**From the Department of Dermatology, Allergology and Venerology**

**of the University of Lübeck**

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# Impact of β1 integrin-mediated signaling on human epithelial progenitor cells *in situ*

**Dissertation**

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Submitted by

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I hereby declare that I prepared the PhD thesis "Impact of β1 integrin-mediated signaling on human epithelial progenitor cells *in situ*" on my own and with no other sources and aids than quoted.

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# Abstract

β1 integrin regulates multiple epithelial cell functions by connecting cells with each other or the extracellular matrix (ECM). While β1 integrin-mediated signaling in murine epithelial stem cells is well-studied, its role in human adult epithelial progenitor cells (ePCs) *in situ* remains to be defined. The current thesis project takes up this challenge.

Microdissected, organ-cultured human scalp hair follicles (HFs), as well as HF epithelium embedded into ECM components that mimic key characteristics of the HF mesenchyme, and experimentally wounded human skin were employed as clinically relevant models for studying β1 integrin-mediated signaling in human ePCs and their progeny within their natural topobiological habitat. In these models, the functional consequences of β1 integrin-mediated signaling for selected ePCs biology read-out parameters *in situ* were explored by β1 integrin siRNA silencing, specific β1 integrin-binding antibodies and pharmacological inhibition of integrin-linked kinase (ILK), a key component of the integrin-induced signaling cascade.

These experiments showed that *β1 integrin* knockdown reduced keratin 15 expression as well as the proliferation of ePCs in the human HF outer root sheath keratinocytes (ORSKs). Embedding of HF epithelium into an ECM environment rich in β1 integrin ligands that mimic the HF mesenchyme significantly enhanced proliferation and migration of ORSKs, while K15 and CD200 gene and protein expression were down-regulated.

Employing ECM-embedded β1 integrin-activating or -inhibitory antibodies allowed identifying functionally distinct human ePC subpopulations in different compartments of the HF epithelium. The β1 integrin-inhibitory antibody mAb13 reduced β1 integrin expression *in situ* and selectively enhanced proliferation of bulge ePCs. Instead, the β1 integrin-stimulating antibody 12G10 decreased hair matrix keratinocyte apoptosis and enhanced transferrin receptor (CD71) immunoreactivity, a marker of transit amplifying cells, but did not affect bulge ePC proliferation.

The putative ILK inhibitor, QLT0267, significantly reduced ORSK migration and proliferation and induced massive ORSK apoptosis. This suggests a key role for ILK in mediating the observed β1 integrin effects.

In order to explore the relevance of β1 integrin signaling for epithelial homeostasis and regeneration activating or the inhibitory β1 integrin antibodies were administered to experimentally wounded, organ-cultured human skin. This showed a reduced skin reepithelization by a diminished proliferation and migration but an increased apoptosis. Mainly the β1 integrin-activating antibody 12G10 strongly inhibited keratinocyte migration as well as differentiation, possibly due to the induction of enhanced ligand binding to the basement membrane.

These findings demonstrate that ePCs and their progeny in human HFs and human skin require β1 integrin-mediated signaling for survival, adhesion, and migration. In addition, the data generated here show that distinct human ePC subpopulations differ in their response to β1 integrin signaling *in situ*, which may help to functionally distinguish these human ePC subpopulations. Taken together, this thesis provides new, physiologically relevant insights into the role of β1 integrin-mediated signaling in human epithelial biology. These may also be utilized for cell-based regenerative medicine strategies that employ human HF-derived ePCs, e.g. for the promotion of cutaneous wound healing.

# Zusammenfassung

Integrine mit einer β1 Untereinheit stellen eine der wichtigsten Gruppe der Rezeptoren dar, die eine Vielzahl von verschiedenen Funktionen epithelialer Zellen steuern, indem sie die Zellen miteinander bzw. mit der extrazellulären Matrix (EZM) verbinden. Anhand verschiedenster Mausmodelle wurde ihre Signaltransduktion bereits sehr genau charakterisiert, aber ihre spezifische Rolle hinsichtlich der humanen, adulten, epithelialen Progenitorzelle (ePZ) ist bisher noch nicht klar beschrieben. Diese vorliegende Doktorarbeit nimmt sich dieser Herausforderung an.

Unter Verwendung und Kultivierung von mikrodissizierten Haarfollikeln (HF) der humanen Kopfhaut oder HF Epithels, welches in HF Mesenchym-nachahmende EZM-komponenten eingebettet wurde, sowie verwundeter Haut, sollte die β1 Integrin-vermittelte Signaltransduktion auf die ePZ und deren Nachkommen *in situ* in ihrer natürlichen Umgebung untersucht werden. Genauer gesagt wurden die funktionellen Konsequenzen durch Manipulation der β1 integrin-vermittelten Signaltransduktion *in situ* auf ePZ untersucht. Eine solche gezielte Veränderung wurde durch spezifische *β1 Integrin* siRNA, durch β1 Integrin-bindende Antikörper, wie auch durch einen pharmakologischen Hemmstoff gegen die intrazellulär gebundene Integrin-verlinkte Kinase (ILK) erreicht. Innerhalb dieser klinisch relevanten Modelle wurde daraufhin das ePZ Verhalten und derer Nachkommen in Bezug auf den Einfluss von β1 Integrin mit Hilfe ausgewählter read-out Parameter analysiert.

Dabei führte der *β1 Integrin* Knockdown zu einer Verringerung der Keratin 15 (K15) Expression und zu einer Abnahme der Keratinozytenproliferation in der äußeren Wurzelscheide. Das Einbetten von isoliertem Haarfollikelepithel in eine β1 Integrin Liganden angereichte, artifizielle EZM erhöhte zwar signifikant die Proliferation und Migration der äußeren Wurzelscheiden Keratinozyten, aber hemmte parallel die Gen- und Proteinexpression der ePZ Marker K15 und CD200.

Die weitere Zugabe des β1 Integrin aktivierenden bzw. des hemmenden Antikörpers zur artifiziellen EZM ermöglichte eine funktionelle Unterscheidung von verschiedenen ePZ

Populationen, die in voneinander getrennten Haarfollikelkompartimenten lokalisiert sind. Der inhibierende Antikörper mAb13 reduzierte *in situ* signifikant die β1 Integrin Expression und wirkte selektiv stimulierend auf die Proliferation der ePZ des Haarfollikelswulsts. Anders jedoch verringerte der aktivierende β1 Integrin Antikörper 12G10 die Apoptose der Haarmatrixkeratinozyten und erhöhte die Immunoreaktivität des Transferrin Rezeptors (CD71 – ein Marker für Zellen die aus den ePZ hervorgehen) im unteren Teil des Haarfollikels, aber beeinflusste nicht die Proliferation der ePZ des Wulstes.

Die als ILK Inhibitor gehandelte Substanz QLT0267 führte zu einer signifikanten Verringerung der Proliferation und Migration der Keratinozyten der äußeren Wurzelscheide, aber demgegenüber zu deren massiven Apoptose. Dies lässt die Schlussfolgerung zu, dass ILK eine Schlüsselrolle in der β1 Integrin-vermittelten Signaltransduktion einnimmt.

Um die Relevanz der β1-Integrin-Signalisierung für die epitheliale Homöostase und Regeneration zu untersuchen, wurden aktivierende bzw. hemmende β1-Integrin-Antikörper zu experimentell verwundeter, Organ-kultivierter, menschlicher Haut zugegeben. Dabei wurde eine gehemmte Reepithelialisierung der verwundeten Haut durch eine verminderte Proliferation und Migration, aber einer erhöhten Apoptose nachgewiesen. Gerade der aktivierende Antikörper 12G10 führte zu einer starken Migrationsreduktion und zeigte ein differenzierungshemmendes Potential aufgrund der gesteigerten Bindung der spezifischen Basalmembranliganden.

Diese Ergebnisse zeigen, dass für ePZ und deren Nachkommen im menschlichen Haarfollikel und in der menschlichen Haut β1 Integrin-vermittelte Signalwege für das Überleben, die Adhäsion und die Migration erforderlich sind. Weiterhin unterscheidet sich die Reaktion unterschiedlicher ePZ Subpopulationen auf die β1 Integrin Signaltransduktion *in situ*.

Zusammengenommen bietet diese Arbeit neue, physiologisch relevante Einblicke in die Rolle von β1 Integrin-vermittelter Signaltransduktion in der humanen epithelialen Biologie. Diese können in der Zell-basierten regenerativen Medizin verwendet werden, die sich mit menschlichen Haarfollikel-abstammenden ePZ beschäftigen, sowie zur Förderung der kutanen Wundheilung.

# Publications

***Parts of the work presented in this thesis are based on the following publication:***

**Nancy Ernst**, Arzu Yay, Tamás Bíró, Stephan Tiede, Martin Humphries, Ralf Paus and Jennifer E. Kloepper

**β1 integrin signaling maintains human epithelial progenitor cell survival *in situ* and controls proliferation, apoptosis and migration of their progeny**

*PLoS One. 2013 Dec 27; 8(12):e84356. doi: 10.1371/journal.pone.0084356.*

***Methological skills acquired during this thesis project also lead to a co-authorship to another hair follicle study:***

Jennifer E. Kloepper*, **Nancy Ernst***, Karsten Krieger, Enikö Bodó, Tamás Bíró, Iain S. Haslam, Ruth Schmidt-Ullrich, Ralf Paus

**NF-κB activity is required for anagen maintenance in human hair follicles**

***contributed equally***

Submitted to J Invest Dermatol, accepted in December, 2013

***Thesis-related meeting presentations published in abstract format:***

**International Investigative Dermatology (IID) 2013, Edinburgh, UK**

**Nancy Ernst**, Arzu Yay, Martin Humphries, Tamás Biró, Jennifer E. Kloepper and Ralf Paus

**β1 integrin signaling regulates maintenance and differentiation of adult human epithelial progenitor cells *in situ***

*Abstract, Journal of Investigative Dermatology (2013) 133, S243, poster 1428*

**European Society for Dermatological Research (ESDR) 2012, Venice, IT**

**Nancy Ernst**, Martin Humphries, Jennifer E. Kloepper and Ralf Paus

**Controlled β1 integrin signaling regulates maintenance and differentiation of adult human epithelial progenitor cells in distinct hair follicle compartments**

*Abstract, Journal of Investigative Dermatology (2012) 132: S105, poster 600*

**Arbeitsgemeinschaft Dermatologische Forschung (ADF) 2012, Marburg, Germany**

**N. Ernst**, S. Tiede, M. Humphries, R. Paus, J. Kloepper

**Influences of regulatory β1 integrin antibodies on epithelial hair follicle progenitor cell activation in an novel ECM model assay**

*Abstract, Experimental Dermatology, 2012, 21, e49, poster P295*

**Arbeitsgemeinschaft Dermatologische Forschung (ADF) 2011, Tübingen, Germany**

**N. Ernst**, S. Tiede, M. Humphries, R. Paus and J. E. Kloepper

**The impact of β1 integrin signaling on adult human hair follicle epithelial progenitor cell viability**

*Abstract, Experimental Dermatology, 2011, 20, e206 , poster P296*

# Table of Contents

# Abbreviations

| | |
|---|---|
| A.dest. | Aqua destillarum |
| aECM | Artificial extracellular matrix |
| AKT | AKT kinase |
| BM | Basement membrane |
| DAPI | 4′,6-diamidin-2′-phenylindol-dihydrochlorid |
| DNA | Deoxyribonucleic acid |
| Col | Collagen |
| CTS | Connective tissue sheath |
| ECM | Extracellular matrix |
| ePC | Epithelial progenitor cell |
| FAK | Focal adhesion kinase |
| FITC | Fluorescein isothiocyanate |
| GFP | Green fluorescent protein |
| HF | Hair follicle |
| IF | Immunofluorescence |
| ILK | Integrin linked kinase |
| IR | Immunoreactivity |
| K | Keratin |
| KC | keratinocyte |
| KD | knockdown |
| KO | knockout |
| K-SFM | Keratinocyte |
| mAb | Monoclonal antibody |
| MG | Matrigel® |
| min | Minutes |
| ORS | Outer root sheath |
| ORSK | Outer root sheath keratinocytes |

| | |
|---|---|
| PBS | Phosphate buffered saline |
| SC | Stem cell |
| SG | Sebacous gland |
| siRNA | silencing ribonucleic acid |
| RNA | Ribonucleic acid |
| RT | Room temperature |
| TBS | Tris-Buffered Saline |
| TdT | Terminal dioxynucleotidyltransferase |
| TGF-β | Transforming growth factor-β |
| TNT | Tris NaCl Tween |
| TSA | Tyramide Signal Amplification |

# List of Figures

# List of Tables

# 1. Introduction

## 1.1. Project overview

The human scalp hair follicle (HF) is an easily accessible mini-organ that represents a prototypic neuroectodermal-mesodermal interaction system. Within this model system, β1 integrin-mediated signaling in human epithelium *in situ*, a medically important area of both integrin and epithelial biology that awaits systematic exploration, can be exemplarily studied. The current thesis attempts this, and complements human HF organ culture with organ-cultured, experimentally wounded human skin as a clinically relevant model for studying epithelial regeneration.

Therefore human HFs are utilized in the current project to study the influence of β1 integrin signaling on key functions of various epithelial progenitor cell (ePC) subpopulations and their progeny within their natural tissue habitat, while the organ culture of wounded human skin is examined to gauge the role of β1 integrin signaling in epidermal reepithelization. Specifically, this study aims to elucidate the impact of manipulating the outside-in signaling of β1 integrin via different ligands on the maintenance, differentiation and/or migration of distinct human ePC subpopulations in the HFs and on the human wound healing *in situ*.

## 1.2. Integrins

Integrins represent a large family of ubiquitously expressed transmembrane receptors, which participate in cell-extracellular matrix (ECM) interactions but also in cell-cell connections (Bouvard et al., 2013; Chin et al., 2013; Legate et al., 2009). Since the first unspecific recognition of an fibronectin-binding glycoprotein (Hansen and Clemmensen,

1982) the structure and function of integrins were more and more characterized (Hynes, 1987; Tamkun et al., 1986). Moreover new insights in their specific ligands and their key role for development, homeostasis, immune response, leukocyte traffic and cancer as well as the position/relevance of integrins in diseases were evaluated (Hynes, 2002). The main action of integrins, which belong to the cell adhesion molecules (CAMs), are mediated via cell-matrix interactions, but they also serve as cell-cell adhesion molecules (blood cells).

All cell types are surrounded or underlaid by an ECM of collagen fibers, proteoglycans and multiadhesive proteins such as laminin or fibronectin (Gilbert, 2010; Kreis and Vale, 1999; Lodish et al., 2012).

Mainly by their linkage of cells to their environment multiple cell functions are coordinated like cell shape, cell migrations or activate classic signal-transduction pathways, including proliferation, cell growth, gene expression and differentiation (Akhtar and Streuli, 2013; Benoit et al., 2009; Brakebusch et al., 2000; Streuli, 2009). Moreover integrins serve as anchors for the attachment of cells to the underlying ECM, which is provided through 2 different types of integrin-dependent junctions – focal adhesions and hemidesmosomes (Lodish et al. 2013).

In contrast to other metazoas (sponges, flies, nematodes), 18 α and 8 β integrin subunits have so far been identified in mammals. Those can assemble non-covalently into 24 different heterodimers with different affinities toward specific ECM components and tissue distributions (Byron et al., 2010; Campbell and Humphries, 2011; Hynes, 2002; Legate et al., 2009) (Figure 1). A β-chain can interact with different α-chains, forming integrins that bind to short amino acid sequences present in different ligands like laminin, collagen or fibronectin.

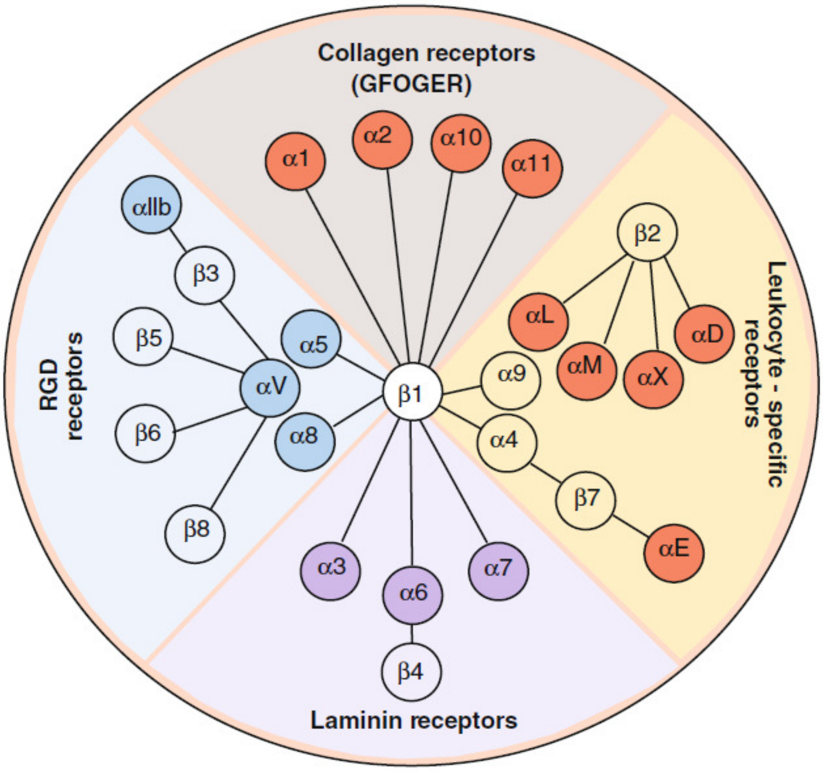



**Figure 1: Heterodimers of the integrin family.**
24 different integrins comprised of 18 α and 8 β mammalian subunits are classified into diverse subfamilies depending on their evolutionary relationships, ligand specificity and tissue expression (β2, β7) (copied from Barczyk et al., 2009).

### 1.2.1. General structure of integrins

Both α and β subunits of integrins are type I transmembrane glycoproteins and consist of a short cytoplasmic tail, a single membrane-spanning helix and a large extracellular domain (see Figure 2A) (Campbell and Humphries, 2011; Fu et al., 2011; Kalli et al., 2013; Zhang and Chen, 2012).

Over the last years it was a challenging task to solve the structure of integrins, because they are large membrane proteins which impede the purification and analysis of the high-resolution structure (Srichai and Zent, 2010). The structure could be identified only separately and for a number of integrin types (Mathew et al., 2012; Mehrbod and Mofrad, 2013). However this knowledge forms the basis for a better understanding of mechanism of integrin activation.

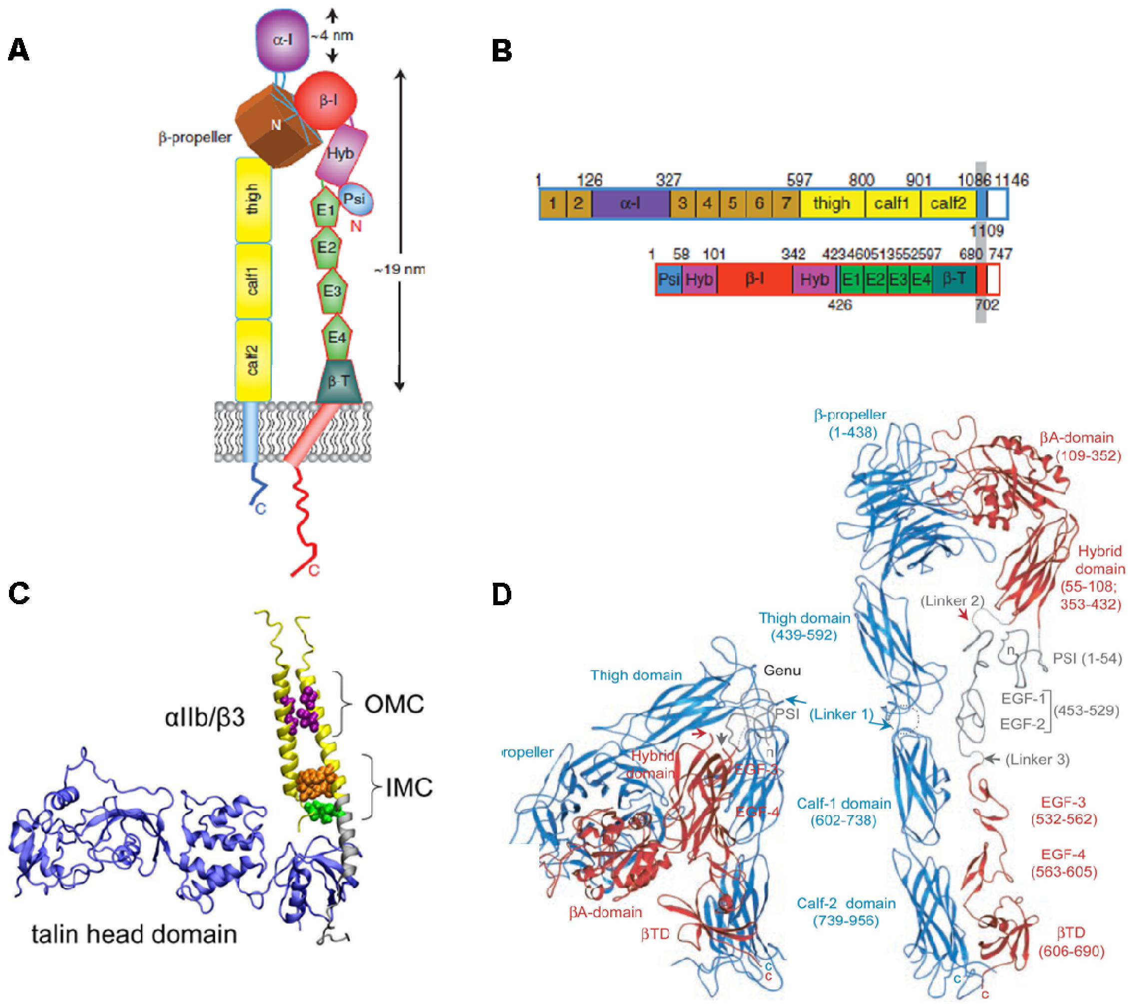


**Figure 2: Integrin structure.**
(A) The unbent integrin conformation showing approximate dimension of the receptor and (B) domain structure of the α-and β-subunits is exemplarily shown for αxβ2 (copied from Campbell and Humphries, 2011). (C) Model of the talin/αβ complex (exemplified in αIIb/β3 integrin) and the representation of the outer (OMC) and inner membrane clasp (IMC) of the transmembrane domain (copied from Kalli et al., 2013). (D) Bent and extended crystallized extracellular domain of αVβ3 integrin [shown in blue (αV chain) and red (β3 chain)] (copied from Xiong et al., 2001).

The short cytoplasmic tails are described as highly unstructured and consist of 10-70 amino acid residues (excluding β4 which comprised of <1000 amino acid residues). The effort to characterize this domain by NMR studies was not consistent. Such discrepancies could be explained by high flexible tails, which form only transient structures in the absence of protein binding partners (Campbell and Humphries, 2011). While the β cytoplasmic tails are highly homologous, α subunit tails are highly divergent (Srichai and Zent, 2010) and both of them are devoid of enzymatic features. Therefore they transduce signals by association with adapter proteins that connect the integrin to the cytoskeleton, cytoplasmic kinases, and transmembrane growth factor receptors. Furthermore there are 2 well-defined motifs within the β integrin tail (proximal NPxY, distal NxxY) which display canonical recognition sequences for phosphotyrosine-binding domains (PTB) and provide binding sites for different integrin binding proteins, like talins and kindlins, which are important for transmitting integrin-mediated intracellular signals (Kalli et al., 2013; Mathew et al., 2012; Srichai and Zent, 2010).

The transmembrane domain is responsible for the transmission of allosteric interactions across the cell plasma membrane (Chua et al., 2012). The use of artificial bilayers realized a characterization of these two interacting helices. The inactive or low-affinity integrins are locked by two transmembrane interactions between the α and β subunits – the outer (OMC) and the inner membrane clasp (IMC) (Figure 2C) (Kalli et al., 2013; Ulmer, 2010). Moreover, a specific motif of these subunits (GXXXG) is considered to keep the two heterodimers in contact (Mehrbod and Mofrad, 2013; Schneider and Engelman, 2004). In contrast, the high-affinity integrins need to separate these subunits, thus allowing the unbending of the ligand-binding headpiece and by this the activation. This activation lead to conformational changes that increase ligand-binding affinity (Kalli et al., 2013; Mehrbod and Mofrad, 2013).

The largest and best-characterized part of integrins represents the extracellular domain, which is up to 150 kDa in size. Most of their structural data arise from high-resolution x-ray crystallography, as e.g. in the case of αVβ3 (see Figure 2D) (Srichai and Zent, 2010; Xiong et al., 2001).

The α subunit comprises four or five different domains – a β-propeller, a thigh, two calf domains and nine of 18 chains have a α-I domain that is placed within the β-propeller (Larson et al., 1989) (for details see Figure 2A, B). This special α-I domain, which is expressed by a ~200 amino acid structure, displays the exclusive extracellular binding site of integrins.

The β subunit has seven domains with flexible and complex interconnections – the β-I (or βA) domain, a hybrid domain, a plexin-semaphorin-integrin (PSI) domain, followed by 4 cystein-rich epidermal growth factor (EGF) modules and a β tail domain (Campbell and Humphries, 2011). The β-I domain is located in the hybrid domain and represents a copy of the α-I domain, whereby this subunit constitutes a important role for ligand binding in α subunits that lack the α-I domain (Srichai and Zent, 2010). In general, the β subunits seem to be more flexible than the α subunit.

The integrin ligand binding is dependent on divalent cations, which can be bound at different sites of the extracellular domain (Campbell and Humphries, 2011; Mould and Humphries, 2004; Srichai and Zent, 2010). The metal-ion-dependent adhesion site (MIDAS) is located in the α-I domain (if present) or in the β-I domain next to the α propeller domain (Chin et al., 2013). Binding of $Mg^{2+}$ on the central MIDAS site stabilises the high-affinity conformation and initiates/supports the ligand binding. Moreover two flanking sites of MIDAS have been identified - the ADMIDAS, where binding of a $Ca^{2+}$ ion leads to an inhibition, but the binding of $Mn^{2+}$ results in activation of the integrin (Humphries et al., 2003; Zhang and Chen, 2012). The other flanking site is the synergistic metal ion binding site (SyMBS), which is responsible for the $Ca^{2+}$ synergy (Mould et al., 2003; Zhang and Chen, 2012; Zhu et al., 2008).

### 1.2.2. Bidirectional signaling of integrins

By serving as a communication tool for cells with their environment integrins are able to transfer signals bidirectionally (Figure 3A). More precisely, the extracellular binding activity is regulated intracellularly (inside-out signaling), while extracellular binding of the ECM triggers signals that are transmitted into the cell (outside-in signaling) (Bouvard et al., 2013; Brizzi et al., 2012; Campbell and Humphries, 2011; Chin et al., 2013; Giancotti and Ruoslahti, 1999; Legate et al., 2009). However, integrins on the cell surface usually cannot efficiently bind to the ECM or other receptors, because they are expressed in an inactive conformation which needs to be activated (Legate et al., 2009).

For the inside-out signaling the binding of various integrin adaptor proteins, such as talin, focal adhesion kinase (FAK) or kindlin are necessary due to their own lack of enzymatic activity (Figure 3B). Changes in the intracellular surrounding lead to the recruitment of talin and FAK to the cytoplasmic tail, which is activated via phosphatidylinositol-4.5-bisphosphate. The binding of talin disrupts the salt bridges between the integrin subunits and activates the integrin receptor. Kindlin as a co-activator improves this activation by its additional binding and further linking with the IPP complex consisting of integrin-linked kinase (ILK), Pinch and

Parvin completed the formation of focal adhesion sites (FA) (Bouvard et al., 2013; Chin et al., 2013; Maydan et al., 2010; Srichai and Zent, 2010; Wickstrom et al., 2009; Widmaier et al., 2012).

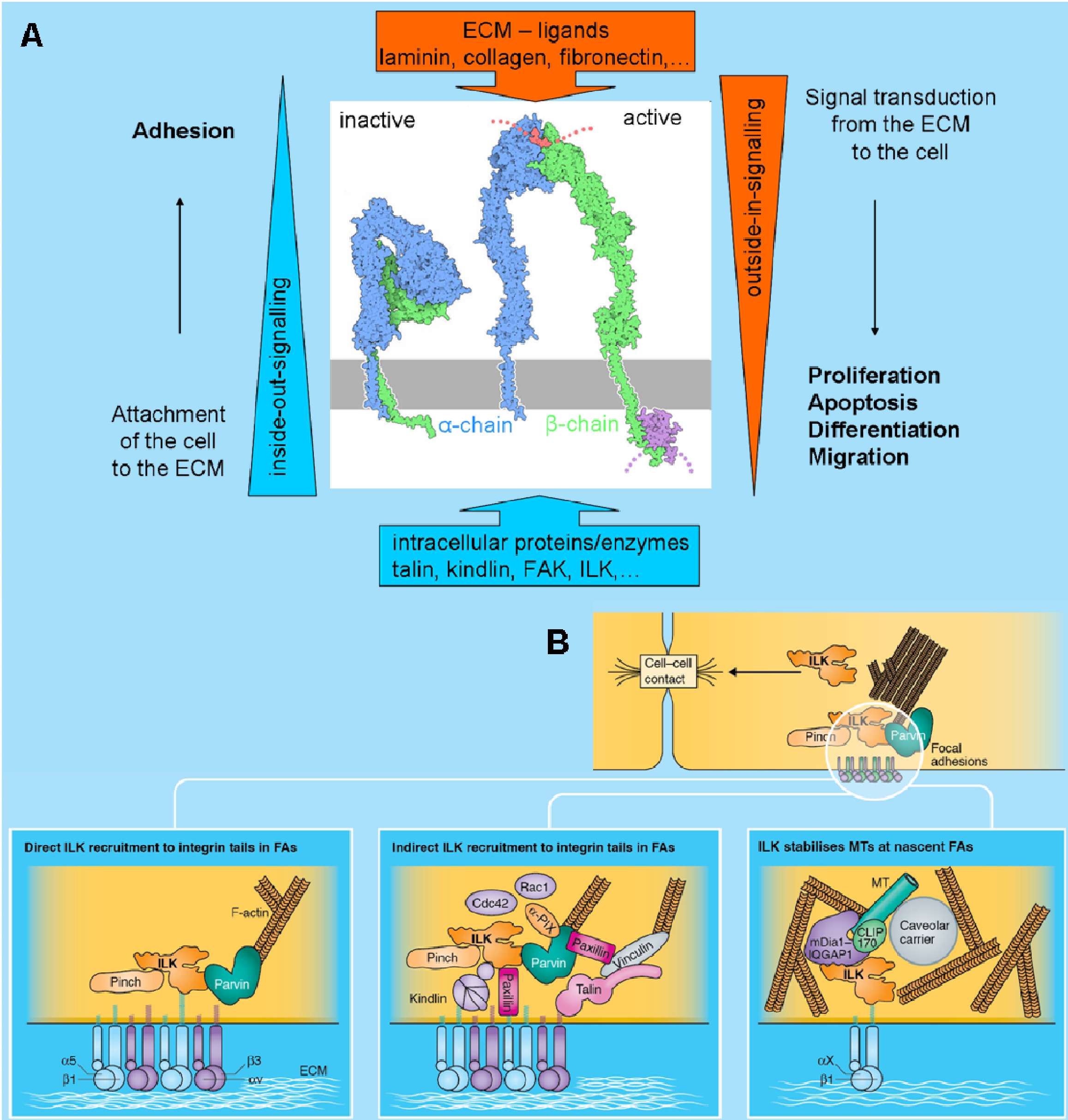



**Figure 3: Bidirectional signaling of integrins.**
(A) Bidirectional signaling exampled by β1 integrin (Schematic drawing of the active and inactive β1 integrin receptor used from February 2011 Molecule of the Month by David Goodsell, http://www.rcsb.org/pdb/education_discussion/molecule_of_the_month/images/mom134_integrins.jpg). (B) The inside-out signaling of integrins depend on the binding of various integrin adaptor proteins, such as talin, focal adhesion kinase (FAK), the IPP complex (integrin-linked kinase/Pinch/parvin complex) or kindlin (copied from Widmaier et al. ,2012).

The other part of the bidirectional signaling via integrins is the outside-in signaling. Various ECM ligands such as laminin, collagen or fibronectin bind to the extracellular region and induce a receptor clustering in the cell membrane, besides the formation and intracellular binding of the adaptor protein complex (as mentioned above). The following signal transduction leads to the actin rearrangement and the formation of focal adhesions (Huttenlocher and Horwitz, 2011; Meves et al., 2013; Morgan et al., 2013).

Such ability of integrins to bind their ligands is dynamically regulated, thereby realizing a controlled adherence to the matrix or the establishment of connections to surrounding cells (Lowell and Mayadas, 2011). Also the characteristically low affinities to their ligands ($K_D$ [dissociation constant] $10^{-6}$ – $10^{-8}$) permit an optimal reaction like migration or adhesion to different ligands (Lodish et al., 2012).

The change in reactivity or function of integrins is enabled by shifting between three main conformational changes (i.e. different affinity states): Structural analysis of diverse integrins demonstrated a low affinity for ligands, if the receptor has a bent or extended-closed headpiece conformation on the cell surface (see Figure 4A, B) (Fu et al., 2011; Luo et al., 2007; Yu et al., 2012). In contrast, activated and clustered integrins with an extended-open headpiece conformation (see Figure 4D) have a high affinity for ligand binding and strongly transduce signals intracellularly via the outside-in signaling pathway (Fu et al., 2011; Luo et al., 2007; Yu et al., 2012).

However, there are some obscurities concerning the different conformation states and how integrins realize by this their many functions, like rolling adhesion of hematopoietic cells. Above, three different conformation states of integrins are described, but these would not explain the phenomenon of rolling adhesion. A study describes a novel extended intermediate conformation of α4β7 integrins on lymphocytes which explains the rolling and not the solid adhesion of these cells on mucosal tissue (Figure 4C) as well as the necessity of activation signals for opening the headpiece of extended integrins (Yu et al., 2012).

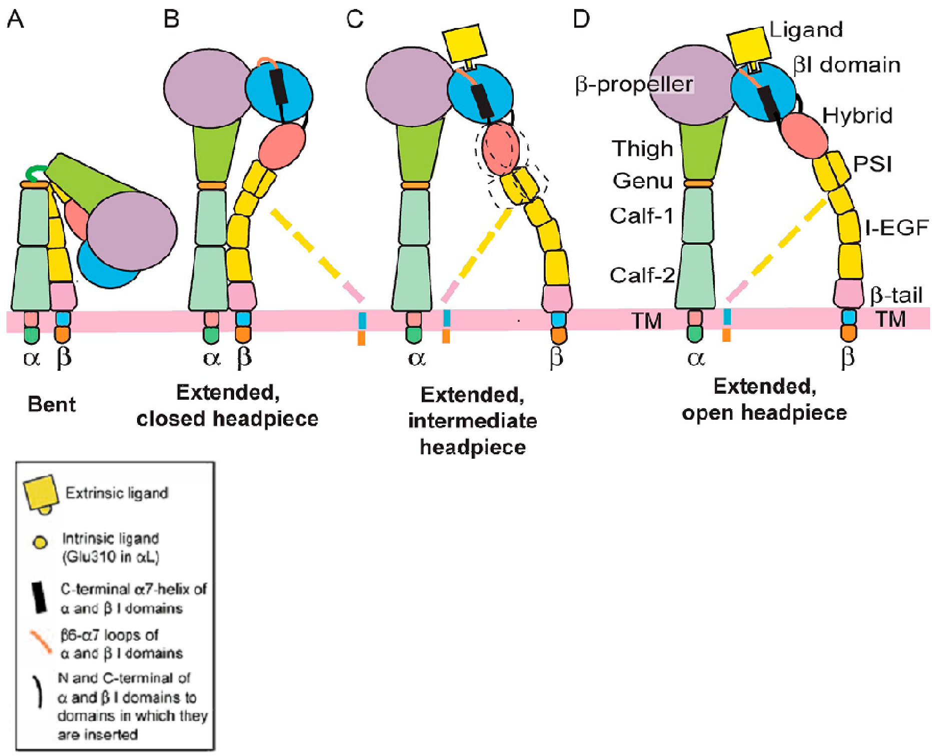



**Figure 4: Schematic drawing of integrin domain organization and conformational states.** The pink loop and black bar in B–D represent the β6-α7 loop and α7-helix of the βI domain, respectively. Broken lines symbolize lower β leg flexibility. The intermediate headpiece state is shown here for the first time (Modified from Fu et al., 2011 and Yu et al., 2012).

### 1.2.3. Integrin ligands - role of the extracellular matrix

Like I mentioned above, the outside-in signaling of the ubiquiteous expressed integrins is realized by connecting cells with specific components of their ECM, such as fibronectin, collagen I, laminin or vitronectin. On the cell surface multiple types of integrins are simultaneously expressed (Figure 5). A characteristic feature of integrins is their ability to bind diverse ligands enabled by different combinations of the monomers (Campbell and Humphries, 2011; Humphries et al., 2006), but their binding activity is modulated by cell-type specific factors, which lead to different ligand-specifity depending on the cell type (Alberts et al., 2008; Humphries et al., 2006).

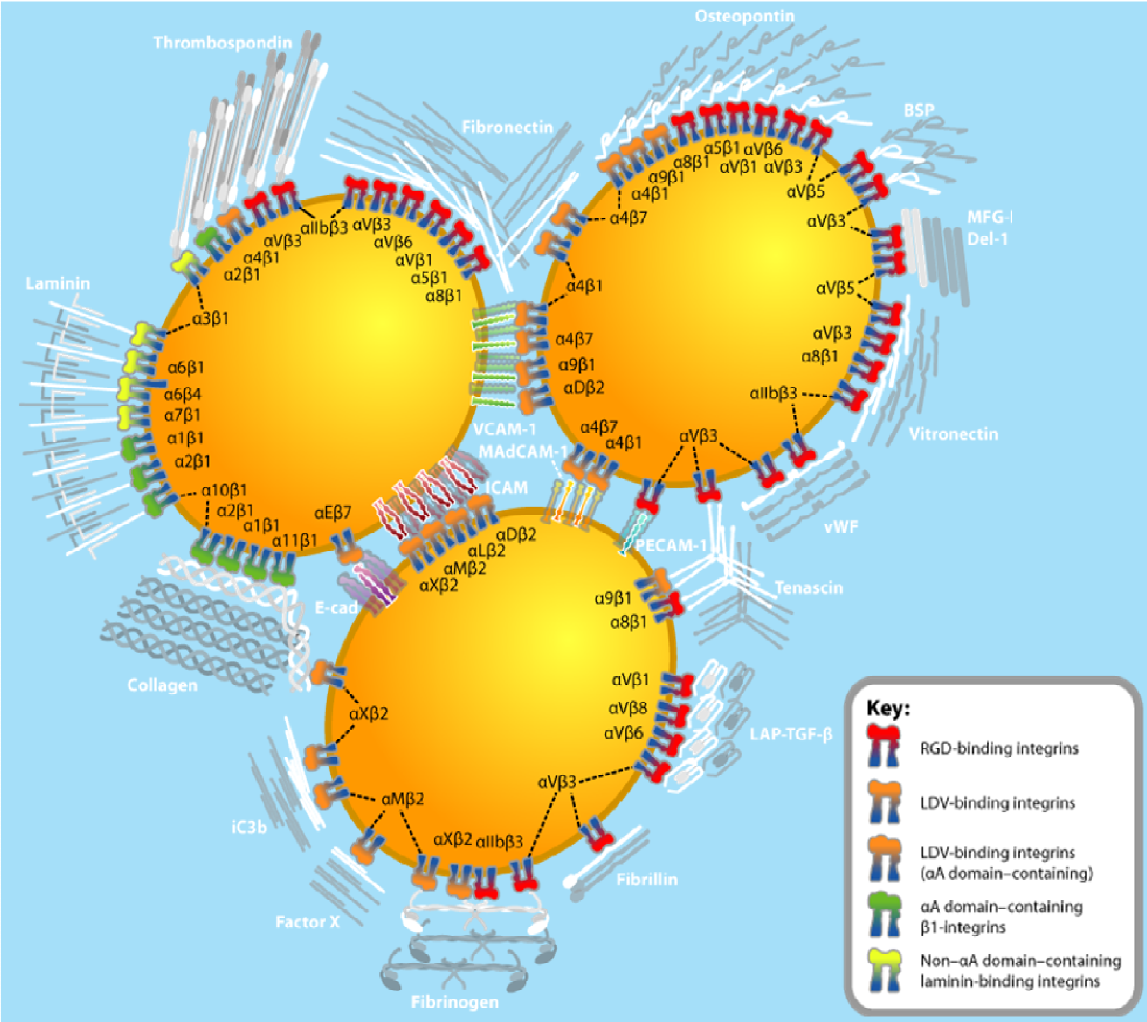



**Figure 5: Different integrin ligands.**
The cartoon demonstrates the simultaneous expression of different integrin types on the cell surface with the same or different ligands specifity (copied from Humphries et al., 2006).

This linkage between integrins and many different ECM proteins physically anchors cells to their environment and realizes a cell reaction after the exposure of mechanical forces, which may induce a cytoskeleton re-organization (Brizzi et al., 2012).

By using the different molecular interactions between integrins and their ligands they can be classified into four main classes. First the RGD-binding integrin family, which binds a large number of different ECM molecules and soluble vascular ligands, like fibronectin, tenascin, vitronectin or fibrinogen (Kloepper et al., 2008a; Plow et al., 2000). These RGD-binding ligands bind at an interface between the α and β subunits of all five αV integrins, α5β1, α8β1 and αIIbβ3 (Humphries et al., 2006).

Similar to the RGD sequence of ligands is the LDV-motif of ligands which were recognized from α4β1, α4β7, α9β1, four members of the β2 family and αEβ7. The structures of this integrin subfamily are not solve, but it is assumed that LDV peptides binds to a similar region like the RGD binding ligands at the junction between the α and β subunits, unless the β2 family where the interaction takes place at the αA-domain (see Figure 5) (Campbell and Humphries, 2011).

β1 integrins coupled with α subunits (α1, α2, α10 and α11), which consist of the ligand binding αI domain, represent a third class of integrin-ligand combinations (Figure 5). These integrins are able to bind laminin and collagen (Campbell and Humphries, 2011)

An exclusively laminin-binding integrin subfamily comprises α3β1, α6β1, α7β1 and α6β4 (Figure 5).

## 1.3. β1 integrin

β1 integrin (CD29, Fibronectin receptor subunit β, Glycoprotein IIa) belongs to the single-pass type I membrane protein, which is encoded by the *ITGB1* gene. It display almost the largest occurrence of this receptor family (Nagae et al., 2012; Shakibaei et al., 2008), since the β1 subunit associate with a large variety of α subunits, like α1, α2, α3, α4, α5, α6, α7, α8, α9, α10, α11 and αV (for details Figure 1 or Figure 5). Because of their high distribution β1 integrins represent a main actor of mediating the cell-matrix and cell-cell interactions with the aim of regulating fundamental processes, like migration, cell polarity, adhesion, proliferation and differentiation (Akhtar and Streuli, 2013; Hehlgans et al., 2007; Shakibaei et al., 2008).

### 1.3.1. Structure

All β subunits consist of different ectodomains (the β-I [or βA] domain, a hybrid domain, a PSI domain, an EGF domain [comprise of 4 modules], a β tail domain), a transmembrane domain and the cytoplasmic tail (see 1.2.1 and Figure 6) (Campbell and Humphries, 2011).

Most information about the ectodomain structure is solved by crystallizing β3 integrins because of its stable bent conformation (Campbell and Humphries, 2011; Takagi et al., 2002). However, structure as well as ligand binding studies of the β1 subunit is primarily done with α5β1 integrin, a fibronectin receptor (Humphries et al., 2000; Nagae et al., 2012; Pan and Song, 2010), but in that case it turned out that crystallizing of the full-length α5β1 ectodomain is complicated because of its flexible character of the lower half (Figure 6) (Nagae et al., 2012). For this reason only shorter fragments were analyzed especially the binding and ligand-recognized pocket/domain of β1 integrins.

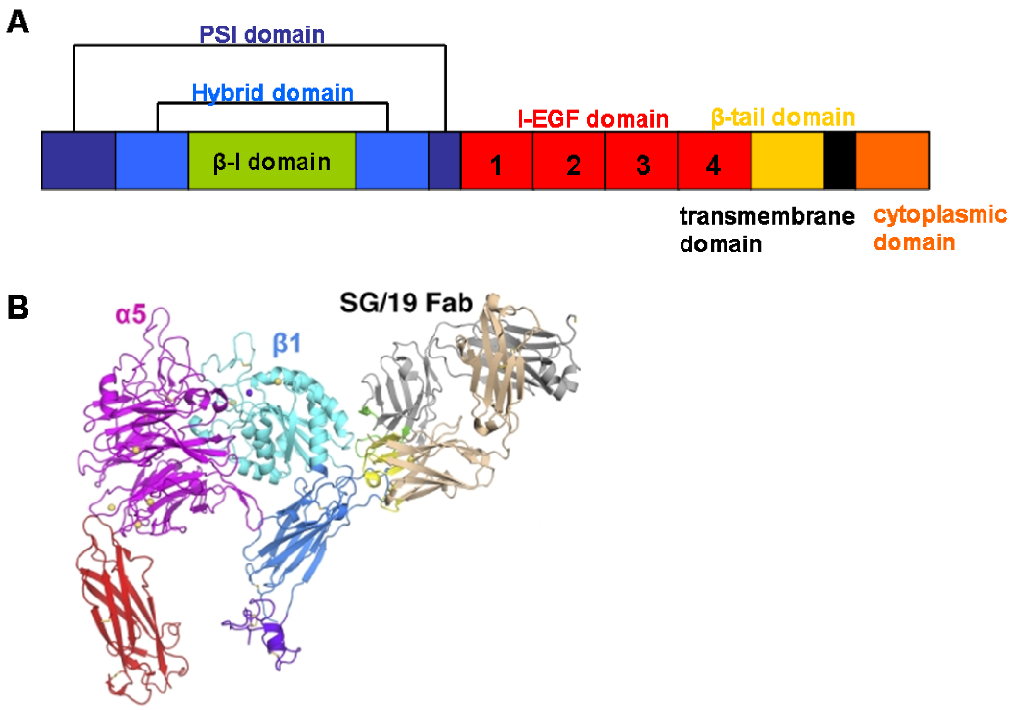



**Figure 6: Structure of the β1 integrin subunit.**
(A) The cartoon shows the different domains of β integrin subunits (modified from Campbell and Humphries, 2011). (B) "Structure of the α5β1 integrin headpiece in complex with SG/19 Fab." SG/19 is an anti-β1 inhibitory antibody (copied from Nagae et al., 2012).

