THE JOURNAL OF CELL BIOLOGY

Distinct roles of Akt1 and Akt2 in regulating cell migration and epithelial–mesenchymal transition

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The Akt family of kinases are activated by growth factors and regulate pleiotropic cellular activities. In this study, we provide evidence for isoform-specific positive and negative roles for Akt1 and -2 in regulating growth factor–stimulated phenotypes in breast epithelial cells. Insulin-like growth factor-I receptor (IGF-IR) hyperstimulation induced hyperproliferation and antiapoptotic activities that were reversed by Akt2 down-regulation. In contrast, Akt1 down-regulation in IGF-IR–stimulated cells promoted dramatic neomorphic effects characteristic of an epithelial–mesenchymal transition (EMT) and enhanced cell migration induced by IGF-I or EGF stimulation. The phenotypic effects of Akt1 down-regulation were accompanied by enhanced extracellular signal–related kinase (ERK) activation, which contributed to the induction of migration and EMT. Interestingly, down-regulation of Akt2 suppressed the EMT-like morphological conversion induced by Akt1 down-regulation in IGF-IR–overexpressing cells and inhibited migration in EGF-stimulated cells. These results highlight the distinct functions of Akt isoforms in regulating growth factor–stimulated EMT and cell migration, as well as the importance of Akt1 in cross-regulating the ERK signaling pathway.

Introduction

The Akt/PKB family of kinases, Akt1, -2, and -3, plays critical roles in regulating growth, proliferation, survival, metabolism, and other cellular activities. Akt kinases control these activities by phosphorylation-mediated regulation of multiple substrates (for reviews see Brazil et al., 2004; Woodgett, 2005). Deregulated, or enhanced, Akt signaling has also been implicated in a variety of human cancers, and may promote tumorigenesis (for review see Bellacosa et al., 2005). However, the specific contribution of Akt isoforms to phenotypes in normal and cancerous cells have not been clearly elucidated.

Akt activity is induced by ligand stimulation of growth factor receptors, such as the insulin-like growth factor-I receptor (IGF-IR) and the EGF family of receptors. Both IGF-I and EGF receptor signaling lead to pleiotropic effects in normal and cancerous cells (for reviews see Hynes and Lane, 2005; Foulstone et al., 2005). Multiple signaling proteins are activated downstream of these receptors, including the extracellular signal–related kinase (ERK)/MAP kinase, phosphatidyl-inositol-3′ (PI 3) kinase, and AKT. Ligand stimulation activates PI 3-kinase, the upstream activator of Akt, by direct binding of PI 3-kinase subunits to either the activated, phosphorylated receptor or to adaptor proteins phosphorylated by receptor kinase activity (Yamamoto et al., 1992; Myers et al., 1993). Phosphoinositides generated by PI 3-kinase activity trigger activation of Akt kinases through direct binding to the pleckstrin homology (PH) domain and the subsequent phosphorylation of Akt at two conserved residues (for review see Woodgett, 2005).

Although the three Akt isoforms are structurally homologous and share similar mechanisms of activation, they also exhibit distinct features. Akt1 and -2 are ubiquitously expressed, whereas Akt3 has been reported to have a more limited tissue distribution (Yang et al., 2003). Emerging evidence supports distinct functions for Akt isoforms in normal cells, as well as in tumor cells. Studies of Akt isoform deficient mice highlight the potentially nonredundant functions of Akt1 and -2. *Akt1*$^{-/-}$ mice are small with significant growth defects, and *Akt2*$^{-/-}$ mice are unable to maintain glucose homeostasis (Cho et al., 2001a,b). Akt2 was also implicated in insulin-stimulated glucose metabo-

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Abbreviations used in this paper: 3D, three-dimensional; EMT, epithelial–mesenchymal transition; ERK, extracellular signal–related kinase; GSK3, glycogen synthase kinase-3; IGF-IR, insulin-like growth factor-I receptor; PH, pleckstrin homology; PI 3-kinase, phosphatidyl-inositol 3′ kinase; shRNA, short hairpin RNA.

The online version of this article contains supplemental material.

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The Journal of Cell Biology, Vol. 171, No. 6, December 19, 2005 1023–1034
http://www.jcb.org/cgi/doi/10.1083/jcb.200505087

lism by studies using $Akt2^{-/-}$ mouse embryo fibroblasts or 3T3-LI adipocytes transfected with isoform-specific short interfering RNA (Bae et al., 2003; Jiang et al., 2003; Katome et al., 2003). The loss or down-regulation of Akt2 impairs glycogen synthase kinase 3α phosphorylation and glucose transporter 4 translocation more significantly than Akt1 down-regulation. However, there is redundancy in function, as the combined down-regulation of Akt1 and -2 results in more severe defects.

All Akt isoforms possess in vitro transformational ability (Mende et al., 2001); however, there may be isoform-specific functions in tumor cells. Akt2 amplification or mutations have been detected in breast, ovarian, and colon tumors (Bellacosa et al., 1995; Parsons et al., 2005), whereas Akt1 amplification has been reported only in a single gastric cancer cell line (Staal, 1987). Thus, Akt2 may exert isoform-specific effects on tumor progression. Overexpression of wild-type Akt2, but not Akt1, in an ErbB2-overexpressing breast cancer cell line enhanced invasiveness in vitro and metastases in animal models (Arboleda et al., 2003). In other studies, overexpression of activated Akt1 in ErbB2 transgenic mouse mammary tumors decreased their metastatic potential but enhanced their proliferation (Hutchinson et al., 2004).

In this paper, we describe surprising isoform-specific functions of Akt revealed by investigations of the role of Akt signaling downstream of the IGF-IR. These studies used a nontransformed breast epithelial cell line, MCF-10A, which displays a stable epithelial morphology in monolayer culture and forms hollow, growth-arrested structures in three-dimensional (3D) cultures (Debnath et al., 2003a). IGF-IR hyperstimulation led to the formation of hyperproliferative 3D structures lacking a hollow lumen caused by the suppression of apoptosis. Initial studies using receptor variants indicated that Akt signaling may be critical for the antiapoptotic and proliferative activities induced by IGF-IR hyperstimulation, as the substitution of tyrosine residue 950 impaired these phenotypes and inhibited Akt activation, but not ERK signaling. To investigate the role of Akt in this system, we examined the consequences of down-regulating expression of two distinct isoforms, Akt1 and -2, on IGF-I–stimulated phenotypes. Akt2 down-regulation reverted all aspects of IGF-IR–induced phenotypic changes in 3D acinar structures. Unexpectedly, we found that the specific down-regulation of Akt1 induced a dramatic phenotype resembling an epithelial–mesenchymal transition (EMT) and enhanced growth factor–stimulated migration. These phenotypic changes were associated with up-regulation of ERK/MAPK signaling. Interestingly, Akt2 expression was required for EMT-like morphological changes induced by Akt1 down-regulation. Our studies suggest that there are isoform-specific, positive and negative effects of Akt isoforms on migration and EMT. Furthermore, these distinct effects may result from isoform-specific cross-regulation of other signaling pathways.

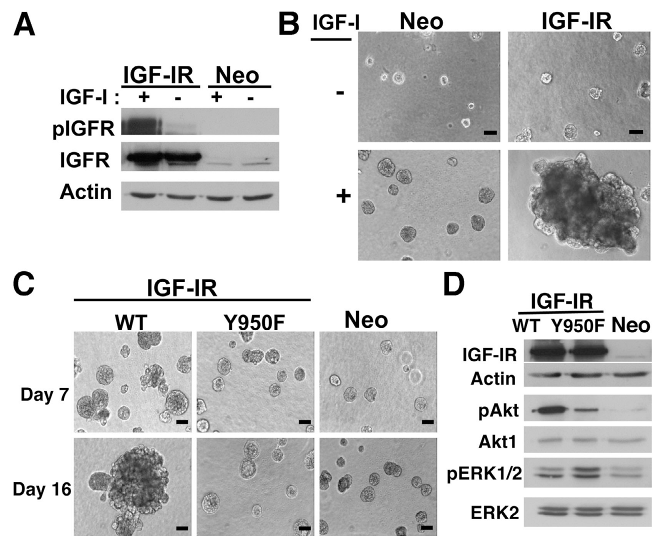

Figure 1. **IGF-IR hyperstimulation induces morphological changes in mammary epithelial cells.** (A) MCF-10A cells overexpressing human IGF-IR or vector control (Neo) were grown in monolayer cultures containing EGF ± IGF (100 ng/ml). Lysates were immunoblotted with antibody against phosphorylated IGF-IR or total receptor. (B) Phase-contrast images of IGF-IR or Neo cells grown in 3D Matrigel cultures for 16 d in media containing 2% horse serum and EGF ± IGF-I (100 ng/ml). B (C) IGF-IR, Neo, or MCF-10A cells overexpressing a variant IGF-IR (Y950F) were grown in 3D Matrigel cultures for 7 or 16 d in 2% horse serum, EGF, and 100 ng/ml IGF-I. (D) Lysates of IGF-IR, Neo, or Y950F cells grown in monolayer cultures in 2% horse serum, EGF, and 100 ng/ml IGF-I were immunoblotted with antibody against phosphorylated Akt or phosphorylated, activated ERK. Bars, 50 μM.

