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# THE DIVERSE ROLE OF LAMININ ISOFORMS IN NEURONAL CELLS, HUMAN MAST CELLS AND BLOOD PLATELETS

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*In memory of my sister in law Bizunesh, my mother in law W/o Taitu, a great mother W/o Yewubdar and my friend Ayalew Astateke*

*“With inflammation we step into a real-life drama, traditionally interpreted as the microscopic equivalent of warfare against true or perceived invaders, with its cellular heroes, villains, casualties, suicides, chemical weapons, and even victims of friendly fire. The setting is a battlefield in which real blood is shed, and the pace may be frantic or sluggish, but the action is always highly programmed, with message flying in all directions.” Guido Majno and Isabelle Joris (2004). Cells, Tissues and Disease. 2nd ed, p.307*

# ABSTRACT

The close interaction of cells with other cells and with extracellular matrix (ECM) components is a prerequisite in a number of biological activities. During development of the nervous system, axons often navigate a considerable distance depending on the type of molecular factors they encounter. Similarly, precursor and mature cells of haematopoietic origin home to specific locations during inflammation, wound healing, immune responses and thrombosis. Laminins (LMs), major components of specialized ECMs known as basement membranes, are large heterotrimeric glycoproteins made of αβγ subunits. To date, 5α, 3β, and 3γ chains have been identified, which by combination yield at least 15 different LM isoforms. These isoforms have a cell-and tissue-specific expression and are recognized by several integrins. The functional role of laminin isoforms has not been fully explored as the majority of early studies focused on LM-111 (α1β1γ1, laminin-1), the first laminin to be identified. The work presented in this thesis was therefore designed primarily to address synthesis, expression and, particularly, function of laminin isoforms in different cell types.

First, we showed synthesis and expression of LM-211 (α2β1γ1, laminin-2) and LM-411 (α4β1γ1, laminin-8) by tooth pulp fibroblasts based on RT-PCR, FACS, immunoprecipitation and Western blot analysis. In functional studies, LM-411, unlike LM-211, was shown to strongly promote neurite outgrowth from sensory trigeminal ganglion (TG) neurons. This activity may be relevant to tooth innervation.

In a following study, strong adhesion and migration promoting activities of α3-and α5-LMs, but not of α1-, α2- and α4-LMs, for human mast cells (CBMCs and HMC.1) were demonstrated. Among the different laminin-binding integrins, α3β1 mediated the cell adhesion and migration. HMC-1 cells expressed transcripts for LM α5, β1 and γ1 and, following stimulation, secreted the corresponding heterotrimer LM-511 (α5β1γ1). These findings demonstrated the pivotal role of α3- and α5-LMs and their α3β1 integrin receptor in mast cell adhesion and migration, and may explain the characteristic tissue localization of these immune cells in close apposition to epithelial, vascular and neural basement membranes.

Synthesis of laminins by erythromegakaryocytic cell lines and their secretion by blood platelets were also investigated. Other than detecting transcripts for LM α3, α5, β1, β2, and γ1 in the cell lines, presence of fully heterotrimeric alpha-3 (LM-311 and LM-321) and alpha-5 (LM-511 and LM-521) laminins was demonstrated in these cells as well as in platelets. Both α3- and α5-LMs were secreted by the platelets following stimulation. Functional studies showed that LM-511 (Lm-10) was the most platelet adhesive isoform followed by LM-411 (LM-8), LM-332 (LM-5) and LM-111 (LM-1). This adhesion was largely mediated by α6β1 integrin. In spite of their adhesive properties, LM-332, LM-411 and LM-511 induced neither P-selectin expression nor cell aggregation, two signs of platelet activation. In addition, we reported expression of α3-LMs in blood vessels of skin, gingiva, lymph nodes and other tissues, mainly as LM-311 and/or LM-321. Moreover, migration promoting and platelet-like particle forming activities of major vascular laminin isoforms LM-411 and LM-511 were demonstrated on *in vitro* differentiated $CD41^+$ megakaryocytes, in comparison to other laminin isoforms. Thus, vascular laminins may contribute, when exposed to the circulation, to platelet adhesion but not activation and, in bone marrow, to megakaryocyte migration and platelet formation.

Altogether, this thesis work illustrates the diverse functional role of laminin isoforms in different cell types.



Key words: laminins, extracellular matrix, integrins, tooth pulp fibroblasts, mast cells, megakaryocytes, platelets, cell adhesion, cell migration, neurite outgrowth

# LIST OF PUBLICATIONS

This thesis is based on the following papers, which will be referred to in the text by their roman numerals:

**I.** Kaj Fried*****, **Wondossen Sime***, Christina Lillesaar, Ismo Virtanen, Karl Tryggvason and Manuel Patarroyo (2005). Laminins 2 (α2β1γ1, Lm-211) and 8 (α4β1γ1, Lm-411) are synthesized and secreted by tooth pulp fibroblasts and differentially promote neurite outgrowth from trigeminal ganglion sensory neurons. *Experimental Cell Research* 307(2): 329-341.

**II.** **Wondossen Sime**, Carolina Lunderious, Randall Kramer, Patricia Rousselle, Gunnar Nilsson and Manuel Patarroyo. The selective role of Laminin-332 (Lm-5) and Laminin-511 (Lm-10) and their integrin receptor α3β1 in human mast cell adhesion and migration. *Manuscript*

**III.** Ayele Nigatu, **Wondossen Sime**, Gezahegn Gorfu, Tarekegn Geberhiwot, Ingegerd Andurén, Sulev Ingerpuu, Masayuki Doi, Karl Tryggvason, Paul Hjemdahl, Manuel Patarroyo (2006). Megakaryocytic cells synthesize and platelets secrete α5-laminins, and the endothelial laminin isoform laminin 10 (α5β1γ1) strongly promotes adhesion but not activation of platelets. *Thrombosis and Haemostasis* 95(1): 85-93.

**IV.** **Wondossen Sime**, Ismo Virtanen, Ingegerd Andurén, Sulev Ingerpuu, Patricia Rousselle, Sergei Smirnov, Peter Yurchenco, Jonathan C.R. Jones, Anna Domogatskaya, Karl Tryggvason, Gunnar Nilsson, Paul Hjemdahl and Manuel Patarroyo. Expression of α3-laminins by platelets and blood vessels and their role in promoting platelet adhesion, megakaryocyte migration and platelet formation in comparison to other laminin isoforms. *Manuscript*

***** Equal contribution.

# CONTENTS

# LIST OF ABBREVIATIONS

| | |
|---|---|
| ADP | Adenosine diphosphate |
| BL | Basal lamina |
| BM | Basement membrane |
| BSA | Bovine serum albumin |
| CBMCs | Cord blood derived mast cells |
| CD | Cluster of differentiation |
| Col | Collagen |
| ECM | Extracellular matrix |
| EHS | Engelbreth-Holm-Swarm |
| EDTA | Ethylenediaminetetraacetic acid |
| EGF | Epidermal growth factor |
| ELISA | Enzyme linked immunosorbant assay |
| FACS | Fluorescence activated cell sorter |
| FBS | Foetal bovine serum |
| FGF | Fibroblast growth factor |
| FITC | Fluorescein isothiocyanate |
| FN | Fibronectin |
| GAPDH | Glyceraldehyde-3-phosphate dehydrogenase |
| HRP | Horse radish peroxidase |
| HSA | Human serum albumin |
| IA | Immunoaffinity |
| Ig | Immunoglobulin |
| IL | Interlukine |
| IMDM | Iscove's modified dulbeco's medium |
| INT | Integrin |
| IP | Immunoprecipitation |
| kDa | Kilodalton |
| KO | Knockout |
| LM | Laminin |
| mAb | Monoclonal antibody |
| MCs | Mast cells |
| MIDAS | Metal ion-dependent adhesion site |
| MKs | Megakaryocytes |
| mRNA | Messenger RNA |
| PBS | Phosphate buffered saline |
| PE | Phycoerythrin |
| PMSF | Phenyl methyl sulfonyl flouride |
| PVP | Polyvinylpyrrolidone |
| rh | Recombinant human |

| | |
|---|---|
| RGD | Arginine-Glycine-Aspartic acid |
| RNA | Ribonucleic acid |
| RT | Room temperature |
| RT-PCR | Reverse transcription polymerase chain reaction |
| SCF | Stem cell factor |
| SDF-1α | Stromal cell derived factor |
| SDS-PAGE | Sodium dodecyl sulphate polyacrylamide gel electrophoresis |
| TG | Trigeminal ganglion |
| TNFα | Tumor necrosis factor α |
| TPO | Thrombopoietin |
| TPA | Tetradecanoyl phorbol acetate |
| VLA | Very late antigen |
| VEGF | Vascular endothelial growth factor |
| VN | Vitronectin |
| WB | Western blotting |

# 1 INTRODUCTION

Laminins (LMs) are a family of multifunctional heterotrimeric glycoproteins largely found in basement membranes. Over 15 additional laminin isoforms have been identified since the first laminin (laminin-1, LM-111, α1β1γ1, EHS-laminin) was discovered some 28 years back. Although identification of the members of the family has been relatively simple, their purification and in-depth structural and functional characterization have proven to be difficult. Since LM-111 is comparatively easy to isolate in large amounts from its source, most previous studies in the field have focused on this prototype laminin isoform. Nevertheless, for some of the laminin isoforms other than LM-111, recombinant laminins are presently available as well as different commercial laminin preparations isolated from human placenta. Unfortunately, the quality of the latter preparations is rather poor.

To date, the accumulated knowledge in the field, using the various available laminin preparations, has indicated how these different isoforms are involved in different biological activities both in normal and pathological conditions. Even if cells from solid tissues, such as epithelial, endothelial, stromal and other cell types, are believed to be the main source of laminins, presence of these multifunctional large glycoproteins has been recently shown in haematopoietic and blood cells. Some of the work presented in this thesis was a continuation of these early encouraging findings in which the synthesis, expression and secretion of laminin isoforms were determined in different cell types of haematopoietic origin. In addition, the diverse role of laminin isoforms in promoting neurite outgrowth, adhesion and migration of human mast cells, platelet activation and aggregation, and megakaryocyte migration and platelet formation were explored.

## 1.1 BASEMENT MEMBRANE

Basement membranes (BMs) are specialized forms of extracellular matrix with a thin sheet like structure (50-100 nm), which are commonly seen beneath epithelial and endothelial cell layers and surrounding muscle, fat, and peripheral nerve cells. Based on electron microscopic observation, most of the basement membranes have a two layered appearance. The layer which is close to the cell membrane and less electron dense is referred as the *lamina lucida* and the other layer, which is electron dense and located close to the connective tissue, is the *lamina densa.* Besides providing some structural support and serving as a barrier, BMs influence cell adhesion, migration, polarization, differentiation, and survival. The retention and sequestration of some growth factors by BM is also one way of influencing the nearby cells. Because of all these essential contributions, BMs have been implicated in tissue maintenance, regeneration, and repair as well as in some pathological conditions like tumor growth and metastasis (Timpl, 1996; Timpl and Brown, 1996; Kalluri, 2003).

Though the composition of BM varies from one tissue type to another, at different developmental stages, and during tissue repairing/remodeling, certain protein families are usually present. These major components are the laminins, type IV collagens, nidogens (entactins) and perlecan. Additionally, some minor components such as agrin, BM-40/osteopontin/SPARC, fibulins, type XV collagen and type XVIII collagen can also be found in BMs. The functional diversity of BMs correlate with the heterogeneous nature of its components coming either from diverse groups of protein families or from the different isoforms of same family, such as for type IV collagens and laminins (Yurchenco, 1990; Timpl, 1996, Timpl and Brown, 1996, Durbeej, 1996; Aumailley and Gayraud, 1998; Collognato and Yurchenco, 2000).

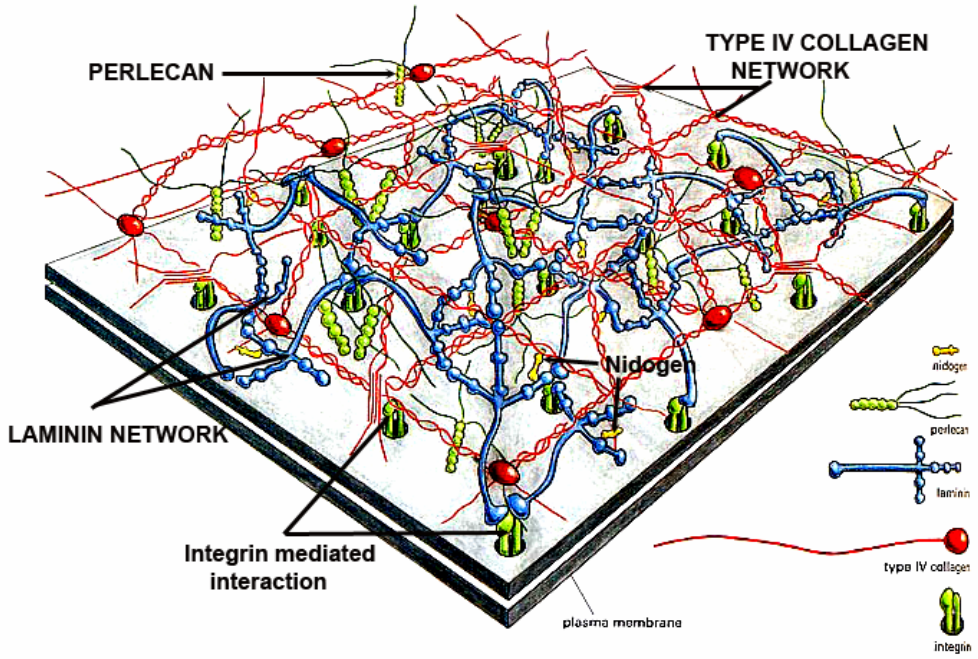


**Figure 1.** Molecular structure of basement membrane. Laminin and type IV collagen networks linked by nidogen and the interaction of cell surface bound integrins with specific BM components are indicated with arrows. Adapted and modified from Molecular biology of the cell.4th ed, Garland Science, 2002.

Assembly and integrity of BM are initiated through interactions between its multiple components, which are capable of making the network, inter-component binding and cell surface interactions. Some laminin isoforms and type IV collagens contain specific domains in their structure that allow them to form intermolecular self-assembly. The ability of laminin isoforms and type IV collagens to form independent networks is generally considered to be essential to create dynamic flexibility and mechanical stability in basement membranes, respectively (Fig.1). These two separate networks are connected to each other by nidogen (Timpl, 1996; Timpl and Brown 1996; Collognato and Yurchenco 2000; Yurchenco 2004).

## 1.2 LAMININS

Laminins (LMs) are large (400-900 kDa) heterotrimeric glycoproteins composed of three genetically different polypeptides, termed as α, β and γ chains. To date, five α (α1–5), three β (β1–3), and three γ (γ1–3) chains have been identified, which by combination constitute the various laminin isoforms. Sixteen different laminin isoforms are known to be assembled, including the major splice variants (Aumailley *et al*., 2005). As shown in Fig.2, LM-111 has a chain composition of α1β1γ1 and is also referred to as laminin-1. This first laminin, discovered some 28 years ago, was isolated from the mouse Engelbreth -Holm-Swarm (EHS) sarcoma tumor (Timpl, 1979) and from the culture supernatant of a mouse embryonic carcinoma cell line (Chung *et al*., 1979). It is also known by other names such as EHS-laminin and "prototype" laminin. Since isolation of LM-111, a number of other laminin isoforms has been identified, and the list keeps growing.

### 1.2.1 Laminin nomenclature

In the early days, when the existing laminin isoforms were few, their designation was rather confusing. For instance, the three chains (α, β and γ) of LM-111 were denominated as A, B1, B2, and for laminin-2 (Merosin), which was discovered

thereafter, M, B1, B2, since the β and γ chains were the same for the two isoforms. Therefore, the importance of having a simplified nomenclature for the various laminin chains, isoforms, and their domains was essential for a common understanding, and also to disseminate the information quite easily. Thus, the first nomenclature was introduced some 12 years ago. This nomenclature has been widely used and largely contributed to the field (Burgeson *et al*., 1994). However, some modification of this nomenclature was still needed because of the increasing number of laminin chains and isoforms, as well as some problems associated with it. To avoid confusion and to better understand the chain composition of the different laminin isoforms, a new simplified nomenclature was recently introduced (Aumailley *et al*., 2005). For instance, the first laminin (α1β1γ1), referred to as laminin-1 (LM-1) in the old nomenclature, is termed laminin-111 (LM-111) in the new nomenclature, to indicate its chain composition α1β1γ1. The same principle is applied to the other laminin isoforms as presented in Table 1.

