

# Prolactin Does Not Require Insulin-Like Growth Factor Intermediates but Synergizes with Insulin-Like Growth Factor I in Human Breast Cancer Cells

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## Abstract

**Insulin-like growth factor (IGF)-II is a required intermediate for prolactin-induced up-regulation of cyclin D1 and proliferation in normal murine mammary epithelial cells *in vivo* and *in vitro*. However, we have recently shown that prolactin can rapidly induce cyclin D1 protein expression and subsequent proliferation in the MCF-7 human breast cancer cell line, suggesting that prolactin actions can be independent of IGFs in breast disease. Here, we investigate the relationship between these factors and show that prolactin up-regulated transcript levels of both IGF-I and IGF-II, but only after increases in cyclin D1 protein were observed. Moreover, prolactin increased cyclin D1 in the presence of the IGF-I receptor neutralizing antibody  $\alpha$ IR3. However, on cotreatment, IGF-I and prolactin elicited cooperative phosphorylation of extracellular signal-regulated kinases 1 and 2 and protein kinase B/AKT, but not signal transducer and activator of transcription 5. This interaction extended to increased activation of activating protein-1 enhancer elements, phosphorylation of glycogen synthase kinase 3 $\beta$ , induction of cyclin D1, and ultimately, increased cell number. It also increased invasive behavior, which correlated with elevated matrix metalloproteinase-2 transcript levels. Interestingly, prolactin augmented phosphorylation at Tyr<sup>1135</sup> and Tyr<sup>1136</sup> of IGF-I receptor on cotreatment with IGF-I, although prolactin alone had no effect. Together, these data indicate that strong cooperative cross talk between prolactin and IGF-I augments biological processes associated with neoplastic progression, with implications for therapeutic strategies. (Mol Cancer Res 2008;6(4):634–43)**

## Introduction

Prolactin is critical for numerous physiologic processes in the mammary gland, including ductal development, alveolar proliferation and differentiation during pregnancy, and expression of genes involved in milk production (1). Prolactin is produced by the anterior pituitary gland. However, it is also produced at numerous extrapituitary sites including mammary epithelium and adjacent adipose tissue, particularly in primates (2). The contribution of prolactin to mammary oncogenesis has become increasingly recognized (3, 4). Recent studies have correlated circulating prolactin levels and breast cancer incidence (5). Multiple studies have shown prolactin receptor expression in a majority of primary breast tumors, and at higher levels than in surrounding normal tissue (4, 6). Finally, prolactin expression targeted to mammary epithelial cells of transgenic mice causes mammary tumors and decreases the latency in combination with another oncogene, TGF- $\alpha$  (7, 8).

Prolactin exerts its biological effects via binding to prolactin receptor, a member of the type I cytokine superfamily of receptors. Ligand binding activates intracellular kinases such as Janus kinase 2 and c-src, creating docking sites to recruit adapter proteins, and resulting in activation of several downstream pathways including signal transducer and activator of transcription proteins (STAT), mitogen-activated protein kinases, and phosphatidylinositol 3-kinase (4). These pathways have been studied extensively and may serve as sites of cross talk with other oncogenic factors.

Recently, it has been reported by two independent groups that prolactin induces transcription of an insulin-like growth factor (IGF)-II intermediate, which is required for prolactin-induced mitogenesis in the developing mouse mammary gland *in vivo* as well as in murine mammary cell lines *in vitro* (9, 10). This locally produced IGF-II acts in an autocrine/paracrine fashion through the IGF-I receptor (IGF-IR) to increase expression of cyclin D1, as well as of other proteins involved in the proliferation and morphogenesis of the developing gland. In normal mammary cells, these processes require nearly 18 hours of prolactin treatment, in contrast to other studies in human breast cancer cells that report increases in cyclin D1 protein in response to prolactin in as little as 4 to 6 hours (11). This suggests that the role of prolactin in normal mammary development is directly tied to the IGF system, but this relationship may be disrupted as cells undergo neoplastic changes.

