Defining the Role of Prolactin as an Invasion Suppressor Hormone in Breast Cancer Cells

Zaynab Nouhi,1,2 Naiia Chughtai,1,2 Strachan Hartley,1,2 Efthia Cocolakis,1 Jean-Jacques Lebrun,1 and Suhad Ali1,2

1Hormones and Cancer Research Unit, and 2The Division of Hematology, Department of Medicine, McGill University, Montreal, Quebec, Canada

Abstract

Prolactin hormone (PRL) is well characterized as a terminal differentiation factor for mammary epithelial cells and as an autocrine growth/survival factor in breast cancer cells. However, this function of PRL may not fully signify its role in breast tumorigenesis. Cancer is a complex multistep progressive disease resulting not only from defects in cell growth but also in cell differentiation. Indeed, dedifferentiation of tumor cells is now recognized as a crucial event in invasion and metastasis. PRL plays a critical role in inducing/maintaining differentiation of mammary epithelial cells, suggesting that PRL signaling could serve to inhibit tumor progression. We show here that in breast cancer cells, PRL and Janus-activated kinase 2, a major kinase involved in PRL signaling, play a critical role in regulating epithelial-mesenchymal transformation (EMT), an essential process associated with tumor metastasis. Activation of the PRL receptor (PRLR), achieved by restoring PRL/JAK2 signaling in mesenchymal-like breast cancer cells, MDAMB-231, suppressed their mesenchymal properties and reduced their invasive behavior. While blocking PRL autocrine function in epithelial-like breast cancer cells, T47D, using pharmacologic and genetic approaches indicated that blocking PRL signaling led to activation of mitogen-activated protein kinase (extracellular signal-regulated kinase 1/2) and transforming growth factor-β/Smad signaling pathways, two major prometastatic pathways. Furthermore, our results indicate that following PRL/JAK2 inhibition, ERK1/2 activation precedes and is required for Smad2 activation and EMT induction in breast cancer cells. Together, these results highlight PRL as a critical regulator of epithelial plasticity and implicate PRL as an invasion suppressor hormone in breast cancer. (Cancer Res 2006; 66(3): 1824-32)

Introduction

Prolactin has been described as the most versatile hormone of the anterior pituitary gland displaying biological activities related to reproduction, behavior, and immunomodulation (1). Prolactin hormone (PRL) is best known for its effect in mammary gland development. It is required for lobuloalveolar formation of the mammary ducts during pregnancy, terminal differentiation of the mammary epithelial cells, and for synthesis of milk components during lactation (2). PRL binding to its receptor, the PRL receptor (PRLR), a member of the class I cytokine receptor family, induces receptor dimerization and activation of cytoplasmic tyrosine kinases (3). Knock-out mice of PRL, its receptor, and components of the Janus-activated kinase-2 (JAK2)/signal transducer and activator of transcription-5a (STAT5a), the main signaling cascade downstream of the PRLR, confirmed the critical role of PRL in mammary gland alveolar growth/differentiation as these mice show limited alveolar growth and are unable to lactate (4–7). Whereas PRL is a potent mammary epithelial differentiation factor, in breast carcinomas, autocrine/paracrine PRL is now recognized to contribute to tumor cell viability (8–10). Both the hormone and its receptor are expressed in mammary tumors and breast cancer cell lines, thereby creating autocrine/paracrine modes of action for PRL (11–13). Interfering with PRL autocrine/paracrine loop in breast cancer cells with the use of PRL (14–16) and PRLR antagonists (17) led to inhibition in cell growth and induction of apoptosis. Furthermore, in transgenic mouse model systems, autocrine PRL led to mammary tumor formation (18). In addition, PRL was shown to play a permissive role in oncogene-induced breast tumors (19). Together, these results indicate a pro-oncogenic effect of PRL in breast cancer. However, this function of PRL may not fully explain its role in breast carcinogenesis, particularly in processes related to tumor progression leading to metastasis.

Cancer is now recognized as a multistep disease resulting from abnormalities in processes related to cell growth and cell differentiation (20). Indeed, epithelial plasticity, a fundamental property of epithelial cells originally described during embryogenesis, also contributes to tumor metastasis for many epithelial tumors including breast cancer (21, 22). During progression of epithelial tumors, cells undergo a complex dedifferentiation process designated as epithelial-mesenchymal transformation (EMT), leading to loss of epithelial polarity and acquisition of mesenchymal phenotype, ultimately resulting in an increase in the migratory/invasion potential of tumor cells. The hallmark of EMT is down-regulation of adherence junction proteins, such as E-cadherin, and gain of various mesenchymal proteins, such as vimentin, leading to the emergence of a fibroblastoid-invasive phenotype. The signaling pathways regulating the motile-invasive phenotype of epithelial tumors are not fully characterized. However, recent studies have clearly implicated robust activation of Ras/mitogen-activated protein kinase [MAPK; extracellular signal-regulated kinase 1/2 (ERK1/2)] pathway and the transforming growth factor-β (TGF-β)/Smad pathway in inducing EMT in vitro and metastasis in vivo (23–26).