**Table I:** Nomenclature of laminin isoforms based on Burgeson *et al*., 1994 and Aumailley *et al*., 2005 .

| **Chain Composition** | ***New nomenclature*** | ***Previously referred to as*** | **References** |
|---|---|---|---|
| α1β1γ1 | LM-111 | laminin-1<br>EHS-laminin | Timpl et al.,1979 |
| α2β1γ1 | LM-211 | laminin-2<br>Merosin | Engvall et al.,1990 |
| α1β2γ1 | LM-121 | laminin-3<br>S-laminin | Green et al.,1992 |
| α2β2γ1 | LM-221 | laminin-4<br>S-merosin | Engvall et al.,1990 |
| α3Aβ3γ2 | LM-332/LM-3A32 | laminin-5/5A<br>kalinin | Rousselle et al., 1991 |
| α3Bβ3γ2 | LM-3B32 | laminin-5B | Yoshinobu *et al*., 2004 |
| α3Aβ1γ1 | LM-311/LM-3A11 | laminin-6<br>K-laminin | Marinkovich et al., 1992 |
| α3Aβ2γ1 | LM-321/LM-3A21 | laminin-7<br>KS-laminin | Champliaud et al., 1996 |
| α4β1γ1 | LM-411 | laminin-8 | Miner et al., 1997 |
| α4β2γ1 | LM-421 | laminin-9 | Miner et al., 1997 |
| α5β1γ1 | LM-511 | laminin-10 | Miner et al., 1997 |
| α5β2γ1 | LM-521 | laminin-11 | Miner et al., 1997 |
| α2β1γ3 | LM-213 | laminin-12 | Koch et al., 1999 |
| α4β2γ3 | LM-423 | laminin-14 | Libby et al., 2000 |
| α5β2γ2 | LM-522 | - | Siler et al., 2002 |
| α5β2γ3 | LM-523 | laminin-15 | Libby et al., 2000 |

### 1.2.2 Laminin structure and domains

Laminins are multidomain glycoproteins with different arms in their structures containing specific binding sites. The short arms, representing the amino terminal (N-terminal) regions of the three laminin polypeptides, are localized at opposite ends, in contrast to the long arm region where the three chains are joined together and positioned on the same plane (Fig.2). Though eleven different laminin chains exist, certain structural similarities are shared by these subunits. These include the presence of laminin epidermal growth factor-like (EGF-like) (LE) domains, small globular domains (LN, L4a, L4b and LF domains) and the α-helical coiled-coil long arm (domain I and II). The three LN-domains are usually found at the very end of the short arm regions (N-terminal). These LN domains are involved in laminin polymerization. The α-helical coiled-coil long arm is located in the middle and has an approximately 75 nm long rod-like structure with two domains designated as I and II. This is the trimeric portion of the chains, joined together and stabilized by disulphide-bridges during the process of laminin assembly. The assembly of the three laminin chains gives to the laminin isoforms their characteristic "T", "Y" or cross shaped appearance under rotary shadowing electron microscope, depending on the laminin chains involved (Beck *et al*., 1990; Beck *et al*., 1993; Colognato and Yurchenco 2000, Aumailley *et al*., 2005).

The laminin-type epidermal growth factor (LE) domain is a cysteine-rich domain of 50-60 residues. These tandem repeats form rod-like structures connecting the larger globular domains on the short arm region of each laminin subunit. Except for serving as a spacer element to give some structural support and having a high affinity binding site for nidogen on the γ chain, no other biological activities have been described to these domains (Beck *et al*., 1990; Hochenester and Engel, 2002).

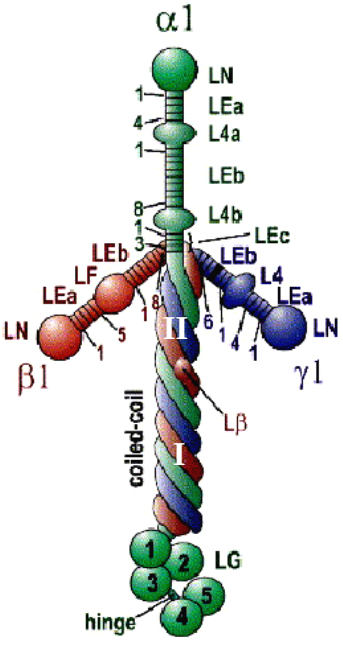


**Fig 2**. Schematic representation of LM-111 structural domains based on the new nomenclature. Changes for some of the domains (e.g. the LN and LE domains etc) were given. The LN, laminin N-terminal domain, on short arm region was referred as domain VI previously.
The approximate position of domain I and II is depicted in white. The black mark on domain LEb represents the nidogen-binding site (Modified from Aumailly et al., 2005).

The triple helical coiled-coil interaction in the long arm region is critical in defining the feature of the laminin isoform and the specificity of the chain assembly. Based on sequence analysis, domains I and II are poorly conserved among the laminin subunits (20-40%). Studies have also shown that the mechanism for this intramolecular assembly is dependent on the ionic interactions between the multiple seven-residues repeats (heptads) embedded in the hydrophobic core of the coiled-coil

polypeptides. Each heptad repeat, “abcdefg” contains hydrophobic or non-polar residues at position “a” and “d”, and charged residues at position “e” and “g” which are involved in the assembly process (Beck et al., 1990; Beck et al., 1993; Engvall and Wewer, 1996).

To compare the first laminin with other isoforms structurally, another classification has been also used (Fig.3). Those laminin isoforms with domain structure similar to laminin-111 are referred to as “classical” laminins, others which lack one short arm are named “topless”, and those devoid of more than one short arm are called “truncated”. Moreover, those laminin isoforms having the α chain longer than the α1 chain are called “long” (Collognato and Yurchenco, 2000). In contrast to the β- and γ- chains, all the α-chains have a characteristic feature known as laminin globular (LG) domain at their C-terminus. The LG domain consists of five repeating homologous modules called LG1-LG5, and the LG1-LG3 modules are connected to the LG4-LG5 modules by a linker domain (hinge). This link domain is sensitive to proteolytic processing, which is known to occur at least in α3- and α4-chains (Beck et al., 1990; Timpl et al., 2000).

A full crystal structure for laminin has not yet been determined, but a hypothetical model has been reported for the LG-domains based on its recently determined partial crystal structure. Other BM molecules like perlecan and agrin also have these domains. LG domains of the five laminin α-chains have very limited sequence identity (20-25%), something that seems to be reasonable considering that the effect of a specific laminin isoform for a particular biological function may not be compensated by others. This may be due to the fact that most of the key biological activities are mediated at the level of the LG domains, which contain the binding sites for cellular receptors such as integrins, dystroglycans, sulfated carbohydrates and other extracellular ligands (Beck et al., 1990; Hohenester et al., 1999; Talts et al., 1999; Timpl et al., 2000). Characteristic and essential domain structures are present in LM β and γ chains as well. At the border of the coiled-coil I/II regions of the β-chain, a small interruption termed as the laminin β-knob (Lβ) domain is found (prior to adoption of the recent nomenclature it was confusingly referred as to”α”). The LM β3 and γ2 chains, components of laminin-5 (LM-3A32), differ quite significantly from other laminin subunits in terms of their domain organization. Structurally, β3 contains the LN domain but lacks the L4 domain, beside its very few LE repeats, whereas γ2 lacks the short arm N-terminal end (LN-domain) but has the L4 domain (Fig.3).

All the LN-domains in the short arm region of the three chains are involved in the intermolecular assembly of laminin and this process of forming an independent laminin network is $Ca^{2+}$-dependent. If one or more of these domains are missing, as in the α3A, α4 and γ2 chains, it might not be possible to form the laminin network. However, an alternative assembly between the three α3A-containing laminin isoforms, LM-3A32 (laminin-5), LM-3A11 (laminin-6), and LM-3A21 (laminin-7) has been described. This intermolecular interaction and assembly occurs through LN domains including the single LN-domain on β3 chain of LM-3A32, and the two LN-domains on β1 and γ1 of LM-3A11, and β2 and γ1 of LM-3A21. Presence of additional binding sites on the laminin chains to connect with other ECM molecules is also important. For instance, the nidogen-binding site on the γ1 chain LE domain (LEb) is essential in linking the laminin network with the type IV collagen network, so that the structural integrity of the basement membranes is properly maintained (Champliaud *et al*., 1996; Engvall and Wewer., 1996; Kleinman *et al*., 2003; Aumailley *et al*., 2005).

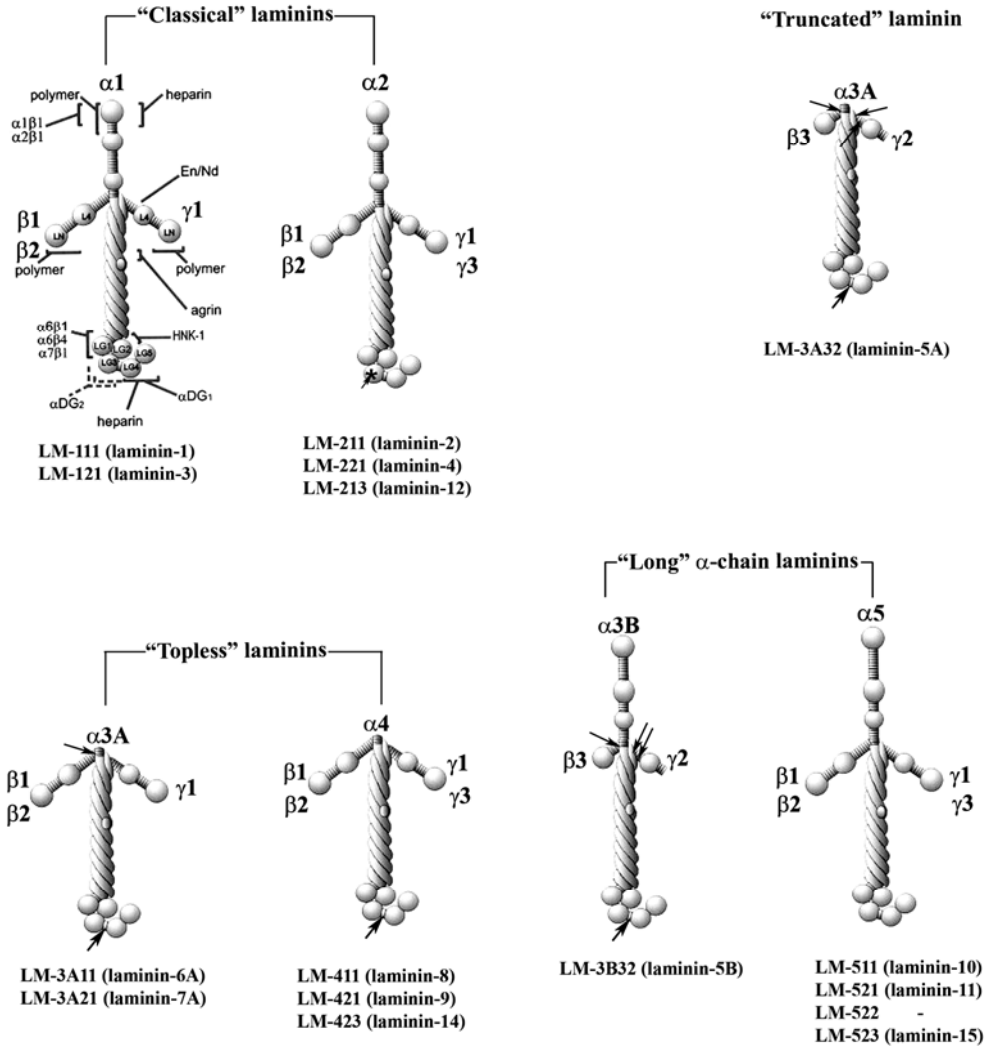


**Figure 3.** Schematic representation of laminin heterotrimers. Some binding sites that map to specific regions of the molecule (mostly LM-111) and the chain compositions in the form of numbers next to the LM abbreviation are presented. Possible cleavage sites are also indicated by arrows. The asterisk (⋆) in α2-containing laminins indicates how the cleaved fragment remains non-covalently associated with the trimer. Modified from (Aumailly et al., 2005; Colognato and Yurchenco, 2000).

### 1.2.3 Laminin isoforms and their tissue distribution and biological roles

As mentioned before, laminin-111 (α1β1γ1) was the first laminin to be found and, since then, over 15 new laminins, assembled by combination of five α, three β and three γ chains have been recognized. These various laminin isoforms are expressed in a tissue-and development-specific manner. The process of laminin assembly is similar to many other secreted proteins and it is known to occur entirely in the endoplasmic reticulum (ER), before secretion of the αβγ heterotrimer. Each of the laminin chains is transported to the endoplasmic reticulum where the first specific interaction between the β and γ chains takes place followed by insertion of the α chain partner. Studies have shown that final inclusion of the α chain is a rate-limiting step in laminin biosynthesis since it triggers the secretion of the whole laminin trimer. Although the laminin α chain was found to be secreted as a monomer, no secretion of homodimers (ββ/γγ), or homotrimers (βββ/γγγ) were seen (Yurchenco *et al*., 1997).

To study expression of laminins in different tissues and other samples, various methods have been used, including Northern blotting, *in situ* hybridization and immunostaining. The latter technique is most widely used, but it has a limitation, namely, that the antibodies recognize laminin chains, not laminin heterotrimers. Therefore, caution must be taken in the interpretation of the results. Despite these limitations, it is still valuable to determine the tissue distribution of the particular laminin chains and, if possible, to extrapolate these results for laminin heterotrimers.

### Laminin β-and γ-chains

LM β1 and γ1 chains, which account for 6/16 and 10/16 of the various laminin isoforms, respectively (Table I), are present in most of the basement membranes studied. The intracellular expression of these two chains has been reported at the two-four-cell stages of fertilized eggs. Mice lacking γ1 chain die at 5.5 days post-coitum, with a complete lack of BMs (Kallunki *et al*., 1992; Thomas and Dziadek, 1993; Smyth *et al*., 1999; Sorokin *et al*., 1997a). The laminin β2-chain is present in perineural BMs and at the neuromuscular junctions (NMJ). In addition, there is a switch from β1 to β2 chain expression during glomerular basement membrane development (Patton *et al*., 1997). Mice lacking LM β2 chain showed as a characteristic phenotype defects in NMJ development and in kidney functions. These mice suffered from massive proteinuria and died between 2-3 weeks of their postnatal period (Noakes *et al*., 1995a and1995b).

β3 and γ2, two components of LM-332 (laminin-5), are expressed in epithelia of skin, kidney, respiratory and gastrointestinal tracts, and the developing tooth. Interestingly, LMγ2 chain is expressed at the invading edge of many cancer forms of epithelial origin, though the biological significance of the finding is presently unknown. On the other hand, mutations of the *LAMB3* and *LAMC2* genes cause junctional epidermolysis bullosa (JEB), a severe skin blistering disease (Kallunki *et al*., 1992; Aberdam *et al*., 1994; Pulkkinen *et al*., 1994; Kivirikko *et al*., 1996; Salmivirta and Ekblom., 1998; Skyldberg *et al*., 1999).

γ3 is the most recently described laminin chain, and it is a component of the newly isolated laminin isoforms LM-213 (laminin-12), LM-423 (laminin-14) and LM-523 (laminin-15). It contains all the expected domains of a γ chain, including a putative nidogen-binding site. Studies on its tissue distribution showed that it is broadly expressed in the skin, retina, heart, lung, and the reproductive tracts. Moreover, this chain has been reported to be expressed on the apical surface of ciliated epithelial cells found in lung, oviduct, epididymis, and seminiferous tubules (Iivanainen *et al*., 1999; Koch *et al*., 1999; Libby *et al*., 2000).

### Laminin α-chains

The spatial specificity of laminin isforms seems to correlate with the α chain expression pattern in a given tissue. All the five α chains are known to be present at early embryonic and adult stages but each has its own distinct expression and distribution pattern in terms of time and tissue sites both at the mRNA and protein levels. These expression patterns of the known α chains and possible laminin isoforms to which these chains are associated with are presented in the following section. In general, laminin α1 chain has the most restricted expression and laminin α2 chain is particularly abundant in mesoderm-derived tissues. Laminin α3 is expressed in most epithelial BMs whereas α4 and α5 chains are the most widely expressed laminin α subunits (Ekblom, 1990; Galliano et al., 1995; Miner et al., 1997; Virtanen et al., 1997).

### *α1-containing LMs*

LMα1-chain is a component of LM-111 (laminin-1) and LM-121 (laminin-3). LM-111, also known as EHS/classical/prototype laminin, is the most extensively characterized of all laminins. As the first laminin to be identified, polyclonal antibodies were raised against it and used for immunostaining. Since a wide tissue staining of BMs was observed by using these reagents, a corresponding wide distribution of LM-111 was concluded (Timpl *et al*., 1979). This conclusion was incorrect, because the polyclonal antisera cross-react with other laminin isoforms containing LMβ1 and γ1 chains. Also mistakenly, a broad distribution of LMα1-chain in adult human tissues was concluded by immunohistochemistry with mAb 4C7 (Engvall *et al*., 1990), which later on was proven to be specific for LMα5 chain. These immunological studies were in clear contrast with *in situ* hybridization studies showing a very restricted expression pattern of LMα1-chain (Vuolteenaho *et al*., 1994; Tiger *et al*., 1997; Kikkawa *et al*., 1998). Thus, the revised knowledge concerning expression of LM α1-chain in adult tissues is that it has the most restricted tissue distribution, namely, a few epithelial BMs including proximal tubules of the kidney, gastrointestinal tract, thyroid and mammary glands, and male and female reproductive organs such as the testis and the endometrium of the uterus. In fetal tissues, presence of LM α1-chain is detected in epithelia of the developing kidney, testis and epididymis, bronchial tubules and developing glands in the gastrointestinal tract already at 16 weeks of gestation (Ekblom *et al*., 1990; Miner *et al*., 1997; Virtanen *et al*., 2000).

LM-111 has been shown to be critical in early embryogenesis and, more particularly, to have a biological role in epithelial morphogenesis of various organs including kidney, lung, intestine, salivary, and mammary glands. Several studies have shown the crucial role of laminin α1, β1, and γ1 chains, as mice which lack either of these chains die during early embryogenesis, shortly after implantation before gastrulation (Smyth *et al*., 1999; Miner *et al*., 2004; Scheel *et al*., 2005;).

