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14. ABSTRACT: We hypothesized that Gpr is a highly specific marker of mammary stem cells or early progenitors that become amplified in basal type tumors and that elimination of Gpr+ cells will lead to tumor regression and eradication. To test these hypothese we aimed to: 1) identify, isolate and characterize Gpr+ cells, determine their potency by tracing their lineage, and kill Gpr+ cells and monitor the effect on mammary development; and 2) determine Gpr expression in human breast cancer, and test if ablating Gpr+ cells affects tumorigenesis in murine mammary tumors. In this grant period we have 1) characterized Gpr expression using lac Z reporters, 2) generated Gpr null mice and documented their mammary and eye developmental defects, 3) genetically demonstrated a requirement for the Gpr cytoplasmic and transmembrane domains, 4) begun to track the Gpr lineage by crossing a mouse where the Gpr promoter drives expression of a tamoxifen induced cre recombinase to an inducible R26R-TdTomato reporter line, 5) we have mined in silico datasets which show that Gpr is most highly expressed in basal breast cancer and that its highest expression correlates with poor

15. SUBJECT TERMS
Cell adhesion, G-protein coupled receptor, stem cell marker, basal breast cancer

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1. INTRODUCTION

Our inability to accurately predict breast cancer prognosis results in unnecessary treatment for those with indolent cancers and treatment that is ultimately ineffective for those with aggressive tumors. This emphasizes the urgent need to identify biomarkers that can precisely distinguish deadly from indolent breast cancer. One school of thought posits that epidemiological links between reproductive history and breast cancer risk relate to stages of rapid proliferation of mammary stem cells (MaSCs) and long-lived progenitors during development, which expose such cells to accumulate replicative errors in their DNA [14]. To date, three distinct regenerative subpopulations have been described, marked by sSHIP, Lgr5 and PROCR, respectively. However, these markers are neither specific for mammary cells, nor do they individually capture all regenerative mammary cells. Our incomplete understanding of the relationships among these regenerative mammary subpopulations remains a critical barrier to defining the mammary hierarchy and utilizing this knowledge to comprehend breast cancer subtypes. Here, we set out to validate a novel adhesion G-protein coupled receptor (Gpr) as a specific marker of mammary stem and cancer stem cells.

2. KEYWORDS

Cell Adhesion, G-protein coupled receptors, Stem cell marker, Basal Breast Cancer

3. ACCOMPLISHMENTS

The major goals of the project were:

Task 1) Determine the role of Gpr+ cells in mammary development. (40% complete)

Task 2) Determine the significance of Gpr expression in human and mouse breast cancers. (20% complete)

- ***What was accomplished under these goals:***

Specific Aim 1. Determine the role of Gpr+ cells in mammary development.

Subtask 1. Validate expression in Gpr-DTR:EGFP-creER^{T2} and Gpr-lacZ mice by reporter expression, immunohistochemistry and FACS analysis. (1-12 months)

We began by documenting Gpr protein expression. To do this we studied a reporter mouse line (Gpr-lacZ) where *lacZ* has been inserted in frame into the Gpr gene such that six of the seven transmembrane domains and the cytoplasmic domain are deleted and beta-galactosidase is expressed as a membrane-bound fusion protein tethered to the Gpr ectodomain. Thus, Gpr-lacZ reports protein expression of the Gpr ectodomain and not just Gpr promoter activity. Gpr-lacZ expression was detected by X-Gal staining of whole mounts and tissue sections over the course of embryonic and postnatal mammary development. Our results show Gpr-lacZ is absent from early E14 mammary placodes and appears in mammary buds at E15. Significantly, this developmental stage is associated with mammary stem cell commitment (1). Gpr-lacZ expression progressively intensifies as

the embryonic rudiment elongates and meets the mammary fat-pad where it branches to form the small mammary tree ~ E18.5. This stage has been shown by transplantation studies to correlate with the time of maximal fetal stem cell content (1).

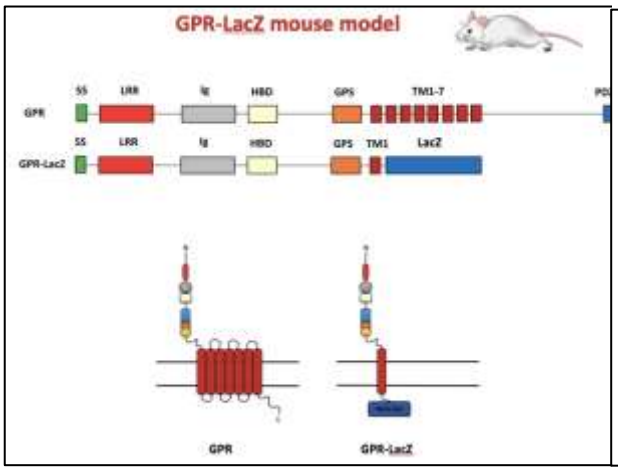

Figure 1. Gpr-lacZ reporter mouse. Sequence encoding *lacZ* was inserted in frame with Gpr, so as to delete sequence encoding the following six transmembrane and cytoplasmic domains. This results in a fusion protein of beta-galactosidase tethered to the first transmembrane region and ectodomain of Gpr.
SS= signal sequence; LRR= leucine rich repeats; Ig= immunoglobulin-like domain; HBD= hormone binding domain; GPS= G-protein cleavage site; TM= transmembrane; PDZ= protein interaction module