Here, we show that PRL, through activation of its downstream kinase JAK2, plays a critical role in regulating the morphogenic program of breast cancer cells, leading to suppression of their

Note: Z. Nouhi and N. Chughtai contributed equally to this work.
S. Ali and J-J. Lebrun are research scientists of the National Cancer Institute of Canada.

Requests for reprints: Suhad Ali, Royal Victoria Hospital, H5-81, 687 Pine Avenue West, Montreal, Quebec, Canada H3A 1A1. Phone: 514-934-1934 ext. 34863; Fax: 514-982-0893; E-mail: suhad.ali@muhc.mcgill.ca.
©2006 American Association for Cancer Research. doi:10.1158/0008-5472.CAN-05-2292
invasion capacity. Furthermore, we show that PRL regulates these epithelial plasticity properties by negatively regulating prometastatic pathways, the MAPK (ERK1/2) and the Smad pathways. Our results point to a dual activity of PRL in breast carcinogenesis as a pro-oncogenic and as a tumor progression suppressor factor.

Materials and Methods

Plasmid constructs, antibodies, and reagents. Expression plasmid encoding the rat long-form PBLR NH-terminal FLAG-tagged was a gift from Dr. Dominic Devost (McGill University, Montreal, Canada). Expression plasmid encoding kinase-inactive form of JAK2 was obtained from Dr. Dwayne Barber (Ontario Cancer Institute, Toronto, Canada). Reporter construct 3TP-Luc was a gift from Dr. Joe Massague (Memorial Sloan-Kettering Cancer Center, NY). Polyclonal antibodies (pAb) against phospho-(ERK1/2)-T202/Y204 and ERK1/2 (New England Biolabs, Pickering, Ontario, Canada), transcription factor IID (TFB; SI-1; Santa Cruz Biotechnology, Santa Cruz, CA), PRL and JAK2 (Upstate Biotechnology, Charlottesville, VA), phospho-Smad2 (kindly provided by Dr. Aris Moustakas, Ludwig Institute for Cancer Research, Sweden) were used. Monoclonal antibodies (mAb) against phospho-STAT5-694 (Intermedico, Markham, Ontario, Canada), vimentin, E-cadherin, STAT3 (BD Transduction Laboratories, Ontario, Canada), FLAG-M2, and β-tubulin (Sigma-Aldrich, Ontario, Canada) were used. Goat anti-mouse horseradish peroxidase (HRP) and goat anti-rabbit HRP were from Santa Cruz Biotechnology. Goat anti-mouse Rhodamine Red X was from Jackson Immunoresearch Laboratories (West Grove, PA). Ovine prolactin (oPRL) was from Sigma-Aldrich. Human prolactin (hPRL) was a gift from Dr. Vincent Goffin (Institut National de la Sante et de la Recherche Medicale (INSERM) Unit 344, Paris, France). Human TGF-β1 was obtained from PeproTech, Inc. (Rocky Hill, NJ).

Phosphorothioated oligodeoxynucleotides were from Alpha DNA (Quebec, Canada). PDP90859 was from Cell Signaling Technology, and AG490 was from Calbiochem (CA). Transwell filter discs (8 μm) for migration assay by Corning were from Fisher Scientific (Nepean, Ontario, Canada). LipofectAMINE 2000 reagent and Hoechst stain 33258 were from Invitrogen (Ontario, Canada). Enhanced chemiluminescence Hyperfilm and Protein A-Sepharose beads were from Amersham Biosciences/GE Healthcare (Quebec, Canada).

Cell culture and transfections. Human breast cancer cell lines, MDA-MB-231, T47D, and MCF7 and Chinese hamster ovary (CHO) cells were grown in DMEM containing 10% fetal bovine serum (FBS) and starved in starvation media. Phase-contrast images were taken with a 10 objective. MDA-MB-231, T47D, and CHO cells were transfected with LipofectAMINE 2000 for 5 hours at DNA/reagent of 1:3 in serum-free media. Cells were recovered in 5% FBS DMEM for an O/N period. The following day, cells were stimulated with 2 μg/mL of pQPLR in 2%FBS DMEM for an O/N period. Conditioned medium (500 μL) was collected and mixed with nonreducing 2× loading buffer. An 8% SDS-PAGE gel was prepared as usual with a final concentration of gelatin at 1 mg/mL. Samples were run using regular running buffer. The gel was renatured in zymogram renaturation buffer for 1 hour at room temperature and developed in zymogram assay buffer O/N at room temperature. The following day, the gel was stained with 0.5%/v/v Cocasmise blue R-250 for 1 hour at room temperature. Gel was destained until white bands appeared against a dark blue background.