IGF-I and IGF-II control proliferation, survival, migration, and differentiation of numerous cell types (12, 13). The major function of the IGFs is to promote growth, and circulating IGF-I

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mediates many effects of growth hormone postnatally. However, IGFs are produced locally in a number of tissues, including the mammary stroma and epithelium (14). This local production plays a critical role in controlling cell cycle progression and apoptosis of mammary epithelial cells in the developing gland (15, 16).

The IGF system has been implicated in malignancies of the breast, prostate, lung, colon, and many others (13, 17). Moreover, IGF-IR can transform various cell types *in vitro* (18) and is overexpressed in human breast tumors compared with normal mammary tissue (19). IGF-IR is a  $\alpha_2\beta_2$  heterotetramer, a structure that is characteristic of the insulin family of receptors (12). Ligand binding induces intrinsic tyrosine kinase activity, recruitment of docking proteins, and activation of downstream pathways including extracellular signal-regulated kinase (ERK)-1/2 and protein kinase B/AKT.

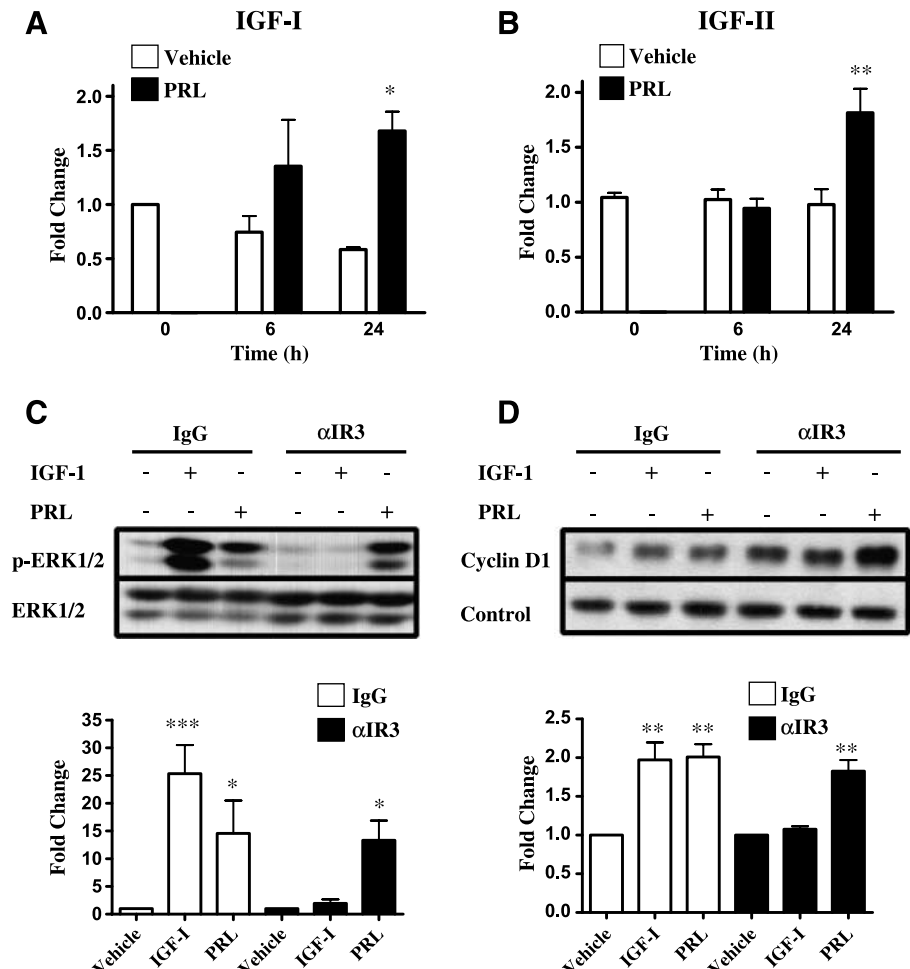
In this study, we have examined signaling interactions between prolactin and IGF-I and the resulting biological responses in MCF-7 breast cancer cells. We found that prolactin and IGF-I cooperate to activate ERK1/2 and AKT, increasing cyclin D1 expression and cell number, as well as promoting invasive behavior. In contrast to reports in normal murine mammary cells, we found no evidence for a required IGF

intermediate for prolactin actions in this model. These findings contribute to a growing body of evidence implicating prolactin in mammary tumorigenesis.

