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TITLE: Role of P13 Kinase Signaling Pathways in Polarity Determination of Human Mammary Epithelial Cells Grown in Three-Dimensional Extracellular Matrix

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Introduction

Metastasis is one of the major characteristics of malignancy, a process by which cancer cells acquire aberrant ability to undergo a complex process in order to become invasive and ultimately metastasize to distant organs. Tumor cells use mechanisms to migrate, which are similar to those that occur in normal, nonmalignant cells during physiological processes such as embryonic morphogenesis, wound healing, and immune-cell trafficking (Friedl and Brocker 2000; Friedl and Wolf 2003). Unlike physiological processes of cell invasion, however, the migration of tumor cells seems to be activated by a dominance of promigratory events in the absence of countering stop signals (Giannelli, Falk-Marzillier et al. 1997; Alper, Bergmann-Leitner et al. 2001). This imbalance of signals allows cancer cells to become continuously migratory and invasive, leading to tumor expansion across tissue boundaries, followed by metastasis. This ability is achieved through the alterations of cellular signaling and gene expression, which provide cancer cells with selective advantages to disrupt and migrate through surrounding tissues and to survive in the alien growth conditions (Zigrino, Loffek et al. 2005). Although abnormal cell proliferation and survival are crucial for cancer development, additional changes of genes or gene products that promote cell motility and invasion are usually required for metastasis.

The evolutionarily conserved serine/threonine kinase Akt is one of the most frequently activated protein kinases in human cancer (Scheid and Woodgett 2001; Vivanco and Sawyers 2002). Hyperactivation of Akt is associated with resistance to apoptosis, increased cell growth, cell proliferation, and cellular energy metabolism by phosphorylating its substrates such as TSC1/TSC2, $P27^{Kip1}$, FOXO, and mTOR (Blume-Jensen and Hunter 2001; Vivanco and Sawyers 2002). The phosphorylation of these substrates by Akt affects their activity, stability, or cellular localization, which furthermore promotes protein synthesis, inhibits cell cycle, and protects cells from apoptosis (Vivanco and Sawyers 2002). Although it is well established that Akt is a crucial component of PI3 kinase signaling pathways to regulate cell proliferation and survival and to dramatically promote tumor growth rate in animals, activation of Akt is correlated with an alteration in cell migration and invasion in several mammalian systems. However, the mechanisms remain largely unknown. In my previous report, I showed that polarity and proliferation are controlled independently by Rac1 and Akt downstream of PI3 kinase. The observations were summarized and published on the Journal of Cell Biology of February, 2004. While I was characterizing other malignant phenotypes induced by expressing constitutively active Akt in human mammary epithelial cell line HMT-3522, I found that up-modulation of Akt activity had profound effects on cell motility, invasion, and actin cytoskeleton in addition to the greatly increased cell proliferation and survival in both in vitro and in vivo assays. The downstream pathways were further characterized, which will provide novel insights into the molecular mechanisms of its role in tumuorigenesis when Akt signaling is dysregulated.

Body

To determine whether deregulated Akt signaling could affect cell motility and invasion in addition to its classical role in the regulation of cell proliferation and survival, we made use of a number of in vitro and in vivo assays to fully asses the possibility. A myristolated Akt or empty vector was expressed in human mammary epithelial cancer cell line, T4-2. In order to demonstrate its effect on cell survival, transduced cells were subjected to anchorage-independent assays. Cells were grown in soft agar or methyl cellulose. As expected, cells overexpressing Myr-Akt survived much better than control cells as Myr-Akt significantly increased colony number and size (Figure 1A and B). When cells were cultured in physiologically relevant 3D lrBM assay, Myr-Akt caused a larger colony size as we showed previously. The increased cell survival and growth are due to up-regulated cell proliferation and suppressed apoptosis assessed by KI-67 and TUNEL immunostaining. As I have shown in my previous report, the tumor size in the group with the Myr-Akt construct was considerably larger and growth rate was much faster than the group without ($p<0.001$, Figure 1C). All these results suggest that Akt signaling pathway is the driving force for cell survival and tumor growth.

