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13. SUPPLEMENTARY NOTES

14. ABSTRACT: We set out to determine if 1) Gpr specifically identifies mammary stem/progenitors that expand in basal type tumors, and 2) whether ablating Gpr+ cells would eradicate tumors. To test these hypotheses, we proposed to: a) identify, isolate and characterize Gpr+ cells, determine their potency by tracing their progeny, and monitor the effects of ablating them on mammary development; b) determine Gpr expression in human breast cancer, and test if ablating Gpr+ cells affects mammary tumorigenesis in mouse models. In this grant period we crossed a mouse where the Gpr promoter drives expression of a tamoxifen induced cre recombinase to an inducible R26R-TdTomato reporter line, and used their progeny to trace the Gpr lineage in 1) pubertal and mature mammary ducts, 2) in ducts and alveoli during pregnancy 3) in the lactating gland and 4) in aged and multiparous mice. The results show that Gpr+ cells are long-lived unipotent basal stem cells during normal postnatal mammary development. 5) We have also traced the progeny of Gpr+ cells in MMTV-Wnt1 tumors and shown that they have acquired bipotency, generating both basal and luminal progeny, within the context of hyperplasia.

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

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1. INTRODUCTION

There is a pressing need for biomarkers that can distinguish aggressive from indolent breast cancer in order to spare women from unnecessary treatment and direct resources toward those who could derive benefit. Epidemiological links between reproductive history and breast cancer risk have led to the view that hormonally stimulated cyclical proliferation of mammary stem cells (MaSCs) and progenitors, caused by ovulation and pregnancy, expose such long-lived cells to replicative error. Several regenerative subpopulations, for example those marked by sSHIP, LGR5 and PROCR, have been described (1-3). However, these markers label mutually exclusive populations, and do not capture all regenerative cells. Moreover, they are not specific for epithelium. Our poor understanding of the relationships among these regenerative subpopulations remains an obstacle to defining the mammary hierarchy and hinders our ability to harness this knowledge to comprehend the cellular origins of breast cancer subtypes. We proposed to validate an adhesion G-protein coupled receptor (Gpr) as a novel and specific marker of mammary stem/progenitors 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. (80% complete)

Task 2) Determine the significance of Gpr expression in human and mouse breast cancers. (40% complete)

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

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

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

In our Year 1 report (Y1) we documented the generation of two reporter mice Gpr-lacZ and Gpr-DTR:EGFP-creERT2 (Y1 Figs. 1 and 4). The Gpr-lacZ reporter (Y1 Fig 1) allowed us to determine the pattern of Gpr expression by staining with X-gal to detect beta-galactosidase fused to the Gpr ectodomain (Y1 Figs. 2, 3, 7). The Gpr-DTR:EGFP-creER^{T2} knock out mouse showed an identical EGFP expression pattern (Y1 Fig. 5). Promoter activity was confirmed by analysis of mRNA by qPCR (Y1 Fig. 7). Colocalization of these reporters with lineage markers by immunofluorescence (Y1 Fig. 6) showed that Gpr is expressed at predicted sites of embryonic, pubertal and pregnancy-induced stem/progenitor activity in a basal subpopulation. FACS analysis of surface markers (Y1 Fig. 8) indicated that Gpr+ cells express CD24 and integrin CD29 and CD49 characteristic of regenerative mammary repopulating units (4-5).

To investigate the heterogeneity of Gpr populations and their potential overlap with other regenerative subpopulations we performed flow cytometry. Gpr+ cells, identified by a fluorescent substrate of beta-galactosidase (FDG-gal) comigrated with cells stained by PROCR antibodies (Fig. 1) and with sSHIP populations expressing an EGFP reporter.

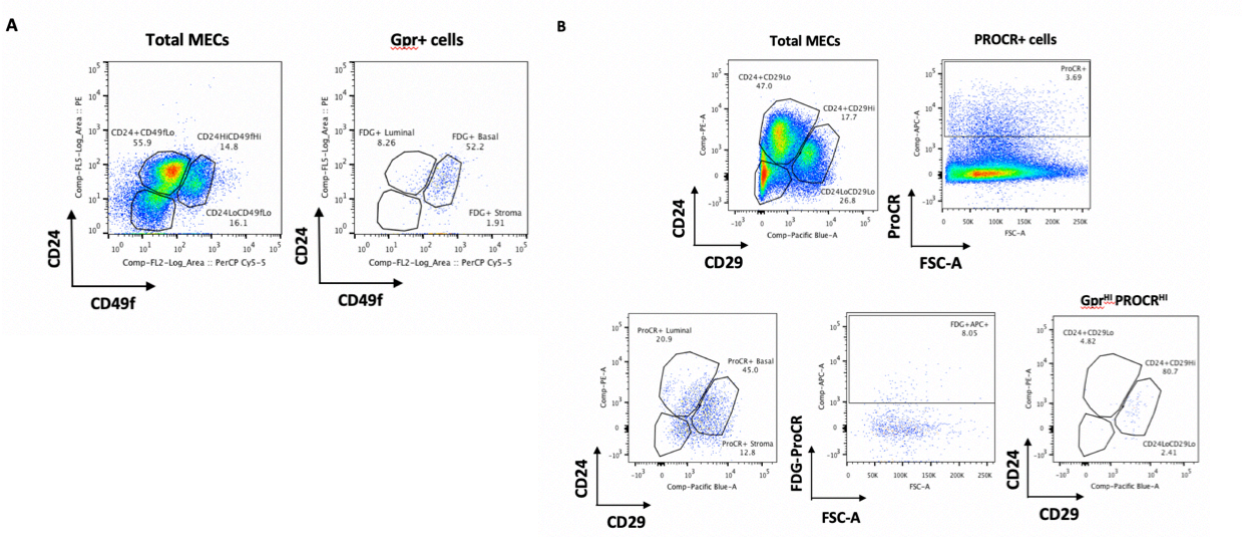

Fig. 1. Gpr+ populations overlap with regenerative PROCR+ cells. A) left: flow cytometry of total mammary epithelial cells stained with CD24 and CD49f define luminal, basal and stromal populations. Right: Gpr+ cells fall within the basal gate. **B)** Flow cytometry of (top) total MECs and PROCR+ cells stained with antibodies against CD24 and CD29; (bottom) followed by selection of FDG+PROCR+ cells. Gpr^{HI} $PROCR^{High}$ cells fall within the $CD24^{+}$ $CD29^{High}$ basal subpopulation.

