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14. ABSTRACT Fibroblast growth factors (FGFs) are vital modulators of development as well as angiogenesis. They play a large role in vascular formation, body axis patterning, cell migration and organ branching. The scientific community is still piecing 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 moderately 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 gland branching. This work supports the hypothesis that overexpressing human BP in a developed mammary gland results in altered mammary gland structure.					
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INTRODUCTION

A secreted fibroblast growth factor-binding protein (BP1) can enhance the activity of locally stored, immobilized 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 BP1 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 BP1 gene was induced indicating a role in vessel regulation and maintenance. Furthermore, other studies have shown BP1 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, BP1 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 BP1 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 than 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.

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 (ER α) (Fendrick et al. 1998). By disrupting the ER α 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 ER α in the stromal compartment during ductal growth. Mueller et al showed that stromal ER α was necessary regardless of the epithelial expression by transplanting wild type epithelial cells into ER α knockout stroma. However, ER α deficient mammary epithelial cells were unable to develop epithelial ductal structures in an ER α positive stroma, indicating that ER α is also required in the epithelium. Interestingly, the decrease in mammary duct elongation due to ER α 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

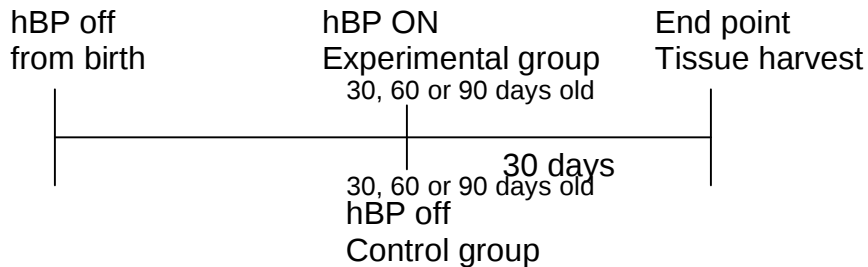
As the mammary gland is subjected to repeated cycles of ovarian stimulation, the ductal tree is filled out completely. During this stage, elongated ducts form lateral branches or buds, which are distinct from TEB bifurcation. Lateral branches form at separate sites along the ducts and represent controlled 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; Briskin 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 (Briskin 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 (Briskin 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)

BODY

To evaluate the effect of hBP on distinct stages of mammary development the following time points were evaluated as depicted below. Puberty begins from 4-6 weeks of age and continues until 12 weeks (Howlin et al. 2006). Mice were induced at 30 days to 60 days, which encompasses the first part of puberty and ductal tree formation. The second group was induced at 60 days of age and harvested at 90. The ductal tree goes through rapid proliferation and extension especially in the first 9 days (from day 30-39 of age) (Atwood et al. 2000). This second time point was chosen well past the initial proliferation spurt to evaluate later pubertal development during which lateral buds are forming in response to estrous cycling. The third time point of 90 days (13 weeks) to 120 days evaluates the mature and fully developed mammary gland.



A. MICE THAT HAVE BEEN INDUCED BY A REGULAR DIET SHOWED SIGNIFICANT LEVELS OF hBP EXPRESSION.

1. QRT-PCR SHOWS EXPRESSION OF hBP TRANSGENE.

These conditional transgenic mice were developed using a CMV promoter for ubiquitous expression of the transgene hBP. To determine if the mammary gland expressed hBP, thoracic mammary glands #3 were harvested and immediately frozen in liquid nitrogen. qRT-PCR was performed using actin to normalize. There is a significant amount of hBP1 poly-A RNA detected in mammary glands from animals induced to express hBP1 (Figure 1.)

2. IHC SHOWS THAT hBP IS PRODUCED IN TRANSGENIC MICE.

Inguinal #4 mammary glands were harvested from transgenic mice on the doxy diet, transgenic mice expressing the protein on regular diet and transgene negative littermates on both diets. Samples were fixed in formalin and embedded in paraffin. Using a human BP specific monoclonal antibody, immunohistochemistry (IHC) was performed (Figure 2). Staining was evident in the ductal stroma in transgenic mice expressing hBP. There was slight staining in transgenic mice on the doxy diet that is expected under the slightly leaky tet expression system. Negative controls showed no staining regardless of diet.

