The Roles of Akt1 and Akt2 Downstream of Src Family Kinases in the Migration and Invasion of Breast Cancer Cells

by

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Abstract

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The leading cause of death in breast cancer patients is metastasis. Cancer metastasis involves a complex series of events, including cell detachment from the primary tumor, invasion into the surrounding tissue, intravasation into the circulatory system, extravasation, and growth in a new organ. Src Family Kinases (SFKs) comprise a group of non-receptor tyrosine kinases that regulate a variety of pathways that promote cell survival, growth, proliferation, motility, and invasion. Previous studies have found a correlation between the activity of SFKs and the progression of breast cancer into metastasis. Since the pathways regulated by SFKs are often activated in metastasis, I investigated whether SFKs play a role in the metastasis of breast cancer cells.

To determine whether SFKs regulate breast cancer metastasis, I analyzed the role of SFKs in the migration and invasion of a metastatic breast cancer cell line, MDA-MB-231 cells. SFKs were inhibited with expression of dominant negative Src or by treatment with the pharmacological inhibitors PP2 and SU6656. The inhibition of SFKs in MDA-MB-231 cells led to decreased cell migration and invasion through transwell migration and invasion chambers. To explore the mechanism by which SFKs regulate cellular migration and invasion, I investigated whether SFKs regulate the Akt1 and Akt2 isoforms in MDA-MB-231 breast cancer cells. Akt isoforms are serine/threonine kinases that are often activated in cancer cells. The activity of Akt isoforms were decreased in SFK-inhibited MDA-MB-231 cells as determined by immunoblot detection of the phosphorylation of Threonine 308 and Serine 473 residues, *in vitro* kinase assays, and immunoblot detection of the phosphorylation of an Akt substrate. To determine whether the Akt isoforms play a role in the migration and/or invasion of breast cancer cells, small interfering RNAs were used to knockdown the expression of each Akt isoform. Akt2 knockdown specifically led

to decreased MDA-MB-231 cell migration and invasion by mechanisms that did not involve the attachment of cells onto extracellular matrix or the regulation of Pak1. Co-immunoprecipitation and mass spectrometry assays were used in an effort to identify an Akt2-specific binding partner that could mediate its role in breast cancer cell migration and invasion. The Arf6-GAP ACAP2 was identified as a protein that co-immunoprecipitated with Akt2-Flag, but additional analysis found that ACAP2 was immunoprecipitated non-specifically by the mouse anti-Flag antibody and does not interact with Akt2.

These results point to a role of SFKs in breast cancer metastasis, specifically in promoting cell migration and invasion. Although SFKs regulate the activation of both Akt1 and Akt2 isoforms in MDA-MB-231 cells, only Akt2 is required for the migration and invasion of MDA-MB-231 cells.

Dedication

To my husband, Gary, for always believing in me.

To my parents, Teresita and Alfredo, and to my siblings, Katrina, Maria, Michael, and Victoria for all your love and support.

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Chapter 1: Introduction

The serine/threonine kinase Akt was first discovered as the cellular homolog of v-*Akt*—the transforming gene of AKT8, a retrovirus isolated from a spontaneous thymoma of an AKR mouse (Bellacosa et al., 1991). Cellular Akt is found in eukaryotic organisms ranging from *C. elegans* to mammals and is activated in response to various growth factors and hormones. Since its discovery, a substantial amount of research has been performed to identify the cellular roles of Akt. It has been found that Akt is an important regulator of many cell processes including survival, proliferation, growth, migration, and invasion. Akt plays a critical role in repressing pro-apoptotic proteins in response to stimulation with growth factors and cell adhesion to the extracellular matrix. Akt regulates cell growth in part by globally activating translation. Akt regulates glucose metabolism downstream of insulin signaling. And Akt promotes cell proliferation by inhibition of cell cycle inhibitors and activation of proteins that promote the progression of the cell cycle. The pathways regulated by Akt are often activated in cancer; therefore, understanding the role of Akt in tumorigenesis could inform efforts to exploit Akt as a drug target in cancer therapy.

Domain Structure of Akt Isoforms

Akt is classified as a member of the AGC family of kinases. The family of AGC kinases consists of 60 kinases that contain a catalytic domain which exhibits a high degree of sequence similarity with the catalytic domains of PKA, PKG, and PKC (Pearce et al., 2010). AGC kinases are prototypically bilobal serine/threonine kinases that require phosphorylation at two amino acid residues for activation. The subfamily of Akt kinases is comprised of three members: Akt1, Akt2, and Akt3, which are transcribed from unique genes. The Akt isoforms have the same domain organization and exhibit over 80% sequence identity (Figure1). At the N-terminus, the Akt isoforms contain a pleckstrin homology (PH) domain. PH domains are typically protein-protein or protein-lipid interacting domains that can regulate the subcellular localization and interaction with binding partners of PH domain-containing proteins. In unstimulated cells, the PH domain maintains Akt in an inactive state by preventing the phosphorylation of Akt (Sable et al., 1998). Upon cell stimulation by growth factors and/or hormones, the levels of phosphatidylinositol-3,4-bisphosphate (PtdIns(3,4)P2) and phosphatidylinositol-3,4,5-trisphosphate (PtdIns(3,4,5)P3) in the cell membrane increase and these phosphoinositides bind to the PH domain of Akt isoforms (Franke et al., 1997). This leads to the localization of Akt isoforms at the cell membrane and induces a conformational change that permits phosphorylation of Akt kinases at a residue required for activation (Alessi et al., 1997a; Andjelkovic et al., 1996). Proximal to the PH domain is a linker region that connects the PH domain to the catalytic kinase domain. Of the different domains in Akt isoforms, the kinase domains exhibit the highest degree of sequence

similarity and contain several elements that are present in other kinases of the AGC family. Within the catalytic domain is an Asp-Phe-Gly motif that positions an ATP molecule, and a critical Lys residue that interacts with the ATP molecule and orients it for phosphoryl transfer (Pearce et al., 2010). The consensus target sequence of Akt kinases is RxRxxS/T where x denotes any amino acid. At the C-terminus is the hydrophobic regulatory tail. In the inactive state, this domain is disordered, but upon phosphorylation becomes ordered and stabilizes Akt in the active conformation (Yang et al., 2002).

Regulation of Akt Isoforms

As with other members of the AGC family of kinases, Akt isoforms require phosphorylation at two amino acid residues for full activation (Figure 1). Phosphorylation of Akt1/2/3 at Thr308/309/305 within the T-loop of the catalytic domain is required for a conformational change that allows the interaction and orientation of an ATP molecule. Additional phosphorylation of Akt1/2/3 at Ser473/474/472, located in the hydrophobic regulatory tail, is required for a disordered to ordered transition and is required for maximal kinase activation. There have also been reports that phosphorylation at the Ser473/474/472 site can regulate Akt substrate specificity (Jacinto et al., 2006).

Growth factors, hormones, and integrins activate the Akt isoforms via the class I Phosphoinositide 3-kinase (PI3K) pathway (Vanhaesebroeck et al., 2010). Class I PI3Ks contain two subunits: a regulatory subunit and a catalytic subunit that phosphorylates phosphoinositides at the 3'hydroxyl position. Receptor tyrosine kinases can activate PI3K directly or through adaptor proteins. Upon binding of growth factors or hormones to their corresponding receptors, the phosphorylated tyrosine of the activated receptor or an associated adaptor protein binds to the SH2 domain of the PI3K regulatory subunit. Interaction of the regulatory subunit with the phosphorylated tyrosine causes a conformational change that relieves its inhibition of the catalytic subunit and localizes the catalytic subunit in close proximity with its substrate phosphoinositides. GPCRs can activate PI3K by either directly binding the catalytic subunit via the GPCR Gβγ subunit or by activation of Ras. Ras can activate PI3Ks by directly binding the Ras binding domain of the catalytic subunit (Vanhaesebroeck et al., 2010). Enrichment of the PI3K products PtdIns(3,4)P2 and PtdIns(3,4,5)P3 at the cell membrane leads to co-localization of Akt isoforms with PDK1, a protein kinase that contains a PH domain that also binds PtdIns(3,4)P2 and PtdIns(3,4,5)P3 (Stephens et al., 1998). PDK1 is a constitutively active serine/threonine kinase that phosphorylates Akt1/2/3 at Thr308/309/305 (Alessi et al., 1997b). The kinase required for Akt1/2/3 phosphorylation at Ser473/474/472 had eluded investigators until recently. mTor-Rictor was discovered as the Akt1/2/3 Ser473/474/472 kinase in a RNAi screen in Drosophila cells in which the effects of knocking down members of the mTor pathway on the phosphorylation status of the hydrophobic motif of Akt were examined. The RNAi

knockdown of mTor or Rictor inhibited the phosphorylation of Akt1 at Ser473 in various cell lines. Conversely, the mTor-Rictor complex phosphorylated the Ser473 residue of Akt1 in an *in vitro* kinase assay (Sarbassov et al., 2005). The role of the PH domain of Akt in the colocalization of Akt with activating kinases at the plasma membrane can be mimicked by attachment of a myristoylation signal at the N-terminus of Akt. Myristoylated-Akt (Myr-Akt) is constitutively targeted to the cell membrane and, thus, exhibits constitutive kinase activity. Myr-Akt is a powerful tool that has been used to elucidate the roles of Akt isoforms.

The proteins that attenuate Akt activity are the phosphatases PTEN, PP2A, and PHLPP. PTEN is a commonly mutated tumor suppressor in cancer. It functions as a phosphatase that dephosphorylates phosphoinositides at the 3'hydroxyl position, thus decreasing the levels of the PI3K products PtdIns(3,4)P2 and PtdIns(3,4,5)P3. PP2A and the PHLPP isoforms directly dephosphorylate Akt1/2/3 at Thr308/309/305 and Ser473/474/472, respectively. PP2A is a multimeric serine/threonine phosphatase that targets a wide range of proteins including protein kinases (Millward et al., 1999). PHLPP phosphatases were discovered in a bioinformatics screen to identify PH domain-containing phosphatases that regulate Akt (Gao et al., 2005).

Akt isoforms contain additional sites that undergo posttranslational modifications that may regulate protein stability and/or activity. Ser124 and Thr450 of Akt1, and the corresponding amino acid residues in Akt2 and Akt3, are constitutively phosphorylated even in unstimulated cells (Andjelkovic et al., 1996). Shao et. al. (2006) found that phosphorylation of Akt at residue Thr450 by the JNK kinase inhibited apoptosis in cardiomyocytes after hypoxic injury. Mutation of Thr450 to Ala prevented the phosphorylation of Akt at residues Thr308 and Ser473 and led to apoptosis (Shao et al., 2006). It remains to be determined whether JNK phosphorylates Akt in other cell types and whether the phosphorylation of Akt at Thr308 and Ser473 is always dependent upon initial Akt phosphorylation at Thr450. Recently, the E3 ubiquitin ligase TRAF6 was discovered to target Akt and regulate its recruitment to the plasma membrane upon growth factor stimulation. Deletion of the PH domain of Akt abrogated TRAF6 induced ubiquitination. Mutational analysis narrowed down the possible residues to K8 or K14. Akt-K8R or Akt-K14R mutants did not translocate to the plasma membrane upon growth factor stimulation and, therefore, were not phosphorylated at Thr308 and Ser473. Mouse embryonic fibroblasts (MEFs) isolated from TRAF6 null mice exhibited attenuated Akt activation and phosphorylation of the Akt substrates Foxo1 and Foxo3a when compared to MEFs isolated from wild type mice (Yang et al., 2009).

The non-receptor tyrosine kinase Src can regulate the kinases and phosphatases that control the activity of Akt isoforms. Src promotes Akt activity by activating PI3K, enhancing the activity of PDK1, and by inhibiting PTEN and PP2A. There are two mechanisms by which Src family kinases (SFK) can bind the regulatory subunit

of PI3K: interaction of the phosphorylated tyrosine of the SFK with the SH2 domain of PI3K or by the interaction of the SH3 domain of SFK with a proline-rich region in the regulatory subunit of PI3K (Pleiman et al., 1994; Vogel and Fujita, 1993). Both mechanisms result in the activation of the PI3K catalytic subunit and co-localization with its phosphoinositide substrates. Activated Src can lead to Ras activation, which directly binds and activates the PI3K catalytic subunit. There is evidence that Src regulates the subcellular localization, level of activity, and protein stability of PDK1. Stimulation of HEK-293 cells with insulin or treatment with the tyrosine phosphatase inhibitor pervanadate led to phosphorylation of PDK1 on Tyr9 and Tyr373/376. Overexpression of v-Src in untreated HEK-293 cells mimicked the increase of phosphorylation of Tyr9 and Tyr373/376 observed in insulin and pervanadate treated cells. Phosphorylation of Tyr373/376 was required for PDK1 activity (Park et al., 2001). Phosphorylation of PDK1 on Tyr9 was required for the formation of a protein complex containing PDK1, Src, and HSP90, which stabilized PDK1 and promoted its kinase activity (Yang et al., 2008). Src-mediated tyrosine phosphorylation of PTEN has been observed and is believed to regulate PTEN activity and stability. The C2 domain of PTEN is required for interaction with phosphoinositides. Pervanadate treatment and ligand stimulation of cells induced phosphorylation of the C2 domain of PTEN, which inhibited the ability of C2 to bind cellular membrane and led to a decrease in PTEN protein levels. Src did not directly phosphorylate PTEN and the kinase required for Src mediated PTEN phosphorylation is unknown (Lu et al., 2003). Tyrosine phosphorylation of PP2A by tyrosine kinases including Src was observed to inhibit PP2A activity (Chen et al., 1992)

Chen et al. (2001) reported that growth factor stimulation induces the phosphorylation of Akt isoforms by Src at Tyr 315 and Tyr 326 near the Akt activation loop. Treatment of a panel of cell lines with Src pharmacological inhibitors abrogated the induction of Akt tyrosine phosphorylation by epidermal growth factor. Additionally, the activation of Akt isoforms was attenuated in cells lacking the Src family kinases Src, Yes, and Fyn, as measured by *in vitro* kinase assays with immunoprecipitated endogenous Akt proteins. This attenuation could be rescued by expression of wild-type but not kinase-inactive Src. To determine whether the phosphorylation of the tyrosine residues affects the function of Akt *in vivo*, cells were co-transfected with constructs encoding the forkhead transcription factor FKHR and constructs encoding either wild-type or Akt mutated at Y315F and/or Y326F. The activity of FKHR was determined with a luciferase construct containing the FKHR recognition motif IRS. Expression of wild-type Akt inhibited the ability of Forkhead to activate the transcription of the luciferase construct. However, Akt constructs containing either single or double mutations of Tyr315F and Tyr326F not only prevented the inhibition of FKHR, but also appeared to act as a dominant negatives, leading to further enhancement of the activity of Forkhead. From these results, the authors concluded that the phosphorylation of Akt by Src is required for the activation of Akt (Chen et al., 2001). A subsequent study from the

same authors found that the SH3 domain of Src interacts with a PxxP motif at the C-terminus of Akt. Mutation of the proline residues to alanine abolished the interaction of Akt with Src and prevented the activation of Akt by EGF.

Physiological Roles of Akt Isoforms

Akt in cell survival

The initial discovery that Akt is the cellular homolog of a viral gene implicated in cell transformation implied that cellular Akt might regulate cell processes required for transformation. One of the characteristics of transformed cells is the ability to survive even in absence of survival signals. Normal cells are dependent on extracellular cues such as signals from growth factors or attachment to cell matrix for continued inhibition of apoptosis. Withdrawal of survival cues results in the activation of the apoptotic pathway, whereas in diseases such as cancer cells may generate endogenous survival signals. The first evidence of a role of Akt in cell survival came from studies of neuronal cells. Dudek et. al. demonstrated that removal of growth factors from the cell culture medium of neuronal cells leads to apoptosis, but treatment of the cells with IGF or high levels of insulin could prevent cell death. Since the PI3K-Akt pathway is activated by both IGF and insulin, the authors investigated whether Akt might regulate neuronal cell survival. Overexpression of dominant negative Akt induced apoptosis in the cultured cells, conversely overexpression of wild-type Akt prevented apoptosis upon growth factor removal (Dudek et al., 1997). The role of Akt in regulating cell survival is not limited to neuronal cells and has been shown in other cell types such as hematopoietic cells and fibroblasts (Kauffmann-Zeh et al., 1997; Kennedy et al., 1997; Songyang et al., 1997).

There are multiple mechanisms by which Akt can regulate cell survival, including pathways regulated by the Akt substrates BAD, Forkhead family proteins, and NF-kappaB. BAD is a member of the BCL2 family of proteins that regulate apoptosis. Unphosphorylated BAD binds to the pro-survival proteins Bcl2 and Bcl-xL at the mitochondrial membrane and induces the release of cytochrome c, which leads to activation of the caspase cascade. The phosphorylation of BAD by Akt leads to the sequestration of BAD by 14-3-3 proteins in the cytosol, and prevents the interaction of BAD with Bcl2 and Bcl-xL (del Peso et al., 1997). Another substrate of Akt, the Forkhead family of transcription factors including FKHRL1, and AFX, can also induce apoptosis. Forkhead transcription factors are cytosolic proteins that translocate to the nucleus upon withdrawal of growth factors or other survival cues. In the nucleus, the Forkhead transcription factors bind to the promoters of genes required for apoptosis such as the Fas ligand. However, phosphorylation of the Forkhead family of proteins by Akt mediates the interaction of Forkhead proteins with 14-3-3, which sequesters Forkhead in the cytosol (Brunet et al., 1999; Kops et al., 1999). The result of Akt phosphorylation of BAD and Forkhead proteins is the inhibition of

pro-apoptotic signals. Another mechanism by which Akt can inhibit apoptosis is by phosphorylation of substrates that activate signals for survival. Activation of the NF-kappaB pathway leads to transcription of proteins including pro-survival members of the Bcl2 family, and inhibitors of caspases (Datta et al., 1999).

Akt and cell growth

In addition to cell survival, one of the first roles attributed to Akt was the regulation of cell growth. The role of Akt in cell growth is primarily mediated by the mTOR-Raptor pathway. mTOR can exist in two protein complexes: mTOR-Rictor, which phosphorylates the Akt isoforms, and mTOR-Raptor, which is activated by Akt isoforms. mTOR-Raptor has an essential role in regulating cell growth through its upregulation of translation of ribosomal proteins and other components of the translation machinery (Schmelzle and Hall, 2000). mTOR-Raptor is a Ser/Thr protein kinase that phosphorylates p70S6K and 4E-BP1. Phosphorylated p70S6K activates the translation of mRNAs that contain an oligopyrimidine track at the 5' translation start site. The 5'TOP mRNAs encode members of the translation machinery such as ribosomal proteins and elongation factors (Dufner and Thomas, 1999). Phosphorylation of 4E-BP1, an inhibitor of translation, results in the dissociation of 4E-BP1 from eIF4E. Once released, eIF4E and the translation complex to which it is bound can bind the 5'cap of mRNA and initiate translation. Akt does not directly phosphorylate mTOR-Raptor, but activates the mTOR-Raptor pathway by phosphorylation of tuberous sclerosis protein 2 (TSC2), an inhibitor of the mTOR-Raptor pathway. TSC2 forms a heterodimer with TSC1 and acts as a GTPase activating protein for Rheb (Zhang et al., 2003). Rheb activates mTOR-Raptor, although the mechanism is still unclear.

Akt and glucose metabolism

The discovery of insulin and its role in regulating glucose metabolism of muscles and adipose tissue was an important breakthrough in medicine. The mechanism by which insulin stimulates glucose import and metabolism has been partially elucidated. It involves the activation of the insulin receptor, which subsequently binds and phosphorylates the insulin receptor substrate (IRS1), which in turn binds and activates the class I PI3K pathway (Cohen, 2006). Activation of Akt is critical for two specific events in the insulin pathway (Burgering and Coffer, 1995). The first involves glucose import mediated by glucose transporters GLUT4 and GLUT1. In unstimulated cells GLUT4 is maintained in vesicles that translocate to the plasma membrane upon stimulation with insulin. Akt directly regulates the translocation of GLUT4 vesicles, which are regulated by Rab GTPases. In the GTP bound form, Rab10 promotes the translocation of GLUT4-containing vesicles to the plasma membrane. This activity is inhibited by the RabGAP protein, AS160 (Eguez et al., 2005). It has been shown that Akt2 binds and phosphorylates AS160, inhibiting its GAP activity on Rab and leading to GLUT4 translocation to the plasma membrane (Gonzalez and

McGraw, 2009; Sano et al., 2003). Akt can also regulate the levels of GLUT1 through mTOR-Raptor. The cellular level of GLUT1 is regulated transcriptionally and increases upon Akt activation (Taha et al., 1999). The second role of Akt in the insulin pathway involves GSK3, the first bona fide substrate identified for Akt. In conditions of cell starvation, GSK3 phosphorylates and inactivates Glycogen Synthase to prevent production of glycogen. Upon stimulation with insulin, Akt phosphorylates GSK3α/β at Ser21/9, which inactivates GSK3 activity. Inhibition of GSK3 leads to activation of Glycogen Synthase and synthesis of glycogen (Cross et al., 1995).