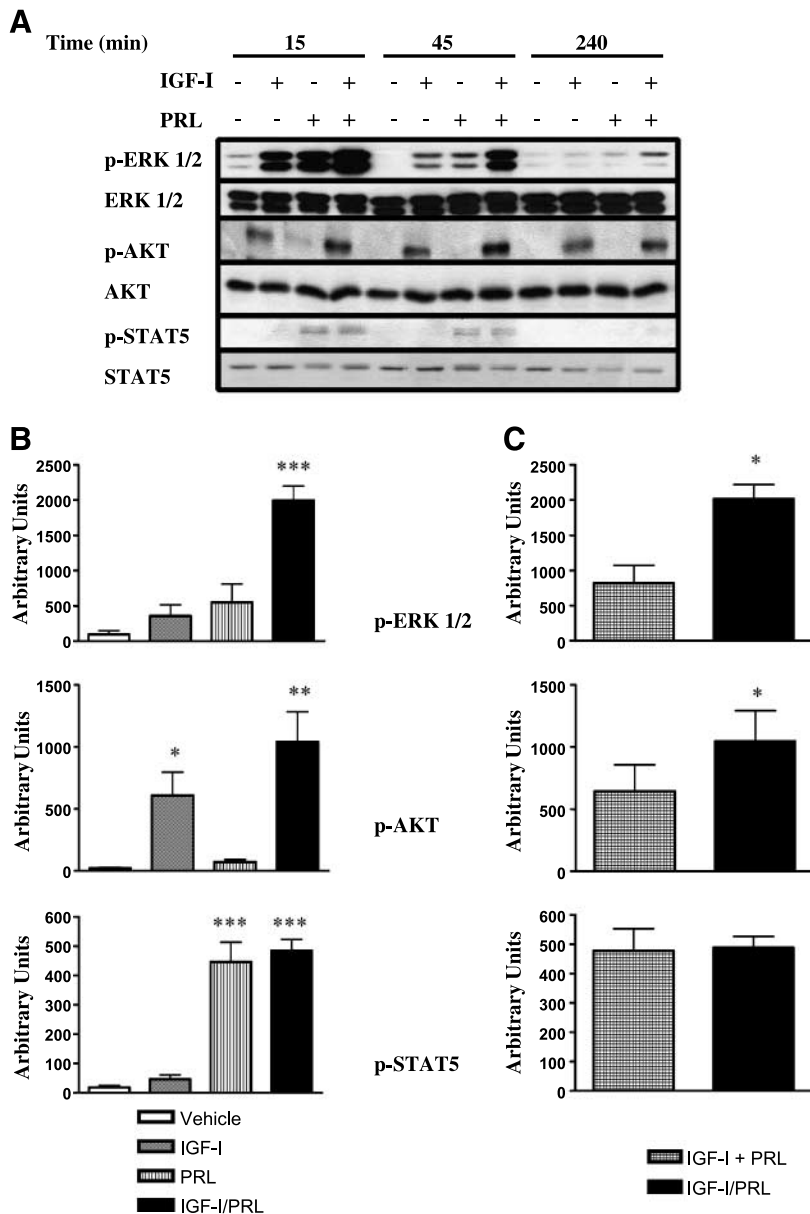
**Results**

*Prolactin Does Not Require an IGF Intermediate to Increase Levels of Cyclin D1*

In light of the apparent disparity between reports in normal mammary epithelial cells and breast cancer cells described above, we examined both IGF-I and IGF-II transcript levels to determine if they can be up-regulated in response to prolactin in MCF-7 cells, and whether any increase in transcript levels is temporally linked with increases in cyclin D1 protein. Prolactin did not induce significant changes in IGF-I or IGF-II transcript levels after 6 hours, but did induce a moderate increase (~2-fold) after 24 hours (Fig. 1A and B). IGF-I protein in the media, after 24-hour exposure to prolactin, was below the limit of detection via ELISA (0.3 ng/mL; data not shown). This suggests that the increase in cyclin D1 protein observed after 6 hours may not be due to an IGF intermediate but instead results from the direct actions of prolactin.



