

Expression of the nidogen-binding site of the laminin γ 1 chain disturbs basement membrane formation and maintenance in F9 embryoid bodies

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Summary

Basement membranes contain two major molecular networks consisting of laminin and collagen IV. Previous antibody perturbation experiments suggest that the interaction between laminin and nidogen-1 is necessary for proper basement membrane formation and epithelial development, whereas results from gene ablation experiments in mice show that both basement membranes and general development are grossly normal in the absence of nidogen-1. To refine the perturbation approach, we produced F9-teratocarcinoma-cell-derived embryoid bodies in the presence of recombinantly expressed nidogen-binding sites localized within the γ III3-5 laminin fragment. We found basement membranes were disrupted

in γ III3-5-expressing embryoid bodies. As a measurement of basement membrane function, we tested permeability and detected drastically increased diffusion rates in correlation with basement membrane disruption. Furthermore, TROMA-1 localization in embryoid bodies expressing the nidogen-binding site was altered, suggesting separation of epithelium-specific gene expression from the formation of the actual epithelium when occurring in the absence of an organized basement membrane.

Key words: Laminin-nidogen complex, Basement membrane permeability, Epithelial differentiation

Introduction

Laminin has been shown to be a prerequisite for the in vivo production of the basement membrane (Smyth et al., 1999), a structure crucial for the normal development of many epithelia (Murray and Edgar, 2000). Results from a multitude of sources (Paulsson et al., 1987; Mann et al., 1988; Aumailley et al., 1989; Fox et al., 1991; Aumailley et al., 1993) suggest a model of the basement membrane in which the two major self-assembling networks, formed by laminin and collagen IV, are connected by nidogen-1 (Yurchenco and Schittny, 1990).

Nidogen-1, a sulphated 150 kDa glycoprotein, co-purifies with laminin-1 upon EDTA extraction (Paulsson et al., 1987). The nidogen-binding site on laminin-1 has been localized to laminin EGF-like (LE) module 4, which has subsequently been determined at atomic resolution (Stetefeld et al., 1996; Baumgartner et al., 1996). It was shown that this interaction might be significant for proper basement membrane formation in organ cultures, as the presence of antibodies raised against the nidogen-binding site on laminin-1 disrupted the basement membrane and reduced branching epithelial morphogenesis in a number of different organs (Ekblom et al., 1994; Kadoya et al., 1997). The results suggested that the formation of the laminin/nidogen-1 complex would be a key event during basement membrane deposition and epithelial differentiation. Detailed analysis of the sites of laminin-1 and nidogen-1 gene expression by in situ hybridization (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, whereas 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 and possibly dictate the site of basement membrane formation (Dziadek et al., 1995).

Surprisingly, the loss of nidogen-1 in the mouse by homologous recombination does not result in any gross changes in development, and the basement membranes in these animals appear to be structurally normal. However, it could be shown that the related protein (nidogen-2) was redistributed and/or upregulated in certain tissues in the nidogen-1-null animals. Nidogen-2 was initially isolated from an osteoblast-like cell line and shown to have 27.4% identity to nidogen-1 at the amino acid level (Kimura et al., 1998). Recombinant human nidogen-2 has a similar structure to nidogen-1 but binds to the nidogen-binding site γ III4 of the murine laminin γ 1 chain with a 100- to 1000-fold lower affinity than murine nidogen-1 (Kohfeldt et al., 1998). Like nidogen-1, nidogen-2 also binds collagens I and IV, and perlecan in vitro (Kohfeldt et al., 1998). These results suggested that nidogen-2 could take over certain of the roles of mammalian nidogen-1 in its absence, but they did not explain why there appeared to be no basement membrane defect in *Caenorhabditis elegans* upon the loss of the single nidogen family member in this species (Kim and Wadsworth, 2000). The possibility cannot be ruled

out that, in the original perturbation experiments, the binding of antibodies prevented other interactions of the laminin molecule by steric hindrance caused by the large immunoglobulin molecule.

To study the importance of laminin-nidogen binding in an alternative and less artefactual manner, we attempted to block the interaction by the introduction of an excess of recombinantly expressed nidogen-binding sites during basement membrane formation in F9 derived embryoid bodies. F9 cells have a very limited differentiation ability and so offer a simpler model of basement membrane development than that seen in vivo or in organ culture systems, presumably with a lesser ability to compensate for molecular defects. Here, we show that differentiation of such cells in the presence of the exogenously added or endogenously recombinantly expressed nidogen-binding site led to defects in the basement membrane, increased permeability and abnormal differentiation suggesting that the laminin-nidogen interaction might be highly significant in epithelial development.

Materials and Methods

Construction of laminin- γ 1/FLAG fusion constructs

Full-length mouse laminin γ 1 cDNA was prepared by reverse transcription of mouse kidney total RNA. LE modules 3-5 of domain III (amino acids 771-932) and 1-3 of domain V (amino acid position 340-492) of the γ 1 chain (SWISS-PROT primary accession number P02468) were amplified by PCR and cloned in frame into the *NheI/NotI* restricted CMV-NFlag vector, a pCEP-Pu-based vector (Kohfeldt et al., 1997) with the FLAG tag inserted between the BM40 signal peptide and the insert. To produce the asparagine to serine mutation at residue 802, which inhibits nidogen binding, the cDNA encoding LE modules 3-5 was subcloned into KS (+) pBluescript and site-directed mutagenesis performed using the primer N802S (5'-GTG TAA CGA CAA TAT TGA CCC CAG CGC GGT TGG C-3'). The three N-terminal FLAG-tagged *HindIII/XhoI* fragments were then subcloned into the *HindIII/SalI* sites of pBKEF (a fusion product of pBK-CMV and pEF BOS; Mizushima and Nagata, 1990) downstream of the EF1 α promoter. All cloning steps were confirmed by sequencing.

Cell culture

Mouse teratocarcinoma F9 (CRL 1720; American Type Culture Collection; DSM ACC 112) cells were maintained in DMEM containing 200 U ml⁻¹ penicillin, 200 μ g ml⁻¹ streptomycin, 2 mM L-glutamine and 10% foetal calf serum (Gibco BRL) and grown at 37°C in a humidified incubator with a 5% CO₂ atmosphere. For embryoid body cultures, F9 cells were maintained in normal growth medium supplied with 5 \times 10⁻⁸ M all-*trans*-retinoic acid (R-2625; Sigma-Aldrich) for 12 days using the cell spin system (Integra Bioscience).

1 \times 10⁶ F9 cells were electroporated with 5 μ g circular plasmid DNA. Selection was carried out with 1 mg ml⁻¹ G418 (Gibco BRL) and G418 resistant clones were picked and grown in 48-well plates. These were screened for expression by immunoblotting with the Bio M2 monoclonal antibody directed against the FLAG epitope (F9291; Sigma-Aldrich).

Immunofluorescence staining

Rabbit polyclonal antisera to laminin-1 (M.P.), perlecan and nidogen-2 (R. Timpl, Max Planck Institute for Biochemistry, Munich), a rat monoclonal anti-nidogen G2 domain antibody (MAB 1884;

Chemicon) and a rat monoclonal TROMA-1 antibody (Kemler et al., 1981) were used as primary antibodies. As secondary antibodies, fluorescein-conjugated goat anti-rabbit IgG, Cy3TM-conjugated goat anti-rabbit IgG or Cy3TM-conjugated goat anti-rat IgG (all from Jackson ImmunoResearch Laboratories) were used. All stainings were performed in PBS, pH 7.4. The embryoid bodies were either fixed with 1% paraformaldehyde for 30 minutes at room temperature and embedded in Tissue tek (Sakura) for subsequent cryosectioning or fixed in ice-cold methanol:acetone (7:3) for 1 hour at -20°C, washed with 0.1% Triton-X 100, stored in PBS at 4°C and used for whole-mount immunostaining.

7 μ m cryosections were briefly fixed with 0.5% paraformaldehyde, blocked with 5% normal goat serum (ICN Biomedicals) and 0.2% Tween for 30 minutes, then the primary antibody was applied for 1 hour followed by three washes with the blocking solution. The sections were incubated with the secondary antibody for 45 minutes, washed, mounted in fluorescent mounting medium (DAKO) and examined using a Axiophot microscope (Carl Zeiss) equipped with a fluorescent light source. Whole embryoid bodies were blocked in 10% milk powder for 1 hour, incubated with the primary antibody for 1.5 hours on a rocking device, washed with 0.01% Triton-X100, incubated with the second antibody for 1 hour, washed again and analysed using a laser scanning confocal microscope (LSM 410; Carl Zeiss) with a Plan-Neofluar 25 \times /0.8NA objective and 4.5 \times zoom.

