Prolactin receptor in breast cancer: marker for metastatic risk

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Abstract

Prolactin and prolactin receptor signaling and function are complex in nature and intricate in function. Basic, pre-clinical and translational research has opened up our eyes to the understanding that prolactin and prolactin receptor signaling function differently within different cellular contexts and microenvironmental conditions. Its multiple roles in normal physiology are subverted in cancer initiation and progression, and gradually we are teasing out the intricacies of function and therapeutic value. Recently, we observed that prolactin has a role in accelerating the time to bone metastasis in breast cancer patients and identified the mechanism by which prolactin stimulated breast cancer cell-mediated lytic osteoclast formation. The possibility that the prolactin receptor is a marker for metastasis, and specifically bone metastasis, is one that may have to be put into the context of the different variants of prolactin, different prolactin receptor isoforms and intricate signaling pathways that are regulated by the microenvironment. The more complete the picture, the better one can test biomarker identity and design clinical trials to test therapeutic intervention. This review will cover the recent advances and highlight the complexity of prolactin receptor biology.

Introduction

The complexity of prolactin (PRL) signaling and function in breast cancer biology is apparent, with multiple reports covering a spectrum of observations that range from a protective nature to the induction of lytic bone cells in the metastatic process of breast cancer. The intricacy of these conflicting observations intriguingly lies somewhere in the spectrum of signaling pathways, involving multiple receptor isoforms and the interaction of the breast cancer cells with the cells and molecules in the tumor microenvironment. This review will explore the potential of the PRL receptor (PRLR) to be used as a biomarker for metastatic risk, within the spectrum of the roles of prolactin signaling, in early breast cancer initiation to breast cancer metastasis to bone.

PRL and the risk of breast cancer and metastasis

There have been some extensive investigations using large prospective cohorts to assess the risk of primary breast cancer in women with high levels of circulating PRL (Tworoger *et al.* 2013, Tikk *et al.* 2014). In the Nurses Heath Study, it was found that PRL levels measured less than 10 years before diagnosis (2468 cases and 4021 controls), as opposed to greater than 10 years (953 cases and 1339 controls), was associated with postmenopausal breast cancer risk (relative risk (RR), >15.7 vs ≤8.1 ng/mL (i.e., top vs bottom quartiles)=1.20; 95% CI, 1.03–1.40; $P_{trend}=0.005$), particularly for estrogen receptor (ER)-positive tumors (RR 1.52) and lymph node-positive disease (RR 1.63). The associations were similar for grade, tumor size, ductal vs lobular tumors, human

epidermal growth factor receptor (HER)-2 status, as well as for luminal A vs B subtypes ($P_{heterogeneity} > 0.46$), but apparently stronger for invasive tumors (RR 1.38, $P_{trend} = 0.0005$) compared with in situ tumors (RR 1.16, $P_{trend} = 0.23$), although the $P_{heterogeneity}$ was not statistically significant ($P = 0.81$). The association with triple-negative tumors, recurrent or fatal disease, was not significant (Tworoger *et al.* 2013).

The association of PRL levels with ER-positive disease is very interesting, given the predominance of ER-positive tumors associated with bone metastasis (Hess *et al.* 2003, Wei *et al.* 2008) and the recent observations of PRL affecting breast cancer cell communication with bone cells (see PRLR and bone metastasis) (Sutherland *et al.* 2016).

Another group investigating a subgroup of the European Prospective Investigation into Cancer and Nutrition (EPIC) cohort (2250 invasive breast cancer patients with equal number of matched control subjects) demonstrated an increase in risk among postmenopausal breast cancer patients (odds ratio $(OR)_{Q4-Q1} = 1.29$ (95% confidence interval (CI) 1.05–1.58), $P_{trend} = 0.09$), particularly those women who had hormone replacement therapy ($OR_{Q4-Q1} = 1.45$ (95% CI 1.08–1.95), $P_{trend} = 0.01$) (Tikk *et al.* 2014).

So there is an association of invasive breast cancer risk in postmenopausal women with high circulating PRL, particularly for ER-positive disease. Interestingly, there was no significant association with disease recurrence, similar to another report using a very small cohort (Coskun *et al.* 2003).

