AN INVESTIGATION OF THE ROLE OF PAK6 IN TUMORIGENESIS

by

JoAnn Roberts

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This thesis was prepared under the direction of the candidate's thesis advisor, Dr. Michael Lu, Department of Biomedical Science, and has been approved by the members of her supervisory committee. It was submitted to the faculty of the Charles E. Schmidt College of Medicine and was accepted in partial fulfillment of the requirements for the degree of Master of Science.

SUPERVISORY COMMITTEE:

Michael Lu, Ph.D.
Thesis Advisor

Vijaya Iragavarapu, Ph.D.

Keith Brew, Ph.D.

Keith Brew, Ph.D.
Chair, Department of Biomedical Science

David J. Bjorkman, M.D., M.S.P.H. ACTING DEAN
Dean, Charles E. Schmidt College of Medicine

Barry T. Rosson, Ph.D.
Dean, Graduate College

July 16, 2012
Date

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ABSTRACT

Author: JoAnn Roberts

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The function and role of PAK6, a serine/threonine kinase, in cancer progression has not yet been clearly identified. Several studies reveal that PAK6 may participate in key changes contributing to cancer progression such as cell survival, cell motility, and invasiveness. Based on the membrane localization of PAK6 in prostate and breast cancer cells, we speculated that PAK6 plays a role in cancer progression cells by localizing on the membrane and modifying proteins linked to motility and proliferation. We isolated the raft domain of breast cancer cells expressing either wild type (WT), constitutively active (SN), or kinase dead PAK6 (KM) and found that PAK6 is a membrane associated kinase which translocates from the plasma membrane to the cytosol when activated. The downstream effects of PAK6 are unknown; however, results from cell proliferation assays suggest a growth regulatory mechanism.

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INTRODUCTION

Prostate cancer is a hormone-dependent disease. The growth, differentiation and secretory functions of the prostate are dependent on androgen signaling through the androgen receptor (AR); where age, diet, family history, and ethnicity are also risk factors in prostate cancer development[1]. This cancer is the second leading cause of cancer-related deaths in men, with anticipated new cases in 2012 of approximately 241,740 resulting in 28,170 deaths[2]. There is currently no effective treatment for late-stage prostate cancer. However, androgen-ablation therapy remains the principal treatment for metastasized prostate cancer. The probability of relapse after hormonal therapy is extremely high, resulting in death for most patients with metastasized cancer[3]. Therefore, much research is focused on identifying potential therapeutic targets for cancer. Among these potential targets are a group of serine/threonine kinases, p21-activated kinases (PAKs), which phosphorylate substrates affecting numerous cellular processes such as cell survival, cell motility and proliferation[4]. This investigation focuses specifically on PAK6 (p21-activated kinase 6), a 75 kDa protein which has been shown to be increased in primary and metastatic prostate tumors as well as those that have relapsed after androgen deprivation therapy[4-6]. The normal and possible tumorigenic function of PAK6 is poorly understood, although recent studies have suggested

positive correlations between PAK6 expression and cell survival and invasiveness[7,8]. However, the role of PAK6 in cancer progression has not yet been clearly identified and will be the long term goal of this investigation.

BACKGROUND

The formation of a tumor is a complex multi-step process driven by a sequence of genetic mutations and epigenetic alterations which progressively transforms normal human cells into malignant cells. Hanahan and Weinberg have defined several phases in the physiology of the cell which together constitute a logical framework for the progression of cancer. These phases include: self-sufficiency in growth signals, insensitivity to growth-inhibitory (antigrowth) signals, replicative immortality, evasion of apoptosis, sustained angiogenesis, tissue invasion and metastasis, deregulation of cellular energetics and evasion of immune destruction[9,10]. Each of these capabilities gained by cancer cells plays a critical role in the autonomous nature and severity of cancer. However, the mechanisms by which cancer cells acquire these abilities often differ. For example, there are three common molecular strategies by which cancer cells may gain self-sufficiency in growth signals: some cancer cells may alter extracellular growth signals, others may alter the proteins involved in the transmission of the signal, and still other cells may alter the downstream proteins that translate the signals into action[9]. The progressive acquisition of these abilities, regardless of the mechanism, dictates the successful breach of normal cellular anticancer defense systems thereby supporting malignancy.

Most cancer deaths are primarily due to failure to manage the metastatic disease. Metastasis involves a succession of cell-biological changes which facilitate local invasion in which the cells invade nearby normal tissue, followed by intravasation where the cells

move through the walls of lymphatic or vascular vessels; and extravasation which ultimately leads to colonization at another site[11]. The initial changes driving these processes include, but are not limited to altered cytoskeletal dynamics, genome instability, and deregulation of cellular energetics[9] contributing to the ability of the cell to become motile. Well-studied orchestrators in these processes essential for cell motility are members of the Rho-family of GTPases (Cdc42, Rac, and Rho). These GTPases cycle between an active GTP-bound state and an inactive GDP-bound state regulated by numerous cellular proteins classified as GEFs (guanosine nucleotide exchange factors) and GAPs (GTPase activating proteins). In their active form, Rho GTPases initiate downstream signaling responses by binding to effector molecules[12]. Of their effector molecules, p21-activated kinases (PAKs) are among the best characterized binding to the active forms of Cdc42 and Rac, with specific links to migration potential, anchorage independent growth and metastasis[4,6]. This literature review section will focus on the PAK family and their contributions to cancer progression with a specific focus on PAK6.

The PAK family

PAKs are a family of serine/threonine kinases that are highly conserved. There are six identified members of this family that are separated into groups I (1-3) and II (4-6) based upon sequence and structural homology[13] (Figure 1). All members of the PAK family have a conserved C-terminal kinase domain and an N-terminal p21-binding domain (PBD) that contains a 20 amino acid Cdc42/Rac interactive binding motif (CRIB)[14]. Group I PAKs contain an auto-inhibitory domain (AID) that overlaps with the PBD at the N-terminus. Group II PAKs however, lack this AID.

The degree of conservation of the kinase and PBD regions differs between the two groups of PAKs. The N-terminal PBD domain is at least 60 percent identical among group II PAKs. However, when comparing group I and group II, there is less than 40 percent identity. The kinase domains of the two groups of PAKs are also diverged when compared to each other, where group I PAKs have a 95 percent identity and group II PAKs have only about 54 percent identity[13]. The differences between the two groups suggest that the regulatory mechanisms for groups I and II PAKs may differ.

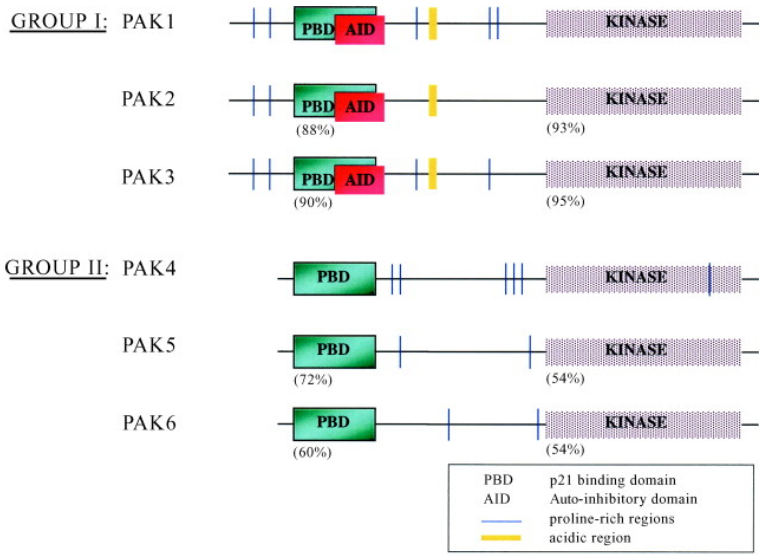

Figure 1. Structural Comparison of the group I and group II PAKs[13]

PAK regulation

Group I PAKs activation is regulated by the binding of active Cdc42 or Rac to the PBD. At resting stage, they form an auto-inhibited dimer, where the AID of one protein interacts with the kinase domain of the other. The binding of GTP-loaded Cdc42 or Rac to the CRIB domain causes the release of the auto-inhibition resulting in an auto-

phosphorylated intermediate dimer thereby enhancing the kinase activity[5,13,14]. Group II PAKs however, lacking an AID, do not depend on active Cdc42 or Rac binding for activation. Rather, the binding of these GTPases are important for group II PAKs localization[15-17]. For example, binding of active Cdc42 to Group II PAKs can result in translocation of PAK4 to the Golgi apparatus and PAK5 to the mitochondrion[15,16]. In addition, recent data suggests that active Cdc42 or Rac binding may be important in facilitating PAK6 membrane localization, but it is not the only contributing factor (M.L. unpublished data).

