Constitutively Active Akt Induces Ectodermal Defects and Impaired Bone Morphogenetic Protein Signaling

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Aberrant activation of the Akt pathway has been implicated in several human pathologies including cancer. However, current knowledge on the involvement of Akt signaling in development is limited. Previous data have suggested that Akt-mediated signaling may be an essential mediator of epidermal homeostasis through cell autonomous and noncell autonomous mechanisms. Here we report the developmental consequences of deregulated Akt activity in the basal layer of stratified epithelia, mediated by the expression of a constitutively active Akt1 (myrAkt) in transgenic mice. Contrary to mice overexpressing wild-type Akt1 (Akt^{wt}), these myrAkt mice display, in a dose-dependent manner, altered development of ectodermally derived organs such as hair, teeth, nails, and epidermal glands. To identify the possible molecular mechanisms underlying these alterations, gene profiling approaches were used. We demonstrate that constitutive Akt activity disturbs the bone morphogenetic protein-dependent signaling pathway. In addition, these mice also display alterations in adult epidermal stem cells. Collectively, we show that epithelial tissue development and homeostasis is dependent on proper regulation of Akt expression and activity.

INTRODUCTION

A large number of processes in the cell are modulated by the protein kinase Akt, also known as protein kinase B (PKB). In particular, this kinase has been widely involved in the control of cell survival and apoptosis, proliferation, cell cycle progression, glucose metabolism, and protein translation (Brazil *et al.*, 2004). Three Akt isoforms sharing common structural features (Hanada *et al.*, 2004) have been found in mammals (Akt1, Akt2, Akt3). Although the relevance of Akt signaling in cancer is widely recognized (Bellacosa *et al.*, 2005; Manning and Cantley, 2007), its involvement in development has only recently been highlighted. Akt1 and Akt2 knockout (KO) mice are viable and exhibit mild phenotypes characterized by growth retardation and diabetes (Chen *et al.*, 2001; Cho *et al.*, 2001), suggesting functional redundancy among Akt isoforms. Further proof of these overlapping roles comes from analysis of compound mutant mice. Akt1 and Akt2 double KO mice displayed a much more severe

phenotype: dwarfism, impaired skin development, delayed bone development, reduced adipogenesis, and early lethality after birth (Peng *et al.*, 2003). More recently, mice with combined mutant alleles of Akt1 and Akt3 have also been generated. Double KO of Akt1 and Akt3 causes embryonic lethality at around embryonic days 11 and 12, and Akt1^{−/−};Akt3^{−/−} mice have severe developmental defects in the cardiovascular and nervous systems (Yang *et al.*, 2005). Akt1^{−/−};Akt3^{+/−} mice display multiple defects in the thymus, heart, and skin and die within several days after birth, whereas Akt1^{+/−};Akt3^{−/−} mice are viable (Yang *et al.*, 2005). These data demonstrate that the three Akt isoforms have overlapping functions but also may have unique functions in specific organs.

Besides the skin phenotype observed in different compound-deficient mice, several lines of evidence have highlighted the importance of the phosphoinositide 3 kinase (PI3K)/Akt pathway in epidermis. Of particular interest is the relevant role of Akt during mouse skin carcinogenesis. We have demonstrated that Akt is a key molecule in insulin growth factor 1 (IGF-1)-mediated mouse skin tumor promotion (Wilker *et al.*, 2005). In addition, Akt exerts essential roles in two-stage carcinogenesis protocols affecting tumor proliferation and apoptosis (Segrelles *et al.*, 2002) and also modulates the tumor-stroma cross-talk leading to an increase in angiogenesis (Segrelles *et al.*, 2004). Recently, using cultured cell systems, we provided evidence indicating that the functions of Akt in epidermal tumors are exerted by transcriptional and posttranscriptional mechanisms and

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Abbreviations used: BMP, bone morphogenetic protein; HF, hair follicle.

show several parallels with human head and neck squamous cell carcinomas (Segrelles *et al.*, 2006). Of relevance, we observed that increased Akt expression modulates β -catenin and Δ Np63 expression, two essential modulators of epidermal development, in this system (Segrelles *et al.*, 2006).

To further explore the role of Akt in skin, we have generated transgenic mice expressing either a wild-type form of Akt1 (Akt^{wt}) or a form of Akt1 that is constitutively activated by means of a myristoylation sequence (myrAkt), directed to the basal layer of the stratified epithelia by the K5 promoter (Segrelles *et al.*, 2007). These approaches, together with the tissue-specific knock out of PTEN tumor suppressor gene, have been widely used to explore the functions of the Akt signaling pathway in vivo (Yang *et al.*, 2004). Notably, there are functional differences among these three approaches. Because PTEN is a negative modulator of PI3K signaling, elimination of PTEN leads to the permanent up-regulation of the PI3K pathway, whereas increased expression of wild-type Akt leads to the amplification of PI3K signaling, and the expression of constitutively active Akt generates a permanent, PI3K-independent signal. Although performing our previous analysis, we observed that many of the founders expressing K5myrAkt, but not those expressing K5Akt^{wt}, displayed developmental defects in multiple ectodermal organs in parallel with the level of transgene expression and Akt activity. Here we have characterized these developmental alterations. We provide evidence that deregulated Akt activity in stratified epithelia mediated by myrAkt expression leads to aberrant bone morphogenetic protein 4 (BMP4) signaling and altered adult epidermal stem cell homeostasis.

