Suprabasal Dsg2 expression in transgenic mouse skin confers a hyperproliferative and apoptosis-resistant phenotype to keratinocytes

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Summary

Desmoglein 2 (Dsg2), a component of the desmosomal cell-cell adhesion structure, has been linked to invasion and metastasis in squamous cell carcinomas. However, it is unknown whether – and if so how – Dsg2 contributes to the malignant phenotype of keratinocytes. In this study, we addressed the consequences of *Dsg2* overexpression under control of the involucrin promoter (*Inv-Dsg2*) in the epidermis of transgenic mice. These mice exhibited epidermal hyperkeratosis with slightly disrupted early and late differentiation markers, but intact epidermal barrier function. However, *Inv-Dsg2* transgene expression was associated with extensive epidermal hyperplasia and increased keratinocyte proliferation in basal and suprabasal epidermal strata. Cultured *Inv-Dsg2* keratinocytes showed enhanced cell survival in the anchorage-independent state that was critically dependent on EGF receptor activation and NF-κB activity. Consistent with the hyperproliferative and apoptosis-resistant phenotype of *Inv-Dsg2* transgenic keratinocytes, we observed enhanced activation of multiple growth and survival pathways, including PI 3-kinase/AKT, MEK-MAPK, STAT3 and NF-κB, in the transgenic skin in situ. Finally, *Inv-Dsg2* transgenic mice developed intraepidermal skin lesions resembling precancerous papillomas and were more susceptible to chemically induced carcinogenesis. In summary, overexpression of Dsg2 in epidermal keratinocytes deregulates multiple signaling pathways associated with increased growth rate, anchorage-independent cell survival, and the development of skin tumors in vivo.

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Introduction

Desmosomes are intercellular junctions found primarily in epithelial tissues but also in certain non-epithelial tissues including the meninges, dendritic reticular cells of lymph node follicles and myocardium (Cheng and Koch, 2004; Franke et al., 2006; McDonald et al., 2004). These adhesion structures are essential to maintaining tissue architecture during embryonic development and in the adult organism (Garrod et al., 2002). Desmosomes establish cell-cell contact through three families of proteins including the desmosomal cadherins, armadillo and plakin proteins (Yin and Green, 2004; Kottke et al., 2006). Desmosomal cadherins include desmogleins (Dsg) and desmocollins (Dsc), which associate by both heterophilic and homophilic interactions (Chitaev and Troyanovsky, 1997; He et al., 2003). On the cytoplasmic side, desmosomal cadherins bind to plakoglobin, plakophilins 1-4, and the intermediate filament binding protein desmoplakin. The hallmark feature of the desmosomes is the linkage of this transmembrane protein complex to keratin intermediate filaments forming a highly organized structure that allows cells and tissues to withstand mechanical stress and disruption (Cowin, 1994; Cowin and Burke, 1996; Green and Jones, 1996).

Four distinct desmogleins (Dsg1, Dsg2, Dsg3, Dsg4) have been reported with differential expression profiles dependent on the tissue type and differentiation state (Cheng and Koch, 2004; Garrod et al., 2002; Mahoney et al., 2006). Unlike Dsg1 and Dsg3 whose expression is restricted to complex stratified epithelia, Dsg2 and Dsg4 are expressed in a wide range of other cell types. For example, Dsg2 has been detected in simple epithelia including the stomach mucosa and intestines as well as in select non-epithelial tissues such as the myocardium (Koch et al., 1992; Schäfer et al., 1994; Schäfer et al., 1996). Despite rapid recent progress in understanding the role of desmogleins in epidermal cell adhesion and morphogenesis, the contribution of Dsg2 to these phenomena is poorly understood. Although elegant work has recently been done using reconstitution studies in cultured cells (Getsios et al., 2004), the adhesive properties of Dsg2 in the context of other co-expressed desmoglein family members in the in vivo context remain largely undefined. Interestingly, in humans, mutations in the *DSG2* gene are associated with

arrhythmogenic right ventricular cardiomyopathy (Pilichou et al., 2006). The development and characterization of *Dsg2*-null mice has reinforced the notion that Dsg2 has important biological roles beyond epidermal cell-cell adhesion, as these mice die at, or shortly after implantation (Eshkind et al., 2002). Although this circumstance precluded an investigation of Dsg2 as it relates to epidermal functions, *Dsg2*-null mice revealed that Dsg2 contributes to embryonic stem cell proliferation particularly in the inner cell mass of the developing blastocyst. In addition to being a structural component of desmosomes, Dsg2 is overexpressed or abnormally expressed in select epithelial malignancies including squamous cell carcinomas (Biedermann et al., 2005; Denning et al., 1998; Harada et al., 1996; Kurzen et al., 2003; Schäfer et al., 1996). Similarly, genetic profiling of prostate cancer cell lines showed increased expression of Dsg2 in a metastatic cell line compared with a non-metastatic syngeneic precursor cell (Trojan et al., 2005).

To investigate Dsg2 functions in epidermal biology, we established transgenic mice expressing *Dsg2* in the differentiating layers of the epidermis under control of the involucrin (*Inv*) promoter (*Inv-Dsg2*). Here, we describe that *Inv-Dsg2* transgenic mice exhibited epidermal hyperplasia associated with an increase in cell proliferation and enhanced activation of the PI3-kinase/AKT, MEK-MAPK, STAT3 and NF-κB signaling pathways. In addition, transgenic keratinocytes acquired resistance to apoptosis incurred in the anchorage-independent state (anoikis). Finally, *Inv-Dsg2* transgenic mice were prone to papilloma development both spontaneously and upon application of chemical carcinogens. Collectively, these results highlight previously unrecognized aspects of Dsg2 in support of malignant transformation of epithelial cells. The *Inv-Dsg2* mice provide a useful model system for in-depth studies of this phenomenon in the in vivo context.

Results

Characterization of transgene expression in *Inv-Dsg2* transgenic mice

To express Dsg2 in the suprabasal epidermis, we subcloned mouse *Dsg2*-Flag cDNA into the pH3700-pL2 parental vector epitope at the *Not*I restriction site downstream of the involucrin promoter (Fig. 1A). We obtained seven male and one female founder mice, bred the male founders back to C57Bl/6 females and screened the F1 generation by PCR. Two independent transgenic lines were maintained showing similar phenotype among all progeny. *Inv-Dsg2* transgenic mice developed normally (healthy, fertile, and normal lifespan) with no gross abnormalities in skin or hair appearance compared to wild-type littermates.

We assessed the expression of the Dsg2-Flag transgene in skin from adult (3 months old) transgenic mice by immunostaining with anti-Flag (Fig. 1B, left panels) and Dsg2-specific MP6 antibodies (Fig. 1B, middle panels). As expected, anti-Dsg2 and anti-Flag antibodies strongly reacted with suprabasal cell layers of the transgenic epidermis revealing similar staining patterns (Fig. 1B, right panels). Immunoblotting analysis of total skin lysates from adult control and transgenic mice confirmed the immunofluorescence data (Fig. 1C) as the MP6 and the anti-Flag antibodies detected a similar size band in adult transgenic skin lysates. As expected, little – if any – endogenous Dsg2 was detected in the wild-type skin lysate by the MP6 antibody (Fig. 1C,B). These results demonstrate expression of the transgene Dsg2-Flag in the differentiated cell layers of the epidermis. Note that MP6 and Flag antibodies cannot be used on paraffin embedded tissues thus limiting the present analysis to frozen sections, which are not ideal for obtaining punctate cell-cell border staining in the differentiated layers of the mouse epidermis.

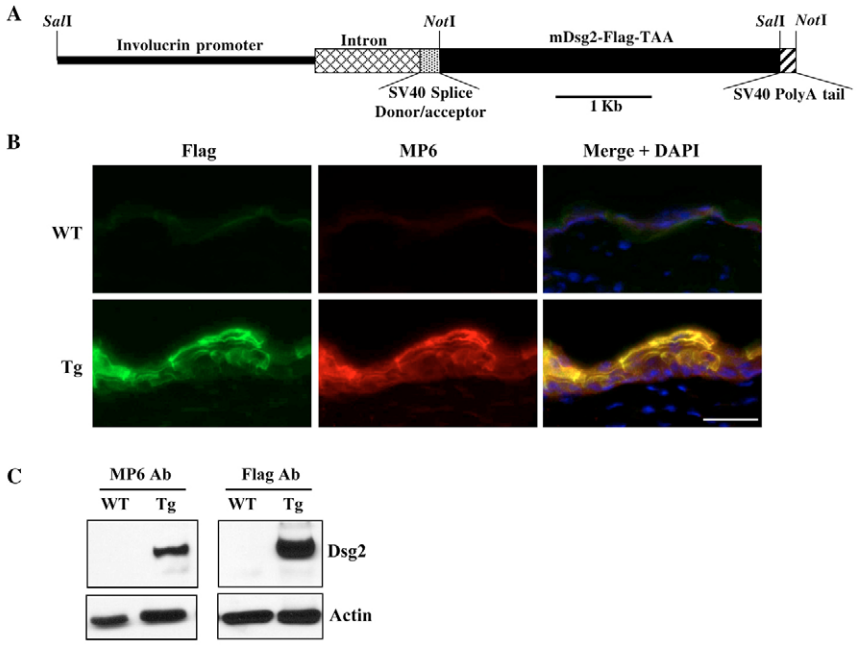

Fig. 1. Targeting construct and *Dsg2*-Flag expression in transgenic mice. (A) Schematic diagram of the mDsg2-Flag transgenic construct inserted at *Not*I restriction sites downstream of the involucrin promoter in the pH2700-pL2 vector mDsg2, mouse desmoglein 2 cDNA; Flag, nucleotide sequence encoding for the Flag octapeptide; polyA, polyadenylation signal. (B) Immunofluorescent staining of wild-type (upper panels) and *Inv-Dsg2* transgenic (lower panels) adult mouse skin with Flag (green, left panels) and Dsg2 MP6 (red, middle panels) antibodies showing expression of Dsg2-Flag in the transgenic, but not control epidermis. Co-staining (merge, right panels) for Dsg2 and Flag in the *Inv-Dsg2* transgenic but not control wild-type skin. Nuclei were stained with DAPI (blue). (C) Immunoblot analysis of adult control and transgenic mouse skin extracts using Flag and MP6 antibodies showing Dsg2 and Flag expression in the transgenic but not wild-type skin. Blots were reprobed with actin antibody as a loading control. In all panels: Tg, transgenic; WT, wild type.

