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The Role of Protein Kinase C β (PKCβ) in Breast Cancer

Honors Research Thesis

Presented in Partial Fulfillment of the Requirements for Graduation *with Honors Research Distinction* in Molecular Genetics in the Undergraduate Colleges of The Ohio State University

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1. Abstract

Although the tumor promoting PKC gene was discovered in the 1980s, the exact effects of its isoforms are still not completely understood. PKCβ in particular has been implicated as having an important role in a multitude of diseases, including cancer, through both cell autonomous and cell non-autonomous mechanisms. Interestingly, PKCβ is thought to be important in blood vessel formation and inflammation, integral aspects of tumor progression. As such, it is critical that the exact function of PKCβ in cancerous tissue be understood. By focusing on the role of PKCβ in the tumor, our project helps to establish more conclusively how PKCβ contributes to breast tumor progression, both in the tumor cells and cells of the surrounding tumor microenvironment, and may potentially lead to the development of more effective therapeutics for treatment of various cancer types, including breast cancer.

2. Introduction

2.1 Protein Kinase C (PKC) Biological Function

The role of protein kinase C (PKC) in cancer has been recognized for over 20 years, with early studies in the 1980s identifying PKCs as targets of tumor promoting agents [1]. This family of serine-threonine kinases is classified into three major groups: the classical, which are Ca^{2+} and DAG (diacylglycerol) dependent; the novel, which are activated by DAG; and the atypical, which are independent of Ca^{2+} and DAG. The β isoform of protein kinase C is part of the classical group, and thus dependent on both Ca^{2+} and DAG. Figure 1 shows a sample classical PKC pathway in which phospholipase C (PLC) is activated by ligand and in turn generate DAG and IP_3 from membrane phospholipids. DAG then moves PKC to the cell membrane while IP_3 potentiates release of intracellular Ca^{2+}, both of which are utilized to activate PKC [9].

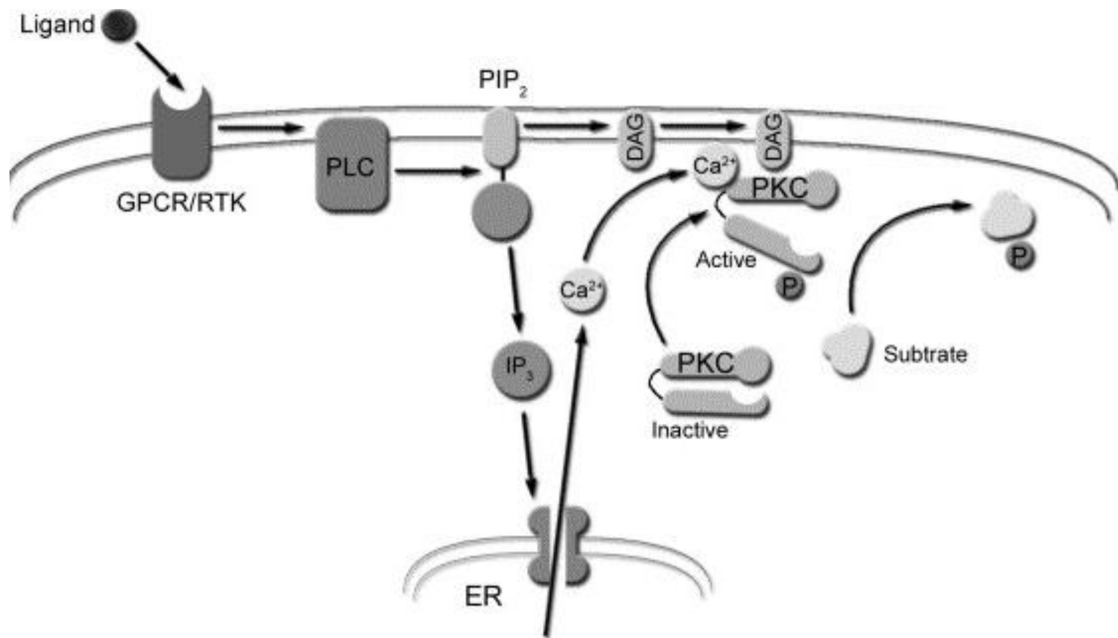

Figure 1: Classical PKC activation pathway. Adapted from "Protein Kinase C(PKC) family in cancer progression" by *Koivunen, J. et al.* [9]

Some downstream targets of PKC include ERK1/2, GSK-3β, and NFκB, which is an important mediator of inflammatory pathways [15]. Moreover, there is extensive crosstalk between the different isoforms of PKC, further complicating an examination of PKCβ activity.

2.2 Relevance

Protein kinase C has been shown to be involved in various physiological processes of cells, including proliferation, differentiation, apoptosis and migration. In fact, PKCβ is overexpressed in many cancers, including colon and prostate cancer [2]. Further studies have also demonstrated an important role for PKCβ in the growth of breast cancer cell lines [3]. In addition to its cell autonomous role in cancer cell growth, PKCβ has also been shown to be an important mediator of vascular endothelial growth factor (VEGF) signaling, which is important for tumor angiogenesis. Because of PKC's obvious role in tumor progression, a multitude of inhibitors targeting this family have been developed and tested in multiple cancer models, both in mice and more recently in humans [9]. In fact, an inhibitor specific for PKCβ, enzastaurin

(LY317615), has been shown to impair VEGF-driven tumor growth in mouse colon cancer and renal cell carcinoma xenografts [10]. Additionally, enzastaurin was shown to have antiangiogenic effects in other various cancer types, including in lung, breast, ovarian, hepatocellular and gastric cancer xenografts [11-13]. Phase II studies are also currently underway using this inhibitor for the treatment of gliomas and lymphomas [9].

Therefore, PKCβ inhibition has a promising future in the field of cancer therapeutics. Further analysis of the role of PKCβ during breast cancer progression may uncover novel functions of this gene in either tumor cells or other cells of the microenvironment, which may lead to more effective therapeutic strategies. The ultimate goal of most cancer researchers is the eventual translation of their findings into a clinical setting. Along the same lines, if we are able to determine compensatory pathways that are activated in the absence of PKCβ, dual inhibitor treatments may be more successful for the treatment of patients.

2.3 Goals and Hypothesis

For this project, we hypothesized that PKCβ deficient mice would have decreased tumor formation and progression in our model of breast cancer. Using genetic mouse modeling, we had preliminary data showing breast tumors in mice without the PKCβ gene (PKCβ$^{ko/ko}$) are smaller than those in mice with one copy of the gene (PKCβ $^{ko/wt}$) or control, wild type mice with both copies of the gene (PKCβ $^{wt/wt}$). Further characterization of these tumors was needed to determine a possible mechanism through which PKCβ could be promoting tumor growth in this model. We determined that the PyMT;PKCβ$^{ko/ko}$ mice produced palpable tumors later than the control PyMT;PKCβ $^{wt/wt}$ mice did. Furthermore, the progression of tumors in the PKCβ$^{ko/ko}$ (the double-knockout) mice was slower than it was in the wild-type mice.