Regulation of insulin-dependent glucose metabolism appears to be an isoform-specific role of Akt2. Engineered knockout mouse models revealed distinct phenotypes associated with the knockout of each Akt isoform. Akt2 knockout mice developed insulin resistance reminiscent of human type 2 diabetes mellitus (Cho et al., 2001a). The Akt2 knockout mice had increased blood glucose levels, decreased glucose uptake by muscle in response to administered insulin, and failure of insulin-induced suppression of glucose production in the liver. Akt1 and Akt3 knockout mice did not exhibit any defects in glucose metabolism, implicating Akt2 as the specific target of insulin in glucose homeostasis. Gonzalez and McGraw (2009) used *in vitro* cell culture to elucidate the difference between Akt1 and Akt2 activation upon insulin stimulation. They found that insulin stimulation led to a more profound increase in Akt2 localization to the plasma membrane compared to Akt1. The localization of Akt2 corresponded to an increase in the phosphorylation of AS160 and with the increased transport of GLUT4 to the plasma membrane. Chimeric constructs of Akt1/2 failed to recapitulate the effects of Akt2 downstream of insulin stimulation, which implies that the role of Akt2 cannot be attributed to a single domain. However, a mutation in the PH domain of Akt1 that leads to increased localization at the plasma membrane was able to mimic the insulin response of Akt2.

Akt and cell proliferation

One of the hallmarks of cell transformation is the ability of cells to proliferate in the absence of mitogenic signals. In normal cells, regulators of the cell cycle are dependent on growth factors to signal progression through the cell cycle. These regulators are cyclin-dependent kinases (CDK) and their corresponding cyclins, and cdk inhibitors. The activity of Cdks is dependent on the levels of their cyclin partners. A decrease in the levels of cyclins by inhibition of transcription or translation, or by enhanced degradation attenuates the activity of Cdks and halts the cell cycle. Cdk inhibitors can also halt the cell cycle by associating with the Cdk-cyclin complex to inhibit Cdk activity.

There are multiple mechanisms by which Akt promotes cell cycle progression. Some of the Akt substrates mentioned previously as regulators of other cell

processes such as cell survival, growth, and metabolism also have roles in regulating the cell cycle. The forkhead family of transcription factors, which control cell survival and growth, regulate the transcription of the cell cycle inhibitor p27 (Medema et al., 2000). Overexpression of AFX, FKHR, or FKHR-L1 lead to cell cycle arrest at the G1 phase due to increased levels of p27, which is a transcriptional target of AFX. Akt directly regulates this pathway since insulin stimulation leads to the phosphorylation of the forkhead family of proteins by Akt and to the subsequent decrease in the levels of p27. mTOR-Raptor promotes cell cycle progression through the activation of 5'cap-dependent translation by eIF4-E; this promotes cell cycle progression by controlling the translation of cell cycle proteins such as cyclin D (Rosenwald et al., 1993). GSK3α/β, an Akt substrate that regulates glucose metabolism, also regulates the degradation of several regulators of the cell cycle. The phosphorylation of cyclin D on Thr-286 by GSK3β promotes nuclear to cytoplasmic relocalization and proteosomal degradation of cyclin D (Diehl et al., 1998). Similarly, the degradation of cyclin E is dependent upon phosphorylation by GSK3β (Welcker et al., 2003). Akt phosphorylation of GSK3 inhibits these effects.

Akt also directly phosphorylates several cell cycle inhibitors. Akt phosphorylates p27 at the amino acid residue Thr157. Upon phosphorylation, p27 localizes to the cytoplasm, which prevents its interaction with Cdk2-cyclin E (Liang et al., 2002). The regulation of p21 appears to differ between Akt1 and Akt2 isoforms. Akt1 phosphorylates p21 on amino acid residue Thr145, which leads to its cytoplasmic retention and inhibition of activity. Akt2, however, binds and sequesters p21 in the nucleus, which promotes p21 function and arrests the cell cycle (Heron-Milhavet et al., 2006).

Akt isoforms in Cancer

The discovery of *v-akt* as a gene that induces malignant transformation led to the hypothesis that cellular Akt may play a role in cancer. Indeed, the PI3K pathway is one of the most commonly mutated pathways in breast and colon cancer (Wood et al., 2007). Mutations that lead to the constitutive activation of growth factor receptors, the PI3K subunits, and the activating kinases PDK1 and mTOR-Rictor all lead to hyperactivation of Akt. Inactivating mutations in the phosphatase PTEN are common in cancer and also result in the hyperactivation of Akt isoforms.

Biochemical analyses of gene copy number, transcript and protein levels of Akt isoforms have shown that Akt isoform levels are increased in various types of cancer. Amplification of Akt2 has been detected in ovarian cancer, breast cancer, pancreatic cancer, non-Hodgkin's lymphoma, and hepatocellular carcinoma (Arranz et al., 1996; Bellacosa et al., 1995; Cheng et al., 1992; Cheng et al., 1996; Xu et al., 2004). Amplification of Akt1 was detected in gastric adenocarcinomas (Staal, 1987). Increased Akt3 protein levels and activity were detected in estrogen receptor deficient breast cancer cells and androgen-independent prostate cancer cells

(Nakatani et al., 1999b). The availability of antibodies that specifically recognize the phosphorylated forms of Akt has allowed for analysis of changes in Akt activity in cancer cells. Increased Akt activity was detected in ovarian, breast, and prostate cancer cells as determine by immunohistochemistry and immune-complex kinase assays (Sun et al., 2001). A mutation in the PH domain of Akt1 has been identified in a subset of breast, colorectal, and ovarian tumor samples. The glutamic acid to lysine substitution in these mutants results in a conformational change of the PH domain that leads to increased localization at the plasma membrane and subsequent phosphorylation and activation. Rat1 cells infected with Akt1-E17K were able to form colonies in soft agar, which is indicative of cellular transformation. Tumorigenicity was determined using a mouse model in which hematopoietic stem cells from Eu-myc mice co-expressing a second oncogene are transplanted into wild type mice. Mice transplanted with cells co-expressing Eu-myc and Myr-Akt1 or Akt1-E17K, but not WT-Akt1, developed leukemia (Carpten et al., 2007).

Akt has been implicated in processes required for cancer initiation and progression. The physiological roles of Akt in preventing apoptosis and promoting cell growth, metabolism, and proliferation are deregulated in cancer cells. There are various examples of the dependence of cancer cells on Akt for survival. Inhibition of oncogene-induced apoptosis is dependent on Akt. C-myc overexpression in fibroblasts triggers the apoptotic pathway, but this is inhibited by co-overexpression of constitutively active Akt created by fusion with the viral gene gag (Kauffmann-Zeh et al., 1997). Akt can also inhibit the tumor suppressor p53, which is activated by signals such as DNA damage and stress. P53 activation leads to cell cycle arrest and possibly to apoptosis. Akt regulates p53 by phosphorylating the ubiquitin ligase MDM2 on residues Ser166 and Ser186. Akt phosphorylation of MDM2 promotes MDM2 nuclear localization and subsequent interaction with p53. The MDM2-p53 complex is then translocated to the cytoplasm where ubiquitinated p53 is degraded by the proteosome (Mayo and Donner, 2001; Zhou et al., 2001).

Akt can regulate the migration and invasion of cancer cells *in vitro*, which correlate with the metastatic capacity of cancer cells *in vivo*. Akt phosphorylates the actin binding protein Girdin, which promotes the organization of actin filaments to promote cell migration. The invasion of cancer cells into the surrounding tissue is an early step in metastasis. Matrix metalloproteinases are expressed and secreted by invasive cancer cells in order to degrade the surrounding extracellular matrix. In several cancer cell lines Akt1 promotes the expression and/or activation of MMPs associated with cell invasion. In a Lewis lung carcinoma cell line, Akt1 mediates the IGF-induced expression of MT1-MMP, and subsequent MMP2 activation and increase in cell invasion (Zhang and Brodt, 2003). Akt1 also regulates the invasive capacity of a pancreatic cancer cell line. Akt1 phosphorylates and activates ARK5, a serine/threonine kinase, which leads to increased expression of MT1-MMP and activation of MMP2 and MMP9 in PANC1 cells (Suzuki et al., 2004). The HT1080 fibrosarcoma cell line is a highly metastatic cancer cell line that expresses high

levels of MMP9. The invasion of HT1080 cancer cells is regulated by Akt1 activation of NF-kB, which transcriptionally activates MMP9 (Kim et al., 2001).

Akt isoform-specific roles in breast cancer migration and invasion

The roles of Akt1 in promoting cell migration and invasion are not conserved in breast cancer cells. The first evidence of an inhibitory role of Akt1 in breast cancer metastasis came from a bi-transgenic MMTV-ErbB2/MMTV-Akt1 mouse model. Mice containing both transgenes exhibited accelerated initiation of tumorigenesis attributed to increased expression of Cyclin D when compared to mice with an ErbB2 or Akt1 transgene alone. However, upon analysis of metastasis, the bi-transgenic mice had fewer metastatic lesions compared to ErbB2 transgenic mice (Hutchinson et al., 2004). The mechanism by which Akt1 inhibited ErbB2-mediated metastasis was not elucidated. Several groups have since proposed various mechanisms by which Akt1 may inhibit the migration and invasion of breast cancer cells. Expression of myristoylated Akt1 in HMT-3522 T4-2 cells promoted cell proliferation and cell survival in 3D laminin-rich extracellular matrix Matrigel cultures. However, experiments with wound-healing assays and Matrigel-coated invasion chambers showed that myr-Akt1 expressing cells had decreased capacities for migration and invasion. Akt1 phosphorylation of TSC2, an Akt substrate that regulates cell growth, was crucial for this effect. TSC2 promotes cell migration and invasion by activating Rho. Akt1 phosphorylation of TSC2 results in a decrease in TSC2 protein levels and a consequent decrease in the activity of Rho (Liu et al., 2006). Akt1 can also decrease breast cancer cell migration through downregulation of NFAT transcription factors. Yoeli-Lerner et. al. (2005) investigated the role of Akt1 in breast cancer cells by either overexpression of myr-Akt1 or knockdown of Akt1 by RNAi. They found that overexpression of myr-Akt1 in the metastatic cell lines MDA-MB-435, MDA-MB-231, and SUM-159-PT, inhibited the ability of cells to invade through Matrigel-coated transwell filters. Conversely, siRNA-mediated knockdown of Akt1 in SUM-159-PT cells resulted in increased invasion. Since a previous study by the same group had found that NFAT is activated downstream of α6β4 integrin clustering in breast cancer cells and is required for breast cancer migration and invasion, and previous studies have shown that activation of PI3K negatively regulates NFAT, the authors investigated whether Akt1 targets NFAT in breast cancer cells. They found that Akt1 phosphorylates and activates the E3 ubiquitin ligase MDM2, which promotes the ubiquitination and proteosomal degradation of NFAT (Yoeli-Lerner et al., 2005).

Unlike Akt1, Akt2 promotes the migration and invasion of breast cancer cells. Irie et. al. investigated the roles of Akt1 and Akt2 isoforms in IGF-IR overexpressing MCF-10a cells. Parental and IGF-IR-overexpressing MCF-10a cells were grown in 3D Matrigel cultures. Both cell types were dependent on stimulation with IGF for morphogenesis, however IGF-IR overexpressing cells formed large disorganized colonies compared to the monolayer spheres formed by parental MCF-10a cells.

Isoform specific knockdown of Akt1 or Akt2 using shRNA showed that the formation of the large disorganized colonies by IGF-IR overexpressing cells was dependent on Akt2, while Akt1 knockdown resulted in cells with increased spindle-shaped morphology. The Akt1-knockdown cells exhibited traits of epithelial to mesenchymal transition (EMT): increased migration, downregulation of E-cadherin, upregulation of N-cadherin, and increased expression of vimentin. Akt1 expression repressed the ERK/MAPK pathway, which was required for the induction of EMT upon Akt1 knockdown. The mechanism by which Akt2 promoted IGF-IR activated EMT was not determined (Irie et al., 2005). Arboleda et. al. investigated the role of Akt2 in the metastasis of ovarian and breast cancer cells. They found that overexpression of Akt2, but not of Akt1 or Akt3, recapitulated the effects of PI3K overexpression on the capacity of breast and ovarian cancer cell lines to invade through Matrigel coated transwell filters. The increase in invasive capacity was accompanied by an increase in cell-matrix adhesion by Akt2-overexpressing cells, and was attributed to an in increase in expression of β1 integrin. Concomitant with the increase of cell invasion through transwell filters *in vitro*, there was an increase in metastatic lesions in mice injected intraperitoneally with MDA-MB-435 or SKOV3 cells overexpressing Akt2 compared to vector control cells (Arboleda et al., 2003).

The Akt isoforms clearly play important physiological roles in cell survival, growth, metabolism, and proliferation. Many of these roles are deregulated in cancer and have made the Akt family an attractive target for cancer therapy. However, isoform-specific roles of Akt1, Akt2, and Akt3 are beginning to emerge and Akt isoforms may have distinct roles in different tissues. For example, the roles of Akt1 in promoting migration and invasion of fibrosarcomas, colon, and pancreatic cancer cells are not preserved in breast cancer cells. Thus, the role of each Akt isoform in different types of cancer needs to be investigated to determine whether targeting an Akt isoform will be of therapeutic value. The work presented here addressed the questions of whether SFKs are required for the migration and invasion of breast cancer cells and whether these roles are mediated by Akt isoforms. Differences in the roles of Akt isoforms in the migration and invasion of breast cancer cells were also investigated and provide evidence that Akt isoforms play distinct but important roles in breast cancer.

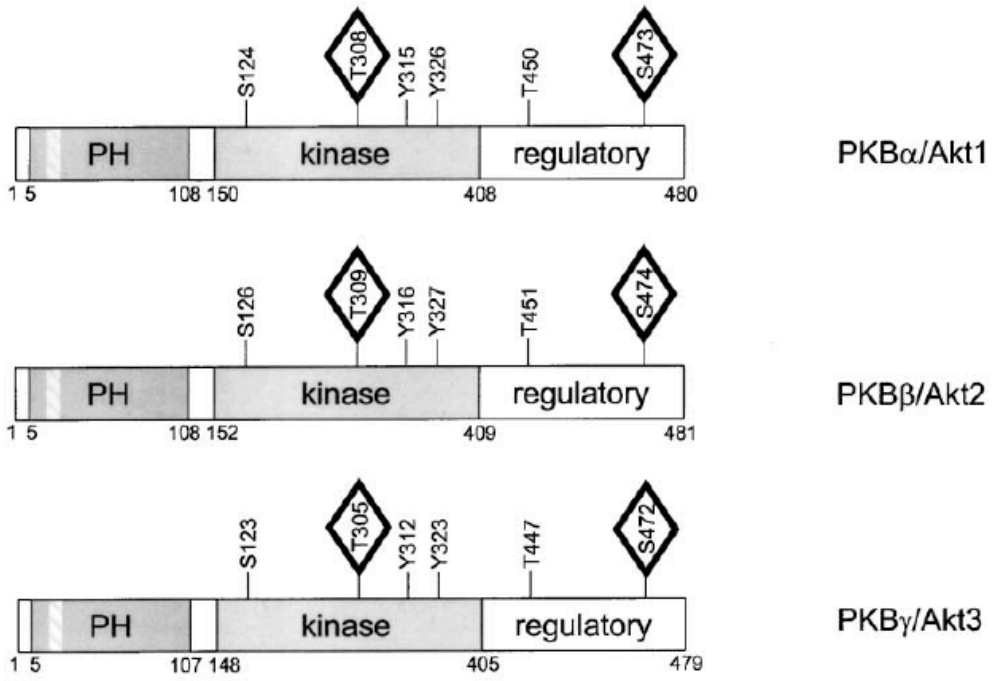


Figure 1: Domain organization of Akt isoforms. The protein domains of Akt isoforms are labeled as follows: (PH)-pleckstrin homology domain, (kinase)-catalytic kinase domain, and (regulatory)-hydrophobic regulatory tail. Hatched bars represent the regions in the PH domain required for binding phospholipids. Major phosphorylated residues are marked. The serine and threonine residues that are phosphorylated upon activation are marked in diamonds. (Nicholson and Anderson, 2002).

Chapter 2: Materials and Methods

Reagents and DNA constructs

The pharmacological inhibitors PP2 and SU6656 were purchased from Calbiochem, Inc. and dissolved in DMSO (Invitrogen). Stealth RNAi™ siRNA negative control and Stealth Select RNAi™ Akt1 siRNA were purchased from Invitrogen. SignalSilence Akt2 siRNA was purchased from Cell Signaling Biotechnologies. The following antibodies were purchased from Cell Signaling Biotechnologies: rabbit monoclonal anti-pan Akt, mouse monoclonal anti-Akt1, rabbit monoclonal anti-Akt2, rabbit monoclonal anti-Akt3, rabbit monoclonal anti-phosphorylated Thr308 Akt, rabbit monoclonal anti-phosphorylated Ser473 Akt, mouse monoclonal anti-HA, anti-mouse conjugated to HRP, and anti-rabbit conjugated to HRP. Mouse monoclonal anti-beta tubulin, mouse monoclonal anti-Flag conjugated to agarose, and Flag peptide were purchased from Sigma-Aldrich. Goat polyclonal anti-ACAP2 was purchased from Abcam.

The following plasmids were purchased from Addgene: Addgene plasmid 9011 pBabe puroL Akt1 and Addgene plasmid 9018 pBabe puroL Myr HA Akt2. pCMV-HA-Akt1 and pCMV-HA-Akt2 constructs were created by amplifying Akt1 with primers 5'-AAACGAATTCGGATGAGCGACGTGGCTATT-3' and 5'-AAATCTCGAGGTTCAGGCCGTGCCGCTGGCCCGA-3' and Akt2 with primers 5'-AAACGAATTCGGATGAATGAGGTGTCTGTC-3' and 5'AAATCTCGAGGTTCACTCGCGGATGCTGGCCGA-3'. The forward primers contained an EcoRI target site and the reverse primers contained a XhoI target site. PCR products and the pCMV-HA vector (Clontech Laboratories) were sequentially digested with EcoRI and XhoI, gel purified, and ligated. Clones were screened by DNA sequencing. The pCMV-HA-Akt2 RNAi mutant construct was created with NEB/Finnzymes Phusion Site-Directed Mutagenesis Kit with phosphorylated primers 5'-GCGACGGCTCATTCATAGGATACAAGGAGA-3' and 5'-TCTTCAGCAGGAAGTACCGTGGCC-3'. The forward primer harbors 3 silent mutations in the Cell Signaling SignalSilence Akt2 siRNA target sequence. Akt2-pFLAG-CMV™-5a was created by PCR amplifying Akt2 from pBabe puroL Myr HA Akt2 with primers 5'-GCCCAAGCTTCCATGAATGAGGTGTCTGTCA-3' and 5'-CCGGATCCCTCGGATGCTGGCCGAGTA-3'. The forward primer contained a HindIII restriction enzyme site and the reverse primer contained a BamHI restriction enzyme site. The PCR product and the pFLAG-CMV™-5a vector (Sigma-Aldrich, Inc.) were digested with HindIII and BamHI, gel purified, and ligated. A positive clone was identified by DNA sequencing. The cDNA corresponding to human ACAP2 was purchased from Open Biosystems and amplified with primers 5'-CCAAAGGCCATGGAGGCCAGGATGAAGATGACTGTG-3' and 5'-CCAAACTCGAGAAATTCAGAATTTCTGTGAATC-3', which contain SfiI and XhoI restriction enzyme sites, respectively. To create pCMV-HA-ACAP2, the PCR product

and the pCMV-HA vector were digested with SfiI and XhoI, gel purified, and ligated. A positive clone was identified by DNA sequencing.

Cell Lines

MDA-MB-231 breast cancer cells cells were purchased from ATCC. MDA-MB-231 cells stably expressing enhanced-Green Fluorescent Protein (eGFP) or eGFP tagged dominant-negative Src (Src K295R, Y527F) were created by L. Kusdra in our lab. Cells were maintained in DMEM supplemented with 10% FBS (Invitrogen) and 1% Penicillin and Streptomycin. Cells were passaged every 2-3 days and incubated in 37°C with 5% CO_2.

Transfections

Transient transfections of MDA-MB-231 cells were performed with Lonza/Amaxa Nucleofector II with Kit V. For siRNA transfections, $5x10^6$ cells were resuspended in 100 ul Solution V with 150 pmol siRNA. The cell solution was then transferred to a cuvette and pulsed at setting X-13 as directed by manufacturer's protocol. The cells were resuspended in 0.5 ml of prewarmed DMEM/10%FBS and transferred to a 1.5 ml Eppendorf tube and incubated in a 37°C waterbath for approximately 10 minutes before plating in a 10 cm tissue culture dish containing 9.5 ml of medium. Transfections with DNA constructs were performed with $2x10^6$ cells in 100 ul Solution V. Cells were pulsed and processed as mentioned previously with the exception that cells were plated in a 6 cm tissue culture dish containing 3.5 ml of medium.

