Outside-in Signaling through Integrins and Cadherins: A Central Mechanism to Control Epidermal Growth and Differentiation?

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The process of epidermal renewal persists throughout the entire life of an organism. It begins when a keratinocyte progenitor leaves the stem cell compartment, undergoes a limited number of mitotic divisions, exits the cell cycle, and commits to terminal differentiation. At the end of this phase, the postmitotic keratinocytes detach from the basement membrane to build up the overlaying stratified epithelium. Although highly coordinated, this sequence of events is endowed with a remarkable versatility, which enables the quiescent keratinocyte to reintegrate into the cell cycle and become migratory when necessary, for example after wounding. It is this versatility that represents the Achilles heel of epithelial cells allowing for the development of severe pathologies. Over the past decade, compelling evidence has been provided that epithelial cancer cells achieve uncontrolled proliferation following hijacking of a "survival program" with PI3K/Akt and a "proliferation program" with growth factor receptor signaling at its core. Recent insights into adhesion receptor signaling now propose that integrins, but also cadherins, can centrally control these programs. It is suggested that the two types of adhesion receptors act as sensors to transmit extracellular stimuli in an outside-in mode, to inversely modulate epidermal growth factor receptor signaling and ensure cell survival. Hence, cell–matrix and cell–cell adhesion receptors likely play a more powerful and wide-ranging role than initially anticipated. This Perspective article discusses the relevance of this emerging field for epidermal growth and differentiation, which can be of importance for severe pathologies such as tumorigenesis and invasive metastasis, as well as psoriasis and Pemphigus vulgaris.

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Introduction

Epidermal renewal is accompanied by continuous reorganization of the keratinocyte's adhesive interactions. This is particularly evident in the basal layer of the epidermis, where the keratinocytes proliferate and, after exiting the cell cycle and commitment to terminal differentiation, disassemble their cell–matrix attachments to move upwards and rebuild the overlying epidermis (for review see Blanpain and Fuchs, 2006;

Editor's Note

In this issue of the JID, the Perspectives Series on keratinocyte adhesion and cell junctions concludes with a thought-provoking review of the potential role of integrins and cadherins as molecules that not only hold the keratinocytes together, but also serve as signaling proteins for the epidermis. In this review, Eliane Müller and colleagues discuss the hypothesis that adhesion proteins may function as signaling molecules that help communicate important extracellular signals to the keratinocytes, and by so doing play an important role in keratinocyte growth and differentiation. This article will provide readers with a different perspective on the function of keratinocyte cell-surface adhesion proteins and stimulate new thought and investigation into how these proteins may affect epithelial biology.

''To raise new questions, new possibilities, to regard old problems from a new angle, requires creative imagination and marks real advance in science''. *Albert Einstein.*

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Abbreviations: Dsg, desmoglein; EGF-R, epidermal growth factor receptor; ERK, extracellular signal-regulated kinase; GSK, glycogen synthase kinase; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor-κB; PI3K, phosphatidylinositol trisphosphate kinase; RTK, receptor tyrosine kinase; STAT, signal transducer and activator of transcription

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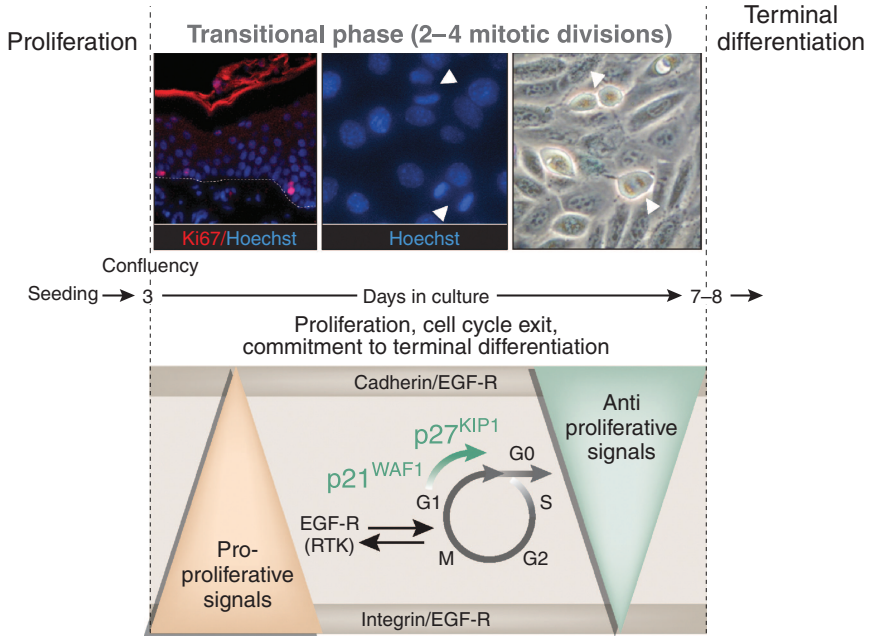

Figure 1. Diagram depicting the time frame in which the "transitional phase" takes place in cultured keratinocytes. Top panel: Left; human epidermis was stained with Ki67 and Hoechst 33258 to show one proliferating cell per approximately 10 basal keratinocytes; middle; cultured mouse keratinocytes at confluency were stained with Hoechst 33258 to show asynchronous division; about one cell in 10 is dividing, similar to what is observed in basal epidermal keratinocytes stained with Ki67. Right: dividing mouse keratinocytes of the transitional phase are loosely attached to the culture dish. Bottom panel: Model discussed in this review. After seeding, keratinocytes proliferate under influence of RTK (specifically discussed for EGF-R) and integrins. Three to four days post-seeding, the keratinocytes reach confluency. Integrin- and cadherin-mediated signals in cooperation then oppose each other to lead the keratinocyte into cell-cycle exit, growth arrest, and onset of terminal differentiation. Figure adapted from Kolly *et al.* (2005).

Watt *et al.*, 2006). These dynamic adhesive interactions could endow epidermal keratinocytes with a unique and potent system to survey the changing environment and direct the cell fate when coupled to cell signaling. Hence, this article tackles the emerging knowledge on outside-in signals emanating from adhesive interactions in epidermal keratinocytes of the "transitional phase", that is from the time point the epidermal keratinocyte exits the stem cell compartment and becomes a transit amplifying cell until it is growth arrested and has committed to terminal differentiation (Figure 1).

The integrins are the main family of adhesion molecules that connect keratinocytes to the underlying basement membrane. In human and mouse epidermis, the primary integrins expressed are α6β4 (adhering to laminin-332 (formerly laminin 5)), α2β1 (adhering to collagen I), α3β1 (adhering to laminin-332), and the less abundant αvβ5 (adhering to vitronectin) (Watt, 2002; Wilhelmsen *et al.*, 2006). Of these, α6β4 assembles with plectin, BP 230, and the collagen-type molecule BP180 to form hemidesmosomes that tether to keratin intermediate filaments, whereas the other αβ-isomers assemble into focal adhesions that link to actin. Like their suprabasal neighbors, basal keratinocytes also adhere tightly to each other via anchoring junctions, that are adherens junctions and desmosomes (for review see Green and Gaudry, 2000; Vasioukhin and Fuchs, 2001). The transmembrane proteins mediating the intercellular adhesive interactions belong to the cadherin superfamily. In basal keratinocytes, E- and P-cadherin are the major adhesion-mediating molecules in adherens junctions. They assemble with plaque proteins $p120^{cnt}$, β-catenin, plakoglobin (γ-catenin), and α-catenin into functional junctions. Alternatively, the strongest intercellular adhesion between these cells is provided by desmosomal cadherins (for example, in basal keratinocytes desmoglein (Dsg) 3 and desmocollin 3 and low levels of Dsg2) forming desmosomes together with plaque proteins such as plakophilins and plakoglobin, whereas desmoplakin anchors desmosomes to the intermediate filament network.

The adhesive interactions of keratinocytes are of crucial importance for the mechanical integrity of the epidermis, as exemplified by bullous autoimmune diseases that disable adhesive functions (for review see Suter *et al.*, 1998; Schmidt and Zillikens, 2000). However, evidence is emerging that the functional units of cell adhesion, termed here adhesion receptors, can in addition serve as transducers of outside-in signals to control proliferation and terminal differentiation in keratinocytes of the transitional phase (for example, integrins, Mainiero *et al.*, 1997; Nikolopoulos *et al.*, 2005; or cadherins, Pece and Gutkind, 2000; Calautti *et al.*, 2005; Williamson *et al.*, 2006; Perrais *et al.*, 2007; Xie and Bikle, 2007).

To date, no enzymatic activity has been attributed to the cytoplasmic tails of adhesion receptors. Their signaling capability emanates from the recruitment of intracellular signaling components such as kinases and phosphatases that physically link to the adhesion receptors' cytoplasmic tail, to adaptor proteins, or plaque proteins (for example, Fuchs *et al.*, 1996; Mainiero *et al.*, 1996; Shaw *et al.*, 1997; Calautti *et al.*, 2005). Although outside-in signals transmitted by ligand-activated integrin receptors have been extensively described and are known to control cell migration, survival, and proliferation in many cell types (for review see Miranti and Brugge, 2002; Guo and Giancotti, 2004; Janes and Watt, 2006; Wilhelmsen *et al.*, 2006), evidence for outside-in signals triggered by the engagement of cadherin receptors through transadhesion is only starting to emerge.