Histological changes associated with *Inv-Dsg2* transgene expression in mouse epidermis

To examine transgene-induced alterations in the epidermis (Fig. 2), we collected back skin (Fig. 2A-D,I,J), ears (Fig. 2E,F), and tongues (Fig. 2G,H) from transgenic (Fig. 2B,D,F,H,J) and wild-type (Fig. 2A,C,E,G,I) littermates at birth and at 3 months of age. Tissue sections were stained with Hematoxylin and Eosin. All transgenic skin samples exhibited hyperplasia and slight hyperkeratosis. This phenotype was most evident in the skin of adult animals (compare Fig. 2D with Fig. 2C and Fig. 2I with Fig. 2J). In addition, keratinocytes in the stratum spinosum of transgenic skin were larger with bigger nuclei. A thicker granular layer with some retention of nuclei was also apparent (Fig. 2B,D). We also observed pink translucent flattened eosinophilic keratinocytes in the transgenic ear (Fig. 2F, open arrow) similar to the stratum lucidum of thick skin (Montagna et al., 1989). Transgenic mice also contained a compacted stratum corneum (Fig. 2B,D, *) similar to that of the Inv-mDsg3 transgenic mice previously described (Elias et al., 2001). The irregular and fragmented stratum corneum of *Inv-Dsg2* transgenic epidermis was similar in appearance to that observed in dry scaly skin where stratum corneum production occurs at a faster rate than normal (Harding, 2004). In addition, we observed slightly hyperplastic epidermis in the tongue of adult transgenic mice but not wild-type mice (Fig. 2G,H). Note that transgenic lines carrying a lower transgene expression developed a less extensive epidermal hyperproliferative phenotype. In addition, compared to heterozygous transgenics, homozygous littermates showed increased transgene expression and as well as a more dramatic hyperproliferation (data not shown). In summary, suprabasal expression of Dsg2 was associated with increased epidermal thickening, a phenotype becoming more prominent in adult animals.

To further examine the transition between the stratum granulosum and stratum corneum, electron microscopy was performed on back skin from wild-type and transgenic mice (Fig. 3). Consistent with the light microscopic observations we observed subtle morphological abnormalities particularly at the stratum granulosum-stratum corneum interface. Furthermore, the corneocytes of the *Inv-Dsg2* skin were much thicker in the axial direction (Fig. 3, brackets). The *Inv-Dsg2* skin was also thicker in the granular cell layer, as evidenced by the presence of more keratohyalin granules. We did not observe any abnormalities in desmosome size and appearance in the granular cell layers of wild-type and transgenic skin. In control epidermis, an abrupt transition was observed from the granular layer to the stratum corneum (Fig. 3A). The desmosomes in the last granular cell layer of wild-type epidermis retained the dense plaque with electron dense cytokeratin filament bundles, whereas a less distinct plaque without cytokeratins was observed on the opposing face (Fig. 3A, arrows). This was less prominent in the *Inv-Dsg2* transgenic skin. Similar to the Inv-mDsg3 transgenic mice (Elias et al., 2001), we observed a high number of electron dense intercellular structures (Fig. 3B, arrowheads) that became detached (Fig. 3B, *) in the cleft of separated corneocytes. Note, other than slightly larger cells, as observed by histological examination, no significant

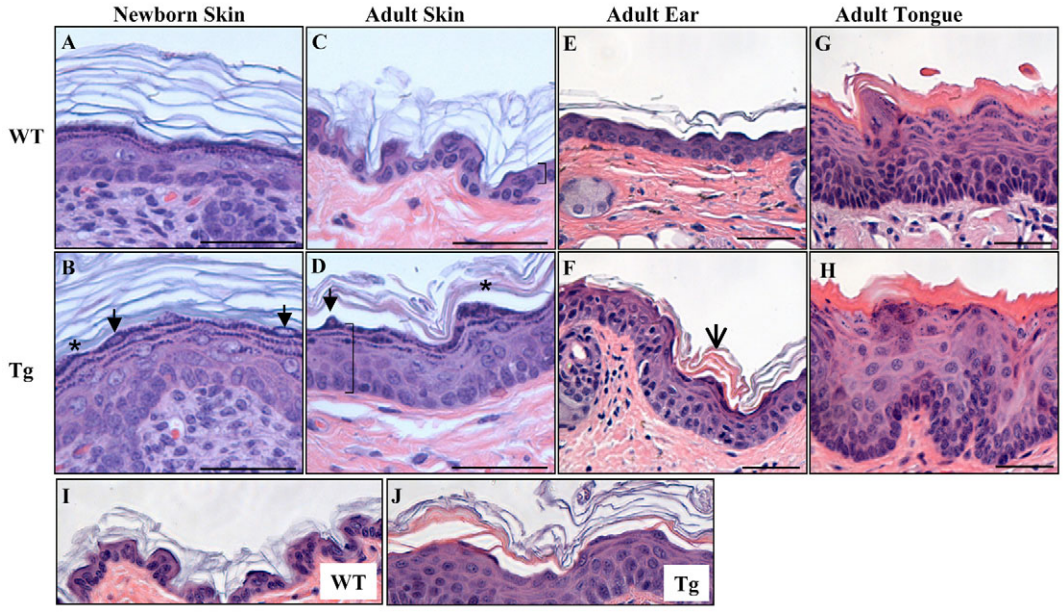

Fig. 2. *Inv-Dsg2* mice develop epidermal hyperplasia. Hematoxylin and Eosin staining of newborn back skin (A,B) and adult (C-J) back skin (C,D,I,J), ear (E,F) and tongue (G,H) of wild-type control (A,C,E,G,I) and *Inv-Dsg2* transgenic (B,D,F,H,J) littermate mice. Newborn and adult mice were 2 days and 3 months old, respectively. Histological analysis shows varying degrees of epidermal thickening in all *Inv-Dsg2* transgenic tissues examined. Minor compaction (B,D; asterisk) and the presence of nuclei (B,D; arrows) was observed in the outermost granular and horny layer interface. A more dramatic difference in epidermal thickness was observed in the adult transgenic mice compared that of control littermates (C,D; brackets) and to newborn transgenic skin. Pink translucent flattened eosinophilic keratinocytes were apparent in the stratum corneum (F; open arrow). Cells and nuclei in the spinous layers of transgenic epithelia appear slightly enlarged relative to those in wild-type epithelia. Bars, 25 μm.

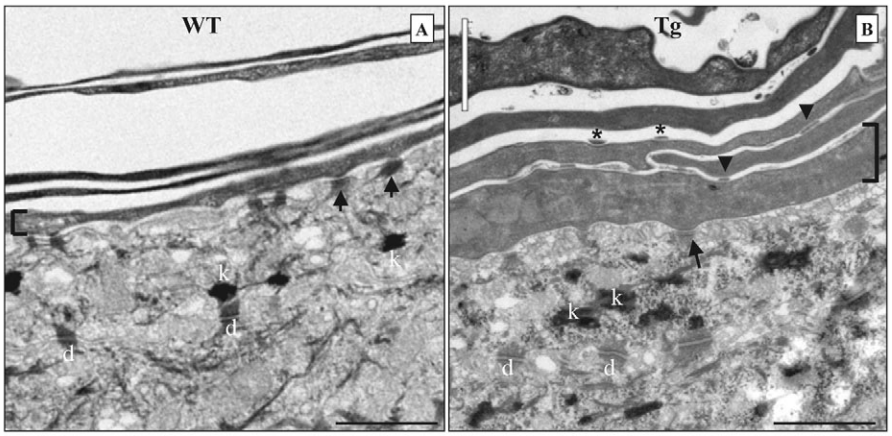

Fig. 3. Transmission electron microscopy of adult control wild-type (A) and *Inv-Dsg2* transgenic (B) epidermis. The granular cells appeared more compact and interconnected and corneocytes were thicker in the axial direction of the *Inv-Dsg2* transgenic epidermis (brackets). Detached desmosomes with intercellular gaps were detected in the stratum corneum (*). Arrows, desmosomes at the granular and stratum corneum interface; arrowheads, desmosome-like electron dense intercellular structures; d, desmosome; k, keratohyalin granules. Bars, 1 μm.

morphological differences were observed between the keratinocytes of the basal and spinous layers of *Inv-Dsg2* transgenic and control wild-type animals (not shown).

