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Mammary Stem Cells

PRINCIPAL INVESTIGATOR: Bart O. Williams, Ph.D.
Charlotta Lindvall, MD, Ph.D.

CONTRACTING ORGANIZATION: Van Andel Research Institute
Grand Rapids, MI 49503

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Introduction

The overarching goal of this proposal is 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.

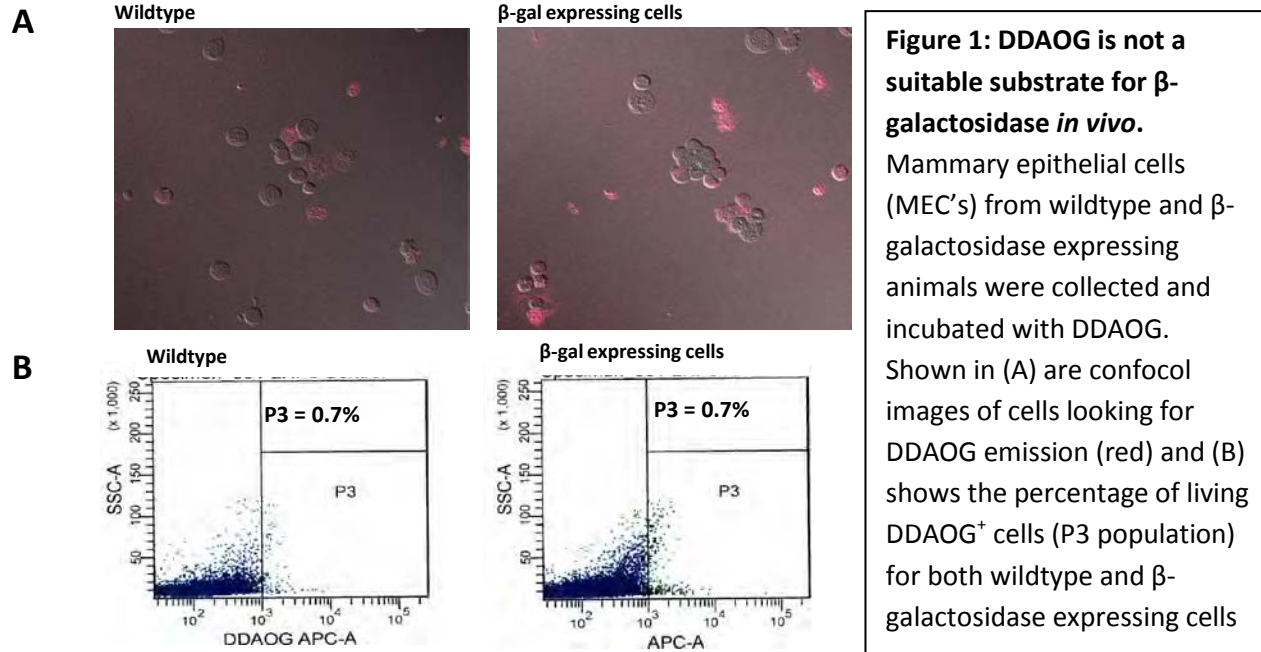
Body

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.

Last year's annual report 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. We also showed a significant increase in the number of cells undergoing canonical Wnt signaling in *MMTV-Wnt1* pre-neoplastic mammary glands, suggesting that this cell population is expanded. 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.

Task 2. 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.