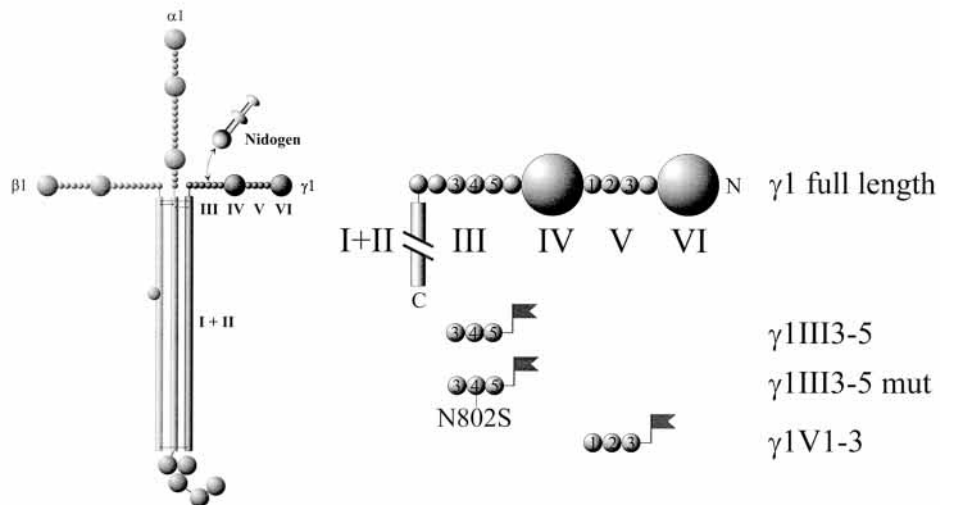
Immunoblot analysis

To determine the expression level of γ 1-FLAG fusions by transfected F9 cells, culture supernatants were TCA precipitated and separated by SDS-PAGE on a 15% polyacrylamide gel. Proteins were transferred onto nitrocellulose membranes and probed with 10 μ g ml⁻¹ 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 lysed in SDS sample buffer (Laemmli, 1970), submitted to SDS-PAGE on a 3-10% polyacrylamide gel in the presence of 5% β -mercaptoethanol. Proteins were 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). As secondary reagents, 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 enhanced chemiluminescent detection system.

Diffusion assay

At day 8 of culture, embryoid bodies were rinsed in E1 solution (135 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl₂.2H₂O, 1 mM MgCl₂.6H₂O, 10 mM glucose, 10 mM HEPES pH 7.5), transferred into 10 μ M rhodamine dextran solution of either 10 kDa (neutral D-1824; Molecular Probes) or 70 kDa (neutral D-1841; Molecular Probes) with a hydrodynamic radius comparable to those of 40 kDa and 280 kDa globular proteins. After incubation for 5 minutes, the embryoid bodies were briefly washed twice in E1 solution to reduce background staining and analysed with a laser scanning microscope (LSM 410; Carl Zeiss) by means of the optical probe technique (Wartenberg et al., 1998a). The diffusion coefficient D was calculated based on the Einstein and Smoluchowski equation $D=x^2 \div 2t$, where x describes the distance of diffusion in a distinct time period t (Wartenberg et al., 1998b).

Fig. 1. Production of nidogen-binding site constructs and controls. The site for interaction between laminin-1 and nidogen-1 has been localized to LE module 4 of domain III of the laminin $\gamma 1$ chain (Mayer et al., 1993). Two different FLAG fusion proteins (indicated with flags) were constructed and modified with the BM40 signal peptide to ensure secretion to the extracellular space, $\gamma 1$ III3-5 coding for LE modules 3-5 and $\gamma 1$ V1-3 comprising three LE modules of domain V.



Treatment of wild-type F9 embryoid bodies with affinity-purified FLAG fusion protein $\gamma 1$ III3-5

The FLAG fusion protein $\gamma 1$ III3-5 was expressed in human embryonic kidney 293-EBNA cells using the CMV promoter (Smyth et al., 2000), loaded onto a anti-FLAG M2 affinity gel (A1205; Sigma-Aldrich) column and eluted with $100 \mu\text{g ml}^{-1}$ FLAG[®] peptide (F3290; Sigma-Aldrich) following the manufacturer's instructions. SDS-PAGE analysis of the purified protein showed a single band after Coomassie staining (data not shown).

Untransfected F9 cells were grown to confluence, trypsinized and transferred into the Cell Spin system (Integra Bioscience), where all-*trans*-retinoic acid (R-2625; Sigma-Aldrich) was added to a concentration of 5×10^{-8} M to induce differentiation. At day two of culture, embryoid bodies were transferred into single wells of a 96-well plate filled with $100 \mu\text{l}$ normal cell culture medium supplemented with affinity purified FLAG fusion protein $\gamma 1$ III3-5 at a concentration of $10 \mu\text{g ml}^{-1}$. Every day, the medium was changed and new protein was added. At day 8 of culture, embryoid bodies were fixed and stained for laminin-1.

Results

Expression of the nidogen-1-binding site of the laminin $\gamma 1$ chain in F9 cells

To block the interaction between laminin and nidogen-1, the nidogen-binding site $\gamma 1$ III4 of the $\gamma 1$ chain was recombinantly expressed in F9 cells. This polypeptide binds to nidogen molecules and so inhibits binding to the intrinsic laminin $\gamma 1$ chains (Mayer et al., 1993; Poschl et al., 1996). The LE modules 3-5 of domain III of the laminin $\gamma 1$ chain ($\gamma 1$ III3-5) were cloned downstream of the elongation factor 1α (EF1 α) promoter (Niimi and Kitagawa, 1997) for constitutive protein expression. Sequences encoding the BM40 signal peptide and the FLAG tag were added to the N-terminus of the polypeptides to enable efficient secretion to the extracellular space (Mayer et al., 1993; Yurchenco et al., 1997) and their detection and purification (Hopp et al., 1988), respectively.

For control purposes, F9 cells were either transfected with the empty expression vector, a similar but inactive set of three LE domains also present in laminin $\gamma 1$ ($\gamma 1$ V1-3) or a mutant-binding-site-expressing construct ($\gamma 1$ III3-5)N802S. The LE domains $\gamma 1$ V1-3 have no known nidogen-binding activity but this fragment has a similar structure and size to $\gamma 1$ III3-5

(Fig. 1). However, biochemical studies have shown that Asn residue 802 is crucial for nidogen binding, with the N802S change (present in the homologous region of the laminin $\gamma 2$ chain) reducing the interaction between nidogen and its binding domain upon laminin by ~ 5000 times (Poschl et al., 1996).

F9 cells electroporated with the empty expression vector and the FLAG fusion constructs were selected for resistance to G418 and surviving clones isolated. Expressing clones were identified by immunoblotting with a monoclonal antibody against the FLAG epitope (Bio M2, Sigma-Aldrich; Fig. 2). For comparison of protein expression levels in these cells, loading of the medium was standardized by the detection of BM40, a calcium-binding extracellular matrix protein (Nischt et al., 1991) that is also secreted by F9 cells (Nishiguchi et al., 1996). For further studies, a representative clone containing the empty expression vector was chosen, along with two independent clones ($\gamma 1$ III3-5/A and $\gamma 1$ III3-5/B) that express