Cell culture and transfections. Human breast cancer cell lines, MDA-MB-231, T47D, and MCF7 and Chinese hamster ovary (CHO) cells were grown in DMEM containing 10% fetal bovine serum (FBS) and starved in DMEM phenol red-free media. Phase-contrast images were taken with a Zeiss Axiovision 135 microscope (Carl Zeiss, Jena, Germany) using a >60 oil immersion lens. Photographs were analyzed using LSM software.

Invasion assay. For Boyden chamber invasion assay, cells were plated in a 24-transwell plates coated with Matrigel (100 μg/cm²; VWR, Montreal, Quebec, Canada). Following 24 hours of treatment (as described for the different experiments), migrated cells were counted using five fields of triplicates for each experimental point using Zeiss Axiovision 135 microscope (Carl Zeiss Canada, Ltd., Toronto, Ontario, Canada) with a ×10 objective and Northern Eclipse program version 6.0 (Empix Imaging, Mississauga, Ontario, Canada).

Luciferase assay. Luciferase activity was measured as described previously (ref. 28; EG&G Berthold Microplate Luminometer LB96V) in relative light units.

Gelatin zymography assay. Gelatinase activity was done as described previously (29). Briefly, MDA-MB-231 cells were transfected with LipofectAMINE 2000 for 5 hours at DNA/reagent of 1:3 in serum-free media. Cells were recovered in 5% FBS DMEM for an O/N period. The following day, cells were stimulated with 2 μg/mL of pQPLR in 2%FBS DMEM for an O/N period. Conditioned medium (500 μL) was collected and mixed with nonreducing 2× loading buffer. An 8% SDS-PAGE gel was prepared as usual with a final concentration of gelatin at 1 mg/mL. Samples were run using regular running buffer. The gel was renatured in zymogram renaturation buffer for 1 hour at room temperature and developed in zymogram assay buffer O/N at room temperature. The following day, the gel was stained with 0.5%/v/v Coomassie blue R-250 for 1 hour at room temperature. Gel was destained until white bands appeared against a dark blue background.

Results and Discussion

Based on estrogen receptor expression, cellular phenotype (i.e., epithelial as opposed to mesenchymal markers expression), and invasion capacity breast cancer cell lines can be divided into epithelial-like cells of low invasion capacity, such as T47D and MCF-7, or mesenchymal-like cells exhibiting high invasion capacity, such as MDA-MB-231 (30). Due to the well-known effects of PRL on induction and maintenance of a differentiated epithelial phenotype in mammary cells, we investigated whether PRL may play a role in regulating the epithelial phenotype and hence the invasion capacity of breast cancer cells. Recently, the MDA-MB-231 cells were shown to lack expression of the PRLR due to gene promoter hypermethylation (31). Using this cell system, we considered whether or not restoring PRL signaling would alter the mesenchymal invasive phenotype of these cells. We restored PRL signaling by transiently co-overexpressing the PRLR and JAK2, as not only these cells fail to express the PRLR but also JAK2 expression level is weaker compared with the epithelial-like breast cancer cells T47D and MCF-7 (data not shown). As shown in Fig. 1A, whereas MDA-MB-231 cells did not respond to PRL, a clear ligand-dependent increase in JAK2 phosphorylation was observed in cells overexpressing the PRLR and JAK2, as detected by Western blotting using a specific antibody to phospho-JAK2 (Y1007/1008) detecting activation of JAK2. In addition, to examine the level of PRLR phosphorylation after restoring PRLR/JAK2 expression in these cells, PRLR was immunoprecipitated, and immunocomplexes were blotted with a mAb to phosphotyrosine. As expected, two phosphorylated proteins were detected corresponding to the PRLR and JAK2 in the MDA/PRLR/JAK2 cells compared with the MDA/pcDNA3 cells (Fig. 1B). Collectively, these results indicate that overexpressing the PRLR and JAK2 in MDA-MB-231 cells is sufficient to restore PRLR/JAK2 activation.