Results

Enhanced IGF-I signaling disrupts normal mammary acinar architecture and morphogenesis

To examine the effects of enhanced IGF-I stimulation on mammary acinar architecture, MCF-10A cells overexpressing IGF-IR cells were generated using a retroviral vector encoding the human IGF-IR complementary DNA. MCF-10A is an immortalized breast epithelial cell line that has been reported to express IGF-IR (Tannheimer et al., 1998); however, levels of endogenous receptor were barely detectable by Western blotting (Fig. 1 A). Despite significant overexpression, activation of the receptor remained ligand dependent (Fig. 1 A). In monolayer cultures, IGF-I stimulation of IGF-IR cells induced a subtle, but recognizable, conversion from a cuboidal, epithelial morphology to a more spindle-shaped morphology (unpublished data).

In 3D basement membrane Matrigel cultures, both IGF-IR cells and MCF-10A cells overexpressing vector control (Neo cells) were able to initiate morphogenesis only in the presence of IGF-I (Fig. 1 B). The inability of vector control cells to form acini in the absence of ligand suggests a critical role for IGF-I stimulation in normal acinar morphogenesis. With ligand addition, IGF-IR cells formed complex structures that were significantly larger than control acini. These differences were apparent as early as day 6 in 3D culture and were sustained throughout the duration of the assay (Fig. 1 B). This phenotype was dependent on IGF-IR kinase activity, as comparable overexpression of a kinase-inactive receptor failed to induce the formation of these large, abnormal structures (unpublished data).

As an initial approach to define pathways that are critical for changes in acinar structure mediated by enhanced IGF-I signaling, we examined the effects of overexpressing variants of IGF-IR with amino acid substitutions at key regulatory sites outside of the catalytic domain that have been implicated

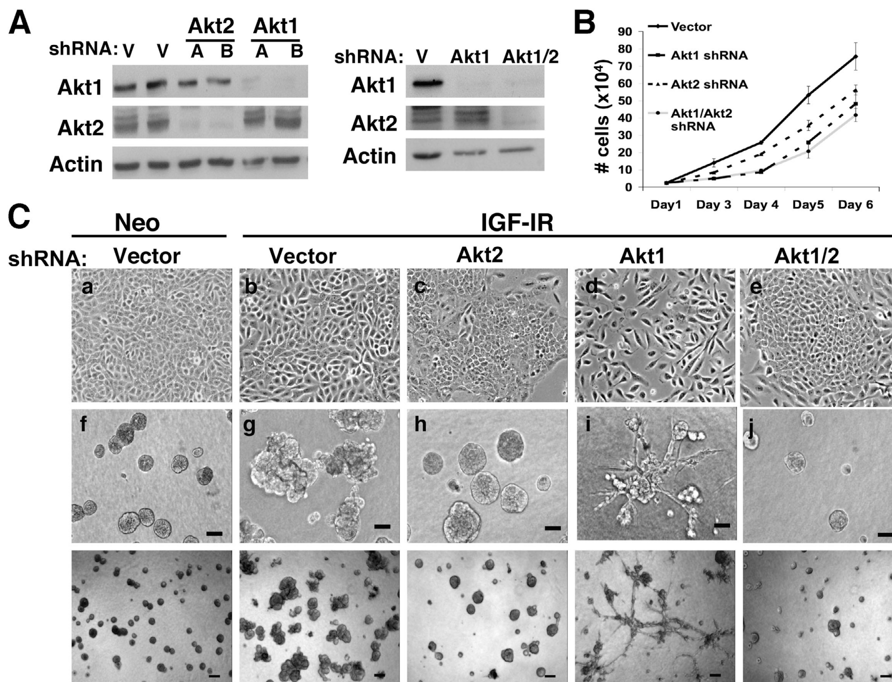

Figure 2. **Isoform-specific down-regulation of Akt results in distinct phenotypes.** (A) IGF-IR cells were superinfected with shRNA vectors targeting Akt1 and/or -2. Empty vectors (V) were used as controls. Immunoblotting with antibody against actin was used to confirm equal loading. Cells in monolayer cultures were lysed and immunoblotted with Akt isoform-specific antibodies. (B) IGF-IR cells superinfected with empty vector, Akt1 shRNA, Akt2 shRNA, or both were grown in triplicate monolayer cultures for the indicated days in media containing EGF and IGF-I (100 ng/ml), with initial seeding of 2.5×10^4 cells. Cells were trypsinized, stained with Trypan blue, and counted. A representative experiment of three independent experiments is shown. Error bars represent means ± SD. (C) IGF-IR cells in which either Akt1 and/or -2 were down-regulated were cultured in monolayer (top) or 3D Matrigel/collagen (50:50) cultures for 8 d (middle and bottom). Monolayer cultures were grown in the presence of EGF and IGF-I (100 ng/ml). 3D cultures were grown in the presence of 5 ng/ml EGF and 100 ng/ml IGF-I. Bars: (top and middle) 50 μM; (bottom) 100 μM.

in IGF-IR signaling (Y1250/1251F, H1293F, Δ1280–1283, K1294L, and Y950F). Y950F was the only variant that was defective in formation of the hyperproliferative 3D structures; it induced structures that were slightly larger, but otherwise indistinguishable, from parental MCF-10A structures (Fig. 1 C and not depicted). Y950 serves as a binding site for Shc and the IRS family of adaptor proteins that are critical in the activation of downstream signaling proteins such as ERK, PI 3-kinase, and Akt (Craparo et al., 1995). MCF-10A cells overexpressing the Y950F variant were severely impaired in activation of Akt, but not ERK, when compared with cells overexpressing wild-type IGF-IR (Fig. 1 D). The lack of effect on ERK activation is consistent with a study reporting the importance of other residues (Y1250/Y1251) in modulating ERK (Leahy et al., 2004). These studies indicate that IGF-IR Y950 is critical for full Akt activation and that Akt may be critical for IGF-IR–induced phenotypic changes.

Differential effects of Akt isoform-specific down-regulation in IGF-IR–overexpressing cells

To investigate whether specific Akt isoforms are critical for the phenotypes induced by IGF-IR in the 3D culture model, we used RNA interference to specifically down-regulate the expression of Akt isoforms. Quantitative analyses using purified Akt1, -2, and -3 as protein standards revealed that Akt1 is present in excess of Akt2 (approximately threefold) and -3 (slightly less than twofold; Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200505087/DC1). Retroviral or lentiviral vectors encoding short hairpin RNA (shRNA) sequences were used to specifically down-regulate the expression of Akt1 and/or -2 in cells overexpressing wild-type IGF-IR (Fig. 2 A). Although several shRNA vectors targeting Akt3 were evaluated, none consistently down-regulated Akt3 expression. The down-regulation of Akt1 or -2 using shRNA vectors was at least 75% (Fig. 2 A and Fig. S1) and confirmed to be stable for at least 2 wks (not depicted).

Down-regulation of Akt1 or -2 had a significant impact on the proliferation of IGF-IR cells in monolayer culture. Reduction of either Akt1 or -2 resulted in decreased cell numbers, although the loss of Akt1 consistently induced a more significant effect (Fig. 2 B). Interestingly, shRNA-induced down-regulation of Akt1 or -2 also caused dramatic differences in the morphology of MCF-10A cells in both monolayer and 3D cultures. In monolayer cultures, down-regulation of Akt2 reverted the spindle-shaped morphology induced by wild-type IGF-IR overexpression (Fig. 2 C, top); in confluent cultures Akt2 down-regulated cells assumed a tightly packed, cuboidal appearance, which is characteristic of parental MCF-10A and normal epithelial cells (Fig. 2 C, compare c with a and b). In contrast, Akt1 down-regulation exaggerated the spindle-shaped, fibroblastic morphology, and many cells displayed ruffled surfaces (Fig. 2 C, d). IGF-IR cells that were dually expressing Akt1 and -2 shRNA vectors displayed a cuboidal morphology similar to the parental MCF-10A cells (Fig. 2 C, e), suggesting that Akt2 is required for the spindle-shaped phenotype induced by Akt1 down-regulation and indicating that the spindle morphology is not a consequence of the more significant reduction in total Akt caused by reduction of the dominant Akt1 isoform.

