Regulation of epidermal homeostasis and repair by phosphoinositide 3-kinase

Sandra Pankow1, Casimir Bamberger1,2, Anke Klippel3 and Sabine Werner1,*

1Institute of Cell Biology, Department of Biology, ETH Zurich, CH-8093 Zurich, Switzerland
2Department of Cell Biology, Harvard Medical School, Boston, MA 02115, USA
3Molecular Oncology, Merck Research Laboratories, Boston, MA 02115, USA

*Author for correspondence (e-mail: sabine.werner@cell.biol.ethz.ch)

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Summary

The epidermis undergoes continuous self-renewal to maintain its protective function. Whereas growth factors are known to modulate overall skin homeostasis, the intracellular signaling pathways, which control the delicate balance between proliferation and differentiation in keratinocytes, are largely unknown. Here we show transient upregulation of the phosphoinositide 3-kinase (PI3K) catalytic subunits p110α and p110β in differentiating keratinocytes in vitro, expression of these subunits in the epidermis of normal and wounded skin, and enhanced Akt phosphorylation in the hyperproliferative wound epithelium in organotypic skin cultures. Activation of PI3K signaling also caused reorganization of the actin cytoskeleton and induced keratinocyte migration in vitro and in skin organ cultures. The identification of 122 genes, which are differentially expressed after induction of PI3K signaling provides insight into the molecular mechanisms underlying the observed effects of active PI3K on keratinocytes and indicates that hyperproliferation may be achieved at the expense of genome integrity. These results identify PI3K as an important intracellular regulator of epidermal homeostasis and repair.

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Key words: Keratinocyte, Organotypic culture, Skin, Wound healing

Introduction

The epidermis is a dynamic tissue, which maintains its barrier function through a continuous self-renewal process, making it a superb model to study tissue homeostasis. During an elaborate terminal differentiation process, keratinocytes leave the basal cell layer, cease to proliferate and lose their metabolic activity until they are finally shed as dead cornified envelopes (Green, 1980). This constant cell loss is compensated by transit amplifying cells, which originate from stem cells residing in specialized niches in the epidermis and in the bulge region of hair follicles (Oshima et al., 2001; Tumbar et al., 2004). Transit amplifying cells are determined to differentiate and escape from the cell cycle when they leave the basal layer, a process which is accompanied by a series of stage-specific morphological and biochemical changes (for reviews, see Eckert et al., 1997; Fuchs and Raghavan, 2002; Fuchs et al., 2004).

Upon skin injury this delicate balance between proliferation and differentiation in the epidermis is severely confounded. In the sequence of events leading to repair of the wounded tissue, keratinocytes exhibit enhanced motility and increased proliferation to form a hyperproliferative epithelium. The latter is characterized by the presence of several layers of proliferation-competent, non-differentiated keratinocytes, which invade the site of injury from the rim. Once the wound area is completely covered with a neo-epidermis, the normal process of keratinocyte differentiation is re-established, and the neo-epidermis is reduced to its initial thickness (Clark and Henson, 1996; Martin, 1997; Werner and Grose, 2003).

Several signaling pathways have been implicated in the regulation of keratinocyte proliferation and differentiation in normal and wounded skin. Of particular importance is the growth factor-mediated signaling via receptor tyrosine kinases, which involves activation of the phosphoinositide 3-kinase (PI3K) pathway. As revealed by knockout mice for the catalytic PI3K subunits p110α and p110β, class IA PI3Ks are crucial for cell proliferation (Bi et al., 2002; Bi et al., 1999), and they regulate a variety of cellular processes such as cell migration or apoptosis, depending on the cell type and signal received (Cantley, 2002). Aberrant activation of PI3K is associated with increased tumor incidence, and mutations leading to enhanced PI3K signaling are frequently observed in human cancers (Ali et al., 1999; Cully et al., 2006; Vivanco and Sawyers, 2002). However, it remains elusive if PI3K can influence skin homeostasis, and its role in cutaneous wound healing has, as yet, not been explored.

Indirect evidence for a relevant role of PI3K signaling in skin homeostasis is provided by the phenotype of mice lacking the phosphatidylinositol-3,4,5-trisphosphate-specific phosphatase PTEN in the epidermis (Backman et al., 2004;
Suzuki et al., 2003). These animals display a ‘wrinkled bear’ phenotype with a shaggy, ruffled coat and wrinkled skin owing to epidermal hyperplasia, hyperkeratosis and hypergranulosis.

Since knockout mice for p110α and p110β are early embryonic lethal, they cannot be used to study the role of PI3K in the regulation of keratinocyte differentiation and epidermal stratification. However, a role of PI3K in keratinocyte biology has been suggested by experiments where keratinocyte differentiation was induced by suspension culture (Janes et al., 2004; Sayama et al., 2002), but overall rather conflicting results have been obtained.

To gain insight into the role of PI3K in the regulation of keratinocyte proliferation and differentiation in vitro and in vivo, we first determined the expression pattern of p110α and p110β in normal and wounded skin. Subsequently, we modulated PI3K signaling in keratinocytes through stable introduction of an inducible, activated form of PI3K (Leenders et al., 2004). Our results reveal a crucial role of PI3K in the regulation of keratinocyte motility, proliferation, and differentiation in vitro and suggest that this enzyme is a key regulator of epidermal homeostasis and repair in vivo.

Results
p110α and p110β are strongly expressed in keratinocytes of normal and wounded skin

To elucidate possible functions of class I PI3Ks in the skin, we first identified the isoforms expressed in this tissue and we determined their regulation after skin injury (Fig. 1). By ribonucleotide protection assay (RPA) we detected transcripts encoding the catalytic subunits p110α, p110β, and p110γ in RNA preparations of total murine skin, but only p110α and p110β were expressed in the epidermis and in cultured murine keratinocytes. During cutaneous wound repair, mRNA levels of p110α and p110β remained unchanged in comparison with those of unwounded skin, whereas the mRNA levels of p110γ, which is expressed in cells of the immune system (Hirsch et al., 2000), were upregulated between day 1 and day 7 after wounding (Fig. 1A). The wound-regulated expression of p110γ was verified by western blotting (Fig. 1B), and the strongest expression was seen during the inflammatory phase of the wound repair process. In addition, strong phosphorylation of the PI3K downstream effector Akt occurred within this time period (Fig. 1B). Akt1 as well as PTEN mRNA levels were unaltered during the wound repair process (data not shown), and we also did not observe changes in the expression levels of the regulatory subunits p55γ and p85α, which were ubiquitously expressed in the dermis and the epidermis (Fig. 1B).