To next investigate whether reestablishing PRLR/JAK2 activation would alter the mesenchymal phenotype of the aggressive breast cancer MDA-MB-231 cells, we examined the effects of
restoring PRLR/JAK2 signaling on the expression level of the mesenchymal marker vimentin. MDA-MB-231 cells overexpressing PRLR/JAK2 or not were not incubated in 2% serum and stimulated or not with PRL for 24 hours. Cell lysates were subsequently subjected to Western blot analysis using a mAb to vimentin. As shown in Fig. 1C, expression levels of vimentin were significantly decreased in cells overexpressing the PRLR and JAK2, and this effect was further marked upon PRL stimulation of the cells. Another hallmark of invasive breast cancer cells is reflected by an increase in expression and activity of matrix metalloproteinases (MMP) that contribute to their invasion potential (32). Therefore, we next examined whether restoring PRLR/JAK2 expression in MDA-MB-231 cells would modulate their MMP activity using gelatinase assay (33). As can be seen in Fig. 1D, wild-type MDA-MB-231 cells (transfected with vector only) exhibited a major gelatinase activity at 72 kDa. However, in cells overexpressing the PRLR/JAK2, a marked reduction in this activity was observed. We then determined whether these PRLR-induced phenotypic changes were associated with changes in the invasion capacity of MDA-MB-231 cells, using Matrigel invasion assays. As illustrated in Fig. 1E, crystal violet staining of the cells revealed that restoring PRLR/JAK2 in these cells dramatically altered their invasion capacity. After counting cells that migrated through the Matrigel, our results indicated that in fact restoring PRLR/JAK2 signaling reduced by 50% the invasion capacity of the MDA-MB-231 cells (Fig. 1E, right). Altogether, these results indicate that restoring PRL signaling strongly suppresses the mesenchymal/invasive phenotype of aggressive breast cancer cells, highlighting PRLR/JAK2 pathway as a negative regulator of EMT process and cell invasion.

Contrary to MDA-MB-231, T47D human breast cancer cells still maintain epithelial-like features and are considered to be of weak invasive phenotype (30). As well, these cells are known to express both PRL and its receptor, thereby creating an autocrine/paracrine growth and survival loop for PRL (8). Using this cell system, we investigated whether blocking JAK2 leads to phenotypic changes typical of EMT process and increase the invasive capacity of these cells. To block JAK2 kinase, we used the pharmacologic inhibitor AG490 (34). As shown in Fig. 2A, treatment of T47D cells with AG490 for 24 hours at a concentration (50 μmol/L), sufficient to significantly inhibit PRL-induced STAT3 phosphorylation (Fig. 2A, bottom), 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 the loss of membrane E-cadherin (Fig. 2B) and increased expression of the mesenchymal marker vimentin (Fig. 2C), also suggestive of an EMT. 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 with control cells (190% of control; Fig. 2D). Thus, our data indicate that blocking JAK2 kinase activity lead to epithelial-mesenchymal phenotypic changes and an increase in the invasive capacity of T47D cells.

To further confirm that blocking JAK2 leads to EMT, we overexpressed a kinase-inactive form of JAK2 (JAK2KI), in which
the predicted type VIII phosphotransferase motif in the COOH-terminal JH1 kinase domain of JAK2 has been mutated (35). As seen in Fig. 2D, expression of the epithelial marker E-cadherin was markedly reduced in T47D cells overexpressing JAK2KI compared with control cells. Similar results were obtained in the other nonaggressive human breast cancer cell line MCF7 upon overexpression of the kinase-inactive form of JAK2 (data not shown). Together, these results indicate that blocking JAK2 kinase activity in breast cancer cells leads to induction of the EMT process and cell invasion. Although JAK2 is a key kinase in mediating PRL signals in mammary cells, it can also be activated by growth factors other than PRL (36). Thus, we next determined whether PRL hormone itself could regulate EMT in a manner similar to JAK2. As shown in Fig. 2E, down-regulation of autocrine PRL levels in these cells clearly led to a significant loss in E-cadherin expression, further strengthening our previous results and confirming the role of PRL itself in this process. Although the functionality of the RRL autocrine loop previously established (8–10), suggests that PRL signaling pathways, such as JAK2 and STAT5a, to be constitutively active; however, under our experimental condition set-up of serum starvation in combination with a possible limitation in detection, we do not observe constitutive activation of this pathway, as measured by phosphorylation of STAT5a, and indeed, STAT5a phosphorylation occurs upon exogenous PRL stimulation as shown in Fig. 2A. Altogether, these results indicate that inhibition of autocrine PRL loop in epithelial-like breast cancer cells leads to EMT and enhances their invasion potential.

