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The Protein Kinase Akt Induces Epithelial Mesenchymal Transition and Promotes Enhanced Motility and Invasiveness of Squamous Cell Carcinoma Lines[1]

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ABSTRACT

Epithelial-mesenchymal transition (EMT) is an important process during development and oncogenesis by which epithelial cells acquire fibroblast-like properties and show reduced intercellular adhesion and increased motility. Squamous cell carcinoma lines engineered to express constitutively active Akt underwent EMT, characterized by down-regulation of the epithelial markers desmoplakin, E-cadherin, and β-catenin and up-regulation of the mesenchymal marker vimentin. The cells lost epithelial cell morphology and acquired fibroblast-like properties. Additionally, E-cadherin was down-regulated transcriptionally. The cells expressing constitutively active Akt exhibited reduced cell-cell adhesion, increased motility on fibronectin-coated surfaces, and increased invasiveness in animals. AKT is activated in many human carcinomas, and the AKT-driven EMT may confer the motility required for tissue invasion and metastasis. These findings suggest that future therapies based on AKT inhibition may complement conventional treatments by controlling tumor cell invasion and metastasis.

INTRODUCTION

Most cancer deaths are due to the spread of tumor cells resistant to conventional therapies (1). Metastatic cells acquire genetic and epigenetic changes that cause their aggressive phenotype. The acquisition of increased motility and invasiveness is essential for metastasis (1). Increased motility and invasiveness are associated with decreased cell-cell adhesion, degradation of basement membranes and stroma, and enhanced local growth of tumor cells. Some of these properties of metastatic cells have been linked to down-regulation of E-cadherin (2, 3), a frequently observed phenotypic change usually caused by transcriptional repression (4–6), and to degradation of basement membranes that is initiated by up-regulation of matrix metalloproteinases and collagenases (7).

The oncogenic serine/threonine kinase AKT1 (also known PKBα) is a downstream effector of the PI3K[5] and is frequently activated in human cancer (8). The gene for the closely related AKT2 is amplified and overexpressed in ovarian, pancreatic, breast, and follicular thyroid carcinomas, and AKT2 kinase activity is high in ovarian cancer (9–13). Furthermore, total AKT kinase activity is activated in non-small cell lung cancer, squamous cell carcinomas of the oral cavity, and breast and prostate carcinomas (14–16). In ovarian cancer, *AKT2* amplification and overexpression are associated with undifferentiated histology and aggressive clinical behavior, suggesting that AKT contributes to tumor progression (10).

AKT activation contributes to the neoplastic phenotype. AKT stabilizes the cell cycle inhibitors $p21^{Cip1}$ and $p27^{Kip1}$ and inhibits the transport of both proteins into the nucleus. AKT also enhances the translation of mRNAs for cyclins D1 and D3. These changes lead to increased cyclin-dependent kinase and E2F activity and promote cell cycle progression (reviewed in Ref. 8). AKT also promotes cell survival. The antiapoptotic function of AKT has been linked to inhibition of cytochrome *c* release from mitochondria, stimulation of glucose uptake and utilization, phosphorylation and inactivation of Bad and (pro)caspase 9, activation of nuclear factor κB, overexpression of Bcl-2 or Bcl-xL, and phosphorylation and nuclear exclusion of FKHRL (8, 17). AKT activation is also associated with enhanced tumor cell invasion. AKT enhances invasiveness of pancreatic carcinoma cells via up-regulation of IGF-I (18) and increases secretion of matrix metalloproteinases 2 and 9 from immortalized mammary epithelial cells and ovarian carcinomas (19, 20).

In this article, we investigate the role of AKT in the biology of human squamous cell carcinoma lines and show that AKT activation causes EMT characterized by down-regulation of numerous epithelial cell-specific proteins, including E-cadherin and β-catenin, and up-regulation of the mesenchymal cell-specific protein vimentin. Interestingly, EMT was accompanied by increased *in vivo* cell motility on fibronectin-coated surfaces and increased invasiveness in animals. These findings expand the spectrum of biological activities of AKT and suggest that therapeutic inhibition of AKT may be a useful strategy to control tumor cell invasion and metastasis.

MATERIALS AND METHODS

Cell Culture. Squamous carcinoma cell lines SCC13 and SCC15 were derived from human tongue carcinomas (21). Cells were cultured at 37°C and 5% CO_2 in DMEM supplemented with 10% fetal bovine serum.

Antibodies, Immunoblotting, and Immunostaining. The mouse monoclonal antibodies used were anti-p130cas, anti-β-tubulin, and anti-desmoplakin I/II (gifts from Drs. E. Golemis and M. Pasdar); anti-vimentin (Biodesign); anti-pan-cadherin (Sigma); and antihuman E-cadherin and anti-β-catenin (Transduction Laboratories). The rabbit polyclonal antibodies used were anti-HEF1 and anti-α-catenin (gifts from Drs. E. Golemis and R. Kemler), anti-Akt (19992), anti-phospho-AKT threonine 308 (Cell Signaling), and anti-γ-catenin (Transduction Laboratories).

Immunoblotting was performed as described previously (22). Anti-AKT and anti-HEF1 antibodies were diluted 1:500 before use, anti-p130cas antibody was diluted 1:750 before use, and anti-β-tubulin and anti-γ-catenin antibodies were diluted 1:2000 before use. Anti-α-catenin, anti-β-catenin, anti-E-cadherin, anti-desmoplakin, and anti-pan-cadherin antibodies were used at final concentrations of 2, 0.2, 0.2, 0.5, and 15 μg/ml, respectively. Enhanced chemiluminescence detection was used (ECL; Amersham).

