

# Deregulated Activity of Akt in Epithelial Basal Cells Induces Spontaneous Tumors and Heightened Sensitivity to Skin Carcinogenesis

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## Abstract

**Aberrant activation of the phosphoinositide-3-kinase (PI3K)/PTEN/Akt pathway, leading to increased proliferation and decreased apoptosis, has been implicated in several human pathologies including cancer. Our previous data have shown that Akt-mediated signaling is an essential mediator in the mouse skin carcinogenesis system during both the tumor promotion and progression stages. In addition, overexpression of Akt is also able to transform keratinocytes through transcriptional and posttranscriptional processes. Here, we report the consequences of the increased expression of Akt1 (wtAkt) or constitutively active Akt1 (myrAkt) in the basal layer of stratified epithelia using the bovine keratin K5 promoter. These mice display alterations in epidermal proliferation and differentiation. In addition, transgenic mice with the highest levels of Akt expression developed spontaneous epithelial tumors in multiple organs with age. Furthermore, both wtAkt and myrAkt transgenic lines displayed heightened sensitivity to the epidermal proliferative effects of the tumor promoter 12-O-tetradecanoylphorbol-13-acetate (TPA) and heightened sensitivity to two-stage skin carcinogenesis. Finally, enhanced susceptibility to two-stage carcinogenesis correlated with a more sustained proliferative response following treatment with TPA as well as sustained alterations in Akt downstream signaling pathways and elevations in cell cycle regulatory proteins. Collectively, the data provide direct support for an important role for Akt signaling in epithelial carcinogenesis *in vivo*, especially during the tumor promotion stage. [Cancer Res 2007;67(22):10879–88]**

## Introduction

Akt is a 60-kDa serine/threonine kinase capable of modulating numerous processes in the cell, including cell survival and

apoptosis, proliferation, cell cycle progression, glucose metabolism, and protein translation through numerous downstream signaling proteins (1). Well-characterized substrates of Akt include anti-apoptotic proteins, such as Foxo, BAD, and IKK- $\beta$ ; cell cycle regulators, such as p27kip1, p21cip1, MDM2, and Myt1; and GSK-3, which is involved in a variety of processes (1). There are three Akt isoforms in mammals (Akt1, Akt2, and Akt3). They all share common structural features with three functionally distinct regions: an NH<sub>2</sub>-terminal pleckstrin homology domain, a catalytic domain in the center, and a COOH-terminal hydrophobic motif (2).

Data generated over the past decade have shown that the activation of Akt kinases is frequent in a wide number of human solid tumors and hematologic malignancies (reviewed in ref. 3). In addition, several mouse models have contributed to our understanding that aberrant Akt signaling plays a predominant role in malignant transformation *in vivo*, either alone or in cooperation with other genetic alterations (4–6). We have shown that Akt is a key molecule in insulin-like growth factor-I (IGF-I)-mediated mouse skin tumor promotion (7). In addition, diverse tumor promoters have been shown to activate epidermal Akt following topical treatment through activation of the EGFr (8). Furthermore, we have shown that Akt exerts tumor-specific effects in response to two-stage carcinogenesis protocols by modulating proliferation and apoptosis (9), and Akt also influences the tumor-stroma relationship by enhancing angiogenesis (10). Recently, using cultured cell systems, we provided evidence indicating that Akt may function differently in epidermal tumors than in other tissues through transcriptional and posttranscriptional mechanisms, which have several parallels with human head and neck squamous cell carcinomas (SCC; ref. 11).

To further explore the role of Akt in skin, we generated transgenic mice that express either a wild-type form of *Akt1* (wtAkt), or a form that is permanently activated by means of a myristoylation sequence (myrAkt), directed to the basal layer of the stratified epithelia using the *bovine K5* (*BK5*) promoter. We show here that deregulated expression of Akt and, concomitantly, increased Akt activity lead to the development of spontaneous tumors in multiple tissues of founders or lines with the highest expression levels. In addition, expression of either wtAkt or myrAkt in epidermal basal cells dramatically enhances susceptibility to two-stage skin carcinogenesis. Collectively, the data show that deregulated expression of Akt and the accompanying alterations in signaling pathways and gene expression can lead to spontaneous tumor development and an enhanced response to chemical carcinogenesis in the skin.

**Note:** Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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## Materials and Methods

**Transgenic mouse production and maintenance.** Wild-type mouse *Akt* cDNA (obtained from Dr. A. Bellacosa, Fox Chase Cancer Center, Philadelphia, PA) or *myrAkt* (obtained from Dr. S. Gutkind, National Institute of Dental and Craniofacial Research, NIH, Bethesda, MD) was inserted into a 5.2-kb fragment of the *BK5* promoter at a *Sna*BI site between the rabbit  $\beta$ -globin intron and polyadenylation sequences from a vector previously described (12). Orientation and integrity of the inserts were confirmed by restriction digests, and purified fragments were used for microinjection. Transgenic mice were generated by microinjection of *BK5.myrAkt* into a (C57BL/6  $\times$  DBA/2) F2 genetic background or by microinjection of *BK5.wtAkt* into the pronucleus of FVB embryos. Genomic DNA was isolated from tail clips, and the presence of the transgene was analyzed by Southern blots or PCR using primers specific for  $\beta$ -globin as previously described (13). Mice were housed in a 12-h/12-h light/dark cycle at 24°C and given standard mouse chow and water ad libitum. In some cases, alterations in oral cavity or teeth necessitated supplementation with a nutritional gel.

**Histologic and immunofluorescence analyses.** For histologic analyses, tissue samples were fixed in formalin and embedded in paraffin before sectioning. Sections of 5  $\mu$ m were stained with H&E. Epidermal thickness was assessed as previously described (14). To analyze epidermal labeling index, mice received an i.p. injection of bromodeoxyuridine (BrdUrd) in PBS (100  $\mu$ g/g body weight) 1 h before sacrifice, and sections were stained using an anti-BrdUrd antibody as previously described (14). Epidermal proliferation was assessed by calculating the percentage of cells in the epidermal basal layer positive for incorporation of BrdUrd. For analysis of expression of activated Akt, the slides were microwaved for 10 min after deparaffinization to enhance staining. Sections were then incubated with 5% horse serum for 45 min and washed thrice with sterile PBS (pH, 7.5) before incubation with a 1/100 dilution of primary rabbit anti-Ser<sup>473</sup> phosphorylated Akt (Cell Signaling Technology Inc.) in bovine serum albumin (BSA)/PBS. Standard protocols were used on deparaffinized sections of samples that had been fixed in either ethanol (70%) or formalin for the antibody against the hemagglutinin (HA) epitope (monoclonal antibody 1/500 dilution, Covance Research Products, Inc.) and for the Texas-Red- and FITC-conjugated secondary antibodies (Jackson Immuno Research Laboratories, Inc.), which were used at dilutions of 1/500 and 1/50, respectively. Control slides were obtained by replacing primary antibodies with PBS (data not shown).