The MAPK (ERK1/2) pathway is a key pathway implicated in epithelial plasticity (EMT), leading to breast cancer progression/metastasis (21, 37, 38). As our results indicated that blocking JAK2 kinase also leads to EMT and cell invasion, we next investigated whether these effects could be mediated through activation of the MAPK (ERK1/2) pathway. Interestingly, blocking JAK2 kinase with AG490 in T47D cells (Fig. 3A, top) or in MCF-7 cells (Fig. 3A, bottom) led to a marked increase in the basal

Figure 2. Blocking PRLR/JAK2 leads to EMT in T47D cells. A. top, phase-contrast microscopic images of T47D cells treated with DMSO or AG490 (50 μmol/L) for 24 hours. Bottom, T47D cells were treated with DMSO or AG490 (50 μmol/L) in starvation media for 24 hours before stimulation with hPRL (100 ng) for 10 minutes. Cells were then lysed, and total cell lysates were analyzed using a mAb to phospho-STAT5 Y694. The membrane was reprobed with a mAb to STAT5. B. top, immunostaining using a mAb to E-cadherin (red). Bottom, immunostaining with a mAb to vimentin (red) of T47D cells treated with either AG490 (50 μmol/L) or DMSO for 24 hours. C, equal number of T47D cells was plated on Matrigel-coated 24-transwell plates and treated with AG490 (50 μmol/L) or DMSO for 24 hours, and invaded cells were quantified. Columns, mean of three separate experiments. Microscopic images of a field of invaded cells (left). D. T47D cells were transiently transfected with empty vector or expression plasmid encoding for JAK2KI. Cell lysates were immunoblotted with a mAb to E-cadherin (middle). The membrane was reprobed with a mAb to β-tubulin (middle). Lysates from the same transfection were immunoprecipitated using a pAb to JAK2 and immunodetected with the same antibody (bottom). E. T47D cells were treated with ASO to hPRL or control oligodeoxynucleotide for the indicated time points. Cell lysates were immunodetected using a mAb to E-cadherin (top) or a pAb to PRL (middle). Equal loading was monitored using a pAb to ERK1/2 (bottom).
activation of MAPK (Fig. 3A, compare DMSO-treated with AG490-treated samples). In addition, our results indicate that whereas PRL is able to stimulate MAPK (ERK1/2) activation as has been previously described (39, 40), however, PRL-induced MAPK activation was greatly amplified following inhibition of JAK2 (Fig. 3A). Moreover, as shown in Fig. 3B, in contrast to PRL, AG490 treatment of T47D cells led to ERK1/2 activation similar to what was observed in cells treated with epidermal growth factor, a known mitogen for breast cancer cells. Furthermore, activation of ERK1/2 was also seen following 5 to 10 minutes of treatment of T47D cells with AG490 (Fig. 3C). Together, these results indicate 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.

We next confirmed these inhibitory effects of JAK2 on MAPK activation using the kinase-deficient form of JAK2 (JAK2KI). Overexpression of JAK2KI in T47D cells or in MCF-7 cells led to a significant increase in basal activation/phosphorylation of ERK1/2 pathway (Fig. 3B). These results indicate that blocking JAK2 kinase in breast cancer cells leads to activation of the MAPK pathway. To specifically address the role of PRL in this process, we next blocked the autocrine/paracrine function of PRL in T47D cells using ASO to hPRL gene. As can be seen in Fig. 3C, blocking PRL expression in T47D cells led to a marked increase in MAPK (ERK1/2) pathway, after 48 and 72 hours of treatment compared with control cells incubated with scrambled oligodeoxynucleotides. Together, these results indicate that blocking PRL expression or PRLR signaling by inhibiting JAK2 kinase activity results in a robust and prolonged activation of MAPK (ERK1/2) pathway in breast cancer cells, suggesting that the PRL/JAK2 pathway serves to mediate negative regulatory signals to MAPK and consequently cell invasion.

Another critical signaling pathway (TGF-β/Smad) has also recently been shown to contribute to EMT and metastasis of mammary tumors (as described in Introduction). In various epithelial cells, including mammary cells, cooperation between Ras-MAPK (ERK1/2) and TGF-β pathways was found to induce EMT (23). As blocking PRL/JAK2 induces ERK1/2 pathway and EMT, therefore, we next examined whether blocking PRL signaling in addition to activation of the ERK1/2 pathway would also activate the TGF-β pathway. As shown in Fig. 4A and B, treatment of T47D cells with AG490 for different time points or overexpressing kinase-inactive form of JAK2 led to Smad2 activation as measured by Western blotting using a specific phospho-Smad2 antibody recognizing the two COOH-terminal serine residues phosphorylated by activated type I receptors of the TGF-β superfamily. Moreover, blocking JAK2 activity in MCF-7 breast cancer cells also led to Smad2 activation (Fig. 4C). To further confirm the activation of Smad proteins, we examined the effects of blocking JAK2 function on the transcriptional activity of Smad2. For this, CHO cells were transfected with TGF-β/Smad-responsive reporter construct 3TP-luc and were treated or not with AG490 for different time points. As shown in Fig. 4D, AG490 treatment of the cells led to a marked increase in luciferase activity. Similarly, as shown in Fig. 4E, blocking