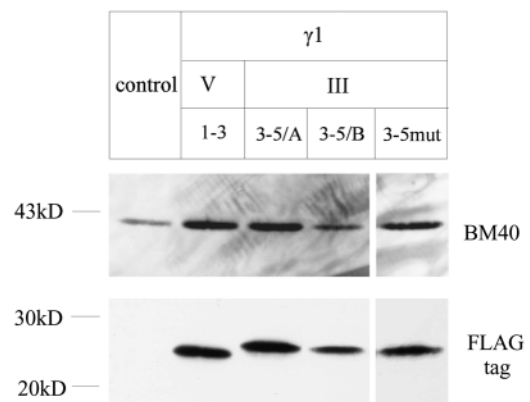
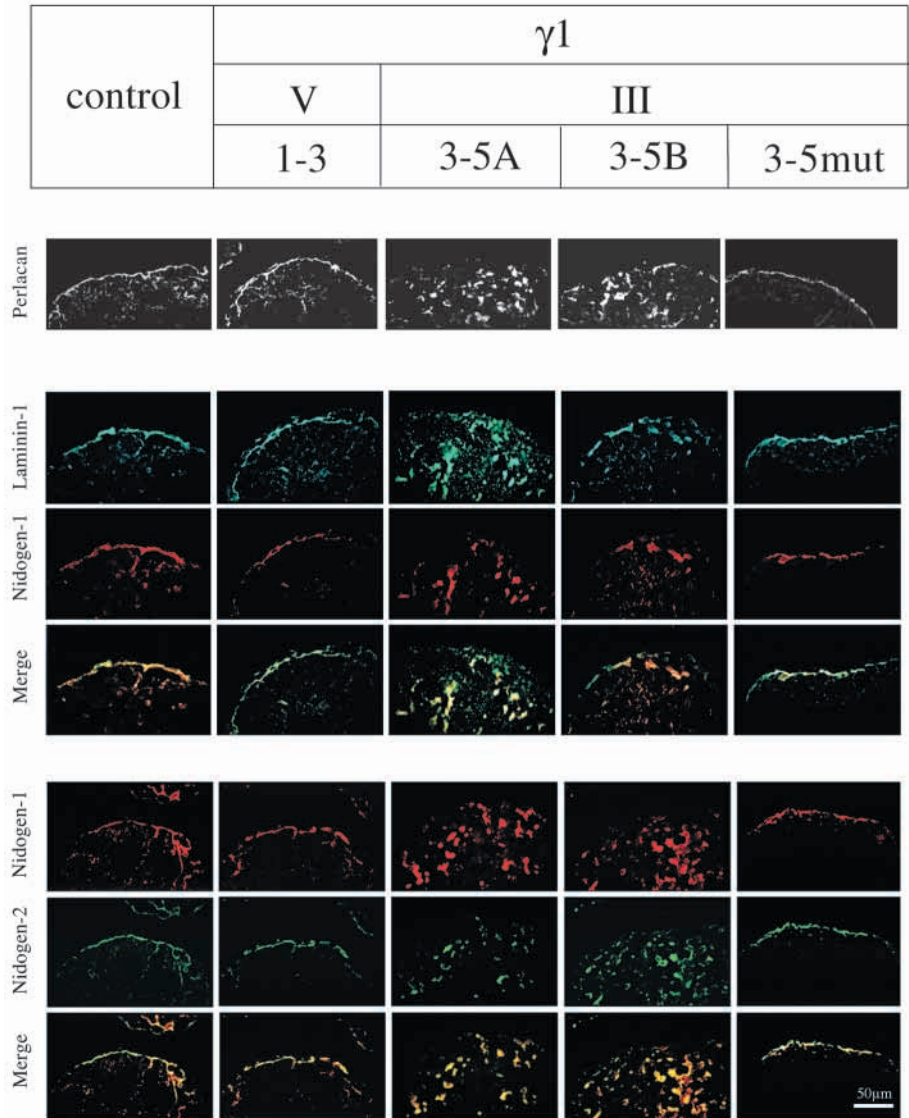


Fig. 2. Levels of exogenous protein expression: western blot analysis of supernatants of stably transfected F9 teratocarcinoma cells with a mouse monoclonal antibody detecting the FLAG tag shows approximately equal expression of both constructs, $\gamma 1$ III3-5 and $\gamma 1$ V1-3. As a control, F9 cells carrying the empty expression vector were analysed. For normalization of the loading of secreted proteins, a rabbit polyclonal antibody against the mouse BM40 protein was used. The two blots shown are derived from the same gel.

Fig. 3. Immunohistochemical localization of basement membrane proteins in 12-day-old F9-cell-derived embryoid bodies: cells stably expressing the constructs γ 1III3-5, γ 1III3-5mut and γ 1V1-3 were differentiated into embryoid bodies with 5×10^{-8} M retinoic acid, cryosectioned and stained for basement membrane components. As a control, F9 cells carrying the empty expression vector were used. γ 1III3-5/A and γ 1III3-5/B represent two different clones from one transfection with the γ 1III3-5 construct. Bar, 50 μ m. Upper panels: staining for perlecan shows disruption of basement membrane formation only in embryoid bodies derived from clones expressing the nidogen binding site (γ 1III3-5/A and /B). Middle panels: double immunofluorescence for laminin-1 and nidogen-1. Cryosections of differentiated 12-day-old embryoid bodies were incubated simultaneously with a rabbit polyclonal antibody against laminin-1 (green) and a rat monoclonal antibody against nidogen-1 (red). Embryoid bodies from clones γ 1III3-5/A and γ 1III3-5/B show a separation in the laminin-1 and nidogen-1 staining not seen in those derived from control or γ 1III3-5 expressing cells. Lower panels: double immunofluorescence for nidogen-1 and nidogen-2. Sections of differentiated 12-day-old embryoid bodies stained with a rabbit polyclonal antibody against nidogen-2 (green) and the rat monoclonal antibody against nidogen-1 (red). Both nidogen isoforms colocalize and nidogen-2 is found in the basement membrane of F9-derived embryoid bodies.



the nidogen-binding site, one expressing the three LE domains γ 1V1-3, and a fifth producing the mutated binding site γ 1III3-5mut, all at similar levels.

Analysis of the deposition of basement-membrane proteins in the F9 embryoid bodies

The embryoid body system offers a well-studied model of basement membrane deposition (Prehm et al., 1982; Cooper et al., 1983). Pluripotent F9 cells (Alonso et al., 1991) form parietal or visceral endoderm only upon treatment with, for example, retinoic acid (Strickland and Mahdavi, 1978) or cAMP (Hogan et al., 1983) accompanied by a 5-20-fold increase in the synthesis of basement membrane proteins. These are secreted to the extracellular surface and deposited between the outer endodermal cell layer and the inner core of the differentiating embryoid body. These rudimentary basement membrane structures contain laminin-1, nidogen-1, perlecan and collagen IV (Carlin et al., 1983; Durkin et al., 1986; Kleinman et al., 1987; Chakravarti et al., 1993).

The cell clones were expanded and used to produce

embryoid bodies in suspension culture. The embryoid bodies from each of the clones grew at a comparable rate and showed a similar gross morphology. After 12 days in culture, these were harvested, cryo-embedded, sectioned and stained with a rabbit polyclonal antibody raised against laminin-1. Sections from two sets of independent experiments and at least 20 embryoid bodies from each culture were examined for the presence of a subepithelial basement membrane. A near-continuous signal for laminin-1 at the periphery of the embryoid body underlying the outer layer of differentiated endodermal cells was considered to be a correctly formed basement membrane, whereas a disrupted, intermittent staining (covering less than 50% of the periphery) was taken as a sign of perturbation.

By these criteria, a formed basement membrane was produced in the empty vector control, γ 1V1-3 and γ 1III3-5mut expressing embryoid bodies (Fig. 3B). In the γ 1III3-5/A and γ 1III3-5/B expressing embryoid bodies the laminin-1 signal had a disrupted appearance, with little continuous staining and immunoreactive material was often deposited irregularly within the more central regions of the aggregates. This punctate

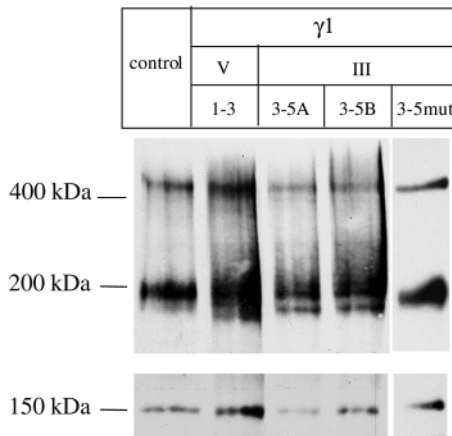


Fig. 4. Immunoblot for laminin-1 and nidogen-1 in embryoid body total lysates. A rabbit polyclonal antibody against laminin-1 and nidogen-1 was used to detect these proteins in total lysates prepared from 12-day-old retinoic-acid-treated embryoid bodies in SDS. Equal loading and transfer to nitrocellulose were confirmed by Ponceau Red staining of the membrane (not shown).

laminin-1 staining pattern suggests a disruption of the basement membrane and/or a defect in cellular differentiation. To analyse the former further, we then stained for another basement membrane component, perlecan. This showed a similar change in its staining pattern in the $\gamma 1$ III3-5 expressing embryoid bodies as described for laminin-1 (Fig. 3A).