Effects of suprabasal Dsg2 expression on expression of epidermal adhesion, structural and differentiation proteins

Next we examined the effect of suprabasal expression of Dsg2 on the molecular composition of desmosomal junctions, by immunohistochemical analysis of desmogleins (Dsg1-α, Dsg1-β and Dsg1-γ, and Dsg3) and desmosomal plaque proteins (desmoplakin and plakoglobin). Overexpression of Dsg2 did not markedly alter the expression pattern of any of these proteins (see supplementary material Fig. S1A). The distribution of adhesion proteins (especially those concentrated in the upper layers such as the three Dsg1 isoforms) appeared to be broader in the transgenic mice, probably due to the increase in the total number of cell layers. Interestingly, the expression of desmoplakin (see supplementary material Fig. S1Ai,j) and plakoglobin (see supplementary material Fig. S1Ak,l) were slightly increased in the transgenic skin. Insets in supplementary material Fig. S1Ai,k show wild-type images taken with the same exposure time as those of the transgenic mice (supplementary material Fig. S1Aj,l). Note that Dsg4 was expressed at low levels in the mouse epidermis and that suprabasal expression of *Inv-Dsg2* in transgenic mice did not affect Dsg4 expression (not shown). Finally, the expression of proteins within the adherens junctions, such as E-cadherin and β-catenin, were not affected in the *Inv-Dsg2* transgenic epidermis (see supplementary material Fig. S1B).

Next we asked whether overexpression of Dsg2 in the superficial epidermis had an effect on the expression and organization of cytoskeletal and differentiation proteins. By immunofluorescence we observed that suprabasal Dsg2 expression was associated with altered expression patterns of several biochemical markers for keratinocyte differentiation, including cytokeratins (CK), involucrin, loricrin and filaggrin (Fig. 4). CK14 was detected only in the basal layer of the control wild-type skin, but its expression extended into the suprabasal cell layers of the transgenic epidermis (Fig. 4A). Similarly, immunoblot analysis of epidermal protein extracts revealed enhanced expression of CK14 in transgenic as compared to wild-type skin (Fig. 4B). CK10 expression was similarly increased in transgenic skin extracts (Fig. 4B) and was localized to the superficial epidermis of transgenic skin (Fig. 4A). Under homeostatic conditions, CK6 expression is restricted to the hair follicles, whereas it extends into the interfollicular epidermis under hyperproliferative conditions, for example psoriatic lesions (Heyden et al., 1994; Mils et al., 1992; Stoler et al., 1988) or during wound healing (Coulombe, 1997; McGowan and Coulombe, 1998). Interestingly, we observed intermittent expression of CK6 in the interfollicular epidermis of the *Inv-Dsg2* transgenic, whereas CK6 was completely absent from interfollicular epidermis of control wild-type littermate (Fig. 4A). Because CK6 expression in the interfollicular epidermis was sparse, but high where it did occur in the hair follicles, immunoblotting detected similar levels of CK6 in both wild-type and transgenic skin lysates (Fig. 4B). Induction of CK6 and CK14 expression in the suprabasal epidermis is a further indication of altered epidermal differentiation in the *Inv-Dsg2* transgenic mice.

Consistent with the thickening of the granular cell layers, we observed an increase in expression of cornified envelope proteins including the differentiation marker involucrin (Fig. 4A,B). Filaggrin, an intermediate filament-associated protein expressed during the late stages of differentiation in the granular cells of the epidermis (Yaffe et al., 1993), was also overexpressed in the transgenic epidermis (Fig. 4A,B). Upon longer exposure, the wild-type filaggrin immunoblot showed a similar protein banding pattern to that of the transgenic blot (not shown). Similar to findings in transgenic mice expressing human DSG3 (hDsg3) under the keratin 1 promoter control (K1-hDsg3), the *Inv-Dsg2* transgenic epidermis had an increase in granular cell layers as shown by the increased number of cells stained positive for loricrin, a cornified envelope precursor protein (Fig. 4A). These results were confirmed by immunoblotting (Fig. 4B).

We next sought to determine the effect of Dsg2 overexpression on the subcellular location of desmosomal components in the *Inv-Dsg2* transgenic skin. First, we extracted adult wild-type and transgenic skin in Triton-soluble and -insoluble fractions. Immunoblotting analysis showed Dsg2-Flag in both Triton-soluble and -insoluble fractions whereas other desmosomal proteins (Dsg1, Dsg3 and Dsg4, and desmoplakin) were found exclusively in the Triton-insoluble fraction (see supplementary material Fig. S2A). It is unclear whether the presence of Dsg2-Flag in the Triton-soluble fraction of transgenic mice is due to high protein expression or

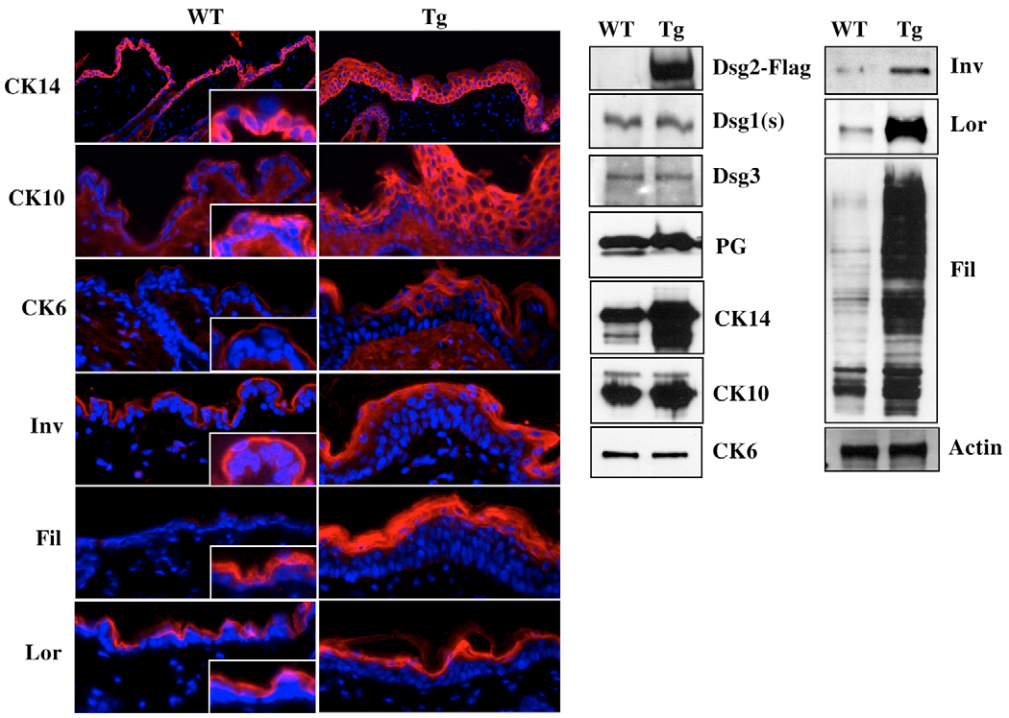

Fig. 4. Changes in expression patterns of cornified envelope and keratin proteins in *Inv-Dsg2* mice. (A) Immunofluorescent staining of 3-month old adult wild-type and *Inv-Dsg2* transgenic mouse skin for cytokeratin (CK14, CK10 and CK6), involucrin (Inv), filaggrin (Fil), and loricrin (Lor). CK14 was observed only in the basal layer of the control but extended into the suprabasal epidermal layers of *Inv-Dsg2* skin. Increased expression of CK10 was observed in the *Inv-Dsg2* epidermis. CK6 expression was observed in the interfollicular epidermis in the *Inv-Dsg2* mice. Suprabasal Dsg2 expression was associated with expression of involucrin, filaggrin, and loricrin. Insets are higher resolution and increased exposure images. Nuclei were stained with DAPI (blue). (B) Immunoblotting analysis of adult control wild-type and *Inv-Dsg2* transgenic mouse skin with antibodies against Flag, Dsg1 (antibody 27B2; recognizes mouse Dsg1-α, Dsg1-β and Dsg1-γ), Dsg3 (AP904), PG, CK6, CK10, CK14, Lor, Fil and actin (loading control).

a novel functional role of Dsg2, independent of desmosomes. As expected, plakoglobin and β-catenin were detected in both Triton-soluble and -insoluble fractions. Thus, suprabasal expression of Dsg2 did not alter the Triton solubility of desmosomal proteins.

To elucidate the potential mechanism underlining epidermal hyperproliferation in the *Inv-Dsg2* transgenic skin, we performed additional subcellular fractionation of wild-type and transgenic skin. Total skin proteins were purified for crude (soluble) cytoplasmic, enriched (wash) cytoplasmic, crude nuclear (high salt), and enriched nuclear and or cytoskeletal (insoluble) proteins. Interestingly, the expressed *Dsg2*-Flag transgene was detected not only in the cytoplasmic but also the nuclear fractions (see supplementary material Fig. S2B). This finding is significant since Dsg2 is overexpressed in squamous cell carcinomas of the skin. It is unknown whether Dsg2 is present in the nuclei of these carcinomas or whether Dsg2 serves any functions in the nucleus. The other desmosomal proteins were localized mainly in the insoluble pool along with cytokeratins (see supplementary material Fig. S2B).

