Cortactin: a novel target of prolactin-activated JAK2 signaling

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A Thesis
entitled

Cortactin- a novel target of prolactin-activated JAK2 signaling

by

Sneha Laghate

Submitted to the Graduate Faculty as partial fulfillment of the
requirements for the Master of Science Degree in Biology

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The University of Toledo
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Prolactin (PRL), is a cytokine/peptide hormone important for mammary gland development and has also been implicated in mammary neoplasia. PRL signals mainly via the receptor associated tyrosine kinase JAK2. It is known that PRL acts as a chemoattractant for breast carcinoma cells. Yet, the role of PRL in breast cancer invasion is not very well studied. We hypothesize that cortactin, an actin binding protein is a novel target of PRL-activated JAK2 leading to enhanced migration and invasiveness of breast cancer cells. Cortactin is an actin nucleation promoting factor (NPF) and accompanies N-WASP in Arp2/3 complex mediated actin polymerization leading to formation of invasive cytoskeletal structures such as invadopodia and podosomes. Cortactin is overexpressed in several cancers owing to the amplification of the EMS1/cortactin gene. Tyrosyl phosphorylation of cortactin downstream of Src and Src family of kinases is well studied and is implicated in breast cancer invasiveness and metastases. In this study we have demonstrated that overexpressed constitutively active JAK2 tyrosyl phosphorylates overexpressed cortactin in non-invasive T47D breast cancer cells. PRL-activated
endogenous JAK2 also stimulates tyrosyl phosphorylation of endogenous cortactin in a time and concentration-dependent manner in invasive TMX2-28 breast cancer cells. Cortactin is an invadopodia marker protein and enhances invadopodia formation through interaction with MMPs which leads to degradation of the extracellular matrix (ECM). We have established the gelatin-matrix degradation assay and demonstrated gelatin-matrix degradation by invasive MDA-MB-231 and TMX2-28 breast cancer cells. The current study focuses on cortactin tyrosyl phosphorylation by PRL-activated JAK2 as a step towards invasion and matrix degradation by breast cancer cells.
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List of Abbreviations

BSA- Bovine serum albumin

CBP90- Cortactin-binding protein 90

cDNA- Complementary deoxyribonucleic acid

Co-IP - Co-immunoprecipitation

ECM – Extracellular matrix

ER- Estrogen Receptor

FBS- Fetal bovine serum

HRP- Horseradish peroxidase

IB- Immunoblotting

IP- Immunoprecipitation

JAK2- Janus Kinase 2

KD - kinase dead

LSB- Laemmlı Sample buffer

MMP – Matrix metalloproteases

MT1MMP- Membrane type1 matrix metalloprotease
MLCK- myosin light chain kinase

NPF - Nucleation promoting factor

PAK1- p21- activated kinase 1

PBS- Phosphate buffered saline

PEI- Polyethyleneimine

PI3-K- Phosphatidylinositol 3-kinases

PRL- Prolactin

PRLR- Prolactin Receptor

pTyr- phosphotyrosine

pY- phosphotyrosine

SDS-PAGE - Sodium dodecylsulfate-polyacrylamide gel electrophoresis

S.E.M. - Standard Error

SH2- Src homology 2

SH3- Src homology 3

SOCS- Suppressor of cytokine signaling

STAT- Signal transducer and activator of transcription

TBS- Tris buffered saline
TBST- TBS Tween

WT- Wild type
Introduction

Breast cancer is one of the most common causes of cancer death in women. Currently, among cancers in women, about 28% are breast cancer. The American Cancer Society has predicted that about 1.3 million women will be diagnosed with breast cancer annually worldwide and about 465,000 will die from the disease (American Cancer Society, 2009-2010). Death usually occurs, not due to primary tumor but due to distant metastases (Chambers et al., 2002). Metastases usually result from aberrant cell signaling, deregulation of cytoskeletal reorganization leading to enhanced cell motility, migration and invasion (Acconcia et al., 2006). Hence it is important to dissect the signaling pathways in breast cancer which promote metastases in order to develop effective therapies against the disease.

PROLACTIN

Prolactin (PRL) is a peptide hormone that is primarily involved in development and differentiation of the mammary gland and has been implicated in mammary tumorigenesis. PRL is mainly secreted by the lactotrophs of the pituitary gland and has diverse reproductive functions beyond lactation, including fertilization, ovulation, and maintenance of corpus lutea, implantation and immunity (Dickson et al., 1992; Horseman et al., 1997; Karasek et al., 2006). PRL is also secreted locally by breast tissue (Zinger et al., 2003). PRL functions in both endocrine, and auto/paracrine fashion and participates
Fig 1. Prolactin signaling. Prolactin is a peptide hormone which binds to the prolactin receptor (PRLR) and activates several pathways downstream. PRLR doesn’t possess intrinsic tyrosine kinase activity and hence several kinases are associated with it which carry out the functions of PRL. The main tyrosine kinase associated with PRLR and primarily activated in the mammary gland is JAK2 kinase. JAK2 gets autophosphorylated and activated on PRL binding to PRLR, and this activated JAK2 in turn tyrosyl phosphorylates the receptor and several downstream target proteins important for PRL function.
in cell proliferation, motility, anti-apoptosis/survival with dysregulation of these pathways leading to cancer progression. PRL enhances the development of chemically induced breast cancers. (reviewed in (Clevenger, 2003),(Clevenger et al., 1995) (Fig. 1). PRL was shown to be expressed in 78% of the breast tumors studied (Bhatavdekar et al., 2000). Hyperprolactinemia results from excessive PRL secretion by tumors, with tumor size correlating to serum PRL levels (reviewed in (Karasek et al., 2006)). In malignant breast carcinoma, PRL leads to enhanced survival and motility as opposed to differentiation of breast epithelial cells (Maus et al., 1999; Miller et al., 2005)

**PROLACTIN RECEPTOR (PRLR).**

PRL carries out its function by binding to the membrane-located PRL receptor (PRLR) (reviewed in (Goffin and Kelly, 1997)). PRL on binding to PRLR induces the dimerisation of the receptor and activates receptor associated tyrosine kinase JAK2 which gets autophosphorylated and in-turn tyrosyl phosphorylates PRLR, recruiting downstream signaling proteins which carry out the functions of PRL (Fig.1) (reviewed in (Clevenger et al., 2003)). Several PRLR isoforms have been identified such as the long form (85-90 kDA) and short (42 kDA) resulting from alternative splicing from a single gene (reviewed in (Goffin and Kelly, 1997)). PRLR mRNA was detected in 95–100% of breast carcinomas (Ormandy et al., 1997; Reynolds et al., 1997; Touraine et al., 1998). A recent study demonstrates the increased expression of PRLR in an aggressive form of lobular carcinoma (Tran-Thanh et al., 2011). PRLR antagonist enhances the effect of the anti-cancer drug cisplatin in breast cancer therapy, thus highlighting the role of PRL and PRLR in chemoresistance (Ramamoorthy et al., 2001). Monoclonal antibodies against
PRL inhibited proliferation of T47Dco (ER negative) and MCF-7 (ER positive) breast cancer cells (Ginsburg and Vonderhaar, 1995). Considering the role of PRL and PRLR in tumorigenesis, it is important to understand the various pathways downstream of PRL leading to breast cancer.