To determine if modulation of Akt activity could affect cell invasiveness, T4-2 cells expressing Myr-Akt and vector control were subjected to invasion assay. Surprisingly, however, we found that active Akt remarkably reduced invasion of T4-2 cells through Matrigel-coated Boyden chambers (Figure 1D, n=8). Since altered cell motility is the driving force of invasiveness, wound-healing and cell migration assays were employed to assess this possibility. We found Myr-Akt significantly inhibited cell motility in both assays (Figure 1E). To see the compromised motility in real time, we monitored the tracks of cell random migration for 24 hours. We found that Myr-Akt cells migrated much less distance than control cells (Figure 1F), an observation further verifies the results of migration assays. These results suggest that hyperactivated Akt signaling negatively and profoundly modulates cell migration and invasion.

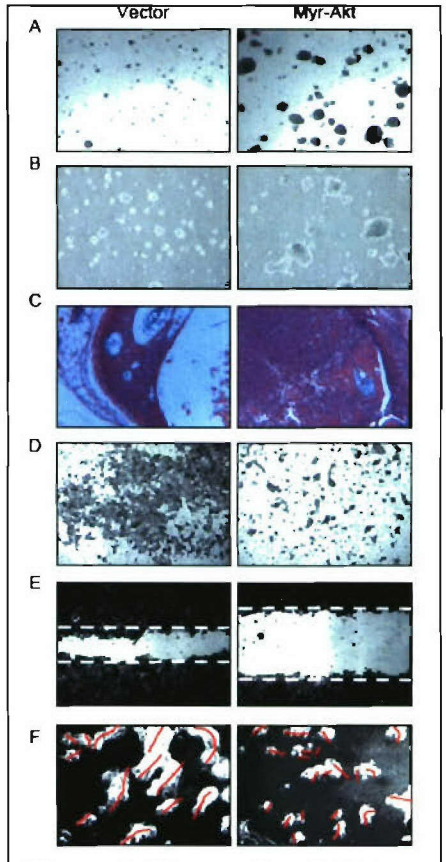

Figure 1. Up-regulated Akt activity enhances cell survival and tumor formation but inhibits cell invasiveness and motility.

To confirm if the catalytic activity of Akt is required for the reduced cell invasion and motility, dominant negative Akt construct mutated on Serine 473 and threonine 308 was transduced into T4-2 cell. Dominant negative Akt did not have significant effect on both cell invasion and motility, even though its overexpression remarkably reduced the phosphorylation of its downstream targets and caused

smaller colonies in 3D lr BM assay, suggesting the kinase activity of Akt is necessary for cell motility and invasion inhibition.

Cell extensions are a prerequisite for the onset and maintenance of cell motility in normal and cancer cells (Friedl and Wolf 2003). We found that cell adhesion to culture plates and spreading was significantly delayed in early stage when active Akt was overexpressed (Figure 2A). These cells formed very little extension throughout the culture and eventually developed into very tight colonies with smooth edges (Figure 2C), suggesting that cell spreading was fundamentally changed.

Cell movement is a dynamic, orchestrated, cyclical process in which moving cells undergo drastic changes in cell shape, and involves the creation of actin-rich protrusions, formation and disassembly of adhesive complexes, and establishment of migration polarity (Friedl and Wolf 2003; Ridley, Schwartz et al. 2003). Malfunction occurring in any of those steps can greatly affect cell motility. As actin polymerization process is one of the major driving forces of cellular movement (Pollard and Borisy 2003), we examined actin patterns and its dynamics. In contrast to vector control cells, cells with active Akt mutant had more cortical actin staining but much reduced stress fibers at 48 hours or early time points after plating (Figure 2B and D). When scratch wounds were made on confluent monolayer to re-initiate cell movement and the healing process, there was much less actin-rich projection toward the closing gap.

Since focal adhesion formation is required for attachment and spreading, and focal adhesions serve as points of contact for cell spreading and of traction over which the body of the cell moves (Horwitz and Parsons 1999), we examined focal adhesion formation by immunostaining for one of the components, paxillin. We discovered that overactivation of Akt significantly decreased the formation of focal adhesion without any effect on endogenous paxillin expression (Figure 2E). These results suggest that overactive Akt signaling interferes with the process of focal adhesion formation, a defect that might be relevant to the less invasive and mobile phenotypes.