Recent studies in various epithelial and endothelial cell types, including keratinocytes, now underscore that cadherins can play a key role in controlling, for example, cell growth, differentiation, and survival (Takahashi and Suzuki, 1996; Carmeliet *et al.*, 1999; Pece *et al.*, 1999; Cabodi *et al.*,

2000; Pece and Gutkind, 2000; Calautti *et al.*, 2002, 2005; Laprise *et al.*, 2002, 2004; Dejana, 2004; Qian *et al.*, 2004; Pang *et al.*, 2005; Liebner *et al.*, 2006; Williamson *et al.*, 2006; Hofmann *et al.*, 2007; Kang *et al.*, 2007; Perrais *et al.*, 2007; Xie and Bikle, 2007). Moreover, there is increasing evidence that cadherins like integrins associate with and control the action of receptor tyrosine kinases (RTKs), in particular of the epidermal growth factor receptor (EGF-R) family (integrins, Miranti and Brugge, 2002; Guo and Giancotti, 2004; Janes and Watt, 2006; Wilhelmsen *et al.*, 2006; cadherins, Hoschuetzky *et al.*, 1994; Takahashi and Suzuki, 1996; Pece and Gutkind, 2000; Qian *et al.*, 2004; Calautti *et al.*, 2005; Kang *et al.*, 2007; Perrais *et al.*, 2007). While signals generated by ligand-activated integrins and RTK are in principle pro-proliferative, signals emanating from cadherins and RTKs can be pro-proliferative or antiproliferative depending on the cadherin and the associated RTK; for instance, N-cadherin was suggested to interact with fibroblast growth factor receptor to promote invasion and tumor-cell behavior, whereas E-cadherin has been described to associate/cooperate with EGF-R to modulate its activity in confluency-dependent events preceding growth arrest (Takahashi and Suzuki, 1996; Pece and Gutkind, 2000; Qian *et al.*, 2004; Calautti *et al.*, 2005; Perrais *et al.*, 2007; Theisen *et al.*, 2007).

Conventional submerged cultures of mouse, human, and canine keratinocytes share many features with epidermal keratinocytes *in vivo,* and thus were instrumental in gaining invaluable insight into the control of epidermal homeostatic processes (for example, Yuspa *et al.*, 1989; Pillai *et al.*, 1990; Poumay and Pittelkow, 1995; Kolly *et al.*, 2005). In low-density cultures and in media containing EGF or related growth factors, keratinocytes proliferate and migrate, and are most similar to keratinocytes in re-epithelializing skin. At confluency, a continuous monolayer is formed and ample cell–cell contacts promote the stabilization of junctional components at the plasma membrane, similar to the situation in basal or transit-amplifying keratinocytes *in vivo*. At that stage the keratinocytes are said to become ''contact inhibited'', and like transit-amplifying cells in the epidermis, undergo two to four mitotic divisions while committing to terminal differentiation (Poumay and Pittelkow, 1995; Kolly *et al.*, 2005; Watt *et al.*, 2006). During their mitotic divisions, the proliferation rate of confluent keratinocyte cultures remains constant in spite of the steady increase of antiproliferative factors such as cyclin-dependent kinase inhibitors $p21^{WAF}$ and $p27^{KIP}$ (Missero *et al.*, 1996; Kolly *et al.*, 2005). This suggests that during the transitional phase pro-proliferative and antiproliferative events occur simultaneously within the same cell, but the antiproliferative effectors accumulate over time and only override pro-proliferative signals when they have reached a certain threshold level (Figure 1; Kolly *et al.*, 2005).

The concept explored in this review is that integrins and cadherins sense the cells immediate environment and cooperate in directing the transitional phase of epidermal renewal by outside-in signaling. As suggested for other epithelial cell types, ligand-activated integrins and cadherins may thereby serve as signaling receptors, which exert their function before assembling into fully organized junctions firmly anchoring to the cytoskeletal network (for example, Mariotti *et al.*, 2001; Kovacs *et al.*, 2002; Laprise *et al.*, 2002; Calautti *et al.*, 2005). This conceivably facilitates lateral movement, supporting their association and cooperation with EGF-R family members in addition to their control.

To begin, this review focuses on the pathways through which integrins can stimulate proliferation, and how groundbreaking work on β4-integrin-mediated signaling using a novel approach (Nikolopoulos *et al.*, 2005; Guo *et al.*, 2006) integrates with the broader body of related literature. The review then continues with an analysis of the emerging knowledge on the involvement of cadherins in growth arrest and commitment to terminal differentiation, and how recent studies on E-cadherin-mediated signaling in keratinocytes (Calautti *et al.*, 2005; Perrais *et al.*, 2007; Xie and Bikle, 2007) combine with established results on E-cadherin and new data on Dsg3-mediated signaling (Williamson *et al.*, 2006) to form a comprehensive picture, which now allows us to simultaneously discuss a possible cross talk between integrins and cadherins with EGF-R family members in epidermal keratinocytes of the transitional phase (Figure 1).

Integrin-mediated pro-proliferative signals

Keratinocytes in the basal proliferative layer of the epidermis can be distinguished from their suprabasal postmitotic neighbors by their cell–matrix attachment (Watt, 2002). If this connection is disrupted by placing human or mouse keratinocytes in suspension, they start to terminally differentiate (Green, 1977; Adams and Watt, 1989; Rodeck *et al.*, 1997). Blocking phosphatidylinositol trisphosphate kinase (PI3K) or alternatively, EGF-R of suspension cells, induces apoptosis. Inversely, if integrins are cross-linked, proliferation is maintained (Adams and Watt, 1989; Watt *et al.*, 1993; Rodeck *et al.*, 1997; Nikolopoulos *et al.*, 2005). This indicates that normal homeostatic keratinocytes require integrin-mediated cell-substrate adhesion to proliferate. Moreover, when cell–substrate adhesion is lost, differentiation-promoting and cell survival cascades are activated, which depend on PI3K and EGF-R. Collectively, these findings suggest that in epidermal keratinocytes, functional integrin receptors either support proliferation or alternatively suppress terminal differentiation or both.

Reports on the lack of a proliferative phenotype in β1-integrin-knockout keratinocytes *in vitro* and more recently in conditional Cre-mediated β1-integrin-knockout epidermis of adult mice appeared to exclude β1-integrin as a regulator of the proliferative process during epidermal turn-over (Raghavan *et al.*, 2003; Lopez-Rovira *et al.*, 2005). Moreover, deletion confined to small stretches (to prevent loss of cell–matrix adhesion) of β4-integrin, the second major β-subunit in the epidermis, also failed to impede normal proliferation and/or terminal differentiation, raising further doubts about a pro-proliferative

activity of integrins during epidermal renewal (Raymond *et al.*, 2005). These findings were surprising in view of the many signaling events reported downstream of integrin receptors (for review see Miranti and Brugge, 2002; Guo and Giancotti, 2004; Janes and Watt, 2006; Wilhelmsen *et al.*, 2006).

Studying the role of adhesion receptors is complicated by the fact that their genetic deletion not only affects signaling but also adhesion. In addition, deletion of adhesion components in skin can trigger secondary events such as inflammation, which induce hyperproliferation as a bystander effect. As an example, varying grades of inflammation were discussed as a reason for conflicting results obtained from different β1-integrin-knockout mice (Lopez-Rovira *et al.*, 2005). Consequently, for many studies on adhesion receptors, it is difficult to discriminate between signals related to homeostatic processes, and those linked to lack of tissue integrity or to secondary events like inflammation. In the case of β4-integrin, this problem was recently elegantly circumvented by expressing a C-terminal deletion mutant (from aa 1,355 onwards) in mouse epidermis, which is not critical for adhesion but abrogates signaling (Nikolopoulos *et al.*, 2005).

Transgenic mice (both newborn and adult) expressing the signaling-defective β4-integrin mutant showed a twofold decrease in the epidermal proliferative index, confirming that β4-integrin actually contributes to proliferation (Nikolopoulos *et al.*, 2005). Furthermore, keratinocytes from β4-integrin mutant mice failed to proliferate and migrate *in vitro* in response to EGF, suggesting an essential cooperation between β4-integrin and an RTK in proliferative epidermal keratinocytes. The follow-up of this study with comparative expression of full-length and signaling-defective β4-integrin in a transgenic mouse mammary carcinoma model with constitutive ErbB2 activation (an EGF-R family member; Ishizawar and Parsons, 2004), confirmed the pro-proliferative activity of β4-integrin; that is, loss of β4-integrin signaling-suppressed tumor onset, and invasive growth. More importantly, this study demonstrated that β4-integrin has to physically associate with an EGF-R family member to amplify pro-proliferative signals, and provided novel insights into the mechanism of the β4-integrin-mediated cooperation (Guo *et al.*, 2006). Specifically, after association, β4-integrin mediates the phosphorylation of ErbB2 on Tyr^{877} by a Src-family kinase. This phosphorylation site resides in the catalytic domain of ErbB2 and corresponds with Tyr^{845} in EGF-R, which is known to be essential for mitogenesis in terms of DNA synthesis (Ishizawar and Parsons, 2004).

