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14. ABSTRACT

We have determined that the Wnt/B-catenin signaling pathway is active within a small subset of cells within the mammary gland. This small subset is entirely contained within the previously identified population that has the ability to repopulate a cleared mammary fat pad. Thus, activation of canonical Wnt signaling may be a very specific marker for mammary stem cells and be a target for transformation that results in the formation of aggressive mammary tumors.

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Introduction

The overarching goal of this proposal was to test the hypothesis **that activated canonical Wnt signaling identifies mammary stem cells and mammary tumor cells with cancer stem cell properties.** Our objective is to determine whether this is the case by transplantation experiments of normal mammary cells and mammary tumor cells that exhibit activated canonical Wnt signaling.

Activation of the Wnt pathway is associated with abnormal mouse mammary development, tumorigenesis, and human breast cancer. In addition, increasing evidence suggests that tumors arise from either normal stem or progenitor cells through the deregulation of self-renewal processes [1]. The existence of mammary stem cells was established by transplantation experiments in mice. For example, a functioning ductal tree can be regenerated using very few transplanted mammary cells carrying CD24 and CD49f cell surface markers: although only 1 in 20 to 90 $CD24^{+}/CD49f^{++}$ mammary cells is a true stem cell [2]. We have found that activated Wnt/β-catenin signaling is restricted to a sub-population of mammary tumor cells that are $CD24^{+}/CD49^{++}$. Since Wnt pathway activation occurs in human breast cancer and is required for proliferation of various other stem cell compartments, addressing how Wnt signaling promotes mammary stem cell renewal and the role this plays in breast cancer will ultimately lead to more effective treatments for breast cancer.

Body

Note: We have organized this section to include each of the eight tasks outlined in the "Statement of Work" (SOW) followed by a summary of the work done toward completing each of these tasks. The original tasks are listed with bold type.

Aim 1: To determine whether canonical Wnt signaling through β-catenin is associated with specific markers of mammary stem cell activity.

Task 1. Collect mammary glands from *BATgal* transgenic female mice [3] at different stages of mammary development: embryonic (day 12.5 and 18.5), newborn (7 day-old), juvenile (5 week-old), adult virgin (12 week-old) and pregnant (day 6.5, 12.5 and 18.5). Mammary glands from four mice per time point will be isolated. A total number of 32 female mice will be included in this part of the study. One abdominal mammary gland will be used for whole mount LacZ staining to determine the expression pattern of β-galactosidase (β-gal). *BATgal* Wnt reporter mice exhibit expression of β-gal in cells that have activated Wnt/β-catenin signaling. Hence, β-gal expression can be used as a marker for canonical Wnt signaling. Mammary whole mount LacZ staining will be performed at the time the mammary gland is isolated.

The other abdominal and both inguinal glands will be processed to single cell mammary cell suspensions. Any lymph nodes will be removed. The isolated glands will be chopped into small pieces using scissors and then dissociated into single cells using a standard protocol that includes digestion with collagenase/hyaluronidase followed by trypsin, dispase/DNase, and ammonium chloride. The

cell suspension will be filtered and then frozen and stored in liquid nitrogen until required for further analysis.

We will similarly collect tissues and cells from *BATgal;MMTV-Wnt1*-induced mammary hyperplasia and tumors [4]. *BATgal;MMTV-Wnt1* mammary glands and mammary tumor tissues will be analyzed for β-gal expression using LacZ and single cell suspension will be isolated, frozen and stored as well. We will collect mammary glands from eight *BATgal;MMTV-Wnt1* females and tumor tissues and cells from eight tumor-bearing mice. Collection of mammary whole mounts and tumor tissues, LacZ staining and isolation of single mammary cell and tumor cell suspensions will take approximately one year. A total number of 48 female mice will be included.

In previous reports we have clearly showed that cells with activated canonical Wnt signaling are present within the mammary epithelium starting at embryonic day 12.5 through adulthood as determined by LacZ staining. There is a significant expansion of cells undergoing canonical Wnt signaling in *MMTV-Wnt1* pre-neoplastic mammary glands compared to controls. This data shows that Wnt-responsive cells reside in the mammary stem cell niche as determined by Lac Z staining of *BATgal* reporter animals.