Our next step was to characterize the tumors to determine the abundance of macrophages and other immune cells present, as well as blood vessel formation by endothelial cells, angiogenesis. In addition, staining for proliferation was done to determine if PKCβ influenced any of the above-mentioned processes. Subsequently, we aimed to determine whether PKCβ action in the tumor cells is important mostly within the cell (cell autonomous) or whether its function is also important in the tumor microenvironment (cell non-autonomous). Although the role of PKCβ in tumor cells and endothelial cells has been well studied, less is known about its role in macrophages and fibroblasts during tumor progression. Our experiments using the orthotopic injection model have the potential to uncover novel functions of PKCβ in these other cell types, which may also be important in tumor progression. During this project, we aimed to a) complete the tumor study with enough mice for each genetic group in order to validate our preliminary data, b) characterize tumors using various different staining techniques and c) determine whether PKCβ action is tumor cell autonomous or whether its function is important in the tumor microenvironment as well.

3. Materials and Methods

3.1 PKC;PyMT Mouse Model

To study the impact of PKCβ on breast cancer initiation and progression, we utilized a genetic mouse model in which conventional PKCβ knockout mice have been crossed with mice containing the polyoma middle T oncoprotein (PyMT) oncogene [14]. The PyMT model of mouse mammary cancer was used because it closely mimics the four stages of human breast cancer. The following figure illustrates the four stages of PyMT-mediated cancer, as well as the morphology of the stages and biomarkers involved at each stage.

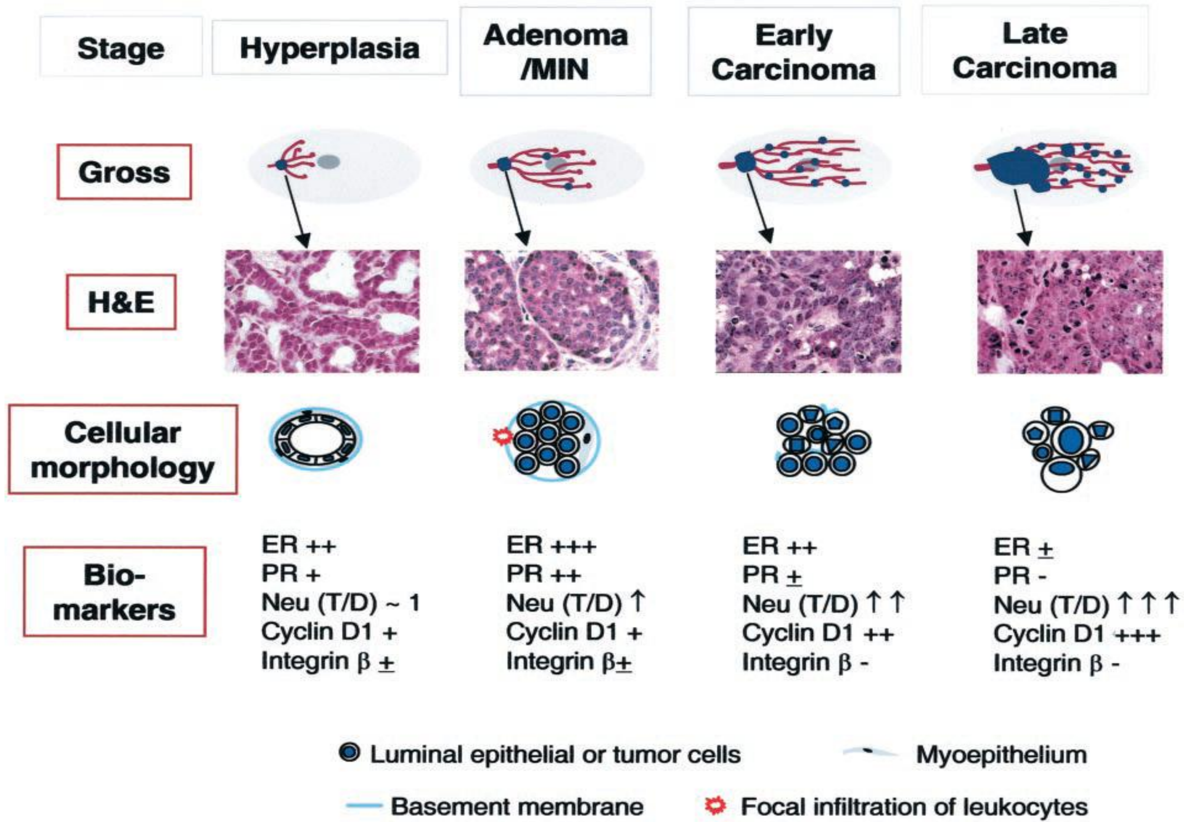

Figure 2: The PyMT model of "breast"/mammary cancer. Adapted from "Progression to Malignancy in the Polyoma Middle T Oncoprotein Mouse Breast Cancer Model Provides a Reliable Model for Human Diseases" by *Lin et al.*

The initial genetic groups of interest are as follows: PyMT;PKC $^{wt/wt}$(control), PyMT;PKC $^{wt/ko}$ (control) and PyMT;PKC $^{ko/ko}$ (experimental).

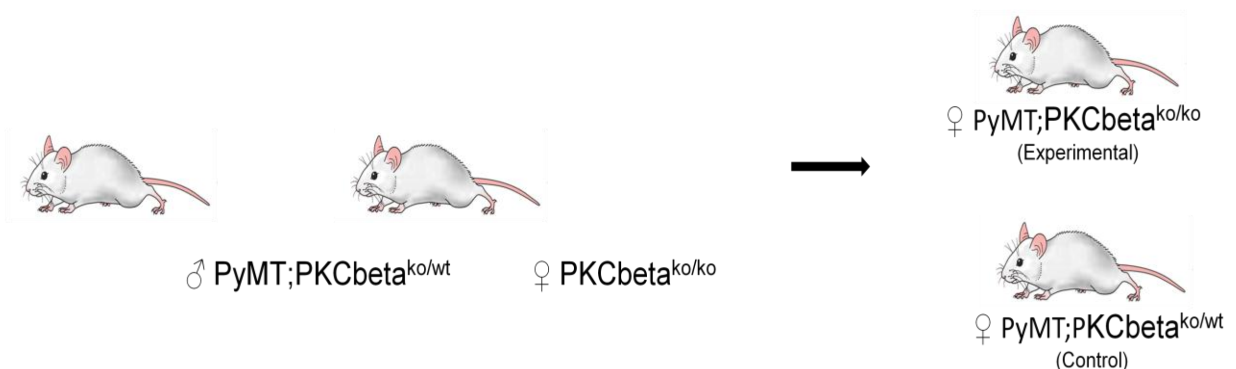

Figure 3: Original Breeding Scheme

Because we saw a significant difference between the PyMT;PKCβ $^{wt/wt}$ and PyMT;PKCβ $^{ko/ko}$ mice and because it was more feasible to investigate differences between these two groups, we bred PyMT male mice with either their knockout experimental or wild type control female non-PyMT counterparts.