The achieved knowledge about the integrin–ligand binding via the core recognition of specific sites, like RGD, and the secondary interaction by synergistic sites such as MIDAS, clarified the high affinity and specificity to their ligands (Nagae et al., 2012). In contrast to β3 ectodomains have the β1 chain two conserved N-glycosylation sites near the ligand binding site. In addiction to the glycosylation state of α5β1 on the cell surface the biological function of the receptor were influenced (Nagae et al., 2012).

The bidirectional signaling of integrins is realized by various splice variants of the subunits; thereby the expression of different β1 integrin cytoplasmic domains realizes an adapted cell reaction. For example, the splice variants β1A and β1D include two conserved NPxY motifs and provide the inside-out activation of integrins neither the outside-in activation of FAK, but in contrast β1B cytoplasmic domain miss the carboxy-terminal end of β1A, which lead to an inactive conformation (Cordes et al., 2006). The inactive β1 integrin achieve cell adhesion but not mediating signals.

### 1.3.2. Ligands

The diversity of β1 integrins (Figure 1) defines the possibility to bind a large variety of ligands, such as fibronectin, laminin, collagen, fibrinogen, vcam-1 or vitronectin (Figure 5). In absence of the α-I domain (see 1.2.1), as in α5β1 integrins, the β-I domain of integrins is responsible for recognizing specifc motifs and binding of these ligands. The small aspartate or glutamates including sequences, like RGD or LDV, were attached with only a low affinity, which can be enhanced by the binding of $Mg^{2+}$ and $Mn^{2+}$ions at the synergistic sites – the MIDAS or the SYMBS (Humphries et al., 2000; Mould et al., 2003; Nagae et al., 2012).

### 1.3.3. β1 integrin-mediated signaling and target genes

Besides the anchor or adhesion function integrins, such as β1 integrin, are able to permit a cell reaction in response to mechanical forces via the outside-in signaling by targeting the transcription of specific genes and by this control the cell survival (Cordes et al., 2006; Danen, 2013). This clarifies that integrins are not only simple receptors which permit the attachment of cells with their environment, but they can influence the transcription of genes by their intracellular signal cascade (Cordes et al., 2006; Legate et al., 2009).

The β1 as well as the β3 integrin-mediated signaling can be divided in three different time periods. First events include the production of second messengers phosphoinositide (PtdIns-4,5-$P_2$ and PtdIns-3,4,5-$P_3$) and the activation of adaptor proteins like ILK, FAK or Src, followed by the stimulation of actin regulatory proteins, including Rho family GTPases. The last signaling stage lead to a regulated transcription of distinct genes (cyclin D1, c-Jun amino-terminal kinase 1), which controls cell proliferation, differentiation or survival (Gagne et al., 2009; Goel et al., 2013; Legate et al., 2009).

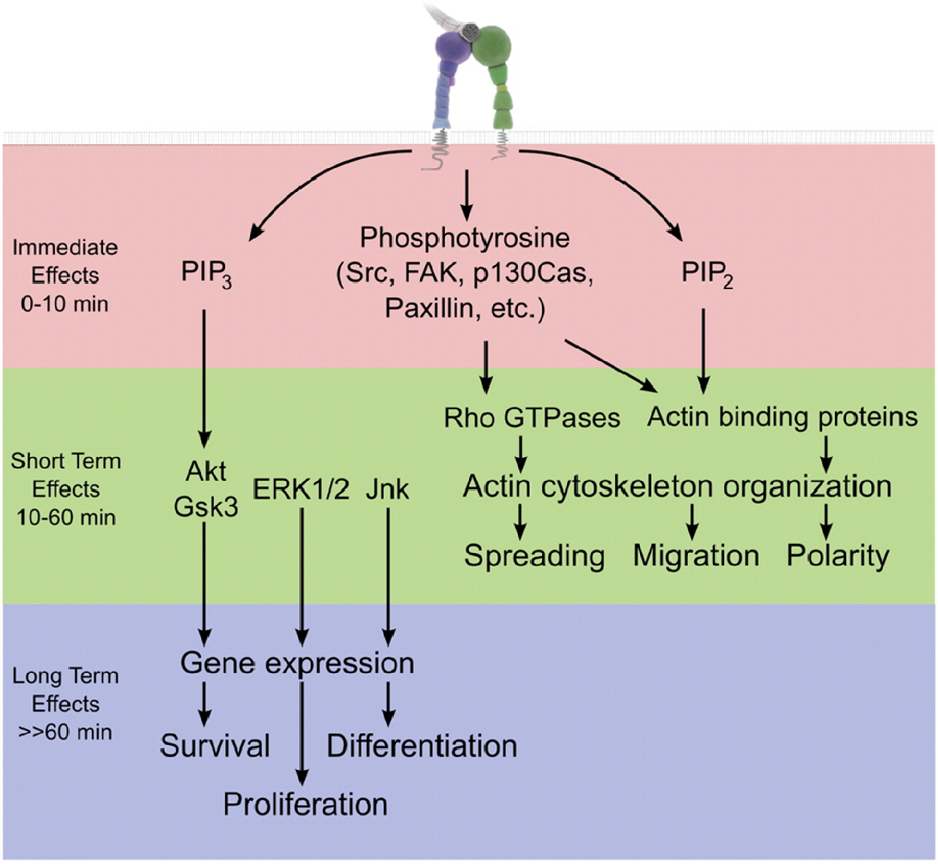



**Figure 7: Intracellular effects of the integrin activation.**
Scheme of time-dependent effects after the activation of β1 integrin (copied from Legate et al., 2009). Abbreviation: PIP = phosphoinositide-P2/P3, FAK = focal adhesion kinase, Src = proto-oncogene tyrosine-protein kinase, GSK3 = glycogen synthase kinase-3, Akt = protein kinase B, ERK 1/2 = extracellular signal-regulated kinases ½, Jnk = c-Jun-N-terminalen Kinasen, p130Cas = Crk-associated substrate.

A peculiarity of β1 integrin-mediated signaling to facilitate resistance to ionizing radiation and cytotoxic drugs was already shown in cell cultures and in mice studies. Based on the inhibition of c-Jun amino-terminal kinase 1 (JNK1) by the β1 integrin downstream targets p130Cas and paxillin, normal cells and transformed cells (cancer cells) develop a resistance to radiation, such as in prostate carcinoma (Cordes et al., 2006; Goel et al., 2013). This prosurvival influence of β1 integrins and namely their cytoplasmic domains after ionizing radiation or genotoxic injury demonstrate the huge relevance for understanding the β1 integrin-mediated signaling for anticancer therapies (Cordes et al., 2006).

### 1.3.4. Recognized functional significance of β1 integrin signaling

The integrin receptor family is generally described as main actor of multiple cell functions and cell-ECM connections. But different mice studies discovered that namely β1 integrin is fundamental already during early stages of mammalian development as well as adult cell/tissue maintenance, such as skin or HFs (Brakebusch et al., 2000; Danen, 2013; Piwko-Czuchra et al., 2009; Raghavan et al., 2000; Stephens et al., 1995). Their ubiquitous distribution and their large variety concerning the heterodimerization with different α subunits connected with multiple of ligand binding sites elucidate the miscellaneous influence of β1 integrin in distinct tissues.

Thus without the β1 integrin-mediated signaling via their crosslinking of cells with the ECM, controlled proliferation or differentiation signals would missing, which induce cell anoikis (Attwell et al., 2000; Danen, 2013; Kamarajan and Kapila, 2007). The ECM attachment is also quite necessary for the maintenance of stem cells (SCs) as well as their controlled differentiation within the SC niche. This communication is realized by receptors like integrins. β1 integrin subfamily is generally used as a marker for epithelial SC populations (Jones and Watt, 1993) and regulate with cadherins the symmetrical and asymmetrical divisions, which represents a key role of SC properties (Marthiens et al., 2010).

Moreover the expression of β1 integrin is imperative for the development and function of the mammary gland, like the control of basal-apical cell polarity, the differentiation or the attachment to the basement membrane (BM) of their epithelial cells and the mammary gland SC self-renewal (Akhtar and Streuli, 2013; Naylor et al., 2005; Taddei et al., 2008; Xu et al., 2009). Also in neuronal progenitors or intestinal epithelial SC β1 integrin participate in cell fate decisions by coordinating different signaling pathways, such as the Notch or the hedgehog signaling (Campos et al., 2006; Jones et al., 2006).

This contribution of β1 integrin-mediated signaling mainly concerning different SCs and their progeny constitute a huge research field/chapter, however many distinct receptor functions are remain unsolved, such as their specific influence/relevance in SCs harbouring regions like the HF. Precisely this point to be examined in detail in this thesis.

## 1.4. Epithelial stem cells and their niche – ECM as key determinant of integrin-mediated outside-in signaling

The behaviour of SCs and their progeny is mainly controlled by the interplay between intrinsic and extrinsic signals (Watt and Driskell, 2009). The extrinsic signals, which are mediated by defined ECMs in SC niches, are likely to be the first molecular components interacting with SCs (Chen et al., 2012; Philp et al., 2005). SCs are classified in 3 main classes: (1) embryonic SCs, which are obtained from embryonic sources; (2) adult SCs, like epithelial or mesenchymal SCs, acting as multipotent progenitor cells which can differentiate into a more or less limited set of cell types of the tissue in which they reside and are critical to the maintenance of tissue homeostasis and tissue repair following injuries; and (3) induced pluripotent SCs, rising from genetic reprogramming of somatic differentiated cells into a dedifferentiated state resembling embryonic SCs (Nava et al., 2012; Takahashi and Yamanaka, 2013).

The surrounding niche regulates adult SC-preservation and/or differentiation and by that impacts on the homeostasis of tissues/organs, like the epidermis and the cyclic activity of the HF (Brizzi et al., 2012; Cotsarelis et al., 1999; Philp et al., 2005). The SC niche is composed of SC and their progeny supporting cells as well as soluble factors such as growth factors, cytokines, enzymes and molecules like transforming growth factors (TGF) or bone morphogenetic protein (BMP), which are embedded into an ECM (e.g collagens, fibronectin, laminin, elastin and vitronectin). This ECM not only serves as a mechanical support and as a reservoir for secreted signaling molecules, but also signals itself, e.g. by outside-in integrin-mediated signaling (Brizzi et al., 2012; Nava et al., 2012). The epithelial SC niche of HFs, the so-called bulge region (see below Figure 8) represents such a typical environment which habours SCs (Solanas and Benitah, 2013).

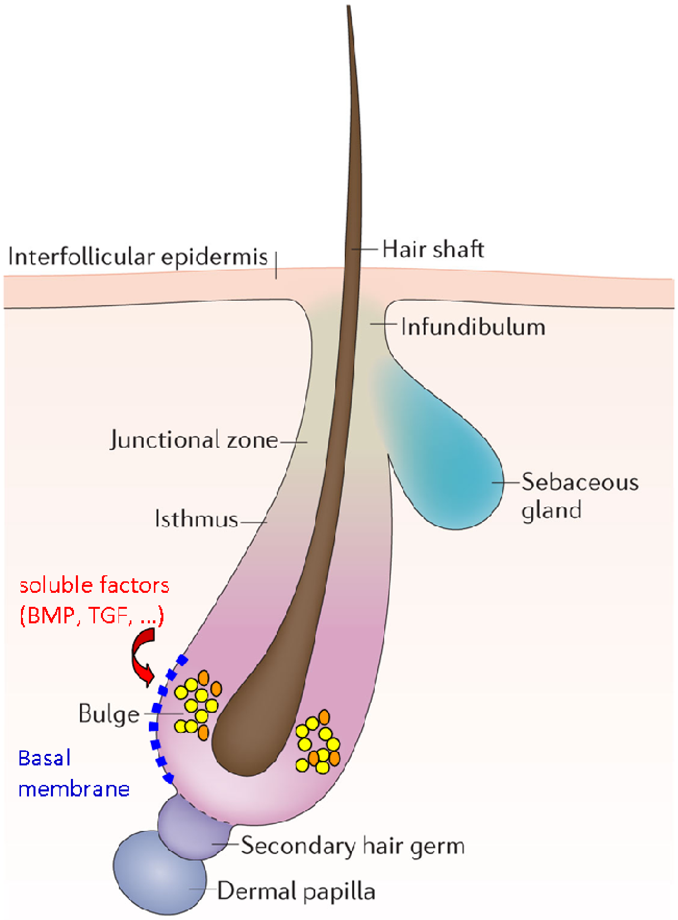



**Figure 8: Epithelial stem cell niche in the hair follicle bulge.**
The cartoon depicts a telogen hair follicle (HF in resting phase) consisting of the infundibulum, the junctional zone, the isthmus and the bulge. The HF bulge is the niche, comprised of stem cells (yellow spheres) and their progeny (orange spheres), which are influenced by surrounding basal membrane components (blue line), like fibronectin, laminin or collagen, and soluble factors (such as transforming growth factors [TGF] or bone morphogenetic protein [BMP]) (picture modified from Solanas and Benitah, 2013).

Thus, there is a growing interest in the role of ECM as a crucial niche constitutent that regulates SC function, differentiation, activation, and survival (Solanas and Benitah, 2013; Watt and Fujiwara, 2011). Namely the regulation of SC activities/maintenance that is controlled by cell binding to their ECM microenvironment through receptors like integrins, which operate as the physical anchors that simultaneously activate cell transduction pathways by extrinsic ECM signals (Nava et al., 2012; Watt and Fujiwara, 2011) has moved into the focus of research interest in epithelial and SC biology (Chen et al., 2012; Gilbert et al., 2010; Lodish et al., 2012; Solanas and Benitah, 2013).

## 1.5. Recognized roles of β1 integrin in mammalian skin biology

It was already proven that integrins, like namely β1 integrin, act not only as cell anchors to the BM or surrounding cells, but they are decisive for normal skin homeostasis by balancing between proliferation, differentiation and migration, but also for HF morphogenesis and BM composition/rearrangement (Brakebusch et al., 2000; Grose et al., 2002; Watt, 2002). These multifactorial influences of β1 integrins justify their main role in development of skin and their appendages, in normal wound healing, in skin aging and also in inflammatory diseases, like scleroderma or psoriasis (Giangreco et al., 2009; Haase et al., 2001; Liu et al., 2009; Liu et al., 2010; McFadden et al., 2012; Raghavan et al., 2000).

### 1.5.1. Epidermal and hair follicle function

Mammalian skin consists of several layers, including the outermost multilayered epidermis and associated structures like the HFs and SGs (Watt and Fujiwara, 2011). β1 integrin is expressed in all these areas (Figure 9) and regulate by this the skin homeostasis, HF morphogenesis or the formation/composition of the BM. A long time ago they could already point out that the expression of β1 integrin on the KC surface is essentiell for the skin and hair follicle integrity in mice (Brakebusch et al., 2000). The KC-restricted deletion of β1 integrin lead to a disturbance of the BM, skin blister formations and an impaired keratinocyte differentiation and proliferation accompanied by a massive hair loss (Brakebusch et al., 2000).

While β1 integrin is mainly expressed in adhesive KCs of the basal layers in unwounded skin and downregulated in the direction to the subrabasal layers (Figure 9), there are not significant differences in distinct compartments of the ORS in HFs (Bose et al., 2012; Kloepper et al., 2008a; Watt, 2002). A previous study verified a functionally important role of β1 integrin-mediated signaling in human HF growth (Kloepper et al., 2008a).

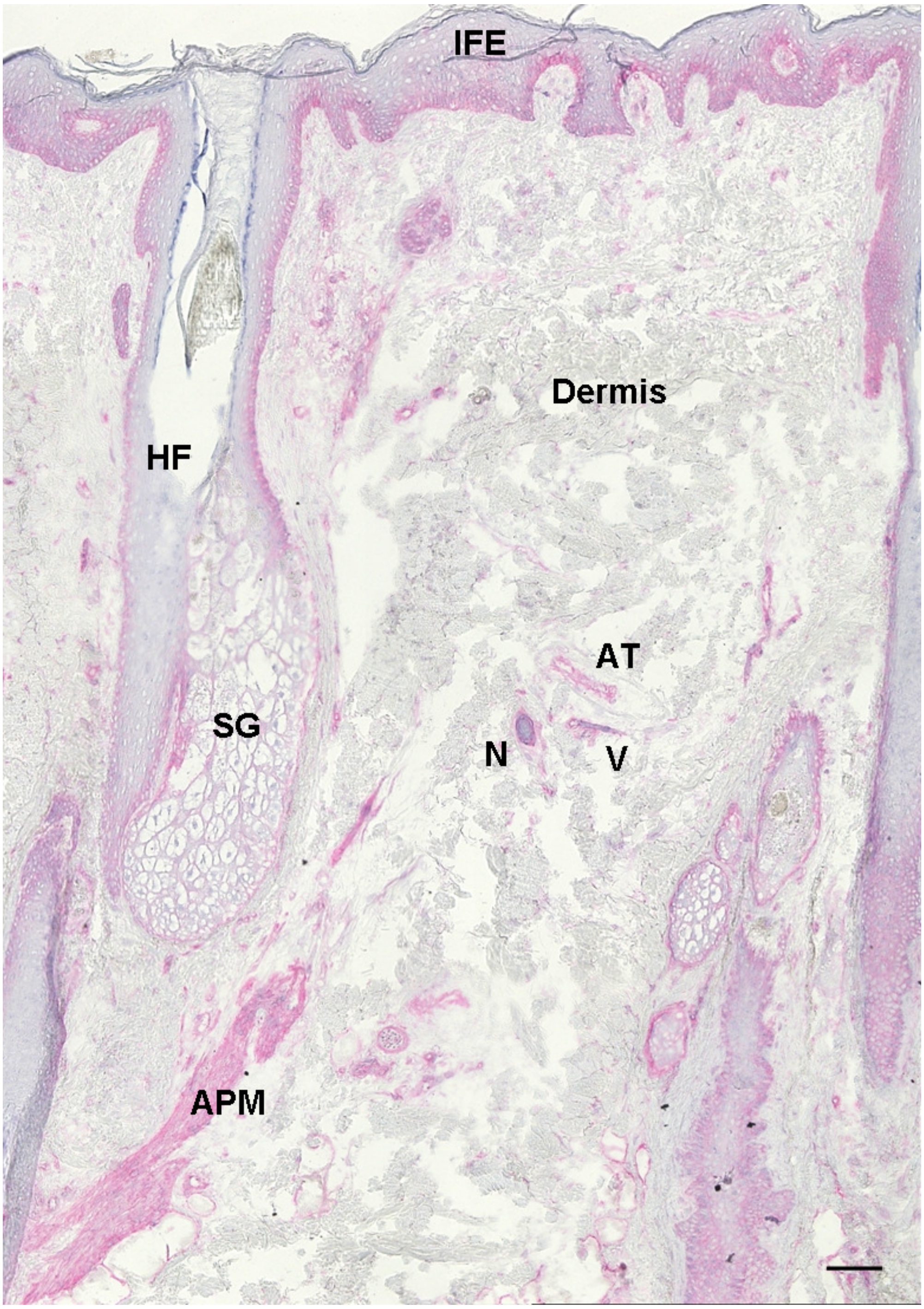



**Figure 9: β1 integrin immunoreactivity pattern in human skin.**
Cryosectioned human skin which were stained by using the β1 integrin specific antibody TS2/16 (1:100, generated by N. Ernst). β1 integrin is expressed in the basal layers of the IFE as well as in skin appendages like the HF or the SG. Black scale bar = 50µm. Abbreviation: APM = arrector pili muscle, AT = arteria, HF = hair follicle, IFE = interfollicular epidermis, N = nerve, SG = sebaceous gland, V = vein.

Potential ligands for integrins expressed on HF keratinocytes (KCs) are components of the BM that separates the HF epithelium from its surrounding mesenchyme; the connective tissue sheath (CTS) and the follicular dermal papilla (DP) which is enclosed by the epithelial hair matrix. These BM-associated integrin ligands include collagen IV, laminin-5, fibronectin, perlecan and nidogen (Brakebusch et al., 2000; Gilcrease, 2007; Peltonen et al., 1989). Thus, ORS keratinocytes (ORSKs) can interact with multiple ECM components of the BM via α2β1, α3β1 and α6β4 integrins, which are differentially expressed in distinct regions of the HF (Commo and Bernard, 1997; Conti et al., 2003; Margadant et al., 2010). α3β1 integrins connect the actin cytoskeleton to the BM via binding laminin-5, whereas the α2β1 integrin is found in basal KCs, where it is thought to mediate cell–cell interactions and the attachment to the BM via collagens (Hynes, 2002; Margadant et al., 2010; Watt, 2002).

### 1.5.2. Epithelial stem cells and their progeny

The skin as a complex organ harbours numerous different SC reservoirs, for example in the interfollicular epidermis (IFE), in the permanent portion of HFs (the bulge) or in sweat glands (Figure 10). ESCs are responsible for the skin maintenance, tissue homeostasis or regeneration by proliferation of SCs and differentiation of their progeny and reside in the basal layer of the epidermis in their specialized niches (Danner et al., 2012; Jaks et al., 2010; Nagel et al., 2013; Watt and Fujiwara, 2011; Watt and Jensen, 2009). Sitting in these niches SCs underdo regularly an asymmetric devision to renew themselves and to produce their progeny cells which differentiate and move outwards to the suprabasal layer or ePCs in other compartments of the skin like the HF, SG or IFE (Blanpain and Fuchs, 2009).

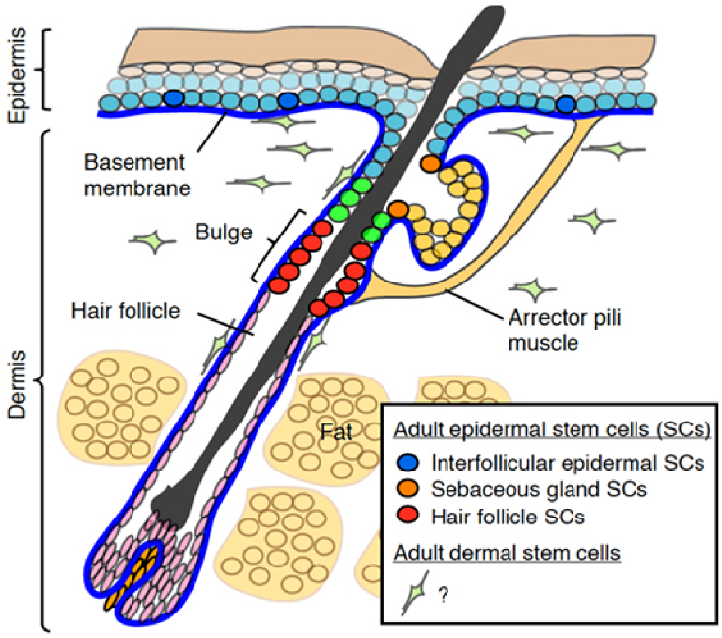



**Figure 10: Basic anatomy of murine and human skin.**
IFE: interfollicular epidermis; HF: hair follicle; SG: sebaceous gland; BM: basement membrane; DP: dermal papilla; APM: arrector pili muscle (copied from Watt and Fujiwara, 2011).

Over the last 2 decades the distribution and relevance of integrins and extracellular matrix proteins in the skin has been characterized extensively. β1 integrin signaling has long been thought to be important in epidermal and HF eSCs (Kloepper et al., 2008b; Roh et al., 2005; Tan et al., 2013; Watt, 2002; Watt and Fujiwara, 2011).

In the HF, eSCs and partially differentiated epithelial progenitor cells (ePCs) can give rise to all epithelial cell types of the hair, the epidermis and the sebaceous gland and are mostly found within the HF bulge (Fujiwara et al., 2011; Ma et al., 2004; Morris et al., 2004; Tiede et al., 2007). The eSCs within this HF compartment (Kloepper et al., 2008b) are slow-cycling, and show clonogenicity as well as proliferative capacity (Cotsarelis, 2006; Lavker and Sun, 2000). Mainly in this eSC-enriched compartment microarray analysis confirmed more marked differences in expression of diverse ECM genes (like insulin-like growth factor binding protein 5, collagen type-VI α1, collagen type-I α2 or tenascin C) in mice than in other epidermal cell populations, but until now the functional relevance of the ECM composition of the bulge is incompletely understood (Wang et al., 2012; Watt and Fujiwara, 2011).

The HF bulge is comprised of SC/ePCs, which differentiate to a progenitor population called the secondary germ cells. These cells, residing adjacent to the bulge, represent the basis to produce a new hair shaft at anagen until catagen (Garza et al., 2011; Panteleyev et al., 2001). Potential markers for the HF SCs and their immediate progeny include β1 integrin, keratin 15 and 19 (K15, K19), α6 integrin, CD71, CD200, p63 and CD34; moreover, gap junctional communication (connexin 43) and MHC class I molecules are markedly down-regulated (Kloepper et al., 2008b). However there is still a considerable debate how to distinguish the least committed eSCs from their immediate progeny from transit amplifying cells (Beck and Blanpain, 2012; Cotsarelis et al., 1990; Inoue et al., 2009; Lyle et al., 1998; Webb et al., 2004). The most relevant markers and their specific expression pattern are described more in detail:

#### 1.5.2.1. β1 integrin

Previous work has suggested that epithelial cells in human epidermis with the highest level of α2β1, α3β1 and α5β1 integrins show a high colony-forming efficiency (CFE) (Jones and Watt, 1993), and β1 integrin signaling is absolutely required for epidermal and HF maintenance (Brakebusch et al., 2000). Whether or not β1 integrin protein is overexpressed on eHFSCs is still a matter of debate. At least in human anagen scalp HFs there is no evidence that β1 integrin proteins are significantly overexpressed in the bulge region compared to other areas of the ORS (see Figure 11 and Figure 12) (Kloepper et al., 2008a). But their high expression in the SC harboring basal layer of the IFE combined with the specific properties of β1 integrin$^{+}$ KCs like clonogenicity identify their importance for eSCs (Tan et al., 2013).

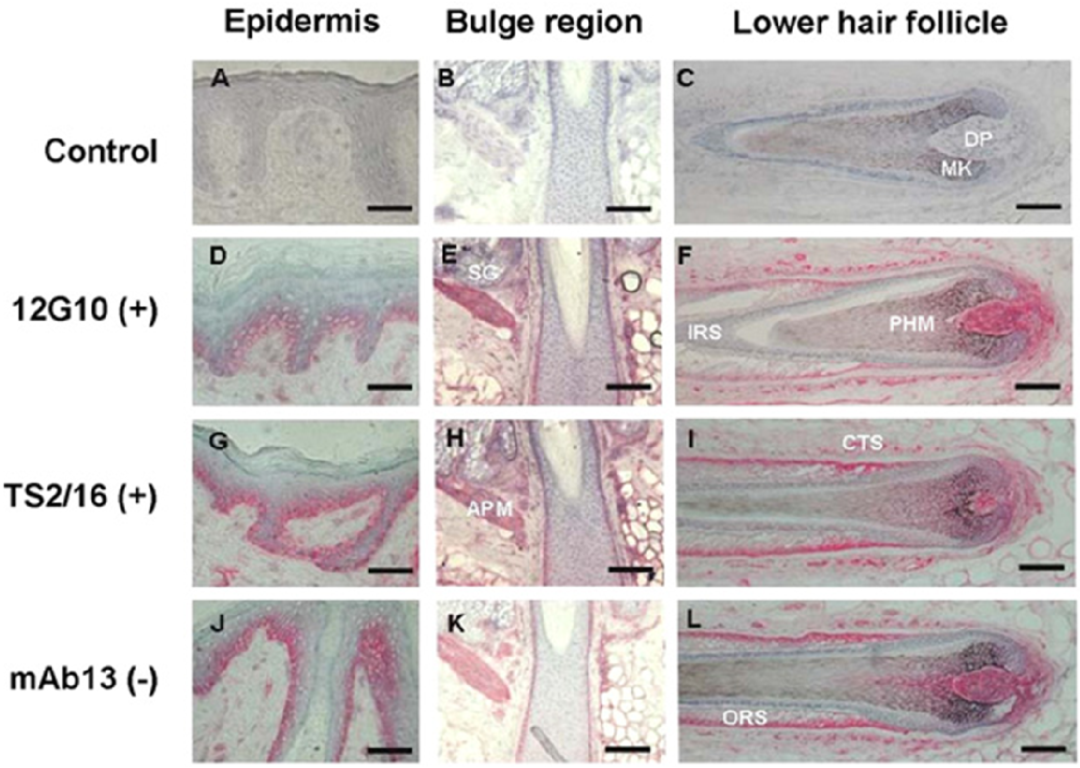



**Figure 11: None remarkable differences in β1 integrin immunoreactivity (IR) on human skin and on distinct hair follicle compartments.**
Pictures show that the using of various β1 integrin specific antibodies detects no significant differences within the skin and the whole hair follicle. A, D, G, J: Epidermis; B, E, H, K: Bulge region; C, F, I, L: Lower hair follicle. A, B, C: negative control without primary antibody, D, E, F: Staining with 12G10 (+) antibody, G, H, I: Staining with TS2/16 (+) antibody, J, K, L: Staining with mAb13 (−) antibody. Abbreviation: DP = Dermal papilla, MK = Matrix keratinocytes, SG = Sebaceous gland, PHM = Precortical hair matrix, IRS = Inner root sheath, CTS = Connective tissue sheath, APM = Arrector pili muscle, ORS = Outer root sheath. Bars: A, D, G, J, C, F, I, L: 100 µm, B, E, H, K: 150 µm (copied from Kloepper et al., 2008).

While the integrin levels may not differ greatly between the SCs and their more differentiated progeny, they are both sufficient and required to guarantee that eSCs are more adhesive to ECM proteins and thus maintained in their undifferentiated state (Jones et al., 1995). Due to its almost ubiquitous expression on surface of many different cell types the description of β1 integrin as part of a SC signature (Jones and Watt, 1993) should thus be used with some caution (Barczyk et al., 2009).

#### 1.5.2.2. Keratin 15

Keratin 15 (K15) is now the accepted standard marker for follicular SCs/PCs in the bulge and in the epidermal basal layer during embryogenesis and throughout adulthood. It identifies these cells at least as highly undifferentiated epithelial cells that can undergo differentiation into various distinct epithelial lineages (Garza et al., 2011; Kloepper et al., 2008b; Liu et al., 2003; Sieber-Blum, 2011; Tiede et al., 2009). Given the appropriate signals and environment, these ePCs may also undergo epithelial-mesenchymal transition, thus contributing e.g. to carcinogenesis, fibrosis, and scarring alopecia (Nakamura and Tokura, 2010; Rao et al., 2013). K15 mRNA as well as protein are overexpressed in the HF bulge, but lower expression levels are also seen in the basal layer of the lower HF ORS (Cotsarelis, 2006; Tiede et al., 2009) (Figure 12). Therefore, K15 is not a selective bulge marker (Lyle et al., 1998; Waseem et al., 1999) but as a marker for ePCs, which encompass eSCs and early transit amplifying cells (TAs)/ePCs. Interestingly, the expression of K15 declines with age (Liu et al., 2003; Webb et al., 2004), suggesting a slowly progressive exhaustion of the pool of K15$^{+}$ ePCs in murine and human skin. However, even the miniaturized vellus HFs in male pattern balding (androgenetic alopecia) still exhibit an essentially normal number of K15$^{+}$ cells (Garza et al., 2011).

#### 1.5.2.3. CD200 - the immunoinhibitory "no danger-signal"

CD200 is a type-1 transmembrane glycoprotein that delivers a negative immunoregulatory signal through the CD200 receptor (CD200R). CD200 is commonly expressed on cells originating from the hematopoietic cells. But by using transcriptional profiling the mRNA of this cell surface protein was identified in the HF SC compartment where it was upregulated and largely restricted to the HF bulge as well as the immediately adjacent ORS in mouse and human epidermis (Figure 12) (Meyer et al., 2008; Rosenblum et al., 2006).

Besides the expression of immunoinhibitory molecules like alpha-melanocyte stimulating hormone, transforming growth factor-beta2 (TGF-β2), macrophage migration inhibitory factor and indoleamine-2,3-dioxygenase is mainly the "no danger signal" of CD200 responsible for the immuneprivilege and by this the protection of the eSC harboring HF bulge (Harries et al., 2013; Meyer et al., 2008). A skin CD200-deficience is associated with inflammatory diseases like alopecia or Lichen planopilaris (LPP) (Harries et al., 2013; Rosenblum et al., 2006).

#### 1.5.2.4. CD34 – a mouse HF SC marker

The expression of this cell surface molecule represents less divided SCs of the HF bulge in mouse (Hsu et al., 2011; Singh et al., 2013), but is not bulge-restricted in humans, where CD34 is prominently expressed in the ORS rather outside of the bulge of human anagen human HFs (Cotsarelis, 2006; Garza et al., 2011; Inoue et al., 2009). Thus, the expression of this surface protein represents a classical KC SC marker in mouse, but demarcates transit-amplifying precursor in human anagen HFs, which is not constantly expressed during the human development and the HF cycle (Gutierrez-Rivera et al., 2010; Poblet and Jimenez, 2008).

#### 1.5.2.5. CD71 – a marker for transit amplifying cells

In contrast to the previously described markers having a high expression in epithelial resting SCs, CD71 shows a low expression in human IFE SCs (Jensen and Watt, 2006; Szabo et al., 2013), but is an optimal opportunity to classify their actively cycling, transient amplifying progeny by a high expression (Figure 12). The gene as well as the protein expression is present in suprabulbar ORS cells of HFs below the sub-bulge (Ohyama et al., 2006) (Figure 12).

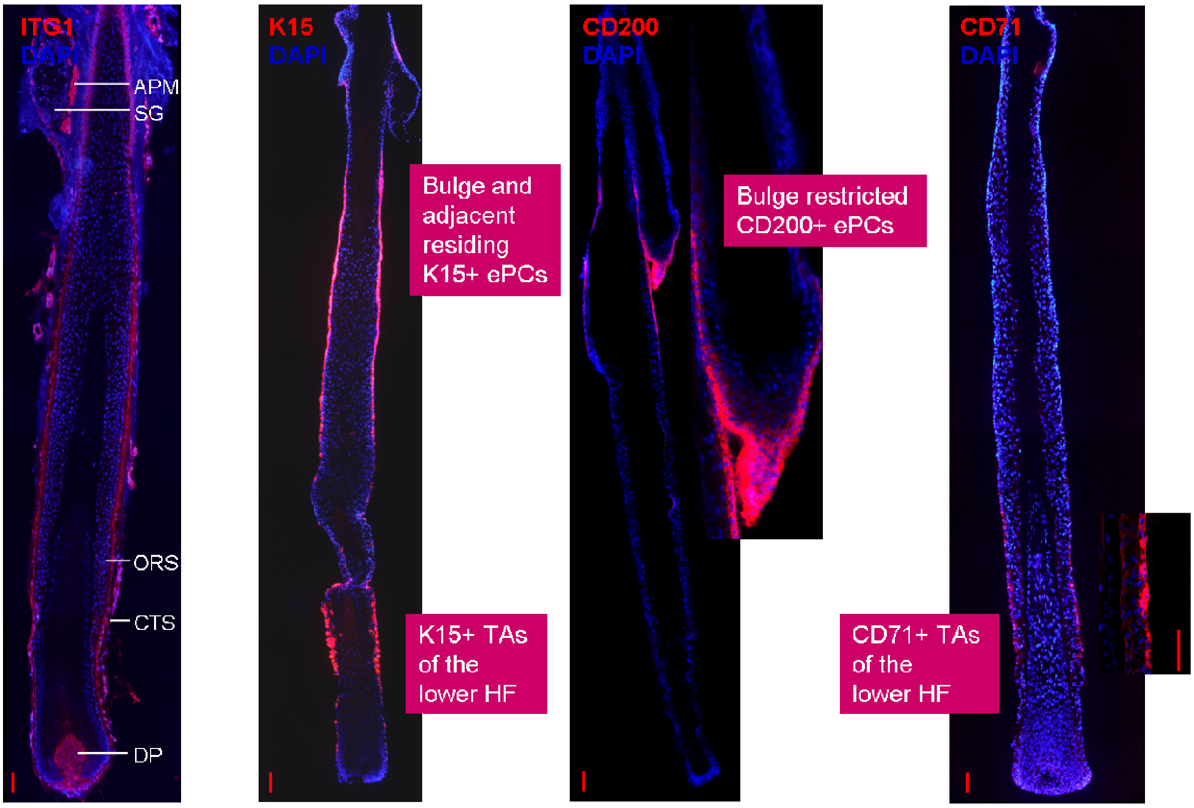



**Figure 12: Overview of immunoreactivity pattern of selected stem cell/epithelial progenitor cell/transit-amplifying marker in human hair follicles (HFs).**
Pictures show the expression pattern of β1 integrin in a full-length HF, Keratin 15 (K15), CD200 and CD71 in dispase-treated HF epithelium (day 0) (generated by N. Ernst). To determine the protein expression of these markers via immunofluorescence specific primary antibodies was applied and further visualized with suitable secondary antibodies. Abbreviation: DP = Dermal papilla, SG = Sebaceous gland, CTS = Connective tissue sheath, APM = Arrector pili muscle, ORS = Outer root sheath. Bars: 100 µm.

### 1.5.3. Integrins and wound healing

After injury or wounding of epithelial tissue a rapidly migration is necessary to form an epithelial cover and by this restore the barrier against infection. This migration-based reepithelization after skin wounding as well as hyperproliferation of KCs is crucially dependent on deposition of ECM and appropriate activation and function of integrin receptors in the epithelial cells (Larjava et al., 2011). Mainly integrins play a major role as transmembrane receptors in these processes, thereby linking the ECM environment with intracellular signaling and regulating multiple cell functions such as cell survival, proliferation, migration, and differentiation (Brakebusch et al., 2000; Iwata et al., 2013; Streuli, 2009;

Widgerow, 2013). Attachment of basal epidermal KCs to the BM is mediated by different heterodimers of the integrin family, like α2β1, α3β1, α9β1 and α6β4, but also *de novo* expression of additional integrins, for example, integrins α5β1, αvβ1, αvβ5 and αvβ6 is induced upon wounding (Margadant et al., 2009; McFadden et al., 2012).

The specific property of integrins of low affinity to their ligands is essential during wound healing, thus avoiding irreversible binding of cells and by this preventing migration (Cohen et al., 2004; Schwartz, 1992). The reepithelization process after epidermal injury is a very complex cooperation of different cell types, growth factors and the remodeling of ECM. Mainly the crosstalk between integrins and TGF-β (transforming growth factor) signaling appears to be important interaction for optimal wound healing (Liu et al., 2010; Margadant et al., 2010). On the one hand, this crosstalk involves the impact of TGF-β on integrin-mediated cell adhesion and migration by regulating the expression of various integrins, their ligands (like tenascin, vitronectin, fibronectin) and integrin-associated proteins (ILK, kindlin 1, paxillin and PINCH); on the other hand several integrins, like αvβ6 or αvβ8 are directly controlling TGF-β activation (Horiguchi et al., 2012; Margadant et al., 2010; Munger and Sheppard, 2011).

Figure 13 illustrates the main phases of wound healing and the supposed crosstalk of integrins and TGF-β within the skin.

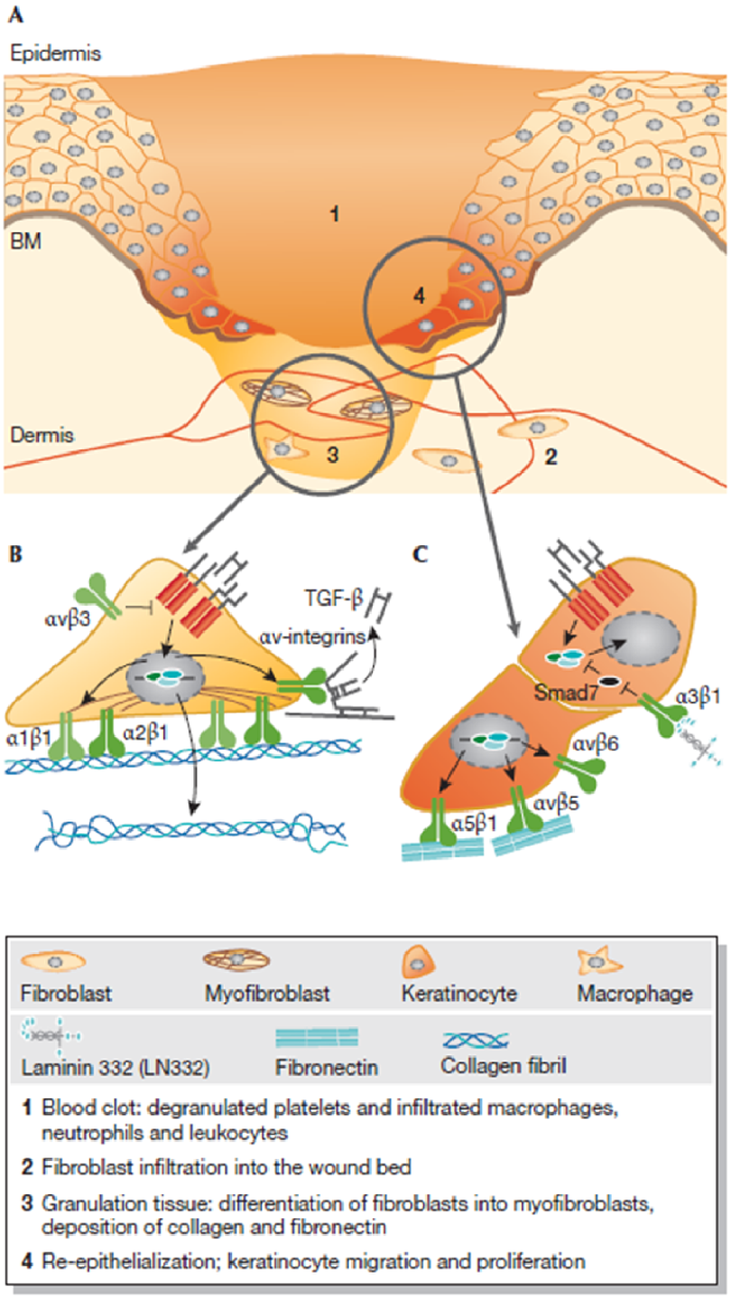



**Figure 13: Schematic drawing of the proposed integrin–TGF-β interactions during wound healing.**

"(A) Represents the major phases in wound healing, which are explained in the figure key. (B) In the granulation tissue, TGF-β induces expression of integrins α1β1 and α2β1, which mediate fibroblast contraction, and of αv-integrins, which activate latent TGF-β. Furthermore, αvβ3 might repress TGF-β signaling by inhibiting TGF-βR expression. (C) During re-epithelialization, TGF-β stimulates the expression of fibronectin and integrins, which mediate keratinocyte migration or activate latent TGF-β. Integrin α3β1 could enhance TGF-β signaling by controlling the expression of Smad7. Abbreviation: BM = basement membrane, Col = collagen, FN = fibronectin, LN332 = laminin 332" (copied from Margandant et al., 2010).

At present, exact mechanisms of interaction are only poorly understood and still controversial. Although TGF-β is a general "player" in every phase of wound healing and reepithelization (e.g., it stimulates the expression of fibronectin and of α5β1, αvβ5 and αvβ6 integrins in KCs and promotes granulation tissue formation), it also exerts important inhibitory function, e.g. by repression of excessive KC proliferation (Fleming et al., 2012; Margadant et al., 2010).

Namely, β1 integrin represents a main actor/receptor of skin wound healing, especially because of its ubiquitous expression and its numerous influences on cell behavior, like proliferation, migration or adhesion. Fibroblast-specific β1 integrin knockout studies in mice and fibroblast cell cultures revealed their major role in fibroblast proliferation, collagen contraction, adhesion, TGF-β1 activation and myofibroblast differentiation during wound healing (Leask, 2013; Liu et al., 2010). Furthermore the general or conditional deletion of β1 integrin in mice leads to cruel defects of development or skin and their appendages, such as skin blistering, hair defects and a disturbed BM formation (Brakebusch et al., 2000; Raghavan et al., 2000; Stephens et al., 1995).

Thus, the β1 integrin-mediated connection of KCs or fibroblasts to their surrounding, like laminin, collagen or fibronectin (Figure 13), should be well-balanced to support and realize a normal wound healing after injury and also HF-derived ePCs are well-appreciated to contribute epidermal regeneration after wounding (Ansell et al., 2011; Fuchs et al., 2013; Plikus et al., 2012; Xiong et al., 2013).

Yet, a role for β1 integrin in human skin reepithelization after wounding remains to be explored and demonstrated.

## 1.6. Methods in β1 integrin research: Manipulation of β1 integrin-mediated signaling

In order to elucidate the complex biological functions of β1 integrin-mediated signaling, several research techniques have proven invaluable, which are briefly summarized here, as some of these are also employed in the current thesis project.

### 1.6.1. β1 integrin knockdown or knockout

The tissue-selective silencing or knockout (KO) of a single receptor like β1 integrin is quite useful to study specific signaling effects of this receptor concerning a reduction in defined cell collectives, especially because of its widespread influences in cell behaviour like proliferation, migration, differentiation, adhesion and remodelling of the ECM (Beaty et al., 2013; Margadant et al., 2010; Wickstrom et al., 2011). Moreover there is a rising interest in integrin-inactivating proteins because of their particular significance for the *in vitro* and *in vivo* integrin function as well as the regulation for the integrin–ligand interactions (Bouvard et al., 2013).

