Impaired Turnover of Prolactin Receptor Contributes to Transformation of Human Breast Cells

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Abstract

Signaling by polypeptide hormone prolactin (PRL) is mediated by its cognate receptor (PRLr). PRLr is commonly stabilized in human breast cancer due to decreased phosphorylation of residue Ser349, which when phosphorylated recruits the βTrcp E3 ubiquitin ligase and facilitates PRLr degradation. Here, we show that an impaired PRLr turnover results in an augmented PRL signaling and PRL-induced transcription. Human mammary epithelial cells harboring degradation-resistant PRLr display accelerated proliferation and increased invasive growth. Conversely, a decrease in PRLr levels achieved by either pharmacologic or genetic means in human breast cancer cells dramatically reduced transformation and tumorigenic properties of these cells. Consequences of alteration of PRLr turnover for homeostasis of mammary cells and development of breast cancers, as well as the utility of therapies that target PRLr function in these malignancies, are discussed.

Introduction

Malignant transformation of cells and development of tumors result from several key events that include stimulation of cell proliferation and inhibition of cell death (1). The pituitary hormone prolactin (PRL), which is also secreted by mammary epithelia, plays a central role in mammary gland development and function. In addition, several lines of evidence strongly implicate the role of PRL in breast tumorigenesis (reviewed in ref. 2). First, PRL promotes growth of human breast cancer cells acting as a survival agent and as a mitogen (3, 4), and up to 95% of primary human breast cancers are positive for PRL and its receptors (PRLr; refs. 5–7). Second, transgenic mice locally expressing PRL within mammary epithelia develop tumors (8, 9), whereas genetic ablation of PRLr severely delays the development of SV40 large T antigen–induced breast carcinomas (10). Third, mutant PRLrs that are characterized by high levels of constitutive signaling have been recently identified in human breast tumors (11, 12). Finally, epidemiologic studies link elevated levels of circulating PRL with increased risk of breast cancer (13, 14) and its metastases (15), as well as with decreased taxane therapeutic efficacy (16–18) that could be reversed by pharmacologic suppression of PRL levels (17).

PRL acts via cell surface receptors that exist as long/ΔS1 isoforms (hereafter called PRLr) and several shorter alternatively spliced variants that often exert dominant-negative effects on signaling via the PRLr. PRL activates the PRLr-associated Janus-activated kinase 2 (Jak2) tyrosine kinase (19) and a series of downstream signaling pathways, including signal transducers and activators of transcription (Stat), extracellular signal-regulated kinase 1/2 (Erk1/2), and phosphatidylinositol 3-kinase (PI3K)–Akt. Because a high proportion of human breast cancer cells secrete their own PRL, the autocrine effects of PRL may account for the limited success of inhibitors of pituitary PRL synthesis/release against human breast cancers (reviewed in ref. 2). Antagonists of PRLr kill human breast cancer cells in vitro and abrogate the tumorigenesis in the xenograft models, showing that persistent signaling induced by locally secreted PRL is essential for growth and survival of these cells (20, 21).

However, PRL also induces proteolytic degradation of PRLr via receptor ubiquitination facilitated by the SCFβTrcp E3 ubiquitin ligase that is recruited to the substrate in a manner that requires phosphorylation of Ser349 within the phosphorylated degron (22, 23). Given that this ligand-induced PRLr down-regulation limits the extent of PRL signaling (2), it is not clear how PRL maintains the survival of breast cancer cells. Whereas levels of PRLr are decreased in the breast cancer intratumoral stromal compartment, the levels of PRLr in tumor cells are not decreased in comparison with benign mammary cells (5, 24), suggesting a possibility that down-regulation and degradation of PRLr in tumor cells might be impaired. Indeed, we have reported that phosphorylation of PRLr on Ser349 within its phosphorylated degron is impaired in breast cancer cells and tissues that exhibit increased stability of PRLr and ensuing high levels of its expression (25).

