The significance of the laminin/nidogen-1 interaction for basement membrane formation and stability in embryoid bodies

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Meinen Lieben

Die Entdeckungen der letzten Zeit lassen praktisch alles, was wir viele Jahre für richtig gehalten haben, als falsch oder nur bedingt richtig erscheinen. Meiner Meinung nach kann man heute nur noch eines mit Sicherheit sagen: Die Lichtgeschwindigkeit ist absolut das Schnellste, was es gibt. Möglicherweise.

Edward Teller

ABSTRACT

Basement membranes are thin layers of extracellular matrix which separate epithelial and endothelial cells from underlying connective tissue and surround nerve, muscle and fat cells. Biochemical data indicate the important role of nidogen-1, a 150kD sulfated glycoprotein, in supporting basement membrane stability by connecting the laminin and type IV collagen network (Aumailley et al., 1989; Yurchenco and Schittny, 1990).

The aim of this study was to analyse the significance of the laminin/nidogen-1 interaction for basement membrane formation and stability in a more complex system. For that purpose F9 embryoid bodies, a cell culture model for basement membrane formation, were grown in the cell spin system after comparison with the conventional hanging drop method. F9 cells are mouse teratocarcinoma cells which differentiate and develop a basement membrane when cultured with retinoic acid.

Recombinant expression of the nidogen-binding site, located in LE module 4 of the γ1 chain of the trimeric laminin molecule, as the γ1III3-5 fragment and its binding to free nidogen-1 molecules should interfere with the laminin/nidogen-1 interaction. As controls, F9 cells were transfected with the empty expression vector, the γ1V1-3 and the γ1III3-5mut fragment. γ1V1-3 is similar to the γ1III3-5 fragment in secondary structure and size while γ1III3-5mut carries a point mutation drastically reducing its affinity to nidogen-1. For detection of recombinant expression and for purification purposes all constructs had been modified with the FLAG peptide at their N-terminal end.

Analysis of basement membrane formation by fluorescence microscopy revealed a different pattern in clones expressing the nidogen-binding site than in the controls. While cells carrying the empty expression vector and such expressing the γ1V1-3 or the γ1III3-5mut fragment developed embryoid bodies with completely or partially continuous basement membranes, only a punctate distribution of basement membrane components could be detected in cell aggregates expressing the nidogen-binding site. This indicated effective blocking of the laminin/nidogen-1 interaction by recombinant expression of the γ1III3-5 fragment. Additional studies on differentiation showed patchy distribution of cells expressing genes specific for visceral endoderm all over the embryoid body. In the controls these cells were organised into an peripheral epithelium which suggests a role of basement membranes in regulating the differentiation of these cells. To evaluate further functional aspects of the basement membrane discontinuity a diffusion assay was developed in which permeability properties could be tested. This revealed higher permeabilities among clones with comparable embryoid body morphologies when the basement membrane was disrupted.

To exclude the possibility that endogenous recombinant expression or the genetic manipulation during transfection was responsible for the observed phenotypes, experiments were also performed with the extraneous addition of the recombinant nidogen-binding site. Wild type F9 cells were grown in suspension culture and treated with recombinantly expressed, affinity purified nidogen-binding site fragment. Microscopy demonstrated a similar basement membrane breakdown upon exogenous addition of the nidogen-binding site as observed before in embryoid bodies derived from F9 cells recombinantly expressing the γ1III3-5 fragment.

ZUSAMMENFASSUNG

Basalmembranen sind dünne Schichten extrazellulärer Matrix, die Epithel- und Endothelzellen vom Bindegewebe trennen und Nerven-, Muskel- und Fettzellen umgeben. Eine auf biochemischen Daten (Aumailley et al., 1989) gestützte Hypothese erklärt die Stabilität der Basalmembran mit Nidogen-1, einem 150kD großen sulfatierten Glykoprotein, als Bindeglied zwischen dem Laminin- und Typ IV Kollagen- Netzwerk der Basalmembran (Yurchenco und Schittny, 1990).

Ziel war es, die Bedeutung der Laminin/Nidogen-1 Wechselwirkung für die Ausbildung und Stabilität von Basalmembranen in einem komplexeren System zu analysieren. Dafür wurde ein Zellkultur-Modell der Basalmembranbildung, das F9 Zellaggregatsystem, nach Vergleichen mit der "Hanging Drop"-Methode in Spinnerflaschen etabliert. Solche "Embryoid Bodies" aus F9 Maus Teratokarzinomazellen bilden bei Retinsäurebehandlung eine Basalmembran aus.

Rekombinante Expression der in LE Modul 4 der γ1 Kette des trimeren Laminin Moleküls lokalisierten Nidogenbindungsstelle im Fragment γ1III3-5 und dessen Bindung an freie Nidogen-1 Moleküle sollte das natürliche Gleichgewicht der Laminin/Nidogen-1 Interaktion stören. Als Kontrollen wurden neben dem leeren Expressionsvektor ein der Nidogenbindungsstelle in Sekundärstruktur und Größe ähnliches Fragment der γ1 Kette, γ1V1-3, sowie eine mutierte Version der Nidogenbindungsstelle γ1III3-5mut in F9 Zellen transfiziert. Zuvor waren alle Konstrukte zum Nachweis rekombinanter Expression und um eine Aufreinigung zu ermöglichen mit einem N-terminalen FLAG Bindungsmodul versehen worden.

Die mikroskopische Untersuchung der die Nidogenbindungsstelle exprimierenden Klone ergab eine von den Kontrollen abweichende Ablagerung der Basalmembranproteine. Während die nur resistente Kontrolle sowie die γ1III3-5mut und γ1V1-3 synthetisierenden "Embryoid Bodies" entweder durchgehend oder wenigstens streckenweise Basalmembran ausbilden konnten, war in den die Nidogenbindungsstelle exprimierenden Klonen nur eine punktförmige Verteilung von Basalmembrankomponenten zu erkennen. Dies sprach für eine effiziente Blockierung der Laminin/Nidogen-1 Wechselwirkung durch die rekombinante Expression des γ1III3-5 Fragments. Die zusätzliche Bestimmung der Fähigkeit zur Differenzierung zeigte, dass in dem Klon mit gestörter Basalmembranausbildung Zellen mit einer für das viscerale Endoderm spezifischen Genexpression über das ganze Zellaggregat verteilt waren. In den Kontrollen hingegen waren solche Zellen vorzugsweise an der äußeren Peripherie in einem Epithel organisiert, was eine Funktion der Basalmembran in der Steuerung der Zelldifferenzierung impliziert. Um die mikroskopisch erkennbare Zerstörung der Basalmembran auf ihre funktionelle Konsequenz hin zu testen, wurden ihre Permeabilitätseigenschaften mit der Diffusions-Messmethode bestimmt. Daraus ergab sich für "Embryoid Bodies" mit vergleichbarer Morphologie eine höhere Permeabiliät bei gestörter Basalmembranausbildung.

Weil die Möglichkeit bestand, dass die den Zellmetabolismus belastende rekombinante Expression oder klonale Selektion ausschlaggebend für die beobachteten Phänotypen waren, wurde außerdem in Suspensionskulturen herangezogene Wildtyp F9 "Embryoid Bodies" in mit rekombinant erzeugten, affinitätsgereinigten Nidogenbindungsstellen versetztem Medium differenziert. Deren mikroskopische Analyse zeigte bei Zugabe der Bindungsstelle von außen dieselbe Störung der Basalmembranausbildung wie sie zuvor bei den Klonen mit endogener rekombinanter Expression beobachtet worden war.

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1. Introduction

1.1. The evolution of extracellular matrices

During the development from uni- to multi-cellularity novel structural requirements were placed upon the evolving organisms. A primary need was the maintenance of the body form. While in primitive organisms this could probably be met by receptor mediated cell-cell contacts, these became insufficient as size and complexity increased leading to the evolution of the extracellular matrix. Although it is uncertain which extracellular matrix function evolved first, it may be speculated that the underlying need was to provide physical support to maintain the integrity of the body and that this finally led to the production of a prototype connective tissue and, particularly, the evolution of the collagen family (van der Rest and Garrone, 1991; Garrone, 1998). Here great tensile strength is produced through the close intramolecular association supplied by the triple helix and intermolecular covalent bonds. With specialisation of cells into tissues, it became necessary to evolve modes of compartmentalisation (Kleinman and Schnaper, 1993). The physical barrier separating cell and tissue types led to the formation of the architechtural framework of higher organisms. To transmit this stabilisation from the extracellular scaffold to the cell, specialised areas may have developed containing receptor molecules anchoring the intracellular skeleton to particular molecules in the surrounding matrix. These receptors were possibly refined to also allow signalling between the matrix and the cells and *vice versa*. So external events were not only conveyed by soluble proteins, which may themselves be bound into the matrix, as is the case with many cytokines, but also by the structural components themselves. This has led to the extracellular matrix having key roles in the presentation of growth and guidance cues to cells, which can in turn influence the secretion of extracellular matrix components. So, cell-matrix contacts may determine cellular differentiation. This has become crucial for most normal developmental processes such as gastrulation (Czaker, 2000), neural crest formation (Poelmann et al., 1990) and whether a cell or axon migrates, where it goes and when it stops migrating, as well as for adult physiology and pathology (e.g. extravasation of white blood cells, metastasis, wound healing). In addition to directing cellular motility, the extracellular matrix controls the formation of tissues like bone, cartilage, and tendon with characteristic structural proteins and growth factors. The latter contribute by establishing gradients of guidance cues in which cells can orientate, differentiate and secrete those extracellular matrix components serving the specific needs of each tissue.

Most studies of the extracellular matrix have been performed on vertebrate organisms, however investigations of invertebrates show that many extracellular matrix components are ancient and highly conserved proteins. For example, the fibril-forming collagens I, II, III and V , providing tensile strength, as well as the non-fibrillar collagen type IV of basement membranes are found in invertebrate organisms like sponges, worms and mussels (Coyne et al., 1997; Kramer et al., 1994; Garrone et al., 1993). However broad the spectrum of identified collagen homologs seems to be, little is known about the mechanisms behind their evolution (Engel, 1997). Ancestor proteins have also been found for molecules presenting information in the extracellular matrix. Perlecan (Laurie et al., 1986), a basement membrane proteoglycan of higher organisms, is also a cartilage component and plays an important role in chondrogenic differentiation probably by binding and presenting growth factors (French et al., 1999). However, closely related proteoglycans are also present in primitive invertebrate organisms such as *C. elegans* and the fruit fly, where an ortholog of perlecan (Mullen et al., 1999; Moerman et al., 1996) and a perlecan-like core protein sequence have been identified (Friedrich et al., 2000), respectively. Since primitive organisms do not have cartilage these findings are an example of how the roles of extracellular matrix proteins may be adapted to new functions during evolution.

Modern extracellular matrix proteins are mosaic proteins consisting of about 65 different domains (Bork, 2000). It has been suggested that they arose by exon shuffling (Patthy, 1996), a good example of which could be the mammalian nidogen-1. The earliest nidogen is seen in ascidians and contains three epidermal-growth-factor (EGF)-like motifs, three thyroglobulin-like motifs and five LDL-receptor YWTD domains (Nakae et al., 1993). Through evolution three extra cysteine-rich epidermal-growth-factor (EGF)-like motifs have been added by exon shuffling and one thyroglobulin-like motif was achieved by combination of three ancestral ones. Further highly conserved homologs of nidogen are observed in fruit flies, nematodes and sea squirt (Mayer et al., 1998). Another example where gene duplication appears to have occurred is the laminin protein family, mainly present in basement membranes. Laminins are heterotrimeric glycoproteins consisting of three genetically different chains. Today eleven distinct chains and fifteen different laminin isoforms are known in mammals. Identification of two laminin chains in *Drosophila* (Fessler et al., 1987; Martin et al., 1999), four laminin chains (Hutter et al., 2000) and the laminin-related netrins (Wadsworth et al., 1996) in *C.elegans* strongly imply the existence of a single ancestral laminin chain which has duplicated further within the vertebrate lineage.

1.2. Basement membrane structure and function

Basement membranes are 40 to 120nm thick sheets of extracellular matrix found throughout the body. They line the basolateral membrane of all epithelial and endothelial cells, separate them from the underlying connective tissue and surround nerve, muscle and fat cells. Basement membranes may interpose between endothelial and epithelial cell sheets as in the lung alveoli and kidney glomeruli. In the kidney, the basement membrane functions as a porous filter, preventing the passage of macromolecules from blood into urine. Basement membranes beneath epithelia prevent the contact between fibroblasts and epithelial cells, but allow the passage of macrophages, lymphocytes or nerve processes. In addition, basement membranes play an important role during development, binding growth factors and hormones which influence cell metabolism, cell growth, cell polarisation, and differentiation (Streuli et al., 1991; Schuger et al., 1997). The ability of certain basement membrane molecules to interact with cell surface receptors allows it to direct cell migration during development or to help to reconstruct original tissue architecture (e.g. guidance of regenerating motor nerve terminals to neuromuscular junctions) after tissue injury. All these different basement membrane functions imply tissue- and time-specific expression of its components from embryogenesis to adulthood. Some of these basement membrane molecules will be discussed below in detail.

1.3. Basement membrane components

1.3.1. Laminins

1.3.1.1. Laminin isoforms

The laminins are large (600-800kD), cruciform glycoproteins (Tunggal et al., 2000) consisting of three genetically different chains (α, β and γ) which share a common domain structure (figure 1.1). The short arms formed by the amino-terminal regions of the β and γ chains contain the domains III and IV instead of domains IIIa/b and IVa/b found in the α chains (Sasaki and Yamada, 1987; Sasaki et al., 1987, 1988). These biologically active domains are separated by flexible rows of EGF-like repeats, the LE motifs, which are stabilised by disulfide bonds. The long arm results from oligomerisation through a triple helix of

domains I and II of the α, β and γ chain. At the carboxy-terminal end of the α chains the large G domain is formed by folding of the five tandemly arranged subdomains LG1-LG5 into separate globes.

Five α-, three β- and three γ- chains have been identified in mammals which could in theory be combined to give 45 cruciform heterotrimers. However due to additional assembly restrictions (e.g. γ2 is never seen connected with β1) only 15 laminin isoforms have been observed although more forms may well exist (Colognato and Yurchenco, 2000; Libby et al., 2000). Studies on the assembly of laminin chains indicate that certain sites within the carboxy-terminal α-helical region of the long arm are important for chain-specific assembly (Utani et al., 1994). Little is known about the mechanism of chain secretion *in vivo*, but cell culture studies demonstrate that the α chain can be secreted as a single subunit while β and γ can not (Yurchenco et al., 1997).

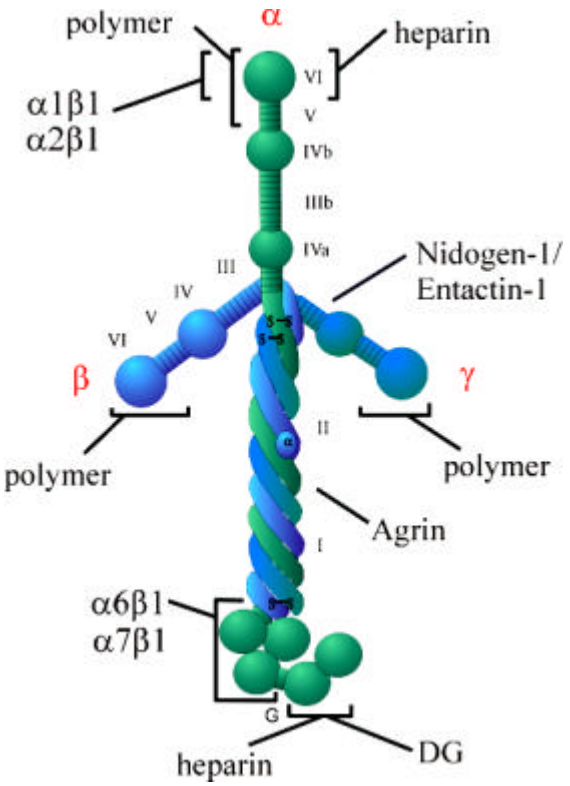


Figure 1.1: Schematic structure of the laminin molecule comprising an α, β and γ chain. The molecule has a cruciform shape (Engel et al., 1981) with three short amino-terminal arms which are involved in polymerisation. The long carboxy-terminal arm consists of domain I and II of all three chains and forms a coiled-coil α-helix (Paulsson et al., 1985; Beck et al., 1993) which is terminated by a globular carboxy-terminal domain (G) contributed by the α chain only. The three-stranded coiled-coil domain is required for high-affinity binding to agrin (Kammerer et al., 1999). α1β1, α2β1, α6β1, and α7β1 indicate integrin binding sites. Other binding partners indicated are dystroglycan (DG), the polysaccharide heparin and nidogen-1/entactin-1.

An overall picture of the time- and tissue-specific deposition of laminin isoforms does not exist at the moment, because immunohistology and *in situ* hybridisations can only describe the distribution pattern of single laminin chains but not of the whole trimer. Still, three classes of epithelial laminins containing either the α1, α3 or α5 chain and the group of endothelial or mesenchymal laminins comprising the α2 chain or the α4 chain can be distinguished (see table 1). So laminins-1, -3, -5, -6, -7, -10 and -11 underly epithelial structures while laminins-2, -4 and -12 are observed in the placenta (Paulsson et al., 1991), in basement membranes surrounding skeletal and cardiac muscles (Leivo and Engvall, 1988; Paulsson et al., 1991) and peripheral nerves (Uziyel et al., 2000). Laminins-8 and -9 are found in endothelial basement membranes of certain blood vessels, e.g. aorta (Frieser et al., 1997). The α4 chain is also expressed by skeletal, cardiac muscle (Lefebvre et al., 1999; Liu and Mayne, 1996) as well as fat cells (Niimi et al., 1997a). Recent data indicate the existence of two additional isoforms, laminin-14 and -15, present in the retinal matrix (Libby et al., 2000). The ongoing identification of new laminin isoforms increases basement membrane complexity not only in tissue-specific composition, but also during development and repair (Erickson and Couchman, 2000).

Name	Chain composition	References
Laminin-1	α1β1γ1	Timpl et al., 1979
Laminin-2	α2β1γ1	Ehrig et al., 1990
Laminin-3	α1β2γ1	Hunter et al., 1989a
Laminin-4	α2β2γ1	Sanes et al., 1990
Laminin-5	α3β3γ2	Rousselle et al., 1991
Laminin-6	α3β1γ1	Marinkovich et al., 1992
Laminin-7	α3β2γ1	Champliaud et al., 1996
Laminin-8	α4β1γ1	Miner et al. 1997
Laminin-9	α4β2γ1	Miner et al., 1997
Laminin-10	α5β1γ1	Miner et al., 1997
Laminin-11	α5β2γ1	Miner et al., 1997
Laminin-12	α2β1γ3	Koch et al., 1999
Laminin-13	α3β2γ3	*
Laminin-14	α4β2γ3	Libby et al., 2000
Laminin-15	α5β2γ3	Libby et al., 2000

* not yet biochemically demonstrated

Table 1. Nomenclature of laminin isoforms (modified from Tunggal et al., 2000)

Basement membrane composition becomes even more complex when considering isoform expression changes during embryogenesis. Such switches can occur very rapidly as in the conversion of mesenchyme to epithelium in the developing kidney (Ekblom et al., 1998; Miner and Li, 2000; Pedrosa-Domellof et al., 2000). The first sign of conversion into epithelium *in vitro* is the condensation of mesenchyme, followed by tubulogenesis with formation of an underlying basement membrane and cell polarisation. Formation of distinct tubules and cell polarisation correspond to the S-shaped stage of *in vivo* development. Studies of α chain expression during this process revealed that the α4 chain is transiently expressed in condensated mesenchyme, followed by α1 and α5 chain expression in S-shaped tubules and their elongations, respectively.

Laminin-1 is the major laminin essential for early embryogenesis (Dziadek and Timpl, 1985; Leivo et al., 1980; Cooper and MacQueen, 1983). The deletion of the LAMC1 gene encoding the γ1 chain results in absence of laminin-1 and embryonic lethality at day 5.5 *post conceptum* (Smyth et al., 1999). In its long arm structure (Aumailley et al., 1987) laminin-1 possesses cell-binding domains as well as sites for cell adhesion and stimulation of neurite-like outgrowth (Powell et al., 2000; Weston et al., 2000). The stimulatory effect of laminin on neurons is further supported by experimental data which demonstrated that laminin in neuron-extracellular matrix interaction prevents hippocampal cell death (Chen and Strickland, 1997). In addition to its growth promoting activities laminin-1 was shown to modify the behavior of growth cones in *Xenopus* (Hoepker et al., 1999) possibly via induction of microtubular bundling in the growth cone of axons (Tang and Goldberg, 2000). Our understanding of laminin-1 as a basement membrane molecule specifically directing cell components is further supported by the discovery that it can organise acetylcholine receptors into clusters during synaptogenesis at the neuromuscular junction (Sugiyama et al., 1997).

1.3.1.2. Laminin receptors

There appear to be two major sets of laminin receptors comprising α-dystroglycan and the integrins. The major binding sites for both receptors on the laminin molecule are located within the five subdomains of the G domain (Hohenester et al., 1999; Talts et al., 2000), however the ones for integrin are believed to also require the adjacent coiled-coil region consisting of the α1, β1 and γ1 chain for proper folding (Aumailley et al., 1987; Goodman et al., 1987; Tisi et al., 2000).

α-Dystroglycan is part of a large transmembrane complex that plays an important role in muscle biology (Ervasti and Campbell, 1993; Hemler et al., 1999). It links laminin-2 to the myofiber cytoskeleton via indirect binding to dystrophin and blocking of this interaction with antibodies induces a dystrophic phenotype (Brown et al., 1999). Dystroglycan is expressed in developing and adult tissues by epithelial and neuronal cells which contact basement membranes (Matsumura et al., 1993; Durbeej et al., 1998) and is involved in kidney epithelial morphogenesis (Durbeej et al., 1995). The absence of dystroglycan leads to an embryonic lethality with structural and functional defects of one of the earliest basement membranes, the Reichert's membrane (Williamson et al., 1997), and analysis of dystroglycan null embryoid bodies shows disrupted basement membranes (Henry and Campbell, 1998).

Integrins are heterodimeric transmembrane molecules containing an α and a β subunit and are involved in signal transfer between the extracellular matrix and the cell interior (Hynes, 1992; Clark and Brugge, 1995). Only a subset of all known integrins, the α7, α6 and α3 integrins seem to specifically interact with the G domain (Belkin and Stepp, 2000). α6β4 integrin is found in hemidesmosomes whereas α6β1 integrins are often localised in focal contacts (Sonnenberg, 1992). For the integrins α1β1 and α2β1 the binding site was identified on the short arm of the laminin-1 molecule (Languino et al., 1989; Goodman et al, 1991), but it remains controversial if corresponding binding sites exist on other laminin isoforms (Pfaff et al., 1994; Colognato et al., 1997). α7β1 integrin is also laminin-specific and expressed highly in myoblasts (Von der Mark, 1991) together with α-dystroglycan. β1 integrin null embryoid bodies exhibit failures of basement membrane formation (Faessler and Meyer, 1995; Aumailley et al., 2000) a similar phenotype can also be observed in skin epithelium after conditional targeting of the β1 integrin gene (Raghavan et al., 2000). Similar phenotypes can be caused by mutations in the α3, α6, β4 integrin subunits (George-Labouesse et al., 1996; Niessen et al., 1996; DiPersio et al., 1997) and in syndecan-2, a heparan sulphate proteoglycan (Klass et al., 2000).