MATERIALS AND METHODS

Transgenic Mouse Production and Maintenance

Wild-type mouse Akt cDNA (obtained from Dr. A. Bellacosa, Fox Chase Cancer Center) or myrAkt (obtained from Dr. S. Gutkind, National Institute of Dental and Craniofacial Research, NIH) were placed under a 5.2-kb fragment of the BK5 promoter into the SnaBI site between the rabbit β -globin intron and polyadenylation sequences from a vector previously described (Bol *et al.*, 1998). Orientation and integrity of the inserts were confirmed by restriction digests. Purified fragments were used in microinjection. Mice were genotyped using primers specific for β -globin on genomic DNA isolated from tails, as previously described (Bol *et al.*, 1998). Transgenic and control mice in a C57BL/DBA/FVB mixed background were housed in a 12/12 light/dark cycle at 24°C and given standard mouse chow and water ad libitum. In some cases, where alterations in oral cavity or teeth were observed, food was supplemented with jelly. In addition to the founders, at least 40 mice of each transgenic line or controls were analyzed in search for developmental defects.

Histology and Akt Kinase Analyses

For histological analysis, samples were fixed in Formalin and embedded in paraffin before sectioning. Sections of 5 μ m were cut and stained with H&E. At least five different samples were analyzed for each time point. The expression of BMP2 (1/200 diluted mAb; Abcam, Cambridge, UK), BMP4 (1/50 diluted goat polyclonal; AbCam) and Δ Np63 (1/200 diluted mAb 4A4; Santa Cruz Biotechnology, Santa Cruz, CA) was monitored in deparaffinized sections using conventional protocols. For expression of active Akt (phospho Ser 473, 1/50 diluted; Cell Signaling, Beverly, MA), phospho Smad1/5/8 (phosphorylated in Ser463 and 465, 1/100 diluted; Cell Signaling), Foxo3a (1/200 diluted; Upstate, Lake Placid, NY), and BMPRIA (1/100; Santa Cruz Biotechnology), the slides were microwaved for 10 min after deparaffinization to enhance the staining. Keratin 15 (mouse mAb LHK15; Neomarkers, Lab Vision, Fremont, CA; diluted 1/50), and CD34 (rat mAb; eBioscience, San Diego, CA; diluted 1/50) were monitored in 70% ethanol-fixed tissues by double immunofluorescence. Before incubation overnight at 4°C with primary antibodies diluted in bovine serum albumin (BSA)/phosphate-buffered saline (PBS), sections were incubated with 5% horse serum for 45 min to block the Fc receptor in tissue and then washed three times with sterile PBS (pH 7.5). Horseradish peroxidase-, Texas red-, or fluorescein isothiocyanate-conjugated secondary antibodies were purchased from Jackson ImmunoResearch (West Grove, PA) and used at dilutions of 1/4000, 1/500, and 1/100, respectively. Peroxidase was visualized using a DAB kit (Vector, Burlingame, CA). Control slides were obtained by replacing primary antibodies with PBS

(data not shown). Akt activity in mouse skin was determined after immunoprecipitation with anti-Akt (Santa Cruz Biotechnology, C-20 antibody 1 ml/25 mg protein) essentially as previously described (Segrelles *et al.*, 2002), using histone 2B (H2B; Roche Molecular Biochemicals, Indianapolis, IN) as the substrate for Akt or by Western blot (see below). Autoradiograms were scanned and subsequently quantified using a Phosphorimager (Bio-Rad, Richmond, CA).

Scanning Electron Microscope Studies of Hair Shafts

Hair shafts from the dorsal skin of 30-d-old transgenic and nontransgenic littermates were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.5). After dehydration, samples were dried by critical point drying (Balzers, Hudson, NH; CPD020), coated with gold palladium by a Jeol JFC-1100 Ion Sputter, and examined with a Jeol JSM-T220A scanning electron microscope (SEM; Peabody, MA). At least 10 samples from each genotype were analyzed.

RNA Purification and Affymetrix Mouse Gene Chip 430A Analysis

Mouse skin tissue, obtained 30 d after birth, was preserved in RNAlater (Ambion, Austin, TX) and disrupted and homogenized using Mixer Mill MM301 (Retsch). Total RNA was extracted and purified from 30 mg of skin using RNeasy Fibrous Tissue Mini kit (Qiagen, Chatsworth, CA) following the manufacturer's recommendations. The integrity of the RNA populations was tested in the Bioanalyzer (Agilent, Wilmington, DE). Two (transgenic mouse lines L60, L84 and LA) or three pools (control mice) from RNA whole skin extracts of same genotype were done and analyzed, individually, in mouse microarrays. We exported CEL files from Affymetrix GCOS software (Santa Clara, CA), and using the web-based tool Gene Expression Profile Analysis Suite (GEPAS, <http://www.gepas.org>; Vaquerizas *et al.*, 2005), we subtracted the background intensity values with RMA (Irizarry *et al.*, 2003), normalized the chips using quantile method (Bolstad *et al.*, 2003), and finally log2-transformed and mean-centered the intensity values. Differentially expressed genes with normal variability were extracted between the four mouse genotypes with CLEAR test (2183 Affymetrix probes, $p < 0.05$; Valls *et al.*, 2007). Further analyses were performed using the MeV software (Saeed *et al.*, 2003). The selection of genes belonging to clusters 1–4 was made using Template Matching (Pearson $R > 0.8$, $p < 0.01$; Pavlidis and Noble, 2001). Genes selected in more than one cluster were included in the cluster in which they displayed the best R and p values. The cluster Gene Ontology (GO) analysis of Biological Processes using DAVID software (Dennis *et al.*, 2003; <http://david.abcc.ncifcrf.gov/home.jsp>) from the National Institute of Allergy and Infectious Diseases/NIH was used to identify functional categories for each cluster. The search for GO terms was made using all themes, listed by p value based on EASE score (Hosack *et al.*, 2003) and manually curated. EASE score identifies functional categories overrepresented in a gene list relative to the representation within the proteome of a given species (Hosack *et al.*, 2003). Pathway Architect software (Stratagene, La Jolla, CA) was used to identify and characterize possible pathways affected by specific alterations in gene expression. Gene expression microarray dataset has been submitted in GEO database under the accession number GSE9054.

