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Pemphigus vulgaris identifies plakoglobin as key suppressor of c-Myc in the skin

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The autoimmune disease pemphigus vulgaris (PV) manifests as loss of keratinocyte cohesion triggered by autoantibody binding to desmoglein (Dsg)3, an intercellular adhesion molecule of mucous membranes, epidermis, and epidermal stem cells. Here we describe a so far unknown signaling cascade activated by PV antibodies. It extends from a transient enhanced turn over of cell surface-exposed, nonkeratin-anchored Dsg3 and associated plakoglobin (PG), through to depletion of nuclear PG, and as one of the consequences, abrogation of PG-mediated *c-Myc* suppression. In PV patients (6/6), this results in pathogenic c-Myc overexpression in all targeted tissues, including the stem cell compartments. In summary, these results show that PV antibodies act via PG to abolish the *c-Myc* suppression required for both maintenance of epidermal stem cells in their niche and controlled differentiation along the epidermal lineage. Besides a completely novel insight into PV pathogenesis, these data identify PG as a potent modulator of epithelial homeostasis via its role as a key suppressor of c-Myc.

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Introduction

Proliferation and terminal differentiation are part of the lifelong renewal of stratified squamous epithelia. During this process, the recruitment of stem cells into the proliferative compartment and subsequent commitment to terminal differentiation is finely regulated by the proto-oncogene c-Myc (Watt, 2001). In basal keratinocytes, c-Myc activation results in stem cell depletion and hyperproliferation as was shown in transgenic mouse models (Arnold and Watt, 2001; Waikel *et al*, 2001; Frye *et al*, 2003). When activated in suprabasal keratinocytes, c-Myc triggers proliferation and disrupts terminal differentiation in addition to premalignant transformations (Pelengaris *et al*, 1999; Waikel *et al*, 1999). Accordingly, in cultured mouse keratinocytes, c-Myc is induced at the onset of proliferation, then repressed as cells enter terminal differentiation (Kolly *et al*, 2005). In contrast to mouse, the consequences of c-Myc overexpression are unknown in human epidermis, as to date no skin disease with generalized c-Myc overexpression has been reported. However in human monolayer keratinocyte cultures, constitutive c-Myc expression is known to cause premature recruitment of epidermal stem cells into the proliferative compartment highlighting the importance of timely *c-Myc* suppression also in human skin (Gandarillas and Watt, 1997). The exact mechanisms of *c-Myc* suppression in both mouse and human keratinocytes, however, are not known.

Pemphigus vulgaris (PV) is a life-threatening autoimmune disease characterized by suprabasal acantholysis (i.e. loss of basal–basal and basal–suprabasal cell adhesion) in stratified squamous epithelia (Beutner and Jordon, 1964; Payne *et al*, 2004). The antigenic target is the desmosomal cadherin desmoglein (Dsg)3, which by virtue of its integration into desmosomes mediates cell–cell adhesion (Amagai *et al*, 1991). Dsg3 is mainly expressed in mucous membranes, the deep layers of epidermis and hair follicles, including epidermal stem cells (Koch *et al*, 1998; Shirakata *et al*, 1998; Mahoney *et al*, 1999; Wan *et al*, 2003). Most PV patient's antibodies target the adhesive interface between juxtaposed Dsg3 molecules of adjacent cells, and are thought to disrupt their trans-adhesion by steric hindrance (Tsunoda *et al*, 2003). Initially, this leads to depletion of nonkeratin–anchored, that is, Triton X-100-soluble Dsg3, and ultimately to loss of Dsg3 from fully assembled desmosomes (Aoyama and Kitajima, 1999; Sato *et al*, 2000). Although the molecular mechanism of Dsg3 depletion and subsequent acantholysis remains unknown, we have shown that the armadillo protein plakoglobin (PG), which associates with the cytoplasmic tail of Dsg3, is crucially involved in this process (Caldelari *et al*, 2001). Our previous work suggested that 'secondary to antibody binding, modulation of available PG at the plasma membrane supports rapid degradation of Dsg3, and subsequently affects the ongoing differentiation process' (Caldelari *et al*, 2001). A number of other studies also indicated that besides adhesion, PG is involved in cell cycle regulation and the control of *c-Myc*. Findings include premature exit from the cell cycle triggered by moderately overexpressed PG in mouse epidermis (Charpentier *et al*, 2000), the ability of PG to limit excessive proliferation (Teuliere *et al*, 2004), the positive correlation between malignancy of epithelial tumors and loss of PG expression (Zhurinsky *et al*, 2000a) as well as the direct regulation of *c-Myc* by PG in hematopoietic cells

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(Muller-Tidow *et al*, 2004). Thus overall, a growing body of evidence now indicates that PG has a much broader biological role than just in adhesion.

In this study, we further characterize the role of PG in epidermal homeostasis suggested by our earlier study. Specifically, we investigate the control PG exerts over c-Myc, which in turn influences the delicate balance of stem cell recruitment, proliferation and terminal differentiation in keratinocytes.

Results

Pemphigus vulgaris antibodies (PVIgG) retain cultured keratinocytes in a proliferative state

The fate of Dsg3 and PG was addressed in PVIgG-exposed mouse keratinocytes at the transition between proliferation and terminal differentiation (i.e. during cell cycle exit/growth arrest). This stage largely reflects the status of basal keratinocytes with established Dsg3-based desmosomes, but not of suprabasal cells, which already are growth arrested (Caldelari *et al*, 2001) (Figure 1A). At 4 and 48 h postincubation with PVIgG or normal human control IgG (nhIgG), total steady-state PG appeared unchanged (Figure 1B). Given that most PG is associated with desmosomes, changes in nondesmosomal PG were masked. In subcellular fractions, the turn over of antibody targeted Triton X-100-soluble (nonkeratin-anchored) Dsg3 and associated PG at the plasma membrane transiently increased within the first hour after exposure to PVIgG and resulted in a significant depletion of these molecules from the cytoplasmic–vesicular pool (Supplementary Figure 1). Concomitantly, soluble PG levels failed to rise in the cytosol/low-detergent soluble membrane fraction and was followed by decreased nuclear import of PG as compared to normal terminally differentiating cells (Figure 1C; Supplementary Figure 2 shows relative levels of membrane, cytoplasmic and nuclear proteins). The reduction in PG protein was further paralleled by a decrease in PG steady-state mRNA (Figure 1D) suggesting the activation of a signaling cascade in response to the initial transient depletion of Dsg3 and PG at the plasma membrane. In contrast to PG, nuclear β-catenin and plakophilin1, two other armadillo-(like) proteins as well as β-catenin mRNA were unchanged (Figure 1C).

The consequences of decreased nuclear PG on transcription of proliferation and terminal differentiation markers were addressed. Consistent with unchanged levels of nuclear β-catenin, the mRNA level of its target gene cyclin D1 (Tetsu and McCormick, 1999) was not reduced in PVIgG-treated cells (Figure 2A). In opposite, cyclin D1 mRNA was moderately but significantly increased. Accordingly, $p21^{WAF1}$ mRNA, which encodes a crucial determinant of the exit from the cell cycle in keratinocytes and suppressor of cyclin D1 (Missero *et al*, 1996), failed to increase. The steady-state level of the corresponding proteins followed a similar pattern with cyclin D1 and $p21^{WAF1}$ persisting at higher levels up to day 15 in PVIgG-exposed cells (Figure 2B). Apparent