Our previous reports outlined a mechanism by which PRLr might get stabilized and accumulated in breast cancers. We found that glycogen synthase kinase 3β (GSK3β) mediates the recruitment of βTrcp and receptor ubiquitination and degradation through phosphorylation of PRLr on Ser349. Constitutive oncogenic signaling downstream of the Ras pathway inactivates GSK3β through phosphorylation of GSK3β on Ser9. Inhibition of GSK3β activity prevents phosphorylation of PRLr on Ser349 and PRLr ubiquitination, ultimately leading to PRLr stabilization (26).

Here, we sought to investigate the outcomes of PRLr stabilization in breast cancer. Our studies reveal that abrogation of PRLr phosphorylation on Ser349 in near-normal human mammary epithelial cells contributes to the development of a transformed phenotype. Furthermore, decreasing the levels of PRLr in human breast cancer cells is detrimental for their growth, invasion, and tumorigenicity. Collectively, these findings suggest that an altered degradation (and resulting accumulation) of PRLr might play a role in human breast cancers and could be targeted for anticancer therapies.
Materials and Methods

Cell lines, DNA constructs, and gene delivery. The MCF10AΔp53 derivative cell line, in which p53 expression is knocked down (MCF10A), was a generous gift from Alan Eastman (Dartmouth University, Lebanon, NH; ref. 27). Generation of the MCF10AΔp53 cells stably expressing wild-type (WT) or S349A mutant PRLr was previously described (28). Human breast cancer MCF7 and T47D cells (gift from Ze'ev Ronai, Burnham Institute, San Diego, CA) were cultured as previously described (29). Negative control short hairpin RNA (shRNA; Sigma) is a lentiviral plKO.1-puro vector containing an irrelevant shRNA insert. shPRLr subcloned within the same vector was purchased (Open Biosystems) and used for transduction of T47D cells followed by selection in puromycin (2 μg/mL). CISH promoter–driven firefly luciferase reporter (30) was kindly provided by C.V. Clevenger (Northwestern University, Chicago, IL). Renilla luciferase expression vector was purchased from Promega.

Chemicals and antibodies. Human recombinant PRL was kindly provided for a fee by Dr. A.F. Parlow (National Hormone and Peptide Program, Bethesda, MD). Antibody against pSer349-PRLr was previously described (21). Antibodies against Flag tag (M2; Sigma), PRLr (H300; Santa Cruz Biotechnology), actin (Affinity BioReagents), phosphorylated Erk, Erk, phosphorylated Stat5, Stat5 (Cell Signaling Technology, Inc.), and cyclin D1 (AB-3; Calbiochem, Inc.) were purchased. Secondary antibodies conjugated with horseradish peroxidase (Chemicon) were purchased. Methylamine hydrochloride, cycloheximide, and anandamide, as well as other chemicals, were purchased from Sigma. Immunoprecipitations and immunoblotting analyses were carried out as previously described (29). Immunohistochemical analysis on tumors was carried out using the anti-PRLr antibody (H300) as previously described (25).

Analysis of cell growth, invasion, and tumorigenesis. Growth in a two-dimensional culture was analyzed using the staining with trypan blue. The number of live cells in each well was counted. Results from three independent experiments are presented as average ± SE. For the analysis of cell growth in a three-dimensional culture, cells were mixed with Matrigel Basement Membrane Matrix (BD Biosciences) and cultured in complete medium for indicated number of days. Growth of cells in soft agar was carried out as previously described (31).

Invasion assays were done in Boyden chambers supplied with polyethylene terephthalate filter inserts containing 0.8-μm pores (BD Company). Filters were coated on ice with 50 μL of Matrigel Basement Membrane Matrix and incubated for 30 min (37°C). Either MCF10AΔp53-derived or T47D-derived cells (5 × 10⁴) were plated in 300 μL of Matrigel (diluted in 0.1% bovine serum albumin–DMEM/F-12, 1:3) into the upper chamber. The lower chamber was filled with 700 μL of DMEM/F-12 medium supplied with 10% fetal bovine serum. Noninvaded cells in the inserts were removed with cotton swabs after either 48 h (for MCF10AΔp53) or 72 h (for T47D) of incubation. Invaded cells on the underside were fixed with absolute methanol for 2 min, stained with H&E solution (Sigma), and photographed using either 5× or 10× objectives.