A double-staining, using a rabbit polyclonal antibody for laminin-1 (laminin subunits $\alpha 1$, $\beta 1$, $\gamma 1$ and nidogen-1) and a nidogen-1-specific monoclonal antibody reacting with the G2 domain of mouse nidogen-1, was used to provide evidence of a successful competition for nidogen-1 binding (Fig. 3A). This revealed colocalization of both proteins in each of the control forms of embryoid bodies, whereas the $\gamma 1$ III3-5 expressing embryoid bodies showed imperfect colocalization indicating the presence of laminin molecules not bound with nidogen. Human nidogen-2 has a similar binding repertoire to nidogen-1 with the exception of a markedly weaker interaction with the murine laminin $\gamma 1$ III4 module. To see whether F9-derived embryoid bodies produced and incorporated nidogen-2 into a basement membrane, they were double labelled using a rabbit polyclonal specific for nidogen-2 and the nidogen-1-specific monoclonal antibody. These showed that nidogen-2 was expressed and was seen present with a similar staining pattern to nidogen-1, being incorporated only into a basement membrane in the three control clones (Fig. 3C).

To ensure that the disrupted appearance of the basement membrane proteins in $\gamma 1$ III3-5 expressing embryoid bodies is caused by efficient blocking of the laminin/nidogen-1 complex and not by changes in nidogen-1 production, mRNA and protein blots were performed. Total RNA was isolated from embryoid bodies and hybridized with cDNA probes for mouse nidogen-1 and human GAPDH. This revealed a similar levels of *nidogen-1* mRNA expression in all sets of embryoid bodies (results not shown). When the embryoid bodies were lysed in SDS sample buffer (Laemmli, 1970), no major variation in laminin-1 or nidogen-1 could be observed between control, $\gamma 1$ V1-3 and $\gamma 1$ III3-5mut or $\gamma 1$ III3-5 expressing embryoid bodies (Fig. 4).

Altered permeability in embryoid bodies expressing the nidogen binding site

An important biological role of the basement membrane is its function as a permeability barrier. To test whether the disruption of the laminin/nidogen-1 interaction resulted in an altered permeability of the embryoid bodies, a diffusion assay was established. Embryoid bodies were incubated with rhodamine-labelled 10 kDa and 70 kDa dextrans, which correspond in their hydrodynamic radius to globular proteins of ~ 40 kDa and ~ 280 kDa, respectively. After 5 minutes the distance of polymer diffusion into the embryoid bodies was determined by confocal microscopy (Fig. 5A) and diffusion coefficients were calculated to compare the permeability properties of the different clones (Fig. 5B). These diffusion coefficients of $\sim 5 \times 10^{-8} \text{ cm}^2 \text{ sec}^{-1}$ for control or $\gamma 1$ V1-3 expressing embryoid bodies, and $1 \times 10^{-7} \text{ cm}^2 \text{ sec}^{-1}$ for the $\gamma 1$ III3-5 expressing clones $\gamma 1$ III3-5/A and $\gamma 1$ III3-5/B, fit to inhibited and facilitated diffusion, respectively, as previously measured for embryonic-stem-cell-derived embryoid bodies (Wartenberg et al., 1998b). This shows that the control and $\gamma 1$ V1-3 expressing embryoid bodies have effective diffusion barriers for both 10 kDa and 70 kDa dextrans, whereas the expression of the nidogen-binding site results in drastically increased diffusion rates (Fig. 5). This suggests that the disruption of the basement membrane upon expression of the nidogen-binding site correlates with an increased permeability of the embryoid body.

Expression of the nidogen-1-binding site results in altered differentiation

The differentiation of F9 cell aggregates in the presence of retinoic acid differentiation induces the formation of an external polarized endoderm. To discover whether this is seen in the transfected F9 cells, TROMA-1 (cytokeratin 8) expression was analysed. This protein is normally synthesized in mature endoderm and would be expected to be seen in the outermost cell layer (Oshima, 1982). The control forms of embryoid bodies, including that expressing the mutated binding domain, showed the expected strong staining in the flattened surface cell layer. The embryoid body was covered with TROMA-1-positive cells but they were only occasionally present internally. However, where the nidogen-binding site was expressed, TROMA-1 signals occurred widely and were not merely restricted to the outer cells (Fig. 6).

Exogenous addition of the nidogen-binding site alters laminin deposition in wild-type embryoid bodies

Recombinant expression, genetic manipulation and cell cloning might lead to artefacts independent of the effect of the induced protein. Although two independent clones gave the same result, changes not seen with our three different types of control, we attempted to further exclude the possibility of such artefacts by the exogenous addition of the fusion protein to wild-type-derived embryoid bodies. The laminin FLAG fusion protein $\gamma 1$ III3-5 was recombinantly expressed in 293-EBNA cells, purified and added to the medium of wild-type embryoid bodies at a concentration of $10 \mu\text{g ml}^{-1}$. Non-supplemented controls were also cultured. Both supplemented and non-supplemented embryoid bodies grew in a similar manner to

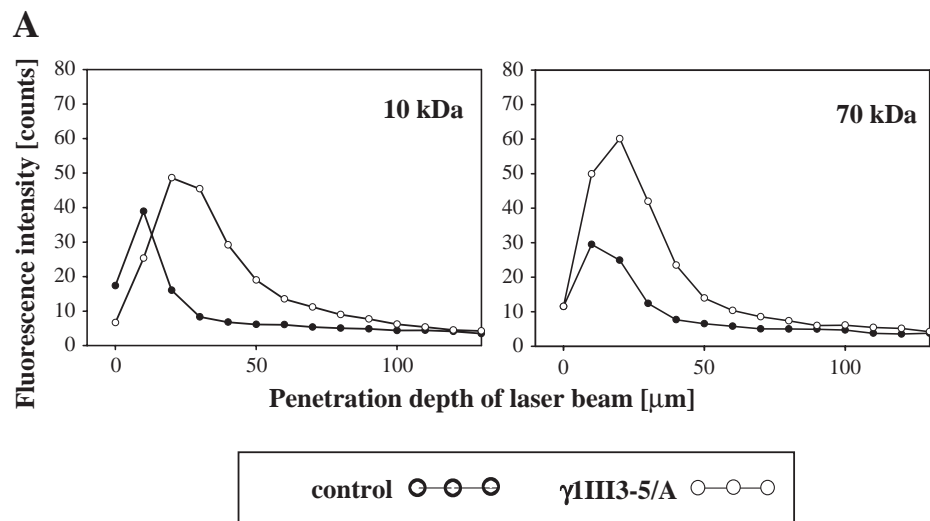
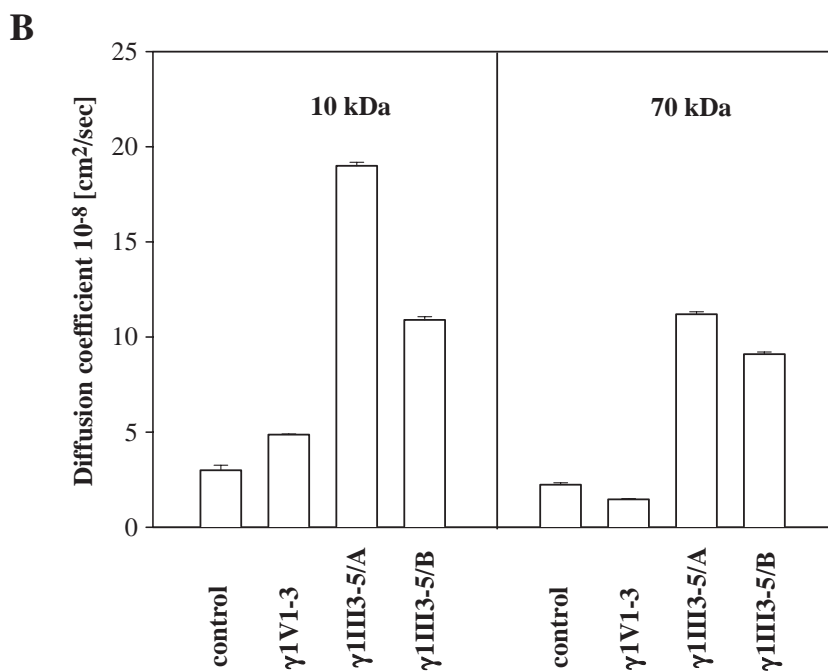


Fig. 5. Comparison of diffusion coefficients obtained from incubation of differentiated F9-cell-derived embryoid bodies with rhodamine-labelled dextrans of 10 kDa and 70 kDa. Embryoid bodies stably expressing the constructs γ 1III3-5, γ 1V1-3 and a control clone were incubated with 10 μM dextrans of either size. Fluorescence intensities were measured inside the embryoid bodies after 5 minutes of diffusion. (A) Representative curves of single embryoid bodies (control and γ 1III3-5/A), incubated with 10 kDa and 70 kDa fluorescent dextrans. (B) From at least six traces of individual embryoid bodies, mean diffusion coefficients plus s.d. were calculated. Two independent experiments were performed with comparable results.



those described earlier. Whole-mount staining for laminin-1 was analysed by confocal microscopy and revealed in wild-type embryoid bodies a network-like laminin-1 pattern (Fig. 7B) similarly organized to that observed on the cell surface of embryonic stem cells (Henry et al., 2001), whereas embryoid bodies treated with exogenous γ 1III3-5 FLAG fusion protein showed a highly disrupted punctate laminin-1 signal (Fig. 7A).