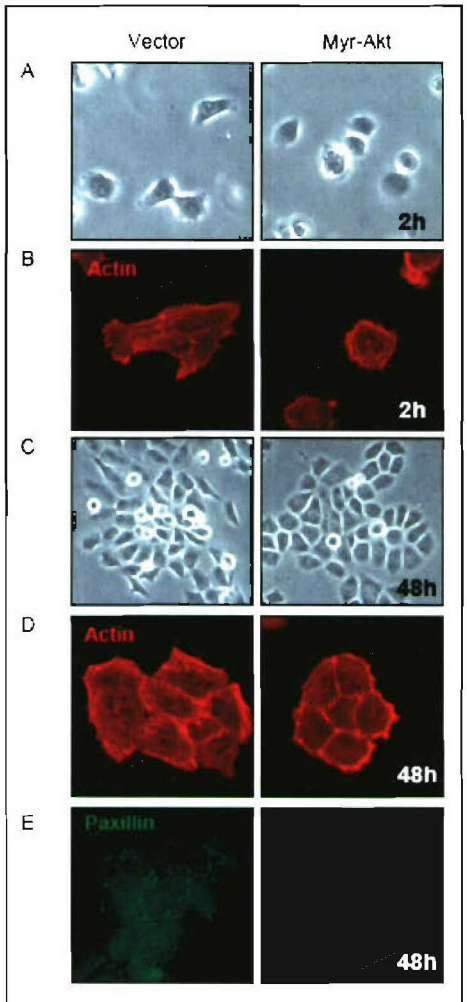

Figure 2. Cell spreading, actin cytoskeleton, and focal adhesions are drastically altered by Akt activation.

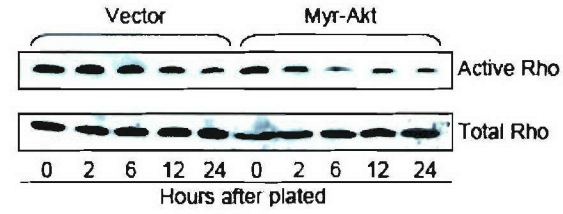

Figure 3. Increased Akt activity down-modulates Rho activity.

Since the assembly and organization of the actin cytoskeleton are controlled by Rho GTPases, and the formation of stress fibers and focal adhesions is one of the hallmarks of Rho activation (Hall 1998), the activity of Rho was measured by using Rhotekin-GST fusion protein pulldown assay. Consistent with loss of stress fiber phenotype, the active Rho levels were significantly lower in Myr-Akt-expressing cells, starting from 2 hours after cells were plated on tissue culture plastic (Figure 3). To determine if the change of actin cytoskeleton correlates with Rho activity, cells plated on coverslips were stained with phalloidin at the same time points as pulldown assays. At 2 hours, vector control, but not Myr-Akt-expressing cells, spread well and already formed stress fibers. There was no significant difference in Rac1 and Cdc42 activities in parallel lysates, even though Akt was found to phosphorylate and inhibit Rac1 activity (Kwon, Kwon et al. 2000). These results suggested that overactivated Akt specifically inhibited Rho activity and that the low Rho activity might play a role in active Akt-mediated defects of motility and invasiveness. However, dominantly negative Akt did not affect cell spreading, focal adhesion formation, and the actin cytoskeleton, which is very similar to vector control T4-2 cells. These results suggest that increased catalytic activity of Akt signaling profoundly affects Rho activity and actin cytoskeleton, and therefore, cell motility.

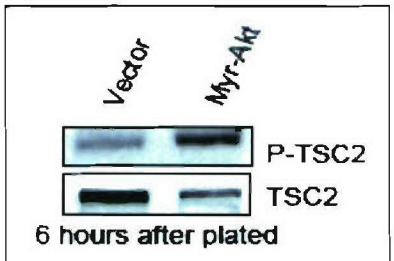

Figure 4. TSC2 was highly phosphorylated and its total level down-modulated by Myr-Akt.