To clarify the important role of different integrins with specific and non-redundant functions in many biological processes, the murine KO experiments gave an elucidating insight. Mice that lack integrin expression either constitutively or in specific cell types display a huge variety of phenotypes (Srichai and Zent, 2010). The KO phenotypes reflect the fundamental roles of the various integrins as they range from a complete block in preimplantation development (β1), through major developmental defects and perinatal lethality, to failings in tissue homeostasis, inflammation, angiogenesis and/or leukocyte function (Bouvard et al., 2013; Hynes, 2002; Liu et al., 2010).

**Table 1: Phenotypes of unchallenged and challenged integrin mutant mice.**
"The knockout data was collected from the Mouse Genome Informatics (MGI) database (http://www.informatics.jax.org/) and, whenever needed, updated with relevant Pubmed-retrieved (http://www.ncbi.nlm.nih.gov/pubmed/) original references" (copied from Barczyk et al., 2009).

| | Integrin | Viability | Unchallenged mutant phenotype | Challenged mutant phenotype |
|---|---|---|---|---|
| Collagen | α1 | + | No phenotype. Cell adhesion defect to collagen IV. | Reduced tumor angiogenesis, increased glomerulosclerosis, diminished callus size in bone fracture model, reduced atherosclerosis in ApoE-/- mice, reduced psoriasis in xenograft model. |
| | α2 | + | Mild mammary gland branching morphogenesis phenotype. Platelet, fibroblast, and keratinocyte adhesion defect to collagen I. | Reduced angiogenesis in tumor and wound healing models, reduced innate immune response to peritoneal Listera infection, reduced thrombi formation increased embolization in thrombosis model. |
| | α10 | + | Mild cartilage defect. | |
| | α11 | + | Incisor eruption defect. | |
| Laminins | α3 | +/- | Defects of kidney and submandibular gland, decreased bronchial branching of the lungs, skin blisters, abnormal layering of the cerebral cortex. | Faster wound healing in a Cre-model. |
| | α6 | +/- | Severe blistering of the skin and other epithelia, absence of hemidesmosomes, altered laminin deposition in the brain. and ectopic neuroblastic outgrowths on the brain and in the eye. Mutants die at birth. | |
| | α7 | - or + | Embryonic vasculature defect, cerebral hemorrhage, and placenta defects. Muscular dystrophy in adult mice. | Fibrotic muscle tissue when crossed with mdx mice. Protective role in exercise-induced muscle injury. |
| RGD | α5 | - | Severe defects in posterior trunk and yolk sac mesodermal structures, lack of epithelialization of somites, reduced numbers of Schwann cells and embryonic lethality at E10-E11. | |
| | α8 | +/- | Absent or reduced kidneys and abnormal steriosilia in the inner ear. | |
| | αv | - or +/- | Placental defects and intracerebral, intestinal hemorrhages and cleft palate. Death varies from midgestation to perinatal. | |
| | αIIB | + | Bleeding disorder, lack of platelet binding to fibrinogen, absence of fibrinogen in platelet alpha granules, and increased numbers of hematopoietic progenitors in yolk sac, fetal liver, and bone marrow. | |
| | α4 | - | Embryonic lethality either due to failure of chorioalloantoic fusion or cardiac abnormalities including defrects in epicardium formation. | |
| | α9 | +/- | Bilaterlal chylothorax causing death within 14 days. | Altered cutaneous wound healing in wound model. |
| Leucocyte spesific | αL | + | Reduced immune response, defects in neutrophil adhesion to endothelium, and in osteoclast adhesion. | Reduced leukocyte adhesion in TNF-α induced inflammation. |
| | αM | + | Reduced immune response, reduced neutrophil adhesion to fibrinogen and reduced degranulation of neutrophils. | Reduced T-cell proliferative response to Staphylococcal enterotoxin, reduced wound healing, reduced cerebral ischemia, reduced encephomyelitis, reduced melanoma rejection. |
| | αX | + | Reduced immune response. | |
| | αD | + | Reduced immune response. | |
| | αE | + | Reduced number of intestinal and vaginal interepithelial lymphocytes, skin inflammation. | Reduced experimental colitis. |
| | β1 | - | Null mutants die soon after implantation due to inner cell mass defects in blastocysts. | |
| | β2 | + | Leukocyte adhesion deficiency with immune, hematopoietic and skeleton defects. | Reduced listeriosis. |
| | β3 | - or + | Platelet defects, extended bleeding times, cutaneous and gastrointestinal bleeding, anemia, increased bone mass, hypocalcemia, reduced survival, and placental defects associated with some fetal loss. | Enhanced wound healing. |
| | β4 | +/- | Extensive detachment of epidermis and other squamous epithelia. Stratified tissues lack hemidesmosomes and simple epithelia are also defective in adherence. | |
| | β5 | + | Age-related blindness due to defective retinal phagocytosis. Cell adhesion defect of keratinocytes to vitronectin. | Reduced lung injury in a ventilator-induced model. |
| | β6 | + | Baldness associated with macrophage infiltration of skin and exaggerated pulmonary inflammation. | Reduced fibrosis in a bleomycin-induced lung model, impaired mucosal mast cell response to nematode infection, reduced wound healing, increased periodontal infection. |
| | β7 | + | Hypoplasia of gut-associated lymph tissue due to defects in lymphocyte migration. | |
| | β8 | + or +/- | Death either at midgestation (E11.5) as a result of circulatory abnormalities in the placenta, or the days around birth due to intracerebral hemorrhaging. | |

As shown in Figure 1 β1 integrin can associate with many different α subunits and is ubiquitously expressed which elucidates the embryonic lethality. Receptors containing the β1 subunit represent the main group of cell-matrix receptors (Hynes, 1992). In contrast the severities of phenotypes, where different α subunits were knocked out, depend on their typical binding partners. Whereas KOs in α subunits combined with a β1 chain and a primary binding to collagens facilitated a significant redundancy and compensation between the collagen receptors, the primary laminin-binding and the primary RGD-binding integrins suggest less redundancy and compensation because of severe phenotypes in mice (Srichai and Zent, 2010).

Most data on the function of β1 integrin-mediated signaling in ePCs and their interaction with the ECM are based on murine models (Brakebusch et al., 2000; Chen et al., 2009; Fassler and Meyer, 1995; Grose et al., 2002; Piwko-Czuchra et al., 2009; Raghavan et al., 2000; Stephens et al., 1995). The severity of symptoms in these β1 integrin KO models very much depends on their mode of inheritance (homozygous or heterozygous), the time point when and where during development the KO becomes functionally active, and whether it is a restricted deletion (like K5 promoter-restricted (Brakebusch et al., 2000)) or a general ("constitutive") β1 integrin deletion (Fassler et al., 1995; Stephens et al., 1995). The KO of β1 integrin adapter proteins in mice, like kindlin, arose new insights in their relevance for the inside-out, as well as the outside-in mediating signaling of β1 integrin (Petzold et al., 2013). Because of their own lack of enzymatic activity β1 integrin rely on different kinases, such as FAK, ILK or kindlin, for realizing functions like platelet adhesion.

The following Table 2 include/summarize relevant insights in the significance of β1 integrin-mediated signaling by using β1 integrin KO phenotypes in mice.

**Table 2: Published β1 integrin knockout (KO) phenotypes in mice**
**Overview of β1 integrin KO phenotypes in mice which demonstrates its major role for the embryo development, but also for formation of the skin including skin appendages (such as hair growth, basement membrane assembly or hemidesmosome formation) and wound healing (migration).**

| β1 integrin KO mice variant | Phenotypes | Reference |
|---|---|---|
| null mutation in the gene of the β1 integrin subunit in mice | β1 integrin-deficiency causes lethality shortly after embryo implantation | Fässler and Meyer, 1995 |
| null mutation in the gene of the β1 integrin subunit in mice | β1-null phenotype results in early postimplantation lethality in utero | Stephens et al., 1995 |
| KC-restricted deletion of the β1 integrin (K5) | mice show a progressive loss of hair, malformations of the HFs, a hyperthickened epidermis, a reduction of hemidesmosomes, a defective BM at the dermal–epidermal junction, blister formation, inflammation and dermal fibrosis | Brakebusch et al., 2000 |
| β1 conditional knock out mice | β1 mutant mice exhibit severe skin blistering and hair defects, accompanied by massive failure of BM assembly/organization, hemidesmosome instability, and a failure of hair follicle KCs to remodel BM and invaginate into the dermis | Raghavan et al., 2000 |
| K5β1-null mice | loss of β1 integrins in KCs caused a severe defect in wound healing; β1-null KCs showed impaired migration and were more densely packed in the hyperproliferative epithelium; proliferation rate was not reduced in early wounds and even increased in late wounds; β1-deficient epidermis did cover the wound bed, but the epithelial architecture was abnormal | Grose et al., 2002 |
| conditional (oral mucosa-specific) β1 integrin KO mice | severe disruption of the BM of the tongue epithelium and developing tooth buds; Primary KO in oral KCs showed defective cell spreading and robust focal adhesions | Chen et al., 2009 |
| *hpm*$KI^{lox}/\beta1^{fl}$, $\beta1^{fl/+}$ or $\beta1^{fl+}$/K5Cre | hypomorphic β1 integrin mice developed similar, but less severe defects than mice with β1-deficient KCs; upon aging these abnormalities were attenuated due to a rapid expansion of cells | Piwko-Czuchra et al., 2009 |
| $\beta1^{TTAA/-}$ (missing Kindlin-3 binding site) and $\beta^{Hpm/-}$ mice | reduced levels of β1 integrins or an activation-deficient β1 integrin show strongly reduced platelet adhesion to collagen *in vitro* and in a carotis ligation model *in vivo;* hypomorphic mice expressing only 3% of β1 integrins on platelets have normal bleeding times even with reduced platelet adhesion | Petzold et al., 2013 |

Mainly in tumor progression there is a clinical interest to analyze the β1 integrin function and reduction, thus many KO or silencing studies are done in cancer cell lines (Beaty et al., 2013; Gama-de-Souza et al., 2008; Walsh et al., 2009). Silencing experiments with the usage of specific siRNA against β1 integrin have elucidated, for example, an elementary role of this receptor for the formation of invadopodia by actin polymerization (abrogation of Arg-dependent cortactin phosphorylation) and matrix degradation in metastatic tumor cells (Beaty et al., 2013), as well as their fundamental role for adhesion and migration mainly in the invasion of cancer cells (Walsh et al., 2009).

Like I mentioned above β1 integrin achieve prosurvival signaling after mechanical stress, ionizing radiation or genotoxic injury which impede anticancer therapies. This was clarified by using specific siRNA against β1 integrin in the transformed cell line A-172 (glioma cells) (Cordes et al., 2006).

However, β1 integrin silencing in intact mammalian tissues *in vitro*, namely in human tissue organ culture, has not yet been achieved.

### 1.6.2. Specific extracellular binding effectors of β1 integrin

As explained above, integrin conformation changes result in modifications of receptor activity and thus can affect the functions of this receptor (Campbell and Humphries, 2011; Hu and Luo, 2012; Widmaier et al., 2012). Such confirmation changes have been analyzed with monoclonal antibodies (mAbs) that detect conformation-dependent epitopes (see Figure 14).

Some mAbs binding β1 integrins that recognize ligand-induced receptor binding sites stimulate the receptor activity. This is possible by stabilizing the ligand-occupied conformation of the integrin (Mould et al., 1996), by inducing the clustering of cell-surface integrins, and the preferential localization of β1 integrins expressing the epitope at cell-cell adhesion sites (Whittard and Akiyama, 2001), like the β1 integrin-activating antibody, 12G10. Besides its stimulatory effect on ligand binding a differentially modulation of α4β1 and α5β1 adhesion was shown by analyzing cell spreading properties. This dual functionality of 12G10 is a unique feature as other activating β1 integrin antibodies, such as TS2/16, displayed no effects on α4β1 and α5β1. Studies like this were able to demonstrate structural specificities such as that the extracellular β-propeller domain of the α-subunit causes the agonistic differences between these different integrins (Humphries et al., 2005).

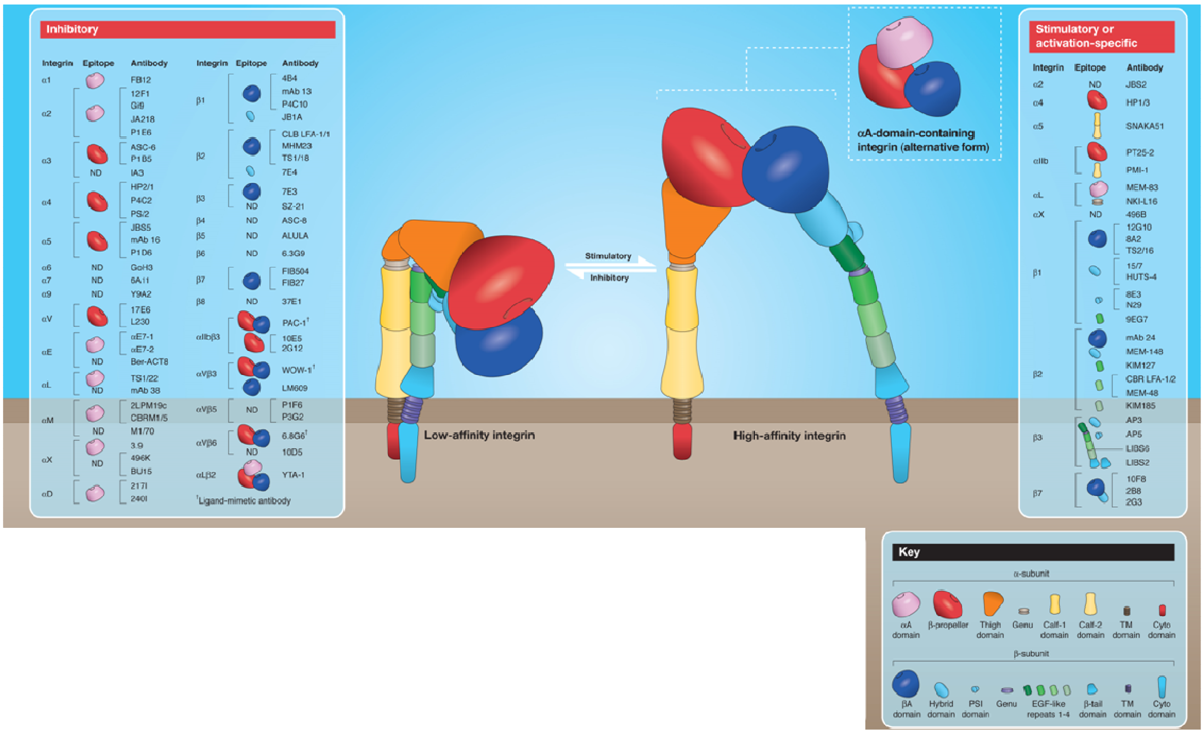



**Figure 14: Anti-integrin monoclonal antibodies**
Schematic drawing of low- and high-affinity integrins with their binding sites for specific stimulatory or inhibitory antibodies (copied from Byron et al., 2009). Specific inhibitory and stimulatory/activation-specific antibodies were designed, which identify different conformation epitopes on the diverse structural components of the heterodimer. Some of them are able to influence the specific receptor function, such as β1 integrin-binding antibodies mAb13 and 12G10 (Humphries et al., 2005; Kloepper et al., 2008a; Mould et al., 1996; Mould et al., 1995).

In contrast, the mAb13 antibody acts as a functional inhibitor of cell spreading and attachment on matrices like laminin or fibronectin, but also inhibits both Akt/PKB and FAK activities and stabilizes the ligand-unoccupied state of β1 integrin (Akiyama et al., 1989; Castello-Cros et al., 2009; Mould et al., 1996; Mould et al., 1995; Strobel and Cannistra, 1999; Veevers-Lowe et al., 2011). These studies proved the specific function of mAb13 as an allosteric inhibitor in cell culture, which leads to a displacement of the ligand and results in the loss of cell adherence. Furthermore the anti-β1 integrin mAb13 inhibits the glandular differentiation of SW1222 cells (61%) and their cellular binding to type I collagen (60%) (Pignatelli et al., 1992).

These activating 12G10 and inhibitory mAb13 antibodies are of particular interest in the current context, since they had already been shown to modulate hair shaft production, anagen duration and hair matrix KC proliferation in organ-cultured human scalp HFs (Kloepper et al., 2008a). These studies had provided the first direct demonstration that β1 integrin-mediated signaling is functionally important for the biology of a complex epithelial human (mini-) organ. Thus, using these specific receptor antibodies β1 integrin-mediated signaling functions and effects can be instructively examined.

### 1.6.3. Specific intracellular binding effectors of β1 integrin

Besides extensive progression in understanding the integrin signaling by using specific antibodies against this receptor, the examination of downstream proteins of the integrin-mediated signaling cascades (Figure 3B) is of fundamental interest. The absence of catalytic function of the β1 integrin receptor caused the necessity of intracellular binding integrin adaptor proteins, like talin, integrin-linked kinase (ILK), focal adhesion kinase (FAK), PINCH or kindlin for the β1 integrin signaling. By β1 integrin KO experiments in mice or transformed cells and by the inhibition or overexpression of specific intracellular binding proteins the inside-out or the downstream outside-in signaling of β1 integrin was investigated (Eke et al., 2009; Montanez et al., 2008; Petrich, 2009; Wedel et al., 2011).

In this context, ILK represents a key signal transduction molecule in the β1 integrin-mediated signaling cascade and a main target for manipulating the downstream signaling of this receptor by stimulation or inhibition. ILK is a key adaptor protein that interacts with the cytoplasmic domains of β1 and β3 integrins and regulates many cellular processes by connecting β1 integrin with other regulatory and adaptor proteins like Pinch, α- and β-parvins (Hannigan et al., 1996; Lange et al., 2009; Leyme et al., 2012; Wickstrom et al., 2011) (Figure 3B). Due to the important role of ILK as a potential therapeutic target in oncogenesis several studies demonstrated an efficient *in vivo* inhibition of ILK using antisense oligonucleotides or siRNA targeting ILK as well as small molecule inhibitors such as KP392, QLT0254, QLT0267 or other chemical compounds. Their application decreased the tumor growth in animal models of different cancer types such as of prostate cancer, pancreatic cancer, melanoma or breast cancers (Kalra et al., 2013; Kalra et al., 2009; Lee et al., 2011; Wong et al., 2007; Yau et al., 2005).

Among these, QLT0267 is of particular interest. It represents a putative, specific second-generation, synthetic ILK inhibitor, which is derived from KP-392, but more potent than the latter and highly selective over different kinases (Kalra et al., 2013). By blocking the ATP binding QLT0267 it seems to inhibit the kinase activity of ILK (Lim et al., 2013; Widmaier et al., 2012). However, the kinase activity of ILK is controversially discussed as some studies in mice and cell cultures suggest that this protein is a pseudokinase and functions only as an essential scaffold protein, rather than as a true kinase (Lange et al., 2009; Wickstrom et al., 2009; Wickstrom et al., 2011).

## 1.7. β1 integrin as a therapeutic target

Due to their strong impact on numerous different signaling pathways and their ubiquitous tissue expression (Cox et al., 2010) it is not surprising that abnormal signaling via various integrins is implicated in a wide range of pathological conditions ranging from, e.g., inflammatory diseases and pathological platelet aggregation via tumor progression to osteoporosis and macular degeneration (Lahlou and Muller, 2012; Lundberg et al., 2006; Millard et al., 2011; Schaffner et al., 2013).

Namely, β1 integrin has been implicated in autoimmune thyroid disorders, fibrocontractive diseases, cutaneous sclerosis and numerous different cancers, including mammary tumor, melanoma or lung cancer (Bredin et al., 1998; Kupper and Ferguson, 1993; Lahlou and Muller, 2012; Liu et al., 2009; Marazuela et al., 1997; Schaffner et al., 2013). In the last years the development of β1 integrin antagonists against tumor progression increased, via their angiogenic potential and their ability for migration or changing adhesion. Antagonists like specific antibodies, small peptides or small non peptidic RGD-like molecules binding to α5β1 are analyzed and explored up to the phase II clinical trial (Schaffner et al., 2013).

Exploration of the function of integrins in cell-ECM interactions in skin is of particular medical relevance because of the wide variety of benign and malignant skin diseases, in which β1 integrin signaling has been implicated (Watt and Fujiwara, 2011). For example, skin scleroderma was determinded in mice and elucidated the particular relevance of β1 integrin in the development of cutaneous sclerosis (Liu et al., 2009). Furthermore mice with a fibroblast-specific deletion of integrin β1 demonstrated an abnormal wound closure accompanied by less granulation tissue formation (Liu et al., 2010). These findings indicate the fundamental role of β1 integrin-mediated signaling for an efficient migration, adhesion and ECM arrangement/composition.

In this context, the following PhD thesis take up the challenge to resolve the significance of β1 integrin in human wound healing by using a model of wounded organ-cultured human skin.

For the targeting of integrins their transmembrane structure permits a good cell "surface accessibility and 'drugability'" (Millard et al., 2011). However, the advantages of integrins as therapeutic targets are tarnished as most diseases with a possible involvement of β1 integrin are multifactorial, where integrins are only one of many types of receptors involved. Furthermore cells express multiple different integrins (Cox et al., 2010). Based on the knowledge of activation, ligand binding, focal adhesion formation or cytoskeletal contacts, which determines the functionality of integrins, the most promising target site is at or near the receptor binding site (Millard et al., 2011).

The development of new pharmacological substances against integrins pursues 3 different approaches: the inhibition of ligand-binding, the blocking of downstream signaling components and the modulation of integrin expression (Cox et al., 2010). Until now many different potential strategies or compounds were described, nevertheless because of the complexity and diversity of integrins an effective drug development represents a huge challenge. One of the main problems constitutes that more than one integrin pathway is involved in diseases like the e.g. cancer progression, but mostly compounds target only a single integrin (Sawada et al., 2012). Thus, a detailed knowledge concerning the activation mechanism and structural features of distinct integrins and their functions in complex human tissue *in situ* is very much needed.

Taken together no integrin inhibitors have shown favourable results so far, but integrin-targeted therapies represent a promising approach for further clinical investigation (Sawada et al., 2012). Namely, the striking modulatory effects that are achievable with β1 integrin activating/inhibitory antibodies on the growth of human HFs *in vitro* (Kloepper et al., 2008a) document that well-targeted and clinically relevant manipulations of human tissue function are possible in principle.

## 1.8. Objectives and specific questions addressed

The role of β1 integrin-mediated signaling in ePC maintenance and differentiation in human HFs *in situ* remains to be clarified, especially since the bulge region of human scalp HFs does not express markedly more β1 integrin protein than other regions of the basal layer of the outer root sheath (ORS) (Kloepper et al., 2008a; Kloepper et al., 2008b).
Since the laboratory in which this thesis was performed had already documented a functionally important role of β1 integrin-mediated signaling in human HF growth (Kloepper et al., 2008a). Here organ-cultured scalp HFs were utilized as an easily accessible and clinically relevant human mini-organ that represents a prototypic neuroectodermal-mesodermal interaction system in which various ePC populations can be studied within their natural tissue habitat. For this, one major challenge that had to be met by the current thesis project was to develop a modified human HF organ culture system in which the interactions of ECM with ePCs from the basal layer of the HF ORS could be analyzed.

Using this novel assay system for exploring the functional roles of β1 integrin-mediated signaling in a compact human epithelial tissue *in situ*, I specifically wished to elucidate the impact of manipulating the outside-in signaling of β1 integrin on human ePC survival, proliferation, migration and adhesion.

In addition, this thesis attempted to clarify the importance of β1 integrin-mediated signaling in epithelial tissue homeostasis and repair in experimentally wounded human skin.

Therefore, the following specific questions were addressed:

1. Does β1 integrin-mediated signaling modulate maintenance, proliferation/apoptosis, migration and/or differentiation of $K15^+$ versus $K15^-$ ePCs and their progeny within the human HF?

2. Does β1 integrin-mediated signaling impact on the HF bulge immune privilege?

3. Does manipulation of the outside-in signaling of β1 integrin via specific ligands (ECM components or β1 integrin activating/inhibiting antibodies) or inhibiton of the intracellular binding kinase ILK also permit new insights into the outside-in signaling of integrins in human epithelial cells *in situ*, which is much less well-understood compared to inside-out signaling?

4. Does a selected cocktail of ECM components that mimics some characteristics of HF mesenchyme (Matrigel®/collagen I) improve cellular outgrowth or exists there any differences in the epithelial outgrowth with respect to different antibodies or different regions of the dispase-pretreated, embedded HFs?

5. Do the effects of this ECM cocktail respectively of β1 integrin activation/inhibition on HF epithelial outgrowth differ between distinct regions of the human HF epithelium?

6. Does β1 integrin-mediated signaling modulate the reepithelization of experimentally wounded human skin *in vitro*?

## 1.9. Experimental design

To evaluate the role of β1 integrin-mediated signaling in ePCs in their intact human HF tissue habitat, including the bulge SC niche, full-length human scalp HFs were microdissected from excess skin obtained during elective plastic surgery procedures and organ-cultured in well-defined, serum-free medium. β1 integrin was silenced by transient transfection with β1 integrin specific siRNA or scrambled oligonucleotides.

In addition, dispase-pretreated organ-cultured adult human scalp HF epithelium (i.e. in the absence of normal HF mesenchyme) was embedded into a mixture of Matrigel®, a latter which is rich in ECM components that are also found in the HF's CTS and BM like laminin or collagen IV (Kleinman et al., 1982; Villa-Diaz et al., 2012) and collagen I. The further manipulatin of the outside-in signaling of β1 integrin were effected in the absence or presence of specific, activating or inhibiting β1 integrin antibodies (kindly given by Prof.

Humphries ) or the putative pharmacological inhibitor of ILK activity QLT0267 (Eke et al., 2009; Kalra et al., 2009; Lim et al., 2013; Wang et al., 2010).

Besides the exploration of the influence of β1 integrin-mediated signaling on the ePC within human HFs, a "punch-within-a-punch" wound healing model of organ-cultured human skin (Meier et al., 2013) was employed to probe whether β1 integrin is involved in the reepithelization of wounded human epidermis. Therefor I quantified the degree of reepithelization by measuring the evolving area and length of the epithelial tongue (ET).

For testing the effect of different ligands on the survival, proliferation, maintenance, differentiation and/or migration of distinct human ePC subpopulations *in situ* several methods/analysis on the gene (qRT-PCR) and protein expression (quantitative immunohistomorphometry, western blot) level were done. Here, was on the analysis of the following markers concentrated: specific ePC marker, like K15, CD200, CD71 and K6 as well as proliferation and apoptosis marker, like Ki-67, EdU, TUNEL and cleaved caspase 3. For the characterization of β1 integrin-mediated migration cortactin serve as a suitable target.

Moreover for the assessment of the outgrowth of HF epithelium within the artificial matrix as well as the further immunohistomorphometry of distinct proteins (Matrigel® + collagen I) new application methods for the measurement (area and largest outgrowth points) as well as the cutting of these embedded HFs were established by myself.

# 2. Material and Methods

## 2.1. Material

### 2.1.1. Reagents/Chemicals

| | |
|---|---|
| Ammoniumpersulfate | *Sigma-Aldrich Chemie, Schnelldorf, Germany* |
| Bovine Serum Albumin | *Carl Roth, Karlsruhe, Germany* |
| Collagen (rat tail) | *Cell Systems, Troisdorf, Germany* |
| Control siRNA (FITC Conjugate)-A | *Santa Cruz (sc-36869), Santa Cruz, USA* |
| Dispase | *Gibco™ (Invitrogen) Corporation* |
| Dimethyl sulfoxide | *Merck, Darmstadt, Germany* |
| Dry milk powder | *Carl Roth, Karlsruhe, Germany* |
| Ethanol | *Th.Geyer, Renningen, Germany* |
| Eukitt® | *Kindler GmbH, Freiburg, Germany* |
| Faramount | *DAKO, Hamburg, Germany* |
| Fluoromount-G | *Biozol, Eching, Germany* |
| Glutamine | *Invitrogen, Karlsruhe, Germany* |
| Glycerol | *Sigma-Aldrich Chemie, Schnelldorf, Germany* |
| Glycine | *Sigma Life Science, USA* |
| Hydrocortisone | *Sigma-Genosys, USA* |
| Insulin | *Sigma-Genosys, USA* |
| Integrin β1 siRNA (h) | *Santa Cruz (sc-35674), Santa Cruz, USA* |
| Lämmli buffer | *Sigma-Aldrich Chemie, Schnelldorf, Germany* |
| Matrigel | *BD Biosciences, New Jersey, USA* |
| Meyers hemalun | *Merck, Darmstadt, Germany* |
| Sodium chloride | *J.T. Baker, Avantor Performance Materials B.V., Deventer, NL* |

| | |
|---|---|
| Periodic acid-Schiff reagent | *Merck, Darmstadt, Germany* |
| Polyacrylamid | *Sigma-Aldrich Chemie, Schnelldorf, Germany* |
| Propanol | *Merck, Darmstadt, Germany* |
| Sodium dodecyl sulfate | *Carl Roth, Karlsruhe, Germany* |
| siRNA Transfection medium | *Santa Cruz (sc-36868), Santa Cruz, USA* |
| siRNA Transfection reagent | *Santa Cruz (sc-29528), Santa Cruz, USA* |
| Temed | *Sigma-Aldrich Chemie, Schnelldorf, Germany* |
| Tissue Tek | *Fisher Scientific, Schwerte, Germany* |
| Triton X-100 | *Carl Roth, Karlsruhe, Germany* |
| TRIreagent | *Applied Biosystems, Warrington, UK* |
| TRIZMA® base | *Sigma Life Science, USA* |
| Tween 20 | *Carl Roth, Karlsruhe, Germany* |
| RNase-free DNase-1 | *Applied Biosystems, Warrington, UK* |

### 2.1.2. Disposable laboratory material

| | |
|---|---|
| Microcentrifuge tubes (1.5 ml, 2 ml) | *Sarstedt, Numbrecht, Germany* |
| Pipette tips | *Eppendorf, Hamburg, Germany, Sarstedt* |
| Cryotubes | *Nunc, Wiesbaden, Germany* |
| Glass cover slips | *Thermo Scientific, Waltham, USA* |
| Immobilon TM-P (PVDF) | *Millipore, Billerica, USA* |
| Petri dish - 35 mm | *Greiner Bio-one, Frickenhausen, Germany* |
| - 100 mm | *Sarstedt, Numbrecht, Germany* |
| Sterile filter 0.2 µm | *Sarstedt, Numbrecht, Germany* |
| Tissue well plates (6, 48, 96) | *PAA, Pasching, Österreich* |

### 2.1.3. Kits

| | |
|---|---|
| APEX Antibody Labeling Kit | *Invitrogen, Karlsruhe, Germany* |
| Click-iT® EdU Alexa Fluor® 488 Flow Cytometry Assay Kit | *Invitrogen, Karlsruhe, Germany* |
| ECL Plus Western Blot Detection | *GE Healthcare, Buckinghamshire, UK* |

| | |
|---|---|
| High Capacity cDNA kit | *Applied Biosystems, Warrington, UK* |
| ApopTag Fluorescein *In Situ* apoptosis detection kit | *Millipore, Billerica, USA* |

### 2.1.4. Instruments

| | |
|---|---|
| Bio Photometer | *Eppendorf, Hamburg, Germany* |
| Centrifuge 5810 | *Eppendorf, Hamburg, Germany* |
| $CO_2$ incubator | *Heraeus, Hanau, Germany* |
| Cryostat | *Leica Microsystems, Heidelberg, Germany* |
| Freezer (-80°C) | *Thermo Scientific, Waltham, USA* |
| Heating block (Thermomixer®) | *Eppendorf, Hamburg, Germany* |
| Laminar air flow | *ScanLaf, Lynge, DK* |
| Microscope | *Biozero Keyence 8000, Itasca, USA* |
| Mini-Protean III cell system | *Bio-Rad Laboratories GmbH, München, Germany* |
| pH-meter | *Knick (Calimatic), Berlin, Germany* |
| Pipettes | *Eppendorf, Hamburg, Germany* |
| Scale/Balance | *Kern EW, Balingen, Germany* |
| Vortex Genie 2 | *Bender & Hobein AG, Bruchsal, Germany* |
| Staple | *Soennecken, Bonn, Germany* |

### 2.1.5. Buffers

#### 2.1.5.1. Histology/Immunohistology

**Table 3: Washing buffers**

| Tris Buffered Saline (TBS) 0.05 M, pH 7.6 | |
|---|---|
| 1x conc. | |
| 6.1 g | Tris-Base |
| 8.8 g | NaCl |
| 1000 ml | A. dest |

| Phosphate Buffered Saline (PBS) 0.05 M, pH 7.2 | |
|---|---|
| 1x conc. | |
| 1.8 g | $NaH_2PO_4*H_2O$ |
| 8 g | NaCl |
| 1000 ml | A. dest |
| Tris NaCl Tween (TNT), pH 7.5 | |
| 15.76 g | Tris-HCl |
| 8.766 g | NaCl |
| 500 µl | Tween 20 |
| 1000 ml | A. dest |

### 2.1.5.2. Western Blot

Protein isolation

**Table 4: Lysis buffer for protein isolation**

| Lysis buffer | For 1 ml Lysis buffer |
|---|---|
| 10 mM Tris-HCl pH 7,2 | 770 µl Tris-HCl |
| 2% Sodium dodecyl sulfate (SDS) | 100 µl of 20% SDS |
| 1% Triton x 100 | 10 µl Triton X 100 |
| 10% Glycerol | 100 µl Glycerol |

SDS-Gel

**Table 5: Separating gel**

| % SDS gel | 10% |
|---|---|
| A. dest | 4.8 ml |
| 30% PAA | 2.5 ml |
| TRIS buffer (1.5 M, pH 8.8) | 2.5 ml |
| 10% SDS | 0.1 ml |
| TEMED | 0.005 ml |
| 10% APS | 0.1 ml |

**Table 6: Stacking gel**

| % SDS gel | 5% |
|---|---|
| A. dest | 3 ml |
| 30% PAA | 0.625 ml |
| TRIS buffer (1.0 M, pH 6.8) | 1.25 ml |
| 10% SDS | 0.05 ml |
| TEMED | 0.003 ml |
| 10% APS | 0.05 ml |

Western blot

**Table 7: Buffers for Western blot**

| Guanidinium isothiocyanate (GIT) buffer (5x conc.) | |
|---|---|
| 15.1 g | TRIZMA® base |
| 72 g | Glycine |
| 1000 ml | A. dest |
| Electophoresis buffer | |
| 200 ml | 5x GIT buffer |
| 10 ml | 10% SDS (in A.dest) |
| 1000 ml | A. dest |
| Transfer buffer | |
| 200 ml | 5x GIT buffer |
| 200 ml | Methanol (4°C) |
| 1000 ml | A. dest |
| 10x TBS buffer | |
| 12.1 g | TRIZMA base |
| 87.7 g | NaCl |
| Fill up to 800ml for adjustment of pH 7.4, then fill up to 1000 ml | A. dest |
| Tris Buffered Saline Tween 20 buffer (TBST) | |
| 100 ml | 10x TBS buffer |
| 900 ml | A. dest |
| 500 µl | Tween 20 |

| TBST buffer milk (5%) | |
|---|---|
| 500 ml | TBST |
| 25 g | Dry milk powder |
| 500 µl | Tween 20 |

### 2.1.6. Software

Microsoft Excel, Word, Power Point

Image J

GraphPad Prism 5.01

Biozero Keyence 8000 Microscope (BZ analyzer, BZ observer)

## 2.2. Methods

### 2.2.1. Human hair follicle and skin organ culture plus ethics approval

For all experiments human scalp skin or corporal skin specimens originated anonymously from patients undergoing plastic or reconstructive surgery with written informed patient consent, Institutional Research Ethics Committee permission (University of Luebeck, license: 06-109) and according to Helsinki Declaration principles. Skin samples were delivered by overnight courier service to the laboratory from the collaborating plastic surgeons. In further, skin specimens were placed in serum-free William's E medium which was supplemented with 2 mmol/l L-glutamine, 10 ng/ml hydrocortisone, 10 µg/ml insulin and 1% antibiotic/antimycotic (Kloepper et al., 2009; Philpott et al., 1990) mixture at 4°C and were used within 24 hours after surgery.

For the isolation of human HFs I used temporal and occipital scalp skin from a total of 12 different female donors with a mean age of 50.2 years (age range:19-75 years; Table 8).

**Table 8: Description of patients skin samples employed for HF organ cultures**

| Patient Number | Sex | Age | Scalp skin location |
|---|---|---|---|
| 1[a] | female | 30 | temporal |
| 2[a] | female | 54 | temporal |
| 3[a] | female | 59 | temporal |
| 4[b] | female | 46 | occipital |
| 5[b] | female | 48 | temporal |
| 6[b] | female | 66 | occipital |
| 7[b] | female | 19 | temporal |
| 8[b] | female | 42 | temporal |
| 9[c] | female | 56 | temporal |
| 10[c] | female | 47 | temporal |

| | | | |
|---|---|---|---|
| 11[c] | female | 60 | temporal |
| 12[c] | female | 75 | temporal |

[a]whole HF culture for β1 integrin silencing, [b-c] HF epithelium embedded in Matrigel®/collagen I/K-SFM, [b]Manipulation via β1 integrin ligands and RGD-motif binding antibodies, [c]Manipulation via QLT0267

Further, a previously established "punch-in-a-punch" assay design (Moll et al., 1998) was combined with our method for full-thickness human skin organ culture assay (Bodo et al., 2010; Lu et al., 2007) to create a model of experimentally wounded human skin. These wound healing skin organ cultures were performed with skin samples of 3 female donors with a mean age of 59.3 years (aged 53-68 years) (Table 9). This assay imitated human skin wound healing conditions as far as this is possible *in vitro*; since our lab had previously shown, and it is optimally suited for studying the reepithelization of wounded human skin (Meier et al., 2013).

**Table 9: Description of patients skin samples employed for wound healing organ cultures**

| Patient Number | Sex | Age | Skin region |
|---|---|---|---|
| 1 | female | 57 | thigh |
| 2 | female | 68 | temporal |
| 2 | female | 68 | occipital |
| 4 | female | 53 | occipital |

#### 2.2.1.1. Isolation of full-length human scalp hair follicles

For the isolation of the whole, intact mini-organ I washed fresh scalp specimens in PBS and cut into thin pieces (approximately 0.5 cm) by using scalpels. These thin scalp skin pieces were placed into petri dishes and the HFs were cut out with caution to remove the surrounding tissue (Figure 15) (comparable (Yoo et al., 2007)). After one additional washing step in sterile PBS the HFs were placed in 6 well plates with 3 ml supplemented William's E and were used for later siRNA transfection.

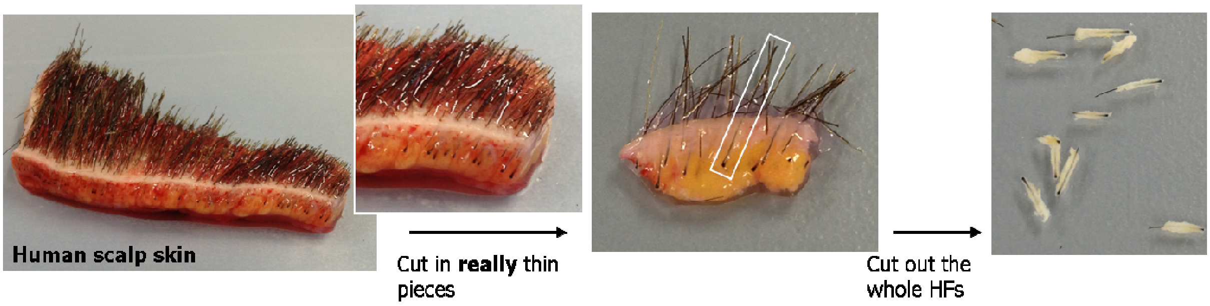



**Figure 15: Isolation of full-length hair follicles from human scalp skin.**
The illustration demonstrates the isolation of single full-length intact HFs from human scalp skin with scalpels and the washing in sterile PBS.

### 2.2.1.2. Isolation of hair follicle epithelium

Scalp skin samples were briefly washed in PBS, dissected into approximately 0.5 $cm^2$ pieces and incubated in 0.1% dispase diluted in William's E, which acts as a selective protease and digests the key BM components fibronectin and collagen IV (Link et al., 1990; Stenn et al., 1989; Tiede et al., 2009), over night at 4°C. After removing the epidermis I plucked out the HF epithelia (see Figure 16) and washed them briefly in sterile PBS. Freshly isolated, intact HF epithelium was placed in supplemented William's E for up to 1 hour until the start of the studies (i.e. embedding into ECM).

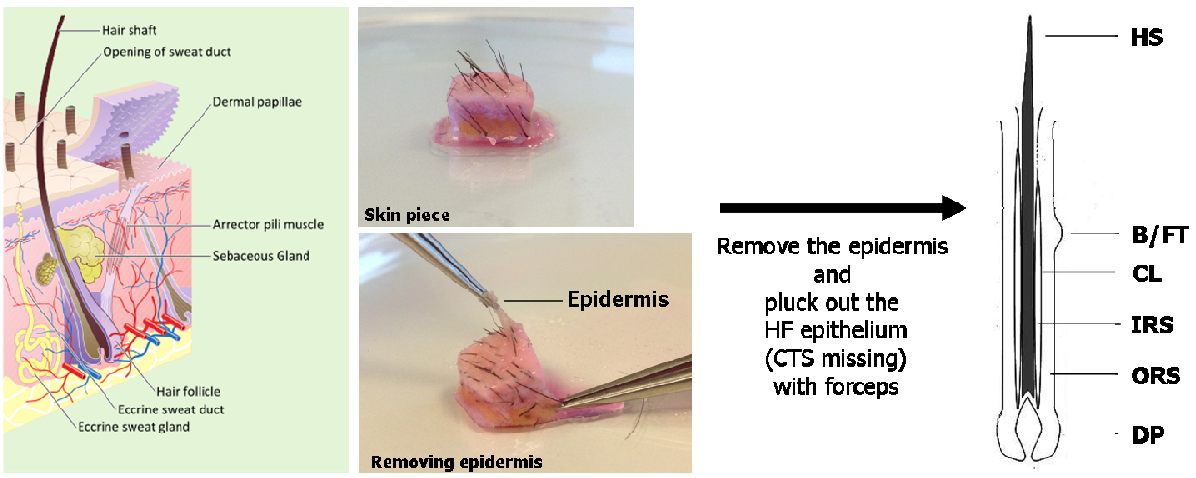



**Figure 16: Isolation of hair follicle epithelium from human scalp skin.**
It is pictured how the epidermis is removed and the HF epithelium is plucked out of human skin after the incubation over night in a 0.1% dipase solution. B/FT: Bulge/ Follicular trochanter; CL: Companion

layer; DP: Dermal papilla, E: Epidermis, HS: Hair shaft, IRS: Inner root sheath, ORS: Outer root sheath. Modified cartoon of human skin on the left is derived from "en.wikipedia.org", pictures in the middle are generated by N. Ernst, and scheme of HF epithelium on the right side is modified from Klöpper and Meyer.

### 2.2.1.3. Human skin wound healing organ culture model

Full-thickness adult human skin (including subcutaneous fat) was cultured under serum-free conditions in William's E medium supplemented with 2 mmol/l L-glutamine, 10 ng/ml hydrocortisone, 10 µg/ml insulin and 1% antibiotic/antimycotic mixture (Lu et al., 2007; Philpott et al., 1990).

First, I made 2 mm punches in the obtained skin specimens, which were followed by a wider (4 mm) punch This was located in the surrounding skin to obtain a "punch-within a punch" skin piece (Figure 17). Skin samples were frozen immediately for analysis (day 0) or transferred to six-well plates containing supplemented William's E medium.

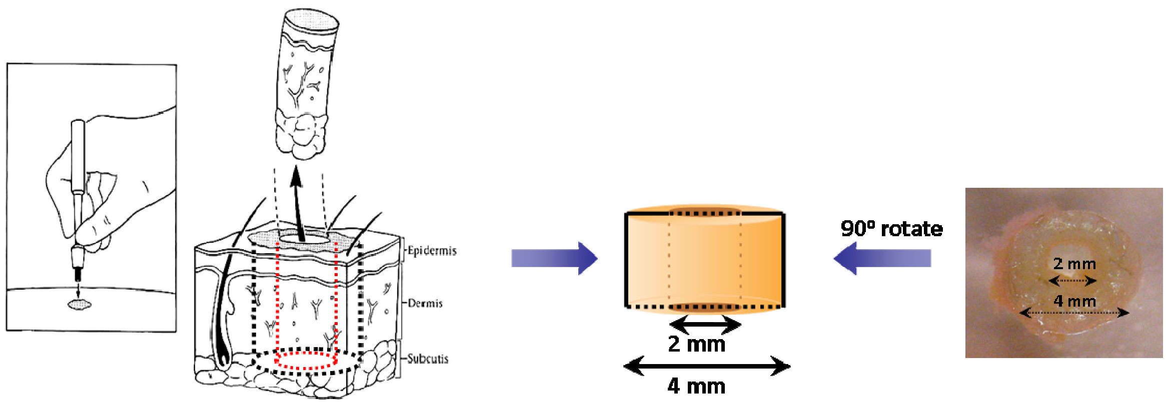



**Figure 17: Human skin wound healing organ culture model.**
Schematic drawing of the punch biopsy on skin modified from "en.wikipedia.org". Picture of human skin is modified from Zhang et al. (Paus lab, manuscript in prep.).

## 2.2.2. Gene silencing via siRNA

To knockdown β1 integrin I placed whole, intact and full-length HFs (2.2.1) in supplemented William's E medium in a 6-well plate (15 HFs per well, 90 HFs for 6 wells). For the transfection of the HFs all required reagents were prepared under the laminar flow box following the manufacturer's guidelines of Santa Cruz using a standardized method (Figure 18) (Samuelov et al., 2012; Sugawara et al., 2012).

During the incubation step of solution A and B, isolated full-length HFs were washed with 2 ml siRNA transfection medium per well to eliminate antibiotic substances, which would inhibit the transfection reaction. After 30 min 800 µl transfection medium was added to the master mix of each transfection condition (control, scrambled control, ITGβ1 KD), mixed gently and the amount of 1 ml was placed into each well. For the silencing of β1 integrin the HFs were transfected with siRNA (sc-35674, Santa Cruz), which consist of three target-specific 19-25 nt siRNAs designed to knockdown the receptor (ITGβ1 KD) (Hu et al., 2010; Teckchandani et al., 2009), scrambled FITC-labelled control RNAs (scrambled control) or transfection medium (control). Detailed information is given in Figure 18.

