Prolactin Stimulates Expression of BP1, a Homeotic Protein Frequently Expressed in Breast Cancer

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Abstract

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Prolactin (PRL) is a well characterized regulatory hormone secreted by the anterior pituitary and other organs, as well as by normal and malignant breast epithelial cells. PRL, referred to as "the master regulatory hormone in breast cancer", has been implicated in breast cancer cell proliferation, survival and metastasis. It is involved in multiple pathways including the Jak/STAT, the MAPK and the PI3K/Akt, one of the major pathways activated in breast cancer. BP1 is a transcription factor belonging to the family of homeobox genes. BP1 protein (pBP1) appears to play a crucial role in cell survival, aggressiveness and metastasis. It has been shown that pBP1 is expressed in 80% of invasive ductal breast tumors, and that 100% of estrogen receptor (ER) negative tumors. The aim of this study is to determine whether there is a relationship between PRL and BP1 expression due to their significant roles in cell survival and to determine any common pathways that both PRL and BP1 may be involved in. If BP1 activation by PRL is required, BP1 may be a potential target for cancer therapy.

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Introduction

1. Prolactin and the Prolactin Receptor

1.1 Prolactin

Prolactin is recognized as the most versatile hormone that is secreted from the anterior pituitary. It is best known for its biological activities related to reproduction and in mammary gland development (Nouhi et al, 2006). Prolactin, (PRL) is a 23kDa single polypeptide hormone involved in normal growth, development and differentiation of breast tissues (Liby et al., 2003). It is a member of growth and differentiation factors including growth hormone, placental lactogens, somatolactin and prolactin-related proteins (Owerbach, et al. 1986). PRL is synthesized and secreted mainly by lactotroph cells of anterior pituitary but also from extrapituitary sites including mammary epithelium, placenta, uterus, brain and the immune system (Duan et al., 2007).

In humans, the gene encoding PRL is located on chromosome 6 (Owerbach, 1981). The gene itself is composed of five exons and four introns and overall length of approximately 10 kilobases (Truong et al., 1984). Prolactin expression is directed by two promoter regions; a proximal promoter region and a distal promoter region. It has been

shown that mRNAs from both promoters were present in human breast cancer cell lines, which suggests a dual promoter usage (Baudhuin et al., 2002).

The human prolactin hormone consists of 199 amino acids with a total molecular mass of approximately 23kDa. PRL has been identified in all vertebrates (Bole-Feysot et al., 1998). In addition, studies show that biological activity and immunoreactivity of PRL are affected by posttranslational modifications of mature PRL such as phosphorylation and glycosylation. Phosphorylated PRL has less activity when compared with unphosphorylated PRL. Phosphorylated PRL is unable to bind its receptor because of conformational changes in the structure (Wicks & Brooks, 1995). Also, glycosylation of PRL is shown to down-regulate PRL activity. Glycosylated PRL has a lower biological activity in target tissues when compared to nonglycosylated form of PRL (Hoffman et al., 1993). In addition, cleaved PRL products, 14kDA and 16kDa, have anti-angiogenic activity and inhibit the vascular endothelial growth factor (VEGF)-induced growth of capillary endothelial cells (Ferrera et al., 1991).

PRL has many biological functions. In humans, the best known function of PRL is to stimulate growth and development of the mammary gland ultimately leading to milk production and secretion. (Goffin et al., 2002). Besides the functions in mammary gland, PRL has approximately three hundred separate functions which can be organized into different categories related to growth and metabolism, endocrinology, reproduction and immunoregulation (Bole-Feysot et al., 1998). Recent studies have shown a role of prolactin in breast cancer with an autocrine/paracrine pathway where PRL either can auto-stimulate the production of more prolactin by those cells or can stimulate the release of PRL by nearby mammary cells (Clevenger et al., 2003).

1.2 Prolactin Receptor

PRL acts through a classic endocrine pathway in which it is secreted by the pituitary gland and transported to its target tissues. When reaching the target tissues, PRL binds to its receptor, PRL receptor (PRLR) on the cell membrane to activate various intracellular pathways. PRLR belongs to the cytokine hematopoietic family of receptors (Brockman et al., 2002). It is separated into three domains an extracellular ligand binding domain, a hydrophobic transmembrane domain and an intracellular domain containing a proline-rich motif (Vonderhaar, 1999).

The gene that encodes PRLR is located on chromosome 5 (Arden et al., 1990). PRLR has mainly three isoforms which differ in their cytoplasmic domains. The two PRLR isoforms, short (40kDA) and long (90 kDa) forms are produced from alternative splicing of the primary transcript (Kelly et al., 1993). The intermediate form has also been shown, which is a deletion product of the long form of PRLR. It is found in Nb2 rat lymphoma cells and is more sensitive to PRL compared with other forms (Ali et al., 1992). All three forms promote mitosis (Das & Vonderhaar, 1995).

Both normal and malignant mammary cells contain both long and short forms of PRLR. The receptors also exist as multiple charged forms. Dimerization of the membrane associated receptor results from the interaction of PRL with its receptor (Goffin & Kelly 1997).

Cytokine receptor family induces receptor dimerization and activation of intracellular signaling pathways by ligand binding. Activation of PRLR also involves ligand induced receptor dimerization. Initially, PRL binds to its receptor and they form an inactive H_1R_1

(one hormone, one receptor) complex. This is followed by binding of an additional receptor molecule forming a trimeric, H_1R_2 complex (Bole-Feysot et al., 1998). PRLR dimerization induces activation of various intracellular signaling pathways. PRLR and other members of cytokine receptor family lack intrinsic kinase activity. Therefore, dimerization also leads to the activation of some other associated kinases. PRL stimulation of PRLR leads to tyrosine phosphorylation of numerous signaling proteins including PRLR itself which in turn activates other kinases that are downstream of PRL such as JAK/STAT kinase, MAPK and Src family of intracellular kinases (Lebrun et al., 1995). Recent studies of human mammary tumors have shown that neoplastic tissue expresses higher levels of PRLR than normal adjacent tissues. (Schroeder et al., 2002)

2. Prolactin and Breast Cancer

Cancer is known as a multifactorial disease resulting from abnormalities in cell growth and cell differentiation (Nouhi et al., 2006). Breast cancer is one of the very complex and multistep diseases in which various different oncogenic factors contribute to initiate tumor formation, transformation and growth (Vonderhaar, 1999). Loss of function mutations results in unregulated cell growth and transformations. In addition to genetic abnormalities, there are several other factors that may increase the risk of breast cancer such as environmental changes, age, geographical location, age at pregnancy, nulliparity, hormones and growth factors. Estrogens, progesterones, androgens and insulin are some of the hormones that have been shown to stimulate tumorogenesis in breast cancer

(Harris et al., 2004). Many of these hormones facilitate tumor growth in autocrine/paracrine pathway. PRL has a role in mammary gland development, differentiation and lactation. It has been shown that PRL may be involved in pathogenesis and progression of breast cancer malignancy (Tworoger & Hankinson, 2008). Furthermore it is shown that PRL acts on mammary epithelial cells directly to increase cell proliferation and accelerate invasion to carcinoma (Harvey et al., 2008).

2.1 Synthesis and Secretion of PRL by Human Breast Cancers

PRL has been shown to be synthesized and secreted by normal and malignant breast epithelial cells (Vonderhaar, 1999). It has been established that human breast tissue and breast cells synthesize and secrete PRL into the medium in significant amounts (Clevenger et al., 2003). A study by Vonderhaar (1999) has revealed that 98% of breast cancers express PRL mRNA and there is a significant increase in PRL mRNA and PRLR mRNA amounts in malignant tissues when compared with breast cancer patients (Vonderhaar, 1999).

PRL secretion was examined in T47Dco breast cancer cell lines. After specific radio-immuno assays for human pituitary PRL, it has been shown that 0.3ng/ml of hPRL protein is produced over a 24 hour period of time; furthermore when the conditioned media from these cells are treated with an anti-pituitary PRL, the PRL activity is abolished (Ginsburg & Vonderhaar, 1995). Also in additional breast cancer cell lines MCF-7 and MDA-MB-231, PRL secretion is observed. It is shown that MCF-7 cell lines

secrete around 0.3ng/ml PRL and MDA-MB-231 cells show approximately 0.1ng/ml PRL secretion over a 24 hour period (Clevenger et al., 2003).

PRL mRNA produced in the mammary glands differs from the PRL mRNA produced in pituitary but the mature hPRL hormone that is secreted from all tissues is identical (Sinha, 1995). The exact mechanism of PRL secretion or the factors affecting the regulation of hPRL synthesis is not known. Several studies indicate that production of PRL by breast cancer tumors and breast cancer cells is greater than of normal breast epithelium tissue.

The effects of PRL are seen through PRL binding to its receptor and dimerization, thus alterations in the PRLR expression can be important to study the biology of breast diseases. Studies have shown that malignant breast tissues have a greater PRLR expression when compared to normal breast tissues (Clevenger et al., 2003). Of the breast cancer cell lines, T47D cells known to have the highest expression of PRLR (Vonderhaar, 1999). It is also shown that PRLR is expressed heterogeneously at different sites in normal, malignant and benign breast epithelial cells. In normal cells it is mainly along the luminal border, in malignant cells, mainly cytoplasmic region whereas in benign tumors the location is either luminal, cytoplasmic or combination of two. It is also shown that PRLR is expressed in more than two thirds of female breast carcinomas which may suggest that PRLR may play a role in pathogenesis of breast carcinoma (Gill et al., 2001). The presence of mRNA for PRL and PRLR for several breast cancer cell lines are shown in Table 1.