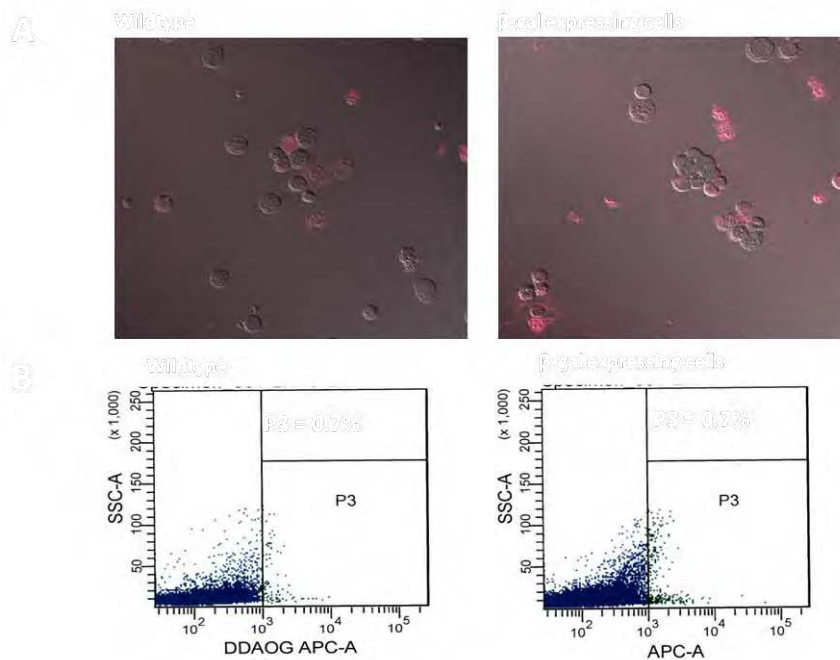


Figure 2: FDG is a suitable substrate for β -galactosidase *in vivo*. Mammary epithelial cells (MEC's) from wildtype and β -galactosidase expressing animals were collected and incubated with FDG (fluorescent substrate for β -galactosidase). Shown in (A) are confocal images of cells looking for FDG (green) and (B) shows the percentage of living FDG positive cells (P3 population) for both wildtype and β -galactosidase expressing cells as determined by FACS.

We previously analyzed Lin⁻ *BATgal* and pre-neoplastic *BATgal/MMTV-Wnt1* mammary epithelial cells by FACS using DDAOG as a substrate for β -gal activity. We had complications optimizing the use of DDAOG in mammary epithelial cells. Others within our institution have used this substrate to detect β -gal activity in other primary cell lines and our initial data with this substrate looked promising. For our optimizations we used a mouse model other than *BATgal* which has a known level of β -gal activity within MEC's. However, after further analysis of cells from this animal we determined that DDAOG⁺ cells were dead (Figure 1A) as determined by confocal imaging. We also saw DDAOG⁺ cells in our control sample in which β -gal was not expressed. This indicates that DDAOG is unable to enter living cells and once a cell dies and its cytoplasm is compromised DDAOG has an opportunity to interact with other endogenous enzymes causing a false positive signal. This was further validated by FACS analysis. MEC's were collected from control and β -gal positive animals and labeled with DDAOG. We collected a total of 20,000 events per samples and only analyzed cells that were negative for PI for the presence of DDAOG. What we found was that there was no difference in the number of DDAOG⁺ cells in both the control and β -gal expressing cells (Figure 1B).