**JAK2**

PRL binding to PRLR activates several pathways such as JAK-STAT, MAPK and PI3-K pathway (reviewed in (Ben-Jonathan et al., 2008)) (Fig.1). PRLR does not possess intrinsic tyrosine kinase activity, and JAK2 kinase associated with PRLR carries out major functions downstream of PRL in the mammary gland (Campbell et al., 1994). As mentioned above, PRL binding to PRLR activates JAK2 which tyrosyl phosphorylates PRLR and other downstream target proteins (reviewed in Clevenger et al., 2003). JAK2 is a non-receptor tyrosine kinase belonging to the Janus kinase protein family and participates in cell cycle progression, apoptosis, mitotic recombination, genetic instability and histone modifications (reviewed in (Rawlings et al., 2004)). JAK2 knockout mice die within 12 days of embryonic development (post coitum) (Neubauer et al., 1998). JAK2 has been implicated in hematopoietic malignancies and myeloproliferative disorders. JAK2 is ubiquitously expressed and is associated with approximately two thirds of the cytokine-hematopoietin receptor family (reviewed in (O'Shea et al., 2002)). Active JAK2 signaling occurs via binding of proteins such as signal transducers and activators of transcription (STATs), actin binding adapter protein SH2B1β to the phosphotyrosine docking sites of JAK2 via their Src-homology2 (SH2) domains and this signaling is blocked by suppressor of cytokine signaling 1 (SOCS1) binding to JAK2 (Argetsinger et al., 2004; Starr et al., 1997; Witthuhn et al., 1993). STATs, upon tyrosine
phosphorylation by JAK2 migrate to the nucleus where they carry out transcription of the lactogenic genes, a response to PRL signaling (Ball et al., 1988). JAK2 is essential for the initiation of mammary cancer and JAK2 functional ablation suppresses PRL-mediated tumorigenesis (Sakamoto et al., 2010). STATs have been implicated in breast cancer progression and PRL activated JAK2 phosphorylation of STAT5 plays a role in PRL mediated onset of mammary tumorigenesis (Cotarla et al., 2004; Nevalainen et al., 2004; Sakamoto et al., 2010). Another recently discovered target of JAK2 is p21-activated serine-threonine kinase PAK1 (Rider et al., 2007). PAK1 is known to be involved in invasion, survival and migration of breast cancer cells (reviewed by (Kumar and Vadlamudi, 2002)). JAK2 tyrosyl phosphorylation of PAK1 is required for maximal cell motility and protection from apoptosis (Rider et al., 2007). A very recent study also demonstrates a PRL-induced multiprotein complex formation of JAK2 targets SH2B1β, PAK1 and Filamin A and this complex participates in PRL-mediated membrane ruffling and migration of breast cancer T47D cells and also melanoma cells (M2 and A7) ectopically expressing the PRLR (Rider and Diakonova, 2011). Several targets downstream of PRL-activated JAK2 are implicated in breast cancer. Thus, studying PRL-activated JAK2 targets is necessary for understanding mechanisms of breast carcinoma development and progression. Hence, we decided to look at the protein cortactin involved in breast cancer metastasis to be a probable target of JAK2.

**CORTACTIN**

Cortactin an actin binding protein that is found to be overexpressed in several cancers and plays a role in metastasis (Li et al., 2001; Ormandy et al., 2003; Wu and Parsons, 1993). Cortactin is a multifunctional cytoskeleton-modulating protein, that is localized to
the cell cortex on growth factor stimulation, and contributes towards cellular processes such as motility, endocytosis, migration, invasion and extracellular matrix (ECM) degradation (reviewed in (Weaver, 2008)). The cortactin gene (EMS1) resides on chromosome locus 11q13 which is commonly amplified in breast cancer prone to metastasis (Bekri et al., 1997). Overexpression of cortactin due to EMS1 gene amplification has been correlated to enhanced tumor aggressiveness and poor prognosis in estrogen receptor (ER) negative breast cancers (reviewed in(Weaver, 2008)). Cortactin plays an important role in connecting growth factor signaling to reorganization of the actin cytoskeleton (reviewed in (Cosen-Binker and Kapus, 2006)). Cortactin is hijacked by pathogens during infections for formation of different actin based cytoskeletal structures (reviewed in (Selbach and Backert, 2005)). Cortactin participates in actin polymerization through direct F-actin binding and also acts as an actin nucleation promoting factor (NPF) through binding and activating actin nucleating Arp2/3 complex (Fig. 2) (Urino et al., 2001; Weaver et al., 2001). This actin polymerization effect is enhanced by cortactin binding to and activating N-WASP, an actin nucleation polymerising factor (NPF) and regulator of Arp2/3 complex (Kowalski et al., 2005). Cortactin localization to the cell cortex and in cell motility structures such as lamellipodia occurs on growth factor stimulation and to invasive structures such as invadopodia and podosomes on tyrosyl phosphorylation by Src kinase family members which will be discussed further (Weed et al., 2000) (reviewed in (Cosen-Binker and Kapus, 2006)).
**Fig. 2. Structure of cortactin.** Cortactin N-terminal domain interacts with actin nucleating Arp2/3 complex to aid in actin polymerisation. It has a 6.5 actin binding repeat domain binding followed by a Pro-Serine-Threonine (PST) rich domain phosphorylated by Serine-Threonine kinases such as PAK and Erk. The PST domain is followed by a tyrosine-rich patch phosphorylated by Src and Src family of tyrosine kinases, Abl and Arg kinase. The SH3 domain at the C-terminus binds several cytoskeleton modifying proteins. (Adapted from Cosen-Binker and Kapus, 2006)
Cortactin tyrosyl phosphorylation.

Cortactin tyrosyl phosphorylation is necessary for its function in cytoskeletal modulation. Cortactin migrates as a doublet band with isoforms p80 and p85 on SDS-PAGE gels and this is due to post-translational modification (because of both tyrosine and serine-threonine phosphorylation of cortactin). It was observed previously that on tyrosyl phosphorylation of cortactin in invasive cancer cells, there was selective conversion of p80 isoform to p85 (Campbell et al., 1999; Schuuring et al., 1998; van Damme et al., 1997). Cortactin is tyrosyl phosphorylated by several kinases such as Src and Src family e.g. v-Src, c-Src and Fyn, by other kinases such as Syk, Fer and c-Met and also by Abl-tyrosine kinase family members Abl-Arg kinase (Boyle et al., 2007; Crostella et al., 2001; Fan et al., 2004; Gallet et al., 1999; Huang et al., 1997; Kapus et al., 2000; Kapus et al., 1999). Tyrosine phosphorylation of cortactin was observed in response to several stimuli such as growth factor stimulation, cell adhesion, and hyperosmotic stress (reviewed in (Daly, 2004)). Cortactin was first discovered as a target of oncogenic v-Src tyrosine kinase in transformed chicken fibroblasts (Wu et al., 1991). Src phosphorylates murine cortactin at Y421, Y466 and Y482 (corresponding to Tyr421, Tyr470, and Tyr486 in human cortactin) in a hierarchical manner (Head et al., 2003; Huang et al., 1998). These 3 tyrosines are also phosphorylated by other kinases, i.e., Fer, Abl and c-Met, the phosphorylation being important for cell migration and motility (Boyle et al., 2007; Crostella et al., 2001; Huang et al., 1998; Sangrar et al., 2007). Tyrosyl phosphorylation of cortactin also promotes its binding via cortactin SH3 domain
Fig 3. **Cortactin is a marker protein for invadopodia.** Invadopodia are actin rich protrusions of the plasma membrane of invasive tumor cells, formed by cortactin mediated actin polymerization along with N-WASP, CDC42 and Arp2/3 complex. These structures extend vertically from the ventral cell membrane into the ECM. Invadopodia are enriched in tyrosyl phosphorylated cortactin where cortactin regulates MMP secretion thus leading to the proteolytic degradation of extracellular matrix (ECM) as the invadopodia contact the ECM. This process consequently leads to intravasation of the tumor cell into the blood stream, thus leading to metastases (adapted from ‘Cell Migration lab, University of Reading).
to adaptor CD2AP and to myosin light-chain kinase (MLCK) (Dudek et al., 2004; Lynch et al., 2003). Stable expression of Src dependent tyrosyl phosphorylation deficient mutant of cortactin (Y3F) in metastatic MDA-MB-231 breast cancer cell lines reduces their osteolytic metastases by 74% when injected in nude mice as compared to cells stably expressing wild type cortactin, thus underscoring the importance of tyrosyl phosphorylation of cortactin in breast cancer metastases. (Li et al., 2001).