Table 6. PRL mRNA and PRLR expression in Breast Cancer Cell Lines (Vonderhaar, 1998)

Cell Line	PRL (mRNA) & PRLR Status
T47D	+
MCF-7	+
T47Dco	+
MCF10A	+
MDA-MB-453	+
HS578T	+
MDA-MB-231	-

2.2 Autocrine/Paracrine Role of PRL in Breast Cancer

The evidence of production and secretion of PRL from mammary tumors and breast cancer cells and elevated levels of PRLR expression leads us to hypothesize that PRL is acting through breast tumors via autocrine/paracrine pathway. Further studies have shown that release of prolactin by breast cancer cells auto-stimulates production of more prolactin as well as stimulating the release of PRL by nearby mammary cells (Clevenger et al., 2003).

There are various studies to support this hypothesis. One study shows that when T47Dco and MCF-7 cells, which are known to secrete PRL and express PRLR, are treated with monoclonal human pituitary PRL antibody, the growth was inhibited 20-90% (Ginsburg & Vonderhaar, 1995). Furthermore, some studies show that a hPRL antagonist, hPRL-G129R, induces apoptosis in T47D and MCF-7 breast cancer cell lines (Chen et al., 1999). Also in vivo studies with inoculation of nude mice with the hPRL antagonist, hPRL-G129R has been carried out and after a 7 week period, it has been seen that hPRL-G129R injected T47D breast cancer cells inhibited tumor growth (Chen et al., 2002). Also inhibiting PRL stimulates proliferation of T47Dco breast cancer cell line (Llovera et al., 2000). Studies above show that PRL produced by human breast cancer cells act via autocrine/paracrine pathway to stimulate cell survival and proliferation.

2.3 Serum PRL levels in Breast Cancer

PRL has been shown to be synthesized and secreted from breast cancer tissues and is circulating in serum of the patients. Yet, it is still not very clear if circulating levels of PRL in serum has an effect on tumorigenesis or if it plays a role in development of breast cancer. Studies examining a correlation between serum PRL levels and breast cancer risk have found contradictory results (Liby et al., 2003). In patients with prolactinoma or breast cancer, no exact correlation has been seen between serum PRL levels and breast cancer and breast tumors (Wennbo & Tornell, 2000). Yet, hyperprolactinemia was seen in 44% of metastatic breast cancer patients (Holtkamp, 1984). Furthermore, treatment of patients with the dopamine antagonist, bromociptine to block transcription of pituitary

PRL had no consistent effect on tumor progression (Schroeder et al., 2002). However, endogenous and exogenous PRL are known to have different effects. Despite the endogenous production of PRL, exogenous PRL has been shown to stimulate proliferation of mammary cells in several studies (Schroeder et al., 2002).

Serum PRL levels in women have been shown to elevate according to the environmental, physical or psychological stress and to the menstrual cycle (Clevenger et al., 2003). Also serum PRL levels decrease after each pregnancy. A study shows that in post-menopausal women, as breast cancer risk increases serum PRL levels also increase. Serum PRL levels which are higher than 20ng/ml are known to be associated with higher risk of developing metastatic breast cancer due to their correlation with tumor size, stage and lymph node status of the carcinoma. Also three quarters of the tumors in this study are shown to express PRL which confirms the idea that PRL may play a role through autocrine/paracrine pathway (Harris et al., 2004).

Although the exact correlation between serum PRL levels and risk of breast cancer is still unclear, with the above data it can be stated that PRL is involved in the etiology of at least some breast tumors in premenopausal and postmenopausal women. Circulating PRL levels still have yet no consistent correlation between tumor size, estrogen receptor status, stage of the tumor or nodal status. Yet, there is a high correlation between serum estrogen and androgen levels with an increased breast cancer risk. Therefore, this may show that in any analysis of serum PRL level correlation with breast cancer, estrogen and androgen would be included to understand each hormones independent association with breast cancer (Tworoger & Hankinson, 2008).

3. Prolactin and Pathways in Cancer

Prolactin is known to signal through various different pathways in cancer such as Jak/STAT, Ras-Raf-MAPK, PI3K pathways and Src family of intracellular kinases (Clevenger et al., 2003). PRL-PRLR dimerization leads to the activation of these intracellular signaling kinases. Stimulation of PRLR leads to tyrosine phosphorylation of several other proteins, including PRLR itself and this activates downstream proteins.

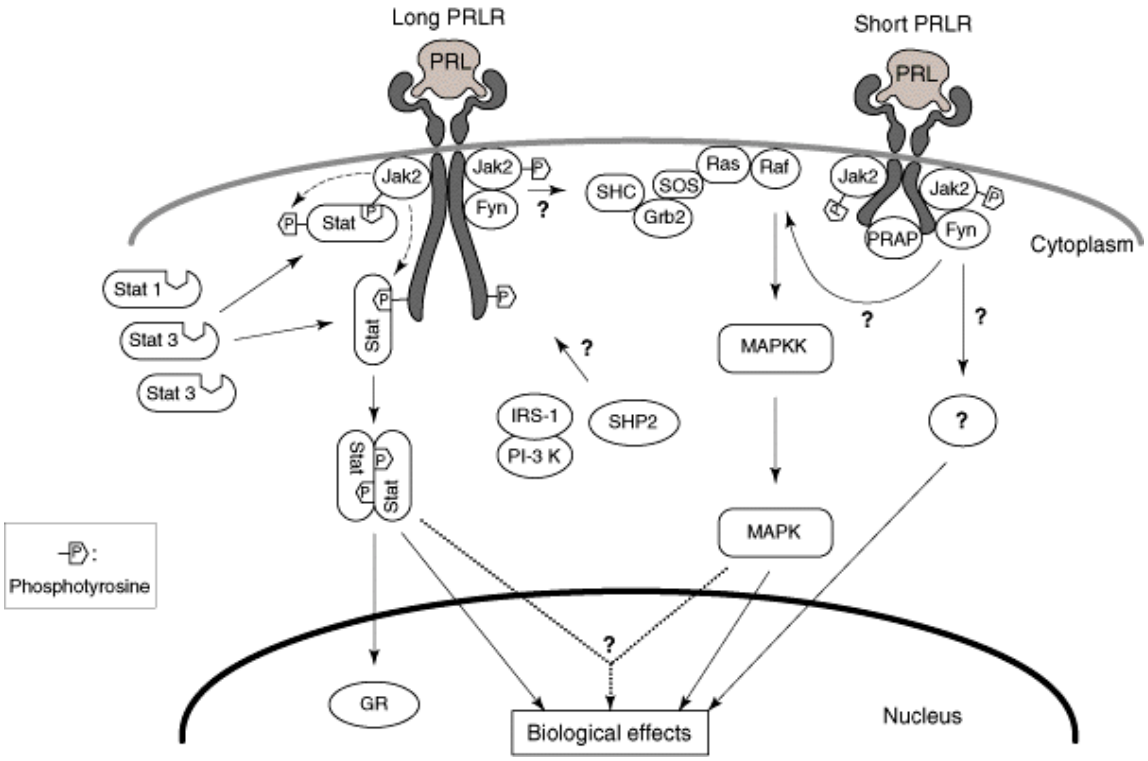


Figure 1. PRL signaling pathway (online reference 1)

Jak/STAT Pathway:

Jak/STAT pathway is one of the main signaling pathways that PRL signals through with dimerization of PRLR. JAK family of kinases consists of four different isoforms, Jak1, Jak2, Jak3 and Jak4. Jak2 has been shown to be the main protein that is associated with PRL-PRLR dimerization (Lebrun et al., 1995) The PRLR-Jak2 activation involves membrane proximal region of PRLR cytoplasmic domain. Ligand binding to PRLR leads to homodimerization of two receptor molecules, transphosphorylation of tyrosine residues and further activation of Jak2 kinases (Brockman et al., 2002). Jak2 activation by PRLR is a very rapid process, Jak2 kinases are seen to be active in one minute of PRLR activation (Acosta et al., 2003). Activation of Jak2 by PRL then leads to tyrosine phosphorylation of wide number of other intracellular signaling molecules such as Stat family of proteins. Stat proteins are essential in mediating the biological effect of PRL. Phosphorylation results in dimerization and retrotranslocation of Stat which then leads to binding of Stat complex to DNA and transactivates the promoter. In primary breast tumors, there is an increase in STAT-1, STAT-3, STAT-5a and STAT-5b. In murine mammary epithelial cell lines as well as some human mammary tumor cell lines, T47D and MCF-7, Jak2 activation of Stat proteins has been observed. (Clevenger et al., 2003).

Ras-Raf-MAPK Pathway:

The mitogen Activated Protein Kinase (MAPK) signaling pathway is one of the essential pathways in regulation of cell growth and differentiation. PRL activation of the MAPK pathway has been seen in several cell systems such as mammary epithelial cells, rat liver

and rat lymphoma Nb2 cell lines. All of the cytokine receptors that activate Jak/STAT pathway are known to activate MAPK pathway as well (Winston & Hunter, 1996). Extracellular regulated kinase ERK-1 and ERK-2 are serine/threonine directed MAP kinases which phosphorylate other kinases and nuclear proteins such as transcription factors and they are involved in proliferation of mammary tumor cells.

PRL activation of Jak2 proteins provides binding sites for adapter proteins Grb2 or erbB2 which recruit SOS to the membrane. Recruitment of these adapter proteins result in activation of MAPK through phosphorylation of both tyrosine and threonine residue in a Thr-X-Tyr motif in the activation loop (Clevenger et al., 2003). ERK (p42/44) MAPKs are known to be involved in cell proliferation through several multiple signaling mechanisms such as phosphorylation of Ets transcription factors, increasing synthesis of fos gene family, etc. MAPKs are also known to be involved in phosphorylation of STATs 1, 3, 5a and 5b (Clevenger et al., 2003).