The combination of the more detailed study in mammary epithelium with the findings in mouse epidermis using the unique approach of signaling-defective (but adhesion competent) β4-integrin now enables a clear definition of signals relevant for the β4-integrin-mediated pro-proliferative and pro-survival activity (Nikolopoulos *et al.*, 2005; Guo *et al.*, 2006). Like in mammary epithelium, β4-integrin was found to cooperate with EGF-R in epidermal keratinocytes to enhance proliferation in a Src- but not Ras-dependent manner. Signaling-wise this involves mainly the mitogen-activated protein kinase (MAPK) kinase module JNK/c-Jun and contributing activation of signal transducer and activator of transcription 3 (STAT3), whereas the PI3K/Akt kinase survival axis (protein kinase B) was suggested to be activated by β4-integrin and/or RTK alone (see Figure 2, figure legend for full names of effectors). The relevance of these signals for the processes governing epidermal proliferation and cell survival is, discussed in more detail in the following three sections.

MAPK activation downstream of β4-integrin/EGF-R: pro-proliferative events. In epithelial cells, three MAPK modules have been identified, which lead up to extracellular signal-regulated kinase (ERK)1/2, c-Jun N-terminal kinase (JNK), or p38 activation, respectively (Cowan and Storey, 2003). Of these, JNK-activated c-Jun was found to centrally control the proliferative activity downstream of β4-integrin and EGF-R in mouse and human keratinocytes plated on laminin-332, as well as downstream of β4-integrin/ErbB2 in the mammary carcinoma model (Mainiero *et al.*, 1997; Nikolopoulos *et al.*, 2005; Guo *et al.*, 2006; Figure 2). Importantly, both β4-integrin/EGF-R and β4-integrin/ErbB2 did not activate/phosphorylate JNK, but instead triggered the nuclear translocation of activated JNK. Although the mechanism for nuclear targeting is currently not known, it implies β4-integrin-independent JNK activation through EGF-R or another RTK (Zenz and Wagner, 2006), followed by β4-integrin/EGF-R-dependent nuclear targeting (Figure 2).

An important role of JNK in epidermal proliferation is compatible with the contribution of JNK1 (but not of JNK2; Sabapathy and Wagner, 2004; Weston *et al.*, 2004) and c-Jun to normal epidermal proliferation as well as pathological conditions like psoriasis and tumor formation (Zenz and Wagner, 2006). Consistently, JNK drives proliferation in embryonic mouse epidermis with NF-κB/RelA deficiency (Zhang *et al.*, 2004), whereas experimentally blocked NF-κB was found to act in a laminin-332 and β4-integrin-dependent manner to promote human epidermal tumorigenesis in cooperation with oncogenic Ras (Dajee *et al.*, 2003). In contrast to the involvement of NF-κB blockade in tumorigenesis, activation but not inhibition of NF-κB/RelA was implicated in β4-integrin/EGF-R-dependent proliferation in addition to JNK/c-Jun in mouse keratinocyte cultures, raising the possibility that NF-κB can function in a bidirectional manner in epidermal keratinocytes (Nikolopoulos *et al.*, 2005).

In parallel to JNK, two other MAPKs, ERK1 and ERK2, were also found to be targeted to the nucleus in a β4-integrin-dependent manner in keratinocytes (Nikolopoulos *et al.*, 2005). Nonetheless, proliferation was not dependent on ERK in either keratinocytes or the mammary carcinoma model downstream of β4-integrin (Nikolopoulos *et al.*, 2005; Guo *et al.*, 2006). This is in line with persistence of hyperproliferation in NF-κB-blocked human keratinocytes treated with pharmacological ERK inhibitors (Zhang *et al.*, 2004), as well as lack of evidence for a contribution of ERK to mitogenesis

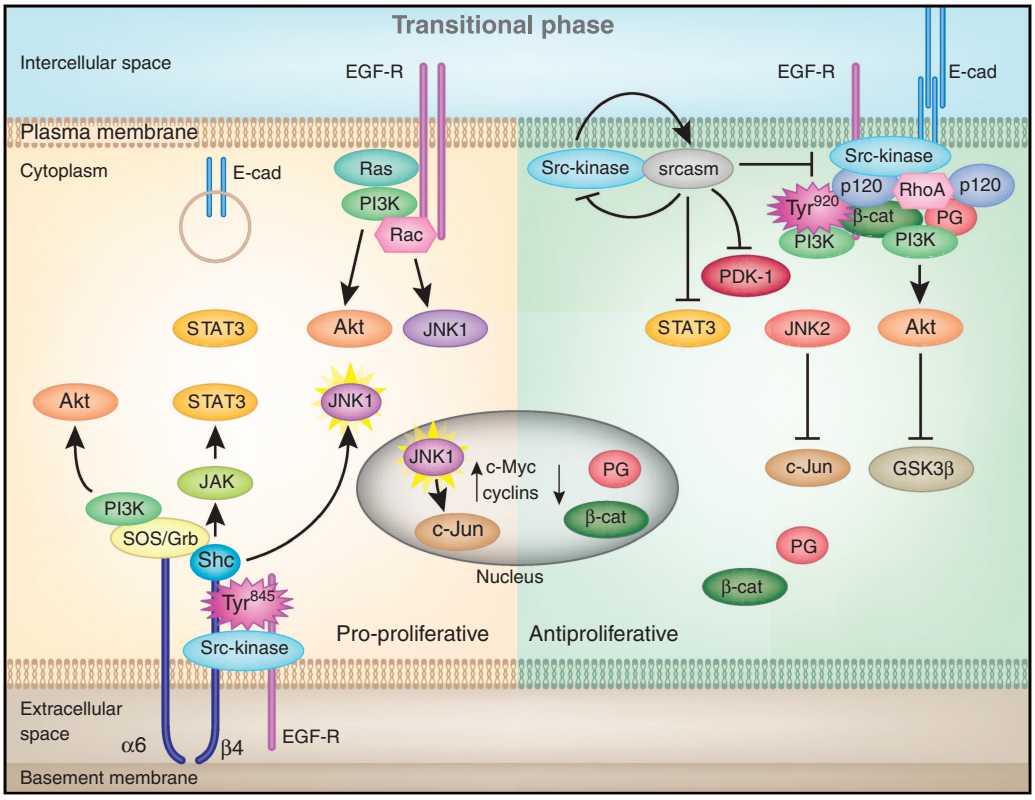

Figure 2. Opposing signaling events discussed in this review to emanate from β4-integrin and E-cadherin receptors within one keratinocyte. Depicted are effectors contributing to proliferation (β4-integrin) and cell-cycle exit (E-cadherin), in addition to cell survival in keratinocytes. Note that the respective cascades were investigated independently of each other but under similar experimental settings. JNK/c-Jun was found to be crucial for proliferation. STAT3 was described to contribute to the disruption of tight junctions and adherens junctions in mammalian carcinoma cells (Guo *et al.*, 2006). Akt generates survival signals, in addition to possibly contributing to proliferation and terminal differentiation depending on the receptor and Akt isoform. Yellow star on JNK1: β4-integrin/EGF-R-mediated nuclear targeting of phosphorylated JNK1; purple stars on EGF-R: Src kinase-mediated phosphorylation of EGF-R. Abbreviations: β-cat, β-catenin; EGF-R, epidermal growth factor receptor; ERK1/2, extracellular signal-related kinase; GSK3, glycogen synthase kinase; c-Jun, Janus kinase; JNK, c-Jun N-terminal protein kinase; PDK-1, phosphoinositide-dependent kinase-1; PG, plakoglobin; PI3K, phosphatidylinositol triphosphate kinase; Srcasm, Src-activating and signaling molecule; STAT; signal transducers and activator of transcription.

downstream of Tyr^{845}EGF-R (Ishizawar and Parsons, 2004).

Janus kinase–STAT activation downstream of β4-integrin/EGF-R: proliferation-dependent events. In confluent mammary carcinoma cells *in vitro*, β4-integrin/ErbB2 activation prevented contact-induced growth inhibition (Guo *et al.*, 2006). Simultaneously, adherens junctions and tight junctions were disrupted in spite of the fact that expression levels of junctional proteins remained unaffected. Junctional disruption was forestalled by signaling-defective β4-integrin or by inhibitors to ErbB2, and in part by a dominant-negative STAT3 mutant. This indicates that in addition to supporting proliferation via JNK, β4-integrin/ErbB2 cross talks to intercellular junctions via STAT3 in the mammary carcinoma cells (Figure 2).