Cortactin, invadopodia and ECM degradation

Extracellular matrix (ECM) degradation is a part of the tumor dissemination process, through which invasive tumor cells intravasate out of the basement membrane or matrix into the blood stream to form tumors at distant sites in the body (reviewed by (Foda and Zucker, 2001)). The metastatic tumor cells have to form invasive structures such as invadopodia and podosomes to carry out ECM degradation. Cortactin is necessary for the assembly of and acts as marker protein for ‘Invadopodia’, which are proteolytically active, actin rich cell protrusions involved in ECM degradation in invasive breast carcinoma (Fig. 3) (Artym et al., 2006; Bowden et al., 1999; Chen, 1989; Coopman et al., 1998). Podosomes are structures similar to invadopodia and are sites of integrin mediated actin polymerization and active ECM modification (Calle et al., 2006; Linder, 2007). In vitro, invadopodia are induced to form by plating cells on 3D matrix cushions in the presence of growth factors- recognized by co-localization of invadopodia markers (eg: cortactin) with proteolytic degradation of fluorescently-labeled ECM (reviewed in (Weaver, 2006)). Fig. 4 lists the proteins important in invadopodia formation and maturation (Artym et al., 2006). MMP2, MMP9 and MT1-MMP (MMP14) are the most crucial MMPs associated with invadopodia and cortactin controls their
secretion thus linking the enzyme secreting machinery to the actin cytoskeleton (Artym et al., 2006; Clark et al., 2007). Invadopodia, as shown through time-lapse video analysis, are formed de novo at the cell border having a lifetime anywhere from minutes to several hours. Short lifetime invadopodia are motile, as compared to long-lasting invadopodia being more immobile (Yamaguchi et al., 2005). Time-lapse video analysis of invadopodia in MDA-MB-231 breast cancer cells revealed the stepwise model for invadopodia formation and maturation with concomitant degradation of ECM. Firstly the recruitment and aggregation of cortactin at pre-invadopodia membrane adhering to the matrix, followed by recruitment of MT1MMP (MMP14) at the site of cortactin aggregation which results in focal degradation of matrix at sites where invadopodia contacts the matrix, and lastly the dissociation of cortactin from mature matrix degrading invadopodia where MMP14 accumulation and matrix degradation continues (Artym et al., 2006). Cortactin depletion diminishes the number of podosomes rings and invadopodia puncta in oncoprotein Src-transformed/overexpressing cells or breast cancer cells overexpressing constitutively active Src (Y527F) (Artym et al., 2006; Webb et al., 2007). Several invadopodia proteins like dynamin and Arg kinase also bind to cortactin via their SH3 domain suggesting that cortactin may play a scaffolding function in invadopodia (Baldassarre et al., 2003; Mader et al., 2011). This suggests that cortactin modulation of cytoskeleton is important in invadopodia formation and cancer cell motility.

High levels of tyrosyl phosphorylated cortactin are found in invadopodia, the levels of phosphorylation correlating to the extent of matrix degradation in breast cancer cells (Fig. 3). Cortactin and phosphotyrosine colocalise at invadopodia and sites of matrix
Multiple proteins are associated with invadopodia. (Artym et al., 2006)

<table>
<thead>
<tr>
<th>Minimal motility machinery (actin and its regulators)</th>
<th>Adhesion proteins</th>
<th>Signaling proteins</th>
<th>Membrane-associated proteases mediating ECM degradation at sites of invadopodia function</th>
</tr>
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<tr>
<td>Arp2/3, N-WASP, Cdc42, Nck, coflin, capping proteins, Cortactin, and Dynamin</td>
<td>Integrins, that mediate invadopodia interaction with ECM</td>
<td>Tyrosine kinases and Ras-related GTPases</td>
<td>MMP14, seprase, MMP-2, and the urokinase-type plasminogen activator (uPA)/uPA receptor proteolytic system</td>
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degradation (Bowden et al., 2006). Tyrosyl phosphorylation of cortactin is also necessary for actin-based podosome formation in osteoclasts (Tehrani et al., 2006)

**Can cortactin be a PRL-activated JAK2 target?**

The role of PRL signaling in cortactin modulation and function is unknown. Cortactin mRNA was found to be slightly increased in response to PRL through 36 days of pituitary grafting. It was suggested that cortactin binding protein (CBP90) plays a role in PRL mediated morphogenesis and growth of mammary epithelial cells through cortactin modulation (Imaoka et al., 2002). As mentioned before cortactin phosphorylation was detected on several other tyrosines by a few phosphoproteomic and mass-spectrometric studies suggesting that it is important to decipher the mechanism behind the phosphorylation at these tyrosines which would enable us link cortactin to other Src-dependent and independent pathways in breast cancer (Amanchy et al., 2005; Hinsby et al., 2003; Rush et al., 2005; Stuible et al., 2008; Wang et al., 2006; Wolf-Yadlin et al., 2007; Wolf-Yadlin et al., 2006; Zhang et al., 2005). We would like to investigate the role of PRL-activated JAK2 in cortactin tyrosyl phosphorylation and function. Though PRL acts as a chemoattractant for human breast carcinoma its effect on motility and actin cytoskeleton is poorly understood (Maus et al., 1999). We intend to unravel more mechanisms involved in PRL-induced breast cancer metastases with cortactin as our target.

We hypothesize that cortactin is a novel substrate of JAK2 in the PRL pathway. We propose a model wherein PRL-activated JAK2 tyrosyl phosphorylation of cortactin mediates invasion, matrix degradation and metastases of breast cancer cells.
Materials and Methods

Cell Culture

293T cells from American Type Cell Culture (ATCC) were grown in Dulbecco’s Modified Eagle’s Medium (DMEM-Cellgro) with 10% calf serum (Cellgro), 1 mM L-Glutamine (Hyclone), 100 units/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml amphotericinB (Cellgro). T47D cells were a gift by Dr. Ethier (Karmanos Cancer Institute, Detroit, MI) and grown in RPMI 1640 medium (Cellgro) supplemented with 10% fetal bovine serum (HyClone), 5 μg/ml Insulin (Sigma), 1 mM L-Glutamine, 100 units/ml penicillin (Cellgro), and 100 μg/ml streptomycin (Cellgro). TMX2-28 cells and MDA-MB231 cells (a gift from Dr. Kathryn Eisenmann, University of Toledo, Health Science Campus, OH) were cultured in DMEM (Cellgro) supplemented with 10% fetal bovine serum (HyClone), 1 mM L-Glutamine (Cellgro), 100 units/ml penicillin and 100 μg/ml streptomycin. All cell lines were kept in an incubator with 5% CO2 at 37°C. Deprivation Media (DMEM for 293T, TMX2-28 and MDA-MB231 cells and RPMI for T47D cells) was made by replacing serum with 1% BSA and supplemented with 100 units/ml penicillin and 100 μg/ml streptomycin.