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[5] The abbreviations used are: PI3K, phosphatidylinositol 3′-kinase; EMT, epithelial-mesenchymal transition; IGF, insulin-like growth factor; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; HEF1, human enhancer of filamentation 1; GSK3, glycogen synthase kinase 3.

Immunostaining was performed as described previously (22). Antibody concentrations were 2.5 μg/ml for anti-desmoplakin, 0.3 μg/ml for anti-vimentin, 10 μg/ml for anti-E-cadherin, and 2.5 μg/ml for anti-β-catenin. Slides were examined under a Leica DM IRB light microscope equipped for epifluorescence or a laser scanning confocal microscope driven by Scanware software (Leica). Images were collected using the same settings and processed with Adobe Photoshop 4.0 at identical thresholds to allow semiquantitative comparisons.

Adenoviral Infection. Recombinant adenoviruses encoding β-galactosidase, Akt T308A, or myristylated Akt (myr-Akt) were amplified in HEK293 cells and purified according to standard procedures (23). SCC15 cells were infected with 50 plaque-forming units/cell in serum-free medium. After 3 h, infection was stopped by adding medium containing 20% fetal bovine serum. The cells were infected again 2 days later by the same procedure. Phase-contrast photographs were taken 2 days later. The proportion of infected cells was estimated from the number of cells producing β-galactosidase, as revealed by X-gal staining.

Retroviral Infection. Inserts harboring c-Akt, v-Akt, or myr-Akt were cloned into the retroviral vectors pLXSN (24) or MSV-SRα (25). Infectious viral supernatants were generated by transfection of the amphotropic packaging cell line PA-137 for pLXSN-based constructs and by transient cotransfection of COS cells with the amphotropic packaging plasmid pSV-A-MLV (a gift of Dr. Landau) for the MSV-SRα-based constructs. Retroviral infections involved treating subconfluent cultures of SCC13 and SCC15 with DEAE-dextran (40 μg/ml) for 1 h and then with viral supernatants overnight. G418 (400 μg/ml) was used for selection 48 h after infection, and resistant colonies were pooled. Several mass cultures from independent infections were generated.

Northern Blot Analysis. Total and poly(A)$^+$ RNA were isolated with Roti-Quick and Quick Prep kits, respectively. Samples (20 μg of total RNA or 4 μg of poly(A)$^+$ RNA) were subjected to electrophoresis in 1% agarose formaldehyde gels, transferred onto Hybond N+ membranes, and hybridized with ^{32}P-labeled probes, as indicated. Signals were quantified with Storm 820 using Image Quant 5.2.

Promoter Reporter Assays. SCC15 and SCC15 v-Akt C cells were transiently transfected with Exgen 500 (Euromedex) in 6-well plates. Each well contained serum-free medium containing 1.5 μg of pPGKβgeopA and 2 μg of either pGL3 basic vector or pGL3 hE-cad prom. Both constructs contain the luciferase gene under no promoter (pGL3) or under the wild-type human *E-cadherin* promoter (pGL3 hE-cad prom) (6). Luciferase was assayed by standard procedures, and transfection efficiency was normalized for β-galactosidase activity.

Cell Migration Assay. Freshly trypsinized cells were plated at 10^5 cells on 35-mm Falcon 3004 dishes, coated with 10 μg/ml fibronectin, 25 μg/ml collagen, or 50 μg/ml laminin. The average speed (μm/h) of locomotion in complete medium, for optimal migration (26), was calculated for 20–30 cells for each experimental condition.

Detachment Assay. Cells were seeded at 1.5×10^5 cells/25-cm^2 flask. After 48 h, cells were washed with 5 ml of warm PBS and then trypsinized with 1 ml of fresh 0.25% trypsin (Life Technologies, Inc.) at 20°C with gentle agitation. The number of detached cells was determined at various times, and the total number of cells/flask was determined after complete trypsinization. One flask was used for each time point, and each experiment was performed at least five times independently.

Growth Curve. Cells were seeded at 1×10^5 cells/25-cm^2 flask, fed every other day, and counted every day. Growth curves were constructed, and doubling times were estimated.

Assays for Tumorigenesis and Invasiveness. To determine tumorigenicity, SCC13 cells (5×10^6) were transferred s.c. into athymic nude mice. Eight weeks after injection, mice were killed and examined for gross evidence of tumors. Tissues were collected from the injection area, stained with H&E, and analyzed.

A tracheal invasion assay was performed as described previously (21, 27, 28). Cells (5×10^5) were injected into the lumen of de-epithelialized rat tracheas that were closed with metal clips at both ends and then transplanted s.c. into nude mice. After 4 or 8 weeks, transplanted tracheas were removed and processed for histopathology. Invasiveness was estimated as the extent of penetration into the tracheal wall from the center of the cell mass to the most distal invasive point (28).

