Sustained Akt Activity Is Required to Maintain Cell Viability in Seborrheic Keratosis, a Benign Epithelial Tumor

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Seborrheic keratoses (SKs) are common benign skin tumors that share many morphological features with their malignant counterpart, squamous cell carcinoma. SKs frequently have acquired oncogenic mutations in the receptor tyrosine kinase/phosphatidylinositol 3-kinase/Akt signaling cascade. We developed a reliable culture system to study SKs in vitro and screened these cells using a library of selective kinase inhibitors to evaluate effects on cell survival. These benign tumors are sensitive to inhibition by ATP-competitive Akt inhibitors, including A-443654 and GSK690693. RNA interference-mediated Akt suppression mimicked the effects of enzyme inhibition in cultured cells. Akt inhibition suppressed phosphorylation of downstream targets of Akt kinase that are critical for cell survival, including MDM2 and FOXO3a, and induced apoptosis. Cell death was also dependent on p53, mutations in which, although common in cutaneous squamous cell carcinoma, have not been identified in SKs. Intact explants of SKs were also sensitive to Akt inhibition. In addition to the obvious therapeutic implications of these findings, identifying the signaling characteristics that differentiate benign and malignant tumors may inform our understanding of the malignant state.

INTRODUCTION

Human skin is continuously exposed to an onslaught of environmental stresses, the most prominent of which is UV light (Armstrong and Kricker, 2001; Hildesheim and Fornace, 2004; Kulms and Schwarz, 2000). Chronic exposure to UV radiation leads to oxidative overload and irreparable DNA damage. The biological consequences of these changes are accelerated aging and benign as well as malignant tumor formation (Agar et al., 2004). Although it is widely accepted that malignant transformation is the result of sequential genomic alterations in oncogenes and tumor suppressor genes, much less is known about the etiology of benign tumors. Recent advances have shown that some benign lesions without malignant potential also accumulate a number of genomic aberrations (Hafner et al., 2006, 2007b). The most common example of this phenomenon is seborrheic keratosis (SK). SKs are highly prevalent, benign tumors. The clinical appearance of these lesions varies, but they usually present as brown warty growths, most often on the trunk or face. Histologically, SKs typically show a discrete proliferation of basaloïd cells in the spinous layer of the epidermis. SK does not represent a reactive epidermal hyperplasia but more likely results from clonal expansion of a somatically mutated keratinocyte (Nakamura et al., 2001). Strikingly, despite their lack of malignant potential, recent studies show that more than 80% of SKs have at least one mutation, and 45% have more than one mutation, in a bona fide oncogene such as FGFR3, PIK3CA, KRAS, and/or EGFR (Hafner et al., 2010c).

Mutations identical to those found in SKs are observed in malignant tumors and, when overexpressed in cells or transgenic animals, can induce malignant transformation. Similarly, our attempt to identify important, specific molecular determinants of SKs resulted in the detection of increased expression of growth factors and other genes thought to be involved in epithelial tumorigenesis or keratinocyte differentiation (Mandinova et al., 2009). Therefore, the observation that SKs rarely, if ever, become malignant is perplexing. In contradistinction, benign melanocytic nevi, although frequently harboring activating mutations in the melanoma-associated signaling molecule b-Raf, associated with malignant melanoma (Taube et al., 2009), do show a risk, albeit small, of malignant transformation (Bevona et al., 2003). Therefore, SKs are a model for studying benign tumors that are resistant to transformation and allow us to investigate how molecular signaling driven by activated oncogenes functions in nontransformed cells.

The most frequently mutated genes in SKs are FGFR3 and the p110 catalytic subunit of phosphatidylinositol 3-kinase.

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Abbreviations: P13K, phosphatidylinositol 3-kinase; SCC, squamous cell carcinoma; SK, seborrheic keratosis

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Both of these proteins impinge on Akt kinase, the stimulation of which enhances the survival of cells by blocking the p53 pathway and the FOXO-mediated proapoptotic cascade (Manning and Cantley, 2007). Although recent reports indicate that SKs show significantly elevated levels of phosphorylated Akt compared to normal skin, little is known about the biological significance of these findings. In the past, efforts to study SKs were hampered by the inability to culture the cells in vitro, a problem common to many benign tumors. We have overcome this obstacle and also developed an explant technique that permits the biopsied SK specimen to be studied en bloc for several days in the laboratory. Taking advantage of these two advances, we utilized panels of specific signaling kinase inhibitors to map out the molecular pathways that are critical for SK cell viability. Small-molecule–based interrogation of key signaling pathways in the past has provided important evidence on the biology of benign tumors in other systems (Arbiser et al., 2002). We find that the signaling kinase Akt is critical in preventing SK cells from undergoing programmed cell death. Both small-molecule Akt inhibitors and Akt small interfering RNA knockdown induce caspase-dependent cell death via FOXO3a. Endogenous wild-type p53 also appears to be critical in directing the apoptotic program after Akt inhibition.