**Western blot analysis.** Primary keratinocytes were prepared from skin excised from newborn mice as described previously (13). Epidermal extracts were prepared as previously described (7) and lysed in 20 mmol/L Tris-HCl (pH, 7.5), 137 mmol/L NaCl, 10% glycerol, 1% Triton X-100, 1 mmol/L phenylmethylsulfonyl fluoride, 20 mmol/L NaF, 1 mg/mL aprotinin and leupeptin, 1 mmol/L disodium PPI, and 1 mmol/L Na<sub>3</sub>VO<sub>4</sub>. To examine alterations in protein status or expression in primary keratinocytes and whole skin, total protein (25  $\mu$ g) was used for SDS-PAGE, transferred to nitrocellulose (Amersham Biosciences), and probed with primary antibodies against Akt1/2, cyclins E, D1, D3, and A, cyclin-dependent kinase 4 (cdk4; Santa Cruz Biotechnology) and phosphorylated Akt (Ser<sup>473</sup>; Cell Signaling Technology Inc).  $\alpha$ -Tubulin (Sigma-Aldrich) was used to normalize the loading. For the experiments in which protein lysates were prepared from epidermis, the following primary antibodies were used for analysis: Akt, p-Akt (Ser<sup>473</sup>), p-GSK3- $\beta$ , p-BAD (Ser<sup>155</sup>), p-Foxo3a (Ser<sup>253</sup>), p-mTOR (Ser<sup>2448</sup>), p-eIF4G (Ser<sup>1108</sup>), p-eIF4E (Ser<sup>209</sup>), p-p70S6K (T389), cyclin D1, and c-myc (Cell Signaling Technology Inc.);  $\beta$ -catenin and Ras (BD Transduction Laboratories); E2F1 and E2F4 (Santa Cruz Biotechnology, Inc.); MDM2 (BD Biosciences); and  $\beta$ -actin (Sigma-Aldrich).  $\beta$ -Actin or Ras was used to normalize the amount of protein. Chemiluminescence was done according to the manufacturer's recommendations (Pierce Biotechnology). Quantification was done using Quantity One software.

**Responsiveness to 12-O-tetradecanoylphorbol-13-acetate.** Akt transgenic or nontransgenic mice (3 per group, 7–9 weeks of age) received topical applications of either 3.4 or 6.8 nmol 12-O-tetradecanoylphorbol-13-acetate (TPA; LC Laboratories) or acetone (0.2 mL) twice a week for 2 weeks. Animals were sacrificed 48 h after the last treatment. Only the

higher dose of 6.8 nmol TPA was used for the experiments using the *BK5.myrAkt*<sup>L60</sup> mice. To determine the time course of epidermal proliferation, mice received a single topical treatment of 6.8 nmol TPA and were sacrificed at the indicated time points. All groups received i.p. injections of BrdUrd 1 h before sacrifice, and epidermal thickness and proliferation were determined as described above.

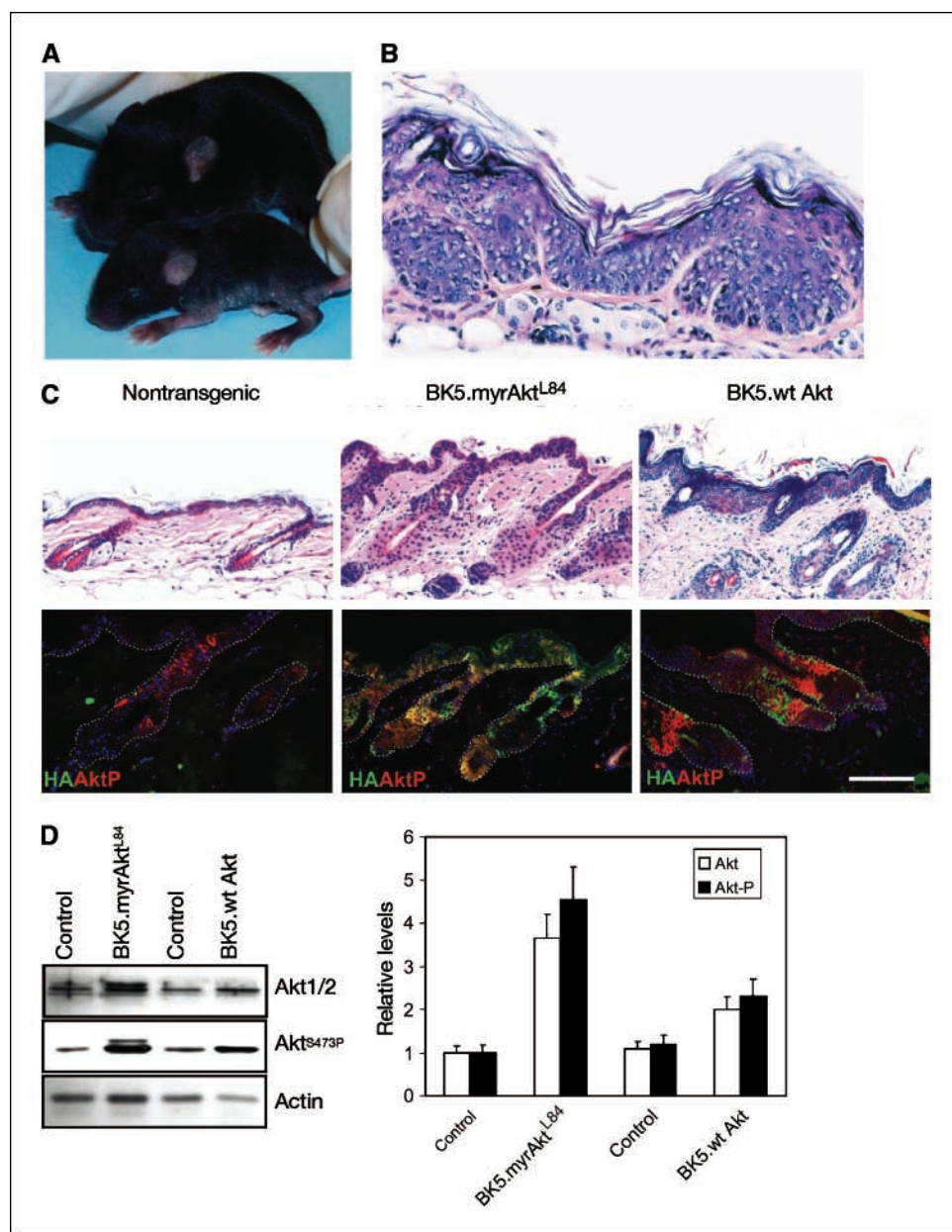
**Two-stage skin carcinogenesis.** At least 15 mice were used per experimental group [either *BK5.wtAkt* (FVB/N background) or *BK5.myrAkt* (C57BL/6 background)]. All solutions of 7,12-dimethylbenz(a)anthracene (DMBA, Sigma-Aldrich) and TPA (LC Laboratories) were prepared in reagent-grade acetone and applied topically in a volume of 0.2 mL. Doses of DMBA and TPA used were based on the responsiveness of the background strain to the two-stage carcinogenesis regimen. Initiation was accomplished by a single application of either 25 nmol (*BK5.wtAkt*) or 100 nmol (*BK5.myrAkt*) DMBA. Two weeks after initiation, groups of mice were subjected to promotion with either twice-weekly applications of 5 nmol (*BK5.wtAkt*) or thrice-weekly applications of 6.8 nmol TPA (*BK5.myrAkt*). Promotion was continued in all groups until the number of tumors reached a plateau or unless otherwise noted. The average number of papillomas per mouse and papilloma incidence was tabulated weekly as well as carcinoma incidence. When carcinoma-bearing mice were moribund, they were killed, and tumors were removed for histologic verification. The carcinoma incidence and the average number of carcinomas per mouse are expressed as a function of the mice at risk at the time of appearance of the first carcinoma and have been adjusted for non-carcinoma-related mortality as described (15, 16).