Major functions of laminin receptors are in cell attachment and in providing signals which are transferred to intracellular signal cascades. However, many results also indicate that laminin receptors facilitate *in situ* laminin deposition on the cell surface as it has been demonstrated for α-dystroglycan (Montanaro et al., 1999). Laminin polymerisation, a requirement for basement membrane formation in turn induces redistribution of dystroglycan, α7β1 integrin and the cortical cytoskeleton of muscle cells (Colognato et al., 1999).

1.3.1.3. Laminin polymerisation

The three-arm interaction model of laminin polymerisation proposes that laminin molecules self-assemble above a critical concentration of 0.1µM through reciprocal, calcium-dependent binding at their amino-terminal short arms leading to the formation of large polymers (Yurchenco et al., 1985; Paulsson et al., 1988; Ancsin and Kisilevsky, 1996). This interaction is conformation-dependent (Yurchenco and Cheng, 1994) and leaves the long laminin arms free for cell contacts. It is unknown whether *in vivo* laminin networks also contain heterogenous isoforms, but theoretically laminin-1 and -2 could co-polymerise (Cheng et al., 1997). Studies on the influence of lipid bilayers on laminin polymerisation suggest that plasma membranes could enhance aggregation of laminin molecules. The concentration critical for assembly was believed to be lowered by binding of laminins to lipid bilayers (Kalb and Engel, 1991). Although the association with cell surface receptors rather than exposed lipids was considered as a possible mechanism of enhancing polymerisation, recent *in vitro* evidence demonstrates that simple acidification of laminin monomer solution induces polymerisation (Freire and Coelho-Sampaio, 2000).

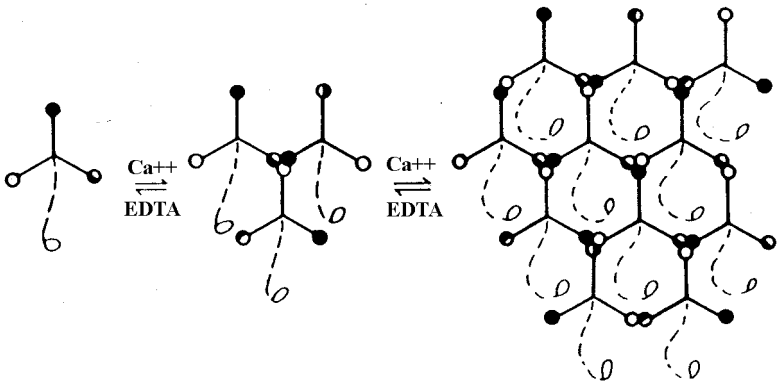


Figure 1.2: Three-arm interaction model of laminin polymerisation (Yurchenco and Cheng, 1994). The short arms of the α, β and γ chain (continuous lines) are represented with globules at their amino-terminal ends. These can interact with each other in a calcium-dependent fashion *in vitro*. In this model the long arm (broken line) is free for interactions with cells.

1.3.2. Nidogens

Nidogen-1, a sulfated 150kD glycoprotein, was first isolated from F9 mouse terato-carcinoma cells and at that time called entactin (Hogan et al., 1980; Carlin et al., 1981). Differentiation of F9 cells with retinoic acid and dibutyryl cAMP induced synthesis of laminin-1 and nidogen-1. As shown by co-precipitation laminin-1 and nidogen-1 were in a complex that could be dissociated with sodium dodecyl sulfate. At the same time a protein which later proved to be identical with entactin was identified as a 80kD fragment in basement membranes of the Engelbreth-Holm-Swarm tumor and named nidogen (Timpl et al., 1983). The full-length, 150kD form of nidogen-1 could be purified with laminin-1 by EDTA extraction (Dziadek et al., 1985; Paulsson et al., 1987). Analysis of large amounts of the intact laminin-1/nidogen-1 complex purified from the extracellular matrix showed that the two proteins occur in an equimolar ratio (Paulsson et al., 1987). Data obtained from electron microscopy confirmed the interaction between laminin-1 and nidogen-1 (Paulsson et al., 1987) and binding studies with proteolytic fragments of the laminin-1/nidogen-1 complex indicated that the carboxy-terminus of nidogen-1 is responsible for binding laminin-1 (Mann et al., 1988). Electron microscopy of recombinant nidogen-1 showed three globular domains, G1 to G3, with G1 and G2 connected by a flexible link and G2 to G3 by a rod-like segment containing a series of EGF-like repeats (Mann et al., 1989; Durkin et al., 1995). The strong binding to laminin-1 with a K_D of 0.5nM was found to be mediated by the C-terminal G3 domain (Fox et al., 1991).

The nidogen-binding site on laminin-1 was localised to LE module 4 within domain III of the laminin γ1 chain (Gerl et al., 1991; Mayer et al., 1993; Poeschl et al., 1996). This part of the γ1 chain could be crystallised (Stetefeld et al., 1996) and structurally analysed (Baumgartner et al., 1996).

A typical EGF-like LE module forms a rigid structure. The rigidity is given by eight non-contiguously arranged cysteines which interact via disulfide bridges (figure 1.3). This leads to formation of four loops as observed in the nidogen-binding site module γ1III4 between the 1st and 3rd cysteine, the 2nd and 4th, and two further between the 5th and 6th and the 7th and 8th. The heptapeptide sequence Asn-Ile-Asp-Pro-Asn-Ala-Val located within the first loop was found to be crucial for binding to nidogen-1 (Poeschl et al., 1994) in combination with hydrogen bonding between the first and third loop providing binding struc-

tures and conformational stability (Stetefeld et al., 1996). Exchange of the central Asn and Val by Ser leads to a 100.000 fold lower binding affinity. Such an exchange has occurred in the corresponding domain γ2III4 of laminin-5 (α3β3γ2) despite the high overall sequence identity between the γ1III4 and γ2III4 motifs (Mayer et al., 1995) and laminin-5 can accordingly not bind nidogen-1.

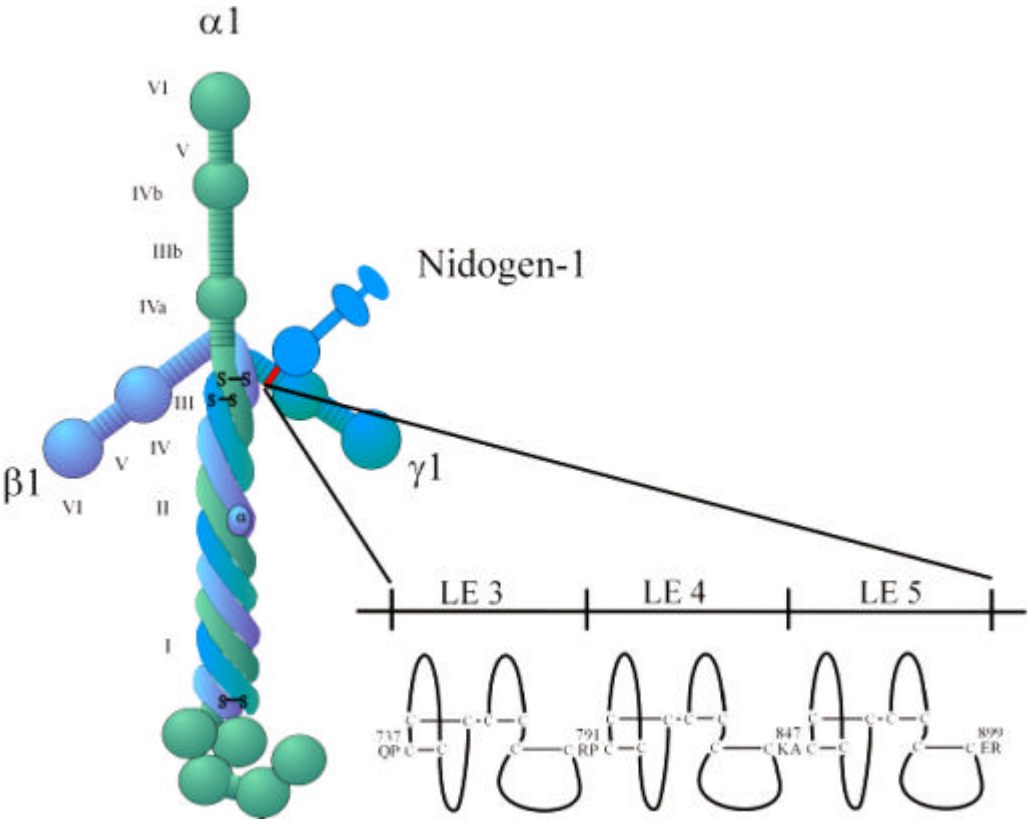


Figure 1.3: The interaction between laminin-1 and nidogen-1. Nidogen-1 binds with its globular domain G 3 to the short arm of the γ1 chain. The nidogen-binding site, LE module 4 of the γ1 chain, is shown enlarged together with the neighbouring LE mocules. LE modules contain eight cysteines residues which upon disulfide bonding form loops similar to those of EGF (Cooke et al., 1987).

In vitro binding assays indicate the ability of nidogen-1 to mediate complex formation between laminin and collagen type IV and also between laminin-1 and the proteoglycan perlecan (Aumailley et al., 1993; Hopf et al., 1999). Nidogen-1 can further bind fibulins, extracellular matrix proteins composed of multiple arrays of epidermal growth-factor like modules similar to the LE modules of laminin (Adam et al., 1997). If the interaction between laminin and nidogen-1 is blocked by antibodies raised against the nidogen-binding site, *in vitro* epithelial development in organ cultures of embryonic kidney and lung gets disrupted (Ekblom et al., 1994). Similar *in vitro* experiments performed with submandibular gland

organ cultures lead to disruption of the basement membrane between epithelium and mesenchyme and reduced branching epithelial morphogenesis (Kadoya et al., 1997). These results suggest that the formation of the laminin/nidogen-1 complex may be a key event during epithelial development. Detailed analysis of the sites of laminin-1 and nidogen-1 gene expression by *in situ* hybridisation (Dong and Chung, 1991; Thomas and Dziadek, 1993; Ekblom et al., 1994, Fleischmajer et al., 1995) have revealed that laminin-1 is predominantly produced by epithelial cells while nidogen-1 is secreted by mesenchymal cells. The binding of mesenchymal nidogen-1 to epithelial laminin-1 is believed to occur at the interface between epithelial and mesenchymal tissues where basement membrane formation could be dependent on complex formation between laminin-1 and nidogen-1 (Dziadek et al., 1995).

Additional evidence for nidogen-1 production by mesenchymal cells was obtained in coculture experiments with rat mesenchymal peritubular and epithelial-like Sertoli cells (Konrad et al., 2000). Even though nidogen-1 was transcribed in peritubular cells and Sertoli cells, its mRNA was only translated by peritubular cells. Antibody perturbation experiments could further show that nidogen-1 is required for cell adhesion of peritubular cells. The finding that nidogen-1 is secreted by mesenchymal cells was also confirmed using a culture microecosystem of mammary epithelium where nidogen-1 is produced by mesenchymal cells but deposited between epithelial cells. Analysis of this *in vitro* system initially designed to investigate the role of nidogen-1 in gene regulation showed that nidogen-1 can regulate β-casein expression in cooperation with laminin-1 (Pujuguet et al., 2000).

The susceptibility of uncomplexed nidogen-1 to proteolytic degradation (Dziadek et al., 1988) allows speculation about such degradation playing a role during embryogenesis, where basement membrane assembly and degradation are tightly regulated (Dziadek, 1995). The growing knowledge about the action of matrix metalloproteases (Werb, 1997; Giannelli et al., 1997) will lead to a better understanding of the mechanisms behind this balance which is crucial for normal growth, morphogenesis and tissue repair.

Nidogen-2 was recently isolated as osteonidogen or entactin-2 from an osteoblast-like cell line and shown to have 27.4% identity to nidogen-1 on the amino acid level (Kimura et al., 1998). Recombinant nidogen-2, a highly glycosylated 200kD protein, has a shape similar to nidogen-1. Electron microscopy shows that it also consists of three globular domains connected by two threads although it is somewhat different in length (figure 1.4). Immuno-

fluorescence and northern blots revealed coexpression and colocalisation of both nidogens in vessel walls and other basement membrane zones, but differences in heart and skeletal muscle (Kohfeldt et al., 1998). Nidogen-2 can bind to the nidogen-binding site γ1III4 of the laminin γ1 chain but with a 100 to 1000 fold lower affinity than nidogen-1 and interacts with a second binding-site of laminin-1 unrelated to the γ1III4 module. Deletion of LE module 4 abolishes binding of both nidogens to the recombinant γ1III3-5 fragment. In addition, nidogen-2 binds type IV and type I collagen and perlecan. However it does not interact with fibulin-1 or -2 like nidogen-1 (Kohfeldt et al., 1998).

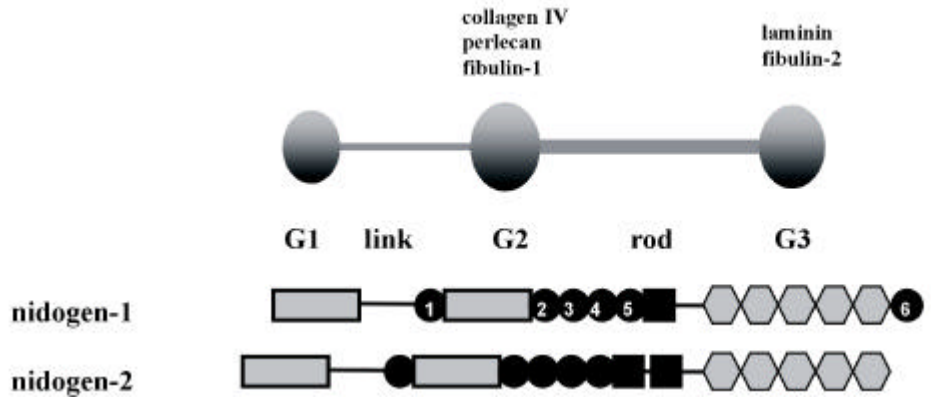


Figure 1.4: The domain organisation and modular structures of nidogen-1 and nidogen-2. Both nidogens comprise three globular domains. G1 and G2 are joined by a link region while G2 and G3 are connected by a rod-like structure. Different basement membrane proteins binding to G2 and G3 domain of nidogen-1 are indicated. Legend: dark circles represent epidermal growth factor (EGF) like motifs, which are numbered in nidogen-1, dark boxes represent thyroglobulin (TG) like motifs and hexagons represent low density lipoprotein (LDL) receptor YWTD motifs (partially taken from Murshed, 2001).

To understand the *in vivo* role of the nidogens in basement membranes, the mouse and worm model were studied. The lack of nidogen-1 in mice does not affect basement membrane formation, instead nidogen-1 -/- mice develop seizures and other neurological defects later in life (Murshed et al., 2000). This phenotype could be explained by the existence of the structurally related nidogen-2/entactin-2 (Kohfeldt et al., 1998; Kimura et al., 1998) and a possible compensatory effect of nidogen-2 in basement membrane assembly of nidogen-1 deficient mice. Although immunostaining for nidogen-2 is stronger in distinct basement membranes, it does not appear to be transcriptionally upregulated in nidogen-1 -/- mice. Nidogen-2 null mutant mice also appear normal. The production of nidogen-1/-2 double null-mutant mice is currently underway (Smyth, personal communication). This will help to explain the importance of nidogen-1 and -2 and their contribution to basement membrane stability. Recent results obtained from loss of function experiments in *C. elegans* support the neurological

phenotype found in nidogen-1 -/- mice. Introducing a stop codon into the homologous *C. elegans* nidogen gene causes irregular neuronal migration (Kim et al., 2000). Deletions in NID-1 however do not affect type IV collagen assembly into basement membranes (Kang and Kramer, 2000).

1.3.3. Type IV Collagen

The collagens are a glycoprotein family with at least 19 genetically distinct types (Prockop and Kivirikko, 1995) which can be divided into fibril-forming and non-fibrillar molecules. Typically, collagens consist of a ropelike superhelix comprising three polypeptide α chains wound round each other. These α chains contain a series of Gly-X-Y repeats. In 20-22% of all triplets the positions X and Y are occupied by proline and hydroxyproline, respectively. The non-fibrillar collagens can form three-dimensional networks (type IV), beaded filaments (type VI), anti-parallel dimers (type VII) or a hexagonal lattice (type VIII).

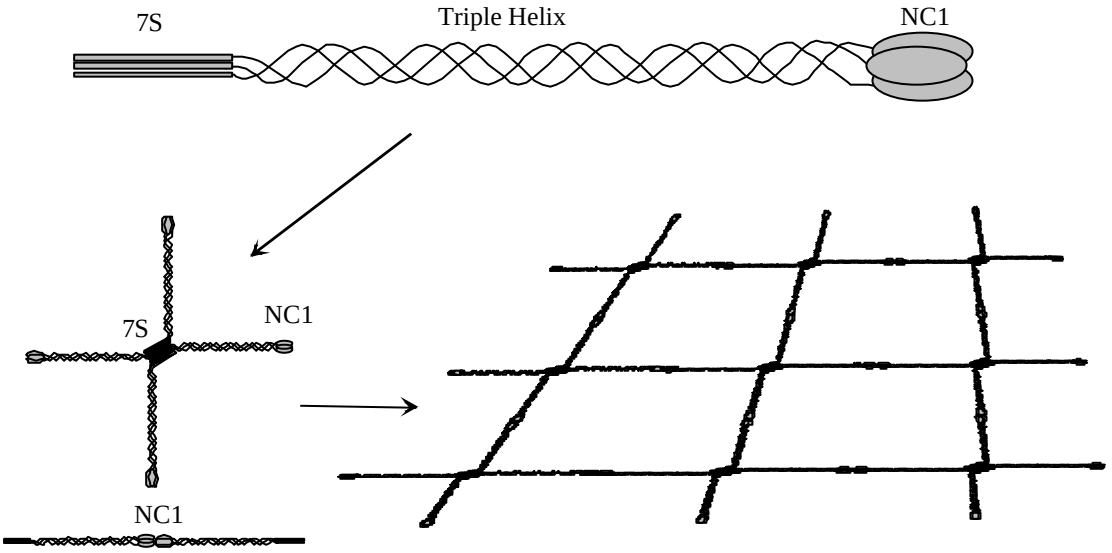


Figure 1.5: Schematic representation of the type IV collagen network. Three α chains interact and form a triple helix with amino-terminal 7S and carboxy-terminal NC1 domains. Covalent interactions between four 7S and two NC1 domains and additional lateral interactions (not shown) result in a lattice which provides mechanical strength to the basement membrane (taken from Tunggal, 2000).

Type IV collagen contains a carboxy-terminal noncollagenous domain (NC-1), a 350nm long triple-helical domain and an amino-terminal 7S domain. It is only present in basement membranes, where it can self-assemble into a network via anti-parallel interactions

between the 7S domains of four molecules (Risteli et al., 1980; Glanville et al., 1985; Tsilibary and Charonis, 1986), the interaction of two molecules at their carboxy-terminal NC1 domains and by lateral aggregation (Timpl et al., 1981; Yurchenco and Furthmayr, 1984). This network (figure 1.5) provides structural support to the basement membrane, facilitates assembly of laminin, nidogen-1/entactin-1 and perlecan (Laurie et al, 1986) and functions as a size-selective filter unit.

Type IV collagen α chains are encoded by six genes, COL4A1-COL4A6 which are arranged in pairwise head-to-head organisation (Soininen et al., 1988; Mariyama et al., 1992; Zhou et al., 1994). Since bidirectional regulatory elements have been identified between each pair, coordinate transcription and expression of COL4A1-COL4A2, COL4A3-COL4A4 and COL4A5-COL4A6 was suggested. This assumption was confirmed by the finding that the most abundant type IV collagen with ubiquitous expression in basement membranes (Hudson et al., 1993) has the chain composition $[\alpha1(IV)]_2\alpha2$ (IV). In addition, colocalisation of the α3 and α4 chains (Kleppel et al., 1989; Miner and Sanes, 1994) as well as the α5 and α6 chains could be demonstrated, although the α5 chain is also expressed without the α6 chain in the glomerular basement membrane (Peissel et al., 1995).

Mutations in the COL4A3, COL4A4 or COL4A5 gene result in a progressive hereditary disease of the glomerular basement membrane, Alport's syndrome (Kashtan and Michael, 1993). In Alport's patients the basement membranes of the kidneys are disorganised and fragile and less resistant to the high hydrostatic pressure. Mouse models which either lack the α3(IV) chain (Cosgrove et al, 1996; Miner and Sanes, 1996) or both the α3(IV) and the α4(IV) chain undergo fibrosis leading to a glomerulonephrosis. This is possibly due to the failure in switching from α1, α2 expression to α3, α4 and α5 expression which occurs during glomerular development (Lu et al., 1999).

1.3.4. Perlecan, a heparan sulphate proteoglycan

Proteoglycans are very heterogenous in composition and are present in the extracellular matrix, on cell surfaces (Bernfield et al., 1999) or in intracellular granules (Burditt et al., 1985). They consist of a protein core and glycosaminoglycan (GAG) chains (Prydz and Dalen, 2000). These long, unbranched, strongly anionic polysaccharide chains can bind

cations as well as H_2O. Thereby hydrated GAG gels are able to resist pressure changes in tissues, a property that is particularly important for joint function. *In vitro* GAGs can bind growth factors, matrix components, enzymes, enzyme inhibitors and cell adhesion molecules. The core proteins themselves may show particular biological activities (Iozzo, 1998). Insights gained from *in vivo* studies in *Drosophila* and mice imply specific functions for heparan sulphate proteoglycans in cell differentiation and morphogenesis (Perrimon and Bernfield, 2000).

In mammals three proteoglycans are found in basement membranes: Perlecan (Murdoch et al.,1994), agrin (Groffen et al., 1998) and bamacan (Couchman et al., 1996). Perlecan, the most widespread and highly expressed proteoglycan of basement membranes and cartilage, comprises a 470kD core protein with a beads-on-a-string-like appearance and often carries three heparan sulphate side chains at its amino-terminal end (Costell et al., 1996; Groffen et al., 1996; Schulze et al., 1996; Friedrich et al., 1999). Domain III of its protein core interacts with cell surfaces (Chakravati et al., 1995, Peng et al., 1998; Hohenester et al., 1999; Talts et al., 1999) while domain IV binds nidogens, the laminin-1/nidogen-1 complex (Battaglia et al., 1992), type IV collagen (Laurie et al., 1986, Villar et al., 1999), fibronectin, fibulin-2, and heparin (Hopf et al., 1999).

Perlecan occurs in preimplantation embryos prior to basement membrane formation (Dziadek et al., 1985; Smith et al., 1997) and inside the blastocyst on the outer surface of trophectoderm cells (Carson et al., 1993). Interestingly, the distribution pattern of perlecan is similar to that of fibroblast growth factor (FGF)-2 in various basement membranes of the mouse embryo (Friedl et al., 1997) which led to speculations about an involvement of perlecan in growth control (Klein et al., 1995; Weiser et al., 1997). Perlecan expression was also demonstrated on the surface of vascular endothelial cells, possibly concentrating thromospondin 1 (Vischer et al., 1997), and in mesenchymal tissues like cartilage where it persists into adulthood (Handler et al., 1997). Mice lacking perlecan show defects in cartilage and cephalic development (Arikawa-Hirasawa et al., 1999) and while basement membranes form they become disrupted with increasing mechanical stress (Costell et al., 1999) which suggests a role of perlecan in regulating basement membrane integrity and permeability (Murdock et al., 1993; Gauer et al., 1996; Groffen et al., 1997; Groffen et al., 1999).