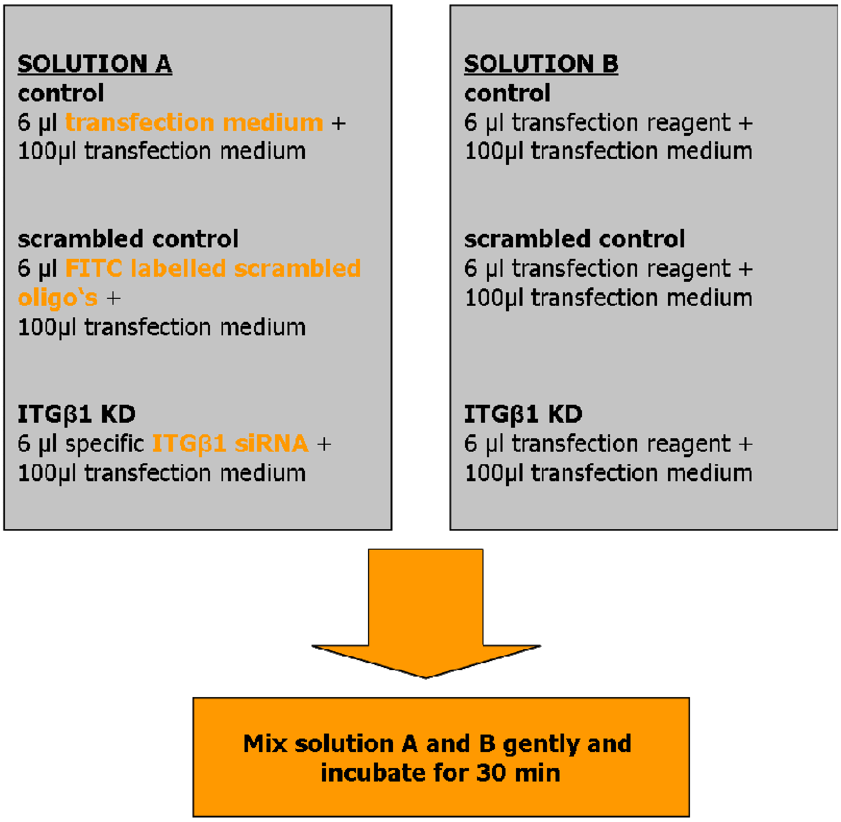



**Figure 18: Preparation of a master mix for the transfection per 1 well of a 6 well-plate.**

These HFs were incubated for 5-7 hours at 37°C in a $CO_2$ incubator to enable the transfection. After this period the transfection medium was removed and replaced with supplemented William's E medium. After 2 days of culture I refreshed the medium and at day 4 I stopped the HF culture by either embedding them into TissueTek or freezing them down in liquid nitrogen for mRNA extraction and subsequent RT-qPCR analysis (Figure 19).

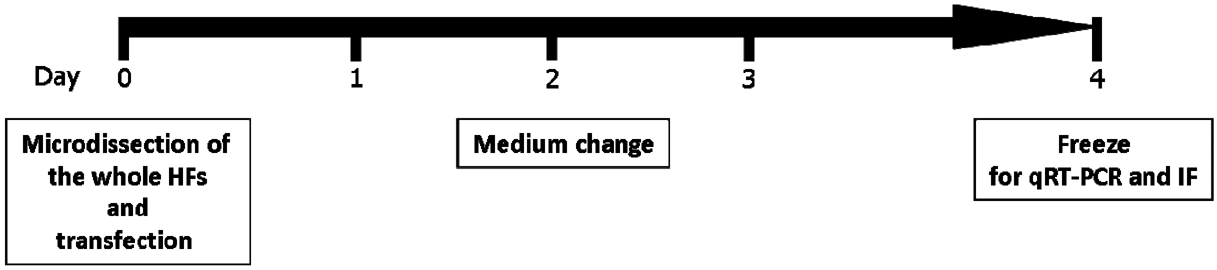



**Figure 19: Experimental design of the β1 integrin silencing using specific siRNA.**
After the isolation of intact full-length HFs (shown in Figure 9) they were devided in 3 different groups and transfected: a control (HFs treated only with the transfection medium), a scrambled control (HFs treated with scrambled FITC-labelled control RNAs) and an ITGβ1KD group (consisting of HFs transfected with three target-specific 19-25 nt β1 integrin siRNAs). During the culture supplemented William's was changed and after 4 days the HFs were frozen for further analysis (like qRT-PCR and IF).

### 2.2.3. Manipulation of the β1 integrin-mediated signaling

For a direct manipulation of the β1 integrin-mediated signaling dispase-pretreated HF epithelium of human scalp skin or human "punch-within a punch" skin specimens were used.

#### 2.2.3.1. Manipulation via β1 integrin ligands

The required lab equipment for the experiments, like sterile pipettes, tips, well-plates, tubes and Matrigel® I put in -20°C overnight. After the isolation of the HF epithelium the CTS- and BM-mimicking ECM was prepared by diluting the Matrigel®, which is rich in key β1 integrin-activating ligands like laminin, collagen type IV, but also heparin sulfate proteoglycans, entactin, and several growth factors such as transforming growth factor beta (TGF-beta), epidermal growth factor (EGF), insulin-like growth factor 1, bovine fibroblast growth factor (bFGF), and platelet-derived growth factor (PDGF) (Dias et al., 2012; Kleinman et al., 1986; Philp et al., 2005). Matrigel® was mixed with collagen I (rat tail, for greater stability) in a ratio of 1:1 under the bench on ice in K-SFM (hereafter termed "aECM" = artificial ECM consisting of Matrigel® and collagen I).

In each well of a 48-well-plate were dropped 100 µl of this cooled matrix and put into an incubator for 30 min at 37°C. After this incubation I placed the HF epithelium in the centre of each "matrix"-containing well by using forceps and covered it with additional 100 µl of Matrigel®/collagen I/K-SFM. Thirty minutes later, when the aECM was solid in the incubator with 37°C, 250 µl supplemented William’s E medium were added and all embedded HF epithelia were checked under the microscope on day 0, day 2 and day 4 to follow up the ORSK outgrowth. The whole experimental course/process is summarized in Figure 20.

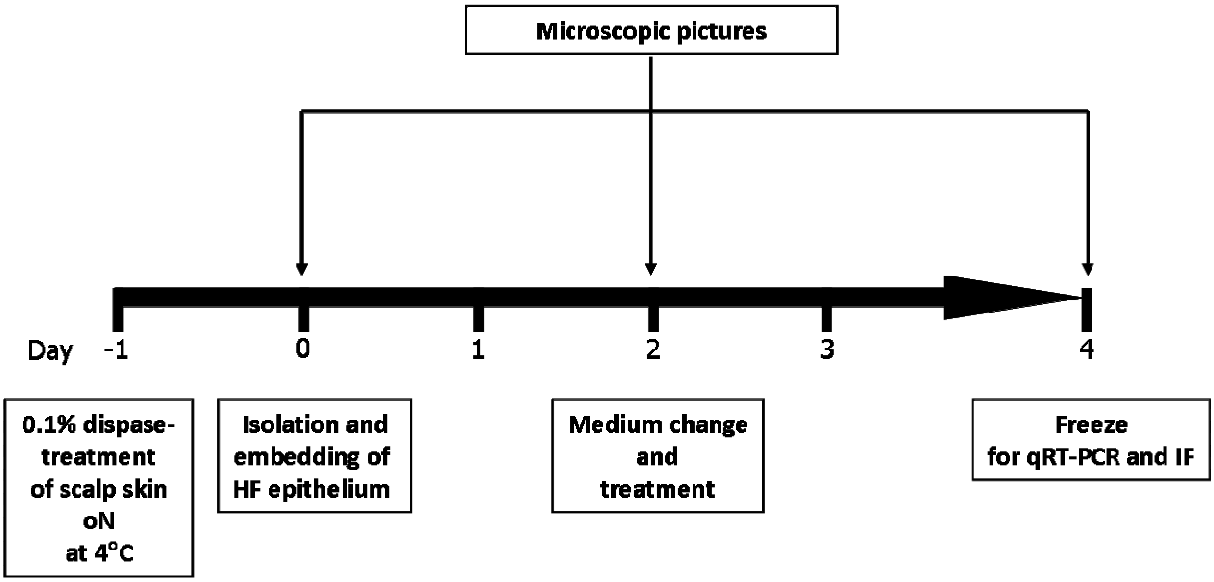



**Figure 20: Experimental design of the manipulation by highly selective β1 integrin ligands.** After the over night incubation in 0.1% dispase the HF epithelia were isolated (as shown in Figure 16) and embedded in an artificial ECM consisting of Matrigel® and collagen I (aECM). The ORSK outgrowth was follow up by checking the HF epithelia under the microscope on day 0, day 2 and day 4. During the experiment supplemented William’s E was changed and at day 4 the HFs were frozen for further analysis (like qRT-PCR and IF).

#### 2.2.3.2. Manipulation via specific RGD-motif binding β1 integrin antibodies

The dispase-pretreated “naked” HF epithelium was also embedded into the aECM (2.2.3.1), which was additional enriched with 10 µg/ml of the specific activating β1 integrin antibody 12G10 or the inhibitory β1 integrin antibody mAb13 (Kloepper et al., 2008a) and was covered during the culture with 200 µl supplemented William's E medium (see experimental design Figure 20). The specific β1 integrin antibodies were kindly given by Prof. Martin Humphries.

### 2.2.3.3. Manipulation via pharmacological inhibition of integrin-linked kinase with QLT0267

For the preparation of the QLT0267 (Dermira) stock solution 0.016 g of the integrin-linked kinase (ILK) inhibitor substance was dissolved in 2 ml DMSO and 3 ml K-SFM. I incubated twenty of the plucked-out HF fragments (HF epithelia) for 2h in 37°C in 5 ml 100 µM QLT0267 dissolved in 0.4 % DMSO/K-SFM in a petri dish (35mm). The subsequent embedding procedure of the HF epithelium was performed as delineated in 2.2.3.1, but including the additional administration of 0.4 % DMSO (control) or 100 µM QLT0267 to the aECM and the culture medium William's E. The experiment was performed as summarized in Figure 20.

### 2.2.3.4. Isolation of embedded HF epithelium for RT-qPCR

By using forceps I picked out twelve embedded and cultured HF epithelia per treatment and put them into a 50 ml Falcon tube. The residual aECM of all 12 wells was washed out twice with sterile PBS and pipetted to the collected HF epithelia in the Falcon tube to add also the ORSKs, which were migrated into the artificial matrix during culture. After a centrifugation step the pellet, consisting of the HFs and the ORSKs, was diluted in 50 µl sterile A. dest, transferred into a 1.5 ml cryo tube and centrifuged again. The resulting supernatant was discarded and the pellets were shock frozen in liquid nitrogen.

### 2.2.3.5. Cutting of embedded HF epithelium

To be able to characterize/verify different structural proteins/markers of the "manipulated" HF epithelium, a new method for cutting these cultured HFs in their artificial surrounding habitat was established. After the culture period of 4 days the embedded HF epithelia were coated with Tissue Tek. By using staples, which were put into every well with forceps, these small blocks could be retrieved for cutting after freezing at -80°C . The frozen small blocks were cut in 8 µm sections with a cryostat (Figure 21).

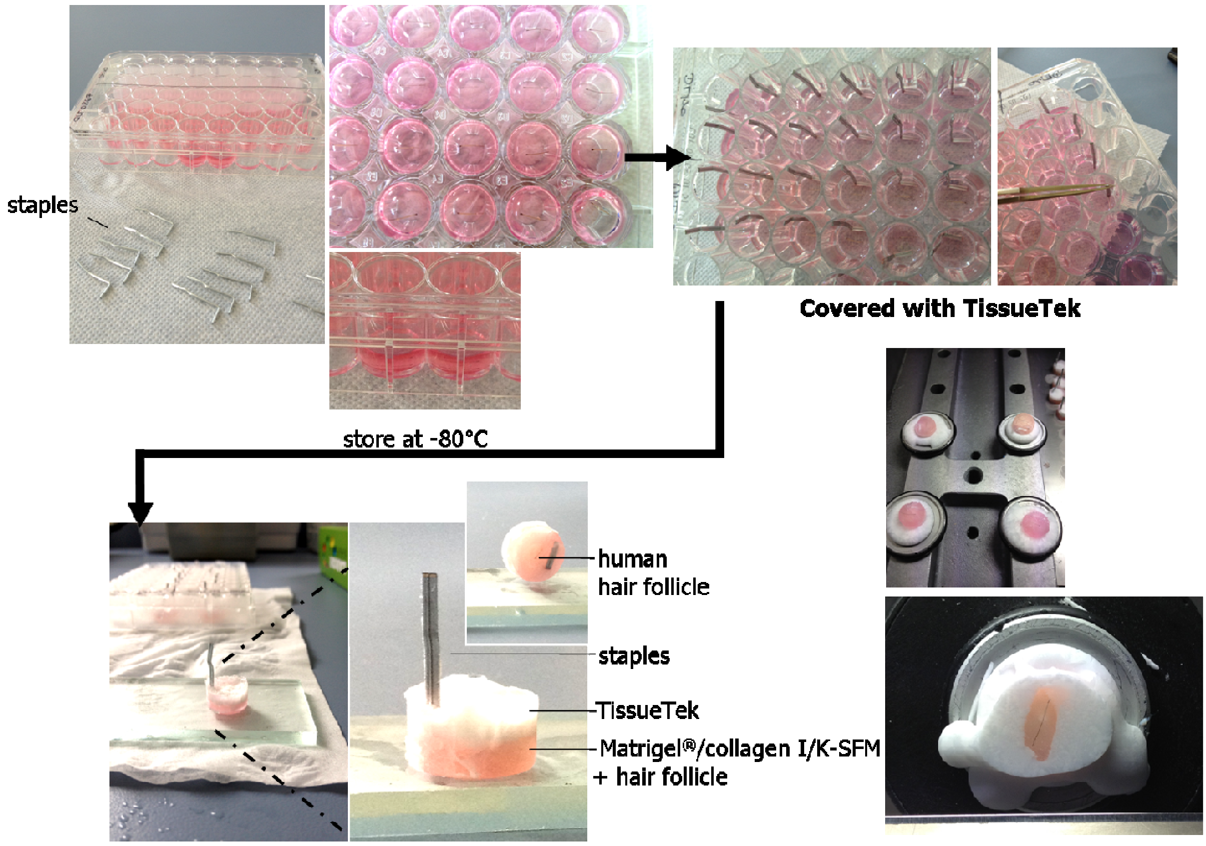



**Figure 21: How to section embedded and cultured HF epithelium after 4 days of culture (generated by N. Ernst).**
This figure depicts the establishment of a new method for cutting the HF epithelium embedded in their artificial matrix (aECM) for further analysis such as immunohistochemistry. By putting staples with forceps in every culture well, the frozen blocks consisting of the embedded HF epithelium and TissueTek were transferred into the cryostat.

#### 2.2.3.6. Manipulation of β1 integrin-mediated signaling in human skin wound healing

2 "punch-within a punch" skin samples were placed in one well of a 6-well-plate containing 3 ml supplemented William's E (vehicle control) or this culture medium additionally laced with 10 µg/ml of the specific activating β1 integrin antibody 12G10 or the inhibitory β1 integrin antibody mAb13 (Kloepper et al., 2008a).

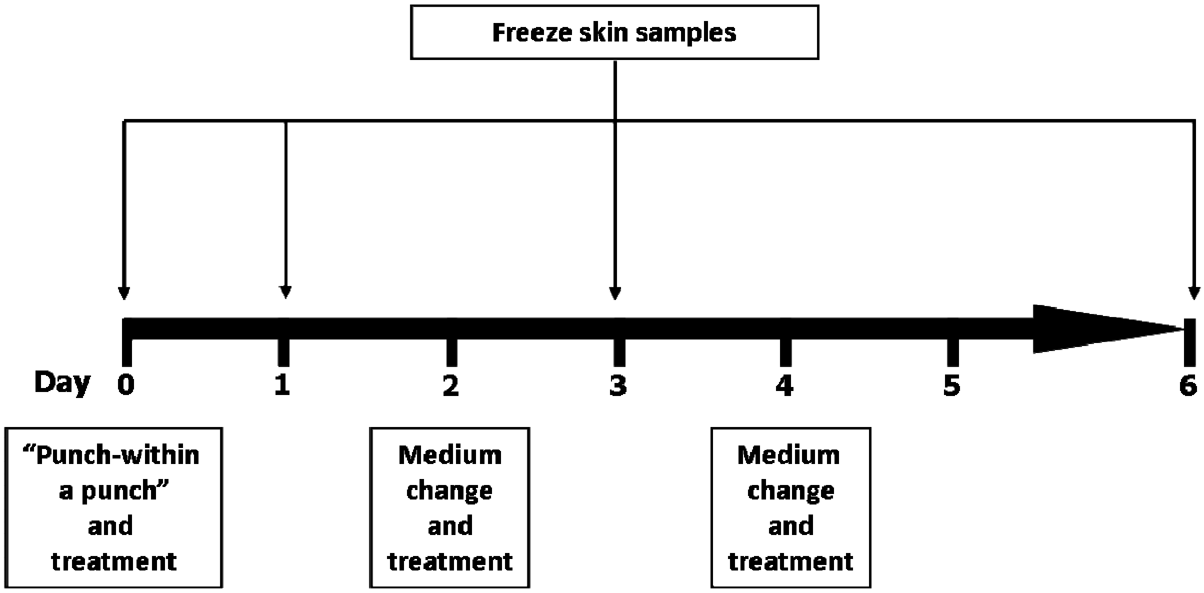



**Figure 22: Experimental design of the wound healing manipulation by given β1 integrin activating and inhibitory antibodies.**
Full-thickness human skin was punched as described befor (see 2.2.3.6) and cultured in supplemented William's E laced with or without the activating (12G10) or inhibitory (mAb13) β1 integrin antibody for 6 days. For further analysis punched skin were frozen on day 0, day 1, day 3 and day 6.

The subsequent wound healing culture was made following the experimental design shown in Figure 22. Before the specimens were frozen on day 0, day 1, day 3 and day 6 an embedding in Tissue Tek was performed for further cryostat cutting (6 µm).

### 2.2.4. Analysis of human hair follicle cultures

#### 2.2.4.1. HF epithelium outgrowth measurements

During the culture period the embedded HF epithelia were photographed every second day for the assessment of the ORSK outgrowth with the Keyence microscope. With the software program Image J **area outgrowth** of every HF was measured by surrounding the whole expanse of the ORSKs on day 0, day 2 and day 4, like presented in Figure 23. Day 0 was set as 0 % and by this the ORSK area outgrowth was analyzed.


**Figure 23: Method for measuring the ORSK area outgrowth during 4 days of culture.**
Dotted lines represent the ORSK area outgrowth.

By using the microscopic pictures of the embedded HF epithelium the **largest outgrowth** points were measured over the course of 4 days in 3 distinct HF compartments – the HF bulb, the lower HF and the upper HF (including the bulge). The calculation of the largest outgrowth points included the distance between the beginnings of the hair shaft to the widest horizontal outgrowth point (for detailed information see Figure 24). This method was designed to detect whether there is a different response of the ORSKs to the given matrix (environment).

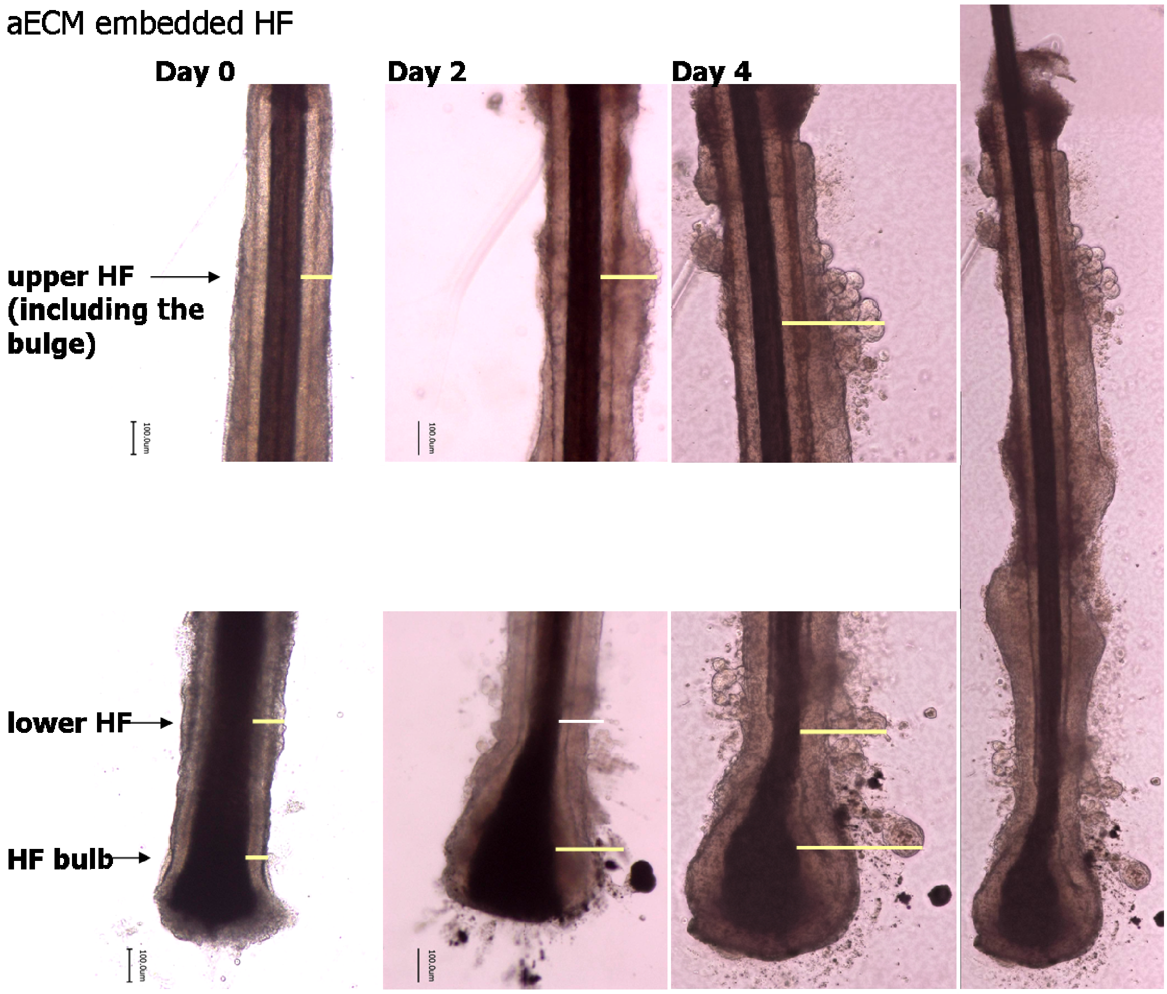



**Figure 24: Method for calculating the largest outgrowth points during 4 days of culture.** Yellow lines demarcate the measurement of the largest outgrowth points. Scale bars represent 100µm.

### 2.2.4.2. Immunohistochemical analysis in human hair follicles

**(A) Assessment of proliferating or apoptotic keratinocytes**

The assessment of proliferating and apoptotic matrix KCs was made by staining the cryosections of organ-cultured and β1 integrin silenced HFs for Ki-67/TUNEL and the further analyzing at 200x magnification. All Ki-67$^{+}$ (red) and TUNEL$^{+}$ matrix cells below the Auber's line (Auber, 1952) were counted as well as the total number of matrix KCs, stained with 4′,6-diamidin-2′-phenylindol-dihydrochlorid (DAPI, blue) (see Figure 25). The number of Ki-67$^{+}$ or TUNEL$^{+}$ cells in relation to DAPI$^{+}$ cells was given in percent.

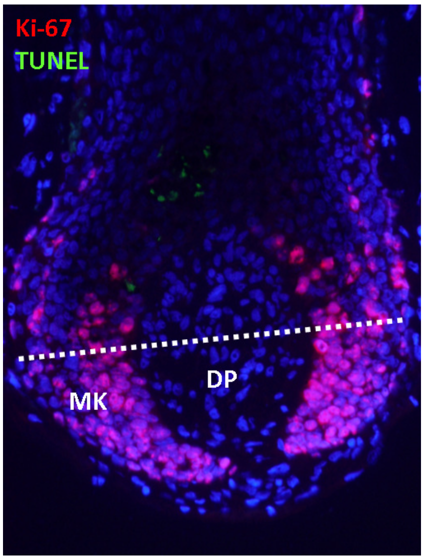



**Figure 25: Auber's line shown in a human anagen VI hair follicle.**
White dotted line defines the Auber's line, which runs through the part with the widest diameter of the HF bulb and denotes the line below most proliferation occurs within the anagen hair matrix (Auber 1952) (picture generated by N. Ernst). Abbreviation: DP = dermal papilla, MK = matrix keratinocytes.

The evaluation of proliferation and apoptosis of HF ORSKs occurred in a different manner. Cryosections of organ-cultured embedded HF epithelia, but also the β1 integrin-silenced HFs were stained for Ki-67/TUNEL and analyzed at 100x magnification. All Ki-67$^+$ (red) or TUNEL$^+$ (green) ORSKs in fixed rectangles were counted on the left and the right side as well as the total number of KCs, stained with DAPI (blue) (see Figure 26) in the HF bulb and the upper HF (including the HF bulge). The number of Ki-67$^+$ or TUNEL$^+$ cells in relation to DAPI$^+$ cells was also given in percent.

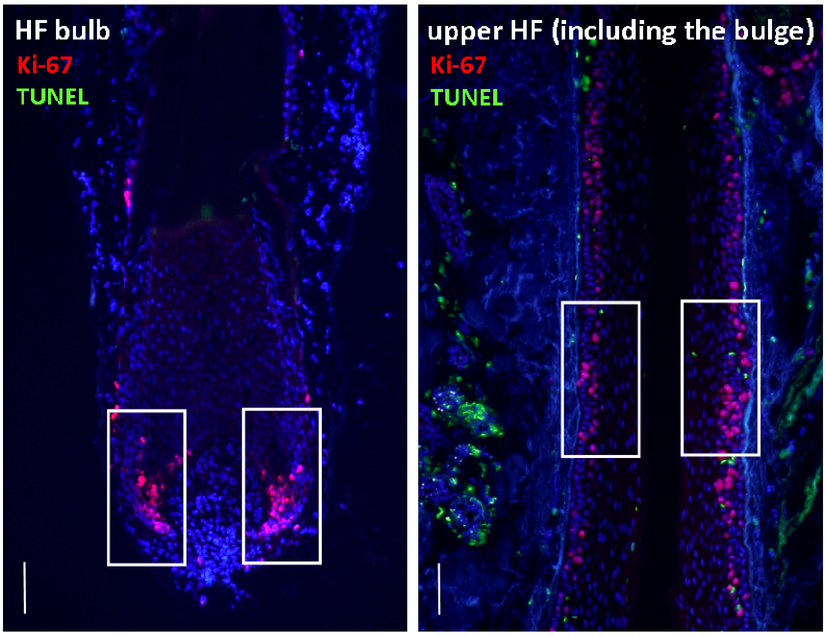



**Figure 26: Reference areas for counting of proliferating and apoptotic ORSKs (quantitative immunohistomorphometry).**
Ki-67$^+$ and TUNEL$^+$ ORSKs were counted in fixed rectangles in the hair follicle bulb and the upper hair follicle (including the bulge) and analyzed at 100x magnification, scale bars = 100µm (generated by N. Ernst).

In addition, the TUNEL results of embedded HFs, which was pharmacologically manipulated by QLT0267, were compared with a complementary, independent method for apoptosis detection. The quantitative immunohistomorphometry of cleaved caspase 3, a cysteine-aspartic acid protease, should confirm the initiation of apoptosis after ILK inhibiton by QLT0267. This assessment was made in the same manner as the evaluation of TUNEL$^+$ ORSKs within the whole embedded HFs. By using fixed rectangles in the HF bulb and upper HF cleaved caspase 3$^+$ cells were counted and and the percentage values given in relation to DAPI$^+$ cells.

### (B) Assessment of immunostaining intensity

The immunoreactivity (IR) intensity of all analyzed parameters was measured in fixed rectangles on the left and the right side of the HF bulb and the upper HF (including the bulge) with Image J software (National Institutes of Health, Bethesda, MD) followed by the normalization of the control to 100%.

## 2.2.5. Analysis of wounded skin

### 2.2.5.1. Assessment of reepithelization

**(A) Assessment of the area and the length of the ET**

For a quantitative evaluation of the reepithelization periodic acid-Schiff (PAS) stained skin sections were used to analyze defined areas of the epithelial tongue (outer and inner epithelial tongue [ET]). PAS stained skin offered the possibility to visualize the basal membrane and by this to measure the length and the area of the newly formed ET (see Figure 27 for details) by using the Image J software (National Institutes of Health, Bethesda, MD).

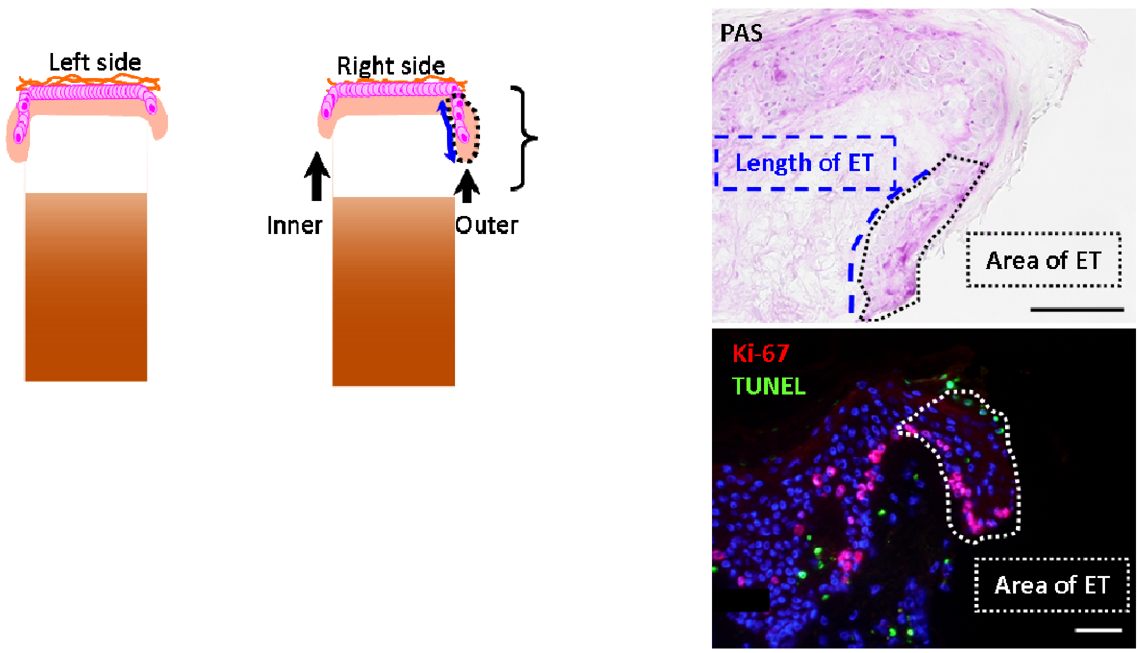



**Figure 27: Assessement of reepithelization in wounded epidermal skin.**
Epithelial tongue (ET) area was measured in the reference area marked with blacked dotted line and ET length was calculated marked with the blue line in PAS stained skin sections. The proliferation or apoptosis were evaluated in the ET of wounded skin by counting Ki-67$^+$ or TUNEL$^+$ in relation to DAPI$^+$ cells (right pictures of ET generated by N. Ernst, scale bars = 50µm). Left cartoon modified after Zhang et al. (Paus Lab, manuscript in prep.).

**(B) Assessment of apoptotic/proliferating cells (Ki-67/TUNEL)**

Already published quantitative immunomorphometrical techniques described the analysis (Gaspar et al., 2009; Holub et al., 2012) of the number of apoptotic (TUNEL) and proliferating (Ki-67) cells in the newly generated human wound epithelial tongue (ET) *in situ*.

Cryosections of organ-cultured, wounded skin were stained for Ki-67/TUNEL and further analyzed at 100x magnification. All Ki-67$^{+}$ (red) or TUNEL$^{+}$ (green) cells in the ET of the outer and inner wound edges were counted as well as the total number of KCs, stained with DAPI (blue) (see Figure 27). The number of Ki-67$^{+}$ or TUNEL$^{+}$ cells in relation to DAPI$^{+}$ cells was also given in percent.

**(C) Assessment of immunoreactivity intensity**

The IR of the analyzed read-out parameters were measured by using the Image J software in the ET as described previously (Knuever et al., 2012). All IR intensities were normalized to 100% of the control.

#### 2.2.5.2. Migration analysis *in situ*

For the reepithelialsation of wounded skin migrating KCs play a major role. Therefore, the skin sections were analyzed for cortactin, an F-actin associated protein and a substrate of the Src kinase, as a sensitive marker for migration (Ceccarelli et al., 2007; Gendronneau et al., 2008; Wang et al., 2011b).

### 2.2.6. Molecular biological methods

#### 2.2.6.1. Nonretroviral transfection of a *K15* promoter GFP expressing plasmid

Isolated HF epithelium were transfected, as described previously by Tiede et al. (2009), with a *K15* promoter GFP expression construct by using lipofectamin (Invitrogen) and were afterwards embedded into Matrigel® (diluted in K-SFM). The successful transfection of HFs were evaluated in comparison to a vehicle control (only treated with lipofectamin) after 24 h by a microscopically checking of the direct immunofluorescence of the K15 promoter–driven expression of GFP (excitation maximum ¼ 475 nm; emission maximum ¼ 505 nm).

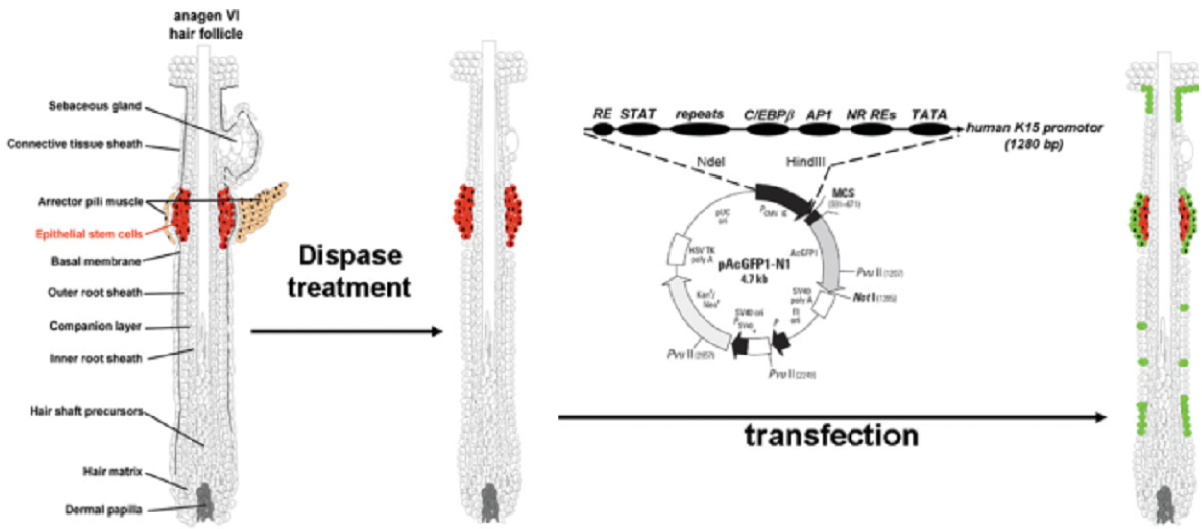



**Figure 28: *K15* promotor-driven GFP expression in ePCs *in situ* by non-viral transient transfection of human HF epithelium (picture copied from Tiede et al., 2009).**
Human scalp HF epithelia were isolated after a dispase-pretreatment and transfected with a plasmid consisting of the *K15* promotor driven GFP/geneticin-resistant expression system (Tiede et al., 2009). By the transfection K15$^+$ keratinocyte population could be visualized within the cultured and embedded HFs and followed microscopically *in situ*.

The embedded and transfected HF epithelium were cultured for 4 days and microscoped every second day to follow up the outgrowth and behaviour of the K15-GFP$^+$ cell population in the surrogate environment (Matrigel®).

### 2.2.6.2. Primers for qRT-PCR

**Table 10: Primers**

| Target Gene Abbreviation | Target Gene | Size of Amplicon | Assay IDs of Applied Biosystems |
|---|---|---|---|
| *GAPDH* | Glyceraldehyde 3-phosphate dehydrogenase (housekeeping gene) | 124bp | Hs99999905_m1 |
| *ACTB* | β-Actin (housekeeping gene) | 171bp | Hs99999903 |
| *PPIA* | Peptidylprolyl-isomerase A (housekeeping gene) | 98bp | Hs99999904 |
| *ITGB1* | β1 integrin | 75bp | Hs00559595_m1 |
| *Krt6* | Keratin 6 (keratin expressed by ORS KCs and wounded/hyperproliferative epidermis) | 83 bp | Hs01699178_g1 |
| *Krt15* | Keratin 15 (ePC marker) | 81 bp | Hs00267035_m1 |
| *CD71* | Transferrin Receptor (ePC marker) | 66bp | Hs00951083_m1 |
| *CD200* | “no danger” signal expressed by immunologically privileged ePCs | 64bp | Hs01033303_m1 |

### 2.2.6.3. QRT-PCR

All qRT-PCR reactions were performed in a collaborating laboratory (Prof. T. Bíró, DE-MTA “Lendulet” Cellular Physiology Group, Department of Physiology, University of Debrecen, Debrecen, Hungary) under well-standardized conditions, while all cultures, RNA isolations, quantifications and data analysis were performed by the PhD candidate.

The total RNA of 12 HFs per condition, cultured in supplemented William's E medium or the ECM assay was extracted by using TRIreagent (Applied Biosystems/Life Technologies) and digested with recombinant RNase-free DNase-1 (Applied Biosystems) to remove interfering DNA according to the manufacturer's protocol. One µg of total, isolated RNA was reverse transcribed into cDNA with High Capacity cDNA kit (Applied Biosystems) following the manufacturer's protocol.

For the qRT-PCR analysis 12 knockdowns HFs or 12 embedded HFs were isolated, placed in a 1 ml cryo tube and shock frozen in liquid nitrogen. Theses samples were sent to our collaborating laboratory where qRT-PCR analysis of different genes was performed. For each qRT-PCR reaction 3 different controls *(GAPDH*, *PPIA*, *ACTB*; see Table 10) were run and the expression of the test gene was normalized to only that housekeeping gene which changed mRNA steady-state levels least under the experimental condition.

## 2.2.7. Biochemical methods

### 2.2.7.1. Antibodies

**Primary antibodies**

**Table 11: Primary antibodies**

| Target | Origin | Clone | Ig-class | Dilution | Method | Source/ Company | References |
|---|---|---|---|---|---|---|---|
| β1 integrin | Mouse<br>Rat | 12G10<br>mAb13 | IgG1<br>IgG2a | 1:500<br>1:500 | Indirect IF<br>Indirect IF | Humphries Lab<br>Humphries Lab | (Kloepper et al., 2008a) |
| Keratin6<br>Keratin6-bt | Mouse<br>Mouse | KA12<br>KA12 | IgG1<br>IgG1 | 1:10<br>1:100 | Indirect IF<br>TSA | Progen<br>Progen | (van Beek et al., 2008) |
| Keratin15<br>Keratin15-bt | Mouse<br>Mouse | LHK-15<br>LHK-15 | IgG2a, kappa<br>IgG2a, kappa | 1:400<br>1:150 | TSA<br>TSA | Chemicon<br>Chemicon | (Tiede et al., 2009) |
| CD200<br>CD200-bt | Mouse<br>Mouse | OX-104<br>OX-104 | IgG1<br>IgG1 | 1:250<br>1:25 | Indirect IF | Serotec<br>Serotec | (Kloepper et al., 2008b) |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | | | | | TSA | | |
| CD71-PE | Mouse | M-A712 | IgG2a, кappa | 1:100 | Direct IF | BD Pharmingen™ | (Ohyama et al., 2006) |
| Cleaved Caspase 3 (Asp 175) | Rabbit | polyclonal | - | 1:400 | Indirect IF | Cell Signaling | (Kleszczynski and Fischer, 2012) |
| Cortactin-AlexaFluor 488 | Mouse | 4F11 | IgG1 | 1:400 | Direct IF | Millipore | (Gendronneau et al., 2008) |
| MHC class Ia | Mouse | W6/32 | IgG2a, кappa | 1:50 | Indirect IF | DAKO | (Ito et al., 2004; Meyer et al., 2008) |
| ILK | Rabbit | EP1593Y | IgG | 1:100<br>1:2000 | Indirect IF<br>Western Blot | Epitomics | (Judah et al., 2012) |
| β1-Actin | goat | sc-1615 | IgG | 1:1000 | Western Blot | Santa Cruz | (Kueper et al., 2007) |

## Secondary antibodies

**Table 12: Secondary antibodies**

| Name | Conjugated with | Cat no. | Dilution | Method | Company |
|---|---|---|---|---|---|
| Goat anti-mouse | biotin | | 1:200 | IF | Beckman Coulter |
| Goat anti-mouse | Rhodamin or FITC | | 1:200 | IF | Jackson Immuno Research |
| Goat anti-rat | Rhodamin or FITC | | 1:200 | IF | Jackson Immuno Research |
| Goat anti-rabbit | Rhodamin or FITC | | 1:200 | IF | Jackson Immuno Research |
| Anti rabbit IgG | HRP linked | 7074 | 1:2000 | Western Blot | Cell Signaling |

| | | | | | |
|---|---|---|---|---|---|
| Anti Biotin (Ladder) | HRP-linked | 7727 | 1:10000 | Western Blot | Cell Signaling |
| Bovine anti-goat IgG | HRP-linked | sc-2350 | 1:5000 | Western Blot | Santa Cruz |

### 2.2.7.2. Labeling of Antibodies

Human HFs, which I manipulated during culture with β1 integrin specific activating or inhibitory antibodies (mouse anti-human 12G10 or rat anti-human mAb13) and were to be analyzed for different structural proteins, like K15 or K6, a direct labeling of these antibodies was necessary. This antibody labeling realized a specific immunofluorescence staining of these proteins and prevented a false-positive staining with the established secondary antibodies descended from the same host like the activating or inhibitory β1 integrin antibodies.

I did the direct labeling of the primary antibodies by biotin-coupling using the APEX Antibody Labeling Kit (A10495, Invitrogen) according to the manufacturer's guidelines. For a successful antibody labeling only small amounts of IgG antibody (10-20 µg) were necessary, which was achieved by lyophilisation of the required antibody amount. By doing different parallel control stainings, like the established staining (Paus lab) of untreated and treated HFs with unlabeled K15 and K6, the successful labeling and the correct IR pattern were checked.

### 2.2.7.3. Staining of cellular/structural proteins by

**(A) Immunohistochemistry**

The periodic acid-Schiff (PAS) staining is a method which detects polysaccharides and mucosubstances like glycogen, glycoproteins or glycolipids and by that is useful to visualize the basal membrane (Roland et al., 2003). I fixed the cryosections of the wounded skin in acetone for 10 min after air drying (10 min). Further free hydroxyl groups were oxidized with 0.5 % periodic acid for 8 minutes, which results in the formation of aldehyde groups. After a washing step in A. dest these aldehyde groups were detected with Schiff reagent (Merck) and later washed in 3 cuvettes with sulfite water (each 2 min) to reduce a pseudo reaction of unbound fuchsine. Ten minutes of running tap water increased the red staining of the detected aldehyde groups. The counterstaining was made with Mayer's hemalum (Merck) for 30 sec with the following "blueing" using running tap water for 10 min. Finally, the sections were dehydrated and mounted with Eukitt® (Kindler GmbH).

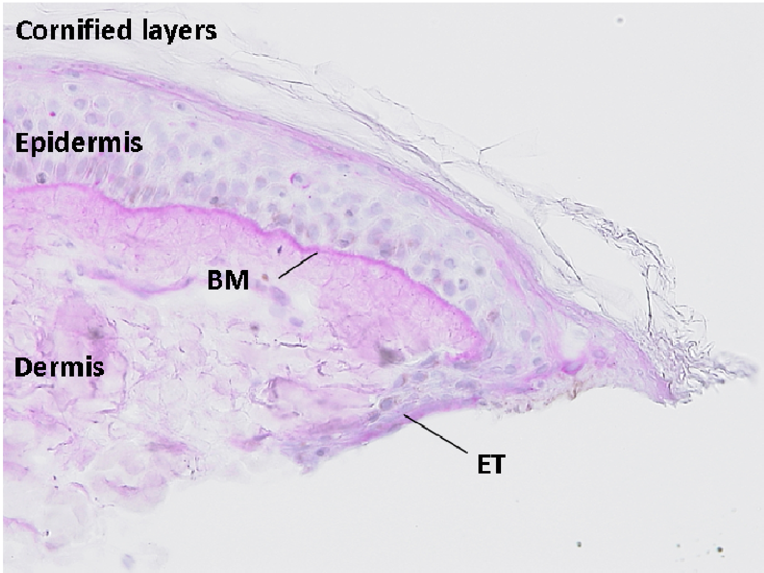



**Figure 29: Example for a PAS-stained skin section (histochemistry).**
PAS-stained wounded skin (vehicle control) after one day of culture was performed to demarcate the basement membrane (BM). ET = newly formed epithelial tongue (generated by N. Ernst), magnification of 200x.

### (B) Immunofluorescence

To determine the protein expression of different cellular proteins via immunofluorescence stainings, primary antibodies and secondary antibodies were applied, which are listed in Table 11 and Table 12.

