Characterizing the role of prolactin-induced Grb2 tyrosine phosphorylation in the regulation of epidermal growth factor signalling in mammary epithelial and breast cancer cells

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Abstract

Multiple hormones and growth factors regulate mammary epithelial and breast cancer cell growth and differentiation. While prolactin (PRL) is a differentiation hormone, epidermal growth factor (EGF) is an important mitogen. Here we examined the PRL and EGF crosstalk mechanism in mammary epithelial cells. Our results indicate that PRL and EGF exhibit antagonistic properties that impacts mammary epithelial cell proliferation and differentiation. While EGF blocked PRL-induced cellular differentiation, PRL suppressed EGF-induced cell growth. EGF was shown to block PRL-induced cellular differentiation without inhibiting PRL proximal signaling cascade such as Jak2 and Stat5a tyrosine phosphorylation. We identified Grb2 as a novel substrate of the PRLR/Jak2 complex. Moreover, the tyrosine phosphorylation of Grb2 was shown to be an essential mechanism utilized by PRL to inhibit EGF signals to MAPK activation and cell growth. Together these results define a novel signaling mechanism underlying PRL and EGF crosstalk in mammary epithelial and breast cancer cell growth and differentiation.

Résumé

La PRoLactine (PRL) et *Epidermal Growth Factor* (EGF) sont des facteurs importants pour la croissance cellulaire, la différenciation et la carcinogenèse mammaire. Tandis qu'EGF est un mitogène puissant, PRL est critique pour l'induction de la différenciation de cellules épithéliales mammaires. Tandis qu'EGF bloque la différenciation cellulaire causée par PRL, PRL supresse la croissance cellulaire dûe à EGF. D'un côté, on a montré qu'EGF bloque la différenciation cellulaire sans inhiber la signalisation proximale de PRL. De l'autre, le traitement avec PRL inhibe l'activation de MAP Kinases par EGF et la prolifération cellulaire. De plus, le traitement avec PRL entraîne une augmentation significative de la phosphorylation en ou sur les tyrosines de Grb2. Pour confirmer ces résultats, nous avons utilisé Grb2YF, une forme de Grb2 sans sites de phosphorylations de tyrosines. En présence de PRL et Grb2YF, nous avons observé un rétablissement de l'activation de Ras et des MAP Kinases. Nos données suggèrent que la PRL réduit l'activation des MAP Kinases et la prolifération cellulaire induite par EGF grâce à la phosphorylation des sites tyrosines de Grb2 empêchant ainsi l'interaction avec Sos. Ensemble ces résultants définissent un nouveau mécanisme de signalisation qui met en évidence la communication entre PRL et EGF dans la croissance et différenciation des cellules épithéliales mammaires et cancérigènes.

Abbreviations

PRLR = Prolactin receptor

PRL = Prolactin

Jak2 = Janus kinase 2

Stat5a = Signal transducer and activator of transcription 5a

SHP-2 = PTPN11 protein tyrosine phosphatase

SH2 = Src homology domain

EGFR = Epidermal growth factor receptor

EGF = Epidermal growth factor

TGFα = Transforming growth factor alpha

AR = Amphiregulin

NRG = Neuregulin

HRG = Heregulin

HB-EGF = Heparin-binding epidermal growth factor

Sos = Son of sevenless

Grb2 = Growth factor receptor binding protein 2

Ras = Ras GTPase

Raf = Rapidly accelerated fibrosarcoma

Mek = Map kinase kinase

MAPK = Mitogen-activated protein kinase

RSK = Ribosomal S6 kinase

PI3K = Phosphoinositide Kinase-3

HI = Hydrocortisone and insulin

HIE = Hydrocortisone, insulin and EGF

HIP = Hydrocortisone, insulin and PRL

HIPE = Hydrocortisone, insulin, PRL and EGF

TEB = Terminal end bud

EMT = Epithelial mesenchymal transformation

FBS = Foetal bovine serum

MTT = Tiazolyl blue tetrazolium bromide

DNA = Deoxyribonucleic acid

RNA = Ribonucleic acid

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Chapter 1: Background

1.1. Breast cancer in the general population

According to the Breast Cancer Society of Canada, 1 in 9 women will be diagnosed with breast cancer in their lifetime and 1 in 28 women will die from the disease. In 2007, approximately 180,000 women and 3000 men in the United States (similar ratio for Canada) were diagnosed with invasive breast cancer and approximately 41,000 women and 500 men will die from the disease. Moreover, among all female breast cancer is the most diagnosed (Jemal et al., 2007). The increase in development of breast cancer therapeutics, better screening techniques, and the vast influx of knowledge on the disease has lead to a substantial decrease in breast cancer-related deaths (Lester, 2007). However, breast cancer remains uncured and the identification of novel therapeutic targets and diagnostic markers is needed.

1.2. Prolactin signalling pathway

Prolactin (PRL) is a hormone that plays an important role in regulatory the development of the breast during pregnancy and lactation, as well as in the synthesis of milk proteins. PRL activates a variety of signalling pathways downstream of its receptor, the PRL receptor (PRLR). While is has been reported that PRL can activate both the Mitogen-activated protein kinase (MAPK) pathway (Erwin et al., 1995) as well as the phosphoinositide-3-Kinase (PI3K) pathway (Bishop et al., 2006), the major pathway activated downstream of the PRLR is the Jak2/Stat5a pathway (Liu et al., 1998). Upon ligand binding, two PRLR molecules dimerize leading the cross-phosphorylation/activation of the constitutively bound Janus kinase 2 (Jak2). The activation of Jak2 results in the phosphorylation of tyrosine residues on the PRLR molecules. The tyrosine phosphorylated residues act as binding sites for the transcription Signal transducer and activator of transcription 5a (Stat5a). Upon binding to the PRLR, Stat5a is tyrosine phosphorylated by Jak2 leading to its homodimerization and translocation into the nucleus where it activates target genes such as β-casein (Liu et al., 1998) (See Figure 1.2.1). See the description below for a detailed discussion of the components of the Jak2/Stat5a pathway).

1.2.1. PRL, the ligand

PRL is a polypeptide hormone originally identified for its role in the development of mammary gland and lactation in rabbits (Stricker P and Gruter F, 1928). PRL is synthesized within the anterior pituitary gland. However, alternative promoters have been shown to drive the synthesis of PRL in non-pituitary sites (Ben-Jonathan et al., 1996). The secretion of PRL is highly regulated by the hypothalamic activity and occurs only upon the inhibition of the dopaminergic system (DeMaria et al., 1999). While PRL is primarily known for its function in mammary development and lactation, PRL has also been shown to play an important role in other biological systems such as maternal care (Lucas et al., 1998), appetite and body weight (Freemark et al., 2001), immune response (Gala, 1991), stress response (Torner et al., 2001), neuropeptide (Ma et al., 2005) and suppression of fertility (Grattan and Selmanoff, 1994).

1.2.2. Prolactin Receptor

PRL exerts its effects through the Prolactin receptor (PRLR). The PRLR is a member of cytokine I superfamily of receptors, which include the receptors for factors such as growth hormone, erythropoietin, and several interleukins. Homology between cytokine receptor type I family members are based on the following features: they are single membrane receptors that form homo or heterodimers and show high (14-44%) homology in their extracellular domain. The primary role of the extracellular domain is ligand-binding and this achieved via its 2 N-terminal cysteine residue pairs within the 2 or more fibronectin III-like modules.The intracellular domain of cytokine I receptor contain a C-terminal WSXWS or equivalent motif involved in the folding and cellular trafficking of the receptor and 2 conserved cytoplasmic regions identified as the Box1 and Box2 domains (Waters et al., 1999). Box1 is a proline rich region required for receptor-Jak family member interactions (Lebrun et al., 1995b). The Box2 region is not well conserved amongst family members and its function is not well defined.

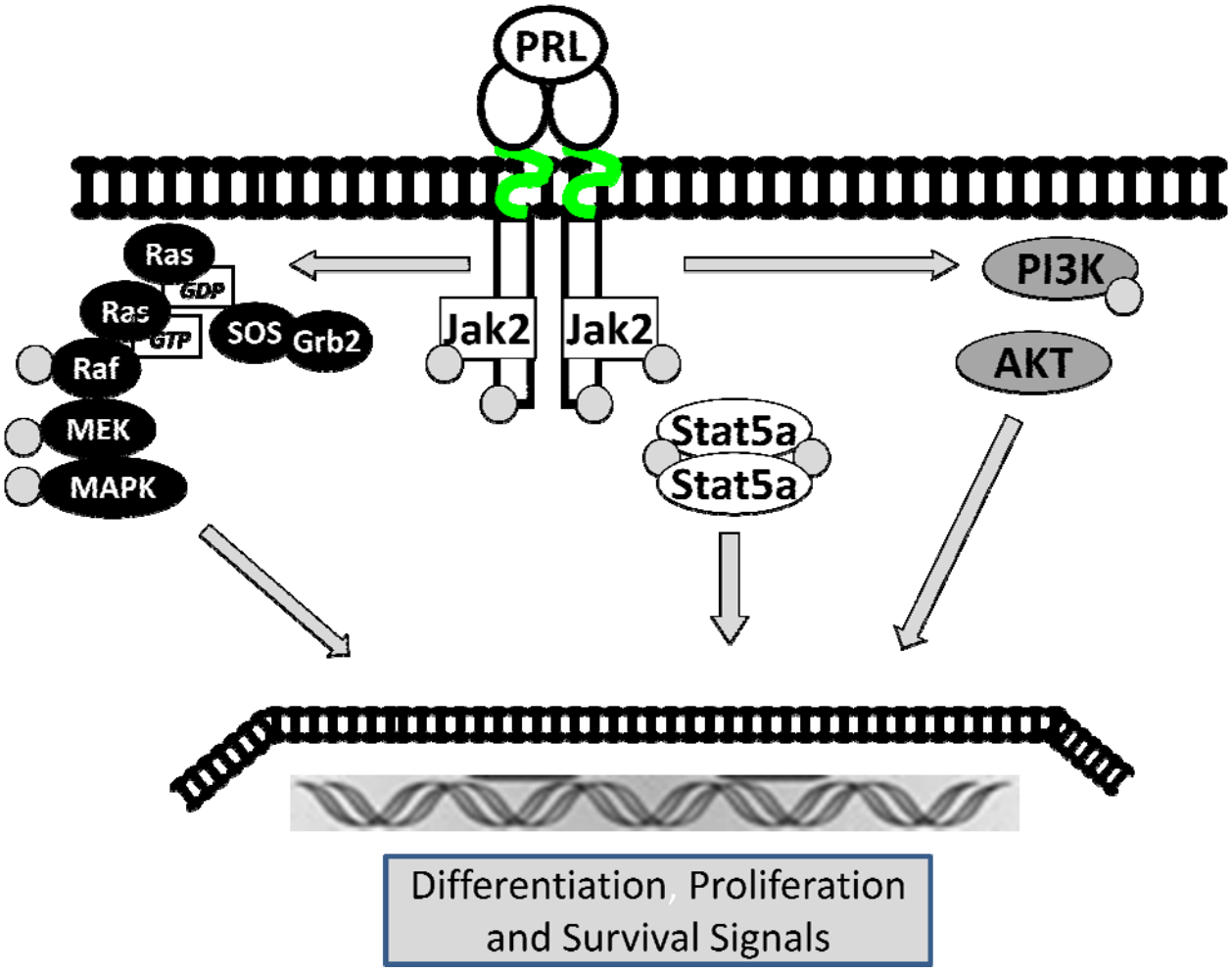

Figure 1.2.1. Signalling pathways activated downstream of the PRLR. The Jak2/Stat5a pathway is the major pathway activated downstream of the PRLR within the mammary gland (Liu et al., 1998). This pathway has been implicated in the differentiation, proliferation and survival of mammary epithelial cells (Oakes et al., 2008). While PRL has been shown to activate both the Ras/MAPK (Erwin et al., 1995) and the PI3K/AKT (Bishop et al., 2006), their role in mammary epithelial cell growth and differentiation has yet to be fully elucidated.

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The PRLR receptor exists in three forms: a short 310 amino acid form that was first cloned from rat liver (Boutin et al., 1988), a long 610 amino acid form cloned from rat ovary (Zhang et al., 1995), human hepatoma and breast cancer cells (Boutin et al., 1989) and an intermediate (Nb2) 412 amino acid form cloned from the Nb2 T lymphoma cell line (Ali et al., 1991). The Nb2 receptor contains a 198 amino acid deletion within the cytoplasmic domain of the long form. While both the long and Nb2 forms signal to the induction of milk protein expression (Ginsburg and Vonderhaar, 1995), the short from acts as a dominant negative receptor and blocks PRL-induced milk protein expression (Berlanga et al., 1997). The PRLR possesses no intrinsic kinase activity and relies on its interaction with the tyrosine kinase Jak2 to relay its signals. The long form of the receptor contains nine cytoplasmic tyrosines that mediate downstream signals (Lebrun et al., 1995a).

1.2.3. Janus Kinase 2

The non-receptor tyrosine kinase Jak family consist of four members: Jak1, Jak2, Jak3, and Tyk2 (Firmbach-Kraft et al., 1990; Harpur et al., 1992; Wilks et al., 1989). Jaks are composed of seven Jak Homology (JH) domains, numbered from the carboxyl to the amino terminus. The carboxyl JH_1 domain is the tyrosine kinase domain; the JH_2 domain contains a catalytically inactive pseudo kinase domain (Yoshikawa et al., 2001); the JH_{3-5} domains contain a domain with high homology to the Src homology 2 (SH2) domain, a highly conserved protein-protein interaction domain that binds tyrosine phosphorylated residues (Koch et al., 1991). However, it has yet to shown that this domain interacts with phosphorylated tyrosine residues. The JH_{6-7} domains contain a 300 amino acid long FERM domain which mediates the interaction between the Jaks and the Box1 region of cytokine I receptor family members (Haan et al., 2002). Even though PRLR has been shown to activate other tyrosine kinases such as Src and Tec (Clevenger and Kline, 2001), majority of its downstream signals are mediated by Jak2. Jak2 is constitutively bound through its FERM domain to the Box1 region of the PRLR. Upon ligand binding, the PRLR homodimerizes and activates Jak2 via auto- and cross-phosphorylation. Jak2 in turn then tyrosine phosphorylates the PRLR creating docking sites for the transcription factor Stat5a (Wood et al., 1995).

1.2.4. Signal Transducers and Activators of Transcription 5a

The Stat family of transcription factors consists of seven members: Stat1, 2, 3, 4, 5a, 5b, and 6. Stat family members have 6 structurally and functional conserved domains: a N-terminal domain involved in both dimerization and nuclear translocation, a coiled-coiled domain involved in protein interactions, a DNA-binding domain, a SH2 domain involved in interactions with phosphotyrosine residues and plays an important role in Stat dimerization, a linker domain that links DNA-binding domain to SH2 domain and a transactivation domain involved its transcriptional activity (Paukku and Silvennoinen, 2004). The transactivation domain is poorly conserved amongst Stat family members allowing for transcriptional specificity (Duncan et al., 1997). While PRL has been reported to activate both Stat1 (Schaber et al., 1998) and Stat3 (DaSilva et al., 1996), the majority of its signals are mediated through Stat5a. Jak2 tyrosine phosphorylates PRLR and creates docking sites for Stat5a; Stat5a is then recruited to the receptor and tyrosine phosphorylated by Jak2 on the highly conserved tyrosine residue Y694. Stat5a molecules homodimerize via their SH2 domains and translocate to the nucleus where it interacts with co-regulators, binds to the Stat5a response elements and activates its target genes (Hennighausen and Robinson, 2008; Litterst et al., 2005).

1.2.5. Tyrosine Phosphatase SHP-2

SHP-2, also commonly known as PTP1D, was the third member of the protein tyrosine phosphatase (PTP) family to be cloned, preceding SHP-1 and the drosophila PTP corkscrew. SHP-2 contains two N-terminal SH2 domains, a central phosphatase domain, and a C-terminal tail containing two tyrosine phosphorylation sites: Y546 and Y584 (Stein-Gerlach et al., 1998). Interactions with the SH2 domains of SHP-2 negatively regulate the its catalytic activity (Lechleider et al., 1993). Moreover, the cooperative binding to both SH2 domains reduces its catalytic activity to a greater extent than individual SH2 interactions (Pluskey et al., 1995). C-terminal phosphorylation of SHP-2 mediates interactions between SHP-2 and SH2 containing proteins (Stein-Gerlach et al., 1998). Furthermore, tyrosine phosphorylation regulates its phosphatase activity (Vogel et al., 1993). SHP-2 is recruited to the PRLR/Jak2 complex by interacting with the tyrosine residues within of C-terminal of the PRLR (Ali, 2000). SHP-2 promotes PRL signalling by increasing the stability of Jak2 (Ali et al., 2003) and increasing the activation and transcriptional activity of Stat5a (Chughtai et al., 2002).

1.2.6. Target Genes

The PRLR signals regulate a selection of genes. During pregnancy, PRL mediates the expression of the estrogen receptor (ER) (Frasor and Gibori, 2003). Whereas, during lactation, PRL induces the expression of milk proteins such as the caseins (Guyette et al., 1979), WAP (Hennighausen et al., 1991), and k-lactoglobulin (Burdon et al., 1994). PRL also induces the expression of the suppressors of cytokine signalling (Socs) family members. Socs family members regulate PRL signals via a negative auto-feedback loop mechanism (Larsen and Ropke, 2002). PRL up-regulates the expression of cycle cell regulators such as Cyclin D1 and the Cyclin-dependent kinases in mammary epithelial and breast cancer cells (Brockman et al., 2002).

1.3. The role of PRL in mammary gland development

1.3.1. Overview of Mammary Gland Development

The mammary gland is a complex structure that undergoes multiple structural and functional changes throughout its development. Most of the mammary gland development occurs post-natally. In mice, prior to birth, underdeveloped ductal structures (containing the terminal end buds (TEB) invade the mammary fat pad. The ductal structures remain quiescent until approximately 3 weeks of age. At this point, an increase in hormone secretion and growth factor expression promotes the ductal elongation originating from the TEB (Oakes et al., 2008). TEBs are specialized cube-like structures at the end of rudimentary ducts characterized by their cellular multi-potent potential, forming both the myoepithelium and luminal epithelium in the mature mammary gland (Williams et al., 1985). During puberty and the onset of the oestrous cycle, ductal branching and alveolar budding begin. During pregnancy, the alveolar buds proliferate and differentiate to form alveolar structures, capable of milk proteins synthesis. At parturition, the alveolar structures are fully differentiated spanning the entire fat pad and are filled with milk proteins. Offspring suckling maintains milk production (Oakes et al., 2008). At weaning, the mammary gland undergoes massive remodelling via the apoptosis of alveolar structures returning the mammary gland to its pubertal state. Similar mammary development is observed in both humans and mice, yet there are some specie specificities. While each nipple in rodents contains a single ductal tree, the

human mammary gland consists of a complex ductal network that coalesces at the nipple. Furthermore, the exposure to maternal hormones during embryogenesis can promote mild secretory activity during late-foetal stages and early childhood in humans, which is not present in rodents. Also, the human mammary is surrounded by a more pronounced dense stromal network compared to the rodent stroma (Russo et al., 1987; Sternlicht et al., 2006).

1.3.2. Pregnancy Hormones and Mammary Development

While, pregnancy hormones do not regulate the pre-pubertal mammary gland (Hens and Wysolmerski, 2005), the post-pubertal development is highly regulated by both ovarian and pituitary hormones. The interplay of hormones is important in regulating the different stages of mammary development. Growth hormone (GH) is synthesized by the pituitary gland and signals primarily through the Jak/Stat pathway. GH does not play a regulator role in the mammary epithelium. However, stromal GH mediates pubertal ductal elongation (Kleinberg, 1997). Estrogens are ovarian steroids that regulate female secondary sexual characteristics and signal through a nuclear steroid receptor. During puberty, estrogens progress the rudimentary ducts to elongated ductal structures (LaMarca and Rosen, 2007). Similar to estrogen, progesterone is an ovarian steroid and signals through the nuclear progesterone receptor. Progesterone promotes ductal out-branching and alveolargenesis during pregnancy (Anderson, 2002). Lactation is marked by a decrease in progesterone levels and an increase in lactogenic hormones such as PRL (Fernandez-Valdivia et al., 2005). Lactogenic hormones regulate alveolar differentiation and promote milk protein synthesis (Pang and Hartmann, 2007). The influence of hormones, steroids and growth factors on mammary gland development is summarized in Table 1.3.1

1.3.3. PRLR/Jak2/Stat5a pathway in Mammary Development

PRL signals through Jak2/Stat5a activation are implicated in alveolar differentiation and milk protein synthesis. The importance PRL and its downstream signalling components in mammary development are demonstrated in a variety of *in vivo* transgenic knockout models. While both terminal end bud formation and ductal elongation was unaffected in PRL-null mice, ductal out-branching and alveolar differentiation was impaired. Moreover, defects in milk synthesis were also observed (Horseman et al., 1997). No impairments in ductal

branching and alveolar bud formation were observed in PRL heterozygotes (PRL+/-) However, lactation remained impaired suggesting a regulatory role for PRL in milk synthesis (Goffin et al., 1999). Impairments in ductal branching, alveolar differentiation and lactation were also observed in PRLR-null mice (Ormandy et al., 2003).

The conventional Jak2 knockout is prenatally lethal (Neubauer et al., 1998). Therefore, role Jak2 in mammary morphogenesis is observed in conditional mammary knockout models. Jak2-null mammary glands showed defects in alveolar differentiation and lactation (Wagner et al., 2004). Stat5a null mice are viable. Similar to PRL, PRLR, and Jak2 knockout models, lactation was impaired in Stat5a null mice (Liu et al., 1997) further emphasizing the importance of the PRL-induced Jak2/Stat5a pathway in lactation. Moreover, the maintenance of ductal development and alveolar bud formation in Jak2 and Stat5a null mice suggests that PRL may utilize another pathway to promote ductal branching and alveolar end bud formation.

The tyrosine phosphatase SHP-2 positively regulates PRL-signalling. SHP-2 null mice are embryonic lethal (Saxton et al., 1997). The conditional knockdown of SHP-2 impaired alveolar buds differentiation and milk synthesis further denoting the positive regulatory role of SHP-2 in Jak2/Stat5a signalling. However, the impairment of alveolar differentiation was less than observed impairments in PRLR, Jak2 and Stat5a null mice. (Ke et al., 2006)

1.4. PRL in breast cancer development and progression

1.4.1. Epidemiology of PRL in breast cancer

Many factors are associated with the promotion and prevention of breast cancer. A decreased occurrence of breast cancer is correlated with early-age of first pregnancy. In fact, women who have undergone a first full term of pregnancy before the age of 20 have 50% less chance of being diagnosed with breast cancer. Whereas, women who have undergone their

Factor	Role in mammary development
Prolactin	Terminal end bud differentiation; milk secretion; alveolar proliferation (Oakes et al., 2008)
Growth hormone	Terminal end bud proliferation, alveolar differentiation; milk synthesis (Perry et al., 2008)
Estrogen	Terminal end bud proliferation; ductal elongation; alveolar development (Mallepell et al., 2006; Perry et al., 2008)
Progesterone	Ductal side branching; alveolargenesis (Fernandez-Valdivia et al., 2005)
Insulin growth factor I	Terminal end bud formation; ductal growth (Rowzee et al., 2008)
Insulin growth factor II	Alveolar proliferation; alveolar differentiation (Brisken et al., 2002)
Epidermal growth factor	Ductal elongation; ductal branching (Troyer and Lee, 2001)
Neuregulin	Differentiation, lactation, alveolargenesis (Troyer and Lee, 2001)
Amphiregulin	Ductal elongation; ductal branching (Troyer and Lee, 2001)
Transforming growth factor α	Ductal elongation; ductal branching (Troyer and Lee, 2001)
Heparin-binding epidermal growth factor	Differentiation, lactation, alveolargenesis (Muraoka-Cook et al., 2008; Troyer and Lee, 2001)
Glucocorticoids	Alveolar differentiation; lactation (Michael et al., 2003)

Table 1.3.1. Regulatory factors in mammary development.

first full term of pregnancy after the age of 35 have a higher risk of being diagnosed with breast cancer (MacMahon et al., 1970). Furthermore, breastfeeding is protective against breast cancer (Ursin et al., 2004). Moreover, post partition women are at higher risk of developing breast cancer (Navrozoglou et al., 2008).

Since PRL plays a pivotal role in lactation, investigators have examined the correlations PRL serum levels and PRLR expression and breast cancer incidence. The epidemiological evidence for the role of PRL in breast cancer remains controversial. There is evidence suggesting that PRL plays a breast cancer promoting role. Whereas, other studies suggest that PRL may play a protective role. There is a third set of evidence that suggests that PRL uninvolved in breast cancer. Breast cancer is heterogeneous in nature and it is hard to draw conclusion about the role of PRL in breast cancer.

1.4.2. PRL in breast tumourgenesis

A vast source of evidence suggests an oncogenic role for PRL. Mammary pathogenesis is associated with the autocrine/paracrine secretion of PRL by the mammary epithelium (Welsch et al., 1970). Moreover, breast cancers cells utilize this autocrine/paracrine mechanism to promote cellular proliferation and tumourgenesis (Clevenger et al., 2003). Transgenic overexpression of PRL ligand in mice induces mammary adenocarcinomas (Rose-Hellekant et al., 2003). Moreover, PRL synergized with transforming growth factor alpha (TGFα), a member of the epidermal growth factor family of growth factors implicated in mammary tumourgenesis, to promote MAPK activation and cell proliferation in breast cancer cells (Arendt and Schuler, 2008). Furthermore, deletion of PRL delayed the onset of polyoma middle T antigen, a protein encoded by the polyomavirus genome known to induce cellular transformation (Griffin et al., 1980) , induced mammary tumourgenesis (Vomachka et al., 2000). Similarly, PRLR deletion delayed Simian Vacuolating Virus 40 Large-T-Antigen (SV40) (Oakes et al., 2007), a transforming factor derived from the polyomavirus SV40, induced mammary tumourgenesis (Griffin et al., 1980). PRL also promotes the cell proliferation and cell survival of breast cancer cells (Perks et al., 2004). Moreover, PRL induced the expression of Cyclin D1, a promoter of cell cycle and breast cancer (Brockman et al., 2002).