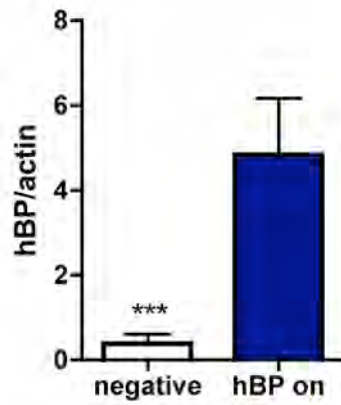


Figure 1. Transgenic mice express significant levels of hBP as shown by qRT-PCR. The #3 mammary gland from transgenic mice 60 days old induced to express hBP1 for 30 days were compared to negative controls. This experiment was done with n=5 for both groups and samples being analyzed in duplicate. Expression levels were normalized for actin expression. *** Indicates p value < 0.01.

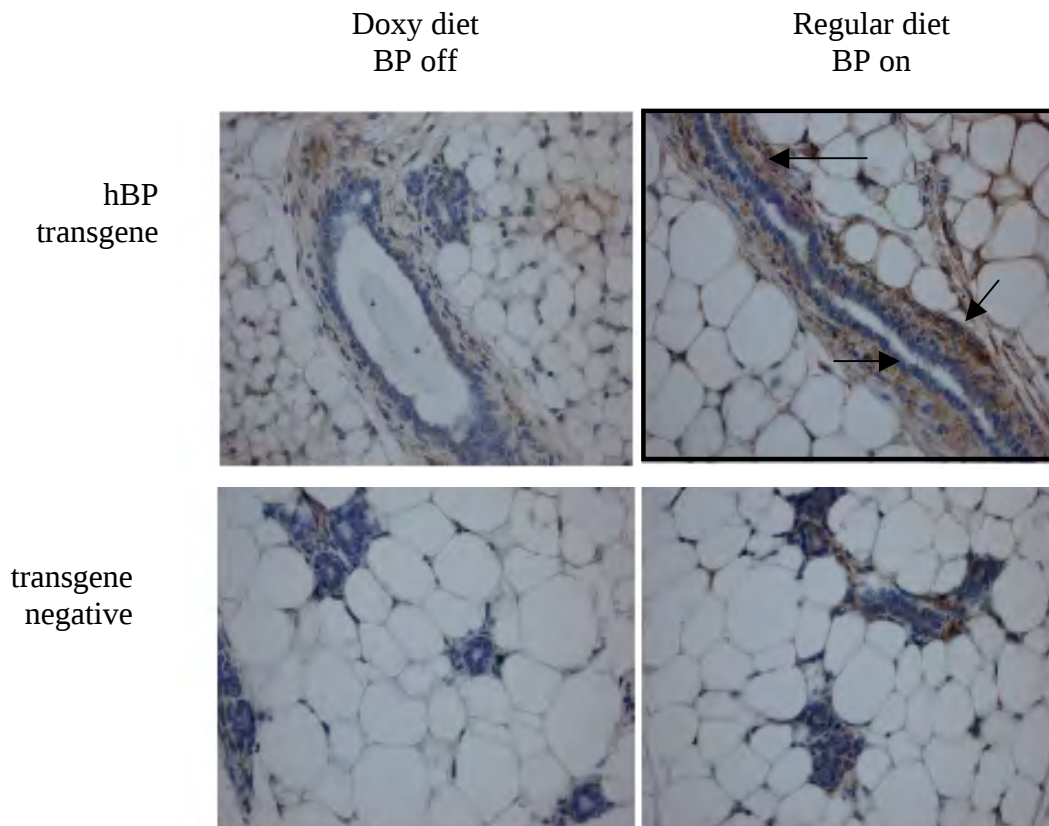


Figure 2. Expression of hBP protein in transgenic mice. Virgin mice were induced at 90 days of age and sacrificed at 120 days. The 4th inguinal gland was removed and formalin fixed for paraffin embedding. Positive staining is outlined in black with arrows.