Three-dimensional cell culture for microscopy

Six-well plates were pre-coated with a thin layer of Matrigel by using the tip of a pipette to evenly coat the bottom of each well. Plates were placed in a tissue culture incubator for 15-30 minutes to allow the Matrigel to polymerize. MDA-MB-231 cells were trypsinized and resuspended at a concentration of 5×10^4 cells per ml of cell culture medium. One ml of MDA-MB-231 cells was seeded per well of the 6-well plate and incubated in the tissue culture incubator for approximately 45 minutes to allow the cells to adhere. After visualizing the cells under the microscope to confirm that they had adhered, an additional 1 ml of cell culture medium containing 10% vol/vol Matrigel and DMSO or pharmacological inhibitor was added to each well. Cell culture medium containing Matrigel and DMSO or pharmacological inhibitor was replaced every 2 days. Images were taken using a Zeiss Axiovert Inverted microscope with QImaging software.

Three-dimensional cell culture for immunoblot

Two days post-transfections, cells were trypsinized and resuspended in phenol red-free DMEM with 10% FBS. Cells were seeded in 6-well dishes or 6 cm dishes pre-coated with a thin layer of Matrigel. After incubation at 37°C with 5% CO_2 for 1-4 hours, cells were harvested for immunoblots as described below.

Immunoblots

Cells were washed twice with ice cold PBS and lysed in RIPA (1x PBS, 1% nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS) or Triton X-100 lysis buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton X-100) supplemented with protease and phosphatase inhibitors (2.5 mM $Na_4P_2O_7$, 25 mM NaF, 1 mM Na_3VO_4, 1 mM β-glycerophosphate, 174 ug/ml phenylmethanesulfonyl fluoride, 1 ug/ml Leupeptin, 1 ug/ml Aprotinin, 1 ug/ml Pepstatin, 1 ug/ml Chymostatin, 1 ug/ml Antipain, 1 ug/ml Phenanthroline, 1.5 ug/ml Benzamidine). Samples were clarified by centrifugation at 16,000 g at 4°C and transferred to new Eppendorf tubes. Protein concentrations were determined with a BCA protein assay. 60-120 ug of protein lysate was separated by SDS-PAGE and transferred onto an Immobilon P® PVDF membrane. Membranes were blocked for 1 hour at room temperature or overnight at 4°C with 5% milk in TBST (10 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% Tween-20). Primary antibodies were diluted in TBST containing 5% milk or 5% BSA and incubated for 1 hour to overnight. After 3 washes with TBST, membranes were incubated with secondary antibodies diluted in 5% milk TBST for 1 hour. Membranes were washed 3x with TBST and incubated with Pierce West Pico ECL reagent and visualized with Kodak Biomax ML film.

Immunoprecipitation and Immune-complex kinase assay

Cells were lysed in Triton X-100 lysis buffer containing protease and phosphatase inhibitors and protein concentrations determined by BCA protein assay. Samples were precleared by incubation with Protein A or G-agarose beads (Santa Cruz Biotechnology) for at least 1 hour. Clarified lysates were incubated with antibody for 2 hours to overnight. Protein A-agarose beads were added for samples incubated with rabbit antibody, Protein G-agarose beads were added for samples incubated with mouse or goat antibody and incubated an additional 2 hours. Immunoprecipitated complexes were then processed for immunoblot or kinase assays. For immunoblots, immunoprecipitated complexes were washed 4x with lysis buffer then resuspended in 1x Laemmli buffer and boiled for 5 minutes. Samples were either loaded on SDS-PAGE for immunoblot or stored at -20°C. The Cell Signaling Biotechnology non-radioactive Akt kinase assay kit was used for immune complex kinase assays. The kit contains a GSK3 fusion protein as the kinase substrate. Phosphorylation level of the GSK3 fusion protein is detected on an immunoblot with a rabbit anti-phospho-GSK3α/β (Ser21/9) antibody.

Migration and Invasion Assays

BD Biocoat™ BD Matrigel™ Invasion chambers, 8 um PET membranes and control inserts were purchased from Becton, Dickinson and Company. Invasion chambers were rehydrated with DMEM for 2 hours as described in manufacturer's protocol. Cells were trypsinized and resuspended in DMEM containing 1% BSA. Cells were washed once with DMEM containing 0.1% BSA and counted using a Beckman Coulter Counter. Cells were resuspended in 0.1 % BSA at a concentration of $1x10^5$/ml. Five hundred microliters of cell suspension was seeded into each filter with 750 ul of DMEM with 10% FBS or NIH3T3-conditioned medium resuspended in 50% DMEM with 0.1% BSA in the well as chemoattractant. Three filters were seeded with each sample of siRNA-nucleofected cells or six filters were seeded with each sample of DNA-nucleofected cells. Cells were incubated overnight. The following day, filters were washed once with PBS then incubated in 4% formaldehyde/PBS for 15 minutes. For each sample, 3 filters were scrubbed on top to remove cells that had not migrated or invaded. The cells were scrubbed from the bottom of the other 3 filters of the DNA-nucleofected cells. Filters were incubated with DAPI (0.5 ug/ml) for 5 minutes to stain nuclei, then washed twice with PBS. Filters were removed from the insert using a razor blade and mounted with Prolong Gold antifade mounting medium. Six images were taken from each filter using a Zeiss AxioImager fluorescence microscope equipped with a digital CCD camera and iVision software. Cells were counted either manually or with Imaris software. For cells that had been transfected with siRNA, nuclei were counted to determine the average number of cells that had migrated or invaded through triplicate filters. For cells that had been transfected with DNA, the number of GFP-positive cells was counted. The percentage of cells migrating or invading was determined by taking the average number of cells on the bottom of 3 filters divided by the average total number of cells (determined by the sum of the cells on the bottom of 3 filters plus the cells on the top of the corresponding 3 filters), multiplied by 100.

Immunoprecipitation and Mass Spectrometry

MDA-MB-231 cells were Amaxa nucleofected with Akt2-pFlag-CMV5a. The following day, cell lysates were harvested in Triton X-100 lysis buffer. Lysates were precleared with Mouse IgG-agarose beads (Sigma) for 2 hours, rocking end-over-end at 4°C. Samples were pulse-centrifuged and the precleared lysates were divided equally between two tubes. Mouse anti-Flag-agarose beads (Sigma) were added to each tube and incubated for 4 hours. The reactions were washed 4x with TBS. One tube was eluted with TBS buffer; the other tube was eluted with 150 ng/ul of Flag peptide (Sigma) for 30 minutes. Eluates were transferred to new tubes. An aliquot of each sample was analyzed by SDS-PAGE and immunoblotting. An aliquot of each sample was analyzed by SDS-PAGE and silver stained with Pierce SilverSNAP Stain kit. The remaining eluates were processed for mass spectrometry according to

protocols provided by the University of California, Berkeley QB3 Vincent J. Coates Proteomics/Mass Spectrometry Laboratory.

Immunofluorescence

Approximately $2x10^5$ cells were seeded in each well of a 6-well dish containing UV-sterilized coverslips. After overnight incubation, coverslips were washed twice with PBS and cells were fixed with 4% formaldehyde in PBS for 15 minutes. Coverslips were washed 3x with PBS and blocked/permeabilized for 1 hour with IF blocking buffer (1x TBS, 0.3% Triton X-100, 5% normal serum, 1% BSA, 1% fish gelatin). Coverslips were rinsed with PBS then incubated with primary antibody diluted in IF blocking buffer for 2 hours to overnight. Coverslips were washed 3x with PBS and incubated with secondary antibody conjugated to an Alexa fluor dye (Invitrogen) for 1 hour in the dark. Coverslips were washed 1x with PBS, incubated with DAPI 0.5 ug/ml in PBS for 5 minutes, washed 2x with PBS and mounted in ProLong Gold antifade medium (Invitrogen). After curing at room temperature overnight, coverslips were store at 4°C. Cells were visualized with a Zeiss 510 Meta Laser Scanning Confocal Microscope and images acquired with the corresponding software.

Cell Attachment Assay

MDA-MB-231 cells were nucleofected with control, Akt1, or Akt2 siRNA. Two days after nucleofection, cells were trypsinized and resuspended in cell culture medium at the same concentration. Cells were seeded in triplicate wells of 12-well plates that were precoated with a thin layer of Matrigel. Identical plates were seeded for various time points and incubated in 37°C with 5% CO_2. Non-adherent cells were harvested after specified incubation times by gently rocking the plate and transferring the cell culture medium into 1.5 ml Eppendorf tubes. The number of cells in each sample was determined with a BD Coulter Counter.

Arf6-GTP Pull Down Assay

MDA-MB-231 cells were nucleofected with control, Akt1, or Akt2 siRNA. Two days after nucleofection, cells were lysed and Arf6-GTP pull down assays were performed with Pierce/Thermo Scientific Active Arf6 Pull-Down and Detection Kit. Briefly, 250-500 ug of protein lysates were incubated with 100 ug of GST-GGA3-PBD for 1 hour at 4°C. Samples were washed three times with lysis buffer, then incubated with reducing sample buffer to isolate pulled down proteins. Samples were boiled and resolved by SDS-PAGE. Proteins were transferred onto PVDF membranes and probed with Mouse anti-Arf6 antibody.

Chapter 3: Src Family Kinases regulate Akt isoforms in MDA-MB-231 breast cancer cells

Introduction

Cancer metastasis is the leading cause of death for breast cancer patients. Unfortunately, the progression of cancer cells through metastasis is a complicated process and is not well understood. Conflicting theories have emerged on how metastases arise. Early work by Fidler using experimental metastasis assays, in which cancer cells cultured *in vitro* were injected intravenously into mice, showed that cells isolated from the resulting metastases had increased metastatic capabilities compared to the parental cancer cells when re-injected intravenously into mice (Fidler and Kripke, 1977). The theory that arose from these experiments proposed that cells of the primary tumor are poorly metastatic but acquire additional somatic mutations that eventually produce highly metastatic clones. The risk of metastasis would correlate with tumor progression and size, which is a prognostic factor currently used in the clinic (Carter et al., 1989). This theory is supported by a recent study that compared the DNA sequences of cells from the primary tumor versus cells from metastatic lesions. Yachida et. al. (2010) performed genomic sequencing of protein-coding exons of metastatic lesions and sections of primary pancreatic ductal adenocarcinoma isolated from the same individual during autopsy. They found that the primary tumor contained multiple subclones, several of which had the same genomic profile as metastatic lesions. Based on the proliferation index of the metastatic colonies and the rate of accumulation of passenger mutations, they proposed a mathematical model that predicted an average of 18.5 years from the initiation of tumorigenesis to the birth of subclones that have metastatic capacity. They concluded that within the primary tumor, subclones evolve that acquire the ability to metastasize to various organs (Yachida et al., 2010).

Results from studies that used mouse models of spontaneous metastasis did not find a difference in the metastatic capacity of the primary tumor and metastatic lesions (Giavazzi et al., 1980). Additionally, an experiment that compared the metastatic capacity of equally sized sections from small and large primary tumors implanted into mice found that there was no difference in the number of metastases that arose from the two samples (Weiss et al., 1983). There are also cases in which metastatic lesions are found in patients who do not have large primary tumors. van't Veer et. al. (2002) used gene expression analysis of cells from the primary tumor of breast cancer patients to identify a gene expression profile that could predict disease outcome more reliably than traditional methods. In a group of patients with the same tumor stage, they identified a gene expression profile that could distinguish which patients were more likely to present with metastasis within 5 years of diagnosis (van 't Veer et al., 2002). The theory that emerged from these results

proposes that highly metastatic clones arise at the onset of tumorigenesis. Therefore, gene expression analysis of the primary tumor could be used to determine the risk of metastasis. Whether the capability to metastasize is acquired at a late stage during tumor evolution or is acquired early during tumorigenesis is still widely debated (Weigelt et al., 2005).

Regardless of the mechanism by which metastatic cells arise, the initial stage of metastasis involves the detachment of cancer cells from the primary tumor, penetration into the surrounding basement membrane, and invasion into adjacent tissues (Friedl and Wolf, 2003). These steps rely on altered cell morphology and expression or activation of proteins that promote cell migration and invasion. Some of the changes include a switch in expression from E-cadherin, which mediates strong cell-cell contacts, to N-cadherin, which permits dissociation of cancer cells from the primary tumor (Gravdal et al., 2007). Cells also exhibit increased expression and turnover of certain integrins (Mukhopadhyay et al., 1999). Integrins are transmembrane proteins that bind the extracellular matrix. The formation of integrin-extracellular matrix adhesion at the front end of the cell and the coordinated cleavage of integrins that are bound to the extracellular matrix at the rear end of the cell is necessary for cell migration. Matrix metalloproteinases, which degrade the extracellular matrix and allows the cell to move through tissue, are also upregulated and activated in metastatic cancer cells (Garbisa et al., 1992; Ueno et al., 1997). One of the signaling proteins that regulate many processes that are hyperactive in metastatic cancer cells is Src.

src is the cellular homolog of *v-src*, which is encoded by the transforming gene of Rous Sarcoma Virus (Martin, 2001). This virus was discovered by Peyton Rous in 1911 as a filterable agent capable of inducing tumors in chickens. Src is the prototypical member of a family of protein kinases known as Src Family Kinases (SFKs), comprising 10 additional non-receptor tyrosine kinases that have the same domain organization (Roskoski, 2004). At the N-terminus, SFKs are myristoylated, generated by hydrophobic residues that mediate binding to lipid membranes (Cross et al., 1984). The N-terminal domain is followed by a unique domain, which, as the name implies, is the area of the highest sequence divergence amongst the SFKs. Following the unique region are the SH3 domain, the SH2 domain, a linker region connecting the SH2 domain to the catalytic domain (SH1), and finally a C-terminal regulatory tail. Src is maintained in an inactive conformation by intramolecular interactions. The SH3 domain of Src binds to a poly-proline motif in the linker region that lies between the SH2 and kinase domains, while the SH2 domain binds to the phosphorylated Tyr 527 in the C-terminal tail (Weijland et al., 1997; Williams et al., 1997). Dephosphorylation of Src at residue Tyr 527 by phosphatases such as PTPα or interaction of the Src SH2 domain with activated receptor tyrosine kinases, G-protein coupled receptors, and integrins displaces the inhibitory intramolecular interactions, thus activating the catalytic activity of Src (Zheng et al., 1992). Full

activation of Src requires the phosphorylation at Tyr416 (Kmiecik and Shalloway, 1987).

Studies using fibroblasts and colon cancer cells have provided evidence that SFKs can regulate cellular processes that promote cell metastasis. The capacity of v-Src to induce metastasis was first determined by Egan et. al. (1987) who injected v-Src transformed NIH3T3 fibroblasts intravenously into T-cell deficient nude mice and found metastatic lesions in the lungs (Egan et al., 1987). Although c-Src and other members of SFKs have lower activity than v-Src, there is evidence that SFKs are required for cellular metastasis (Johnson et al., 1985; Parker et al., 1984). In the Fidler model of colon cancer metastasis, spontaneously metastatic cell lines derived from non-metastatic KM12C cells exhibited increased Src protein and activity levels (Mao et al., 1997). Additionally, overexpression of constitutively active Src (Src-Y527F) in KM12C cells blocked formation of cell-cell contacts, enhanced cell-matrix adhesion and formation of integrin complexes, but did not affect cell proliferation (Avizienyte et al., 2002; Jones et al., 2002). The decrease in cell-cell contacts was due to an impairment of E-cadherin localization in cells that was dependent on the phosphorylation of myosin light chain (MLC). Src regulates the phosphorylation of MLC through MEK/ERK and treatment with inhibitors targeting MEK/ERK, ROCK, and MLCK proteins restored E-cadherin localization in KM12C cells expressing Src-Y527F (Avizienyte et al., 2004).

Although mutations in SFKs have not been detected in breast cancer, it has been shown that SFKs are required for breast cancer tumorigenesis and SFKs exhibit increased levels of activity in breast cancer cells versus normal breast tissue. c-Src is essential for the induction of mouse mammary tumors by polyomavirus (PyV) middle T as evidenced by the lack of mammary tumors in PyV middle T transgenic mice in which the *c-src* gene has been inactivated (Guy et al., 1994). Comparison of the levels of protein tyrosine kinases from a panel of breast tumor specimens versus non-malignant tissue showed that all of the tumors had increased protein tyrosine kinase activity. Approximately 70% of this activity was attributed to c-Src, as determined by immunoprecipitation assays (Ottenhoff-Kalff et al., 1992). The protein levels and kinase activity of SFKs are increased in human breast carcinoma tissue versus matched non-tumor tissue from the same donor (Reissig et al., 2001). Increased activation of SFKs in breast cancer cells may be due to the activation of receptor tyrosine kinases, such as EGFR and ErbB2, which are commonly amplified and/or overexpressed in breast cancer, or to the expression of activating phosphotyrosine phosphatases (Bjorge et al., 2000; Egan et al., 1999; Ro et al., 1988; Slamon et al., 1987).

The Akt isoforms, which play a role in Src-mediated cell survival, have recently been shown to regulate the migration and invasion of breast cancer cells. Interestingly, the roles of Akt1 and Akt2 in breast cancer cells differ from their roles in other cell types. In fibroblasts, fibrosarcomas, and colon cancer cells, Akt1 promotes cell

migration and invasion by activating Pak1 or by promoting the expression and/or activation of MMPs (Kim et al., 2001; Suzuki et al., 2004; Zhou et al., 2006). In fibroblasts, Akt2 inhibits cell migration by inactivating Pak1 through an uncharacterized mechanism (Zhou et al., 2006). In breast cancer cells, however, Akt1 inhibits cell migration and invasion by promoting the degradation of NFAT, by inhibiting TSC2, and by inhibiting the Raf/MEK pathway (Irie et al., 2005; Liu et al., 2006; Yoeli-Lerner et al., 2005). Akt2 promotes cell migration and invasion, although the mechanism by which this occurs has not been determined (Arboleda et al., 2003; Irie et al., 2005).

To determine whether the SFKs are required for the migration and invasion of metastatic breast cancer cells, I inhibited SFKs and measured the effects of this inhibition on migration and invasion *in vitro*. *In vitro* assays for migration and invasion recapitulate the metastatic capacity of cells and allow for more detailed analysis of cellular processes involved in metastasis. I found that SFKs are required for the protrusion of breast cancer cells into the extracellular matrix, and the inhibition of SFKs leads to decreased migration and invasion of breast cancer cells. Since the SFK substrates Akt1 and Akt2 have recently been shown to have opposite roles in breast cancer migration and invasion, I investigated whether SFKs regulate Akt isoforms in breast cancer cells. I found that the phosphorylation and activation of Akt isoforms are dependent on SFKs. However, there was no difference in the regulation of different Akt isoforms by SFKs, which suggests that the mechanism by which Akt1 and Akt2 perform different functions in breast cancer cells does not involve differential activation by SFKs.

Results

Inhibition of SFKs leads to decreased migration and invasion of breast cancer cells

The MDA-MB-231 cell line was isolated from the pleural effusion of a female breast cancer patient. MDA-MB-231 cells exhibit high metastatic capability in mouse models of breast cancer metastasis. When grown in 3D cultures of laminin-rich extracellular basement matrix, such as Matrigel, the MDA-MB-231 cells assume a stellate morphology and exhibit high invasive capacity. To determine whether inhibition of SFKs affects the morphology of MDA-MB-231 cells grown in 3D cultures of Matrigel, MDA-MB-231 cells were seeded on Matrigel and treated with either vehicle control DMSO or 2 uM PP2, a small molecule pharmacological inhibitor of SFKs that competes with the binding of ATP. The concentration of PP2 used in this experiment had previously been shown to inhibit the activity of SFKs in cultured cells (Kusdra, 2007). Cell culture medium containing fresh DMSO or PP2 was replenished every two days. After four days in 3D cultures, cells that had been treated with DMSO were spindle-shaped and formed protrusions into the extracellular matrix. However, cells that had been treated with PP2 were round and did not form protrusions into the Matrigel (Figure 2a). To confirm that the change

in the ability of MDA-MB-231 cells to form protrusions in Matrigel was due to the inhibition of SFK and not to off-target effects of the pharmacological inhibitor, I used MDA-MB-231 cells that were stably expressing enhanced-green fluorescent protein (eGFP) or an eGFP-tagged dominant-negative mutant of Src, Src- K295R, Y527F (eGFP-dnSrc). The Y527F mutation keeps Src in an open conformation and allows binding to upstream activators and downstream substrate proteins, while the K295R mutation renders the protein catalytically inactive. Hence, this mutant acts as a dominant-negative, presumably because it can bind and sequester activators and/or effectors of Src, thereby preventing the activation or function of endogenous SFKs. The cells were seeded on Matrigel and maintained in 3D culture for 4 days. The cells expressing eGFP-dnSrc did not form protrusions in Matrigel, but the cells expressing eGFP were spindle shaped and formed protrusions (Figure 2b).