RESULTS

Constitutively Active Akt Alters the Morphology of Squamous Cell Carcinoma Lines. The human squamous cell carcinoma line SCC15 was infected with recombinant adenoviruses expressing β-galactosidase, Akt T308A (kinase-dead) or myristylated Akt [myr-Akt, constitutively active (29)]. The titer of all three adenoviruses was similar. X-gal staining showed that about 45% of the cells exposed to the β-galactosidase adenovirus were actually infected (Fig. 1*A*). β-Galactosidase- or Akt T308A-infected cells displayed no morphological changes (Fig. 1, *A, B, D, E, G,* and *H*). Cells infected with myr-Akt lost their epithelial cell morphology; they were dispersed and

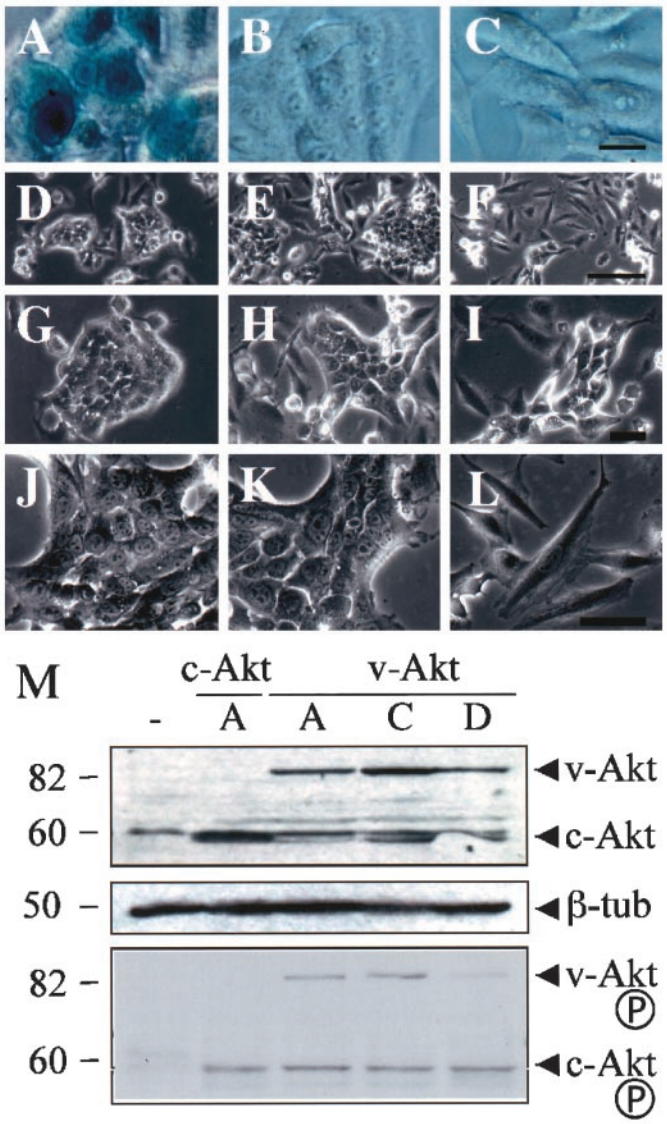

Fig. 1. Akt affects the morphology of epithelial cells and induces loss of cell-cell adhesion after transient (*A–I*) or stable (*J–M*) expression. Morphology of SCC15 cells after infection with an adenovirus expressing either the β-galactosidase marker (*A, D,* and *G*), a kinase-dead form of Akt (T308A; *B, E,* and *H*), or a constitutively active form of Akt (myr-Akt; *C, F,* and *I*). Cells were either fixed and stained with X-gal (*A–C*) or directly observed by phase-contrast microscopy (*D–I*). Phase-contrast microscopy of G418-resistant SCC15 cells infected with retrovirus expressing v-Akt (*L*), empty vector (*K*), or parental cells (*J*). Note loss of the epithelial shape and paucity of cell-cell contacts in cells expressing active Akt (*F, I,* and *L*). *Scale bars* represent 10 μm (*A–C*), 40 μm (*D–F*), and 20 μm (*G–L*). *M*, Western blot analysis confirmed the production of viral (v-Akt) and cellular (c-Akt) forms of Akt (*top panel*) in SCC15 cells (−), in one c-Akt cell line called (*A*), and in three independent v-Akt cell lines called (*A, C, D*). β-Tubulin (*middle panel*) is a loading control. Western blotting with anti-phospho-AKT T308 antibody showed that unlike endogenous AKT, exogenous Akt is active (*bottom panel*).

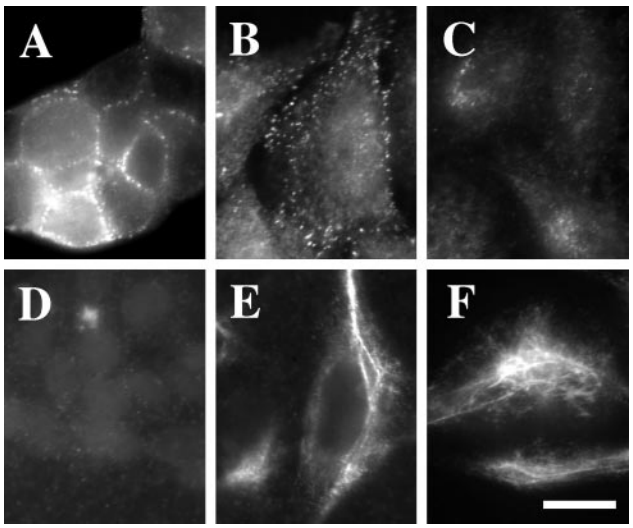

Fig. 2. Akt induces cellular changes associated with EMT in SCC15 cells. Parental SCC15 cells (*A* and *D*), SCC15 v-Akt B cells (*B* and *E*), and SCC15 v-Akt D cells (*C* and *F*) were fixed and processed for immunofluorescence with antibodies recognizing desmoplakin (*A–C*) and vimentin (*D–F*). *Scale bar*, 10 μm.

assumed a fibroblast-like appearance (Fig. 1, *C*, *F*, and *I*). Similar results were obtained with NBT-II, a rat carcinoma cell line (data not shown).