PI3K Pathway:

The PI3K pathway enables PRL to signal cell survival, inhibit apoptosis and modulate cell cycle regulation. It is one of the most essential pathways in cancer studies. PRL is known to signal through this pathway. Once PRL binds to PRLR, several kinases are phosphorylated which results in phosphorylation of PRLR. Phosphorylated PRLR serves as a docking site for Protein Kinase B (PKB/Akt), active phosphorylated form of Akt, PTEN, PI1K and PI2K (Clevenger et al., 2003).

PI3K pathway is known to participate in cell growth, transformation, differentiation, motility, insulin action and cell survival (Toker, 2000). PI3-Kinase consists of two subunits, p110 catalytic subunit and p85 regulatory subunit. Active p110 domain can phosphorylate downstream signaling targets on serine and threonine residues (Rameh & Cantley, 1999). In mammals several p110 subunit isoforms have been identified such as p110, 110β and 110δ each encoded by a separate gene (Vanhaesebroeck & Alessi, 2000).

p110 and p85 subunits are shown to generate phosphtidylinositol (PtdIns), PtdIns(4,5)P_2 to produce PtdIns(3,4,5)P_3. Then through the action of PTEN and SH2 phosphatases, Akt, multifunctional serine/threonine directed kinase is activated by phosphorylation. This pathway initiates survival and inhibits proapoptotic signals (Clevenger et al., 2003). Akt in humans have three isoforms, Akt1 (PKBα), Akt2 (PKBβ) and Akt3 (PKBδ) which are all similar in structure and size. All three isoforms have been expressed in most tissues in humans. Akt1 is mainly expressed in human gastric adenocarcinoma; Akt2 in ovarian, breast and pancreatic carcinoma (Dennis, 2008). Phosphorylation of threonine 308 and serine 473 in Akt results in full kinase activity (Frogne et al., 2005). Activated Akt is responsible for phosphorylation of some other downstream regulators which are involved in apoptosis, cell growth and proliferation, all of which promote tumorigenesis (Bose et al., 2006). Some of the downstream effectors of Akt are Bcl-X_L-Associated Death Inducer (Bad), Human Caspase-9, Forkhead (FH) transcription factors, etc, (Scheid & Woodgett, 2001). Additionally, p-Akt expression and loss of PTEN expression were frequently observed in patients with advanced breast cancer which states that p-Akt and PTEN expression status can be a definite prognostic factor in clinical breast cancer. (Yamamoto et al., 2006).

Through Jak/Stat pathway, PRL is known to activate transcription factor, activating protein-1 (AP-1). AP-1 has been implicated in many types of human cancer including breast cancer. C-Jun and c-Fos activity and expression are correlated with tumor grade, cell cycle regulator protein expression estrogen receptor expression and tamoxifen resistance. It has been shown that PRL directs a signaling pathway by activating AP-1 through Jak/Stat pathway with the contribution of c-Src kinase, PI3K and protein kinase C. Furthermore, ERK ½ are the primary downstream activators of c-Jun and c-Fos. (Gutzman et al., 2005).\

4. BP1 and Breast Cancer

Breast cancer is the second most common type of cancer found in women. In 2008, estimated 180,000 cases of invasive breast cancer were diagnosed among women. Yet, the estimated number of new breast cancers now will be lower than 2007 since in 2007 the number of breast cancer issues were lower than 2005 due to the better understanding of the disease and new treatments developed (www.cancer.org). Furthermore, it is known than the rate of breast cancers have been decreasing since 1990 with the development in medicine and technology (www.acs.org). About 5-10% of breast cancers are caused by inherited gene mutations. Yet, about 90% of breast cancers are due to genetic abnormalities that occur as a result of aging and environment in general (www.breastcancer.org).

There are several genes that have been observed in initiation and proliferation of cancer cells. Homeobox genes are transcription factors that are known to play an important role in many types of cancers. Homeobox was first discovered in *Drosophila* and then identified in many metazoans ranging from sponges to humans (Gehring et al., 1994). Homeobox genes share a highly conserved region of DNA with 180 base pairs and 60 amino acids (Lewis, 2000). Homeobox genes play an important role in many processes in eukaryotic cells. They have been implicated in controlling cell identity, regulating cell growth, differentiation and cell proliferation (Pattyn et al., 2000). Changes in homeobox gene expression are related to several cancers such as breast, leukemia, prostate, skin and brain.

Beta Protein 1 (BP1) is a homeobox gene that belongs to Distal-less (DLX) subfamily of Class II vertebrate homeobox genes (Chase et al., 2002). BP1 was cloned by Dr. Berg's laboratory and was shown to be a repressor of the β-globin gene in normal erythroid cells (Chase et al., 2002). BP1 is located on a region near chromosome 17q, 17q21-22, which is known to be amplified in breast cancer (Fu et al., 2001). BP1 has been shown to be upregulated in 47% of adult and 81% of pediatric acute myeloid leukemia cells (Haga et al., 2000) also in 80% of invasive ductal breast tumors (Fu et al., 2003). Furthermore, BP1 expression strongly correlates with the estrogen receptor status of the cells where 100% of estrogen receptor negative (ER-) tumors were BP1 positive while 73% of estrogen receptor positive (ER+) tumors were found to be BP1 positive (Fu et al., 2003). Also, there is a correlation between BP1 status of the cells with breast cancer progression and invasion; 21% of hyperplasias, 46% of ductal carcinomas in situ, 81% of invasive ductal carcinomas and 100% of inflammatory breast cancers were found to be BP1

positive by immunostaining (Man et al., 2005). There is also disparity in BP1 expression where 89% of African-American women with breast cancer expressed BP1 mRNA, whereas only 57% of Caucasians expressed BP1 mRNA (Fu et al., 2003).

BP1 has been shown to increase the expression of the anti-apoptotic gene bcl-2, where the cells overexpressing BP1 were more resistant to treatment with TNF-α than the cells containing empty vector. (Stevenson et al., 2007) Other downstream targets of BP1 include Twist, Met, c-Fos and MMP9 (J.Rheey & P.E. Berg, unpublished data).

The mechanisms causing activation of BP1 are beginning to be elucidated (Cavalli et al., 2008). In this thesis, the possibility that BP1 may be regulated by PRL is studied due to their significant roles in cell survival and proliferation. If BP1 is activated by PRL and if they are involved in the same pathways in cancer, BP1 would be a strong target for therapy.

Materials and Methods

Mammalian Cell Lines

MCF-7 and T47D breast cancer cell lines were grown in RPMI 1640 media containing 10% FBS and 1% penicillin/streptomycin. Stably BP1 overexpressing MCF-7 cells (O2 and O4) and empty vector MCF-7 cells (V1 and V2) were also grown in RPMI1640 (Invitrogen, 11875-093) media containing 10% FBS and 1% penicillin/streptomycin (pen/strep) in the presence of 500µg/ml G418 sulfate (Fisher, MT-30-234-CI). Normal breast epithelial MCF-10A cells were grown in MEBM media supplemented with 0.4% Bovine Pituitary Extract, 0.1% human epidermal growth factor, 0.1% insulin, 0.1% hydrocortisone and 0.1% gentamycin (Lonza, CC-3150). MDA-MB-435 empty vector (435-EV) and stably prolactin overexpressing MDA-MB-435 (435-PRL) cells (kindly gifted by Dr. Barbara Vonderhaar, National Cancer Institute, Bethesda, MD, USA) were grown in RPMI 1640 media with 10% FBS, 1% penicillin/streptomycin and 1 µg/ml insulin. All the cells were incubated at 37°C with 5% CO_2 and humidity. For the V1, V2, O2, O4 cells that are used for western blotting for p-Akt, phospho-p44/42, bcl-2 comparison, after incubating the cells in RPMI media with 10%FBS and 1% pen/strep for 48 hours, the cells were washed with PBS and the media was replaced with phenol-red

free, serum-free RPMI 1640 media (Invitrogen, 11835-030) containing 1% pen/strep and the cells are incubated for 48 hours and cell lysis is performed.

Prolactin Treatment of Cells

MCF-7 and T47D cells were thawed in regular RPMI1640 media with 10% FBS and 1% penicillin/streptomycin. 24-48 hours after transferring the cells to T-75 flasks the media was replaced with RPMI1640 with 5% charcoal-stripped serum (CSS) and 1% pen/strep. (CSS RPMI 1640 media) (Biswas, Vonderhaar, 1987) The cells were grown in this media for 7 days with changing the media to fresh RPMI 1640 with 5% CSS, 1% pen/strep on day 4. At the end of 7 days, the cells were counted using a hemocytometer; 300,000 cells/well were seeded on 6-well plates. The cells were allowed to attach to the 6-well plate for 36 hours in CSS RPMI 1640 media. After 36 hours (day 0), the media was replaced to fresh CSS RPMI 1640 media containing 0 ng/ml, 250 ng/ml and 500 ng/ml ovine prolactin (Sigma, L6520). After performing the experiments once with ovine prolactin, the results were verified using human prolactin (Sigma, L4021) with 0ng/ml, 100ng/ml and 250 ng/ml concentrations. Both human and ovine prolactin is stored at -20°C freezer at 50µg/ml concentration. The cells were treated for 1, 2, 3 and 4 days. RNA or protein extractions were performed on corresponding days.