In 3D Matrigel/collagen (50:50) cultures (Fig. 2 C, middle and bottom), Akt2 down-regulation suppressed the IGF-IR–induced hyperproliferative phenotype, resulting in structures that resembled parental MCF-10A acini, although slightly larger (Fig. 2 C, compare h with g and f). We also examined the effects of Akt2 down-regulation on IGF-IR–induced hyperproliferation, by examining expression of Ki67, a marker of cycling cells, and on luminal apoptosis by examining caspase-3 cleavage. IGF-IR structures exhibited significant Ki67 staining

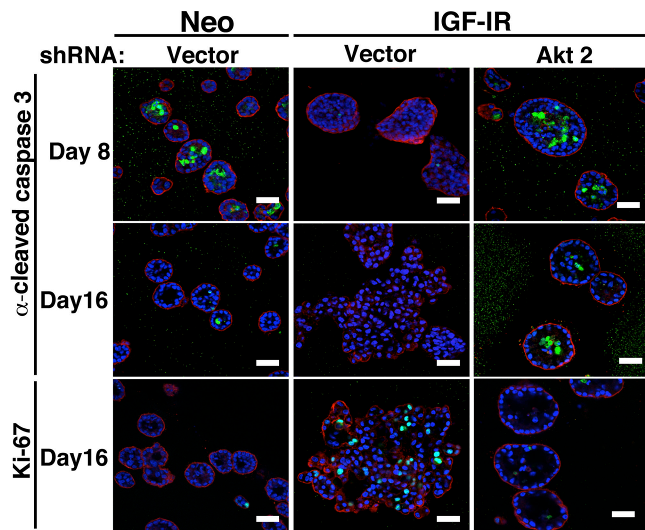

Figure 3. **Akt2-specific down-regulation inhibits IGF-IR–induced changes in 3D cultures.** IGF-IR cells expressing Akt2 shRNA vector were cultured in 3D Matrigel cultures for 8 or 16 d in the presence of EGF and IGF-I (100 ng/ml). Structures were stained with antibodies to cleaved caspase-3 or Ki-67 (green), α6 integrin (red), and TOPRO (blue). Equatorial confocal images are shown. Bars, 50 μM.

at day 16, unlike parental MCF-10A cultures, which have arrested proliferation by this time period (Debnath et al., 2002). In contrast, Akt2–down-regulated IGF-IR structures underwent proliferative arrest similar to that of parental MCF-10A acini, as demonstrated by the absence of Ki67 staining at day 16. (Fig. 3, bottom). IGF-IR 3D structures cultured in the presence of IGF-I also exhibit filled lumen because of their failure to undergo cavitation via apoptosis of centrally localized cells, a key feature of morphogenesis of parental MCF-10A acini (Debnath et al., 2002). Suppression of Akt2 expression restored lumen formation and luminal apoptosis, with intense activated caspase-3 staining in the presumptive luminal space (Fig. 3).

In contrast, Akt1 down-regulation dramatically disrupts acinar morphogenesis, thus precluding evaluation of lumen formation or growth arrest. Furthermore, Akt1 down-regulation induced a dramatic neomorphic effect involving the production of protrusive extensions that invaded the basement membrane gel (Fig. 2 C, i). This effect resembles organotypic structures that have undergone EMT. These protrusions were evident as early as day three of 3D cultures. Finally, IGF-IR acini that dually express Akt1 and -2 shRNA vectors did not form protrusions and their replicative potential in 3D cultures was significantly compromised, resulting in the formation of small, irregular structures that did not resemble normal acini (Fig. 2 C, j). Thus, down-regulation of either Akt1 and/or -2 isoforms influences IGF-I induced morphological changes in distinct ways.

Akt1 down-regulation enhances migration of IGF-IR–overexpressing cells and enhances expression of EMT markers

To establish whether Akt1 down-regulation also affects the migratory behavior of IGF-IR cells, as well as their invasive activity in basement membrane cultures, we examined cell motility in transwell assays. Ligand stimulation of control or IGF-IR cells did not stimulate migration in transwell assays. (Fig. 4 A, top). However, down-regulation of Akt1 with either shRNA sequence (sequence A or B) caused a dramatic enhancement of cell migration relative to IGF-IR cells superinfected with control empty vectors. In contrast, down-regulation of Akt2 did not enhance migration of IGF-IR cells. The increase in migration observed with Akt1 down-regulation occurred under basal conditions, but was significantly enhanced by IGF-I stimulation. The enhanced basal and IGF-I–stimulated migration induced by Akt1 down-regulation was impaired with concomitant Akt2 down-regulation (Fig. 4 A, bottom), suggesting that Akt2 is required for this effect of Akt1 down-regulation.

To address whether the effect of Akt1 down-regulation is because of more significant loss of this dominant Akt isoform, we generated IGF-IR cells that overexpress wild-type Akt2, such that Akt1 is no longer present in excess of Akt2. Akt1 down-regulation in these cells also resulted in enhanced migration (Fig. S2 A, available at http://www.jcb.org/cgi/content/full/jcb.200505087/DC1), thus supporting the isoform specificity of the effect on migration.

The enhanced migration observed with Akt1 down-regulation was accompanied by changes in protein expression that are consistent with EMT (Fig. 4 B). In EMT, the expression of epithelial proteins (e.g., E-cadherin) is suppressed and the expression of mesenchymal genes (e.g., vimentin and N-cadherin) is enhanced (for reviews see Grunert et al., 2003; Thiery, 2003). Ligand stimulation of IGF-IR cells resulted in minimal changes in the expression of these proteins when compared with parental MCF-10A cells, despite the morphological changes. However, Akt1 down-regulation repressed E-cadherin expression and enhanced N-cadherin expression. Parental MCF-10A cells express basal levels of vimentin; however, Akt1 down-regulation consistently induced a small increase in its expression in IGF-IR cells. In contrast, Akt2 down-regulation in IGF-IR–overexpressing cells had no detectable effect on E-cadherin levels. Interestingly, vimentin expression was significantly decreased, even below the baseline observed with parental MCF-10A cells. Collectively, these results suggest that Akt1 down-regulation in IGF-IR cells promotes phenotypic changes associated with EMT, as demonstrated by alterations in epithelial and mesenchymal protein expression and migratory capacity. In contrast, Akt2 down-regulation does not alter expression of E-cadherin or N-cadherin, but does reduce expression of vimentin to levels lower than the basal levels detected in control MCF-10A cells, suggesting that Akt2 expression may be necessary for vimentin expression.

ERK activation is enhanced by Akt1 down-regulation

Hyperactivation of the ERK/MAP kinase pathway, via either activation of Ras or a growth factor receptor, plays a cooperative role in many models of EMT (for review see Grunert et al., 2003). Akt1 overexpression has previously been reported to suppress ERK activation (Rommel et al., 1999; Zimmermann and Moelling, 1999). Therefore, we examined the effect of down-regulating Akt1 or -2 on activation of ERK signaling.

