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Award Number: W81XWH-08-1-0708

TITLE: Fibroblast Growth Factor 2: an Epithelial Ductal Cell Growth Inhibitor That Drops Out in Breast Cancer

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REPORT DATE: October 2010

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
October 2010	Annual	15 September 2009 – 14 September 2010

4. TITLE AND SUBTITLE

Fibroblast Growth Factor 2: an Epithelial Ductal Cell Growth Inhibitor That Drops Out in Breast Cancer

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-08-1-0708

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

University of California, San Diego
La Jolla, CA 92093-0657

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

The overall objective of this work is to establish whether the growth factor called FGF merits investigation as a drug development target in a breast cancer therapeutic drug discovery program. We are now questioning the idea that FGF2 is involved in the "dysinhibition" model of breast cancer progression. If so the better approach would be to create FGF2 antagonists. To meet the overall idea, we are measuring the natural onset and progression of cancer in a mouse model of spontaneous mammary tumor development. We show that in a new line of PyVT mice that are cancer prone and lack one of the presumed regulators of tumor growth, FGF2 have altered tumor development kinetics

15. SUBJECT TERMS
transgenic mice, growth factors, gene knockout, PyVT mammary cancer

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	11	19b. TELEPHONE NUMBER *(include area code)*

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Fibroblast Growth Factor-2: an Epithelial Ductal Cell Growth Inhibitor that Drops Out in Breast Cancer

Andrew Baird, PhD,
CDMRP-BCRP IDEA Award

1. Introduction

Many factors present in the breast are thought to be involved in the progression of breast cancer. If this was not complicated enough, each of these factors can have different activities depending on where they are produced, what cell they are acting upon and whether they are present in small or large amounts. The growth factor called "FGF" stands for fibroblast growth factor and it is a good example of this medical problem. Although FGF was originally named because it was discovered to stimulate fibroblast cell growth, it is most famous for its ability to make blood vessels grow. But depending on how it is tested in cell culture, it can stimulate cells, inhibit cells or even change how these cells respond to other factors. So what is it really doing in the breast? And is it involved in the progression of breast cancer?

To answer these questions, we turned to a genetic mouse model of breast cancer where mammary tumors develop with very high predictability and at very predictable times after birth. The model, called the "PyVT mouse", was created by introducing a cancer-causing gene from polyoma virus (PyVT) into the genome of the mouse mammary gland. These mice are otherwise normal except that they all get mammary tumors by 60-85 days of age.

Our idea was to ask two very straightforward questions: (1) what happens to the FGF that is naturally found in the mammary gland when these cancers develop? and (2) what happens to these tumors if there is no FGF in the mammary gland? We reasoned that if FGF is involved in the progression of cancers, then the levels may change as cancer develops. We also reasoned that if there was no FGF present then maybe the natural course of cancer development would change. If it did, then the results would point us to a new target for drug discovery: FGF-dependant breast epithelial cells.

2. Body

This project is roughly on schedule for successful completion at the end of the funding period with minimal slippage and in some areas, acceleration of the proposed tasks. The progress is summarized in the SOW table presented below but also highlighted in the key research accomplishments section. In this funding period, we got the genetically modified line going, began to acquire data on tumor progression in the genetically modified animals and initiated the staining of different markers of tumor growth and angiogenesis.

Methods.

Mice: Experiments were conducted under the oversight of the Institutional Animal Care and Use Committee of the University of California, San Diego. This project used three strains of mice: (1) wild-type, (2) PyVT mice developing spontaneous mammary tumors, (3) FGF2 deficient mice. Hemizygous PyVT (t/+) males and FGF-/- females were crossed to generate male offspring that are heterozygous for FGF2 and expresses the transgene. PyVT/FGF2+/- males are crossed with female FGF2+/- mice to yield PyVT/FGF2-/- , PyVT/FGF2+/- , and PyVT/FGF2+/+ mice. Genotypes were identified from tail DNA by slot blot analysis using a probe for PyVT and FGF2.