1.4.3. PRL a suppressor of breast cancer progression

While PRL as a promoter of breast carcinogenesis remains the stronghold in the literature, recent evidence suggests a role for PRL in the suppression of breast cancer progression. Overexpression of Stat5a in invasive breast cancer cells promoted cellular adhesion and blocked invasive characteristics (Sultan et al., 2005). Moreover, Stat5a expression is correlated with good prognosis (Nevalainen et al., 2004) as well as increased survival rate and response to endocrine therapy in breast cancer patients (Yamashita et al., 2006). Furthermore, while PRL increased the occurrence of mammary tumours in transgenic mice, the tumours were highly differentiated and expressed the estrogen receptor (Rose-Hellekant et al., 2003) suggesting that PRL may prevent the formation of undifferentiated, invasive tumours. Moreover, the overexpression of PRL in differentiated mammary glands promoted hyperplasia, benign growths, however, no carcinomas were detected (Manhes et al., 2006) suggesting that tumourgenic properties of PRL is highly dependent on differentiation state of the mammary tissue.

The overexpression of either PRLR/Jak2 or Jak2/Stat5a in invasive breast cancer cells reversed the process of epithelial-mesenchymal transformation (EMT) (Nouhi et al., 2006; Sultan et al., 2008). EMT is a process by which a differentiated epithelial cell loses its epithelial characteristics and transforms into an invasive undifferentiated mesenchymal cell. EMT is required for tumour cells to migrate and metastasize to secondary sites (Moustakas and Heldin, 2007). PRL blocked EMT by inhibiting both the MAPK and transforming growth factor beta (TGFβ) pathways (Nouhi et al., 2006), two pathways involved in the promotion of EMT (Janda et al., 2002; Pardali and Moustakas, 2007). Moreover, inhibition of Jak2 kinase activity in epithelial breast cancer cells induced EMT (Nouhi et al., 2006) emphasizing the importance of the PRLR/Jak2 pathway in the suppression of breast cancer progression.

1.5. Role of EGF in mammary morphogenesis

Growth factors are molecules within the body that promote the growth of their target tissues. The stereotypical example of a growth factor are the epidermal growth factors (Raven PH and Johnson GB, 2002). These factors regulate mammary morphogenesis and carcinogenesis (Brisken et al., 2002; Kim and Muller, 1999; Rowzee et al., 2008; Troyer and Lee, 2001). Characterizing negative regulatory mechanisms of growth factor induced mammary carcinogenesis can identify new highly specific therapeutic targets, as well as improve present breast cancer therapies. The present study characterizes a novel, negative signalling mechanism downstream of the PRLR in mammary epithelial and breast cancer cells. Using EGF, as the prototypical growth factor (Riese and Stern, 1998), we demonstrate that PRL inhibits growth factor-mediated MAPK activation and cell proliferation. A discussion of the EGF-Ras/MAPK pathway as well as an illustration of EGF-mediated signalling pathways (Figure 2.1.1) is depicted below.

1.5.1. EGF ligands and receptor family

The epidermal growth factor receptor (EGFR) family are members of the superfamily of receptor tyrosine kinases (RTKs). The family consists of four receptors: ErbB1 (EGFR, Her1), ErbB2 (Her2), ErbB3 (Her3), and ErbB4 (Her4). Several peptide growth factors act as ligands for these receptors. Ligands include: epidermal growth factor (EGF), transforming growth factor-alpha (TGFalpha), heparin-binding EGF-like growth factor (HB-EGF), amphiregulin (AR), heregulin (HRG), and four neuregulins (NRG) (Dreux et al., 2006). The extracellular domain of EGFR family is required for ligand binding and is divided into 4 domains: I, II, III, and IV. Domains I and III play a role in ligand specificity, while domain II and IV (cysteine rich regions) play a role in target the receptor to the membrane (Jorissen et al., 2003; Lax et al., 1989). The trans-membrane domain plays an anchorage role for the receptor and has no influence on ligand-induced signalling. The

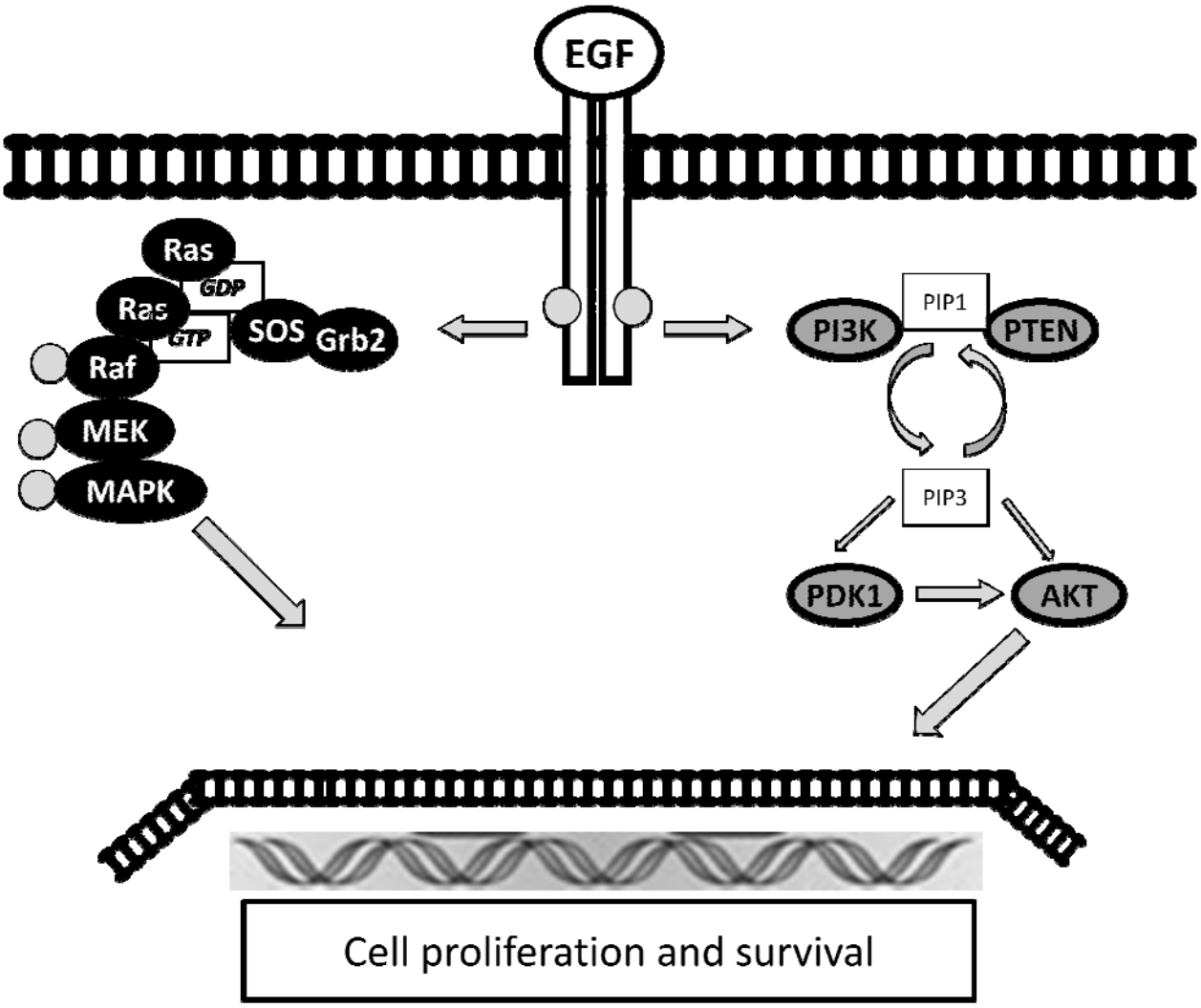

Figure 1.5.1. Signalling pathways activated downstream of the EGFR. The two major pathways activated downstream of the EGFR are the Ras/MAPK and PI3K/AKT pathways (Grant, 2008; Troyer and Lee, 2001). These pathways regulate the growth and survival of mammary epithelial cells (Grant, 2008) (Ferrer-Soler et al., 2007). Moreover, over-activation of these two pathways often results in breast carcinogenesis (Ferrer-Soler et al., 2007) (Kim and Muller, 1999).

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juxtamembrane domain is highly conserved amongst EGFR family members and regulates receptor internalization and down-regulation (Jorissen et al., 2003; Wells, 1999). The tyrosine kinase domain is located in its cytoplasmic domain and is responsible for receptor signalling. Furthermore, tyrosine phosphorylation of cytoplasmic-tail residues creates docking sites for downstream signalling modulators (Yarden, 2001).

1.5.2. Ras-MAPK Pathway

Upon ligand binding, the kinase domain of the EGFR family is activated by both homo and heterodimerization. The EGFR family primarily activate two downstream pathways the PI3K and the MAPK pathway (Zahnow, 2006). Moreover, EGF signalling is activated by cytokine-induced Jak/Stat pathway (Yamauchi et al., 1997) and via the G-protein coupled receptor transactivation of the EGFRs (Daub et al., 1996). Activation of these pathways is involved in multiple cellular responses such as proliferation, apoptosis, differentiation and migration. Activation of the receptor creates docking sites for SH2 containing proteins such as growth-factor receptor bound protein (Grb) 2. Grb2 recruits the guanine exchange factor (GEF) Sos which activates Ras. Ras activates a signal cascade leading to the activation of the MAPKs Erk1/Erk2.

1.5.2.1. Sos

Son of Sevenless (Sos) is a GEF involved in the activation of the small GTPase Ras downstream of growth factor receptors (Quilliam et al., 2002). Sos contains conserved functional domains such as the pleckstrin homology (PH) domain involved in the recruitment Sos to the plasma membrane, the Dbl homology (DH) domain involved in catalytic activity, the CDC25 domain involved Ras displacement and GDP release, and a Ras exchange motif (REM) involved in the GTP loading of Ras (Quilliam et al., 2002). The interaction between Grb2 and Sos involves both the N and C-terminal SH3 domains of Grb2. However, it was shown that the N-terminal SH3 domain contributes more to Sos binding than its C-terminal counterpart. The SH3 domains of Grb2 have been shown to interact with the proline rich region of SOS (Buday and Downward, 1993).

1.5.2.2. Grb2

Grb2 is an adaptor protein involved in signalling events downstream of activated growth factor receptors. Grb2 contains a SH2 domain that interacts with phosphotyrosine residues containing proteins such as the activated form of growth factor receptors, and a c-terminal and n-terminal SH3 domain that interacts with proline-rich regions such as that of the guanine exchange factor Sos (Lowenstein et al., 1992). In the presence of either excess Ras or growth factor receptors, Grb2 has been shown to promote cell proliferation and tumourgenesis (Tari and Lopez-Berestein, 2001).

1.5.2.3. Ras

Ras is a member of the small GTPase superfamily known to contain a conserved G box GDP/GTP binding motif (Bourne et al., 1991). There are three human Ras molecules: HRas, NRas, and KRas which act as GDP/GTP molecular switches regulating a variety of signalling mechanism involved in cell proliferation, survival and differentiation (Mitin et al., 2005; Wennerberg et al., 2005). However, Ras is primarily characterized for their role in oncogenesis (Repasky et al., 2004). Ras activity is highly dependent on GTP and regulated by GEFs and GTPase activating proteins GAPs. Activated Ras binds the serine/threonine kinase Raf-1 and promotes Raf-1 translocation to the plasma membrane where further phosphorylation events lead to its activation (Mitin et al., 2005).

1.5.2.4. Raf

Rapidly accelerated fibrosarcoma (Raf) is a serine/threonine kinase involved in the Ras-MAPK signal cascade leading to cell proliferation and survival. Similar to Ras, Raf is implicated in cancer. The highly transforming viral RAF, v-Raf, containing a constitutively active kinase domain, induces tumourgenesis (Leicht et al., 2007). Moreover, C-Raf alone or in combination with v-Raf promotes tumour formation (Leicht et al., 2007). There are three mammalian Raf isoforms: A-, B-, and C-Raf. Raf members contain three conserved regions: the CR1 domain consisting of the Ras-binding domain and a zing-finger/cysteine-rich region, the CR2 domain containing a conserved phosphorylation site involved in the regulation of its kinase activity and CR3 domain consisting of the kinase domain (Leicht et al., 2007). Mek kinases are the only characterized targets of Raf (Howe et al., 1992). However, recent evidence suggests Mek-independent Raf signalling (Baccarini, 2005).

1.5.2.5. Mek Kinase Family

The Mek kinases in response to mitogenic signals and environmental stress regulate multiple cellular responses such as cell cycle progression and apoptosis (Bodart et al., 2002). Over-activation of Mek kinases is associated with tumourgenesis (Mansour et al., 1994). Mek kinase family is composed of 7 members: Mek 1 through 7; all of which are known to activate members of the MAPK family. Mek 1 and 2 activate the MAPKs Erk1 and Erk2; while Meks 3, 4 and 6 regulate p38 MAPK (Derijard et al., 1995; Raingeaud et al., 1996). Mek 7 regulates stress activated protein/ Jun kinase (Derijard et al., 1995) whereas Mek 5 activates Erk5 (Zhou et al., 1995).

1.1.5.6. MAPK Family

The MAPK family is serine/threonine kinases involved in the modulation of transcription downstream of growth factor signalling pathways. There are 11 known human MAPK family members divided into 6 groups based on sequence similarity. These include: the extracellular signal-regulated protein kinases (Erk1 and Erk2); the c-Jun N-terminal kinases (JNK1, JNK2 and JNK3); the p38 MAPKs (p38a, p38b, p38c and p38d); the Erk3s (Erk3, p97 MAPK and Erk4) and the Erk7s (Erk7 and Erk8) (Pearson et al., 2001; Schaeffer and Weber, 1999). The activation of MAPKs depends on the dual phosphorylation of threonine and tyrosine residues within its activation loop (Canagarajah et al., 1997; Pearson et al., 2001). Moreover, dephosphorylation of these residues by specific phosphatases terminates its kinase activity (Zhou et al., 2002). Upon its activation, MAPKs phosphorylate cytoplasmic and nuclear substrates such as RSK and MSK which in turn regulate gene expression (Cheung et al., 2000; Vicent et al., 2006).

1.1.5.7. RSK

RSKs are a family of serine/threonine kinases activated specifically by Erk MAPKs (Nebreda and Gavin, 1999). RSK interacts with a variety of transcription factors and thus connects the growth factor/MAPK signals to transcription and gene expression. RSK contains two linked kinase domains. The N-terminal domain is essential for the phosphorylation of its nuclear and cytoplasmic substrates such as the transcription factors CREB and c-fos (Poteet-

Smith et al., 1999). While phosphorylation of RSK by the MAPKs is essential for its activation, complete activation of RSK is dependent on the N-terminal phosphorylation by PDK1 suggesting that RSK may act by integrating MAPK and PDK signals (Sherwood et al., 1999). RSK is implicated in tumourgenesis based on its promotion of cell proliferation as well as cell survival (Bonni et al., 1999; Nebreda and Gavin, 1999).

1.5.3. EGF in mammary development

The four EGF receptors and their multiple ligands are expressed differentially throughout the development of the mammary. While EGFR and ErbB2 receptors are involved in ductal morphogenesis, the NRG receptors (ErbB3 and ErbB4) regulate alveolargenesis (Troyer and Lee, 2001). The role of EGFR family members and their ligands in mammary gland development has been characterized in multiple mice knockout models. Defects in ductal morphogenesis were observed in mice lacking EGFR or ErbB2 as well as the ligands EGF, TGFα and AR (Jones et al., 1996; Sebastian et al., 1998) In contrast, defects in alveolargenesis and milk protein synthesis were observed in mice lacking ErbB3 or ErbB4 as well as their ligand NRG (Darcy et al., 2000; Sebastian et al., 1998) While the role of each receptor in mammary gland is dependent on the stage of development, recent evidence suggests a more complex role for the EGFRs. The EGFR and ErbB2 have been recently shown to play an influential in alveolar proliferation as well as ductal morphogenesis. However, the exact function of these receptors in alveolar proliferation has yet to be fully elucidated (Troyer and Lee, 2001).

1.5.4. EGF in breast tumourgenesis

The over-activation of the EGFR receptor family is strongly associated with increased risk and progression of human cancers (Kim and Muller, 1999). The overexpression of both the EGFR and ErbB2 has been observed in a significant portion of breast cancer patients. (Kim and Muller, 1999; Lacroix et al., 1989; Slamon et al., 1989). Moreover the overexpression of ErbB2 is correlated with a decreased survival rate (Ross and Fletcher, 1999). Furthermore, overexpression of EGFRs ligands is associated with an increased risk of breast tumourgenesis (Normanno et al., 1996). Transgenic mouse models have also demonstrated a key role for EGFRs signalling in mammary carcinogenesis. The

overexpression of EGFR ligands (Kim and Muller, 1999) or constitutively active forms of the receptors (Kim and Muller, 1999) within the mammary gland promoted tumourgenesis. The knockdown of the EGFR, (Sibilia and Wagner, 1995) as well as the overexpression of a kinase dead mutant receptor (Sibilia et al., 2003) suppressed tumourgenesis. Moreover, inhibition of EGF signalling with specific monoclonal antibodies reduced tumour growth as well as block cancer progression (Burgess, 2008).

Chapter 2: Results

2.1. Rationale and Hypothesis

The Ras-MAPK pathway is an important signal transduction cascade that regulates cell growth, differentiation and carcinogenesis. Multiple kinases, phosphatases, adaptor and scaffold proteins regulate Erk1/Erk2 activation (Morrison and Davis, 2003; Sebolt-Leopold and Herrera, 2004). The adaptor protein growth factor bound protein-2 (Grb2) is characterized for its contribution to Ras activation downstream of growth factor receptors (Downward, 1994). The Grb2 SH3 domains interact with proline rich regions of the Ras exchange factor – Sos. This interaction induces GTP loading and Ras activation (Downward, 1994), Activated Ras then interact with the N-terminal domain of the kinase Raf leading to the activation of the MAPK (Erk1/Erk2) cascade (Mitin et al., 2005). While Grb2 association with growth factor receptors is mediated through phosphotyrosine/SH2 domain interactions it is generally believed that Grb2 itself does not undergo tyrosine phosphorylation in response to growth factor stimulation such as EGF and PDGF (Lowenstein et al., 1992; Rozakis-Adcock et al., 1992). However, Grb2 tyrosine phosphorylation was recently observed Bcr/Abl transformed cells and was characterized as a negative feedback mechanism of MAPK activation by interfering with Grb2/Sos interaction (Li et al., 2001). However, the biological context of Grb2 tyrosine phosphorylation in signal regulation of growth factor induced- MAPK activation and cell proliferation has yet to be defined.

Recently our laboratory demonstrated that PRL suppresses EGF-induced MAPK in breast cancer cells and thus blocks the progression of breast cancer by blocking EMT (Nouhi et al., 2006). Moreover, we identified a PRL-induced tyrosine phosphorylated protein in immunoprecipitates of Grb2 (Minoo et al., 2003) that we further characterized to be a tyrosine phosphorylated form of Grb2. Therefore, we were interested in defining the mechanism through which PRL blocks EGF signaling in mammary epithelial and breast cancer cells and how this inhibition of EGF-induced MAPK activation correlates with mammary epithelial cell proliferation. Since we have previously demonstrated that PRL can induce the tyrosine phosphorylation of the adaptor protein Grb2 and the tyrosine phosphorylation of Grb2 negatively regulates the activation of the MAPK pathway (Li et al., 2001), we proposed that

PRL utilizes the tyrosine phosphorylation Grb2 to blocked EGF-induced MAPK activation and thus cell proliferation.

2.2. Materials and Methods

2.2.1. Reagents and DNA constructs

Reagents used in this study were: ovine PRL (Sigma-Aldrich), human PRL provided by Dr. Vincent Goffin (Institut National de la Sante et de la Recherche Medicale, Paris France), mouse EGF (Sigma-Aldrich), human EGF (Sigma-Aldrich) and the monoclonal antibodies were obtained from Upstate: phospho-tyrosine (4G10), BD Transduction Laboratories: Stat5, Ras, Sos1, SHP-2, Santa Cruz: Myc, GST, Sigma-Aldrich: b-tubulin and Zymed Laboratories Invitrogen:. phospho-Stat5a (Y694). Polyclonal antibodies were obtained from Upstate: Jak2, Myc, BD Transduction Laboratories: phospho-tyrosine, Santa Cruz: Grb2, RSK-1, Phospho-RSK1/2 (T359/S363), ACK-1, Cell Signaling: Phospho-p44/42 (Erk1/Erk2), p44/42 (Erk1/Erk2). Other reagents used: goat anti-mouse HRP and goat anti-rabbit HRP (Santa Cruz); Jak2 inhibitor II (Calbiochem), G418 (Sigma-Aldrich), super signal kit (Pierce).

DNA constructs used consisted of expression plasmids encoding the long form of the PRLR and Jak2 previously characterized (Lebrun et al., 1995a). Briefly, upon PRL stimulation the tyrosine phosphorylation of both PRLR and Jak2 was observed when over expressed in 293 cells at concentrations lower than 2.5 μg. Non-specific, ligand independent phosphorylation of PRLR and Jak2 occurred at higher concentrations (Lebrun et al., 1995a). The Myc-Grb2WT, the human wild type Grb2 tagged with 3 tandem myc-tags and the Myc-Grb2YF, the human wild type Grb2 tagged with 3 tandem myc-tags in with its 4 tyrosine residues (Y7, Y37, Y52, Y209) mutated to phenylalanine, blocking tyrosine phosphorylation, plasmids were kindly provided by Dr. R. Van Etten, (Tufts-New England Medical Center, Boston MA) and Dr. S. Li (The Jackson Laboratory, Bar Harbor, ME). The expression plasmid encoding catalytically inactive form of SHP-2, SHP-2CA was previously characterized (Minoo et al., 2003).

2.2.2. Cell culture

HC11 and HC11-Lux cells (transfected stably with the β-casein gene promoter luciferase reporter construct) were obtained from Dr. N. Hynes (Friedrich Miescher Institute, Basel, Switzerland) and Dr. B. Groner (Institute for Biomedical Research, Frankfurt am Main, Germany). They were grown to confluency in RPMI 1640 (Wisent) supplemented with 10% foetal bovine serum (FBS), mouse EGF (10 ng/mL) and bovine insulin (5 μg/mL); cells were then differentiated 3 days in RPMI 1640 media supplemented with 10% FBS, insulin (5 μg/ml) and hydrocortisone (1 μM) and starved overnight in RPMI 1640 media supplemented with fetuin (0.5 μg/ml), transferin (10 μg/ml), insulin (5 μg/ml), and hydrocortisone (1 μM) . HC11-vector and HC11-GrbYF clones were cultured in the same media described above for parental HC11 cells supplemented with the antibiotic G418 (250 mM). NMuMG cells were obtained from Dr. Peter Siegel (McGill University, Montreal, Quebec, Canada) and grown in Dulbecco's modified Eagle's Medium (DMEM) (Wisent) supplemented with 10% FBS and insulin (5 μg/ml) and starved in DMEM/F12 (Invitrogen). The human embryonic kidney 293 cells were cultured in DMEM (Wisent) supplemented with 10% FBS and starved in DMEM/Hams F12 (Wisent). MCF7 and T47D cells were cultured in DMEM supplemented with 10% FBS and starved in DMEM. 4T1 cells were obtained from Dr. Fred Miller (Michigan Cancer Foundation and were cultured in DMEM supplemented with 10% FBS and starved in DMEM.

2.2.3. Cell lysis and western analysis

Following ligand stimulation, cells were washed in phosphate buffered saline (PBS) on ice to stop the stimulation period Cells were then lysed with a lysis buffer solution (10 mM Tris-HCl pH 7.5, 5 mM EDTA pH 8.0, 150 mM NaCl, 30 mM sodium pyrophosphate, 30 mM sodium fluoride, 1 mM activated sodium orthovanadate, 2 mM leupeptin, 5 mM aprotinin, 0.4 mM Pefabloc and 0.5 % Triton X-100) for 2 minutes on ice. Lysed cells were then collected and cellular debris was pelleted for 10 minutes at 4 ^{o}C and 13,000 rpm. Bradford assay was used to quantify protein levels of cell lysates. Equal proteins were then denatured with a loading buffer (5 mL of 1.0 M Tris-HCl pH 6.8, 5 mL 20% SDS, 0.1 M DTT in 10 mL of 50% glycerol) at 100 ^{o}C for 10 minutes. Denatured proteins were then separated on an accrylamide SDS-Page gel. Separated proteins were then transferred to nitrocellulose membranes and processed by western analysis.

Nitrocellulose membranes were blocked overnight at 4 ^{o}C in a blocking buffer solution of either 0.25% gelatin or 5% milk in TBST (500 mM Tris-HCl pH 7.6, 2 M NaCl and 0.5% Tween-20). Membranes were then equilibrated to room temperature and incubated in primary antibody solution at concentrations of 1:1000 to 1:10000 in 0.25% gelatin solution for 1 hour. Membranes were then washed in TBST twice for 20 minutes. Membranes were then incubated in a secondary antibody (HRP-tagged) solution at a concentration of 1:10000 in 0.25% gelatin solution for 1 hour. Membranes were then washed 4 times in TBST solution for 20 minutes. ECL luminescence substrate was then added to the membrane for 1 minute and luminescence was then illustrated on film.