The glands from *BATgal* and pre-neoplastic and tumorigenic *BATgal;MMTV-Wnt1* animals that were not used for LacZ staining were isolated and frozen as single cell suspensions of mammary epithelial cells (MEC's). These cells are being used to accomplish Aim 3 of the SOW.

<u>Task 2.</u> We will analyze single mammary cell suspension by FACS using cell surface markers and the β-gal substrate DDAOG. We will first label single cell suspensions with CD31, CD45, and TER119. These antibodies mark endothelial and hematopoietic cells (Lin^+) and will be excluded from further analysis. The remaining Lin^- mammary cells will be labeled with CD24 and CD49f which marks the stem cell enriched cell population. We will identify cells with canonical Wnt signaling by staining for β-gal reporter gene activity using the β-gal substrate DDAOG. DDAOG is detectable in far red. FACS analysis of Wnt canonical signaling activity at different time points of mammary development and of Wnt1-induced mammary tumors will take approximately two months to complete.

As discussed in last years report we are using FDG (fluorescein di-β-D-galactopyranoside) as a substrate for β-gal activity. We are currently characterizing the expression levels of CD24 and CD49f within FDG+ cells to determine if this population of cells is enriched for stem cell activity. Our past data has shown that there is a 2-fold increase in the number of FDG^+ MEC's in *BATgal* animals compared to controls which is further supported by our LacZ staining. We are currently determining the percentage of FDG^+ cells within pre-neoplastic and tumorogenic *BATgal/MMTV-Wnt1* MEC's by FACS analysis.

<u>Task 3.</u> Determine the hormone receptor status of mammary cells with activated Wnt signaling. We will isolate cells with and without active canonical Wnt signaling onto microscopic slides and perform immunohistochemistry for the estrogen and the progesterone receptors using commercially available antibodies from Cell Signaling Technologies. These experiments will be performed in adjunction to the FACS experiments described in Task 2 and will take two months to complete.

We have optimized antibodies against the estrogen and the progesterone receptor for use on the Ventana automated staining system. We have stained sections of *BATgal/MMTV-Wnt1* tumor tissue and found that these tumors express estrogen receptor. Previously we have using LacZ staining to determine β-gal activity within the mammary gland, which renders the tissue unusable for immunohistochemistry. We are currently optimizing a new antibody for β-galactosidase on the Ventana system in the hopes of co-staining our slides for β-gal, ER and PR to determine the hormone receptor status of β-gal positive cells.

Aim 2. To determine the stem cell activity of mammary cells with activated Wnt/β-catenin signaling.

<u>Task 1</u>. We will test whether Wnt responsive cells possess the majority of the stem cell activity by limiting dilution transplantation experiments. $Lin^-/CD24^+/CD49f^{++}/DDAOG^+$ (test) and $Lin^-/CD24^+/CD49f^{++}/DDAOG^-$ (control) mammary cells from adult virgin *BATgal* transgenic females will be isolated by FACS as described under Aim 1. Cells with canonical Wnt signaling will be identified as $DDAOG^+$. Based on our previous experience, we will need approximately 8 *BATgal* transgenic female mice to get enough sorted mammary cells for the transplantation experiments.

We are currently working with our FACS core to sort sort $Lin^-/CD24^+/CD49f^{++}/FDG^+$ (test) and $Lin^-/CD24^+/CD49f^{++}/FDG^-$ (control) cells for transplantation into the cleared fat pad of NSG mice. Our FACS core has undergone a change in management leading to a delay in completing this work. Currently our facility is considering purchasing a newer BD FACS Aria, which should help with our inconsistencies in cell viability post sort.

<u>Task 2</u>. We will inject 10, 50, 100, and 1000 test and control cells into the cleared fat pads of 3-week-old *Rag2*-deficient females. We will inject 10 $Rag2^{-/-}$ females with 10 test cells into one abdominal mammary fat pad and 10 control cells into the contralateral fat pad, 30 $Rag2^{-/-}$ females with 50 test and 50 control cells, 10 $Rag2^{-/-}$ females with 100 test and 100 control cells, and 10 $Rag2^{-/-}$ females with 1000 test and 1000 control cells. We will isolate the host fat pads after six weeks and determine ductal out-growth. We estimated the number of animals to be used for this via the following rationale. Limiting dilution experiments have found that on average 1/64 $Lin^-/CD24^+/CD49f^{++}$cells is a mammary stem cell. Hence, transplanting 10 $Rag2^{-/-}$ females with 10 test and control cells should result in around 3 out-growths with control cells. If we assume that the presence of canonical Wnt signaling will be associated with a three-fold enrichment of stem cells, we would predict that 90% (9