Tumor measurements: We followed cohorts of female PyVT/FGF2-/-, PyVT/FGF2+/-, and PyVT/FGF2-/- mice to evaluate mammary tumor onset, incidence, growth and progression. After weaning, body weights of the mice were recorded weekly and the presence of palpable lesions in the mammary glands were determined. Blinded assessments were done with calipers to measure tumor size in two dimensions. Tumor volumes were calculated using a formula of axb2/2 with a being the length and b the length. Following excessive weight loss or the presence of tumors in excess of 20 mm in length, the mice were killed. Tumor volumes at various time points and tumor weights at necropsy were compared between the three groups using ANOVA followed by a Wilcoxon-Rank test.

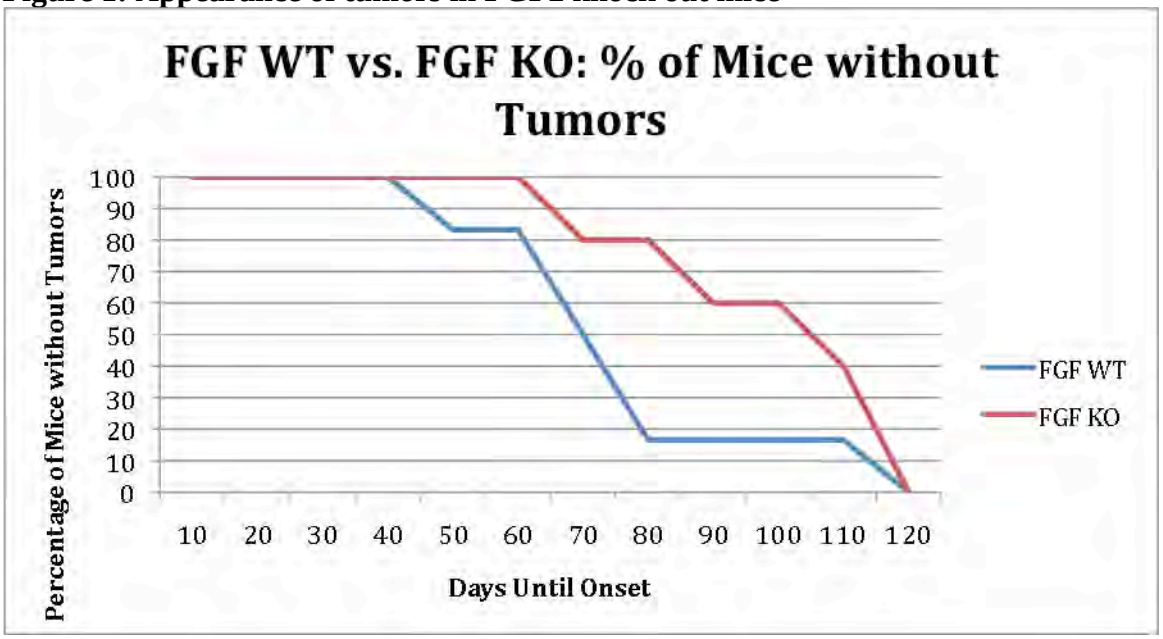
Immunohistochemistry: To further characterize mammary tumor development in the absence of FGF2, we performed histological characterization of primary tumors at the early stages of tumor development. Mouse mammary fat pads (MFP) were obtained following euthanasia, perfused with PBS and then fixed with 4%

paraformaldehyde (PFA) in PBS, pH 7.4. At the time of immunohistological (IHC) staining, paraffin sections were first deparaffinized in xylene and in progressively more dilute solutions of ethanol. Following this, sections were incubated with Proteinase K (Millipore Cat # 21627 0.2 mg/ml) for 10 minutes. These sections were then blocked in normal goat serum (ABC Rabbit Kit PK-4002) for 1 hour and incubated with either anti-Factor VIII (Biocare) or anti-FGF2 and anti-FGFR1, R2, R3 and R4 (Sigma) at concentrations of 1:100 overnight at 4 C. After washing, sections were then incubated with biotin-conjugated secondary antibody for 30 minutes at room temperature. Between each of the following steps, three separate washes were conducted for 3 minutes each. Endogenous peroxidase activity was quenched with 0.3% hydrogen peroxide in distilled water before the sections were treated with Avidin Biotin Complex (ABC) kit (Vectastatin, Burlingame Ca). For visualization, the sections were incubated with diaminobenzidine substrate for 30 minutes. Following washes, the sections were successively counterstained by incubating in, Hematoxylin, 2% acetic acid, bluing reagent, with separate washes between. Sections were then dehydrated in solutions of progressively more concentrated ethanol and xylene. The cover slips were mounted with Vectamount Mounting Solution. Images were taken with an Olympus FXS100-BSW microscope.