### *α2-containing LMs*

LMα2-chain is a component of LM-211 (laminin-2), LM-221 (laminin-4) and LM- 213 (laminin-12). Though closely related to laminin α1-chain and also capable of undergoing polymerization, this chain is different in that it is proteolytically cleaved at its LG3 domain (Fig.3) and has different binding sites for dystroglycan and heparin. Under reducing conditions, two fragments of 300 and 80 kDa, specific to α2 polypeptides, are resolved in SDS-gel electrophoresis (Paulsson *et al*., 1991). The LMα2-chain is particularly abundant in mesoderm-derived tissues, and it is localized in BMs of developing and adult skeletal muscle, cardiac muscle, endoneurium of peripheral nerves and thymus, and in some tubular BMs of kidney in mouse (Miner *et al*., 1997; Patton *et al*., 1997; Sorokin *et al*., 2000). Studies carried out on human tissues have also shown expression in BMs of myocytes, Schwann cells, placenta and mesangium of mature kidney (Leivo and Engvall., 1988; Virtanen *et al*., 1995a).

Evidence for the critical role of LMα2 chain has been demonstrated in spontaneous and targeted disruption of the corresponding gene in mice, which resulted in severe muscular dystrophy and in death of the mice some weeks after birth. Similarly, mutations in *LAMA2* gene in humans cause congenital muscular dystrophy (CMD). In addition to the severe muscular weakness, the peripheral nervous system is also affected. Recently, lack of laminin α2 chain in BM of testis in α2-deficient mice was shown to affect male fertility. The defect was due to lack of

laminin incorporation into the BM of testis (Sunada *et al*., 1994; Guo *et al*., 2003; Miyagoe *et al*., 1997; Mattias *et al*., 2005).

### *α3-containing LMs*

Alternative splicing occurs for some laminin chains such as α3, α4, and γ2. Hence, there are two variants of the α3 chain, namely, α3A (the short variant) and α3B (the full length), which are transcribed from the same gene (*LAMA3*) by using two different promoters (Airenne *et al*., 1996; Ferrigno *et al*., 1997; Xiao *et al*., 1997). The two products, α3A and α3B, differ in their amino terminal region as depicted in Fig.3. The most widely studied α3-containing laminin is LM-3A32 (laminin-5A), previously referred to as epiligrin/nicein/kalinin. This isoform consists of three polypeptides of 200 kDa (α3A), 140 kDa (β3) and 155 kDa (γ2), and it is the only laminin known so far to have three very short arms, thereby the term "truncated" is used to indicate its structural features (Fig.3). Extracellular proteolytic processing can influence the biological role of laminin-5. Cleavage of α3A from 190 kDa to 165 kDa is mediated by plasmin, and a further processing to 140 kDa may still occur. The LMγ2 chain also undergoes processing from 155 kDa to 105 kDa at the N-terminus by metalloproteinases (Marinkovich *et al*., 1992; Goldfinger *et al*., 1998; Pirila *et al*., 2003). LMα3A chain is also a constituent of LM-311 (laminin-6A) and LM-321 (laminin-7A). The α3B chain has an expected molecular size of about 360 kDa, based on the sequence analysis, but the protein isolated from mouse lung extract demonstrated an apparent molecular weight of 280-300 kDa (Ryan *et al*., 1994; Galliano *et al*., 1995; Miner *et al*., 1997). Though not much is known about its biological role, assembly of a α3B-containing laminin as LM-3B32 or laminin-5B has been recently reported. Unlike the α3A chain, α3B posseses a LN-domain at the N-terminal region, which allows self polymerization (Galliano *et al*., 1995; Kariya et al., 2004).

Studies on mouse tissue using *in situ* hybridization demonstrated that both laminin α3A and α3B chains were detected in the basal membrane of the upper alimentary tract and urinary and nasal epithelia. The α3A chain appeared to be predominantly expressed in the skin and, specifically, in hair follicles and developing neurons of the trigeminal ganglion (13.5 days postcoitum). Strong expression of the α3A and α3B chain was also demonstrated in the salivary glands and teeth. On the other hand, α3B transcripts were exclusively found in bronchi and alveoli, stomach and intestinal crypts, whisker pads, and central nervous system. In general, broader tissue distribution of α3B, when compared to α3A, has been reported (Doliana et al., 1997 Yoshiba et al., 1998).

LM-3A32 (laminin-5A) is not only restricted to promote stable anchorage of keratinocytes to the underlying connective tissue as commonly seen in skin. Its relevance in mediating cell motility is also essential for wound-healing and may contribute to tumor invasion. In contrast to LM-3A32 (laminin-5A), little is known about the biological role of LM-3A11 (laminin-6A) and LM-3A21 (laminin-7), though their co-expression and association with LM-3A32 have been observed in human amnion and keratinocytes (Ryan *et al*., 1994; Champliaud *et al*., 1996). Mutations in LM α3, β3 and γ2 chains are found in patients suffering from junctional epidermolysis bullosa (JEB). Though severe skin blistering appears as the major defect, abnormalities in mucous membranes and in internal organs are also observed in these patients. Likewise, LMα3 deficient mice developed progressive skin blistering, and eventually died a few days after birth (Pulkkinen *et al*., 1994; Christiano and Uitto, 1996; Ryan *et al*., 1999).

### *α4 -containing LMs*

Laminin isoforms containing α4 chain are LM-411 (laminin-8), LM-421 (laminin-9) and LM-423 (laminin-14). Studies from human cell lines have shown presence of two splice variants of laminin α4, namely, α4A and α4B (Miner *et al*., 1997; Libby *et al*., 2000; Hayashi *et al*., 2002). Interestingly, a condroitin sulphate modification has been reported on the short arm region of laminin α4-chain (α4IIIa), both in a mammalian expression system and in certain tissues (Sasaki *et al*., 2001; Kortesmma *et al*., 2002). LMα4 chain expression in mice has been described in peripheral nerves, developing kidney, lung alveolar septa, around cardiomyocytes, in blood vessels and in bone marrow. In human tissues, the LMα4 chain was found in BMs of skeletal and smooth muscle, developing heart, in some parts of adult kidney such as the mesangium, and in capillaries associated with tubules, but not in glomerular basement membrane (GBM). Its presence has also been reported in BMs of adipocytes, axons of developing and mature nerves, and in all types of endothelial cells.

Synthesis and/or expression of LM-411/421 (laminin-8/9) in non-BM sites, including bone marrow stromal cells, blood platelets, monocytes, lymphocytes and granulocytes, have been demonstrated by different groups (Miner *et al*., 1997; Gu *et al*., 1999; Geberhiwot *et al*., 1999; Siler *et al*., 2000; Gu *et al*., 2003,; Geberhiwot *et al*., 2000 ; Geberhiwot *et al*., 2001; Wondimu *et al*., 2004). Mice with targeted disruption of the *LAMA4* gene were viable and fertile but displayed uncoordinated movements of the hind limbs due to neuromuscular dysfunction. Impaired myelination and radial sorting, transient hemorrhages at birth, impaired neutrophil extravasations to inflammed tissue and defects in the organization of endothelial BM were other abnormalities identified in various studies (Patton *et al*., 2001; Thyboll *et al*., 2002; Wondimu *et al*., 2004).

### *α5-containing LMs*

Laminin α5 is a component of LM-511 (laminin-10), LM-521 (laminin-11), and LM-523 (laminin-15) and is the most widely expressed among the five laminin α chains (Miner *et al*., 1995; Miner *et al*., 1997; Ferletta and Ekblom, 1999; Libby *et al*., 2000). This broad distribution of the LMα5 chain occurs in both embryonic and adult tissues. It is expressed in developing epithelia and larger vessels of the embryo and in adult tissues with very high level of expression in the lung, skin, kidney, heart, intestine, blood vessels, bone marrow and neuromuscular synaptic clefts (Durbeej *et al*., 1996; Miner *et al*., 1998; Sorokin *et al*., 1997a, Sorokin *et al*., 1997b). Deletion of laminin α5 in mouse leads to early embryonic lethality at E13.5-16.5 and multiple defects including, impaired limb patterning, lung lobe and digit septation (syndactyly), neural tube closure as well as placental defects, breakdown of the glomerular BMs and exencephaly (Miner *et al*., 1998; Kikkawa *et al*., 2003).

## 1.2.4 Laminin receptors

Existence of multiple laminin chains and isoforms creates functional diversity, in addition to the molecular and structural heterogeneity. Laminins have been shown to interact with numerous cell-surface molecules and to participate in a number of biological activities like cell adhesion, differentiation and migration. These and other biological effects are largely mediated via specific cell surface receptors, such as integrins, dystroglycans, syndecans and Lutheran blood group glycoproteins (Henry and Campbell, 1996; Hoffman et al., 1998; Colognato and Yurchenco, 2000; Patarroyo *et al*., 2002; Suzuki *et al*., 2003). Since integrins are the best characterized laminin-binding proteins, a brief summary of the integrin family is presented here.

### Integrins

Integrins are heterodimeric transmembrane glycoproteins composed of non-covalently associated α and β chains. At present, 24 different integrin are known in man, which results from specific combination of 18 α and 8 β subunits. Each integrin subunit has three major domains based on their cellular location, namely, the long N-terminal extracellular domain, a single transmembrane spanning domain and, in almost all cases, a short cytoplasmic domain (Hynes, 1992; Humphries, 2000). The extracellular domains of both the α and β chains are responsible for their specific ligand-binding properties. The N-terminal region of the α chain has a domain with conserved "heptad" repeats and of these three have putative cation binding sites. In addition, some integrin α chains contain a particular domain known as I-domain (A-domain), consisting of 180-200 amino acids, which is inserted between two N-terminal repeats.

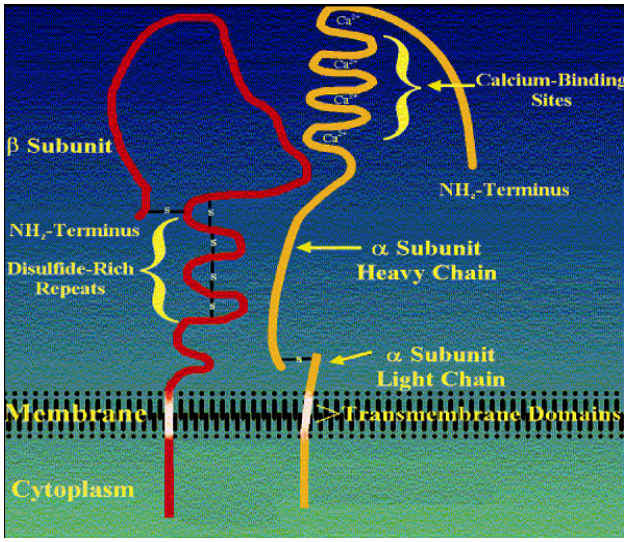


**Figure 4**.
Schematic outline of integrin structure.
The two subunits α-(yellow) and β-(red), the three domains and binding sites are shown.
Source: Anne Cress, Arizona Cancer Center (http://student.biology.arizona.edu/)

The I-domain has a metal ion dependent adhesive site (MIDAS) and plays a major role in recognition and binding of the particular ligands, whereas the cytoplasmic domain connects the extracellular domain of the integrin with cytoskeletal and other proteins involved in signalling processes. The pattern of integrin expressin on the cell surface seems to be highly regulated, and several factors appear to influence the ligand binding ability of integrins (Mercurio, 1995; Mercurio *et al*., 2001).

Integrins can signal in two directions: from outside into the cell (outside-in signalling) and from inside the cell (inside-out signalling). In the latter case, several stimuli, after binding to their cell-surface receptors, induce signals which activate integrins by inducing their clustering and/or conformational changes from a low to a high ligand affinity state. An example for this type of signalling is integrin αIIbβ3 (GpIIb/IIIa) which is present in resting platelets in a low affinity state. However, following vascular injury, the platelets get activated, for instance by ADP, and this leads to activation and clustering of αIIbβ3 which then binds soluble fibrinogen efficiently to mediate platelet aggregation (Mecham, 1991). In contrast, the "outside-in" signalling is initiated after binding of the integrin to specific ligands, such as the ECM molecules, and this leads to induction of signalling through the cytoplasmic domain and generation of cellular responses. Presence of cations such as $Ca^{2+}$ and $Mg^{2+}$ is critical during the process of integrin-ligand interaction.

Integrins are clasified in subfamilies defined by a common β subunit. The β1 subfamily (CD29 or VLA) is the largest one, as the β1 chain can combine with at

least 12 different α chains. The β2 (CD18)-subfamily, commonly known as the leukocyte integrins, is composed by αLβ2 (CD11a/CD18, LFA-1), αMβ2 (CD11b/CD18, MAC-1), αXβ2 (CD11c/CD18, p150, 95), and αDβ2 (CD11d/CD18). Many cells express different kinds of integrins, each capable of interacting with its specific ligand. Integrins are able to recognize cell-surface ligands, such as ICAM-1 and VCAM-1, as well as ECM proteins, such as fibronectin (FN), collagens (Col), vitronectin (VN) and laminins (LM). Several integrins recognize the RGD (Arginine-Glycine-Aspartic acid) motif in ECM proteins (Ruoslahti, 1996).

LMs have been reported to interact so far with α1β1, α2β1, α3β1, α6β1, α6β4, α7β1, α9β1, αVβ3, αVβ5 and αVβ8 integrins ( Kramer *et al*., 1991; Mercurio,1995; Sasaki and Timpl, 2001; Patarroyo *et al*., 2002). Among these, α3β1, α6β1, α6β4 and α7β1 are considered to be the "classical" laminin binding integrins, as some of the other members are capable of recognizing additional ECM molecules. Integrins bind to specific domains of laminins. As stated earlier, the laminin binding sites for integrins and for other non-integrin molecules are mainly located on the α chains. Nevertheless, evidence is now emerging for the ability of integrins to bind laminin subunits other than the α chains (Decline *et al*., 2000).

The laminin globular (LG) domain at the C-terminal region and the LN domain at the N-terminal region of the short arm of LM α chains are functionally active sites which are involved in specific interactions with cell surface receptors. As shown in Table II, each of the different laminin isoforms is recognized by one or more integrin receptors. Among all the different laminin isoforms tested for laminin-binding integrins, α5-containing laminin, particularly LM-511 (laminin-10), seems to be the most preferred ligand and, on the other hand, integrin α6β1 has been shown to be the most promiscuous laminin binding integrin (Table II.). Likewise, laminin-5 has been reported to interact with α2β1, α3β1, α6β1, α6β4, especially with the latter three integrins. Recently, integrin α2β1 was found to bind the short arm of the γ2 subunits of LM-332 (laminin-5), and its role in promoting keratinocyte migration and spreading was demonstrated in an *in-vitro* study (Decline *et al*., 2000).

Interestingly, presence of two RGD sequences on the short arm region of LMα5 in the globular domain L4b (previously called domain IVa) and the binding capacity of these sites for integrin αVβ3 have been demonstrated (Sasaki and Timpl, 2001). Even though the RGD sequences are present in other ECM proteins like fibronectin and vitronectin, they are rarely found in laminins (Flier *et al*., 2001; Sasaki and Timpl, 2001). Several studies have elucidated that laminin ligand-integrin receptor interactions are dependent on the nature of the ligand (either precursor or processed form), the physiological state of the cells, and alternative splicing in some of the laminin-binding integrins (α3, α6, α7, β1 and β4). The unprocessed form of LMα3A (190/200KDa), a component of LM-332 (laminin-5), which is usually found in a cell-associated form, has been shown to interact with both α3β1 and α6β4 integrins and to play a major role in migration of epithelial cells, required for wound healing. On the contrary, the processed form of LMα3A (165 KDa), which is found in a cell-free form such as in epithelial BMs, is shown to interact with α6β4 integrin and to be involved in the formation of specific junctions between the epidermis and the underlying dermis known as hemidesmosomes (Jones *et al*., 1991; Goldfinger *et al*., 1998a; Goldfinger *et al*., 1998b).

Studies of alternative spliced integrins have verified that distinct biological roles can be generated by the different variants. As shown in Table II, integrin α7, which is expressed in three cytoplasmic (A, B and C) and two extracellular (X1 and X2) splice

variant forms, interact with the different laminin isoforms distinctly (Ziober *et al*., 1993; Mark *et al*., 2002).

**Table II:** Laminin-binding integrins

| Laminin | Integrins | References |
|---|---|---|
| LM-111 (laminin-1) | α1β1, α2β1, α3β1, α6β1, α6β4, α7X2β1 α7X1β1, α9β1, αvβ3 | Wayner and Carter, 1987; Sonnenberg *et al*., 1988 Tomaaselli *et al*., 1988; Kramer *et al*., 1989 Languino *et al*., 1989; Lotz *et al*., 1990 Sonnenberg *et al*., 1990; Forsberg *et al*., 1994; Mark *et al*., 2002 |
| LM-211 (laminin-2) | α1β1, α2β1, α3β1, α6β1, α7X1β1 α7X2β1 | Delwel *et al*., 1993; Delwel *et al*., 1994 Colognato *et al*., 1997; Mark *et al*., 2002 |
| LM-332 (laminin-5) | α2β1, α3β1, α6β1, α6β4 | Carter *et al*., 1991; Delwel *et al*., 1993; Delwel *et al*., 1994; Kikkawa *et al*., 1994; Decline *et al*., 2000 and Paper II and IV |
| LM-411 (laminin-8) | α3β1, α6β1, α6β4, α7X1β1 | Geberhiwot *et al*., 1999; Kortesmaa *et al*., 2000; Fujiwara *et al*., 2001; Mark *et al*., 2002; Wondimu *et al*., 2004 and Paper III and IV |
| LM-511 (laminin-10) | α3β1, α6β1, α6β4, α7X1β1, αvβ3 | Kikkawa *et al*., 1998; Kikkawa *et al*., 2000; Sasaki *et al*., 2001; Mark *et al*., 2002 and Paper II, III and IV |

The integrin binding sites of each laminin isoform are mainly restricted to the LG-domains (mostly LG1-3 modules), and there is an overlapping interaction between some integrins with different laminin isoforms. Moreover, there is emerging evidence that integrins can bind to other molecules such as tetraspanins and that this might have some impact on their biological roles (Nishiuchi et al., 2006). Despite all this progress, there are many questions remaining to be answered. For instance, is compensation for a particular biological activity possible between different laminin isoforms assuming that they are recognized by the same integrins? Is it the same or different signaling pathways initiated during those overlapping recognition patterns? How about the signaling process when a given cell is capable of expressing more than one laminin binding receptors (both integrin and non-integrin) at the same time?

