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Role of Fibroblast Growth Factor Binding Protein-1 in Mammary Development and Tumorigenesis

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14. ABSTRACT

Fibroblast growth factors (FGFs) are vital modulators of development as well as angiogenesis. The play a large role in vascular formation, body axis patterning, cell migration and organ branching. The scientific community is stillpiecing together the role that distinct FGFs play due to the complexity of the FGF network, which involves 22 distinct members that signal through 4 receptors to activate 3 major signaling pathways. FGFs act as major angiogenic factors and have therefore been of interest for therapeutic targeting. Success may rely on further elucidation of the regulation involved. The redundancy of FGF has made current FGF targeted therapies only moderatly effective. Overexpression of human BP in a conditional mouse model leads to decreased tertiary mammary ductal branching caused by increased epithelial apoptosis. This phenotype is seen only in mature mice that have fully developed mammary glands as opposed to pubertal mice developing altered mammary glands. FGFs have been strongly implicated in both dorsal/ventral axis patterning as well as mammary glad branching. This work supports the hypothesis that overexpressing human BP in a developed mammary glad results in altered mammary gland structure.

15. SUBJECT TERMS
Fibroblast Growth Factor Binding Protein-1, mammary gland development, reduced tertiary branching, apoptosis, HER2/neu mice

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INTRODUCTION

A secreted fibroblast growth factor-binding protein (FGFBP1) can enhance the activity of locally stored, immobilized fibroblast growth factors (FGFs) in the extracellular membrane. FGFs act as potent mitogens during embryogenesis, cell differentiation, and proliferation. Through its function as an angiogenic switch, we hypothesize that BP1 can support tumor growth through facilitation of new vessels to feed the growing tumor and through inducement of cell proliferation. To further study the role that this protein has on tumorigenesis, transgenic mice were generated containing human FGFBP1 (hFGFBP1) under a tetracycline inducible system. Preliminary data indicate a striking decrease in the lateral budding of mammary glands in animals expressing BP1. Matrigel plug assays, wound healing studies and ischemic models in the transgenic mice showed increased angiogenesis, fibroblast and keratinocyte proliferation and macrophage invasion. Drastic changes were seen in arterial blood pressure as soon as 24 hours after the FGFBP1 gene was induced indicating a role in vessel regulation and maintenance. Furthermore, other studies have shown FGFBP1 is upregulated in the progression from normal to *in situ* carcinoma of the breast as well as in further progression to invasive breast cancer in patients. Normally down regulated in adults, FGFBP1 is seen at high levels in cell lines derived from squamous cell carcinomas and some colon cancers as well as xenografted squamous cell carcinomas. Other studies have indicated that BP is the second most predominant protein purified from bovine mammary secretions on a heparin column during the last trimester of gestation. FGF-2 has been shown to be an important signal pathway in pregnancy dependent lobuloalveolar development in the mammary gland. This data suggests that FGFBP1 has an important role in the biology of the mammary gland.

As the only branched organ that undergoes the majority of its development during adolescence and adulthood rather that during the embryonic state, the mammary gland offers a fascinating opportunity to study organ formation. Mammary development, including branching, is largely directed by hormonal and growth factor signaling. While the hormonal interactions have been extensively investigated, effects due to growth factor signaling, specifically FGF, have yet to be completely elucidated. To that end, we have used a conditional transgenic mouse model that expresses hBP and evaluated the effects that modulation of FGF signaling has on murine mammary glands. To evaluate the effects of FGFBP expression on mammary tumorigenesis, we bred FGFBP1 transgenic mice with HER2/neu mice.