Preliminary results

1. Tumor appearance in FGFKO mice: It took a prolonged period of mating and back crossing into PyVT background mice to generate a genetically stable line suitable to monitor the appearance of tumors in KO mice. The primary issue was creating a control line to which the experimental line could be compared. Second, the experiments could only use female mice and the tumors sometimes developed so rapidly that we could not cross breed. This was a major stumbling block that we had to overcome. We did so by creating and maintaining a carrier lines of male and female to optimize animal yields. During this funding period we were able to monitor tumor development, a primary objective of the proposal. As shown in Figure 1 below , we began to observe that there was delayed progression of tumorigenesis and decreased tumor size in knockout and heterozygous mice for FGF2 as compared to normal mice. As illustrated, the simplest question was asked: i.e. when do tumors arise? We began too see a difference between the wild type line (containing PyVT and FGF) vs. the knock out line expressing no FGF. All of these experiments were conducted blinded to the investigator but clearly data collected during different stages of mammary development show that mice lacking the FGF2 gene have delayed onset of tumorigenesis (see figure below).

Figure 1: Appearance of tumors in FGF2 knock out mice



As shown the wild-type mice begin to have palpable tumors by day 65 and by day 80, all have tumors. Some FGF2 KO mice begin to have palpable tumors around that same time. However, most FGF knockout mice show a delayed progression of tumorigenesis, with some only beginning to have palpable tumors around day 110.

Similarly, we have begun to evaluate the preliminary data arising from other "het-lines" of mice that only have one copy of FGF2 knocked out and preliminary analyses show an intermediate phenotype with an onset of tumorigenesis that is earlier than was seen in FGF2 KO mice, but later than was seen in FGF2 WT. These data are being collated for analyses in the final report and were presented at the Era of Hope poster presentation. The preliminary analyses presented at this meeting also show that mammary tumors in FGF2 knockout mice are also significantly smaller than those in normal mice. Accordingly there are also different ways of evaluating tumor progression: the appearance, location and size of tumors generated. Earliest data generated during this funding period suggest that tumors in "FGF-normal" PyVT mice are also larger as compared to mice lacking FGF2 at time of necropsy.

2. Morphology of mammary cancers in FGF+/+ and FGF-/- animals that develop tumors: As expected ductal morphology in PyVT- is comparable to PyVT+ by Masson's Trichrome staining of mammary gland from a normal lactating mouse harvested at 26 weeks of age. As shown in Figure 1 of Supporting Data, the ductal cells are readily evident in lactating mothers because of their post partum status. They are difficult to localize in normal mice mammary glands and this approach allowed us to obtain sufficient control tissue for comparative purposes. Also shown in this figure is the histological pattern of tumor development in the FGF++ and het mice which lack one FGF2 gene. They appear histologically similar and are characterized by dense areas of tumor cell growth (arrows). In contrast the normal ductal cell epithelial in a non tumor mouse shows a normal layer of ductal epithelial cells. The loss of FGF2 appears to result in decreased tumor vascularization but we have sought to characterize markers for each of these endpoints.

3. FGF receptors in mammary tumor development: One of our objectives was to characterize FGF receptor expression in mammary tissues of these mice and we obtained five kinds of antibodies for FGF receptors (Figure 2). One antibody we developed ourselves (AB6) recognizes all forms of the FGF receptor and has the advantage of informing the investigator of receptor presence. Its disadvantage is that it does not inform as to specific receptor gene. To this end, we turned to antibodies generated against different receptors. As shown in Figure 2, it is clear that the FGFR pathway is present in these cancers. The pan-specific antibody (Panel A) and each of the anti FGFR1, R2, R3 and R4 react positively in the tissue sections. There is also an increase in staining in the tumor cell compared to neighboring area (which we did not yet quantify). These data certainly support our hypothesis that FGF-FGFR pathways are active in mouse mammary cancer. The lower signal in adjacent normal tissue points to the receptors as potential markers as well.