RESULTS
Small-molecule screening identifies essential survival pathways for benign SK lesions
Cells from benign tumors often undergo senescence or enter a nonreplicative quiescent state when placed in culture. We were able to establish reliable culture conditions (see Materials and Methods) to circumvent this problem with SKs. Preparation of the cells included an initial physical dissociation step and extended exposure to dispase. This procedure allowed us to expand SK cells in culture through at least three cell passages, an adequate time to conduct many in vitro studies. Figure 1a shows cells from three typical SK lesions and control keratinocytes in culture. Sequencing the commonly mutated exons of FGFR3 and PI3K within individual cultures identified four distinct cell populations: cells with mutations in either the FGFR3 or PI3K genes, in both of these genes, or in neither of these two genes. Specific mutations in FGFR3 and PI3KCA have been well characterized and occur, on average, in 71% (FGFR3) and 50% (PI3KCA) of all sporadic SK tumors (Hafner et al., 2010a). As in internal control, the cultured cells can be distinguished as SK cells and not, for example, contaminating normal keratinocytes, by the presence of high levels of FOXN1 mRNA and protein in quantitative reverse transcriptase-PCR and western blots assays, respectively (Figure 1b and c). FOXN1 is abundantly expressed in all tested SKs compared to normal skin. In malignant keratinocytic tumors, such as squamous cell carcinoma (SCC), FOXN1 RNA is suppressed to levels even further below those seen in normal skin cells (Mandinova et al., 2009). Thus, an abundance of FOXN1 RNA is a specific marker for SK cells, both in vivo and in cell culture.

Several lines of evidence suggest that SKs have a higher proliferative rate than normal keratinocytes and that apoptosis is suppressed in SKs compared to normal skin (Hafner et al., 2007a; Simionescu et al., 2012). It seemed reasonable to assume that because the established SK–associated mutations are known to drive survival in malignant tumors, they would also be intrinsically related to abnormal growth and survival in SKs. To determine the relative importance of the signaling molecules within the receptor tyrosine kinase/PI3K/Akt/mTOR and Ras/mitogen-activated protein kinase kinase/extracellular signal–regulated kinases pathways on SK cell survival, cultured SK cells were exposed to a panel of specific inhibitors targeting this pathway (see Supplementary Table S1 online). Strikingly, only ATP-competitive Akt inhibitors, including A-443654 (A44) had a strong effect on cell viability (Figure 2a) (Luo et al., 2005). Two other kinase inhibitors, namely, PI-103 and Torin2, had much weaker, albeit reproducible, effects on SK cell viability. PI-103 is an ATP-competitive inhibitor of PI3K and DNA-PK kinases, whereas Torin is an ATP-competitive inhibitor of mTOR (Liu et al., 2011). Because FGFR3 and PI3KCA are frequently mutated in SK, we anticipated that inhibition of these enzymes would impact SK cell survival in culture, but, surprisingly, highly potent inhibitors of both FGFR3 tyrosine kinase (FGF1-1 and PD1739074) and PI3K kinase (GDC-0941) were largely ineffective in impacting cell viability. None of the ATP-dependent Akt inhibitors tested affected the viability of primary human keratinocytes (Figure 2b). Additionally, these inhibitors did not acutely affect the survival of SCC cell lines (Figure 2c). SK lesions have similar, often overlapping genomic alterations with SCC cells (Mandinova et al., 2009) and yet exhibit an exclusively benign clinical phenotype in contrast to the well-known malignant nature of cutaneous SCC. This suggests that small-molecule inhibition of Akt disables a critical survival pathway essential only for benign lesions and not for malignant tumors, at least in keratinocytes.