To localize phosphorylated Akt in wounded skin, we performed immunofluorescence staining with an antibody against phosphorylated (Ser473) Akt. Overall levels of phosphorylated Akt in normal adult mouse skin were low, as indicated already by western blotting. Phosphorylated Akt was only present in hair follicle keratinocytes of the outer root sheath (Fig. 1Ca, arrowhead). One day after wounding, we detected phosphorylated Akt in some epidermal keratinocytes in close proximity to the wound rim (Fig. 1Cb,e,d) and in the wounded, subcutaneous muscle panniculus carnosus (Fig. 1Cb). At day 3-5 after injury, high levels of phosphorylated Akt were seen in suprabasal, nucleated keratinocytes of the hyperproliferative wound epithelium (Fig. 1Cd,e, and data not shown). At 14 days after wounding, when the wound was covered by a neoeperidermis, phosphorylated Akt was no longer detected (Fig. 1Cf). In 4-day human incisional wounds, a similar phospho-Akt staining was observed and signals were predominantly seen in suprabasal keratinocytes of the hyperproliferative wound epidermis (Fig. 1Ch, arrows). No signal was observed in human unwounded epidermis, except for occasional single cells (Fig. 1Cg). Non-radioactive in situ hybridization localized p110α and p110β mRNA in keratinocytes at the rim of day 5 wounds. As depicted in Fig. 1D, p110α mRNA was found in basal and particularly in suprabasal keratinocytes of the hyperproliferative epithelium (HPE) as well as in intact skin adjacent to the wound (Fig. 1Da,b, arrowheads), whereas p110β mRNA was only detected in suprabasal keratinocytes of the HPE (data not shown).

Furthermore, we detected p110α mRNA in hair follicles and – in agreement with the data obtained by RPA – also in dermal fibroblasts (Fig. 1Da,b, arrows). Hybridization of serial sections with the corresponding sense probes did not reveal a specific signal (Fig. 1Dc).

Transient upregulation of p110α and p110β during terminal differentiation of human keratinocytes

The predominant suprabasal expression of p110α and p110β suggested a role of PI3K in the control of keratinocyte differentiation. To address this possibility, we first determined the expression of these catalytic subunits during differentiation of primary human keratinocytes in vitro. Northern blot analysis revealed a transient upregulation of p110α and p110β mRNAs (4.6-fold and 9.2-fold, respectively), which preceded the expression of the differentiation marker involucrin (Fig. 2A). Consistent with the northern blot experiments, we also detected transient upregulation of p110α protein during the differentiation process (Fig. 2B). The upregulation of p110α and p110β was not reflected by increased Akt phosphorylation though; rather, a continuous reduction in the levels of phosphorylated Akt was observed. This might be explained by the slight increase in PTEN expression during the differentiation process (Fig. 2B).

Enhanced PI3K activity promotes keratinocyte proliferation

Is the transient induction of PI3K expression during keratinocyte differentiation in vitro and in the hyperproliferative wound epidermis in vivo of functional importance? To address this question we determined the consequences of enhanced PI3K activity in keratinocytes by generating HaCaT keratinocyte cell lines stably expressing a 4-OHT-inducible, active form of PI3K (Fig. 3A). The two cell clones 1 and 17 expressing different amounts of the fusion protein after 4-OHT treatment of these cells was monitored by detection of Ser473 phosphorylated Akt1 as well as PTEN mRNA levels were unaltered during the wound repair process (data not shown), and we also did not observe changes in the expression levels of the regulatory subunits p55γ and p85α, which were ubiquitously expressed in the dermis and the epidermis (Fig. 1B).

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Possible compensatory alterations in the expression levels of PTEN, Akt1, p110α or mTOR in response to PI3K activation were not observed as assessed by RPA (data not shown). Proliferation rates of these cell lines were determined by [3H]thymidine incorporation after treatment with 4-OHT or solvent (Fig. 3E). Myr-p110α*-mER cells that were treated
**Fig. 1.** Expression of p110α, p110β, and p110γ in normal and wounded mouse skin. (A) mRNA levels of the PI3K catalytic subunits p110α, p110β, and p110γ were assessed by RPA using 10 µg total RNA from murine back skin, tail epidermis, tail dermis and skin-derived primary keratinocytes and fibroblasts or from full-thickness excisional mouse wounds at the indicated time points after injury. Hybridization with a GAPDH-specific riboprobe was performed as a loading control. -p, undigested riboprobes. (B) 30 µg total cellular protein from non-wounded and wounded skin at different time points after injury were analyzed by western blotting for the presence of p110γ, Ser473-phosphorylated Akt or p85/p55. Coomassie staining of the gel was performed to verify equal loading. High levels of immunoglobulins in early wounds result from invading immune cells. (C) Sections from tail skin and 1- to 14-day full-thickness excisional wounds were stained with an antibody detecting Ser473-phosphorylated Akt. (a) Tail skin showing phosphorylated Akt (red) in the outer root sheath keratinocytes of hair follicles (arrowhead). 1-day wounds harbor high levels of phosphorylated Akt in keratinocytes at the wound rim (arrow, b and higher magnification in c), and in the panniculus carnosus (arrow, b). The eschar (Es) stains non-specifically. (d) Phosphorylated Akt is detected in suprabasal cells of the hyperproliferative epithelium of 5-day wounds (higher magnification in e, arrowheads) but not of 14-day wounds (f). No phosphorylated Akt was detected in normal human epidermis (g), but suprabasal cells were phospho-Akt positive in the hyperproliferative wound epidermis of a 4-day human incisional wound (h, arrow). The basal lamina is indicated by the white dotted line. Nuclei were counterstained with Hoechst 33342 (blue). Bars, 50 µm. (D) In situ hybridizations with a digoxigenin-labeled antisense riboprobe (a,b) show p110α mRNA in basal and suprabasal keratinocytes (arrowheads in b) of the normal epidermis and of the hyperproliferative epithelium of 5-day wounds and in dermal fibroblasts (arrows in b). (c) Serial sections incubated with the sense riboprobe revealed only nonspecific background (c). Bars, 100 µm (a); 50 µm (b,c). D, dermis; E, epidermis; Es, eschar; G, granulation tissue; HF, hair follicle, HPE, hyperproliferative epithelium, M, muscle panniculus carnosus.
with 4-OHT showed a threefold increase in proliferation rate ($P=0.0004$) compared with Myr-p110α*-mER cells treated only with solvent or vector-transfected control cells treated with either 4-OHT or solvent. Successful activation of PI3K in the same experiment was monitored by western blotting for phosphorylated Akt. Consistent with this result, a twofold increase in the number of cells in S-phase and a decrease in the number of cells in G1 was found after 4-OHT treatment of Myr-p110α*-mER expressing cells as revealed by FACS analysis of propidium iodide-stained cells (data not shown).