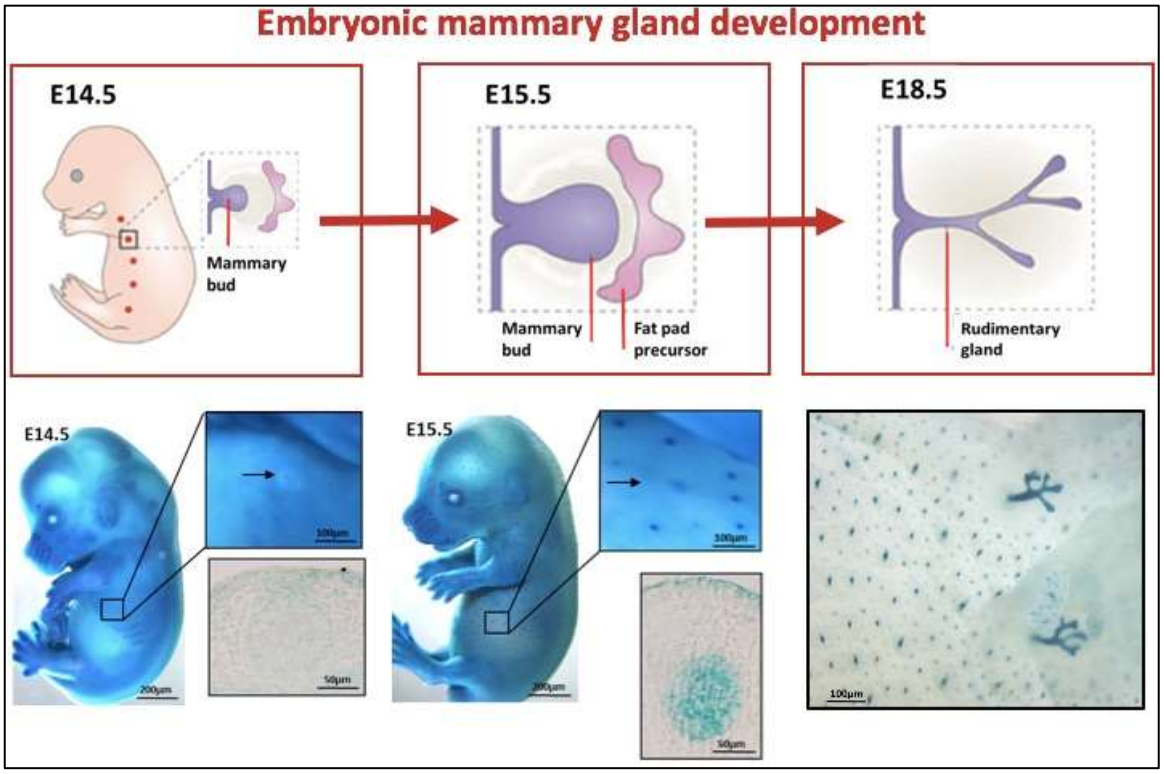

Figure 2. Gpr-lacZ expression during embryonic mammary development. Gpr was detected by X-Gal staining for expression of the Gpr-lacZ reporter. E= embryonic stage. N.b. Gpr is expressed diffusely throughout the ectoderm at E14.5, becomes focally concentrated into the E15.5 mammary bud (black arrows in lower panel) at the time of mammary specification and is maximally expressed at E18.5 in the mammary tree when fetal stem cells are maximal. Gpr-lacZ is also found in the bulge stem cell compartment of hair follicle appendages (spots in lower panels).

During puberty, Gpr-lacZ is expressed moderately in ducts lying proximal to the nipple and intensely within bulbous terminal end buds, which are proliferative structures that generate the permanent ductal system. Lgr5+ regenerative cells have been described at nipple proximal locations and sSHIP+ regenerative cells have been found in terminal end buds (2,3). Histological sections through terminal end buds shows that Gpr-lacZ is confined within these structures to the outer layer of cap cells. These cells have been proposed to be basal cell progenitors.

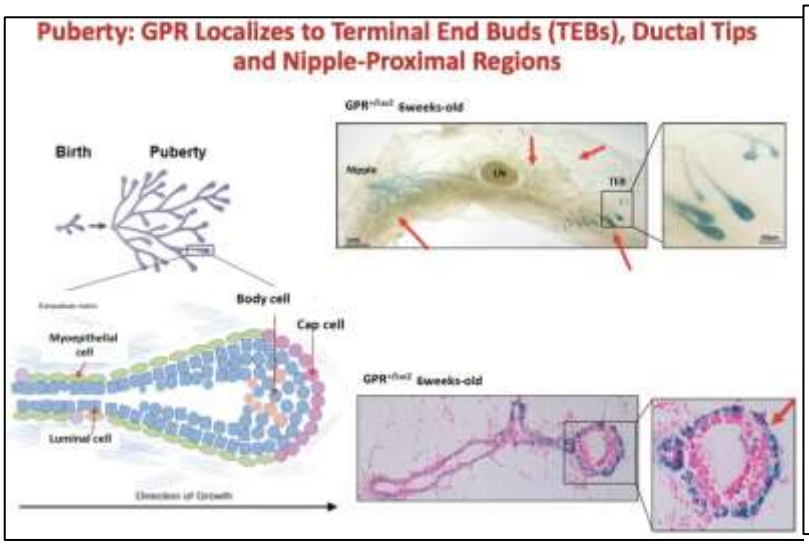

Figure 3. Pubertal Gpr-lacZ expression. Gpr expression detected by X-Gal staining of mammary whole mounts (top panel) and sections (bottom panel). NB Gpr-lacZ is found in ducts proximal to the nipple and strongly expressed in Terminal End Buds (TEB). Sections show expression is restricted to the outer cap cells (red arrow) and to single cells dispersed along

In addition to detecting Gpr-lacZ we have also examined Gpr expression in a second mouse reporter line. In this line we inserted a cassette (DTR:EGFP-creER^{T2}) following the codon for the first methionine of Gpr. This cassette comprises sequence encoding: Human Diphtheria Toxin Receptor (DTR), which allows Gpr cells to be killed, fused to Enhanced Green Fluorescent Protein (EGFP), which allows Gpr+ cells to be visualized, followed by cre recombinase (cre) fused to a Tamoxifen-sensitive mutated form of Estrogen Receptor (ER^{T2}), which allows Gpr+ cells and their progeny to be genetically marked and tracked.

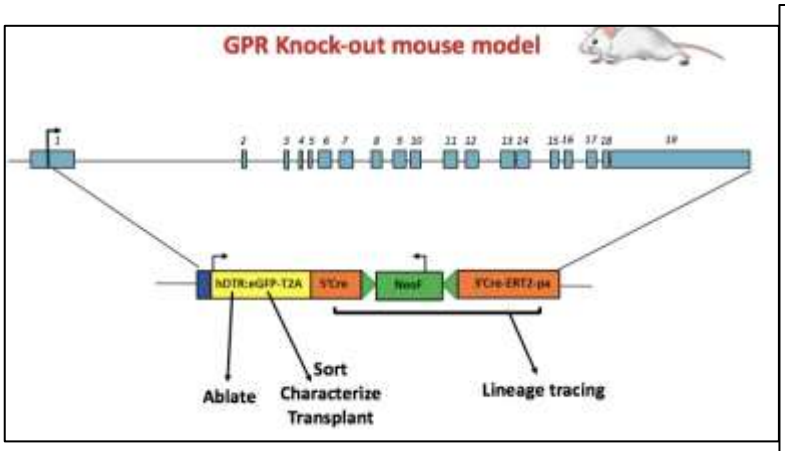

Figure 4. Gpr-DTR:EGFP-creER^{T2} knock-out mouse. A cassette comprising Human Diphtheria Toxin Receptor (DTR), fused to Enhanced Green Fluorescent Protein (EGFP), followed by cre recombinase (cre) fused to a Tamoxifen-sensitive mutated form of Estrogen Receptor (ER^{T2}) was inserted in frame after the first methionine codon of Gpr.

We examined Gpr-promoter activity in mammary whole mount mammary from Gpr-DTR:EGFP-creER^{T2} mice by detecting expression of the EGFP reporter. Whole mounts were cleared of fat using established procedures CUBIC and See-DB, then stained with antibodies specific for E-cadherin or Smooth Muscle Actin (SMA) to detect luminal and basal cell types respectively (4). Gpr expression was detected by 3D-confocal microscopy within pubertal terminal end bud structures, where the EGFP reporter co-localized with the cap cell layer which encapsulated the EGFP-negative E-cadherin-positive body cells.