Western Blot Analysis

Extracts, prepared from skin of 30-d-old mice, were ground with a mortar on liquid nitrogen and homogenized in buffer P (Tris, pH 7.5, NaCl 150 mM, EDTA 1 mM, EGTA 1 mM, β -glycerophosphate 40 mM, sodium orthovanadate 1 mM, PMSF 0.1 mM, aprotinin 2 mg/ml, leupeptin 2 mg/ml, NP-40 1%). Total protein (35 μ g) from at least three age- and genotype-matched pooled skin samples was used for NuPAGE 4–12% Bis-Tris Gel (Invitrogen, Carlsbad, CA), transferred to nitrocellulose membrane (Invitrogen), and probed with primary antibodies against Akt1/2 (Santa Cruz Biotechnology; 1/500), phosphorylated Akt (Ser 473, diluted 1/50 and Thr 308, diluted 1/10; Cell Signaling), BMPR1 (Santa Cruz Biotechnology; 1/100), BMP4 (Abcam; 1/300), p63 (Santa Cruz Biotechnology; 1/500), and phospho-Smad1/5/8 (recognizing Smad1/5/8 phosphorylated in Ser463 and 465; Cell Signaling; 1/500). Actin (Santa Cruz Biotechnology; 1/1000) was used to normalize protein loading. Secondary antibodies (anti-rabbit, anti-mouse, or anti-goat IgG) were purchased from Jackson ImmunoResearch. Chemiluminescence was performed using manufacturer's recommendations (Pierce, Rockford, IL).

Label-retaining Cell Analysis

Ten-day-old nontransgenic and myrAkt pups (six mice of each class) were injected with BrdU (20 μ l of a 12.5 mg/ml dilution in 0.9% NaCl every 12 h for a total of four injections). Skin sections were collected at 30 d after the last injection and bromodeoxyuridine (BrdU) incorporation was measured as percentage of hair follicles (HF) containing positive cells as previously reported (Ruiz *et al.*, 2004). Experiments were performed in triplicate ($n \geq 3$ per group) and at least 100 follicles were scored in each section.

Fluorescence-activated Cell Sorting Analysis

For each experiment, dorsal skin of four adult animals of each genotype was shaved and treated with trypsin to separate dermis from epidermis. Cell suspensions were strained (40- μ m nylon, Falcon, Oxnard, CA) and stained in PBS containing 2% fetal bovine serum with anti-CD34 coupled to biotin (BD-PharMingen, San Diego, CA) and anti- α 6 integrin (CD49f) coupled to PE (PharMingen). Cells were washed and stained with streptavidin-Tricolor (Caltag Laboratories, Burlingame, CA) and washed again. Cells were resuspended in PBS containing 2 μ g/ml propidium iodide to exclude dead cells and analyzed in an EPICS XL flow cytometer (Coulter Electronics, Hialeah, FL).

RESULTS

Ectodermal Alterations in myrAkt Transgenic Mice

The K5 promoter was used to target the expression of wild-type Akt1 (Akt^{wt}) or permanently activated Akt1 (myrAkt) to the basal layer of the mouse stratified epithelia. The overall epidermal phenotype and tumor susceptibility of these mice has been reported elsewhere (Segrelles *et al.*, 2007). It is worth mentioning that, because of the deleterious effects of myrAkt or wtAkt expression, it was impossible to establish lines from many of the founders. Eventually, we were able to establish two lines with different expression levels of the myrAkt transgene (lines 60 and 84, hereafter L60 and L84) and one line expressing the wtAkt transgene (Line A, hereafter LA). Our analysis was then focused on these three lines and six myrAkt founders.

MyrAkt founders and L84 transgenic mice showing high levels of Akt kinase activity (Figure 1, A and A') displayed developmental defects that affected multiple ectodermally

derived organs such as hair, teeth, nails and several ectodermal glands. Of note, these alterations were not observed in mice expressing wtAkt or low levels of myrAkt, associated with lower Akt activity (Figure 1, A' and B). This suggests that a certain threshold of Akt activity is necessary to promote the deregulated development of ectodermal organs. As expected, the increased kinase activity was restricted to those tissues expressing the transgene, such as epidermis (Figure 1C' and data not shown).

With respect to hair, we observed alopecia in the snout, face, and back skin and loss of vibrissae in myrAkt founders with high levels of Akt activity, in myrAkt L84 mice, and sporadically in L60 mice (Figure 2A). Compared with control mice (Figure 2B), the vibrissae of myrAkt mice were structurally distorted in L60 (Figure 2B') and overall degeneration was evident in L84 mice (Figure 2B''). In parallel, the back skin pelage showed alterations in *awl* hair shafts (the predominant type of overhairs in the mouse) consisting of an enlarged cortex with the medulla cells arranged in a single row of septa, instead of the four to five septules observed in the controls (compare Figure 2C, control non-transgenic, with C', L84 transgenic mice). Examination of *awl* hair shafts with SEM showed that the cuticle is made up of more densely compacted overlapping scales (compare Figure 2D, nontransgenic with D', L84 transgenic mice). Finally, we also analyzed the distribution of different hair types. We found a decrease in *zig-zag* hairs, whereas all the other hair types (*awl*, *auchene*, and *guard*) were increased in transgenic mice compared with nontransgenic mice (Table

Figure 1. Akt Kinase activity in transgenic mice. (A) Top panel, representative kinase assay using skin extracts and histone H2B as substrate; middle and bottom panels, Western blot of the same extracts blotted against Akt and Actin. (A') Western blot of pooled skin samples from Control, L84, and LA transgenic mice obtained by pnd 28 probed against total and phosphorylated Akt (Ser 473 and Thr 308). The increased phosphorylation of Akt in L84 indicates the increased activation of Akt. (B) Summary of three independent kinase assays using skin extracts from the indicated mice. Transgenic mice displaying ectodermal organ development are denoted. Representative example of immunohistochemical detection of phosphorylated Akt (ser473) in skin section of control nontransgenic (C) and myrAkt founder 82 transgenic mouse. Note that only epithelial cells are positive for phospho-Akt in transgenic sample (C'). Bar, 250 μ m.

