to create a mouse where the $\alpha 4$ LG4-5 fragment is not proteolytically cut off. The enzymes that participate in the cleavage are not known, although three sites of proteolytical processing within the linker region

between LG3 to LG4 have been recognised. It would be helpful in the design of the construct to be aware of what enzymes actively cut in the linker. Identification of the enzymes would also add new information to the biology of the LM α 4 chain. Without further knowledge on the players in this proteolytical process it could still be possible to create a mouse with a LM α 4LG4-5 fragment that is not liberated. Simply mutating the known cutting sites to change the translated amino acid sequence and hopefully disrupt recognition sites for the cleavage could do this. Another way to create such a mutant would be to replace the linker region of the LM α 4 chain with the linker from the LM α 1 chain. This LG4-5 domain has not been shown to be released *in vivo* (Aumailley et al., 2005), although one report has indicated separated LM α 1LG4-5 in the ectoplacental cone (Scheele et al., 2005). As the intact LM α 4 chain can be considered pro-angiogenic (Li et al., 2006) and the released LM α 4LG4-5 is anti-angiogenic, a speculation on the outcome of phenotype of mice carrying a non-liberated LM α 4LG4-5 could be that these mice would be presented with a mild vascular defect as seen for LM α 4 null mice. These defects are increased vascular sprouting and delayed vessel maturation both during development and in experimentally induced situations of neo-vascularisation (Thyboll et al., 2002).

Another transgenic animal that could be created to give insights into the LM α 4LG4-5 effect on vascular modelling is to genetically delete the LG4-5 domain. Again, a similar phenotype as seen for the LM α 4 deficient mice could perhaps be expected, but since there are suggestions that the disorganised angiogenesis in LM α 4 null mice is due to the replacing LM α 5 chain being more pro-angiogenic than the LM α 4 chain, this phenotype would perhaps not occur (Zhou et al., 2004a). Moreover, in neonatal mice with the LM α 4 chain genetically deleted, an abnormal composition of the vascular BM was seen. This BM contained reduced amounts of LMs and collagen IV, but retained normal levels of perlecan (Thyboll et al., 2002). By creating these mice lacking the anti-angiogenic LG4-5 domain but expressing the rest of the LM α 4 chain we would be able to test whether the hypothesis concerning the differential pro-angiogenic activities of the different LM α chains holds. If vascular phenotypes still occur, this would strongly propose that the LM α 4 chain within itself is important for modulating neo-vascularisation.

A third animal model that could be proposed is to create a general overexpression of the LM α 4 chain by putting the gene in front of a chicken β -actin promoter as was done for the LM α 1 chain (Gawlik et al., 2004). These LM α 4 overexpressing animals could be used to study the effect of LM α 4LG4-5 as an endogenous angiogenesis inhibitor. After determining if the circulating levels of LM α 4LG4-5 truly are elevated *in vivo*, angiogenesis assays such as matrigel plug assays can be done on mice overexpressing LM α 4 with the transgenic LM α 1 overexpressing mice as an appropriate control. If the LM α 4 overexpressing mice would show reduced vascular response, growth of engrafted tumours could be tested.

This animal model could also be used to investigate whether the LM α 4 chain can compensate for loss of LM α 2 in a mouse model of congenital muscular

dystrophy. There are indications that functional compensation would be possible from the studies on EOM, where a high LM $\alpha 4$ expression sustained integrin $\alpha 7 X 1$ presence at the sarcolemma (paper III). The integrin $\alpha 7 X 1$ isoform has previously been considered to be absent from mature limb muscles (Ziober et al., 1993), but later results (Kaariainen et al., 2002) along with ours indicates its persistence in mature muscles. Therefore, it is likely that introduction of a high expression of LM $\alpha 4$ in limb muscle could rescue LM $\alpha 2$ deficient muscles.

