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Regulation of basement membrane formation in skin-organotypic coculture

Dissertation
zur Erlangung des Grades eines Doktor
der Naturwissenschaften

der Mathematisch-Naturwissenschaftlichen Fakultät
der Christian-Albrecht-Universität Kiel

von

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Heidelberg 2003

*The opposite of a profound truth may
very well be another profound truth*

Niels Bohr

DANKSAGUNG

Herrn Prof. B.W. Müller danke ich ganz herzlich für die Vertretung meiner Doktorarbeit an der Universität Kiel.

Mein besonderer Dank gilt **Herrn P.D. Dr. Dirk Breitkreutz**.

Vielen Dank für die Überlassung des spannenden Themas und die Betreuung. Bedanken möchte ich mich für das Vertrauen, das es mir ermöglichte selbständig zu arbeiten, eigene Ideen zu entwickeln und zu verfolgen. Die konstruktive Kritik und Diskussionsbereitschaft unterstützten die Bearbeitung meiner Arbeit maßgeblich.

Herrn Prof. N. E. Fusenig möchte ich herzlich für seine Unterstützung danken und dass ich in seinem hervorragend ausgerüstetem Labor arbeiten durfte.

Regina Beck und **Iris Martin** danke ich für die engagierte Hilfe vor Ort und ihr exzellentes Fachwissen im Bereich der Zellkulturen.

Meinen Kollegen und Mitstreitern im Labor möchte ich für die freundliche Zusammenarbeit ganz herzlichen Dank aussprechen.

Dr. Hans Jürgen Stark danke ich für seine Ratschläge und Diskussionsbereitschaft, **P.D. Dr. Nicole Maas-Szabowski** danke ich für die Hilfe bei der in-situ-Hybridisierung und bei der molekularbiologischen Arbeit, und **Dr. Nicolae Mirancea** danke ich für die elektronmikroskopische Analysen meiner Proben.

Herzlich bedanken möchte ich mich auch bei **Sonja Depner, Nicole Daum, Eva Obermüller, Dr. Sylvia Vosseler, Dr. Wiltrud Lederle, Martina Koci** und allen andere in den Abteilungen Fusenig und Boukamp, die mit mir den Alltag an der Bench und den Weg zur Promotion geteilt haben. Ich danke auch unserem Praktikant **Holger Russ** für die Mithilfe bei der Wiederholung „unzähliger“ Versuche.

Mein ganz persönlicher Dank geht an **Björn Wagner** für seine Unterstützung und Zuversicht, für seinen Glauben an mich auch in schwierigen Phasen dieser Arbeit.

Nicht zuletzt möchte ich meine Eltern danken, die mir immer zur Seite standen und mich in jeglicher Hinsicht unterstützten.

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1. INTRODUCTION

1-1. The epidermis

The epidermis is a multilayered squamous epithelium that forms the interface between the organism and its environment. The keratinocytes account for more than 80% of the cells in the epidermis. They provide barrier properties and participate in the epidermis repair and regeneration. Other cells in the epidermis have their own specific function; the Langerhans cells provide immunological protection, the melanocytes absorb UV-light, and the Merkel cells are sensors for mechanical events at the skin surface and within the epidermal compartment.

In the epidermis, the keratinocytes are organized into four layers (Fig. 1.1); the basal layer (stratum basale), the spinous layer (stratum spinosum), the granular layer (stratum granulosum) and the cornified layer (stratum corneum). Each layer is defined by position, shape, polarity, morphology and state of differentiation of the cells. Correspondingly, the ultrastructure of the keratinocytes correlates with their position within the epidermis and their state of differentiation (reviewed in Holbrook, 1994).

Stratum basale

The cells of the basal layer have a columnar shape. They are anchored to the basement membrane via the hemidesmosomes, and this is crucial to restrain their differentiation. In addition, the contact to neighboring basal cells as well as differentiating suprabasal cells is important for defining the polarity of the basal cells. Proliferation in the epidermis is normally restricted to the basal layer.

Typical keratin expressed in the basal cells are keratins K5 and K14, forming a pair as the basic subunit. Keratins are cytoskeletal proteins composing the intermediate filaments in epithelial cells. 20 different keratins are described for the human skin (Moll et al., 1982, Steinert et al., 1985, 1988). Prominent keratins in human skin in addition to K5/K14 are the keratins K1/K10, representing early markers of differentiation.

Further adhesion proteins with basal localization are the integrins, which are essential for cell-cell and cell-matrix interactions. The major types in the epidermis are the integrins $\alpha 2\beta 1$, $\alpha 3\beta 1$ and $\alpha 6\beta 4$ (Carter et al., 1990, Klein et al., 1990, Sonnenberg et al., 1990).

Stratum spinosum

The cells of the three- to four layers above the basal cells show a gradient in size, shape and cytoplasmic structure. Lower spinous cells have more oval shape than the flattened spinous cells adjacent to the granular cell zone. The spinous cells are connected to each other by desmosomes, with associated bundles of keratin filaments. These contacts appear like “spines” in histological preparations, giving these layers their name. In this compartment, the differentiation specific proteins keratins K1 and K10 arise, which mechanically stabilize the epidermis together with the desmosomal connections (Schwarz et al., 1990).

Stratum granulosum

The hallmark of the two or three layers of granular keratinocytes are the dense “keratohyalin granules” which are mainly composed of the protein profilaggrin, further processed to filaggrin. Granular cells also synthesize the precursors of cornified envelopes, keratolinin and loricrin, whereas the enzyme responsible for the cross-linking process, transglutaminase is made already in spinous cells. Filaggrin and loricrin are also called late differentiation markers (Steinert et al., 1981, Lonsdale-Eccles et al., 1984, Mehrel et al., 1990, Magnaldo et al., 1990, Hohl et al., 1991). The granular zone is the last of the viable epidermal layers, and metabolically very active. While granular cells are engaged in the synthesis of new structural proteins, at the same time the first steps are initialized for the destruction of cytoplasmic organelles and cell nuclei, which takes place during the abrupt transition from the uppermost granular to the first cornified cell layer.

Stratum corneum

The stratum corneum is a multilayered zone of terminally differentiated keratinocytes embedded in an extracellular lipid matrix. These dead keratinocytes have the greatest dimensions of the keratinocytes and finally flake off from the skin. They differ substantially in structure depending on their position relative to the granular layer and the skin surface. Keratins may account for as much as 85% of the protein content in these cells. The keratins K1 and K10 are here associated with filaggrin, forming the final compact protein structure of the stratum corneum.

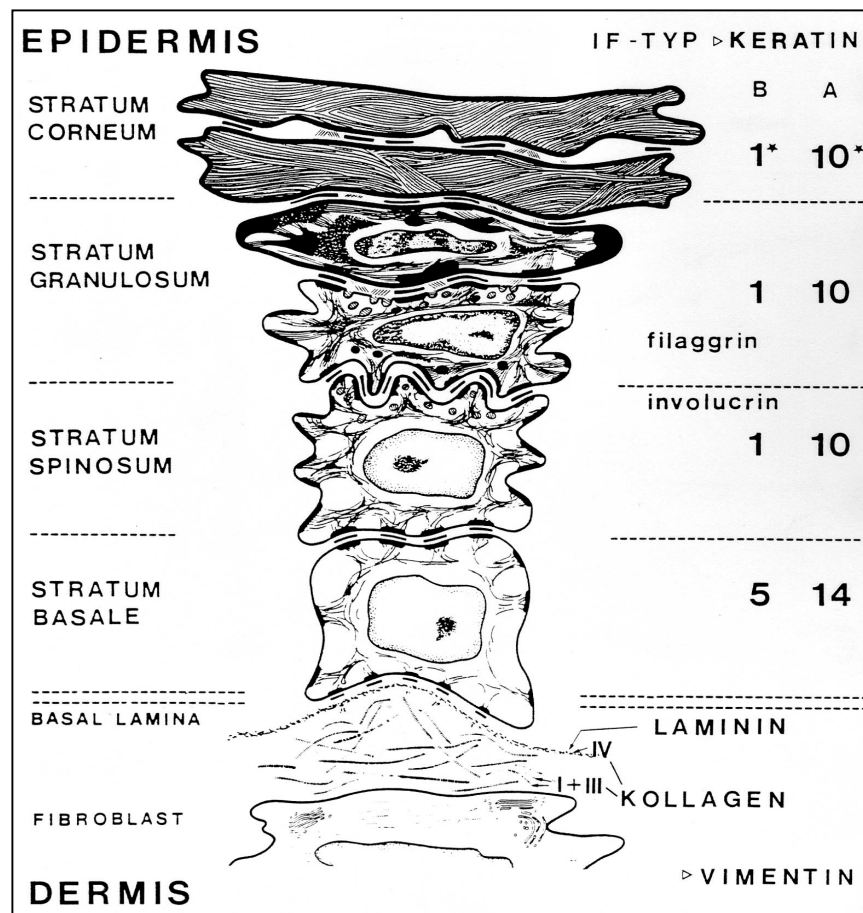


Fig 1.1. Schematic view of skin section

1-2. In vitro models of the epidermis

1-2.1 Skin-organotypic three-dimensional (3D-) coculture

The particular characteristics of the epidermis are generally not very well reflected in cultures of keratinocytes in vitro. The epidermis is a surface epithelium, with its upper cell sheet exposed to the outer environment. Formation and maintenance of the mature epidermis consisting of four layers rely on a continuous process of keratinocyte proliferation and terminal differentiation. The epidermal organization and tissue homeostasis are regulated by mesenchymal influences (Fusenig, 1994). In addition, the proliferating basal cells are attached to a specialized extracellular matrix, the basement membrane. This system is completely altered in conventional cultures on plastic, where epithelial cells grow submerged in medium, mostly forming only a single-layer. Such cultures are nourished from above and lack the basement membrane, being deprived of mesenchymal support.

The first attempts to create more natural growth conditions for epidermal cells in vitro involved placing pieces of skin in culture. While this allowed the keratinocytes to migrate from the explants, the differentiation program was only rudimentary, and the cells eventually became senescent or detached. Pioneering work was done by Rheinwald and Green, 1975, demonstrating that single cell suspensions of keratinocytes could be grown on feeder layers of irradiated fibroblasts. Although keratinocytes could be further propagated in such cultures, neither the cell polarization and structural organization, nor the expression of the differentiation markers filaggrin, keratin K1/10 and loricrin were improved (Holbrook et al., 1983, Breitkreutz et al., 1984, Fusenig 1986, Bowden et al., 1987, Watt et al., 1982).

Since then, multiple versions have been developed to mimic the in vivo situation of normal skin. In skin organotypic 3D-cultures, keratinocytes are cultivated at the air-liquid interface on various substrates serving as dermal equivalents. These consist of porous membranes either cell-free, coated with extracellular matrix, or seeded with fibroblasts (3D-coculture) at the lower side of the filter (Rosdy and Clauss, 1990; Limat et al., 1996; Poncic et al., 1997).

Alternatively and closest to skin physiology, dermal equivalents consisting of type I collagen gels populated with fibroblasts provide an appropriate substratum for the development of a polarized and stratified epithelium (Bell et al., 1981, Asselineau et al., 1986; Parenteau et al., 1991). As in the other described models (Fig. 1.2), the contact of the cultures with the medium is restricted to the base of the gel, so that fibroblasts and keratinocytes are nourished by diffusion from below, similar to the in vivo situation. While the fibroblasts seem to proliferate in a controlled fashion like in the dermis, they produce readily extracellular matrix components. Furthermore, the function of diffusible factors mediating epithelial-mesenchymal interactions has been demonstrated (Smola et al., 1998, Maas-Szabowski et al., 2000). The keratinocytes grown in the organotypic culture can develop into a stratified epidermis-like epithelium, consisting of several nucleated cells as well as fully keratinized layers, closely resembling the epidermal strata.

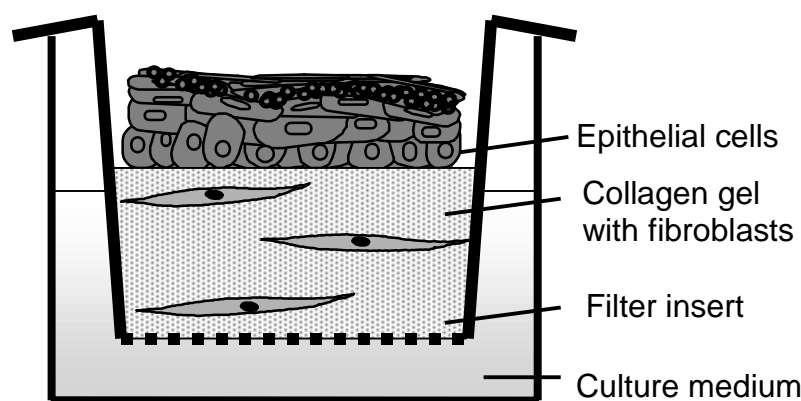


Fig. 1.2. The skin organotypic 3D-coculture

1-2.2 The HaCaT cell line as relevant model

In our laboratory, a human skin keratinocyte line named HaCaT, has been developed and characterized (Boukamp et al., 1988). This cell line originated from the histological normal “non-involved” epidermis on the edge of a melanoma removed from a 62-year old patient. The cultivation of these cells took place under low calcium (0,2 mM) and high temperature conditions, thereby the name HaCaT – H(uman)-a(dult)-(low)-Ca(lcium)-(high)-T(emperature). The HaCaT cell line is one of the few spontaneous, immortalized human skin cell lines developed.

It is assumed that the initial crucial event for immortalization of the HaCaT cells was the mutation of both alleles of the cell cycle control gene p53 leading to an inactivation of the “tumor suppressor function” of p53 (Lehmann et al. 1993). In addition, the special cultivation conditions probably lead to increasing non-repaired DNA damages and chromosomal aberrations, verified by the appearance of characteristic marker chromosomes (Boukamp et al., 1993).

The HaCaT cells display some of the characteristics seen in transformed cells, namely growth under clonal conditions and in semisolid nutrition-substrates (soft agar) (Hülsen et al., 1990). The cells are able to differentiate like normal keratinocytes, also in high passages after continuous growth in culture. The cells do not develop tumors after transplantation or subcutaneous injection (Boukamp et al., 1988).

Despite their genetic alterations, HaCaT cells have conserved many morphological and biochemical features of normal epidermal cells. In addition, HaCaT cells develop epidermis-like epithelia with the typical differentiation markers. Thus, keratins K5/14 develop in the basal layers followed by keratin 1/10, involucrin, loricrin and filaggrin in suprabasal layers when transplanted on the dorsal muscle fascia of nude mice. As in the transplants of normal human keratinocytes, the HaCaT cells also display the keratins K6/16, which implies an activated tissue, similar to the wound situation. In vitro, the HaCaT cells show a modified keratin pattern, which in addition to the mentioned in vivo expressed keratins also includes expression of keratins mostly of simple epithelia, such as K7, K8, K18 and K19 in the exponential growth phase (Ryle et al., 1989, Breitkreutz et al., 1989, 1991). However, these keratin patterns are

normalized at high cell density, also being modulated by retinoids and Ca^{2+} -levels comparable to normal cells (Breitkreutz et al., 1993).

In addition, organotypic cocultures of HaCaT cells on a collagen substrate containing human dermal fibroblasts are able to produce an epidermis-like epithelium (Schoop et al., 1999), with respect to morphology and expression of the characteristic differentiation markers. Taken together, all these data suggest the HaCaT cell line as an ideal substitute for normal human keratinocytes in studies of epidermal proliferation and differentiation.

1-3. The basement membrane

Basement membranes are flexible, thin (40-120 nm) mats of specialized extracellular matrix underlining all epithelial and endothelial cell sheets or tubes, and surrounding individual muscle cells, fat cells, or pericytes. Basement membranes thus separate these cells and tissue compartments from the underlying or surrounding connective tissue. However, basement membranes serve more than simple structural and filtering roles. They are able to determine cell polarity, influence cell metabolism, organize the proteins in adjacent plasma membranes, control cell differentiation, and serve as specific tracks for cell migration. The basement membrane can also act as a selective barrier to the movement of cells. It usually prevents fibroblasts in the underlying connective tissue from making contact with the epithelial cells. On the other hand, it does not stop macrophages, lymphocytes, or nerve processes from passing through. Basement membranes play an important part in tissue regeneration after injury. When tissues such as muscles, nerves and epithelia are damaged, the basement membrane often survives and provides a scaffold along which regenerating cells can migrate. In this way the original tissue architecture is readily reconstructed. In some cases, as in the skin or cornea, the basement membrane becomes chemically altered following injury, for example by infiltration of fibronectin, which promotes cell migration required for wound repair.

By ultrastructure, two layers can be distinguished, an electron-lucent layer (lamina lucida) adjacent to the plasma membrane of the cells that rest on the basement membrane, and an electron-dense layer (lamina densa) situated below.

In the skin the basement membrane separates the epidermis from the dermis but also secures the epidermis to the underlying connective tissue. Within the basal cells specialized anchoring complexes are represented by the hemidesmosomes visible in the electron microscope as multiple electron dense plaques at the ventral pole (Ellison and Garrod, 1984, Eady 1986).

This highly complex architecture contributes to the resistance of the epithelia to applied forces and it is built up by specific collagenous and non-collagenous proteins. The main components of the basement membrane are the glycoproteins laminin, nidogen and type IV collagen, and the large heparan sulfate proteoglycan perlecan (Fig. 1.3). Basement membrane-laminin (representing several isoforms) consists of a number of functional domains, one binds to type IV collagen, one to heparan sulfate, one to nidogen and two or more to other laminin receptors on the surface of cells. Laminin and type IV collagen both self-assemble into two independent feltlike sheets (Yurchenco and Schittny, 1990, Timpl and Brown 1996). These networks are connected by the monomeric nidogen molecule, acting as a bridge between the distinct domains (Fox et al. 1991, Aumailley and Smyth, 1998).

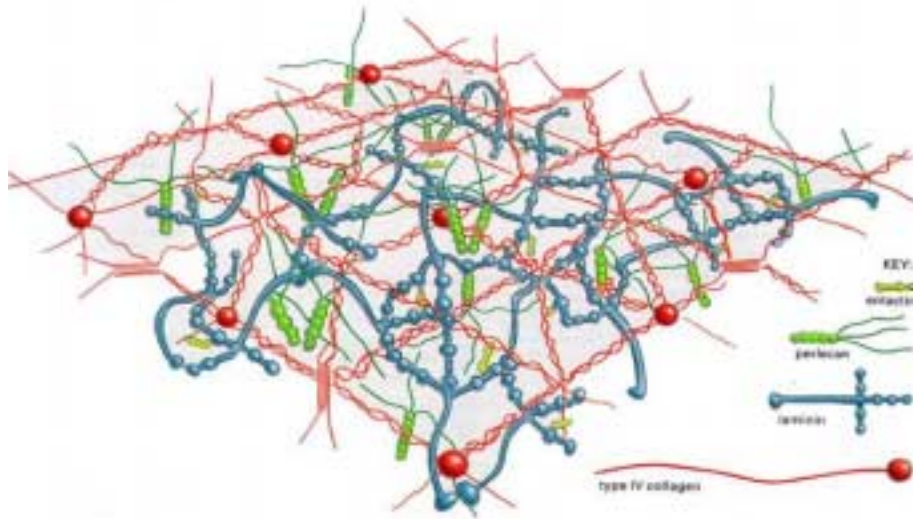


Fig. 1.3. The molecular structure of the basement membrane. Specific interactions between the proteins type IV collagen, laminin, perlecan and nidogen (entactin) form the basement membrane of the skin. (Taken from Alberts, 1994)

The precise mechanisms how basement membranes are assembled and how the biological function of the proteins is maintained within the basement membrane *in vivo*, are still unclear. The basic molecular interactions can be recapitulated *in vitro* with purified basement membrane components (Ruben and Yurchenco 1994, Yurchenco and Furthmayr 1984, Yurchenco and Ruben 1987, 1988). However, although these interactions are apparently crucial for supramolecular matrix architecture, they seem to be insufficient *per se* to insure formation of a thin sheet-like matrix. Presumably, cell surfaces are required in addition to ensure this tissue- and site-specific process, being largely mediated by matrix receptors (Bloch et al., 1997, DiPersio et al., 1997, Henry and Campbell et al., 1998, Sasaki et al., 1998). Integrins are dimeric ($\alpha\beta$) transmembrane proteins in skin acting as receptors for e.g. laminin (Belkin and Stepp, 2000). Several members of the integrin family, including $\alpha1\beta1$, $\alpha2\beta1$, $\alpha3\beta1$, and $\alpha6\beta4$ not only serve as anchors that target basement membrane deposition, but in response to ligation can redistribute within the plasma membrane and facilitate redistribution of intracellular proteins, creating a new organization of matrix components, receptors and cytoskeleton (Cognato et al., 1999).

1-3.1 The laminins, structure and genetic regulation

Laminins are heterotrimeric molecules, containing one α , β and γ chain (Beck et al., 1993). Since the original discovery of laminin (Timpl et al., 1979) five different α chains ($\alpha1$ - $\alpha5$), three different β chains ($\beta1$ - $\beta3$), and three different γ chains ($\gamma1$ - $\gamma3$) have been identified. This allows theoretically the formation of 45 different trimeric laminin isoforms. However, there are limitations in which trimer combinations are actually realized. For example, the $\gamma2$ chain only assembles with the $\alpha3$ and the $\beta3$ chain, forming laminin-5. So far, 15 different laminin isoforms have been clearly

identified (Burgeson et al., 1994, Miner et al., 1997, Koch et al., 1999, Champliand et al., 1996, Marinkovich et al., 1992a, Rouselle et al., 1991, Ehrig et al., 1987, Hunter et al., 1989).

The general structure of laminins can be described by the structure of laminin-1 ($\alpha1\beta1\gamma1$), the first discovered and best-studied isoform. Laminin-1 resembles a cross with three short arms and one long arm. The long arm ends in a globular structure called the G domain. While the N-terminal regions of each of the three chains form the three short arms, their C-terminal portions associate in a triple coiled-coil α -helix, forming the rod-like part of the long arm. The G domain is formed exclusively by the $\alpha1$ chain, making it the largest chain in the molecule with a mass of about 400 kDa, as compared to approximately 200 kDa of the $\beta1$ and $\gamma1$ chains. The basic features of this structure hold true for all laminin isoforms, with the exception that laminins 5-9 contain one to three of the N-terminally shortened chains $\alpha3A$, $\alpha4$, $\beta3$, or $\gamma2$, which yield truncated short arms (reviewed in Tunggal et al. 2000, and Colognato and Yurchenco, 2000)).

Most laminin chains are distinct gene products. Exceptions are the alternatively spliced $\alpha3A$ and $\alpha3B$ variants (Ryan et al., 1994, Galliano et al., 1995, Miner et al., 1997), while for the $\alpha5$ chain it is not clear yet whether the different forms are the result of alternative splicing or extracellular processing (Miner et al., 1997, Sorokin et al., 1997a). Intracellular assembly of $\alpha/\beta/\gamma$ trimers involves two major steps, first stable, disulfide-linked β/γ dimers are formed, followed by incorporation and disulfide cross-linking between the dimers and the α chain, which drives secretion of the heterotrimers (Marinkovich et al., 1992b, Utani et al., 1994, Yurchenco et al., 1997). Except for cleavage of the signal peptide, processing has so far not been observed for the $\alpha1$, $\beta1$, $\beta2$, $\beta3$ or $\gamma1$ chains. In contrast, the $\alpha2$, $\alpha3A$, possibly the $\alpha5$, and the $\gamma2$ chains are extracellularly processed.

Laminin chain expression is tissue- and developmental stage specific, certain chains being synthesized by both mesenchymal and parenchymal cells, and others by parenchymal cells only (Simo et al., 1992, Thomas and Dziadek 1993, Schuler and Sorokin, 1995, Tiger et al., 1997). Expression of laminin chains has been shown to start very early in the mouse embryo and is absolutely required for its development, laminin-1 representing the first isoform expressed (Wu et al., 1983). At the two- to four-cell stage, the $\beta1$ and $\gamma1$ chains have been detected (Dziadek and Timpl, 1985), and at the 16-cell stage, with a visible basement membrane, the $\alpha1$, $\beta1$ and $\gamma1$ chains of laminin-1 have been found extracellularly (Cooper and McQueen, 1983). Laminin-1 plays further an important role in morphogenetic epithelial-mesenchymal interactions (Ekblom 1996, Schuger et al., 1997), and deletion of the LAMC1 gene precludes the formation of laminin trimers and of a basement membrane, and the development of embryos (Smyth et al., 1999). At later stages of development, the laminin $\alpha1$ chain is transiently expressed during mesenchymal-epithelial transition (Ekblom, 1996). It is synthesized by both epithelial and mesenchymal cells, but its synthesis by epithelial cells requires contact between epithelial and mesenchymal cells (Schuger et al., 1997). Expression of laminin $\alpha3$, $\beta3$ and $\gamma2$ chains is restricted to certain epithelial cells, while the $\beta1$, $\beta2$, $\gamma1$ and $\alpha5$ chains are produced by a broader repertoire of cells (Miner et al., 1997, Tiger et al., 1997). The expression of the $\alpha5$ chain seems to succeed that of the $\alpha1$ chain in diverse mature epithelia (Durbeej et al., 1996, Sorokin et al., 1997b), and thus, laminin-10 ($\alpha5\beta1\alpha1$) represents the predominating form in the adult organism.

1-3.2 The laminins of the dermo-epidermal junction

Laminin-5 ($\alpha3\beta3\gamma2$) was the first isoform shown to be specific of the dermo-epidermal basement membrane zone where it localizes to anchoring filaments (Rouselle et al., 1991). Other isoforms present at this location are laminin-6 ($\alpha3\beta1\gamma1$) and presumably laminin 7 ($\alpha3\beta2\gamma1$), while laminin-10 ($\alpha5\beta1\gamma1$) is in adult skin an integral part of the basement membrane itself (for review see Aumailley and Rouselle, 1999).

1-3.2.1 Laminin-10

Laminin-1 is known to self-associate and believed to form a hexagonal network. This assembly model involves the aminoterminal LN modules of the α , β , and γ chains (Yurchenco and Cheng, 1993), and applies probably for the other isoforms with homologous domain organization, i.e. with LN modules of each of the short arms (Cheng et al., 1997). Such a network is likely to be present in the basement membrane of the dermo-epidermal junction since it contains laminin-10 with the full-length $\alpha5$, $\beta1$ and $\gamma1$ chains.

Besides self-assembly, laminin-10 forms a stable complex with nidogen (Paulsson et al., 1987, Brown et al., 1994, Lindblom et al., 1994). This is the result of a high affinity interaction between one LE motif in domain III of the laminin $\gamma1$ chain and the carboxy-terminal G3 domain of nidogen (Fox et al., 1991, Mayer et al., 1993). By its G2 domain nidogen binds to type IV collagen and perlecan (Fox et al., 1991, Battaglia et al., 1992, Aumailley et al., 1993). So, despite the fact that type IV collagen and laminin-10 do not directly interact, by the formation of ternary complexes the two major networks become connected (Fig. 1.4).

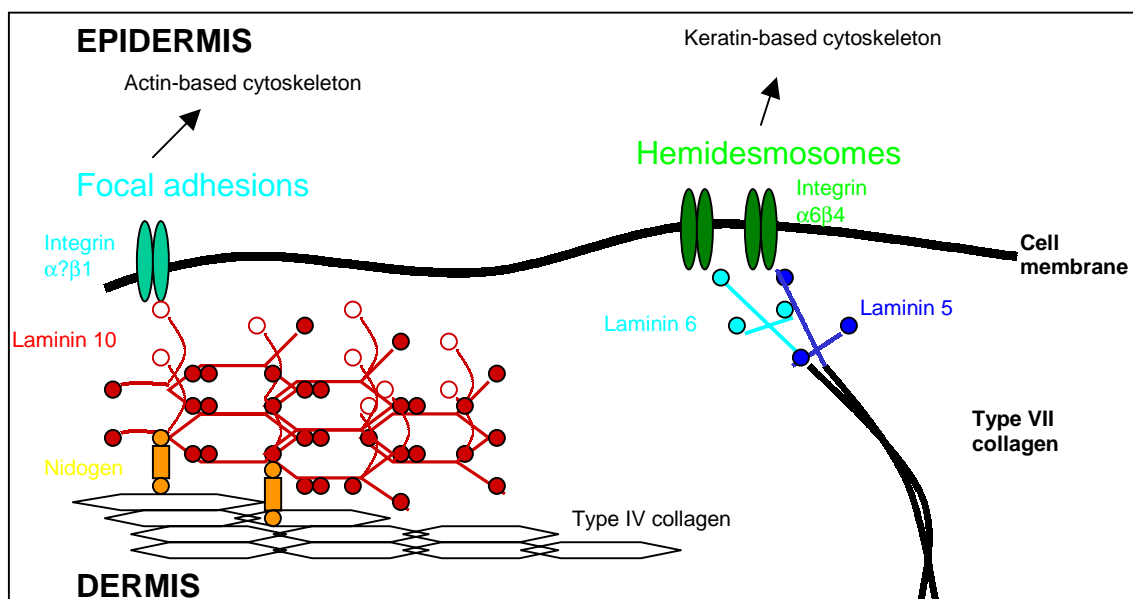


Fig. 1.4. Model for laminin assemblies at the dermo-epidermal junction. The theoretical assembly of laminin-10 is based on the three-arm polymerization hypothesis described for laminin-1 (Yurchenco and Cheng, 1993). For clarity the bended rod-like regions of laminin-10 (pointing out of the plane) are drawn as thinner lines.

1-3.2.2 Laminin-5

The three-arm based laminin assembly model apparently does not apply to isoforms with less than three LN modules, in particular those containing the $\alpha 3$, $\alpha 4$ or $\gamma 2$ chains (Cheng et al., 1997). Thus, laminin-5 ($\alpha 3\beta 3\gamma 2$) cannot form a two-dimensional network. Another important aspect is the very low affinity of the $\gamma 2$ chain to nidogen which in turn cannot form a bridge to type IV collagen. The replacement of two critical residues exposed at the surface of the $\gamma 11114$ motif is responsible for the low affinity (Mayer et al., 1995, Baumgartner et al., 1996).

Laminin-5 can therefore only form heteropolymers, which involves dimerisation with laminin-6 (Champlaud et al., 1996) and direct interactions with type VII collagen (Chen et al., 1997, Rouselle et al., 1997). Rotary shadowing images of laminin-5 have revealed that it interacts with the short arm of its $\beta 3$ chain with an amino-terminal site close to the intersection of the $\alpha 3$ short arm of laminin-6. These complexes are presumably stabilized by a disulfide bridge between a conserved unpaired cysteine in the LN module of the laminin-5 $\beta 3$ chain and domain III of the processed $\alpha 3$ chain in laminin 6 (Marinkovich et al., 1992a, Champlaud et al., 1996). The second mechanism for anchoring laminin-5 within the scaffold of the basement membrane is the interaction between laminin-5 localized in the anchoring filaments and type VII collagen (Fig. 1.4). Type VII collagen binds to laminin-5 or to the laminin-5/6 dimer, but not to laminin-6 alone (Rouselle et al., 1997, for review see Aumailley and Rouselle, 1999). Furthermore, laminin-5 can bind to the $\alpha 6\beta 1$, $\alpha 6\beta 4$, and $\alpha 3\beta 1$ integrins, presumably through its G domain (Delwel et al., 1994, Marchisio et al., 1993, Rouselle and Aumailley, 1994). Especially the interaction with the $\alpha 6\beta 4$ integrin seems to be very critical for the stability of the dermo-epidermal junction.

The dermal-epidermal junction contains plaque-like hemidesmosomes that connect to keratin tonofilaments in the keratinocyte cytoplasm (for review see Colognato and Yurchenco, 2000, Gerecke et al., 1994, Green and Jones, 1996). In the adjacent basement membrane, laminin-5 and integrin $\alpha 6\beta 4$ are enriched at these sites. Type VII collagen fibrils are connected to this complex arising from locations deep down in the dermis (Sakai et al., 1986). This array of linking molecules from keratin filaments to hemidesmosomes to laminins and finally to anchoring filaments insures a firm bond between keratinocyte and dermis that provides firm resistance against surface friction.

Because laminin-5 and laminin-6 contribute to this dermal-epidermal cohesion, mutations in the constituent chains result in the blistering disease junctional epidermolysis bullosa. In these patients the abnormally fragile skin is prone to vesicles, bullae and erosions as a result of mechanical trauma or friction. Blisters also occur on the mucous membranes and may in addition affect internal organs. The skin separates directly below the basal keratinocytes on the epidermal side of the basement membrane (McGowan and Marinkovich, 2000).

1-3.3 Laminin interactions

The physiological relevance of the interaction repertoire of laminins can be deduced from the phenotypes of patients affected with inborn or acquired diseases and of mice bearing site-directed mutations. Targeted extinction of the genes coding for the

laminin $\gamma 1$ chain (Smyth et al., 1999) led to prenatal death of the embryo. An important finding was that the null embryos lacked basement membranes. Embryonic stem cells cultured to form embryoid bodies provide a model for this process. This demonstrated that in the absence of laminin $\gamma 1$, embryoid bodies fail to form a basement membrane under the visceral endodermal cell layer. The inner cell mass have a disorganized distribution of type IV collagen and perlecan, and nidogen is secreted into the medium. In addition, it has been shown that the loss of laminin subunits $\alpha 5$, $\alpha 3$ or $\beta 2$ gives rise to intrinsically weaker basement membranes, affecting different tissues. (Miner et al., 1998, Ryan et al., 1999, Xu et al., 1994). These findings strongly suggest that laminin expression and transport into the extracellular space is a prerequisite for assembly of the other major components into the basement membrane.

Further studies provided strong evidence for the crucial role of laminin. An epithelial cell line CaCo2 can form a basement membrane when cultured on top of fibroblasts (DeArchangelis et al., 1996). Cells that failed to express laminin did not form a detectable basement membrane, and type IV collagen and nidogen remained associated with the fibroblast layer.

Genes coding for the integrin $\beta 1$ chain (Fässler et al., 1995, Stephens et al., 1995, Aumailley et al., 2000) or dystroglycan (Williamson et al., 1997) have also been targeted and deleted. This led to early embryonic lethality and indicates that not only laminin-1 but also its receptors are absolutely required for embryonic development.

It is also clear that there is no compensation for the structural and mechanical roles of laminin-5 and for its binding to $\alpha 6\beta 4$ integrins at the cell surface and to collagens in the lamina densa. Absence of laminin-5 or $\alpha 6\beta 4$ integrin, or alterations of their interactions are associated in man as well as in mouse with a dramatic loss of cohesion between epidermis and dermis and with severe complications mostly being lethal (Aumailley and Krieg, 1996). The phenotypes resulting from the absence of laminin-5 (e.g. the above mentioned junctional epidermolysis bullosa) are, at least in man, more severe than those resulting from the absence of $\alpha 6\beta 4$ integrin. This indicates that other laminin isoforms cannot replace the structural function of laminin-5, while that of the integrin $\alpha 6\beta 4$ is apparently compensated partially by other linkages with cell surface components, e.g. other integrins, type XVII collagen or α -dystroglycan (reviewed in Aumailley and Rouselle, 1999).

1-3.4 Nidogens, structure and regulation

Nidogen is a sulphated glycoprotein that is an integral part of the basement membrane. It associates with both laminin (in a 1:1 molar ratio) and type IV collagen and is thought to play an important role as bridge between these molecules.

When isolated under denaturing conditions, the molecule resembles a dumbbell when viewed by electron microscopy after rotary shadowing. However, the molecule under non-denaturing conditions appears to contain three rather than two globular domains separated by rod-like segments.

However, recently it was discovered that several isoforms of nidogen exist. cDNA studies first indicated the existence of related proteins in lower animals (Nakae et al., 1993, Mayer et al., 1994), and further cloning and sequencing revealed evidence of such isoforms (Kohfeldt et al., 1998, Kimura et al., 1998). The two mammalian

isoforms of nidogen are referred to as nidogen-1 and nidogen-2, and are similar in several but not all of their structural and functional aspects. Ultrastructural studies of nidogen-1 have revealed three globular domains (G1, G2, G3). G1 and G2 are connected by a thread-like structure, whereas that between G2 and G3 is rod-like (Nagayoshi et al., 1989, Olsen et al., 1989). Each domain contains particular motifs: in G2 it is similar to a cysteine-rich epidermal growth factor; the rod-like structure contains four EGF-motifs and one that is thyroglobulin-like (Carpenter and Cohen, 1979, Gray et al., 1983); G3 possesses an EGF-like motif and a region homologous to the LDL-receptor (Yamamoto et al., 1984). It was shown that nidogen-1 could potentially bind to laminin by its G3 domain (Fox et al., 1991, Mann et al., 1988) and to type IV collagen and perlecan by G2 (Reinhardt et al., 1993), resulting in formation of ternary complexes (Chakravarti et al., 1990). When this was compared to nidogen-2, high homology was observed between their individual G2 and G3 domains and rod-like structures, being all rich in cysteine residues. Thus, within the G2 domain of both nidogen-2 and nidogen-1, one EGF-like motif is located at the N-terminus, and the EGF precursor/LDL receptor homologous region was found in the G3 domain. In contrast, low homology was seen between the G1 domains and thread-like regions of both molecules (Kimura et al., 1998).

Analysis of the distribution in various tissues showed mostly co-expression of the two isoforms, although their relative amounts apparently differ. The two isoforms co-localize in various basement membranes, including renal glomeruli and tubuli, perineural structures and blood vessels and the dermo-epidermal junction. In contrast, the expression differs in cardiac and skeletal muscle (Kohfeldt et al., 1998).

Nidogen-1 has been shown to have a broad binding repertoire for major basement membrane proteins such as laminins, type IV collagen, perlecan and fibulin (Mayer and Timpl, 1994, Timpl and Brown, 1996). These interactions are considered to connect and stabilize the basement membrane, and to target mesenchymal nidogen to epithelial basement membranes (Dziadek, 1995). Nidogen-2 seems to share some of these functions, since it binds in a comparable manner to type IV collagen, perlecan and type I collagen, but fails to bind fibulins (Kohfeldt et al., 1998).

The only nidogen-1 high-affinity binding site of laminins has been localized to a single LE module γ 1III4 in the short arm of the γ 1 chain (Mayer et al., 1993). Nidogen-2 binds to this epitope with a 100 to 1000-fold lower affinity, indicating a distinct functional difference. Just three residues located in a narrow ridge of the γ 1III4 epitope contribute to the high-affinity binding of nidogen-1, suggesting that there may be three donors for electrostatic, hydrogen bond and van der Waals interactions in the complementary binding site of nidogen-1 domain G3 (Pöschl et al., 1996). Probably is the same region of nidogen-2 domain substantially different. Several data have indicated that nidogen-2 can bind to laminin through at least a second epitope not related to γ 1III4 (Kohfeldt et al., 1998).

Furthermore, nidogen-1 has been reported to be a cell-adhesive protein, based on its interaction with several integrins (Dedhar et al., 1992, Senior et al., 1992, Yelian et al., 1993, Dong et al., 1995, Gresham et al., 1996). These integrins were identified as α V β 3 and the α 3-like leukocyte response integrin, which recognize a RGD sequence in the rod-domain (Dong et al., 1995, Gresham et al., 1996), and α 3 β 1 integrin, which binds to the G2 domain (Dedhar et al., 1992, Gresham et al., 1996). Nidogen-2 also promoted cell adhesion and spreading even more strongly than nidogen-1, but this

interaction could not be inhibited by a synthetic RGD peptide, a sequence not present in nidogen-2, or by blocking antibodies against $\beta 1$ and $\alpha 3$ integrin subunits. This suggests that different integrins or possible non-integrin receptors are involved in cell adhesion to the two nidogen isoforms (Kohfeldt et al., 1998).

1-3.5 Nidogen interactions

The relevance of nidogen in the basement membrane has been investigated by the generating of transgenic mice with null mutations in the NID-1 gene or the NID-2 gene.