Similar experiments were performed using empty retroviruses and retroviruses expressing c-Akt or another constitutively active mutant, v-Akt. The v-Akt oncoprotein contains NH_2-terminal viral Gag sequences that provide a myristylation site (30, 31). Three mass cultures of cells infected with pLXSN c-Akt (called SCC15 c-Akt A, B, and C), four mass cultures of cells infected with pLXSN v-Akt (SCC15 v-Akt A, B, C, and D), and two mass cultures of cells infected with empty retrovirus (SCC15-pLXSN A and B) were subjected to G418 selection. Lysates from uninfected and infected cultures were analyzed by immunoblotting using an antibody that recognizes both endogenous human AKT and exogenous murine Akt. β-Tubulin was used as loading control (Fig. 1*M*). Uninfected SCC15 produced the M_r 60,000 endogenous AKT. SCC15 c-Akt cells contained at least three times more Akt than SCC15 cells. Various amounts of a protein larger than M_r 85,000 corresponding to v-Akt were detected in SCC15 v-Akt A, C, and D cells. AKT activation status was examined by Western blotting with anti-phospho-AKT threonine 308 antibody, a marker of AKT activation. Total AKT activity was very low in parental SCC15 cells and high in SCC15 cells expressing exogenous Akt (Fig. 1*M*). Expression of constitutively active Akt (v-Akt) was associated with the transition from an epithelial to a fibroblast-like morphology (Fig. 1, *J–L*).

Constitutively Active Akt Promotes a Shift in Expression from an Epithelial to a Mesenchymal Repertoire. The morphological effect of v-Akt expression on squamous cell carcinoma lines suggested that active Akt promotes an EMT. The morphological changes characteristic of cells undergoing EMT are accompanied by a shift in gene expression from an epithelial to a mesenchymal repertoire. To determine whether Akt promotes such a shift, we used immunofluorescence to examine the expression and subcellular distribution of desmoplakin and vimentin, two markers of EMT. In parental SCC15 cells, desmoplakin was mostly in spots at the sites of cell-cell contact (Fig. 2*A*). All v-Akt-infected cells contained desmoplakin, but only in cytoplasmic granules (Fig. 2, *B* and *C*), and desmoplakin was less abundant in v-Akt-infected cells than in SCC15 cells, as assessed by Western blot analysis (data not shown). The parental SCC15 cell line did not produce vimentin, a mesenchymal marker (Fig. 2*D*), whereas all v-Akt cell lines produced vimentin microfilaments (Fig. 2, *E* and *F*). The heights of SCC15, SCC15 v-Akt C, and SCC15 v-Akt D cells were determined by confocal microscopy using E-cadherin as marker (data not shown). The mean height of SCC15 cells was 6.0 μm, and the mean heights of SCC15 v-Akt C and SCC15 v-Akt D cells were similar and equal to about 3.8 μm. We conclude that constitutively active Akt indeed induces EMT.

Constitutively Active Akt Alters the Expression of Cell Adhesion Molecules. The integrity of adherent junctions and other structures involved in cell-cell contact is essential for the maintenance of epithelial structures. To determine whether Akt disrupted such structures, lysates from SCC15 cells expressing active Akt were probed with antibodies against proteins involved in cell adhesion (Fig. 3*A*). E-cadherin, α-catenin, β-catenin, and p130cas were all dramatically down-regulated in v-Akt-infected cells, but not in c-Akt- or vector-infected cells. The level of plakoglobin was slightly down-regulated, and the level of the p130cas substrate HEF1 was not down-regulated. N-cadherin was slightly up-regulated (data not shown). Similar experiments with SCC13, another human squamous cell carcinoma line, and MSV-SRα retroviruses encoding myr-Akt revealed changes in expression indistinguishable from those in SCC15 v-Akt (data not shown).