β4-integrin STAT3-induced junctional disruption has as yet not been addressed in other cell types. It is however of particular interest as it differs from endocytosis and down-regulation of junctional components known to accompany Src/PI3K-induced snail-related tumorigenic transformations (Thiery, 2002). It instead resembles normal mitosis-induced disruption and endocytosis of adherens junctions, which occurs without degradation of junctional proteins in dividing Madin–Darby canine kidney cells at confluency (Bauer *et al.*, 1998). Consistent with the activation of β4-integrin signaling during mitotic processes (Nikolopoulos *et al.*, 2005; Guo *et al.*, 2006), cell–substrate adhesion was reduced in dividing Madin–Darby canine kidney cells. Reduced cell–substrate adhesion is a long known phenomenon of dividing cells and is also observed in confluent mouse keratinocyte cultures (Figure 1, top). In several epithelial cell types, including HaCaT keratinocytes, reduced cell-substrate adhesion was ascribed to Src-family kinase-induced disassembly of hemidesmosomes preceding β4-integrin signaling (Mariotti *et al.*, 2001), a phenomenon that might also account for Madin–Darby canine kidney cells. Disassembly of hemidesmosomes was discussed to uncouple α6β4-integrin from the adhesion complex and from the underlying cytoskeletal, to favor its colocalization with EGF-R in signaling platforms known as lipid rafts (Mariotti *et al.*, 2001; Gagnoux-Palacios *et al.*, 2003).

In vivo, β4-integrin amplifies pro-proliferative signals in basal epidermal keratinocytes (Nikolopoulos *et al.*, 2005), where STAT3 is expressed and

can be activated by EGF/EGF-R (Nishio *et al.*, 2001; Chan *et al.*, 2004; Li *et al.*, 2007). This raises the possibility of a β4-integrin/EGF-R to cadherin cross talk via STAT3 also *in vivo*. Although this needs to be confirmed, transient dissolution of intercellular junctions without degradation of junctional proteins would perfectly meet the requirements of dividing keratinocytes in epidermal tissue. These cells need to rapidly reorganize their cell–matrix and cell–cell attachment to preserve the integrity of the epidermal sheet. Such reorganizational processes would however only concern one dividing cell per epidermal proliferative unit (10–20 basal keratinocytes; Figure 1; Gambardella and Barrandon, 2003), and possibly only in selected areas of the plasma membrane. Consistently, inspection of basal keratinocytes by electron microscopy did not reveal apparent junctional disruption (Baker and Garrod, 1993). Altogether, this suggests that the β4-integrin/EGF-R/STAT3-induced junctional disruption without degradation could be a general mechanism, which reversibly disassembles intercellular junctions during mitosis (Figure 2). This would further permit β4-integrin to simultaneously control pro-proliferative signals and STAT3-related antiproliferative signals that emanate from transadhering E-cadherin (as will be discussed below).

PI3K/Akt activation downstream of β4-integrin/EGF-R: survival and potential pro-proliferative events. Akt is a serine/threonine kinase with a well-known role in cell survival (for review see Vivanco and Sawyers, 2002). Accordingly, Akt is activated and ensures cell survival in keratinocytes devoid of cell–matrix attachment (Frisch and Screaton, 2001). In addition to its pro-survival function, Akt has also been involved in cellular processes ranging from migration, proliferation, to terminal differentiation (for review see Vivanco and Sawyers, 2002; Toker and Yoeli-Lerner, 2006). These functions might depend on the Akt isoform and the activating receptor. Class IA PI3K, the main activators of Akt, can be recruited to receptors such as activated RTK (for review see Vivanco and Sawyers, 2002; Engelman *et al.*, 2006), integrins (Mainiero *et al.*, 1997; Shaw *et al.*, 1997; Miranti and Brugge, 2002), but also transadhering cadherins (as further discussed below).

As outlined above, β4-integrin signaling involves reduced cell–substrate adhesion secondary to disruption of hemidesmosomes (Mariotti *et al.*, 2001). Accordingly, Akt is activated/phosphorylated to ensure survival in mouse keratinocytes and mammary carcinoma cells with signaling active β4-integrin, and this activation in part depends on RTK (Nikolopoulos *et al.*, 2005; Guo *et al.*, 2006). In case of signaling-defective β4-integrin, cellular stress such as starvation or enhanced proliferation resulted in apoptosis in both cell types. This implies that under circumstances requiring high Akt activity, such as stress, the Akt pool becomes limiting in absence of β4-integrin signaling. Hence, this provides circumstantial evidence that β4-integrin itself can activate Akt in these experimental settings. A possibility, which so far lacks experimental proof, is that, in addition to supporting cell survival, β4-integrin-mediated Akt activation might be involved in nuclear targeting of JNK and thereby contribute to the proliferative process. This is compatible with the observation that Akt can amplify growth factor-induced signals (Toker and Yoeli-Lerner, 2006).

In normal homeostatic mouse epidermis, Akt activation is mainly confined to suprabasal cells, with the exception of scattered keratinocytes in the basal layer with a loosely attached appearance (Calautti *et al.*, 2005). Overall, this is consistent with the fact that Akt is activated in keratinocytes without cell–substrate attachment to promote cell survival (Frisch and Screaton, 2001). Hence, Akt-positive cells in the basal layer can comprise keratinocytes committed to terminal differentiation that have disassembled their hemidesmosomes to start migrating upwards. Alternatively, as outlined above, Akt can be activated in actively dividing cells that have disassembled their hemidesmosomes in presence of β4-integrin signaling. In the basal layer, the Akt-positive cells could therefore also result from and indicate β4-integrin signaling (one out of 10–20 cells in the epidermis; Figure 1), a possibility, which warrants further investigations.

Conclusion on α6β4-integrin signaling in epidermal keratinocytes. In summary, in epidermal keratinocytes of the transitional phase (Figure 1), ligand-activated β4-integrin receptors cooperate with EGF-R to amplify pro-proliferative and survival signals. This occurs in an Src- but not Ras-dependent manner and mainly involves JNK and Akt (Figure 2). The cooperation between β4-integrin and EGF-R may be brought about by uncoupling α6β4-integrin from hemidesmosomes, which facilitates the association with EGF-R in lipid rafts (Mariotti *et al.*, 2001). In addition to pro-proliferative signals, β4-integrin/EGF-R may control antiproliferative events emanating from transadhering E-cadherin receptors via STAT3 (Figure 2). The collective findings on β4-integrin signaling in keratinocytes are supported by complementary observations in a mammary carcinoma model, while the evoked signaling pathways align with current knowledge on pro-proliferative and survival signals driving epidermal renewal.

With respect to the mechanisms, a number of important questions remain to be answered, including how JNK is targeted to the nucleus, whether STAT3 is activated in a β4-integrin/EGF-R-dependant manner in the epidermis and whether it contributes to junctional disassembly, as well as to which extent Akt is involved in the pro-proliferative process.

Based on the studies using signaling-defective β4-integrin, the central query ''does β-4-integrin-mediated signaling amplify epidermal proliferation *in vivo*'' seems to have been answered in the affirmative. Outstanding is whether and to which extent other β-integrin subunits and eventually also α6-integrin (Owens *et al.*, 2003) contribute to this event, and whether they act synergistically or redundantly with β4-integrin. Redundancy is actually suggested by the discrepant observation that the signaling-defective β4-integrin but not its gene deletion displays a proliferative

phenotype in the epidermis (Nikolopoulos *et al.*, 2005; Raymond *et al.*, 2005). Signaling-defective β4-integrin might compete with wild-type integrins for the binding of essential proliferation-promoting factors, without being able to propagate their activity. This conceivably fails to happen in the knockout situation. Redundancy is also supported by the fact that the amplification of pro-proliferative signals in the manner described for β4-integrin equally applies for β1-integrin. β1-integrin phosphorylation by Src results in EGF-R activation (Miranti and Brugge, 2002), and β1-integrin expression is required for the initiation of mouse mammary tumors expressing the polyoma virus middle T antigen (White *et al.*, 2004). Furthermore, both, β4- and β1-integrin amplify signals of other RTKs than of ErbB2 and EGF-R, such as RON, the hepatocyte growth factor receptor Met, and tumor necrosis factor receptor, and all have been involved in carcinogenesis also in the skin (for review see Miranti and Brugge, 2002; Guo and Giancotti, 2004; Janes and Watt, 2006). Collectively, these results strongly argue in favor of an obligatory role of the integrin family members in controlling and propagating pro-proliferative and survival signals in basal keratinocytes of the transitional phase.

E-cadherin-mediated antiproliferative events

In contrast to integrins, evidence for cadherin-induced outside-in signaling came into focus only slowly. Published over the last 10 years, a number of studies in both epithelial and endothelial cells now appear to agree that signaling cascades emanating from transadhering or ligated E-cadherin or vascular (V) E-cadherin play an important role in confluency-dependent growth arrest and differentiation (Takahashi and Suzuki, 1996; Carmeliet *et al.*, 1999; Pece *et al.*, 1999; Cabodi *et al.*, 2000; Pece and Gutkind, 2000; Calautti *et al.*, 2002, 2005; Laprise *et al.*, 2002, 2004; Qian *et al.*, 2004; Liebner *et al.*, 2006; Hofmann *et al.*, 2007; Kang *et al.*, 2007; Perrais *et al.*, 2007; Xie and Bikle, 2007). Proof of concept that E-cadherin actually acts as an adhesion receptor in keratinocytes as well as in other epithelial cells, was provided by homophilic ligation of E-cadherin with recombinant protein, which results in direct activation of signaling cascades and growth arrest (Kovacs *et al.*, 2002; Pang *et al.*, 2005; McLachlan *et al.*, 2007; Perrais *et al.*, 2007), and inversely, by function disrupting E-cadherin antibodies, which abrogate for instance E-cadherin signaling, growth arrest, or terminal differentiation (Takahashi and Suzuki, 1996; Pece *et al.*, 1999; Noren *et al.*, 2001; Laprise *et al.*, 2002; Qian *et al.*, 2004; Calautti *et al.*, 2005; McLachlan *et al.*, 2007).