To quantify the decrease in migratory and/or invasive capacity of SFK-inhibited MDA-MB-231 cells, I used modified Boyden chambers, also known as transwell migration and invasion chambers. These chambers are commercially available for use in determining the migratory and invasive capacities of cells *in vitro*. The bottom of the chambers is composed of a filter with pores of a specified size that may be overlayed with extracellular matrix such as Matrigel. Cells are placed in the chamber, typically in serum-free cell culture medium, and the chamber is placed in a well of a multi-well dish containing the appropriate chemoattractant. Migratory capacity is determined by the number of cells that are able to pass through an uncoated filter towards the chemoattractant. Invasive capacity is determined by the number of cells that are able to transverse an extracellular matrix-coated filter. MDA-MB-231 cells were seeded in transwell migration and Matrigel-coated invasion filters and allowed to adhere, treated with DMSO or 2 uM PP2 and incubated overnight. The following day, the filters were harvested and the number of cells that had migrated or invaded was counted. Inhibition of SFKs with PP2 led to decreased migration and invasion compared to cells treated with DMSO (Figure 2c). This is consistent with the decrease in migration and invasion of MDA-MB-231 cells stably expressing dominant negative eGFP-Src versus eGFP alone (Kusdra, 2007). These results suggest that SFKs are required for the invasive morphology of MDA-MB-231 breast cancer cells, and the inhibition of SFKs reduces the efficiency by which breast cancer cells can migrate and invade.

Decreased phosphorylation of Akt1 and Akt2 isoforms upon inhibition of SFKs

There are various mechanisms by which SFKs can regulate the proteins involved in the activation of Akt isoforms. However, whether any of these mechanisms are active in metastatic breast cancer cells has not been determined. An interesting observation from Okano et. al. (2000) was that EGF stimulation of the EGF-receptor induced the activation of Akt isoforms in a panel of esophageal cancer cell lines in a cell-line specific fashion. Akt1 was activated in all the cell lines, however Akt2 and Akt3 were only activated in a subset of the cell lines. This cell-line specificity was

attributed in part to differences in Ras function since the activation of the Akt isoforms was prevented by inhibition of Ras in some of the cell lines but not in others (Okano et al., 2000). Since SFKs promote breast cancer cell migration and invasion, I investigated whether SFKs preferentially activate Akt2 in breast cancer cells.

To determine whether SFKs differentially regulate the activation of Akt isoforms in MDA-MB-231 cells, I analyzed the phosphorylation status of Akt isoforms in MDA-MB-231 cells in which SFKs had been inhibited. Phosphorylation at residues Thr308 and Ser473 is required for activation of Akt and is used as an indirect measure of Akt activation. MDA-MB-231 cells were treated with DMSO, 5 uM PP2 or 5 uM SU6656, another SFK pharmacological inhibitor, for 1 – 24 hours. Cell lysates were prepared and Akt was immunoprecipitated with antibodies specific for Akt1 or Akt2 isoforms, then blotted with antibodies that detect Akt isoforms phosphorylated at Thr308 or Ser473. Inhibition of SFKs with pharmacological inhibitors led to decreased levels of phospho-Thr308 and phospho-Ser473 Akt1 (Figure 3a) and Akt2 (Figure 3b). Thus, the activation of Akt isoforms in breast cancer cells is dependent on SFKs, and does not vary between Akt1 and Akt2.

Decreased kinase activity of Akt1 and Akt2 isoforms upon inhibition of SFKs

A decrease in the activity of Akt1 and Akt2 can be inferred from the decrease in the phosphorylation at Thr308 and Ser473 residues. This result was confirmed with an *in vitro* kinase assay. MDA-MB-231 cells were treated with DMSO, 5 uM PP2, or 5 uM SU6656. Cell lysates were harvested and immunoprecipitated with an Akt1-isoform specific antibody. A negative control reaction contained lysates from MDA-MB-231 treated with DMSO subjected to immunoprecipitation without Akt1 antibody (lane 7, Figure 4a). The kinase activity of Akt1 was measured with a non-radioactive kinase assay kit using a substrate that corresponds to a portion of GSK3 encompassing the Akt-target sequence. Recombinant activated Akt1 served as a positive control reaction for the kinase assay (lane 8, Figure 4a). The levels of phospho-GSK3 were measured by immunoblot with an antibody that detects phospho-GSK3. Akt1 immunoprecipitated from 231 cells treated with PP2 or SU6656 was less active than Akt1 from cells treated with DMSO (Figure 4). A similar experiment was performed with cell lysates from MDA-MB-231 cells stably expressing eGFP or eGFP-dnSrc. Akt1 from eGFP-dnSrc MDA-MB-231 cells had decreased activity compared to Akt1 from eGFP MDA-MB-231 cells (Figure 4b). These results confirm that the inhibition of SFKs leads to decreased activity of Akt isoforms.

Inhibition of SFKs leads to decreased phosphorylation of Akt substrates

Inhibition of SFKs in MDA-MB-231 cells led to decreased phosphorylation and activity of Akt isoforms as measured by an *in vitro* kinase assay. To determine

whether the decrease in activity of Akt isoforms measured *in vitro* corresponds to decreased Akt activity *in vivo*, I analyzed the phosphorylation status of an endogenous substrate of Akt isoforms upon the inhibition of SFKs. Protein lysates were harvested from MDA-MB-231 cells that were treated with DMSO, 5 uM PP2, or 5 uM SU6656, or were stably expressing eGFP or dominant negative Src. The lysates were resolved by SDS-PAGE and transferred onto a PVDF membrane for immunoblot. Several members of the Forkhead family, including FoxO3a, are Akt substrates. The levels of FoxO3a phosphorylated at Thr32, an Akt target site, were decreased in cells treated with PP2 and SU6656 (Figure 5, lanes 2 and 3) and in cells expressing dominant negative Src (lane 5) versus control cells (lanes 1 and 4). This confirms that the endogenous activity of Akt isoforms is decreased upon inhibition of SFKs.

Discussion

The goals of this study were to determine whether SFKs are required for the migration and invasion of breast cancer cells, and to determine whether the Akt isoforms are regulated by SFKs in breast cancer cells. Numerous studies have provided evidence that SFKs regulate invasion by non-breast cancer cells, but whether SFKs have the same role in breast cancer cells has not been elucidated. I used the MDA-MB-231 cell line because this cell line was obtained from the metastasis of a human breast cancer patient and is highly metastatic when injected or implanted into mice in *in vivo* assays of metastasis. When cultured *in vitro* in 3D extracellular matrix, such as Matrigel, MDA-MB-231 cells form invadopodia—actin rich protrusions that degrade the extracellular matrix. Invadopodia are found in invasive cancer cells, but not in non-invasive cells. Although invadopodia were initially observed *in vitro,* they have also been observed *in vivo*, which suggests that invadopodia structures are indeed used by metastatic breast cancer cells (Yamaguchi et al., 2005). Invadopodia contain a high concentration of proteins involved in cell-extracellular matrix adhesion and matrix degradation, such as integrins and matrix metalloproteinases. Many of the proteins that localize in invadopodia are also found in related actin-rich structures called podosomes, which are found in Src-transformed cells, macrophages, and osteoclasts; podosomes frequently assemble into ring-like aggregates called rosettes. Although invadopodia and podosomes differ in morphology, they both contain focal adhesion proteins, integrins, and proteases, and both invadopodia and podosomes promote extracellular matrix degradation (Weaver, 2006). Since Src has an essential role in podosomes, it is not entirely surprising that the invadopodia formed by MDA-MB-231 cells in Matrigel cultures were absent when cells were treated with the pharmacological inhibitors PP2 and SU6656 or in cells overexpressing a dominant-negative form of Src.

Invasive cancer cells, such as the MDA-MB-231 cell line, that exhibit a mesenchymal morphology during invasion are able to degrade the extracellular matrix to facilitate

invasion of a tissue. Inhibition of extracellular matrix degradation with protease inhibitor cocktails can lead to a shift from a mesenchymal morphology to an ameboid shape. However, this does not necessarily lead to a decrease in cell migration and/or invasion as demonstrated by Wolf et. al. (2003), who showed that when HT1080 and MDA-MB-231 cells were treated with protease inhibitor cocktails they could carry out an ameboid type of cell migration and invasion. The efficiency of cell migration and invasion was only slightly decreased following the transition to an ameboid morphology (Wolf et al., 2003). Inhibition of SFKs with pharmacological inhibitors led to decreased formation of invadopodia and decreased migration and invasion of MDA-MB-231 cells. Therefore, inhibition of SFKs inhibits the mesenchymal-type of MDA-MB-231 cell migration and prevents MDA-MB-231 cells from carrying out ameboid form of migration. Inhibition of SFKs with the pharmacological inhibitor PP2 had a drastic effect on both cell migration and invasion through transwell filters. Since PP2 decreased the migration of cells by almost 100%, any additional effects of the inhibition of SFKs on the invasion of cells could not be detected.

Both Akt1 and Akt2 isoforms exhibited decreased phosphorylation and activity upon inhibition of SFKs. This suggests that SFKs regulate the activity of Akt isoforms in invasive breast cancer cells, however the mechanism was not identified. This finding was somewhat surprising because MDA-MB-231 cells contain a constitutively active Ras due to a *K-ras* codon 12 mutation (von Lintig et al., 2000). Ras can be activated by SFKs and can directly activate PI3K. The activating mutation might be expected to bypass a requirement for SFKs in the activation of the PI3K pathway. One possible explanation is that activation of Akt requires other Src-dependent pathways in addition to Ras activation. Src may be required for the phosphorylation of Akt isoforms by SFKs or for the phosphorylation, stabilization, and activation of PDK1. Chen et. al. (2001) found that Src phosphorylates Akt isoforms at two tyrosine residues, and that this phosphorylation is required for the phosphorylation of Akt at Thr308 and Ser473 (Chen et al., 2001). Although I analyzed the phosphorylation status of Akt at Thr308 and Ser473, I did not investigate phosphorylation of Akt at these tyrosine residues. I also did not investigate whether these tyrosine residues are required for the activation of Akt isoforms in MDA-MB-231 breast cancer cells. Yang et. al. (2008) found that Src forms a complex with PDK1 and HSP90. The Src-PDK1-HSP90 complex increased PDK1 activity towards Akt and SGK, and protected PDK1 from degradation (Yang et al., 2008). I did not investigate whether the activity and/or protein levels of PDK1 are decreased upon the inhibition of SFKs in breast cancer cells. The mechanism by which SFKs regulate the phosphorylation and activation of Akt isoforms in MDA-MB-231 cells remain to be identified.

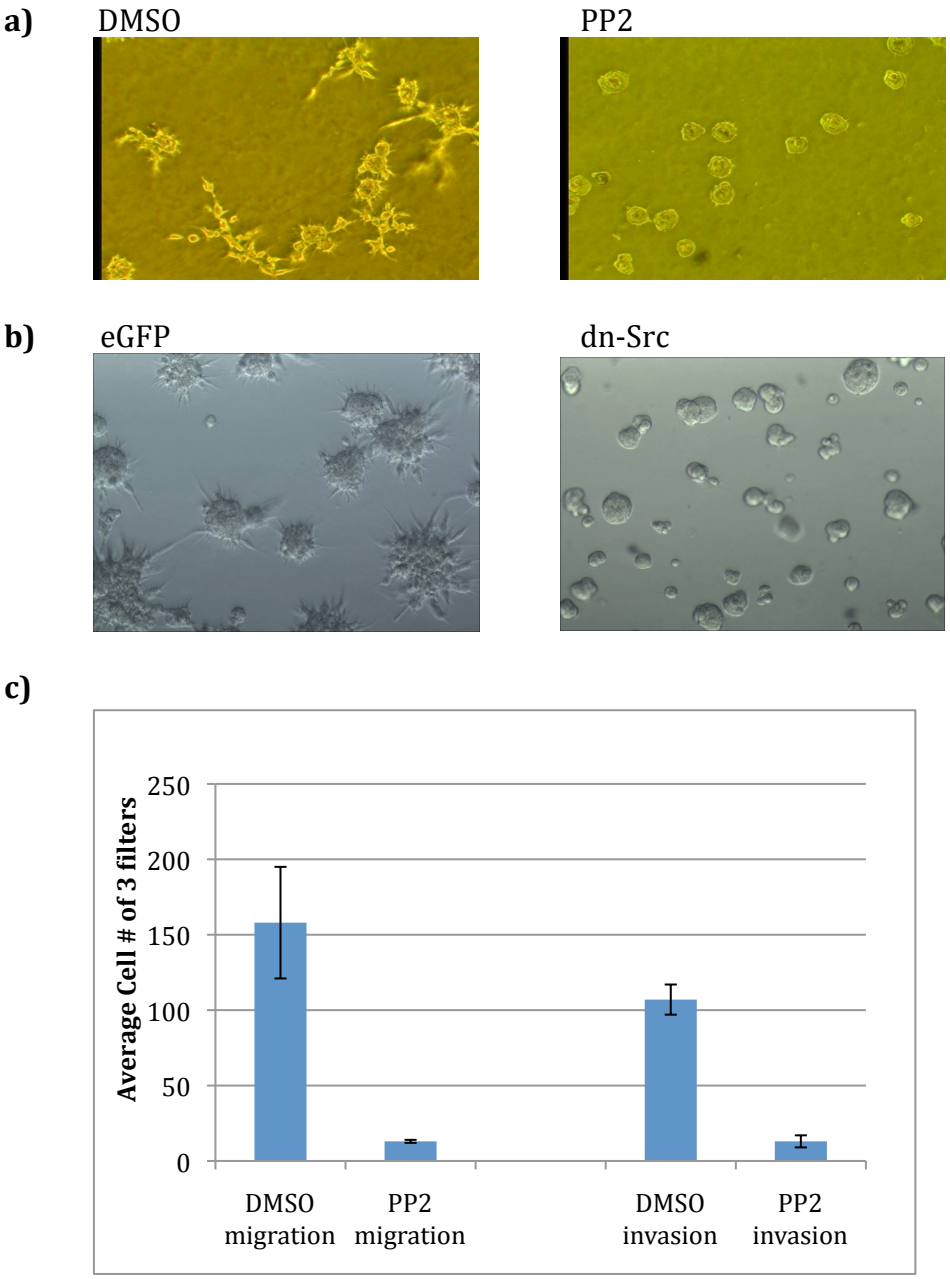


Figure 2: Inhibition of SFKs leads to decreased migration and invasion of MDA-MB-231 breast cancer cells. a) MDA-MB-231 cells were seeded in 3D Matrigel cultures and treated with DMSO or 2 uM PP2 for 4 days. b) MDA-MB-231 cells stably expressing eGFP or eGFP-dnSrc were seeded in 3D Matrigel and cultured for 4 days. c) MDA-MB-231 cells were seeded in triplicate migration or invasion filters, treated with DMSO or PP2, and harvested the following day. The average numbers of cells that migrated or invaded through triplicate filters (±S.D.) are shown.

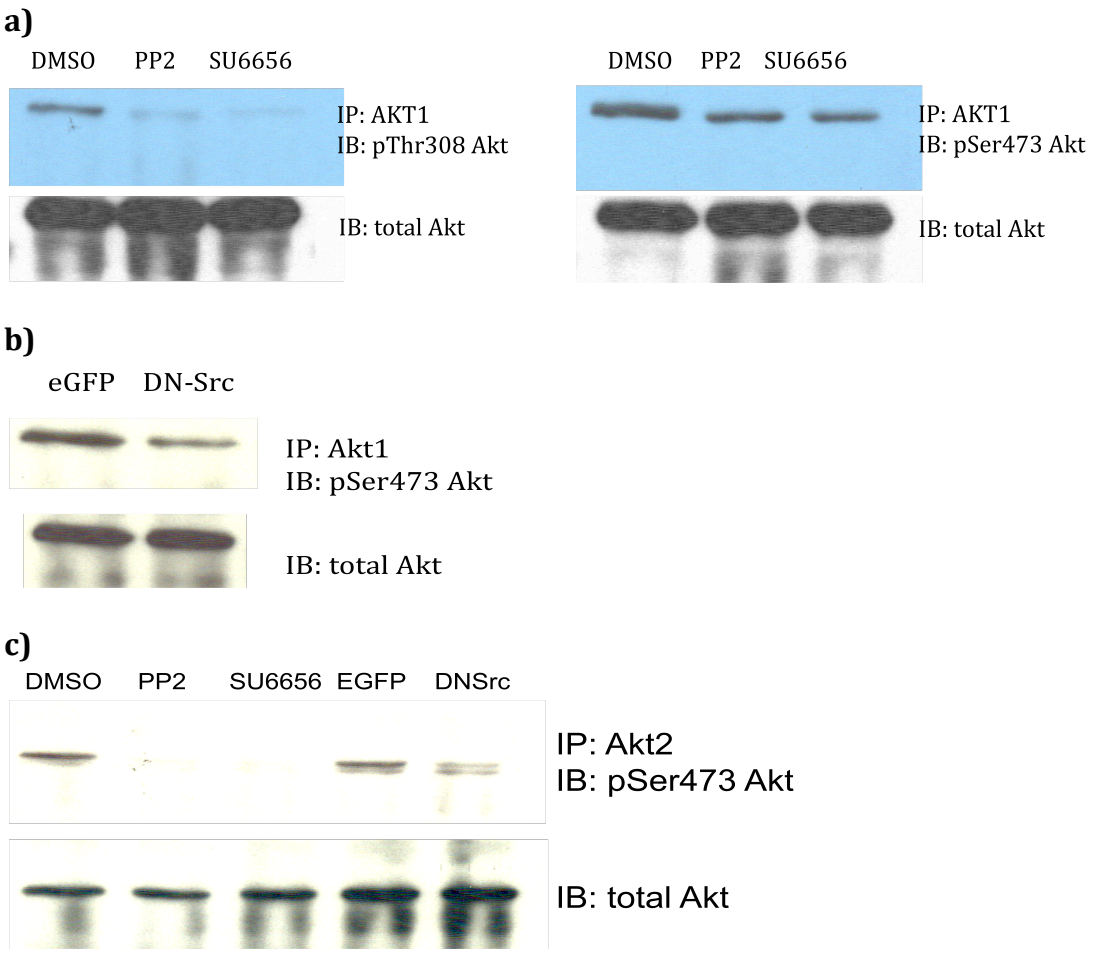
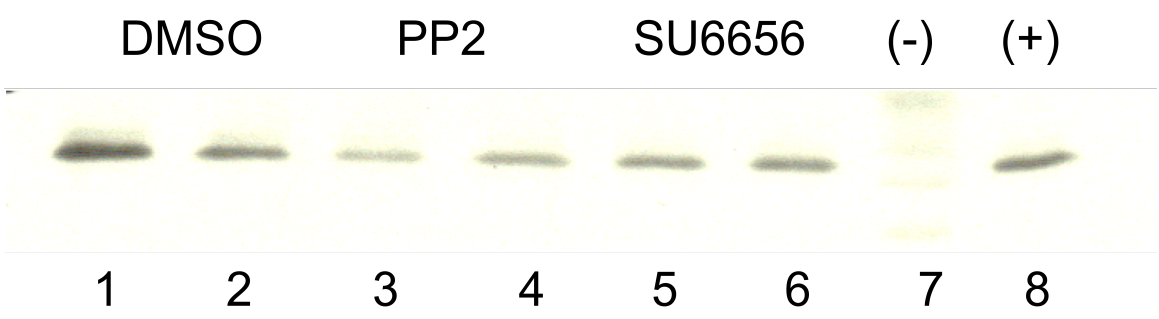


Figure 3: Inhibition of SFKs leads to decreased phosphorylation of Akt isoforms at residues Thr308 and Ser473. a) MDA-MB-231 cells were treated with DMSO, 5 uM PP2, or 5 uM SU6656. Akt1 was immunoprecipitated with mouse anti-Akt1 and subjected to SDS-PAGE and immunoblotting with rabbit anti-phosphoThr308 Akt (top left panel) or rabbit anti-phosphoSer437 Akt (top right panel). The immunoblots were subsequently stripped and reprobed with rabbit anti-pan Akt as a loading control (corresponding bottom panels). b) Akt1 was immunoprecipitated from MDA-MB-231 cells stably expressing eGFP or dominant-negative Src. The immunoprecipitates were resolved on an SDS-PAGE gel and immunoblotted with rabbit anti-phosphoSer473 Akt. The immunoblot was subsequently stripped and reprobed with rabbit anti-pan Akt as a loading control. c) Akt2 was immunoprecipitated from the cell lysates of MDA-MB-231 cells treated with DMSO, 5 uM PP2, or 5 uM SU6656, or from MDA-MB-231 cells stably expressing eGFP or dominant negative Src. Immunoprecipitates were subjected to SDS-PAGE and transferred to a PVDF membrane. The membrane was probed with rabbit anti-phosphoSer473 Akt antibody, stripped, and reprobed with rabbit anti-pan Akt as a loading control.

a)



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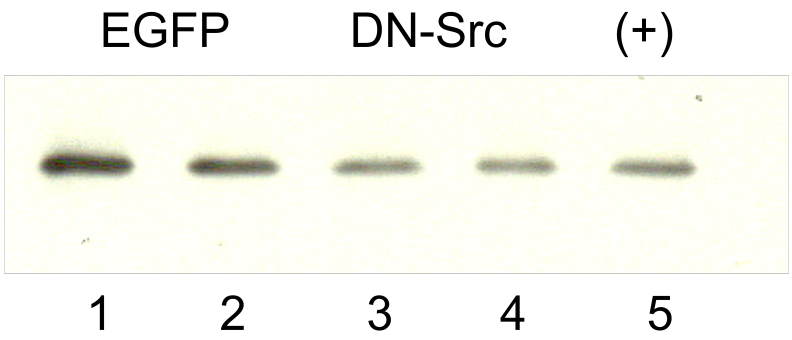


Figure 4: Inhibition of SFKs leads to decreased kinase activity of Akt1. a) MDA-MB-231 cells were treated with DMSO, 5 uM PP2, or 5 uM SU6656. Cell lysates were processed and immunoprecipitated with mouse anti-Akt1 in duplicate reactions. An immune-complex kinase assay was performed using the Cell Signaling Non-Radioactive Akt Kinase Assay Kit with a GSK3 fusion protein as the kinase substrate. The products of immune-complex kinase assay reactions were subjected to SDS-PAGE, transferred onto PVDF membranes and blotted with rabbit anti-phospho GSK3 antibody. (-) denotes the negative control reaction in which MDA-MB-231 DMSO lysate was immunoprecipitated without the mouse anti-Akt1 antibody. (+) is a positive control kinase assay reaction performed with purified recombinant active Akt1. b) MDA-MB-231 cells stably expressing eGFP or dominant negative Src was immunoprecipitated with mouse anti-Akt1 and subjected to a kinase assay and immunoblot as described in part (a).