PRLR and bone metastasis

Although there is a known association of PRL levels with invasive breast cancer, information about the role of PRL in metastasis has been lacking until recently (Yonezawa *et al.* 2015, Sutherland *et al.* 2016). We recently reported the involvement of PRL signaling in breast cancer bone metastases (Fig. 1) (Sutherland *et al.* 2016). In a retrospective cohort (134 patient samples) of breast cancer primary tumor samples, we identified, using quantitative immunohistochemistry, an association of the PRLR (monoclonal antibody 1A2B1 to receptor extracellular domain) with a shorter time to metastasis that includes bone ($PRLR_{AQUA\ Max-per\ 100\ unit}$ hazard ratio = 1.04, 95% CI = 1.00–1.07, $P = 0.03$). There was no association with any other location, and the PRLR was an independent marker. We also observed that the PRLR was present in a second set of primary breast tumor tissues with matched bone metastases. Interestingly, there was variable PRLR staining between the matched samples, such that there could be high or low staining in the primary tumor, with either high or low staining in the matched bone metastasis. This may be due to technical issues common to bone preparations, but it may also be explained by heterogeneous PRLR levels also detected in advanced breast cancer patient circulating tumor cells (Sutherland *et al.* 2016).
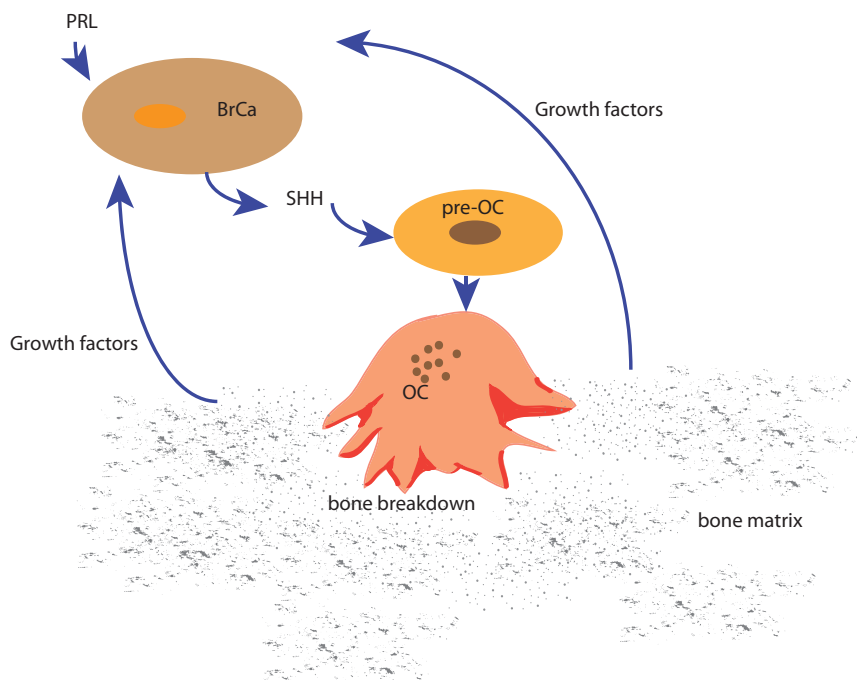
Figure 1
PRL stimulates breast cancer cells to secrete soluble factors, such as SHH, which instructs pre-osteoclasts to differentiate and become lytic, mature, multi-nucleated osteoclasts. The breakdown of the bone has been reported to release calcium and growth factors, which stimulate tumor cell reproduction, resulting in a vicious cycle.

Given the association of high PRLR with a shorter time to bone metastases, we hypothesized that high PRLR may be involved in creating a more serious bone lesion upon metastasis (Fig. 1) (Sutherland *et al.* 2016). We asked if the PRLR present on the breast cancer cells could contribute to bone breakdown (osteolysis) and investigated whether there would be a direct contribution of PRL-induced breast cancer cells to the induction of bone osteoclasts. As osteoclast cells do not express the PRLR at the gene or protein level (Clement-Lacroix *et al.* 1999, Coss *et al.* 2000), the PRL effect would be mediated by the PRLR on the breast cancer cells. Indeed, PRL was capable of inducing osteoclast differentiation when the breast cancer cells were either co-cultured with osteoclasts or when breast cancer conditioned medium was used, and PRL also contributed to breast cancer-mediated osteolysis. Interestingly, different PRLR-positive breast cancer cell lines responded somewhat differently, with PRL-treated MCF7 cells producing the greatest number of osteoclasts only when in co-culture with osteoclasts, in comparison with PRL-treated MCF7-conditioned medium. SKBr3 and BT-483 were able to induce osteoclasts via conditioned media in a PRL-dependent manner. MCF7 cells have been identified as luminal breast cancer cells (Neve *et al.* 2006, Kao *et al.* 2009), luminal B in particular (Prat *et al.* 2013). SKBr3 was identified as a luminal molecular subtype (Kao *et al.* 2009), particularly luminal B with *HER2* overexpression (Prat *et al.* 2013). BT-483 cells have been reported as luminal (Neve *et al.* 2006), luminal B without *HER2* expression (Kao *et al.* 2009) or luminal B with *HER2* overexpression (Prat *et al.* 2013). The differences in cellular response of MCF7 from other PRLR-positive cell lines may reflect alternate PRLR signaling pathways, different post-transcriptional processing of PRL-regulated targets such as sonic hedgehog (SHH) or a difference in qualitative response between the cell lines.

In this investigation, SHH was identified as PRL-enhanced in the conditioned medium of different PRL-responsive cell lines (Fig. 1), but not in MCF7 cells. SHH has been demonstrated to stimulate osteoclast differentiation (Das *et al.* 2011, Sutherland *et al.* 2016), and a hedgehog pathway (smoothened) inhibitor, cyclopamine (Chen *et al.* 2002), reduced PRL-induced breast cancer cell-mediated osteoclastogenesis (Sutherland *et al.* 2016).

The two retrospective cohorts in this study were chosen with an interest in bone metastasis and contained a number of advanced breast cancer patients. As such, it will

be interesting to follow-up this study with a prospective cohort such as the Breast to Bone Metastasis (B2B) cohort (Brockton *et al.* 2015). The results indicate that PRLR signaling may be an important marker for breast cancer bone metastasis, and that the PRLR is present in such bone metastases and likely to have a deleterious impact on the level of osteolysis *in vivo*. This builds on other work that has identified a vicious cycle of bone metastasis (Fig. 1), whereby breast cancer-secreted factors can act on the osteoblast or osteoclasts of the bone to induce bone breakdown. The release of bone-matrix factors stimulates cancer cell reproduction and begins a vicious cycle of bone breakdown and tumor cell growth, which PRL would accelerate or perpetuate. It will be interesting to identify the key PRLR isoforms and contributing pathways involved in this process.