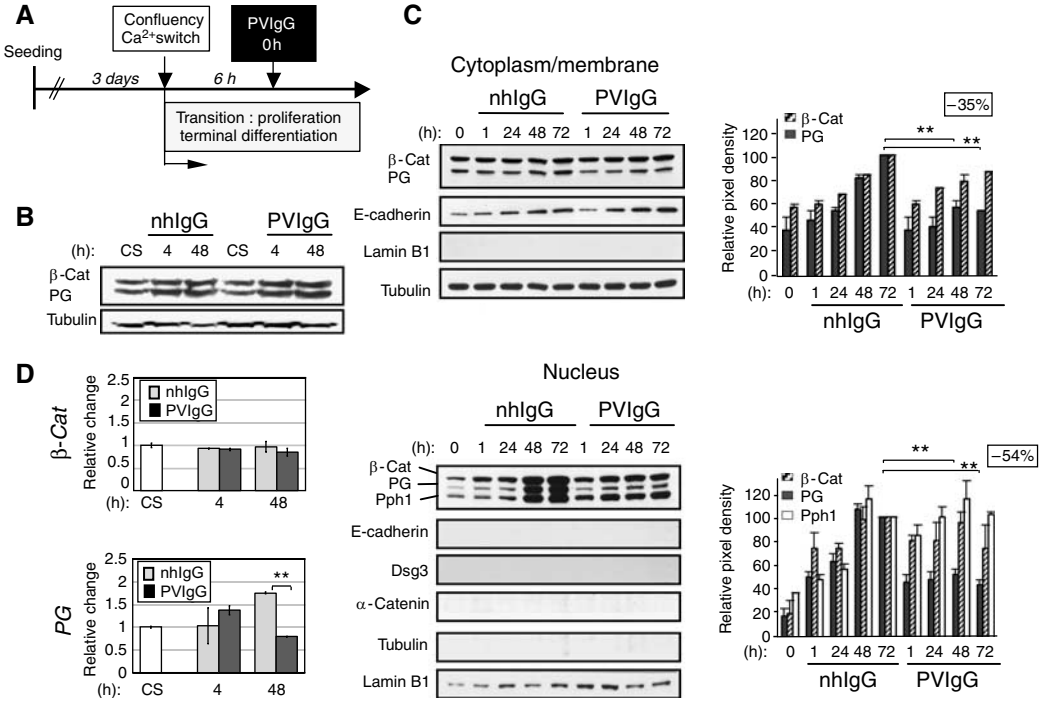

Figure 1 Nuclear PG is reduced in PVIgG-treated mouse keratinocytes. (**A**) Experimental setup used in this and our previous study (Caldelari *et al*, 2001). (**B**) Western blot analysis of total lysates from keratinocytes treated as indicated. CS designates the sample collected at calcium switch. Tyrosine tubulin was used as a loading control. (**C**) Western blot analyses of cytoplasmic/membrane and nuclear fractions. Proportionally 10 times more protein (in terms of cell equivalents) was loaded for the nuclear than the cytoplasmic/membrane fractions (see Supplementary Figure 2A for respective amounts of adhesion components in each fraction). Tubulin, E-cadherin, Dsg3 and α-catenin and the nuclear envelope protein lamin B1 served as a loading or purity control, respectively. Graphs depict the relative change of indicated proteins with respect to control cells at 72 h (set as 100). Insets indicate percentage PG reduction in PVIgG-treated cells at 72 h as compared to control keratinocytes. Note that nuclear β-catenin was similar in control and PVIgG-treated cells. Nuclear accumulation of armadillo proteins coincides with the phosphorylation of GSK3β on Ser9 (Calautti *et al*, 2005). Pph1 = plakophilin1. $^{**}P<0.009$, $n=5$. Error bars are ±s.d. (**D**) Graphs show steady-state mRNA levels determined by Q-PCR presented as relative change compared to CS. $^{**}P<0.01$, $n=6$. Error bars are ±s.d.

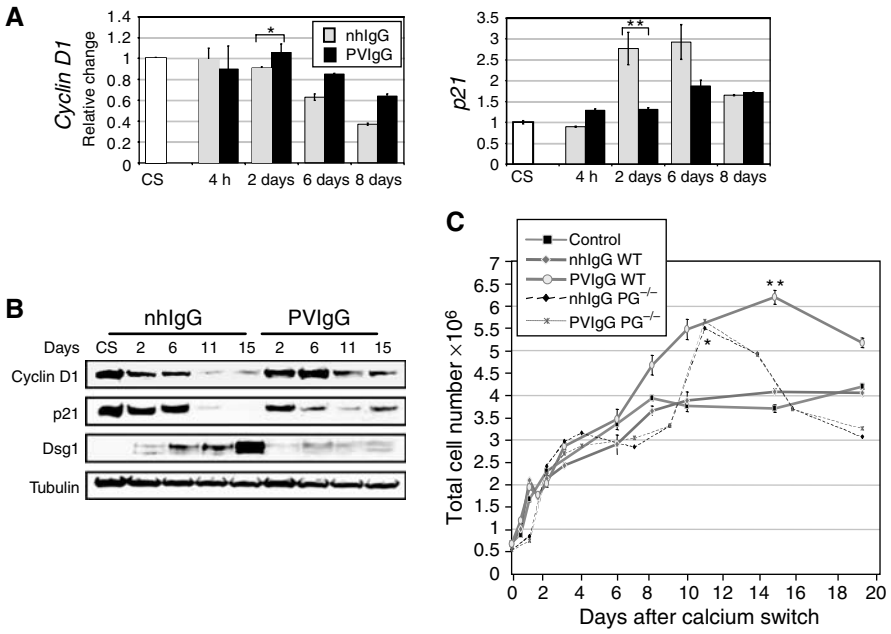

Figure 2 PVIgG retain keratinocytes in a proliferative state. (**A**) Relative change of indicated mRNA levels in wild-type keratinocytes assessed by Q-PCR. $*P<0.05$; $**P<0.01$; 4 h, 2 days, $n=8$; 6 days, 8 days, $n=2$. Error bars represent the $\pm$s.d. or range (6 days, 8 days). (**B**) Western blot analysis from parallel cultures to those in (A). Tubulin was assessed on the same blot than cyclin D1 and $p21^{WAF1}$. (**C**) Proliferation curves are shown for indicated cell types. Counts started at confluency when the calcium switch was introduced. Note that untreated wild-type keratinocytes undergo growth arrest between days 4 and 6 after calcium switch as seen by a plateau. * = detachment of intact cellular sheets; ** = detachment of single cells. Error bars are $\pm$s.d. A representative experiment of two carried out in triplicates is shown. WT = wild-type keratinocytes.

sustained proliferation in these cells was paralleled by hampered accumulation of Dsg3 mRNA and protein during the first 48 h (Supplementary Figure 3). Indicative for a role of PG in the process, $PG^{-/-}$ keratinocytes showed similar mRNA and protein levels as PVIgG-treated cells (Supplementary Figure 3), and noticeable were unresponsive to PVIgG as shown in our previous study (Caldelari *et al*, 2001).

Consistently, growth curves with PVIgG-treated keratinocytes showed sustained proliferation as compared to control cells (Figure 2C). A similar fate was also observed for $PG^{-/-}$ keratinocytes in 3D cultures (Supplementary Figure 4) as well as conventional submerged cultures (Figure 2C). Remarkably, the growth rate of $PG^{-/-}$ cells was strictly independent of PVIgG. In line with their unresponsiveness to PVIgG but despite weaker adhesion than wild-type cells (Caldelari *et al*, 2001), the $PG^{-/-}$ keratinocytes detached from the culture dish in large intact cell sheets around day 10, as opposed to single cells in PVIgG-exposed wild-type cultures. All cultures entered at least a transient plateau around growth arrest (days 4–6 after calcium switch). This could be owing to cell–cell contact-induced signals like Notch-1 (Kolly *et al*, 2005) that may precede the major changes (as defined in the following), leading to sustained proliferation in $PG^{-/-}$ as well as PVIgG-treated keratinocytes. Together, these results suggest that proliferation is sustained in cultured keratinocytes with reduced or lack of nuclear PG.

The status in PV patients reflects the findings in cultured keratinocytes

The proliferation status was addressed in PV patients using immunofluorescence analysis performed on perilesional and nonlesional biopsies from six patients with variable duration of disease. For comparison, biopsies from healthy donors, patients with other autoimmune bullous diseases such as pemphigus foliaceous (PF) and bullous pemphigoid (BP) as well as chronic eczematous dermatitis (as an example of an inflammatory skin disorder with epidermal hyperproliferation (Altekrueger and Ackerman, 1994)) were also investigated.