BRIEF SUMMARY OF NORMAL STAGING IN MURINE MAMMARY DEVELOPMENT

1. EMBRYONIC

Mouse mammary gland development begins after mid-gestation with the formation of milk lines, which consist of two bilateral epidermal ridges that run from the hindlimb up to the forelimb on both sides of the embryo (Veltmaat et al. 2003; Hens and Wysolmerski 2005). At the future site of each nipple, five disk-like placodes line up and invade into the underlying mesenchyme. Referred to as the anlage, this bud enters a knot of preadipocytes that are destined to become what is considered the mammary fat pad. The anlage then branches more than 10 times to form a rudimentary ductal tree (Hinck and Silberstein 2005). There are multiple signaling pathways implicated in this process. Those that are required for normal embryonic gland formation include the Wnt pathway (Andl et al. 2002) the FGF signaling pathway (Mailleux et al. 2002) and parathyroid related hormone (Wysolmerski et al. 1998). Here we have focused on the effects that FGF may have on the mammary gland and know regulation by sex hormones. Signaling pathways that are not required for embryonic gland development include the estrogen receptor (Couse and Korach 1999) and the progesterone receptor (Hovey et al. 2002) as shown by knockout models. Unlike the human breast, which forms several trees, the mouse mammary gland forms a single ductal tree leading to each nipple. The nascent mammary gland remains quiescent until puberty where the introduction of sex hormones (estradiol/progesterone) and the resulting growth factor signaling induces explosive growth and differentiation (Howard and Gusterson 2000).

2. ADOLESCENCE/PUBERTY

The ovarian secretion of estrogen and progesterone occurs in response to a rise in the level of gonadotrophins during this stage. The result in the mammary gland is a rapid invasion of ductal epithelium into the surrounding stromal fat pad. This phase generally begins when the mouse is 4-6 weeks and ends around 12 weeks of age (Howlin et al. 2006). Terminal end buds (TEBs) form at the periphery of the immature ducts and are the leading edge of the penetrating ducts. As the ducts elongate into the fat pad, the majority of cellular proliferation takes place at the tip of the TEB. Elongation continues until the entire fat pad has been overrun. New primary ducts are formed by bifurcation of the TEB and secondary branches begin sprouting off perpendicularly from the primary ducts. (Hennighausen and Robinson 1998; Hennighausen and Robinson 2001; Sternlicht et al. 2006)

Studies have shown that although the nascent mammary gland is refractory to estrogen/progesterone treatment, mammary gland growth and elongation of epithelial ducts is stimulated and regulated by estradiol and the estrogen receptor α (ERα) (Fendrick et al. 1998). By disrupting the ERα in a mouse model, it was shown that estrogen receptor α is required for ductal elongation during puberty (Korach et al. 1996; Bocchinfuso and Korach 1997) Further studies defined the requirement for ERα in the stromal compartment during ductal growth. Mueller et al showed that stromal ERα was necessary regardless of the epithelial expression by transplanting wild type epithelial cells into ERα knockout stroma. However, ERα deficient mammary epithelial cells were unable to develop epithelial ductal structures in an ERα positive stroma, indicating that ERα is also required in the epithelium. Interestingly, the decrease in mammary duct elongation due to ERα knockout could be rescued by the treatment of high doses of estradiol and progesterone(Cunha et al. 1997; Mueller et al. 2002).

3. ADULT MATURATION

As the mammary gland is subjected to repeated cycles of ovarian stimulation, the ductal tree is filled out completely. During this stage, elongated ducts form lateral branches or buds, which are distinct from TEB bifurcation. Lateral branches form at separate sites along the ducts and represent controlled sprouting of the epithelium into the surrounding fat pad. They are also referred to as tertiary branches or side branches with end buds or alveolar sprouts (Robinson et al. 1999; Brisken 2002; Hennighausen and Robinson 2005; Lu et al. 2006). This strategy of using a branched system to acquire a large epithelial surface in a limited tissue volume is seen in multiple organs and organisms and represents an evolutionarily conserved process.

Functional deletion of progesterone receptor (PR) led to defects in side branching in virgin mice (Humphreys et al. 1997). Further transplant studies showed prevention of normal lobuloalveolar development and the formation of tertiary branching in virgin mice where PR was absent in transplanted donor epithelium. Defects in development were not seen when PR was absent in recipient stroma indicating that PR is necessary in the epithelium but not the stroma (Brisken et al. 1998). Atwood et al. showed that administration of progesterone (by pellet) does indeed induce increased side branching normal mice. Furthermore, normal side branching corresponds with an increase in progesterone serum levels (Atwood et al. 2000). This data supports earlier studies indicating that progesterone stimulates branching (Haslam 1988b; Haslam 1988a). As noted earlier, other factors play a role in mammary gland branching and it should be noted that Humphreys et al. claimed that there was a possible role for a secondary but not yet identified growth factor signal in conjunction with progesterone signaling (Humphreys et al. 1997).

