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Cover Page Footnote

This project was made possible by financial support from the Samuel Mayhugh Research Fund as well as the Honors Program and Biology Department at Olivet Nazarene University. I would like to thank my mentor, Dr. Greg Long, for his guidance, expertise, and constant support throughout this project and my time at Olivet. Throughout the last few years, I have experienced tremendous growth as a scientist and an individual, and I owe much of it to him. I'd also like to give a special "thank you" to Dr. Daniel Sharda for his assistance, review and support. Finally, I owe thanks to the entire faculty of the Biology Department at Olivet Nazarene University for their sincere desire to foster an environment of academic and spiritual growth. It has made such a difference in my life—in my pursuit of science and my walk with Christ—and I am so grateful

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ABSTRACT

Signaling molecules have important roles in many cellular functions, but because these pathways are incredibly complex, the exact mechanisms often remain unknown. One signaling molecule, protein kinase C alpha (PKCα), is involved in cell proliferation and is expressed at high levels in many cancers. Interestingly, its activity as a tumor promoter or tumor suppressor varies depending on the cell type for reasons not yet fully understood. This study aimed to investigate the role of PKCα in cell proliferation in order to better understand its function as a signaling molecule. To assess this, a knockout line was generated using CRISPR-Cas9 and human embryonic kidney (HEK) cells. After confirmation of knockout, proliferation studies were conducted on untreated knockout and wild-type cells and upon addition of 0-0.5 μg/mL of PMA and 0-5 μg/mL of sphingosine-1-phosphate to induce proliferation. Our results indicate that compared with wild-type cells, untreated PKCα knockout cells exhibited reduced proliferation and had high percentages of cell death when treated with proliferative agents. This supports the hypothesis that PKCα knockout reduces proliferation in human embryonic kidney cells and suggests that PKCα has an important role in normal cell function in a tumor-promoting context.

Keywords: protein kinase C alpha (PKCα), proliferation, cancer, knockout, cell signaling

INTRODUCTION

Protein kinase C alpha (PKCα), a signaling molecule involved in many cellular processes, has an important role in cell growth and proliferation. The exact details of this pathway, however, are unknown, and further elucidation on the effects of PKCα on cell proliferation is needed. In this study, HEK cells were genetically modified using CRISPR-Cas9 to generate a cell line lacking PKCα. Together with a wild-type HEK cell line, these knockout cells were treated with varying levels of proliferative agents, and differences in the resulting growth rates were assessed. Developing a PKCα gene knockout cell line and assessing proliferation of these cells increases our understanding of the PKCα signaling pathway, helps to elucidate its involvement in cell growth and division, and contributes to hypotheses of the role it may play in the abnormal proliferation that is characteristic of cancer cells. In short, our findings contribute to the foundation for future applications of PKCα involvement in cell proliferation.

REVIEW OF LITERATURE

PKCα function

Cancer is the result of uncontrolled cell growth and is the second largest cause of death in the United States [5]. This abnormal cell growth is attributed to cancer-causing mutations that typically involve malfunction of tumor-suppressor genes and the activation of proteins involving cell proliferation. In fact, many cancers are known to involve a mutation in TP53, a tumor-suppressor gene [12]. Without the proper function of the protein produced by that gene, p53, cell growth and division are largely unregulated [12]. This results in abnormal growth, often initiated by cell signaling molecules that have a role in normal, non-cancerous cell proliferation. One of these

signaling molecules, an enzyme called protein kinase C alpha (PKCα), is activated in normal cells by diverse signaling pathways. Various stimuli, often involving interaction with a membrane, can lead to PKCα involvement in differentiation, apoptosis, cellular transformation, motility, cell growth, proliferation, and many other cellular functions [13]. Importantly, PKCα has been linked to proliferation, survival, differentiation and motility in tumor growth but can also act as a tumor suppressor depending on the context [4]. In fact, past research has shown that PKCα activation leads to the progression of many cancers (for example, bladder and breast cancer), while it may act as a growth-inhibitory kinase in others (such as colon cancer and non-small cell lung cancer) [1], [4], [10]. Its role in cell proliferation as an application of tumor promotion is, however, not well understood, but targeting PKCα as a potential therapy for many cancers has been suggested [4]. In order to understand more about how aberrant PKCα signaling may be contributing to the rapid growth and division of cancer cells, a better understanding of the functional diversity of its proliferative role in cells is needed.