2.2.4. Immunoprecipitations

Cells were lysed as described above. Protein-A-Sepharose (PAS) beads (10 μL) and antibody (1 μL) are added to cell lysates (800 μL) and lysates were incubated at 4 ^{o}C on a rotor for 3 hours. To reduce non-specific binding and background signal, PAS beads were then washed 3 times in a HNTG solution (20 mM Hepes pH 7.5, 150 mM NaCl, 0.1% Triton X-100, and 10% glycerol). Next, Proteins were isolated from the PAS beads via denaturation as described above and separated on an accrylamide SDS-PAGE gel. Separated proteins were then transferred to nitrocellulose membranes and processed by western analysis as described above.

2.2.5. Transient transfections and Grb2YF stable cell line

For 293 cells transient transfections, approximately 5 x 10^5 cells were plated and co-transfected with indicated cDNA plasmids using the calcium phosphate method. Briefly, 1 μg of expression plasmids were precipitated in 225 μL water, 25 μL of 2M $CaCl_2$ and 250 μL of 2X BBS pH 6.96 (50 mM BES, 280 mM NaCl and 1.5 mM Na_2HPO_4) and added to cells for an overnight transfection period. The next day cells were starved overnight left unstimulated or stimulated with PRL (1 μg/mL) for indicated time points. For the Transient transfection HC11 cells, cells were grown to confluency and transfected with indicated expression plasmids using a Lipofectamine tranfection kit (Gibco-BRL) according to manufacturer's protocol. For the development of the myc-Grb2YF stable clones, HC11 cells were grown to 75% confluency and transfected with 8 μg of either vector for HC11-vector clones or Grb2YF

for HC11-Grb2YF clones using a Lipofectamine 2000 transfection kit (Gibco-BRL) according to manufacturer's protocol. Cells were then grown overnight to confluency. Stable expressing myc-Grb2YF clones were then selected with the antibiotic G418 (250 mM) (Sigma-Aldrich). Western analysis confirmed stable expression of myc-Grb2YF.

2.2.6. Ras pulldown assay

The Ras binding domain of human c-raf-1 (amino acids 1 to 149) was expressed as a GST-fusion protein in bacteria (construct provided by Dr. James Woodgett, Samuel Lunenfeld Research Institute, Toronto) and then bound to glutathione-sepharose beads (40 μg proteins for each 15 ul of packed beads). Starved differentiated HC11 cells were lysed in RIPA buffer (50 mM Tris-HCl, pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 μg/ml aprotonin, 1 μg/ml leupeptin, 1 μg/ml pepstain, 1 mM sodium orthovanadate, 1 mM NaF). Cell lysates were then incubated with GST-fusion protein coupled beads at 4 °C for 30 minutes. Beads were collected, washed 3 times with HNTG buffer and resuspended in loading buffer and processed for western blotting as described above.

2.2.7. Luciferase reporter assay

HC11-Lux cells were grown as described above and then starved overnight. Starved cells were then either left untreated or treated either with PRL (1 μg/ml) or PRL (1 μg/ml) and EGF (10 ng/ml) for 16 hours. Cells were then lysed in a lysis buffer solution (15 mM $MgSO_4$, 4 mM EGTA, 1 mM DTT and 1% Triton X-100). Luciferin substrate (25 mM luciferin in 6 mL of 0.1 M KH_2PO_4 pH 7.8) and an ATP solution (1 mM ATP, 15 mM KH_2PO_4 pH 7.8 and 15 mM $MgCl_2$) were then added to cell lysates and samples were processed for luciferase activity using a luminometer (FLUOstar OPTIMA). Luminescence values were equalized to cell lysate protein content.

2.2.8. MTT and cell counting assays

HC11 cells were plated in a 96-well plate at a concentration of $1.5x10^3$ cells and grown overnight in the absence or presence of PRL (1 μg/mL) in RPMI 1640 media supplemented with 2% FBS, insulin (5 μg/mL) and hydrocortisone (1 μM). Cells were then left untreated or treated with EGF (10 ng/mL) for 72 hours. Following the 72 hour treatment period, MTT

substrate (tiazolyl blue tetrazolium bromide) (5 μg/mL) was added to the cells for 2 hours. The MTT reaction was stopped by adding a lysis solution (20% SDS in 50% Dimethyl-formamide solution pH 4.7) for an overnight period and absorbance at 560 nm using a plate reader. Statistical significance was assessed through one-way ANOVA comparing absorbance across treatments.

For the NMuMG experiment, $2.5x10^3$ cells were grown overnight in the absence or presence of PRL (1 μg/mL) in DMEM media supplemented with 2% FBS, insulin (5 μg/mL) and hydrocortisone (1 μM). Cells were then left untreated or treated with EGF (10 ng/mL) for 72 hours. MTT assay was performed and statistical significance was assessed through one-way ANOVA as described above.

For 4T1 experiments, $5x10^3$ cells were grown overnight in DMEM supplemented with 2% FBS Cells were then left untreated or treated with EGF (10 ng/mL), PRL (1 μg/mL) or PRL (1 μg/mL) and EGF (10 ng/mL) for 48 hours. MTT assay was preformed and statistical significance was assessed through one-way ANOVA as described above.

For T47D and MCF7 cells experiments, $5x10^3$ cells were grown in the absence or presence of human PRL (100 ng/mL or 1000 ng/mL as indicated) in DMEM supplemented with 2% FBS for 48 hours. MTT assay was then preformed and statistical significance was assessed through one-way ANOVA as described above.

For stable HC11-Grb2YF experiments, $1.5x10^3$ HC11-vector or HC11-Grb2YF cells were grown overnight in the absence or presence of PRL (1 μg/mL) in RPMI 1640 media supplemented with 2% FBS, insulin (5 μg/mL) and hydrocortisone (1 μM). Cells were then left untreated or treated with EGF (10 ng/mL) for 72 hours. MTT assay was then preformed and statistical significance was assessed through one-way ANOVA as described above.

In Jak2 inhibitor experiments, HC11 cells ($2.5x10^3$) were pre-treated either with DMSO or inhibitor (1 μM) and grown overnight in the absence or presence of PRL (1 μg/mL) in RPMI 1640 media supplemented with 2% FBS, insulin (5 μg/mL) and hydrocortisone (1 μM). Cells were then left untreated or treated with EGF (10 ng/mL). MTT assay was then preformed and statistical significance was assessed through one-way ANOVA as described above.

For cell counting assays, HC11, HC11-vector and HC11-Grb2YF ($2.5x10^4$ cells) were grown overnight in the absence or presence of PRL (1 μg/ml) in RPMI 1640 supplemented

with 2% FBS, insulin (5 μg/ml) and hydrocortisone (1 μM). Cells were then left untreated or treated with EGF (10 ng/mL) for 24 hours and 48 hours. Cell number was determined following trypan blue stain to exclude dead cells. Results are presented as mean ± standard deviation of triplicates of three separate experiments. Statistical significance was assessed through one-way ANOVA.

2.2.9. RNA isolation and quantitative real-time PCR

Differentiated HC11 cells were left untreated or treated with EGF, (10 ng/mL), PRL (1 μg/ml) or PRL (1 μg/ml) and EGF (10 ng/mL) for 16 hours. Cells were then lysed in 500 μl of TRIZOL and purified; total RNA was isolated as described by manufacturers (Invitrogen Life Technologies, Burlington, Ont). Samples were quantified by absorbance at 260 nm. Aliquots of (300-400ng) of total RNA were used for reverse transcription and PCR amplification in one step using Brilliant II SYBR Green QRT-PCR Master Mix Kit 1-Step (Stratagene Amsterdam, Zuidoost, Netherlands) according to the manufacturer's recommendation. The Q-PCR amplification was done under the following conditions: 50 ºC, 30 minutes; 95 ºC, 10 minutes, followed by 40 cycles at 95 ºC, 30 seconds; 55 ºC, 1 minute; 72 ºC, 30 seconds and then the specificity of the primers is tested by dissociation program 95 ºC, 1 minute, ramp down to 55 ºC then ramp up to 95 ºC (at the instrument default rate of 0.2 ºC/second). Results were normalized to GAPDH. The primer sequences of GAPDH and Beta Casein are described in Table 2.2.1

2.2.10. Nuclear Extracts and electromobility shift assay (EMSA)

Nuclear extracts were prepared as previously described (Dignam et al., 1983). Briefly, cells were lysed with a hypotonic buffer (10 mM HEPES-KOH, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol, 1 mM Na_3VO_4, 20 mM NaF, 4 mM pefabloc, 5 μg/mL aprotonin, and 2 μg/mL leupeptin) and 3 freeze-thaw cycles of 15 minutes each. Cells were pelleted at 13,000 rpm for 10 minutes at 4 °C, and supernatant (cytoplasmic fraction) was discarded. The pellet was then washed 3 times with PBS and lysed with a high salt buffer (20 mM HEPES-KOH, pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 1 mM Na_3VO_4, 20 mM NaF, 4 mM pefabloc, 5 μg/mL aprotonin, and 2 μg/mL leupeptin). Nuclear extracts were processed for EMSA using the Stat5a binding site from bovine β-casein promoter (5'-

AGATTTCTAGGAATTCAATCC-3') as described previously. (Cocolakis et al., 2008). The EMSA gels were dried and revealed using a phosphoimager Cyclone Storage Phosphoscreen (Packard Canberra).

Primer name	Sequence
GAPDH-AS	CTCAGTGTAGCCCAGGATGC
GAPDH-Sense	ACCACCATGGAGAAGGCTGG
Beta Casein- Forward	CTATTGCTCAACCCCCTGTG
Beta Casein- Reverse	AGAGTTTATGAGGCGGAGCA

Table 2.2.1. Primers used in quantitative real-time PCR experiment

2.3 Results and discussion

2.3.1. PRL regulation of EGF-mediated cell growth

Since both PRL and EGF are important regulators of mammary epithelial cell growth and differentiation, as well as mammary carcinogenesis, it is important to better understand the co-regulation of signalling events downstream of these two ligands within the mammary epithelia and in breast cancer. While EGF inhibits lactogenic hormone-induced milk protein expression (Marte et al., 1995), PRL blocks EGF-mediated DNA synthesis and MAPK activation (Fenton and Sheffield, 1993, 1997). However, the mechanism of crosstalk between these two factors has yet to be fully elucidated. To investigate the underlying mechanism of cross-regulation within the mammary epithelia we utilized the HC11 cells, a cell line originally isolated from the mammary gland of mid-pregnant mice (Danielson et al., 1984), and NMuMG cells, a cell line isolated from a lactating mouse mammary gland (Sizemore and Cole, 1982). Moreover, we characterized the role of the two ligands in mammary carcinogenesis utilizing the mouse breast carcinoma cell line 4T1 (Aslakson 1992) and the PRL responsive human breast carcinoma MCF7 and T47D cell lines(Keydar et al., 1979; Levenson and Jordan, 1997)

Since EGF regulates mammary epithelial cell proliferation (Troyer and Lee, 2001) and the role of PRL in cellular proliferation remains controversial, we first examined the role of each ligand and the combination of both ligands in regulating mammary epithelial cell growth. HC11 cells were plated in media supplemented with 2% FBS, insulin and hydrocortisone in the absence (HI) or presence of PRL (HIP) for an overnight period before being left untreated or treated with EGF for 72 hours (Figure 2.3.1.1). The regulation cell growth by the two ligands was then determined by MTT assay, an assay commonly used to examine cell growth and survival by staining the mitochondria of cells (Mosmann, 1983). As expected, EGF (HIE) significantly induced the growth of HC11 cells (p=0.043). PRL-treated samples (HIP) were not significantly different to control cells suggesting that PRL does not promote mammary cell growth. More interestingly, in samples co-treated with PRL and EGF (HIPE) did not significantly induce the proliferation of HC11 cells compared to EGF-treated samples suggesting that PRL is a negative regulator EGF signals to cellular proliferation.

Next, we confirmed the negative regulatory role of PRL on EGF-induced mammary epithelial cell proliferation; we examined the influence of the ligands on cell proliferation of HC11 cells by cell counting assay (Figure 2.3.1.2). Cells were plated as described above for MTT assays and then left untreated or treated with EGF for 24 and 48 hours as indicated. Trypan blue stain was used to exclude dead cells from the analysis. EGF significantly induced cell proliferation at both 24 (p=0.07*)* and 48 hours (p=0.049). Interestingly, PRL slightly suppress the natural growth of mammary epithelial cells, more evident at 48 hours supporting an anti-proliferative role for PRL in mammary morphogenesis. More importantly, no significant growth was observed in samples co-treated with PRL and EGF further confirming an anti-proliferative role for PRL in mammary epithelial cells.

Next we characterized the role of PRL and EGF in the cellular growth of another mammary epithelial cell model, the NMuMG model (Sizemore and Cole, 1982) (Figure 2.3.1.3). The proliferation of NMuMG cells was measured via MTT assay. Cells were plated overnight in the absence or presence of PRL in media supplemented with 2 % FBS, hydrocortisone and insulin before being left unstimulated or stimulated with EGF (10 ng/mL or 100 ng/mL) for 72 hours. While EGF, at both concentrations, significantly induced the growth of NMuMG cells (p=0.0189; p=0.0165), PRL did not significantly induce cell proliferation. In samples co-treated with both ligands, no significant induction of cell proliferation was observed further suggesting that PRL is a negative regulator of EGF signals mammary epithelial cells proliferation.

Our results thus far illustrated a negative regulatory role for PRL in mammary epithelial cell proliferation. Next, we used the mouse mammary carcinoma 4T1 cell line (Figure 2.3.1.4) and the human breast carcinoma MCF7 and T47D cell lines (Figure 2.3.1.5) to examine the influence of PRL in breast cancer cell proliferation. In 4T1 experiments, cells were left unstimulated or stimulated with EGF, PRL or PRL and EGF for 48 hours. While, EGF significantly increased 4T1 cell growth (p = 0.047), PRL slightly reduced the proliferation of

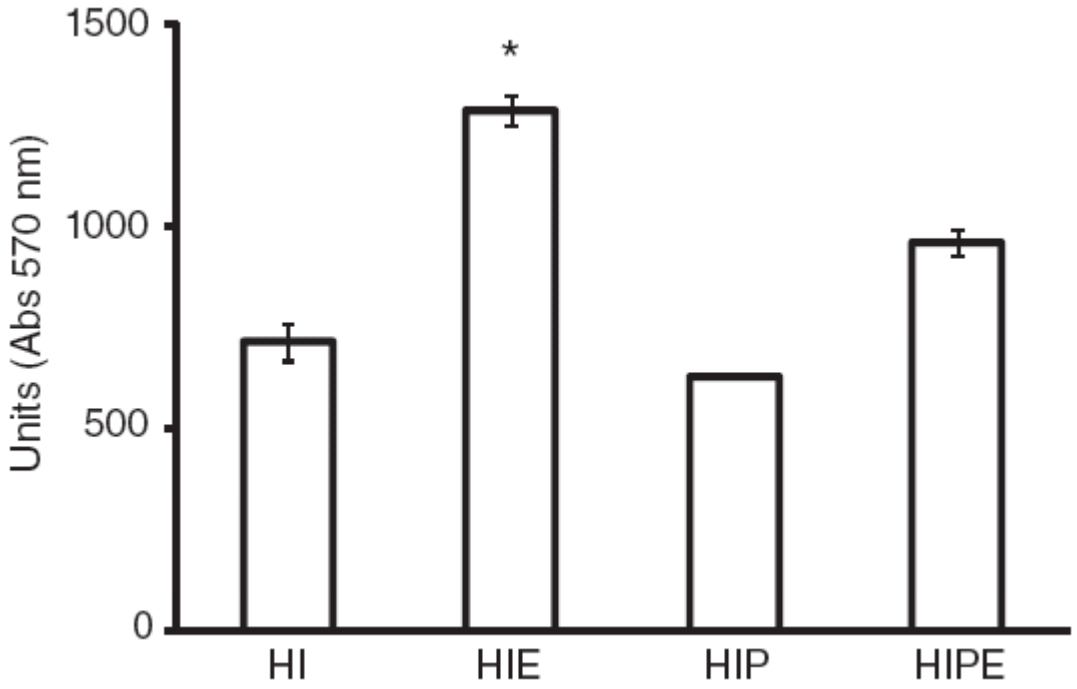

Figure 2.3.1.1.: PRL attenuates EGF-induced HC11 cell growth. HC11 cells were plated in assay media supplemented with 2% FBS, hydrocortisone (1 μM) and insulin (5 μg/mL) in the absence or presence of PRL (1 μg/mL) for an overnight period. Cells were then left untreated or treated with EGF (10 ng/mL) for 72hrs. MTT assays were performed in HC11 as described in Materials and Methods. Results are the mean ± SEM of triplicates of three different experiments HI = hydrocortisone and insulin; HIE = hydrocortisone, insulin, EGF; HIP = hydrocortisone, insulin, PRL; HIPE = hydrocortisone, insulin, PRL, EGF. (* $p<0.05$).

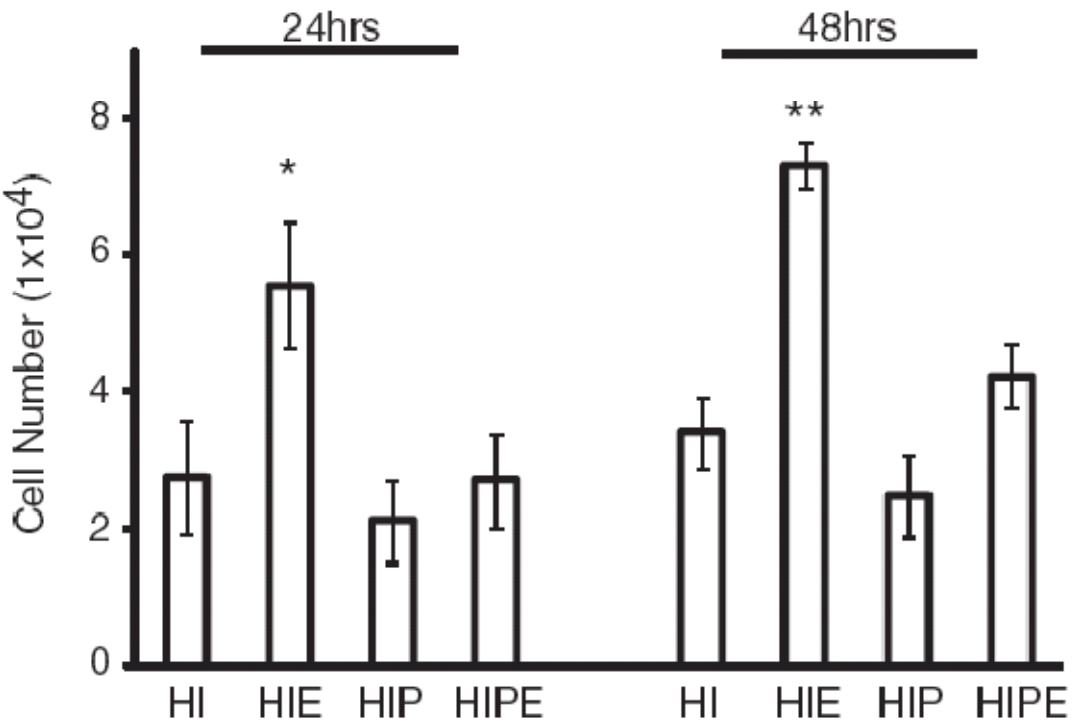

Figure 2.3.1.2.: PRL attenuates EGF-induced HC11 cell proliferation. HC11 cells were plated in media supplemented with 2% FBS, hydrocortisone (1 μM) and insulin (5 μg/mL) in the absence or presence of PRL (1 μg/mL) for an overnight period. Cells were then left unstimulated or stimulated with EGF (10 ng/mL) for 24 and 48 hours as indicated above. Cell counting assay was performed as described in Materials and Methods. Results are the means ± SEM of triplicates of three different experiments. HI = hydrocortisone and insulin; HIE = hydrocortisone, insulin, EGF; HIP = hydrocortisone, insulin, PRL; HIPE = hydrocortisone, insulin, PRL, EGF. (*p<0.1, **p<0.05).

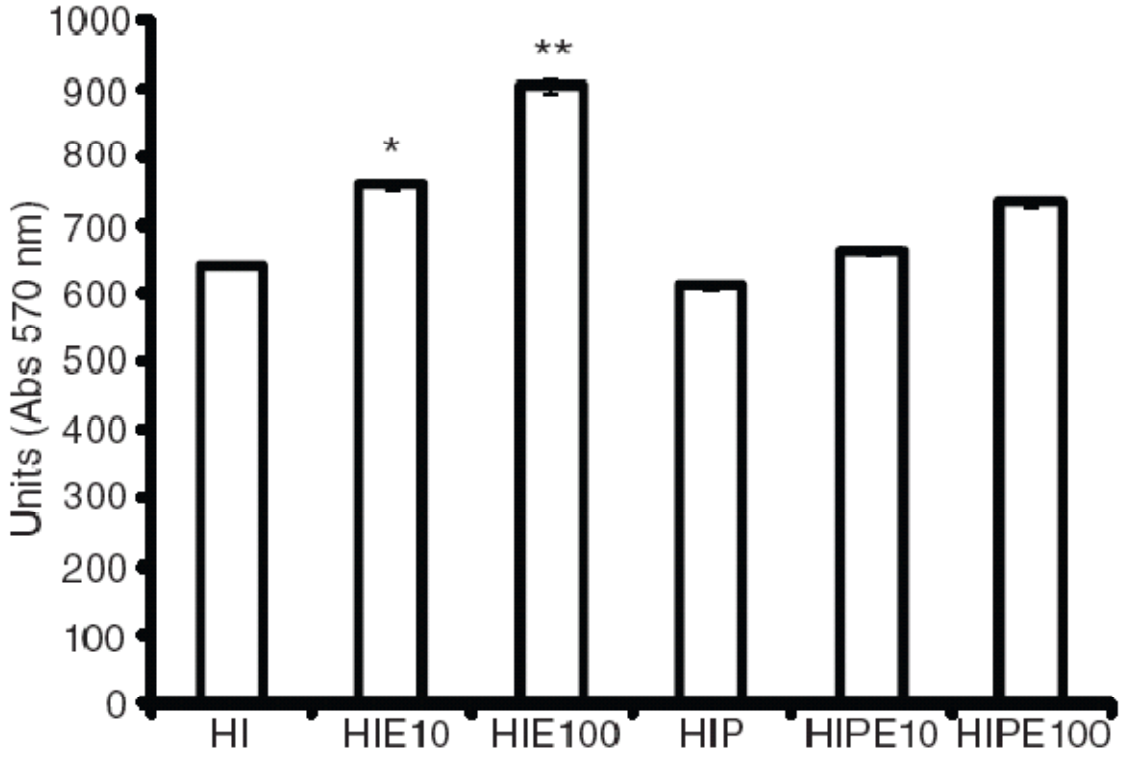

Figure 2.3.1.3: PRL attenuates EGF-induced NMuMG cell growth. NMuMG cells were plated in media supplemented with 2% FBS, hydrocortisone (1 μM) and insulin (5 μg/mL) in the absence or presence of PRL (1 μg/mL) for an o/n period. Cells were then left unstimulated or stimulated with EGF (10 ng/mL) or EGF (100 ng/mL) for 72 hours. MTT assay was performed as described in Materials and Methods. Results are the means ± SEM of triplicates of three different experiments. HI = hydrocortisone and insulin; HIE10 = hydrocortisone, insulin, EGF (10 ng/mL); HIE100 =hydrocortisone, insulin, EGF (100 ng/mL); HIP = hydrocortisone, insulin, PRL; HIPE10 = hydrocortisone, insulin, PRL, EGF (10 ng/mL); HIPE100 = hydrocortisone, insulin, PRL, EGF (100 ng/mL). (*p<0.05; **p<0.05)

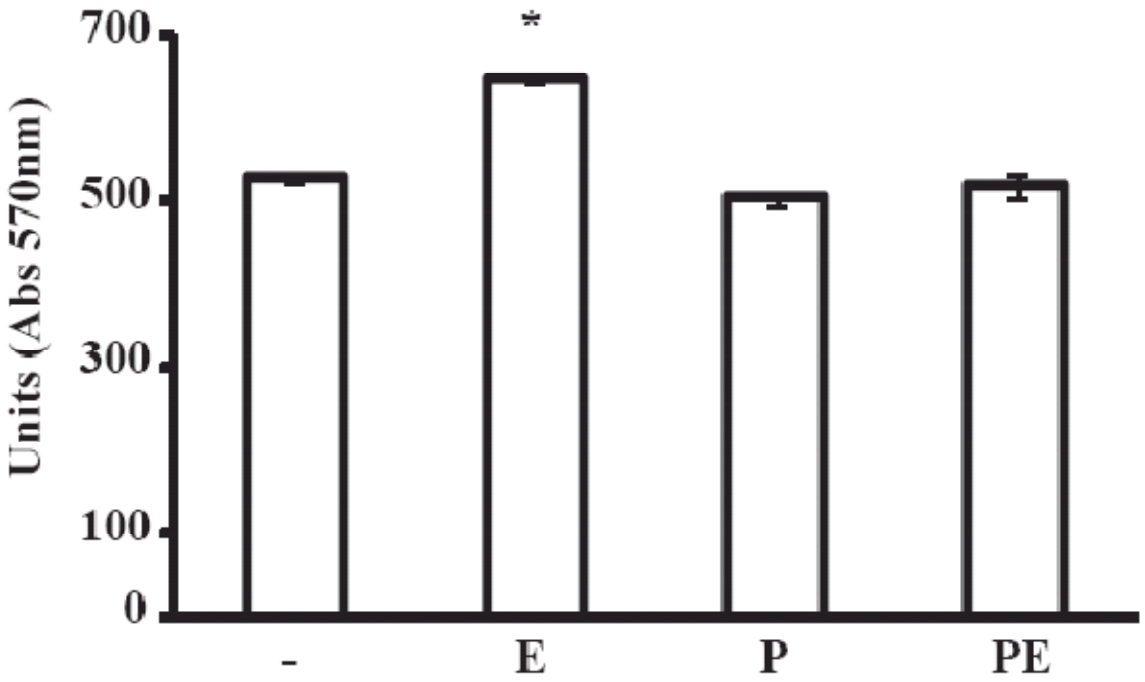

Figure 2.3.1.4: PRL attenuates EGF-induced 4T1 cell growth. 4T1 cells were left unstimulated or stimulated with EGF (10 ng/mL), PRL (1 μg/mL) or PRL (1 μg/mL) and EGF (10 ng/mL) for 48 hours. MTT assay was performed as described in Materials and Methods. Results are the means ± SEM of triplicates of three separate experiments. (*p<0.05)