Direct immunofluorescence confirmed the typical clustered staining pattern of IgG deposits and PG in the epidermis and oral mucosa of PV patients (Carlotti *et al*, 1993; A de Bruin, unpublished) (Figure 3A and Supplementary Figure 5). Staining for PG appeared weaker while that of β-catenin was unchanged. Consistent with the results obtained in culture, the number of cycling keratinocytes (Ki67-positive cells) was diffusely upregulated (up to 15 times) in perilesional and nonlesional epidermis as well as in mucosa of all PV patients analyzed (Figure 3B). Moreover, the proliferating Ki67-positive cells localized to basal and suprabasal layers, a typical feature of hyperproliferative epidermis (Andreadis *et al*, 2001). Despite this, differentiation markers were still expressed, but their expression pattern was disrupted in particular in patients with longstanding disease (Supplementary Figure 5). None of these features were observed in PF, an autoimmune bullous disease so far considered closely related to PV. BP and eczematous dermatitis showed some increased Ki67-positive cells, probably depending on the relevant inflammatory exocytosis involved.

PVIgG induce a generalized c-Myc overexpression in keratinocytes

The PV phenotype with hyperproliferation despite continuing expression of differentiation markers in suprabasal cells was

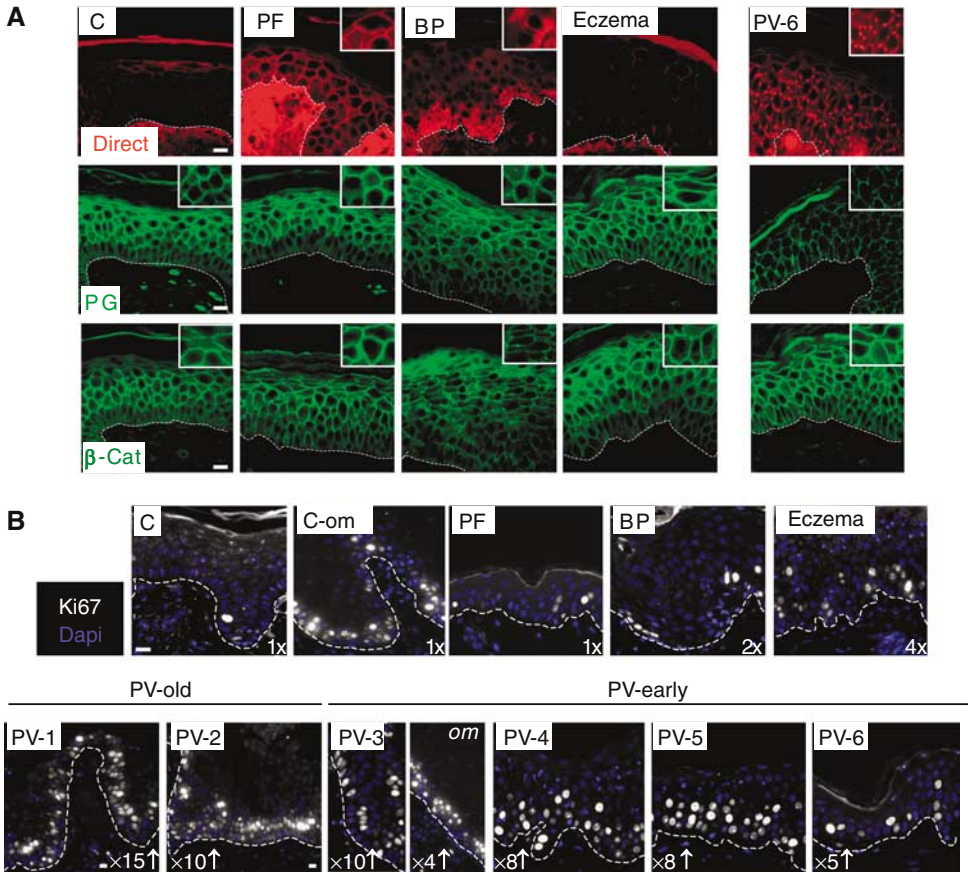

Figure 3 The status in PV patients reflects results obtained in cultured keratinocytes. (**A**) Paraffin-embedded tissue from nonlesional skin of a PV patient (PV6) and patients with PF, BP and chronic eczematous dermatitis (Eczema) were investigated by immunofluorescence microscopy and compared to control biopsies (epidermis (C)). Direct labeling demonstrates IgG deposits. Insets represents a three times higher magnification of representative areas to depict clustered desmosomal, but not adherens junction (β-catenin) proteins in PV patient six (nonlesional epidermis; PV patients 1–5, see Supplementary Figure 4). Note that biopsies were processed simultaneously and photographic procedures held constant to obtain semiquantitative results. Scale bars, 200 μm. (**B**) Consecutive cuts of the biopsies in (A) and in Supplementary Figure 4 were stained with Ki67 and counterstained with Hoechst. Numbers indicate the increase of Ki67-positive cells (in nonacantholytic areas) as compared to the normal situation (set as 1 for C and C-om (oral mucosa)). Scale bar, 200 μm (scale was reduced for PV-1 and PV-2 to give a broader overview).

reminiscent of that described for transgenic mice with activated c-Myc in basal keratinocytes (Arnold and Watt, 2001; Waikel *et al*, 2001). Consistent with this, the relative *c-Myc* mRNA levels were generally up to 1.5 times higher (maximally up to two-fold (data not shown)) in PVIgG-treated keratinocyte cultures than in control cells (Figure 4A). During the 8 days investigated, *c-Myc* levels in PVIgG-treated cells always exceeded those of confluent control cells at calcium switch and, importantly, reached to the level reported in proliferating keratinocytes (Kolly *et al*, 2005). On the protein level, the change in mRNA was paralleled by a substantial increase in the 64 and 46 kDa c-Myc isoforms (Figure 4B). The 46 kDa isoform of c-Myc likely represents the unphosphorylated c-Myc, which is characteristic for rapidly growing cells and is constitutively overexpressed in colon tumor cells (Tao *et al*, 2002). In human (Figure 4B) and mouse (Figure 4C, WT) keratinocytes, the increase in both c-Myc isoforms was already substantial after 1 day of PVIgG exposure, and was observed in both the cytoplasm and the nucleus (Figure 4C, WT). C-Myc accumulation correlated with progressive weakening of intercellular adhesive strength, as examined by an adhesion assay that applies mechanical stress to monolayer cultures (Figure 4D).

$PG^{-/-}$ keratinocytes had 1.5 times higher *c-Myc* mRNA levels than normal differentiating wild-type cells (data not shown). This correlated with a high protein level predominantly of the cytoplasmic 46 kDa c-Myc isoform (Figure 4C, $PG^{-/-}$). Furthermore, cytoplasmic c-Myc was not regulated after calcium switch or in response to PVIgG. Consistent with a 2-day delay of enhanced growth as compared to PVIgG-treated cells (Figure 2C), nuclear accumulation of the 64 kDa isoform was only increased in the $PG^{-/-}$ keratinocytes at day 6 after calcium switch and in both nhIgG- and PVIgG-treated cells. This suggests that the PVIgG-induced enhanced turn over of PG in wild-type cells (Supplementary Figure 1), which does not occur in $PG^{-/-}$ cells, amplifies c-Myc activity by increasing its nuclear accumulation. In contrast to $PG^{-/-}$ cells, c-Myc levels in β-$catenin^{-/-}$ keratinocytes corresponded to those of wild-type cells and were upregulated in response to PVIgG (Figure 4C, β-$cat^{-/-}$). This is consistent with the finding that proliferation and onset of terminal differentiation proceed normally in these cells (Posthaus *et al*, 2002), that epidermal deletion of β-catenin did not upregulate c-Myc (Teuliere *et al*, 2004) and 3D cultures of β-$catenin^{-/-}$ keratinocytes grew to the thickness of wild-type cultures in contrast to $PG^{-/-}$ keratinocytes, which form