---

**Figure 3.** Blocking PRL autocrine loop induces ERK1/2 activation in breast cancer cells. A, T47D (top) or MCF7 (bottom) cells were treated with DMSO or AG490 (50 μmol/L) for 24 hours before stimulation with hPRL (100 ng/mL) for the indicated time points. B, T47D cells were treated with DMSO, hPRL (100 ng/mL), AG490 (50 μmol/L), AG490 (50 μmol/L) and hPRL (100 ng/mL), or epidermal growth factor (EGF; 10 ng/mL) for 24 hours. C, T47D cells were treated with AG490 (50 μmol/L) for the indicated time points. D, T47D or MCF-7 cells were transiently transfected with empty vector or expression plasmid encoding JAK2KI. E, T47D cells were treated with ASO to hPRL or control oligodeoxynucleotide for 48 and 72 hours. A-E, cell lysates were immunodetected using a phosphospecific pAb to ERK1/2 (top). The membranes were reprobed with a pAb to ERK1/2 (bottom). E, immunoblot of PRL was done using a pAb to PRL (middle).

---

**Figure 4.** EMT and TGF-β/Smad pathway activation. A, T47D or MCF-7 cells were transiently transfected with empty vector or expression plasmid encoding JAK2KI. B, these cells were stimulated with DMSO or AG490 for 24 hours. C, these cells were stimulated with DMSO or AG490 for 24 hours. D, these cells were stimulated with DMSO or AG490 for 24 hours. E, these cells were left untreated or were treated with DMSO or AG490 for 24 hours. F, these cells were left untreated or were treated with DMSO or AG490 for 24 hours. G, these cells were left untreated or were treated with DMSO or AG490 for 24 hours. H, these cells were left untreated or were treated with DMSO or AG490 for 24 hours. I, these cells were left untreated or were treated with DMSO or AG490 for 24 hours. J, these cells were left untreated or were treated with DMSO or AG490 for 24 hours. K, these cells were left untreated or were treated with DMSO or AG490 for 24 hours. L, these cells were left untreated or were treated with DMSO or AG490 for 24 hours. M, these cells were left untreated or were treated with DMSO or AG490 for 24 hours. N, these cells were left untreated or were treated with DMSO or AG490 for 24 hours. O, these cells were left untreated or were treated with DMSO or AG490 for 24 hours. P, these cells were left untreated or were treated with DMSO or AG490 for 24 hours. Q, these cells were left untreated or were treated with DMSO or AG490 for 24 hours. R, these cells were left untreated or were treated with DMSO or AG490 for 24 hours. S, these cells were left untreated or were treated with DMSO or AG490 for 24 hours. T, these cells were left untreated or were treated with DMSO or AG490 for 24 hours. U, these cells were left untreated or were treated with DMSO or AG490 for 24 hours. V, these cells were left untreated or were treated with DMSO or AG490 for 24 hours. W, these cells were left untreated or were treated with DMSO or AG490 for 24 hours. X, these cells were left untreated or were treated with DMSO or AG490 for 24 hours. Y, these cells were left untreated or were treated with DMSO or AG490 for 24 hours. Z, these cells were left untreated or were treated with DMSO or AG490 for 24 hours.
JAK2 activity by overexpressing kinase-inactive mutant form of JAK2, JAK2KI, in both CHO and MCF-7 cells also led to a significant increase in 3TP-luc reporter activity. Together, these results indicate that blocking JAK2 leads to increased Smad phosphorylation and Smad-mediated transcriptional activity. Thus, inhibiting PRL/JAK2 signaling induces activation of two well-characterized prometastatic pathways ERK1/2 and TGF-β/Smad and promotes high-invasion capacity of human breast cancer cells.

The mechanism by which Ras pathway cooperates with TGF-β/Smad to induce EMT is still not fully characterized. It has been shown previously that in epithelial cells transformed with Raf autocrine production of TGF-β leads to Raf-induced EMT (41). In addition, it was shown in squamous carcinoma cells of murine keratinocytes that activation of Ras led to Smad2 nuclear accumulation, and this effect was required for EMT and metastasis (42). Furthermore, RON receptor tyrosine kinase activation was recently shown to induce Smad2 activation through ERK1/2-dependent mechanism leading to EMT in epithelial cells (43), suggesting that MAPK (ERK1/2) and TGF-β/Smad pathways may contribute to EMT and cell invasion in a sequential and temporal manner. Therefore, we next investigated whether ERK1/2 pathway is required for Smad2 activation and EMT induction following inactivation of PRL/JAK2 cascade in breast cancer cells. For this, we blocked ERK1/2 activation using PD98059, a chemical inhibitor of MAP/ERK kinase-1 (MEK-1) kinase. As shown in Fig. 5A, AG490-induced ERK1/2 activation was blocked in the presence of PD98059. Interestingly, PD98059 also inhibited AG490-induced Smad2 activation (Fig. 5B). In addition, AG490-induced epithelial to mesenchymal morphology changes and loss of E-cadherin expression was reversed in the presence of PD98059 (Fig. 5C and D) in T47D cells.