Plasmids

cDNA encoding FLAG-tagged Cortactin (in pcDNA 3.1) was kindly provided by Dr. Alan Mak (University of Queen's University, Kingston, Ontario, Canada). Myc-tagged Cortactin was provided by Dr. Edward Seto (Department of Pathology & Cell Biology,
College of Medicine, University of South Florida, Tampa, FL. cDNAs for prk5myc, JAK2 WT, JAK2 K882E (in prk5 vector) and GFP (in pEGFP C1) were provided by Dr. Carter-Su (University of Michigan, Ann Arbor, MI).

**Transfection**

Cells were plated the previous day. 2 six well dishes (~60mm well) were transfected for a single condition and harvested together during cell lysis to extract proteins. Each well was transfected with ~ 3.5μg of total DNA. DNA was added to plain medium followed by PEI transfection reagent (Polysciences). The DNA-reagent mixture was gently pipetted up and down for mixing (vortexing was avoided). After a 10 minute incubation time, the DNA-reagent complex was added to the cells and the plate was gently rocked and returned to the CO2 incubator. The media was replaced the next day with either complete medium or deprivation medium according to the experiment. For deprivation, the cells were washed twice with Dulbecco’s Phosphate buffered saline (DPBS) and then deprivation media was added to the wells. Deprivation times differed as per the experiment. For a 72 hour deprivation period, deprivation medium was changed after 48 hours.

**Antibodies**

Monoclonal anti-phosphotyrosine (anti-pY, clone 4G10) antibody from Upstate Biotechnology, Inc. (Millipore), monoclonal anti-cortactin (clone 4F11) from Upstate Biotechnology, Inc (Millipore), monoclonal anti-myc (9E10) from Santa Cruz Biotechnology, Inc. were used for immunoprecipitation and immunoblotting. Monoclonal
anti-cortactin (clone 4F11) was also used for immunofluorescence. Monoclonal anti-JAK2 antibody from Biosource (number AHO1352, clone 691R5) was used for immunoblotting. Anti-JAK2 antiserum was provided by Dr. Carter-Su and used for immunoprecipitation. Monoclonal anti-cortactin (clone 4F11) was also used for immunofluorescence. A polyclonal rabbit- anti-mouse antibody (Millipore) was used for immunoprecipitations with monoclonal antibodies. Human PRL was purchased from Dr. Parlow (National Hormone and Peptide Program, National Institute of Diabetes and Digestive and Kidney Diseases).

**Immunoprecipitation**

After 48 hours of transfection, the cells were washed two times with 7 ml of PBSV (10 mM Na3PO4, 150 mM NaCl, 1 mM Na3VO4, pH 7.3). Lysis buffer was prepared by supplementing L-RIPA (50 mM Tris-HCl, 150 mM NaCl, 2 mM EGTA, 0.1% Triton X-100, pH 7.5) with 1 mM Na3VO4, 1mM PMSF, 10 μg/ml aprotinin and 10 μg/ml leupeptin. Ice cold lysis buffer (0.8 ml of ice cold for a 10 cm dish) or (0.2 mls for one well of a 6 well plate) was added and cells were scraped off the dish and transferred to a microcentrifuge tube. For endogenous cortactin IPs, two wells were harvested together. The cell lysates were incubated on ice for 10 minutes and then centrifuged at 13,000 rpm at 4°C for ten minutes. 50 μl of the cell lysate was placed in a new microcentrifuge tube alongwith 12.5 μl of 4X Laemmli sample buffer (LSB)(containing 100μl β-mercaptoethanol/ml). The remainder of the cell lysate was pipetted into a new microcentrifuge tube and incubated with the indicated antibodies (1:100 dilution) at 4°C for 2 hours. Bridge antibody was added after one hour of
incubation. At the end of two hours, 30 μl of protein A beads were added and rotated at 4°C for 1 hour. The protein A beads were pelleted by centrifugation at 13,000 rpm and were washed three times in lysis buffer. 75 μl of 1X LSB sample buffer was added to the beads and they were boiled for 5 minutes. The samples were run on a 10% acrylamide gel at 10 mA overnight (big gel) or at 30 mA for one hour (minigel).

Western Blot Analysis

The proteins from the gel were transferred to PVDF membrane (Millipore) at 100 V for one and a half hours. The membrane was incubated for 4 hours at 4°C or one hour at room temperature in 3% BSA blocking buffer. The indicated primary antibody was added to the membrane to incubate overnight. The membrane was washed 3 times in Tris Buffered Saline (TBS)-Tween and rinsed in TBS. Then secondary antibody (goat anti-mouse-HRP or anti-rabbit-HRP, 1:7500, diluted in TBS) was added to the membrane and incubated for 1 hour. The membrane was then washed three more times in TBS-Tween. After rinsing the membrane in TBS, the membrane was incubated in a chemiluminescent solution for 5 minutes before exposing to film to visualize the protein bands.

Fluorescent Gelatin degradation assay

I] Conjugation of gelatin with Texas Red

Gelatin conjugation with Texas-Red was done by modifying protocol described previously (d'Ortho et al., 1998; Hembry, 2001). 10 mg gelatin (Sigma) was dissolved (at 37°C) in 1 ml 0.1 M sodium bicarbonate pH 9.0. 1mg of Texas-Red sulfonil chloride
(Molecular Probes) was dissolved in 100 µl extra dry dimethyl formamide (Fisher) on ice. When completely dissolved, Texas-Red dye in DMF solution was added dropwise to the gelatin solution and stirred (30 min, room-temperature, light protected). Conjugated gelatin-Texas-Red was separated from unconjugated dye by desalting, i.e. passing through a Sephadex G25 (Superfine range from Aldrich) column (Pierce) equilibrated with PBS following the Pierce desalting protocol. It was stored in 50µl aliquots at 4ºC (protected from light) until use. Unconjugated gelatin was filtered through a 0.22µm filter and stored at 4ºC until use.

II] Coating coverslips with fluorescent Gelatin

Protocol was modified from - (Artym et al., 2009).

Glass coverslips were treated with 1M HCL at 50ºC for 3 hours, cooled and sonicated at half hour intervals with increasing concentrations of ethanol (50% - 95%) in a water-bath sonicator (Fisher Scientific) and stored in 95% ethanol until use. Coverslips were flamed before use. All the further coating steps were carried out in a humidified chamber on parafilm at room-temperature. Coverslips were initially coated with 50ug/mL of pre-chilled poly-L-lysine (Sigma) solution in DPBS for 20 mins followed by washing 3 times with DPBS upto 25 mins. Next, 0.5% Glutaraldehyde (Fisher) solution in DPBS was pipetted onto coverslips for 15mins followed by a 25 min wash with DBPS. This was followed by coating coverslips with pre-warmed (at 37ºC), Texas-Red-gelatin thoroughly mixed with unconjugated gelatin at a ratio of 1:8 (conjugated:unconjugated) for 10 mins followed by 25 min wash with DPBS. The free aldehyde groups were quenched with freshly prepared solution of sodium-borohydride (5mg/ml) solution in DPBS, again
followed by 3 washes in DPBS for 45mins. These coverslips were either used immediately or stored in DPBS (light protected) at 4°C.