## 1.3 TOOTH PULP TISSUE AND ITS FIBROBLASTS

Dental pulp is a loose connective tissue uniquely situated within the rigid encasement of dentin. There is a characteristic cellular arrangement within the pulp cavity. The most peripheral portion is occupied by a single layer of odontoblasts, which are the sources of dentin, and next to this layer, inwards, is a relatively cell-free zone but still rich in networks of unmyelinated nerve fibers and blood capillaries. More towards to the center of the pulp is the area known as the cell-rich zone, since it has a relatively higher number of cells. A mixture of cells found in this particular zone includes fibroblast-like cells (the most abundant cell type), cells of the immune system (lymphocytes, macrophages, dendritic cells), and undifferentiated mesenchymal cells (Hargreaves and Goodis, 2002). In addition, the tooth pulp is also a highly innervated and vascularized tissue. Like several regions of the face, teeth are supplied with rich sensory innervations from the peripheral branches of the trigeminal

system (TG) (Dodd and Kelly, 1991). Depending upon the type of stimulus they encounter, the nerve fibers convey sensory information from peripheral receptors to the brain. Pain and temperature sensations are conveyed by thin myelinated nerve fibers (Aδ fibers) and unmyelinated axons (C fibers). The pulp has very few innervations of myelinated axons (Aβ) and is rather rich in C nerve fibers (70-90%), followed by some myelinated (Aδ) axons. Aδ axons are suggested to be responsible for acute and sharp pain, whereas the C fibers mediate a dull ache (Fried and Hildebrand, 1981; Narhi, 1990; Hildebrand *et al*., 1995).

Pulp fibroblasts are the predominant cell type with a broad distribution throughout the pulp tissue, and particularly in high densities in the cell-rich zone. They are responsible for the synthesis and release of both collagenous (type I and II) and non-collagenous (proteoglycans and FN) ECM proteins. Apart from this, fibroblasts actively participate in providing some structural framework for other cells, and play a major role in wound healing. Their vital immunoregulatory role is being strengthened by emerging evidence (Fries *et al*., 1994; Smith RS *et al*., 1997; 2001; Laura Koumas *et al*., 2001; Nosrat et al., 2004). Under normal and pathological conditions, fibroblasts are known to release a number of mediators, including cytokines, growth factors and proteases, which might influence their various biological activities. Interestingly, recent studies have shown that fibroblasts from different anatomical regions or even within a single tissue are heterogeneous, despite sharing some structural characteristics and the ability to synthesis matrix proteins.

Various attempts have been made to understand the physiological role of pulp fibroblasts and their released molecular factors, from different perspectives. The capacity of pulpal cells to produce some neurotrophic agents and their neurite outgrowth promoting effect were demonstrated in a co-culture study by using trigeminal ganglion explants, though antibodies against some of the known neurotrophic factors did not show any inhibitory effect (Lillesaar *et al*., 1999). Likewise, the release of angiogenic factors was demonstrated in another study where human pulp fibroblasts were co-cultured with human umbilical vein endothelial cells, and formation of tubular structures corresponding to capillaries *in vivo* was revealed. The latter effect was blocked by neutralizing antibodies against FGF-2 and VEGF, and the finding was suggested to be relevant to pulp healing (Tran-Hung *et al*., 2006).

## 1.4 HUMAN MAST CELLS

Mast cells (MCs) were first described by Paul Ehrlich in 1878, who named them "Mastzellen" (well-fed cells) because of their numerous large cytoplasmic granules with unique staining characteristics (Crivellato *et al*., 2003). Despite various speculations about the origin of mast cells in the past, their haematopoietic origin was confirmed quite recently (Kirshenbaum *et al*., 1991; Fodinger *et al*., 1994). Like blood cells, human mast cells originate from $CD34^+$ hematopoietic pluripotent stem cells but, unlike them, they do not mature in the bone marrow. Hence, the committed progenitors, which circulate in peripheral blood, enter into tissues of different anatomical sites, and homing of these progenitor cells is followed by final differentiation and maturation within the tissues. The circulating progenitors have been reported to be $CD34^+$, $Kit^+$, $CD13^+$, $Fc\varepsilon RI^-$, and $CD14^-$ cells (Agis *et al*., 1993; Rottem *et al*., 1994; Kirshenbaum *et al*., 1999). Compared to leukocytes, mast cells are long-lived and re-enter the cell cycle to undergo proliferation locally. Differentiation and survival mechanisms of MCs are known to be highly dependent on the presence of growth factors, among which stem cell factor (SCF/ c-Kit ligand) is the single most crucial one. SCF plays a major role in MC growth, migration,

differentiation, survival, adhesion and degranulation (Kitamura *et al*., 1991; Galli *et al*., 1994; Nillson *et al*., 1994; Galli, 2000).

Under normal conditions, MCs are distributed in close proximity to blood and lymphatic vessels, and beneath the epithelial surfaces, nerves and smooth musle cells. These strategic positioning of mast cells makes them "goal-keepers" to encounter various pathogens and environmental antigens invading skin and mucosal surfaces (Kitamura, 1989; Metcalfe *et al*., 1997; Galli *et al*., 2005). Although single progenitors of MCs originally migrate to different locations, there is heterogeneity of matured mast cells with distinct phenotypes, depending on the local environmental factors they are exposed to. These discrete groups of MCs can be distinguished on the basis of their tissue location, dependence on T lymphocytes, and their granular contents. For instance, rodent MCs are divided into two groups based on their tissue distribution, namely, connective tissue MCs (CTMC) and mucosal MCs (MMC). Likewise, human MCs are classified into two major subsets according to their distinct protease composition, such as human mast cells containing only tryptase ($MC_T$) and those with both tryptase and chymase ($MC_{TC}$) (Irani *et al*., 1986; Hogan and Schwartz, 1997; Metcalfe *et al*., 1997). $MC_T$ are usually localized in mucosal surfaces (e.g. alveolar wall, bronchi, intestinal mucosa) and they are T-cell dependent, whereas $MC_{TC}$ are found in skin, gastrointestinal tract and subcutaneous tissue (Krishnaswamy *et al*., 2006).

MCs express different types of immunoreceptors which allow them to respond to various stimuli. These cells are well known for their activation during allergic reactions, in which multivalent antigens (e.g. allergen) breach the barrier and interact with specific IgE-antibodies bound to the high affinity receptor, FcεRI, on the surface of the MCs. In addition, MCs can be activated by biological substances such as products of complement activation (C3a and C5a), lipopolysaccarides (LPS), and some neuropeptides. MCs are also known to release a wide variety of mediators following cellular activation. They can be categorized as preformed mediators (histamine, serotonin, proteoglycans such as heparin and chondroitin sulphate, proteases, some cytokines like tumor necrosis factor/TNF-α, and basic fibroblast growth factors/bFGF) and as newly synthesized mediators (lipid-derived mediators including prostaglandin $D_2$, leukotrienes, thromboxanes, cytokines and chemokines such as TGF-β, IL-3, IL-4, IL-5, IL-8) (Metcalfe, 1997; Marshall, 2004; Galli, 2005). Though traditionally known for their role in allergic reactions, emerging evidence has shown the multifunctional properties of MCs, because of their large varieties of mediators and strategic locations (Fig 5.). Thus, MCs have been suggested to participate in many normal biological processes and in different pathological conditions, including allergy, asthma, defense against parasitic infection, inflammation, wound healing, pulmonary fibrosis, atherosclerosis, tumor development and angiogenesis (Metcalfe, 1997; Kobayashi et al., 2000; Wedmeyer., 2000; Marshall, 2004; Galli, 2005).

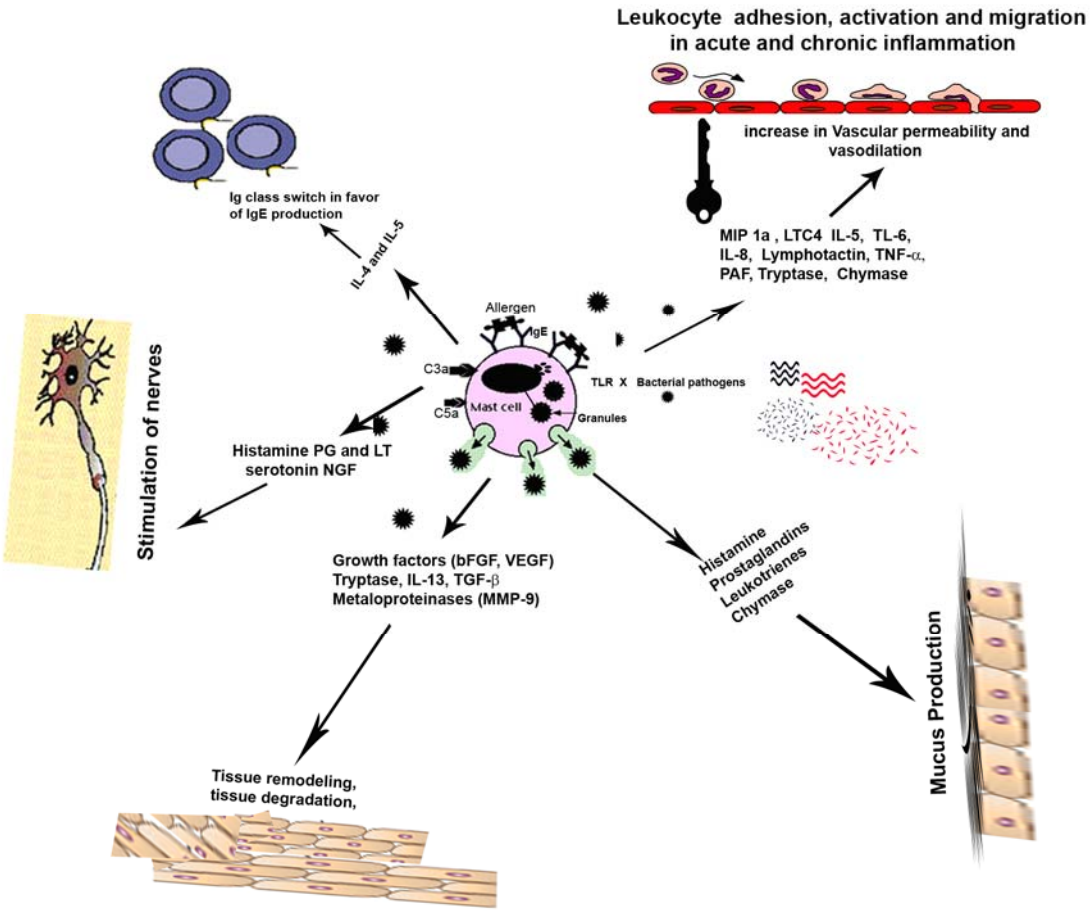


**Figure 5**. Schematic diagram showing the various mediators released by mast cells following stimulation and their multifunctional properties.

## 1.5 MEGAKARYOCYTES AND PLATELETS

### 1.5.1 Megakaryocytes (MKs)

Thrombopoiesis (platelet formation) is a multi step process which begins with the differentiation and maturation of megakaryocytes (MKs) from $CD34^+$ multipotent haematopoietic progenitors through complex differentiation stages driven primarily by the hormone thrombopoietin (TPO) and other cellular or environmental factors in the bone marrow. The developmental hierarchy of megakaryocytic lineage consists of megakaryoblast, promegakaryocytes and mature MKs, before blood platelets are released at the end. Mature MKs are large (20-70 μM) polyploid cells with big lobulated nuclei (Mega=large, karyo=nucleus, cyte=cell). These highly specialized platelet precursors are rare myeloid cells representing only about 0.02%-0.05% of all the nucleated cells in normal human marrow. However, after undergoing polyploidization and cytoplasmic changes associated with formation of demarcation of the membrane system, MKs are able to shed and release from the cytoplasm hundreds of platelets from each cell. The expression pattern of MKs- platelet specific glycoproteins such as CD41a (GPIIb), CD42 (GPIb), von Willebrand factor (vWF) and CD61 (GPIIIa) are commonly used to follow the differentiation of MKs (Gewirtz *et al*., 1995; Nagahisa *et al*., 1996; Debili *et al*., 1993; Levine *et al*., 1982; Tomer, 2004).

Normally, developing megakaryocytes are distributed characteristically in the bone marrow environment. It has been suggested that the more mature MKs (c-mpl$^+$ CD41a$^+$ CD42b$^+$ and CXCR4$^+$) are recruited to vascular niches by SDF-1a and FGF-4 and become localized to the abluminal side of sinusoidal bone marrow endothelial cells (BMECs) (Tavassoli *et al*., 1989; Zuker-Franklin., 2000; Larson and Watson 2006a). This initial close interaction between MKs with BMECs was illustrated to be essential for the subsequent translocation of the intact MKs into the sinusoidal lumen, and for the MK cytoplasm penetration of the endothelial lining at the time of platelet release (Fig 6) (Lichtman *et al*., 1979; Tavassoli *et al* 1981; Avecilla *et al*., 2004; Goro Kosaki, 2005).

Two theories of platelet formation have been proposed and are equally well evidenced, though still in debate, namely, the "proplatelet" theory and the "explosive-fragmentation" theory (Becker *et al*., 1976; Kosaki, 2005). The "proplatelet" theory put forward the concept that MKs are able to form cytoplasmic extension processes (proplatelets) from which platelets are released after protruding into sinusoids located in the bone marrow haematopoietic compartment. In contrast, "explosive fragmentation" of the MK cytoplasm, composed of platelet territories, has been documented from liquid-cultured MKs kept in suspension, which showed platelet formation without proplatelets, and also from other findings revealing fragmentation of MK cytoplasm in bone marrow and lung capillaries (Becker *et al*., 1976; Topp *et al*., 1990; Kosaki G, 2005).

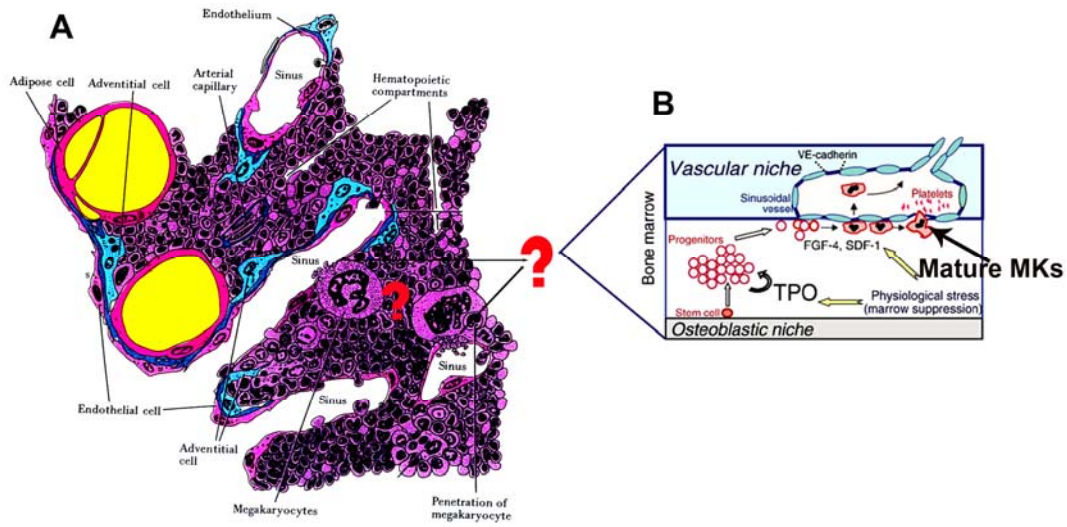


**Figure 6**. (A) Schematic diagram of a cross section through the marrow showing active hemopoiesis. To be noted are the megakaryocytes discharging platelets into the sinuses. Adapted from Histology: A text and atlas, 3rd ed. Baltimore, Wiliams & Wilkins, 1995, p208. (B) Generation of megakaryocytes and platelets: Differentation and proliferation of MKs progenitors followed by their migration to the vascular niche. The close apposition of mature MKs to the abluminal side of the sinus before platelets are released or mature intact MKs transmigrated. Adapted from Avecilla et al., 2004.

In the past, working with MKs was challenging due to the scarcity of these cells. More recently, the possibility of *in vitro* culturing and expanding MKs from various sources has facilitated these studies. Though TPO and its receptor *c-mpl* are major factors to regulate MKs proliferation and platelet number, mice deficient of both

($TPO^{-/-}$ and c-$mpl^{-/-}$) are still able to produce functional platelets, but at much lower levels (80-90% reduction) (Gurney *et al*., 1996; Bunting *et al*., 1997). This has prompted the interest to do further research, trying to identify other molecular factors which might be involved and could compensate for MK maturation and thrombopoiesis. The ability of chemokines and growth factors such as SDF-1 and FGF-4 to promote TPO-independent platelet production and to restore thrombopoiesis in $TPO^{-/-}$ and c-$mpl^{-/-}$ mice has been reported (Avecilla *et al*., 2004). Larson and Watson (2006b) have also recently shown the contribution of fibrinogen-binding receptor αIIbβ3 in proplatelet formation and platelet release using MKs expanded from mouse bone marrow.