**FIGURE 1.** Prolactin increases IGF-I and IGF-II transcript levels, but IGF-IR binding is not required for induction of cyclin D1. **A** and **B.** Serum-starved MCF-7 cells were treated with vehicle or prolactin (PRL) and RNA was extracted at each time point indicated. Quantitative real time-PCR for IGF-I or IGF-II was done on samples from independent experiments as described in Materials and Methods. Columns, mean ( $n = 3$ ); bars, SE. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ , between vehicle and prolactin treatment at each time point (two-way ANOVA, Bonferroni post-test). **C** and **D.** Serum-starved MCF-7 cells were pretreated for 1 h with 100 ng/mL  $\alpha$ IR3 or mouse IgG before treatment with vehicle, IGF-I, or prolactin for 15 min (**C**) or 6 h (**D**). Immunoblots were done with the indicated antibodies (representative experiments shown). Signals from independent experiments were quantified by densitometry and results expressed as fold change relative to vehicle treatment. Columns, mean [ $n = 5$  (**C**) and  $n = 3$  (**D**)]; bars, SE. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ , compared with vehicle (one-way ANOVA, Neuman-Keuls post-test).



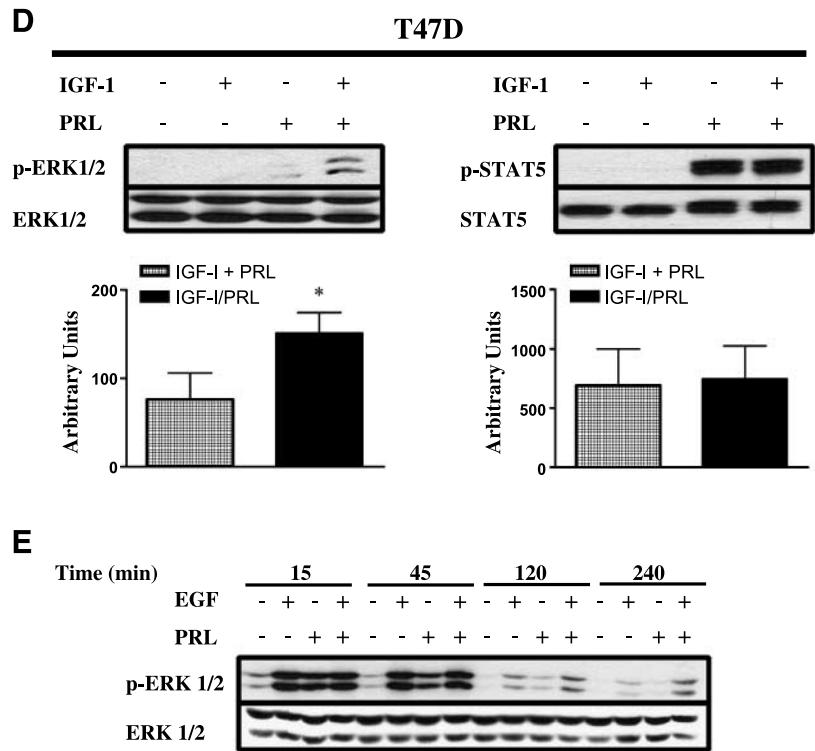
**FIGURE 2.** IGF-I and prolactin synergistically activate multiple signaling pathways. **A.** Serum-starved MCF-7 cells were treated with vehicle, IGF-I, prolactin, or IGF-I/prolactin for the indicated time periods. Immunoblots were done with the indicated antibodies (representative experiments shown). **B.** Signals from 45-min treatments of independent experiments were quantified by densitometry. Columns, mean ( $n = 4$ ); bars, SE. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ , compared with vehicle (one-way ANOVA, Neuman-Keuls post-test). **C.** Comparison of the sum of individual IGF-I and prolactin treatments to IGF-I/prolactin cotreatment. Signals from 45-min treatments were quantified as described in Materials and Methods. Columns, mean ( $n = 4$ ); bars, SE. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ , compared with the sum of IGF-I and prolactin single hormone treatments (paired  $t$  test).