Other signaling during branching involves prolactin, which has been shown to act directly on mammary epithelium to induce alveolar development but acts through an indirect mechanism to influence ductal branching (Brisken et al. 1999; Ling et al. 2000). This is likely tied to prolactin's ability to stimulate synthesis and secretion of progesterone (Bole-Feysot et al. 1998). Although estrogen is not directly associated with side branching, it indirectly affects ductal development by elevating both prolactin and progesterone levels and

inducing progesterone receptors in mammary epithelium (Edery et al. 1985; Imagawa et al. 1985; Bocchinfuso et al. 2000)

4. PREGNANCY/LACTATION/ INVOLUTION

This stage involves massive tissue remodeling with rapid and global proliferation of epithelial cells within the ductal branches and alveoli (Richert et al., 2000). The single epithelial cell layer of the alveoli is surrounded by a discontinuous layer of myo-epithelial cells capable of contraction. Contact with the ECM is required for complete differentiation of the epithelial cells (Fata et al., 2004). As the mouse nears parturition, tight junctions in the alveoli close and milk and cololstrum proteins move into the alveolar lumen in preparation for milk secretion. Along with the epithelial expansion, adipocytes lose their lipid content and the vasculature remodeling occurs to provide the necessary energy, sugars, amino acids etc needed for milk production (Neville et al., 1998). This cycle requiring explosive epithelial expansion after which massive programmed cell death occurs indicates the existence of persistent self renewing mammary stem cells capable of expanding into separate lineages necessary for mammary epithelial maintenance and milk production (Smalley and Ashworth, 2003; Smalley and Clarke, 2005; Stingl et al., 2005).

BODY

The MMTV-Her2/neu (Human epidermal growth factor receptor 2) mice are a widely used model that involves a transgene that introduces the rat Erb2 (neu) receptor that contains a mutation such that dimerization occurs without binding of ligand similar to what is seen in many human breast cancers. To address Task 2, we first determined that mouse FGFBP1 is expressed in the normal adult strain of C57/BL6 mouse mammary glands but not in hyperplastic MMTV-Her2/neu mammary tissue (Fig.1). There is some expression of FGFBP1 in Her2/neu mammary tumors but it is lower than normal tissue.

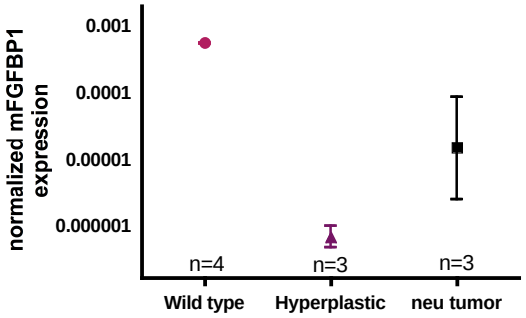


Figure 1. Real-time polymase chain reaction (RT-PCR) shows decreased expression of mFGFBP1 in HER2/Neu tumors and hyperplastic tissue as compared to wild type littermate levels of mFGFBP1 in normal mammary gland tissue. Samples were collected and frozen at necropsy. Samples were homogenized in Trizol and RNA was extracted using Qiagen RNeasy kit. Sample number is indicated in each column.

Decreased levels of FGFBP in hyperplastic tissue indicated that FGFBP1 might play a role in tumor progression. Our earlier data indicated that FGFBP1 plays a role in apoptosis in mammary gland biology therefore; low expression of FGFBP1 in the HER2/neu model suggested over expression of FGFBP1 may impact normal tumor development. To this end, tet inducible FGFBP mice (tTA/tetBP) mice were bred with HER2/Neu mice per Task 2 part 1. However, tumor incidence as determined by weekly palpation, was dramatically below reported levels (Fig 2) and may be due to strain differences. To determine if this was the case, RT-PCR was used to determine expression levels of the neu transgene as part of Task 2 part 3 (Fig 3). The thoracic mammary glands were harvested, homogenized in Trizol and the RNA was extracted using RNA easy kit (Qiagen). Levels of target RNA were normalized to levels of actin RNA as shown below. Mice were evaluated for neu expression levels at 9 months of age.

