Epithelial cell migration on laminins

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To my family

ABSTRACT

At the epidermal-dermal junction of the skin, the basal keratinocytes are stably anchored to the underlying basement membrane by specific anchoring structures, the hemidesmosomes. For tissue remodelling, like during wound healing or the cycling growth of the hair follicle, epithelial cell migration occurs and the epithelial cells are forced to reorganize their anchoring structures in order to respond to the new environment.

Here, we report that components of the hemidesmosomes, in particular collagen XVII and the tetraspanin CD151, are involved in the regulation of keratinocyte migration in cooperation with the laminin-binding integrins. We observe that collagen XVII-deficient cells are more motile than normal keratinocytes (Tasanen et al., 2004). The tetraspanin CD151 is known to form stable complexes with laminin-binding integrins. In the epidermis, CD151 is associated with $\alpha3\beta1$ integrins at cell-cell contacts and with $\alpha6\beta4$ integrins in hemidesmosomes. We used a monoclonal antibody against CD151, TS151r, the epitope of which overlaps with the tetraspanin integrin-binding site, to investigate the role of CD151 in the migration of epithelial cells. Under standard culture conditions, the migratory capacity of epithelial HaCaT cells on laminins is low compared to that of Wi26 fibroblasts, apparently due to the production of endogenous laminin 5. However, HaCaT cells treated with TS151r respond to laminin 1 by an enhanced migration. This event is associated with a disruption of cell-cell contacts and a redistribution of $\beta1$ and $\beta4$ integrins. In the hair follicle, free CD151 is present in cell-cell contacts in the epithelial sheet lining the hair bulb, which is considered to contain migrating cells. Together, these results strongly suggest that integrin-bound CD151 inhibits cell migration and that dissociation of the CD151-integrin complex permits cell migration.

In contact with the extracellular matrix, the cells capture information through their integrin membrane receptors, which further transduce the signals to the actin cytoskeleton. Two different laminin isoforms, laminin 1 and 5, activate $\alpha6\beta1$ and $\alpha3\beta1$ integrins, respectively, in fibroblasts and epithelial cells. Upon adhesion, these cells develop laminin-isoform specific morphology and cell-matrix adhesions, which influence the migration pattern of the cells. On laminin 5, the cells develop lamellipodia and very small vinculin patches and migrate in a polarized manner, while on laminin 1, the same cells form filopodia-like structures and thick vinculin aggregates and migrate in an uncoordinated manner, as shown by immunofluorescence staining and time-lapse videomicroscopy, respectively. In HaCaT epithelial cells, these differences can be explained by the activation of different Rho GTPases as shown by pull-down assays of activated Rho GTPases. Upon adhesion on laminin 1, Cdc42 is primarily activated and in the absence of Cdc42 the cells are not able to spread correctly and to develop strong focal adhesions. In contrast, laminin 5 is an activator

of Rac1 and a down-regulator of RhoA. In a complementary approach, RhoA and Rac1 were down-regulated in epithelial cells using siRNA. RhoA-deficient HaCaT cells are flat and present small focal complexes, even when plated on laminin 1, while Rac1-deficient HaCaT cells have numerous and large focal adhesions on laminin 1 and laminin 5. An enhanced migration on laminin 1 is obtained when RhoA (to a smaller extent Cdc42) is down-regulated, leading to the development of lamellipodia and tiny focal adhesions.

These results show that laminins differentially regulate epithelial cell migration by transducing defined signals via specific membrane receptors. These signals induce the activation of the small Rho GTPases, which participate in the formation of cell morphology and behavior.

ZUSAMMENFASSUNG

Die basalen Keratinozyten der Epidermis sind stabil an der unterliegenden dermo-epidermalen Basalmembran durch spezifische Verankerungsstrukturen, den Hemidesmosomen, verbunden. Nur während einer Umgestaltung des Gewebes, wie bei der Wundheilung oder dem Haarfollikel-Zyklus, tritt epitheliale Zellmigration auf, wobei die Epithelzellen gezwungen sind ihre Verankerungsstrukturen zu reorganisieren. In dieser Studie haben wir untersucht, inwieweit die Komponenten der Hemidesmosomen, insbesondere Kollagen XVII und Tetraspanin CD151, in die Regulation der Keratinozyten-Migration involviert sind.

Wir haben beobachtet, dass Kollagen XVII-defiziente Zellen beweglicher sind als normale Kontrolle-Keratinozyten (Tasanen et al., 2004). Das Tetraspanin CD151 bildet stabile Komplexe mit Laminin-bindenden Integrinen. In der Epidermis ist CD151 mit $\alpha3\beta1$ Integrin an den Zell-Zell-Kontakten und mit $\alpha6\beta4$ Integrin in den Hemidesmosomen assoziiert. Wir benutzten einen monoklonalen Antikörper TS151r, der gegen die Integrin-Bindenstelle des CD151 gerichtet ist, um die Rolle des CD151 bei der Migration von epithelialen Zellen zu untersuchen.

Unter normalen Kulturbedingungen ist die Migrationskapazität von epithelialen HaCaT-Zellen auf Laminin gering im Vergleich zu der von Wi26-Fibroblasten. Dies liegt an der endogenen Laminin 5 Produktion der HaCaT-Zellen. HaCaT-Zellen, die mit TS151r behandelt wurden, reagieren auf Laminin 1 mit verstärkter Migration. Dieser Prozess ist mit einem Auseinanderbrechen der Zell-Zell-Kontakte und einer Umverteilung von $\beta1$- und $\beta4$-Integrinen assoziiert. *In vivo*, im unteren Bereich des Haarfollikels, das migrierende Zellen enthält, befindet sich freies CD151. Zusammengenommen wiesen diese Ergebnisse auf eine Hemmung der Zellmigration durch Integrin-gebundenes CD151 hin. Eine Dissoziation von CD151-Integrin-Komplex fördert die Zellmigration.

Bei Kontakt mit der extrazellulär Matrix fangen die Integrin-Rezeptoren der Zellen Information auf, die sie weiter im innere der Zellen zu der Aktin-Netzwerke vermitteln. Zwei verschiedene Laminin-Isoformen, Laminin 1 und Laminin 5, aktivieren $\alpha6\beta1$- bzw. $\alpha3\beta1$-Integrin in Fibroblasten und Epithelzellen. Nach der Adhäsion bilden diese Zellen Laminin-Isoform spezifische Morphologien und Zell-Matrix-Kontakte aus, was das Migrationsmuster der Zellen beeinflusst. Auf Laminin 5 entwickeln die Zellen Lamellipodien mit diskreten Fokalkomplexen und wandern in polarisierter Form, während die gleichen Zellen auf Laminin 1 Filopodien-ähnliche Strukturen mit dicken fokalen Kontakten ausbilden und in unkoordinierter Art wandern. In HaCaT-Zellen können diese Unterschiede durch die Aktivierung verschiedener Rho GTPasen erklärt werden. Bei der Adhäsion auf Laminin 1 wird hauptsächlich Cdc42 aktiviert und in Abwesenheit von Cdc42 sind die Zellen dann nicht in der Lage, sich korrekt auszubreiten und starke Zell-Matrix-Kontakten zu bilden. Im Gegensatz dazu ist Laminin 5 ein Aktivator von Rac1 und reguliert die Aktivität von RhoA herunter. Dies erlaubt der Zelle sich komplett auszubreiten und kleine Fokalkomplexe zu entwickeln. Diese Ergebnisse wurden durch das Herunterregulieren von Rho GTPasen in Epithelzellen mittels SiRNA bekräftigt. RhoA-defiziente HaCaT-Zellen sind flach und zeigten kleinen Zell-Matrix-Kontakten, auch wenn sie auf Laminin 1 ausplattiert sind. Rac1-defiziente HaCaT-Zellen dagegen haben zahlreiche und dicke Zell-Matrix-Kontakte auf Laminin 1 und Laminin 5. Eine verstärkte Migration auf Laminin 1 wird erreicht, wenn die RhoA-Aktivierung (in einem schwächeren Ausmaß auch Cdc42-Aktivierung) unterdrückt wird. Dies führt zur Entwicklung von Lamellipodien und kleinen Zell-Matrix-Kontakten.

Diese Ergebnisse zeigen, dass Laminine die Zellmigration mittels definierten Integrin-vermittelten Signaltransduktion regulieren können. Diese Signale führen zur Aktivierung von kleinen Rho GTPasen, die die Zellmorphologie und das Zell-Verhalten beeinflussen.

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Erklärung

1. INTRODUCTION

1.1. The extracellular matrix

The extracellular matrix is a complex network of secreted macromolecules, one function of which is to hold together cells and tissues and so to maintain the structural integrity and the plasticity of the body. Another function of the extracellular matrix is to be a conductor of information and to control the behaviour of the cells in contact with it. It can influence cell shape, differentiation, migration, survival and proliferation as well as the expression of specific genes. This is due to the fact that some of the extracellular matrix proteins are ligands for adhesive and/or signalling receptors on the cell surfaces. Here, we will focus on the interplay between the expression of laminin isoforms that have different biological functions and the response of the cells to it.

1.1.1. The laminins

The laminins are ubiquitous components of basement membranes (Aumailley and Smyth, 1998; Aumailley and Rousselle, 1999; Colognato and Yurchenco, 2000). They are large N-glycosylated proteins composed of three genetically distinct chains α, β, γ of 130 to 400 kDa. The molecules have a cross- or T-shaped form when visualized by transmission electron microscopy after rotary shadowing. The long arm of laminins is formed by the three chains joined together through a central coiled-coil domain (about 600 residues) consisting of heptad repeat sequences **(Fig. 1)**. The C-terminal end of the α chain, longer than that of the β and γ chains, forms five globular LG modules, LG1 to LG5 (Timpl and Brown, 1995). Much more variable than the C-terminal region are the short N-terminus arms of the laminin chains. They consist of three types of modules: the LE modules (EGF-like motifs with four instead of three disulfide bridges) forming rod-like structures and globular modules, the L4 (domain IV) and LN (domain VI) motifs. The L4 motifs are inserted between LE motifs and the LN motifs are N-terminal (Aumailley and Rousselle, 1999).

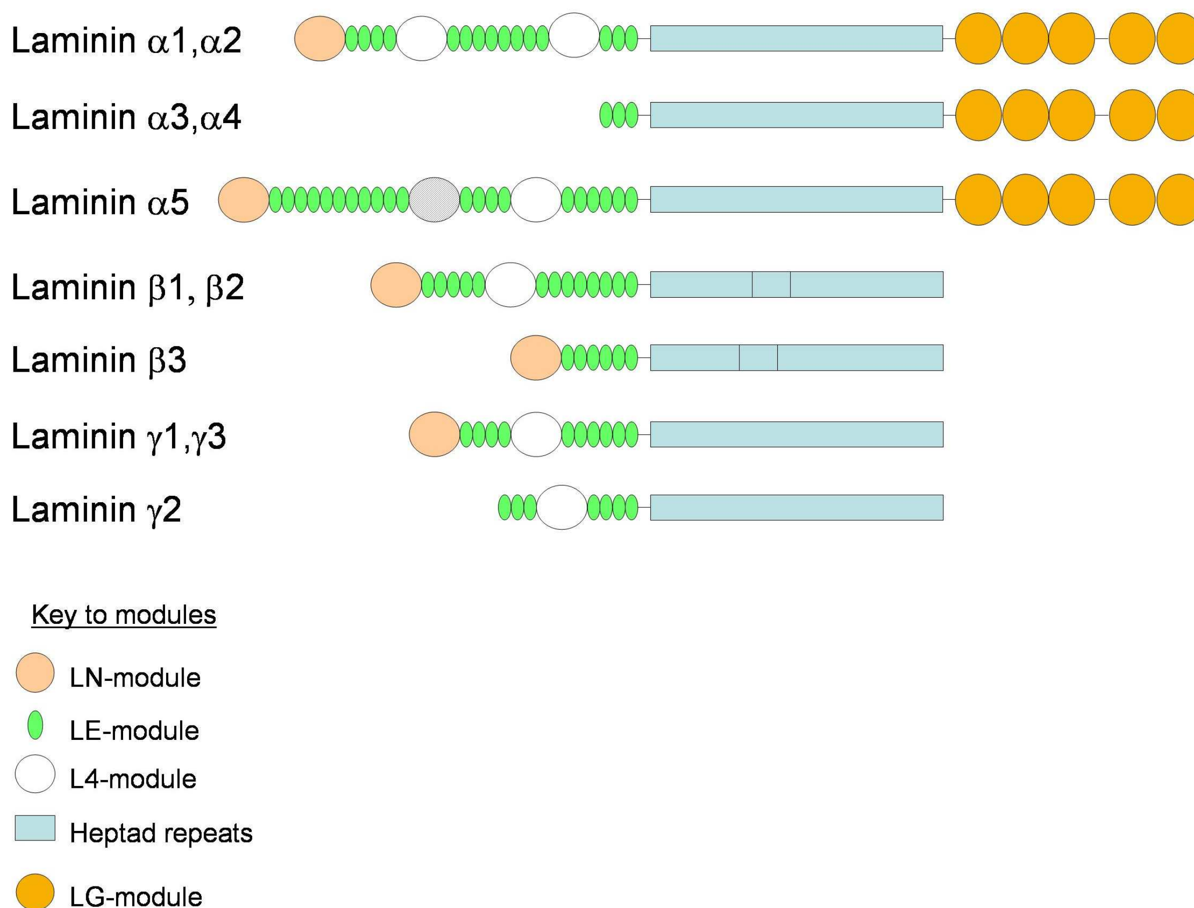

Fig. 1: Structural modules of the laminin chains (inspired from Timpl and Brown (1995))

So far, from five variants of α chains, three for the β chains and three for the γ chains, only fifteen different laminin heterotrimers were found in mammals **(Table 1)**. There are two different variants in fly and in nematode.

Table 1: Combination of the 15 heterotrimeric mammalian laminins

Laminin 1	α1β1γ1	Laminin 9	α4β2γ1
Laminin 2	α2β1γ1	Laminin 10	α5β1γ1
Laminin 3	α1β2γ1	Laminin 11	α5β2γ1
Laminin 4	α2β2γ1	Laminin 12	α2β1γ3
Laminin 5	α3β3γ2	Laminin 13	α3β2γ3
Laminin 6	α3β1γ1	Laminin 14	α4β2γ3
Laminin 7	α3β2γ1	Laminin 15	α5β2γ3
Laminin 8	α4β1γ1		

The expression of the laminin α chains is spatially and temporally regulated (Ekblom, 1996; Ekblom et al., 1998). Expression of the α1 and α3 chains is limited to epithelial cells. The α2 and α5 chains are primarily expressed by mesenchymal cells and the α4 chain is more specific of endothelial cells (Miner, 1995).

Alternative spliced forms were found for the α2, α3, α5 and γ2 chains. In addition, the α2, α3, α4 and α5 chains of laminin, but not α1, undergo proteolytic cleavage within the G domain between LG3 and LG4.

1.1.2. Laminin 1, a model for the organization of basement membrane

Laminin 1 from the mouse Engelbreth-Holm-Swarm tumor was the first laminin variant to be described (Timpl et al., 1979). Composed by the association of the α1 (400 kDa), β1 (200 kDa) and γ1 (200 kDa) chains, laminin 1 has a prominent expression during early epithelial development. Already at 8- and 16-cell stage during mouse embryo development, laminin 1 is the first isoform to be expressed by epithelial cells. In adult tissues, it is expressed in only few epithelial basement membranes. The highest amount of α1 chain is found in male and female reproductive organs, liver, kidney and eye (Falk et al., 1999; Virtanen et al., 2000; Ekblom et al., 2003).

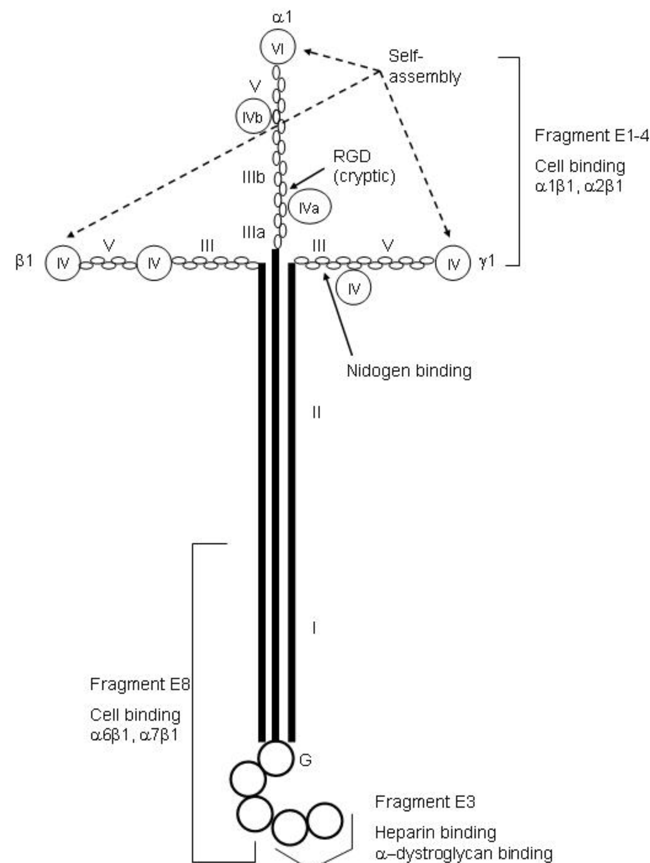

Fig. 2: Schematic view of laminin 1 and its interactions with cellular and extracellular components.
The different domains (roman numbers) and the sites for protein interactions are indicated (from Aumailley and Gayraud, 1998).

The short arms of laminin 1 contain binding sites for α1β1 and α2β1 integrins **(Fig. 2)**. However, their contribution to cell adhesion is minor and their role as laminin 1 receptors *in vivo* is not clear. In the N-terminal region of the α1 chain, there is, in addition, a cryptic and inaccessible RGD sequence. In contrast, the α6β1 integrin is the main adhesion receptor for laminin 1 (Aumailley et al., 1990). It binds to the E8 fragment composed of the C-terminal part of the coiled-coil region and the globular domains LG1 to LG3 of the α1 chain. In addition, the α1LG4 domain is recognized by cell surface protein dystroglycan, as well as by cell surface sulfatides and heparin (Talts et al., 1999).

The organization of basement membranes was initially described based on studies performed with laminin 1. Laminin 1 is able to self-associate into a quasi-hexagonal network by means of interactions between the N-terminal globular LN domains **(Fig. 3).** The formation of the network is reversible and controlled by temperature, calcium and laminin concentration. All laminins with three short arms containing a N-terminal LN module, (i.e. laminins 1, 2, 3, 4, 10 and 11) can form networks. In contrast, laminins lacking a full set of LN modules, such as laminins 5, 6 and 7 cannot form these networks, but they can covalently associate to form dimers (Colognato and Yurchenco, 2000; Odenthal et al., 2004).

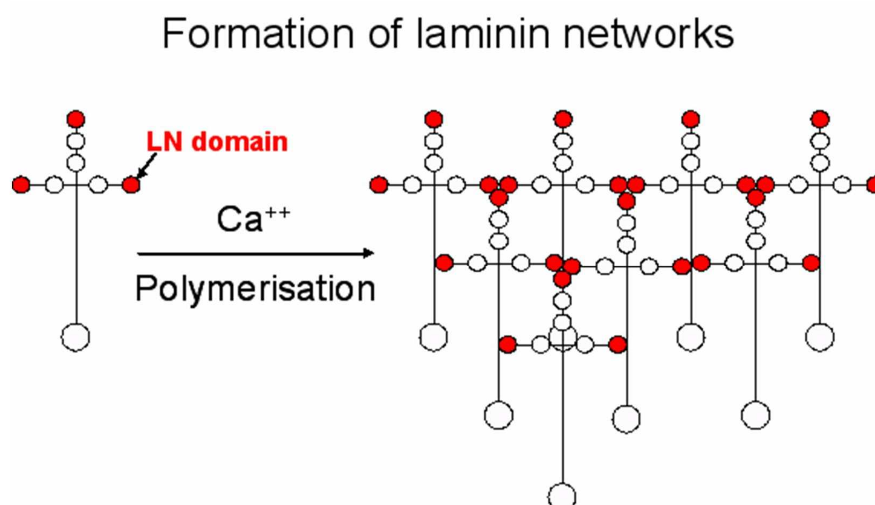

Fig. 3: Schematic representation of the formation of laminin networks through interaction of the N-terminal LN modules.
The assembly of the chains is a reversible process controlled by calcium and laminin concentration.

In addition, laminin 1 is a binding partner for extracellular matrix components contributing to the formation of supramolecular networks. The C-terminal LG4-5 domains have a recognition motif for perlecan and fibulin-1, the N-terminal part of the α1 chain for perlecan, the LEIII4 module of the γ1 chain for nidogen, while the coiled-coil domain binds to agrin. **Figure 4** shows a model for the assembly of basement membrane in the presence of laminin 1. In this model, nidogen-1 forms a bridge between the laminin network and the collagen IV network (Mayer et al., 1998).

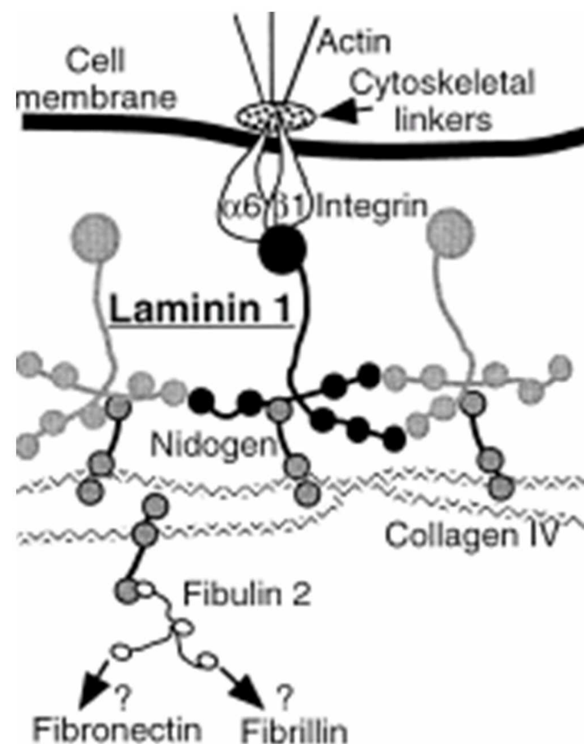

Fig. 4: Model of protein-protein interactions in basement membrane involving laminin 1 (from Aumailley and Gayraud, 1998).

Interestingly, the genetic ablation of either the laminin γ1 chain, the integrin β1 subunit or α-dystroglycan leads to a failure of basement membrane assembly and the mutant mouse embryos die at the peri-implantation stage (Smyth et al., 1998; Colognato and Yurchenco, 2000; Fässler and Meyer, 1995; Williamson et al., 1997). This suggests that there is a reciprocity between laminin polymerization and receptor clustering and that laminin binding to its cellular receptors enables its self-polymerization (Colognato et al., 1999; Colognato and Yurchenco, 2000).

1.1.3. Supramolecular organization of the cutaneous basement membrane

The dermal-epidermal junction (DEJ) is a 1 µm thick-zone separating the epidermis from the dermis. It is a special basement membrane as it contains specific anchoring structures which provide mechanical stability between the two layers (Burgeson and Christiano, 1997). The DEJ can be divided in four zones recognized in transmission electron micrographs **(Fig. 5).** In the upper layer of the junction, intermediate filaments of the basal keratinocytes insert into an electron dense plaque, the hemidesmosome, located in the basal plasma membrane of the cells (Zone 1). Below, is an electron-translucent zone, the lamina lucida, traversed by anchoring filaments (Zone 2) extending to an electron dense structure, the lamina densa (Zone 3). At last, banded structures, the anchoring fibrils, extend from the lamina densa into the underlying dermis (Zone 4).

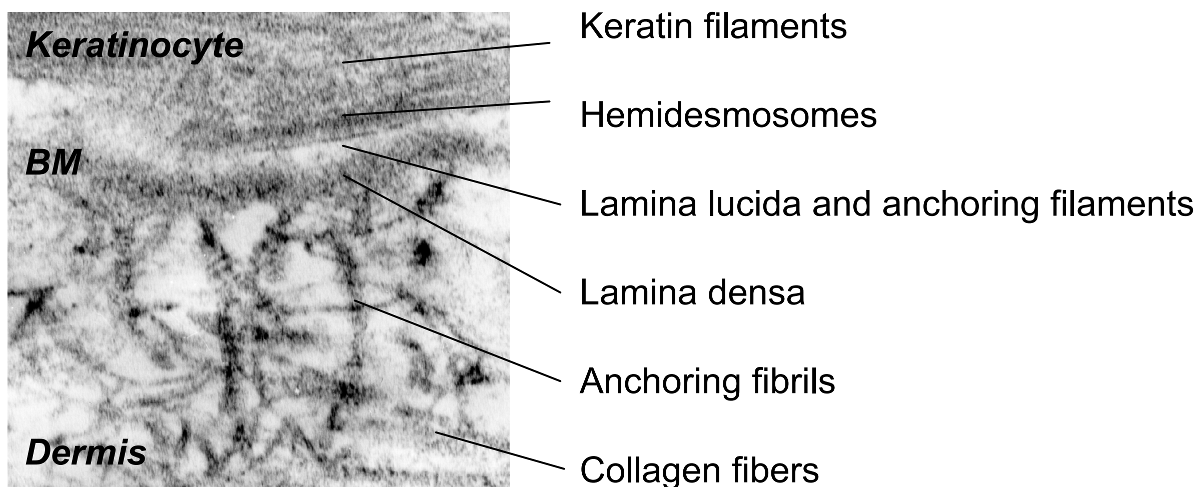

Fig. 5: Transmission electron microscopy of the dermal-epidermal junction of human skin.

1.1.3.1. The hemidesmosomal plaque

The hemidesmosomal plaque is formed by intracellular components, the 230 kDa bullous pemphigoid antigen (BP230) and plectin, and by transmembrane components, the $\alpha6\beta4$ integrin, collagen XVII (or 180 kDa bullous pemphigoid antigen BP180) and the CD151 tetraspanin (Koster et al., 2004b) **(Fig 6)**.
Inside the cell, the intermediate filaments linked to the hemidesmosomes consist of keratin 5 and 14 dimers. The keratins interact via their rod domain with the 230 kDa

bullous pemphigoid antigen (BP230) and plectin, which are cytoskeletal linker proteins belonging to the plakin family. Both proteins bind to keratin filaments via their C-terminus and to transmembrane proteins collagen XVII/BP180 and integrin β4 subunit via their N-terminal head domain.

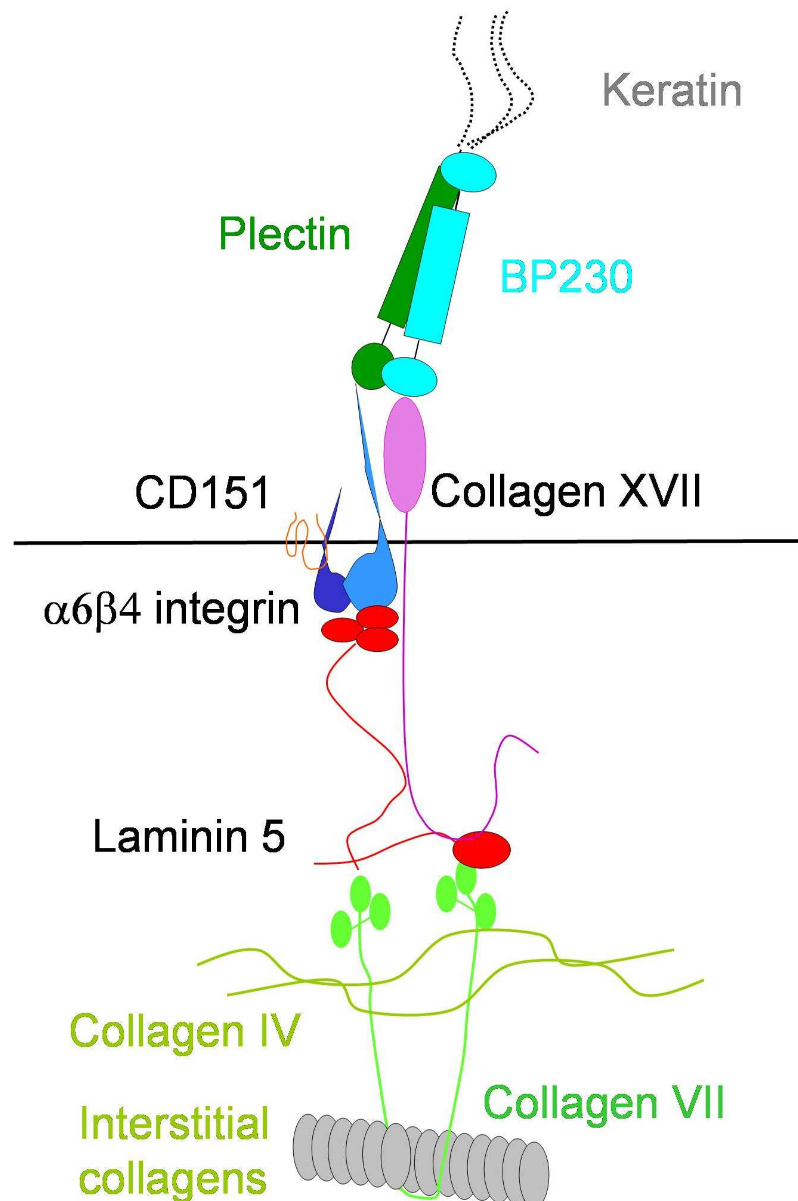

Fig. 6: Protein-protein interactions at the dermal-epidermal junction.
Inside the cells, plectin and BP230 link the keratin bundles with the transmembrane proteins α6β4 integrins and collagen XVII. Outside the cells, α6β4 integrins and collagen XVII interact with laminin 5, itself linked to collagen VII in the anchoring fibrils.

The $\alpha6\beta4$ integrin is an essential component of the hemidesmosomes as mice lacking either the integrin $\alpha6$ or $\beta4$ subunits do not have stable attachment of the epidermis to the dermis, resulting in death shortly after birth (Georges-Labouesse et al., 1996; Van der Neut et al., 1996). The integrin $\beta4$ subunit differs from the other integrin β subunits in that it has an unusually long cytoplasmic domain (about 1000 residues). The $\beta4$ endodomain binds to plectin and BP230 and to the intracellular domain of collagen XVII. In addition, the extracellular domain of the integrin $\alpha6$ subunit can interact *in vitro* with a sequence of collagen XVII situated next to the transmembrane domain. The integrin $\alpha6$ subunit also binds to another constituent of the hemidesmosome, the tetraspanin CD151 (Sterk et al., 2000) **(Fig. 6)**.

The stabilisation of the hemidesmosomes requires the recruitment of all constituents. Koster and collaborators have proposed a hierarchical interaction model for the assembly of the hemidesmosomes (Koster et al., 2004b). First, the $\alpha6\beta4$ integrin interacts with plectin. Together, they recruit collagen XVII. Finally, BP230 is included into the complex via its interaction with collagen XVII and the integrin $\beta4$ subunit.

CD151 is a 32 kDa glycosylated protein with four transmembrane domains, TM1 to TM4, one small hydrophilic extracellular loop, EC1, between TM1 and TM2 and a major extracellular loop, EC2 (106 amino acids), between TM3 and TM4 **(Fig. 7)**. Both the N-and C-terminus are intracellular. CD151 has a single N-glycosylation site in the EC2 loop and is palmitoylated on several cysteins at its C-terminus. According to these features, CD151 is a member of the tetraspanin superfamily (Stipp et al., 2003). The majority of the tetraspanin proteins are implicated in the so called "tetraspanin web", regrouping a certain number of different proteins of the superfamily and others like immunoglobulin superfamily proteins, proteoglycans, complement regulatory proteins, integrins, growth factors, growth factor receptors and signalling enzymes (Maecker et al., 1997; Boucheix and Rubinstein, 2001).
CD151 is a particular tetraspanin as it preferentially associates with laminin-binding integrins $\alpha3\beta1$, $\alpha6\beta1$, $\alpha6\beta4$ and $\alpha7\beta1$ (Serru et al., 1999, Sterk et al., 2002). The formation of the complexes require an interaction between a short sequence, QRD, in the large extracellular EC2 loop of CD151 and the membrane proximal stalk region of the integrin α subunit (Yauch et al., 2000; Berditchevski et al., 2001; Kazarov et al., 2002). In human skin, expression of CD151 is restricted to basal keratinocytes,

colocalizing with α3β1 integrins at the lateral membranes and with α6β4 integrins at the basal membranes (Sincock et al., 1997, Peñas et al., 2000; Sterk et al., 2000; 2002) **(Fig. 6)**. CD151 may play a role in the spatial organization of the hemidesmosomes through initial binding to α3β1 integrins in pre-hemidesmosomes, then switching to α6β4 integrins as the hemidesmosome assembles.

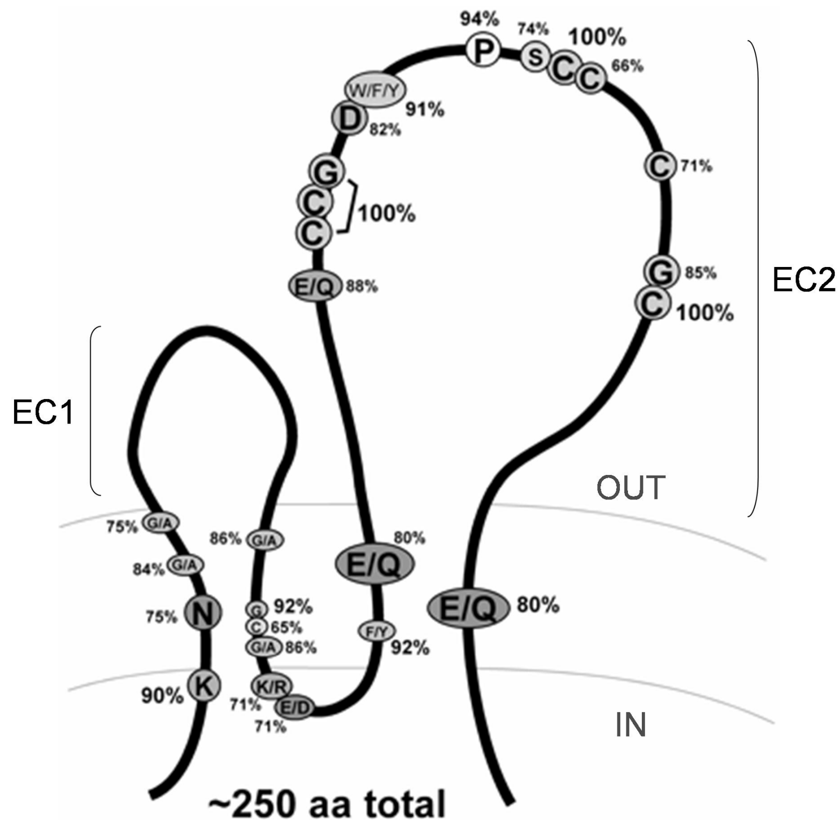

Fig. 7: Schematic representation of tetraspanin.
26 key residues conserved in human and *drosophila* tetraspanins are indicated (from Hemler, 2001). In particular, 4 cysteins in the large extracellular loop EC2 are supposed to form two disulfide bridges. CD151, with 6 cysteins in EC2, shows the consensus CCG, PXXC and EGC motifs.

Very recently, Karamatic Crew and collaborators have reported a new role of CD151 in the assembly of human basement membranes in kidney and skin (Karamatic Crew et al., 2004). Indeed, patients homozygous for a mutation resulting in the absence of the integrin-binding domain in CD151, present hereditary nephritis, pretibial epidermolysis bullosa and β-thalassemia minor. However, at the same time, Wright and collaborators have characterized mice lacking CD151. These mice are viable, healthy and fertile and present no apparent skin phenotype (Wright et al., 2004).

1.1.3.2. The anchoring filaments

Collagen XVII is part of the hemidesmosomal complex and of the anchoring filaments. Collagen XVII is inserted in the cell plasma membrane in a type II orientation: its N-terminal domain is intracellular and localized in the hemidesmosomal plaque and its large and flexible C-terminal is extracellular and colocalizes with laminin 5 in the anchoring filaments **(Fig. 6)**. With its 193 nm long C-terminus extending across the lamina lucida into the lamina densa, it is the largest transmembrane collagen. In human, collagen XVII ectodomain contains 15 collagenous domains interrupted by 16 non-collagenous domains (Franzke et al., 2003) **(Fig. 8)**. It can be shed within its juxtamembrane linker region by proteinases of the ADAMs family (Franzke et al., 2002). After shedding, the ectodomain may be denatured, leading to the accessibility of new cell adhesion site for β1 integrins and to a positive effect on keratinocyte cell migration (Nykvist et al., 2001) **(Fig. 8)**.

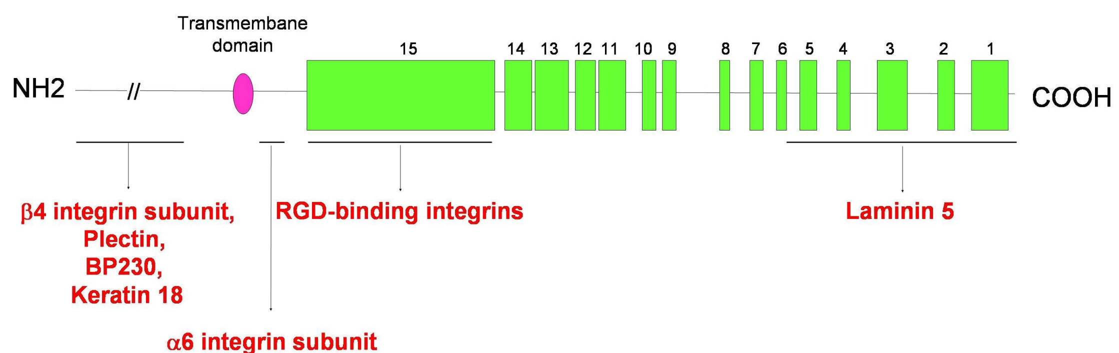

Fig. 8: Schematic representation of human collagen XVII.
The ectodomain contains 15 collagenous (green boxes) and 16 non-collagenous (black lines) segments. The sites for interactions with components of the hemidesmosomes and with laminin 5 are indicated.

Intracellularly, collagen XVII binds to the cytoplasmic domain of the integrin β4 subunit and recruits BP230 into hemidesmosomes **(Fig. 8)**. Extracellularly, its NC16a domain binds to the integrin α6 subunit (Koster et al., 2003) and its C-terminal region interacts with laminin 5 (Tasanen et al., 2004). The importance of collagen XVII in the maintenance of epithelial cell adhesion is demonstrated by the phenotype of patients with inherited and acquired skin bullous diseases targeting collagen XVII. Indeed, patients with inherited generalized atrophic benign epidermolysis bullosa (GABEB) lack collagen XVII or have a mutated collagen XVII and they present tissue separation at the level of the lamina lucida due to immature hemidesmosomes

(Pulkkinen and Uitto, 1999). In acquired diseases, the ectodomain of collagen XVII contains the epitopes recognized by bullous pemphigoid autoantibodies. The patients suffering from this autoimmune disease also present a subepidermal blistering. These observations suggest that collagen XVII contributes to maintaining epithelial adhesion.

Laminin 5 is the major adhesion protein for basal keratinocytes in the skin and it acts as a bridge between the basal epithelial cells and the anchoring fibrils **(Fig. 6)**. Mutations of the LAMA3, LAMB3 and LAMC2 genes encoding the α3, β3, γ2 chains of the laminin 5 are associated with Herlitz Junctional Epidermolysis Bullosa (H-JEB), a form of epidermolysis bullosa characterized by a detachment of the basal keratinocytes from the basement membrane (Pulkkinen and Uitto, 1999). Mice lacking the α3 subunit of laminin 5 display normal embryonic development but die within the first days after birth due to severe blistering (Ryan et al., 1999). This shows that laminin 5 is required to maintain anchorage of the epithelium to the dermis in the adult.

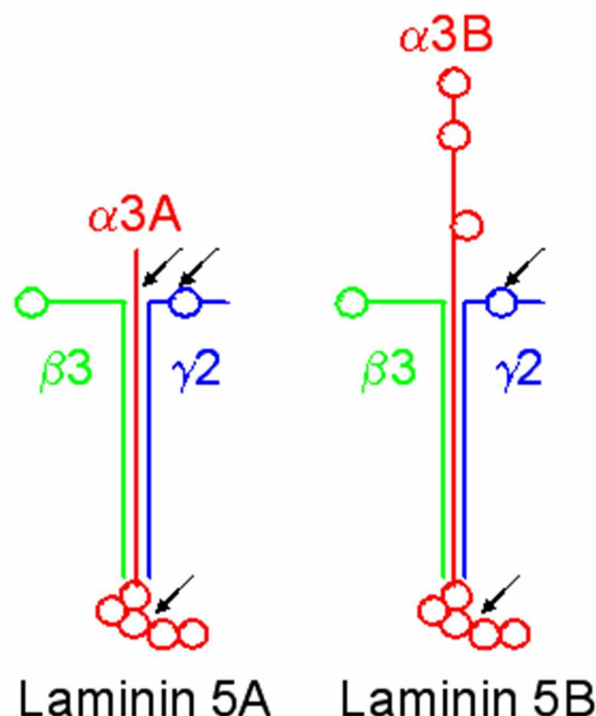

Fig. 9: Diagram of laminin 5A and 5B showing the $\alpha3\beta3\gamma2$ chains.
The arrows indicate the location of the cleavage sites.

Laminin 5A and 5B are synthesized and secreted by epithelial cells as 480 kDa and 625 kDa heterotrimer precursors, respectively. The precursors can be extracellularly

processed to form mature laminin 5 **(Fig. 9)**. At the C-terminal end of the α3A and α3B variants, the LG4-5 region is enzymatically removed (Tsubota et al., 2000). This processing leads to the 165kDa form of α3A chain and 310-kDa form of α3B chain. The 165-kDa form of the α3A chain can be further reduced to 145-kDa by truncation of the LE1-3 modules at its N-terminal end. Also the N-terminal region of the γ2 chain can be cleaved in the globular domain L4m. In the basement membrane of human adult skin, the laminin 5 is fully processed (Tunggal et al., 2002).

The C-terminus of the laminin α3 chain binds to epithelial cell receptors, while the short arms of the γ2 and β3 chains associate with extracellular proteins (Aumailley et al., 2003). The LG1-3 domains interact with α3β1, α6β1 and α6β4 integrins **(Fig. 10)** (Carter et al., 1991; Niessen et al., 1994). The LG4-5 region has affinity for cell surface syndecan-2 and -4 and perhaps for α-dystroglycan (Utani et al., 2001; Aumailley et al., 2003). The truncation of the LG4-5 modules can therefore modulate the interactions of laminin 5 with cells. The isolated LG4-5 fragment itself has no significant activity (Tsubota et al., 2000), however, when unprocessed, the C-terminal LG4-5 domains could sterically hinder the interaction between integrins and the LG1-3 domains or trigger a specific signalling leading to different biological behaviour of the cells (Fleischmajer et al., 1998, Kariya et al, 2003; Tunggal et al., 2002).

Laminin 5, with γ2 and α3 chains lacking LN modules, is not able to self-polymerize like other laminins. However, laminin 5 can associate covalently with laminin 6 (Champliaud et al., 1996). Therefore, its binding to laminin 6 can be the initiation of small oligomers. A further interaction takes place between the N-terminal region of laminin 5A and the NC1 domain of collagen VII, contributing to the stability of the anchoring plaques (Rousselle et al., 1997). Also, nidogen-1, fibulin-1 and -2 are ligands for recombinant forms of the N-terminal region of the γ2 chain (Sasaki et al., 2001). These binding regions might have a biological role like permitting the integration of laminin 5 into the extracellular matrix before the maturation of the γ2 chain (Sasaki et al., 2001; Gagnoux-Palacios et al., 2001).