Patients, in particular those, who have hormone receptor-positive (ER and/or progesterone receptor) bone-only breast cancer metastases have an excellent clinical outcome (Lee *et al.* 2011). Patients with bone metastases have high levels of morbidity, and better identification of these patients, combined with better treatment, could significantly improve their quality of life. The studies by Sutherland and coworkers assessed all metastases that included bone. In future, it will be important to stratify PRLR-positive patients based on organ-specific relapse. A report on 85 breast cancer patients and 25 controls showed that there was no association of serum PRL levels with visceral or bone metastases (Coskun *et al.* 2003), so information regarding the PRLR is essential. Identifying the roles of PRL-PRLR in specific stages of metastases, whether the metastases are preferentially boney or visceral and if PRL-PRLR is involved in homing to any specific secondary site will help clarify the observations in the literature and highlight the importance of proper patient identification when using anti-PRL or anti-PRLR cancer treatments.

The long form of the PRLR is responsible for metastasis

The PRLR gene has the potential to produce multiple isoforms (see PRLR structure and isoforms). Recently, the role of the long PRLR isoform was investigated (Yonezawa *et al.* 2015). Splice-modulating oligomers were delivered *in vivo* to block splicing between *Prlr* exons 9 and 10, reducing the amount of the full-length PRLR, but not SF1a–c, and not of the related growth hormone receptor. The intermediate form of the receptor was not considered an abundant isoform in

the study. There was a knockdown of the long isoform in the mammary gland, liver and ovary. Interestingly, specific knockdown of the long isoform resulted in a sharp decrease in metastatic colonization of the lungs and liver from orthotopic mammary fat pad transplants of syngeneic 4T1 cells in Balb/c mice. The observations were replicated in a humanized xenograft model, using BT474 cells in non-obese diabetic severe combined immunodeficiency (NOD-SCID) mice (Yonezawa *et al.* 2015) (BT474, luminal B-like with Her2 overexpression (Neve *et al.* 2006, Prat *et al.* 2013)).

The use of both the xenograft and syngeneic models allowed an interesting opportunity to study the role of the native immune system also. It was observed that knockdown of the PRLR long form resulted in decreased immune infiltration of the metastatic sites by neutrophils and macrophage and increased anti-tumor cytotoxic CD8+ cells. Interestingly, a lack of ability of oligomer-treated 4T1 cells to form primary spheres in non-adherent culture, and an apoptotic reduction in cells with the tumor-initiating phenotype, Lin– CD24+CD29hi ALDH+, was also noted. This study demonstrated that the long form of the PRLR contributed significantly to the development of both lung and liver metastases in these mouse models. It would be very interesting to assess its contribution to bone and brain metastases.

A number of different mouse models of increased mammary PRL, including mouse models of mammary cancer, have been studied for the effects of PRL on mammary cancer and metastasis and interaction with specific oncogenes. These have been comprehensively reviewed (Arendt & Schuler 2008*b*).

PRLR and clinical outcome by breast cancer molecular subtype

What is known about the PRLR in breast cancer molecular subtypes? Can their biology inform our understanding of the different roles of the PRLR in breast cancer? There have been two recent reports of PRL and PRLR levels and gene expression (Hachim *et al.* 2016*a*,*b*).

Interrogation of a small tissue micro-array (TMA) (100 patients) for PRL and PRLR levels showed an association of PRL with lymph node-positive patients, although there was no detection of the PRLR in the cancer samples (polyclonal antibody H-300 to the PRLR intracellular domain) (Hachim *et al.* 2016*b*). Interrogation of the KM-Plotter database using the PRL gene identified an association with prolonged relapse-free survival. A gene signature, of PRL, PRLR, Janus kinase (JAK)-2 and signal

transducer and activator of transcription (STAT)-5a (derived from 1881 GOBO (Gene expression-based Outcome for Breast cancer Online (GOBO) patients)), was used to assess the GOBO database. This analysis demonstrated an association with ER-positive and luminal A subtypes, as well as with prolonged relapse-free survival (Hachim *et al.* 2016*b*). A PRL gene signature derived from differentiated PRL-induced mammary epithelial HC11 cells was also found to be associated with well-differentiated tumors and luminal A tumors (Hachim *et al.* 2016*b*). This is as may be expected, given the association of this pathway with mammary epithelial cell differentiation and early tumorigenesis (see PRL-JAK2-STAT5 signaling).

Using a small TMA (102 patients) with immunohistochemistry, analysis of PRLR levels and its gene expression was undertaken (Hachim *et al.* 2016*a*). Cores of the small TMA were reclassified on the basis of immunohistochemical markers, as surrogates of the molecular subtype, and found the PRLR (polyclonal antibody H-300 to the receptor intracellular domain) was present in luminal, A, luminal B and Her2-enriched, but not in triple-negative patient tumors. A larger analysis was carried out at the mRNA levels in a cohort of the GOBO (1881 patients) database (Hachim *et al.* 2016*a*). There was no evidence of association of PRLR expression with hormone receptors, although an association of *PRLR* expression and the luminal A subtype was observed, and there was no evidence of association with the basal subtype. Analysis of 21 subtypes (in a subset of 1379 patients) resulted in a significant association of *PRLR* expression with better distant metastasis-free survival in luminal B (PAM50 and Hu classifications) patients (Hachim *et al.* 2016*a*). In future, it would be very interesting to discover which receptor variants are predominantly expressed in each of these molecular subtypes and which pathway may be preferentially activated within each subtype.