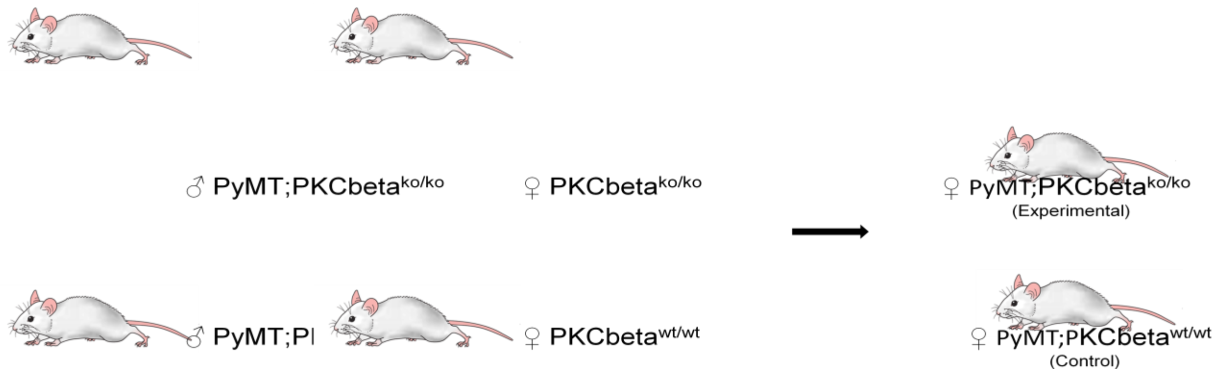

Figure 4: Current Breeding Scheme

Since the PyMT oncogene affects *breast* or in this case, *mammary* tumor progression, the male mice in breeding cages were chosen to carry the PyMT oncogene because it would take them much longer, if at all, to grow tumors.

3.2 Genotyping

After the mice were bred, they were tattooed approximately ten days after birth and a small sample of their tails were taken to be genotyped. The tails were digested overnight with proteinase K and isolated the next day following a standard DNA isolation protocol. Polymerase Chain Reaction (PCR) was used to genotype the mice to determine if they had the PyMT oncogene and which PKC genotype (ko/ko, ko/wt, wt/wt) they were. In order to look for the genes, we used primers specific and antisense to the DNA sequence of the genes. The primers were added to the mix of buffer, DNA, dNTPs, and the enzyme Taq polymerase.

3.3 Tumor Study Protocol: The Genetic Model

Starting at three months of age, mice with the aforementioned genotypes are palpated bi-weekly. When palpable tumors were felt, we allowed three weeks for the tumors to progress, after which the mammary glands from the mice were harvested. Using this strategy, we were able to determine how long it takes the mice to get palpable tumors, as well as the rate of progression of the tumors over the three-week time point. At the time of harvest, tumor burden was measured as a ratio of tumor weight/total body weight of the mouse, and the total tumor volume was calculated for each mouse. To calculate the total tumor volume (v), the tumor length (l), width (w), and height (h) were measured and subsequently, the measurements were multiplied to determine volume (lxwxh=v). Each tumor was then either frozen or fixed in formalin for later histological analysis, or saved to extract DNA, RNA or protein for further analysis. In addition to the primary mammary tumors, the lungs from the mice are also fixed in formalin for histological analysis.

3.4 Injection Study Protocol

For the orthotopic injection study, in which we aimed to determine if the function of PKCβ is tumor cell autonomous, we injected 3.0 x 10^6 tumor cells (from the B6 PyMT cell line) that were wild type for the PyMT gene into the mammary fat pad (both mammary glands 4 and 9) of either PKCβ $^{ko/ko}$ or PKCβ $^{wt/wt}$ mice. Tumor progression was then monitored, with the tumors being palpated once every week. Tissue was harvested between two-three weeks after injection. We used the same harvesting protocol for collection of the injected tumors as we used in the genetic model. The harvested mammary gland 4 was frozen in OTC sections and mammary gland 9 was fixed in formalin. The sections were then cut, with one hematoxylin and

eosin (H/E) stained slide per section, and stained for angiogenesis via the Meca32 antibody, proliferation via Ki67, and macrophage activity via F4/80 antibody.

The B6 PyMT cells to be injected were initially frozen tumor cells. We thawed and cultured the cells in media, incubating them at 37°C. The tumor cells were usually split 1:3 once they became confluent on the plate. The cells were passaged approximately three or four times before they were injected into the mammary fat pads of the mice.

3.5 Immunofluorescent Staining Protocol

Six sections from three pairs of mice born at the same time were stained for the tumor study, with three of the sections from knockout mice and three from wild type mice. Six sections were also stained for the injection study in the second part of the project, with three of the sections from knockout mice and three from wild type. In order to probe for macrophages, proliferation, and angiogenesis, immunofluorescent staining was used on these paraffin sections of tissue. The F4/80 primary antibody was utilized to look for inflammation and macrophage activity, Ki67 primary antibody for proliferation, and the Meca32 antibody for angiogenesis.

3.6 F4/80 and Ki67 Analysis

As mentioned, the F4/80 primary antibody was utilized to probe for macrophages. Once the sections were stained, they were photographed using a fluorescent microscope. Five pictures were taken for each of the slides. The macrophages in each picture were counted using the counting tool in Adobe Photoshop CS5. The total nuclei in each picture, which were stained with DAPI, were also counted and the number of macrophages was taken as a percent of the total number of cells in the picture ((100%)(#macrophages/#DAPI)).

Ki67 was used to probe for proliferating cells. Again, the slides for each section were photographed using a fluorescent microscope, with five photos of each slide. The cells positive

for Ki67 were counted using the counting tool in Adobe Photoshop CS5. The total number of nuclei, stained with DAPI, was also counted and the positive Ki67 cells were taken as a percentage of the total number of cells stained with DAPI ((100%)(#Ki67/#DAPI)).