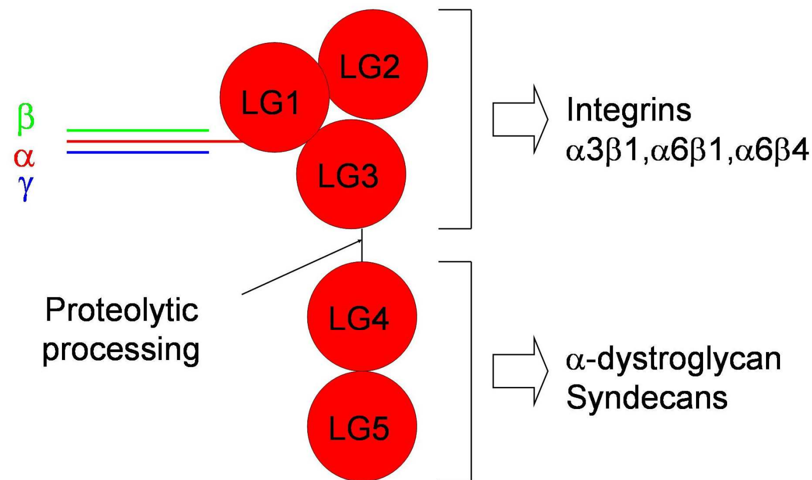

Fig. 10: The C-terminus of the laminin α chain binds to cell receptors.
In laminin 5, the LG1-3 region interacts with α3β1, α6β1 and α6β4 integrins, while the LG4-5 domain binds to α-dystroglycan and syndecans. The protolytic cleavage site between LG3 and LG4 domain is shown.

In the anchoring filaments, the α5 chain of laminin is also present and can associate with β1γ1 dimers to form laminin 10 (Miner et al., 1997). It presumably assists laminin 5 and 6 in forming stable complexes with other DEJ components. Furthermore, the study of mouse skin deficient in laminin α5 chain has demonstrated a crucial role for laminin 10 for hair follicle development (Li et al., 2003).

1.1.3.3. The lamina densa and the anchoring fibrils

The major lamina densa molecule, collagen IV, provides the core of the basement membrane through self-assembly and formation of large networks **(Fig. 6)** (Aumailley and Gayraud, 1998), to which other matrix proteins like collagen VII, nidogens and perlecan are attached. Among the six genetically distinct α chains (α1(IV) to α6(IV)), the α1(IV), α2(IV), α5(IV) and α6(IV) chains are present in collagen IV heterotrimers of the adult DEJ (Tanaka et al., 1998). A collagen IV trimer has a 400 nm long helicoidal domain with one globular non-collagenous domain at its N-terminus (NC1) and a cystein-rich region (7S domain) at the C-terminus. The formation of tetramers by association of four 7S domains and of dimers by interactions between two NC1 domains lead to the chicken wire structure of the collagen IV network (Ortega and Werb, 2002). Collagen IV has also a function in cell adhesion via its central triple-helical domain (Aumailley and Timpl, 1986), which has affinity for α1β1 and α2β1 integrins (Vandenberg et al., 1991).

Collagen VII is the major constituent of the anchoring fibrils. It is a homotrimer composed of three identical α1(VII) chains. Its N-terminal end is a large non-collagenous NC1 domain composed of two vWA modules followed by 10 fibronectin type III motifs. Its C-terminus is formed by a small globular NC2 domain involved in the anti-parallel self-assembly of dimers, further associated laterally into fibrils (Van der Rest and Garrone, 1991; Aumailley and Gayraud, 1998). Because of the flexibility of the molecule, it loops and reinserts into the lamina densa. In doing so, it entraps dermal matrix proteins stabilizing the basement membrane zone. The NC1 N-terminal domain of collagen VII binds to collagen IV in the lamina densa (Burgeson et al., 1990) and alternatively also to laminin 5 short arms. Through these protein-protein interactions, collagen VII has a role as a linker between anchoring filaments and anchoring fibrils **(Fig. 6)**. Indeed, a large number of mutations in the collagen VII gene (COL7A1) leads to the dystrophic forms of epidermolysis bullosa (EBD) characterized in the most severe form by an absence of anchoring fibrils, resulting in skin blistering (Pulkkinen and Uitto, 1999).

1.2. Cell migration and integrins

Cell movement is essential for embryogenesis and morphogenesis, and also occurs in pathological conditions like metastasis, inflammation and during tissue repair. According to the cell types, cells can either migrate in coherent masses as do epithelial and endothelial cells or independently as single cells like leukocytes, lymphocytes, fibroblasts and neuronal cells.

Single cell migration can be defined as cycling events divided in several steps **(Fig. 11)** (Huttenlocher et al., 1995). Migration is initiated in response to extracellular cues by the formation of an extension of the cell plasma membrane. This implies a spatial asymmetry or a polarized phenotype with a cell front and a cell rear (Lauffenburger and Horwitz, 1996). At the cell front, polymerization of an actin network take place and cell protrusions like a filopodium, a ruffle or a lamellipodium are formed. At the same time, new adhesion contacts form just behind the cell extension to stabilize it by connecting the substratum with the actin cytoskeleton. This occurs through the sequential recruitment of individual adhesion components beginning with aggregation

of integrins after ligand binding. These adhesion plaques serve as points of traction over which the cell body moves and contractile forces pull the cell body forward (Horwitz and Parsons, 1999). In a final step, the adhesions at the rear of the cells are released and this permits the retraction of the tail and the subsequent forward movement of the cell.

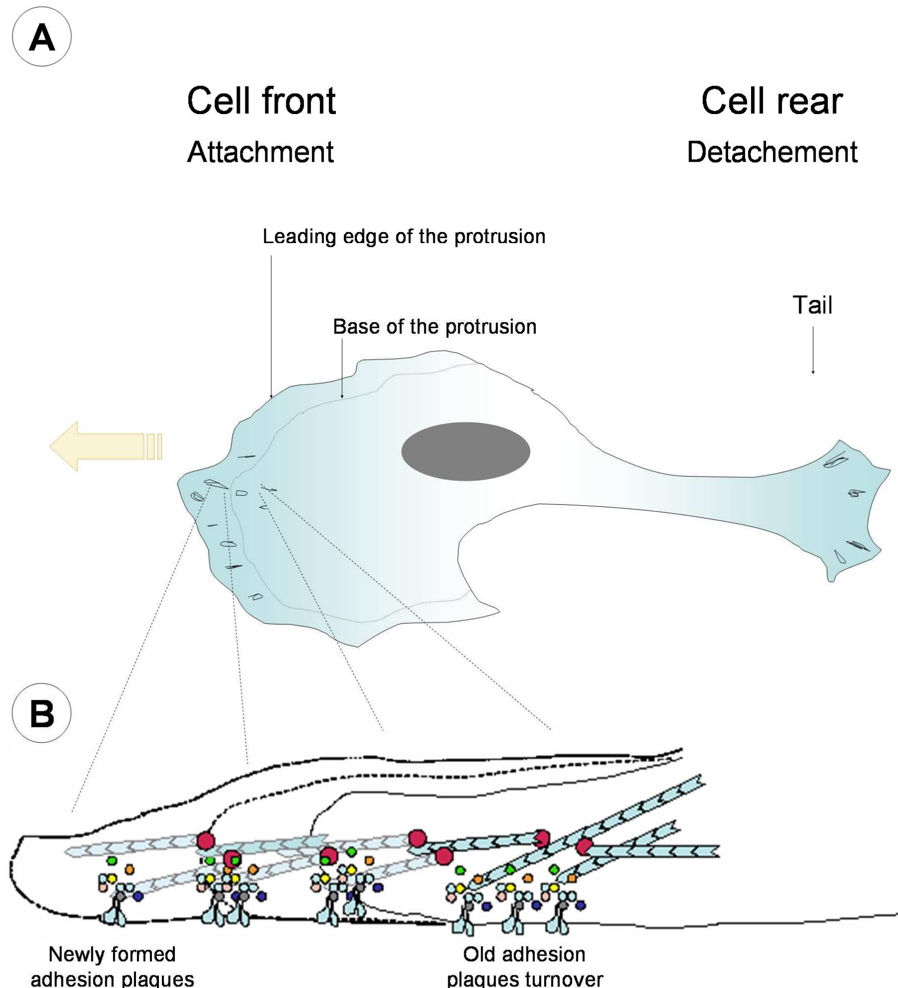

Fig. 11: (A) Schematic side view of a migrating fibroblast.
The cell projects at its front protrusions in form of lamellipodia or filopodia. As the cell body move forward, there is release of the adhesions at the cell rear.
(B) Actin network polymerization drives the extension of the protrusion.
Subsequently, there is formation of new adhesion plaques through recruitment of plaque components while the old adhesion plaques dismantle.

The process of collective cell migration is less understood than the one of individual cells. It occurs for example during embryonic morphogenesis, when cell sheets in the blastoderm or the ectoderm migrate to close the neural tube (Bronner-Fraser, 1994) or during branching morphogenesis, e.g. during the formation of new vessels by

endothelial cells. In pathological conditions, like in wound healing and in carcinoma, cells can also disseminate as cell clusters (Thiery, 2002; Friedl and Wolf, 2003).
Some aspects of the single-cell migration process, as it is described in the precedent paragraph, are shared by migrating cell sheets. For example, the polarization of one single cell can extend across the sheet. The leading cells at the front of the cluster extend protrusions or lamellae (Kolega, 1981). They also engage and cluster β1 integrins. Specific to collective cell migration is the maintenance of cell-cell adhesions through cadherin and other receptors, as well as cell-cell communications via gap-junctions. This is lost during epithelial-mesenchymal transitions (EMT), where an epithelial cell acquires the phenotype of a mesenchymal cell (Thiery, 2002).

1.2.1. Cell-matrix adhesions

The cell-matrix adhesions are structurally complex as they integrate at least 50 different proteins with different functions, from structural linker proteins which interact with membrane receptors and the actin cytoskeleton, to kinases, phosphatases and other adaptor molecules needed to spread downstream signalling cascades (Geiger et al., 2001, Zamir and Geiger, 2001a). Most components have multiple binding sites for different partners and the protein-protein interactions are modulated spatially as well as temporally.
There are several kinds of cell-matrix adhesions, at least in cultivated cells (Geiger et al., 2001b). Focal complexes (FC) are small adhesions assembled at the periphery of lamellipodia and filopodia. They represent a kind of immature and transient cell-matrix adhesions. They drive rapid cell migration or they grow and strengthen into larger and stable focal adhesions (FA) that tend to inhibit cell migration (Rottner et al., 1999). A third structure, termed fibrillar adhesion, is generated after cell binding to extracellular fibronectin fibrils. These adhesions have an elongated structure and their typical components are α5β1 integrins and the structural protein tensin. Finally, the hemidesmosomes, containing α6β4 integrins, are, in contrast to the other adhesion contacts, large and stable adhesion plaques found exclusively in epithelial cells. They are the only adhesion contacts connecting the intracellular keratin intermediate filament system, instead of the actin filaments as in case of the others, with the extracellular matrix. These points of connection are, however, also dynamic

as they disassemble during cell migration. How this happens when the cells switch to more dynamic, β1-containing cell-matrix adhesions, is still unknown.

1.2.2. Receptors for cell migration: the integrins

Among the different cell surface receptors, the integrins are the ones who play a central role in cell migration as they function as a link between the extracellular environment and the cytoskeleton inside the cell. In addition, they serve as a signalling machinery regrouping a network of enzymes and adaptors proteins. During the cyclic events of cell migration, integrins are required at many if not all steps.

Integrins are transmembrane glycoproteins, composed of one α and one β subunit non covalently linked (Shimaoka et al., 2002; Hynes, 2002). In mammals, 18 α and 8 β subunits form 24 different heterodimers. Five integrin α subunits are found in *Drosophila.* Nematodes such as *C. elegans* also have integrins while none have been found in prokaryotes, plants or fungi.
The extracellular N-terminal part of the α and β subunit is about 940 and 640 residues, respectively, and is composed of a globular domain and a membrane-proximal stalk region. The cytoplasmic tails of the integrins are short, composed of 30 to 50 residues (except for the β4 subunit) and have no catalytic properties **(Fig. 12)**.
The globular domain of an α subunit is formed by seven repeated motifs folded into a seven-bladed propeller structure. The upper surface of the globular domain represents the binding site for the ligands, and divalent cations bind to the lower surface. Several integrin α subunits contain an insertion of 200 amino acids, the I domain between blade 2 and 3 of the propeller. In this I domain is a metal ion Mg^{2+}-dependent adhesion site (MIDAS) critical for ligand binding as Mg^{2+} forms a bridge between integrin and ligand (Plow et al., 2000; Liddington and Bankston, 2000). A second cation binding site is found on the α subunit EF-hand motif where Ca^{2+} binding can then promote or inhibit ligand interaction (Plow et al., 2000). The extracellular N-terminal part of the β subunit is made up of a cystein-rich region which shares sequence homology with plexins and semaphorins and is therefore called the PSI domain. The seven cysteins of this domain form disulfide bridges with the C-terminal cystein-rich region, or EGF-like domain, in the β stalk region. In this conformation, the globular head domain is bent and restrained in an inactive state.

Next to the cystein-rich region is a domain similar to the I-domain, called I-like domain. It participates in ligand binding.

Both α and β subunits are subject to posttranslational modifications like glycosylation. In addition, α subunits lacking the I domain, undergo at their membrane proximal region a cleavage resulting in a disulfide-linked heavy and light chains. Some of the integrin subunits can be alternatively spliced in particular the β chains ($\beta1_{A-D}$, $\beta3_{A-C}$ and $\beta5_{A-B}$) (Van der Flier and Sonnenberg, 2001). The $\beta1$ variants are all spliced at their cytoplasmic regions, loosing their cyto-2 and -3 domains.

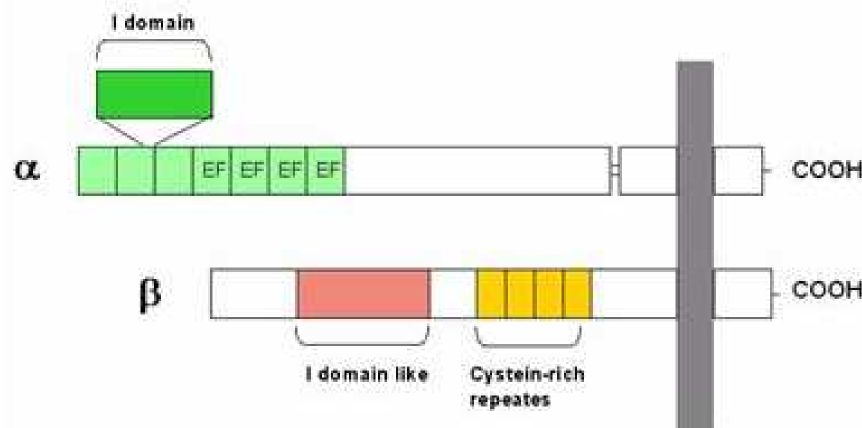

Fig. 12: Schematic representation of the integrin α and β subunits.
The C-terminal region is located intracellularly.

The combination of the integrin α and β subunits forms a heterodimeric receptor with binding specificities for an extracellular ligand (Hynes, 2002) **(Fig. 13)**. Many integrins interact with the same ligand. However, according to the integrin expression or activation, as well as the availability of the ligand, specific interactions occur *in vivo*. $\alpha1\beta1$, $\alpha2\beta1$, $\alpha10\beta1$ and $\alpha11\beta1$ integrins are receptors for native collagens. $\alpha3\beta1$, $\alpha6\beta1$, $\alpha6\beta4$ and $\alpha7\beta1$ integrins are laminin-binding receptors. Extracellular components containing non-cryptic RGD sequences like fibronectin or vitronectin are recognized by $\alpha4\beta1$, $\alpha5\beta1$, $\alpha8\beta1$, $\alpha9\beta1$, $\alpha_{IIB}\beta3$ and αv containing integrins. The integrins of the haematopoietic cells ($\beta2$ subgroup) bind to vascular cell adhesion molecule 1 (VCAM-1) and intercellular adhesion molecule 1 (ICAM1) receptors on other cells.

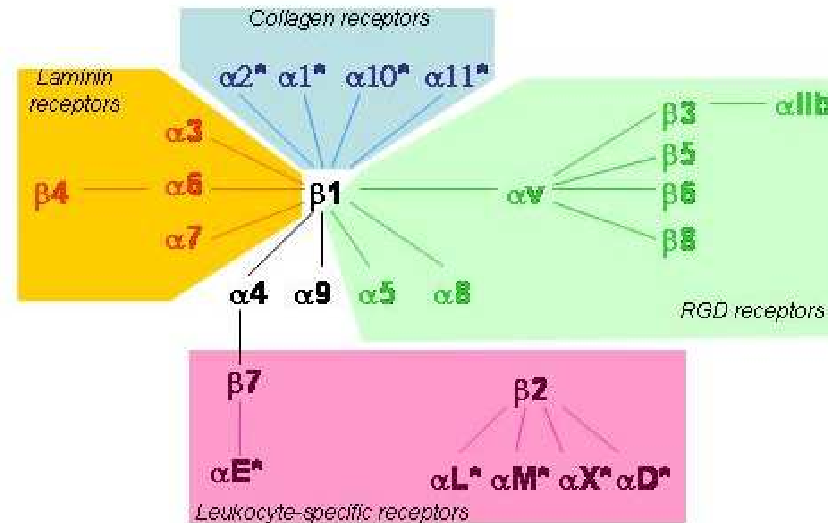

Fig. 13: Mammalian αβ integrin subunit associations.
Integrins are divided in four main subfamilies based on ligand specificity and expression pattern. Asterisks denote a subunit containing one I domain (inspired from Hynes, 2002).

1.2.3. Integrins and signal transduction

Using mutational analysis, Hughes and colleagues could show that both integrin α and β subunit membrane proximal intracellular domains are required for the regulation of the integrin affinity state (Hughes et al., 1996). Indeed, these two domains interact with each other through a salt bridge, maintaining the dimer in a default low affinity state. Based on electron microscopy, αIIβ3 integrin shows two conformations: a closed and an open conformation. The integrin has low affinity for ligand under the closed conformation that is stabilized by the bond between α and β subunits. In the open conformation, the bond is broken (Liddington and Bankston, 2000). This change creates an allosteric so called "shapeshift" across the membrane in which the extracellular binding surface becomes complementary to the ligand and the integrin has a high affinity to the ligand (Liddington and Ginsberg, 2002). Based on the bent conformation of the β-I-like domain that was crystallized in absence of ligand, the integrin in its bent form is presumed to be inactive **(Fig. 14)** (Hynes, 2002). Upon ligand binding or allosteric conformational changes coming from inside the cell, the extracellular arms straighten and the intracellular legs separate, which brings the integrin in an active state.

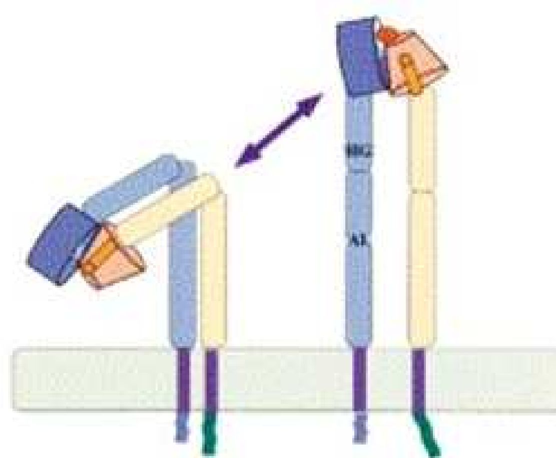

Fig. 14: Hypothetical model for active and inactive integrin conformations.
The integrin in its bent form is presumed to be inactive. Activation can occur either by ligand binding or by effects on the cytoplasmic domains, leading to straightening and separation of the legs. When the cytoplasmic domains separate, cytoplasmic proteins can bind to them and engage in signalling events. All these changes are reversible and can operate in either direction, allowing both outside-in and inside-out signalling (from Hynes, 2002).

Most of the integrin α or β subunits have conserved intracellular sequences proximal to the membrane. These motifs are phosphorylation sites and binding sites for intracellular proteins (Sakai et al., 1998). The binding of intracellular proteins to the integrin cytoplasmic region provokes the separation of the two subunits and activation of the integrin. At the proximity of the transmembrane region, three conserved and functional sequences are found in β subunits: the sequence KLLxxxxD, Cyto-1, where x represents any amino acid, and two NPXY motifs, Cyto-2 and Cyto-3 (O´toole et al., 1995). Also, the sequence KxGFFKR, found in all α subunits maintains a default inactive state when salt bridges are created with the adjacent β subunit.

Using beads coated with substrates or anti-β1 integrin subunit antibodies, Miyamoto and colleagues looked at the accumulation of intracellular proteins at the adhesion plaques via indirect immunofluorescence staining. The results showed that both receptor occupancy and clustering through extracellular molecules induce the accumulation of most cytoskeletal proteins around the activated integrins (Miyamoto et al., 1995). The initiation of a signal transduction cascade and the reorganization of the actin cytoskeleton are essentially orchestrated through the β subunit cytoplasmic domain that binds directly to several cytoskeletal proteins (Dedhar and Hannigan, 1996; Hemler, 1998, Van der Flier and Sonnenberg, 2001). Using peptide-binding or

immunoprecipitation studies, a direct binding of the β1A subunit to α-actinin, talin and filamin was demonstrated. These actin-binding proteins bridge integrins to the actin cytoskeleton directly or indirectly via vinculin or tensin.
Several kinases also bind to the β subunit cytoplasmic tail. The interaction of tyrosine kinase focal adhesion kinase (FAK) and paxillin with integrins was demonstrated *in vitro* with peptides derived from the cytoplasmic domains of β1A, β2 and β3 integrins (Schaller et al., 1995). However, no co-immunoprecipitation experiments *in vivo* could confirm that FAK or paxillin bind directly to integrins. ILK, integrin linked kinase, was shown to bind to β1A and β3A in the yeast-two hybrid interaction assay and in co-immunoprecipitation (Hannigan et al., 1996). ILK interacts with β-parvin in focal adhesions. ICAP-1, integrin cytoplasmic-domain-associated protein, interacts with β1A integrins, and shows phosphorylation upon cell adhesion to fibronectin (Hemler, 1998). Rack1, receptor for activated protein kinase C, binds to β1, β2 and β5 integrins (Liliental and Chang, 1998). Its binding with β1 integrins could be a bridge to protein kinase C (PKC).
In addition to tyrosine kinases such as FAK, the cellular adhesions possess high concentrated tyrosine phosphorylated proteins like Src and C-terminal Src kinase (CSK) and are rich in serine/threonine kinase (PKC, PAK), tyrosine phosphatases, modulator of small GTPases and other enzymes (PI3K, calpain). FAK, Src kinase, paxillin and vinculin have a pivotal role as multidomain proteins that interact with many structural as well as signalling proteins (Thomas and Brugge, 1997; Hanks and Polte, 1997; Schlaepfer et al., 1999; Turner, 2000; Zamir and Geiger, 2001b).

1.2.4. Transdominant negative effect of integrin cytoplasmic domains

Clustering of the receptors, through direct interaction between the β1 cytoplasmic regions, increases their avidity leading to signals different to those elicited by occupation of an isolated receptor. A potent trans-dominant negative effect of the β1 cytoplasmic domain was shown in studies where chimeras of cytoplasmic domains were used. When expressed at high level, β1 and β3 chimeric receptors function as dominant negative mutants by reducing integrin affinity leading to perturbation of cell spreading, inhibition of migration and ECM assembly (Chen et al., 1994; Laflamme et al., 1994, Lukashev et al., 1994; Mastrangelo et al., 1999). *In vivo*, different integrin subunits compete for common cytoplasmic binding partners that may be present in

limiting amount. Most cell types express a combination of several different integrins with an identical β subunit cytoplasmic domain. Thus, the presence of one dominant integrin may limit the activity of the others by competing for the same cytoplasmic binding partners.

The cytoplasmic tails of the integrin α subunits play also a critical role in the regulation of integrin-induced signal transduction. In particular, the intracellular tail of the integrin α3 subunit controls in a transdominant negative manner the α2β1, α5β1 and α6β1 integrins in the formation of adhesion complexes in cells adhering to laminins, collagens and fibronectin (Nguyen et al., 2000; Laplantine et al., 2000; Hodivala-Dilke et al., 1998). In another example, β1-null keratinocytes can adhere on fibronectin by recruiting alternative integrins like αvβ6, but their focal adhesions are enlarged and present reduced FAK/Src activity, this probably being the cause for the impaired cell migration (Grose et al, 2002; Raghavan et al., 2003).

1.2.5. Functions of laminin-binding integrins

In the epidermis, integrin expression is confined to the basal layer of keratinocytes (Watt, 2002). The two laminin-binding integrins, α3β1 and α6β4, are recruited to distinct locations. The α6β4 integrins are at the basal surface of keratinocytes in contact with laminin 5, while the α3β1 integrins are found at cell-cell contact sites. In human, loss of α6β4 integrins through mutations in the integrin α6 or β4 subunit leads to defective hemidesmosomes and to blistering skin diseases called junctional epidermolysis bullosa with pyloric atresia (Pulkkinen and Uitto, 1999).

In mice, ablation of either the α3, α6 or β4 gene results in perinatal lethality (Kreidberg et al., 1996; Georges-Labouesse et al., 1996; van der Neut et al., 1996; Dowling et al., 1996). Newborn mice lacking integrin α3 subunits display epidermal-dermal microblisters on footpads. Newborn mice with α6 or β4 gene mutations (Georges-Labouesse et al., 1996; Van der Neut et al., 1996) present more severe skin blistering due to impairment in the development of stable adhesion structures, the hemidesmosomes. Analysis of the basement membrane (BM) of the α3-deficient mice indicates a disorganization and rupture of the BM. α3 null keratinocytes do not spread on laminin 5, showing that α3β1 integrins are required for cell spreading on laminin 5 although the expression of α6β4 integrins is normal (DiPersio et al., 1997).

In addition, $\alpha3\beta1$ integrins also play a role in hair follicle morphogenesis (Conti et al., 2003).

Interestingly, mutant embryos that lack both the integrin $\alpha3$ and $\alpha6$ subunits displayed epidermal blistering but they show normal stratification and differentiation of the epidermis suggesting that these integrins are not essential for skin morphogenesis during embryonic development (DiPersio et al., 2000). As the adhesion of keratinocytes is in some way maintained while the epidermis matures, there must be an alternative receptor that accounts for cell adhesion. This receptor is not the $\alpha6\beta1$ integrin but possibly the $\alpha2\beta1$ integrin or dystroglycan, a non-integrin receptor for laminin (Henry and Campbell, 1998; Dipersio et al., 2000).

These results indicate that the function of the $\alpha3\beta1$ and $\alpha6\beta4$ integrins is to maintain the integrity of the epidermis, primarily in adult skin. They suggest that a function of $\alpha3\beta1$ integrins is to maintain the organization of the basement membrane, and that the $\alpha6\beta4$ integrins are responsible for stable cell adhesion and formation of hemidesmosomes.

1.2.5.1. $\alpha3\beta1$ integrin signalling

In vitro, $\alpha3\beta1$ integrins are found in small focal complexes in keratinocytes and other cells like fibroblasts (Dogic et al., 1998). The $\alpha3\beta1$ integrins show a particularly high affinity for laminin 5 and laminin 10/11, but not for other laminins, including laminin 1 (Eble et al., 1998). In contrast, the $\alpha6\beta1$ integrins bind to all laminin isoforms (Van der Flier and Sonnenberg, 2001).

Both the $\alpha3\beta1$ and $\alpha6\beta4$ integrins are involved in epithelial migration during wound healing (Goldfinger et al., 1999; Lotz et al., 2000). Just after wounding, the quiescent epidermal keratinocytes are activated and the ones located at the wound edge, i.e. exposed to the interstitial connective tissue, are transformed in migratory keratinocytes. In this situation of cellular remodelling, $\alpha3\beta1$ integrins are found at the basal surface of the cells and mediate cell motility in addition to promote gap junctional intercellular communication (Lampe et al., 1998).

In $\alpha3$-deficient keratinocytes, the formation of actin stress fibres and focal contacts is enhanced and there is an increase in fibronectin and collagen IV receptor activity (Hodivala-Dilke et al., 1998). Microinjection of peptides representing the cytoplasmic

tail of the integrin $\alpha3$ subunit in cells adhering to laminin 1 through $\alpha6\beta1$ integrins resulted in the disassembly of the $\alpha6\beta1$ integrin-specific thick adhesion complexes (Laplantine et al, 2000). Together these data suggest that the $\alpha3\beta1$ integrin is a regulator, in a transdominant manner, of cytoskeletal assembly. This regulatory role involves the cytoplasmic domain of the $\alpha3$ subunit that is able to specifically disrupt interactions between the cytoplasmic tail of the integrin $\beta1$ and structural proteins like vinculin (Laplantine et al., 2000).

In addition to focal complexes, $\alpha3\beta1$ integrins are frequently found at cell-cell contacts, where they colocalize with the tetraspanin CD151 and the urokinase receptor uPAR, a glycosylphosphorylinositol (GPI)-linked surface protein. Both proteins interact extracellularly with the integrin $\alpha3$ subunit (Wei et al., 2001; Zhang et al., 2003). The role of these interactions is still elusive. The engagement of $\alpha3\beta1$ and uPAR seems to lead to a loosening of cell-cell contacts and to an altered distribution of E-cadherin (Zhang et al., 2003). Interactions between $\alpha3\beta1$ and CD151 seem to be implicated in the formation of intercellular junctions (Chattopadhyay et al., 2003) as CD151 binding to $\alpha3\beta1$ positively regulates the association of cadherin-catenin complex with the actin cytoskeleton and cadherin-mediated cell-cell adhesion.

1.2.5.2. $\alpha6\beta1$ and $\alpha6\beta4$ integrin signalling

The $\alpha6\beta1$ integrin was first shown to be a receptor for the E8 fragment of laminin 1 (Aumailley et al., 1990). Later it was shown to be a receptor for all laminin isoforms. After treatment with PMA, the $\alpha6A$ subunit becomes phosphorylated on serine residues by the ser/thr kinase PKC (Gimond et al., 1995). This phosphorylation event is not required for the activation of the $\alpha6\beta1$ integrin, but rather for the modulation of the receptor affinity as cells with an $\alpha6A$ subunit mutated on serine residues adhere more extensively on laminin than the controls (Hogervorst et al., 1993).

The integrin $\beta4$ subunit differs from the other β subunits by the size of its cytoplasmic domain, which is much larger, approximately 1000 amino acids **(Fig. 15)**. It contains four fibronectin type III repeats grouped in two pairs separated by a connecting segment.

Although the involvement of α6β4 in the formation of hemidesmosomes is its predominant function, recent studies have revealed that it may be involved also in the migration of epithelial and epithelial-derived carcinoma cells **(Fig. 15)**. In invasive carcinomas, the expression of α6β4 integrin is maintained, although the hemidesmosomes are dismantled. In carcinoma cells, the localization of the α6β4 integrins is diffuse or located in membrane protrusions like lamellipodia and ruffles in comparison to a basal localization in normal epithelial cells (Mercurio and Rabinovitz, 2001). During wound healing, the hemidesmosomes disassemble and the α6β4 integrins are observed in lamellae of the cells at the wound edges, suggesting that they contribute to cell migration together with α3β1 integrins (Goldfinger et al., 1999; Lotz et al., 2000).

Figure 15 shows different signalling pathways involving the integrins α6β4 and leading to cell migration. Upon engagement of α6β4 integrins, the cytoplasmic domain of the β4 subunit is phosphorylated on serine, threonine and tyrosine residues (Mainiero et al., 1995). Under stimulation by growth factors, the β4 subunit is PKC-dependently phosphorylated on its serine residue leading to the dissociation of the hemidesmosomes and chemotactic migration (Rabinovitz et al, 1999). The α6β4 integrin has been shown to activate phosphoinositol-3-kinase (PI3Kinase) more effectively than other integrins and α6β4-mediated stimulation of PI3Kinase promotes α3β1-dependent cell spreading (Nguyen et al., 2000a; Shaw et al, 1997; Shaw, 2001). Two signalling events were shown to induce PI3Kinase **(Fig. 15)**. On the one hand, α6β4 integrins interact with erbB2, a receptor of the epidermal growth factor protein family on the surface of carcinoma cells. Expression of erbB2 is required for PI3Kinase activation. On the other hand, upon ligation of α6β4 integrin to laminin, phosphorylated β4 subunit associates with the insulin receptor substrate that binds and activates PI3Kinase. Finally, phosphorylated β4 subunit interacts with tyrosine phosphorylated Shc and Grb-2 adaptor protein, leading to the Ras and MAPK signalling cascade, which could participate in acto-myosin contraction and cell migration (Mercurio and Rabinovitz, 2001).

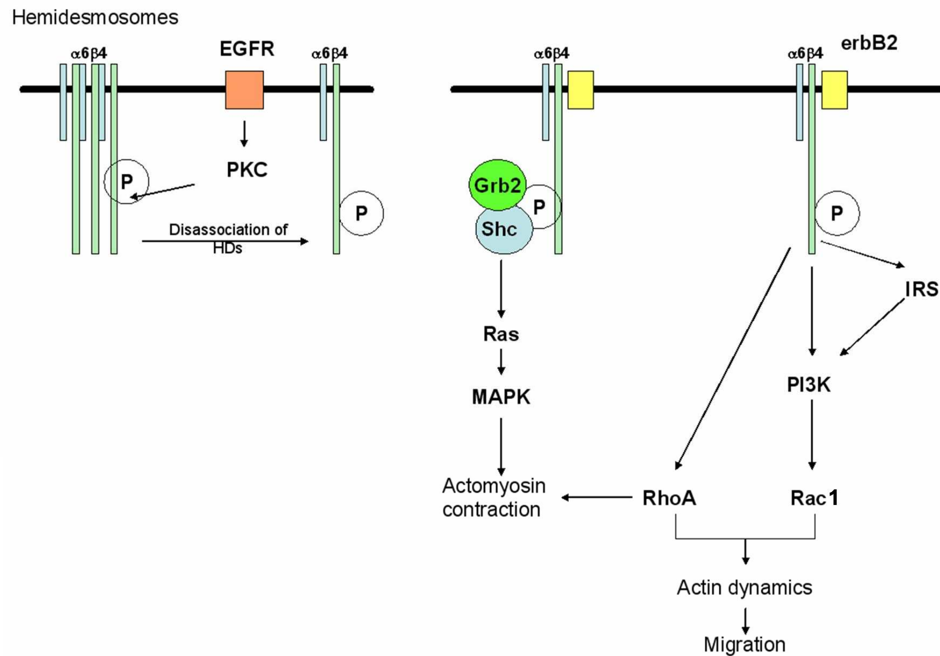

Fig. 15: Proposed signalling pathways initiated by α6β4 integrin activation and leading to cell migration.

Activation of Rac1 is linked to α6β4 stimulation of PI3Kinase. RhoA is also activated by α6β4 in carcinoma cells through a mechanism that involves cAMP and PKA (O´connor et al., 2000). RhoA is known to participate in acto-myosin contraction. In relation with it, using a traction-force detection assay, it was demonstrated that α6β4 integrins could transmit forces while remodelling the laminin 1-containing BM underneath the cells (Rabinovitz et al., 2001).

1.2.6. Modulation of cell migration

Cell migration, in term of dynamic cell adhesion, is dependent on the amount of immobilized substrate, cell surface integrin (avidity) and the activation state of the appropriate integrin (affinity) and follows a ball curve (Palecek et al., 1997). When the cell adhesiveness is too weak, the cells do not generate enough traction to move significantly. A too strong attachment to the underlying ECM results in the incapability of the adhesion plaques to dissociate. This means that there is an optimal adhesion level leading to an optimal migration. The extracellular matrix-integrins-cytoskeleton linkage is an important factor in the cell contraction process during cell migration (Choquet et al., 1997; Palecek et al., 1997). The contractile forces are transmitted

through the binding of the intracellular tail of integrin β1 subunits to cytoskeletal proteins. In case of a strong linkage, an efficient force can be applied.

The adhesiveness is also a matter of interactions between cell surface adhesion receptors and substratum as well as between receptors and the cytoskeleton. For example, during cell invasion, cells release proteases that degrade and remodel the ECM promoting the cell passage into the tissue. Also a change in integrin surface expression and distribution in cancer cells contributes to the invasive property (Hood and Cheresh, 2002). The extracellular cues like cytokines (hepatocyte growth factors/scatter factor, epidermal growth factors) and extracellular components (fibronectin, collagen, laminin and others) influence cell spreading and cell migration.

1.2.7. Models of epithelial cell migration *in vivo*

1.2.7.1. Wound healing

Skin injury is normally followed by rapid wound closure that can be divided in three phases: inflammation, granulation tissue formation and reepithelialization, tissue remodelling (Singer and Clark, 1999). Immediately after injury, there is extravasation of blood constituents from damaged blood vessels leading to the formation of a blood clot and later of a granulation tissue. In this provisional matrix, the cytokines secreted by platelets, macrophages, endothelial cells and fibroblasts, play a role in the initiation of wound vascularization, fibroblast proliferation and chemoattraction as well as reepithelialization. The latter is accomplished by the migration of epidermal cells in order to regenerate an epidermis and a dermal-epidermal junction. Epidermal cells begin to migrate between the collagenous dermis and the fibrin clot **(Fig. 16)**. To do this, they have to undergo phenotypic alteration such as dissolution of their hemidesmosomes from the basal surface and remodelling of their cell-matrix interactions, i.e. change in the expression of integrin receptors allowing interaction with the provisional extracellular matrix (dermal collagens, fibrin and fibronectin).

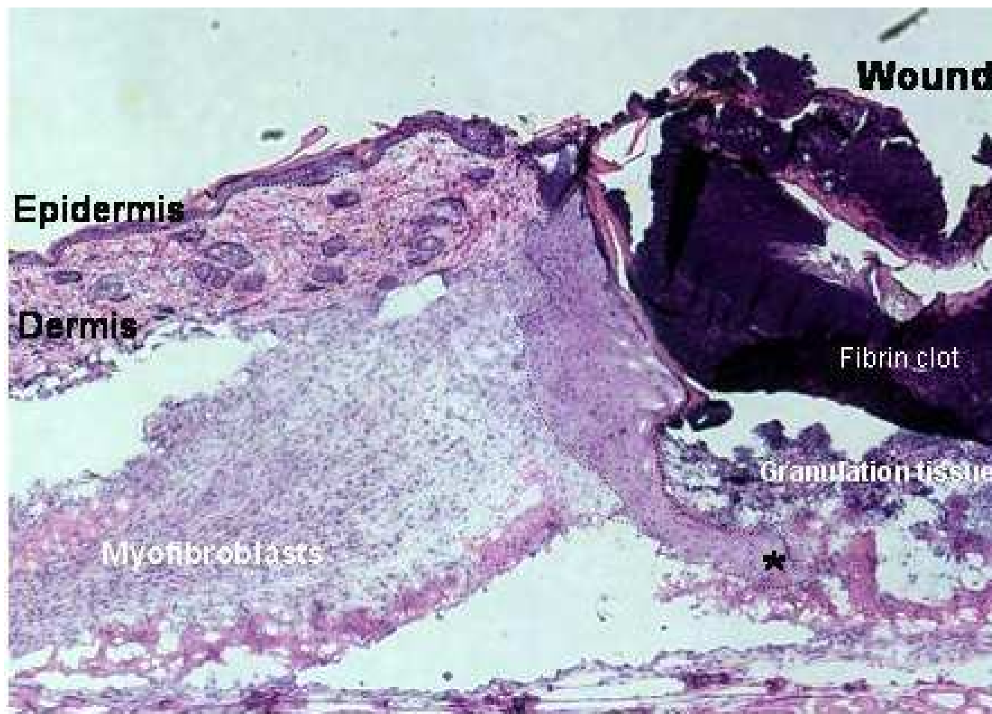

Fig. 16: Haematoxylin-eosin staining of a mouse wounded skin.
Five days after injury, the leading keratinocytes have infiltrated the dermis and the granulation tissue in form of a tongue (*) in order to close the wound.

One to two days after injury, epidermal cells behind the actively migrating cells at the wound margin begin to proliferate. As reepithelialization occurs, basement membrane proteins reappear, in particular laminin 5, the first keratinocyte secreted protein that accumulates in the basement membrane zone (Kainulainen et al., 1998; Singer and Clark, 1999; Nguyen et al., 2000, Laplante et al., 2001). Once firmly attached to the re-established basement membrane, epidermal cells revert to their normal phenotype.

1.2.7.2. The hair follicle

Like the epidermis, the hair follicle is a self-renewing tissue. However, the hair follicle regeneration depends on a tightly controlled growth cycle. This cycle is divided in 3 phases: anagen, catagen and telogen, characterised by a period of active growth, a period of regression and a resting phase, respectively (Stenn and Paus, 2001). Morphologically, the hair follicle in the anagen phase, as it reaches its full length, can be subdivided in four regions **(fig. 17)**: from the top to the bottom, the sebaceous gland region, the bulge region, the intermediate region and the bulb region with the papilla. From the hair shaft to the capsula, it consists of an epithelial core (inner root sheath to outer root sheath) surrounded by a mesenchymal sheet. The mesenchymal

cells are also found in the follicular papilla embedded in a loose connective tissue stroma.

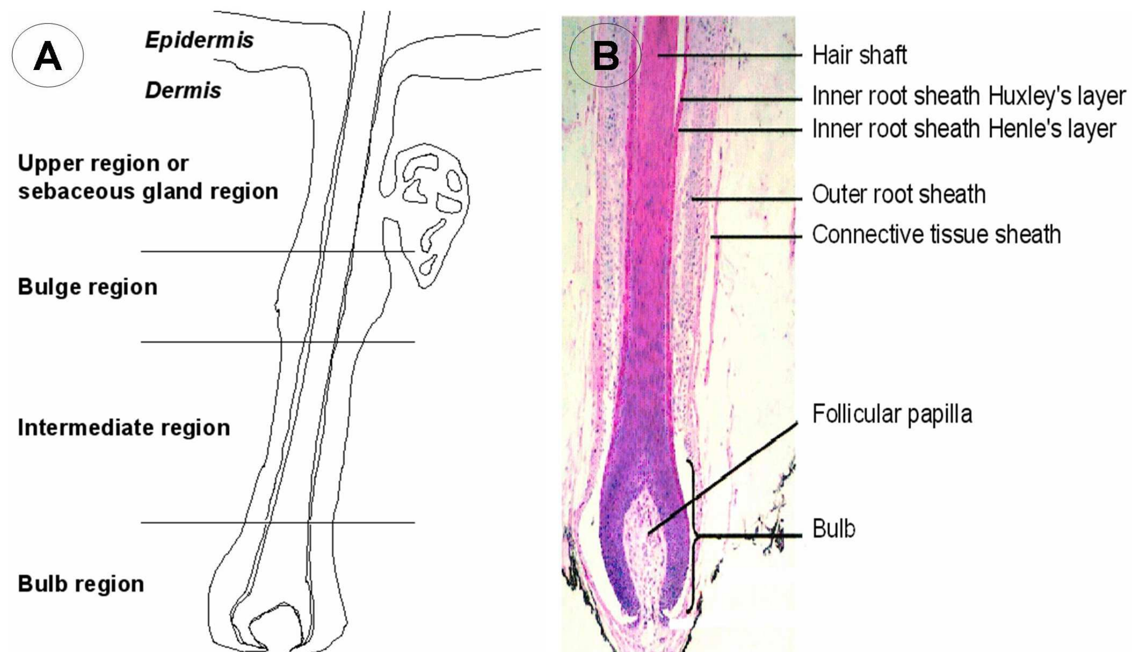

Fig. 17: (A) Schematic view of a hair follicle in anagen phase.
The different regions are indicated in the figure. **(B) Human skin follicle morphohistology** (from Stenn and Paus, 2001).

The morphogenesis and renewal of hair follicles are dependent on the cells located in the bulge region, which is a niche for multipotent stem cells capable of responding to signals to generate the hair follicle, the sebaceous glands and the epidermis (Lavker et al., 1991; Oshima et al., 2001). By studying the fate of the keratinocytes of the bulge, Oshima and collaborators showed that they have a high proliferative capacity characteristic of stem cells (Rochat et al., 1994; Oshima et al, 2001). These cells can migrate from the bulge region where they were issued along the entire length of the follicle until the papilla. Arrived in the root of the follicle, they would receive appropriate signals to contribute to the hair growth. Moreover, these multipotent stem cells also migrated to the upper part of the follicle to generate sebaceous glands and the epidermis.