Group II PAKs activation requires the auto-phosphorylation of a specific serine in the activation loop located in the kinase domain (Ser-474 in PAK4; Ser-602 in PAK5; Ser-560 in PAK6)[13,15]. In addition to the auto-phosphorylation on serine-560 in the activation loop of PAK6, studies also indicate PAK6 activity to be stimulated by p38 MAP kinase and MKK6 by phosphorylation on serine-165 and tyrosine-566 respectively[18]. To date, the kinase activity regulation of group II PAKs remains to be determined.

PAKs and cancer

Group I PAKs have been involved in the progression of cancers such as glioblastoma, breast, liver, kidney, colon, bladder, and ovarian with implications in cell migration and invasion. For example, PAK1 forms a complex with LIM-kinase, which is involved in reorganization of the actin cytoskeleton by inactivating the ADF/cofilin family of proteins. PAK2 can directly phosphorylate myosin II regulatory light chain resulting in the activation of myosin II (an actin interacting motor protein that can drive

cell contractility)[19]. Group I PAKs can also mediate cytoskeletal changes through Rho GTPases by binding specifically to Cdc42/Rac[6].

Group II PAKs have also had implications in cancer progression. For example, PAK4 expression is increased in lung, breast, prostate, pancreas, and colon cancer cells. The expression of activated PAK4 in cancer has been shown to result in decreased stress fibers and focal adhesions and an increase in the formation of actin clusters. In addition, binding of PAK4 to Cdc42 induces filopodia formation and actin polymerization[15]. These actions result in the "rounding" of the cell, indicative of a particular cell migration mode (amoeboid) specific for individual cell movement, allowing cells to migrate through the extracellular matrix[20]. Activated Pak4 has also been shown to protect cells from apoptosis by delaying caspase activation or through the phosphorylation of BAD[21]. Therefore, PAK4 contributes to cancer progression through the promotion of cell-survival, motility and anchorage independent growth in various cancer cell lines[17].

PAK5 has also been recently implicated in increased cancer progression and metastatic potential in colorectal carcinoma cells[22]. Other studies reveal that PAK5 phosphorylates BAD similar to PAK4, thus contributing to apoptosis-resistance[23]. However, the role and mechanisms by which PAK5 may influence cell survival, motility, and proliferation in various cancers has not yet been fully investigated.

PAK6 is localized on the membrane (M.L. unpublished data) and its increased expression has been detected in both prostate and breast cancer cell lines[5]. It was first identified as an AR and ER-interacting protein involved in mediating the inhibition of AR-mediated signaling[21]. However, there have also been several recent implications of PAK6 involvement in increased cell survival, radiation-induced apoptotic resistance, and

invasiveness. Wen et al. silenced PAK6 using siRNA in prostate cancer cell lines and found that PAK6 silencing resulted in reduced proliferation *in vitro* and *in vivo*, decreased invasiveness *in vitro* and cell cycle arrest at the G2/M phase. These findings suggest the promotion of cell proliferation, invasive ability, and cell cycle progression as possible mechanisms by which PAK6 may contribute to tumorigenesis[8]. Another study, by Zhang et al., investigated the effect of PAK6 shRNA inhibition on the radiosensitivity of prostate cancer cells (PCa). Their findings showed increased levels of PAK6 in irradiated PCa cells versus the control PCa cells, suggesting a role of PAK6 in the survival of the cell and resistance to radiation-induced apoptosis. In order to further investigate PAK's role in prostate cancer radioresistance, they analyzed the effect of PAK6 inhibition and irradiation on the phosphorylation of BAD, a pro-apoptotic protein which when phosphorylated can initiate events resulting in the suppression of cell death. Their findings showed irradiated cells expressing PAK6 had a slight increase in phosphorylated-BAD, however, irradiated shRNA PAK6 cells showed decreased levels of phosphorylated-BAD; suggesting that PAK6 may protect PCa cells from radiation-induced apoptosis[7].

PAK6 has also been linked to increased prostate cancer motility as revealed in a Transwell Haptotatic Migration Assay conducted previously using LNCaP cells (M.L. unpublished data). In addition, cell morphological changes such as extended lamellipodia, actin filament reorganization and clustering at the tips of lamellipodia were observed in response to PAK6 activation (M.L. unpublished data). These findings suggest that PAK6 may mediate the changes necessary for cell movement; however, the mechanisms by which PAK6 may promote the migratory capabilities of the cell are

unknown. Shepelev and Korobko recently found that PAK6 is a binding partner for an atypical member of the Rho GTPase family, Chp/RhoV[24]. As previously mentioned another member of this family, Cdc42 binds to PAK6 via the CRIB domain. Similarly, GTP-bound Chp interacts with PAK6 at the CRIB domain and also has no effect on its kinase activity. The function of Chp is poorly understood however current research has implicated its involvement in lamellipodia and focal adhesion formation, as well as regulation of the actin cytoskeleton[24]. Therefore, the interaction of Chp and PAK6 may provide insight into biological mechanisms by which PAK6 may promote cell motility.

Further exploration of the effect of PAK6 expression on the ability of the cell to acquire functional capabilities promoting cancer revealed increased expression levels of genes associated with epithelial-mesenchymal transition (EMT), cell motility and proliferation. These results retrieved from a microarray analysis conducted in MCF7 breast cancer cells ectopically expressing either wild-type PAK6, PAK6 constitutive active mutant (S531N) or kinase dead mutant (K436M), provide insight into possible mechanisms by which PAK6 may be involved in tumorigenesis (Figure 2).

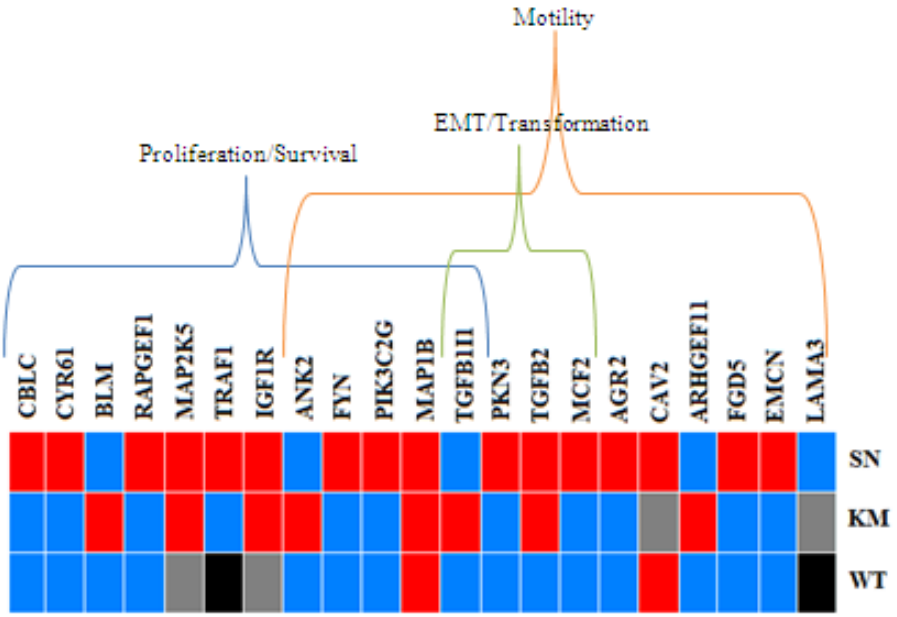

Figure 2. Heat Map of MCF7 Overexpression Model Microarray.

Gray indicates no data was collected. Black indicates no change. Blue indicates less expression, red indicates increased expression as compared to the parental cell.

Abbreviations: CBLC, Cbl proto-oncogene E3-ubiquitin protein ligase C; CYR61, cysteine-rich angiogenic inducer 1; BLM, Bloom Syndrome, RecQ helicase-like; RAPGEF1, Rap guanine nucleotide exchange factor 1; MAP2K5, mitogen-activated protein kinase kinase 5; TRAF1, TNF receptor-associated factor 1; IGF1R, insulin-like growth factor receptor 1; ANK2, ankyrin 2; FYN, Fyn oncogene related to Src, FGR, YES; PIK3C2G, phosphoinositide -3-kinase class 2 gamma polypeptide; MAP1B, Microtubule associated protein 1B; TGFβ1I1, transforming growth factor beta 1 induced transcript 1; PKN3, protein kinase N3; TGFβ2, transforming growth factor beta 2; MCF2, MCF.2 cell line derived transforming sequence; AGR2, Anterior Gradient Homolog 2; CAV2, caveolin 2; ARHGEF11, Rho guanine nucleotide exchange factor 11;

FGD5, FYVE RhoGEF and PH domain containing 5; EMCN, endomucin; LAMA3, laminin alpha 3[25].