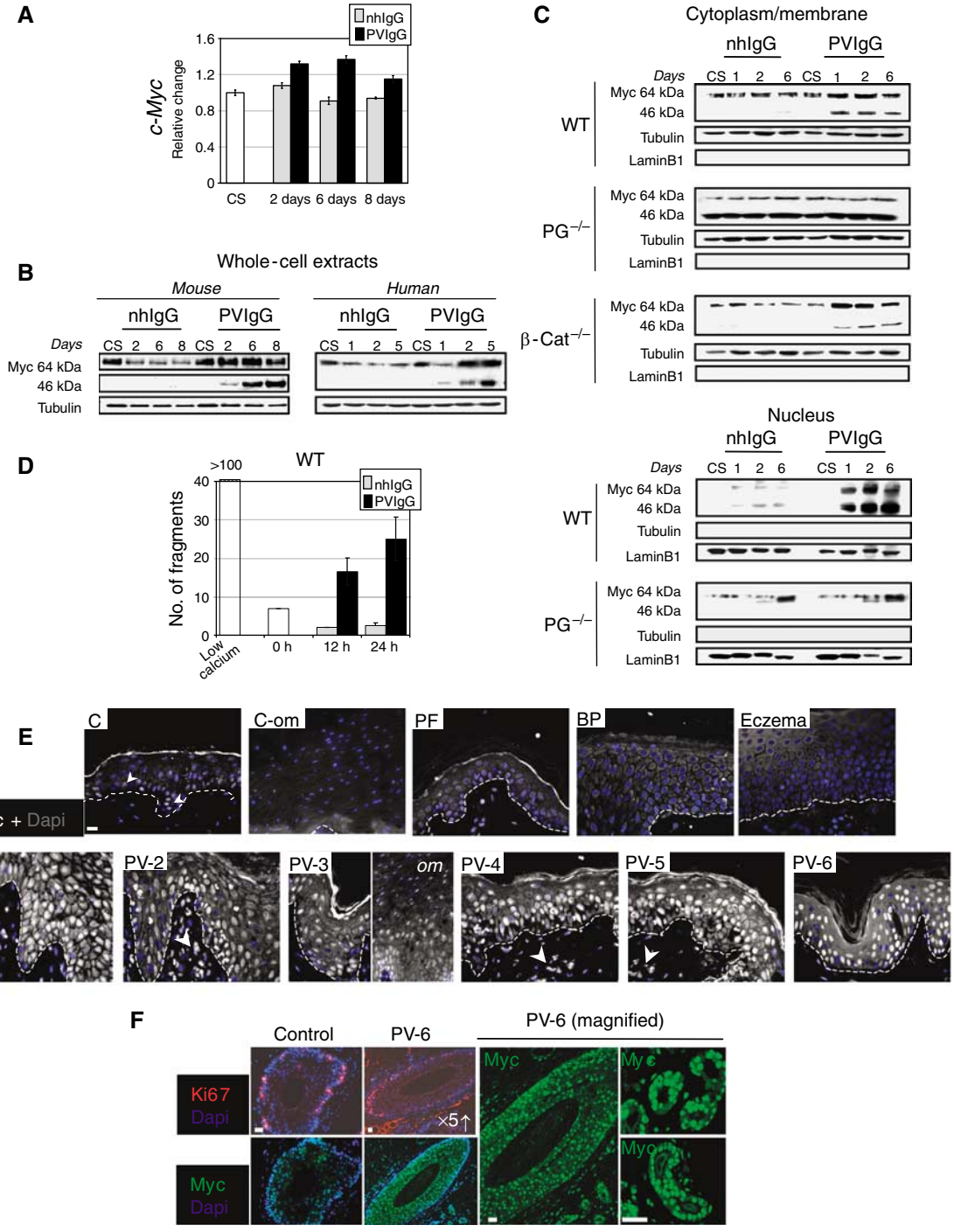

Figure 4 PVIgG upregulate c-Myc. (**A**) Graph indicates the relative change in *c-Myc* mRNA levels as compared to CS. One representative result carried out in duplicates of three independent experiments is shown. Error bars represent the range. (**B**) Western blot analyses for c-Myc was performed on total cell lysates obtained from parallel cultures to those in (A) (mouse) or from human keratinocytes. (**C**) Western blot analyses of cytoplasmic/membrane and nuclear fractions. (**D**) Graph indicates number of fragments generated after the application of mechanical stress to wild-type mouse monolayer cultures. Zero hours indicate beginning of PVIgG or nhIgG treatment (6 h after calcium switch). One experiment of two carried out in duplicates is shown. Scale bars represent the range. (**E**) Consecutive sections of paraffin-embedded PV and control biopsies, as in Figure 3 (B), were stained for c-Myc, counterstained with Hoechst. c-Myc-positive cells in the dermis (arrow-heads) likely are leukocytes as judged from H&E stains (data not shown), which is consistent with their absence from nonlesional skin (PV-6). Arrows point to faint c-Myc staining in control skin. (**F**) Hair follicles stained with Ki67 or c-Myc and Hoechst (left panel). The right panel (PV-6 magnified) is a two-fold magnification of the hair follicle in the left panel and a six-fold magnification of sebaceous glands. All biopsies were processed simultaneously and photographic procedures held constant to obtain semiquantitative results. Scale bars, 200 μm.

hyperplastic sheets (Supplementary Figure 4). Accordingly, β-catenin$^{-/-}$ cells responded to PVIgG with loss of intercellular adhesion like wild-type cells (data not shown) while PG$^{-/-}$ cells did not (Caldelari *et al*, 2001).

Consistent with results obtained in cell culture, the six PV patients revealed a generalized (diffuse) strong c-Myc staining in all layers of the epidermis and oral mucous membrane, with highest intensity in the nucleus as demonstrated by

semiquantitative immunofluorescence analysis (Figure 4E). Importantly, c-Myc was as significantly upregulated in non-lesional PV skin, hair follicles and sebaceous glands (patient 6; Figure 4E and F). In each case, the c-Myc levels exceeded those in basal keratinocytes of healthy donors, and in particular in biopsies of patients with other autoimmune bullous diseases or eczematous dermatitis.

Like in the two transgenic mouse models with c-Myc activation in basal keratinocytes (Arnold and Watt, 2001; Waikel *et al*, 2001), staining for β1-integrin was substantially lower and keratins 6 and 14 were increased in the epidermis of all six PV patients (Supplementary Figure 5). Impaired wound healing and in particular enhanced exit of epidermal stem cells from their niche were attributed to these changes (Arnold and Watt, 2001; Waikel *et al*, 2001; Frye *et al*, 2003). Together, these results revealed for the first time that PVIgG trigger c-Myc accumulation and hyperproliferation, which is accompanied by reciprocal regulation of β1-integrin, and keratins 6 and 14.

PG is a suppressor of c-Myc in epidermal keratinocytes

In mouse embryonic stem cells and keratinocytes, a dual mode of c-Myc regulation has been proposed that consists of transcriptional regulation in conjunction with post-translational stabilization (Cartwright *et al*, 2005; Kolly *et al*, 2005). Transcription of *c-Myc* can be regulated by Tcf/Lef transcription factors together with PG (Kolligs *et al*, 2000; Muller-Tidow *et al*, 2004). These factors are expressed in mouse keratinocytes (Posthaus *et al*, 2002), of which Lef-1 was related to hyperproliferation in mouse epidermis if functionally inactivated (Niemann *et al*, 2002).