As far as analyzed, the mode by which E-cadherin transmits growth-inhibiting outside-in signals seems to follow a strikingly similar scheme than that of integrins involved in proliferation. Discussed in more detail below, signaling is initiated through homophilic transadhesion between E-cadherin molecules at points of cell–cell contact (Figure 2). The E-cadherin complex then associates and cooperates with an EGF-R family member to activate the PI3K/Akt pathway and possibly MAPK in a Src-family kinase-dependent manner, as indicated in a number of complementary studies on keratinocytes and other cell types (Hoschuetzky *et al.*, 1994; Takahashi and Suzuki, 1996; Pece and Gutkind, 2000; Qian *et al.*, 2004; Calautti *et al.*, 2005; Perrais *et al.*, 2007). Ultimately, this results in the loss of the cells' responsiveness to EGF through inhibition of the EGF-R pro-mitotic activity. Recently reported in A431 cells, this can occur through E-cadherin-mediated inhibition of transphosphorylation on Tyr^{845}EGF-R (Perrais *et al.*, 2007), the site, which catalyzes EGF-induced mitogenesis (Ishizawar and Parsons, 2004).

As outlined above also, studies in human and mouse keratinocytes highlight the involvement of E-cadherin in outside-in signaling. In addition, they demonstrate cooperation between E-cadherin and EGF-R in activating PI3K/Akt and inducing growth arrest (Figure 2; Pece and Gutkind, 2000; Calautti *et al.*, 2005; Perrais *et al.*, 2007; Xie and Bikle, 2007). Although not yet confirmed more broadly, this perfectly fits the observations that inhibition of PI3K or EGF-R is sufficient to prevent terminal differentiation in keratinocytes in suspension (Rodeck *et al.*, 1997; Nikolopoulos *et al.*, 2005), and that function disrupting antibodies to E-cadherin abrogate Akt phosphorylation inducing growth arrest and terminal differentiation in conventional submerged mouse keratinocyte cultures at confluency (Calautti *et al.*, 2005). The underlying mechanism of the apparent joint signaling through E-cadherin/EGF-R is currently not known. In mouse keratinocytes, it was however shown to result in Src kinase-mediated phosphorylation of EGF-R, presumably on Tyr^{920} (Calautti *et al.*, 2005). This can generate a docking site for p85, a regulatory subunit of PI3K (Stover *et al.*, 1995; Calautti *et al.*, 2005), with potential activation of diverse signaling cascades (for review see Engelman *et al.*, 2006), including the MAPK described in human HaCaT cells (Pece and Gutkind, 2000).

In support of a possible involvement of E-cadherin in growth control in keratinocytes *in vivo*, E-cadherin-knockout epidermis displays some hyperproliferation, which however appears to be limited due to compensatory upregulation of P-cadherin (Young *et al.*, 2003; Tinkle *et al.*, 2004; Tunggal *et al.*, 2005) or (as will be further discussed below) compensatory desmosomal cadherins. Furthermore, compatible with a dual contribution of EGF-R, PI3K, and Akt to pro-proliferative and antiproliferative events in epidermal keratinocytes (Figure 2), EGF-R- and Akt1/2-knockout mice as well as mouse keratinocytes overexpressing PI3K class IA catalytic subunits display a broad range of epidermal phenotypes, which extend from impaired migration and proliferation to impaired terminal differentiation (Miettinen *et al.*, 1995; Peng *et al.*, 2003; Pankow *et al.*, 2006).

PI3K/Akt activation downstream of cadherins/EGF-R: antiapoptotic and antiproliferative events. In keratinocytes, exogenous expression of membrane tethered, constitutively active Akt was found to be sufficient in driving cell-cycle exit and onset of terminal differentiation, underscoring

the importance of Akt in this process (Calautti *et al.*, 2005). Accordingly, Akt activation downstream of E-cadherin engagement has been analyzed in quite some detail in mouse and human keratinocytes (Figure 2). The sequence of events outlined in the following was mainly established in several follow-up studies in mouse keratinocytes by Calautti and co-workers, and was sequentially confirmed by other investigators for mouse or human keratinocytes.

What is known so far is that transadhering E-cadherins are stabilized through association with $p120^{cnt}$ (for review see Anastasiadis, 2007), which results, in the context of confluent mouse keratinocytes, in recruitment and activation of the small GTPase RhoA (Calautti *et al.*, 2002; Anastasiadis, 2007) necessary for the association of a Src-family tyrosine kinase with the nascent cadherin tails at the plasma membrane (Cabodi *et al.*, 2000; Calautti *et al.*, 2002). Src-family kinases then phosphorylate the plaque proteins β-catenin, plakoglobin, $p120^{cnt}$, and α-catenin, which is required for their association with the E-cadherin tail (Calautti *et al.*, 1998, 2005). In mouse keratinocytes, Src kinase-mediated phosphorylation was found to generate a docking site for PI3K (p85) on plakoglobin in an EGF-R-dependent manner, to a minor extent on $p120^{cnt}$, but not on β-catenin, which apparently lacks a PI3K (p85)-docking motive (Calautti *et al.*, 2005). Consistently, in the former study the catalytic subunit of PI3K (p110) was mainly associated with plakoglobin upstream of PI3K/Akt activation. This contrasts with data from human keratinocytes in which β-catenin and $p120^{cnt}$ but not plakoglobin were found to be involved in PI3K recruitment to transadhering E-cadherin upstream of Akt (Xie and Bikle, 2007). On the other hand, PI3K recruitment was related to β-catenin but not $p120^{cnt}$ in SW480 human colon carcinoma cells (Perrais *et al.*, 2007).

The discrepant results on the involvement of β-catenin or plakoglobin in tethering PI3K to the E-cadherin tail in keratinocytes might be explained by a fundamental difference in the experimental set ups (Calautti *et al.*, 2005; Xie and Bikle, 2007). In the context of human keratinocytes, PI3K association was investigated in the first minutes after E-cadherin engagement, whereas it was addressed 1 hour later in mouse keratinocytes (Calautti *et al.*, 2005; Xie and Bikle, 2007). Hence, it is conceivable that the PI3K recruitment occurs in a biphasic time course, at first for E-cadherin-mediated signaling involving $p120^{cnt}$ and β-catenin or β-catenin-dependent molecules, and then second for Dsg3-mediated signaling involving plakoglobin (SID Meeting abstract; Schulze *et al.*, 2007), while potentially underlying the cross talk between E-cadherin and desmosomal cadherins that plakoglobin was described to mediate many years ago (Lewis *et al.*, 1997). Although this hypothesis needs to be confirmed, it is noteworthy that quite a few studies on various mouse cell types have concluded on the association of PI3K with β-catenin based on co-immunoprecipitation experiments using E-cadherin antibodies (for example, Espada *et al.*, 1999; Woodfield *et al.*, 2001). Given that plakoglobin and β-catenin coexist in adherens junctions (Butz *et al.*, 1992), they co-precipitate and hence cannot be distinguished (for example, Calautti *et al.*, 2005). Although the docking partners for PI3K on E-cadherin in keratinocytes require further detailed investigations, the studies on human and mouse keratinocytes agree on Akt activation following PI3K recruitment to transadhering E-cadherin (Calautti *et al.*, 2005; Xie and Bikle, 2007).

Two possible targets inhibited through Akt-induced phosphorylation are glycogen synthase kinase (GSK3) α and β (for review see Vivanco and Sawyers, 2002). This inhibition is known to promote, among many other activities, the stabilization and nuclear translocation of β-catenin and plakoglobin in various cell types (Aberle *et al.*, 1997; Kodama *et al.*, 1999). Accordingly, confluent mouse and human keratinocytes subjected to Akt activation exhibit phosphorylated GSK3α and GSK3β (Calautti *et al.*, 2005; Thrash *et al.*, 2006), which might trigger the confluency-dependent translocation of β-catenin and plakoglobin into the nucleus that was described in an unrelated study on mouse keratinocytes (Figure 2; Williamson *et al.*, 2006). One of the reported consequences of this translocation is the plakoglobin-mediated suppression of the pro-proliferative proto-oncogene *c-Myc*, which is critical for growth inhibition in these cells (Arnold and Watt, 2001; Waikel *et al.*, 2001; Kolly *et al.*, 2005; Figure 2). Consistently, maximal accumulation of nuclear plakoglobin as well as nuclear β-catenin chronologically correlates with reduced cyclin D1 levels and keratinocyte growth arrest (Williamson *et al.*, 2006). It is noteworthy that nuclear accumulation of plakoglobin and/or β-catenin has not yet been functionally linked to transadhering E-cadherin, plakoglobin was however related to Dsg3-induced signaling as will be further discussed below (Williamson *et al.*, 2006).