### 1.5.2 Platelets

Platelets are the smallest of human blood cells (1.5-2.5μm diameter), though they originate from MKs, which are the largest of all. These anucleate, subcellular fragments display a characteristic discoid shape under normal resting conditions. There are about 150 to 400 x $10^9$ platelets/L with a circulating half life of about 10 days (George, 2000). Structurally, the outer most part of the platelet is called glycocalyx, which is composed of glycoproteins and it is the site where various receptors are localized. Moreover, the surface connected membrane invagination, an open canalicular system (OCS), is a channel through which granular contents of platelets are released, and also serves as storage for membrane-bound receptors and proteins (Rendu and Brohard-Bohn, 2001). The dense tubular system (DTS) is an endomembrane system which stores calcium and metabolic enzymes, including adenylate cyclase (cAMPase), phospholipid-modifying enzymes and other mediators involved in the control of platelet activation.

Three types of secretory granules are found in platelets, namely, lysosomes, dense granules and α-granules. Lysosomes (175-250 nm in diameter) contain different enzymes which are active under acidic conditions, such as glycosidase and proteases, as well as cationic proteins with antibacterial activity. Dense granules are the smallest platelet granules (mean diameter of 150 nm) and contain ADP and pro-aggregating factors, including nucleotides, amines (serotonin, histamine) and bivalent cations ($Ca^{2+}$, $Mg^{2+}$). The α-granules are the largest (200-400 nm) and most abundant of all. They contain high molecular weight substances categorized as proteoglycans (e.g. platelet specific beta-thromboglobulin/βTG and platelet factor IV/PFIV), adhesive glycoproteins (fibronectin, vitronectin, vWF, thrombospondin), haemostasis factors (fibrinogen, factors V,VII, XI and XIII), cellular mitogens (PDGF, VEGF, TGFβ), protease inhibitors ($\alpha_2$-antitrypsin, $\alpha_2$-antiplasmin) and miscellaneous components (IgG, IgA, GPIa). Not all of these granular contents are synthesized by MKs. α-granule constituents such as immunoglobulin and albumin ar, for instance, the results of passive fluid phase endocytosis, while fibrinogen is taken up through receptor-mediated (GPIIb-IIIa) endocytosis (Harrison and Cramer, 1993; Niewiarowski *et al*., 1994; Rendu and Brohard-Bohn, 2001).

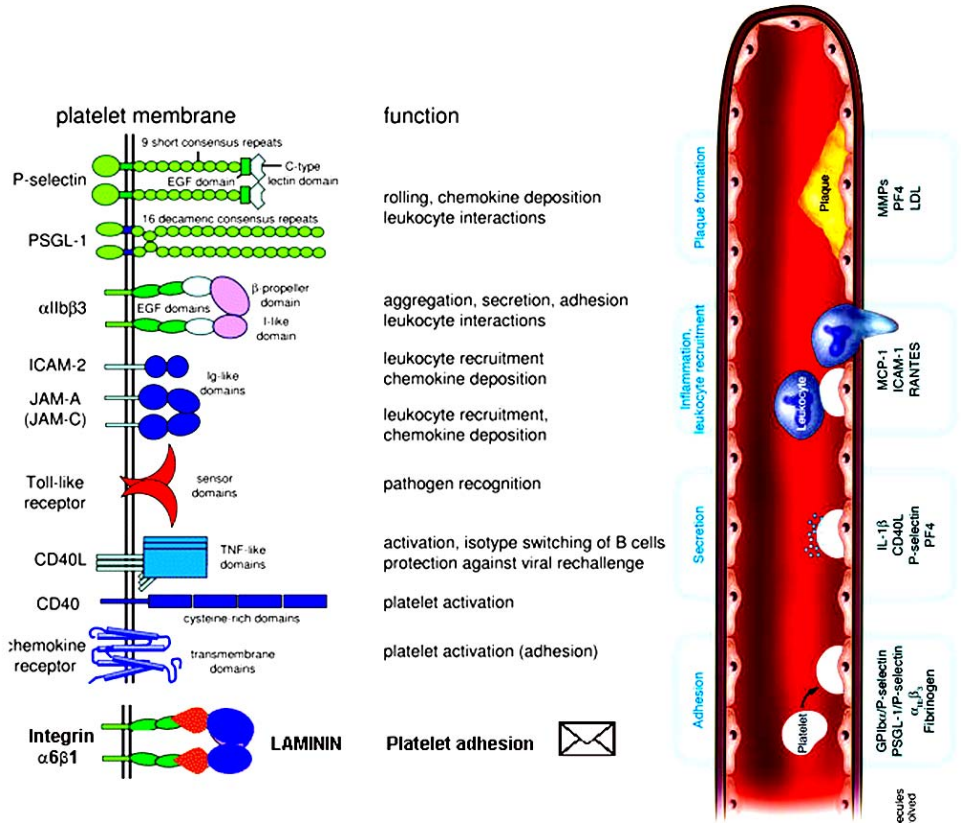


**Figure 7**. Pictures showing the various platelet surface-bound and secreted molecules and their roles during inflammatory reactions, wound healing and thrombosis. The envelope indicates the information generated from our studies and previous reports. (adapted and modified from Gawaz et al., 2005; Hundelshausen and Weber, 2007)

Platelets are capable of secreting a large number of modulators and active compounds stored in their granules. Moreover, presence of various cell surface receptors on platelets has been reported to be essential in various biological processes. Recently, the functional relevance of platelets in inflammation, immune response, angiogenesis, atherosclerosis, and cancer metastasis is becoming more evident, in addition to their well established role in maintaining the vascular integrity (Fig 6) (Hundelshausen and Weber, 2007). There are now a number of studies demonstrating the close interactions between platelets and leukocytes. Traditionally, in response to inflammatory stimuli, there is recruitment of leukocytes to the inflamed tissue following the multistep process of extravasation. Interestingly, part of this same course of actions such as leukocyte tethering, rolling, leukocytes activation and firm adhesion has been described on activated platelets. P-selectin, which is normally localized in the seretory α granules of platelets and Weibel-Palade bodies of endothelial cells, is rapidly redistributed to the cell surface upon platelet activation.

Besides the various platelet secreted chemokines and lipid mediators, P-selectin was suggested to be responsible for the heterotypic interaction through its ligand, P-selectin glycoprotein ligand-1 (PSGL-1), expressed on leukocytes (McEver R, 2001; Weber C and Springer T, 1997; Hundelshausen and Weber, 2007). In general, as shown in Fig 6., these small cellular fragments, once thought to be responsible for haemostasis only, have been found to be equipped with multiple adhesion molecules and receptors, which allow them to participate in several biological processes.

## Platelets–vessel wall interactions

The endothelium has an active role in haemostasis, vascular response to injury, and inflammatory reactions. Under normal conditions, platelets are known to circulate freely without adhering to the vascular wall since various anti-thrombotic or anti-adhesive molecules, such as prostacyclin and NO are secreted by endothelial cells (Maschio *et al*., 1997; Cines *et al*., 1998). However, this well regulated normal process can be affected by different factors, such as vascular injury, shear rate/stress, and some pharmacological agents. Hence, during vascular injury, platelets become responsive and get involved in this complex multi-step process.

Platelet adhesion to the injured vessel wall is the first step that promotes primary haemostasis. Additional steps include degranulation and platelet aggregation, before the formation of a haemostatic plug. During the process, platelets change shape, from discoid to extensive pseudopodia formation, which allows them to adhere, release a number of secretory granular contents, and cover the injured site (Shattil *et al*., 1994). Secondary haemostasis is also essential, as the initial platelet plug is further reinforced by fibrin deposition *via* thrombin. Immobilization of platelets at the site of vascular injury requires the presence of various membrane-bound adhesion molecules and soluble molecular and cellular factors involved in either platelet-vessel wall adhesion or platelet-platelet adhesion (aggregation).

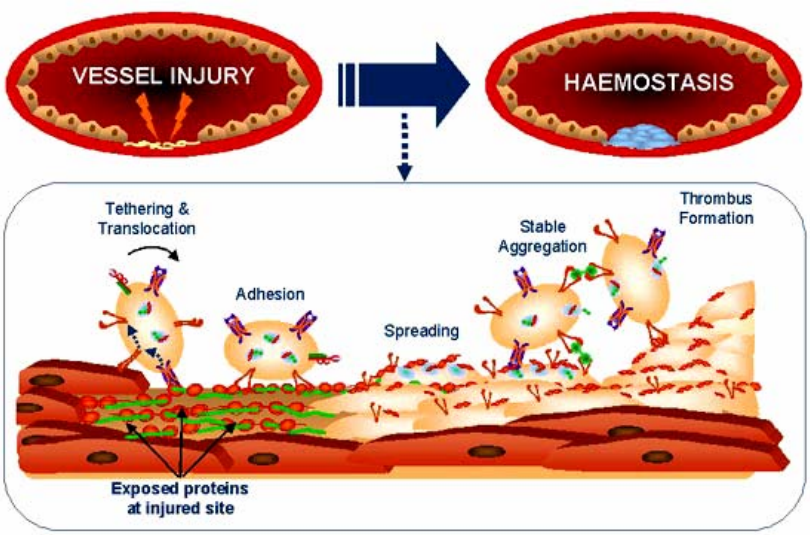


**Figure 8.** The multistep process of haemostasis after vascular injury: Close interaction between platelets and the vessel wall.

Depending on the degree of vascular injury, various matrix proteins, known to be recognized by distinct platelet membrane-bound receptors, can be exposed and become accessible for interactions. The first interaction between the multimeric vWF released by endothelium and platelet GPIb/IX/V complex was reported to be weak and transient under low shear conditions. In contrast, the interaction of collagen from the exposed sub-endothelium with platelet α2β1 integrin and/or GPVI is known to be strong under static or low shear conditions (Clemetson *et al*., 1995; Kehrel, 1995). This early adhesion step promotes spreading of platelets, activation of αIIbβIII integrin to a high affinity state enabling binding of soluble fibrinogen, platelet aggregation, and granule secretion. Subsequently, release of ADP from platelet dense granules further activates the platelets in an autocrine manner promoting thrombus formation (Beumer *et al*., 1994; Kehrel *et al*., 1998; Jurk and Kehrel, 2005). During

vascular damage, subendothelial laminins can be also exposed to platelets, which express the laminin-binding integrin $\alpha 6\beta 1$, but only a few studies have attempted to explore the relevance of this process. Early studies by Tryggvason *et al*. (1981) and Hindriks *et al*. (1994) targeted LM-111 (laminin-1), which is now known not to be expressed by most blood vessels. In contrast, the recently identified LM-411 (laminin-8) and LM-511 (laminin-10) constitute the major vascular endothelial laminin isoforms and are widely expressed by the vasculature. A platelet adhesion promoting activity has been demonstrated for LM-111, even though this isoform had no effect on platelet aggregation (Timpl and Brown, 1994; Frieser *et al*., 1997; Sorokin *et al*., 1997).

# 2 AIMS

The aims of the work presented in this thesis are summarized as follows:

- To identify laminin isoforms synthesized, expressed and secreted by human dental pulp fibroblasts, and to determine their neurite outgrowth promoting activity.

- To investigate the effect of different laminin isoforms and their integrin receptors in the adhesion and migration of human mast cells.

- To explore whether human mast cells are capable of synthesizing and expressing laminins.

- To examine expression of α3-laminin(s) in various blood vessels.

- To study synthesis of α3- and α5-laminins by human megakaryocytes and their secretion by blood platelets.

- To explore the functional role of different laminin isoforms in platelet adhesion and activation (P-selectin expression and cell aggregation), and in megakaryocyte migration and platelet formation.

# 3 MATERIALS AND METHODS

## 3.1 CELLS

### 3.1.1 Establishment of primary tooth pulp fibroblasts (Paper I)

A primary pulp fibroblast cell culture was established after the tooth pulp was extracted from third molars with incomplete root formation from healthy patients aged 18-20 years. All teeth were free from caries and confirmed healthy based on clinical examinations. Once the tooth was carefully dissected out, the pulp was minced and treated for 120 minutes with 0.25% trypsin-EDTA, and collagenase A (Sigma-Aldrich, Stockholm, Sweden) at 37°C. The digested tissue was subsequently passed through a Cell strainer (Falcon, BD Biosciences, Stockholm), and the cell suspension was then plated in 25 $cm^2$ culture flasks in L-modified Eagle's medium (Gibco, Invitrogen, Carlsbad, CA) with 10% fetal bovine serum, penicillin (50 U/ml) and streptomycin (50 μg/ml), and incubated at 37°C in humid air supplemented with 5% $CO_2$. The medium was changed every 2-3 days. When the primary outgrowth of pulpal fibroblast-like cells had reached confluence, cells were rinsed in 0.02 % EDTA and detached using a 0.1 % trypsin/EDTA solution. After centrifugation, cells were sub-cultured in 75 $cm^2$ culture flasks.

Fibroblast-like cells of third to eighth passages were used for experiments. For pulp tissue immunohistochemistry assay, immediately after extraction, the teeth were transferred to tissue culture medium and then cracked open in a vice. Subsequently, pulps were dissected out while soaked in medium and then rapidly frozen. Specimens were stored at–80°C until sectioning and immunohistochemistry assays were done.

*Methodological comments*

Although, the morphology of primary cells established resemble that of fibroblast's and no morphological heterogeneity was observed in our culture set up, still we considered the possibility of having cells other than fibroblasts coming from the pulp tissue. To address this issue, the expression of human Thy-1 antigen (CD90), which is reported to be expressed on fibroblasts of different anatomical sites, (Saalbach *et al*., 1998) was determined by flow cytometry analysis. Over 98 % of the cells were stained positive.

### 3.1.2 Isolation of trigeminal ganglions (Paper I)

Trigeminal ganglion (TG) neurons were prepared from neonatal Sprague-Dawley rat pups as described previously (Lillesaar *et al*., 2003). Briefly, the TGs were collected in Leibovitz 15-medium, rinsed in 0.02% EDTA and treated with 0.125% collagenase II (30 min, 37°C; Sigma) and with 0.25% trypsin (30 min, 37°C; Invitrogen AB, Lidingö, Sweden). The tissue was triturated and dissociated cells were spun (100 x g) and preplated on polystyrene dishes for 4 x 1 h to remove glial cells and fibroblasts. For co-cultures with TG neurons, pulpal cells were trypsinized, replated in Falcon Chamber slides (Becton Dickinson Labware, Franklin Lakes, NJ, USA) and allowed to form confluent monolayers.

### 3.1.3 Purification of CD34+ cells from cord blood (Paper II and IV)

Umbilical cord blood was obtained from normal full term birth after obtaining an informed consent from mothers. CD34+ cells were enriched following the procedure given in the MACS Direct CD34 progenitor cell isolation kit (Miltenyi Biotech GmbH, Bergisch Gladbach, Germany, MACS). Briefly, heparin-treated cord blood

was diluted with PBS and layered over Ficoll-Hypaque (density 1.077; Amersham Bioscience, Uppsala, Sweden) at room temperature. After gradient centrifugation and washing twice with MACS buffer (PBS containing 0.5% BSA and 2 mM EDTA), the mononuclear cells pellet was suspended in 0.5 ml of MACS buffer. Fc-receptors were blocked first by using human IgG, and then cells were magnetically labelled with MicroBeads conjugated to CD34 antibodies (100 µl/$10^8$ mononuclear cells) for 30 min at 4$^o$C. After washing carefully, the cells were re-suspended in MACS buffer and loaded to a MiniMACS column which was attached to MACS separators. Cells were allowed to pass through twice and the unbound cells were washed out with MACS buffer. The retained CD34+ cells were then eluted after the column was separated from the magnet. Finally, purified CD34+ cells were re-suspended in plain RPMI 1640 from which a small fraction was taken for total cell counting, viability checking using trypan blue and determining degree of purity of the selected population by using flow cytometry with an anti-human $CD34^+$ antibody conjugated with fluorescein isothiocyanate (HPCA-2) (Becton Dickinson, BD Bioscience, Stockholm, Sweden). Purity was consistently in between 90 to 95%.

Purified cells were finally used for *in vitro* expansion of both cord blood derived human mast cells (CBMCs) (*Paper II*) and megakaryocytes (MKs) (*Paper IV*) as described below.

### 3.1.4 Cord blood derived mast cells (CBMCs) (Paper II)

CBMCs were generated by seeding CD34+ cells in RPMI 1640 medium supplemented with 10 % FBS, 50 µM 2-mercaptoethanol, 10 mM HEPES, 2m M L-glutamine, 0.1 mM non essential amino acids, 100 U/ml penicillin, and 50 µg/ml streptomycin (Sigma). The cell density was 10 x $10^6$ cells per 10 ml in each culture flass grown in the presence of 100 ng/ml SCF (Biosource, International, Camaeillo,CA) and 50 ng/ml IL-6 (Amgen, Thousand Oaks, CA).

The CBMCs at 4-5 weeks and at 8-10 weeks were used in different assays as stated in *Paper II.*

#### *Methodological comments*

In addition to verifying the viability of *in-vitro* generated cord blood mast cells, purity of the cells were checked by using tryptase staining and by FACS analysis using monoclonal antibody against the c-Kit (CD-117) (Southern biotech).

