

## ORIGINAL ARTICLE

**AKT induces senescence in primary esophageal epithelial cells but is permissive for differentiation as revealed in organotypic culture**K Oyama<sup>1</sup>, T Okawa<sup>1</sup>, H Nakagawa<sup>1</sup>, M Takaoka<sup>1</sup>, CD Andl<sup>1</sup>, S-H Kim<sup>2</sup>, A Klein-Szanto<sup>3</sup>, JA Diehl<sup>4</sup>, M Herlyn<sup>5</sup>, W El-Deiry<sup>2,4,6</sup> and AK Rustgi<sup>1,4,6</sup><sup>1</sup>Gastroenterology Division and Department of Medicine, University of Pennsylvania, Philadelphia, PA, USA; <sup>2</sup>Hematology-Oncology Division and Department of Medicine, University of Pennsylvania, Philadelphia, PA, USA; <sup>3</sup>Department of Pathology, Fox Chase Cancer Center, Philadelphia, PA, USA; <sup>4</sup>Abramson Cancer Center, University of Pennsylvania, Philadelphia, PA, USA; <sup>5</sup>Wistar Institute, University of Pennsylvania, Philadelphia, PA, USA and <sup>6</sup>Department of Genetics, University of Pennsylvania, Philadelphia, PA, USA

**Epidermal growth factor receptor (EGFR) overexpression and activation is critical in the initiation and progression of cancers, especially those of epithelial origin. EGFR activation is associated with the induction of divergent signal transduction pathways and a gamut of cellular processes; however, the cell-type and tissue-type specificity conferred by certain pathways remains to be elucidated. In the context of the esophageal epithelium, a prototype stratified squamous epithelium, EGFR overexpression is relevant in the earliest events of carcinogenesis as modeled in a three-dimensional organotypic culture system. We demonstrate that the phosphatidylinositol 3-kinase (PI3K)/AKT signaling pathway, and not the MEK/MAPK (mitogen-activated protein kinase) pathway, is preferentially activated in EGFR-mediated esophageal epithelial hyperplasia, a premalignant lesion. The hyperplasia was abolished with direct inhibition of PI3K and of AKT but not with inhibition of the MAPK pathway. With the introduction of an inducible AKT vector in both primary and immortalized esophageal epithelial cells, we find that AKT overexpression and activation is permissive for complete epithelial formation in organotypic culture, but imposes a growth constraint in cells grown in monolayer. In organotypic culture, AKT mediates changes related to cell shape and size with an expansion of the differentiated compartment.**

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**Keywords:** AKT; EGFR; proliferation; differentiation; organotypic culture

**Introduction**

Epidermal growth factor receptor (EGFR) is a transmembrane molecule that binds cognate ligands with subsequent activation of its tyrosine kinase residues. This results in the activation of multiple downstream signaling pathways, such as phosphatidylinositol 3-kinase (PI3K) pathway, Ras/mitogen-activated protein kinase (MAPK) pathway, focal adhesion kinase (FAK) pathway and signal transducer and activator of transcription-3 pathway (Hunter, 2000; Prenzel *et al.*, 2001). Activation of PI3K leads to the conversion of phosphatidylinositol-4, 5-diphosphate (PIP2) to phosphatidylinositol-3, 4, 5-triphosphate (PIP3) and activates effector molecules, notably AKT. EGFR is highly expressed in certain epithelial cancers, including breast cancer (Lo *et al.*, 2006), skin cancer (Chan *et al.*, 2004), cervical cancer (Kim *et al.*, 2002) and esophageal cancer (Mandard *et al.*, 2000; Okano *et al.*, 2003), and the activation of AKT is likely important in mediating some, if not many, of the effects of EGFR overexpression. AKT is a serine/threonine kinase and is known as protein kinase B (PKB). AKT facilitates not only antiapoptotic functions, but is involved in other functions, including cell size, glycogen metabolism, cell cycle regulation and cell proliferation. AKT activation is also important in cancer development. Activation of PI3K and AKT are reported to occur in breast (Kirkegaard *et al.*, 2005), ovarian (Xing and Orsulic, 2005), pancreatic (Asano *et al.*, 2004), esophageal (Okano *et al.*, 2000) and other cancers.

The investigation of molecules and pathways, even canonical ones, is hindered at times by the heterogeneity of transformed cell lines and the paucity of primary cells. We and others have developed and characterized three-dimensional (3D) organotypic culture systems to investigate primary, non-transformed esophageal epithelial cells, permitting elucidation of mechanisms that underlie proliferation, differentiation, senescence and apoptosis in a physiologic context (Andl *et al.*, 2003; Harada *et al.*, 2003; Zahir and Weaver, 2004; Debnath and Brugge, 2005; Nelson and Bissell, 2005). For example, Ras-dependent hyperproliferation in

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mammary epithelial cells could not be detected in monolayer culture, but the Ras-activated PI3K signaling pathway is required and sufficient for epithelial cell hyperproliferation in an organotypic culture system (Janda *et al.*, 2002). We find that AKT is preferentially activated, compared to MAPK activation, in the context of EGFR overexpression and activation, suggesting dichotomous roles for these two pathways with EGFR activation. In particular, inducible AKT overexpression and activation results in expansion of the differentiated layers with increased cell size and partial suppression of terminal differentiation as assessed in organotypic culture (3D culture). However, AKT imposes growth arrest upon cells grown in monolayer. These results reveal a specific role for AKT in epithelial stratification and maintenance of the differentiated compartments in the stratified squamous epithelium.

## Results

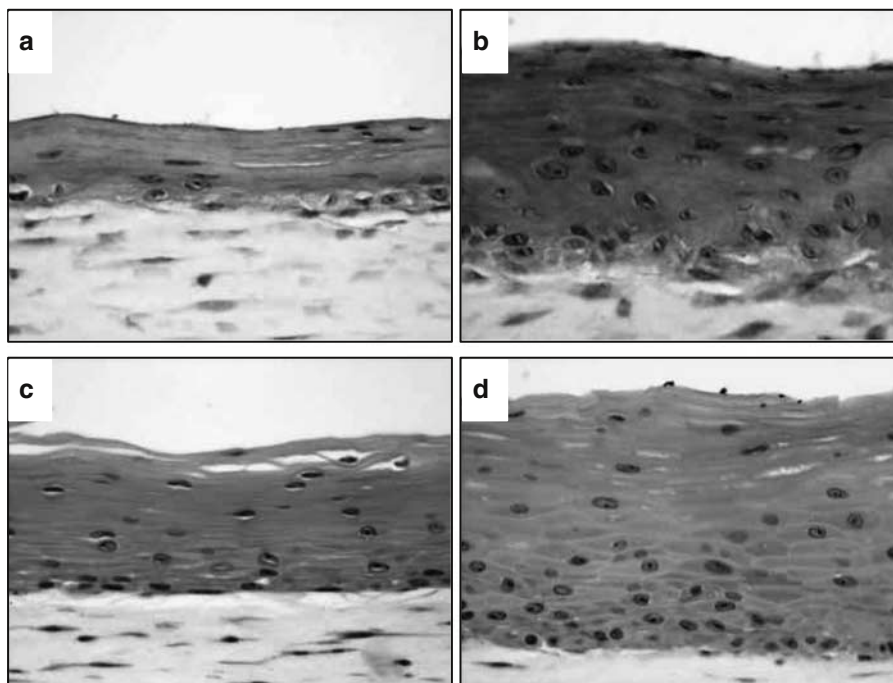
Primary esophageal epithelial cells (EPC) and immortalized EPC-hTERT cells were transduced with a retroviral vector containing wild-type human EGFR with resulting EGFR tyrosine kinase activation. We next cultured EPC-neo (empty vector control), EPC-EGFR (EGFR-induced cells), EPC-hTERT-neo and EPC-hTERT-EGFR in an organotypic culture. Compared with control cells (EPC-neo, EPC-hTERT-neo), EGFR-overexpressing cells resulted in basal cell hyperplasia (Figure 1) and a thicker epithelium (Table 1), both of which are canonical features of preneoplasia.