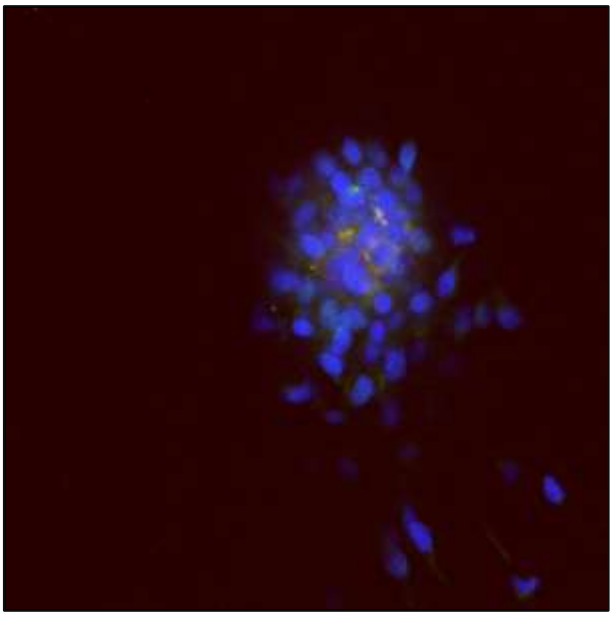

Figure 5. Movie of Gpr-EGFP expression in Terminal End Bud (TEB)
Double click on the image to play.
Mammary whole mounts, cleared then stained with anti-E-cadherin (Red) to detect luminal and basal cell types respectively and DAPI (blue) to visualize the nuclei. Gpr expression was detected by anti-EGFP (yellow) expression in 3D confocal microscopy within the cap cells of pubertal terminal end bud structures encapsulating the E-cadherin-positive body cells.

Previous studies have shown that ductal fragments from any part of the adult rodent mammary gland can regenerate a mammary tree when transplanted into cleared fat-pads. demonstrating that multipotent cells are dispersed throughout the ductal system (5). In glands from adult mice, Gpr-lacZ was observed in single cells interspersed along the basal layer of the ducts. In both TEB and proximal ducts these cells were positive for p63 and SMA, but showed low expression of keratin (K) 14. Low K14 expression has also been described for a dispersed population of regenerative cells expressing PROCR (6).

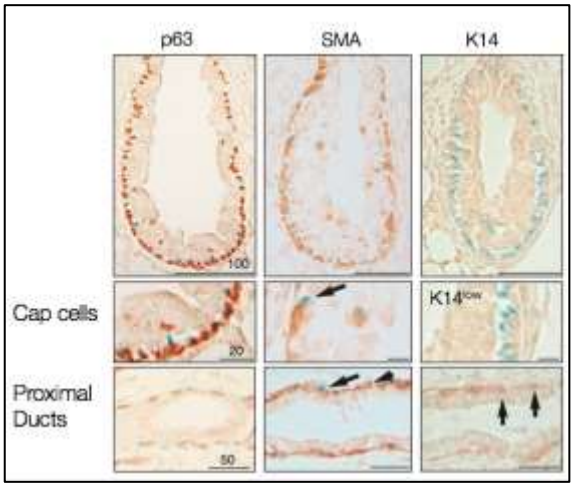

Figure 6. Localization of Gpr-LacZ to the cap cells and a basal subpopulation of ductal cells.
Sections through TEBs (top and center panels) and proximal ducts of pubertal mammary glands (bottom panel) show expression of Gpr-lacZ (blue) detected by X-Gal staining and processed for immunohistochemistry with antibodies against p63, SMA and K14 as indicated. N.b. Gpr-lacZ+ cells are p63+, SMA+ but K14-low.

A similar pattern was also detected in Gpr-DTR:EGFP-creER^{T2} mice immediately after Tamoxifen (TAM) induced reporter expression during lineage tracing (see Figure 11B, C below). During pregnancy Gpr-lacZ reporter expression intensifies within these dispersed cells and becomes focally concentrated at the tips of invading side-branches. Reporter expression is however conspicuously absent from differentiated alveoli.

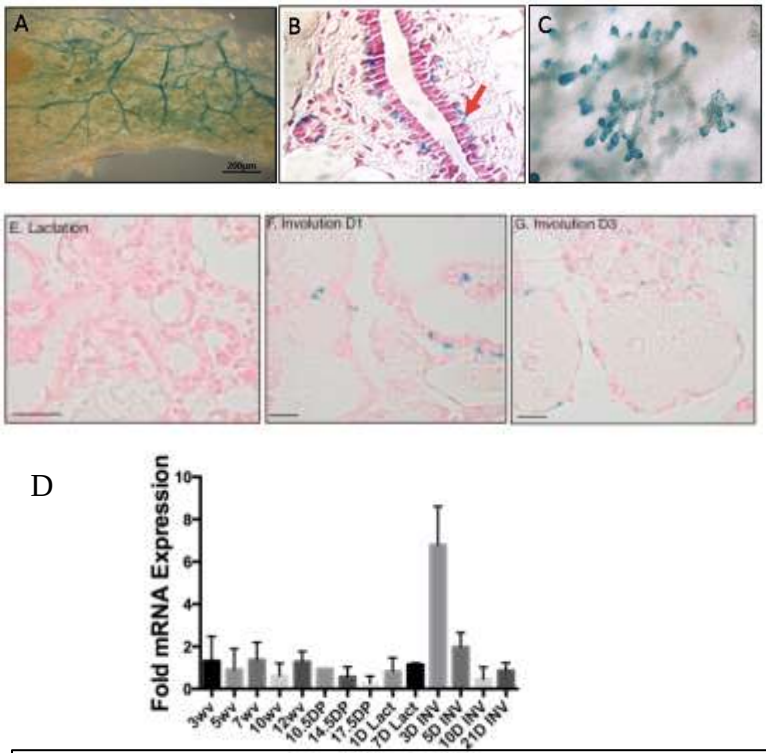

Figure 7. Gpr expression in adult mammary glands.
Mammary whole mounts (A, C) and sections (B, E-G) taken from pregnant (A-C) lactating (E) and involuting (F,G) mice. During pregnancy Gpr-lacZ reporter expression intensifies within a basal subpopulation of cells dispersed along the permanent ductal system (A, B). Gpr-lacZ becomes focally concentrated at the tips of invading side-branches (A, C). Reporter expression is however conspicuously absent from differentiated alveoli (E) but is again seen along ducts of the involuting glands (F).
(D) shows expression of Gpr mRNA detected by q-PCR. wv=weeks old in virgin animals; DP= days of pregnancy; Lact = days of lactation; INV = days of involution

Thus the spatio-temporal pattern of Gpr expression is consistent with that expected of a mammary stem cell or very early progenitor population and encompasses each of the distinct patterns of subpopulations expressing Lgr5, sSHIP and PROCR. Its high expression at ductal tips is also consistent with a potential role in detecting guidance signals or promoting polarized ductal outgrowth.

To probe further the potential overlap between Gpr+ cells and other putative stem cell subpopulations we performed FACS analysis and looked for co-expression of Gpr with markers of stem cells, progenitors and differentiated cell types. We utilized a fluorescent substrate of β-galactosidase (FDG), to detect and sort Gpr-lacZ+ cells from the general MEC population. In 2006 two studies defined a population of mammary repopulating units (MRU), capable of regenerating a mammary tree following transplantation into cleared fat-pads, by their high levels of integrin expression, detected by antibodies CD29 and CD49f, and low levels of CD24 expression (7,8). Our results show Gpr+ (FDG+) cells fell within the $CD24^{+}CD49f^{high}$ and $CD24^{+}CD29^{high}$ gates. Consistent with the profile ascribed to MRU's, Gpr+ cells expressed the very highest levels of CD29 and CD49f and showed low or no expression of Sca1 and CD61, which define more limited progenitor populations.