**Immunofluorescence**

The cells on coverslips were fixed in CFA buffer containing 4% formaldehyde for 15 min followed by permeabilisation in CFB buffer containing 0.1% Triton X-100 for fifteen minutes followed by blocking with 2% goat serum in PBS at room temperature. Then the coverslips were incubated with mouse monoclonal anti-cortactin antibody (4F11) from Upstate Biotechnology, Inc (Millipore) at a dilution of 1:500 in goat serum overnight at 4°C. The next day, coverslips were washed with goat serum for 15 mins at room temperature followed by goat-α-mouse Alexa Fluor 488 (Invitrogen) at 37°C for one hour. All the incubations were carried out in a humidified chamber. The coverslips were then mounted onto microscope slides using 5% propyl gallate (in 90% glycerol). Images were taken on an Olympus IX51 fluorescent microscope using a Zeiss Plan Neofluar 100X oil immersion objective.
RESULTS

Overexpressed JAK2 phosphorylates endogenous cortactin in 293T cells.

Endogenous cortactin migrates as a doublet band of p80 and 85kDa on SDS-PAGE, due to post-translational modification. Tyrosyl phosphorylation of cortactin is one of the events leading to conversion of p80 to p85 isoforms of cortactin. These events are also accompanied by translocation of cortactin from cytoplasm to cell-matrix contact sites (van Damme et al., 1997). In a preliminary experiment to test the tyrosyl phosphorylation of endogenous cortactin (Fig. 5), we overexpressed either WT JAK2 or kinase-dead JAK2 in 293T cells. The lysates were separated by SDS-PAGE gel and analysed by Western blotting. When probed with anti phophotyrosine (anti-PY) antibody, we could see a doublet band around the molecular weight of 80-85kDa phosphorylated in the presence of JAK2 overexpression, but unphosphorylated with overexpression of kinase dead JAK2 or vector alone. Later, this doublet band was identified as cortactin by probing with anti-cortactin antibody which recognizes both cortactin isoforms. Surprisingly, there was no selective conversion of isoform p80 to p85 on tyrosyl phosphorylation of cortactin as was observed in previous studies (Van Damme H, et al., 1997).
**Fig. 5. Overexpressed JAK2 tyrosyl phosphorylates endogenous cortactin in 293T cells.**

293T cells were transfected with either vector alone (lane1), WT JAK2 (lane2) or kinase-dead JAK2 (lane3). Lysates were separated on SDS PAGE gel and subjected to Western blot analysis with anti-PY antibody and reblotted with anti-cortactin and anti-JAK2 antibodies. The arrows in the anti-PY blot (for cortactin-lower panel) point towards the two isoforms of cortactin (p80 and p85).
Overexpressed constitutively active JAK2 tyrosyl phosphorylates overexpressed cortactin in non-invasive T47D breast cancer cells.

Next, in order to confirm our preliminary results we chose to use T47D breast cancer cells. T47D is a human ductal breast epithelial tumor cell line and possesses a large number of PRL receptors (PRLR) thus making it easier to stimulate these T47D cells with PRL to study signaling pathways downstream (Canbay et al., 1997; Peirce and Chen, 2001). Wild type myc-tagged cortactin was co-expressed either with constitutively active (JAK2 V617F) or kinase dead (JAK2 KD) mutants of JAK2 or GFP as a control (Fig. 6). Myc-tagged cortactin was then immunoprecipitated with anti-myc antibody. A non-specific IgG negative control (lane 4) was also used to show that pull down of cortactin was specific with the anti-myc antibody. The immunoprecipitates were separated by SDS-PAGE and analysed by Western blotting. On immunoblotting the membrane with anti phosphotyrosine (anti-PY) antibody, strongly tyrosyl phosphorylated cortactin was seen to be immunoprecipitated (lane2-upper left portion) in the presence of kinase-active JAK2 (V617F) but not in the presence of kinase-dead JAK2 (KD) or GFP alone. However, JAK2 and cortactin did not co-IP. To confirm that, we immunoprecipitated constitutively active JAK2 (V617F), which was not co-IP’d with cortactin (data not shown).

PRL activates endogenous JAK2 in T47D breast cancer cells.

To determine PRL-activation of endogenous JAK2 in T47D cells and its co-IP with cortactin, cortactin was overexpressed in T47D cells, followed by 72 hours of serum
Fig. 6. Overexpressed constitutively active JAK2 tyrosyl phosphorylates overexpressed cortactin in non-invasive T47D breast cancer cells.

WT Cortactin was transiently co-transfected with either GFP (lane 1), kinase active JAK2 [V617F] (lane 2 and 4), or kinase dead JAK2 [K882E] (lane 3). Cells were deprived of serum and cortactin was immunoprecipitated with anti-myc ascites antibody (lanes 1-3) or control non-specific IgG (lane 4). Left portion shows the immunoprecipitates and the right portion shows the corresponding lysates for each lane on the left.
deprivation in order to deplete endogenous JAK2 phosphorylation (Fig. 7). This was followed by stimulation with PRL. Cells were harvested at 20 min of PRL stimulation and endogenous JAK2 was immunoprecipitated from both PRL treated and untreated cells. At a concentration of 150ng/ml PRL for a time period of 20 min, we observed a strong activation of endogenous JAK2 when compared to untreated, which did not show any phosphorylation. This was followed by immunoblotting with anti-cortactin antibody to assess cortactin and endogenous JAK2 co-IP on PRL stimulation. We did not observe a co-IP of JAK2 and cortactin on PRL activation of JAK2.

**PRL does not stimulate endogenous cortactin phosphorylation in non-invasive T47D breast cancer cells.**

Next, we wanted to investigate cortactin tyrosyl phosphorylation on PRL stimulation. We did a time course experiment by immunoprecipitation of cortactin at different times with PRL treatment in T47D cells. This would tell us whether cortactin is getting tyrosyl phosphorylated on PRL stimulation and also the accurate timing for cortactin tyrosyl phosphorylation by PRL. Another factor to consider is that the activation of JAK2 by PRL (150ng/ml, 20 min) (Fig. 7) may not coincide temporally with PRL phosphorylation of cortactin (which might be occurring downstream in the PRL pathway) hence, a time-course of cortactin phosphorylation by PRL was necessary. T47D cells were deprived for 72 hours followed by stimulation with 150ng/ml PRL at five different time-points from 0 to 60 min (Fig. 8). Endogenous cortactin was immunoprecipitated followed by SDS-PAGE and Western blotting. The membrane was first probed with anti-PY antibody.
Fig. 7. JAK2 activation with PRL in T47D breast cancer cells.