The genes displayed in Figure 2 are grouped into three major groups: cell proliferation and survival, cell motility, and EMT/transformation. In each group, the expression of cancer-promoting genes is increased when PAK6 is constitutively active (SN mutant). For example, genes promoting proliferation such as CBLC, a regulator of epidermal growth factor receptor[25]; CYR61, a promoter of cell proliferation and adhesion[25]; FYN, a Src-related kinase overexpressed in human prostate cancer, where it is involved in a number of different signaling pathways, interacting with regulators of cell proliferation, morphology and motility[26,27]; and PIK3C2G, a member of the PI3K family of kinases involved in proliferation and cell survival[25], are increased in PAK6SN and decreased in wild-type (WT) and kinase dead (KM) cell lines.

Genes involved in the epithelial-mesenchymal transition (EMT) are also increased with PAK6 activation (SN). For example, TGFβ2 is a well-established cytokine associated with promoting EMT through contributions to cell motility and adhesion by increasing transcription factors such as Snail and Slug[28]. The microarray reveals that TGFβ2 is also upregulated in kinase dead (KM) PAK6 as compared to the parental cell. Numerical evaluations of the gene increase however, reveal that TGFβ2 is increased 6 fold more in SN than in KM (data not shown). The increased expression of TGFβ2 may therefore not be solely dependent on PAK6. However, constitutively active PAK6 (SN) does enhance its expression. Another example of a protein involved in EMT is MCF2, an oncogenic protein which exerts control over Rho GTPases promoting their conversion

from the GDP-bound state to the GTP-bound state[25]. Rho GTPases, although established in driving changes involved in cell motility, have also had implications in the disruption of cell-cell junctions and loss of polarity attributed to the change of cells from epithelial to mesenchymal[12].

Figure 2 also reveals the promotion of genes important for driving cell motility when PAK6 is constiutively active (SN), such as FGD5, which plays a role in the regulation of cell shape and the formation of filopodia[25]; AGR, a proto-oncogene believed to play a role in cell migration and differentiation[25]; and CAV2 (caveolin-2), a structural component of caveolae lipid rafts associated with metastasis, tumor growth and the aggressiveness of prostate cancer through facilitation of intracellular signaling[29]. Therefore, the activation of PAK6 is either directly or indirectly involved in the upregulation of genes important in proliferation, EMT, and motility in cancer progression.

There are a few genes displayed, however, that are decreased in SN and increased in KM and/or WT. For example, transforming growth factor beta 1 induced transcript 1 (TGFB1I1) is expressed higher in KM and less in SN and WT. This protein is involved in regulation of the Wnt and TGFβ signaling pathways as well as processes of migration and differentiation[25]. Another example is ARHGEF11, which promotes the activation of RhoA, a protein important in various biological changes important for cell motility such as the assembly of focal adhesions[25]. The low expression levels of these genes, although contrary to what would be expected, does not negate the possible function of PAK6 in promoting cell motility and EMT, but rather suggests that it may function through an alternative mechanism.

The increased expression of genes involved in cell motility and EMT displayed in the microarray are supported by current literature that implicates the expression of PAK6 to be important for the migratory and invasive capabilities of the cancer cell.

Therefore, both the positive correlation between proliferative/EMT-related gene expression and PAK6 activation as well as studies previously discussed, provide evidence that PAK6 may promote cancer progression by increasing the ability of the cell to proliferate, migrate, and promote cell survival. However, little is known about the biological mechanisms and specific role of PAK6 in tumorigenesis. Based on the possible functions of PAK6 discussed previously, we speculate that PAK6 localizes in the membrane lipid raft domains.

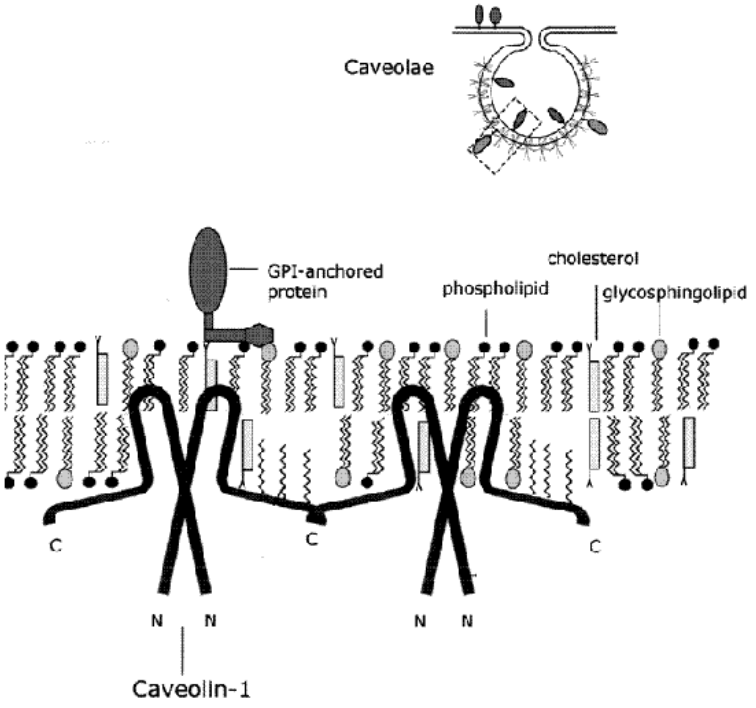

Figure 3. Caveolae Lipid Rafts Composition[30]

Lipid rafts are membrane microdomains which function as organizing centers for signal transduction thereby influencing membrane protein, trafficking, endocytosis, and membrane fluidity[31]. Caveolae originate from lipid rafts and are thus nearly identical in composition, containing GPI-anchored proteins, phospholipids, cholesterol, glycosphingolipids and caveolin-1[30] (Figure 3). However, there are a few key differences that give caveolae unique properties different from lipid rafts. First, the side chains of the phospholipids and glycosphingolipids are enriched in saturated fatty acids as compared to non-raft regions. These fatty acids allow for close packing of all components of the caveolae lipid raft making the lipid raft more ordered than the rest of the plasma membrane, while still allowing for lateral movement. Second, the lipid raft has a higher lipid to protein ratio than non-raft regions or bulk membranes, giving it a lower density in comparison to the rest of the plasma membrane[32,33]. Third, the presence of caveolin-1 is unique to caveolae but absent from lipid rafts.

Caveolin-1 is a 22-24 kDa protein essential for the formation of the invaginated structure unique to caveolae, which form as a result of the polymerization of caveolin-1. This protein is located on the inner leaflet of the plasma membrane with both N and C termini on the cytosolic side. Caveolin-1 contains several palmitoylation sites that allows for close packing of the protein by binding to the fatty acid side chains of the phospho- and glycosphingolipids. Caveolin-2 is another member of the family of caveolins and is often co-expressed with caveolin-1 forming heterooligomers. However, the expression of caveolin-2 alone will not form caveolae[20,34]. Therefore, caveolin-1 is used as a marker indicating caveolae lipid rafts.

The differential properties of caveolae, as compared to the rest of the plasma membrane (i.e. high cholesterol and sphingolipid content; lower density), allows them to be isolated using non-ionic detergents and a density gradient[34]. The membrane raft domain of breast cancer cells expressing wild type PAK6 (WT), constitutive active PAK6 (SN), and kinase dead PAK6 (KM) as well as prostate cancer cells expressing endogenous PAK6 were isolated in order to determine if the localization of PAK6 was in the raft domain where it may modify proteins involved in cancer progression.

MATERIALS AND METHODS

Materials and Reagents

Taqman gene expression assay and probes for PPIA (4333763F), Caveolin-1(HS00971716_m1), Caveolin-2 (HS00184597_m1) were purchased from Applied Biosystems (Foster City, CA). Anti-PAK6 polyclonal antibody (34B9) was custom generated by Covance. Anti-Caveolin polyclonal antibody was purchased from BD Biosciences. DTSSP, (3,3´-dithiobis[sulfosuccinimidylpropionate]), was purchased from ThermoScientific (Rockford, IL).