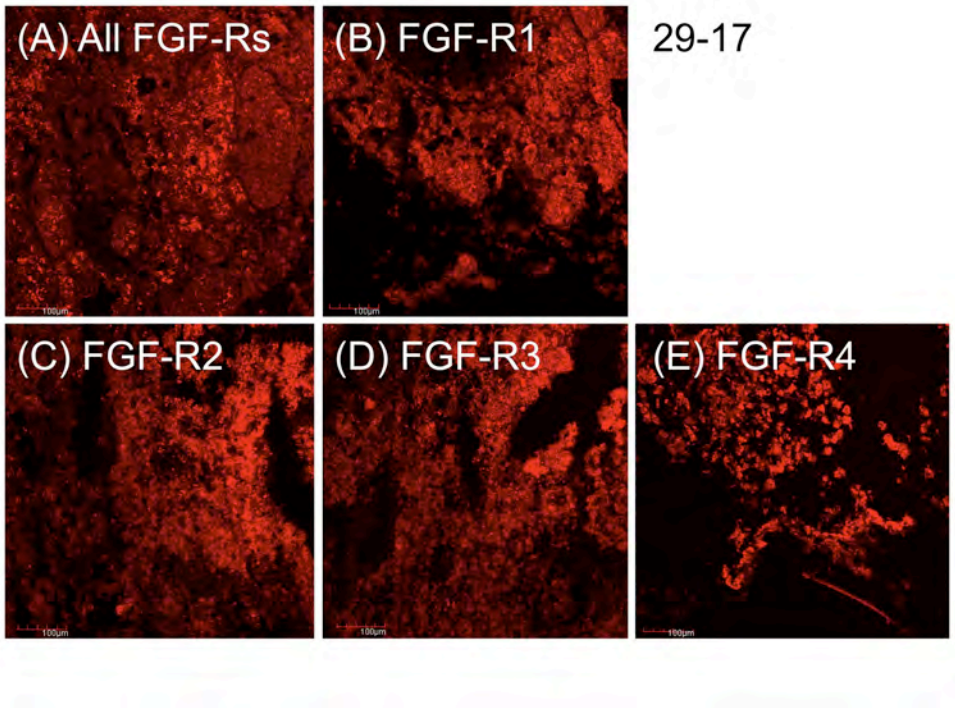


FIGURE 2 FGF receptors in mammary tumors

4. Markers of normal mammary ductal cells and mammary tumors in FGF+/+ and FGF-KO mice: We used IHC to evaluated markers like smooth muscle actin (SMA), myeloperoxidase (MPO), collagen lV (Col-lV)

Von Willebrand's factor (VWF) and an newly recognized marker called Ecrg4 in normal lactating mouse mammary fat pad at 17 weeks of age and compared it to FGF2 knockout mouse mammary fat pad at 19 weeks of age, a PyVT+ mouse mammary fat pad at 22 weeks of age and a PyVT+, FGF knockout mouse mammary fat pad at 14 weeks of age. We were generally dissatisfied with these markers as illustrated by the patterns of staining of normal ductal cells shown Figure 2 of Supporting Data, Section 8 of this report and in tumors as shown in Figure 3 here. While positive staining was indeed obtained and the pattern was better then that observed on normal mouse mammary tissue, the results were overall unimpressive. The tumors, which are highlighted by an arrow in each of the panels below did not react significantly (Panels B, C, D and E) although some patterns were observed (for example vessels with VWF in panel D). In contrast, we noted that a newly recognized epithelial cancer tumor suppressor gene called Ecrg4 to which we were fortunate enough to find antibodies, was readily staining the mammary cancer epithelium (Panel F). This led us to determine whether this peptide could be a marker for the FGF knock out mice while we monitored FGF staining.