Identification of novel PI3K target genes in keratinocytes

To determine if the increased rate of keratinocyte proliferation upon PI3K activation is associated with altered gene expression, RNAs of Myr-p110α*-mER cells and vector-transfected cells were subjected to microarray analysis before and after treatment with 4-OHT or solvent for 5 hours. A total of 122 genes were differentially expressed in 4-OHT-treated Myr-p110α*-mER cells compared with expression in solvent-treated and untreated Myr-p110α*-mER cells as well as with untreated, ethanol-treated or 4-OHT-treated vector-transfected cells. Of these, 78 genes were downregulated and 44 were upregulated by a factor >2 in at least one comparison and at least by >1.5-fold in all appropriate comparisons (supplementary material Table S1). These genes were assigned to one or more of eight functional groups corresponding to specific biological processes. Genes involved in regulation of cell motility and cell adhesion were highly represented as well as genes involved in cell-cycle control, of which most also play crucial roles in DNA repair (Table 1).

As a possible mediator of the observed effects of PI3K on keratinocyte proliferation, we identified RTP801/REDD1. As determined by western blot analysis and confirmed by RPA (data not shown), RTP801 expression was induced in Myr-p110α*-mER cells 2 hours after 4-OHT treatment in contrast to controls treated either with solvent or 4-OHT (Fig. 4A). In addition, RTP801 expression levels were enhanced following the upregulation of p110α/H9251 and p110α/H9252 during terminal differentiation of keratinocytes (Fig. 2A).

To validate additional effector genes, RNAs from different cell lines and from independent experiments were subjected to real-time RT-PCR. These genes include the α-thalassemia/mental retardation syndrome X-linked gene (ATRX), which encodes a protein involved in chromatin remodeling and DNA methylation (Gibbons et al., 2000; Xue et al., 2003). Levels of ATRX transcripts were downregulated on average 2.5 times as determined by microarray analysis. Real-time RT-PCR confirmed ATRX downregulation (14.5 times) in an

<table>
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<th>Process</th>
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<th>Number of genes upregulated &gt;twofold</th>
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<td>Cell motility, adhesion, polarity</td>
<td>9</td>
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<td>15 (2)</td>
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<tr>
<td>Cell-cycle control, DNA repair</td>
<td>10 (1)</td>
<td>3 (3)</td>
<td>13 (4)</td>
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<tr>
<td>Epidermal development, differentiation and integrity</td>
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<td>Apoptosis</td>
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<tr>
<td>Unknown function</td>
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Table 1. PI3K target genes are involved in cytoskeleton organization, cell-cycle control, DNA repair and epidermal homeostasis

Genes that are differentially regulated by PI3K (>twofold in at least one comparison) were assigned to nine functional groups. Gene products with unknown function are classified as such. The number of differentially regulated genes involved in a certain process is displayed. Numbers in parentheses indicate additional genes with only proposed or uncertain function in a particular process.
PI3K in epidermal homeostasis

independent Myr-p110α*-mER clone, and downregulation was confirmed by simultaneous addition of 4-OHT treatment during this time period. (E) Vector-transfected and Myr-p110α*-mER expressing keratinocytes were treated with 4-OHT (+) or solvent (−) as indicated. DNA replication was measured by [3H]thymidine incorporation. Untreated control was arbitrarily set as 1.0. Western blot analysis, which was carried out in parallel, verifies phosphorylation of Akt after 4-OHT-treatment in Myr-p110α*-mER cell clones. Error bars indicate s.e.m. Significance was determined with the Student’s t-test (two-tailed). **P<0.01; ***P<0.001.

Fig. 3. Expression of an inducible active form of PI3K in HaCaT keratinocytes. (A) The retroviral vector used for expression of the Myr-p110α*-mER fusion protein is shown. IRES, internal ribosomal entry site; iSH2 (p85), inter SH2 domain of the murine p85α protein; L, linker sequence; LTR, long terminal repeat; mER, cDNA encoding the mutated murine estrogen receptor ligand binding domain; mpl10α*, murine p110α cDNA; Myr, myristoylation signal sequence; neo, neomycin-resistance gene. The position of the riboprobe p110α*-p used for RPA is indicated as a black line. (B) Western blotting with 30 μg total cellular protein for detection of endogenous p110α and overexpressed Myr-p110α*-mER protein in HaCaT cell clones stably transfected with a retroviral Myr-p110α*-mER construct. (C) Western blotting using 30 μg total cellular protein revealed increased amounts of phosphorylated Akt after 4-OHT-treatment of the stable cell clones 1 and 17. The amount of β-actin in each lane served as a loading control. (D) As shown by western blot analysis of phosphorylated Akt, induction of PI3K signaling by 4-OHT was blocked by simultaneous addition of LY294002. Simultaneous detection of PCNA indicates that cell proliferation is not negatively influenced by LY294002 treatment during this time period. (E) Vector-transfected and Myr-p110α*-mER expressing keratinocytes were treated with 4-OHT (+) or solvent (−) as indicated. DNA replication was measured by [3H]thymidine incorporation. Untreated control was arbitrarily set as 1.0. Western blot analysis, which was carried out in parallel, verifies phosphorylation of Akt after 4-OHT-treatment in Myr-p110α*-mER cell clones. Error bars indicate s.e.m. Significance was determined with the Student’s t-test (two-tailed). **P<0.01; ***P<0.001.