B. ADULT TRANSGENIC MICE EXPRESSING hBP1 SHOWED A DECREASE IN TERTIARY BRANCHING AND LATERAL BUDDING IN THE DEVELOPED MAMMARY GLAND. EARLIER DEVELOPMENTAL STAGES SHOWED NO SIGNIFICANT DIFFERENCE IN BRANCHING AFTER CHRONIC INDUCTION.

1. DECREASED BUDDING PHENOTYPE OF MAMMARY GLANDS IN ADULT MICE

Mammary glands from mice that were chronically induced to express hBP1 from 90 to 120 days of age were analyzed by whole mounting the 4th inguinal mammary gland. While the controls showed no difference in branching, a significant difference was seen in adult animals expressing the hBP transgene (Figure 3). There was a dramatic decrease in lateral budding and tertiary branching. Interestingly, the mouse mammary gland is fully developed at 90 days of age. Earlier time points with the same period of induction resulted in a few samples (not statistically significant) demonstrating the reduced branching but the majority were unaffected by transgene expression (see next section 2.)

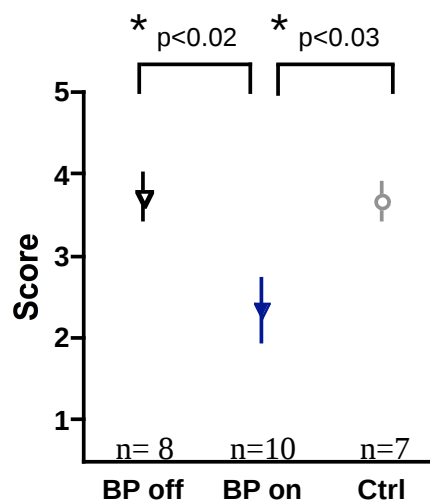
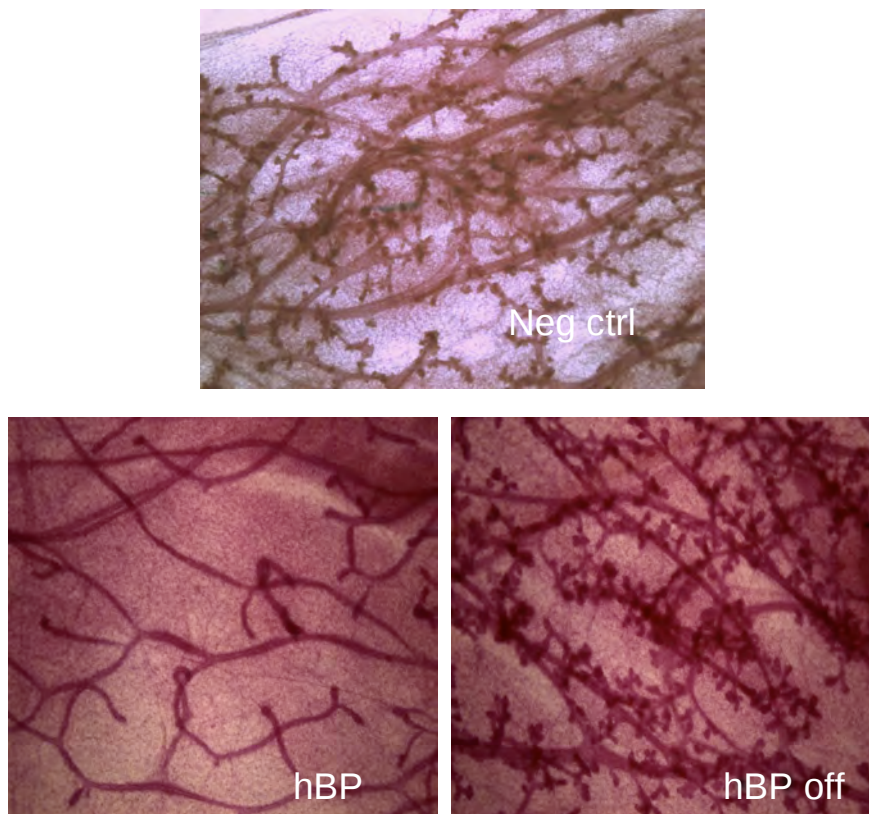
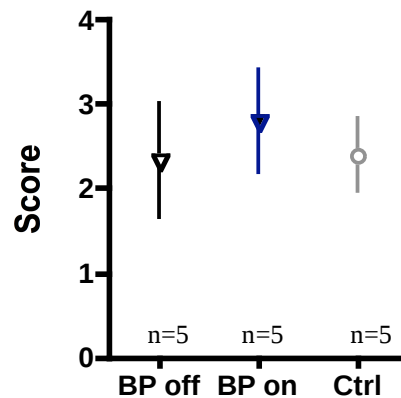