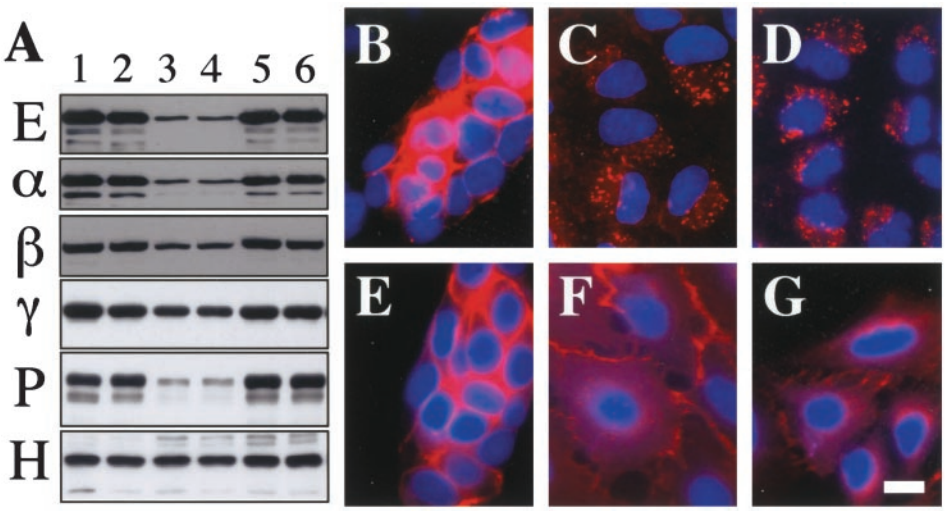

Fig. 3. Akt down-regulates adherent junction and epithelial markers and induces the relocation of E-cadherin and β-catenin. *A*, Western blot analysis (*E*, E-cadherin; α, α-catenin; β, β-catenin; γ, plakoglobin; *P*, p130cas; *H*, HEF1) of total lysates of parental SCC15 cells (*Lane 1*) and independent mass cultures of SCC15 cell lines infected with retroviruses expressing c-Akt (*Lane 2*), v-Akt (*Lanes 3* and *4*), or empty vector pLXSN (*Lanes 5* and *6*). SCC15 parental cells (*B* and *E*), SCC15 v-Akt C cells (*C* and *F*), and SCC15 v-Akt D cells (*D* and *G*) were fixed and immunostained with antibodies directed against E-cadherin (*B–D*) or β-catenin (*E–G*); nuclei were labeled with 4′,6-diamidino-2-phenylindole. *Scale bar*, 5 μm.

Constitutively Active Akt Down-Regulates E-Cadherin and β-Catenin and Alters Their Subcellular Localization. To confirm the immunoblot findings and determine the localization of E-cadherin and β-catenin in the SCC15 cell lines, we used laser scanning confocal microscopy (Fig. 3, *B–G*). Parental SCC15 cells produced both E-cadherin and β-catenin, and both proteins were at contact sites between cells (Fig. 3, *B* and *E*). v-Akt-expressing-SCC15 cells contained less β-catenin and E-cadherin: most of the E-cadherin was in cytoplasmic granules, with only a small amount at the plasma membrane (Fig. 3, *C* and *D*); and β-catenin was diffuse in the cytoplasm, although some remained at the membrane (Fig. 3, *F* and *G*). Slight quantitative differences in β-catenin distribution were observed between SCC15 v-Akt C and SCC15 v-Akt D cells; the amount of β-catenin at the membrane was slightly higher in SCC15 v-Akt C cells. In the cytoplasm of Akt-expressing cells, E-cadherin and β-catenin did not colocalize (Fig. 3, *F* and *G*; data not shown).

Constitutively Active Akt Down-Regulates the Expression of the *E-Cadherin* Gene Transcriptionally by Inducing *SNAIL*. During EMT there is a massive shift of gene expression from a pattern characteristic of epithelial cells to that of mesenchymal cells. There may be genes that are the primary targets of the EMT-inducing signal(s) and others whose expression is affected secondarily. E-cadherin and β-catenin are potential candidate genes whose down-regulation by Akt may be direct, so we examined their mRNAs in v-Akt-expressing and parental SCC15 cells. Akt did not significantly affect the level of β-catenin mRNA but substantially reduced the level of E-cadherin mRNA (Fig. 4*A*). Parental SCC15 and v-Akt-expressing SCC15 cells were transiently transfected with pGL3 or pGL3 hE-cadherin promoter luciferase constructs to determine whether Akt modulates the activity of the *E-cadherin* promoter. Luciferase assays of extracts from the transfected cells revealed that the activity of the *E-cadherin* promoter was repressed by Akt (Fig. 4*B*). Similar results were obtained for the murine *E-cadherin* promoter (data not shown). Thus, Akt appears to down-regulate *E-cadherin* gene transcription.

The *E-cadherin* promoter is a direct target of the transcriptional repressor *SNAIL* (32), and we therefore tested whether Akt regulates *SNAIL* expression or activity. We examined the abundance of *SNAIL* mRNA in parental and v-Akt-expressing cells and found that *SNAIL* is indeed induced by constitutively active Akt (Fig. 4*A*).

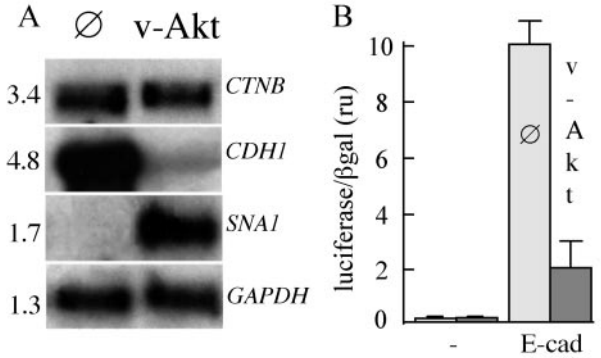

Fig. 4. Activation of *SNAIL* transcription is associated with repression of *E-cadherin* transcription in the presence of active Akt. *A*, poly(A)$^+$ Northern blot analysis of the mRNA for β-catenin (*CTNB*), E-cadherin (*CDH1*), SNAIL (*SNAI1*), and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) in SCC15 cells expressing v-Akt (SCC15 v-Akt C) or empty vector (designated Ø). The approximate size of the transcripts (in kb) is indicated to the *left*. *B*, parental SCC15 (*light gray box*) and SCC15 v-Akt C cells (*dark gray box*) were cotransfected with pGL3 (−) or pGL3 hE-cad prom (*E-cad*) as a reporter and with pPGKβgeopA as an internal standard. Two days after transfection, the level of reporter gene transcription was measured as the ratio of luciferase activity:β-galactosidase activity (*luciferase/β-gal*) and is expressed in relative units (*r.u.*).