To elucidate which pathway may contribute to EGFR-induced epithelial hyperplasia, we focused our attention upon PI3K/AKT and MAPK. Whereas phospho-AKT was confined to the basal cell compartment in control cells, it extended beyond this compartment to the mid-zone of the epithelium compartment in EGFR-overexpressing cells (Figure 2). Western blotting indicated that phosphorylated AKT was more enhanced in the EPC-EGFR epithelium than in the EPC-neo epithelium (Figure 2) and in EPC-hTERT-EGFR epithelium than in EPC-hTERT-neo epithelium (data not shown). By contrast, both immunohistochemistry and Western blotting failed to reveal any differences in activated MAPK in these epithelia (Figure 2), suggesting the potential preferential activation of PI3K/AKT.

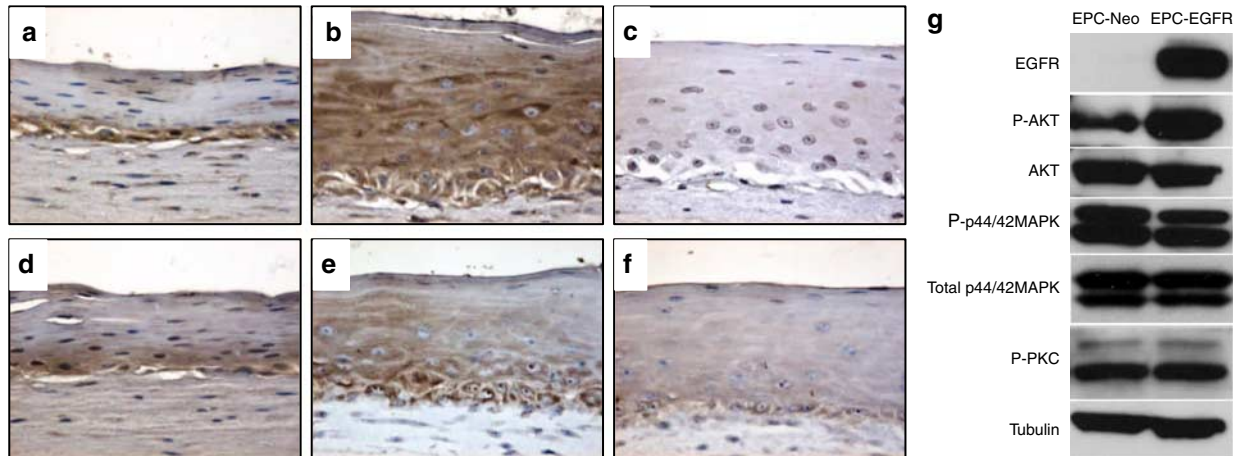
Motivated by these findings, we next determined the influence of LY294002, a PI3K inhibitor, upon the phenotypes that were a consequence of EGFR overexpression. The epithelium shows a gradual decrease in the number of basal keratinocytes and a decrease in overall epithelial thickness in a dose-dependent manner with LY294002 (Figure 3). We also employed an AKT specific inhibitor (designated as AKT<sub>i</sub> 1/2), which yielded the same results (Figure 3). In a manner that was complementary to the MAPK immunohistochemistry in Figure 2, inhibition with U1026, a MEK

**Table 1** Epithelial thickness ( $\mu\text{m}$ )

	<i>Control</i>	<i>EGFR</i>	<i>P-value (t-test)</i>
EPC	0.056 ± 0.008	0.1032 ± 0.026	0.0057
EPC-hTERT	0.096 ± 0.019	0.144 ± 0.020	0.0046



**Figure 1** EGFR overexpression results in increased thickness of the epithelium in EPC and EPC-hTERT cells in organotypic culture. Cell lines used are EPC-neo (a), EPC-EGFR (b), EPC-hTERT (c) and EPC-hTERT-EGFR (d).



**Figure 2** Phosphorylated AKT is overexpressed in EGFR-overexpressing esophageal epithelial cells in organotypic culture. EPC-neo cells transduced with a retroviral vector control form an epithelium with pAKT (a) and pMAPK (d) immunohistochemical staining confined to the basal cell compartment. EGFR-overexpressing cells (EPC-EGFR) demonstrate basal cell hyperplasia (d) with preferentially increased pAKT immunohistochemical staining extending from the basal cell compartment to the mid-zone of the epithelium (b) but pMAPK confined to the basal cell compartment (e). Note, in panels b and e, there are some cells that have increased size (400 $\times$ ). Specificity for pAKT and pMAPK was established with the appropriate blocking peptides (data not shown) and absence of primary antibodies (c and f). Panels a–f, 400 $\times$ . (g) Western blotting from organotypic EPC-neo and EPC-EGFR epithelia reveals increased pAKT in EPC-EGFR. Antibodies specific for EGFR, pAKT, total AKT, pMAPK, total MAPK and tubulin (loading control) were used.