1.3. Signalling through small GTPases

Previous studies have shown that different extracellular ligands can induce specific signalling involving small Rho GTPases and that the interplay between integrins and

the small Rho GTPases controls cell morphology (Schwartz and Shattil, 2000; Burridge and Wennerberg, 2004) and cell behavior (Zondag, 2000). However, these matrix-dependent signallings are not fully understood.
The small GTPases are monomeric G proteins of about 20 to 40 kDa. Expressed only in eukaryotes, they possess a GDP/GTP binding and a GTPase activity.
The small GTPase family can be divided in 5 main groups represented by Ras, Ran, Rab, Rho and Sar1/Arf (Kaibuchi et al., 1999; Takai et al., 2001). Of particular interest here, the Rho subfamily in human is composed of 20 members (Mackay and Hall, 1998; Burridge and Wennerberg, 2004). The Rho family of GTPases can be divided again in 5 subgroups which share high sequence similarities and exert related functions (Burridge and Wennerberg, 2004). Here, we will consider only RhoA, Rac1 and Cdc42. RhoA shares 45% homology with Rac1 and Cdc42. These two share 68% identity and bind to common effectors.

1.3.1. General properties of Rho GTPases

1.3.1.1. Structure of Rho GTPases

RhoA, Cdc42 and Rac1 crystallographic analysis have shown that they consist of 5 α-helices (A1 to A5), 6 β-strands (B1 to B6) and 5 most structurally conserved loops (G1 to G5). These features, regrouped in the 20-kDa catalytic G-domain, are conserved with other related small GTPases (Ihara et al, 1998; Paduch et al., 2001) **(Fig. 18)**.

Fig. 18: The most conserved structural elements of the G domain are the loops.
The common amino-acids to RhoA, Rac1 and Cdc42 are indicated in colour (inspired from Paduch et al., 2001).

The consensus NKXD sequence responsible for specific interaction with GDP/GTP and for GTPases activity is found in the G3 loop or Switch II. The P-loop or G1 domain has a consensus Walker A-motif GXXXXGK(S/T) responsible for the binding of α- and β-phosphate groups. The G3 loop or Switch II domain provides binding sites for Mg^{2+} and the γ-phosphate group. Mg^{2+} ion plays a key role in bringing

together the functional regions for phosphate binding of Switchs I and II domains. The Switch I and II loops are the effector binding regions (Fujisawa et al., 1998).
At the C-terminus, posttranscriptional modifications, in form of attachment of a lipid moiety and proteolysis, confer to the proteins the ability to anchor in the plasma membrane where they interact with their effectors. RhoA, Rac1 and Cdc42 possess a –CAAX sequence recognized by geranyl-geranyl-transferase 1 which covalently attaches 20-carbongeranyl-geranyl moieties to the cystein via a thioesther linkage (Casey and Seabra, 1996).

1.3.1.2. Localization of Rho GTPases

The Rho GTPases are believed to be widely distributed in cells and tissues although there are few suitable isoform-specific antibodies for immunolocalization.
According to Yonemura (Yonemura et al., 2004), Rho (referred as RhoA, B and C) is ubiquitously expressed in all tissues tested (small intestine, brain, salivary gland, lung, kidney, spleen). Only the smooth and skeletal muscles as well as cells in the cerebellum show a low level of Rho expression. In epithelia and cultivated epithelial cells, Rho is found at the lateral membranes. In fibroblasts, Rho is distributed all over the cytoplasm. Interestingly, there is accumulation of Rho at the cleavage furrow of cells during cytokinesis (Kosako et al., 2000; Yonemura et al., 2004).
Through video imaging using Fluorescent Resonance Energy Transfer method (FRET) in living cells, Itoh and colleagues (Itoh et al., 2002) could detect the highest Rac1 activity immediately behind the leading edge of HT1080 cells while active Cdc42 was concentrated at the tip of the leading edge of the cells. These results confirm those of Kraynov (Kraynov et al., 2000) on migrating Swiss 3T3 fibroblasts. These authors also pointed out to a localization of active Rac in the nucleus.
The distribution of Rho GTPases differs according to the cell type, suggesting different functional roles in various cells. Moreover, the localization of Rho GTPases depends on their functional state as they localize to the cytoplasm in the inactive form and translocate to the membranes when activated by extracellular stimuli (Yonemura et al., 2004; Fleming et al., 1996). At this level, an important role is played by the C-terminal posttranslational modifications in form of the isoprene moity (see before). This permits the active Rho GTPases to bind to the membrane and meet their downstream effectors. Integrin signalling induced upon cell adhesion to the ECM

regulate the distribution of lipid rafts and so the targeting of Rac1 and RhoA to the plasma membrane (Guan, 2004; del Pozo et al., 2004; del Pozo, 2004). When the cells detach, the small GTPases are internalized in the cytoplasm.

1.3.1.3. Cycling between active and inactive form

The small GTPases are monomeric G proteins related to the heterotrimeric G proteins such as Gs and Gi as both groups serve as molecular intermediates that transduce an upstream signal to downstream effectors. They both can switch between an active state, when GTP (guanosine 5´-triphosphate) is bound, and an inactive state, when GDP (guanosine 5´-diphosphate) is bound. However, contrary to the heterotrimeric G proteins, the monomeric G proteins need regulatory proteins to modulate the transition between the active and the inactive state **(Fig. 19)**. The regulatory proteins are of three sorts: the GTPase-Activating Proteins (GAPs), the Guanine Exchange Factor (GEFs) and the GTPase Dissociating Inhibitors (GDIs). Some regulators are specific for one small GTPase, others are common to several of them.

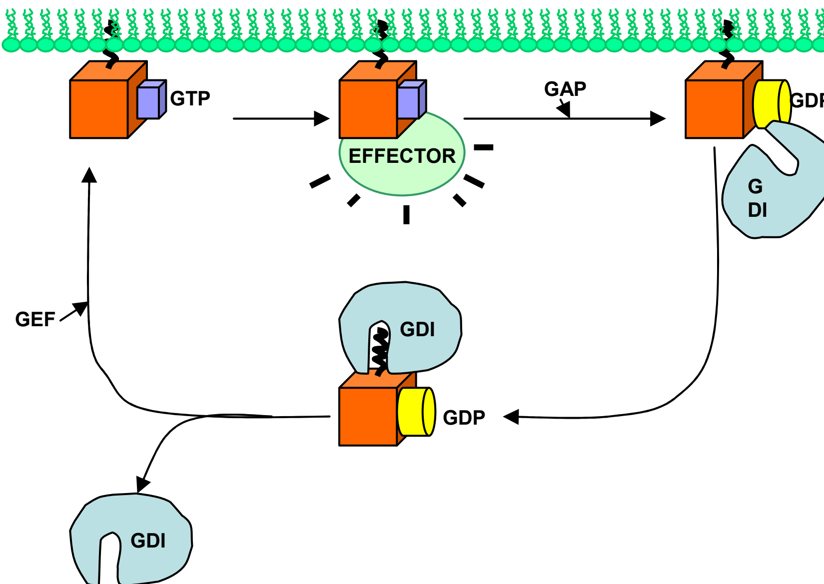

Fig. 19: Activation and inactivation of small GTPases.
GAPs stimulate the intrinsic low GTPase activity to promote the conversion of the GTP-bound to the GDP-bound form. GDIs associate with GDP-bound form and maintain the GTPases in their inactive state in the cytosol. After release of GDI, GEFs can catalyse the exchange of GTP to GDP, permitting the activation of the GTPases and binding to effectors at the cell membrane. (GTP: Guanosine-5'-triphosphate, GDP: Guanosine-5'-diphosphate, GAP: GTPase-activating protein, GDI: GTPase dissociating inhibitor, GEF: Guanine exchange factor)

In resting cells, small G proteins are inactive in the cytosol complexed with RhoGDI. The latter can bind to the Rho protein which is anchored in the cellular membrane and it stimulates its release through transfer of its isoprenoid moiety from the membrane to a hydrophobe pocket within the GDI (Olofsson, 1999; Hoffman et al., 2000). Some unknown proteins (probably of the ERM family) liberate RhoGDI from the small GTPase that can then interact with a GEF.

GEF interacts with GDP-bound form as an intermediate complex and stimulates the dissociation of GDP and the subsequent uptake of GTP from the cytosol. GEFs share a common sequence motif, the Dbl-homology (DH) domain, which catalyses nucleotide exchange, and a pleckstrin homology (PH) domain, which localizes Rho GEFs near their downstream effectors. Various PH domains bind to membrane phosphoinositides, filamentous actin (Yao et al., 1999), or actin-binding proteins (Umikawa et al., 1999; Bellanger et al., 2000). So GEFs can act as a scaffold to guide the G proteins to a specific subcellular localisation where specific receptors are present.

As GTP is present in the cytosol at a 10 times higher concentration than GDP, the small GTPase would remain constantly active. Therefore, the inactivation of small G proteins is made by GAPs, which are negative regulators of the Rho protein pathways. They increase the rate of hydrolysis of bound GTP to GDP by stimulating the intrinsic GTPase activity of the small G proteins. The GAPs share a related GAP homology domain that is responsible for the binding of GTP-bound Rho protein and the accelerating of their GTPase activity.

1.3.1.4. Effector binding domains

When activated, i.e. in GTP-bound form, the Rho GTPases can interact with their specific effectors. The binding domain of the effectors is not unique but differ depending on their binding to RhoA, Rac1 or Cdc42 or if they are common to Rac1 and Cdc42.

Proteins containing the CRIB motif are potential binding partners for active Rac1 and Cdc42 (Burbelo et al., 1995; Cotteret and Chernoff, 2002). CRIB stands for Cdc42/Rac-interactive binding domain, also known as PBD domain (p21binding domain), which sequence is I-S-X-P-$(X)_{2\text{-}4}$-F-X-H-X-X-H-V-G. It interacts with the

domains called switch I and switch II of Rac1 and Cdc42, which are highly similar (Morreale et al., 2000).
Using yeast two–hybrid screening with RhoA as a bait, different RhoA target proteins and, subsequently, three classes of Rho-binding motifs could be identified: the class I Rho-binding domain (RBD) or REM-1 (for Rho effector motif class 1) is 70 amino acid-long and is located at the N-termini of PKN (protein kinase N), Rhophilin and Rhotekin. The class II Rho-binding motif is 81 amino acid-long and is located between a coiled-coil structure and a pleckstrin homology domain of ROCK (Rho-associated coil-coiled containing protein kinase). Finally, the third class of Rho-binding motifs is found within the coiled-coil region of Citron (Reid et al., 1996; Tapon and Hall, 1997; Fujisawa et al., 1998):

1.3.2. Role of RhoA, Rac1 and Cdc42 in cell migration

Because the members of the small Rho GTPase family interact with many different downstream effectors, they are involved not only in regulation of actin-containing structures but also in phagocytosis, neuronal morphogenesis (Luo, 2000), gene expression, cell cycle progression through G1 (Olson et al., 1995), differentiation, endocytosis and tumour progression (Sahai and Marshall, 2002). Rho GTPases and integrins, which both are connected to actin-containing structures, play a key role to integrate signals from the front to the back of the cell during cell migration (Ridley et al., 2003). An important role of the Rho proteins is to modulate not only cell-substrate adhesion but also cell-cell adhesion.
Early in the 90´s, **RhoA** was shown to mediate maturation of focal adhesions and actin stress fibre-associated contractility (Ridley and Hall, 1992) **(Fig. 20)**. RhoA activates the Rho-Kinase and so stimulates contractility. On the one hand, Rho-Kinase phosphorylates and therefore inhibits the myosin light chain (MLC) phosphatase and on the other hand, Rho-Kinase activates the MLC kinase leading to increased MLC phosphorylation. The result of both effects is an increase in the tension which promotes the assembly of actin stress fibres and integrin clustering in focal adhesions (Chrzanowska-Wodnicka and Burridge, 1996). Dia 1, LIM Kinase (LIMK) and phosphatidyl inositol 4- phosphate 5-kinase (PI(4)P5K) are targets of RhoA and contribute also to actin polymerization and stress fibre formation. Dia1, from the formin family, is a factor capable of initiating new actin filaments (Higashida

et al., 2004). It binds to actin-monomer-binding protein profilin and to IRSp53 (53 kDa insulin receptor substrate protein), which interacts with WAVE/Scar, and so stimulates actin nucleation (Watanabe, 1999; Ridley, 2001). LIMK, a target of Rho-Kinase, inhibits through phosphorylation Cofilin/ADF, a severing protein, permitting the stabilisation of existing actin filaments. Rho stimulates the activity of PI45K leading to the production of the lipid PIP2 (phosphatidylinositol-4,5-bisphosphat), a partner of several actin-binding proteins, in particular of the actin capping protein gelsolin. The interaction between PIP2 and gelsolin leads to the liberation of the barbed ends of actin filaments and the addition of new monomers of actin.

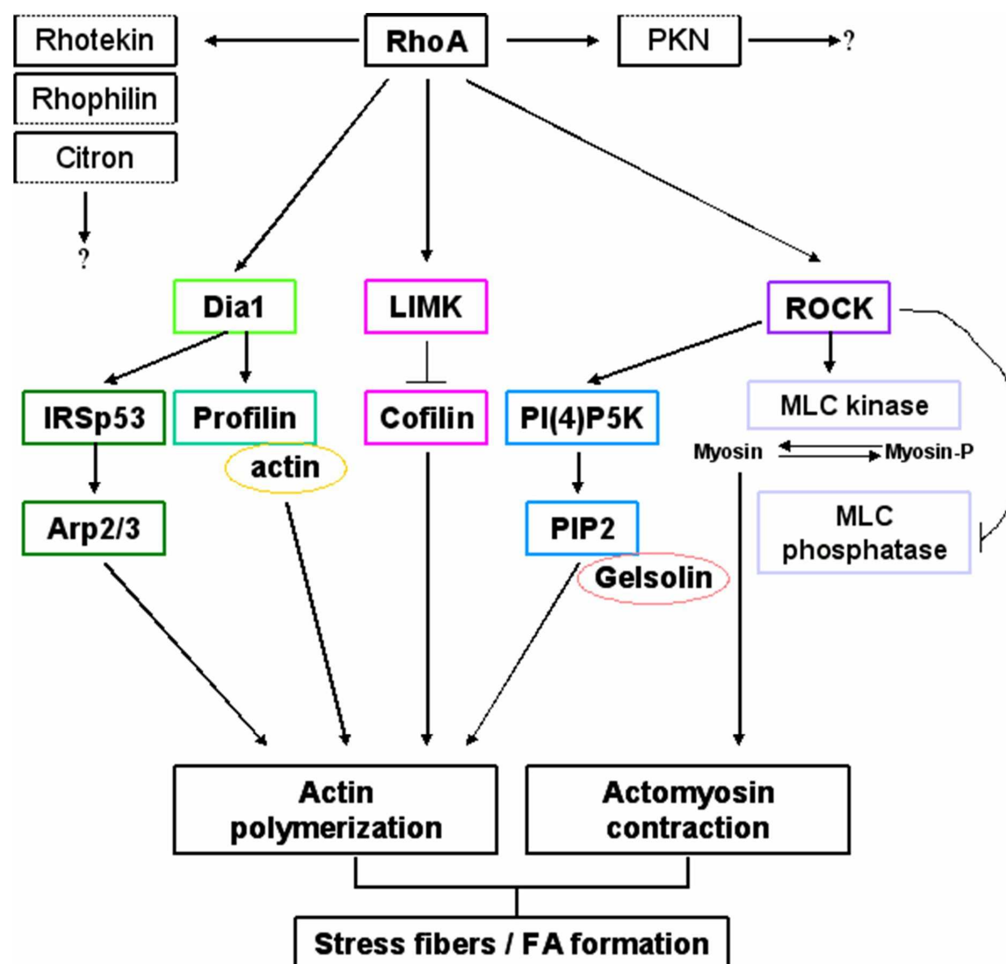

Fig. 20: RhoA downstream signalling involved in the formation of actin stress fibers and focal adhesions.

Rac1 regulates the formation of cellular protrusions, such as lamellipodia and ruffles, associated with focal complexes (Ridley et al., 1992). The formation of lamellipodia and ruffles is directed by different signalling pathways, all leading to actin polymerization **(Fig. 21)** and inhibition of stress fibre formation (Burridge and

Wennerberg, 2004). Several of these pathways are common to Rho signalling pathways (activation of PI(4)P5-K, IRSp53 and LIMK) or antagonizing them (Inhibition of MLCK).

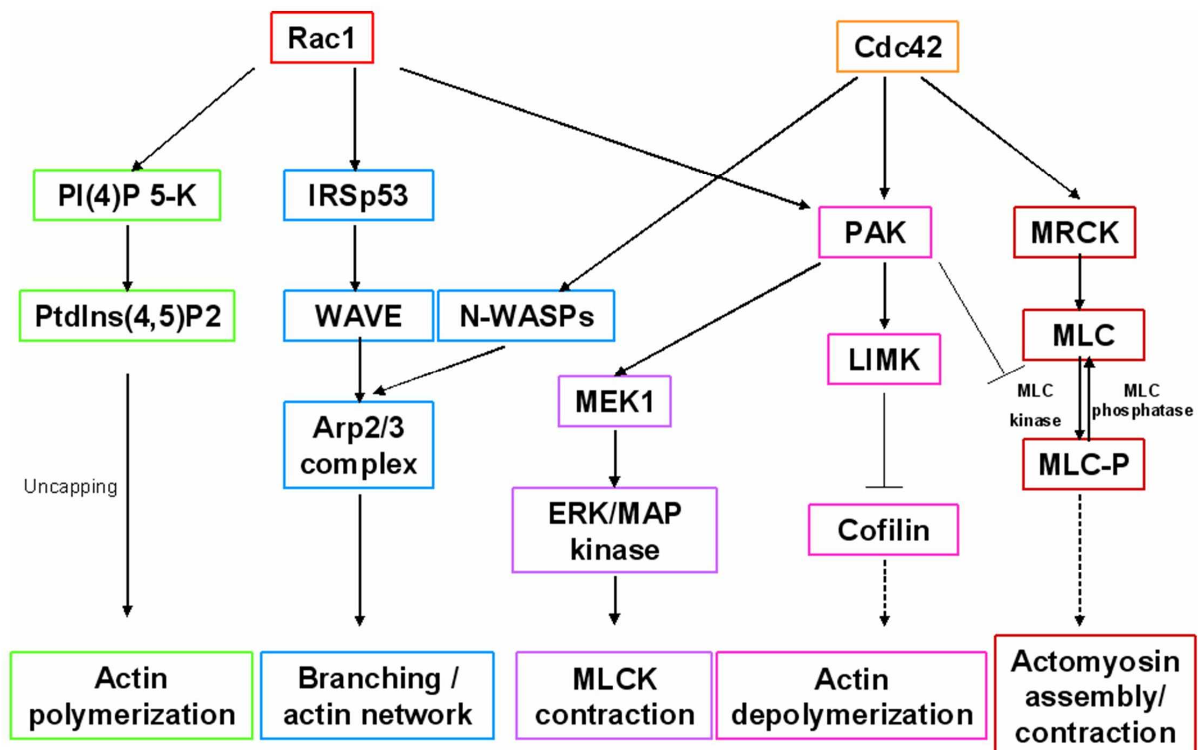

Fig. 21: Rac1 and Cdc42 act upstream of several effectors leading to actin polymerization and actomyosin contraction.

Rac1 modulates the initiation of actin polymerization by interacting with actin regulatory proteins like the scaffold protein IRSp53 (insulin receptor substrate of 53 kDa) or Nap125 (NCK-associated protein of 125 kDa) and PIR121 (p53 inducible RNA), which all bind to the nucleation promoting factor WAVE (Takenawa and Miki, 2001). Active WAVE will activate the Arp2/3 complex and promote the formation of a branched actin filament network towards the plasma membrane (Ridley, 2001). **Cdc42** pathway meets the one of Rac1 as it interacts with the Wiscott-Aldrich syndrome proteins WASPs. These proteins in turn bind to the Arp2/3 complex **(Fig. 21)** (Erickson and Cerione, 2001). An important partner of Rac1 is PAK (p21-activated kinase) which is also an effector for Cdc42. It participates in the formation of filopodia and ruffles and of focal complexes (Schmitz et al., 2000). It has been demonstrated that PAK phosphorylates and, in doing so, inactivates the MLC kinase, which result in a decrease in MLC phosphorylation. This leads to the disassembly of actin stress fibres and focal adhesions.

1.3.3. Rho GTPases and epithelial cells

In epithelial cells, the Rho GTPases modulate cell morphology and migration by regulating also the formation of cell-cell adhesions (Evers et al., 2000; Fukata and Kaibuchi, 2001; Jamora and Fuchs, 2002).

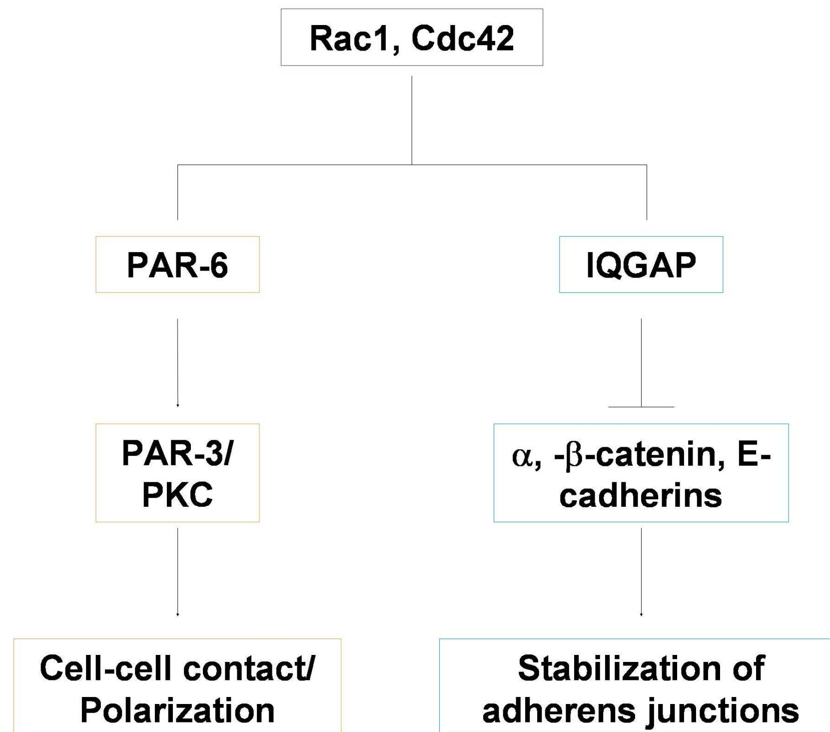

Fig. 22: Rac1 and Cdc42 common effectors implicated in the maintenance of cell-cell adhesion.

Rac1 and Cdc42 activation is linked to the establishment of polarity and cell-cell contacts and, therefore, they inhibit epithelial migration **(Fig. 22)**. On the one hand, Rac1 and Cdc42 form a complex with PAR6 (partitioning defective 6) and PAR3 (partitioning defective 3) and participate in the maintenance of tight junctions (Erickson and Cerione, 2001). On the other hand, activated Rac1 and Cdc42 are regrouped at E-cadherin containing adherens junctions by upstream effectors like Tiam-1 (Hordijk, 1997; Sander et al, 1998). Furthermore, phosphoinositol-3-phosphate (PI3Kinase), activated by E-cadherin, recruits Rac1 as well as Cdc42 at this location (Kodama et al., 1999). Together, Rac1 and Cdc42 trigger signals, which positively regulate cell-cell adhesions through indirect action on the cadherin-catenin complex. This occurs by the association of activated Rac1 and Cdc42 with IQGAP-1 (IQ motif containing GTPase activating protein 1), preventing it from interacting with β-catenin and so destabilizing the cadherin-catenin complex (Fukata et al., 1999).

RhoA influence on cell-cell contacts is not clear; it might affect E-cadherin mediated adhesive activity through an effect on the actin cytoskeleton (Fukata and Kaibuchi, 2001). As the cells become confluent and form cell-cell contacts, RhoA activity is drastically reduced (Noren et al., 2001; Noren et al., 2003).

1.3.4. Extracellular matrix, integrins and Rho GTPases

Several ECM molecules have been shown to modulate the activity of Rho GTPases. In the case of ECM reorganization like during wound healing, the new extracellular matrix or provisional matrix, could influence the behaviour of the cells by activating the Rho GTPases (Nguyen et al., 2001). In quiescent epidermis, the keratinocytes adhere on a matrix rich in laminin 5 via $\alpha 6\beta 4$ integrins and the activity of RhoA is low. After wounding, the leading keratinocytes migrate over the provisional matrix, principally made of collagens, via $\alpha 2\beta 1$ integrins, and the activity level of RhoA is increased. Another example of ECM molecules regulating Rho GTPases was reported: laminin 2 signalling leads to reduced activity of RhoA, promoting smooth muscle myogenesis (Beqaj et al., 2002).
The modulation of Rho GTPases through integrin-mediated adhesion to fibronectin was intensively studied. It has been shown that on fibronectin, Rac1 and Cdc42 participate in cell spreading (Price et al., 1998), while the formation of focal contacts is RhoA-dependent (Barry et al., 1997; Ren et al., 1999). Interestingly, the GTPase signalling cascade activated by $\alpha 5\beta 1$ integrins is distinct from that activated by $\alpha v\beta 3$ integrins, which are both receptors for fibronectin. The organization of cell-matrix adhesions and matrix remodelling involving either one of those receptors are also different (Danen et al., 2002). Thus, stimulation of the Rho GTPases by initial cell adhesion enhances integrin function and clustering which could subsequently promotes a wave of integrin-dependent signals leading for example to FAK activation or phosphorylation of paxillin and p130Cas resulting in cell migration.

1.4. Aim of the work

Epithelial cells maintain a stable connection to the basement membrane through interactions of transmembrane proteins with extracellular matrix and cytosolic components. In resting epidermis, the basal keratinocytes are firmly attached to the underlying matrix through epithelium-specific attachment points, the

hemidesmosomes, the loss of which is a prerequisite for cell motility (Jones et al., 1998). In the hemidesmosomes, one of the transmembrane receptor is the $\alpha 6\beta 4$ integrin connecting the intracellular keratin intermediate filament system to the extracellular matrix, in particularly to laminin 5 (Jones et al., 1998; Nievers et al., 1999). The other transmembrane proteins within the hemidesmosomes are collagen XVII and the tetraspanin CD151 (Borradori and Sonnenberg, 1996, Sterk et al., 2000). Based on genetic studies of inherited human disorders, it is predicted that these transmembrane proteins are involved in cell adhesion to the basement membrane.

In the first part of this work, I adressed the question whether these transmembrane proteins, which must be dynamic during tissue remodelling, are involved in the regulation of keratinocyte migration. I first analysed by immunofluorescence the distribution of the transmembrane proteins *in vivo* in resting epidermis and in remodullating tissue like in wound healing or in the hair follicle. To test whether collagen XVII can regulate keratinocyte behavior, I compared the migration of collagen XVII-deficient keratinocytes from non-lethal junctional epidermolysis bullosa patients with that of normal human keratinocytes using a novel migration assay. The role of CD151 in the migration of epithelial cells was investigated *in vitro* using a monoclonal antibody against CD151, TS151r, which epitope overlaps with the tetraspanin integrin-binding site.

In the second part of this work, the goal was to study the role of Rho GTPases in cell migration on laminins. In the laboratory, it has been previously shown that particular adhesion complexes are formed according to the laminin isoform involved in cell adhesion (Dogic et al., 1998, 1999, Laplantine et al., 2000). More specifically, fibroblasts and epithelial cells adhering on laminin 5 (activation of $\alpha 3\beta 1$ integrins) develop lamellipodia and tiny vinculin patches, while on laminin 1 (activation of $\alpha 6\beta 1$ integrins), the same cells form distinct actin stress fibers and thick vinculin aggregates. We hypothesized that these differences could be explained by the activation of different Rho GTPases, which act on the actin cytoskeleton and adhesion plaque formation. I therefore tested whether laminin 1 and laminin 5 activate RhoA, Rac1 and Cdc42 differently during cell adhesion using pull-down assays. To better dissect the molecular mechanism implying different Rho GTPases in the specific signalling by laminin isoforms, I used RNAi down-regulation of RhoA,

Rac1 and Cdc42 in epithelial cells to determine their role in epithelial cell adhesion, spreading and migration on laminins.

2. MATERIALS AND METHODS

2.1. Materials

2.1.1. Reagents

Reagent	Supplier
Acetic acid	*Merck KGaA, Darmstadt, Germany*
Acrylamide Protogel 30:0.8AA/Bis-AA	*National Diagnostics, Biozym Scientific, Oldendorf, Germany*
Ammoniumpersulfate	*Serva, Heidelberg, Germany*
Ampicilline	*Serva, Heidelberg, Germany*
Aprotinin	*Sigma-Aldrich Chemie, Schnelldorf, Germany*
Bromophenol blue	*Sigma-Aldrich Chemie, Schnelldorf, Germany*
BSA Bovine Serum Albumin	*Sigma-Aldrich Chemie, Schnelldorf, Germany*
Coomassie-brilliant blue	*Serva, Heidelberg, Germany*
DAKO mounting medium	*DAKO, Hamburg, Germany*
DMSO Dimethyl sulfoxide	*Merck KGaA, Darmstadt, Germany*
DMEM	*Biochrom, Berlin, Germany*
DTT	*Sigma-Aldrich Chemie, Schnelldorf,Germany*
ECL chemiluminiscence detection system	*Amersham Biosciences, Braunschweig, Germany*
EDTA	*Merck KGaA, Darmstadt, Germany*
EGTA	*Roth GmbH, Karlsruhe, Germany*
Ethanol	*Riedel-de-Haen, Seelze, Germany*
Fetal Calf Serum (FCS)	*Biochrom, Berlin, Germany*
Glucose	*Merck KGaA, Darmstadt, Germany*
Glutathione Sepharose™ 4B	*Amersham Biosciences, Braunschweig, Germany*
Glutaraldehyde 25%	*Serva, Heidelberg, Germany*
Glycine	*Merck KGaA, Darmstadt, Germany*
Glycerol	Fluka, *Sigma-Aldrich, Schnelldorf, Germany*
β-glycerophosphate	*Sigma-Aldrich Chemie, Schnelldorf, Germany*
IPTG	*Sigma-Aldrich Chemie, Schnelldorf, Germany*
Isopropanol	*Fluka, Sigma Aldrich, Schnelldorf, Germany*
HEPES	*Serva, Heidelberg Germany*
Hydrochloric acid	*Merck KGaA, Darmstadt, Germany*
LB medium	*Invitrogen, Karlsruhe, Germany*
Low molecular weight marker for proteins	*Amersham Biosciences, Braunschweig, Germany*
β-mercaptoethanol	*Fluka, Sigma Aldrich, Schnelldorf, Germany*
Methanol	*Riedel-de-Haen, Seelze, Germany*
Milk powder	*Heirler Cenovis, Radolfzell, Germany*
Paraformaldehyde	*Fluka, Sigma Aldrich, Schnelldorf, Germany*
PEG	*Serva, Heidelberg, Germany*
PMSF	*Sigma-Aldrich Chemie, Schnelldorf, Germany*
Ponceau S Solution for electropheresis	*Serva, Heidelberg, Germany*
SDS	*Serva, Heidelberg, Germany*
Sodium deoxycholat	*Sigma-Aldrich Chemie, Schnelldorf, Germany*
Sucrose	*Merck KGaA, Darmstadt, Germany*
TEMED	*Sigma-Aldrich Chemie, Schnelldorf, Germany*

Triton-X100 *Fluka, Sigma Aldrich, Schnelldorf, Germany*
TRIZMA Base *Sigma-Aldrich Chemie, Schnelldorf, Germany*
Developper solution *Fujifilm Europe GmbH*
Fixer solution *Fujifilm Europe GmbH*

2.1.2. Laboratory materials

Centrifugation tubes, 15 and 50 ml *Greiner Bio-one, Frickenhausen, Germany*
Microcentrifuge tubes, 0.5, 1.5 and 2.2 ml *Sarstedt, Numbrecht, Germany*
Micropipette tips Bio-One Cellstar *Greiner Bio-one, Frickenhausen, Germany*
Filter micropipette tips *Greiner Bio-one, Frickenhausen, Germany*
Pipette Falcon *Amersham Biosciences, Braunschweig, Germany*
Glass pasteur pipette *VWR International GmbH, Darmstadt, Germany*
Sterile filter 0.2 µm *Schleicher and Schüll, Dassel-Relliehausen, Germany*
3MM filter paper *Whatman, Hagen, Germany*
Nitrocellulose transfer membrane Protran *Schleicher and Schüll, Dassel-Relliehausen, Germany*
X-film cassette *Rego, Augsburg, Germany*
Biomax XAR film 18x24 cm *Kodak, Stuttgart, Germany*
Glass coverslips *Marienfeld, Lauda-Königshofen, Germany*
Histoslides 75x25 mm *Shandon, Frankfurt, Germany*
Cell culture dish Ø60mmx17mm *Falcon, Heidelberg, Germany*
Cell culture dish Ø90mmx17mm *Falcon, Heidelberg, Germany*
Tissue culture plates 6, 24, 96 wells *Costar, Bodenheim, Germany*
Cryotubes, 1 ml *Nunc, Wiesbaden, Germany*
Neubauer chamber *Brand, Wertheim, Germany*
Tissue-Tek *Sakura, Vogel GmbH, Giessen, Germany*

2.1.3. Equipment

Microcentrifuge 5415C *Eppendorf, Hamburg, Germany*
Centrifuges (table-top, cooling, low speed)
Megafuges 1.0R *Heraeus, Hanau, Germany*
Ultracentrifuge (cooling) Biofuge primo R *Heraeus, Hanau, Germany*
Ultracentrifuge (cooling) J2-21 *Beckman Coulter GmbH, Krefeld, Germany*
Ultracentrifuge (cooling) J2-HS *Beckman.CoulterGmbH,Krefeld, Germany*
Ultracentrifuge (cooling) 3K-12 *Sigma-Aldrich, Schnelldorf,Germany*
Centrifuge rotor JLA-10500 *Beckman Coulter GmbH, Krefeld , Germany*
Centrifuge rotor JA-20 *Beckman Coulter GmbH, Krefeld , Germany*
Centrifuge rotor JS-13.1 *Beckman Coulter GmbH, Krefeld , Germany*
Leica CM3050 Cryotom *Leica Microsystems, Heidelberg, Germany*
Electrophoresis power supply *Bio-Rad Laboratories GmbH, München, Germany*
Power-pac-200 *Bio-Rad Laboratories GmbH, München, Germany*
Glass plates *Bio-Rad Laboratories GmbH, München, Germany*
Heating block DB-3D *Techne, England*
Incubator Lab-Therm *Kühner, Burkhardtsdorf, Germany*

CO_2-incubator	*Heraeus, Hanau, Germany*
Laminar flow, Hera-safe	*Heraeus, Hanau, Germany*
Water bath Type 1083	*GFL, Burgwedel, Germany*
Cryostat CM 3050	*Leica instruments, Heidelberg, Germany*
Freezer -80°C	*Heraeus, Hanau, Germany*
Vortex Genie 2	*Scientific Industries*
pH-meter Delta 320	*Mettler Toledo, Niederlande*
Sonicator, Ultra turrax T25	*IKA Labortechnik, Staufen, Germany*
X-ray film developing machine RGII	*Fujifilm Europe GmbH*
Biophotometer	*Eppendorf, Hamburg, Germany*
Elisa Reader	*Labsystems Multiskan MS*
Ice machine	*Ziegra, Isernhagen, Germany*
Light Microscope Axiovert S100TV	*Zeiss, Oberkochen, Germany*
Fluorescence Microscope Axiophot	*Zeiss, Oberkochen, Germany*
Confocal laser scanning microscope	*Leica Microsystems, Heidelberg, Germany*
Video Camera Xillix Microimager	*Zeiss, Oberkochen, Germany*
Tempcontrol 37-2 digital	*Zeiss, Oberkochen, Germany*
CTI-controller 3700	*Zeiss, Oberkochen, Germany*
Camera Power-Shot G5	*Canon, Krefeld, Germany*
PC P3	*DDS, Siemens*
Power Macintosh G3	*AppleVision, USA*
Microsoft Powerpoint	*Microsoft, USA*
Microsoft Excel	*Microsoft, USA*
Photoshop	*Adobe, USA*
Openlab 1.7.8.	*Improvision, Heidelberg, Germany*
Scion Image software	*Scion Corporation, USA*

2.2. Cell culture

2.2.1. Cell lines

Two cell lines have been used in most of the experiments: spontaneously immortalized human keratinocytes HaCaT and SV40-transformed lung Wi26 fibroblasts. The HaCaT cells present the phenotype of normal keratinocytes and are suited for *in vitro* studies of regulatory mechanisms of keratinocytes (Breitkreutz et al., 1998). Wi26 cells are a non-tumor cell line closely resembling dermal fibroblasts.

Both cell lines were cultured in Dulbecco´s modified Eagle medium (DMEM) containing 4.5 g/l glucose and supplemented with 10% fetal calf serum, 2 mM glutamine, penicillin (100 U/ml) and streptomycin (100 µg/ml) at 37°C in humidified air containing 5% CO_2. The growth and the morphology of the cells were regularly controlled using an inverted phase contrast micoscope. When the cells reached confluency, they were passaged.

Primary keratinocytes and collagen XVII-deficient keratinocytes were kindly provided by Dr. L. Bruckner-Tuderman and maintained in low-calcium, serum-free, keratinocyte growth medium. Cells in the second and third passages were used for experiments.

2.2.2. Cell culture methods

Passaging:

When the cells had grown to confluency, they were washed once with sterile phosphate-buffered saline (PBS) (137 mM NaCl, 2.7mM KCl, 4.3 mM $Na_2HPO_4.7H_2O$, 1.4 mM KH_2PO_4, pH 7.4) and detached from the surface using a minimal volume of 0.05% trypsin and 0.02% EDTA in PBS, pH 7.4. Fresh medium supplemented with serum was then added to neutralize the action of the trypsin. An appropriate volume of cells were redistributed and diluted in new tissue culture dishes in either 3 ml or 10 ml final volume for 6-cm dishes or 10-cm dishes, respectively.

Storage:

Confluent cells were detached from their culture support as described above. The cells were collected by centrifugation at 1000 rpm for 5 min and resuspended in 90% serum and 10% DMSO, used as cryoprotective agent, and transferred to cryotubes. Cells were then slowly cooled down to -80°C in a cryobox containing isopropanol. 24 hours later they were placed in liquid nitrogen for longer conservation.

Thawing:

Frozen cryotubes containing the cells were rapidly defrosted in a 37°C water bath. The suspension was diluted in complete DMEM medium, and centrifuged at 1000 rpm for 5 min to remove DMSO. The cells were then resuspended in fresh complete medium.

Cell counting:

Quantification of cell numbers was needed for some experiments like the adhesion assays, which required identical assay conditions. Cells were trypsinized, collected

upon centrifugation (1000 rpm, 5 min) and resuspended in a small volume of medium (5 ml for a 10-cm plate). An aliquot was introduced in a Neubauer chamber with a pipette via capillarity. With a x10 objective, cells were counted within 8 fields of a 1 mm^2 area. The averages of the counts were multiplied by 10^4 giving the cell density in 1 ml.

2.3. Immunofluorescence labelling

2.3.1. Antibodies

Antibody	Source
FITC-phalloidin	*Sigma-Aldrich Chemie, Schnelldorf, Germany*
mAb P1B5, anti-α3 integrin	*Chemicon Europe, Hofheim, Germany*
pAb 1920, anti-α3 integrin	*Chemicon Europe, Hofheim, Germany*
mAb P1E6, anti-α2 integrin	*Chemicon Europe, Hofheim, Germany*
mAb GoH3, anti-α6 integrin	*A. Sonnenberg, Division of Cell Biology, Cancer Institut, Amsterdam, The Netherlands*
mAb 9EG7, anti-activated β1 integrin	*D. Vestweber, Institut for Cell biology, ZMBE, Münster, Germany*
mAb P4C10, anti-β1 integrin	*Chemicon Europe, Hofheim, Germany*
mAb 1997, anti-β1 integrin	*Chemicon Europe, Hofheim, Germany*
K20-FITC, anti-β1 integrin	*DAKO Dianova, Hamburg, Germany*
pAb C-20, anti-β4 integrin	*Santa-Cruz Biotechnology, USA*
Cy3-conjugated Donkey Anti-Rabbit	*Jackson Immuno Research Laboratories, USA*
Cy3-conjugated Goat Anti-Mouse	*Jackson Immuno Research Laboratories, USA*
Cy3-conjugated Goat Anti-Rat	*Jackson Immuno Research Laboratories, USA*
Cy2-Goat anti-mouse	*Jackson Immuno Research Laboratories, USA*
Cy3-Donkey anti-goat	*Jackson Immuno Research Laboratories, USA*
TS151r, anti-CD151 free	*C. Boucheix, INSERM, Villejuif, France*
TS151, anti-CD151 complexed	*C. Boucheix, INSERM, Villejuif, France*
mAb BM165, anti-laminin 5 α3 chain	*R.E. Burgeson, CBRC, Boston*
pAb anti-laminin γ2 L4m	*R. Timpl, T. Sasaki, Max-Plank-Institut for Biochemistry, München, Germany*
pAb anti-laminin α3 LG4-5 chain	*R. Timpl, T. Sasaki, Max-Plank-Institut for Biochemistry, München, Germany*
pAb anti-laminin γ2 LE4-6 chain	*R. Timpl, T. Sasaki, Max-Plank-Institut for Biochemistry, München, Germany*
mAb GB3, anti-laminin 5 γ2 chain	*Biozol Diagnostica, Echin, Germany*
mAb 4C7, anti-laminin α5 chain	*Chemicon Europe, Hofheim, Germany*
pAb anti-laminin β1 and γ1 chains	*M. Aumailley, Institut for Biochemistry II, Cologne, Germany*
mAb anti-α-actin	*Sigma-Aldrich Chemie, Schnelldorf, Germany*
pAb anti-β-catenin	*Sigma-Aldrich Chemie, Schnelldorf, Germany*
mAb Rac1	*Upstate Biotechnology, USA*
mAb RhoA	*Santa-Cruz Biotechnology, USA*
mAb Cdc42	*Transduction laboratories, USA*
mAb F-VII anti-vinculin	*Dr. M. Glukhova, Institut Curie, Paris, France*
Purified mouse IgG	*Sigma-Aldrich Chemie, Schnelldorf, Germany*

2.3.2. Cultivated cells

Sterile glass cover slips were washed with 1 N HCl, sterile water and 70% ethanol. After drying, they were placed in a 24 well-culture plate and sterilized under UV light for 20 min.
If the cells were to be seeded without serum, they needed a substrate to attach to the glass coverslip. Therefore, the latter was either coated with 50 µl of laminin1 (15 µg/ml) or laminin 5 (5 µg/ml) and incubated overnight at 4°C or with poly-L-lysine (10 µg/ml) prepared just prior to seeding the cells. The cells were trypsinized and resuspended in DMEM with serum before centrifugation for 5 min at 1000 rpm. The medium was replaced with DMEM without serum and the cells were then resuspended through several pipetting and vortexing to get a single cell suspension. HaCaT cells were allowed to attach and spread for two hours while Wi26 cells adhere for 1 hour.
At room temperature, cells were fixed with a freshly prepared 2% paraformaldehyde solution in PBS for 15 min and permeabilized with 1% Triton-X100 in PBS for 1 min. Immediately, cells were washed 3 times with PBS. Subsequently, to block unspecific binding of the antibodies, cells were incubated in 1% BSA in PBS for 30 min. Afterwards, they were incubated with the first antibody diluted appropriately in PBS for 1 hour. Cells were washed 6 times with PBS before applying the second fluorochrome-conjugated antibody dilute appropriately in PBS for 45 min. In the case of double immunfluorescence, the wash step was repeated and the successive incubations with first and second antibodies were repeated. Care was taken to avoid cross-reaction between antibodies. After the last wash with PBS, coverslips were mounted on histoslide with DAKO mounting medium.