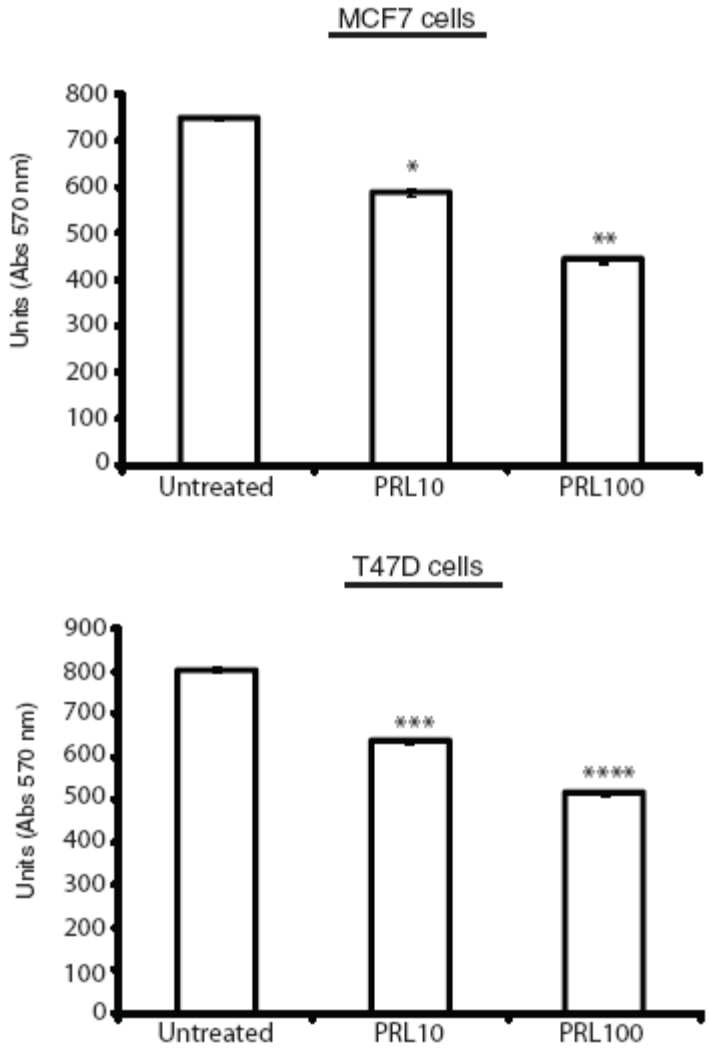

Figure 2.3.1.5: PRL suppresses the proliferation of human breast cancer cells. Top panel - MCF7 cells were plated in media supplemented with 2% FBS in the absence or presence of human PRL (10 ng/mL) or human PRL (100 ng/mL) for 48 hours. MTT assay was performed as described in Materials and Methods. Results are the means ± SEM of triplicates of three different experiments. PRL10 = PRL (10 ng/mL; PRL100 = PRL (100 ng/mL) (**p*<0.05**;** ***p*<0.01). Bottom panel – T47D cells were plated in media supplemented with 2% FBS in the absence or presence of human PRL (10 ng/mL) or human PRL (100 ng/mL) for 48 hours. MTT assay was performed as described in Materials and Methods. Results are the means ± SEM of triplicates of three different experiments. PRL10 = PRL (10 ng/mL; PRL100 = PRL (100 ng/mL) (****p*<0.05**;** *****p*<0.01).

4T1 cells compared to control cells suggesting that PRL may be anti-proliferative in mouse breast cancer cells. More importantly, PRL significantly attenuated EGF-induced 4T1 cell proliferation.

Together our results suggest that PRL is anti-proliferative and suppresses EGF signalling in both mammary epithelial and breast cancer cells. These results support the notion that PRL is differentiative in mammary epithelial cells and thus does not induce cellular growth (Oakes et al., 2008). Furthermore, our results counter the ongoing dogma in breast cancer research: PRL as a proliferative and tumourgenic factor (Clevenger et al., 2003). We clearly demonstrate that PRL significantly suppresses human breast cancer cell growth (Figure 2.3.1.5). Moreover, PRL suppressed EGF-mediated mammary epithelial and breast cell growth. Since EGFR is overexpressed in a large population of breast cancer patients (Kraus et al., 1989), it is important to determine mechanisms to counter the effects of EGF in these patients. While monoclonal antibodies have been developed against the EGFR, many patients are unresponsive or experience a variety of adverse events when treated with these antibodies (Modjtahedi et al., 1998). Therefore, enhancing PRL signals in EGFR overexpressing breast tumours may be an interesting treatment alternative.

2.3.2. PRL attenuates EGF-induced MAPK activation

Next we sought out to determine the mechanism through which PRL suppressed EGF-induced cell proliferation. Since the MAPK pathway mediates EGF-induced cell proliferation and our laboratory previously demonstrated that PRL treatment attenuated EGF-induced MAPK and inhibiting PRL signalling using the Jak2 inhibitor potentiated MAPK activation in breast cancer cells (Nouhi et al., 2006), we sought out to determine whether PRL inhibited EGF-induced cell proliferation by attenuating MAPK activation.

We examined the influence of PRL, EGF as well as the combination of both ligands in the activation of the MAPK pathway in the mammary epithelial cell lines. Starved differentiated HC11 cells were left untreated or treated with EGF, PRL or PRL and EGF for the indicated time points (Figure 2.3.2.1A). Cells were lysed and whole lysates were immunoblotted with a phospho-specific polyclonal antibody to Erk1 and Erk2. EGF influenced a substantial, prolonged activation of MAPK compared to the transient moderate activation observed in PRL-treated cells. More importantly, PRL suppressed EGF-mediated

MAPK activation. Equal protein levels of Erk1 and Erk2 were confirmed by immunoblotting with a polyclonal antibody to Erk1 and Erk2.

Next, we examined the regulation of the two ligands in the NMuMG cell model. Starved cells were left untreated or treated with EGF, PRL or PRL and EGF for the indicated time points (Figure 2.3.2.1B). Cells were then lysed and whole cell lysates were immunoblotted with a phospho-specific antibody detecting activated Erk1 and Erk2. Similar to what was observed in HC11 cells, EGF-induced MAPK activation and PRL did not influence the activation of the MAPK pathway. Once again, PRL was shown to suppress EGF-induced MAPK activation. Equal Erk1 and Erk2 levels were confirmed by reprobing the same membrane with a polyclonal antibody to Erk1 and Erk2. Together our results suggest that the ability of PRL to suppress EGF-induced cell growth is correlated with its ability to suppress EGF-induced MAPK activation.

To further characterized the regulation of PRL and EGF on MAPK activation, we next examined the regulation of the activation of RSK, a downstream target of MAPK (Erk1/Erk2) (Blenis, 1993), in mammary epithelial cells. Starved differentiated HC11 cells were left unstimulated or stimulated with PRL, EGF and PRL/EGF for the indicated time points (Figure 2.3.2.2A). Cells were lysed and whole cell lysates were immunoblotted with a polyclonal antibody to phospho-RSK. EGF significantly induced the activation of RSK; PRL did not induce RSK activation suggesting that the PRL-induced MAPK activation observed at 10 minutes in Figure 2.3.2.1A is not sufficient enough to activate a downstream target of MAPK. Moreover, PRL-induced MAPK activation was not correlated with an increase in cellular proliferation (Figure 2.3.1.1). PRL-induced MAPK may exert its cellular effects independent of RSK. Therefore, PRL-mediated MAPK pathway requires further characterized to determine whether PRL influences the activity of other downstream targets of MAPK such as c-MYC and MSK (Turjanski et al., 2007), as well as other MAPK dependent cellular functions. PRL also suppressed EGF-induced activation of RSK further emphasizing potency of the negative regulatory role of PRL. Equal RSK protein levels were confirmed by immunoblotting whole lysates with a polyclonal antibody to RSK.

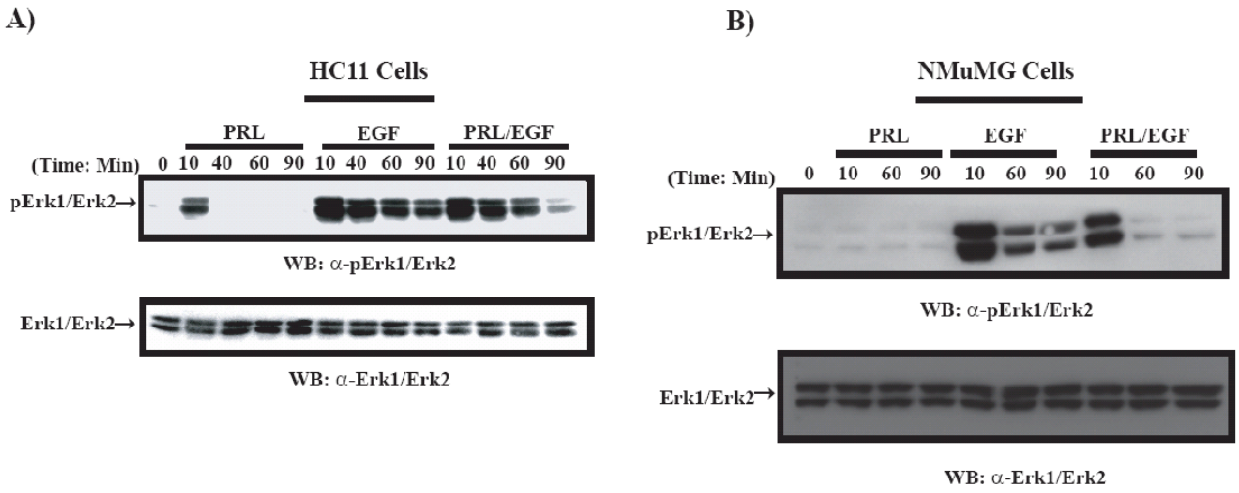

Figure 2.3.2.1: PRL attenuates EGF-induced MAPK activation in mammary epithelial cells. (A) Serum starved differentiated HC11 cells were left untreated or treated either with PRL (1 μg/mL), EGF (10 ng/mL) or PRL (1 μg/mL) and EGF (10 ng/mL) for the time points indicated. Cell lysates were immunoblotted with a polyclonal antibody to phospho-Erk1/Erk2 (upper panel) and to Erk1/Erk2 (lower panel). Results were confirmed by repeating the experiment 3 times. **(B)** Serum starved NMuMG cells were left untreated treated either with PRL (1 μg/mL), EGF (10 ng/mL) or PRL (1 μg/mL) and EGF (10 ng/mL) for the time points indicated. Cell lysates were immunoblotted with a polyclonal antibody to phospho-Erk1/Erk2 (upper panel) and to Erk1/Erk2 (lower panel). Results were confirmed by repeating the experiment 2 times.

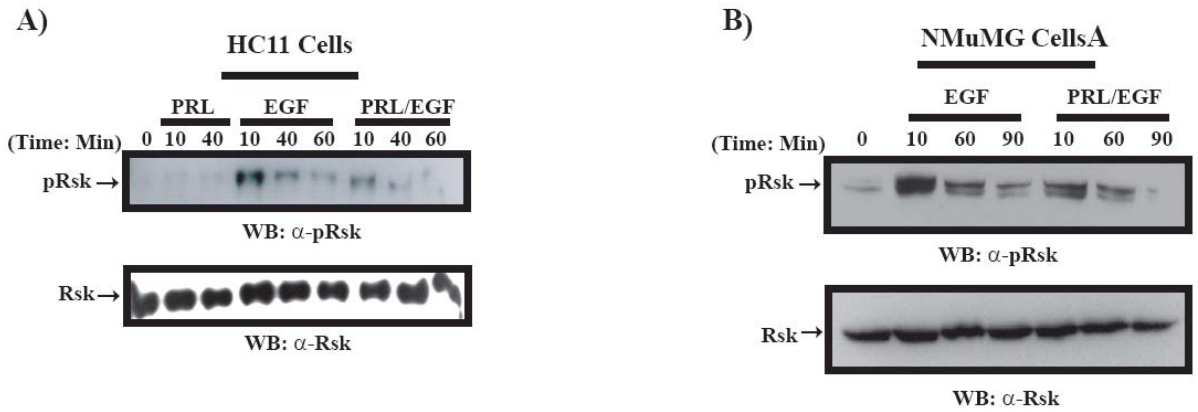

Figure 2.3.2.2: PRL attenuates EGF-induced RSK activation in mammary epithelial cells. (A) Serum starved differentiated HC11 cells were left untreated or treated either with PRL (1 μg/mL), EGF (10 ng/mL) or PRL (1 μg/mL) and EGF (10 ng/mL) for the time points indicated. Cell lysates were immunoblotted with a polyclonal antibody to phospho-RSK (upper panel) and to RSK (lower panel). Results were confirmed by repeating the experiment 3 times. **(B)** Starved NMuMG cells were left untreated or treated either with EGF (10 ng/mL) or PRL (1 μg/mL) and EGF (10 ng/mL) for the time points indicated. Cell lysates were immunoblotted with a polyclonal antibody to phospho-RSK (upper panel) and to RSK (lower panel). Results were confirmed by repeating the experiment 2 times.

Next we examined RSK activation in the NMuMG cell model. Cells were treated with either EGF alone or in combination with PRL for the indicated time points (Figure 2.3.2.2B). Whole cell lysates were immunoblotted with a polyclonal antibody to phospho-RSK. EGF significantly induced the activation of RSK. PRL was shown to block EGF-induced RSK activation. Equal RSK protein levels were confirmed by immunoblotting whole lysates with a polyclonal antibody to RSK.

Together, our results suggest that both PRL and EGF regulate MAPK/RSK activation. While EGF substantially activated both MAPK and RSK, PRL only moderately and transiently activated MAPK and was unable to activate RSK. More importantly, PRL negatively regulated EGF signals to MAPK and RSK activation. Furthermore, PRL-induced suppression of MAPK and RSK activation correlated the ability of PRL to inhibit EGF-induced cell proliferation as observed in Figures 2.3.1.1 and 2.3.1.3.

2.3.3. EGF regulation of PRL-induced cellular differentiation

To further characterize the signalling crosstalk between PRL and EGF in mammary epithelial cells, we next examined the influence of the ligands in mammary epithelial cell differentiation. Using the HC11-lux cell line, HC11 cells stably overexpressing the β-casein promoter luciferase construct, we examined the influence of PRL and the combination of PRL and EGF on β-casein gene expression, a well characterized marker of mammary cell differentiation (Figure 2.3.3.1) (Ball et al., 1988). This β-casein promoter construct is known to be activated by PRL, so we sought out to examine the influence of EGF PRL-induced β-casein gene expression. HC11-lux cells were stimulated with PRL or the combination of PRL and EGF for 16 hours and luciferase activity was measured as described in material and methods to determine the induction of β-casein promoter activity. As expected, PRL induced a robust activation of the β-casein gene promoter (more than 25 folds induction) whereas, in agreement

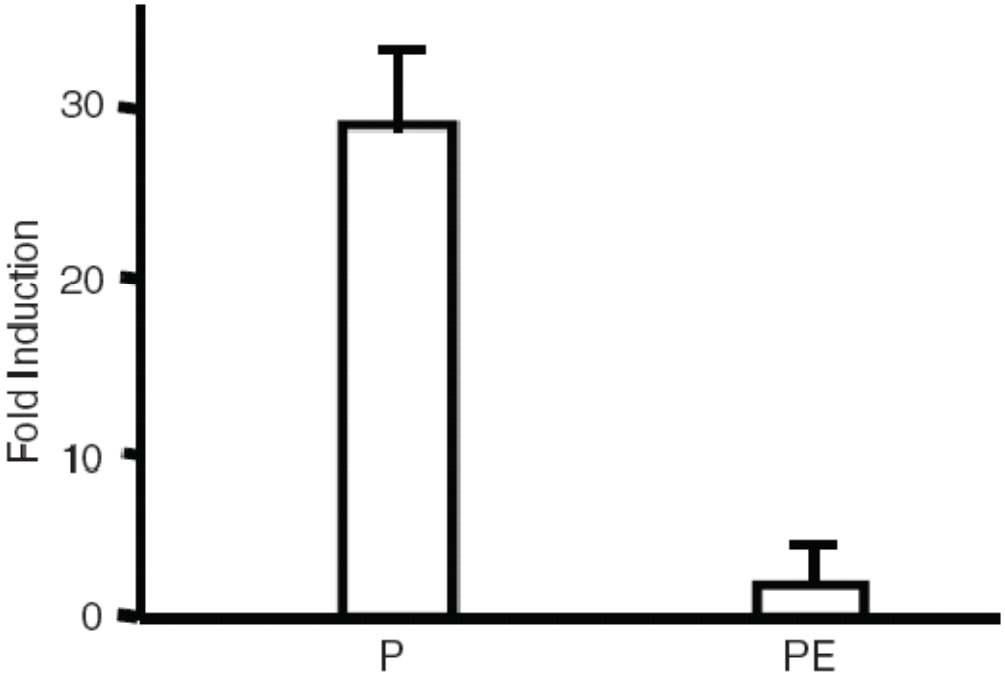

Figure 2.3.3.1: EGF suppresses PRL-induced β-casein expression (Luciferase assay) Differentiated HC11-Lux cells were left untreated or treated either with PRL or PRL (1 mg/mL) and EGF (10 ng/mL) for 16 hours. Luciferase activity of the β-casein gene promoter was measured as in Materials and Methods. Results are the means ± SEM of quadruplicates of two different experiments. P = PRL; PE = PRL and EGF

with previous studies (Horsch et al., 2001; Marte et al., 1995), EGF abolished the ability of PRL to induce β-casein gene expression suggesting that EGF is inhibitory to PRL signals to milk protein expression and thus cellular differentiation. Together, our data suggests that both PRL and EGF exert opposing negative regulatory signals. While PRL inhibits EGF signals to cell proliferation, EGF inhibits PRL-induced milk protein expression and cellular differentiation.

Next, we used quantitative real-time PCR (qRT-PCR) to measure the regulation of PRL-induced differentiation signals as measured by induction of β-casein gene expression. Serum starved differentiated HC11 cells were left untreated or treated overnight with PRL, EGF or PRL and EGF. As can be seen in Figure 2.3.3.2, PRL treatment led to a significant induction of β-casein gene expression. In contrast, EGF treatment of HC11 cells did not increase β-casein gene expression. Importantly, treatment with both factors led to a significant inhibition in PRL-induced β-casein gene expression. Values were normalized to GAPDH levels.

2.4.4. PRL proximal signalling is unaffected by EGF

Since EGF was shown to negatively regulate PRL-induced cellular differentiation, we next examined the influence of EGF on the Jak2/Stat5a pathway, the major pathway downstream of the PRLR receptor involved in the induction of milk proteins (Ball et al., 1988) (Oakes et al., 2008). To characterize this negative regulatory mechanism, we first examined influence of EGF, PRL and the combination of PRL and EGF on the activation of Jak2 (Figure 2.3.4.1). HC11 cells were left untreated or treated with each condition for 15 minutes. Cells were lysed and Jak2 was immunoprecipitated from cell lysates using a polyclonal antibody to Jak2. The presence of activated Jak2 (tyrosine phosphorylated Jak2) in the immunopreciptates was quantified by immunodetecting phosphorylated Jak2 with a monoclonal recognizing phosphorylated tyrosine residues. As expected, PRL-induced the activation of Jak2. In samples treated with EGF, EGF was shown to mildly activate Jak2 in this western analysis. However, it can be reasoned that this mild activation is due to the saturation of the western signal. As in other experiments, EGF-induced Jak2 tyrosine phosphorylation was undetected and EGF still was unable to affect PRL-induced Jak2 activation. In samples co-treated with PRL and EGF, PRL still induced the activation of Jak2 suggesting the EGF does not block

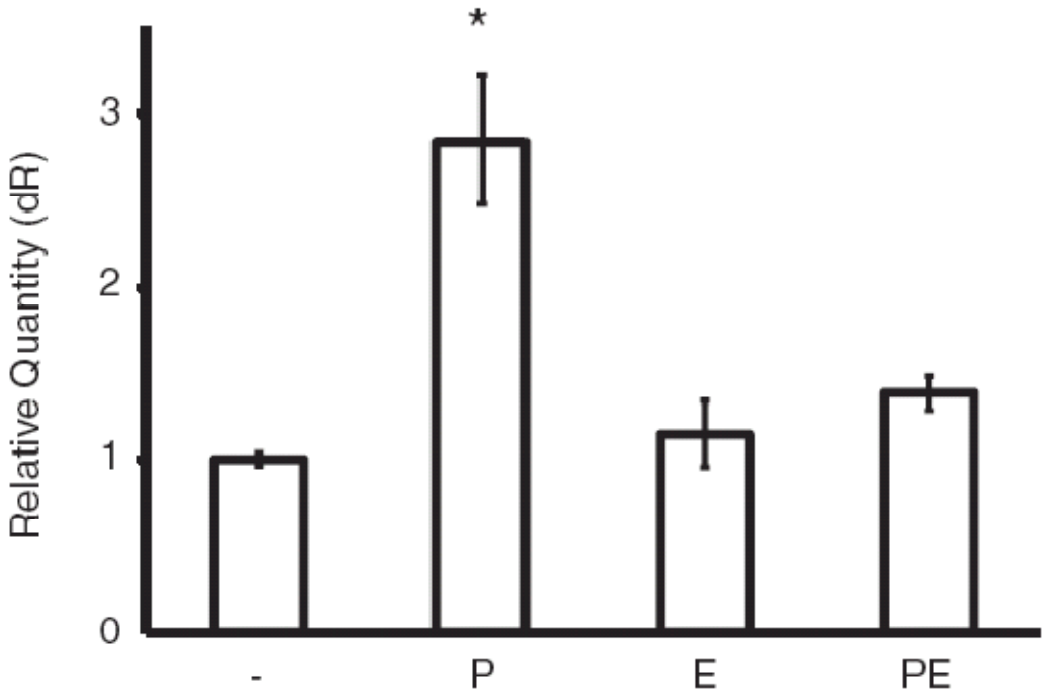

Figure 2.3.3.2: EGF suppresses PRL-induced β-casein expression (qRT-PCR). Differentiated HC11 cells were left untreated or treated either with PRL (1 μg/mL), EGF (10 ng/mL), or PRL (1 μg/mL) and EGF (10 ng/mL) for 16 hours. qRT-PCR of the β-casein gene was performed as described in Materials and Methods. Results are the mean of three independent experiments. P = PRL; E = EGF; PE = PRL and EGF (*$p \leq 0.05$).

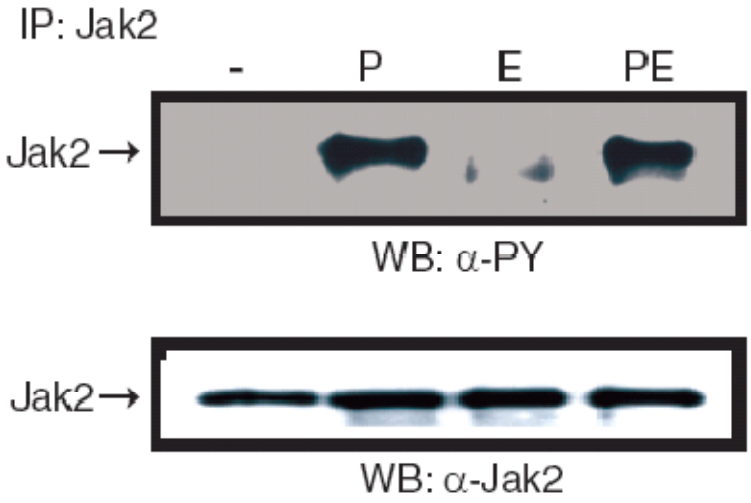

Figure 2.3.4.1: Jak2 activation is unaffected by EGF. Serum starved differentiated HC11 cells were untreated or treated either with PRL (1 μg/mL), EGF (10 ng/mL) or PRL (1 μg/mL) and EGF (10 ng/mL) for 15 minutes. Cell lysates were immunoprecipitated with a polyclonal antibody to Jak2, immunoblotted with a monoclonal antibody to phospho-tyrosine (upper panel) and reprobed with a polyclonal antibody to Jak2 (lower panel). Results were confirmed by repeating experiment 5 times. P = PRL; E = EGF; PE = PRL and EGF.

PRL-induced cellular differentiation by inhibiting Jak2 activation. Equal presence of Jak2 in immunoprecipitates was determined by reprobing with a polyclonal antibody to Jak2. Long-term treatment with EGF was shown to induce the expression of the tyrosine phosphatase PTP-PEST. Increased expression of PTP-PEST resulted in the dephosphorylation of Jak2 and blockade of PRL signals to milk protein expression (Horsch et al., 2001). While our data does not rule out this mechanism as a possible mean of negative regulation of PRL signalling, immediate PRL-induced Jak2 activation is unaffected by EGF.

Next, we examined the effects of EGF on the activation of Stat5a (Figure 2.3.4.2). Using the same lysates obtained in Figure 2.3.4.1, we examined the Stat5a activation using a monoclonal antibody recognizing the phosphorylated tyrosine residue Y694, the tyrosine residue essential for Stat5a activity (Hennighausen and Robinson, 2008). PRL stimulation induced the activation of Stat5a, whereas Stat5a was not activated by EGF. Moreover, EGF did not inhibit the ability of PRL to activate Stat5a suggesting EGF exerts a nuclear regulation of PRL signals to milk protein gene expression. Equal Stat5a expression in lysates was shown by reprobing with a monoclonal to Stat5a.