Since nidogen-1 interacts with all other major basement membrane components, particularly type IV collagen and laminin, it was suggested that nidogen-1 plays a mainly structural role within the basement membrane. Furthermore, embryonic basement membranes do not have a fully developed ultrastructural architecture until nidogen-1 is present (Misogoe et al., 2000). Moreover, since mice lacking the LAMC1 gene coding for the laminin $\gamma 1$ chain lack basement membrane (Smyth et al., 1999), it was believed that lack of nidogen-1 would affect the basement membrane. But surprisingly, it turned out that this was not the case. Thus, nidogen-1 null-mice appeared to develop normally and not to have any structural anomalies of the basement membranes (Murshed et al., 2000), neither in the skin nor in other tissues. The most likely explanation is that nidogen-2 completely compensates for the loss of nidogen-1, as the nidogen-2 expression is increased in certain basement membranes where it is low or virtually absent in wild-type mice (Murshed et al., 2000, Miosge et al., 2002).

Accordingly, mice with a null mutation in the nidogen-2 gene display no alterations in their basement membranes (Schymeinsky et al., 2002). Nidogen-1- and nidogen-2 null-animals develop normally and are fertile, which allows also cross-breeding to generate double-null animals. However, the double-null mice are born with very severe defects and die at the perinatal stage due to kidney and lung arrest (Nischt, unpublished data). Ultrastructural analysis of the skin have not revealed anomalies so far, but dermal fibroblasts have been isolated for further investigations.

1-3.6 Type IV collagen, structure and regulation

Type IV collagen is found exclusively in basement membranes where it provides major structural support. The molecule contains a long (approximately 350 nm) triple-helical domain containing approximately 20 short interruptions that are thought to introduce flexibility into the helix portion. Type IV collagen self-assembles into a meshwork by the anti-parallel interaction and extensive disulfide bonding of four molecules at their NH_2 -termini to form the 7S domain, by the interaction of two molecules at their COOH-terminal-non-collagenous (NC1) domains, and by lateral aggregations.

This type IV collagen network may provide a scaffold for the assembly of other basement membrane components through specific interactions with laminin, nidogen and perlecan. In addition, this network endows the basement membrane with a size-selective filtration property.

Type IV collagen molecules are composed of three α chains selected from the translated product of six genetically distinct genes. The most abundant form of type

IV collagen has the composition $\alpha1(IV)_2\alpha2(IV)$ (Soininen et al., 1997, Hostikka and Tryggvason, 1988, Mariyama et al., 1994). The composition of the molecules containing the less abundant $\alpha3(IV)$ - $\alpha6(IV)$ is not certain, although the chains are expressed in tissue-specific manner. Thus, the chains $\alpha3$ - $\alpha6$ are predominantly expressed in kidney, and absolutely required for development and kidney function. Cysteine residues in the NC1 domains of all chains sequenced to date are conserved and participate in both intra-chain and inter-molecular disulphide bonding (Leinonen et al., 1994, Zhou et al., 1992, Oohashi et al., 1994). The trimeric, disulphide-bonded CB3 cyanogen bromide fragment of type IV collagen has been shown to support cell adhesion via the integrins $\alpha1\beta3$ and $\alpha2\beta1$ (Vandenberg et al., 1991). With regard to skin (and the described 3D-coculture system), it should be noted that type IV collagen (in contrast to nidogen) can be synthesized by the epidermal keratinocytes and dermal fibroblasts.

1-3.7 Perlecan, structure and regulation

Perlecan is a large proteoglycan, and a specific and integral component of all basement membranes. It provides basement membranes with a fixed negative electrostatic charge and is responsible, in part, for the charge-selective ultra-filtration properties of this extracellular matrix. It interacts with the other basement membrane components such as laminin and type IV collagen, and also nidogen, which binds its G2 domain to perlecan. Comparable to type IV collagen, perlecan is probably synthesized by different cell types (keratinocytes and fibroblasts) in complex tissues as the skin.

Perlecan consists of a large protein core of 470 kDa molecular mass bearing three heparan sulfate glycosaminoglycan side-chains. The heparan sulfate side chains are located near one end of the protein core (N-terminus), and their size varies depending upon the source of the proteoglycan. The perlecan core protein has been divided into five domains I-V according to their amino-acid sequence (Paulsson et al., 1987a, Kallunki et al., 1991, Kallunki and Tryggvason, 1992). This domain structure becomes visible by electron microscopy after rotary shadowing and can be seen as series of globular domains separated by thin segments.

1-3.8 Perlecan interactions

Embryos carrying a targeted null mutation for perlecan show a different phenotype from that of mice lacking any described biosynthetic enzyme, as demonstrated in independent studies (Costell et al., 1999, Arikawa-Hirasawa et al., 1999). Many perlecan-deficient embryos die at prenatal day 10-17 due to rupture of basement membranes in the heart. Embryos that survive this critical stage go on developing exencephaly or other brain defects and die perinatally. These defects are due to stress-sensitive basement membranes, which are obviously not correctly assembled in the absence of perlecan. Generally, the structural importance of perlecan can be attributed to both the core protein, which binds to type IV collagen and nidogen-1, and to the heparan sulfate chain, which interact with laminins among other extracellular matrix proteins (Timpl and Brown, 1996). However, the analysis of the skin has revealed no abnormalities.

Chondrodysplasia has also been observed in perlecan-null animals surviving the early critical period. The phenotype is characterized by an abnormal fibrillar collagen network and altered expression of other cartilage extracellular matrix genes, suggesting that perlecan protects the cartilage extracellular matrix from degradation (Costell et al., 1999). Other results indicate that defective growth factor signaling may also contribute to chondrodysplasia in perlecan-null embryos (Arikawa-Hirasawa et al., 1999). Growth factor binding primarily occurs to the heparan sulfate chains, but the perlecan core protein has been shown to interact with fibroblast growth factor 7, FGF-7 (Mongiat et al., 2000) and also with FGF-binding protein (Mongiat et al., 2001), a modulator of FGF-2 activity.

Recently, mutations for perlecan have been associated with human Schwartz-Jampel syndrome (chondrodystrophic myotonia) (Nicole et al, 2000). While these patients have a myotonic phenotype and skeletal dysplasia, they do not show craniofacial abnormalities. Since the N-terminal part of perlecan is still present in patients with this disease, and this region carries the heparan sulfate chains, a lack of heparan sulfate might be crucial for the craniofacial phenotypes.

2. OBJECTIVE

The basement membranes are ubiquitous extracellular protein membranes, which are adjacent to or surround distinct formations or individual cells. They form stable sheets through self-assembly mechanisms and provide ligands for specific interactions with cellular receptors. They have a variety of biological functions, such as providing tissue compartmentalization by acting as selective barrier to cell penetration, supporting tissue, and controlling cell migration and proliferation.

In 3D-coculture systems mimicking the situation in skin, the basement membrane can be closer investigated. These skin organotypic 3D-cocultures consist of keratinocytes growing air-exposed on a type I collagen gel with incorporated fibroblasts. In this system, a stratified, squamous epithelium develops displaying all differentiation characteristics of epidermis within a period of 1-2 weeks. Furthermore, a basement membrane forms at the interface between the keratinocytes and the collagen matrix. Finally, the 3D-coculture provides a closed system, where the input and output can be controlled, and regulatory or compensatory mechanisms can be avoided.

The goal of this study was to provide more insight into (i) the role of individual components on basement membrane assembly and (ii) the importance of regular interactions between these components.

In particular the aims of the investigations were:

- 1) The specific role of nidogen-1 or nidogen-2 for basement membrane formation. For this purpose, nidogen knockout fibroblasts from transgenic mice of various genotypes were incorporated into the 3D-coculture system, fibroblasts being the only source of nidogen herein.
- 2) The effect of exogenous nidogen-1 and nidogen-2 in a nidogen-deficient environment. In order to restore normality in the presence of nidogen knockout fibroblasts, recombinant nidogen was applied to the 3D-coculture system.
- 3) The cell source of perlecan, made by epidermal or dermal cells in basement membrane and its role on assembly. To assess that, first perlecan deficient fibroblasts were grown in the 3D-coculture system with normal keratinocytes (HaCaT cells). Second, perlecan antisense mRNA transfected HaCaT cells were combined with normal fibroblasts. Third, the perlecan deficient cell types were applied together in this system.
- 4) The importance of molecular interactions, exemplified by the laminin-nidogen complex. To interfere with complex-formation, a laminin fragment L γ 1f, exhibiting high affinity to nidogen, was used in the 3D-coculture system.
- 5) Challenge the current model on laminin network formation. A N-terminal γ 1 fragment LN+4EGF supposed to interfere with the three-arm bond self-assembly of laminin, was applied to the 3D-coculture system.

3. MATERIALS

3-1. Cell culture

3-1.1 Cells

3-1.1.1 The HaCaT cell line

The spontaneously immortalized, human keratinocyte cell line HaCaT, was established in our research group from a normal primary culture of human epidermal cells in the vicinity of a melanom in a 62-year-old patient (Boukamp et al., 1988). A genetic instability due to UV-specific mutations in both alleles of the tumor suppressor gene p53 (Lehman et al., 1993) as well as an elevated initial culture temperature is considered as determinations for the immortalization. Despite their genetic alterations and prolonged life in culture, HaCaT cells conserve many morphological and biochemical criteria of normal epidermal cells (Ryle et al., 1989). Many variants have been derived from the standard HaCaT cell line. The following cell lines were used in this study in addition to the standard HaCaT cell line:

HaCaT subclone 6: Standard HaCaT cells were cloned in the 10th passage and further recloned after the 16th passage. From this population, the highly differentiating HaCaT subclone 6 was derived. In this study, cells from HaCaT subclone 6 (HaCaT clone 6) were used in the passages 10-17.

HaCaT diploid: This line was selected early during establishment of HaCaT. HaCaT diploid cells were used in the passages 12-15.

HaCaT expressing perlecan antisense mRNA: Low (control) and high expressors of perlecan antisense mRNA cells was kindly provided from D. Ron, Israel.

3-1.1.2 Normal primary keratinocytes

Human keratinocytes were isolated from healthy human skin obtained from operations in the University hospital of Heidelberg (Smola et al., 1993).

3-1.1.3 Fibroblasts

In this study, human as well as mouse fibroblasts were used. Human fibroblasts were isolated from the dermis of healthy human skin obtained from the University hospital of Heidelberg (Smola et al., 1993). Mouse fibroblasts were isolated from skin from various knockout mice. The nidogen knockout fibroblasts were kindly provided from Dr. R. Nischt, Dept. of Dermatology, University of Cologne. The perlecan knockout fibroblasts were kindly provided from R. Faessler, University of Lund, Sweden. The fibroblasts used in the study were all primary, with one exception, perlecan knockout fibroblasts were SV-40 transfected.

3-1.2 Media

DMEM: Dulbecco`s Modified Eagles Medium (BioWhittaker, Serva).

FAD: FAD consists of 1 part Ham`s F12 (Serva), 3 parts DMEM, supplemented with 5 µg/ml insulin, 18,2 µg/ml adenin, 0,4 µg/ml hydrocortisone, 10^{-10} M Cholera toxin and 10 ng/ml EGF (all Sigma).

rFAD: reduced FAD, consists of 1 part Ham`s F12, 3 parts DMEM, 0,4 µg/ml hydrocortisone and 10^{-10} M Cholera toxin.

SKDM: SKDM consists of KBM (keratinocyte basal medium, Clonetics) supplemented with 0,1 ng/ml EGF, 5 µg/ml insulin, 0,5 µg/ml hydrocortisone, 1 mg/ml bovine serum albumin (PAA Laboratories GmbH), 100 µg/ml transferrin (Serva) and 100 µg/ml gentamicin (Clonetics).

FAD, rFAD and DMEM also contained 100 U/ml penicillin, 50 µg/ml streptomycin and 1mM sodium pyruvat (all Hoechst).

3-1.3 Further media supplements

Ascorbic acid	Sigma
Recombinant nidogen 1 and 2	Dr. Neil Smyth, University of Cologne
Recombinant laminin γ 1LN+4EGF fragment	Dr. Neil Smyth, University of Cologne
Recombinant laminin γ 1III3-5 fragment	Aventis Germany, Frankfurt am Main

3-1.4 Reagents

EDTA	0,05% in PBS-, + 1 µg/ml phenolred (Serva)
Fetal Calf Serum (FCS)	heat-inactivated, 30 min, 56°C (Biochrom)
Geneticin	100 mg/ml in PBS; filter sterilized (Gibco-BRL)
Hank`s salts	with 8 mg/ml phenolred without NaHCO ₃ (Seromed)
PBS-buffer	with or without (-) Mg ²⁺ and Ca ²⁺ (Serva)
Phenolred	0,5% in PBS (Biochrom)
Trypsin	0,1% in PBS-, + 1 µg/ml phenolred

3-2. Protein analysis

3-2.1 Primary antibodies and secondary antibodies for immunofluorescence

The following primary antibodies were used for immunofluorescence (Tab. 3.1):

Antigen/ Nr.	Specificity	Type	Dilution	Obtained from	Catalogue nr.
Type IV collagen Nr. 153	Type IV collagen, human	Rabbit polyclonal	1:500	Heyl	-----
Type IV collagen Nr. 495	Type IV collagen, human	Mouse monoclonal	1:200	Progen	10710
Type IV collagen Nr. 536	Type IV collagen, mouse	Rabbit polyclonal	1:600	J.M. Foidart	-----
Type VII collagen Nr. 478	Type VII collagen, human	Rabbit polyclonal	1: 10 000	L. Tudermann, Uni. Muenster	-----
Fibronectin Nr. 180	Fibronectin, human	Rabbit polyclonal	1:600	Telios	-----
Integrin alpha 6 Nr. 370	Integrin alpha 6 chain	Rat monoclonal	1:400	A. Sonnenberg	-----
Integrin beta 1 Nr. 385	Integrin beta 1 chain, human/ CD 29	Mouse monoclonal	1:200	Immunotech	1151
Integrin beta 1 Nr. 373	Integrin beta 1 chain, A II B2, human	Rat monoclonal	1:5	C. Damsky	-----
Keratin 1,10,11 Nr. 638	Keratin 1,10,11, human	Mouse monoclonal	1:100	Sigma	C7284
Laminin Nr. 414	Laminin, mouse	Rabbit polyclonal	1:160	Hartmann, Inst. Pasteur, Lyon	-----
Laminin-5 Nr. 328	Laminin-5 (Kalinin)	Rabbit polyclonal	1:200	P. Marinkovich	-----
Laminin alpha 1 Nr. 720	Laminin alpha 1 chain	Mouse monoclonal	1:1	I. Virtanen, Helsinki	-----
Laminin alpha 5 Nr. 723	Laminin alpha 5 chain, human	Mouse monoclonal	1:3000	I. Virtanen, Helsinki	
Laminin Nr. 496	Laminin EHS, mouse	Rabbit polyclonal	1:100	Progen	10765
Laminin gamma 1 Nr. 528	Laminin gamma1 III5, human	Mouse monoclonal	1:2000	HMR Gerl Coop	-----
Laminin gamma 2 Nr. 466	Laminin gamma 2, human	Rabbit polyclonal	1:1000	Meneguzzi, Nice	-----
Loricrin Nr. 204	Loricrin, a-CREP, AF-62	Rabbit polyclonal	1:8	D. Hohl, Lausanne	-----
Nidogen-1 Nr. 416	Nidogen-1, human	Rabbit polyclonal	1:2000	R. Nischt, Cologne	-----
Nidogen-2 Nr. 593	Nidogen-2, human	Rabbit polyclonal	1:1000	R. Nischt, Cologne	-----
Perlecan Nr. 177	Perlecan	Mouse monoclonal	1:50	Biermann GmbH	-----
Perlecan Nr. 640	Perlecan, human	Rat monoclonal	1:4000	Chemicon	1948
Transglutaminase Nr. 423	Transglutaminase BC1	Mouse monoclonal	1:20	Harbor BioProd Cellsyst.	5003
Vimentin Nr. 628	Vimentin, human	Guinea pig, polyclonal	1:200	Progen	GP53
Vinculin Nr. 357	Vinculin, human	Mouse monoclonal	1:500	Sigma	V-9131

Table 3.1. Primary antibodies for immunofluorescence

The following secondary antibodies were used for immunofluorescence (Tab. 3.2):

Specificity	Fluorochrom	Obtained	Catalogue nr.
Guinea pig	Fluorescein (DTAF)	Dianova	706-015-148
Mouse	Alexa 488 (green)	Molecular Probes	A-11029
Mouse	Alexa 568 (red)	Molecular Probes	A-11031
Rabbit	Cy3	Dianova	111-165-144
Rabbit	Alexa 488 (green)	Molecular Probes	A-11034
Rat	Cy3	Dianova	712-166-152

Table 4.2. Secondary antibodies for immunofluorescence

3-2.2 Primary and secondary antibodies for Western blotting

The following primary antibodies were used in Western blotting (Tab. 3.3):

Antigen/ Nr.	Specificity	Type	Dilution	Obtained	Catalogue nr.
Type IV collagen Nr. 153	Type IV collagen, human	Rabbit polyclonal	1:500	Heyl	-----
Laminin-5 Nr. 328	Laminin-5 (Kalinin)	Rabbit polyclonal	1:200	P. Marinkovich	-----
Laminin alpha 5	Laminin alpha 5 chain, human	Rabbit polyclonal	1:200	Santa Cruz Biotechnology	SC-20145
Laminin gamma 1	Laminin gamma 1 chain, human	Mouse monoclonal	1:100	Santa Cruz Biotechnology	SC-17751
Laminin gamma 2	Laminin gamma 2 chain, human	Mouse monoclonal	1:200	Chemicon	19562
Nidogen-1 Nr. 416	Nidogen-1, human	Mouse monoclonal	1:500	R. Nischt, Cologne	

Tab 3.3. Primary antibodies for Western blotting

The following secondary antibodies were used in Western blotting (Tab. 3.4):

Specificity	Detection	Obtained	Catalogue nr.
Rabbit	POX	Dianova	711-035-152
Mouse	POX	Dianova	800-367-5296

Tab. 3.4. Secondary antibodies for Western blotting

3-2.3 Protein analysis reagents

Acrylamid (T40%-C3,7%)	Biorad
Ammonium persulfate (APS)	Serva
Aprotinin	Roche
Bradford reagent	Biorad
ECL	Amersham-Pharmacia Biotech
Leupeptin	Roche
Ortho-vanadate	Serva

4-(2-aminoethyl)-benzene-sufonylfluoride (Pefablock)	Roche
Pepstatin	Roche
Ponceu solution	Sigma
Rainbow marker	Amersham-Pharmacia Biotech
N,N,N,N,-Tetra-methyl-ethylen-diamine (TEMED)	Sigma

3-3. In situ hybridisation

3-3.1 Plasmids, cDNA and bacteria

Nidogen-1 cDNA cloned into plasmid pSPORT 1 vector was generously provided from Dr. R. Nischt, Dept. of Dermatology, University of Cologne. Antisense probes were generated by restriction with Not I and transcription from T7 promotor, sense probes by restriction with Sal 1 and transcription from SP6 promotor.

Nidogen-2 cDNA cloned into plasmid pBLUEScript vector was generously provided from Dr. B. Bader, Dept. Protein Chemistry, Max-Planck-Institute of Biochemistry, Munich. Antisense probes were generated by restriction with EcoRI and transcription from T7 promotor, sense probes by restriction with XhoI and transcription from T3 promotor.

All plasmids were propagated in E.coli HB101

3-3.2 Reagents, kits and enzymes

RNA molecular weight marker	AGS
DNA molecular weight markers	AGS
RNase inhibitor	Roche
DIG-RNA labeling mix	Roche
Anti-digoxigenin fragment	Roche
TRNA	Sigma
BCIP	Gibco BRL
NBT	Gibco BRL
10x loading buffer	Roche
Plasmid Maxiprep kit	Qiagen
DNase I	Boehringer Mannheim
RNase A	Boehringer Mannheim
Proteinkinase K	Boehringer Mannheim
Restriction endonucleases	Roche
RNA polymerases	Boehringer Mannheim

3-3.3 Standard solutions for molecular biology

TE buffer, pH 7,5	50x TAE
10 mM Tris-HCl (pH 7,5)	Tris-base 2M
1 mM EDTA (pH 8,0)	Glacial acetic acid 0,95 M
	EDTA 50 mM
20x SSC, pH 7,4	DEPC-H ₂ O
NaCl 3M	Treat water overnight with 0,1% DEPC,
Na-citrate 0,3 M	autoclave
Adjust pH with 10 M NaOH	

Deionized formamide

Mix 1l formamide and 50 g of ion-exchanger resin and stir 30 min at room temperature. Filter through filter paper and keep at room temperature.

3-4. Additional material

Cell scraper	Costar
Cell culture dishes	Becton Dickinson/ Falcon
Deep-well plates	Becton Dickinson/ Falcon
Micron polycarbonate filter	Becton Dickinson/ Falcon
Blotting membrane	Schleicher & Schuell
Films	Kodak
Slides	Menzel Gläser
Gel blotting apparatus	Amersham Biosciences

3-5. Special equipment

CASY cell counter and analyzer system	Schaerfe System GmbH
Cryotom Frigocut 2700	Reichert und Jung
Electron microscope	Zeiss
ELISA reader	SLT Labinstruments
Fluorescence and light microscope DM RD	Leica
Film analyzer	Optimax TR
Gelanalyse-System Multimanager™ 4.400	Alpha Innotech Corp
Incubator with CO ₂	Heraeus
Laser confocal microscope	Leica
Light microscope BX51	Olympus
Sorvall RC-2-B (GSA, SS-34)	DuPont
Spectral photometer	Eppendorf
Sterile bench	Baker Company Inc.

3-6. Chemicals

Acetic acid	Riedel-de Haen	Lead citrate	Serva
Acetic anhydride	Sigma	Magnesium chloride	Merck
Acetone	Baker	Mayer`s hemalaun solution	Merck
Agarose	FMC	β - Mercaptoethanol	Serva
Ammonium acetate	Merck	Methanol	Baker
Ampicillin	Sigma	Moviol	Calbiochem
Bacto agar	Difco	Levamisol	Sigma
Bisbenzimidazole Hoechst 33258	Sigma	Paraformaldehyde	Merck
Calcium chloride	Merck	PBS	Serva
Chloroform	Merck	Phenol	Roth
Dextran sulfate	Pharmacia	Polyvinylpyrrolidone	Sigma
Diethyl pyrocarbonate (DEPC)	Sigma	Osmium acetate	Serva
DL-dithiothreitol (DTT)	Sigma	Sodium acetate	Merck
EDTA	Serva	Sodium cacodylate	Serva
Eosin	Sigma	Sodium carbonate	Merck
Ethanol	Riedel-de Haen	Sodium chloride	Fluka
Ethidium bromide	Sigma	Sodium citrate	Merck
Ficoll 400	Sigma	Sodium dodecyl sulfate (SDS)	Gerbu
Formaldehyde, 37%	Merck	Sodium hydrogen carbonate	Merck
Formamide	Fluka	Sodium hydrogen phosphate	Merck
Glutaraldehyde	Serva	Sodium hydroxide	Baker
Glycerol	Roth	Triethanolamine	Merck
Glycin	Roth	Tris buffer	Sigma
Glycid ether	Serva	Triton X-100	Serva
Hydrochloric acid	Merck	Tryptone	Difco
Ion exchanger V	Merck	Tween 20	Merck
Isomylalcohol	Merck	Uranyl acetate	Serva
		Yeast extract	Difco

4. METHODS

4-1. Cell culture

The cell lines were grown in standard media, and medium was changed 3 times a week. Cell culture dishes were from Falcon (Becton Dickinson).

4-1.1 Cells and culture conditions

The keratinocyte cell line HaCaT and its variants were cultured in DMEM medium with 10 % fetal calf serum (FCS). For the HaCaT perlecan antisense clone the medium was additionally supplemented with 400 µg/ml geneticin, G418. Primary human keratinocytes were cultured in FAD medium with 5 % FCS, and fibroblasts were grown in DMEM medium with 10% FCS. The growth media for the different cell types are listed in Table 4.1.

Cells were routinely grown in a humidified atmosphere of 5% CO₂/95% air at 37°C. When confluence was reached, cells were subcultured. Standard HaCaT cultures were normally subcultured at a split ratio 1:10 every 10 days. Cells were dissociated from each other by incubation with 0,05% EDTA for 7 min at 37°C, and subsequently detached from the growth surface by incubation with a 0,05% Trypsin/0,025% EDTA mixture for 3 min at 37°C. Trypsin was neutralized by addition of 2 volumes of FCS-containing medium. Quantitative analysis of cell numbers was performed with a CASY cell counter and analyzer system.

Cell type	Medium	FCS
HaCaT clone 6	DMEM	10%
HaCaT standard	DMEM	10%
HaCaT diploid	DMEM	10%
HaCaT antisense perlecan	DMEM + 400 µg/ml geneticin	10%
Primary human keratinocytes	FAD	5%
Primary human dermal fibroblasts	DMEM	10%
Primary mouse dermal fibroblasts	DMEM	10%

Table 4.1. Cell types and corresponding culture mediums

4-1.2 Cryopreservation

The cells were detached from the culture dish as described for routine subculture method. The cells were counted, centrifuged at 1000 rpm for 5 min to remove trypsin and the cell pellet was resuspended in freeze medium (growth medium with 20% FCS and 10% glycerin) at final concentration of 2x10⁶ cells/ml. 1 ml aliquots were dispensed in Cryo-tubes and pre-cooled for 1 h at 4°C. The freezing was preformed

gradually (1°C/min) in an automatic cell freezer. Frozen samples were stored in liquid nitrogen.

Rapid thawing was achieved in a 37°C waterbath and cells transferred immediately to a 10- or 15-cm culture dish containing culture medium. Medium was changed 24h after thawing.

4-1.3 Test for mycoplasma contamination

All cell lines were prior to work tested for mycoplasma contamination. Cells were cultivated for two passages in antibiotic-free medium and then seeded on slides. When 80% confluence was reached, cells were washed with PBS and fixed in freshly prepared Carnoy's fixative (methanol/glacial acidic acid 3:1) for 15 min at room temperature. Air dried cells were stained with Hoechst bisbencimide (0,5 µg/ml), a DNA intercalating dye, for 30 min at room temperature in the dark. Following 3 washes in dH₂O cells were mounted in moviol and examined under UV epifluorescence at high magnification for positive signals in the cytoplasm.

4-1.4 Growth kinetics

HaCaT cells were seeded in a 12-well plate (8000 cells/well) and allowed to settle and spread. The first medium change with addition of growth factors occurred 8 h after plating. Proliferation in culture was evaluated by trypsination and counting of cells every 24 hour over a period of 10 days. Medium was changed every 2 days. Plotting cell counts versus time completed a growth curve.

4-2. Skin equivalents

4-2.1 Preparation of 2D cultures

Human or mouse dermal fibroblasts (150 000) were seeded in a 10 cm culture dish and incubated at 37°C. After 24 hours, HaCaT cells or primary human keratinocytes (100 000) were seeded on top of the fibroblast layer. Medium was changed after another 24 h, and then 3 times a week. After 10 days, the cultures were harvested by scraping the cells of the dish and lysed directly in 200 µl RIPA buffer (see 4-3.4)

4-2.2 Preparation of collagen

Collagen type I was isolated from rat tail tendons. The rat tails were washed in 80% ethanol and sterile H₂O, then the skin was peeled away and the tendons pulled out and collected in PBS. After removing the small blood vessels, the tendons were dried and dissolved in 0,1% acidic acid (4°C) to a concentration of 4 mg/ml. The solution was stirred for 24 h at 4°C, centrifuged 10000 g for 20 min until the supernatant was clear and freeze-dried. The freeze-dried collagen was stored at room temperature.

4-2.3 Preparation of 3D-organotypic cocultures

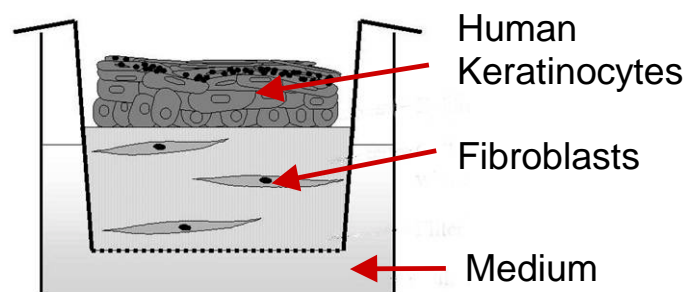
A collagen type I gel with integrated fibroblasts was prepared by mixing the 4 mg/ml collagen solution with 10x Hank`s balanced salt solution. After neutralization with NaOH, FCS containing human or mouse dermal fibroblasts in suspension ($1,5-2 \times 10^5$ cells/ml) was added. All solutions added to the collagen were ice-cold and the mixture was kept on ice during manipulation. 2,5 ml of this mixture was poured on to polycarbonate filter inserts (3 μ m pore size) placed in deep-well plates (both Becton-Dickinson). After 1 h incubation at 37°C, glass rings (20 mm diameter) were gently pressed on to the gels to press out excess liquid and to provide a defined area of epithelial growth. After another 15 min incubation at 37°C, the excess liquid was removed and the gels were incubated in 15 ml medium (DMEM, rFAD or SKDM supplemented with 50 μ g/ml ascorbic acid) in each well and 1 ml medium on top of the gel.

Collagen gel mixture

<i>Collagen solution (4 mg/ml)</i>	<i>80%</i>
<i>Hank`s solution (10x)</i>	<i>10%</i>
<i>FCS</i>	<i>10%</i>
<i>NaOH (5M)</i>	<i>several drops; until the gel changes color from yellow to light pink</i>

After 24 h, HaCaT cells or primary human keratinocytes in 1 ml medium were seeded on to the gels (1×10^6 cells/insert). After another 24 h, the glass rings were removed and the cultures were lifted to the air-liquid interface by removing all medium from the surface and incubate the cultures in only 10 ml medium in each well (Fig. 4.1). From this point on the cultures were only nourished through the collagen gel. Medium was changed 3 times a week. After 10, 12 and 14 days, the cultures were harvested and processed for cryosections, histology, electron microscopy or western blotting.

Fig. 4.1: The 3D-organotypic coculture



4-3. Tissue processing

4-3.1 Cryostat sectioning

Specimen were embedded in Tissue Tec, slowly frozen in evaporating liquid nitrogen and further stored in liquid nitrogen. Sections, 5-7 μm thick, were made on a cryostat, collected on sylan-coated slides, air-dried and stored at -80°C for immunofluorescence or in situ hybridization, respectively.

4-3.2 Histology

Specimen were prefixed in buffered formalin (3,7%) overnight, embedded in agar (2%) and postfixed in formalin. After paraffin embedding, 5 μm sections were cut, stretched at a waterbath at 40°C before collected on slides and dried. Before staining with hematoxilin and eosin, the paraffin was removed.

4-3.3 Electron microscopy

Specimen were fixed in 2,5% glutaraldehyde in 0,05 M sodium cacodylate buffer (pH 7,2) at 4°C . After several washes in 0,05 M sodium cacodylate buffer, specimens were post-fixed in 2% osmium tetroxide for 2 h at 4°C . The samples were incubated in 0,5% aqueous uranyl acetate, dehydrated and embedded in glycid ether, equivalent to EPON 812. Ultrathin sections were counterstained with 4% uranyl acetate and lead citrate and viewed under an electron microscope operated at 100 kV.

4-3.4 Preparation for western blotting

Specimen were incubated 1 h in 0,01 mg/ml DL-dithiothreitol (DTT) at 4°C . After incubation the epithelia was gently separated from the collagen gel, both parts were cut into small pieces and collected in 200 μl RIPA buffer supplemented with protease inhibitors (see below). Lysates were incubated 1 h on ice before centrifuged at 13 000 rpm 10 min at 4°C . The supernatants were transferred to clean tubes and the protein amount was quantified. In order to prevent protein degradation through the action of proteases, all the steps in the isolation were carried out on ice and using ice-cold solutions. Protein quantification was determined by a colorimetric assay (Bradford reagent from Bio-Rad) using a standard BSA concentration curve as reference, covering the range from 2,5 to 20 $\mu\text{g/ml}$. The reaction was measured in an ELISA-reader at 595 nm.

<i>RIPA buffer</i>		<i>Protein inhibitors</i>	
<i>Tris-HCL pH 7,2</i>	<i>10 mM</i>	<i>Leupeptin</i>	<i>1 µg/µl</i>
<i>NaCl</i>	<i>150 mM</i>	<i>Pepstation</i>	<i>1 µg/ml</i>
<i>Triton X-100</i>	<i>0,1%</i>	<i>Aprotinin</i>	<i>1 µg/ml</i>
<i>SDS</i>	<i>0,1%</i>	<i>Ortho-vanadate</i>	<i>100 mM</i>
<i>Sodiumdeoxycholatl</i>	<i>0,1%</i>	<i>Pefablock</i>	<i>10 mM</i>
<i>EDTA</i>	<i>5 mM</i>		

4-4. Indirect immunofluorescence

4-4.1 Basic protocol

Cryostat sections were made (5-7 µm), collected on sylan-coated sections, air-dried and used immediately or stored at -80°C. For direct staining of fibroblasts, cells were grown directly on the slides. Slides were fixed in methanol (-20°C) for 1 min, then acetone (4°C) for 2 min, and then rehydrated in PBS for 2 min at room temperature before blocking with 0,02% BSA in PBS for 15 min with gently agitation. Slides were placed in moist chamber and primary antibody (15 µl) was applied. The antibodies were always diluted in PBS+1% BSA+0,02% NaN₃. Primary antibody was incubated at room temperature for 2 h or alternatively overnight at 4°C. Slides were rinsed in dH₂O and blocked with 5% milk and 0,02% Tween-20 in PBS for 15 min with gently agitation. The secondary antibody conjugated to the green or red fluorochrome was diluted 1:800 or 1:1000 respectively, and incubated 40 min at room temperature in a moist chamber in the dark. If desired, counterstaining was performed by staining nuclei with 20 µg/ml intercalating dye bisbencimide (Hoechst) together with the secondary antibody. Following 3x10 min washes in PBS, the slides were rinsed in H₂O and mounted in moviol. Stained sections were kept at 4°C. If primary antibodies from different species were available, two or more antigens were revealed on the same section by multiple-labeling. The technique was the same as for single-labeling, except that primary as well as the secondary antibodies were applied as mixture. Sections were viewed and photographed on a microscope equipped with epi-fluorescence or a laser scan microscope.

Mounting medium (Moviol)

Moviol (20 g) was resuspended in 80 ml PBS/0,02% NaN₃ by stirring for several hours at room temperature. After addition of 40 ml 86% glycerin the solution was allowed to stir overnight. Then pH was adjusted to 7,5-8,0 with 5 M NaOH. Centrifugation for 30 min at room temperature and 27000 g resulted in precipitation of non-soluble material. The clear supernatant used for mounting was stored at 4°C.

4-5. Protein analysis

4-5.1 SDS-polyacrylamide-gel-electrophoresis

Proteins from the lysates (4-2.1 and 4-3.4) were separated in SDS-polyacrylamide gels, according to their size. The separation-gel mixture (Table 4.2) was poured between two glass plates (10 x 10,5 cm) and allowed to polymerize for 30 min.

	Separation gel					Collection gel
Concentration	6%	7,5%	10%	12%	15%	2,8%
Glycerol (50%)	0,75 ml	0,75 ml	0,75 ml	0,75 ml	0,75 ml	-----
Water	5,25 ml	4,9 ml	4,25 ml	3,75 ml	3 ml	
Separation gel buffer	2,5 ml	2,5 ml	2,5 ml	2,5 ml	2,5 ml	-----
Collection gel buffer	-----	-----	-----	-----	-----	0.95 ml
Acrylamid (T40%-C3,7%)	1,5 ml	1,9 ml	2,5 ml	3 ml	3,75 ml	0,3 ml
APS (10% w/v)	100 µl	100 µl	100 µl	100 µl	100 µl	22,5 ml
TEMED	10 µl	10 µl	10 µl	10 µl	10 µl	7,5 ml

Table 4.2. Separation and collection gel

The collection gel was poured on top of the separation gel. After polymerization, 30 µg of each protein sample, diluted in sample buffer (see below), was loaded pro lane. Also a protein molecular-weight standard sample was loaded on to each gel. Gels were submerged in running buffer and the proteins were allowed to separate for 2-3 h at constant 60 V.

Separation gel buffer

Tris-HCl pH 8,8 1,5 M
SDS 0,4%

Collection gel buffer

Tris-HCl pH 6,8 0,5 M
SDS 0,4%

2x Sample buffer

Tris-HCl pH 6,8 130 mM
SDS 6%
Glycerol 20%
β-mercaptoetanol 10%
Bromophenol blue 0,1%

Running buffer

Tris 33 mM
Glycin 194 mM
SDS 0,1%

4-5.2 Western blotting

Following protein electrophoresis, the separated proteins were transferred to a nitrocellulose membrane. Putting the gels in direct contact with a nitrocellulose membrane did this. The gels and the membranes were mounted in a transfer

chamber and submerged in transfer buffer. The proteins were transferred at 48 V for 2-3 h. After transfer, the membrane was stained with Ponceu solution in order to detect the bound proteins and to check the relative amount of protein in each lane. Washing with blocking buffer eliminated Ponceu solution.

Transfer buffer

Tris 33 mM
Glycin 194 mM
Methanol 20%

Blocking buffer

PBS-
Low-fat milk 10%
Tween-20 0,1%

4-5.3 Immunological detection of proteins

Primary antibodies, diluted in blocking buffer, were used to detect the transferred proteins. First, the nitrocellulose membranes with transferred proteins were incubated in blocking buffer for 16 h at 4°C. Incubation with the different primary antibodies was performed at room temperature for 1-3 h. Membranes were washed in blocking buffer (5 x 5 min) and incubated in the presence of a horseradish peroxidase-coupled secondary antibody (1:10 000) for 45 min at room temperature. After washing the membranes for at least 5 x 5 min in blocking buffer, enhanced chemiluminescence (ECL) detection was performed according to the manufacturers instructions (Amersham-Pharmacia Biotech), and further densitometric analysis of the films.

4-6. Cultivation of bacteria

To expand the amount of plasmids keeping the probe for in situ hybridization, bacteria were transfected with this plasmid. Bacteria were either grown in liquid media or on agar plates.

4-6.1 Growth in liquid media

A small freshly saturated culture of E.coli was prepared by inoculating 5 ml LB medium with a single bacterial colony or 50 µl frozen bacteria and incubating at 37°C in 15 ml tube at least 6 h or overnight. Large cultures were generally inoculated with overnight cultures diluted 1:100 or 1:200 in an Erlenmeyer flask. For adequate aeration cultures were vigorously agitated on a roller drum at 250 rpm.

4-6.2 Growth on solid media

To ensure that each cell population is descended from a single cell, bacteria were grown from single colonies on agar plates. A small amount of bacterial culture (50-500 µl) was spread evenly on petri dish. Dishes were dried and incubated with bottom side up at 37°C overnight. Well-separated single colonies were picked with sterile toothpicks and grown further in liquid media.

LB medium

For 1l: tryptone 10 g
yeast extract 5 g
NaCl 5 g

Autoclave

Store at 4°C

LB plates

LB medium 1l
Agar 15 g

Autoclave, pour into sterile petri dishes
and dry well

Store at 4°C, bottom side up

Before adding antibiotics medium was cooled to 50°C.

Medium with antibiotics was stored no longer than 2 weeks

4-6.3 Storage of bacteria

For permanent storage bacteria were grown to saturation, the cell suspension mixed with glycerol (end concentration 30%) and stored at -80°C. Before use bacteria were thawed rapid on ice. For short-term storage petri dishes or bacteria suspension were kept at 4°C for several weeks.

4-6.4 Preparation of competent cells

E.coli were grown to log phase ($OD_{600} = 0,4$). Cells were harvested by centrifugation at 4°C and 5000g for 10 min. The pellet was suspended in 1/10 volume 0,1 M $CaCl_2$ and kept on ice for 30 min. After a second centrifugation step, the supernatant was discarded and the pellet suspended in 1/30 volumes 0,1 ml $CaCl_2$ solution containing 15% glycerol. Cells were aliquot (200 μ l) and frozen immediately at -80°C.