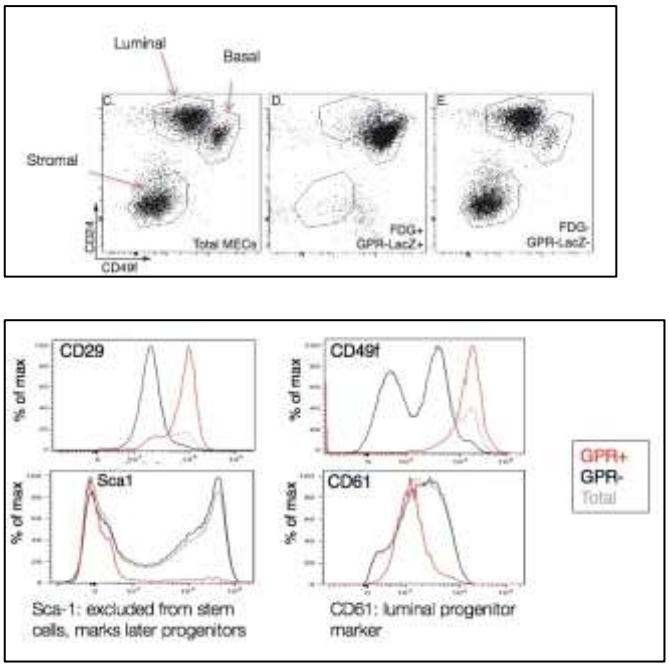

Figure 8. Isolation and characterization of Gpr+ cells.

Top panel: Flow cytometry of: (Left) total MECs; (Center) Gpr+ cells identified by FDG-Gal, which segregate into the basal gate; (Right) Gpr-depleted MECs.

Bottom panel: Flow cytometry of Gpr+ cells (red line) show the highest expression of expression of CD29 and CD49f, typical of the profile of regenerative MRU cells. Gpr+ cells show low expression of luminal progenitor markers Sca1 and CD61.

We have been unable to sort Gpr+ cells from Gpr-DTR:EGFP-creER^{T2} mice by FACS using the EGFP reporter. This is likely due to the DTR:EGFP fusion protein being 100x lower in brightness than free EGFP. As the DTR:EGFP is tethered to the cytoplasmic side of the membrane its epitope is also unavailable in non-permeable living cells precluding from using antibodies to enhance the signal. We now plan to use a second more robust reporter, TdTomato, by activating the creER^{T2} module in bi-transgenic Gpr-DTR:EGFP-creER^{T2} x Rosa26R-STOP-TdTomato mice. Although this module was originally designed with lineage tracing in mind, at early time points following induction, the reporter detects expression within parental Gpr+ cells prior to being additionally passed on to their progeny. This will permit us to sort and transplant viable Gpr+ (TdTomato-positive) and Gpr-depleted cells (TdTomato-negative) into empty fatpads to test their relative regenerative potential at comparable limiting dilutions.

Subtask 2. Cross Gpr-DTR:EGFP-creER^{T2} to Rosa26-STOP-reporter lines (1-18 months)

Although transplantation experiments described above will measure the ability of cells to acquire regenerative capacity in an artificial experimental setting they do not address whether cells actually operate as stem cells during normal physiological development. To address this question requires lineage tracing, where a permanent genetic change is introduced that allows progeny to be traced within the normal physiological context. We have crossed our Gpr-DTR:EGFP-creER^{T2} mice to several Rosa26R-STOP-reporter lines, where reporter expression is blocked by a STOP sequence. This block is removed when Tamoxifen (TAM) is administered, which causes cre recombinase to enter the nucleus and recombine loxP sites flanking the stop sequence. This results in the reporter being expressed in the Gpr+ parental cell at initial time points and since this is a permanent genetic change reporter expression continues under the control of the open ROSA locus in all cellular progeny. We proposed to use R26R-STOP-lacZ to produce a permanent histological record of Gpr progeny by means of X-Gal detection of Gpr-LacZ expression. After rederiving and establishing sufficient numbers of adults of each parental strain we have established six breeding pairs and litters of progeny were born two weeks ago. From these we predict approximately 15 mice will be useful (~ 60 pups = 30 females = 15 bi-transgenic females). These bi-transgenic progeny and controls and subsequent litters will be treated +/-TAM and their lineage traced over the course of mammary development.

Although X-gal staining offers the advantage of a permanent record, during the course of our experiments, we have also become aware that it also has limitations. In particular, assigning cells to the basal or luminal layer is sometimes open to ambiguity when performing 2D analysis on tissue sections due to a) oblique sections and b) leaching of the X-Gal stain during histological processing. Moreover, once the lineage tracing progresses to the point of clonal analysis, which demands very low levels of recombination, then tracing the few rare cells in 2D sections becomes very difficult. To address these issues we decided to cross the Gpr-DTR:EGFP-creER^{T2} mice to additional fluorescent reporter lines that will facilitate superior methods of clonal analysis and 3D confocal imaging of whole mounts. As a technical positive control of TdTomato expression and this technique of clearing and imaging we first examined lactating glands, from GATA3-cre; ROMA; RosaR26R-TdTomato mice, which have abundant epithelium and express the reporter in the alveolar population.

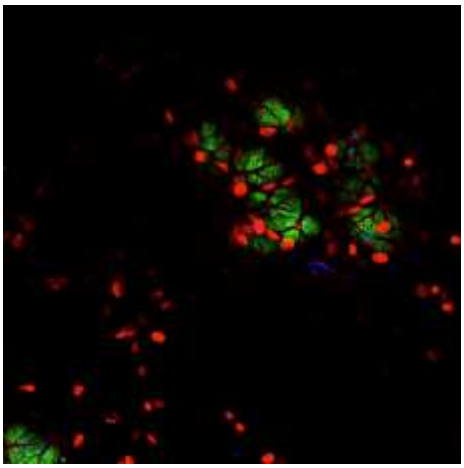

Figure 9A: Control of the tissue clearing and 3D-imaging technique.
Double click on the image to play the movie.
Whole mammary glands from lactating ROMA; TdTomato mice were cleared of fat by CUBIC techniques and processed for 3D confocal fluorescence microscopy. Glands were stained with anti-E-cadherin (Green) to visualize the borders of luminal cells; anti-SMA (blue) to visualize the basal myoepithelial cells; and DAPI (red) to visualize the nuclei. NB TdTomato is not expressed as there is no cre present in these mice.

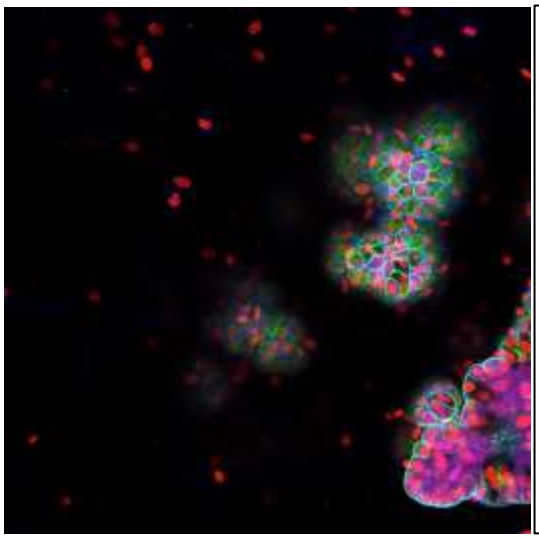

Figure 9B: Control of TdTomato detection after clearing and 3D imaging technique.
Double click on the image to play the movie.
Whole mammary glands from lactating GATA3-cre; ROMA; TdTomato mice were cleared of fat by CUBIC techniques and processed for 3D confocal fluorescence microscopy. Glands were stained with anti-E-cadherin (Green) to visualize the borders of luminal cells; anti-SMA (blue) to visualize the basal myoepithelial cells; and DAPI (red) to visualize the nuclei. TdTomato (yellow) was detected in many luminal cells indicative of GATA3-cre activity in these mice.