1.4. Basement membrane assembly and structure

Although many different basement membrane components and numerous isoforms of these have been identified, our understanding how molecules become deposited, locally concentrated and organised into basement membranes after secretion is comparatively poor. *In vitro* experiments demonstrate that dermal fibroblasts can produce type IV collagen, perlecan and nidogen, but they need keratinocyte integrins to bind type IV collagen in order to initiate basement membrane formation (Fleischmajer et al., 1998). Keratinocytes are also necessary for relocalisation of nidogen, which is of dermal origin during early basement membrane formation of *in vitro* skin models (Fleischmajer et al., 1995). Retinal basement membrane instead seems to require collagens for reassembly of neuroepithelia (Halfter et al., 2000) while alveolar epithelial cells can synthesise all major basement membrane components but still need exogenous laminin-1 for complete basement membrane assembly (Furuyama and Mochitate, 2000).

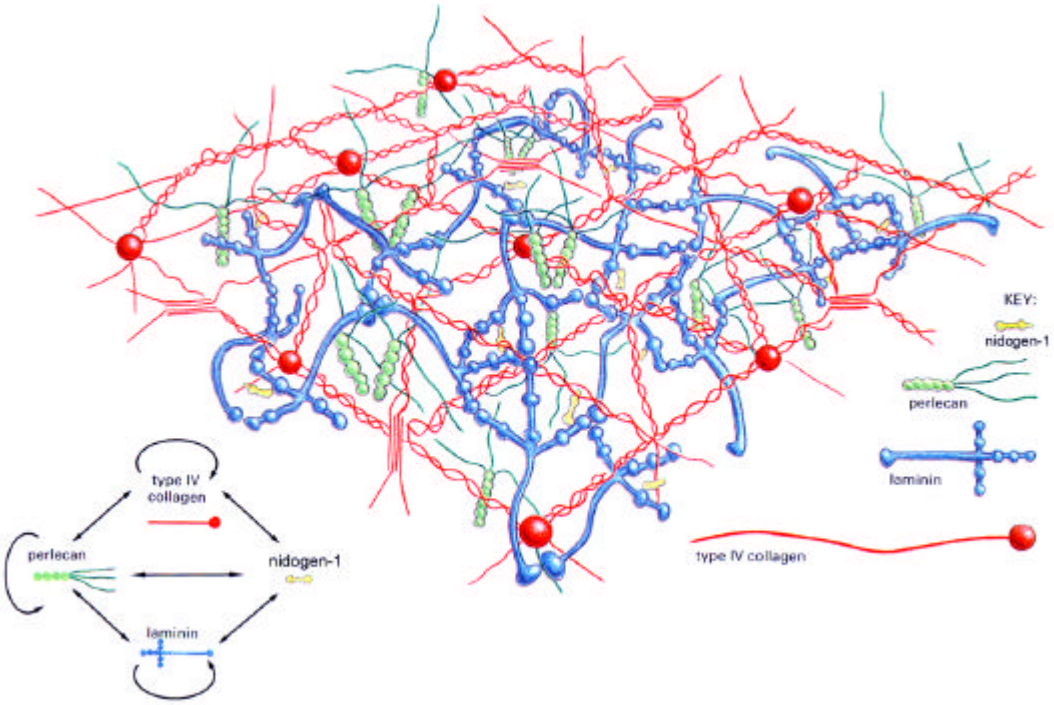


Figure 1.6: Molecular model of the basement membrane based on *in vitro* data (modified from Yurchenco and Schittny, 1990).The two major basement membrane networks are formed by laminin (blue) and type IV collagen (red) and are bridged by nidogen-1 (yellow). Perlecan (green) is a heparan sulphate proteoglycan which can interact with itself, type IV collagen and laminin.

Most of these studies on basement membrane formation are descriptive or *in vitro* experiments. In an *in vivo* approach the deletion of nidogen-binding module γ1III4 interferes with the formation of the laminin-1/nidogen-1 complex (Mayer et al., 1998). Although embryonic stem cells homozygous for this defect secrete mutant laminin-1 which fails to associate with nidogen-1, they are still able to form embryoid bodies with a differentiation pattern similar to wild type. However complex the mechanisms of basement membrane formation might be, the self assembly of laminin and type IV collagen (described above) are still considered the main principles of basement membrane formation and the proposed function of nidogen-1 as the linker module between the type IV collagen and laminin networks has dominated the model of basement membrane structure (Aumailley et al., 1989; figure 1.6).

1.5. Embryoid bodies

Embryoid bodies are cellular aggregates derived from either embryonic stem cells or embryonic carcinoma cells (Martin, 1980), which resemble early stages of mouse development (figure 1.7). The pluripotent F9 cells (Bernstine et al., 1973; Alonso et al., 1991) have a limited differentiation repertoire forming endoderm-like structures which develop only upon treatment with e.g. retinoic acid (Strickland and Mahdavi, 1978) or dibutyryl cyclic AMP (cAMP; Hogan et al., 1983). In F9 derived embryoid bodies treated with retinoic acid cells are found on the outer surface which are morphologically similar to visceral endoderm while F9 monolayers treated with retinoic acid and dibutyryl cAMP instead differentiate into parietal endoderm (Strickland et al., 1980; Damjanov et al., 1994). Parietal and visceral endoderm are two distinct populations of extra-embryonic endoderm found in the normal mouse embryo shortly after implantation (Hogan et al., 1994).

Since embryonic carcinoma cell lines can participate in the formation of chimeric mice, they were an important tool for the first gene transfer experiments in mouse before it was possible to establish totipotent embryonic stem cell lines *in vitro* (Martin et al., 1980). Today embryoid bodies grown from F9 cells represent a well studied *in vitro* system of early mouse embryogenesis which can help to understand the nature of endodermal and epithelial differentiation (Coucouvanis and Martin, 1995). For example, it has been shown that Indian

hedgehog, which is upregulated during extra-embryonic endoderm differentiation in F9 embryoid bodies is also increased in 6.5 day old mouse embryos (Becker et al., 1997). Similarly, α-Fetoprotein is synthesised by visceral endoderm (Hogan et al., 1981; Grover et al., 1983) in F9 embryoid bodies and it is restricted to the visceral endoderm during post-implantation development in mouse (Dziadek and Adamson, 1978). Differentiation of F9 cells into parietal or visceral endoderm upon treatment with retinoic acid and cAMP or retinoic acid alone, respectively, is accompanied by increasing synthesis of basement membrane proteins. At the transcriptional level a coordinate increase of the laminin α1, β1 and γ1 chains and the type IV collagen α1 chain (Durkin et al., 1986; Kleinman et al., 1987) has been demonstrated, whereas at the protein level the synthesis of the laminin β1 and γ1 chains is first induced followed by the α1 chain of type IV collagen and α-fetoprotein (Rogers et al., 1990). Analysis of the gene regulation of the β1 and γ1 chains revealed that both contain DNA regulatory elements which are activated during F9 induction (Chang et al., 1996; Li and Gudas, 1996). Differentiation into visceral or parietal endoderm leads to a 5-10 fold and 15-20 fold increase in the synthesis of basement membrane proteins (Howe and Solter, 1980; Prehm et al., 1982; Cooper et al., 1983) and in parietal endoderm the production of laminin and nidogen-1 occurs independently. Laminin and nidogen-1 are secreted to the medium and deposited at the cell surface and at cell junctions (Carlin et al., 1983; Chung et al., 1993).

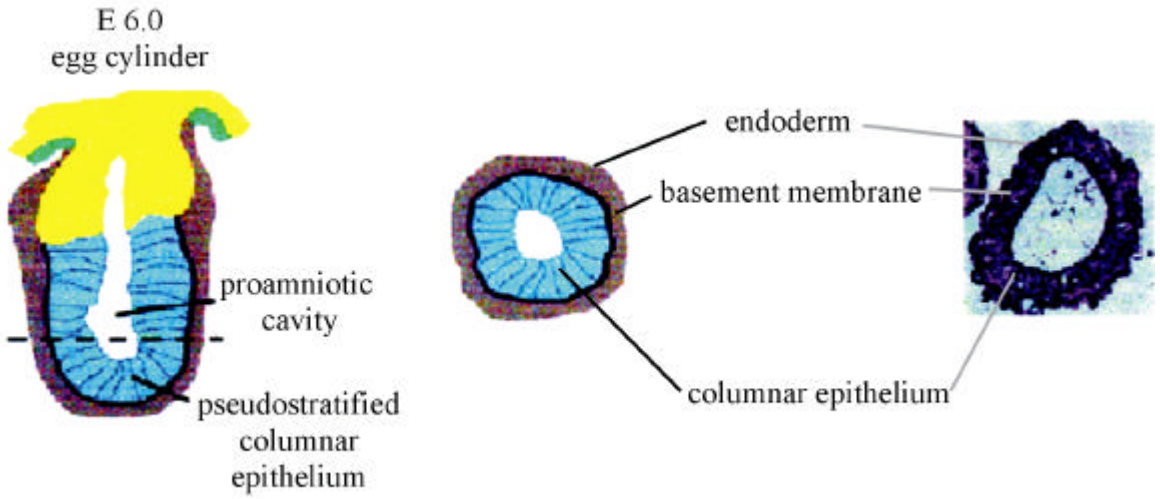


Figure 1.7: Embryoid bodies undergo differentiation processes analogous to early mouse development. On the left a saggital section through a mouse embryo at embryonic day 6.0, when the proamniotic cavity has been formed, is depicted. To the right a cross section (dashed line) through the egg cylinder is shown and compared to an embryoid body after cavitation. (modified from Coucouvanis and Martin, 1995).

1.6. Aim of the present study

The purpose of this study was to analyse the importance of the laminin/nidogen-1 interaction for proper basement membrane formation and stability. The contribution of nidogen-2 to stabilisation of major basement membrane networks is not clear at the moment.

Based on the basement membrane model designed by Yurchenco and Schittny (1990) nidogen-1 links the laminin lattice to the type IV collagen network. In order to test this hypothesis, I planned to disturb the laminin/nidogen-1 interaction by introducing an excess of recombinantly expressed nidogen-binding sites. In theory these additional binding-sites should compete with intrinsic laminin molecules presenting their binding-sites to nidogen molecules.

For time-specific interference with the laminin/nidogen-1 interaction at different stages of basement membrane development inducible expression systems such as the ecdysone-inducible (Invitrogen) or the retroviral tet on/off system (Hofmann et al., 1996) could be applied. It was also decided to use the EF1α promotor for constant protein expression which was previously shown to work well in F9 cells (Niimi and Kitagawa, 1997b). F9 and D3 (Evans and Kaufman, 1981) cells were chosen as they represent an *in vitro* system of basement membrane formation very similar to early stages of mouse development where the basement membrane separates the inner ectodermal cell mass from the outer endodermal epithelium. To rule out that any phenotype might be caused by artefacts due to clonal selection of overexpressing cells, the laminin γ1III3-5 FLAG fusion protein was also added extraneously to developing wild type embryoid bodies. This required the nidogen-binding site to be expressed in 293-EBNA cells using the CMV promotor, affinity-purified and added to differentiating embryoid bodies derived from wild type F9 cells. For analysis of basement membrane formation and stability molecular biological, biochemical, microscopical, and physiological techniques were used and the expression of markers for cell differentiation were determined in the embryoid bodies.

2. Results

2.1. Cloning of nidogen-binding site constructs and controls

All constructs (figure 2.1) were derived by PCR from full-length mouse laminin γ1 cDNA which was obtained by reverse transcription of mouse kidney total RNA. Considering the complex secondary structure of LE 4, which contains the nidogen-binding site, it was expressed together with its neighbouring LE modules 3 and 5 (amino acid position 771-932). Previous work had shown that such a polypeptide folds correctly (Stetefeld et al., 1996) and binds to nidogen-1 in a similar manner to native laminin (Mayer et al., 1993). For control purposes, the construct γ1III3-5mut with the point mutation N802S in LE 4, which decreases binding affinity for nidogen-1 by 46,000 fold (Poeschl et al., 1996) and the construct γ1V1-3 containing the LE modules 1-3 (amino acid position 340-492) of domain V of the γ1 chain were also expressed. To enable secretion of the expressed proteins to the extracellular space and their detection and purification, sequences encoding the the BM40 signal peptide and FLAG tag (Hopp et al., 1988), respectively, were added to the N-terminus of the polypeptides.

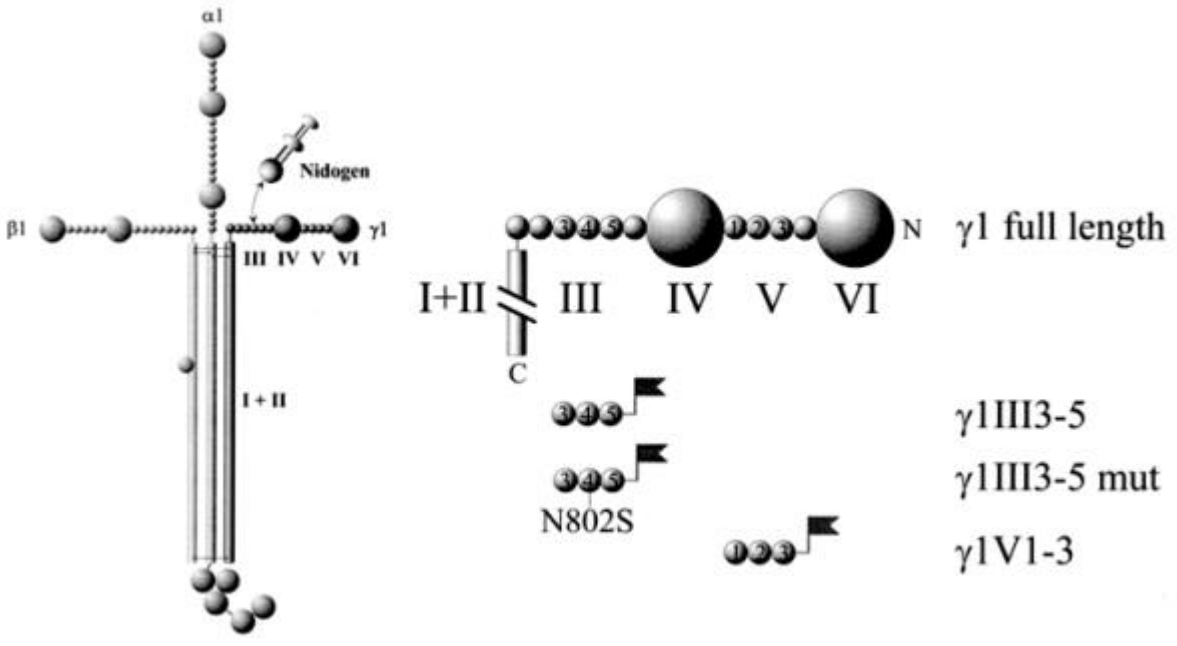


Figure 2.1: Production of nidogen-binding site constructs and controls: The site for interaction between laminin-1 and nidogen-1 has been localised to LE module 4 of domain III of the laminin γ1 chain (Mayer et al., 1993). Three different FLAG fusion proteins (⚑) were constructed and modified with the BM40 signal peptide to ensure secretion to the extracellular space, γ1III3-5 coding for LE modules 3-5, γ1III3-5mut which differs from γ1III3-5 in a point mutation of amino acid 802 from N to S a mutation causing a 46000 fold loss of nidogen binding activity (Poeschl et al., 1996) and γ1V1-3 comprising 3 LE modules of domain V.

Although LE modules 3-5 had earlier been cloned into pCEP-Pu (Kohfeldt et al., 1997) which added the BM40 signal peptide, to insert the FLAG sequence the LE 3-5 coding sequence had to be subcloned into NheI/NotI restricted CMV-NFlag (a pCEP-Pu based vector with the FLAG tag placed 3 prime to the BM40 signal peptide sequence). To obtain LE modules 1-3 a PCR was performed on the γ1 cDNA using the sense primer AAT T**GC TAG C**CC TTC CTT GTG ACT GCA ATG GC and the antisense primer AAT A**GC GGC CGC** CTA GGG TGT GCA GCC CTT AGG containing a **NheI** and **NotI** site, respectively. After digestion the PCR product was cloned into the restriction digested CMV-NFlag plasmid. To mutate amino acid 802 in γ1III3-5 from an asparagine to a serine, the sequence for the FLAG tagged LE modules 3-5 was subcloned into pBluescript KS (+) by HindIII/XhoI digestion. Then site-directed mutagenesis was performed using the Transformer™ Site-Directed Mutagenesis Kit (Clontech Laboratories Inc.) following the manufacturer's instructions with TransSCA (5' GTG ACT GGT GAG GCC TCA ACC AAG TC 3') as the selection primer and the primer N802S (5' GTG TAA CGA CAA TAT TGA CCC CAG CGC GGT TGG C 3') as the mutagenic primer. Constructs were verified by DNA sequencing and correspond to γ1 sequences available in the SWISS-PROT data base (primary accession number P02468).

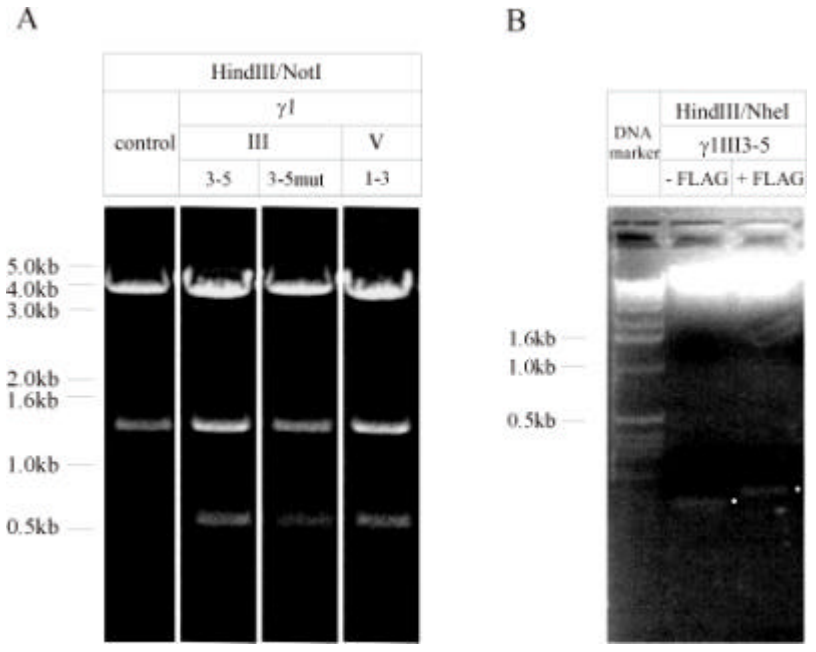


Figure 2.2: Restriction digest on nidogen-binding site constructs and controls cloned into pBKEF-5 (A) and pCEP-Pu (B). A) HindIII/NotI restriction of the empty pBKEF-5 expression vector yields two fragments of 4.0kb and 1.4kb separated on 0.7% agarose gel. Upon insertion of the nidogen-binding site constructs γ1III3-5, γ1III3-5mut and the control region γ1V1-3 an additional 0.6kb band appears. Lane γ1 III3-5mut is derived from a later experiment. B) HindIII/NheI restriction of γ1III3-5 cloned into pCEP-Pu excises the BM40 signal peptide while a slightly shifted fragment coding for the BM40 signal peptide plus the FLAG tag is obtained from γ1III3-5 inserted into CMV-NFlag. Restriction digests were separated on a 1.5% agarose gel and for better comparison the fragments of interest are marked with white dots.

The DNA coding for γ1III3-5mut, γ1III3-5 and γ1V1-3 were excised with HindIII/XhoI from pBluescript KS (+) or CMV-NFlag respectively and inserted into pBKEF-5 linearised with HindIII/SalI. This vector is a fusion plasmid formed of pBK-CMV and pEF BOS (Mizushima and Nagata; 1990) and contains the EF1α promotor which drives protein expression in F9 cells. For expression of the nidogen-1 binding site in 293-EBNA cells (Smyth et al., 2000) γ1III3-5 cloned into CMV-NFlag was used (figure 2.2).

The constructs described above were expected to lead to constitutive protein expression. For inducible expression with the mammalian ecdysone-inducible system (Invitrogen) and the retroviral tet on/off system (Hofmann et al., 1996), γ1III3-5 was also cloned into the expression vectors pIND and pGEM-IRES respectively. To test the ecdysone system, F9 cells were transfected with pINDγ1III3-5 and pVgRXR, the transactivator encoding plasmid. Resistant F9 cells were grown either under differentiating or non-differentiating conditions and protein expression induced with muristerone A, a synthetic analog of the steroid hormone ecdysone. Cell supernatants and extracts were tested for γ1III3-5 expression by immunoblotting but no recombinant protein was detected. Cloning of the γ1III3-5 construct into pGEM-IRES was stopped when control infections with pGEM-IRES lacZ containing viruses showed extremely low efficiency (results not shown).

2.2. g1III3-5 expression in 293-EBNA cells

293-EBNA cells were electroporated with γ1III3-5 CMV-NFlag, selected for puromycin resistance and grown to confluency. Serum-free supernatant was collected, TCA precipitated and tested for expression of the recombinant protein. Polyacrylamide gel electrophoresis showed that the fusion protein was expressed without degradation (figure 2.3). Immunoblotting with the anti-FLAG Bio M2 antibody on the reduced supernatant of transfected 293-EBNA cells identified a single, 25kD band of a FLAG tagged protein. Cells expressing the γ1III3-5 construct were grown in large scale and 500ml supernatant was collected for affinity purification. The supernatant was centrifuged and after overnight dialysis into TBS, pH 7.4 was loaded on to a anti-FLAG M2 agarose affinity column. The column was washed four times with TBS, pH 7.4 to remove unspecifically bound proteins and the FLAG tagged protein was eluted with 100µg/ml FLAG peptide.

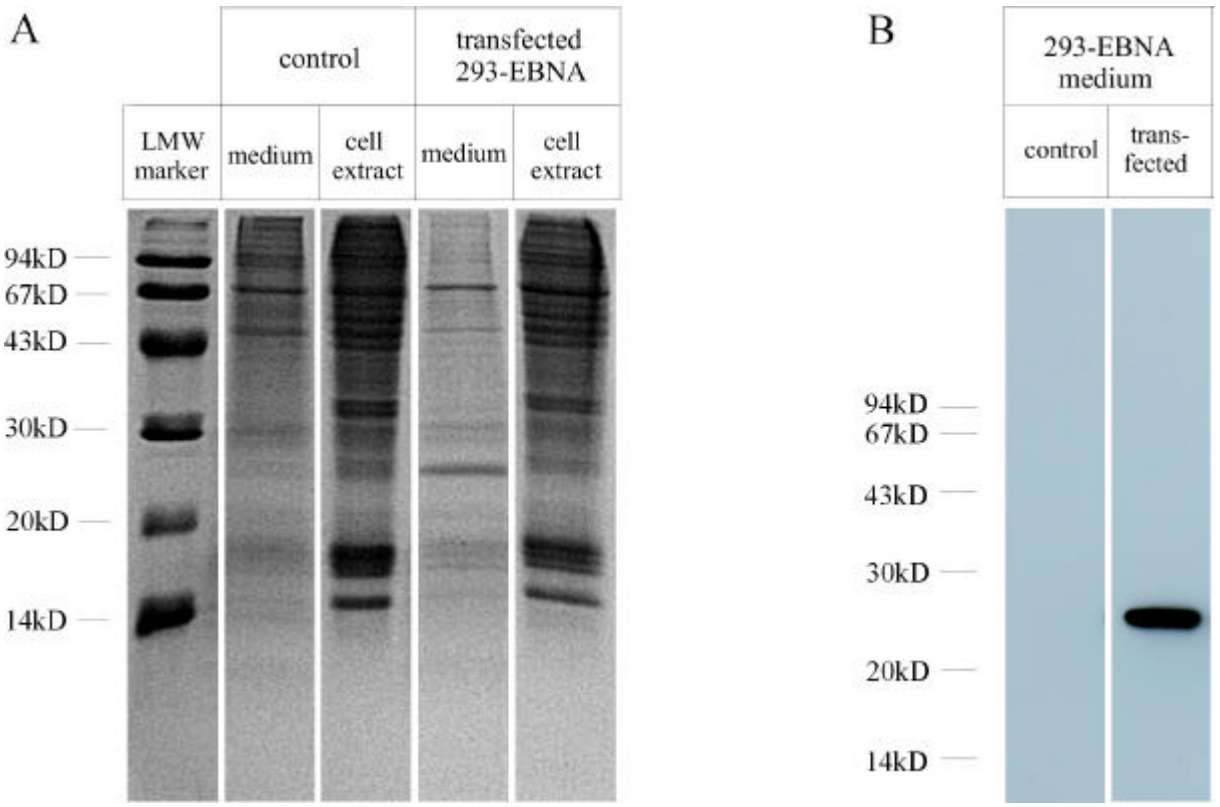


Figure 2.3: Expression of γ1III3-5 in 293-EBNA cells. A) Coomassie staining of a 15% polyacrylamide gel loaded with reduced supernatants from transfected and untransfected cells reveals an additional band of about 25kD in the medium of resistant cells while no recombinant protein is found in the cell extracts. All lanes are excised from one gel. B) Western blot analysis with the anti-FLAG Bio M2 antibody under reducing conditions identifies a FLAG tagged protein of 25kD in the supernatant of transfected cells. Representative lanes are derived from one experiment.