4-6.5 Transformation

Competent bacteria were thawed rapidly on ice and used immediately. Plasmid DNA (100 ng – 1 μ g) was added to 200 μ l bacteria and incubated on ice for 20 min. Negative control (only E.coli) was included. Subsequently, bacteria were heat-shocked by placing the tubes into a 37°C heat block for 5 min, and then on ice for 2 min. To each tube 450 μ l LB medium without antibiotics was added and bacteria were incubated on a roller drum at 250 rpm, 37°C for 30 min. Several dilutions of each transformation culture were plated on appropriate antibiotic-containing dish. Dishes were incubated overnight at 37°C. Well-isolated colonies were picked and grown in liquid medium for plasmid maxi preparation.

4-6.6 Plasmid maxi preparation

Plasmid DNA was isolated and purified with Qiagen plasmid kit according to manufacturers instructions. Briefly, large bacterial cultures (200 ml) were grown in standard LB medium. Cells were harvested by centrifugation and lysed under alkaline conditions. Chromosomal DNA, denatured proteins and cellular debris were precipitated and the supernatant solution neutralized with acidic potassium acetate.

Precipitated debris was removed by centrifugation and a cleared lysate loaded on to the Qiagen column. Eluted plasmid DNA was desalted and concentrated by isopropanol precipitation. After wash in 70% ethanol DNA was air dried, suspended in H₂O and stored at -20°C.

4-6.7 Digestion of plasmid DNA with restriction endonucleases

Reaction conditions: DNA sample (0,1 – 5 µg)
1 x restriction endonuclease buffer
restriction endonuclease (1-5 U/µg DNA)
total volume 30-50 µl

Reaction mixture was incubated 1-2 h at 37°C. Multiple digestions were achieved by simultaneous incubation in appropriate buffer.

4-6.8 Agarose gel electrophoresis

Restriction digests were analyzed on 1% agarose gels. Ethidium bromide was added to the gel at 0,5 µg/ml final concentration. Loading buffer was added to the samples to final volume of 1/10 and total volume was adjusted with H₂O. Appropriate DNA molecular weight markers were included. The gel was run in 1 x TAE running buffer at 60 V for 1-2 h and analyzed on a UV-transilluminator.

4-7. RNA in situ hybridization

4-7.1 Cryosectioning and fixation

During the whole procedure extreme care was taken to prevent RNase contamination as described elsewhere (Ausubel et al., 1984). Sections (5-7 µM) were cut in a cryostat at -30°C, dried on sylan-coated slides and fixed in 4% paraformaldehyde for 20 min at room temperature. After dehydration in a graded ethanol series sections were dried and either immediately processed or stored at -80°C. Prior to further use, frozen slides were immersed in 100% ethanol and dried.

Paraformaldehyde (PFA) fixative

Heat 450 ml 2 x SSC to 70°C and add 20g PFA to make a 4% solution. Maintain on a heating plate at 70°C with stirring. After the solution has become clear remove from heat and allow cooling to room temperature. Add 2,5 ml MgCl₂ (final concentration 5 mM) and filter. Always use freshly prepared.

4-7.2 Synthesis of digoxigenin (dig-) labeled RNA probes

Single-stranded dig-labeled RNA probes were generated by *in vitro* transcription. The gene of interest was inserted into a polylinker site of a plasmid vector containing an SP6, T3 or T7 bacteriophage promoter. Prior to transcription, the plasmid was cleaved with a restriction enzyme at the end of the insert in order to synthesize transcripts that terminate in the end of the DNA template. Antisense and sense RNA probes were generated by digestion at the restriction site closest to the gene of interest upstream and downstream of the coding sequence, respectively. Linearized plasmid was purified with a phenol/chloroform/isoamylalcohol (25:24:1) extraction, precipitated in 100% ethanol at -80°C overnight, centrifuged 10 000 rpm 10 min at 4°C, washed in 70% ethanol, centrifuged again, air dried and dissolved in H₂O.

RNA probes were generated during 2 h incubation time at 37°C of 1 µg of the DNA template in the following reaction mix:

<i>10x Transcription buffer</i>	2 µl
<i>DIG-RNA labeling mix</i>	2 µl
<i>SP6, T3 or T7 polymerase</i>	2 µl
<i>RNase inhibitor</i>	1 µl
<i>H₂O until final volume is 20 µl</i>	

Subsequently, the DNA template was digested with 2 µl DNase I for 15 min at 37°C. The RNA probes were precipitated with 4 M LiCl and 100% ethanol at -80°C overnight, centrifuged and washed with 80% ethanol (-20°C), centrifuged again, air dried and dissolved in hybridization mixture. The concentration of the probes was calculated on a 2% agarose gel against control RNA (E.coli r RNA). Riboprobes were either used immediately or stored at -80°C for up to one week.

4-7.3 Limited alkaline hydrolysis

As the size of the probe length affects hybridization efficiency and probes of 30-300 nucleotides being optimal, RNA probes longer than 400 bp were shortened by limited alkaline hydrolysis. The RNA pellet was dissolved in 50 µl TE and 50 µl bicarbonate buffer and incubated at 60°C. Incubation time was calculated according to the following equation:

$$t = \frac{l_0 - l_d}{K \times l_0 \times l_d}$$

t = incubation time
 k = constant (0,11)
 l₀ = original length of the transcript
 l_d = desired length of the transcript

Reaction was stopped with equal volume of neutralization buffer and RNA precipitated at -80°C with 3 volumes of cold 100% ethanol with 17 µl tRNA and 1/10 volumes 3M sodium acetate. Following centrifugation (30 min, 20 000 g, 4°C) RNA pellet was washed with 80% ethanol, centrifuged again and dissolved in hybridization buffer as described previously.

Bicarbonate buffer

NaHCO_3 80 mM
 Na_2CO_3 120 mM, pH 10,2

Neutralization buffer

CH_3COOH 1%
 CH_3COONa 0,2 M

4-7.4 Pretreatment of sections

4-7.4.1 Proteinase K digestion

Sections were digested in 0,5 µg/ml proteinase K in PBS for 5 min at 37°C in water bath with gentle agitation. Rinsing slides in PBS for 5 min at room temperature stopped proteolysis. Sections were post fixed in 4% PFA 5 min and rinsed in PBS.

4-7.4.2 Acetylation

Sections were acetylated with acetic anhydride diluted 1:100 with 0,1 M triethanolamine for 10 min, rinsed in PBS for 5 min and immediately used for hybridization.

4-7.5 Hybridization and washing

Sections were prehybridized with hybridization mixture without probe in a moist chamber for 60 min at room temperature. Then the hybridization mixture with labeled RNA-probes (2,5-5 µg/ml) were heated 7 min at 70°C for denaturation. Sections were incubated with 15 µl hybridization mixture in a moist chamber at 52°C overnight.

Hybridization mixture (1 ml)

10x salt 100 µl
 deionized formamide 500 µl
 tRNA (c = 10 ml/ml) 2 µl
 50% dextran sulfate 200 µl
 1 M DTT 100 µl
 H_2O 100 µl

10x salt

0,2% polyvenylpurrolidone
 0,2% Ficoll 400
 1,38% NaH_2PO_4 , pH 6,8
 50 med mer EDTA
 3 M NaCl
 0,1 M Tris, pH 8,0

Sections were washed 2 x 5 min in 5xSSC at room temperature, then 30 min in 10xSSC/formamide (1:1) with temperature increasing from 37°C to 60°C, then 60 min in 4xSSC/formamide (1:1) at 37°C. Formamide was washed out in NTE (5M NaCl/1 M Tris pH 8,0/0,5 EDTA, 1:0,1:1) 15 min at 37°C. Further, sections were digested with RNase (20 µg/ml RNase A in NTE) 30 min at 37°C. Sections were washed 30 min in 2xSSC at room temperature and incubated with anti-digoxigenin-AP-fragment solution (1:250) at 4°C in moist chamber overnight.

4-7.6 Staining

Sections were incubated 10 min in B1DIG at room temperature and further 10 min in NTMT+2 mM Levamisol. Sections were coated with staining solution until a color reaction developed (overnight), washed in PBS and mounted in moviol. Sections were visualized and photographed under an Olympus bright-field microscope.

NTMT+ 2 mM Levamisol

5 M NaCl	20 ml
Tris pH 9,5	100 ml
Tween 20	1 ml
1 M Levamisol	2 ml
H ₂ O	879 ml

B1DIG

1 M Tris pH 7,5	100 ml
5 M NaCl	30 ml
H ₂ O	870 ml

Staining solution

NTMT + 2 mM Levamisol	1 ml
BCIP	3,5 μ l
NBT	4,5 μ l

5. RESULTS

5-1. Growth behavior of different HaCaT cell variants in the 3D-coculture

The basis of this work on the basement membrane was provided by the 3D-organotypic coculture system. The 3D-organotypic coculture has proved to be a sensitive assay for *in vitro* studies on epithelial-mesenchymal interactions (Schoop et al., 1999, Stark et al., 1999). Herein, keratinocytes or HaCaT cells are grown on fibroblast-populated type I collagen gels, which allows the development and growth of a stratified epithelia for a period of 1-4 weeks (see Methods).

In order to provide a reliable model for standard conditions, different HaCaT clones were tested and compared with normal human skin and 3D-coculture of normal human keratinocytes. The hypotetraploid parental cell line HaCaT-standard was tested against HaCaT-subclone 6 and a nearly diploid subclone HaCaT-diploid. HaCaT-standard is a well-known keratinocyte substitute in 3D-cultures (Baur, 1995, Schoop et al., 1999) as well as in transplants (Breitkreutz et al., 1998), and was because of this, a self-evident choice. HaCaT-clone 6 was chosen because of excellent differentiation abilities in transplantation studies (Baur et al., 1995, Breitkreutz et al., 1998), after only two to three weeks HaCaT-clone 6 developed a highly differentiating epithelium. HaCaT-diploid, on the other hand, was quite a blank page in transplantation- and *in vitro* coculture system studies, but was chosen because of good proliferation and differentiation capacity in monoculture. Beside differentiation and epithelial development abilities, the main aspect to be investigated was the synthesis and assembly of basement membrane components. Previous studies had revealed that not all HaCaT clones synthesize or assemble basement membrane components, and for these studies, an absolute caution was the ability to develop a basement membrane.

5-1.1 Epithelial development and differentiation

Immunofluorescence examination of HaCaT-standard, HaCaT-clone 6 and HaCaT-diploid in 3D-coculture revealed significant differences between the three cell variants. After 14 days, HaCaT-standard formed thinner, less developed epithelia than HaCaT-clone 6 and HaCaT-diploid (Fig. 5.1). In particular, no distinct cell layers could be distinguished in the HaCaT-standard 3D-cocultures. By contrast, HaCaT-clone 6 developed epithelia close to what is seen in 3D-cocultures with normal human keratinocytes or in human skin. The single, homogeneous basal layer could be recognized with typical columnar shape, and further defined spinous- and granulous layer. HaCaT-diploid also generated complete, but less polarized epithelia. The cells did not organize into epithelial layers and the basal cells spread in the whole epithelia.

In epidermis, the expression of the keratins K1 and K10 starts with the differentiation and correlates with the translocation of the keratinocytes from the basal layer to the spinous layer. The epithelia of the HaCaT variants showed differences in the localization of these suprabasal keratins (Fig. 5.1 A-C). HaCaT-standard expressed the keratins K1 and K10 throughout the epithelia, also in the basal layer, HaCaT-

clone 6 regularly only in the suprabasal layers, while HaCaT-diploid showed generally less expression of keratins K1/ K10 than the other two variants.

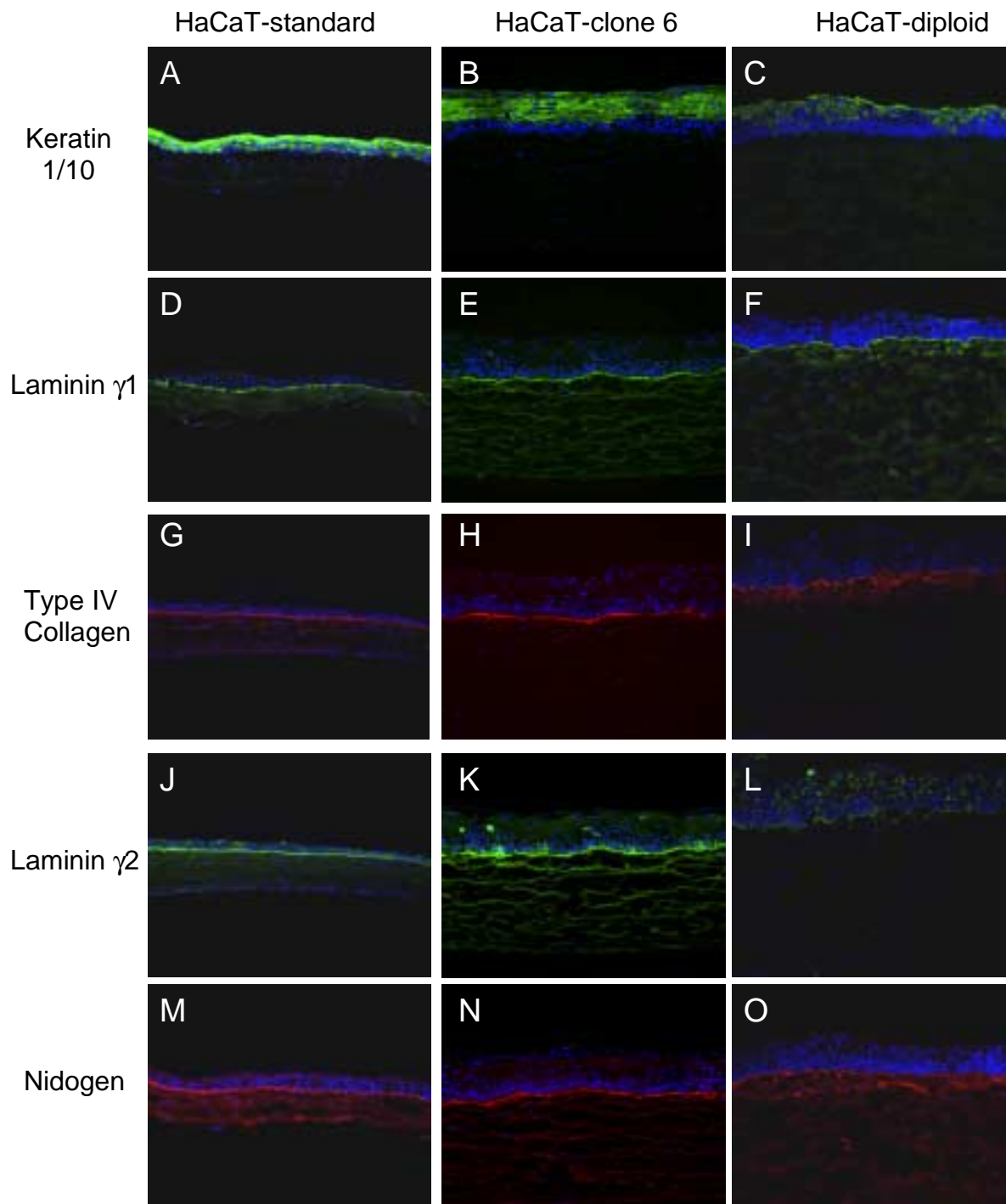


Fig. 5.1. Staining of keratin 1/10 (638 – antibody-number), laminin γ 1 (528), type IV collagen (495), laminin γ 2 (466) and nidogen (416) in 3D-cocultures day 14 of the HaCaT cell variants: HaCaT-standard (A, D, G, J, M), HaCaT-clone 6 (B, E, H, K, N) and HaCaT-diploid (C, F, I, L, O). Cell nuclei stained with bisbenzimidazole DNA dye (blue).

5-1.2 Proteins of the basement membrane zone

For this initial screening general basement membrane components as well as the epidermis-specific laminin-5 were visualized by immunofluorescence. Laminin γ 1 was expressed in 3D-cocultures of all cell variants (Fig. 5.1 D-F). The staining showed laminin γ 1 on the basal pole in the HaCaT-standard, the HaCaT-clone 6 as well as in the HaCaT-diploid 3D-cocultures. There was no significant difference between the clones in the laminin γ 1 expression and assembly.

This was not the case for type IV collagen (Fig. 5.1 G-I). Both HaCaT-standard and HaCaT-clone 6 showed a normal expression and distribution of type IV collagen, whereas HaCaT-diploid showed diffuse localization and no sharply stained line in the basal zone. Although the type IV collagen seemed to be there in an amount comparable to HaCaT-standard and HaCaT-clone 6, it remained partly in the upper part of the collagen gel.

Nidogen was expressed in all 3D-cocultures, but again HaCaT-diploid showed deficiencies in the assembly (Fig. 5.1 M-O). Nidogen was partly in the collagen gel, as seen for type IV collagen. This led to insufficient staining in the basement membrane zone, and no continuous line of nidogen could be seen.

Laminin γ 2 (Laminin-5) was expressed in 3D-cocultures of both HaCaT-standard and HaCaT-clone 6, but was almost completely abolished in the HaCaT-diploid cocultures (Fig. 5.1 J-L).

In summary, HaCaT-standard synthesized and assembled the necessary basement membrane components, but the epithelial development was after 14 days insufficient. HaCaT-diploid showed serious deficiencies in regular basement membrane development and marked reduction of epithelial differentiation. HaCaT-clone 6 on the other hand, not only developed stratified epithelia but also synthesized and assembled all components of the basement membrane in a normal pattern. On the background of these tests, it was clear that HaCaT-clone 6 was the most suitable candidate for further experiments (from now on referred to as HaCaT).

5-2. The influence of nidogen on the basement membrane assembly

The basement membrane is composed of two major molecular networks consisting of laminin and type IV collagen (see Introduction). Of the major constituents, the two nidogens known in mice and humans seem to be present in most adult basement membranes (Erickson et al., 2000). Nidogen-1 appears early during basement membrane assembly in the embryo in several tissues (Timpl et al., 1996), but expression of nidogen-2 during embryogenesis has not yet been studied in detail. Based on biochemical and structural studies of nidogen-1, it has been proposed that nidogens act as connecting elements for basement membrane assembly (Timpl et al., 1996). Nidogen-1 is a 150-kD basement membrane protein consisting of three globular domains connected by a link-region. It forms a stable complex with laminin (Paulsson et al., 1987b) and has been shown to bind to most of the known basement membrane components, including perlecan and type IV collagen. Nidogen-2, having the same molecular domain structure, shows 27,4% identity to nidogen-1 on the amino acid level (Kimura et al., 1998). Like nidogen-1, nidogen-2 also binds type IV collagen and perlecan in vitro (Kohfeldt et al., 1998).

Knockout mice lacking either nidogen-1 (Murshed et al., 2000) or nidogen-2 (Schymeinsky et al., 2002) show no obvious phenotype during embryogenesis. The two nidogens could therefore have complementary functions in basement membrane assembly or organ development. While nidogen-2 represents the minor isoform, in nidogen-1 deficient mice a significant up-regulation of nidogen-2 in the absence of nidogen-1 has been shown (Miosge et al., 2002).

The cellular origin of nidogens in skin has also been discussed. Initially, transplantation studies with epidermal tissues had suggested that keratinocytes alone are responsible for the formation of the basement membrane. Thus, keratinocytes can synthesize laminin (Stanley et al., 1982), type IV collagen and perlecan (Peltonen et al., 1989). However, using dermal tissue models it could be shown that mesenchymal cells, the fibroblasts, are the exclusive source of nidogen (Fleischmajer et al., 1995).

The purpose of this study was to investigate whether lack of nidogen-1 or nidogen-2 or both in the 3D-coculture had an influence on the synthesis and deposition of the basement membrane, and as consequence of that, on the formation and differentiation of the epithelia. In the following experiments 5 different fibroblast genotypes from knockout mice were used (nidogen-1/nidogen-2), +/-+, +/--, -/++, -/-+ and -/-/- in the 3D-coculture. The +/-+ genotype, missing just one allele of both nidogen was used as control ("wild-type") fibroblasts, because mice with this genotype did not show any physical or physiological deficiencies.

5-2.1 Expression of basement membrane components in nidogen knockout fibroblasts

Further, to investigate the expression of some basement membrane components in mouse nidogen knockout fibroblasts, the fibroblasts were grown on slides and stained directly with antibodies against basement membrane components.

Fig. 5.2 shows the expression of nidogen-1 and nidogen-2, vimentin and laminin in the knockout fibroblasts. Nidogen-1 was expressed as expected; thus a fair amount was seen in the +/-+ fibroblasts (Fig. 5.2 A), which was also the case for the +/-- fibroblasts (Fig. 5.2 E). Neither the -/++, -/+ nor the -/- fibroblasts expressed nidogen-1 (Fig. 5.2 I, M Q). Nidogen-2 was less expressed than nidogen-1 in the +/-+ fibroblasts (Fig. 5.2 B), well correlating to previous studies on nidogen expression in mouse tissue (Murshed et al., 2000). Fig 5.2 J shows a certain upregulation of nidogen-2 in the absence of nidogen-1.

Vimentin was employed as cell-specific marker. It is an intermediate filament protein, which is a characteristic of non-epithelial cells. Fig 5.2 shows no difference in the expression of vimentin between the fibroblast genotypes. The staining of laminin was also unchanged in spite of the different fibroblast genotypes' ability of nidogen expression.

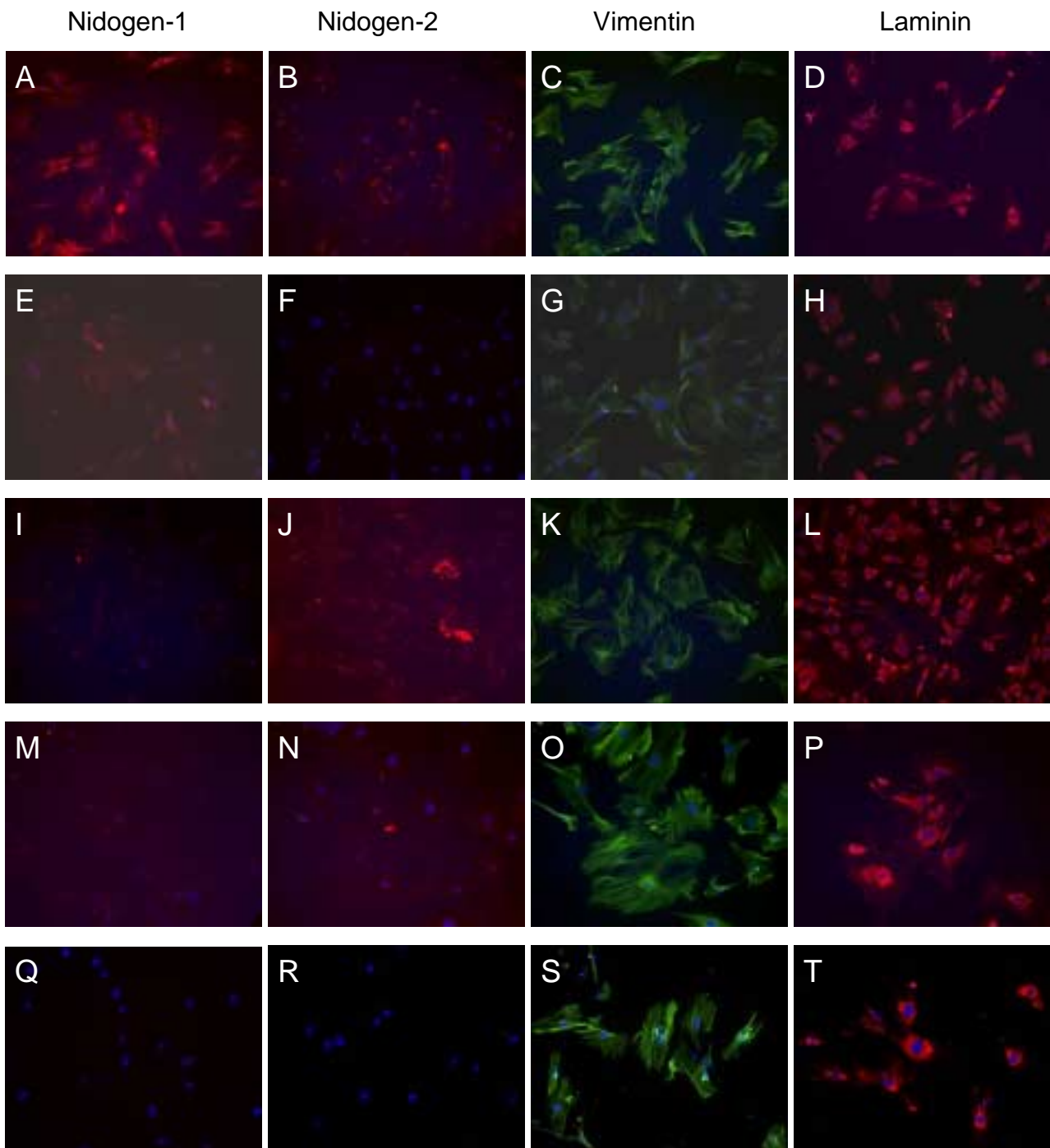


Fig. 5.2. Expression of nidogen-1 (416), nidogen-2 (593), vimentin (628) and laminin (414) in nidogen knockout fibroblasts. Fibroblast genotype +/+ : A-D, +/- : E-H, -/+ : I-L, +/- : M-P, -/- : Q-T.

5-2.2 The epithelial development of 3D-cocultures with nidogen knockout fibroblasts and HaCaT cells

For the following experiments, the 5 different fibroblast genotypes were used in 3D-coculture experiments together with HaCaT-clone 6 as epithelial cells. Generally, the development of a differentiated epithelium in a 3D-coculture is dependent on a keratinocyte – fibroblast “crosstalk”. Furthermore, it has been shown that this works also by combining human epithelial cells and mouse fibroblasts– the result is a so-called heterogeneous 3D-coculture (Maas-Szabowski et al., 1999).

Surprisingly, no significant differences could be seen between the 3D-cocultures with the different fibroblast genotypes (Fig. 5.3). The epithelia in the 3D-coculture with the $-/-$ fibroblasts (Fig 5.3 E) developed just as well as the epithelia where the $+/+$ -control fibroblasts (Fig 5.3 A) were used, thus all giving rise to normally stratified epithelia.

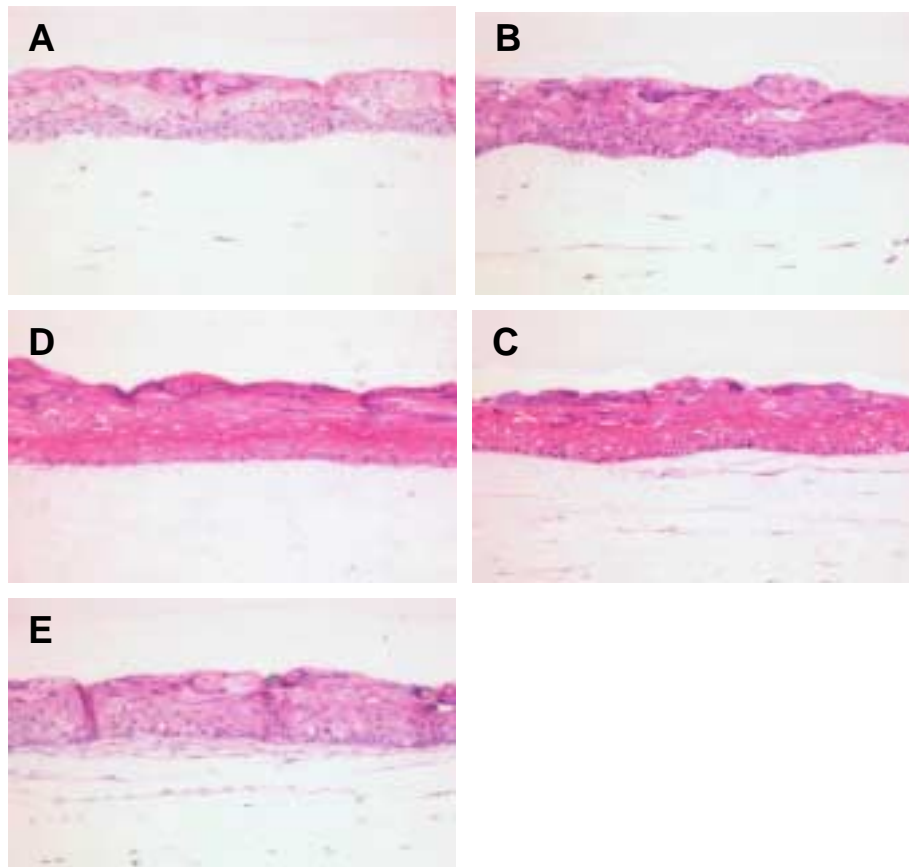


Fig. 5.3. Histology of 3D-cocultures of nidogen knockout fibroblasts and HaCaT cells, day 12 (the color of the epithelia is dependent on duration of eosin staining and independent of the epithelial development). Fibroblast genotype $+/+$: A, $+/-$: B, $-/+$: C, $-/+$: D, $-/-$: E.

5-2.3 Expression of basement membrane proteins in 3D-cocultures with nidogen knockout fibroblasts and HaCaT cells

5-2.3.1 Nidogen

The next step to be investigated was whether the expression of nidogen in the 3D-cocultures correlated to the genotype, and furthermore if this was corresponding to the observations in the respective knockout mice.

Fig 5.4 shows immunofluorescence staining of nidogen-1 and nidogen-2 in the 3D-cocultures. The “wild-type” (+/+) fibroblasts expressed more nidogen-1 than nidogen-2 (Fig. 5.4 A, B). This was also observed in skin sections from mice. A “normal” situation requires more nidogen-1 than nidogen-2. The +/- fibroblasts also expressed high levels of nidogen-1 and, as expected, no nidogen-2 (Fig. 5.4 C, D). The situation was reversed in the 3D-cocultures with the -/+ fibroblasts. In these cultures, more nidogen-2 was expressed than in the +/- cultures (Fig. 5.4 F). This may reflect an upregulation of nidogen-2 in the absence of nidogen-1. In the -/+ 3D-cocultures, nidogen-2 was almost abolished, and the -/- 3D-cocultures expressed as expected neither nidogen-1 nor nidogen-2 (Fig. 5.4 G-J).

In the 3D-cocultures with less or no nidogen (-/+ and -/-) partial detachment of the epithelia from the collagen gel was frequently observed. This was an indication for a strong influence of nidogen on basement membrane formation, or the anchorage of basal cells.

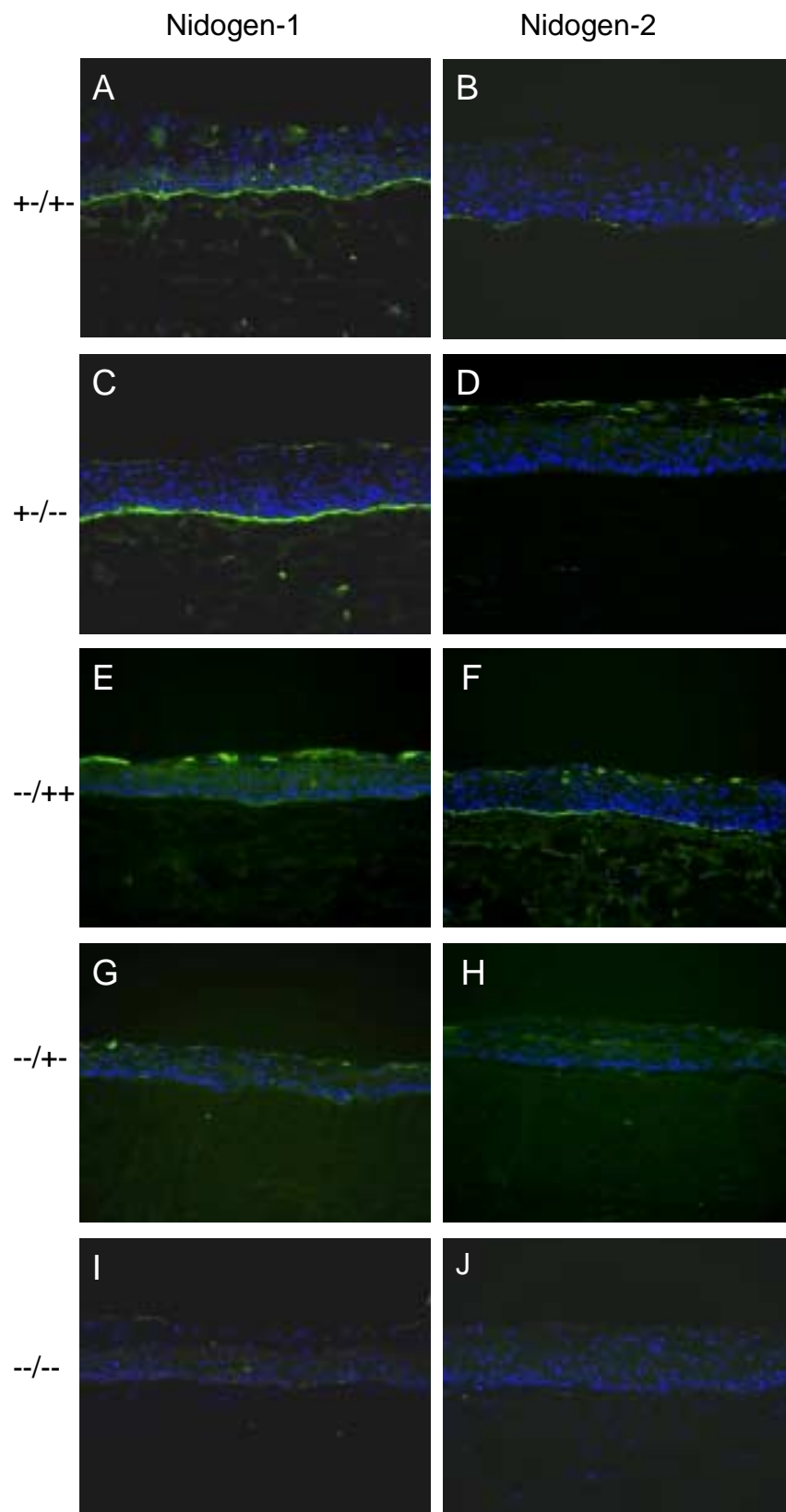


Fig. 5.4. Differential expression of nidogen-1 (416) and nidogen-2 (593) in HaCaT 3D-cocultures with nidogen knockout fibroblasts, day 12.

These results could be verified on western blots detecting nidogen-1 in the 3D-cocultures with some of the most relevant fibroblast genotypes. The epithelia were separated from the collagen gels. 2D-cultures on conventional culture dishes were used as control.

The blot showed that nidogen-1 was expressed in 3D-cocultures with control fibroblasts, both in the epithelia and in the gel as well as in 2D-cocultures (Fig 5.5.). By contrast, nidogen-1 could be detected neither in 3D- nor 2D-cocultures with --/-- fibroblasts, correlating to the immunofluorescence results.

The blot also clearly demonstrated that nidogen-1 is exclusively produced by the fibroblasts. Thus, no nidogen-1 was detected in the epithelia-fraction of the 3D-coculture with the --/-- fibroblasts.

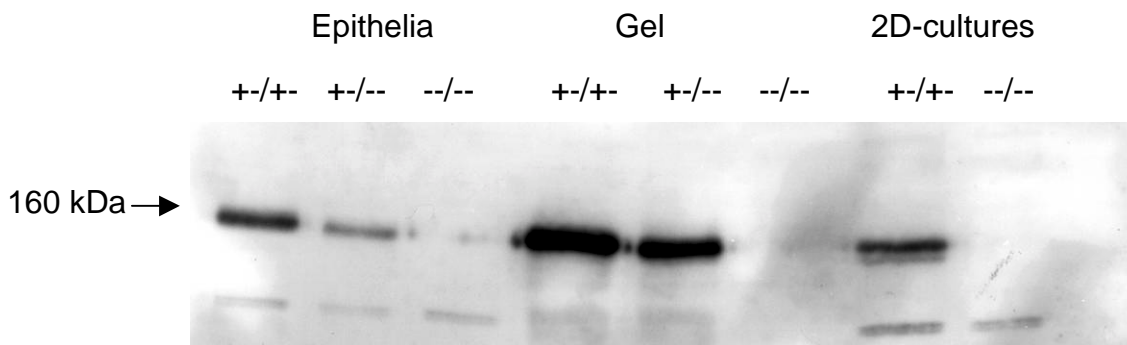


Fig. 5.5. Western blot of nidogen-1 in HaCaT 3D- and 2D-cocultures with nidogen knockout fibroblasts, day 14. Since the lower band is present throughout, it is considered unspecific and not a nidogen degradation product.

5-2.3.2 Laminin

An important aspect was the consequence of nidogen depletion for expression of the other basement membrane components in the 3D-cocultures. Among the others, nidogen has a particular high binding affinity to laminin. This became already apparent by the initial isolation of stable nidogen-laminin complexes from the Engelbreth-Holm-Swarm (EHS) mouse tumor (Paulsson et al., 1987b). Laminin-10 is the main laminin in the adult basement membrane in skin and forms a network of homopolymers. Nidogen is supposed to connect the laminin network and the type IV collagen network in the basement membrane (see Introduction), and although nidogen-1 and nidogen-2 knockout mice do not show laminin deficiency in the skin (Nischt et al., Schymeinsky et al., 2002), this is not necessarily the case in 3D-cocultures, in which the regulatory mechanisms should be different from the mouse.

In the following immunofluorescence experiments, three different antibodies against laminin were used. Both the laminin antibodies against laminin $\alpha 5$ and $\gamma 1$ chains recognize laminin-10 ($\alpha 5\beta 1\gamma 1$), but the laminin $\gamma 1$ antibody also detects other laminins as well, e.g. laminin-1 ($\alpha 1\beta 1\gamma 1$) and laminin-6 ($\alpha 3\beta 1\gamma 1$). Laminin-1 is probably not present in adult basement membranes in the skin, so the fact that the laminin $\gamma 1$ detects more than laminin-1 and -10 should not have large influence on

the results. The general basement membrane or EHS-laminin antibodies on the other hand, should detect more laminin types, since those antibodies are raised against laminins isolated from mouse EHS tumors. They normally cross-react with other laminins than laminin-1 or -10.

The immunofluorescence staining of 3D-cocultures with the nidogen knockout fibroblasts in Fig. 5.6 certainly revealed that nidogen deficiency had an influence on the laminin expression. The staining with the laminin $\alpha 5$ antibody (Fig. 5.6 A, D, G, J, M) showed that there was a dose-dependent decrease in laminin-10 deposition correlating to fibroblast genotypes. 3D-cocultures with +/- and +/-- showed a normal continuous staining pattern in the basal zone. This was also the case in the 3D-cocultures with the -/+ fibroblasts, providing strong evidence that nidogen-2 can compensate for nidogen-1. The 3D-cocultures with the -/+ and the -/- fibroblasts showed no laminin $\alpha 5$ deposition in the basal zone. The laminin $\gamma 1$ antibody pattern showed more or less the same result, Fig. 5.6 B, E, H, K, N revealed that the laminin deposition decreased proportionally with the degree of nidogen deficiency.

The staining with the EHS laminin antibody showed a slightly different result (Fig 5.6 C, F, I, L, O) although the dose-dependency was also in this case clear recognizable. Traces of laminin could be detected in the 3D-cocultures with the -/+ and the -/- fibroblasts. This was probably due to the fact that the EHS laminin antibody detects not only laminin-10 but also other laminins.

These results may appear surprising, because as seen in Fig. 5.2, chapter 5-2.1 the fibroblasts of all genotypes were able to synthesize matrix protein recognized by EHS laminin antibodies when grown on slides. Although this seems to be in sharp contrast to what was seen in the 3D-cocultures, it could indicate failure in laminin assembly or synthesis.

Fig. 5.6 also shows staining for the differentiation markers keratins K1 and K10 and loricrin. Keratins K1 and K10 were described in 5.1.1, representing early markers. There was no difference between the genotypes in the keratins K1 and K10 staining. Loricrin is a protein accumulating in the stratum granulosum. It participates in the envelope formation and is suggested to be a major envelope constituent and a late differentiation marker.

Taken together, the regular expression of early and late markers and the histological analysis of the 3D-cocultures in Fig. 5.3 show that the fibroblast genotypes were able to support the epithelial development despite the degree of nidogen deficiency.