Currently, we have generated an active breeding colony of Gpr-DTR:EGFP-creER^{T2} x ROSA26R-STOP-TdTomato mice and have imaged a cohort of their progeny (see below figure 10). We are improving the efficiency of this breeding colony by intercrossing breeders to produce both male and female parents that are homozygous for both Gpr-DTR:EGFP-creER^{T2} and the reporter strain. By doing this we will a) remove the need for genotyping and b) reduce use of animals by generating a greater proportion of genetically useful female progeny in each generation c) to increase the reporter expression thereby improving signal detection.

We have also established a ROSA26R-Confetti reporter line in our colony and begun to cross them to the Gpr-DTR:EGFP-creER^{T2} mice. The R26R-Confetti conditional allele comprises a CAG promoter followed by a STOP sequence flanked by loxP sites and multicolor fluorescent reporters targeted into the Gt(ROSA)26Sor locus. Cre recombinase activity leads to stochastic expression of each of the four reporters allowing a way to label and distinguish the progeny of individual / adjacent cells.

Subtask 3. Trace the Gpr lineage by inducing cre activity with TAM and detecting Rosa26R-reporter in Gpr descendants in conjunction with differentiation markers (18-30 months).

As described above we have generated the first sets of Gpr-DTR:EGFP-creER^{T2}/ROSA26R-STOP-TdTomato progeny. To test the efficacy of Gpr-driven cre recombinase to produce reporter activation we have administered TAM over a range of doses that have been used in published protocols for other mammary promoters/drivers. Cognizant that TAM can induce abortion and cause issue with delivery when administered to pregnant mice we decided to delay analysis of embryonic stages of Gpr+ cells until we have satisfactorily established other parameters and to begin with pubertal stages. We have administered a range of TAM during early stages of puberty and assessed mammary whole mounts at intervals thereafter. To test whether TAM administration would adversely affect

expression of the Gpr promoter we examined whole mounts from Gpr-LacZ mice one week after administering a range of TAM from1.5- 15 mg/mouse. Gpr-lacZ expression remained robust in nipple proximal ducts and terminal end buds. However, TAM reduced the size of the terminal end buds. Therefore, we have chosen to proceed using the lowest dose of TAM.

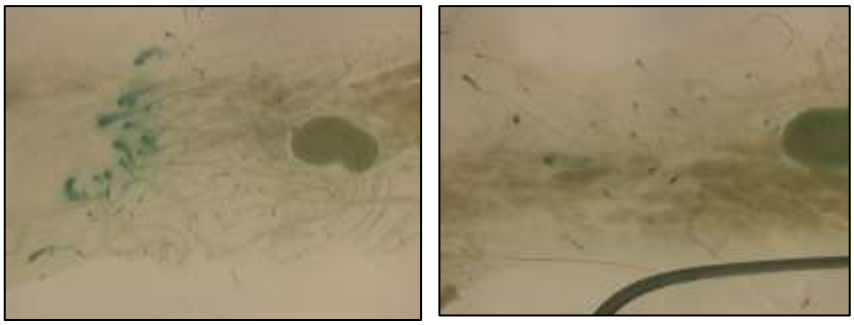

Figure 10. Effects of tamoxifen on pubertal Gpr-lacZ expression.
Mice were treated with either vehicle (left panel) or TAM 2.5 mg (right panel) to mid pubertal 6-week old mice.

Having established that the Gpr promoter would remain active following TAM and therefore drive expression of the creERT2 recombinase we proceeded to test the effects of TAM on Gpr-DTR:EGFP-creER^{T2} /ROSA26R-STOP-TdTomato progeny. Mice were treated with TAM at either 4 or 6 weeks and their mammary glands harvested after 1 and 3 week intervals and processed for whole mount CUBIC clearing, followed by immunofluorescence and 3D confocal imaging. This imaging was technically more challenging than in our lactating controls above (Figure 9A, B) because there is far less epithelium in pubertal glands and the ducts weave into the fat-pad and are frequently out of range of focus for confocal imaging. Nevertheless, we were able to detect labelling of both cap cells in the terminal end buds and sporadic cells dispersed along the ducts at early time points. These cells showed the classical hallmarks of basal cells: being flat and spindle shaped; and positive for SMA and negative for E-cadherin. At later times in some ducts we also observed TdTomato expression within a subset of what appear to be columnar E-cadherin luminal cells.

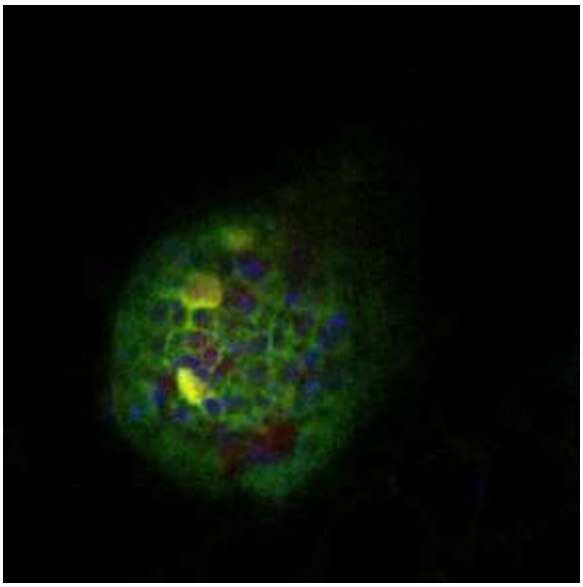

Figure 11A. 3D confocal images of mammary whole mounts from pubertal Gpr-DTR:EGFP-creER^{T2}/ROSA26R-STOP-TdTomato.
Double click on image o to play movie: This movie shows TdTomato (yellow) labeling of cap cells in the terminal end buds at 6 weeks, in this image Dapi (blue) marks nuclei, E-cadherin (red) marks luminal cell borders and SMA (green) marks caps cells.