Figure 2. Hair phenotype of myrAkt transgenic mice. (A) Example of alopecia in L60 mice affecting the snout and some patches of dorsal skin. (B) Histology of vibrissae from control (B), L60 (B'), and L84 (B'') mice. Note the progressive degeneration. Bar, 250 μ m. Overall macroscopic (C and C') and scanning electron microscopic (D and D') appearance of awl back skin hairs from control (C and D) and L84 (C' and D') transgenic mice. Bars, 50 μ m. (E–G) Representative histology of skin by postnatal day (pnd) 28 from Control (E), LA (F), and L84 (G) mice. Note the absence of true anagen in L84 transgenic mice. (H) Representative histology of transgenic L84 skin by pnd 45. Note the enlarged anagen HF structures (denoted by arrows). Bars, 250 μ m.

1). These alterations prompted us to analyze possible hair cycle defects; however, this study had to be limited to those mice that allowed the establishment of transgenic lines, namely L60 and L84, expressing myrAkt, and LA expressing wtAkt. By 28–30 d after birth (the time at which the first postnatal hair anagen is evident) we observed that HF in control (Figure 2E), L60 (not shown), and LA (Figure 2F) displayed clear anagen features. On the contrary, the HF of L84 mice showed clear signs of outer root sheet hyperplasia and remained in telogen (Figure 2G), thus demonstrating that an increase in Akt activity above a certain level produces hair cycle defects. By 45 d after birth most of control, L60, and LA hairs are in telogen (not shown, see also Supplementary Figure 1), whereas L84 HF displayed clear signs of anagen (Figure 2H) with enlarged HF structures (denoted by arrows in Figure 2H). Subsequent analysis of multiple skin sections of different transgenic mice at different time points after birth indicated a loss of synchrony in

the first postnatal hair cycle in the L84 mice (Supplementary Figure 1).

Another characteristic displayed by L84 transgenic mice and some founders were long and fragile nails (Figure 3A). Histological analysis revealed that the nails of these transgenic mice (Figure 3B) had normal eponychium (e) and hyponychium (h), but an enlarged nail root (denoted by r in Figure 3B) and hyperplasia of the nail bed epithelium (nb, see insets in Figure 3, B and B') compared with controls (Figure 3B').

With respect to teeth, most of L84 transgenic mice and some founders showed overgrowth and thinning of the incisors (Figure 4A), which in a few cases was also accompanied by the development of supernumerary teeth located in the palate (arrow in Figure 4B). Additionally, in most cases the second or third molar primarily in the lower jaw, was missing (arrow in Figure 4C).

We also characterized alterations in other ectodermally-derived structures. We observed a significant hyperplasia of the meibomian glands (denoted by mb in Figure 5, A and A'). These are a special kind of sebaceous glands at the rim of the eyelids that have no association with adjacent hair follicles and produce a lipid secretion that forms a component of the tear film. We also observed a marked dysplasia of salivary glands (denoted by arrows in Figure 5, B and B') and a marked hypoplasia of the sweat glands of the foot pads in the transgenic L84 mice (denoted by sg in Figure 5, C and C'). Collectively, these data demonstrate that the expression of constitutively active Akt in the basal layer of stratified epithelia leads to altered development of multiple ectodermally derived organs in a dose-dependent manner.

Table 1. Hair type distribution

Hair type	MyrAkt line 84 ^a	Control ^a
Zig-zag	75.3 $\pm$ 2.1%	82.4 $\pm$ 2.5%
Awl	19.7 $\pm$ 1.3%	15.4 $\pm$ 1.5%
Auchene	3.6 $\pm$ 1.1%	1.9 $\pm$ 1.0%
Guard	1.3 $\pm$ 0.2%	0.4 $\pm$ 0.2%

^a At least 300 hairs per mouse of each type were scored from at least five different age- and sex-matched mice.

Figure 3. Nail phenotype of myrAkt transgenic mice. (A) Example of nail overgrowth in founder 23. Also the fragility of nails is depicted (inset). Similar observations were obtained in L84 mice. Histology analysis of nails from L84 transgenic (B) and nontransgenic (B') mice. Note the normal eponychium (e) and hyponychium (h), but an enlarged nail root (r) and hyperplasia of the nail bed epithelium (nb, insets B and B') in the transgenic mice.

Alterations in Gene Expression Mediated by De-regulated Akt Activity in Skin

The development of ectodermal organs is controlled by multiple pathways involving numerous genes. Most of these pathways modulate the process of hair cycling. The ectodermal and hair cycle defects observed in the transgenic mice suggested that increased and deregulated Akt activity leads to altered expression of genes involved in these developmental pathways. To gain a deeper insight into this aspect, we performed global expression profiling of paired RNA samples from whole skin extracts of transgenic (L60, L84, and LA) and nontransgenic mice at 30 d after birth. The LA mice did not display the ectodermal alterations observed in L84, and in L60 mice only sporadic alopecia was detected in the snout, face, and back skin; none of these transgenic lines displayed hair cycle defects (Figure 2F; see also Supplemen-

Figure 4. Tooth phenotype of myrAkt transgenic mice. Representative examples of transgenic mice showing overgrowth and fragility of the incisors (A), supernumerary teeth located in the palate (B), and lack of the second molar in the lower jaw (C).

tary Figure 1). Consequently, the microarray data were first processed to find specific clusters that included genes associated with the modest changes observed in L60 and the more dramatic alterations observed in L84 (Figure 6, A and B; clusters 1 and 2, down- and up-regulated, respectively). Subsequently, we also searched for genes only deregulated in L84 compared with LA, controls, and L60 (Figure 6, A and B; clusters 3 and 4, down- and up-regulated, respectively). It is worth mentioning that, from the total 173 genes identified, the largest number of changes correspond to those showing alterations only in L84 skin (clusters 3 and 4), which includes 127 genes (see Supplementary Tables 1–4 for the complete list of identified genes).