Cell Culture

MCF7 breast cancer cells were grown in Dulbecco's modified Eagle's medium (DMEM) and supplemented with 10% fetal bovine serum and antibiotics. LAPC4 prostate cancer cells were grown in Iscoves modified Dulbecco's medium (IMDM) and supplemented with 10% fetal bovine serum and antibiotics. Both cell lines were stably transfected with WT, S531N, and K436M PAK6 mutants, which were cloned into an N-terminal HA-tag containing pCDNA3.0 vector (Invitrogen, CA). PC3 prostate cancer cells were grown in RMPI1640 and supplemented with 10% fetal bovine serum and antibiotics.

RNA extraction, cDNA synthesis, and Real-Time PCR

Total RNA of MCF7 cells stably expressing PAK6 WT and mutants were extracted using Qiagen RNeasy Kit spin protocol for isolation from animal cells (Valencia, CA). The RNA integrity number (RIN) was determined using Agilent and was then subsequently reverse transcribed using a High Capacity DNA Reverse transcriptase kit (Applied Biosystems, Foster City, CA). Real-Time PCR reactions were performed using Taqman gene expression assay kits specific for caveolin-1 and caveolin-2 (Applied Biosystems, Foster City, CA) with pre-developed Taqman assay probes. Probes were tagged with the reporter fluorophore FAM (emission maximum at 515nm) and quencher TAMRA (emission maximum at 579 nm) with ROX as the passive reference dye. Thermal cycling conditions for all reactions were the same, consisting of 1 cycle at 95˚C for 10 minutes, then 40 cycles at 95˚C for 15 sec, and 60˚C for 1 min. All measurements were performed in triplicate for each sample and normalized to internal control gene Cyclophilin (PPIA) and comparative analysis normalized to the parental cell.

Cell Proliferation

MCF7 and LAPC4 PAK6-tranfected cells were plated at the same initial concentration of 1.13×10^4 cells and supplemented with one milliliter of complete media. The cells were not fed or any growth stimulatory agent added during the course of the experiment. Cell quantity was determined every 24 hours over a period of 5 days using an Invitrogen Cell Counter (Invitrogen, CA). Measurements were performed in triplicate and averaged. Doubling times were calculated based on the equation $h \times \ln(2) / \ln(c_2/c_1)$,

where h is the number of hours, c_2 is the initial concentration and c_1 is the final concentration.

Membrane Isolation

Cells were grown in complete media to 90 percent confluency in four 100 mm plates. The media was aspirated and the cells washed twice with cold 1X Phosphate Buffered Saline (PBS) solution. The PBS was removed and the cells scraped, using a rubber policeman, into 1 mL of 2.25mM DTSSP cross-linking solution (plates were kept on ice). This solution was pipetted into a 1.5 mL eppendorf tube and incubated in ice for 10 minutes. The cells were then pelleted by centrifugation for 10 minutes at 4˚C, 11,200 g. The supernatant was removed and the cells were frozen at -80˚C overnight.

The following day, cells were thawed on ice, the pellet broken up and washed with cold 1M Tris-buffered saline. The cells were subsequently centrifuged for 10 min at 11,200 g at 4˚C. The supernatant was removed and 800 µL of 0.1% Triton X-100 plus protease (AEBSF, pepstatin, PMSF), phosphatase (NaF, Na_3VO_4, β-glycerolphosphate) inhibitors, and benzonase (2 units/mL), was added to the cell pellet. The pellet was pipetted, vortexed for 10 seconds, and homogenized by forcing cells through a 23 gauge needle ten times. Lysed cells were then centrifuged in solution for 10 minutes at 1120 g at 4˚C. The postnuclear supernatant was collected (500µL) and mixed with 1000µL of OptiPrep Separation medium (60% iodixanol) in a new 1.5 mL eppendorf tube. In an empty rotor tube, an iodixanol step gradient of 5, 30, and 40% (the lysed cells solution) was layered, totaling 3 mL. The gradient was centrifuged in a Beckman Coulter ultracentrifuge for 5 hours at 132,000 g (35,000 rpm) at 4˚C. Five equal fractions of 600

µL volume were collected from the rotor tube, collecting the visible raft domain first (fraction 2). Treated cells were stimulated for 60 minutes with Forskolin at a final concentration of 5nM and then the isolation protocol was conducted as previously described.

Western Blot

Fractions from the membrane isolation were solubilized in 150 µL of 5X Sample Buffer Proteins. Each sample (40 µL/lane) was loaded and separated by SDS-page using either a 10 or 12 percent Bis-Tris gel. Post-electrophoresis, proteins were electroblotted onto a nitrocellulose membrane (0.22 uM). The blots were blocked by 5% non-fat dry milk, 0.01% Tween-20 in Tris-buffered saline [50 mM Tris Base, pH 7.6; 150 mM NaCl] for 1 hour. Immunoblotting was performed with designated antibodies for either PAK6 (34B9) or Caveolin at 4˚C overnight. Secondary antibodies used were either anti-rabbit HRP visualized using an enhanced chemiluminescence detection system (ECL, Pierce, Supersignal, Rockford, IL), or anti-rabbit infrared dye antibody visualized using Licor Odyssey imaging system (Licor, Lincoln, NE), following the manufacturer protocol.

Statistical Analysis

All experiments were repeated three times with significance being determined by a student's paired t-test/ two-tail t-test. Data is represented as mean +/- SE. A probability level of $P < 0.05$ was considered significant. Statistical output was generated using statistical software, SPSS 20.

RESULTS

As previously indicated in the microarray, constitutively active (SN) PAK6 results in the upregulation of caveolin-2. In order to confirm the upregulation of caveolin-2 seen in the microarray and co-expression of caveolin-1, real-time PCR was performed using Taqman gene expression assays as described in **Materials and Methods** in the PAK6-transfected MCF7 breast cancer cell model; wildtype PAK6, PAK6 mutant SN (constitutive active kinase) and PAK6 mutant KM (kinase dead). Figure 4 displays the results of the qPCR which indicates that both caveolin-1 and -2 mRNA expressions are significantly increased approximately 3-fold in PAK6 constitutive active mutant (SN) as compared to normal. Caveolin expression is decreased in WT and comparable in KM cell lines as compared to normal. The increased expression in PAK6SN is consistent with the results seen in the microarray. However, the decrease in caveolin-2 gene expression in cells expressing PAK6WT contradicts the microarray data which shows an increase of caveolin-2 in this same cell type. These differences were determined to be significant for both caveolin-1 and -2 at $p < 0.05$ as compared to normal.

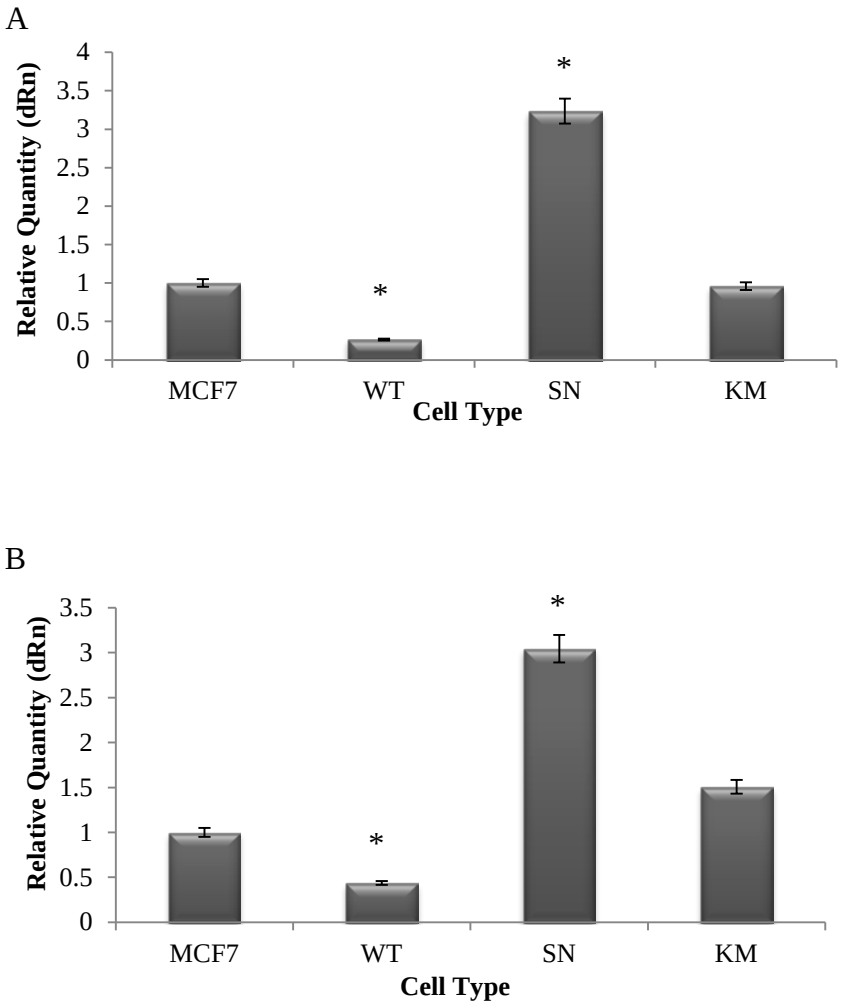

Figure 4. Taqman qPCR Comparative Analysis of Caveolin mRNA expression in PAK6 transfected MCF7 cells. (A) Caveolin-1 mRNA Expression. (B) Caveolin-2 mRNA expression. *$p < 0.05$