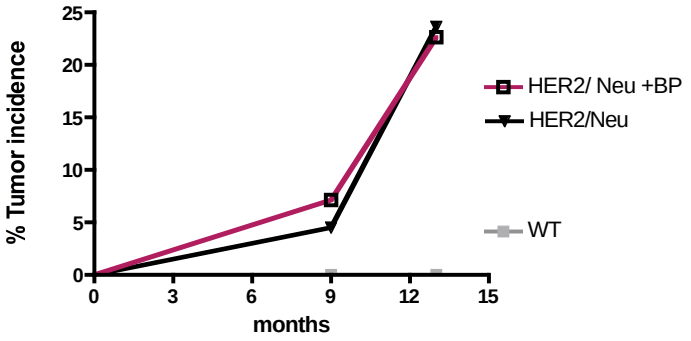


Figure 2.
tTA/tetBP mice were bred with HER2/Neu mice and were monitored weekly for palpable tumors for 15 months. No difference in tumor incidence was seen however, for both HER2/Neu mice and HER2/Neu/BP mice, the percentage of tumors was much lower than has been previously reported.

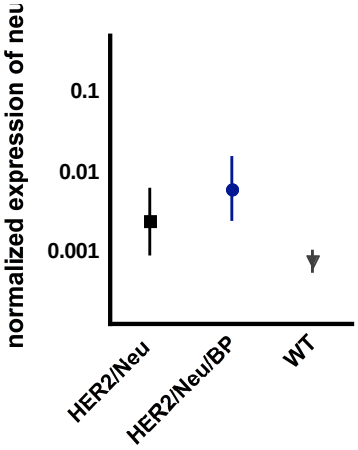


Figure 3. Expression of Her2/neu transgene in both HER2/neu and HER2/neu/BP1 mice is low. HER2/neu mice bred with tTA/tetBP mice to generate HER2/neu/BP1 mice. These mice were induced to produce hFGFBP1 for 30 days and mammary glands were harvested, processed and evaluated using RT-PCR for the neu transgene normalized to actin as described previously.

Because neu transgene expression was low and tumor incidence correlated with expression levels, an alternate model was utilized. I trained in Bernd Groner's lab for 3 weeks learning to transplant mammary glands, grow primary mammary epithelial cultures and to make lentivirus capable of transducing primary mammary epithelial cells. This system has the added benefit of determining local versus systemic effects that a transgenic animal under a CMV promoter may not be able to determine. Figures 5-6 show cloning of hFGFBP1 by polymerase chain reaction (PCR) from LS174T human colon cancer cells and subsequent insertion into the pCDH-CMV-MCS-EF1-copGFP vector from System Biosciences. Transduced human embryonic kidney cells HEK293Ts were transduced with virus and shown to produce both copepod green fluorescent protein (copGFP) and hFGFBP1 at the RNA and protein level.

Wild type murine mammary glands were analyzed for expression of mFGFBP1 during developmental stages (Fig 4). Mice were placed in breeding couples and monitored every morning for vaginal plugs. After observation of the plug, females were separated and sacrificed at day 7 and day 15. After birth, pups were weaned at 21 days and separated from mother. 3 and 5 days later, mammary glands were harvested for

evaluation. Expression of mFGFBP1 was high in virgin adult mammary glands but levels dropped during pregnancy and went back up during involution. Our earlier data showing that in the tTA/tetBP mice, FGFBP plays a role in apoptosis would be supported by this expression pattern. As the mammary gland goes through dramatic proliferation stages preparing for lactation, FGFBP1 expression is lost and as the mammary gland goes through apoptotic remodeling, expression of FGFBP1 goes back up to earlier levels. These data are also important to address Task 1 part 3 as to whether FGFBP1 has a local or systemic effect. Background levels of mFGFBP1 are low in virgin mice but even lower during involution.