Key factors influencing PRLR biology

The link of PRL levels to invasive breast cancer risk (Tworoger *et al.* 2013, Tikk *et al.* 2014) implies a connection to primary tumor initiation and also to metastatic capacity. PRL and the long form of the PRLR also contribute to the metastatic process (Yonezawa *et al.* 2015). These results are consistent with the reports of PRLR-mediated chemotherapeutic resistance (Howell *et al.* 2008, LaPensee *et al.* 2009) and the role of PRL-PRLR in osteolytic bone metastases of breast cancer cells (Sutherland *et al.* 2016). The recent demonstration of PRL and PRLR mRNA and protein in tumor-initiating cells

warrants additional follow-up, and their roles in apoptosis and sphere formation may indicate a contribution to early tumorigenesis, metastasis and chemotherapy resistance, consistent with the role of tumor-initiating cells in cancer. Interestingly, the recent evidence also indicates that *PRL* and *PRLR* expression is associated with relapse-free survival and distant metastasis-free survival of breast cancer patients (Hachim *et al.* 2016*a*,*b*).

So how can these findings be reconciled, aside from the potential differences resulting from different experimental platforms and breast cancer cohort selection? The key to understanding these results that appear to be conflicting on the surface of our knowledge, likely lies in what we do not yet fully understand: the intricate nature of PRL and PRLR biology, discussed in the following sections.

Pituitary and extra-pituitary PRL, PRL isoforms and alternative ligands

The complexity of PRL signaling and the spectrum of biological response start with the potential for different prolactin isoforms to be produced (Bernard *et al.* 2015). Prolactin is produced in an endocrine manner in the anterior pituitary gland, as well as secreted in an extra-pituitary, autocrine/paracrine manner from normal breast and breast tumor tissue. The major isoform, the 23 kDa protein, is proteolytically cleaved to 14 kDa, 16 kDa and 22 kDa isoforms (Bernard *et al.* 2015). Although the 16 kDa fraction cannot interact with the PRLR, it functions as an anti-angiogenic molecule (vasoinhibin) by interacting with endothelial cells, in contrast to the full-length form of PRL, which is vasoactive (Clapp *et al.* 2008).

Both normal breast tissue and breast tumors secrete extra-pituitary prolactin, which is thought to act in an autocrine/paracrine manner in the microenvironment (Ben-Jonathan *et al.* 2002). Although the two sources produce identical proteins, the level, post-translational modifications, and microenvironmental function of autocrine prolactin is not taken into consideration in the majority of studies. Autocrine *PRL* was detected in nearly half of 144 breast cancer samples by cDNA array, in situ hybridization or PCR, albeit generally at very low levels (Nitze *et al.* 2013).

Although PRL appears to only bind to the PRLR, the PRLR can bind three different ligands (PRL, placental lactogen and growth hormone (Brooks 2012)) leading to additional complexity regarding PRLR function. Therefore, studies assessing the role or association of PRL with tumorigenic potential are more specific to PRL biology than studies investigating the PRLR, which can bind additional ligands. This, however, does not reduce their importance.

When assessing PRL or PRLR function with respect to a biomarker for metastasis or other, it is apparent that there is a complex picture unfolding and often one observes only a glimpse of the intricacy involved. The potential for the prolactin inducible protein (PIP) to be a marker for breast cancer metastasis has been explored (Clark *et al.* 1999, Mitas *et al.* 2001). The biology and involvement of PIP is also complex, involves the induction predominantly by other hormones and has been the subject of a recent review (Ihedioha *et al.* 2016).

PRLR structure and PRLR isoforms

Greater insight into the structure of the PRLR was recently determined, with the PRLR serving as the archetype of the structure of cytokine class-1 receptors (Haxholm *et al.* 2015, Bugge *et al.* 2016) (Fig. 2). Class-1 cytokine receptors, share a common structure, consisting of an extracellular domain, transmembrane domain and an intracellular domain. The receptor chains are already dimerized in the absence of ligand (Gadd & Clevenger 2006, Tallet *et al.* 2011). The extracellular domain is divided into two fibronectin 3 domains, D1 and D2, of which the latter contains a WS motif (Fig. 2) that acts as a molecular switch for the PRLR during ligand-bound activation (Dagil *et al.* 2012). The receptor chain does not possess kinase activity, and it is dependent upon associated kinases for its ability to transduce phosphorylation-based signal cascades. The intracellular domain of the PRLR is disordered and does not appear to be involved in receptor dimerization (Haxholm *et al.* 2015). The intracellular domain includes Box-1 and Box-2 domains (Fig. 2), the first of which is known to interact with JAK2 and Rous sarcoma oncogene cellular homolog (SRC) family kinases such as FYN (Haxholm *et al.* 2015).