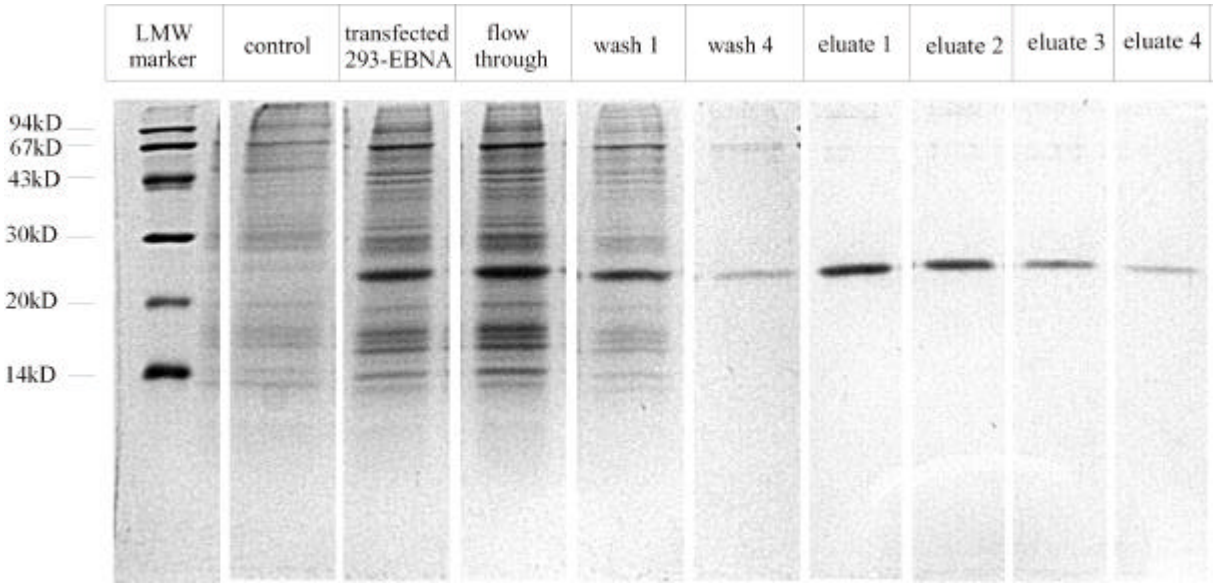


Figure 2.4: Affinity purification of recombinantly expressed nidogen-binding site construct γ1III3-5. Collected supernatants from untransfected (control) and transfected cells, flow-through and wash fractions were TCA precipitated and separated on a 15% polyacrylamide gel after addition of 5% ß-mercaptoethanol. The gel elution is documented with 1/50 of each fraction. Lanes are derived from two gels.

Comparison of the flow-through with the supernatant loaded onto the column showed that not all protein bound to the affinity matrix (figure 2.4). Therefore the purification was repeated after regeneration of the anti-FLAG M2 agarose gel. In total 400µg protein could be purified from 500ml of cell supernatant. Before use in cell culture experiments the protein was dialysed against unsupplemented DMEM F9 growth medium.

2.3. Expression of g1III3-5, g1III3-5mut and g1V1-3 in F9 and D3 cells

F9 cells were electroporated with the FLAG fusion constructs γ1III3-5, γ1III3-5mut and γ1V1-3 and selected for resistance with G418. A total of 25 clones per construct were picked and expanded. Expressing clones (six for γ1III3-5, nine for γ1III3-5mut and four expressing γ1V1-3) were identified by immunoblotting with the Bio M2 monoclonal antibody against the FLAG epitope. For comparison of the levels of protein expression in these cell clones, loading was standardised with the detection of BM40, a calcium binding extracellular matrix protein (Nischt et al., 1991), also produced by undifferentiating F9 cells (figure 2.5).

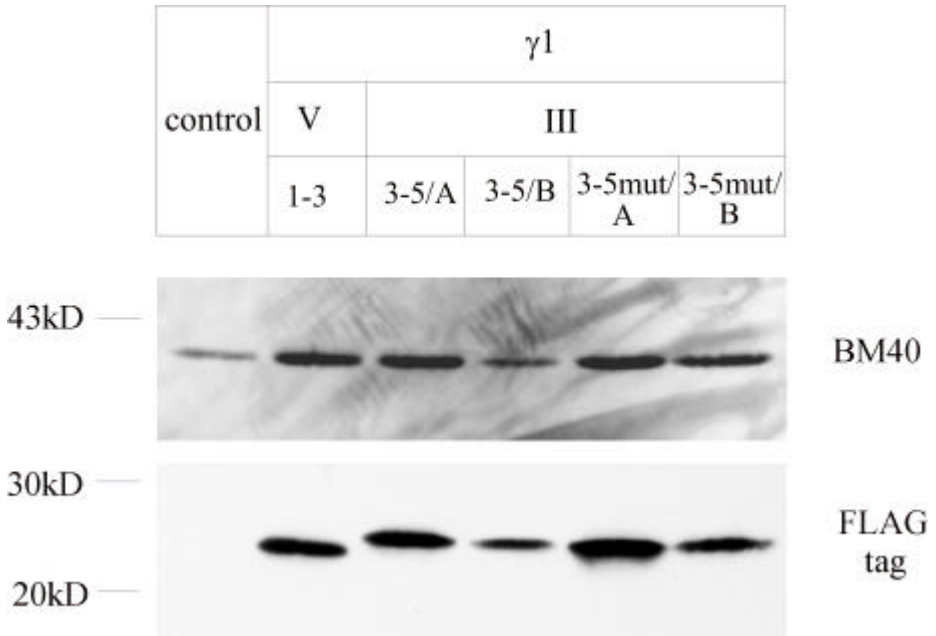


Figure 2.5: Levels of exogenous protein expression: Western blot analysis on supernatants of stably transfected F9 teratocarcinoma cells with a mouse monoclonal antibody detecting the FLAG tag, shows approximately equal expression of all three constructs, γ1III3-5, γ1III3-5mut and γ1V1-3. In case of the γ1III3-5mut construct γ1III3-5mut/B cells were choosen for further studies. As a control F9 cells carrying the empty expression vector were analysed. For normalisation of loading a rabbit polyclonal antibody against the mouse BM40 protein was used.

Electroporation of the embryonic stem cell line D3 with the same constructs used for the transfection of F9 cells gave many G418 resistant clones, but none expressed the FLAG tagged recombinant proteins (results not shown).

2.4. Comparison of embryoid body phenotype in two different cell culture systems

Two systems, the hanging drop method and the cell spin system have been established as embryoid body culture techniques. However direct comparison, especially for the production of F9 derived embryoid bodies has not been carried out. To test which one was the most appropriate, F9 cells which carry the empty expression vector and are G418 resistant, were cultured as embryoid bodies in both systems. These cells are designated the control clone below.

The drop method uses 20µl of a cell suspension containing $1.5x10^5$ cells/ml hanging on an inverted petri-dish. After two days in culture each drop contained a small aggregate of cells which were released from the drops and kept in suspension cultures of 10ml in a bacterial petri-dish until harvest. During this period, medium was changed every second day.

The cell spin system in contrast started with a density of $1.2x10^5$ cells/ml. 100ml of this cell suspension was maintained in siliconated glass bottles overnight under continuous gentle stirring. The next day when small aggregates had formed, 200ml of fresh medium was added and thereafter changed daily.

The control clone appeared to grow equally well in both systems (data not shown) and embryoid body morphology showed similar results. In both systems, bright central areas indicative of cavity formation could be observed (figure 2.6A).

To analyse the expression of basement membrane components induced by retinoic acid in both systems, total homogenates were prepared from differentiated control embryoid bodies after 12 days culture. Polyacrylamide gel electrophoresis of these homogenates and analysis for laminin-1 and nidogen-1 by immunoblotting was performed with a rabbit polyclonal antibody raised against laminin-1/nidogen-1 complexes isolated from the EHS tumor. Bands were seen for the β1 (220kD) and γ1 (210kD) chains of laminin-1 as well as

nidogen-1 (150kD) with similar expression levels for all three proteins in both systems. No band corresponding to the α1 laminin chain (400kD) was seen (figure 2.6B). Basement membrane structure was analysed by immunohistochemistry on embryoid body cryosections with the same antibody (figure 2.6C). This revealed continuous stretches of laminin and nidogen-1 present directly basal to the most peripheral cells in the embryoid body. These signals were interpreted as intact basement membranes and occurred in both systems.

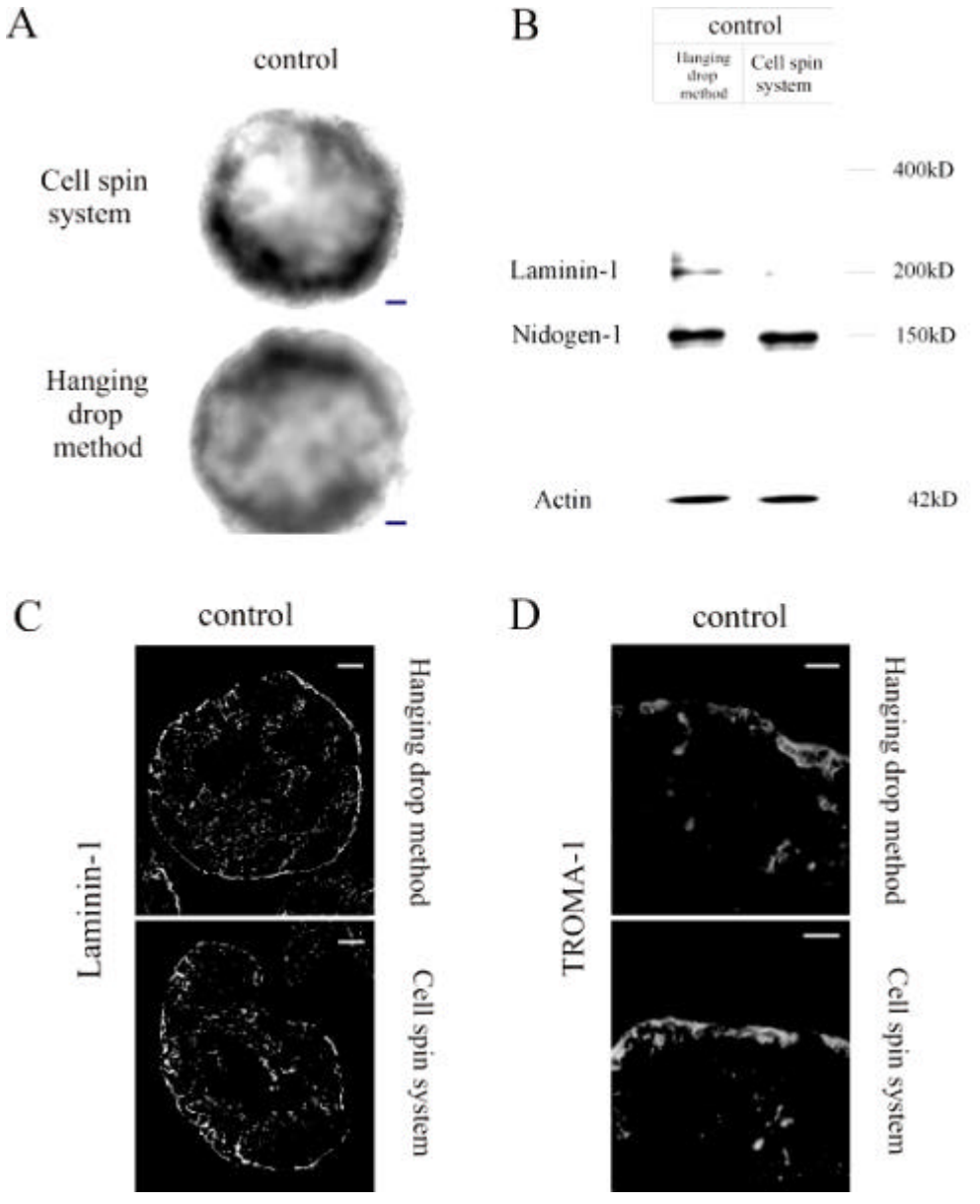


Figure 2.6: Comparison of embryoid bodies formed by the hanging drop technique or in the cell spin system. F9 cells carrying the empty expression vector represent the control clone and were raised for 12 days with both methods in culture with retinoic acid. Analysis by transmission light microscopy is shown in representative images in A (Bar = 100µm). Immunoblotting for laminin-1 and nidogen-1 with a rabbit polyclonal antibody on total homogenates prepared from 12 day old embryoid bodies and standardisation with a mouse monoclonal antibody directed against actin are depicted in B. C shows immunohistochemical localisation of laminin-1 in cryosections of 12 day old cell aggregates (Bar = 100µm). In D similar cryosections were stained for TROMA-1. (Bar = 50µm).

To determine if retinoic acid induced epithelial differentiation into visceral endoderm, control embryoid bodies raised in the two systems were compared by staining with TROMA-1 antibodies. These are directed against the intermediate filament component Endo A (cytokeratin 8) which is produced by endodermal cells in the mouse embryo. It stained cells primarily at the embryoid body periphery in both cultures (figure 2.6D) which indicates the expected differentiation into the visceral endoderm.

The experiments performed to compare the two embryoid body culture systems suggested similar growth and differentiation in both. The only obvious difference at the end of culture was in the yield of embryoid bodies which was approximately 100 fold higher in the spinner flask than in the petri-dish for comparable amounts of labour. However, the cell spin system is expensive to run requiring a high throughput of media and needing specialised equipment as well as occupying much incubator space. Thus initially the hanging drop method was used to set up embryoid body cultures from the F9 cell lines expressing the laminin γ1 derived peptides. However, when the increase in embryoid body diameter was measured during twelve days of culture it became apparent that the embryoid bodies expressing the recombinant polypeptides were smaller in size than those formed by the control clone. This observation is documented for the F9 cell lines carrying the γ1V1-3 and γ1III3-5/B construct and the control in figure 2.7.

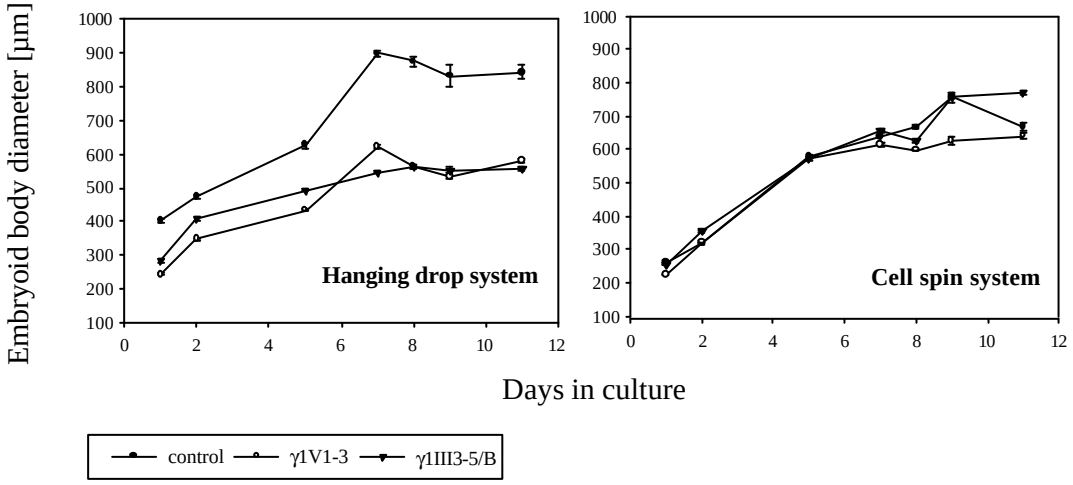


Figure 2.7: Comparison of growth rates of F9 derived embryoid bodies from two different cell culture systems. Diameters of embryoid bodies grown from clones expressing the constructs γ1III3-5/B and γ1V1-3 and from a control clone carrying the empty expression vector were measured. Mean values were calculated and are depicted in the graph.

Assuming that recombinant expression might put pressure on the cell metabolism, resulting in growth retardation, providing an improved nutrition and oxygen supply to the developing embryoid body might overcome this problem. Therefore control, clone γ1V1-3 and γ1III3-5/B embryoid bodies were test-cultured in the spinner flask system and here showed a far more similar growth rate than in the petri-dish (figure 2.7). Daily exchange of medium combined with continuous agitation apparently could compensate for metabolic pressure caused by recombinant expression. Therefore it was decided to use the cell spin system for further experiments to ensure that possible phenotypes are specific to recombinant expression of the laminin γ1 constructs and are not cell culture dependent.

2.5. Comparison of F9 embryoid bodies expressing the laminin g1 constructs

2.5.1. Analysis of embryoid body growth rate and morphology

Growth rates were determined every second day for the various F9 cell clones cultured in the cell spin system (figure 2.8). All of them grew at similar rates in the spinner flask until day 8. However embryoid bodies formed by the control showed an obvious divergence in size which ranged from 800 to 1000µm at day 12.

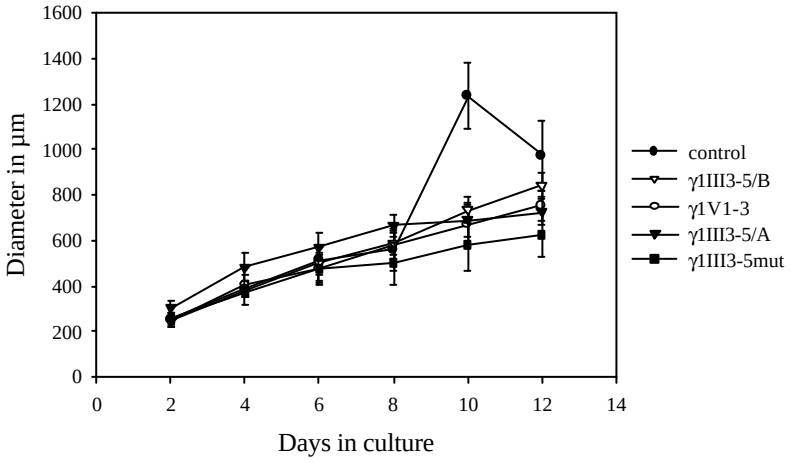


Figure 2.8: Comparison of growth rates of F9 derived embryoid bodies γ1III3-5/A, γ1III3-5/B, γ1III3-5mut and γ1V1-3. F9 cell clones stably expressing γ1III3-5, γ1III3-5mut and γ1V1-3 were grown in the cell spin system and differentiated with $5x10^{-8}$M retinoic acid. The control represents F9 cells transfected with the empty expression vector.

Staining of these embryoid bodies with Lucifer Yellow VS, a necrosis specific marker and SYTOX, a fluorescent dye which stains single dead cells in intact tissue showed that the dark central regions observed by light microscopy in γ1III3-5/A and γ1III3-5/B expressing clones were areas of necrosis (figure 2.9). Both clones expressing the nidogen-binding site developed larger necrotic areas than γ1V1-3 expressing embryoid bodies. Where the embryoid bodies expressed the mutated nidogen-binding site they were morphologically similar to the control clone which does not express any recombinant protein. SYTOX staining could be observed in the periphery of all clones, particularly in the control clone and γ1III3-5mut which showed strong SYTOX signals in regions of low or no Lucifer Yellow VS staining, indicating programmed cell death, possibly an effect induced by differentiation.

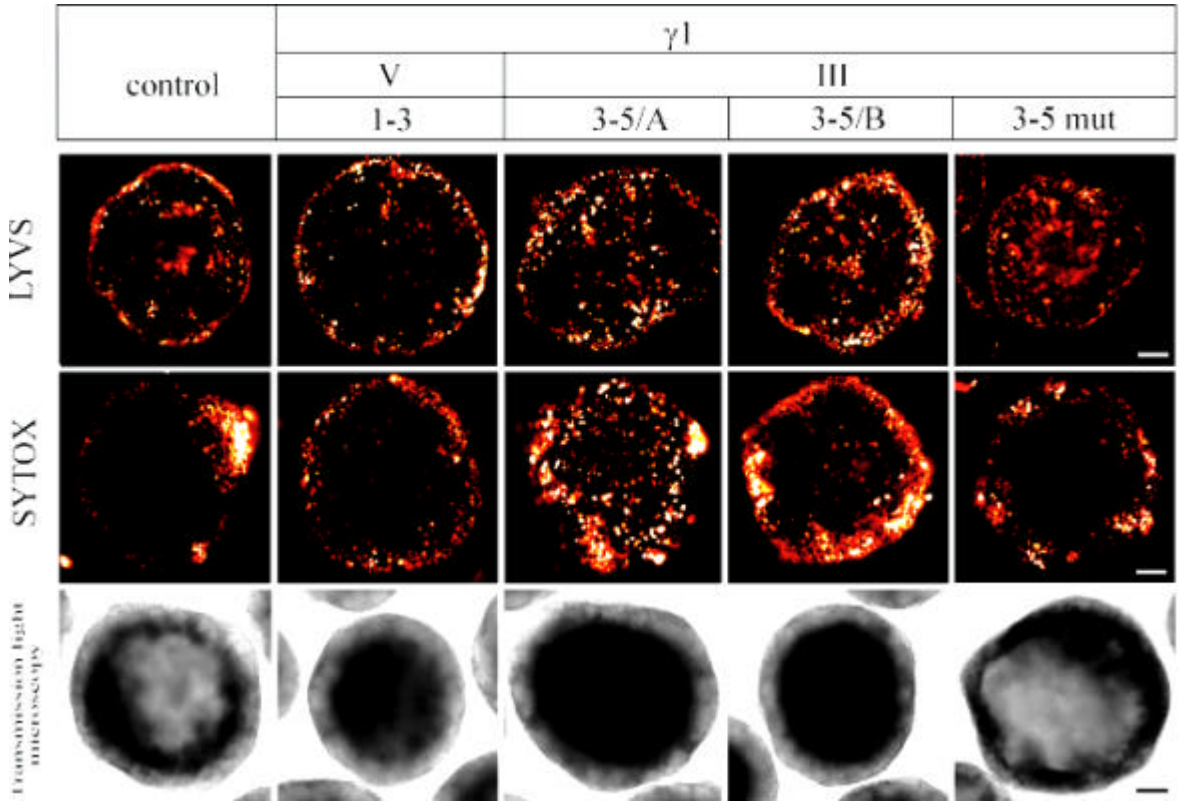


Figure 2.9: LYVS and SYTOX staining on differentiated, F9 embryoid bodies expressing the laminin γ1 FLAG fusions γ1III3-5, γ1III3-5mut and γ1V1-3. Viability of differentiated embryoid bodies was assessed by transmission light microscopy (lower panel), Lucifer Yellow VS staining (LYVS; upper panel) shows strongest staining in the inner core of clone γ1III3-5/A and γ1III3-5/B. SYTOX staining (middle panel) also gives strong signals in the inner core of γ1III3-5/A and in peripheral areas of γ1III3-5/B, γ1III3-5mut and the control clone (Bars = 100µm).