### 3.1.5 In vitro differentiated megakaryocytes (ivMKs) (Paper IV)

Enriched $CD34^+$ cells (5 x $10^4$/ml) were cultured in serum-free medium Stemspan H3000 (StemCell Technologies Inc) at 37°C in humid air supplemented with 5% $CO_2$ in the presence of TPO at 50 ng/ml and SCF at 10 ng/ml (Biosource International, Inc). After one week, the same fresh medium was replaced and cells were cultured for one more week. After 14 days of culture, the cells were used for various assays as described in *Paper IV.*

#### *Methodological comments*

The morphology, efficiency of expansion and purity of MKs were checked by using Giemsa-Maygrunauld staining and by FACS using PE-conjugated anti CD41a antibody (BD Bioscience), both at day 0 and after 14 days of culturing period.

### 3.1.6 Isolation of platelets (Paper III and IV)

Human platelets were isolated from buffy coat prepared from healthy donors (Karolinska Hospital at Huddinge). Buffy coats were diluted 1:1 or 1:2 with PBS, depending on viscosity, and centrifuged at 170 *g* for 30 min to spin down blood

leukocytes and red cells. The supernatant was collected and platelets were sedimented after centrifugation at 1900 *g* for 10 min. Thereafter, platelets were washed by centrifugation three times with 10 mM EDTA/PBS and once with PBS alone (at 280*g* for 10 min). Finally, isolated cells were counted in a haemocytometer and used in various assays. For stimulation, isolated platelets were resuspended at $10^9$/ml in RPMI 1640 medium with 10 mM HEPES. After preincubation for 15 min, the cells were stimulated with 200 nM tetradecanoyl phorbol acetate (TPA) (Sigma-Aldrich) for 20 min at 37$^o$C in the presence of protease inhibitors (aprotinin, leupeptin and pepstatin) (Amersham Pharmacia Biotech), phenylmethylsulfonyl fluoride (PMSF) (Sigma) and EDTA. Following centrifugation of the cells, supernatants were collected, concentrated twenty times by centrifugation using Macrosep 30 K Omega filters (Pall Life Sciences, Ann Arbor, MI), and analyzed for secreted laminins in the presence of 1% Triton X-100.

### 3.1.7 Cell lines (Paper II, III and IV)

Human mast cell line, namely HMC-$1^{560}$ (HMC 1.1) (Butterfield,1988; Nilsson, 2003) was maintained in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% FBS, 2 mM L-glutamine, 100 IU/ml penicillin, 50 μg/ml streptomycin and 1·2 mM α-thioglycerol, and passaged every 3-4 days (*Paper II*). The human megakaryocytic leukaemia cell lines HEL and DAMI were obtained from the American Type Culture Collection (ATCC) (Martin *et al*., 1982; Greenberg *et al*., 1988) (Rockville, MD) and cultured in RPMI 1640 supplemented with 10% FBS, penicillin (50 U/ml), and streptomycin (50 μg/ml) (Sigma-Aldrich) **(***Paper III and Paper IV***)**.

## 3.2 EXPRESSION AND IDENTIFICATION ANALYSES

### 3.2.1 Immunohistochemistry (Paper I and IV)

As mentioned earlier in section 3.1, longitudinal 14 μm sections were made from samples stored at –80$^o$C and incubated for 24-48 hrs in a humid atmosphere at 4°C with mouse monoclonal antibodies (mAbs) against the different laminin chains. For double-labelling, some sections were incubated with a selected LM antibody and either a mixture of rabbit polyclonal antibodies against neural antigens peripherin (dilution 1:400, Chemicon, Temecula, CA, USA) and PGP9.5 (dilution 1:2000, UltraClone, Isle of Wight, UK) or with FITC-conjugated *Ulex europaeus* agglutinin I lectin (UEA-I) (20 μg/ml; Sigma-Aldrich, Stockholm, Sweden). This lectin, which is specific for α-L-fucose-containing compounds, selectively binds human vascular endothelium.

Similarly, detection of laminin-binding integrins was carried out using various mAbs against the specific integrin subunits. After rinsing with 0.01M PBS, sections were incubated with Cy3-conjugated donkey anti-mouse antiserum (diluted 1:250, Jackson ImmunoResearch, USA) in a humid atmosphere at room temperature for 1 hour. Sections for double-labelling incubated with peripherin and PGP 9.5 antiserum were incubated with a mixture of the Cy3-conjugated donkey anti-mouse antiserum and Cy2-conjugated donkey anti-rabbit antiserum (diluted 1:250, Jackson ImmunoResearch, USA). All reagents were diluted in 0.01M PBS containing 0.3% Triton X-100, 5% bovine serum albumin (BSA), 3% normal donkey serum and 0.1% sodium azide. Control sections were incubated as above but either with mouse IgG or without a primary antibody and gave no immunostaining. Subsequently, the sections were rinsed in 0.01M PBS, mounted in a mixture of glycerol and 0.01M PBS (3:1),

cover slipped and examined in a Nikon E600 fluorescence microscope equipped with proper filter combinations.

To determine LMα3 expression in the vasculature (*Paper IV*), specimens of normal human tissues were obtained from surgical operations, biopsy and autopsy, after approval from ethical committees, as previously described (Petäjäniemi *et al*., 2002). For indirect immunohistochemistry, 6 μm tissue sections fixed in acetone at -20$^{o}$C were used. The specimens were exposed to mAb BM165 to LMα3 (Rousselle *et al*., 1991), followed by fluorescein isothiocyanate (FITC)-coupled goat anti-mouse IgG (Jackson Immunoresearch, West Grove, PA), and tetramethylrhodamine-isothiocyanate-(TRITC)-coupled Ulex Europaeus I-lectin to identify vessel endothelia

### 3.2.2 Immunofluoresence flow cytometry (FACS) (Paper I-IV)

Both cell surface and intracellular expression of LM chains and their binding integrins were investigated by indirect immunofluorescence flow cytometry. For intracellular staining, trypsinized and washed pulp fibroblasts, HMC 1.1, HMC 1.2, CBMCs, DAMI, HEL and human platelets were permeabilized with IntraStain Kit (DAKO) as recommended by the manufacturer. Fc-receptors (FcR) were blocked by incubating 5-10 x $10^6$ cells with 1 mg/ml of heat aggregated hIgG (Sigma). The cells were washed once, re-suspended in cold PBS (at 0.5-1x$10^6$ cells/well) and incubated with different mAbs against laminin and integrin subunits at 20 μg/ml, as stated in each paper. Bound Abs were detected with fluorescein isothiocyanate (FITC)-conjugated F(ab´)$_2$ fragments of rabbit anti-mouse Ig (1:20) (DAKO). All the incubations, with primary and secondary antibodies, were done for 30 min at 4ºC. After 2-3x washing steps, cells were fixed with 1% paraformaldehyde, resuspended in PBS and analyzed in a FACScan flow cytometer (BD Biosciences, San Jose, CA). Each of the specific cell types, mentioned above, was identified based on expression of particular lineage specific markers, in addition to the forward and side scatter properties. Except for human platelets where 50 x $10^3$ events were counted, 10x$10^3$ events were collected for flow cytometry of cells using Cell Quest software. The results were presented as histograms of mean fluorescence versus cell count.

### 3.2.3 RNA extraction and RT-PCR (Paper I-IV)

Total ribonucleic acid (RNA) was extracted from cultured pulp fibroblasts (using, TRIzol Extraction Kit, Invitrogen, Carlsbad, CA), HMC 1.1, DAMI, and HEL (Total RNA Extraction kit, Amersham Bioscience, Sweden) and CBMCs (RiboPure$^{TM}$ RNA extraction kit, Ambion, AB Applied Biosystems) as recommended by the manufacturer. After oligo(dT)$_{18}$ priming, mRNA was reverse-transcribed into cDNA after incubating for 60 minutes at 42°C with M-MLV reverse transcriptase in 20 μl reaction buffer containing 20 units of recombinant RNase inhibitor, and 10 μM of each dNTP. The reactions were terminated by heating samples at 94°C for 5 minutes and the mixture was diluted to 100 μl with DEPC-treated water. Single-stranded cDNAs in 20 μl of reaction buffer containing 1.5 mM $MgCl_2$, 0.5μM of each primer and dNTP mix (200μM each) were amplified by 30 cycles of PCR using 1.25 units of Ampli Taq DNA polymerase (Perkin Elmer/Roche Molecular Systems, Inc., Branchburg, NJ, USA). The conditions for PCR were 94°C, 20 sec; 60°C, 30 sec; 72°C, 2 min. PCR products were analysed by electrophoresis in 2% agarose gels stained with ethidium bromide.

#### *Methodological comments*

To avoid and control possible genomic contamination, total RNA was treated with DNase as recommended by the manufacturer (Ambion, AB Applied Biosystems) and

PCR reactions without prior reverse transcriptase product were performed in parallel as negative controls.

### 3.2.4 Metabolic labeling (Paper III)

Erythromegakaryocytic HEL cells maintained in culture were washed with PBS, resuspended in methionine-and cysteine-free RPMI 1640 medium containing 10% dialyzed FBS and incubated for 30 min at 37ºC. Afterwards, cells were labelled with 0.20 mCi/ml Trans $^{35}$S-label (ICN Radiochemical Inc.) for 4 h at 37ºC. After three washes with cold PBS, cells were lysed in 1% Triton X-100 containing protease inhibitors. The soluble fraction (cell lysate) was used for immunoprecipitation as described in section 3.13.

### 3.2.5 Immunoaffinity chromatography (IA) (Paper IV)

A monoclonal antibody against laminin α3 (BM165) was coupled to CNBr-activated Sepharose 4B (approx. 2 mg of IgG to 2.0 ml of sepharose) (Amersham Pharmacia Biotech) according to the manufacturer's instructions. Following pre-adsortion with Sepharose CL-4B (1:1) under continuous mixing at 4ºC overnight, platelet lysate was passed through the column twice with a flow rate of 35 µl-75 µl/min. The unbound material was removed by successive washing using 0.1 % Triton X-100 in PBS, 0.1 % Triton X-100 /0.5% Na-deoxycholate/0.5 M NaCl/0.01 M Tris-HCl (pH 8), 0.1 % Triton X-100/0.05M Tris (pH 10), and finally 0.05 M Tris (pH 10). Expected target proteins bound to the BM165 column were eluted using high pH elution buffer (50 mM diethylamine/150 mM NaCl) and analyzed by SDS-PAGE and western blotting as described under immunoprecipitation.

### 3.2.6 Immunoprecipitation (IP) and SDS-PAGE/WB (Paper I-IV)

Cell lysate preparations, concentrated culture supernatants, and platelet secreted materials were used to identify laminin isoforms employing the IP/WB assay. Sample lysates were prepared as stated in each paper for the particular cell types by using 1% Triton X-100 lysis buffer containing protease inhibitors (1 µg/ml of aprotinin, 2 µM leupeptin, 2 µM pepstatin, 1 mM PMSF, and 2 mM EDTA) (Amersham Pharmacia Biotech and Sigma). The lysate was pre-cleared by incubating with protein G Sepharose for 1 h. Aliquots of pre-cleared cell lysate were incubated with specific mAbs against a particular laminin chains for 1 h where the expected Ag-Ab complexes were formed. In parallel, protein G Sepharose beads were incubated with rabbit anti-mouse Ig and, following removal of the excess secondary Ab by washing with lysis buffer, the precoated beads and the Ag-Ab complexes were incubated for 2 h more. All incubations were done at 4ºC under continuous mixing. Finally, unbound complexes were washed off and precipitated proteins were eluted by incubating at 100ºC for 5 min in SDS sample buffer containing β-mercaptoethanol. The immunoprecipitated material was separated by SDS-PAGE using 4.5 to 6% polyacrylamide gels and electroblotted to nitrocellulose membrane. The blots were incubated with specific primary Abs against various LM chains and detected with HRP-conjugated secondary antibodies (DAKO). ECL (Amersham Pharmacia Biotech) was used as developer.

## 3.3 FUNCTIONAL ASSAYS

### 3.3.1 Neurite outgrowth assay (Paper I)

Neurite outgrowth assays were performed on laminin substrates using Falcon Chamber slides coated with different laminin substrates (LM-111/laminin-1, LM-

211/laminin-2 or LM-411/laminin-8) at 20μg/ml for 2 h at room temperature followed by brief rinsing in PBS. Slides were then blocked with human serum albumin (HSA) (5 mg/ml) for 30 min at room temperature and washed twice with PBS. Wells coated with HSA or FN (20μg/ml) were used as negative controls.

In both co-cultures with pulpal cells and cultures on pre-coated slides, approximately 2 x $10^3$ non-adherent TG cells (putative neurones) were added in each well. Cultures were incubated for 24 h and then fixed in 4% paraformaldehyde with 0.2% picric acid, rinsed in PBS and incubated overnight at 4°C with rabbit polyclonal antibodies against peripherin and PGP9.5 (see above). The cultures were then rinsed in PBS and incubated for 1 h at 20°C with polyclonal Alexa Fluor 499-conjugated donkey-anti-rabbit antibodies (2 μg/ml; Jackson ImmunoResearch, Pennsylvania, USA). Subsequently, the cultures were rinsed in PBS and mounted in glycerol-PBS (3:1) for microscopical examination.

### 3.3.2 Cell adhesion assay (Paper II, III and IV)

To analyze the role of laminin isoforms in promoting cell adhesion of human mast cells (HMC 1.1 and CBMCs) and blood platelets, 96 well flat bottomed polystyrene plates (BD Biosciences, Heidelberg, Germany) were coated with 50 μl/well of PBS (Dulbecos with $Ca^{2+}$ and $Mg^{2+}$) or of HSA (Sigma), LM-111, LM-211, LM-332, LM-411 and LM-511 at 20 μg/ml at 37ºC for 3 hr (or 4°C overnight). After rinsing the wells with PBS, non-coated space was blocked with 2% polyvinylpyrrolidone (PVP, molecular weight 360 kDa, Sigma) or 0.5 % HSA for 1 h at RT. Plates were then washed twice with PBS and once more with RPMI 1640, and 100 μl of cultured HMC 1.1 or CBMCs (1x$10^6$ cells/ml), or isolated platelets (3 x $10^8$cells/ml) suspended in RPMI-1640 were plated in the coated wells. Cells were allowed to adhere for 1 h at 37°C. Thereafter, plates were washed with RPMI medium to remove unattached cells, and adherent cells were fixed with 4% formaldehyde in PBS for 15 min at RT and stained overnight with 0.5% toluidine blue in PBS (Sigma) also at RT. After extensive washing with distilled water to remove excess dye, adherent cells were solubilized with 100 μl of 2% sodium dodecyl sulfate (SDS) and then quantified in a plate reader (Multiskan MS; Labsystems, Helsinki, Finland) at 620 nm. To identify integrin laminin receptors with function-blocking mAbs, cells in suspension were pre-incubated with 20 μg/ml of mIgG (negative control) or mAbs P4C10 or 13 to INTβ1, P1E6 to INTα2, P1B5 to INTα3, GoH3 to INTα6 or LM609 (αVβ3INT) (all from Chemicon). The effect of 10 mM EDTA was also tested.

### 3.3.3 Cell migration assay (Paper II and IV)

To determine the migration promoting activity of exogenous LM isoforms on CBMCs, HMC 1.1 and *in vitro* differentiated MKs, polycarbonate Transwell culture inserts with 5- or 8-μm pore size and 6.5 mm diameter (Costar, Cambridge, MA) were coated and blocked as for the cell adhesion assay. After washing the cultured cells twice with sterile PBS, 100 μl of cell suspension (1 x $10^6$/ml) prepared in RPMI 1640 medium was added into the upper chamber of the coated inserts and 600 μl of medium into the lower chamber. Cells were then incubated for 3 h (CBMCs and HMC 1.1) or 4 h (MKs) at 37°C in the absence (spontaneous migration) or presence (stimulated migration) of chemoattractants. In HMC 1.1/CBMCs transmigration assay, Stem cell factor/c-Kit ligand (SCF, 50 ng/ml), Stromal cell derived factor 1α (SDF-1α, 500 ng/ml), and complement 3a (C3a, 10 ng/ml) were used. For MKs migration, only Stromal cell derived factor 1α (SDF-1α, 300 ng/ml) was included.

To identify integrins mediating the cell migration, cells were pre-incubated with function-blocking mAbs (20 μg/ml) for 15 min at 37°C. To remove all transmigrated cells efficiently from the lower chamber, a final concentration of 10 mM EDTA was added to each well and the cells were vigorously resuspended and collected. Thereafter, the cells were fixed with 1% formaldehyde and counted microscopically using a haemocytometer at 400 x magnification or alternatively by flow cytometry.

*Methodological comments*

Different commercial and recombinant LM preparations were used in functional studies (*Paper I, II, III, and IV*), following extensive molecular characterization of the preparations to determine possible degradation or contamination with laminin isoforms or other ECM proteins (Wondimu *et al*., 2006). LM-111 was isolated from mouse EHS tumor (Chemicon). Both commercial LM-211 (laminin-2, merosin) isolated from human placenta (Chemicon) and recombinant human LM-211 (LM-2, furin-processed form) (Smirnov *et al*., 2002) were tested for comparison. α3-laminin LM-332 (α3β3γ2, laminin-5) was obtained from the culture supernatant of human oral squamous carcinoma (SCC25) (Patricia *et al*., 1994). Recombinant LM-411 (rhLM-8) and LM-511 (rhLM-10) were produced in a mammalian expression system as full length heterotrimers and purified by affinity chromatography as previously described (Kortesmaa *et al*., 2000; Doi *et al*., 2002; Koretesmaa *et al*., 2002). To evaluate the functional relevance of chondroitin sulphate modifications in LM-411, two forms of rhLM-8 were used in the neurite outgrowth experiments (Paper I), namely, non-chondroitin sulphated (hrLM-8A) and chondroitin sulphated (hrLM-8B) forms (Kortesmaa *et al*., 2002). Commercial α5-laminin LM-511 (α5β1γ1, laminin-10) (Sigma, St.Louis, MO) was isolated from human placenta and purified by immunoaffinity chromoatography.

