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The Role of Protein Kinase D (PKD) Signaling in Breast Cancer Cell Migration and Invasion

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14. ABSTRACT

The goal of my project as outlined in the original application is to analyze the role of Protein Kinase D (PKD) in breast cancer cell motility, the phenotype critical for metastasis. The work I have conducted includes the demonstration that loss of PKD in a number of highly metastatic breast cancer cell lines results in a migration defect. I have also discovered a potential isoform specificity of PKD in the control of breast cancer cell motility.

15. SUBJECT TERMS
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Introduction

The goal of my project as outlined in the original application is to analyze the role of Protein Kinase D (PKD) isoforms in breast cancer cell motility, the phenotype critical for metastasis. PKD is a serine/threonine kinase that has been best characterized as a key regulator of vesicular fission in golgi trafficking (1), a process is necessary for the delivery and secretion of molecules destined for the plasma membrane. This highlights its importance in homeostatic cell signaling, but despite four independent studies (2),(3),(4),(5) identifying upregulation and mutations of PKD in cancer tissue, a potential role for PKD in cancer progression has not been well explored. Based in part on a recent study which links PKD to fibroblast cell migration (6), I hypothesize that a PKD signaling network controls cytoskeletal reorganization and cellular adhesion, thereby regulating cell motility. I am testing the invasive migration of PKD1 and PKD2-knockdown and -overexpressing cells in transwell motility assays using 3T3-conditioned media as chemoattractant. I aim to determine the specific mechanism and PKD substrates that control this phenotype. Mutant PKD constructs refractory to silencing by shRNA will be introduced into PKD-knockdown cells to determine the role of the specific motifs and phosphorylation sites that I have mutated. To identify the contribution of a panel of both known and putative PKD substrates towards this phenotype, these substrates, mutated at known/putative sites of modification by PKD will be introduced into PKD-knockdown cells. I will also use live cell microscopy and immunofluorescence as additional methods to better elucidate the mechanism by which PKD controls invasive migration. This work will determine the regulation and function of a previously uncharacterized signaling pathway that is critical for breast cancer progression, the PKD signaling axis, and how it impacts invasive migration. The results of this research will yield an increased understanding of mechanisms that control the metastatic phenotype of breast carcinoma cells, subsequently allowing for new therapeutic strategies targeted to advanced stage tumors.

Body

The following tasks from the Statement of Work for this project were the focus for the research period from 30 September 2007-30 September 2008:

To determine whether PKD controls cytoskeletal reorganization and a migratory/invasive phenotype in epithelial cells (months 1-15).

a. Analyze the ability of each individual PKD isoform to control invasive migration and influence the dynamics of the actin cytoskeleton (months 13-15).

b. Characterize actin stress fiber formation and focal adhesion assembly using immuno-fluorescence microscopy and immunohistochemistry (months 10-12).

To test whether Rho family GTPases cooperate with PKD signaling to control breast cancer cell migration and invasion (months 16-29)

Progress

a. Analyze the ability of each individual PKD isoform to control invasive migration and influence the dynamics of the actin cytoskeleton (months 13-15).

To ensure that PKD isoforms are specifically targeted in these experiments, I tested the ability of two distinct shRNAs for PKD1 and PKD2 to efficiently and specifically knock down exogenously expressed alleles (**FIGURE 1**). Knockdown of both PKD1 and PKD2 in a number of cancer cell lines including MDA-MB-231 breast cancer cells significantly impairs cell migration (**FIGURE 2**). However, knockdown of PKD2 but not PKD1 in Sum159PT and BT549 breast cancer cells lines impairs cell migration (**FIGURE 3**). This result implicates the PKD2 signaling network as dominant in these breast cancer cell types. I have therefore discovered a potential isoform specificity of PKD in the control of breast cancer cell motility.

This would be the first discovery of PKD isoform specificity in any biological context. I have conducted RT-PCR to determine relative expression of PKD isoforms. This experiment has only been conducted once and therefore needs to be repeated, but I have confirmed that both PKD isoforms are present in significant quantities in at least one of these cell types (**FIGURE 4**). In order to determine whether the migration phenotype might be a consequence of decreased cell viability, I conducted FACS analysis of Propidium Iodide-stained PKD-knockdown LnCap prostate cancer cells. I compared the profiles of control and PKD1 knockdown cells at the time at which I would run a migration assay (three days post-infection). This analysis indicates that PKD1 knockdown does not impact cell viability (**FIGURE 5**).

b. Characterize actin stress fiber formation and focal adhesion assembly using immuno-fluorescence microscopy and immunohistochemistry (months 10-12).