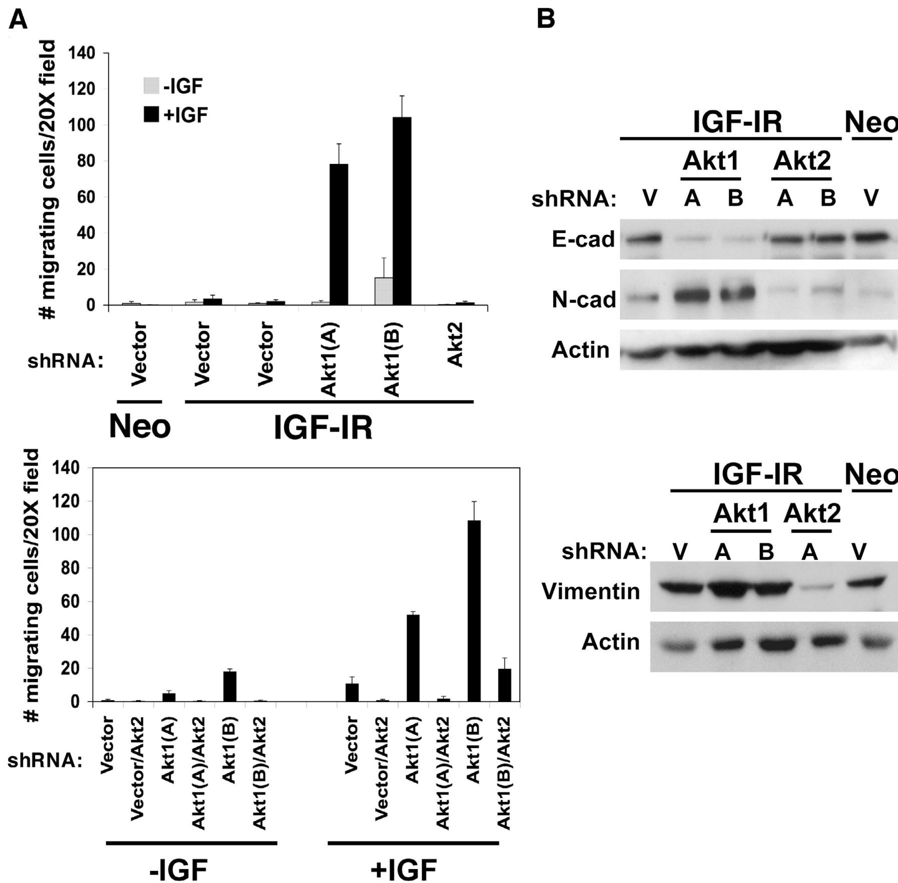

Figure 4. **Down-regulation of Akt1 in IGF-IR cells induces migration and EMT.** (A, top) The motility of IGF-IR cells with isoform-specific down-regulation of Akt was assessed using transwell assays. Cells were starved overnight in 2% serum in the absence of EGF and IGF. Migration was assessed after 16–20 h in the presence or absence of 100 ng/ml IGF-I. The histogram displays the mean number of migrated cells obtained by counting 10 separate fields in three independent experiments. (bottom) IGF-IR cells with down-regulation of both Akt1 and -2 were starved and migration in the presence or absence of IGF-I was assessed. Shown is a representative experiment displaying the mean number of migrated cells in five separate fields. Error bars represent means ± SD. (B) IGF-IR cells infected with empty vector control (V) or shRNA vectors targeting Akt1 or -2 were grown in 2% horse serum and 100 ng/ml IGF-I and lysed in NP-40 (for E- and N-cadherin) or RIPA buffer (Vimentin). Lysates were immunoblotted with the indicated antibodies.

Immunoblotting with antibody to activated ERK1/2 showed that Akt1 down-regulation caused an activation of ERK in nonstimulated cells and enhanced ERK activation under IGF-I–stimulated conditions (Fig. 5 A). Enhanced ERK activation was also observed with Akt1 down-regulation in cells overexpressing Akt2 (Fig. S2 B). In contrast, down-regulation of Akt2 in IGF-IR cells had no effect on ERK1/2 activation. Cells in which both Akt1 and -2 were down-regulated exhibited enhanced ERK activation, comparable with that observed with Akt1 down-regulation alone (Fig. 5 B).

To assess whether enhanced ERK activation was sufficient to induce phenotypic alterations in IGF-IR cells similar to those caused by Akt1 down-regulation, we overexpressed an activated variant of MEK2 (MEKDD) in IGF-IR or Neo cells (Fig. 6). Expression of MEKDD in IGF-I–stimulated IGF-IR cells enhanced the spindle-shaped morphology of these cells in monolayer cultures (Fig. 6 A). MEKDD overexpression also enhanced migration of both IGF-IR and Neo control cells in the presence of IGF-I (Fig. 6 B). However, in 3D cultures, only cells expressing both ectopic IGF-IR and MEKDD were capable of forming structures with invasive protrusions that resembled those observed with Akt1 down-regulation (Fig. 6 C). Despite their migratory capacity in transwell assays, MEKDD-overexpressing parental MCF-10A cells formed normal acinar structures. We have previously shown that MEKDD cells produce EGF receptor ligands, which could be responsible for the migration observed with MEKDD cells (Debnath et al., 2003b).

The effect of MEKDD expression on epithelial markers was also assessed. MEKDD expression alone was sufficient to enhance expression of N-cadherin (Fig. 6 D). However, consistent with the 3D assays, only coexpression of MEKDD in ligand-stimulated IGF-IR, but not control MCF-10A, cells resulted in significant repression of E-cadherin expression (Fig. 6 D). Thus, ERK hyperactivation, driven by activated MEK, in ligand-stimulated, IGF-IR–overexpressing cells was sufficient to phenocopy both the morphological alterations, loss of

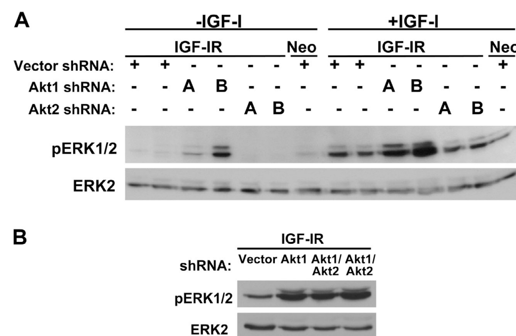

Figure 5. **ERK activation is enhanced by Akt1 down-regulation.** (A) Neo or IGF-IR cells with isoform-specific down-regulation of Akt were grown in monolayer cultures, starved, and stimulated with 2% horse serum ± 100 ng/ml IGF-I in the absence of EGF. Lysates were immunoblotted with the indicated antibodies. (B) IGF-IR cells overexpressing empty vector control, Akt1 shRNA, or both Akt1 and -2 shRNA vectors were starved and stimulated with 100 ng/ml IGF-I in the absence of EGF. Lysates were immunoblotted as indicated.

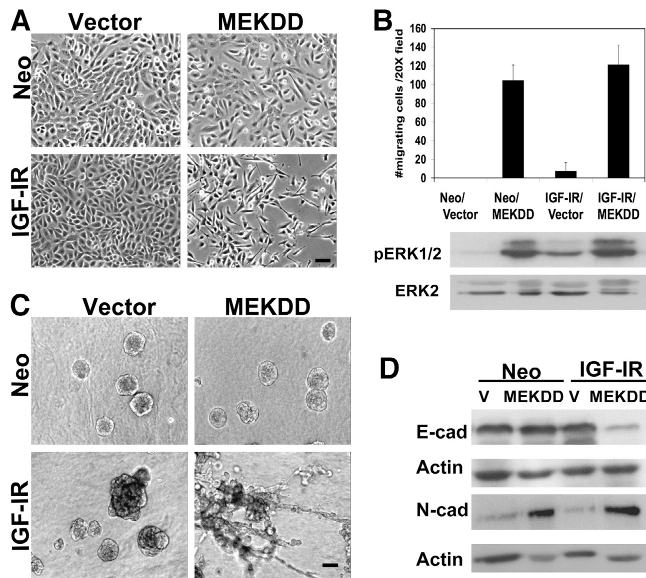

Figure 6. **Expression of constitutively active MEK in IGF-IR cells results in invasive structures and repression of E-cadherin.** (A) IGF-IR or Neo cells overexpressing vector control or constitutively active MEK2 (MEKDD) were grown in monolayer cultures containing EGF and IGF-I (100 ng/ml). Bar, 50 μM. (B) Transwell migration assay and western analyses of IGF-IR or Neo cells overexpressing MEKDD were performed. Cells were starved in the absence of EGF for 24 h. Migration was assessed after 16–20 h in media containing 2% horse serum and 100 ng/ml IGF-I. Lysates were immunoblotted as indicated. Error bars represent means ± SD. (C) IGF-IR or Neo cells overexpressing MEKDD were grown in 3D Matrigel/collagen (50:50) cultures for 12 d. All cells were maintained in 2% horse serum, EGF, and 100 ng/ml IGF-I. Phase-contrast images are shown. Bar, 50 μM. (D) IGF-IR or Neo cells overexpressing vector control (V) or MEKDD were grown in monolayer cultures with 2% horse serum and 100 ng/ml IGF-I. Cells were lysed in NP-40 lysis buffer and lysates were immunoblotted with the indicated antibodies.