The armadillo family of adhesion/signaling proteins found in desmosomal and adherens junctions include β-catenin, plakoglobin and plakophilin 1. Plakoglobin was recently shown to play an important role in negatively regulating the expression of the proto-oncogene Myc (Williamson et al., 2006). Thus, we performed subcellular fractionation to determine whether epidermal hyperproliferation in *Dsg2* transgenic mice is due to downregulation of the nuclear pool of plakoglobin (see supplementary material Fig. S2B). Immunoblotting analysis showed the subcellular pools including the nuclear fraction of plakoglobin were relatively unchanged in transgenic compared to wild-type skin. Unexpectedly, whereas the cytoskeletal pool of β-catenin was unchanged between the wild-type and transgenic animals, both the cytoplasmic and nuclear pools of β-catenin were significantly reduced in *Inv-Dsg2* transgenic compared to control wild-type skin (see supplementary material Fig. S2B). Thus, suprabasal expression of Dsg2 did not alter plakoglobin expression or subcellular localization but did reduce the cytoplasmic and nuclear levels of β-catenin. It remains to be determined in future studies how this observation relates to the upregulation of Myc expression in transgenic mice.

To further investigate the thickening of the granular layers, we isolated cornified envelopes (CEs) from wild-type and *Inv-Dsg2* transgenic newborn (1-2 days) and adult (3 months) skin and ear (Table 1). This analysis revealed only a marginal increase in CE production in transgenic newborn dorsal and in adult mouse ear skin as compared to wild-type skin. These results are consistent with the histology data showing only minor hyperplasia in newborn skin and adult ear epithelia (Fig. 2A,B). By contrast and consistent with the age-dependent increase in hyperplasia (Fig. 2C,D), a dramatic increase in CE

Table 1. Increase in cornified envelopes in Inv-Dsg2 transgenic skin

Tissue	Percentage (Tg to WT)	Number of animals	
		WT	Tg
Newborn dorsal skin	117.0±16.7	5	8
Adult ear	127.9±40.7	7	18
Adult dorsal skin	386.4±156.0	5	9

Tg, transgenic; WT, wild type.

production was seen in adult back skin of *Inv-Dsg2* transgenic relative to wild-type animals (Table 1). Although the number of CEs increased in the transgenic skin, we observed no significant difference in their susceptibility to stress by ultrasound treatment under the conditions chosen (data not shown).

Suprabasal expression of Dsg2 altered the adhesive strength of the stratum corneum

In newborn *Inv-Dsg2* transgenic mice, we observed abnormal organization and fragmentation of the corneal layers (see supplementary material Fig. S3A, arrows and bracket). To assess functional consequences of the morphological changes in the stratum corneum, we tape-stripped transgenic and control wild-type littermates. The stratum corneum of control mice detached in organized sheets whereas that of transgenic mice detached in scattered fragmented sheets of corneocytes (see supplementary material Fig. S3B). After ten tape strips, the stratum corneum in transgenic mice and normal littermates appeared more similar although there were still more corneocytes removed from the control compared to the transgenic skin. These results demonstrate moderate changes in morphology and mechanical properties of the stratum corneum in *Inv-Dsg2* transgenic mice.

Epidermal hyperproliferation in *Inv-Dsg2* transgenic mice

To assess the proliferative state of the *Inv-Dsg2* epidermis, skin sections of wild-type and transgenic mice were stained with an antibody to the proliferating cell nuclear antigen (PCNA); an increase in PCNA staining generally correlates with mitotic activity and cell proliferation. In wild-type skin, we observed intermittent staining of nuclei in the basal cell layer (arrow in Fig. 5A). By contrast, virtually all nuclei in the basal layer of the transgenic epidermis were stained with the PCNA (Fig. 5B). Additionally, some transgenic suprabasal cells were also positive for PCNA (Fig. 5B, arrows). These results are consistent with epidermal hyperproliferation. To further investigate the proliferative state of the transgenic epidermis, we injected wild-type and transgenic mice with bromodeoxyuridine (BrdU), a halogenated thymidine analog that integrates into the DNA of actively dividing cells progressing through S-phase of the cell cycle. We identified actively dividing cells within an hour of BrdU treatment and observed markedly enhanced numbers of BrdU-labeled cells in the transgenic (13±6 per 100 basal cells) over wild-type epidermis (2±2 per 100 basal cells) (Fig. 5C,D). In further support of epidermal hyperproliferation in transgenic epidermis we observed enhanced expression of Myc relative to that in wild-type epidermis (Fig. 6). Thus, overexpression of Dsg2 in the superficial epidermis results in epidermal hyperplasia associated with enhanced proliferation of keratinocytes.

Deregulated signal transduction pathways in the epidermis of *Inv-Dsg2* transgenic mice

Next, we investigated activation states of signaling pathways commonly implicated in epidermal hyperplasia, i.e. the mitogen-activated Raf-MEK-MAPK and the PI3-kinase/AKT

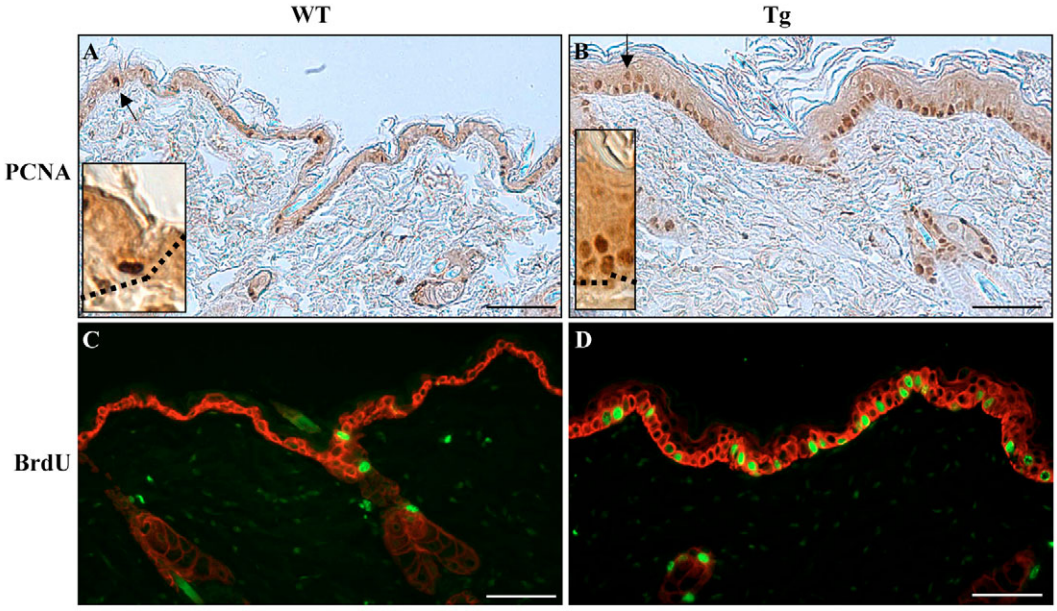

Fig. 5. *Inv-Dsg2* transgenic mice show hyperproliferative epidermis and increase in mitotic activity. Paraffin embedded sections of adult wild-type (A,C) and *Inv-Dsg2* transgenic (B,D) skin were stained with antibodies to detect either PCNA expression (A,B) or BrdU incorporation (C,D; green). Insets show higher resolution images. Also shown in red in C and D is immunostaining for CK14.

pathways. This analysis revealed slight increase in total AKT, markedly higher levels of AKT phosphorylation on both Ser473 and Thr308, and of the AKT target GSK-3β on Ser9 in transgenic epidermis as compared to epidermis from wild-type mice (Fig. 6). AKT-Thr308 is phosphorylated by the phosphoinositide-dependent protein kinase (PDK) 1 (Mora et al., 2004) which also showed higher phosphorylation levels in *Inv-Dsg2* transgenic epidermis. Additionally, we examined phosphorylation level of PTEN, a negative regulatory phosphatase of the PI3-kinase/AKT pathway. Phosphorylation of PTEN has been described to sequester PTEN in the cytoplasm, thus preventing recruitment of this negative regulator of PI 3-kinase to the cell membrane (Das et al., 2003). Consistent with increased PI3-kinase/AKT activity in transgenic epidermis we observed an increase in both total PTEN and PTEN Ser308 phosphorylation (Fig. 6). The Raf-MEK-MAPK signaling axis was similarly deregulated in transgenic epidermis with increased steady-state phosphorylation of Raf, MEK1/2, and p42/44 MAPK as well as the MAPK target p90RSK (Fig. 6).

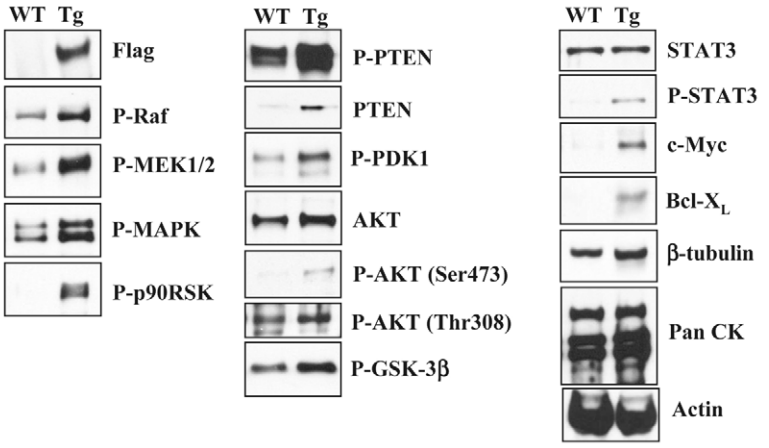

Fig. 6. Characterization of the signaling events in *Inv-Dsg2* epidermis. Total protein lysates of wild-type and *Inv-Dsg2* transgenic adult skin were analyzed by western analysis to assess activation (phosphorylation) states of signaling intermediates. Immunoblotting for actin, pan cytokeratin (CK) and β-tubulin expression showed equal loading of lanes.