We also mined two published single cell sequence datasets (6). t-SNE plots generated from *Tabula Muris* dataset confirms our results by showing that Gpr+ cells have a basal phenotype (Fig. 2). t-SNE plots from the second dataset, which shows RNA expression by mammary epithelial cells over the course of mammary development, show that Gpr+ cells co-cluster with PROCR and LGR5 populations (Fig. 3) (7)

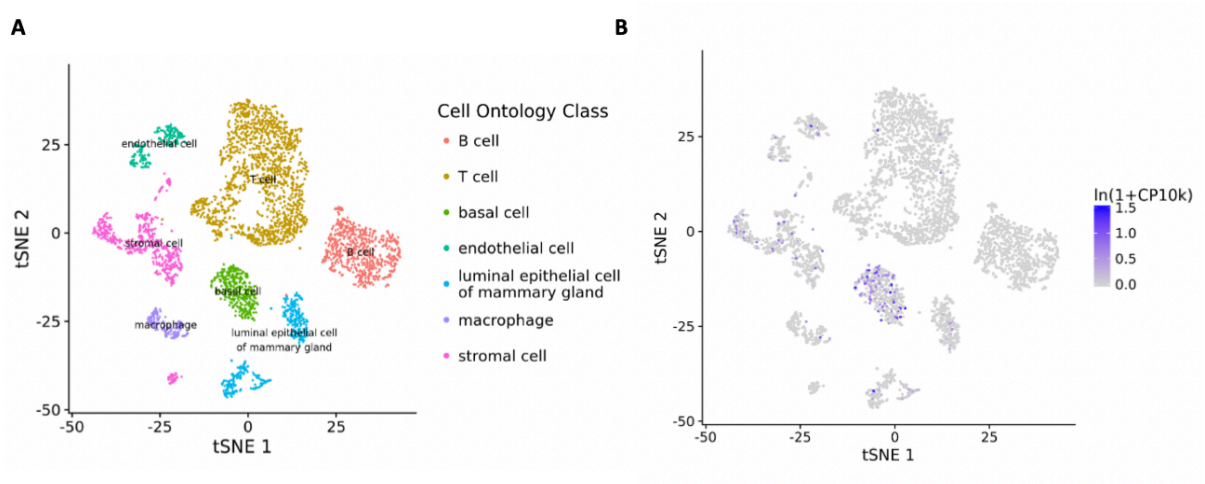

Fig. 2. Gpr+ cells cluster with basal mammary cells. **A)** t-SNE plot of 4,481 mammary cells **B)** t-SNE plot of cells expressing Gpr.
Dataset Source: Tabula Muris Consortium (6)

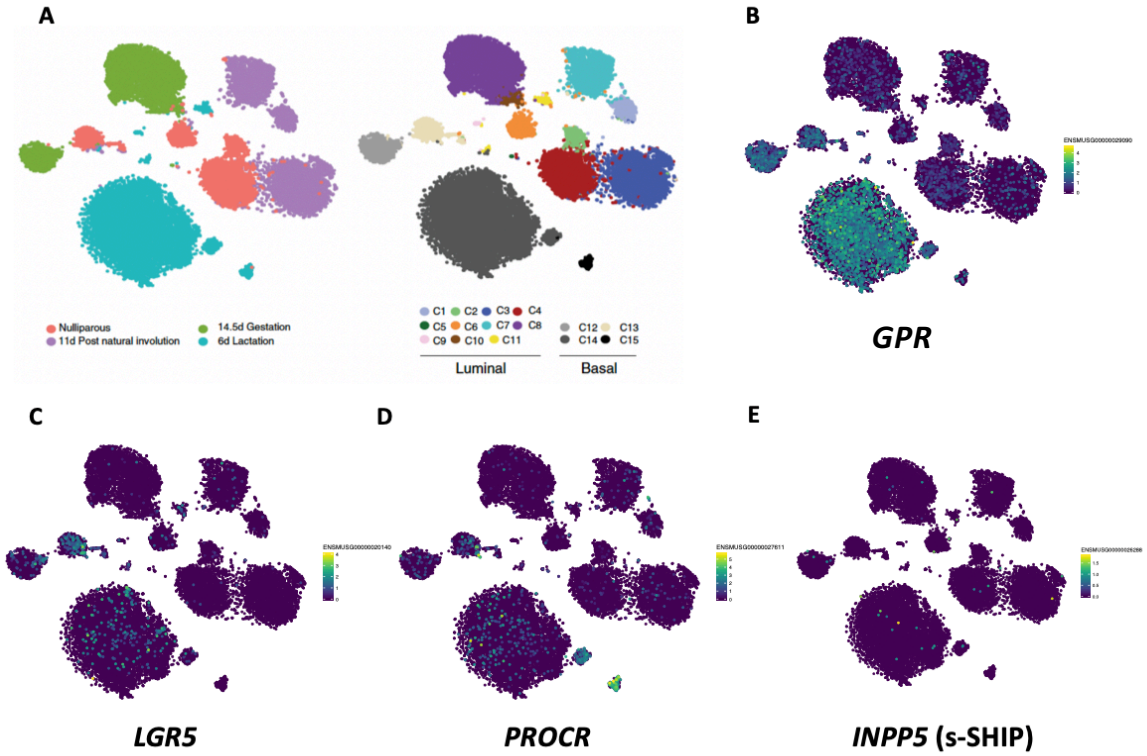

Fig. 3. Gpr localizes with basal clusters of mammary epithelial cells over the course of mammary development. t-SNE plot of single cell sequencing of 23,184 cells **A) (left)** colored according to developmental time points: pink=NP=nulliparous, dark green G=gestation, light green L= lactation, purple PI= post-involution; **A) (right)** clusters colored for luminal and basal markers as indicated below figure. t-SNEs plot clusters colored for the expression of **B)** GPR, **C)** LGR5, **D)** PROCR and **E)** INPP5. **Dataset Source: ref 7**