out of 10) of the transplants with test cells will repopulate the cleared mammary fat pad. Power analysis (powered to 80% for a Type I error of 0.05) suggests that it will be necessary to use 10 animals in each group. If we assume that 75% of cleared mammary fat pads will be repopulated via injection of 50 control cells and all will be repopulated with test cells (assuming at least a three fold enrichment), power calcuations (again powered to 80% with a Type I error of 0.05) suggest we will need to use at least 27 *Rag2*$^{-/-}$ females for each group. We predict that virtually all cleared fat pads will be repopulated when either 100 or 1000 control cells are injected. Therefore, we will inject 5 *Rag2*$^{-/-}$ females with test and control cells at 100 cells/gland and 1000 cells/gland and evaluate these as positive controls for these experiments. These experiments will take one year to complete and will include 60 *Rag2*$^{-/-}$ females.

As mentioned in our previous report we have switched our recipient animal model from *Rag2*-deficient females to NOD/SCID/IL-2 receptor gamma females (NSG) as transplantation recipients. NSG mice have recently been shown in a variety of settings to be a superior and more efficient choice for these types of studies (for example see [6]). We have acquired NSG and are currently expanding the colony. We have a sufficient number of animals to perform the transplantation experiments once cells have been sorted.

As stated in task 1 of this aim once the FACS core is up and running we will be able to sort cells for transplantation experiments.

Aim 3. To determine the tumorigenicity of *MMTV-Wnt1* tumor cells with activated Wnt/β-catenin signaling.

<u>Task 1</u>. We will test whether β-gal^{+} cells isolated from *BATgal/MMTV-Wnt1* mammary tumors specifically confer tumorigenesis in a transplantation model. When tumors develop in *BATgal/MMTV-Wnt1* females we will dissect the tumors and isolate single cell suspensions using the automated mechanical disaggregation system Medimachine from Becton Dickinson. The sorted tumor cells will be frozen and stored in liquid nitrogen until needed for further analysis.

We have taken another approach to understanding how changes in Wnt/β-catenin signaling affect the tumorigenic potential of *MMTV-Wnt1* transgenic cells. We generated a mouse line in which β-catenin is conditionally deleted in the mammary epithelium of *MMTV-Wnt1* transgenic animals. Using MMTV-cre animals in which cre recombinase is expressed in the mammary epithelium, we created MMTV-cre$^{Tg/+}$-β-catenin $^{flox/flox}$ mice. In our previous report, we confirmed that MMTV-cre transgenic animals can lead to recombination of our genes of interest within the mammary gland epithelium. As documented previously, loss of β-catenin in the mammary epithelium leads to ductal abnormalities; however, the phenotype was not as severe as expected. To more thoroughly examine cre recombination in the mammary glands, we analyzed MMTV-cre-mT/mG glands by confocal microscopy. MMTV-cre-mT/mG mice express red (Tomato) fluorescence, but when cre recombinase is expressed, the mT cassette is

deleted and GFP is expressed. By confocal analysis, we observed that cre recombination was incomplete in the mammary epithelium (Figure 1). Approximately 5% of the mammary epithelial cells contain normal levels of β-catenin and we believe these 'normal cells' are able to form the impaired ductal structure that exists in MMTV-cre$^{Tg/+}$-β-catenin $^{flox/flox}$ mice.

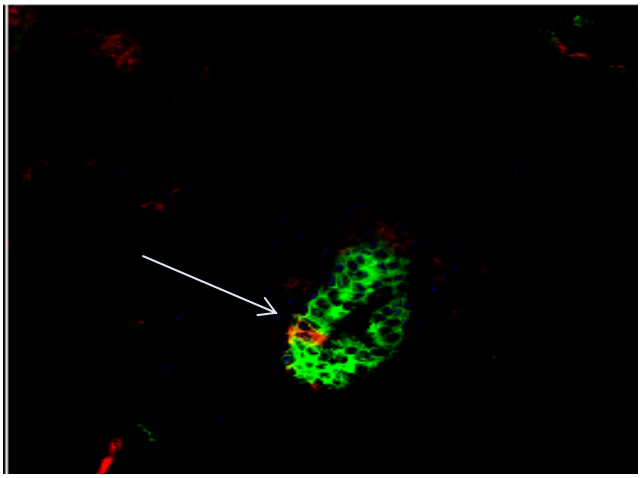

Figure 1. Using the Cre reporter mT/mG mouse we can detect Cre recombination in mammary epithelium based on expression of GFP. The arrow shows the lack of GFP expression in a single cell within the mammary epithelium of a MMTV-cre-mT/mG gland.