We next examined the expression and phosphorylation levels of STAT3, a cytokine-activated transcription factor that upregulates oncogenes such as Myc and Bcl-X_L. STAT3 has been proposed to be a key player in regulating cell cycle and tumor progression in squamous cell carcinomas. Increased STAT3 phosphorylation was detected in transgenic relative to wild-type skin (Fig. 6). No change in total STAT3 was detected in wild-type and transgenic skin. Collectively, these results demonstrate that increased epidermal proliferation in *Inv-Dsg2* transgenic mice was associated with enhanced activation of at least three major signaling pathways including PI3-kinase/AKT, MEK-MAPK and STAT3.

Dsg2 overexpression enhances anchorage-independent keratinocyte survival

In order to determine functional aspects of Dsg2 overexpression for keratinocyte biology in vitro, we established keratinocyte cultures from back skin of *Inv-Dsg2* transgenic and wild-type newborn mouse. This was accomplished by initiating cell lines in culture medium containing low Ca^{2+} (0.08 mM). Under these conditions, the *Dsg2*-Flag transgene was not expressed because the involucrin promoter was not induced (Fig. 7A,B). However, addition of Ca^{2+} (1 mM) induced both differentiation and transgene expression as determined by immunoblot and immunofluorescence analysis of the Flag tag (Fig. 7A,B). Note that Dsg1 isoforms were not expressed until 2-3 days after calcium treatment (data not shown). Similar to the in vivo model, overexpression of Dsg2 also enhanced STAT3 phosphorylation in cultured keratinocytes (Fig. 7A). Cellular differentiation upon calcium treatment was confirmed by upregulation of involucrin expression (Fig. 7A). An alternative method to induce keratinocyte differentiation is to place these cells into forced suspension culture, which prevents anchorage to the extracellular matrix. Loss of cell matrix adhesion leads to robust induction of involucrin expression in human keratinocytes (U.R., unpublished observation). Thus we expected that the *Dsg2* transgene, under control of the involucrin promoter, would be similarly induced. Indeed, we observed robust involucrin and *Dsg2* transgene expression in forced suspension culture in transgenic but not wild-type keratinocytes (Fig. 7C).

This circumstance allowed us to monitor the effects of Dsg2 overexpression on keratinocyte biology in the anchorage-independent state. In the absence of extracellular matrix attachment, epithelial cells including keratinocytes undergo a specialized programmed cell death referred to as ‘anoikis’ (Frisch and Francis, 1994). To ascertain potential effects of Dsg2 overexpression on cell survival in the anchorage-independent state, transgenic and wild-type cells were seeded on top of an agarose layer, which prevents attachment of the cells to substrate (forced suspension culture), followed by transfer of cells to cell culture plastic; upon reseeding only viable cells will reattach and commence proliferation (Mahoney et al., 2002b; Rodeck et al., 1997b). Survival and proliferation of rescued cells was scored by observing colony formation of reseeded cells visualized by Crystal Violet staining. These experiments revealed a clear survival advantage of the *Inv-Dsg2* transgenic over wild-type keratinocytes (Fig. 8A). Not only did more transgenic keratinocytes reattach after prolonged (3 days) forced-suspension culture, but they also formed larger colonies than wild-type keratinocytes upon further incubation (Fig. 8A). Collectively these results suggest that Dsg2 overexpression increases resistance of keratinocytes to anoikis. They are consistent with high level of expression of the pro-survival protein Bcl-X_L in transgenic epidermis (Fig. 6) and raise the question of which signaling pathways supports anchorage-independent cell survival.

This question was addressed by performing forced suspension/rescue experiments in the presence of pharmacological inhibitors of signaling pathways targeting activation of EGF-R, PI3-kinase, MEK1/2 and NF-κB. Activation of the NF-κB pathway has previously been implicated in epithelial cell survival in three-dimensional

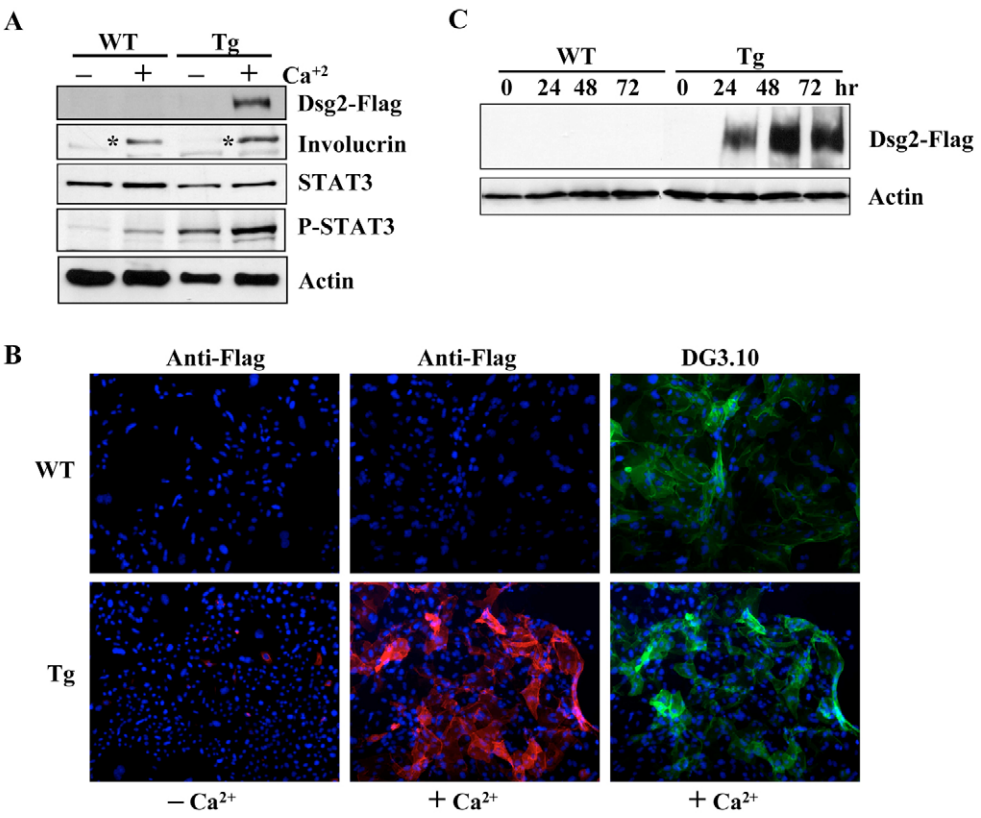

Fig. 7. Establishment of cultured primary keratinocytes from *Inv-Dsg2* transgenic and wild-type newborn mouse skin. (A) Immunoblotting analysis for the Flag tag of wild-type and transgenic cell lines grown in low (80 nM; –) or high (1 mM; +) calcium for 5 days. Dsg2-Flag (160 kDa) expression was detected in the calcium-treated transgenic but not the untreated transgenic or the wild-type control keratinocytes. Differentiation was confirmed by increased involucrin expression (*) in response to calcium. Upregulation of Dsg2 enhanced *P*-STAT3 level but not total STAT3 in transgenic compared with wild-type skin. Actin was used as a loading control. (B) Wild-type and transgenic keratinocytes were grown to confluency in low-calcium-containing CnT medium and then 1 mM calcium was added for 24 hours. Cells were fixed and stained with Flag and DG3.10 antibodies. Transgenic but not wild-type keratinocytes expressed the Dsg2-Flag protein. DG3.10 recognized the endogenous Dsg2 in the wild-type and as well as the Dsg2-Flag in the transgenic cells. (C) Wild-type and *Inv-Dsg2* transgenic cells were trypsinized and put in suspension culture for up to 72 hours. Cells were collected at the time points indicated. Dsg2-Flag was detected by immunoblot analysis in *Inv-Dsg2* cells within 24-72 hours in suspension culture. Immunoblotting analysis for actin expression showed equal loading. Similar results were observed with three independent wild-type and transgenic cell lines.

mammary cell tissue reconstructs (Rodeck et al., 1997a; Weaver et al., 2002; Zahir et al., 2003) and in human keratinocyte suspension cultures (Ren et al., 2006). To assess the importance of NF-κB signaling in growth and survival of *Inv-Dsg2* transgenic keratinocytes, we used the pharmacologic inhibitor, Bay11-7082, which acts by inhibiting IκB-alpha phosphorylation thus preventing nuclear translocation of NF-κB dimers. Bay11-7082 abolished proliferation and survival of *Inv-Dsg2* transgenic as well as wild-type keratinocytes in the anoikis setting (Fig. 8B). The activation of epidermal growth factor receptor (EGFR) has been shown to provide a measure of protection against death of keratinocyte cells in suspension (Jost et al., 2001b). Here we show that activation of EGF-R with exogenous EGF enhanced growth and survival of transgenic keratinocytes, but was unable to reverse the effect of Bay11-7082 (Fig. 8B). Similar results were obtained with MG132, a proteasome inhibitor that prevents IκB degradation (not shown). Interestingly, inhibitors of MEK1/2 (U0126) and PI3-kinase had only marginal effects on mouse keratinocyte survival regardless of transgene expression (not shown). To assess the activation state of NF-κB in *Inv-Dsg2* transgenic epidermis, we examined the expression and subcellular localization of NF-κB p65 protein in situ. We observed a dramatic increase in p65 expression in transgenic compared to wild-type skin by immunoblotting (Fig. 8C) and immunofluorescent staining (Fig. 8D). In addition, NF-κB p65 protein was detected in cell nuclei, which is indicative of activation, in transgenic but not wild-type skin (Fig. 8D). Collectively, these results demonstrate that Dsg2-mediated cell survival is controlled, in part, by EGF-R- and NF-κB-dependent signaling events.