The EOM is spared from muscle wasting in both LM $\alpha 2$ and integrin $\alpha 7$ deficiency. This indicates that occupancy of the integrin $\alpha 7$ receptor by LM $\alpha 2$ may not be as crucial for the EOM to function as it is for the limb muscles. However, the mice deficient in integrin $\alpha 7$ are presented with a fairly mild but progressive dystrophy and the report of spared EOM are to my knowledge on 20-day-old mice. One possibility is that, at this early time point, the dystrophy has not yet manifested in the EOM and that therefore, the EOMs in LM $\alpha 2$ deficient mice can indeed be spared from muscle wasting by the high levels of LM $\alpha 4$. A mean to verify this hypothesis would be to cross LM $\alpha 2$ deficient mice with LM $\alpha 4$ deficient mice creating a double knockout. If these mice would turn out to be viable and some of them have the expected lifespan of mice lacking LM $\alpha 2$, which is about a 4-5 weeks, it would be possible to see if the EOMs in these mice were spared or not. A sparing of the EOMs would indicate that additional receptors other than integrin $\alpha 7$ and α -dystroglycan are important for the sustaining a healthy EOM in particular and for other muscles in general. One such receptor could be $\alpha 5 \beta 1$. Integrin $\alpha 5$ -null mice die early in development around embryonic day 8 of development. However, chimaeras of integrin $\alpha 5$ -null and WT mice have been made and those having a high contribution of $\alpha 5$ -null myoblasts show signs of muscle degeneration (Taverna et al., 1998).

The finding that VSMC lost expression of LM $\beta 2$ and started to produce LM $\beta 1$ as they became activated to migrate and proliferate during plaque formation (paper I), points at something often overlooked in the LM field, that not only the LM α chains are important for the ability of the LMs to regulate cell behaviour although they contain the major cell binding sites. A regulatory switch between the LM $\beta 1$ chain in developing, active, and remodelling tissues to expression of the LM $\beta 2$ chain in more static, stiff organs, such as the mature kidney glomeruli, synapses and VSMC in vessels occur. Cells from these organs revert to a LM $\beta 1$ chain expressing phenotype in some pathological conditions, such as in atherosclerosis shown here (paper I) and in malignant brain tumours (Ljubimova et al., 2004). Interestingly, also in congenital muscular dystrophy resulting from deficiency of the LM $\alpha 2$ chain, the LM $\beta 2$ chain expression is lost from the muscle BMs. When muscles are rescued by introduction the of LM $\alpha 1$ chain, the LM $\beta 2$ chain expression was restored (Gawlik et al., 2004). These events could indicate that also a sustained LM $\beta 2$ expression is important for skeletal muscle function. In the reports on the LM $\beta 2$ to $\beta 1$ switch in brain tumours, a high LM 411 expression gave a bad prognosis with increased occurrence of metastasis, whereas in the cases of sustained LM 421 expression the

cancers were less malignant (Ljubimova et al., 2004; Ljubimova et al., 2001). The reports on changes from a LM 421 to a LM 411 expressing phenotype mimics the situation observed in atherosclerosis, although during this progress the LM α 5 expression was also altered. Further, the malignant tumour could be made more benign by si-RNA directed against LM β 1 (Khazenzon et al., 2003). This hints to that the reversion from LM β 2 expression to LM β 1 in VSCM could be important for the progression of atherosclerotic plaque formation. Further studies should be made on the changes in behaviour and signalling activity of VSMC on β 2 rich LMs vs. β 1 rich LMs. One problem in performing such studies is that there are no pure LM extractions containing only the LM β 2 chain. A way to come around this problem would be to knock down LM β 1 in cultured VSMC to get these cells to produce purer β 2-LMs. The most crucial study sprouting out from our observation of shifts in LM expression patterns in VSMC should deal with what causes the reversion of LM expression and how expressions of different LMs are regulated. Very few studies have concerned that aspect in LM biology.

As a last general note, I think that it would be important to perform thorough studies on how LM gene expression is regulated, both on the transcriptional as well as on the translational level. This could lead to creation of therapeutics useful in reducing neo-intimal formation after e.g. insertions of stents to increase the lumen size in patients suffering from atherosclerosis. It could also be beneficial for treatment of other diseases such as congenital muscular dystrophy, where an increased synthesis of LM α 4 could potentially spare the muscles from wasting, or in different forms of cancers as exemplified by malignant brain tumours.