Besides β-catenin and plakoglobin, another potential effector activated downstream of E-cadherin/Akt and GSKβ is NF-κB/RelA, which also can control cell-cycle exit in human and mouse keratinocytes (Dajee *et al.*, 2003; Zhang *et al.*, 2004). Upstream of Akt, E-cadherin/PI3K activates phospholipase-C-γ1, which is a crucial element in the mobilization of intracellular calcium and terminal differentiation (Xie and Bikle, 2007). The relative contribution of the Akt pathway and PI3K/phospholipase-C-γ1 to growth arrest *versus* terminal differentiation in keratinocytes downstream of transadhering E-cadherin, remains to be investigated.

MAPK activation downstream of cadherins/EGF-R: potential antiproliferative events. Only a small number of reports have investigated the activation of MAPK modules downstream of transadhering E-cadherin and cooperating EGF-R in keratinocytes and other epithelial cells. However, some evidence exists that ERK1/2 and p38 are transiently activated (Pece and Gutkind, 2000; Laprise *et al.*, 2004). In human HaCaT keratinocytes for instance, ERK1/2 phosphorylation increases in dependence of EGF-R recruitment to transadhering E-cadherin, and E-cadherin-mediated activation

of MAPK was also reported in intestinal epithelial cells (Pece and Gutkind, 2000; Laprise *et al.*, 2004). Of particular interest is that JNK2 was found to be necessary for cell-cycle arrest in keratinocytes, as exemplified by hyperproliferation of JNK2-knockout epidermis (Sabapathy and Wagner, 2004; Weston *et al.*, 2004). In light of the central pro-proliferative role attributed to JNK/c-Jun downstream of β4-integrin/EGF-R in keratinocytes (Nikolopoulos *et al.*, 2005), it is noteworthy that JNK2 was suggested to destabilize JNK1-induced c-Jun (Figure 2). As E-cadherin ligation or transadhesion is sufficient for growth arrest and onset of terminal differentiation in cultured keratinocytes (Calautti *et al.*, 2005; Perrais *et al.*, 2007; Xie and Bikle, 2007), JNK2 might potentially be activated via Src kinase-mediated phosphorylation of EGF-R on Tyr^{920} (Calautti *et al.*, 2005), to counteract the pro-proliferative c-Jun activity, a hypothesis requiring further detailed investigations.

Inhibition of ERK, Janus kinase/STAT3, and JNK1: antiproliferative events. E-cadherin signaling seems to be able to oppose keratinocyte proliferation by transient activation of the PI3K/Akt/GSK3β axis (Calautti *et al.*, 2005; Figure 2). It can also contribute to destabilizing JNK1-induced c-Jun by JNK2 (Sabapathy and Wagner, 2004), and to silencing the mitotic activity of EGF-R by inhibiting Tyr^{845} phosphorylation (Perrais *et al.*, 2007). Furthermore, in human keratinocytes, E-cadherin/PI3K also activates the terminal differentiation-inducing phospholipase-C-γ1 (Xie and Bikle, 2007), whereas the Src tyrosine kinase Fyn can activate protein kinase-Cδ, which was suggested to be the major protein kinase-C isoform involved in terminal differentiation downstream of EGF-R (Denning *et al.*, 1995). The latter downregulates, for example, α6β4-integrin expression, an event necessary to support the upward migration of keratinocytes (Alt *et al.*, 2004). Furthermore, the increasing activity of the Src-family kinase Fyn measured during the transitional phase (Calautti *et al.*, 1998, 2002) was found to exert a negative feedback on EGF-R-triggered pro-proliferative effectors via the Src-activating and signaling molecule (Srcasm) in mouse keratinocytes (Li *et al.*, 2007). Downstream of EGF-R activation, Srcasm was shown to block ERK1/2 and STAT3 in addition to PDK-1, the latter being a crucial activator of the PI3K/Akt axis (Vivanco and Sawyers, 2002). In other terms, Srcasm appears to be able to inhibit all pro-proliferative signals described downstream of integrin/EGF-R, except c-Jun, which was not addressed and might be destabilized by JNK2 (Figure 2). Finally, Srcasm itself exerts a negative feedback, as it inhibits its activator, the Src kinase Fyn (Li *et al.*, 2007). Although not investigated so far, this could potentially terminate both β4-integrin and E-cadherin-mediated signaling, which in each case is transient and depends on Src-family kinases (Figure 2). Following inhibition of Src-family kinases, phosphatases likely dephosphorylate adherens junction components to allow their assembly into stable junctions at the plasma membrane (Gumbiner, 2000), similarly as was suggested for the termination of β4-integrin-mediated signaling in HaCaT keratinocytes (Mariotti *et al.*, 2001).

It has long been known that Src-mediated tyrosine phosphorylation shifts cadherin-based cell adhesion from a strong to a weak adhesive state (Takeda *et al.*, 1995; for review see Gumbiner, 2000). As Src activation is a prerequisite for E-cadherin-induced signaling (Calautti *et al.*, 2005), this would imply that signaling active E-cadherin receptors are of an intermediate state between no and strong intercellular adhesion. The transient nature of signaling cascades triggered by E-cadherin actually entails the formation of an intermediate state and would best explain the bidirectional activity of Src-family kinases that are involved in junction assembly as well as disassembly (Takeda *et al.*, 1995; Calautti *et al.*, 1998; McLachlan *et al.*, 2007).

Indeed, based on observations in Chinese hamster ovary and intestinal cells by immunofluorescence analyses, signaling activity was assigned to E-cadherin molecules without cytoskeletal anchorage because E-cadherin-mediated signaling preceded the assembly of adherens junctions (Kovacs *et al.*, 2002; Laprise *et al.*, 2002). A similar possibility was discussed for mouse keratinocytes (Calautti *et al.*, 2005) and would correspond with the properties evoked for the signaling-active β4-integrin pool (Mariotti *et al.*, 2001). Although this topic needs to be addressed in detail, the possibility that the signaling pool of E-cadherin resides outside of junctions and has no or weak cytoskeletal connection is compatible with the notion that transadhesion between E-cadherins (and desmosomal cadherins) does not necessarily involve cytoskeletal anchorage (Roh and Stanley, 1995; Marcozzi *et al.*, 1998). In addition, the capacity of sensing the environment by ''ultrafast probing'' was attributed to C-cadherin, which has no cytoskeletal interaction site (Pierres *et al.*, 2007), while the lateral movement thought to facilitate the association of E-cadherin with EGF-R in lipid rafts (Seveau *et al.*, 2004), was ascribed to E-cadherin receptors without cytoskeletal anchorage, which reside outside of cell–cell contacts in a mouse keratinocyte cell line (Kusumi *et al.*, 1993). Support for the possibility that non-junctional E-cadherin without strong cytoskeletal anchorage exists *in vivo* was provided by the observation that E-cadherin antibodies bind to the plasma membrane between adherens junctions in mouse epidermis (Boller *et al.*, 1985).

Conclusion on E-cadherin/EGF-R signaling in epidermal keratinocytes. Compelling but so far limited studies implicate transadhering E-cadherin receptors and cooperating EGF-R in outside-in signaling during growth arrest and terminal differentiation in keratinocytes, principally via PI3K/Akt and potentially MAPK (Figures 1 and 2). More ample results from other epithelial cell types and also from V(E)-cadherin in endothelial cells corroborate these findings, suggesting a potent cadherin-mediated mechanism to sense cell–cell contact and regulate cell fate. The capability to sense the environment might be an attribute of non-junctional E-cadherin, which in the absence of cytoskeletal anchorage

has some leeway that allows the association with EGF-R in signaling platforms known as lipid rafts.

As all investigations to date were conducted with cultured cells, it cannot currently be judged whether E-cadherin signaling is of relevance for growth arrest *in vivo*. Indirect support is provided, as already mentioned, by E-cadherin gene deletion, which results in some hyperproliferation in the epidermis (Young *et al.*, 2003; Tinkle *et al.*, 2004; Tunggal *et al.*, 2005). Furthermore, gene ablation of α-catenin in the epidermis resulted in a severe phenotype resembling pre-neoplastic transformations (Vasioukhin *et al.*, 2001), whereas, unexpectedly, $p120^{cnt}$ knockout did not impact tissue homeostasis if inflammation was prevented (Perez-Moreno *et al.*, 2006). The α-catenin gene deletion correlated with ultrastructurally disturbed adherens junctions as well as reduced desmosomes and tight junctions. In contrast, only adherens junctions were affected in the case of the $p120^{cnt}$ knockout. Collectively, these findings may suggest that if E-cadherin-mediated growth inhibition and onset of terminal differentiation cannot occur in the epidermis *in vivo*, it can be compensated by other cadherins, including desmosomal cadherins and, as discussed below, possibly by Dsg3.