For zymography analysis, cells were seeded as described in “invasion assay.” The medium was collected at 24 h after seeding, centrifuged, and concentrated using Amicon Ultra (Millipore; cutoff at 10 kDa), and 10 μg of the proteins were resolved by gelatin-contained Novex 10% Zymogram (gelatin) gel (Invitrogen) and analyzed in accordance to the manufacturer's protocol. Tumorigenesis assays were carried out in NCRNU-M (Taconic) or in NSG mouse model (NOD-SCID, IL2Rγnull; The Jackson Laboratory) female mice, which also obtained pellets of 17β-estradiol and PRL (purchased from Innovative Research of America). Cells were implanted s.c. or into abdominal mammary glands, and the growth of tumors was measured by caliper at indicated days after cell injection.

Signal quantification and statistical analysis. Digital images were processed with Adobe Photoshop 7.0 software. For some experiments, band intensities and percentage of surface covered by cell growth were quantified by densitometry (Image; software). The statistical differences were analyzed using two-tailed Student’s t test.

Additional details are provided in Supplementary Data.

Results

Stabilization of PRLr augments the extent of PRL signaling in human mammary epithelial cells. Stabilization of PRLr in human breast cancers occurs via oncogene-mediated inhibition of PRLr phosphorylation on Ser349 by GSK3β (26). We sought to investigate the consequences of impaired PRLr phosphorylation on Ser349 in human mammary epithelial cells by expressing PRLrS349A mutant that cannot be phosphorylated by GSK3β (26). This approach (rather than modulating overall GSK3β activity, which affects cell functions via numerous diverse mechanisms) reviewed...
in ref. 32) was implemented in MCF10AΔp53 cells (27), which express low levels of endogenous PRLr (Fig. 1A). Stable cell lines transduced with PRLrS349A mutant (S349A) exhibited higher levels of PRLr expression compared with those that received WT PRLr or empty vector (puro; Fig. 1A). This result is consistent with inefficient degradation of PRLr proteins whose phosphorylation within the phosphodegron is impaired (22, 25, 26). Abrogated phosphorylation of PRLr on Ser349 is expected to impair receptor ubiquitination leading to impaired PRLr endocytosis and, therefore, increased stability (23, 28, 33). Indeed, turnover of PRLr was noticeably impaired in S349A cells (Supplementary Fig. S1).

It is plausible that stabilization and accumulation of PRLr are expected to augment the magnitude and duration of PRL signaling (22, 23). Indeed, expression of PRLrS349A led to a robust increase in magnitude and duration of signaling events, including activation of Stat5 and Erk triggered by a pulse treatment with PRL when compared with cells expressing WT PRLr (Fig. 1B). Furthermore, the S349A cells displayed the highest levels of cyclin D1 (a key regulator of cell cycle progression and induced by PRL; ref. 34) among all cell lines tested. Accordingly, these cells exhibited much higher levels of PRL-induced transcripational activity, as evident from the analysis of the CISH promoter–driven luciferase reporter (Fig. 1D). Together, these results indicate that abrogation of PRLr phosphorylation on Ser349 augments the cellular responses of mammary epithelial cells to PRL.