Next we investigated whether elimination of Wnt/β-catenin signaling at the receptor interface would create the same phenotype as the MMTV-cre$^{Tg/+}$-β-catenin $^{flox/flox}$ mice. Our past work using knockout models showed that loss of either Lrp5 or Lrp6 alone significantly delayed mammary development, with Lrp6 heterozygous knockout animals demonstrating the more severe phenotype. Since homozygous Lrp6 knockout animals are embryonic lethal, we are utilizing conditional mouse models. We have generated conditional models of both Lrp5 and Lrp6 using the same MMTV-cre transgenic mouse model mentioned above. We focused on how loss of Lrp6 or loss of Lrp5 and Lrp6 impacts the developing mammary gland. Our results showed MMTV-cre$^{Tg/+}$-Lrp6$^{flox/flox}$ mice develop functional mammary glands, but have a significant decrease in the number of terminal end buds at 5 weeks of age (Figure 2). These animals also show a statistically significant delay in ductal branching, which was present at 11 weeks of age (Figure 3). Mice lacking both Lrp5 and Lrp6 genes (MMTV-cre$^{Tg/+}$-Lrp5$^{flox/flox}$-Lrp6$^{flox/flox}$) have a very primitive ductal structure with no evidence of branching at 11 weeks of age (Figure 4) compared to controls. Interestingly, pregnancy stimulates expansion of the mammary ductal network and allows these animals to nurse their young (Figure 5). This result suggests that Wnt/β-catenin signaling plays an important role in establishing the mammary epithelium during development, but pregnancy-induced mammary proliferation is dependent on other signaling pathways.

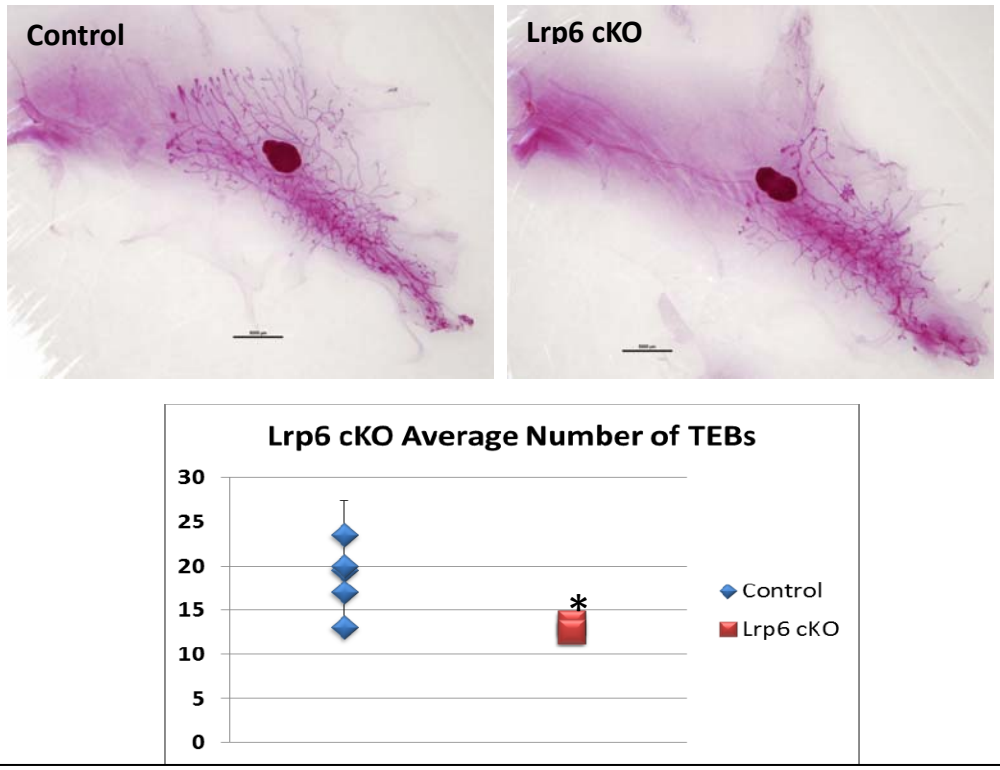

Figure 2. Mammary whole-mount preparations from 5 week control and Lrp6 cKO animals are shown. Morphometric analysis of the average number of terminal end buds (TEBs) shows a statistically significant (*P≤.01; n=10) decrease in the number of TEBs in Lrp6 cKO animals.