Gene knockout and human embryonic kidney cells

There are two common methods used to understand protein function: genetically modifying a cell line to render the gene nonfunctional, and utilizing drug inhibition to block protein function. In the inhibition method, proteins are still present, so although function is impaired, it is still possible for the drug-inhibited protein to interact with binding partners. Drug inhibition often also has off-target effects [16], and thus an agent used to inhibit PKCα function may also interfere with molecules involved in other proliferation pathways. For example, UCN-01 is a PKCα inhibitor with anti-tumor applications, but because it does not work exclusively via the PKCα pathway it cannot be used to understand the specific role of PKCα in proliferation and tumor promotion [4]. In contrast, gene knockout prevents the production of the protein altogether and eliminates the problems associated with inhibition. Because it exclusively targets the gene that codes for a protein, gene knockout yields more specific results. Knocking out the gene for PKCα in human embryonic kidney (HEK) cells would generate a cell line that could serve as a comparison to wild-type HEK cells with a normal functioning PKCα gene.

HEK cells are commonly used in cell biology and cancer research for many reasons: they are easy to grow and transfect, relatively inexpensive, and tumorigenic [15]. These properties make HEK cells a practical and meaningful tool for use in a study involving gene-knockout and cell proliferation. Additionally, PKCα does not act as an anti-proliferative factor in HEK cells, so this cell line serves as an excellent model for better understanding the functional role of PKCα in proliferation and tumor promotion [13]. A common concern involving gene-knockout involves cell viability. Removing a gene can have drastic effects and is often too significant of an alteration for the cells to survive. In 2002, researches developed a method of targeting PKC isotypes in HEK-293 cells and demonstrated that subtype-specific differentiation was possible [6]. Specifically, they performed subtype-specific knockdown of PKC on HEK cells using short interfering RNA and grew them in normal conditions. Although this is not the same as gene knockout with CRISPR-Cas9, the survival of these HEK cells without active PKCα suggests that CRISPR-Cas9-generated PRKCA knockout HEK cells would be viable for use in proliferation studies.

CRISPR-Cas9

CRISPR-Cas9 is a relatively new technology that can be used for gene knockout. Since the first discovery of a CRISPR locus in 1993, CRISPR-Cas9 has been researched extensively [7]. In 2013, researchers used it to genetically modify the genes of mice and mark them with a fluorescent reporter. Analysis verified that the CRISPR-Cas9 mechanism rarely makes errors and the use of this technology in eukaryotic cells is effective, efficient, and accurate [17]. Continuing this work, in 2014 CRISPR-Cas9 was successfully used for gene knockout in HEK-293 cells [18]. Similarly, this system could be employed to create a HEK cell line without PRKCA, the PKCα gene. Because of its use and reliability in genetic modification, CRISPR-Cas9 is important for cancer research and understanding the role of specific proteins, such as PKCα, in cell proliferation. Additionally, our use of CRISPR-Cas9 in this study makes this method accessible as a research and teaching tool at Olivet. The successful use of the CRISPR-Cas9 mechanism will contribute to the development of laboratory protocol so that this tool could be widely used by Olivet biology students.