Figure 3. Chronic induction of BP results in decreased mammary branching and lateral budding. Transgenic mice were induced at 90 days of age and sacrificed at 120 days. The inguinal #4 mammary gland was whole mounted and stained with Carmine Alum.

2. EARLIER TIME POINTS RESULTED IN NO SIGNIFICANT DIFFERENCE IN BRANCHING.

To evaluate hBP effect on pubertal branching, mice were given regular diet at 30 days of age to induce hBP1 and were sacrificed at 60 days. The fourth inguinal mammary gland was harvest and stained with Carmine alum. Branching was analyzed as described previously. No significant difference was found between the BP-on group and the BP-off (Figure 4A). Scoring was similar to the corresponding non-transgenic littermates suggesting that mice not expressing hBP1 show a wild type phenotype. A second time point to evaluate branching during lateral bud formation as they cycle through multiple estrous cycles, mice were induced at 60 days of age and sacrificed at 90 days showed results similar to the earlier time frame of 30-60 days (Figure 30B). No difference was seen in terminal end buds from induced mice as compared to wild type controls.

A. Induction from 30 to 60 days of age



B. Induction from 60 to 90 days of age

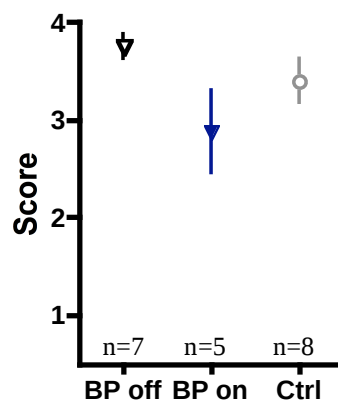


Figure 4. Chronic induction of BP does not impact branching in mice younger than 3 months. **A.** Transgenic mice were induced at 30 days and sacrificed at 60 days. The inguinal #4 mammary gland whole mount was scored and evaluated in a blind manner. There were several glands with diminished branching but the phenotype was seen in all groups including controls. **B.** Mice induced at 60 days and sacrificed at 90 days showed no difference in lateral budding or tertiary branching.

C. EFFECTS OF hBP RESULTED IN TRANSIENTLY INCREASED APOPTOSIS BUT NOT INCREASED PROLIFERATION.

1. CHRONIC INDUCTION OF hBP1 DOES NOT INCREASE PROLIFERATION

The reduction of mammary branching may be explained by reduced epithelial proliferation or increased apoptosis or both. Mice that were induced at 90 days and sacrificed at 120 days were analyzed by IHC specific for PCNA to evaluate the number of proliferating cells. Animals that were induced chronically in this manner and expressed hBP1 showed no difference in the percent of dividing cells as compared to un-induced control or transgene negative animals (Figure 5).