3.7 Meca32 Analysis

In order to examine any angiogenesis occurring in the tumor sections, the Meca32 primary antibody was utilized. Once the sections were stained, five pictures of each slide were taken using a fluorescent microscope. The level of staining in each of the pictures was then measured using the photo analysis program Fiji. The amount of staining was quantified as percent area stained with Meca32. There were three pairs of mice (three knockouts and three wild types), on which tumors were palpated around the same time for the genetic tumor study, and three pairs again for the orthotopic injection study.

3.8 Statistical Analyses

All statistical analyses were conducted by the statistics department. Because the raw data sets for this project contained non-normal data points, the Mann-Whitney test was utilized to measure statistical significance. This test accounts for the variation present in the data set, and gives a p-value to describe the confidence level present in the trends seen in the data. We used a p-value of 0.05 as a cut off for confidence level because this cut-off indicated that essentially, the trends we saw in data would only have happened 5% of the time spontaneously.

4. Results

4.1 Preliminary results

During the preliminary phase of this study, the data indicated that there was potentially a significant difference in the tumor burdens and tumor volumes of the wild type mice with PKCβ,

PyMT;PKCβ $^{wt/wt}$, and the experimental knockout mice, PyMT;PKCβ $^{ko/ko}$. In order to further examine this difference, PyMT;PKCβ $^{wt/wt}$ and PyMT;PKCβ $^{ko/ko}$ mice were bred. From preliminary results, we had determined that tumors could be palpated at approximately three months of age and, therefore, we utilized that age marker (three months) to begin palpating for tumors.

4.2 PKCβ in the genetic model

For this project, we hypothesized that deletion of PKCβ would cause decreased tumor formation and progression in a mouse model of breast cancer. Using genetic mouse modeling, we determined that the PKCβ $^{ko/ko}$ mice produced smaller palpable tumors than the control PKCβ $^{wt/wt}$ mice did.

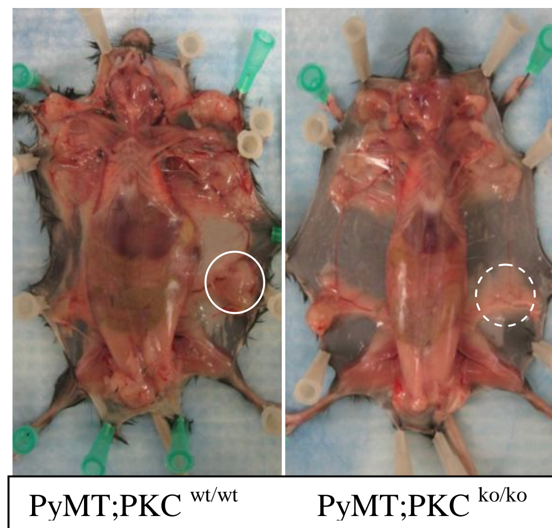

Figure 5: Representative pictures of wild type and experimental mice for the genetic model. The circle indicates the tumor in mouse mammary gland 1.

Figure 5 shows a representative example of the visual difference in tumor size. The circle on the dissection pictures shows the mammary tumor in mammary gland 1 of the mouse. From the pictures, it is evident that the PyMT;PKCβ $^{wt/wt}$ control genotype has a much greater tumor load than the PyMT;PKCβ $^{ko/ko}$ experimental genotype. Furthermore, analysis of data for tumor

burden and tumor volume for the genetic model revealed a statistically significant difference between our two groups of interest, PyMT:PKCβ $^{wt/wt}$ and PyMT:PKCβ $^{ko/ko}$, with a p-value of 0.0418.

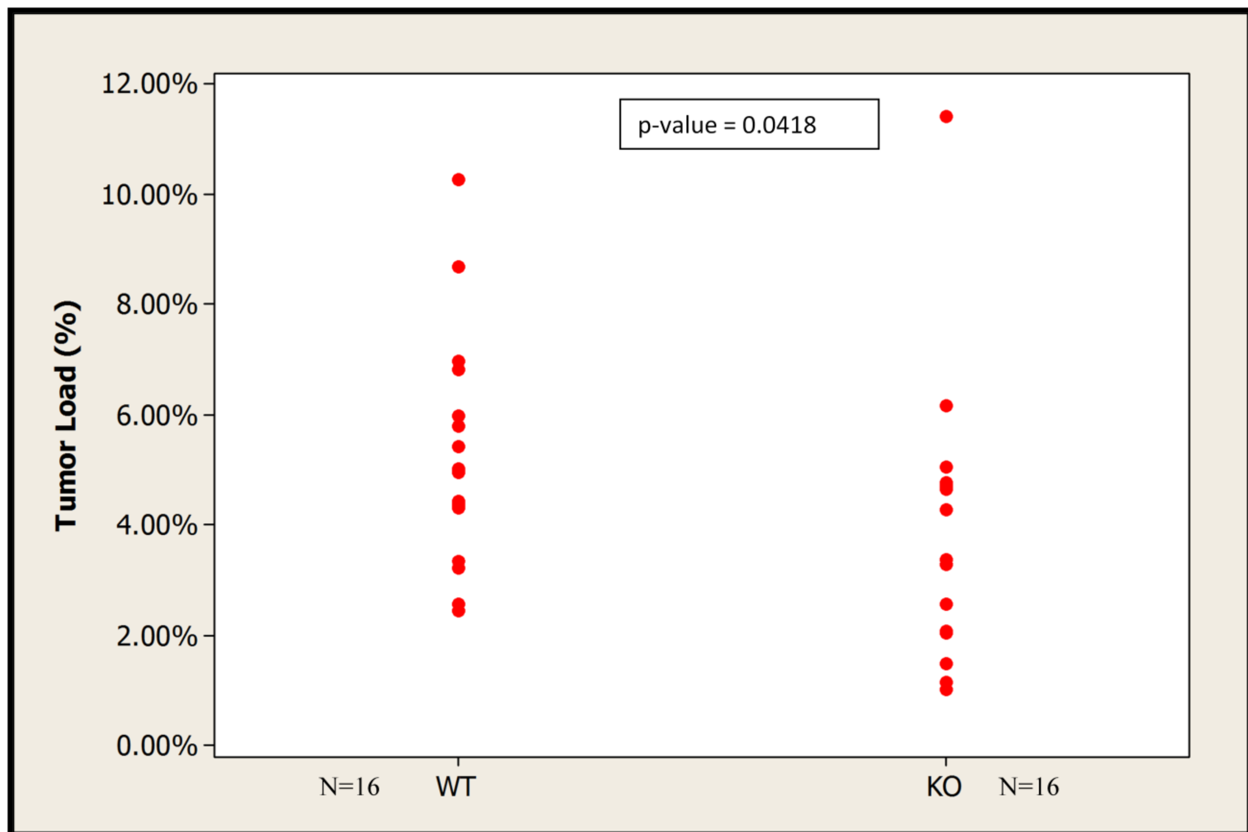

Figure 6: Graphical representation of the differences in tumor burden observed during the tumor study, using the Mann-Whitney statistical test.