Modulation of PI3K activity results in cytoskeletal reorganization

As indicated by the data obtained from microarray experiments, PI3K activation regulates several genes involved in actin cytoskeleton remodeling, for example PTPL1-associated RhoGAP 1 (PARG1) and Slit-Robo Rho-GTPase-activating protein (GAP) 2 (supplementary material Table S1). To determine if the observed regulation of these genes by PI3K is of functional importance, we visualized the F-actin cytoskeleton before and after 4-OHT treatment of Myr-p110α*-mER expressing HaCaT cells. Phalloidin staining revealed that F-actin filaments were predominantly located at the cell periphery in 4-OHT-treated Myr-p110α*-mER cells (Fig. 5C,F arrowheads). The filament pattern probably reflects lamellipodial protrusions or cortical actin formation, which provides the contractile force to release the cell from the substratum and enables its subsequent movement (Ehrenreiter et al., 2005; Jimenez et al., 2000; Lim et al., 2004; Miki et al., 1996). By contrast, in control cells treated with 4-OHT or solvent, F-actin filaments were more evenly distributed throughout the cell (Fig. 5A,B,D,E). Focal adhesions were unaltered as revealed by paxillin co-staining (Fig. 5E,F). As actin cytoskeletal rearrangement is a prerequisite for cellular motility and proliferation (Kubler and Watt, 1993), we asked whether PI3K affects these processes. The influence of PI3K on cell movement was first determined in scratch assays with primary human keratinocytes in the presence or absence of the PI3K inhibitor LY294002. Treatment with 25 μM LY294002 reduced keratinocyte motility by a factor of three in comparison to cells treated with the LY294002 solvent DMSO (P<0.048, Student’s t-test). The reduced motility was...
dependent on the concentration of the inhibitor, and treatment with 50 μM LY294002 resulted in a marked reduction of the average migrated distance (P<0.0004, Student’s t-test) (Fig. 6A). This effect of LY294002 was seen at different time points after wounding (Fig. 6A and data not shown). Staining of the wounded monolayer for phosphorylated Akt revealed the efficiency of the inhibitor treatment and demonstrated the presence of phospho-Akt-positive cells at the wound edge of DMSO-treated cells (Fig. 6B). No obvious cell death was observed at this concentration of LY294002 during the time frame of the experiment (data not shown).

In Trans-well migration assays with Myr-p110α*-mER expressing HaCaT cells, stimulation of PI3K activity by addition of 4-OHT significantly enhanced the rate of migration, whereas 4-OHT had no effect on migration in control cells (Fig. 6C).

An explant assay originally developed by Mazzalupo et al. (Mazzalupo et al., 2002) emphasized the necessity of PI3K signaling for keratinocyte migration: in this defined ex vivo experiment, mitomycin-C-treated keratinocytes migrated several cell diameters from the murine skin biopsy (Fig. 6Da-c). Under the same conditions, LY294002 completely suppressed keratinocyte migration (Fig. 6Dd). The mTOR inhibitor rapamycin did not influence keratinocyte migration significantly, demonstrating that PI3K but not mTOR controls keratinocyte movement (Fig. 6Dc).

PI3K is a negative regulator of keratinocyte differentiation

The expression pattern of PI3K in the epidermis and the analysis of PI3K-regulated target genes suggested that keratinocyte differentiation is controlled by PI3K activity. To test this possibility, Myr-p110α*-mER cell clones were cultured in suspension, which is known to rapidly induce keratinocyte differentiation (Green, 1977). Expression of the differentiation marker involucrin was upregulated in solvent-treated Myr-p110α*-mER cells as expected. By contrast, 4-OHT-treated Myr-p110α*-mER cells failed to induce involucrin protein expression under these conditions (Fig. 7A). Since in suspension culture differentiation is coupled to anoikis, we also induced differentiation of Myr-p110α*-mER expressing HaCaT cells by autocrine cell growth to distinguish between differentiation and apoptosis. Subconfluent cells were serum-starved in the presence or absence of 4-OHT, and induction of differentiation was monitored by upregulation of involucrin. In 4-OHT-treated Myr-p110α*-mER cells involucrin expression was markedly delayed under these conditions in comparison with controls and even after 9 to 11 days under serum-free culture conditions involucrin protein levels were very low (Fig. 7B). By contrast, inhibition of PI3K activity by LY294002 in Myr-p110α*-mER overexpressing cells as well as in primary

![Fig. 4. Validation of PI3K target genes. (A) Western blotting reveals upregulation of RTP801 following PI3K-activation. Unaltered RTP801 levels were seen in vector-transfected control clones. Equal loading was verified by treatment of the membranes with a β-actin antibody. (B) RNAs isolated from independent Myr-p110α*-mER expressing or vector-transfected cell clones were subjected to real-time RT-PCR. Genes encoding α-thalassemia/mental retardation syndrome X-linked protein (ATRX), periphilin1 protein (PPHL1), ets-homologous factor 3 protein (EHF) and Cockayne syndrome 1 protein (CKN1) were tested for differential expression in 4-OHT-treated Myr-p110α*-mER cells as expected. By contrast, 4-OHT-treated Myr-p110α*-mER cells failed to induce involucrin protein expression under these conditions (Fig. 7A). Since in suspension culture differentiation is coupled to anoikis, we also induced differentiation of Myr-p110α*-mER expressing HaCaT cells by autocrine cell growth to distinguish between differentiation and apoptosis. Subconfluent cells were serum-starved in the presence or absence of 4-OHT, and induction of differentiation was monitored by upregulation of involucrin. In 4-OHT-treated Myr-p110α*-mER cells involucrin expression was markedly delayed under these conditions in comparison with controls and even after 9 to 11 days under serum-free culture conditions involucrin protein levels were very low (Fig. 7B). By contrast, inhibition of PI3K activity by LY294002 in Myr-p110α*-mER overexpressing cells as well as in primary

![Fig. 5. PI3K affects actin reorganization. FITC-coupled phalloidin detected polymerized F-actin filaments (green) in 4-OHT- or solvent-treated Myr-p110α*-mER cell clones (clone #17, A,C,E,F) or vector-transfected control clones (B,D). Arrowheads indicate cells with accumulated actin filaments at the cell periphery (C,F). Distribution of paxillin is depicted in red (E,F). Nuclei were counterstained with Hoechst 33342 (blue). Bars, 50 μm.
human keratinocytes resulted in premature induction of differentiation as determined by earlier expression of differentiation markers (data not shown).

Epithelial hyperthickening and invasive-like growth of Myr-p110α*-mER expressing HaCaT cells in organotypic cultures

Since the differentiation capacity of HaCaT cells in monocultures is limited, we next used a three-dimensional organotypic culture system, which allows terminal differentiation of HaCaT cells (Maas-Szabowski et al., 2000; Schoop et al., 1999). We tested whether PI3K activation would be sufficient to induce hyperproliferation and concomitantly delay differentiation in this in vivo-like situation. Organotypic cultures of Myr-p110α*-mER HaCaT cells were treated with 4-OHT or solvent, or left untreated. After 16 days of continuous air exposure and treatment, we observed a dramatic effect of PI3K activation on epidermal morphology. In contrast to controls, the epithelium formed by keratinocytes with chronically active PI3K signaling was strongly hyperplastic and disorganized. In particular, the number of nucleated cell layers increased significantly and keratinocytes invaded the collagen gel (Fig. 8Ab,e,h,l), resulting in a disorganized border between collagen gel and epithelium.