Discussion

Nidogen-1 is unique in the basement membrane in having such a wide protein-binding repertoire and, in particular, in having a strong interaction with laminin (Mayer et al., 1993). Nidogen is in high molar excess over laminin (Schymeinsky et al., 2002), suggesting that other nidogen interaction partners play a role in its anchorage into the basement membrane, but its binding to laminin has been considered to be a key step in

basement membrane formation. This view originated from biochemical interaction studies (Fox et al., 1991; Aumailley et al., 1989; Mayer et al., 1993; Poschl et al., 1996) and cell biological evidence, the most significant results being derived from antibody perturbation experiments that induced structural changes in the basement membrane and caused defects in the differentiation of epithelial derived tissues in organ culture (Ekblom et al., 1994; Kadoya et al., 1997). However, this assumption has recently been questioned because of findings from organisms lacking nidogen genes. Mice lacking *nidogen-1* have ultrastructurally normal basement membranes and fail to display any great developmental defects (Murshed et al., 2000). However, this might be explained by the redistribution and/or upregulation of *nidogen-2*, the remaining homologue in this species.

Very recently, a mouse line with a deletion of the *nidogen-2* gene has been reported to have no obvious phenotype (Schymeinsky et al., 2002). Similar results have been described in the case of *C. elegans*, whose genome encodes for only one member of the nidogen family. Deletion of this gene again resulted in no obvious effects upon basement membranes, with the only phenotype discovered being altered axonal pathfinding (Kang and Kramer, 2000; Kim and Wadsworth, 2000). One possibility for this variance in results could be an interference with other binding partners in antibody blocking experiments by steric hindrance. To determine by an independent method whether the disruption of the laminin/nidogen-1 complex influences basement membrane formation or maintenance, we introduced the binding area (LE modules 3-5 of the γ 1 laminin chain) into differentiating F9 embryoid bodies. The F9 system was chosen for its simplicity, and appears to reflect the previous biochemical results pointing to the ability of the mouse to

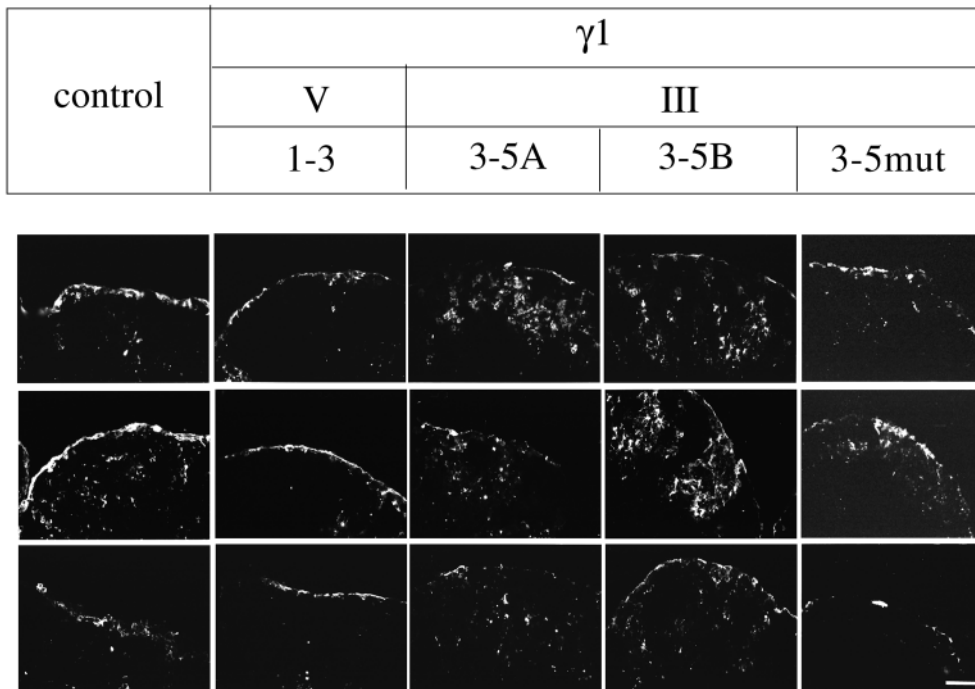


Fig. 6. Immunofluorescence detection of TROMA-1. Cryosections of differentiated 12-day-old embryoid bodies derived from control, $\gamma 1$ V1-3, $\gamma 1$ III3-5/A and $\gamma 1$ III3-5/B expressing F9 cells were stained with a rat monoclonal antibody against TROMA-1 (Kemler et al., 1981). Bar, 50 μ m.

basement membrane formation. That receptors for individual basement membrane components are significant in the organization of the basement membrane has been shown by basement membrane defects in α -dystroglycan and integrin deletion mice. Laminin $\alpha 1$ chain expression, which is required for laminin secretion, is regulated by $\beta 1$ integrin (Aumailley et al., 2000; Li et al., 2002). Hence, basement membrane defects in the absence of $\beta 1$ integrin might

partially compensate for basement membrane defects by unknown mechanisms.

We chose to use a method of continuous agitation for growth of the F9 aggregates, rather than the more conventional hanging drop method, after preliminary experiments showed that the control embryoid bodies in such a system were far more uniform in size and growth characteristics. Cell differentiation of these embryonic carcinoma cells is limited, in embryoid bodies this results in an ordered outer single endodermal layer sitting upon a basement membrane. The disorganization in the basement membrane and the reduced colocalization between laminin and nidogen-1 in embryoid bodies expressing the binding site suggest the successful competition by the recombinant nidogen-binding-site fragment for the binding to nidogen-1. Indeed, where laminin is completely absent, nidogen appears to be partially lost from the tissue (De Arcangelis et al., 1996; Smyth et al., 1999). In our competition experiments, redistribution of nidogen-1 was more marked than actual loss from the embryoid body (Figs 3, 4). This might indicate that nidogen-1 released from laminin finds other binding partners, with which it interacts using an independent site. The possibility cannot be excluded that the severity of the defects described here were exacerbated by the culture system used, and that the embryoid bodies were placed under shear stress during spinner culture. This could result in the changes seen here occurring owing to a less stable rather than unformed basement membrane, and indeed no alteration was described in embryonic stem cells carrying genetic defects in the nidogen-binding site (Willem et al., 2002) when grown in hanging drops. However, in initial experiments with the F9 derived cells cultured in hanging drops, we produced changes similar to those described from the spinner culture.

Disruption or weakness of the basement membrane has been a consistent effect seen upon the loss of other basement membrane components and receptors, although only the lack of laminin $\gamma 1$ or $\beta 1$ integrin have led to a complete absence of

be due to the lack of laminin rather than to abnormalities in basement membrane organization per se. Our results, together with those derived from the antibody perturbation experiments, suggest strongly that the nidogen-1-binding region of laminin plays a highly significant role in the molecular organization of the basement membrane.

Basement membranes with varying ultrastructure have been identified *in vivo* (Ogawa et al., 1999; Eyden, 1999), and developmental stages have been shown in basement membrane formation. For example, in the six-day-old mouse embryo, although a classical basement membrane has formed under the ectoderm in the extraembryonic egg cylinder, the basement membrane is unstructured within the embryo itself even though laminin-1 is deposited in a linear pattern (Miosge et al., 1993). In fact, only upon the full ultrastructural development of the basement membrane is nidogen-1 found by immunogold histochemistry, suggesting that in the early embryo nidogens might have a role in the maturation of basement membranes (Miosge et al., 2000), albeit one that is not crucial for embryonic survival or that might be compensated for by other molecules.