To identify possible functional alterations in the different skin samples, we analyzed the biological functions of the 173 genes mentioned above using DAVID software (Dennis *et al.*, 2003; Table 2). Interestingly, the concept “development” appears as significantly represented in the clusters including those genes that only showed altered expression in L84 skin

Figure 5. Ectodermal gland phenotype of *myrAkt* transgenic mice. Representative histology sections from meibomian (denoted by mb in A and A'), salivary (denoted by arrows in B and B'), and sweat (denoted by sg in C and C') glands from control (A–C) and L84 transgenic mice (A', B', and C'). Note the hyperplasia of the meibomian, dysplasia of the salivary and hypoplasia of the sweat glands in transgenic mice. Bars, (A' and C') 250 μ m; (B') 100 μ m.

samples (clusters 3 and 4). Utilizing the Entrez gene database, we narrowed the dataset to those genes that were involved in skin or ectodermal morphogenesis (Table 3). Overall, these data indicate that deregulated Akt activity results in gene expression changes that can modulate epithelial development.

Microarray data were further analyzed using Pathway Architect (Stratagene) to identify and characterize possible signaling pathways affected by specific alterations in gene expression. This analysis revealed that the global expression changes modify BMP4 signaling. In this regard, not only was the *Bmp4* gene specifically down-regulated, but also negative regulators of BMP signaling such as *Egf* (Nonaka *et al.*, 1999) and *Twsg1* (Scott *et al.*, 2001) were up-regulated. In addition, several genes found in these clusters are involved in BMP signaling (Figure 6C and highlighted in bold in Table 3) either as integral pathway effectors or as downstream targets (Figure 6C), thus suggesting that this developmental pathway is altered by *myrAkt* expression.

The forkhead transcription factor family has been widely described as a target of Akt (Burgering and Kops, 2002). Direct phosphorylation of these factors by Akt results in their cytoplasmic retention and inactivation, inhibiting the expression of forkhead transcription factor-regulated genes (Burgering and Kops, 2002). We thus studied whether the expression and localization of Foxo3a, a representative member of this family, is affected in the epidermis of the different transgenic mice by postnatal day (pnd) 28. In control, nontransgenic mice (Figure 6D), we observed a positive staining throughout the epithelium, and a number of positive nuclei were detected in the hair follicle and the basal layer of the interfollicular epidermis (denoted by arrows in Figure 6D). A similar staining pattern was observed in L60 (Figure 6E) and LA (Figure 6F) transgenic mice epidermis, although a minor decrease in the interfollicular epidermal positive nuclei was observed in L60 samples. On the con-

trary, in L84 skin (Figure 6G), the overall staining was decreased and very few positive nuclei were observed (denoted by arrows in Figure 6G). Therefore, in agreement with our previous data (Segrelles *et al.*, 2006), the expression of Akt, upon a certain threshold, leads to the reduction and cytoplasmic localization of Foxo3a in keratinocytes (Segrelles *et al.*, 2006). This might help to explain, at least in part, some of the changes observed in microarray analyses.

Altered BMP Signaling Mediated by *myrAkt* in Skin

BMP signaling is thought to perform multiple functions in the regulation of skin appendage morphogenesis and the postnatal growth of HFs (Botchkarev, 2003). BMPs function by binding type 1 (BMPRI1A and BMPRI1B) and type 2 (BMPRI2) transmembrane serine/threonine kinase receptors, resulting in phosphorylation of the intracellular proteins Smad 1, 5, and 8. Therefore, to further confirm the possible alterations in BMP signaling, we carried out a detailed histology analysis of several components of this pathway.

As shown in Figure 7, expression of BMP4 (Figure 7A) and BMP2 (Figure 7B) was predominant in the HF matrix and outer and inner root sheath (denoted by ORS and IRS, respectively) in nontransgenic mice with very little expression observed in the interfollicular epidermis (denoted by IE). With respect to *myrAkt* transgenic mice, we observed altered distribution and localization of both morphogens (Figure 7, C and D, respectively), because scattered expression was observed in only a few cells. Western blot analysis of whole skin extracts (Figure 7E) confirmed the dramatic decrease in the level of BMP4.

We also examined the expression and localization of BMPRI1A and phospho-Smad1 (Ser463 and Ser465). By full anagen (pnd 28), phospho-Smad staining was present in cells of the medulla, cortex, and cuticle of the hair shaft in nontransgenic and LA mice (Figure 8m A and B, respectively). These results are similar to previous reports (Kobiela *et al.*, 2003;

Figure 6. Summary of microarray data and Foxo3a expression. (A) Heatmap showing the relative expression of different genes selected for the analysis. (B) Clusters of genes that were regulated in transgenic mice according to the pattern denoted by red (Up) or green (Down) lines, extracted using Template Matching ($R > 0.8$, $p < 0.01$). (C) Summary of pathway integration of microarray analyses using Pathway Architect software (Stratagene). (D–G) Representative