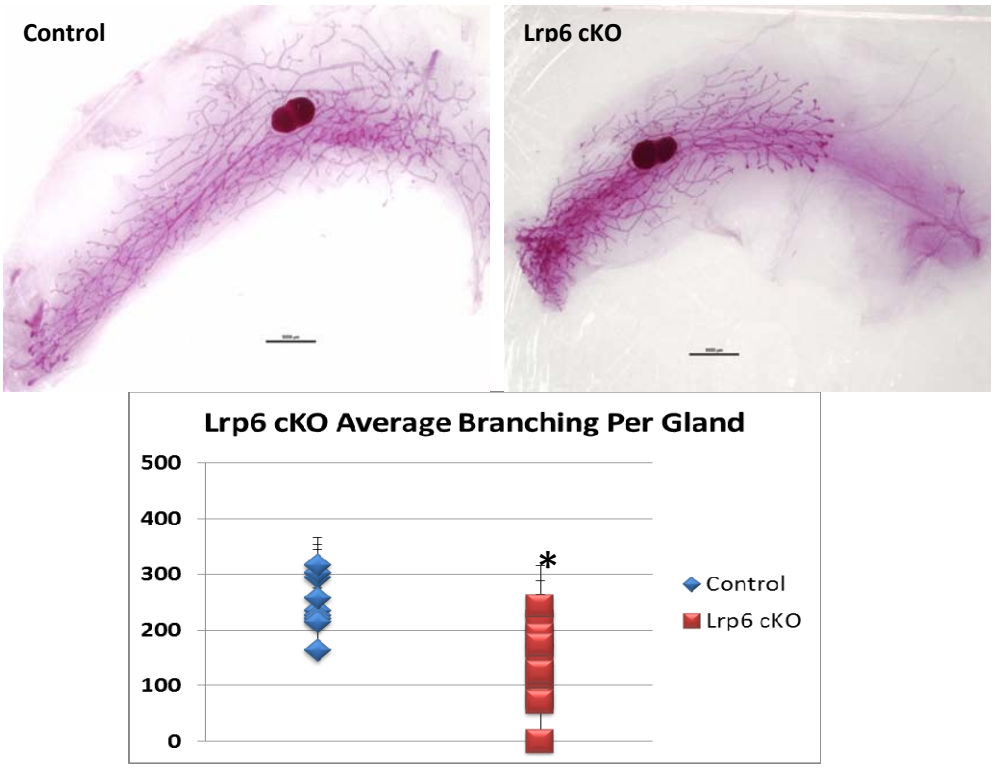

Figure 3. Mammary whole-mount preparations from 11 week control and Lrp6 cKO animals are shown. Morphometric analysis of the average branches per gland shows a statistically significant (*P≤.01; n=10) decrease in the glands of Lrp6 cKO animals