To confirm that secreted IGF-I or IGF-II was not mediating the actions of prolactin, we used the neutralizing antibody  $\alpha$ IR3 (20) to block ligand binding to IGF-IR. We treated cells with IGF-I to ensure that the signals examined were transduced solely by IGF-IR, as IGF-II may bind both IR and IGF-IIR (12).  $\alpha$ IR3 inhibited acute phosphorylation of ERK1/2 in response to IGF-I, but had no such effect on prolactin action (Fig. 1C). Additionally, both IGF-I and prolactin elicited 2-fold increases in cyclin D1 protein after 6 hours.  $\alpha$ IR3 abolished this induction in response to IGF-I but had no effect on the prolactin-induced increases in cyclin D1 (Fig. 1D), although  $\alpha$ IR3 raised unstimulated levels, consistent with its weak agonistic properties (21). These data suggest that although prolactin can elevate IGF-I and IGF-II mRNA expression, these growth factors do not mediate the prolactin-induced increase in

cyclin D1 protein, which we have shown to subsequently lead to mitogenesis in these cells (11).

#### *IGF-I and Prolactin Synergistically Activate Multiple Signaling Pathways*

Although prolactin is not dependent on IGF intermediates, the possibility of cross talk between IGFs and prolactin in breast cancer remains. Both are available from the circulation as well as local production within the mammary gland. We therefore examined cross talk to shared downstream pathways. As shown in Fig. 2A, IGF-I and prolactin cotreatment (IGF-I/prolactin) cooperatively activated ERK1/2 and AKT, but not STAT5. This cooperation was most robust at 45 minutes. Quantification of signals at this time revealed that activation of ERK1/2 and AKT in response to IGF-I/prolactin was



**FIGURE 2 Continued.** **D.** Serum-starved T47D cells were treated with vehicle, IGF-I, prolactin, or IGF-I/prolactin for 45 min. Immunoblots were done with the indicated antibodies (representative experiment shown). Signals from independent experiments were quantified as described in Materials and Methods. Columns, mean ( $n = 3$ ); bars, SE. \*,  $P < 0.05$ , compared with the sum of IGF-I and prolactin single hormone treatments (paired  $t$  test). **E.** Serum-starved MCF-7 cells were treated with vehicle, EGF, prolactin, or EGF/prolactin for the time points indicated. Immunoblots were done with the indicated antibodies (representative experiment shown).

significantly greater than the predicted sum of signals for IGF-I and prolactin alone, although cooperation at ERK1/2 was greater than that observed for AKT (Fig. 2B and C). Because prolactin only weakly activates STAT5 in this cell line, we repeated this experiment in T47D cells, which have a more robust STAT5 response (22). We found that IGF-I and prolactin also synergistically activated ERK1/2, but not STAT5, in these cells (Fig. 2D), confirming our results in MCF-7 cells. Although the strength of interaction at ERK1/2 in T47D cells was slightly lower than that observed in MCF-7 cells (2.5- versus 2.0-fold), these data show that the greater-than-additive interactions are a feature of prolactin-IGF-I cross talk in another breast cancer cell line.

To determine if prolactin could potentiate signals from other type I receptor tyrosine kinases, we examined prolactin interactions with epidermal growth factor (EGF; Fig. 2E). Although EGF/prolactin also sustained ERK1/2 activation, apparent after 2 hours, the IGF-I/prolactin interaction evolved more rapidly. This difference in kinetics suggests distinct underlying mechanisms of interaction for these two growth factors with prolactin.

#### *IGF-I and Prolactin Cooperate at Key Regulators of Processes Important in Oncogenesis*

ERK1/2 and AKT are upstream of central regulators that coordinate proliferation and invasion and are implicated in progression of many cancers including breast cancer. To confirm that the sustained activation of these kinases resulted in altered downstream signaling events, we examined activation of activating protein 1 (AP-1) enhancer elements and phosphorylation of glycogen synthase kinase (GSK)-3 $\beta$ .