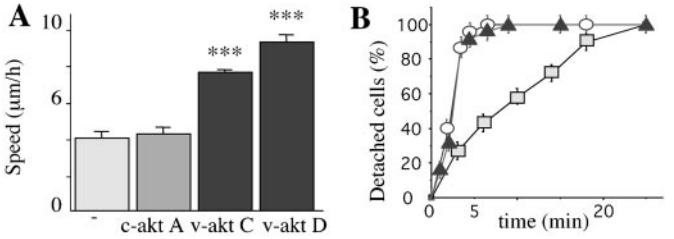

Fig. 5. Akt affects cell migration and attachment to substratum. *A*, random cell migration is increased in SCC15 cells expressing v-Akt. SCC15 cells expressing or not expressing exogenous Akt were allowed to attach onto plates coated with 10 μg/ml fibronectin. Cell motility was evaluated by tracking at least 20 cells. The mean ± SE of three independent experiments are shown. Data from migration assays of SCC15, SCC15 c-Akt A, SCC15 v-Akt C, and SCC15 v-Akt D cell lines (ANOVA; $F_{(3,88)} = 53.39$; $P < 0.0001$) reveal a statistically significant increase in migration induced by v-Akt compared with endogenous or c-Akt [SCC15 *versus* SCC15 v-Akt C, $t[41] = 8.7$ (***, $P < 0.0001$); SCC15 *versus* SCC15 v-Akt D, $t[44] = 9.1$ (***, $P < 0.0001$)]. Migration induced by v-Akt is significantly different from all other cases (Fisher's analysis: ***, $P < 0.0001$). *B*, the strength of attachment to substratum was estimated by the rate of detachment after trypsinization. □, SCC15 cells; ○, SCC15 v-Akt C cells; ▲, SCC15 v-Akt D cells.

Constitutively Active Akt Alters Cell Motility on Different Substrates and Cell Attachment to Plastic. Motility of parental and Akt-expressing SCC15 cells (SCC15, SCC15 c-Akt A, SCC15 v-Akt C, and SCC15 v-Akt D cells) was examined on uncoated plastic and plastic that had been coated with fibronectin, collagen, or laminin. On fibronectin, SCC15 v-Akt C and v-Akt D cells migrated more rapidly than parental SCC15 cells, whereas SCC15 c-Akt A cells exhibited the same motility as the parental SCC15 cells (Fig. 5*A*). On laminin, SCC15 v-Akt C cells migrated more slowly than SCC15 cells, whereas on collagen, the speed of migration of all cell lines was similar (data not shown).

The strength of cell anchorage to plastic substratum was also determined. SCC15 v-Akt C and v-Akt D cells detached after 9 min in trypsin, whereas parental SCC15 cells detached after 25 min in trypsin (Fig. 5*B*). Thus, constitutively active Akt attenuates the attachment of SCC15 cells to plastic.

Constitutively Active Akt Promotes Proliferation, Tumorigenicity, and Invasiveness of Squamous Cell Carcinoma Lines. E-cadherin suppresses tumor invasiveness (3, 33), so we examined whether Akt promotes the tumorigenic potential and invasiveness of epithelial cells (Fig. 6). Growth curves were constructed for SCC15, SCC15 v-Akt B, and SCC15 v-Akt D cells (Fig. 6*A*). The doubling time of the parental cells was 23 h, and those of SCC15 v-Akt B and v-Akt D were only 13 and 12 h, respectively. Similar experiments were performed with SCC13 cell lines transfected with empty vector (Srα), v-Akt, and myr-Akt. The respective doubling times were estimated to be 28, 13, and 17 h for SCC13-Srα, SCC13-v Akt, and SCC13-myr Akt, respectively (data not shown).

SCC13 cells are not tumorigenic in athymic nude mice (27). To determine whether Akt is sufficient to render SCC13 cells oncogenic, we evaluated tumor formation by vector-infected SCC13 cells and c-Akt-, v-Akt-, or myr-Akt-expressing SCC13 cells. Parental cells and vector- and c-Akt-infected SCC13 cells could not produce tumors in nude mice, whereas SCC13 cells expressing v-Akt and SCC13 myr-Akt cells produced tumors (Fig. 6*B*). Microscopic examination of these tumors revealed evidence of invasion of the surrounding muscular tissue. Unlike parental SCC13 cells, parental SCC15 cells are fully tumorigenic in nude mice (21) and therefore were not used to score for the effect of constitutive Akt activation on tumorigenesis.

We next measured the ability of parental and Akt-expressing SCC15 cells to pass through the *pars membranacea* of tracheal walls to assess invasiveness (Fig. 6, *C–F*). The cells expressing ectopic v-Akt migrated more efficiently through tissues than did the parental