Originally, we proposed to sort living Gpr+ cells from Gpr-DTR:EGFP-creER^{T2} mice by FACS using the EGFP reporter. Although we could detect EGFP in fixed tissue (Y1 Fig. 5) we were unable to detect it by FACS in unfixed living cells. This is likely due to the DTR:EGFP fusion protein being 100-fold less bright than free EGFP. As the EGFP module is tethered to the cytoplasmic side of the membrane its epitope is unavailable in non-permeabilized living cells, thus precluding the use of antibodies to enhance the signal. To circumvent this problem we attempted to activate a more robust reporter, TdTomato, by delivering a brief dose of tamoxifen (TAM) to bi-transgenic Gpr-DTR:EGFP-creER^{T2};Rosa26R-STOP-TdTomato mice. This creER^{T2} module was originally designed with lineage tracing in mind. However, at early time points following TAM induction, the TdTomato reporter should detect expression within parental Gpr+ cells. Unfortunately, the rate of cre recombination was too low to permit sufficient numbers of parental cells to be detected by FACS. We are now taking two other approaches. First, we will increase the EGFP signal two-fold by breeding homozygous Gpr-DTR:EGFP-creER^{T2} mice. Second, we will attempt to use expression of the human Diptheria toxin receptor (hDTR) that was knocked into the Gpr locus as a surrogate tag for Gpr expression. hDTR antibodies are available commercially and we are currently screening these for their specificity in detection of hDTR and lack of cross reaction to mouse. Success with these approaches will permit us to proceed to sort and transplant viable Gpr+ and Gpr- depleted cells into empty mammary fat-pads to functionally test their regenerative potential at limiting dilutions.

In conclusion, our results this year show that Gpr+ cells are heterogenous and encompass multiple regenerative populations. We will extend these analyses to determine the parent-progeny relationship among these cell populations i.e. are Gpr+ cells parental and give rise to the stage-specific LGR5, PROCR and sSHIP progeny, or is Gpr marking activated progeny of multiple parental subpopulations.

Subtask 2 and 3. Cross Gpr-DTR:EGFP-creER^{T2} to Rosa26-STOP-reporter lines (1-18 months). 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).

Although the 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 we proposed to carry out lineage tracing, where a permanent genetic change is introduced that allows progeny to be traced within the normal physiological context. Last year we 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 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 cells at initial time points but, as this is a permanent genetic change, reporter expression continues under the control of the open ROSA locus in all cellular progeny.

Originally we proposed to use R26R-STOP-lacZ to produce a permanent histological record of Gpr progeny by X-Gal detection of Gpr-lacZ expression. However, during the course of our

experiments we became aware of its limitations in assigning cells to the basal or luminal layer when performing 2D analysis on tissue sections. To address these issues we switched to crossing the Gpr-DTR:EGFP-creER^{T2} mice to fluorescent reporter lines and also established 3D confocal imaging of whole mounts to follow the cellular progeny and assign them to basal or luminal lineages and subpopulations (Y1 Fig 9 and 10). Over the last year we have improved the efficiency of this breeding colony by generating parental homozygous Gpr-DTR:EGFP-creER^{T2} mice and crossing them to homozygous ROSA26R-STOP-TdTomato mice and ROSA26R-STOP-confetti mice. This a) removed the need for genotyping and b) reduced use of animals by generating a greater proportion of genetically useful female progeny in each generation c) increased the reporter expression thereby improving signal detection. We have been able to successfully trace the progeny of pubertal Gpr+ cells throughout mammary development using the Gpr-DTR:EGFP-creER^{T2}/ROSA26R-STOP-TdTomato mice. We administered TAM during early puberty (4 weeks) to avoid effects of depleting estrogen on ductal extension and assessed mammary whole mounts at intervals thereafter. Mammary glands were harvested at intervals over the course of mammary development and processed for whole mount CUBIC clearing, followed by immunofluorescence and 3D confocal imaging. In Year 1 we showed labelled progeny in pubertal terminal end buds (Y1 Fig. 10) and ducts of pubertal mice (Y1 Fig. 11). We have extended these studies to trace the progeny of pubertal Gpr+ cells in mature ducts (Fig. 4), in alveoli and ducts during pregnancy (Fig. 5), in lactating glands (Fig 6.). Gpr progeny show classical spindle-shaped features typical of ductal basal cells and the characteristic spider web morphology of contractile myoepithelia in lactating glands. They colocalize with basal-specific markers by immunofluorescence (Fig 7), such as keratin 5 (K5), keratin 14 (K14) and smooth muscle actin (SMA) and lack expression of luminal markers (Fig 8), such as E-cadherin (E-cad). These data show that pubertal Gpr+ cells generate basal progeny throughout mammary development and therefore pubertal Gpr+ cells are unipotent basal stem/progenitors.