Though I have not yet optimized the conditions for immuno-fluorescence and have not yet acquired high quality pictures, it appears as though knockdown of each isoform causes a unique morphology change and actin profile in MDA-MB-231 breast cancer cells.

To test whether Rho family GTPases cooperate with PKD signaling to control breast cancer cell migration and invasion

As a result of the discovery that PKD controls cell migration in an isoform specific manner in SumPT159 and BT549 breast cancer cells, I decided to broaden the scope of my studies to focus on isoform specific mechanisms and a number of potential substrates that control this phenotype. I have modified my Statement of Work accordingly. To investigate the role of PKD isoforms and specific PKD sites and domains, I am conducting migration defect rescue experiments. I have generated mutant

alleles of both PKD1 and PKD2 that are refractory to silencing by shRNA and contain mutations in domains of interest and phosphorylation sites. I am also investigating the contribution of a variety of both known and putative PKD substrates in PKD1 and PKD2-specific signaling networks towards invasive migration. This panel, most of which I have already generated, includes phospho-mimetic and non-phosphorylatable alleles of RIN1, HSP27, and PI4KIIIβ, all of which are known PKD substrates and similarly mutated alleles of B-Raf and Rac1, a member of the Rho family of GTPases, both of which are putative PKD substrates.

Additionally, my thesis committee and I have decided that live cell microscopy would be an optimal method by which to identify the morphologic and mechanistic defect behind the inability of PKD-deficient cells to migrate. This method would allow me to better understand the specific cytoskeletal changes that occur as a result of PKD loss. I have also added this to my amended Statement of Work as my third task.

Figure 1: Efficiency and specificity of PKD1 and PKD2 shRNAs in Hela cells expressing exogenous isoforms

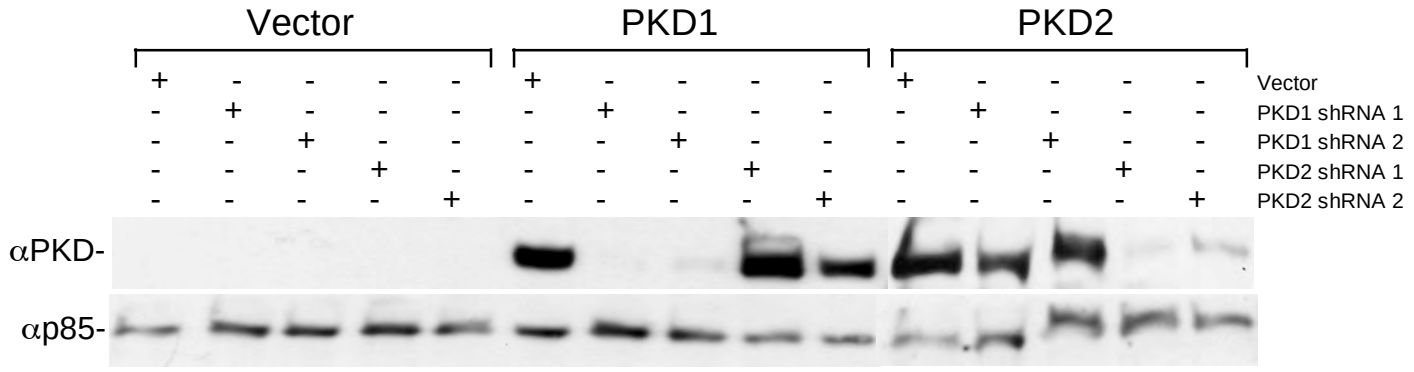


Figure 2: Knockdown of PKD1 and PKD2 results in impaired cancer cell migration.

