NOTE TO USERS

Prolactin Plays a Dual Role in Breast Cancer; Promoting Formation of Breast Tumour while Inhibiting its Metastasis

by

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Table of **Contents**

1 Abstract

Prolactin is a key mammary gland differentiation factor. However, the contribution of prolactin (PRL) to breast carcinogenesis is less clear. Accumulating evidences indicate that in established breast carcinomas autocrine/ paracrine PRL can enhance growth/viability of breast cancer cells. Still, it is not known whether the ascribed pro-oncogenic activity of PRL describes fully the role of PRL in regulating breast carcinogenesis. On the other hand a critical role for Ras- Erk1/2 and TGF-β (Transforming Growth Factor β) pathway in breast cancer progression has already been established. Our results indicate that blocking PRL signal leads to activation of Ras-Erk1/2 pathway and TGF-β pathway, two key pathways contributing to breast cancer metastasis. I showed that modulation of PRL signaling in breast cancer cells alters their morphogenic program. My results highlight a critical role for PRL in regulating epithelial plasticity and implicate PRL as invasive suppressor hormone in breast cancer cells

2 Resumé (French)

La prolactine est un facteur principal de différentiation de glande mammaire. Cependant, la contribution de PRL à la carcinogenèse de sein est moins claire. Les evidence bien montrent que PRL comme an facteur autocrine/ paracrine peut augmenter la croissance des cellules de cancer de sein. On ne connaît pas encore, si l'activité pro-oncogène attribuée à PRL décrit completement le rôle de PRL dans la reglulation de carcinogenèse de sein. En outre, un rôle critique pour la voie de Ras- Erk1/2 et de TGF-β dans la progression de cancer de sein a été déjà établi. Mes résultats indiquent que le blocage du signal de PRL mène à l'activation la voie Ras-Erk1/2 ainsi que la voie du TGF-β (Le facteur-β de croissance transformant), deux voies principales contribuant à la métastase de cancer de sein. J'ai prouvé que la modulation de la voie de PRL dans les cellules de cancer de sein change leur programme morphogène. Mes résultats accentuent un rôle critique pour PRL en réglant la plasticité épithéliale et impliquent PRL en tant qu'une hormone qui empeche l'invasion des cellules de cancer de sein.

3 Literature Review

3.1 Prolactin

Prolactin (PRL), a 23 kDa polypeptide hormone is synthesized and secreted by the lactotroph cells of the anterior pituitary gland [1]. However it can be produced by other cell types and organs such as the mammary epithelium, placenta, uterus, lacrimal gland, adrenal gland, corpus luteum, prostate, testes, pancreas, brain, immune system,[1] human umbilical vein, endothelial cells, myeloid leukemic cells[2], bone and lymphocytes [3].

Prolactin gene (10 kb) is located on chromosome 6 in human and contains 6 exons, encoding signal peptide (28 amino acids) and mature prolactin. Prolactin secretion from pituitary is negatively regulated by dopamine and positively by estrogen and TRH (Thyroid Releasing Hormone)[4].

Mature PRL polypeptide contains 199 amino acides. Alternative splicing, cleavage, phosphorylation, glycosylation and other post-transcriptional modifications, lead to differences in its molecular weight and function. For example, glycosylated PRL has lower affinity to bind to its receptor, phosphorylated PRL may act as antagonist and a cleaved form of PRL (designated as 16 kDa PRL) has antiangiogenic properties. Moreover, in serum of patients with hyperprolactinemia big (50-60 kDa) and macro (150-170 kDa) forms of PRL also can be detected as a result of polymerization and conjugation to IgG[5]. In fact these post-translational modifications are not useful for hormone function[4].

Prolactin (pro-lactin) has been discovered 70 years ago as a stimulator of mammary gland development and lactation in rabbits[2]. It has been shown that it plays an important role in mammary gland development, growth and differentiation[3] as it is important for lobuloalveolar development, alveolar cell growth and milk production. "It acts as a growth factor, neurotransmitter, or immunoregulator"[3].

Transgenic mice studies revealed that PRL -/- mice are infertile with no immune system deficiency (contradictory to the known role of prolactin in immune system) pointing its critical function in reproduction[4] (Table-1).

3.1.1 Prolactin Receptor

Prolactin receptor (PRLR) belongs to the class I cytokine receptor super family which also contains, growth hormone receptor, leptin receptor, erythropoietin receptor and several interleukin receptors[1]. PRLR is encoded by a unique gene located on chromosome 5 of the human genome containing at least 10 exons[2].

To date 6 different isoforms of PRLR have been identified in human cells (Fig 1):

1. Long form; hPRLR:

The human PRLR (hPRLR) is 85 kDa and as other members of the class I cytokine receptor superfamily is a transmembrane receptor with an extracellular domain, a transmembrane domain and an intracellular domain. Its extracellular domain contains two type III fibronectin-like domains (S1, S2), which are connected by a linker of five amino acids. This domain is important for ligand

binding and receptor dimerization. The transmembrane domain comprises of 24 amino acids with still unknown function. The intracellular domain consists of a juxtamembrane region containing Box1, Variable Box (V-Box), Box2 and extended Box2 (X-Box), conserved between the cytokine receptor superfamily, as well as a C-terminal region. Box1 is important for Janus kinase2 (Jak2) activation. The function of the C-terminal region of the receptor is still under investigation. However it has been shown that tyrosine residues in this region are important for activation of the signal transducer and activator of transcription 5 (STAT5) and the SH2 domain–containing protein tyrosine phosphatase SHP2.

2. Intermediate hPRLR (50 kDa):

It is an out-of- frame splicing variant of hPRLR long form, lacking all of the C-terminal to the X-Box and addition of 13 amino acids. While it has the same affinity to bind ligand as the hPRLR long form and it is able to activate Jak2, it is unable to activate Fyn tyrosine kinase.

3. ΔS1 isoform (70 kDa):

This isoform lacks S1 domain because of splicing out of exons 4 and 5 in frame from mRNA of the hPRLR. ΔS1 has a 7-fold less affinity to ligand compared to hPRLR long form and is unable to self-associate in high concentrations. However it is able to associate and differentially regulates integrin-associated signaling cascades (not observed in hPRLR long or intermediate forms).

4. Prolactin Binding Protein (PRLBP) (32 kDa):

PRLBP is the shortest PRLR isoform identified, containing only the extracellular domain of the hPRLR long form. It is present in the serum, medium of human

breast cancer cells and hematopoietic cells overexpressing the hPRLR long form. This isoform is a result of proteolysis. *In vivo*, it acts as antagonist of PRL. While *in vivo* it seems to limit PRL secretion and degradation, increasing its half-life and function.

5 & 6. hPRLR S1a (56 kDa) and hPRLR S1b (42 kDa):

In these isoforms some or all of exon 10 is replaced by some or all of exon 11. hPRLR S1a contains both the Box1 and 2 while the hPRLR S1b contains only the Box 1. They may be important in ligand internalization of down-regulation of PRL signaling[4]

Since PRLR- /- female mice but not males, are infertile like PRL -/- mice, effect of lacking PRLR on mammary gland growth has been studied by transplantation of PRLR-/- mammary epithelium into the cleared fat pads of wild type mice. These studies showed that lack of PRLR lead to marked deficiencies in lobuloalveolar differentiation during pregnancy and milk production[4] and less STAT5 phosphorylation. However ductal formation was normal indicating a critical role for prolactin in alveolar development rather than ductal structure.

PRLR -/- and PRLR +/- mice showed also deficiency in maternal behavior, ossification, bone formation and maintenance of bone mass[2]. Moreover PRLR +/- mice were not able to lactate after their first, but not subsequent pregnancy.

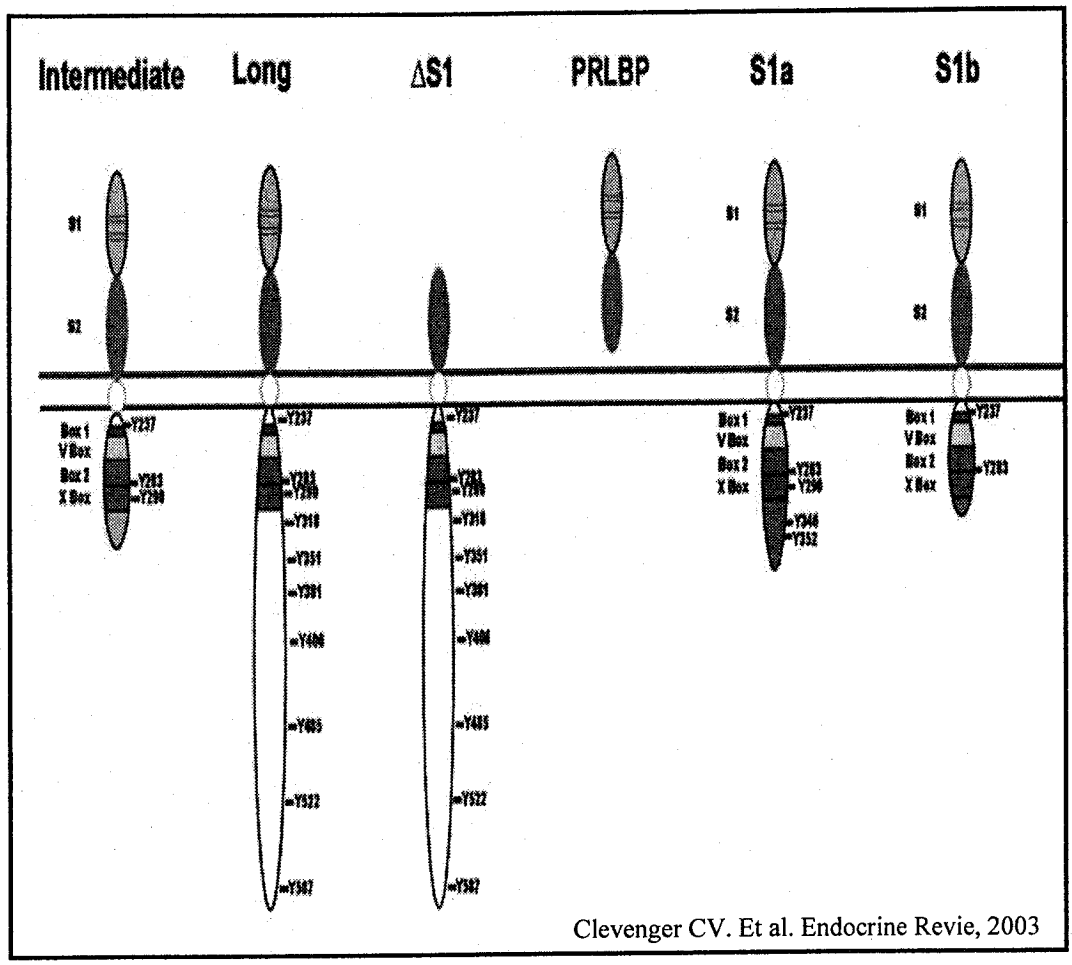
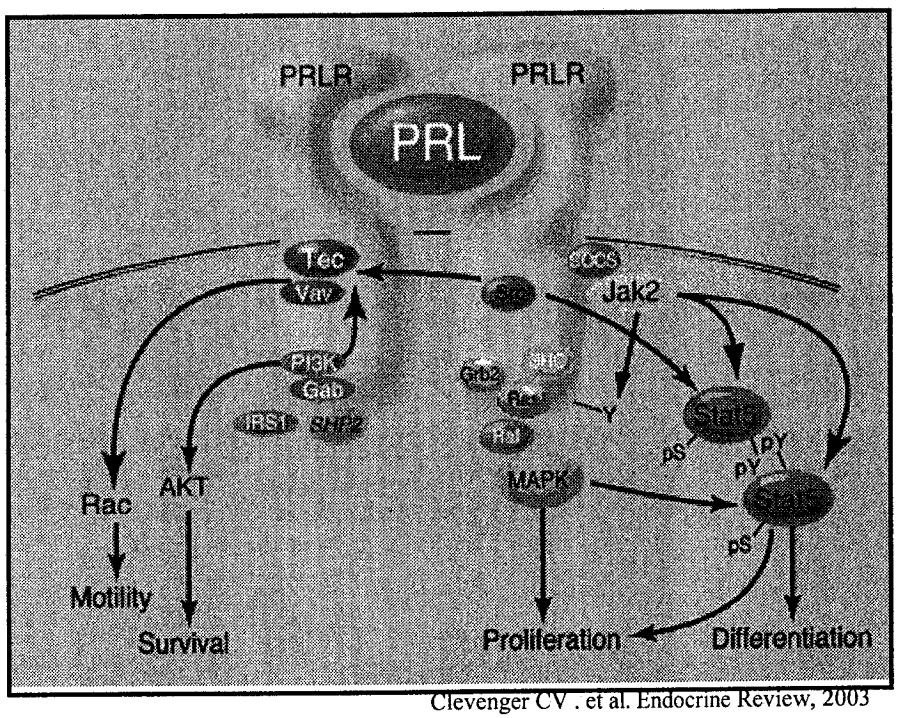


Figure 1. Schematic Representation of the Human Prolactin Receptor Isoforms

3.1.2 Prolactin and Prolactin Receptor Signaling

The PRLR is able to interact with PRL, primate growth hormone and placental lactogen[1, 2]. PRL binding to its receptor leads to receptor dimerization and activation. Heterodimerization of PRLR isoforms inhibits its signaling. The PRLR lacks instinct kinase activity, so it signals through activation of associated kinases like Jak family, Src family, etc. Activation of these kinases leads to

activation/ phosphorylation of downstream molecules such as the receptor itself[2] (Fig 2). Here I summarized some of these molecules/ pathways that are activated upon receptor dimerization:



Clevenger CV . et al. Endocrine Review, 2003

Figure 2. Prolactin Receptor Signaling Mechanisms

3.1.2.1 Jak2:

Since type I cytokine receptors lack intrinsic kinase activity, they act by intermediation of a protein tyrosine kinase. Among these protein tyrosine kinases, Janus kinases (Jaks) are essential for the first steps of cytokine-mediated signaling.

Jak family contains four members: Tyk2, Jak1, 2 and 3. Jak1, 2 and Tyk2 are ubiquitously expressed, while Jak3 expression is limited to hematopoietic lineage.