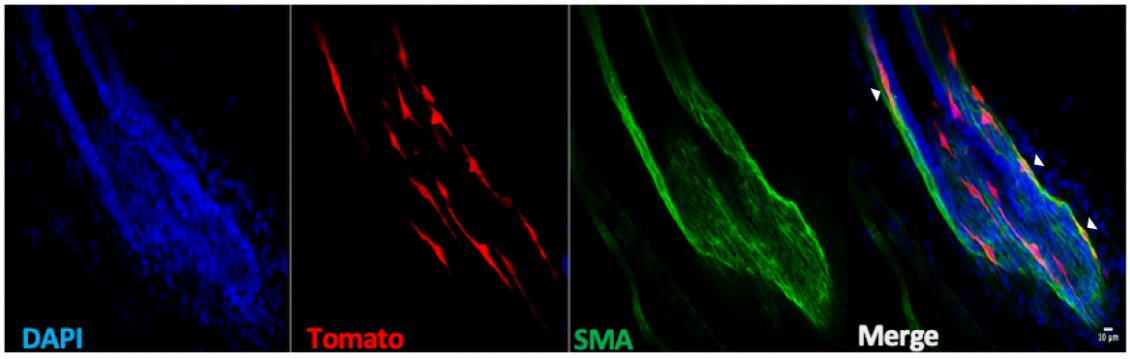

Fig. 4. Pubertal Gpr+ cells give arise to basal myoepithelial cells in the mature duct. Immunofluorescence of 3D-mature ductal system from 7-week old mice shows spindle-shaped basal tomato (red) cells positive for SMA (green). DAPI = nuclear staining.

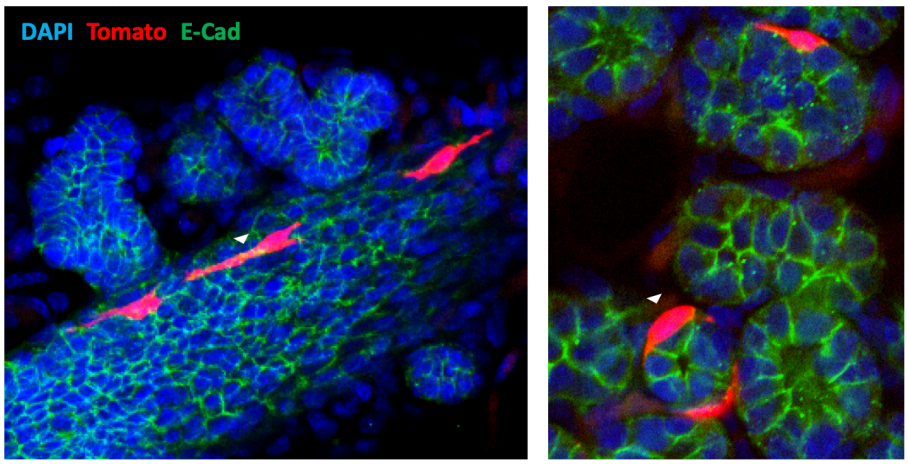

Fig. 5. Pubertal Gpr+ parental cells generate ductal and alveolar basal cells in pregnant gland. Female injected with TAM at 4 weeks of age were mated ~10 weeks of age and analyzed at day 14.5-15.5 of pregnancy. (left) Spindle shaped Tomato cells along the ducts and (right) surrounding new formed alveoli are negative for luminal E-cadherin expression (green). DAPI = nuclear staining.

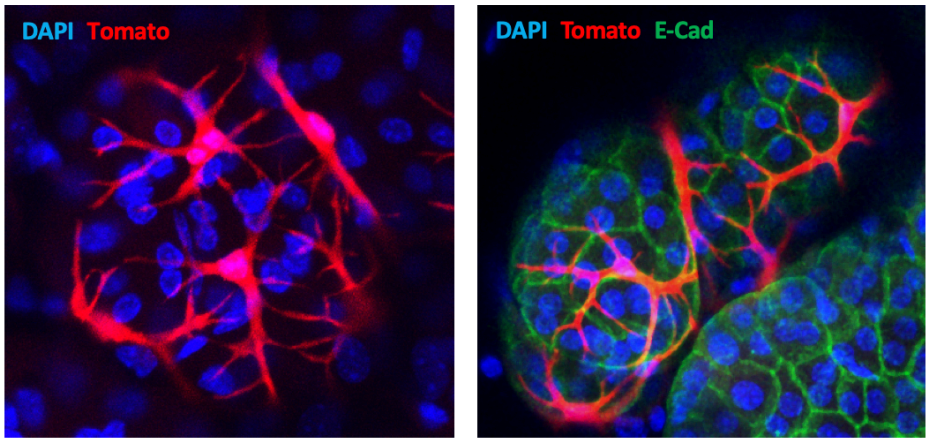

Fig. 6. Pubertal Gpr+ parental cells give rise to fully differentiated basal, myopithelial cell progeny in lactating gland. 3D analysis of lactating mammary gland at day 6 after birth shows fully differentiated tomato+ basal cells (Red) with characteristic myoepithelial shape surrounding E-cadherin-positive alveoli responsible for milk secretion (E-cad, green). DAPI = nuclear staining.