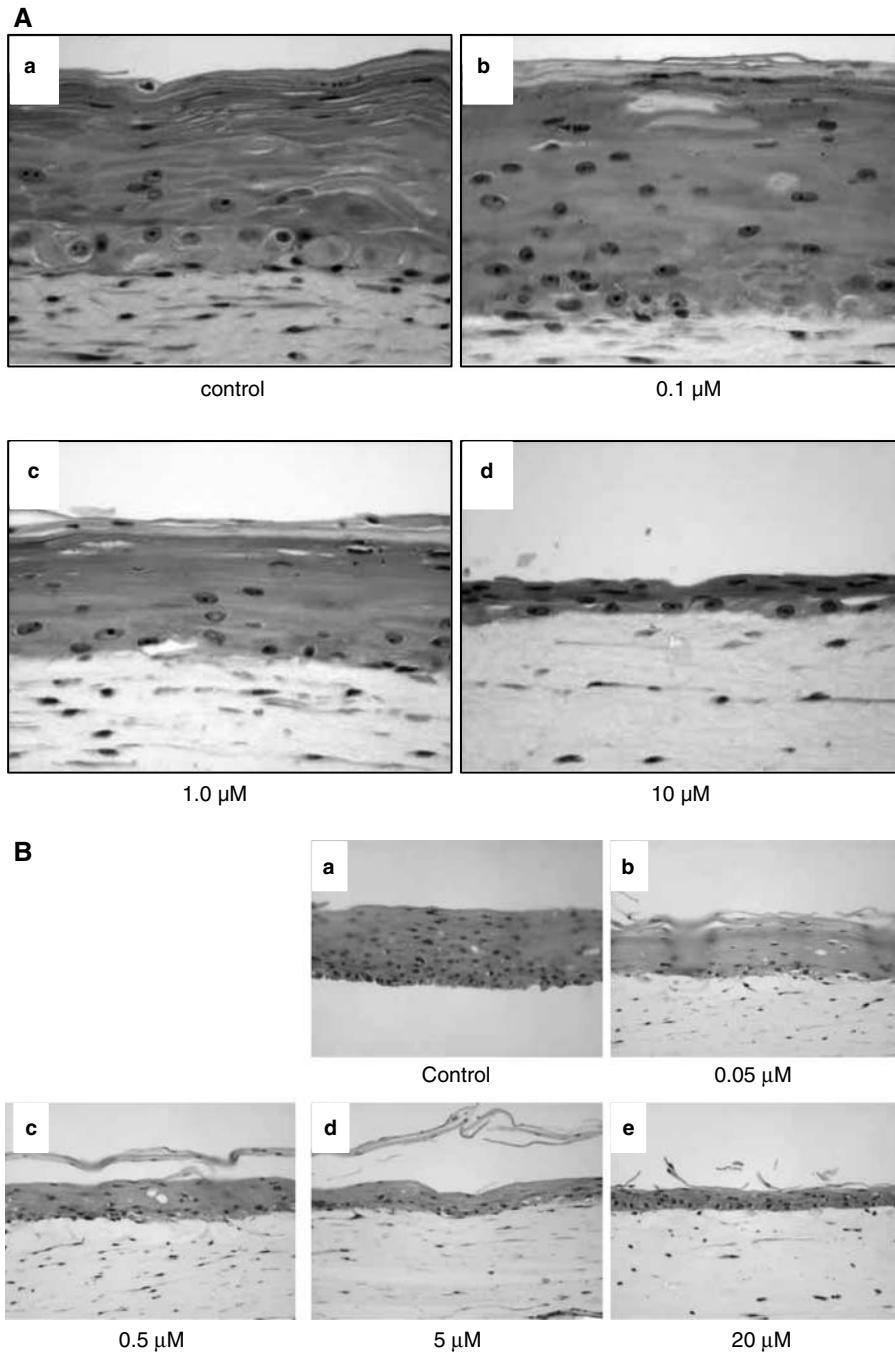
inhibitor, did not affect the thickness of the organotypic cultures (Supplementary Figure 1), suggesting that AKT activation is important. We next undertook a genetic approach. To that end, we transduced a 4-hydroxytamoxifen (4-HT)-inducible AKT vector (designated as pWXL-myrAKT-HA-ER) in EPC-neo and EPC-hTERT-neo cells. Importantly, maximum induction of phospho-AKT was achieved at a concentration of 10 nM 4-HT (Figure 4).

Striking differences were observed between cells grown in monolayer versus those in organotypic culture. Interestingly, cells transduced with the ER-AKT-expressing retrovirus vector grew slower in the presence of 4-HT than those without 4-HT (Figure 5), indicating an effect upon the replicative lifespan of the cells. By contrast, control cells infected with pWXL-A2myrAKT-ER, a mutant version of AKT, did not show any differences in cell morphology and growth in the presence of 4-HT (Figure 5), thereby underscoring the specificity of AKT-mediated effects in this system. Senescence-associated beta-galactosidase (SABG) staining, an established marker of senescence, was increased in a statistically significant manner in EPC-ER-AKT and EPC-hTERT-ER-AKT cells compared to control cells (Figure 5). In order to determine the molecular underpinnings of the senescence, Western blotting revealed the induction of the cyclin-dependent kinase inhibitors, p16, p21 and p27, known to be induced with senescence, (Figure 6) in EPC-ER-AKT and EPC-hTERT-ER-AKT cells compared with control cells.

Interestingly, inducible EPC-ER-AKT cells, by day 14, p21 were increased five-fold and p27 was increased four-fold compared to untreated or uninduced EPC-ER-AKT cells. P16 levels were relatively unchanged. In inducible EPC-hTERT-ER-AKT cells, p27 was

increased over 2700-fold, p21 was increased two-fold and p16 was relatively unchanged. Overall, the morphological changes, increased SABG staining and induction of p21 and p27 are all compatible with senescence of EPC-ER-AKT and EPC-hTERT-ER-AKT cells when compared to control cells, thereby establishing the effects of AKT in these genetically engineered cell lines in monolayer.

We next cultivated EPC cells and EPC-hTERT cells infected with pWXL-myrAKT-HA-ER, or control vector, pWXL-A2myrAKT-ER in organotypic culture. In the absence of AKT induction, EPC and EPC-hTERT showed an essentially identical epithelial phenotype with EPC-neo and EPC-hTERT-neo, respectively. However, with induction of AKT, EPC and EPC-hTERT cells formed a thicker epithelium (Figure 7) as observed in EPC-EGFR cells. Furthermore, phospho-AKT extended to the mid-zone of the epithelium in EPC, reminiscent of what was observed in EPC-EGFR cells, and to the entire epithelium of EPC-hTERT cells, when all cells were induced with 10 nM of 4-HT (Figure 8). For each epithelium, five random fields were selected and statistically analysed. The epithelium of EPC-AKT cells without 4-HT was  $81.6 \pm 18.0 \mu\text{m}$  and that with 10 nM of 4-HT was  $115.7 \pm 18.0 \mu\text{m}$  and statistically thicker ( $P < 0.05$ ) (Table 2). The epithelium of EPC-hTERT-AKT cells without 4-HT was  $132.5 \pm 13.9 \mu\text{m}$  and that with 10 nM of 4-HT was  $216.5 \pm 34.5 \mu\text{m}$  and statistically thicker ( $P < 0.05$ ) (Table 2). In the AKT-induced epithelium, larger cells were observed from the mid-zone to the luminal surface. To characterize further these cells, alcian blue and periodic acid-Schiff stain (PAS) staining were performed (Supplementary Figure 2). PAS staining was negative. However, with alcian blue staining, which

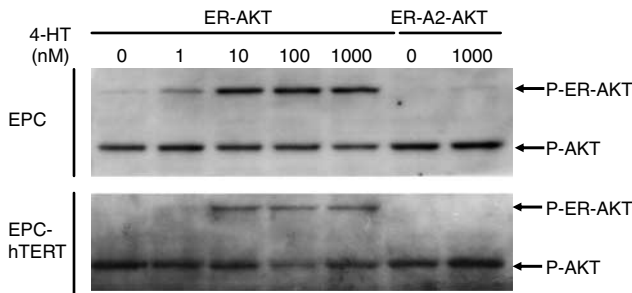


**Figure 3** Inhibition of PI3K and AKT attenuates the phenotype of EPC-EGFR cells in organotypic culture. (A) Hematoxylin–eosin staining of EPC-EGFR cells with the PI3K inhibitor, LY294002. The epithelium shows a gradual decrease in the number of basal keratinocytes and a decrease in overall epithelial thickness in a dose-dependent manner with LY294002. (a) No LY294002; (b–d) 0.1, 1.0  $\mu\text{M}$  and 10  $\mu\text{M}$  of LY294002 compound, respectively. (B) The AKT-specific inhibitor, AKT<sub>1/2</sub>, decreases the epithelial thickness also in a dose-dependent manner (b–e). Dimethyl sulfoxide is used as a control vehicle (a).

detects acid sulfated mucosubstances and hyaluronic acid, the cell membranes in the upper half of the epithelia were positive. In contrast to cells without AKT induction that are flat and with little nuclear content, EPC-hTERT cells with AKT induction are large and retain nuclei, suggesting that normal terminal differentiation is disrupted.