ATP-competitive inhibition of Akt signaling leads to cell death in primary SK cells
Surprisingly, MK2206, the other Akt inhibitor present in our compound collection, did not affect the viability of SK cells (Figure 3a). In contrast to A44, which inhibits the Akt signaling pathway by competing with ATP for the active kinase site, MK2206 is an allosteric inhibitor. These results are similar to differences that have been observed for allosteric mTOR inhibitors relative to the newer generation of ATP-competitive mTOR inhibitors. Here it has been shown that the ATP-competitive inhibitors result in more complete suppression of mTORC1 and exhibit substantially enhanced antiproliferative activity relative to the allosteric rapalogs (Hsu et al., 2011; Liu et al., 2012; Thoreen et al., 2009). In order to verify the activity of A44 in our assay, we studied the phosphorylation status of Akt in the treated SK cells and confirmed the paradoxical hyperphosphorylation of Akt at Thr308 and Ser473 characteristic of ATP-competitive Akt inhibitors. As recently reported, this hyperphosphorylation is due to the docking of the compound within the ATP-binding site, which blocks the interaction of Akt with its downstream targets (Okuzumi et al., 2009). SK cells treated with the Akt inhibitor A44 have supranormal levels of phospho-Akt, whereas the allosteric inhibitor MK2206 suppresses phosphorylation of Akt (Figure 3b). Akt downstream signaling was shut down in SK cells upon treatment with A44, whereas
suppression of substrate phosphorylation with MK2206 was weaker (Figure 3c). This weaker effect on blocking phosphorylation of Akt substrates might underlie the differences seen with the various Akt inhibitor subclasses in the SK viability assay (Figure 3d). Next, we extended our observations on the specific effects of both classes of Akt inhibitors and demonstrated that another allosteric Akt inhibitor (KIN102) is not toxic to SK cells, whereas an additional ATP-competitive molecule (GSK690693 [“GSK69”]) decreased the survival of SK cells (Figure 3d). Because our data indicate that SK lesions are dependent on activated Akt signaling for survival, at least in cell culture, we sought to confirm that primary lesions from our patients had evidence of pathway activation, as reported previously by Hafner et al. (2007b). Protein lysates from paired normal skin and SKs from individual patients were blotted and probed with antibodies to Akt and the Ser473-phosphorylated form of Akt (see Supplementary Figure S1 online). In accordance with previous results, most SKs show elevated baseline levels of activated Akt.

Although the ATP-competitive Akt inhibitors A44 and GSK69 both killed SK cells in culture (Figure 4a), their chemical structures are quite different (Figure 4b), as are the magnitudes of their effects on p-Akt levels (Figure 4c). Because many ATP-competitive AGC kinase inhibitors block multiple kinases, we compared the spectrum of kinase targets of the two most potent Akt inhibitors, A44 and GSK69. Apart from strong inhibition of the three Akt isoforms, the only significant overlap in kinase inhibition between the two compounds was to three relatives of protein kinase C (Figure 4d). To rule out a significant killing effect related to PKC inhibition, we were able to demonstrate that the pan-PKC inhibitor sotraspaurin did not significantly affect SK cell viability in our colorimetric SRB assay (Figure 4e), suggesting that Akt kinase is indeed the functional target of both A44 and GSK69. Additionally, RNA interference-mediated suppression of the Akt-1, Akt-2 and Akt-3 isoforms mimicked the effect on SK killing seen with A44 and GSK69, confirming our hypothesis that Akt activity is necessary for SK viability (Figure 4f).