Cell proliferation and proliferative agents

PKC isoforms are classified into several groups. PKCα falls into the category of classical PKCs (cPKCs). cPKCs are activated at a C1 domain and a C2 domain. The C1 domain is activated by diacylglycerol (DAG), a secondary messenger that is stimulated by membrane receptors, and the C2 domain allows for the binding of other molecules in the presence of calcium [13]. A phorbol ester, such as phorbol 12-Myristate 13-Acetate (PMA), is a proliferative agent known to utilize the PKCα pathway by binding at the C1 domain [11]. Another proliferative agent, sphingosine-1-phosphate, causes proliferation independent of PKCα signaling [2]. The mechanism of proliferation via PKCα can differ depending on the cell type and activity. Activated PKCα has been documented to upregulate cyclin-D1, cdk4 and p21cip1, all of which can enhance proliferation [13]. Commonly, PKCα phosphorylates Raf-1, which leads to the activation of ERK-MAPK (extracellular signal-regulated kinase-mitogen-activated protein kinase cascade) [13]. This enhances proliferation in many cell types, including HEK-293 cells [19]. PKCα dependent and independent proliferation in HEK cells can be evaluated through visual observation and the generation of a cell growth curve with doubling times. This can be calculated using a hemocytometer over a period of several days. In order to evaluate the effects of proliferative agents on cell proliferation, cells can be treated with various concentrations of the proliferative agent, counted, and then used to construct a concentration vs. cell proliferation curve.

PKCα has an important role in cell proliferation, but it is functionally diverse and largely not understood. Because of its involvement in tumor promotion, understanding this protein and its signaling pathway is important and relevant for cancer research and treatment. Investigating the effects of PRKCA knockout on proliferation in a cell line where PKCα is not tumor-suppressive can help fill this gap in research. Specifically, attempting to induce abnormal proliferation by mechanisms that are PKCα independent and dependent in a PRKCA knockout cell line will yield further understanding of its exact role in tumor promotion. We hypothesized that proliferation studies of wild-type and knockout cells under both normal conditions and when treated with PKCα-independent and PKCα-dependent proliferation agents would demonstrate that knocking out protein kinase C-alpha reduces proliferation in HEK-293 cells.

METHODOLOGY

Generation of PRKCA knockout cells

PRKCA knockout cells were produced following the manufacture protocol provided by Santa Cruz Biotechnology [3]. Briefly, HEK-293 cells were seeded at a density of 50,000 cells/well and grown in 24-well culture dishes in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum. Approximately 48 hours later, cells reached 60-75% confluence, and culture media was replaced with media generated for optimal transfection of HEK-293 cells [14]. This media contained both the CRISPR-Cas9 KO plasmid and the homology-directed repair (HDR) plasmid with a fluorescent tag and also a puromycin-resistant gene insert prepared according to the manufacture's protocol [3]. Briefly, three tubes were incubated then combined and added to each well. Tube A contained 240 µl of Optimem medium and 3.2 µg of the CRISPR-Cas9 KO plasmid. Tube B contained 240 µl of Optimem medium and 3.2 µg of the HDR plasmid. Tube C contained 400 µl of Optimem medium and 16 µl of Lipofectamine 2000. These tubes were incubated individually for 5 minutes at room temperature, then mixed gently and the combined solution was incubated for 20 minutes at room temperature. Following incubation, 115 µl of the final solution was added to each well (twelve total) with rocking. After 24 hours, this media was replaced with standard DMEM and cells were grown in normal culture conditions.

Approximately seven days post-transfection, cells were observed with fluorescent microscopy to confirm transfection efficiency by fluorescence. Cells were then grown and treated with 2.5 µg/mL of puromycin to kill cells lacking the transfected puromycin-resistant gene insert. This selected for cells likely to be PRKCA knockout cells. After growing cells for an additional seven days, they were treated with 10 µg/mL of puromycin to select for highly-expressing homozygous knockout cells.