The increase in epithelial thickness correlated with the transgene expression in different clones and reached a maximum of 2- to 2.5-fold (Fig. 8B). BrdU-labeling experiments revealed that proliferation rates of keratinocytes in 4-OHT-treated Myr-p110α*-mER cultures were enhanced up to threefold ($P<0.001$) in comparison with 4-OHT-treated vector-transfected controls as well as ethanol-treated Myr-p110α*-mER cells (Fig. 8A,B). Furthermore, BrdU-positive cells were not restricted to the basal layer, but were also present in the suprabasal layers (Fig. 8Ae,l, arrowheads). PI3K hyperactivation did not induce additional cell death as determined by TUNEL staining (data not shown). To verify the activation of PI3K in the organotypic cultures, sections were stained with an antibody to Ser473-phosphorylated Akt. As expected, strong phospho-Akt staining was seen in the epithelium of 4-OHT treated cultures, but was only weak in solvent- or untreated cultures (Fig. 9Aa,b).

Keratinocyte differentiation in 4-OHT-treated 3D cultures of Myr-p110α*-mER-expressing HaCaT cells was delayed as revealed by immunostaining for differentiation-specific proteins (Fig. 9A). The number of cell layers expressing high levels of K14, a marker for basal, non-differentiated keratinocytes, was strongly enhanced in 4-OHT-treated cultures (Fig. 9Ac,d), and the onset of expression of the early differentiation marker K10 was shifted upwards concomitantly (Fig. 9Af). Later differentiation was also retarded in comparison with the controls as assessed by immunostaining for involucrin (Fig. 9g,h). Both differentiation markers exhibited a patchy distribution instead of the typical uniform distribution in suprabasal layers. Late differentiation though was not abrogated, because cells in the most suprabasal layers started to express loricrin (Fig. 9Al,m), a marker for terminally differentiated keratinocytes (Hohl et al., 2000).

**Fig. 6. PI3K enhances keratinocyte motility.**

(A) Motility of mitomycin C-treated primary human keratinocytes after 12 hours or 60 hours of treatment with LY294002 in growth medium was monitored by in vitro scratch assay.

(B) Immunofluorescence staining of DMSO- and LY294002-treated primary human keratinocytes after scratch wounding using an antibody against phosphorylated Akt. (C) Quantitative analysis of Transwell migration assays using 4-OHT- or solvent-treated Myr-p110α*-mER cell clones (left panel) or vector-transfected cells (right panel). Error bars in B and D indicate s.d. Significance was determined by one-way ANOVA with Bonferroni post-correction. *$P<0.05$; **$P<0.01$; ***$P<0.001$.

(D) Explant cultures from back skin biopsies of 1-day-old mice were established (a) and treated with DMSO (b), rapamycin (c) or LY294002 (d) in combination with mitomycin C. 10 days after establishment of the culture, the biopsy was removed and the cells, which had grown out from the culture, were stained with hematoxylin and eosin and photographed.
In addition, expression of α6 integrin, which has been described as a marker for epidermal stem cells (Tumbar et al., 2004), was extended from the basal layer to the suprabasal layers in the epithelia of 4-OHT-treated Myr-p110α*-mER cultures (Fig. 9Ai,k). To confirm that cells in the suprabasal layers exhibited typical features of basal, proliferation-competent keratinocytes, we determined expression of p63, a marker for the proliferative potential of keratinocytes (Bamberger et al., 2002; Green et al., 2003; Mills et al., 1999; Yang, A. et al., 1999). Indeed, p63 expression was confined to the basal layer in the controls, but was extended to the suprabasal layers in the epithelia of 4-OHT-treated cultures, resembling the situation found in skin wounds (Bamberger et al., 2005) (Fig. 9B).

To test if PI3K is able to induce a hyperplastic response in an already established epithelium as seen during wound healing, 3D organotypic cultures were first established in the absence of 4-OHT, and 10-day-old cultures were then treated for a further 15 days with either 4-OHT or solvent. Under these conditions, the 4-OHT-treatment also resulted in the formation of a hyperthickened epithelium (Fig. 10A) with a twofold increase in keratinocyte proliferation (Fig. 10B). In these cultures, however, suprabasal, BrdU-positive cells were very rare, and the suprabasal keratinocytes were well differentiated as revealed by K10, involucrin and loricrin immunostaining (Fig. 10C and data not shown). This finding suggests that the basal cell compartment was predominantly expanded in these cultures. The invasive growth phenotype was also less pronounced in these cultures compared with cultures immediately treated with 4-OHT.
PI3K in epidermal homeostasis

Taken together, enhanced PI3K activity in keratinocytes resulted in enhanced proliferation, delayed differentiation and formation of a hyperthickened epithelium in developing as well as established epithelia, demonstrating that PI3K is an important regulator of epidermal homeostasis.

Discussion
We used a combination of in vitro and in vivo studies to determine the expression and function of PI3K in the epidermis. An interesting in vivo finding was the correlation between Akt phosphorylation in keratinocytes and the proliferative potential of these cells. Thus, phospho-Akt was found in basal cells of highly proliferative newborn mouse skin (Calautti et al., 2005; Umeda et al., 2003, Di-Poi et al., 2005), but not in thin adult mouse epidermis (this study). Most importantly, we found enhanced phosphorylation of Akt in the epidermis of mouse wounds, most likely as a result of altered expression of PI3K catalytic subunits. These results prompted us to determine the functional consequences of high PI3K activity in keratinocytes in vitro by generation and characterization of keratinocyte cell lines, which express an inducible form of constitutively active PI3K (Leenders et al., 2004).