Total RNA was isolated for the analysis of nidogen-1 mRNA levels, to exclude the possibility that nidogen-1 expression in the embryoid bodies could be effected by the transfection procedure and/or recombinant expression of the various polypeptides. However, levels

of expression of nidogen-1 mRNA were similar in all clones irrespective of which construct they contained. Equivalent loading was tested by subsequent probing of the blot with GAPDH (figure 2.10).

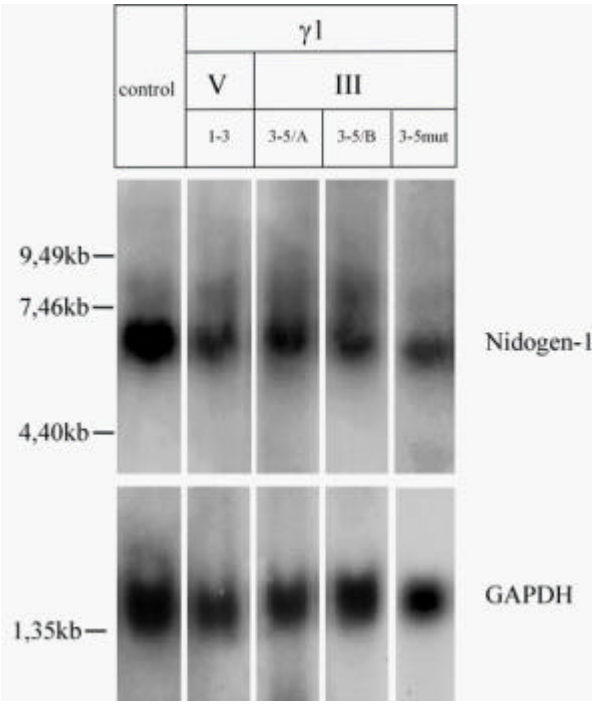


Figure 2.10: Expression of FLAG fusion proteins does not affect nidogen-1 mRNA level: Total mRNA was prepared from 12d old retinoic acid-treated embryoid bodies, separated on a 1% agarose gel and hybridised with a cDNA probe for nidogen-1. Probing for GAPDH message showed that comparable amounts of total mRNA had been loaded.

2.5.2. Detection of basement membrane proteins in g1III3-5/A, g1III3-5/B, g1III3-5mut and g1V1-3 expressing embryoid bodies

To see if differentiation with $5x10^{-8}$M retinoic acid induced the expected production of basement membrane components total homogenates were prepared from the various 12 day old embryoid bodies. After separation on a 3-10% polyacrylamide gradient gel and subsequent transfer to a nitrocellulose membrane, protein extracts were tested for laminin-1 and nidogen-1 (figure 2.11). This revealed similar nidogen-1 levels in γ1V1-3, γ1III3-5/A, γ1III3-5/B, γ1III3-5mut and the control clone. This was surprising since one possible consequence of blocking the binding of nidogen-1 to the laminin network by expression of the nidogen-binding site could be the loss of free nidogen-1 from the embryoid body and its release into the cell culture medium. Further attempts to detect nidogen-1 in the growth

medium by radioactive labeling with ^{3}H-leucine failed. Unlike the levels of nidogen-1, levels of laminin-1 varied markedly between the clones. The homogenates prepared from γ1V1-3, γ1III3-5/A and B and that expressing the γ1III3-5mutant all appeared to contain greater amounts of laminin β1 and γ1 chains than those of the control (nonexpressing) clone.

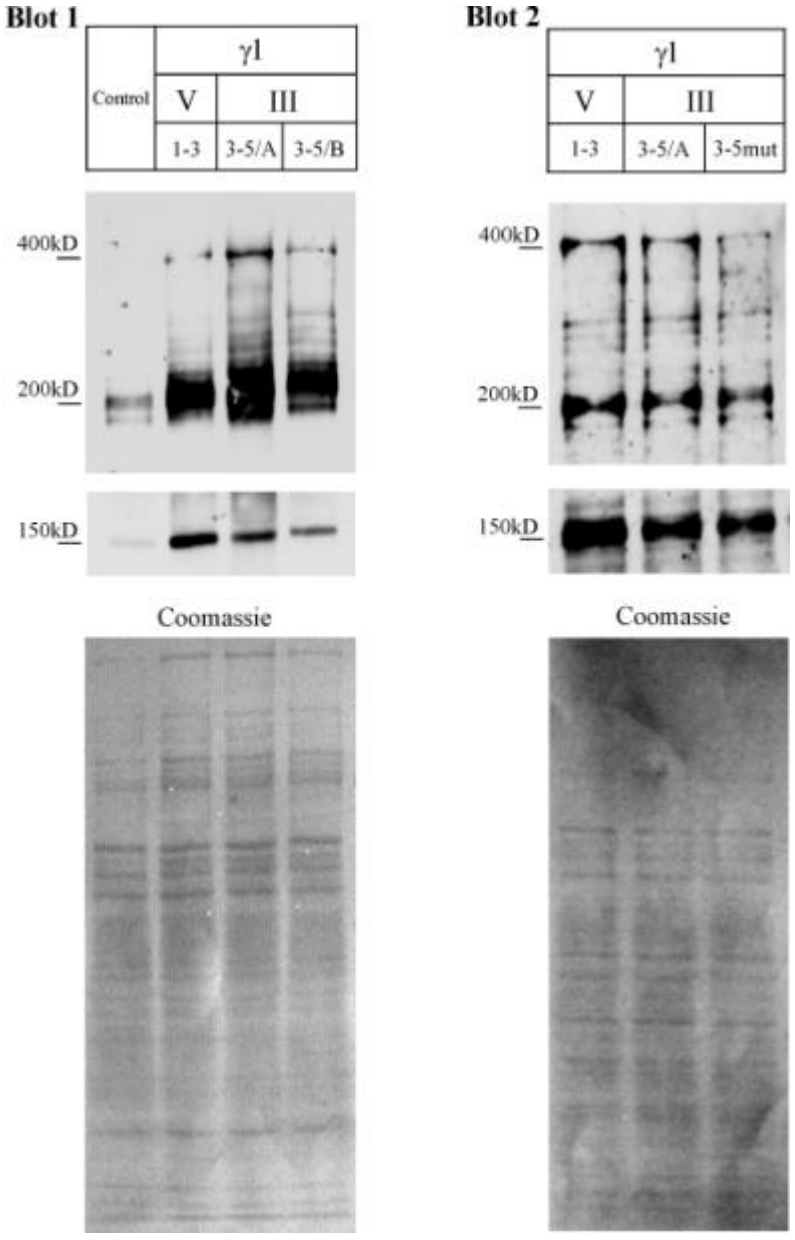


Figure 2.11: Immunoblot for laminin-1 and nidogen-1 on embryoid body total homogenates. A rabbit polyclonal antibody against laminin-1 and nidogen-1 was used to detect these proteins in total homogenates prepared from 12d old retinoic acid-treated embryoid bodies. Coomassie staining of the membrane shows that equal amounts had been loaded and transferred to the nitrocellulose. Blot 1 and 2 compare homogenates prepared from two different spinner cultures. Control describes embryoid bodies carrying the empty expression vector, γ1III3-5/A and γ1III3-5/B represent two different clones from a single transfection with the γ1III3-5 construct. Embryoid bodies designated γ1V1-3 and γ1III3-5mut result from F9 clones stably expressing constructs of the same designation.

Since these differences may be due to changes in basement membrane assembly, its structure was analysed by immunohistochemistry on embryoid body cryosections with a rabbit polyclonal antibody against laminin-1. This revealed regions of continuous laminin-1 signal at the periphery of control, γ1V1-3 and γ1III3-5mut expressing embryoid bodies, while in γ1III3-5/A and γ1III3-5/B laminin-1 signals have a highly disrupted appearance (figure 2.12). Double staining for laminin-1 and nidogen-1 using a monoclonal antibody reacting with the G2 domain of mouse nidogen-1 (figure 2.13) revealed complete colocalisation of both proteins in γ1III3-5mut, γ1V1-3 and control embryoid bodies, while embryoid bodies γ1III3-5/A and γ1III3-5/B derived from the nidogen-binding site expressing cells showed imperfect colocalisation with some laminin not occurring together with nidogen-1.

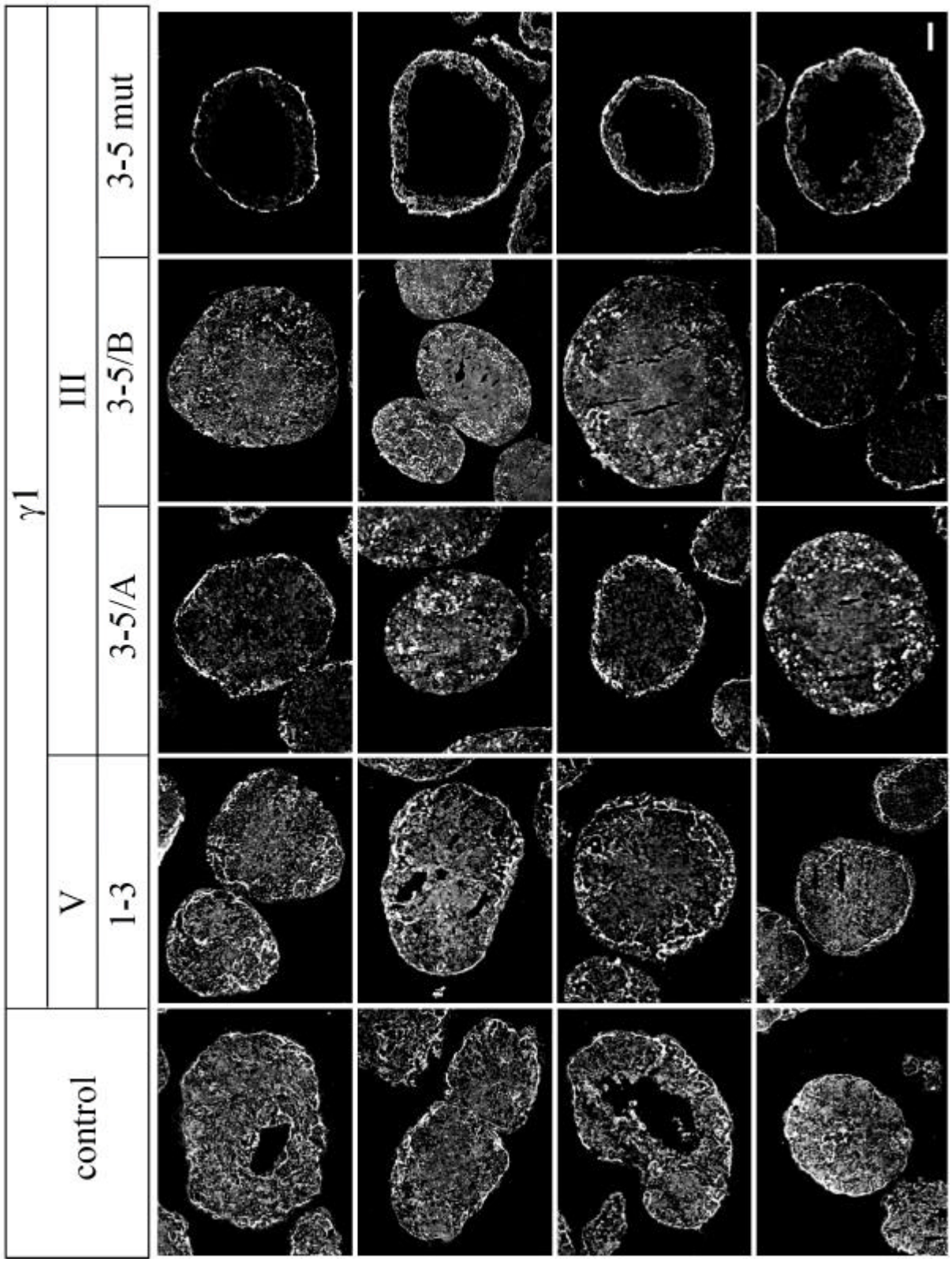


Figure 2.12: Immunohistochemical localisation of laminin-1 in 12d old F9 cell derived embryoid bodies: Cells stably expressing the constructs γ1III3-5, γ1III3-5mut and γ1V1-3 were differentiated into embryoid bodies with $5x10^{-8}$ M retinoic acid, cryosectioned and stained for laminin-1. As a control F9 cells carrying the empty expression vector were used. γ1III3-5/A and γ1III3-5/B represent two different clones from one transfection with the γ1III3-5 construct. Note the pronounced cavitation seen in control embryoid bodies and, particularly, in those derived from the clone γ1III3-5mut. Bar = 100µm.

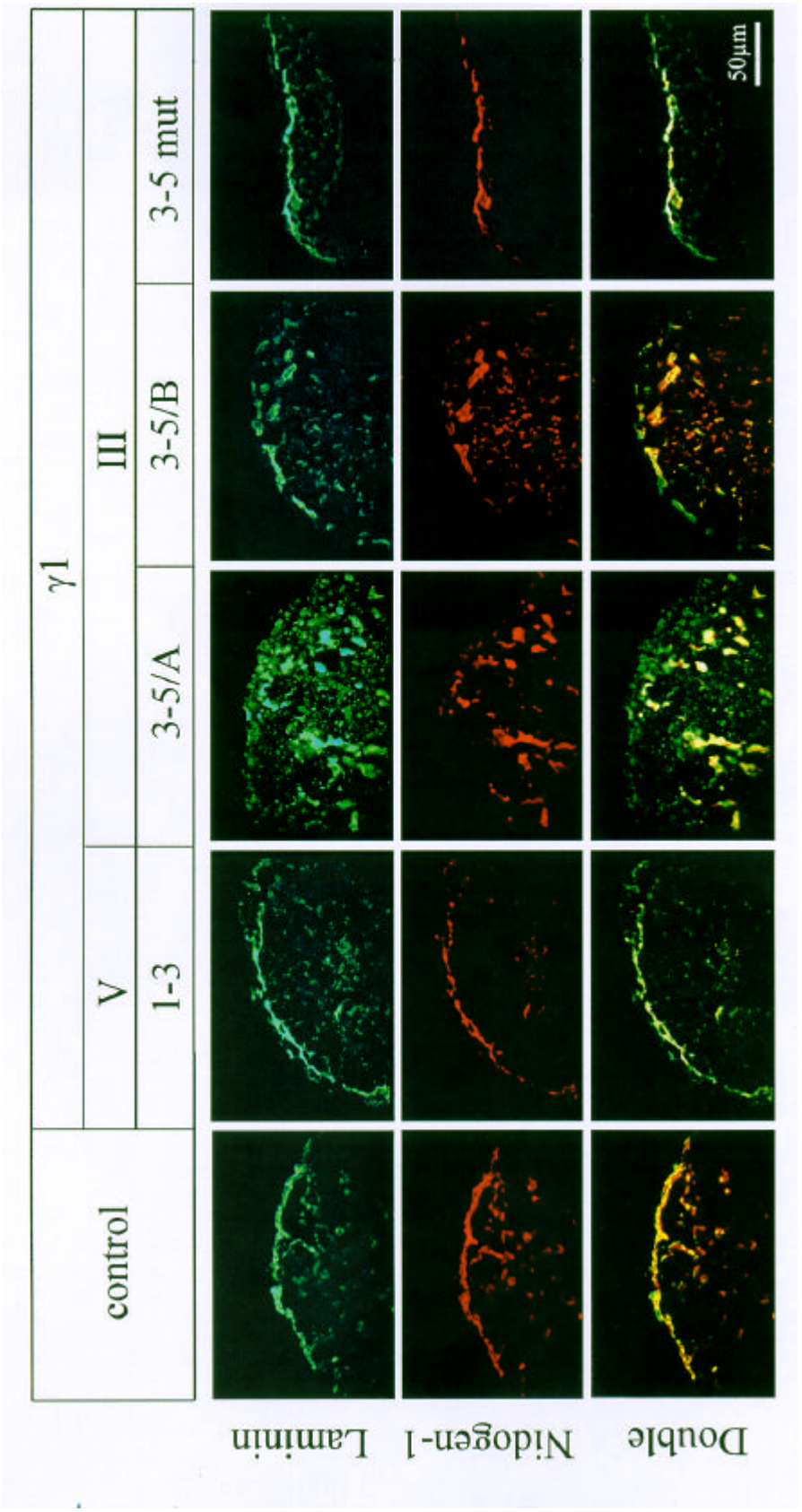


Figure 2.13: Double immunofluorescence for laminin-1 and nidogen-1. Cryosections of differentiated 12d old embryoid bodies were incubated simultaneously with a rabbit polyclonal antibody against laminin-1 (green) and a rat monoclonal antibody against nidogen-1 (red). F9 cells carrying the empty expression vector were used as a control. γ1III3-5/A and γ1III3-5/B represent two different clones from a single transfection with the γ1III3-5 construct. Embryoid bodies described as γ1V1-3 and γ1III3-5mut result from F9 clones stably expressing constructs carrying the same designation. Bar = 50µm.

Attempts to show if the FLAG fusion proteins colocalised with laminin-1 staining in the F9 derived embryoid bodies using a biotinylated mouse anti-FLAG monoclonal failed in both cryosections and whole mount embryoid bodies (results not shown).

2.5.3. Differentiation and permeability properties of F9 embryoid bodies expressing g1III3-5, g1III3-5mut and g1V1-3

Embryoid body differentiation was studied morphologically using semithin sections. Embryoid bodies were fixed, dehydrated and embedded in araldite. After polymerisation, 1µm sections were prepared and stained with methyl blue. Transmission light microscopy of these sections revealed no striking morphological differences between any of the clones (figure 2.14) despite distinct changes in basement membrane formation (figure 2.13). All embryoid bodies showed a differentiated external cell layer often displaying microvilli, a morphological marker of endodermal differentiation (figure 2.14, lower panel, 2x magnification).

Epithelial differentiation was studied using the TROMA-1 monoclonal antibody directed against mouse cytokeratin 8. Both the control clone and γ1V1-3 expressing embryoid bodies showed strong staining in the flat surface cell layer. However, in embryoid bodies expressing the nidogen-binding site, TROMA-1 signals were seen occuring widely over the embryoid body and not merely restricted to the outer cells (figure 2.15). While TROMA-1 positive cells were occasionally present internally in control embryoid bodies and those derived from the γ1III3-5mut and γ1V1-3 expressing cells, these were far rarer. While certain cells appear to show markers for endodermal development in embryoid bodies with a disrupted basement membrane, these are expressed in abnormal localities.

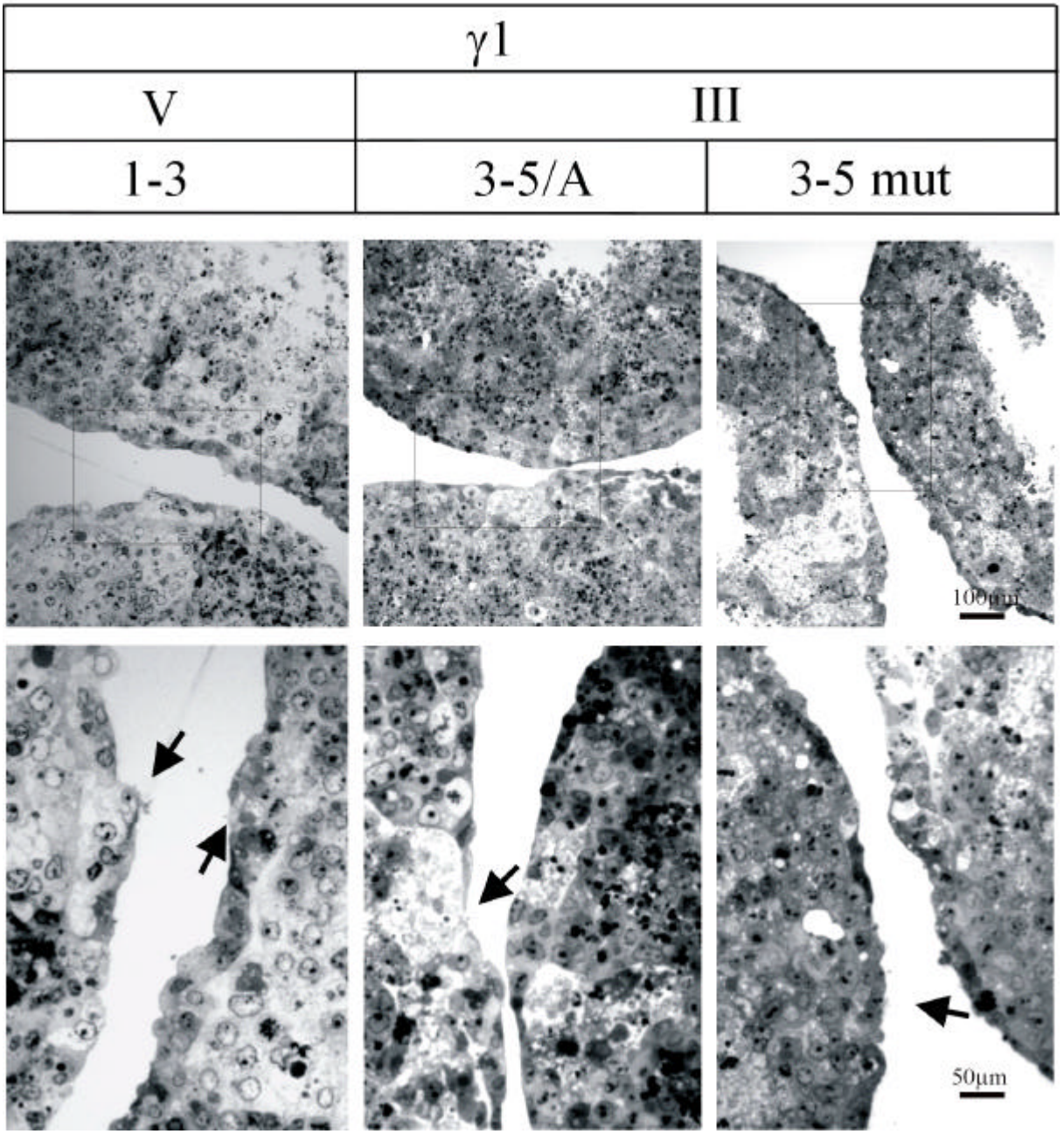


Figure 2.14: Semithin sections of F9 cell derived embryoid bodies. Embryoid bodies expressing the laminin γ1 FLAG fusions γ1III3-5, γ1III3-5mut and γ1V1-3 were maintained in the cell spin system supplemented with retinoic acid for differentiation. After 12 days of culture they were harvested for the preparation of semithin sections. After staining these were analysed by transmission light microscopy and representative images are shown in upper panel (bar = 100µm) . Zoomed pictures depicted in the lower panel (bar = 50µm) correspond to image areas surrounded by boxes in the upper panel. Arrows point to microvilli.