RNA Extraction, Cleanup and cDNA synthesis

MCF-7 and T47D cells were lysed from 6-well plates using 800µL of Trizol Reagent. (Invitrogen, 15596-018). 160µL of chloroform was added to the Trizol and the sample was vortexed followed by a 15min centrifuge at 12,000x g at 4°C. After centrifuge RNA was in the top, aqueous layer. This layer was collected into a new centrifuge tube; RNA was precipitated with 0.4ml of room temperature (RT) isopropyl alcohol by centrifugation at 12,000x g for 15min at 4°C. RNA pellet was then washed with 800µl of 75% room temperature ethanol and centrifuged at 7500x g for 5 min at 4°C. Ethanol remaining above the precipitated RNA pellet was removed and dried off by evaporating the remaining ethanol at 65°C heat block for ~5min. RNA pellet was then dissolved in 20µL RNase free H_20. Total RNA was treated with RNase-free DNase (Qiagen, 79254) for DNase digestion before RNA purification. RNA is then purified using RNeasy Mini Kit (Qiagen, 74104) according to the manufacturer's protocol. After DNAse digestion and RNA purification, RNA concentration is determined using Nanodrop. The concentration of RNA is then diluted to 1µg/µl using RNase-free water. 1 µg of RNA is then used for reverse transcription for cDNA synthesis using SuperScript III First-Strand cDNA synthesis kit. (Invitrogen, 18080-051).

Real-Time PCR

Real-time PCR of the cDNA samples is performed using ABI Prism 70000 real-time instrument (Applied Biosystems) SYBR Green master mix (Biorad, 1725850) is prepared for each sample. BP1, 18S master mixes were prepared using 12.5 µl SYBR Green

Master Mix, 9.5 µl of DEPC water and 1 µl of each forward and reverse primer for each reaction tube which makes a total of 24 µl of master mix for each reaction. Final concentration of the primers in master mixes is set up to be 20µmol/µl. After addition of the master mixes to each tube, 1 µl of the appropriate cDNA is added to the tubes. The samples are run in triplicates. Non-template controls, which are composed of only master mixes with no cDNA added are also used to test DNA contamination. Samples are then placed in the ABI prism 7000 instrument and the reaction is run for 50°C for 2min for 1 cycle, 95°C for 10min for 1 cycle, 95°C for 15 sec and 60°C for 1 min up to 40 cycles.

Primers of c-Fos, Met, MMP9 and Twist were purchased from SA Biosciences (PPH00094A). And the other primer sequences that are used in the experiments are as follows:

Table 7. Primer Sequences used for real-time PCR

18S (F)	GCC GCT AGA GGT GAA ATT CTT G
18S (R)	CAT TCT TGG CAA ATG CTT TCG
BP1 (F)	CCT CCC CCA ATT TGT CCT ACT C
BP1 (R)	GGT TGC TGG CAG GAC AGG TA

Protein Extraction and Quantification

Protein lysates of MCF-7 and T47D cells from 6-well plates were prepared by first trypsinizing cells with 800 µl of trypsin followed by a wash with cold PBS pH 4. Then, the cells were lysed in 100~150 µl of RIPA lysis buffer with freshly added 1X protease inhibitors (Roche, 04693124001). RIPA lysis buffer is composed of 50mM Tris-HCl pH 7.5, 2mM EDTA, 100 mM NaCl and 1% NP-40 and stored at 4°C refrigerator. Furthermore, for detection of phosphorylated proteins, phosphatase inhibitor set is used with RIPA lysis buffer. Phosphatase inhibitor set is purchased from Calbiochem (Phosphatase Inhibitor Cocktail Set III, 524627) and used in the cell lysate with 1:5 dilution. The cell lysis was incubated on ice for 10 min followed by a 30 min rotation on a rotating wheel in cold room. The protein extracts were then centrifuged at 13,000rpm for 30 min at 4°C. The supernatant was transferred to a new microcentrifuge tube, glycerol was added to the cells with the volume of 1/10 of the volume of RIPA lysis buffer. The cell lysates were then stored at -80°C freezer. Protein concentrations of the lysates were determined using BCA Protein Assay Kit (Pierce, 23227).

Western Blots

After quantifying the protein concentration of the cell lysates, 10 µg of protein was loaded on a 12% Tris-HCl precast gel (Biorad, 161-1102). The protein samples were prepared first with 30 µl of total protein (10 µg of protein + filled up to 30 µl with RIPA lysis buffer). Then, 15 µl of 3X SDS loading buffer was added to the samples and the samples were then heated at 100°C for 10 min. After 10 min, the samples were taken out

of the heating block and kept outside until they reach room temperature. 45 µl of each sample is then loaded to the appropriate wells of the 12% Tris-HCl gels and the proteins are separated for about 1 hour at 30 mA for one membrane. When the running was complete, polyvinyludene fluoride (PVDF) membranes (Perkin Elmer, NEF1000) were first activated in methanol for 2min followed by incubation in 1X transfer buffer. Transfer cassettes were prepared and the proteins were transferred to the PVDF membrane either at 100V for 1 hour, 80V for 2 hours or 30V overnight in cold room. The membrane was taken out after the transfer and was washed in wash buffer for 10 min. Wash buffer was poured out and the membrane was incubated in blocking buffer for 2-3 hours at room temperature on a shaking platform. After blocking, blocking buffer was discarded and the primary antibody buffer was added and incubated overnight at cold room on a rocking platform. The next day, primary antibody solution was collected and stored at 4°C, the membrane was washed three times, 10 min each with washing buffer and incubated in secondary antibody solution for 2-3 hours. The blots were then washed three times for 10 min each and exposed for band detection. For exposure, Pierce West Dura Kit was used. 1 ml of each reagent was mixed in a 15ml centrifuge tube and added evenly onto the membrane. The membrane tip box was covered with foil and incubated for 5min. The blots were then developed on Kodak 2000mm Image Station.

For calculation of relative protein amounts and controlling equal amounts of proteins loaded, the blots were then probed for β-actin antibody. The membrane was stripped with 10 ml of stripping buffer with 20 min incubation at 45°C water bath. Membrane was then washed for at least three times for 10 min each in PBS/Tween to rinse off any remaining β-mercaptoethanol. After washings were complete, the membrane was incubated in

mouse monoclonal β-actin antibody (Sigma) 1:10,000 dilution in PBS/Tween on a rocker in cold room overnight. Next day, membrane was washed in PBS/Tween three times, 10 min each. Secondary goat-anti mouse antibody (Thermo Scientific, 32430) was diluted 1:2500 in PBS/Tween and the membrane was incubated in this solution for 2-3 hours followed by three times 10 min PBS/Tween washes. Then the blot was developed as described above. Relative band intensities were determined using region of interest (ROI) data. The results were normalized against β-actin data.

Wash buffers, blocking buffers, primary and secondary antibody dilutions for each antibody used is listed in Table 3.

Table 8. Wash/Blocking/Primary Antibody/Secondary antibody buffers

Protein	Wash Buffer	Blocking Buffer	Primary Antibody	Secondary Antibody
BP1	TNET	5% milk in TNET	-Novus - Rabbit -1:2000 in TNET	-Pierce (#1858415) -Goat anti-Rabbit -1:15000 in TNET
Bcl-2	PBS/T	5% milk in PBST	-Santa Cruz -Rabbit -1:1000 in PBS/T	- Pierce (#1858415) -Goat anti-Rabbit -1:15000 in TNET
t-Akt	TBS/T	TBS/T 5% BSA	-Cell Signaling (#9272) -Rabbit -1:1000 in TBS/T 5% BSA	-Cell Signaling (#7074) - Goat anti-Rabbit -1:2000 TBS/T 5%milk
p-Akt	TBS/T	TBS/T 5% BSA	-Cell Signaling (#9271) -Rabbit -1:1000 in TBS/T 5% BSA	-Cell Signaling (#7074) - Goat anti-Rabbit -1:2000 TBS/T 5%milk
Total-p44/42	TBS/T	TBS/T 5% BSA	Cell Signaling (#4695) -Rabbit -1:1000 in TBS/T 5% BSA	Cell Signaling (#7074) - Goat anti-Rabbit -1:2000 TBS/T 5%milk
Phospho-p44/42	TBS/T	TBS/T 5% BSA	Cell Signaling (#4377) -Rabbit -1:1000 in TBS/T 5% BSA	Cell Signaling (#7074) - Goat anti-Rabbit -1:2000 TBS/T 5%milk
β-Actin	PBS/T	5%milk in PBS/T	-Sigma -Mouse -1:10,000 PBS/T	-Thermo (#32430) -Goat anti-mouse -1:2500 in PBS/T

Table 4 lists the components of the running buffer, transfer buffer, stripping buffer, wash buffers.

Table 9. Components of the buffers

BUFFER	
Running Buffer (10X)	30.3g Tris,144g Glycine,50mL 20% SDS buffer, Total vol. 1L
Transfer Buffer(10x)	30.3g Tris, 144g Glycine, Total vol. 1L
TNE (20X)	200mM Tris/HCl, 50mM EDTA, 1M NaCl, total vol.500ml (pH 7.4)
TNET (1X)	500µl of Tween-20 in 500ml of 1X TNE (pH 7.4)
TBS (1X)	2.7mM KCl, 138mM NaCl, 20mM Tris, pH7.4, total vol 1L (pH 7.4)
TBS/T (1X)	500µl of Tween-20 in 500ml of 1X TBS (pH 7.4)

RT^2 Profiler Array

RNA is extracted from MCF-7 cells grown in 100ng/ml PRL for 2 days and from MCF-7 cells grown in CSS RPMI media (Day 0 samples, explained in prolactin treatment of cells section). RNA extraction is performed using Trizol (Invitrogen) as described above. DNase-treatment is carried out using RNase-Free DNase Set (Qiagen) and RNA cleanup is performed with RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. RNA concentration and purity is determined with NanoDrop and further examined by Agilent BioAnalyzer using a RNA 6000 Nano Labchip to ensure the RNA is not degraded. Then, 1 µg of RNA is reverse transcribed using Reaction Ready First Strand cDNA synthesis Kit (SuperArray).