The use of the kinase-inactive form of JAK2 confirmed these results. As shown in Fig. 5E, JAK2KI-induced phosphorylation of Smad2 was markedly blocked in the presence of the MEK-1 inhibitor. In addition, loss of E-cadherin expression in cells overexpressing JAK2KI was reversed in the presence of PD98059 (Fig. 5F). Collectively, these results indicate that induction of EMT in response to a loss of PRL/JAK2 signaling is a multistep process and that following inhibition of PRL/JAK2, activation of Smad2, and induction of EMT are mediated through an ERK1/2-dependent mechanism.

PRL has long been known as an important factor in regulating mammary epithelial cell growth and differentiation. Furthermore,
it is now accepted that PRL functions as an autocrine factor promoting the growth/survival of breast cancer cells. Our studies here describe for the first time a novel function of the hormone PRL and its downstream signaling 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 our study, STAT5a, a downstream mediator of PRL in mammary cells, was recently shown to display invasion suppressive activity in breast cancer (44). Therefore, in light of the current study, the hormone PRL and its main downstream effector molecules JAK2 and STAT5a are critical regulators of human breast cancer cell invasion and suggest that PRL may suppress invasion through multiple mechanisms.

We now propose a new model for the role of PRL in breast carcinogenesis. PRL is required as a growth and survival factor in normal mammary epithelial cells and in breast cancer but functions as an invasion/metastasis suppressor hormone by blocking the prometastatic MAPK (ERK1/2) and TGF-β/Smad pathways (Fig. 6).

Tumor metastasis significantly contributes to death in cancer patients. Although precise criteria have been defined to classify oncogenes/proto-oncogenes and tumor suppressor genes, no such variables exist to characterize metastasis suppressor genes/pathways. Recently, based on the identification of several metastasis suppressor genes, such as NM23 and KAI-1, certain criteria for metastasis suppressor genes/pathways have been proposed (45, 46). Interestingly, PRL fulfills these variables. It is proposed that metastasis suppressor genes are involved in regulating/maintaining differentiation of target cells, and indeed, PRL is known as a differentiation factor in mammary epithelial cells. In

Figure 5. Blocking JAK2 leads to activation of ERK1/2 resulting in activation of Smad2 and EMT. T47D cells were treated with DMSO, AG490 (50 μmol/L), PD98059 (10 μmol/L), or AG490 and PD98059 for an overnight period. A, cell lysates were immunoblotted with a pAb to phospho-ERK1/2 (top), and the membrane was reprobed with a pAb to ERK1/2 (bottom). B, nuclear extracts were immunoblotted using a pAb to phospho-Smad2 (top), and the membrane was reprobed with a pAb to TBP (bottom). C, phase-contrast microscopic images. D, fluorescent images of immunostained cells with a mAb to E-cadherin. E, T47D cells were transiently transfected with empty vector (10 μg) or expression plasmid encoding JAK2KI (10 μg). Twenty-four hours following transfections, cells were used for immunoprecipitation using a pAb to JAK2 and immunoblotted using the same antibody (bottom). F, T47D cells were transiently transfected with vector alone (3 μg) or expression plasmid encoding JAK2KI (3 μg). The day after, cells were either treated or not with PD98059 overnight. Nuclear fractions were immunodetected using a pAb to phospho-Smad2. The membrane was reprobed with a pAb to TBP (middle). Parallel transfections were carried out, and cells were used for immunoprecipitation using a pAb to JAK2 and immunoblotted using the same antibody (bottom). F, T47D cells were transiently transfected with vector alone (3 μg) or expression plasmid encoding JAK2KI (3 μg). The day after, cells were either treated or not with PD98059 for an overnight period. The membrane was immunodetected using a mAb to E-cadherin (top). The membrane was reprobed with a mAb to β-tubulin (bottom).
addition, it is proposed that the expression level of metastasis suppressor genes is reduced/lost in aggressive cancers compared with nonaggressive cancers. Similarly, loss of PRLR expression was shown in aggressive breast cancer cells (31). Finally, functionally, these metastasis suppressor genes affect pathways that are known to be prometastatic. Both NM23 and KAI-1 metastasis-inhibitory activity was related to their ability to negatively regulate MAPK (ERK1/2) pathway (47, 48). Likewise, our data presented here clearly show the negative role of PRL and JAK2 kinase in MAPK and Smad activation in breast cancer cells. Thus, PRL/JAK2 signaling cascade can now be included in the growing list of metastasis suppressor pathways.