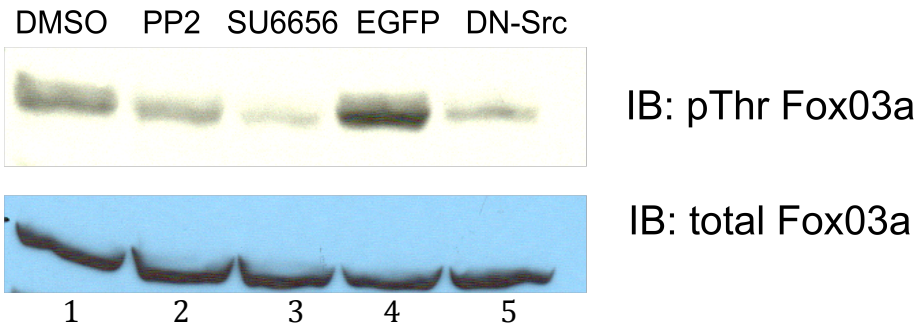


Figure 5: Inhibition of SFKs leads to decreased phosphorylation of Akt substrates in MDA-MB-231 cells. Protein lysates from MDA-MB-231 cells treated with DMSO, PP2, or SU6656, or from MDA-MB-231 cells stably expressing eGFP or dominant-negative Src were subjected to SDS-PAGE and transferred onto a PVDF membrane. The membrane was probed with a rabbit anti-phosphoThr 32 FoxO3a antibody to detect the levels of phosphorylated FoxO3a. The membrane was subsequently stripped and reprobed with rabbit anti-FoxO3a to determine the total levels of FoxO3a protein.

Chapter 4: The Akt2 isoform specifically regulates the migration and invasion of MDA-MB-231 breast cancer cells

Introduction

A question that emerged from the discovery of three Akt isoforms was whether the Akt isoforms have unique or redundant roles. This is particularly important since the majority of studies have been performed on Akt1 and have not distinguished among the different Akt isoforms. Since Akt is frequently activated in several types of cancer, pharmacological inhibitors targeting Akt are currently being developed for clinical use (Carnero, 2010). Some of these Akt inhibitors inhibit all Akt isoforms whereas others are isoform-specific. A more thorough understanding of the functions of individual Akt isoforms is needed to determine whether targeting individual or multiple Akt isoforms would be most effective. Analysis of the activity of Akt isoforms *in vitro* or the use of constitutively membrane targeted and activated Akt isoforms have not clarified functional differences between Akt isoforms. Akt isoforms are able to phosphorylate the same substrates in *in vitro* kinase assays. Individual myristoylated Akt isoforms have the same oncogenic effects when expressed in fibroblasts (Mende et al., 2001). These results suggest that there may be no differences in the functions of Akt isoforms, or that the intrinsic substrate specificity of each Akt isoform is not preserved in these conditions. Perhaps Akt-interacting proteins expressed *in vivo* regulate the differential localization and/or activation of Akt isoforms, and such differences in localization and/or activation may be required for differences in function.

The tissue expression profiles of Akt isoforms provided initial evidence that Akt isoforms may have different roles. Akt1 and Akt2 are ubiquitously expressed, although at varying levels, but Akt3 expression is restricted to several tissues (Masure et al., 1999). Akt1 is expressed highly in most tissues. Akt2 is expressed highly in skeletal muscle, heart, liver, and kidney. Akt3 is expressed highly in the brain but is also present in the pancreas, skeletal muscle, and kidney (Brodbeck et al., 2001; Nakatani et al., 1999a). The relevance of these differences was not revealed until mice lacking each Akt isoform were generated.

Genetically engineered knockout mouse models lacking individual Akt isoforms have provided evidence that there are Akt isoform-specific roles. Akt1 knockout mice have defects in growth as evidenced by their smaller size and weight compared to wild type counterparts. At birth, Akt1 knockout mice have an average weight of 1.21 ± 0.038 g whereas wild type mice have an average weight of 1.51 ± 0.058 g. The difference in size and weight of Akt1 knockout and wild type mice persisted through adulthood (Cho et al., 2001b). Akt2 knockout mice are of normal size but exhibit defects in glucose homeostasis, which was not affected in Akt1 knockout mice. Akt3 knockout mice are the same size and weight as wild-type and do not

have defects in glucose homeostasis. However, the knockout of Akt3 leads to decreased size of the brain caused by a decrease in the number of cells and smaller cell size (Easton et al., 2005; Tschopp et al., 2005).

I investigated whether the Akt isoforms play unique or redundant roles in the migration and/or invasion of breast cancer cells. I used siRNA targeted against each Akt isoform to knockdown the levels of Akt1 or Akt2 in MDA-MB-231 cells. I analyzed the effects of Akt1 or Akt2 knockdown on the migratory and invasive capacity of MDA-MB-231 cells by using transwell migration and invasion assays. Endogenous Akt2 specifically regulated cell migration and invasion through a mechanism that does not involve cell attachment to extracellular matrix. I also investigated whether the Akt isoforms bind to Pak1, an Akt1 binding partner that regulates migration in fibroblasts, however, neither Akt1 nor Akt2 co-immunoprecipitated Pak1. These results show that there are differences in the roles of Akt isoforms in breast cancer cells, and the Akt2 isoform specifically promotes the migration and invasion of breast cancer cells.

Results

The knockdown of Akt2 leads to decreased MDA-MB-231 cell migration and invasion

Several studies have analyzed the role of Akt2 in the migration or invasion of mammary epithelial cells. Irie et. al. (2005) found that overexpression of IGF-IR in non-tumorigenic MCF-10a cells led to enhanced cell migration upon stimulation with IGF-I. This response was dependent on Akt2 since siRNA-mediated knockdown of Akt2 abrogated the effect of IGF-I on the migration of MCF-10a-IGF-IR cells (Irie et al., 2005). Arboleda et. al. (2003) analyzed the effects of overexpression of Akt2 on the invasive capacity of a panel of ovarian and breast cancer cell lines. They found a statistically significant increase in the invasion of Caov3 ovarian and MDA-MB-435 breast cancer cells through Matrigel-coated transwell filters. To determine whether the increase in invasion of cells could be attributed to a role of Akt2 in regulating the attachment of cells to the extracellular matrix, they used MDA-MB-435 cells stably overexpressing the Her2 oncogene. They found that overexpression of Akt2 enhanced the expression of β1 integrin and this led to increased attachment onto laminin rich matrix. The cells also exhibited enhanced metastatic capacity when injected intraperitoneally in mice (Arboleda et al., 2003). However, a possible drawback with these studies is that they did not use spontaneously derived metastatic breast cancer cells. Irie et. al. (2005) used a non-malignant mammary cell line that was engineered to overexpress IGF-IR, and although this cell line is valuable in elucidating the role of IGF-IR in the uncontrolled proliferation of MCF-10a-IGF-IR cells, it does not recapitulate the genetic and morphological characteristics of spontaneously metastatic breast cancer cells (Bruchim et al., 2009). Arboleda et. al. (2003) used the MDA-MB-435 cell line engineered to overexpress the oncogene HER2, but karyotyping, comparative

genomic hybridization, microsatellite polymorphism analysis, and analyses of gene expression and single nucleotide polymorphisms have confirmed that the MDA-MB-435 cell line is a melanoma cancer line (Rae et al., 2007).

To determine whether Akt2 has a role in the migration or invasion of metastatic breast cancer cells, I used the MDA-MB-231 cancer cell line, which was derived from the pleural effusion of a breast cancer patient. I used small interfering RNA (siRNA) to knockdown the levels of Akt2. Immunoblot analysis shows that the siRNA is specific to Akt2 and does not target Akt1 or Akt3 isoforms (Figure 6a). Non-targeting control siRNA or Akt2 siRNA Amaxa-nucleofected cells were seeded in transwell migration or Matrigel coated transwell invasion filters and allowed to migrate or invade overnight. The cells that migrated or invaded towards the chemoattractant were counted. The MDA-MB-231 cells in which Akt2 had been knocked down exhibited decreased migration and invasion when compared to cells nucleofected with a control non-specific siRNA (Figure 6b).

Since previous studies on breast cancer cells have not revealed a role for Akt3 in migration or invasion, I focused my analysis on Akt1 and Akt2. To determine whether Akt1 or Akt2 has a role in migration or invasion of breast cancer cells, I used siRNA specific for each isoform to knockdown Akt1 or Akt2 in MDA-MB-231 cells (Figure 7a). siRNA-mediated knockdown of Akt2, but not of Akt1, led to decreased migration and invasion of MDA-MB-231 breast cancer cells (Figure 7b).

To confirm that inhibition of migration and invasion of MDA-MB-231 breast cancer cells was due to the knockdown of Akt2, and not to off-target effects of the siRNA, the migration and invasion of MDA-MB-231 Akt2 siRNA cells were rescued by expression of an Akt2 construct containing silent mutations in the siRNA target sequence (Figure 8a). Expression of RNAi-mut Akt2, but not of Akt1 to near endogenous levels rescued the decrease in invasion of MDA-MB-231 Akt2 siRNA cells (Figure 8b). These results suggest that Akt2 has a unique isoform-specific role in regulating the migration and invasion of breast cancer cells.

Overexpression of Akt2 leads to increased invasion of MDA-MB-231 cells

The decrease in migration and invasion of MDA-MB-231 cells upon knockdown of Akt2 showed that Akt2 is necessary for the migration and invasion of MDA-MB-231 cells. To determine whether the expression of Akt2 is sufficient to promote cell migration and invasion, I co-nucleofected MDA-MB-231 cells with pMaxGfp vector and pCMV-HA, pCMV-HA-Akt1, or pCMV-HA-Akt2 and seeded the cells in migration and invasion transwell filters. In parallel with the migration and invasion assays aliquots of nucleofected cells were seeded in tissue culture dishes and lysed for immunoblot analysis. The immunoblots confirmed the expression of wild-type Akt1 and Akt2 (Figure 9a). The percentage of gfp-positive cells that had migrated or invaded was determined (Figure 9b). Overexpression of Akt1 or Akt2 did not lead

to increased migration of MDA-MB-231 cells. However, overexpression of Akt2, but not of Akt1, led to increased invasion through Matrigel-coated transwell filters. This was not due to higher expression of Akt2 because comparison of Akt isoform bands in the immunoblot shows that Akt1 was overexpressed at a higher level than Akt2. These results suggest that endogenous levels of Akt2 regulates both migration and invasion of MDA-MB-231 breast cancer cells. However, overexpression of Akt2 does not enhance the migratory capacity of MDA-MB-231 cells, suggesting endogenous levels of Akt2 are sufficient for cell migration. Since Akt2 overexpression increased the ability of MDA-MB-231 cells to invade, endogenous Akt2 protein levels, and or activation, appears to be limiting in the process of invasion in MDA-MB-231 cells.

Akt2 does not regulate the attachment of MDA-MB-231 cells to extracellular matrix

One of the mechanisms by which Akt isoforms have been shown to promote the migration and/or invasion of cancer cells is by regulating the attachment of cancer cells to extracellular matrix. This is achieved by regulating the levels or recycling of β1 integrin. To determine whether Akt isoforms differentially regulate the ability of MDA-MB-231 cells to adhere to extracellular matrix, I used siRNA to knockdown Akt1 or Akt2 and seeded the cells on Matrigel-coated well of a 12-well dish. At the specified time points post-seeding, I transferred a sample of cell culture medium from each well into a cuvette and counted the non-adherent cells with a BD Coulter Counter. There was no significant change in the number of cells that adhered to Matrigel upon knockdown of Akt1 or Akt2 (Figure 10).

Akt2 does not bind to Pak in MDA-MB-231 cells

In an attempt to identify an Akt2 substrate or binding partner that might regulate breast cancer cell migration and/or invasion, I analyzed whether Akt2 binds to proteins that interact with Akt1 and mediate its effects on the migration and invasion of other cell types. In fibroblasts Akt1 binds to Pak1 and enhances the function of Pak1 in the formation of dorsal ruffles and in cell migration and invasion. To determine whether Akt2 binds and enhances the activation of Pak1 in MDA-MB-231 breast cancer cells, I nucleofected MDA-MB-231 cells with pCMV-HA empty vector, pCMV-HA-Akt1, or pCMV-HA-Akt2 and immunoprecipitated cell lysates with a mouse anti-HA antibody. The immunoprecipitates were subsequently subjected to immunoblots for Pak1 (Figure 11). I did not detect Pak1 in samples immunoprecipitated with mouse anti-HA antibody (Figure 11, lanes 1-3). Pak1 was detected in the supernatants of the immunoprecipitates of cell lysates containing HA-Akt1 or HA-Akt2, respectively (Figure 11, lanes 4 and 5). The immunoblot was stripped and reprobed with mouse anti-HA, to confirm that HA-Akt1 and HA-Akt2 were expressed and successfully immunoprecipitated.

Discussion

The goal of this study was to determine whether Akt1 and Akt2 have distinct roles in the migration and/or invasion of MDA-MB-231 cells. Previous studies have shown that Akt1 has an inhibitory role in the migration and invasion of breast cancer cells. However, the role of Akt2 in promoting migration and invasion of breast cancer cells has not been clearly elucidated, and the mechanism by which Akt2 can perform a role distinct from that of Akt1 has not been determined. I found that the knockdown of Akt2 but not of Akt1 impeded the ability of metastatic breast cancer cells to migrate and invade through transwell filters. This inhibition can be rescued by expression of an RNAi-resistant mutant Akt2 but not by Akt1, which confirms that the regulation of migration and invasion is specifically dependent on the Akt2 isoform, and not on the total level of Akts. Knockdown of Akt2 resulted in a greater inhibition of invasion than of migration. Hence, Akt2 has additional roles in MDA-MB-231 cell invasion over and above those that it plays in migration. This was the first study to demonstrate regulation of migration and invasion by endogenous Akt2 in spontaneously metastatic human breast cancer cells.

Previous studies have shown that the knockdown of Akt1 by siRNA can promote the migration and/or invasion of SUM-159PT and MCF-10-IGF-IR cells (Irie et al., 2005; Yoeli-Lerner et al., 2005). Interestingly, I did not find an increase in MDA-MB-231 cell migration or invasion upon the knockdown of Akt1. The different results from this study and previous results may be due to biological differences between the cell lines used in these studies. Since the role of endogenous Akt1 in breast cancer cell migration and invasion has only been analyzed in the three cell lines mentioned previously, a more extensive analysis of a panel of breast cancer cell lines is needed to elucidate the role of Akt1 in breast cancer cells.

The overexpression of HA-Akt2 led to increased invasion of MDA-MB-231 cells but did not increase cell migration. This was a surprising finding since endogenous Akt2 regulates both migration and invasion. A possible explanation is that the level of endogenous Akt2 is sufficient to maximally activate the pathways by which it regulates cell migration. Conversely, endogenous Akt2 levels may be a rate-limiting factor in the process of cell invasion.

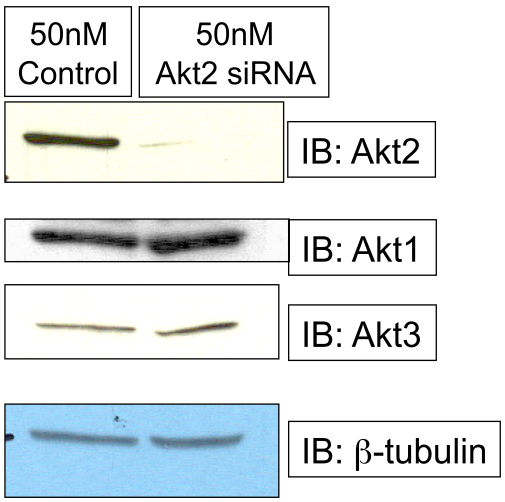
Overexpression of wild type Akt1 did not inhibit the migration and invasion of MDA-MB-231 cells. Although this seems to contradict previous studies in which overexpression of myristoylated Akt1 in breast cancer cells led to a decrease in migration and invasion, a possible explanation is the difference in Akt1 constructs: HA-tagged wild-type Akt1 would be under the same regulation of activity and localization as endogenous Akt1, while myristoylated Akt1 is constitutively active and targeted to the plasma membrane. Constitutive localization of hyper-activated Akt1 at the plasma membrane could lead to interaction with membrane-localized proteins that do not interact with endogenous wild-type Akt1. Since endogenous

Akt1 is not mutated in MDA-MB-231 cells, the use of wild-type Akt1 should better recapitulate the endogenous function of Akt1.

Previous studies have shown that Akt isoforms can regulate the recycling and expression of integrins that promote the attachment of cells onto extracellular matrix. In particular, β1 integrin is often upregulated in cancer cells that exhibit invasive capacity. Arboleda et. al. (2003) found that Akt2 promotes the expression of β1 integrin in MDA-MB-435 cells, and that this increase was required for the enhanced adhesion of MDA-MB-435HER2-Akt2 cells onto laminin or collagen-rich extracellular matrix (Arboleda et al., 2003). However, I did not find any change in the attachment of MDA-MB-231 cells onto Matrigel upon the inhibition of Akt1 or Akt2 by siRNA. To determine whether Akt2 regulates β1 integrin expression in MDA-MB-231 cells, I am currently investigating whether the level of β1 integrin is altered upon Akt2 knockdown by siRNA.