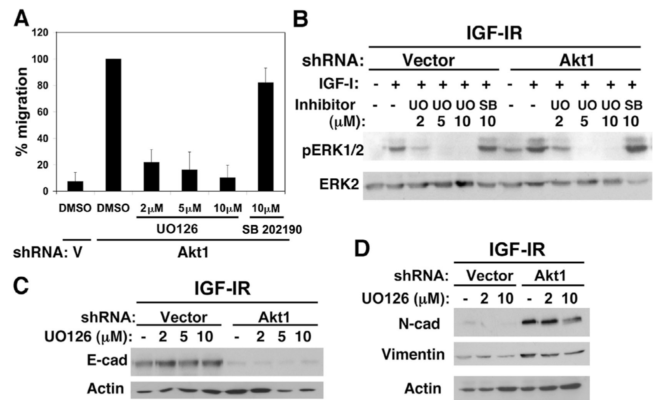

Figure 7. **Inhibition of ERK signaling inhibits migration of Akt1 down-regulated cells.** (A) Migration of IGF-IR cells expressing Akt1 shRNA or empty vector (V) control treated with DMSO, 2–10 μM UO126, or 10 μM SB 202190 was assessed using transwell migration assay. Cells were starved in the absence of EGF. Migration was assessed after 16–20 h in media containing 2% horse serum, 100 ng/ml IGF-I, and DMSO or inhibitor. The histogram displays the mean percentage migration relative to Akt1 down-regulated IGF-IR cells treated with DMSO within the same experiment. The mean values were derived from three independent experiments. Error bars represent means ± SD. (B) Akt1 down-regulated IGF-IR cells were grown in 2% serum, 100 ng/ml IGF-I, and DMSO, 2–10 μM UO126, or SB 202190 for 72 h, with the inhibitor replaced after 48 h. Lysates were immunoblotted as indicated. (C) IGF-IR cells expressing Akt1 or empty vector shRNA were cultured in 2% serum, IGF-I, and DMSO or 2–10 μM UO126 for 72 h, lysed in NP-40 lysis buffer, and immunoblotted with antibodies against E-cadherin. (D) IGF-IR cells expressing Akt1 or empty vector shRNA and cultured in 2% serum, IGF-I, and DMSO or 2–10 μM UO126 for 72 h were lysed in RIPA lysis buffer and immunoblotted with the indicated antibodies.

E-cadherin expression and increase in N-cadherin expression, observed with Akt1 down-regulation.

Enhanced ERK activation is necessary for migration induced by Akt1 down-regulation

To determine whether enhanced ERK activation is required for the phenotypes induced by Akt1 down-regulation, we examined the effects of inhibiting MEK-induced ERK activation using UO126, a specific MEK inhibitor. Treatment with UO126 significantly inhibited migration of Akt1 down-regulated cells, whereas treatment with a p38 inhibitor (SB 202190) or DMSO vehicle control had no effect (Fig. 7 A). Furthermore, this inhibition was observed at low concentrations of UO126 (2 μM), which reduced IGF-I–stimulated ERK activation in Akt1 down-regulated IGF-IR cells to the levels observed in IGF-IR control cells (Fig. 7 B); thus, these effects are detectable at levels of ERK inhibition that would not be expected to affect basal cell functions.

The effect of MEK/ERK inhibition on epithelial and mesenchymal protein expression was also examined (Fig. 7, C and D). Interestingly, sustained treatment with UO126 over 3–5 d did not reverse the down-regulation of E-cadherin. Furthermore, the enhancement in N-cadherin expression observed with Akt1 down-regulation was also resistant to UO126 treatment. These results suggest that although enhanced ERK activity observed with Akt1 down-regulation is necessary and sufficient for migration of IGF-IR cells, the effects on EMT gene expression may be irreversible or that other pathways may contribute to the sustained maintenance of EMT gene expression changes.

Down-regulation of Akt1 enhances migration and ERK activation induced by EGF

The phenotypes observed with Akt1 down-regulation in IGF-IR cells suggested that Akt1 has an inhibitory effect on IGF-I–triggered migration and ERK activation. To determine whether similar effects are observed when cells are stimulated by other growth factor receptors, we examined migration and ERK activation in response to EGF stimulation (Fig. 8). Endogenous levels of EGF receptor are sufficient to robustly induce these activities upon EGF stimulation. The effects of Akt isoform down-regulation was examined in the context of subsaturating EGF concentrations. Akt1 down-regulation enhanced EGF-induced ERK activation and migration. In contrast, Akt2 down-regulation inhibited EGF-stimulated transwell migration at all concentrations evaluated. This inhibition is more readily apparent as EGF, in contrast to IGF-I, robustly induces migration. Thus, Akt1 down-regulation has similar effects on ERK activation and migration when cells are stimulated by another growth factor.

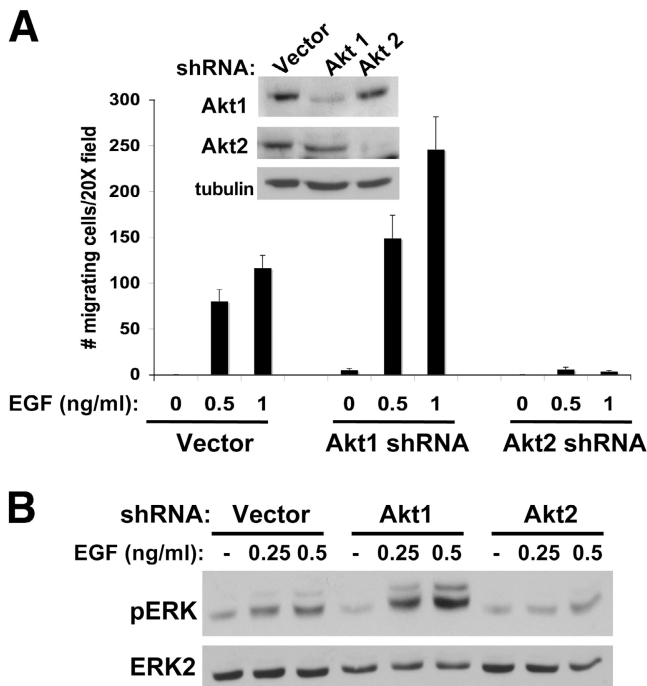

Figure 8. **Down-regulation of Akt1 enhances EGF-stimulated migration and ERK activation.** (A) MCF-10A cells overexpressing empty vector or Akt1 or -2 shRNA vectors were generated. Isoform-specific down-regulation was confirmed by Western analysis and migration in response to EGF stimulation was assessed. Before plating in transwell assays, cells were starved overnight in the absence of EGF. Cells were stimulated with EGF at the indicated concentrations and migration was assessed. The histogram displays a representative experiment with mean values obtained by counting 10 independent fields. Error bars represent means ± SD. (B) MCF-10A cells overexpressing empty vector control or Akt1 or -2 shRNA vectors were starved in the absence of EGF. Cells were stimulated with the indicated concentrations of EGF, lysed, and immunoblotted with the indicated antibodies.

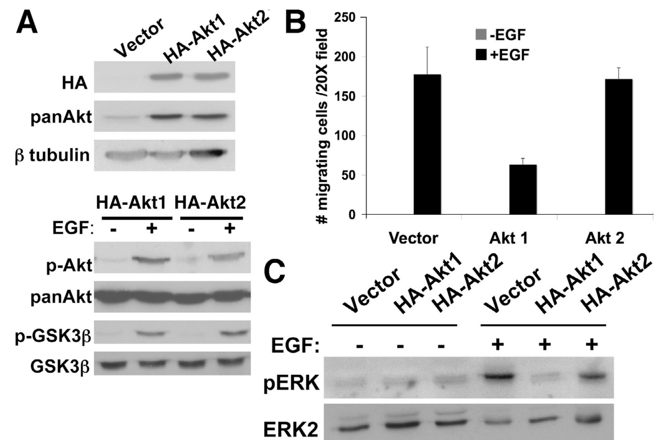

Figure 9. **Overexpression of Akt1 suppresses EGF-stimulated migration and ERK activation.** (A) MCF-10A cells overexpressing HA-tagged wild-type Akt1 or -2 were generated. (top) Levels of overexpression were confirmed using antibodies against the HA tag or a panAkt antibody that recognizes all isoforms. (bottom) Levels of activated, phosphorylated Akt and phosphorylated GSK3β were assessed after EGF stimulation. (B) Migration of MCF-10A cells overexpressing vector control or wild-type HA-tagged Akt1 or -2 was assessed by transwell assay. Cells were starved in the absence of EGF for 24 h. Migration was assessed after 16–20 h in media with 2% horse serum ± 5 ng/ml EGF. A representative experiment is shown with mean values obtained by counting 10 separate fields. Error bars represent means ± SD. (C) MCF-10A cells overexpressing vector control or wild-type Akt1 or -2 were starved for 24 h and stimulated with media containing 2% horse serum ± 5 ng/ml EGF. Cells were lysed and immunoblotted with the indicated antibodies.