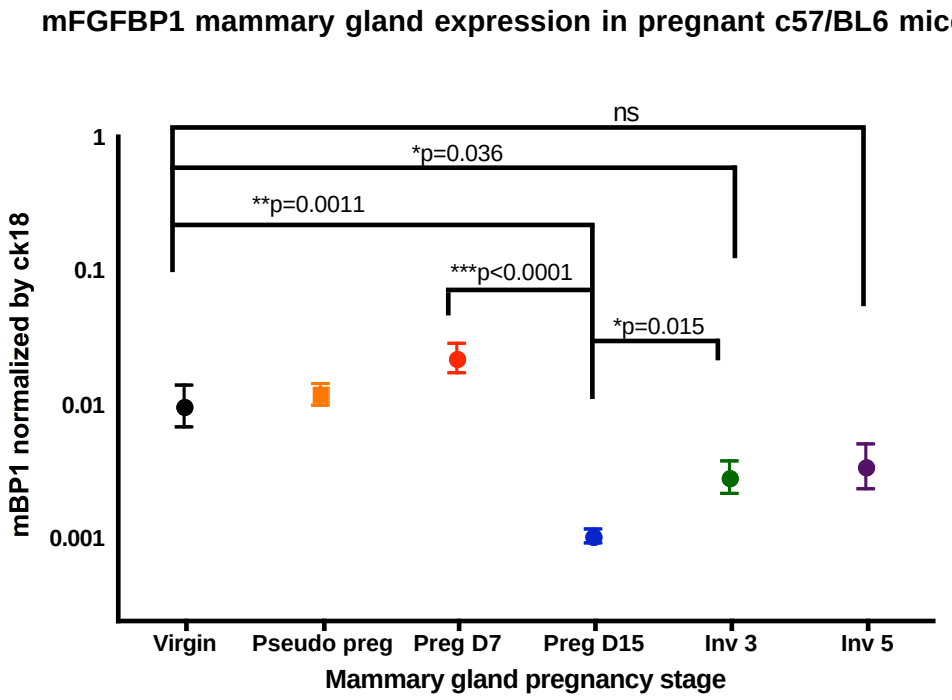


Figure 4. Virgin mammary glands show expression of mFGFBP1 but expression decreases during later stages of pregnancy (Day 7, Day 15)and increases during involution stages (3 days post weaning, Inv 3 and 5 days post weaning, Inv 5). Mammary glands were harvested and RNA was extracted for RT-PCR analysis and normalized using cytokeratin-18 expression levels

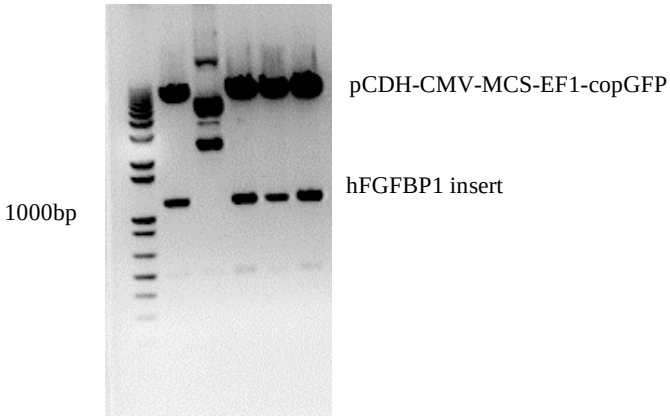
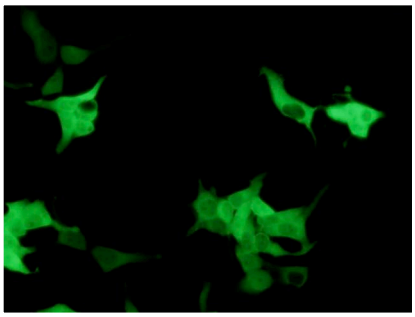


Figure 5. Cloning of hFGFBP1 into pCDH-CMV-MCS-EF1-copGFP vector from System Biosciences with GFP marker. cDNA from LS174T cells was used to PCR the hFGFBP1 insert, which was confirmed by sequencing of the plasmid.

A.



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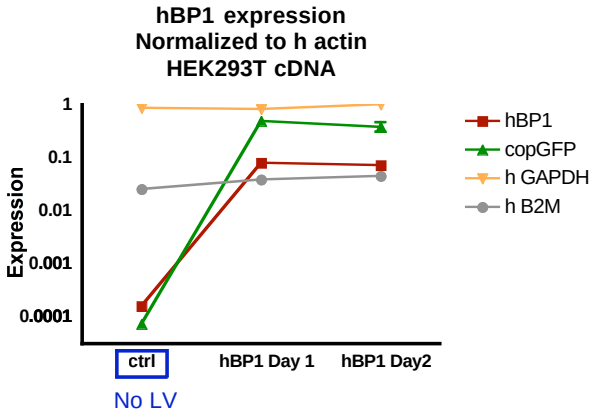

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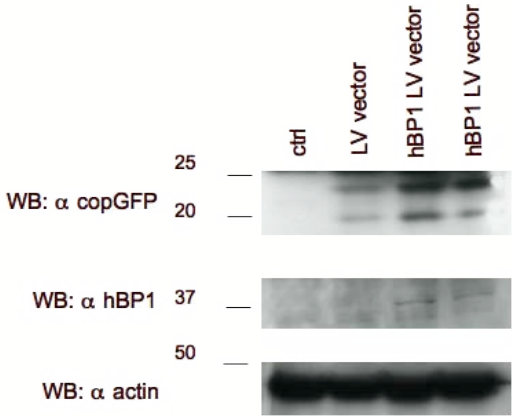

Figure 6. Transduced HEK293T human embryonic kidney cells express hFGFBP1. A. GFP localization of transduced cells as seen by an inverted stereomicroscope with 400x magnification. B. RT-PCR of cells transduced with hFGFBP1. C. Western blot showing both GFP and hBP1 proteins are present in transduced cells.