Interestingly, there are three lipid-interacting domains in the intracellular portion of the molecule (Fig. 2), one of which overlaps with the Box-1 domain, although the interactions are not necessarily mutually exclusive (Haxholm *et al.* 2015). These lipid-interacting domains (LIDs) may function to focus on signal transduction or have another role in creating specificity or signal transduction pathway choice, and the elucidation of their function will certainly shed light on the cellular context of PRLR function. The lipid content of the membrane may even influence signaling pathway choice downstream of the PRLR. It is not yet known if association of the receptor with the membrane is constitutive or regulated or if
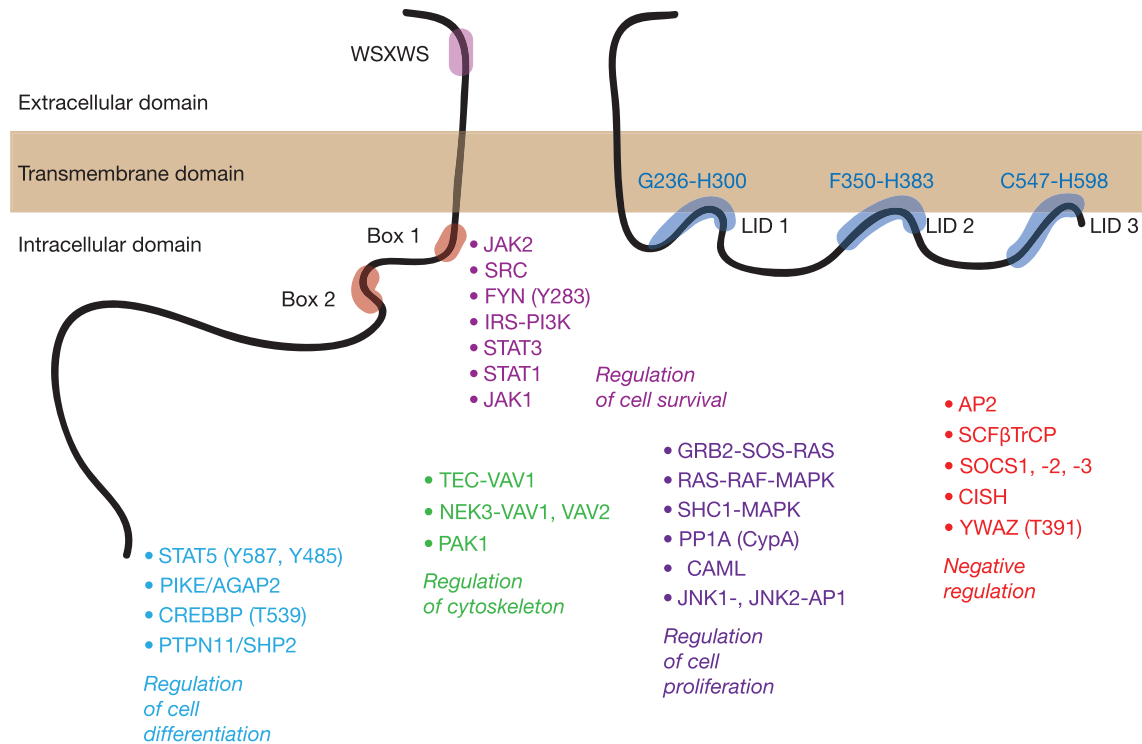
Figure 2

The PRLR consists of three main domains, the extracellular domain, which contains a WS motif that acts as a molecular switch for activation, a transmembrane domain and a disordered intracellular domain. Shown are two different views and two possible orientations of identical receptor chains, showing Box-1, which interacts with JAK2 and SRC family kinases, Box-2 and three lipid-interacting domains (LID) (shown with the amino acids that the LIDs span). The interactions of the LIDs with the plasma membrane are not specific for the state of activation. A number of different proteins listed with some signaling pathway members help perform and regulate activities related to regulation of cell differentiation, the cytoskeleton, cell proliferation, cell survival or the overall function or activity of PRLR signaling (negative regulation). If known, the PRLR amino acid with which they interact is identified.

sub-domains of JAK2 may be involved in mediating the interaction with the receptor (Haxholm *et al*. 2015).

Multiple human PRLR isoforms add additional complexity to the biology, with up to 8 mRNA variants encoding transmembrane receptors, and 3 possible soluble isoforms (Tsai-Morris & Dufau 2012) created by alternative splicing or proteolytic cleavage. For the most part, the isoforms differ in length of their intracellular domain, creating differential signal transduction capacity along with only some unique intracellular domains. The major human mRNA variants are capable of encoding a transmembrane isoforms including the long form, short form (SF)1a, SF1b (Trott *et al*. 2003) and SF1c (Pujianto *et al*. 2010). The intermediate form of the receptor has some capacity for unique signaling within the intracellular domain (Kline *et al*. 1999), and the deltaS1 PRLR has reduced signaling capacity due to a shorter extracellular domain (Kline *et al*. 2002). The soluble forms include delta7/11 (and delta4/11 or delta7/11) (Trott *et al*. 2003). PRL transcript variants

and PRLR isoforms have been covered in a recent review (Bernard *et al*. 2015).