RT^2 Profiler Human Cancer Pathway Finder PCR Array is a 96-well plate array with 84 wells coated with QPCR primers for targeting genes and 6 wells are coated with primers for housekeeping genes and the rest of the wells for cDNA contamination checking, efficiency of reverse transcription and PCR. To each well of the Cancer Pathway Finder PCR Array, 1µl of 0.2x cDNA, 12.5µl of 2x Superarray Master Mix and DEPC water is added with a total of 25µl volume. Later, the reactions are run on an ABI Prism 7000 instrument as described in Real-Time PCR section. The data is then analyzed using $\Delta\Delta C_t$ method that is recommended by SuperArray (RT^2 Profiler Human Cancer Pathway Finder Booklet) to determine expression levels of the genes based on the housekeeping genes.

Results

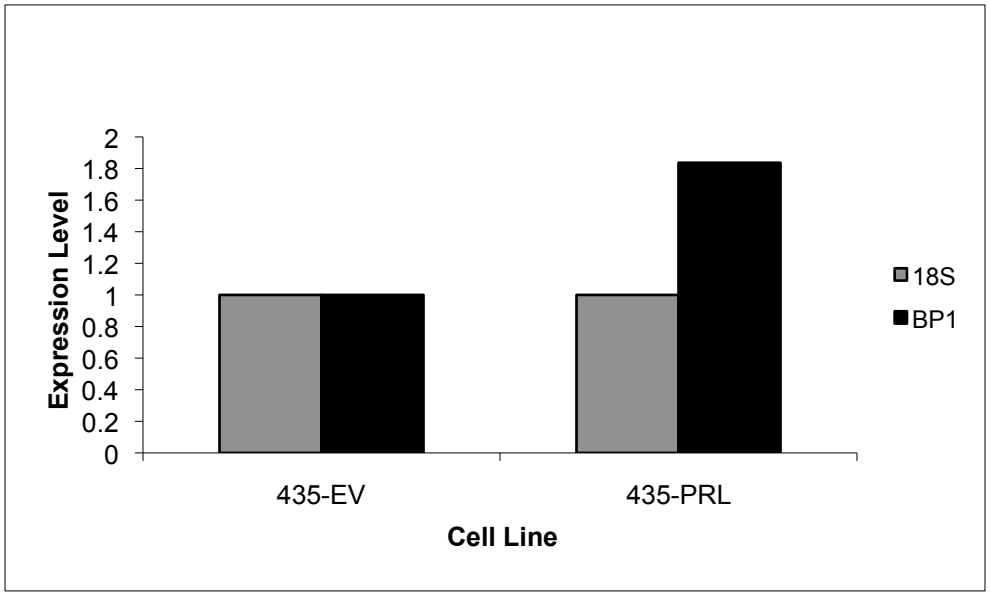
1. Endogenous PRL stimulates BP1

MDA-MB-435 breast cancer cell lines that stably overexpress PRL (435-PRL) were compared with an empty vector (435-EV) to detect the effects of endogenous PRL on BP1. These cell lines were a kind gift from Dr. Barbara Vonderhaar's laboratory at NCI, Bethesda. 435-PRL cells are shown to express PRL whereas 435-EV cells do not have any detectable PRL levels, as shown by Dr. Vonderhaar's laboratory; the data was confirmed by Cynthia Chatterjee from Dr. Berg's Laboratory (data not shown). Real-time PCR experiments were carried out to detect the effects of the endogenous presence of PRL on BP1 mRNA. Figure 2A shows the real-time PCR data of BP1 mRNA levels when 435-EV and 435-PRL are compared. It is seen that there is approximately 1.8 fold increase in BP1 mRNA levels normalized to 18S with increased endogenous PRL. Real-time PCR was repeated with 3 independent experiments in triplicates.

Upregulation of BP1 in the presence of endogenous PRL was confirmed by performing Western blots. 435-EV and 435-PRL cell lines were compared. Figure 2B shows a representative Western blot for BP1 protein relative BP1/β-actin ratios normalized to

435-EV cell lines. It is shown that in the presence of PRL, BP1 protein is upregulated about 1.8 fold. Western blots were repeated 3 times.

A.



B.

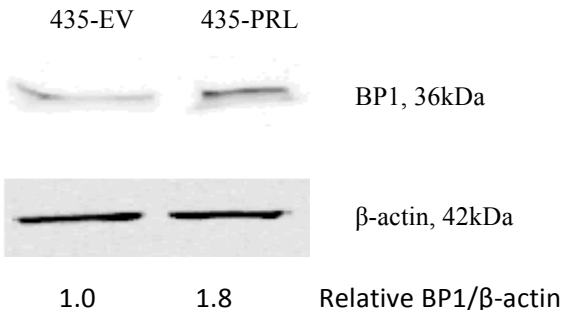


Figure 2 Activation of BP1 with endogenous PRL. (A) Real-time PCR and (B) Western blots.

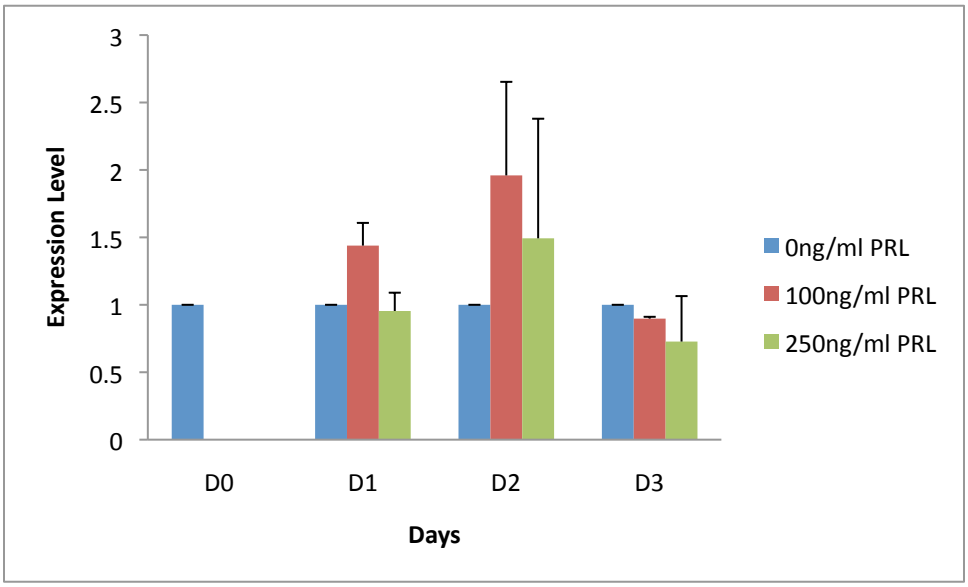
2. Exogenous PRL stimulates BP1

The effect of exogenous PRL on BP1 expression was determined next. For this purpose, both MCF-7 and T47D breast cancer cell lines were treated with PRL. Before treatment, the cells were first grown in RPMI 1640 media with 5% charcoal-stripped serum (CSS) and 1% pen/strep for 7 days instead of growing them in fetal bovine serum. Fetal bovine serum is known to contain bovine prolactin and, in the presence of bovine prolactin, the growth response of human prolactin is inhibited. Use of charcoal-stripped serum removes endogenous lactogens that inhibit the effects of human prolactin. (Biswas & Vonderhaar, 1987)

After growing MCF-7 and T47D cells in 5% CSS media, the cells were seeded in 6-well plates and treated with different concentrations of PRL. In the first set of experiments, 250ng/ml and 500ng/ml of ovine prolactin was used. Often ovine PRL is used since it is less expensive than hPRL. Later, the results were confirmed with using 100ng/ml and 250ng/ml human PRL. A higher concentration of ovine PRL is needed to get the same effects on the cells with human PRL.

Figure 3A and 3B show the BP1 mRNA levels after treatment of MCF-7 and T47D cells with 100ng/ml and 250ng/ml hPRL for 4 days with real-time PCR experiments. It is seen that after day 2, there is no constant increase in BP1 mRNA levels in MCF-7 or T47D cells. Also, it is seen that there is more increase in BP1 mRNA in MCF-7 cells when compared to T47D cells. Real-time PCRs are repeated with 2 independent experiments in triplicates.

A.



B.