Desmosomal cadherin-mediated antiproliferative events

Dsg3-mediated antiproliferative events. Anti-Dsg3 adhesion-disrupting antibodies are produced in the majority of Pemphigus vulgaris patients (Amagai *et al.*, 1991; Payne *et al.*, 2004) or experimentally in the mouse (monoclonal anti-Dsg3 (AK23)) (Tsunoda *et al.*, 2003). When added to cultured mouse keratinocytes at the beginning of the transitional phase, they induce, besides other events (for review see Amagai *et al.*, 2006), sustained proliferation, in correlation with a dramatic upregulation of c-Myc (Williamson *et al.*, 2006). Moreover, these antibodies disrupt the desmosomal organization, but initially do not disrupt the oranization of adherens junctions (de Bruin *et al.*, 2007).

In agreement with the *in vitro* data, high c-Myc expression, sustained proliferation (in terms of increased Ki67-positive cells), and consistently, transepidermal keratin-14 expression was observed as a general feature of epidermis and oral mucosa in all human and canine Pemphigus vulgaris patients analyzed so far ((Williamson *et al.*, 2006, 2007a, b; de Bruin *et al.*, 2007; Muller *et al.*, 2007) and Lietti *et al.*, unpublished observations). Discovered only recently, the fact that sustained proliferation does not result in hyperplasia in the patient's tissue might explain why this phenomenon has not been recognized previously. Hyperproliferation without hyperplasia can for instance result from more rapid outward migration of basal epidermal keratinocytes, as was observed in keratin-10-knockout mice in presence of increased c-Myc expression (Reichelt and Magin, 2002).

In analogy to results on E-cadherin (for example, Calautti *et al.*, 2005; Perrais *et al.*, 2007), the phenotype of sustained proliferation induced by anti-Dsg3 antibodies suggested a link between transadhering Dsg3 and cell-cycle control. Recent data on Dsg3-mediated signaling in keratinocytes are in agreement with such a possibility (Figure 3, left panel).

At the beginning of the transitional phase, desmocollin 3 and Dsg3 are the main desmosomal cadherins displayed at the cell surface of keratinocytes (Green and Gaudry, 2000; Dusek *et al.*, 2007). Similar to E-cadherin, they are stabilized following RhoA activation (Waschke *et al.*, 2006), Dsg3-associated plakoglobin becomes tyrosine phosphorylated in a Src-family kinase-dependent manner (Calautti *et al.*, 1998) and PI3K (p85) associates with the Dsg3/plakoglobin complex (SID Meeting abstract; Schulze *et al.*, 2007). The formation of a Dsg3/PI3K complex is followed by an increase in Akt activity and growth arrest (Lietti *et al.*, unpublished observation) (Figure 3, left panel). If cultured keratinocytes are challenged at that stage with antibodies from Pemphigus vulgaris patients, they in contrast exhibit (with regards to Dsg3-mediated signaling) (a) reduced RhoA activity (demonstrated in Pemphigus vulgaris antibody-treated organotypic cultures; Waschke *et al.*, 2006), (b) enhanced turnover of membrane-soluble, non-junctional Dsg3 (Calkins *et al.*, 2006; Williamson *et al.*, 2006), and plasma membrane-anchored non-junctional Dsg3-associated plakoglobin (Williamson *et al.*, 2006), (c) decreased non-junctional Dsg3 protein levels (Aoyama and Kitajima, 1999; Calkins *et al.*, 2006; Williamson *et al.*, 2006; Yamamoto *et al.*, 2007), (d) reduced nuclear import of plakoglobin (Williamson *et al.*, 2006), (e) abrogation of plakoglobin-mediated transcriptional repression of c-Myc, and (e) sustained proliferation concurrent with reduced expression of differentiation markers (Figure 3, right panel) (Williamson *et al.*, 2006, 2007a, b). Furthermore, inhibition of both GSK3 and c-Myc prevents loss of intercellular adhesion in neonatal mice injected with Pemphigus vulgaris antibodies or anti-Dsg3 mouse monoclonal antibodies AK23. This firstly provides circumstantial evidence that GSK3 remains activated in presence of pathogenic anti-Dsg3 antibodies and secondly underscores the pathogenic nature of this event (Williamson *et al.*, 2006; Figure 3).

Taken collectively, the signaling pathways downstream of transadhering Dsg3 and changes upon binding of Pemphigus vulgaris antibody or AK23 are reminiscent of events triggered by transadhering E-cadherins. They suggest Dsg3-mediated activation of the PI3K/Akt pathway and growth arrest contrasting with disruption of the PI3K/Akt pathway and continuing proliferation at the expense of terminal differentiation in presence of anti-Dsg3 antibodies (compare Figures 2 and 3). At that stage, Pemphigus vulgaris antibodies neither visibly affect the localization or turn-over in individual components of adherens junctions, nor do they prevent the nuclear import of β-catenin (Calkins *et al.*, 2006; Williamson *et al.*, 2006; de Bruin *et al.*, 2007; Muller *et al.*, 2007). This suggests that Dsg3 engagement is synergistic with and possibly amplifies E-cadherin-mediated signaling to induce growth arrest via PI3K/Akt/GSK3 and potentially EGF-R.

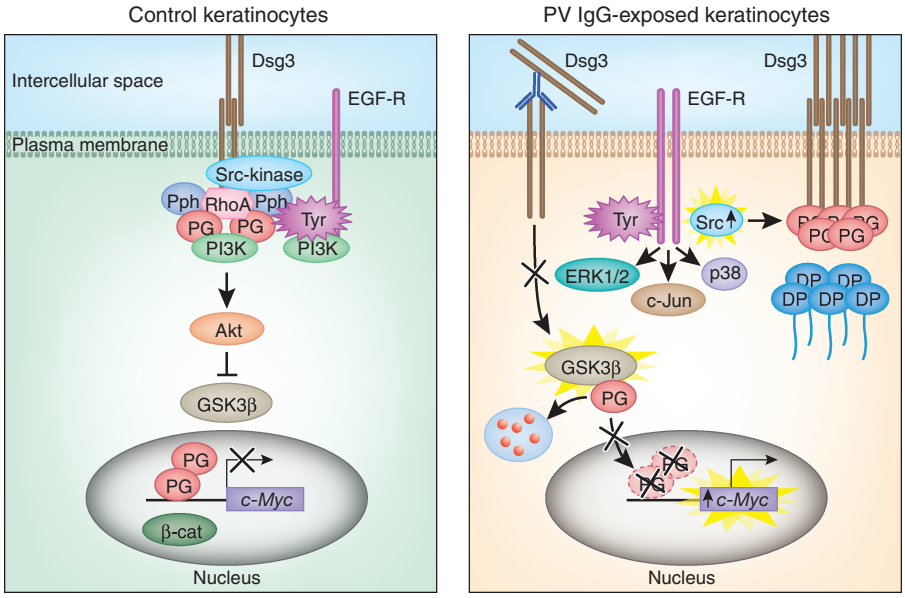

Figure 3. Dsg3-mediated signaling pathways discussed to be activated during the transitional phase, under control conditions or in keratinocytes exposed to Pemphigus vulgaris antibodies. Note that increased proliferation and c-Myc accumulation is observed in epidermis and oral mucosa of all human and canine patients analyzed so far (Williamson *et al.*, 2006, 2007a, b). Yellow stars, constitutively activated proteins. Note that the association of Dsg3/EGF-R in control cells as well as the disrupted association between PG and DP of fully assembled desmosomes is based on circumstantial evidence, as is constitutive activation of GSK3 in Pemphigus vulgaris-exposed keratinocytes. Results on Dsg3-mediated activation of the PI3K/Akt axis upstream of PG are unpublished (Lietti *et al.*, Schulze *et al.*, unpublished observations). For abbreviations see Figure 2. DP, desmoplakin.

In support of a role of Dsg3 in growth control *in vivo*, a twofold increase in the proliferative index was noted in terms of Ki67 staining in the basal layer of Dsg3-knockout mice (our unpublished observation, Koch *et al.*, 1997). Consistently, expression of a truncated Dsg3 transgene in basal epidermal keratinocytes, which lacks the transadhesion domain, induced a hyperproliferative phenotype (Allen *et al.*, 1996). Moreover, suprabasal mis-expression of full-length mouse Dsg3 under the control of an involucrin promoter disrupted the barrier function but failed to induce hyperproliferation, which is expected if Dsg3 has anti-proliferative activity (Elias *et al.*, 2001). In contrast, human Dsg3 transgene expression driven by the keratin-1 promoter displayed a hyperplastic phenotype (Merritt *et al.*, 2002). The exact reason for this discrepancy is currently not known.