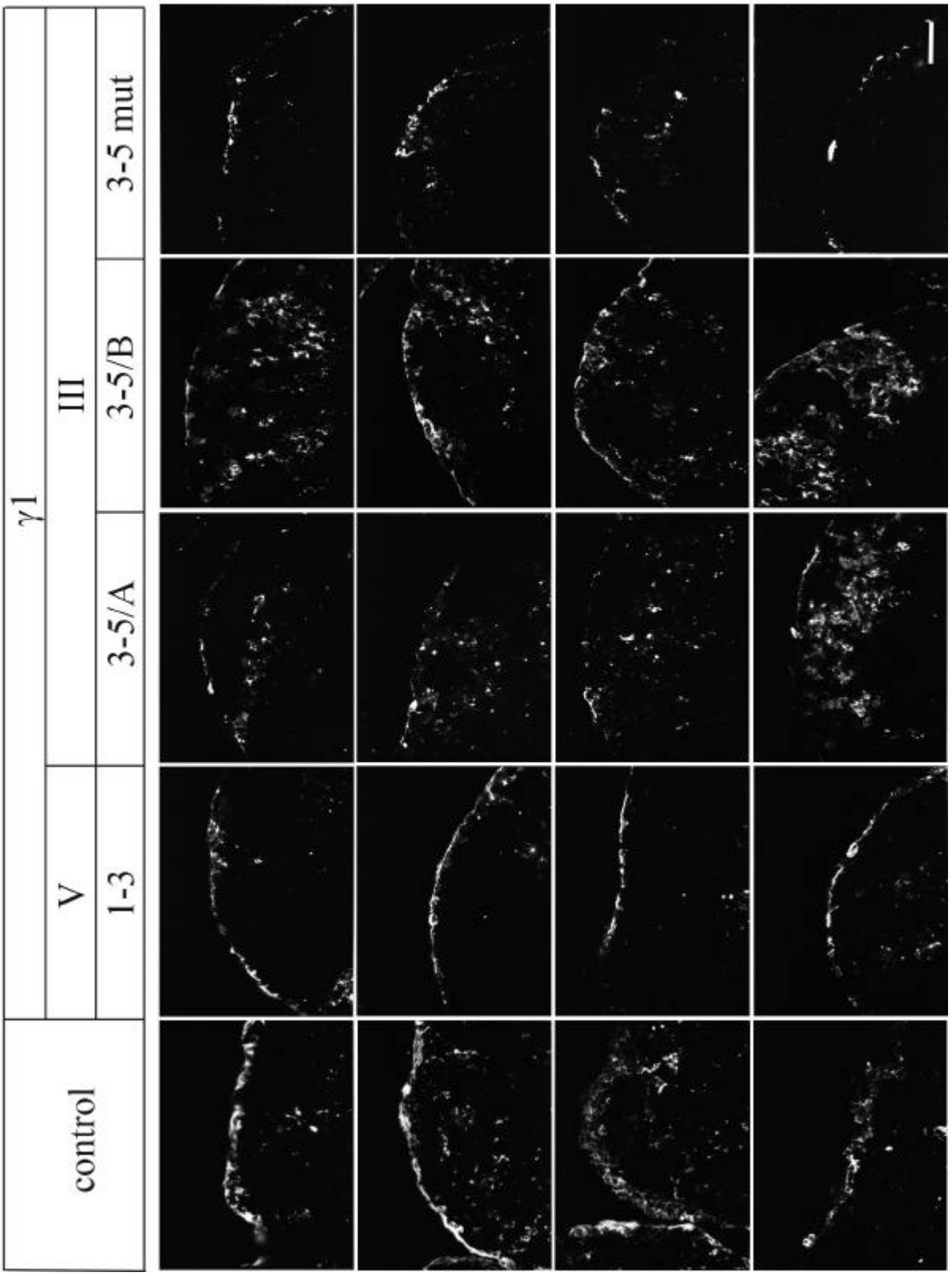
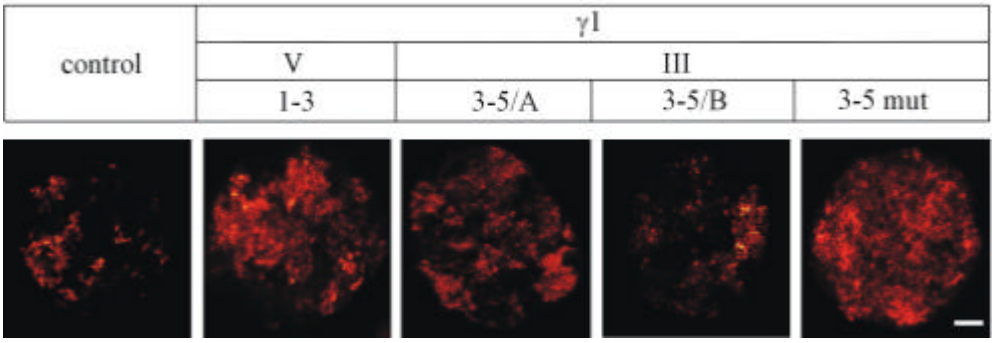


Figure 2.15: Immunofluorescence for TROMA-1: Cryosections of differentiated 12d old embryoid bodies derived from control, γ1V1-3, γ1III3-5/A and γ1III3-5/B and γ1III3-5mut expressing F9 cells were stained with a rat monoclonal antibody against TROMA-1 (Kemler et al., 1981). Bar = 50µm.

The expression of PECAM-1 (Tang and Honn, 1995), the platelet/endothelial cell adhesion molecule-1, was studied to provide a more comprehensive analysis of differentiation. PECAM-1 is frequently used as a marker for vascularisation in embryonic stem cell derived embryoid bodies as it is concentrated along the borders between endothelial cells. F9 cells have a lower differentiation potential and embryoid bodies derived from them do not vascularize as extensively as those derived from embryonic stem cells. For four clones, those expressing the γ1V1-3 or γ1III3-5 polypeptides and the control, a similar pattern of staining was observed with a marked increase between days 10 and 12. However, the clone derived from γ1III3-5mut had a generally far higher PECAM-1 staining, perhaps due to its altered morphology (figure 2.16).

A



B

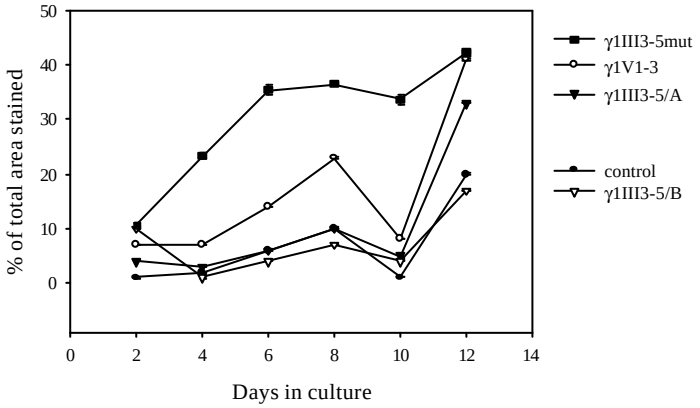
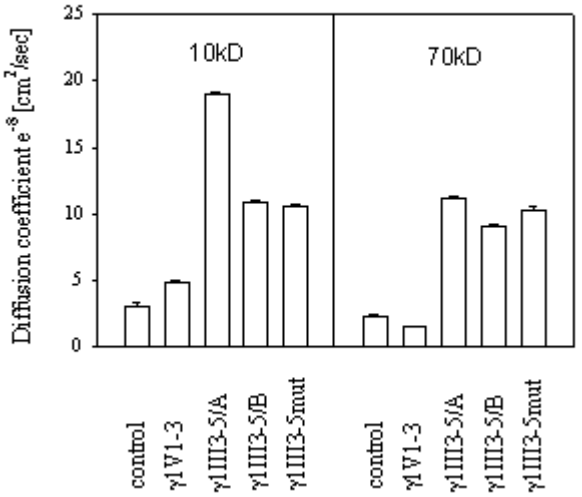


Figure 2.16: PECAM-1 signal intensities during 12 days of F9 embryoid body culture treated with $5x10^{-8}$M retinoic acid. Whole mount stainings using a rat monoclonal antibody against mouse PECAM-1 were performed with embryoid bodies expressing the constructs γ1III3-5/A, γ1III3-5/B, γ1III3-5mut and γ1V1-3. F9 cells carrying the empty expression vector were included as a control. Representative images of 12 day old embryoid bodies are shown in A (bar = 100µm). The percentage of total area stained was calculated for all embryoid bodies by measuring PECAM-1 signals above a common treshold level. Average signal intensities during day 12 days of culture are summarised in B.

Other differentiation markers for neuronal, skeletal and muscular cell types were tested. However, anti-NF200 and anti-myosin did not give any signal in differentiated F9 embryoid bodies, possibly due to the limited differentiation repertoire of these.

To determine if expression of the nidogen-binding site affects the function of basement membranes as permeability barriers (Williams, 1994) polymer diffusion into the embryoid bodies was studied.

A



B

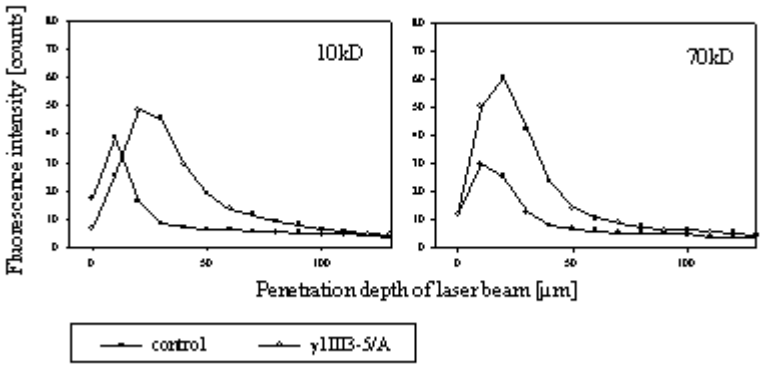


Figure 2.17: Comparison of diffusion coefficients obtained from incubation of differentiated F9 cell derived embryoid bodies with rhodamine-labeled dextrans of 10kD and 70kD size. A) Embryoid bodies stably expressing the constructs γ1III3-5, γ1III3-5mut, γ1V1-3 and a control clone were incubated with 10µM dextrans of either size. γ1III3-5/A and γ1III3-5/B represent two different clones from a single transfection with the γ1III3-5 construct. Fluorescence intensities were measured inside the embryoid bodies after 5min of diffusion. Mean diffusion coefficients + SD of ≥6 traces of individual embryoid bodies were calculated. Two independent experiments were performed with comparable results. In B representative curves of single embryoid bodies (control and γ1III3-5/A) incubated with 10kD and 70kD fluorescent dextrans are shown.

Kidney glomerular basement membranes are critical in the restriction of the passage of serum proteins into the urine, allowing the passage only of those with a molecular weight below 70kD. Therefore embryoid bodies expressing the various polypeptides were washed and maintained in the physiological E1 solution (see Materials and Methods). Rhodamine-labelled dextrans of 10kD and 70kD were added to the embryoid bodies at a concentration of 10µM. Diffusion of the fluorescent molecules into the embryoid body was compared after 5min by analysis of serial optical sections with the laser scanning microscope. These sections were of distinct fluorescence intensity at different planes from the embryoid body`s surface to its center and dependent on the distance of diffusion of the rhodamine-labeled dextrans. Subsequent calculation of the diffusion coefficients (see Materials and Methods) showed for control embryoid bodies and γ1V1-3 expressing embryoid bodies relatively low permeability for 10kD and 70kD dextrans compared to both clones expressing the nidogen-binding site, γ1III3-5/A and γ1III3-5/B (figure 2.17). Clone γ1III3-5mut which expresses a mutated version of the nidogen-binding site demonstrates a diffusion behavior similar to clone γ1III3-5. However differences in its morphology (see figure 2.12) made a direct comparison unreliable.

2.6. Addition of affinity-purified laminin g1 FLAG fusion protein g1III3-5 to wild type embryoid bodies

Artefacts due to the recombinant expression, genetic manipulation, or cloning could not be excluded as the causes of these differences upon expression of the nidogen-binding site. Therefore, the affinity-purified laminin γ1 FLAG fusion protein γ1III3-5, expressed in 293-EBNA cells (section 2.2.) was added to the medium of wild type embryoid bodies. F9 cells were maintained with retinoic acid in a spinner flask for two days and then transferred into single wells of a 96-well plate, and were grown further in the presence of the affinity-purified laminin γ1 FLAG fusion protein at a concentration of 10µg/ml. Nonsupplemented controls were also cultured. Both supplemented and nonsupplemented embryoid bodies grew in a similar manner to those in the spinner flask (figure 2.18).

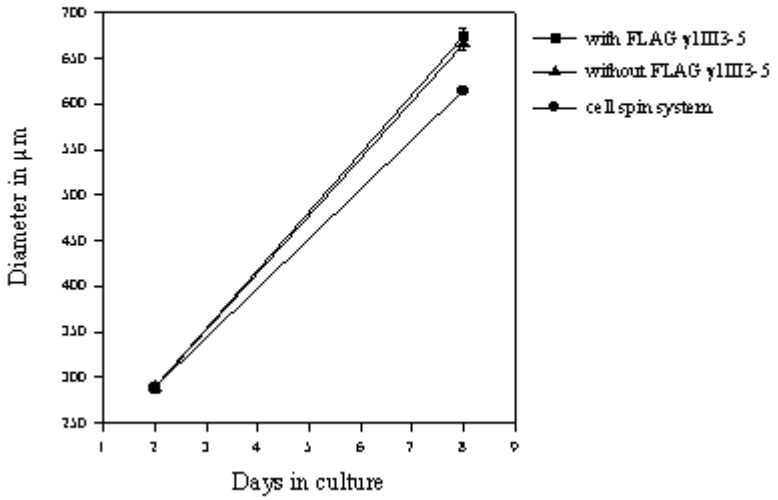


Figure 2.18: Comparison of growth of F9 wild type embryoid bodies in the cell spin system and 96-well plates. Diameters of embryoid bodies either differentiated in the cell spin system or in 96-well plates with $5x10^{-8}$M retinoic acid were measured at day two and eight of culture. The graph summarises growth data of three sets of embryoid bodies: wild type embryoid bodies cultivated in the spinner flask (cell spin system) and wild type embryoid bodies cultivated in the spinner flask for two days and then transferred into single wells either with (with FLAG γ1III3-5) or without (without FLAG γ1III3-5) addition of affinity-purified FLAG tagged γ1III3-5.

After 8 days of culture whole mount stainings for laminin-1 were performed and revealed in wild type embryoid bodies a network-like laminin-1 staining, whereas embryoid bodies treated with exogenous γ1III3-5 FLAG fusion protein showed a highly disrupted laminin-1 signal (figure 2.19).

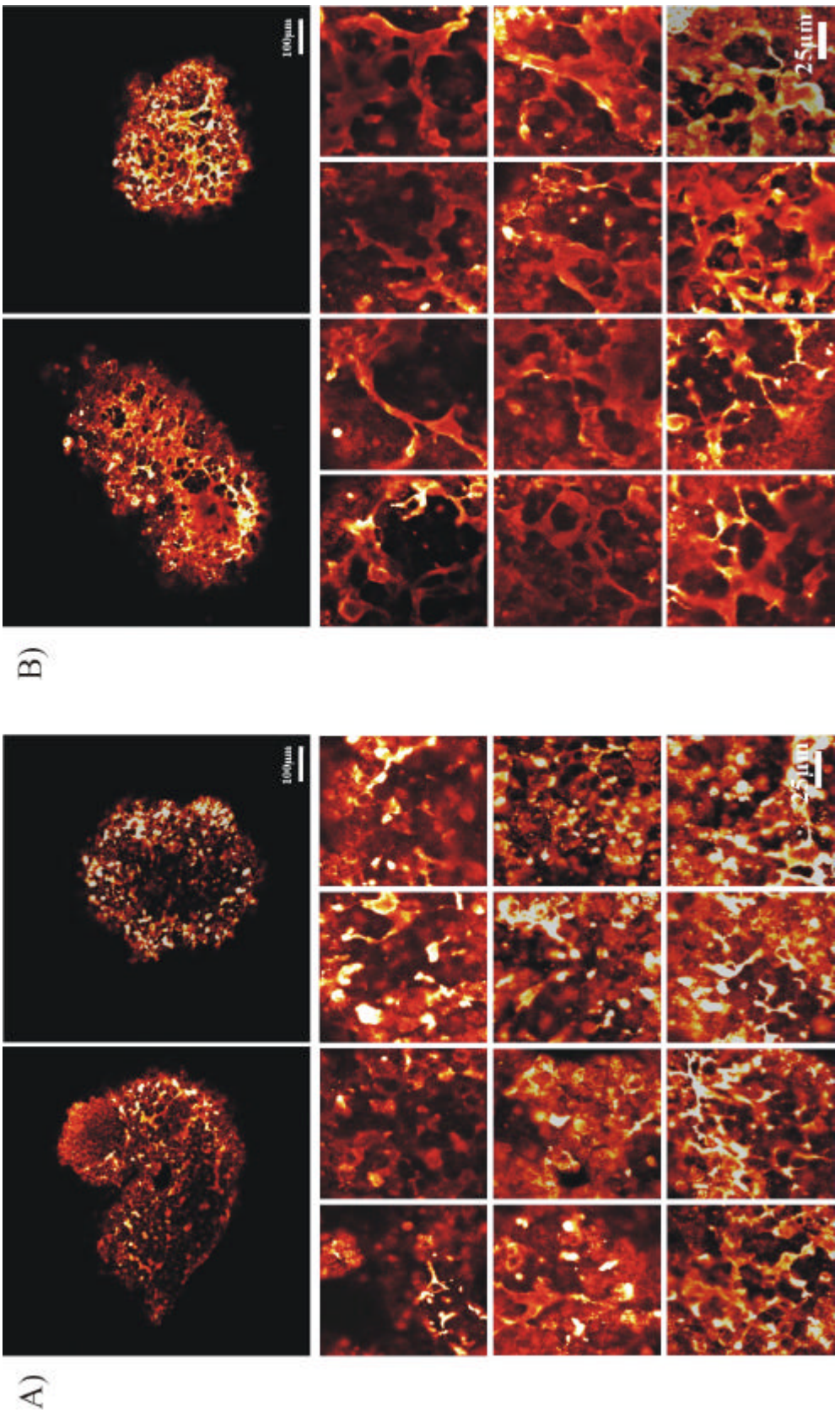


Figure 2.19: Laminin-1 localisation in F9 wild type embryoid bodies treated with the FLAG fusion protein γ1III3-5. Embryoid bodies were transferred from a spinner culture system into single wells at day two and then either supplied with additional FLAG fusion protein γ1III3-5 at a concentration of 10µg/ml (A) or not (B). At day 8 of culture whole mount stainings with a rabbit polyclonal antiserum against laminin-1 were performed. Representative images show either the embryoid body top (upper panel; bar = 100µm) or part of a section through the embryoid body periphery (lower panel; bar = 25µm).

3. Discussion

3.1. Establishment of cells expressing laminin g1 FLAG fusion proteins

3.1.1. Cloning of the constructs

The aim of this study was to discover if disruption of the laminin/nidogen-1 complex influences basement membrane formation and, if so, to analyse the consequences for cellular differentiation. To interfere with the laminin/nidogen-1 interaction, the nidogen-binding site located in LE module 4 of domain III of the laminin γ1 chain (Mayer et al., 1993) was expressed in the F9 embryoid bodies, a model for basement membrane formation. The correct folding of the nidogen-binding site was ensured by the additional expression of the flanking LE modules γ1III-3 and -5. Such a polypeptide has been previously shown to fold (Stetefeld et al., 1996) and interact with nidogen-1 in a manner similar to native laminin-1 (Mayer et al., 1993). In addition it was necessary to include controls which allow the distinction of specific phenotypes from artefacts produced by either clonal selection or through stressing the cellular metabolism due to expression of the recombinant protein.

Three sets of controls were produced, F9 cells transfected with the empty expression vector, ones expressing a mutated, presumably inactive form of the nidogen-binding site (γ1III3-5mut) and those expressing a similar but non-active set of three LE domains also present in laminin γ1 (γ1V1-3). The polypeptide γ1III3-5mut carries the N802S point mutation (Poeschl et al., 1996), which reduces its binding affinity to nidogen-1 by approximately 50,000 fold. The domain V of the laminin γ1 chain is comprised of four LE modules and has no known nidogen-binding activity. Expression of the γ1V1-3 construct produces a laminin γ1 fragment similar in secondary structure and size but of a different amino acid sequence to γ1III3-5 (figure 2.1).

If the present theory of basement membrane assembly is correct, i.e. nidogen-1 is a crucial linker between the collagen type IV and laminin networks (Yurchenco and Schittny, 1990), these controls should distinguish a phenotype due to the disruption of laminin/nidogen-1 binding from one caused by expression of LE rich polypeptides.

Since specific antibodies are available only for the nidogen-binding site fragments but not for the detection of γ1V1-3, all three laminin γ1 proteins were modified by fusion with a N-terminal FLAG peptide which could also be utilised for affinity purification. To produce these constructs, the PCR amplified laminin γ1 cDNA fragments were subcloned into CMV-

NFlag (see appendix) and then transferred into pBKEF-5 plasmid downstream of the EF1α promoter.

The FLAG peptide (Hopp et al., 1988) consists of eight amino acids (AspTyrLysAspAspAspAspLys) of which especially Lys and Asp contribute to high hydrophilicity, increasing accessibility of the tag by bringing it to the protein surface (Hopp et al., 1986). To minimise any possible interference with the adoption of native conformation by the expressed protein, a small linker was placed between the FLAG tag and the LE domains. Similar systems have been used before for the expression of other extracellular matrix proteins (Yurchenco et al., 1997).

3.1.2. Promoter activity in different cell lines

F9 mouse teratocarcinoma cells are a frequently used *in vitro* model for the study of early embryogenesis, since they differentiate into primitive, visceral or parietal endoderm upon treatment with retinoic acid or retinoic acid and cAMP (Hogan et al., 1981; Calogero et al., 1991; Damjanov et al., 1994). Here various laminin γ1 constructs were expressed in differentiating F9 cells, and their effects on basement membrane formation studied in the resulting embryoid bodies.

Initially inducible expression of the polypeptides was attempted and two different systems were assessed. Generally such systems consist of two vectors, one encoding the gene of interest and the other containing so called transactivator sequences. For ecdysone-inducible expression the transactivator is an ecdysone receptor heterodimer comprising the VgEcR (ecdysone receptor modified with the VP16 transactivation domain) and the RXR (retinoid X receptor) receptor subunits. Transcription of these sequences is regulated by the cytomegalovirus (CMV) and the Rous sarcoma virus (RSV) promoters, respectively. Both promoters need to be active in F9 cells to induce transcription of the gene of interest. Possibly one or both promoters are non-functional in F9 cells as no recombinant protein could be detected in the cell supernatants or extracts of differentiated and undifferentiated F9 cells transfected with the pINDγ1III3-5 and pVgRXR plasmids. Indeed, attempts to obtain F9 subclones using CMV promoter-based lac repressor or tet responsive expression systems were unsuccessful (Miller and Rizzino, 1995). This suggests that the CMV promoter may be silenced when integrated in the genome of embryonic carcinoma cells, especially as in 293-EBNA cells the γ1III3-5 construct was successfully expressed using the CMV promoter.

In a second attempt to obtain inducible protein expression, a viral vector was evaluated in F9 cells. Unlike the conventional two plasmid system, the tet on/off retrovirus (Hofmann et al., 1996) contains all the components necessary for reversible induction in a single cassette. However, control infections with pGEM-IRES lacZ containing viruses yielded only weak expression in few cells. This is in agreement with one earlier study which suggested that embryonic carcinoma cells are difficult to transfect with retroviral vectors (Linney et al., 1987).