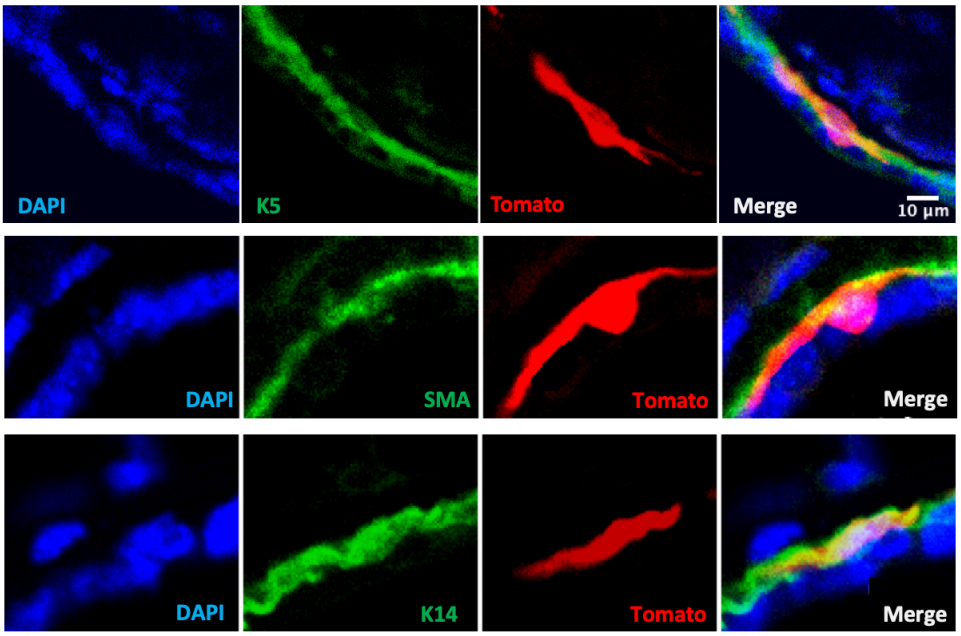

Fig. 7. Gpr derived tomato cells colocalize with basal markers. Representative tomato cells (red) in mammary gland of virgin mice after TAM induction at 4 weeks of age. Immunofluorescence for the basal markers K5 (green), SMA (green) and K14 (green) as indicated. DAPI = nuclear staining.

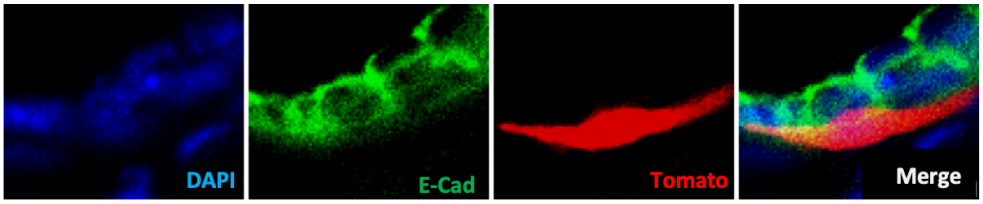

Fig. 8. Gpr progeny lack expression of the luminal marker E-Cadherin. Immunofluorescence of mammary gland of virgin mice after TAM induction at 4 weeks shows tomato cell progeny (red) are negative for luminal marker E-cad (green). DAPI = nuclear staining.

We addressed the longevity of Gpr+ progenitors by tracing their progeny after 6 months in virgin mice and after completing two cycles of pregnancy and involution. Clusters of TdTomato cell progeny elongated morphology were localized along the basal ductal borders in 6-month virgin mice (Fig. 9A). TdTomato cells with a more rounded appearance were also present after 2 pregnancy cycles (Fig 9B). These results indicate that Gpr+ cells are long-lived basal stem/progenitors.

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. We found that this line did

not undergo cre recombination efficiently. This has also been reported recently by others using different promoters to drive cre expression in the mammary gland (8, 9). Nevertheless, as the rate of recombination with the Gpr-driven creER^{T2} was low we no longer think that confetti will be needed to study clonality.

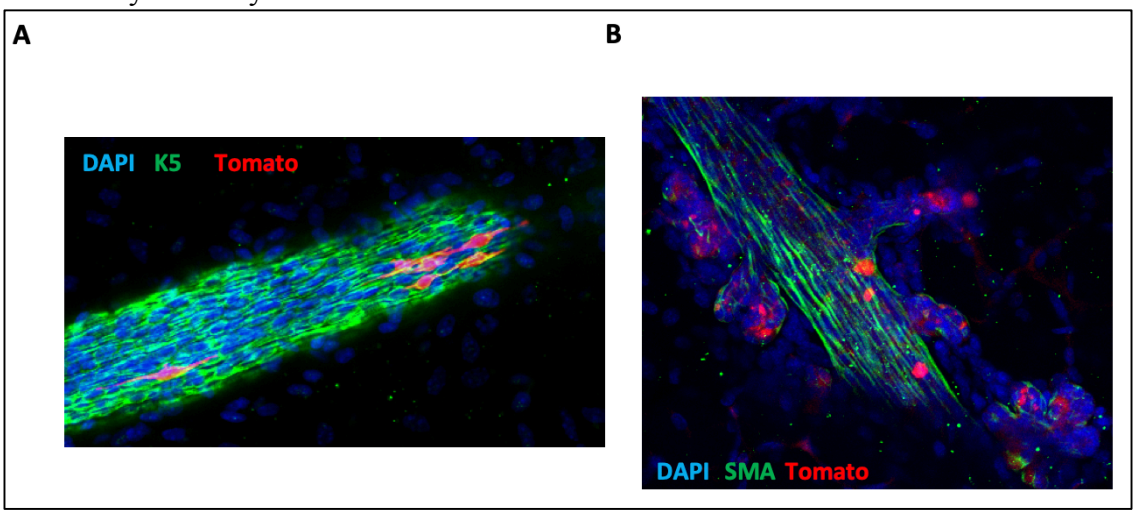

Fig. 9. Gpr basal stem/progenitors are long-lived. Mice were injected with TAM at 4 weeks of age. Clusters of tomato cell progeny (red) that are positive for the basal marker K5 (green) by immunofluorescence are found in mammary gland 6-months later (**A**) and after 2 cycles of pregnancy (**B**). DAPI = nuclear staining.

We are extending these analyses by administering TAM to mid-pregnant females in order to trace the progeny of embryonic Gpr+ cells and of Gpr+ cells that appear at ductal tips during pregnancy. Two attempts to labelling mid-pregnant dams resulted in their failure to deliver live pups due to effects of TAM on the PR/PRL hormonal axis. We are addressing this by following two published protocols that have successfully overcome this problem by 1) delivering progesterone concurrently with TAM 2) performing C-section on the dams just prior to term and fostering the pups (10,11).