We initially addressed whether PG can inhibit Lef-1 or Tcf4-mediated transcription from the human *c-Myc* promoter described previously (Kolligs *et al*, 2000). In a reporter gene assay, PG inhibited transcription together with Lef-1 in a dose-dependent manner, while it activated transcription in the presence of Tcf4 or, as reported, on its own (suggesting the use of endogenous Tcf/Lef family members) (Kolligs *et al*, 2000) (Figure 5A). PG-mediated suppression was Lef-1 dependent (as demonstrated by truncated Lef-1, which fails to bind PG (Huber *et al*, 1996)) and was exerted by the C-terminus of PG known to harbor the transactivation site (Hecht *et al*, 1999) (as shown by deletion mutants of PG (Zhurinsky *et al*, 2000b)). PG/Lef-1-mediated suppression was comparable in transfected wild-type and β-catenin$^{-/-}$ keratinocytes, further demonstrating that this effect was directly mediated by PG and not indirectly via endogenous β-catenin displaced by PG from its cellular binding partners (Figure 5A shows selected transfections). In addition, suppression by Lef-1/PG further was characteristic for the *c-Myc* promoter as it was not seen when using an artificial Tcf-responsive promoter (Molenaar *et al*, 1996) (Figure 5B). In line with a role of Lef-1 in basal keratinocytes, Lef-1 protein was mainly confined to the nuclei in the deep mouse and human epidermis (Figure 5C).

Using chromatin immunoprecipitation (ChIP), we further examined whether PG/Lef-1 is recruited to the *c-Myc* promoter at cell cycle exit, and if β-catenin is involved in this process. Before growth arrest (1 day after calcium switch (Figure 2C and Kolly *et al* (2005)) neither PG nor β-catenin was detectably bound to the TCF/LEF binding site in the mouse *c-Myc* promoter, as seen by lack of DNA amplification above back ground with a primer set immediately adjacent to the TCF/LEF binding site (chosen according to quantitative real-time PCR (Q-PCR) requirements) (Figure 5D, left panel; compare primer set a to b). Lef-1 antibodies precipitated a small amount of DNA from the TCF/LEF domain. Results were identical for untreated cells as well as for cells 1 day before calcium switch (data not shown). In contrast, in cells undergoing growth arrest (4 days after calcium switch (Figure 2C and Kolly *et al* (2005)), TCF/LEF-specific amplification increased over 100-fold in Lef-1 and PG antibody precipitates, while significantly less TCF/LEF-specific fragments were precipitated from PVIgG-treated keratinocytes. Compatible with our findings so far, TCF/LEF-specific β-catenin precipitates were in any case below the background defined with the upstream primers in the same sample. No specific amplification of *c-Myc* promoter fragments was obtained from PG$^{-/-}$ cells (Figure 5D, right panel).

This set of experiments shows that at exit from the cell cycle and onset of terminal epidermal differentiation, PG but apparently not β-catenin represses the *c-Myc* promoter in an Lef-1-dependent manner and that PVIgG compromise this repression by reducing PG/Lef-1 binding.

PVIgG-triggered c-Myc overexpression weakens intercellular adhesion

To establish the link between PV antibody binding to Dsg3, c-Myc activity and loss of intercellular adhesion, lesion formation was examined in a passive transfer study (Anhalt *et al*, 1982) in the presence or absence of c-Myc inhibitors that prevent the Myc–Max interaction required for c-Myc activity (Yin *et al*, 2003). The importance of Dsg3 as trigger of the signaling cascade leading to c-Myc activation was addressed by the mouse monoclonal Dsg3-specific antibody AK23 (Tsunoda *et al*, 2003). AK23 is a pathogenic PV antibody, which targets the adhesive interface between juxtaposed Dsg3 molecules. The mouse monoclonal antibody NAK3 was used as a negative control (M Amagai *et al*, unpublished). NAK3 also targets Dsg3, is unable to induce blisters on its own, but has weak pathogenic activity in combination with other blister-inducing antibodies.

NhIgG and PVIgG, AK23 and NAK3 were subcutaneously injected in the lumbar area of neonatal mice that had been preinjected at the same site with c-Myc inhibitors or vehicle. After 24 h, blisters were observed in PVIgG/vehicle- and AK23/vehicle-injected mice but not in their siblings pretreated with c-Myc inhibitors (Figure 6A). Despite binding of NAK3 to the surface of keratinocytes, no blisters developed under any condition, as seen in nhIgG-injected mice (Figure 6A shows NAK3). However, consistent with its weak pathogenic activity (M Amagai *et al*, unpublished), c-Myc levels seen by semiquantitative immunofluorescence microscopy were higher in NAK3-injected than in control mice, but significantly lower than in PVIgG- and AK23-injected mice with or without c-Myc inhibitors (Figure 6B). At that early time point, c-Myc was mainly localized in the cytoplasm and only to some extent in the nucleus.

To further confirm that abrogation of PG's nuclear import is key for c-Myc accumulation and consequently PV pathogenesis, the passive transfer study was repeated in the presence or absence of the GSK3 inhibitors SB216763 (Coghlan *et al*, 2000) and LiCl (Beaulieu *et al*, 2004). GSK3β is a serine/threonine kinase that targets PG to the