Given the potential interactive capacity of the different PRLR isoforms that influences signal transduction, one can hypothesize that the ratio in cellular expression of these variants could lead to altered signaling. A soluble form of the receptor is reported to potentially inhibit PRL signaling by binding to PRL (Trott *et al*. 2003). Additionally, some short forms, in particular SF1b, act as a dominant negative to the long form of the PRLR (Saunier *et al*. 2003, Tan *et al*. 2005, Tan & Walker 2010) and negatively influence STAT5 signaling (Qazi *et al*. 2006). A low ratio of short-to-long form of the PRLR was also associated with cancerous breast tissue rather than normal matching breast tissue, indicating that the reduction in dominant negative regulation may result in unrestrained PRLR signaling in cancerous breast tissue (Meng *et al*. 2004).

The presence of these different PRLR isoforms creates an additional layer of complexity with respect to signaling capacity, differential signaling and interactive

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responses leading to altered signaling. In general, it is difficult to ascribe the different published observations to any specific isoform or ratio of isoforms in most reports, unless there were specific mRNA variant analyses, and this is not generally performed with large tissue microarrays. Unfortunately, antibodies specific to individual isoforms are lacking.

PRLR signal transduction

Few of the potential tyrosine amino acid docking sites in the PRLR have been identified as a specific docking site for a specific signaling protein. Of note, the docking sites for STAT5 (Pezet *et al*. 1997*b*, Mayr *et al*. 1998, Rui and Nevalainen 2003), FYN (Haxholm *et al*. 2015), CREB-binding protein (CREBBP) (Ma *et al*. 2010) and 14-3-3 protein zeta/delta (YWAZ) (Olayioye *et al*. 2003) on the human PRLR, or equivalent site, have been identified (Fig. 2). The distal STAT5 docking site is absent in the majority of the isoforms except for the long form of the PRLR receptor. Therefore, multiple signaling pathways exist downstream and differ, in part, based on the PRLR isoform expressed in the cell. Signaling pathways include those triggered by receptor-bound kinases (Fig. 2) such as JAK2 (DaSilva *et al*. 1994), SRC, FYN, NIMA (never in mitosis gene a)-related kinase 3 (NEK3) (with VAV1, -2 and -3) (Miller *et al*. 2005, Miller *et al*. 2007) and tyrosine protein kinase (TEC) (with VAV1) (Kline *et al*. 2001). JAK2 and SRC family kinases FYN and LYN are constitutively associated with the PRLR (Clevenger *et al*. 1995, Lebrun *et al*. 1995, Pezet *et al*. 1997*a*, Fresno Vara *et al*. 2001). Activation of the PI3K pathway has been reported via JAK2 and insulin receptor substrate (IRS) recruitment (Yamauchi *et al*. 1998), as well as via SRC and focal adhesion kinase (FAK) (Acosta *et al*. 2003). Additional proteins that interact with the receptor to regulate cell survival include JAK1 (Gao *et al*. 1996, Neilson *et al*. 2007), STAT1 and STAT3 (DaSilva *et al*. 1996).

These and other interacting proteins have been recently summarized according to protein–protein interactions in NetSlim (Radhakrishnan *et al*. 2012) and in BioGRID (Stark *et al*. 2006). These include proteins that function in cellular differentiation (Fig. 2), such as STAT5 (Wakao *et al*. 1994), Arf GTPase-activating protein (AGAP-2)/PIKE-A (Chan *et al*. 2010) and CREBBP, which acetylates the receptor and promotes receptor dimerization and STAT5 activity (Ma *et al*. 2010). Src Homology Phosphatase-2 (SHP2)/PTPN11 protein tyrosine phosphatase, non-receptor type 11 (Ali & Ali 2000), promotes STAT5 activation by promoting JAK2 stability

(Ali *et al*. 2003). Proteins that function in the regulation of the cytoskeleton include TEC-VAV1 and NEK3-VAV-1 and -VAV-2. The p21-activated kinase-1 (PAK1) is an important central modulator that also regulates the cytoskeleton (Fig. 2) (for review see Hammer & Diakonova 2015).

Regulation of cell proliferation is controlled by pathways involving calcium-modulating cyclophilin ligand (CAML) (Lim *et al*. 2011), PP1A/cyclophilin peptidylprolyl isomerase-A (cyclophilin A) (Syed *et al*. 2003), Src Homology-2 Domain-Containing protein (SHC) (Erwin *et al*. 1995, Das & Vonderhaar 1996), SHC-Transforming Protein C1 (SHC1), growth factor receptor-bound protein 2 (GRB2)-Son of Sevenless (SOS)-rat sarcoma (RAS) (Erwin *et al*. 1995, Das & Vonderhaar 1996), cjun N-terminal kinase-1 (JNK1) and JNK2 (Olazabal *et al*. 2000, Schwertfeger *et al*. 2000) (Fig. 2).

PRLR signaling is negatively regulated by a number of proteins and pathways (Fig. 2), such as 14-3-3 protein zeta/delta (YWAZ) (Olayioye *et al*. 2003), cytokine-inducible SH2-containing protein (CISH) (Endo *et al*. 2003), suppressors of cytokine signaling (SOCS2) (Pezet *et al*. 1999), SOCS3 (Dif *et al*. 2001), Skp, Cullin, F-box-containing complex (SCF)βTrCP/F-box and WD repeat domain-containing 11 FBXW2 (Li *et al*. 2004), beta-transducin repeat containing E3 ubiquitin protein ligase (BTRC) (Li *et al*. 2006) and general transcription factor IIIA (GTF3A)/AP2 (Varghese *et al*. 2008), which controls lysosome targeting post ubiquitination. Degradation of the PRLR is regulated by the phosphorylation of serine-349 (Li *et al*. 2004) by glycogen synthase kinase-beta (Plotnikov *et al*. 2008), and polyubiquitination by SCFβTrCP (Plotnikov *et al*. 2008, Varghese *et al*. 2008). Control over the PRLR protein levels can therefore vary from mRNA levels under different cellular circumstances. Additionally, the natural regulation of PRL induction of PRLR degradation appears to be impaired in breast cancer cells (Li *et al*. 2006).