Subtask 4. Ablate Gpr+ cells in Gpr-DTR:EGFP-creER^{T2} mice by administration of DTA

We administered 50 ng/g body weight of Diptheria Toxin (DTA) or vehicle to a small cohort of 6-week old mice expressing Gpr-DTR:EGFP-creER^{T2} and to wild-type and Gpr-lacZ controls that both lack expression of hDTR. All controls continued to thrive. However, all Gpr-DTR:EGFP-creER^{T2} lost weight rapidly and adopted a staggering gait, forcing us to end the experiment after one week for humane reasons. A second attempt was made using reduced doses (25 ng/g and 10ng/g body weight). Again the mice lost weight and showed abnormal gait and had to be euthanized due to reaching human endpoint criteria. We investigated the reasons for this morbidity by examining Gpr expression by X-Gal staining of other organs and found high Gpr expression in salivary glands, parotids, testis, kidney, ear and brain (Fig. 10). The latter were the most likely cause of the ataxia and morbidity. These results, however, have led to a productive collaboration with a research group in Denmark, who are working on a possible role for Gpr as a receptor for the Mumps SH protein. We have contributed the expression data, which shows a pattern consistent with the spectrum of tissues affected by Mumps. We have also supplied this group with post-mortem tissue of our knock out Gpr-DTR:EGFP-creER^{T2} mice for them to carry out binding assays.

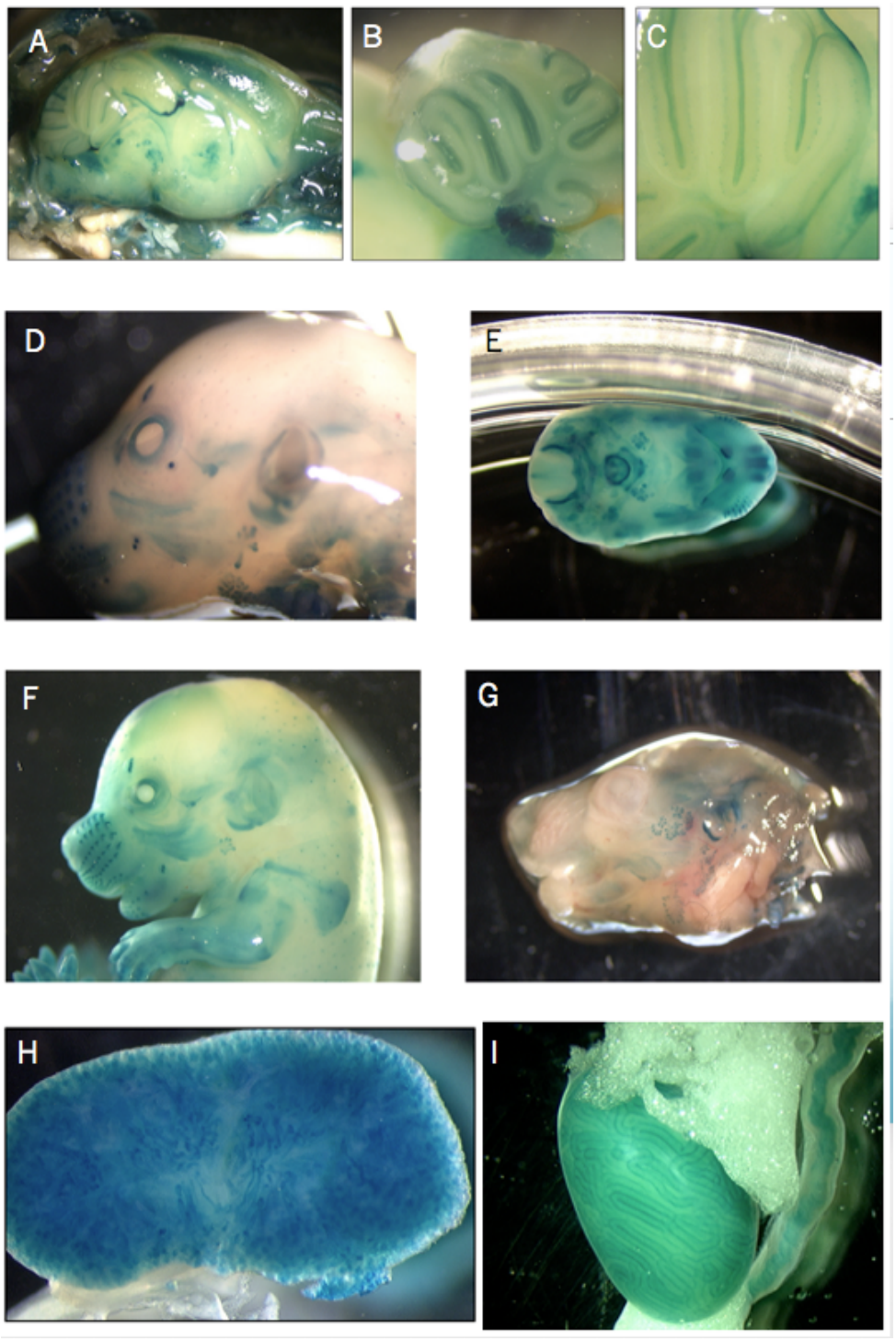

Fig. 10. Gpr-lacZ expression in mouse tissue.
X-Gal staining of tissues from Gpr-LacZ mice shows the pattern of Gpr expression (blue) in (A) whole brain; (B,C) cerebellum; (D, F, G) lacrimal glands, parotids and ear canal (E) submandibular glands; (H) kidney; (I) Testis

Subtask 5: Validate cell death by loss of Gpr expression and monitor the effect on mammary development.