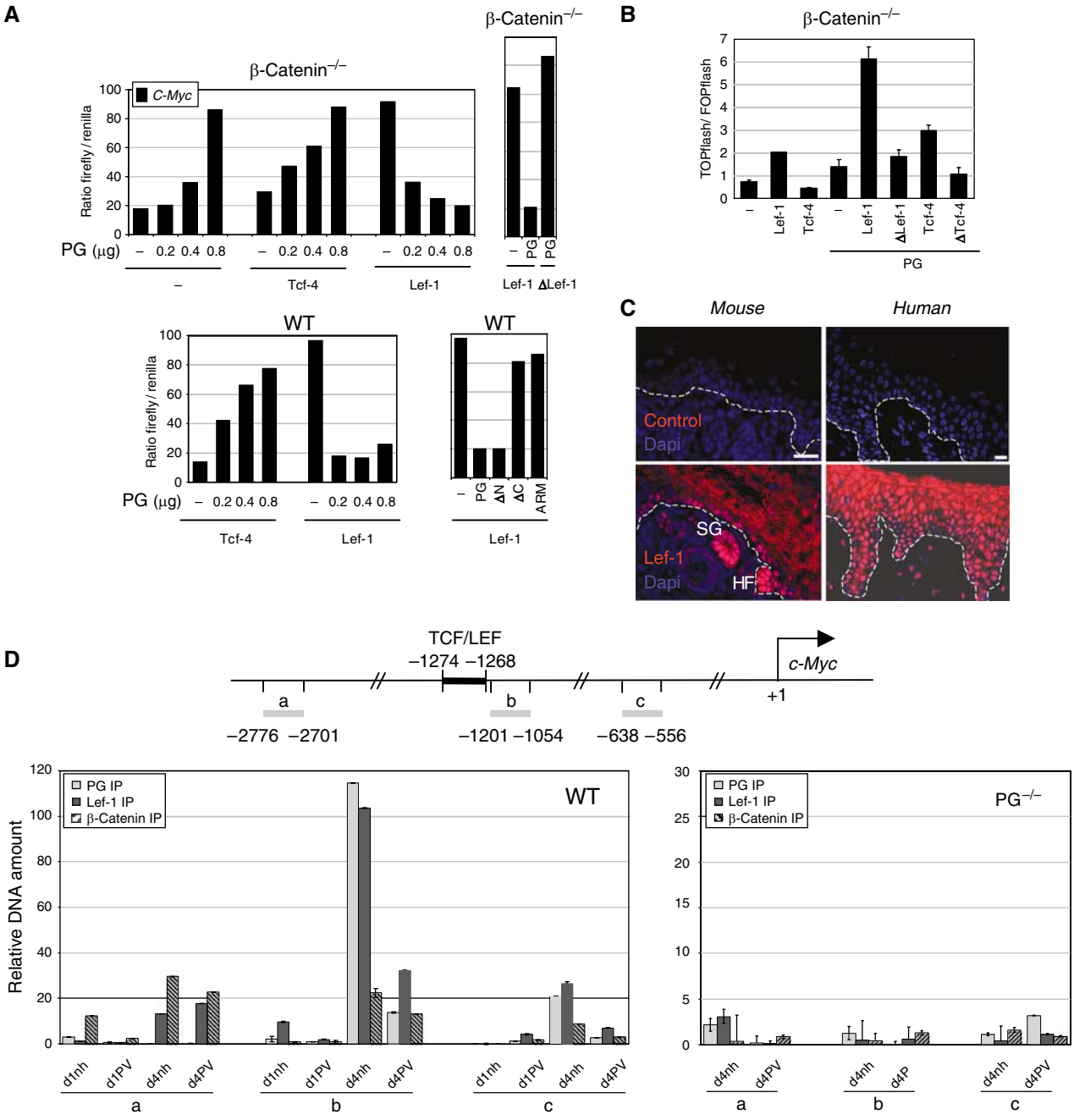

Figure 5 PG is a suppressor of c-Myc. (**A**) Graphs of reporter gene assays using the human *c-Myc* promoter on indicated cell types. The ratio of firefly over renilla luciferase activity is indicated. One representative experiment of at least four independent experiments carried out in single measures per cell type is shown. ΔLef-1 lacks the armadillo-binding domain; PG ΔC, ΔN and ARM lack the C-terminal, N-terminal domain or both, respectively. Individual mutation of three LEF/TCF binding sites in the *c-Myc* promoter (two reported, one unreported) reduces Lef-1-mediated activation by over 50% (C Kolly *et al*, unpublished). (**B**) TOPflash/FOPflash promoter activity in the presence of indicated exogenous factors. One representative experiment of three is shown. Error bars represent the range. (**C**) Paraffin-embedded tissue from normal newborn mouse epidermis and human skin stained with Lef-1 antibodies. Controls were incubated with secondary antibody and processed for enhancement. Lef-1 staining in mouse epidermis is highest in remnants of hair follicles (HF) and sebaceous glands (SG), and fainter staining is confined to the nuclei of mainly basal keratinocytes. Human epidermis shows strong Lef-1 nuclear staining in basal and suprabasal keratinocytes. Scale bars, 200 μm. (**D**) Top panel: Scheme of the mouse *c-Myc* promoter and TCF/LEF binding site. Gray bars indicate positions of primer sets relative to the transcription initiation site which were used to amplify precipitated DNA by Q-PCR. Bottom panel: ChIP. Graphs show relative amounts of amplification products obtained by Q-PCR with indicated primer sets. One of three (WT) and two ($PG^{-/-}$) independent experiments measured in duplicates is shown. Error bars indicate the range.

proteasome degradation machinery (Aberle *et al*, 1997). Its inhibition in keratinocytes results in PG stabilization and nuclear accumulation (Kodama *et al*, 1999). Furthermore, GSK3β was found to be inactivated at cell cycle exit in cultured mouse keratinocytes (Calautti *et al*, 2005), suggesting that this event might be responsible for the nuclear trafficking of PG at growth arrest (Figure 1C). In presence of GSK3β inhibitors, AK23-induced blister formation was abrogated and c-Myc levels were significantly decreased (Figure 6C and D).

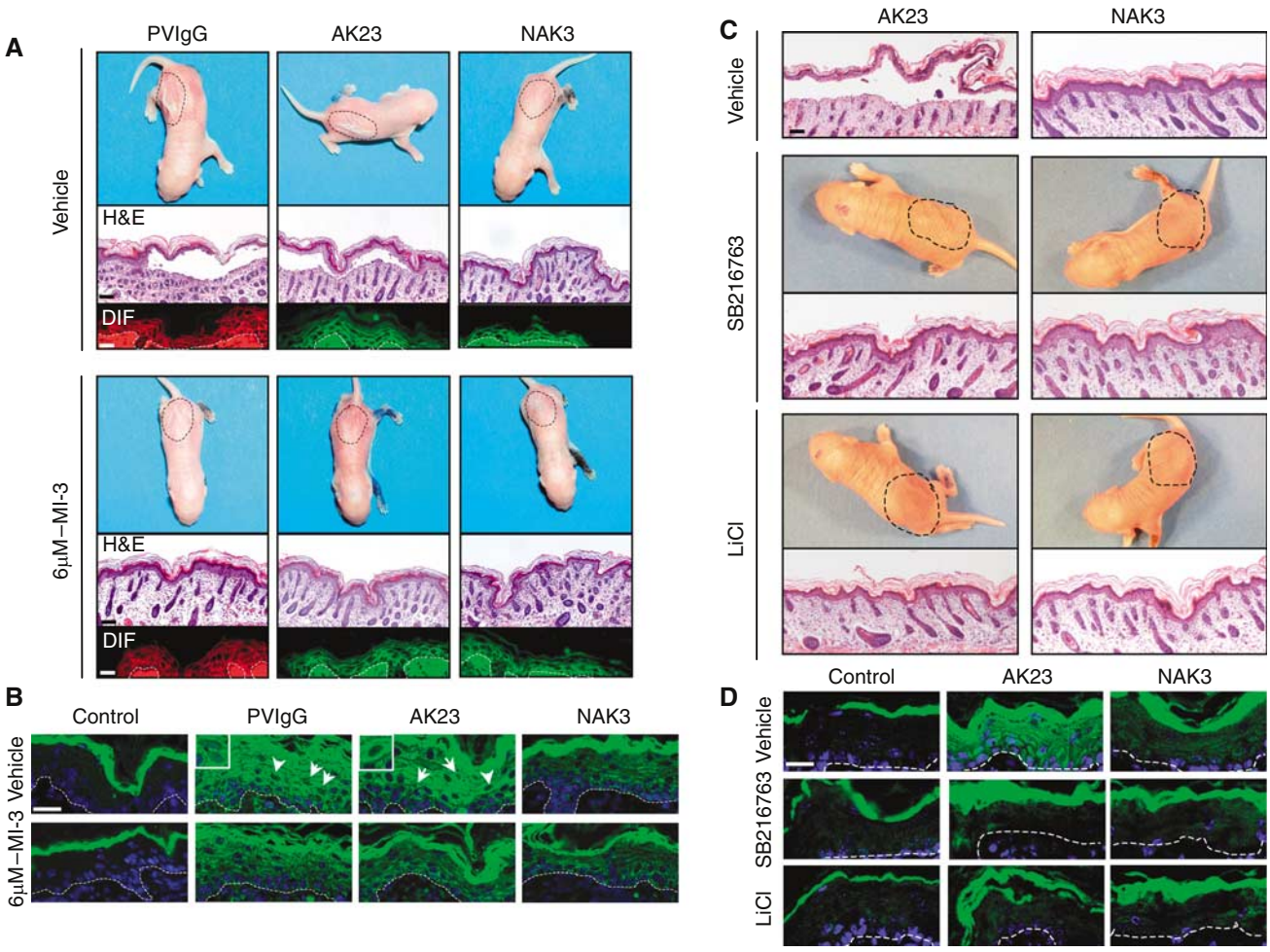

Figure 6 c-Myc accumulation accounts for the loss of intercellular adhesion. (**A**) Top pictures show neonatal mice subcutaneously injected with vehicle (top panel) or 6 μM c-Myc inhibitor 3 (bottom panel) followed by PVIgG, AK23 or NAK3 together with half the minimal dose of a human PF serum. Discontinued lines indicate the area of injection. Micrographs underneath show lesions (H&E) and IgG binding in the perilesional area assessed by direct immunofluorescence (DIF) (red = anti-human antibodies; green = anti-mouse antibodies). (**B**) Consecutive sections of the perilesional areas shown in (A) were stained for c-Myc. c-Myc was mainly present in the cytoplasm of PVIgG- and AK23-injected mice, and to some extent also of NAK3-injected mice. Arrows point to the nuclei (insets) with slightly elevated c-Myc. (**C**) As in (A), except that mice were preinjected with inhibitors to GSK3. (**D**) Immunofluorescence analysis of c-Myc in consecutive sections of perilesional areas shown in (C). Scale bars, 1.6 mm for H&E, otherwise 200 μm.

Taken together, these results demonstrate that constitutive c-Myc activation initiated by PVIgG-bound Dsg3 directly affects intercellular adhesive strength in mouse epidermis and underscore that this event is critically dependent on de-regulated nuclear trafficking of PG.