Western blot analysis of PKCα knockout

Puromycin resistant cells were grown and tested for PKCα presence using standard Western blot techniques [8]. Wild-type and knockout cells were grown in normal conditions in 6-well culture dishes until they reached confluence. Upon reaching confluence, cells were washed with PBS and treated with a lysis buffer containing RIPA buffer (Sigma R0278) and four protease inhibitors: PMSF (100 mM), Luepeptin (10 mg/mL), Aprotinin (10 mg/mL), and Pepstatin A (1 mg/mL). Lysed cells were collected, washed and centrifuged, and a BCA assay was performed to determine protein concentration in the cell lysate for wild-type and knockout cells. Lysates were diluted to a concentration of 1 mg/mL with SDS-PAGE loading buffer. 10% SDS-PAGE gel (Lonza) was loaded with 20 µl of cell lysate per lane. Three lanes were loaded for each cell type, and two lanes were loaded with protein molecular weight ladders. This procedure was run at a constant voltage of 125 V for approximately ninety minutes. Western blot protocol was taken from the Xcell II Blot Module User's Manual. Transfer to the Western blot membrane was performed overnight in a cold room at a constant voltage of 12 V. Following this, the membrane was removed, treated with blocking buffer, and incubated in primary antibody overnight. Primary antibody was prepared at a dilution of 1:500. Following this, the membrane was washed and treated with the secondary antibody for one hour. The secondary antibody was prepared at a dilution of

1:12,500. Remaining western blot procedures took place in a photography dark room. The membrane was incubated in ECL Western Blotting Substrate (ThermoFischer) then exposed to film for times ranging from 10 to 45 seconds. Film was developed in developer solution for two to three minutes, placed in water for 30 seconds, placed in fixer for three minutes, and then rinsed under running water for five minutes.

Treatment with proliferative agents: sphingosine-1-phosphate and phorbol ester

PRKCA knockout and wild-type HEK-293 cells were first grown in normal conditions to determine viability and disruption of the normal cell cycle. Cells were grown in the absence of puromycin to prevent altered proliferation compared to wild-type cells. Cell counts were recorded for each group in duplicate over a four day period. Next, knockout and wild-type cells were treated with proliferative agents. The first of these proliferative agents, sphingosine-1-phosphate, is known to work via PKCα-independent mechanism in HEK-293 cells [2]. Knockout and wild-type cell cultures were treated with concentrations of sphingosine-1-phosphate ranging from 0-5 μg/mL, and observations of percent confluence were recorded. The second proliferative agent, phorbol ester, is known to activate PKCα by mimicking diacylglycerol, the physiological ligand for PKCα that binds along with calcium for normal PKCαactivation [11]. Unlike diacylglycerol, phorbol ester does not also require the binding of calcium to induce proliferation [11]. Knockout and wild-type cell cultures were treated with concentrations of phorbol ester ranging from 0-0.5 μg/mL, and observations of percent confluence were recorded.

Statistical analysis

A student's t-test was used to assess significance of PKCα in cell proliferation, with a p-value less than or equal to 0.05 considered statistically significant.