Jak proteins contain seven highly conserved domains with yet unknown function called JH1to JH7 (Fig. 3). The C-terminal region of Jaks contains tandem kinase and kinase-like modules (JH1,2). The domain JH1 consists of conserved residues identified in tyrosine specific kinases. Furthermore the JH1 domain is the site for autophosphorylation/ phosphorylation, which is essential for full kinase activity. It seems that the JH2 domain has an inhibitory function on the catalytic activity of the kJaks. The N-terminal (JH3-JH7) region is important in "coordinating Jak functions and are essential for the receptor interaction"[6]. Jak2 is the member of this family known to be associated with the PRLR.

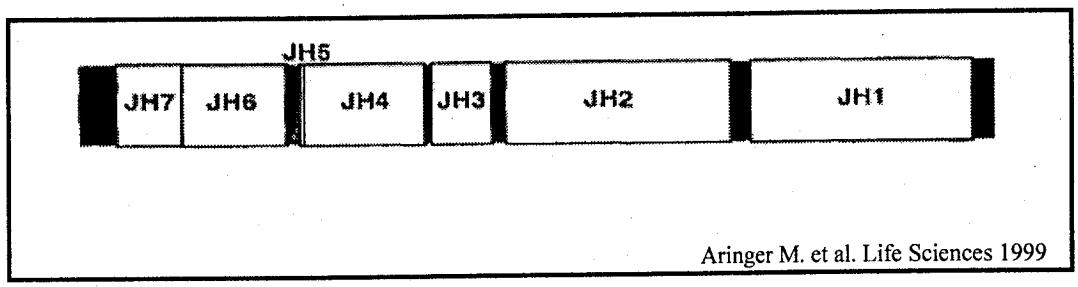


Figure 3. Schematic Representation of the Domain Structure of the Jak Kinases

Jak2 activation leads to its transphosphorylation as well as receptor tyrosine phosphorylation, which in turn can serve as docking site for SH2-domain containing proteins like STATs. Jak2 activation is negatively regulated by suppressor of cytokine signaling/ cytokine-inducible inhibitor of signaling (SOCS/ CIS) family.

Jak2 -/- mice are not viable. Because of late embryonic lethality of these mice, *in vivo* studies examining the role of Jak2 in mammary gland growth were

performed using transplantation techniques. Transplantation of knock out Jak2 mammary epithelium in the cleared fatpad of wild-type mice revealed impaired mammary gland development and deficiencies in lobuloalveolar formation during pregnancy, indicating an important role of Jak2 during lobuloalveolar development and furthermore milk secretion[1, 4]. *In vitro* and *in vivo* studies provide a contradictory role for Jak2 in the proliferation of the mammary epithelium. *In vitro* it has been shown that HC11 cells expressing antisense oligonucleotide to Jak2 has an increased growth rate compared to control cells. However transplantation studies showed a reverse effect of Jak2 on mammary epithelial cell proliferation as the knock out of Jak2 showed 95% less proliferation compared to WT.

3.1.2.2 STATs

STAT family of transcription factors were identified 12 years ago as transcription factors which bind to GAS motifs in promoter region of genes activated by interferon (IFN)-α and-γ. They are critical integrators of cytokine and growth factor receptor signaling. They play important role in cell growth, survival, differentiation and motility. Moreover STAT3 and 5 have key role in breast tumorigenesis.

"STAT proteins consist of numerous distinct functional domains" [7] (Fig. 4): The N-terminal domain that is necessary for STATs interaction with coactivators and dimerization; a central DNA- binding domain; which is DNA-recognition domain, an SH2 domain, which is bound to DNA-binding domain by

a linker domain and is important in binding to phospho-tyrosine residues, which is in turn important for receptor binding and STAT dimerization and a C-terminal transactivation domain. Between the SH2-domain and the transactivation domain, there is a conserved tyrosine residue, which plays a critical role in the dimerization of STATs[7].

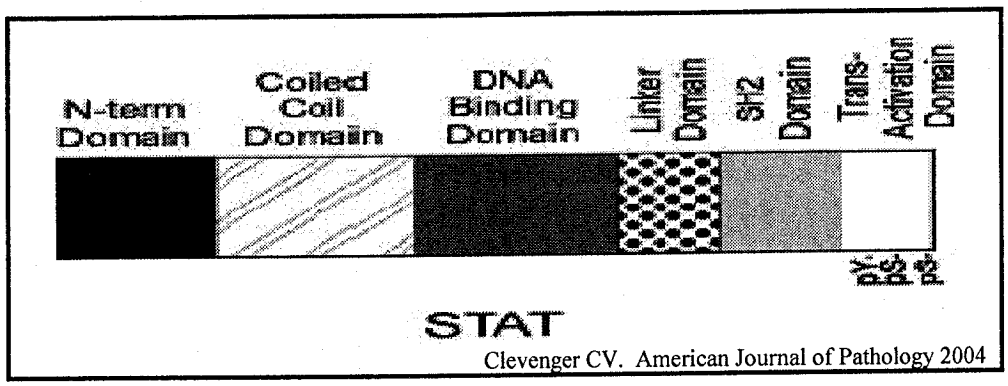


Figure 4. Schematic Representation of the Domain Structure of STAT Proteins

Stat1, 3, 5a and 5b have been shown to be tyrosine phosphorylated by Jak2 upon activation of the PRLR. Phosphorylation of STATs leads to their dimerization/multimerization and nuclear translocation where they can act as transcriptional regulators to enhance transcription of some genes like cyclin D1, etc[5]. SOCS/ CIS family, Peptide Inhibitors of Activated STAT (PIAS) are known to negatively regulate STATs activation. Critical role of Stat5 in milk protein production and secretion is well known, as Stat5a knock out mice showed impaired lactation after first pregnancy. In fact mammary phenotype of the

STAT5a knock out mice is similar to PRL and PRLR null mice which is revealed with profound loss of alveolar maturation and milk production[7].

3.1.2.3 Ras/ MAPK

The Mitogen Activated Protein Kinase (MAPK) family consists of five groups; Erk1 and Erk2 (Extracellular signal-regulated kinases1 and 2); c-Jun amino terminal kinases (JNKs) 1, 2 and 3; p38 isoforms α, β,γ and δ; Erk3, 4 and Erk5. However the most studied groups are Erk1/2, JNK and p38 kinases.

Erk1/2 are activated in response to growth factor stimuli, which are transmitted to the cells through cell surface receptors such as tyrosine kinase receptors (RTK) and G-protein coupled receptors or cytokine receptors such as the PRLR. Receptor activation results in activation of small GTP-binding protein of Ras/Rho family. Moreover activation of Ras/Rho family members lead to activation/phosphorylation of a Ser/ Thr containing kinases called MAPKK kinase which in turn activates/phosphorylates MAPKK. Actiavation of MAPKK finally leads to activation/phosphorylation of MAPKs. In the case of Erk1/2, phosphorylation of Ser and Thr residues in the kinase domain is mediated by MEK1/2 (as MAPKK), which are activated by A-Raf, B-Raf and Raf1 (MAPKKKs) [8]. (Fig. 5)

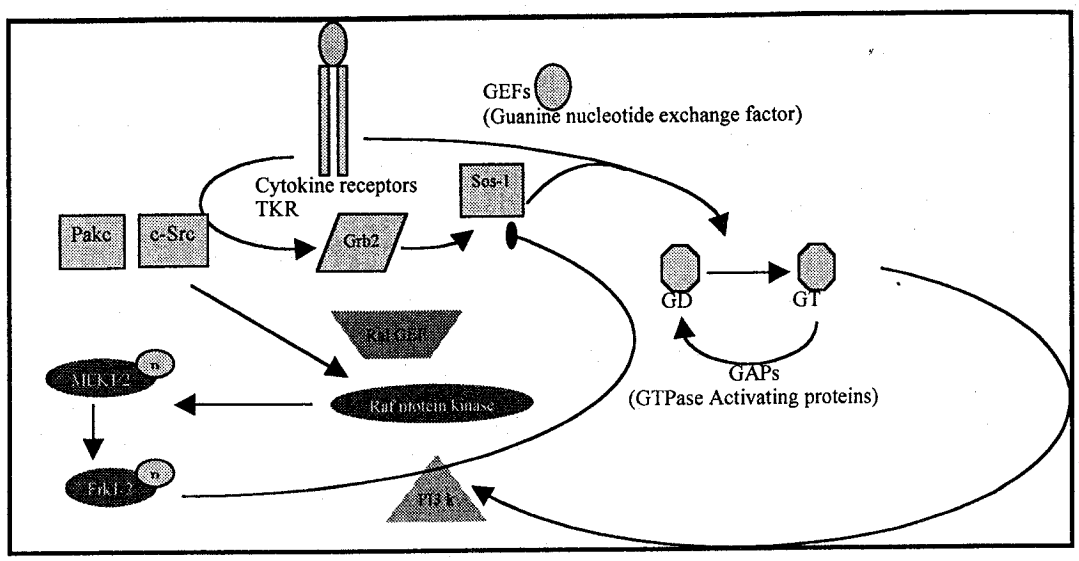


Figure 5. Activation of Erk1/2 by Cytokine Receptors

"Erk1 and Erk2 have 83% amino acid identity and are expressed in all tissues" [8]. Their activation leads to phosophorylation, activation, suppression of some proteins such as Elk1, MEF2, paxillin and MKs (RSKs). In addition they may affect protein expression by modulating mRNA translation.

Hyper activation of Ras and Raf results in oncogenesis. Thus regulation of Ras and Raf is essential for the proper maintenance of cell proliferation. Furthermore to stop tumor growth the use of Erk1/2 inhibitors has been suggested. U0126 and PD98059 are chemical inhibitors used to inhibit Erk1/2 activation through inhibition of MEK1/2 activity[8]. It has been shown that PRL is among hormones able to activate Erk1/2 linking prolactin to proliferation of many cell system especially mammary cells.

3.1.2.4 PI3-Kinase

Activation of PI3K leads to activation of Pleckstrin-Homology (PH) containing proteins. Class I PI3Ks contain one catalytic (p110) and one regulatory (p85) subunit. Activation of the catalytic subunits leads to generation of phosphatidylinositol (PtdIns) (3) P, PtdIns (3,4) P2, PtdIns (3,4,5) P3 that play an important role in oncogenesis, proliferation, cytoskeletal rearrangement, apoptosis and angiogenesis. PI3K can be activated by PRL as its regulatory domain can be associated by downstream effectors of PRLR signaling like; STAT5, 3, IRS 1, Gab1, Gab2 and SHP2. Phosphoinositides will serve as docking site for Akt/ PKB and PtdIns-dependent kinases 1 and 2, resulting in threonine/serine phosphorylation/ activation of Akt. This pathway is important in survival, apoptosis and cell cycle arrest[9].

Mouse model	Signaling molecule	Mammary gland development	Mammary gland cancer
Germ-line disruption of the PRL gene	Loss of PRL	Arrested mammary gland development	Reduced growth of polyoma middle T antigen-induced mammary cancers
Metallothionein promoter-rat PRL transgene	PRL overexpression in mammary gland	Development of lactation type morphology in virgin mice	Mammary cancers at 11–15 months of age
Germ-line disruption of the PRLR gene	Loss of functional PRLR	Loss of one functional PRLR gene results in impaired lactation at the first pregnancy. Loss of two functional PRLR genes results in loss of alveolar development during pregnancy. Loss of functional PRLR in mammary stroma impairs differentiation of wild type	

		mammary epithelial cells during puberty.	
Germ-line disruption of the Jak2 gene	Loss of Jak2	No alveolar development during pregnancy	
Germ-line disruption of the Stat5a gene	Loss of Stat5a	Impaired mammary gland development and lactation Decreased survival of mammary epithelial cells	Decreased survival of premalignant and malignant mammary epithelial cells Reduced tumor growth of TGFα and SV40 TAg induced cancers
Germ-line disruption of both Stat5a and Stat5b genes	Loss of functional Stat5a and Stat5b	No alveolar development during pregnancy	
ß-Actin-CIS1/SOCS1/SSI1 transgene	Overexpression of CIS1/SOCS1/SSI1 in mammary gland	Impaired mammary gland development and lactation	
Germ-line disruption of CIS1/SOCS1/SSI1	Loss of functional CIS1/SOCS1/SSI1	Accelerated mammary gland development during pregnancy Loss of one functional CIS1/SOCS1/SSI1 gene rescues the lactation defect found in mice that carry only one functional PRLR gene.	

Clevenger CV . et al. Endocrine Review, 2003

Table 1. Effects on Specific Signaling Molecules in the PRL Pathway on Mammary Gland Development and Mammary Gland Cancer in Mouse Models

3.1.3 Breast Cancer and Prolactin Receptor Signaling

While the role of PRL and PRLR has been extensively evaluated in mammary gland development, their role in tumor formation/progression is not very well studied. In fact the role of PRL in breast cancer is controversial[3].

Although there is a correlation between PRL serum level and breast cancer in rodent, such a correlation has not been observed in human as bromocriptine treatment (a blocker of PRL release from the anterior pituitary gland) is not effective[3-5] to treat breast cancer. Real time RT-PCR results revealed that a high malignant tumor cells such as MDA MB-231 expresses little PRLR compared to non aggressive or less aggressive human breast tumor cells such as MCF-7 or T47D cells[10]. In fact in MDA MB-231 breast cancer cells promoter of the PRLR gene is highly methylated[10] so the receptor expression is silenced. Although, PRLR isoforms are expressed in almost 98% of breast cancer samples, there is no established study on their function in tumor progression[1-5, 11]. PRL and its receptor are detected in many breast cancer cell lines[3, 5] however transcription of PRL is different in tumor cells versus pituitary, so its regulation is different and this, may explain failure of bromocriptine treatment[5]. Furthermore, there is no reports on the differences between the levels of PRL in tumor versus normal cell lines[3].

Tumor development requires cell proliferation, survival, angiogenesis and acquisition of mesenchymal characteristic to be able to invade to other sites. PRL has been shown to have a role in all of these important features *in vitro*[5]. However it is yet unknown if these effects of PRL on nonlactating mammary gland are direct or they are mediated by another factor(s) such as TGF-α or TGF-β[4].

In vivo studies revealed a positive role for PRL in formation of breast cancer (Table-1) as polyoma middle-t antigen (PyMT) induced tumor later in PRL-/- mice comparing to WT. As well mice overexpressing PRL from the metallothionein promoter, developed mammary tumors. However these studies

didn't rule out the possibility of indirect action of PRL or its crosstalk with other pathways such as estrogen or progesterone. Furthermore Her2 (ERBB2) expressing mammary tumors that express PRL as well witll have higher migratory/proliferation capacity[5].