Other promoters used for constitutive expression in F9 cells include the simian virus (SV) 40 promoter (Mueller and Wagner, 1984; Gorman et al., 1985; Kindregan et al., 1994), the human β-actin promoter (Xu et al., 1998) and the elongation factor 1 α (EF1α) promoter (Niimi and Kitagawa, 1997). After testing for successful transfection and expression of a EF1α-GFP reporter plasmid in F9 cells (results not shown), this promoter was selected for expression of the laminin γ1 fragments. Recombinant protein was found to be expressed in 24% (γ1III3-5), 36% (γ1III3-5mut) and 16% (γ1V1-3) of all the G418 resistant cell lines tested. This compares well with the SV40 or human β-actin promoter when used in F9 cells where only 4% and 2% of all resistant clones expressed detectable protein (Espeseth et al., 1989; Kindregan et al., 1994). D3, embryonic stem cells, were also transfected with these constructs, such cells having a wider differentiation capacity than F9 cells. However, while G418 resistant clones were obtained they failed to express the fusion proteins.

3.1.3. Protein expression in F9 mouse teratocarcinoma cells

The expressed proteins were transported to the extracellular space by the presence of the BM40 signal peptide which has been shown previously to give highly efficient secretion of extracellular matrix proteins (Mayer et al., 1993; Yurchenco et al., 1997). Cell supernatants were collected, precipitated and tested by immunoblotting with a biotinylated mouse monoclonal antibody against the FLAG tag. To ensure a comparable level of expression in all stable F9 cell lines loading had to be standardised. Housekeeping genes like mouse β-actin or human GAPDH frequently used for such purposes could not be analysed since they are intracellular proteins. BM40 is an extracellular matrix protein constitutively expressed in undifferentiated F9 cells (Nishiguchi et al., 1996). Normalisation with this protein showed that all F9 derived cell lines synthesised and secreted approximately equal levels of the

laminin γ1 FLAG fusion proteins (figure 2.5). It would have been interesting to know the molar ratio between intrinsic γ1 chains and recombinantly expressed laminin γ1 FLAG fusion proteins to assess the theoretically possible intensity of interference with the laminin/nidogen-1 interaction. Therefore supernatants of stably expressing F9 cells lines which produce basal levels of laminin-1 also in the undifferentiated state (Grover et al., 1983) should be analysed by immunoblotting. However, this experiment could not be performed due to lack of an antibody which recognises the nidogen-binding site on the laminin γ1 chain under reducing conditions. These conditions were necessary to break the disulfide bonds between the α1, β1 and γ1 chain of laminin-1 which as a whole (800kD) is too big to migrate into a 3-10% polyacrylamide gradient gel.

The observation of equal expression of the laminin γ1 FLAG fusion constructs was made with undifferentiated F9 cell lines while the analysis of basement membrane formation was done in differentiated F9 embryoid bodies. It is known that some promoters e.g. SV40 or adenovirus type 5 alter their activity pattern during differentiation (Niwa, 1985; Kellermann and Kelly, 1986). Therefore and to localise the recombinant γ1III3-5 polypeptides with respect to nidogen-1, the expression and distribution of the FLAG tagged recombinant proteins were also followed in differentiated F9 embryoid bodies. With the use of a primary antibody directly conjugated to biotin together with a streptavidin-rhodamine detection system possible background problems due to the application of a mouse monoclonal antibody on murine tissue should be avoided. However, probably due to intrinsic biotin naturally occurring in mouse tissue this detection system also failed and protein expression and distribution in F9 embryoid bodies could not be documented. Another reason for obtaining no specific signals for the FLAG fusion proteins may be found in the nature of the experiment itself. Possibly, the produced fusion constructs were secreted to the extracellular space but not anchored to the pericellular matrix and therefore lost to the medium. This speculation also holds true for the nidogen-binding site construct γ1III3-5, which could have been secreted to the medium either bound or not bound to nidogen-1. Metabolic labeling of nidogen-1 to determine any altered accumulation in the cell culture medium upon loss of anchorage to the basement membrane however was unsuccessful (see section 2.5.2.).

3.1.4. Expression and purification of the laminin g1III3-5 FLAG fusion protein from 293-EBNA cells

As observed for the FLAG tagged polypeptides γ1V1-3 (Mr 18.6kD) and γ1III3-5 (Mr 19.1kD) expressed in F9 cells (figure 2.5), the γ1III3-5 FLAG fusion protein expressed in 293-EBNA cells migrated at about 25kD in SDS-PAGE. This discrepancy may be explained by the negative charge of the hydrophilic FLAG tag which binds fewer SDS molecules than marker proteins of comparable molecular weight and therefore runs higher than predicted in the gel (figure 2.3). To confirm the identity of the protein, serum-free supernatant of transfected 293-EBNA cells was collected, precipitated and tested for the presence of the FLAG tag by immunoblotting (figure 2.3B).

The FLAG peptide allowed single step affinity purification of the γ1III3-5 protein. Analysis of this affinity chromatography showed that the amount of the recombinant protein in the supernatant was higher than the binding capacity of the column. However, 400µg of FLAG tagged protein could be purified from 500ml of supernatant and only a faint band at 67kD (figure 2.4) contaminated the eluates. Since this band probably corresponds to bovine serum albumin and because the FLAG fusion protein had been purified for later addition to fetal calf serum supplemented cell culture medium, this purity was considered sufficient.

3.2. Comparison of two different embryoid body cell culture systems

The need for a reproducible system to grow F9 embryoid bodies was a prerequisite. To find the most appropriate culture method, control cells carrying the empty expression vector were maintained in two different embryoid body systems. The conventional hanging drop technique (Hogan et al., 1983) was simple, albeit time-consuming and the numbers of embryoid bodies produced were low as on the average only 30 could be harvested from one petri-dish. Cell spin cultures were far simpler to produce in bulk. Initially the embryoid bodies produced were of a similar size and number, per quantity of starting cells, in both systems. The embryoid bodies of the cell spin system were maintained under continuous movement by a magnetic stirrer, which guaranteed equal distribution of oxygen and nutrients, in contrast to the hanging drop system where embryoid bodies were only agitated during medium changes.

Control embryoid bodies, grown in both systems, showed no phenotypic differences when analysed morphologically, for the expression and localisation of laminin-1 and nidogen-1 or for differentiation into visceral endoderm (figure 2.6). However, when embryoid bodies expressing recombinant laminin γ1 FLAG fusion constructs were cultured in the hanging drop system it became apparent, already after two days, that such embryoid bodies were smaller than the control (figure 2.7), while in the cell spin system there was far less divergence in embryoid body growth between the different clones.

Other morphological parameters varied between the two systems, in particular in the formation of central cystic structures, suggesting that expression of recombinant protein apparently evokes non-specific morphological changes under the less optimised culture conditions of the hanging drop system. The greater nutrient and oxygen supply provided to the embryoid bodies by continuous agitation in the cell spin system, possibly compensated for changes in the cell metabolism exerted by recombinant expression. So to eliminate cell culture artefacts, it was decided to use the cell spin system for further experiments.

3.3. The influence of laminin g1 FLAG fusion proteins on basement membrane formation and function

3.3.1. Changed expression and structural organisation of basement membrane components

Daily harvesting and immunohistochemical staining for basement membrane components showed that by eight days of culture, F9 embryoid bodies grown in the presence of retinoic acid have a distinct basement membrane directly underneath the outer layer of cells (results not shown). By day 12, expression had become stronger, a continuous basement membrane could be seen (figures 2.12 and 2.13) and the outer cells had differentiated towards visceral endoderm expressing cellular markers such as cytokeratin 8 (figure 2.15). Earlier studies suggested that F9 monolayers and cell aggregates produce laminin-1 at basal levels in the undifferentiated state, but treatment with $5x10^{-8}$M retinoic acid leads to a great increase in expression (Grover et al., 1983). Results obtained by *in situ* hybridisations show induction of laminin β1 and γ1 chain and collagen type IV(α1) mRNA after four days of differentiation, with maximal levels occurring by day eight and followed by a decline in expression (Rogers

et al., 1990). However in the control embryoid bodies tested here there was little laminin seen expressed before six days in culture. The action of retinoic acid is probably mediated by the retinoic acid receptors (RARs) present in most cell nuclei. These receptors bind specific DNA sequences located in the promoter regions of responsive genes, some of which have been identified in the promoter of the laminin β1 gene (Vasios et al., 1991).

In the spinner flask all F9 derived cell lines expressed laminin-1, however at varying levels, the embryoid bodies expressing recombinant protein apparently having a higher laminin-1 production than the control (figure 2.11). Strangely, upregulation of laminin-1 does not correlate with the presence of intact basement membranes. Laminin-1 upregulation seems to be independent of the recombinant protein expressed and its influence on basement membrane formation. All the embryoid bodies which express recombinant fragments have increased laminin-1 protein levels, although those from γ1V1-3 and γ1III3-5mut cell clones can form relatively intact basement membranes unlike ones expressing the γ1III3-5 polypeptide. Possibly laminin-1 upregulation is an nonspecific side effect of recombinant expression in the F9 embryoid body system or it is due to a feature common to all laminin γ1 fusion constructs. This question could be addressed in further expression studies.

It may be possible that the absence of an intact basement membrane induces the cells to increase the production of basement membrane components via alteration in basement membrane receptor signalling. Indeed the receptor mediated regulation of the laminin α1 chain has been observed in embryoid bodies raised from embryonic stem cells deficient for laminin receptors such as β1 integrin (Aumailley et al., 2000) or dystroglycan (Henry and Campbell, 1998).

Surprisingly, nidogen-1 expression was unchanged at both the mRNA (figure 2.10) and the protein levels (figure 2.11). Recombinant expression of the nidogen-binding site might be expected to lead to loss of nidogen-1 into the growth medium of γ1III3-5 derived embryoid bodies. However it is probable that nidogen-1 molecules occupied by recombinant nidogen-binding sites remain bound in the extracellular matrix by interactions with other basement membrane molecules, such as type IV collagen.

Although loss of nidogen-1 from the embryoid bodies could not be detected, immunofluorescent analysis of the distribution of laminin-1 and nidogen-1 in the γ1III3-5 expressing clones revealed breakdown of the basement membrane. Only control and γ1V1-3

and γ1III3-5mut expressing embryoid bodies showed continuous stretches of laminin-1 staining (figures 2.12 and 2.13). This is as expected as these polypeptides cannot bind nidogen-1 and therefore should not disturb the laminin/nidogen-1 interaction. The basement membrane disorganisation observed for γ1III3-5/A and γ1III3-5/B is apparent as widespread punctate laminin staining all over the embryoid bodies. At higher magnification (figure 2.13) these clones also show a partial lack of colocalisation between laminin and nidogen-1 while embryoid bodies derived of clones γ1V1-3, γ1III3-5mut or from control cells reveal precise codistribution. The occurrence of free laminin suggests the successful competition by the recombinant nidogen-binding site fragment for the binding to nidogen-1.

To eliminate the possibility that this basement membrane change could be an artefact caused by recombinant expression *per se* or by the preceeding genetic manipulation, purified FLAG tagged γ1III3-5 fusion protein was extraneously added to differentiating wild type F9 embryoid bodies. Two day old spinner culture derived embryoid bodies were transferred into single wells of a 96-well plate containing 10µg/ml (0.5µM) of the affinity purified nidogen-binding site (figure 2.4). The embryoid bodies maintained in the multi-well plate grew as rapidly as those in the cell spin system (figure 2.18). However, whole mount staining of these embryoid bodies for laminin after eight days culture in the presence of the polypeptide, showed a markedly disrupted laminin network (figure 2.19), indicating that the changes observed upon the endogenous expression of the nidogen-binding site were not artefactual (figure 2.12 and 2.13).

These data confirm the role of nidogen-laminin binding in basement membrane formation since interference with this interaction causes basement membrane defects. It has been supposed that nidogen-1 acts as a linker molecule between the laminin and collagen type IV network (Aumailley et al., 1989; Yurchenco and Schittny, 1990). *In vitro* experiments using antibodies directed against the nidogen-binding site perturbed basement membrane formation and inhibited epithelial development in organ cultures of embryonic kidney, salivary gland and lung (Ekblom et al., 1994; Kadoya et al., 1997). Recent *in vivo* manipulation of the laminin/nidogen-1 interaction by the targeted removal of the nidogen-binding γ1 LE 4 module in murine embryonic stem cells (Mayer et al., 1998) leads to lethality in the resultant mice. While 50% of all these mutant animals die of unknown causes at or before embryonic day 7, possibly due basement membrane changes, the other 50% survive until birth, but die then or shortly afterwards. These mice show alterations in blood vessel,

brain, eye, kidney and lung formation, including changes in epithelial development (Mayer, personal communication). Surprisingly changes in basement membrane ultrastructure in these mice are limited, though there appears to be defects in the endothelial basement membrane in capillaries as well as alterations in the basement membranes of the eye (Miosge, personal communication). It should be noted, however, that this deletion in the γ1 chain also leads to shortening of one of the laminin arms, which may interfere with laminin polymerisation and hence basement membrane assembly.

Mice lacking nidogen-1 have also been produced and again show few alterations in basement membrane assembly (Murshed et al., 2000), although the retinal inner limiting membrane undergoes disruption (Smyth and Nischt, personal communication). Instead they develop seizures and other neurological defects. These surprisingly restricted changes could be explained by a compensatory effect of nidogen-2 (Kohfeldt et al., 1998; Kimura et al., 1998).

In the embryo, the basement membrane formed between the trophoectoderm and the endoderm, Reichart`s membrane (Salamat et al., 1995), is structurally similar to that formed under the differentiating endoderm in the F9 embryoid body both being markedly thicker than other basement membranes. It is interesting to speculate whether defects in this structure, which could be common to those in the F9 cells described here, are the cause of the 50% early embryonic lethality in the mice lacking the nidogen-binding site. Embryos lacking the basement membrane receptor α-dystroglycan die at the same time in gestation due to rupture of Reichart`s membrane.

The ultrastructure of F9 cell derived basement membranes has not been as well characterised as that of many other basement membranes (Miosge et al., 1999). However basement membranes with varying ultrastructure have been identified *in vivo* (Ogawa et al., 1999; Eyden, 1999), and developmental stages have also been shown in basement membrane formation. For example, in the six day old mouse embryo, while a classical basement membrane had formed between the ectoderm and the yolk sac cells in the extraembryonic part of the egg cylinder, no basement membrane occurred within the embryo although laminin-1 was deposited in a linear pattern. Even at seven days the basement membrane between the ectodermal and the endodermal cell layers is poorly ordered (Miosge et al., 1993). In fact only upon the full ultrastructural development of the basement membrane was nidogen-1 found to be present by immungold histochemistry, suggesting that in the early embryo nidogen-1 may have a role in the maturation of this structure (Miosge et al., 2000a), albeit a role which is not

always crucial for embryonic survival. Possibly nidogen-1 is compensated for by nidogen-2 when absent, as both proteins are often found in the same basement membranes (Kohfeldt et al., 1998; Miosge et al., 2000b), although to date no expression studies have been carried out for nidogen-2 in early embryonic development. Even though nidogen-2 synthesis by F9 cells has not been studied, it was reported that undifferentiated F9 cells produce a 190kD protein, in addition to the 150kD nidogen-1, which appeared to be either associated with, or be immunologically related to, nidogen-1 (Carlin et al., 1983). Synthesis of this protein stopped upon differentiation and only the 150kD form of nidogen could be detected at later stages. This suggests that nidogen-2 may be absent from the F9 embryoid bodies and so could not compensate for the loss of nidogen-1 upon expressing the γ1III3-5 polypeptide, hence explaining the defects seen in the embryoid bodies described here. It should be noted that mice lacking nidogen-2 appear to be phenotypically normal and although only few litters from matings possibly resulting in animals lacking both proteins have been produced, no such animals have yet been born (Smyth, personal communication).

3.3.2. Influence of the various polypeptides on differentiation

For analysis of epithelial development, semithin sections were prepared from control, γ1III3-5/A and γ1III3-5mut derived embryoid bodies. Transmission light microscopy showed on all embryoid bodies, regardless of their ability to form intact basement membranes, smooth surfaces and in places microvilli (figure 2.14). This suggests that the organisation into an outer epithelial layer is independent of the presence of an intact basement membrane. Studies in embryonic stem cell derived embryoid bodies lacking the LAMC1 gene, and so unable to produce an intact laminin trimer, also showed the ability to form a polarised endoderm in the absence of a basement membrane (Murray and Edgar, 2000). The laminin receptor α-dystroglycan, expressed by endodermal cells, is also required to produce an intact basement membrane, but in its absence epithelial differentiation can proceed (Henry and Campbell, 1998). In contrast, loss of β1 integrin receptors in either F9 embryonic carcinoma or D3 embryonic stem cell derived embryoid bodies prevented epithelial differentiation of the visceral endoderm as well as leading to a disruption of the basement membrane (Aumailley et al., 2000; Stephens et al., 1993). This suggests that signalling from certain receptors is required for endodermal differentiation rather than the presence of an intact basement membrane. It should be noted that these peripheral cells did display α-fetoprotein, an early

marker in epithelium formation, even in the absence of β1 integrins (Stephens et al., 1993), so the early stages of endoderm formation appear to be divorced from both the expression of these receptors and the presence of a basement membrane.

TROMA-1, also called Endo A, the murine homologue of cytokeratin 8, is synthesised in mature visceral endoderm, after seven or eight days of culture (Oshima, 1982). γ1III3-5 embryoid bodies express the nidogen-binding site and show an altered localisation of TROMA-1 positive cells (figure 2.15). While control and γ1V1-3 expressing embryoid bodies reveal strong peripheral staining, γ1III3-5/A and γ1III3-5/B producing clones show patchy staining distributed over the embryoid bodies and a reduced staining in the marginal cells. γ1III3-5mut derived cell aggregates, which produce continuous laminin-1 deposits at their periphery, were difficult to interpret and showed fewer TROMA-1 positive cells than all the other clones (figure 2.12). However, these cavitated embryoid bodies were difficult to manipulate during fixation and embedding and this possibly led to epithelial detachment.

Ectopic (stromal) TROMA-1 production in the γ1III3-5 expressing embryoid bodies indicates a separation of expression of this usually tissue-specific gene from epithelial formation. In F9 embryoid bodies changes in the expression pattern of integrins (Morini et al., 1999), Indian hedgehog (Becker et al., 1997), and extracellular matrix components such as laminin-1 and the collagen type IV(α1) (Rogers et al., 1990) characteristically occur concurrent with the formation and subsequent organisation of the visceral epithelium. However it is unknown if laminin is necessary to trigger TROMA-1 synthesis. A colocalisation of TROMA-1 and laminin in γ1III3-5 derived embryoid bodies could indicate a role for laminin in influencing cytokeratin 8 expression. TROMA-1 typically occurs late in endoderm formation (Kemler et al., 1981) and the change of its expression pattern in the embryoid bodies producing the γ1III3-5 polypeptide, suggests alterations in differentiation despite the normal appearance of the endoderm. This implies that TROMA-1 synthesis does require the presence of an organised basement membrane. Cytokeratin 8 is expressed by early embryonic epithelia, however in its absence differentiation in these tissues appears to occur normally (Baribault et al., 1993; Baribault et al., 1994; Brock et al., 1996; Hesse et al., 2000), this suggests that the endoderm formed in these embryoid bodies could in other respects be normal. Immunostaining for α-fetoprotein, a visceral endoderm marker for which many publications report differentiation-specific upregulation at the mRNA level, failed, probably due to its loss into the growth medium.

Transmission light microscopy of 12 day old embryoid bodies revealed cavity formation, seen as bright central regions in the control, γ1V1-3 or γ1III3-5mut expressing clones (figures 2.9). No cavities occured in γ1III3-5/A and γ1III3-5/B derived embryoid bodies which show disrupted basement membranes. Lucifer Yellow VS staining showed large central necrotic cell areas in these clones, while this was absent or far less apparent in the cavity forming embryoid bodies (figures 2.9). Further necrosis appears only to occur where intact basment membranes are absent (figures 2.12 and 2.13).

There are two views of cavitation in the early mouse embryo, based upon experiments in embryoid bodies. In the first a soluble signaling molecule, possibly BMP-4 (Coucouvanis and Martin, 1999), originating from the outer endodermal cell layer of the implanted blastocyst, is thought to induce apoptosis of the inner ectodermal cells. An opposing signal, mediated by contact with the basement membrane, leads to survival of the more peripheral ectodermal columnar cells which line the cavity and contact the basement membrane (Coucouvanis and Martin, 1995). In the second theory, the polarisation of the ectoderm requires a basement membrane, this induces separation and the apoptotic death of the underlying central non differentiated cells. Here BMP-4 either plays no role, or without a basement membrane is unable to induce differentiation or apoptosis (Murray and Edgar, 2000).

The results presented here show that with the disruption of the basement membrane, there is impairment of cavity formation which finally results in the embryoid body exceeding its nutritional supply and undergoing central necrosis (figure 2.9). This is in agreement with the second theory of cavity formation based upon studies with LAMC1-/- embryoid bodies which are also unable to form basement membranes and so fail to cavitate.

PECAM-1 (platelet/endothelial cell adhesion molecule-1; Tang and Honn, 1995) staining was performed despite a lack of reports on endothelial differentiation in F9 embryoid bodies. These experiments revealed PECAM-1 expression in all clones but at varying levels. Embryoid bodies formed by γ1V1-3 or γ1III3-5 expressing cells and the control show a similar pattern of staining with a marked increase between days 10 and 12. γ1III3-5mut shows highest signal intensities, but its differentiation state might be distinct from the other clones due to pronounced cavity formation. However, the PECAM-1 staining did not show vessel-like structures due to the limited differentiation repertoire of F9 embryoid bodies. Still, it was a valuable tool in determining the reproducibility of F9 cell differentiation in the cell spin system.

3.3.3. Influence on the permeability properties of basement membranes

The function of basement membranes as a filtration barrier was tested by use of a diffusion assay. Embryoid bodies expressing the laminin γ1 FLAG fusion proteins as well as control ones were incubated with dextran molecules of 10 and 70 kD. Analysis of diffusion distances into the embryoid bodies by laser scanning microscopy and calculation of the diffusion coefficients were used to compare the permeability properties of all F9 derived clones (figure 2.17). This showed that basement membranes synthesised by control and γ1V1-3 expressing embryoid bodies were the most effective diffusion barriers for both 10 and 70kD dextrans. Expression of the nidogen-binding site construct γ1III3-5 increased diffusion rates, which can be explained by the disrupted basement membranes (figure 2.12 and 2.13). Analysis of clone γ1III3-5mut, which shows continuous laminin-1 deposition all around the embryoid body, was impaired by its extensive cavitation at the time of analysis (figure 2.12) The diffusion coefficients of about $5x10^{-8}cm^2/sec$ for control or γ1V1-3 derived embryoid bodies and $1x10^{-7}cm^2/sec$ for the γ1III3-5 expressing clones fit to inhibited and facilitated diffusion, respectively, as previously measured for embryonic stem cell embryoid bodies (Wartenberg et al., 1998a).

3.4. Perspectives

Before starting further complex experiments investigating *in vivo* the role of the laminin/nidogen-1 interaction, it would be interesting to determine if F9 embryoid bodies produce nidogen-2. While this has a 100 to 1000 fold lower affinity for the nidogen-1 binding module γ1III4, it can also bind to the laminin γ1 chain via a second epitope, as well as interact with collagens type I and IV and perlecan (Kohfeldt et al., 1998). If nidogen-2 is expressed, this would indicate that the recombinant nidogen-binding site is very efficient in blocking both nidogens in their basement membrane stabilising function. If not, detailed studies of basement membrane formation in nidogen-1/nidogen-2 double null mutant mice will help to understand further the interplay between both nidogens and laminin and the type IV collagen network.