**Statistical analysis.** Differences in tumor multiplicity between each treatment group were evaluated using the Mann-Whitney *U* test, and differences in tumor incidence between each group were evaluated by  $\chi^2$  analysis. Significance was set at a *P* value of 0.05 or less.

## Results

**Generation and epidermal characteristics of Akt transgenic mice.** The expression of *wtAkt* or *myrAkt* was targeted to the basal layer of the mouse-stratified epithelia as noted above. Injection of either transgene construct yielded a reduced number of transgenic mice, and those generated had few copies of the transgene. Of the several founders obtained, those bearing the highest copies of transgene displayed progressive weakening and had to be sacrificed, precluding the establishment of lines from these founders. These results indicate that expression of high levels of either *wtAkt* or *myrAkt* in the basal layer of stratified epithelia is deleterious in mice. Consequently, only three lines were established, two expressing *myrAkt* (*BK5.myrAkt*<sup>L60</sup> and *BK5.myrAkt*<sup>L84</sup>) and one expressing the *wtAkt* (*BK5.wtAkt*) transgene. Further analyses were focused primarily on these three lines and the original founders.

The phenotype of the various founders was characterized by reduced size, and in some cases, reduced vigor, scaly (hyperkeratotic) skin, and a sparse hair coat (see Fig. 1A). The epidermal phenotype varied from mild hair follicle hyperplasia (*BK5.myr*<sup>L60</sup>, and 2 *myrAkt* founders; data not shown) to generalized hyperplasia of the interfollicular epidermis as well as marked follicular hyperplasia and enlargement of sebaceous glands in the *BK5.myrAkt*<sup>L84</sup> and *BK5.wtAkt* lines (Fig. 1C) and in three additional founders, two of which expressed the *myrAkt* transgene and one which expressed the *wtAkt* transgene (data not shown). Immunohistochemical analysis of the epidermis revealed the expansion of the cell layers expressing K5 and K10 (Supplementary Fig. S1A) and the presence of cells coexpressing both K5 and K10 (Supplementary Fig. S1D, left, arrows). In addition, we also observed that the hair follicle hyperplasia was associated with expansion of K5-expressing cells, and interestingly, expression of K10 was observed in scattered cells (Supplementary Fig. S1D, right, arrows), indicating



**Figure 1.** Gross epidermal phenotype of Akt transgenic mice. *A*, nontransgenic littermate and an Akt transgenic mouse (runted) at 10 d of age. Small size and apparent hyperkeratosis in the skin is evident. *B*, histologic analyses of ear epidermis revealed an area displaying *in situ* carcinoma. *C*, top, histology of the adult epidermis of two Akt transgenic lines (BK5.myrAkt<sup>L84</sup>, BK5.wtAkt) compared with nontransgenic (control) mice. Bottom, immunofluorescence analysis of transgene expression using antibodies against the HA tag (HA, green), and phosphorylated Akt (Ser<sup>473</sup>, AktP, red). Dashed lines, epidermal-dermal border. *D*, Western blot analysis of whole skin extracts showing the expression of the noted proteins in the different genotypes and normalized quantification of Akt and phosphorylated Akt expression from Western blot analysis. Bars, 100  $\mu$ m.

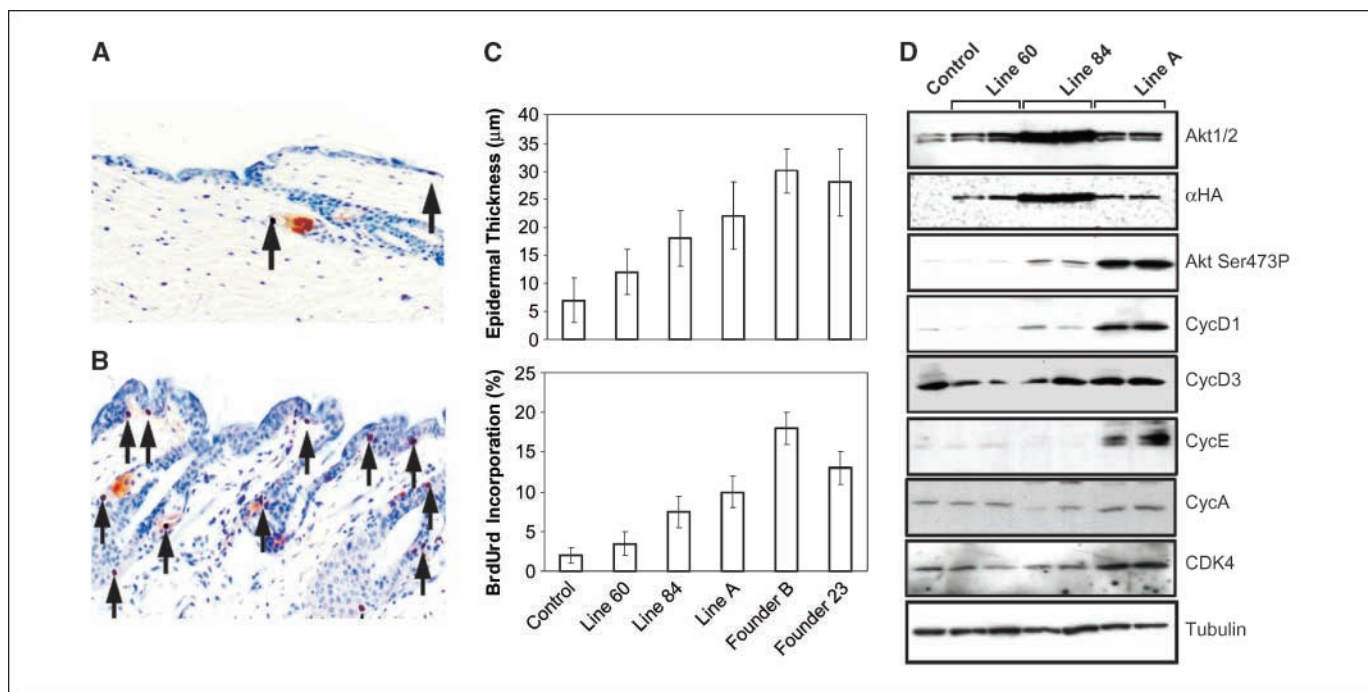
that although these cells were localized in the hair follicle, they had entered into an interfollicular epidermal differentiation program. We also observed increased expression of K6 in the hair follicles in accordance with the observed hyperplasia of these structures. In addition, patches of cells displaying K6 and K10 coexpression could be found throughout the hyperplastic interfollicular epidermis (Supplemental Fig. S1D, center; arrows). Finally, the expression of two markers of terminal differentiation, filaggrin (Supplementary Fig. S1C) and loricrin (data not shown), was reduced in transgenic mice.