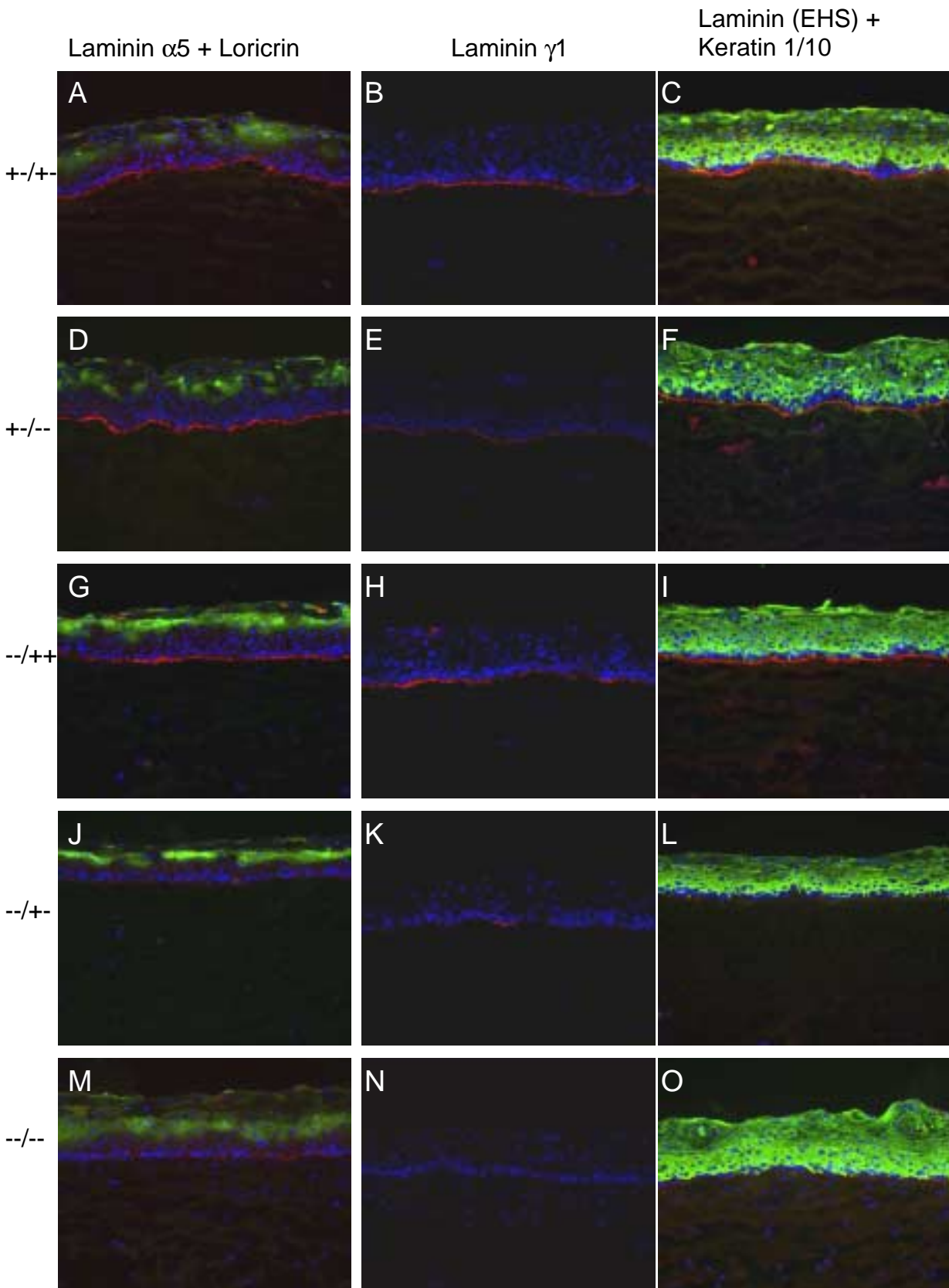


Fig. 5.6. Staining for the laminin α 5 chain (723), the γ 1 chain (528), and EHS laminin (496) together with keratin 1/10 (628) and loricrin (204) in HaCaT 3D-coculture with nidogen knockout fibroblasts, day 12. Fibroblast genotype +/+ : A-C, +/- : D-E, -/+ : G-H, -/+ : J-L, -/- : M-O. Photos A, D, G, J and M show laminin α 5 in red and involucrin in green, photos C, F, I, L and O show laminin (EHS) in red and keratins K1 and K10 in green.

The laminin deficiency was further analyzed by confocal laser scan microscopy. With this method, the intensity of the fluorochrom signal of the secondary antibody can be measured, providing a semi-quantitative result.

Two fibroblast genotype 3D-coculture samples were chosen for analysis, the control 3D-cocultures (+/-) and the 3D-cocultures with the -/+ fibroblasts.

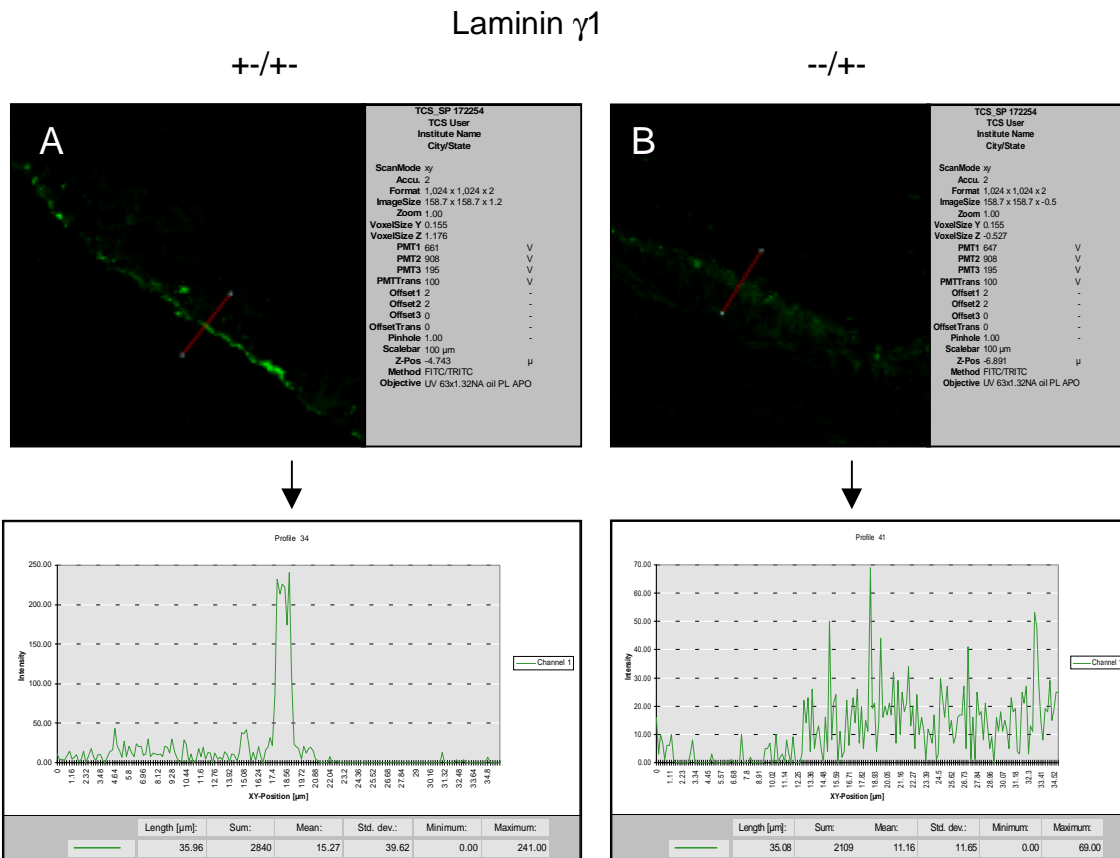


Fig. 5.7. Confocal laser scan analysis of laminin $\gamma 1$ in HaCaT 3D-cocultures with high (A) and low (B) nidogen expressing fibroblasts, day 12. The red marker shows the measured area. For the weaker signals the scale is expanded for (B), which also indicate more diffuse laminin $\gamma 1$ distribution.

The records in Fig. 5.7 verified at higher magnification the laminin $\gamma 1$ deficiency in the 3D-cocultures with the -/+ fibroblasts, indicating also a much broader, less defined localization.

In order to distinguish if the laminin deficiency seen in the 3D-cocultures with nidogen knockout fibroblasts was due to lower protein levels, degradation, or altered distribution, western blots were performed detecting two of the laminin-10 chains, laminin $\alpha 5$ (appr. 400 kD) and laminin $\gamma 1$ (204 kD). This also provided the proof that in this system, the laminin-10 isoform was synthesized.

The blots showed Fig 5.8 A, B revealed that the laminin $\alpha 5$ and $\gamma 1$ chains were clearly detectable in the 3D-cocultures with the nidogen knockout fibroblasts. This indicated that laminin-10 was produced also in the nidogen deficient 3D-cocultures, though not assembled to the basement membrane zone. While this was also the case in 2D-cocultures, separation of the tissue compartments of the 3D-cocultures demonstrated that the laminin $\alpha 5$ and $\gamma 1$ chains were exclusively present in the epithelial part and not in the collagen gel.

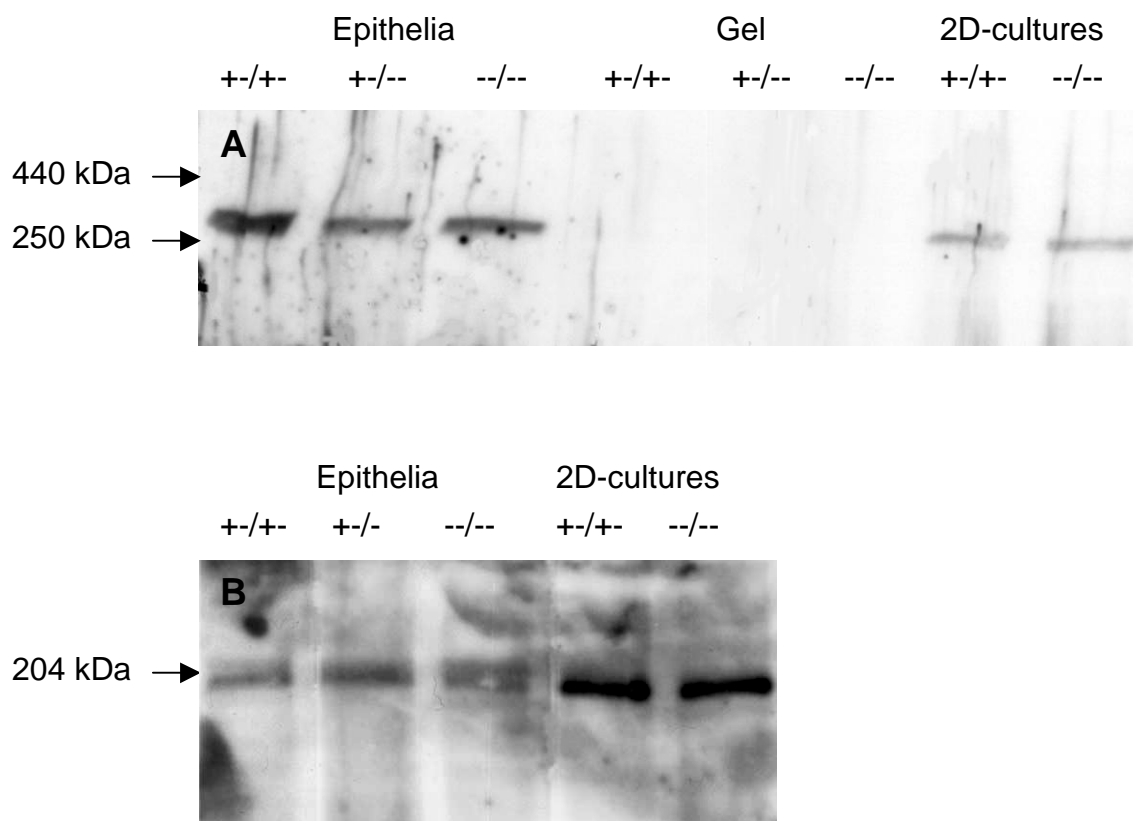


Fig. 5.8. Western blot of laminin-10 in HaCaT 3D-cocultures with nidogen knockout fibroblasts, day 14. A: Laminin $\alpha 5$ chain, B: Laminin $\gamma 1$ chain. Both chains were detectable in all epithelia independent of the fibroblast status.

5-2.3.3 Alterations of Laminin-5, Type IV Collagen and Perlecan

In addition to the laminin network, the basement membrane also consists of a type IV collagen network, which is interlinked to the laminin network via nidogen (see Introduction). Perlecan is another basement membrane protein, while laminin-5 ($\alpha3\beta3\gamma2$) as the major keratinocyte adhesion ligand, is also located in the lower part of the basement membrane zone.

Thus, the influence of the nidogen deficiency on the expression pattern of these proteins was also investigated.

The mildest effect was seen for laminin $\gamma2$ (Fig 5.9 A, D, and G). The expression pattern was normal in the 3D-cocultures with the fibroblast genotypes $+/+$, $+/-$ and $-/+$. By contrast, in the 3D-cocultures with the $-/+$ and $-/-$ fibroblasts, the expression of laminin $\gamma2$ was almost abolished (Fig. 5.9 J and M). Some protein was localized in the basement membrane zone, but it is not a continuous stained line.

This was also the case for type IV collagen and perlecan, these proteins were normally expressed in the 3D-cocultures with the fibroblasts $+/+$, $+/-$ and $-/+$, but failed complete in the $-/+$ and the $-/-$ 3D-cocultures.

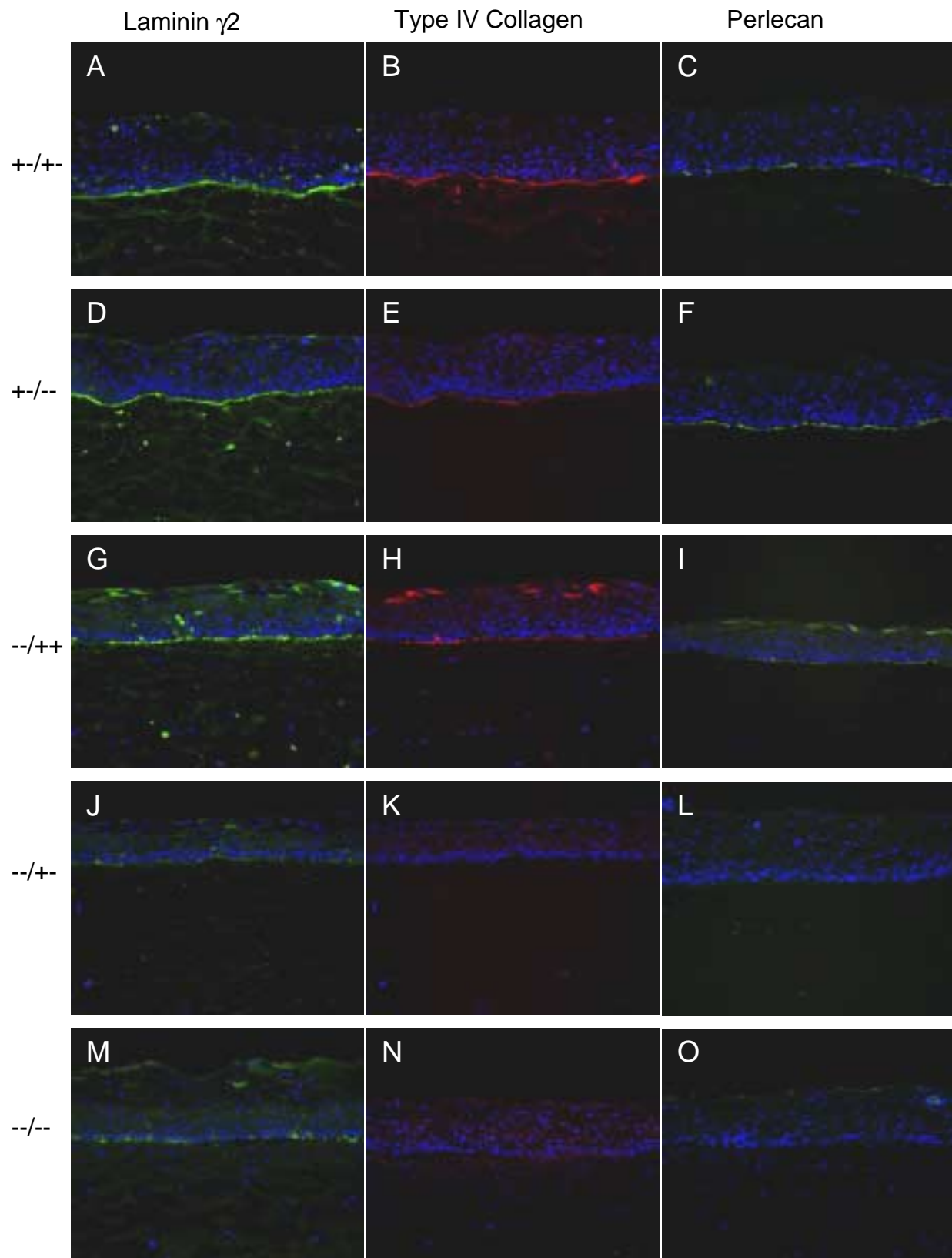


Fig. 5.9. Staining for laminin γ 2 (466), type IV collagen (495) and perlecan (177) in HaCaT 3D-cocultures with nidogen knockout fibroblasts, day 12. Fibroblast genotype +/-+: A-C, +/- -: D-E, -/+ +: G-H, -/+ -: J-L, -/- -: M-O.

This was further analyzed by western blots detecting type IV collagen and laminin-5, to investigate if the situation was the same for these proteins as for laminin-10. However, it was not possible to detect perlecan on a western blot because an appropriate antibody was not available.

The western blot in Fig. 5.10 shows that type IV collagen (approximately 180 kDa), as with laminin-10, could be detected in a western blot of the 3D-cocultures with nidogen knockout fibroblasts. This revealed that type IV collagen was also produced in the 3D- and 2D-cocultures with the $-/+$ and the $-/-$ fibroblasts, although it was hardly not detectable in the immunofluorescent staining.

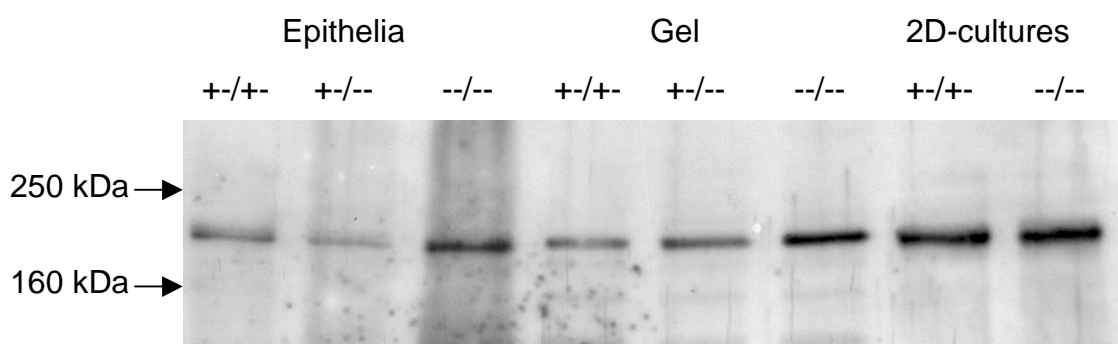


Fig 5.10. Western blot for type IV collagen in HaCaT 3D-cocultures with nidogen knockout fibroblasts, day 14.

In contrast to the type IV collagen situation, the western blot in Fig. 5.11 shows that laminin $\gamma 2$ (160 kDa, processed form 105 kDa) was absent in the 3D-cocultures of $-/-$ fibroblasts. The processed form was detectable in the collagen gel of the $-/-$ cultures. This showed that laminin-5 was eventually being produced also in the $-/-$ cultures, but presumably degraded by proteases as it was not properly assembled.

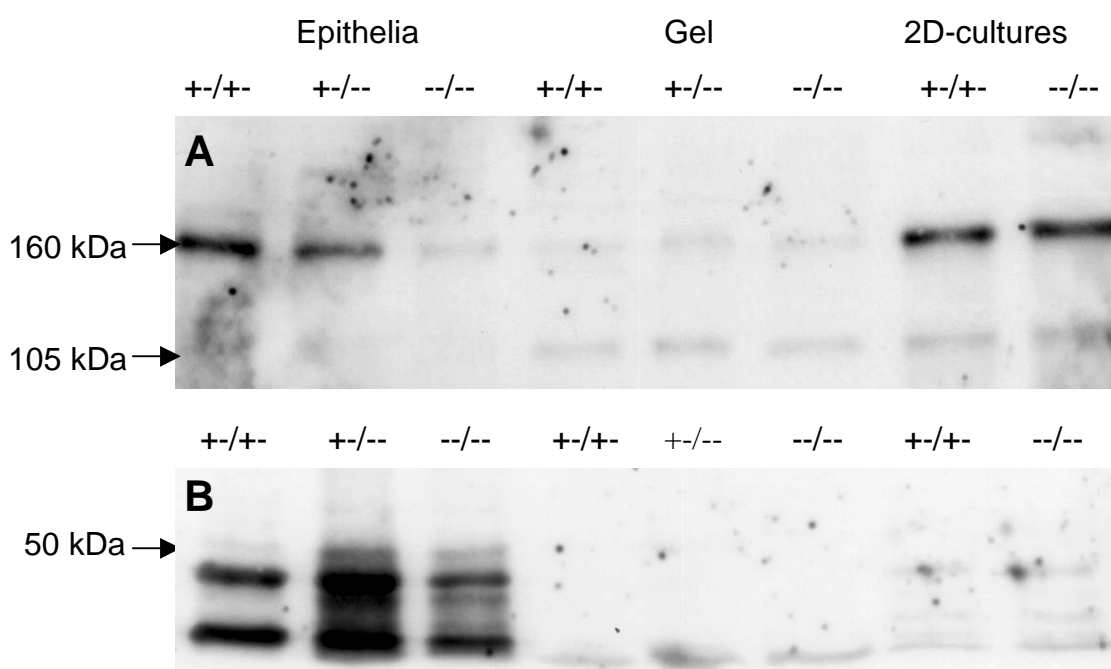


Fig. 5.11. Western blot of laminin $\gamma 2$ in HaCaT 3D-cocultures with nidogen knockout fibroblasts day 14. A: Laminin $\gamma 2$, B: Loading control showing unspecific staining.

5-2.3.4 Integrins

Integrins are heterodimeric transmembrane proteins composed of one α and one β subunit. Integrin $\alpha6\beta4$ is one of the major epidermal integrins and laminin-5 is its ligand (see Introduction).

The immunofluorescent staining in Fig 5.12 show that also the expression of integrin chain $\alpha6$ was influenced by the nidogen deficiency. The expression is almost as strong in the knockout samples as in the control samples, but the pattern is slightly more irregular.

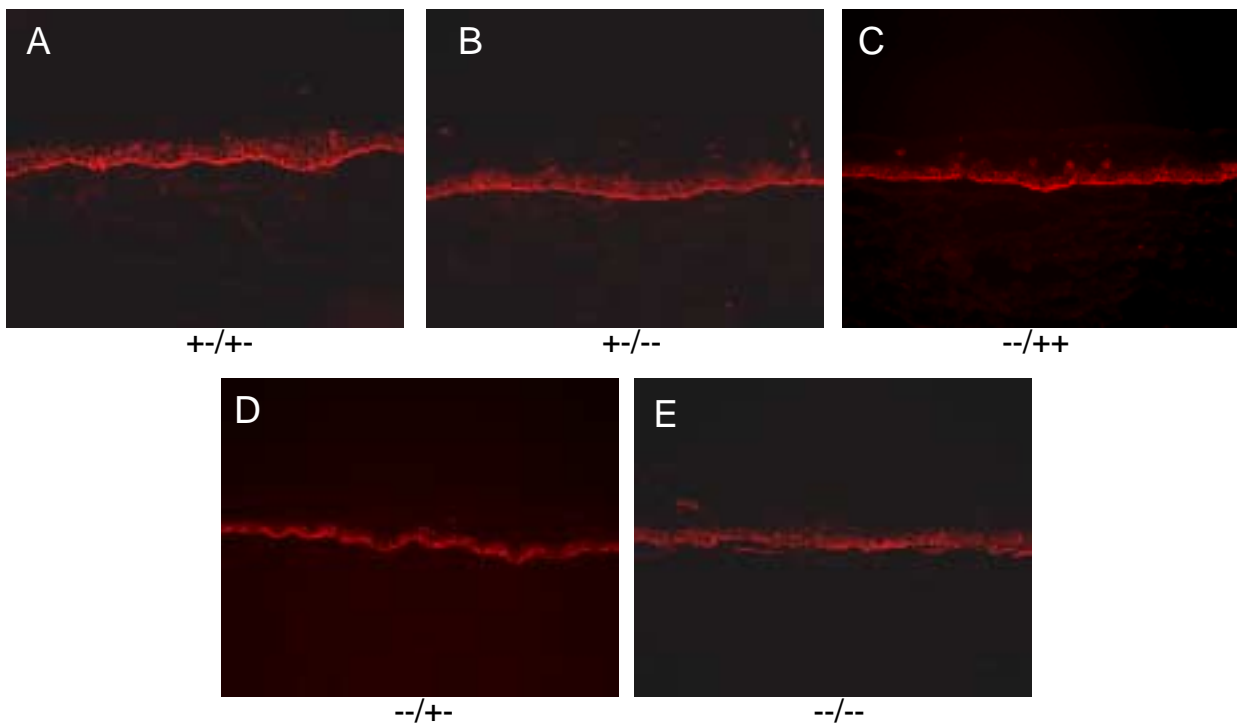


Fig. 5.12. Expression of the integrin chain $\alpha6$ (370) in HaCaT 3D-cocultures with nidogen knockout fibroblasts, day 12. Fibroblast genotype $+/+$: A, $+/-$: B, $-/+$: C, $-/+$: D, $-/-$: E.

This was also the case for the integrin chain $\beta1$. Fig. 5.13 shows that the integrin chain $\beta1$ is expressed also in the knockout cultures, but as for $\alpha6$ in a more irregular and less extent than in the control samples.

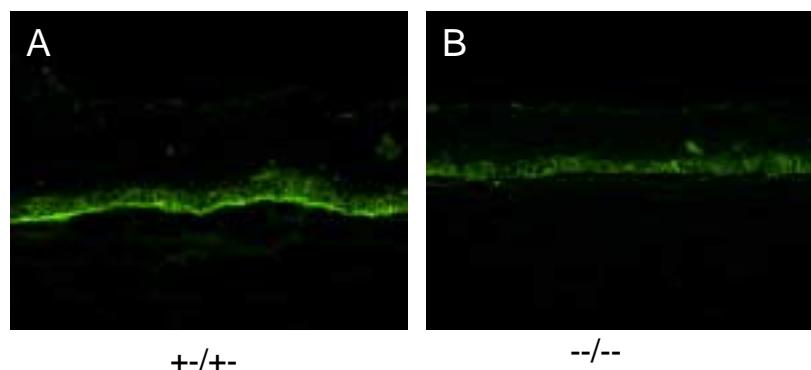


Fig. 5.13. Expression of the integrin chain $\beta1$ (385) in HaCaT 3D-cocultures with nidogen knockout fibroblasts, day 12. Fibroblast genotype $+/+$: A, $-/-$: B.

5-2.4 Influence of nidogen deficiency on 3D-cocultures with normal human keratinocytes

The next step was to prepare 3D-cocultures with the nidogen knockout fibroblasts and normal human keratinocytes. This was to ensure that the lack of basement membrane development and assembly seen in the 3D-cocultures with HaCaT cells was due to the nidogen deficiency, and not due to the fact that the HaCaT cells did not behave as normal keratinocytes.

Three of the fibroblast genotypes were chosen for these experiments, the control $+/+$ fibroblasts, the $-/+$ fibroblasts and the $-/-$ fibroblasts.

Fig. 5.14 shows the nidogen expression in the 3D-cocultures with normal human keratinocytes. These results correlated to what was seen in Fig. 5.4 (Chapter 5-3.2). The 3D-cocultures with the $+/+$ fibroblasts expressed nidogen-1 and nidogen-2, the 3D-cocultures with the $-/+$ fibroblasts expressed only nidogen-2, although in lower amounts than seen in Fig. 5.4. The 3D-cocultures with the $-/-$ fibroblasts expressed neither nidogen-1 nor nidogen-2.

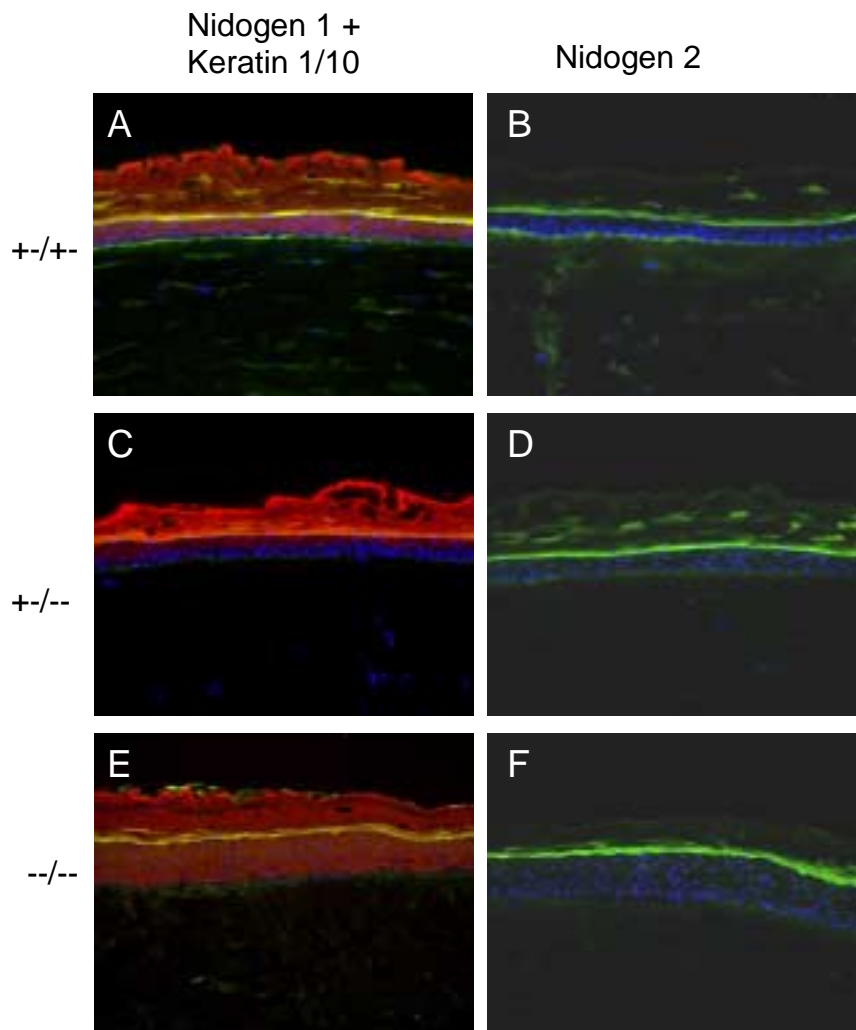


Fig. 5.14. Staining for nidogen-1 (416) and nidogen-2 (593) day 12 in 3D-cocultures of normal human keratinocytes with fibroblast genotype $+/+$: A, B, $-/+$: C, D, $-/-$: E, F. In A, C, E: Nidogen in green, keratin 1/10 in red.

The expression of type IV collagen, laminin-10 (represented by laminin γ 1) and perlecan were further investigated. Fig. 5.15 shows only minor differences in comparison to Fig. 5.6 (chapter 5-2.3.2) and Fig. 5.9 (chapter 5-2.3.3). Type IV collagen was normally expressed in the basement membrane zone in 3D-cocultures with +/-/+ and -/+ fibroblasts, whereas in the -/- 3D-cocultures only traces could be seen. Laminin γ 1 was totally abolished in the same cultures, and traces of perlecan could be detected in the collagen gel.

These results provided further evidence that the changes seen in chapter 5-2.3 were due to the nidogen deficiency.

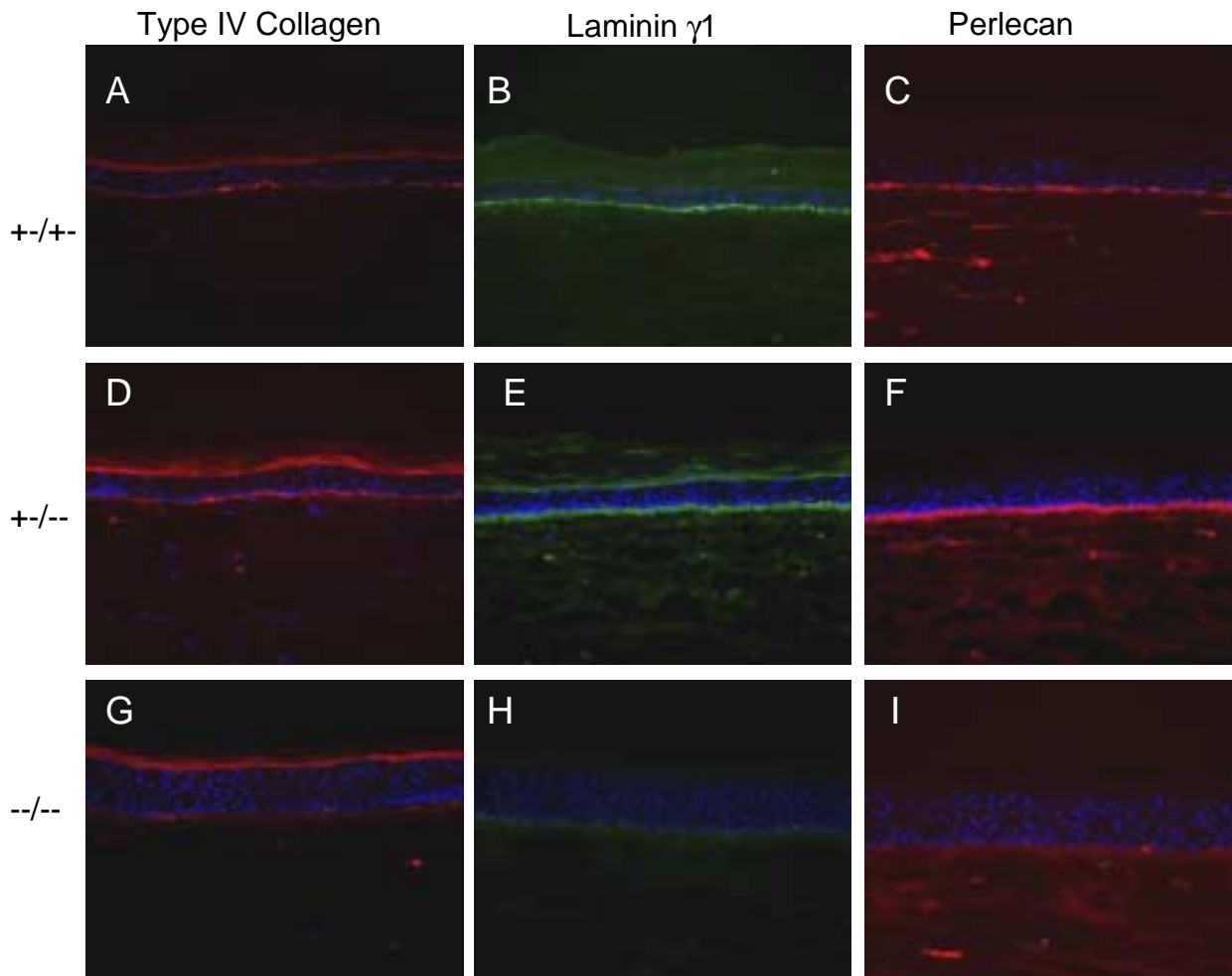


Fig. 5.15. Staining for type IV collagen (495), laminin γ 1 (528) and perlecan (640) in 3D-cocultures day 12 of normal human keratinocytes, fibroblast genotype +/-/+ : A-C, -/+ : D-F, -/- : G-I.

5-2.5 Restoration of the nidogen deficient HaCaT 3D-cocultures with recombinant nidogen-1 and nidogen-2

A critical aspect was whether addition of recombinant nidogen to the 3D-cocultures would change the deficiency situation back to normal. By applying 5 µg/ml recombinant (mouse) nidogen-1 or (human) nidogen-2 to the medium of the 3D-coculture throughout the whole growth period, this question could be investigated. For these experiments only 3D-cocultures with the control fibroblasts (+/+/-) and the complete knocked out genotype (-/-/-) were used.

In principle, these investigations were interesting from three different angles. First, if the applied exogenous nidogen would be transported from the medium to the basal zone, as the recombinant protein was added in the media compartment and not directly onto the 3D-coculture. Second, if the laminin assembly would then proceed as normal and lead to restoration of the nidogen-laminin complex. Third, if the other basement membrane components type IV collagen, perlecan and laminin 5, which were all influenced by the nidogen-deficiency in the knockout 3D-cocultures, would reassemble on the dermo-epidermal interface.

5-2.5.1 Nidogen-1 and Nidogen-2

As depicted in Fig. 5.16, addition of nidogen-1 and nidogen-2 lead to full recovery of the deposition of nidogen in the dermo-epidermal interface of the 3D-cocultures with the -/-/- fibroblasts. This meant that the applied nidogen was transported from the medium compartment through the collagen gel to the basement membrane zone.

After addition of nidogen-1, the nidogen-1 antibody detected a continuous line in the basal zone (Fig. 5.16 C, D), but also the nidogen-2 antibody had a clear signal after addition of nidogen-1. Apparently, the nidogen-1 and nidogen-2 antibodies might not be as specific as presumed; this was particularly the case for anti-nidogen-1, strongly staining nidogen-2 (Fig. 5.16 E). The addition of nidogen-2 also led to recovery of nidogen-2 in the basal zone.

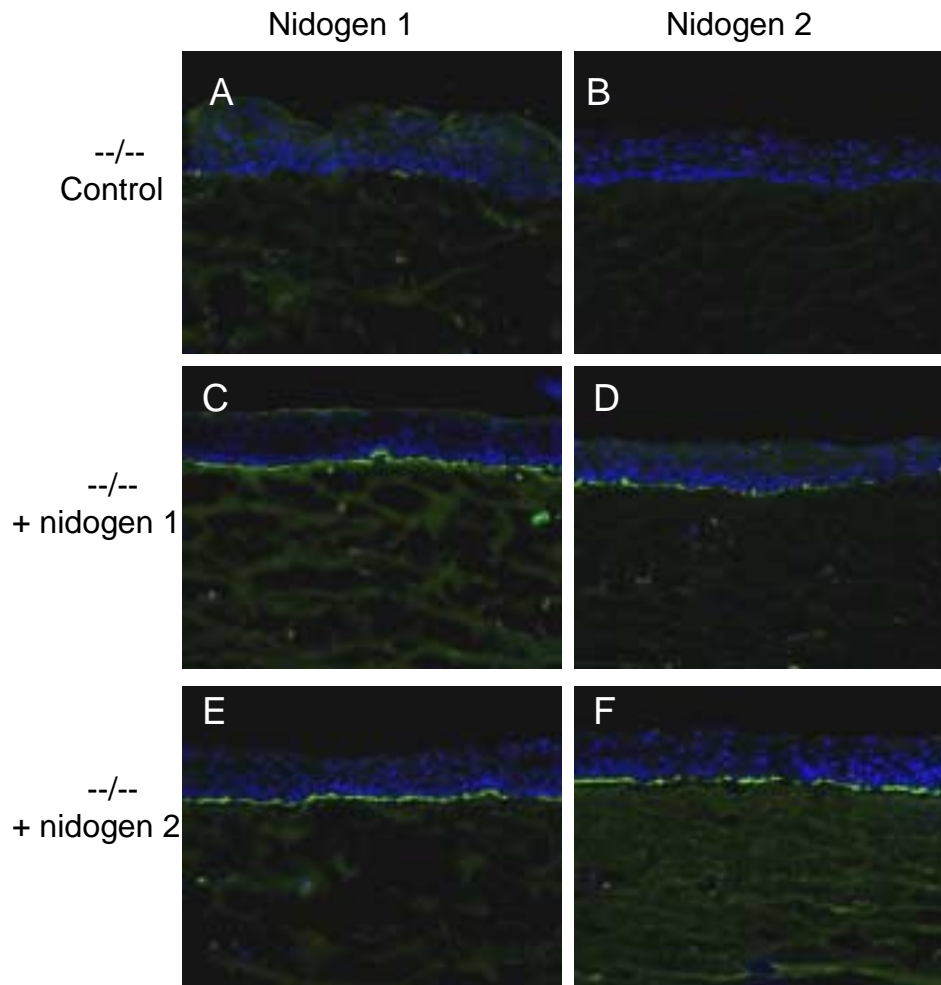


Fig. 5.16. Regular deposition of exogenous nidogen-1 (416) and nidogen-2 (593) in HaCaT 3D-cocultures with $-/-$ fibroblasts, day 12. Control: A, B, addition of 5 $\mu\text{g/ml}$ nidogen-1: C, D, addition of 5 $\mu\text{g/ml}$ nidogen-2: E, F.