To determine whether AKT overexpression is related to proliferation or not, epithelia were cultured with

5-bromo-2'-deoxyuridine (BrdU) just before they were harvested. BrdU-positive cells were localized only in basal layer and there was no significant difference between AKT-induced epithelium and control epithelium in the percentage of BrdU-positive cells within basal cells both in EPC and EPC-hTERT epithelia (Supplementary Table 1). The lack of differences was also apparent with proliferating cell nuclear antigen



**Figure 4** AKT is induced by 4-HT in EPC and EPC-hTERT cells at day 7. EPC cells (top panel) and EPC-hTERT (bottom panel) infected with pWXL-myrAKT-HA-ER (designated EPC-ER-AKT and EPC-hTERT-ER-AKT, respectively) were stimulated by 4-HT at variable concentrations. As a control, cells infected with pWXL-A2myrAKT-HA-ER (designated EPC-ER-A2-AKT and EPC-hTERT-ER-A2-AKT) were stimulated at concentrations of 0 and 1000 nM. Three hours after stimulation, cells were harvested and protein lysates were assayed by Western blotting.

(PCNA) and caspase-3 immunohistochemistry (Supplementary Figure 3). AKT induction and activation consistently resulted in increased cell size and retained cell nuclei extending from the mid-zone of the epithelium to the luminal surface, consistent with an expansion of the differentiation compartment as normally cells lose their nuclei and slough off into the lumen. Furthermore, we observed fewer keratohyaline granules in the superficial layer with a reduction of 50% of such granules with AKT induction (Figure 7). Reduction in keratohyaline granules indicates a tendency towards less terminal differentiation.

Given that AKT activation appeared to modulate differentiation, we assessed profillagrin, a precursor to fillagrin, a marker of late differentiation. Profillagrin accumulates in keratohyaline granules, and thus, is decreased in expression in EPC and EPC-hTERT epithelia, with 4-HT stimulation (Supplementary Figure 4). The bands corresponding to profillagrin reveal multiple phosphorylated species and cannot interact with intermediate filaments in the mouse. However, while the differentiation compartment and late differentiation may be retarded, late differentiation is not disrupted or irreversibly suppressed in our system. Profillagrin is proteolytically cleaved by excision of the linker to fillagrin, and can interact still with intermediate filaments. This is consistent with our finding that fillagrin protein levels are not decreased and that basic cellular structural integrity is not impaired (Supplementary Figure 4). As separate markers of differentiation, tissue transglutaminase and involucrin were not altered (Supplementary Figure 4).

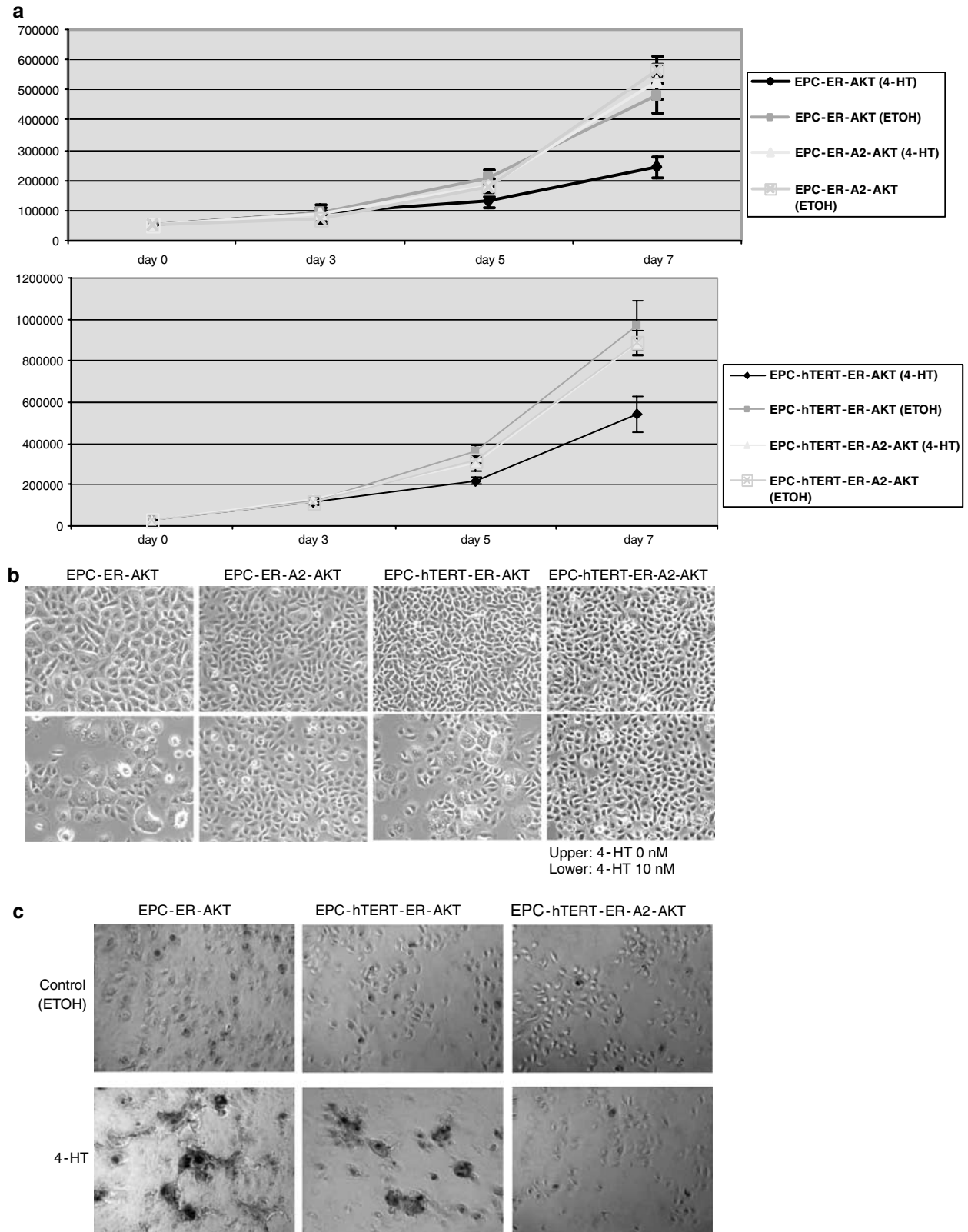
## Discussion

The esophageal epithelium is a prototype stratified squamous epithelium that undergoes an exquisitely regulated program of proliferation, differentiation and apoptosis. Proliferating cells in the basal compartment