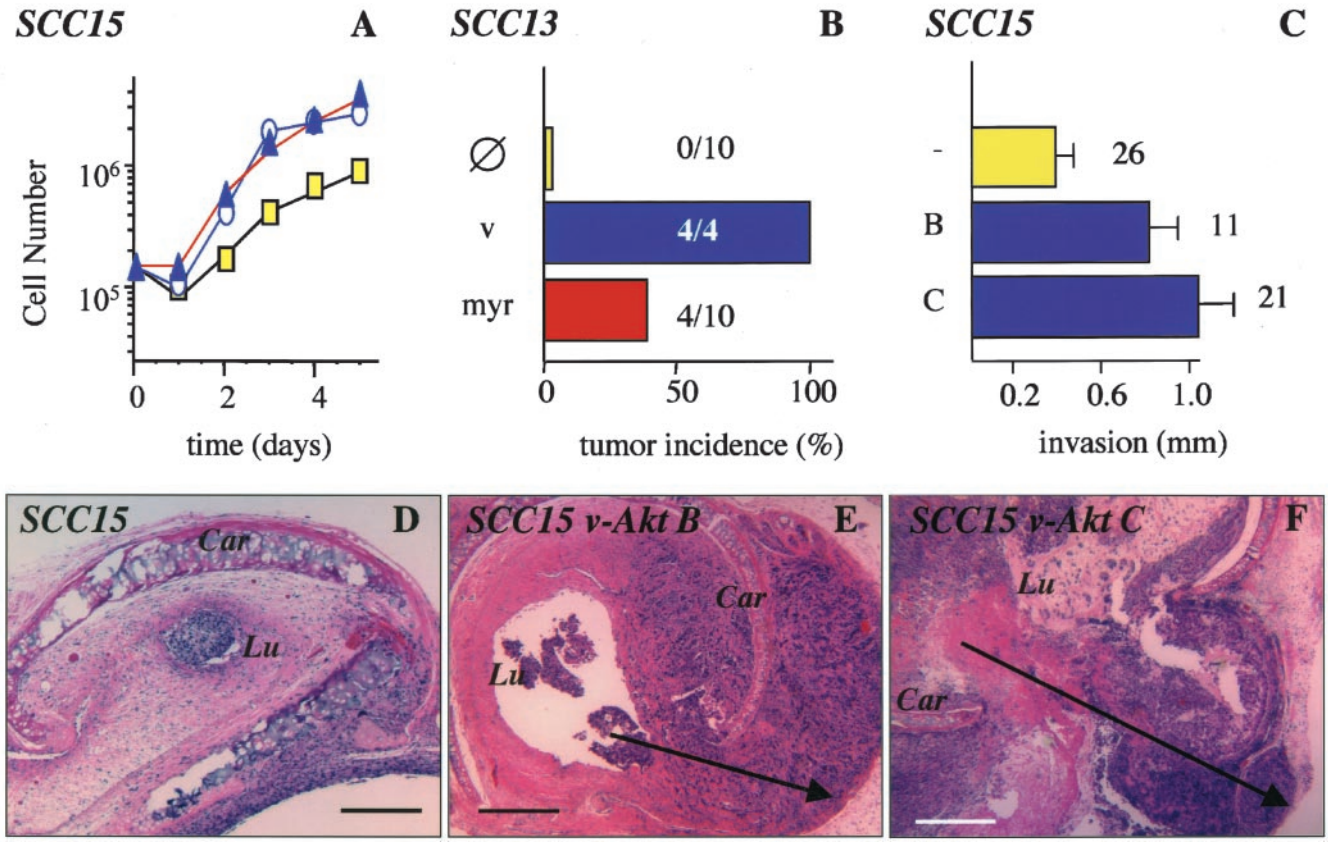

Fig. 6. Akt induces cell proliferation, tumorigenicity, and invasiveness. *A*, doubling times for SCC15 cells expressing and not expressing exogenous Akt were estimated from growth curves as follows: 23 h for SCC15 cells (■), 13 h for SCC15 v-Akt B cells (○), and 12 h for SCC15 v-Akt D cells (▲). *B*, oncogenic potential of squamous cell carcinoma lines. The percentage of tumor incidence in nude mice of SCC13 cells infected with retroviruses expressing v-Akt (*v*), myr-Akt (*myr*), or empty vector pLSN (Ø) is shown in the *bar graph*. The number of tumors per animal injected is indicated for each cell line. *C–F, in vivo* invasion assay. Micrographs of tracheal transplant cross-sections, stained with H&E, showing the representative growth pattern of SCC15 cells (*D*), SCC15 v-Akt B cells (*E*), and SCC15 v-Akt C cells (*F*). Cells were initially placed in the lumen (*Lu*) of the trachea that is surrounded by the cartilage (*Car*) and the *pars membranacea*. The v-Akt-expressing cells invaded the tracheal wall and grew in the direction of the *arrow* after crossing the *pars membranacea* (*E* and *F*). *Scale bars*, 0.22 mm. *C*, the extent of invasion in tracheal transplants [parental SCC15 (−) and SCC15 v-Akt B and C (*B* and *C*)] was measured (in mm) and is shown in the *histogram* along with SDs. The number of tumors analyzed (range, 11–26) is indicated on the *right*.

SCC15 cells. The test of invasiveness was also performed with the various SCC13 cell lines yielding virtually identical results: SCC13 cell lines expressing v-Akt or myr-Akt, but not SCC13-Srα cells, were able to pass through the *pars membranacea* (data not shown). Therefore, constitutively active Akt is a potent promoter of tumorigenicity and invasion.

DISCUSSION

During EMT, epithelial cells acquire fibroblast-like properties and exhibit reduced cell-cell adhesion and increased motility. The plasticity afforded by EMT is central to the complex remodeling of embryo and organ architecture during gastrulation and organogenesis. In pathological processes such as oncogenesis, EMT may endow cancer cells with enhanced motility and invasiveness. Indeed, oncogenic transformation may be associated with signaling pathways promoting EMT (34). AKT activation is frequent in human epithelial cancer (8–13, 15, 16). Interestingly, in ovarian carcinomas, AKT2 activation has been linked to aggressive clinical behavior and loss of histological features of epithelial differentiation (10). These findings are consistent with AKT directly affecting epithelial cell morphology, tumorigenicity, cell motility, and invasiveness.