2.3.3. Skin cryosections

The tissue sections of human skin containing hair follicles were kindly provided by Prof. C. Mauch (Dept. Dermatology, University of Cologne) and the tissue sections of mice wounded skin were generously provided by Dr. B. Eckes (Dept. Dermatology, University of Cologne). The sections were stored at -80°C before fixation and staining.

2.3.3.1. Preparation of tissues for immunocytochemistry

The tissue section of human skin was embedded in a cryoprotectant tissue matrix and stored at -80°C. Before sectioning, the cryochamber as well as sectioning block fitted on an ultramicrotome was cooled down until -20°C. The tissue was then placed on the block and sections were cut with a thickness of 7 µm and immediately placed on uncoated glass histoslides, which were stored at -80°C before staining.

Cryosections were thawed and blocked with 1% BSA in PBS for 60 min. The solution was drained and the first antibody was applied. The histoslides were placed in a humid chamber for 60 min of incubation. Subsequently, they were washed 6 times with PBS and the second antibody conjugated with a fluorochrome was applied for 45 min in a humid and dark chamber. After the last wash step, the tissue sections were covered with DAKO mounting medium and a glass coverslip.

2.3.3.2. Haematoxylin and eosin

Alternatively, cryosections were stained with haematoxylin and eosin. The sections were rinsed with distilled water and stained with haematoxylin for five minutes. The specimens were then dipped in 1% HCl-70% ethanol for one minute and washed in tap water to remove the excess of stain. At last, they were stained with eosin for 30 seconds, drained in 70% ethanol and rinsed in distilled water before being covered with DAKO mounting medium and a glass coverslip.

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2.3.4. Image analysis

The immunofluorecence stainings were observed with an inverted fluorescence Axiophot microscope (Zeiss) or with a laser scanning confocal microscope TCS-SP (Leica). The types of laser used in confocal microscopy were a 488 nm Argon laser for excitation of FITC or Cy2 fluorescence and a 543 nm HeNe laser for excitation of Cy3 fluorescence. Digital images were obtained and saved with the Leica software, and then processed using Adobe Photoshop software running on a

PC. In particular, concerning the double stainings, the images from green and red channels were independently acquired in the confocal microscope and the images were then superimposed using Adobe Photoshop software. For the analysis of the size of single cells stained for fibrillar actin, the Scion Image software was used.

2.4. Cell adhesion assay

Tissue culture 96-multiwell plates were coated in triplicate with a serial dilution of laminin 1 (20 to 0.31 µg/ml) or laminin 5 (from 5 to 0.1 µg/ml) and incubated at 4°C overnight. The first lane was kept uncoated to serve as negative control. Unspecific binding sites were blocked by 1% BSA in water for 3 hours at 4°C.
The cells were detached and resuspended in DMEM supplemented with serum. A sample was taken to count the cells after centrifugation at 1000 rpm for 5 min. The cells were resuspended in DMEM without serum with a final concentration of 40 to 80.10^4 cells/ml and seeded on the coated 96-well-plates. The cells were allowed to adhere for 15 to 30 min.
Cells were then washed once with PBS and adherent cells were fixed with 1% glutaraldehyde in PBS for 15 min. The cells were stained with 0.1% crystal violet in water for 25 min and washed with tap water. Afterwards, 1% Triton-X100 dissolved in water was added and the absorbance at 540 nm was determined using an enzyme-linked immunosorbent assay (ELISA) reader. The blank value corresponding to BSA-coated wells was automatically subtracted. The graphs and histograms were done using Microsoft Excel software. Adherent cells were photographed using a phase contrast microscope equipped with a camera (Canon PowerShot G5).

2.5. Cell migration and time-lapse video microscopy

2.5.1. Preparation of the cells

Confluent plates of cells were suspended, collected by centrifugation (1000 rpm for 5 min) and resuspended in 800 µl of complete DMEM in order to get a high cell density (5.10^5 cells/ml). Cells were deposited dropwise (10 µl) in the center of

wells of a 24-multiwell-plate and allowed to adhere for one hour at 37°C. The wells were washed several times before being filled with 400 µl serum-free medium containing or not laminin 1 (15 µg/ml) or laminin 5 (5 µg/ml). In some experiments, the medium was supplemented also with mAb TS151r (10 µg/ml) or purified mouse immunoglobulins (10 µg/ml).

2.5.2. Equipment and software

For conventional migration assays, a first photograph of each colony was taken immediately (T0) and further photographs of the colonies were recorded after 2, 3, 5 and 6 hours (Tx) **(Fig. 23)**. The cultures were maintained at 37°C between the photographs. In order to make photographs at the identical position, a mark underneath the plate was made. In some cases, the cells were fixed, stained with crystal violet and photographed at the end of the experiment.

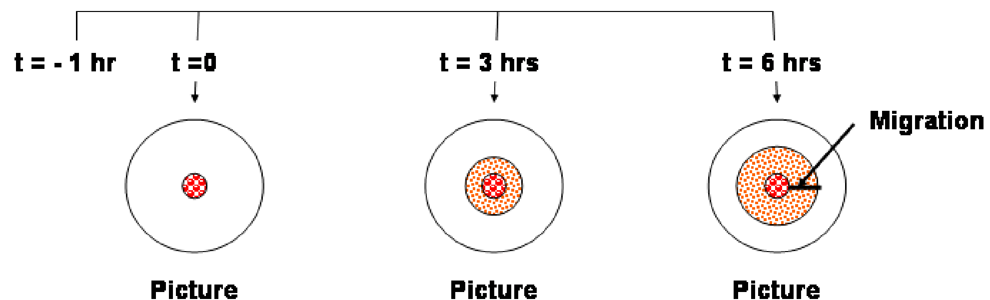


Fig. 23: Principle of the cell migration assay.
A 10 µl-cell suspension was seeded in the centre of wells in 24-well plates and allowed to adhere for 1 hour (t = - 1 hr). The wells were then filled with medium containing laminin (t = 0) and a first picture was taken. Afterwards, several other pictures were taken at different time intervals (t= 3 hrs, t = 6 hrs…) to observe the cell migration at the border of each cell colony.

The images were captured with a CCD camera placed on an inverted phase contrast microscope. They were then stored on a computer and processed using the Openlab 1.7.8. and Adobe Photoshop softwares.

To record cell migration by time-lapse video microscopy, the 24-well plate containing the cells was placed on the heated microscope stage in a humidified chamber with a 5% CO_2 supply **(Fig. 24)**.

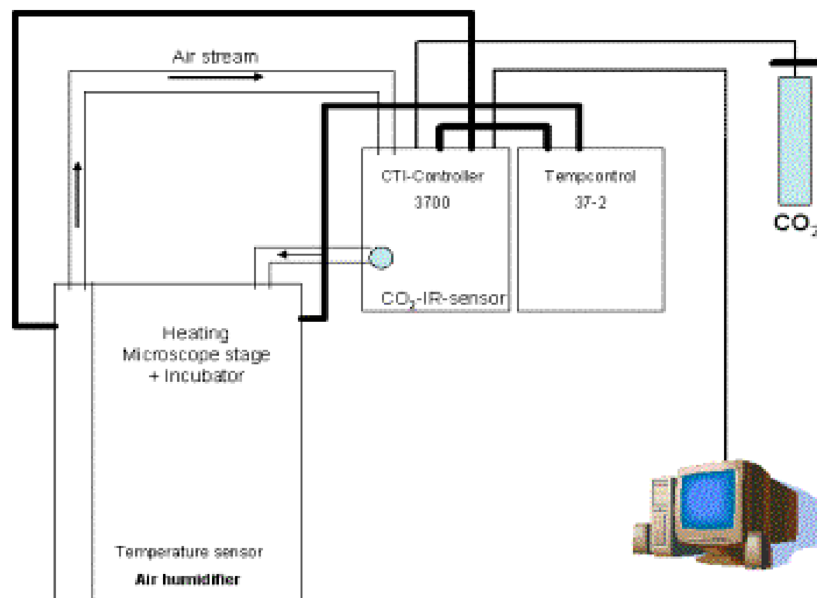


Fig. 24: Video microscope facility.

The time-lapse video recording was for 13 hours. Using the software Openlab 1.7.8, a programme was written using a visual programming language that allows the creation of a schedule of tasks running automatically. Pictures were taken every 5 min during the 13 hours.

The schedule was as follows:

- "Start" task: Begin the programme.

-"Target image window" task: The active layer window, in which the images will be saved, is recognized.

- "Loop" task: Series of following tasks will be executed 160 times:

  - "Delay" task: This task permits the automation to wait 300 s before going to the next step.
  - "Capture" layer: A picture is captured and a name for the captured layer is created.

-"End" task: When the conditions of the "Loop" task are filled, the automation stops.

The video recording was viewed as a Quick Time movie after conversion of all the layers into an appropriate format. Alternatively, the itinerary of a particular cell was followed by digitally labelling the cell body in a transparent layer in the software

Openlab, while, like turning the pages of a book, pictures are viewed at 50 min intervals. The migration tracks of single cells allow to determine the velocity and the progression index of the cell. The progression index represents the ratio of the linear distance between start and end to the real distance travelled by the cell (Frank and Carter, 2004). A progressive index equal to 1 represents a linear migration.

2.6. SDS-PAGE

SDS-PAGE was carried out on 12% acrylamide gels in electrophoresis buffer (25 mM Tris, 250 mM Glycine pH 8.3, 0.1% SDS). The samples were mixed with Laemmli buffer (60 mM Tris/HCl, pH 6.8, 10% Glycerol, 2% SDS, 0.1% bromophenol blue) supplemented with 5% β-mercaptoethanol.
After electrophoresis, the proteins in the gel were visualized by staining with Coomassie Brilliant Blue solution (0.1% Coomassie Brilliant Blue R250, 50% ethanol, 10% acetic acid). The gel was incubated with the staining solution with gentle agitation at room temperature for two hours. In order to get rid of the background and until the protein bands were clearly visible, the gel was washed with a destaining solution (10% acetic acid, 20% ethanol) under gentle agitation.

2.7. Western Blotting

Proteins were transferred electrophoretically onto nitrocellulose membrane in transfer buffer (25 mM Tris-HCl, 192 mM Glycine containing 20% methanol) at constant current (100 mA) overnight at 4°C. After transfer, the membrane was washed in distilled water and the proteins on the membrane were stained with Ponceau Red solution for two minutes. The position of the molecular weight makers was marked with a pencil. The Ponceau Red stain was then washed off with TBS under gentle agitation.
The nitrocellulose membrane was blocked with 5% low fat milk powder for one hour. The membrane was incubated one to two hours at room temperature with a first antibody diluted in 1.5% BSA/TBS. Then, the membrane was washed several times for 10 min in TBS, before being incubated with the appropriate horseradish

peroxidase-coupled secondary antibody. Finally, the membrane was rinsed in TBS and the proteins detected using an enhanced chemiluminescence (ECL) detection system.

2.8. Pull-down assay

The preparation of the fusion-proteins and the pull-down assay were based on the protocol of XD. Ren and M.A. Schwartz (2000).

2.8.1. Expression and purification of GST-CRIB and GST-TRBD fusion proteins

The Rhotekin Rho-Binding Domain (TRBD) was used to affinity-precipitate endogenous activated RhoA from cell lysates (Ren et al., 1999). The coding sequence of TRBD (aa 1-90) cloned into a pGEX-2T vector at the BamH1 and EcoR1 sites was kindly provided by M.A. Schwartz. The Cdc42-Rac1-Interaction Binding domain (CRIB) was used to precipitate endogenous activated Rac1 and Cdc42 (Sander et al., 1998). The coding sequence of CRIB (aa 56-141), cloned into a pGEX-2T vector was kindly provided by JG. Collard.

Transformation of Escherichia Coli cells:
Before introducing the plasmids pGEX-2T-CRIB and pGEX-2T-TRBD into BL21 and DH5α strain of E. Coli, respectively, the bacteria cells were made competent.
For this, the bacteria cells from bacterial stocks maintained in 10% glycerol at -80°C, were allowed to grow in 100 ml LB medium overnight at 37°C under vigorous shaking at 250 rpm until an OD_{600} of 0.5 was reached. The cells were centrifuged at 4000 rpm for 10 min and resuspended in 10 ml cold TSS (85% LB medium, 10% PEG, 5% DMSO, 5 mM $MgCl_2$, pH 6.5). The cells were then aliquoted and shock-frozen in liquid N_2 to be stored at -80°C.
One µl of vector pGEX-2T-CRIB or pGEX-2T-TRBD was given in 100 µl of competent cells and incubated on ice for 30 min. Immediately, the cells were incubated at 42°C for 90 seconds and subsequently cooled on ice for 2 min. The cells were then mixed with 900 µl LB medium for one hour at 37°C under shaking.

The selection of transformed bacteria was done on ampicillin treated agar plates, where the bacteria were allow to grow at 37°C overnight. Only the bacteria, which had incorporated the plasmid, survived and were picked up for growth.

Expression of GST-fusion proteins:

The bacteria cells were allowed to grow overnight at 37°C under shaking in a large volume of LB-selection medium containing 100 µg/ml ampicillin until an OD_{600} of 0.8 was reached. Protein expression was then induced with 0.5 mM isopropyl βD-thiogalactopyranoside (IPTG) for 3 hours at 37°C for GST-CRIB and 30°C for GST-TRBD. The bacteria cells were collected by centrifugation at 4000 g for 15 min. The pellet was resuspended in the lysis buffer (50 mM TRIS pH7.5, 1% Triton-X100, 150 mM NaCl, 5 mM $MgCl_2$, 10 µg/ml leupeptin and aprotinin each and 1 mM Pefablock) before sonication. To regain the protein, the solution was ultracentrifuged at 27000 g for 30 min at 4°C. The lysat was then mixed with glutathione Sepharose beads at 4°C for one hour. Afterwards, the beads were collected and washed by repetitive brief centrifugation (Wash buffer: 50 mM TRIS pH 7.5, 0.5% Triton-X100, 150 mM NaCl, 5 mM $MgCl_2$, 1 µg/ml leupeptin and aprotinin each, 0.1 mM Pefablock). For qualitative and quantitative analysis, the products were electrophoresed on a 10% acrylamide gel under reduced conditions and visualized after staining with Coomassie Blue. To quantify the amount of purified proteins, the bands were compared with known quantity of BSA (4, 8, 16 µg).

2.8.2. Pull-down assay

All pull-down assays were performed with HaCaT and Wi26 cells, which were serum-starved for at least 24 hours. After treatment with trypsin-EDTA in PBS, the cells were collected through centrifugation and resuspended in DMEM without serum before seeding on 6-cm diameter culture dishes coated with different substrates (laminin 1 EHS (15 µg/ml), laminin 5 (5 µg/ml) or fibronectin (15 µg/ml)). After 30, 45 or 60 min (short assay) or 1, 2, 3 or 4 hours (long assay) incubation, the cells were washed with cold PBS and lysed on ice in 500 µl of lysis buffer (25 mM Hepes pH 7.3, 150 mM NaCl, 5 mM $MgCl_2$, 0.5 mM EGTA, 0.5% Triton-X100, 4% Glycerol, 20 mM β-glycerophosphate, 10 mM NaF, 5 mM DTT, 10

µg/ml aprotinine, leupeptine and PMSF each). The lysat was centrifuged at 13000 g for 10 min at 4°C. An aliquot from the supernatant (40 µl) was kept as loading control and the rest was incubated with 20-30 µg beads binding to the fusion proteins. The latter rotated for 45 min at 4°C. The beads were then washed 3 times with the lysis buffer on ice through successive centrifugation step at 1000 rpm for 1 min. After the last wash, about 30 µl of the wash buffer was left and supplemented with sample buffer. The samples were heated at 95°C for 10 min.
The total cell lysates and the affinity-precipitated products were run on a 12% acrylamide-gel, transferred to nitrocellulose membrane and blotted for Cdc42, Rac1 or RhoA. The amount of activated GTP-bound RhoA, Rac1 or Cdc42 was normalized to the total amount of the corresponding small GTPase in cell lysate.

2.9. siRNA silencing

2.9.1. siRNA annealling and storage

All steps of siRNA handling were carried out under sterile, RNAse free conditions.
The sequences of 21 nucleotides siRNA of the small GTPases Rac1, RhoA and Cdc42 are as followed: Rac1: 5´CACCACUGUCCAACACUCTT 3´, RhoA: 5´ GAAGUCAAGCAUUUCUGUCTT 3´, Cdc42: 5´ GAUAACUCACCACUGUCCATT 3´. As control for all transfections, a scramble of the Cdc42 oligos was designed: 5´AUACUUACGCACGCUCCAATT 3´ (Deroanne et al., 2003). These RNA oligos were chemically synthesized and purified through HPLC (Eurogentec, Liège, Belgium). Analysis of these sequences using the software BLAST (NCBI) showed that they are specific for the given proteins.
Single-stranded sense and anti-sense siRNA pairs were annealled to get complementary siRNA duplex: siRhoA as well as siCdc42, siRac1, siScramble oligos were diluted in DEPC-treated water to a concentration of 50 µM and 100 µM respectively. 30 µl of each siRNA oligo solution was then combined with 15 µl of 5X annealling buffer (50 mM Tris pH 7.5-8.0, 100 mM NaCl in DEPC-treated water). The final concentration was 20 µM for siRhoA and 40 µM for siRac1, siCdc42 and siScramble. The solutions were incubated for 1 minute in a water-

bath of 95°C and allowed to cool down for 1 hour at room temperature. The duplex siRNA were then stored at -20°C or -80°C for long storage.

2.9.2. Lipofectamine transfection

Lipofectamine transfection of siRNA duplex was executed according to the manufacturer's protocol (Invitrogen Life Technologies, USA) using Lipofectamine^{TM} 2000 reagent. HaCaT cells were grown until 80% confluency. Before transfection, the cells were washed with DMEM exempt of serum. 200 nM or 400 nM siRac1 were mixed in 1 ml medium without serum supplemented with 2% lipofectamine and incubated for 20 minutes at room temperature. The mixture was then given to the cells in 2 ml medium. After 4 hours incubation at 37°C and 5% CO_2, the medium was replaced with fresh medium supplemented with serum and the cells grown 48 hours before being harvested.

2.9.3. Calcium-phosphate transfection

The small double-stranded RNAs were introduced into the cells by transfection using calcium phosphate precipitates. Based on DNA/calcium phosphate precipitates (Loyter, 1982; Chen and Okayama, 1987), this transfection method is commonly used for the transfer to and expression of genetic information like siRNA duplex (Weil et al., 2002; Donzé and Picard, 2002). It is a simple and low cost method to transfect efficiently mammalian cells. The principle is that siRNA duplex is mixed to a $CaCl_2$-solution before adding a phosphate buffer, which initiates the formation of calcium-phosphate precipitate. The siRNA-calcium-phosphate precipitate are then added in the culture medium and adsorbed at the cell surface through phagocytosis.

24 hours prior transfection, subconfluent HaCaT cells were split 1:5 in order to get a 50 % confluent plate the next day. One hour before transfection, the medium was replaced by fresh DMEM containing serum. At this time, the siRNA-calcium-phosphate solution was prepared in a sterile, RNAse free tube placed on ice.

For a 10-cm diameter dish and a final volume of 7 ml medium:

292.5 - x µl DEPC-treated water
32.5 µl of a 2.5 M $CaCl_2$ in DEPC-treated water
x µl siRNA duplex (final concentration: 100-200 nM)

For a 6-cm diameter dish and a final volume of 2 ml medium:

135 - x µl DEPC-treated water
15 µl of a 2.5 M $CaCl_2$ in DEPC-treated water
x µl siRNA duplex (final concentration: 100-200 nM)

One volume of this Ca-RNA mix solution was gently mixed with one volume of sterile 2xHBSP solution (280 mM NaCl, 1.5 mM Na_2HPO_4, 12 mM Glucose, 10 mM KCl, 50 mM HEPES, pH 7.05). The calcium-phosphate-RNA mixture was kept on ice for 1 hour before adding to the cell culture medium. 24 hours after transfection, the cells were washed several times with PBS supplemented with 0.5% glucose. This solution was then replaced with DMEM with serum and the cells were placed in the incubator for at least 24 hours. To quantify the level of protein expression, the cells were lysed and the products separated by SDS-PAGE on a 12% acrylamide gel. The protein expression was analysed by Western Blotting and normalized to the amount of actin in the total cell lysate.

3. RESULTS

The control of cell migration by the extracellular matrix involves several factors, including the tissue-specific composition of the extracellular matrix, the proteolytic processing of some of the extracellular matrix constituents and the concerted action of different cell surface proteins. Integration of these different parameters allows the extracellular matrix to promote or inhibit cell migration.

3.1. Remodelling of cellular interactions at the dermal-epidermal junction and keratinocyte migration

Epithelial laminins are key structural elements of basement membranes. In the skin, they are found at the epidermal-dermal junction where their main functions are to provide a structural support to adjacent epithelial cells and provide them with extracellular cues. These cells anchor to the laminin via stable adhesion structures, the hemidesmosomes, containing transmembrane proteins such as $\alpha6\beta4$ integrins, CD151 and collagen XVII. The hemidesmosomal complex must be dynamic during tissue remodelling and thus presumably participates to the regulation of cell migration.

3.1.1. Expression of integrins and laminin 5 in *in vivo* models of keratinocyte migration

3.1.1.1. Resting epithelia and wounded skin

The repair of a wounded epithelial layer is a process that forces static epithelial cells to migrate and proliferate in order to close the wound. The quiescent epidermis is rapidly activated and a sub-population of cells forms the epidermal outgrowth containing leading and following cells. The wounding of epidermis transform a stable anchoring system involving $\alpha6\beta4$ integrins binding to laminin 5 into a dynamic transient adhesion system involving $\beta1$ integrins (Nguyen et al., 2000).

In the epidermis, only the basal keratinocytes express the $\alpha2\beta1$, $\alpha3\beta1$ and $\alpha6\beta4$ integrins (Watt, 2002). To compare the distribution of these integrins in quiescent and

wounded epidermis, skin cryosections were stained by indirect immunofluorescence with integrin-specific antibodies.

In quiescent epidermis, the integrin $\alpha2$ (not shown), $\alpha3$ and $\beta1$ subunits are present in intercellular junctions while the integrin $\alpha6$ subunit is localized at the basal surface of keratinocytes **(Fig. 25A)**. Interestingly, the monoclonal antibody 9EG7 that recognizes activated $\beta1$ integrins did not stain the epidermis **(Fig. 25A)** indicating that in resting epidermis, the $\beta1$ integrins are not in the activated state.

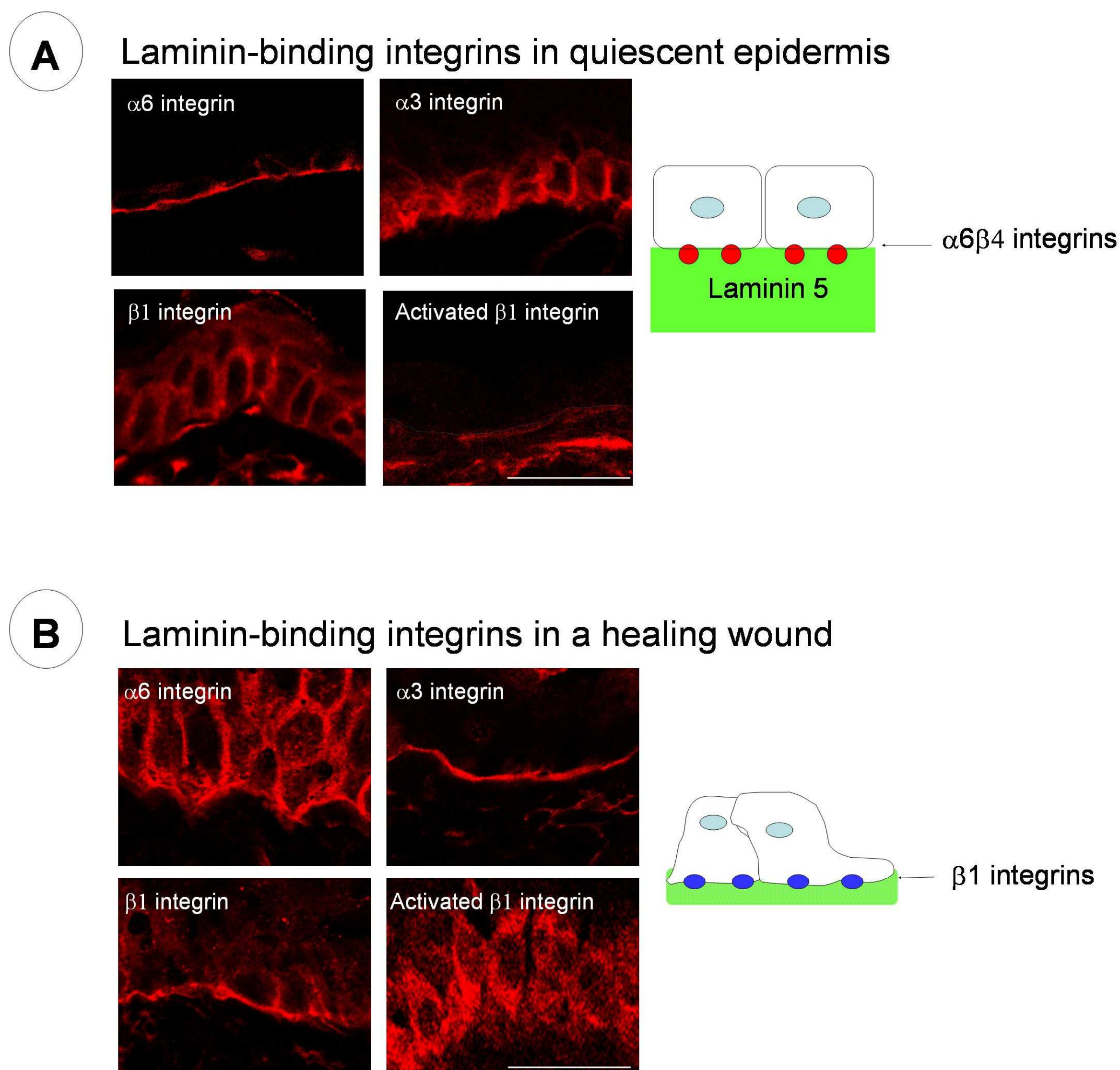


Fig. 25: Labelling of laminin-binding integrins in quiescent epidermis and in a healing wound.
Cryosections of mice skin two days after wounding. The keratinocytes in the quiescent epidermis **(A)** and in the wound **(B)** were analysed using antibodies against the integrins as indicated: $\alpha6$ (mAb GoH3), $\alpha3$ (mAb 1920) and $\beta1$ subunits (pAb 1997 and mAb 9EG7) followed by Cy3-conjugated anti-rabbit or anti-rat IgG secondary antibodies. Schematic representation of quiescent keratinocytes shows that $\alpha6\beta4$ integrins are located at the dermal-epidermal interface in contact with laminin 5. In contrast, in the wound bed, the $\beta1$ integrins are found in contact with the provisional matrix. Bar: 20 µm

In wounded skin, the α3 and β1 integrins are targeted to the dermal-epidermal interface **(Fig. 25B)**. In contrast to the quiescent epidermis, the antibody 9EG7 stained strongly the cytoplasm of the leading cells, indicating that at this location, β1 integrins are upregulated. The integrin α6 subunit was detected both at basal and apico-lateral membranes of the cells, indicating that the hemidesmosomes containing α6β4 integrins were dissociated **(Fig. 25B)**.

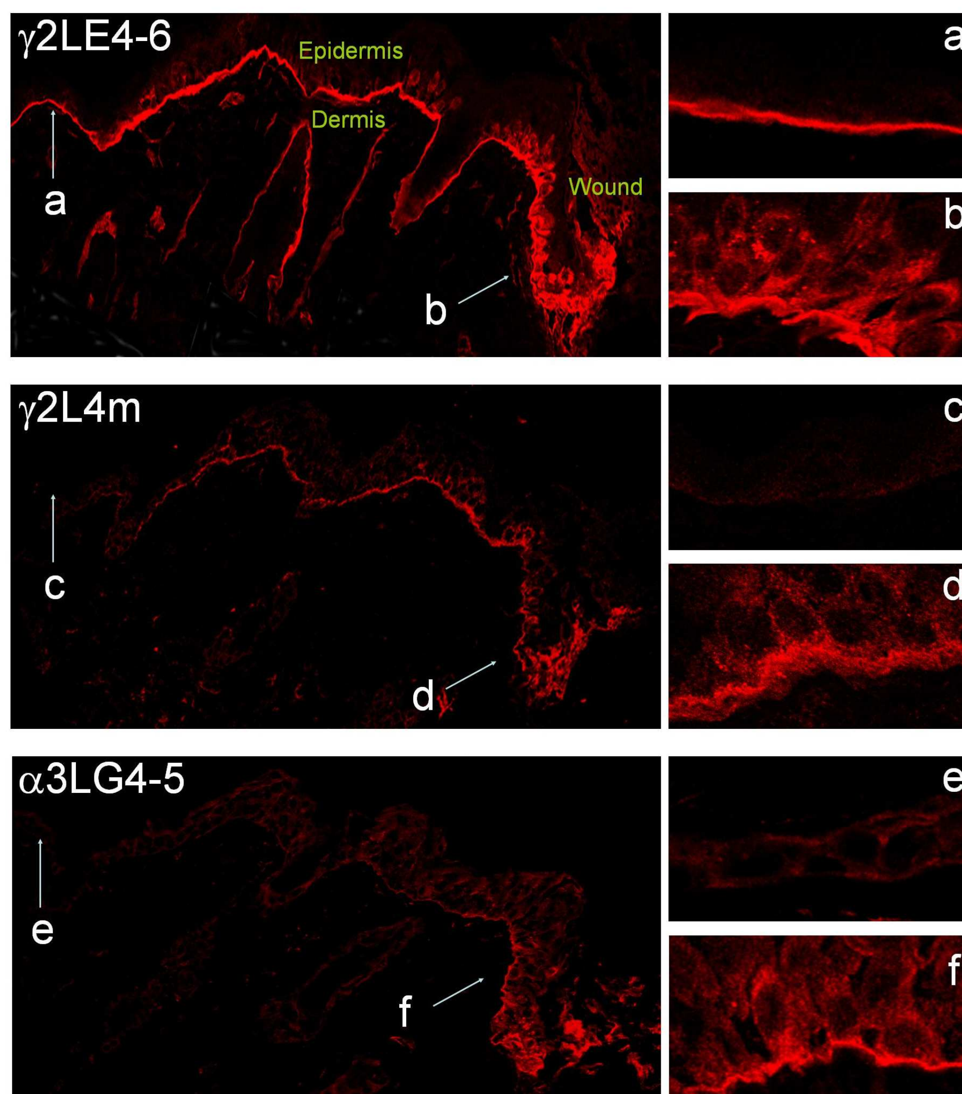


Fig. 26: Expression of processed and unprocessed laminin 5 in resting and wounded epidermis.
Polyclonal antibody to the domain LE4-6 of the γ2 chain of laminin 5 shows a linear staining at the dermal-epidermal junction in quiescent epidermis (**a**). In the wound bed, laminin 5 is also observed in the cytoplasm of the leading cells (**b**). Staining with polyclonal antibodies to the domains L4m and LG4-5 of unprocessed γ2 and α3 chain, respectively, show that laminin 5 is fully processed in quiescent epidermis (**c,e**), while unprocessed laminin 5 is present in the leading cells migrating over the wound (**d,f**).

We next analysed laminin 5 in quiescent and wounded mouse skin by indirect immunofluorescence staining. The antibody against the domain LE4-6 of the $\gamma 2$ chain recognizing the processed and unprocessed laminin 5 gives a linear staining along the dermal-epidermal junction. In contrast, antibodies recognizing the L4m domain or the LG4-5 domains present in unprocessed laminin $\gamma 2$ or $\alpha 3$A chains, respectively, do not stain the dermal-epidermal junction **(Fig. 26)** underneath the intact epidermis away from the wound site.

In the wound bed, the antibody against the domain LE4-6 stains very strongly the cytoplasm of the cells, especially the ones at the leading edge, indicating an up-regulation of the expression of laminin 5 at this location **(Fig. 26)**. The staining against the LG4-5 domain, a marker for the immature laminin 5 with unprocessed $\alpha 3$ chains, is positive at the cell-matrix interface where the leading keratinocytes migrate over a provisional matrix **(Fig. 26)**. Interestingly, unprocessed $\gamma 2$ chain of laminin, stained with the antibody against the L4m domain, is detected in the wound bed but also underneath the keratinocytes flanking the wound site **(Fig. 26)**.

3.1.1.2. The hair follicle in the anagen phase

The hair follicle in the anagen phase represents an *in vivo* model to follow active remodelling of tissue and cellular interactions (Oshima et al., 2001). Sections of human skin biopsies were processed for indirect immunofluorescence staining of the laminin-binding $\alpha 3\beta 1$ and $\alpha 6\beta 4$ integrins and of the laminins. Observation of the stainings in the epidermis showed that the integrin $\alpha 3$ subunit, which associates with the integrin $\beta 1$ subunit, is present at intercellular junctions and the integrin $\alpha 6$ subunit, which associates with the $\beta 4$ subunit, is at the dermal-epidermal interface **(Fig. 27A-C, Epidermis)**. This was also true along the upper part of the hair follicle **(Fig. 27A-C, Upper region)**. In these regions, the basement membrane contains laminin 5 as well as laminins containing $\alpha 5$ and $\beta 1$ chains (**Fig. 27C-E**).

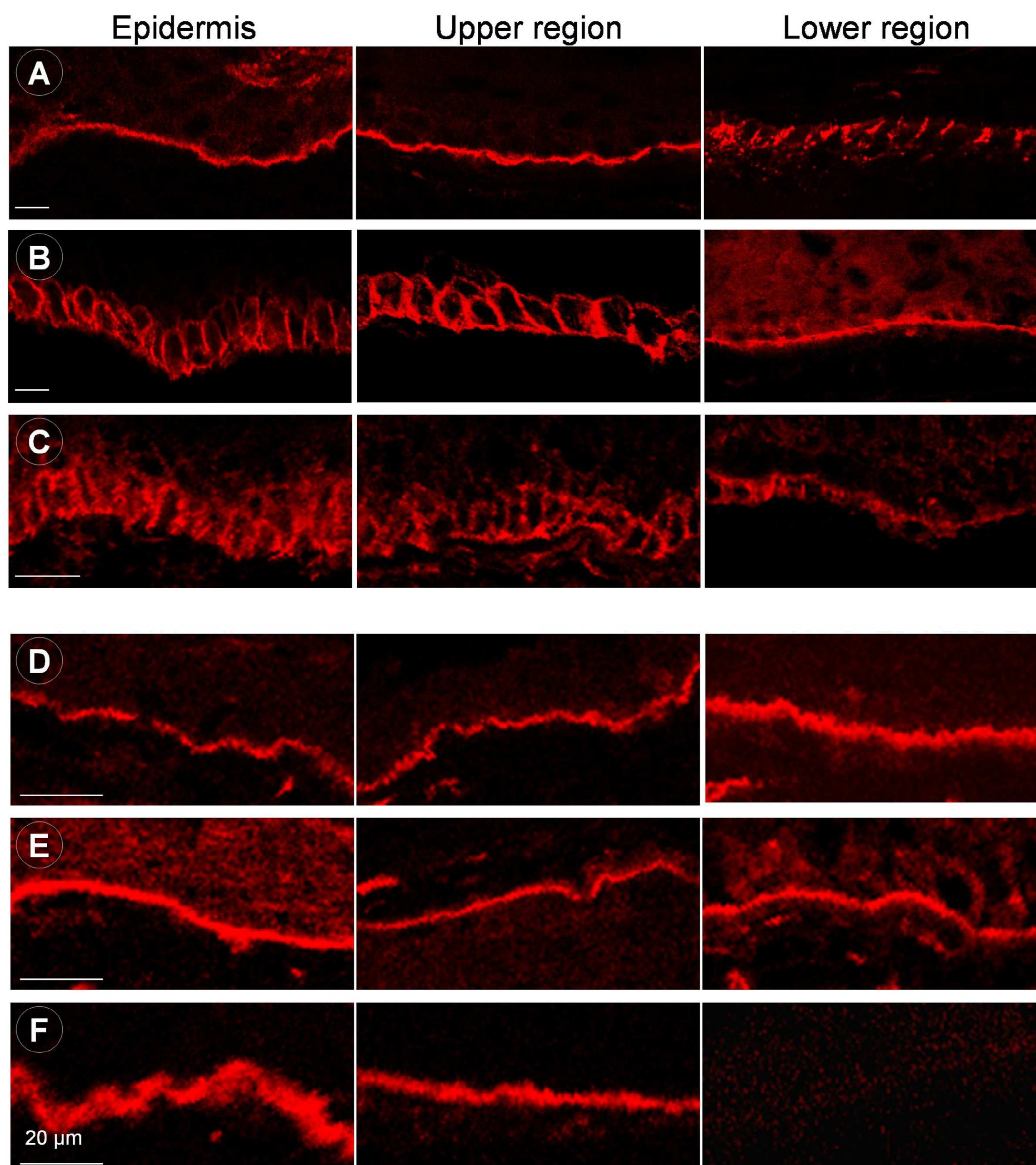


Fig. 27: Expression of laminin-binding integrins and laminin chains in epithelial cells of the epidermis and the hair follicle.
Tissue cryosections were processed for indirect immunofluorescence staining of the integrin α6 (mAb GoH3, **A**), α3 (mAb P1B5, **B**) and β1 (mAb P4C10, **C**) subunits as well as laminin α5 (mAb 4C7, **D**), β1 (pAb 1006, **E**) and γ2 chains (pAb GB3, **F**). Representative pictures of the epidermis and upper and lower region of the hair follicle are shown. In the epidermis and upper region of the hair follicle, the integrin α6 subunit is located at the dermal-epidermal junction facing laminin 5 and laminin 10, while the integrin α3 and β1 integrins are found at the cell-cell junctions. In the lower part of the hair follicle, the integrin α6 subunit is barely detectable at the cell-cell junctions. Instead, it is replaced by the integrin α3 and β1 subunits at the dermal-epidermal junction, where laminin α5 and β1 chains, but not α3 chains, are found. Bar: 20 µm

In contrast, in the lower part of the hair follicle, i.e. at the level of the hair bulb, the integrin α3 and β1 subunits are targeted to the dermal-epidermal interface (**Fig.**

27B,C, Lower region) while the integrin $\alpha6$ subunit is weakly stained at cell-cell junctions (**Fig. 27A, Lower region**). The laminin $\beta1$ and $\alpha5$ chains, representing laminin 10, are present over the entire hair follicle from the top to the bulb (**Fig. 27D,E**). In contrast, laminin 5, which is the primary adhesion factor in the epidermis, is absent or expressed below the detection level in the hair bulb (**Fig. 27F**).

These observations indicate that, in the lower region of the hair follicle and during wound healing there is rearrangement of the integrins. In resting keratinocytes, the integrin $\alpha3$ and $\beta1$ subunits are at the sites of cell-cell interactions. In contrast, during tissue remodelling, the receptors are targeted to cell-matrix interactions, suggesting that keratinocytes may utilize the $\alpha3\beta1$ integrin for motility or for reorganization of the BM. The integrin $\alpha6$ subunits, they switch from a location at the dermal-epidermal interface in resting epidermis to cell-cell junctions in remodelling tissues. In quiescent epidermis, the transcription of laminin 5 is repressed, in contrast to its strong expression in the wound bed.

3.1.2. Migration of keratinocytes on laminin 1 and laminin 5 *in vitro*

The integrin receptors, in direct contact with laminins in the dermal-epidermal junctions, participate in the control of cell adhesion and migration. The rearrangement of the integrin receptors, that we observed *in vivo,* allows keratinocytes to migrate over the provisional matrix. The nature of the integrins and extracellular matrix involved may therefore regulate the behavior of the cells. In order to study the migration of epithelial cells, we performed migration assay *in vitro* on laminin 1 and 5, which recruit two different subsets of integrins: $\alpha6\beta1$ and $\alpha3\beta1$, respectively.

The migration of epithelial cells was explored using the spontaneously immortalized human HaCaT cells. To that aim, we established an assay, in which cells are allowed to escape from a confluent colony in the presence of laminin 5 or laminin 1. To verify that laminin 1 and laminin 5 were evenly deposited on the support **(Fig. 28)**, the HaCaT cells were seeded on glass coverslips and after one hour adhesion, laminin 1 or 5 were added to the medium without serum. The deposition of exogenous laminin 1 and 5 was observed after immunofluorescence staining while the cell bodies were visualized by staining fibrillar actin. The stainings show that laminin 1 and 5 were

deposited homogenously all over the bottom of the plates. Also the endogenous laminin 5 is detected in the cytoplasm of the cells.

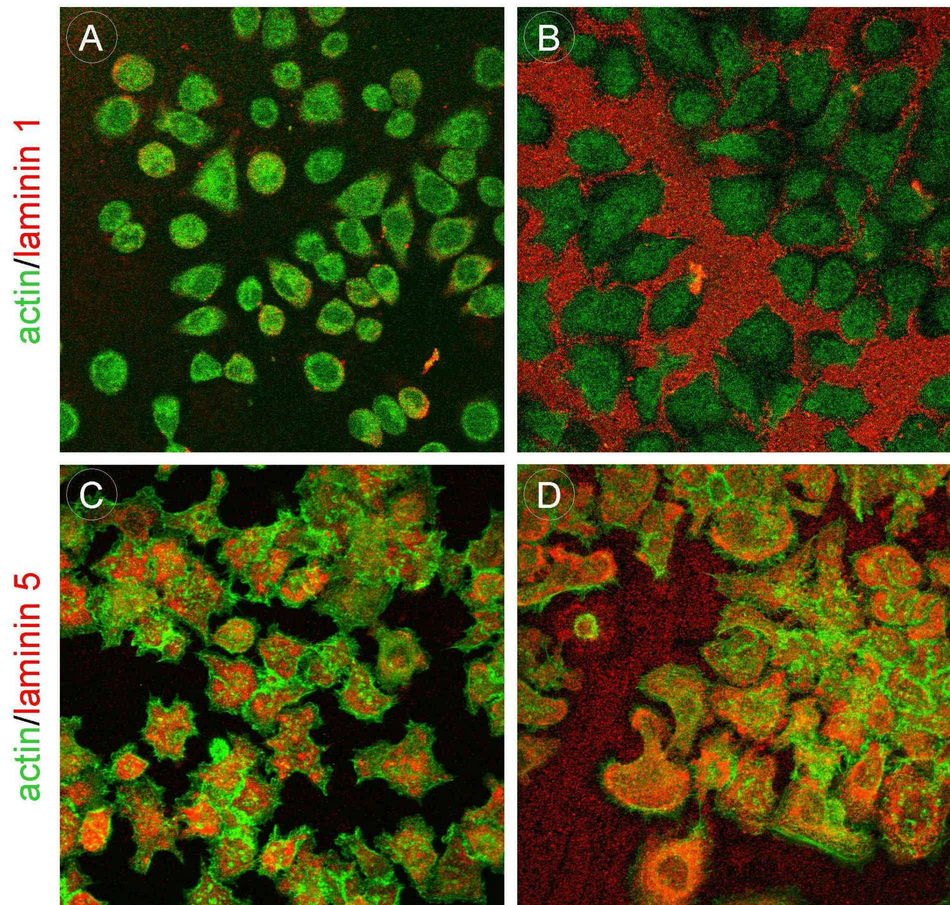


Fig. 28: Detection of exogenous laminin 1 and laminin 5 on the culture support.
After HaCaT cell adhesion to glass coverslips, exogenous laminin 1 (15 µg/ml) **(B)** or laminin 5 (5 µg/ml) **(D)** were added to the medium. After three hours of incubation, the cells were fixed and coimmunostained for either laminin 1 (mAb 1006, red) **(A-B)** or laminin α3 chain (mAb BM165, red) **(C-D)** and fibrillar actin (FITC-phalloidin, green). Exogenous laminin 1 **(B)** or laminin 5 **(D)** are homogenously deposited all over the bottom of the plates. Note that in figure C and D, the endogenous laminin 5 is also seen.