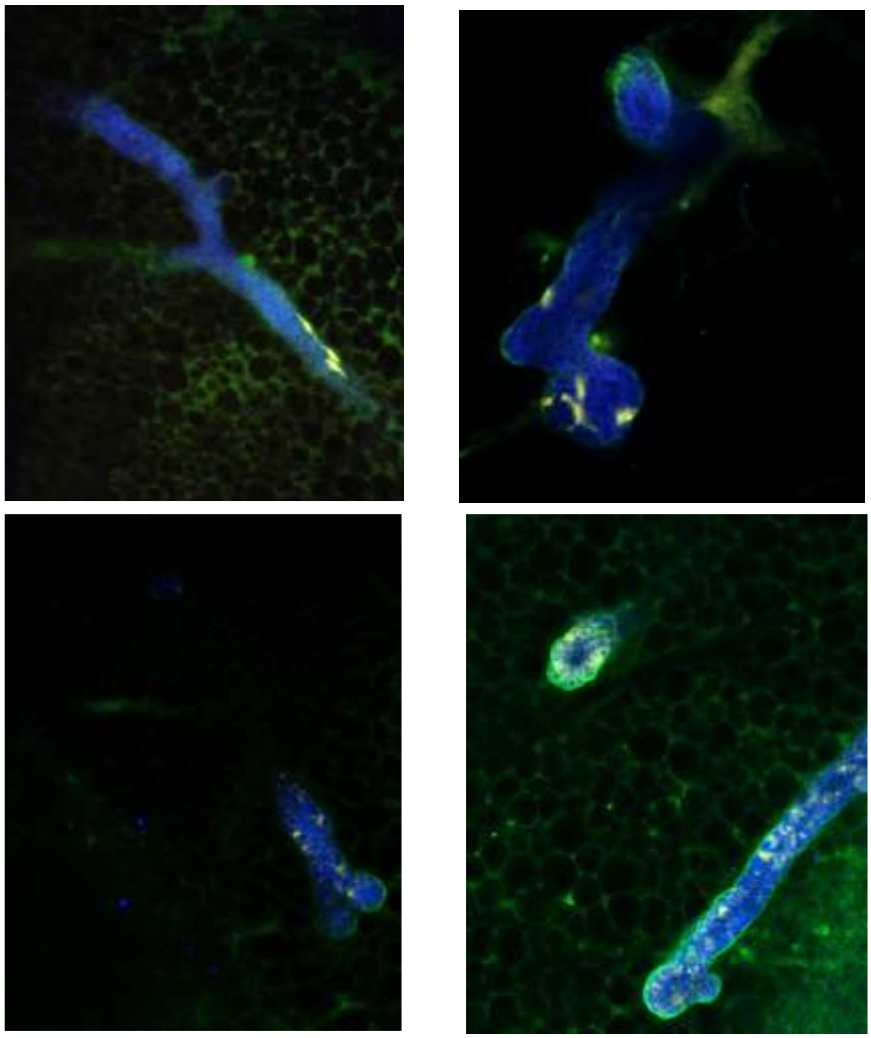

Figure 11B. 3D confocal images of mammary whole mounts from pubertal Gpr-DTR:EGFP-creER^{T2}/ROSA26R-STOP-TdTomato.
(TOP) At later times (7-weeks) TdTomato (yellow) was seen in small groups of flat and spindle shaped basal cells positive for SMA (green) and negative for E-cadherin (blue) dispersed along the ducts. (BOTTOM) In some ducts we also observed TdTomato expression within a subset of what appear to be columnar E-cadherin positive luminal cells.

Further clonal analysis is required to trace the full spectrum of cell types that originate over the course of mammary development from the Gpr+ cells, however, our preliminary data suggest that Gpr+ cells are multipotent giving rise to basal and luminal cells.

Subtask 4 and 5: Kill Gpr+ cells in Gpr-DTR:EGFP-creER^{T2} mice by administration of DTA. Validate cell death by loss of EGFP expression and monitor the effect on mammary development.

This aim has been delayed due to difficulty in detecting the DTR:EGFP fusion protein in living cells. Once we have the parameters for use of TdTomato at early times of lineage tracing established we will be able to substitute use of this reporter to ensure that DTA eradicates the GPR+ cell population and test the effect of this on development.

We have, however, been able to study the effects of total loss of Gpr protein expression by examining mice homozygous for the Gpr-DTR:EGFP-creER^{T2} allele as well as to determine the effects of loss of the cytoplasmic domain in mice homozygous for the *lacZ* allele. Both sets of mice are viable and fertile although the males have a late onset sterility due to loss of testicular Gpr expression. In the mammary gland Gpr-lacZ homozygotes show retarded ductal elongation during puberty and we are investigating this phenotype in the Gpr-DTR:EGFP-creER^{T2} mice.

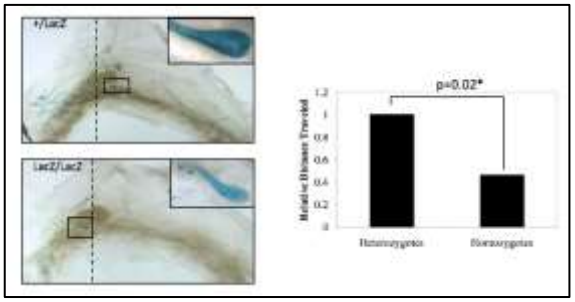

Figure 12. Retarded ductal elongation phenotype.
Left panels show X-Gal stained whole mounts from 6-week old Gpr-lacZ mice. Right panel shows quantitation of ductal extension, measured by distance of TEB from the lymph node. N=5; *p=0.02

Both lines of mice show a pronounced eye phenotype. The mice show inflammation of the eye suggesting that they could provide a useful model for human "Dry Eye Syndrome". The fact that Gpr-lacZ homozygotes phenocopy complete loss of Gpr in Gpr-DTR:EGFP-creER^{T2} mice indicates a requirement for the transmembrane and cytoplasmic domains and their downstream signaling functions. Several antagonists of G-protein signaling are in use to treat Dry Eye Syndrome and could therefore be useful in directing our investigation to define the signaling pathways and identify drugs to decrease high Gpr activity seen in some breast cancer.

Figure 13A. "Dry Eye" phenotype.
Mice wildtype (left Gpr$^{+/+}$) and heterozygous (right Gpr$^{+/-}$) for null for the Gpr-DTR:EGFP-creER^{T2} allele have normal eyes. Homozygous mice (center GPR$^{-/-}$) show pronounced squinting and blinking and show inflammation of their eyes.

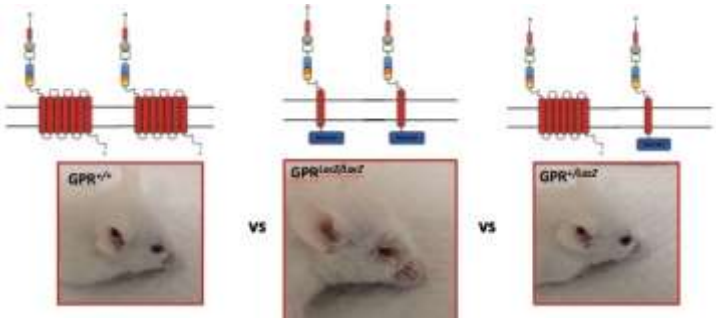

Figure 13B. "Dry Eye" phenotype.
Mice wildtype (left $Gpr^{+/+}$) and heterozygous (right $Gpr^{+/-}$) for null for the Gpr-lacZ allele have normal eyes. Homozygous mice that lack 6 transmembrane and cytoplasmic domains (center $GPR^{LacZ/LacZ}$) show pronounced squinting and blinking and show eye inflammation.

Gpr-lacZ is expressed in the conjunctiva, lacrimal glands and meibomian glands, which contribute to tear formation. Its expression at the ductal tips of the invading lacrimal and salivary glands is highly reminiscent of its expression in the terminal end buds of the mammary gland, suggesting a more global requirement for its role in the ductal extension during development of secretory glandular structures.

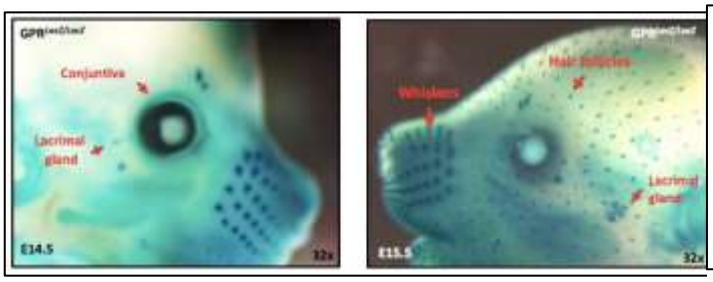

Figure 14. Gpr expression in ectodermal appendages and eye.
X-Gal stained E14 (left) and E15 (right) embryos show Gpr-lacZ expression in the conjunctive, lacrimal gland hair follicle and whiskers.