As the mice aged, we also noticed the enlargement of the ears, with clear signs of hyperkeratosis and sparse and matted hair. Histologic analysis confirmed these observations and also showed areas of *in situ* carcinoma (Fig. 1B). Notably, many of these characteristics were similar to *HK1.IGF-1* and *BK5.IGF-1* transgenic mice (17, 18). We analyzed the expression of the transgene and Akt activity in the epidermis of transgenic mice using antibodies

specific for the HA tag and phosphorylated Akt (Ser<sup>473</sup>). Expression correlated with the epidermal phenotype and varied from generalized in the outer root sheath of hair follicles and the vast majority of basal cells (*BK5.myrAkt<sup>L84</sup>*, Fig. 1C) to scattered and/or limited in basal keratinocytes and hair follicles (*BK5.wtAkt*, Fig. 1C, and *BK5.myrAkt<sup>L60</sup>*, data not shown). These data were confirmed by Western blot analysis using whole skin extracts (Fig. 1D). Stronger expression was also observed in founders with a more dramatic phenotype (data not shown).

The results indicated that increased activity of Akt in epidermis was associated with hyperplasia of the epidermis and was most likely due to increased proliferation. To analyze this aspect, we monitored BrdUrd incorporation (Fig. 2A–C). We found in every case an increase in proliferation in epidermis of transgenic mice compared with nontransgenic control animals. Increased epidermal proliferation was associated with epidermal hyperplasia quantified as epidermal thickness (ref. 14; Fig. 2C). Notably, we





**Figure 2.** Proliferation in epidermis and keratinocytes of *Akt* transgenic mice. Epidermal incorporation of BrdUrd as determined by staining with an anti-BrdUrd antibody (arrows) in control (A) and *BK5.myrAkt<sup>L84</sup>* (B) mice illustrating the increase in proliferation in response to increased expression of Akt. C, quantitative analysis of epidermal thickness showing the hyperplasia of the various *Akt* transgenic lines and quantitative analyses of epidermal proliferation obtained from BrdUrd incorporation. Columns, mean data derived from the analysis of three different sections; bars, SD. D, Western blot analysis of cultured primary keratinocytes showing the expression of the noted proteins in the different *Akt* transgenic lines relative to control levels (Line 60, Line 84, and Founder 23, expressed a *myrAkt* transgene; Line A and Founder B, expressed a *wtAkt* transgene). An anti-HA antibody was used to detect the presence of the transgene.  $\alpha$ -Tubulin was used to normalize protein loading. Bar, 100  $\mu$ m.

did not observe alterations in epidermal apoptotic index, probably due to the fact that this process is very infrequent in epidermis without inducing treatments (data not shown).

To study the possible molecular mechanisms responsible for the phenotypic alterations observed in the skin of *Akt* transgenic mice, we examined transgene expression together with the expression of several cell cycle regulatory molecules in pooled samples of primary cultured keratinocytes derived from each of the three *Akt* transgenic lines compared with nontransgenic control samples. Protein lysates from primary keratinocytes derived from transgenic mice showed an increase in Akt expression and in phosphorylated (Ser<sup>473</sup>) Akt (Fig. 2D). Although *BK5.myrAkt<sup>L84</sup>* had the highest level of transgene expression, interestingly, the highest Akt activity was found in *BK5.wtAkt* keratinocytes, contrary to the data with whole skin extracts. This would indicate that *wtAkt* is more susceptible to activation by the primary culture conditions, such as growth factors and/or serum, whereas *myrAkt* is not significantly affected by these conditions. The increased Akt activity was associated with increased cyclin D1, cyclin E, and *cdk4* expression with the most marked alterations observed in the *BK5.wtAkt* line. No significant alterations were observed in cyclin D3 or cyclin A protein levels.

**Sporadic tumors in epithelial organs in transgenic mice.** As noted above, lines could not be established from several founders due to sterility and/or inability to transmit the transgene. Progressive weakening was observed in many of these founders with age. Upon necropsy, multiple sporadic tumors were discovered in sections of various tissues, likely contributing to the rapid deterioration of the health of these animals. A detailed description and histologic analysis is given in Supplementary Table S1 and Supplementary Fig. S2. In the male reproductive tract, seminal

vesicle adenoma (data not shown) and prostate intraepithelial neoplasia were observed (Supplementary Fig. S2). In the female reproductive tract, sporadic SCCs of the vagina and cervix and adenocarcinomas and ductal carcinomas (data not shown) in the mammary gland were observed (Supplementary Fig. S2). In addition, a number of other tumors were observed, including preputial gland tumors (data not shown), SCCs of the salivary glands and urinary bladder, cholangiocarcinoma, sebaceous adenoma and trichoepithelioma of the skin, and papillomas of the forestomach (Supplementary Fig. S2). Of note, trichoepitheliomas and *in situ* SCCs were also observed in the oral cavity and lips of *BK5.myrAkt<sup>L84</sup>* mice (data not shown). A more detailed description of the oral cavity phenotype in these mice will be presented elsewhere.