The PRLR has crosstalk interactions with several different receptors that can influence signal transduction. The PRLR crosstalks with integrin via the signal regulatory protein alpha (SIRPa) transmembrane glycoprotein and SHP2 (Galbaugh *et al*. 2010). PRL and estrogen synergistically regulate gene expression (Rasmussen *et al*. 2010) and constitutive active PRLR variant can inhibit estrogen-induced cellular proliferation (Huang *et al*. 2015). There are also reports of PRL activating the unliganded estrogen receptor (Arendt & Schuler 2008*a*, Gonzalez *et al*. 2009, O'Leary *et al*. 2013). PRL and estrogen induce extracellular regulated kinase (ERK)-1, -2

phosphorylation and activate the transcription factor, activator protein-1 (AP-1). Crosstalk of the PRLR with the ER has several important implications with respect to breast cancer progression, chemotherapeutic resistance and in particular bone metastases. The PRLR was also shown, via JAK2, to activate HER2 signaling (Yamauchi *et al.* 2000), as well as estrogen receptor signaling (Arendt & Schuler 2008*b*).

It is clear that PRLR signaling must be specific and that cellular context and the microenvironment may very well contribute to pathway choice and function. Cellular context likely plays a critical role in the selection or predominance of particular pathways, which can include cell type, PRLR isoform expression, crosstalk with other pathways and the specific contents of the microenvironment.

Cellular context and the tumor microenvironment

The stiffness of collagen matrices has been identified as a key factor in PRL-PRLR-pathway choices (Barcus *et al.* 2013) and crosstalk with estrogen (Barcus *et al.* 2015). Using *in vitro* collagen matrices, it was observed that compliant collagen matrix promoted the activation of PRL-STAT5 and cell differentiation, whereas a stiff collagen matrix activated the PRL-SRC-FAK-MAPK signaling and invasive behavior (Barcus *et al.* 2013, 2016). These observations go a long way to explain why in some cases PRL or PRLR is associated with a good or alternatively poor prognosis. The precise timing of JAK2-STAT5 signaling with respect to early cellular transformation is also likely important, and discussed in the following sections.

A stiff collagen-I matrix modulates the PRL response to involve an increase in the crosstalk of PRL with estrogen (Barcus *et al.* 2015). High-density collagen-I gels increased the invasive properties of the breast cancer cells in response to estrogen and PRL. The stiff matrix also reduced the sensitivity of the cells to 4-hydroxy-tamoxifen in the presence of both estrogen and PRL. SRC family kinases were implicated in the proliferative responses of the cell lines in stiff matrix. Together, these results show that the microenvironmental conditions, such as matrix stiffness, affect gene regulation and signaling pathway choice that encourages cancer progression, such as proliferation, invasion and drug resistance, rather than encouraging differentiation.

Despite the importance of the tumor micro-environment in PRLR signal pathway choice and crosstalk,

the stiffness of the tumor microenvironment is not often investigated when assessing patient samples and PRL signaling. Mammographic density (Li *et al.* 2005), due to increased fibrillar collagen, would be an interesting clinical feature to additionally analyze when assessing prognosis associated with PRL serum levels or *PRLR* expression or protein levels. Circulating PRL levels have been linked to mammographic density in postmenopausal women in some studies (Boyd *et al.* 2002, Greendale *et al.* 2007, Rice *et al.* 2015), but not all (Bremnes *et al.* 2007, Maskarinec *et al.* 2007), as well as with an inverse correlation to low-density breast tissue (McCormack *et al.* 2009, Rice *et al.* 2015). It would be intriguing to assess this association with metastasis and survival outcomes.

PRL-JAK2-STAT5 signaling: protective or oncogenic or both?

There is one major PRLR signal transduction pathway that has received a great deal of attention and has demonstrated to be intricate in its contribution to cancer, with apparent conflicting results presented, until it was understood well. The evidence indicates that the PRL-PRLR-JAK2-STAT5 pathway is important in tumor initiation but not in later progression.

A mouse model of PRL-mediated tumorigenesis showed a role for JAK2 in PRL-induced mammary cancer (Sakamoto *et al.* 2010) in a timing-dependent manner. Using a mouse model that constitutively expresses rat *Prl* under the neu-related lipocalin promoter (Rose-Hellekant *et al.* 2003) crossed to a mouse mammary tumor virus (MMTV)-Cre-recombinase model that conditionally ablates *Jak2* in the mammary gland before or after tumor initiation, demonstrated a contributory role for JAK2 in tumor initiation but not post-transformation. This work illustrates a key molecular timing of PRL-JAK2-STAT5 in tumor initiation and puts other data exploring this pathway in post-transformation breast cancer cells into context with regard to proliferation. It also illustrated the idea that erythroblastic leukemia viral oncogene (ERBB) family member crosstalk with PRLR signaling may supersede JAK2 functionality.