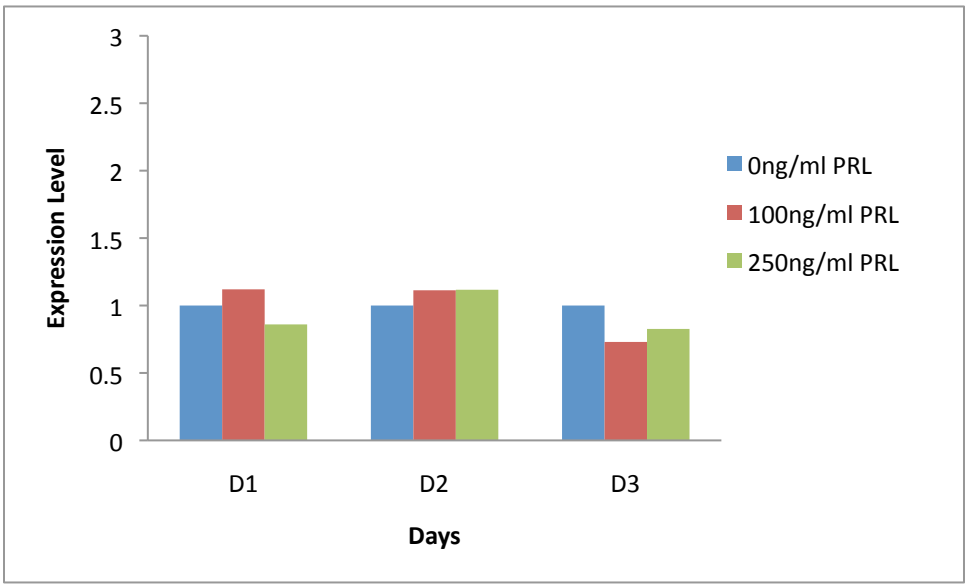
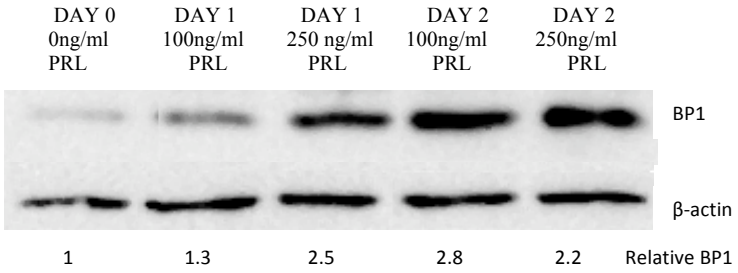


Figure 3 Real-Time PCR of MCF-7 (A) and T47D (B) cells after treatment with PRL

Western blots were performed with protein extracts from MCF-7 and T47D cells after 4 days of PRL treatment with 100ng/ml and 250ng/ml PRL. As with the real-time PCR data, no further increase in BP1 protein was observed after day 2 (data not shown). Figure 4A and 4B show the Western blots for BP1 with MCF-7 and T47D cells on days 1 and 2. It is seen that in MCF-7 cells there is more than 2 fold increase after 2 days, and the highest BP1 protein amount is seen on day 2 with 100ng/ml PRL with a 2.8 fold increase, in agreement with the real-time PCR data. Also, it is seen that 100ng/ml PRL has a larger effect on BP1 levels than 250ng/ml PRL. Figure 4B displays Western blots of T47D cells with 2 days of 100ng/ml and 250ng/ml PRL treatment. Again there is a larger increase in BP1 protein with 100ng/ml PRL when compared to 250ng/ml PRL and the BP1 protein reaches up to 3 fold at the end of day 2. Also, it has been observed that for T47D cells, real-time PCR data do not correlate with Western blot data where BP1 protein expressions are higher than RNA expression. Real-time PCR needs to be repeated for more accurate results. Western blots are repeated with 2 independent experiments and a representative figure is shown.

A.MCF-7 cells



B.T47D cells

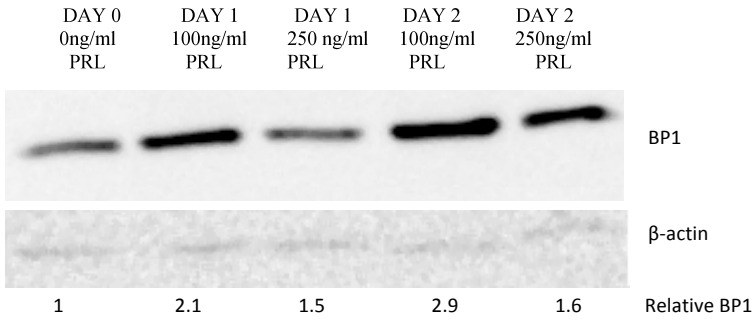


Figure 4 Western Blots of (A) MCF-7 and (B) T47D cells with 2 days of 100ng/ml and 250ng/ml PRL treatment

It has been shown that MCF-7 breast cancer cells do not express as much endogenous PRL as T47D cells and are therefore more sensitive to exogenous PRL (Gutzman et al., 2004). Therefore, in further experiments we have used MCF-7 cells with exogenous 100ng/ml hPRL treatment for 2 days, where we have seen the biggest effect.

3. Effects of PRL and BP1 on the MAPK and PI3K pathways

PRL, known as the 'master regulatory hormone' in breast cancer is known to be involved in several different pathways in cancer such as the JAK/STAT pathway, the MAPK pathway and the PI3K pathway. The MAPK pathway is an important pathway involved in cell growth and cell differentiation. PRL is known to signal through this pathway via phosphorylation of Erk1/2 (p44/42) (Acosta et al., 2003). The PI3K pathway is an essential pathway in cell survival, apoptosis and modulating cell cycle regulation. PRL signals through this pathway through phosphorylation of Akt (Clevenger et al., 2003). The serine-threonine kinase Akt is a downstream target of PI3-kinase and phosphorylated Akt (p-Akt) inhibits apoptosis (Yamamoto et al., 2006).

To determine the effects of BP1 on phospho-p44/42 and p-Akt, BP1 overexpressing MCF-7 cells (O2) were compared with empty vector cells (V2). Cells were grown in RPMI 1640 media with 10% FBS and 1% pen/strep, but 48 hours before the protein extraction, the media was replaced with serum-free and phenol-red free media since the serum itself is also known to stimulate MAPK and PI3K pathways. Figure 5A and 5B show the Western blots of p-Akt and phospho-p44/42, respectively in V2 and O2 cells. In Figure 5A, it is seen that there is no significant change in total Akt (t-Akt) amount when V2 and O2 cells are compared, whereas in V2 cells there is no significant p-Akt but O2 cells show a p-Akt band. It can be stated that t-Akt amount does not vary with BP1 levels but p-Akt increases in O2 cells compared with V2 cells. Figure 5A was repeated 3 times with independent experiments and a representative figure is seen.

Figure 5B was done by Cynthia Chatterjee (unpublished data, Dr. Berg's laboratory, one experiment). It shows the Western blots for V2 and O2 cells comparing levels of both total-p44/42 and phospho-p44/42. It is seen that both total and phospho- p44/42 expressions, when normalized to β-actin, remain constant in V2 cells compared to O2 cells.

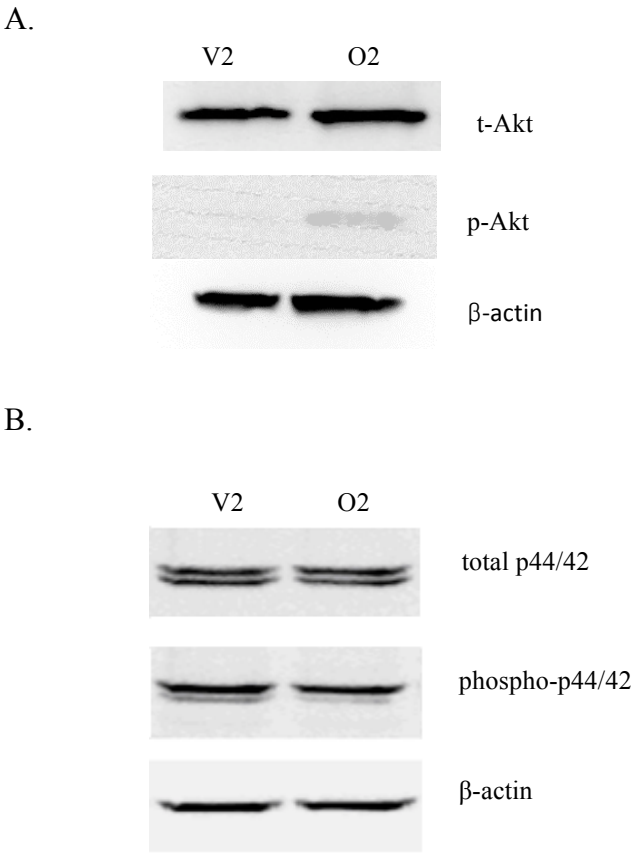


Figure 5. Western Blots of t-Akt and p-Akt (A) and total-p44/42 and phosphor-p44/42 (B) expressions with V2 and O2 cells comparison

The effects of endogenous PRL on Akt and p44/42 protein expression were then assessed. For this purpose p-Akt and phospho-p44/42 protein levels were compared in 435-PRL and 435-EV cells. Figure 6A displays the Western blot of p-Akt for 435-EV and 435-PRL. The relative values of p-Akt normalized to β-actin do not vary significantly when these cells are compared. Figure 6B shows the phospho-p44/42 Western blots for 435-EV and 435-PRL cells and again there is no significant change of phospho-p44/42 protein levels in the presence of endogenous PRL. The upper band is the 44kDa phosphorylated form of p44 and lower band is the 42kDA phosphorylated form of p42. Figures are repeated twice and a representative figure is shown.