### 3.3.4 Platelet P-selectin expression (Paper III and IV)

Once whole blood was collected in a citrated tube from healthy donors, P-selectin expression on the cell surface of platelets was measured by FACS (Coulter EPICS XL-MCL flow cytometer), as described previously (Hjemdahl et al., 1994). Briefly, within three min of collection, 5 μl of blood was added to 45 μl of HEPES-buffered saline (150 mM NaCl, 5 mM KCl, 1 mM Mg $SO_4$, 10 mM HEPES, pH 7.4) containing FITC-conjugated mAb AC1.2 to P-selectin (CD62P) (BD Biosciences, Stockholm, Sweden) and different concentrations of adenosine diphosphate (ADP) (Sigma-Aldrich) in the presence or absence of LM-332 (LM-5), LM-411 (rhLM-8) or LM-511 (rhLM-10), and incubated for 20 min at RT. Following gating of the platelet population, the percentage of P-selectin positive cells and their mean fluorescence intensity were determined. The assay was carried out in duplicate in each experiment.

### 3.3.5 Platelet aggregation (Paper III and IV)

Platelet aggregation studies were performed using platelet-rich plasma (PRP) with a four-channel platelet aggregation profiler (PAP-4, Bio/Data Corporation, Hatboro, PA, USA). Briefly, an initial dose-response was determined first to establish ADP concentration eliciting 60-70% aggregation so that tested laminins could be evaluated for their ability to either inhibit or further stimulate platelet aggregation. In this assay, 200 μl of PRP were used in duplicate. After adding PBS or different concentrations of LM-332 (LM-5), LM-411 (rhLM-8) or LM-511 (rhLM-10) in the absence or presence of ADP, light transmission was measured during 4 minutes of incubation at 37 °C.

*Methodological comments*

At the same time when PRP was prepared for the aggregation assay, a portion of the collected whole blood was used to prepare platelet-poor plasma (PPP), which served as a reference between each measurement to set the aggregometry to record 100% light transmission (100% aggregation).

### 3.3.6 Platelet-like particle formation (Paper IV)

*In vitro* differentiated megakaryocytes (ivMKs) were allowed to migrate for 24 h at 37$^{o}$C through Transwells coated with different laminin isoforms representing all α-chains and HSA as a control. All cells which migrated through the filters (inserts with 5 μm pore size and 6.5 mm diameter Costar, Cambridge, MA), including platelet-like cells, were collected from both the upper and the lower chambers, double stained with platelet specific markers PE-CD41a and FITC-CD42 mAbs, washed in PBS, and analyzed by FACS.

*Methodological comments*

Prior to adding the *in vitro* differentiated MKs to the upper chamber of the inserts, platelet-like cells or cell fragments were excluded by centrifugation at 170 *g* for 15 min. All procedures were performed with minor physical manipulations due to the fragile nature of mature MKs. For platelet like-events counting by FACS, first normal blood platelets were prepared from buffy coats, and treated with the CD41 and CD42 antibodie. Once the platelet population gate was defined, based on the FSC and SSC properties and platelet markers, the same gate was used to count the platelet-like events. A fixed time was used to determine the number of platelet-like events obtained from each laminin substrate and HSA under low flow rate (12μl ±3/min) in a FACScan flow cytometer (BD Biosciences, San Jose, CA).

Statistical analysis

For statistical analysis, student's t-test, mean, and SD values were calculated and the level of significance (*, $p<0.05$; **, $p<0.01$; ***$p<0.001$) was determined by comparing laminin isoforms with HSA, or mAbs with mIgG.

# 4 RESULTS AND DISCUSSION

## 4.1 PAPER I

### 4.1.1 Tooth pulp fibroblasts synthesize and and secrete LM-211 and LM-411

To identify laminin isoforms present in human dental pulp tissues, we first performed immunohistochemistry by using mAbs against different laminin chains and tissue-specific markers. Despite unambiguous stainining of peripheral nerves (LM α1, α2, α4, α5, β1 and γ1 chains) and blood vessels (LM α3, α4, α5, β1, β2 and γ1 chains), the pulp fibroblasts *in situ* were either negative or faintly positive. Employing established primary fibroblast-like cells, expression of transcripts for laminin chains was investigated by RT-PCR, and amplified products of the expected size for LM α2, α4, β1 and γ1 chains were easily detected. Moreover, following permeabilization, the fibroblasts were found to react with mAbs to LM β1 and LM γ1 chains and, to a lower extent, with mAbs to LM α2 and LMα4 by immunoflorescence flow cytometry (FACS).

In order to demonstrate physical association of the expressed laminin chains and to ascertain the presence of fully heterotrimeric laminin molecules in human dental pulp tissues, IP/WB experiments were performed on both pulp fibroblast cell lysates and their culture supernatants. Using immunoprecipitating mAbs to LM α2 and α4 chains, assembly of both LM-211 (α2β1γ1/LM-2) and LM-411 (α4β1γ1/LM-8) was confirmed in the cell lysates and culture supernatants, following immunoblotting with mAbs specific to each α, β, and γ chain. In the cell lysates, LMα2 chain was visualized by WB, and polypeptides of 230 (LMβ1), 190 (LMβ2) and 220 (LMγ1) kDa were detected in the LMα2-immunoprecipitates. Likewise, in the mAb α4-immunoprecipitates, a 200 kDa band corresponding to LMα4 chain was found, in addition to the LM β1, β2 and γ1 chains.

The laminin isoforms detected in the cell lysates were similarly detected in the culture supernatants. Here, the LMα2 chain was visualized as a sharp 350 kDa band, and no fragment(s) of 60-80 kDa were detected with mAb 5H2 against the LMα2 carboxyterminus, unlike previous reports by Engvall *et al*. (1990). In contrast, LMα4 appeared as a smeary 200-250 kDa band. Since similar observations made by others indicated presence of chondroitin sulphate modifications in the LMα4 chain (Sasaki *et al*., 2001; Kortesmaa *et al*., 2002), we addressed the same phenomenon in the fibroblast laminin. Following treatment of the immunoprecipitated LM-411 with chondroitinase ABC, the LMα4 chain band was compressed and reduced to an apparent molecular weight of nearly 200 kDa, demonstrating chondroitin sulphate modification(s) in the α chain.

To date, very few studies have explored laminin expression in human dental tissue, and most of them focused on the dental epithelium, where contribution of LM-332 (LM-5) to tooth morphogenesis has been reported. Presence of LMα2 in odontoblasts has also been described (Sahlberg *et al*., 1998; Yoshiba *et al*., 2000; Lesot *et al*., 2002; Yuasa *et al*., 2004). Despite an apparent heterogeneity within fibroblasts, irrespective of their tissues of localization (Koumas *et al*., 2001), a recent study on periodontal ligament derived fibroblasts has documented findings similar to ours (Ohshima *et al*., 2006). In addition, the authors investigated the migration promoting effect of isolated LM-211 (LM-2) and LM-411 (LM-8) on epithelial cells. These

findings indicated the multi-functional properties of fibroblast derived laminins which, besides promoting neurite outgrowth, might be involved in other biological activities.

### 4.1.2 LM-411, but not LM-211, strongly promotes neurite outgrowth from TG neurons

The presence of unknown molecular factors promoting neurite outgrowth from TG neurons in co-culture with tooth pulp fibroblasts has been reported earlier (Lillesaar et al., 1999). Since the observed neuritogenic effect could not be inhibited by blocking antibodies against neurotrophic factors such as NGF and GDNF (Lillesaar et al., 2003), we investigated whether pulpal fibroblast laminin isoforms could contribute to neurite outgrowth. TG sensory neurons were, thus, incubated for 24 h on substrates of isolated LM-211 (LM-2) and LM-411 (LM-8). LM-111 (LM-1) was also tested, and FN and HSA were used as controls. Mouse LM-111 and recombinant human (rh) LM-411 (LM-8) strongly promoted the neurite outgrowth. These results were reproducible when different batches of rhLM-411 (LM-8) were used. In contrast, LM-211 (LM-2), either as recombinant or natural protein isolated from human placenta, exerted minimal effects, if any. Both FN and HSA were inactive, and the neurite outgrowth promoting effect of pulp fibroblast-like cells co-cultured with TG neurons was reproduced.

Since chondroitin sulphate proteoglycans and their glycosaminoglycan have been reported to modulate neurite outgrowth (Margolis andMargolis, 1997), contribution of the chondroitin sulphate modification of LM-411 (LM-8) to its biological activity was analyzed. We took here the advantage of using two forms of rhLM-411: a non-chondroitin sulphated form (rhLM-8A), and chondroitin sulphated form (rhLM-8B). No major differences were seen between these forms, as both of them promoted neurite outgrowth to a similar extent.

Strong neurite promoting activities of various LM isoforms, including α1-, α2-, α3- and, maybe, α5-laminins on neurons isolated from different anatomical locations have been reported by various groups (Engvall et al., 1986; Brandenberger and Chiquet, 1995; Powell and Kleinman, 1997; Culley et al., 2001). The functional difference observed between LM-211 (rhLM-2) and LM-411 (rhLM-8) could be adscribed to their distinct α-chain, since both share the same LMβ1 and LMγ1 subunits. The diverse effects might indicate preference of laminin isoforms for particular laminin-binding integrins.

Currently, the possibility of using dental pulp derived cells as a co-adjuvant therapy for different neurological disorders such as Parkinson's disease and spinal cord injuries is under consideration, since these cells have been shown to release neurotrophic factors which promote neurite outgrowth and survival (Nosrat et al., 2004). More recently, Chen et al. (2007) reported the beneficial effect of cultured fibroblast-like cells from bone marrow stromal cells (BMSCs) on sciatic nerve regeneration. These fibroblast-like cells were shown to express various neurotrophic factors and supporting substances, including LM. Therefore, the information generated through our findings might have broader implications for nerve and tissue regeneration.

## 4.2 PAPER II

### 4.2.1 α3-(LM-332) and α5-(LM-511) laminins selectively promote adhesion and migration of human mast cells

Mast cells are often found in close contact with basement membranes of epithelia, nerves and blood vessels. In order to explore the possible contribution of laminins, we performed cell adhesion and migration assays using a human mast cell line (HMC1.1) and cord blood derived mast cells (CBMCs) on a panel of different laminin substrates, representing all α-chains. Constitutive adhesion was readily detected for HMC-1 cells and CBMCs (8 weeks) on α3- and α5-laminins, but not on α1-, α2-, and α4-laminins, and various stimuli substancially increased the isoform-specific cell adhesion. TPA was the most effective stimulus, followed by SDF-1α and SCF. Plasma fibronectin (pFN) was also adhesive for the MCs. The adhesion promoting effect was more pronounced on α5- than on α3-laminins.

In cell migration assays, constitutive migration of both HMC-1 cells and CBMCs (10 weeks) was significant on α5-laminin and pFN only. However, in presence of chemoattractants, migration of both populations through filters coated with α3- and α5-laminins dramatically increased and, still, the other laminin isoforms had no effect. SDF-1α and SCF were more active than C3a as chemoattractants. As for cell adhesion, cell migration on α5-laminin was significantly higher than on α3-laminin, and native LM-511 isolated from placenta behaved similarly to rhLM-511. The observed adhesion and migration of MCs on LM-511 and, to a lower extent, α3-LM may be relevant during extravasation of MC precursors, and for migration and localization of MCs within tissues, apposing to BMs.

### 4.2.2 Integrin α3β1 mediates adhesion and migration of human mast cells on LM-332 and LM-511

Before identifying integrins involved in the cell adhesion and migration, we analysed HMC-1 cells and CBMCs for integrin expression using a panel of mAbs and immunofluorescence flow cytometry. Both MC populations, identified as CD117 (c-kit) positive, expressed integrins α2β1, α3β1, α9β1 and αVβ3, but lacked α1β1, α6β1, α7β1 and α6β4. Following treatment of HMC-1 cells with TGFβ for 3 days, the levels of α9β1 and αVβ3 increased.

Thereafter, the effect of function-blocking mAbs to INTα2, α3, α6, β1 and αVβ3 was investigated in both constitutive and stimulated adhesion of HMC-1 and CBMCs on LM-332 and LM-511. mAbs P1B5 (Intα3) and 13 (Intβ1) were strongly and significantly inhibitory, indicating a major and selective participation of α3β1 integrin. As in other integrin-mediated activities, EDTA completely inhibited the cell adhesion. Similarly, the same mAbs significantly reduced SDF-1α- and SCF-induced migration of both HMC-1 cells and CBMCs on α3- and α5-laminins by half or more, demonstrating participation of the same integrin in the cell migration.

Previous studies by other groups have demonstrated MC adhesion and/or migration promoting effects of LMs by using LM-111 (LM-1) and placental laminin preparations. Thus, mouse MCs have been reported to adhere to LM-111 and to placenta laminins, and to migrate on the former laminin isoform (Thompson *et al*., 1989a; Thompson *et al*., 1989b; Fehlner-Gardiner *et al*., 1996; Vliagoftis and Metcalfe, 1997; Rosbottom *et al*., 2002). In contrast, human MCs were found unable to adhere to and migrate on LM-111, but were responsive instead to a placenta laminin preparation (Nilsson *et al*., 1994; Columbo *et al*., 1995; Kruger-Krasagakes

*et al*., 1996; Hartmann *et al*., 1997). Moreover, MC integrin expression has been reported to differ between mice and humans, with participation of INT α6β1 and α7β1 in mouse and of INT α3β1 in man during MC-laminin interactions. Because of this controversy, we also included INTα6 function blocking antibodies in our functional studies, but we could not observe any inhibitory effect. To our knowledge, no previous studies have looked into integrins mediating MC migration on LMs. The selective adhesive and migratory responses of human mast cells on LM-332 and LM-511, but not on other laminin isoforms, may be explained by the specificity of the integrin α3β1, as recently shown by Nishiuchi and co-authors (Nishiuchi *et al*., 2006), where a selective binding of laminin-332 (α3β3γ2) and laminin-511 (α5β1γ1)/521 (α5β2γ1) was shown among laminin isoforms covering all α-chains.

### 4.2.3 Human mast cells synthesize and secrete LM-511

Encouraged by previous findings from our group indicating expression and synthesis of LMs by blood cells, we further asked whether the same holds true for human mast cells. Our approach was first to check presence of transcripts for all eleven LM chains in HMC-1 cells and CBMCs by RT-PCR. Accordingly, both cell populations were found to express transcripts for LMα3AB (common to truncated and full length), LMα3B (full length only), LMα5, LMβ1 and LMγ1. In addition, HMC-1 cells expressed transcript for LMβ3. No other LM transcripts were detected. We then tested the reactivity of mAbs to LM chains with HMC-1 cells and CBMCs (8 weeks) by immunofluorescence flow cytometry. Both MC populations, identified as CD-117 (c-kit) positive, reacted with mAbs to LMα5, LMβ1 and LMγ1, but only after cell permeabilization, suggesting intracellular localization of LM-511.

Would these identified laminin chains assemble together and form a full heterotrimer? Since IP/WB analysis of laminins demands a large number of cells, we used HMC1.1 cell line to address this question. mAb 4C7 (LMα5)-immunoprecipitates from cell lysate and culture supernatant were tested for Western blotting with mAbs to LM α5, β1 and γ1 chains. From the cell lysate, double bands of nearly 230 and 220 kDa corresponding to LMβ1 and LMγ1 chains, respectively, were obtained. Though no LMα5 band was visible by WB analysis, a corresponding band of 350 kDa was detected, in addition to the LMβ1 (230 kDa) and LMγ1 (220 kDa) chains, in the mAb 4C7 (LMα5)-immunoprecipitate from concentrated culture supernatant of HMC-1 cells stimulated with TPA for 18 h. Minimal amounts of LM chains were detected in the culture supernatant of non-stimulated HMC-1 cells (data not shown). Though we found expression of laminin α3 chain by RT-PCR and flow cytometry, we were unable to see any band following IP/WB using mAb P3H9-2 (LMα3)-immunoprecipitates. Under these experimental conditions, LM bands were obtained only with mAb 4C7 (LMα5)-immunoprecipitates.

Supporting our findings, the ability of murine mast cells to synthesis laminins was reported formerly, though the study failed to give evidence for the α-chain (Thompson *et al*., 1991). Moreover, another study showed by immunohistochemistry the colocalization of strong LM staining with mast cells in the gut of patients with Crohn's disease (Gelbmann *et al*., 1999). The close association of mast cells with laminins may influence other function and activities of these cells, such as costimulation of cytokine secretion, in addition to the mere promotion of adhesion and migration.

## 4.3 PAPER III AND IV

### 4.3.1 Erythromegakaryocytic cells synthesize and express α3-laminins ((LM-311 and LM-321) and α5-laminins (LM-511 and LM-521) (Paper III and IV)

The first demonstration of a fully trimeric laminin isoform (LM-411, LM-8) in blood cells and MKs came from the work by Geberhiowt *et al*. (1999, 2000). In these reports and based on RT-PCR results, the authors suggested that additional laminin isoforms could be present in MKs and blood platelets. Thus, further studies were performed searching for α3- and α5-LMs in these cells. Erythromegakaryocytic HEL and DAMI cell lines, known to express megakaryocytic lineage markers such as integrin GPIIb/IIIa, were used as MK homologues (Martin and Papayannopoulou, 1982; Greenberg *et al*., 1988).