It has been proven that PRL acts through an autocrine/paracrine system to promote tumorigenesis, as transgenic mice expressing PRL under control of the hormone-independent neu-related lipocalin 2 (Lcn2/Nrl) promoter developed mammary tumors independent of circulating level of PRL, however PRL had a stimulatory effect on the proliferation of these tumors[1]. Eventually, normal range of PRL and its continuous secretion can lead to tumor formation [1]. "PRL, increases the size and number of spontaneous and virus-induced mammary tumors"[5]

One of the important regulators of tumor progression is apoptotic pathways. Apoptosis leads to death of cells with high malignancy potential. In normal mammary gland development, PRL leads to proliferation and survival of lobuloalveolar cells especially during lactation. PRL withdraw, in the end of lactation period leads to activation of apoptotic pathways and shrinks of mammary gland. It has been shown that PRL antagonist hPRLG129R induces apoptosis in T47D cells through caspase 3 activation, indicating a positive role for PRL in the survival of breast cancer cells. Furthermore suppression of PRL by its antagonist, antibody or antisense oligonucleotides leads to decreased proliferation of T47D cells[4, 5, 12].

In another report, it has been shown that PRL treatment increases the cell mobility of ER- and + tumor cells in a PI3-kinase dependent manner, pointing to a metastatic role for PRL in tumorigenesis[4].

In T47D and MCF7 cells activation of PRL leads to activation of STAT family members (STAT1, 3 and 5) however their role in tumor progression and their target genes are not studied[4, 11]. PRL is also known to activate MAPK pathways in mammary tumor cell lines, linking the PRLR signaling to cell proliferation. In the MCF7 cells it has been shown that inhibition of MAPK activation by MEK inhibitors lead to decreased cell proliferation[4]. PRL can also activate the PI-3 kinase pathway in breast tumor cell lines, permitting activation of Rho family members (RhoA and Rac), which are important, factors in motility of cells (formation of stress fiber and lamellipodia). As well, PRL leads to tyrosine phosphorylation of focal adhesion kinase and paxillin in T47D and MCF7 cells, which are important proteins in cell adhesion and migration. However the pathways are unknown[4, 5].

Contradictory to all the evidence pointing toward a positive role for PRL in tumorigenesis are reports indicating negative effect of PRL on tumor progression, for example it has been documented that PRL leads to increased activation of BRCA1 which is a tumor suppressor factor[5]. Moreover Stat 5a nuclear translocation is associated with a more differentiated breast tumor (better prognosis)[13]. While contradictory to this finding, is a report in rat mammary tumor showing correlation of poorly differentiated breast tumor with nuclear accumulation of Stat5a)[14]. However differences in species may explain the

differences. So far, to date the role of PRL in breast cancer progression is contradictory and not well established.

3.2 Epithelial- Mesenchymal Transition (EMT) and Metastasis

Metastasis is a feature of tumor cells to invade to a distinct site/tissue. In fact metastasis is a process in which epithelial cells become able for "local invasion, intravasation, survival in circulation, arrest in capillaries, extravasation and growth in a distant organ"[1]. So one of the steps in metastasis, is loss of epithelial polarity and cell-cell contacts, changes in cytoskeletal and gain of mesenchymal components, which refers to epithelial –mesenchymal transition (EMT) (Fig. 6). In fact epithelial cells have to go through EMT to be able to migrate to distant tissue. EMT occurs during embryogenesis for formation of different tissues and organs[1, 15], in tissue repair, and tumorigenesis. Moreover, genes implicated in embryogenesis, often are important players of tumor formation and progression. Some of these genes are TGF-β, E-cadherin, vimentin and metaloproteases. It is important to note that each of these, alone is not sufficient for migration of cells and for cells to go for metastasis or EMT.

Some of the known factors inducing EMT are: scatter factor/ hepatocyte growth factor, fibroblast growth factors, epithelial growth factor (EGF) family members (HER2, ErbB2, Neu...) and insulin-like growth factors 1 and 2[15]. In most cases these factors, function through Ras/ MAPK pathway that plays a key

role in EMT. However depending to the cell type, different pathways resulting in EMT may be activated such as Src, PI-3K and TGF-β. Moreover PI3K and MAPK have been directly or indirectly implicated in TGF-β signaling. In fact it seems that cooperation of TGF-β and MAPK is the main event resulting in EMT at least in skin squamous and breast cancer. It has also been shown that in Ras transformed EpH4 mouse mammary epithelial cell line, treatment with low doses of TGF-β leads to invasive growth but "retain an epithelial phenotype when untreated". As well, mouse mammary epithelial NMUMG cells expressing TGF-β become invasive and metastatic[16]. So, "abrogation of apoptotic effect of TGF-β" by Ras/ MAPK and/ or PI3 kinase leads to EMT and progression of cancer[15, 16]. So far, it has been shown that the activation of the MAPK or the PI-3K in collaboration with TGF-β is sufficient and necessary for EMT in mammary epithelial cells[17].

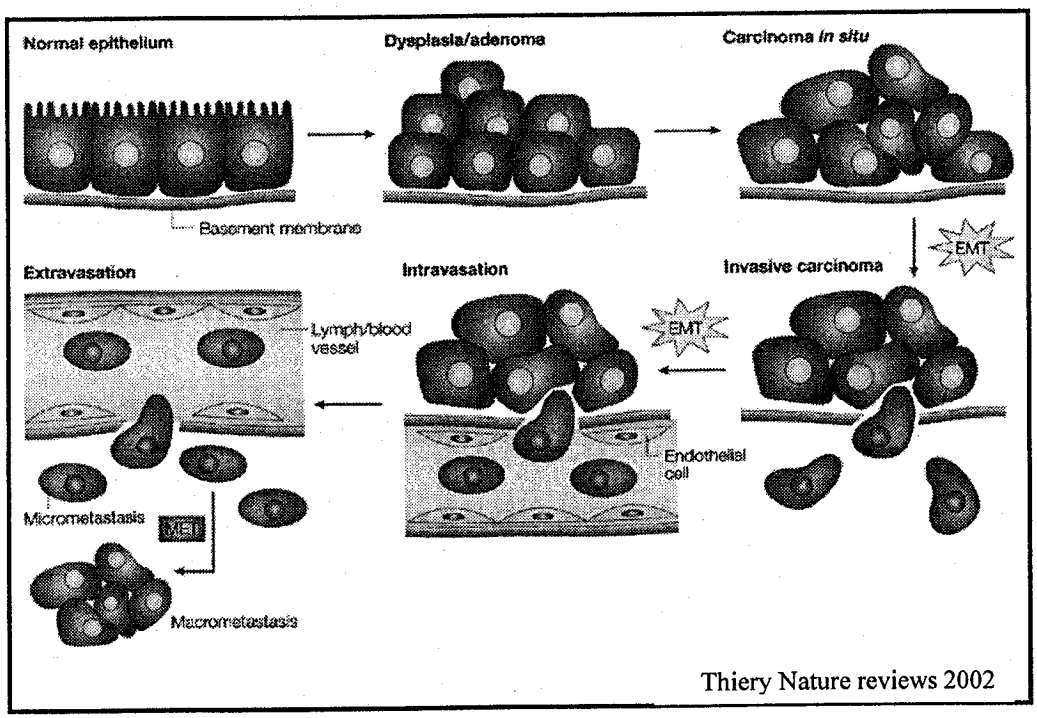


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Figure 6. Sites of EMT and MET in the Emergence and Progression of Carcinoma

3.2.1 E-cadherin

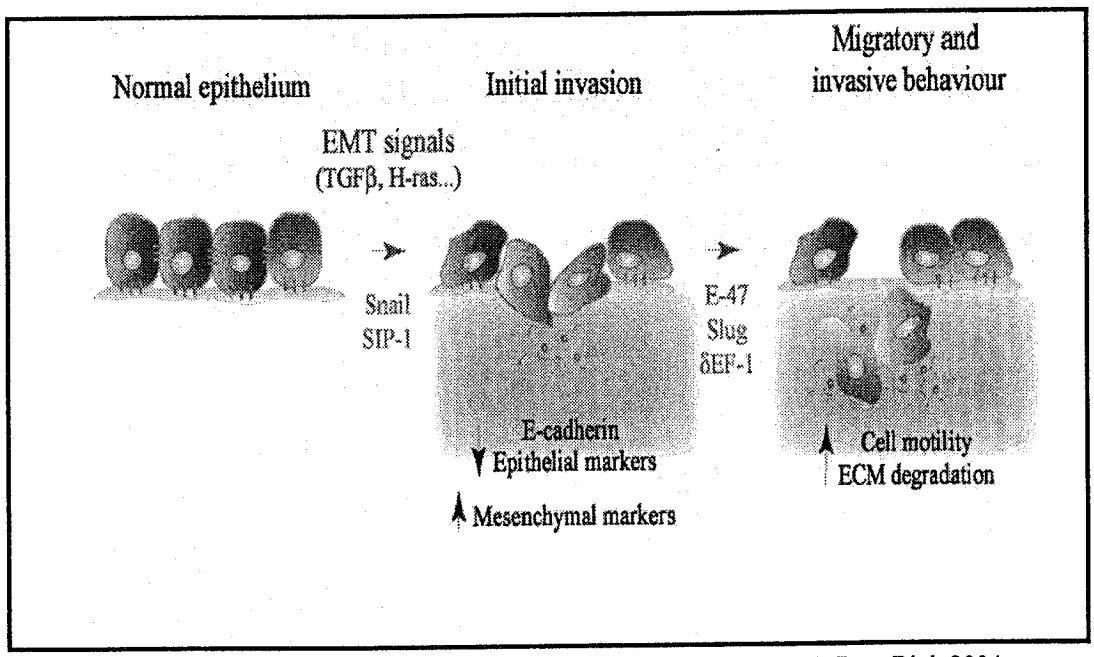
" Type I cadherins mediate homophilic interactions by forming adhesive bonds between one or several immunoglobulin (Ig) domains in their extracellular region and connecting to actin microfilaments indirectly via α- catenin and β-catenin in the cytoplasm"[16].

"Loss of E-cadherin expression is a hallmark of EMT". E-cadherin is a key component of cell-cell junction in epithelial cells. Over-expression of E-cadherin in mesenchymal cells, leads to acquisition of epithelial cell phenotype and adherence junctions[16]. As well, treatment of epithelial cells with anti- E-cadherin antibodies disrupts epithelial contacts and changes to a mesenchymal phenotype. In vivo and in vitro studies revealed that loss of E-cadherin is normally accompanied by tumor progression and acquisition of migratory phenotype in tumor and the loss of E-cadherin correlates with grade/ stage of tumor[1]. Thus, E-cadherin expression count as a good prognostic factor/marker in tumorigenesis[16]. During tumor progression, normally, E-cadherin promoter becomes hyper methylated or its translation is repressed. In some cases an E-cadherin inactive mutant has been expressed or a miscent (silence gene) mutation[16].

Snail, Slug and SIP1 (Zinc Finger-containing transcription repressors) negatively regulate E-cadherin expression and are shown to induce EMT. Another

negative regulator of E-cadherin is basic helix-loop-helix transcription factor E12/E47, the E2A gene product. Thus expression of this transcription factor leads to progression of cancer. Moreover it is thought that Snail expression may initiate EMT while E12/47 maintains mesenchymal phenotype (Fig. 7). It is now clear that E-cadherin is involved also in cell proliferation via β- catenin, which is in turn involved in transcriptional activation of TGF 4 or LEF transcription factor.

In conclusion "loss of E-cadherin is central to EMT, both in cancer cells and in normal development"[16]. However E-cadherin is not the only factor, which is necessary for metastasis.



Peinado H. et al. Int. J. Dev. Biol. 2004

Figure 7. Model of the Participation of Different E- cadherin Repressors During Tumor Invasion

3.2.2 Vimentin

As EMT occurs, remodeling of the cytoskeleton can be observed in which cytokeratin intermediate filaments changes to vimentin[15]. However because of the sensitivity of vimentin expression to serum deprivation, this marker is not used as a reliable marker of EMT (comparing to E-cadherin)[15].

3.2.3 Metaloproteases

Another characteristic of EMT is acquisition of local invasion ability. To be able to invade, tumor cells have to produce matrix metaloprotease to degrade extracellular matrix (ECM) components including collagens and laminin 5. Moreover in some breast cancer cell lines it has been shown that expression of matrix metaloprotease-3 (MMP-3/ stromelysin1) is sufficient to induce EMT *in vitro* and tumorigenesis *in vivo*[15].

3.3 TGF-β

TGF-β was first identified as a growth factor inducing malignancy in fibroblasts[18]. However later on it has been shown that it has strong inhibitory effect on the cell growth in epithelial cells, so it considered as a tumor suppressor. TGF-β is a member of cytokine family with two subfamilies: TGF-β/ Activin/ Nodal subfamily and the BMP (Bone Morphogenetic Protein)/ GDF (Growth and Differentiation Factor)/ MIS (Muellerian Inhibiting Substance) subfamily[19-21].

3.3.1 TGF-β Receptor

The TGF-β receptor is a member of serine/threonine kinase receptor family. It consists of two subunits, both are transmembrane receptors containing an extracellular binding domain, a transmembrane and a C-terminal cytoplasmic domain. Type I receptors lack autokinase activity however it has a SGSGSG sequence refers as the GS domain, located immediately N-terminal to the kinase domain. Phosphorylation of the GS domain by the Type II receptor is necessary for Type I activation. Thus dimerization or ligand binding of both Type I and II is important for TGF-β signaling.

"TGF-β and activin have high affinity for type II receptor, while they don't interact with the type I receptor" [20]. Ligand bonded type II receptor will then bind to type I. In fact in such a case the ligand will bind to two type II and type I receptors, forming a large ligand-receptor complexes. This, results in conformational changes and phosphorylation of the GS domain of the Type I by constitutively active kinase domain of type II[20].

3.3.2 TGF-β signaling

The TGF-β signaling pathway plays an important role in normal and malignant cells. It is involved in cell proliferation, differentiation, apoptosis, migration, adhesion, embryogenesis and tumorigenesis[19, 20].