Discussion

Here we show that PG is a crucial suppressor of c-Myc in keratinocytes and that PV antibodies disrupt this activity by selective depletion of PG from soluble Dsg3 pools and reduced nuclear PG resulting in abolished PG/Lef-1-mediated *c-Myc* suppression. Given that the c-Myc upregulation in PV at the protein level was larger than would be expected from the observed increase in mRNA, it appears that post-transcriptional control mechanisms also contribute to c-Myc regulation in these cells. Such mechanisms were already discussed for normal keratinocyte proliferation and differentiation (Kolly *et al*, 2005) and fit well with both our current results and emerging reports of regulatory pathways in keratinocytes that link intercellular adhesion to c-Myc regulation via PG (as discussed below).

Pathogenic PV antibodies (like the monoclonal antibody AK23) are thought to disrupt trans-adhesion between Dsg3 molecules (Tsunoda *et al*, 2003), which results, as shown here, in sustained proliferation. Under normal culture conditions, proliferation is stopped in keratinocytes by confluency, that is, cell–cell contact that results in the stabilization of cadherins through trans-adhesion (Kolly *et al*, 2005). Hence, the current study suggests that the disruption of trans-adhesion between surface-exposed soluble Dsg3 and associated PG is sufficient to generate a signal of 'no cell contact' in confluent monolayers.

Under normal conditions, the stabilization of cadherins through trans-adhesion is accompanied by recruitment of activated phosphoinosite 3-kinase (PI3K) to the cadherin–catenin complexes, which is followed by GSK3β inhibition (Calautti *et al*, 2005). PG thereby serves as the main docking partner for PI3K in the cadherin–catenin complex, while GSK3β inhibition stabilizes cytoplasmic PG and β-catenin

(Kodama *et al*, 1999), prompting their nuclear translocation and the PG-mediated *c-Myc* suppression shown here. Recruitment of PI3K to trans-adhering cadherins also activates, among others, the small GTPase Rac-1 (Fukuyama *et al*, 2006). Deletion of Rac-1, as we show for PG, results in c-Myc overexpression (Benitah *et al*, 2005), suggesting that PG and Rac-1 are in the same pathway. Accordingly, as demonstrated in $Rac^{-/-}$ mice (Benitah *et al*, 2005), abolished negative regulation by p21-activated kinase 2 might contribute to c-Myc post-translational accumulation in PV. Collectively, our findings along with these other reports support the notion that cadherin-bound PG links the status of intercellular adhesion to c-Myc transcriptional and possibly post-translational regulation via a singling cascade involving PI3K and GSK3β, which in turn regulates nuclear trafficking of PG. Although this cascade of events must be investigated more deeply, our findings in PV support this possibility. Downstream of cadherin-bound PG, PVIgG negatively interfere with a signaling pathway that relates to terminal differentiation and involves GSK3β: the transiently enhanced turn over of Dsg3 and PG in response to PVIgG was followed by delayed terminal differentiation and consequently sustained proliferation; GSK3β inhibition was sufficient to abrogate blister formation in PVIgG-injected neonatal mice, supporting the possibility that the PVIgG-targeted signaling pathway involves the PI3K/GSK3β axis that in turn is in control of PG nuclear trafficking and consequently *c-Myc*.

The cascade of events from cadherin-bound PG at the plasma membrane through to PG-mediated transcriptional control mechanisms underscores the notion that PG acts as an integrator of environmental cues (Calautti *et al*, 2005; Yin *et al*, 2005a, b). Our results also demonstrate that PG can integrate these cues when bound to trans-adhering soluble Dsg3, but conversely is restricted from doing so in the absence of the Dsg3 stabilization seen in confluent monolayers in the presence of PV antibodies. Further support of PG-mediated crosstalk between desmosomal adhesion and c-Myc is evident in that observation that $PG^{-/-}$ keratinocytes fail to respond to environmental cues in the same manner as wild-type cells, that is, they are unresponsive to PVIgG for all parameters analyzed, fail to integrate growth factor-induced signals (Yin *et al*, 2005a), have elevated Src activity (Yin *et al*, 2005b), a tyrosine kinase that is activated upstream of PI3K (Calautti *et al*, 2005), and consequently constitutively express c-Myc and hyperproliferate.

The fact that PV antibodies trigger c-Myc overexpression in basal and suprabasal epidermis suggests that PG acts as a suppressor of c-Myc both at cell cycle exit and in terminally differentiated cells. Moreover, the basal compartment also harbors stem cells, which crucially depend on *c-Myc* suppression (Owens and Watt, 2003). Stem cells express Dsg3 and therefore are likely targets of PV antibodies (as also judged from the continuous pattern of IgG deposits). Hence, *c-Myc* suppression by PG in the stem cell compartment cannot currently be ruled out. Further indications that PG regulates c-Myc also in the stem cell compartment are evident when considering that tumor development in epithelial cells likely is initiated by stem cells (Owens and Watt, 2003). Lack of PG was found to be involved in a range of tumors, including loss of heterozygosity in breast and ovarian carcinomas (Aberle *et al*, 1995), and reduced PG levels predict an adverse outcome in patients with epithelial tumors (Zhurinsky *et al*, 2000b). The opposite is generally reported for β-catenin, which is considered a tumor promoter in many tissues (Conacci-Sorrell *et al*, 2002), and this is in line with the lack of tumor formation in β-catenin-deleted skin (Watt, 2001). Thus, the idea that PG expression is crucial both to preserve epithelial homeostasis and prevent carcinogenesis is not new. However, our finding that PG is a key suppressor of the proto-oncogene *c-Myc*, both at cell cycle exit in keratinocytes and probably also in the stem cell compartment, provides a molecular basis for these observations for the first time. Thus, the molecular mechanism of PV pathogenesis described here provides insights into an unexpected role of PG in epidermal homeostasis that will have consequences for epidermal stem cell and tumor biology.

Materials and methods

Keratinocyte cultures

Isolation and characterization of mouse wild-type, $PG^{-/-}$ and $\beta\text{-catenin}^{-/-}$ keratinocytes (all C57BL/6 genetic background) were described previously (Caldelari *et al*, 2001; Posthaus *et al*, 2002). Human keratinocytes from foreskin were a kind gift form CELLnTEC Advanced Cell Systems AG, Switzerland. Conventional submerged cultures and stimulation conditions were according to our previously established protocol for studies on PV (Figure 1) (Caldelari *et al*, 2001), except that the medium was changed to CnT-02 (CELLnTEC, Switzerland) or Cnt-02 supplemented with 1.2 mM calcium (referred to as 'high calcium medium'). Human cells were cultivated in CnT-07 (CELLnTEC, Switzerland). For 3D cultures, cells were grown at the air–liquid interface for 14 days as described earlier (Suter *et al*, 1991).

Passive transfer studies using neonatal mice

Around 16 h after birth, alebino DDY mice were subcutaneously injected in the lumbar area with 6 μM c-Myc inhibitors (5521700 (MI-1) or 5404711 (MI-3); Chembridge) or vehicle (DMSO/PBS). After 2 h, 15 mg PVIgG or nhIgG, or alternatively 75 μg mouse monoclonal AK23 (Tsunoda *et al*, 2003) or NAK3 antibody (M Amagai, unpublished) were injected in the same area in combination with half the minimal dose of a human PF serum inducing blister formation (Mahoney *et al*, 1999). Vehicle or c-Myc inhibitors alone were used as negative controls. After 24 h, the animals were evaluated for macroscopic blistering. Biopsies were taken from lesional areas, fixed in 4% buffered formaldehyde overnight at 4°C and paraffin embedded. Biopsies were deparaffinized and stained with H&E or processed for direct immunofluorescence as described under immunofluorescence analyses. Four animals per treatment (PVIgG, AK23, NAK3, nhIgG, no treatment), each with vehicle or c-Myc inhibitors, were analyzed in two independent experiments, which showed consistent results. The time-course and injection protocols using chemical GSK3 inhibitors, SB216763 (100 μM; 2 μg/g body weight; Calbiochem) and LiCl (200 μg/g body weight) (Beaulieu *et al*, 2004), were identical to the c-Myc inhibitor experiment. In total, 18 animals were preinjected with the inhibitors and subsequently with AK23 or NAK3 and two with PBS. Six animals were preinjected with vehicle followed by AK23, NAK3 or vehicle alone. Results obtained were identical per treatment group.