F9 cells also produce and incorporate nidogen-2 into their basement membrane, but this did not prevent basement membrane disorganisation in the presence of the nidogen-binding site. Our results indicate that nidogen-2 indeed has an affinity for this site despite that fact previous results suggested the use of an alternative binding surface upon the laminin trimer (Kohfeldt et al., 1998).

In glomerular capillaries, the basement membrane has been identified as the primary filtration barrier to graded dextrans (Caulfield and Farquhar, 1974). Many studies have been carried out characterizing the role of basement membrane components, such as proteoglycans and collagen IV in the permselectivity process (Morello et al., 2001; Tryggvason and Wartiovaara, 2001). The function of the basement membrane as a barrier to molecules diffusing into the embryoid body was

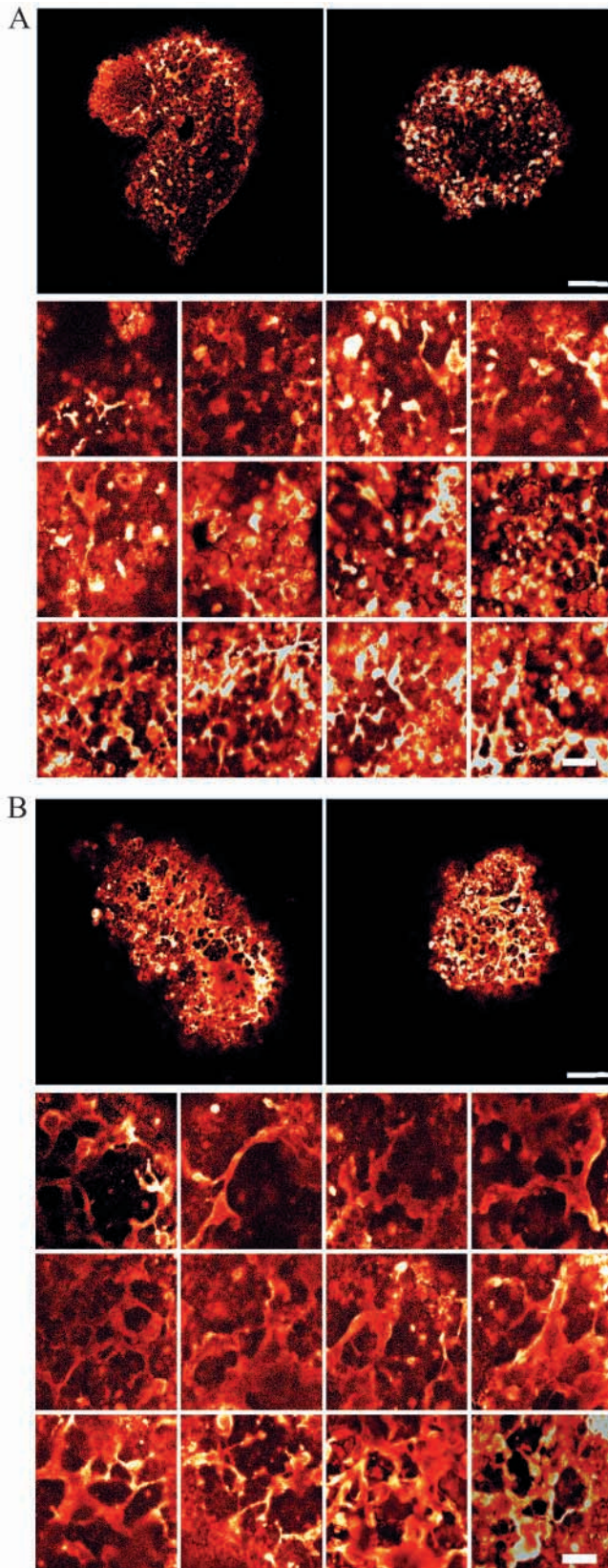


Fig. 7. Laminin-1 localization in F9 wild-type embryoid bodies treated with the FLAG fusion protein γ 1III3-5. Embryoid bodies were transferred from a spinner culture system into single wells at day 2 and then either supplied with additional FLAG fusion protein γ 1III3-5 at a concentration of $10 \mu\text{g ml}^{-1}$ (A) or not (B). At day 8 of culture, whole-mount staining with a rabbit polyclonal antiserum against laminin-1 was performed. Representative images show either the embryoid body surface in the upper panel (two images; bar, $100 \mu\text{m}$) or part of a section through the embryoid body periphery in the lower panel. Images from 12 different embryoid bodies from each culture condition are shown. Bar, $25 \mu\text{m}$.

permeability for embryoid bodies expressing the nidogen-binding site than for controls. This might either be caused directly by the disruption of the basement membrane proteins or be produced by secondary changes in the development of the outer epithelial layers.

To study the consequences of basement membrane disruption on cellular differentiation, the embryoid bodies were stained for TROMA-1, which is synthesized by mature endoderm (Oshima, 1982). All embryoid body types had TROMA-1 positive cells, but there was a marked alteration in their localization. Although control embryoid bodies revealed the expected strong peripheral staining, γ 1III3-5 producing clones show patchy staining distributed over the embryoid bodies and a reduction in staining of the marginal cells. Ectopic (stromal) TROMA-1 production in the γ 1III3-5 expressing embryoid bodies indicates a separation of expression of this usually epithelium-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 collagen IV(α 1) (Rogers et al., 1990) characteristically occur concurrently with the formation and subsequent organization of the epithelium. In the absence of laminin, parietal endoderm differentiation occurs in embryonic-stem-cell-derived embryoid bodies with the peripheral cells showing the morphological markers and high matrix production usual for such cells. However, parietal endodermal cells are found in greater numbers and as here in a disorganized manner through the embryoid body (Murray and Edgar, 2001). Hence not only is laminin itself a prerequisite for ordered parietal endodermal differentiation but its regulated deposition is also needed. Cytokeratin 8 (the TROMA-1 antigen) is expressed by embryonic epithelia but, in its absence, differentiation in these tissues is able to occur (Baribault et al., 1994; Brock et al., 1996). It is possible that the endoderm formed upon the surface of the nidogen-binding-site-expressing embryoid bodies could in other respects be normal.

A mouse line in which the nidogen-binding site (LE module γ 1III4) has been deleted by homologous recombination has been very recently described (Willem et al., 2002). These animals show far more severe phenotypes than that seen in the nidogen-1 knockout. Even so, basement membrane defects often appeared to be discrete or confined to certain tissues, suggesting that the requirement for the laminin-nidogen interaction is not needed for the formation of all basement membranes. The early lethality of \sim 40% of mutant animals lacking the nidogen-binding site could be a defect, similar to that described here, which is partially overridden by a compensatory mechanism in a proportion of embryos.

tested with 10 kDa and 70 kDa dextran molecules. Both sizes of dextran were excluded from the control embryoid bodies with diffusion coefficients clearly indicating higher

Furthermore, the lack of basement membrane alterations in *C. elegans* mutants lacking nidogen suggests that requirements of a basement membrane alter between tissues, through development and during evolution.

Together, the present results, the binding-site deletion (Willem et al., 2002) and the *nidogen-1* gene inactivation (Murshed et al., 2000) suggest that the roles of nidogen-1 can be compensated for. This is possibly through the action of nidogen-2 because both proteins are generally found in the same basement membranes (Kohfeldt et al., 1998; Miosge et al., 2000) and, in the absence of nidogen-1, there is obvious redistribution of this protein in particular within the basement membranes of striated muscles (Murshed et al., 2000). Although initial in vitro studies indicated that the binding of the murine γ 1III3-5 region to human nidogen-2 to be at least 100 times weaker than that occurring with nidogen-1 (Kohfeldt et al., 1998), it is conceivable that this interaction, in vivo, is sufficient to provide this compensation. Indeed, recent in vitro results suggest that the murine nidogen-2 can bind recombinant and proteolytic fragments of laminin in a manner comparable to murine nidogen-1, suggesting a species variation in binding activity (Salmivirta et al., 2002). Alternatively other proteins might interact with this region and affect the molecular structure of the basement membrane. One further, less likely, possibility is that free nidogen is itself able to direct aberrant differentiation, an effect not seen in the total absence of this protein. The discrepancy between the in vitro results and the in vivo deletion experiments might be a result of altering requirements for a stable basement membranes in different differentiation systems. A resolution of the apparent discrepancies between these experiments is likely to come from our ongoing studies of the mice lacking both *nidogen-1* and *nidogen-2* genes. However, it is evident that different tissues and different basement membranes show varying susceptibility to the loss of the laminin-nidogen interaction.