Cryosections (6 µm thick) of human skin or whole HFs stored at -80°C were air dried for 10 min before starting the fixation (acetone, 1% Formalin, methanol). In contrast, the cryosections of the embedded HF epithelia (8 µm thick) were directly fixed in the solutions (mentioned above) to prevent the dehydration of the Matrigel®/collagen I which would result in tissue destruction. Depending on immunofluorescence staining TBS, PBS (both followed by a direct or indirect IF) or TNT (followed by a TSA) was used to wash the slides three times for 5 min (produced as described before, see Table 3).

After a preincubation step with 10% goat normal serum (GNS) diluted in the used washing solution for 20 min, the primary antibodies (see Table 11) in their appropriate dilution were directly applied on the sections and incubated overnight at 4°C. On the next day, after washing three times for 5 min in the washing buffer (TBS, PBS or TNT), slides which need an indirect immunofluorescence were stained with their suitable secondary antibody (see Table 12) for 45 min at RT. Later three washing steps for 5 min were done, followed by the counterstaining with DAPI (4′,6-diamidin-2′-phenylindol-dihydrochlorid) for 1 min. Finally the sections were washed three times in their corresponding washing buffer and mounted with Fluoromount-G (Southern Biotechnologies).

As negative controls, the primary antibodies were omitted, performing the same staining steps as described before. Besides this classical control the absence and presence of immunoreactive cells in already known human skin locations served as additional internal negative or positive controls. Dependent on the analyzed protein the specific IR (beyond background) was evaluated, or the number of positive immunoreactive cells was counted in relation to $DAPI^+$ cells.

**(C) Tyramide signal amplification**

Besides the standard immunofluorescence (indirect or direct) stainings I used the tyramide signal amplification (TSA) to amplify and quantify some cellular proteins in cryosections. The slides were washed in TNT buffer (prepared as described in Table 3) for 5 min after standard fixation and further incubated with 3% H2O2 in PBS (phosphate buffered saline) for 15 min to block the endogenous horseradish peroxidase. Preincubation was performed with the treatment of avidin and biotin for 15 min and 5% GNS (DAKO) in TNT for 30 min, including washing steps in between (three times for 5 min in TNT). Furthermore, the primary antibodies (see Table 11) diluted in TNT and 2% GNS were incubated overnight at 4°C followed by a biotinylated secondary antibody (see Table 12) for 45 min at room temperature. If the primary antibody was already labeled with biotin (see Table 11) the secondary antibody step was recessed. Streptavidin horseradish peroxidase (TSA kit; Perkin-Elmer) was administrated (1:100 in TNT) for 30 min at room temperature and later amplified by tetramethylrhodamine- or FITC-tyramide amplification reagent at room temperature for 5 min (1:50 in amplification diluent provided with the TSA kit). Finally the sections were stained with DAPI (Boehringer Mannheim) for 1 min and mounted with Fluoromount-G (Southern Biotechnologies).

As negative controls, the primary antibodies were also omitted and the absence and presence of immunoreactive cells in already known human skin locations were checked as additional internal negative or positive controls. The evaluation of specific cellular proteins carried out by analyzing the specific IR (beyond background), or the number of positive immunoreactive cells in relation to $DAPI^+$ cells.

**(D) Ki-67/TUNEL**

For the demarcation of apoptotic cells in co-localization with the proliferation marker Ki-67, the Ki-67/TUNEL (terminal dUTP nickendlabeling) double-staining was performed (van Beek et al., 2008). Ki-67 is an antigen, which is located in the nucleus and identified by its reactivity with the monoclonal antibody from Ki-67 clone (Gerdes et al., 1984). It is expressed during all active phases of the cell cycle (G1-, S-, G2- and M-phase), but not in resting cells (G0-phase) (Gerdes et al., 1984).

Apoptosis is a process of a programmed cell death and is normally involved in the tissue homeostasis. Endonucleases cut the DNA into fragments detectable by the terminal deoxynucleotidyltransferase (TdT), which labels enzymatically the free 3′-OH termini with modified nucleotides. TdT catalyzes a template-independent addition of nucleotide triphosphates to the 3′-OH ends of double-stranded or single-stranded DNA. The digoxigenin-nucleotide labeled DNA fragments can be recognized with an anti-digoxigenin antibody that is conjugated to FITC.

Cryosections were fixed in paraformaldehyde for 10 min at RT and followed by incubation in ethanol-acetic acid (2:1) for 5 min at -20°C after three washing steps in PBS (each 5min; produced as described before, see Table 3). Then the slides were first labeled with a digoxigenin-deoxyUTP (ApopTag Fluorescein *In Situ* Apoptosis detection kit) in the presence of TdT (60 min, 37°C) and further incubated with a mouse anti-Ki-67 antiserum (DAKO) overnight at 4°C. Between the different steps the cryosections were regularly washed in PBS. The next day the TUNEL-positive cells were visualized by an anti-digoxigenin FITC-conjugated antibody (ApopTag kit) for 30 min at RT, whereas Ki-67 was detected by a rhodamine-labeled goat anti-mouse antibody for 45 min at RT. The final counterstaining with DAPI for 1 min where followed by mounting the sections with Fluoromount-G.

Since the TUNEL technique can also demarcate terminally differentiating KCs (Magerl et al., 2001) only those TUNEL$^{+}$ cells were counted that showed a shrunken nucleus and/or TUNEL$^{+}$ aoptotic bodies.

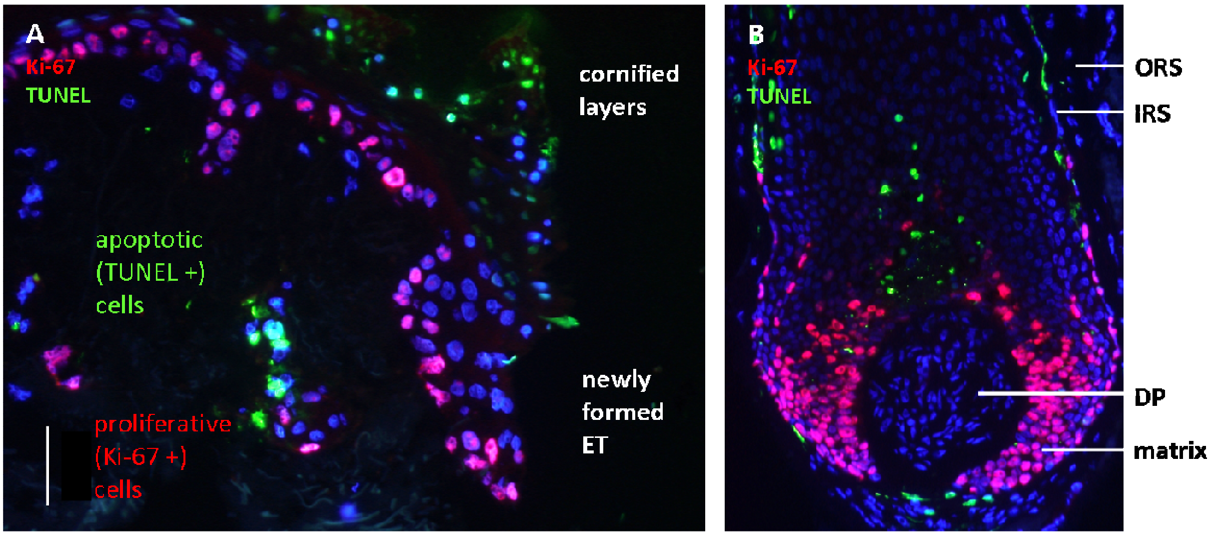



**Figure 30: Example for a Ki-67/TUNEL stained skin and HF sections.**
(A) The picture represents Ki-67/TUNEL stained wounded skin after 3 days of culture. (B) shows a Ki-67/TUNEL stained HF bulb. Red nuclei demarcate proliferative keratinocytes, green nuclei delimitate apoptotic keratinocytes, 200x magnification. Abbreviation: ORS = outer root sheath, IRS = inner root sheath, DP = dermal papilla (generated by N. Ernst)

### 2.2.7.4. S-Phase analysis via EdU incorporation

A more selective method to detect cell proliferation is the quantification of only those cells that are in the S-phase of the cell cycle and actively engage in DNA-synthesis (Kotogany et al., 2010). By using 5-ethynyl-2'-deoxyuridine (EdU), a terminal nucleoside analog of thymidine, its incorporation into newly synthesized DNA (Wang et al., 2011a) can be visualized because of its labeling with a stabile fluorescence dye (Figure 31). The whole method was performed following the manufacturer’s guidelines (Click-iT® EdU Alexa Fluor® 488 Flow Cytometry Assay Kit), whereas the EdU incorporation time of 2 hours was adapted on the HF cultures.

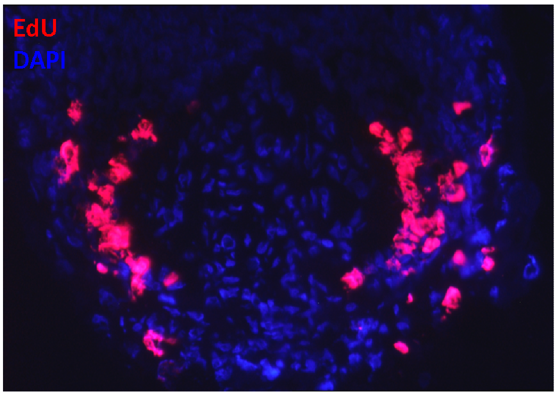



**Figure 31: S-phase marked HF matrix cells via EdU incorporation.**
Red cells demarcate matrix keratinocytes of the HF bulb that have incorporated EdU and thus are in the S-phase of the cell cycle. 400x magnification (generated by N. Ernst)

#### 2.2.7.5. Western Blot

Twelve to fifteen dispase-pretreated HFs were homogenized in 50 µl lysis buffer (prepared as described before, see Table 4) for 5-10 min. To achieve a complete homogenization of the lysate samples they were later treated with ultrasound for 15 sec (cycle 0.5, power 60). After centrifugation at 10.000 rpm for 5 min two phases were obtained: a clear supernatant with isolated proteins and a black pellet consisting of cellular debris. The supernatant was used for the quantification of proteins.

The total protein amount of the HF epithelium was measured by using the BCA assay (bicinchoninic acid assay) (Smith, 1985), where temperature dependent the peptide bonds in proteins reduced $Cu^{2+}$ ions from the cupric sulfate to $Cu^{+}$. The resulting $Cu^{+}$ is proportional to the amount of protein contained in the sample. In the next step of this reaction each $Cu^{+}$ ion formed a purple-colored product with two molecules of bicinchoninic acid chelate and adsorbed light at a wavelength of 562 nm. This method was done by according the manufacturer's manual (ThermoFischer) in 96-well-plates. The protein amount of the solution was quantified by measuring the absorption spectrum and by comparing this with BSA (bovine serum albumin) solutions of known concentration.

Samples were separated in a 10% denaturating Tris/Tricine SDS polyacrylamide (PAA) gel electrophoresis system, as described by (Schagger and von Jagow, 1987) and were done by using the Mini-Protean III Cell System (Biorad). The 10% separating gel solution (see Table 5) was added directly between the glasses with a 1000 µl pipette and coated with methanol for avoiding any evaporation. After 30 min the gel was polymerized, methanol could be decanted and the separating gel was carefully washed with A. dest. Next, the 5% stacking gel (see Table 6) was prepared, and added to the polymerized separating gel in glasses. Approximately after 30 min the polymerization was achieved, the PAA gel was directly used or stored in damp sheets over night at 4°C.

The prepared gels were put into the Biorad equipment and filled with electrophoresis buffer. Before loading the samples (10 µg proteins) were boiled with the same amount of Lämmli buffer for 5 min. Lämmli buffer allowed a proper separation of the proteins not by shape (β2-mercaptoethanol) and charge (SDS), but by size.

Western blotting enables the specific detection of proteins (Towbin et al., 1989), which are separated by size with the help of SDS-PAGE. The fractionated proteins were transferred to an Immobilon TM-P (PVDF - Polyvinylidene Difluoride) membrane, detected with specific antibodies and quantified with a colorimetric method (Nelson and Cox, 2013). The blot was performed using the Mini Trans-Blot cell (Biorad) following the manufacturer’s guidelines. The transfer was carried out for 60 min at 100 V.

The blotted membranes were blocked in 5% TBST-milk (prepared as described before, see Table 7) for 1 h at RT under shaking for blocking of non-specific binding and further incubated overnight 4°C with the appropriate dilutions of the primary antibody diluted in 5% TBST-milk (rabbit anti-ILK; EP1593Y, Epitomics, 51kDA; β-Actin; sc-1615, Santa Cruz, 42kDa). After 3 washing steps in 5% TBST-milk (10 min each) the blots were incubated with horseradish peroxides-conjugated secondary antibodies (Table 12; diluted 1:2000-1:10000) for 2 h at room temperature. The protein bands were visualized and detected with the enhanced chemiluminescence (ECL) system (PerkinElmer LAS, Inc., Boston) according manufacturer’s manual.

### 2.2.8. Statistical analysis

All harvested data were given as means ± SEM (standard error of the mean) and the evaluation of statistical significance was performed by using GraphPad Prism 5.01 (Graph Pad software, Inc., San Diego, CA, USA). Student's t-test or one-way ANOVA by appropriate post hoc comparison (depending on a given Gaussian distribution) was used at single time points.

# 3. Results

## 3.1. β1 integrin-mediated signaling impacts on the proliferation and the maintenance of human hair follicle epithelial progenitor cells

### 3.1.1. Transient knockdown lead to a significant reduction of β1 integrin gene but not protein expression

First, it was asked whether β1 integrin silencing can be achieved in a complex human tissue, as this had not been accomplished before. Therefor intact, full-length, organ-cultured human scalp HFs were transfected with a cocktail of three *β1 integrin*-specific siRNAs or scrambled control RNAs following the manufacturer's guidelines (Santa Cruz) and, using a standardized method that had previously been employed successfully in the lab for silencing two other genes expressed in the HF epithelium (Samuelov et al., 2012; Sugawara et al., 2012).

I analyzed the knockdown of *β1 integrin* at two different time points (day 1 and 4) with RT-qPCR by using intact, full-length HFs. At the first time point the transfection reaction alone had a strong influence on the *β1 integrin* gene expression on HFs of one of the two patients, but on day 4 a significant *β1 integrin* silencing effect in human anagen scalp HFs was demonstrated at the transcript level (Figure 32) on all analyzed patients (n=3 individuals).

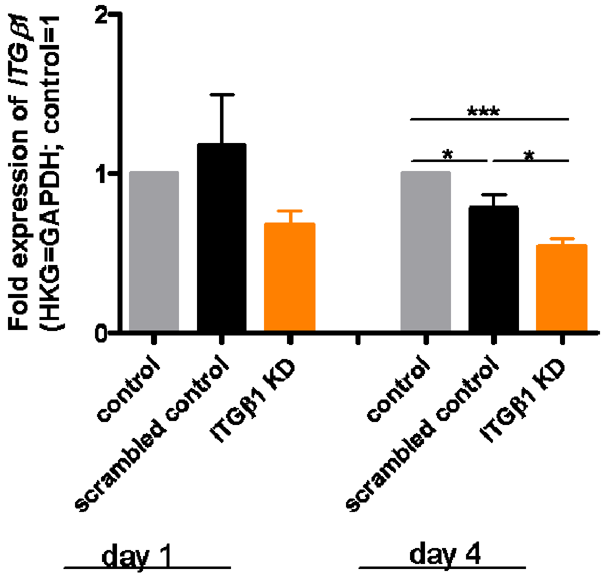



**Figure 32: Gene silencing of *β1 integrin* in anagen human hair follicles.**
*β1 integrin* gene expression was analyzed with qRT-PCR using full-length hair follicles (HFs). At day 1 the silencing had a strong influence on the *β1 integrin* gene expression of HFs, but PCR results on day 4 confirmed a significant silencing. Day1: n=2 individuals, day4: n=3 individuals. Statistical analysis were performed with the One way ANOVA, Dunns comparison test; Mean +/- SEM (*p<0.05, ***p<0.001). Abbreviation: HKG = housekeeping gene, GAPDH = Glyceraldehyde3-phosphate dehydrogenase, ITGβ1 KD = knockdown of *β1 integrin*.

Next, I investigated if this corresponded to a reduction in the intrafollicular expression of β1 integrin protein. For this, the immunreactivity (IR) pattern of β1 integrin in the whole HF was analyzed from day 4 with 2 different specific antibodies - the β1 integrin activating 12G10 (Figure 33) and the inhibitory mAb13 (Figure 34) antibody, which recognize distinct conformation-dependent epitopes. Disappointingly, this showed that the knockdown did not change β1 integrin protein immunoreactivity (IR) in any of the compartments of the silenced HFs compared to scrambled control after 4 days (Figure 33, Figure 34). This suggested that knockdown had only been successful in reducing the mRNA steady-state level, but had failed to translate into significant effects at the β1 integrin protein level up to this point in time (day 4).

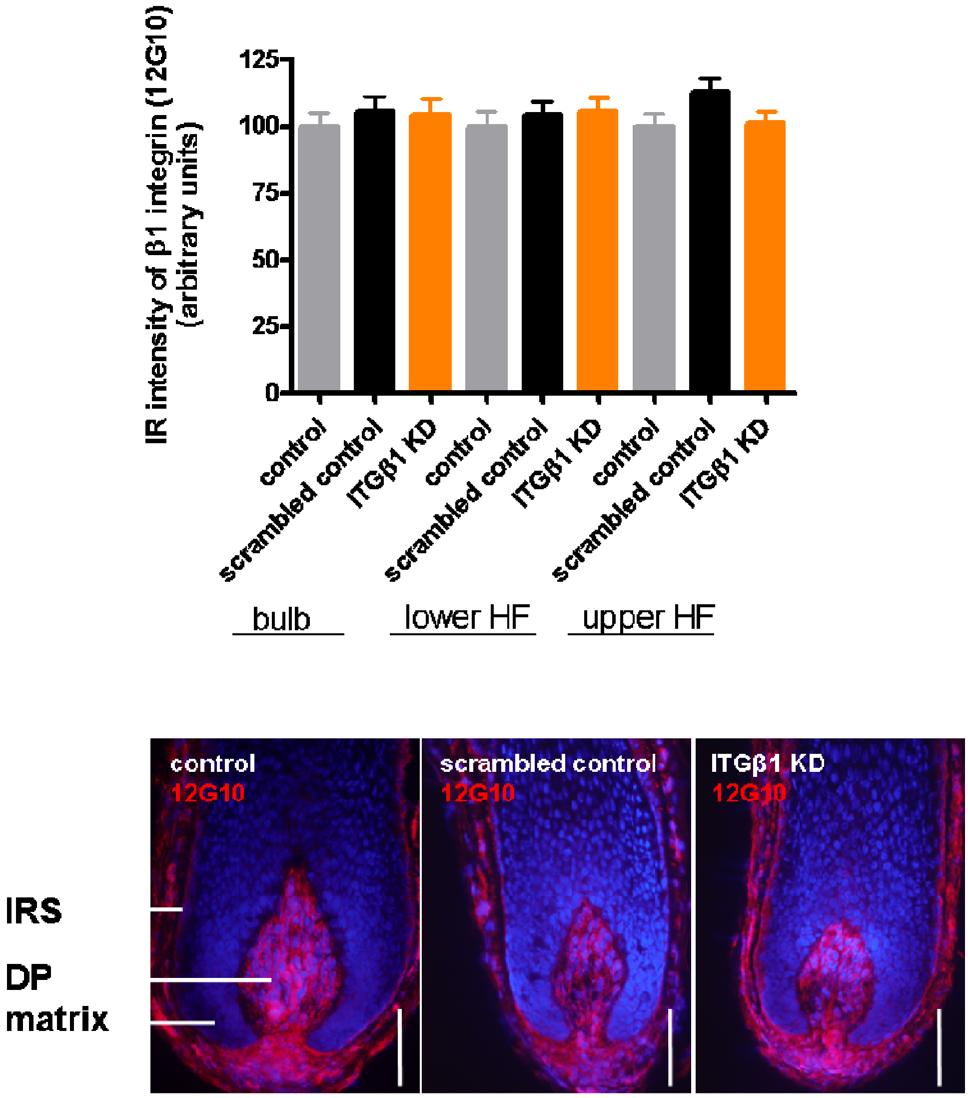



**Figure 33: The immunoreactivity (IR) pattern of 12G10 stained full-length HFs.**
The IR pattern was analyzed on day 4 using the β1 integrin-activating antibody 12G10 (n=3 individuals [17-26 HFs]). The IR intensity displayed no differences between the scrambled control and ITGβ1 KD group as well as in the different measured HF compartments [bulb, the lower HF and the upper HF (including the bulge)]. The control is normalized to 100%; representative photos of HF bulbs on day 4, white scale bars = 100 µm. Abbreviation: IRS = inner root sheath, DP = dermal papilla.

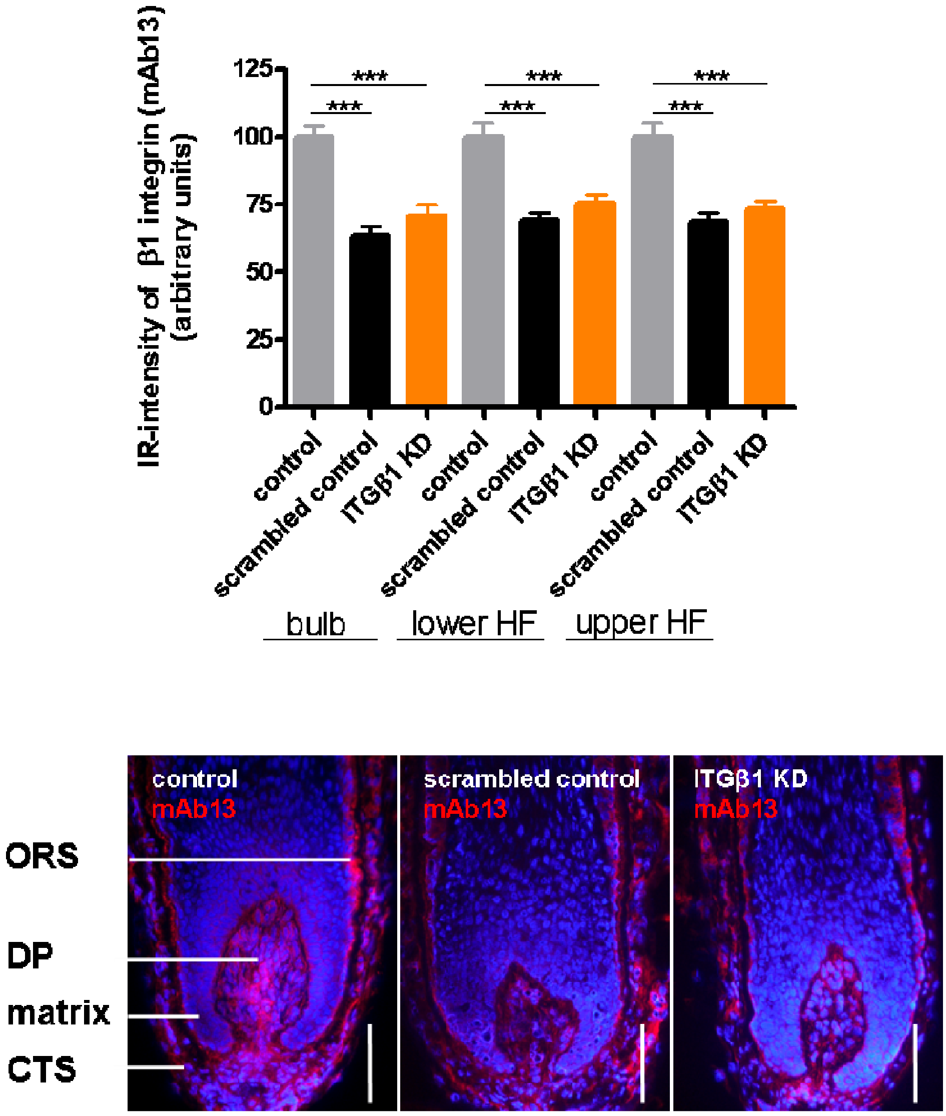



**Figure 34: The immunoreactivity (IR) pattern of mAb13 stained full-length HFs**
The IR pattern was analyzed on day 4 using the β1 integrin-inhibiting antibody mAb13 (n=2 individuals [12-20 HFs]). The IR intensity displayed no differences between the scrambled control and ITGβ1 KD group as well as in the different measured HF compartments [bulb, lower HF and the upper HF (including the bulge)]. The control is normalized to 100%; representative photos of HF bulbs on day 4, white scale bars = 100 µm. Statistical analysis were performed with the One way ANOVA, Bonferroni comparison test; Mean +/- SEM (***p<0.001). Abbreviation: ORS = outer root sheath, DP = dermal papilla, CTS = connective tissue sheath.

But these analysis arose the question whether the silencing might have a different influence on the conformation-dependent epitopes of β1 integrin, because mAb13 detects the bent and low-affinity β1 integrin domain, while 12G10 binds at the extended and activated (Byron et al., 2009) receptor.

The stainings revealed that the knockdown did not display any changes in the IR pattern of 12G10, but the transfection as such appeared to reduce significantly the number of β1 integrin epitopes/receptors recognized by the mAb13 in the analyzed compartments (Figure 33, Figure 34).
This suggested that only by the transfection reaction low-affinity β1 integrins were diminished at the surface of HF KCs.

### 3.1.2. β1 integrin silencing reduces proliferation and DNA synthesis in different progenitor cell populations of the human hair follicle epithelium

Arguing that integrin protein may have been too long-lived under assay conditions for a reduction in β1-associated intrafollicular IR to become visible by IF after silencing, it was next searched for functional evidence whether β1 integrin silencing had any impact on intrafollicular ePCs. As explained above (see 1.5), HF epithelium contains different progenitor cell populations with distinct proliferation capacities, such as slow-cycling, intermittently proliferating ePC populations in the bulge versus rapidly proliferating, transient amplifying cells in the hair matrix (Watt and Jensen, 2009; Xu et al., 2003).

Therefore, it was subsequently assessed whether β1 integrin silencing modulated proliferation and apoptosis in the HF epithelium, using quantitative Ki-67/TUNEL immunohistomorphometry. In the counting of proliferative or apoptotic KCs I been supported by our trainee Arzu Yay.

This showed that, compared to scrambled oligo-treated control HFs, β1 integrin-specific silencing significantly reduced the number of Ki-67$^+$ cells (10% less than scrambled control) in the maximally proliferating epithelial hair matrix which is mainly composed of transient amplyfing cells (Figure 35).

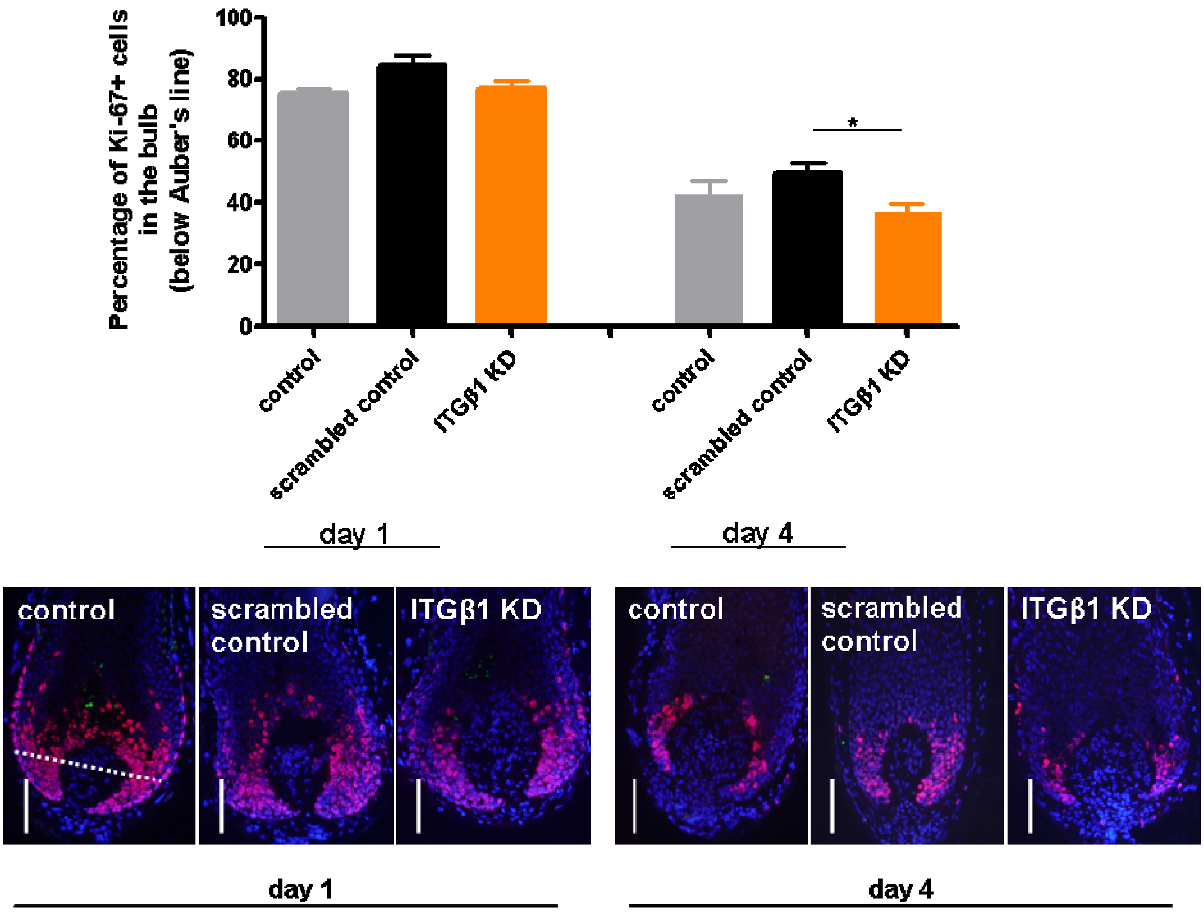



**Figure 35: β1 integrin silencing caused significant reduction of keratinocyte proliferation.** The specific silencing significantly reduced Ki-67$^{+}$ cells in HF bulbs treated with *β1 integrin* siRNA compared to the scrambled control on day 4, n=3 individuals (13-16 HFs). Matrix keratinocytes of anagen HFs were counted below Auber's line (dotted white line); white scale bars = 100µm. Statistical analysis were performed with the One way ANOVA, Bonferroni comparison test; Mean +/- SEM (*p<0.05).

Further quantification of Ki-67/TUNEL in other defined areas of the HF (bulb, lower HF and upper HF [including HF bulge]) confirmed the hair matrix data and also demonstrated a significant reduction of the number of Ki-67$^{+}$ cells in the bulge with its slow-cycling ePCs (Figure 36). By counting Ki-67$^{+}$ cells in the lower HF no differences were detectable in the analyzed groups (data not shown).

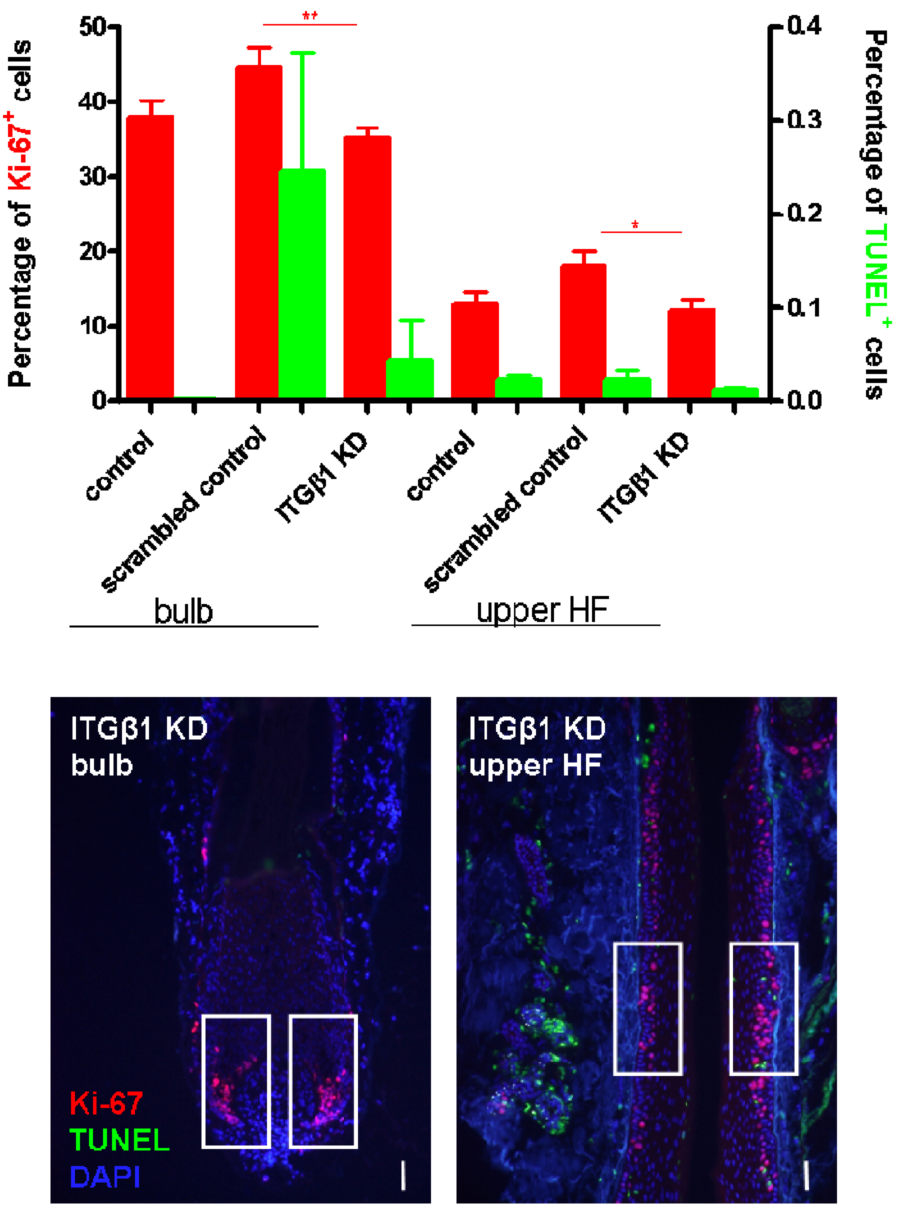



**Figure 36: β1 integrin silencing decreased the proliferation capacity in HF bulb as well as in the HF bulge.**
To dissect the proliferation capacity of slow-cycling ePCs of the HF bulge Ki-67$^{+}$ cells were counted in defined rectangles (representative photos, white scale bars = 100µm). β1 integrin silencing caused a significant reduction of Ki-67$^{+}$ cells in the HF bulb, but also in the HF bulge on day 4. n=3 individuals (13-24 HFs). Statistical analysis were performed with the One way ANOVA, Bonferroni comparison test; Mean +/- SEM (*p<0.05, **p<0.01).

These proliferation results were double-checked by measuring 5-ethynyl-2'-deoxyuridine (EdU) incorporation, a cell cycle S-phase specific marker to determine active DNA synthesis (Wang et al., 2011a). Counting $EdU^+$ cells in defined reference areas in the HF bulb, the lower HF (data not shown) and HF bulge, the same proliferation-inhibitory tendency after *β1 integrin* knockdown could be confirmed (Figure 37).

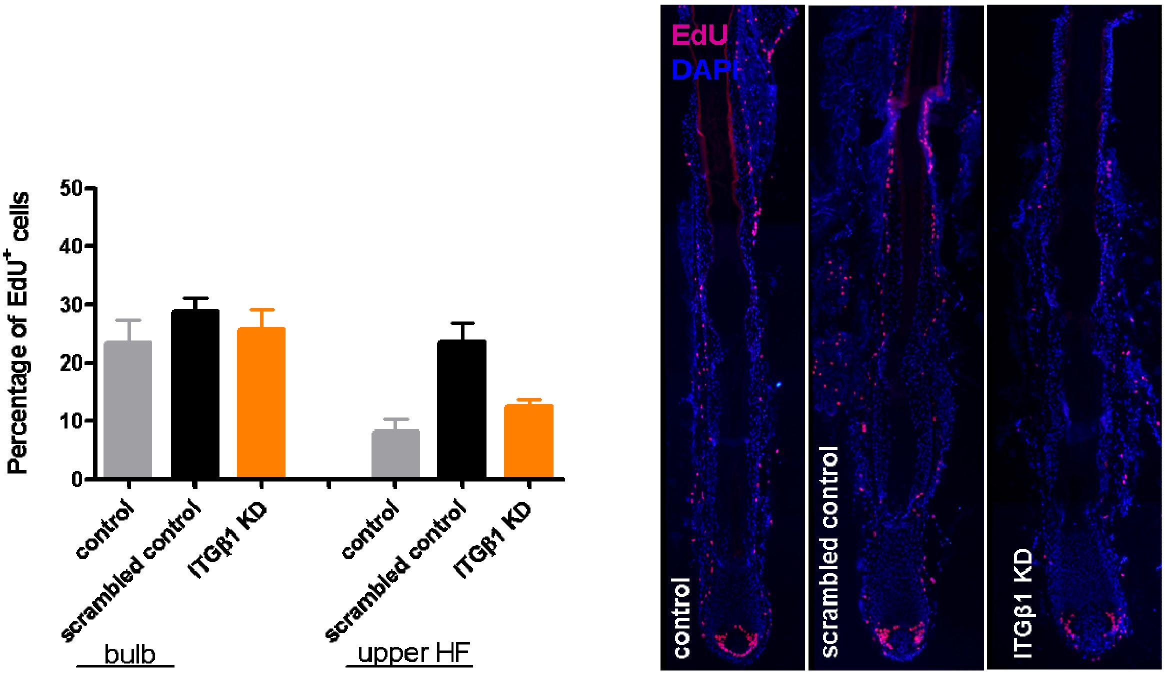



**Figure 37: The knockdown decreased the S-phase active ePCs on the HF bulge.**
By analyzing the proliferating status in a human HF via counting $EdU^+$ cells in defined rectangles in the HF bulb and HF bulge the same tendency for proliferation in β1 integrin-mediated signaling were showed (representative photos, 100x magnification). n = 1 individual (2-3 HFs).

Instead, *β1 integrin* knockdown did not significantly affect apoptosis in the HF compartments, as measured by TUNEL assay (Figure 36).

Thus, even though successful *β1 integrin* knockdown was documented only on the mRNA level (perhaps due to extended β1 integrin protein stability within the human HF), silencing was functionally effective since it reduced proliferation and DNA synthesis in both slow-cycling human bulge ePCs and rapidly proliferating human hair matrix KCs *in situ*. This suggests that β1 integrin may indeed operate as an important niche receptor that regulates proliferation activity in human ePCs and their more committed progeny in the hair matrix.

### 3.1.3. β1 integrin-mediated signaling is required for epithelial progenitor cells maintenance *in situ*

To verify if β1 integrin-mediated signaling is indeed needed for the maintenance and differentiation of ePCs, the effects of *β1 integrin* knockdown on the expression of the ePC markers K15 and CD200 was analyzed (Cotsarelis, 2006; Garza et al., 2011; Kloepper et al., 2008b; Ohyama et al., 2006) in human HFs *in situ.*

Initially, i.e. one day after knockdown, *β1 integrin* silencing slightly enhanced *K15* and *CD200* gene expression in human scalp HFs (Figure 38A, B), possibly as a temporary compensatory phenomenon. Subsequently, however, *K15* transcription was significantly reduced 4 days after silencing by *β1 integrin* siRNA compared to scrambled controls (Figure 38A, B). In contrast, overall intrafollicular *CD200* transcription was not significantly altered by *β1 integrin* silencing.

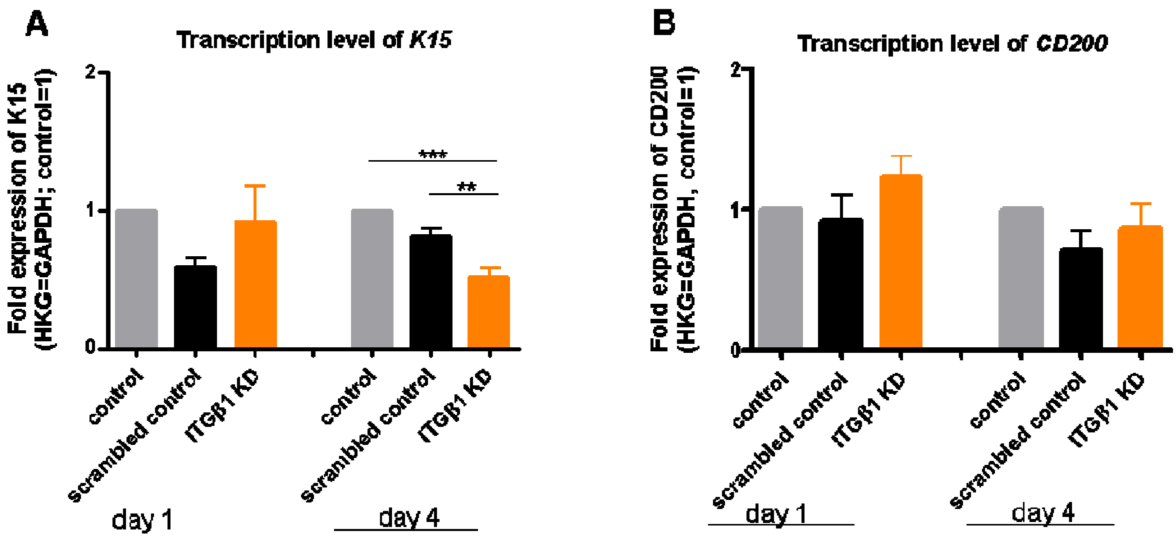



**Figure 38: β1 integrin-mediated signaling significantly reduced *K15* gene expression.**
(A) K15 transcription was significantly reduced at day 4 by *β1 integrin* siRNA compared to the scrambled control. (B) *β1 integrin* silencing slightly enhanced the gene expression of CD200 in the full-length HF. Fold expression of all analyzed genes were normalized to GAPDH. n=2-3 patients (for RNA extraction 12 HFs/patient were used and cultured over 4 days). Statistical analysis were performed with the One way ANOVA, Bonferroni comparison test; Mean +/- SEM (**p<0.01, ***p<0.001). Abbreviation: ITGβ1 KD = knockdown of *β1 integrin*, IR = immunoreactivity.

The main eSC region, the HF bulge, also showed a significant reduction of K15 and CD200 protein IR (Figure 39 A, B). Taken together, *β1 integrin* knockdown impacts on K15 and CD200 ePC population and suggests the concept that uninterrupted β1 integrin signaling is required to maintain the human HF eSC niche within the HF bulge.

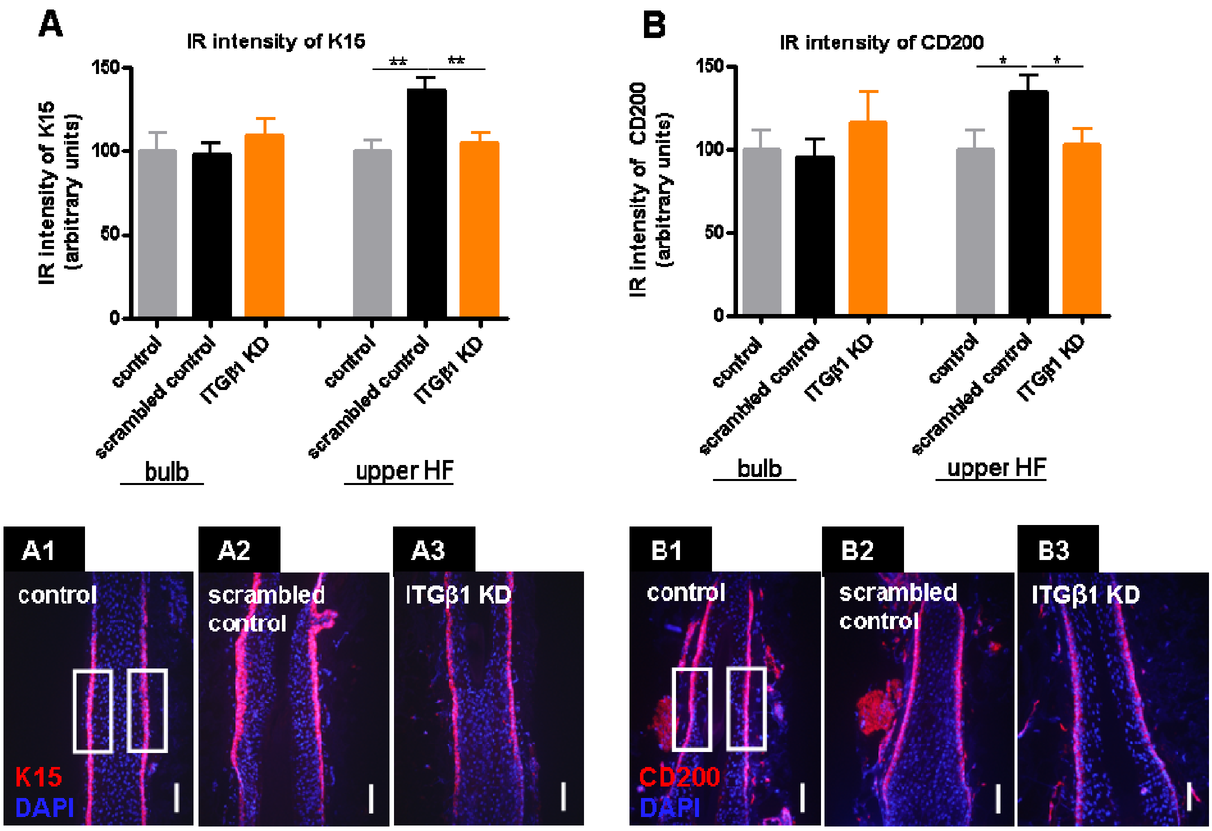
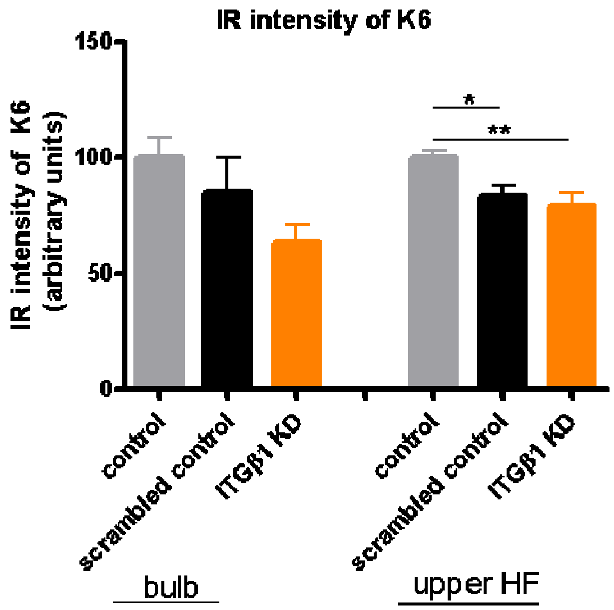



**Figure 39: β1 integrin-mediated signaling maintained the ePC properties in the HF bulge.** (A) K15 immunoreactivity (IR) was most downregulated in *β1 integrin* siRNA silenced HFs in the upper HF (including bulge region), n=3 individuals (19-26 HFs). (A1-3) Representative photos demonstrating the reference areas in the upper HF, white scale bars = 100µm. (B) CD200 IR in the HF bulge was significantly reduced compared to the scrambled control at day 4; n=3 patients (17-28 HFs). (B1-3) Representative photos which show the reference areas in the upper HF. Statistical analysis were performed with the One way ANOVA, Bonferroni comparison test; Mean +/- SEM (*p<0.05, **p<0.01). Abbreviation: ITGβ1 KD = knockdown of *β1 integrin*, IR = immunoreactivity.