Under these conditions, cell migration was assessed first by comparing the margin of the colonies on photographs taken at different time points. During 6 hours of migration, more Wi26 fibroblasts than HaCaT cells had migrated out of the colonies in the presence of either one of the laminins **(Fig. 29A)**. Most of the fibroblasts had an elongated morphology, reflecting a distinct motile phenotype, while only few HaCaT cells were bipolar on laminin 5 and round on laminin 1 **(Fig. 29A)**. Quantification of the results showed that migration of the fibroblasts was at least two-fold that of the HaCaT cells under all conditions **(Fig. 29B)**.

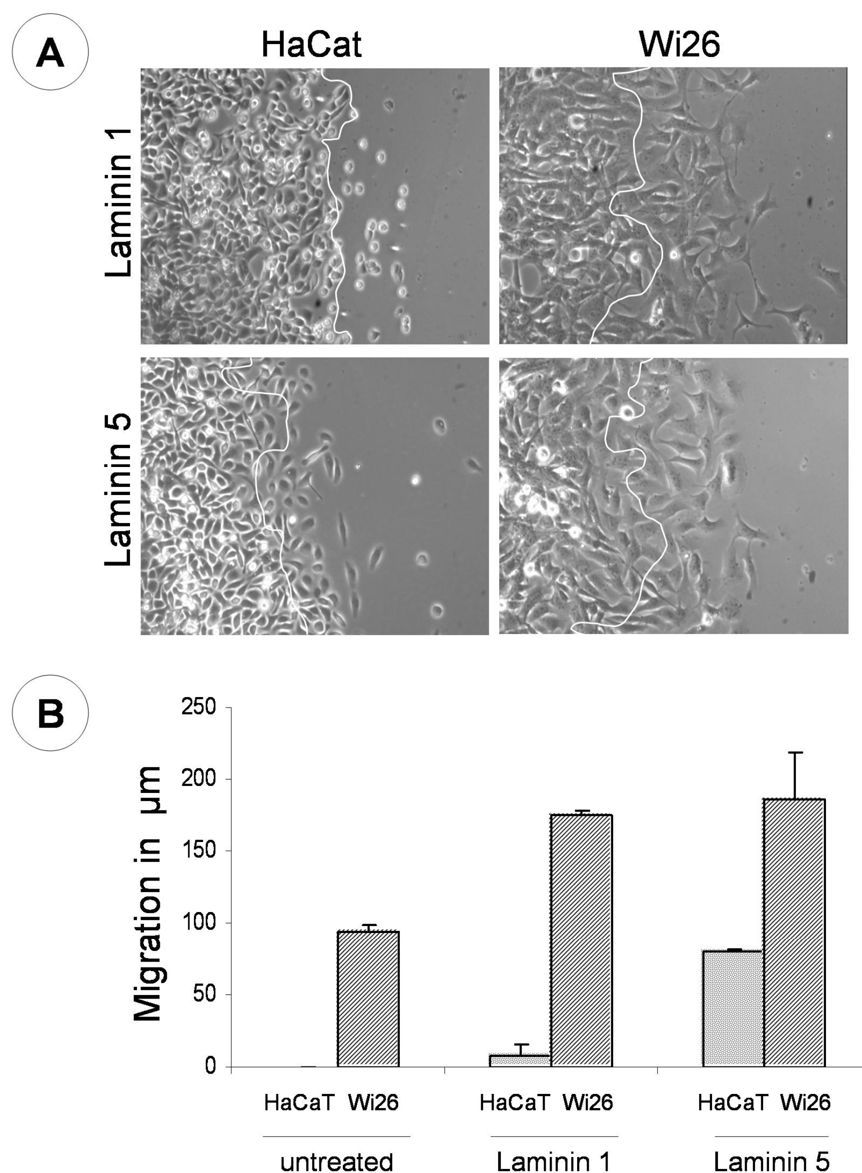


Fig. 29: Migration of HaCaT epithelial cells and Wi26 fibroblasts on laminin 1 and laminin 5.
(A) Small colonies (10 µl) of HaCaT and Wi26 cells were seeded at high density in serum-containing medium in the center of culture wells and allowed to attach for one hour. Afterwards, the wells were washed and filled with serum-free medium alone (untreated) or containing laminin 1 (15 µg/ml) or laminin 5 (5 µg/ml) as indicated. The cells were immediately photographed. The pictures show the margins of the colonies after 6 hours and the white line indicates the margin at the beginning of the experiment. **(B)** Quantification of cell migration within 6 hours. The distance covered by the cell front in 6 hours was measured on photographs. Each column represents the average of three experiments and the bars indicate standard deviation. The migration of HaCaT epithelial cells (column depicted with dots) is lower than that of the fibroblasts (column depicted with lines) under all conditions tested.

Using time-lapse video microscopy, we monitored the progression of the cells during 13 hours. With the help of the software Openlab, we labeled the cell body digitally to visualize the tracks of the cells and the cell velocity was determined. The fibroblasts

migrated with an average velocity of 0.47 µm/min (± 0.09) on laminin 5 and 0.36 µm/min (±0.09) on laminin 1 during the entire length of the assay. On exogenous laminin 5, HaCaT cells had emigrated from the colonies with an average velocity of 0.28 µm/min (± 0.08) and of 0.12 µm/min (± 0.03) on laminin 1. In addition, the epithelial HaCaT cells progressed during the first hours and then slowed down until resuming migration in contrast to the fibroblasts, which migrate with a constant velocity on laminin 5 **(Fig. 30)**.

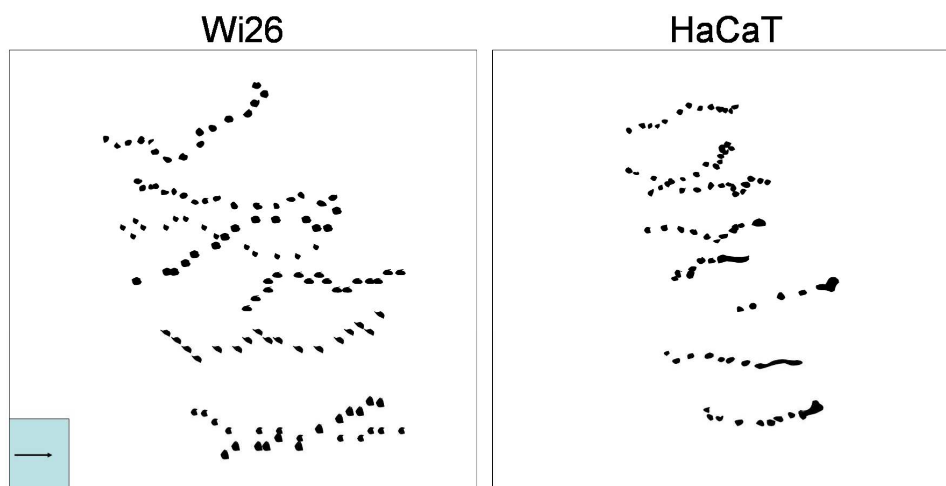


Fig. 30: Migration tracks of HaCaT and Wi26 cells on exogenous laminin 5.
Using time-lapse video microscopy, cells escaping from the colony were photographed every 10 min during 13 hours with an inverted phase contrast microscope equipped with a CCD camera. After recording, the translocation of single cells was followed in successive frames using the software Openlab and is represented as black points. The migration tracks show that the Wi26 cells migrate with a constant velocity while the HaCaT cells slow down over the time. The arrow indicates the direction of the migration.

The analysis of the cell migration on laminin 1 in shorter assays also showed that the HaCaT cells migrate at a moderate speed during the two first hours and decrease their velocity (not shown). As the HaCaT epithelial cells have the ability to produce their own laminin, in particular laminin 5 as seen in figure 28, we examined the expression of the latter by indirect immunofluorescence staining at different time points after the onset of the migration assay in the presence of laminin 1 **(Fig. 31)**. Laser scanning confocal microscopy observation showed a strong intracellular staining of the cells labelled one or two hours after the onset of the experiment **(Fig. 31, T1 and T2)**, indicating high expression levels of endogenous laminin 5. After three hours and later on, laminin 5 staining appears as well defined rings of protein deposited underneath the cells on the surface of the culture support **(Fig. 31, T3 to T5)**. These observations suggest that endogenous laminin 5 accumulating

underneath the epithelial cells provides strong adhesion structures impairing cell locomotion.

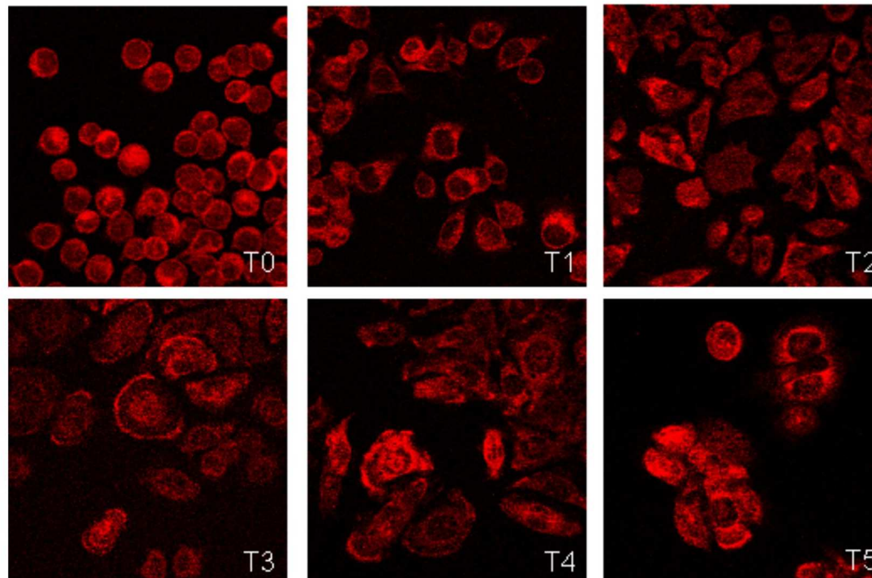


Fig. 31: Synthesis and deposition of laminin 5 by HaCaT cells.
HaCaT cells were seeded on glass coverslips and treated with serum-free medium containing laminin 1. Cells were fixed before treatment (T0) and after 1 to 5 hours (T1 to T5). Endogenous laminin 5 was stained by indirect immunofluorescence using mAb BM165 against the α3 chain of laminin 5. The stainings were observed by laser scanning confocal microscopy. At T0, T1 and T2, laminin 5 is mostly intracellular while at T3 and later, the typical staining pattern indicates extracellular deposition of laminin 5.

3.1.3. Collagen XVII as a regulator of keratinocyte motility

Patients with generalized atrophic benign epidermolysis bullosa (GABEB) caused by mutations in the gene coding for collagen XVII or with bullous pemphigoid autoimmune disorders caused by autoantibodies against collagen XVII present severe skin blistering and immature hemidesmosomes (Van den Bergh and Giudice, 2003). In addition, the transmembrane collagen XVII is part of the hemidesmosomal complex where it co-localizes with $\alpha 6\beta 4$ integrins and anchoring filaments together with laminin 5. These observations suggest that collagen XVII may function as a cell-matrix adhesion molecule or participate in the stability of keratinocyte anchorage. Recently, it was shown that collagen XVII is proteolytically shed from the cell surface and the release of the ectodomain is associated with keratinocyte motility *in vitro* (Franzke et al., 2002). Based on these observations, we tested whether collagen XVII could be involved in the regulation of cell migration.

To do this, we performed *in vitro* migration assays using keratinocytes issued from patients lacking collagen XVII (Col17-/-) and compared them to normal human keratinocytes (NHK).

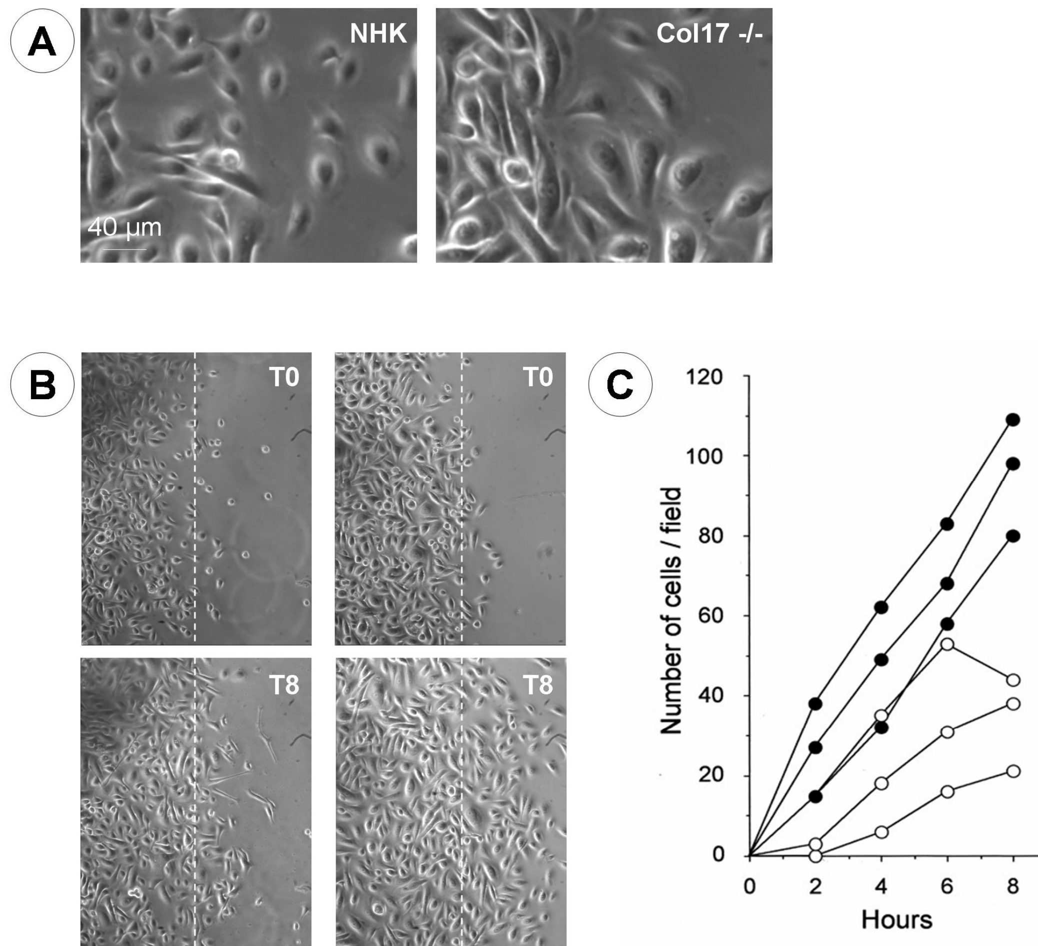


Fig. 32: Migration of NHKs and C17-/- keratinocytes.
(A) Phase-contrast photographs of living NHKs and Col17-/- keratinocytes. The C17-/- keratinocytes are larger and flatter than NHKs. **(B)** Equal numbers of cells were seeded as small colonies in the center of tissue culture wells and the cells were photographed (T0) immediately and after 2, 4, 6 and 8 hours (T8). The white line marks the margin of the colonies at T0. **(C)** The number of the cells that had emigrated from three individual colonies of NHKs (open circles) and C17-/- keratinocytes (filled circles) was counted on photographs taken at the indicated time after onset of the experiment. The results show that the C17-/- keratinocytes are more motile than the control NHKs.

The Col17-/- keratinocytes were flatter than normal keratinocytes and presented larger lamellipodia **(Fig. 32 A)**, a characteristic for motile cells. The migration assays show that the mutant keratinocytes were more motile than normal keratinocytes and, in particular, migrate constantly over time while the control cells slow down **(Fig. 32B,C)**. These results unravel an unexpected role of collagen XVII in the regulation of keratinocyte migration.

3.1.4. Role of CD151 in epithelial cell migration

The laminin-binding integrins directly and specifically interact with the tetraspanin CD151 (Serru et al., 1999). Little is known about the physiological role of CD151-integrin complexes. In tissues, it is the only tetraspanin which always colocalizes with one or several of the various laminin-binding integrins at cell-cell or cell-matrix contacts (Sincock et al., 1997; Serru et al., 1999, Sterk et al., 2002). We used a particular monoclonal antibody, mAb TS151r, the epitope of which is confined to the QRD sequence of CD151 involved in the binding to integrins. This epitope is masked in many tissues, including the skin, indicating that most CD151 is complexed with laminin-binding integrins *in vivo* (Sterk et al., 2002).

3.1.4.1. Localization of CD151 in HaCaT cells

In resting HaCaT epithelial cells grown overnight as monolayer under standard culture conditions, the tetraspanin CD151 is complexed with integrins since it is detected only with the mAb TS151 that recognizes both free and integrin-bound CD151 **(Fig. 33A-C)** and not with mAb TS151r which only binds to free CD151 **(Fig. 33E)**. Under these conditions, CD151 is located at the cell-matrix contacts together with $\alpha6\beta4$ integrins (not shown), where it colocalizes with deposited laminin 5, and in cell-cell contacts, where it colocalizes with $\alpha3$ integrins **(Fig. 33)**, in agreement with previous reports (Sterk et al., 2000). In cell culture as *in vivo*, CD151 is therefore part of two structures associated with cell immobility: the hemidesmosomes and the stable intercellular junctions.

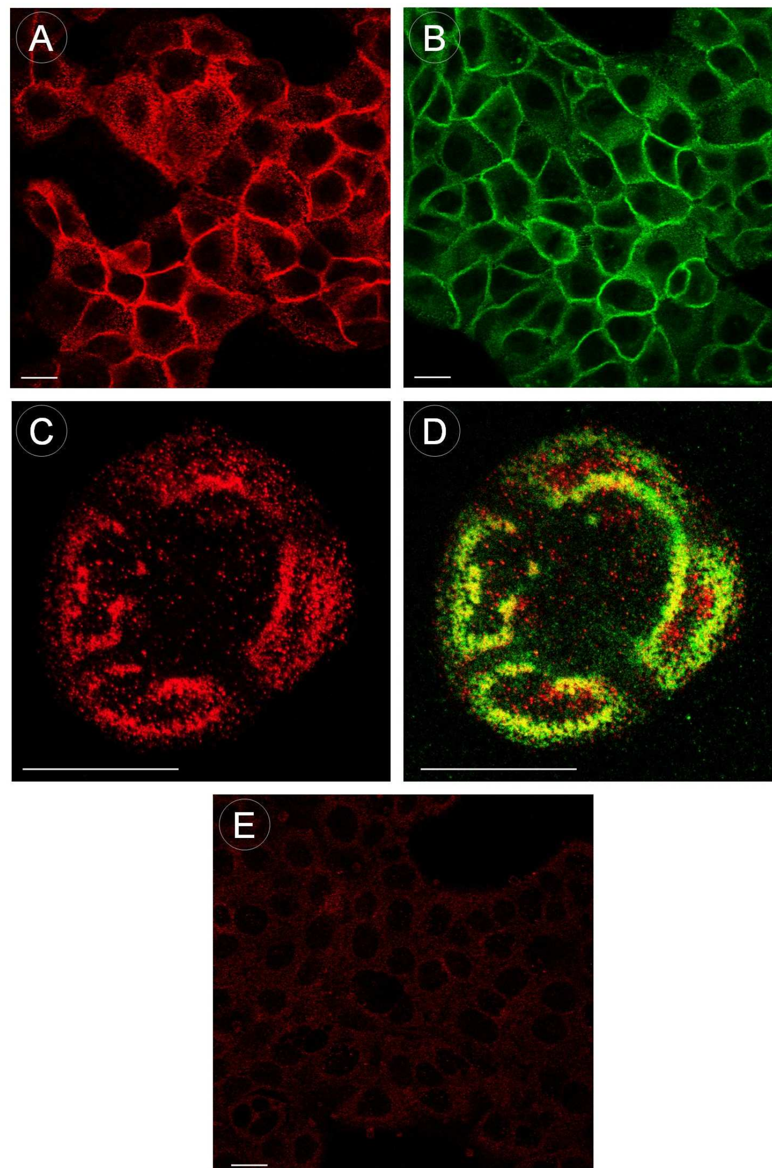


Fig. 33: Absence of free CD151 in resting HaCaT epithelial cells.
HaCaT cells cultivated under standard conditions were stained with mAb TS151 antibody recognizing both free and complexed CD151 (**A**) and with mAb P1B5 against α3 integrins (**B**). Both CD151 and α3 integrins are located at cell-cell contacts. CD151 is found also at the basal side of the cells **(C)** where it colocalizes with laminin 5 (pAb1097) **(D)**. All CD151 is complexed to integrins and is not recognised by mAb TS151r recognizing free CD151 only **(E)**. Bar: 20 µm

3.1.4.2. Cell treatment with mAb TS151r against the integrin-binding site of CD151 induces cell migration on laminin 1

To examine how CD151 could play a role in the control of epithelial cell motility, we performed migration assays in the presence of mAb TS151r, the epitope of which overlaps with the integrin binding site of CD151. Cells treated with mouse immunoglobulins were taken as control. In the presence of the antibody TS151r, a

distinct migration of HaCaT epithelial cells was observed on laminin 1 **(Fig. 34A)**. Interestingly, the enhancing effect was not observed in the presence of laminin 5 **(Fig. 34B)** or for Wi26 fibroblasts **(Fig. 34C)**.

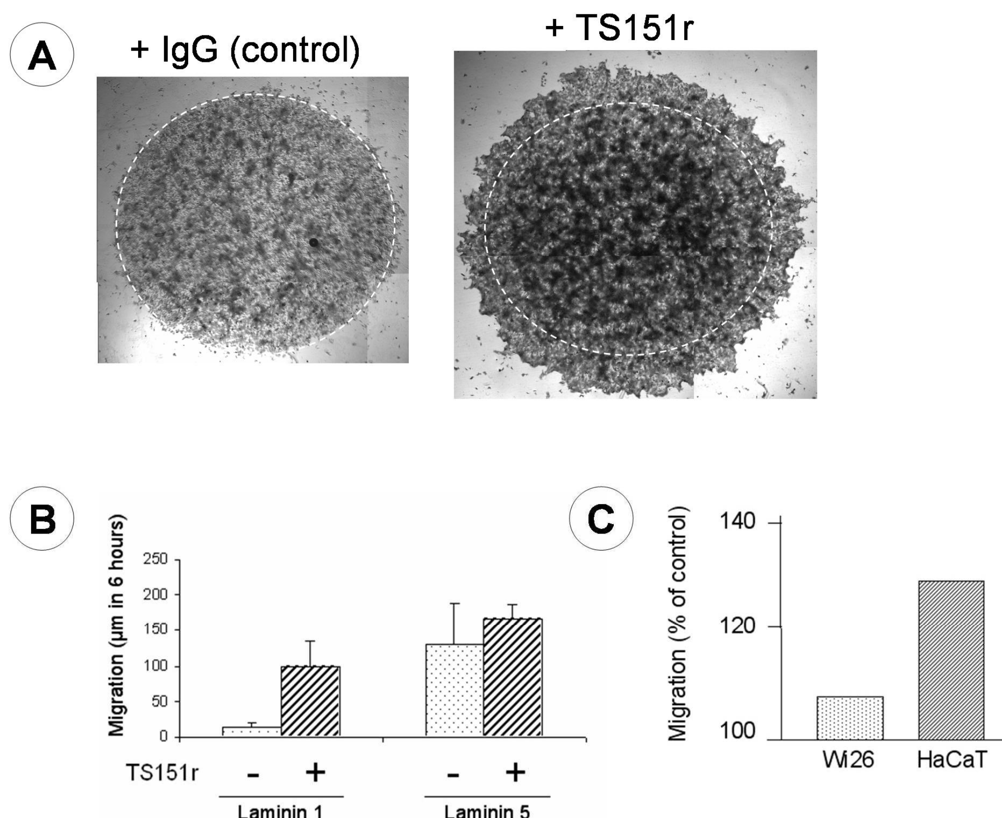


Fig. 34: Treatment with mAb TS51r induces HaCaT epithelial cell migration on laminin 1. (A) Small colonies of cells were allowed to adhere in tissue culture wells for one hour. After wash, fresh medium deprived of serum, but containing laminin 1 (15 µg/ml) and either mAbTS151r or mouse immunoglobulins (10 µg/ml) was added to the wells. Photographs of the HaCaT colonies were immediately taken (white dotted line). After 17 hours of incubation at 37°C, the cells were fixed and stained with crystal violet and photographed. **(B)** Small colonies of HaCaT cells were seeded and treated with laminin 1 or 5 as indicated, and either with mAbTS151r (**+**) or control mouse immunoglobulins (**-**). Photographs were taken immediately after the onset of the experiments and six hours later and the distance covered by the cell front within 6 hours was measured on photographs. Each column represents the average of four experiments and the bars indicate the standard deviation. **(C)** HaCaT cells and fibroblasts Wi26 were treated with laminin 1 and with mAbTS151r or control immunoglobulins. Migration is expressed as percentage of migration of cells treated with control IgG. mAb TS151r has a marginal effect on the migration of Wi26 fibroblasts while it distinctly induces HaCaT to migrate.

After 6 hours of migration, the epithelial HaCaT cells treated with mAb TS151r or with the control immunoglobulins in presence of laminin 1 or 5 were fixed and stained with crystal violet before being photographed. In the presence of laminin 5, the morphology of the cells is not affected by the treatment with mAb TS151r **(Fig. 35)**. In contrast, the cells treated with laminin 1 and mAb TS151r were much more elongated

than the control cells **(Fig. 35)**. Furthermore, the migrating leading cells present protrusions in the form of lamellipodia **(Fig. 35 arrows)**.

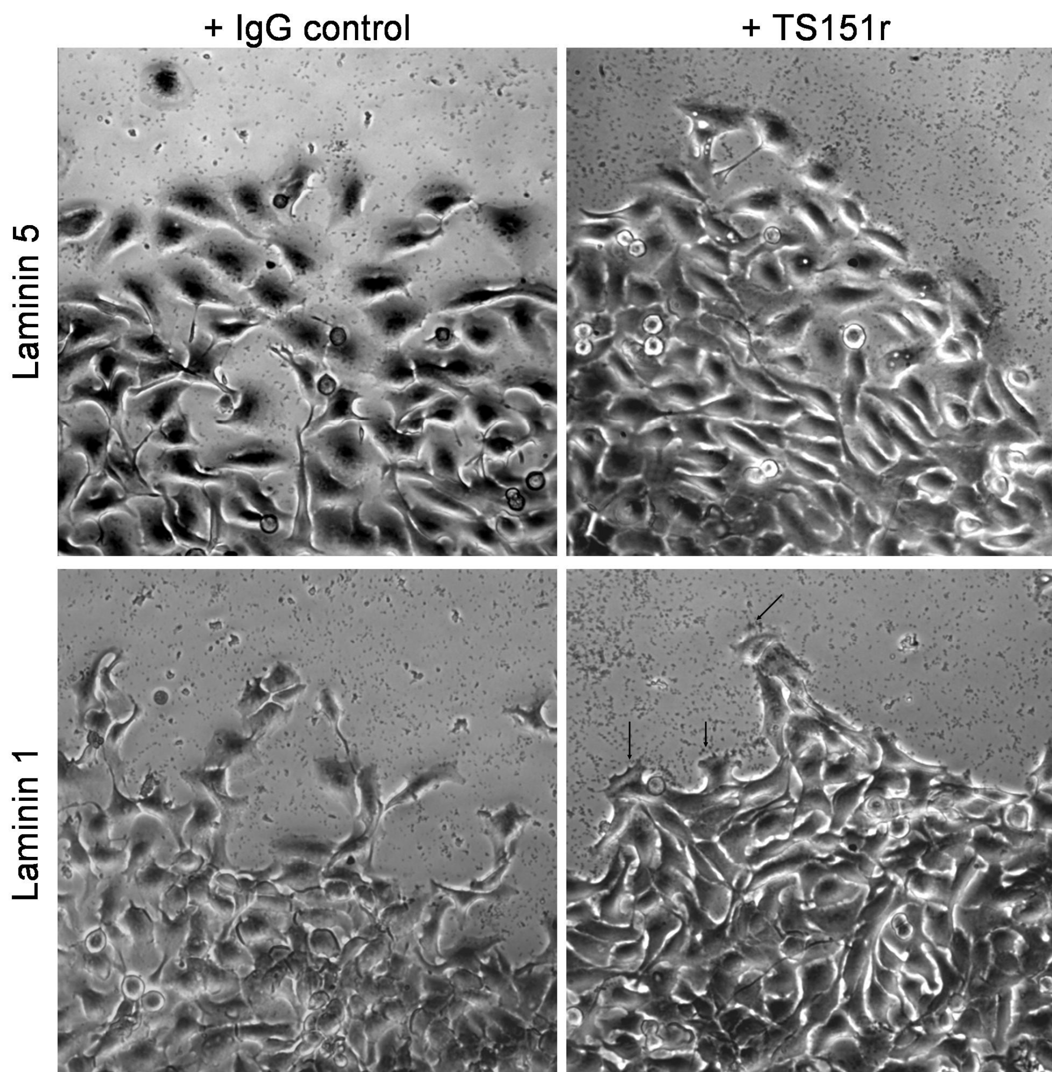


Fig. 35: Morphology of HaCaT epithelial cells treated with the control IgG or mAb TS151r.
After 6 hours of treatment with control IgG or mAb TS151r in presence of laminin 5 (5 µg/ml) or laminin 1 (15 µg/ml), the cells were fixed, stained with crystal violet and photographed. The arrows show the protrusions of the leading migrating cells treated with mAb TS151r and laminin 1.

To analyse in more details this phenomenon, HaCaT epithelial cells treated for 6 hours with mAb TS151r or control mouse immunoglobulins were fixed and stained with FITC-conjugated phalloidin to visualize fibrillar actin. In the presence of laminin 1, cells treated with mAb TS151r displayed lamellipodia and alterations in the morphology of the cell-cell contacts with numerous microspikes seen between the cells instead of close intercellular junctions while the cells exposed to control immunoglobulins had a typical polygonal shape and cell-cell contacts **(Fig. 36)**.

Staining of β-catenin, a marker of cell-cell junctions, confirmed that intercellular contacts were rearranged in HaCaT cells treated with mAb TS151r and migrating on laminin 1 **(Fig. 36)**.

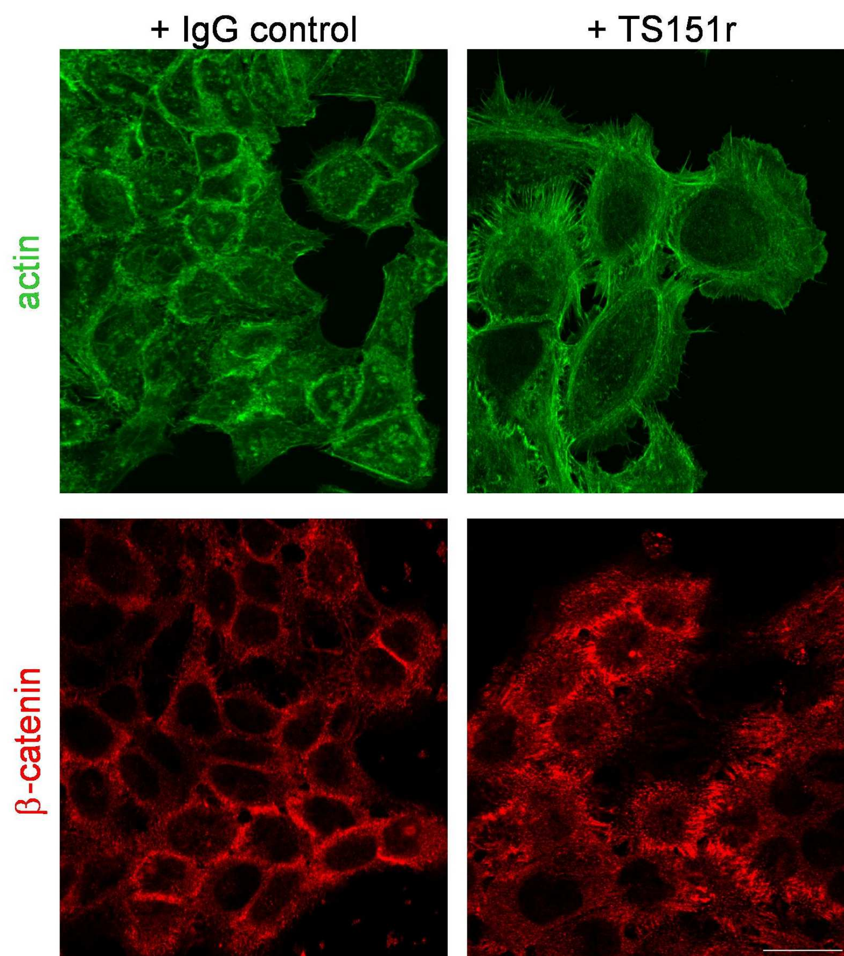


Fig. 36: Reorganization of the actin cytoskeleton and the cell-cell junctions in HaCaT cells treated with mAb TS151r.
HaCaT cells were seeded on glass coverslips and treated with serum-free medium containing laminin 1 and either mAb TS151r or control mouse immunoglobulins. Six hours later, the cells were fixed and stained for fibrillar actin with FITC-phalloidin and for β-catenin. HaCaT cells treated with control IgG have a typical polygonal shape while the actin network is especially cortical and β-catenin is observed along the cell-cell junctions. In opposite, TS151r-treated cells are more elongated, have less cortical actin network and present lamellipodia. In these cells, β-catenin has lost its linear localization along cell-cell junctions. Scale bar: 20 µm.

3.1.4.3. mAb TS151r releases a fraction of CD151 from integrin association

After the cells had migrated for 6 hours on laminin 1 in the presence of mAb TS151r, the cells were fixed and directly incubated with Cy-3 conjugated secondary antibodies against mouse immunoglobulins. Observation of the stainings revealed that the mAb TS151r was bound to the cells at the level of cell-cell-junctions while the control

immunoglobulins were not **(Fig. 37A-B)**. Since this antibody binds to CD151 only when it is not associated with integrins, it indicates that there is a pool of non ligated CD151, i.e. CD151 not complexed with the integrins expressed at the surface of the treated cells.

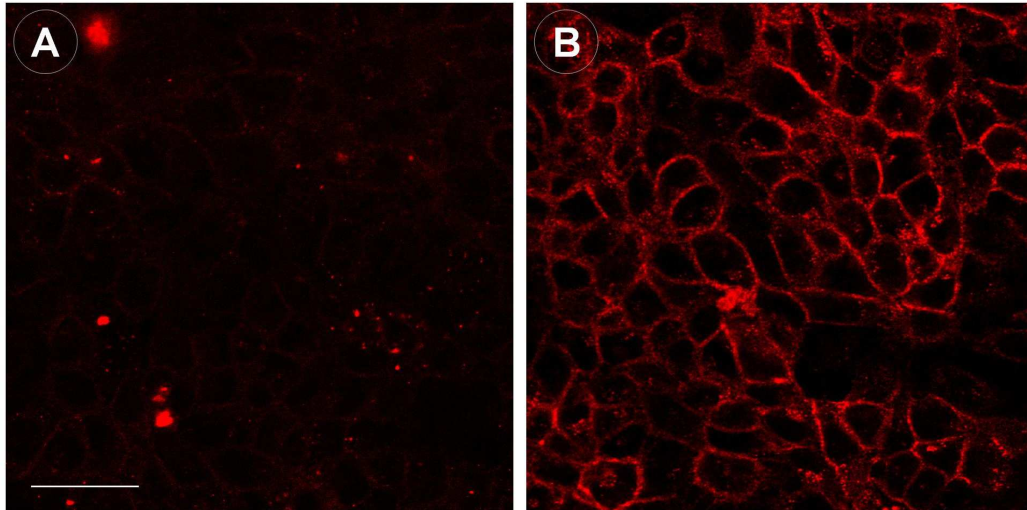


Fig. 37: Localization of CD151 in cells treated with mAb TS151r.
After 6 hours of treatment with control mouse immunoglobulins **(A)** or mAb TS151r **(B)**, HaCaT cells were fixed and directly stained with Cy-3 conjugated secondary antibodies against mouse immunoglobulins. This latter recognizes the antibody TS151r at cell-cell contacts while it does not recognized any control mouse IgG. This suggests that the antibody TS151r is bound to CD151 not associated with integrins. Scale bar: 40 µm.

3.1.4.4. Treatment with mAb TS151r against the integrin-binding site of CD151 induces a redistribution of laminin-binding integrins

To understand the relationship between CD151-binding to integrins and migration of HaCaT cells on laminin in the presence of the mAb TS151r, the distribution of integrins was examined using indirect immunofluorescence staining and laser scanning confocal microscopy. When treated with control mouse immunoglobulins, β1 integrins were typically located in cell-cell contacts **(Fig. 38A)** while cell membrane staining was absent at the border of the colonies after treatment with mAb TS151r **(Fig. 38B)** and β1 integrins were found in discrete cell-matrix contacts (not shown).

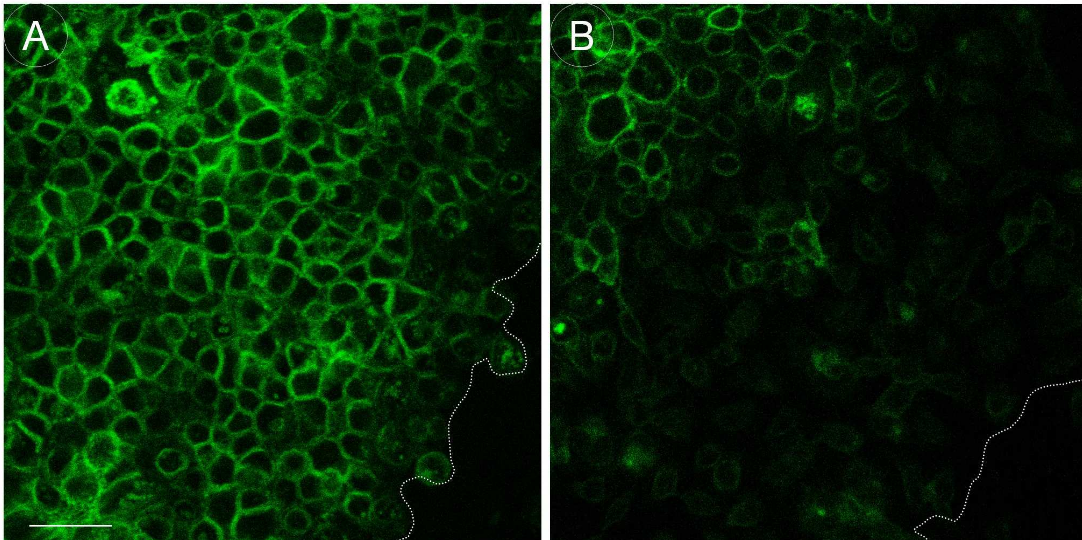


Fig. 38: Localization of β1 integrins TS151r-treated cells.
After six hours of treatment with control mouse immunoglobulins **(A)** and mAb TS151r **(B)**, HaCaT cells were stained for integrin β1 subunits using fluorescein-coupled mAb K20. The integrin β1 subunits were typically found at cell-cell junctions in control cells **(A)** but not in TS151r-treated cells **(B)**. The white lines indicate the margins of the cell colonies. Scale bar : 40 µm

The staining with antibodies against integrin β4 subunit revealed that these integrins were targeted to the surface of the cells in the presence of the control immunoglobulins **(Fig. 39A-B)**, in particular to the basal surface where they form clusters in the hemidesmosome-like structures **(Fig. 39C)**. In contrast, β4 integrins are present in the cytoplasm of the cells treated with mAb TS151r, in particular in the leading cells migrating out of the colony **(Fig. 39D-E)**. In these cells, the basal cell surface in contact with the substratum is devoid of β4 integrins, indicating the absence of stable anchorage structures.

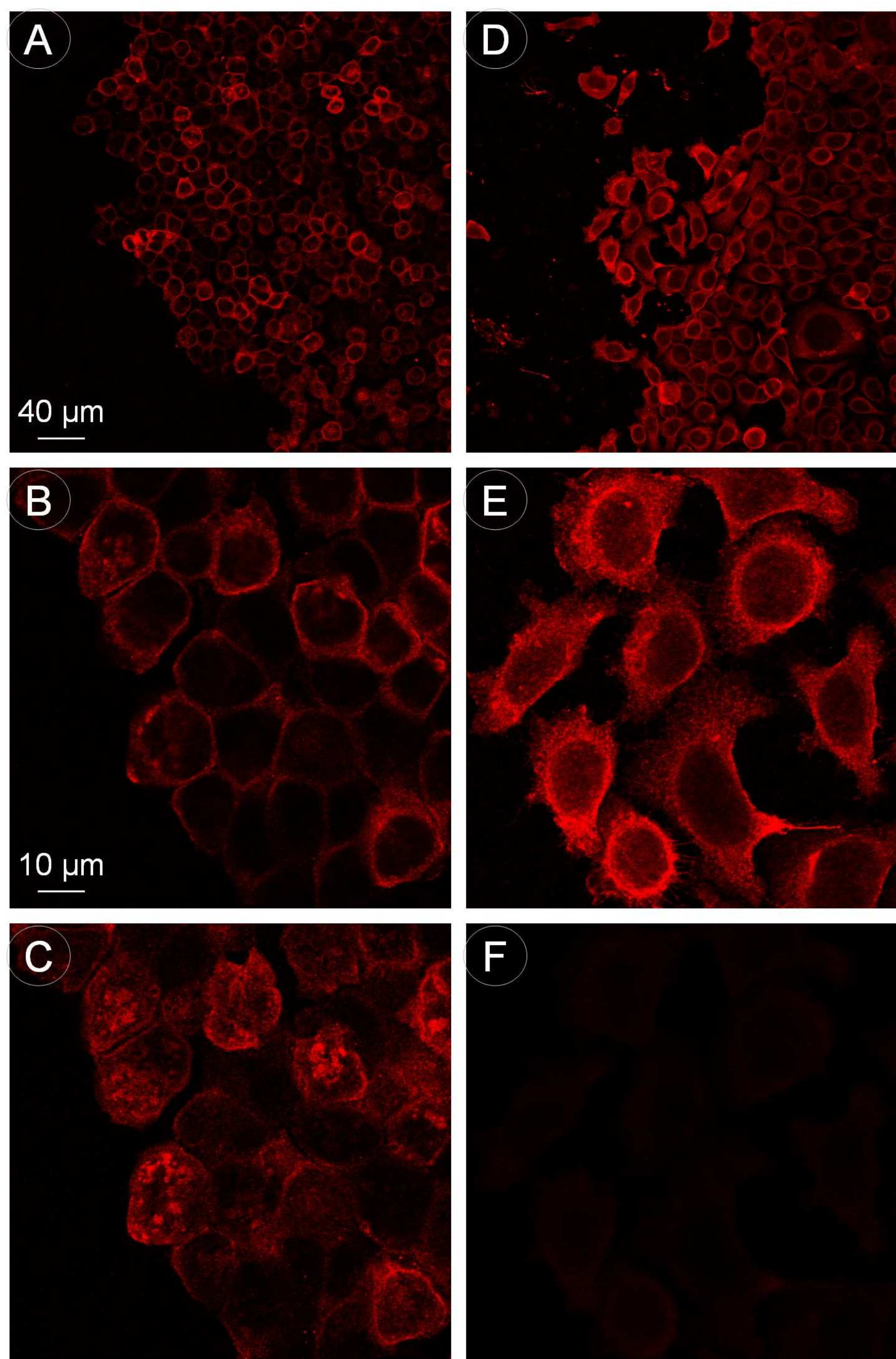


Fig. 39: Localization of integrin β4 subunits in TS151r-treated cells.
HaCaT cells treated for six hours with control mouse immunoglobulins **(A,B,C)** or mAb TS151r **(D,E,F)** were stained with antibody C-20 against β4 integrin. The sections A and D show epithelial cells located at the border of the cell colonies. B, C and E, F: high-power magnification of panel **A** and **D**, respectively. Pictures in B and E show confocal sections within the cell bodies; pictures in C and F show confocal sections at the cell-matrix interface. In control cells, the integrin β4 subunits are associated with the cell surfaces (**B**) and with hemidesmosome-like structures at the cell-matrix interface (**C**). In opposite, in the TS151r-treated cells, the integrin β4 subunits are observed in the cytoplasm **(E)** and are absent from the basal cell surface **(F)**.