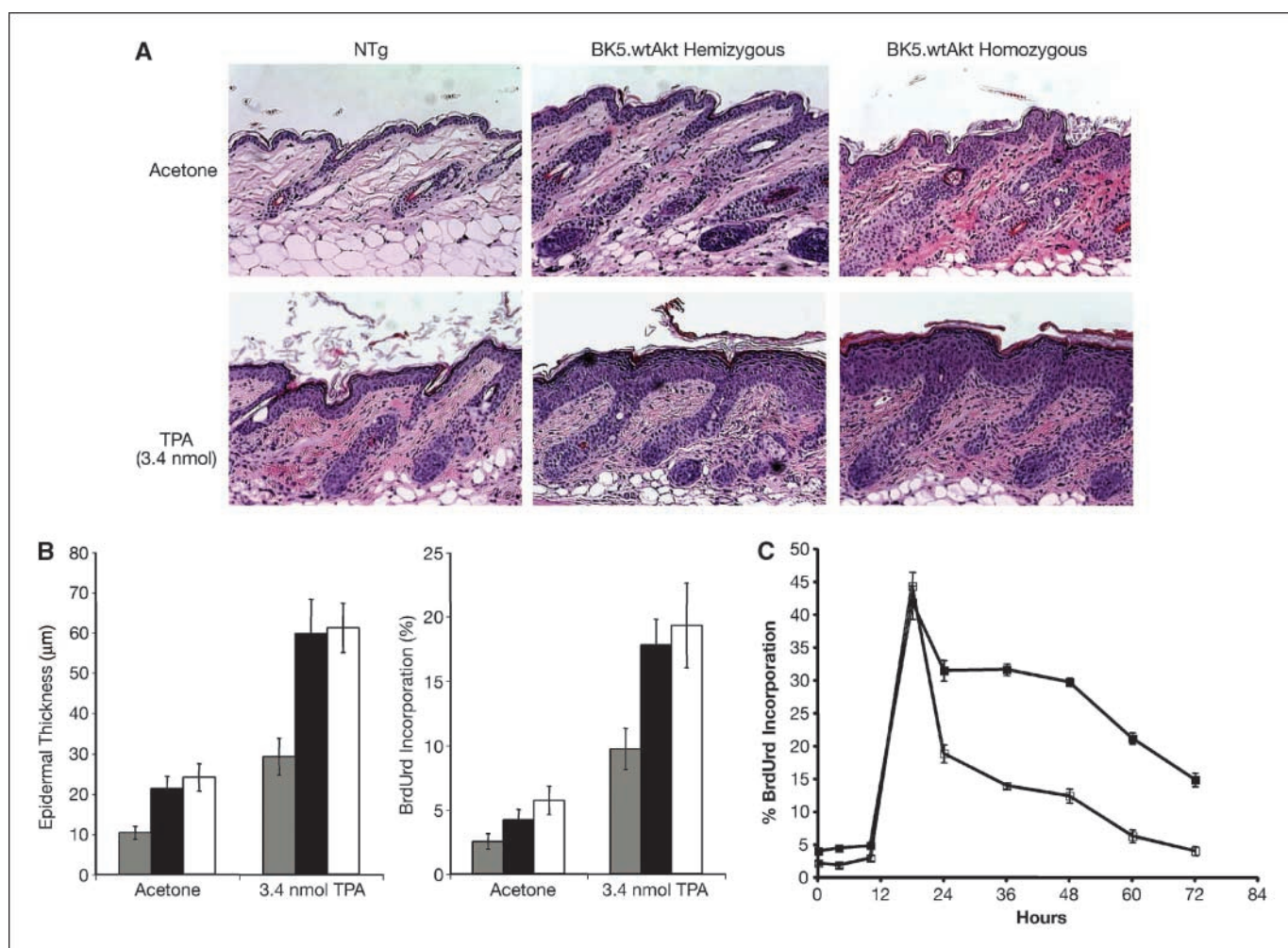
**Analysis of responsiveness to TPA in *BK5.wtAkt* and *BK5.myrAkt<sup>L60</sup>* mice.** The data described above suggested that *Akt* transgenic mice may be more susceptible to tumor promotion and two-stage skin carcinogenesis. To explore this hypothesis, we examined the sensitivity of the *BK5.wtAkt* mice and *BK5.myrAkt<sup>L60</sup>* mice to TPA-induced epidermal hyperproliferation. These lines were chosen because they had the mildest skin phenotype and lowest incidence of spontaneous tumors. Although similar results were observed in both lines of mice, the data shown in Fig. 3 were generated in the *BK5.wtAkt* mice. Figure 3A shows representative H&E-stained sections of the skin 48 h after the last of four treatments of either acetone or TPA (3.4 nmol). Both hemizygous and homozygous *BK5.wtAkt* mice showed significantly ( $P < 0.05$ ) greater (~2-fold) epidermal thickness and labeling index (LI; Fig. 3B) compared with nontransgenic littermates. Similar results were obtained using a higher (6.8 nmol) dose of TPA.

To further evaluate the responsiveness of *BK5.wtAkt* mice to TPA-induced epidermal hyperproliferation, we compared their response to that of nontransgenic mice following a single application of 6.8 nmol TPA over a 72-h time course. As shown in Fig. 3C, TPA treatment led to an increase in epidermal LI with a peak of ~40% at 18 h in both nontransgenic and transgenic mice. In nontransgenic mice, the epidermal LI then declined rapidly over the next 48 h and returned to control levels at 72 h after treatment. In contrast, the epidermal LI in *BK5.wtAkt* mice remained significantly elevated for at least 48 h after reaching the peak. Thus, Akt transgenic mice were more sensitive to the effects of TPA, exhibiting sustained epidermal proliferation and indicating that they may be more sensitive to a two-stage skin carcinogenesis regimen.

**Two-stage skin carcinogenesis experiments.** Two-stage skin carcinogenesis experiments were done in groups of *BK5.wtAkt* as well as *BK5.myrAkt<sup>L60</sup>* mice. As shown in Fig. 4A, *BK5.wtAkt* transgenic mice were significantly more sensitive to two-stage skin carcinogenesis using the DMBA/TPA protocol. In this regard, *BK5.wtAkt* transgenic mice developed 50.4 papillomas per mouse

(100% incidence), whereas the nontransgenic mice had 10.85 papillomas per mouse (100% incidence). Interestingly, the papillomas arising in *BK5.wtAkt* transgenic mice developed very rapidly and were considerably larger than those produced in nontransgenic mice. In addition, *BK5.wtAkt* transgenic mice treated with the DMBA/TPA regimen had to be sacrificed at 11 weeks due to the very high tumor burden. At this time, there were already a significant number of grossly observable SCCs on these mice. Histologically, we confirmed the presence of SCC in 13/17 (76%) transgenic mice treated with DMBA/TPA. Only 1/13 (7.7%) nontransgenic mice initiated with DMBA and promoted with TPA for 20 weeks possessed SCCs. These latter data suggest that overexpression of Akt may also enhance the progression of papillomas to SCCs. These data are summarized in Table 1.

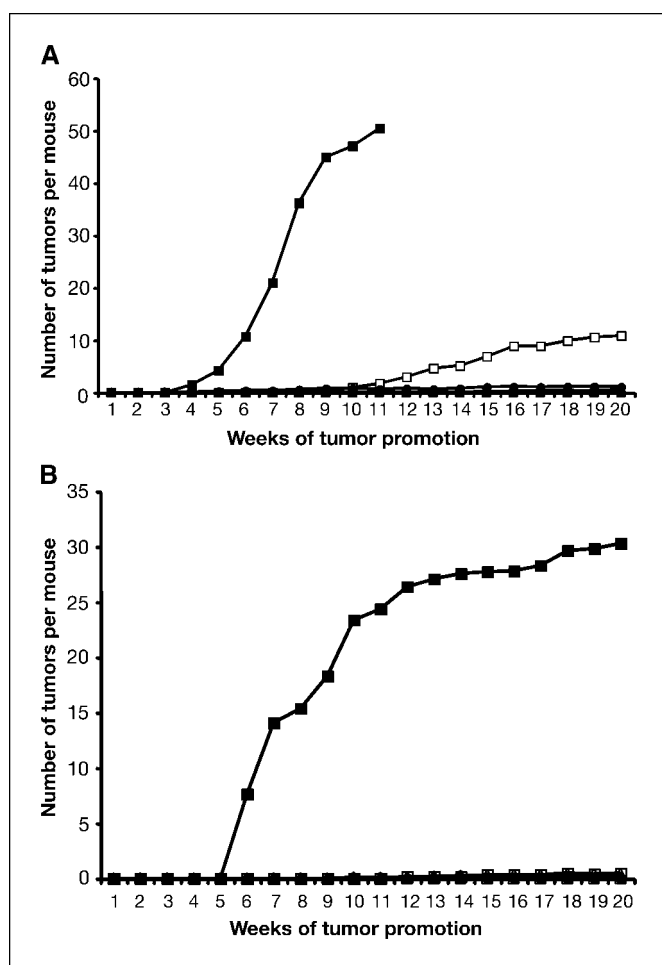
Analysis of the data in Table 1 shows that 20% of the *BK5.wtAkt* mice treated with only DMBA developed papillomas. In addition, 57% of the *BK5.wtAkt* mice treated only with TPA developed papillomas. These data are quite similar to data obtained in IGF-I transgenic mice (17) as well as in transforming growth factor- $\alpha$  (TGF $\alpha$ ) transgenic mice (19, 20). Development of skin tumors in