T47D cells were deprived for 72 hours (were transfected with myc-cortactin in deprivation medium (24 hours after plating) and then harvested, followed by immunoprecipitation of endogenous JAK2 followed by SDS-PAGE and Western blotting. The membrane was probed with anti-PY antibody and further reprobed with anti-JAK2 and anti-cortactin antibodies. (The band for IP’d JAK2 in the PRL treated lane looks darker than the band for IP’d JAK2 in the untreated lane because the immunoblot wasn’t stripped after probing with anti-PY antibody before re-probing with anti-JAK2 antibody).
Fig. 8. PRL does not stimulate cortactin tyrosyl phosphorylation in non-invasive T47D breast cancer cells. Cells were deprived for 72 hours and treated with 150ng/ml PRL for different times. Endogenous cortactin was immunoprecipitated and immunoprecipitates were subjected to SDS-PAGE and western blotting. The blot was initially probed with anti-PY antibody and reprobed with anti-cortactin antibody. The fold change in tyrosyl phosphorylation of cortactin over 60 min was calculated by band densitometric analysis. The above blot is representative of 3 different experiments. Bars represent mean ± S.E.M.
The membrane was then re-probed with anti-cortactin antibody to calculate the total amount of immunoprecipitated cortactin. Band densitometric analysis was performed (Image J software) to calculate fold change in the tyrosyl phosphorylation of cortactin. The fold change was calculated by comparing the phospho-cortactin/total cortactin value in the treated (10 min to 60 min) versus untreated cells (0min) at all 5 time points. No difference in the tyrosyl phosphorylation of cortactin was observed on PRL stimulation at any of the time points. A strong basal level of tyrosyl phosphorylation was observed (0min) as shown previously in T47D cells (Campbell et al., 1996). This suggests that PRL stimulation does not enhance the overall tyrosyl phosphorylation of cortactin in T47D cells. It is important to note that there is a strong tyrosyl phosphorylation of cortactin downstream of JAK2 in T47D cells when both cortactin and JAK2 are overexpressed, but no enhancement in tyrosyl phosphorylation of endogenous cortactin on PRL stimulation.

**PRL stimulates cortactin tyrosyl phosphorylation in invasive TMX2-28 cells in a concentration dependent manner.**

PRL differentially regulated MMP transcription in invasive TMX2-28 cells as opposed to non-invasive T47D cells (unpublished lab data). On PRL stimulation, there was no difference in the MMP transcription in T47D cells, but a stark difference in the MMP transcript profile of TMX2-28 cells was observed, i.e. MMP2, MMP9 and MMP3 (unpublished lab data). The two MMPs, i.e. gelatinases MMP2 and MMP9 are regulated by cortactin expression (Clark et al., 2007). Thus, based on this observation of differential MMP secretion in invasive vs. non-invasive cells, and the premise that
cortactin also regulates MMP secretion, we hypothesized that stimulation via PRL might phosphorylate and regulate cortactin function differently in the more invasive TMX2-28 cells as opposed to non-invasive T47D cells through tyrosyl phosphorylation of cortactin. Hence, PRL stimulation of cortactin tyrosyl phosphorylation (as assessed for T47D cells, Fig. 8) was done in TMX2-28 cells. Initially, a concentration curve was performed in which TMX2-28 cells were treated with different concentrations of PRL for 20 min after being deprived for 72 hours. Cortactin was immunoprecipitated using anti-cortactin antibody followed by SDS-PAGE and Western blotting. When the membrane was probed for anti-PY antibody, there was a statistically significant increase in tyrosyl phosphorylation of cortactin (approximately 1.6 fold over unstimulated) starting at 200ng/ml PRL concentration when compared to 0ng/ml (untreated) (Fig. 9, upper panel). The tyrosyl phosphorylation seemed to increase beyond 200 ng/ml up to 500 ng/ml, but the increase was not statistically significant between 200 to 500 ng/ml. This suggests that in invasive TMX2-28 cells, PRL stimulates tyrosyl phosphorylation of cortactin in a concentration dependent manner, with 200ng/ml being sufficient to significantly enhance the phosphorylation.

Endogenous cortactin co-IP’d with proteins such as paxillin and PKCµ in invasive cells as opposed to non-invasive cells and this complex was enriched in the invadopodia fraction of invasive cells as compared to cytosolic fractions (Bowden et al., 1999). Thus to see whether this complex formation occurs in more invasive TMX2-28 cells, the immunoblot for endogenous cortactin immunoprecipitates following PRL treatment (Fig. 9) were re-probed with anti JAK2 antibody. We did not observe a co-IP of endogenous cortactin and JAK2 (data not shown). When the same was repeated with both
Fig. 9. PRL stimulates tyrosyl phosphorylation of cortactin in a concentration dependent manner in invasive TMX2-28 breast cancer cells. TMX2-28 cells were deprived for 72 hours and treated with different concentrations of PRL (from 0 ng/ml to 500 ng/ml) for 20 min. Endogenous cortactin was immunoprecipitated and immunoprecipitates were subjected to SDS-PAGE and Western blotting. The blot was initially probed with anti-PY antibody and reprobed with anti-cortactin antibody. The fold change in tyrosyl phosphorylation of cortactin over 60 min was calculated by band densitometric analysis using Image J software. Bars represent mean ± S.E.M, *, p<0.05, n=3.
proteins overexpressed, i.e. JAK2 and cortactin in TMX2-28 cells, we still did not observe their co-IP (data not shown).

**PRL stimulates tyrosyl phosphorylation of cortactin in a time-dependent manner in invasive TMX2-28 breast cancer cells.**

Since phosphorylation and de-phosphorylation events govern the course of most signaling pathways, it is necessary to determine the accurate timing and concentration at which a ligand has its optimal effect in initiating and sustaining phosphorylation of target proteins before the de-phosphorylation events set in. These values of optimal concentration and timing can be further kept constant while studying the pathway and manipulating other factors in the pathway. Hence, we decided to also perform a time course experiment for cortactin tyrosyl phosphorylation by PRL in TMX2-28 cells. The same conditions as in the concentration curve were repeated for time-course; TMX2-28 cells were deprived for 72 hours and then treated with 200 ng/ml PRL at different timings starting from 0 to 60 min (Fig. 10). In accordance with the concentration curve, the tyrosyl phosphorylation of cortactin peaked at 20 min (2.5 fold increase in phosphorylation over basal) and declined upto 60 min. Thus, 200 ng/ml PRL concentration at 20 min was sufficient to induce maximal tyrosyl phosphorylation of cortactin.

Currently, we are in the process of cloning His-tagged cortactin. It would enable us to look at direct tyrosyl phosphorylation of purified recombinant His-cortactin by purified recombinant JAK2 in an *in vitro* kinase assay using $\gamma^{32}$P ATP.
Fig. 10. PRL stimulates tyrosyl phosphorylation of cortactin in a time-dependent manner in invasive TMX2-28 breast cancer cells. TMX2-28 cells were deprived for 72 hours and treated with 200 ng/ml PRL at different times. Endogenous cortactin was immunoprecipitated and immunoprecipitates were subjected to SDS-PAGE and Western blotting. The blot was initially probed with anti-PY antibody and re-probed with anti-cortactin antibody. The fold change in tyrosyl phosphorylation of cortactin over 60 min was calculated by band densitometric analysis.
Invasive MDA-MB-231 cells and TMX2-28 breast cancer cells degrade fluorescent gelatin matrix.