3.1.4.5. Remodelling of cellular interactions at the dermal-epidermal interface along hair follicles coincides with expression of free CD151

Observation of human skin cryo-sections after indirect immunofluorescence staining of CD151 confirmed that CD151 colocalizes with laminin-binding integrins in the epidermis (Sterk et al, 2002). Indeed, using the antibody TS151 that recognize both free and integrin-bound CD151, CD151 colocalized with $\alpha6\beta4$ integrins along the dermal-epidermal interface and with $\alpha3$ integrins at the cell-cell contacts **(Fig. 40D)**. In the epidermis, staining for CD151 with mAb TS151r is negative, indicating that CD151 is ligated to integrins **(Fig. 40A)**. Along the hair follicle in anagen phase, however, the staining of free CD151 becomes positive at the dermal-epidermal interface **(Fig. 40B)**. At the level of the hair bulb, CD151 is recognized by both mAb TS151 and mAb TS151r, and it is mainly located in cell-cell contacts **(Fig. 40C,F)**. This shows that, in this region where the cells migrate, CD151 is free and localized at intercellular junctions.

These observations suggest that in resting epidermis all CD151 is complexed with laminin-binding integrins, while it is not in regions of remodelling and cell movement.

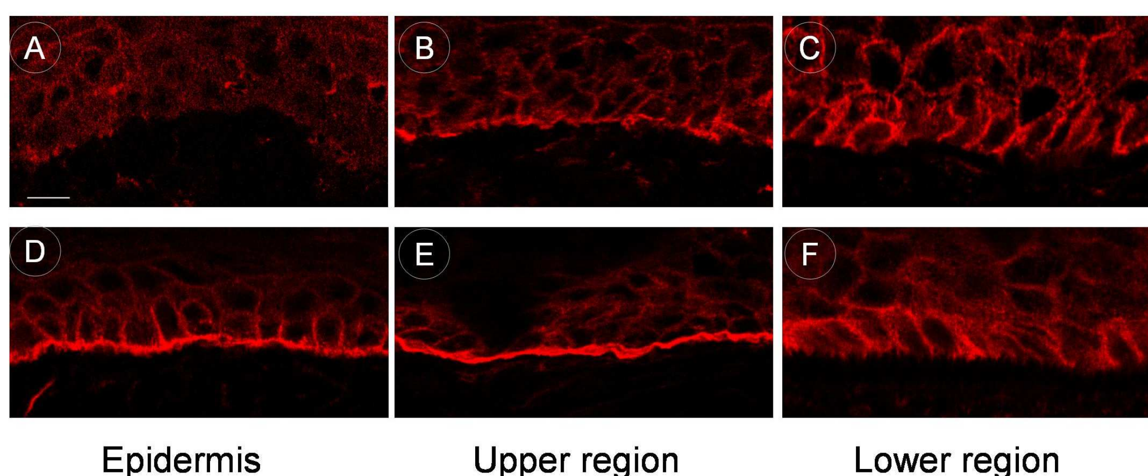


Fig. 40: Localization of CD151 in the epidermis and hair follicle.
Cryosections of human skin were processed for indirect immunofluorescence staining with mouse monoclonal antibodies TS151r **(A,B,C)** or TS151 **(D,E,F)** against the free CD151 and integrin-associated CD151, respectively. Mouse immunoglobulins were detected with Cy3-conjugated secondary antibodies. In the epidermis, most of the CD151 is localized at cell-matrix interface, some at cell-cell junctions **(D)**. The absence of staining using mAb TS151r **(A)** indicates that in the epidermis, all CD151 is complexed with integrins. In the upper part of the hair follicle, integrin-complexed CD151 **(E)** as well as unbound CD151 **(B)** are observed at the dermal-epidermal interface and cell-cell junctions. In contrast, in the hair bulb, most CD151 is located at cell-cell junctions **(**not in complex with integrins **C, F)**. Scale bar: 20 µm

3.2. Role of RhoA, Rac1 and Cdc42 in epithelial cell migration

Extracellular matrix components affect biological process such as morphogenesis, differentiation and cell migration. Cell attachment to the extracellular matrix at focal contacts is mediated by integrins, which then transduce the signals from the matrix to the actin cytoskeleton (Brakebusch and Fässler, 2003). This event triggers cell spreading and re-organization of cell-matrix adhesions. During the process of cell migration, the integrin-mediated adhesions and their release need to be regulated (Lauffenburgen and Horwitz, 1996). Some signalling pathways, which depend on integrin activation, involve the activation of the small GTPases RhoA, Rac1 and Cdc42, which are intermediate partners to the dynamic actin cytoskeleton (Ren et al., 1999; Del Pozo et al., 2002). It is not known whether integrins trigger activation of small GTPases at these sites or if the focal contacts are the sites of GTPase-dependent events. In addition, in epithelial cells, the small GTPases may modulate cell migration by regulating cell-cell junctions (Jamora and Fuchs, 2002). Here, we studied the role of laminin 1 and laminin 5 in modulating the behavior of epithelial cells via the small GTP-bound proteins RhoA, Rac1 and Cdc42.

3.2.1. Small GTPase activation on cells plated on laminins

3.2.1.1. Morphology of Wi26 fibroblasts and HaCaT epithelial cells adhering on laminin 1 or laminin 5

Fibroblasts and epithelial cells were plated on surfaces coated with laminin 1 or laminin 5, which are ligands for $\alpha6\beta1$ and $\alpha3\beta1$, respectively. Under serum-free conditions, the morphology of both cells types was similar whether they adhere on one laminin isoform or the other. On laminin 1 fibroblasts Wi26 and epithelial HaCaT cells presented an elongated morphology with filopodia–like protrusions while on laminin 5, both types of cells were flatter and had lamellipodia **(Fig. 41)**. To further investigate the effect of these laminins, we analyzed the kinetic of the adhesion plaque formation by indirect immunofluorescence staining using a monoclonal antibody against vinculin, a marker of focal contacts. The experiments were performed in the absence of serum to avoid the cross talk between integrin- and

growth-factor-receptor–mediated signaling and the cells were fixed at different time points (30, 60 and 120 minutes).

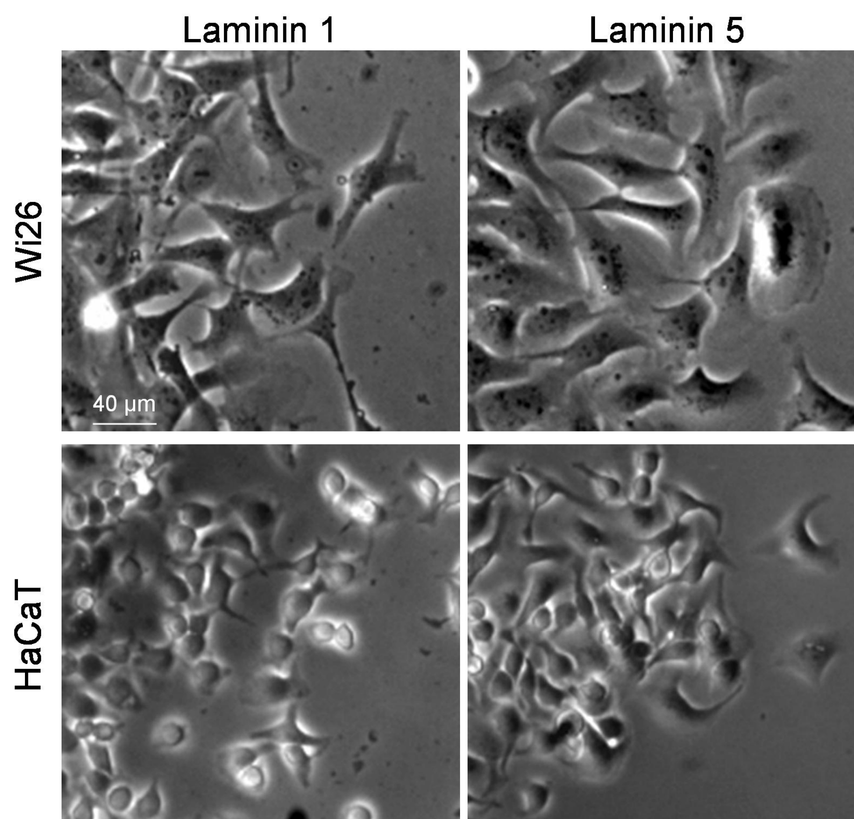


Fig. 41: Morphology of fibroblasts and epithelial cells adhering to laminin 1 and laminin 5.
Wi26 and HaCaT cells were plated on laminin 1 (15 µg/ml) or laminin 5 (5 µg/ml) for 6 hours and photographed under phase contrast microscopy. Wi26 and HaCaT cells present filopodia when plated on laminin 1 and lamellipodia when plated on laminin 5.

Thirty min after cell adhesion to the substrates, the Wi26 fibroblasts had begun to spread while the HaCaT cells had adhered but not spread **(Fig. 42A, 30 min)**. In addition, isoform-specific focal adhesions were already formed at this time point in the case of Wi26 fibroblasts **(Fig. 42A, 30 min)**. On cells adhering on laminin 1, vinculin appeared as short and thick patches, while on laminin 5, vinculin staining was thin with punctuate structures localized at the periphery of the lamellipodia. Up to 60 min of spreading, the fibroblasts presented the morphology typical for laminin 1 or laminin 5 and the difference could be observed even better after a longer incubation period **(Fig. 42A, 120 min)**.

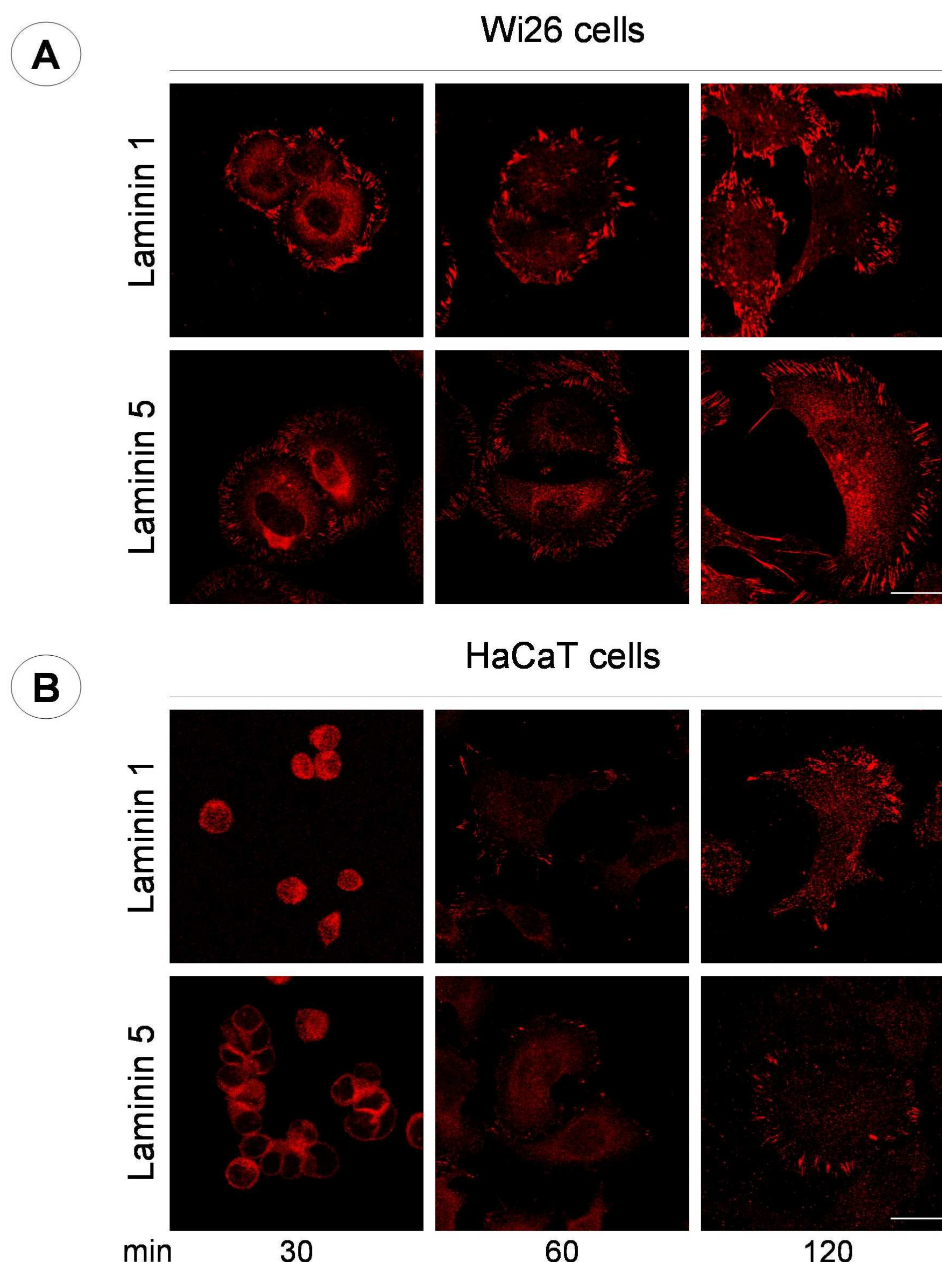


Fig. 42: Kinetics of adhesion complex formation in Wi26 fibroblasts and HaCaT epithelial cells on laminin 1 and 5.
Wi26 cells **(A)** and HaCaT cells **(B)** were plated on glass coverslips coated with laminin 1 (15 µg/ml) or laminin 5 (5 µg/ml). After 30, 60 or 120 min, adherent cells were fixed and stained with a monoclonal antibody against vinculin followed by Cy3-conjugated secondary antibody. Although the kinetics of the adhesion complex formation is different for the two cell lines, HaCaT and Wi26 cells develop strong focal adhesions on laminin 1 and thin focal complexes on laminin 5. Scale bar: 20 µm

While fibroblasts already were fully spread already after 30 min, HaCaT epithelial cells plated on laminin 1 and laminin 5 took on a characteristic morphology between 30 and 60 min of adhesion **(Fig. 42B, 30-60 min)**. Only after one hour of spreading, the epithelial cells begin to engage isoform-specific focal contacts that were better

observed at later time point (120 min). At this time, the focal adhesions were similar to those observed for fibroblasts under the same conditions **(Fig. 42B, 120 min)**.

Focal adhesions are sites where β1 integrins cluster and organize signalling complexes that control integrin-mediated changes in cell behavior. In order to assess whether the focal adhesions formed on laminin 1 and laminin 5 contained clustered activated integrins, we used the monoclonal antibody 9EG7, which recognizes ligand-bound β1 integrins only (Lenter et al., 1993). As HaCaT epithelial cells spread slower than fibroblasts, as demonstrated by the kinetic assay described above, they were allowed to spread for two hours before fixation instead of one hour for Wi26 cells. The activated β1 integrins were in both cell lines found in focal contacts colocalizing with vinculin on laminin 1 as well as on laminin 5 **(Fig. 43)**.

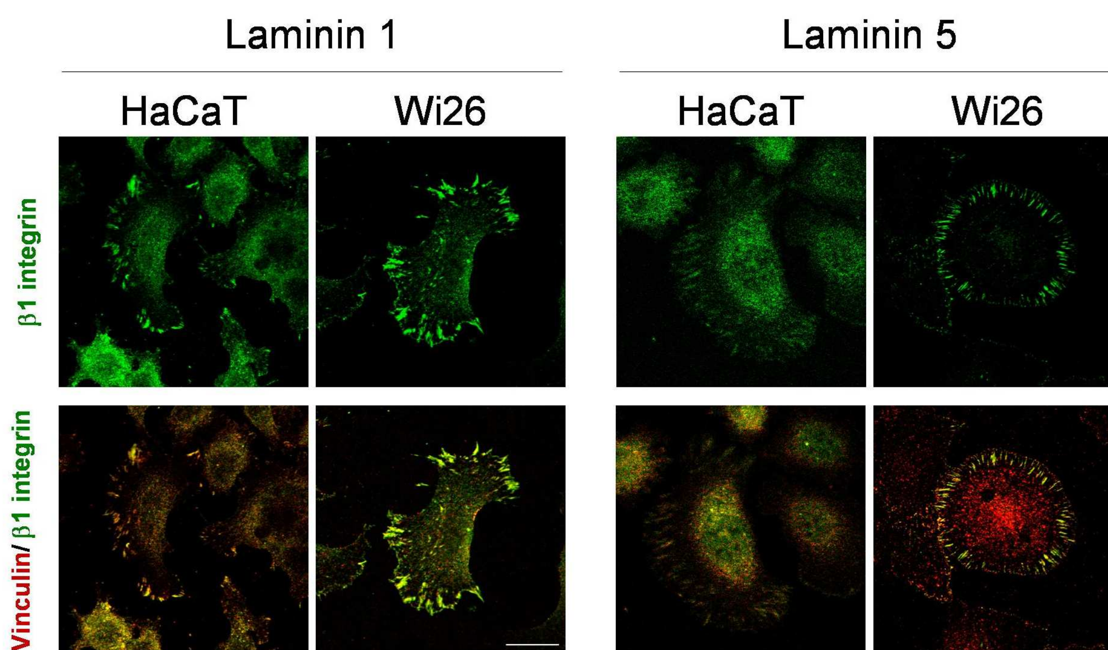


Fig. 43: Indirect immunofluorescence staining of vinculin and activated β1 integrin in HaCaT epithelial cells and Wi26 fibroblasts adhering on laminin 1 and 5.
Cells were plated on glass coverslips coated with either laminin 1 (15 µg/ml) or laminin 5 (5 µg/ml) for 1 hour (Wi26 cells) or 2 hours (HaCaT cells). They were then fixed and processed for immunofluorescence staining using antibodies against activated β1 integrins (mAb 9EG7) and vinculin (mAb F-VII). Activated β1 integrins are found in the vinculin-containing adhesion complexes. Bar: 20 µm

Together these results show that cell adhesion to laminin 1 or laminin 5 induces isoform-specific clustering of activated β1 integrins associated with cytoskeletal proteins like vinculin and a specific morphology for fibroblasts and epithelial cells.

However, the kinetics of signalling activation is different according to the cell type and the substrate. It occurs much earlier in fibroblasts than in epithelial cells.

3.2.1.2. Cell migration on laminin 1 and laminin 5

We next characterized the migration pattern of the two cell types on laminin 1 and laminin 5 by time-lapse video microscopy. The cells were plated in medium supplemented with serum to allow fast adhesion. When the cells had adhered and spread, they were washed with medium lacking serum and further incubated in serum-free medium containing either laminin 1 or laminin 5. At this time point, we started to record cell migration every five minutes for 13 hours. In the presence of laminin 5, the Wi26 fibroblasts, as well as the HaCaT epithelial cells, show a polarized, unidirectional migration pattern **(Fig. 44A)**. In contrast, laminin 1 induces a random migration of the fibroblasts, which change their polarity several times during the 13 hours of recording. The migration pattern of epithelial cells on laminin 1 was similar to that of fibroblasts, although they migrate slower **(Fig. 44A)**.

The cell directionality was quantified by determining the processive index (Frank and Carter, 2004), which is defined as the ratio between the linear distance from starting to end points and the real distance covered by the cells. As shown in **fig. 44B**, the epithelial cells and the fibroblasts migrating on laminin 5 had a processive index close to 1 (0.75 ± 0.08 for Wi26 and 0.78 ± 0.05 for HaCaT), meaning that the cells moved with a nearly linear mode. In contrast, laminin 1 induced a multidirectional migration as shown by the processive indexes (0.58 ± 0.19 for Wi26 and 0.33 ± 0.15 for HaCaT). These results indicate that laminin 1 and laminin 5 coordinate cell migration in a different manner.

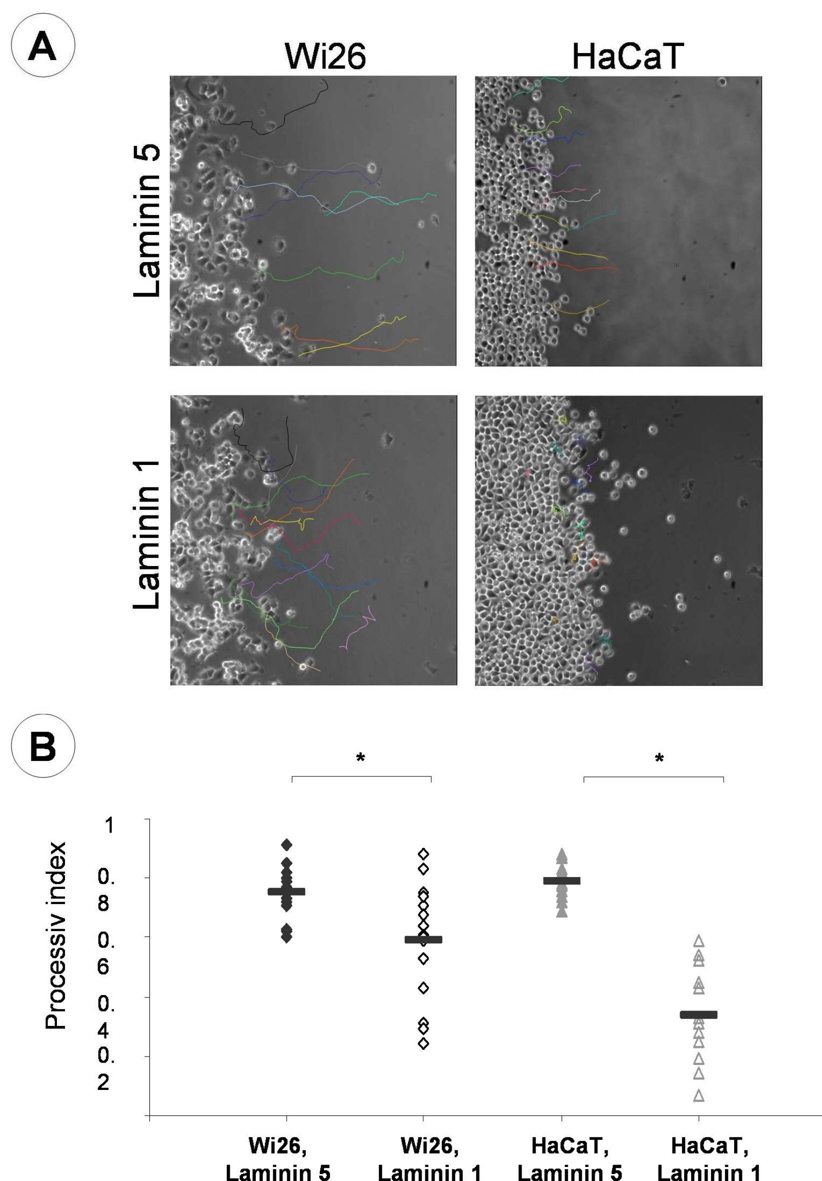


Fig. 44: Laminin 5 induces a polarized, directional migration in contrast to laminin 1. **(A)** Wi26 fibroblasts and HaCaT epithelial cells were submitted to migration assays on laminin 1 (15 µg/ml) and laminin 5 (5 µg/ml). The migration of the cells was recorded by time-lapse imaging during 13 hours. The tracks of several cells were followed using Openlab software and are visualized with the colored lines. **(B)** The processive index for cells in A was calculated as the ratio between the linear and the real distance covered by the cells (Measurements of 15 cells for each condition). The asterisk indicates significant differences ($p < 0.05$).

3.2.1.3. Laminin 1 and laminin 5 differentially activate the Rho GTPases

It is well established that the small GTPases RhoA, Rac1 and Cdc42 affect cell migration by acting on actin polymerization and on the formation of the adhesion plaques (Machesky and Hall, 1996; Ridley, 2001).

As described in a previous paragraph, laminin 1 and laminin 5 have the property to induce isoform-specific focal adhesions. These are very comparable to the ones induced by the activation of the small GTPases. We therefore wanted to analyse the activity of RhoA, Rac1 and Cdc42 in cells exposed to laminins. For this purpose, we first established the pull-down assays enabling to detect activated GTP-bound RhoA, Rac1 and Cdc42 (Ren et al., 1999; Sander et al., 1998). In these assays, the RhoA-binding domain of Rhotekin (RBD) and the CRIB domain of PAK were used to precipitate activated RhoA, Rac1 and Cdc42, respectively.

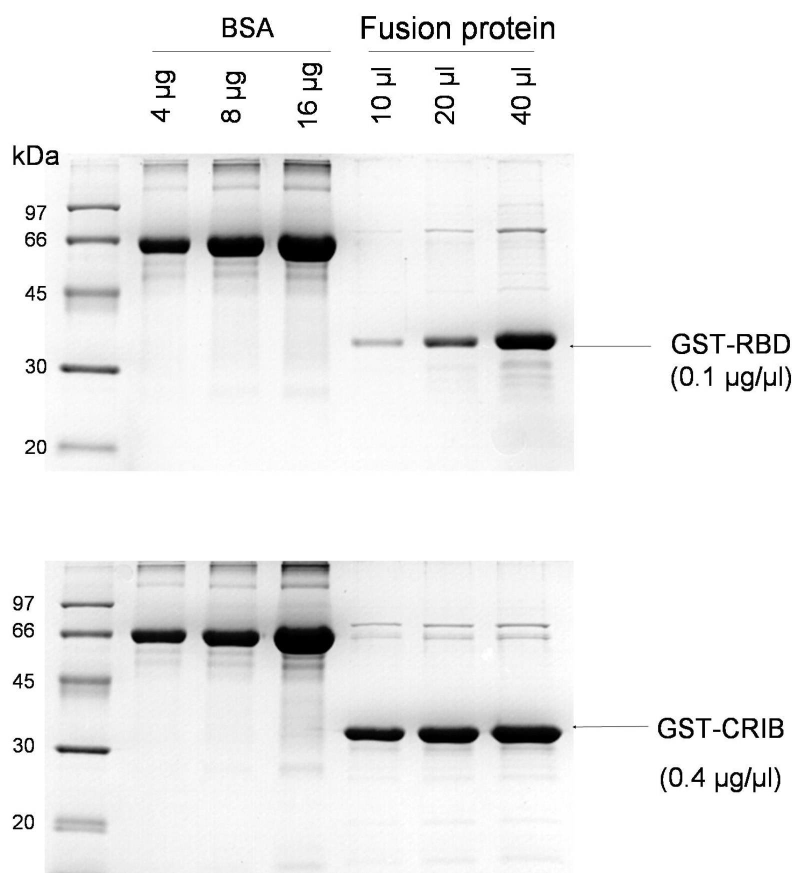


Fig. 45: Purification of GST-RBD and GST-CRIB fusion proteins expressed in BL21 and DH5α bacteria, respectively.
Different volumes (10, 20 and 40 µl) of the products were fractionated on a 12% acrylamide gel under reducing conditions and visualized after staining with Coomassie blue. To quantify the amount of fusion proteins, band intensity was compared to that of lanes loaded with a known quantity of BSA.

The plasmids pGEX-2T-CRIB and pGEX-2T-TRBD, containing the Cdc42-Rac1-Interaction Binding Domain (CRIB) and the Rhotekin Rho-binding domain (RBD), respectively, were introduced in E. coli cells. The expression of glutathione S-transferase (GST)–fusion proteins in the bacteria was induced by adding IPTG in the growth medium. After cell lysis, the GST-fusion proteins were affinity precipitated by

coupling to glutathione beads resolved by SDS-PAGE under reducing conditions. Staining the gel with Coomassie blue revealed that the fusion proteins were efficiently purified **(Fig. 45)**. By comparing band intensities to that of bands with known amounts of BSA, the yield was 0.1 mg/ml GST-RBD and 0.4 mg/ml for GST-CRIB. For the pull-down assays, 30 µg of fusion proteins were used.

To test whether the fusion proteins GST-CRIB and GST-RBD were able to precipitate endogenous GTP-bound proteins, we performed pull-down assays with the HaCaT cells. Previous experiments have shown that the small GTPases can be activated by components of serum such as lysophosphatidic acid or epidermal growth factor (EGF) for RhoA and Rac1, respectively (Ridley and Hall, 1992; Ridley et al., 1992; Kozma et al., 1995). The cells were therefore serum-starved for 24 hours and then stimulated or not with serum. After cell lysis and incubation with either GST-RBD or GST-CRIB beads and the bound proteins were separated by SDS-PAGE and analysed by immunoblotting **(Fig. 46)**. The amount of activated RhoA and Rac1 were increased after treatment with serum, consistent with previous reports (Ren et al., 1999) and our results demonstrate that the GST-RBD and GST-CRIB beads that we have purified can effectively precipitate activated RhoA and Rac1.

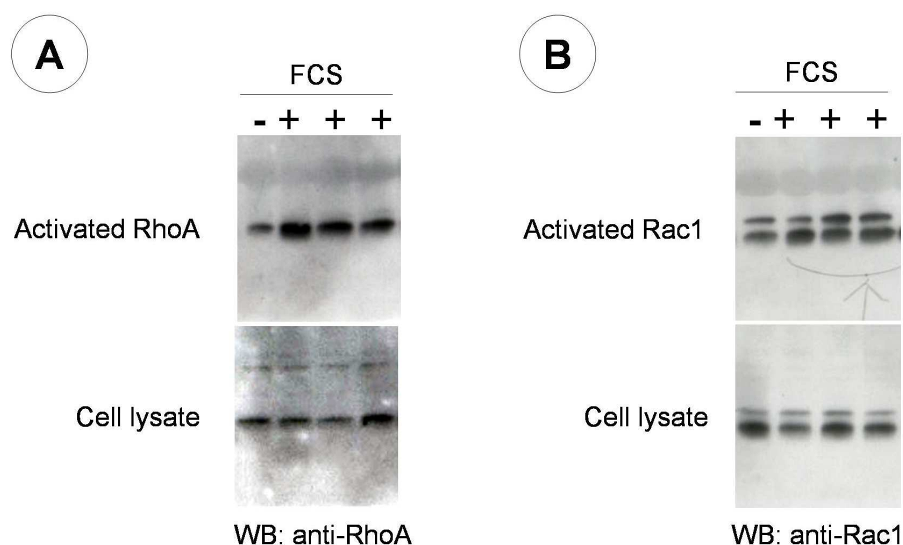


Fig. 46: Control experiment to test GST-RBD and GST-CRIB activity.
HaCaT cells were serum starved for 24 hours and then treated with FCS. Without treatment (**-**) or after incubation with 10% serum (**+**), the cells were lysed and incubated with GST-RBD **(A)** or GST-CRIB beads **(B)**. Bound proteins were fractionated by SDS-PAGE and detected by western blotting with monoclonal antibodies against RhoA and Rac1. The amount of activated GTP-bound RhoA (A, upper panel) and Rac1 (B, upper panel) was normalized to the total amount of the corresponding small GTPase in cell lysate (lower panel). The treatment with serum induced the activation of RhoA and Rac1 in HaCaT cells.

In order to investigate the role of laminin isoforms in regulating small GTP-binding proteins, their activation was analyzed in HaCaT epithelial cells and Wi26 fibroblasts adhering on laminin 1 and laminin 5. The cells were serum-starved for 24 hours before being plated on laminin 1 and laminin 5. After 30, 45 and 60 minutes of incubation, the cells were lysed and either GST-CRIB or GST-RBD was used to precipitate endogenous activated Rac1, Cdc42 or RhoA, respectively. The precipitated proteins, as well as an aliquot of the whole cell lysate, were analyzed by SDS-PAGE followed by immunoblotting with specific monoclonal antibodies against RhoA, Rac1 and Cdc42 **(Fig. 47A)**. The amount of activated proteins was measured by scanning densitometry and normalized to the amount of proteins in whole cell lysates **(Fig. 47B)**.

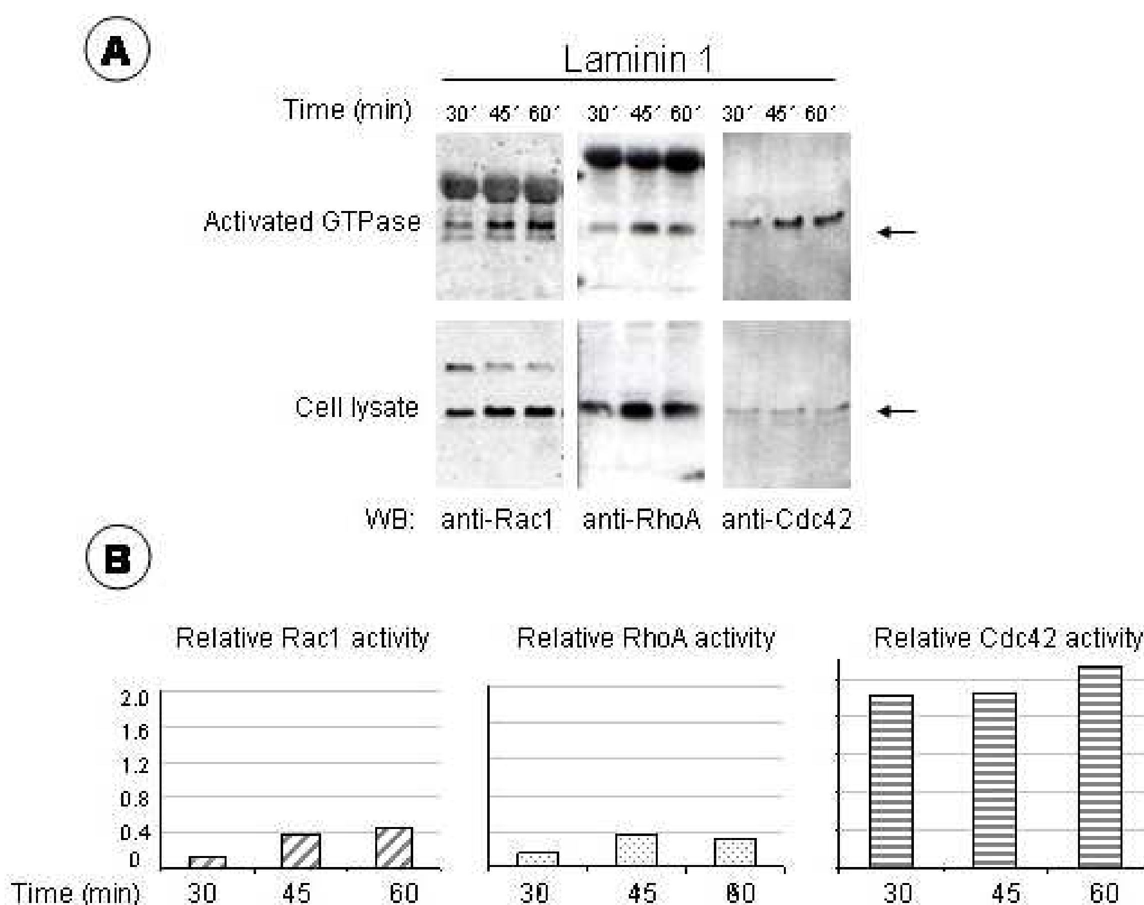


Fig. 47: Activation of small GTPases in HaCaT epithelial cells adhering on laminin 1. HaCaT cells were serum-starved for 24 hours and then stimulated with laminin 1 for 30, 45 or 60 minutes. GST-CRIB or GST-RBD was used to precipitate endogenous GTP-Rac1/Cdc42 and GTP-RhoA, respectively. **(A)** Immunoblots were performed using monoclonal antibodies to Rac1, RhoA and Cdc42. The whole cell lysates were immunobloted with the same antibodies. **(B)** The autoradiographs shown in A were quantified using Scion Image software, and the level of GTP-bound proteins normalised to

the total proteins in the lysate. The results shown are representative of three independent experiments.

The results showed that on laminin 1 the levels of activated Rac1 and RhoA were low after 30 min and then increased up to 60 min of adhesion. The level of activated Cdc42 was already high at the beginning of the experiment and slightly increased during the first hour of adhesion.

In HaCaT cells plated on laminin 5 **(Fig. 48A,B)**, the activation of Rac1 and Cdc42 was rapid while no endogenous GTP-RhoA could be precipitated in three independent assays.

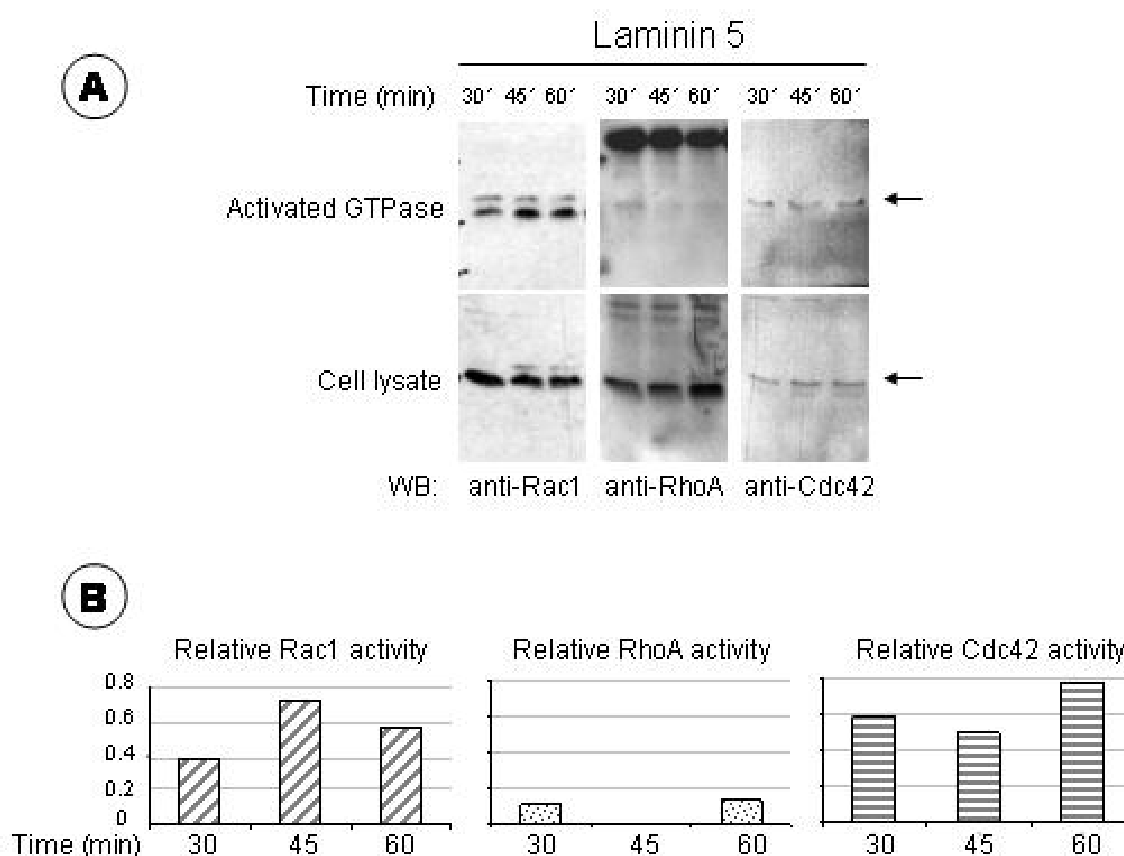


Fig. 48: Activation of small GTPases in HaCaT epithelial cells adhering on laminin 5. HaCaT cells were serum-starved for 24 hours and then stimulated with laminin 5 forr 30, 45 or 60 minutes. GST-CRIB or GST-RBD was used to precipitate endogenous GTP-Rac1/Cdc42 and GTP-RhoA, respectively. **(A)** Immunoblots were performed using monoclonal antibodies to Rac1, RhoA and Cdc42. The whole cell lysates were immunobloted with the same antibodies. **(B)** The autoradiographs shown in A were quantified using Scion Image software, and the level of GTP-bound proteins normalised to the total proteins in the lysate. The results shown are representative of three independent experiments.

In Wi26 fibroblasts plated on laminin 1, the activation of RhoA was high already after 30 min of adhesion and was maintained up to one hour **(Fig. 49A,B)**. In contrast, the level of activated Rac1 was low at 30 min and increased progressively **(Fig. 49A,B)**. In the case of GTP-bound Cdc42, the pull-down assay showed a decrease of its level between 30 and 45 min **(Fig. 49)**.

Adherence of the Wi26 fibroblasts to laminin 5 induced a strong activation of Rac1 after 30 minutes, as observed for HaCaT cells, which then decreased progressively **(Fig. 50A)**. Laminin 5 induced the activation of RhoA in the fibroblasts already after 30 minutes and, in addition, the activation of RhoA increased gradually over the time. Analysis of the activation of Cdc42 on laminin 5 **(Fig. 50)** showed that, in contrast to laminin 1, laminin 5 stimulates a progressive increase of Cdc42 activity.

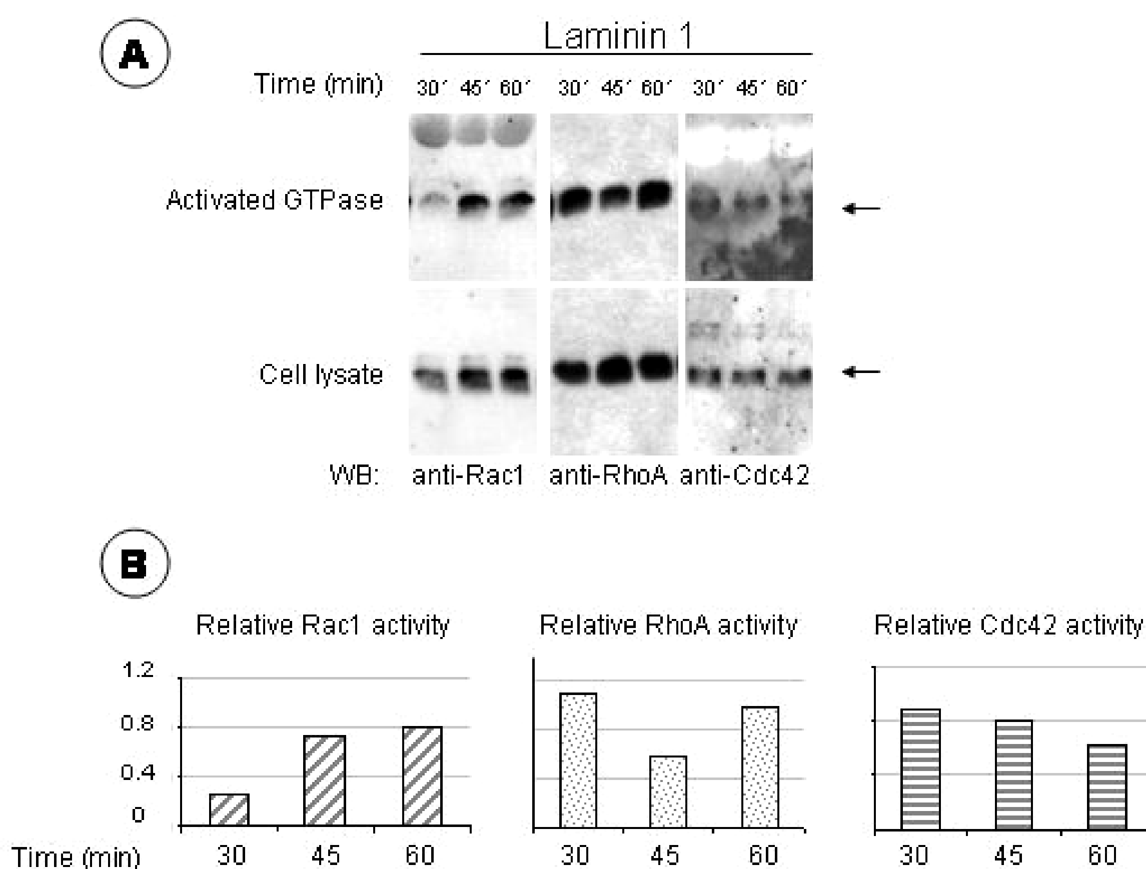


Fig. 49: Activation of the small GTPase in fibroblasts adhering on laminin 1.
Fibroblasts Wi26 cells were serum-starved for 24 hours and then with laminin 1 for 30, 45 or 60 minutes. GST-CRIB or GST-RBD was used to precipitate endogenous GTP-Rac1/Cdc42 and GTP-RhoA, respectively. **(A)** Immunoblots were performed using monoclonal antibodies to Rac1, RhoA and Cdc42. The whole cell lysates were immunobloted with the same antibodies. **(B)** The autoradiographs shown in A were quantified using Scion Image

software, and the level of GTP-bound proteins normalised to the total proteins in the lysate. The results shown are representative of two independent experiments.