**Figure 3.** Response to TPA-induced epidermal proliferation in Akt transgenic mice. Hemizygous or homozygous *BK5.wtAkt* mice and nontransgenic (*NTg*) littermates 7 to 9 wk of age were treated with either acetone or 3.4 nmol TPA twice a week for 2 wk ( $n = 3$  per group). A, H&E staining of dorsal skin sections. B, quantitative analysis of proliferation by measurement of epidermal thickness and incorporation of BrdUrd (% positive cells). Gray bars, *NTg*; black bars, hemizygous *BK5.wtAkt*; white bars, homozygous *BK5.wtAkt*. C, time course of epidermal proliferation following TPA treatment. Groups of hemizygous *BK5.wtAkt* (■) and nontransgenic (□) mice were treated with a single application of 6.8 nmol TPA and sacrificed at various time intervals thereafter. Percent of BrdUrd incorporation was calculated as the number of BrdUrd-positive cells in the basal layer of the epidermis relative to the total number of cells. Bars, SE.

mice initiated with DMBA suggests that elevated Akt activity possesses tumor-promoting activity in skin keratinocytes. Conversely, the development of skin papillomas in BK5.wtAkt mice treated only with TPA suggests that elevated Akt activity may have promoted the growth of previously initiated cells residing in the epidermis that were further promoted by TPA. The development of small *in situ* skin tumors in BK5.myrAkt<sup>L84</sup> mice supports this hypothesis. Conversely, elevated activity in keratinocytes may confer phenotypic properties on keratinocytes similar to initiated cells.

The BK5.myrAkt<sup>L60</sup> transgene is on a predominantly C57BL/6 genetic background. C57BL/6 mice are relatively resistant to two-stage carcinogenesis using TPA as the promoter (14, 21). Therefore, a regimen comprised of a higher dose of DMBA (100 nmol) and more frequent application of TPA (thrice per week) was used. As shown in Fig. 4B, the DMBA/TPA-treated wild-type (C57BL/6 mice) mice developed only 0.46 papillomas per mouse (23% incidence),



**Figure 4.** Response of BK5.wtAkt and BK5.myrAkt mice to two-stage carcinogenesis. **A**, BK5.wtAkt (Tg) and nontransgenic (NTg) littermates were initiated with either acetone or 25 nmol DMBA and promoted with either acetone or 5 nmol of TPA twice a week for 20 wk. ■, Tg, DMBA/TPA; □, NTg DMBA/TPA; ●, Tg TPA only; ○, NTg TPA only; ▲, Tg DMBA only; △, NTg DMBA only; -+-, Tg acetone only; +, NTg acetone only. **B**, BK5.myrAkt (Tg) and nontransgenic (NTg) littermates were initiated with 100 nmol DMBA and promoted with 6.8 nmol TPA thrice per week for 20 wk. ■, Tg DMBA/TPA; □, NTg DMBA/TPA; ●, Tg TPA only; ○, NTg TPA only; ▲, Tg DMBA only; △, NTg DMBA only. At least 15 mice were used for each group for both experiments. Differences in the number of tumors per mouse were analyzed using the Mann-Whitney *U* test.

whereas the BK5.myrAkt<sup>L60</sup> mice under the same regimen developed ~30 papillomas per mouse (100% incidence) after 20 weeks of promotion. These values are also highly significant when compared with the nontransgenic control mice. These data indicate that overexpression of constitutively active Akt in epidermis can overcome the high resistance to skin tumor promotion associated with the C57BL/6 genetic background. Furthermore, the BK5.myrAkt<sup>L60</sup> transgenic mice treated with DMBA alone also had a tumor incidence of 25% and a multiplicity of 0.33 papillomas per mouse. Similar to results with the BK5.wtAkt mice, this suggests that overexpression of Akt acts as a skin tumor promoter. The data from this experiment are also summarized in Table 1. Note that no SCCs were observed in BK5.myrAkt<sup>L60</sup> mice at the time the experiment was terminated (20 weeks).

**Altered cell cycle and signaling molecules in the epidermis of Akt transgenic mice.** The increased proliferation in the epidermis of Akt transgenic mice as well as the hyperproliferative response of the Akt transgenic mice following treatment with TPA led us to investigate the expression and status of cell cycle regulatory proteins as well as downstream targets of the Akt signaling pathway. Groups of transgenic mice and nontransgenic littermates were treated with a single topical dose of either TPA or acetone on the dorsal skin and then sacrificed at various time points up to 60 h. Similar results were obtained in both BK5.wtAkt (Fig. 5) and BK5.myrAkt<sup>L60</sup> mice (data not shown). As shown in Fig. 5A, levels of Akt, phospho-Akt, and downstream signaling components, including cell cycle proteins, were examined in epidermal lysates by Western blotting. In the epidermis of nontransgenic mice, Akt phosphorylation was induced at 4 h post-treatment with TPA, but returned to basal levels at ~24 h. There was an apparent increase in the phosphorylation of GSK3- $\beta$  that was sustained over the time course until a return to basal levels by 60 h. We also observed increases in phosphorylated mTOR (Ser<sup>2448</sup>) and its downstream targets, p70S6K and eIF4E. In addition, significant elevations were observed in p-Foxo3a, p-Bad, and p-eIF4G. These data are consistent with our recent data showing that diverse tumor promoters activated epidermal Akt and downstream signaling pathways following a single topical treatment (8). In the epidermis of BK5.wtAkt transgenic mice, many of these proteins were elevated or had elevated phosphorylation (activation) even in the absence of TPA treatment compared with nontransgenic mice. In particular, p-GSK3- $\beta$ , p-Bad, p-Foxo3a, p-mTOR, p-eIF4G, and p-p70S6K were all constitutively elevated in the epidermis of Akt transgenic mice.  $\beta$ -Catenin levels were also constitutively elevated. Following TPA treatment, the activation of Akt and its downstream effectors was significantly elevated above that seen in nontransgenic mice (see Fig. 5A).

The expression of a number of cell cycle regulatory proteins was also examined following treatment with TPA (Fig. 5B). In nontransgenic mice, the levels of cyclin D1, cyclin E, E2F1, and c-myc in the epidermis initially dropped at 4 h and then returned to levels at or above those seen in untreated mice between 10 and 18 h in response to TPA. These data are consistent with Rodriguez-Puebla et al. (22). In contrast, expression of cell cycle related proteins, particularly cyclin D1, cyclin E, E2F1, and E2F4 was constitutively elevated in the epidermis of Akt transgenic mice treated with acetone only (see Fig. 5B). Following TPA treatment, the same initial decreases in levels of cyclin D1, cyclin E, E2F1, and c-myc observed in the nontransgenic mice were also observed in the BK5.wtAkt mice. This initial decrease was again followed by a return to or sustained increase over basal levels. Another