These data indicate that the absence of PKCβ in the mouse model has a measurable impact on the mammary tumor phenotype.

Furthermore, we also noted the time when the first tumor was palpated, the time-to-tumor ratio, in order to calculate a survival curve. With a p-value of 0.057, the survival plot appears to be trending towards significance, indicating that there is likely a trend between the knockout

experimental mice and the wild-type control mice. This data suggested that the mammary tumors in the wild-type mice tend to progress faster than they do in the knockout mice.

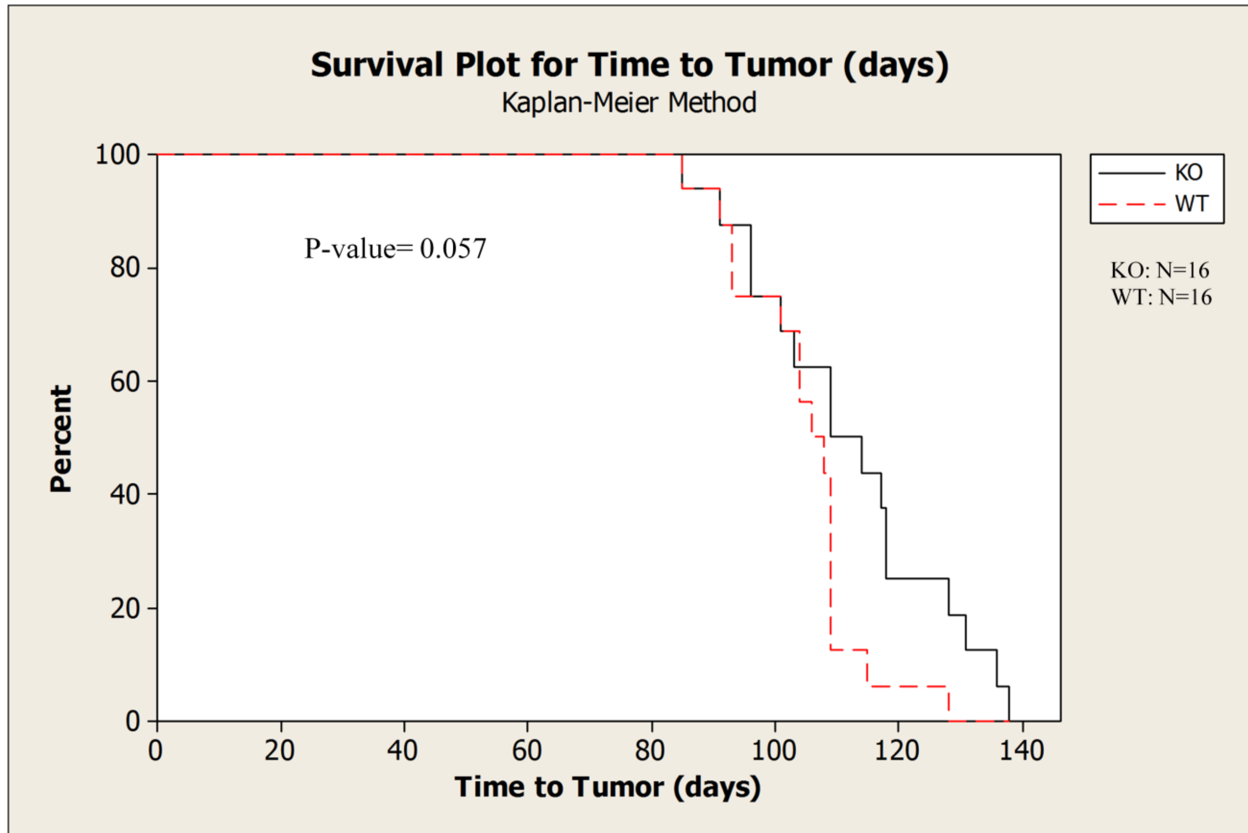

Figure 7: Survival plot for time to tumor for the PyMT;PKCβ $^{wt/wt}$ control and PyMT;PKCβ $^{ko/ko}$ mice.

4.3 PKCβ in the orthotopic injection model

In order to determine if PKCβ function was cell autonomous or non-cell autonomous, we injected B6 PyMT tumor cells into the mammary fat pad of either PKCβ $^{ko/ko}$ or PKCβ $^{wt/wt}$ mice. The cultured cells went through approximately three to four passages before being injected into both mammary glands four and nine; 3.0×10^6 PyMT B6 cells were injected. Once tumors were palpated to 1-cm and harvested, tumor burden and tumor volume were measured. There were 15 mice in the PKCβ $^{wt/wt}$ control group and 15 mice in the PKCβ $^{ko/ko}$ experimental group. We

observed similar differences in tumor volume, strong evidence supporting the tumor-promoting role of PKCβ in the tumor microenvironment.