Table 2. Functional annotation (GOBP) of genes selected from microarray analyses

Cluster (no. of genes)	Gene ontology biological processes category	No. of genes (%)	p value
1 (32)	Cell-cell signaling	4 (11.8%)	9.1E-3
	Establishment of localization	10 (29.4%)	4.6E-2
	Gametogenesis	3 (8.8%)	4.6E-2
	Transport	9 (26.5%)	7E-2
2 (14)	Immune response	5 (31.2%)	7.1E-3
	Defense response	5 (31.2%)	1.6E-2
3 (64)	Cell differentiation	10 (13.7%)	4.5E-3
	Development	14 (19.2%)	2.5E-2
4 (63)	Regulation of development	4 (5.5%)	2.9E-2
	Tube development	5 (7.6%)	3.4E-4
	Macromolecule metabolism	19 (28.8%)	4.5E-2
	Biosynthesis	8 (12.1%)	6.5E-2

Andl *et al.*, 2004; Yuhki *et al.*, 2004; Sharov *et al.*, 2005). In contrast, phospho-Smad staining in myrAkt transgenic mice was faint, and only a few cells expressed very low amounts at the tip of the hair (Figure 8C). The apparent decrease in phospho-Smad was confirmed by Western blot of whole skin extracts (Figure 7E). In nontransgenic mice and mice expressing the wtAkt transgene (LA), BMPRIA expression was restricted to a subset of cells in the outer root sheath and hair matrix (Figure 8, D and E), as previously reported (Kobielak *et al.*, 2003; Andl *et al.*, 2004; Yuhki *et al.*, 2004; Sharov *et al.*, 2005), whereas in myrAkt L84 mice (Figure 8F), BMPRIA expression was observed in the outer and inner root sheath (denoted by ORS and IRS, respectively in Figure 8F) and almost absent in hair tips. Moreover, immunohistochemistry also suggested a possible increased expression of BMPRIA that was confirmed by Western blotting (Figure 7E).

Finally, we also studied the possible changes in the expression and localization of Δ Np63. This protein is an ectoderm-specific direct transcriptional target of BMP signaling (Bakkers *et al.*, 2002) and is essential for skin development (Mills *et al.*, 1999; Yang *et al.*, 1999), and it can be induced by increased Akt activity (Barbieri *et al.*, 2003; Segrelles *et al.*, 2006). Compared with nontransgenic mice (Figure 8G) and LA mice (not shown), L84 mice did not display any obvious difference in localization, although L84 mice also displayed some Δ Np63-positive cells located in the inner root sheath (denoted by IRS in Figure 8H) in addition to the outer root sheath and the interfollicular epidermis (denoted by ORS and IE in Figure 8H), or overall expression level (Figure 7E). Notably, we found that in control mice Δ Np63 expression correlated with increased phospho-Akt in the bulge areas (Figure 8I), but not in the hair bulb (Figure 8I'), whereas in myrAkt L84 mice, Δ Np63 was coexpressed with phospho-Akt throughout the HF (Figure 8J). These data indicated that, despite the alterations in BMP signaling observed in mice with high expression of the myrAkt transgene the overall expression of Δ Np63 was not affected and discounted the possibility that the phenotypic alterations were primarily mediated by altered Δ Np63 expression.

examples of the expression and distribution of Foxo3a analyzed by immunohistochemistry in Control (D), L60 (E), LA (F), and L84 (G) mouse epidermis by pnd 28. Note the decreased staining and the reduced number of positive nuclei (denoted by arrows) in L84. Bar, 150 μ m.

Table 3. Representative genes involved in ectoderm development found in clusters

Cluster	Gene symbol	Fold change in L84
1	Axin2	-5.14
	Cxcl12	-4.81
	Ddr1	-5.49
	Dlgh4	-5.66
	Gcm2	-3.10
	Pou2f1	-5.70
	Rgl1	-5.20
	Snai3	-5.60
	Snx11	-5.77
	Tff2	-2.86
	Vtn	-3.39
	Wnt8b	-4.01
2	Akt1	4.83
	Sema5a	3.43
	Twsg1	6.22
3	Bmp4	-4.82
	Capn6	-3.53
	Epim	-2.96
	Gja4	-2.94
	Heyl	-3.64
	Hp	-6.03
	Map3k7	-5.11
	Mknk1	-5.11
	Pla2g1b	-4.91
	Runx1	-3.77
	Sellh	-5.27
	Sfrp5	-5.03
	Sox10	-4.19
	Syt7	-4.67
	Tbx19	-4.00
	Tiam2	-3.15
	Tmepai	-5.19
	Traf3	-3.21
	Traip	-3.23
4	Pik3r1	5.83
	Ccl27	5.52
	Dlgh2	4.37
	Egf	5.32
	Enah	4.70
	Fgf1	4.76
	Fgf18	5.34
	Gas1	3.21
	Gpld1	5.12
	Itm2a	4.28
	Stat1	5.39
	Vsnl1	4.73
	Wwp1	5.07

Bold face indicated genes that are involved in BMP signaling.

Deregulated Akt Activity Alters Epidermal Stem Cell Homeostasis

There are several lines of evidence indicating that BMP signaling may influence the behavior of epidermal stem cells (Sharov *et al.*, 2006; Zhang *et al.*, 2006b; Kobielak *et al.*, 2007). It has also been shown that other adult stem cell populations, for example, as in hematopoietic tissue (Rossi and Weissman, 2006) may be impacted by PTEN/Akt pathway signaling. Because the K5 promoter is also active in these cells, we have thus studied possible alterations in the stem cell population in the skin of L84 transgenic mice. Initially we determined the expression of two putative epidermal stem cell markers, K15 and CD34 (Liu *et al.*, 2003; Cotsarelis, 2006). The pattern of double immunofluorescence staining suggested an increase in the population of cells in the hair