Tumor formation in *Inv-Dsg2* transgenic epidermis

Previously, overexpression of Dsg2 has been described in squamous cell carcinomas of the skin (Denning et al., 1998; Harada et al., 1996; Schäfer et al., 1996). Interestingly, histological analysis revealed sporadic and spontaneous appearance of benign papillomas in over half of our *Inv-Dsg2* transgenic animals at 6 weeks (Fig. 9A) and 3 months of age (Fig. 9B) in the absence of carcinogen treatment. By contrast,

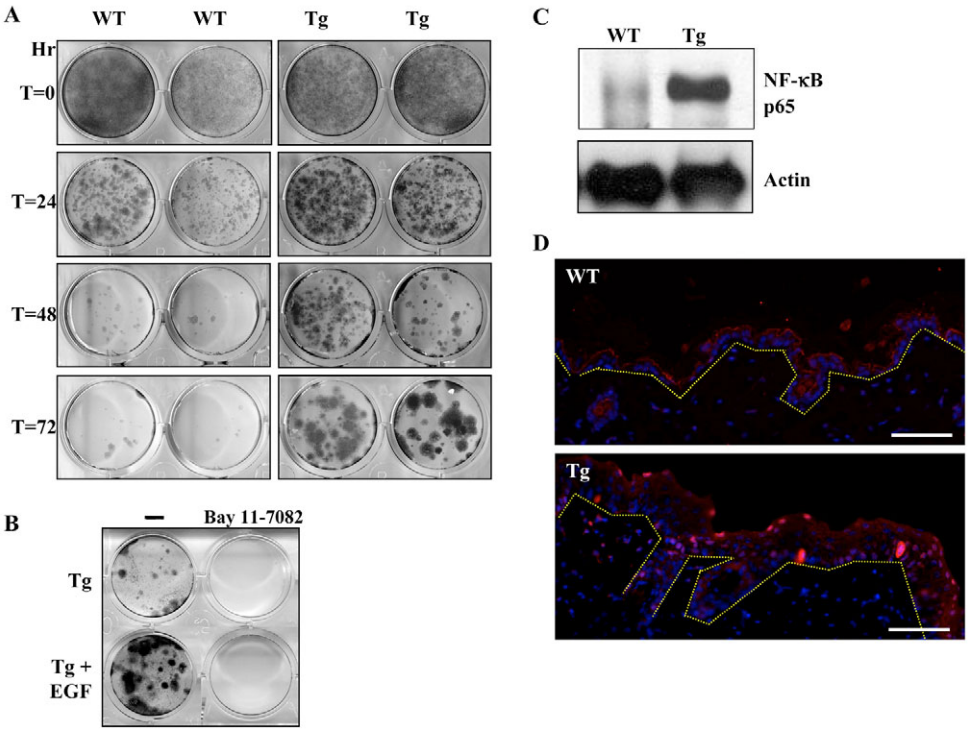

Fig. 8. Enhanced survival of *Inv-Dsg2* keratinocytes in forced suspension culture is dependent on NF-κB activation. (A) Clonal growth of control and *Inv-Dsg2* cell lines after 0, 24, 48 and 72 hours in forced suspension culture was assessed by replating cells on tissue culture plastic and allowing cell proliferation for 7-9 days after replating. *Inv-Dsg2* transgenic cells showed dramatically increased survival and re-growth compared to wild-type cells, in this setting. (B) *Inv-Dsg2* transgenic cells were subjected to cell suspension culture for 48 hours with EGF and/or Bay11-7082, an IκB-alpha phosphorylation inhibitor. Cell survival was assessed by replating cells on tissue cultured-treated plastic for 7 days. Activation of the EGF receptor with exogenous EGF further enhanced survival of *Inv-Dsg2* transgenic cells in forced suspension cultures. Bay11-7082 inhibition of NF-κB completely abolished cell survival in suspension culture. EGF-R activation was unable to counteract the effect of Bay11-7082. (C) Immunoblot analysis of NF-κB p65 expression in wild-type and transgenic skin. Actin was used as a loading control. (D) Immunoblot results were confirmed by immunofluorescent staining, which showed the presence of NF-κB p65 in nuclei of transgenic but not wild-type skin. Dotted lines demarcate dermal-epidermal junction. Bars, 50 μm.

none of the wild-type mice examined developed epidermal hyperplasia or papillomas (Fig. 9A,B, upper panels). In the *Inv-Dsg2* transgenic animals, we observed extensive hyperkeratosis (box), marked hyperproliferation (arrows) with epidermal outgrowths extending over the existing epidermis forming two complete skin layers. When these outgrowths came in contact with each other, they appeared to merge, often encapsulating (*) the stratum corneum.

The hyperproliferative/apoptosis resistant phenotype of *Inv-Dsg2* transgenic keratinocytes raised the question of whether the transgenic mice would be prone to carcinogen-induced tumor development. To determine whether the transgenic mice were more susceptible to chemical-induced carcinogenesis, we treated transgenic and wild-type control littermates once with 7,12-dimethylbenz[*a*]anthracene (DMBA) followed by twice weekly 12-*O*-tetradecanoylphorbol 13-acetate (TPA) treatments to promote tumor formation. The transgenic mice showed significantly increased susceptibility to skin tumor formation compared to wild-type littermates (Fig. 9C). The transgenic mice developed a higher total number of tumors 12.4±6.1 (mean ± standard deviation at week 25) compared with wild-type mice at the same time point with only 4.8±4.1 tumors (Student's *t*-test, P=0.0152; Fig. 9D). This difference was even more pronounced when we counted only papillomas with diameters greater than 2 mm with transgenic mice averaging 8.9±4.5 tumors per animal compared to wild-type with only 2.1±2.1 tumors (Student's *t*-test, P=0.0063; Fig. 9E). These results are consistent with a functional role of Dsg2 overexpression by keratinocytes in tumor development. Despite enhanced tumor development in the transgenic mice, histological analysis showed no significant differences in the architecture of the transgenic and wild-type papillomas (Fig. 9F). At 25 weeks, the papillomas in both groups were well differentiated with minor hyperkeratosis. No gross signs of invasiveness or secondary tumors were observed in the livers or lungs, known target organs of invasive carcinomas.

Discussion

This report describes the establishment and characterization of *Inv-Dsg2* transgenic mice, in which Dsg2 expression was directed by the involucrin promoter to the differentiating cell layers of the epidermis. Ectopic Dsg2 expression resulted in subtle perturbation of epidermal differentiation and alterations in expression of some desmosomal and terminal differentiation proteins. Furthermore, mild structural abnormalities in the stratum corneum were evident morphologically as determined by tape stripping.

The rather moderate changes in differentiation and

hyperkeratosis of *Inv-Dsg2* transgenic epidermis were contrasted by epidermal hyperplasia, which was most pronounced in adult animals. Epidermal hyperplasia manifested as enhanced thickness due to an increase in cellularity up to 10 cell layers. The hyperplasia in the transgenic adult mouse skin was associated with increased PCNA staining and BrdU labeling indicative of accelerated cell cycle progression and proliferation. The hyperproliferative phenotype in situ was accompanied by multiple molecular alterations consistent with accelerated cell cycle progression. We observed a marked increase in Myc expression in the transgenic relative to wild-type skin. Activation of Myc in basal keratinocytes has been previously linked to hyperproliferation (Arnold and Watt, 2001; Frye et al., 2003; Waikel et al., 2001) whereas suprabasal Myc activation is linked to proliferation and disruption of terminal differentiation (Pelengaris et al., 1999; Waikel et al., 1999). Interestingly, epidermal hyperplasia and hyperkeratosis observed in *Inv-Dsg2* transgenic mice is reminiscent of the ML-*myc2* transgenic mice overexpressing Myc in the differentiated epidermis under control of the loricrin promoter (Waikel et al., 1999).