To date the role of basement membrane components involved in epithelial differentiation has been studied by antibody perturbation in organ culture experiments. Also recent results from skin organ cultures have shown that the recombinant γ1III3-5 nidogen-binding site fragments could interfere with basement membrane formation between keratinocytes and fibroblasts in a manner similar to that described in this thesis (Breitkreutz, personal communication). It would be interesting to understand, how the basement membrane influences cellular differention *in vivo* and how this is effected by basement membrane disruption. This could be addressed by the production of a transgenic animals expressing the nidogen-binding site under the control of e.g. a mammary/lactation-specific promoter. Mammary-specific disruption of the basement membrane has the additional advantage that it is unlikely to be lethal, and that male animals can transmit the transgene in the event that females are unable to lactate and so fail to rear offspring. Further the mammary epithelium highly active can be monitored throughout its cyclical development.

4. Materials and Methods

4.1. Culture and maintenance of tissue culture cell lines

4.1.1. Mouse teratocarcinoma cell line

Mouse teratocarcinoma F9 (CRL 1720; American Type Culture Collection; DSM ACC 112) cells were plated on gelatinised dishes, cultured in DMEM containing 200U/ml penicillin, 200µg/ml streptomycin, 2mM L-glutamine and 10% fetal calf serum (GIBCO BRL™) and grown at 37°C in a humified incubator with a 5% CO_2 atmosphere. For embryoid body cultures, untransfected F9 cells were maintained in normal growth medium supplied with $5x10^{-8}$M all-trans retinoic acid (R-2625; Sigma-Aldrich) for 12 days either using the cell spin system (Integra Bioscience) or the hanging drop method (Hogan et al., 1994).

In the cell spin system embryoid body cultures are started with 100ml of single cell suspensions of about $1.2x10^5$ cells/ml in siliconated spinner flasks. After one day when small aggregates have formed, more growth medium is added up to a volume of 250ml and changed daily. In this system embryoid bodies are maintained under continuous agitation by magnetic stirring which guarantees optimal nutrition and oxygen support.

The hanging drop method starts with 20µl cell suspensions of $1.5x10^5$ cells/ml which hang on a petri-dish. After two days when these drops contain small cell aggregates 10ml medium is added and changed every second day till the embryoid bodies are harvested.

4.1.2. Human embryonic kidney cell line

293-EBNA cells (Invitrogen) were cultured in DMEM-F12 containing 200U/ml penicillin, 200µg/ml streptomycin, 20mM L-glutamine, 10% FBS (GIBCO BRL™) and 50µg/ml G418 (GIBCO BRL™) at 37°C in a humified incubator with a 5% CO_2 atmosphere. After transfection, to maintain expression in 293-EBNA cells transfected with the γ1III3-5 construct, G418 was removed and the cells were treated with 0.5µg/ml puromycin (Sigma-Aldrich).

4.1.3. Establishment of stably transfected cell lines

$1x10^6$ cells were electroporated with 5µg circular plasmid DNA in 800µl growth medium using the Gene Pulser® II and 0.4cm cuvettes (Biorad). Resistance and voltage were set to 500µF and 230V, respectively and time constants of approximately 7sec were obtained. For F9 cells, selection was initiated 24 h after electroporation with medium containing 1mg/ml G418 (GIBCO BRL™). G418 resistant clones were picked, expanded and screened by immunoblotting and positive clones were frozen in liquid nitrogen. 293-EBNA cells which do not allow clonal selection were expanded during selection with puromycin (Sigma-Aldrich) at a concentration of 0.5µg/ml.

4.1.4. Thawing and freezing cells

Frozen cells were quickly transferred from liquid nitrogen into a 37°C waterbath, thawed, washed with prewarmed growth medium and seeded on tissue culture plates (Greiner). For the purpose of freezing, cells were trypsinised, resuspended in growth medium supplemented with 20% fetal calf serum (GIBCO BRL™) and 10% DMSO (Sigma-Aldrich) and slowly cooled to -80°C. After overnight incubation at -80°C, cells were kept in liquid nitrogen for long term storage.

4.2. Molecular cloning

4.2.1. Bacterial cell culture

The bacterial strain DH5α was grown following the instructions of Sambrook et al. (1989). For transformation and production of competent *E.coli* cells the method of Hanahan (1983) was applied.

4.2.2. DNA preparation

Small amounts of plasmid DNA (10µg) were isolated from 2ml *E.coli* cultures following the protocol of Birnboim (1983). For production of up to 100µg of plasmid DNA the Midi-Prep Kit (Macherey and Nagel) was used. The DNA concentration was measured photometrically at 260nm and calculated on the basis of the assumption that an optical density of 1 equals a concentration of 50µg/ml double stranded DNA. DNA gel electrophoresis with 1kb DNA ladder (GIBCO BRL™) was performed in 1x TAE buffer as described in Sambrook et al. (1989).

4.2.3. RNA preparation and northern blot analysis

Total RNA was isolated from 12 day old, differentiated F9 derived embryoid bodies following the protocol of Chomczynski and Sacchi (1987) and resuspended in diethyl pyrocarbonate (DEPC)-H_2O. The concentration was calculated from the optical density at 260nm assuming that an OD value of 1 corresponds to 40µg/ml RNA. Gel electrophoresis of 20µg RNA on a 1% formaldehyde agarose gel with 0.24-9.5 kb RNA Ladder (GIBCO BRL™), RNA transfer to a nylon membrane by vacuum blotting and northern blot analysis were performed as described in Sambrook et al. (1989). Membranes were probed with a [α^{32}P]dCTP labeled DNA probes for mouse nidogen-1 and human GAPDH which served as a loading control. Bands were detected using autoradiography.

4.2.4. Labeling of DNA probes

DNA probes were labeled with [α^{32}P]dCTP (Hartmann Analytic) by random priming as reported by Feinberg and Vogelstein (1983) using the Ladderman™ Labeling Kit (TaKaRa Shuzo Co., Ltd.). For standardisation of loading a 500bp HindIII/XbaI fragment of human GAPDH (gene bank accession number AJ005371) was isolated and labeled. A 1154-bp PCR product of mouse nidogen-1 cDNA using AGA ATC CAT GCT ACA TTG GC (sense) and TGG GTG CCA TCC ATC TTT GC (antisense) primers of the protein coding region served as a DNA probe for nidogen-1 mRNA (gene bank accession number: X14480).

4.2.5. Polymerase chain reaction (PCR)

Oligonucleotides were ordered at MWG-Biotech AG in highly purified salt free quality and primer concentration was determined photometrically at 260nm assuming that an optical density of 1 equals 37µg/ml single stranded DNA. Since only fragments below 2kb were amplified, Taq polymerase (Perkin Elmer; Roche) was used at a concentration of 0.05U/µl in combination with 0.25µM primer, 0.125mM dNTP mix (Pharmacia), 1x reaction buffer supplemented with 0.15mM $MgCl_2$ (Perkin Elmer; Roche) and 10ng plasmid DNA in 100µl reaction volume. Using the RoboCycler® Gradient 40 (Stratagene) the template DNA was denatured at 95°C for 2min followed by 30 amplification cycles each comprising 1min incubation at 95°C, 1min at annealing temperature and 1min at 72°C. Per kb fragment length an elongation time of 1min was calculated and the annealing temperature was defined by addition of 3°C to the lowest melting temperature $T_m = 4x(G+C) + 2x(A+T)$.

4.2.6. Recombinant techniques

All DNA modifying enzymes (e.g. restriction enzymes, T4 DNA ligase and T4 polynucleotide kinase) were obtained from New England Biolabs and used following the manufacturers instructions. Site directed mutagenesis was performed using the Transformer™ Site-Directed Mutagenesis Kit (Clontech Laboratories, Inc.). DNA sequences were determined in the service laboratory of the ZMMK (Center for Molecular Medicine Cologne) using an ABI Prism™ 377 DNA Sequencer (Applied Biosystems). Sequences were analysed using the Wisconsin Sequence Analysis Package, Version 8.1 (Genetics Computer Group, Inc.).

4.3. Microscopy

4.3.1. Preparation of cryosections

After a brief wash in 1x PBS, pH 7.4, embryoid bodies were incubated in 1% paraformaldehyde for 30min at room temperature, rinsed in 1x PBS, pH 7.4, twice, transferred into Tissue Tek (Sakura) via stepwise incubation in 5% sucrose for 24h at 4°C, in 20%

sucrose for 2h at 37°C, and for 1h at 37°C in a 1:1 mix of Tissue Tek and 20% sucrose. All sucrose solutions were prepared in 1x PBS, pH 7.4, supplemented with 0.05% sodium azide. After 1h incubation in Tissue Tek embryoid bodies were frozen in liquid nitrogen and stored at -80°C. Subsequent cryosectioning with a LEICA CM3050 cryostat was performed to obtain 7µm sections which were collected on Shandon histoslides (Life Science) and stored at -20°C.

4.3.2. Preparation of semithin sections

After overnight fixation in 2% paraformaldehyde, 1% glutardialdehyde, 0.2% picric acid (Sigma-Aldrich) in 0.1M HEPES buffer and three subsequent washes in 0.1M HEPES buffer each for 20min, embryoid bodies were dehydrated in 50% ethanol for 10min, in 70% ethanol supplemented with 1% uranyl acetate overnight followed by 10min incubation in 90% ethanol and 3x 10min in 100% ethanol. After dehydration the embryoid body material was immersed into a 1:1 mix of propylene oxide and ethanol for 2x 10min, into propylene oxide for 2x 10min followed by a 1:1 mix of araldite and propylene oxide for 6h and 2h incubation in araldite at 40°C. Subsequent polymerisation proceeded for 12h at 45°C and 48h at 60°C. Semithin sections of 1µm thickness were produced with an ultramicrotome (type OMU 3, Reichert), stained with methlyene blue (Romeis, 1989) and analysed with transmission light microscopy using an Axiovert Microscope (Zeiss). In addition transmission light microscopy with an Achrostigmat 10x/0.25NA objective was used to determine morphology of embryoid bodies still floating in growth medium.

4.3.3. Immunofluorescence staining

A rabbit polyclonal antiserum to laminin-1 (M. Paulsson, Cologne), a rat monoclonal anti-nidogen [entactin] G2 domain antibody (MAB 1884; Chemicon), a rat monoclonal TROMA-1 antibody (Kemler et al., 1981) and a purified rat anti-mouse CD31 (PECAM-1) monoclonal antibdoy (Cat.No. 01951D; Pharmingen) served as primary antibodies. As secondary antibodies DTAF-conjugated goat anti-rabbit IgG, Cy3™-conjugated goat anti-rabbit IgG, Cy3™-conjugated goat anti-rat IgG and Cy5™-conjugated syrian hamster anti-rat IgG (all Jackson Immuno Research Laboratories, Inc.) were used. All stainings were performed using 1x PBS, pH 7.4, as a buffer system.

For whole mount stainings embryoid bodies were fixed in ice-cold methanol:acetone (7:3) for 1 h at -20°C, washed with 0.1% Triton-X 100 and either stored in PBS at 4°C or blocked in 10% milk powder for 1h. After that embryoid bodies were incubated with the primary antibody for 1.5 h on a rocking device, washed with 0.01% Triton-X 100, incubated with the second antibody for 1h, washed again and stored in PBS at 4°C. For analysis a laser scanning confocal microscope (LSM 410; Zeiss) with a Plan-Neofluar 25x/0.8NA objective and 4.5x zoom was used in case of laminin-1. PECAM-1 staining was scanned using a 16x/0.5NA objective and a method called "extended type of focus". For that purpose five serial sections of 20µm thickness through single embryoid bodies were examined for PECAM-1 signals and their overlay used to detect three dimensional vascularised tissues. The percentage of total area stained for PECAM-1 was determined with the computer programme LSM DUMMY (Zeiss) which was also used for the determination of embryoid body size.

7µm cryosections were briefly fixed with 0.5% paraformaldehyde and blocked with 5% normal goat serum (ICN, Biomedicals) and 0.2% Tween for 30min. The primary antibody was applied for 1h followed by three washes with the blocking solution. The sections were incubated with the secondary antibody for 45min, washed, mounted in fluorescent mounting medium (DAKO) and examined using a Axiophot Microscope (Zeiss) equipped with a fluorescent light source.

4.3.4. Staining for cell death and viability

Two fluorescent dyes were used to detect dead cells, Lucifer Yellow VS (Sigma-Aldrich) and SYTOX (Molecular Probes, Inc.). Lucifer Yellow VS is a highly charged, hydrophilic dye which cannot pass through hydrophobic membranes and is excited at a wavelength of 450nm (Stewart, 1981). It diffuses into dead cells with disrupted plasma membranes and remains in necrotic cell areas due to high binding affinity to SH-groups. Lucifer Yellow VS was added to embryoid bodies floating in growth medium to a final concentration of 180µM, incubated for 40min at room temperature in darkness and removed in two washing steps with growth medium. Embryoid bodies were then incubated overnight in normal growth conditions to remove any Lucifer Yellow VS incorporated by endocytosis into viable cells. Embryoid bodies were analysed by means of laser scanning microscope with a Plan-Neofluar 25x/0.8NA objective at a wavelength of 488nm.

SYTOX green nucleic acid stain penetrates cells with compromised plasma membranes, intercalates into DNA and upon binding to double stranded DNA its fluorescence intensity increases 1000 times. Necrotic cell areas where DNA has mostly been degraded do not react with SYTOX. Thus it can be used to detect single dead cells in otherwise intact tissue. SYTOX was added to embryoid bodies floating in growth medium at a final concentration of 0.5µM, incubated for 10min at 37°C and analysed by means of laser scanning microscope with a Plan-Neofluar 25x/0.8NA objective when excited with the 488nm wavelength of the argon-ion laser.

4.4. Diffusion assay

After 8 days of culture, embryoid bodies were rinsed in E1 solution (135mM NaCl, 5.4mM KCl, 1.8mM $CaCl_2$ x 2 H_2O, 1mM $MgCl_2$ x 6 H_2O, 10mM glucose, 10mM HEPES, pH 7.5), transferred into 10µM rhodamine dextran solution of either 10kD (neutral D-1824; Molecular Probes, Inc.) or 70kD (neutral D-1841; Molecular Probes, Inc.), incubated for 5 minutes, briefly washed twice in E1 solution to reduce background staining and analysed with a laser scanning microscope (LSM 410; Zeiss) by means of the optical probe technique (Wartenberg et al., 1998b). Based on the property of the laser scanning microscope to produce series of optical sections of defined thickness and precise distance from each other throughout the embryoid body, small regions of interest (600µm^2 = 40x40 pixel) are analysed in z-orientation at a distance of 10µm. The mean fluorescence intensity in each region of interest is measured from the embryoid body surface to its center, plotted against the penetration depth of the laser beam and depicted in a graph from which the diffusion distance can be derived. The diffusion coefficient may then be calculated based on the Einstein and Smoluchowski equation $D = x^2/2t$, where x describes the distance of diffusion in a distinct time period t.

4.5. Protein chemistry

4.5.1. Affinity chromatography

The FLAG fusion protein laminin γ1III3-5 was expressed in 293-EBNA cells (see 6.1.3.) using the CMV promotor (Smyth et al., 2000) and by use of the BM40 signal peptide secreted to the medium after passing the Golgi apparatus. Serum-free supernatant was dialysed overnight (molecular weight cut off of 12-14kD), centrifuged and loaded onto a anti-FLAG M2 agarose affinity column (A1205; Sigma-Aldrich) buffered in TBS, pH 7.4. Non-binding proteins were washed away using TBS, pH 7.4, while bound FLAG tagged protein was eluted with 100µg/ml FLAG peptide (F3290; Sigma-Aldrich) following the manufacturers instructions.

4.5.2. Treatment of wild type F9 embryoid bodies with affinity purified FLAG fusion protein g1III3-5

Untransfected F9 cells were grown to confluency, trypsinised and transferred into the cell spin system (Integra Bioscience) where $5x10^{-8}$M all-trans retinoic acid (R-2625; Sigma-Aldrich) was added to induce differentiation. At day two of culture embryoid bodies were transferred into single wells of a 96-well plate filled with 100µl normal cell culture medium supplemented with affinity purified FLAG fusion protein γ1III3-5 at a concentration of 10µg/ml. The medium was changed daily to ensure a maintained concentration of retinoic acid and peptide.

4.5.3. Immunoblot analysis

To determine the expression level of laminin γ1-FLAG fusion proteins in supernatants of transfected F9 cells these were TCA precipitated and separated by SDS-PAGE on a 15% polyacrylamide gel. Proteins were transferred onto nitrocellulose membranes and probed with 10µg/ml BioM2 monoclonal antibody against the FLAG epitope (F9291; Sigma-Aldrich) and a rabbit polyclonal antiserum against mouse BM40 (Nischt et al., 1991). For determination of the endogenous level of laminin-1 and nidogen-1, embryoid bodies were homogenised with

0.15M NaCl in 50mM Tris/HCl, pH 7.4. The homogenates were diluted 1:1 (v/v) in Laemmli sample buffer (1970), submitted to SDS-PAGE on a 3-10% polyacrylamide gel in the presence of 5% β-mercaptoethanol, transferred to nitrocellulose and incubated with a rabbit polyclonal antiserum against laminin-1, a rat monoclonal antibody against the nidogen [entactin] G2 domain (MAB 1884; Chemicon) and a mouse monoclonal antibody against human actin (sc-8432; Santa Cruz Biotechnology, Inc.). As secondary antibodies either streptavidin-biotinylated horseradish peroxidase (HRP) complexes (RPN1051; Amersham Life Science) or HRP conjugated immunoglobulins from swine anti-rabbit (P0399; DAKO), rabbit anti-rat (P0450; DAKO) or rabbit anti-mouse (P0260; DAKO) immunoglobulin G antisera were used. Immunoreactive proteins were detected using the ECL chemiluminescent detection system.

5. Appendix

5.1. Abbreviations

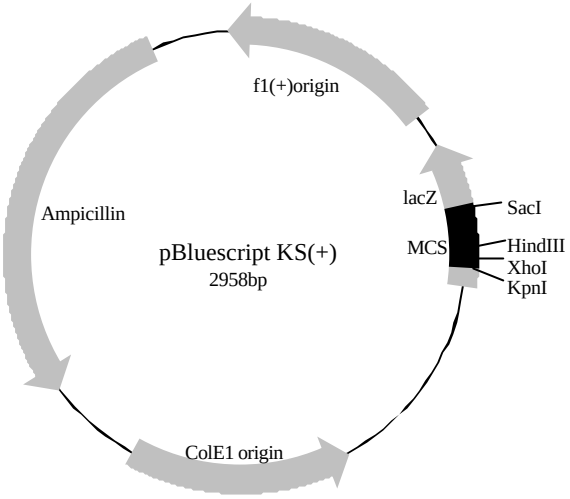
A	adenosine
BM40	basement membrane protein 40kD
C	cytidine
cDNA	complementary DNA
CMV	cytomegalovirus
dCTP	desoxycytosintriphosphate
DMEM	Dulbecco`s modified Eagle medium
DMSO	dimethylsulfoxide
DNA	desoxyribonucleic acid
dNTP	desoxyribonucleotidtriphosphate
EBNA	Epstein Barr nuclear antigen
EDTA	ethylenediaminetetraacetic acid
EF 1α	elongation factor 1 α
EGF	epidermal growth factor
EHS	Engelbreth-Holm-Swarm
F	Farad
FBS	fetal bovine serum
G	guanosine
G418	geneticin
GAPDH	glycerinaldehydephosphate dehydrogenase
GFP	green fluorescent protein
HRP	horseradish peroxidase
kB	kilobases
kD	kilodalton
LE	laminin EGF-like
MCS	multiple cloning site
mRNA	messenger RNA
NF200	neurofilament 200
OD	optical density
PAGE	polyacrylamide gel electrophoresis

PBS	phosphate buffered saline
PCR	polymerase chain reaction
PECAM-1	platelet/endothelial cell adhesion molecule 1
RNA	ribonucleic acid
RSV	Rous sarcoma virus
SDS	sodium dodecyl sulfate
T	thymidine
Taq	Thermophilus aquaticus
TBS	tris buffered saline
TCA	trichloric acid
T_m	melting temperature
TROMA-1	trophoectodermal marker 1

5.2. Amino acid code

A	Ala	Alanine	M	Met	Methionine
C	Cys	Cysteine	N	Asn	Asparagine
D	Asp	Aspartic acid	P	Pro	Proline
E	Glu	Glutamic acid	Q	Gln	Glutamine
F	Phe	Phenylalanine	R	Arg	Arginine
G	Gly	Glycine	S	Ser	Serine
H	His	Histidine	T	Thr	Threonine
I	Ile	Isoleucine	V	Val	Valine
K	Lys	Lysine	W	Trp	Tryptophane
L	Leu	Leucine	Y	Tyr	Tyrosine

5.3. Vectors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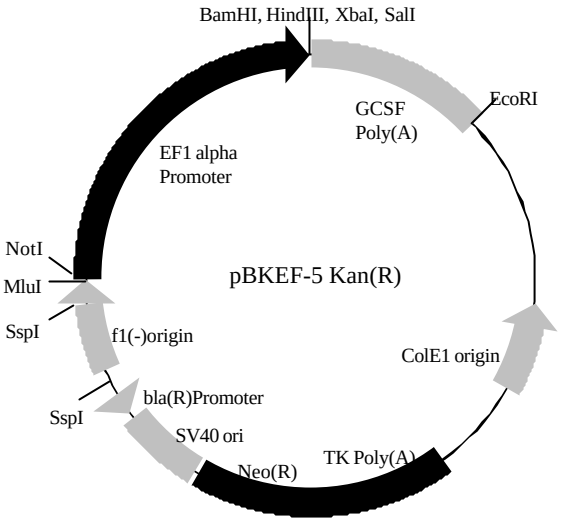
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NotI
MluI
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f1(-)origin
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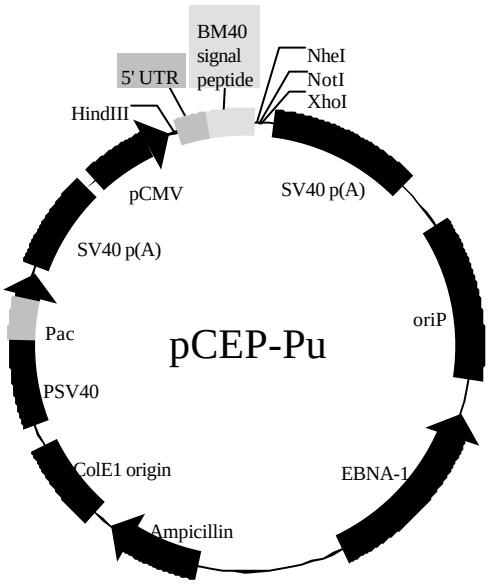
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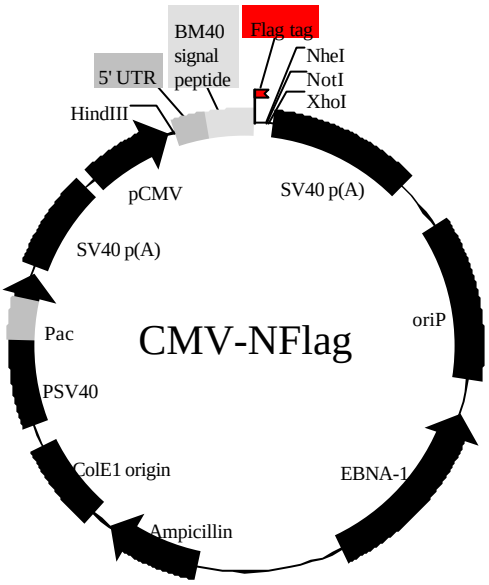
Ampicillin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pCMV
SV40 p(A)
SV40 p(A)
oriP
Pac
CMV-NFlag
PSV40
ColE1 origin
EBNA-1
Ampicillin

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