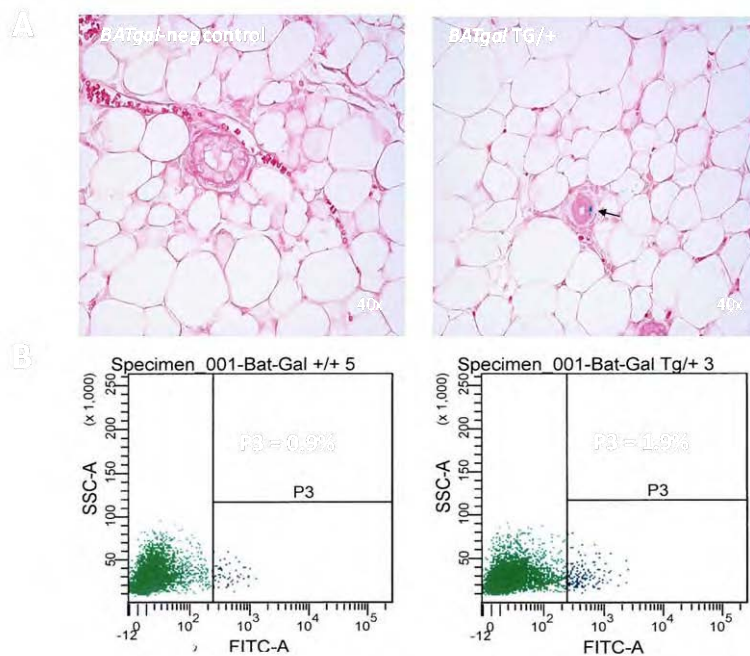


Figure 3: Detection of Wnt responsive cells by Lac Z staining and FDG. Mammary glands were collected from wildtype and *BATgal* transgenic animals and used for either Lac Z staining or MEC isolation. Shown in (A) are images of Lac Z stained tissues and (B) shows the percentage of living FDG positive MEC's (P3 population) for both wildtype and *BATgal* transgenic animals as determined by FACS.

We searched the literature and found that Duncan et. al. used FDG (fluorescein di- β -D-galactopyranoside) as a substrate for β -gal in primary hepatocytes [7]. We first tested FDG as a substrate of β -gal in our β -gal expressing cells and our control cells. After incubating the cells with FDG we took confocal images (Figure 2A) and saw that the FDG signal was specific for cells that were expressing β -gal. We also analyzed these same cells by FACS analysis for the presence of FDG. We collected a total of 20,000 events and only analyzed the PI negative cells for FDG expression. The data indicates that 15% of the total population of cells were FDG⁺ in the β -gal expressing cells (Figure 2B) as compared to 1.8% FDG⁺ cells in the control. This result is expected based on previous data for our β -gal expressing test model.

We are currently using FDG as a substrate to characterize β -gal positive cells in both *BATgal* and pre-neoplastic *BATgal/MMTV-Wnt1* MEC's. Our initial data shows that there is a 2-fold increase in the number of FDG⁺ MEC's in *BATgal* animals compared to controls (Figure 3B). This is also supported by our LacZ staining (Figure 3A) which shows there is a small proportion of β -gal positive cells within the mammary gland of *BATgal* animals. We are currently determining the percentage of FDG⁺ cells within pre-neoplastic and tumorigenic *BATgal/MMTV-Wnt1* MEC's by FACS analysis.

We are also currently determining whether cells with activated Wnt signaling (FDG⁺) express moderate levels of CD24 and high levels of CD49f which is the determined expression pattern of a cell population enriched for stem cell activity [5].

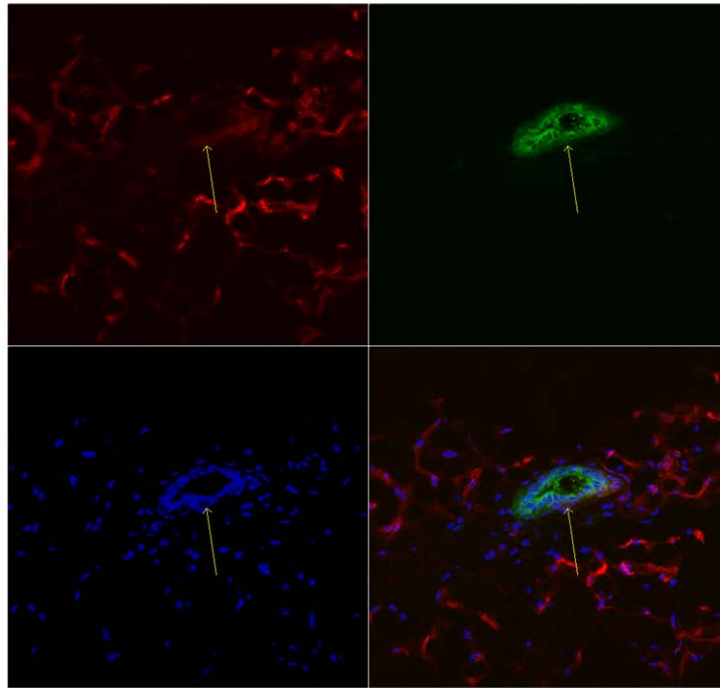


Figure 4: Cre recombinase is expressed in the mammary epithelium of MMTV-Cre transgenic animals.