Co-immunoprecipitation assay results show that the Akt isoforms do not interact with Pak1 in MDA-MB-231 cells (Figure 11). Although an Akt1-Pak1 interaction promotes the migration of fibroblasts, this does not appear to be the case in breast cancer cells and could partially explain why Akt1 does not promote migration in these cells. The mechanism that regulates the interaction of Pak1 with Akt isoforms has not been determined, but is relevant to the question of why Akt isoforms have different functions in different tissues.

a)



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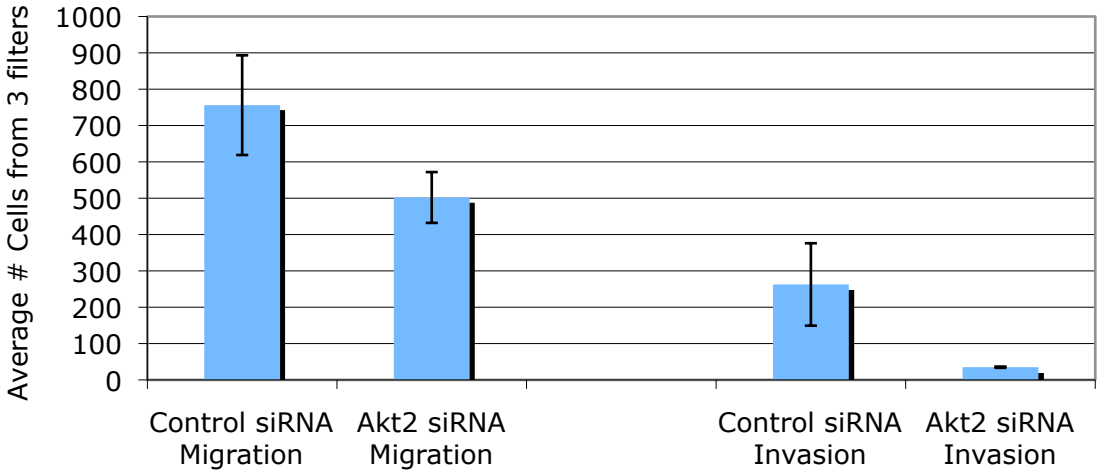
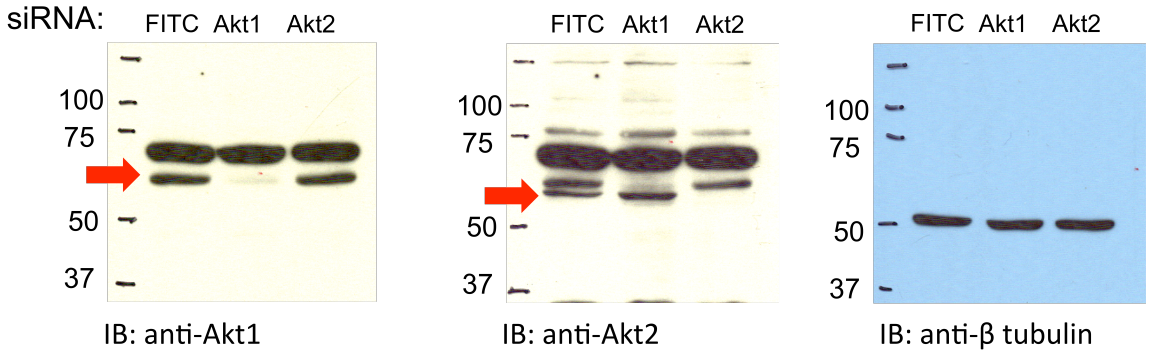


Figure 6: siRNA-mediated knockdown of Akt2 leads to decreased migration and invasion of MDA-MB-231 breast cancer cells. a) Knockdown of Akt2 in MDA-MB-231 cells nucleofected with control or Akt2 siRNA. Cell lysates were resolved by SDS-PAGE and transferred onto a PVDF membrane. The membrane was probed with rabbit anti-Akt2, stripped and reprobed with mouse anti-Akt1, stripped and probed with rabbit anti-Akt3, and finally probed with mouse anti-beta tubulin as a loading control. The Akt2 siRNA is specific for Akt2 and does not lead to decreased levels of Akt1 or Akt3. b) Migration and invasion assays of MDA-MB-231 cells nucleofected with control-FITC labeled siRNA or Akt2 siRNA. The average numbers of cells that migrated or invaded in triplicate wells (±S.D.) are shown.

a)



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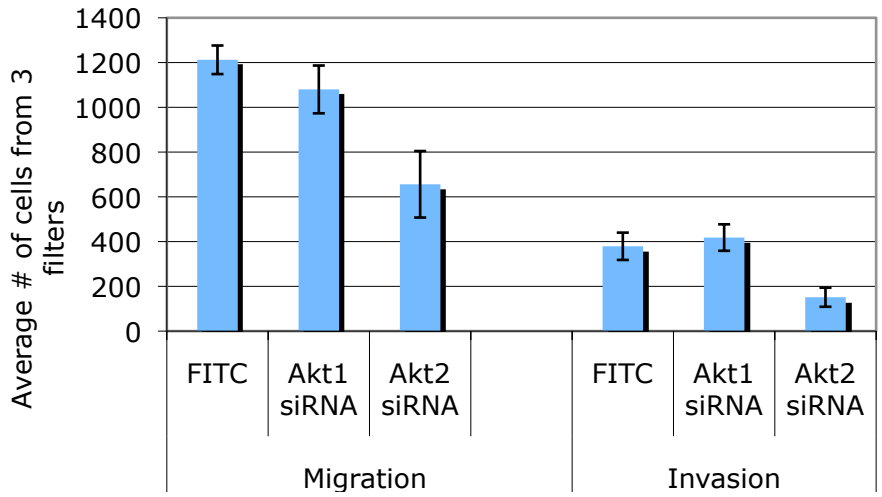
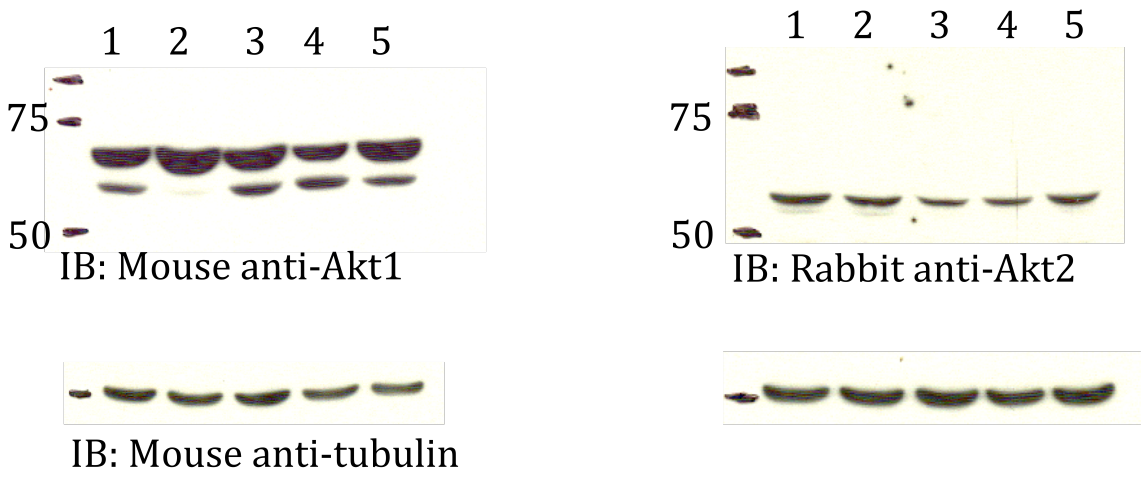


Figure 7: siRNA-mediated knockdown of Akt1 does not affect MDA-MB-231 cell migration or invasion. a) Knockdown of Akt1 or Akt2 in MDA-MB-231 cells nucleofected with FITC-labeled control, Akt1, or Akt2 siRNA. Cell lysates were subjected to SDS-PAGE and immunoblotted with mouse anti-Akt1, rabbit anti-Akt2, or mouse anti-beta tubulin as loading control. Arrows denote the specific bands. b) Migration and invasion of MDA-MB-231 cells nucleofected with control, Akt1, or Akt2 siRNA. The average numbers of cells from triplicate migration and invasion filters (±S.D.) are shown.

a)



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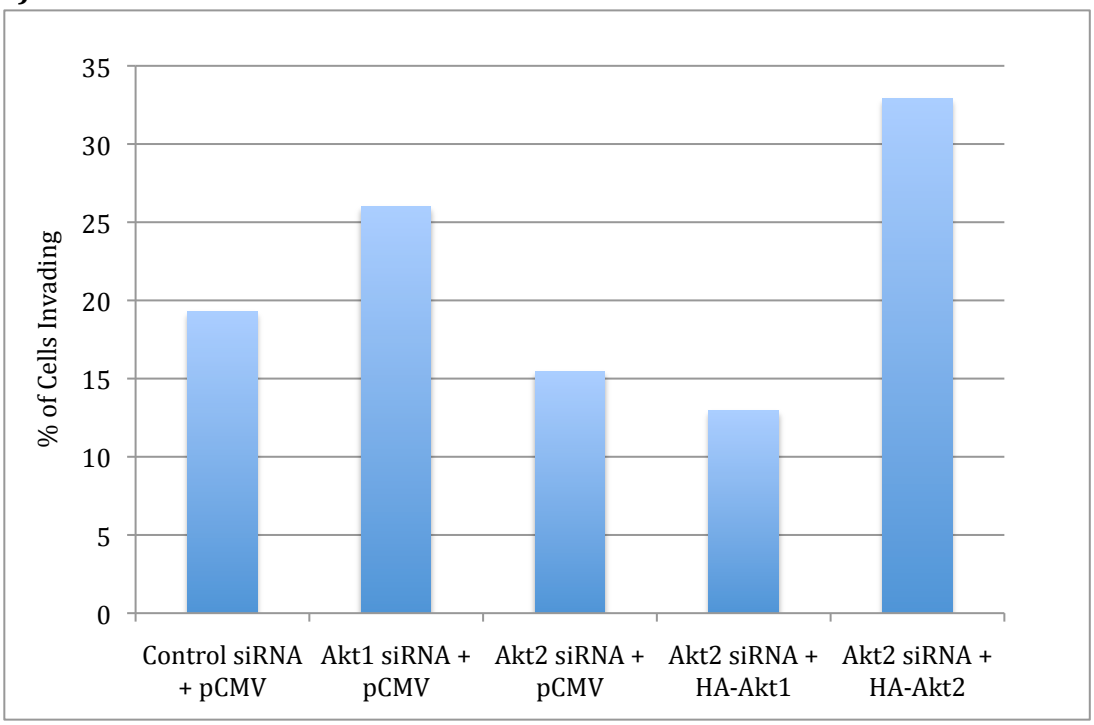
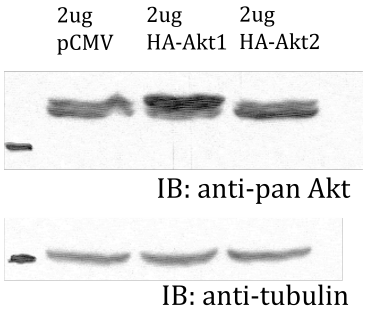


Figure 8: **The decrease in invasion of MDA-MB-231 cells upon siRNA knockdown of Akt2 can be rescued by expression of Akt2, but not Akt1**. a) Knockdown of Akt1 or Akt2 in MDA-MB-231 cells used for invasion assays. The top left panel was probed with mouse anti-Akt1. The top right panel was probed with rabbit anti-Akt2. Lanes were loaded as follows: 1. Control siRNA with pCMV-HA, 2. Akt1 siRNA with pCMV-HA, 3. Akt2 siRNA with pCMV-HA, 4. Akt2 siRNA with pCMV-HA-Akt1, 5. Akt2 siRNA with pCMV-HA-Akt2. b) Invasion assay of MDA-MB-231 cells co-nucleofected with the indicated siRNA and DNA constructs.

a)



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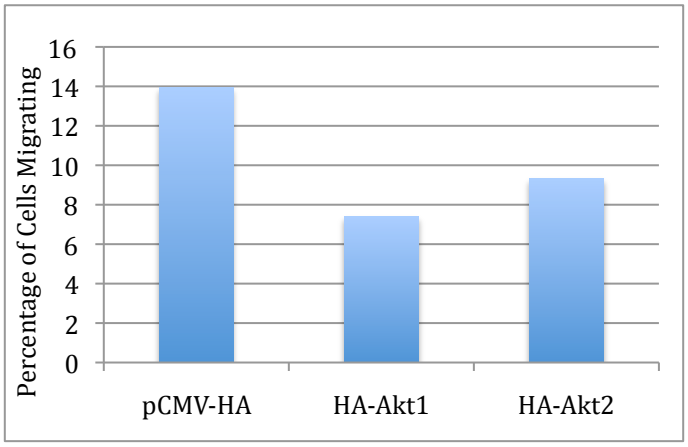

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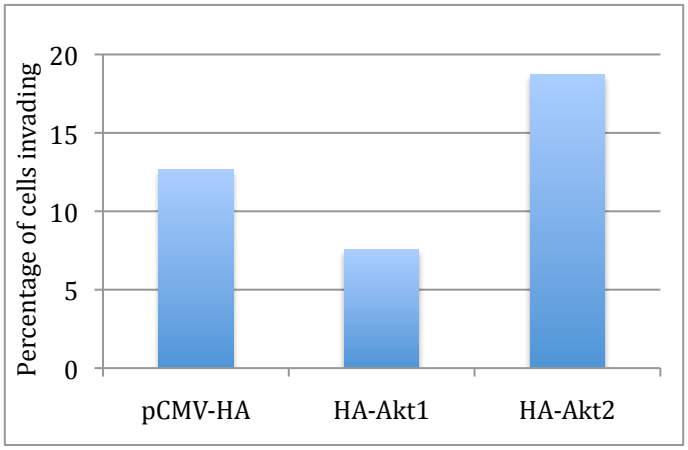


Figure 9: Overexpression of Akt2, but not of Akt1, leads to increased invasion of MDA-MB-231 cells. a) MDA-MB-231 cells nucleofected with pCMV-HA, HA-Akt1, or HA-Akt1 were lysed and cell lysates subjected to immunoblotting with mouse anti-HA. Membranes were stripped and reprobed with mouse anti-beta tubulin as a loading control. b) Cells nucleofected as in (a) were loaded onto migration filters. c) Cells nucleofected as in (a) were loaded onto invasion filters.

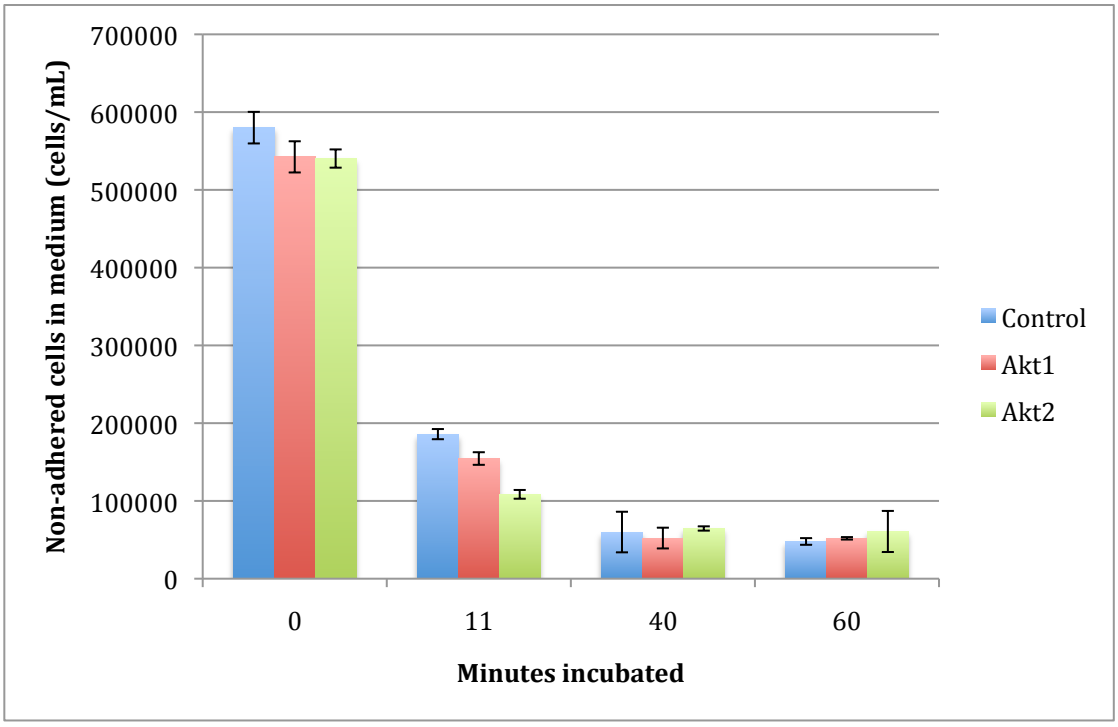


Figure 10: **siRNA mediated knockdown of Akt2 does not lead to decreased cell attachment to laminin-rich extracellular matrix**. MDA-MB-231 cells were nucleofected with control, Akt1, or Akt2 siRNA and seeded on Matrigel for 0, 11, 40, or 60 minutes. Non-adherent cells were harvested from the cell culture medium and counted with a Coulter Counter. The average numbers of non-adherent cells from triplicate filters (±S.D.) is shown.

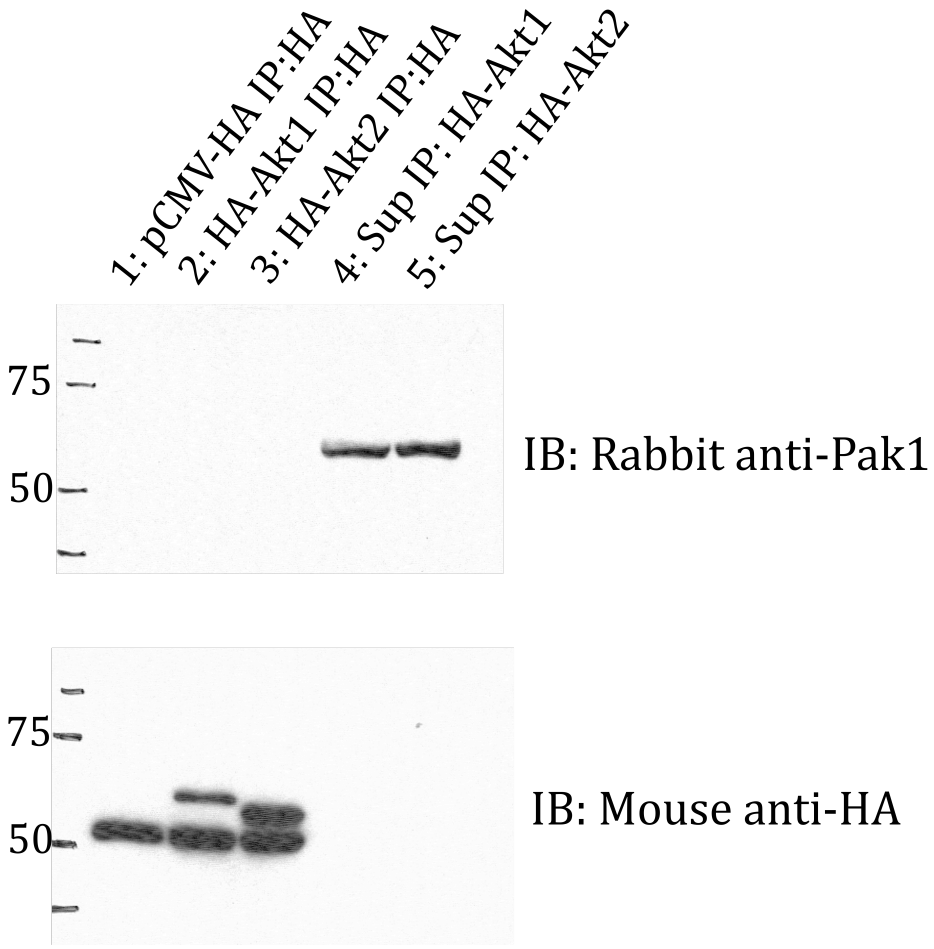


Figure 11: Akt2 and Pak do not interact in breast cancer cells. MDA-MB-231 cells were nucleofected with pCMV-HA, pCMV-HA-Akt1, or pCMV-HA-Akt2. Cell lysates were immunoprecipitated with mouse anti-HA and subjected to SDS-PAGE and immunoblotting. The molecular weights ($x10^{-3}$) of the protein markers are shown. Lanes 1-3 correspond to the immunoprecipitate samples. The supernatant from the mouse anti-HA immunoprecipitated from the lysates containing HA-Akt1 and HA-Akt2 were loaded on lanes 4 and 5, respectively. The immunoblot was probed with rabbit anti-Pak1 (top panel), stripped and reprobed with mouse anti-HA (bottom panel) to confirm the immunoprecipitation.

Chapter 5: Identifying Akt2-specific binding partners in metastatic breast cancer cells

Introduction

The ability of Akt2 to promote the migration and invasion of metastatic breast cancer cells is, hypothetically, mediated by proteins that are not regulated by other Akt isoforms in the same manner. Previous studies on the role of Akt2 in breast cancer cells did not reveal Akt2 isoform-specific substrates. In non-breast cancer cells, several proteins have been identified that are either differentially regulated by Akt isoforms, or exhibit preferential binding to and activation of specific Akt isoforms. Zhou et. al. (2006) found that mouse embryonic fibroblasts (MEFs) isolated from Akt1 knockout mice have decreased formation of dorsal ruffles as well as decreased migration and invasion through transwell filters. Conversely, MEFs isolated from Akt2 knockout mice have enhanced formation of dorsal ruffles, and higher rates of migration and invasion through transwell filters. The effects of Akt1 and Akt2 inactivation on fibroblast migration are due at least in part to the differential regulation of Pak1 by Akt1 and Akt2: the interaction of Akt1 with Pak1 activates Pak1, as determined by *in vitro* kinase assays, whereas the interaction of Akt2 leads to decreased activity of Pak1 (Zhou et al., 2006). Akt isoforms have been found to differentially interact with the TCL1 proto-oncogene family, which is made up of TCL1, MTCP1, and TCL1b (Laine et al., 2002). The TCL1 family is expressed in pre-T cell and B cells and is implicated in T-cell leukemia. Akt1 and Akt2 isoforms can interact with all TCL1 family members, although to varying degrees. However, Akt3 only interacts with TCL1. Since interaction with the proteins in the TCL1 family enhances the activation of Akt proteins, and is postulated to mediate hetero-oligomerization of Akt isoforms, overexpression of particular TCL1 family members in T-cell leukemia may cause overactivation of certain Akt isoforms (Kunstle et al., 2002; Laine et al., 2002; Pekarsky et al., 2000).

As previously mentioned, several studies have uncovered different mechanisms by which Akt1 inhibits breast cancer cell migration and invasion. Yoeli-Lerner et. al. (2005) reported that Akt1 downregulates the levels of NFAT transcription factors, while Liu et. al. (2006) reported that Akt1 inhibits TSC2-mediated migration and invasion through the Rho pathway. Irie et. al. (2005) found that Akt1 inhibits the Ras/MAPK pathway in MCF-10a IGF-IR cells, and that this pathway is required for the migration and invasion of these cells. However, the mechanism by which Akt2 promotes cell migration and invasion has not been identified. Irie. et. al. (2005) analyzed whether Akt2 antagonizes the effect of Akt1 on the Ras/MAPK pathway, but did not find a link between Akt2 and Ras/MAPK. Hence, the identity of the Akt2 substrates in breast cancer migration and invasion has remained a mystery.