Even though TGF-β is known as a growth-inhibitory cytokine, it doesn't have any growth inhibitory function during embryogenesis. Receptor-ligand complex formation activates/phosphorylates downstream proteins such as the

Smad transcription factors (Fig. 8). To date, Smads are the most important mediators of TGF-β signaling. However TGF-β can also signal independently of Smads for example through the MAPK pathway (Erk1, 2, p38 or JNK)[19-21].

Smads are members of a family of transcription factor consisting of 8 members; (Smad1-8) that are classified in three groups. One group is the receptor regulated Smad (R-Smad: Smad 1,2, 3, 5 and 8). This group of Smads "is directly phosphorylated and activated by the type I receptor kinases and undergoes homotrimerization and formation of heteromeric complexes with the Co-Smad" [21]. another group is the co-mediator Smad (Co-Smad: Smad4). Smad4 heteromerization with R-Smads enable nuclear translocation of Smads where with other cofactors, they regulate transcription of target genes. The fact that same Smad can act as a repressor or activator of transcription, further increases the complexity of this pathway.

Inhibitory Smads (I- Smad) include Smad6 and 7. I-Smads negatively regulate TGF-β signaling by inhibiting the binding of R-Smads to the receptor or their interaction with Smad4 or by triggering receptor degradation.

TGF-β signaling is highly regulated by different mechanisms. While its activation and degradation is regulated by I-Smads, it is also regulated by ligand binding to its receptor by two different classes of molecules:

1- A group of proteins that by binding to ligand inhibit ligand access to the receptor. For example "proregion of TGF-β precursor, which after cleavage in the secretory pathway remains noncovalently bound to the bioactive domain as a latency-associated polypeptide (LAP)[20, 22]

2- Co-receptors are the membrane-anchored proteins like the membrane anchored proteoglycan betaglycan (TGF-β type III receptor), Connective Tissue Growth Factor (CTGF), etc. These proteins by binding to the ligand induce or inhibit (depending on the ligand and receptor) its binding to the signaling receptor.

Moreover receptor activation is negatively regulated by binding of FKBP12 to the unphosphorylated GS domain of type I receptors, thus preventing receptor activation/phosphorylation[20].

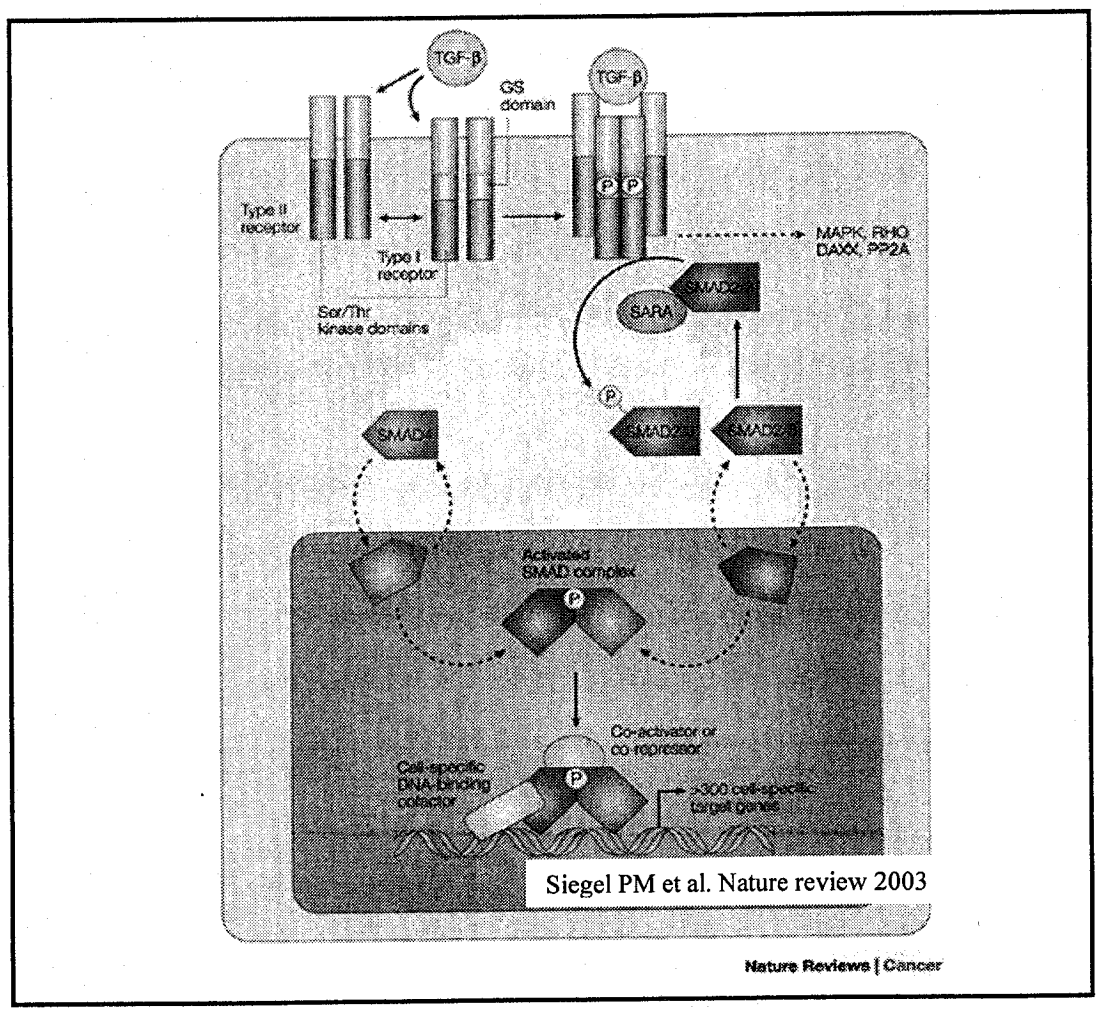


Figure 8. TGF-β Signaling Pathway

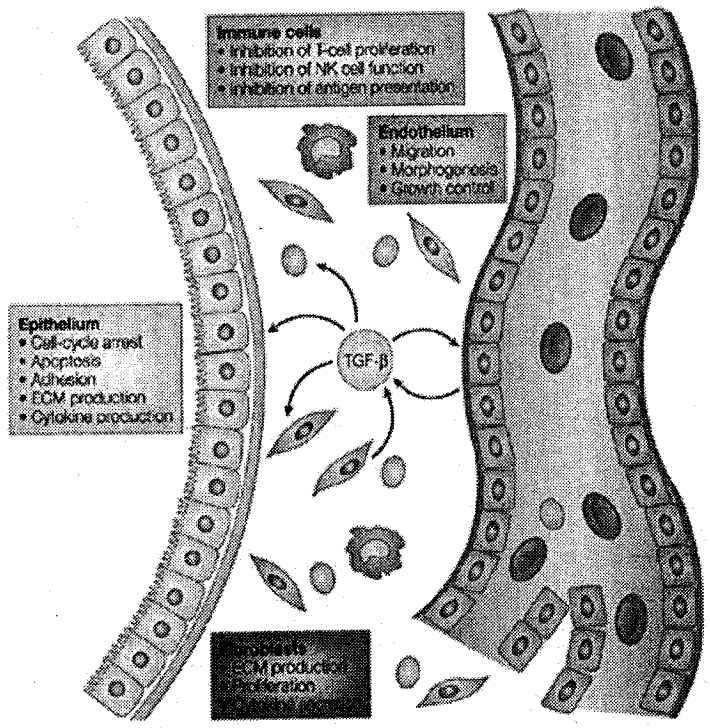
3.3.3 TGF-β and mammary gland

TGF-β 1,2 and 3 are expressed in the mammary gland at different stages of mammary gland development except during lactatin. TGF-β activation leads to cell growth arrest in ductal epithelial cells, while it increases apoptosis in the alveolar cells. In early stages of breast cancer it exerts growth inhibitory function. However as tumor progresses, TGF-β becomes a potent inducer of metastasis of breast cancer[23, 24]. "Loss of TGF-β type II receptor in the mammary epithelium results in lobular-alveolar hyperplasia in the developing mammary gland and increased apoptosis". While loss of TGF-β type II receptor "in the context of PyVmt (mammary tumor virus-polyomavirus middle T antigen) expression results in a shortened median tumor latency and an increased formation of pulmonary metastases" pointing to a dual role of TGF-β in breast cancer[25-27].

3.3.4 TGF-β and tumorigenesis

Since TGF-β pathway is important in apoptosis in adult/normal epithelial cells, it is not surprising that its signaling is disrupted in a variety of tumor cells. However tumor cells with activated TGF-β pathways have a worse prognosis. It has been shown that transgenic mice expressing TGF-β1 in the skin developed less skin papillomas in response to carcinogen treatment, but had an increased incidence of locally invasive spindle carcinomas compared with non-transgenic controls"[19]. Furthermore "mice expressing TGF-β 1 in the liver are more

susceptible to chemical hepatocarcinogenesis[18]. In mammary tumors it has been shown that activation of TGF-β signaling delays the appearance of primary mammary tumors proofing the inhibitory function of TGF-β on tumor formation. However these tumors showed increased lung metastasis indicating a positive role for TGF-β in invasion and later stages of tumorigenesis[18]. " Consistent with this observation, treatment of metastatic breast cancer cell lines with TGF-β in vitro enhanced metastases, whereas suppression of TGF-β receptor function markedly reduced their ability to metastasize"[18]. Overall, it is now clear that tumor response to TGF-β increases as tumor progress[19]. The positive effect of TGF-β on the tumorigenesis is on tumor cells themselves or on the microenvironment (stromal fibroblasts, endothelial cells and immune cells)[19, 28] (Fig.9).



Siegel PM. et al Nature review 2003

Figure 9. TGF-β Targets and Its Actions in Mature Tissues

In fact the role of TGF-β in EMT and tumor invasion is well established [19]and there is a time difference between the inhibitory effect of TGF-β on tumor growth and the time that TGF-β positively affects invasion/metastasis. Indeed TGF-β has a dual role in oncogenesis; a negative role in early stages of cancer as a growth inhibitor and a positive role in late stages of tumor progression as an inducer of metastasis[28, 29]. In addition, in a metastatic carcinoma model, Muraoka RS. et al. showed that blocking TGF-β leads to a decrease in tumor cell survival and metastasis[30] (Fig. 10).

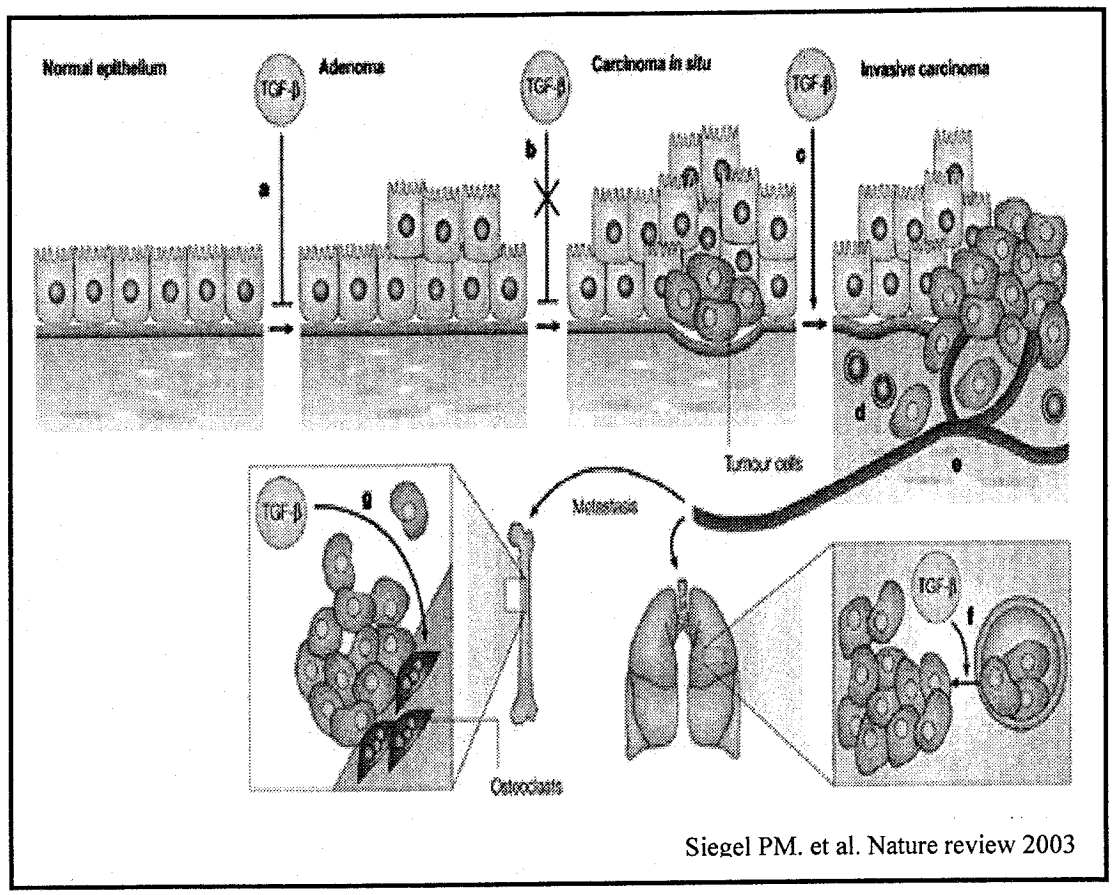


Figure 10. Points of TGF-β Action During Cancer Progression

3.3.5 TGF-β and Ras/ MAPK

TGF-β and Ras/ MAPK pathways play important roles during embryogenesis and tumor progression. Even though they exert opposing role in proliferation, but their cooperation is also known to be important. In fact for TGF-β to exert its positive effect on invasion of tumor cells to a distant site, activation of the Ras/MAPK pathway is necessary[20, 31-33]. It has been shown that MAPK (Erk1/2) as opposite effect on R-Smads depending on the cell type. It is known that in epithelial cells, Erk1/2 phosphorylate Smad1, 2 and 3 on the linker domain, inhibiting their translocation to the nucleus and their activation subsequently.