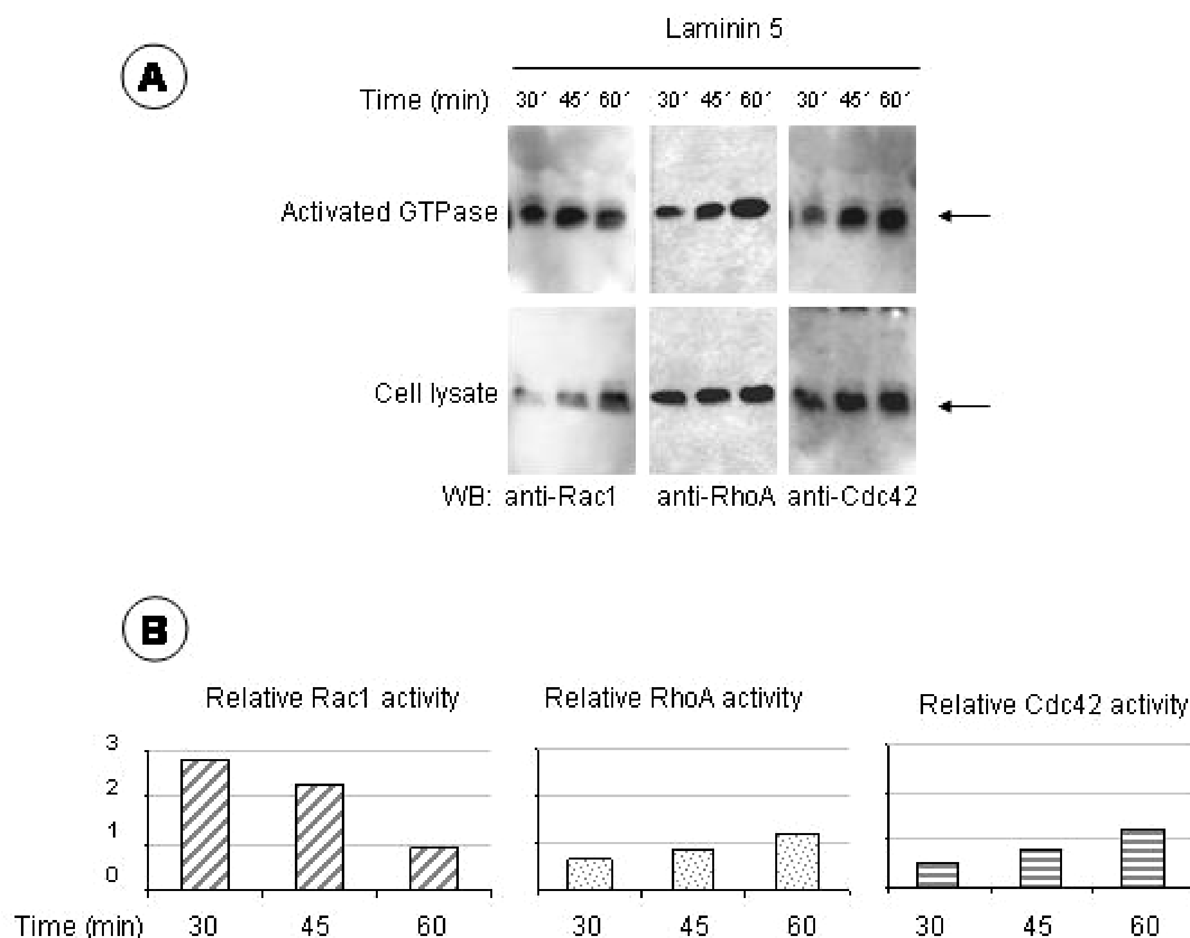


Fig. 50: Activation of small GTPases in fibroblasts adhering on laminin 5.
Fibroblasts Wi26 cells were serum-starved for 24 hours and then with laminin 1 for 30, 45 or 60 minutes. GST-CRIB or GST-RBD was used to precipitate endogenous GTP-Rac1/Cdc42 and GTP-RhoA, respectively. **(A)** Immunoblots were performed using monoclonal antibodies to Rac1, RhoA and Cdc42. The whole cell lysates were immunobloted with the same antibodies. **(B)** The autoradiographs shown in A were quantified using Scion Image software, and the level of GTP-bound proteins normalised to the total proteins in the lysate. The results shown are representative of two independent experiments.

To test the possibility that one hour of adhesion on laminin 5 was too short to activate RhoA in HaCaT cells, we increased the adhesion time to 1, 2, 3 and 4 hours **(Fig. 51)**. As positive controls, we used HaCaT cells adhering to fibronectin, which is known to enhance RhoA activity (Bourdoulous et al., 1998; Hotchin et al., 1999; Ren et al., 1999) and fibroblasts transfected with constitutively activated RhoA (QL). In addition, the amount of activated RhoA in lysate of HaCaT cells deprived of serum for 24 hours and untreated was assessed. In these cells, there was a basal level of activated RhoA, (as shown also in figure 41) which was downregulated upon

adhesion on laminin 5. This inhibition of the activation of RhoA in HaCaT cell adhering on laminin 5 was maintained up to four hours of adhesion while the levels of activated RhoA were high in cells adhering on fibronectin.

In fibroblasts, fibronectin also induced a high level of GTP-RhoA as expected, but there was a reduction of laminin 5-induced RhoA activation in cells adhering for longer than one hour. The level of GTP-loaded RhoA at these later time points was similar to that in untreated, serum-starved cells **(Fig. 51)**.

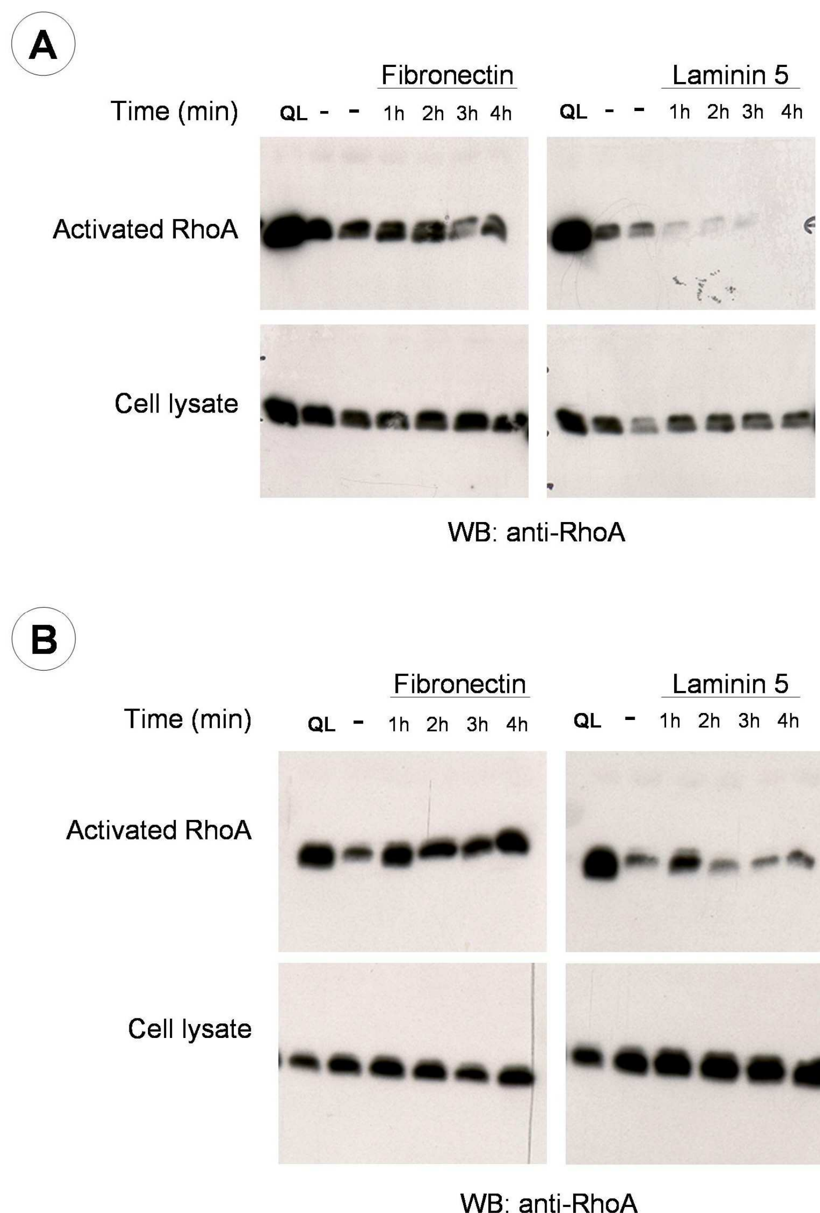


Fig. 51: Activation of RhoA in HaCaT and Wi26 cells adhering on fibronectin and laminin 5.
HaCaT **(A)** and Wi26 **(B)** cells were serum-starved for 24 and then stimulated with fibronectin or laminin 5 for 1, 2, 3 and 4 hours as indicated. GST-RBD was used to precipitate endogenous GTP-RhoA. Immunoblots were performed using monoclonal antibodies to RhoA. The whole cell lysates were immunobloted with the same antibodies. Fibroblasts transfected with constitutively activated RhoA (QL) were used as positive control.

The amount of GTP-bound RhoA in untreated, serum-starved cells is shown (-). The results shown are representative of two independent experiments.

Together, these results suggest that laminin isoforms differentially regulate the activation of RhoA, Rac1 and Cdc42 during adhesion and that the regulation of the Rho-GTPases is not only laminin-isoform specific but also cell-type specific. Laminin 1 promotes a fast activation of Cdc42 in HaCaT epithelial cells, while Rac1 and RhoA activities increase progressively over time. In contrast, on laminin 5, these same cells present a high Rac1 activity, while the activities of Cdc42 and, to a greater extent, of RhoA are down-regulated. In fibroblasts, laminin 1 promotes a fast activation of RhoA, which is maintained over time, and a progressive activation of Rac1. On laminin 5, Wi26 cells have a high activity of Rac1, which decreases gradually, while the activities of RhoA and Cdc42 are slowly upregulated. Although both type of cells present the same phenotype when plated on laminin 1 or on laminin 5, the kinetics of adhesion, spreading, formation of focal contacts and migration are more rapid for the Wi26 cells than for the HaCaT cells. This probably reflects the differences in the kinetics of the activation of the small GTPases, which are known to be time-regulated (Ren et al., 1999; Price et al., 1998).

3.2.2. Epithelial cell adhesion and migration after downregulation of Rho GTPases

3.2.2.1. Downregulation of Rho GTPases in HaCaT cells by siRNA

Introduction of double-stranded small interfering RNA (siRNA) in a cell is an efficient method to repress post-transcriptionally the expression of a gene (Elbashir et al., 2001). We used this method to ablate specifically the small GTP-binding proteins RhoA, Rac1 and Cdc42 in HaCaT epithelial cells.

The HaCaT cells were transfected with siRNA duplexes of small GTPases and with a siScramble, which sequence is an amalgam of the sequence of the Cdc42 duplex. After 24, 48 and 60 hours post-transfection, the cells were harvested and the protein levels of Rac1, Cdc42 and RhoA were analysed by immunoblotting. In contrast to the Lipofectamine method (not shown), the calcium-phosphate transfection leads to efficient silencing of the Rho GTPase protein expression **(Fig. 52)**. A 90 to 100% reduction of either one of the three small GTPases was observed already 24 hours

after transfection, and was maintained at least until 60 hours post-transfection **(Fig. 52)**.

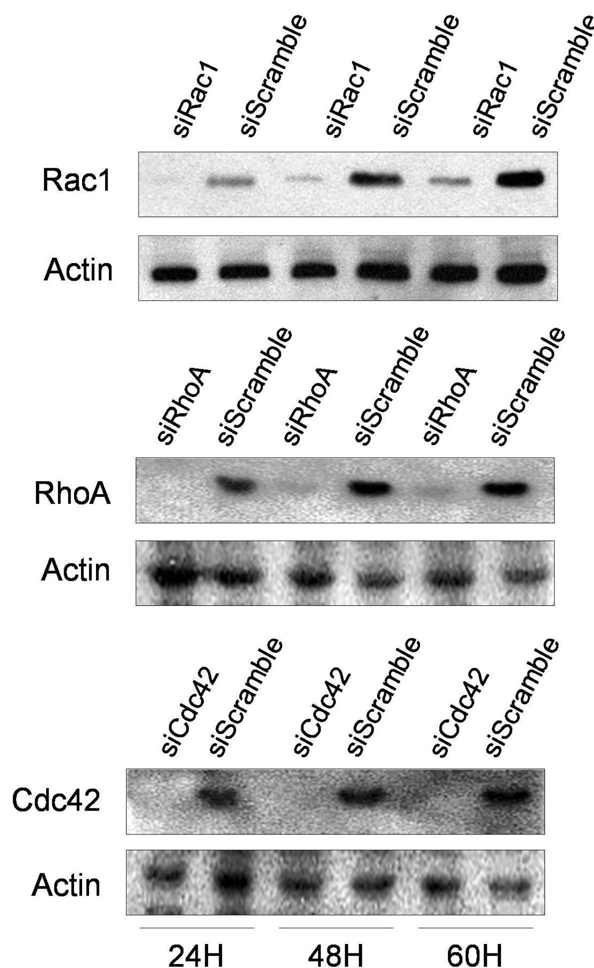


Fig. 52: Downregulation of RhoA, Rac1 and Cdc42 by siRNA transfection in HaCaT cells.
HaCaT epithelial cells were transfected with control siRNA duplex siScramble or siRNA duplex of siCdc42 (100 nM), siRhoA (100 nM) or siRac1 (200 nM) using calcium phosphate precipitation. The cells were harvested at the indicated times post-transfection. The protein expression was analysed by immunoblotting and compared to the expression level of actin in the same cell lysate.

In order to assess whether the silencing of one small GTP-binding protein could affect the expression of the two others, the HaCaT cells were transfected either with siRhoA, siRac1 or siCdc42 and the protein expression levels of all three GTPases was examined by immunoblotting. As shown in **Fig. 53**, the repression of one GTPase did not significantly alter the expression of the two others.

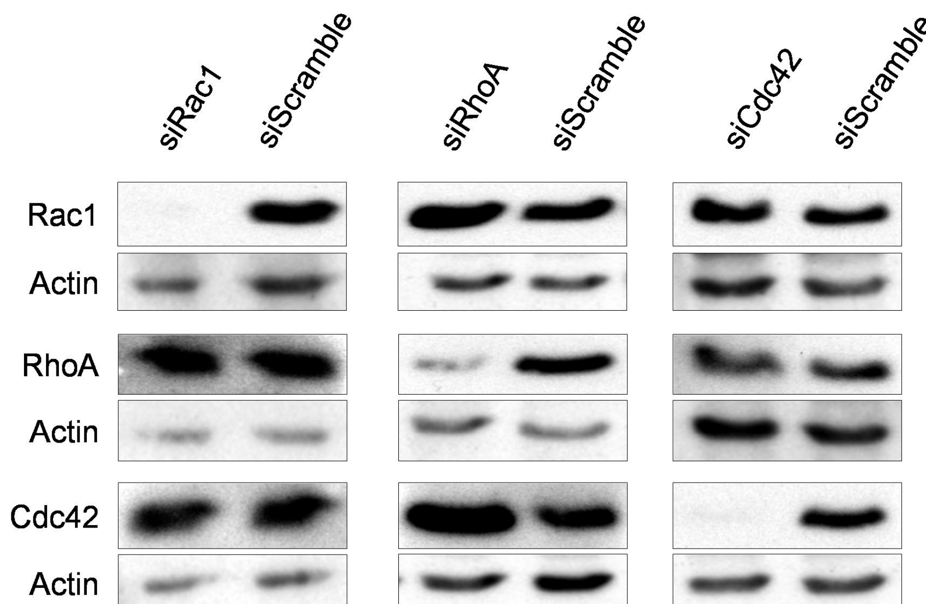
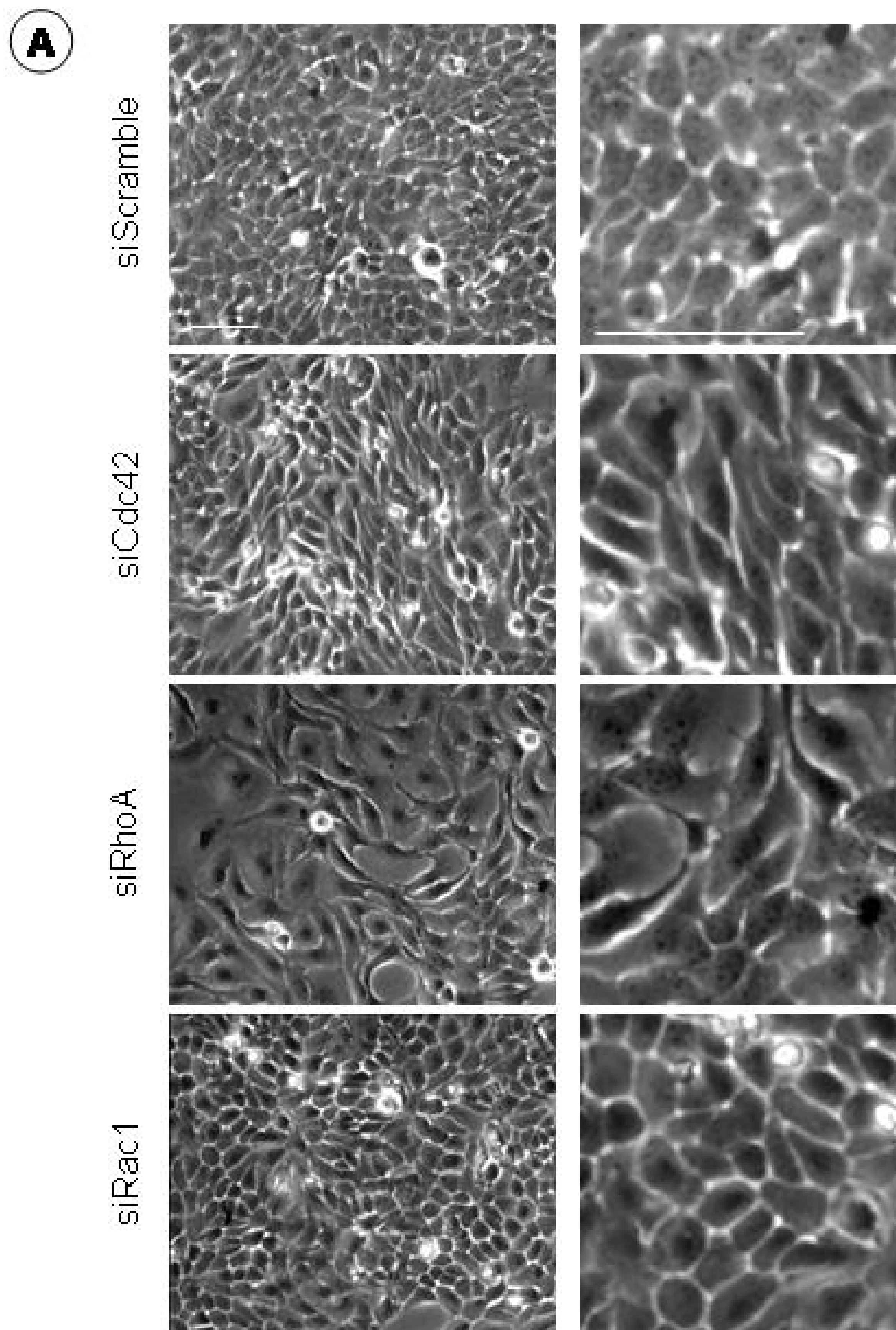


Fig. 53: Comparison of Rac1, Cdc42 and RhoA proteins expression in siRNA-transfected HaCaT cells.
HaCaT epithelial cells were transfected with control siScramble (100 or 200 nM) or siCdc42 (100 nM), siRhoA (100 nM) or siRac1 (200 nM). The expression of RhoA, Rac1, Cdc42 and actin were analysed by immunoblotting in the same lysate. The suppression of one small GTPase does not influence the expression level of the two others.

3.2.2.2. Morphology of HaCaT epithelial cells after downregulation of Rho GTPases

We examined the morphology under phase contrast microscopy of non-confluent and confluent HaCaT cells 24 and 48 hours post-transfection. As shown in **Fig. 54A-B**, transfection with siRhoA, siRac1 or siCdc42 induced distinct changes in the morphology of the cells. At confluency, the Cdc42-deficient cells showed loosening of their cell-cell junctions and an elongated shape as compared to the control siScramble cells. When non confluent, the cells appeared flatter than the controls and displayed lamellipodia **(Fig. 54B)**. SiRNA targeting of RhoA induced a disorganization of sub-confluent HaCaT islets **(Fig. 54B)**, which were flanked with cells showing protrusions like lamellipodia and filopodia. Although the cell-cell contacts also seemed to be affected, neighbouring cells still maintained contacts. HaCaT cells transfected with siRac1 duplex seemed to have tighter cell-cell junctions and to lack protrusions **(Fig. 54AB)**.


A
siScramble
siCdc42
siRhoA
siRac1

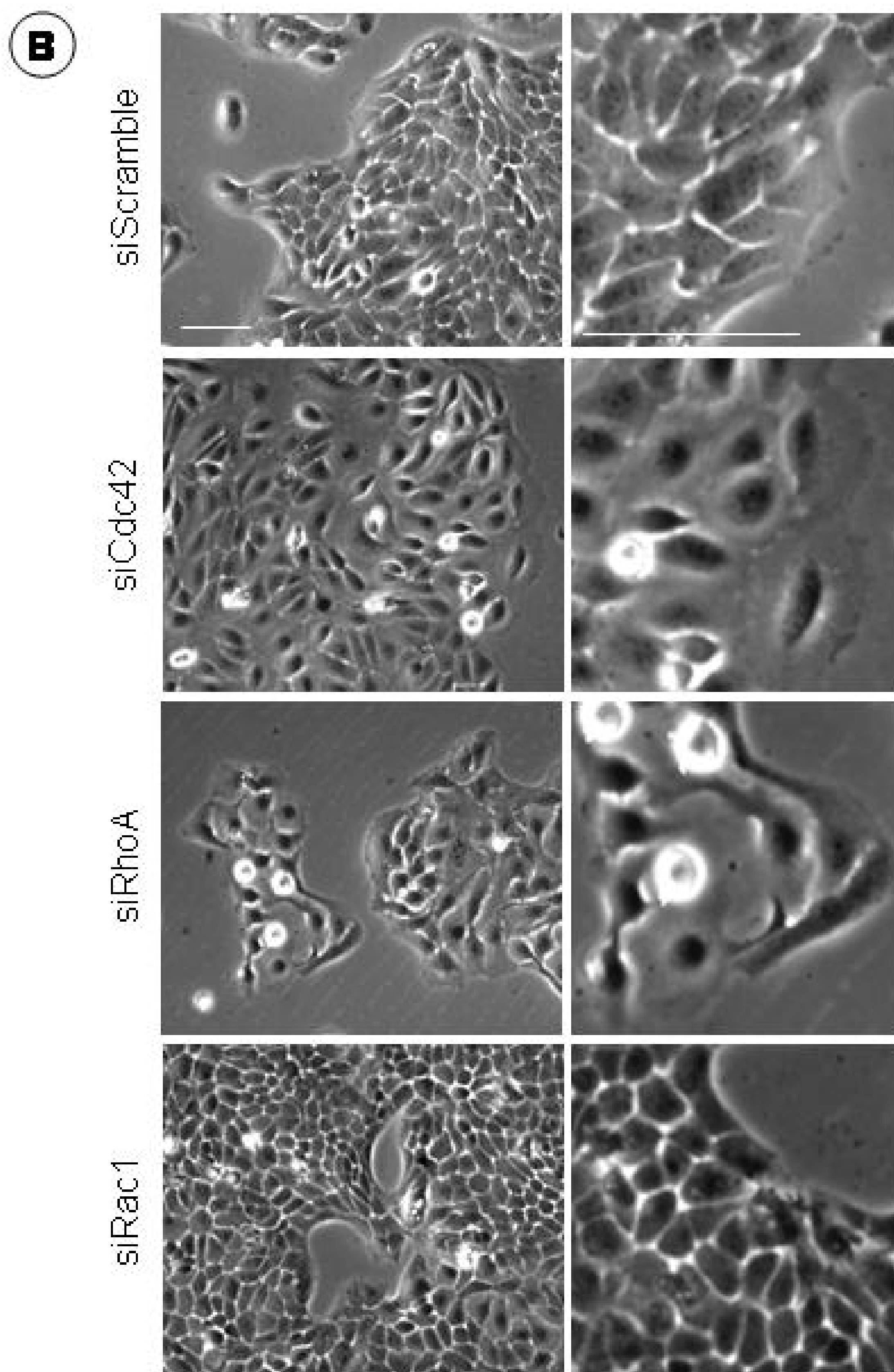


Fig. 54: Morphology of HaCaT epithelial cells transfected with GTPase siRNAs.
Phase contrast photographs of living HaCaT cells after transfection with siScramble, siCdc42, siRhoA or siRac1. After 24 hours (**A**-non-confluent state) and 48 hours transfection (**B**-confluent state), HaCaT cells were photographed. Pictures to the right are higher magnifications of the pictures to the left. Scale bar: 100 µm.

3.2.2.3. Adhesion of HaCaT epithelial cells to laminin 1 and laminin 5 after downregulation of Rho GTPases

Among their multiple functions, Rho-GTPases were reported to regulate integrin avidity (Schwartz and Shattil, 2000). We therefore analysed the impact of the suppression of small GTPase expression in HaCaT epithelial cells on cell attachment and spreading. The transfected HaCaT cells were allowed to adhere for 30 min on tissue culture plates coated with laminin 5 or laminin 1.
There was no significant difference between the adhesion of Cdc42-deficient and control cells plated on laminin 5, which indicates that the lack of Cdc42 protein does not affect the affinity of epithelial cells for laminin 5 **(Fig. 55A)**. The Cdc42-deficient cells spread and developed lamellipodia similarly to the control cells **(Fig. 55B)**. In contrast, the HaCaT cells lacking Cdc42 protein did not bind to laminin 1 as well as the control cells, they stayed round and did not spread **(Fig. 55B)**.

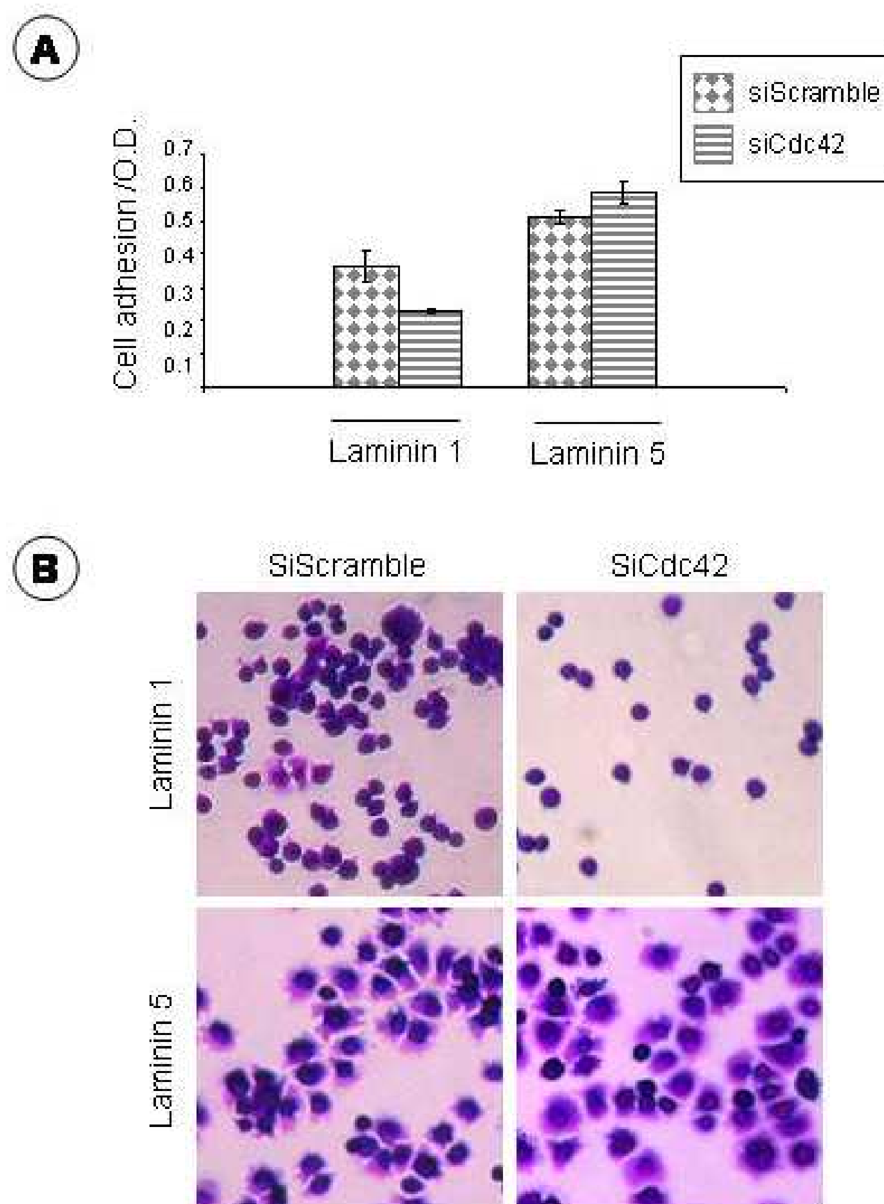


Fig. 55: Adhesion and spreading of siCdc42-transfected HaCaT cells.
(A) Adhesion of Cdc42-deficient and control siScramble cells to laminin 1 and laminin 5. Cell adhesion was measured after 30 min using a colorimetric assay as described in Materials and methods. **(B)** Phase contrast microscopy of HaCaT cells allowed to spread on laminin 1 and laminin 5. The absence of Cdc42 expression affects the cell adhesion and spreading on laminin 1 only.

The adhesion of RhoA-deficient cells to laminin 1 and laminin 5 was comparable to that of siScramble-transfected cells **(Fig. 56),** indicating that the absence of RhoA did not affect integrin affinity for these laminins. The morphology of the RhoA-deficient cells was similar to that of control cells on laminin 5, they were however flatter than the control cells when plated on laminin 1 **(Fig.51B)**.

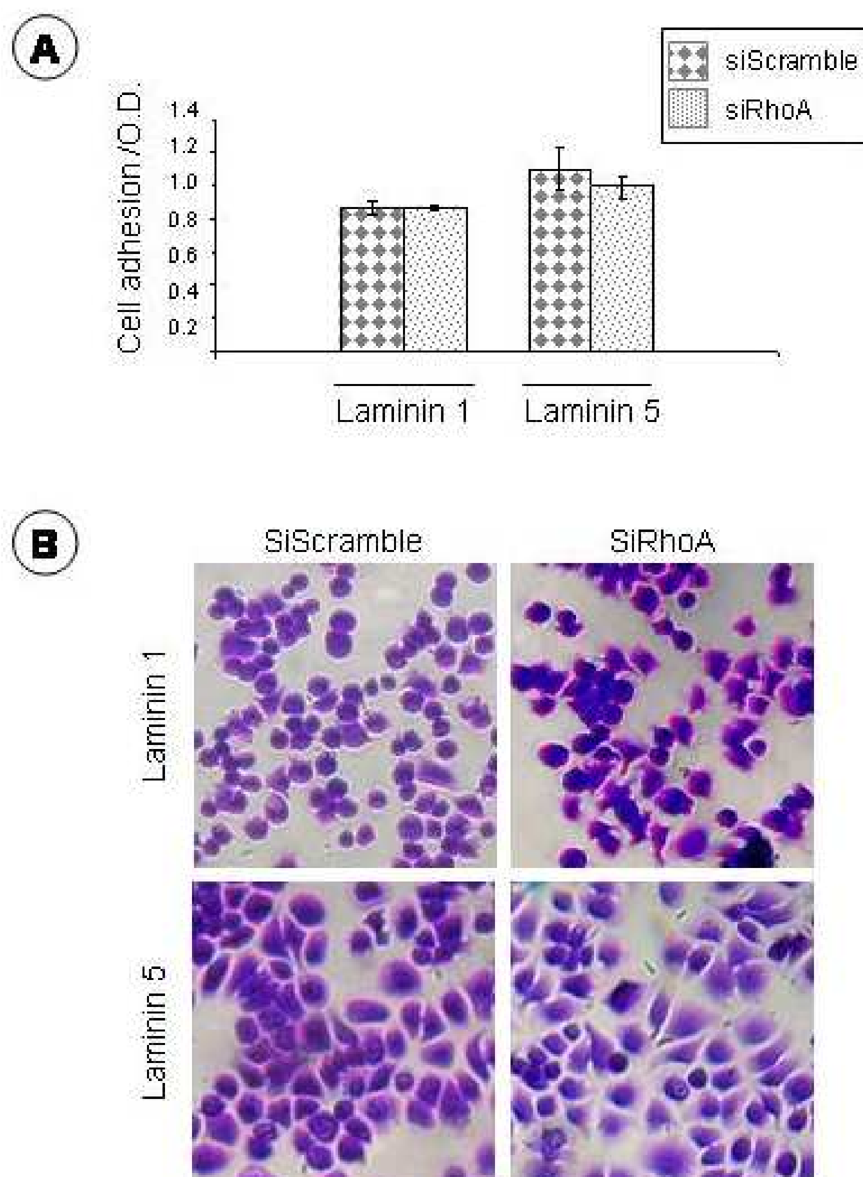


Fig. 56: Adhesion and spreading of siRhoA-transfected HaCaT cells.
(A) Adhesion RhoA-deficient and control siScramble cells to laminin 1 and laminin 5. Cell adhesion was measured after 30 min using a colorimetric assay as described in Materials and methods. **(B)** Phase contrast microscopy of HaCaT cells allowed to spread on laminin 1 and laminin 5. The absence of RhoA expression does not affect the cell adhesion on either laminin 1 and laminin 5.

The adhesion of the Rac1-deficient cells was equivalent to that of control transfected cells on laminin 1 and slightly reduced on laminin 5, suggesting that the absence of Rac1 affects the initial adhesion process on laminin 5. On both substrates, the morphology of the Rac1-deficient and control cells was comparable **(Fig. 57B)**.

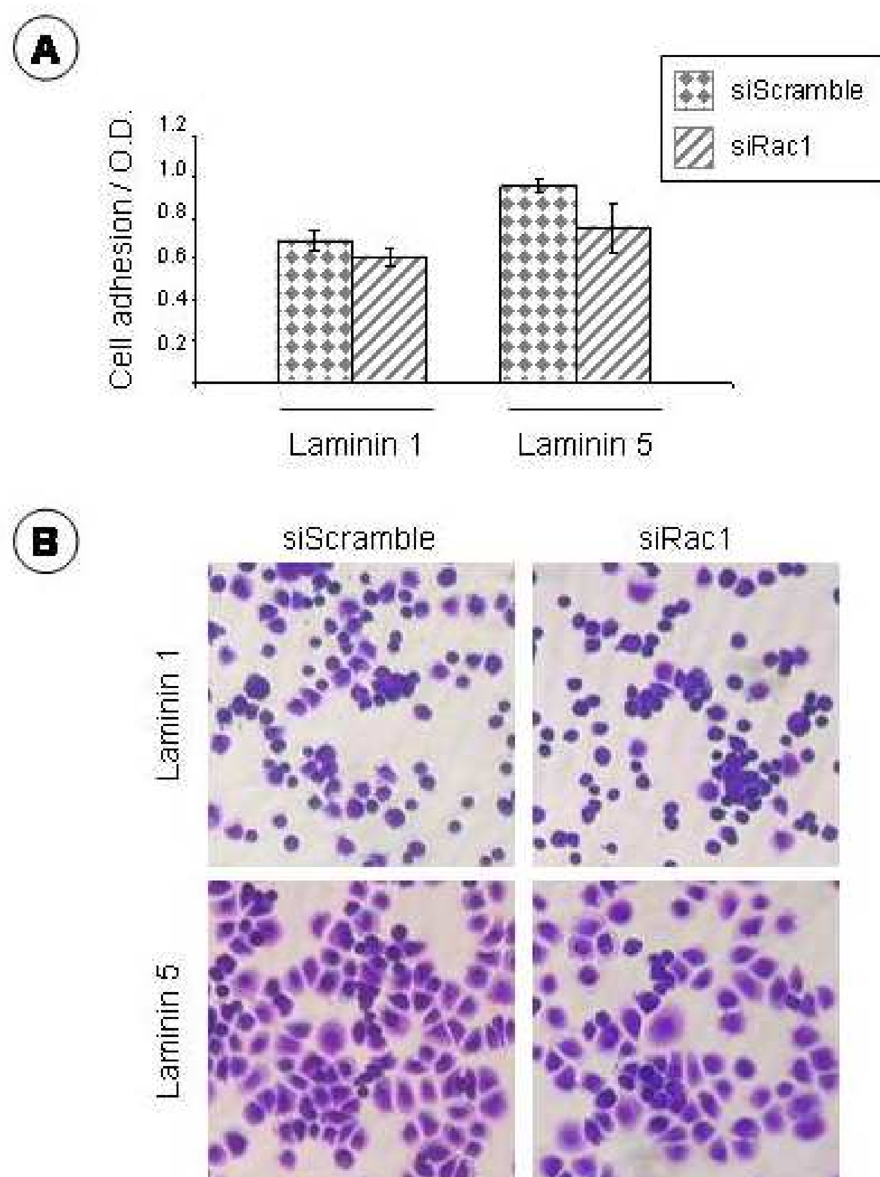


Fig. 57: Adhesion and spreading of siRac1-transfected HaCaT cells.
(A) Adhesion efficiency Rac1-deficient and control siScramble cells to laminin 1 and laminin 5. Cell adhesion was measured after 30 min using a colorimetric assay as described in Materials and methods. **(B)** Phase contrast microscopy of HaCaT cells allowed to spread on laminin 1 and laminin 5. The absence of Rac1 expression does not affect the cell adhesion on laminin 1, slightly on laminin 5.

3.2.2.4. Spreading of HaCaT cells on laminin 1 and laminin 5 after downregulation of Rho GTPases

To characterize the spreading pattern of HaCaT cells on laminin 1 and 5 when RhoA, Rac1 or Cdc42 protein expression is down-regulated, we stained fibrillar actin and vinculin.

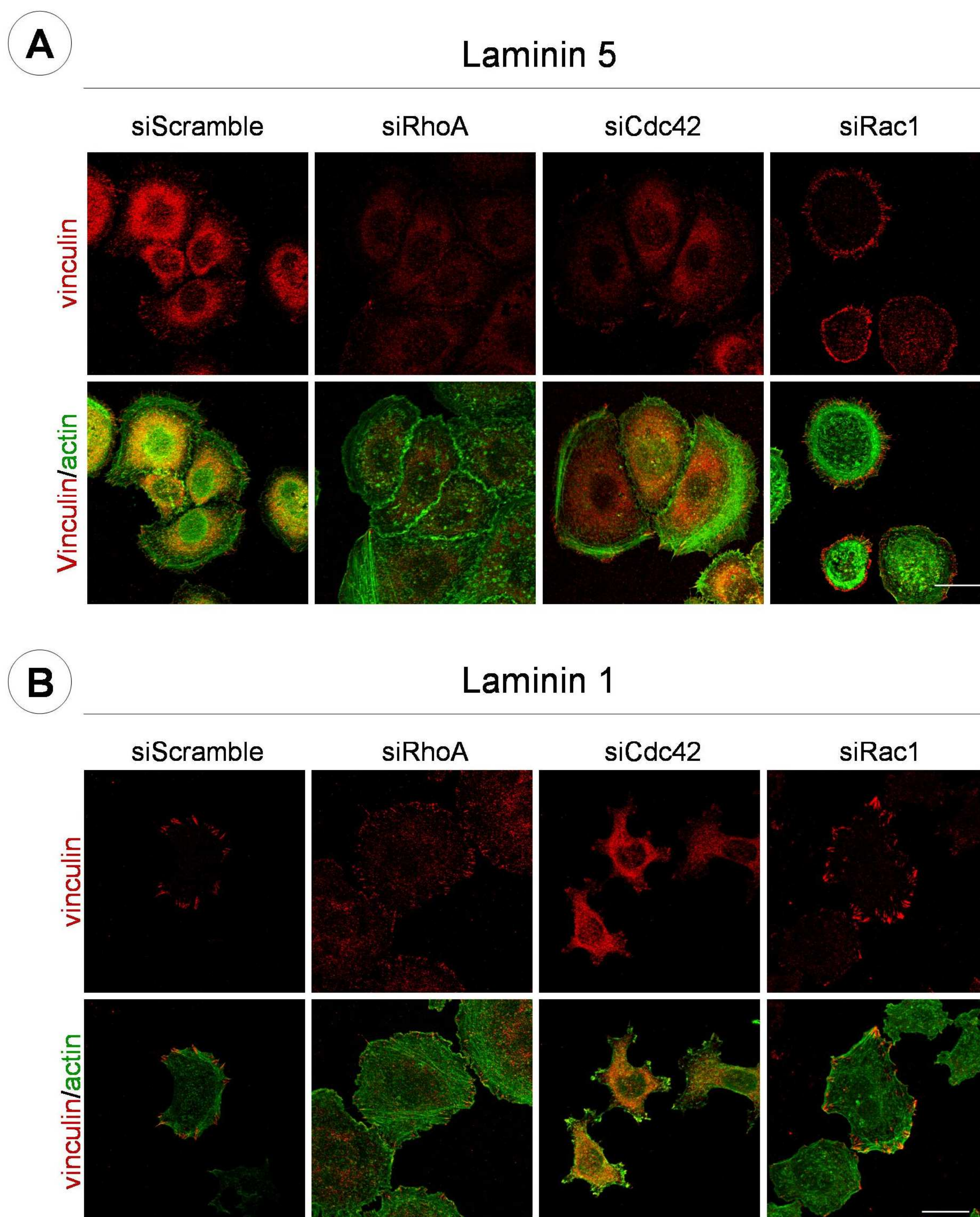


Fig. 58: Immunofluorescence staining of actin and vinculin in siRNA-transfected HaCaT cells adhering to laminin 1 and laminin 5.
SiScramble, siRac1, siRhoA or siCdc42 transfected cells were plated on laminin 5 **(A)** or laminin 1 **(B)** and stained for fibrillar actin (green) and vinculin (red), as indicated. The stainings were visualized using confocal microscopy. Scale bar: 20 µm

The siScramble transfected control cells adhering on laminin 5 were flatter and presented thin vinculin staining at their periphery while, on laminin 1, they showed large vinculin-containing focal contacts associated with actin stress fibers, as

previously observed on wild-type HaCaT cells (see Fig. 42). Similar to the control transfected cells, the Cdc42-deficient HaCaT cells, when plated on laminin 5, had a thin and punctuate vinculin distribution and a distinct network of cortical actin at the periphery of lamellipodia. This observation confirms the previous results showing that Cdc42 is apparently not needed for cell spreading on laminin 5. In contrast, the stainings of Cdc42-deficient cells plated on laminin 1 was very different to that of the control cells **(Fig. 58B)**. Moreover, the Cdc42-deficient cells presented large protrusions, had very small if any focal contacts and the vinculin staining was mainly cytoplasmic.

The siRhoA transfected HaCaT cells plated on laminin 5 presented large lamellipodia like the control cells but showed less cortical actin. Instead, the actin was found over the cytoplasm dispersed in short bundles or concentrated at the cell-cell junctions **(Fig. 58A)**. On laminin 1, RhoA-deficient cells were also very flat and, as on laminin 5, the focal contacts containing vinculin were very short and discrete **(Fig. 58AB)**. To quantify the extent of cell spreading, the area occupied by cells was measured. The control cells adhering on laminin 1 were the smallest and the RhoA-deficient cells, irrespective of if they were adhering on laminin 1 or laminin 5, had a size comparable to the control cells plated on laminin 5 **(Fig. 59)**. The results confirm that RhoA has to be downregulated in order to allow the cells to fully spread, as they do on laminin 5.

Rac1-deficient cells plated on laminin 5 were round and showed more and thicker focal contacts at the cell periphery than the control cells and, on laminin 1, the vinculin-containing focal contacts were also thick.