PI3K activation delays keratinocyte differentiation
Constitutive activation of PI3K signaling delayed keratinocyte

Fig. 9. Delayed differentiation of keratinocytes expressing Myr-p110α*-mER in organotypic cultures. (A) 4-OHT-treated and solvent-treated (control) organotypic cultures of Myr-p110α*-mER expressing HaCaT cells were analyzed for phosphorylated Akt (a,b, green), K14 (c,d, green), K10 (e,f, green), involucrin (g,h, blue), loricrin (l,m, green) and α6 integrin (c,d,i,k, red) by immunofluorescence. Arrowheads in d indicate strong K14 expression in the upper suprabasal layers. Nuclei were stained with Hoechst 33342 and are shown in blue in e and f. Note the apical α6 integrin expression in suprabasal cells (k, arrowheads), which is not observed in the controls (i). Counterstaining in l and m was performed with propidium iodide (red). (B) Paraffin sections of 4-OHT- (c,d) or solvent-treated (a,b) Myr-p110α*-mER expressing organotypic cultures were subjected to immunofluorescence staining using an antibody recognizing p63. Nuclei are counterstained in a and c with Hoechst 33342. The basement membrane is indicated with a dotted line. Bars, 50 μm.
differentiation in autocrine growth culture and in a suspension-induced differentiation model (Fig. 5), demonstrating that the effect is independent of anoikis or apoptosis. Our result is consistent with data from Sayama et al. (Sayama et al., 2002), who detected reduced expression of differentiation markers in suspension-cultured primary human keratinocytes harbouring constitutively active PI3K.

LY294002 treatment, by contrast, rapidly induced differentiation of keratinocytes, resulting in premature expression of differentiation markers (this study). Most importantly, HaCaT cells with enhanced PI3K activity also formed a strongly hyperplastic epithelium in organotypic cultures (Figs 7-9), suggesting that the differentiation-inhibiting effect of PI3K is also important in vivo.

The identification of PI3K target genes indicates that PI3K exerts its differentiation-inhibiting effect on the one hand by promoting expression of proteins preserving the undifferentiated state of a keratinocyte, such as RTP801/REDD1 (Ellisen et al., 2002), and on the other hand by suppressing transcription of differentiation-promoting genes such as ese-3 and periphilin1. Since RTP801/REDD1 overexpression was shown to abolish keratinocyte differentiation (Ellisen et al., 2002) and also contributed to PI3K-dependent malignant growth of prostate cancer cells (Schwarzer et al., 2005), its upregulation after PI3K activation may be important for the preservation of the proliferation-competent state of keratinocytes as observed in the 4-OHT-treated organotypic cultures. The concomitant downregulation of periphilin1 and of other differentiation-inducing genes may enhance this effect, and this may be further enforced by enhancement of cell-cycle progression, since periphilin1 acts as a repressor of Cdc7 expression and induces S-phase arrest (Kazerounian and Aho, 2003; Kurita et al., 2004).

PI3K promotes keratinocyte proliferation
The retarded keratinocyte differentiation was accompanied by enhanced proliferation in monolayer cultures and in the 3D organotypic culture system (Figs 7-9). In the latter, BrdU-positive cells were still present in suprabasal layers, and suprabasal keratinocytes inappropriately expressed p63 and the α6 integrin subunit. Both proteins are characteristic for non-differentiated keratinocytes and are discussed as markers of epidermal stem cells (Bamberger et al., 2002; Georges-Labouesse et al., 1996; Green et al., 2003; Mills et al., 1999; Yang, A. et al., 1999). Thus, in the presence of active PI3K, early suprabasal cells are retained longer in a proliferative state, demonstrating that the proliferative potential of keratinocytes is coupled to PI3K signaling.

These activities of PI3K are most likely of major importance for the in vivo situation, especially under proliferation-demanding conditions such as during cutaneous wound repair or epidermal development, where a transient increase in PI3K activity was observed (Di-Poi et al., 2005) (this work). However, chronically active PI3K signaling is likely to contribute to the pathogenesis of hyperproliferative skin.
disorders such as psoriasis where increased PI3K activity was detected in the epidermis (Pike et al., 1989).

Regulation of DNA repair and cell cycle checkpoint control: a novel function of PI3K?
Strikingly, PI3K-regulated genes in keratinocytes are involved in cell-cycle control and DNA repair, and downregulation of the genes encoding Rad25, Rad26L, Msh2 and NFAT5 by activated PI3K may provide a way to overcome cell-cycle checkpoints. This could enable keratinocyte proliferation even under unfavorable, potentially DNA-damaging conditions, e.g. in wounded or inflamed skin or after exposure to toxic chemicals or UV irradiation, to secure epidermal barrier function. This idea is supported by results from Shitivelman et al. (Shitivelman et al., 2002), who demonstrated crosstalk between the PI3K pathway and Chk1, one of the key regulators of the DNA damage checkpoint machinery. Further evidence for this unexpected role of PI3K comes from the observation that activated Akt can override DNA damage-induced G2-M checkpoints (Kandel et al., 2002). PI3K signaling may even provoke epigenetic changes as indicated by downregulation of ATRX, which is involved in chromatin remodeling, and, when mutated, causes diverse changes in DNA methylation patterns (Gibbons et al., 2000; Xue et al., 2003). The identification of a putative negative role in the regulation of DNA damage checkpoint controls provides new insight into PI3K function as a tumor promoter. However, under most circumstances, PI3K activation will not lead to tumor formation in the skin per se because it is not sufficient to completely prevent the differentiation process and subsequent shedding of dead keratinocytes. Furthermore, albeit delayed, keratinocyte differentiation occurs in vivo in the presence of active PI3K, such as in wounded or embryonic skin. Similarly, overexpression of p110α or Akt in other tissues was not sufficient to induce tumor formation unless combined with an oncogenic mutation (Bernal-Mizrachi et al., 2001; Holland et al., 2000; Hutchinson et al., 2001; Tuttle et al., 2001). Thus, we speculate that PI3K-mediated hyperproliferation occurs at the expense of the genomic integrity, a situation that is temporarily tolerated, for example under stress conditions such as in wound repair, but enhances in the long run, the risk of tumor formation, as seen in mice deficient for the PI3K antagonist PTEN in keratinocytes (Backman et al., 2004; Suzuki et al., 2003).

PI3K is a potent inducer of keratinocyte migration
Finally, our findings reveal that PI3K is a potent regulator of the actin cytoskeleton in keratinocytes. Rearrangement of the cytoskeleton after PI3K stimulation facilitates keratinocyte motility and could explain the inability of keratinocytes to cover cell-free areas in scratch assays when PI3K signaling is blocked by LY294002. Assessment of the keratinocyte reepithelialization potential in explant assays confirmed the important role of PI3K in keratinocyte migration, and strongly suggests that PI3K also enhances wound reepithelialization by inducing both the initial migration of keratinocytes and their subsequent proliferation. Additional signaling pathways are likely to determine if PI3K activates migration, proliferation or both processes in vivo.