HEK293T cells were used as a means of virus production and analysis of viral titer. Figure 6 shows successful viral infection, DNA integration into the genome and hFGFBP1 protein production. Cells were transduced with virus MOI = 10 in a volume of 3 mL in a 10cm dish. After 8 hours, media was replaced and cells were grown for 48 hours, observed by fluorescence microscopy and harvested for RT-PCR as described previously and for Western Blot. The copGFP antibody (Evrogen) was used as recommended and the hFGFBP1 monoclonal antibody was used at 1:300. This data indicates that hFGFBP1 was successfully cloned into a lentiviral expression vector capable of producing protein in target cells.

KEY RESEARCH ACCOMPLISHMENTS

Figure 1. FGFBP1 is expressed in wild type mammary glands but is not significantly expressed in HER2/neu tumors

Figure 2 and 3. HER2/neu mice crossed with tTA/tetBP transgenic mice show a decreased tumor incidence due to strain decreased neu expression and is not an appropriate model with out further backcrossing.

Figure 4. mFGFBP1 is highly expressed in adult virgin mammary glands but during times of significant proliferation (prior to lactation) FGFBP1 is down regulated but is again expressed during apoptotic stages (involution).

Figure 5. hFGFBP1 was successfully cloned into a lentiviral expression system to allow targeted manipulation of primary mammary epithelial and tumor cells.

Figure 6. hFGFBP1 is expressed in HEK293T cells transduced with lentivirus.

REPORTABLE OUTCOMES

1. This work was presented to the Georgetown Medical Graduate School on October 23, 2007.
2. This work was also presented in poster format at the Era of Hope 2008 conference.
3. This work was presented at CABTRAC 2008 meeting in Park City, UT.

CONCLUSION

IMPLICATIONS OF FGFBP1 INDUCED APOPTOSIS IN MAMMARY GLAND BRANCHING

Epithelial branching has long been associated with FGF signaling. FGFs are the most documented mesenchymal factors and while prevalent, overall pathway complexity has left gaps in our understanding of their exact role and implications. Nevertheless, FGF is known to be necessary for the induction of kidney, lung and salivary gland branching. This is shown by loss of FGF10 or FGFR2 expression in the mouse embryonic lung epithelium, which prevents primary budding and causes organ failure (Peters et al. 1994; Min et al. 1998). Implantation of a bead soaked in FGF10 attracts ectopic branches indicating a role for FGFs in the direction of branching events (Bellusci et al. 1997).

The role of FGF is largely to induce migration and proliferation. However, FGF has been shown to have apoptotic effects in certain instances (Ramos et al. 2006). Specifically, FGF2 induces apoptosis when over expressed in breast cancer cell lines. Furthermore, low levels of FGF2 are associated with a more malignant phenotype in human breast cancer (Luqmani et al. 1992; Lai et al. 1995; Yiangou et al. 1997). Maloof et al. showed that expression of FGF2 decreases expression of the anti-apoptotic protein, Bcl-2, in breast cancer cells as opposed to the survival effects seen in fibroblasts, endothelial cells, smooth muscle cells, bladder cancer cells etc (Maloof et al. 1999). The apoptotic effect caused by BP is surprising due to its normal function as an activator of FGF leading to increased proliferation and survival. However, the implications that FGF2 plays a pro-apoptotic role in some settings in breast cancer cells may be reflected in our model. It should also be noted that during progression towards malignancy in mammary tumors, expression of stromal factors FGF2, FGF7 and FGF10 is lost and expression of FGF1, FGF3 and FGF4 is upregulated (Imagawa et al. 2002).