Since the cytoplasmic signalling events downstream of the PRLR were unaffected by EGF, we next investigated the influence of EGF on Stat5a nuclear events such as Stat5a nuclear translocation and Stat5a binding activity to its DNA response element on the β-casein gene promoter. First, we examined the nuclear translocation of Stat5a in nuclear extracts isolated from HC11 cells treated with PRL alone or the combination of PRL and EGF for the indicated time points (Figure 2.3.4.3). Detection of nuclear Stat5a was carried out using either phospho-specific antibody to Stat5a detecting active Stat5a (Figure 2.3.4.3, upper panel) or a monoclonal antibody to Stat5a (Figure 2.3.4.3, lower panel). As can be seen, PRL-induced a rapid nuclear translocation of Stat5a within 15 minutes of treatment. Importantly, EGF did not block PRL-induced Stat5a nuclear translocation even after 1 hour of co-treatment. The levels of nuclear phosphorylated Stat5a were unaffected by EGF suggesting that EGF does not play a role in the nuclear dephosphorylation of Stat5a. Moreover, as can be seen in Figure 2.3.4.4, using EMSA PRL led to the rapid binding of Stat5a to its response element on the β-casein gene promoter. EGF was unable to block PRL-induced Stat5a DNA binding activity to its response element. Together, the above results suggest that the regulation of PRL signaling to β-casein

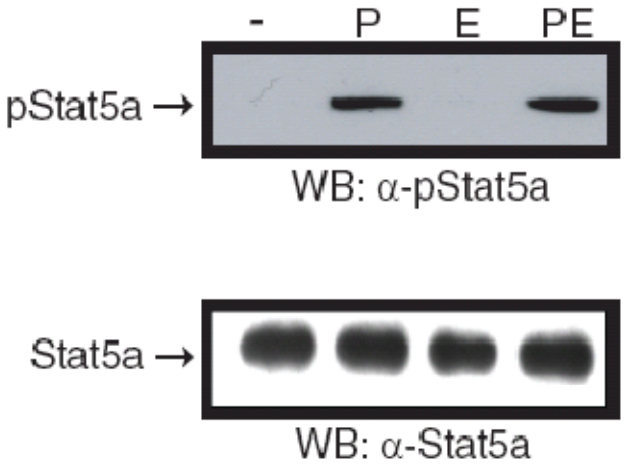

Figure 2.3.4.2: Stat5a activation is unaffected by EGF. Serum starved differentiated HC11 cells were untreated or treated either with PRL (1 μg/mL), EGF (10 ng/mL) or PRL (1 μg/mL) and EGF (10 ng/mL) for 15 minutes. Cell lysates were immunoblotted with a monoclonal antibody to phospho-Stat5a (upper panel) and reprobed with a monoclonal antibody to Stat5a (lower panel). Results were confirmed by repeating the experiment 5 times. P = PRL; E = EGF; PE = PRL and EGF.

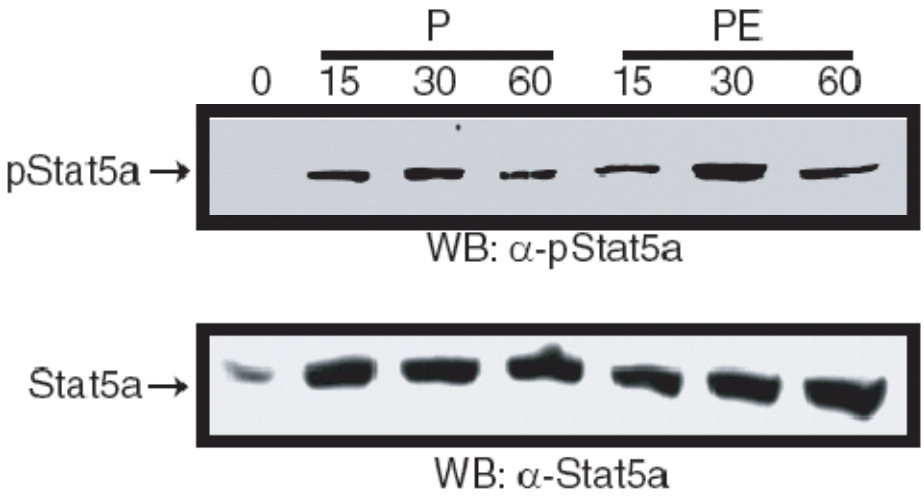

Figure 2.3.4.3: Stat5a nuclear translocation is unaffected by EGF. Serum starved differentiated HC11 cells were untreated or treated either with PRL (1 μg/mL) or PRL (1 μg/mL) and EGF (10 ng/mL) for the indicated time points. Nuclear extracts were immunoblotted with a monoclonal antibody to phospho-Stat5a (upper panel) and to monoclonal antibody to Stat5a (lower panel). Results were confirmed by repeating the experiment 3 times. P = PRL; PE = PRL and EGF.

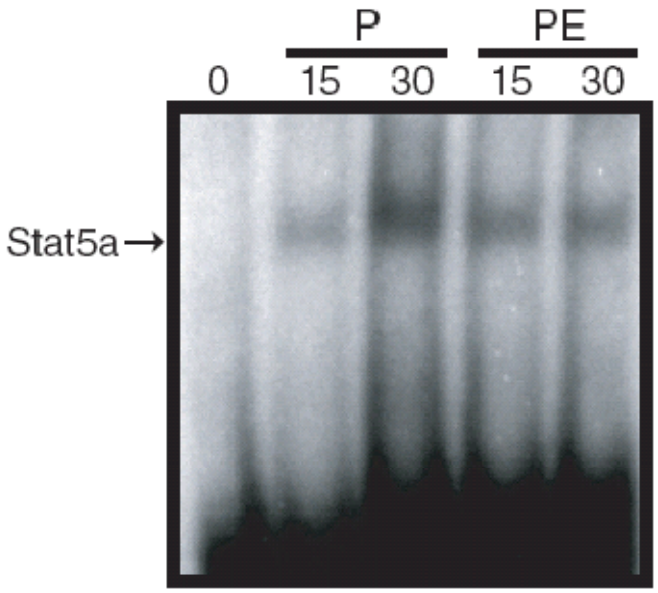

Figure 2.3.4.4: Stat5a DNA binding is unaffected by EGF. Serum starved differentiated HC11 cells were untreated or treated either with PRL (1 μg/mL) or PRL (1 μg/mL) and EGF (10 ng/mL) for the indicated time points. EMSA was performed using the Stat5a-binding site of the β-casein promoter as described in Materials and Methods on nuclear extracts isolated in Figure 2.3.4.3. Results were confirmed by repeating the assay repeated 3 times. P = PRL; PE = PRL and EGF.

gene promoter activation by EGF is independent of PRL-induced proximal signaling events such as Jak2/Stat5a tyrosine phosphorylation as well as Stat5a nuclear translocation and DNA binding activity.

In agreement with previous studies, our study shows that EGF signals are inhibitory to PRL induced mammary epithelial cellular differentiation as measured by induction of milk protein gene expression (Horsch et al., 2001; Marte et al., 1995). The mechanism by which EGF blocks PRL signals in mammary cells to milk protein synthesis has yet to be fully defined. Our data suggests that the PRL-induced activation of the Jak2/Stat5a pathway as well as the nuclear translocation and DNA binding capability Stat5a is unaffected by EGF. It has been suggested that serine phosphorylation of Stat5a on residues S725 and S779 suppresses Stat5a DNA binding (Beuvink et al., 2000; Yamashita et al., 2001). However, more recently ErbB4-induced serine phosphorylation of Stat5a on residues S127 and S128 has been shown to enhance Stat5a DNA binding (Clark et al., 2005) suggesting EGF may utilize serine phosphorylation of Stat5a to negatively regulate the functions of Stat5a. However, we have been unable to detect the serine phosphorylation of Stat5a using phospho-specific antibodies to S725 and S779. Moreover, long-term EGF treatment (Marte et al., 1995) as well as the overexpression of constitutively active Ras (Cerrito et al., 2004), has been previously shown to attenuate Stat5a DNA binding. Further characterizing of our model system is required to identify the EGF negative regulatory mechanism responsible for attenuating PRL-induced cellular differentiation.

2.3.5. Jak2 kinase activity is essential to attenuate EGF-induced growth

The above results suggest that Jak2/Stat5a activation is maintained in cells co-treated with PRL/EGF. This led us to determine if the Jak2/Stat5a pathway is required to suppress EGF signals to cell proliferation (Figure 2.3.5.1). We utilized the Jak2 inhibitor to suppress the Jak2/Stat5a and examined the regulation of cell growth by PRL and EGF via MTT assay. First, we confirmed the ability of the Jak2 inhibitor to block the tyrosine phosphorylation/activation of Jak2 in HC11 cells. To do this we pre-treated HC11 cells with either DMSO (control) or Jak2 inhibitor (25 μM) for 2 hours before leaving cells untreated or treated with PRL for 15 minutes. Cell lysates were than immunoprecipitated with a polyclonal

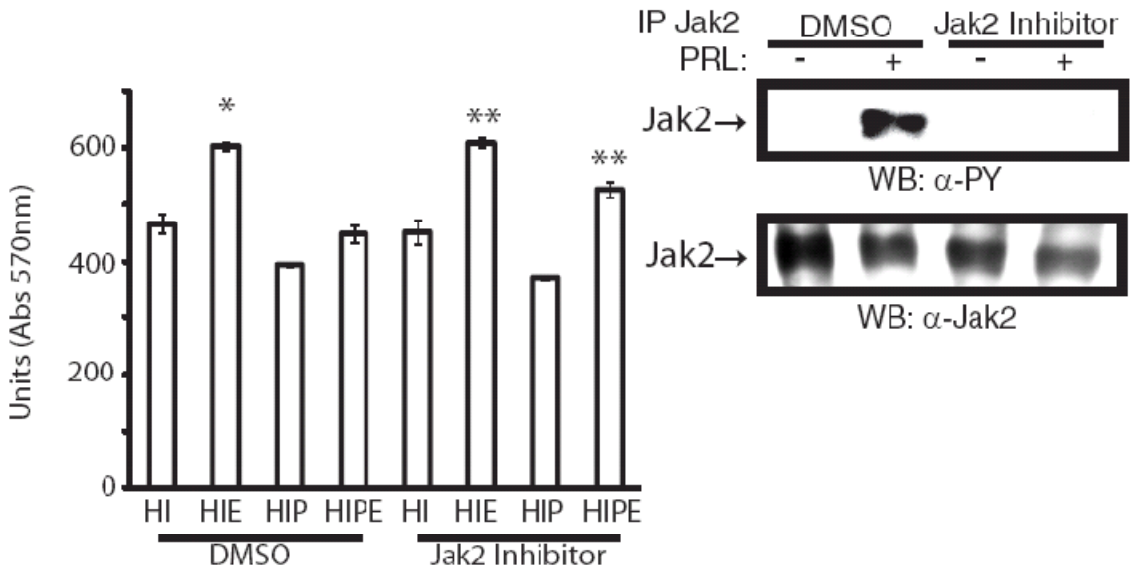

Figure 2.3.5.1: Jak2 kinase is essential to mediate PRL-induced attenuation of EGF-induced cell growth. Left panel - HC11 cells were treated either with DMSO or Jak2 inhibitor (1 μM) and grown overnight in media supplemented with 2% FBS, hydrocortisone (1 μM) and insulin (5 μg/mL) in the presence or absence of PRL (1 μg/mL). Cells were then left untreated or treated with EGF (10 ng/mL) for 48 hours. MTT assay was performed as described in Materials and Methods. Results are the means ± SEM of triplicates of three different experiments (* $p<0.01$ ** $p<0.05$). HI = hydrocortisone, insulin; HIE = hydrocortisone, insulin, EGF; HIP = hydrocortisone, insulin, PRL; HIPE = hydrocortisone, insulin, PRL, EGF. Right panel - HC11 cells were pre-treated either with DMSO or the Jak2 inhibitor (25μM) for 2 hours. Cells were then left untreated or treated with PRL for 15 minutes. Cell lysates were immunoprecipitated with a polyclonal antibody to Jak2 and immunoblotted with a monoclonal antibody to phospho-tyrosine (top panel). The membrane was then reprobed with a polyclonal antibody to Jak2 (lower panel).

antibody to Jak2 and immunoblotted with a monoclonal antibody to phosphotyrosine residues. In DMSO control pre-treated cells PRL induced the activation of Jak2. The PRL-induced Jak2 activation was abolished in samples pre-treated with the Jak2 inhibitor. Now knowing that the inhibitor is effective in blocking Jak2 activation, we next examined the ability of the Jak2 inhibitor to regulate cell proliferation. We were only able to detect a reduction in Jak2 activation during short-term stimulation at inhibitor concentrations of 10 μM and over. However, since previous studies have shown that prolonged treatments of the Jak2 inhibitor (1 μM) is sufficient to inhibit Jak2 kinase activity (Sandberg et al., 2005) and we wanted to avoid inhibiting cell growth via inhibitor toxicity we used a lower concentration of the Jak2 inhibitor (1 μM) for the MTT assays. HC11 cells were grown in 2% serum media containing hydrocortisone and insulin (HI) or HI and PRL (HIP) in the presence of DMSO (as control) or the Jak2 inhibitor (1 μM) for an overnight period before being left unstimulated or stimulated with EGF for 48 hours. In the absence of the Jak2 inhibitor, significant induction of cell proliferation was only observed in samples treated with EGF alone suggesting that DMSO does not interfere with the ability of PRL to block EGF-induced cell growth. However, in the presence of the Jak2 inhibitor, significant induction of cell proliferation was observed in samples treated with EGF alone as well as samples co-treated with both PRL and EGF. This data suggests that Jak2 kinase plays an important role in mediating the inhibitory effect of PRL on EGF-induced mammary cell growth.

2.3.6. Grb2 is a novel substrate of the PRLR/Jak2 complex

Due to the well-known effect of EGF as a promoter of mammary epithelial cell proliferation and tumourgenesis and based on our data indicating the ability of PRL to significantly suppress-EGF induced cell proliferation, we were interested in defining the mechanism by which PRL exerts this effect on EGF-mediated signaling. Our results indicate that PRL through inhibition of MAPK pathway suppresses EGF-induced cell proliferation. Therefore, we decided to examine the regulatory role of PRL on the Ras-MAPK signalling pathway.

To delineate a possible mechanism through which PRL/Jak2 blocks EGF-signaling, we analyzed the contribution of the adaptor protein Grb2. Previously within our laboratory we demonstrated that Grb2 or a protein associated with Grb2 of 29 kDa was a substrate of the

tyrosine phosphatase SHP-2 and was tyrosine phosphorylated following the activation of the PRLR/Jak2 signaling cascade (Minoo et al., 2003). To confirm that the protein p29 was indeed a tyrosine phosphorylated form of Grb2, we overexpressed PRLR, Jak2, Grb2 (29 kDa) or myc-Grb2 (45 kDa) in presence and absence of catalytically inactive SHP2 (SHPCA) (Figure 2.3.6.1). A 29 kDa tyrosine phosphorylated form of Grb2 was present only in the sample overexpression SHP2CA. Interestingly, in the sample overexpressing myc-Grb2 in presence of SHP2CA no band was identifiable at 29 kDA. However, a tyrosine phosphorylated band was observed at 45 kDA suggesting that protein p29 was either a tyrosine phosphorylated protein of 45 kDA that interacts with Grb2 or a tyrosine phosphorylated form of Grb2. While it is generally believed that Grb2 itself does not undergo tyrosine phosphorylation in response to growth factor stimulation (Lowenstein et al., 1992; Rozakis-Adcock et al., 1992), tyrosine phosphorylation of Grb2 on residues Y7, Y37, Y52, Y209 within its SH3 domains has been recently reported to negatively regulate growth factor-induced Ras/MAPK activation (Li et al., 2001). Therefore, we hypothesized that Grb2 is a substrate of the PRLR/Jak2 complex and may contribute to PRL inhibitory role in EGF-induced MAPK activation and cell proliferation.

To investigate whether PRL induces phosphorylation of Grb2 on tyrosine residues present within its SH3 domains, we overexpressed in 293 cells PRLR, Jak2 and either Grb2WT or Grb2YF mutant form in which the four tyrosine residues Y7, Y37, Y52, Y209 were substituted by phenylalanine (Figure 2.3.6.2). Upon PRLR activation by stimulating with PRL for 15 minutes, tyrosine phosphorylation was observed in samples overexpression Grb2WT but not Grb2YF suggesting that PRL phosphorylates Grb2 on one or multiple SH3 domain tyrosine residues. It is suggested that all four tyrosine residues are capable of being phosphorylated, but the tyrosine phosphorylation of residue Y209 is essential for the suppression of the MAPK pathway (Li et al., 2001). However, more experiments using single YF point mutations and combinational YF mutations are required to determine which residues are phosphorylated by the PRLR/Jak2 complex. Moreover, when Grb2 was overexpressed in presence of PRLR and either Jak2 or Src kinase, Grb2 tyrosine phosphorylation was only detected in cells overexpressing the Jak2 kinase and not Src kinase, indicating that Grb2 is a specific substrate of the PRLR/Jak2 complex and that other tyrosine kinases such as Src are

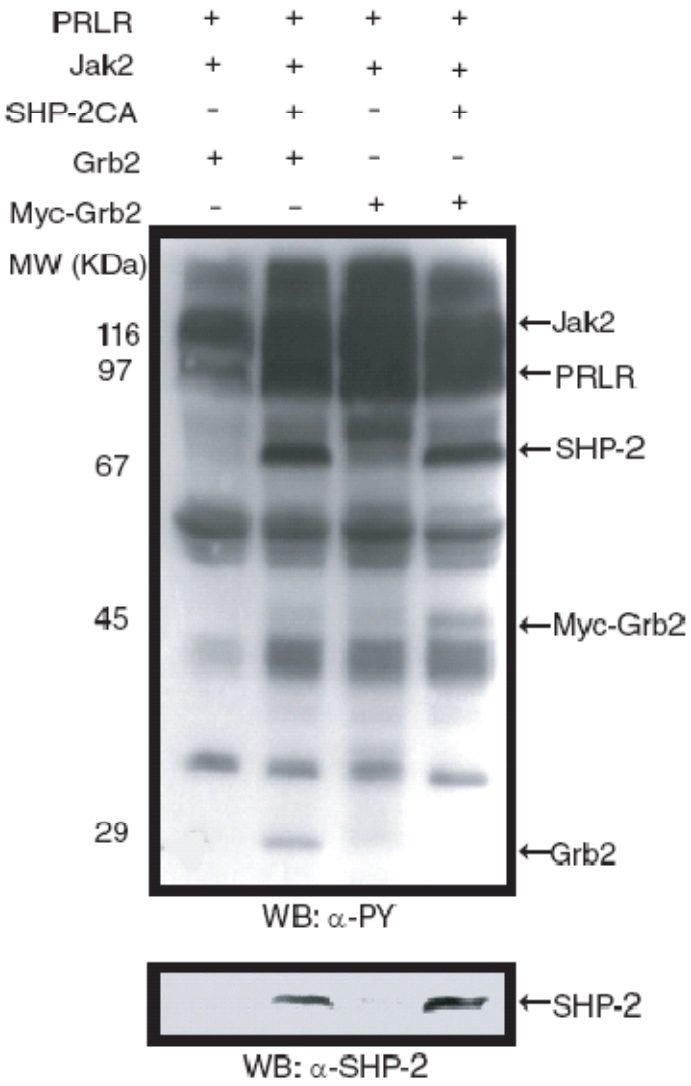

Figure 2.3.6.1: p29 is either a tyrosine phosphorylated protein that interacts with Grb2 or a tyrosine phosphorylated form of Grb2. Expression plasmids encoding the long form of the PRLR, Jak2, SHP2CA, Grb2 or myc-Grb2 were transiently transfected in 293 cells as indicated above. Following an overnight starvation period, cells were treated with PRL for 10 minutes. Cell lysates were immunoblotted with a monoclonal antibody to phospho-tyrosine (upper panel) and a monoclonal antibody to SHP2 (lower panel). Results were confirmed in another experiment performed by another investigator.

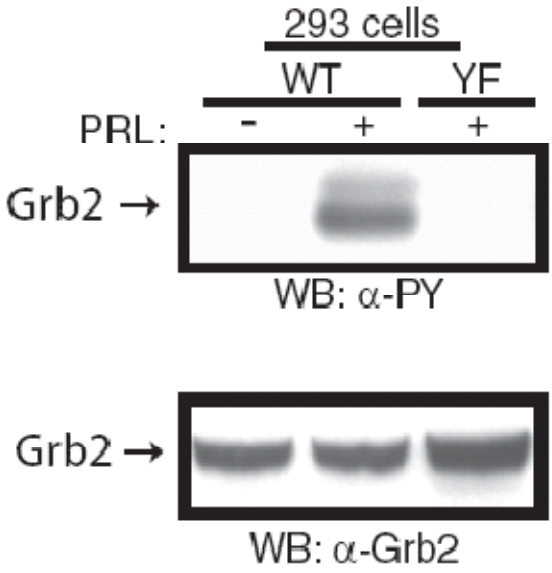

Figure 2.3.6.2: PRL induces the tyrosine phosphorylation of overexpressed Grb2. Expression plasmids encoding the long form of the PRLR, Jak2 and indicated form of Grb2 were transiently transfected in 293 cells. Following an overnight starvation period, cells were treated with PRL for 10 minutes. Cell lysates were immunoblotted with a monoclonal antibody to phospho-tyrosine (upper panel) and a polyclonal antibody to Grb2 (lower panel).

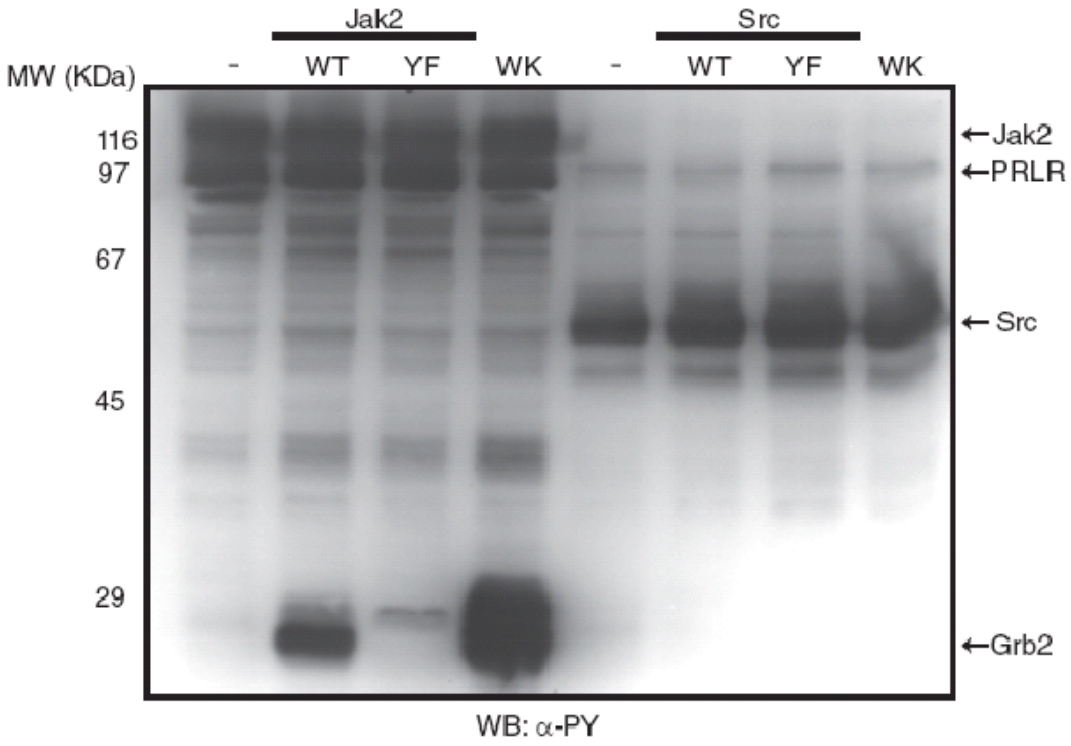

Figure 2.3.6.3: Jak2, but not Src kinase is required for Grb2 tyrosine phosphorylation. Expression plasmids encoding the long form of the PRLR, Jak2 or Src and indicated forms of Grb2 were transiently transfected in 293 cells. Following an overnight starvation period, cells were treated with PRL for 10 minutes. Cell lysates were immunoblotted with a monoclonal antibody to phospho-tyrosine.

unable to phosphorylate Grb2 (Figure 2.3.6.3). The PRLR/Jak2-induced Grb2 tyrosine phosphorylation is further emphasized in a mutant form of Grb2 in which the two tryptophan residues W36 and W193 are mutated to lysine (Grb2WK) (Figure 2.3.6.3). The Grb2WK mutant form is unable to interact with Sos and thus incapable of activating Ras. It is suggested that the inability of Grb2WK to interact with Sos stems from a loss of SH3 domain functionality (Gupta and Mayer, 1998). PRLR, Jak2 and the indicated Grb2 forms (Grb2WT, Grb2YF, and Grb2WK) were overexpressed in 293 cells and tyrosine phosphorylation of the three Grb2 forms was assessed using a monoclonal antibody recognizing phosphorylated tyrosine residues. As expected, the tyrosine phosphorylation was observed in the sample overexpressing Grb2WT, but not Grb2YF. More interestingly, the samples overexpressing the Grb2WK mutant form exhibited dramatic, more potent compared to Grb2WT, tyrosine phosphorylation, suggesting the inability of Grb2WK to interact with Sos and activate the Ras-MAPK pathway may be the result of an increased tyrosine phosphorylation of the SH3 domains. It is known that tyrosine residues within the SH3 domains of Grb2 (N-terminal SH3 domain, Y7 & Y52, and C-terminal SH3 domain, Y209) along with W36 and W193 form part of the SH3-ligand binding site required for Sos interaction. (Calero et al., 2004; Chardin et al., 1995). Therefore, one may speculate that the inability of Grb2WK to bind Sos is due to its highly stable phosphorylation state further emphasizing tyrosine phosphorylation of Grb2 as a regulatory mechanism to modulate signalling.