migrate in an outward direction towards the luminal surface, and in so doing, undergo early differentiation and eventually terminal differentiation. Cells slough into the lumen, which triggers continuous renewal of the epithelium. EGFR-mediated signaling is critical to the homeostatic maintenance of the proliferation–differentiation gradient. Furthermore, EGFR overexpression and activation is a key parameter in the induction of a preneoplastic state. There is emerging evidence that EGFR-mediated signaling in response to mitogenic growth factors induces preferential activation of different cascades of pathways to execute cellular responses that culminate in proliferation, differentiation, senescence or apoptosis depending upon cellular conditions. Previously, we have demonstrated that in esophageal cancer cell lines, EGF-mediated activation of AKT isoforms is both overlapping and distinctive (Okano *et al.*, 2000). The mechanism by which EGFR recruits the PI3K/AKT pathway was in part differentially regulated at the level of ras but independent of heterodimerization of EGFR with either ErbB2 or ErbB3 based upon functional dissection of pathways in esophageal cancer cell lines (Okano *et al.*, 2000).

To appreciate the role of EGFR in a physiological microenvironment, 3D culture systems assist in the recapitulation of the normal esophageal epithelium, designated as organotypic culture. To that end, EGFR overexpression results in basal cell hyperplasia and expansion of the proliferative compartment to the differentiating suprabasal layer (Andl *et al.*, 2003). Although EGFR activation may trigger a proliferative response, our current studies demonstrate that attenuation of AKT activity abolishes this response, and is not observed with inhibition of MAPK. Inducible activation of AKT through a genetic approach is permissive for an orderly stratification of the esophageal epithelium in organotypic culture, but this is accompanied by increased cell size and partial, but not irreversible, retardation of terminal differentiation as illustrated by decreased keratohyaline granules that in turn contain less profillagrin, and also, increased retained nuclei. The decreased amount of profillagrin in keratohyaline granules may underlie that notion that cells are delayed in a postmitotic state (Kohn *et al.*, 1998). Normal terminal differentiation is associated with increased keratohyaline granules and a reduction in nuclei in superficial layer cells. The amount of keratohyaline granules determines the extent of the keratinization process (Li *et al.*, 2005). Also, in psoriasis, the decrease or absence of keratohyaline granules is associated with abnormal terminal differentiation (parakeratinization) (Li *et al.*, 2005). In leukoplakias of the oral mucosa, parakeratinized and non-keratinized leukoplakic epithelia stained less than orthokeratinized epithelium with fillagrin. These lesions with little or no fillagrin are usually more proliferative and are less keratinized (Murphy, 2005).

AKT is a serine–threonine kinase that has three family members: AKT1, AKT2 and AKT3, which are encoded by three different genes (Itoiz *et al.*, 1985). They are ubiquitously expressed, but their levels are variable



**Figure 5** AKT induces growth arrest and a senescent morphology in EPC and EPC-hTERT cells by day 7 in monolayer culture. **(a)** Growth curves. **(b)** The tamoxifen inducible myrAKT-HA-ER induces morphological changes in EPC-ER-AKT and EPC-hTERT-ER-AKT consistent with senescence that are not apparent in the A2myrAKT-HA-ER overexpressing cells, namely the EPC-ER-A2-AKT and EPC-hTERT-ER-A2-AKT. Upper panel: 4-HT 0 nM; Lower panel: 4-HT 10 nM. **(c)** SABG staining of cells reveals positive staining as a function of AKT activation. **(d and e)** SABG staining over 21 days reveals increased scoring in cells with inducible AKT treated with 4-HT compared to control cells treated with ethanol ( $P < 0.05$ ).

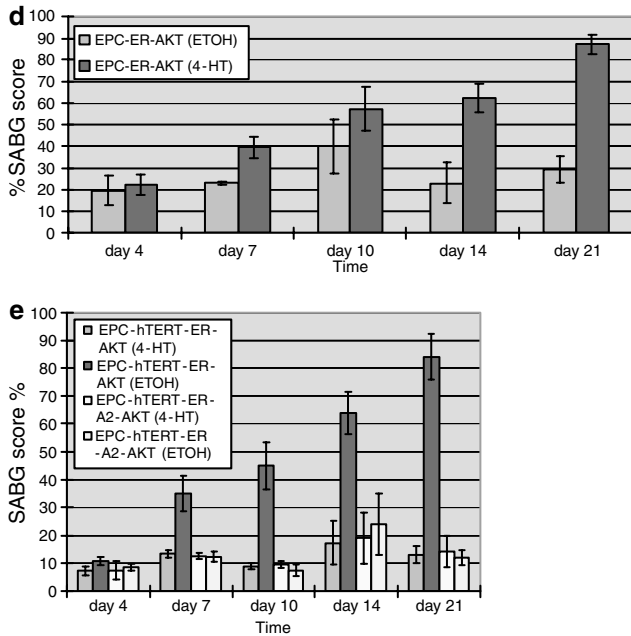


Figure 5 Continued.

depending upon the tissue type. The amino terminus contains a pleckstrin (PH) domain that binds phospholipids, a central kinase domain, and a regulatory serine phosphorylation site in the carboxy terminus. AKT activity is regulated by PI3K, which recruits AKT to the cell membrane, permitting its activation by PDK1 (Itoiz *et al.*, 1985). AKT is phosphorylated and activated by growth factors, including insulin and insulin-growth factor-1. AKT also plays an important role in promoting cell survival (Kandel and Hay, 1999). A number of substrates have been identified, including the proapoptotic proteins BAD and pro-caspase 9. Other substrates include the forkhead transcription factors, chiefly AFX, FKHR and FKHL1, resulting in the inhibition of their transcriptional activities (Datta *et al.*, 1997). The forkhead family of transcription factors plays a pivotal role in the regulation of cell proliferation and differentiation. It has been shown that AKT phosphorylates FKHR1, an event that prevents FKHR1 entry into the nucleus. A potential link between AKT activation and differentiation has also been implied (Kops *et al.*, 1999).

Growth factors and hormones may activate both PI3K and the Raf–Mek–Erk signaling pathways. Cross-talk between the two pathways and inhibition of the Raf–Mek–Erk pathway by AKT may be responsible for switching the biological response from growth arrest to proliferation and may also regulate differentiation. In cancers, AKT may be amplified: AKT1 in gastric adenocarcinomas, AKT2 in pancreatic, breast and ovarian cancers. AKT roles may be quite divergent potentially in primary or normal versus transformed cells. Activation of the serine/threonine kinase AKT/PKB positively impacts on three cellular processes relevant to tumor progression: proliferation/differentiation, survival and cell size/growth.