Specific Aim 2
Determine the significance of Gpr expression in human and mouse breast cancers.
Task 1. Screen breast cancer cell lines for Gpr expression by qPCR and mine bioinformatics datasets of human breast cancers.
We have collected and cultured a panel of breast cancer cell lines shown below, prepared mRNA and tested them for expression of Gpr by qPCR. Expression of Gpr was found in both luminal and basal cell lines.

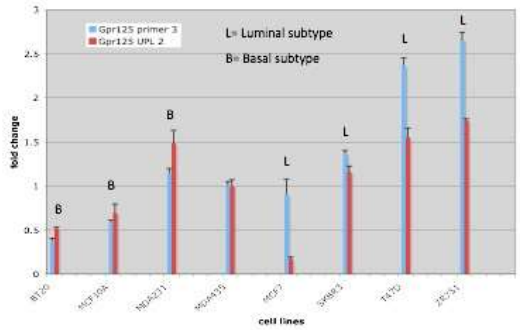

Figure 15. qPCR analysis of Gpr expression in human breast cancer cell lines.

We are continuing to expand the panel to include more representatives of the different basal subtypes.

We have further investigated Gpr expression and its association with patient outcome in the different breast cancer subtypes using the publicly available gene expression database

Kaplan-Meier Plotter (9). This tool allows analysis of changes in relapse-free survival (RFS) and distant metastasis-free survival (DMFS) curves based on Gpr expression for four breast cancer subtypes – Basal-like, Luminal A, Luminal B, and HER2-overexpressing (n=4142 total). Notably, high expression of Gpr predicted for poor prognosis in RFS exclusively in the basal breast cancer subtype (p=0.0054) (Figure D, red line), while high expression of Gpr did not have significant prognostic value in Luminal A, Luminal B, or HER2-overexpressing (p=0.069-0.1) (Figure E-G red lines). Furthermore, high Gpr expression was positively associated with poor outcome in DFMS for basal-like (p=0.0043), Luminal B (p=0.045), and Luminal A (p=0.015) (Figure D-F), suggesting that increased expression of Gpr signified increased metastatic capability in multiple human breast cancer subtypes, and that Gpr may have prognostic significance to identify advanced metastatic disease as well. We are now extending this analysis databases of TNBC human breast tumor that distinguish subtypes (TNBCtype).

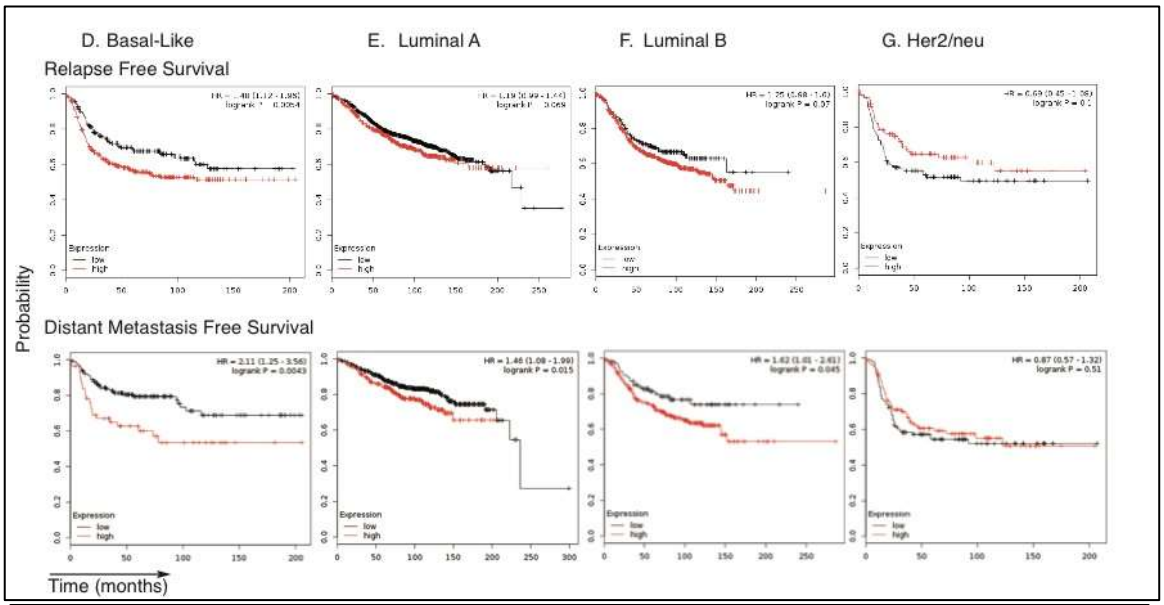

Figure 16. Gpr expression and its association with outcome in different subtypes of human breast cancer.
D-E) Relapse-free survival (RFS) and Distant Metastasis Free Survival (DMFS) for high Gpr expression (red lines) and low expression (black lines) in human breast cancer subtypes (D) Basal-like, (E) Luminal A, (F) Luminal B, and (G) Her2-neu. N=4142 total data points, sourced from publicly available database Kaplan-Meier Plotter (9) HR=Hazard Ratio, indicating positive association between poor prognosis and gene expression. p values for each Hazard Ratio (HR) >0.05 indicate significant association between high expression and poor prognosis. High Gpr is positively associated with poor prognosis in RFS for basal-like cancer, and DMFS for basal-like, Luminal A, and luminal B

Task 2 Test whether eradicating Gpr+ cells affects tumorigenesis and/or regression
Subtask 1: Generate bi-transgenic Gpr-DTR:EGFP-creER^{T2}/MMTV-Wnt1 mice.
We have generated the first crosses to introduce the necessary ROSA26R-TdTomato reporter onto the Gpr-DTR:EGFP-creER^{T2} and MMTV-Wnt1 mice and are in the process of breeding these lines together to generate cohorts of experimental progeny.

Subtask 2: Ablate Gpr cells by DTA administration and assess effects on tumor onset, progression, regression and histology.
This subtask has been delayed due to the necessity to introduce the ROSA26R reporters onto the background in order to track the effectiveness of DTA ablation of the Gpr-positive cell population.

- **What opportunities for training and professional development has the project provided.**

- **Dr. Cowin** acquired skills in tissue clearing and 3D-confocal imaging from colleagues in the Pathology Department, Cambridge who have pioneered this technique in the mammary gland (4).
- **Dr. Spina** joined the lab in March 2018. She is both a fully trained pharmacist and carried out a Ph.D. on "The Role of Estrogen Receptor Alpha and Notch4 Axis in the Regulation of Breast Cancer Stem Cells". She arrived with expertise in cell culture and FACS analysis and a strong background in breast cancer. Since joining she has acquired new skills in a) mouse genetics and breeding, b) whole mount and histological analysis, c) tissue clearing and 3D-confocal imaging, and d) *in vivo* survival surgery for future fat-pad clearing and transplantation experiments.
- **Dr. Spina** has taken a courses in ethics, animal welfare and professional career development. She has joined the molecular oncology and immunology training program and also attends the "works in progress" presentations of the stem cell training program the pharmacology training program and the cancer center. She is scheduled to present in these forums and the departmental retreat. She has assembled a committee to guide her professional development and provide additional expert guidance on the topic of her project. Her committee comprises: Dr. Mayumi Ito Ph.D. an expert in the lineage tracing of hair follicle and nail ectodermal appendages; Dr. Dimitris Placontonakis MD, an expert on adhesion-GPCR 133 in glioblastomas and Dr. Konstantin Itchenko, an expert in Adhesion-GPCR signal transduction.