Altogether, these results suggest that the adaptor protein Grb2 is a substrate of the PRLR/Jak2 complex and the tyrosine phosphorylation of Grb2 may be a mechanism utilized by PRL to negatively regulate EGF signalling. These results prompted us to determine whether PRL stimulation of mammary epithelial cells led to the tyrosine phosphorylation of Grb2. For this starved mammary epithelial HC11 and NMuMG cells were left untreated with PRL for 15 minutes. Next we immunoprecipitated Grb2 in HC11 (Figure 2.3.6.4) and NMuMG (Figure 2.3.6.5) cell lysates using a polyclonal antibody against Grb2 and immunoblotted tyrosine phosphorylated Grb2 immunoprecipitates using a monoclonal antibody against phosphorylated tyrosine residues. In untreated samples no tyrosine phosphorylate Grb2 was detected in both mammary epithelial cell lines. However, treated with PRL induced the tyrosine phosphorylation of Grb2 in both cell lines. Equal expression of Grb2 in immunoprecipitates was confirmed by reprobing with a polyclonal antibody against Grb2.

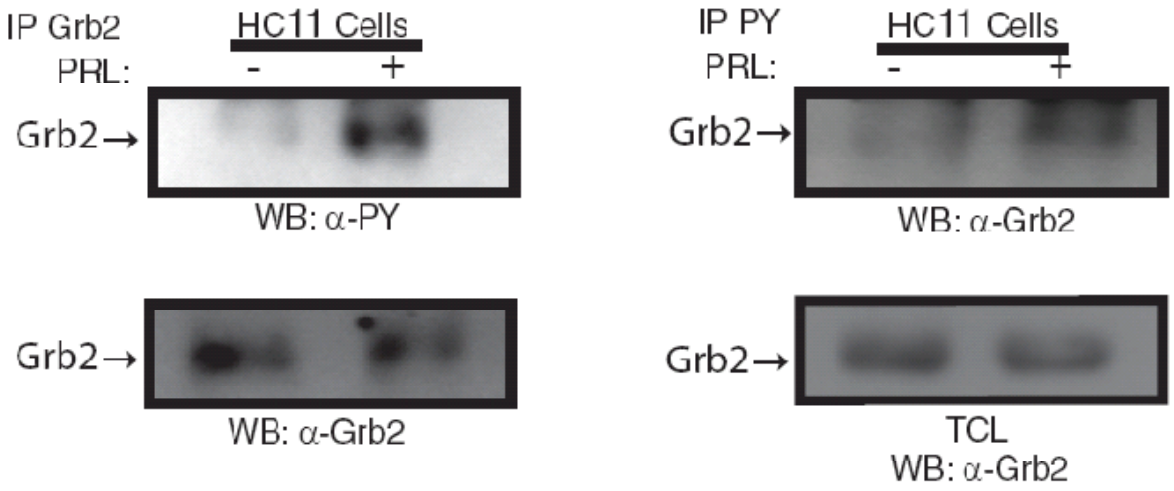

Figure 2.3.6.4: Grb2 tyrosine phosphorylation in HC11 cells. Left panel - Starved differentiated HC11 cells were left untreated or treated with PRL (1 μg/mL) for 15 minutes. Cell lysates were immunoprecipitated with a polyclonal antibody to Grb2 and immunoblotted with a phospho-specific monoclonal antibody to phosphotyrosine (upper panel). The membrane was stripped and reprobed with a polyclonal antibody to Grb2 (lower panel). Right panel – Starved differentiated HC11 cells left untreated or treated with PRL (1 μg/mL) for 15 minutes. Cell lysates were immunoprecipitated with a polyclonal antibody to phosphotyrosine and immunoblotted with a polyclonal antibody to Grb2. Whole lysates were immunoblotted with a polyclonal antibody to Grb2 (lower panel). Results were confirmed in another experiment and in experiments using the phosphatase inhibitor sodium vanadate.

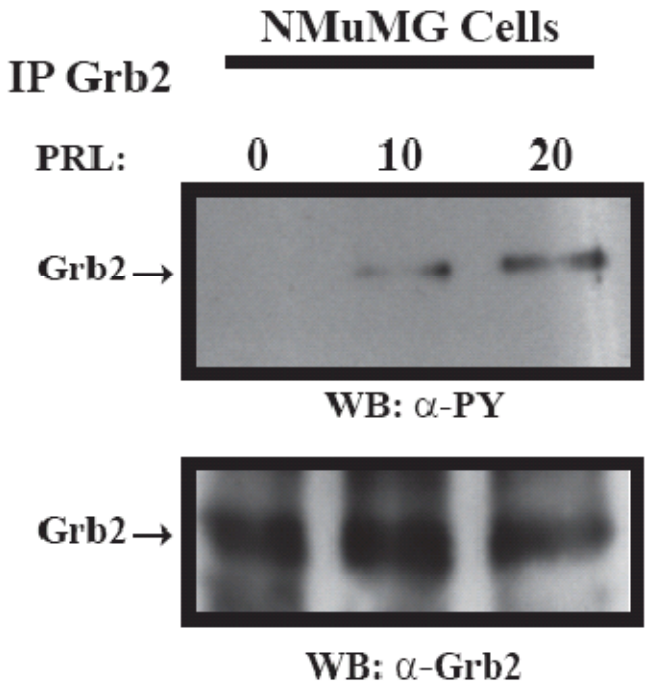

Figure 2.3.6.5: Grb2 tyrosine phosphorylation in NMuMG cells. Starved NMuMG cells were left untreated or treated with PRL (1 μg/mL) for the indicated time points. Cell lysates were immunoprecipitated with a polyclonal antibody to Grb2 and immunoblotted with a phospho-specific monoclonal antibody to phosphotyrosine (upper panel). The membrane was stripped and reprobed with a polyclonal antibody to Grb2 (lower panel). Results were confirmed in another experiment.

To further confirm PRL-induced tyrosine phosphorylation of Grb2 in mammary epithelial cells, we performed the reverse co-immunoprecipitation (Figure 2.3.6.4). To accomplish this, we immunoprecipitated the similar HC11 lysates described above using a polyclonal antibody against phosphorylated tyrosine residues and immunoblotted using a polyclonal antibody against Grb2. Compared to untreated cells, PRL was shown to induce the tyrosine phosphorylation of Grb2, further suggesting that PRL may induce the tyrosine phosphorylation of Grb2 in mammary epithelial cells. Equal expression of Grb2 in cell lysates was confirmed by immunoblotting whole cell lysates with a polyclonal antibody to Grb2. To potentiate the tyrosine phosphorylation of Grb2 in HC11 cells, we next overexpressed myc-tagged Grb2 (Figure 2.3.6.6). Since it is previously suggested that tyrosine phosphorylation of Grb2 is tightly regulated (Lowenstein et al., 1992; Rozakis-Adcock et al., 1992) and our data suggests that Grb2 tyrosine phosphorylation is negatively regulated by the catalytic activity of tyrosine phosphatase (Figure 2.3.6.1), cells were pre-treated with the tyrosine phosphatase inhibitor (sodium orthovanadate) before PRL stimulation to further potentiate the tyrosine phosphorylation detection. Myc-tagged Grb2 was immunoprecipitated using a polyclonal against myc-tag and then immunoblotted with a monoclonal antibody to phosphotyrosine residues. Interestingly, tyrosine phosphorylation of Grb2 was detected within 5 minutes of PRL stimulation and was still detected for up to 60 minutes.

Next, we examined the endogenous tyrosine phosphorylation of Grb2 in sodium orthovanadate pre-treated PRL-responsive HC11, MCF7 and T47D cell lines (Figure 2.3.6.7). Starved HC11 were pre-treated 30 minutes with sodium orthovanadate before being left untreated or treated with ovine PRL for 15 minutes. Grb2 was then immunoprecipitated from cell lysates using a polyclonal antibody against Grb2 and immunoblotted with a monoclonal antibody against phosphotyrosine residues. Similar to unpretreated HC11 cells (Figure 2.3.6.4); PRL was shown to induce the tyrosine phosphorylation of Grb2. Equal expression of Grb2 in immunoprecipitates was confirmed by reprobing the immunoblot with a polyclonal antibody to Grb2. For experiment using the human breast cancer cells MCF7 and T47D (Figure 2.3.6.7), cells were pre-treated with sodium orthovanadate for 30 minutes before being left untreated or treated with human PRL (100 ng/mL) for 15 minutes. Immuno -precipitation and –detection were completed as described above for HC11 cells. PRL was shown to induce

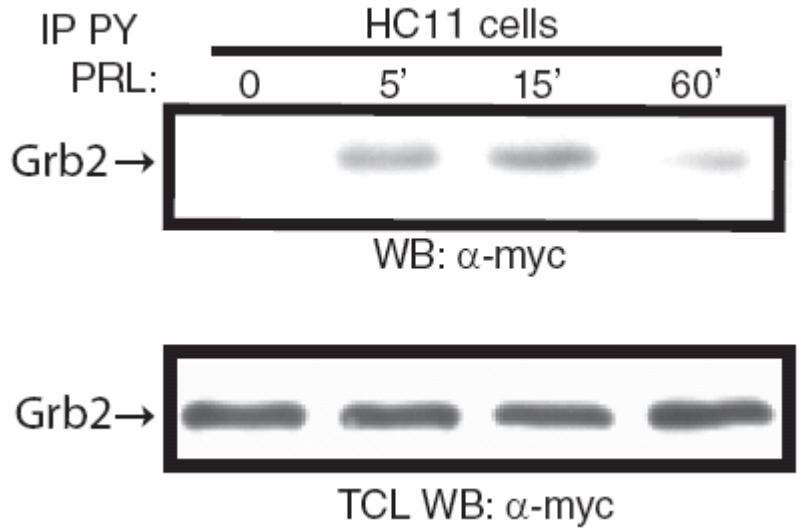

Figure 2.3.6.6: Tyrosine phosphorylation of myc-Grb2 in HC11 cells. HC11 cells were transiently transfected with the myc-tagged Grb2. Cells were pre-treated with the phosphatase inhibitor sodium orthovanadate for 30 minutes. Cell were then left untreated or treated with PRL (1 μg/mL) for the indicated time points. Cell lysates were immunoprecipitated with a polyclonal antibody to phosphotyrosine residues and immunoblotted with a monoclonal antibody to myc-tag (upper panel). Equal transfection was confirmed by immunoblotting whole lysates with a monoclonal antibody to myc-tag (lower panel).

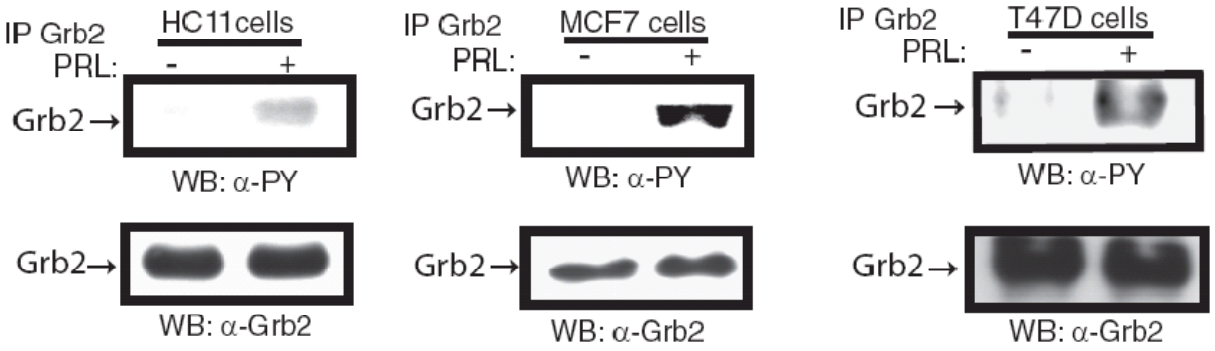

Figure 2.3.6.7: Tyrosine phosphorylation of Grb2 in PRL-responsive cells. Left panel - HC11 cells pre-treated with the tyrosine phosphatase inhibitor sodium orthovanadate. Cells were then left untreated or treated with PRL (1 μg/mL) for 15 minutes. Cell lysates were immunoprecipitated with a polyclonal antibody to Grb2 and immunoblotted with a monoclonal antibody to phosphotyrosine (upper panel). The membrane was reprobed with a polyclonal antibody to Grb2 (lower panel). Centre panel – MCF7 cells pre-treated with the tyrosine phosphatase inhibitor sodium orthovanadate. Cells were then left untreated or treated with PRL (1 μg/mL) for 15 minutes. Cell lysates were immunoprecipitated with a polyclonal antibody to Grb2 and immunoblotted with a monoclonal antibody to phosphotyrosine (upper panel). The membrane was reprobed with a polyclonal antibody to Grb2 (lower panel). Right panel – T47D cells pre-treated with the tyrosine phosphatase inhibitor sodium orthovanadate. Cells were then left untreated or treated with PRL (1 μg/mL) for 15 minutes. Cell lysates were immunoprecipitated with a polyclonal antibody to Grb2 and immunoblotted with a monoclonal antibody to phosphotyrosine (upper panel). The membrane was reprobed with a polyclonal antibody to Grb2 (lower panel). Results were confirmed in two other experiments.

the tyrosine phosphorylation of Grb2 in both breast cancer cell lines suggesting that PRL can induce the tyrosine phosphorylation in both normal and cancerous mammary cells and that tyrosine phosphorylation of Grb2 may be a mechanism utilized to suppress breast cancer cell proliferation (Figure 2.4.1.4). Together, the above results suggest that Grb2 is a substrate of the PRL signalling pathway in both mammary epithelial and cancer cells and that the tyrosine phosphorylation of Grb2 is tightly regulated by tyrosine phosphatises such as SHP-2.

2.3.7. Characterizing the mechanism of PRL-induced Grb2 tyrosine phosphorylation

Next, we sought to characterize the mechanism of Grb2 tyrosine phosphorylation. As Jak2 kinase but not Src kinase mediates PRL-induced Grb2 tyrosine phosphorylation (Figure 2.3.6.3) and PRL generally signals are mediated through Jak2, we hypothesized that Jak2 is the kinase involved in Grb2 phosphorylation. Previously, our laboratory examined the ability of Jak2 to interact with Grb2. However, we were unable to detect a direct Jak2-Grb2 interaction by far western analysis. Our results suggest that Jak2 is involved in Grb2 phosphorylation, but an intermediate protein(s) link Jak2 to Grb2 phosphorylation.

We next blocked Jak2 kinase activity using the Jak2 inhibitor to assess whether Jak2 kinase activity is required to induce Grb2 tyrosine phosphorylation (Figure 2.3.7.1). HC11 cells were pre-treated with either DMSO (control) or Jak2 inhibitor (25 mM) for 2 hours before being left untreated or treated with PRL for 15 minutes. Tyrosine phosphorylated Grb2 was then immunoprecipitated using a polyclonal antibody to Grb2. Immunoprecipitates were immunodetected with a monoclonal antibody to phosphotyrosine residues. As expected, PRL-induced Grb2 tyrosine phosphorylation in control DMSO pre-treated cells. More interestingly, pre-treatment with the Jak2 inhibitor blocked PRL-mediated tyrosine phosphorylation of Grb2 suggesting that Grb2 phosphorylation requires Jak2 kinase activity. The ability of the Jak2 inhibitor (25 μM) to inhibit Jak2 kinase activity was demonstrated above in Figure 2.3.5.1. Together, our results suggest that Grb2 is recruited to Jak2 via an intermediate protein or Jak2 is involved in the activation of a signalling cascade leading to Grb2 tyrosine phosphorylation. Previously in our laboratory, we demonstrated that Grb2 is

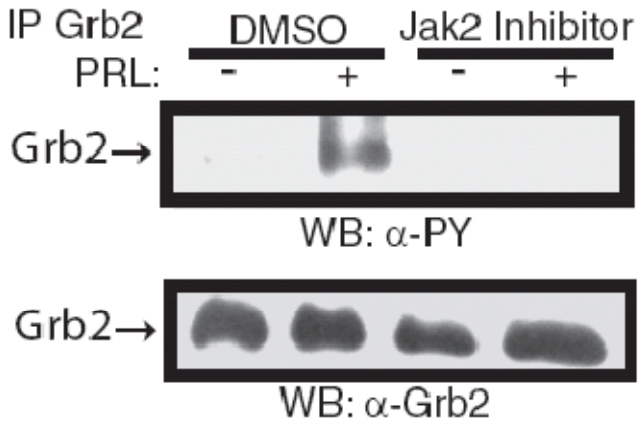

Figure 2.3.7.1: Jak2 kinase activity is essential for the PRL-induced Grb2 tyrosine phosphorylation. Serum starved differentiated HC11 cells were pre-treated with, DMSO or the Jak2 inhibitor (25μM) for 90 minutes, and sodium orthovanadate for 30 minutes. Cells were then left unstimulated or stimulated with PRL (1 μg/mL) for 15 minutes. Cell lysates were immunoprecipitated with a polyclonal antibody to Grb2 and immunoblotted with a monoclonal antibody to phosphotyrosine residues. The membrane was reprobed with a polyclonal antibody to Grb2. Results were confirmed in one other experiment.

recruited to Jak2 through the C-terminal SHP-2 (Minoo et al., 2003). However, further experiments are needed to confirm if the C-terminal of SHP-2 is essential for Grb2 tyrosine phosphorylation in mammary epithelial cells. Moreover, the role of SHP-2 in mammary cell proliferation should also for be determined.

Another mechanism of PRL-induced Grb2 tyrosine phosphorylation may involve the tyrosine kinase activated CDC42 kinase-1 (ACK-1). ACK-1 is a regulator of EGF signalling (Shen et al., 2007; Worby and Margolis, 2000). Since ACK-1 interacts with the SH3 domains of Grb2, the domains phosphorylated by PRL (Yokoyama and Miller, 2003) and negatively regulate the MAPK pathway (Shen et al., 2007; Worby and Margolis, 2000), ACK-1 was considered a potential kinase involved in PRL-mediated Grb2 tyrosine phosphorylation. Preliminary results, not shown here, suggest that PRLR/Jak2 complex tyrosine phosphorylates/activates ACK-1. In immunoprecipitations of ACK-1, basal activation of ACK-1 was detected in HC11 cells. However, PRL enhanced ACK activation. Moreover, our preliminary data suggests that PRL mediates ACK-1-Jak2 and ACK-1-Grb2 interactions in HC11 cells. These results are preliminary in nature and the role of ACK-1 in mediating Grb2 tyrosine phosphorylation and EGF signaling needs to be confirmed. Moreover, future experiments are required to determine whether the kinase activity of ACK-1 is essential for Grb2 tyrosine phosphorylation and whether ACK-1 regulates mammary epithelial cell proliferation.

Together, PRL-induced Jak2 activation was shown to lead to Grb2 tyrosine phosphorylation. However, the mechanism of Jak2-induced Grb2 tyrosine phosphorylation is yet to be fully elucidated. We hypothesize that Grb2 is recruited to Jak2 via SHP-2, thus allow Jak2 to phosphorylate Grb2 (Minoo et al., 2003) or Jak2 promotes Grb2 phosphorylation by activating an intermediate tyrosine kinase, possibly ACK-1.

2.3.8. PRL utilizes Grb2 tyrosine phosphorylation to attenuate EGF signalling

Since Grb2 tyrosine phosphorylation is implicated in the negative regulation of the MAPK pathway by interfering with the Grb2-Sos interaction (Li et al., 2001) and our results suggest that Grb2 is a substrate of the PRLR/Jak2 complex, we next determined whether PRL utilizes Grb2 tyrosine phosphorylation as a mechanism to inhibit EGF-induced MAPK activation in mammary cells. To characterize the role of the tyrosine phosphorylation of Grb2

in the PRL/EGF crosstalk, we first examined the pattern of Grb2 tyrosine phosphorylation in HC11 cells stimulated with PRL, EGF or a combination of PRL and EGF (Figure 2.3.8.1). HC11 cells were pre-treated with the phosphatase inhibitor sodium orthovanadate for 30 minutes before being left untreated or treated either with PRL, EGF or PRL and EGF for 15 minutes. Cell lysates were immunoprecipitated with a polyclonal antibody against Grb2 and immunoblotted with a monoclonal antibody to phosphotyrosine residues. While PRL potently induced tyrosine phosphorylation of Grb2, EGF was unable to significantly induce Grb2 tyrosine phosphorylation. Furthermore, the level of Grb2 phosphorylation in cells co-treated with the combination of PRL and EGF was comparable to that observed in PRL treated cells suggesting that PRL-induced Grb2 phosphorylation is unaffected by EGF. Equal Grb2 immunoprecipitates were confirmed by reprobing with a polyclonal antibody to Grb2. Therefore, within the PRL/EGF signalling crosstalk Grb2 is tyrosine phosphorylated and may be used by PRL to negatively regulate EGF signalling.

To confirm that PRL-induced Grb2 tyrosine phosphorylation is unaffected by EGF, we overexpressed myc-tagged Grb2 in HC11 cells (Figure 2.3.8.2). Cells were pre-treated with sodium orthovanadate before being left untreated or treated with PRL, EGF or the combination of PRL and EGF. Cell lysates were immunoprecipitated with a polyclonal antibody to Myc-tag and immunoblotted with a monoclonal antibody to phosphotyrosine residues. PRL induced the tyrosine phosphorylation of myc-tagged Grb2, whereas; EGF did not promote Grb2 phosphorylation. Moreover, PRL-induced tyrosine phosphorylation was maintained when co-treated with EGF. Together, our data suggests that PRL is more effective in inducing Grb2tyrosine phosphorylation compared to EGF and that in samples co-treated with PRL and EGF, the ability of PRL to induce Grb2 tyrosine phosphorylation is unaffected by EGF.

Next, we characterized the role of PRL-induced Grb2 phosphorylation in mediating EGF-induced MAPK activation. (Figure 2.3.8.3). To do this, we utilized a mutant form of Grb2 (Grb2YF) that is unable to be tyrosine phosphorylated. HC11 cells were transiently

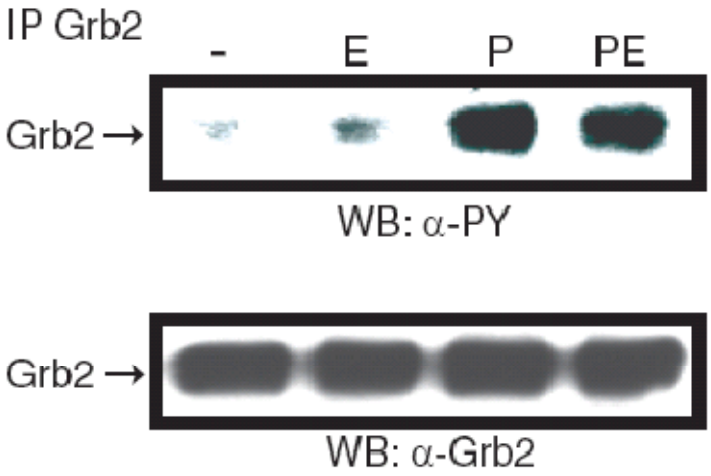

Figure 2.3.8.1: EGF is unable to inhibit PRL-induced Grb2 tyrosine phosphorylation. Serum starved differentiated HC11 cells were initially pre-treated with the tyrosine phosphatase inhibitor sodium orthovanadate for 30 minutes before being left untreated or treated with EGF (10 ng/mL), PRL (1 μg/mL) or PRL (1 μg/mL) and EGF (10 ng/mL) for 15 minutes. Cell lysates were immunoprecipitated with a polyclonal antibody to Grb2 and immunoblotted with a monoclonal antibody to phosphotyrosine (upper panel). The membrane was stripped and reprobed with a polyclonal antibody to Grb2 (lower panel).

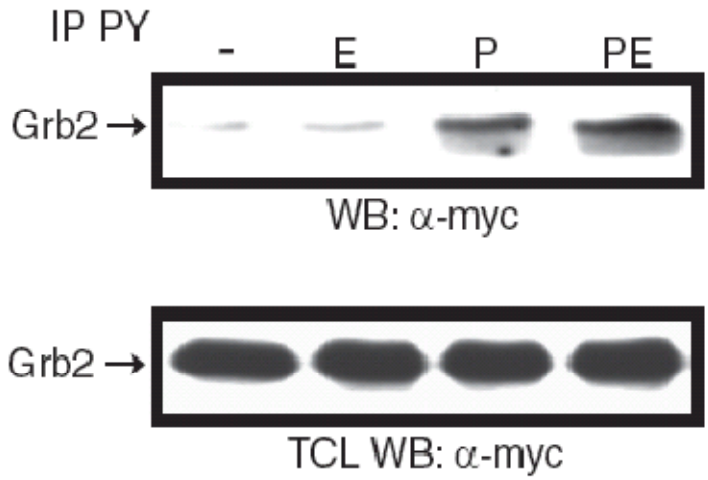

Figure 2.3.8.2: EGF is unable to inhibit PRL-induced tyrosine phosphorylation of myc-Grb2. Differentiated HC11 cells were transfected with an expression vector encoding myc-Grb2. Serum starved cells were pre-treated with sodium orthovanadate (50 μg/ml) for 30 minutes and then stimulated with PRL (1 μg/mL) for the time points indicated. Cell lysates were immunoprecipitated using a polyclonal antibody to phosphotyrosine followed by immunoblotting with a monoclonal antibody to myc-tag (upper panel). Total cell lysates were immunoblotted with a monoclonal antibody to myc-tag (lower panel).

mock-transfected (control) or transfected with Grb2YF. Cells were then treated with either EGF or the combination of PRL and EGF for the indicated time points. As expected in mock-transfected cells, EGF induced a robust activation of the MAPK and PRL was capable of attenuating EGF-induced MAPK activation. In cells overexpressing Grb2YF, EGF activated MAPK to a similar extent as observed in mock-transfected cells. However, PRL was unable to attenuate EGF-induced MAPK activation suggesting that PRL utilizes Grb2 tyrosine phosphorylation as a negative regulatory mechanism to EGF signals leading to MAPK activation. Alternative mechanisms of PRL-mediated suppression of EGF-induced MAPK activation have been proposed. For example, PRL has been shown to act through a protein kinase C (PKC) dependent mechanism (Fenton and Sheffield, 1997), as well as induce EGFR threonine phosphorylation (Quijano and Sheffield, 1998). Together, PRL negatively regulates EGF-induced MAPK activation via multiple signalling mechanisms; one of which is through the tyrosine phosphorylation of Grb2.