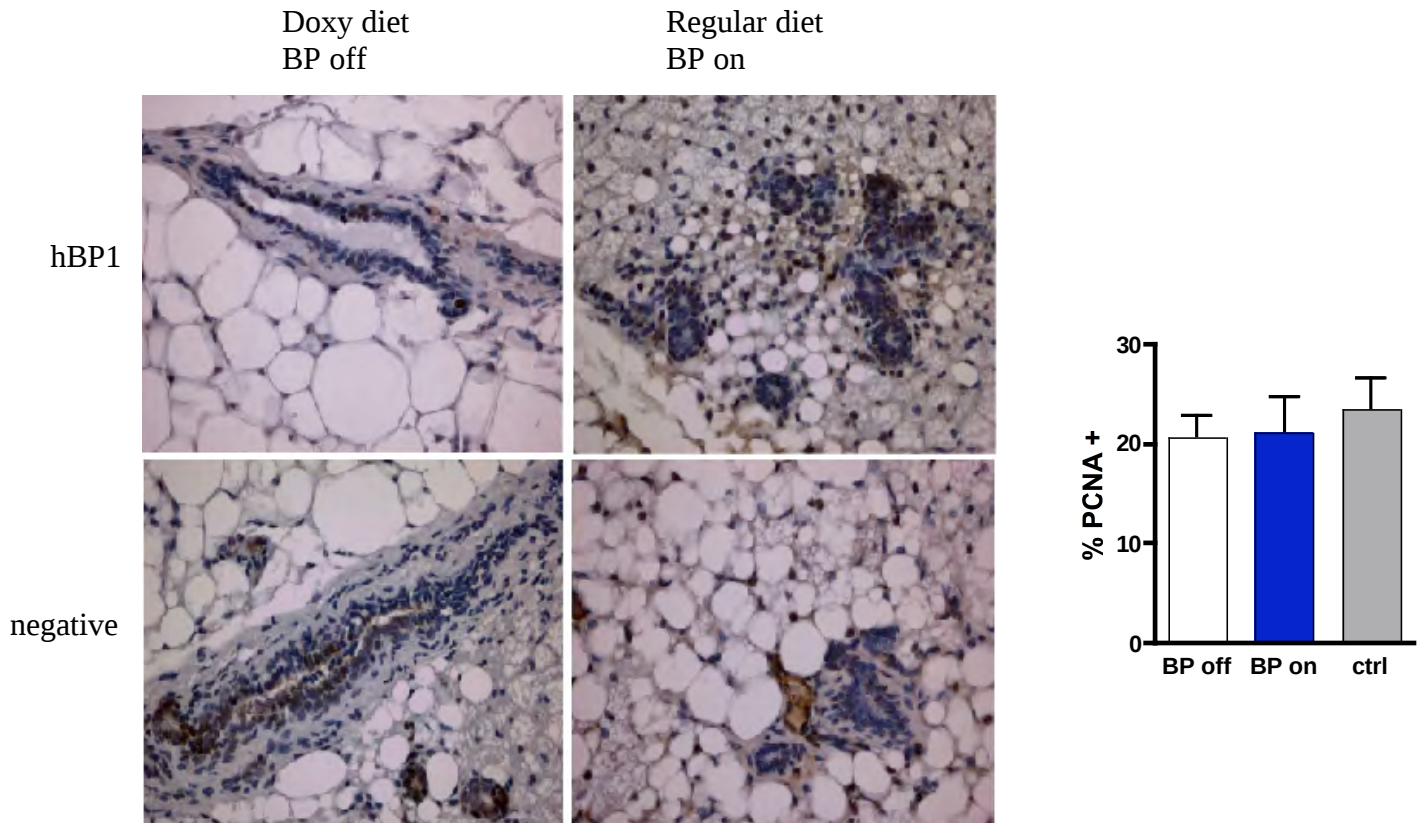


Figure 5. Proliferation of ductal epithelial cells is not increased by chronic induction of BP. Transgenic mice induced at 90 days and sacrificed at 120 had their left 4th inguinal mammary gland removed and fixed in formalin for paraffin embedding. These slides were analyzed by IHC using a PCNA monoclonal antibody to identify proliferating cells in the ductal epithelia. Sample numbers involved two experiments as follows: BP on (n=6), BP off (n=6), transgene negative (n=4)

2. APOPTOSIS INCREASED AFTER AN ACUTE INDUCTION OF 1 WEEK AND RETURNED TO BACKGROUND LEVELS BY 30 DAYS OF INDUCTION.

At 90 days the mammary gland will have invaded the fat pad completely and development is complete for virgin mice. Because proliferation levels do not change as shown in Figure 5, glands were evaluated for increased apoptosis to explain diminished mammary gland branches. Slides already evaluated for PCNA were analyzed for cleaved caspase-3, which acts as a marker of apoptosis. After 30 days of induction, the decrease in branching is seen and there is no increase in apoptosis detected (Figure 7).