Furthermore, based on our results, it is evident that PRL/JAK2 limits ERK1/2 activation in breast cancer cells. We can envisage that this PRL/JAK2 activity would categorize PRL as a negative regulator of potentially many oncogenic signals that operate through the Ras-ERK1/2 cascade. Therefore, it is of importance to define the mechanisms by which PRL negatively regulates the MAPK pathway. Indeed, multiple kinases, phosphatases, and scaffold proteins regulate ERK1/2 activation (49, 50). In addition to GTP hydrolysis by GTPase-activating protein (Ras/GAP) to convert Ras from GTP-bound active form to GDP-bound inactive form and ERK1/2 inactivation by MAPK phosphatases (MKP-1 and MKP-3; ref. 51), there exist several other negative regulators of the Ras/MAPK pathway. For example, the protein Ras-interacting protein-1 (RIN-1) competes with Raf for binding to Ras thereby blocking MAPK (ERK1/2) activation (52), the protein Raf kinase inhibitor protein (RIKP) competitively disrupts the interaction between Raf and MEK kinases leading to suppression in MAPK activation (53) and the protein impedes mitogenic signal propagation (IMP; ref. 54) blocks the functional assembly of MEK and Raf kinases through kinase suppressor of Ras (KSR). Other studies have shown that adaptor proteins, such as sprouty (spry), sprouty-like protein sprd (55, 56), and members of the protein family of the Ras/GAP-associated p62 downstream of tyrosine kinase (Dok; refs. 57, 58) also inhibit the MAPK pathway. One mechanism by which JAK2 was previously shown to inhibit ERK2 activation downstream of the angiotensin II hormone is by modulating the expression levels of the MAPK phosphatase MKP-1 (59). The mechanism by which PRL negatively regulates the MAPK pathway in mammary and breast cancer cells is unknown and is currently under investigation.

Moreover, our results also highlight an important role for JAK2 kinase in negative regulation of EMT and cell invasion. Chromosomal translocations and, recently, activating mutations involving JAK2 have been described and were found to cause myeloproliferative diseases (60). In addition, activation of JAK2 has been implicated in solid tumors in addition to breast, such as ovarian and prostate (61). Therefore, efforts are being devoted to target JAK2 for cancer treatment (62). In light of our study, it will be important to determine whether JAK2-induced signaling would also modulate epithelial plasticity and invasion in these types of tumors.

In summary, our results here define a novel mechanism controlling invasiveness/metastasis of breast cancer cells through PRL/JAK2 pathway that could be exploited for therapeutic manipulation.

Acknowledgments

Received 6/29/2005; revised 10/31/2005; accepted 11/15/2005.

Grant support: Canadian Institutes of Health Research grants MOP-13681 (S. Ali) and MOP-33141 (J-J. Lebrun), FRQ studentship award (E. Cocolakis), and Canadian Cancer Society (S. Ali and J-J. Lebrun).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank Drs. Dominic Devost; Dwayne Barber, Vincent Goffin, Joan Massague, and Aris Moustakas for providing us with various reagents used in this study and Eric Lizotte for technical help with confocal microscopy (Hormones and Cancer Research Unit core facility).


Defining the Role of Prolactin as an Invasion Suppressor Hormone in Breast Cancer Cells

Zaynab Nouhi, Naila Chuhtai, Strachan Hartley, et al.


**Updated version**

Access the most recent version of this article at:

[http://cancerres.aacrjournals.org/content/66/3/1824](http://cancerres.aacrjournals.org/content/66/3/1824)

**Cited articles**

This article cites 62 articles, 17 of which you can access for free at:

[http://cancerres.aacrjournals.org/content/66/3/1824.full.html#ref-list-1](http://cancerres.aacrjournals.org/content/66/3/1824.full.html#ref-list-1)

**Citing articles**

This article has been cited by 12 HighWire-hosted articles. Access the articles at:

[http://cancerres.aacrjournals.org/content/66/3/1824.full.html#related-urls](http://cancerres.aacrjournals.org/content/66/3/1824.full.html#related-urls)

**E-mail alerts**

Sign up to receive free email-alerts related to this article or journal.

**Reprints and Subscriptions**

To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

**Permissions**

To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.