Both p53 and FOXO3a participate in SK cell death after Akt inhibition

Akt kinase activity in cells is known to elicit prosurvival effects through activation/suppression of several pathways (Manning and Cantley, 2007). One of the common mechanisms for cell death upon Akt inhibition is induction of apoptosis, or programmed cell death. This mode of cell death was observed upon treatment of SK cells with A44, as evidenced by high levels of cleaved poly ADP ribose polymerase (Figure 5a) and positive TUNEL staining (Figure 5b). Akt activation enhances cell survival in part by phosphorylating MDM2 and FOXO3a (Brunet et al., 1999; Gottlieb et al., 2002). Phosphorylated MDM2 binds p53, leading to ubiquitination and concomitant p53 degradation (Gottlieb et al., 2002). Although SKs do not harbor mutations in p53 (Hafner et al., 2007b), decreased levels of the p53 protein were identified in patient SK samples (see Supplementary Figure S2 online). FOXO3a is transcription factor that regulates the level of several proapoptotic proteins, among them the Bcl2-related protein BIM (Finnberg...
Phosphorylation of FOXO3a blocks nuclear localization and hence transcriptional activity. Both MDM2 and FOXO proteins showed diminished phosphorylation after exposure to A44 (Figure 5c). In addition, levels of p53 were seen to increase with decreased phospho-MDM2 levels, as expected (Figure 5c). Although we did not observe elevation of p53 mRNA levels upon A44 treatment (see Supplementary Figure S3 online), the increase of p53 protein levels was comparable to the effects of a pharmacological inhibition of MDM2 by the small molecule Nutlin-3 (Figure 5d). Similarly, treatment of SK cells with Nutlin-3 induced a dose-dependent increase in cell death (Figure 5e). Importantly, when we depleted FoxO3 expression or p53 through small interfering RNA in primary SK cells, we were able to partially rescue the SK cells from the killing activity of A44 (Figure 5f and g). Inhibition of Akt leads to a decrease of FOXO3a phosphorylation and concomitant increase of baseline p53 levels, possibly through decreased MDM2-mediated degradation (Gottlieb et al., 2002; Zhou et al., 2001). Apoptosis ensues after the blockade of Akt, as seen in some malignant nonkeratinocytic cell lines (Fala et al., 2008).

**Akt inhibitors induce apoptotic cell death in intact SK lesions**

In order to assess whether the killing effect of Akt inhibitors was related to the specific state of the SK cells induced by the process of culture or whether in vivo SKs would also be sensitive to Akt inhibition, an explant system was developed to allow us to monitor cell morphology in intact tumors (Kolev et al., 2008). Whole SKs were removed from patients and placed with the basal side (basement membrane) down onto filters that were in contact with growth media. The edges of the lesions were sealed with semisoft agar, and the apical surface (stratum corneum) was in contact with air, recapitulating keratinocyte cell polarity in vivo. The SK explants were exposed on the apical surface to a 1 mM DMSO solution of A44. After 48 hours, distinct morphological changes became apparent. SK cells began to lose their intercellular connections, and spongiosis (intraepithelial edema) and acantholysis (breakdown of cell-cell contact) were visible with hematoxylin and eosin staining (Figure 6a). Activated caspase-3, a marker for programmed cell death, was identified by immunocytochemistry (Figure 6b). Explants exposed to A44 also showed dramatic increases in TUNEL-positive staining (Figure 6c). In contrast, normal human skin explants and SCCs exposed to a similar dose of A44 did not show significant increases in TUNEL-positive staining or activated caspase, mirroring the lack of toxicity of this compound in normal keratinocytes observed in vitro (Figure 6c and Supplementary Figures S4 and S5 online). These findings suggest that the A44-induced cell death in SKs is not simply due to the mechanical disruption of the lesions before cell culture or an artifact of the culture system, but that the in vitro culture system likely preserves many of the critical attributes of SK cells in vivo.

**DISCUSSION**

Our laboratory is investigating the molecular differences between benign and malignant epidermal tumors. SK and SCC are both clonal tumors derived from keratinocytes, but they have very different clinical behaviors. Whereas SCC can locally invade and sometimes metastasize, SK remains in situ indefinitely. SK is not a precursor lesion to SCC. This stands in contrast to the relationship between nevi and melanoma. Although rare, nevi may devolve into melanoma, and many melanomas are histologically contiguous with nevi that share the same “driver” mutations, the most prominent of which is the V600E B-Raf mutation (Thomas, 2006). In benign...
nevi, excess B-Raf activity arrests proliferation, increases expression of the tumor suppressor p16, and induces senescence-associated β-galactosidase (Michaloglou et al., 2005), a state termed oncogene-induced senescence. Additional mutations are required to reverse this state, including activation of the PI3K pathway by loss of the PTEN tumor suppressor, an event that may precede the development of melanoma.