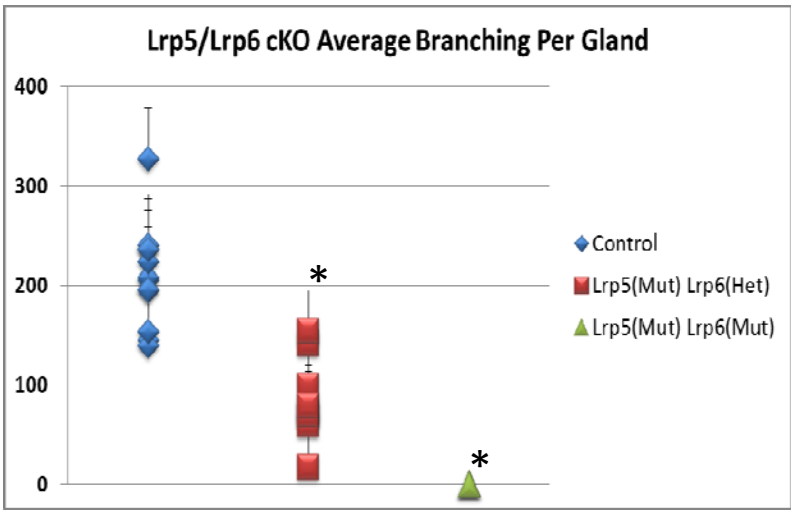

Figure 4. Morphometric analysis of the average branches per gland from 11 week control, Lrp5 cKO/Lrp6 heterozygous cKO, and Lrp5/Lrp6 double cKO animals are shown. No branching was observed(*P≤.01; n=5) in Lrp5/Lrp6 double cKO animals compared to controls.

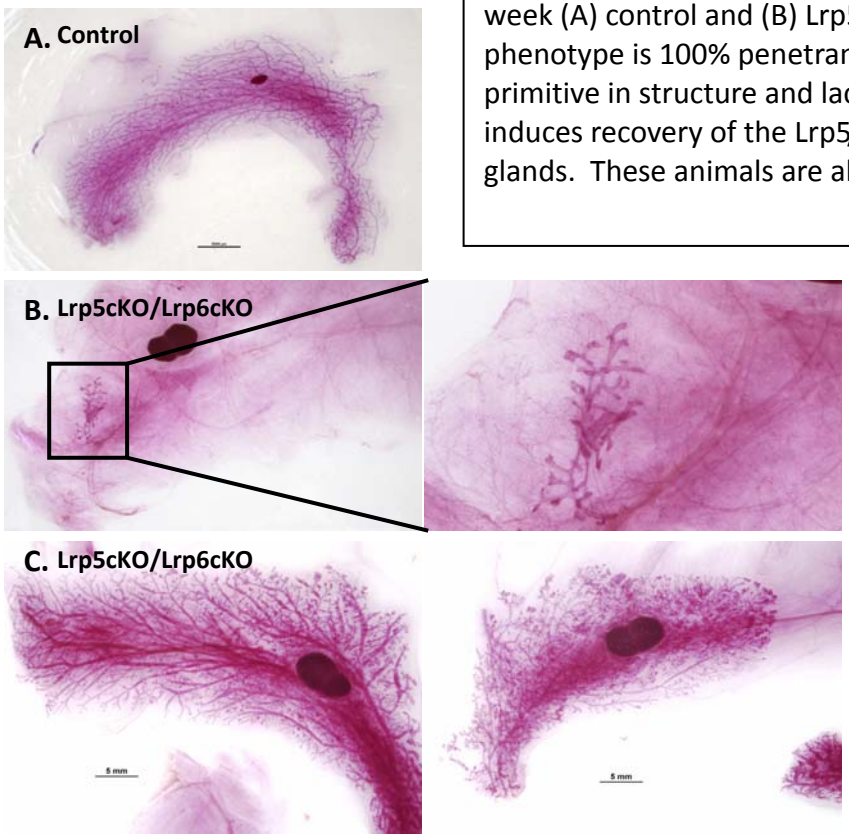

Figure 5. Mammary whole-mount preparation from an 11 week (A) control and (B) Lrp5/Lrp6 double cKO animal. This phenotype is 100% penetrant and the developed gland is primitive in structure and lacks branches. Pregnancy induces recovery of the Lrp5/Lrp6 double cKO (C) mammary glands. These animals are able to nurse their young.

Because there is incomplete recombination in the MMTV-cre transgenic models, we will use lentiviral cre infection of primary MECs isolated from $Lrp6^{flox/flox}$, $Lrp5^{flox/flox}$-$Lrp6^{flox/flox}$, and β-$catenin^{flox/flox}$ animals for the following experiments. Mammosphere development will be measured to determine how loss of Lrp5, Lrp6, or β-catenin impacts self-renewal. Cre-infected cells will be used in transplantation assays to determine if complete loss of these genes in all MECs have a more severe phenotype than the MMTV-cre models. We will also use the same approach above to decipher how loss of β-catenin in

Wnt1 overexpressing MECs impacts their tumorigenicity. To do this we have generated *MMTV-Wnt1*-β-catenin$^{flox/flox}$ animals from which we can collect MECs for delivery of cre by lentiviral infection *in vitro*.