Overexpression of Akt1 suppresses EGF-dependent migration and ERK activation

To more directly assess the potential inhibitory effect of Akt1 on growth factor–induced migration and ERK activation, MCF-10A cells overexpressing wild-type or HA-tagged Akt1 or -2 were generated by retroviral infection. Comparable levels of overexpression were confirmed using antibodies that recognize the HA tag or all Akt isoforms (panAkt; Fig. 9 A). Because ligand-stimulated IGF-IR cells are not motile, we examined the effects of Akt1 or -2 overexpression on parental MCF-10A cells treated with EGF, which induces robust migration and strong activation of ERK. Comparable total levels of activated, phosphorylated Akt were induced after EGF stimulation (Fig. 9 A). Phosphorylation of glycogen synthase kinase-3 (GSK3)β, a known Akt substrate (Brazil et al., 2004), was also comparable in cells overexpressing Akt1 or -2, further confirming equivalent levels of Akt activity. In comparison to control MCF-10A cells, wild-type Akt1-overexpressing cells exhibited decreased migration in response to EGF stimulation (Fig. 9 B). These results are consistent with previous studies in which overexpression of wild-type or activated Akt1 in breast cancer cell lines slightly decreased migration and invasion (Shaw et al., 1997; Arboleda et al., 2003). In contrast, overexpression of wild-type Akt2 did not affect EGF-induced migration.

The effect of Akt1 overexpression on EGF-stimulated ERK activation was also examined. Lysates of EGF-stimulated vector control or Akt1-overexpressing MCF-10A cells were immunoblotted with an antibody against activated ERK1/2. The level of activated ERK detected in lysates of Akt1-overexpressing cells was decreased when compared with parental MCF-10A cells (Fig. 9 C). These results are consistent with a negative, inhibitory effect of Akt1 on migration and ERK activation. In contrast, overexpression of wild-type Akt2 in MCF-10A did not affect ERK activation induced by EGF. These results indicate that Akt1 may exert an isoform-specific suppressive effect on growth factor–stimulated ERK activity.

Discussion

Akt is activated downstream of the growth factor receptors and oncogenes implicated in human cancer and plays a critical role in normal development, as well as in tumor pathogenesis, via effects on metabolism, survival, and proliferation. The role of Akt in cell migration and metastases is less clear because of conflicting studies suggesting either positive or negative regulatory roles (Shaw et al., 1997; Park et al., 2001). Previous studies have largely relied on overexpression strategies in cancer cell lines or pharmacological inhibitors of PI 3-kinase activity, which would inhibit activity of all Akt isoforms. We describe studies that reveal isoform-specific roles for endogenous Akt1 and -2 in both positive and negative regulation of processes

downstream of growth factor receptors and provide insights into mechanisms that may be partly responsible for those conflicting conclusions. In addition, we provide evidence for the importance of Akt1 in a cross-regulatory control circuit between the PI 3-kinase and ERK signaling pathways, two of the major pathways that regulate diverse cellular activities. Differential regulation of signaling pathways by Akt isoforms may critically contribute to their distinct roles in normal development and disease pathogenesis.

The 3D culture system used in this study provides an in vitro model to investigate phenotypic effects that resemble events that take place during breast cancer initiation and progression, such as escape from proliferative suppression, filling of the hollow acinar luminal space, and induction of protrusive, invasive behavior (Debnath et al., 2003a). We demonstrate that enhanced IGF-I signaling leads to the formation of constitutively proliferating structures with low apoptotic activity and filled lumen. These structures share features with noninvasive breast carcinoma in situ, including maintenance of basement membrane architecture, absence of a hollow lumen, and hyperproliferation. These results extend our understanding of the phenotypic effects of IGF-IR hyperstimulation derived from previous studies in monolayer cultures.

The use of shRNA vectors has made it feasible to investigate cellular pathways required for the distinct IGF-IR–induced phenotypic effects in our model and revealed interesting, isoform-specific functions of Akt. Akt2 down-regulation caused a near complete inhibition of the IGF-IR phenotype in 3D cultures; the structures failed to escape proliferation arrest, to suppress apoptosis of centrally localized cells, and to fill the luminal space. These results indicate that either reduction of Akt2 specifically, or reduction in total Akt levels, suppresses all of the phenotypic effects observed in 3D culture. In contrast, Akt1 down-regulation resulted in a surprising conversion of IGF-IR structures from large, misshapen, solid masses to invasive structures that displayed features associated with EMT (fibroblast-like morphology, enhanced migration, loss of epithelial markers, and acquisition of mesenchymal gene expression). These results are consistent with an inhibitory role for endogenous Akt1 in these processes.

Our data suggest that one target of the inhibitory activity of Akt1 is the ERK signaling pathway, as specific down-regulation of Akt1 enhanced ERK activation both in response to IGF-I or EGF stimulation. These data are consistent with previous studies in which overexpression of activated Akt1 decreased ERK activity (Rommel et al., 1999; Zimmermann and Moelling, 1999). Our studies highlight the isoform-specific nature of this effect and establish a role for endogenous Akt1 protein in modulating ERK under conditions of growth factor stimulation. The ability of endogenous Akt to cross-regulate Ras/Raf/MEK/ERK signaling may be conserved across species, as Akt down-regulation in *Drosophila melanogaster*, which express only one isoform, enhances insulin-stimulated ERK activation (Freedman, A., and N. Perrimon, personal communication).

Several lines of evidence suggest that inhibition of ERK and migration is specific for Akt1 and unlikely because of the different degrees of residual Akt activity after down-regulation. First, comparable degrees of overexpression of Akt1, but not Akt2, inhibits ERK activation and migration stimulated by EGF. Second, reduction of Akt2 in the background of Akt1 shRNA expression does not enhance ERK activation or migration. Dual down-regulation of Akt2 suppresses enhanced migration in cells overexpressing Akt1 shRNA vectors. Finally, overexpression of Akt2 to levels comparable or greater than Akt1 does not prevent the effects of Akt1 down-regulation, thus supporting an isoform-specific effect of Akt1 that occurs regardless of total levels of residual Akt.

Akt1-mediated inhibition of the ERK pathway could occur at multiple levels. Constitutively active Akt1 is able to phosphorylate a residue (Ser259) of Raf, which mediates binding to 14-3-3 proteins, causing inhibition of Raf activity (Zimmermann and Moelling, 1999). In preliminary studies, we have not observed significant changes in Ser259 phosphorylation of Raf with Akt1 down-regulation (unpublished data). PI 3/Akt kinase signaling has also been shown to regulate ERK upstream of Raf at the level of IRS-1–Grb2 complex formation (Choi and Sung, 2004). Furthermore, constitutively active Akt1 has been reported to suppress ERK activity downstream of Raf and MEK and independent of ERK phosphorylation (Galetic et al., 2003). Thus, there may be multiple levels of ERK regulation and studies are underway to delineate these mechanisms.

The importance of enhanced ERK activation in migration and induction of EMT is supported by studies in which ERK activity was found to be critically involved in EMT induced by other stimuli, such as Ras/TGFβ (Janda et al., 2002), EGF/TGFβ (Grande et al., 2002), and HGF/ErbB2 (Khoury et al., 2005). Pharmacological inhibition of ERK signaling has been shown to decrease invasion or inhibit specific biochemical changes consistent with EMT induced by these stimuli. In our study, enhancement of ERK activation, via a constitutively active MEK2, appears to be sufficient to induce migration, conversion to an invasive phenotype in 3D cultures, repression of E-cadherin, and induction of N-cadherin expression in collaboration with IGF-I hyperstimulation. Furthermore, the enhanced migration induced by Akt1 down-regulation is sensitive to pharmacological inhibition of MEK/ERK signaling.