follicle of L84 (Figure 9A') compared with control mice (Figure 9A). We next determined whether the epidermal stem cell compartment was affected by myrAkt expression using a label-retaining (LR) technique (Cotsarelis *et al.*, 1990; Taylor *et al.*, 2000). In L84 skin we found a consistent increase in the number of labeled cells 30 d after BrdU administration (Figure 9, B' and C) compared with control mice (Figure 9, B and C). To further substantiate these observations, we also performed fluorescence-activated cell sorting (FACS) analysis to determine the proportion of cells expressing integrin $\alpha 6$ and CD34, which is considered characteristic of epidermal bulge stem cells (Blanpain *et al.*, 2004; Blanpain and Fuchs, 2006). These analyses demonstrate that the proportion of $\alpha 6$ +CD34+ cells is similar between control and L84 skin samples; however, L84 epidermis had a dramatic increase in the population characterized as $\alpha 6$ low CD34+ at the expense of a partial reduction in the $\alpha 6$ high CD34+ cell population (Figure 9, D and E). This result is in agreement with the presence of BrdU positive cells with clear suprabasal localization observed during the LR experiments (denoted by arrows in Figure 9B'). Finally, we also performed a colony-forming efficiency experiment using adult epidermis as the source of primary keratinocytes. Five days after plating, multiple abortive colonies were observed in cultures from control mice (Figure 9F), and very few rendered productive colonies after long-term culture (Figure 9G). In contrast, cells from L84 epidermis displayed undifferentiated morphology (Figure 9F') and produced multiple colonies (Figure 9G). Collectively, these data indicate that constitutively active Akt expression results in an expansion of the stem cell population.

DISCUSSION

In the current study we present data that strongly support the hypothesis that increased and deregulated Akt activity, triggered by myrAkt expression, provokes dramatic alterations in ectodermal organ development. Collectively, these findings support the conclusions that Akt is an important mediator of epithelial homeostasis. The fact that increased wild-type Akt expression (as in wtAkt mice), which requires upstream control elements to become fully activated (Segrelles *et al.*, 2007), does not lead to a similar phenotype points to a finely regulated mechanism of control affecting Akt in the development of these organs. Our results are in agreement with the reported alterations found in different mouse models of Akt deficiency (Di-Poi *et al.*, 2002; Peng *et al.*, 2003; Yang *et al.*, 2005) and the keratinocyte-specific null mutation of epidermal *Pten* (Suzuki *et al.*, 2003). Of note, data obtained from double Akt KO mice, besides arguing for partially overlapping functions of Akt isoforms in vivo, also revealed that some functions of Akt are only discernible when total Akt levels are below a critical threshold in specific cell types and tissues (Dummler *et al.*, 2006). Further evaluation of ectodermal derived organs in compound Akt KO mice may reveal additional roles.

The Akt signaling pathway has been widely studied in the context of carcinogenesis (Manning and Cantley, 2007) and is associated with increased cell proliferation and survival. Consistently, these functions are altered in L84 mice and also in LA and in L60 (Segrelles *et al.*, 2007); however, the major developmental defects are primarily found in L84 mice and several founders. This would indicate that the observed development defects due to increased Akt activity can be delineated from proliferative or antiapoptotic effects. We have taken this aspect as a starting hypothesis for the analysis of the microarray data. Indeed, the consideration of

Figure 7. Altered BMP expression and localization in hair follicles of *myrAkt* transgenic mice. Representative examples of the expression of BMP4 (A and C) and BMP2 (B and D) in control (A and B) and L84 (C and D) transgenic mice. Bars, 250 μ m. IE, interfollicular epidermis; IRS, inner root sheath; ORS, outer root sheath; SG, sebaceous gland. (E) Western blot of whole skin extracts probed for the expression of the indicated proteins.

genes that do not display altered expression in LA epidermis certainly restricts the analyses. The list of genes found is relatively small and includes multiple genes previously associated with ectoderm or skin development. Furthermore, in unconstrained analysis of possible pathways involved, we detected the BMP-dependent pathway, indicating that this may be a major mediator in the skin phenotypic alterations found in *myrAkt* mice. The possibility that this pathway is also a target mediating other ectodermal alterations seems plausible but undoubtedly will require further investigations.

It is widely recognized that BMP signaling is required for proper development of several ectodermal structures (reviewed in Jernvall and Thesleff, 2000; Botchkarev, 2003). Of note, *cre*-mediated mutation of the *BMPR1A* gene causes altered tooth morphogenesis, defective postnatal development of HFs, and abnormal nail growth (Andl *et al.*, 2004) and leads to the formation of epidermal tumors (Kobielak *et al.*, 2003; Andl *et al.*, 2004; Sharov *et al.*, 2006; Zhang *et al.*, 2006b), probably through the altered homeostasis of epidermal stem cells (Sharov *et al.*, 2006; Zhang *et al.*, 2006b; Ko-

Figure 8. Altered BMP signaling in hair follicles of myrAkt transgenic mice. Representative examples of the expression of phospho Smad1/5/8 (A–C) and BMPRIA (D–F) in control (A and D), LA (B and E), and L84 (C and F) transgenic mice. (G and H) Expression and localization of ΔNp63 in control (G) and L84 (H) transgenic mice. (I and J) Double immunofluorescence of ΔNp63 (green) and phospho-Akt (Ser473; red) in bulge (I) and bulb (J) regions of control HF and in L84 (J) HF by pnd 28. Bars, 250 μm. IE, interfollicular epidermis; IRS, inner root sheath; ORS, outer root sheath.

bielak *et al.*, 2007). In agreement, we also observed the development of epithelial tumors in Akt transgenic mice that in many cases were associated with hair follicle structures (Segrelles *et al.*, 2007). Although epidermal-specific deletion of the *Bmpr1a* gene or *Noggin* overexpression caused severe alterations in the expression of several genes associated with development and cell cycle (Kobielak *et al.*, 2003; Andl *et al.*, 2004; Sharov *et al.*, 2006; Zhang *et al.*, 2006b), our microarray analysis did not produce similar results. This difference may be due to the fact that ablation of *Bmpr1a* or *Noggin* overex-

pression completely abrogates BMP signaling, whereas our data support a possible deregulation together with decreased signaling rather than complete inhibition. As an alternative explanation, searching for genes displaying a selected pattern of expression may obscure or lead to an incomplete analysis of the data. Indeed, as mentioned above, the genes found through the unrestricted analysis of the microarray data also included most of the genes reportedly altered in *Bmpr1a* conditional KO and *Noggin* transgenic mice. Nevertheless this group of genes was