As mentioned above, recombinant nidogen-1 and nidogen-2 were also added to the 3D-cocultures with the control fibroblasts

In this case the basement membrane zone seemed to reach saturation levels, leading to accumulation also in the collagen gel. Nidogen-1 and nidogen-2 were detected throughout the gel (Fig. 5.17). Except for this, no other changes could be observed.

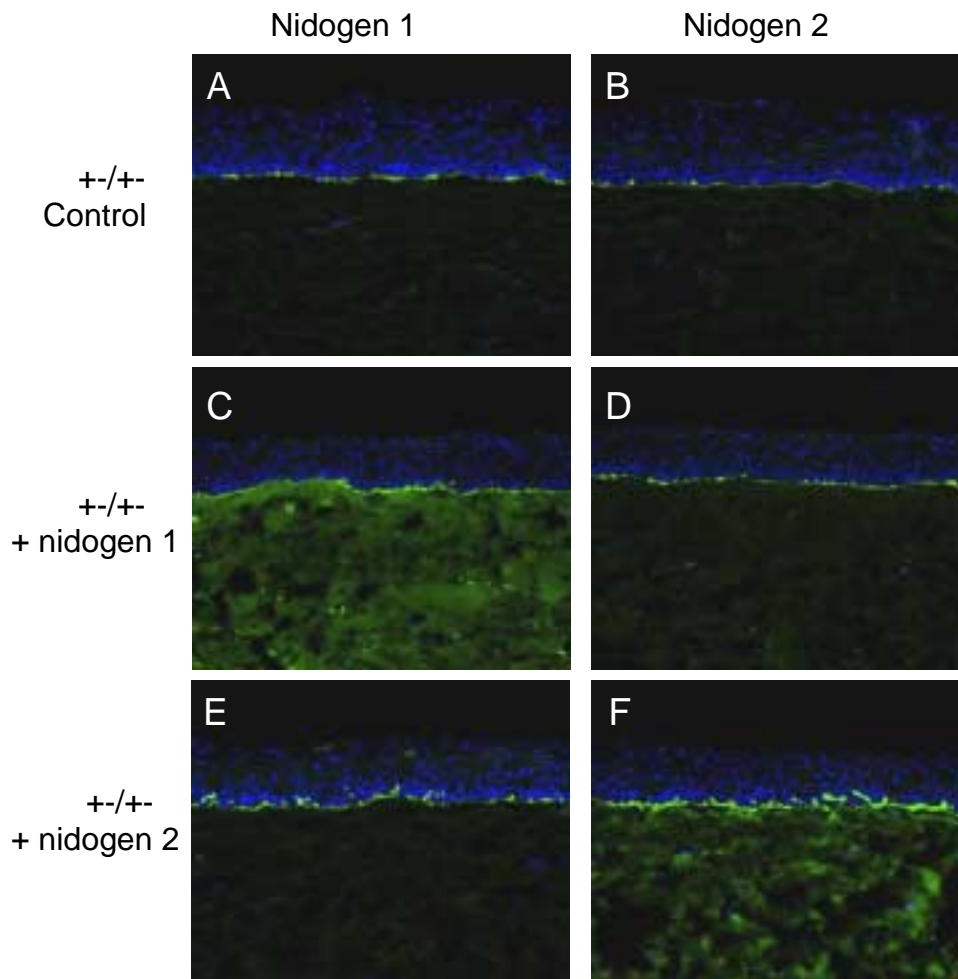


Fig. 5.17. Excess of recombinant nidogen in HaCaT 3D-cocultures with +/-/- nidogen fibroblasts, day 12. Staining for nidogen-1 (416) and nidogen-2 (593). Control: A, B, addition of 5 $\mu\text{g/ml}$ nidogen-1: C, D, addition of 5 $\mu\text{g/ml}$ nidogen-2: E, F.

5-2.5.2 Laminin-10

The second interesting issue was laminin-10. It was observed in chapter 5-2.3.2 that the nidogen deficiency in 3D-cocultures clearly led to disturbance in the laminin-10 assembly. Thus, the nidogen-laminin complex seems to be crucial for basement membrane formation.

After addition of 5 $\mu\text{g/ml}$ nidogen-1 or nidogen-2 to these nidogen deficient 3D-cocultures, the deposition of laminin-10 was normalized. Fig. 5.18 shows staining of laminin 10, represented by the laminin $\alpha 5$ chain, the laminin $\gamma 1$ chain and EHS laminin in the 3D-cocultures with addition of nidogen-1 (B, E, H) and nidogen-2 (C, F, I). A continuous line can be observed for all three laminin antibodies after addition of nidogen-1, providing evidence that exogenous nidogen-1 can restore the laminin-10 self-assembly and the laminin-nidogen complex. Furthermore, since nidogen-2 restored laminin deposition equally to nidogen-1, this supported the nidogen-2 compensation hypothesis.

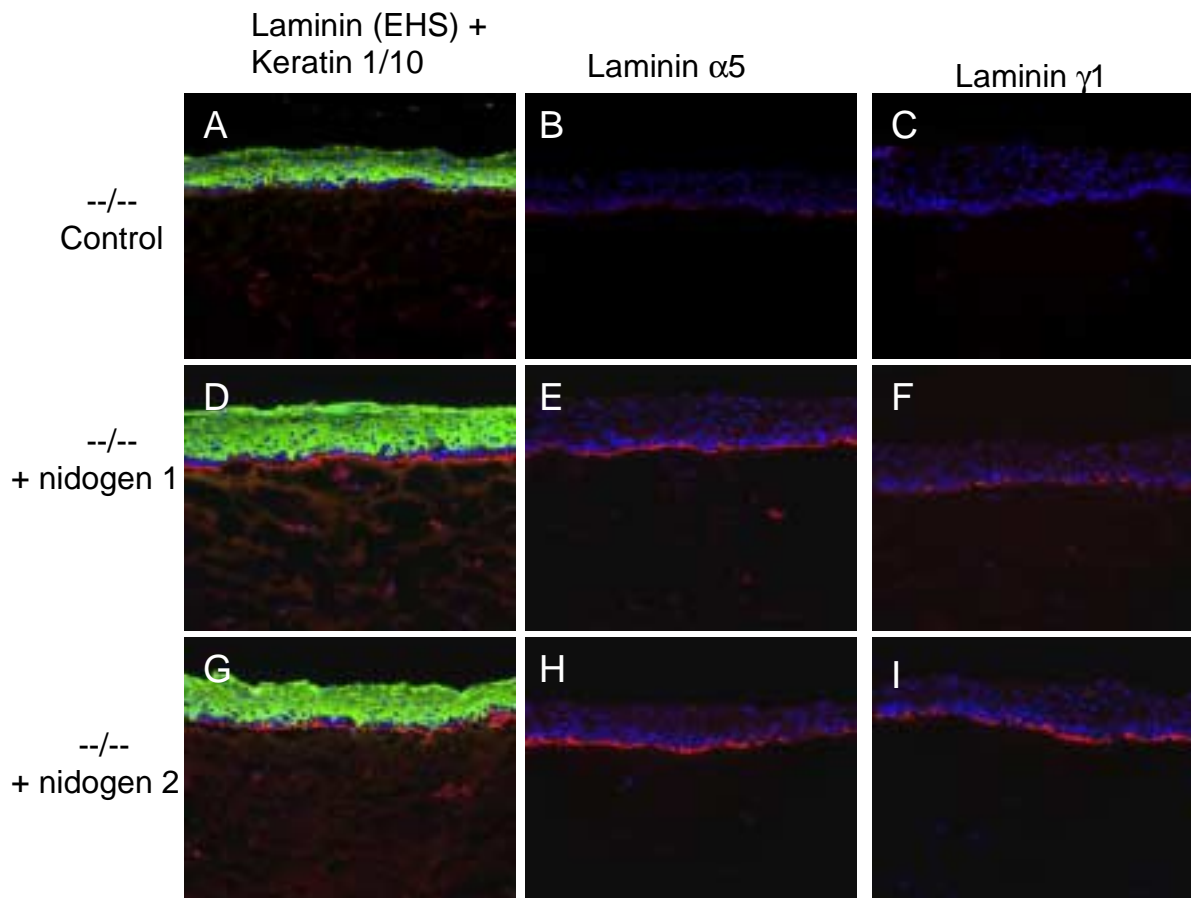


Fig. 5.18. Restoration of laminin deposition in HaCaT 3D-cocultures with the --/-- fibroblasts, day 12. Staining for EHS laminin (496), the laminin α 5 chain (723), γ 1 chain (528) and keratin 1/10 (638). Control: A-C, addition of 5 μ g/ml nidogen-1: D-F, addition of 5 μ g/ml nidogen-2: G-I. A-C: EHS laminin in red, keratin K1/K10 in green.

5-2.5.3 Type IV Collagen, Perlecan and Laminin-5

Chapter 5-2.3.3 revealed that also the assembly of the basement components type IV collagen, perlecan and laminin 5 were disturbed by nidogen deficiency. Both type IV collagen and perlecan are closely connected to nidogen. Type IV collagen self-assembles into a network, which connects to the laminin-10 network via nidogen. Perlecan binds to nidogens G2 globular domain (see Introduction).

The immunofluorescent staining in Fig. 5.19 revealed that also these three components assembled at the dermo-epidermal junction after addition of exogenous nidogen-1 or nidogen-2.

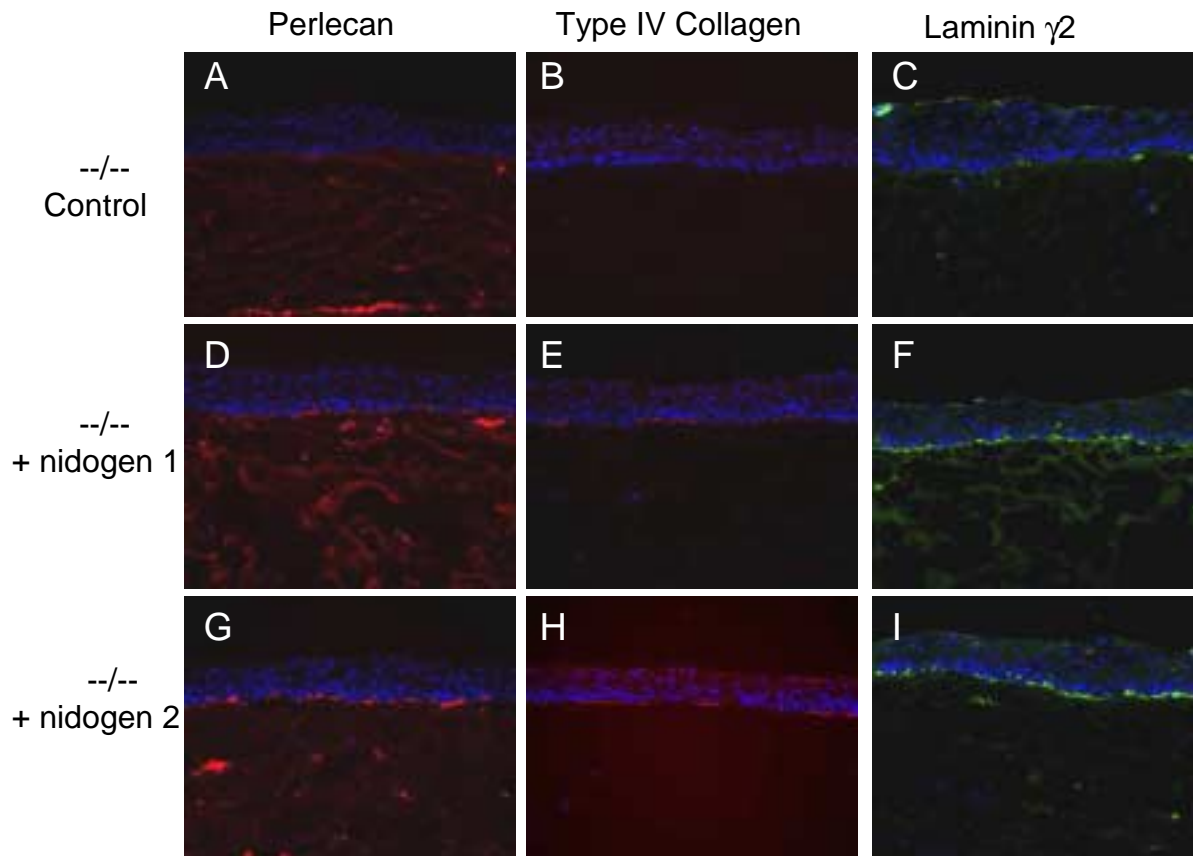


Fig. 5.19. Restoration of perlecan (640), type IV collagen (495) and laminin-5 (laminin γ 2, 466) deposition in HaCaT 3D-cocultures with $-/-$ nidogen fibroblasts, day 12. Control: A-C, addition of 5 μ g/ml nidogen-1: D-F, addition of 5 μ g/ml nidogen-2: G-I.

The HaCaT 3D-cocultures with $+/+$ or $-/-$ nidogen fibroblasts and the “rescued” cultures supplemented with exogenous nidogen-1 or nidogen-2 were also analyzed at the ultrastructural level. In contrast to the positive control 3D-coculture ($+/+$), the electron microscopy clearly demonstrated the complete lack of a defined basal lamina in the $-/-$ 3D-cocultures (Fig. 5.20). Furthermore, stable epidermal attachment complexes, the hemidesmosomes, were absent, but instead basal cells became frequently added to the collagen fibers. In contrast, after addition of either nidogen-1 or nidogen-2 to the 3D-cultures, both basement membrane and hemidesmosomal components were clearly visible (Fig. 5.20, arrows). Compared to the less advanced positive control (Fig. 5.20 B, $+/+$) showing still visible only sparse basement membrane structures and hemidesmosomes (arrow), both nidogens seemed to accelerate the assembly dramatically.

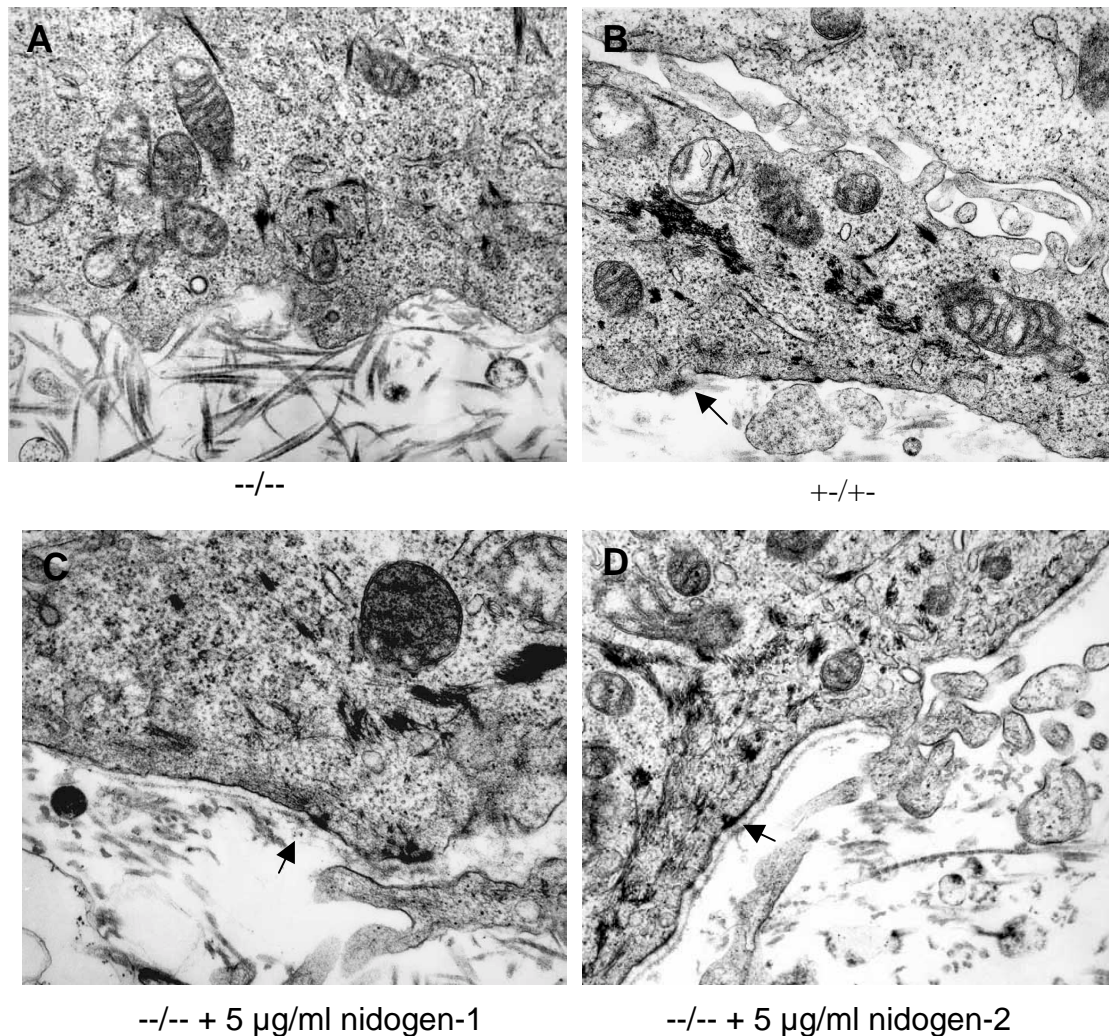


Fig. 5.20. Ultrastructural characteristics of A: 3D-cocultures with --/-- fibroblasts, B: Control 3D-cocultures with +/- fibroblasts. C: --/-- 3D-cocultures after addition of 5 µg/ml nidogen-1, D: --/-- 3D-cocultures after addition of 5 µg/ml nidogen-2. All day 12.

5-2.6 Summary to nidogen deficiency

Taken together, these data showed that nidogen deficient 3D-cocultures were able to develop a stratified epithelium displaying all the normal differentiation markers. But the deposition of the basement membrane components laminin-10, type IV collagen and perlecan were absent, indicating that either the synthesis or the assembly of the basement membrane was highly influenced. Localization of the same components in the epithelia by western blotting, revealed that the components were indeed synthesized but not transported to and assembled on the basement membrane zone. Addition of recombinant nidogen-1 or nidogen-2 led to fully recovery of the basement membrane including the hemidesmosomal components, which was confirmed at the ultrastructural level. Both exogenous nidogens clearly improved deposition of all basement membrane components, but particularly laminin-10. This may indicate the preferential interaction between nidogen and laminin γ 1.

5-3. The influence of perlecan on the basement membrane assembly

Perlecan is a heparan sulfate proteoglycan expressed in all basement membranes, in cartilage, and several other mesenchymal tissues during development. Although several biochemical and structural properties of perlecan have been reported, its *in vivo* function remains largely unknown (see Introduction). The core protein is composed of several different sequence motifs that are also found in the low density lipoprotein receptor and in other extracellular matrix proteins such as laminin (Murdoch et al., 1992).

A significant increase in perlecan expression occurs during organogenesis of the kidney, lung, liver, spleen, gastrointestinal tract and cartilage (Handler et al., 1997). The levels of perlecan are low in precartilaginous tissues (French et al., 1999), but high in mature cartilage. In contrast to the well characterized expression pattern, only a few functional properties of perlecan are known. One feature that perlecan shares with several other proteoglycans, is its ability to bind and store growth factors, e.g. fibroblasts growth factor 2 (FGF-2) and platelet deriving growth factor B (PDGF-B). The presence of perlecan in basement membranes and its ability to interact with other basement membrane components such as type IV collagen, laminin, and nidogen-1 *in vitro* suggests that it is involved in basement membrane assembly (Reinhardt et al., 1993). It also binds cell adhesion molecules such as β 1- and β 3-type integrins (Hayashi et al., 1992). Although both keratinocytes and fibroblasts synthesize perlecan, it has not been shown where perlecan in the basement membrane come from and if it is crucial for assembly in skin.

To answer this question, the 3D-coculture system offers a suitable model using either perlecan deficient epithelial cells or fibroblasts or combining both.

5-3.1 Expression of basement membrane components in perlecan knockout ^{SV}fibroblasts

To examine the role of dermal cells on perlecan deficiency, the human fibroblasts in 3D-cocultures were replaced by fibroblasts from perlecan knockout mice. Three different perlecan genotypes were used, control fibroblasts +/+, heterozygous knocked out fibroblasts +/-, and complete knocked out (homozygous) fibroblasts -/-. Since the homozygous null-fetuses only survived until day 12, cells were cultured from fetal carcasses and immortalized by SV-40 transfection (^{SV}fibroblasts). Generally these cells divided more rapidly and had an atypical morphology (in part resembling endothelial cells) showing frequent cell contacts and forming colonies.

However, Fig. 5.21 shows that basement membrane proteins were expressed in these ^{SV}fibroblasts grown on slides. Except for perlecan, the genotypes showed no difference in the ability to synthesize the basement membrane proteins.

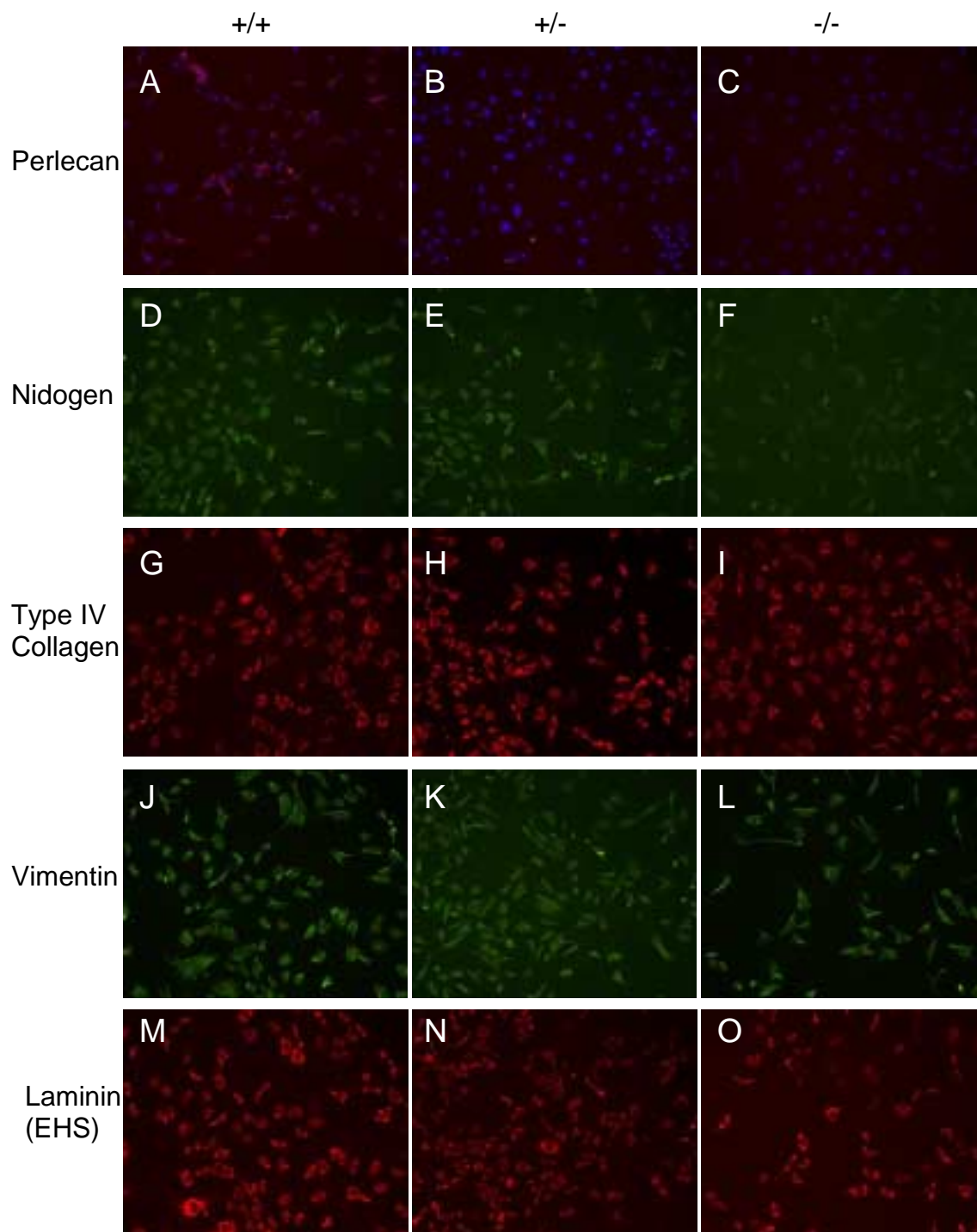


Fig. 5.21. Expression of perlecan (640), nidogen (416), type IV collagen (536), vimentin (628) and EHS laminin (414) in control and perlecan knockout^{SV} fibroblasts.

5-3.2 Epithelial development in 3D-cocultures with perlecan deficient ^{SV}fibroblasts

The SV-40 transfected ^{SV}fibroblasts were, as earlier mentioned, rapidly growing and tended to form cell clusters. In low passages it was still possible to incorporate them into the collagen gels in the 3D-coculture system. Cells that were passaged more than 5-6 times started to grow uncontrollable. Approaches to irradiate the ^{SV}fibroblasts were made, but the irradiation at even very low doses turned out to be mortal. This was in sharp contrast to human fibroblasts from adult dermis, which normally tolerate the irradiation well. Because of this, the following experiments were all with cells in the passages 3-5.

The epithelia in the 3D-cocultures developed rather normal. The collagen gels were overgrown with ^{SV}fibroblasts, but within low passages, the ^{SV}fibroblasts did not approach to invade the epithelia. As depicted in Fig. 5.22, the different ^{SV}fibroblast genotypes did not show significant difference in the development of the epithelia. They all produced quite thick epithelia, only the degree of differentiation was slightly reduced in comparison to human fibroblasts. With all ^{SV}fibroblast genotypes, basal cells were organized on the basement membrane, but no clear cornified layer could be detected, and the epithelial cells tended to form whirls instead of horizontal strata in the upper layers. While the effects of an early fetal cell type cannot be ruled out, this was most likely due to the SV-40 transfection.

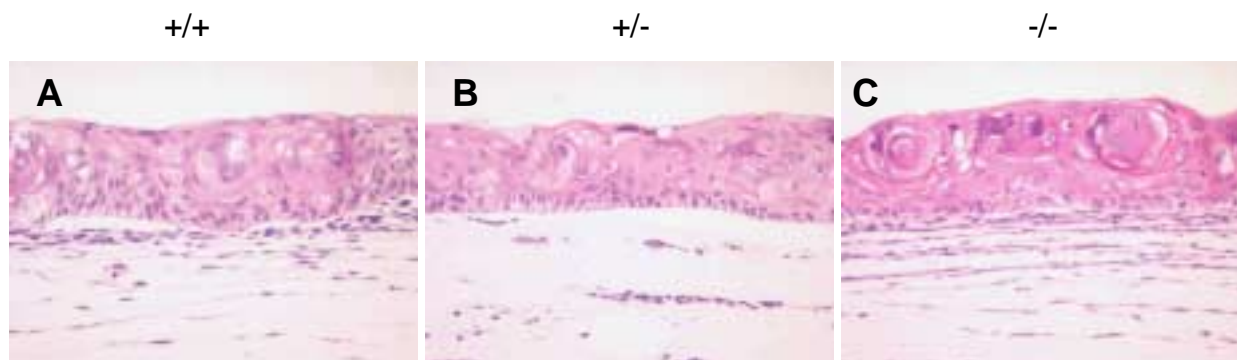


Fig. 5.22. Histology of HaCaT 3D cocultures with perlecan knockout ^{SV}fibroblasts, day 12. ^{SV}Fibroblast genotype +/+/-: A, +/-: B, -/-: C.

5-3.3 Expression of basement membrane proteins in 3D-cocultures with perlecan knockout^{SV} fibroblasts and HaCaT cells

5-3.3.1 Perlecan

The 3D-cocultures were further analyzed for basement membrane proteins. An interesting aspect to investigate was if perlecan was expressed in the 3D-cocultures with the $-/-$ ^{SV} fibroblasts. Unlike the situation with nidogen, it was not clear whether perlecan in the basement membrane was synthesized by keratinocytes or by fibroblasts, or by both (separately or in combination). In Fig. 5.21, chapter 5-3.1 it was shown that the $+/+$ and the $+/-$ ^{SV} fibroblast genotypes were able to synthesize perlecan. However, the findings from ^{SV} fibroblasts growing on solid support could differ from those in the 3D-coculture or the skin where the cells are embedded in a collagen- or an extracellular matrix. If perlecan was only synthesized by fibroblasts, no perlecan should be expressed in the 3D-cocultures with the $-/-$ ^{SV} fibroblasts. If perlecan was produced by the keratinocytes or by keratinocytes and fibroblasts in combination, the expression in the 3D-cocultures should not necessarily be influenced by the $-/-$ ^{SV} fibroblasts.

Immunofluorescent staining of 3D-cocultures with the three different ^{SV} fibroblast genotypes revealed that perlecan was expressed also when $-/-$ ^{SV} fibroblasts were used (Fig. 5.23). Nevertheless, these 3D-cocultures exhibited clear differences in the collagen gels. In Fig. 5.23 A, the collagen matrix was stained red because of antibody signals all over the gel. In Fig. 5.23 B, this was not so clear as this ^{SV} fibroblast genotype tended to build “islands” in the gel, but red staining in the gel around the fibroblasts could still be seen. Fig. 5.23 C shows that although perlecan was expressed in the basement membrane zone, no staining could be seen around the fibroblasts. The explanation of this must be that the perlecan seen at the interface here was produced by the keratinocytes alone.

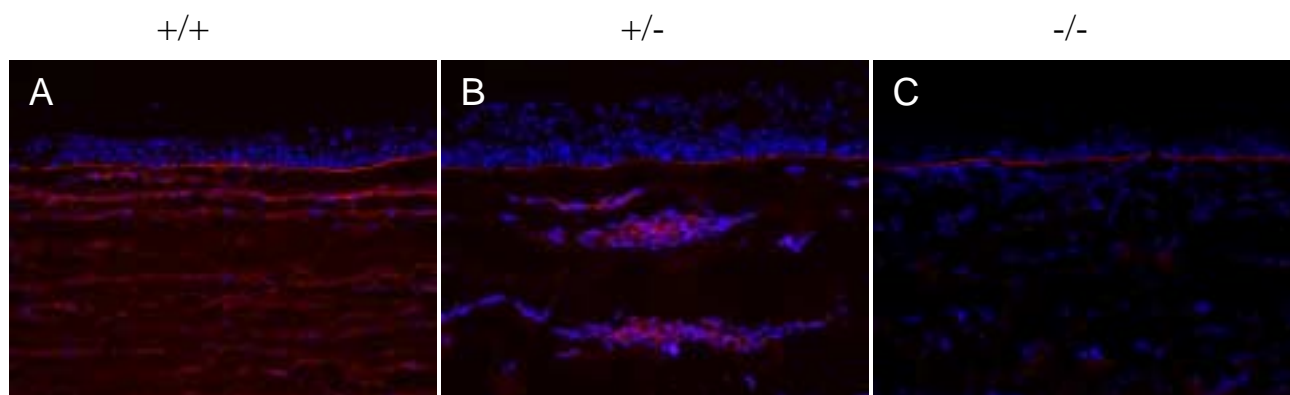


Fig. 5.23. Staining for perlecan (640) in perlecan deficient HaCaT 3D-cocultures, day 12. ^{SV}Fibroblast genotype $+/+$: A, $+/-$: B, $-/-$: C. In B, the cluster formation can be observed.

5-3.3.2 Laminin

Although the 3D-cocultures with all three genotypes of the ^{SV}fibroblasts expressed perlecan, it had to be determined if the expression of the other basement membrane components was normal as well.

In Fig. 5.24, the deposition of laminin γ 1, laminin α 5 and EHS laminin together with the keratins K1/K10 can be seen. As expected, the 3D-cocultures did not reveal any significant difference between the ^{SV}fibroblast genotypes in laminin expression. Also in the -/- 3D-cocultures, laminin α 5 and γ 1 were produced and assembled onto the basal zone (Fig. 5.24 C, F, I). This showed that the perlecan produced from the keratinocytes was sufficient to compensate for the deficiency in the ^{SV}fibroblasts.

Although the degree of differentiation in these 3D-cocultures was decreased when compared to those with human fibroblasts, the distribution of keratin K1/K10 appeared quite normal being mainly expressed in the suprabasal layers (Fig. 5.24 C, F, I). This, together with the histology in Fig. 5.22, clearly indicates maintenance of a normal basal cell compartment.

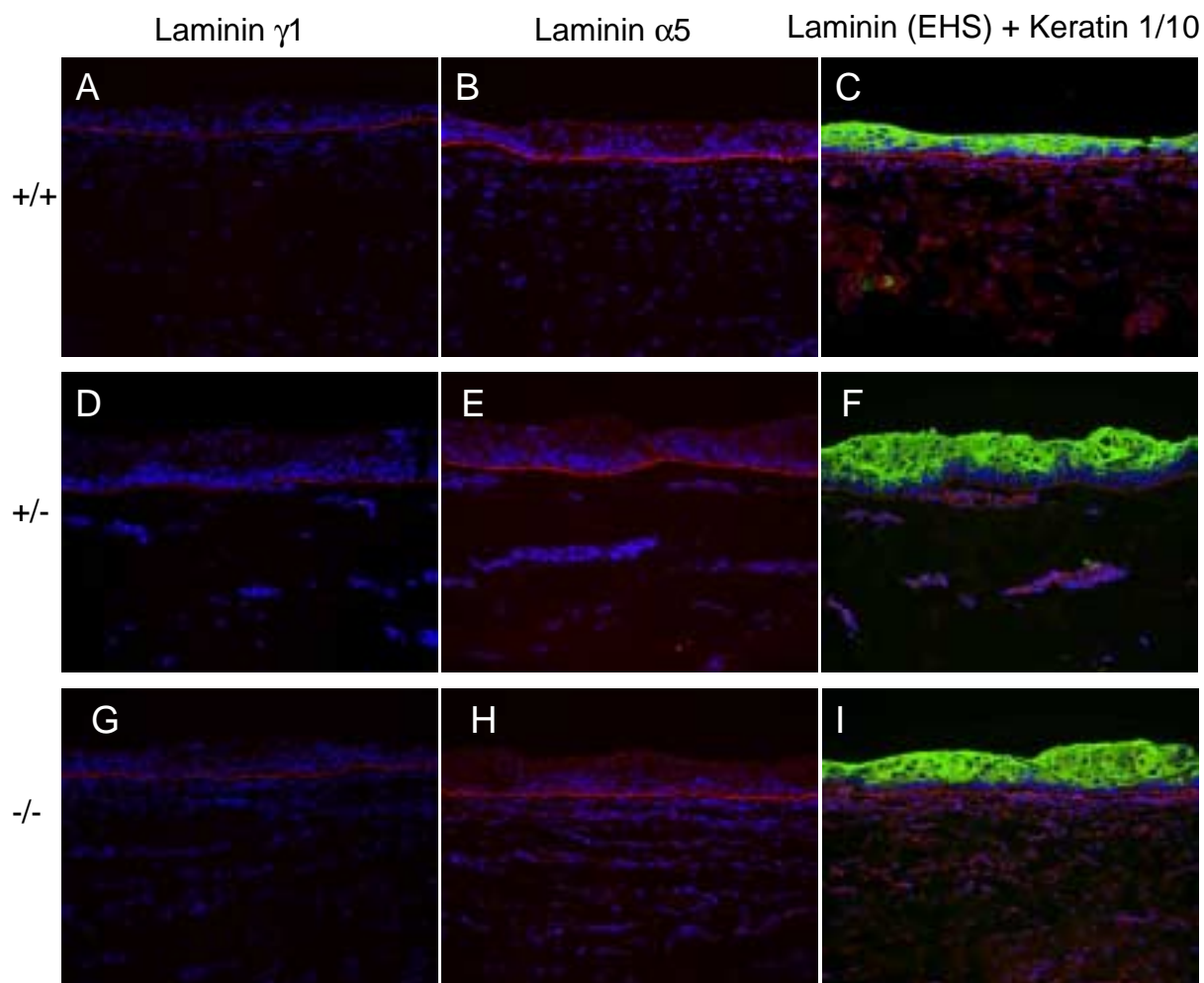


Fig. 5.24. Staining for the laminin α 5 chain (723), γ 1 chain (528), EHS laminin (496) and keratin K 1/10 (638) in perlecan deficient HaCaT 3D-cocultures, day 12. ^{SV}Fibroblast genotype +/+ : A-C, +/-: D-F, -/-: G-I. In C, F, I; EHS laminin in red, keratins K 1/10 in green.

5-3.3.3 Nidogen-1, Laminin-5 and Integrin chain $\alpha 6$

From the experiments with the nidogen knockout fibroblasts it became clear, that the synthesis or regular deposition of the basement membrane components are not strictly coordinated. In the following experiments, the components nidogen-1, laminin $\gamma 2$ and integrin chain $\alpha 6$ were investigated.

Fig. 5.25 revealed that nidogen-1, laminin-5 and the integrin chain $\alpha 6$ remained unchanged also with the null homozygous SV fibroblasts (Fig. 5.25 C, F, I).

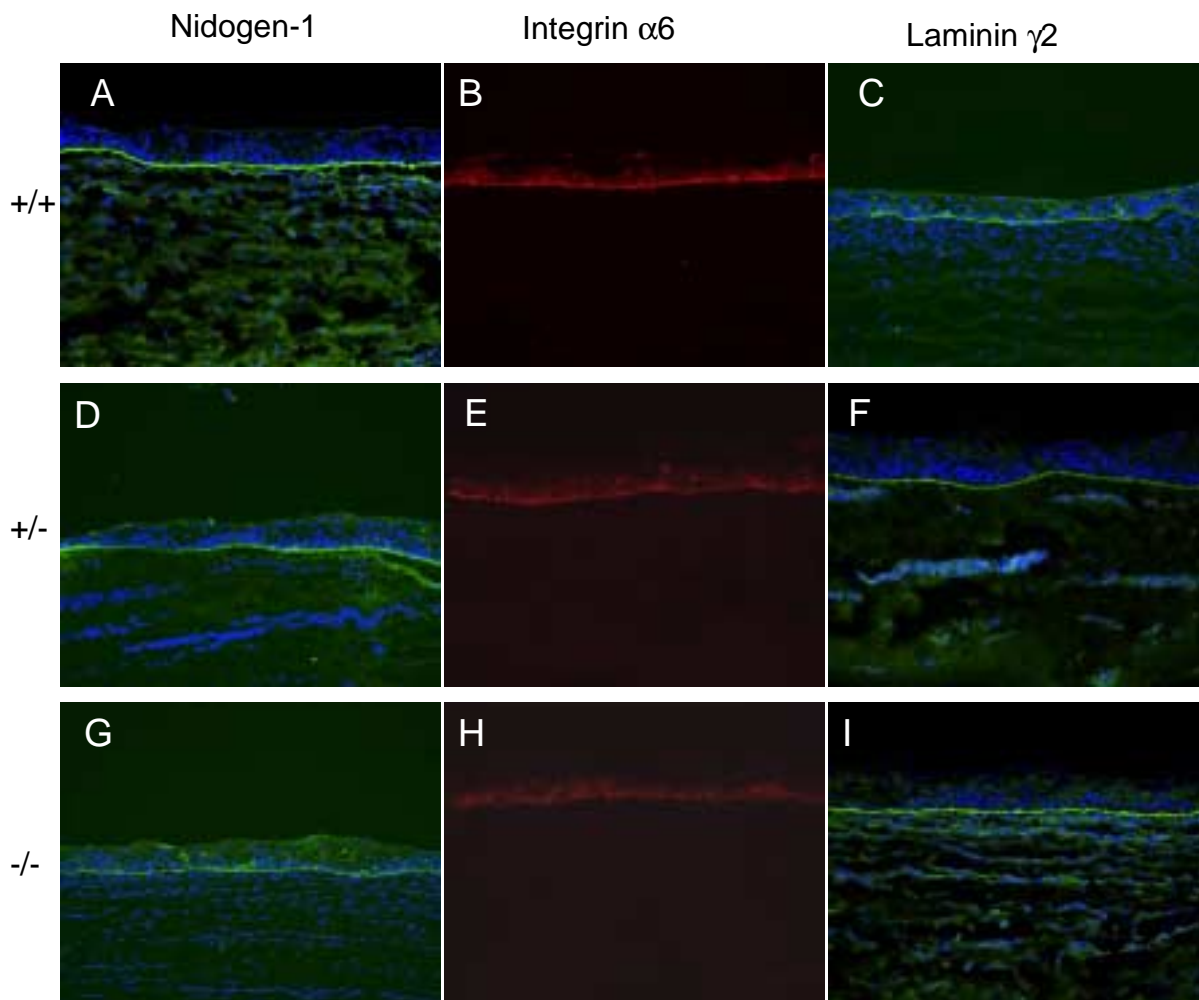


Fig. 5.25. Staining for nidogen-1 (416), the integrin chain $\alpha 6$ (370) and laminin $\gamma 2$ (466) in perlecan deficient HaCaT 3D-cocultures, day 12. SV Fibroblast genotype +/+ : A-C, +/- : D-F, -/- : G-I.