Figure 3. Differential effect of small-molecule Akt inhibitors on primary seborrheic keratosis (SK) cells. (a) Primary SK cells were treated with either DMSO control, equal doses of the ATP-competitive A44 inhibitor, or the allosteric MK2206 inhibitor for 48 hours, and cell morphology was observed. Bar = 32 μm. (b, c) Primary SK cells were treated with compounds as in panel a for 4 hours and subjected to analysis of the Akt signaling pathway by western blotting for relevant proteins. Anti vinculin antibodies were used to assess equal protein loading in the lanes (left panels). Western blot signals were quantified using the UN-SCAN-IT software (Silk Scientific, Orem, UT) (right panels). (d) Primary SK cells were treated as in panel a, and cell viability was measured by alamarBlue assay. Viable cells are presented as percentage of the control treatment group.
SKs never cross the threshold into malignancy, although they usually contain one or more activating mutations in well-known oncogenes, notably FGFR3, KRAS, and PI3KCA. Unlike the situation in the melanocytic nevus, oncogenic activation in SK does not lead to senescence. The cells are not growth-arrested and do not produce b-galactosidase, a common senescence marker (Hafner et al., 2007b). Although one report showed increased staining for p16 in SK (Nakamura and Nishioka, 2003), we have not been able to confirm this finding (data not shown).

This study demonstrates that SK cells are characterized by overactive Akt signaling. In other cell types, unregulated Akt signaling is associated with oncogenesis (Carnero, 2010). Even with SCC and basal cell carcinoma, both of which are derived from keratinocytes, Akt is hyperactivated (Hafner et al., 2010b). It is surprising then that SKs, which harbor malignancy-associated mutations, remain clinically inert indefinitely.

SKs have a defect in cornification. Cornification is a genetic program of epithelial differentiation unique to skin cells, whereby basal keratinocytes undergo an orderly transition to become the enucleated structural building blocks of the stratum corneum. This form of programmed cell death is distinct from apoptosis, which is characterized by the activation of a caspase cascade leading to cytochrome c release from the mitochondria, chromatin condensation and internucleosomal DNA cleavage, breakdown of the cytoskeleton, cell membrane blebbing, and production of apoptotic bodies. In SK, the cornification process is dysfunctional, with some cells living “too long” and some cells undergoing inappropriate intraepithelial cornification, forming so-called pseudocysts. High levels of Akt activity seem to underlie this pathological condition. We have shown that the abrupt inhibition of Akt in SK leads not to reentry into the cornification program but to caspase-associated apoptosis. As with other processes that lead to apoptosis in keratinocytes, most prominently the UV-induced DNA damage response, p53 plays an important role.

One feature that seems uniform in SK is the preservation of wild-type p53 (Hafner et al., 2010c). Strong inhibition of Akt, specifically by ATP-competitive Akt inhibitors, leads to rapid cell death via activation of intrinsic apoptotic...
cascades in both cultured SK cells and ex vivo organotypic cultures of SK lesions. The presence of wild-type p53 appears to be at least partially required for this effect because knockdown of p53 reverses the effect of Akt inhibition. Akt is known to regulate p53 by activating MDM2, which when phosphorylated shunts p53 toward proteolytic degradation. In untreated SK cells, p53 is difficult to detect, whereas A44-treated cells have abundant protein levels (Figure 5 and Supplementary Figure S2). Why p53 mutations, which are extremely common in sun-exposed skin, are not seen in SKs is unclear. It may simply be that keratinocytes with p53 mutations do not or cannot become SKs—they are more likely to become precancerous actinic keratoses or SCC. However, this does not explain why