A second experiment was conducted where the 4th inguinal mammary gland was removed as a biopsy before induction to use as an internal control. Mice were induced to express hBP at 90 days and tissue was harvested at 97 days. Half of this gland was whole mounted to evaluate branching as described previously and half was

fixed in formalin for further IHC analysis. After staining with cleaved caspase-3, an increase in apoptosis was seen after 1 week of hBP induction that dropped to background levels after 5 weeks (Figure 7B). No impact on the branching phenotype was apparent after one week of hBP1 induction (Figure 6).

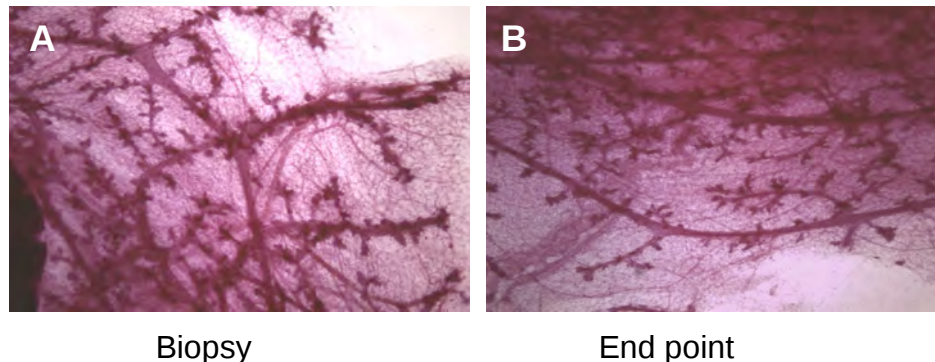
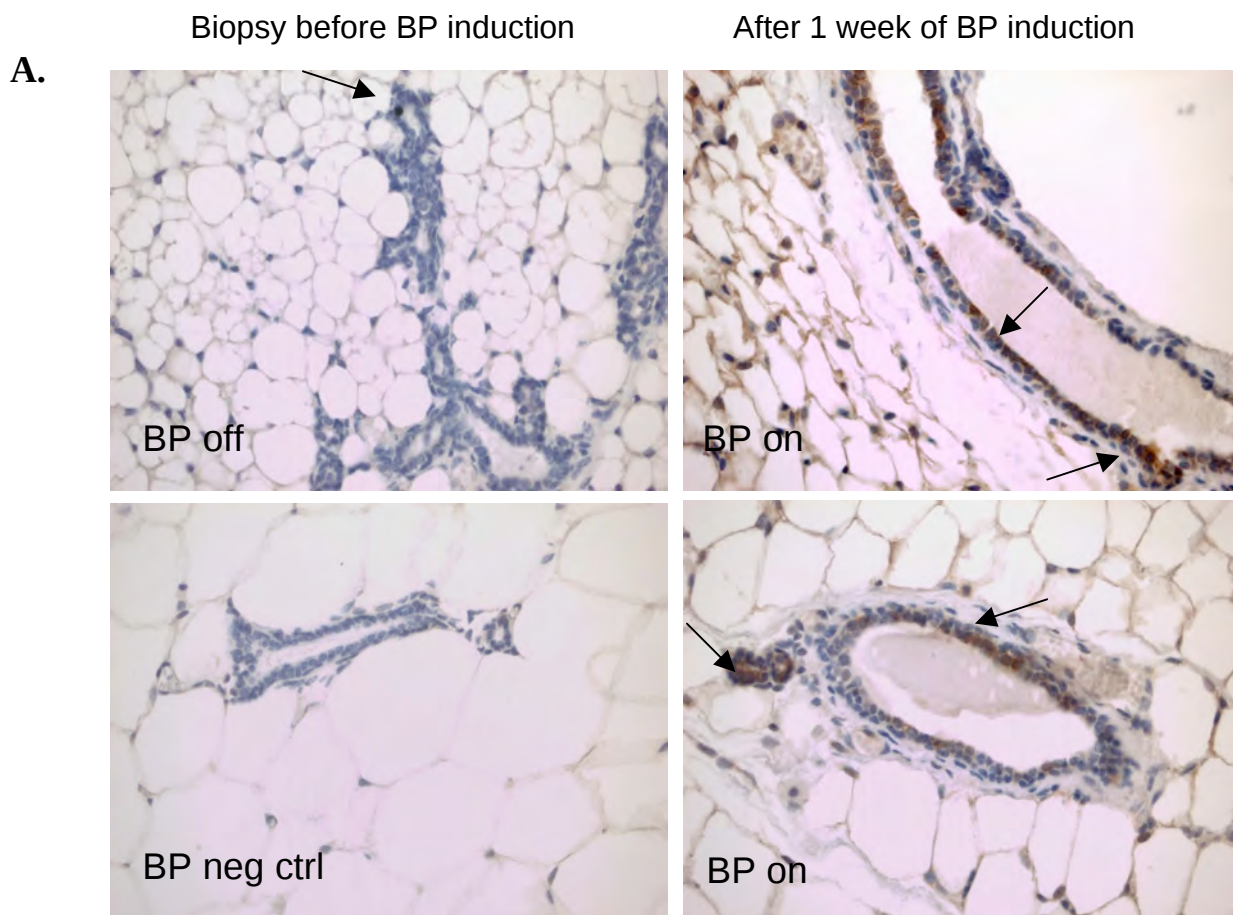