To circumvent the lethality observed in the experiment described above we harvested pre-pubertal mammary glands from progeny of homozygous Gpr-DTR:EGFP-creER^{T2} mice crossed to Gpr-lacZ mice, which carry both alleles (Gpr$^{DTR/lacZ}$), and transplanted them contralaterally together with whole mammary glands from control Gpr-lacZ mice into immunocompromised Foxn1 nu mice. Host mice were given 50 ng/g body weight of DTA or vehicle. All mice survived this dose. The glands were harvested and examined one week later and all glands from Gpr$^{DTR/lacZ}$ mice treated with toxin showed elimination or reduction of lacZ expression whereas lacZ expression continued in all controls. We are now expanding and repeating these experiments to permit quantitative data to be gathered on the effects of ablation of the Gpr+ cells on subsequent ductal outgrowth. In addition, we have harvested and frozen mammary organoids from Gpr$^{DTR/lacZ}$ *and* Gpr-lacZ mice to enable us to carry out functional analyses of mammosphere formation with and without toxin.

In the previous report (Y1) we documented the phenotypic effects of loss of the Gpr on mammary ductal elongation (Y1 Fig. 12), male sterility and also the pronounced eye phenotype seen in homozygous Gpr-DTR:EGFP-creER^{T2} and Gpr-lacZ mice (Y1 Fig. 13, 14). In year 2 we investigated further if the mice recapitulated features of human "dry eye" syndrome. Immunofluorescence analysis showed inflammatory infiltration of CD4, CD8 and macrophages into lacrimal glands as observed in human "dry eye" (Fig. 11 below). This analysis could be useful in defining signaling pathways downstream of Gpr and help to identify drugs to decrease the effects of high Gpr activity seen in some breast cancer.

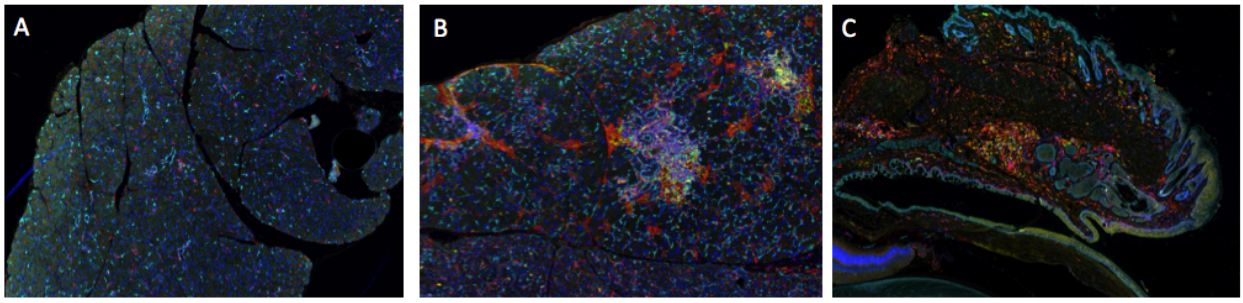

Fig. 11. Gpr loss leads to inflammation of the lacrimal gland and eyelids.
Immunofluorescence analysis on eye sections of: (A) wild-type (B and C) Gpr-DTR:EGFP-creERT2 mice. (A and B) lacrimal glands. (C) eyelids. Markers: DAPI stained nuclei (blue); F480 stained macrophages (red); CD4 T-helper (green); FoxP3 T-regs (purple); B220 B-cells (orange); CD8 cytotoxic T cells (yellow); Keratin 5 (cyan). Note immune infiltration of the tear-producing lacrimal glands and stroma surrounding the meibomian glands in the eyelids of mice lacking Gpr expression (B,C).

Specific Aim 2. To determine the significance of Gpr expression in breast cancers.
Task 1. Screen breast cancer cell lines for Gpr expression by qPCR and mine bioinformatics datasets of human breast cancers.
In year 1 we documented Gpr RNA expression in human breast cancer cell lines (Fig 15) and mined publicly available dataset to show that high expression of Gpr predicted for poor prognosis in relapse free survival exclusively in the basal breast cancer subtype in human breast cancers and was associated with poor outcome in distant metastasis free survival for basal-like (p=0.0043), Luminal B (p=0.045), and Luminal A (p=0.015) suggesting that increased expression of Gpr signified increased metastatic capability in multiple human breast cancer subtypes. We are continuing to expand these studies to include additional cell lines representative of the different basal subtypes.

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 now generated homozygous Gpr-DTR:EGFP-creER^{T2} and crossed them to MMTV-Wnt1 and Gpr-lacZ mice to generate the first experimental cohorts of compound Gpr$^{DTR/lz}$;MMTV-Wnt1 female progeny. Currently, we are permitting this first cohort to develop tumors. MMTV-Wnt1 tumors have an average latency of 7 months. Once the tumors form we will harvest the cells, treat them with and without DTA, monitor for ablation of Gpr+ cells by loss of Gpr-lacZ expression, and transplant them into Foxn1 nu mice to monitor the effects of ablating Gpr+ cells on tumor propagation. Tumors arising from cells transplanted in this manner generally arise within 1 month.

Subtask 2: Ablate Gpr cells by DTA administration and assess effects on tumor onset, progression, regression and histology. In the meantime, we have begun lineage tracing experiments to identify the progeny of Gpr+ cells present in the tumor setting. TAM was administered to compound Gpr$^{DTR/lacZ}$;MMTV-Wnt1 females at 5 weeks of age and their glands were harvested at 12 weeks of age when hyperplastic. The glands were processed for whole mount CUBIC clearing, followed by immunofluorescence and 3D confocal imaging. TdTomato+ progeny cells were present in both basal and luminal positions and colocalized with both basal keratin 5 and luminal E-cadherin markers by 3D immunofluorescence analysis (Fig. 12). Thus, in contrast to the unipotency of Gpr+ cells in the normal gland, in the context of the MMTV-Wnt1 hyperplasia Gpr+ cells acquire bipotency.