Figure 5. Inhibition of Akt induces apoptosis in seborrheic keratosis (SK) cells. (a) SK cells were treated with A44 (1 μM) for 24 hours, and levels of cleaved-poly ADP ribose polymerase (cl-PARP) were analyzed by western blotting using actin as an equal loading control. (b) SK cells were treated with A44 (1 μM) for 48 hours, and apoptosis was detected by TUNEL staining (right panel). Bar = 32 μm. The percentage of TUNEL-positive cells from all DAPI (4',6-diamidino-2-phenylindole) (nuclear staining-positive cells was calculated using ImageJ software (U. S. National Institutes of Health, Bethesda, Maryland, http://imagej.nih.gov/ij/) (left panel). (c) SK cells were treated with A44 (1 μM) for 24 hours, and proapoptotic Akt targets were analyzed by western blotting using actin as a loading control. (d) SK cells were treated with Nutlin-3 (20 μM) or A44 (1 μM) for 24 hours, and p53 levels were analyzed by western blotting using vinculin as a loading control. (e) SK cells were treated with the indicated doses of Nutlin-3 or a single dose of A44 (1 μM) for 48 hours, and cell viability was measured by alamarBlue assay. (f) SK cells were transiently transfected with control, FOXO3a, or p53 small interfering RNAs (RNAi) and 48 hours later were treated with A44 (1 μM) for another 48 hours. Cell viability was checked by bright-field microscopy. Bar = 32 μm. (g) Effectiveness of FOXO3a and p53 small interfering RNA (RNAi) transfection for the experiment in panel f was established by assessing protein levels by western blot analysis, with actin or vinculin as loading controls.
Figure 6. Topical treatment of human seborrheic keratosis (SK) explants induces prompt apoptotic cell death. (a) Human SK explants were grown on filter inserts in contact with air (apical surface up) and topically treated with 10% DMSO (control) or 1 mM A44 for 48 hours. The edges of the tissue explants were sealed with semisolid agarose to prevent test compound diffusion to the liquid phase below the filter. The basal sides of the explants were in contact with nutrients from the well. Hematoxylin and eosin staining was performed on frozen sections of the treated explants. Bar = 64 μm (left panels) and 161 μm (right panels). (b) SK explants were treated with DMSO or A44 as in panel a. Immunofluorescent labeling for activated caspase-3 was performed 48 hours after treatment on frozen tissue sections using DAPI (4',6-diamidino-2-phenylindole) as a nuclear counterstain. Bar = 64 μm. (c) SK explants were treated as in panels a and b, and apoptotic response was assessed using TUNEL staining on frozen sections. Bar = 64 μm. The ratio of TUNEL-positive cells to DAPI-stained cells was determined using ImageJ software (U. S. National Institutes of Health, Bethesda, Maryland, http://imagej.nih.gov/ij/). The values in the graph represent the average of three independently analyzed sections.
existing SKs do not accrue new p53 mutations and become malignant tumors.

In the past, studying the common SK has suffered from the lack of practical animal models and the inability to culture SK cells from patients. In this article, we establish simple culture conditions for manipulating SK cells in vitro and present the descriptions of the dominant signaling pathways that are required for SK cell viability. Specific inhibition of Akt kinase activates an intrinsic keratinocyte program leading to apoptosis. We hope that a more complete understanding of the benign tumor state will shed light on how cells resist malignant transformation, providing potential insight into the management of life-threatening malignancies.

MATERIALS AND METHODS

SK cells isolation and culture

SK lesions were removed by curettage and put immediately in DPBS containing an antibiotic and antifungal cocktail. After physical dissociation and washing twice in 70% ethanol and 1× DBS, SK specimens were kept in 5 U/ml dispase (STEMCELL Technologies) for up to 24 hours. After incubation, the specimens were chopped into smaller pieces using forceps and put in 0.25% trypsin/EDTA (Gibco, Waltham, MA) for 5–10 minutes at 37 °C. During the incubation, the samples were mixed carefully with pipetting several times. DMEM was then added to block the trypsin. After passing the suspension through a 100-μm cell strainer (Fisher Scientific, Pittsburgh, PA), the suspension was centrifuged for 5 minutes at 1,000 rpm. Cell pellets was resuspended in an appropriate amount of Keratinocyte-SFM (Gibco) containing MycoZap Plus-PR (Lonza, Basel, Switzerland). Plates coated with collagen solution (STEMCELL Technologies) were used, and the medium was changed every day. In order to generate a sufficient number of cells for more complex experiments, we pooled cells from multiple SK lesions of individual donors. We routinely checked levels of FOXL1 (as measured by quantitative reverse transcriptase-PCR) in these pooled cells as a reliable marker for SK cells.

SK explants

Whole curetted SKs were removed from patients and placed dermal side down onto membranes (8.0-μm polycarbonate membranes, Transwell Permeable Supports, Costar, Pittsburgh, PA) that were in contact with the growth medium RPMI, containing 10% human serum (Gemini Bio Products, West Sacramento, CA) and antibiotics/antimycotics. The edges of the lesions were sealed with semisoft agar, and the apical surface was kept in contact with air.

All studies involving normal human skin, SK-, and SCC-patient derived samples were reviewed by the Institutional Review Board of Massachusetts General Hospital. It was determined that these research activities use discarded material and are exempt from the regulations of studies using human subjects.

Cell viability

SK cells in culture were treated with compounds for 24 hours, and cell viability was measured using alamarBlue reagent (Life Technologies, Waltham, MA) and/or CytoScan SRB Cytotoxicity Assay (G-Biosciences, St. Louis, MO).

Detection and quantification of apoptosis

Western blotting after compound treatment was detected using antibodies to cleaved poly ADP ribose polymerase (Cell Signaling, Danvers, MA), and apoptotic DNA fragmentation (TUNEL) was quantitated with the In Situ Cell Death Detection Kit, TMR red (Roche, Indianapolis, IN) as described by the manufacturer.