Although this negative effect is in the presence of low levels of TGF-β stimulation and in the higher levels of stimulation phosphorylated Smads by Erk1/2 do accumulate in the nucleus[21]. Moreover, in the same cell type (epithelial cells) Erk1/2 activation induces Smad4 degradation. Also, it may stabilize Smad corepressor TGIF[21]. So, overall in epithelial cells Erk1/2 negatively regulate TGF-β signaling[20]. However mostly in the mesenchymal cells Erk1/2 has opposite effect, as their activation, leads to phosphorylation, nuclear accumulation and increased activity of Smads[20, 23, 31, 34-37] or increased expression of TGF-β[38–41]. Siegel et al crossed mice expressing activated TGF-β type I receptor or dominant negative TGF-β Type II receptor under the control of the mouse mammary tumor virus promoter with mice expressing activated forms of the Neu receptor tyrosine kinase that selectively couple to the Grb2 or Shc signaling pathways. They showed that activated type I TGF-β receptor delayed tumor formation but increased tumor metastasis to the lung[42].

In addition Seton-Rogers et al showed that expression of TGF-β is increased following activation of Erk1/2 by ErbB2 activation in MCF10A mammary cells. Moreover increased activation of TGF-β then leads to increased migratory behavior of these cells pointing a central role for TGF-β and MAPK pathway in tumorigenesis[42, 43].

3.4 TGF-β and prolactin

Although TGF-β and prolactin signaling pathways, both, play a critical role in breast cancer progression, their crosstalk is not very well studied. There is one

report in which Philips et al showed that stimulation of T47D cells with prolactin inhibited the expression of TGF-β and metaloproteases[44]. Moreover there is only a published paper in which the author proposed a cross talk between PRL and TGF-β at AKT level[12].

4 Thesis Objectives

Prolactin hormone (PRL) is well characterized for its role as a terminal differentiation factor for mammary epithelial cells. As well, studies have implicated PRL as an autocrine/ paracrine growth factor contributing to the growth/ survival of breast cancer cells. However, this role of PRL in breast carcinogenesis may not be complete.

Metastasis, the spread of tumor cells to distant sites, involves a complex interplay leading to altered cell adhesion, survival, proteolysis, migration, angiogenesis, immune evasion and extravasations in target organs. Although metastasis represents the main cause of death in human cancer the signaling mechanisms governing this process are still not well defined. Accumulating evidence indicates a critical role for Ras/ Erk1/2 and transforming growth factor-β (TGF-β) ligands/ Smad pathway as the key pathways contributing to breast cancer metastasis. The specific aim of this thesis project was to assess the role of PRL/Jak2 in regulation of breast cancer progression/ metastasis.

To address this objective, I showed that by blocking prolactin receptor (PRLR) signaling pathway, ERk1/2 activation is significantly increased. This in turn was found to lead to activation of TGF-β/ Smad pathway. Cooperation of TGF-β/ Smad and MAPK (Erk1//2) pathways result in tumor progression. As tumor progresses it gains a mesenchymal property to be able to invade to a distant site. Since PRLR signaling is not functional in more aggressive breast tumor, restoring back the signal of PRLR can reverse the phenotype of tumor cells to a

less aggressive phenotype (epithelial like phenotype which are representative of a more differentiated tumor with better prognosis). Altogether, these data suggest that PRLR/ Jak2 act as negative regulator of the metastasis potential of breast cancer cells. Indeed the studies described in this thesis will help define a novel regulatory role for PRL hormone and describe a new mechanism controlling and preventing breast cancer metastasis.

5 Materials and Methods

5.1 Materials, Plasmid constructs and Antibodies:

Expression plasmid encoding the flag tagged long form of rat prolactin receptor was a gift from D. Devost (McGill University, Montreal, Canada) Expression plasmid encoding the kinase inactive Jak2 (KIJ) was kindly provided by Dr. Dewayne Barber (Ontario Cancer Institute, ONT, Canada) Expression plasmid encoding 3TP-Lux was a gift from Dr. J. Massague (Memorial Sloan-Kettering Cancer Center, New York, USA). Polyclonal antibodies to phosphorylated Thr308 of Akt, Akt, Phosphorylated Thr202/Tyr204 of p44/42 MAPK and p44/42 MAPK, as well as MEK1 inhibitor (PD98059) and Jak2 inhibitor (Ag490) were from Cell Signaling Technology (MA, USA). Monoclonal antibody to phosphorylated Tyr 694 of Stat5 was from Zymed laboratories Inc, (San Francisco, CA). Polyclonal antibody to TFIID (TBP) (SI-1), Goat anti mouse HRP and Goat anti rabbit HRP were from Santa Cruz biotechnology, Inc. (CA, USA). Monoclonal antibodies to mouse anti-vimentin, E-cadherin and Stat5 were from BD Transduction Laboratories (ONT, Canada). Polyclonal antibody to c-terminal phosphorylated Smad2, was kindly provided by Aris Moustalkas (Ludwig Institute for Cancer Research, Sweden). Polyclonal antibody to PRL, Jak2 and its SiRNA was from Upstate Biotechnology (NY, USA). Monoclonal antibodies to Flag- M2 and B- tubulin clone TUB2.1 as well as ovine prolactin were from Sigma-Aldrich, Inc (ONT, Canada). Matrigel matrix was purchased from Collaborative Biomedical Products (MA, USA). Transwell filter disks

(8μm) for migration assay by Corning were from Fisher Scientific. Cell proliferation ELISA, BrdU (colorimetric) kit as well as Lumilight plus kit were purchased from Roche (Laval, Canada). Human prolactin (hPRL) was a gift from Dr. Goffin (Institut National de la Santé et de la Recherche Médicale (INSERM), Paris, France). ECL Hyperfilm and Protein-A sepharose beads were purchased from Amersham-Pharmacia Biotech, Inc (QUE, Canada). Phosphothiolated oligonucleotides were made by Alpha DNA (QUE, Canada). Goat anti-mouse Rhoda mine Red X was from Jackson Immuno Research Laboratories Inc. (USA). Lipofectamine 2000, Lipofectine and Lipofectamine reagents as well as Hoechst Stain (33258) were purchased from Invitrogen (Canada).

5.2 Cell Culture:

Human breast cancer cell lines, MDA MB-231, T47D, MCF7 (Provided by Morag Park, McGill University, Montreal, Canada) were grown in Dulbecco's modified Eagle's Medium (DMEM) containing 10% FBS, 0.1 M L-glutamine and 50 units/ml penicillin, 50μg/ml streptomycin and starved in DMEM phenole-red free media containing L-glutamine. Cell treatments were carried out using DMSO (1:1000), AG490 (50μM), PP2 (10μM), or PD 98059 (10μM) as described. Phase contrast images were taken with a Zeiss Axiovision 135 microscope (Carl Ziess Canada Ltd, Toronto, ON) with a 10X objective and Northern Eclipse program version 6.0 (Empix Imaging, Mississauga, ON)

5.3 Cell transfection

T47D cells were transfected by electroporation. 10 X 10^6 cells were resuspended in 500 μl PBS in a 0.4 cm electroporation cuvette (Bio-Rad Laboratories) on ice.

Appropriate amounts of DNA were added then to the cuvettes containing the cells and electroporated at the high capacity of 0.975 μF, and the voltage of 0.240 kv with an ideal time constant of 18-20 ms (Bio-Rad Gene Pulser III). Immediately after electroporation the transfected cells were put on ice for 5min then resuspended in 4ml DMEM growth media and plated in a 6cm cell culture plates, cells were incubated for 6-8hrs and then treated or not in starvation media for overnight.

MDA MB-231 cells were transfected for 5hrs using Lipofectamine 2000 at a ratio of DNA/ Reagent of 1/3 following manufacturer's instructions (Invitrogen) For phosphorylation studies cell were then incubated in media containing 2% FBS for an overnight period prior to stimulation (oPRL 2μg/ml, for 10min) and lysis. For vimentin expression cells were then incubated in media containing 2% FBS in the presence or absence of OPRL (2μg/ml) for 24 hrs prior to cell lysis.

5.4 Cell lysate

Cells were lysed in RIPA lysis buffer (50mM Tris-HCL, PH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1mM EDTA, 1mM PMSF, 1μg/ml aprotinin, leupeptin and pepstatin, 1mM sodium orthovanadate, 1mM NaF). The lysates were cleared by centrifugation at 13000 x g for 10 min at 4°C to remove insoluble material. Protein concentrations were measured using the Bradford technique.

5.5 Immunoprecipitation and Western Blotting:

Immunoprecipitation was done for 3hrs at 4°C using polyclonal antibody to Jak2 and protein-A sepharose beads. Immunoprecipitates were washed with RIPA buffer and separated on SDS-PAGE gel.

Equal whole cell lysate, nuclear extract or immunoprecipitated lysate were used to run on SDS-PAGE, separated proteins then transferred to nitrocellulose membrane and subjected to western blot using appropriate antibody, membrane was stripped and immunostained for other proteins.

5.6 Nuclear Extract

Nuclear extracts were prepared by initially lysing cells with a hypotonic buffer (10mM HEPES-KOH, PH 7.9, 1.5m M $MgCl_2$, 10mMKCl, 0.5mM dithiothreitol, 20mMNaF, 1mMpeftabloc, 5µg/ml aprotinin and 2µg/ml leupeptin), Cells were pelleted at 13000 rpm for 10min at 4°C, supernatant was discarded. The pellet was washed 3 times with phosphate buffered saline and lysed with a high salt buffer (20mM HEPES-KOH, PH 7.9, 25% glycerol, 420mM NaCl, 1.5mM $MgCl_2$, 0.2mM EDTA, 20mMNaF, 5µg/ml aprotinin and 2µg/ml leupeptin) for 30 min at 4°C by vortexing.

5.7 BrdU Assay

BrdU colorimetric assay was performed as described in the protocol (Roche, Laval, Canada) briefly 3000 cells per well were seeded in 96 well plate in DMEM growth media containing 2% serum after O/N cells were treated or not in 2% FBS. BrdU was added as indicated in the protocol and absorbance of the samples was measured after 24hrs. For longer treatment, media was changed every 48hrs.

Results are a mean of three experiments and each experiment is done as a triplicate.

5.8 Invasion Assay

24-transwell plate were coated will Matrigel on ice with a concentration of 100 µg/ cm^2, then incubated at 37°C for 30min (5% CO_2). After 15 min RT, 50000 cells (T47D) or 100000 (MDA MB-231) were added to each well in 200µl media containing 5% FBS with or without treatment. Cells then were incubated for 24hrs (T47D) or 48hrs (MDA MB-231) at 37°C (5% CO_2) and then fixed in formalin phosphate for 30min at RT. After 3 times washing by PBS cells were stained by 0.1% crystal violet in 20 % MetOH for 30min at RT. Staining solution was washed by PBS and membrane was scraped off by a Q-tip. Migrated cells were counted using five fields of triplicates for each experimental point using Zeiss Axiovision 135 microscope (Carl Zeiss Canada Ltd, Toronto, ON) with a 10X objective and Northern Eclipse program version 6.0 (Empix Imaging, Mississauga, ON)

5.9 Immunostaining

T47D cells were plated at 50% confluency on coverslips precoated with poly-L-Lysine in a 24-well plate. After a few hours, cells were treated with DMSO, 50 µM Ag490, 10µM PD98059 and a combination of AG490/PD98059 for an overnight period in phenol red free serum starved DMEM. Cells were fixed in 2%PFA/PBS, blocked in 5% BSA/PBS, and incubated with monoclonal e-cadherin antibody (1: 500) or monoclonal vimentin antibody (1:500) for overnight. Goat anti-mouse Rhodamine Red X (1:200) was used as secondary

antibody for 1hr at RT. Hoechst was used to stain nucleus with a concentration of 1:2000 for 10min at room temperature. Coverslips were mounted on Geltol mounting medium (Immunon Thermo Shandon, PA, USA) and observed under confocal microscope (Zeiss LSM-510 META laser scanning microscope, Carl Zeiss, Jena, Germany) using a 60X oil immersion lens. Photographs were analyzed using LSM software.

5.10 Antisense Oligonucleotide

Control-5′- GATGAATTACGTAACTGGCCCGCCC-3′

Anti hPRL mRNA- 5′- TGGCGATCCTTTGATGTTCAT-3′

Control oligonucleotide was generated by a random sequence of nucleotides. The anti hPrl mRNA phosphorothioated oligonucleotide was antisense to hPRL mRNA, beginning at the start codon (AUG) site of the hPRL mRNA and extended to a total length of 21 nucleotides.

T47D cells were plated in a 24 well plate at a concentration of 300000 per well. Cells were treated with 200μM of AntihPRL or control oligo in starvation media. Fresh media containing oligonucleotides was replenished every 24hrs for up to 48hrs or 72hrs. Cells were lysed in 250μl Laemmli sample buffer.

6 Results

Blocking Jak2 Inhibits Growth of T47D Cells

Prolactin role in mammary gland development/ differentiation is very well established. However its role in breast cancer is not very well understood. PRL is known for its effect as a survival factor in breast cancer cells. It has been shown that blocking PRLR signaling by a PRL antagonist in T47D cells results in cell growth inhibition and cell death[45]. Since Jak2 is the most important kinase mediating PRLR signaling, we thought to verify the effect of inhibition of Jak2 on T47D cell growth. T47D cells were treated with 50μM of AG490, a specific pharmacological inhibitor of Jak2, for up to 5days. Growth of cells was monitored by trypan- blue counting after 24, 48 and 72 hrs (Fig 1A). BrdU assay was performed to further confirm our counting results. As can be seen in Figure 1A and B, treatment of cells with AG490 led to inhibition of cell growth after 48hrs, supporting a survival role for PRL since prolonged blocking of its signaling by Jak2 inhibitor, led to cell growth inhibition. However a short time treatment (24hrs) didn't have any effect on survival/ proliferation of cells. To further investigate the effect of prolactin receptor/ Jak2 signaling in cancer, and to avoid the apoptotic/ growth inhibition effect of prolonged treatment of cells by Jak2 inhibitor, we used 24 hrs of treatment (24hrs) in subsequent experiments.

Prolactin Inhibits Erk1/2 Activation via Jak2 in T47D and MCF7 Breast Cancer Cell Lines.