<u>Task 2</u>. Isolation of Lin$^-$ tumor cells with (DDAOG$^+$) and without (DDAOG$^-$) canonical Wnt signaling will be achieved using FACS as described in Aim 1. Limiting dilutions of 10, 100, 1000, 50,000 of Lin$^-$/DDAOG$^+$ (test) and Lin$^-$/DDAOG$^-$ (control) tumor cells in liquid matrigel will be injected into the mammary glands of *Rag2*$^{-/-}$ immunocompromised female mice. For these experiments, we will inject mice with either test or control cells (not both into contralateral sides as in Aim 2). We used similar power calculations as described above for Aim 2, Task 2 as a base, except for doubling the amount of mice since we are injecting them with either test or control cells (not both). Therefore, we will inject 10 *Rag2*$^{-/-}$ females with 10 test cells and 10 *Rag2*$^{-/-}$ females with 10 control cells. 30 *Rag2*$^{-/-}$ females with 50 test cells and 30 *Rag2*$^{-/-}$ females with 50 control cells. We will also inject 20 *Rag2*$^{-/-}$ females (5 for each group) with either 100 or 1000 test cells and either 100 or 1000 control cells. As a further positive control, we will inject 10 mice with 100,000 unsorted cells. The *Rag2*$^{-/-}$ host mice will be monitored for tumor development. We expect to see tumor out-growths in most *Rag2*$^{-/-}$ females inoculated with 50,000 tumor cells. If canonical Wnt signaling is indeed required for cancer stem cell activity, we expect to see more tumor out-growths when limiting dilutions of DDAOG$^+$ tumor cells relative to DDAOG$^-$ cells are inoculated. Tumor growth will be measured using a caliper, and when the mice have to be sacrificed to ensure the humane treatment of animals. The tumors will then be isolated and analyzed by different means including histopathology, FACS, and gene expression profiling. These experiments will take one to two years to complete and will include approximately 8 *BATgal/MMTV-Wnt1* and 110 *Rag2*$^{-/-}$ female mice.

We will need to perform this study using Lin$^-$/CD24$^+$/CD49f^{++}/FDG$^+$ (test) and Lin$^-$/CD24$^+$/CD49f^{++}/FDG$^-$ (control) cells in limiting dilution. Once our FACS core re-established, we will sorting and characterizing these cells before performing our transplantation experiments in NSG females.

<u>Task 3.</u> We will determine the gene expression pattern of Wnt responsive *BATgal/MMTV-Wnt1* tumor cells. We will isolate mammary tumor cells with (DDAOG$^+$) and without (DDAOG$^-$) canonical Wnt signaling by FACS. We have found that a 5 minute exposure is enough to identify the Wnt responsive cell population by FACS. After staining with DDAOG we will fix the cells in order to preserve the relative mRNA levels and then isolate Lin$^-$/DDAOG$^+$ and Lin$^-$/DDAOG$^-$ cells by FACS. mRNA will be isolated from the cell isolates using the Trizol total RNA extraction method. We expect we will need to amplify the mRNA since our preliminary data suggest that only 0.2% of the BATgal/MMTV-Wnt1 tumor cells are Wnt responsive. For the expression profiling, we will use GeneChip Mouse Genome 430 2.0 Array from Affymetrix. We will perform these experiments in collaboration with the microarray core facility at the Van Andel Research Institute. We expect the microarray experiment to take approximately 3-4 months to complete.

We have outlined this experiment in detail with Dr. SokKean Khoo of the microarray core facility at the Van Andel Research Institute. Once we are able to properly sort and collect Lin^-/FDG^+ and Lin^-/FDG^- MECs we will perform this analysis.

Key Research Accomplishments

1. We have determined that high β-catenin expressing cells (as measured by positive staining for BATgal) are highly enriched in mammary progenitor cells. These cells were identified by flow cytometry to detect cells that were positive for CD24 and CD49f.
2. We have established that activation of Wnt signaling expands the total number of cells in the mammary gland but does not alter the ratio of cells showing activation of canonical signaling relative to those without activation of the pathway.
3. We have shown that loss of Wnt/β-catenin signaling during mammary gland development significantly impacts mammary gland development, but Wnt/β-catenin signaling does not seem to play as significant of a role in pregnancy-induced ductal expansion.
4. We have found that mammary transplantation of cells with activated levels of canonical Wnt signaling do not appear to induce tumorigenesis. There could be several explanations for this observation. Determining the reason for this will have important implications for understanding the role of Wnt signaling in breast tumorigenesis and will be the focus of the remaining work on this grant.