Here we show that constitutively active Akt induces EMT and stimulates proliferation and motility of squamous cell carcinoma lines plated on fibronectin-coated surfaces (Fig. 5). Also, active Akt promotes invasiveness (Fig. 6). Cells expressing a constitutively active mutant of Akt (v-Akt or myristylated Akt) displayed several features typical of EMT: reduction in cell-cell adhesion; and flattening and spreading or scattering (Fig. 1). We detected Akt-induced EMT in cells stably infected with retroviral vectors and in those transiently infected with adenoviral vectors. EMT occurred a minimum of 72–96 h after transient infection, which may reflect the time required for the reprogramming of gene expression and/or structural reorganization associated with EMT (34).

At least two separate correlates of EMT have been identified, namely, cell-cell dissociation and cell movement (34). Akt activation appears to mediate both processes. In particular, expression of active mutants of Akt increases cell migration on fibronectin-coated plates but reduces migration on laminin-coated plates. This pattern is compatible with induction of $\alpha_4\beta_1$ integrin that interacts specifically with fibronectin (35). Indeed, integrin activation often follows EMT (36, 37).

In several cell culture models, EMT is induced by transforming growth factor β or by peptide growth factors via receptor tyrosine kinase signaling (22, 38–41). In both cases, PI3K is a critical mediator of EMT. Oncogenic SRC and RAS, both inducers of EMT, also activate PI3K (42). Our data suggest that AKT kinases are major effectors of EMT signals downstream of PI3K. Potential targets of the P13K/AKT pathway include Rac and Rho, two small G proteins involved in cytoskeletal reorganization, cell migration, and invasiveness (34).

Akt-induced EMT involves a large down-regulation of E-cadherin and β-catenin protein levels (Fig. 3). Down-regulation is specific for E-cadherin because the closely related N-cadherin is not affected. E-cadherin and β-catenin are also relocalized to separate compartments, an indication that their interaction is disrupted. E-cadherin is internalized and displays a punctate cytoplasmic staining pattern, compatible with a vesicular localization. Localization of E-cadherin in vesicles has been described during IGF-I-induced EMT and may point to alterations in protein trafficking, possibly induced by activation of Rab5-mediated endocytosis (22, 43–45).

Akt also down-regulated β-catenin, a result that appeared paradoxical. In the Wnt pathway, GSK3 phosphorylates axin and β-catenin, causing degradation of the latter. Upon Wnt-induced inhibition of GSK3, β-catenin accumulates in the cytoplasm and translocates into the nucleus, acting as a cofactor for the transcription factor LEF/TCF (lymphoid enhancer factor/T cell factor), affecting the transcription of genes that promote cell survival and proliferation (46–50). AKT, activated by peptide growth factor signals, phosphorylates and inhibits GSK3 (51), so we expected stabilization and nuclear translocation of β-catenin in squamous cell carcinoma lines expressing active Akt. The apparent discrepancy can be resolved by recent crystallographic and biochemical studies elucidating the mechanisms of GSK3 regulation (52, 53): Wnt and insulin/AKT signaling pathways affect two distinct pools of GSK3 that in turn target different substrates, thereby giving selective responses and differential substrate phosphorylation (54). In the presence of sustained Wnt signaling, phosphorylation of GSK3 by AKT potentiates the Wnt pathway leading to β-catenin stabilization; however, AKT signaling alone cannot initiate the Wnt signaling process (55–57). Consequently, in our system, activation of Akt alone, *i.e.*, in the absence of Wnt signaling, may not be able to phosphorylate and inhibit the GSK3 pool involved in β-catenin up-regulation.

This does not, however, explain the down-regulation of β-catenin by active Akt. This appears to be a posttranscriptional effect. Possibly, β-catenin is destabilized and even degraded as a secondary consequence of the down-regulation of E-cadherin. After Akt activation, β-catenin was displaced and did not colocalize with E-cadherin (Fig. 3), and in both *Drosophila* and mouse development, binding to E-cadherin stabilizes β-catenin (58, 59).

In addition to internalization of E-cadherin, Akt activation represses *E-cadherin* gene transcription (Fig. 4). In the presence of active Akt, the *E-cadherin* promoter is less active, and this repression appears to be the consequence of up-regulation of the transcription repressor SNAIL. Indeed, SNAIL induces EMT by repressing *E-cadherin* transcription (4, 5). Other potential modulators of *E-cadherin* transcription are SNAIL-related repressors, such as Slug, Smuc, and SIP1 that bind to E-boxes in the *E-cadherin* promoter (6, 32, 60). The mechanisms by which Akt activates transcription of *SNAIL* remain unclear.

Akt-induced EMT endows squamous cell carcinoma lines with an invasive phenotype as demonstrated by an *in vivo* assay of invasion (Fig. 6). The identical assay has demonstrated that *AKT2* antisense RNA can inhibit invasiveness in cancer cells that amplify/overexpress the *AKT2* gene (11). Although the invasiveness may be due in part to the ability of Akt to stimulate cell cycle progression (Fig. 6), it is more likely to result from loss of cell-cell adhesion (Figs. 1–3), increased motility (Fig. 5), and tissue degradation. Akt activation can lead to increased production of matrix metalloproteinases (19, 20), and, in turn, low levels of E-cadherin are associated with stromelysin 1 activation (61). Overexpression of IGF-I receptor in the pancreas *in vivo* leads to transformation and invasion associated with down-regulation of E-cadherin (62). An intriguing possibility is that this effect of IGF-I receptor is mediated by AKT.

In summary, we show that activation of the AKT pathway in cancer cells leads to EMT and invasion *in vivo*. Thus, an important consequence of the AKT activation often detected in human carcinomas is the acquisition of an invasive phenotype. Therapy based on AKT inhibition may therefore complement conventional treatments to control tumor cell invasion and metastasis.

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