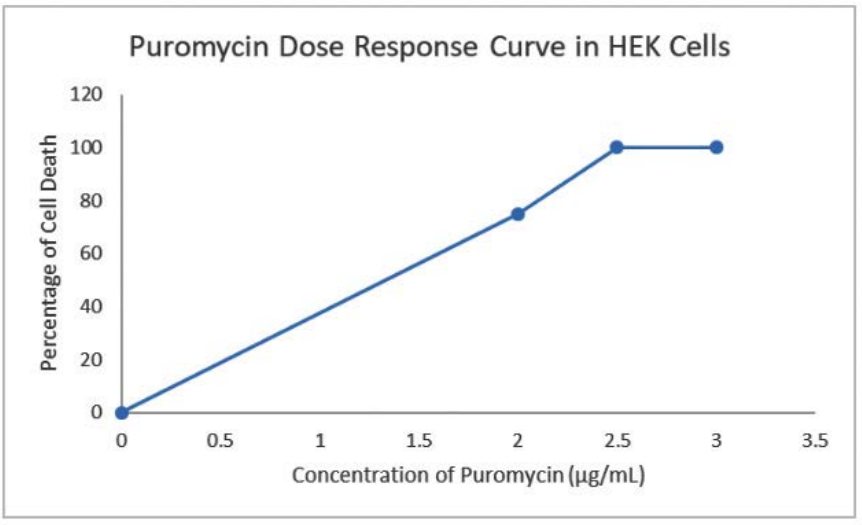


Figure 1. Puromycin dose response curve in HEK cells two days after treatment. Cells were treated with concentrations of 0, 2, 2.5 and 3 μg/mL of puromycin. A concentration of 2.5 μg/mL resulted in 100% cell death.

RESULTS

Establishing a working concentration of puromycin

In order to select for potential knockout cells using puromycin, it was important to establish a minimum level of puromycin that is toxic to wild-type HEK-293 cells. Based on previous literature, a treatment was conducted using concentrations of 0, 2, 2.5 and 3 μg/mL puromycin. Within two days of treatment, concentrations of 2.5 and 3 μg/mL resulted in 100% cell death. A puromycin concentration of 2.5 μg/mL was then selected to be used to treat transfected cells.

Confirmation of knockout

All cells were observed for RFP expression 24 hours after transfection. As expected, wild-type cells demonstrated a complete absence of RFP expression, and RFP was observed at varying levels in transfected cells. Transfected cells treated with 2.5 μg/mL of puromycin resulted in some cell death, but surviving cells displayed normal, healthy growth. Control cells were treated simultaneously with 2.5 μg/mL puromycin and resulted in 100% cell death. The remaining transfected cells were then observed under fluorescent microscopy to analyze RFP expression. **Figure 2** shows photographs depicting RFP expression in transfected cells before treatment with puromycin and one week post-treatment.

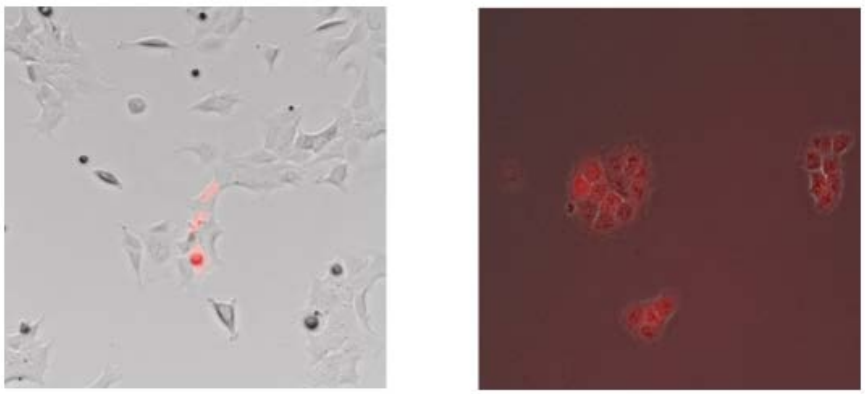


Figure 2. RFP expression using fluorescent microscopy in transfected cells before and after puromycin selection. Few transfected cells expressed RFP before puromycin selection (left), and RFP was observed at much higher levels in transfected cells after selection with puromycin (right).

Observations and cell counts for untreated cells

To monitor the general health of the knockout cell line, observations were made in comparison to wild-type HEK-293 cells. Transfected cells maintained consistent appearances with the wild-type cells and grew without much difficultly. They did, however, appear to grow at a slower rate than control cells. Cell counts were recorded to confirm this observation, where the cell doubling time of wild-type HEK-293 cells was determined to be 1.11 ± 0.04 days, while the doubling time of PRKCA KO cells was over twice as long at 2.27 ± 0.44 days (**Figure 3**, p=0.045).