In *Paper III*, we confirmed presence of α5-containing LMs in MKs first by RT-PCR, where we found transcripts for LMα5, LMβ1, LMβ2 and LMγ1. Thereafter, by metabolic labeling of HEL cells followed by IP with mAbs to either LMβ1 (DG10), LMγ1 (2E8) or LMα5 (4C7), which showed bands of 350, 230 and 220 kDa, representing the expected size of LMα5, LMβ1 and LMγ1, respectively. To strengthen our findings, non-radioactive HEL cell lysate was further analyzed by IP/WB assay using mAb 4C7 (LMα5)-immunoprecipitates. Similar to the metabolic labeling studies, mAbs DG10 (LMβ1), 22 (LMγ1) and 15H5 (LMα5) reacted with bands of 230, 220 and 350 kDa, respectively, whereas mAb C4 to LMβ2 chain demonstrated a band of 190 kDa, the expected size of LMβ2.

In *Paper IV*, the transcripts for LMα3AB (for both truncated and full-length forms), Lmα3B (for full-length only) and LMβ3 were detected, in addition to the previously reported LMβ1, LMβ2 and LMγ1 transcripts (Geberhiwot *et al*., 2000; *Paper III)*. Thereafter, reactivity of DAMI cells with mAbs to LM chains was determined by flow cytometry. Following their permeabilization, mAbs P3H9-2 (LMα3), DG10 (LMβ1), C4 (LMβ2), CAF-2 (LMγ1) and B-2 (LMγ2), but not 17 (LMβ3), were clearly reactive with DAMI cells. No or minimal staining was observed on intact cells, and similar intracellular staining was also obtained with mAb BM165 against another LMα3 epitope (data not shown). To establish physical association of the LM chains and heterotrimeric formation, the mAb P3H9-2 (LMα3)-immunoprecipitate obtained from DAMI cell lysate was analyzed by WB with mAbs against LM α, β and γ chains. Polypeptides of nearly 200 (LMα3), 230 (LMβ1), 200 (LMβ2) and 220 (LMγ1) kDa were obtained, indicating presence of LM-311 (α3β1γ1, laminin-6) and LM-321 (α3β2γ1, laminin-7) in the cells. LM-332 (α3β3γ2, laminin-5) and full-length form (300 kDa) of LMα3 (LMα3B) were not detected.

### 4.3.2 Blood platelets contain and secrete α3-laminins (LM-311 and LM-321) and α5-laminins (LM-511 and LM-521) (Paper III and IV)

As an initial approach to determine expression of LM-311 (LM-6) and LM-321 (LM-7) in *Paper IV* and of LM-511 (LM-10) and LM-521 (LM-11) in *Paper III* in isolated platelets, immunofluorescence flow cytometry analysis with mAbs to LMα3 (both P3H9-2 and BM165), LMα5 (11D5), LMβ1 (2G6), LMβ2 (C4) and LMγ1 (LM-41) was performed. mAb SZ.22 to αIIb INT chain (CD41) was used as platelet marker. Minimal, if any, reactivity was observed with intact cells. However, following permeabilization, platelets reacted with mAbs to LMα3, LMα5, LMβ1,

LMβ2 and LMγ1, and also to LMγ2, but not LMβ3 (*Papers III and IV*). To demonstrate presence of α3- and α5-containing laminin heterotrimers in platelets, we performed IP followed by WB.

In *Paper IV*, attempts to demonstrate presence of α3-laminins by IP/WB assays often resulted in weak bands for LMβ1 and LMγ1, and no detectable LMα3 chain (data not shown). Therefore, immunoaffinity (IA) purification with a mAb BM165 (LMα3)-sepharose column was introduced. Like in DAMI cells, WB analysis of the isolated material demonstrated polypeptides of approximately 190-200 (LMα3), 230 (LMβ1), 190-200 (LMβ2) and 220 (LMγ1) kDa, indicating presence of LM-311 (α3β1γ1, LM-6) and LM-321 (α3β2γ1, LM-7) in platelets. As additional evidence for the presence of α3-laminins in the platelet lysate, we also detected LMα3 in a laminin preparation isolated from platelets by using mAb DG10 (LMβ1)-Sepharose column (Geberhiwot et al., 1999). By Western blotting with mAb 10B5 (Goldfinger et al., 1998) and other LMα3 mAbs, a polypeptide of nearly 200 kDa, the expected size of unprocessed LMa3A, was detected in this preparation. In the same material, the intensity for LMα3 and LMα4 bands was compared. The 180 kDa LMα4 band was much more intense, suggesting much larger amounts of LM-411 than of LM-311 in the platelet lysate.

In *Paper III*, mAb 4C7 (LMα5)-immunoprecipitate from platelet cell lysate was analyzed by Western blotting. mAbs C4 (LMβ2), 22 (LMγ1) and 15H5 (LMα5) reacted with bands of 190, 220 and 300/350 kDa, respectively. A weak 230 kDa band reactive with mAb DG10 to LMβ1 was also observed. This indicated presence of LM-521 (LM-11) and, at lower amounts, LM-511 (LM-10).

We also addressed the question of laminin secretion from platelets following stimulation, as described for other adhesive secretory products. Previously, release of LMγ1-containing laminins from platelets stimulated with thrombin and TPA had been reported (Geberhiwot *et al*., 1999). To establish secretion of particular LM isoforms, the supernatant of TPA-stimulated platelets was resolved with WB. In *Paper III*, bands corresponding to LMβ1 (230 kDa), γ1 (220 kDa), LMα5 (300/350 kDa) and LMβ2 (190 kDa) polypeptides were readily detected, in addition to the previously described LMα4 (180 kDa). In *Paper IV*, mAb P3H9-2 (LMα3)-immunoprecipitate from the supernatant of TPA-stimulated platelets was tested for Western blotting. This immunoprecipitate revealed polypeptides of 230 (LMβ1), 220 (LMγ1) and 190 (LMβ2) kDa. However, under these experimental conditions, no LMα3 band could be detected by mAb BM165, which normally has weaker reactivity than that of the other mAbs by WB. No bands were detected in the mIgG immunoprecipitate, used as control (data not shown).

### 4.3.3 α3-laminin is expressed by certain blood vessels (Paper IV)

Platelets execute their function normally in the vasculature and their close interaction with vascular matrix proteins is essential in the process of hemostasis. The α4-and α5-laminins have been reported to be the primary vascular LM isoforms (Hallmann *et al*., 2005). But what about α3-laminins? Are they expressed by blood vessels so that platelets might interact with them following vascular damage?

In order to answer these questions, expression of α3-LMs by blood vessels was investigated based on the reactivity of mAb BM165 (LMα3) and Ulex Europaeus I-lectin (endothelial marker) with a large panel of adult human tissues by confocal microscopy. As shown in Table II (*Paper IV*), both capillaries and larger vessels of several tissues, including skin, gingiva, tonsils, lymph nodes, mammary gland and

others, do express LMα3 chain. In the same paper, one representative figure (Fig.3) showing staining of gingiva is presented and illustrates the LMα3 antibody reactivity of blood vessels beside the epithelial basement membrane (BM). However, most blood vessels stained by LMα3 were not accompanied by LMβ3 and LMγ2 reactivity (data not shown). Hence, this finding suggests that the vascular LMα3 appears to be mainly associated with LMβ1 and/or LMβ2 and LMγ1 chains, forming LM-311 (LM-6) and/or LM-321(LM-7), and only exceptionally LM-332 (LM-5), such as in some blood vessels of lymphoid tissue. Interestingly, LM-332 in lymphatic organs was suggested to be important in immune responses after observing the deposition of LMγ2 chain around small arteries and veins of the thymus and spleen (Mizushima *et al*., 1998). The source of the LMα3 identified in our study might be the endothelial cells or the pericytes, or some stromal cells in the vicinity. LM-411 and LM-511 are the major isoforms of vascular endothelial cells.

### 4.3.4 α5-laminin (LM-511) is the most active laminin isoform in promoting constitutive platelet adhesion via INTα6β1 (Paper III and IV)

In general, vascular BM components and their interaction with endothelial cells are though to be essential in maintaining vascular integrity and to influence endothelial cell proliferation, migration, differentiation and maturation. The close interaction between blood platelets and the vessel wall involves different membrane-bound and soluble factors as a highly regulated process. Following vascular damage, within a fraction of seconds, platelets adhere, get activated, spread and aggregate. Platelets express integrins α2β1, α6β1 and αVβ3, but lack α3β1 and α6β4 (Shattil and Newman, 2004), and their adherence to LM-111 *via* α6β1 early defined the laminin-binding activity of this integrin (Sonnenberg *et al*., 1988). Considering the fact that LM-411 (LM-8) and LM-511 (LM-10) are the major components of vascular BM (Hallmann *et al*., 2005) and that LMα3 can be also expressed, we investigated the platelet adhesion promoting activity of the various laminin isoforms representing all LMα chains and then approached the participating integrins. Though the perfect match in this functional study would have been to include LM-311 and/or LM-321, based on our findings, these isoforms were not currently available. Therefore, we used instead LM-332 as a source of LMα3.

To summarize the findings, the most platelet adhesive laminin was LM-511 (LM-10) followed by LM-411 (LM-8), and almost equally LM-332 (LM-5) and LM-111 (LM-1). LM-211 (LM-2) did not promote constitutive adhesion. Nonetheless, after TPA stimulation, platelets were found to adhere to all tested laminins, including LM-211 (LM-2). Constitutive adhesion was found to be inhibited by EDTA and function blocking mAbs GoH3 (Intα6) and 13 (Intβ1) or P4C10 (Intβ1), indicating the divalent cations dependent nature of the process and the critical role of Intα6β1. Though sensitive to EDTA, stimulated platelet adhesion was resistant to function-blocking integrin mAbs, including those against INTβ1 and INTα6. On the other hand, mAb P2 to INTgpIIbβ3 reduced the platelet adhesion to LMs by nearly one fourth, but also the platelet adhesion on HSA.

The contribution of Intα6β1 in mediating platelet adhesion to LM-332 and to LM-111, LM-411 and LM-511, which only differ in their α chains, might indicate the promiscuous nature of this laminin-binding integrin. Although the broader specificity of Intα6β1 has been reported by different groups (Nishiuchi *et al*., 2006), lack of constitutive platelet adhesion to either native or recombinant LM-211 (laminin-2) is

intriguing since its recognition by α6β1 integrin variants has been previously described (Delwel *et al*.,1995).

### 4.3.5 α3-(LM-332), α4-(LM-411), and α5-(LM-511) laminins induce neither P-selectin expression nor cell aggregation in platelets (Paper III and IV)

Adhesion of platelets to the subendothelial matrix is the first step in primary hemostasis. Various matrix components have been reported to be involved in this initial step, either in a transient or stable manner. Under static or low shear conditions, collagens are potent inducers of platelet adhesion, which leads to spreading, activation and platelet aggregation following vascular injury (Jurk *et al*., 2005). Persuaded by the adhesion promoting effect of the various laminins tested, we extended our work to explore the contribution of these laminin isforms in promoting platelet activation. In our hands, both LM-411 (LM-8) and LM-511 (LM-10) (*Paper III*), and LM-332 (LM-5) (*Paper IV*) were unable to induce/modulate activation of either resting or ADP-stimulated human platelets, as measured by P-selectin expression and cell aggregation in platelet reach plasma (PRP). It had been previously reported that LM-111 (LM-1) and other BM components such as perlecan were incompetent in triggering platelet activation and aggregation (Tryggvason *et al*., 1981). However, in a single study, a human placental laminin preparation was shown to induce platelet aggregation in almost one out of three donors, compared to the inactive LM-111 and LM-211. Interestingly, the observed platelet response was reported to be inhibited by mAbs to Int α6β1 (Willette *et al*., 1994). Unfortunately, no information was given about the nature of the commercial placenta laminin preparation, making difficult a comparison with our results obtained with recombinant proteins and fully characterized native laminin isoforms.

### 4.3.6 Vascular LM isoforms LM-411 and LM-511 and, to a lower extent, LM-332 promote migration and platelet-like particle formation in *in vitro*-differentiated megakaryocytes (ivMK) (Paper IV)

As mentioned in section 1.5.1, several studies have provided evidence for participation of bone marrow environmental factors, such as SDF-1 and FGF-4, in promoting migration of immature MKs to vascular niches as well as the transendothelial migration of mature and $CXCR4^+$ MKs through bone marrow endothelial cells (BMEC). Moreover, the very close contact between mature MKs and the abluminal side of the BMEC was proven to be critical before platelets were released into the marrow-intravascular sinusoidal space (Tavassoli *et al*., 1989; Zuker-Franklin and Philipp, 2000; Avecilla *et al*., 2004). Considering these facts and the presence of laminins in the sinusoidal basement membrane, and in other locations of the bone marrow (Gu *et al*., 1999; Siler *et al*., 2000; Gu *et al*., 2003), we investigated the ability of LM-332 and other LM isoforms to promote migration of *in vitro*-differentiated megakaryocytes (ivMKs) and formation of platelet-like particles from these cells. ivMKs, generated from CD34+ progenitors by treatment with TPO and SCF for two weeks, expressed high levels of the MK/platelet markers CD41a and CD42a.

In the transmigration assays, LM-332 (LM-5) and, to a higher extent, LM-411 (LM-8) and LM-511 (LM-10) promoted migration of ivMKs in the presence of the chemokine SDF-1. Migration of other cells present in the culture but lacking the CD41/CD42 markers was also promoted. In a model of platelet formation, generation of platelet-like particles was tested in parallel. These particles, defined by CD41/CD42 expression and forward and side scatter properties similar to platelets

obtained from healthy donors, were quantified by flow cytometry in both the upper and lower chambers after 24 h incubation of the ivMKs. In presence of LM-332, a higher number of platelet-like particles were observed, most of them in the lower chamber, when compared to HSA. However, the number of particles was even higher for LM-411 and LM-511. The ranking of activity for the LM isoforms was similar in the assays for ivMK migration and platelet-like particle formation, namely, LM-511>LM-411>LM-332. Thus, α3-laminins and, to a higher extent, α4- and α5-laminins promote ivMK migration and platelet-like particle formation.

Using Matrigel, which contains laminin and other ECM proteins, augmentation of proplatelet formation was reported earlier (Topp *et al*., 1990). Nonetheless, another study failed to see difference between the cells plated on a Matrigel vs control (Choi *et al*., 1995). More recently, fibrinogen was shown to promote proplatelet formation more effectively than any other matrix protein. In this study, a placenta laminin preparation was also active, and to higher levels that VN and FN. Expression of INTα6β1 at different stages of MKs differentiation and maturation has been reported (Molla *et al*., 1999), and the MKs response to bone marrow laminins might be executed through this receptor. We do not know which mechanism is favored by laminins for induction of platelet formation, "proplatelet" or 'explosive-fragmentation".

# 5 CONCLUSIONS AND FUTURE PERSPECTIVES

In general, the work presented in this thesis has generated the following essential information clarifying the diverse roles of laminins in several biological activities:

- Dental pulp fibroblasts synthesize and secrete LM-211 (LM-2) and LM-411 (LM-8).
- LM-411, but not LM-211, promotes neurite outgrowth from trigeminal sensory neurons.
- Human mast cells adhere and migrate selectively on LM-332 (LM-5) and LM-511 (LM-10) *via* integrin α3β1.
- Human mast cells synthesize and secrete LM-511 (LM-10).
- α3- and α5-containing laminins are present in erythromegakaryocytic cells and platelets.
- α5-laminin (LM-511) is the most effective isoform in promoting constitutive adhesion of platelets, followed by α4-(LM-411), α3-(LM-332), and α1-(LM-111) laminins.
- The observed constitutive adhesion of platelets to laminins is mediated by integrin α6β1.
- α3-laminins are present in certain blood vessels in the form of LM-311 and/or LM-321 and, exceptionally, as LM-332 such as in blood vessels of lymphoid organs.
- Vascular LM isoforms LM-411 and LM-511 and, to a lower extent, LM-332 promote migration of *in vitro*-differentiated megakaryocytes (ivMK) and formation of platelet-like particles.

Taken as a whole, this work attempts to put forward some new information but it also poses some questions, as it is common in science, which needs to be answered in future studies. Hence, it will be important to further explore issues regarding:

- The role of endogenous laminins shown to be secreted after stimulation. Particularly, investigating the contribution of platelet-derived laminins in the heterotypic interaction between platelets and leukocytes or other cell types, which is becoming more recognized in inflammation.
- The physiological relevance of the selective interaction of human mast cells with LM-332 and LM-511, other than the adhesion and migration promoting effects.
- Further understanding of the involvement of bone marrow-expressed laminins in MKs physiology, other than in the migration promotion, like in induction of cell differentiation and maturation (polyploidyzation). Moreover, identifying the exact mechanism of platelet formation and the functionality of *in vitro* generated platelet–like particles.
- Based on the findings reported by others and those presented in this thesis, α5-containing laminin (LM-511), among all the laminin isoforms available and tested in different functional studies, seemed to play a major role in most of the *in vitro* studies. It will be of great interest to perform *in vivo* studies and to determine the significance of α5-laminins in the physiology of various types of haematopoietic cells by using conditional knockout mice.

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