Stabilized PRLr contributes to transformation of human mammary epithelial cells. We have noticed that MCF10AΔp53 derivatives that express stabilized PRLr grow faster in tissue culture (Fig. 2A). Furthermore, analysis of cell growth in three-dimensional cultures in Matrigel revealed significant differences in both the rate of growth and morphology between all examined cell types. Whereas vector-transduced puro cells grew slowly and formed well-defined spherical aggregates, WT cells formed numerous smaller spheroids. Remarkably, cells expressing mutant PRLrS349A rapidly deviated from spherical growth to a pattern of irregular and poorly defined masses, forming a network of branches and meshes and, eventually, filling the entire culture space (Fig. 2B). Three other independent S349A individual clones displayed similarly fast tumor-like growth and morphology (Supplementary Fig. S2), indicating that differences in cell growth were not clone specific but mediated by the PRLrS349A mutant. A greater transformed phenotype of cells expressing stabilized receptor was further tested in another transformation assay, such as growth in semisolid medium. Cells expressing PRLr, but not parental MCF10AΔp53 cells, formed colonies in soft agar. Furthermore, consistent with the results obtained in a two-dimensional culture or in Matrigel, S349A...
clones formed larger colonies and showed a statistically significant increase in colony number when compared with cells expressing PRLr^WT (Fig. 2C). In summary, these data indicate that increased stability of PRLr contributes to a transformed phenotype in human mammary epithelial cells.

Aggressive and irregular growth of S349A cells in Matrigel and their ability to form colony in soft agar point to changes in their ability to grow invasively. Indeed, in vitro invasion assays revealed a superior ability of S349A cells (compared with puro or WT cells) to penetrate through Matrigel and insert pores in Boyden chamber assays (Fig. 3A). Cell motility and invasiveness is a complex process positively regulated among others by pathways that involve mitogen-activated protein kinase (MAPK), P38K, and Rho family GTPases, all of which are activated by PRL (reviewed in refs. 2, 35, 36). One of the consequences of PRL signaling may be an increased expression of matrix metalloproteinase (MMP)-2 and MMP-9, which are the critical enzymes for cell invasiveness (37). Zymography analysis of levels of MMP-2/MMP-9 expression in a MCF10AΔp53-derived cell revealed that S349A cells expressed a significantly higher level of MMP-9 compared with cells harboring WT PRLr (Fig. 3B). Expression of MMP-2 followed a similar pattern (data not shown). Together, these data suggest that stabilization and increased levels of PRLr in breast cells contribute to a transformed in vitro phenotype, which is reflected by accelerated cell growth and increased motility and/or invasive abilities.

We next compared the tumorigenic growth of various MCF10AΔp53 derivatives injected into the flanks of the NCRNU-M immunocompromised mice that were implanted with pellets releasing estradiol and PRL. MCF7 breast cancer cells (positive control) grew rapidly and continuously, and the mice that were injected with these cells developed large tumors and had to be sacrificed by day 24. Although MCF10AΔp53 derivatives displayed a period of growth and formed distinct tumors (Supplementary Fig. S3), this growth was relatively short and was followed by tumor regression within 4 weeks after injection. Intriguingly, tumor regression proceeded significantly slower in S349A cells compared with either WT or puro cells (P < 0.05; Fig. 3C). Similar results were obtained when NSG immunodeficient mice were used as hosts on either intraflank or intramammary gland injection of human cells (data not shown). These data suggest that stabilization of PRLr promotes growth of MCF10AΔp53 cells in vivo but is not sufficient for maintaining the tumorigenic phenotype.

Down-regulation of PRLr is detrimental for growth and tumorigenicity of human breast cancer cells. Our observations in nontumorigenic human mammary epithelial cells show that stabilization and accumulation of PRLr augments PRL signaling and contributes to the transformed phenotype, suggesting that high levels of PRLr might be important for the development of breast cancers. To corroborate this conclusion, we undertook a diagnostically opposite approach by seeking to investigate whether down-regulation of PRLr in human breast cancer cells that otherwise display a stabilized PRLr would affect their transformed properties.