While the role of PRL in mediating cell survival in breast cancer is known the role of PRL in regulating other properties of breast cancer cells such as epithelial plasticity and invasion is not well studied. In order to determine the role of PRL in pathways important for EMT, we first looked at Erk1/2 activation as the promoter of cancer progression/metastasis. As can be seen in Fig 2A blocking of Jak2 by AG490 (50μM for O/N) in MCF7 and T47D cells led to a significant increase and prolonged phosphorylation of Erk1/2 with no effects on Akt activation. As can be seen in Fig 2B, it is clear that PRL is still able to increase Erk1/2 activation, indicating that PRL induced activation/phosphorylation of MAPK is Jak2 independent. To determine the effect of inhibition of Src, another kinase mediating PRLR signaling, versus Jak2 we used PP2, a chemical inhibitor of Src activation. Western blot analysis of phosphorylated Akt revealed that while Src inhibition has no effect on Erk1/2 activation, basal but not PRL induced Akt phosphorylation/ activation is Src dependent (Fig2A). Western blotting for Stat5 phosphorylation revealed that treatment of AG490 was sufficient to significantly inhibit PRL-induced Stat5 phosphorylation (Fig2A). Furthuermore, we observed that the effect of Jak2 inhibition on Erk1/2 activation/phosphorylation can be observed as soon as 5 min after treatment reaching a maximum after 10min and again appearing with O/N inhibition of Jak2 (Figure 2C). This activation of MAPK (Erk1/2) is compatible with activated MAPK (Erk1/2) following EGF stimulation, a known activator of MAPK pathway (Fig 2D) while overnight

treatment with PRL alone has no effect (Fig 2D) on MAPK. We next confirmed this result using a kinase inactive form of Jak2 (KIJ), in which the predicted type VIII phosphotransferase motif in the C-terminal JH1 kinase domain of Jak2 has been mutated[46]. Overexpression of KIJ in T47D cells, led to a significant increase in phosphorylation of Erk1/2 (Fig 2E). Same results were obtained in MCF7 cells overexpressing KIJ (Fig 2E) or by using SiRNA against Jak2 to knock down expression of Jak2(Fig 2E). Since Jak2 can be also activated by other cytokines such as growth factors, to determine that this effect of inhibition of Jak2 is in fact related to inhibition of PRLR signaling rather than other cytokines, we used antisense oligonucleotide to hPRL. Blocking autocrine PRLR signaling in T47D cells using anitsense oligonucleotide for PRL (AntiPRL) led to activation of Erk1/2 after 48hrs of treatment (Figure 2F).

These results indicate that blocking PRLR signaling results in high and prolonged activation of MAPK (Erk1/2).

Inhibition of Prolactin Receptor Signaling by Jak2 Inhibitor Leads to Activation of the TGF-β/ Smad Pathway

TGF-β has been shown to contribute to EMT and metastasis of mammary tumors. However, the cross talk of Prolactin and TGF-β pathway is not documented. This prompted us to see if TGF-β pathway is also regulated by PRL. We investigated the activation of this pathway by looking at activation of its downstream protein, Smad2. As shown in Figure 3A, treatment of T47D cells with AG490 for different time points led to Smad2 activation as measured by western blotting using a specific phsopho-Smad2 antibody recognizing the two C-

terminal serine residues phosophorylated by the activated TGF-β Type I receptor. This has been confirmed by KIJ overexpression in T47D cell line. As can be seen in Fig3B, in cells overexpressing KIJ, Smad2 phosphorylation was significantly induced. These results revealed a negative effect of Jak2 on TGF-β/ Smad2 pathway.

Increased Activation of TGF-β/Smad Pathway by Blocking PRLR Signaling is Erk1/2 Dependent.

The mechanism by which Ras pathway cooperates with TGF-β to induce EMT is still not fully characterized. However the positive effect of Erk1/2 (Ras) activation on TGF-β/Smad has been shown in different cell systems[47, 48]. To further investigate the crosstalk of these two pathways, involved in PRL function on cell plasticity, we blocked Erk1/2 activation using PD98059, a chemical inhibitor of MEK-1. To determine if concentration of PD98059 is effective to inhibit Erk1/2 activation, we did a control experiment (Fig4A). As can be seen in Figure 4B and C, blocking of Erk1/2 activation with 10μM of PD98059 for 24 hrs, inhibits induced activation of Smad2 by AG490 or KIJ.

These results indicate that PRLR/ Jak2 signaling leads to inhibition of MAPK (Erk1/2) activation and in turn, inhibition of TGF-β pathway, two key players of EMT process.

Blocking Prolactin Receptor/ Jak2 Signaling Induces EMT

T47D cells maintain epithelial-like features and are considered to be of weak invasive phenotype[10]. As well these cells are known to express both PRL and its receptor thereby creating an autocrin/ paracrine growth and survival loop

for PRL[4]. Since Erk1/2 and TGF-β/ Smad2, two major regulator of EMT, are significantly activated by inhibition of PRLR signaling in these cells, we investigated whether blocking the PRL loop would lead to phenotypic changes typical of EMT process and increase the invasive phenotype of these cells. The activity of Jak2 was blocked using a 50μM of AG490. As shown in Figure 5A, treatment of T47D cells with AG490 for 24hrs led to changes in cell morphology, from epithelial/cuboidal growing as tight colonies to mesenchymal/fibroblastic phenotype growing as dispersed colonies. Furthermore, this was found to be associated with deorganization of E-cadherin from the membrane to the cytoplasm and loss of E-cadherin. As well, vimentin, an intermediate filament which is known as a marker of mesenchymal cells was more expressed in treated cells with AG490 compared to control cells treated with DMSO (Figure5A). Invasion assays using Matrigel covered transwell plates proved that these phenotypic changes were accompanied with an augmented invasive capacity of T47D cells when treated with Jak2 inhibitor compared to control cells (190% of control) (Fig5B). Effect of inhibition of Jak2 on E-cadherin expression which serve as a reliable marker of EMT, was confirmed by overexpression of KIJ, As shown in Figure 5C, E-cadherin expression was significantly lost compared to control. Finally, As shown in Figure 5D, downregulation of autocrine PRL levels using antisense oligonucleotide to mRNA of hPRL, in these cells led to a significant loss of E-cadherin expression. Altogether, these results indicate that inhibition of PRLR/ Jak2 signaling in epithelial-like breast cancer cells leads to EMT and enhances their invasion potential following activation of Erk1/2.

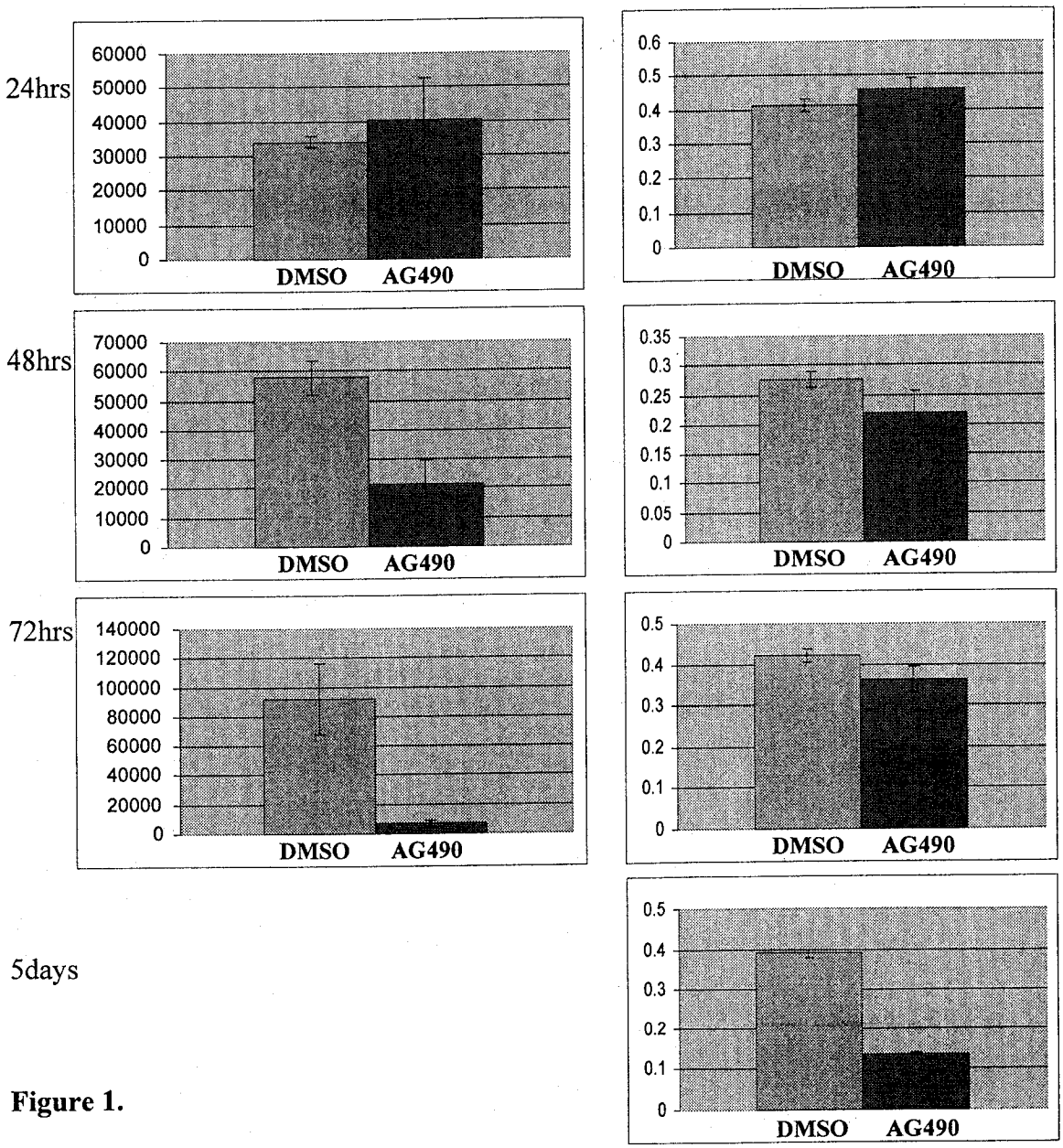
dependent increase in Jak2 phosphorylation was observed in cells overexpressing the PRLR and Jak2, as detected by western blotting of whole cell lysates using a polyclonal antibody to phosphor-Jak2 (Y1007/1008) detecting activation of Jak2. However, Jak2 phosphorylation was observed in cells overexpressing PRLR/Jak2 indicating an autocrine function of PRLR. Moreover, cell lysates overexpressing PRLR/Jak2 or empty vector were immunoprecipitated with monoclonal antibody to Flag. Immune complexes were blotted with a monoclonal antibody to phosphotyrosine. As expected two phosphorylated band was detected corresponding to PRLR and Jak2. To confirm this result membrane was reprobed by polyclonal antibody to Jak2 and then with monoclonal antibody to Flag (Figure 7C) however, antibody for Flag detected a non-specific band right bellow the receptor. Again, these results confirm an autocrine and paracrine function of PRLR followed transfection of PRLR/ Jak2 and stimulation with PRL. Next we investigated the expression level of vimentin, which is highly expressed in MDA MB-231 cells. Cells were transfected with PRLR and Jak2 or empty vector. After transfection, cells were serum starved, left unstimulated or stimulated with PRL for 72 hrs. Cell lysates were then subjected to western blot using monoclonal antibody to vimentin. As can be seen in Figure7D, cells overexpressing PRLR/Jak2 expressed less vimentin comparing to parental cells transfected with empty vector. Stimulation of these cells with PRL led to a more decrease in vimentin expression, indicating that PRL is a suppressor of EMT. To determine the effect of restoring PRLR signaling on cell invasion we next examine invasion capacity of these cells. Same transfection was performed and 24hrs after

transfection cells were plated on matrigel in transwell plates for invasion assay (Fig 7E). After 48 hrs we detected a significant loss of invasion capacity (50%) in cells transfected with PRLR/Jak2 compared to parental cells. Altogether, these results confirm that PRLR signaling pathway is an inhibitor of EMT process and cell invasion in breast cancer cell lines.

7 Figures

A.

B.


24hrs
60000
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0
DMSO
AG490
0.6
0.5
0.4
0.3
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DMSO
AG490
48hrs
70000
60000
50000
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10000
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DMSO
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0.35
0.3
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0.2
0.15
0.1
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DMSO
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72hrs
140000
120000
100000
80000
60000
40000
20000
0
DMSO
AG490
0.5
0.4
0.3
0.2
0.1
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DMSO
AG490
5days
0.5
0.4
0.3
0.2
0.1
0
DMSO
AG490

Figure 1.