Mammary glands were collected from MMTV-Cre tg/+; mT/mG animals and were counterstained with DAPI (blue) and imaged for the expression of both Tomato (red) and GFP (green) proteins.

Task 3. 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 (ER) and progesterone (PR) receptors for use on the Ventana automated staining system. We have stained sections of *BATgal/MMTV-Wnt1* tumor tissue and found that these tumors express ER. Previously we have used LacZ staining to determine β -gal activity within the mammary gland, which renders the tissue unusable for subsequent 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.

Task 1. We will test whether Wnt responsive cells possess the majority of the stem cell activity by limiting dilution transplantation experiments. $\text{Lin}^-/\text{CD24}^+/\text{CD49f}^{++}/\text{DDAOG}^+$ (test) and $\text{Lin}^-/\text{CD24}^+/\text{CD49f}^{++}/\text{DDAOG}^-$ (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 have collected and transplanted $\text{Lin}^-/\text{CD24}^+/\text{CD49f}^{++}/\text{DDAOG}^+$ and $\text{Lin}^-/\text{CD24}^+/\text{CD49f}^{++}/\text{DDAOG}^-$ mammary cells. Based on the data shown in Figure 1 DDAOG is not a suitable substrate for β -gal activity. Our transplantation experiments showed the presence of an abscess-like structure at the injection site 6 weeks post transplantation. This observation makes sense based on our finding in Figure 1, which indicate DDAOG^+ cells are dead. Our new approach is to isolate and sort $\text{Lin}^-/\text{CD24}^+/\text{CD49f}^{++}/\text{FDG}^+$ (test) and $\text{Lin}^-/\text{CD24}^+/\text{CD49f}^{++}/\text{FDG}^-$ (control) cells and transplant these cells immediately after sorting into the cleared fat pad of NSG mice. This work is currently underway.

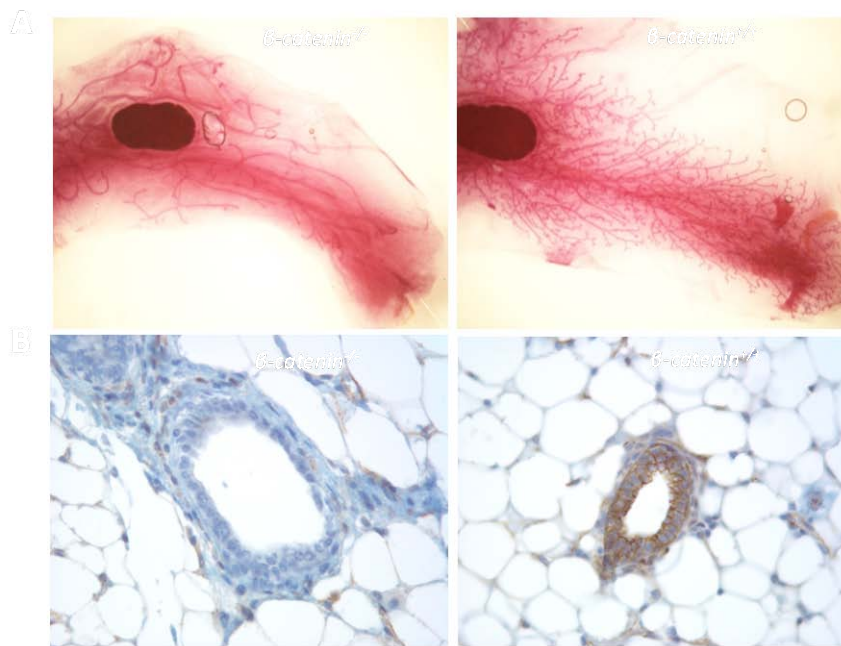


Figure 5: β -catenin deficient mammary glands have developmental defects. Mammary glands were collected from 11 week old wildtype (β -catenin $+/+$) mice and those carrying a mammary-specific deletion of β -catenin (β -catenin $-/-$). Shown in (A) are whole mount images of Carmine stained glands and (B) shows the immunohistochemical staining for β -catenin in these glands.