We initially used pharmacologic approaches based on the published observation that endogenous cannabinoid anandamide decreased the levels of PRLr and slowed down growth of MCF7 human breast cancer cells (38–41). Similarly, treatment with anandamide inhibited growth of T47D breast cancer cells (Supplementary Fig. S4) and also led to a rapid down-regulation of PRLr (Fig. 4A) in these cells known to contain hypophosphorylated PRLrWT (Fig. 2A). In all other cell lines, anandamide inhibited growth (data not shown). Expression of MMP-2 followed a similar pattern (data not shown). Together, these data suggest that anandamide stimulated PRLr degradation upon blocking protein synthesis by cycloheximide (Supplementary Fig. S5). Intriguingly, anandamide inhibited growth of T47D breast cancer cells (Supplementary Fig. S4) and also led to a rapid down-regulation of PRLr (Fig. 4A) in these cells known to contain hypophosphorylated and stabilized PRLr (25, 26). Intriguingly, an increase in the level of S349 phosphorylation was observed in T47D cells upon treatment with anandamide, along with a lysosomal inhibitor, to prevent the degradation of phosphorylated PRLr species. These data are consistent with the hypothesis that anandamide affects growth of breast cancer cells via accelerating the phosphorylation-dependent degradation of PRLr.

To test this hypothesis, we investigated the effect of anandamide on MCF10AΔp53-derived cells expressing PRLr. Treatment of WT cells with anandamide led to an increase in Ser349 phosphorylation of PRLr when its degradation was blocked by lysosomal inhibitor (Supplementary Fig. S5). Accordingly, anandamide stimulated PRLr degradation upon blocking protein synthesis by cycloheximide treatment (Supplementary Fig. S6). Intriguingly, anandamide

![Figure 3](image-url)
dramatically down-regulated PRLr in WT cells but did not affect PRLr levels in S349A cells (Fig. 4C; Supplementary Fig. S7). Furthermore, an inhibitory effect of anandamide on cell growth was much more pronounced in WT cells than in S349A cells (Fig. 4D). This result indicates that down-regulation of PRLr in response to anandamide is mediated by the Ser349 phosphorylation–dependent degradation of PRLr and that impaired degradation of PRLr contributes to the resistance of mammary cells to cannabinoid-induced growth inhibition.

We next used a genetic approach to independently verify a potential link between the levels of PRLr expression with the growth of human breast cancer cells. To this end, we investigated an effect of knocking down the PRLr levels by RNA interference on the extent of PRL signaling and transformed phenotype. Human breast cancer T47D cells harboring shRNA against PRLr displayed noticeably decreased levels of PRLr (Fig. 5A). Knockdown of PRLr almost entirely prevented ligand-induced phosphorylation of Stat5 (Fig. 5B) and dramatically attenuated an activation of the
PRL-responsive CISH promoter (Fig. 5C). Accordingly, PRL-induced induction of cyclin D1 was noticeably impaired in T47D cells that harbor shRNA against PRLr (shPRLr; Fig. 5D).

We next determined how down-regulation of PRLr affects the transformed phenotype of T47D cells. We noticed that shPRLr-containing cells grew slower when cultured under normal conditions (Supplementary Fig. S8). Conversely, an ability to form colonies in three-dimensional culture was noticeably attenuated (Fig. 6A). Knockdown of PRLr dramatically impaired the ability of T47D cells to migrate through Matrigel-covered filters in Boyden chambers (Fig. 6B) and decreased the expression of MMP-9 (Fig. 6C), confirming the role of PRLr levels in promoting invasiveness and motility of human breast cells seen in Fig. 3A. Furthermore, cells harboring shPRLr formed visibly smaller tumors when injected either in the flank or into the mammary gland of immunocompromised mice (Supplementary Fig. S9). Cells transduced with shRNA constructs against PRLr formed tumors that displayed a pronounced knockdown of PRLr levels (Supplementary Fig. S10) and exhibited a statistically significant decrease in growth rate (Fig. 6D). These results indicate that maintenance of high levels of PRLr plays an important role in tumorigenicity of human breast cancer cells.