Since the roles of Akt1 and Akt2 in breast cancer migration and invasion are different from their roles in migration and invasion of other cell types, determining how Akt2 regulates breast cancer cell migration and invasion cannot necessarily be inferred from studies performed in other cell types. Therefore, I performed co-immunoprecipitation and mass spectrometry experiments in MDA-MB-231 breast cancer cells to find proteins that specifically bind to Akt2. ACAP2, an Arf6-GTPase activating protein, was identified as a potential Akt2 co-immunoprecipitating protein. To determine whether ACAP2 has a role in Akt2-mediated breast cancer migration and invasion, I performed migration and invasion assays with MDA-MB-231 cells that were either nucleofected with control or ACAP2 siRNA. I also examined whether ACAP2 regulates the levels of Arf6-GTP in MDA-MB-231 cells and whether ACAP2 regulates the localization of Akt2 in Rab11-positive vesicles. However, after further scrutiny, I found that ACAP2 was non-specifically immunoprecipitated by the Flag antibody used to immunoprecipitate the Flag-tagged Akt2 and does not interact with Akt2.

Results

Mass Spectrometry of proteins co-immunoprecipitated with Akt2-Flag in MDA-MB-231 cells

To identify Akt2 binding partners I decided to use a Flag-tagged Akt2 overexpression construct. I had previously attempted to isolate Akt2 binding partners by immunoprecipitating MDA-MB-231 cell lysates with a mouse anti-Akt2 antibody or a peptide-blocked mouse anti-Akt2 antibody (negative control). Analysis of the immunoprecipitates on a GelCode Blue stained gel did not yield any bands that were specific for the mouse anti-Akt2 immunoprecipitate (data not shown). Since this may be due to a low abundance of Akt2 protein in complex with different binding partners, I decided to overexpress Akt2 to increase the yield of Akt2-binding partner complexes. Additionally, the Flag tag permits the use of commercially available monoclonal anti-Flag antibodies crosslinked to agarose beads to isolate Akt2 and any bound proteins. An excess of Flag peptide allows for the elution of Akt2-Flag and its binding partners, which is more amenable for processing the samples for mass spectrometry. I Amaxa-nucleofected MDA-MB-231 cells with Akt2-pFlag-CMV™-5a. The following day, I harvested the cells for batch immunoprecipitation with an antibody specific for the Flag peptide. I eluted Akt2-Flag and interacting proteins with either TBS buffer alone as a negative control or Flag peptide at a concentration of 150 ng/uL of TBS. To determine whether Akt2-Flag was expressed, and whether the immunoprecipitation and subsequent Flag elution were successful, 2% of the input lysate, the immunoprecipitation supernatant, the eluted sample, the remaining beads after Flag elution, the TBS-buffer only negative control elution, and the TBS-buffer eluted beads were all run on an SDS-PAGE gel and immunoblotted with an anti-Flag antibody (Figure 12a). The immunoblot shows that Akt2-Flag was expressed (lane 1), and was

immunoprecipitated and eluted efficiently (lanes 3 and 4). Half of the remaining eluates were loaded on a 10% acrylamide gel and silver stained (Figure 12b). There were multiple bands that were present in the Flag eluate lane that did not appear in the TBS buffer eluate lane. Importantly, the most prominent band in the Flag eluate lane corresponded to the molecular weight of Flag-tagged Akt2. The remaining eluates that were not loaded on the silver-stained gel were processed for mass spectrometry. The samples were digested with trypsin and Glu-C, purified through a C18 column and vacuum dried. Mass spectrometry was performed at the UC Berkeley QB3 mass spectrometry facility.

Confirmation of Akt2 binding partners by co-immunoprecipitation and immunoblot

Mass spectrometry of the immunoprecipitated Akt2-Flag yielded a list of 107 proteins. To narrow down the list of potential Akt2 partners in breast cancer cell migration and invasion, I focused on proteins that have roles in processes that regulate migration and invasion. I eliminated proteins that were also present in the negative control immunoprecipitation reaction and proteins that were present in a published list of common false positive proteins in Flag immunoprecipitation mass spectrometry experiments (Chen and Gingras, 2007). I focused on proteins for which antibodies are commercially available (Table 1). To confirm that the proteins identified by mass spectrometry are indeed Akt2-interacting proteins, I performed co-immunoprecipitation of Flag-tagged Akt2 and binding partners as described for the samples processed for mass spectrometry. The immunoprecipitates were boiled and subjected to SDS-PAGE, transferred onto PVDF membrane, and probed with specific antibodies. I did not detect Annexin II, Plectin, Vimentin, or Tropomodulin (data not shown). I was able to confirm the presence of cofilin and Centaurin β2/ACAP2 (Figure 13). Comparison of the levels of cofilin and ACAP2 in the immunoprecipitates versus whole cell lysates showed that a larger amount of ACAP2 is found in complex with Akt2. Therefore, I decided to focus my analysis on ACAP2.

The knockdown of ACAP2 has no effect on cell migration, but leads to increased invasion of MDA-MB-231 cells

ACAP1 and ACAP2 are Arf6 GTPase activating proteins (GAPs) that are activated by interaction with PtdIns(3,4)P2. ACAP2 has been reported to bind PtdIns(3,5)P2 *in vitro* although the significance of this interaction and whether it occurs *in vivo* have not been analyzed (Dowler et al., 2000). Overexpression of ACAP1 or ACAP2 in NIH-3T3 fibroblasts leads to decreased cellular protrusions and formation of dorsal ruffles, which is dependent on intact GAP activity (Jackson et al., 2000). ACAP1 has additional roles in regulating endocytic recycling of transferrin receptors in HeLa cells, however this function is not shared by ACAP2 (Dai et al., 2004). It has also been shown that the migration of HeLa cells requires the recycling of β1 integrin and this process is dependent on the regulation of ACAP1 by Akt (Li et al., 2005).

The phosphorylation of ACAP1 by Akt is required for the interaction of ACAP1 with β1 integrin, and is crucial for the localization of β1 integrin into endosomes.

To determine whether ACAP2 has a role in the migration and/or invasion of breast cancer cells and whether ACAP2 is involved in mediating the role of Akt2 in breast cancer migration and/or invasion, I used siRNA to knockdown the levels of ACAP2 in MDA-MB-231 cells. I compared the effects of control non-specific siRNA with ACAP2 siRNA on the migration and invasion of MDA-MB-231 cells through transwell filters. I found that the knockdown of ACAP2 did not have an effect on the migration of MDA-MB-231 cells, however, the knockdown of ACAP2 led to a small increase in the invasive capacity of cells (Figure 14).

Knockdown of ACAP2 by siRNA in MDA-MB-231 cells does not alter the levels of Arf6-GTP

Arf6 is a member of the Ras superfamily of small GTPases. Like other members of the Ras family of GTPases, Arf6 cycles between the GTP-bound and GDP-bound forms. Arf6 is activated by guanine nucleotide exchange factors (GEFs), which promote exchange of the nucleotide bound to Arf6 to GTP, while the GTPase-activating proteins (GAPs) inactivate Arf6 by promoting the hydrolysis of the GTP into GDP. Arf6 localizes to the plasma membrane where it regulates the activation and endosomal trafficking or proteins involved in actin remodeling, cell migration, and cell invasion (D'Souza-Schorey and Chavrier, 2006). For example, Arf6 promotes the membrane localization and activation of Rac1. Arf6 regulates the localization of Rac1 to the plasma membrane from Arf6 endosomal vesicles, leading to enhanced formation of lamellipodia (Radhakrishna et al., 1999). Additionally, the Arf6-GEF ARNO binds the Rac1-GEF DOCK180-ELMO to promote Rac1 activation (Santy et al., 2005). In epithelial cells, Arf6 promotes the internalization of E-cadherin, which leads to disassembly of adherens junctions and enhances cell migration (Palacios et al., 2001). A recent study found that ligand-stimulated EGFR binds to an Arf6-GEF GEP100, which promotes MDA-MB-231 cell invasion in an Arf6-dependent manner (Morishige et al., 2008). Co-overexpression of Arf6 and GEP100 was sufficient to induce the invasion of the non-invasive MCF7 cell line. Conversely, genetic ablation of GEP100 inhibited the metastatic capacity of the mouse mammary tumor cell line 4T1/luc (Morishige et al., 2008). Akt2 could potentially regulate the activity and/or localization of ACAP2 to promote Arf6-GTP induced breast cancer cell invasion. To determine whether the increase of cell invasion upon the knockdown of ACAP2 was mediated by an increase in the levels of Arf6-GTP, I nucleofected MDA-MB-231 cells with control, Akt2, or ACAP2 siRNA and performed an Arf6-GTP pull down assay using Pierce/Thermo Scientific Active Arf6 Pull-Down and Detection Kit. GST-tagged GGA3-protein binding domain selectively binds Arf6-GTP in protein lysates allowing for the relative comparison of active Arf6 in MDA-MB-231 samples. Since ACAP2 functions as an Arf-GAP, the knockdown of ACAP2 by siRNA would be expected to lead to increased Arf6-GTP levels. However

the knockdown of ACAP2 did not lead to significant changes in Arf6-GTP (Figure 15). This was not due to changes in overall protein levels of Arf6 (Figure 15, lanes 5-7).

Low levels of Rab11 co-immunoprecipitate with Akt2-Flag

ACAP1 regulates the recycling of several transmembrane proteins such as the transferrin receptor and β1 integrin (Dai et al., 2004; Li et al., 2005). This function is not dependent on the Arf6-GAP activity of ACAP1, but depends on the localization of ACAP1 in endosomal recycling vesicles. To determine whether the interaction of ACAP2 and Akt2 mediates the recycling of Akt2 substrates or of Akt2 itself, I analyzed the mass spectrometry data for Rab proteins. Rab proteins belong to the Ras superfamily of small GTPases. There are over 70 Rab and Rab-like proteins that regulate various cellular processes. Rab proteins have critical roles in regulating endocytic and exocytic membrane trafficking, and are commonly used as markers to distinguish specific transport compartments (Schwartz et al., 2007). The list of proteins identified by mass spectrometry from the Akt2-Flag immunoprecipitation included Rab11. Rab11 is localized in the Golgi apparatus and in recycling endosomes where it regulates transport through the compartments of the Golgi, and endocytic recycling, respectively (Schwartz et al., 2007). Recently, it has been found that a Rab11-interacting protein, FIP3, regulates the migration of MDA-MB-231 cells. FIP3 co-interacts with Rab11 and Arf6, and regulates the localization of Arf6 at the plasma membrane (Jing et al., 2009).

The observations described above suggested that the Akt2-ACAP2 interaction might regulate the invasion of MDA-MB-231 cells by affecting Rab11 function. To determine whether Akt2 and Rab11 are associated *in vivo* MDA-MB-231 cells were nucleofected with Akt2-Flag and immunoprecipitated with mouse anti-Flag. The immunoprecipitate was resolved on an SDS-PAGE gel and immunoblotted with an antibody against Rab11. A band corresponding to Rab11 was detected in the Flag immunoprecipitate, and was not detected in the immunoprecipitate generated with nonimmune mouse IgG (Figure 16). The level of Rab11 detected in the anti-Flag immunoprecipitate is much lower than the level of Rab11 detected in the whole cell lysate, indicating that only a minor population of Rab11 exists in complex with Akt2.

An immunofluorescence assay was performed to determine whether the colocalization of Akt2, ACAP2, and Rab11 could be visually detected. Since there were no antibodies available that could be used for the immunofluorescence staining of endogenous ACAP2, MDA-MB-231 cells were nucleofected with pCMV-HA-ACAP2 and a mouse anti-HA antibody was used to detect overexpressed HA-ACAP2. The individual staining patterns of Akt2, HA-ACAP2, and Rab11 were mostly distinct and the majority of staining did not overlap (Figure 17). However, there were a few areas of punctate staining (denoted by arrows) in which Akt2, HA-

ACAP2, and Rab11 appeared to overlap. This is consistent with the small amount of Rab11 protein detected from the Akt2-Flag immunoprecipitate.

ACAP2 does not co-immunoprecipitate with HA-Akt2

Elution with TBS buffer alone was the negative control in the Akt2-Flag immunoprecipitations for the initial mass spectrometry experiments. This reaction controls for proteins that disassociate from the immunoprecipitation pellet regardless of the interaction of Akt2-Flag. Additionally, in subsequent confirmation experiments, it was shown that ACAP2 is precipitated from cells expressing Akt2-Flag by anti-Flag antibody but not by control IgG. This latter experiment controls for any proteins that are non-specifically bound to immunoglobulin but does not control for proteins that are weakly recognized by the anti-Flag antibody. To confirm that Akt2 and ACAP2 interact, I performed additional immunoprecipitation experiments with a different epitope tag. I nucleofected MDA-MB-231 cells with HA-tagged ACAP2 or empty vector pCMV-HA negative control. Incubation with mouse anti-HA and subsequent immunoblot with anti-Akt2 antibody did not yield a band corresponding to Akt2 (Figure 18). Re-probing the immunoblot with a mouse anti-HA antibody confirmed that HA-ACAP2 was expressed and efficiently immunoprecipitated. Since the co-immunoprecipitation of Akt2 and ACAP2 was not detected by use of an alternate peptide tag, a possible explanation is that ACAP2 was recognized by the mouse anti-Flag antibody. To determine whether the Flag antibody cross-reacted with Akt2 or ACAP2 I immunoprecipitated non-nucleofected MDA-MB-231 cell lysates with the mouse anti-Flag antibody and subjected the reactions to SDS-PAGE and immunoblotting with rabbit anti-Akt2 or goat anti-ACAP2 antibodies (Figure 19). The immunoblot of Akt2 shows that purified recombinant Akt2 non-specifically bound to mouse IgG and mouse anti-Flag antibodies, albeit at lower levels than to mouse anti-Akt2. Immunoprecipitation of the MDA-MB-231 lysate shows that endogenous Akt2 from MDA-MB-231 lysate was immunoprecipitated by mouse anti-Akt2 and a small amount was immunoprecipitated nonspecifically by mouse anti-Flag. More importantly, the immunoblot with goat anti-ACAP2 shows that ACAP2 was immunoprecipitated by goat anti-ACAP2 and albeit at low levels by mouse anti-Flag although not by mouse IgG. Furthermore, immunoprecipitation of endogenous Akt2 did not yield an ACAP2 band in the goat anti-ACAP2 immunoblot (right panel, lane 6). Immunoprecipitation of endogenous ACAP2 did not yield an Akt2 band in the rabbit anti-Akt2 immunoblot (left panel, lane 7). These results confirm that Akt2 and ACAP2 do not interact, but rather, ACAP2 cross-reacted with the mouse anti-Flag antibody during the immunoprecipitation experiment for mass spectrometry.

Discussion

The goal of this project was to determine the mechanism by which Akt2 regulates breast cancer cell migration and/or invasion by identifying an Akt2-isoform-specific

binding partner that regulates this process. Although kinase-substrate interactions are generally low affinity interactions, several Akt substrates form stable complexes with Akt that are preserved through immunoprecipitation assays and can be detected by western blots. I used a Flag tag to mediate immunoprecipitation of Akt2, since it is a small tag that will not interfere with the function of Akt2 nor will it block the interaction with binding partners. The Flag tag also allows for elution with excess Flag peptide as opposed to elution buffers with detergent, which must be dialyzed before processing the samples for mass spectrometry. The Flag peptide elution also prevents isolation of proteins that are non-specifically bound to the agarose beads. As shown in figure 12a the immunoprecipitation with the mouse anti-Flag antibody conjugated to agarose and the subsequent elution with Flag peptide were completely successful. Unfortunately, the mouse anti-Flag antibody cross-reacted with proteins that do not contain a Flag tag, including Akt2 and ACAP2. I would have expected to find Akt2 and ACAP2 on the published list of common false-positive proteins from Flag-immunoprecipitation mass spectrometry (Chen and Gingras, 2007). However, the Flag immunoprecipitation from Chen and Gighras was performed with lysates from HEK293 cells, which may contain lower levels of Akt2 and ACAP2, and the method of Flag immunoprecipitation may have differed, resulting in a different set of non-specific binding proteins.

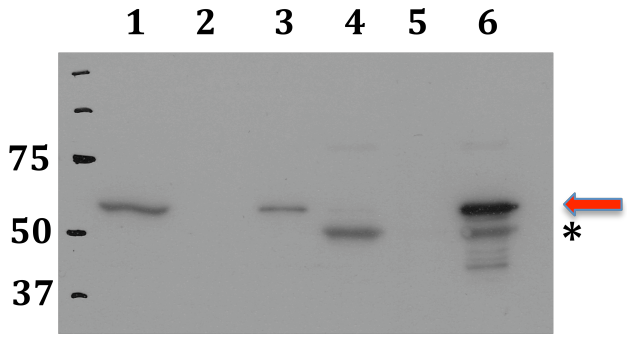
Since the mass spectrometry results contain proteins that bind non-specifically to the Flag antibody or that cross-react with the Flag antibody, the interactions between Akt2 and cofilin or Rab11 will need to be re-confirmed with co-immunoprecipitations performed with HA-tagged Akt2 or with endogenous proteins. Although there seemed to be a small amount of Rab11 that co-localized with Akt2 in the immunofluorescence assay, this does not confirm that the two proteins interact.

Thus far, the only role that has been attributed to ACAP2 is the regulation of Arf6-GTP levels. However, in an Arf6-GTP pull down assay, I did not detect a change in levels of Arf6-GTP upon the knockdown of ACAP2 by siRNA. Since the knockdown of ACAP2 from MDA-MB-231 cells led to increased cell invasion through Matrigel-coated transwell filters, it appears that ACAP2 may have a role in breast cancer cell invasion that does not involve its function as an Arf6-GAP. More studies are needed to identify the mechanism by which ACAP2 regulates MDA-MB-231 cell invasion and whether ACAP2 is a novel tumor suppressor.

The mass spectrometry result, on which the experiments presented in this chapter were based, was an experimental artifact. This remained undiscovered for approximately one year, even though the results from the mass spectrometry were verified with co-immunoprecipitation and immunoblot experiments. Unfortunately, the negative control used for the immunoprecipitation mass spectrometry experiment did not control for the possibility of the Flag antibody binding non-Flag proteins. The mouse IgG control used for the co-immunoprecipitation immunoblot

experiments, which were performed to confirm the findings from the mass spectrometry, did not control for the possibility that the Flag antibody weakly recognizes other proteins, or was not of the correct isotype. This highlights the importance of designing experiments to have the appropriate negative controls. This also shows the importance of verifying results by use of different types of experimental methods.

a)



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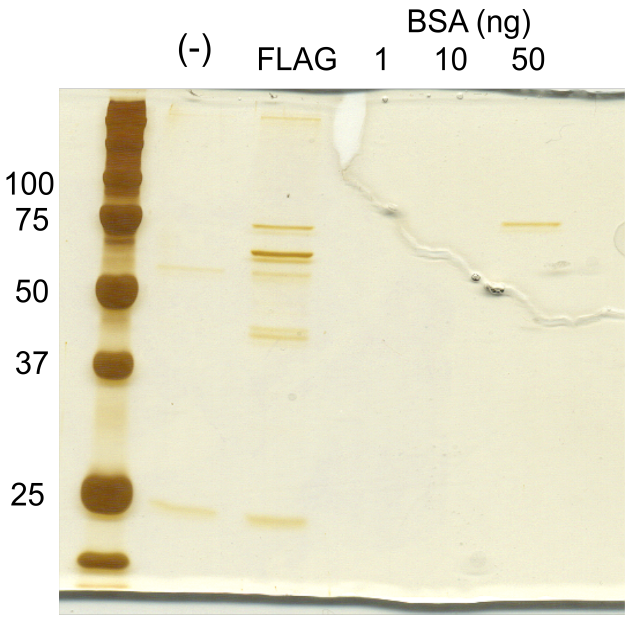


Figure 12: Immunoprecipitation Akt2-Flag immunoblot and silver stain for mass spectrometry. a) Immunoblot analysis to confirm the immunoprecipitation and elution of Akt2-Flag samples for mass spectrometry. The molecular weight ($x10^{-3}$) of each band is noted on the left. Two percent of the following were loaded, lane 1: Input, 2: Supernatant from IP, 3: Flag peptide eluate, 4: mouse anti-Flag beads after elution with Flag peptide, 5: TBS eluate, 6: mouse anti-Flag beads after elution with TBS. An arrow denotes the specific band, (*) denotes mouse IgG band. b) Approximately 50% of the eluates from the immunoprecipitations for mass spectrometry were resolved by SDS-PAGE and detected with silver stain. The molecular weights ($x10^{-3}$) of the protein ladder bands are noted on the left of the gel. (-) is the TBS buffer eluate. FLAG is the Flag peptide eluate. The BSA lanes contain the noted amounts of BSA standards.