Figure 9. Alterations in L84 epidermal stem cell population. (A and A') Skin sections from control (A) and L84 (A') mice by pnd 28 stained with color-coded antibodies showing the expression and localization of keratin K15 (red) and CD34 (green). Note the apparent increase in the double positive cells in L84 hair follicle. (B and B') Skin sections from control (B) and L84 (B') after label retaining cell (LRC) experiments. Bars, 250 μ m. Brackets denote bulge region (b). Sebaceous glands are denoted by sg. Arrowheads denote BrdU-positive follicle cells. (C) Summary of three independent LRC experiments showing the percentage of hair follicles containing positive cells as previously reported (Ruiz *et al.*, 2004). (D, D', and E) FACS analyses of control (D) and L84 (D') sorted by $\alpha 6$ -integrin and CD34 expression. D and D' are representative examples showing the CD34-positive $\alpha 6$ -integrin low and CD34-positive $\alpha 6$ -integrin high expressing cells (boxes). (E) Summary of three independent FACS experiments showing the percentage of CD34+ $\alpha 6^{high}$ and CD34+ $\alpha 6^{low}$ from control (□) and L84 (■) mice (* $p \leq 0.005$). (F and F') Representative examples showing the appearance of the colonies derived from control (F) and L84 (F') 5 d after plating. Bar, 150 μ m. (G) Representative example of the colony-forming efficiency of primary keratinocytes derived from control and L84 adult (8 wk) mice 3 wk after plating. Data in C and E are shown as mean $\pm$ SEM.

also altered in wtAkt transgenic mice that do not display ectodermal defects.

Several lines of evidence have previously shown an association between BMP and PI3K/Akt signaling pathways (Waite and Eng, 2003; He *et al.*, 2004; Tian *et al.*, 2005). In particular it has been shown that altered BMP signaling can, through the modulation of PTEN expression and activity, control the activity of the PI3K/Akt signaling pathway (Tian *et al.*, 2005; Zhang *et al.*, 2006b; Kobiela *et al.*, 2007). The present data complement these observations and show

through microarray and histology analyses that deregulated Akt activity affects BMP signaling and, as an overall consequence, the BMP pathway is at least partially inhibited. Furthermore, the existence of an autoregulatory loop between BMP and PI3K/PTEN/Akt signaling may exist such that each element is subject to the control of the other, and importantly, disruption of this balance may lead to altered ectodermal development and tumor formation. The molecular bases of this alteration in *Bmp4* expression are not known at present; however, among the multiple regulators

of *Bmp4* gene expression there are several putative candidates that can be modulated by Akt activity. In this regard, p65 *RelA*, which can be activated by Akt (Madrid *et al.*, 2000, 2001), is a transcriptional repressor of *Bmp4* gene expression in vivo (Zhu *et al.*, 2007), whereas *Bmp4* transcription is activated by forkhead and NKX2 transcription factors (Zhu *et al.*, 2004; Begum *et al.*, 2005), which are inactivated by Akt (Burgering and Kops, 2002; Naito *et al.*, 2003). In this regard, the altered expression and distribution of Foxo3a observed in L84 epidermis might explain the reduced expression of *Bmp4*. Further studies will help to clarify the functional impact of Akt activity on *Bmp4* expression.

Many of the alterations observed in the ectodermal organs of myrAkt mice were similar to the defects present in mice resembling human ectodermal dysplasia syndromes (Thesleff, 2006). The possibility that Akt may be involved in these disorders is very intriguing and would certainly merit further investigations. In some cases this group of diseases is associated with altered Δ Np63 expression (Koster and Roop, 2004). The finding that expression level of this protein was not altered in myrAkt transgenic mice relative to nontransgenic mice indicates that other targets of Akt kinase may be responsible for the observed phenotype. In this regard, the previously reported cross talk between the glucocorticoid receptor and Akt (Leis *et al.*, 2004) and the involvement of the glucocorticoid receptor in certain ectodermal dysplasia syndromes (Perez *et al.*, 2001; Cascallana *et al.*, 2005) reinforces the possibility that Akt may also be involved in some of these disorders.

In the current study we observed that deregulated Akt activity results in altered homeostasis of adult epidermal stem cells. This result is in agreement with the reported involvement of the PTEN/Akt pathway in the maintenance of other adult stem cells (Li *et al.*, 2002, 2003; Cheung and Mak, 2006; Rossi and Weissman, 2006; Yilmaz *et al.*, 2006; Zhang *et al.*, 2006a). On the other hand, the alterations in stem cells observed in the epidermis of myrAkt mice also agree with the reported modulation of these cells by BMP signaling in epidermis and other tissues (Kobiela *et al.*, 2003; Rajan *et al.*, 2003; He *et al.*, 2004; Zhang *et al.*, 2006b; Kobiela *et al.*, 2007). Unexpectedly, we also observed that expression of myrAkt specifically affects the subpopulation of epidermal stem cells characterized by low integrin $\alpha 6$ expression. It has been reported that this suprabasal cell population is derived from that which maintains basal lamina contact and arises only after the start of the first postnatal hair cycle (Blanpain *et al.*, 2004). Our data implicate that Akt may affect the transition between these two cell populations and would suggest that Akt may control the cross talk between stem cells and the niche microenvironment.

Collectively, we present evidence that ectodermal organ development is dependent on accurate Akt signaling and that deregulation of this activity results in altered development of these organs, which in the case of skin proceeds through altered BMP signaling and affects epidermal stem cell population.

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