It has been suggested that the ability of Grb2 tyrosine phosphorylation to suppress MAPK activation is through the inhibition of Ras activation. Therefore, we next examined the effects of overexpressing the Grb2YF mutant on the mediation of EGF-induced Ras activation by PRL. To measure the levels of activated Ras, we pulldown activated Ras using the Ras binding domain of Raf, which interacts only with activated form of Ras. HC11 cells were mock-transfected or transfected with Grb2YF before being left untreated or treated with either EGF or PRL and EGF for the indicated time points (Figure 2.3.8.4). In control mock-transfected cells, EGF was shown to activate Ras and PRL attenuated EGF-induced Ras activation. Overexpression of Grb2YF reversed the PRL-induced suppression of EGF-mediated Ras activation. Equal presence of the GST-Ras binding domain of Raf was confirmed by reprobing the membrane with a monoclonal antibody to GST. Equal Ras protein levels were confirmed by immunoblotting whole lysates with a monoclonal antibody to Ras. Together, our results thus far suggest that PRL-induced Grb2 phosphorylation inhibits EGF-induced MAPK activation via the suppression of Ras activation.

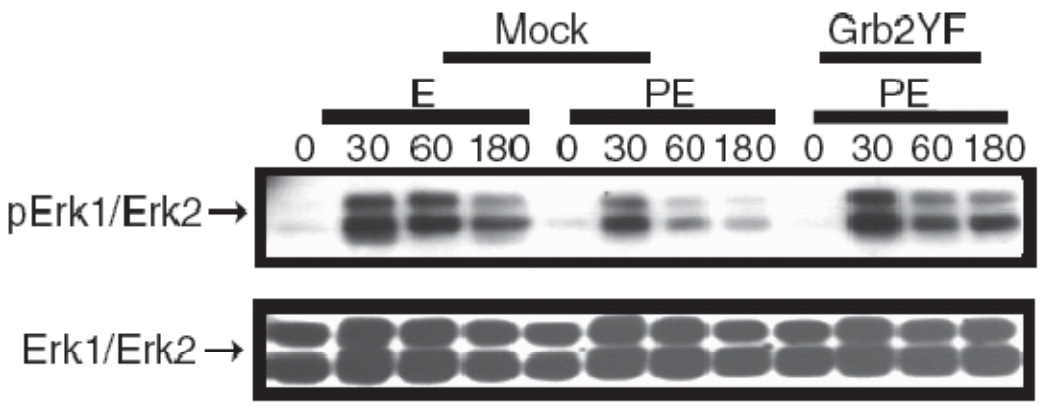

Figure 2.3.8.3: Grb2 tyrosine phosphorylation is essential to inhibit EGF-induced MAPK activation. Differentiated HC11 cells were transfected with vector alone or an expression plasmid encoding myc-Grb2YF. Serum starved cells were left unstimulated or stimulated with EGF (10 ng/mL) or PRL (1 μg/mL) and EGF (10 ng/mL) for the indicated time points. Total lysates were immunoblotted using a polyclonal antibody to phospho-Erk1/Erk2 (upper panel). The membrane was stripped and reblotted with a polyclonal antibody to Erk1/Erk2 (lower panel).

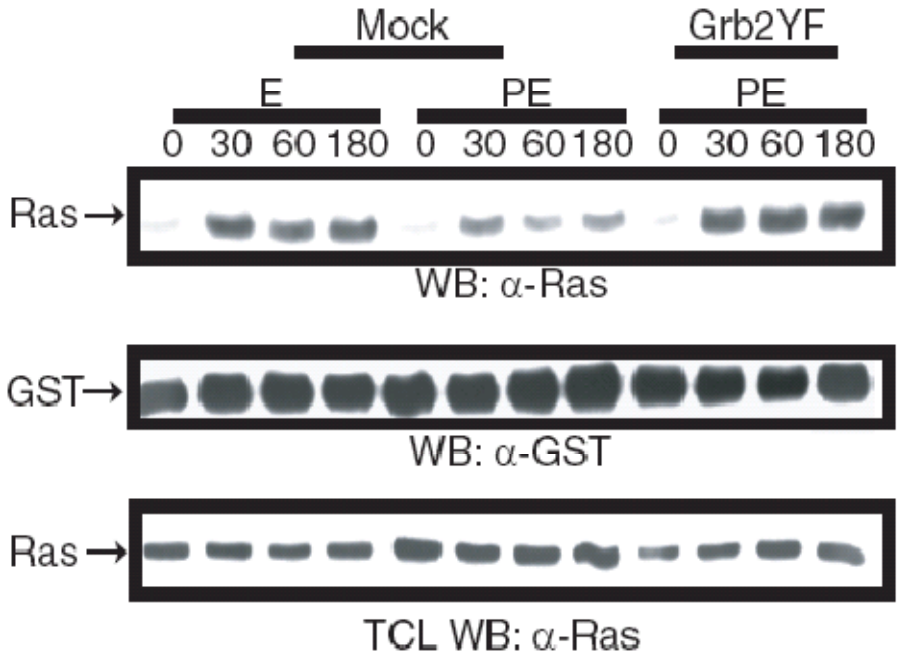

Figure 2.3.8.4: Grb2 tyrosine phosphorylation blocks EGF-induced Ras activation. Differentiated HC11 cells were transfected with vector alone or an expression plasmid encoding myc-Grb2YF. Serum starved cells were left unstimulated or stimulated with EGF (10 ng/mL) or PRL (1 μg/mL) and EGF (10 ng/mL) for the indicated time points. Pulldown assay of Ras-GTP was performed using RBD-GST-fusion protein and immunodetected using a monoclonal antibody to Ras (upper panel). The same membrane was stripped and reprobed with a polyclonal antibody to GST (middle panel). HC11 cell lysates were immunodetected with monoclonal antibody to Ras (lower panel).

The ability of Grb2 tyrosine phosphorylation to attenuate Ras activation is attributed to the inability of Grb2 to interact through its SH3 domains with Sos (Downward, 1994). Therefore, we next examined the influence of PRL-induced Grb2 phosphorylation on the Grb2/Sos interaction (Figure 2.3.8.5). HC11 cells were mock-transfected or transfected with the Grb2YF mutant. Cells were then left untreated or treated with either EGF or the combination of PRL and EGF. Mock-transfected cell lysates were immunoprecipitated using a polyclonal antibody to Grb2 and immunoblotted with a monoclonal antibody to Sos. Grb2YF-transfected cell lysates were immunoprecipitated with a polyclonal antibody to myc-tag and immunoblotted with a monoclonal antibody to Sos. In control mock-transfected cells, while EGF led to a significant increase in Grb2/Sos complex formation, PRL interfered with the ability of EGF to induce Grb2/Sos interaction. Interestingly, in myc-Grb2YF expressing cells, we observed no significant difference in the amount of Sos in complex with Grb2YF in samples treated with EGF or PRL/EGF suggesting that PRL interferes with EGF-induced Grb2/Sos interaction through the tyrosine phosphorylation. All together, our results suggest that PRL suppresses EGF signals to the MAPK activation at the level of Ras activation by blocking Grb2/Sos interaction.

2.3.9. Grb2 tyrosine phosphorylation is essential to inhibit EGF-induced cell proliferation

We demonstrated that PRL attenuates EGF signals to the MAPK activation through Grb2 tyrosine phosphorylation, so next we examined whether the influence of Grb2 phosphorylation in PRL-mediated suppression of EGF-induced cell proliferation. To examine the role of Grb2 tyrosine phosphorylation in cell proliferation we established a HC11 cell line stably overexpressing the myc-Grb2YF mutant (Figure 2.4.9.1). We selected 3 positively expressing HC11-Grb2YF clones (HC11-Grb2YF1, -Grb2YF2 and –Grb2YF3) and 2 control vector clones (HC11-vector1 and HC11-vector2). Positive Myc-Grb2YF expression was confirmed in whole cell lysates by immunoblotting with a monoclonal to Myc-tag.

Before examining the proliferative properties of the stable clones, we first determined whether the ligands activated MAPK in HC11-Grb2YF clones in a similar fashion as the transient expression experiments described above in Figure 2.3.8.3. HC11-vector and HC11-

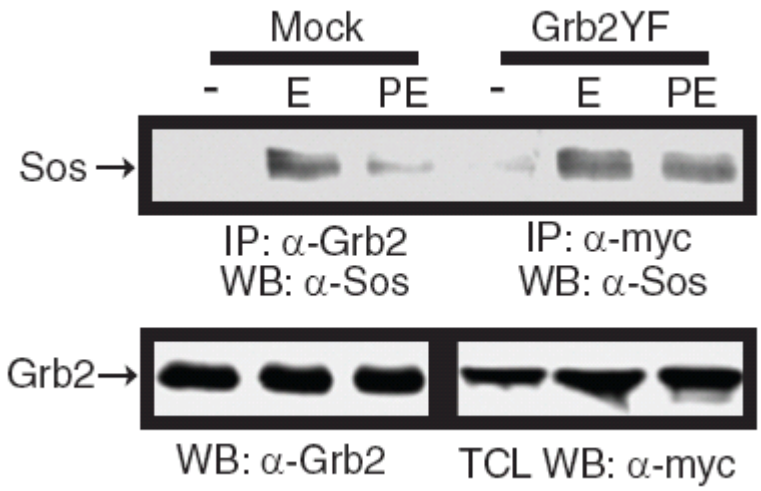

Figure 2.3.8.5: Grb2 tyrosine phosphorylation interferes with Grb2-Sos interaction. Differentiated HC11 cells were transfected with vector alone or an expression vector encoding myc-Grb2YF. Serum starved cells were left unstimulated or stimulated with EGF (10 ng/mL) or PRL (1 μg/mL) and EGF (10 ng/mL) for 15 minutes. Cell lysates were immunoprecipitated with a polyclonal antibody to Grb2 or a monoclonal antibody to myc and immunoblotted with a monoclonal antibody to Sos (upper panel). The membrane was stripped and reblotted with polyclonal antibody to Grb2 (left lower panel) or a monoclonal antibody to myc-tag (right lower panels).

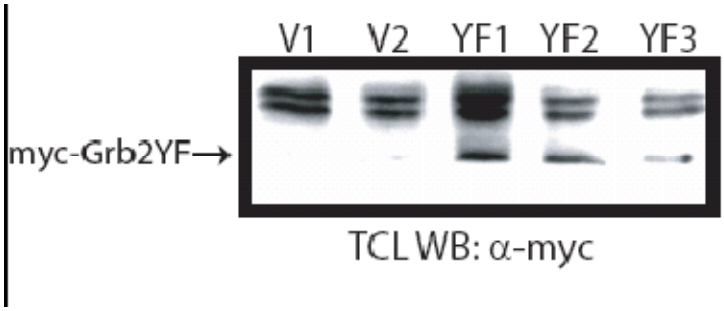

Figure 2.3.9.1: Positively expressing Grb2YF clones. HC11 cells were transfected with vector alone or an expression plasmid encoding myc-Grb2YF. Stable clones were selected by supplementing media with the antibiotic G418 (250 mM). Cell lysates of isolated stable clones were immunoblotted with a monoclonal antibody to myc-tag.

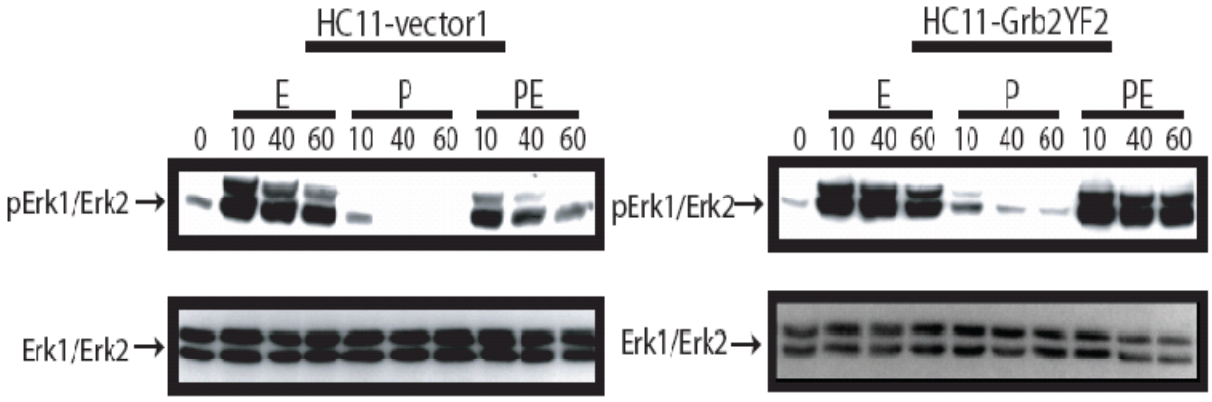

Figure 2.3.9.2: PRL is unable to block EGF-mediated MAPK in HC11-Grb2YF clones. Starved differentiated HC11-vector1 (Left panel) or HC11-Grb2YF2 (Right panel) clones were then left untreated or treated with PRL (1 μg/mL), EGF (10 ng/mL) or PRL (1 μg/mL) and EGF (10 ng/mL) for the indicated time points. Cell lysates were immunoblotted with a polyclonal antibody to phospho-Erk1/Erk2 (upper panels) and to Erk1/Erk2 (lower panels). Blots shown are of representative of all HC11-vector and HC11-Grb2YF isolated clones.

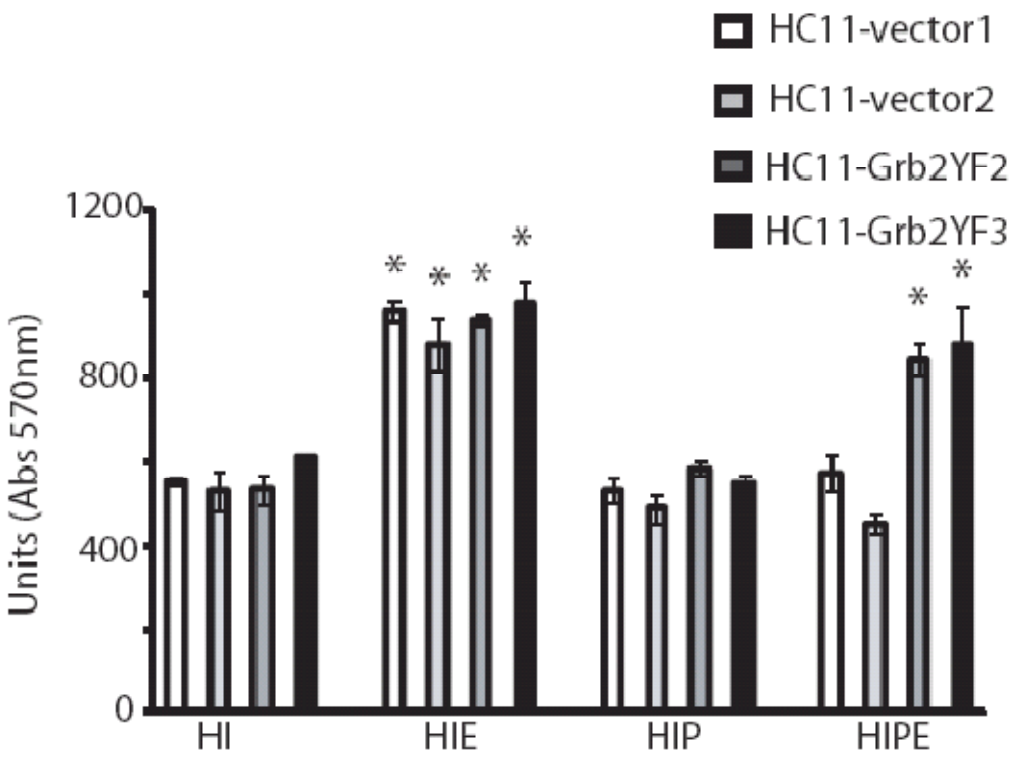

Figure 2.3.9.3: Grb2 tyrosine phosphorylation is essential to block EGF-induced cell growth. HC11-vector1, HC11-vector2, HC11-Grb2YF2 and HC11-Grb2YF3 ($1.5x10^3$ cells) were grown for an overnight period in the absence or presence of PRL (1 μg/mL) in media supplemented with 2% FBS, insulin (5 μg/mL), and hydrocortisone (1 μM). Cells were then left untreated or treated with EGF (10 ng/mL) for 72 hours. MTT assay was performed as described in Materials and Methods and absorbance was read at 570nm. Results are expressed as the means ± standard error of the means of triplicates of three different experiments. HI = hydrocortisone, insulin; HIE = hydrocortisone, insulin, EGF; HIP = hydrocortisone, insulin, PRL; HIPE = hydrocortisone, insulin, PRL, EGF. (*p<0.05)

Grb2YF cells were stimulated with EGF, PRL or PRL/EGF for the indicated time points. The activation of MAPK (Erk1/Erk2) was then immunodetected in whole cell lysates using a polyclonal antibody to phospho-Erk1/Erk2 (Figure 2.3.9.2). Similar to in parental HC11 cells, vector transfected cells (HC11-vector) EGF was shown to significantly induce MAPK activation, whereas; PRL weakly activated the MAPK pathway. Moreover, PRL attenuated EGF-induced MAPK activation. In Grb2YF overexpressing cells (HC11-Grb2YF), while EGF robustly activated the MAPK pathway, PRL only mildly activated the MAPK pathway. Most importantly, the stable expression of the Grb2YF mutant reversed the ability of PRL to block EGF-induced MAPK pathway further confirming the importance of PRL-induced Grb2 phosphorylation in attenuating EGF signalling.

After confirming that PRL was unable to suppress EGF-induced MAPK in Grb2YF-expressing HC11 cells, we next determined whether PRL-induced tyrosine phosphorylation was essential for PRL-induced suppression of EGF-induced cell proliferation. To do this, we first characterized the ligand-induced regulation of cell proliferation of both HC11-vector and HC11-Grb2YF clones via MTT assay. HC11-vector (HC11-vector1 and –vector2) and HC11-GrbYF (HC11-Grb2YF2 and –Grb2YF3) clones where grown overnight absence or presence of PRL in media supplemented with 2% FBS, hydrocortisone and insulin. Clones were then left untreated or treated with EGF for 48 hours (Figure 2.3.9.3). In control HC11-vector clones, while EGF induced cell proliferation (p=0.048), PRL was non-proliferative. Moreover, PRL significantly blocked EGF-mediated cell growth (p=0.048). In HC11-Grb2YF clones, EGF was shown to promote cell proliferation to a similar degree as in control cells. Moreover, PRL was unable to induce cell proliferation. Most interestingly, the stable expressing of Grb2YF impeded the ability of PRL to block EGF signals to cell growth. While if tyrosine phosphorylation one could speculate that overexpression of the Grb2YF mutant would be transforming and promote cellular growth independent of ligand stimulation, we were unable to detect constitutive cellular growth. By observation, the HC11-Grb2YF clones grew at a higher rate than HC11-vector clones. Therefore, we speculate that the sensitivity of the MTT assay may be a marked limitation on our ability to observe the constitutive growth of these clones. Moreover, one would speculate that the combination of Grb2YF and EGF stimulation should potentiate the proliferative signal, yet we were unable to observe a potentiation effect. We speculate that EGF and Grb2YF combinatory effect was undetectable because of

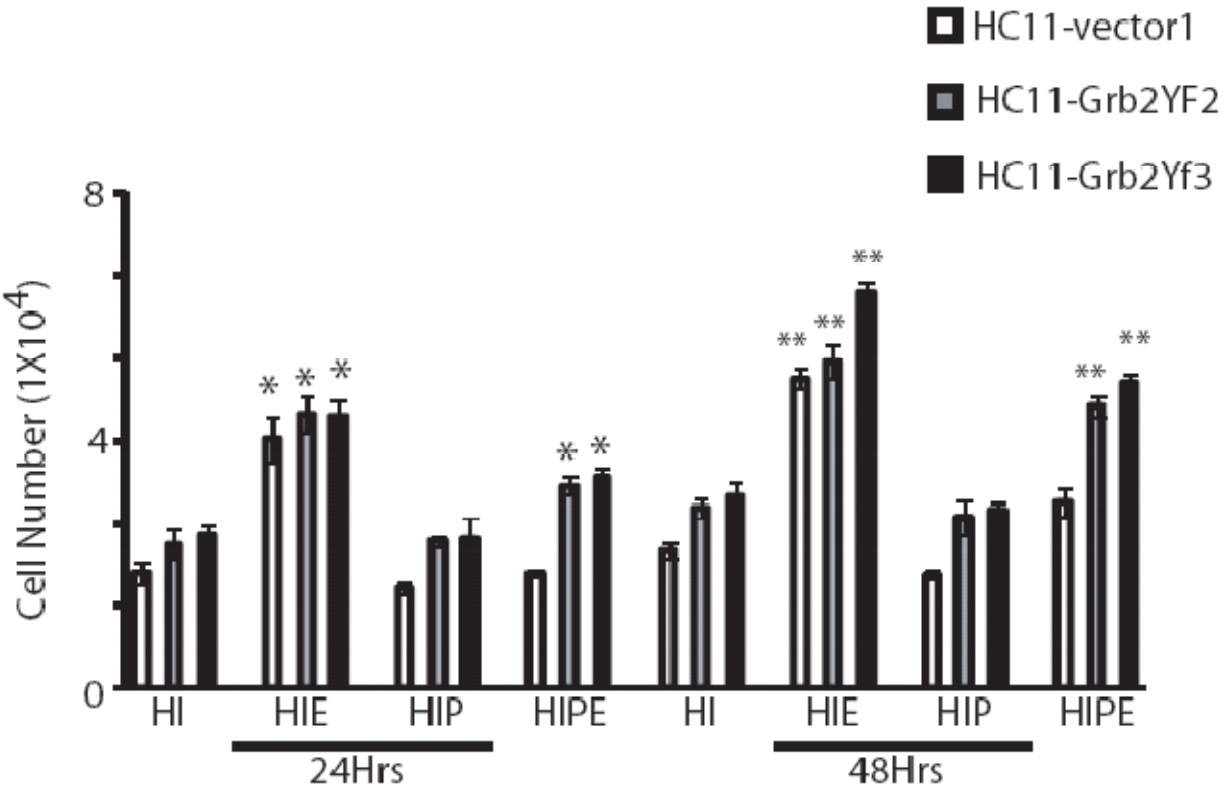

Figure 2.3.9.4: Grb2 tyrosine phosphorylation is essential to block EGF-induced cell proliferation. HC11-vector1, HC11-Grb2YF2 and HC11-Grb2YF3 ($2.5x10^4$) cells were grown for an overnight period in the absence or presence of PRL (1 μg/mL) in media supplemented with 2% FBS, insulin (5 μg/mL), and hydrocortisone (1 μM). Cells were then left untreated or treated with EGF (10 ng/mL) for 24 hours and 48 hours as indicated above. Cell number was determined and results are expressed as the means ± standard error of the means of triplicates of three different experiments (*p<0.05; **p<0.05).

the saturation limitation of the assay. These results suggest that PRL mediates EGF-induced cell proliferation is mediated via the tyrosine phosphorylation of Grb2.

To further confirm the role of PRL-induced phosphorylation of Grb2 in regulating EGF-induced cell growth, we examined the effects of PRL, EGF and PRL/EGF on cell proliferation via a cell counting assay (Figure 2.3.9.4). HC11-vector (HC11-vector1) and HC11-Grb2YF (HC11-Grb2YF2 and -Grb2YF3) clones were grown overnight in the absence or presence of PRL in media supplemented with 2% FBS, hydrocortisone and insulin before treatment with EGF for 24 and 48 hours as indicated. Similar to parental HC11 cells, while EGF-induced the growth of HC11-vector cells, PRL was non-proliferative. Moreover, PRL suppressed EGF-induced proliferation in this cell line. The overexpression of the Grb2YF mutant was shown to enhance the ligand-independent growth of HC11 cells. Moreover, while EGF significantly induced the growth of HC11-Grb2YF clones at both 24 (p=0.034) and 48 (p=0.022) hours, PRL do not enhance the growth of this cell line. Most importantly, the stable expression of Grb2YF blocked the ability of PRL to attenuate EGF-mediated cell proliferation further supporting the role for of tyrosine phosphorylation of Grb2 in the PRL-induced negative regulation of EGF signals to cell growth. Since the limitations of signal saturation and sensitivity present in the MTT assay is not a factor in cell counting assays, we were able to better characterize the proliferative effects of the Grb2YF mutant. HC11-Grb2YF clones were shown to have a higher ligand-independent growth compared to control HC11-vector clones. Moreover, a synergistic effect of Grb2YF and EGF stimulation on cellular proliferation was also observed in the HC11-GrbYF3 clone further emphasizing an anti-proliferative role for Grb2 tyrosine phosphorylation.