- **How were the results disseminated to communities of interest?**
- **Dr. Cowin** presented this work in an invited seminar to the Department of Veterinary Medicine, Cambridge, UK in June 2017
- **Dr. Cowin** presented this work in an invited seminar to the Department of Matrix Biology and Regenerative Medicine, University of Manchester, UK in Sept 2017
- **Dr. Spina** was selected to present this work in an invited seminar at the 9th Adhesion GPCR Workshop Sept 13-15, 2018 in Portland, OR.

- **Dr. Cowin** is scheduled to present this work to the Skirball Institute Faculty Presentations NYUSOM in Oct 2018.
- **Dr. Cowin** is scheduled to present this work to the Breast Biology Group at UCSF in spring 2019.

- **What do you plan to accomplish during the next reporting period to accomplish the goals and objectives?**

- Our major focus will be to complete the lineage tracing as this will be essential to validate and publish our expression data on the position of Gpr+ cells in the mammary hierarchy.

- Our second major goal will be to treat the mice with DTA and examine the effects.

4. IMPACT

- **What was the impact on the development of the principal discipline of the project?**

- Our results have shown that mice lacking this adhesion GPCR show a breast developmental phenotype (delayed ductal elongation).

- Our expression studies support the concept that Gpr demarcates stem cells and sites of ductal invasion.

- Domain analysis indicates downstream signaling of this orphan receptor is essential for normal mammary development.

- Our results have shown that high levels of Gpr occur in aggressive forms of basal positive breast cancer, and that patients with higher levels within these groups have particularly poor outcome. The unpublished data reported herein introduce the concept that Gpr expression has value as a prognostic indicator of patient outcome in basal type breast cancer. This has pioneered a new field in breast cancer research since there are no studies besides our own on this Gpr.

- **What was the impact on other disciplines?**
- The homozygous Gpr-DTR:EGFP-creER^{T2} mice, which have a pronounced eye phenotype show that Gpr is essential for normal eye development and is expressed in several structures responsible for tear production. These mice display features of "Dry Eye Syndrome" and could have utility as a model for this human inflammatory disease.
- Collectively these phenotypes suggest that Gpr is required for the function of several related secretory glandular structures that share common ectodermal origin and undergo similar patterns of ductal branching and secretory differentiation.
- Our results show that Gpr demarcates the stem cell compartments of several other ectodermal appendages including the bulge and secondary germ compartments of hair follicles.

- **What was the impact on technology transfer?**
- Nothing to report

- **What was the impact on society beyond science and technology?**
- Linking breast development factor to breast cancer risk opens the door to preventative strategies linked to reproductive history.

5. CHANGES/PROBLEMS

- Changes in approach and reasons for change:
  - We were unable to detect the EGFP reporter in living cells even though we can image it in permeabilized cells by using antibodies. This prevented our original plan to isolate living cells for functional assays and to monitor cell Gpr cell ablation. We have therefore changed to using a more robust TdTomato reporter activated at early time point br TAM induction of cre recombinase.
  - We have decided to carry out 3D-confocal imaging of whole mammary glands rather than rather than 2D imaging of tissue sections as the former offers superior detection of rare cells within the natural context of the whole gland.

- **Actual or anticipated problems or delays and actions or plans to resolve them**
  - The inability to detect EGFP and swith to the use of the inducible TdTomato reporter as delayed the generation of the Wnt1 tumor mice as additional generations are required to introduce the second reporter onto the strain background.

- **Changes that had a significant impact on expenditures –**
  - Delays in Hiring: Dr. Chandramouli, a long- time research associate skilled in transplantation techniques unfortunately left just prior to the start of the project. She was replaced by Dr. Spina in March 2018, however there was a delay due to obtaining the necessary visa entry requirements for Dr. Spina to come from Italy. A second fellow was scheduled to join from Australia in May 2018 but was forced to decline the position at the last minute due the severe illness and death of his father, which necessitated him returning to Sri Lanka to care for his relatives. He will be replaced shortly by one of three candidates currently under consideration for this position.

- **Significant changes in use or care of human subjects, animals, biohazards or select agents**
  - Nothing to report

6. PRODUCTS

- **Publications, conference papers and presentations**

- ***Manuscripts:*** Nothing to report

- ***Books etc:*** Nothing to report

- ***Other publications, conference papers and presentations***
  Abstract from Dr. Spina's presentation to the 9th Adhesion GPCR workshop
  Acknowledgement of Federal Support: YES

- **Website(s) or other internet site (s):** Nothing to report

- **Technologies or Techniques:** Nothing to report

- **Inventions, patent applications and/or licenses:** Nothing to report

- **Other products:**
  - **Research material:** Generation of Gpr-DTR knock out mouse model

7. PARTICIPANTS AND OTHER COLLABORATING ORGANIZATIONS
Individuals working on the project:

Name	Pamela Cowin
Project Role	P.I.
Research Identifier	
Nearest person month worked	12
Contribution to project	Directed research
Funding Support	DOD BC123572 40%

Name	Elena Spina
Project Role	Postdoctoral fellow
Research Identifier	
Nearest person month worked	6
Contribution to project	Performed work on Aim 1
Funding Support	DOD BC123572 100%

Has there been a change in the active support of the PI or senior key personnel since the last reporting period
P.I. Dr. Pamela Cowin – No change
Postdoc Dr. Elena Spina – No change

Postdoctoral Fellow –Dr. Chandramouli's postdoctoral training ended and she was replaced by Dr. Elena Spina in March 2018. A second postdoctoral fellow will join in the fall of 2018.

What other organizations were involved as partners?
Nothing to report

8. SPECIAL REPORTING REQUIREMENTS N/A

9. APPENDICES:

a) References cited.

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b) Abstract from Dr. Spina's presentation to the 9th Adhesion GPCR workshop

Abstract from Dr. Spina's presentation to the 9th Adhesion GPCR workshop in Portland Oregon Sept 13-15, 2018

Roles of the ADGRA family in glandular development, lineage commitment and tumorigenesis.

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Abstract: We have generated reporter and knock out mouse models to investigate the expression of members of the ADGRA family in ectodermal appendages. Our knock-out mice show defects in ductal elongation and secretory differentiation of lacrimal and mammary glands. Genetic domain analyses demonstrate that the cytoplasmic and transmembrane domains are essential. Expression patterns demarcate early stem/progenitors in hair follicles and in glandular structures at ductal tips that sense directional and growth cues from macrophages. ADGRA-positive cell populations show stem and early progenitor profiles and are amplified in Wnt1 tumors suggesting they lie towards the top of the ductal lineage hierarchy. Tumors with high expression are associated with lineage skewing in favour of stem/progenitors at the expense of differentiated cell populations and with significantly earlier onset in mice. In humans, expression is associated with aggressive tumor subtypes. Within these, high expression correlates with particularly poor survival outcome. Our data indicate that ADGRA are essential for normal ductal development and have potential use as biomarkers of poor prognosis in cancer.