TSC1 and TSC2 are tumor suppressors and TSC2 is the substrate of Akt. Their stability and functions are influenced by binding to protein 14-3-3 as the consequence of Akt-mediated phosphorylation (Krymskaya 2003). Since TSC2 or TSC1 has been shown to activate Rho activity and induce active Rho phenotypes (Lamb, Roy et al. 2000; Astrinidis, Cash et al. 2002), we postulate that Akt overactivation may lead to dysregulation of TSC2 or TSC1, which in turn could reduce Rho activation.

To test the possibility, we examined the levels and modification of TSC1 and TSC2 in T4-2 cells expressing Myr-Akt or vector control and found TSC2 was highly phosphorylated at threonine 1462 (Figure 4). We found also that its total expression level significantly decreased in Myr-Akt expressing cells but not in DN-Akt cells (Figure 4). TSC2 levels are correlated with the levels of Myr-Akt expression negatively and Rho activity as a function of time (Figure 3). Surprisingly, however, there was no measurable change in TSC1 expression.

These results suggest that active Akt signaling significantly phosphorylates TSC2, causes down-modulation of TSC2 expression, implying that low level of TSC2 might cause insufficient Rho activation.

To determine if down-modulated TSC2 might play a role in active Akt-mediated defects of cell motility and invasion, wild type TSC2 was stably expressed in vector control and Myr-Akt cells (Figure 5). Overexpression of TSC2 significantly decreased phosphorylation of p70S6K (Figure 5) and decreased colony size when cells were grown in 3D lr BM, consistent with the results that TSC2 is upstream of mTor and p70 S6K and negatively regulates cell proliferation and growth (Brazil, Park et al. 2002; Inoki, Li et al. 2002; Manning, Tee et al. 2002; McManus and Alessi 2002; Hay and Sonenberg 2004). Re-supplementing of Myr-Akt cells with TSC2 significantly rescued their motility and invasiveness (Figure 5). These results indicate that TSC2, even though acts as a tumor suppressor that inhibit cell growth and proliferation, is required for cell motility and invasion.

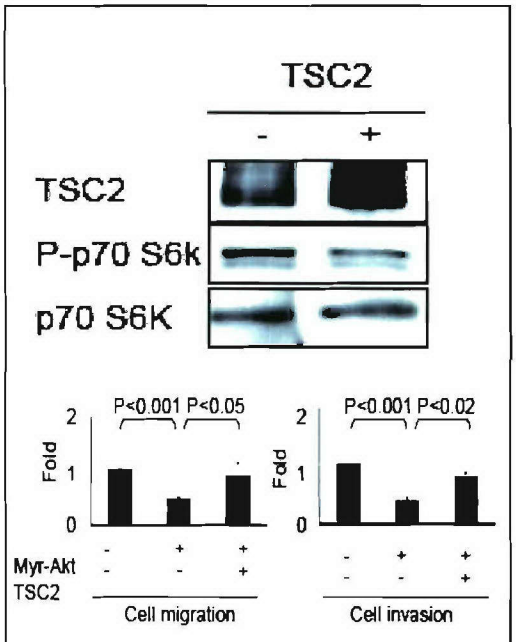

Figure 5. TSC2 acts as downstream effector of Akt to regulate cell invasiveness and motility.

Key research accomplishments

1. Determine the roles of active and dominant negative Akt in the regulation of cell motility and invasion.
2. Determine the effects of active and dominant negative Akt on cell spreading, actin cytoskeleton, and focal adhesion formation.
3. Determine the alteration of Rho activity when active Akt is overexpressed.
4. Determine that modulation of Akt signaling affects the stability of TSC2.
5. Determine that TSC2 acts as the downstream target of Akt to control cell motility and invasion.

Reportable outcomes

The observations will be summarized and submitted for publication after several ongoing experiments are finished. 1) motility and invasion assays when overexpressing active Rho construct in active-Akt-expressing cells or inhibiting Rho activity in vector control cells; 2) measuring Rho activity when TSC2 is overexpressed in active Akt-expressing cells.

Conclusion

Our results demonstrate that active Akt signaling pathway has profound influence over cell invasiveness. The inhibited invasiveness might be due to the effects of active Akt on cell motility and other cell behaviors through TSC2/Rho signaling pathways. The elucidation of novel targets may shed some light on the molecular mechanisms how these processes are regulated by Akt.

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