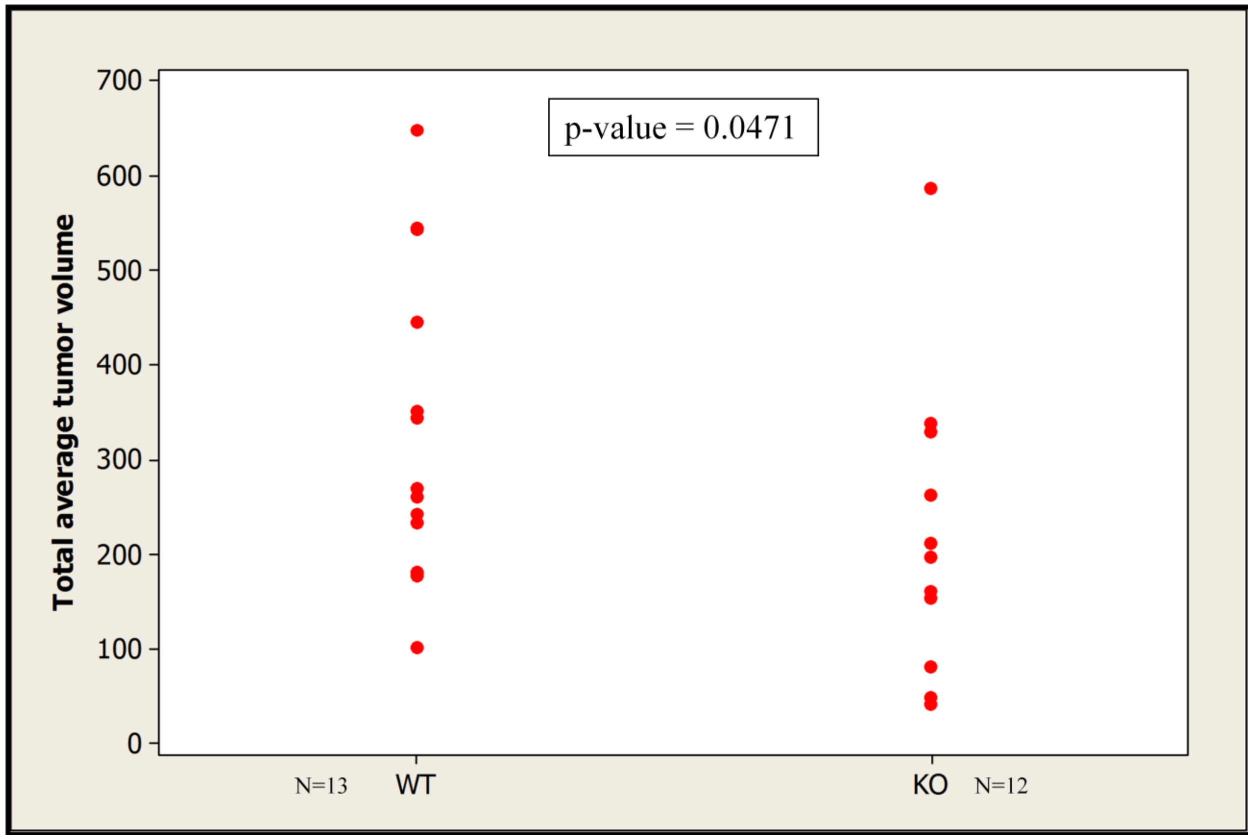

Figure 7: Graphical representation of the differences in total average tumor volume observed during the orthotopic injection study, using the Mann-Whitney statistical test.

Analysis of these data revealed that on average the PKCβ $^{wt/wt}$ mice had a greater tumor volume than the PKCβ $^{ko/ko}$ mice did. This result was statistically significant with a p-value of 0.0471.

4.4 PKCβ does not appear to influence angiogenesis

A review of past literature about PKCβ had indicated its importance in various cellular processes, including angiogenesis. Using immunofluorescent staining, we probed for vascularization with the Meca32 antibody.

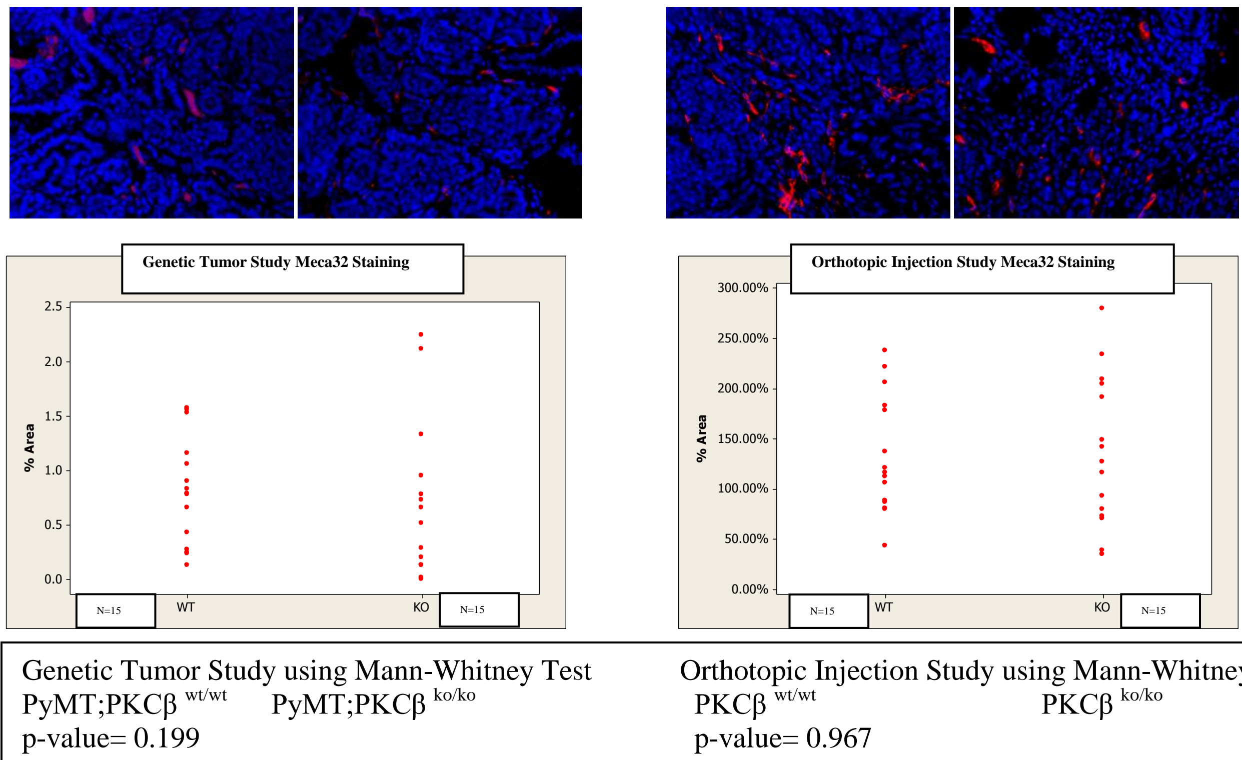

Figure 8: Representative pictures of Meca32 Immunofluorescent staining for angiogenesis in the genetic tumor study and orthotopic injection study.

We had initially hypothesized that vascularization of the tumor tissue might be able to explain, at least in part, the difference in tumor burden observed between the two groups of interest (PyMT;PKCβ $^{wt/wt}$ and PyMT;PKCβ $^{ko/ko}$) in the genetic model. However, further analysis of the staining revealed little difference in vascularization between the two groups. In fact, with a p-value of 0.199, any difference that was present was not statistically significant.

Furthermore, the tumor samples from the injection model were stained with the Meca32 antibody and subsequently analyzed for their level of angiogenesis. With a p-value of 0.967, any differences between the control PKCβ $^{wt/wt}$ and the experimental PKCβ $^{ko/ko}$ groups for angiogenesis were not statistically significant for the injection model as well. For both the genetic model and the injection model, the staining was analyzed using the Fiji program to measure the percent area covered by the staining compared to the area covered in the picture.