Akt-dependent and independent activities of PI3K
Although the mechanisms by which PI3K can exert its pro-motogenic effect are largely unclear, the observed downregulation of Rho-GTPase-activating proteins like slt-robo-GAP2 and PARG1 suggests activation of the Rac/Rho pathway, which has been described before to be crucial for PI3K-mediated invasion in vitro (Shaw et al., 1997). This also implicates new mechanisms by which PI3K could influence keratinocyte behavior independently of Akt (see also Shaw et al., 1997), and it is tempting to speculate that PI3K influences keratinocyte differentiation at least in part via regulation of Rac (Benitah et al., 2005). Distinct effects of mTOR and PI3K inhibition on keratinocyte migration further point in this direction. Akt-independent signaling of PI3K has emerged during the last years as an important mechanism of PI3K action (Vivanco and Sawyers, 2002; Zhao et al., 2003). The existence of such pathways implies that the expression of the catalytic subunits of PI3K in normal epidermis is functionally important, despite the lack of significant Akt phosphorylation. Akt-independent functions of PI3K could also explain the different effects seen upon overexpression of Akt1 (Janes et al., 2004) or PI3K in keratinocytes (Sayama et al., 2002) (this work).

In summary, the results of this work point to a crucial role of PI3K in the regulation of epidermal homeostasis and support the hypothesis that chronic activation of PI3K in keratinocytes is causally involved in the pathogenesis of hyperproliferative skin disease.

Materials and Methods
Preparation of skin wounds
Four full-thickness excisional wounds were generated on the back of Balb/C mice (Werner et al., 1994). At different time points after injury mice were sacrificed and the wounds plus 2 mm of the epidermal margins were collected for isolation of DNA or preparation of protein lysates. For immunohistological analysis, excised wound halves were embedded in tissue freezing medium (OCT, Jung, Nussloch, Germany) or fixed in 4% paraformaldehyde in 1× PBS at 4°C overnight and embedded in paraffin. All experiments were carried out with permission from the local veterinary authorities.

Cell culture
Human primary keratinocytes (passage 3-5) were maintained in defined keratinocyte serum-free medium (Gibco, Basel, Switzerland). HaCaT cells (Boukamp et al., 1988) were maintained in DMEM, 1% penicillin/streptomycin, 10% fetal calf serum (FCS) (Sigma, Buchs, Switzerland). Stable HaCaT cell lines were generated by retroviral infection of HaCaT cells with the vector pLXIN-Myr-p110α*+mER (Leenders et al., 2004). Transfectants with stably integrated Myr-p110α*+mER were selected and subsequently cultured in DMEM, 1% penicillin-streptomycin, 10% FCS supplemented with G418 (400 mg/l; Invitrogen, Basel, Switzerland) or puromycin (200 mg/l; Invitrogen). Activation of Myr-p110α*+mER was achieved by addition of 4-hydroxytamoxifen (4-OHT) (200 nM to 1.0 μM; Sigma) to the medium.

Autoinocrine cell growth and suspension culture
Human keratinocytes were seeded at 30% confluence. The medium was then changed to keratinocyte basal medium supplemented with hydrocortisol and phorbamycin (Cambrex, Verviers, Belgium) to induce terminal differentiation by autoinocrine cell growth (Bamberger et al., 2002; Ponnaym et al., 1999). Medium was changed every second day. To induce differentiation by suspension culture, keratinocytes were seeded into poly-2-hydroxyethyl-methacrylate (Sigma) coated plastic dishes in DMEM, 1% penicillin-streptomycin (Frisch and Francis, 1994; Sayama et al., 2002). Cells were harvested at different time points after induction of differentiation.

Organotypic cultures
Human primary dermal fibroblasts at passage 4-9 and Myr-p110α*+mER-HaCaT cell lines were cultured in an organotypic tissue culture model (Maas-Szabowski et al., 2000; Maas-Szabowski et al., 2003; Schoop et al., 1999). Fibroblasts were treated with 8 μg/ml mitomycin C for 4 hours and washed with 1× PBS before seeding into collagen gels (3×10^6 fibroblasts/ml; HaCaT keratinocytes (7×10^5 cells) were seeded into a glass ring on top of the collagen gel. After 8-10 hours, the glass ring was removed and keratinocytes were left exposed to the air. DMEM, 10%
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FCS, 1% penicillin/streptomycin, 0.1% ascorbic acid was added to the lower part of the chamber. The cultures were maintained for at least 16 days in medium supplemented with either 4-OHT or the solvent ethanol. Alternatively, cultures were grown for 10 days before addition of 4-OHT or solvent.

5-bromo-2'-deoxyuridine (BrdU) labeling and detection

BrdU (65.1 μM; Sigma) was added to the culture medium 12 hours before harvesting the organotypic cultures (Maaß-Szabowski et al., 2000; Maaß-Szabowski et al., 2003). Incorporated BrdU was detected with a horseradish peroxidase-conjugated monoclonal antibody against BrdU (Roche).

\[ ^{3}H \text{thymidine incorporation} \]

Cells were seeded into 24-well plates, serum-starved for 24 hours and treated with 1.0 μM 4-OHT or solvent for additional 24 hours. Addition of 1 μCi/ml \[^{3}H\text{thymidine} \] (Amersham, Buckinghamshire, UK) for 3 hours allowed radioactive labeling of the newly synthesized DNA. After washing, \[^{3}H\text{thymidine} \] incorporation was measured in triplicate wells using a 1500 Tri-Carb liquid scintillation analyzer (Shimazdu, 1999). Statistical analysis was performed with unpaired t-test included in the GraphPad Prism3 software package (GraphPad Software Inc.).

In vivo motility assay

Scratch assays were performed as described (Nasca et al., 1999). Briefly, human keratinocytes at passage three were seeded at high density into culture dishes. After an attachment time of 4 hours, a ‘scratch’ was made in the monolayer by removing cells with a pipette tip. Motility was determined by measuring the newly covered area per mm length of the initial scratch using OpenLab Software 3.5 (Improvement, Germany) immediately after the injury and 12, 24, 48 and 60 hours later.

Transwell migration assay

Transwell migration assays with mitomycin-C-treated cells or untreated cells were performed in triplicates as described by Andl et al. (Andl et al., 2004) and analyzed 12 hours after onset of migration by hematoxylin and eosin staining. Undersides of the filter insert were coated with fibronectin/collagen.

Mouse skin explant culture and analysis of keratinocyte outgrowth

Mouse skin explant cultures were established as described by Mazzalupo et al. (Mazzalupo et al., 2002). 16 hours after establishment of the culture LY294002 (25 μM), rapamycin (20 nM) or solvent DMSO were added and keratinocyte outgrowth was monitored after 10 days. Four to six skin biopsies were taken per mouse (n=18).