Further I investigated if *β1 integrin* silencing impacted on the expression of K6, which is prominently and constitutively expressed by differentiated KCs throughout the human ORS, but not by HF bulge eSCs (Rothnagel et al., 1999; Vollmers et al., 2012) and CD71, a marker of transit amplifying cells, the immediate progeny of ePCs (Kaur et al., 2004).

With the support of Arzu Yay, a practical candidate in our lab, I stained and measured K6 IR in the HF bulb and the upper HF. This showed that *β1 integrin* silencing lead to a non-specific repression of K6 IR in both HF compartments (Figure 40). But these data reflect a mild reduction of K6 protein expression in those bulge-region ORS cells that are no ePCs and does not support the concept that the knockdown of β1 integrin-mediated signaling drives their differentiation towards K6$^+$ ORS cells.



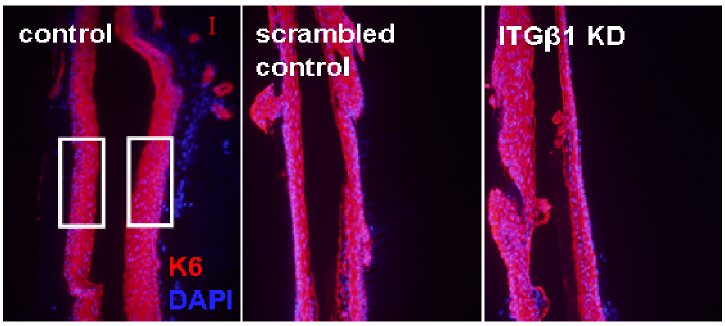




**Figure 40: The specific *β1 integrin* knockdown had no differentiation-modulatory effect, but tendentially reduced K6 protein expression.**
The IR intensity of K6 demonstrated a non-specific repression in every analyzed HF compartment (including the HF bulb and the upper HF) by the silencing procedure, but not specifically by *β1 integrin* silencing in different HF compartments. n=2 individuals (17-18 HFs). (A1-3) Representative photos which show the reference areas in the upper HF (100x magnification). This IR intensity of the HF bulb and the upper HF was measured with defined rectangles with ImageJ (250x125). Statistical analysis were performed with the One way ANOVA, Bonferroni comparison test; Mean +/- SEM (*p<0.05, **p<0.01). Abbreviation: ITGβ1 KD = knockdown of β1 integrin, IR = immunoreactivity.

Since CD71 protein IR was only measurable in the lower HF, this required a change in the analysis method (defined rectangles 200x2000). Quantitative immunohistomorphometry of CD71 protein IR showed no significant IR reduction in the lower HF epithelium (Figure 41).

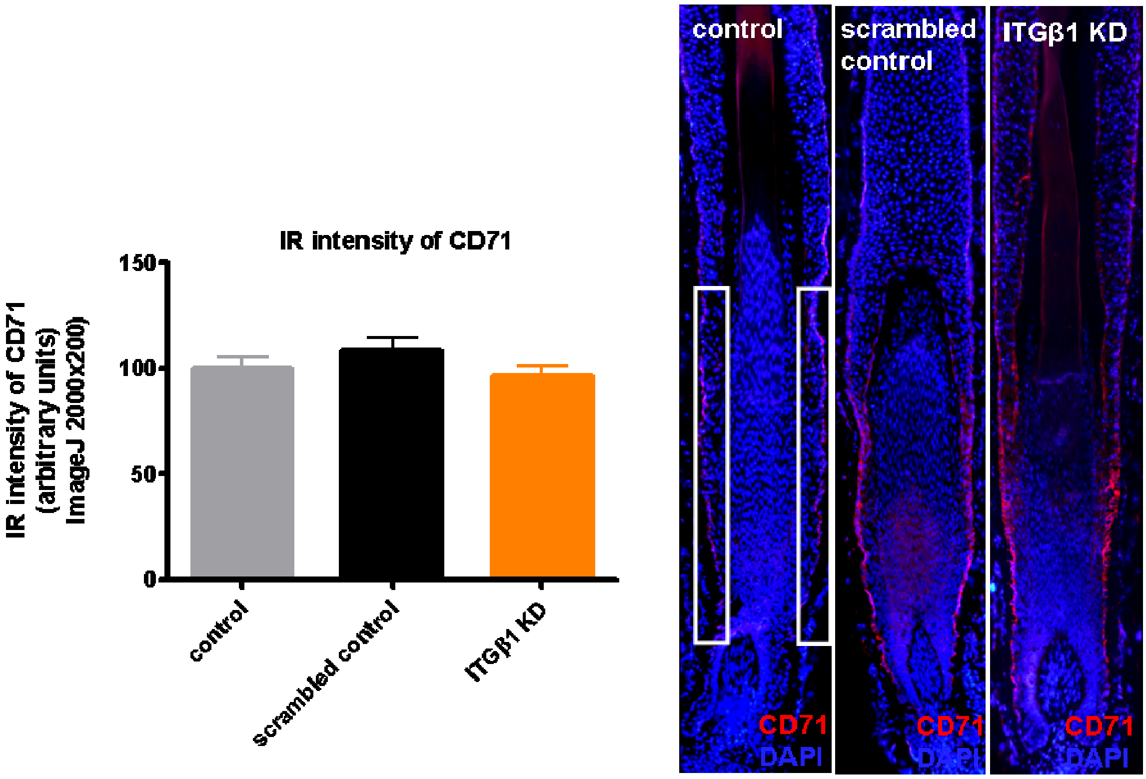



**Figure 41: The *β1 integrin* silencing has no impact on the CD71 IR intensity.**
The knockdown of *β1 integrin* did not alter the intensity of CD71 IR in the specified reference area (rectangles, 200x2000) of the lower HF epithelum (200x magnification). n=2 patients (11-18-HFs). For every analysis of the IR intensity was normalized to the control, set at 100%. Abbreviation: ITGβ1 KD = knockdown of β1 integrin, IR = immunoreactivity

While β1 integrin-mediated signaling is required to preserve the adult ePCs pool in adult human HFs, the current observations do not support the concept that β1 integrin silencing has a major overall differentiation-modulatory impact, as assessed by measuring intrafollicular K6 and CD71 IR.

### 3.1.4. β1 integrin silencing does not influence the hair follicle immune privilege in the bulge

The prominent expression of the immunoinhibitory "no danger-signal", CD200 in the HF bulge (Rosenblum et al., 2006) not only demarcates ePCs (Garza et al., 2011; Kloepper et al., 2008b; Meyer et al., 2008), but also constitutes part of the relative immune privilege of the HF bulge, which may protect the HF eSC niche against autoimmune attacks and is characterized by an extremely low expression of major histocompatibility complex (MHC) class Ia (Harries et al., 2013; Meyer et al., 2008; Paus et al., 2005). Therefore, it was studied whether *β1 integrin* knockdown impacts on the HF bulge immune privilege by analyzing the IR of MHC class Ia.

As revealed by quantitative immunohistomorphometry, CD200 IR was significantly reduced in the HF bulge (Figure 39B, B1-3), but the *β1 integrin* silencing did not further reduce the already minimal MHC class Ia IR within this HF compartment (Figure 42). This suggests that intact β1 integrin signaling is not essential for maintenance of the MHC class Ia-based immune privilege of the human bulge.

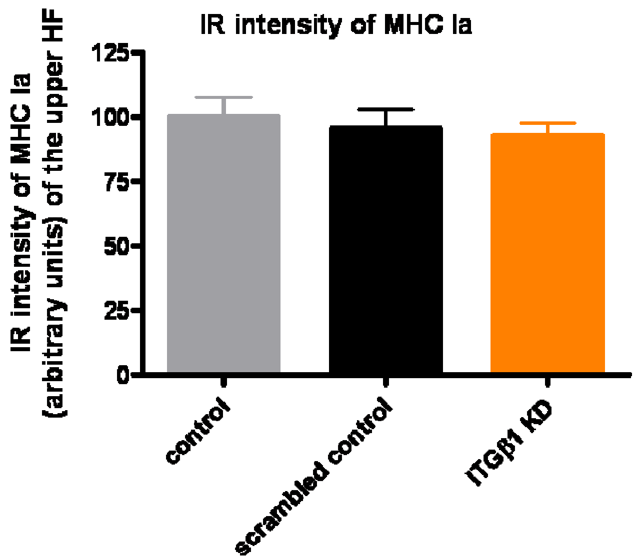



**Figure 42: The immune privilege is maintained in the HF bulge after the *β1 integrin* knockdown.**
MHC Ia IR intensity demonstrated that the silencing reaction and the specific knockdown of *β1 integrin* had no influence on the immune privilege of the HF bulge. n=2 patients (12-19 HFs).

## 3.2. Manipulation of the β1 integrin-mediated outside-in signaling affects ORSK proliferation, migration and ePC maintenance

Besides the knockdown of *β1 integrin* and their related effects on ePC maintenance and differentiation in the human HF, the functionally role of the ECM-β1 integrin-mediated outside-in signaling on various ePC populations within their natural tissue habitat should be considered.

One major challenge that had to be met by the current thesis project was to establish an artificial reconstituted BM for studying the β1 integrin receptor signaling concerning specific cell populations and the influence of ECM ligands of the human HF mesenchyme (BM, CTS) that are likely to interact with β1 integrin.

For this purpose, human scalp HFs were treated with dispase, which cleaves collagen IV and fibronectin (Link et al., 1990; Stenn et al., 1989) to digest and remove the HF BM and CTS. Guided by a previous study (Aasen and Izpisua Belmonte, 2010) the remaining denuded HF epithelium was then embedded into an artificial ECM Matrigel® (MG®), diluted in keratinocyte-serum-free medium (K-SFM), which is optimized for the isolation and expansion of human KCs (Liu et al., 2011). MG® is rich in the β1 integrin ligands laminin, collagen IV, heparin sulfate proteoglycans, entactin, and selected growth factors (Dias et al., 2012; Kleinman et al., 1986; Philp et al., 2005). Thus it is expected to partially mimic aspects of the native HF mesenchyme and BM. This surrogate environment, therefore, should provider an optimal surrounding for enrichment and outgrowing of ePCs of the HF ORS *in vitro*. Moreover the additional usage of an established *K15 promoter*–driven GFP plasmid (Tiede et al., 2009) was chosen to follow a specific ePC population *in situ* regarding their cell number and outgrowth potential (migration). This also permitted monitoring of the outgrowth/migration of GFP-negative, $K15^{+}$ ePCs and their K15-negative progeny within the HF during the culture (Figure 37B).

Some of the embedded HFs were stimulated to produce epithelial outgrowths in this “pseudo-HF mesenchyme” matrix environment (Figure 43A). However, the most of the HFs lost their adhesion to the surrogate matrix and swam in the medium, thus failing to exhibt any ORSK outgrowth (data not shown), and the cells that had emigrated from the HFs (incl. the GFP$^+$K15$^+$ ePCs) were washed out with every medium change (Figure 43B).

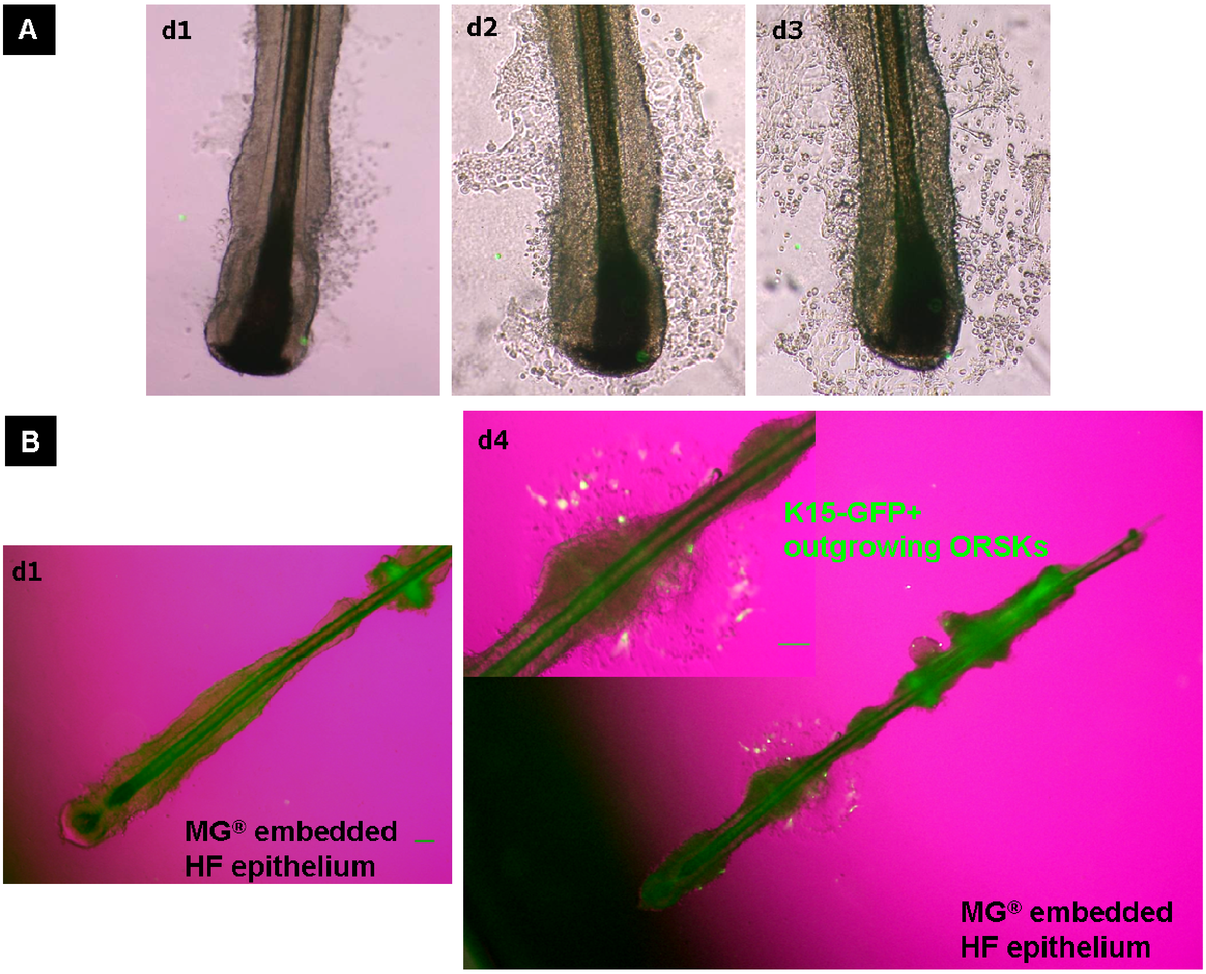


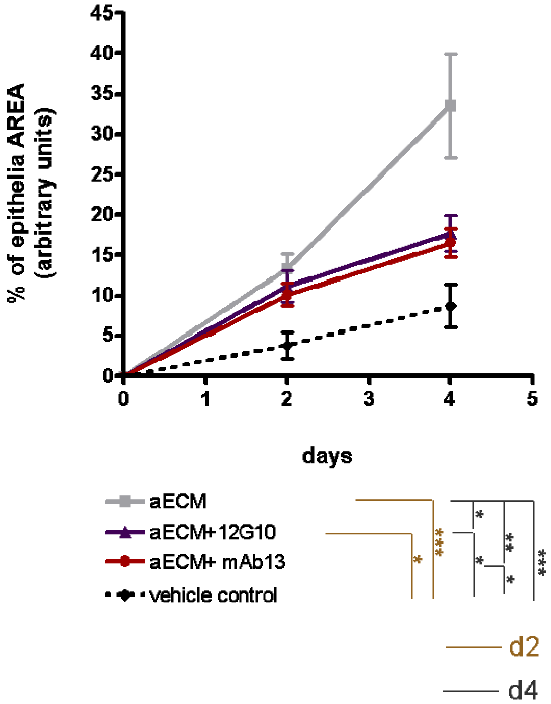

**Figure 43: K15$^+$ ORSK outgrowth into MG$^®$ followed by using *K15 promoter*–driven GFP plasmid.**
Dispase-treated HFs embedded in MG$^®$. (A) ORSK outgrowth of embedded HFs into MG$^®$ during 3 days of culture. (B) Dispase-treated HFs embedded in MG$^®$, which were transfected with a *K15 promoter*–driven GFP plasmid showed an outgrowth of K15$^+$ ePCs in the given matrix.

### 3.2.1. β1 integrin ligands enhance human hair follicle keratinocyte outgrowth *in situ*

To circumvent these HF embedding and cell culture problems, MG® was therefore combined with collagen I, which represents the main dermal collagen, in a ratio of 1:1 and in K-SFM, so as to generate a mechanically more stable ECM and more KC growth-supporting environment. This methodological approach proved to be successful, since it retained the mesenchyme-denuded HFs at the bottom of the culture dishes and facilitated ORSK emigration (Figure 44).

After the establishment of the artificial reconstituted HF-like ECM (aECM, MG® 1:1 collagen I in K-SFM) the outgrowth of ORSKs was measured planimetrically during three different time points (detailed description 2.2.4.1). This demonstrated that only the HFs embedded in aECM showed significantly ORSK outgrowth (Figure 44), suggesting that ECM-mediated signaling via integrins or other ECM receptors expressed on ORSKs is indispensable for ORSK migration *in situ*.

Next, the question was addressed whether a further administration of specific activating or inhibiting β1 integrin antibodies influence the ORSK behaviour within the aECM. Interestingly, the addition of anti-β1 integrin antibodies [namely, the specific ß1 integrin activating (12G10) or inhibitory (mAb13) antibodies (Mould et al., 1996; Mould et al., 1995)] enhanced the ORSK outgrowth area compared to the dispase-pretreated HF epithelium without aECM (vehicle control). Surprisingly, both activating and inhibitory β1 integrin antibodies had very similar stimulatory effects on the ORSK outgrowth area (Figure 44). However, administration of these antibodies resulted in significantly reduced ORSK outgrowth compared to denuded HFs embedded only in aECM.



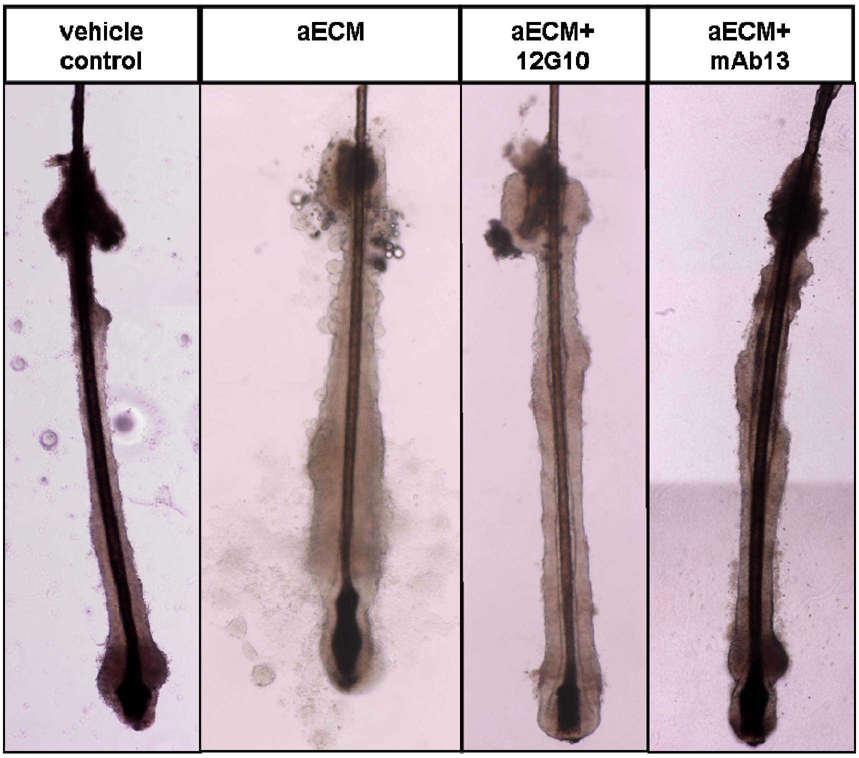




**Figure 44: β1 integrin ligands stimulated the ORSK outgrowth area.**
Epithelial outgrowth area of outer root sheath keratinocytes (ORSKs) over 4 days was measured. While the vehicle control hair follicles (HFs) showed no ORSK outgrowth in the culture dishes, the embedded HFs (aECM) showed a 30% larger ORSK outgrowth area. Activating and inhibiting β1 integrin antibodies had very similar stimulatory effects on ORSK outgrowth area. n=3-4 individuals (20-41 HFs). Brown lines and stars mark the significances of day2; black lines and stars mark the significances of day4. Mean +/- SEM, using unpaired t-test (*p<0.05, **p<0.01, ***p<0.001).

In the other, the largest outgrowth points (longest centrifugal migratory distance of compact epithelial sheets) were measured in defined areas of the embedded HF epithelium (i.e. in the HF bulb, the lower HF and the upper HF [including the HF bulge]). This confirmed the large outgrowth stimulating capacity of the aECM alone in comparison to the vehicle control and also revealed that the β1 integrin activating antibody 12G10 enhanced ORSK outgrowth mainly in the HF bulb (Figure 45A). Interestingly and unexpectedly, in the upper HF (including the bulge) epithelial cell outgrowth was stimulated by the inhibitory antibody mAb13 (Figure 45C).

This suggests that dependent on the type of analysis manipulation-related effects on the β1 integrin-mediated outside-in signaling could be observed. The usage of β1 integrin activating or inhibitory antibodies demonstrated a different outgrowth depth in distinct HF compartments only by measuring the largest outgrowth points but not the outgrowth area. Moreover the results provide a higher proliferation or migration rate of the KCs located in the HF bulb.

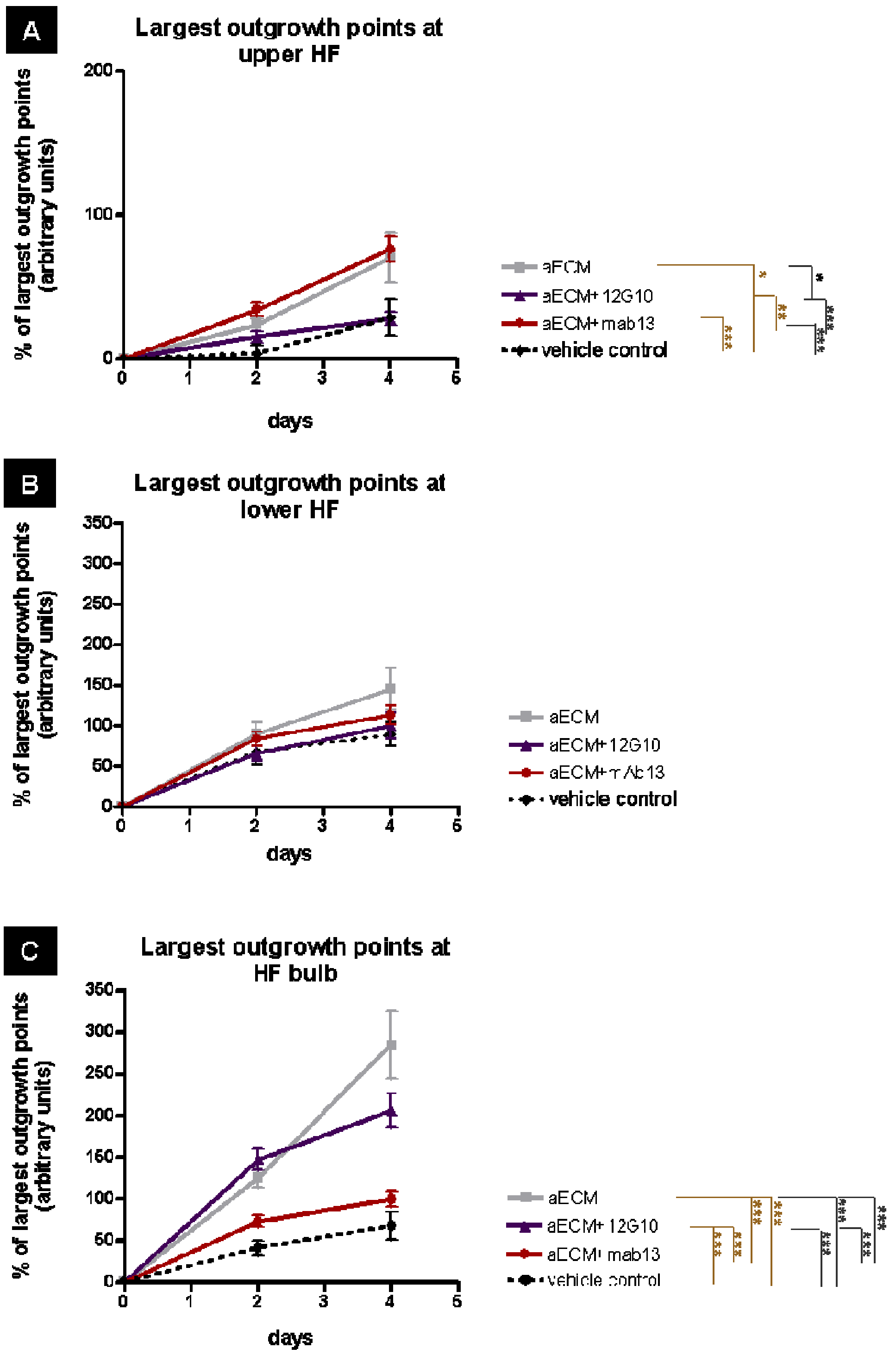



**Figure 45: β1 integrin ligands stimulated mainly the largest outgrowth of ORSKs in the HF bulb.**
Measurement of the largest outgrowth points in the HF bulb, the lower HF and upper HF (including the HF bulge) over 4 days. The large influence of the aECM but also the antibody treatment is demonstrated and realized a distinguishing between the different epithelial progenitor cell populations via their response to β1 integrin antibody stimulation. In the upper HF region (including the bulge) the inhibitory β1 integrin antibody mAb13 significantly stimulated epithelial outgrowth,

whereas in the HF bulb the activating antibody 12G10 antibody stimulated epithelial outgrowth. n=4 patients (18-33 HFs). Mean +/- SEM, using unpaired t-test (*p<0.05, **p<0.01, ***p<0.001).

### 3.2.2. β1 integrin receptor ligands differentially regulate epithelial cell proliferation and apoptosis in different human hair follicle compartments

Since outside-in signaling via β1 integrin regulates many fundamental epithelial cell functions (Hehlgans et al., 2007; Legate et al., 2009; Wickstrom et al., 2011), we sought to correlate the observed differences in ORSK outgrowth to proliferation and apoptosis markers. When dispase-pretreated HF epithelium, embedded in the CTS- and BM- mimicking aECM, was compared with standard organ-cultured but also dispase-pretreated HFs, removal of the BM and CTS promoted epithelial cell apoptosis in human HF epithelium *in situ*. The contact of dispase-pretreated HFs with the aECM alone already significantly reduced apoptosis and up-regulated proliferation of the HF epithelium (Figure 46). Notably, the number of proliferating cells in the upper HF was 3 times higher than in the HF bulb. This suggests that the composition of aECM activated the outside-in signaling mediated by β1 integrin and thus prolonged survival of the embedded HF epithelium; moreover, this enhanced the proliferation rate in the HF bulge, the SC-rich and slow-cycling HF compartment.

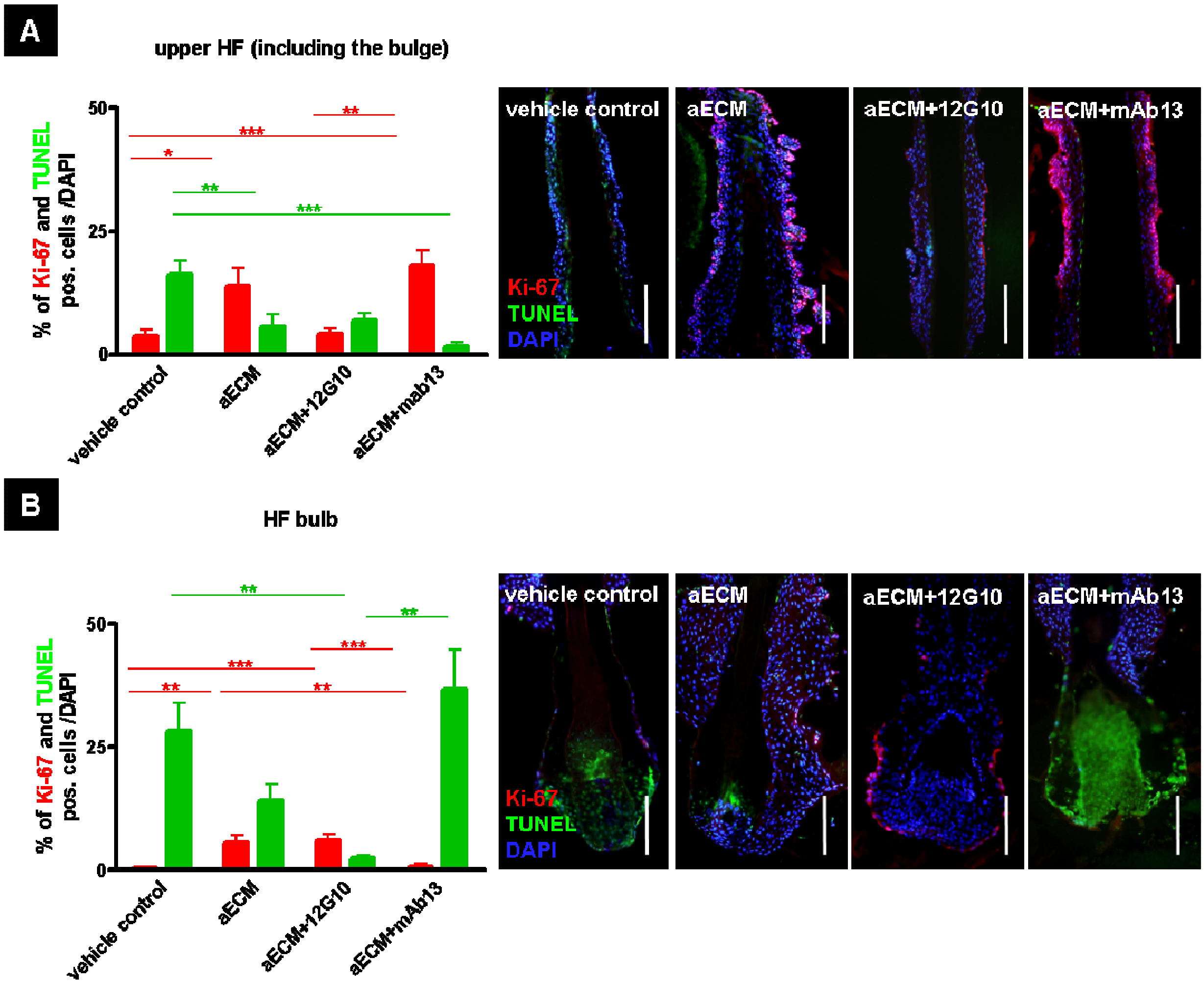



**Figure 46: β1 integrin ligands had a differentially influence on the proliferation and apoptosis of HF epithelium.**
This comparison of the Ki-67/TUNEL-staining results demonstrated differentially influences of β1 integrin ligands, like extracellular matrix components, and the specific receptor antibodies, on distinct HF compartments. (A) The embedding into aECM and the administration of β1 integrin antibodies decreased apoptosis in the upper HF. However, in the aECM and aECM+mAb13-treated group the proliferation rate is up-regulated, but aECM+12G10 is similar to the vehicle control. n=2-3 individuals (7-15 HFs). (B) Ki-67/TUNEL-staining confirmed the influence of β1 integrin ligands on HF bulb cells. In the aECM and aECM+12G10-treated group the number of proliferative cells was significantly higher than in aECM+12G10 and the vehicle control. The inhibitory antibody mAb13 increased apoptosis in HF bulb cells. n=2-3 individuals (8-16 HFs). White scale bars in the representative photos=100µm

Besides I wanted to clarify whether the aECM-incorporated β1 integrin antibodies modify the manipulation related effects on proliferation and apoptosis of the embedded HF epithelium.

Testing these effects showed that the β1 integrin-stimulatory antibody (12G10) reduced apoptosis in the HF bulb, and reduced proliferation in the upper HF compared to the aECM group (Figure 46). Instead, the β1 integrin-inhibitory antibody (mAb13) had the opposite effect and up-regulated apoptosis, yet only in the hair bulb; unexpectedly, it induced proliferation in the upper HF compartments including the bulge (Figure 46).

These antibody stimulation experiments suggest that distinct ePCs in the human HF show a differential proliferation/apoptosis response *in situ* to β1 integrin-mediated signaling and that this differential response may be utilized to functionally distinguish these ePC subpopulations from each other.

### 3.2.3. Extracellular matrix environment stimulates migration mostly in the hair bulb

Next, by focusing on the phenomenon of largest outgrowth mainly in the HF bulb (Figure 45) without a higher ORSK proliferation of the matrix KCs this observation should be examined in detail. Besides proliferation and apoptosis, ORSK outgrowth is likely to be dominated by ORSK migration events. This was gauged by cortactin immunohistomorphometry, since activated cortactin accumulates in actin-enriched lamellipodia and membrane ruffles at the moving edge of migrating epithelial cells, signifying a role in actin network formation (Gendronneau et al., 2008).

Mostly the hair bulbs of dispase-pretreated and subsequently aECM-embedded HFs showed strong activated cortactin IR, prominently expressed in a larger number of focal adhesion-like structures (Murphy and Courtneidge, 2011).

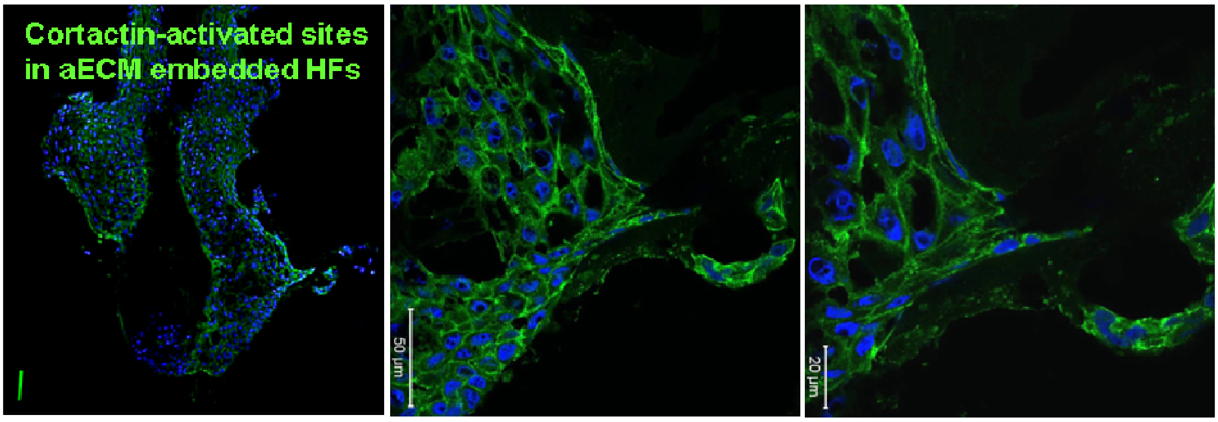



**Figure 47: Cortactin-activated sites are responsible for the largest outgrowth of the ORSKs in the HF bulb.**
Confocal microscopy pictures demonstrating the cortactin-activated sites/filaments (Fraunhofer) in the embedded HF epithelium. Cortactin revealed activated migration mainly in the HF bulb of the aECM-treated group. Green scale bar: 50µm.

ORSKs showed enhanced migration into the provided aECM (Figure 47), which may explain why the largest outgrowth of ORSKs was measured around the HF bulb (Figure 46) although the highest proliferative (Ki-67$^{+}$) capacity of ORSKs was mainly seen in the upper HF (Figure 46). Therefore, the massive ORSK outgrowth seen in our CTS- and BM- mimicking ECM system likely also enhanced ORSK migration in the presence of β1 integrin ligands**.**

### 3.2.4. Different human epithelial progenitor cell populations differ in their dependence on β1 integrin signaling *in situ*

Once the manipulation of β1 integrin-mediated signaling elucidated their impact on proliferation and migration of ORSKs, the outside-in mediated effects of β1 integrin on ePC should be clarify. To dissect the role of β1 integrin ligands in the aECM outgrowth approach with respect to differentiation of adult ePCs in the human HF bulge, the markers K15, CD200, CD71 and K6 (Cotsarelis, 2006; Garza et al., 2011; Kaur et al., 2004; Kloepper et al., 2008b; Ohyama et al., 2006; Sieber-Blum, 2011; Tiede et al., 2009) were analyzed on the gene and protein expression level (Figure 48 - Figure 50). For qRT-PCR the entire dispase-pretreated, embedded and cultured HF epithelium was used. Whereas the K15 and CD200 IR was only present and by this measurable in the upper HF (including the HF bulge; Figure

48), differences of the CD71 and K6 IR was detectable in different HF compartments (Figure 49, Figure 50).

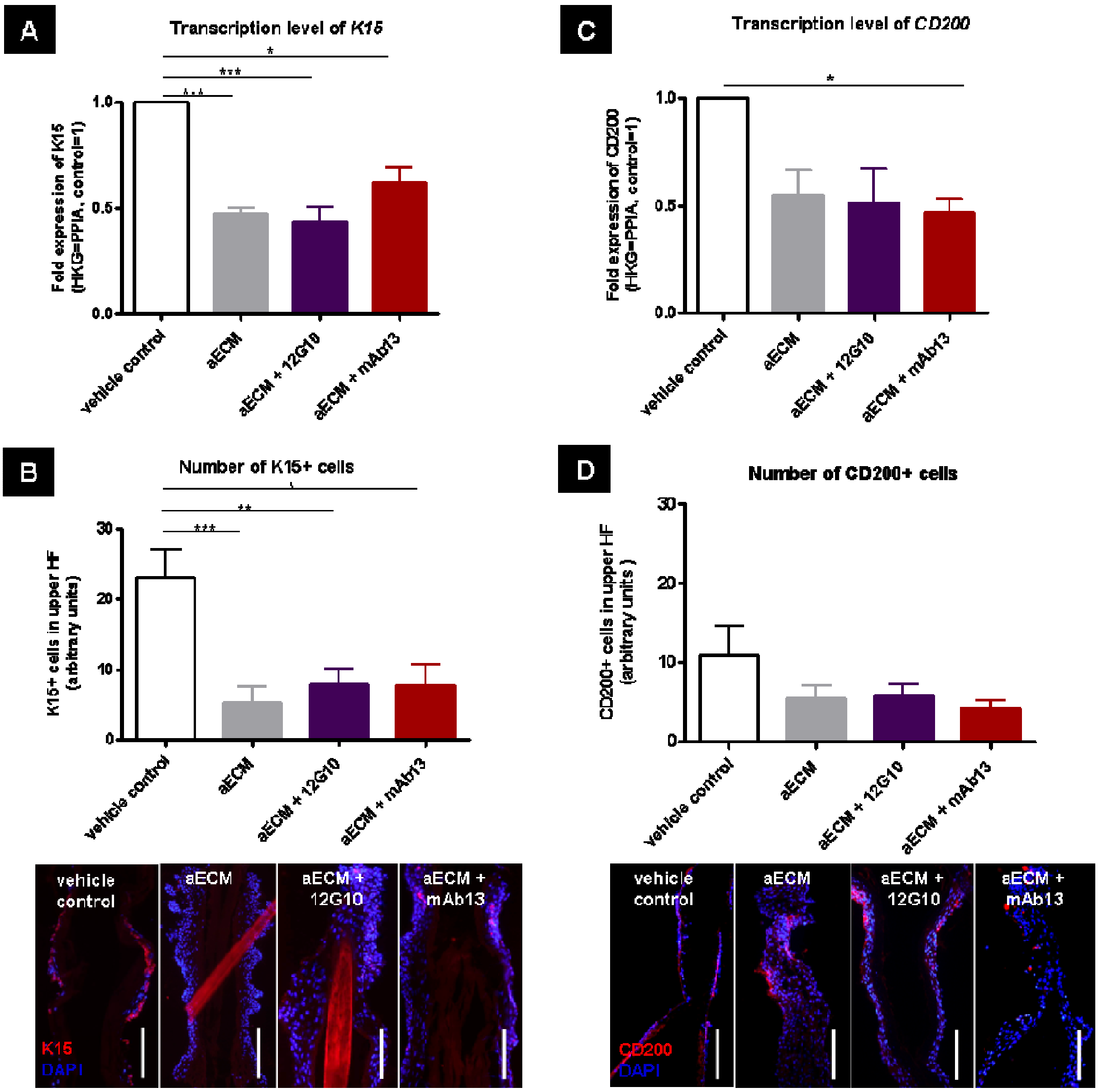



**Figure 48: The embedding into the aECM lead to a tremendous decrease of ePC markers.**
(A) Embedding into the niche mimicking aECM (artificial extracellular matrix) system significantly downregulated the gene expression of the HF (hair follicle) progenitor marker Keratin 15 (K15). The β1 integrin inhibiting antibody mAb13 increased the K15 transcription. (B) Immunoreactivity of K15 was only found in the upper HF including the bulge. By counting K15$^+$ cells in a specified area (250x125) the decrease of this progenitor marker was measurable. n=7-15 HFs of 3-4 individuals. (C) Embedding into the aECM system β1 integrin antibodies significantly reduced the gene expression of CD200. (E) CD200$^+$ cells were also only found in the upper HF including the bulge. The CD200$^+$ cells were counted in a specified area (250x125) and confirmed the gene expression results of this progenitor marker. n=7-10 HFs of 2-3 individuals. White scale bars in the representative photos=100µm. All data were analysed by using the One way ANOVA, Bonferroni post hoc test, mean

+/- SEM (*p<0.05, **p<0.01, ***p<0.001). Abbreviation: aECM = artificial ECM consisting of Matrigel®, collagen I and K-SFM (keratinocyte-serum free medium), aECM+12G10=aECM supplemented with the activating β1 integrin antibody 12G10, aECM+mAb13=aECM supplemented with the inhibitory β1 integrin antibody mAb13, HKG=housekeeping gene, PPIA=peptidylprolyl isomerase A.

The upper HF including the bulge showed that the HF-ECM mimicking system significantly down-regulated the expression of the ePC markers K15 and CD200 on the gene and protein level in contrast to the vehicle control (dispase-pretreated and cultured without MG®/collagen I) (Figure 48A-D). Supplementation of β1 integrin inhibitory antibody mAb13 to HFs, embedded in the ECM system, slightly increased again the *Keratin15* (*K15*) gene expression (Figure 48A), whereas 12G10 demonstrated no strong influence on the epithelial progenitor cell markers K15 and CD200 gene and protein expression (Figure 48A-D).

These results imply the differentiation–inducing capacity of the employed artificial reconstituted HF-like ECM (aECM, MG® 1:1 collagen I in K-SFM). Thus, embedding of the HF epithelium diminished significantly the $K15^{+}$ ePC population regardless of the usage of β1 integrin antibodies.

Further, the differentiation-inducing capacity of aECM should be investigated by the analysis of the early differentiation marker K6. The dispase-pretreated HF epithelium embedded in aECM and 12G10-treated embedded HFs showed a reduction of the transcription level of K6 (Figure 49 A), while the IR pattern demonstrated a strong differentiation inducing capacity in the whole HF (Figure 49 B) – mainly in the HF bulb and lower HF. Opposite results were obtained for the standard organ-cultured denuded HFs (vehicle control) compared to denuded, embedded and mAb13-treated HFs with regard to qRT-PCR and IR of K6 (Figure 49 A,B).

The analysis of the K6 gene and protein expression displayed a biphasic signaling effect like in the vehicle control. But it seemed that these two β1 integrin antibodies acted as opponents in the K6 expression independently from the investigation of the gene or protein expression (Figure 49 A,B). Interestingly, the β1 integrin inhibitory antibody mAb13 enhanced the K6 gene expression (Figure 49 A), but lead to a K6 immunoreactivity decrease in the whole HF (Figure 49 B).