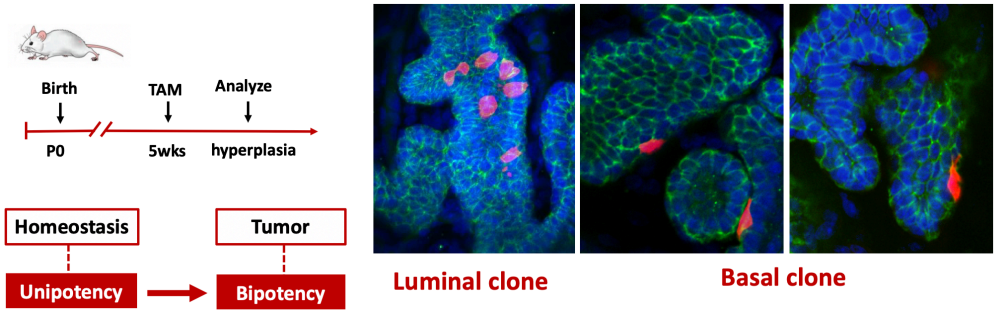

Fig. 12. Gpr progenitors acquire bipotency in MMTV-Wnt1 tumors. Immunofluorescence of hyperplastic mammary glands show tomato expression (red) in both spindle-shaped basal cells as well as in luminal cells expressing E-cad (green). DAPI = nuclear staining.

- **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 (8).
- **Dr. Spina** is a fully trained pharmacist and Ph.D. During the course of this project she has augmented her existing skills in breast cancer cell culture by acquiring new skills in a) mouse genetics b) histological analysis, c) tissue clearing and 3D-confocal imaging d) *in vivo* survival surgery and fat-pad clearing and transplantation. e) lineage tracing f) organoid isolation and mammosphere culture
- **Dr. Spina** has taken a courses in ethics, animal welfare and professional career development. She attends the "works in progress" presentations of the stem cell and pharmacology and molecular oncology training programs as well as the cancer center. She is scheduled to present in these forums and the departmental retreat. She has met with a committee comprising: 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. She made her first public presentation as an invited speaker at the 9th Adhesion GPCR Workshop Sept 13-15, 2018 in Portland, OR and was very well received.

- **How were the results disseminated to communities of interest?**
- **Dr. Cowin** has been invited to present these findings at the Gordon Conference on Mammary Gland Biology in April 2020
- **Dr. Cowin** has been invited to present these findings to the Skirball Institute, NYUSOM in October 9, 2019
- **Dr. Cowin** has been invited to present these findings to the Department of Opthalmology, NYUSOM on November 5, 2019
- **Dr. Cowin** has been selected to present this work to the Annual Skirball Retreat in October 2020
- **Dr. Spina** presented this work as an invited speaker at the 9th Adhesion GPCR Workshop Sept 13-15, 2018 in Portland, OR.
- **Dr. Spina** presented this work as a selected speaker for the Postdoctoral Association Research Day NYUSOM on September 23, 2019

- **What do you plan to accomplish during the next reporting period to accomplish the goals and objectives?**
- Our major focus will be to publish the lineage tracing of Gpr+ cells in the mammary hierarchy by completing the embryonic and pregnancy tracing.
- Our second major goal will be to treat the mice with DTA and examine the effects on ductal outgrowth and tumor propagation

4. IMPACT

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

- Our expression and linage tracing studies support the concept that Gpr identifies unipotent basal stem cells of the mammary gland.
- Our results in Y1 showed that mice lacking this adhesion GPCR show delayed mammary ductal elongation and that the presence of the cytoplasmic signaling domain of this orphan receptor is essential. In Y2 we have shown that Gpr and its signaling domain are required for glandular development more generally.
- Our results in Y1 showed 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. These support 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. In Y2 we have addressed the mechanism by showing that Gpr+ cells acquire bipotency in the tumor situation.

- **What was the impact on other disciplines?**
- The homozygous Gpr-DTR:EGFP-creER^{T2} mice, which have a pronounced eye phenotype display several features of "Dry Eye Syndrome" and could have utility as a model for this human inflammatory disease.
- Our studies show that the pattern of Gpr expression is consistent with those affected by Mumps virus supporting the hypothesis that it functions as a receptor.
- The expression of Gpr in several secretory glandular structures that share common ectodermal origin suggests it may play in ductal branching and secretory differentiation. Its presence in the stem cell compartments of several other ectodermal appendages including the bulge and secondary germ compartments of hair follicles (see Y1 report) suggest it may be an indicator of a more generalized stem cell function.

- **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**:
  - As we were unable to detect the EGFP reporter in living cells and the recombination was too infrequent to permit substitution of the lineage tracer reporter, TdTomato. This prevented our original plan to isolate living cells for functional assays and to monitor cell Gpr cell ablation. We have therefore changed to breeding mice homozygous for the DTR:EGFP to double the expression of the reporter and are testing the specificity of antibodies against the DTR ectodmain to distinguish the human form expressed specifically on Gpr+ cells.

- We have substituted Rosa26R-STOP-TdTomato for Rosa26R-STOP-confetti and Rosa26R-STOP-lacZ lineage reporters as the former is more efficient and permits 3D imaging of the cellular progeny

❖ **Actual or anticipated problems or delays and actions or plans to resolve them**

- The inability to detect EGFP reporter expression discussed above delayed the generation of the Wnt1 tumor mice as additional generations were required to introduce the second *lacZ* reporter onto the strain background so that we could monitor that Gpr+ cells are indeed ablated.
- Administration of TAM during pregnancy led to abortion or the delivery of dead pups. We are therefore co-administering progesterone, performing caesarian section just prior to delivery and supplying foster mothers.

❖ **Changes that had a significant impact on expenditures –**

- Delays in Hiring: Dr. Spina joined the lab in March 2018. A second fellow was scheduled to join 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. We have not found another suitable candidate. However, as Dr. Spina is now well skilled in transplantation and in vivo surgery we have requested a no cost extension to use the remaining unexpended funds to support her salary to complete the proposed experiments.

❖ **Significant changes in use or care of human subjects, animals, biohazards or select agents**

- Nothing to report

6. PRODUCTS

- **Publications, conference papers and presentations**
  See appended abstract from Dr. Spina's presentation to the 9th Adhesion GPCR workshop

- ***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

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

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.