PAK6 localization on the membrane and its suggested functions in cancer progression discussed previously, suggest that PAK6 may localize in raft domains to facilitate changes necessary for tumorigenesis. To establish if active PAK6 localized in the caveolae raft domain, membranes were isolated from MCF7 PAK6-transfected cells (WT, SN, and KM) as described in **Materials and Methods**. The cells were cross-linked

using a membrane-impermeable cross-linker (DTSSP) that reacts with the primary amines of proteins in order to stabilize the raft domain, therefore preventing loss of associated proteins. A recent study conducted also indicates that overnight freezing of the isolated cells prior to lysis, promotes the affinity of weak raft-associated proteins to the raft fraction of the membrane isolation[35]. Therefore, using the method described in the diagram in Figure 5, a qualitative assessment of the presence of PAK6 in the raft domain was conducted.

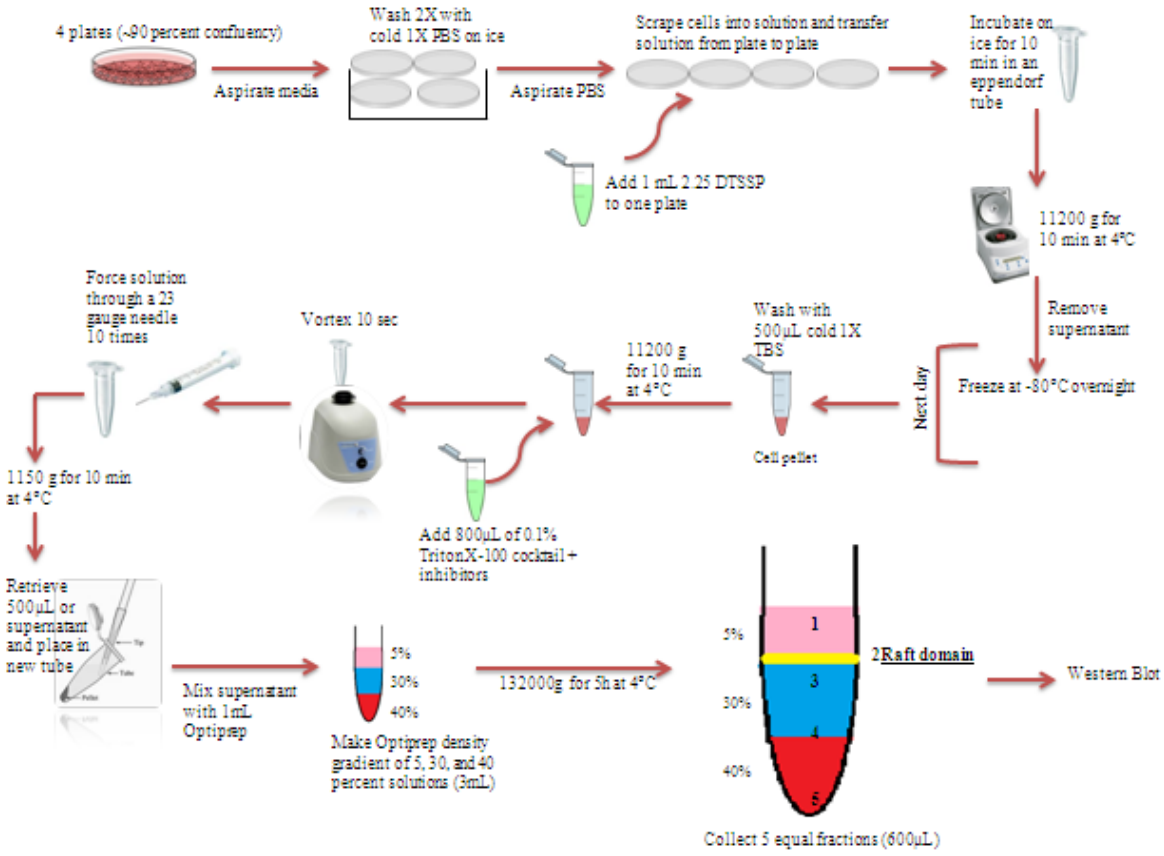

Figure 5. Diagram of Membrane Isolation Protocol.

The results of the western blot analyses of the five fractions collected for each PAK6-expressing cell type (WT, SN and KM) are displayed in Figure 6. Caveolin is used as raft domain marker to confirm the raft domain fraction (Fraction 2). Figure 6A shows PAK6 is colocalized with caveolin in the raft domain in wild-type PAK6-expressing MCF7 cells. Similarly, the isolation results of the PAK6KM mutant (kinase dead) reveal the presence of PAK6 is also predominantly in the raft domain (Figure 6C). However, when PAK6 kinase is constitutively active (SN), there is an increased level of PAK6 in soluble fractions (Figure 6B). This suggests that PAK6 relocalizes from the raft domain to the cytosolic fraction upon activation.

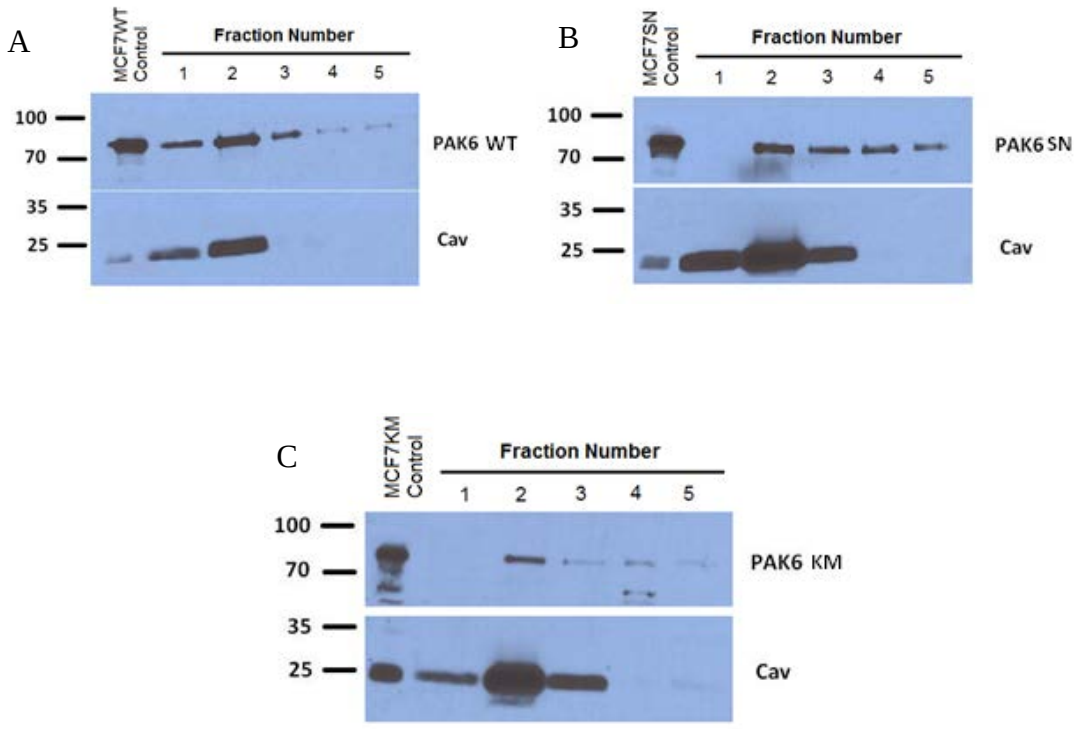

Figure 6. Raft Domain Membrane Isolation of MCF7 cells expressing transfected PAK6. (A) Wild-type PAK6 is mostly present in the raft domain. (B) Constitutively

active PAK6 (SN) is present in both the raft domain and non-membrane fractions. (C) PAK6KM is present majorly in the raft domain.