Interestingly, however, ERK inhibition did not restore expression of epithelial markers or significantly down-regulate mesenchymal markers in Akt1 down-regulated cells that had undergone EMT. These results are consistent with previous ones showing that, although pretreatment or concomitant treatment with a pharmacological MEK inhibitor is able to prevent invasion or the development of EMT (Grande et al., 2002; Janda et al., 2002; Khoury et al., 2005), treatment after the establishment of EMT did not (Khoury et al., 2005). The failure to completely reverse EMT may be caused by irreversible changes induced by enhanced ERK activation or to ERK-independent pathways that are sufficient to maintain the mesenchymal phenotype induced by Akt1 down-regulation. GSK3β signaling has previously been implicated in E-cadherin suppression (Zhou et al., 2004); however, we have not observed significant changes in GSK3β phosphorylation after Akt1 down-regulation (unpublished data). Induction of EMT by both

Ras and FosER has been reported to induce an autocrine TGFβ loop that stabilizes the mesenchymal phenotype (Gotzmann et al., 2002; Janda et al., 2002; Eger et al., 2004). EMT induced by Akt1 down-regulation may lead to the production of a similar stabilizing soluble factor. Thus, combined inhibition of multiple signaling pathways may be required for full reversion of EMT induced by Akt1 down-regulation.

Akt2 may play a role in growth factor–stimulated migration and invasion that is distinct, if not contrasting, to that of Akt1. This is based on our observations that Akt2 down-regulation suppressed migration stimulated by EGF or Akt1 down-regulation, and Akt2 down-regulation reverted the spindle-shaped morphological changes induced by Akt1 down-regulation. These observations are consistent with previous studies that reported that Akt2 overexpression in breast cancer cell lines enhanced their invasive potential and inhibition of Akt2 (via overexpression of dominant-negative constructs) suppressed invasion and metastases triggered by ErbB2 overexpression (Arboleda et al., 2003). Although differential localization and regulation of adhesion molecules (e.g., β1 integrin) was implicated in these Akt2 isoform-specific effects, additional studies to examine endogenous Akt2 functions are ongoing.

The present studies do not allow us to establish whether Akt2 preferentially regulates the antiapoptotic activities of Akt because the loss of Akt1 disrupted acinar morphogenesis to such an extent that analysis of apoptosis in the presumptive luminal space could not be evaluated. For similar reasons, we were unable to examine escape from proliferative arrest in Akt1 down-regulated 3D acini. However, we did observe that proliferation of IGF-I–stimulated cells in monolayer cultures was significantly impaired after down-regulation of either Akt1 or -2, indicating that both proteins contribute to IGF-I–stimulated proliferation. Several targets of Akt family proteins have been shown to regulate cell proliferation and apoptosis through effects on the expression or activity of several proteins including cyclin D, cyclin-CDK inhibitors, mTOR, and proapoptotic proteins Bad and FOXO transcription factors (for review see Brazil et al., 2004). None of the Akt substrates that regulate these proteins have been shown to be specifically phosphorylated by Akt1 or -2. In our preliminary studies, IGF-IR–stimulated phosphorylation of GSK3, FOXO3a, and S6 is not significantly affected by Akt1 or -2 suppression (unpublished data). Previous studies in adipocytes indicated that isoform-specific loss of Akt2 has a more substantial impact on insulin-stimulated glucose uptake than does loss of Akt1 (Bae et al., 2003; Jiang et al., 2003; Katome et al., 2003). Because glucose and other nutrient transporters regulate metabolic processes that affect cell proliferation, effects of Akt2 down-regulation on this pathway may contribute to the reduction in cell proliferation and survival. The precise contributions of Akt1 and -2 to IGF-IR–induced proliferation and antiapoptotic activity require further investigation. In addition, it will be important to examine whether changes in the level of expression of Akt isoforms during morphogenesis contribute in part to the effects of Akt1 and -2 down-regulation in 3D cultures.

The specific mechanisms responsible for the distinct roles of Akt1 and -2 are not known; however, there are a few explanations extrapolated from previously published studies. Differential subcellular localization or binding partners may determine isoform-specific functions. Akt2 expression was reported to be most prominent in regions of cell–matrix contact (Arboleda et al., 2003). Preferential localization of Akt2 to areas of cell–matrix contact may therefore enable interactions with molecules required for motility and invasion. Differential localization may result from distinct protein-binding interactions. Although the Akt isoforms exhibit significant sequence homology and possess similar domain structure, the greatest variation is located within the phosphoinositide-binding PH domain. Indeed, the L-jun NH_2-terminal kinase scaffold proteins POSH and L-jun NH_2-terminal kinase interacting protein 1 interact selectively with the PH domains of Akt2 and -1, respectively (Kim et al., 2002; Figueroa et al., 2003). Chimeric variants of Akt1 and -2 may be useful in establishing which domains of each protein are required for the regulation of ERK activation and cell migration.

The balance between Akt isoform activation downstream of IGF-IR and other growth factor receptors may influence the invasive or metastatic potential of tumors or tumor cell lines. The relative abundance or activation of Akt isoforms may be dynamic and change depending on different cellular contexts. Whether migration or invasion is stimulated may depend on the extent to which a particular agonist activates Akt1 and the extent to which ERK is influenced by Akt1. For example, in tumor cells carrying mutations in Ras or Raf, which activate ERK constitutively, IGF-I may promote cell migration and invasion. Likewise, the ability of wild-type or constitutively activated Akt1 to modulate migration and invasion may depend on whether migration is ERK dependent. The ability of Akt to cross-regulate Ras/Raf/MEK/ERK signaling may also be influenced by mediators that are contextually expressed; e.g., differentiation status in myocytes correlated with the ability to form inhibitory Akt–Raf complexes (Rommel et al., 1999).

The suppressive effects of Akt1 on invasive activity reported previously are consistent with evidence that coexpression of activated Akt1 with oncogenic ErbB2 in mouse mammary epithelial cells decreases the metastatic activity of oncogenic ErbB2 (Hutchinson et al., 2004). However, further studies are required to investigate the precise mechanisms for this decrease. Interestingly, activated Akt1 can rescue the tumor-inducing potential of a mutant form of the polyomavirus middle T antigen that lacks a PI 3-kinase binding site, but does not rescue its invasive activity (Hutchinson et al., 2001). This could result from Akt1-mediated suppression of ERK activation or from a critical requirement for Akt2 or other downstream PI 3-kinase targets. Acute, inducible knockout of Akt isoforms in transgenic tumor models should be informative in establishing the role of specific Akt isoforms in tumorigenesis in vivo.

Efforts are underway to develop pan- or isoform-specific Akt inhibitors as cancer therapeutics (for review see Barnett et al., 2005). Akt2 is amplified in breast and ovarian tumors and, more recently, mutations thought to be activating have been detected in colon cancer (Bellacosa et al., 1995; Parsons et al., 2005). Thus, Akt2 may be a particularly attractive candidate for therapeutic inhibition. The consequences of isoform-specific

inhibition will need to be carefully evaluated in different cellular contexts, especially as there may be unanticipated, differential effects on other signaling pathways, as observed in our studies. The effect of isoform-specific inhibition on different aspects of the tumorigenic phenotype may also vary. Although Akt1 and -2 both contribute to proliferation, isoform-specific pharmacological inhibition may have a differential impact on migration and invasion. Understanding these differences will be key to the development of improved targeted therapeutic strategies.

Materials and methods

Cell culture

MCF-10A cells were obtained from American Type Culture Collection and cultured, as previously described in Debnath et al. (2003a), in DME/F12 (Invitrogen) supplemented with 5% horse serum, 20 ng/ml EGF, 10 μg/ml insulin or 100 ng/ml IGF-I (R&D Systems), 0.5 μg/ml hydrocortisone, 100 ng/ml cholera toxin, 50 U/ml penicillin, and 50 mg/ml streptomycin.

Reagents

Antibodies against phosphorylated IGF-IR/insulin receptor, phosphorylated Akt (Ser 473), phosphorylated MEK (Ser217/221), phosphorylated GSK3β (Ser9), and GSK3β were purchased from Cell Signaling Technology. Phosphorylated ERK1/2 (Threonine185/Tyrosine187) was obtained from Biosource International. IGF-IR β chain, ERK2, MEK, and actin were purchased from Santa Cruz Biotechnology, Inc. Akt1 was purchased from Upstate Biotechnology; Akt3 and panAkt antibody were obtained from R&D Systems; and E-cadherin, N-cadherin, and vimentin were purchased from BD Biosciences. The Akt2-specific antibody and purified Akt1, -2, and -3 were gifts of M. Birnbaum (University of Pennsylvania, Philadelphia, PA). MEK inhibitor UO126 and p38 inhibitor SB 202190 were purchased from Calbiochem.