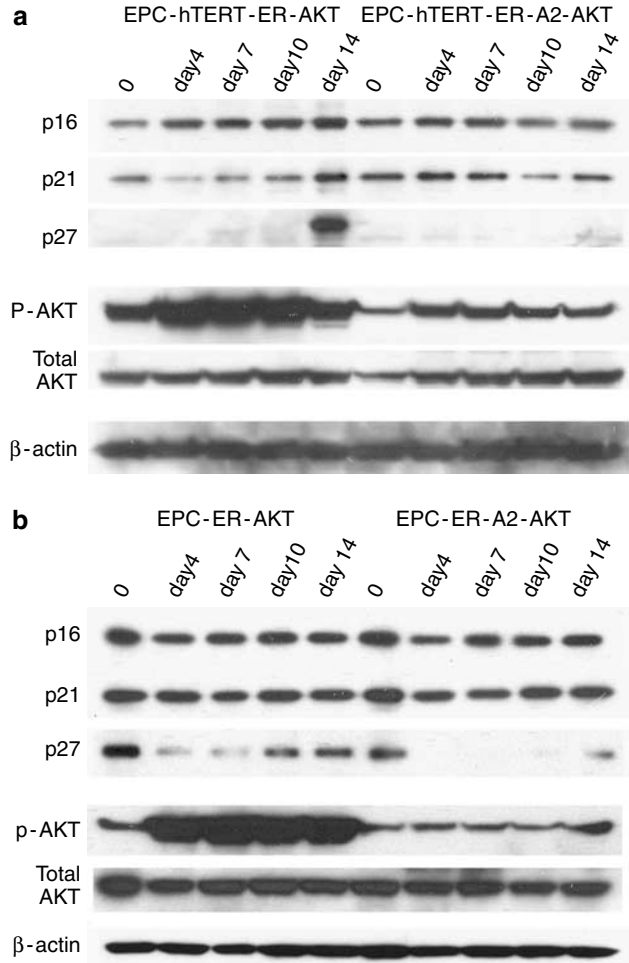
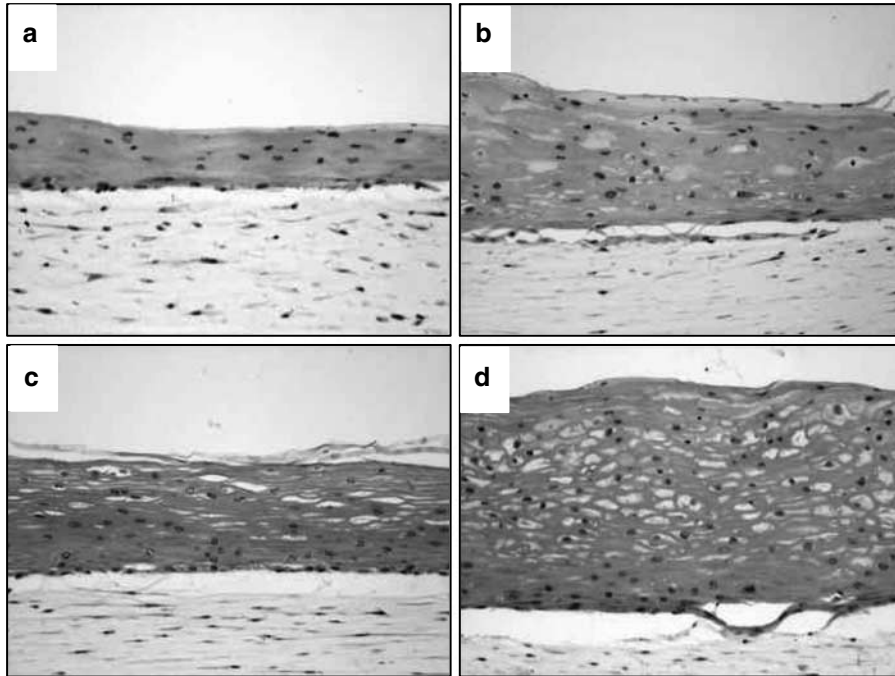
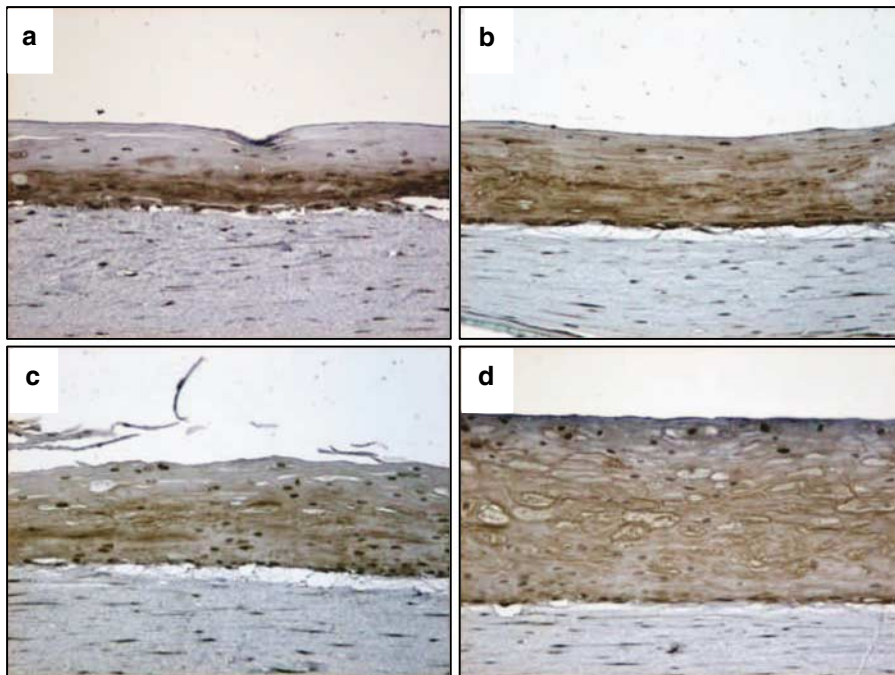


Figure 6 Western blotting of cyclin-dependent kinase inhibitors (p16, p21, p27) with induction of AKT treated with 4-HT in EPC-hTERT-ER-AKT and EPC-ER-AKT cells compared with control treatment (ethanol).

Inhibition of AKT can prolong the lifespan of primary cultured human endothelial cells (Janes *et al.*, 2004), whereas constitutive activation of AKT can foster senescence-like growth arrest via a p53/p21-dependent pathway and inhibition of FOXO3a transcription (Janes *et al.*, 2004). AKT-induced growth arrest was inhibited by a mutated forkhead transcription factor that was resistant to AKT-mediated phosphorylation. We also observe that inducible activation of AKT results in growth arrest and a senescent phenotype in normal primary human epithelial cells as revealed by increased SABG staining and induction of p21 and p27. That AKT activation induces growth arrest in primary esophageal epithelial cells is reminiscent of the effects of oncogenic Ras in primary human cells (Serrano *et al.*, 1997; Miyauchi *et al.*, 2004). Indeed, as with oncogenic Ras, activation of AKT in primary cells may represent an anti-tumorigenic effect. However, in esophageal organotypic culture, AKT does not confer any advantage in proliferation, and has no effect on apoptosis, but



**Figure 7** Organotypic culture of AKT-induced EPC and EPC-hTERT cells. EPC cells with activated myrAKT-HA-ER (designated as EPC-ER-AKT) harbor a thicker epithelium (c) compared with control cells (a) (without 4-HT stimulation; treatment with ethanol). Similarly, the EPC-hTERT cells with activated myrAKT-HA-ER (designated as EPC-hTERT-ER-AKT) form a thicker epithelium (d) compared with control cells (c) (without 4-HT stimulation; treatment with ethanol) (200 $\times$ ).