To confirm that the activation of PAK6 kinase results in activation-dependent translocalization from the raft domain, Forskolin [5mM], a PKA agonist, which has been previously shown to increase the phosphorylation of PAK6 at serine-560 (data not shown), was used to activate wild-type PAK6 transfected into MCF7 cells. Figure 7 displays western blot results for membrane isolations of both forskolin treated and untreated cells. Consistent with previous results, PAK6 in WT-expressing MCF7 cells is present in the insoluble raft domain fraction (Figure 7A). Post-forskolin stimulation, PAK6 presence is decreased in the raft domain and translocates into the soluble fractions of the membrane isolation (Figure 7B). These results confirm that PAK6 localizes to caveolae and translocates into the cytosol when activated.

The membrane isolation (forskolin treated versus untreated) was repeated in prostate cancer PC3 cells expressing endogenous PAK6 (Figure 8). However, the results show that PAK6 is present outside of the raft domain in both the control and forskolin treated PC3 cells. The PC3 cell results do not negate the findings in the MCF7 cells, but rather suggests that endogenous PAK6 may already be in an activated state and thus additional activation through forskolin stimulation had less effect. However, further study is needed to confirm this. Immunocytochemistry results also support the data shown in Figures 6-8, where there is a cytoplasmic staining pattern after forskolin stimulation in wild-type-PAK6-transfected MCF7 breast cancer cells and in LAPC4 prostate cancer cells expressing endogenous PAK6 (data not shown). Immunocytochemistry results also

support the PC3 results where PAK6 cytoplasmic staining was predominant in both the control and forskolin-treated cells (data not shown).

Comparing the membrane isolations in figures 6-8, two bands can be seen for caveolin-1 in figures 7 and 8, whereas there is only one in figure 6. These two bands corresponds to the two isoforms of caveolin-1, alpha (24kDa) and beta (21kDa)[34]. The gel percentage for each analysis (10 percent in Figure 6 and 12 percent in Figures 7-8) accounts for the differences seen. The greater gel percentage (12%) allowed for a more distinct separation of the two isoforms whereas in the 10 percent gel the two isoforms appear as one band. In addition, there appears to be different levels of the two isoforms in MCF7 cells and PC3 cells, where MCF7 cells appear to have more of the beta isoform and PC3 cells appear to have more of the alpha isoform. However, there is no published data on the expression of the two caveolin isoforms in these two cell lines specifically.

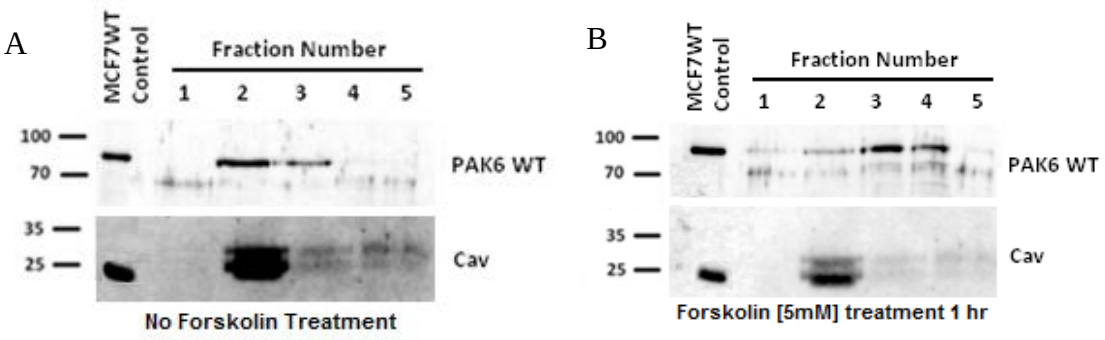

Figure 7. MCF7WT Raft Domain Membrane Isolation. (A) PAK6WT without Forskolin treatment. (B) PAK6WT stimulated by Forskolin [5mM] for 1 hour.

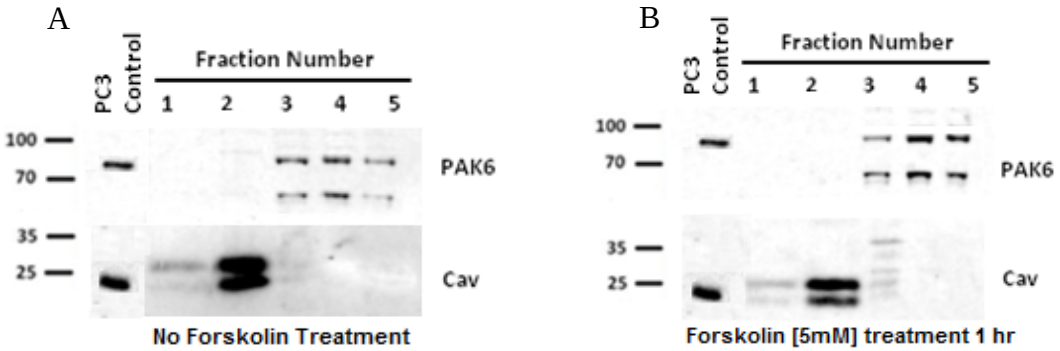

Figure 8. PC3 cells Raft Domain Membrane Isolation. (A) Endogenous PAK6 without Forskolin treatment. (B) Endogenous PAK6 stimulated by Forskolin [5mM] for 1 hour.

Recent studies showed that the inhibition of PAK6 resulted in decreased cell proliferation[10]. These findings combined with the the increase of proliferative genes seen in the microarry analysis (Figure 2) suggest that PAK6 may function in promoting cell proliferation. Therefore, the effects of PAK6 expression on cancer cell growth were tested using PAK6WT and mutants (SN and KM). Cell counts were conducted using an

Invitrogen Cell Counter to generate a growth curve for both MCF7 and LAPC4 overexpression model cell lines. The cells were plated at the same initial concentration and counted at 24 hour intervals for 5 days. The doubling time in hours was calculated for each cell line using the equation h x ln (2) / $\ln(c_2/c_1)$, where h is the number of hours, c_2 is the initial concentration and c_1 is the final concentration.

As shown in Figure 9A-B, the growth rate of MCF7 cells expressing PAK6WT is comparable to the parental MCF7, in that they have a doubling time of 24.6 and 24.4 hours respectively. In the PAK6KM (kinase dead) mutant, a slightly increased growth rate is observed with a doubling time of 22.8 hours. In contrast, the doubling time of MCF7 cells expressing constitutive active SN mutant slows down to 25.5 hours. However, these results are not significant at $p< 0.05$. Similar results are seen in the LAPC4 prostate cancer cells (Figure 10A-B) where the doubling times are 39.8, 39.6, 47.9, and 31.5 hours for LAPC4, WT, SN, and KM cell lines respectively. The LAPC4 results however are also not significant at $p< 0.05$. Although the changes in growth rate are not statistically significant, the results do suggest that PAK6 may have a growth regulatory function rather than growth promoting as initially proposed.