Does Dsg3 control EGF-R signaling?. Indirect evidence for a control of Dsg3 over EGF-R has been provided by activation of EGF-R sustained in Pemphigus vulgaris antibody-treated keratinocytes and A431 epithelial cells. Upon exposure to Pemphigus vulgaris antibodies, EGF-R becomes rapidly hyperphosphorylated, and in addition to Src, all three MAPK modules, ERK1/2 and c-Jun, followed by p38, are activated, (Sanchez-Carpintero *et al.*, 2004; Berkowitz *et al.*, 2005; Frusic-Zlotkin *et al.*, 2006; Chernyavsky *et al.*, 2007; Figure 3). Simultaneously, EGF-R was found to be internalized (Frusic-Zlotkin *et al.*, 2006), reminiscent of vascular endothelial growth factor receptor 2-mediated signaling from intracellular stores (Lampugnani *et al.*, 2006). These collective signaling events involving EGF-R are characteristic of proliferating cells, which correlates with increased proliferation observed in Pemphigus vulgaris antibody-challenged mouse keratinocytes as well as in the patient (Williamson *et al.*, 2006, 2007a, b). Alternatively, Pemphigus vulgaris antibodies can also activate apoptotic pathways in cultured keratinocytes, as is supported by the presence of apoptotic keratinocytes in the blister roof of Pemphigus vulgaris patients (Frusic-Zlotkin *et al.*, 2005, 2006; Chernyavsky *et al.*, 2007). Induction of apoptotic effectors subsequent to inhibition of PI3K/Akt is expected, but only if EGF-R is blocked, that is in growth-arrested keratinocytes (Adams and Watt, 1989; Watt *et al.*, 1993; Rodeck *et al.*, 1997; Calautti *et al.*, 2005; Nikolopoulos *et al.*, 2005). Such a correlation can for instance be observed in cultured human keratinocytes challenged with Pemphigus vulgaris antibodies (Chernyavsky *et al.*, 2007).

In agreement with sustained EGF-R activation, basal keratinocytes in the Pemphigus vulgaris patient's epidermis as well as in culture ultimately show acantholysis or loss of intercellular adhesion (Payne *et al.*, 2004). This is a known phenomenon in epithelial cells treated with EGF and was termed ''cell rounding'' (Peppelenbosch *et al.*, 1993). Mechanistically, EGF-induced modulation of intercellular adhesion was suggested to rely on EGF-dependent tyrosine phosphorylation of plakoglobin that compromises the link between desmosomal cadherins and the intermediate filament cytoskeleton (Gaudry *et al.*, 2001). This appears to correspond with the ''keratin retraction'' described in human Pemphigus vulgaris antibody-treated mouse keratinocytes (Jones *et al.*, 1984; Caldelari

et al., 2001), as well as with the plakoglobin-mediated disruption of the desmosomal plaque (Caldelari *et al.*, 2001; de Bruin *et al.*, 2007) (Figure 3; right panel).

Cell rounding in EGF-treated HaCaT keratinocytes can be prevented simply by application of GSK3 inhibitors (Koivisto *et al.*, 2006), exactly as was shown in neonatal mice exposed to Pemphigus vulgaris antibodies or AK23 (Williamson *et al.*, 2006). This underscores that cell rounding relies on GSK3 activation related to EGF-R signaling. Hence, these events are consecutive and interdependent, with GSK3 being upstream of EGF-R (Figure 3). This would agree with the finding that c-Myc gene transcription (downstream of GSK3β) is required to propagate Ras/MAPK induced uncontrolled proliferation (Oskarsson *et al.*, 2006), whereby Ras activates the MAPK pathways downstream of EGF-R. Further supporting the GSK3–EGF-R hierarchy, plakoglobin-knockout keratinocytes do not undergo keratin retraction in response to Pemphigus vulgaris antibodies, despite elevated c-Myc levels (Caldelari *et al.*, 2001; Williamson *et al.*, 2006), because plakoglobin was found to integrate EGF-R-mediated signaling in mouse keratinocytes in terms of loss of keratin anchorage (Gaudry *et al.*, 2001). Furthermore, c-Myc inhibition alone is sufficient to prevent acantholysis (without inhibiting EGF-R) (Williamson *et al.*, 2006). Finally, the fact that only basal keratinocytes are responsive to Pemphigus vulgaris antibodies in terms of ''cell rounding'' could relate to the finding that Ras activation downstream of EGF-R is dependent on cell–substrate adhesion (Mainiero *et al.*, 1997) and hence is switched off in suprabasal keratinocytes (Dajee *et al.*, 2002).

Is Dsg3 signaling triggered by non-junctional Dsg3? Pathogenic Dsg3 antibodies target the N-terminal domain (patients or mouse monoclonal (AK23); Tsunoda *et al.*, 2003) and interfere with growth arrest. E-cadherin antibodies that bind to the N-terminal domain, disrupt E-cadherin engagement (Hoschuetzky *et al.*, 1994) and prevent PI3K/Akt activation (Pece *et al.*, 1999; Calautti *et al.*, 2005). Due to the similarity of the evoked signaling pathways, pathogenic Dsg3 antibodies may also exert their activity by disrupting transadhesion between Dsg3 molecules. This mechanism was addressed as ''steric hindrance'' (Futei *et al.*, 2000; Tsunoda *et al.*, 2003). Several lines of evidence suggest that Dsg3 antibodies further activate cell signaling by binding to transadhering non-junctional Dsg3, like discussed here for integrin and E-cadherin receptors; (a) non-keratin-anchored Dsg3 is the primary target of Pemphigus vulgaris antibodies observed in time-lapse studies *in vitro* (Sato *et al.*, 2000), (b) Pemphigus vulgaris antibodies bind to the interdesmosomal space as observed in Pemphigus vulgaris patients biopsies (Suter *et al.*, 1990; Bedane *et al.*, 1996), (c) split formation between epidermal keratinocytes in mice injected with Pemphigus vulgaris antibodies first occurs between desmosomes (Takahashi *et al.*, 1985), and (d) in contrast to non-junctional Dsg3, the desmosomal fraction is not affected at the time changes in diverse effectors including nuclear targeting of plakoglobin are measured (Williamson *et al.*, 2006). Dsg3 in the desmosomal fraction is reduced at later time points, which likely represents an important event preceding acantholysis (Koch *et al.*, 1997; Aoyama and Kitajima, 1999; Williamson *et al.*, 2006; Yamamoto *et al.*, 2007).

Conclusion on Dsg3/EGF-R signaling in epidermal keratinocytes. Reminiscent of studies on E-cadherin, investigations on Dsg3 highlight its capability to activate signaling cascades involving PI3K/Akt and potentially EGF-R, to exert growth control *in vitro* and *in vivo*. Although still limited, the current view on Dsg3-mediated signaling is supported by the consequences of anti-Dsg3 antibodies on cell growth seen in Pemphigus vulgaris patients.

The emerging concept is that anti-Dsg3 antibodies oppose Dsg3-mediated signaling by disrupting transadhesion between non-junctional Dsg3 through steric hindrance. This provides a signal of ''no cell'' contact, GSK3 and EGF-R fail to be inhibited, and the keratinocytes in the basal layer of the epidermis continue to proliferate. As a consequence, the desmosomal anchorage is reduced through a plakoglobin/EGF-R-mediated process (Figure 3), Dsg3 is depleted from desmosomes, and adhesiveness is weakened resulting in the formation of blisters. Noticeable, hypotheses other than the one discussed here have been raised for Pemphigus vulgaris pathogenesis. They do however not address Dsg3-mediated growth control, and have been discussed in detail elsewhere (for review see Amagai *et al.*, 2006).

With respect to the detailed mechanism of Dsg3-mediated signaling, some aspects described here are confirmed, some are based on circumstantial evidence, while many others such as Akt inhibition in Pemphigus vulgaris challenged keratinocytes, disruption by steric hindrance, and the relationship GSK3–EGF-R need to be filled in. Nonetheless, these collective results assembled with respect to Dsg3 raise the possibility that non-junctional Dsg3 represents an extraordinary sensory system of basal keratinocytes in the way suggested for E-cadherin.

The relative importance of E-cadherin, Dsg3, and other desmosomal cadherins in mediating antiproliferative signals in basal epidermal keratinocytes requires further detailed studies. Importantly, however, E-cadherin distribution and β-catenin nuclear translocation are not initially affected by Pemphigus vulgaris antibodies (Williamson *et al.*, 2006; de Bruin *et al.*, 2007; Muller *et al.*, 2007), suggesting that the pathways downstream of E-cadherin and Dsg3 are separable, as could also be suggested by the results on PI3K recruitment to the E-cadherin tail (Calautti *et al.*, 2005; Xie and Bikle, 2007).

Conclusion and outlook

Epidermal homeostasis requires a balance of pro- and antiproliferative signals in addition to survival signals (Figures 2 and 3). The current knowledge on adhesion receptor signaling summarized herein highlights the critical control the integrin and cadherin receptor network in cooperation with EGF-R (or other RTK) can impose on

most of these crucial signaling events. It is further proposed that the adhesion receptors exert their control by communicating with the cell's immediate environment in absence of their cytoskeletal anchorage. This would facilitate the association with EGF-R in lipid rafts. Although we are far from understanding the complex interplay between the entire adhesive network, the fact that experimental disruption of adhesion or mimicry of adhesion is sufficient to change cell fate, strongly suggests that adhesion receptors have a fundamental role in regulating important homeostatic processes also *in vivo*. Consistent with such a scenario, constitutive activation or disruption of these receptor functions *in vivo*, including disturbed expression of adhesion components, leads to uncontrolled proliferation, resulting in severe pathologies such as cancer, psoriasis, and as suggested more recently, also Pemphigus vulgaris. Further investigations of the interdependent signaling pathways between integrins, cadherins, and RTK during epidermal renewal will contribute to a better understanding of their complex interplay and provide novel insights into the mechanisms underlying these severe pathologies.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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