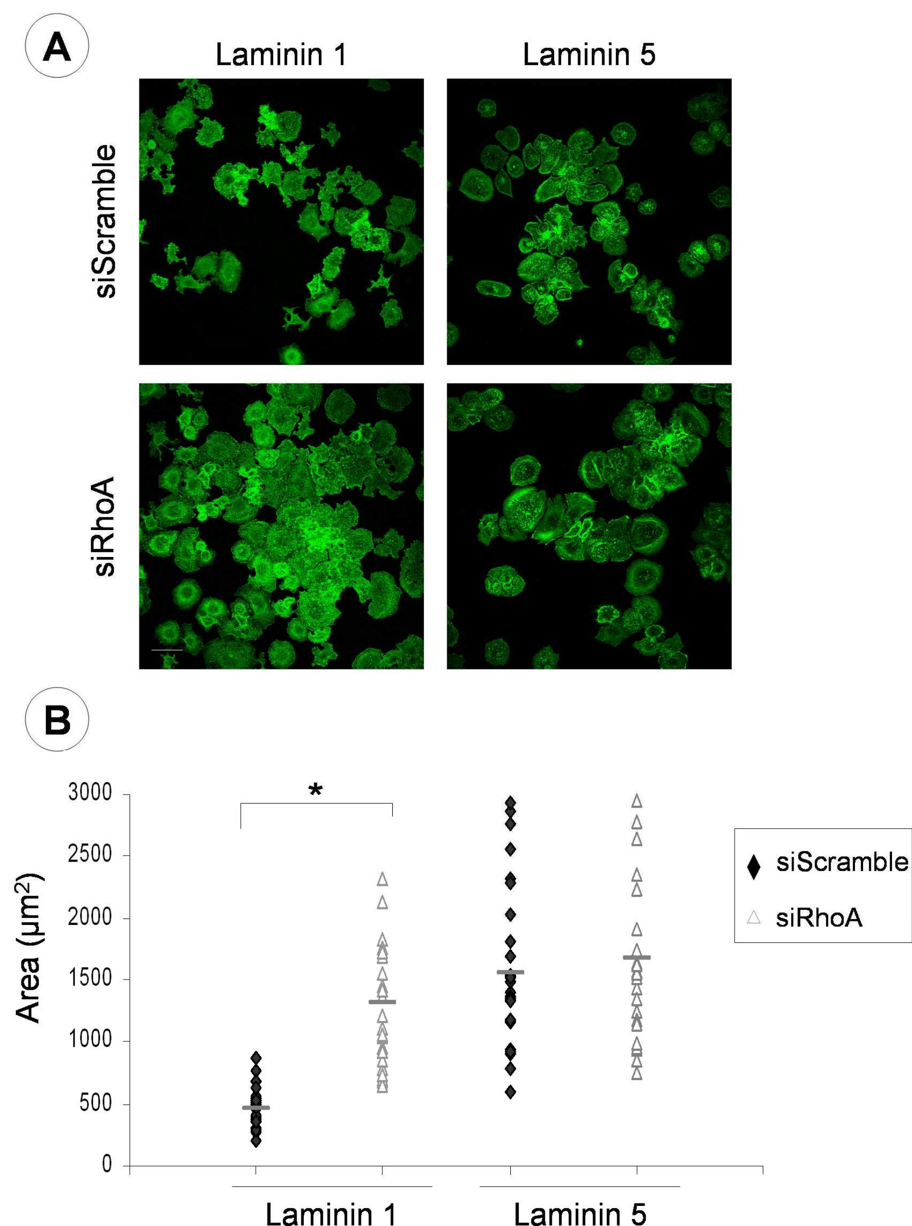


Fig. 59: Size of HaCaT cells transfected with siRhoA or siScramble.
(A) After 30 min of adherence to laminin 1 and laminin 5, the cells were fixed and stained with FITC-phalloidin. Bar: 40 µm. **(B)** Using Scion Image software, the area of 30 cells was measured for each conditions. The asterisk indicates significant differences ($p<0.05$). The RhoA-deficient cells adhering to laminin 1 present the same size than the cells plated on laminin 5.

3.2.2.5. Migration of HaCaT cells after downregulation of Rho GTPases

To determine the relevance of small GTPase deficiency on epithelial cell migration, the migratory behavior of the transfected HaCaT cells on laminin 1 was studied using time-lapse video microscopy. After 13 hours of recording, the translocation of single cells was followed in successive frames and represented as lines. The results

reveal that the cells deficient for RhoA and siCdc42 presented the typically disoriented migration pattern similar to that of the control cells, in presence of laminin 1 and only the Rac1-deficient cells was not able to move properly **(Fig. 60A)**. This hindrance seems to be due to cell-cell-contact inhibition, which was increased in these cells (not shown). The measurement of the average velocity of the cells confirmed that, indeed, RhoA- and Cdc42-deficient cells were able to move more rapidly than the control cells, while Rac1-deficient cells had an impaired cell movement **(Fig. 60B)**.

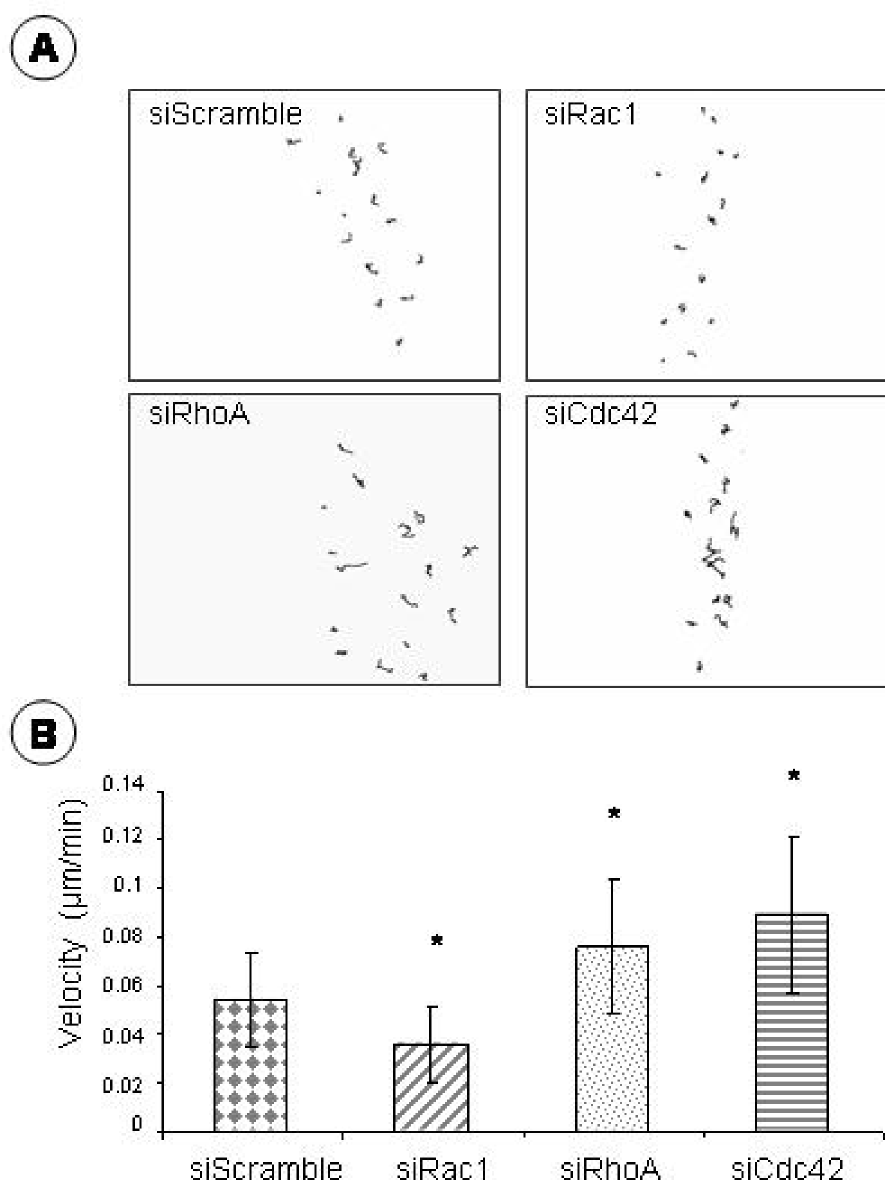


Fig. 60: Migration of siScramble, siRac1, siRhoA and siCdc42-transfected HaCaT cells on laminin 1.
(A) The transfected cells were seeded as single colonies in the center of tissue culture wells as described in Materials and Methods. One hour later, the cell movement was recorded by time-lapse video microscopy for 13 hours. The overall trajectory of single cells was followed in successive frames as shown. **(B)** The velocity of single cells (in µm/min) was determined and the data represent the average velocity for 15 cells. HaCaT cells deficient for Rac1

show impaired migration, while the absence of RhoA or Cdc42 increases the migration rate. Error bars represent s.e.m. Asterisks show significant difference with siScramble ($p<0.05$).

As epithelial cells do not significantly migrate unless activated by external stimuli (Haase et al., 2003), we performed migration assay in the presence of the antibody TS151r of laminin 1 (See 3.1.6.2.). This resulted in induction of siScramble-transfected HaCaT cell migration **(Fig. 61AB)**. Under these conditions, the RhoA-deficient cells and, to some extend the Cdc42-deficient cells also, were more motile than the control cells and Rac1-deficient cells.

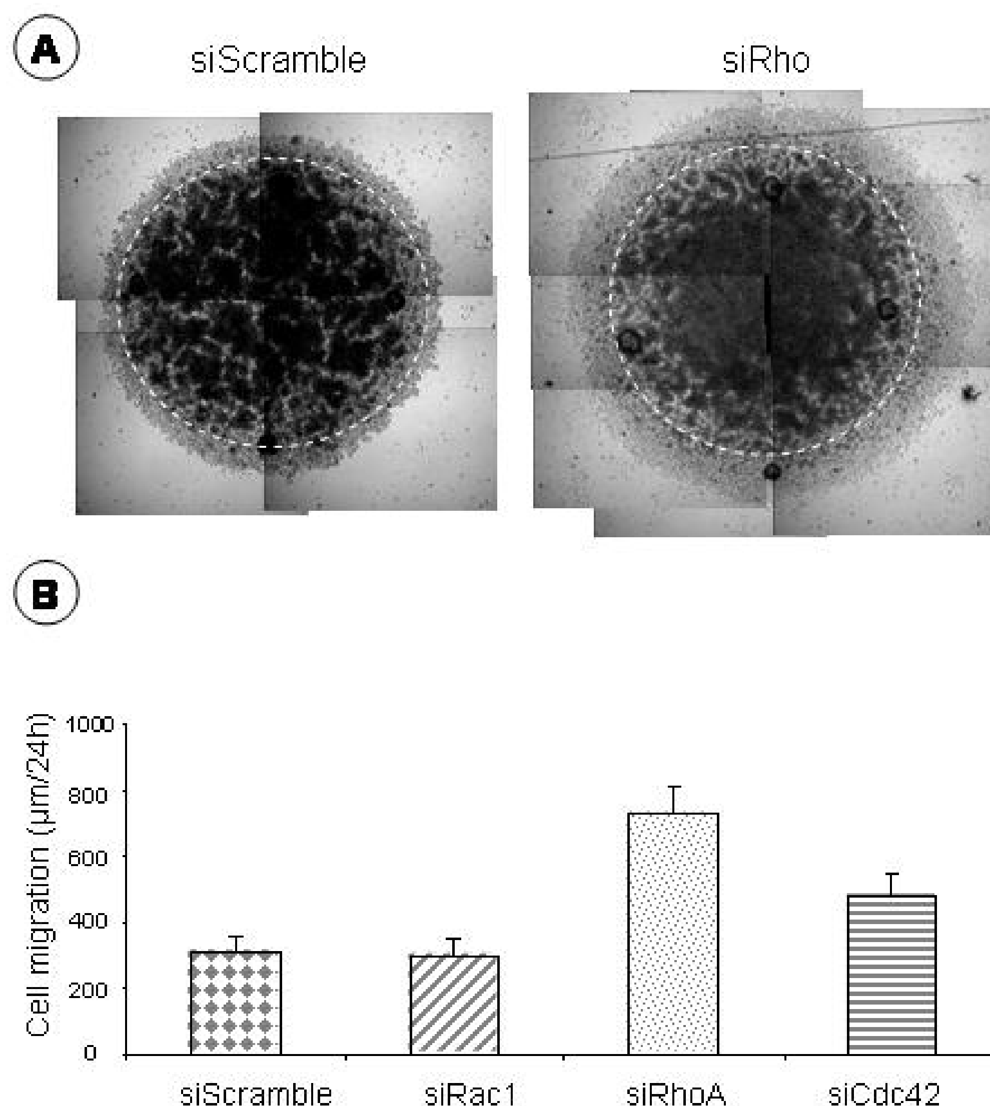


Fig. 61: RhoA-deficient HaCaT epithelial cells are more motile than control cells.
(A) Equal numbers of transfected cells were seeded as small colonies in the center of tissue culture wells. After one hour the wells were filled with medium containing laminin 1 (15 µg/ml) and mAb TS151r. The colonies were photographed immediately and after 24 hours. The white line indicates the position of the colony margin at the beginning of the experiment. **(B)** Cell migration was measured on the photographs as the distance covered by the cell front during 24 hours. Mean of at least 60 measurements ± s.e.m.

4. DISCUSSION

Laminins are major components of basement membranes and their functions are to be part of the architecture of the dermal-epidermal junction and to act as interaction partners for cell receptors influencing their organization and activation and therefore the cell behavior via outside-in signalling (Aumailley and Rousselle, 1999; Colognato et al., 1999; Colognato and Yurchenco, 2000). In a reciprocal manner, the cell receptors are required to target the deposition of basement membrane components (Aumailley et al., 2000; Dipersio et al., 1997). This interconnection must be tightly regulated as a dysregulation of basement membrane architecture can lead to developmental arrest.

4.1. Keratinocytes reorganize their cellular receptors during cell migration *in vivo*

In the resting epidermis, only the basal keratinocytes, in contact with the basement membrane, express integrins (Watt, 2002). Two distinct populations of integrins are present, one is located in cell-matrix adhesions and the other at cell-cell contacts. We have confirmed through indirect immunofluorescence staining of quiescent epidermis that β1 integrins (α2β1 and α3β1) are principally found at the intercellular junctions, while α6β4 integrins are in hemidesmosomes at the basal surface of keratinocytes, where they interact with laminin 5, the major adhesion component of the cutanous basement membrane (Watt, 2002; Aumailley et al., 2003).

The α3β1 and α6β4 integrins are both receptors of laminin 5. Their difference in localization reflects distinct functions in the epidermis. In the quiescent epidermis, the α6β4 integrins, in the hemidesmosomes, are required for stable adhesion of the epidermal cells to the basement membrane, as deletion of the α6 or β4 subunits leads to severe skin blistering due to detachment of the epidermis from the dermis (Georges-Labouesse et al., 1996; Van der Neut et al., 1996). When tissue remodelling occurs, like during wound healing and morphogenesis, there is a position switch and the β1 integrins are found at the basal surface of the cells, as shown in this report, while the cell-cell-contacts loosen and the hemidesmosomes dismantle (Carter et al., 1990; Nguyen et al., 2000). The stable adhesion structures are

replaced by dynamic and transient cell-matrix contacts. These adhesion complexes are formed by integrins of the β1 family linked to the actin-based cytoskeleton (Zamir and Geiger, 2001). During this process, the α3β1 integrins mediate initial attachment of epithelial cells to newly deposited laminin 5, after which ligation is transferred to α6β4 integrins, by an unknown mechanism, which then mediate long-term stable adhesion through induction of hemidesmosome assembly (Carter et al., 1990; Nguyen et al., 2000). Mice with β1-null keratinocytes have a severe defect in wound healing demonstrating a crucial role of β1 integrins in keratinocyte migration (Brakebusch et al., 2000) while mice lacking α3β1 integrins only display a poorly organized BM (Dipersio et al., 1997), supporting a role of this integrin in the maintenance of basement membrane integrity. Furthermore, *in vitro* studies have shown that α3 integrin-null cells are not able to properly assemble the laminin 5-matrix deposited by the epithelial cells and leading to an enhanced motility of the cells as compared to the wild-type cells (deHart et al., 2003).

We have used two *in vivo* models to study the integrin redistribution during cell migration: wound healing and the growing hair follicle. In the lower part of the hair follicle, the epithelial cells, escaping from their niche in the bulge region, migrate downwards to the papilla where they get the appropriate signals to differentiate (Oshima et al., 2001). This happens during the anagen phase, when the follicle matures and reaches its full length. In this model, we observed that the interactions of epithelial cells with the matrix are modified and that a redistribution of the laminin-binding β1 integrins from the cell-cell contact to the cell-matrix contact occurs. At this location, laminin 5 and α6 integrins are absent (Commo and Bernard, 1997; Chuang et al., 2003) while α3β1 integrins are facing the extracellular matrix, as we show here. This suggests that at this location laminin 5 is not used as a migratory substrate and that α3β1 integrins might participate in cell migration through interactions with other ligands. As the hair follicle is a constantly remodelling tissue (Stenn and Paus, 2001), it is not surprising that in the lower part of the hair follicle where the cells migrate, laminin 5 and α6β4 integrins are poorly expressed, reflecting an absence of stable anchoring complexes.

In contrast to the bulb region of the hair follicle, laminin 5 is strongly expressed in the wound bed, while its transcription is repressed in the quiescent epidermis, which is in agreement with previous results (Kainulainen et al., 1998; Ryan et al., 1994). In this region, the new laminin 5 is deposited in order to restore a new matrix. Laminin 5 is indispensable for the stability of the DEJ (Aumailley et al., 2003) as human and mice lacking any of the three α3, β3 or γ2 chains of laminin 5 display junctional epidermolysis bullosa characterised by skin blisters. In quiescient epidermis, laminin 5 is completely processed in the BM in agreement with previous reports (Goldfinger et al., 1999; Tunggal et al., 2002). In contrast, analysis of the wound bed shows that at this location, laminin 5 is unprocessed. The C- and N-terminal processing of the α3 and γ2 chains, respectively, is incomplete and this may lead to a hindrance of the interaction between the LG1-3 module with the α6β4 integrins and between the laminin N-terminal γ2 chain with collagen VII, permitting the cells to migrate and reepithelialize the wound.

4.2. Autocrine laminin 5 inhibits HaCaT epithelial cell migration *in vitro*

In vitro, epithelial cells, like HaCaT cells, are not spontaneous migrating cells, unless activated by external stimuli like EGF or TGFβ (Decline et al., 2003, Haase et al., 2003). In previous reports, it has been suggested that the ability of laminin 5 to regulate cell motility could depend on its processing (Goldfinger et al., 1998) and organizational state (deHart et al., 2003). In contrast, adding mature laminin 5 to wounds accelerates the healing process (Nishiyama et al., 2000; Kainulainen et al., 1998; Takeda et al., 1999). Here, we show that, in contrast to fibroblasts, adding laminin 1 or laminin 5 is not sufficient to stimulate the migration of HaCaT epithelial cells, probably because they rapidly produce and deposit their own matrix composed of endogenous laminin 5 on which they form strong adhesive structures not compatible with migration.

The study of the hair follicle and wound healing reported in the present work shows that there are two physiological models where basal keratinocytes reorganize their adhesion systems to migrate. During this process, the disorganization of the hemidesmosomal adhesion system takes place and is replaced by dynamic adhesions mediated by β1 integrins.

However, integrins are not the only transmembrane proteins present in hemidesmosomes- Two other components, CD151 and collagen XVII are also present at this location, and could therefore play a role in cell migration.

4.3. Novel role of collagen XVII and CD151 as regulators of keratinocyte migration

Collagen XVII colocalizes with laminin 5 in the anchoring filaments and with $\alpha6\beta4$ integrins and CD151 in the hemidesmosomal complex. We have here unraveled a new role of collagen XVII in cell migration by studying keratinocytes from patients with nonlethal junctional epidermolysis bullosa (nJEB). These patients are deficient for collagen XVII and have subepidermal skin blistering and immature hemidesmosomes (Jonkman et al., 1995). The C17-/- keratinocytes are larger and flatter than normal keratinocytes and display a migratory phenotype. Further investigations have shown that collagen XVII binds to laminin 5 and that the C17-/- cells have a poorly organized laminin 5 deposition (Tasanen et al., 2004). The initial adhesion of C17-/- keratinocytes to laminin 5 is mediated by $\alpha3\beta1$ integrins and at a later stage, $\alpha6\beta4$ integrins are targeted to cell-matrix contacts like in normal keratinocytes (Tasanen et al., 2004). However, this latter process does not lead to cell immobilization as it has been thought before. In addition, it was shown in a previous report that collagen XVII is diffusely distributed in migrating keratinocytes in healing wound (Gipson et al, 1993). Together, it suggests that collagen XVII is required for the stabilisation of the hemidesmosomal complex and this is made through interaction with laminin 5 leading to immobilisation (Tasanen et al., 2004). This shows the importance of protein-protein interactions in the hemidesmosomal apparatus of keratinocytes.

CD151, a protein of the tetraspanin family (Yauch et al, 1998; Peñas et al., 2000), is a new member of the hemidesmosomal complex (Sterk et al., 2000). It is the only tetraspanin that associates with high stoichiometry with the α subunit of laminin-binding integrins $\alpha3\beta1$, $\alpha6\beta1$ and $\alpha6\beta4$ in keratinocytes (Sterk et al., 2002). We have confirmed that, in resting epidermis, all CD51 is fully complexed with these integrins, i.e. with $\alpha3\beta1$ integrins in cell-cell junctions and with $\alpha6\beta4$ integrins in hemidesmosomes. In opposite to the epidermis, in the lower part of the hair follicle in

anagen phase, CD151 is not complexed with integrins. This suggests that CD151 interaction with laminin-binding integrins is associated with keratinocyte immobilisation.
To test this hypothesis, we have treated quiescent HaCaT cells with mAb TS151r, the epitope of which corresponds to the integrin-binding site on the extracellular loop EC2 of CD151 (Kazarov et al., 2002) and shown that the treatment induces migration of HaCaT epithelial cells on laminin 1. This was associated with a disruption of cell-cell contacts and a redistribution of β1 and β4 integrins. Unlike the control cells which had β4 integrins at their basal surface associated with hemidesmosome-like structures, the treated cells had no β4 integrins at their basal surface, suggesting that stable adhesion structures are not formed. Therefore, CD151 association with integrins appears as a major regulator of keratinocyte immobilization.

It is not known how CD151 regulates the migration of HaCaT cells and why the effect of the antibody TS151r was observed on laminin 1 but not on laminin 5. Previous studies have proposed an inhibitory role of CD151 in cell migration (Yauch et al., 1998; Peñas et al., 2000). These studies, however, were performed with antibodies that bind to the CD151-integrin complex and therefore the observed effects were not due to CD151 alone. In the case of mAb TS151r, the antibody may interfere with the formation of new complexes between CD151 and integrins, at least on laminin 1. Indeed, keratinocytes have a stronger affinity for laminin 5 than for laminin 1, probably because laminin 1 is a ligand for α6β1 integrins but not α3β1 integrins, while laminin 5 has a high affinity for α3β1 integrins (Rousselle and Aumailley, 1994; Eble et al., 1998). The binding of the mAb TS151r to CD151 may be easier when the cells adhere on laminin 1, involving α6β1 integrins, than on laminin 5 involving α3β1 integrins. In addition, CD151 presents the tightest association with α3 integrins (Serru et al., 1999). Interestingly, it has been proposed that CD151 plays a key role in strengthening α6β1 integrin-mediated adhesions when this integrin is bound to its ligand laminin 1 but not when other integrins are engaged (Hemler, 1998; Lammerding et al., 2003). These results indicate that CD151-α6β1 and CD151-α3β1 integrin complexes are functionally distinct. In our experiments, impairment of complex formation between α6 integrins and CD151 may result in decreased strength of α6 integrin-mediated adhesion and induction of cell migration.

Alternatively, if mAb TS151r impairs the formation of new complexes between $\alpha3\beta1$ integrins and CD151, the transdominant negative regulation of $\alpha3\beta1$ integrins over $\alpha6\beta1$ integrins (Laplantine et al, 2000) and others integrin receptors (Hodivala-Dilke et al., 1998) may be released.

A negative regulation of cell migration by CD151-integrin complexes was suggested by the fact that in resting basal keratinocytes of the epidermis, all CD151 is complexed with the laminin-binding integrins and no free CD151 is found (this report, Sterk, 2000; 2002). Moreover, it has been reported that down-regulation of CD151 in breast cancer cells is associated with the highest invasiveness of the cells (Sauer et al., 2003) and overexpression of CD151 in epidermoid carcinoma cells enhanced intercellular adhesiveness and significantly retards cell migration (Shigeta et al., 2003). Furthermore, recently, patients with functionally deficient CD151 have been described to present, among others defects, epidermolysis bullosa, a disease characterized by skin blistering and detachment of the epidermis from the dermis. This disease is also caused by mutations in the genes encoding laminin 5, $\alpha6$ and $\beta4$ integrins and collagen XVII (Aberdam et al., 1994; Vidal et al., 1995; Ruzzi et al, 1997; McGrath et al.,1995). In the case of CD151, the mutation affects the large extracellular loop (EC2) which contains the binding domain of CD151 for integrins (Karamatic Crew et al., 2004). From these results, CD151 and its binding to integrins, seems to have a functional significance in the skin. In contrast, mice carrying CD151-null alleles are viable, fertile and do not show morphological differences in skin architecture and in integrin expression (Wright et al., 2004). However, this phenotype can be explained by the discovery of a new murine tetraspanin BAB22942 (Hemler, 2001), which has a remarkable 57% identity to the mouse CD151, in particular in the large extracellular loop where the integrin binding site is located, and which probably compensates for some functions of CD151. At last, a recent report supports the role of CD151 in the maintenance of epithelial cell integrity. In fact, targeting of the tetraspanin protein TSP-15, which corresponds to the mammalian tetraspanin CD151 in C. elegans, using siRNA method, affects the stability of the hypodermis by formation of blisters and loss of cell adhesion (Moribe et al., 2004).

We show that during physiological tissue remodelling like in the hair bulb, CD151 is free in cell-cell contacts where $\beta1$ integrins are absent. We propose ae model in

which CD151 is a partner of laminin-binding integrins in resting cells, but not in epithelial cells involved in migration and morphogenesis. In resting epithelial cells, CD151 is associated with α3- and α6- integrins, restricting the integrin movement in the plasma membrane and therefore the migration of the cells. When CD151 is dissociated from the integrins, the latter can move freely to the basal surface and participate to cell migration.

4.4. Involvement of Rho GTPases in epithelial cell adhesion and migration on laminin

The extracellular matrix controls the activity and level of GTP-bound proteins in order to maintain a specific cell phenotype and behavior *in vivo* (Beqaj et al., 2002; Danen et al., 2002; Zahir et al, 2003). For example, in the lung, when peribronchial undifferentiated mesenchymal cells adhere and spread on autocrine laminin 2, they differentiate to become smooth muscle cells. Beqaj et collaborators (2002) have shown that this transformation is in part due to the down-regulation of the activity of RhoA in these cells after adhesion to laminin 2.

We have used laminin 1, a ligand for $\alpha6\beta1$ integrins, and laminin 5, a ligand for $\alpha3\beta1$ integrins, to study two different intracellular signalling pathways influencing the morphology and the migratory behavior of HaCaT epithelial cells. These two laminin variants differ in their biological properties, laminin 5 being a uniquely strong substrate for keratinocyte adhesion, while laminin 1, primarily present in embryonic tissue, was shown to be a poor substrate for the adhesion of keratinocytes (Rousselle and Aumailley, 1994). These two laminin isoforms therefore provide good models to investigate whether $\alpha3\beta1$ and $\alpha6\beta1$ integrins play different roles in signal transduction.

We confirmed that the morphology of two different cell lines, HaCaT epithelial cells and Wi26 fibroblasts, is laminin-isoform specific as described earlier (Dogic et al., 1998; 1999). On laminin 5, the cells present lamellipodia, while on laminin 1 they develop filopodia-like protrusions. Also the focal contacts containing activated $\beta1$ integrins associated with vinculin are morphologically different on these two isoforms: on laminin 1, they are thick and short, while on laminin 5, they appear at the periphery of the lamellipodia as dots or thin streaks. Although the difference in the

overall cell shape was comparable for both cell types according to the substrate, the kinetics of cell spreading and focal contact formation as well as the acquirement of the cell morphology were different. The Wi26 fibroblasts responded more rapidly than the HaCaT epithelial cells to cell adhesion signalling. After 30 minutes on the substrate, these cells had already spread and formed thin vinculin-containing patches on laminin 5 and thick and short focal adhesions on laminin 1. After one hour of adhesion, the Wi26 fibroblasts had acquired the laminin-isoform characteristic cell shape while at this time point the HaCaT cells had just begun to spread. The formation of focal contacts in HaCaT cell took place between the first and the second hour of adhesion. The faster kinetics of Wi26 fibroblasts reflects their ability to rapidly integrate and respond to information from the outside in order to migrate, in contrast to epithelial cells which are not migrating cells.

We suspected that the induction of distinct adhesion complexes and morphologies by laminin 5 and laminin 1 may influence specifically the migration pattern of the cells. Indeed, time-lapse video microscopy analysis of the migratory behavior of the cells showed that these two substrates coordinate cell migration in distinct manners. Laminin 1 induces a disorganized migration pattern, probably due to the numerous filopodia-like protrusions induced upon adhesion to this laminin. The cells extend these protrusions as if they search for directional cues and therefore their migration is not constant but interrupted by directional changes. This observation is consistent with another report revealing that carcinoma cell migration on laminin 1 was characterized by filopodial extensions (Rabinovitz and Mercurio, 1997). One biological role of laminin 1 is to promote adhesion and neurite outgrowth of neuronal cells (Edgar et al., 1984). Filopodia are also required during wound healing, gastrulation and dorsal closure where they explore the extracellular matrix and the surfaces of the other cells (Jacinto et al., 2001; Vasioukhin and Fuchs, 2001). In contrast, Wi26 fibroblasts and HaCaT epithelial cells migrate straight ahead in presence of exogenous laminin 5. These cells have distinct lamellipodia leading the cells to one direction. The processive index near to one confirms that theses cells migrate in a linear manner, parallel to each other. Recently, it has been shown that the deposition of laminin 5 by an epithelial outgrowth regulates a polarized and linear migration of the cells possibly reflecting the process of reepithelialization during wound healing (Frank and Carter, 2004).

It is well established that cells plated on extracellular matrix proteins such as fibronectin rapidly develop protrusive structures triggered by the activation of the small GTPases (Ridley et al, 2003). Integrins are the major receptors for extracellular ligands. After ligand binding, integrin activation involves conformational change and association of the cytoplasmic tail with structural and signalling proteins resulting in the induction of specific signalling cascades (Schoenwalder and Burridge, 1999). This process takes place in cell-matrix contacts. In addition, integrin-mediated cell adhesion can also modulate the formation of adherens junctions (Gimond et al., 1999; Yano et al., 2004). The small Rho GTPases are found at the intersection between integrins and the actin cytoskeleton as well as between the actin cytoskeleton and cell-cell junctions (Schwartz and Shattil, 2000; Jamora and Fuchs, 2002). They regulate the actin dynamics inducing morphological changes during cell adhesion and spreading on extracellular matrix. Measurements of GTP-loading have shown that the GTPases are activated in response to cell adhesion (Ren et al., 1999; Del Pozo et al., 2000). Therefore, we looked at the activation of RhoA, Rac1 and Cdc42 after adhesion to laminin 1 or laminin 5. HaCaT cell adhesion to laminin 1 was accompanied by an early elevated Cdc42 activity. In contrast, Rac1 and RhoA activity were low at the beginning of adhesion and increased progressively within the first hour, consistent with the slow spreading and formation of focal adhesions in HaCaT cells plated on laminin 1. As laminin 1 triggers the extension of filopodia, a process known to be under the control of Cdc42 (Castellano et al., 1999), it is not surprising that laminin 1 activates Cdc42. In fibroblasts, the activation of RhoA and Cdc42 was already high after 30 minutes of adhesion and decreased slightly afterwards. Wi26 cells, which were already spread at this time, had a high RhoA activity probably to permit the assembly of large focal adhesions typical for laminin 1. In both cell lines, GTP-Rac1 level gradually increases, probably reflecting the process of cell spreading as described in previous reports (Price et al., 1998).

Although the HaCaT epithelial cells are not fully spread on laminin 5 after 30 min of adhesion, the activity of RhoA was already down-regulated and this down-regulation was maintained up to four hours. Fibronectin, which induces a high level of GTP RhoA (Danen et al., 2000) was used as a positive control. The fibroblasts, however, showed stimulated RhoA activity on laminin 5 at early stages of adhesion and then

downregulation after one hour of adhesion. HaCaT cells and WI26 cells present the same cell shape, with large lamellipodia and thin patches of vinculin-containing focal adhesions, when plated on laminin 5. The difference in GTP-loading between the two cell lines can be explained by the fact that the Wi26 cells are migrating cells and that they therefore need constant remodelling of their adhesion plaques for contraction and traction of the cell body. RhoA is required for actomyosin contraction and maintenance of cell adhesion during cell movement (Chrzanowska-Wodnicka and Burridge, 1996; Nobes and Hall, 1999). Furthermore, it has been previously shown that keratinocyte adhesion to laminin 5 initially involves $\alpha3\beta1$ integrin signalling to initiate cell movement. The $\alpha3\beta1$ transduction pathway is RhoA-independent but induces the activation of PI3Kinase (Nguyen et al., 2000; 2001) which is itself engaged by Rac1 (Keely et al., 1997). A cross-talk between GTPases has been demonstrated and one explanation for the inhibition of RhoA by laminin 5 may involve the inhibition of Rac1 over RhoA (Sander et al., 1999). Our current findings show that laminin 5, while strongly inhibiting RhoA activity, promotes Rac1 activity in epithelial cells. Recently, a model was proposed in which $\alpha3\beta1$ integrins direct the stabilization of the polarized lamellipodium in epithelial cells through activation of Rac1 (Choma et al., 2004). In this report, the authors show that the $\alpha3\beta1$ integrins are necessary for lamellipodium formation as $\alpha3\beta1$-deficient cells are not able to form large protrusions.

4.5. Knock-down of RhoA, Rac1 and Cdc42 affect cell migration on laminin 1

So far, our understanding of the role of Rho-GTPases is essentially based on results using constitutively active or dominant negative proteins by which the exchange factors are supposed to be sequestered (Feig, 1999). These i*n vitro* studies show that the small GTPases play a role in controlling cell morphology (Schwartz and Shattil, 2000; Burridge and Wennerberg, 2004) and cell behavior (Zondag, 2000), while affecting the organization of the actin cytoskeleton in migratory cells. Moreover, in epithelia, Rac1 and Cdc42 activation is linked to the establishment of polarity and modulation of E-cadherin cell-cell adhesion and, therefore, are supposed to regulate epithelial migration (Fukata and Kaibuchi, 2001).

The study of the role of Rho-GTPases using animal models has been difficult because, in most cases, homozygous null mutations are embryonically lethal

(Sugihara et al., 1998; Chen et al., 2000). We therefore decided to analyze the effects of depleting GTP-binding proteins RhoA, Rac1 and Cdc42 by siRNA. We achieved over 90% suppression of either one of the three Rho-GTPases up to at least 60 hours post-transfection with the respective siRNA duplexes. The introduction of one specific siRNA duplex did not interfere with the expression level of the two other GTPases. Compared to control cells transfected with siScramble, cells transfected with specific siRNAs show visible change in cell morphology, with either loose cell-cell adhesions associated with membrane ruffling for RhoA- and Cdc42-deficient cells or tighten cell-cell junctions and loss of cell protrusions for Rac1-deficient cells.

Depletion of the Rho-GTPases, except for Cdc42-deficient cells on laminin 1, does not affect cell adhesion to laminin 1 and laminin 5 although the spreading patterns of the cells were different. This is in agreement with previous reports showing that cell spreading, but not cell adhesion, was affected by the inhibition of Cdc42, Rac1 and RhoA activity (Clark et al, 1998). It seems that the integrin affinity modulation for extracellular matrix is not under the control of the small GTPases but instead, integrins, upon cell adhesion, modulate the activation of Rho proteins, may be by controlling their membrane targeting (Del Pozo et al., 2004). However, Cdc42-deficient HaCaT cell adhesion to laminin 1 was significantly impaired. As initial adhesion on laminin 1 induces strong activation of Cdc42 in epithelial cells as it does for neuronal cells (Weston et al., 2000), Cdc42 might be needed for enhanced cell adhesion to laminin 1 through a feedback loop. The Cdc42-deficient HaCaT cells plated on laminin 5 had thin and discrete focal contacts comparable to those in the control cells. However, when plated on laminin 1 for two hours, Cdc42-deficient cells hardly spread and presented a distorted cell morphology characterized by numerous membrane protrusions. The thick and large focal adhesions seen on control cells were absent in Cdc42-deficient cells. Surprisingly, these cells did not have an impaired migration on laminin 1, probably due to the fact that only the early steps of cell adhesion are affected by the absence of Cdc42. As the speed of cell locomotion is dependent on cell-substratum adhesiveness (Gumbiner, 1996; Palecek et al., 1997), a weakening of too strong interactions on laminin 1 could account for the enhanced cell migration.

The depletion of RhoA in HaCaT cells induces a disorganization of cell islets although the cells do not disassemble. The RhoA-deficient cells were flatter than the

control cells due to extensive membrane ruffling. The RhoA-deficient cells did not acquire the shape typical for laminin 1: they were more spread than the control cells as shown by a greater cell size and the vinculin-containing adhesion contacts were thin and very similar to those of HaCaT cells on laminin 5. Early reports have shown that the inhibition of RhoA activity by stimulation of p190RhoGAP, which hydrolyzes GTP to GDP and renders RhoA inactive, leads to an augmentation of cell spreading and migration by promoting membrane protrusions (Arthur and Burridge, 2001). Furthermore, treatment of cells with Y27632, an inhibitor of the Rho-Kinase, induces membrane ruffling (Rottner et al., 1999) and down-regulation of RhoA activity results in perturbation of cell-cell adhesion in MDCK cells (Kuroda et al., 1997). In fact, consistent with these results, we observed an increase in cell motility in RhoA-deficient cells adhering on laminin 1, which was much enhanced when the cells were stimulated to migrate by the treatment with mAb TS151r.
Rac1 has been shown to be required for cell migration in a wide variety of cell types, as it promotes lamellipodia extension and formation of small, immature adhesion plaques called focal complexes (Nobes and Hall, 1995; Ridley et al., 2001). Here, we observed that Rac1-deficient cells plated on laminin 5 and laminin 1 develop thicker vinculin containing focal adhesions than the control cells. These results are consistent with our observation that Rac1-deficient cells had an impaired migration. The targeted disruption of the Rac1 gene in mice lead to embryonic lethality during the gastrulation process (Sugihara et al., 1998) and it is therefore difficult to analyse the physiological role of Rac1 in mammalian tissue. However, epiblast cells from Rac1 mutant embryos were isolated and shown to have a reduced cell adhesion and cell motility due to the lack of lamellipodia and membrane ruffles (Sugihara et al., 1998).

In conclusion, keratinocyte migration involves a switch from hemidesmosomal adhesion complexes to a dynamic system associated with the actin cytoskeleton. The $\alpha6\beta4$ integrins, collagen XVII and CD151 are components of the hemidesmosomes and participate to their integrity. Perturbation of this cohesion, as shown here for collagen XVII and CD151, leads to cell migration *in vitro* probably due to the weakening of cell anchorage. Moreover, keratinocytes develop a migratory phenotype suggesting a further process intrinsic to the cells. Finally, laminin 1 and laminin 5, ligands for $\alpha6\beta1$ and $\alpha3\beta1$ integrins, respectively, induce isoform-specific

phenotypes, adhesion contacts and migratory behavior in fibroblasts and epithelial cells, which are linked to a different activation of the small GTPases RhoA, Rac1 and Cdc42. Specifically, a low RhoA activity with a high Rac1 activity leads to an increased cell migration on laminin, which is even more enhanced when the hemidesmosomal complex is perturbed.

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6. Abbreviations

ADAM	a disintegrin and metalloproteinase
Arp2/3	actin-related protein complex
BM	basement membrane
BMZ	basement membrane zone
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
CRIB	Cdc42-Rac-interactive binding domain
csk	carboxyl-terminal Src kinase
DEJ	dermal-epidermal junction
DEPC	diethylpyrocarbonate
DH	Dbl-homology
DMEM	Dulbecco´s minimal Eagle medium
DMSO	dimethylsulphoxide
DNA	Deoxyribonucleic acid
DTT	1,4-dithiothreitol
EC	extracellular
ECL	enzymatic chemiluminiscence
ECM	extracellular matrix
E coli	Escherichia coli
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
EGTA	ethyleneglycol-bis (2-amino-ethylene) N,N,N,N-tetraacetic acid
EHS	Engelbreth-Holm-Swarm
EMT	epithelial-mesenchymal transition
Erk	extracellular signal-related protein kinase
ERM	ezrin/radixin/moesin
ES	embryonal stem
FA	focal adhesion
FACIT	fibril-associated collagen with interrupted triple helix
FAK	focal adhesion kinase
FC	focal complexe
FCS	fetal calf serum
FITC	fluoresceinthiocyanat
FN	fibronectin
FRET	fluorescent resonance energy transfer
GABEB	generalized atrophic begnin epidermolysis bullosa
GAP	GTPase-activating protein
GDI	GTPase dissociating inhibitor
GDP	guanosine-5'-diphosphate
GEF	guanine nucleotide releasing factor
GFP	green fluorescent protein
Grb-2	growth factor receptor-bound protein 2
GST	glutathione S-transferase
GTP	guanosine-5'-triphosphate
GTPase	guanosine-5'-triphosphatase
HD	hemidesmosome
HEPES	N-[2-Hydroxyethyl] piperazine-N´-2-ethanesulphonic acid
HRP	horse radish peroxidase
ICAM	intercellular adhesion molecule 1
ICAP-1	integrin cytoplasmic-domain-associated protein
Ig	immunoglobulin
IgG	immunoglobulin G
ILK	integrin linked kinase

IPTG	isopropyl-β-D-thiogalactopyranoside
IQGAP1	IQ motif containing GTPase activating protein 1
IRSp53	insulin receptor substrate of 53 kDa
JEB	junctional epidermolysis bullosa
LDL	low density lipoprotein
LE	laminin EGF
LG	laminin globular
LIMK	LIM domain kinase
LN	laminin N-terminal
mAb	monoclonal antibody
MAP	mitogen activated protein
MAPK	mitogen activated protein kinase
MEK	mitogen-activated or extracellular signal-regulated protein kinase
MIDAS	metal ion dependent adhesion site
MLCK	myosin light chain kinase
MMP2	matrix metalloproteinase 2
MT1MMP	membrane-type-1 matrix metalloproteinase
NC	non collagenous
Nap125	NCK-associated protein of 125 kDa
PAGE	polyacrylamide gel electrophoresis
PAK	p21-activated kinase
PBD	p21 binding domain
PBS	phosphate buffer saline
PEG	polyethylene glycol
PH	pleckstrin homology
PKA	protein kinase A
PMA	phorbol-12-myristate-13-acetate
CAS	Crk-associated substrate
pAb	polyclonal antibody
PETA_3	platelet-endothelial tetraspan antigen 3
PI3K	phosphoinositide-3 kinase
PI4P5K	phosphatidyl inositol 4-phosphate 5 kinase
PIP2	phosphatidylinositol-4,5-bisphosphat
PIR121	p53 inducible RNA
PKC	protein kinase C
PMSF	phenylmethylsulphonylfluoride
PTP-PEST	protein tyrosine phosphatase PEST
Rack1	receptor for activated protein kinase C
RDS	retinal degeneration slow
RNA	ribonucleic acid
SDS	sodium dodecyl sulphate
Shc	Src homologous and collagen protein
TBS	Tris buffered saline
TM	transmembrane
TRBD	Rho-binding domain of rhotekin
TRIS	(hydroxymetyl)aminomethane
uPAR	urokinase-type plasminogen activator receptor
UP	uroplakin
UV	ultra violet
VCAM1	vascular cell adhesion molecule 1
vWA	von Willebrand A
WASP	Wiskott-Aldrich syndrome protein

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