Task 2. 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 $1/64$ $\text{Lin}^-/\text{CD24}^+/\text{CD49f}^{++}$ 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 calculations (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 mice and are currently expanding the colony. We have a sufficient number of animals to perform the transplantation experiments once cells have been sorted.

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

Task 1. We will test whether β -gal⁺ cells isolated from *BATgal/MMTV-Wnt1* mammary tumors specifically confer tumorigenesis in 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 can be frozen and stored in liquid nitrogen until needed for further analysis.

We have prepared and frozen single cell suspensions of *BATgal/MMTV-Wnt1* mammary tumor cells. Based on our previous experiments we have determined frozen MEC's have a very low cell viability. We will collect new cells to perform these studies and transplant them immediately after sorting. We have also noticed that a large number of tumor cells are dead after processing them to a single cell suspension. This is most likely due to the hypoxic nature of the tumor and once the cells are at normoxia they undergo cell death. This was further confirmed by HIF1 α immunohistochemistry staining of *MMTV-Wnt1* tumor sections. The bulk of the *MMTV-Wnt1* tumor positively stained for HIF1 α indicating a hypoxic environment. We are currently working on processing these cells under hypoxic conditions to increase our cell viability.

We have also taken another approach to understanding how changes in Wnt signaling affects the tumorigenic potential of *MMTV-Wnt1* transgenic cells. We have generated a mouse line in which β -catenin is conditionally deleted in the mammary epithelium of *MMTV-Wnt1* transgenic animals. We have done this by using MMTV-cre animals in which cre recombinase is expressed in the mammary epithelium. To first evaluate the expression of cre within the mammary epithelium we crossed our MMTV-cre animals to mT/mG cre reporter animals. These mice express a membrane-targeted tdTomato (mT) cassette, which is flanked by loxP sites and shows a strong red fluorescence in all tissues. When these animals are bred to a cre recombinase mouse, the mT cassette is deleted in cre expressing tissues and the membrane-targeted EGFP (MG) cassette is turned on. We would expect to see red fluorescence prior to cre-mediated recombination and green fluorescence following in the tissue expressing cre recombinase. We performed confocal imaging of MMTV-cre-mT/mG mammary glands to

determine the expression of cre recombinase in the mammary epithelium. We can detect EGFP but not Tomato in the mammary epithelium of these animals indicating cre recombinase activity in these cells (Figure 4). We next crossed our β -catenin conditional animals to MMTV-cre mice to see how the loss of β -catenin in the mammary epithelium affects the mammary duct. What we see is that loss of β -catenin in the mammary epithelium leads to ductal abnormalities (Figure 5A) and immunohistochemical staining for β -catenin on sections from these glands shows a loss of β -catenin expression in the mammary epithelium (Figure 5B). We hope to collect MEC's from MMTV-Wnt1/ β -catenin⁻ animals and test their tumorigenic potential by transplantation experiments.

Task 2. 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. We are in the process of sorting and characterizing these cells before performing our transplantation experiments in NSG females.

Task 3. 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 mapped out this experiment in detail with Dr. SokKean Khoo of the microarray core facility at the Van Andel Research Institute. We need to collect new samples that are Lin⁻/FDG⁺ and Lin⁻/FDG⁻ to carry out this analysis based on the data presented in Figure 1.

Key Research Accomplishments

1. We have found that cells expressing high levels of active β -catenin (as measured by positive staining for BATgal) are highly enriched in the fraction of cells that have previously been identified as 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 found that cells with activated levels of canonical Wnt signaling do not appear to enrich for the ability to stimulate tumorigenesis when transferred to clear fat pads. 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

Publications supported by this funding

1. KC Valkenburg, Graveel CR, CR Zystra-Diegel, Z Zhong, and BO Williams. Wnt/B-catenin signaling in normal and cancer stem cells (submitted)

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-initiating 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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