Figure 6. Acute induction of BP does not impact mammary branching. The 4th inguinal mammary gland was biopsied (A) and removed before hBP induction in transgenic mice. The biopsies indicated normal branching before transgene induction. Furthermore, after 1 week of induction, the decrease in branching was not seen (B).



B.

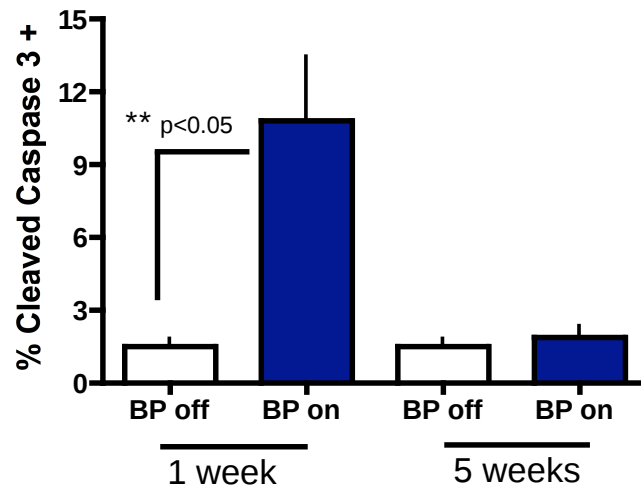


Figure 7. Apoptosis is increased after 1week acute induction of hBP and returns to background levels during a chronic induction of 5 weeks. Paraffin embedded samples were analyzed by IHC of cleaved caspase-3. BP off at 1 week includes the samples that were biopsied before induction. This experiment was performed with an n=4 for each group. **A.** Shows representative samples. Arrows show positive cells and **B.** Quantitation of cleaved caspase-3 positive cells.

D. THE WEIGHT OF THE MICE HAD NO IMPACT ON THE BRANCHING PHENOTYPE.

Initial experiments indicated that the first observed phenotype was decreased mammary gland size. Mice were evaluated for overall body weight as a control. In mice 4 months of age or less, body weight was not significantly different (Figure 8). These mice were evaluated for a branching phenotype and were part of the experiment where adult transgenic animals were induced at 90 days of age. Mammary glands were then evaluated to see whether the larger size impacted the branching phenotype and no significant difference was found (Figure 9).