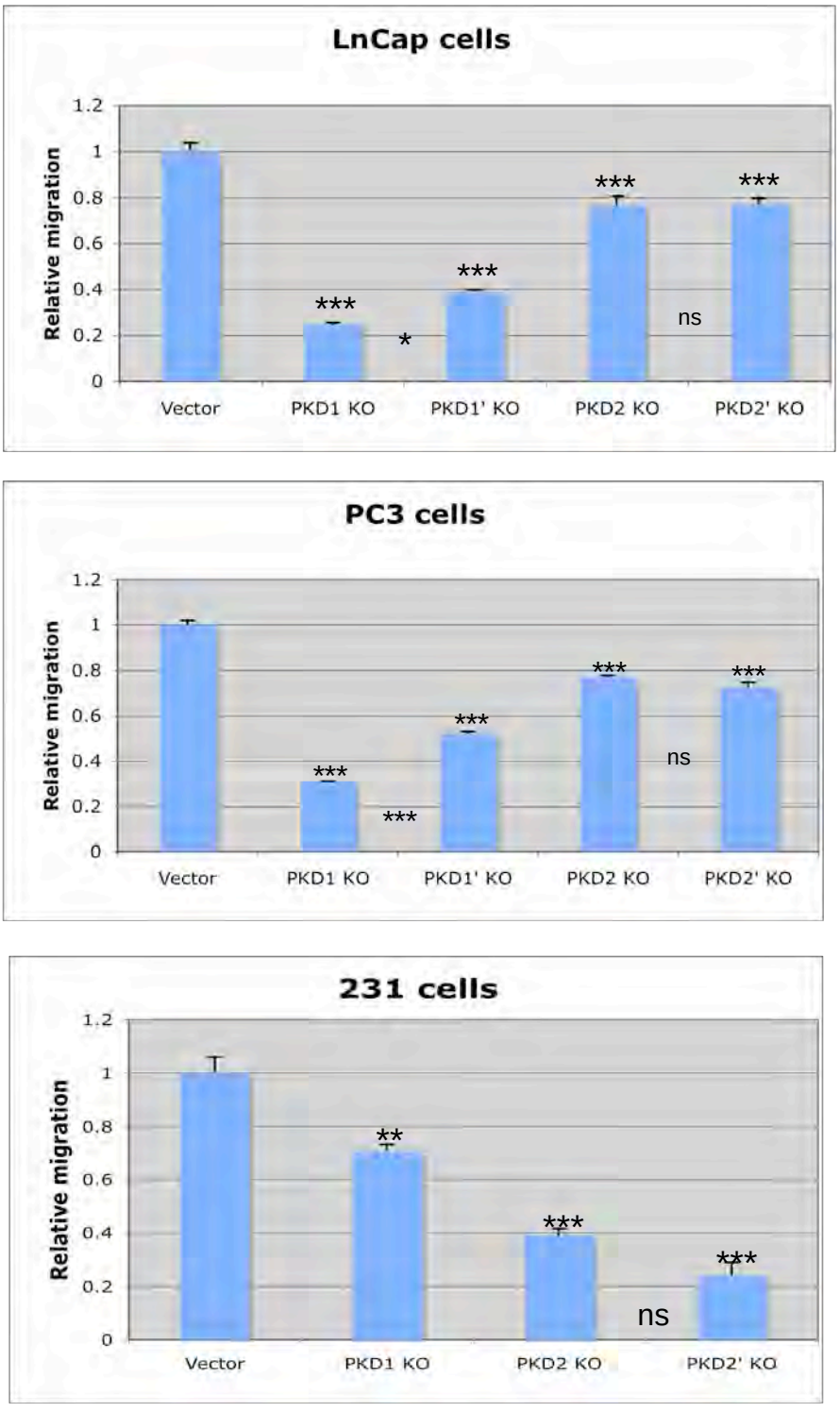


Figure 3: Knockdown of PKD2 impairs breast cancer cell migration.