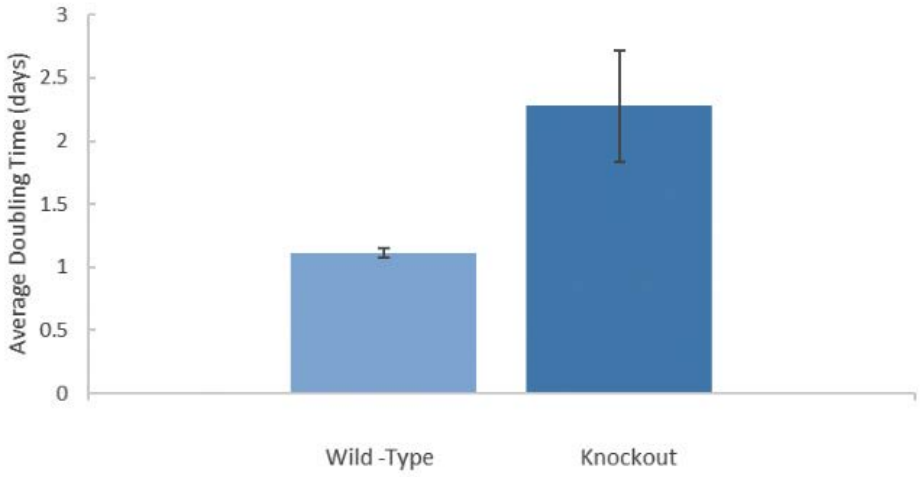


Figure 3. Cell doubling times in PRKCA knockout and wild-type HEK-293 cells. Doubling times were calculating from cell counts over a four day period. Wild-type: mean = 1.11 days, SE = 0.04. KO: mean = 2.27 days, SE = 0.44.

Observations of cells treated with sphingosine and PMA

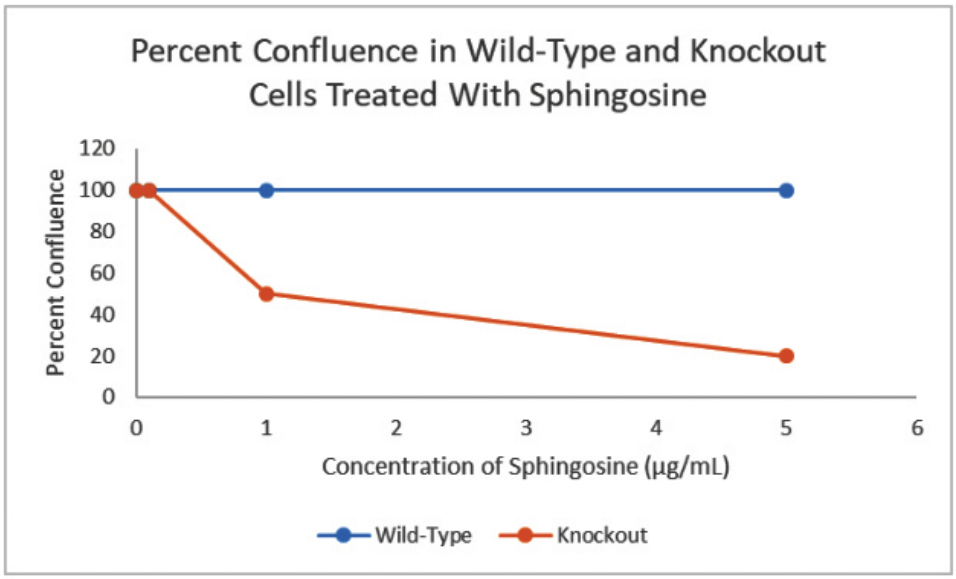
As quiescent PRKCA knockout cells grew more slowly, we also sought to determine if inducing proliferation would result in aberrant growth in PRKCA knockout cells. Treatments with sphingosine (Figure 4, top) and PMA (Figure 4, bottom) were completed. As demonstrated in **Figure 4**, these observations resulted in very high levels of growth for control cells treated with all concentrations of PMA and sphingosine. All wild-type cells reached confluence within 48 hours (data not shown). Contrary to this observation, transfected cells treated with PMA and sphingosine did not demonstrate observable growth within 48 hours. By 96 hours (four days), treated transfected cells demonstrated high levels of death ranging from 75-100% (Figure 4, top and 4, bottom). These results indicate that PKCα plays a central role in proliferative induction by both PMA and sphingosine signaling pathways.