Table 1: A selection of proteins identified by mass spectrometry from the immunoprecipitation of Akt2-Flag from MDA-MB-231 cells

Descriptive Name	Unique peptides	% coverage	MW (kDa)	pI
v-akt murine thymoma viral oncogene homolog 2	34	44.5	55.8	6.4
cofilin 1	4	31.9	18.5	8.1
tropomodulin 3	4	17.3	39.6	5.2
vimentin	5	14.4	53.7	5.1
annexin A2 isoform 2	3	11.5	38.6	7.8
plectin 1 isoform 3	17	4.9	513.7	5.8
centaurin, beta 2 (ACAP2)	2	4.9	88.1	6.7

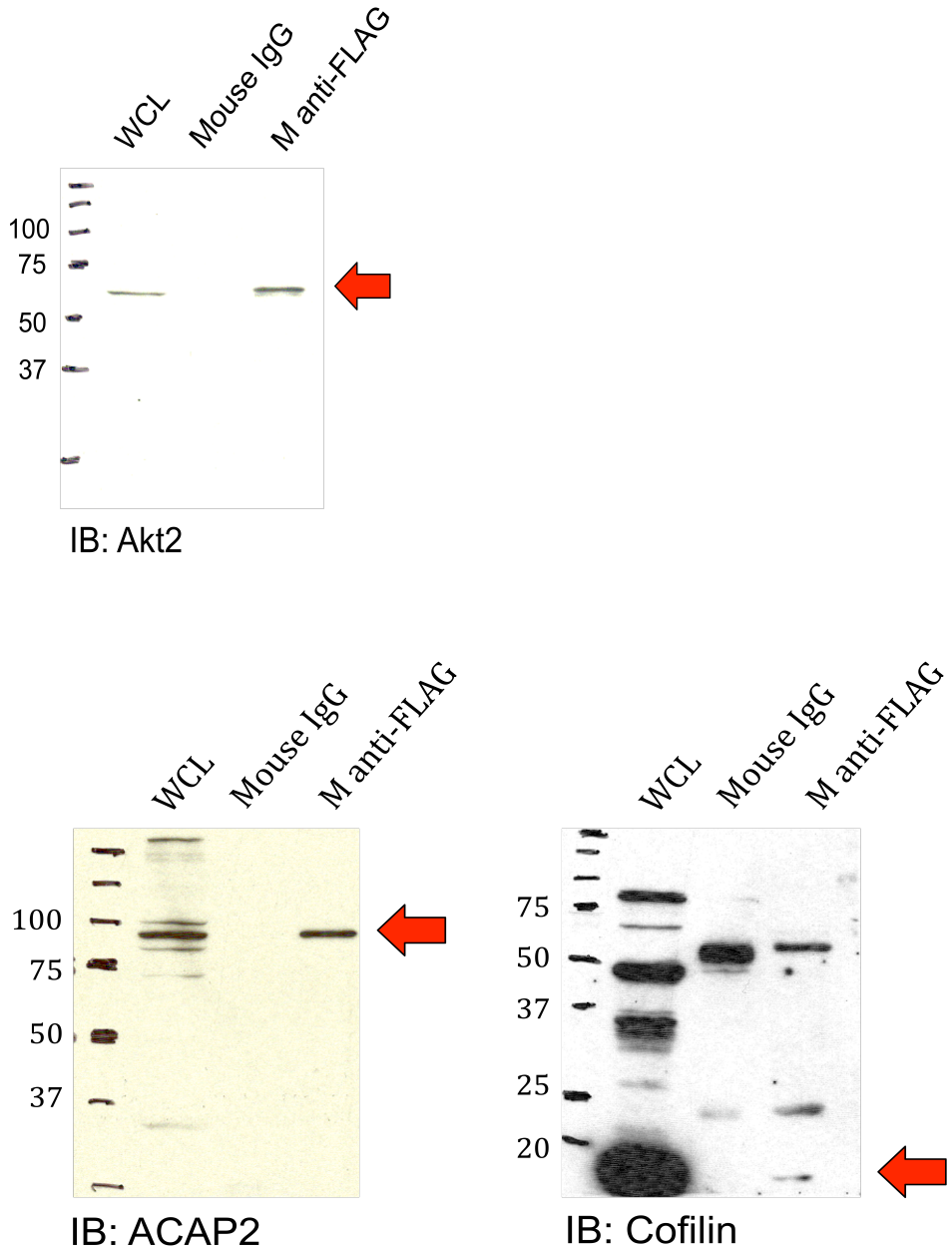


Figure 13: ACAP2 and Cofilin co-immunoprecipitate with Akt2-Flag. MDA-MB-231 cells were nucleofected with Akt2-Flag and immunoprecipitated with mouse IgG (negative control) or mouse anti-Flag. Immunoprecipitated samples were resolved by SDS-PAGE and immunoblotted with antibodies against Akt2 (top panel), ACAP2 (bottom left panel), or Cofilin (bottom right panel). The arrows point to the specific bands on each blot. WCL: whole cell lysate used for immunoprecipitation.

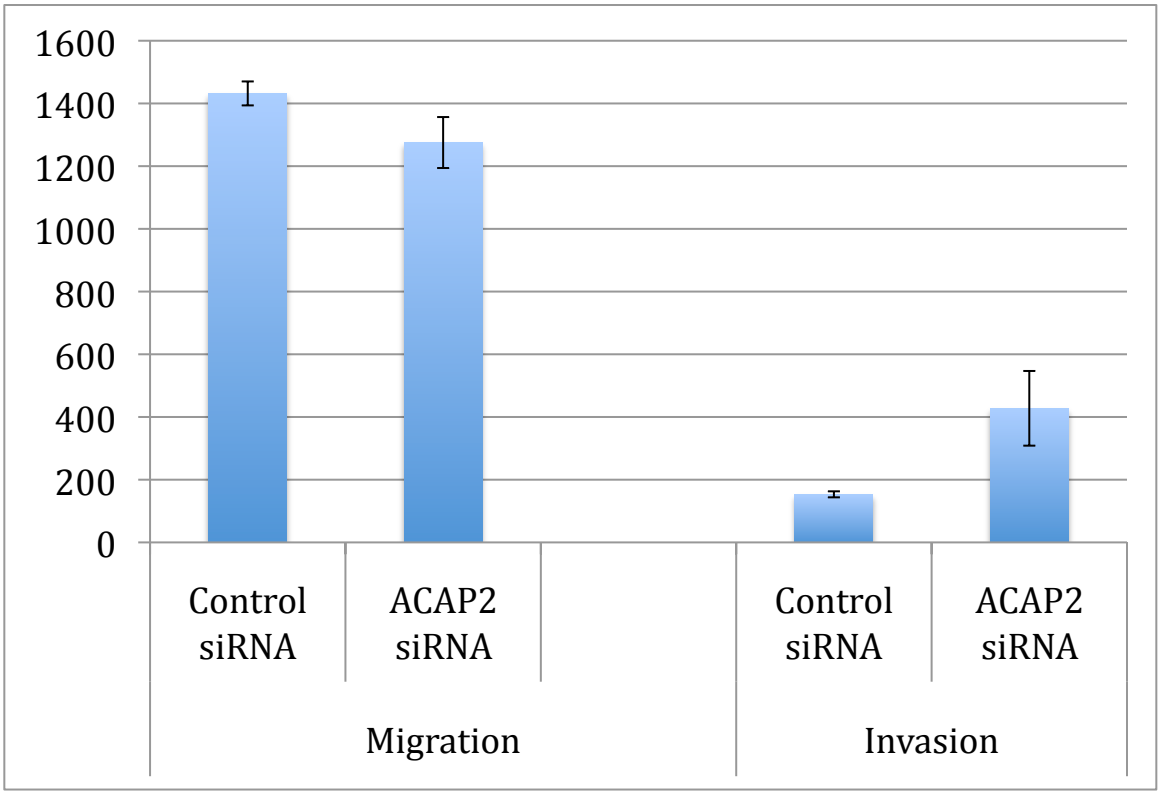
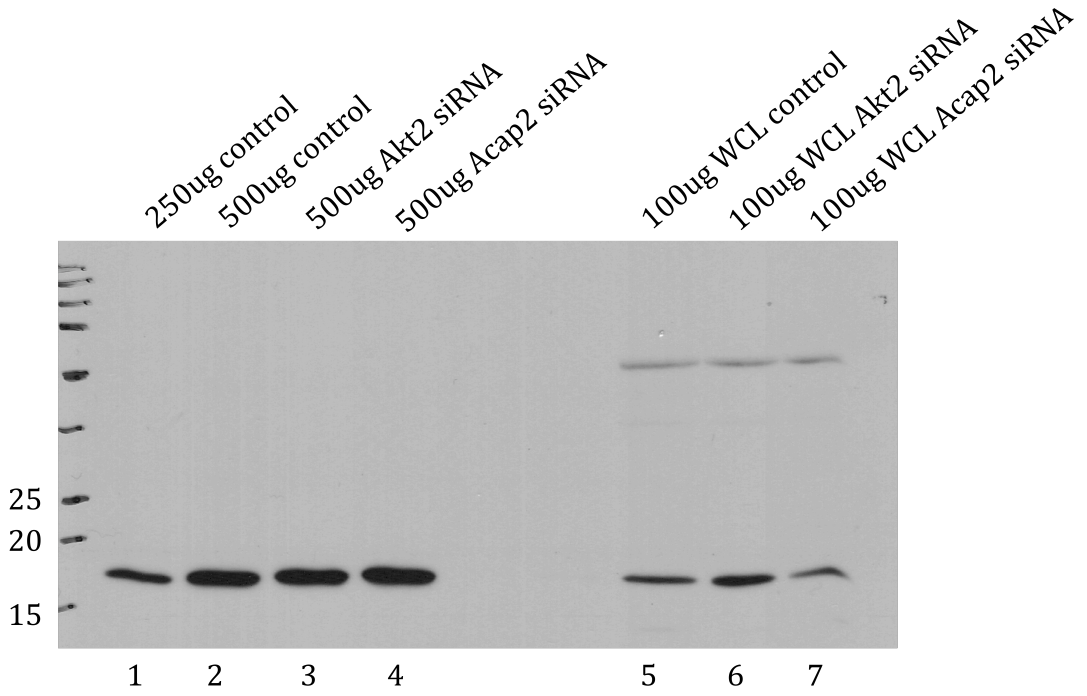


Figure 14: RNAi knockdown of ACAP2 affects the invasion, but not the migration, of MDA-MB-231 cells. MDA-MB-231 cells were nucleofected with control or ACAP2-specific siRNA. The cells were seeded on triplicate migration and invasion filters and incubated overnight. The average numbers of cells that migrated or invaded through triplicate filters (± S.D.) is shown.



IB: Mouse anti-Arf6

Figure 15: ACAP2 does not regulate the levels of Arf6-GTP in MDA-MB-231 breast cancer cells. MDA-MB-231 cells were nucleofected with control, Akt2, or ACAP2 siRNA. Cell lysates were subjected to Arf6-GTP pull-down assays with GST-GGA3-PBD as bait and analyzed by immunoblot against Arf6. The protein molecular weight markers ($x10^{-3}$) are noted on the left. The pull-down performed in Lane 1 used 250 ug of lysate protein to confirm that changes in the levels of Arf6-GTP can be detected. Lanes 2-4 were loaded with pull-down samples from lysates of cells nucleofected with control, Akt2, or ACAP2 siRNA. Lanes 5-7 were loaded with equal amounts of whole cell lysates to confirm that equal amounts of Arf6 were present in the lysates used for the pull-down experiment.

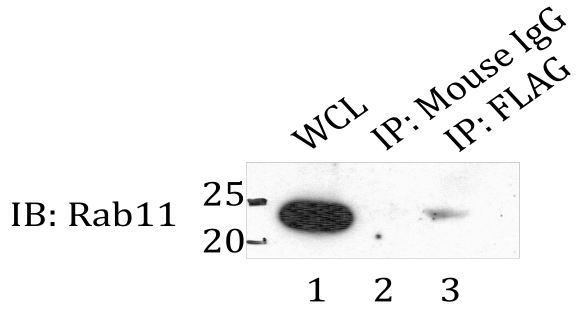


Figure 16: Rab11 co-immunoprecipitates with Akt2-Flag. MDA-MB-231 cells were nucleofected with Akt2-pFlag-CMV5a. Cell lysates were harvested and immunoprecipitated with mouse IgG (negative control, lane 2) or mouse anti-Flag (lane 3). Immunoprecipitates were subjected to SDS-PAGE and immunoblotted with rabbit anti-Rab11 antibody. Lane 1: an aliquot of whole cell lysate (WCL) corresponding to the input used for the immunoprecipitations.

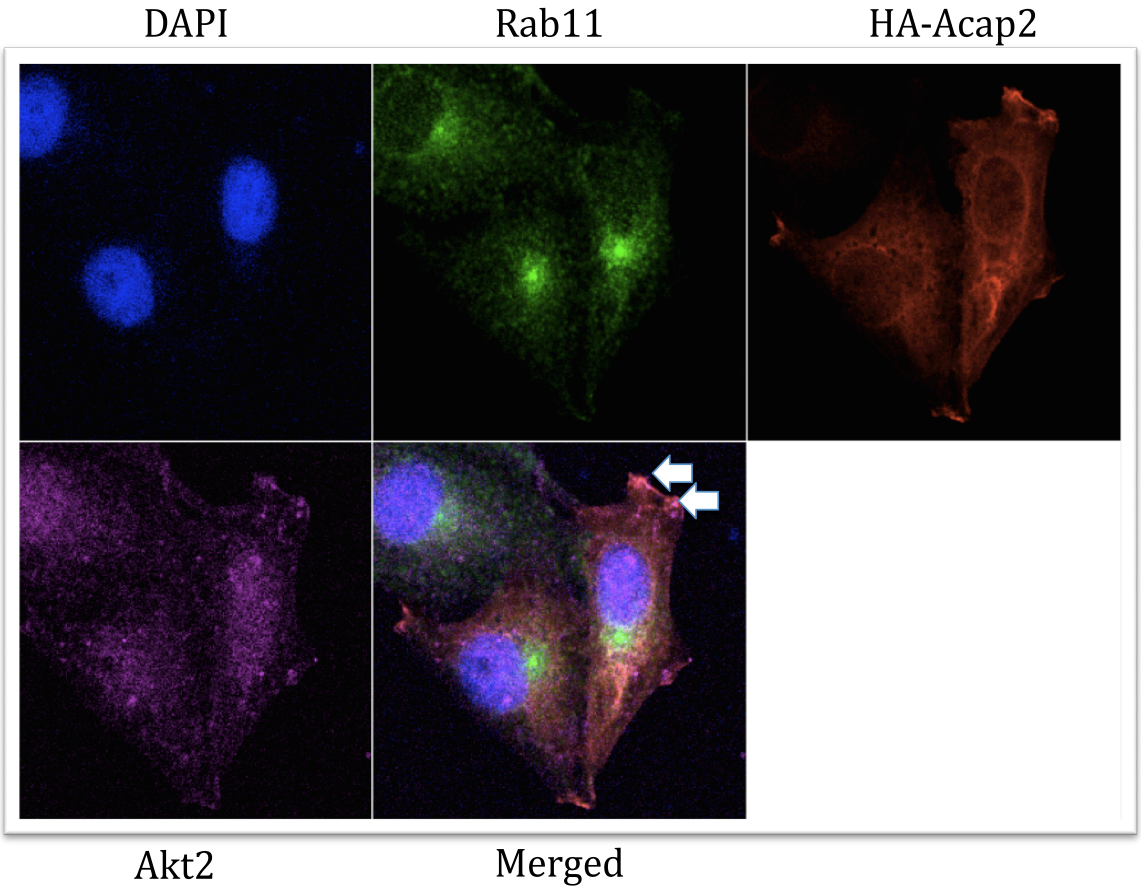


Figure 17: Immunofluorescence staining of endogenous Rab11, HA-ACAP2, and endogenous Akt2. MDA-MB-231 cells were nucleofected with pCMV-HA-ACAP2. Immunofluorescence staining was performed with rabbit anti-Rab11, mouse anti-HA, and goat anti-Akt2. DNA was visualized with DAPI. Arrows in the "Merged" panel point to areas of colocalization of Rab11, HA-ACAP2, and Akt2.

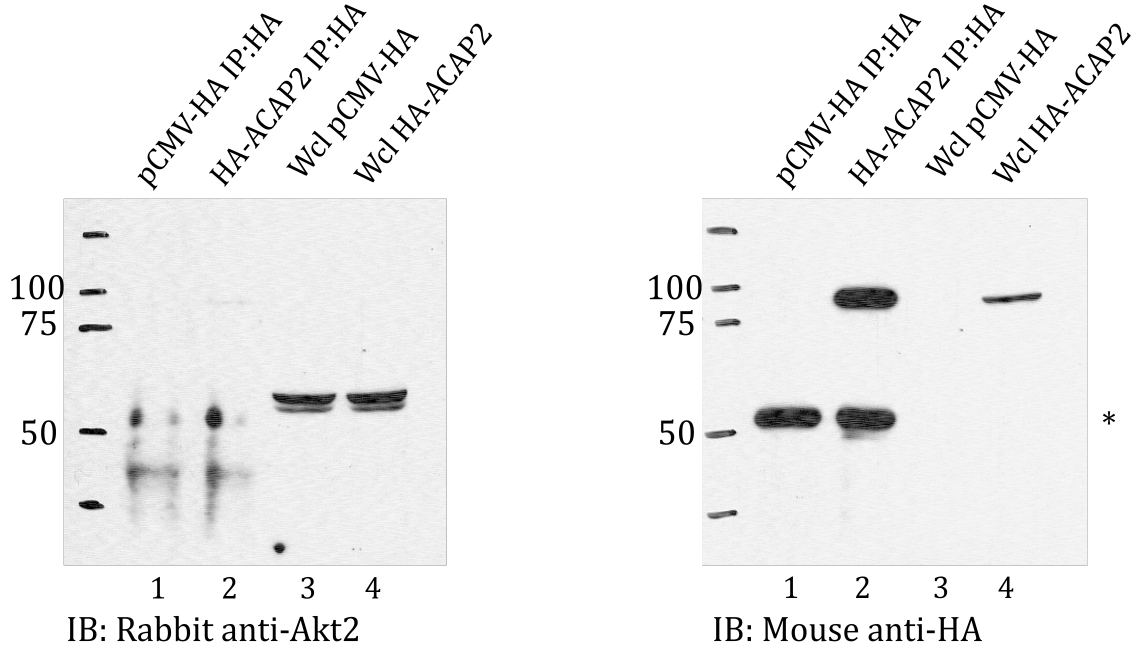


Figure 18: Akt2 does not co-immunoprecipitate with HA-tagged ACAP2. MDA-MB-231 cells were nucleofected with pCMV-HA empty vector or pCMV-HA-ACAP2. The cell lysates were immunoprecipitated with mouse anti-HA, resolved by SDS-PAGE, and immunoblotted with rabbit anti-Akt2 (left panel) to detect co-immunoprecipitated Akt2, or mouse anti-HA (right panel) to confirm the immunoprecipitation of HA-ACAP2. Lanes 1 and 2 (IP:HA) contain the immunoprecipitates. Lanes 3 and 4 (Wcl) contain the whole cell lysate input used for the immunoprecipitation. (*) denotes the bands corresponding to mouse IgG.

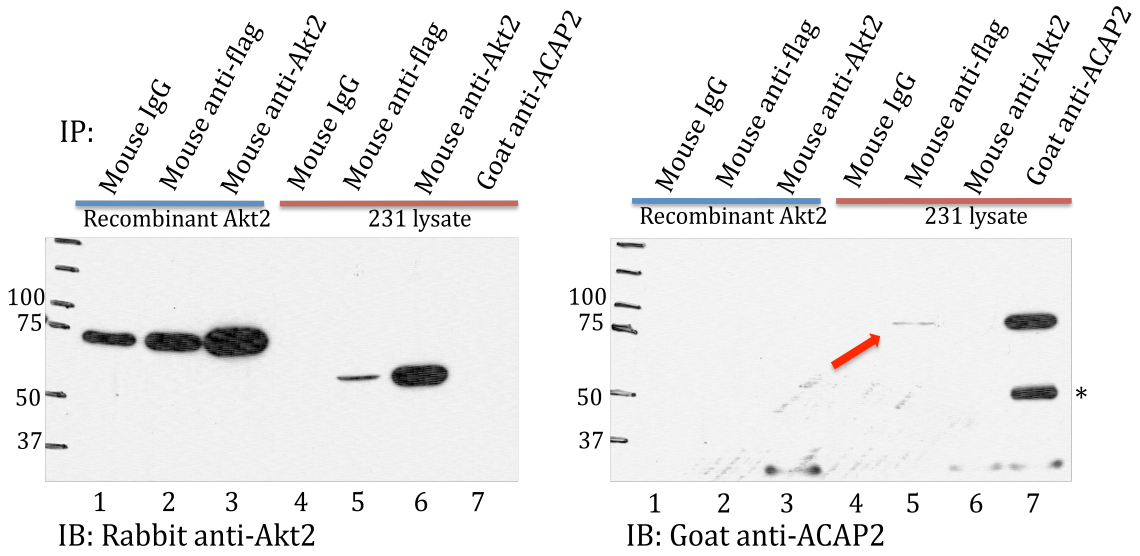


Figure 19: ACAP2 and Akt2 are non-specifically immunoprecipitated by mouse anti-Flag. Purified recombinant Akt2 (approximately 74 kDa) and non-nucleofected MDA-MB-231 cell lysates were immunoprecipitated with mouse IgG, mouse anti-Flag, mouse anti-Akt2, or goat anti-ACAP2 antibodies. The immunoprecipitates were resolved by SDS-PAGE and probed with rabbit anti-Akt2 (left panel) or goat anti-ACAP2 (right panel). The arrow points to the ACAP2 band in the mouse-anti-FLAG immunoprecipitate lane. (*) denotes the band corresponding to goat IgG.

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