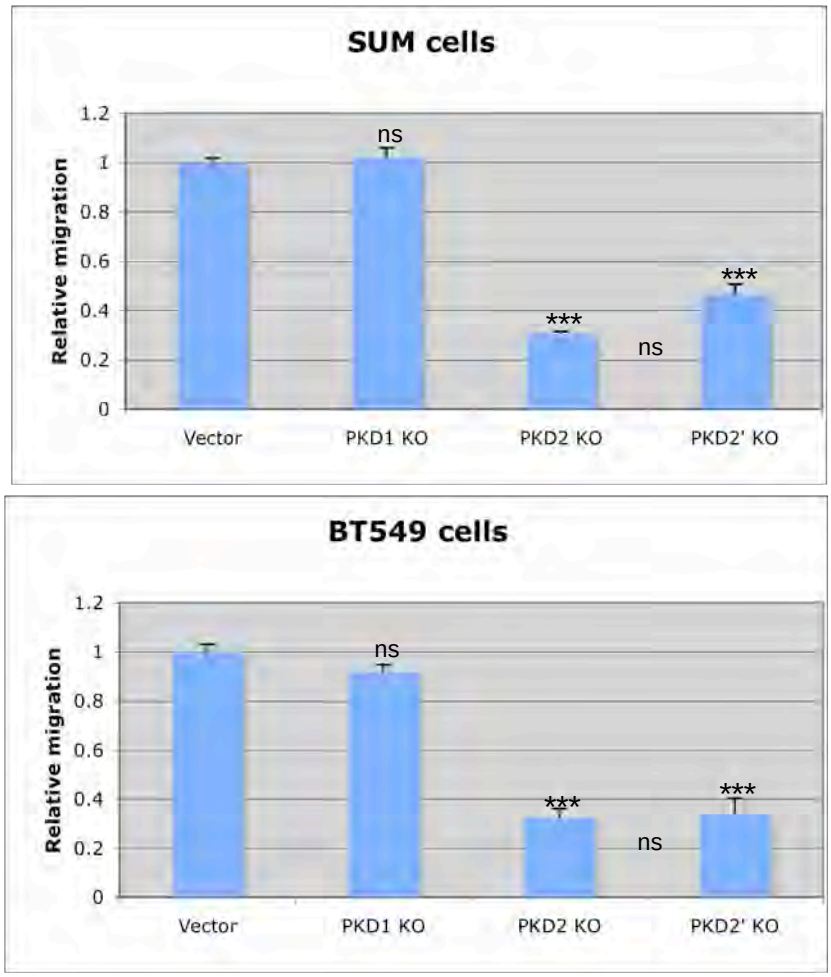


Figure 4: RT-PCR of PKD isoforms.

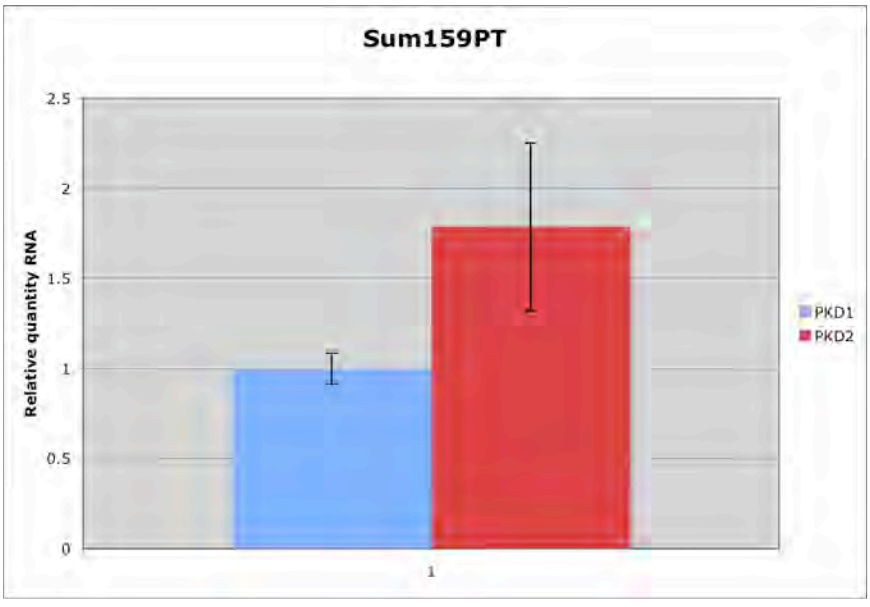
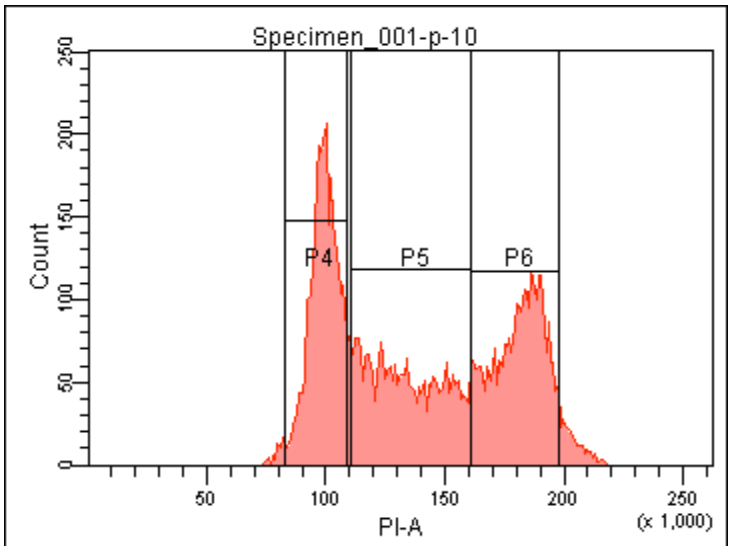
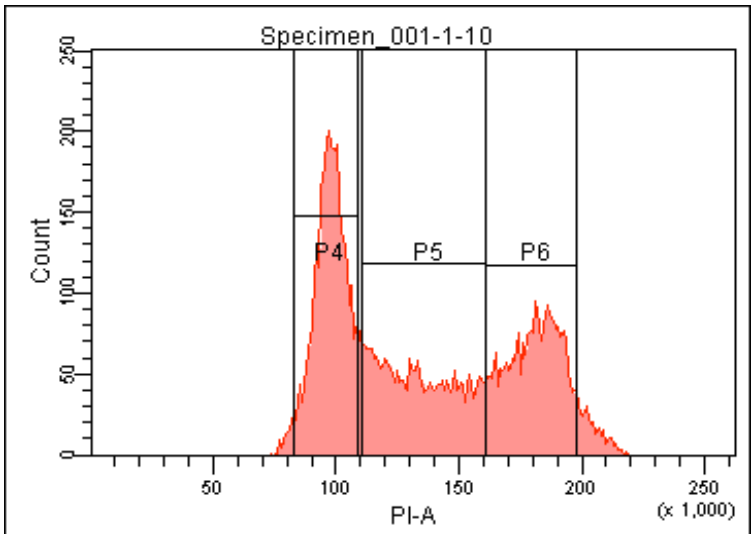