In support of that early role in transformation, PRL-STAT5 has been reported to interfere with the tumor-suppressive function of breast cancer-1 (BRCA1). PRL and the selective PRLR modulator, S179DPRL, (from both the long and short (SF1b) PRLR isoforms), were able to induce BRCA1 levels, although only PRL was able to interfere with BRCA1-mediated transcriptional

activation of the cyclin-dependent kinase inhibitor, p21, via its interaction with STAT5 (Chen & Walker 2016). Therefore, PRL-STAT5 signaling interferes with BRCA1 tumor-suppressive function of cell cycle inhibition, while allowing proliferation in the presence of PRL.

In vitro studies have indicated that the long form of the PRLR and JAK2 signaling contributed toward an epithelial phenotype, rather than a mesenchymal phenotype (Nouhi *et al.* 2006). This was shown in T47D cells, which are a luminal subtype (Neve *et al.* 2006), possibly the more aggressive luminal B (Prat *et al.* 2013). Overexpression of the *PRLR* resulted in a reversal of the mesenchymal phenotype in the MDA-MB-231 cells, shown to be similar to the basal-B subtype (Neve *et al.* 2006). These *in vitro* results obtained from 2D cell culture are in agreement with the work by Barcus and coworkers, who demonstrated that breast cancer cells in compliant (vs stiff) collagen matrices activated JAK2-Stat5 signaling rather than MAPK signaling. The microenvironment would explain the *in vivo* results recently reported, which show that the PRLR long form is important in metastasis (Yonezawa *et al.* 2015).

In established T47D xenograft tumors in nude mice, PRL-STAT5 activation resulted in increased amounts of E-cadherin (Sultan *et al.* 2005). These results are consistent with a protective role for STAT5 in cancerous progression, although the effects on early tumor formation or metastasis were not examined. PRL-STAT5 activation was also associated with reduced invasive characteristics *in vitro* in T47D cells (Sultan *et al.* 2005).

STAT5 activation, which is also downstream of other cytokine receptors, appears to be associated with a better prognosis in some human studies, where STAT5 activation decreases with cancerous progression and lymph node positivity, and a favorable marker in lymph node-negative disease (Nevalainen *et al.* 2004, Peck *et al.* 2011). This could be consistent with the idea that the JAK2-STAT5 pathway favors early cellular transformation, a stage that is generally not captured in tissue microarrays and is later associated with a more differentiated cancer cell phenotype.

The PRLR as a therapeutic target

Small clinical trials combining antiprolactinemia drugs (bromocriptine or cabergoline), in combination with other chemotherapies (taxotere or triptorelin and tamoxifen) were promising for the treatment of metastatic breast cancer (Lissoni *et al.* 2002, Lissoni *et al.* 2003, Frontini *et al.* 2004). PRLR antagonists are also in

development for PRL-related disorders and have been reviewed (Goffin *et al.* 2005).

A recent informative phase 1 clinical trial for the PRLR antagonist, LFA102, a humanized monoclonal antibody, showed good toleration and apparent efficacy of target, as measured by a surrogate marker, but did not show any anti-primary-tumor activity as a monotherapy in the study of 73 patients with metastatic breast cancer or castrate-resistant prostate cancer (Agarwal *et al.* 2016). This could be for several reasons within the clinical trial, including a lack of prior selection of the 73 patients for their PRLR expression and protein levels, lengthy dosing intervals with respect to half-life, resulting in reduced drug exposure. A maximum-tolerated dose was not determined due to a lack of dose-limiting toxicities. It was not reported if the tumors were assessed for any compensatory pathways or other compensatory molecular mechanism. There was a large (6-fold) increase in serum prolactin upon PRLR inhibition, apparently similar to what has been reported in mouse models (above). This increase in serum prolactin was used as a surrogate marker for PRLR inhibition, although it was not possible to know if the inhibition was complete with the dose provided. It is possible that better results would be seen if PRLR isoform identification and tumor molecular subtype were taken into consideration (O'Sullivan & Bates 2016). The utility of LFA102 for hyperprolactinemia, or in combination therapy, in conjunction with HER2 or ER treatments, is still promising for breast cancer.

Impact

So how does one interpret the potentially conflicting reports of PRLR as a potential biomarker for metastases? Is PRL signaling in breast cancer protective or detrimental? One must take into account the myriad of factors that influence PRL signaling, particularly *in vivo*. We are just starting to understand the impact of these factors and the role that the PRL-PRLR pathways have on the spectrum of cellular processes that range from a protective nature to accelerating the vicious cycle of bone metastasis.

Evidence shows that high PRL levels are associated with invasive and lymph node-positive disease, but not disease recurrence, in postmenopausal women. Other reports also show that high *PRLR* expression is associated with better relapse-free survival and better distant metastasis-free survival. This raises the question of whether the PRLR has a role in metastasis that occurs early in the disease, as opposed to one that lies dormant for a period. Certainly, once metastasis to the bone occurs, the likelihood of a

lytic bone lesion is increased, due to the PRLR in breast cancer cells in the secondary bone site.

Unraveling these factors and pathways in the near future will help define both the protective aspects and the therapeutic targets. It is critical that the network of signaling be well defined to better deliver treatment for PRL-responsive cancers. In some ways, it is not really a question of *if* PRL could be a biomarker for metastatic risk, but *when* and *how*.

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