In order to determine the physiological relevance of tyrosyl phosphorylation of cortactin in TMX2-28 cells, we established a gelatin matrix degradation assay. In this assay cells are plated on 3D fluorescent gelatin matrix cushions that allow invadopodia to form and proteolytically (via MMP secretion) degrade the gelatin underneath the cells. Foci of degraded matrix are identified as black holes with a 0.2 µm to 1.2 µm diameter in the background of bright fluorescent gelatin matrix (Artym et al., 2006). These matrix degraded areas co-localize with invadopodia puncta formed by the cells. These invadopodia puncta are identified by staining with either anti-cortactin antibody (cortactin is a marker protein for invadopodia) or with phalloidin to look at actin puncta, or both cortactin and actin. There are different methods of quantification for degradation i.e. by either counting the holes underneath each cell in random fields or measuring the zones of degradation depending on the nature of degradation (Artym et al., 2006; Nakahara et al., 1997). Different matrices such as gelatin, fibronectin, collagen etc. have been used to study matrix degradation depending on the cell type used and the MMPs involved (Artym et al., 2006; Lu et al., 2010; Nakahara et al., 1997; Rosel et al., 2008). We decided to use gelatin matrix conjugated with fluorescent Texas-Red dye since cortactin is known to regulate secretion of gelatinases (MMP2 and MMP9) and MMP14 at invadopodia (Artym et al., 2006; Clark and Weaver, 2008). Currently, we have performed only preliminary experiments to test the degradation of gelatin matrix by metastatic MDA-MB-231 cells (positive control) and TMX2-28 cells. MDA-MB-231
cells showed invadopodia formation, indicated by cortactin staining at punctate structures (green) (in Fig. 11A) which co-incide with extensive matrix degrading areas (black) (in Fig. B) after 24 hours of plating. There is no information about TMX2-28 cells in the context of invadopodia formation and matrix degradation, so, initially, we wanted to test their matrix degrading capability. In a preliminary experiment, when TMX2-28 cells were plated on Texas-Red-gelatin coated coverslips for 48 hours, gelatin degradation was observed in certain areas (Fig. 12).
Fig. 11. MDA-MB-231 cells form invadopodia and degrade gelatin matrix.

Metastatic MDA-MB-231 breast cancer cells were plated on Texas-Red-gelatin matrix and fixed after 24 hours. (A) Green punctuate structures indicate cortactin accumulation at invadopodia. (B) Corresponding gelatin matrix degradation (black holes) indicated by arrows. (C) Co-localisation of invadopodia puncta with areas of matrix degradation.
Fig. 12. TMX2-28 cells degrade gelatin matrix. TMX2-28 breast cancer cells were plated on Texas-Red-gelatin matrix fixed after 48 hours. (A) Green staining indicates cortactin. (B) Gelatin matrix degradation (arrow points towards black degraded regions) underneath and around the cell (C) Overlay of A and B.
Discussion

Cortactin tyrosyl phosphorylation by Src and Src family of kinases in breast cancer metastases has been extensively studied. It is important to study the contribution of other tyrosine kinases in phosphorylation of cortactin which might reveal novel mechanisms by which cortactin promotes invasiveness and metastases in breast cancer. Prolactin hormone and its activation of PRLR associated JAK2 kinase has been implicated in mammary carcinoma. Prolactin acts as a chemoattractant for breast cancer cells and enhances breast cancer cell motility through cytoskeletal modulation (Maus et al., 1999). We hypothesized that PRL plays a role in invasion through JAK2 dependent tyrosyl phosphorylation of cortactin, since cortactin tyrosyl phosphorylation is critical for invasion. Our study shows for the first time that cortactin is tyrosyl phosphorylated by overexpressed JAK2 and by PRL-activated endogenous JAK2 signaling, thus activating a potential pathway for breast cancer metastases. We have demonstrated that overexpressed JAK2 strongly tyrosyl phosphorylates cortactin but JAK2 and cortactin do not co-IP. The complex of JAK2 and cortactin cannot be isolated in the presence of either PRL-activated endogenous JAK2 or with overexpressed kinase-active JAK2. This might be explained by an indirect effect of JAK2 or that the co-IP conditions might not be optimal for the stability of cortactin-JAK2 complex or that the complex formation might be temporary. Previous studies on the well-known JAK2-STAT5 pathway also couldn’t demonstrate the isolation of the complex of JAK2 and STAT5 from cell culture but could isolate such a
complex when both proteins were overexpressed and purified from insect cells using a baculovirus expression system (Flores-Morales et al., 1998).

In order to study whether JAK2 directly phosphorylates cortactin, we are planning an \textit{in vitro} kinase assay using $\gamma^{32}\text{ATP}$, to look at direct tyrosyl phosphorylation of purified His-cortactin by immunoprecipitated kinase active JAK2 or commercially available purified kinase active JAK2 protein.

As mentioned in the introduction, cortactin promotes invasion, ECM degradation and metastases of breast cancer cells and tyrosyl phosphorylation of cortactin is essential for these processes. Cortactin also regulates matrix-metalloprotease (MMP) secretion leading to invadopodia formation and ECM degradation, thus, connecting the modulation of actin cytoskeleton to an enzyme secretory machinery (Artym et al., 2006; Clark and Weaver, 2008; Clark et al., 2007). PRL also affects MMP transcription as observed in a few previous studies (Flint et al., 2006; Hirsch et al., 1999). Our results demonstrate that endogenous cortactin gets tyrosyl phosphorylated in a time and concentration-dependent manner downstream of PRL in invasive TMX2-28 cells as opposed to non-invasive T47D cells (Figs. 8-10). The concentration of 200 ng/ml at 20 min shows a peak in the tyrosyl phosphorylation of cortactin in response to PRL in TMX2-28 cells. This indicates that a 20 min treatment with PRL maybe critical in cortactin tyrosyl phosphorylation and modulation and further study should focus on physiological changes downstream for this time of PRL treatment, eg. cytoskeletal modification and cell motility. For events such as cell invasion or matrix degradation probably, a longer treatment time might be necessary for cortactin to regulate MMPs.
TMX2-28 cells are a recently developed derivative of MCF-7 breast cancer cells, and lack the estrogen receptor. (Fasco et al., 2003; Gozgit et al., 2006). In a previous study done on comparison of EMS1-gene (cortactin-protein) expression across several breast cancer cell lines, it was observed that cortactin was upregulated in MCF-7 as compared to T47D, but the extent of tyrosyl phosphorylation of cortactin was greater in T47D than in MCF-7 (Campbell (Campbell et al., 1996). This might explain the high basal level of tyrosyl phosphorylation of cortactin in T47D cells (0 min PRL) even after 72 hours of serum deprivation. Thus it seems that PRL is unable to over-ride the already existing higher basal levels of cortactin tyrosyl phosphorylation in T47D cells (Fig. 8).

A previous study demonstrated that the extent of JAK2 tyrosyl phosphorylation by PRL stimulation varied across breast cancer cell lines having different amounts of PRLR. The fold increase in JAK2 tyrosyl phosphorylation in response to PRL was the highest in T47D (2.5 fold) followed by MCF7 (1.3 fold) and MDA-MB-231 (1.2 fold), which is also in accordance with the number of PRLR receptors these cell lines possess respectively, being the highest in T47D and lowest in MDA-MB-231. It was interesting to note that the basal level of JAK2 tyrosyl phosphorylation (unstimulated cells) was higher in MCF-7 and MDA-MB-231 as compared to T47D cells (Canbay et al., 1997). It would be useful to find out the endogenous PRLR and PRL levels in TMX2-28 cells as compared to MCF-7 and T47D cells, which would also enable us to co-relate the response to exogenous PRL with the tyrosyl phosphorylation of cortactin. Since PRL also functions in an autocrine paracrine loop, high levels of endogenous PRL can also be responsible for not letting exogenous PRL have an effect.
It is difficult to study PRL response in mammary cancer cells where endogenous PRL interferes with exogenous PRL effect at the molecular level (Schroeder et al., 2002).