RNA purification and ribonuclease protection assay (RPA)

Isolation of total RNA and RPA were performed as described (Bamberger et al., 2002; Chomczynski and Sacchi, 1987; Sambrook, 2001). The riboprobes used to detect murine PI3K catalytic subunits correspond to nt 2929-3150 of p110α (GB: NM_008839), nt 3221-3373 of p110β (GB: NM_029094) and nt 2266-2577 of p110γ (GB: AF208345) cDNAs. A 120 bp fragment corresponding to nt 566-685 of glyceraldehyde-3-phosphatase dehydrogenase (GAPDH; GB: NM_008084) was simultaneously detected.

Northern blot

\[ {^{32}P}\text{deoxyctydine-triphosphate-labeled cDNA fragments were used as probes to detect p110α mRNA (nt 82-402; GB: HSU791434) and p110β mRNA (nt 2996-4279; GB: NM_006219). Involutrin expression was analyzed with a 1647 bp probe (nt 183-1830; GB: M13902), β-actin mRNA with a 377 bp probe (nt 1418-2234 excluding intron C; GB: M10277). Labeling of the probes was carried out using the Oligolabelling Kit according to the manufacturer’s recommendations (Amersham). } \]

Northern blotting was performed according to standard procedures.

In situ hybridization

Tail skin and excised wound halves were either immediately embedded in OCT and stored at −80°C or fixed in 4% PFA for 3 minutes at room temperature (RT). Blocking was performed with PBS for 3 minutes at RT, and subsequently cells were incubated with FITC-coupled phalloidin (Molecular Probes, The Netherlands) and Hoechst 33342.

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Microarray analysis

Total RNA from Mryr-p110α-mER expressing HaCaT cell clone 17 and from a vector-transfected control clone was isolated before and after 5 hours of treatment with 4-OHT or solvent as described above and additionally purified with the RNeasy Microclean up kit (Qiagen, Hombrechtikon, Switzerland). cRNA preparation and hybridization to GeneChip® Human Genome U133 Plus 2.0 arrays (Affymetrix, High Wycombe, UK) as well as raw data processing with Affymetrix GCOS 1.2 software was performed according to standard procedures. Briefly, probe cell intensities were calculated and summarized for the respective probe sets by means of the MAS5 algorithm (Hubbell et al., 2002). Global scaling was performed, resulting in the normalization of the trimmed mean of each chip to a target intensity of 500 as detailed in the statistical algorithm description document of Affymetrix. Quality was controlled by adequate scaling factors and appropriate numbers of present calls calculated by application of a signed-rank call algorithm (Li et al., 2002). Present calls and optimal 3′/5′ hybridization ratios for the housekeeping genes (gapdh and actb), and for the polya spike in controls and the proapoptotic control (biob, bioc, crex, biod) were checked before performing further analysis with Genespring 7.2 software (Agilent Technologies, Palo Alto, CA) using custom script possibilities.

Western blot analysis

Keratinocytes were lysed on ice in lysis buffer [20 mM HEPES, 150 mM NaCl, 10% glycerol, 1% Igepal CA-630, 1 mM EDTA, 0.5 mM 4-(2-aminoethyl)-benzenesulfonil fluoride, 0.01 μg/μl pepstatin, 0.01 μg/μl leupeptin, 0.01 μg/μl aprotinin, 200 mM sodium orthovanadate, 20 mM phenylarsine oxide, 50 mM sodium fluoride and serine/threonine phosphatase inhibitor cocktail II (Sigma)]. Protein lysates were sonicated, and 10-60 μg of total protein were separated by SDS-PAGE and transferred to nitrocellulose membranes. The following primary antibodies were used: Mouse monoclonal antibodies against p110α (Klippel et al., 1994), involucrin (clone SYN5), β-actin (Sigma), or keratin 10 (Dako Cytometion, Baar, Switzerland), and rabbit polyclonal antibodies against p110γ (kindly provided by M. Wymann, Basel, Switzerland), p85/55 (Klippel et al., 1994), Pten (Cell Signaling), phosphorylated Akt (serine 473 of Akt1; Sigma), PCNA (Santa Cruz Biotechnology, Santa Cruz, CA), or RT801/RED1 (Schwarzer et al., 2005). Horseradish-peroxidase-conjugated secondary antibodies were from Promega, Wallisellen, Switzerland. Bound immunocomplexes were detected using the enhanced chemiluminescence detection system (Amersham).

Immunofluorescence

For immunofluorescence paraffin sections fixed in 4% PFA or 95% ethanol/1% acetic acid were blocked in 10% BSA/1× PBS and incubated with the following antibodies: anti-Ser473 phosphorylated Akt (Cell Signaling), anti-keratin 14 (Covance, Berkeley, CA), anti-loricrin (Covance), anti-keratin 10 (Dako Cytometion), anti-involucrin (Sigma), anti-α6-integrin (PharMingen, San Diego, CA), anti-p63 (4A4, Santa Cruz). Nuclei were stained with Hoechst 33342.

Photographs of sections mounted in Mowiol (Sigma) were taken using a Zeiss Axioscope2 microscope coupled to an Axiocam HRc camera (Zeiss) or a Leica TCS SP2 AOBS confocal microscope using the Leica confocal software (Leica Microsystems, Wetzlar, Germany).

Histological analysis

Dewaxed paraffin sections were stained with hematoxylin and eosin according to standard procedures. Measurement of the epithelial thickness of organotypic cultures was carried out with the Openlab 3.1.2 software (Improvement) excluding 25% towards the end of the chamber.

Staining of the actin cytoskeleton

Keratinocytes were fixed in 4% PFA and permeabilized with 0.1% Triton X-100 in PBS for 3 minutes at room temperature (RT). Blocking was performed with 10% BSA/1× PBS (1 hour, RT), and subsequently cells were incubated with FITC-coupled phalloidin (Molecular Probes, The Netherlands) and Hoechst 33342.

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influenceable with the microarray experiments and Dr Matthias Wymann, University of Basel, for kindly providing the p110γ antibody. Especially, we would like to thank Dr L. Vindevoghel, who generated some of the Myr-p110α*-MEK clones, and Drs. C. Fuchs and M. Fussenegger, ETH Zurich, for production of the retrovirus. This work was supported by grants from the Swiss National Science Foundation (31-G1558.00 and 3100A0-109340/1 to S.W.). C.B. was supported by a fellowship from the Deutsche Forschungsgemeinschaft (BA 2156/1-1). The data presented here form part of a PhD thesis (S.P.).

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