DISCUSSION

Determination of the role of PKCα in cell proliferation was desired, so the PRKCA gene knockout was attempted, growth in normal conditions assessed, and the effects of treating knockout cells with PKCα-dependent and independent proliferation agents observed.

Knockout or homozygosity?

Within two days of transfection, RFP expression was observed in transfected cells in varying amounts. Some of this can be attributed to the fact that transfection was not successful in all treated cells. In cells displaying at least some level of RFP, however, one possible explanation for differing levels of expression involves transient expression of the HDR plasmid. Transient expression occurs when the plasmid has not been incorporated into the genome but is actively being transcribed. To account for this possibility, transfected cells were grown for an additional two weeks and



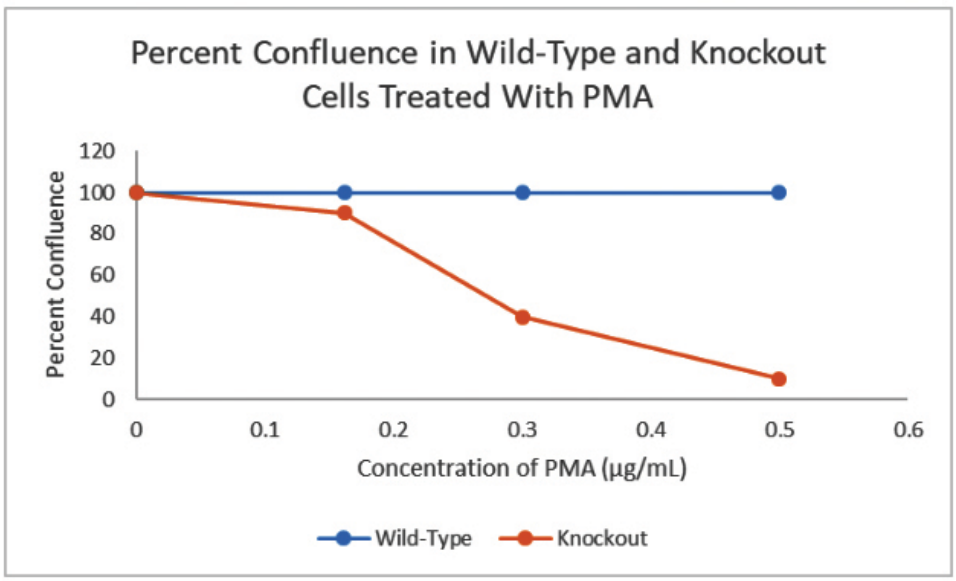


Figure 4. PKCα knockout cells treated with sphingosine (top) and PMA (bottom) exhibit reduced viability over time. Top: Cells were treated with concentrations of 0, 0.1, 1 and 5 µg/mL of sphingosine-1-phosphate. Bottom: Cells were treated with concentrations of 0, 0.162, 0.3 and 0.5 µg/mL of PMA. Confluence was assessed four days post-treatment and was observed in two cell culture wells for each cell type and treatment group.

underwent an additional puromycin treatment. Because transient expression of the HDR plasmid from Santa Cruz Biotechnology lasts a maximum of seven days, any expression of RFP over two weeks post-transfection should be attributed exclusively to genomic expression of the plasmid [3]. The vast majority – approximately 90-95% – of remaining cells demonstrated RFP after this additional treatment, which very strongly suggested transfection success.