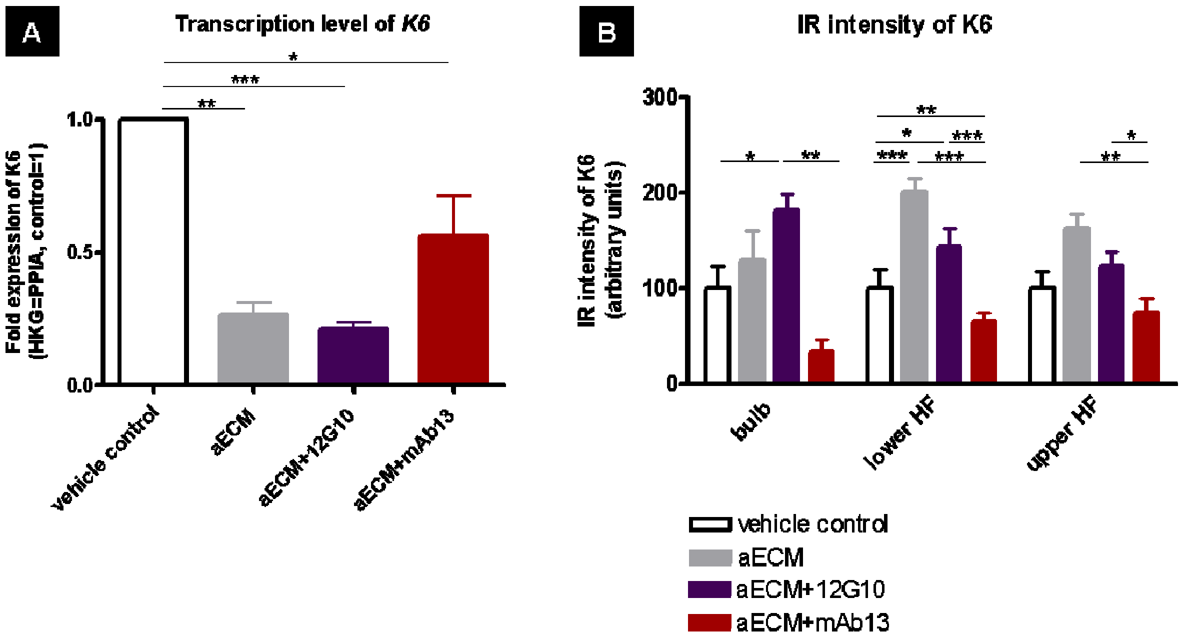



**Figure 49: K6 displayed a biphasic signaling effect on the gene and protein expression.** Differentiation of epithelial progenitor cells is regulated by β1 integrin ligands. (A) The *Keratin 6 (K6)* gene expression was strongly repressed in the aECM and aECM+12G10-treated group, while this reduction is not so high in the mAb13-treated group. n=1 (2) individuals in experimental triplicates (12-15 HFs). (B) The IR expression pattern of K6 was analyzed in the HF bulb, lower HF and upper HF by quantitative immunohistochemistry in fixed rectangle. The supplementation of the inhibitory antibody mAb13 reduced the differentiation inducing capacity of the artificial ECM system in the whole HF. n=3 patients (4-7 HFs). All data were analysed by using the One way ANOVA, Bonferroni post hoc test, mean +/- SEM (*p<0.05, **p<0.01, ***p<0.001).

Finally, the analysis of the transit amplifying cell marker CD71 should completed the question whether manipulation of the β1 integrin-mediated signaling impact on the ePC differentiation.

This evaluation showed the embedding of HF epithelium into the aECM alone, but also the further treatment with the activating antibody 12G10 or the inhibitory mAb13 reduced the CD71 transcription (Figure 50 A) in the same manner. By analyzing the CD71 IR (and thus the number of transit amplifying cells) a significant increase in the HF bulbs could be demonstrated mainly by the activating antibody 12G10, but not the inhibitory mAb13 (Figure 50 B). These controversial data on the gene and protein level may be explained by the half-life of protein moiety of CD71, which is only degraded after about 60 h, but their mRNA is controlled by binding to ribosomes (Omary and Trowbridge, 1981; Ralston et al., 1997).

The given data suggested that inhibiting or activating β1 integrin signaling elicits differential responses in adult human HF ePCs compared to their more committed epithelial progeny.

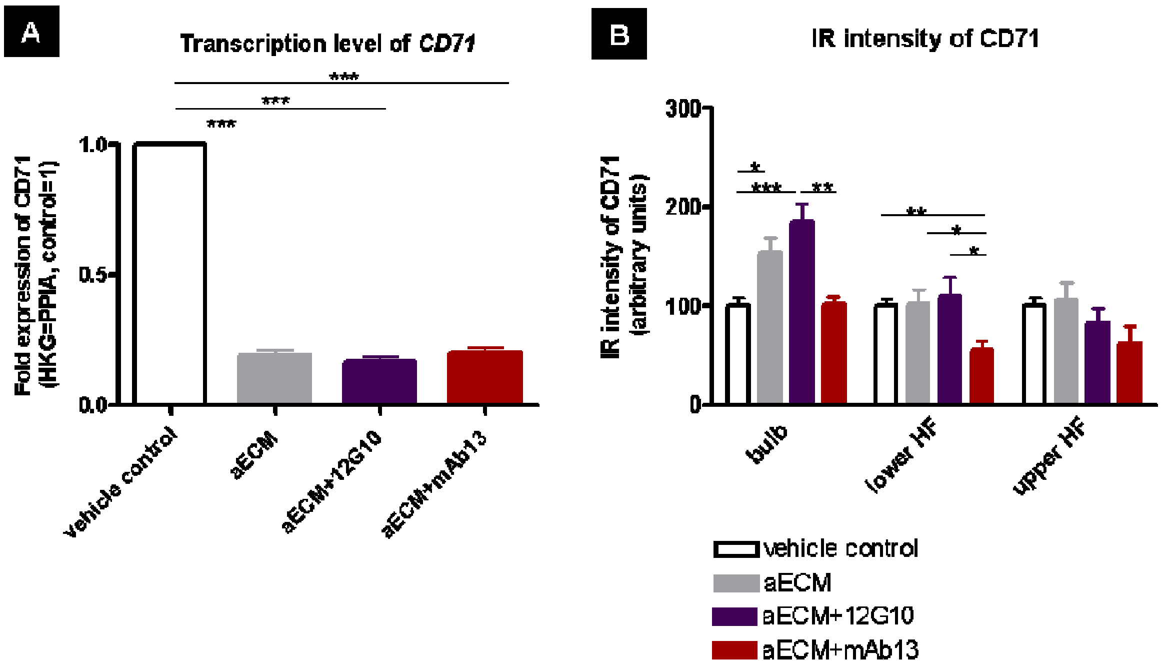



**Figure 50: The activating antibody 12G10 significantly enhanced the CD71 IR in the HF bulb.**
(A) *CD71* transcription was significantly reduced by the embedding into the CTS- and BM mimicking environment (aECM). (B) The analysis of the CD71 IR showed a strong influence of the activating antibody 12G10 on the transit amplifying population mainly in the HF bulb. All data were analyzed by using the One way ANOVA, Bonferroni post hoc test, Mean +/- SEM (*p < 0.05, **p < 0.01, ***p < 0.001). Abbreviation: aECM = artificial ECM consisting of Matrigel®, collagen I and K-SFM (keratinocyte-serum free medium), aECM+12G10 = aECM supplemented with the activating β1 integrin antibody 12G10, aECM+mAb13 = aECM supplemented with the inhibitory β1 integrin antibody mAb13, HKG = housekeeping gene, PPIA = peptidylprolyl isomerase A.

Thus, although our CTS- and BM- mimicking ECM components, which are expected to mimic endogenous β1 integrin ligands, optimize the survival of HF epithelium, the same ligands reduce the ePC reservoir in the human HF bulge and push this rapidly proliferating compartment of the HF epithelium towards differentiation.

### 3.2.5. Inhibiting or activating β1 integrin signaling allows stimulation of epithelial progenitor cells and their progeny located in distinct hair follicle compartments

Since this had never been tested before in human epithelium *in situ*, I also wanted to examine if anti-integrin antibodies impact on β1 integrin transcription in adult human scalp HFs *in situ*. qRT-PCR showed that the aECM-incorporated stimulatory β1 integrin antibody (12G10) demonstrated no further upregulation on day 4 in comparison to the aECM group (Figure 51). Instead, the inhibitory mAb13 antibody down-regulated β1 integrin gene expression in human HFs *in situ* (Figure 51). This is the first demonstration of a direct transcriptional effect of the inhibitory antibody on β1 integrin gene expression in a human mini-organ.

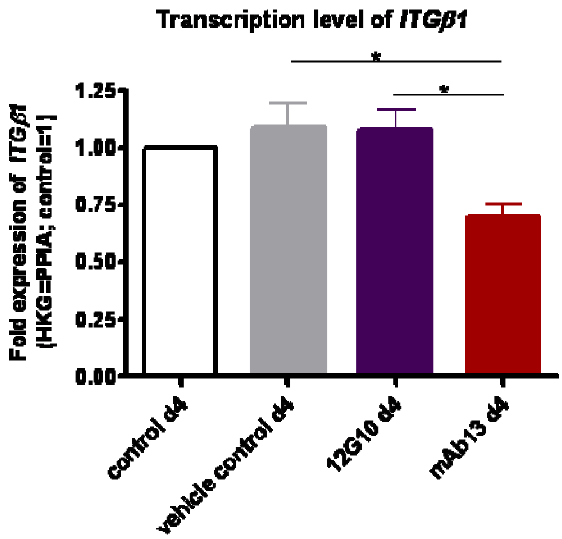



**Figure 51: The inhibtory antibody mAb13 significantly decreased the *β1 integrin* expression.**
The β1 integrin-activating antibody 12G10 did not alter *β1 integrin* gene expression, whereas the inhibition of the receptor via mAb13 significantly reduced *β1 integrin* expression. n=1-2 individuals in experimental triplicates (15 hair follicles).

Next, I wanted to clarify whether distinct subpopulations of human ePCs and their progeny *in situ* showed a differential response pattern to the stimulation with antibodies that either stimulate or inhibit β1 integrin-mediated signaling (Akiyama et al., 1989; Gibson et al., 2005; Kloepper et al., 2008a; Tuckwell et al., 2000). Indeed, this was the case (Figure 46A,B; Figure 50A-D). Especially the β1 integrin signaling in the HF bulb was influenced by the activating antibody 12G10 concerning the proliferation (Figure 46), K6 (Figure 49) and CD71 IR (Figure 50) as well as corresponding differences in ORSK outgrowth in two defined HF compartments (HF bulb and the upper HF). While the β1 integrin activating antibody 12G10 enhanced ORSK outgrowth mainly in the HF bulb (Figure 52), interestingly in the upper HF (including the bulge), epithelial cell outgrowth was stimulated by the inhibitory antibody mAb13 (Figure 52).

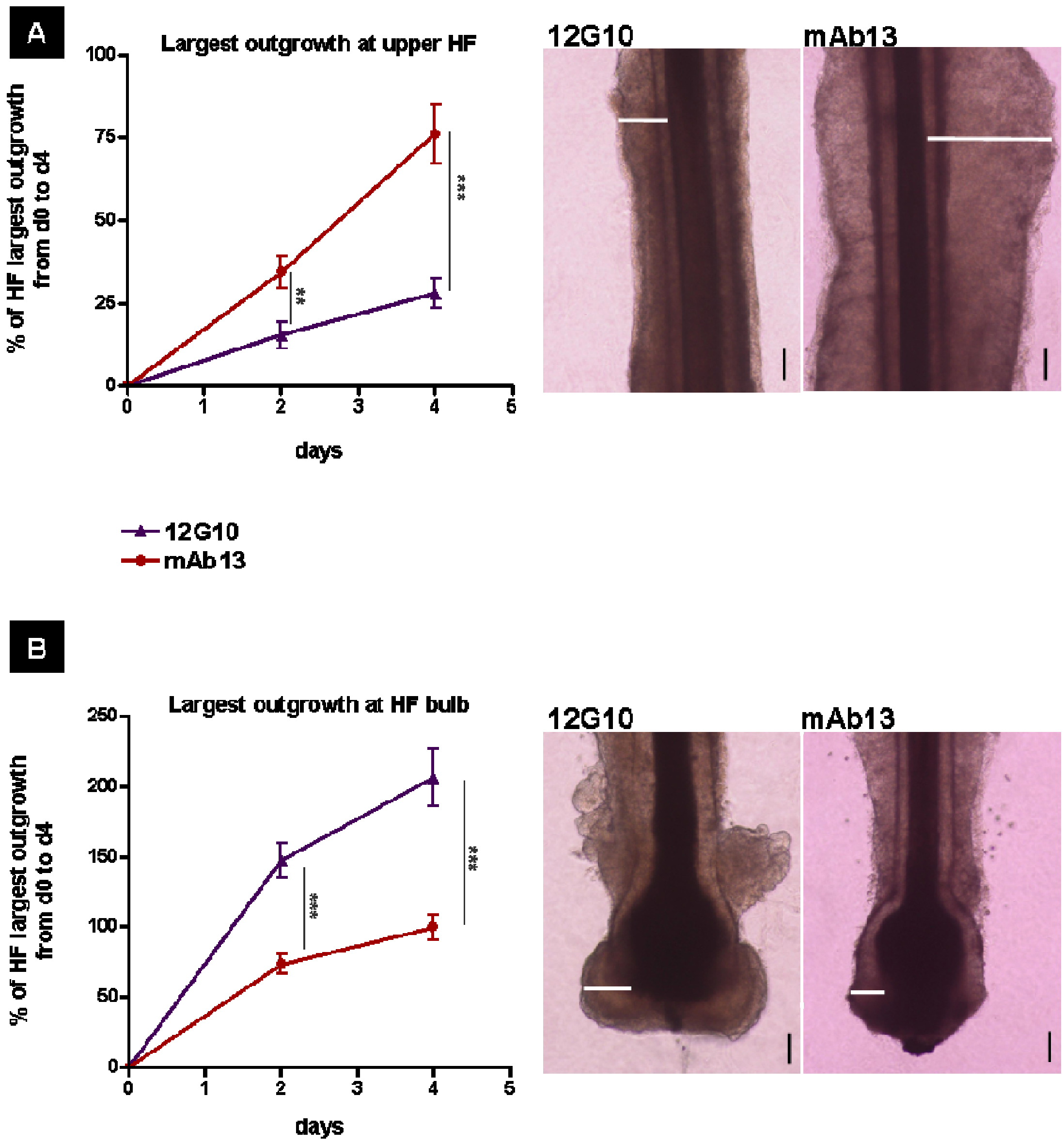



**Figure 52: Largest outgrowth presents a differential response of HF ORSKs by activating or inhibiting the β1 integrin-mediated signaling.**
(A) Measurement of the largest outgrowth in the upper HF and (B) HF bulb over 4 days displayed the large influence of the β1 integrin antibody treatment and distinguished between the different epithelial progenitor cell populations via their response to β1 integrin antibody stimulation. In the

upper HF region (including the bulge) the inhibiting β1 integrin antibody mAb13 significantly stimulated epithelial outgrowth, whereas in the HF bulb the activating antibody 12G10 antibody stimulated epithelial outgrowth. Photos show dispase-pretreated upper HFs and HF bulbs after embedding into the aECM (artificial extracellular matrix) system and treated with β1 integrin antibodies at day 4. White lines demarcate the reference areas. n = 4 patients (18-33 HFs). Mean +/- SEM, using unpaired t-test (**p < 0.01, ***p < 0.001). Scale bars: 100 µm. Abbreviation: aECM = artificial ECM consisting of MG®, collagen I and K-SFM (keratinocyte-serum free medium), aECM+12G10 = aECM supplemented with the activating β1 integrin antibody 12G10, aECM+mAb13 = aECM supplemented with the inhibiting β1 integrin antibody mAb13.

So, the closer examination of the manipulated β1 integrin-mediated signaling via specific activating and inhibitory antibodies suggested a different reponse capacity of the ePC population within the HF. The ligand-occupied-binding β1 integrin antibody 12G10 had a differentiation-inducing capacity by enhancing the K6 and CD71 protein expression as well as the largest outgrowth in the HF bulb and the lower HF. In contrast, the ligand-unoccupied-binding β1 integrin antibody mAb13 stimulated the largest outgrowth and reduced significantly the K6 protein expression in the upper HF (including the bulge).

### 3.2.6. QLT0267 impacts on β1 integrin-mediated signaling in human hair follicle epithelium

As a first step towards dissecting the mechanisms by which β1 integrin-mediated signaling impacts on human ePCs and their progeny *in situ*, I used the putative ILK inhibitor QLT0267 (Eke et al., 2009; Wang et al., 2010) to probe the role of ILK. This cytoplasmic adaptor protein of β1 and β3 integrin plays a key role in many β1 integrin-mediated cellular processes, including actin rearrangement, cell adhesion, migration, proliferation, apoptosis and differentiation by associating with different regulatory proteins (Azimifar et al., 2012; Judah et al., 2012; Leyme et al., 2012; Sayedyahossein et al., 2012; Widmaier et al., 2012; Yu and Luo, 2011). Since ILK protein expression has not yet been demonstrated in human HFs, this was first tested by Western blot. Indeed, human dispase-pretreated HF epithelium expressed ILK protein as the expected 53kDa band was detected (Figure 53).

Using QLT0267 for this study the ILK blocking effects on human HFs *in situ* should analyzed with focussing on ORSK survival and migration. HF epithelium was embedded in aECM, supplemented with 100µM QLT0267 or without (vehicle control). Already during HF culture it became evident that approximately 40% of the aECM-embedded and QLT0267-treated HFs lost their adhesion to the Matrigel®/collagen milieu after 4 days of culture, a first overt evidence for massively reduced ILK activity to create FAs.

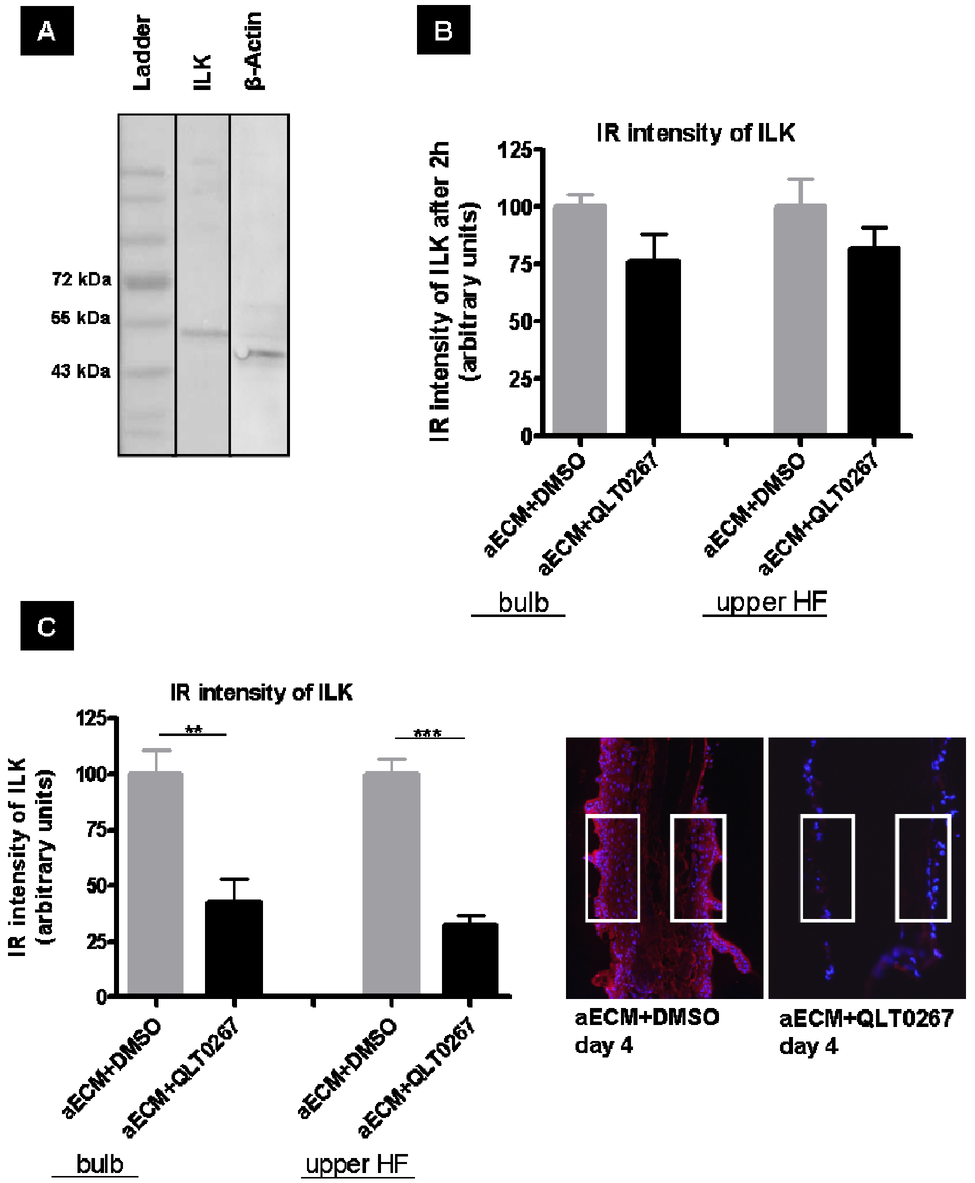



**Figure 53: Significant pharmacological inhibiton of ILK.**
(A) Integrin-linked kinase (ILK) is expressed in human dispase-pretreated HF (hair follicle) keratinocytes, which was demonstrated by using the Western blot method in comparison to the protein expression of β-Actin. (B) Intergin-linked kinase (ILK) IR intensity in human HFs after 2h incubation in 37°C with or without QLT0267 before embedding into the artificial extracellular matrix demonstrated the fast reduction/inhibiton of ILK in outer root sheath keratinocytes. (C) In the HF bulb and in the upper HF a significant reduction of ILK immunoreactivity could be demonstrated with QLT0267 treatment in comparison to our control HFs, which were dispase-pretreated and embedded

in the artificial extracellular matrix with DMSO. Representative photos show the reference areas in the upper HF, n=3 individuals (7-10 HFs). All statistical analyses were done by using Mann-Whitney test, (*p<0.05, **p < 0.01, ***p < 0.001).

The reduction of ILK protein expression was already 2 hours after QLT0267 incubation (100µM) detectable by a slightly reduction in ILK IR within the isolated HFs (Figure 53B) and further documented by analyzing ILK protein IR at day 4. Both in the HF bulb and in the upper HF QLT0267 induced a significant reduction of ILK IR (by 60-70%) (Figure 53C). Furthermore, QLT0267 treatment also induced substantial HF dystrophy, and almost abolished both ORSK outgrowth (Figure 54A,B) as well as cortactin-activated migration (Figure 55).

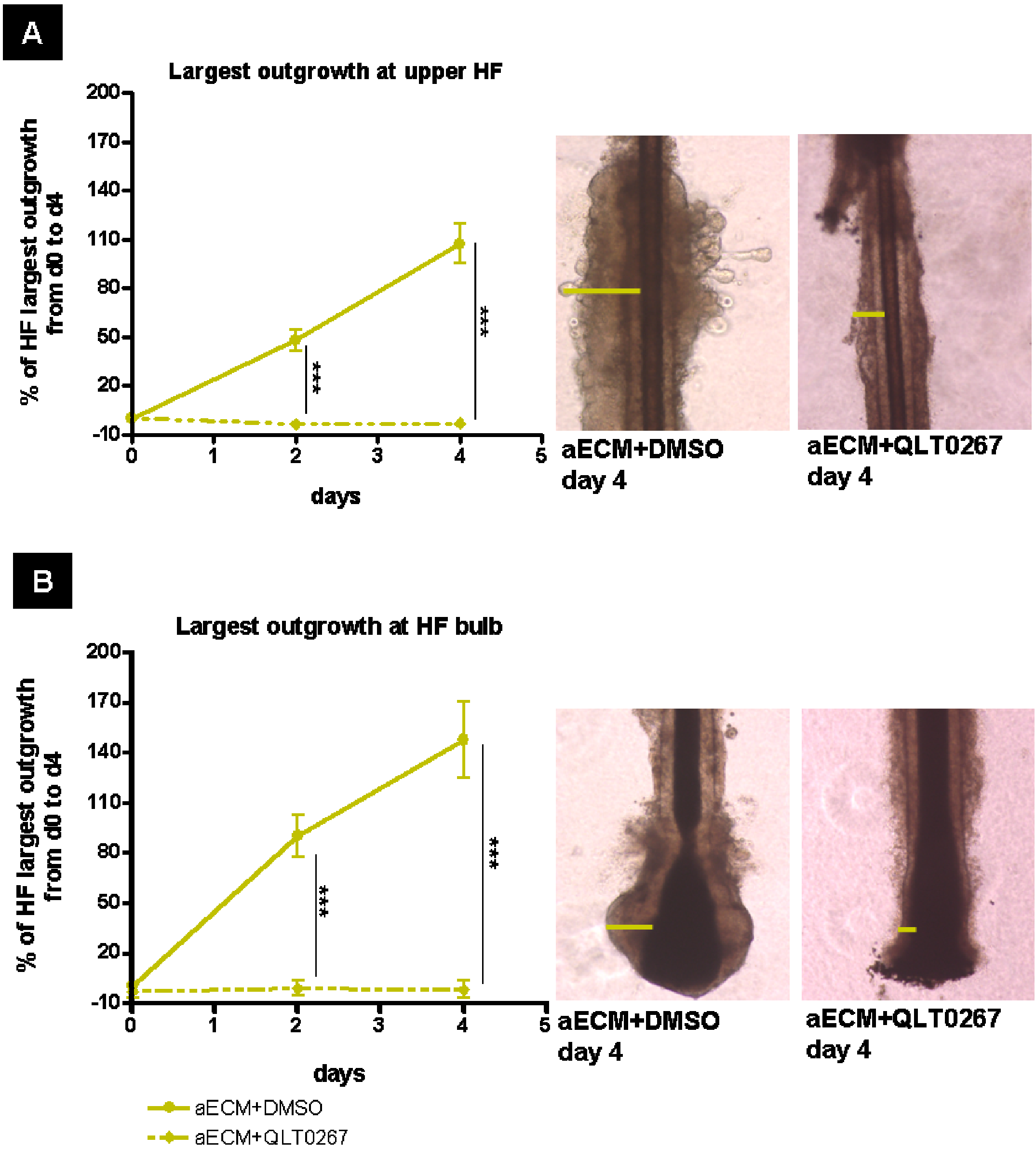



**Figure 54: Assessment of the largest outgrowth revealed the inhibitory effect of QLT0267.** (A) The analysis of the largest outgrowth in the upper HF and (B) the HF bulb revealed the strong inhibitory effect of the pharmacological substance QLT0267 for the proliferative and migrative capacity. Representative photos of the embedded HF epithelium after 4 days of culture. n=3 individuals (24-28 HFs). All statistical analyses were done by using Mann-Whitney test, ***$p < 0.001$). Abbreviation: aECM+DMSO= artificial ECM consisting of Matrigel®, collagen I and K-SFM (keratinocyte-serum free medium), aECM+QLT0267 = aECM supplemented with the 100µM pharmacological inhibitor QLT0267, DMSO = Dimethyl sulfoxide.

This demonstrated that human ORSK migration *in situ* and F-actin cytoskeleton remodelling (Azimifar et al., 2012) critically depend on ILK-mediated signaling via a Src activation of proteins like cortactin, which is mandatory for their phosphorylation and thereby for actin assembly (Tehrani et al., 2007).

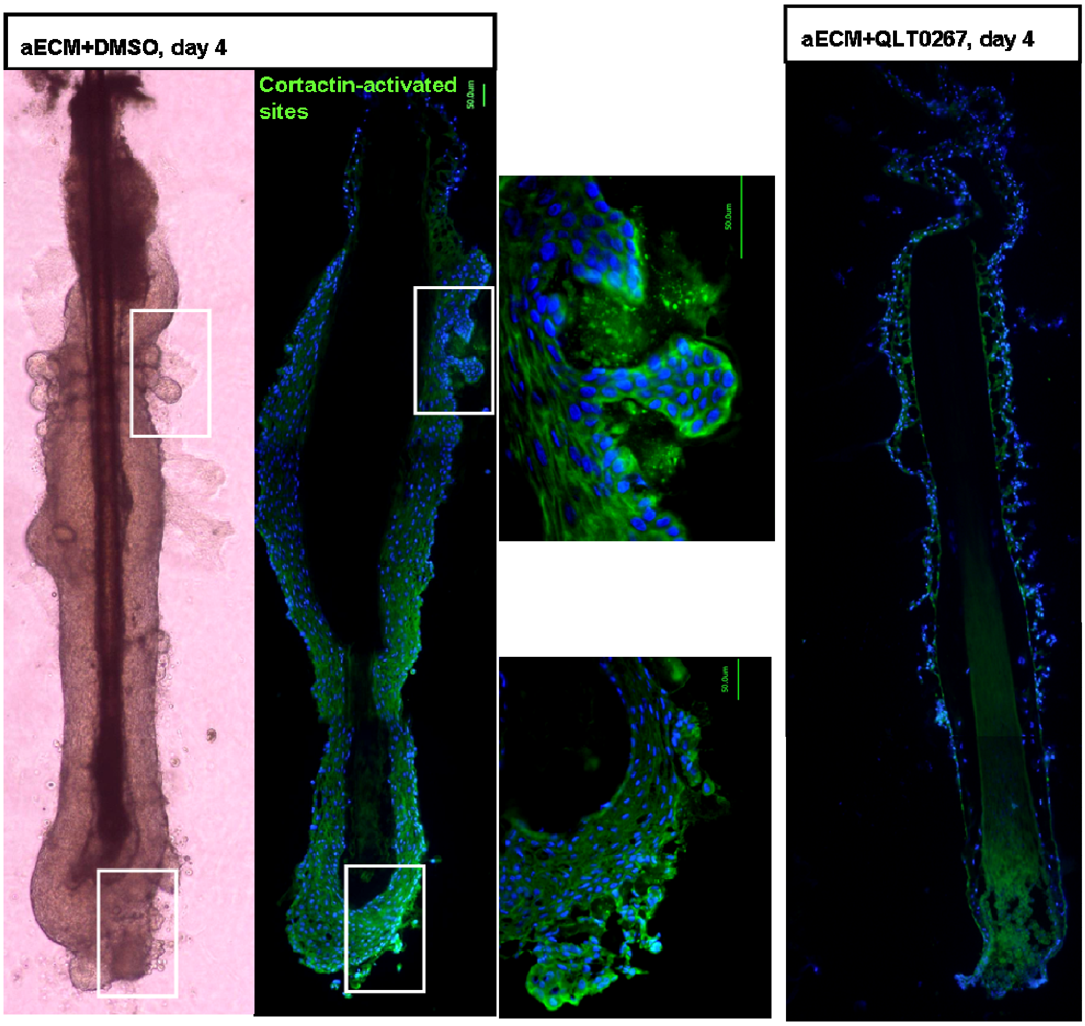



**Figure 55: Integrin-linked kinase inhibition via QLT0267 inhibits keratinocyte migration.** The cortactin immunoreactivity was nearly absent in the aECM+QLT0267-treated HFs compared to the aECM+DMSO-treated group on day 4. This demonstrated that human outer root sheath keratinocyte migration *in situ* and F-actin cytoskeleton remodelling [59] depend on integrin-linked kinase (ILK)-mediated signaling via a Src (Proto-oncogene tyrosine-protein kinase) activation of proteins like cortactin. Abbreviation: aECM+DMSO= artificial ECM consisting of Matrigel®, collagen I and K-SFM (keratinocyte-serum free medium), aECM+QLT0267 = aECM supplemented with the 100µM pharmacological inhibitor QLT0267, DMSO = Dimethyl sulfoxide.

In further, the question should be clarified whether the absent outgrowth potential was not only caused by a disruption of the ILK-mediated cortactin activation via the Src pathway but also by a modified survival capacity.

Already the DAPI staining revealed numerous pyknotic nuclei in the HF epithelium which indicated the high level of HF dystrophy and apoptosis induced by QLT0267 treatment (data not shown). This was confirmed by quantitative immunohistomorphometry for cleaved caspase 3 (Figure 56A), TUNEL and Ki-67, which documented massive intraepithelial apoptosis and cessation of ORSK proliferation (Figure 56B). Therefore, pharmacological inhibition of ILK likely induced anoikis, i.e. cell death due to a loss of connection with the ECM or adjacent cells (Attwell et al., 2000; Kim et al., 2012), thereby destroying the entire ORS.

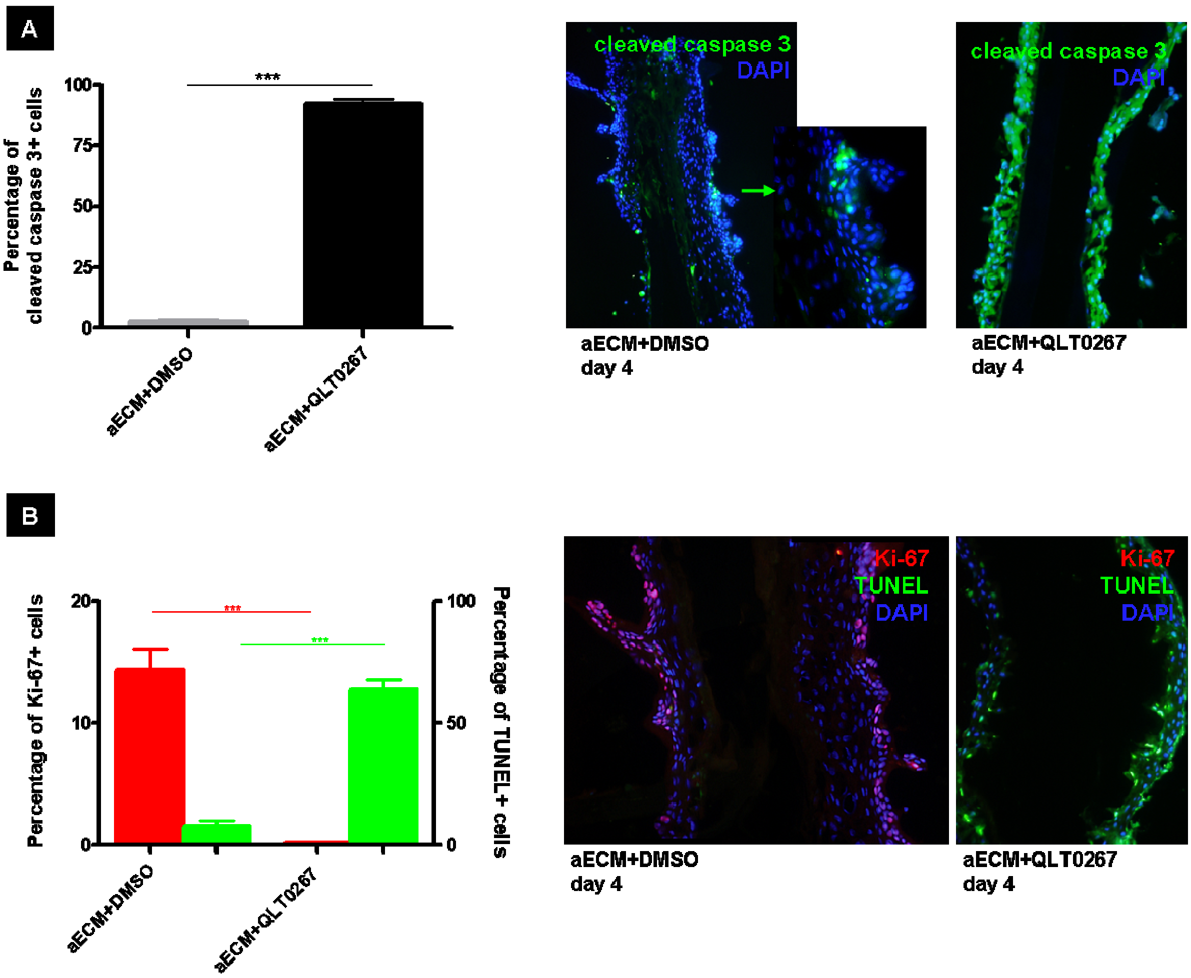



**Figure 56: The QLT0267 treatment caused a tremendous apoptotic effect in the HF epithelium.**

(A) Graphic showing a high cell death with nearly 100% cleaved caspase 3+ outer root sheath keratinocytes. n=3 patients (5-10 HFs). (B) Ki-67/TUNEL-staining confirmed the caspase 3 staining, while the QLT0267-treated HFs showed no proliferation, but a high apoptosis rate in comparison to the DMSO-treated control. n=3 patients (7-12 HFs). All statistical analysis were done by using Mann-Whitney test, (***$p < 0.001$); Mean +/- SEM. Abbreviation: aECM+DMSO= artificial ECM consisting of Matrigel®, collagen I and K-SFM (keratinocyte-serum free medium), aECM+QLT0267 = aECM supplemented with the 100µM pharmacological inhibitor QLT0267, DMSO = Dimethyl sulfoxide, ILK = integrin-linked kinase, IR = immunoreactivity.

In summary, this study provide the first evidence in a human complex system that ILK-dependent β1 integrin-mediated signaling is mandatory for the adhesion of basal layer ORSK to the ECM thus stabilizing cell-ECM connection via FAs as well as promoting survival of human HF epithelium.

## 3.3. The role of β1 integrin-mediated signaling in human skin wound healing

The established model of wound healing in full-thickness adult human skin (including subcutaneous fat) demonstrated (Meier et al., 2013) a gainful method to analyze human reepithelization. β1 integrin is described to have a tremendous role in wound healing because of their signaling effects on KCs concerning migration, proliferation and adhesion the severity of receptor manipulation should be evaluated.

### 3.3.1. β1 integrin binding antibodies display different states of activation of β1 integrin receptors in wounded skin

The specific β1 integrin binding antibodies 12G10 (mouse anti-human) and mAb13 (rat anti-human) are established as functional activators or inhibitors of its receptor binding on the β-I domain. By adding these antibodies into the serum-free "punch in a punch" assay I want to analyze their influences on β1 integrin-mediated signaling in the human reepithelization.

First, the question should be clarified whether the administered antibodies are abled to penetrate into the skin punch, which would allow a uniform influence on the β1 integrin-mediated signaling. For that the antibody-treated wounded skin were counterstained with the specific secondary antibodies (rat against mAb13-treated skin and mouse against 12G10-treated skin).

The staining displayed different activation states of β1 integrin in the skin after 3 days of culture (as well as to all other study days of culture – day1, day 6, but data not shown). While the IR pattern of 12G10 reflected the ligand-occupied state (Figure 57 A1) to BM components like laminin or fibronectin (Figure 57 A2+A3), showed the counterstaining of mAb13-treated skin that the number of ligand-unoccupied β1 integrins (Figure 57 B1) increased upwards of the epidermis (Figure 57 B2+B3).

Further I wanted to clarify if the Ki-67/TUNEL staining confirmed these binding patterns of the different β1 integrin binding antibodies (Figure 57 A4+B4) by using the anti-mouse binding rhodamin-linked secondary antibody. Indeed, this was the case.

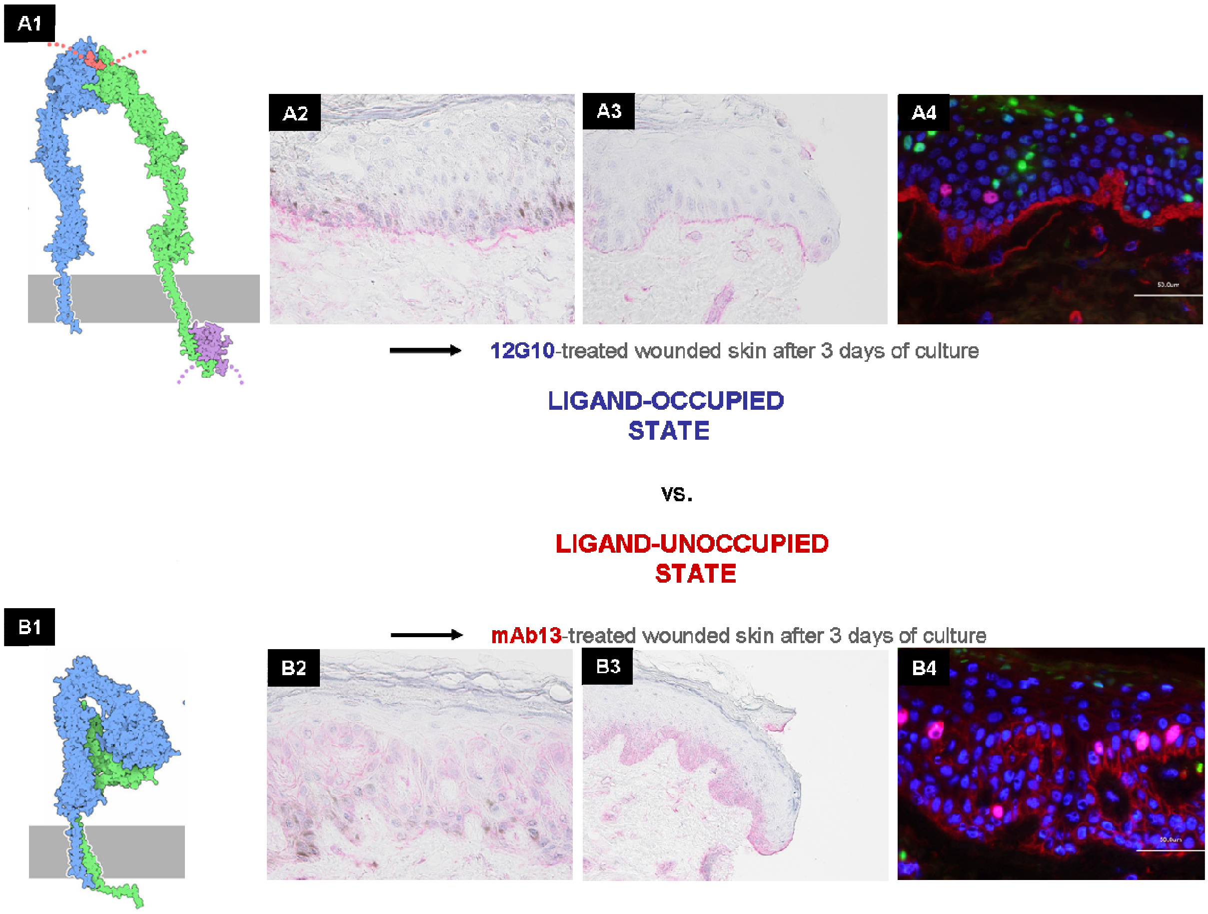



**Figure 57: Different ligand-occupied states of β1 integrin shown with the activating and inhibitory antibody.**
(A1) This schematic drawing depicts a high affinity and (B1) a low affinity integrin (http://www.rcsb.org/pdb/education_discussion/molecule_of_the_month/images/mom134_integrins.jpg). Wounded skin treated with the β1 integrin activating antibody 12G10 (A2-4) or inhibitory antibody mAb13 (B2-4) were stained with their specific secondary antibody or stained for Ki-67/TUNEL and demonstrated a distinct distribution of different activation states of β1 integrin.

These stainings suggested that the β1 integrin antibodies, adiministred to the serum-free medium, penetrate into the wounded skin and bind on their specific receptor point. Besides these different activation states of β1 integrin within the IFE the downregulation of this receptor was observed which demonstrated the initiation of terminal differentiation through the suprabasal layers.

### 3.3.2. Manipulation of β1 integrin-mediated signaling lead to a disturbed reepithelization of wounded skin

The measurement of the reepithelization should discover if the manipulation via the activating and inhibitory β1 integrin antibodies may change the normal wound healing under culture conditions. The reepithelization of the wounded skin was followed over 6 days of culture to verify the influences of the specific β1 integrin antibodies on the human wound healing in periodic acid-Schiff (PAS) stained skin sections.

The measurement of the area as well as the length of the formed epithelial tongues (ETs) demonstrated a strong inhibitory effect on the reepithelization during the observed period. Both the activating antibody 12G10 and the inhibitory antibody mAb13 strongly repressed the growth of the outer ETs (Figure 58A, B) in the same way as the inner ETs (data not shown). Mainly the activating antibody 12G10 disturbed the skin reepithelization and lead to a destruction of the epithelial tissue by increasing the ligand-occupied conformation/binding and stabilizing the cell-ECM connection via the β1 integrin receptor.

This specific manipulation of the β1 integrin-mediated signaling displayed the important role of a well-balanced receptor signaling to realize the skin homeostasis and wound healing.