RNA isolation, cDNA synthesis, and quantitative reverse transcriptase-PCR

RNA from SKs in cell culture was isolated with QIAcube using the RNeasy Mini Kit (QIAGEN, Hilden, Germany). For RNA isolation from tissues we used TRizol reagent (Life Technologies), and the samples were disrupted in Tissue lyser II (QIAGEN) for 5 minutes at 30 oscillations per second. After the extraction, the RNeasy Mini Kit was used to improve the quality of the isolated RNA. RNA 2 μg was used for cDNA synthesis with the Maxima First Strand cDNA Synthesis Kit (Thermo Scientific, Cambridge, MA). Each quantitative PCR reaction contained 8 μl cDNA (100 ng/μl), 12 μl SYBR Green (Roche), and 2 μl primer mix (20 pm/μl). 36B4 was used as a housekeeping gene: sense: GCAATGTTGCCAGTGTCCTG; anti-sense: GCCCTGACCTTTCAGCAAG.

Primers for the human FOXN1 gene were as follows: sense: GTCTTCCACCTTCTCGAAGGAC; antisense GGAAAGCTGCTCTCATGGAA. Primers for the p53 gene were as follows: sense: AGGCCTTGGAACTCAAGGAT; antisense CTAGTCAGGCCCCTTCTGTC.

Mutational analyses

We used cDNAs synthesized as described in the section on “RNA isolation, cDNA synthesis, and quantitative reverse transcriptase-PCR” as a template to obtain PCR products that after gel purification were sequenced for mutations in PIK3CA and FGFR3 as follows:

PIK3CA primers to sequence region E542(E545)/C1624/33 (PIK3CA F: TGCCCAAGTATCTCATGGAT; PIK3CA R: GGCC AATCTTACCAAGCA)

FGFR3 primers to sequence region 724 (R248C) (FGFR3 742 F: CCATCTCCTGCGTGAAGAAC; FGFR3 742 R: ATTACCTCCACTGCTTGG) and FGFR3 primers to sequence region 652 (1948) (FGFR3 652 F: caccctacaagctgtgt; FGFR3 652 R: cgagctgctagctgctgt)

The sequencing was performed at the DNA Core Facility at Massachusetts General Hospital, and results were analyzed using the BLAST program from the National Center for Biotechnology Information.

Western blotting

Protein from SK cells in culture was isolated with RIPA (radio-immunoprecipitation assay) buffer containing protease and phosphatase inhibitors (all reagents purchased from Boston BioProducts, Ashland, MA). The same buffer was used for tissue protein extraction, but in this case the specimen was chopped in small pieces and disrupted in Tissue lyser II (QIAGEN). Protein concentration in the clear lysates after centrifugation was measured with the Pierce BCA Protein Assay (Pierce Biotechnology, Grand Island, NY). Western blots were quantified using the UN-SCAN-IT software (Silk Scientific, Orem, UT).

Antibodies used in this study, obtained from Cell Signaling, were as follows: phospho-Akt (Thr308) #9275, phospho-AKT (Ser473) #4058, total Akt #9272, pMDM2, p-GSK-3b (Ser9) #9323, GSK-3α/β (D75D3) #5676, cleaved caspase-3 (Asp 175) #9661, phospho-FOXO1(Thr24)/FOXO3a, and phospho-MDM2 (Ser166) #3521.

MDM2 (SMP14) sc-965 was from Santa Cruz Biotechnology (Dallas,
RNA interference transfection
Primary SK cells were transfected with On Target plus Smartpool siRNAs: Human FOXO3 ( #2309) and Human TP53 ( #7157), purchased from Thermo Scientific, and validated Silencer Select AKT1 (s661), AKT2 (s1215), and AKT3 (s 19428) siRNAs from Life Technologies. HiPerPerfect reagent (QIAGEN) was used according to the protocol, and efficiency of transfection was checked after 48 hours with Western blot.

CONFLICT OF INTEREST
VN, SL, and AM are listed as inventors on a patent application entitled “Agents and Methods for Treating and Preventing Seborheic Keratosis.”

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SUPPLEMENTARY MATERIAL
Supplementary material is linked to the online version of the paper at www.jidonline.org, and at http://dx.doi.org/10.1016/j.jid.2015.12.023.

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