Western blot analysis detected PKC expression in both cell lines (data not shown). Bands were observed around 80 kDa, the molecular mass of PKCα and other isoforms of PKC, in lanes containing wild-type cell lysate as well as lanes containing knockout cell lysate. There were no noticeable differences in size or expression between the bands in any exposure times. Though this appeared to contradict other indicators of knockout success, there are many possible explanations for this observed result. The quantity of secondary antibody appropriate for use in this procedure was estimated based on previous literature; however, it is highly likely that the amount used was too high and resulted in non-specific binding. Because the secondary antibody contains the horseradish enzyme that indicates expression during x-ray exposure, non-specific binding of this antibody to other forms of PKC would yield similar band expression on the final x-ray film and result in a false positive. Future studies could replicate this procedure with varying levels of secondary antibody to yield more meaningful results.

An additional explanation for varying levels of expression in RFP positive cells and the Western blot result involves the genomic location of RFP. Expression of PKCα is biallelic. Because of this, transfected cells may have been a combination of hemizygous and homozygous PRKCA knockout cells. In an attempt to select for highly-expressing homozygous knockout cells, we treated transfected cells with 10 αg/mL of puromycin (four times the concentration that is toxic to wild-type cells) before conducting proliferation studies. Cells that survived this treatment were determined to be probable knockout cells and were used for all remaining procedures; however, it is still possible that some of these surviving cells were hemizygous knockout, and this could explain the observed expression of PKCα in the Western blot analysis.

Role of PKCα in homeostatic proliferation

Cell growth calculations yielded doubling times of 1.11 ± 0.04 days and 2.27 ± 0.44 days for untreated wild-type cells and knockout cells, respectively. HEK cells are typically expected to have a doubling time of twenty-four hours, so the calculation of approximately a one day doubling time for wild-type cells is consistent with previous literature. A statistical analysis comparing the mean doubling times resulted in a p-value of 0.045. This suggests that the doubling time of PRKCA knockout cells is significantly slower than the doubling time of wild-type cells. Upon further analysis of the data, there are two main factors that contributed to the p-value: the small sample size and the high level of variance in knockout cells. Though all individual doubling times for knockout cells were slower than wild-type cells, they were less consistent. They appeared to take an especially long time to grow immediately after attachment. This observation is important in our understanding of how PRKCA knockout influences proliferation in HEK cells and how varying growth among knockout cells effects the significance of the data. Overall, observations and cell counts consistently demonstrated reduced proliferation in knockout cells, and a statistical analysis of the data yielded a significant result. In further studies, this data should be replicated with a larger sample, but our preliminary data demonstrating slowed growth supports the hypothesis that PRKCA gene knockout reduces proliferation in HEK cells.

Role of PKCα during induction of proliferation

Treatments of knockout cells in high concentrations of sphingosine and PMA resulted in abnormal levels of cell death. This result was observed in two cell culture wells for each group alongside treatment of the same concentrations on wild-type cells that experienced rapid growth and reached confluence. To assess the significance of this data, it should be replicated with a larger sample size and a wider range of concentrations for each proliferation agent. However, we are still able to discuss several possible explanations for this observation within our study. Cells that have experienced gene knockout are often less stable; gene knockout is an intensive process, and a protein such as PKCα is important in many cell processes [4]. Inducing proliferation in these cells may be received as a stressful signal that the weakened cell cannot endure. Additionally, PKCα often acts as an anti-apoptotic factor, restricting the cell's tendency to default toward death [13]. Cells without PKCα are, then, more likely to begin to progress toward apoptosis after receiving an external cellular signal. Because HEK cells are tumorigenic and PRKCA knockout cells became apoptotic when stimulated with high concentrations of proliferation agents, these data suggest that PKCα is important for normal cell function in a tumor-promoting context. Moreover, since untreated PRKCA knockout cells grew significantly slower than wild-type cells, together, these data further highlight the importance of PKCα as a key regulator of cellular proliferation.

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