**Discussion**

Whereas numerous epidemiologic and experimental data support important roles of PRL signaling in human breast cancers, the mechanisms that lead to constitutive activation of PRLr signaling that occurs in primary human mammary tumors are poorly understood. Recent identification of gain-of-function mutations in PRLr in women with benign breast tumors (12) and the fact that PRLr levels are elevated in human breast carcinoma...
is required for angiogenesis in these tumors; under this scenario, in vitro studies should reveal additional genetic events that cooperate with the mechanisms, by which stabilized PRLrS349A mutant stimulates growth and activation of Src, MAPK, and PI3K-Akt rather than toward the modulatory signals, might skew elevated PRL signaling toward metastatic progression of human breast cancer was correlated with MMP-9. Given that the loss of Stat5 activation detected during suppress growth of human breast cancer cells. As shown here, an augmented PRL signaling increases the expression of cyclin D1 and MMP-9. Given that the loss of Stat5 activation detected during metastatic progression of human breast cancer was correlated with poor prognosis (42) and reduced differentiation (43, 44), it is plausible that PRLr stabilization, along with further alterations in modulatory signals, might skew elevated PRL signaling toward activation of Src, MAPK, and PI3K-Akt rather than toward the canonical Jak2-Stat5 pathway. Additional studies aimed at defining the mechanisms, by which stabilized PRLrs stimulate growth and invasiveness of human mammary epithelial cells, are under way. Whereas our current data clearly point to the importance of maintaining PRLr levels for breast cancer cell tumorigenicity, future studies should reveal additional genetic events that cooperate with stabilized PRLr during formation of tumors. Although stabilization of PRLr, along with knockdown of p53 tumor suppressor protein, temporarily allowed near-normal MCF10A to grow in nude mice, these genetic changes were clearly insufficient to sustain tumorigenesis (Fig. 3C). Given an aggressive phenotype of these cells in vitro, they seem to lack a systemic factor when implanted in mice. It is plausible that activation of other oncogenes (e.g., c-Myc) is required for angiogenesis in these tumors; under this scenario, stabilized PRLr is likely to promote survival of tumor cells deprived of nutrition and oxygen. On the other hand, the fact that expression of stabilized PRLr slowed down tumor regression may reflect prolonged PRL signaling, which might be insufficient in transplanted human cells given that mouse PRL poorly activates human PRLr (45). Generation of human PRL knock-in mice will enable testing of this possibility.

Data showing that decreasing levels of PRLr in breast cancer cells were detrimental for their tumorigenicity provide a justification for the development of the agents that promote PRLr degradation. Treatment of cells with endogenous cannabinoid anandamide stimulated down-regulation of PRLr via promoting phosphorylation of this receptor on Ser349 (Fig. 4; Supplementary Fig. S5).

Furthermore, although anandamide can affect human breast cancer cell growth by various pathways (e.g., by affecting cyclic AMP/protein kinase or MAPK pathways; ref. 39), the phosphorylation-dependent degradation of PRLr seemed to be important for the antiproliferative effects of anandamide. Intriguingly, anandamide did not stimulate activity of GSK3β,4 indicating the existence of another kinase pathway that promotes Ser349 phosphorylation and PRLr degradation. As opposed to constitutively active GSK3β, this alternate kinase is likely to be induced by PRL in a Jak2-dependent manner to mediate ligand-stimulated phosphorylation, ubiquitination, endocytosis, and degradation of PRLr (23, 28, 33). We speculate that anandamide may activate this yet-to-be-identified ligand-sensitive kinase to promote Ser349 phosphorylation and subsequent PRLr ubiquitination and degradation. Studies aimed at the identification of this putative kinase and the mechanisms of its activation might be of translational value; these studies are currently in progress. Although endogenous cannabinoids are too unstable and pleiotropic to be used as drugs, identification of other types of small molecules that stimulate PRLr phosphorylation and turnover in a GSK3-independent manner should benefit those patients whose malignancies depend on PRL signaling.

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Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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