**Table 1.** Summary of results from two-stage carcinogenesis in Akt transgenic mice

Mouse	Group	Incidence (%)	Papillomas per mouse	SCC incidence
Experiment 1				
FVB/N	DMBA/TPA	100	10.85 ± 1.9*	7.7% (1/13) at 20 wk
	DMBA/acetone	0	0	0
	Acetone/TPA	0	0	0
	Acetone/acetone	0	0	0
<i>BK5.wtAkt</i> (Line A)	DMBA/TPA	100	50.4 ± 4.2 <sup>†</sup>	76% (13/17) at 11 wk
	DMBA/acetone	20	0.6 ± 0.38 <sup>‡</sup>	0
	Acetone/TPA	57	1.14 ± 0.42 <sup>‡</sup>	0
	Acetone/acetone	0	0	0
Experiment 2				
C57BL/6	DMBA/TPA	23	0.46 ± 0.97	ND <sup>§</sup>
	DMBA/acetone	0	0	ND <sup>§</sup>
	Acetone/TPA	0	0	ND <sup>§</sup>
<i>BK5.myrAkt</i> (Line 60)	DMBA/TPA	100	30.27 ± 8.61 <sup>  </sup>	ND <sup>§</sup>
	DMBA/acetone	25	0.33 ± 0.65	ND <sup>§</sup>
	Acetone/TPA	0	0	ND <sup>§</sup>

\*Statistically significant when compared with all treatment groups in nontransgenic mice.  
<sup>†</sup>Statistically significant when compared with all groups examined.  
<sup>‡</sup>Statistically significant when compared with Acetone/acetone control group.  
<sup>§</sup>ND, none detected at termination of experiment.  
<sup>||</sup>Significant when compared with all groups.

interesting observation was that Mdm-2 protein levels were elevated in the epidermis of *BK5.wtAkt* mice compared with nontransgenic mice (see Fig. 5C). Mdm-2 levels remained elevated in the epidermis of Akt transgenic mice after TPA treatment, although there did not seem to be any significant changes related to treatment.

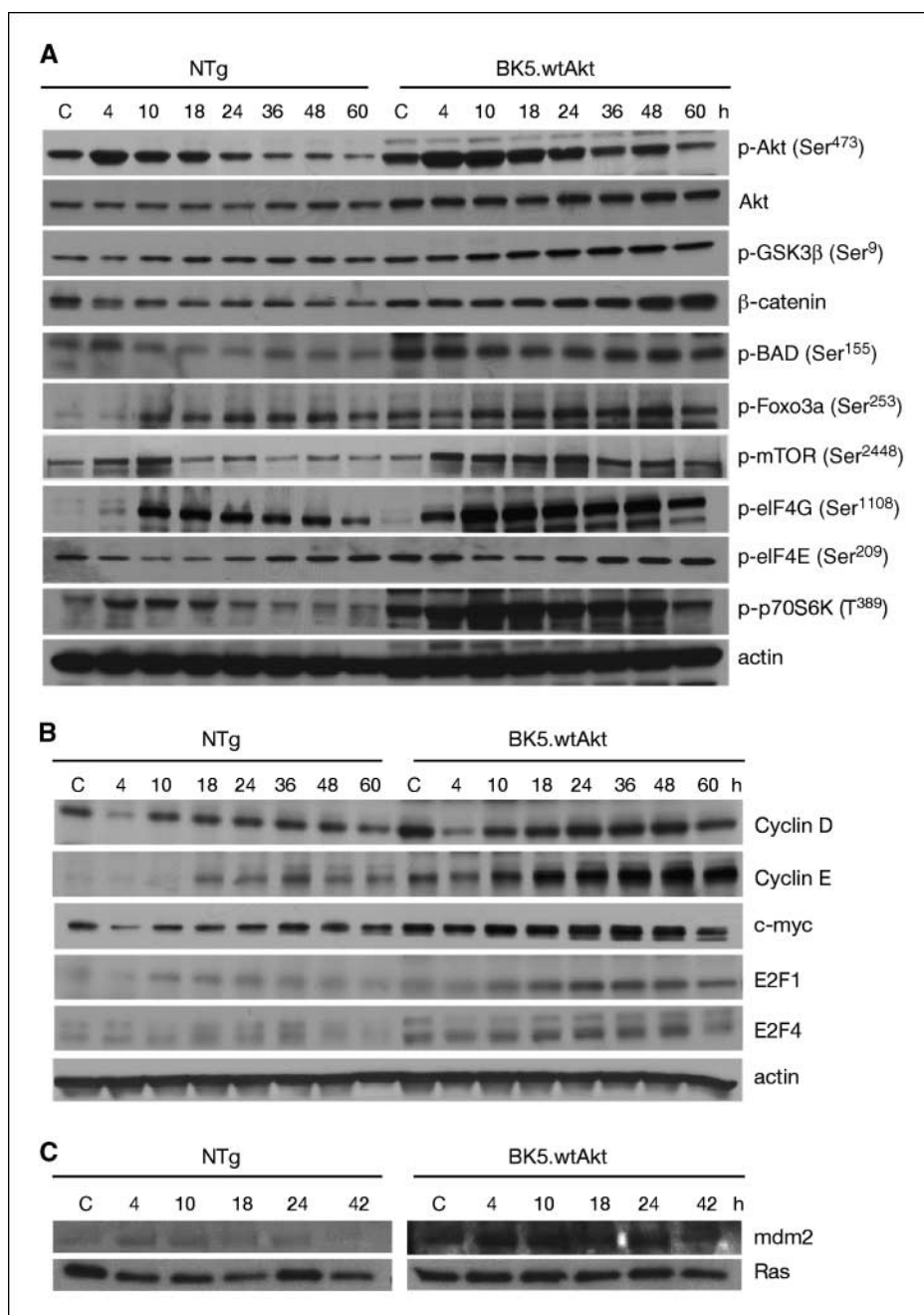
We also did a second set of experiments in which hemizygous *BK5.wtAkt* transgenic mice and age-matched nontransgenic littermates were treated topically with TPA twice a week for 2 weeks. Again, levels of Akt and downstream signaling components were determined by Western blotting (data not shown). In general, the overall trends were similar to those seen after a single application of TPA. Collectively, these data indicate that the enhanced susceptibility to two-stage carcinogenesis and sustained proliferative response following treatment with TPA correlated with sustained alterations in Akt downstream signaling pathways and elevated levels of cell cycle regulatory proteins.

## Discussion

In the present study, we have shown that deregulation of Akt activity mediated through increased expression of either the wild-type form (wtAkt) or an activated form of Akt (myrAkt) in the epidermis of transgenic mice leads to an increase in epidermal proliferation, which led to alterations in epidermal differentiation and hyperplasia. These effects of Akt correlated with changes in cell cycle regulatory proteins, especially those involved in G<sub>1</sub> to S phase transition, as well as altered signaling downstream of Akt. In addition, deregulated Akt activity dramatically enhanced susceptibility to two-stage skin carcinogenesis using a DMBA-TPA protocol, which correlated with increased susceptibility to TPA-induced epidermal hyperproliferation. Finally, in founders and lines (both wtAkt and myrAkt) with the highest expression levels,

spontaneous tumors developed in multiple epithelial tissues that are known to express the *BK5* promoter (23). Collectively, these data provide direct evidence for a critical role for Akt signaling in two-stage skin carcinogenesis and skin tumor promotion. In addition, elevated Akt activity above a certain threshold seems capable of driving spontaneous tumor development in multiple epithelial tissues.