**Figure 8** pAKT is localized to the basal compartment in EPC-control cells (a) but extends to the mid-zone (c: EPC-ER-AKT) and from the mid-zone (b: EPC-hTERT-control) to the entire epithelium in EPC-hTERT-ER-AKT (d) upon inducible AKT activation with 10 nM 4-HT.

does lead to sustained differentiation. In support of our findings, Epstein–Barr viral LMP2A overexpression in HaCaT cells results in hyperproliferation in raft cultures

and tumorigenicity in nude mice and involves PI3K-dependent activation of AKT with subsequent suppression of differentiation (Lin *et al.*, 1998).

**Table 2** Epithelial thickness ( $\mu\text{m}$ )

Cell type	n	Average	Standard deviation	P-value
EPC-AKT-control (ETOH)	5	0.08160	0.01796	0.01734
EPC-ER-AKT (4-HT)	5	0.11568	0.01799	
EPC-hTERT-AKT-control (ETOH)	5	0.13248	0.01393	0.000999
EPC-hTERT-ER-AKT (4-HT)	5	0.21648	0.03446	

A potential link between AKT and promotion of differentiation may be evident through the discoordinated regulation of ribosomal S6 kinase. Ribosomal S6 kinase is essential in the control of the protein translation machinery and has been linked to the control of cell proliferation. Although activation of both AKT and S6 kinase is PI3K dependent, S6 kinase was not activated significantly in LMP2A-expressing tissue culture cells and its activated form was not detected in LMP2A tumors (Lin *et al.*, 1998). This finding suggests that PI3K-dependent activation of AKT by LMP2A may be uncoupled from S6 kinase activation and induction of proliferation (Lin *et al.*, 1998). We too find there is no AKT-mediated upregulation of S6 kinase in organotypic culture (data not shown), which may help to explain further the discordance between suppression of late differentiation and lack of hyperproliferation.

Further evidence for the role of AKT in differentiation is revealed by the finding that FOXN1 controls interfollicular epidermal differentiation. To that end, FOXN1 induces the early stages of differentiation while increasing the pool of total AKT (Kops *et al.*, 1999). Subsequent activation of AKT triggers completion of the differentiation program and formation of the differentiated granular and cornified layers. By analogy, this may be evident in our system where the consequences of AKT activation are more pronounced in differentiated cells.

It has been demonstrated that PI3K/AKT modulates the differentiation of trophoblast cells (Lin *et al.*, 1998). In particular, activation of Lyn, an Src-related non-receptor tyrosine kinase, is associated with trophoblast giant cell differentiation. Lyn displays a differentiation-dependent association with PI3K/AKT. At least part of the activation of AKT in differentiating trophoblast giant cells involves an autocrine growth arrest-specific Sky (regulator of PI3K/AKT) signaling pathway (Scholle *et al.*, 2000). In osteoclast cells, AKT may play a critical role in differentiation, but is indeed dispensable for survival of isolated osteoclast precursors, indicating a potential uncoupling between AKT-mediated differentiation from initiation of proliferation (Kamei *et al.*, 2002). Instead, mammalian target of rapamycin and Bim, a proapoptotic Bcl-2 family member, are critical for cell survival (Kamei *et al.*, 2002).

In a 3D culture model of MCF-10A mammary cells, it has been demonstrated that AKT regulates the

morphogenesis of polarized epithelial structures. Activation of a conditionally active variant of AKT elicits rather large, misshapen units, which essentially arise from the combined effects of AKT on proliferation and cell size (Sugatani and Hruska, 2005). However, while AKT activation fosters proliferation during the early stages of morphogenesis, it cannot overcome signals suppressing proliferation in late-stage cultures (Sugatani and Hruska, 2005). Our organotypic culture have to be harvested at late-stages (days 12–14) as is defined for keratinocytes, which would be analogous to the inability of AKT to overcome antiproliferative signals in late-stage cultures of MCF-10a mammary cells. We would note the changes in cell size and shape in our model system.

Thus, there is compelling evidence for the activation and role of AKT in differentiation but not proliferation in different cellular contexts. Why does AKT activation in organotypic culture result in partial retardation of terminal differentiation? One explanation may relate to the suppression of both p53 and p21. AKT is known to promote the cytoplasmic localization of p21 and promote nuclear translocation of Mdm2, a negative regulator of p53, thereby causing a reduction in p53 transactivation and levels (Debnath *et al.*, 2003).

In summary, the divergent roles of AKT in primary versus transformed cells are further revealed when primary cells are investigated in the context of two-dimensional as opposed to 3D cultures. It is likely that AKT activation is necessary for the maintenance of differentiation as a function of the initiation of pro-proliferative signals, a necessary sequence of events in the organization and homeostasis of the stratified squamous epithelium.

## Materials and methods

### Cell lines

Primary human esophageal cells (EPC) cells were grown at 37°C and 5% CO<sub>2</sub> with keratinocyte serum-free media (KSFM), with 40  $\mu\text{g}/\text{ml}$  bovine pituitary extract, 1.0 ng/ml EGF, 100U/ml penicillin and 100  $\mu\text{g}/\text{ml}$  streptomycin as described previously (Andl *et al.*, 2003).

### Retroviral vectors

pFB-neo retroviral vectors (Stratagene, La Jolla, CA, USA) were used to infect EPC. In addition, we subcloned into the pFB-neo vector the entire coding sequence for the human EGFR. The pBabe hygromycin-resistant retrovirus vector expressing hTERT was obtained from R Weinberg (Whitehead Institute, Cambridge, MA, USA). The retroviral vector pWZLneo was used to express the conditionally active AKT construct myrAKT  $\Delta 4$ -129-ER (myrAKTER) and the control construct A2myrAKT  $\Delta 4$ -129-ER (A2myrAKTER) (Mayo and Donner, 2001). This myristolated AKT, fused to estrogen receptor, permits constitutive localization to the cell membrane with activation by tamoxifen and activation of ectopically expressed AKT, whereas the mutant construct inhibits cell membrane localization and there is no activation of ectopically expressed AKT by tamoxifen. The plasmids were transfected into Phoenix-Ampho cells by the calcium-phosphate precipitation method (Calphos; Clontech, Palo Alto, CA, USA),

according to the manufacturer's instructions. In brief, culture supernatants from individual Phoenix-Ampho cells were used to infect EPC cells. These cells were infected with filtered (0.45- $\mu$ m pore size) supernatant from an overnight culture of Phoenix-Ampho cells, producing the retroviruses encoding EGFR, hTERT or ER-AKT. Cells were passaged 48 h after infection and selected in 300  $\mu$ g/ml G418 for 14 days or in 10  $\mu$ g/ml hygromycin B (Roche, Basel, Switzerland) for 4 days.