Figure1. Blocking Jak2 Inhibits Growth of T47D Cells

T47D cells were treated with 50μM of AG490 or DMSO as control for 24hrs, 48hrs, 72hrs or 5days. A; live cells were counted using trypan blue staining. B; BrdU assay indicates proliferation of T47D cells treated with AG490 compared to control (DMSO). Graphs are representative of three individual experiments

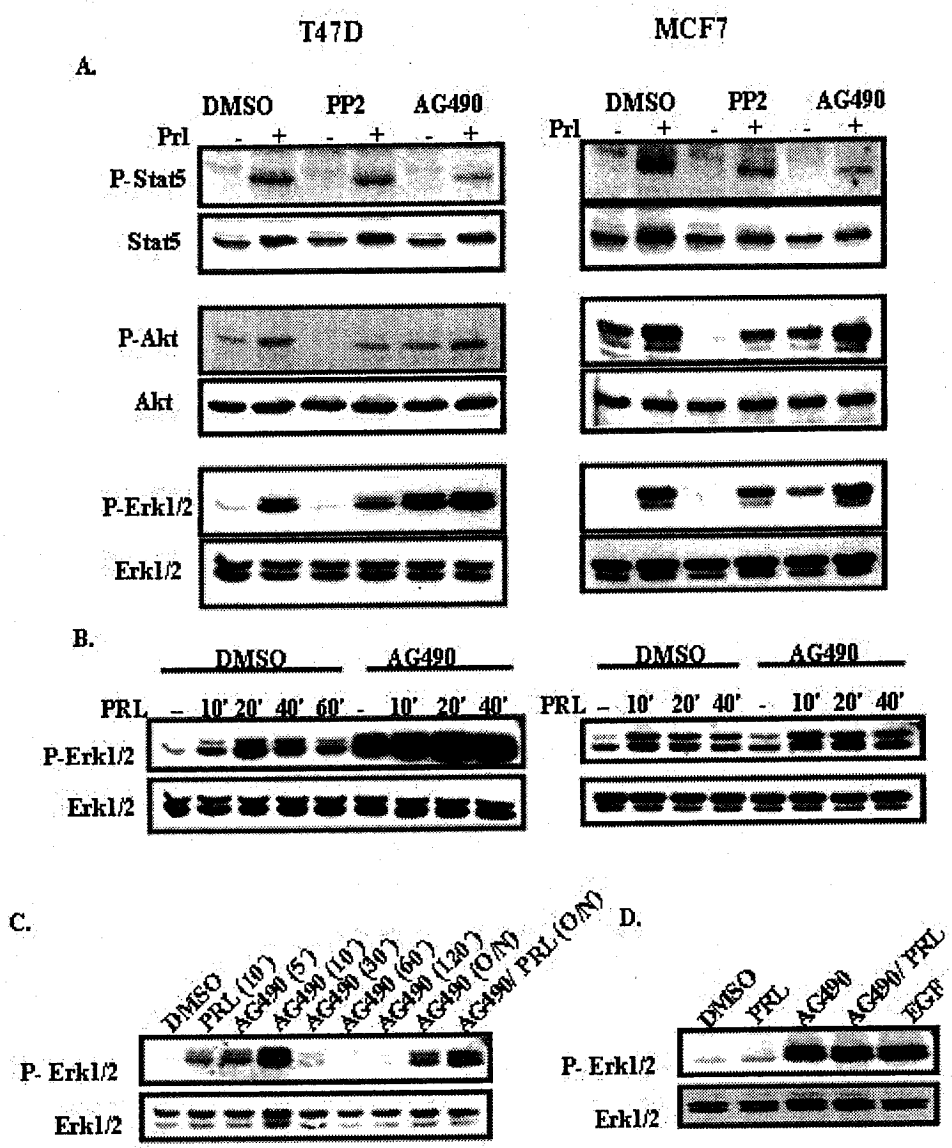
T47D
MCF7
A.
DMSO
PP2
AG490
Prl
P-Stat5
Stat5
P-Akt
Akt
P-Erk1/2
Erk1/2
B.
DMSO
AG490
PRL
10' 20' 40' 60'
C.
DMSO
PRL (10')
AG490 (5')
AG490 (10')
AG490 (30')
AG490 (60')
AG490 (120')
AG490 (O/N)
AG490/ PRL (O/N)
D.
DMSO
PRL
AG490
AG490/ PRL
EGF

Figure 2.

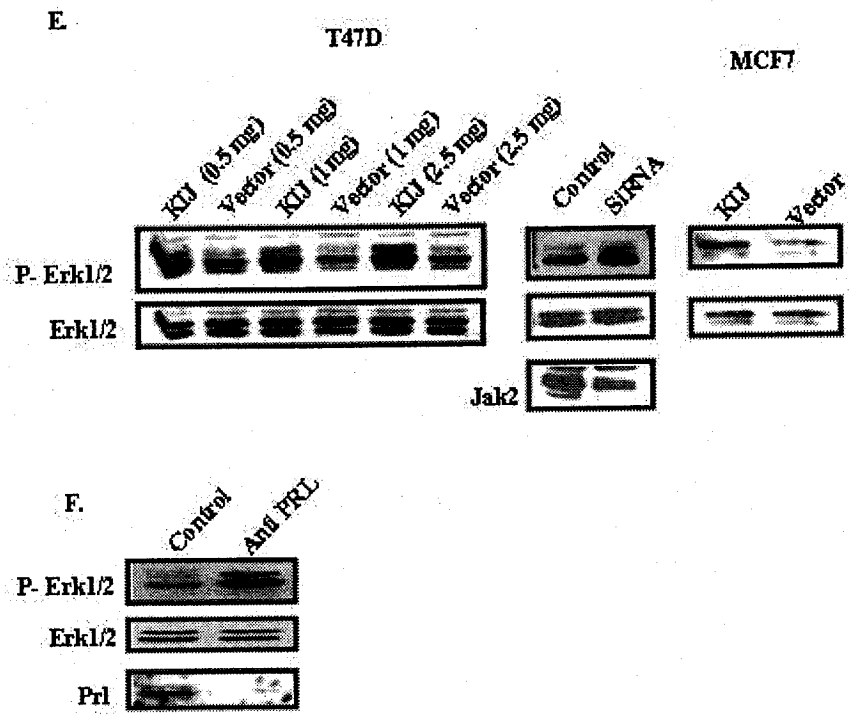

Figure 2. continued

Figure 2. Prolactin Inhibits Erk1/2 Activation via Jak2 in T47D and MCF7 Breast Cancer Cell Line.

A-D; T47D and MCF7 cells were treated with DMSO, AG490, EGF for overnight and treated or not with hPRL for 10min or as indicated. Whole cell lysates were used for immunoblotting with monoclonal antibodies to phospho- Stat5 and phospho- Akt as well as polyclonal antibody to phospho-Erk1/2. The membrane was then reprobed with appropriate antibody to Stat-5, Akt or Erk1/2.

E; T47D or MCF7 cells were transfected with the indicated amount of KIJ or SiRNA, whole cell lysate were used for immunoblotting with polyclonal antibody to phospho-Erk1/2. Membrane was reprobed with polyclonal antibody to Erk1/2 and in the case of SiRNA transfection with a polyclonal antibody to Jak2.

F; T47D cells were treated with antisens oligonucleotide to mRNA of hPRL for 24hrs and whole cell lysates were used as above. As well PRL expression was monitored using polyclonal antibody to hPRL.

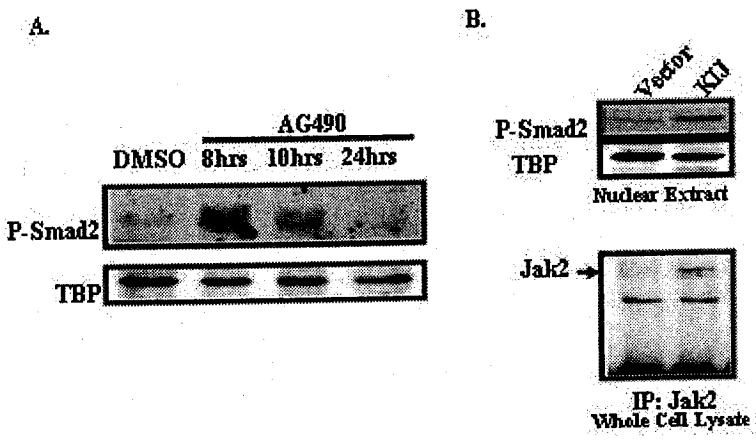

Figure 3. Inhibition of Prolactin Receptor Signaling by Jak2 Inhibitor Leads to Activation of the TGF-β/ Smad Pathway

T47D cells were treated for indicated time with AG490 or DMSO as control (A) or transfected with 10μg KIJ or empty vector (B). Nuclear fractions were immunoblotted using a polyclonal antibody to phosphor-Smad2. The membrane was then stripped and reprobed with a polyclonal antibody to TBP. Parallel transfection was carried out, cells were used for immunoprecipitation analysis using a polyclonal antibody to Jak2 and immunoblotting was performed using a polyclonal antibody to Jak2 (B).

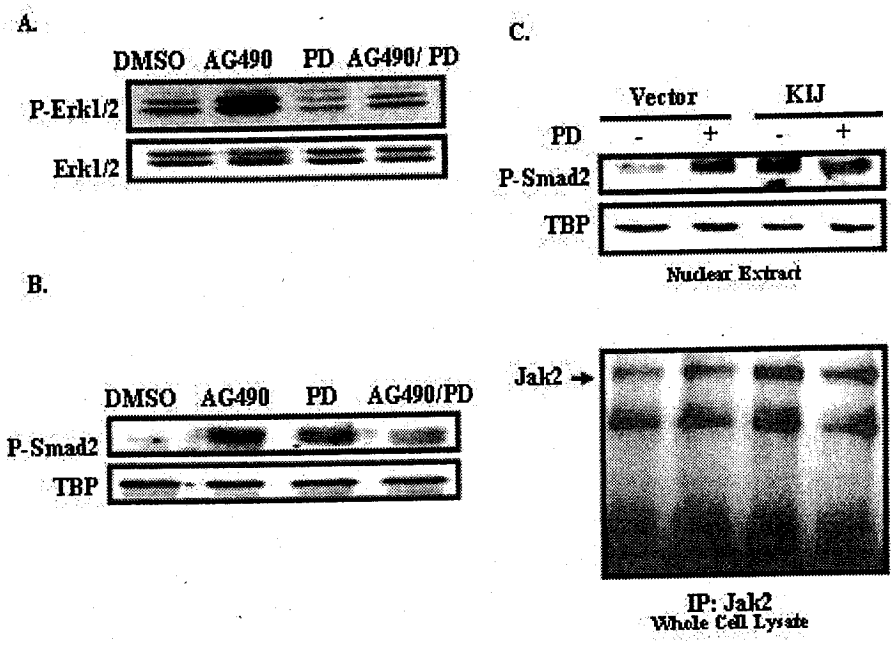
A.
DMSO AG490 PD AG490/ PD
P-Erk1/2
Erk1/2
B.
DMSO AG490 PD AG490/PD
P-Smad2
TBP
C.
Vector KIJ
PD - + - +
P-Smad2
TBP
Nuclear Extract
Jak2
IP: Jak2
Whole Cell Lysate

Figure 4.

Figure 4. Increased Activation of TGF-β/Smad Pathway by Blocking PRLR Signaling is Erk1/2 Dependent.

A; T47D cells were treated with AG490, DMSO, PD98059 or AG490 and PD98059 for an overnight period and whole cell lysates were subjected to immunostaining using polyclonal antibody to phosphor-Erk1/2. Membrane was reprobed with polyclonal antibody to Erk1/2.

B: T47D cells were treated as A, nuclear fractions were immunoblotted with polyclonal antibody to phosphor-Smad2. Membrane was then stripped and reprobed by polyclonal antibody to TBP.

C; T47D cells were transiently transfected with empty vector or indicated amount of KIJ, Nuclear fractions were separated on SDS/PAGE and western blotted with a polyclonal antibody to phosphor-Smad2. Membrane was then reprobed with TBP. Parallel transfections were carried out and cells were used for immunoprecipitation analysis using a polyclonal antibody to Jak2 and immunoblotted with same antibody.

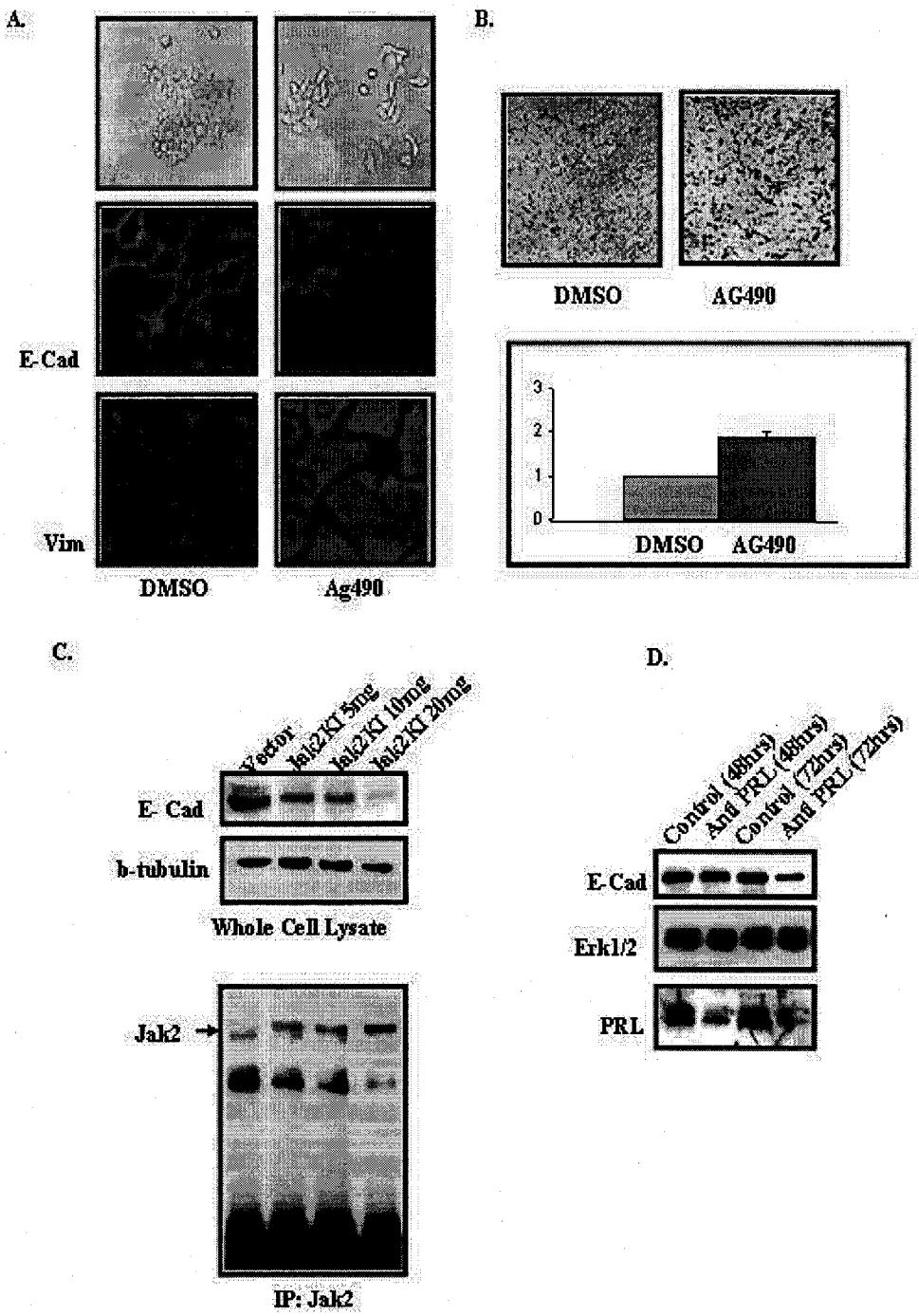
A.
E-Cad
Vim
DMSO
Ag490
B.
DMSO
AG490
3
2
1
0
DMSO
AG490
C.
Vector
Jak2KI 5mg
Jak2KI 10mg
Jak2KI 20mg
E- Cad
b-tubulin
Whole Cell Lysate
Jak2
IP: Jak2
D.
Control (48hrs)
Anti PRL (48hrs)
Control (72hrs)
Anti PRL (72hrs)
E-Cad
Erk1/2
PRL

Figure 5.