4.5 PKCβ does influence inflammation

Since PKCβ is also involved in inflammatory and immune responses, we examined the populations of macrophages present in the tumor cells. We utilized the F4/80 primary antibody and an immunofluorescent secondary antibody to probe for macrophages. While counting the number of macrophages in each picture, we observed a marked difference between the average numbers of macrophages present in the PyMT;PKC $^{wt/wt}$ control group and the PyMT;PKC $^{ko/ko}$ experimental group.

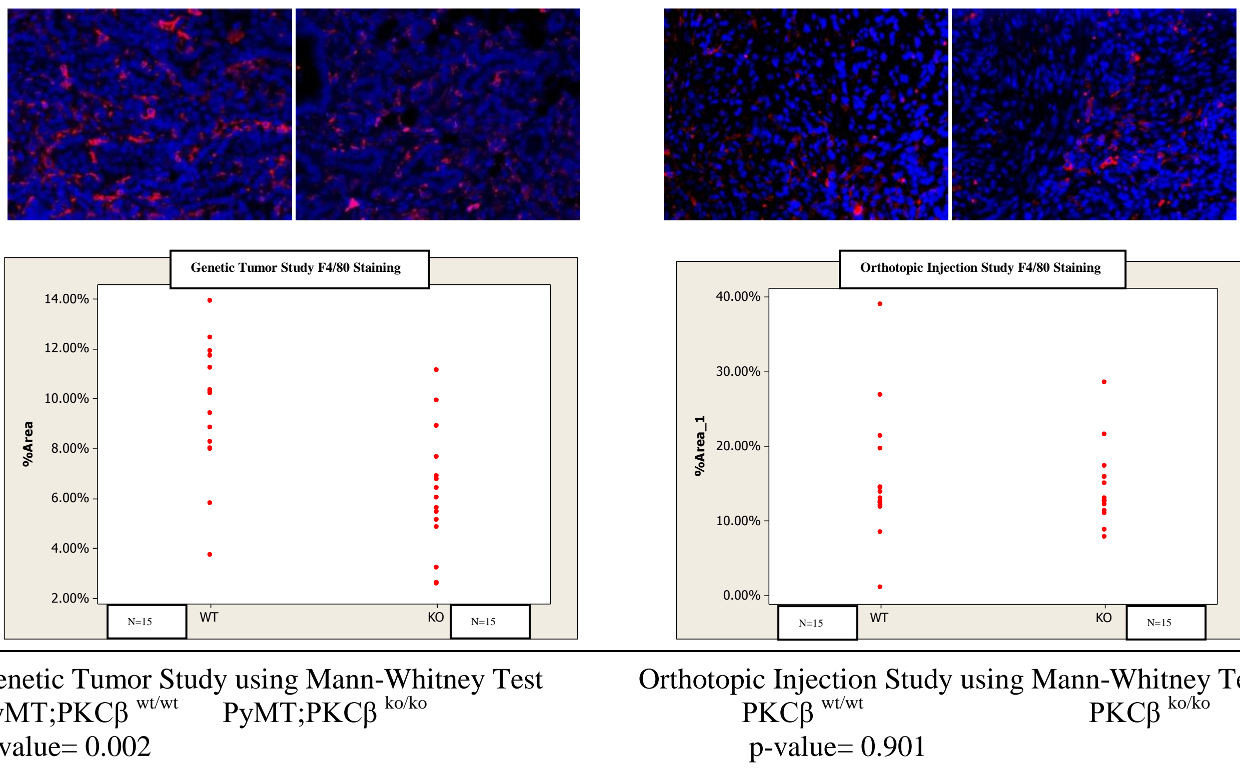

Figure 9: Representative pictures of F4/80 Immunofluorescent staining for inflammation and immune response in the genetic tumor study and orthotopic injection study.

Analysis of the F4/80 staining data revealed a significant difference in the average number of macrophages in each of the groups. On average for the genetic tumor study, the wild-type control mice appeared to have markedly greater inflammation in terms of macrophages than

the experimental knockout mice did. The analysis supported our observations during the analysis/counting phase.

Similarly, we examined the number of macrophages in the injection study. Counting the macrophages for the two groups and subsequent analysis of the data revealed that there was no significant difference between the PKCβ$^{wt/wt}$ and PKCβ$^{ko/ko}$ groups. Figure 9 shows representative pictures of the F4/80 macrophage staining for both the genetic tumor study and for the orthotopic injection study.

4.6 PKCβ function does appear to affect proliferation of tumor cells

In addition to probing for angiogenesis via the Meca32 antibody and inflammation via the F4/80 antibody, we also examined the levels of proliferation in the tumor tissue.