5-3.4 Expression of basement membrane components in 3D-cocultures with Perlecan deficient HaCaT cells and normal fibroblasts

As the findings with the perlecan deficient fibroblasts in chapter 5-3.3 had shown that keratinocytes were perfectly able to produce and assemble perlecan, the intention of the following experiments was to block synthesis in keratinocytes instead, to prove if also fibroblasts could compensate for perlecan deficiency in the keratinocytes. For this purpose, HaCaT deficient perlecan cells were employed in 3D-cocultures together with normal human fibroblasts.

The HaCaT deficient perlecan cells were expressing high levels of antisense perlecan mRNA which interferes with the synthesis of perlecan core protein. Another variant of antisense-transfected HaCaT cells expressing only low levels of antisense perlecan mRNA was used as a vector control.

The results in Fig. 5.26 demonstrated that also normal fibroblasts could provide enough perlecan for deposition. The pictures A, C, E, and G depict the control 3D-cocultures with low antisense mRNA expressors, and the pictures B, D, F and H the 3D-cocultures with high expressors. There was no difference between the 3D-cocultures with the different cells neither in the epithelial development nor in the expression of basement membrane components. In the 3D-cocultures with perlecan deficient HaCaT cells there was no decrease of perlecan, and regular expression was seen for nidogen-1, laminin γ 1 and laminin γ 2. Also there was no major change in the differentiation patterns as exemplified for keratins K1/10, visible regularly in all suprabasal layers.

These results revealed that the fibroblasts also could synthesize and assemble basement membrane components independent of the synthesis in keratinocytes.

Taken together, the results in 5-3 showed that perlecan is a basement membrane component synthesized by both keratinocytes **and** fibroblasts, and there is no need for cooperation in this regard. Furthermore, in situations where one of the cell types is perlecan deficient, this has no influence on synthesis or assembly of the other basement membrane components.

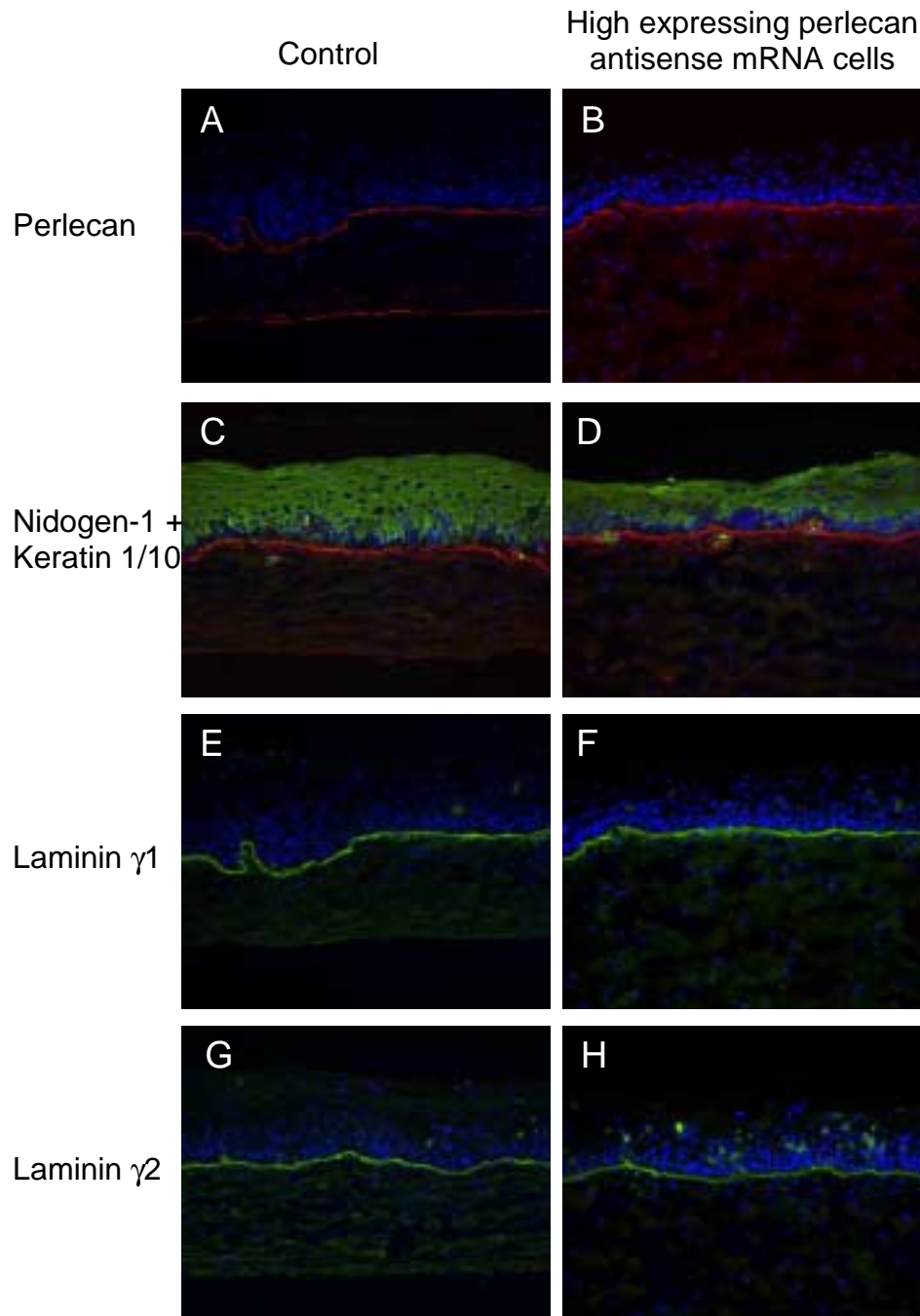


Fig. 5.26. Staining for perlecan (177), nidogen-1 (416), laminin γ 1 (528), laminin γ 2 (466) and keratins K1/10 (638) in 3D-cocultures with HaCaT perlecan deficient cells and normal human fibroblasts, day 21. HaCaT low expressing mRNA cells (control): A, C, E, and G, HaCaT high expressing mRNA cells: B, D, F and H. In C-D: nidogen-1 in red and keratins 1/10 in green.

The findings in chapter 5-3.3 and 5-3.4 revealed that to mimic a complete knockout situation, both cell types have to be perlecan deficient. Several attempts were made in 3D-cocultures to accomplish this. But as the ^{SV}fibroblasts were extremely fast growing and the antisense HaCaT cells very slowly growing, the tissue balance was disturbed. Consequently, the ^{SV}fibroblasts tended to overgrow the epithelia after short time, interfering with epithelial development. Thus, the resulting 3D-cocultures were hardly or not differentiated. Nevertheless, basement membrane components were expressed, which is shown in Fig. 5.27. The deposition of perlecan, nidogen-1 and

laminin $\gamma 1$ was compared between 3D-cocultures of high antisense perlecan HaCaT cells with perlecan (-/-) knockout ^{SV}fibroblasts and control cocultures consisting of low perlecan antisense HaCaT cells and perlecan +/+ ^{SV}fibroblasts.

The immunofluorescence staining demonstrated that in general, nidogen-1 and laminin $\gamma 1$ were expressed on the interface in the controls as well as in the perlecan deficient 3D-cocultures. Nevertheless, some leakiness of high antisense perlecan HaCaT cells can be observed in Fig. 5.27 B. Furthermore, the staining of laminin $\gamma 1$ in Fig. 5.27 C shows an eventually weaker polarity possible due to more staining in cytoplasm.

However, this suggested that lack of perlecan does not significantly influence neither the expression of basement membrane components, nor the formation of the basement membrane, at least as seen in the light microscope.

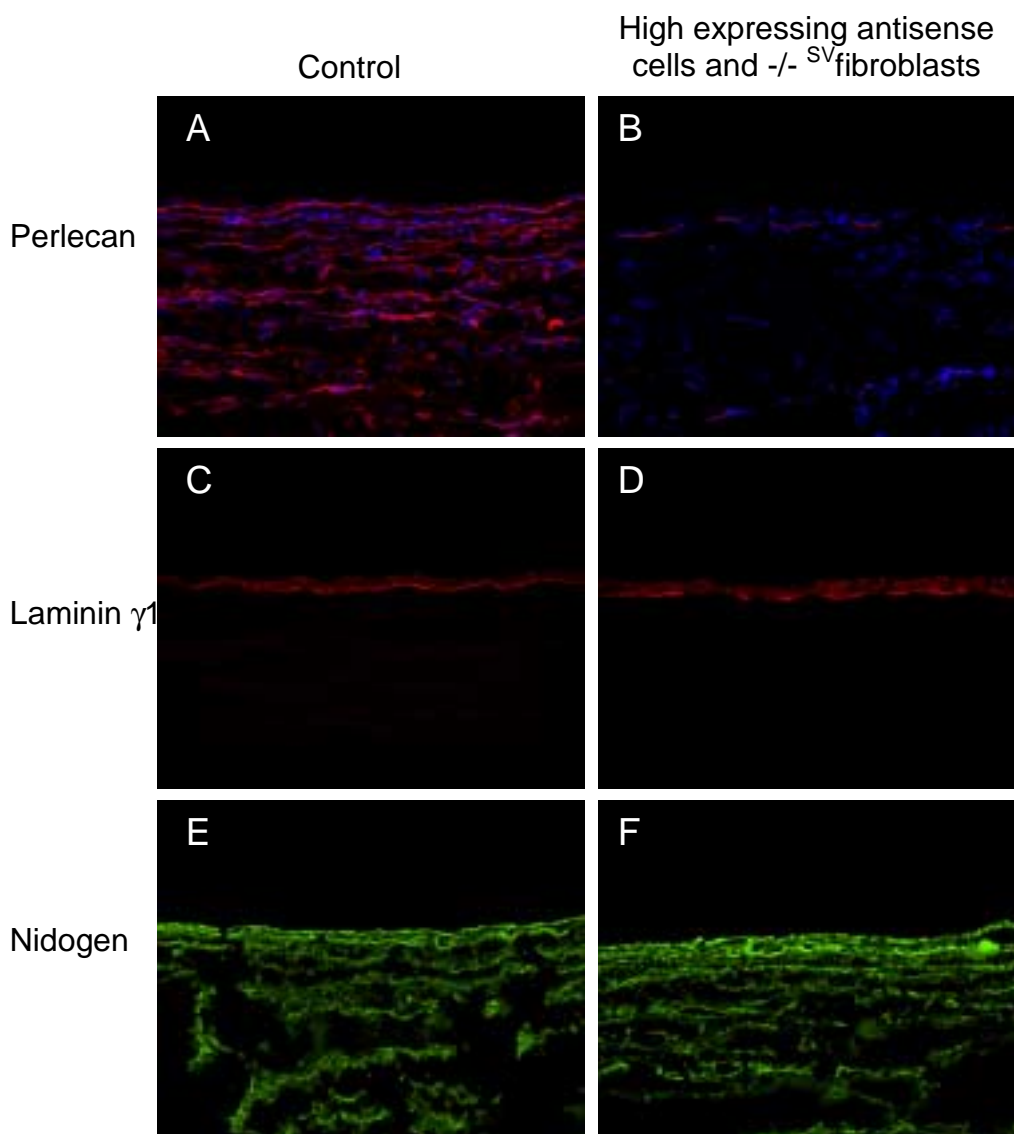


Fig. 5.27. Staining for perlecan (640), laminin $\gamma 1$ (528) and nidogen-1 (416) in 3D-cocultures of high expressing antisense perlecan mRNA HaCaT cells and perlecan -/- ^{SV}fibroblasts, day 14. Control (HaCaT low antisense and +/+ ^{SV}fibroblasts: A, C and E, Perlecan deficient cultures: B, D and F.

5-4. Blockade of basement membrane assembly in 3D-cocultures with HaCaT cells by a L γ 1f-laminin fragment

As already outlined in the Introduction and chapters 5-1 and 5-3, common in the skin basement membrane structures are type IV collagen, laminin-10, nidogen and perlecan. In contrast to type IV collagen and laminin-10, nidogen shows no self-assembly, while it harbours many potential binding sites to the other basement membrane components. Nidogen connects the integrated mesh works of laminin-10 and type IV collagen (Mayer et al., 1995, Timpl 1996), which are supposed to form independent homopolymeric entities (Yurchenco et al., 1992). In addition, nidogen contributes to the integration of perlecan into the basement membrane.

The exact localization of the nidogen binding site is within the short arm of the laminin γ 1 chain in the III4 module (Mayer et al., 1993, Pöschl et al., 1996). This region has been deleted in transgenic mice (Willem et al., 2002). The most severe effects in these mice were observed in kidney and lung development, leading to embryonic or perinatal death. Nevertheless, skin and vasculature seemed to be less affected or normal. Obviously the laminin γ 1-mutation can be compensated in those tissues, at least temporarily.

The following experiments were designed to investigate the consequences of interference of the laminin-nidogen binding for assembly of the basement membrane. For this purpose a recombinant fragment of the laminin γ 1 chain was applied (γ 1III3-5, addressed as L γ 1f), which includes the binding site for nidogen (Fig. 5.28). Basically, conditions very recently used for normal human keratinocytes (Breitkreutz et al., 2003, submitted), were adjusted to HaCaT 3D-cocultures.

The L γ 1f fragment was able to bind to the nidogen-binding site of the laminin γ 1 chain with very high affinity. This was leading to competitive inhibition of the nidogen – laminin binding in 3D-cocultures of normal skin cells (IC_{50} of 0,1 nM; concentration of half-maximal inhibition of laminin binding).

According to the existing protocol, 40 μ g/ml L γ 1f was added to the 3D-coculture in the medium from the first day on, and further 3 times a week. Alternatively, L γ 1f was added from the 7 day of the 3D-coculture to investigate if already synthesized basement membrane could be disturbed. L γ 1f was bio-compatible and did not cause any morphological changes or reduction of the mitotic rate when tested in conventional 2D-cultures of HaCaT cells and normal human fibroblasts.

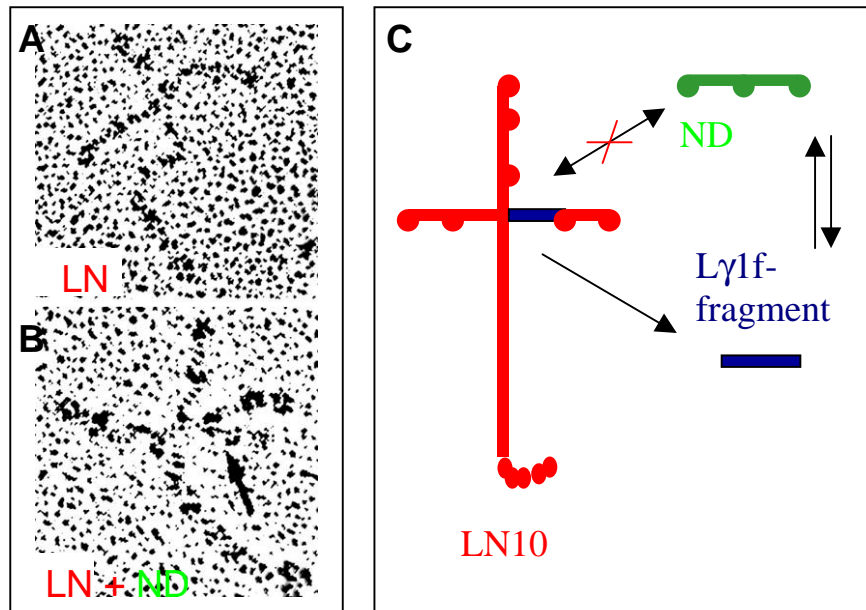


Fig. 5.28. The $L\gamma 1f$ fragment. A, B: Rotary shadowing images of laminin-10 and the binding of nidogen to laminin-10 (arrow). C: $L\gamma 1f$ binds to the nidogen binding site on laminin and thereby inhibits the laminin-nidogen binding.

5-4.1 Epithelial development of $L\gamma 1f$ -treated 3D-cocultures

Similar observations as in the 3D-cocultures with the nidogen deficient fibroblasts were made in the 3D-cocultures with $L\gamma 1f$. The epithelia developed normally in the presence of $L\gamma 1f$ as in the controls, applying $L\gamma 1f$ either after one or seven days (Fig. 5.29). The only clear difference was an increased tendency for splitting at the epidermal-collagen interface with $L\gamma 1f$, which indicated weakening of the epidermal-matrix junction. This observation was also made in the 3D-cocultures with the nidogen deficient fibroblasts.

The epithelial stratification was not impaired and the regular granular and cornified layers implied a normal course of differentiation. This strongly indicated that $L\gamma 1f$ did not interfere with epithelial development.

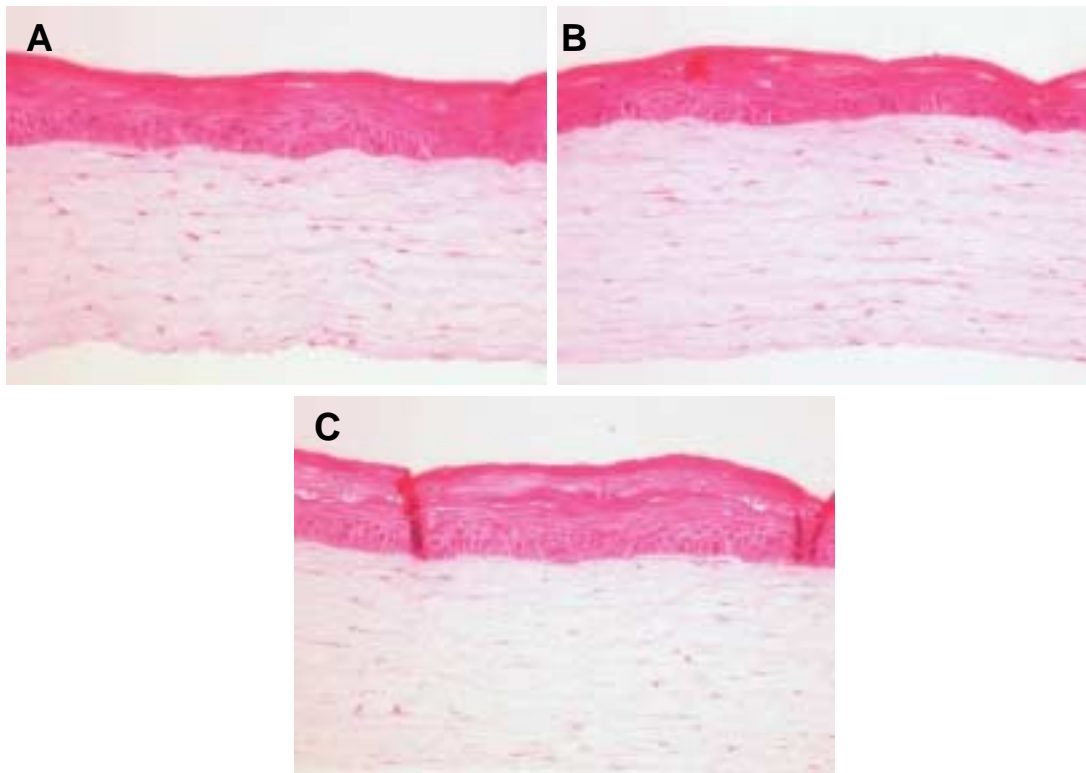


Fig. 5.29. Histology of 3D-cocultures treated with 40 µg/ml Ly1f, day 12. A: Control, B: Ly1f added after 7 days, C: Ly1f present throughout the experiment.

5-4.2 Effects of Ly1f on the deposition of basement membrane components

Earlier experiments had shown that the deposition of the basement membrane in the 3D-coculture with HaCaT cells is still low but clearly visible after 7 days, nearly optimal after 12 days, and then persisting throughout the whole observation period (up to day 21). Therefore, Ly1f was, in addition to continuous treatment from the beginning, also added after 7 days. This should answer the question if already developed basement membrane structures would vanish again with Ly1f.

5-4.2.1 Laminin, Nidogen-1 and Nidogen-2

Immunofluorescent staining of nidogen-1 and nidogen-2 showed that Ly1f completely abolished the deposition of nidogen-1 and nidogen-2 in the 3D-cocultures treated for the whole period, but also in the 3D-cocultures treated just from day 7 on (Fig 5.30 C, F and B, E). This was also the case with laminin-10 as seen by staining for laminin γ 1 and EHS laminin (Fig. 5.30 H, K and I, L).

The staining against keratins K1/10 (Fig. 5.30 J-L) further underlined the normal course of differentiation indicated by histology (Fig. 5.29).

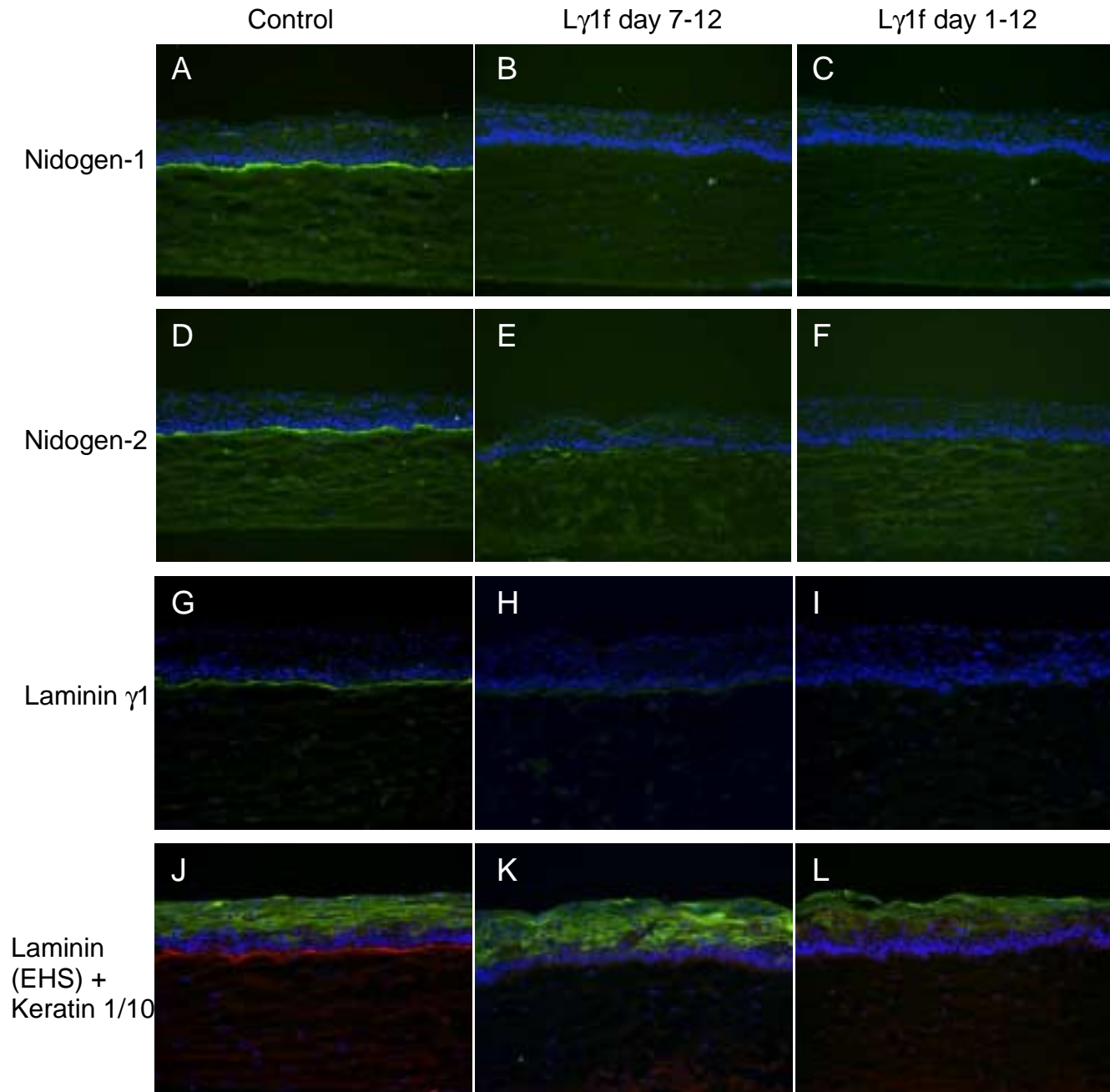


Fig. 5.30. Effect of fragment L γ 1f on basement membrane components. Staining for nidogen-1 (416) and nidogen-2 (593), the laminin γ 1 chain (528), EHS laminin (496) and keratin 1/10 (638) in 3D-cocultures treated with 40 μ g/ml L γ 1f, day 12. Control: A, D, G, J, L γ 1f from day 7 to 12: B, E, H, K, L γ 1f throughout the experiment: C, F, I, L. In J-L: EHS laminin in red, keratins K1/10 in green.

5-4.2.2 Integrin chains $\alpha 6$ and $\beta 1$

Integrin chain $\alpha 6$ was mentioned above (5-2), as part of the integrin $\alpha 6\beta 4$ complex. Other common epidermal integrins are the $\alpha 2\beta 1$ and the $\alpha 3\beta 1$ (Carter et al., 1990, De Luca et al., 1990). Since the epithelia showed an increased tendency to split from the matrix, and since the basal cells seemed to adhere to a great part directly to the collagen gel in the absence of basement membrane structures, it seemed reasonable to examine some of the integrin patterns.

Fig. 5.31 revealed that the integrin chain $\alpha 6$ was variably influenced depending on the duration of $L\gamma 1$ treatment. For the 3D-cocultures treated from day 7 on, no influence at all could be observed. However, the 3D-cocultures treated the whole period showed an irregular, less polarized pattern. Regarding the integrin $\beta 1$ chain, no significant difference between the control and the 3D-cocultures treated 5 days or continuously with $L\gamma 1$ could be observed. The difference in the $\beta 1$ and $\alpha 6$ response suggests subtle changes in the cell-matrix interactions.

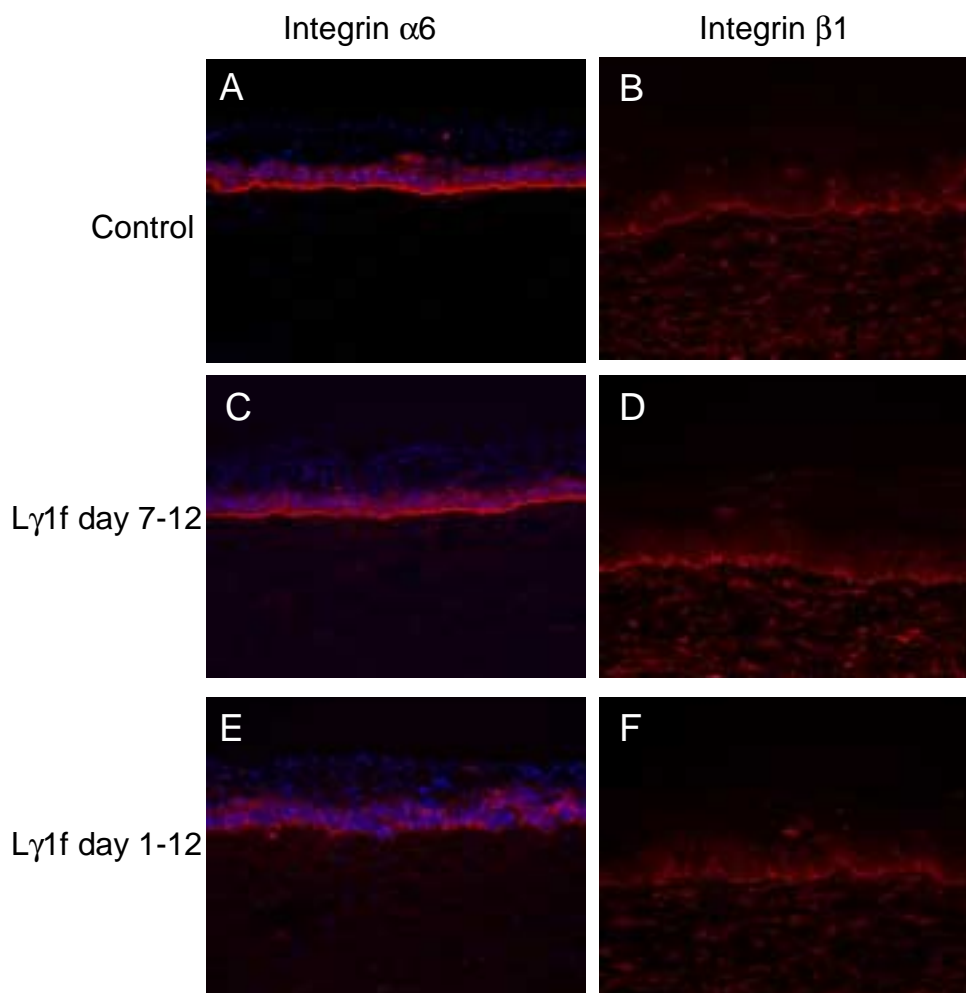


Fig. 5.31. Expression of the integrin $\alpha 6$ chain (370) and $\beta 1$ chain (385) in 3D-cocultures treated with 40 $\mu\text{g/ml}$ $L\gamma 1$, day 12. Control: A-B, $L\gamma 1$ from day 7 on: C-D, $L\gamma 1$ the whole period: E-F. It should be noted that also the fibroblasts in the matrix are $\beta 1$ and not $\alpha 6$ positive.

For a closer examination of $\text{L}\gamma 1\text{f}$ -mediated changes of integrin $\alpha 6$ in the 3D-cocultures treated with $\text{L}\gamma 1\text{f}$, some immunofluorescence samples were further analyzed with the confocal laser scan microscope. The advantage was that this allowed more precise correlation with deposition of basement membrane components such as laminin-10

Because the laser scan recordings could be done at higher magnification, this allowed a much clearer resolution of the integrin $\alpha 6$ pattern, showing it even more disturbed than initially assumed. Thus, in the lower part of the basal cells, the integrins seemed to be shed into the collagen gel. Altogether, the laser confocal recording revealed that the integrin $\alpha 6\beta 4$ was significantly influenced by the $\text{L}\gamma 1\text{f}$ treatment.

The recording of the laminin $\gamma 1$ samples verified findings by regular light microscopy in Fig. 5.30. No signal of laminin $\gamma 1$ in $\text{L}\gamma 1\text{f}$ -treated samples could be recorded in this system (Fig. 5.32, B, C).

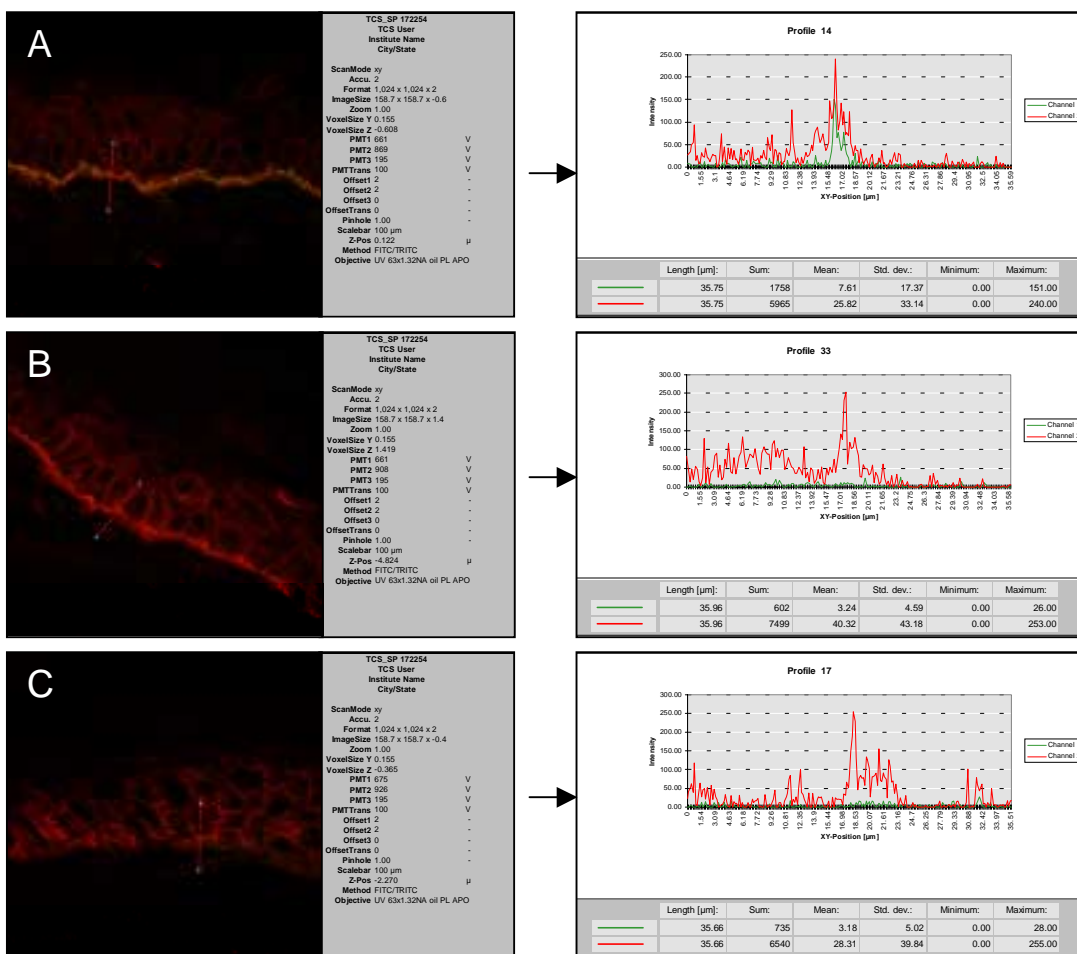


Fig. 5.32. Laser confocal analysis of laminin $\gamma 1$ (green) and integrin chain $\alpha 6$ (red) in 3D-cocultures treated with 40 $\mu\text{g}/\text{ml}$ $\text{L}\gamma 1\text{f}$, day 12. Control: A, $\text{L}\gamma 1\text{f}$ day 7-12: B, $\text{L}\gamma 1\text{f}$ throughout the experiment: C. Marked in red is the area measured from top to bottom (inside-out) corresponding to the recording from left to right.

5-4.2.3 Western blot analysis of laminin-10 and nidogen-1

The analysis by immunofluorescence and laser confocal microscopy had revealed that laminin-10, represented by laminin γ 1 and α 5 totally disappeared with L γ 1f applied to the 3D-coculture. There are several possible explanations for this. The break-up of the laminin-10-nidogen binding, but also the formed L γ 1f-nidogen complex, could result in a downregulation of laminin-10 synthesis, processing or secretion. Another explanation could be induction of protease activity, causing rapid degradation of laminin-10 by proteolysis. Matrix metalloproteinases have been reported to be present in 3D-monocultures of epidermal cell lines, growing within type I collagen matrix (Baumann et al., 2000), but also in 3D-cocultures produced by both keratinocytes and fibroblasts.

If one of these explanations would hold true, no laminin-10 would be detected by immunoblotting of the 3D-cocultures, and this was challenged by western blots. For this analysis, protein extracts were prepared from 3D-cocultures after splitting the epithelial part from the collagen gel.

Fig. 5.33 shows immunoblotting of these fractions after 12 and 14 days with an antibody against the laminin γ 1 chain (appr. 204 kDa). Surprisingly, the expression of laminin γ 1 was just as strong in the L γ 1f treated- as in the control 3D-cocultures. This demonstrated that laminin-10 had to be present despite treatment, although it was neither detected with normal immunofluorescence nor with confocal laser scan microscopy. An explanation could be that laminin-10 is not assembled to the basement membrane zone, but remained intracellularly, being present in the whole epithelial part. Accordingly, this meant that the situation was comparable to the 3D-coculture with nidogen deficient fibroblasts (chapter 5-2).

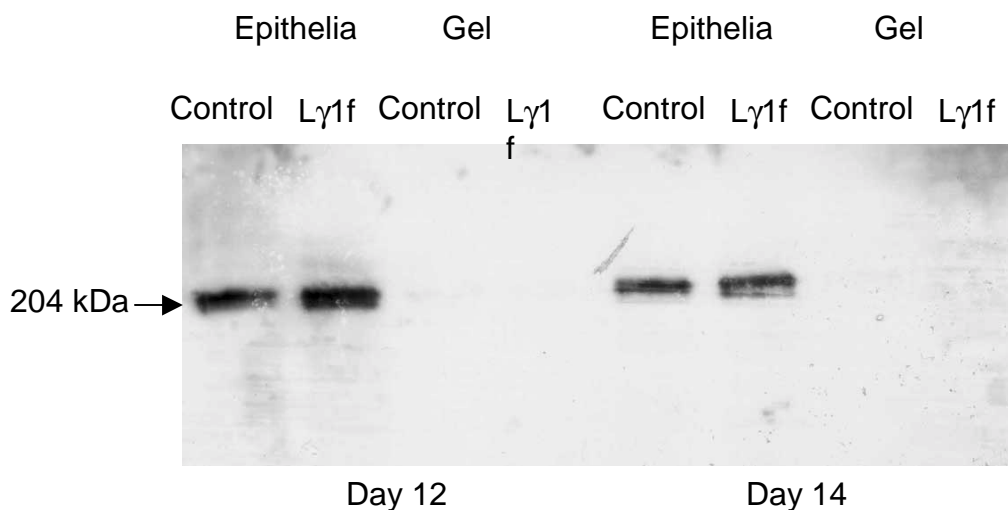


Fig. 5.33. Western blot of the laminin γ 1 chain in 3D-cocultures treated with 40 μ g/ml L γ 1f, day 12 and 14. The blot was performed with protein extracts of the two tissue compartments from 3D-cocultures of normal human keratinocytes with human fibroblasts.

This could be further confirmed by immunoblots with an antibody against the laminin $\alpha 5$ chain (nominal molecular mass appr. 400 kDa), shown in Fig. 5.34.