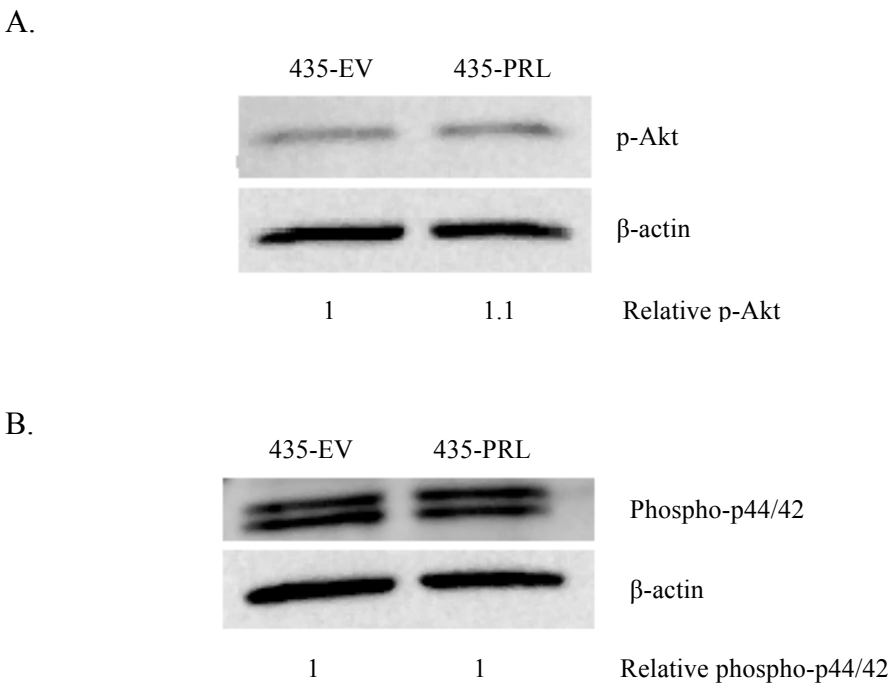


Figure 6 Western blots of p-Akt (A) and phospho-p44/42 (B) for 435-EV and 435-PRL cells comparison

4. Genes regulated by both PRL and BP1

RT^2 Profiler Human Cancer Pathway Finder PCR Arrays, which enables profiling of 84 genes (SuperArray) were used to determine the genes that are up- and down-regulated with exogenous PRL. MCF-7 cells were grown in RPMI 1640 with 5% CSS and 1% pen/strep for 7 days and treated with 500ng/ml ovine PRL for 2 days. PRL treated MCF-7 cells were compared with MCF-7 cells grown in CSS (without PRL) as a control. The gene expression was then normalized to three housekeeping genes: hypoxanthine phosphoribosyltransferase 1, ribosomal protein L13a and glyceraldehydes-3-phosphate dehydrogenase. This set of RT^2 Profiler Human Cancer Pathway Finder PCR Array needs to be repeated with MCF-7 cells treated with 100ng/ml of hPRL for 2 days.

To determine the genes that are upregulated with BP1, another set of RT^2 Profiler Human Cancer Pathway Arrays was performed by Jinguen Rheey (Dr. Patricia Berg Laboratory, 2006). In this set of arrays, BP1 overexpressing MCF-7 cells (O2) cells were compared to empty vector (V2) cells as a control.

Table 5 below lists the genes that are up-regulated in both sets of RT^2 Profiler Human Cancer Pathway Arrays.

Table 10. Genes upregulated in MCF-7 cells by both overexpression of BP1 and treatment with PRL

	FOLD CHANGE		
	GENE	O2 RELATIVE TO V2 CELLS	MCF-7 PRL TREATED CELLS
	c-FOS	1.8	1.8
	MET	1.8	3.0
	MMP9	2	3.6

Upregulation of these genes was confirmed in O2 cells relative to V2 cells with real-time PCR analysis (Figure 7). The values are the values of the gene expression relative to 18S gene expression. Twist does not appear in the list of the genes that are up-regulated in both sets of RT^2 Profiler Arrays; however, since in V2 and O2 cells comparison array, we have seen a very high fold increase in Twist expression, we wanted to confirm this data with real-time PCR. This Real-time PCR has been performed by Jinguen Rheey (Dr. Patricia Berg Laboratory, 2006).

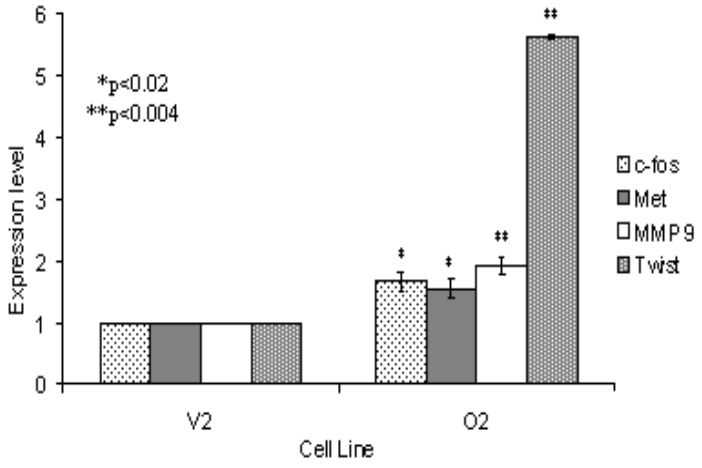


Figure 7 Real-time PCR of O2 cells compared to V2 cells looking at c-Fos, Met, MMP9, Twist

To test the effects of endogenous PRL on c-Fos, Met, MMP9 and Twist, real-time PCR was performed comparing 435-PRL cells with empty vector 435-EV cells as a control. Figure 8 shows the real-time PCR data of the genes in 435-PRL and 435-EV cells. It is seen that there is approximately a 1.6, 1.4, 1.5 and 4.6 fold increase for c-Fos, Met, MMP9 and Twist respectively when 435-PRL cells are compared to 435-EV cells. This real-time PCR was repeated once.

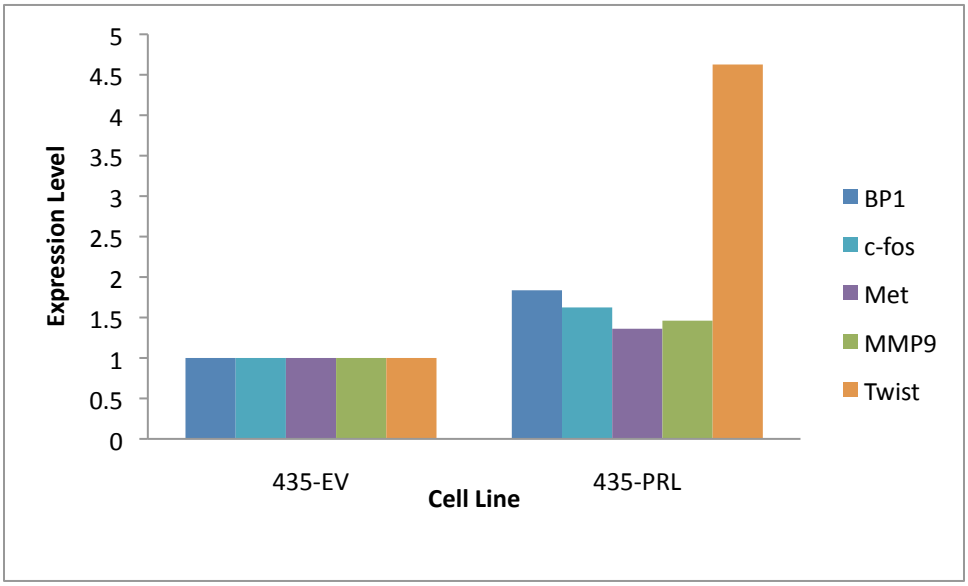


Figure 8 Real-time PCR of c-Fos, Met, MMP9 and Twist with 435-PRL and 435-EV cells

To verify the effects of exogenous PRL on the oncogenes c-Fos, Met, MMP9 and Twist, real-time PCR was performed with MCF-7 cells treated with 100ng/ml PRL for 2 days compared with MCF-7 cells grown in RPMI 1640 media with 5% CSS and no PRL as a control. Figure 9 shows the real-time PCR data where it is seen that BP1, c-Fos, Met and

Twist expressions increase with exogenous PRL whereas MMP9 expression decreases. This real-time PCR was repeated once and needs to be repeated again.

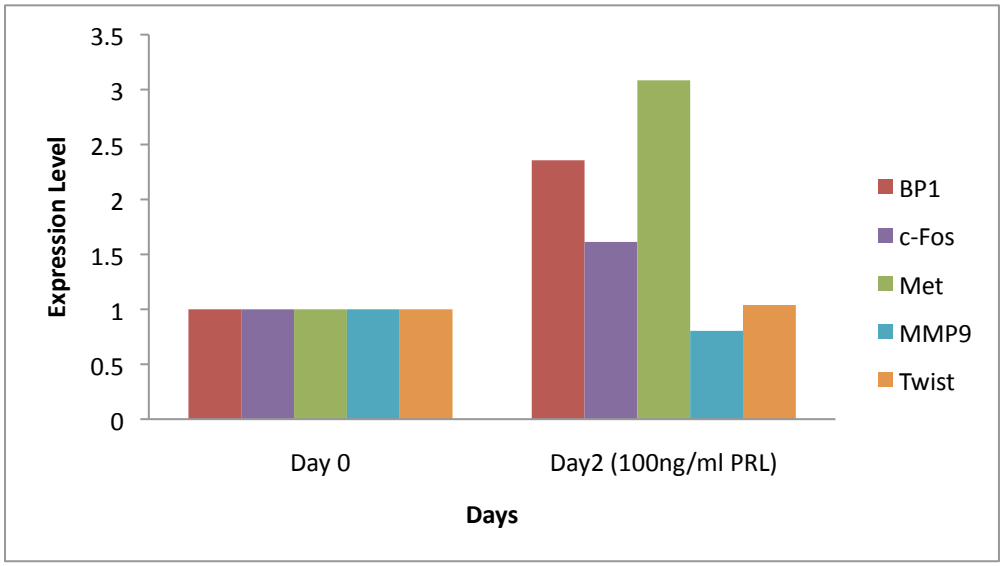


Figure 9 Real-time PCR of c-Fos, Met, MMP9 and Twist comparing untreated MCF-7 cells with MCF-7 cells treated with 100ng/ml PRL for 2 days

Discussion

Prolactin (PRL) is known to be one of the most versatile hormones in mammary gland development. It is a 23kDa hormone, secreted mainly by anterior pituitary gland. It induces cell proliferation, cell survival, differentiation and motility of mammary epithelial cells. Besides its endocrine effects, PRL is known to be synthesized and secreted from cells which make PRL act as an autocrine/paracrine factor (Clevenger et al, 2003). Estrogen, progesterone and PRL act synergistically for mammary gland growth, development and differentiation (Gutzman et al., 2004). Furthermore, serum PRL levels correlate with breast cancer progression in both premenopausal and postmenopausal women (Tworoger & Hankinson, 2008).