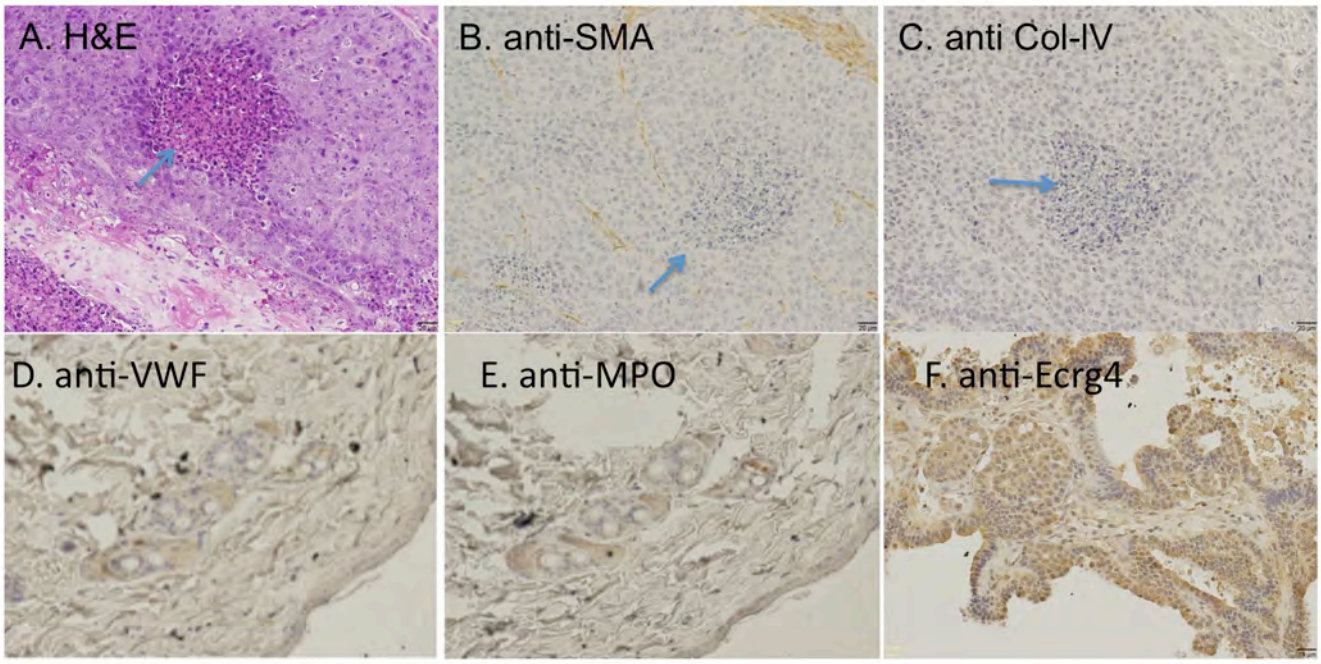


Figure 3 Markers of normal mammary ductal cells and mammary tumors in FGF+/+ and FGF-KO mice

5. Growth factor and tumor suppressor gene staining in FGF-KO mice. As planned to be described at the Era of Hope meeting in the summer, there were positively stained endothelial cells, myoepithelial cells, luminal breast epithelial cells. FGF2 in PyVT+ localizes to MECs whereas its receptor (FGFR1) localizes to BECs (Supporting Figure 3). We also compared IHC of FGF2 in a normal mouse mammary fat pad at 17 weeks of age, in a PyVT+ mouse, a PyVT+, FGF2 KO mouse mammary tumor at 19 weeks of age to FGFR1 in a PyVT+, FGF2 KO mouse mammary tumor, a PyVT+ mouse mammary tumor at 22 weeks of age, a PyVT+, FGF2 KO mouse mammary tumor at 14 weeks of age. In light of the findings described above regarding the candidate tumor suppressor gene Ecrg4 marker (Figure 3 Panel F), we began compared staining for Ecrg4 in normal mammary pad and mammary tumors. As shown in Figure 4 below, we found that there is a dramatic difference in the profile of tumor suppressor staining in normal mammary ductal epithelium and in mammary cancers. In Panel A/B the normal ductal cells show presence of the Ecrg4 protein (arrow in Panel B) but in each of the tumors of FGF++ and FGF—mice the tumors are devoid of this peptide (see arrow of Panels D and F). This unexpected discovery leads to the possibility that it can be applied to look at differences in Ecrg4 as a marker for tumor progression in mammary cancer in the FGF+/+ and FGF-/- mice.