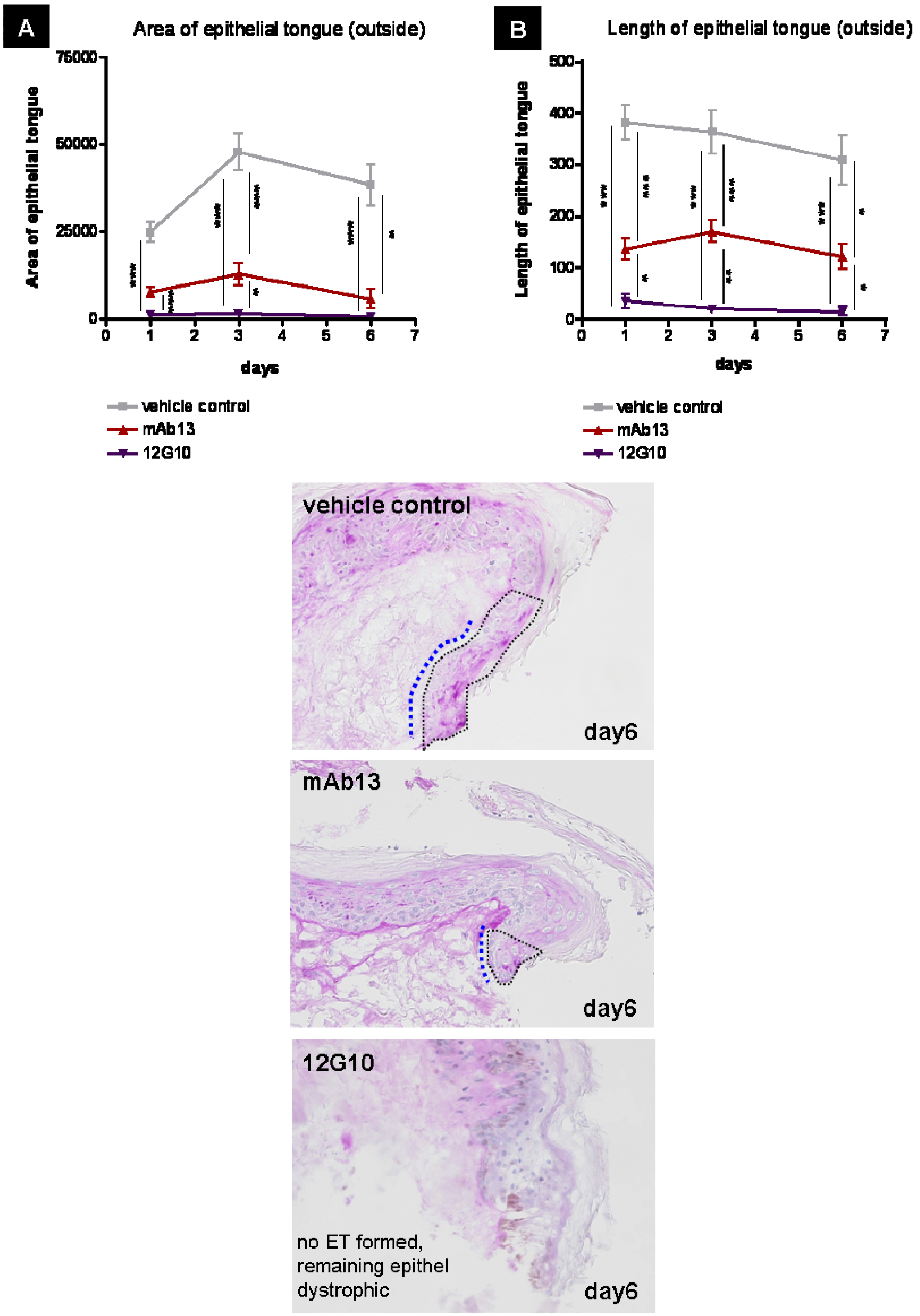



**Figure 58: Both the activating (12G10) and inhibitory (mAb13) β1 integrin antibody inhibit reepithelization.**
By measuring the (A) area (n=4 cultures [ETs = 12-20]) and the (B) length (n = 3 cultures [ETs = 10-18]) of the ET in defined areas the reepithelization was analyzed in periodic acid-Schiff (PAS) stained skin sections. The activating as well as the inhibitory β1 integrin antibodies repressed reepithelization. All data were analyzed by using the One way ANOVA, Bonferroni post hoc test, Mean +/- SEM (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). Representative photos of outer ETs after 6 days of culture, 200x magnification.

### 3.3.3. Specific β1 integrin antibodies inhibit the proliferation and have an apoptosis inducing capacity

Having already been revealed the key role of β1 integrin-mediated signaling for the proliferation of ORSKs and thus the particular relevance of this receptor was confirmed, the influence of β1 integrin should be examined in the wound-stimulated proliferation. For this purpose, I sought to correlate the observed effects on ET formation to proliferation and apoptosis markers.

The Ki-67/TUNEL staining attested the measurements of the reepithelization (area and length of the outer and inner ETs), because both 12G10 and mAb13 reduced the proliferation, but enhanced the apoptosis of KCs in comparison to the vehicle control (Figure 59) after 6 days of culture (similar results were achieved at day 1 and 3, but data not shown). Moreover, by stimulating β1 integrin via the activating antibody 12G10 the proliferation was nearly blocked.

Regardless of whether the activating or inhibitory antibodies were supplemented for manipulation of the β1 integrin-mediated signaling, the wound-activated proliferation has been reduced.

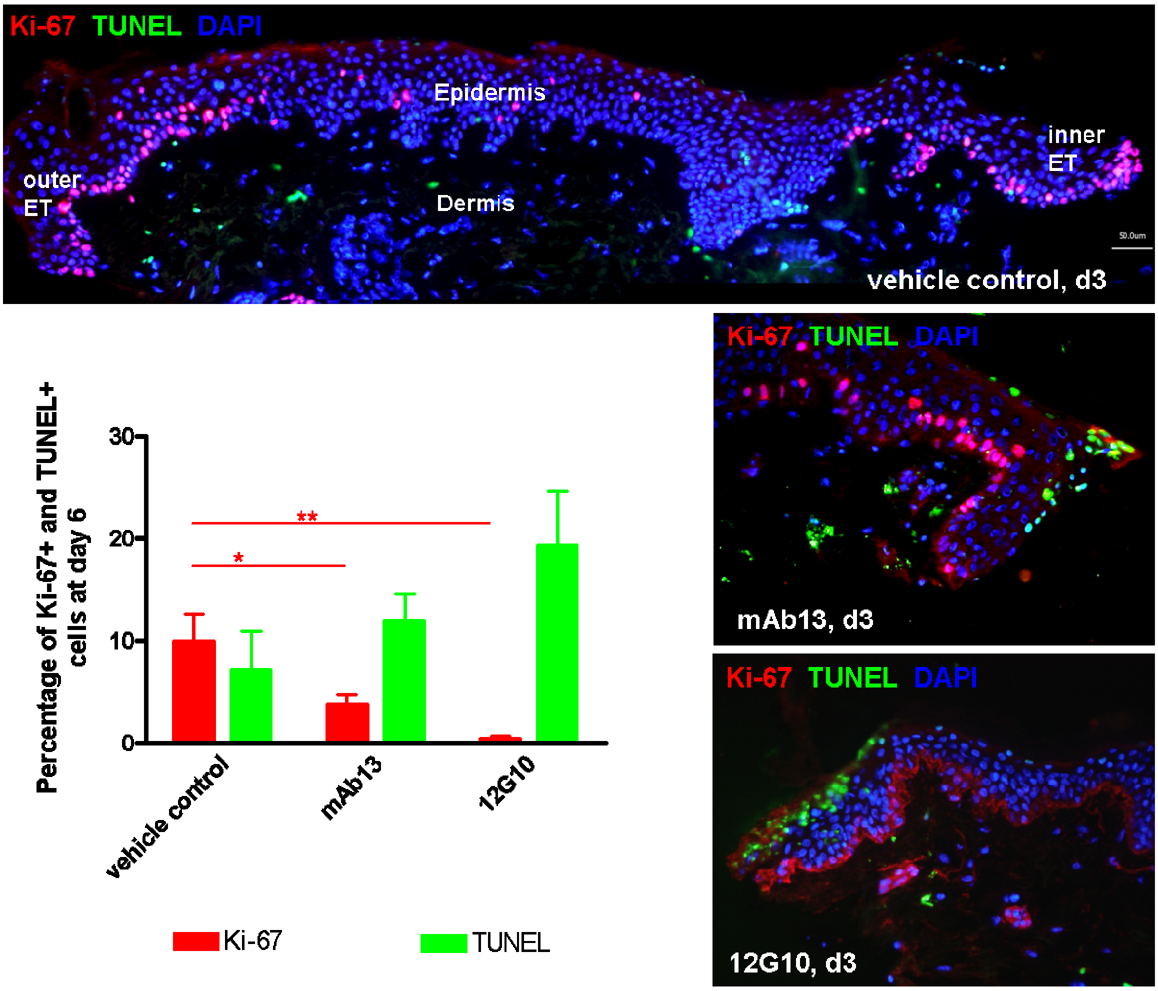



**Figure 59: 12G10 displays the strongest inhibitory effect on wound-activated proliferation.** The number of apoptotic (TUNEL) and proliferating (Ki-67) cells in the newly generated epithelial tongue (ET) was calculated as the percentage to the total number of DAPI positive cells in the same ET area (n=3 cultures [ETs = 8-10]). The activating β1 integrin antibody 12G10 displayed the strongest proliferation-inhibiting and apoptosis-inducing effect. All data were analyzed by using the One way ANOVA, Bonferroni post hoc test, Mean +/- SEM (*p < 0.05, **p < 0.01). Representative photos of outer ETs after 3 days of culture, 200x magnification.

### 3.3.4. Reduced migration-active sites in the epithelial tongues of β1 integrin antibody-treated wounded skin

The reepithelization is based, besides proliferative KCs, on an efficient migration of these cells for closing wounds, which is mainly controlled via β1 integrin-mediated signaling (Margadant et al., 2010; Raja et al., 2007). Hence, the question arose whether the lack of wound healing in antibody-treated skin is also caused by a disturbed KC migration.

Therefore, the assessment of cortactin immunoreactivity (IR), as a sensitive marker of migrating KCs (Gendronneau et al., 2008) was used. The following exemplary pictures of day 3 and day 6 showed that the manipulation of β1 integrin via the activating and inhibitory antibodies reduced the cortactin activation around the periphery of the ET KCs.

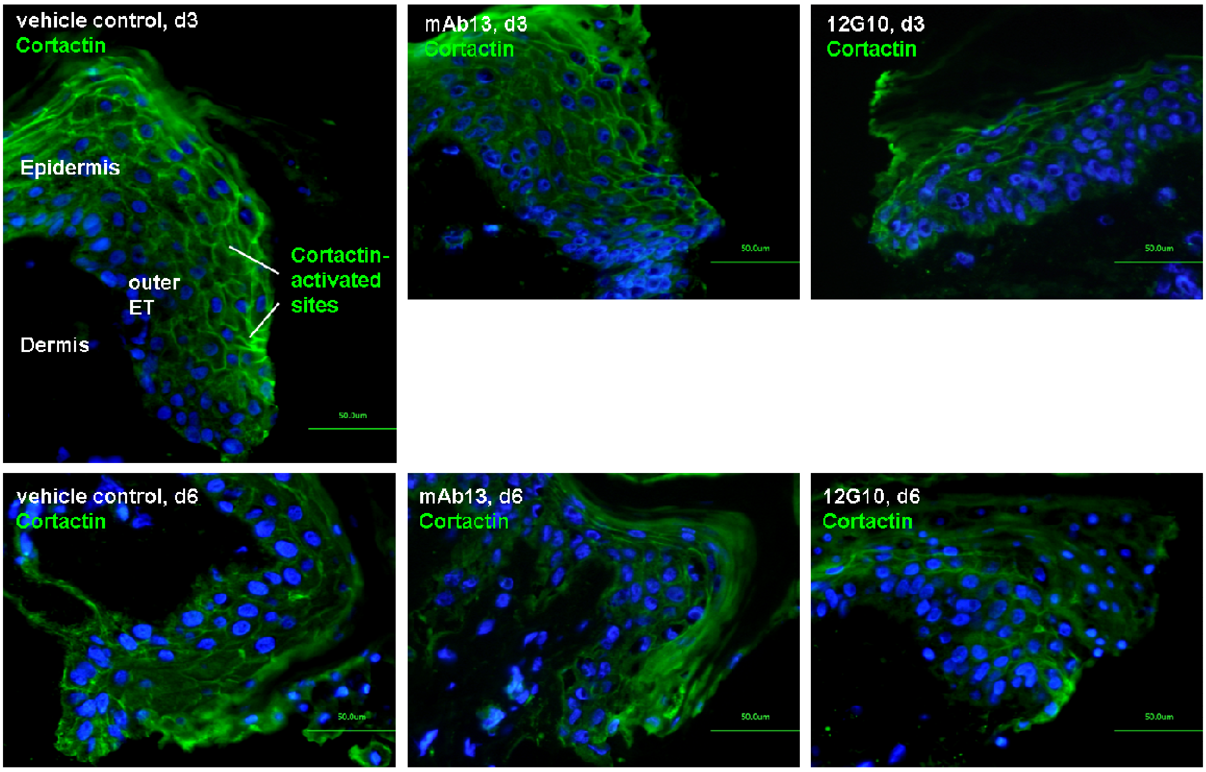



**Figure 60: Manipulation of the β1 integrin-mediated signaling inhibits the migration.** Cortactin-activated sites are decreased in the analyzed outer and inner ETs of the antibody-treated wounded skin at day 3 and 6. Representative pictures of the outer ETs are shown.

These data facilitated, that the combination of inhibited proliferation and migration, as well as increased apoptosis is responsible for the significantly reduced formation of ETs in human wounded skin. So, the usage of manipulative antibodies against a specific receptor interrupted the tissue homeostasis and by this demonstrated its well-balanced necessity concerning proliferation and migration.

### 3.3.5. Differentiation is inhibited by the activating β1 integrin antibody

The already proven particular relevance of β1 integrin-mediated signaling for the maintenance of ePCs within the human HFs led to the issue if the administered β1 integrin manipulating antibodies stimulate/influence the ePC population in the IFE. The assessment of ePC markers, like K15 and CD200, and of the early differentiaton marker K6 should clarify a possible activation of distinct cell populations by the β1 integrin-mediated signaling. To prevent non-specific IR biotin-coupled primary antibodies were used for these determinations of the wounded skin.

Interestingly, the K15 IR as well as the CD200 IR was not activated in the outer and inner ETs of the vehicle control (n=3 individuals) after wounding of human skin. Also the antibody manipulation did not lead to an increasing of the analyzed ePC markers during the culture period of 6 days (data not shown).

As distinct from the non-regulated ePC markers the K6 protein expression was significantly inhibited by the activating antibody 12G10 in the inner ETs after 3 days of culture, but not in the vehicle control and mAb13-treated wounded skin (Figure 61 A1-A3). The same tendency was observed in the outer ETs, but without any significance (data not shown).

These analyses confirmed the strongest reepithelization-inhibitory effect by the β1 integrin activating antibody 12G10 because of its reduction of the wound healing-associated epithelial differentiation.

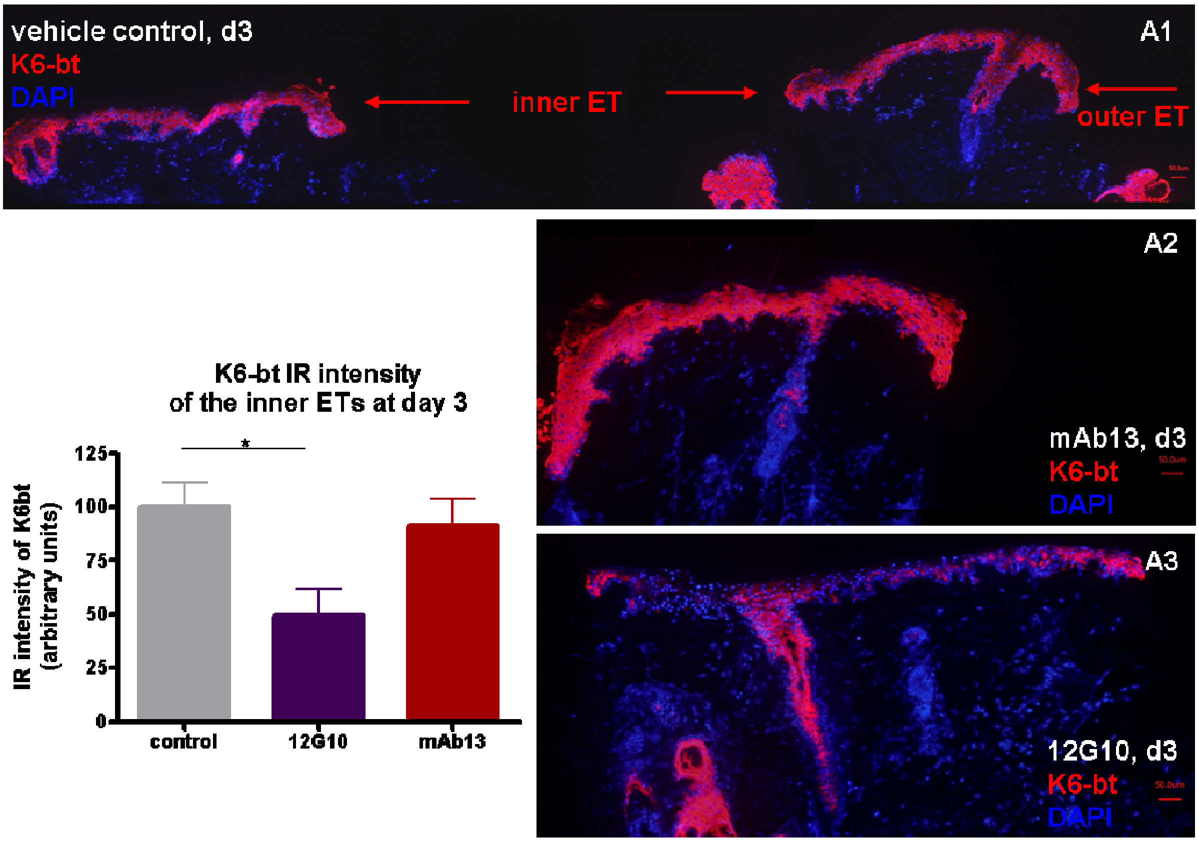



**Figure 61: Differentiation is inhibited by 12G10 treatment.**
The activating β1 integrin antibody 12G10 lead to a reduction of the K6 immunoreactivity in ETs after 3 days of culture (A1-3); n = 2 individuals (8ETs). Representative photos of inner ETs after 3 days of culture. Abbreviation: K6bt = Keratin 6-biotin coupled.

# 4. Discussion

Until now most data on β1 integrin-mediated ePC behaviour or wound healing derive from murine models (Fassler and Meyer, 1995; Nakrieko et al., 2008; Piwko-Czuchra et al., 2009; Raghavan et al., 2000; Stephens et al., 1995) or cell culture experiments (Goel et al., 2010; Kariya et al.; Meng et al., 2009; Mould et al., 1996). However, the role of β1 integrin-mediated signaling in human epithelial cells growing within their natural tissue habitat had been largely unknown. The current thesis project helps to close this gap and demonstrates that human HF organ culture provides an excellent research tool in this context.

Using organ-cultured human scalp HFs as a clinically relevant model for studying human ePCs *in situ*, I show here that β1 integrin signaling control survival, adhesion, and migration in distinct ORS cell populations, including human HF eSCs and their progeny. Moreover, the current data suggest that β1 integrin signaling is fundamental for maintenance of the HF bulge eSC niche, while different human HF ePC subpopulations differ in their response to β1 integrin signaling. This may help to functionally distinguish these human ePC subpopulations from each other. The examined HF effects of β1 integrin signaling are ILK-dependent. Taken together, this thesis provides new, physiologically relevant insights into the role of β1 integrin-mediated signaling in human epithelial biology. These may also be utilized for cell-based regenerative medicine strategies that employ human HF-derived ePCs, e.g. for the promotion of cutaneous wound healing.

The current thesis project also provides the first pilot data that suggest that β1 integrin-mediated signaling is a functionally important parameter in human skin reepithelization. The manipulation of β1 integrin-mediated signaling via the activating and the inhibitory antibody reduce the reepithelization of human wounded skin. The activating antibody 12G10 displayed the strongest migration and differentiation inhibiting potential, likely because of the enhanced ligand binding to the basement membrane.

The first challenge of my thesis was the specific reduction of β1 integrin expression in the complex human HF. This transient transfection are done with a cocktail of three β1 integrin-specific siRNAs or scrambled control RNAs by using a commercial, established protocol (Santa Cruz) to optimize the knockdown in this full-length HFs. Also other studies used this knockdown method and already demonstrated a silencing of specific HF proteins, like P-cadherin (Samuelov et al., 2012) or cannabinoid receptor 1 (CB1) (Sugawara et al., 2012). Thus, contrary to conventional wisdom, gene silencing within a human (mini) organ, rather than only in cultured cells, can be achieved. This greatly extends the range of mechanistic studies that can be performed in human HF organ culture (Kloepper et al., 2008a).

Though my method of siRNA *β1 integrin* silencing was functionally effective and was documented on the mRNA level, on the protein level, no significant change in the immunoreactivity intensitiy was detectable in comparison to the scrambled control 4 days after transfection. While the half-life of integrins on the surface of cultured human KCs *in vitro* reportedly is about 12 h (Hotchin et al., 1995), their half-life *in situ* and *in vivo* is much longer. For example, in murine epidermis, β1 integrin can still be detected *in vivo* 10 days after Cre activation and in some HFs β1 integrin IR is even visible after 1-2 weeks (Brakebusch et al., 2000; Lopez-Rovira et al., 2005). This likely explained the discrepancy of the mRNA and protein results after knockdown.

Interestingly, by using the activating (12G10) and inhibitory (mAb13) β1 integrin antibodies a different silencing effect on conformation-dependent epitopes was observed. The β1 integrin staining with mAb13 detecting the bent and low-affinity β1 integrin domain reveal that the transfection reaction alone lead to a reduction of low-affinity β1 integrins on the cell surface of ORSKs in all HF compartments.

That β1 integrin might be needed as a niche receptor for regulating proliferation activity in distinct ePC populations in the human HF bulb and bulge is consistent with the demonstration that neonatal K5Cre β1 null-mice show HF and sebaceous gland loss, and greatly reduced epithelial proliferation, likely all due to a loss of eHFSCs (Lopez-Rovira et al., 2005). The current silencing results in this complex human mini-organ also are in line with our previous finding that the stimulation of β1 integrin-mediated signaling enhances the proliferation of hair matrix KCs in organ-cultured human HFs, using the β1 integrin-activating antibody 12G10 (Kloepper et al., 2008a). Reduction on the mRNA level of this receptor which was quite important for the niche cell-ECM interaction decreased the proliferation capacity of the active HF matrix cells, but also the slow-cycling ePCs in the HF bulge. Thus also in this complex human organ the β1 integrin-regulated proliferation is proven.

Surprisingly, when measured the strictly S-phase specific incorporation of EdU (a "false" nucleotide similar to BrdU) (Wang et al., 2011a) the same proliferation rate like Ki-67 in the HF bulge were implied. Though the number of $EdU^+$ KCs should be considerably lesser in comparison to the widely expressed protein Ki-67(G(1)-, S-, G(2)- und M-phase) (Kee et al., 2002). However, this was only quantified for a small amount of HFs from one individual. Therefore, these should be interpreted with caution. So, further studies need to clarify these results.

One main challenge of this thesis was the clarification of the relevance of β1 integrin for the ePC maintenance in a very complex human (mini-)organ. K15 and CD200 are well accepted as ePC markers of the HF bulge (Cotsarelis, 2006; Cotsarelis et al., 1990; Fujiwara et al., 2011; Kloepper et al., 2008b; Ohyama and Kobayashi, 2012; Ohyama et al., 2006; Sellheyer and Nelson, 2012), but whether the expression of these markers is β1 integrin-dependent in this compartment remains unclear.

The current knockdown data now show that β1 integrin signaling is necessary for keeping human ePCs in an undifferentiated state *in situ*, i.e. for maintenance of $K15^+$ and $CD200^+$ ORSKs in the HF bulge. The K15 gene and protein expression was restricted to the ORSKs of the full-length HFs, but mainly in the HF bulge. And primarily in this HF compartment K15 were significantly reduced. In contrast, CD200 is, besides the fact that it demarcates ePCs of the HF bulge, an immunoinhibitory membrane protein which is expressed by a broad range of cell types, including thymus, nervous system, vascular endothelium, ovary, and various cells of the immune system (Gorczynski, 2012; Rosenblum et al., 2006). The absent of the significant reduction of the CD200 mRNA level was possibly due to the substantial presence of residual CD200 transcripts also in the HF mesenchyme, whose mRNA was also included in the extracts used for qRT-PCR analysis from whole, intact human HFs. These mesenchymal CD200 transcripts may have masked an effect on β1 integrin-dependent CD200 transcription within the HF epithelium (especially of the HF bulge).

The role of β1 integrin in the maintenance of eSCs or ePCs is still controversially discussed. Jones and Watt proposed a role of β1 integrin signaling for the maintenance of human skin eSCs *in vitro*, because these cells expressed high levels of β1 integrin and showed typical SC properties like high CFE (Jones and Watt, 1993). Also recent literature of Watt claims β1 integrin as a typical marker for highly undifferentiated human epidermal cells (interfollicular epidermal SCs) within the basal layer of the epidermis which is downregulated in the suprabasal, differentiating cell layers (Giangreco et al., 2009; Tan et al., 2013).

In contrast, a direct link between the loss of β1 integrin skin-specific conditional KO to an ePC or eSC reduction could not be elucidated in mutant mice (Piwko-Czuchra et al., 2009). Moreover β1 integrin protein levels *in situ* are not markedly higher in the human bulge than elsewhere in the human ORS (Kloepper et al., 2008a). Nevertheless, this thesis project shows that β1 integrin silencing impacts on K15 and CD200 expression in a complex human mini-organ, the HF. Taken together; this suggests that β1 integrin-mediated signaling is indeed required for ePC maintenance in adult human HFs.

In contrast, the analysis concerning the differentiation-modulatory capacity of the β1 integrin KD demonstrated no significant influence on the K6 or CD71 expression. This represented deductive evidence that a reduction of the β1 integrin transcription impacts on the proliferation and ePC population in the HF bulge, but doesn't accelerate the differentiation of the KCs within the HF.

Since the human bulge likely represents an immunologically privileged SC niche (Harries et al., 2013; Harries and Paus, 2010), it was interesting that β1 integrin silencing also reduced HF bulge protein expression of the immunoinhibitory "no danger"-signal, CD200. Future functional experiments, therefore, will need to clarify whether this reduced CD200 protein expression compromised the relative HF bulge immune privilege in human HFs. Clinically, this may be relevant for irreversible forms of human hair loss characterized by a loss of K15$^{+}$/CD200$^{+}$ bulge cells and a collapse of the HF bulge immune privilege (Harries and Paus, 2010), like cicatricial alopecia and lichen planopilaris (Harries et al., 2013), where insufficient β1 integrin-mediated signaling may contribute to the CD200-

dependent component of the HF bulge immune privilege collapse demonstrated in this scarring hair loss disorder (Harries and Paus, 2010).

Thus, the usage of human, transient silenced HFs to study the influence of β1 integrin signaling on various ePCs within their natural tissue habitat clarifies that the receptor effects are different depending on the ePC population and localization within the HF.

This thesis project also aimed at obtaining new insights into the β1 integrin-mediated signaling by manipulating the outside-in signaling of the receptor (Boscher and Nabi, 2013; Fu et al., 2012; Hu and Luo, 2012; Xue et al., 2013) via different ligands on the maintenance, differentiation and/or migration of distinct human ePC subpopulations within the HF. For the direct manipulation of β1 integrin-mediated signaling on ePC populations of the HF epithelium removal of the HFs BM and CTS by dispase appeared necessary. This, however, artificially disrupts cell-ECM connections and greatly dysregulates the surrounding ECM environment, likely inducing a broad range of abnormalities (Byron et al., 2013). Among the employed HF organ culture conditions, this unphysiological environment of denuded HF epithelium is further cultured by the absence of serum components such that may promote β1 integrin signaling, like EGF (Boscher and Nabi, 2013), thus severely compromising the normal conditions for outgrowth and survival of the HF epithelium.

To optimize this defective ECM environment, I used only Matrigel® for embedding the HF epithelium which contains important niche factors like laminin and collagen type IV, guided by previous work (Aasen and Izpisua Belmonte, 2010). They embedded plucked hairs into Matrigel® to generate induced pluripotent stem (iPS) cells. However, in contrast to their study most HFs lost their adhesion to this provided surrogate matrix after several days. This might result from the activity of enzymes like matrix metalloproteinases (MMPs) which are expressed in HFs for degrading ECM components and by this contribute to HF growth and cycling (Morisaki et al., 2013; Paus et al., 1994; Stenn and Paus, 2001; Yuspa et al., 1993). But such enzymes require a physiological balance between their activity and their specific inhibitors for a controlled function (Stenn and Paus, 2001). Adding collagen I to Matrigel® (1:1) may support the embedding and further culturing of HF epithelium due to its fibrillar structure because it enhances the strength and stability of my given matrix. Consequently, the composition of this artificial environment mimicked at least some of the lost ECM signals that normally arise from the HF's CTS and BM and constitutively stimulate β1 integrin-expressing basal layer ORS KCs, and thus enabled human HF ORSK outgrowth *in situ* and ORSK emigration.

The observed huge outgrowth mainly in the HF bulb were correlated with the proliferative activity of these cells, but interestingly the opposite effects on proliferation and apoptosis in the HF bulb and bulge was noticed. Consequently, the activated cortactin in the HF bulbs embedded in the aECM, which accumulates in actin-enriched lamellipodia moving edge of migrating epithelial cells (Gendronneau et al., 2008) appears to be the key for the maximal outgrowth in this HF compartment. The components of the surrogate matrix induce/enhance the β1 integrin-mediated migration of the matrix KCs within the HF epithelium.

Moreover, also the reduction of the ePC markers, like K15 and CD200 in these embedded HFs, confirmed the widely appreciated effect of Matrigel® as a stimulator of proliferation and differentiation (Ma et al., 2008). Thus, this mouse sarcoma-derived matrix enhanced the migratory and survival potential of the embedded HF epithelium, but does not appear to be an optimal surrogate of the HF ECM for mimicking the human SC niche *in situ* and for keeping human ePCs in an undifferentiated state.

Another disadvantage of this artificial ECM is its highly variable composition, not only in the quantity and mixture of individual ECM compounds, such as laminin and collagen IV (Hughes et al., 2010; Villa-Diaz et al., 2012) but also in its greatly fluctuating contents and activity of growth factors like the transforming growth factor beta (TGF-beta), epidermal growth factor (EGF), insulin-like growth factor 1 (IGF-1), bovine fibroblast growth factor (bFGF), and platelet-derived growth factor (PDGF) (Vukicevic et al., 1992). Because of the close cooperation of growth factors and integrins carefulness is needed to interpret the influence on cellular behaviour related to the used Matrigel®/collagen I matrix (Ivaska and Heino, 2011; Vukicevic et al., 1992).

With this background, better-defined human-derived alternative ECM composites are urgently needed (Hughes et al., 2010; Uemura et al., 2009). Alternatively, it may be advisable to use "growth factor reduced (GFR)" Matrigel® for further studies dealing with signaling pathway. This specialized matrix is more expensive but more defined and characterized.

Interestingly, inhibiting or activating β1 integrin signaling via specific antibodies modulated the functions of human ePCs and their progeny in a highly differential manner, depending on where these cells are located within the HF epithelium. A strong effect on the differentiation-inducing capacity of our aECM environment alone could be shown. But the additional manipulation with the activating β1 integrin antibody (12G10) mainly stimulated ePCs in the HF bulb, by inducing differentiation (K6, CD71), whereas the inhibitory β1 integrin antibody (mAb13) keeps the ePCs in a more undifferentiated state. Yet, the opposite effects seen in the more distally located HF compartment, i.e. the upper HF including the bulge (Figure 62 for details). These results obtained with manipulating β1 integrin activity via specific activating and inhibitory antibodies, therefore, invite the intriguing hypothesis that β1 integrin receptor-mediated signaling in HF matrix cells primarily regulates and stimulates the proliferative capacity and differentiation of the bulb of the HF epithelium. In contrast, β1 integrin signaling in the eSC niche (HF bulge) appears to operate as quiescence signal by outside-in signaling via the surrounding ECM.

mAb13 antibodies are able to inhibit specific kinases, like the AKT (AKT kinase) and FAK (focal adhesion kinase) activity (Castello-Cros et al., 2009), which are intracellular adaptor proteins of β1 integrin and by this mediate the survival, proliferation and migration signals in the cell (Huang et al., 2011; Rho et al., 2010). By using this inhibitory antibody the β1 integrin-mediated signaling in the hair matrix cells of HFs was changed followed by a tremendous apoptosis and no migrative capacity, mainly caused by the inhibition of the AKT and FAK activity. As opposed to this the proliferation quiescence function in the HF bulge altered with mAb13, which lead to an increased Ki-67$^+$ cell number. These results support my hypothesis previously supplied of differentially regulated ePC populations via β1 integrin signaling within the HF. Due to the β1 integrin inhibition by the antibody mAb13 the proliferative capacity as well as the differentiation and migration of the matrix KCs was disturbed which induced apoptosis. Compared with this, mAb13 disrupt the quiescence signal of the HF bulge and thus increased the proliferative activity within this eSC harbouring compartment.

In any case, manipulation of β1 integrin-mediated signaling appears to be one of the means by which the surrounding ECM profoundly and differentially modulates epithelial cell behaviour and distinct ePC subpopulations in human HFs.

The following schematic drawing (Figure 62) of the HF epithelium illustrates/summarizes this differential regulation via β1 integrin antibodies. It compares the clearest differences of activated and inhibited signaling via β1 integrin specific antibodies on the protein expression of different immunoreactivity markers achieved with this study.

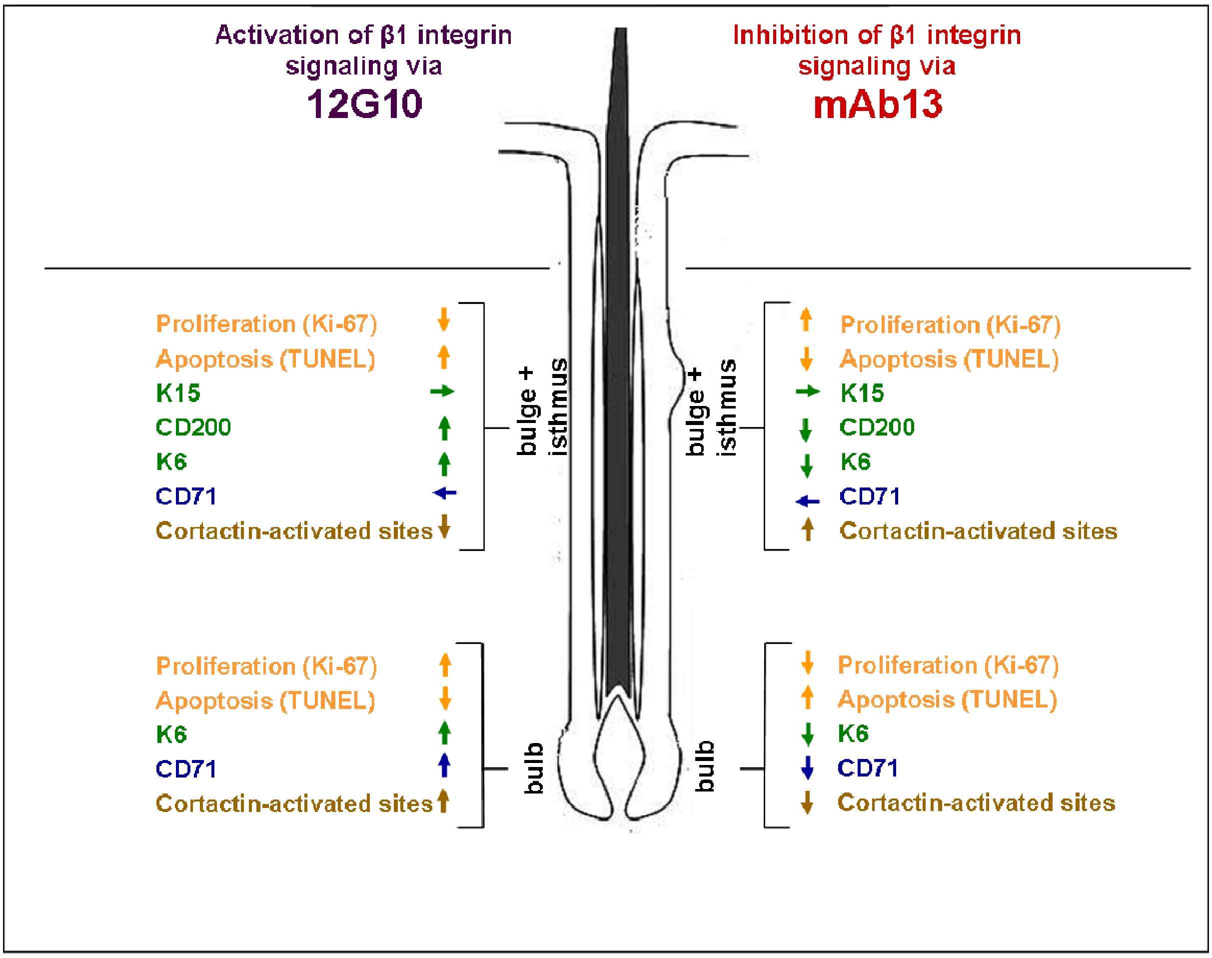



**Figure 62: Comparison of the influences of activated and inhibited signaling via β1 integrin specific antibodies on the protein expression of different immunoreactivity markers.**
The immunoreactivity analysis of the β1 integrin-activating (12G10) or -inhibitory (mAb13) antibody-treated and artificial extracellular matrix medium (aECM) embedded HF epithelium suggested a different response of the endothelial progenitors cell subpopulations on β1 integrin signaling. The application of aECM-incorporated β1 integrin antibodies allowed distinguishing adult human endothelial progenitor cell subpopulations with distinct amplifying capacities *in situ*,

which are located in separate epithelial compartments of human scalp hair follicles. Picture modified from (Kloepper et al., 2008b).

It is controversially debated whether ILK really is a true kinase or just a scaffolding protein. Because there is a researcher community who is still convinced of the mandatory role of ILK-mediated phosphorylations for cell adhesion-mediated cell survival (anoikis), proliferation and mitosis, apoptosis, migration, invasion, and vascularization as well as tumor angiogenesis (Hannigan et al., 1996; Hannigan et al., 2011; Lim et al., 2013; Maydan et al., 2010; Nakrieko et al., 2008; Serrano et al., 2012). But another part published some studies proposing ILK as a pseudokinase and only an essential scaffold protein because mutational, knockin and genetic analysis revealed no kinase activity (Lange et al., 2009; Mackinnon et al., 2002; Wickstrom et al., 2009; Widmaier et al., 2012; Zervas et al., 2001). Furthermore, it has been questioned how the pharmacological inhibitor QLT0267, which was developed to inhibit ATP binding of ILK, really works (Younes et al., 2007).

Until now the role of ILK in cellular processes has been primarily studied in transformed and/or tumorigenic cells (Eke et al., 2009; Lim et al., 2013; McDonald et al., 2008; Wedel et al., 2011), as well as in mouse models (Assi et al., 2011; Judah et al., 2012; Lange et al., 2009; Oloumi et al., 2010), but not in a complex human mini-organ. In defiance of the controversial discussed function of QLT0267 concerning their specifity (Eke et al., 2009) the usage of this putative pharmacological inhibitor of ILK seemed to be the most pragmatic choice for the experimental setup of my thesis. During the culture of 4 days it was necessary to maintain the transient ILK inhibition or silencing by administering an ILK-inhibitory substance to the artificial matrix including HF epithelium.

An efficient reduction of ILK expression *in situ* via QLT0276, the potent apoptosis-inducing capacity (Eke et al., 2009; Lim et al., 2013) and the loss of adhesion (abrogates ORSK migration) in pharmacologically ILK-blockaded human HFs was demonstrated. Former studies with this pharmacological inhibitor also confirmed an effective reduction of ILK activity as well as a decrease of AKT and FAK phosphorylation in tumor cell lines (Eke et al., 2009; Kalra et al., 2009; Younes et al., 2007). β1 integrin-mediated signaling, transmitted via the adaptor protein ILK among others, is already proven to be quite necessary for adhesion and proliferation on Matrigel® (Rowland et al., 2009). Its reduction is followed by the loss of connection to the surrounding matrix. Taken together, the ILK-mediated signaling is mandatory for the HF KCs adhesion to their surrounding matrix.

Importantly, these human HF organ culture data are also in line with the results obtained in ILK-K5 knockout mice concerning the impaired directional migration followed by a missing forming of stable lamellipodia-like structures, as well as the detachment through the surrounding environment (Lorenz et al., 2007). Thus, the high level of ORSK apoptosis could be caused by missing AKT phosphorylation after QLT0267 treatment, as previously described in a tumor cell line (Eke et al., 2009) or the impaired formation of FA because of a reduced ILK expression (Devalliere et al., 2012; Sakai et al., 2003). Irrespective of these considerations, our data suggest that ILK protein is functionally important for β1 integrin-mediated signaling in the human HF and for the survival of human ORSKs *in situ*.

After a key role of β1 integrin-mediated signaling for ePC maintenance, proliferation, adhesion and migration within the human HF had been documented, another clinically relevant human model should be used to clarify the influence of β1 integrin signaling on skin wound healing after injury. β1 integrin has been already described as very important for human wound healing and as a important therapeutic target for different skin diseases (Liu et al., 2009; Watt and Fujiwara, 2011). A recently published study also described a modulation of β1 integrin-mediated signaling in lung tissue repair by using 3 different β1 integrin specific antibodies (JB1a, AIIB2, K20) in a cell culture model (Aljamal-Naylor et al., 2012). They evaluated one antibody which is able to induce matrix remodelling and to improve cell survival. However, the influences of specific β1 integrin-binding antibodies on human skin wound healing are unknown.

As already shown the β1 integrin-binding antibodies 12G10 and mAb13 are able to manipulate the signaling of the receptor in different human HF ePC subpopulations. By supplementation of the β1 integrin activating (12G10) and inhibitory (mAb13) antibody in the "punch in punch" assay (Meier et al., 2013) different β1 integrin expression pattern dependent on the epidermal layer is demonstrated. This is in line with the recognized reduction of this receptor upon terminal differentiation in murine epidermis, where the KCs migrate to the suprabasal layers of the epidermis and then terminally differentiate in order to be transformed to an anuclear corneocyte (Brakebusch et al., 2000). However, my current data enriches additionally the knowledge of changing activation states of β1 integrin within the skin.

The area and length measurements of the formed ETs demonstrate a strong inhibitory effect on the reepithelization by the antibody treatment, and mainly the 12G10-treated skin show progressing tissue destruction during the culture period. The activating antibody 12G10 which binds to the βI-domain of extended β1 integrins and by this stabilizes the ligand-occupied conformation of the integrin (Mould et al., 1996) seem to enhance the binding activity to components of the BM. By stabilizing the cell-ECM connection KCs were nearly immobilized and the normally low affinity of β1 integrin to their ligands which allows the controlled adherence and migration (low KD [dissociation constant] $10^{-6}$ – $10^{-8}$) (Lowell and Mayadas, 2011) is further disturbed. In contrast, the inhibitory antibody mAb13 act as a functional inhibitor of cell spreading in cell culture experiments (Akiyama et al., 1989; Mould et al., 1996; Mould et al., 1995). Thus the missing or reduced reepithelization of the wounded skin is possibly caused by the decreased cell movement towards the wounding edge.

The reduced ET formation after wounding had to be correlated to the proliferation marker Ki-67 and to the apoptosis marker TUNEL, and by assessing the migratory activity via cortactin immunofluorescence microscopy. Both proliferation and migration were disturbed mainly by the activating antibody 12G10, which is in line with the demonstrated strong inhibitory reepithelization effect. This could be due to the reinforcement of the cell-ECM connection to the underlying BM components, like laminin or fibronectin, which may have caused a disturbance of the necessary well-balanced homeostasis of cell-cell and cell-matrix connections which is mandatory for a normal wound healing (Guo and Dipietro, 2010; Leask, 2014). The consequences of the changed ligand affinity of antibody-activated β1 integrin via 12G10 might be the intracellularly induction of apoptosis.

The effects on reepithelization of 12G10-treated wounded skins were more severe than in mAb13-treated skins. In contrast to the activating β1 integrin antibody inhibit mAb13 β1 integrin adaptor protein AKT and FAK activities which were demonstrated in a breast cancer cell line (Castello-Cros et al., 2009). Normally AKT promotes cell survival and proliferation by phosphorylating downstream molecules (Rho et al., 2010) and FAK is necessary for cellular adhesion and cell spreading (Huang et al., 2011). By the reduction of their activity the normal signaling for migration and proliferation is inhibited and delayed in mAb13-treated wounded skin but not totally stopped like it seem to be in 12G10-treated skin.

While the protein expression of ePC markers was not affected in the analyzed ETs, K6 IR demonstrated a delayed differentiation in 12G10-treated wounded skin. K6 is normally expressed after stressful stimuli, like wounding (Ramot et al., 2009; Windoffer et al., 2011) and described to be induced during reepithelization in wound-proximal KCs (Rotty and Coulombe, 2012; Wojcik et al., 2000). The stabilisation and reinforcement of the ligand-binding conformation of β1 integrin by the activating antibody 12G10 (Mould et al., 1996) inhibited the differentiation stimuli for the KCs in the wound edges. These 12G10-mediated effects disturb any possibility for reepithelization and wound closure. In contrast, the inhibitory antibody mAb13 did not have any impact on the ePC population or the differentiation-inducing stimuli.

Again, the usage of these two antibodies in different clinical relevant models – the HF and the wounded skin - signifies that their influences on the β1 integrin-mediated signaling depend on the location of ePCs and their progeny.

Mainly the only delayed effects of mAb13 on reepithelization can be utilized for the creation of therapeutically targets against keloid scar formation, because the normally increased activation of FAK (Wang et al., 2006) during this disease could be possibly inhibited via mAb13. Moreover the 12G10-mediated delayed wound reepithelization should be considered in more detail for their using in the treatment of cutaneous scleroderma. One mouse study proved the connection of the loss of β1 integrin and the resistance to skin scleroderma (Liu et al., 2009). A targeted activation or inhibition of integrins, like β1 integrin, and by this influencing wound healing constitutes an important role in an early intervention during the wound healing cascade and represents a cost-effective opportunity for a controlled wound healing process (Widgerow, 2013).

Taken together, this thesis demonstrates that ePCs in human HFs require β1 integrin-mediated signaling for survival, adhesion, and migration, and that different human HF ePC subpopulations differ in their response to β1 integrin signaling. Mechanistically, this effect is likely ILK-dependent. In addition, to maintain the skin homeostasis in human wounded skin the well-balanced β1 integrin signaling is necessary for a functional reepithelization. These new insights into the β1 integrin-dependence of distinct human ePC populations significantly enrich our as yet very fragmentary understanding of the integrin-dependent topobiology of human ePCs *in situ*, and (Ohyama and Kobayashi, 2012; Ohyama and Veraitch, 2013; Plikus et al., 2012).

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„Wenn man so will
Bist du das Ziel einer langen Reise
Die Perfektion der besten Art und Weise
In stillen Momenten leise
Die Schaumkrone der Woge der Begeisterung
Bergauf, mein Antrieb und Schwung

Wenn man so will
Bist du meine Chill-Out Area
Meine Feiertage in jedem Jahr
Meine Süßwarenabteilung im Supermarkt
Die Lösung, wenn mal was hakt
So wertvoll, dass man es sich gerne aufspart
Und so schön, dass man nie darauf verzichten mag

Ich wollte dir nur mal eben sagen
Dass du das Größte für mich bist
Und sichergehen, ob du denn dasselbe für mich fühlst
Für mich fühlst"
(copied from Sportfreunde Stiller)