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References

- Alonso, A., Breuer, B., Steuer, B. and Fischer, J. (1991). The F9-EC cell line as a model for the analysis of differentiation. *Int. J. Dev. Biol.* **35**, 389-397.
- Aumailley, M., Wiedemann, H., Mann, K. and Timpl, R. (1989). Binding of nidogen and the laminin-nidogen complex to basement membrane collagen type IV. *Eur. J. Biochem.* **184**, 241-248.
- Aumailley, M., Battaglia, C., Mayer, U., Reinhardt, D., Nischt, R., Timpl, R. and Fox, J. W. (1993). Nidogen mediates the formation of ternary complexes of basement membrane components. *Kidney Int.* **43**, 7-12.
- Aumailley, M., Pesch, M., Tunggal, L., Gaill, F. and Faessler, R. (2000). Altered synthesis of laminin 1 and absence of basement membrane component deposition in β 1 integrin-deficient embryoid bodies. *J. Cell. Sci.* **2**, 259-268.
- Baribault, H., Penner, J., Iozzo, R. V. and Wilson-Heiner, M. (1994). Colorectal hyperplasia and inflammation in keratin 8-deficient FVB/N mice. *Genes Dev.* **8**, 2964-2973.
- Baumgartner, R., Czisch, M., Mayer, U., Poschl, E., Huber, R., Timpl, R. and Holak, T. A. (1996). Structure of the nidogen binding LE module of the laminin γ 1 chain in solution. *J. Mol. Biol.* **257**, 658-668.
- Becker, S., Wang, Z. J., Massey, H., Arauz, A., Labosky, P., Hammerschmidt, M., St-Jacques, B., Bumcrot, D., McMahon, A. and Grabel, L. (1997). A role for Indian hedgehog in extraembryonic endoderm differentiation in F9 cells and the early mouse embryo. *Dev. Biol.* **187**, 298-310.
- Brock, J., McCluskey, J., Baribault, H. and Martin, P. (1996). Perfect wound healing in the keratin 8 deficient mouse embryo. *Cell. Motil. Cytoskeleton* **35**, 358-366.
- Carlin, B. E., Durkin, M. E., Bender, B., Jaffe, R. and Chung, A. E. (1983). Synthesis of laminin and entactin by F9 cells induced with retinoic acid and dibutyryl cyclic AMP. *J. Biol. Chem.* **258**, 7729-7737.
- Caulfield, J. P. and Farquhar, M. G. (1974). The permeability of glomerular capillaries to graded dextrans. Identification of the basement membrane as the primary filtration barrier. *J. Cell Biol.* **63**, 883-903.
- Chakravarti, S., Hassell, J. R. and Phillips, S. L. (1993). Perlecan gene expression precedes laminin gene expression during differentiation of F9 embryonal carcinoma cells. *Dev. Dyn.* **197**, 107-114.
- Cooper, A. R., Taylor, A. and Hogan, B. L. (1983). Changes in the rate of laminin and entactin synthesis in F9 embryonal carcinoma cells treated with retinoic acid and cyclic AMP. *Dev. Biol.* **99**, 510-516.
- De Arcangelis, A., Neuville, P., Boukamel, R., Lefebvre, O., Kedinger, M. and Simon-Assmann, P. (1996). Inhibition of laminin alpha 1-chain expression leads to alteration of basement membrane assembly and cell differentiation. *J. Cell Biol.* **133**, 417-430.
- Dong, L. J. and Chung, A. E. (1991). The expression of the genes for entactin, laminin A, laminin B1 and laminin B2 in murine lens morphogenesis and eye development. *Differentiation* **48**, 157-172.
- Durkin, M. E., Phillips, S. L. and Chung, A. E. (1986). Control of laminin synthesis during differentiation of F9 embryonal carcinoma cells. A study using cDNA clones complementary to the mRNA species for the A, B1 and B2 subunits. *Differentiation* **32**, 260-266.
- Dziadek, M. (1995). Role of laminin-nidogen complexes in basement membrane formation during embryonic development. *Experientia* **51**, 901-913.
- Eklblom, P., Eklblom, M., Fecker, L., Klein, G., Zhang, H. Y., Kadoya, Y., Chu, M. L., Mayer, U. and Timpl, R. (1994). Role of mesenchymal nidogen for epithelial morphogenesis in vitro. *Development* **120**, 2003-2014.
- Eyden, B. (1999). Perivascular amorphous matrices containing laminin and type IV collagen not organized as a conventional basal lamina: identification by electron microscopy and implications for the control of cell biological processes. *Ultrastruct. Pathol.* **23**, 355-357.
- Fleischmajer, R., Schechter, A., Bruns, M., Perlsh, J. S., Macdonald, E. D., Pan, T. C., Timpl, R. and Chu, M. L. (1995). Skin fibroblasts are the only source of nidogen during early basal lamina formation in vitro. *J. Invest. Dermatol.* **105**, 597-601.
- Fox, J. W., Mayer, U., Nischt, R., Aumailley, M., Reinhardt, D., Wiedemann, H., Mann, K., Timpl, R., Krieg, T. and Engel, J. (1991). Recombinant nidogen consists of three globular domains and mediates binding of laminin to collagen type IV. *EMBO J.* **10**, 3137-3146.
- Henry, M. D., Satz, J. S., Brakebusch, C., Costell, M., Gustafsson, E., Fassler, R. and Campbell, K. P. (2001). Distinct roles for dystroglycan, β 1 integrin and perlecan in cell surface laminin organization. *J. Cell Sci.* **114**, 1137-1144.
- Hogan, B. L., Barlow, D. P. and Tilly, R. (1983). F9 teratocarcinomas as a model for the differentiation of parietal and visceral endoderm in the mouse embryo. *Cancer Surv.* **2**, 115-140.
- Hopp, T. P., Prickett, K. S., Price, V. L., Libby, R. T., March, C. J., Cerretti, D. P., Urdal, D. L. and Conlon, P. J. (1988). A short polypeptide marker sequence useful for recombinant protein identification and purification. *Biotechnology* **6**, 1204-1210.
- Kadoya, Y., Salmivirta, K., Talts, J. F., Kadoya, K., Mayer, U., Timpl, R. and Eklblom, P. (1997). Importance of nidogen binding to laminin γ 1 for branching epithelial morphogenesis of the submandibular gland. *Development* **124**, 683-691.
- Kang, S. H. and Kramer, J. M. (1990). Nidogen is nonessential and not required for normal type IV collagen localization in *Caenorhabditis elegans*. *Mol. Biol. Cell* **11**, 3911-3923.
- Kemler, R., Brulet, P., Schnebelen, M. T., Gaillard, J. and Jacob, F. (1981). Reactivity of monoclonal antibodies against intermediate filament proteins during embryonic development. *J. Embryol. Exp. Morphol.* **64**, 45-60.
- Kim, S. and Wadsworth, W. G. (2000). Positioning of longitudinal nerves in *C. elegans* by nidogen. *Science* **288**, 150-154.
- Kimura, N., Toyoshima, T., Kojima, T. and Shimane, M. (1998). Entactin-2: a new member of basement membrane protein with high homology to entactin/nidogen. *Exp. Cell Res.* **241**, 36-45.
- Kleinman, H. K., Ebihara, I., Killen, P. D., Sasaki, M., Cannon, F. B., Yamada, Y. and Martin, G. R. (1987). Genes for basement membrane