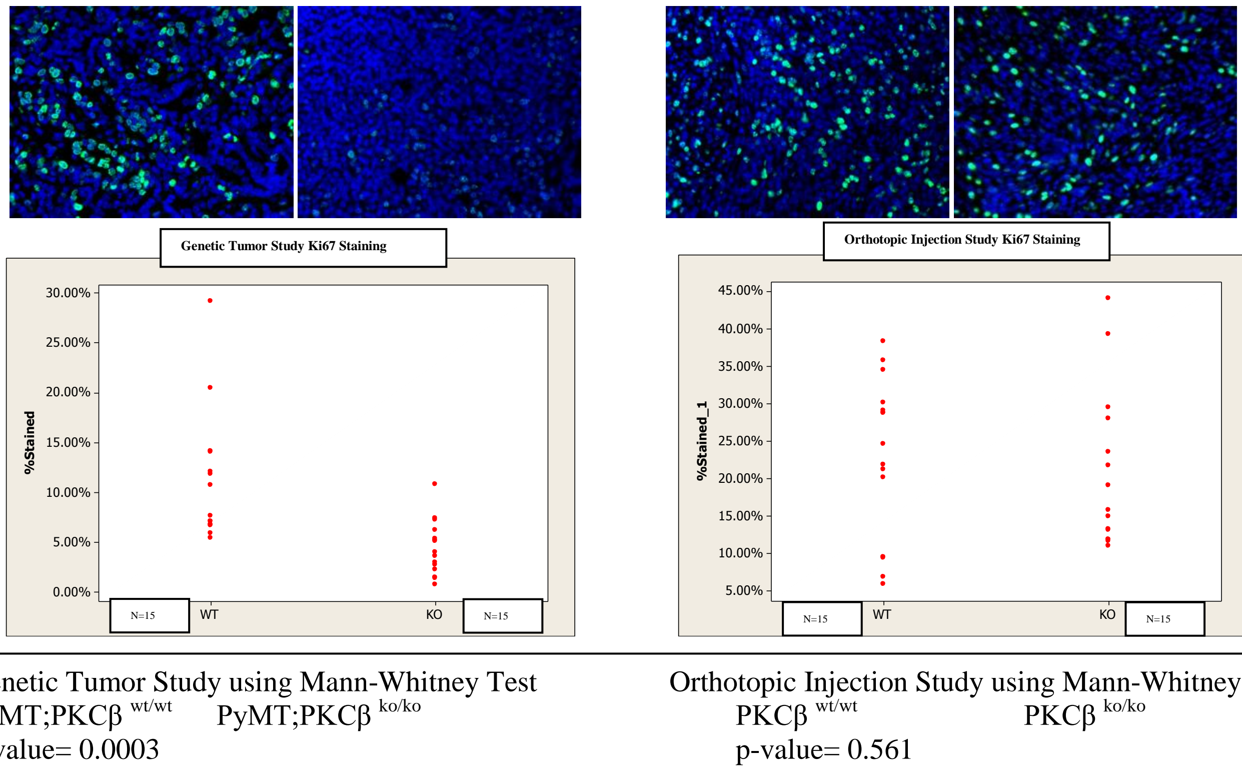

Figure 10: Representative pictures of F4/80 Immunofluorescent staining for inflammation and immune response in the genetic tumor study and orthotopic injection study.

We utilized the Ki67 antibody to probe for proliferating and cell division. As can be seen from the representative pictures, in the genetic model, we found a large difference between the control PyMT;PKCβ $^{wt/wt}$ mice and the experimental PyMT;PKCβ $^{ko/ko}$ mice. The wild-type mice appeared to have much more Ki67 staining, indicating that those tumor cells were more proliferative than those of the knockout mice. In fact, further analysis of the data revealed a p-value of 0.0003, indicating that the disparity between the two groups of interest was statistically significant. Additionally, we examined proliferation in the orthotopic injection study using the same counting method we had utilized for analysis of the Ki67 tumor study data. There appeared to be no significant difference between the PKCβ $^{wt/wt}$ control group and the PKCβ $^{ko/ko}$ experimental group, as seen from the p-value of 0.561.

5. Discussion

5.1 The genetic basis of PKCβ function

In addition to being involved in various physiological and cellular processes, PKCβ has been found to play an important role in various types of cancer. PKCβ expression has been shown to be upregulated in colon and prostate cancers, and downregulated in bladder cancer. Additionally, transgenic mice overexpressing PKCβII have hyperproliferation of the colonic epithelium and are sensitive to carcinogen-induced colon cancer [7]. Furthermore, PKCβ appears to be activated in response to VEGF receptor activation, and is important mediator of VEGF induced proliferation of endothelial cells. *In vitro* studies found that inhibition of PKCβ inhibited the growth of several breast cancer cell lines, but not normal mammary epithelial cells [3]. As such, PKCβ is an obvious target for further study.

The *in vivo* studies conducted in this project revealed that PKCβ has a measurable impact on mammary tumors. The significant difference between the control PyMT;PKCβ $^{wt/wt}$ and

experimental PyMT;PKCβ $^{ko/ko}$ groups indicated that PKCβ deficiency leads on average to smaller total tumor volumes. Moreover, PKCβ also leads to a decreased tumor burden in these mice. These results imply that the presence of PKCβ affects important aspects of tumor growth to be able to produce such significant differences in the *in vivo* genetic mouse model of breast cancer.

While conducting the genetic study, we also recorded the time in days when the first mammary tumor was palpated. The p-value for the time-to-tumor ratio, p=0.057, indicated that although not statistically significant, the data set was trending towards significance. The wild-type mice appeared to have a much faster progression of the tumors once they were first palpated, whereas the knockout mice appeared to have slower progression of the disease after first palpation. Additionally, a large number of the PKCβ wild-type mice (PyMT;PKCβ $^{wt/wt}$) had palpable tumors at 110 days, while tumor initiation in the knockout mice (PyMT;PKCβ $^{ko/ko}$) was more variable, although generally later than 110 days as seen in Figure 7. Even though this data is not significant with a p-value of 0.057, it is important to note that it is trending towards significance, indicating that the potential delay in tumor progression observed in the knockout might be a demonstrable effect.

In order to explicate these results, we conducted further staining and subsequent analysis. The three main areas we examined were angiogenesis due to the link between PKCβ and VEGF, inflammation since PKCβ function also affects NFκB, and proliferation, as overexpression of PKCβ was previously shown to induce hyperproliferative colonic epithelium [7]. In the genetic model, we discovered that PKCβ wild-type mice produced hyperproliferative mammary tumor cells and tended to have more inflammation in the tumor tissue. The PKCβ deficient mice did not

exhibit either of these phenotypes in their mammary tumor tissues, indicating that PKCβ function is likely influencing cell division and immune/inflammatory responses in the mice.

5.2 PKCβ function in the tumor microenvironment

To further examine whether PKCβ function in our mouse model was cell-autonomous or non cell-autonomous, we conducted the orthotopic injection study. The mammary tumor burden in this study tended to be significantly higher for the PKCβ $^{wt/wt}$ mice than for the PKCβ $^{ko/ko}$ mice. However, in contrast to the results of the staining for the tumor study, although we found that there was a statistically significant different between the PKCβ $^{wt/wt}$ mice and PKCβ $^{ko/ko}$ mice in the injection study, our staining revealed no significant difference in the levels of endothelial cells (angiogenesis and vascularization), macrophages (inflammation), and cell division (proliferation). Nevertheless, the fact that there appeared to be a difference in tumor burden for the injection study indicates that PKCβ may play a more involved role in the tumor microenvironment, hinting at a non cell-autonomous function for this tumor promoting gene. Further research is needed to determine conclusively if PKCβ functions largely in a cell autonomous or non cell-autonomous manner in breast cancer models.

5.3 Future Directions

Since the injection study implied that PKCβ might function non cell-autonomously in the tumor stroma, conditional knockout mice for various stromal compartments are necessary. Once those mice have been obtained, a genetic tumor study, similar to the tumor study conducted during this project, is needed.

In addition, PKC β is further implicated in obesity as previous work from the Mehta lab has shown that loss of PKC β function protects mice against diet-induced obesity. Since it has been established that obesity is a risk factor for breast cancer in post-menopausal women, PKC β

may contribute to development of breast cancer. As such, the link between PKCβ and obesity needs to be further explored. In the future, with PyMT;PKCβ$^{wt/wt}$ control mice and PyMT;PKCβ $^{ko/ko}$ mice on a high fat, obesity-inducing diet, a genetic study could be conducted. This study would reveal whether PKCβ function in obese mice induces more aggressive, more frequent, or earlier mammary tumors.

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