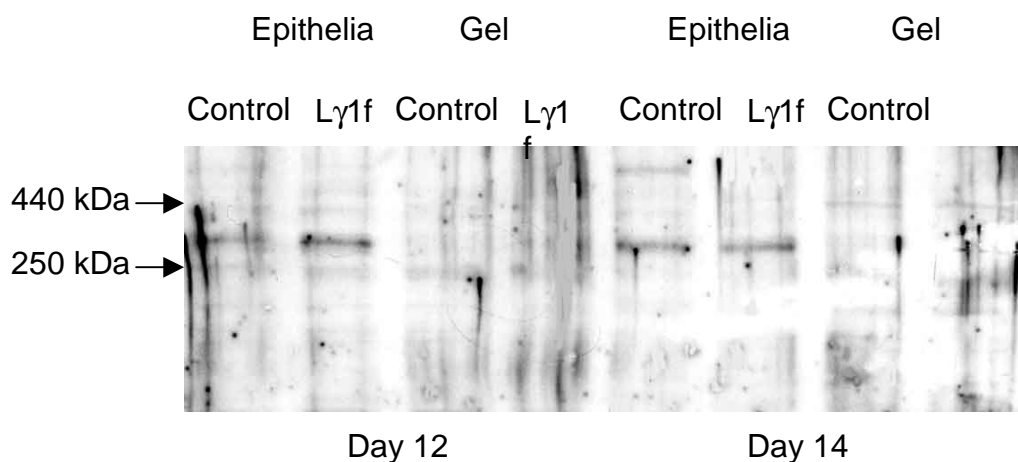


Fig. 5.34. Western blot of the laminin $\alpha 5$ chain in 3D-cocultures treated with 40 $\mu\text{g}/\text{ml}$ L γ 1f, day 12 and 14. The blot was performed with the same extracts of normal human keratinocytes with human fibroblasts, shown in Fig. 5.33.

Further, western blots were performed with nidogen-1, since also nidogen-1 was not detectable in the immunofluorescence samples in Fig. 5.30, chapter 5-4.2.1. But again, also nidogen-1, like laminin-10, was still present in the L γ 1f treated 3D-cocultures. However, although the major part was seen in the collagen fraction as expected for its synthesis by fibroblasts, comparable amounts were associated with the epithelia of both treated and control cultures.

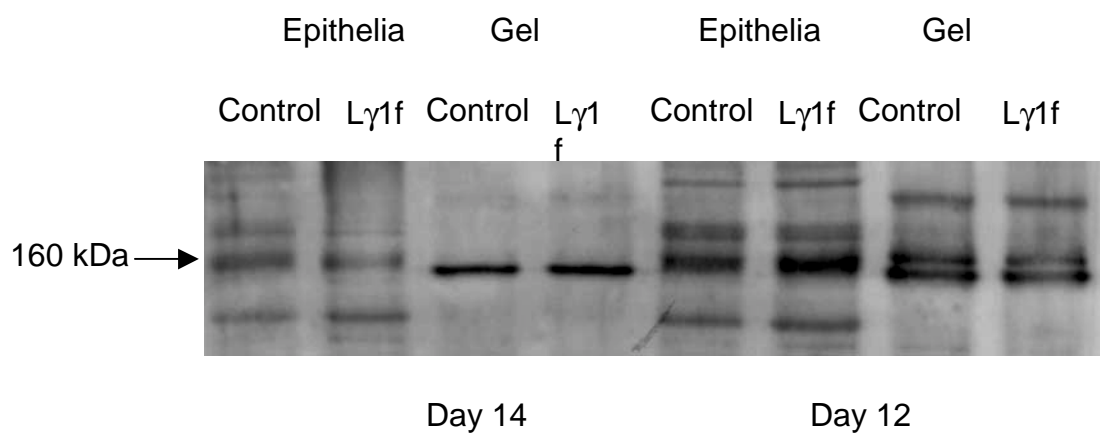


Fig. 5.35. Western blot of nidogen-1 in 3D-cocultures treated with 40 $\mu\text{g}/\text{ml}$ L γ 1f, day 12 and 14. Extracts as in Fig. 5.33 and 5.34.

5-4.2.3 Perlecan, Type IV Collagen and Laminin-5

While the previous data confirmed that nidogens and laminin-10 were directly interacting partners interlinking their appearance and fate, the relationship differs individually towards the other basement membrane components perlecan, type IV collagen and laminin-5.

Thus, L γ 1f did not influence laminin-5, here represented by the laminin γ 2 chain (Fig. 5.36 A, D, G). The 3D-cocultures treated with L γ 1f from day 7 on and the whole period expressed laminin γ 2 strongly and did not show any significant difference with the control cultures. This underlines that the deposition of laminin-5 is virtually independent of the basement membrane status, although it is a crucial element of anchoring fibrils. While perlecan is also not forming homopolymers, it is in contrast to laminin-5 not bound to the basal cells either. Thus, perlecan disappeared from the junctional interface in the L γ 1f treated 3D-cocultures like laminin-10 and nidogen. It could, however, be observed in the collagen gel, which implies different binding properties and diffusion rates (Fig. 5.36 B, E, H).

Type IV collagen was not as strongly influenced as perlecan, but a clear difference could be seen between the control cultures and the L γ 1f treated 3D-cocultures. In contrast to the control cultures where a strong, clear line was observed, the staining in the L γ 1f treated cultures was rather weak and not continuous. These findings raise some doubts on the hypothesis that type IV collagen and laminin-10 form independent networks. At least this indicates that for compact structure both elements are absolutely required.

These 3D-cocultures were also stained for the general differentiation marker transglutaminase. Transglutaminase is the enzyme responsible for the cross-linking process leading to the formation of the cornified envelope, and is considered as one of the late differentiation markers. As already mentioned (Fig. 5.29, chapter 5-4.1), the L γ 1f treatment did not seem to have any influence on the epithelial development or differentiation. This was confirmed with the transglutaminase staining, as the L γ 1f treated 3D-cocultures showed the same staining pattern as the control cultures.

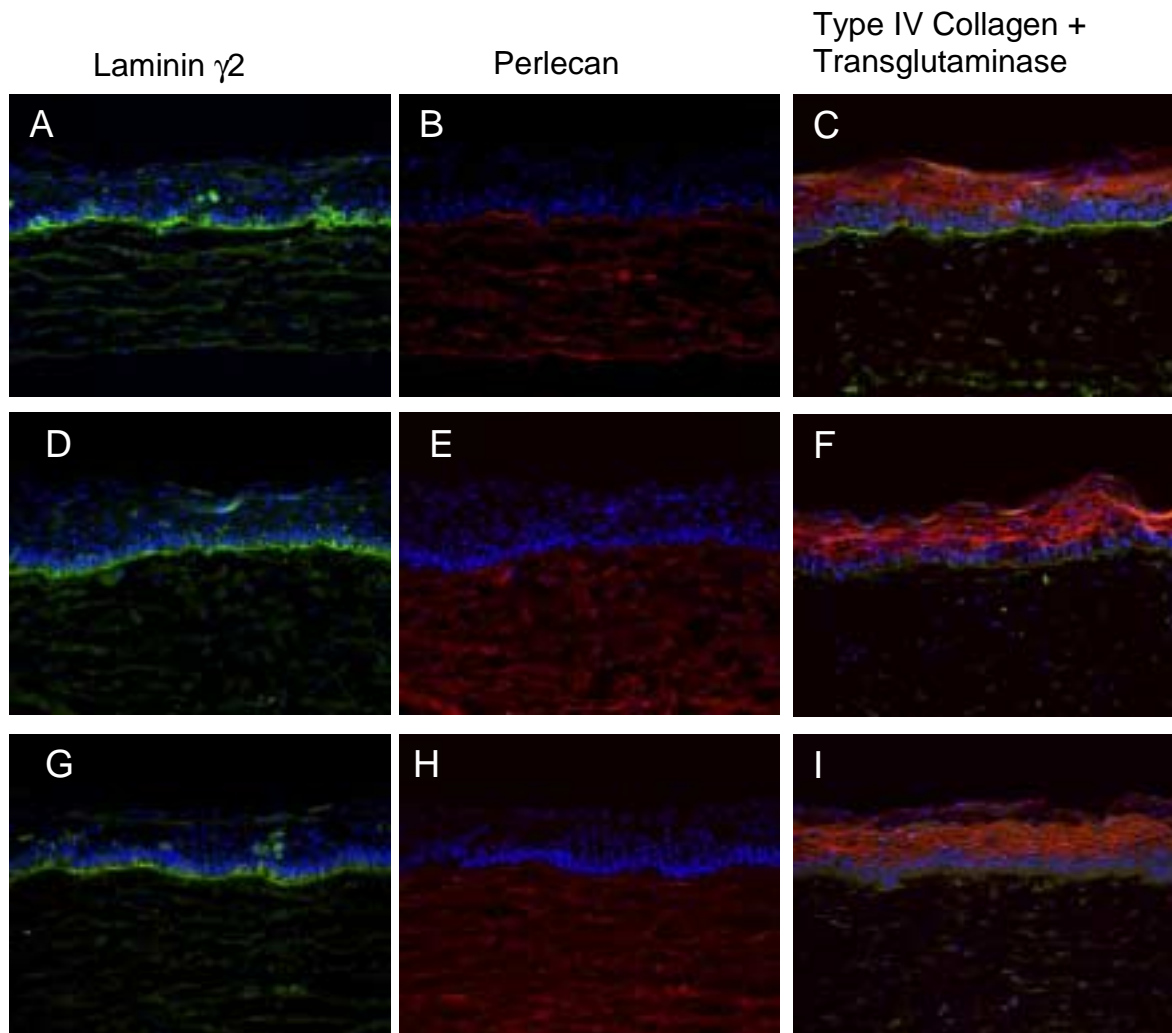


Fig. 5.36. Staining for laminin $\gamma 2$ (466), perlecan (640) and type IV collagen (495) together with transglutaminase (423) in 3D-cocultures treated with 40 $\mu\text{g/ml}$ Ly1f, day 12. Control: A, D, G, Ly1f day 7-12: B, E, H, Ly1f throughout the experiment: C, F, I. In J-L: Type IV collagen in green, transglutaminase in red.

The distribution of perlecan was closer analyzed with the confocal laser scan microscope (Fig. 5.37). Herein, the integrin $\beta 1$ chain was used as a marker for the basal cells and the interface. This revealed that perlecan could just be detected in very small amounts even at this high magnification. In contrast, the integrin $\beta 1$ chain was obviously apparently not influenced by the Ly1f treatment.

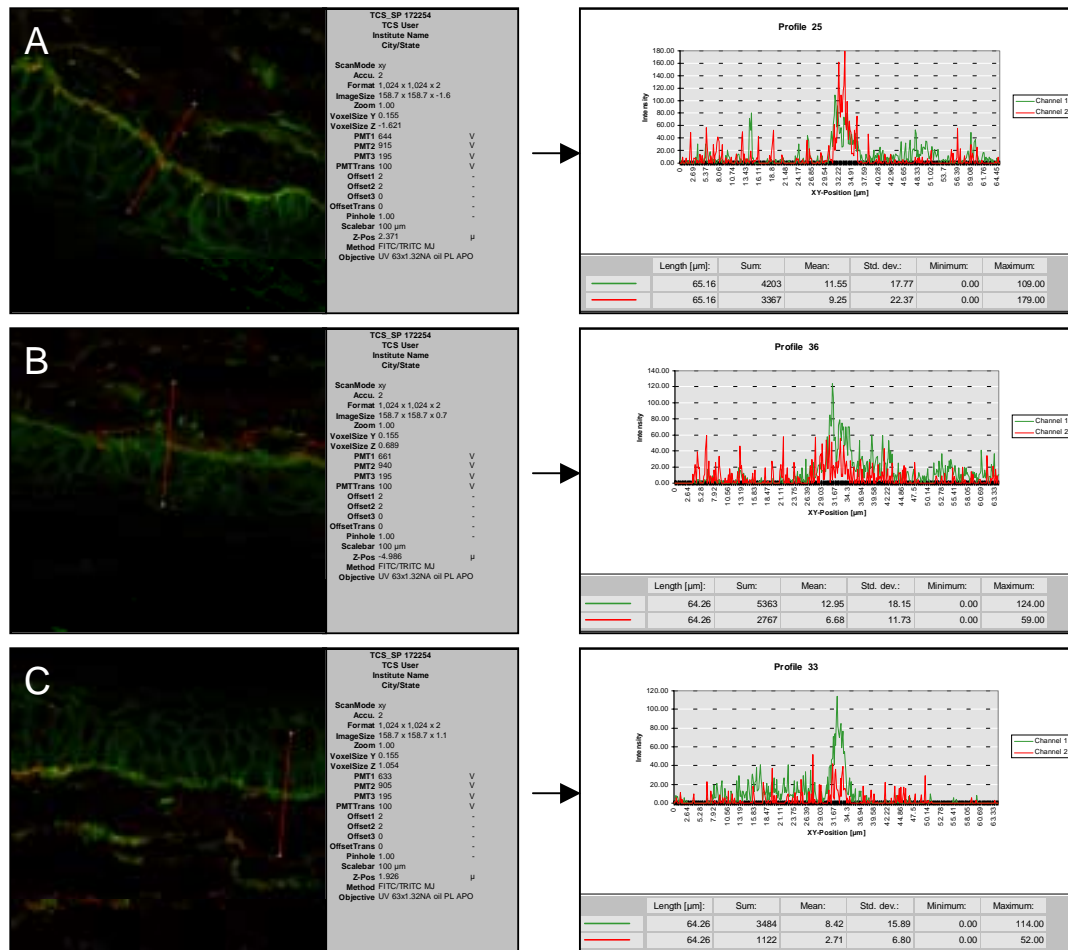


Fig. 5.37. Laser confocal analysis of the integrin β 1 chain (green) and perlecan (red) in 3D-cocultures treated with 40 μ g/ml $L\gamma$ 1f, day 12. Control: A, $L\gamma$ 1f day 7-12: B, $L\gamma$ 1f throughout the experiment: C. The red marker shows the measured area. In A,B the epithelium is oriented upside down.

Taken together, these results showed that the laminin-nidogen binding could be broken up in the 3D-coculture by the $L\gamma$ 1f fragment. $L\gamma$ 1f was competitively inhibiting this bonding and thereby breaking the connection between the laminin- and the type IV collagen meshwork. This led to abolished deposition of laminin 10, nidogen and perlecan in the 3D-cocultures, less deposition of type IV collagen, while the laminin-5 pattern remained unchanged. The integrin chain α 6 showed an irregular and less polarized deposition, while the integrin chain β 1 distribution pattern seemed quite normal. This implies a powerful feedback of the matrix scaffold on cell polarity and structure of the basal cell membranes.

The changes were also true for the 3D-cocultures treated from day 7 on, which indicate that initial basement membrane structures would vanish.

The complexity of regulation is illustrated by western blotting of laminin-10 and nidogen, which revealed that these components could be still found in the epithelia of treated 3D-cocultures, and that apparently no downregulation or proteolysis occurred. The abolishment of the basement membrane components were probably due to assembly problems as the nidogen-laminin bridge was missing, leading to a diffuse distribution. This suggests that the formation of the laminin-nidogen complex is an important event in the basement membrane deposition.

5-5. Blockade of basement membrane assembly in 3D-cocultures with HaCaT cells by a LN+4EGF fragment

All characterized laminin variants are heterotrimeric molecules formed by covalent bonds of one polypeptide from the α , β and γ laminin subunit families (see Introduction), each of which comprises multiple members encoded by individual genes (Maurer and Engel, 1996). Laminin $\gamma 1$ is one of the earliest laminin subunits found in development. Together with the $\alpha 1$ and $\beta 1$ subunits of laminin-1, it is expressed in the preimplantation embryo (Shim et al., 1996) before appearance of the first basement membrane of the trophectodermal epithelium (Dyadek and Timpl, 1985). Furthermore, $\gamma 1$ is the most ubiquitous laminin subunit, being present in 10 of the (until now) 12 known laminin isoforms (Burgeson et al., 1994). Laminin $\gamma 1$ is also present in laminin-10, the main laminin isoform in the adult skin basement membrane.

A strict heterologous dimerisation of the N-terminal-short-arm fragments of laminin-1 has been demonstrated using different tags (N. Smyth, unpublished results), which should apply for most of the $\gamma 1$ type laminins. In the test tube, self-assembly of $\gamma 1$ -type laminin has been successfully blocked by N-terminal fragments.

From these investigations, it could be assumed that laminin-10 builds its meshwork by repetitive trimerisation of the laminin monomers via their short arms. This was the basis for the following experiments; applying the N-terminal fragment LN+4EGF to the 3D-cocultures. Fig. 5.38 shows how this fragment was supposed to work; by binding to the N-terminal end of the $\gamma 1$ chain, the $\gamma 1$ chain should get disabled to bind to the $\alpha 5$ and $\beta 1$ short arms, thus preventing regular meshwork formation.

Finally, the LN+4EGF fragment was chosen because it would also interfere with other laminin isoforms such as laminin-1 and -2. These are found in similar skin models (Fleischmajer et al., 1998), and compensatory mechanisms of laminin-10 function could not be excluded. It also has to be noted that this N-terminal fragment did not contain the nidogen binding site.

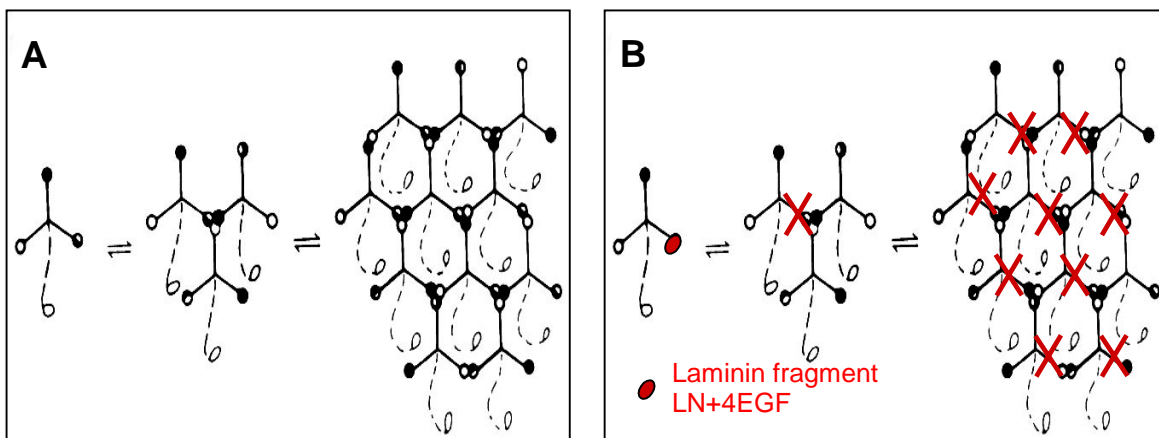


Fig. 5.38. A: The self-assembly of the laminin network (Yurchenco and Cheng, 1993). B: LN+4EGF breaking up the $\alpha 5\beta 1\gamma 1$ trimerisation by binding to the N-terminal end of the $\gamma 1$ chain

Practically, the fragment LN+4EGF was applied in a concentration of 10 µg/ml to the culture medium of the 3D-coculture 3 times a week, similarly to the Lγ1f fragment in chapter 5-4.

5-5.1 Epithelial development of LN+4EGF treated 3D-cocultures

As with the Lγ1f fragment, LN+4EGF did not seem to significantly influence the epithelial development in the treated 3D-cocultures. Fig. 5.39 shows that in the control 3D-coculture as well as in the treated 3D-coculture the basal cells were polarized forming a distinct basal zone. Further, the squamous and the granular layer were well developed and could be clearly distinguished. This showed that LN+4EGF did not influence the differentiation.

The only difference that could be observed between the controls and the LN+4EGF treated 3D-cocultures, was that in the LN+4EGF treated cultures, the cells slightly tended to invade the collagen gel. Nevertheless, this was not observed in all samples, and it could not be concluded if this was a significant incident.

In contrast to the experiments in chapter 5-2 and 5-4 where splitting of the epithelia from the collagen matrix occurred at high incidence, this was not the case in the 3D-cocultures treated with LN+4EGF. The treated cultures were just as stable as the controls.

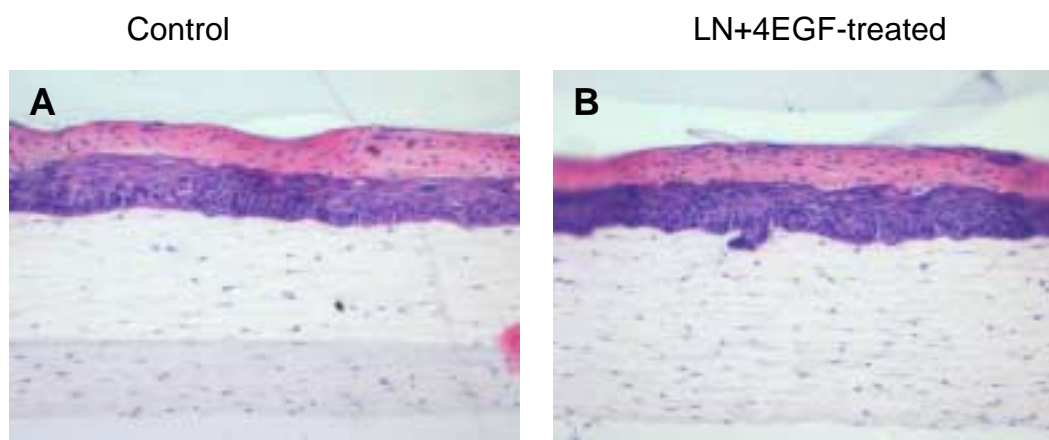


Fig. 5.39. Histology of 3D-cocultures after addition of 10 µg/ml LN+4EGF, day 12. A: Control, B: Addition of LN+4EGF

5-5.2 Effects of LN+4EGF on the deposition of basement membrane components

5-5.2.1 Laminin

Since LN+4EGF was supposed to interfere with a regular laminin-10 meshwork, the deposition of laminin-10, but also the other basement membrane components after treatment with LN+4EGF was investigated by immunofluorescence.

Fig. 5.40 shows the laminin-10 deposition in control 3D-cocultures and 3D-cocultures treated with LN+4EGF the whole experiment period (12 days). However, the addition of LN+4EGF did not seem to destroy the deposition of laminin in the 3D-cocultures. The staining of the laminin $\alpha 5$ - and $\gamma 1$ chain and EHS laminin was just as continuous in the treated cultures as in the control cultures staining.

The regular positioning of keratins K1/K10 indicated a normal basal cell layer in both samples.

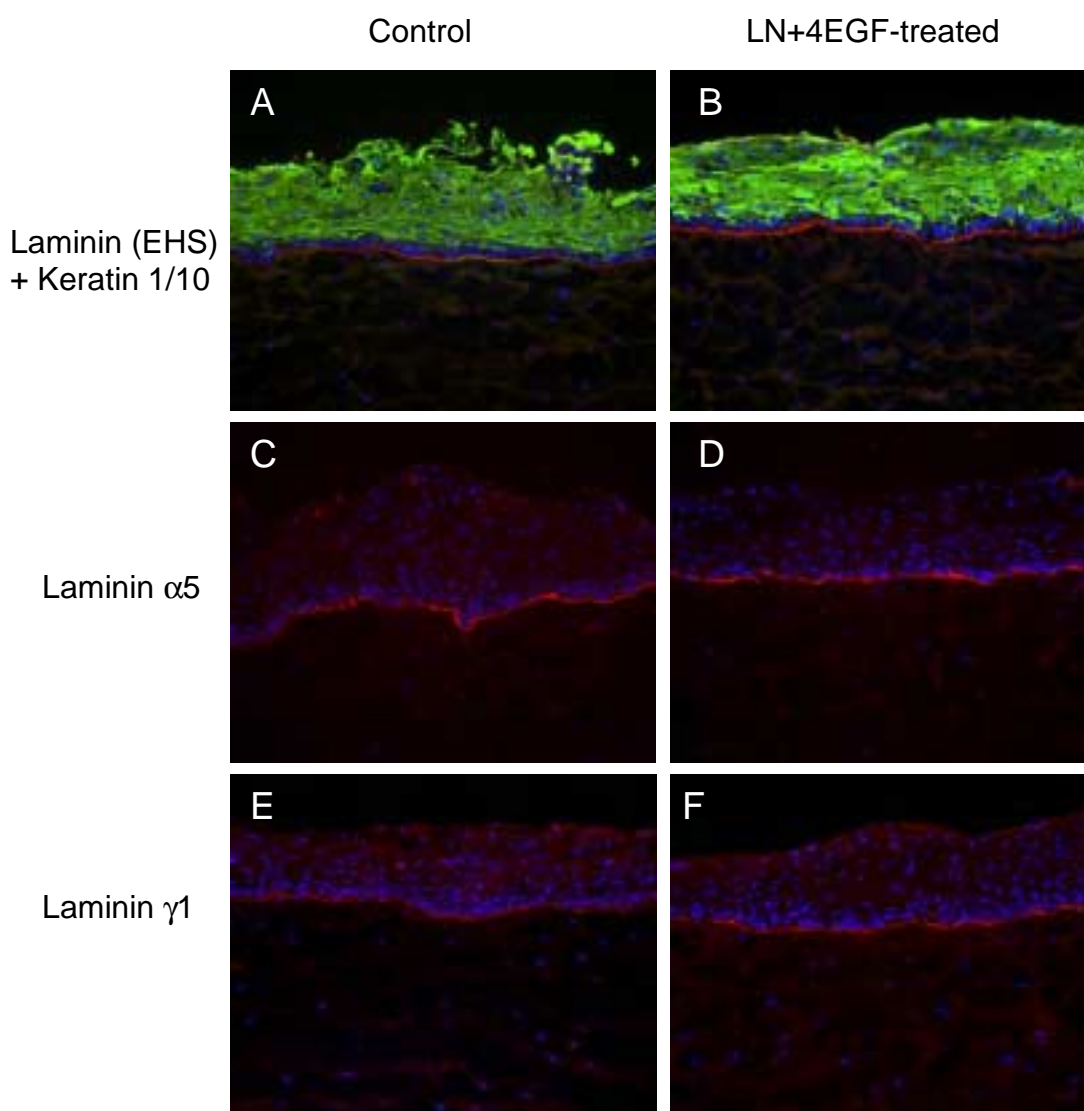


Fig. 5.40. Staining for EHS laminin (496), laminin $\alpha 5$ chain (723), $\gamma 1$ chain (528) and keratins K1/10 (638) in 3D-cocultures treated with 40 $\mu\text{g/ml}$ LN+4EGF, day 12. Control: A, C, E. Treatment with LN+4EGF: B, D, F. In A-B: Keratins K1/K10 in green, laminin (EHS) in red.

5-5.2.2 Other components of the basement membrane zone

The samples were stained for additional basement membrane components such as nidogen-1 and nidogen-2, laminin-5, type IV collagen and perlecan. Neither nidogen-1 nor nidogen-2 seemed to be influenced by the addition of LN+4EGF in the 3D-cocultures (Fig. 5.41 A-D) and their expression appeared fairly normal. This was also the case for laminin-5, represented by the laminin γ 2 chain (Fig. 5.41 E-F).

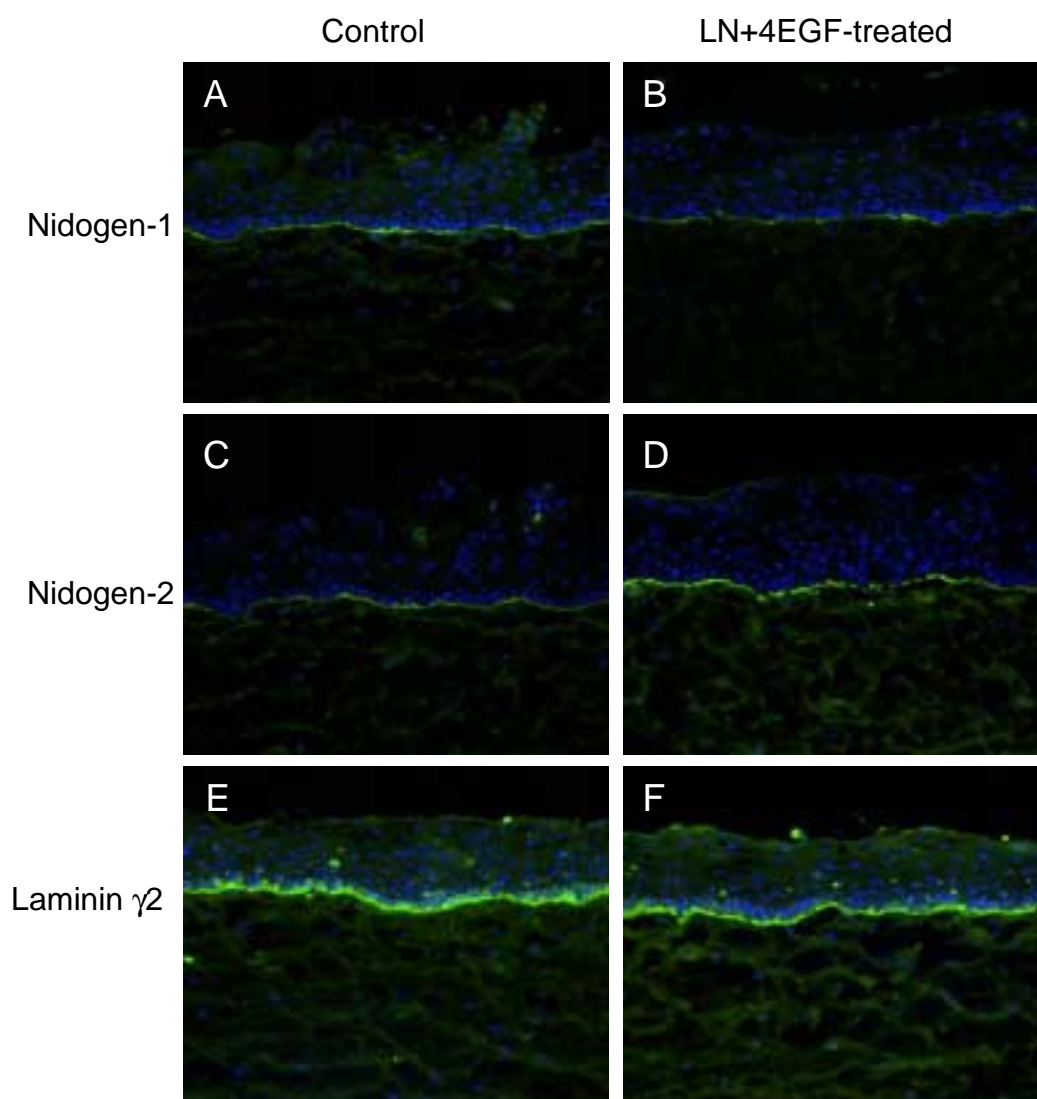


Fig. 5.41. Staining for nidogen-1 (416) and nidogen-2 (593), and laminin γ 2 (466) in 3D-cocultures treated with 40 μ g/ml LN+4EGF, day 12. Control: A, C, E. Treated with LN+4EGF: B, D, F.

Finally, deposition of perlecan, type IV collagen and integrin $\alpha 6$ were analyzed. Fig. 5.42 revealed that none of these components showed any irregularities in the distribution after addition of LN+4EGF.

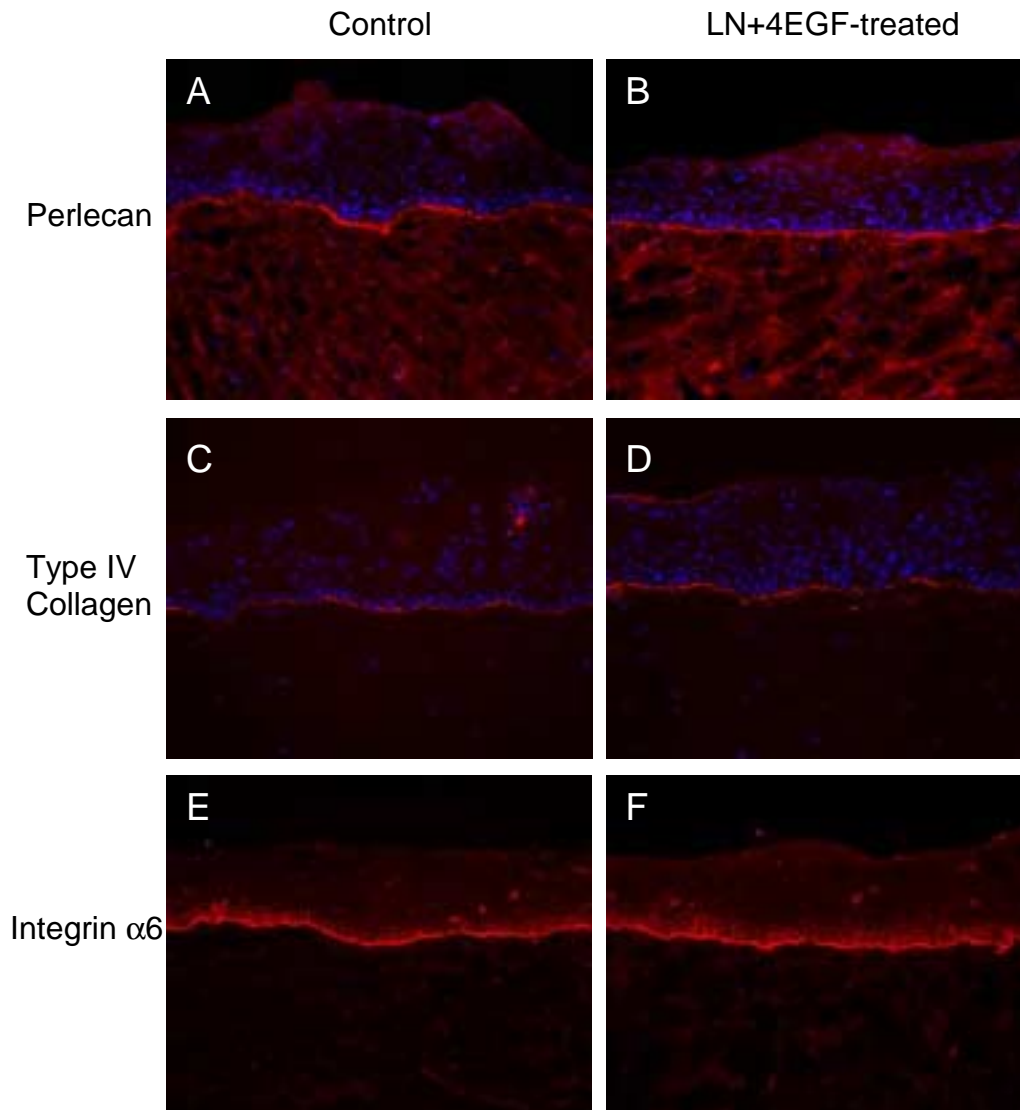


Fig. 5.42. Staining for perlecan (640), type IV collagen (495) and the integrin $\alpha 6$ chain (370) in 3D-cocultures treated with 40 $\mu\text{g}/\text{ml}$ LN+4EGF, day 12. Control: A, C, E. Treated with LN+4EGF: B, D, F.

In summary, the results from 5-5 showed that the attempt on breaking up the bonding between the laminin $\gamma 1$ chain and the laminin $\alpha 5$ and $\beta 2$ chain by the N-terminal laminin fragment LN+4EGF, did neither influence the expression and assembly of laminin-10, nor of the other basement membrane components.

No final answer can be given to these results. One possible explanation could be that the dimerisation between the $\alpha 5$ and the $\beta 2$ chain were sufficient to keep the laminin network stable in the 3D-cocultures, but since no effect was seen at all, it could also be that the affinity of the LN+4EGF fragment was too low to inhibit the binding.

In addition, a critical point could be the local concentration. Both laminin fragments applied here (chapter 5-4 and 5-5) had to diffuse from the medium compartment through the collagen matrix. But while $L\gamma 1f$ could react on the way with the nidogen from the fibroblasts, the LN+4EGF actually has to reach the dermo-epidermal junction interface to be effective. So the concentration gradient may get too low for a strong effect in the latter case.

6. DISCUSSION

6-1. The role of nidogen in basement membrane assembly

Nidogen-1 and nidogen-2 are highly conserved 160 kDa and 200 kDa proteins in mammals. It has been proposed that nidogen-1 connects the laminin and type IV collagen networks and integrates other proteins, including perlecan, into the basement membrane. Thus, nidogen-1 is believed to maintain a role in the structural integrity of the basement membrane. Nidogen-2 is typically enriched in endothelial basement membranes. Except for this, very little is known about the functions of nidogen in basement membranes.

In this study, the role of nidogen-1 and nidogen-2 in the development and assembly of the basement membrane in the skin was investigated. This was done in an *in vitro* 3D-coculture system, which closely resembles the situation in the skin. The advantage of this system over other models is the possibility to include cells from different species to a so-called heterogeneous system. In this study, nidogen deficient fibroblasts from nidogen knockout mice were used in the 3D-coculture system. Previous studies (Fleischmajer et al., 1995) had demonstrated that in skin, fibroblasts are the only source of nidogen. This made it possible to investigate consequences of nidogen deficiency in the skin-like system.

Knockout mice lacking nidogen-1 are viable, and homozygous animals appear phenotypically normal when compared to their heterozygous or wild-type littermates (Murshed et al., 2000). Furthermore, these mice do not show any overt anatomical abnormality and are fertile. In all tissues of nidogen null-mice, the staining for laminin-1 or perlecan was unchanged compared to wild-type mice, indicating a compensation of this deficiency presumably by nidogen-2. Thus, the staining pattern of nidogen-2 was more intense than normal and reminiscent of that of nidogen-1. These results were correlating to our 3D-cocultures. When nidogen-1 were lacking, the other basement membrane components were synthesized and assembled as long as nidogen-2 was present in the system. Nidogen-2 was also stronger expressed than normal in this situation. Miosge et al., 2002 concluded that nidogen-2 is the most obvious candidate for the compensation of nidogen-1 deficiency, as a three- to sevenfold increase in nidogen-2 was observed in heart and muscle of mice with nidogen-1 deficiency. Accordingly a corresponding increase was seen in the intensity of immunogold staining of these tissues.

These results could be fully confirmed in the *in vitro* system. Nidogen-2 was completely compensating for the nidogen-1 deficiency. When the situation was reversed and nidogen-2 deficient instead of nidogen-1, similar results were observed. In the 3D-coculture system, lack of nidogen-2 did not lead to changes in the basement membrane. This again resembled the situation *in vivo*. Nidogen-2 knockout mice were fully viable and no obvious histological changes or cellular architecture alterations could be observed (Schymeinsky et al., 2002). Furthermore, basement membranes appeared also normal by ultrastructure.

The results in the 3D-coculture system further demonstrated that a lack of both nidogen-1 and nidogen-2 lead to dramatic changes in the basement membrane assembly. When neither nidogen-1 nor nidogen-2 were present in the system, other important basement components were completely abolished. Laminin-10, type IV collagen as well as perlecan totally disappeared from the basement membrane zone. The total loss of these structures was confirmed by confocal laser scan microscopy and ultrastructurally analysis. It was assumed that this would have major consequences on the distribution of integrins as the major cell matrix-receptors. But the distribution of integrins $\alpha6\beta4$ and $\alpha3\beta1$ were just slightly irregular and not significantly influenced by the lack of nidogen.

Interestingly, these results do not completely correlate with the situation in the knockout mouse. Double knockout (nidogen-1 and nidogen-2) mice have been generated (Nischt et al., unpublished data). These transgenic mice had severe basement membrane defects in several organs and also skeletal malformations leading to neonatal death. However, the analysis of their skin basement membrane showed that all other components were present. This was in clear contrast to the in vitro findings. But compared to the in vitro model, skin represents a multifunctional, complex organ, which should be able to activate a variety of compensatory mechanisms. One striking difference is the more robust texture of dermal extracellular matrix in comparison to the soft collagen "hydro gel". Nevertheless, more recent, though still preliminary ultrastructural analysis of the skin from these mice, strongly suggest that the epidermal basement membrane is more fragile than first assumed (Mirancea, unpublished data).

Furthermore, double knockout fibroblasts grown on glass cover slips (not in the 3D-coculture system) synthesized some basement membrane components, such as EHS-laminin and type IV collagen. Because of this, it was assumed that these components had to be present in the 3D-coculture system at least at certain time-points. This indicated that the basement membrane components, which were missing could be either downregulated or degraded, or still present in the epithelia but not properly assembled onto the basement membrane zone. Immunoblot investigations of the epithelia showed that the basement membrane components laminin-10 and type IV collagen were present, but probably distributed all over the epithelia instead of on the basement membrane zone. An exception was laminin-5. The laminin $\gamma2$ chain was not detectable in the double knockout 3D-cocultures; this was actually indicating a proteolytic degradation of the chain. It is known that laminin-5 undergoes processing of its $\gamma2$ chain (Marinkovich et al., 1992b). Several proteases have been implicated in laminin-5 processing, e.g. matrix metalloprotease 2 (Gianelli et al., 1997) and membrane type I matrix metalloprotease (Koshikawa et al., 2000). But this is still under debate. Other studies have suggested that other groups of proteases are responsible for this cleavage, e.g. bone morphogenetic protein 1 (Veitch et al., 2003). Fact is that laminin-5 is quite sensitive for proteases, and as the assembly mechanism was disturbed in the nidogen knockout 3D-cocultures, the absence of the laminin $\gamma2$ chain could be possibly due to increased protease activity, including degradation in addition to the normal processing of the chain.