Together, our results demonstrate that there is an important negative crosstalk between EGF-Ras/MAPK and the PRL-Jak2/Stat5a pathways regulating the growth and differentiation of mammary epithelial and breast cancer cells. While PRL was shown to suppress EGF-induced MAPK activation and cell proliferation, EGF inhibited PRL-induced milk protein expression and cellular differentiation. We also indentified a novel role for the PRL-induced tyrosine phosphorylation of Grb2 in this negative regulatory crosstalk. PRL was shown to utilize the tyrosine phosphorylation of Grb2 as mechanism to suppress EGF-induced Ras/MAPK activation and thus cell proliferation.

Chapter 3: Further discussion

Mammary morphogenesis and carcinogenesis are regulated by multiple hormones and growth factors. In the above study we have examined the signalling crosstalk between the hormone PRL and the growth factor EGF in mammary epithelial and breast cancer cells. Our results indicate that PRL and EGF exhibit antagonistic properties that impacts mammary epithelial and breast cancer cell proliferation and differentiation. We characterized a negative crosstalk mechanism between the PRL-Jak2/Stat5a and EGF-Ras/MAPK pathways. EGF was shown to block PRL-induced cellular differentiation by interfering with Stat5a-mediated gene transcription without inhibiting PRL proximal signaling cascade such as Jak2 activation. PRL was shown to attenuate EGF-induced MAPK activation and cell proliferation through the Jak2 dependent tyrosine phosphorylation of the adaptor protein Grb2. (Figure 3.1.1).

3.1. Implication of PRL/EGF crosstalk in mammary gland development

During mammary gland development EGF plays a crucial role in ductal formation and outgrowth during puberty (Troyer and Lee, 2001) and PRL regulates the differentiation of terminal end buds to alveolar structures and the synthesis milk proteins during pregnancy and lactation (Oakes et al., 2008). In agreement with previous studies (Horsch et al., 2001; Marte et al., 1995), our study demonstrated that EGF signals are inhibitory to PRL-induced mammary epithelial cellular differentiation as measured by the induction of milk protein gene expression. Our study also demonstrated that PRL-induced proximal signalling events such as Jak2 activation and Stat5a activation, nuclear translocation and DNA binding activity was unaffected by EGF suggesting that EGF mediates PRL signals via the up- or down-regulation of co-regulators or by regulating the transcriptional activity of Stat5a. Serine phosphorylation of Stat5a is implicated in the negative regulation of its transcriptional activity (Beuvink et al., 2000; Yamashita et al., 2001). However, we were unable to detect the serine phosphorylation of Stat5a using phospho-specific antibodies to serine residues on Stat5a. The mechanism through which EGF mediates PRL signals is yet to be fully elucidated and future research will be required to define this mechanism.

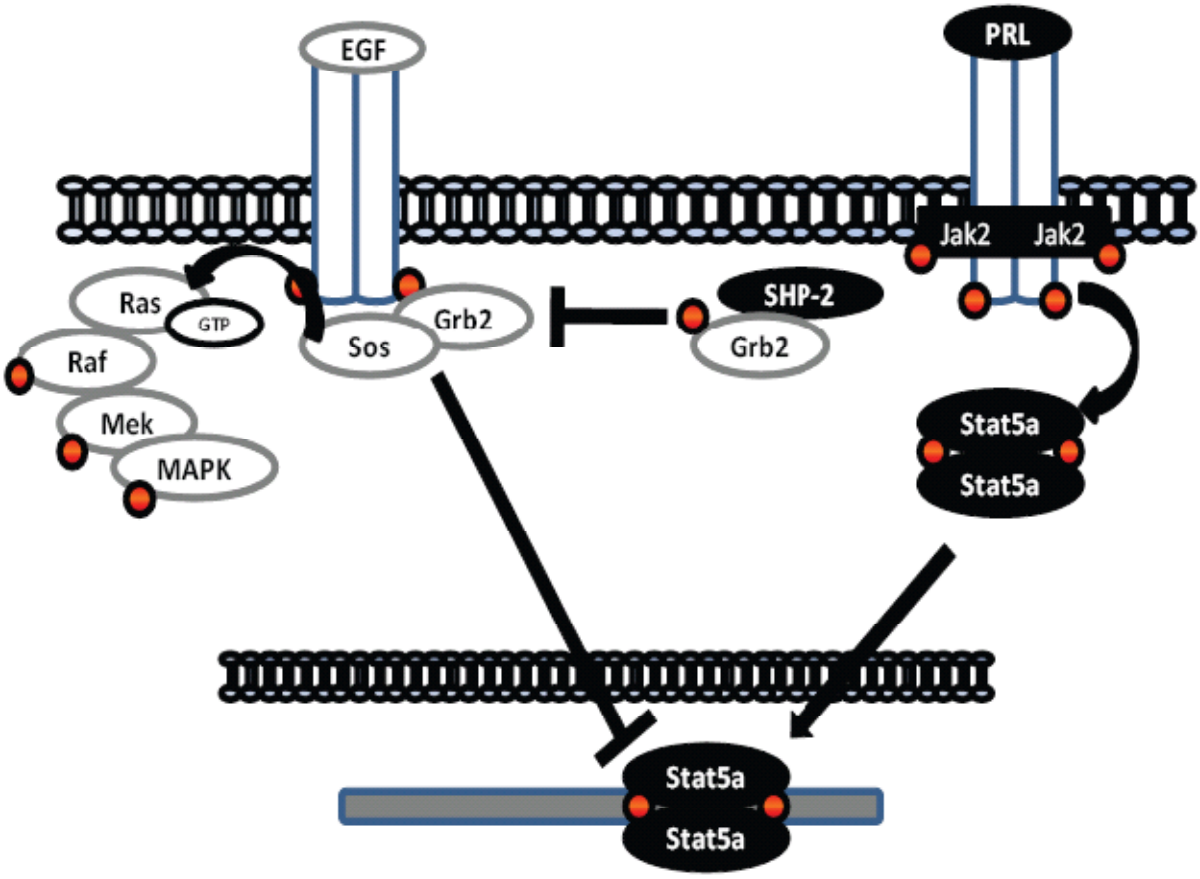

Figure 3.1.1: PRL/EGF negative crosstalk in mammary epithelial cells. Both PRL and EGF play important roles in mammary epithelial cell growth and differentiation. We propose that EGF blocks PRL-induced cellular differentiation by interfering with Stat5a transcriptional activity without inhibiting PRL proximal signaling cascade, suggesting a nuclear signaling event, whereas PRL suppressed EGF-induced MAPK activation and cell proliferation through tyrosine phosphorylation of Grb2 implicating a cytoplasmic signaling event.

We demonstrated that PRL suppresses EGF-induced MAPK activation. However, a previous study (Marte et al., 1995) as well as findings in our laboratory suggests that EGF mediates PRL-induced milk protein synthesis via MAPK activation. This suggests that the attenuation of MAPK activation induced by PRL (Figure 2.4.2.1A) is not substantial enough to inhibit the ability of EGF to abolish PRL-induced cellular differentiation.

Altogether, this suggests that EGF is a potent inhibitor of PRL signalling and may act at multiple levels to block PRLR/Jak2/Stat5a-induced mammary epithelial cell differentiation. Moreover, the PRL-induced attenuation of MAPK activation is insufficient to block EGF-mediated negative regulation of cellular differentiation. Based on these findings, one could speculate that during puberty EGF-induced inhibition of PRL is required to promote the proper growth of the ductal network within the mammary and prevent the premature differentiation of the mammary gland. Furthermore, one could also speculate that EGF promotes breast cancer progression by blocking PRL-induced differentiation.

PRL-induced suppression of EGF signals may also ensure proper mammary morphogenesis. PRL is essential for the differentiation of terminal end buds and milk synthesis during pregnancy and lactation (Oakes et al., 2008). To promote differentiation of the mammary gland, one could hypothesize that PRL negatively regulates EGF-proliferative signals allowing for alveolar differentiation. This confirmed by the inability of PRL to exert its negative effects on EGF-mediated proliferation in the absence of the differentiation factors hydrocortisone and insulin. When HC11 cells were left untreated or treated with EGF, PRL or PRL and EGF for 72 hours, no significant difference in cell proliferation was observed in samples treated with EGF alone and the combination of PRL and EGF suggesting that within the undifferentiated mammary epithelium PRL does not exert a negative regulation of EGF-mediated cell growth. Since Hydrocortisone and insulin play an important role in the differentiation of mammary epithelial cells (Brisken et al., 2002; Michael et al., 2003), the anti-proliferative properties of PRL are highly dependent on the differentiation state of the mammary gland. One could speculate that PRL exerts its negative effects on EGF-mediated cell proliferation and transformation (Nouhi et al., 2006) in breast cancer cells by inducing cellular differentiation.

3.2. The regulatory role of PRL in mammary epithelial cell proliferation

Our results demonstrate that in mammary epithelial (HC11 and NMuMG cells) and breast cancer cells (4T1 cells), while EGF is proliferative, PRL is non-proliferative and suppresses EGF-mediated signals to cell proliferation. The anti-proliferative role of PRL in mammary epithelial cells confirms previous findings in NMuMG cells (Fenton and Sheffield, 1993; Johnson et al., 1996). The role of PRL mediating cell proliferation is highly cell-context specific. While no impairments in ductal proliferation was observed in cleared mammary gland transplanted with mammary epithelial cells from PRLR, Jak2 or Stat5a-null mice, defects in alveolar proliferation were observed. This suggests that PRL signalling plays a regulatory role in alveolar proliferation, but not ductal proliferation (Miyoshi et al., 2001; Shillingford et al., 2002). PRL-induced mammary cell proliferation is also highly dependent on the availability of steroid hormones (Mallepell et al., 2006; Rosen, 2003). In fact, ERα null mammary epithelial cells were found, even upon lactogenic hormone stimulation, to be arrested and thus non-proliferative. The differentiation state of the mammary gland also depicts the proliferative role of PRL. While the conditional overexpression of PRL within the mammary epithelium has been shown promote proliferation and exert a tumourgenic phenotype (Rose-Hellekant et al., 2003), the overexpression of PRL in the differentiated mammary epithelium produced defects in alveolargenesis, but no carcinomas were detected (Manhes et al., 2006). The importance of differentiation state of the mammary is always emphasized by the requirement for differentiation factors such as hydrocortisone and insulin for PRL to exert its anti-proliferative. The influence of cellular differentiation is also depicted when comparing NMuMG and HC11 cells. NMuMG cells are isolated from lactating mice and are highly differentiated, whereas, HC11 cells are isolated from mid-pregnant mice and are not fully differentiated. The ability of PRL to attenuate EGF-mediated MAPK activation was stronger in NMuMG cells (Figure 2.3.2.1B) compared to HC11 cells (Figure 2.3.2.1A) further stressing the regulation of PRL signals via cellular differentiation. Together, these findings suggest that the role of PRL in proliferation is highly context specific and complex. However, our data suggests that within the differentiated mammary epithelial cells PRL is non-proliferative and negatively regulates EGF-mediated proliferation.

3.3. PRL-induced tyrosine phosphorylation of Grb2

We identified Grb2 as a novel downstream target of the PRLR/Jak2 complex. However, the mechanism by which PRL phosphorylates Grb2 needs further investigation. We demonstrated the essential role of Jak2 kinase activity in mediating Grb2 tyrosine phosphorylation (Figure 2.3.7.1). Moreover, the overexpression of Jak2, but not Src induced the phosphorylation of Grb2 (Figure 2.3.6.3). However, we were unable to detect a direct interaction between Jak2 and Grb2 by far western suggesting while Jak2 is required for tyrosine phosphorylation of Grb2, intermediate signalling events are required. The phosphorylation level of Grb2 is regulated by the catalytic activity of SHP-2 as well as C-terminal tyrosine residues of SHP-2 (Minoo et al., 2003). This suggests that Grb2 tyrosine phosphorylation steps from the recruitment of Grb2 to the PRLR/Jak2 complex via the C-terminal tyrosine residues of SHP-2 (Figure 3.3.1). The recruitment by SHP-2 brings Grb2 in contact with Jak2, inducing tyrosine phosphorylation

Another possibility Jak2-dependent mechanism may stem from the requirement of Jak2 to activate the tyrosine kinase or to induce a signalling cascade involved in Grb2 phosphorylation. Upon PRL-induced Jak2 activation, Jak2 activates directly activates the tyrosine kinase that phosphorylates Grb2 or activates a signalling cascade leading to Grb2 phosphorylation (Figure 3.3.2). Future work within our laboratory will focus on identifying and characterizing the kinase involved in Grb2 tyrosine phosphorylation.

Preliminary results suggest that ACK-1 may regulate the phosphorylation of Grb2 (Figure 3.3.3). PRL activates ACK-1 and promotes ACK-1-Jak2 and ACK-1-Grb2 interactions. ACK-1 was considered a good potential tyrosine kinase involved in the tyrosine phosphorylation of Grb2 because ACK-1 negatively regulate EGF-induced MAPK activation and interacts with the SH3 domains of Grb2 (Shen et al., 2007; Yokoyama and Miller, 2003). However, future experiments are required to determine if the kinase activity of ACK-1 is essential for the phosphorylation of Grb2. Moreover, it would also be interesting to determine whether the ACK-1 plays a role in PRL-mediated suppression of EGF-induced MAPK activation and cell proliferation. Furthermore, it would be important to show that the ability PRL to suppress EGF signalling is abolished when ACK-1-Grb2 interactions are interrupted.

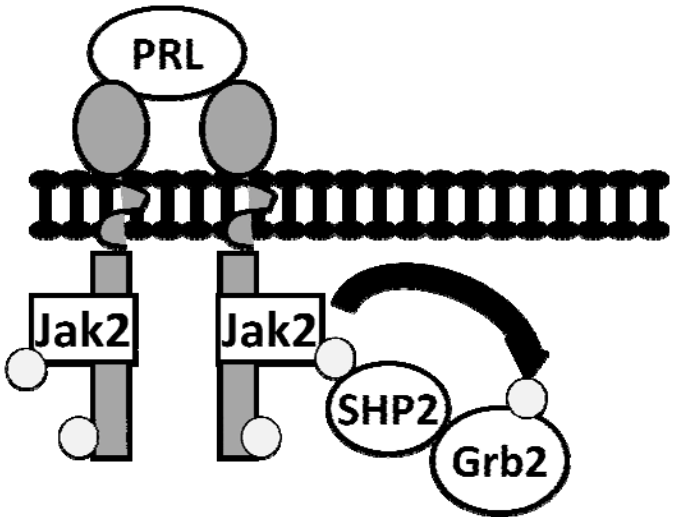

Figure 3.3.1: Grb2 is recruited to Jak2 via the c-terminal tyrosine residues of SHP-2. Upon activation of the PRLR/Jak2 complex, SHP-2 recruits Grb2 to the complex via its c-terminal residues. Upon recruitment, Grb2 is tyrosine phosphorylated by Jak2 which interferes with the ability of Grb2 to interact with Sos and thus blocks the ability of EGF to activate the Ras/MAPK pathway and induce cell proliferation.

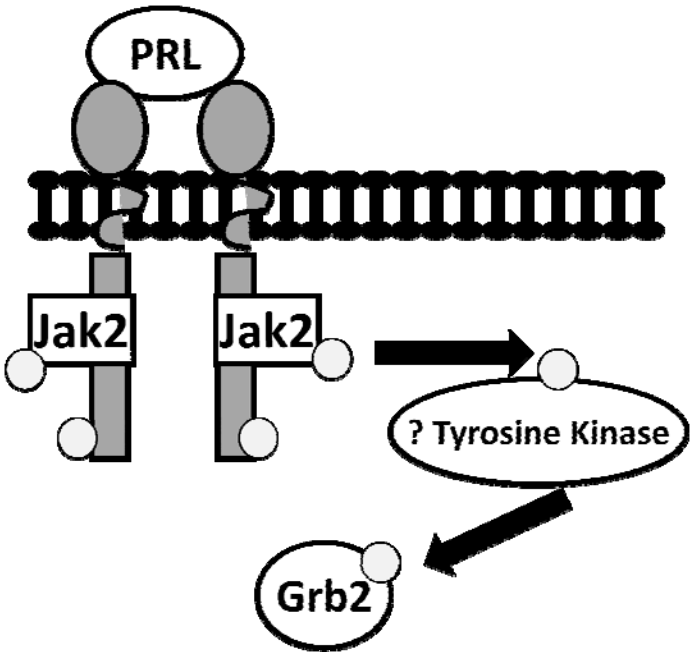

Figure 3.3.2: Jak2 plays an essential role in the signalling cascade leading to Grb2 tyrosine phosphorylation. Upon activation of the PRLR/Jak2 complex, Jak2 activates either the kinase or a signalling cascade involved in the tyrosine phosphorylation of Grb2 which interferes with the ability of Grb2 to interact with Sos and these block the ability of EGF to induce the activation of the MAPK pathway and cell proliferation.

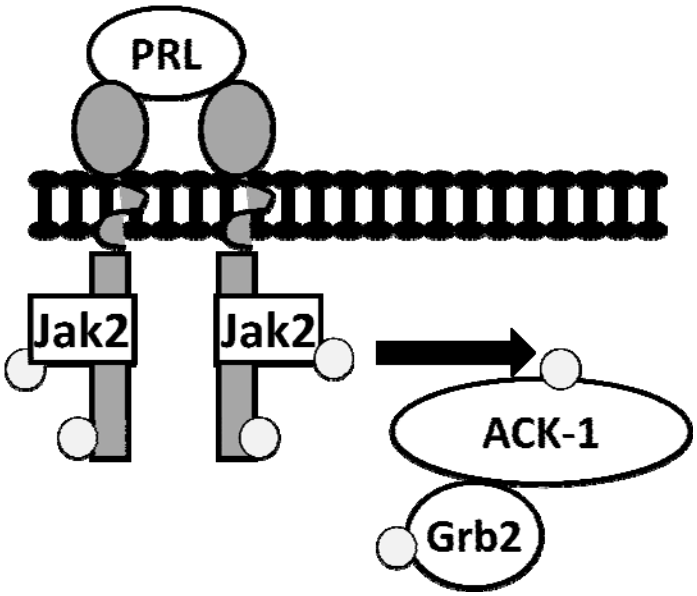

Figure 3.3.3: Jak2 through ACK-1 tyrosine phosphorylates Grb2. Upon activation of the PRLR/Jak2 complex, Jak2 activates the tyrosine kinase ACK-1. ACK-1 may play two roles in the tyrosine phosphorylation of Grb2: It can act as a tyrosine kinase and phosphorylate Grb2 directly or it may also play a recruitment role. ACK-1 may recruit Grb2 to the PRLR/Jak2 complex allowing Jak2 to tyrosine phosphorylate Grb2.

Together, we have demonstrated that PRL-induces the tyrosine phosphorylation of Grb2 via a Jak2 dependent mechanisms. However, we were unable to detect a direct interaction between Jak2 and Grb2 by far western analysis. Future research will focus on further characterizing a possible interaction between Jak2 and Grb2. The exact mechanism of PRLR/Jak2-induced Grb2 tyrosine phosphorylation remains to be fully elucidated. Future experiments within the laboratory will be oriented to defining this mechanism and determining a possible role for ACK-1 within the PRL/EGF crosstalk.

3.4. A role for PRL-induced Grb2 tyrosine phosphorylation in mediating EGF signalling

Tyrosine phosphorylation of Grb2 within its SH3 domains was previously observed in cells transformed by BCR/ABL oncogene as well as following EGFR activation in A431 cells (Li et al., 2001). However, the physiological role for the tyrosine phosphorylation of Grb2 had yet to be elucidated. Our results demonstrate the Grb2 phosphorylation is an important mechanism utilized by PRL to attenuate EGF signals to MAPK activation and cell proliferation in mammary epithelial and breast cancer cells. PRL has been previously shown to utilize other mechanisms to suppress EGF-induced signalling such as through a protein kinase C (PKC) dependent mechanism (Fenton and Sheffield, 1997). or via the threonine phosphorylation of the EGFR (Quijano and Sheffield, 1998). However, the observations that the overexpression of Grb2YF mutant form was able to recover the ability of EGF signals to Grb2/Sos complex formation as well as Ras/MAPK activation and cell proliferation emphasizes the importance of this mechanism in PRL suppression of EGF signalling in mammary epithelial cells.

While the tyrosine phosphorylation of Grb2 was previously proposed as an auto-feedback mechanism to inhibiting EGF-induced MAPK activation (Li et al., 2001), in accordance with previous studies (Lowenstein et al., 1992; Rozakis-Adcock et al., 1992), our results demonstrates that, even in cells pre-treated with the phosphatase inhibitor sodium orthovanadate, EGF is unable to induce the tyrosine of phosphorylation of Grb2 (Figure 2.4). This suggests that Grb2 phosphorylation might represent a cross-regulatory mechanism rather than an auto-regulatory one for EGF in mammary epithelial cells. Therefore, ligands that incorporate Grb2 in the signalling network can utilize Grb2 to negatively regulate EGF

signalling. Our results suggest a model in which, the presence of PRL significantly increase the pool of phosphorylated Grb2, which impairs the ability of EGF to induce the activation of the Ras/MAPK pathway and hence cell proliferation.

Chapter 4: Conclusion and implications

4.1. Implication in the use of PRL antagonists in breast cancer

We demonstrated that PRL and EGF cross-regulate in mammary epithelial and breast cancer cells. While EGF is proliferative and inhibits PRL-induced cellular differentiation, PRL is a differentiation factor and inhibits EGF-mediated cell growth. The anti-proliferative and transformational inhibitory (Nouhi et al., 2006) properties of PRL make it a prospective therapeutic agent in the treatment of breast cancer. Furthermore, its ability to block EGF signalling, a pathway commonly overactive in breast cancer patients (Modjtahedi et al., 1998) further stresses its potential therapeutic benefits.

Currently, no clinical trials are utilizing PRL as a therapeutic agent for breast cancer. In fact, a group of PRL antagonists are being developed and trialed (Clevenger et al., 2008). The tumourgenic properties of PRL (Clevenger et al., 2003) make PRL a favourable therapeutic target in breast cancer. While we do not deny the role of PRL in breast tumourgenesis, we suggest that PRL may play a dual role in breast cancer: a tumourgenic role and a cancer suppressor role. Our data suggests that PRL has differentiative properties that can suppress the growth of differentiated mammary cells. Therefore, treatment of differentiated breast tumours may suppress the growth of tumours and one may speculate reduce tumour size. Moreover, its ability to suppress EGF-induced MAPK activation and cell proliferation suggests that patients with over active EGF signalling may benefit from PRL treatment.

The role of PRL in breast tumourgenesis is high dependent on cellular context (Manhes et al., 2006; Rose-Hellekant et al., 2003; Rosen, 2003). Therefore, it is important to look at breast cancer patients on an individual basis before considering PRL antagonistic treatments; treatments with these antagonists may block the anti-proliferative role of PRL in differentiated and over EGF-signalling tumours. Moreover, inhibition of PRL signalling in breast cancer cells promotes the transition from a differentiated epithelial breast cancer to a metastatic, progressed mesenchymal breast cancer. Therefore, treatments with PRL antagonist can also dedifferentiate breast tumours. In fact, antagonizing estrogen, another differentiating factor, with tamoxifen has also been shown to promote cellular dedifferentiation and to select for more aggressive non-responding breast cancer cells (Borley et al., 2008).

4.2. Grb2 tyrosine phosphorylation: a novel prognostic marker and therapeutic

Grb2 promotes the activation of the MAPK pathway downstream of growth factor receptors (Buday and Downward, 1993). Moreover, Grb2 promotes cellular proliferation (Tari and Lopez-Berestein, 2001) and mammary tumourgenesis (Galliher-Beckley and Schiemann, 2008). Therefore, characterizing negative regulatory mechanism to Grb2 functionality can lead to the development of novel breast cancer therapeutics. Our data stresses the importance of the tyrosine phosphorylation of Grb2 in the negative regulation of Grb2/Sos interaction leading to growth factor-induced MAPK activation and cell proliferation in mammary epithelial and breast cancer cells.

A variety of promising Grb2 inhibitors have been developed. While inhibitors have been developed against the SH3 domain of Grb2, most inhibitors target the SH2 of Grb2 (Dharmawardana et al., 2006; Li et al., 2003; Long et al., 2003). The Grb2-SH2 domain is essential for growth factor receptor interaction and Ras activation, whereas; the SH3 domain is essential for Grb2/Sos interactions (Tari and Lopez-Berestein, 2001). These inhibitors significantly reduce signals to MAPK activation (Li et al., 2003) and mouse mammary tumourgenesis (Giubellino et al., 2007; Kim et al., 2005). Our results stress the importance of SH3 domain functionality in promoting EGF signalling. We demonstrated the tyrosine phosphorylation of Grb2 within its SH3 domains attenuated EGF signals to MAPK activation and cell growth. Our results define a therapeutic role for Grb2-SH3 targeting therapeutics in breast cancer. Moreover, we demonstrated that PRL suppresses Grb2-SH3 functionality suggesting that PRL may play a therapeutic role in breast cancer.

While the mortality rates due to breast cancer has significantly reduced (Lester, 2007) due to the development of specific targeting treatments and better screening techniques, breast cancer is yet to be curable. Therefore, identifying novel diagnostic markers will allow physicians to detect and treat breast cancer patients earlier. Grb2 tyrosine phosphorylation was shown to negatively regulate EGF signalling and suppress cellular proliferation, suggesting that tyrosine phosphorylated Grb2 acts as a tumour suppressor. Quantifying the levels Grb2 tyrosine phosphorylation in breast cancers could act as a prognostic tool for physicians. In that, breast cancers with higher levels of Grb2 phosphorylation would be diagnosed more favourably than breast cancers with lower or absent Grb2 phosphorylation.

Knowledge of mechanisms regulating mammary cell growth and differentiation is vital as many of these mechanisms are implicated in breast carcinogenesis. Together in this study we have identified a novel cross regulatory signaling mechanism operating downstream of PRL and EGF in mammary epithelial cells influencing mammary epithelial cell growth and differentiation

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