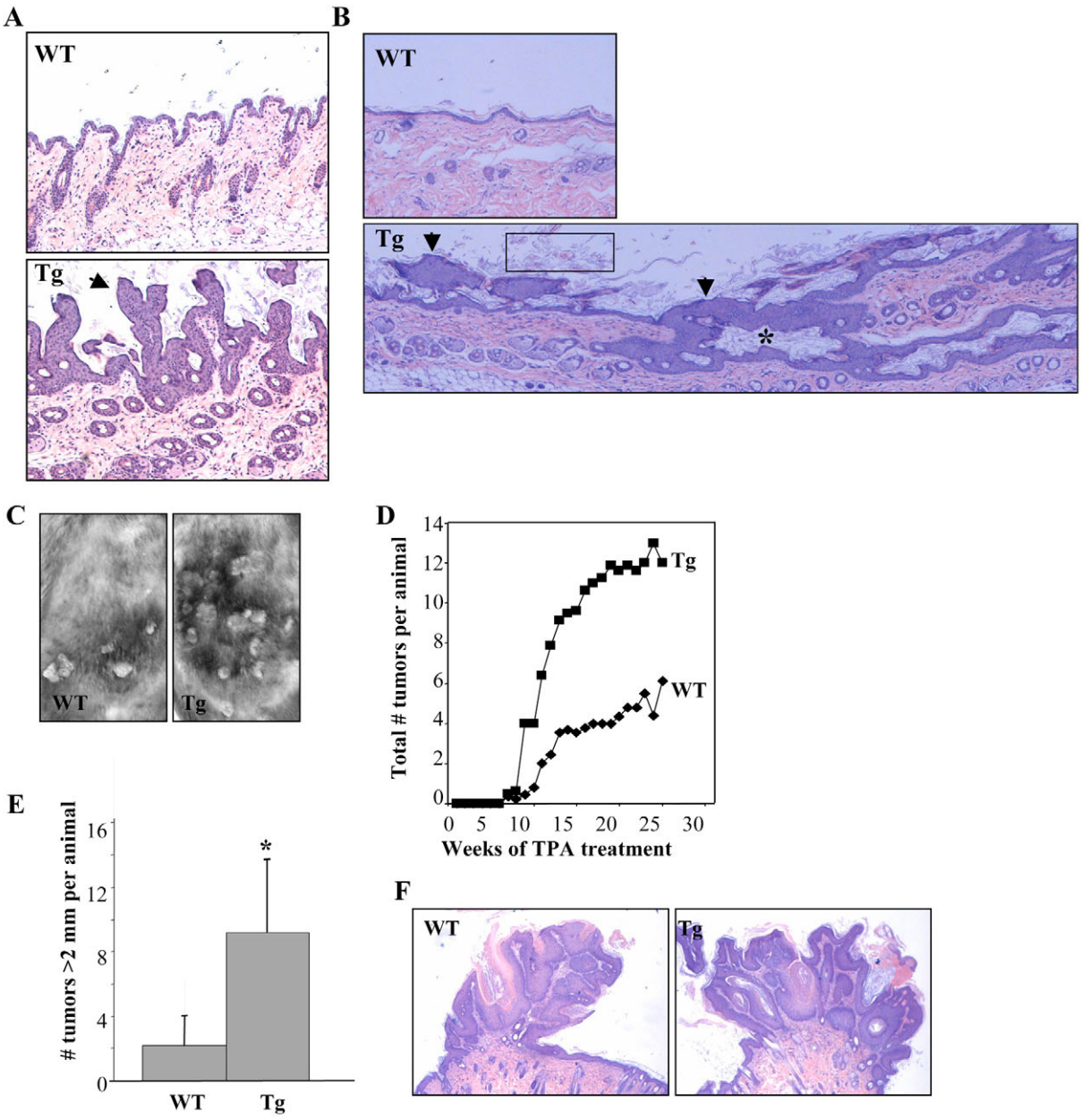

Fig. 9. Increased tumor formation in *Inv-Dsg2* transgenic mice. (A,B) Histological analysis of epidermal hyperplasia and early forms of papillomas in *Inv-Dsg2* transgenic but not control wild-type mice at 6 weeks (A) and 3 months (B) (10× magnification). Arrows indicate hyperproliferation; box, hyperkeratosis; asterisk, encapsulated stratum corneum. (C) After 25 weeks of DMBA and TPA treatment, the *Inv-Dsg2* transgenic mice developed more tumors than control wild-type mice. (D,E) The frequency and average number of papillomas in wild-type and transgenic mice after 25 weeks of tumor promotion. For tumors greater than 2 mm, **P*=0.0063. (F) Histopathological appearance of carcinogen-induced tumors from wild-type and transgenic mice showing differentiated and hyperkeratotic papillomas.

Consistent with the hyperproliferative phenotype, we observed high activation states of the PI 3-kinase/AKT, Raf-MEK-MAPK, STAT3 and NF-κB signaling pathways in transgenic skin. Previous work has implicated these signaling events not only in keratinocyte proliferation but also in enhanced keratinocyte survival in experimental conditions that induce apoptosis of normal keratinocytes, notably deprivation of extracellular matrix interaction (Jost et al., 2001b; Ren et al., 2006). Indeed, we observed markedly higher rates of anchorage-independent survival of *Inv-Dsg2* transgenic when compared to wild-type keratinocytes. This effect was, at least in part, due to activation of EGF-R and NF-κB signaling as demonstrated by use of EGF and pharmacological inhibitors of NF-κB pathways. Previous work has implicated both signaling pathways in aberrant anchorage-independent survival of human keratinocytes (Jost et al., 2001a; Jost et al., 2001b; Ren et al., 2006). Furthermore, deregulated EGF-R (Chan et al., 2004; Hansen et al., 2000; Merlino et al., 1985; Ozawa et al., 1989; Sibilia et al., 2000; Woodworth et al., 2000) and NF-κB (Chung et al., 2004; Dong et al., 2001; Duffey et al., 1999; Loercher et al., 2004) signaling have been described in human squamous cell carcinomas, and both pathways have been linked to upregulation of the anti-apoptotic Bcl-2 family member Bcl-X_L in epithelial cells. Consistent with these earlier results, we observed high expression of Bcl-X_L in transgenic but not wild-type epidermis. Most importantly, we also observed high rates of spontaneous and carcinogen-induced papilloma formation in the epidermis of *Inv-Dsg2* transgenic mice. To our knowledge, we are the first to report that misexpression of a desmosomal cadherin in the epidermis affects susceptibility to skin tumor development. This result, however, is consistent with previous reports of Dsg2 overexpression in human squamous cell carcinomas. We propose that the inappropriate Dsg2 expression contributes to epidermal tumorigenicity by deregulating signal transduction pathways altering the hyperproliferative and apoptosis-resistance phenotype of keratinocytes. We submit that the *Inv-Dsg2* mice described here provide an excellent in vivo model system to further investigate signaling events downstream of Dsg2 overexpression as they relate to roles of desmosomal cadherins beyond maintenance of cell adhesion and tissue integrity.

These results contrast with previous observations of decreased Dsg2 expression during tumor development (Rieger-Christ et al., 2005; Yashiro et al., 2006). Specifically, restoring PG expression in bladder carcinoma cells resulted in elevated level of Dsg2 expression and suppression of migration and tumorigenic potential (Reiger-Christ et al., 2005). Of note, their study primarily focused on functional roles of PG in heterogeneous bladder carcinoma cell lines whereas the present study is concerned with deregulated Dsg2 expression in keratinocytes. It is possible that these contradictory findings reflect the transformed state of the particular cell lines studied. Alternatively, Dsg2 expression may play different roles in different tissues.

Previous work has implicated desmosomal cadherins in epidermal proliferation, differentiation and morphogenesis (Chidgey et al., 2001; Elias et al., 2001; Eshkind et al., 2002; Hardman et al., 2005; Merritt et al., 2002). For example, expressing Dsc1 in the proliferative basal layer, where it is not normally expressed, using the K14 promoter did not affect epidermal morphogenesis and development (Henkler et al., 2001) whereas ectopic expression of Dsc3, which normally is restricted to the basal cell layer, in the spinous layers using the K1 promoter altered epidermal differentiation (Hardman et al., 2005). Targeting Dsg3 expression to the spinous layers using the K1 promoter also affected epidermal proliferation and differentiation (Merritt et al., 2002). Furthermore, expressing Dsg3 in the upper spinous and granular layers using the involucrin promoter disrupted epidermal barrier function (Elias et al., 2001). The histological and ultrastructural changes observed in the *Inv-Dsg2* mice described here were significantly different from those of the Inv-mDsg3 transgenic mice (Elias et al., 2001). Shortly after birth the Inv-mDsg3 mice die of dehydration due to transepidermal water loss whereas the *Inv-Dsg2* mice described in this report survived to adulthood without any obvious loss of epidermal barrier function. The outer stratum corneum of the newborn Inv-mDsg3 transgenic skin resembled that of the oral mucosa, whereas the *Inv-Dsg2* transgenic stratum corneum displayed only minor compaction in the early transition between the granular to corneal layers. The remaining upper layers of the stratum corneum had the appearance of the normal 'basket weave' pattern although it was fragmented and unorganized upon further inspection. Thus, overexpression of either Dsg3 or Dsg2 in the differentiating epidermal strata has dramatically different consequences for epidermal barrier function. A possible compensatory mechanism to prevent this severe skin barrier phenotype may be the upregulation of CE components such as SPRRP2D and SPRRP2H, members of the small proline-rich family of proteins (Jarnik et al., 1996), which were observed to be overexpressed in *Inv-Dsg2* transgenic compared to wild-type skin (M.G.M., unpublished RT-PCR results). In addition, both early and late differentiation proteins, such as CK10 and filaggrin, respectively, were perturbed in adult *Inv-Dsg2* transgenic mice. In particular, we observed significant induction of CK14 into suprabasal layers of *Inv-Dsg2* possibly indicating a more proliferative basal-like phenotype of those cells. Thus, the aberrant and fragile phenotype of the stratum corneum in our *Inv-Dsg2* transgenic mice may be due to the high suprabasal expression of CK6 and CK14 mimicking that of the oral mucosal epithelium (Lindberg and Rheinwald, 1990).