Figure 5. Blocking Prolactin Receptor signaling induces EMT

A; Phase contrast microscopic images (upper panel) and immunostaining using a monoclonal antibody to E-cadherin (middle panel), immunostaining with a monoclonal antibody to vimentin (lower panel) of T47D cells treated with either AG490 or DMSO for 24hrs.

B; Equal number of T47D cells were plated on Matrigel coated 24-transwell plates and treated with AG490 or DMSO as control, for 24hrs and invaded cells were quantified as described in Material and Methods. The mean of three separate experiments is presented. Microscopic images of a field of invaded cells are shown in parallel.

C; T47D cells were transiently transfected with empty vector or expression plasmid encoding for KIJ. Whole cell lysates were immunoblotted with a monoclonal antibody to E-cadherin. The membrane was then reprobed with a monoclonal antibody to β-tubulin. Lysates from the same transfection were immunoprecipitated using a polyclonal antibody to Jak2 and subjected to immunodetection with the same antibody.

D; T47D cells were treated with antisene oligonucleotide to mRNA of hPRL or control oligodeoxynucleotide for the indicated time points. Whole cells lysates were immunodetected using a monoclonal antibody to E-cadherin (upper panel) or a polyclonal antibody to PRL (lower panel). Equal loading was monitored using a polyclonal antibody to Erk1/2 (middle panel).

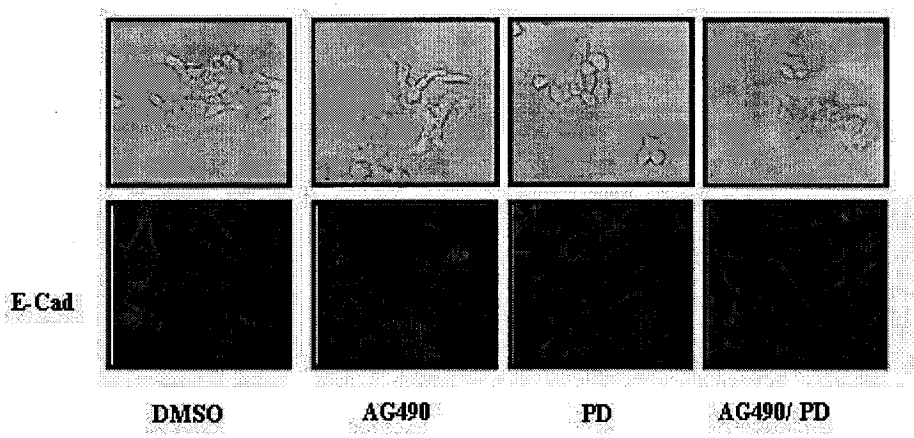

Figure 6. Increased EMT by Blocking PRLR Signaling is Due to Elevated Erk1/2 Activation

Phase contrast microscopic images (upper panel) and immunostaining using a monoclonal antibody to E-cadherin (lower panel) of T47D cells treated with AG490, PD98059, DMSO or AG490 and PD98059 for 24hrs are presented.

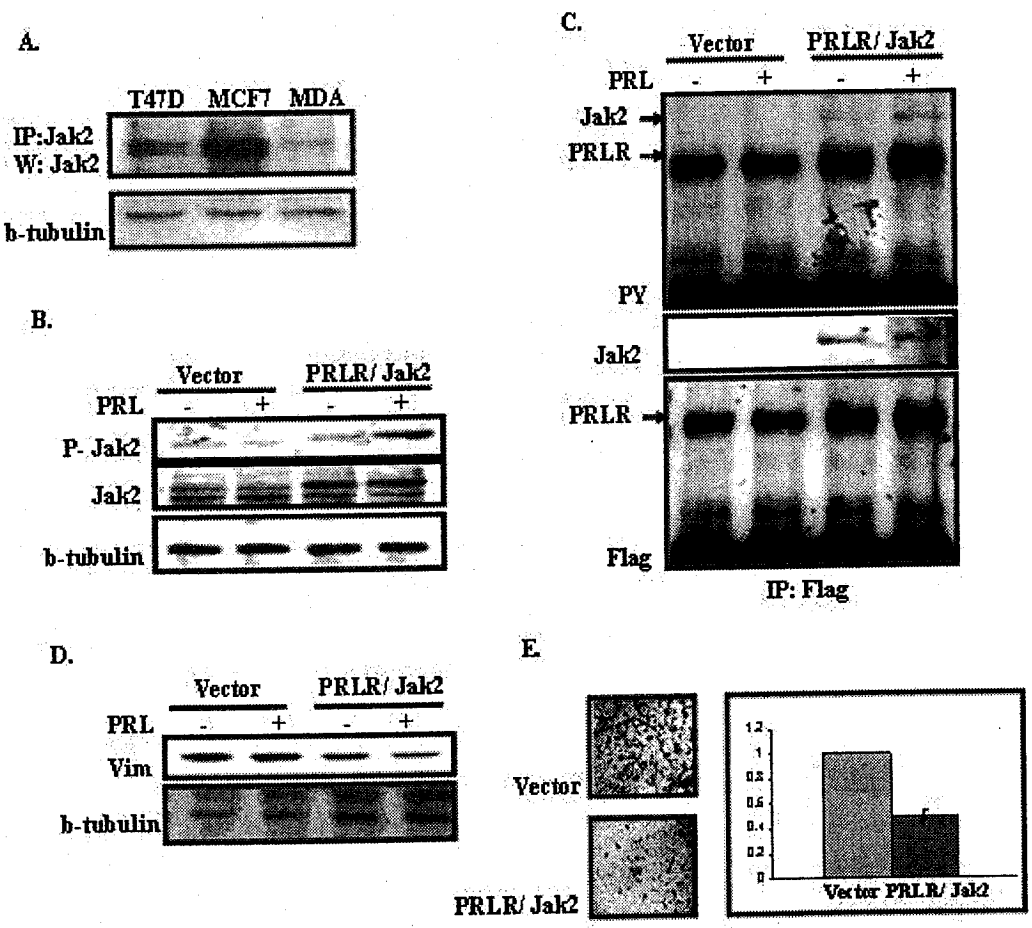
A.
T47D MCF7 MDA
IP:Jak2
W: Jak2
b-tubulin
B.
Vector
PRLR/ Jak2
PRL
- + - +
P- Jak2
Jak2
b-tubulin
C.
Vector
PRLR/ Jak2
PRL
- + - +
Jak2
PRLR
PY
Jak2
PRLR
Flag
IP: Flag
D.
Vector
PRLR/ Jak2
PRL
- + - +
Vim
b-tubulin
E.
Vector
PRLR/ Jak2
Vector PRLR/ Jak2

Figure 7.

Figure 7. Prolactin Signaling Inhibits the Invasiveness of Breast Cancer Cell Line MDA MB-231

A; Equal whole cell lysate of T47D, MCF7 and MDA MB-231 cells were separated on SDS/PAGE gels and immunoblotted using polyclonal antibody to Jak2, membrane was then reprobed with β-tubulin.

B; MDA MB-231 cells were transiently co-transfected with plasmids encoding a FLAG- tagged PRLR long form and Jak2 or with vector alone. Cells were then stimulated or not with oPRL for 10min. Whole cell lysates were subjected to immunodetection using a polyclonal antibody to phospho- Jak2. Membrane was then reprobed with a polyclonal antibody to Jak2 and then with a monoclonal antibody to β- tubulin.

C; Similar transfections and cell treatments were performed as in A. and whole cell lysates were immunoprecipitated using the monoclonal antibody M2 to FLAG epitope. Immunoprecipitated lysates were separated on SDS/ PAGE and immunoblotted with a monoclonal antibody to phosphotyrosine. Membrane was then reprobed with a polyclonal antibody to Jak2 and then with the monoclonal antibody to FLAG epitope.

D; same transfections as A. were performed. Cells were then stimulated or not with oPRL for 24hrs. Immunodetection was then carried out on whole cell lysates using a monoclonal antibody to vimentin. Equal loading was monitored by reprobing the membrane with a monoclonal antibody to β-tubulin.

E; Similar transfections as in A. were performed. The following day cells were trypsinized and equal cell numbers were plated for invasion assay in the presence

of oPRL as described in Material and Methods. The mean of three different experiments is shown. Microscopic images of a representative field of invaded cells are shown in the right panel

8 Discussion

Prolactin has long been known as an important factor in regulating mammary epithelial cell growth and differentiation. Furthermore, it is now accepted that prolactin functions as an autocrine factor promoting the growth/survival of breast cancer cells. It has to be mentioned that while the functionality of the PRL autocrine loop previously established, suggests that PRL signaling pathways such as Jak2 and Stat5a to be constitutively active in breast tumor cell lines like T47D and MCF7 cells, under my experimental condition set-up of serum starvation in combination with a possible limitation in detection, I do not observe constitutive activation of this pathway, as measured by phosphorylation of Stat5a and indeed Stat5a phosphorylation occurs upon exogenous PRL stimulation (Fig 2). Here I showed that the positive effect of PRLR signalling on growth/survival of breast cancer is through Jak2 as inhibition of Jak2 the main kinase activated by PRLR signalling resulted in inhibition of growth/survival of T47D cell line after 48 hrs of treatment (Fig 1).

Furthermore my studies indicate for the first time that in human breast cancer cells Jak2 exerts a negative regulatory effect on the MAPK pathway and that Jak2 may limit PRL-dependent activation of the MAPK (Erk1/2) cascade (Fig 2). Blocking of autocrine/ paracrine function of PRL in T47D cells using anitsense oligonucleotide to hPRL gene, also led to a marked increase in MAPK (Erk1/2) phosphorylation/activation after 24hrs of treatment (Fig 2). Therefore, blocking PRL expression or PRLR signaling by inhibiting Jak2 kinase activity, results in a robust and prolonged activation of the MAPK pathway in breast

cancer cells, suggesting that PRL/ Jak2 pathway serves to mediate negative regulatory signals to MAPK pathway. The inhibitory effect of Jak2 on the MAPK pathway has already been shown by Sandberg et al. They reported that activation of Angiotensin II type 2 receptor leads to activation of Jak2, which in turn inhibits Erk1/2 activation/phosphorylation by increasing MAPK phosphatase-1 activity[49]. My results suggest that Jak2 is not required for PRL-induced Erk1/2 activation. Rather, Jak2 is required for PRL-induced Erk1/2 inactivation (Fig 2). However the mechanism by which this negative regulation is conducted, remains unknown.

Moreover, here is the first report of negative effect of Jak2 (the main kinase of PRLR signalling) on TGF-β/ Smad pathway (Fig 3). It has been shown that TGF-β ligand is negatively regulated by PRL in T47D cells[44]. However, there was no further experiment on the involved signalling pathway. Indeed my results are in agreement with reports on positive effect of Erk1/2 on Smad2 phsophorylation/ activation, since using of PD, Mek1 inhibitor resulted in decreased phsophorylation of Smad2 (Fig 4). However Erk1/2 independent activation of Smad2 is also possible because of the fact that Smad2 phsophorylation is partially inhibited following treatment with PD at a dosage that Erk1/2 phosphorylation is completely blocked. These results indicate a negative effect of Jak2 on TGF-β/ Smad2 pathway partially through Erk1/2.

On the other hand, I clearly showed that blocking of PRLR signalling by Jak2 or antisens oligonucleotide to hPRL gene, leads to a loss of E-cadherin expression as well an increase in Vimentin expression (Fig 5). This leads to an increase in invasiveness potential of T47D cells (Fig 5). This event is dependent

to MAPK and TGF-β/ Smad2 activation since blocking MAPK activation by PD which blocks partially Smad2 phosphorylation resulted in inhibition of loss of E-cadherin observed by blocking Jak2 (Fig 6). These results indicate that however the observed elevated MAPK activation has no positive effect on proliferation/ survival of T47D cells as can be seen in figure 1, its cooperation with TGF-β increases invasion of breast cancer.

My results here describe for the first time a novel function of the hormone PRL and its downstream signalling kinase Jak2 as critical regulators of EMT and invasion of breast cancer cells by negatively regulating biochemical pathways involved in tumor invasion and metastasis. Consistent with my study, Stat5a, a downstream mediator of PRL in mammary cells, was recently shown to display invasion suppressive activity in breast cancer[13]. In fact my hypothesis is that as breast tumor progresses it lose PRLR responsiveness. It has to be clarified that even 98% of breast tumors overexpress PRL and its receptor, the functional analysis of PRL and its receptor never been studied. The loss of responsiveness to PRL might be by expressing unfunctional isoform of PRL. It has been reported that BT474, an aggressive breast tumor, expresses a transcript of PRLR encoding only extracellular domain, so it is unable to signal and negatively regulates PRL function[1]. Evidence supporting my data is the hypermethylation of PRLR gene promoter in MDA MB-231, which is a highly malignant breast tumor cell line with a mesenchymal property. Restoring back PRLR/ Jak2 signaling in this cell type resulted in a gain of epithelial property and a significant loss of invasion protential, indicting again a suppressive effect of PRLR/ Jak2 signaling in tumorigenesis (Fig 7).

My studies show that TGF-β/ Smad pathway is negatively regulated by PRL. TGF-β has been shown to be a central player of tumor progression as it switches fro ma growth inhibitory factor to a metastatic factor in various tumors. TGF-β is normally down regulated in the beginning of tumor formation, while its pathway becomes activated during tumor progression. There fore it is not surprising to conclude that PRL has an opposite effect comparing to TGF-β on breast tumorigenesis, switching fro ma survival factor to a suppressor of metastasis.

My data describe for the first time a novel function of PRL and its signaling through Jak2 as critical regulators of EMT and invasion of breast cancer cells. In fact here, I suggest a new model fro the role of PRL in breast carcinogenesis. PRL is required as a growth and survival factor in normal mammary epithelial cells as well as in breast cancer, however, PRL functions as an invasion/ metastasis suppressor hormone by inhibiting the MAPK (Erk1/2) and TGF-β/ Smad pathway.

Tumor metastasis significantly contributes to death in cancer patients. PRL/ Jak2 signaling cascade can now be included in the gorwing list of metastasis suppressor pathways. Moreover, my data define a novel mechanism controlling invasiveness/ metastasis of breast cancer cells through PRL/ Jak2 pathway that could be exploited for therapeutic manipulation.

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