Retroviral/lentiviral DNA vectors and viru2s production

The pMSCV-Neo–based retroviral vectors encoding wild-type human IGF-IR complementary DNA and IGF-IR variants were gifts of R. Baserga (Thomas Jefferson University, Philadelphia, PA). pBABE-MEK2DD was a gift of S. Meloche (Institut Recherches Clinique de Montreal, Montreal, Quebec, Canada). The pLNCX-based retroviral vector encoding wild-type HA-tagged Akt1 was generated from a construct obtained from P. Tsichlis (New England Medical Center-Tufts, Boston, MA). pBABE-puro–encoding wild-type HA-tagged human Akt2 was generated from a construct obtained from J. Cheng (University of South Florida, Tampa, FL). VSV-pseudotyped vectors were produced by transfection of the VSV-GPG producer cell line (a gift from R. Mulligan, Children's Hospital, Boston, MA; Ory et al., 1996) with 10 μg DNA using Lipofectamine 2000 (Invitrogen). Retrovirus-containing supernatants were collected at days 5–7 after transfection and were stored at −80°C.

Retroviral (pMKO.1puro) or lentiviral (pLKO.1puro) vectors encoding hairpin RNA sequences were used to down-regulate specific Akt isoforms. The hairpin sequences used for Akt1 were as follows: Akt1(A), forward, 5′-CCGGTGCTGCTTCCTCCTCAAGAATGTTCAAGAGACATTCTTGAGGAGGAAGTAGCTTTTTGGAAG-3′; reverse, 5′-AATTCTTCCAAAAAGCTACTTCCTCCTCAAGAATGTCTCTTGAACATTCTTGAGGAGGAAGCAGCA-3′; Akt1(B; a gift of W. Hahn, Dana-Farber Cancer Institute, Boston, MA), forward, 5′-CCGGGAGTTTGAGTACCTGAAGCTCCTCGAGGAGCTTCAGGTACTCAAACTCTTTTTG-3′; reverse, 5′-AATTCAAAAAGAGTTTGAGTACCTGAAGCTCCTCGAGGAGCTTCAGGTACTCAAACTC-3′. The hairpin sequences used for Akt2 were as follows: Akt2 (A), forward, 5′-CCGGTGCGTGGTGGATACATCAAGACTTCAAGAGAGTCTTGATGTATTCACCACGCTTTTTGGAAG-3′; reverse, 5′-AATTCTTCCAAAAAGCGTGGTGAATACATCAAGACTCTCTTGAAGTCTTGATGTATCCACCACGCA-3′; Akt2(B), forward, 5′-CCGGTGAGGTGTTTGTCATCAAAGAATTCAAGAGATTCTTTGATGACAGACACCTCTTTTTGGAAG-3′; reverse, 5′-AATTCTTCCAAAAAGAGGTGTCTGTCATCAAAGAATCTCTTGAATTCTTTGATGACAAACACCTCA-3′.

VSVG-pseudotyped retroviral or lentiviral vectors were generated by the cotransfection of GP-2 (BD Biosciences) or 293-T packaging cells.

Generation of MCF-10A cell lines

MCF-10A cells (5×10^5 cells) were infected with the retroviral viruses, and stable populations were chosen by selection with 2 μg/ml puromycin or 300 μg/ml G418 (Sigma-Aldrich). Hairpin RNA–encoding retroviruses and lentiviruses were used to superinfect IGF-IR–overexpressing MCF-10A cells, and stable populations were obtained by dual selection with 2 μg/ml puromycin and 300 μg/ml G418.

3D cultures

3D Matrigel (BD Biosciences) morphogenesis assays were performed as previously described (Debnath et al., 2003a). Acini were cultured in assay media (2% horse serum, 5 ng/ml EGF, 0.5 μg/ml hydrocortisone, 100 ng/ml cholera toxin, 50 U/ml penicillin, and 50 mg/ml streptomycin ± 100 ng/ml IGF-I). Cultures were re-fed every 4 d. For collagen/Matrigel assays, a 50:50 mixture of growth factor–reduced Matrigel and bovine dermal collagen I (Vitrogen Cohesion Technologies) was used as the underlay. Before mixing, collagen I was neutralized as previously described (Seton-Rogers et al., 2004).

Phase-contrast microscopy

Cells grown in monolayer cultures, as well as 3D acinar structures, were visualized at 20°C using a microscope (model TE300; Nikon) equipped with a camera (model MTI CCD-300T-RC; Dage), using a 4×, 0.13 NA, or 10×, 0.3 NA, objective. Images were acquired using IP Lab Spectrum software (BD Biosciences), converted to TIFF images, and arranged using Photoshop 7.0 (Adobe).

Immunofluorescence analyses and confocal microscopy

Acinar structures were fixed in 2% formalin (Sigma-Aldrich) at room temperature for 20 min and permeabilized in 0.5% Triton X-100 in PBS for 10 min at 4°C. Immunostaining of acinar structures was performed as previously described (Debnath et al., 2002, 2003a) and imaged at 20°C. Confocal analyses were performed using a microscope (model TE2000; Nikon) with the C1plus confocal microscope system equipped with krypton-argon (488 line) and HeNe (543 and 633 lines) lasers (Nikon). Structures were analyzed with a 40×, 1.3 NA, objective, and images were acquired using C1 confocal software (Nikon). For each time point, the images presented are representative of four or more independent experiments. All images were converted to TIFF format and arranged using Photoshop 7.0 (Adobe).

Western blot analysis

Cells were lysed in NP-40 lysis buffer (1% NP-40, 50 mM Tris, pH 7.6, and 150 mM NaCl) or RIPA lysis buffer (1% Triton X-100, 1% NaDOC, 0.1% SDS, 20 mM Tris, pH 7.5, 150 mM NaCl, and 1 mM EDTA) supplemented with protease and phosphatase inhibitors (1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 μg/ml pepstatin, 10 μg/ml PMSF, 1 mM $NaVO_4$, and 1 mM NaF) for 30 min at 4°C. Lysates were clarified by centrifugation and supernatants were collected. Proteins were resolved by 8–10% SDS-PAGE and immunoblotted using standard techniques.

Transwell migration assay

MCF-10A cells were starved overnight in assay media containing no EGF and only 2% horse serum ± 100 ng/ml IGF-I. The cells were trypsinized and 10^5 cells were added to the top chambers of 24-well transwell plates (8 μm pore size; BD Biosciences), and assay media (± IGF-I) was added to the bottom chambers. For assays examining migration in the presence of subsaturating concentrations of EGF, EGF was added after overnight starvation. For assays using pharmacological inhibitors, the inhibitors were added to the media at the initiation of the assay. After overnight incubation, the nonmotile cells at the top of the filter were removed and the motile cells at the bottom of the filter were fixed with 70% ethanol and stained with 5 μg/ml DAPI to visualize nuclei. The number of migrating cells in each chamber was quantified by counting five fields under 20× magnification. Each condition was performed in duplicate and the average number of cells per field is represented. Experiments were repeated a minimum of three times.

Online supplemental material

Fig. S1 shows the relative levels of expression of Akt1, -2, and -3 in MCF-10A cells, as well as the levels of down-regulation achieved with the isoform-specific shRNA vectors used in the study. Fig. S2 shows enhancement in migration and ERK activation observed with Akt1 down-regulation in IGF-IR cells overexpressing Akt2. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200505087/DC1.

We thank Dr. William Hahn and the RNAi Consortium for Akt shRNA vectors and Craig Deloughery for help with shRNA vector construction. We thank Drs. Renato Baserga, Morris Birnbaum, Jin Cheng, Philip Tsichlis, Sylvan

Meloche, and Richard Mulligan for various reagents. We thank Steven Bushnell, Randall King, and John Blenis for helpful discussions.

This work was supported by grants from the National Institutes of Health (CA105134 and CA80111), the Department of Defense (DAMD 17-02-1-0692), and Sanofi-Aventis Pharmaceuticals to J.S. Brugge; and an American Cancer Society–Edith A. Pistorino–Ann L. and Herbert Siegel Postdoctoral Fellowship (PF-04-072-01-TBE) and an American Society of Clinical Oncology Young Investigator Award to H.Y. Irie.

Submitted: 16 May 2005
Accepted: 9 November 2005

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