Reportable Outcomes

Work supported by this funding has been presented in the following venues

1. Translational Genomics Research Institute Annual Retreat – June 2009, Phoenix, AZ
2. Van Andel Research Institute Annual Retreat – May 2009 – Thompsonville, MI
3. Wnt Meeting – June 2009 – Washington, DC
4. Van Andel Research Institute Annual Retreat – May 2010 – Thompsonville, MI
5. Translational Genomics Research Institute Annual Retreat – September 2010, Phoenix, AZ

Two trainees supported by this funding have been accepted into clinical training programs

1. Charlotta Lindvall – admission to the Grand Rapids Medical and Research Center Internal Medicine Residency Program
2. Audrey Sanders – admission to the Michigan State University College of Osteopathic Medicine

Conclusion

"So-what section"

For decades, oncologists have focused on developing therapeutic approaches that shrink tumor mass. Unfortunately, while many treatments can dramatically shrink tumors initially, recurrence of the initial tumor is common. Recently, a new model has been proposed that may explain these observations. This "cancer stem cell" model postulates that, in many cases, the cell that is transformed is a cell with pluripotent ("stem-cell like") capabilities. In other words, the cell is capable of both self-renewal and producing progeny of many diverse cell types. The consequence of this is that tumors are mostly composed of cells that are descendants of the original tumor cell, but which are no longer capable of forming tumors themselves. Treatments that shrink the majority of the tumor by attacking the differentiated cells may not affect the small population of pluripotent cells that actually give rise to the tumor. Thus, the tumor recurs and eventually becomes resistant to any known treatment, leading to metastatic progression and, ultimately, the death of the patient. A great deal of experimental evidence published from many laboratories supports this model. This has tremendous implications for how we treat tumors and the types of drugs that we should try to develop to treat tumors. That is, we need to better understand the characteristics of these tumor-forming (cancer stem/progenitor) cells and develop treatments that can kill them while minimizing side effects.

We, and others, have published experiments showing that the Wnt signaling pathway is associated with normal mammary development and with mammary tumorigenesis. Recently, we have developed some very exciting data that suggests that activation of this pathway may provide a very specific marker with which to identify normal and cancer stem cells. These are based on both mouse models and on analysis of genes expressed in human breast tumors. Specifically, we have found that human mammary tumors of the basal-like class (a type thought to be the most "stem-cell like" in origin) have increased activation of this pathway. Tumors of this class do not typically respond well to currently available therapies, and therefore an urgent need exists to identify new potential targets for therapy. If our preliminary results are confirmed, it would provide an excellent target (the Wnt signaling pathway) that could be used to specifically treat this tumor type.

In this proposal, we have initiated a detailed characterization of the role of Wnt signaling in normal and tumorigenic mammary stem/progenitor. In one aim, we have focused on determining that cells with activation of this pathway co-purify with cells carrying previously reported markers for mammary stem cells. In the second aim, we are focused on determining whether cells in which this pathway is turned on have an increased ability to form the ducts within a mammary gland (a direct measure of stem cell activity). We will do this by creating mammary glands in which the cells that would normally make the ducts are removed and then injecting small numbers of mammary cells in which Wnt signaling is activated to see if they are capable of dividing and forming a whole set of ducts in that mammary gland. This will tell us whether cells with activated Wnt signaling are true mammary stem cells, and therefore likely targets in which mutations could cause cancer. In the final aim, we will determine whether activation of this pathway is a marker for mammary tumor stem cells. We will do this by isolating small numbers of these cells and seeing if they can form tumors when transplanted into other mice. This will tell us whether activation of this pathway can be used as a specific marker for mammary tumor stem

cells. Our current work suggests that while these cells are a subset of cells within the “mammary stem cell” fraction, that they are not capable of initiating tumors when injected into cleared fat pads. There are several potential explanations for this. One is that these cells represent a portion of cells within this fraction that is not a true “tumor-initating cell” (TIC) fraction. Another is that in this model system, these cells do represent a true TIC fraction, but that the process of sorting and isolating them removes the cells that secrete the Wnt1 ligand and that this is required for continued tumorigenesis. We will pursue this work in an improved transplant system utilizing immune deficient mice referred to as NSG mice (Nod-Scid-Gamma) as described in the body of this report [6].

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