Figure 5: FACS cell cycle analysis of PKD1 knockdown LnCap prostate





Tube: p-10

Population	#Events	%Parent	%Total
All Events	10,000		100.0
P1	8,437	84.4	84.4
P4	2,632	31.2	26.3
P5	2,603	30.9	26.0
P6	2,748	32.6	27.5
P2	0	0.0	0.0
P3	42	0.4	0.4
P7	313	3.1	3.1

Tube: 1-10

Population	#Events	%Parent	%Total
All Events	10,000		100.0
P1	8,134	81.3	81.3
P4	2,870	35.3	28.7
P5	2,398	29.5	24.0
P6	2,350	28.9	23.5
P2	0	0.0	0.0
P3	46	0.5	0.5
P7	330	3.3	3.3

Current research and future directions

A primary ongoing effort is to investigate the mechanism and signaling by which PKD isoforms control cellular migration. I plan to repeat these experiments in transwells with matrigel coating to determine whether PKD loss results similarly in impaired invasion. I am using the panel of mutant PKD alleles refractory to silencing that I generated to determine their ability to rescue the migration defect phenotype. I will test whether the migration defect and impaired viability of PKD-knockdown cells can be rescued by the introduction of activated alleles which I have generated that are potentially involved in the PKD-signaling pathway. Additionally, I plan to visualize PKD-knockdown cells using live cell microscopy and immunofluorescence to determine both the localization of PKD and the specific morphological defects that account for the impaired migration phenotype.

Key Research Accomplishments and Reportable Outcomes

- **Established reproducible migration defect phenotype in PKD-knockdown cells**
- **Discovered potential isoform specificity of PKD1 and 2 in the control of breast cancer cell migration**
- **Negated the contribution of decreased cell viability towards this phenotype**
- **Created a number of PKD1 and 2 non-silenceable and PKD substrate mutant alleles for PKD-knockdown/rescue analysis**

Conclusion

I have demonstrated that PKD loss by lentiviral shRNA knockdown results in a migration defect phenotype in metastatic breast cancer cell lines. I have further demonstrated that the PKD2 signaling

network is dominant in the control of this phenotype in breast cancer cell types Sum159PT and BT549. I have therefore discovered a potential isoform specificity of PKD in the control of breast cancer cell motility. I have confirmed by RT-PCR that both isoforms are present in significant quantities in at least one of these cell types. I conclude that this defect is not due to decreased viability and am investigating the signaling and mechanistic cause of this phenotype by means of PKD and PKD substrate rescue analysis, immunofluorescence, and live cell microscopy.

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