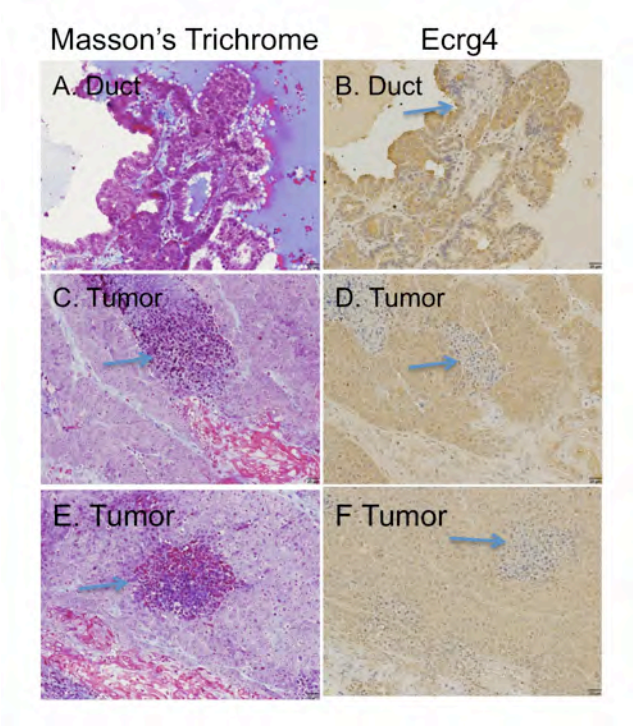


Figure 4. Ecrg4, a marker for tumor development

Task #1 Characterize the distribution of FGF2 and FGFR in mammary wild type mice. (Months 1-12):
In progress
We created baseline colony to map normal distribution of FGF and FGR in wt mice

Task #2 Characterize the distribution of FGFR in mammary of FGF2 null mice. (Months 6-12):
In progress
Create baseline FGF null colony & map changes in distribution of FGFRs

Task #3 Characterize the distribution of FGFR in mammary of FGF2 over-expressing mice (Months 6-12):
In progress and modified to create backcrossed colony.
Animal health precluded creation of baseline FGF2 over-expressing colony to map FGF2 & FGFRs distribution.
Strategy modified to remove animal health variable from tumor growth experiments: back crossing.

Task #4 Characterize the distribution of FGF2 and FGFR in mammary of PyMt mice (Months 6-12):
In progress
Created baseline PyMt over-expressing colony & map changes in FGF2 & FGFRs distribution.

Task #5 Characterize the distribution of FGFR in mammary of PyMt x FGF null mice (Months 12-24):
In progress
Created baseline PyMt$^{+/+}$ FGF$^{-/-}$ colony & map changes in FGF2 & FGFRs.

Task #6 Characterize the distribution of FGF2 and FGFR in mammary of PyMt x FGF over-expressing mice (Months 12-24):
In progress and modified to create backcrossed colony.
See task 3 above. Animal health precluded creation of baseline FGF2++ over-expressing colony to map FGF2 & FGFRs distribution.
Strategy modified to remove animal health variable from tumor growth experiments: back crossing.

Task #7 Determine kinetics, onset and progression of mammary tumors in wild type mice (Months 1-36):
In progress
Described baseline incidence of spontaneous mammary tumor development in wt/wt mice

Task #8 Determine kinetics, onset and progression of mammary tumors in FGF2 null mice (Months 18-36): In progress

Baseline described for incidence of spontaneous mammary tumor development in FGF2$^{-/-}$ mice

Task #9 Determine kinetics, onset and progression of mammary tumors FGF2 over-expressing mice (Months 18-36): In progress and modified to create backcrossed colony.