Materials and Methods

Antibodies

Antibodies used were: Flag M2 (1:2000, Sigma); pan-cytokeratin (1:1000; Biomeda, Foster City, CA); β-actin (1:5000) and cytokeratin 14 (1:1000; Calbiochem, San Diego, CA); MP6 (1:5000); BrdU-FITC (neat), E-cadherin (1:2500), γ-catenin (1:2000) and β-catenin (1:500; BD Biosciences, San Jose, CA); PCNA (1:1000; Oncogene Science, Cambridge, MA); AP61 Dsg1-α (1:50); AP498 Dsg1-β (1:2); Ab15 Dsg1-γ (1:10,000); 4B3 Dsg3 (1:64,000) and AP904 Dsg3 (1:200); involucrin (1:1000; BabCo, Berkeley, CA); loricrin (1:1000) and filaggrin (1:2000; Covance Research Labs, Cumberland, VA); cytokeratin 6 (1:20, Novocastra, Newcastle upon Tyne, UK); cytokeratin 10 (1:500, NeoMakers, Fremont, CA); 27B2 Dsg1, 6F9 β-catenin, 23F4 desmoplakin and 11E4 plakoglobin (1:100) (Wahl et al., 1996); all cell signaling antibodies (1:1000; Cell Signaling Technology, Danvers, MA): *P*-Raf (9421), *P*-MEK1/2 (9121), *P*-MAPK (9101), *P*-p90RSK (9341), PTEN (9552), *P*-PTEN (9551) *P*-PDK1 (3061), STAT3 (9132), P-STAT3 (9131), AKT (9272), *P*-AKT (Ser 473) (9271), *P*-AKT (Thr 308) (9275), *P*-GSK-3β (9336), and Myc (9492); Bcl-X_L (1:1000; Signal Transduction Lab); NF-κB p65 (1:1000; Santa Cruz Biotech, Santa Cruz, CA); FITC- and Texas-Red (Tx-R)-conjugated secondary antibodies (1:200; Molecular Probes, Eugene, OR); HRP-conjugated secondary antibodies (1:5000; Jackson Labs, Bar Harbor, ME).

Generation of transgenic mice

Mouse *Dsg2* (mDsg2) cDNA was cloned using methods previously described (Mahoney et al., 2002a). The nucleotide primer (5′-GGC GGC CGC CTA CTT

GTC ATC GTC GTC CTT GTA GTC GGA GTA AGA ATG CTG TAC AG-3′) was used by polymerase chain reaction (PCR) to add the Flag epitope (underlined) to the 3′ end of mDsg2 cDNA. Sequence was confirmed by automated nucleotide sequencing (ABI, Foster City, CA). The mDsg2-Flag cDNA was inserted in place of the β-galactosidase gene (excised with *Not*I) in the involucrin promoter vector pH3700-pL2 (Fig. 1A). The involucrin promoter-mDsg2-Flag transgene was excised with *Sal*I and microinjected into the male pronuclei of B6C3F1 mice zygotes, which were then implanted into CD-1 foster mothers.

Genotyping of progeny was established by PCR using DNA extracted from tail clippings according to the manufacturer's protocol (PureGene kit, Gentra system, Minneapolis, MN). Primers 5′-CAC TAG CAT TCT TGA CCG G-3′ (exon 4) and 5′-GCA-TTC-AGA-GTC-TCC-GGG-T-3′ (exon 6) were used to generate a 1406 bp PCR product from genomic DNA or a 243 bp product from the *Inv-Dsg2* transgenic cDNA. PCR reactions were performed in 50 μl reaction volume containing 10 ng of mouse genomic DNA, 200 μM of each nucleotide, 0.25 μM primers, 20% (vol/vol) buffer Q, 2.5 U of Taq polymerase, and standard reaction buffer (Qiagen Inc., Valencia, CA). The PCR conditions were 95°C for 3 minutes, followed by 35 cycles of 95°C for 30 seconds, 58°C for 1 minute, and 72°C for 1 minute.

Newborn, 6-week old and adult (3 months) wild-type and transgenic littermates were used for these studies. All animals were back-crossed to C57Bl/6J five times. Some immunohistochemistry experiments were performed on animals between crosses three to five. Similar results were observed.

Histology and transmission electron microscopy

Unless otherwise indicated, all chemicals were from either Sigma (St Louis, MO) or Fisher (Waltham, MA). For histology, skin tissues were fixed at room temperature overnight in a 10% formalin solution. Tissues were then processed for paraffin embedding, sectioned (4 μm), mounted on glass slides, and stained with Hematoxylin and Eosin. For electron microscopy, skin samples were collected in 4% paraformaldehyde, 2.5% gluteraldehyde in 0.1 M sodium cacodylate pH 7.4, with 8.0 mM $CaCl_2$. After several washes in 0.1 M sodium cacodylate, pH 7.4, the tissue was post-fixed in 1% osmium tetroxide in 0.1 M sodium cacodylate, pH 7.4 for 1 hour, dehydrated through a graded ethanol series, followed by propylene oxide, infiltrated and embedded in a mixture of EMbed 812, nadic methyl anhydride, dodecenyl succinic anhydride and DMP-30 (Electron Microscopy Sciences, Hatfield, PA). Thin sections were cut using a Reichert UCT ultramicrotome and post-stained with uranyl acetate and bismuth subnitrate. Tissue sections were examined using a Tecnai 12 transmission electron microscope equipped with a Gatan Ultrascan US1000 2 K digital camera (FEI Company, Hillsboro, OR).

Tissue extraction

Mouse back skin was pulverized in liquid nitrogen in RIPA buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS and protease inhibitor (PI) cocktail (Roche Diagnostics, Indianapolis, IN) or urea lysis buffer for high molecular mass proteins such as desmoplakin [9 M urea, 1% SDS, 10% glycerol, 63 mM Tris (pH 6.8), 0.01% pyronin, and 0. 5% β-mercaptoethanol].

To extract Triton X-100-soluble and -insoluble proteins, adult wild-type and transgenic dorsal skin was snap-frozen in liquid nitrogen and processed as previously described (Vasioukhin et al., 2001). Tissues were homogenized in Triton solubilization buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride (PMSF), and PI. The Triton-insoluble pellet was solubilized in the same buffer plus 9 M urea.

Extraction of cytoplasmic and nuclear proteins was prepared as previously described (Corsini et al., 1996). Buffers used were hypotonic lysis buffer (10 mM Hepes, pH 7.9, 10 mM KCl, 10 mM EDTA, 1 mM DTT, 0.4% IGEPAL (NP-40), 1 mM PMSF, and 1× protease inhibitor mix), high salt buffer (10 mM Hepes, pH 7.9, 0.2 M NaCl, 0.5 mM EDTA, 5% glycerol, 1 mM DTT, 1 mM PMSF and PI) and urea buffer (9 M urea containing high salt buffer).

Immunoblotting and immunohistochemistry

Protein concentration was determined (Pierce BCA kit, Pierce Biotech, Rockford, IL) and immunoblotting was performed as described previously (Brennan et al., 2004) with 5-20 μg of protein in each lane resolved over 5-10% SDS-PAGE (Bio-Rad Laboratories, Hercules, CA). Signals were detected with chemiluminescence (ECL; Amersham Biosciences, Piscataway, NJ).

For immunofluorescence, OCT-fixed tissue sections (5 μm) were prepared as previously described (Mahoney et al., 2006) with DAPI (100 ng/ml) for DNA counterstaining. OCT-fixed tissues were used with the following antibodies: Flag, MP6, CK6, CK10, 23F4, 11E4 and β-catenin. For some antibodies, tissue sections (4 μm) from formalin-fixed and paraffin-embedded tissues were used as previously described (Mahoney et al., 2006). Antibodies used with this method were: AP61, AP498, Ab15, AP904, involucrin, filaggrin, K14 and bromodeoxyuridine (BrdU).

Keratinocyte proliferation in vivo

Newborn and adult wild-type and transgenic mice were injected subcutaneously in the back with BrdU at 50 mg/kg and sacrificed 1 hour later. Back skin was fixed in 10% formalin, paraffin embedded, and sections (4 μm) were subsequently co-stained with BrdU and CK14 antibodies. BrdU-labeled nuclei in the interfollicular regions were counted in each field and the total number of nuclei was determined.

Anchorage-independent survival assay

We established cultured keratinocytes from newborn mouse skin and maintained them in CnT medium (CELLnTEC, Bern, Switzerland) as previously described (Caldelari et al., 2000). Forced suspension cultures were performed as described previously (Jost et al., 2001b; Mahoney et al., 2002b). Growth factors and inhibitors of signal transduction events were used at the following concentrations: EGF, 10 ng/ml; Bay11-7082 9, Biomol, Plymouth Meeting, PA), 0.5-5 μM; and MG132 (Assay Designs, Ann Arbor, MI), 10-100 μM.

Two-step chemical-induced carcinogenesis

Adult wild-type and transgenic mice (6- to 8-weeks old) from three different litters were treated once with DMBA (7,12-dimethylbenz[a]anthracene) followed by TPA (12-*O*-tetradecanoylphorbol 13-acetate) twice weekly according to established protocols (Guo et al., 2005). Briefly, experimental mice were shaved once on the dorsal skin with an electric razor and DMBA (400 nmol in 200 μl acetone) was painted onto the exposed skin 24 hours later. One week after initiation with DMBA, TPA (17 nmol in 200 μl acetone) was applied twice weekly for up to 25 weeks. The statistical significance of differences in papilloma formation between transgenic and wild-type mice was determined with a Student's *t*-test.

Tape stripping and cornified envelope extraction

For tape stripping analysis, newborn mice were tape stripped 10 times using D-Squame disks (CuDerm Corp., Dallas, TX) as previously described (Dreher et al., 1998). Tapes were adhered onto glass slides and photographed under phase-contrast microscopy. For purification of cornified envelopes (CEs), skin biopsies (6 mm) were boiled for 20 minutes in isolation buffer (20 mM Tris-HCl, pH 7.5, 5 mM EDTA, 10 mM DTT and 2% SDS) essentially as previously described (Jarnik et al., 1996). After centrifugation (5,000 ***g***, 10 minutes), CEs were washed twice at room temperature with a wash buffer (20 mM Tris-HCl, pH 7.5, 5 mM EDTA, 10 mM DTT and 0.2% SDS). CEs were photographed and concentration determined using a hemacytometer.

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