By utilizing a targeted lentiviral approach where the target gene is specifically expressed in the epithelial compartment, we can better dissect out the role of FGFBP1 in normal mammary gland biology and in a tumor setting. Our next step will be to transplant hFGFBP1 expressing primary mammary epithelial cells into cleared fat pads of wild type mice to determine the role that hFGFBP1 plays in the epithelial compartment and whether epithelial stromal interactions are necessary for increased apoptosis and decreased tertiary branching in adult mice. To determine the role of hFGFBP1 in tumor progression, we will utilize a PyMT model system and harvest primary mammary tumor epithelial cells, which will then be transduced with hFGFBP1 and transplanted into wild type mice. Tumor size will be monitored and harvested as described in this original proposal.

Apoptosis at regular intervals is a normal part of mammary physiology. In murine models, cyclic proliferative activity has been shown with the highest rate observed during late proestrous and estrous. The highest rate of apoptosis is seen during the diestrous phase involving entire alveolar structures (Andres et al. 1995). Although this may account for the increase in caspase-3 positive cells it was seen only in mice that expressed the hBP gene. Later studies should take this into consideration and perhaps monitor cycling to eliminate possible artifacts. Apoptosis during the diestrous phase has been noted largely in the alveolar buds where we saw apoptosis in ductal structures as well as alveolar structures indicating a different effect than that seen during cycling alone. The cyclic regulation of Bcl-2 which inhibits apoptosis, is down regulated during metestrous which occurs immediately prior to diestrous (Andres et al. 1995). Since FGF2 has been shown to alter normal expression of Bcl-2 in breast cell lines, it might explain the increased apoptosis seen in BP induced mammary glands.

Other explanations for the increased apoptosis may be due to up regulation of the inflammatory response by FGF signaling. Welm et al. developed a inducible FGFR1 mammary mouse model that results in increased lateral budding to the point of hyperplasia due to increased proliferation, activation of MAPK and Akt and recruitment of macrophages (Welm et al. 2002). When these mice were crossed with a mouse model that has reduced macrophages, the lateral budding was remarkably reduced. Although this model does not reflect normal FGF signaling due to the receptor design (contains intracellular domain only and uses a Src myristylation sequence to anchor to the membrane), the resulting osteopontin production and macrophage recruitment may be indicative of an FGF response (Schwertfeger et al. 2006). FGFBP1 has been shown to recruit increased numbers of macrophages during a wound healing study (data not shown) and it may have a similar effect in the mammary gland that would show a similar reduction in tertiary budding.

It should be noted that alterations in FGF signaling in the mammary gland could lead to inappropriate cellular behavior or pathology. In the murine mammary gland, FGF3, FGF4 and FGF8 have been identified as oncogenes after evaluating the effects of proviral insertion of mouse mammary tumor virus (MMTV) (Peters et al. 1983; Peters et al. 1989; MacArthur et al. 1995; Callahan and Smith 2000). Human breast cancer has also shown elevated levels of FGF8 (Marsh et al. 1999) and amplification of FGFR1, FGFR2 and FGFR4 has been identified in breast cancer (Koziczak et al. 2004). While FGFBP1 has not been shown to interact with all FGFs, it has been shown to bind FGF1, FGF2, FGF4, FGF7, FGF10, and FGF22 among others. Through this modulation, we had expected similar pathologies due to disrupted FGF signaling. The redundancy and further regulation of FGF signaling resulted in altered lateral budding and no malignant phenotypes. We conclude that although FGFBP may act to modulate FGF signaling in murine mammary glands, it is not sufficient to induce a malignant phenotype but it does alter normal branching resulting in a phenotype similar to mammary glands without hormonal stimulation. This alteration may have an impact on tumorigenesis and may lead to better understanding of the overall impact of FGF signaling on breast cancer.

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Appendices:

Poster Presentations:

- Gibby, K., Tassi, E., and A. Wellstein. 2008. *Fibroblast Growth Factor Binding Protein Induces Apoptosis and Leads to Decreased Mammary Gland Ductal Branching in Adult Mice* – Era of Hope-Baltimore, MD.
- Gibby, K., Tassi, E., and A. Wellstein. 2008. *Fibroblast Growth Factor Binding Protein Induces Apoptosis and Leads to Decreased Mammary Gland Ductal Branching in Adult Mice* – Georgetown Research Days.
- Gibby, K., and A. Wellstein. 2008. *Fibroblast Growth Factor Binding Protein Induces Apoptosis and Ruduces Tertiary Branching in Transgenic Mice -CABTRAC Conference, Park City, UT.*