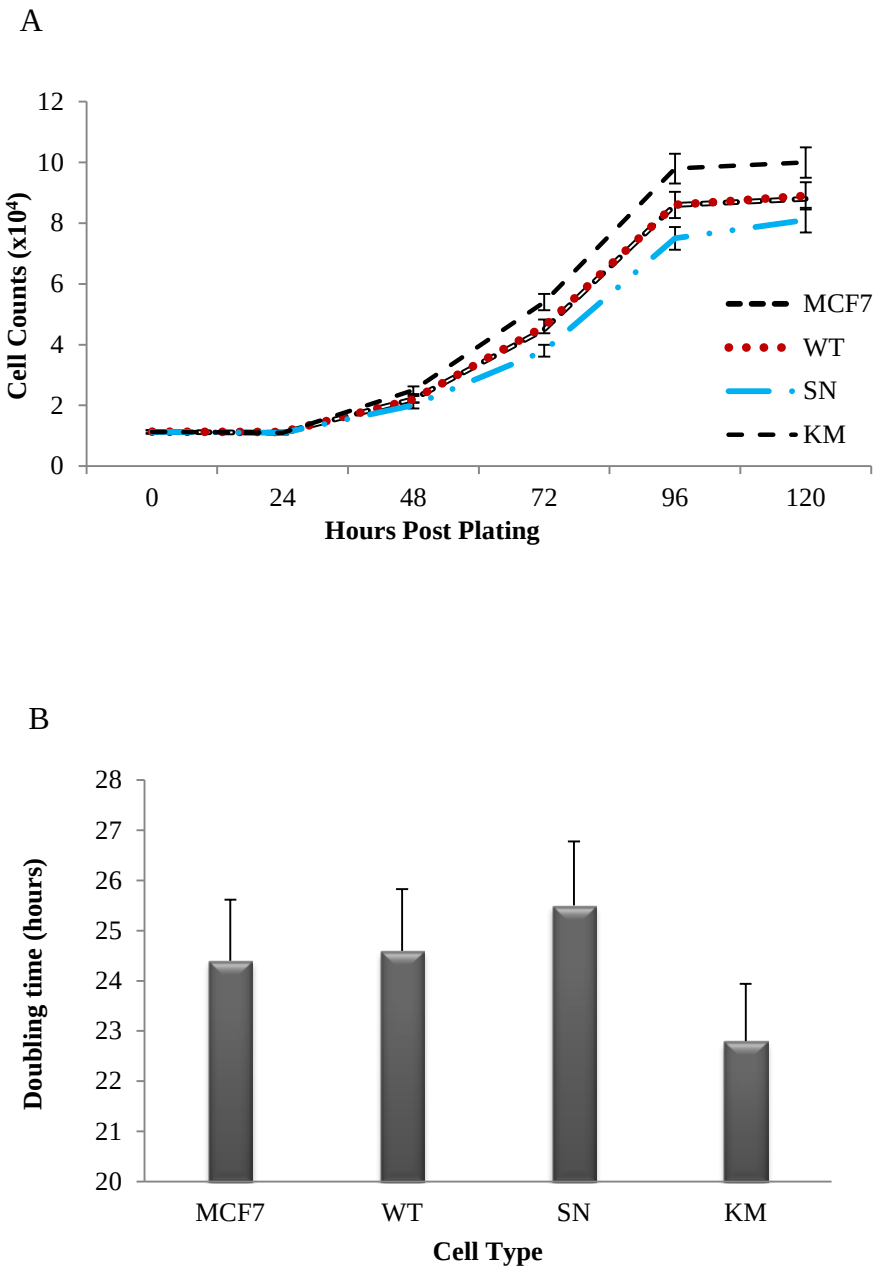

Figure 9. Growth curve and doubling time in PAK6 MCF7 overexpression model

(A) Growth Curve of MCF7, -WT, -SN, and -KM. (B) Doubling time of MCF7 cells determined empirically from growth data.

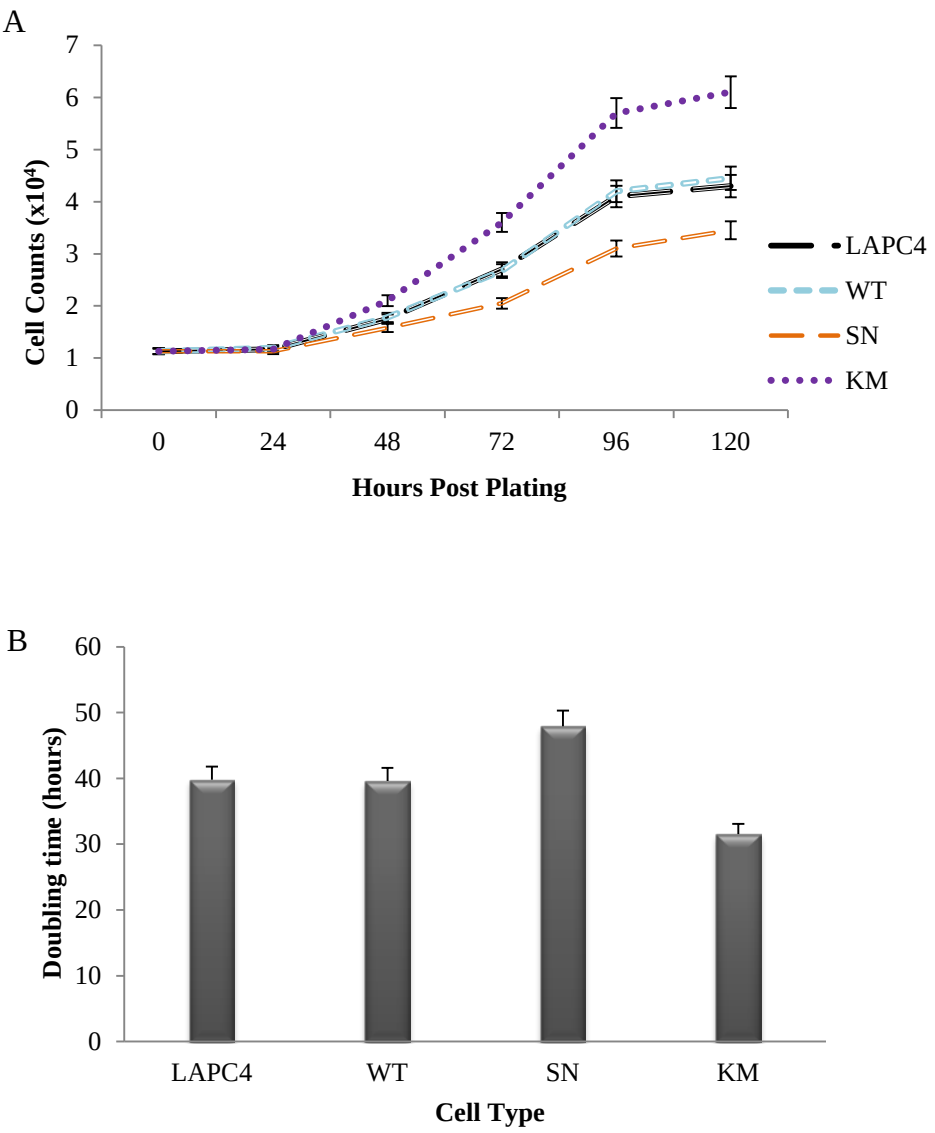

Figure 10. Growth curve and doubling time in LAPC4 PAK6 overexpression model. (A) Growth Curve of LACP4, -WT, -SN and -KM. (B) Doubling time of LAPC4 cells determined empirically from growth data.

DISCUSSION

It was initially postulated that PAK6 localizes to the caveolae raft domain. However, the data provided herein indicates that PAK6 is associated with the membrane in its wild-type form, but is activated in the caveolin-rich lipid rafts, resulting in translocation of PAK6 from the plasma membrane (Figures 6-8). The membrane isolation method used to obtain this data was used as a qualitative assessment of the presence of PAK6 in the lipid raft domain, using caveolin-1 as a raft domain marker. As previously mentioned, there are two visible bands for caveolin-1 in the membrane isolation western blots. These correspond to caveolin-1 isoforms, alpha and beta, which differ by the N-terminal 31 amino acids found only in the alpha isoform[36]. The data appears to suggest a varied level of the two isoforms in prostate cancer PC3 cells (more alpha) and breast cancer MCF7 cells (more beta). Although current literature has not explored the expression of the two caveolin-1 isoforms in PC3 and MCF7 cells specifically, recent studies have shown that they may affect caveolar structure, where expression of the alpha isoform is more efficient in the formation of caveolae than beta[36]. In addition, the ratio of the two caveolin-1 isoforms affects the shape of the caveolae, forming either deep or shallow depressions in the membrane[34,36]. Therefore, the varied levels of the two isoforms in the PC3 and MCF7 cells could suggest a prevalence of deep depression in the PC3 cells having greater alpha isoform, and shallow depressions in MCF7 cells. It has not yet been investigated, but rather suggested that the deep and shallow caveolae may have different functions, which could affect the signaling pathways mediated by them. Thus,

the presence of PAK6 in caveolae with a predominance of the alpha-isoform or the beta-isoform could suggest different downstream targets of PAK6 dependent on caveolae shape and cell type. The downstream targets of PAK6 are not known, however recent literature has identified several PAK6-interacting proteins which may provide insight into its potential function in tumorigenesis.