- proteins are coordinately expressed in differentiating F9 cells but not in normal adult murine tissues. *Dev. Biol.* **122**, 373-378.
- Kohfeldt, E., Maurer, P., Vannahme, C. and Timpl, R.** (1997). Properties of the extracellular calcium binding module of the proteoglycan testican. *FEBS Lett.* **414**, 557-561.
- Kohfeldt, E., Sasaki, T., Gohring, W. and Timpl, R.** (1998). Nidogen-2: a new basement membrane protein with diverse binding properties. *J. Mol. Biol.* **282**, 99-109.
- Laemmli, U. K.** (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.
- Li, S., Harrison, D., Carbonetto, S., Fassler, R., Smyth, N., Edgar, D. and Yurchenco, P. D.** (2002). Matrix assembly, regulation, and survival functions of laminin and its receptors in embryonic stem cell differentiation. *J. Cell Biol.* **157**, 1279-1290.
- Mann, K., Deutzmann, R. and Timpl, R.** (1988). Characterization of proteolytic fragments of the laminin-nidogen complex and their activity in ligand-binding assays. *Eur. J. Biochem.* **178**, 71-80.
- Mayer, U., Nischt, R., Poschl, E., Mann, K., Fukuda, K., Gerl, M., Yamada, Y. and Timpl, R.** (1993). A single EGF-like motif of laminin is responsible for high affinity nidogen binding. *EMBO J.* **12**, 1879-1885.
- Miosge, N., Gunther, E., Becker-Rabbenstein, V. and Herken, R.** (1993). Ultrastructural localization of laminin subunits during the onset of mesoderm formation in the mouse embryo. *Anat. Embryol.* **187**, 601-605.
- Miosge, N., Kother, F., Heinemann, S., Kohfeldt, E., Herken, R. and Timpl, R.** (2000). Ultrastructural colocalization of nidogen-1 and nidogen-2 with laminin-1 in murine kidney basement membranes. *Histochem. Cell Biol.* **113**, 115-124.
- Mizushima, S. and Nagata, S.** (1990). pEf-Bos, a powerful mammalian expression vector. *Nucleic Acids Res.* **18**, 5322.
- Morello, R., Zhou, G., Dreyer, S. D., Harvey, S. J., Ninomiya, Y., Thorner, P. S., Miner, J. H., Cole, W., Winterpacht, A., Zabel, B. et al.** (2001). Regulation of glomerular basement membrane collagen expression by LMX1B contributes to renal disease in nail patella syndrome. *Nat. Genet.* **27**, 205-208.
- Morini, M., Piccini, D., de Santanna, A., Levi, G., Barbieri, O. and Astigiano, S.** (1999). Localization and expression of integrin subunits in the embryoid bodies of F9 teratocarcinoma cells. *Exp. Cell Res.* **247**, 114-122.
- Murray, P. and Edgar, D.** (2000). Regulation of programmed cell death by basement membranes in embryonic development. *J. Cell Biol.* **150**, 1215-1221.
- Murray, P. and Edgar, D.** (2001). Regulation of laminin and COUP-TF expression in extraembryonic endodermal cells. *Dev. Mech.* **101**, 213-215.
- Murshed, M., Smyth, N., Miosge, N., Karolat, J., Krieg, T., Paulsson, M. and Nischt, R.** (2000). The absence of nidogen 1 does not affect murine basement membrane formation. *Mol. Cell. Biol.* **20**, 7007-7012.
- Niimi, T. and Kitagawa, Y.** (1997). Distinct roles of mouse laminin $\beta 1$ long arm domains for $\alpha 1\beta 1\gamma 1$ trimer formation. *FEBS Lett.* **400**, 71-74.
- Nischt, R., Pottgiesser, J., Krieg, T., Mayer, U., Aumailley, M. and Timpl, R.** (1991). Recombinant expression and properties of the human calcium-binding extracellular matrix protein BM-40. *Eur. J. Biochem.* **200**, 529-536.
- Nishiguchi, S., Sakuma, R., Nomura, M., Zou, Z., Jearanaisilavong, J., Joh, T., Yasunaga, T. and Shimada, K.** (1996). A catalogue of genes in mouse embryonic carcinoma F9 cells identified with expressed sequence tags. *J. Biochem. Tokyo* **119**, 749-767.
- Ogawa, S., Ota, Z., Shikata, K., Hironaka, K., Hayashi, Y., Ota, K., Kushiro, M., Miyatake, N., Kishimoto, N. and Makino, H.** (1999). High-resolution ultrastructural comparison of renal glomerular and tubular basement membranes. *Am. J. Nephrol.* **19**, 686-693.
- Oshima, R. G.** (1982). Developmental expression of murine extra-embryonic endodermal cytoskeletal proteins. *J. Biol. Chem.* **257**, 3414-3421.
- Paulsson, M., Aumailley, M., Deutzmann, R., Timpl, R., Beck, K. and Engel, J.** (1987). Laminin-nidogen complex. Extraction with chelating agents and structural characterization. *Eur. J. Biochem.* **166**, 11-19.
- Poschl, E., Mayer, U., Stetefeld, J., Baumgartner, R., Holak, T. A., Huber, R. and Timpl, R.** (1996). Site-directed mutagenesis and structural interpretation of the nidogen binding site of the laminin $\gamma 1$ chain. *EMBO J.* **15**, 5154-5159.
- Prehm, P., Dessau, W. and Timpl, R.** (1982). Rates of synthesis of basement membrane proteins by differentiating teratocarcinoma stem cells and their modulation by hormones. *Connect. Tissue Res.* **10**, 275-285.
- Rogers, M. B., Watkins, S. C. and Gudas, L. J.** (1990). Gene expression in visceral endoderm: a comparison of mutant and wild-type F9 embryonal carcinoma cell differentiation. *J. Cell Biol.* **110**, 1767-1777.
- Salmivirta, K., Talts, J. F., Olsson, M., Sasaki, T., Timpl, R. and Ekblom, P.** (2002). Binding of mouse nidogen-2 to basement membrane components and cells and its expression in embryonic and adult tissues suggest complementary functions of the two nidogens. *Exp. Cell Res.* **279**, 188-201.
- Schymeinsky, J., Nedbal, S., Miosge, N., Poschl, E., Rao, C., Beier, D. R., Skarnes, W. C., Timpl, R. and Bader, B. L.** (2002). Gene structure and functional analysis of the mouse *nidogen-2* gene: *nidogen-2* is not essential for basement membrane formation in mice. *Mol. Cell. Biol.* **22**, 6820-6830.
- Smyth, N., Vatansver, H. S., Murray, P., Meyer, M., Frie, C., Paulsson, M. and Edgar, D.** (1999). Absence of basement membranes after targeting the *LAMC1* gene results in embryonic lethality due to failure of endoderm differentiation. *J. Cell Biol.* **144**, 151-160.
- Smyth, N., Odenthal, U., Merkl, B. and Paulsson, M.** (2000). Eukaryotic expression and purification of recombinant extracellular matrix proteins carrying the Strep II tag. *Methods Mol. Biol.* **139**, 49-57.
- Stetefeld, J., Mayer, U., Timpl, R. and Huber, R.** (1996). Crystal structure of three consecutive laminin-type epidermal growth factor-like (LE) modules of laminin $\gamma 1$ chain harboring the nidogen binding site. *J. Mol. Biol.* **257**, 644-657.
- Strickland, S. and Mahdavi, V.** (1978). The induction of differentiation in teratocarcinoma stem cells by retinoic acid. *Cell* **15**, 393-403.
- Thomas, T. and Dziadek, M.** (1993). Genes coding for basement membrane glycoproteins laminin, nidogen, and collagen IV are differentially expressed in the nervous system and by epithelial, endothelial, and mesenchymal cells of the mouse embryo. *Exp. Cell Res.* **208**, 54-67.
- Tryggvason, K. and Wartiovaara, J.** (2001). Molecular basis of glomerular permselectivity. *Curr. Opin. Nephrol. Hypertens.* **10**, 543-549.
- Wartenberg, M., Hescheler, J., Acker, H., Diershagen, H. and Sauer, H.** (1998a). Doxorubicin distribution in multicellular prostate cancer spheroids evaluated by confocal laser scanning microscopy and the 'optical probe technique'. *Cytometry* **31**, 137-145.
- Wartenberg, M., Gunther, J., Hescheler, J. and Sauer, H.** (1998b). The embryoid body as a novel in vitro assay system for antiangiogenic agents. *Lab. Invest.* **78**, 1301-1314.
- Willem, M., Miosge, N., Halfter, W., Smyth, N., Jannetti, I., Burghart, E., Timpl, R. and Mayer, U.** (2002). Specific ablation of the nidogen-binding site in the laminin $\gamma 1$ chain interferes with kidney and lung development. *Development* **129**, 2711-2722.
- Yurchenco, P. D. and Schittny, J. C.** (1990). Molecular architecture of basement membranes. *FASEB J.* **4**, 1577-1590.
- Yurchenco, P. D., Quan, Y., Colognato, H., Mathus, T., Harrison, D., Yamada, Y. and O'Rear, J. J.** (1997). The α chain of laminin-1 is independently secreted and drives secretion of its β - and γ -chain partners. *Proc. Natl. Acad. Sci. USA* **94**, 10189-10194.