BP1 is a transcription factor belonging to Homeobox gene family and distal-less (DLX) subfamily (Chase et al., 2002). BP1 is expressed in 80% of invasive ductal breast tumors, 100% of estrogen receptor negative (ER-) tumors and 73% of estrogen receptor positive (ER+) tumors (Fu et al., 2003). BP1 has been shown to increase the expression of the anti-apoptotic gene bcl-2 in MCF-7 cells, where the cells overexpressing BP1 are more resistant to treatment with TNF-α than the cells containing and empty vector (Stevenson

et al., 2007). Other downstream targets of BP1 include Twist, Met, c-Fos and MMP9 (J.Rheey & P.E. Berg, unpublished data). An important question is how BP1 is upregulated in breast cancer cells.

We found that endogenous PRL was able to stimulate both BP1 mRNA and BP1 protein. The effect of endogenous expression of PRL was tested using the estrogen receptor negative (ER-) breast cancer cell line MDA-MB-435. Stably prolactin overexpressing MDA-MB-435 cells (435-PRL) were compared with cells containing an empty vector (435-EV). Both BP1 mRNA and BP1 protein were stimulated with endogenous expression of PRL.

Exogenous PRL was also able to stimulate BP1 in both MCF-7 and T47D breast cancer cell lines. For this purpose, these cell lines were treated with 100ng/ml and 250ng/ml human PRL (hPRL) after 7 days of charcoal-stripped serum treatment. With real-time PCR and Western blotting data, it can be concluded that both BP1 mRNA and BP1 protein were increased in the presence of exogenous hPRL. Furthermore, in both cell lines, the greatest effect of PRL was seen on day 2 with 100ng/ml hPRL. There was a slightly less increase in BP1 mRNA and BP1 protein levels in T47D cells when compared to MCF-7 cells. This might be for two reasons. First, the fact that T47D cells express more BP1 mRNA when compared to MCF-7 cells may contribute to this result (Berg lab, unpublished data). Also, T47D cells have a higher amount of endogenous PRL when compared to MCF-7 cells so that the effect of exogenous PRL might be diminished (Perks et al., 2004).

PRL is involved in several pathways in cancer including the Jak/STAT pathway, the Ras-Raf-MAPK pathway and the PI3K pathway. Binding of PRL to PRLR dimer leads to the activation of Jak2 protein, this in turn phosphorylates PRLR itself and phosphorylated PRLR serves as a docking site for several other kinases such as Stats, Shc, Grb2, etc. (Goffin et al., 2002). The MAPK pathway is one of the most important pathways involving PRL. The MAPK pathway is a significant pathway in cancer involved in cell growth, differentiation and proliferation (Kohno & Pouyssegur, 2003). It has been shown that MAPK expression is upregulated in malignant epithelial cells, which may lead to metastasis (Sivaraman et al., 1997). PRL is involved in the MAPK pathway via phosphorylation of p-44/42 (Erk1/2) protein (Gutzman et al., 2005) and also exogenous PRL treatment on MCF-7 and T47D cells leads to phosphorylation of p44/42 (Acosta et al., 2003). In this study, to see the effects of BP1 on this pathway, BP1 overexpressing MCF-7 cell lines (O2) were compared with an empty-vector (V2). No significant change in either total-p44/42 or phospho-p44/42 was seen. These results suggest that exogenous PRL is involved in the MAPK pathway through phosphorylation of p44/42 independent from BP1. We also looked at the effects of endogenous PRL on phospho-p44/42 levels. Western blots were performed to detect phospho-p44/42 levels with comparison of 435-PRL and 435-EV cells. No difference in phospho-p44/42 levels in these two samples was seen. This difference in data might be because of the differences in functions of endogenous and exogenous PRL. For example, it has been shown that endogenous PRL induces estrogen receptor α whereas exogenous PRL does not (Gutzman et al., 2004).

BP1 overexpression does not lead to a change in phospho-p44/42 expression in the MAPK pathway; however, BP1 may be involved in another pathway that is known to be regulated by PRL. The PI3K pathway is another very significant pathway in cancer studies where PRL is known to be involved. This pathway plays an important role in cell survival, proliferation and motility by generating phosphoinositide metabolites. PI3K and other phosphoinositides activate Akt via phosphorylation (Clevenger et al, 2003). Phosphorylated-Akt (p-Akt) results in activation of different protein targets involved in apoptosis, cell growth and proliferation which all promote tumorigenesis. The two most frequently mutated components of this pathway are Akt and PTEN proteins. P-Akt and loss of PTEN expression is frequently seen in patients with advanced breast cancer, therefore p-Akt/PTEN expression can be a prognostic factor in clinical breast cancer patients (Yamamoto et al., 2006). Exogenous treatment of MCF-7 and T47D cells with PRL results in increase in p-Akt expression (Acosta et al., 2003). To check the effects of BP1 on p-Akt expression, Western blots were performed comparing O2 cells with V2 cells to look for total Akt (t-Akt) and p-Akt levels. Although t-Akt levels do not vary between V2 and O2 cells, there was an increase in expression of p-Akt in O2 cells. This data suggests that PRL may be involved in the PI3K pathway through activation of BP1. Again, there was no change in p-Akt levels when 435-PRL cells were compared to 435-EV cell lines. The reason might be the same for both MAPK and the PI3K pathways, as stated above.

Besides the two pathways we have looked at, we have also observed some oncogenes that are upregulated by the presence of both BP1 and PRL. Analysis of two sets of human Cancer Pathway Finder PCR Arrays demonstrated overexpression of the oncogenes Met,

c-Fos and MMP9 which are involved in invasion and metastasis. This data was then confirmed with real-time PCR comparing MCF-7 cells treated with 100ng/ml PRL for 2 days with untreated MCF-7 cells to see the effects of exogenous PRL, as well as comparing O2 cells with V2 cells to determine BP1 specific effects (Daniel Rheey, Berg Lab). We verified that, expression of Met, c-Fos and Twist were upregulated in the presence of both BP1 alone and exogenous PRL. Whereas MMP9 is only upregulated in the absence of exogenous PRL. Interestingly bcl-2 is a very important anti-apoptotic oncogene that is known to be upregulated by both PRL and BP1 (Liby et al., 2003; Stevenson et al., 2007). It has been shown that overexpression of PRL stimulates bcl-2 expression (Liby et al., 2003) and inhibiting PRL leads to a decrease in bcl-2 expression therefore increasing apoptosis (Howell et al., 2008).

Below is a model showing the possible relationship between PRL and BP1.

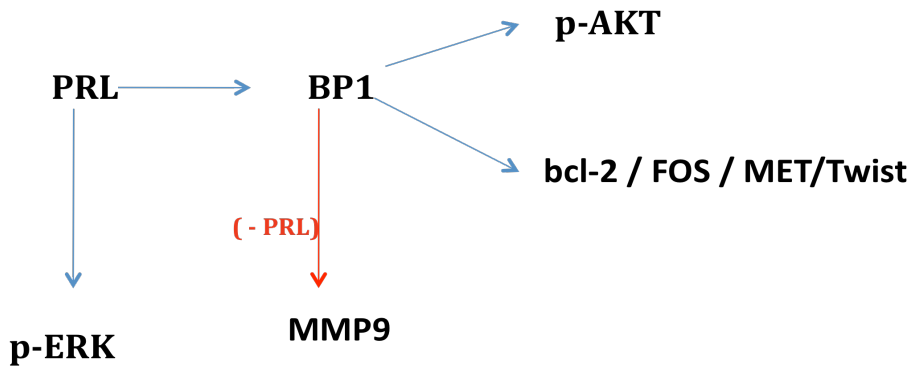


Figure 10. Model representing PRL and BP1 pathway

To test this model, future studies include RNA interference experiments using siBP1. First, siBP1 will be used to knockout BP1 from O2 cells and gene expression will be compared both in the presence and in the absence of BP1. Furthermore, siBP1 will be

introduced into MCF-7 cells before and after treating the cells with PRL. If our model is correct, in the presence of PRL we would expect to see decrease in bcl-2, Met, c-Fos, Twist and p-Akt levels whereas no change in p-Erk levels.

The mechanism by which BP1 may be upregulated by PRL is still unclear. It has been shown that Jak2/Stat5 is one of the main pathways that is activated by PRL (Clevenger et al., 2003). Stat5 proteins bind to Gas elements on DNA to further activate expression of other genes (Soldaini et al., 2000). We have identified a potential Gas element in BP1 DNA sequence. Therefore, activated Stat protein via PRL may bind to the Gas element on BP1 DNA and activate BP1.

As a conclusion, it can be stated that both endogenous and exogenous PRL are able to stimulate BP1 mRNA and BP1 protein expression. Because of their significant roles in cell survival and apoptosis, the aim of this project was to determine if there was a relationship between PRL and BP1 and whether they involved in any same pathways. We have also shown that PRL and BP1 appear to be involved in the PI3K pathway and both lead to overexpression of the oncgoenes bcl-2, Met, c-Fos and Twist. This is the first time it has been shown that PRL stimulates expression of Met and Twist. Determining the relationship between PRL and BP1 may play an important role in better understanding breast cancer progression. Our data also suggest that BP1 may be a potential strong therapeutic target in breast cancer.

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