Human biopsies

Lesional and perilesional biopsies of skin, scalp and oral mucosa performed for clinical purposes were used according to the rules of the Medical Faculty. The diagnosis of PV (six patients), PF (two patients) and BP (five patients) relied on clinical, light microscopical, immunohistological (DIF) and serological (IIF) criteria. At the time of biopsy, all patients with autoimmune bullous disease showed positive antibody titers in IIF. Chronic eczematous dermatitis (two patients) was confirmed by light microscopy. Control biopsies were obtained from two healthy donors. All biopsies were fixed with 4% formaldehyde, paraffin embedded and consecutive sections of the same biopsy were used for

immunofluorescence analyses. The PV biopsies were classified according to light microscopy in 'old' lesions (patients 1 and 2), presenting with extensive suprabasal acantholysis and mixed inflammatory infiltrates, and 'early' lesions (patients 3–6), exhibiting only focal acantholysis and/or eosinophilic spongiosis. For patient 6, a biopsy far from any blister was analyzed (nonlesional).

Sera and antibodies

Protein A-Sepharose-purified PVIgG contained antibodies against Dsg3 but not Dsg1 (Caldelari *et al*, 2001) or any other major protein (Supplementary Figure 4). nhIgG were derived from >1000 healthy donors (Sandoglobulin) and purified by Protein A-Sepharose. The affinity-purified mouse monoclonal anti-Dsg3 antibodies AK23 (Tsunoda *et al*, 2003) and NAK3 (M Amagai, unpublished) were kind gifts of M Amagai (Keio University School of Medicine, Tokyo, Japan). Primary antibodies were against the extracellular domain EC5 of Dsg3 (J Stanley, University of Philadelphia, Philadelphia, PA; Koch *et al*, 1998), Dsg1/2 (DG3.10 Progen), E-cadherin (gp-48, R Kemler, Max-Planck Institute, Freiburg, Germany), β-catenin and PG (Transduction Laboratories), Ki67 and lamin B1 (Zymed), $p21^{WAF1}$ and cyclin D1 (BD PharMingen), tubulin-tyrosine (Sigma), c-Myc (Upstate Biotechnology, sc-764 Santa Cruz and BD PharMingen; the first was used for immunofluorescence analysis of human biopsies, the second for mouse biopsies and the first and third in combination for immunoblots) and Lef-1 (R Grosschedl, Max-Planck Institute, Freiburg, Germany; Travis *et al*, 1991).

Immunofluorescence analyses

Biopsies were deparaffinized and antigens retrieved for DIF by trypsinization (1 × trypsin (S2012 DAKO) in 0.1% CaCl (pH 7.8), 1.5 h at 37°C) and otherwise by microwaving three times 5 min at 720 W in 0.01 M sodium citrate buffer (pH 6.0). Nuclei were counterstained with Hoechst 33258 (H-1398, Molecular Probes). Lef-1 was revealed with the TSA-Plus Cyanine 3 enhancer kit (Perkin-Elmer Life Sciences) according to the manufacturer's protocol. Experimental procedures and microscopical processing as described (Caldelari *et al*, 2001) were held constant for the same antibody to obtain semiquantitative results.

Extraction of cellular proteins

Total cell lysates were obtained by scraping keratinocytes into SDS loading buffer. Cytoplasmic/low-detergent soluble membrane and nuclear extracts were prepared as described for keratinocytes by Corsini *et al* (1996) with some modifications. Briefly, cells were lysed with hypotonic lysis buffer containing 0.58% NP-40, scraped and centrifuged. The supernatant (referred to as cytoplasm/membrane fraction) was removed. Pelleted nuclei were resuspended with the aid of a 23 G needle and additionally centrifuged through a 0.85 M sucrose cushion for 15 min at 11 700 *g* to remove nucleus-associated endoplasmic reticulum and cellular debris. Western blot analysis was performed and the purity of nuclear fractions was confirmed by the absence of E-cadherin, Dsg3, α-catenin, keratin 14 and tyrosine tubulin from the extracts; lamin B1 was used as loading control.

Q-PCR

Total RNA was extracted and analyzed using TaqMan™ Q-PCR technology and primers designed (Supplementary Table 1) and validated as described previously (Kolly *et al*, 2005). All samples were normalized against cyclophilin S1 mRNA and results are reported as *n*-fold change relative to the sample at calcium switch. Analyses were carried out in duplicates of at least three independent experiments.

Proliferation assay

A total of 3×10^4 cells/cm^2 were seeded into 8.8 cm^2 culture dishes and incubated with PVIgG, nhIgG or high calcium medium alone as described (Caldelari *et al*, 2001). Attached cells were trypsinized and counted in a hemacytometer. Two independent experiments were carried out in triplicates.

Reporter gene assay

The assay was performed using the DualLuciferase™ Reporter Assay System (Promega, Wallisellen, Switzerland). One day after seeding, wild-type or β-catenin$^{-/-}$ cells were transfected with polyethylenimine (PEI, linear, MW-25 000; Polysciences Inc., Warrington, PA). Briefly, 50 µl medium was mixed with 2 µg of DNA per 15 µl of PEI (1 mg/ml), incubated for 8 min, mixed with 450 µl medium/10% FCS and added onto cells in six-well plates 24 h postseeding. After 2 h, cells were washed with PBS, and incubated with fresh medium for 24–28 h prior to lysis. In each case, 1.25 µg for reporter genes (kind gifts of E Fearon, Ann Arbor, MI (Kolligs *et al*, 2000) and H Clevers, Netherlands Institute for Developmental Biology, Utrecht, Netherlands (Molenaar *et al*, 1996)) were transfected and, unless stated otherwise, 0.4 µg of plasmids encoding Lef-1, Tcf-4, ΔLef-1 and PG (all kind gifts of R Kemler, Freiburg, Germany (Huber *et al*, 1996)), respectively, and deletion mutants of PG (kind gift A Ben-Ze'ev, Rehovot, Israel (Zhurinsky *et al*, 2000a)). In each case, vector was added to obtain a total of 0.8 µg plasmid encoding exogenous factors. Seven nanograms of renilla luciferase-encoding plasmid were co-transfected as normalizer for transfection efficiency.

ChIP

The ChIP assay was carried out according to Frank *et al* (2001). Briefly, cells were fixed with formaldehyde to crosslink DNA and associated proteins. Samples were sonicated to obtain DNA fragments between 500 and 1000 bp in length as confirmed by gel electrophoresis (data not shown). Prior to immunoprecipitation, samples were adjusted for DNA input as measured by photospectrometry and confirmed by Q-PCR. Each sample was split and immunoprecipitated with Lef-1, PG or β-catenin antibodies, or rabbit IgG as negative control. Thirty percent of the precipitated material was controlled by Western blotting, the remaining reverse crosslinked and the DNA subjected to Q-PCR using three sets of primers to the mouse *c-Myc* promoter (Figure 5A, Supplementary Table 1). The MatInspector program was used to define the TCF/LEF binding site in the mouse *c-Myc* promoter and primers to areas of interest were designed as described (Kolly *et al*, 2005). The primer express program (Applied Biosystems) did only allow one to design Q-PCR primers adjacent to the TCF/LEF binding site. Results are presented as relative change compared to the IgG control. The experiment was carried out three times with PVIgG- and nhIgG-treated wild-type mouse keratinocytes and two times with PG$^{-/-}$ cells.

Adhesion assay

The adhesion assay for PVIgG- and nhIgG-exposed keratinocytes was carried out as described (Yin *et al*, 2005b). Experiments were carried out in duplicates and repeated two times.

Statistical analysis

Using the Lilliefors test, normal distribution of data was excluded. Statistical significance ($P<0.05$) was then assessed by the Kruskal–Wallis test for multiple group comparisons, followed by the Mann–Whitney test for comparisons of two groups.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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