The other basement membrane components laminin-10 and type IV collagen were, as mentioned above, present in the epithelia also in the nidogen knockout 3D-cocultures approximately in the same amount as in the control cultures. This was not visible by immunofluorescence, because the components seemed to be freely

distributed in the epithelia instead of anchored to the lower basal surface. For perlecan, this could not be established, as it was not detectable in immunoblots due to lack of suitable antibodies. But it is quite probable that also perlecan was still present in the epithelia in the knockout cultures.

The above findings revealed that absence of nidogen led to complete collapse of the basement membrane assembly in the in vitro system. Laminin-10 and type IV collagen are both proposed to be cell-adhesive and able to self-assemble into networks (Mann et al., 1988, Aumailley et al., 1993, Yurchenco and Furthmayr 1984, Yurchenco and Schittny, 1990, Yurchenco et al., 1992), this being the basic skeleton of the basement membrane (Timpl and Brown, 1996, Kühn, 1994, Yurchenco and O'Rear, 1994). Interestingly, neither laminin-10 nor type IV collagen could accomplish this assembly as soon as nidogen was missing. From this, it was clear that nidogen must have more functions than just being a bridging molecule between these two networks. Furthermore we could not detect any hemidesmosomes, in contrast to the control cultures. Hemidesmosomes are asymmetrical structures that link cells to the underlying matrix. They are particularly found in epithelial tissues subject to mechanical stress, such as the skin (Cowin, 1994). Not even type II hemidesmosomes (Guo et al., 1995, Fontoa et al., 1999, Green and Jones, 1996, Nievers et al., 1999), representing immature or truncated forms (seen in regenerating epidermis or skin tumors), were present.

It has also been suggested that the basement membrane and nidogen might supply morphogenetic stimuli for development, tissue remodeling and epithelial differentiation (Ekblom 1993, Kleinman et al., 1993, Streuli, 1996). But this was not the case in the in vitro skin model. The nidogen deficiency did not lead to any morphological differences in the 3D-coculture. Thus, the epithelia developed normally in spite of lacking nidogen and basement membrane. These findings showed that an intact basement membrane might not have a direct role in the development of certain tissues such as the skin, but rather a maintenance or stabilizing function.

A direct approach to examine the role of nidogen in the basement membrane assembly was the supplementation of recombinant nidogen to the knockout 3D-cocultures. The question was whether added nidogen in the system would be able to bring the situation back to normal and rescue the entire basement membrane zone. Both recombinant nidogen-1 (mouse) and recombinant nidogen-2 (human) were able to completely restore the basement membrane in the knockout 3D-cocultures. There are four major points to be stressed in this rescue experiment. First, the recombinant nidogen was applied to the 3D-coculture media, and not directly on top of the epithelia. Thus, nidogen had to diffuse through the collagen gel up to the junctional zone to participate on basement membrane formation.

Secondly, the network of laminin-10 and type IV collagen and also perlecan, were fully restored into the basement membrane zone. In addition, the regular pattern of laminin-5 was restored. Together with the synchronous early appearance of nidogen and laminin-5 in cell transplants or regenerating epithelia (Larjawa et al., 1993, Breitzkreutz et al., 1997), this strongly suggests a functional connection.

Third, exogenous nidogen also led to formation of hemidesmosomal components. It is not known whether nidogen has an influence on the formation of hemidesmosomes, but these findings strongly suggest a correlation between nidogen deficiency and lack of hemidesmosomes. We could observe that the tendency of the epithelia to detach from the collagen gel seen in the knockout 3D-cocultures was reduced as

soon as nidogen was added to the culture. This was obviously due to the formation of hemidesmosomes.

The fourth interesting finding the “rescue” revealed was that it did not matter whether recombinant mouse nidogen-1 or recombinant human nidogen-2 was added to the 3D-cocultures. Both nidogen types were able to fully restore the basement membrane and the anchoring elements in the skin model. Taken together, these data support the hypothesis from the different studies concerning the complementary functions of the two nidogens (Salmivirta et al., 2002, Miosge et al., 2002).

In this context, the binding activities of recombinant human nidogen-2 have been extensively studied, also in comparison to mouse- and human nidogen-1 (Kohfeldt et al., 1998). This revealed nearly identical binding affinities for type IV collagen and perlecan, but a 100-1000 fold lower binding affinity to the mouse laminin γ 1 chain (Mayer et al., 1993, Pöschl et al., 1994). The laminin γ 1 (laminin-10) in the 3D-coculture model should be mostly produced by the human keratinocytes, since it was only detectable in the epithelial fraction by western blotting. This is in accordance with most studies suggesting that basement membrane laminins are mainly of epithelial origin, although both epithelial and mesenchymal cells can apparently express or synthesize laminin (Simo et al., 1992, Thomas and Dziadek, 1993, Marinkovich et al., 1993, Fleischmajer et al., 1993, Smola et al., 1994, Fleischmajer et al., 1998). The binding activity of human nidogen-2 to human laminin γ 1 is supposed to be even lower due to one critical amino acid exchange, while the γ 1 chain binding domain is identical in mouse and human laminin (Pikkarainen et al., 1988). However, unlike in the mouse, both human nidogens are found in comparable amounts in tissue extracts (Kohfeldt et al., 1998) suggesting some natural compensation for the binding deficiency by increased production of nidogen-2. This was probably also the case in our system, as both nidogen-1 and nidogen-2 were added in excess. The excess of nidogen-2 probably compensated for its lower binding activity to laminin and could in spite of this restore the basement membrane.

Taken together, these investigations on the role of nidogen deficiency in the 3D-coculture system raised some interesting suggestions for the functions of nidogen-1 and nidogen-2 on the basement membrane development in the skin. The results showed clearly that neither laminin-10 nor type IV collagen could regularly self-assemble when nidogen-1 or nidogen-2 was absent. Hemidesmosomes and anchoring fibrils were also not detectable. The fact that all these components reappeared in the presence of either exogenous nidogen-1 or nidogen-2, indicates that both nidogens might have additional functions than only being a bridging molecule in the basement membrane.

Molecular interaction studies have already pointed out the binding of nidogen to laminin should be a key event in basement membrane formation (Fox et al., 1991, Aumailley et al., 1989, Mayer et al., 1993, Pöschl et al., 1996). The presented data suggest that nidogen acts as a kind of coordinator in the basement membrane assembly. Thus, there is clear indication that without nidogen, the basement membrane components lose their ability to self-assembly and form an epidermal basement membrane under more stringent conditions than in the skin.

6-2. The role of perlecan in the basement membrane assembly

Perlecan, the large heparan sulfate proteoglycan (400 kDa) has been shown to be present in all basement membranes including the skin. Ultrastructurally, the core protein of perlecan has been shown to reside primarily within the lamina densa decorating occasional anchoring filaments as well as overlying hemidesmosomes (Fine, 1994). In addition, differences in the ultrastructural localization of perlecan, which are dependent on the age of the organism, have been reported. For example, a much more discrete basement membrane localization has been noted in adult skin in comparison to neonatal foreskin.

A number of in vitro studies suggest multiple functions of perlecan in cell growth, differentiation and tissue organization. The molecular interactions include binding to the basement membrane proteins laminin (γ -isoforms), type IV collagen and nidogen, but also other extracellular matrix proteins such as fibulin and fibronectin (Hopf et al., 1999). Nidogen-perlecan binding studies have revealed protein interactions at atomic resolution, which are supposedly involved in the basement membrane assembly (Kvansakul et al., 2001). Perlecan binds with its domain IG3 to the G2 globular region of nidogen-1 (Reinhardt et al., 1993, Hopf et al., 2001). However, the exact role of perlecan in the skin basement membrane is so far not fully understood.

For functional studies on perlecan interactions with the other basement membrane components, the 3D-coculture seemed to provide an ideal system. In contrast to nidogen, which is exclusively produced by the mesenchymal cells, the cell source for perlecan in the basement membrane has not been determined. Therefore, more approaches to analyze this were carried through, including perlecan knockout fibroblasts from mice and an antisense expressing perlecan mRNA HaCaT cell line.

Perlecan knockout mice have been generated (Costell et al., 1999). While the heterozygous phenotype appeared normal, not displaying any overt anatomical or behavioral abnormalities, the homozygous phenotype died around embryonic day 17 due to hemopericardium and heart arrest. A few mutants survived, but had developed severe brain and skeletal defects. Nevertheless, with the exception of perlecan, all basement membrane components were similarly expressed in mutant tissue.

This correlated to the biosynthetic potential of the knockout fibroblasts. Growing on glass cover slips, these fibroblasts showed that all fibroblast genotypes were able to produce basement membrane components, except perlecan in the case of the homozygous phenotype. This indicated that lack of the ability to produce perlecan did not influence the expression of the other basement membrane components.

However, when the perlecan null fibroblasts were integrated into the 3D-coculture system, again all basement membrane components were expressed at the basal zone, including perlecan as well. In addition, the development of the epithelia was fairly normal, displaying the normal differentiation markers. Since those fibroblasts were definitely unable to express perlecan, it was clear that perlecan had to be synthesized exclusively by the keratinocytes in the system.

To prove the reverse, if fibroblasts could provide the necessary perlecan for the basement membrane, HaCaT cells expressing antisense perlecan mRNA were employed. Also in these 3D-cocultures, the basement membrane components

laminin-10, nidogen and type IV collagen were synthesized and deposited on the basal zone together with perlecan. Furthermore, these cultures developed well differentiated epithelia. Taken together, this showed that the epithelial cells as well as the mesenchymal cells could produce enough amounts of perlecan to supply the basement membrane formation and eventually the epithelial development. Interestingly, no cooperation seemed to be necessary.

Perlecan was also thought to be important for basement membrane assembly and function (Timpl, 1993, Timpl and Brown, 1996). Studies have shown that perlecan interacts with several basement membrane components, adhesion molecules involved in basement membrane assembly, and growth factors (Timpl, 1993). In spite of this, studies on the knockout mice showed that most basement membranes form in the absence of perlecan and appear morphologically normal, which was underlined by ultrastructural investigations on the skin (Costell et al., 1999).

As it seemed impossible to combine the perlecan knockout fibroblasts and the antisense expressing perlecan mRNA HaCaT cells in the 3D-cocultures system, it could not be clearly establish if this was also the case in the skin model. Because of SV-40 transfection, the perlecan deficient fibroblasts had a rather endothelial phenotype and grew faster than normal fibroblasts. In contrast to the situation in the culture dish, the growth of normal fibroblasts are controlled in the collagen environment of the 3D-coculture, and overgrowth do not occur. This was not the case for the SV-40 fibroblasts. Nevertheless, the approaches to combine the perlecan deficient cell types revealed that the basement membrane components nidogen and laminin-10 were expressed in the basal zone, in spite of poorly developed epithelia due to over-growth of the fibroblasts,.

This was pointing in the direction that perlecan might not be of great importance for the basement membrane formation, at least not in the skin. Perlecan seems to be crucial in the cartilage development and bone formation (Arikawa-Hirasawa et al., 2002, Costell et al., 1999), and also for localizing acetylcholine esterase at the neuromuscular junction (Peng et al., 1999, Arikawa-Hirasawa et al., 2002, Forsberg et al., 2001). In contrast, the in vitro findings together with the situation in the knockout mice, imply that the formation and assembly of the skin basement membrane is not much influenced by the perlecan loss. A possible explanation could be that other heparan sulfate proteoglycans substitute, at least partially, for the loss of perlecan.

From this one may conclude that besides a minor role in structure, the major role of perlecan in basement membrane is to attract growth factors and modulate their availability, for example a fast release in the wound situation (as wound response).

6-3. The importance of the nidogen-laminin complex in basement membrane assembly

Laminin has been shown to be one of the prerequisites for the in vivo production of the basement membrane. Lack of the laminin $\gamma 1$ chain after targeting the LAMC1 gene in mice alters the formation of all known integral basement membrane laminin isoforms and leads to embryonal death (Smyth et al., 1999). Laminin chains $\gamma 1$ and $\beta 1$ were the first network-forming component detected during early embryonic development (Cooper and Mc Queen, 1983, Dziadek and Timpl, 1985), presumably

being a part of the initiation complex of the basement membrane assembly (Aumailley et al., 2000).

The two major networks of the basement membrane, laminin and type IV collagen are supposed to be connected by nidogen-1, as already outlined in chapter 6-1. Nidogen-1 connects to the laminin $\gamma 1$ chain of laminin-10 and this leads to the formation of a stable, equimolecular complex (Paulsson 1987b, Brown et al., 1994, Lindblom et al., 1994). The complex results form a high affinity interaction, $K_d = 0,5$ nM, between one LE motif in domain III of the laminin $\gamma 1$ chain, $\gamma 1III4$, and the carboxy-terminal G3 domain of nidogen (Fox et al., 1991, Mayer et al., 1993).

One major goal of this work was to elucidate the importance of this interaction in skin. Thus, the consequences of the interference with laminin-nidogen binding for the assembly and stability of the basement membrane were investigated in the 3D-coculture system. For this purpose, a recombinant fragment of the laminin $\gamma 1$ chain was applied ($\gamma 1III3-5$, named $L\gamma 1f$), which harbors the binding site for nidogen-1. According to binding studies, the $\gamma 1III4$ motif was supposed to inhibit the interaction between nidogen-1 and laminin-10 with high efficiency.

The outcome was that the addition of $L\gamma 1f$ to the 3D-coculture system efficiently blocked the formation of a basement membrane. Immunofluorescence and confocal laser scan analysis showed that $L\gamma 1f$ affected the majority of the basement membrane components like nidogen-1 and nidogen-2, laminin-10, type IV collagen and perlecan when added from the beginning of the culture time. $L\gamma 1f$ was also able to break up an already existing basement membrane when added at a later time-point. Previous experiments with normal keratinocytes instead of HaCaT cells (Breitkreutz et al., submitted 2003) have shown that in addition to the complete absence of basement membrane structures, also any recognizable elements of hemidesmosomes or intermediate forms were abolished. There was no linkage of keratin filaments to the basal cells. In spite of this, the epithelial development was maintained, including epithelial stratification and complete differentiation, which was also the case seen in the HaCaT cell 3D-coculture.

Comparable to the situation with the nidogen knockout fibroblasts in 3D-cocultures in chapter 6-1, in the $L\gamma 1f$ –treated 3D-cocultures the matrix-epidermal junction appeared more fragile, but adhesion was obviously still sufficient to allow regular growth and epithelial stratification. Although the frequently observed separation of the tissue compartments presumably happened mainly during sample preparation and processing, it certainly reflects weaker epithelial attachment to the extracellular matrix. This mimics blister formation under tension in skin of patients suffering from epidermolysis bullosa forms with severe defects in specific basement membrane molecules (Burgeson and Christiano, 1997, Pulkkinen and Uitto, 1999, McGowan and Marinkovich, 2000).

As mentioned above, staining patterns of laminin-10, nidogen-1 and nidogen-2, type IV collagen and perlecan seen by immunofluorescence disappeared in the $L\gamma 1f$ treated 3D-cocultures. This was further confirmed by laser confocal microscopy and partly ultrastructural analysis by electron microscopy. However, the components were detected associated with the epithelial fraction by western blotting. This revealed that laminin-10 and type IV collagen were not able anymore to self-assemble and were not deposited within the basal zone. Furthermore, nidogen and perlecan were also

redistributed in the epithelia instead of anchoring to the basal surface. This was correlating to what was seen in the experiments with nidogen knockout fibroblasts in chapter 6-1. Other studies using antibodies raised against the nidogen-binding site of laminin-1 (Ekblom et al., 1994, Kadoya et al., 1997), and F9-teratocarcinoma-cell-derived embryoid bodies in the presence of recombinantly expressed nidogen binding sites localized within the γ 1III3-5 laminin fragment (Tunggal et al., 2002), also revealed disrupted basement membranes. Tunggal et al. further reported the profound redistribution of nidogen-1 and laminin in the embryoid bodies rather than loss of the proteins, well correlating to the findings in the 3D-coculture model.

Disruption or weakness of the basement membrane has been a consistent effect seen upon the loss of other basement membrane components and matrix receptors, although only the lack of laminin γ 1 or the integrin β 1 chain have led to complete absence of 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 deficient mice. Laminin α 1 chain expression, which is required for laminin secretion, is regulated by β 1 integrin (Aumailley et al., 2000, Li et al., 2002). Hence, basement membrane defects in the absence of β 1 integrin might be actually due to the lack of laminin rather than to abnormalities in the assembly process. The nidogen-blocking experiments in our model or those derived from the F9-teratocarcinoma-cell-derived embryoid bodies, together with the antibody perturbing experiments, strongly suggest that the nidogen-1 binding to laminin plays a crucial 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), which also correlates to developmental stages. For example, in the six-day-old mouse embryo, although a classical basement membrane has formed under the ectoderm in the extraembryonic egg cylinder, within the embryo itself the basement membrane is unstructured, even though laminin-1 is deposited in a linear pattern (Misoge et al., 1993). In fact, only in the fully developed basement membrane was nidogen-1 found by electron microscopy. This suggests that in the early embryo nidogens might play a role in the maturation of basement membranes (Miosge et al., 2000), while not being crucial for embryonic survival. Thus, other molecules might compensate for nidogen deficiency.

In contrast to laminin-10, nidogen-1 and nidogen-2, laminin-5 was just moderately affected and showed almost regular staining in the immunofluorescence analysis upon treatment with L γ 1f. Although it was less ordered than normal, compared to the other basement membrane components it was clearly detectable in the basal zone. This was also contrary to the findings in the 3D-cocultures with the nidogen knockout fibroblasts in chapter 6-1, where analysis of the laminin γ 2 chain revealed that laminin-5 was completely abolished. Nevertheless, it cannot be excluded that the epitopes for recognition by immunofluorescence or western blotting were lost. This issue needs certainly further investigations.

Laminin-5 is known as the major keratinocyte adhesion ligand and is found in the lamina lucida subregion of the basement membrane (Spirito et al., 2001, Rousselle and Aumailley, 1994). It is specifically associated with the extracellular anchoring filaments that connect the hemidesmosomes to the anchoring fibrils of the underlying matrix (Rousselle et al., 1991, Verrando et al., 1987). The biological relevance of laminin-5 in dermal-epidermal adhesion has been demonstrated by the identification

of genetic mutations affecting laminin-5 in patients with junctional epidermolysis bullosa, an inherited geno-dermatosis characterized by fragility of the skin and blistering in response to minor trauma (Aberdam et al., 1994, Pulkkinen et al., 1994). Several studies have been done on laminin-5 and its functions in the dermal-epidermal junction. It has been reported to influence morphogenetic events (Baker et al., 1996, Stahl et al., 1997), to affect cell motility (Zhang and Kramer 1996, O'Toole et al., 1997), to enhance invasiveness in human cancers (Kikkawa et al., 1994, Pyke et al., 1994, Giannelli et al., 1997), and to control cell growth (Ryan et al., 1999).

All these findings demonstrate the essential role of laminin-5 in the dermo-epidermal junction and basement membrane function. The more interesting it is, that in our model, laminin-5 is strongly affected by complete lack of nidogen (chapter 6-1), but just moderately affected by the break-up of the laminin-nidogen binding. In both cases, nidogen and laminin-10 are completely lost from the basement membrane zone. Even if the effect would be due to excessive proteolytic processing and not complete loss of laminin-5, this would completely change its functions. Laminin-5 has been reported to appear very early at the epidermal junction in a transplantation model, immediately followed by nidogen and by far preceding the deposition of laminin and type IV collagen (Breitkreutz et al., 1997, Breitkreutz et al., 1998). One possible explanation for the different laminin-5 patterns in vitro, could be that in the first case, the early laminin-5 deposition is disturbed because of the complete lack of nidogen, which may result in extensive laminin-5 degradation. In the second case, as laminin-5 occur before the nidogen-laminin complex formation and laminin-10 assembly, laminin-5 deposition might be disturbed to a lower degree, due to pronounced tissue polarity.

Laminin-5 is also proposed to mediate epithelial cell adhesion via integrin $\alpha 3\beta 1$ in focal adhesions and via integrin $\alpha 6\beta 4$ in hemidesmosomes (Carter et al., 1991, Sonnenberg et al., 1993, Mainiero et al., 1995, Baker et al., 1996, Goldfinger et al., 1998). It has been suggested that monomeric laminin-5 molecules within the anchoring filaments bridge integrin $\alpha 6\beta 4$ to type VII collagen, thereby providing cohesion of the dermis and epidermis, whereas laminin-5 complexed to laminin-6 or laminin-7, interacts with integrin $\alpha 3\beta 1$, contributing to basement membrane assembly and stability (DiPersio et al., 1997, Rousselle et al., 1997).

The immunofluorescence staining for integrins revealed significant changes in the distribution of the integrin $\alpha 6$ chain, while the $\beta 1$ chain remained undisturbed in the $\text{Ly}1\text{f}$ treated 3D-cocultures. Thus, the closer examination with confocal laser scan microscopy showed clearly a redistribution of the integrin $\alpha 6$ chain from a preferentially basal position to a more pericellular localization. Since both laminin-5 and integrin $\alpha 6\beta 4$ are integrated into hemidesmosomes, integrin $\alpha 6\beta 4$ as part of the inner plaque and laminin-5 as part of the hemidesmosome-anchoring filament complex, the disturbance of these components was one possible explanation for the increased epithelial splitting in $\text{Ly}1\text{f}$ treated 3D-cocultures.

In summary, these findings demonstrate that the blockade of nidogen binding to laminin-10 by $\text{Ly}1\text{f}$ has far-reaching consequences for the formation and maintenance of the whole junctional zone. They further indicate that the laminin-nidogen complex represents an integrated functional unit, which actually links the internal keratin cytoskeleton with the dermal extracellular matrix.

Furthermore, these results provide strong evidence that apart from its bridging properties, the interaction of nidogen-1 with laminin-10, possibly in a cell-bound complex, may be an initially predominating event in basement membrane formation.

6-4. The laminin network and the basement membrane assembly

Until now, one of the most carefully investigated laminin isoforms has been laminin-1. Laminin-1 was first discovered of the laminins and it is first expressed in the developing embryonic basement membranes (Tunggal et al., 2000). Laminin-10, which is found in adult skin basement membranes, and laminin-1, have the $\gamma 1$ and the $\beta 1$ chain in common. Self-association in vitro and formation of hexagonal networks, independent of the presence of type IV collagen networks (Yurchenco et al., 1982, 1992) was first shown for laminin-1, but should apply to the other laminins with homologous domain organization, including laminin-10.

The basic principle of self-assembly is a strictly heterologous di- and trimerisation of the three different laminin chains, α , β and γ . This polymerization process is reversible and concentration- and divalent cation-dependent; it requires the three N-terminal LN domains to give a Ca^{2+} -dependent gelation (Paulsson, 1988, Yurchenco et al., 1985) and can be inhibited in vitro by the presence of excess free short arm fragments (Schittney and Yurchenco, 1990, Yurchenco and Cheng, 1993).

In order to investigate the role of the laminin network in basement membrane formation in a more physiological context, we took advantage of these studies and added the laminin $\gamma 1$ short arm fragment LN+4EGF to the 3D-coculture system. This fragment binds to the N-terminal end and thereby inhibits the binding interaction between the α and β short arms and the regular $\gamma 1$ chain. The goal was to inhibit laminin polymerization with an excess of this short arm fragment and disturb basement membrane assembly.

However, the LN+4EGF treated 3D-cocultures did not display any significant abnormalities compared to the control cultures. A stratified, squamous epithelium developed, as already seen in the other manipulated cultures in chapter 6-1 and 6-3, which was to be expected. The only notable difference was that the epithelial cells in the treated cultures showed some tendency for invasion into the gel. However, this observation has still to be confirmed to draw any major conclusions.

Nevertheless, the general outcome was that the control- and the treated cultures showed no difference in the expression and deposition of the basement membrane components.

Thus, the immunofluorescence analysis of the LN+4EGF treated 3D-cocultures revealed normal deposition of the laminin network, type IV collagen, nidogen and perlecan, in addition to laminin-5 and integrin $\alpha 6\beta 4$ and $\alpha 3\beta 1$. The staining was continuous for all components and the treated cultures showed no deficiencies compared to the controls in this regard.

Previous studies have shown that laminin indeed should be necessary for basement membrane formation, at least in embryoid bodies. Smyth et al., 1999 reported, after targeting the LAMC1 gene coding for the laminin $\gamma 1$ chain, that the laminin $\gamma 1$ chain was necessary for laminin assembly and that laminin in turn was necessary for the organization of the other basement membrane components. In fact, in the absence of the $\gamma 1$ chain, differentiating embryo stem cells produced no covalently bonded

laminin subunits. The connection between the laminin subunits and the secretion and assembly has also been reported in other studies (Peters et al., 1985, Hunter et al., 1990, De Arcangelis et al., 1996, Yurchenco et al., 1997).

However, in our system the laminin network was targeted in a different way, and unfortunately, the attempt to break up the network was not successful. The fact that laminin was still present indicated that a network could form without the heterodimerisation between the three different laminin chains. Our results may indicate that the binding between one α chain and one β chain could eventually be sufficient to build a laminin network and that a double binding instead of a triple binding could keep the network stable. But in addition, the presence of an ordered tissue assembly should enable a regular array of laminin molecules by the attachment to the basal cell surface by the C-terminal globular region. The fact that it is possible to break up the laminin network in the test tube with excess short arm fragments, may not be transferable to the 3D-coculture system, which is more complex than other in vitro cell systems. However, it can also not be excluded that the affinity of LN+4EGF in the system was not sufficient, or that the local concentration in the 3D-coculture was too low due to diffusion problems.

On the other hand, the observed increased tendency to invasion could be possibly explained with this dimer-binding model. These weakened domains in the basement membrane may lower cell-matrix interactions and cell polarity, and in turn, activate the migratory machinery of the keratinocytes.

Taken together, the attempt to disrupt the laminin network showed no visible influence on the basement membrane formation, probably because the network still existed, being held together by the binding between one α chain and one β chain, while the whole individual laminin molecules stay in place by anchorage to the basal cell membrane.

In conclusion, the findings described in chapter 6-1 – 6-4 demonstrate that the 3D-coculture system provides a model with a defined input and output, which allows specifically dissecting distinct steps in the complex assembly process of the basement membrane in the skin. This simplification avoids also all possible, but hardly predictable, responses of the host faced by in vivo experiments.

Despite some discrepancy to developmental studies, an important role in the basement membrane assembly can be postulated for nidogen. Based on the presented skin-organotypic model, there is strong evidence that nidogen acts as a molecular adapter facilitating binding of laminin to cells or as a coordinator for the initial polymerization of the basement membrane components. The mutual replacement of the isoforms nidogen-1 and nidogen-2, although nidogen-2 exerts a much lower affinity to laminin, could indicate a coordinated interaction of nidogen and laminin on defined cell surface areas. This could also explain the coordination of the synchronous appearance of nidogen and laminin-5 for preceding basement membrane assembly, while absence of nidogen also severely disturbed the laminin-5 localization.

Furthermore, the laminin-nidogen complex represents an integrated functional unit being crucial for the dermal-epidermal stability and an initially predominant event in the basement membrane assembly.

Perlecan on the other hand, might not play such an essential part in the structural basement membrane assembly, being possibly substituted for by analogue proteins, but have a more regulatory function in the growth factor scenario. Although supporting data are not at hand yet, these options for the role of perlecan is feasible by defined supplements in this 3D-coculture model.

7. SUMMARY

The basement membrane of the skin and its main components (laminin, nidogen, type IV collagen and perlecan) are involved in numerous biological functions. In this study, the basement membrane development and assembly and the interactions between the single components, were investigated in a skin-organotypic 3D-coculture model. This in vitro system consisting of epidermal cells (or HaCaT cells) growing on a type I collagen matrix with incorporated fibroblasts, closely resembles the in vivo situation of the skin. This approach makes it possible to generate knockout situations of specific components, targeting either one or both tissue compartments, by incorporating cells from transgenic animals or transfected human cells.

Nidogen deficiency turned out to be fatal for the basement membrane formation in this skin model. When both nidogen-1 and nidogen-2 were absent, the other basement membrane components laminin-10, type IV collagen, and perlecan were unable to assemble and failed to form a distinct basement membrane zone. Also the epithelial anchoring complexes, the hemidesmosomes, did not develop in basal cells facing the collagen matrix. In addition, the laminin-5 assembly was clearly disturbed. Although these proteins were mostly present and detectable in extracts of the epidermal or dermal tissue compartment with no sign of any major degradation, they could not be assigned to defined locations. Application of recombinant nidogen-1 or nidogen-2 to the deficient 3D-cocultures fully restored the basement membrane, underlining the importance of nidogen in this process. These results suggested that nidogen might act as a molecular adapter facilitating binding of laminin to basal cells, or catalyze the initial polymerization steps of the basement membrane components.

Another approach to disturb the basement membrane formation was to inhibit nidogen binding to laminin which was accomplished by a recombinant laminin γ 1-fragment spanning the nidogen binding site. This further emphasized the importance of this complex in the assembly process. When the laminin-nidogen interaction was prevented, neither the laminin-10 network (nor type IV collagen, with HaCaT cells) nor other associated proteins such as perlecan were detectable in the dermo-epidermal junction,. Also in this case, the proteins were still present and detectable in extracts of the respective tissue compartments. Moreover, the integrin α 6 β 4 polarity and consequently the hemidesmosomal adhesion complexes were lost in the absence of a normal basement membrane formation.

In contrast, an attempt to disturb the laminin network by inhibiting the three-arm polymerization process of laminin had no obvious effect on the basement membrane. Based on the classical model of heterotypic binding of laminin-10 short arms by the N-terminal of the α 5, β 1, and γ 1 chains, a recombinant N-terminal γ 1 fragment was used. However, upon treatment in 3D-coculture laminin-10, type IV collagen, nidogen-1, nidogen-2 and perlecan were normally expressed and assembled. This was probably due to sufficiently stable laminin network formed by the available laminin short arms, not blocked by the peptide, and the maintained regular laminin configuration at the epithelial interface due to stable binding by integrins.

Finally, perlecan deficiency in either cell type, keratinocytes or fibroblasts did not interfere with basement membrane assembly. Herein, a HaCaT clone stably expressing perlecan antisense RNA and SV40-immortalized fibroblasts from perlecan knockout mouse embryos were employed. Thus, with perlecan from either source in the system, the other basement membrane components did not display any disturbance in expression or location. This confirms the findings of the nidogen experiments; the location of synthesis of matrix molecules can be quite distant from the place of their assembly, which seems to be tightly regulated by the particular cellular context. However, a skin equivalent where perlecan is completely missing (or its levels could be controlled) is still under development, needing better cells, in particular defined fibroblasts.

Taken together, these findings indicate that the basement membrane components nidogen-1 and nidogen-2 are crucial for basement membrane formation in the skin, and that the nidogen-laminin complex may be not only an initial trigger for the assembly of other extracellular components, but also for intracellular adhesion complexes of the epidermis. The coordinated interaction of fibroblasts (nidogen producers) and keratinocytes (producers of most other basement membrane components) may represent another level of control which could be responsible for timely formation or remodeling of functional basement membrane structures in the skin.

8. ZUSAMMENFASSUNG

Basalmembranen und ihre Hauptkomponenten (Laminin, Nidogen, Typ IV Kollagen und Perlecan) sind in zahlreiche biologische Funktionen involviert. In dieser Arbeit wurden Entwicklung und Aufbau der Basalmembran der Haut in einem organotypischen 3D-Kokulturmodell untersucht, insbesondere die Interaktionen zwischen den einzelnen Komponenten. Dieses System besteht aus einem Rasen epidermaler Zellen (oder der HaCaT Zell-Linie) auf einer Typ I Kollagen Matrix mit inkorporierten Fibroblasten und ähnelt damit im Wesentlichen der in vivo Situation der Haut. Mit diesem in vitro Ansatz lässt sich durch Inkorporieren von Zellen transgener Tiere oder transfizierter humaner Zellen eine knockout Situation für spezifische Basalmembrankomponenten erzeugen, und zwar gezielt in einem oder beiden Gewebekompartimenten.

Das völlige Fehlen von Nidogen war offensichtlich fatal für die Basalmembran-Bildung in diesem 3D-Kokulturmodell. In Abwesenheit von den beiden Isoformen Nidogen-1 und Nidogen-2 war die Interaktion der Basalmembrankomponenten Laminin-10, Typ IV Kollagen und Perlecan und somit die Bildung einer distinkten Basalmembranzone völlig unterbunden. Auch die epithelialen Verankerungskomplexe, die Hemidesmosomen, entwickelten sich nicht in den Basalzellen, die mit der Kollagenmatrix konfrontiert sind. Darüber hinaus war die Strukturierung von Laminin-5 deutlich gestört. Obwohl alle diese Proteine größtenteils in Extrakten der epidermalen oder dermalen Gewebekompartimente ohne erkennbare Degradation vorhanden waren, konnten sie keiner definierten Lokalisation zugeordnet werden. Die externe Zugabe von rekombinantem Nidogen-1 oder Nidogen-2 restaurierte vollständig die Basalmembran im 3D-Kokulturmodell, und unterstrich damit die Bedeutung des Nidogens in diesem Strukturierungsprozeß. Diese Ergebnisse legen nahe, dass Nidogen als molekularer Adapter agiert, der die Bindung von Laminin an die Zielzellen erleichtert und/oder die anfänglichen Polymerisationschritte katalysiert.

Eine andere Methode um die Basalmembran-Bildung zu stören war die Blockierung der Bindung zwischen Nidogen und Laminin. Dies war ein weiterer Hinweis auf die Bedeutung dieses Komplexes im Strukturierungsprozeß der Basalmembran. So führte die Verhinderung der Laminin-Nidogen Interaktion dazu, dass weder das polymere Netzwerk von Laminin-10 (oder Typ IV Kollagen bei HaCaT Zellen) noch die übrigen assoziierten Proteine, wie etwa Perlecan, in der Verbindungszone von Epidermis und Dermis erkennbar waren. Auch in diesem Fall waren die Proteine selbst noch anwesend und in Extrakten der entsprechenden Gewebekompartimente nachweisbar. Darüber hinaus gingen in Abwesenheit einer normalen Basalmembran die Polarität der Verteilung von Integrin $\alpha 6 \beta 4$ verloren, was die Bildung der hemidesmosomalen Adhäsionskomplexe verhinderte.

Es wurde ferner versucht, die Ausbildung des Laminin Netzwerks durch Hemmung der Interaktion der drei Bindungsarme dieses Moleküls mit einem rekombinanten Peptidfragment ($\gamma 1$ N-terminal) zu stören. Im Gegensatz zu den schon erwähnten Versuchen, hatte dieser Ansatz keinen deutlichen Effekt auf die Basalmembran-Bildung. So wurden Laminin-10, Typ IV Kollagen, Nidogen-1, Nidogen-2 und

Perlecan normal exprimiert und zeigten reguläre Strukturen. Dies beruht wahrscheinlich auf einem ausreichenden stabilen Laminin Netzwerk, welches über die restlich verfügbaren Laminin „short-arms“ (das heißt nicht vom Peptid blockiert) gebildet wird, sowie auf dem Erhalt der regulären Laminin Konfiguration an der epithelialen Grenzfläche aufgrund der stabilen Bindung durch die Integrine.

Als Gegenstück zu Nidogen wurde die Rolle von Perlecan auf die Basalmembran-Bildung untersucht, wobei diese Komponente sowohl in Keratinozyten als auch in Fibroblasten produziert werden kann. Hierzu wurden HaCaT Klone mit stabiler Expression von Perlecan antisense RNA und SV40 immortalisierte Fibroblasten von Perlecan knockout Mäusen eingesetzt. Hierbei machte es keinen Unterschied, ob Perlecan ausschließlich aus Keratinozyten oder aus Fibroblasten stammte, und zwar weder bei der Expression noch der Lokalisierung der anderen Basalmembrankomponenten. Dies bestätigte insofern die Ergebnisse der Nidogen-Experimente, dass der Syntheseort extrazellulärer Matrixmoleküle relativ weit vom Ort des „Assembly“ entfernt sein kann, und dies anscheinend im besonderen zellulären Kontext reguliert wird (hier die basalen Keratinozyten). An einen Hautmodell, in dem Perlecan vollständig reguliert werden kann, wird derzeit noch bearbeitet, wozu besser definierten Fibroblasten nötig sind (Vermeidung von SV40).

Zusammenfassend deuten diese Ergebnisse darauf hin, dass die Basalmembrankomponenten Nidogen-1 und Nidogen-2 entscheidend für die Basalmembran-Bildung der Haut sind. Zusätzlich zur Funktion als Initialkeim für die Assoziation der extracellulären Komponenten, dürfte der Nidogen-Laminin-Komplex essenziell für die intrazellulären Adhäsionkomplexe der Epidermis an extrazelluläre Matrix sein. Die koordinierte Interaktion zwischen Fibroblasten (Nidogen Produzenten) und Keratinozyten (vermutlich Quelle des Großteils der Basalmembrankomponenten) dürfte eine andere Ebene der Kontrolle darstellen, die für die Basalmembran-Bildung oder Ultrastrukturierung zum rechten Zeitpunkt verantwortlich ist.

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10. ABBREVIATIONS

%	Percent
°C	Celsius degree
µg, µm, µl, µM	Micro-gram, -meter, -liter, -Molar
APS	Ammonium persulfate
BSA	Bovine serum albumin
Ca ²⁺	Calcium ions
CO ₂	Carbon dioxide
Cy3	Indocarbocyanin (fluorochrom)
cDNA	complementary DNA (to mRNA)
DEPC	Diethylpyrocarbonat
DIG-RNA	Digoxigenin-labeled RNA
DMEM	Dulbecco`s MEM
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside 5´-triphosphate
DTT	DL-dithiothreitol
ECL	Enhanced chemiluminescence
EDTA	Ethylen-diamin-tetra-acetate
EHS	Engelbreth-Holm-Swarm
ELISA	Enzym-linked immunoabsorbent Assay
EGF	Epidermal growth factor
FAD	Hanks`F12 and DMEM
FCS	Foetal calf serum
FGF	Fibroblast growth factor
Fig.	Figure
g	Gram
G418	Geneticin
H	Hours
HaCaT	Human adult calcium temperature (keratinocyte cell line)
IL	Interleukin
KDa	Kilo Dalton
KBM	Keratinocyte Basal Medium
KGf	Keratinocyte growth factor
LB	Luria-Bertani medium
LDL	Low-density lipoprotein
M	Molar
MEM	Modified Eagle`s Minimun Essential Medium
Mg ²⁺	Magnesium ions
mg, mm, ml, mM	Milli-gramm, milli-meter, milli-liter, milli-Molar
min	Minute
MMP	Matrix metalloproteinase
mRNA	Messenger RNA
N ₂	Nitrogen
NaCl	Sodium chloride
NaOH	Sodium hydroxide
ng, nM	nano-gramm, nano-Molar
OD	Optical density
PBS	Phosphate buffer saline

PDGF	Platelet-derived growth factor
RIPA	Radio-immuno-precipitation assay
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Rotation per minute
RT	Room temperature
SDS	Sodium dodecyl sulfate
SKDM	Supplied KDM
SV-40	Simian virus 40
TEMED	N, N, N, N –Tetra-methyl-ethylen-diamine
TIMP	Tissue inhibitor of metalloproteinase
Tris	Tris-(hydroxymethyl)-aminomethan
Triton X-100	Octylphenol-polyethylenglycol ether
Tween-20	Polyoxyethylen-sorbitan-monolaurat
UV	Ultraviolet light
V	Volt

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Erklärung

Ich versichere, dass die vorliegende Dissertation von mir selbst ohne unerlaubte fremde Hilfe und ohne Benutzung anderer als der angegebenen Hilfsmittel angefertigt wurde.

Heidelberg, 31.10.2003

Cathrine Schmidt