In the fluorescent gelatin degradation assay (Figs. 11 and 12), we observed that MDA-MB-231 (positive control) and TMX2-28 cells degrade the Texas-Red labeled gelatin matrix. MDA-MB-231 cells have a mesenchymal type of cell motility and are highly metastatic, extensively degrade gelatin matrix and were thus used as a positive control. TMX2-28 cells have an amoeboid mode of motility, and we observed that they degrade gelatin, but it was not as extensive as that caused by MDA-MB-231 cells. As mentioned above, although TMX2-28 cells are a derivative of minimally invasive (almost non-invasive) MCF-7 cells, TMX2-28 are very invasive, their invasiveness being close to that of metastatic MDA-MB-231 breast cancer cells as observed through Matrigel invasion assay (Gozgit et al., 2006). TMX2-28 cells overexpress mRNA for genes involved in cytoskeletal modulation and morphoregulatory genes. In TMX2-28 cells, Tissue inhibitors of matrix-metalloproteases-1 and -2 (TIMPs) were down-regulated by approximately 3 fold and so were MMP-like 1 and cathepsins F and S as demonstrated through a c-DNA microarray (Gozgit et al., 2006). Yet, there is limited knowledge about the MMP profile, i.e. the MMPs that are secreted and involved in invasion and matrix degradation. Since the TMX2-28 cell line was recently developed, further studies need to be done on the MMP regulation in these cells which confer them the highly invasive phenotype and an amoeboid motility. Cancer cells can adopt both amoeboid and mesenchymal mode of cell motility in order to move through the extracellular matrix. With a mesenchymal motility like that of MDA-MB-231 cells, the movement through basement membrane occurs through secretion of proteases such as MMPs and urokinase-
type plasminogen activator. It has been observed that MMP inhibitor therapy administered to cancer patients has been proven ineffective since cancer cells resort to a compensatory mechanism by switching from a mesenchymal type of motility to an amoeboid motility. This plasticity of cancer cell motility is currently an impediment to therapy against metastases. This amoeboid motility of these cancer cells has often been compared to migration of leukocytes and Dictyostelium. Cells moving in an amoeboid fashion are characterized by an amorphous cell shape and change direction rapidly. They do not secrete MMPs to degrade the matrix but can squeeze through the gaps in the matrix. Rho-ROCK signaling which aides in quick remodeling of the cortical actin cytoskeleton has also been implicated in the amoeboid movement of cells (Rosel et al., 2008; Wolf et al., 2003)(reviewed in (Friedl and Wolf, 2003; Pankova et al., 2010; Sahai, 2005)). But, this mesenchymal amoeboid transition or (MAT) is still a subject of controversy since it is difficult to co-relate in vitro 3D basement membrane degradation to actual human physiology, especially since the MMP inhibitor therapy uses lower concentrations (plasma concentrations of 10–40 ng/ml) of MMP inhibitors as compared to that used in the in vitro matrix degradation assays (500 ng/ml). Also, it was suggested that the protease-independent amoeboid mode of migration occurs when the collagen matrix used to study the migration doesn’t possess the covalent cross-links which are present in normal tissues (Sabeh et al., 2009)(reviewed in (Pankova et al., 2010)). Thus, these controversies regarding the different modes of migration of cancer cells in vitro versus in vivo, necessitates the study of mechanism of migration and matrix degradation of TMX2-28 cells and also with in different basement membrane matrices.
Our data of enhancement in tyrosyl phosphorylation of cortactin on PRL stimulation in TMX2-28 cells (Fig. 9) suggests that PRL may promote gelatin degradation via cortactin tyrosyl phosphorylation in these cells. We initially would assess the degradation of gelatin matrix by TMX2-28 cells overexpressing constitutively active c-Src (Y527F) as a positive control, since c-Src (Y527F) is known to promote invadopodia formation and matrix degradation (Artym et al., 2006). If we observe extensive matrix degradation on c-Src (Y527F) overexpression, next we plan to look at matrix degradation by TMX2-28 cells overexpressing constitutively active JAK2 (V617F) or both constitutively active JAK2 and cortactin. This will be followed by PRL stimulation of TMX2-28 cells to look at matrix degradation by PRL-activated endogenous JAK2 dependent tyrosyl phosphorylation of endogenous cortactin. We also plan to generate TMX2-28 cell lines stably overexpressing cortactin cDNA which would be used to study the effect of PRL dependent tyrosyl phosphorylation of cortactin in migration, invasion and matrix degradation. We also plan to look at the PRL dependent invadopodia formation and matrix degradation in the metastatic MDA-MB-231 breast cancer cells by ectopically expressing the PRLR (since they have very low levels of endogenous PRLR) followed by treatment with PRL (Canbay et al., 1997). This would enable us to understand whether PRL can accelerate the process of invadopodia formation and matrix degradation in these metastatic breast cancer cells.

We propose a model (Fig. 13) wherein PRL-activated JAK2 tyrosyl phosphorylation of cortactin promotes invasion and metastases of breast cancer cells. Our long-term goals are to study the mechanism of JAK2 tyrosyl phosphorylation of cortactin...
and identify the other proteins which might also be involved in forming a complex with JAK2 and cortactin in promoting invasiveness of breast cancer cells. Lastly, we would like to extend our research to *in vivo* systems.
Fig. 13. Proposed model for cortactin as a PRL-activated JAK2 target. PRL on binding to PRLR activates receptor associated JAK2 which tyrosyl phosphorylates cortactin leading to actin modulation, invasion and matrix degradation further leading to metastases of breast cancer cells.
Conclusions

1. Overexpressed JAK2 tyrosyl phosphorylates endogenous cortactin in 293T cells.

2. In non-invasive T47D breast cancer cells, overexpressed constitutively active JAK2 tyrosyl phosphorylates overexpressed cortactin, although on PRL activation of endogenous JAK2 signaling, we couldn’t detect an enhancement in tyrosyl phosphorylation of endogenous cortactin.

3. PRL-activated endogenous JAK2 signaling tyrosyl phosphorylates endogenous cortactin in invasive TMX2-28 cells, in a time and concentration dependent manner.

Dependencies


but normal hematopoiesis, in mice with a targeted disruption of the prolactin

Huang, C., J. Liu, C.C. Haudenschild, and X. Zhan. 1998. The role of tyrosine
phosphorylation of cortactin in the locomotion of endothelial cells. *J Biol Chem.*
273:25770-25776.

Huang, C., Y. Ni, T. Wang, Y. Gao, C.C. Haudenschild, and X. Zhan. 1997. Down-
regulation of the filamentous actin cross-linking activity of cortactin by Src-

binding protein 90 (CBP90) expression in the mouse mammary glands during

Cell volume-dependent phosphorylation of proteins of the cortical cytoskeleton
and cell-cell contact sites. The role of Fyn and FER kinases. *J Biol Chem.*
275:32289-32298.

Src kinases and induces tyrosine phosphorylation of cortactin, independent of the

Karasek, M., M. Pawlikowski, and A. Lewinski. 2006. [Hyperprolactinemia: causes,

regulates cell migration through activation of N-WASP. *J Cell Sci.* 118:79-87.


Wolf-Yadlin, A., N. Kumar, Y. Zhang, S. Hautaniemi, M. Zaman, H.D. Kim, V.