Ramneet Kaur showed nucleolin, a nucleolar protein involved in the ribosome biosynthesis, to be a PAK6-interacting protein[37]. The phosphorylation of nucleolin results in increased rRNA transcription and cell proliferation where it's increased expression results in reduced doubling time of cells, and decreased expression of nucleolin increases the doubling time of cancer cells[38]. The interaction of PAK6 with nucleolin would suggest that PAK6 may promote cell proliferation and growth. However, the cell proliferation findings suggest rather that PAK6 may regulate cancer cell growth (Figure 9-10). Therefore, it is plausible to speculate that multiple mechanisms may be used by PAK6 to regulate the proliferation and growth of the cell. For example, PAK6 has also been shown to interact with PP1B, a phosphatase involved in cell cycle regulation by dephosphorylating cyclin-dependent kinases, suggesting PAK6 may regulate the cell cycle through the PP1B/cyclin pathway. Furthermore, studies indicate that inhibition of PAK6 expression results in cell cycle arrest in the G2/M phase suggesting that PAK6 expression could be involved with cell progression through this phase checkpoint[7,8].

PAK6 may also play a role in the promotion and maintenance of epithelial-mesenchymal-transition (EMT) through TGFβ2 which was previously shown (Figure 2) to be increased when PAK6 is constitutively active (SN) and kinase dead (KM). This was confirmed using qPCR (data not shown). However, the expression of TGFβ2 in SN is 6

fold greater than in KM, suggesting that PAK6 may be involved in the potentiation of EMT through TGFβ2. EMT involves several changes to the characteristics of epithelial cells including the loss of polarity and dissolution of adherens junctions, desmosomes, and tight junctions[11]. TGFβ2 can regulate EMT transcriptionally through the Smad pathway resulting in increased expression of mesenchymal markers such as vimentin and N-cadherin and decreased expression of epithelial markers. These changes can also be induced by TGFβ2 in a Smad-independent manner and autocrine TGFβ2 loop[39,40]. Therefore, although PAK6 may not be involved in the initiation of the pathway it may propagate autocrine signaling of TGFβ2 in TGFβ-induced EMT.

In addition, recent studies suggest that PAK6 functions in cell survival where it has been shown to sustain levels of phosphorylated BAD in irradiated prostate cancer cells. The phosphorylation of BAD renders the protein unable to translocate to the mitochondrion, thereby sequestering it in the cytoplasm of the cell, allowing for the inhibition of cytochrome *c* release and subsequently evasion of apoptosis[7].

Each identified PAK6-binding protein lends insight into potential functions of PAK6 in the regulation of the cell cycle and proliferative capabilities, cell motility, and/or cell survival in cancer cells through activation-induced translocation to the nucleus, nucleolus, or cytoplasm.

Another kinase activated in the raft domain that is increased in high grade malignant prostate cancer cells and may contribute to the hormonal independence found in many aggressive prostate tumors (similar to PAK6), is phosphatidylinositol 3-kinase (PI3K). A brief look into the regulation of this pathway (PI3K/Akt) and downstream

functions in cancer progression and metastasis may provide insight into PAK6 function and possible additional downstream targets.

The activation of the PI3K/Akt pathway initiates in the plasma membrane where Akt is recruited to the membrane. Once at the membrane, Akt is phosphorylated by PDK1 (phosphoinositide-dependent kinase 1). Akt, after activation, then proceeds to phosphorylate a variety of protein substrates. These steps to activate Akt have been shown to be faster and stronger in the raft domain. PTEN, a lipid phosphatase that plays an important role in negatively regulating this pathway was discovered to be localized in non-raft domains. The localization of PTEN to non-raft domains is important in that if it were mistakenly localized to the raft domain, downstream signaling of the PI3K/Akt pathway would be abolished[41]. A negative regulator of PAK6 has not yet been identified. However, the localization of the wild-type form to the raft domain (Figure 6A) could suggest an inhibitory mechanism, similar to that of PI3K/Akt, which is released by PAK6 activation and translocalization.

The PI3K/Akt pathway also has several downstream substrates involved in proliferation, cell survival, and metastasis. Among these targets are GSK-3 and BAD[42]. As previously mentioned, PAK6 phosphorylates the pro-apoptotic protein BAD, protecting the cell from apoptosis. PAK6 activation has also been shown to increase the phosphorylation of GSK3-β (M.L. unpublished data), releasing β-catenin, a multifunctional protein capable of promoting cell proliferation, motility and EMT[42]. The common substrates ofPAK6 and the PI3K/Akt pathway warrant a closer look at additional common substrates that may elucidate PAK6 function in tumorigenesis.

One particular function of PAK6 that is beginning to emerge is its importance in the motility of the cell as indicated by several studies. To briefly review what was previously discussed, the activation of PAK6 results in increased migration potential evident from morphological changes and a migration assay. Also, the inhibition of PAK6 resulted in reduced invasive capabilities[8]. One possible mechanism by which activated PAK6 may drive cell motility is through the upregulation of caveolin expression among other genes shown in the microarray (Figure 2 and 4). Although caveolin has had implications in both tumor progression and suppression, its expression in prostate cancer has been regularly reported to be increased. Studies have shown that the overexpression of caveolin-1 enhances androgen-dependent growth and proliferation in a prostate cancer mouse model, and induces filopodia formation in lung adenocarcinoma[43]. Furthermore, caveolin has been shown to be polarized in migrating cells and compartmentalize signaling molecules relevant to migration[29]. Thus, the upregulation of caveolin revealed in this study, could play an important role in PAK6-driven motility. It was previously mentioned that qPCR results (Figure 4) differed from the microarray, where caveolin-2 expression in PAK6WT is decreased in the qPCR results, but increased in the microarray. It has often been reported that variations between microarray and qPCR data can occur for genes having small degrees of change, usually less than 2-fold. The fold change for caveolin-2 in PAK6WT is borderline at exactly 2-fold. There are other possible explanations for the variations in regards to the preparation of samples (i.e. RNA quality, frozen versus fresh tissue) and different efficiency levels of the methodologies used[44]. However, these possibilities are less likely and do not negate the upregulation of caveolins as a potential mechanism for PAK6-driven motility.

Although this study provides insight into understanding another factor in terms of the activation of PAK6, it is only the first step to identifying specific mechanisms by which PAK6 is involved in tumorigenesis and cell motility. These possibilities must be further explored in both *in vitro* and *in vivo* models.

This can be further explored by the characterization of shRNA PAK6 transduced into prostate cancer cell lines. This model could be used to investigate the effect of down-regulated PAK6 on gene expression as well as physiological changes of the cancer cell. A comparison of the overexpressed (WT, SN, and KM) and shRNA down-regulated models *in vivo* using prostate cancer cell lines in nude mice will allow for the monitoring and comparison of tumor growth, volume, and signs of metastasis. Tumor isolation and immunohistochemistry staining of PAK6 in the overexpression model would not only allow for *in vivo* verification of membrane localization and activated translocation in epithelial cells, but would also provide insight into other cell types in which PAK6 may be expressed. In addition, both models can be stained for EMT markers such as E-cadherin, Snail, and Twist. The tissue may also be stained for cancer stem cell markers (i.e. CD133) which are increasingly being investigated as initiators for tumorigenesis and its reoccurrence. Immunocytochemistry of cells isolated from the tumor tissue could also be stained for PAK6 and co-stained with other EMT markers such as vimentin, ZO-1, and β-catenin, seeing it has been previously discussed that PAK6 interacts or upregulates genes (GSK3β, TGFβ2) that would influence the expression of these markers. Staining for morphological features of motility can also be conducted using vinculin as a marker for focal adhesions.

In addition to staining, RNA isolation from the tumor tissue can also be used to look at the gene expression of caveolins 1 and 2 and TGFβ2 and compared to the *in vitro* results using the MCF7 breast cancer PAK6 overexpression model. Biochemical analysis of protein expression in the cells isolated from tumor would allow for a quantitative assessment of the presence of proteins involved in EMT and motility. It would also be useful to determine the effect of PAK6 overexpression and inhibition on various stress responses such as radiation, inflammatory cytokines, osmotic shock, UV, chemotherapeutics, and other environmental stresses.

Understanding the mechanisms of PAK6 and its role in cancer progression may provide valuable insight into potential therapeutic targets in the future.

CONCLUSION

The precise role of p21-activated kinase 6 in tumor progression remains to be determined. The data provided demonstrated that wild-type PAK6 is a membrane-associated kinase in the lipid raft domain which translocates upon activation, suggesting a myriad of possible functions in intracellular signaling and modification of proteins involved in cancer progression. However, the specific downstream target(s) of PAK6 are still unknown. PAK6 was also shown to regulate cell growth rather than promote it. In addition, based on the array data, it is also suggested that PAK6 may function predominantly in promoting EMT in cancer cells and possibly migration. Furthermore, PAK6's interaction with caveolae may serve as a point of signal transduction.

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