See task 3 and 6 above. Animal health precluded creation of baseline FGF2++ over-expressing colony.
Strategy modified to remove animal health variable from tumor growth experiments: back crossing.
Proceedurally valid experiments performed with F2 backcrossed and beyond generations.

Task #10 Confirm kinetics, onset and progression of mammary tumors in PyMt mice (Months 12-24): In progress

Described baseline incidence of spontaneous mammary tumor development in PyMT$^{+/+}$ mice

Task #11 Determine kinetics, onset and progression of mammary tumors in PyMt x FGF-null mice (Months 12-36): In progress

Described baseline incidence of spontaneous mammary tumor development in PyMT$^{+/+}$ mice

Task #12 Determine kinetics, onset and progression of mammary tumors after PyMt x FGF over-expression (Months 12-36) In progress and modified to create backcrossed colony.

See task 3, 6 and 9 above. Animal health precluded creation of baseline FGF2++ over-expressing colony.
Strategy modified to remove animal health variable from tumor growth experiments used back crossing.
Proceedurally valid experiments performed with F2 backcrossed and beyond generations.

3. Key Research Accomplishments (bulleted list of important research findings resulting from the achievement of project milestones)

• FGF2-/- mice were shown to have delayed time to tumor development compared to PyVT controls with endogenous levels of FGF2,
• FGF2-/- mice were shown to have decreased tumor size compared to PyVT controls with endogenous levels of FGF2,
• Effects of FGF2 are dependant on gene dosage.
• PyVT mammary tumors are sensitive to FGF2 signaling and identify FGF (and its receptor) as a target for antagonism therapy
• No dysinhibition due to FGF2 removal was observed confirming the conventional view that FGF2 is a pro-tumorigenic growth factor.

4. Reportable Outcomes (published or in-press manuscripts, abstracts, presentations, products, patents, grant funding awarded or applied for, and career developments that resulted from this award during the reporting year)
None

5. Conclusion
FGF2 acts as a growth factor in the mouse mammary gland and not a pro-differentiation factor. The new mice lines created are validated as experimental models of breast cancer.

6. References
None

7. Appendices
None

8. Supporting Data

Figure S1: Morphology of mammary cancers in genetically modified animals. Panels A and B show and normal mammary epithelial ducts and tumor bed respectively in FGF+/+ while panels C and D show the tumor beds in the FGF+/- het mice. The arrows serve to orient section as to location of tumor cells.