#### Cell growth

EPC cells infected with pWXL-myrAKT-HA-ER or pWXL-A2myrAKT-ER were seeded onto six-well plates at the concentration of  $5 \times 10^4$ . EPC-hTERT cells were seeded at the concentration of  $3 \times 10^4$ . Those cells were cultured with KSFM containing 4-HT (Sigma Aldrich, St Louis, MO, USA). The numbers of cells were counted by triplicate after 3 days, 5 days and 7 days by Coulter Particle Counter (Beckman Coulter™ Inc., Miami, FL, USA). The Student's *t*-test was performed and  $P < 0.05$  was considered statistically significant.

#### SABG staining

The Senescence beta-Galactosidase Staining (SABG) Kit (Cell Signaling Technology Inc., Beverly, MA, USA) was used to assess cellular morphological changes consistent with senescence, according to the manufacturer's protocol. Cells stained for SABG activity were scored by counting five high-power fields ( $\times 200$ ) under phase contrast microscopy.

#### Organotypic culture

To grow human esophageal epithelial cells (keratinocytes),  $5 \times 10^5$  cells were seeded on to the type I collagen matrix, containing 1  $\times$  minimal essential medium with Earle's salts (Bio-Whittaker, Walkersville, MD, USA), 1.68 mM L-glutamine (Cellgro, Herndon, VA, USA), 10% fetal bovine serum (Hyclone, Logan, UT, USA), 0.15% sodium bicarbonate (Bio Whittaker), 76.7% bovine tendon acid-extracted collagen (Organogenesis, Canton, MA, USA) and  $7.5 \times 10^4$  human skin fibroblast cells. Cells were fed with Epidermalization I medium for 2 days, which is Dulbecco's modified Eagle's medium (JRH Biosciences, Lenexa, KS, USA)/Ham's F-12 (Invitrogen) (3:1) supplemented with 4 mM L-glutamine, 0.5  $\mu$ g/ml hydrocortisone, 0.1 mM *O*-phosphorylethanolamine, 20 pM triiodothyronine, 0.18 mM adenine, 2.4 mM CaCl<sub>2</sub>, 4 pM progesterone (Sigma); 10  $\mu$ g/ml insulin, 10  $\mu$ g/ml transferrin, 10 mM ethanolamine, 10 ng/ml selenium (ITES) (Bio Whittaker); and 0.1% chelated newborn calf serum (Hyclone). For the following 2 days, cells were grown in Epidermalization II medium, which is identical to Epidermalization I except that it contains 0.1% unchelated newborn calf serum. Then cells were raised to a liquid-air interface and cultured in Epidermalization III medium for 6 days containing the same growth supplements as Epidermalization II, except for 2% newborn calf serum. Cells were fixed with 10% formaldehyde and embedded in paraffin. Sections were stained with hematoxylin and eosin. Tissues from organotypic culture were incubated with 0.6 U/ml of Dispase 1 (Roche) for 15 min at 37°C, and epithelia were isolated from collagen plates and used for extracting protein.

#### Antibodies and inhibitors

Antibodies against EGFR and tubulin were obtained from Neo Marker (Union City, CA, USA). Antibodies against phospho-AKT (Ser473), phospho-p44/42MAPK (Thr202/Tyr204), phospho-PKC (pan) and caspase-3 were purchased from Cell Signaling Technology (Beverly, MA, USA).

The antibody against AKT was purchased from Rockland Inc. (Gilbertsville, PA, USA). The antibody against PCNA was purchased from DAKO Co. (Carpenteria, CA, USA). The antibodies for p16, p21 and p27 were obtained from Cell Signaling Technology (Beverly, MA, USA). The antibody against Profilin was purchased from Novo Astra Laboratories Ltd (UK). The AKT-specific inhibitor AKT1/2, the PI3K inhibitor LY294002 and the MEK inhibitor U0126 were obtained from Calbiochem (San Diego, CA, USA).

#### Immunohistochemistry

Tissues were fixed in 10% formaldehyde, embedded in paraffin and 6  $\mu$ m micron thick sections were generated. Sections were mounted on adhesive-coated slides, deparaffinized and rehydrated through xylene and alcohol. For antigen retrieval, tissues were incubated in 10 mM of citric acid buffer (Fisher, pH 6.0) for 14 min in a microwave oven. After rinsing in tap water and phosphate-buffered saline (PBS), endogenous peroxidase was blocked with 1.5% H<sub>2</sub>O<sub>2</sub> in dH<sub>2</sub>O for 15 min at room temperature. Sections were blocked with Avidin D blocking reagent and Biotin blocking reagent (Brocking kit, Vector Laboratories Inc., Burlingame, CA, USA) for 15 min each at room temperature, then blocked with Protein Blocking Agent (Immunotech a Coulter Company, Marseille, France) for 10 min at room temperature. They were incubated with primary antibodies at 4°C for overnight. After PBS washes, they were incubated with biotinylated secondary antibody at 37°C for 30 min. After PBS washes, they were incubated with ABC Elite reagent (Vectastain ABC kit Vector Laboratories Inc.), at 37°C for 30 min, and reaction products were developed using diaminobenzidine tetrahydrochloride (DAB substrate kit, Vector Laboratories Inc.) and counterstained with hematoxylin.

#### Western blotting

Epithelial tissues were lysed in lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS and a protease inhibitor mixture tablet (Roche Molecular Systems, Pleasanton, CA, USA)). Protein concentration was determined by the BCA protein assay (Pierce Biotechnology Inc., Rockford, IL, USA). The solution was subsequently solubilized in NuPAGE lithium dodecyl sulfate sample buffer (Invitrogen, Carlsbad, CA, USA) containing 50 mM dithiothreitol. Total protein samples (10  $\mu$ g) were separated on a 4–12% SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Immobilon-P; Millipore Corp., Bedford, MA, USA). The membrane was blocked in 5% non-fat milk (Bio-Rad) in Tris-buffered saline with 0.1% Tween (TBST) (10 mM Tris, 150 mM NaCl, pH 8.0 and 0.1% Tween 20) for 1 h at room temperature. Membranes were probed with primary antibody at appropriate dilutions in 5% TBST milk overnight at 4°C, washed three times in TBST, incubated with anti-mouse or anti-rabbit horseradish peroxidase antibody diluted 1:2000 in TBST for 30 min at room temperature and then washed three times in TBST. The signal was visualized by an enhanced chemiluminescence solution (ECL Plus; Amersham Pharmacia Biotech, Piscataway, NJ, USA) and was exposed to Eastman Kodak Co. X-Omat LS film.

#### Statistical analysis

The differences between the epithelial thickness of EPC and EPC-hTERT cells expressing EGFR or AKT or control vectors were tested using the paired samples *t*-test. A difference was considered statistically significant when  $P < 0.05$ .

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Supplementary Information accompanies the paper on the Oncogene website (<http://www.nature.com/onc>).