Populärvetenskaplig sammanfattning

I den här avhandlingen presenteras studier vilka berör delar i sjukdomsförloppen för de två vanligaste dödsorsakerna i västvärlden, åderförkalkning och cancer. Dessutom presenteras en studie på ett medfött muskelförtviningsyndrom som leder till en alltför tidig död, oftast redan i tonåren. Sjukdomarna som studeras kan vid en första anblick te sig vitt skilda åt, men det finns en gemensam nämnare för delarbetena och det är den grupp av äggviteämnen som jag valt att studera. Äggviteämnen utgör tillsammans med socker och fetter byggstenarna för vår kropp. Arvsmassan bär anlag som styr vilka äggviteämnen som bildas, vår kropp bär anlag för runt 30 000 olika äggviteämnen. En vuxen kropp består till en femtedel av celler vilka är de aktivt levande enheterna i kroppen. Den resterande delen av kroppen utgörs av utomcellulärt material. Detta utomcellulära material består till stor del utav socker och äggviteämnen. Materialet sammanfogar celler med varandra och sänder även in signaler som styr hur cellerna ska bete sig. Dessa signaler kan vara nödvändiga för cellens överlevnad, delning eller förflyttning. Signalerna kan även styra utmognaden av olika celltyper specialiserade för kroppens organ. Jag har undersökt en grupp av äggviteämnen i det utomcellulära materialet, lamininer. Lamininerna är särskilt viktiga för ovanstående processer och finns i olika former. De olika formerna har skilda och organunika uttryck, formerna påverkar cellernas beteende på skilda sätt. En förändring i lamininuttryck kan således vara av vikt vid vissa sjukdomar.

Åderförkalkning berör större blodkärl som försör vävnader med syre och näring. Ett stort blodkärl består av flata celler fästa i kärlväggen som beklär hålrummet, där blodet transporteras, och yttre lager av muskelceller. Storleken på kärlet regleras genom att muskelcellerna spänns eller slappnar av. Åderförkalkning kan ses som en inflammation i kärlväggen orsakad av inlagringar av fett. Inflammationen leder i flera steg till att muskelcellerna börjar dela sig och vandrar in till det innersta rummet i kärlväggen. Där hjälper de aktiverade muskelcellerna till att bygga upp en beläggning som i slutändan förminskar hålrummet av kärlet. Ett krympt hålrum leder till ett sämre blodflöde. Ett försämrat blodflöde kan bland annat yttra sig som kärlkramp. Beläggningen kan spricka upp vilket kan orsaka blodproppar som i sig kan leda till hjärn- eller hjärtinfarkt. Jag har studerat hur äggviteämnen i lamininer ändras i uttryck och form runt muskelceller under åderförkalkning. Jag har funnit att en sådan reglering sker. Detta fynd är av betydelse för fortsatta studier och leda till ökad förståelse om det bildningsförlopp som leder till åderförkalkning.

För att en tumör ska kunna få näring och växa krävs blodkärl. Blodkärlen bidrar även till att sprida tumören till olika delar av kroppen. Om rekryteringen av blodkärl hämmas kan även tumörens tillväxt och spridning förhindras. I det här projektet har jag funnit att en del av en specifik laminin dämpar nybildningen av blodkärl. Således kan denna upptäckt vara viktig vid framtagandet av nya cancerterapi.

Avsaknad av en unik laminin leder till medfödd muskelförtvinning som yttrar sig i förtidig död. Jag har studerat en muskelgrupp som förutom den ovan nämnda

lamininen även innehåller en andra lamininform. Resultaten visar att denna muskelgrupp ej förtvinar och är intressanta ur grundforskningssynpunkt då de visar att lamininerna kan kompensera varandra och i förlängningen eventuellt leda till framtida behandlingar som lindrar detta medfödda syndrom, kallat kongenital muskeldystrofi.

Resultaten av de tre studierna visar än en gång att lamininer är av stor betydelse för styrandet av den mänskliga fysiologin, både i den friska kroppen och vid uppkomst av sjukdom

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