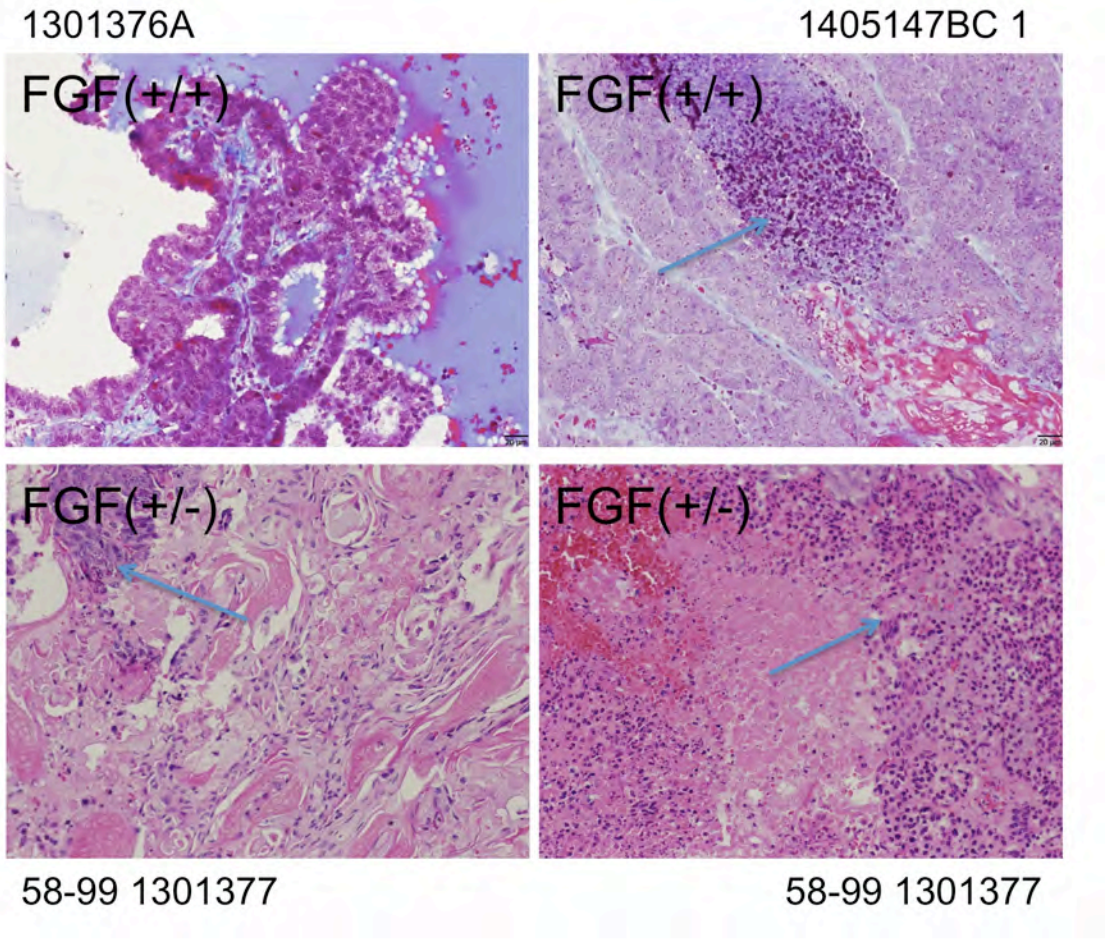


Figure S2: Markers staining of normal ductal epithelium in mouse mammary cancer. To validate markers we used anti smooth muscle actin (SMA) and anti collagen lV (Col-lV) expecting striking results. We were unable to reproduce the kinds of staining reported although light patterns of immunoreactivity were observed.

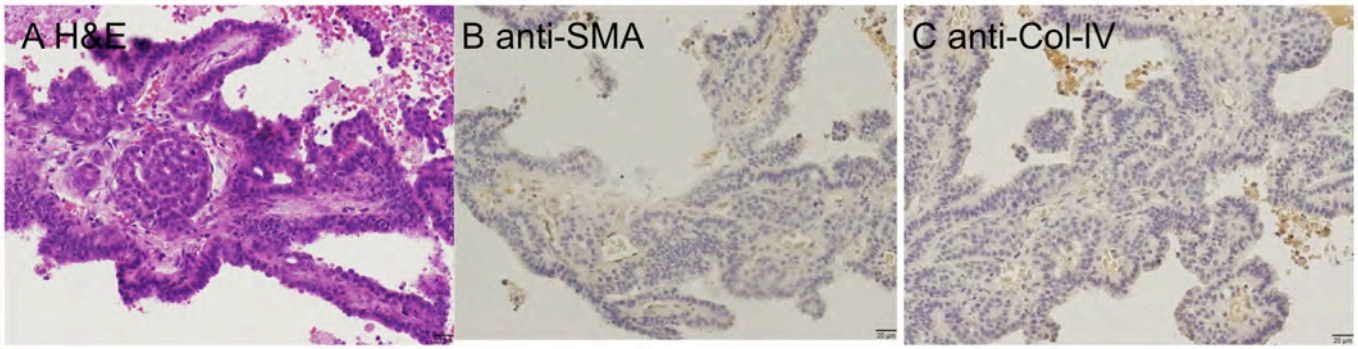


Figure S3: Distribution of FGF in BEC and MEC. The panels below show the complementary staining of FGF in mammary pads and the tumor bed in three lines of mice. The wild type controls, the hetereozygous (one copy of FGF) and the FGF knock out animal. This is the pattern being compared between groups with and without FGF2 and serves to show that the KO animals are devoid of FGF immunoreactivity. These are the samples in which we are proposing to examine markers and FGF receptors.