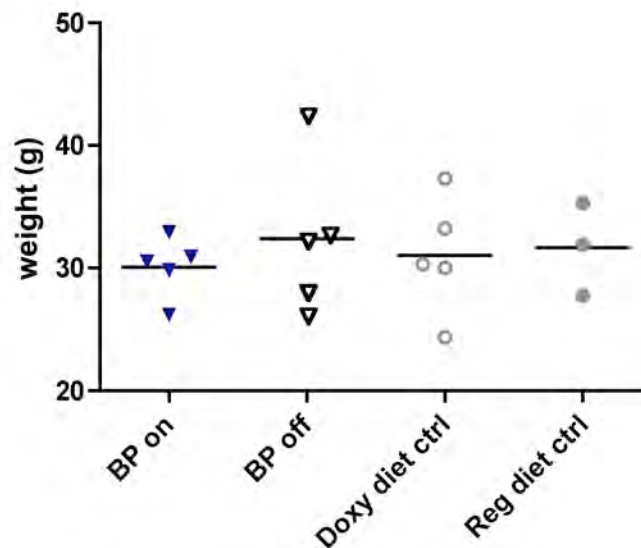


Figure 9. Doxycycline diet does not significantly impact weight in mice 4 months old. Mice were evaluated for overall body weight at the time mammary glands were harvested. No significant difference in body weight was determined.

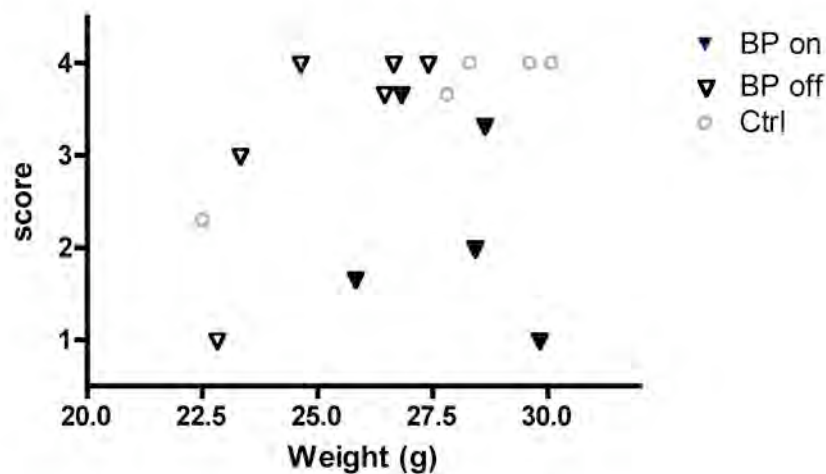


Figure 9. Body weight does not impact branching phenotype. By comparing the branching score with overall body weight there is no significant difference in branching that correlates with weight.

KEY RESEARCH ACCOMPLISHMENTS

- Figures 1 and 2. Expression of hBP1 transgene in mammary gland in tTA/tetBP transgenic mice
- Figure 3. Decrease in tertiary branching in adult non-pregnant female mice expressing hBP1
- Figure 4. No change in branching development during pubertal mammary developmental stages
- Figure 5. Mice with decreased tertiary branches show no difference in proliferation
- Figure 6 and 7. Apoptosis increases after 1 week of hBP1 expression
- Figure 8 and 9. Mouse weight does not significantly impact branching phenotype

REPORTABLE OUTCOMES

This work was presented to the Georgetown Medical Graduate School on October 23, 2007. Training for procedures outlined in this work was completed at the Georg Speyer Institute in Frankfurt, Germany in February 2007.

CONCLUSION

IMPLICATIONS OF BP 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 Bcl-2 expression 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).

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). BP 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 lateral budding similar to what is seen in Figure 29.

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 BP 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 BP 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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