To further investigate the mechanism(s) underlying the effects of elevated Akt activity in epidermis of Akt transgenic mice, we examined the status of Akt and several downstream signaling components after a single treatment with TPA. As expected, we found elevated phosphorylation of Akt following TPA treatment in nontransgenic mice with a peak at 4 h and a return to basal levels by 24 h (8). Interestingly, Akt transgenic mice showed a constitutively elevated level of Akt phosphorylation in the absence of TPA treatment and prolonged activation of Akt after TPA treatment. The results were similar for a variety of downstream targets of Akt, including p-GSK3-β, p-Bad, p-Foxo3a, p-mTOR, and targets downstream of mTOR. We also found that levels of cyclin D1, cyclin E, and c-myc protein were constitutively elevated and remained elevated compared with nontransgenic mice throughout the time course examined following TPA treatment. In addition, basal protein levels of E2F1 and E2F4 were relatively low in nontransgenic mice, but again, epidermis of Akt transgenic mice showed constitutively elevated expression of these proteins and greater increases after TPA treatment. These data are consistent with published reports showing elevated levels of these proteins in hyperplastic epidermis and skin tumors (22, 24–26). Levels of Mdm-2 were also elevated in the epidermis of Akt transgenic mice in response to TPA and remained elevated throughout the time course. Generally, similar results were obtained when the effects of multiple TPA treatments were examined, and nearly identical results were obtained with either transgenic line.



**Figure 5.** Status of Akt signaling and cell cycle regulatory molecules in transgenic mice. Transgenic (*BK5.wtAkt*) and nontransgenic (NTg) mice received a single application of 6.8 nmol of TPA, and epidermal lysates were prepared at the indicated time points following treatment with TPA. Western blot analysis was used to examine the status of (A) Akt and downstream target proteins and (B) cell cycle regulatory proteins. Actin was used as the loading control for these experiments. C, the status of total mdm2 protein was also analyzed, and Ras was used as the loading control for this experiment.

Collectively, the data from Western blot analyses support the following conclusions: (a) spontaneous tumor promotion in epidermis of Akt transgenic mice is likely due to the constitutive up-regulation of cell cycle proteins including cyclin D1, cyclin E, c-myc, E2F1, and E2F4, which leads to an increase in epidermal proliferation; (b) enhanced sensitivity to TPA promotion is due to the fact that the epidermis of Akt transgenic mice exhibits a more sustained and elevated proliferative response after treatment with the tumor promoter, and this response correlates with sustained elevations in cell cycle regulatory proteins; and (c) the spontaneous tumor development observed in founders and those lines with the highest expression of transgene likely occurred as a result of the combined effects of enhanced Akt signaling on both cell proliferation and cell survival pathways.

Deregulated Akt activity mediated by increased expression of wtAkt or myrAkt promoted several alterations in the epidermis characterized by increased proliferation and hyperplasia, as noted above. It has been shown that mice lacking *Akt1*<sup>-/-</sup>; *Akt2*<sup>-/-</sup> (27) or *Akt1*<sup>-/-</sup>; *Akt3*<sup>+/-</sup> (28) exhibit, among other phenotypic alterations, a hypoplastic epidermis due to reduced proliferation of keratinocytes. This observation, together with our present data, clearly suggests that Akt signaling is an essential mediator of epidermal morphogenesis and contributes to epidermal homeostasis by modulating keratinocyte proliferation. Several other reports have indicated that Akt is also important for controlling keratinocyte apoptosis (29); however, we did not observe significant changes in apoptotic rate in the epidermis of untreated transgenic mice *in vivo*. Evidence exists in the literature supporting the idea that



elevated expression of Akt in cells leads to enhanced cell cycle progression through an increase in the number of cells in the S phase and G<sub>2</sub>-M phase (30, 31). Overexpression of activated Akt enhanced cell proliferation in T cells (32) and was shown to override the G<sub>2</sub>-M cell cycle checkpoint after gamma irradiation in Rat1a cells (33). Collectively, these results suggest a role for the activation of Akt in cell cycle progression, consistent with our data in *BK5.wtAkt* transgenic mice. The up-regulation of multiple G<sub>1</sub> to S phase regulatory proteins in epidermis of Akt transgenic mice as shown in Fig. 5 is consistent with this conclusion.

Recently, several other mouse models have been developed that overexpress an *Akt* transgene or have elevated Akt activity (34–41). For example, overexpression of constitutively active Akt (*HAPKB-308D/473D*) in mouse mammary gland via the mouse mammary tumor virus (*MMTV*) promoter led to the interference with normal involution and, when crossed with *MMTV/MTY315/322F* transgenic mice, led to enhanced tumor progression (36). More recently, overexpression of myrAkt1 driven by the *MMTV* promoter led to enhanced susceptibility to DMBA-induced mammary carcinogenesis (37). In both of these models, overexpression of Akt did not significantly induce spontaneous mammary tumorigenesis. Transgenic expression of two different constitutively active forms of Akt in thymocytes led to the development of either thymic lymphomas or lymphomas in peripheral lymphoid organs (38). In prostate, the expression of myrAkt led to the development of PIN lesions as well as prostate enlargement (39). Furthermore, PTEN loss leads to elevated Akt activity and spontaneous tumorigenesis in both prostate and skin and increased sensitivity to chemical carcinogenesis in the latter tissue (40, 41). In both of these tissues, PTEN loss associated with elevated Akt activity was accompanied by increased proliferation in the target tissue (40, 41). The underlying mechanisms involved in Akt-mediated spontaneous tumorigenesis and the enhanced susceptibility to chemically mediated carcinogenesis as seen in some of these models remain to be fully established. In our current and earlier studies, elevation of Akt activity in the skin epidermis either through overexpression of IGF-I (17) or overexpression of Akt led to enhanced epidermal

proliferation that correlated with significant elevations of G<sub>1</sub> to S phase cell cycle proteins. In conjunction with these changes, a marked increase in signaling downstream of mTOR was observed, suggesting that protein translation was also up-regulated. These changes, possibly in concert with alterations in survival pathways (e.g., p-Bad, p-Foxo3a), may drive both spontaneous tumor development and increased sensitivity to skin tumor promotion.

In conclusion, we have generated two novel mouse models of Akt overexpression/elevated activity. Transgenic mice with the highest levels of Akt expression/activity developed spontaneous tumors demonstrating the oncogenic attributes of this signaling molecule. Analysis of mice with lower levels of expression/activity has revealed a dramatic and heightened sensitivity to two-stage skin carcinogenesis, especially to the tumor promotion stage. Elevated Akt activity leads to many changes in downstream signaling pathways that likely contribute to the phenotypic changes observed in these mice. In particular, the changes in cell cycle regulatory proteins, among others, and the similarity of these changes to those seen in *BK5.IGF-I* transgenic mice (17), likely contributed significantly to increased sensitivity to two-stage skin carcinogenesis and skin tumor promotion. Further mechanistic studies are under way using these mice to fully understand the impact of elevated Akt activity during epithelial carcinogenesis.

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## Deregulated Activity of Akt in Epithelial Basal Cells Induces Spontaneous Tumors and Heightened Sensitivity to Skin Carcinogenesis

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