IGF-I/prolactin cotreatment increased expression of an AP-1-driven reporter gene 1.5-fold over predicted additive effects of these factors after both acute (6 hours) and long-term (24 hours) treatments (Fig. 3A and B; data not shown), consistent with upstream signaling cross talk. GSK-3 $\beta$  is a downstream target of both AKT and ERK1/2. Like signals to AP-1, IGF-I/prolactin cotreatment modestly elevated p-GSK-3 $\beta$  to levels significantly greater than the sum of the individual effects (Fig. 3C-E). The interaction observed at these signaling nodes is less robust than observed for ERK1/2 and AKT; however, the greater-than-additive effects even to these end points are highly reproducible and may contribute to biological processes associated with neoplastic progression, which we describe below.

#### *IGF-I and Prolactin Cooperatively Augment Processes Associated with Neoplastic Progression*

Levels of the cell cycle regulator cyclin D1 are determined by multiple signals including GSK-3 $\beta$  and AP-1 (23, 24). Elevated levels of cyclin D1 promote progression through the G<sub>1</sub>-S checkpoint, which is often deregulated in tumors (25). As shown in Fig. 4A and B, IGF-I/prolactin elevated protein levels nearly 2-fold over predicted additive effects. IGF-I/prolactin also increased phosphorylation of ERK1/2, AKT, and their downstream effectors (Figs. 2 and 3), which are associated with cancer cell proliferation and survival (24, 26). Therefore, we investigated the biological outcome of these signaling interactions. First, we measured the net effect of these hormones on cell number using the 3-(4,5-dimethyl-thiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay. IGF-I/prolactin cotreatment caused a 2-fold greater

absorbance value compared with the sum of individual IGF-I and prolactin treatments (Fig. 4C). We have shown that absorbance is directly proportional to MCF-7 cell number (data not shown). The robust increase observed here could be the result of increased proliferation, survival, and/or metabolic activity. The latter may reflect an overall increase in protein and RNA synthesis. This process is enhanced in cancer cells to meet the biosynthetic requirements mandated by rapid proliferation and is facilitated by augmented activation of the phosphatidylinositol 3-kinase/AKT pathway (26, 27). The dramatic increase over additive effects here was observed after a little over one cell cycle. Continuation of this rate of proliferation/survival and/or metabolism would have profound effects on tumor growth, which would be functionally important in the context of a primary breast tumor.

Among the targets of AP-1 are matrix metalloproteinases (MMP), which degrade extracellular matrix components and are associated with tumor progression. We examined the effect of these growth factors on MMP-2, a family member strongly associated with an invasive phenotype (28, 29). IGF-I or prolactin alone did not cause significant changes in levels of MMP-2 mRNA. However, IGF-I/prolactin cotreatment resulted in a nearly 3-fold increase in MMP-2 transcripts (Fig. 5A and B). To investigate the functionality of this biochemical end point, we measured the invasive behavior of these cells in transwell invasion assays. IGF-I and prolactin alone do not significantly stimulate invasion, but IGF-I/prolactin together dramatically promote invasive behavior (Fig. 5C and D). This response would be very significant clinically; the ability to metastasize is a key determinant of survival.

#### *Prolactin Augments Phosphorylation of IGF-IR*

Positive IGF-I/prolactin cross talk could occur at the level of the receptors and/or at a convergent point(s) in the downstream signaling pathways. We examined the effect of these factors on the acute phosphorylation of IGF-IR using an antibody detecting p-Tyr<sup>1135</sup> and p-Tyr<sup>1136</sup>, which are two of the three tyrosines that constitute the activation loop of IGF-IR (12). Levels of p-IGF-IR in response to IGF-I/prolactin were 2-fold greater than IGF-I alone (Fig. 6A-C), despite the inability of prolactin alone to induce detectable activation of IGF-IR. EGF can also synergize with IGF-I (30) but did not enhance phosphorylation of the active site of IGF-IR, indicating that this phenomenon is not shared by all growth factors (Fig. 6D). Next, we investigated the reciprocal phenomenon for prolactin receptor by examining total levels of tyrosine phosphorylation. Levels of phospho-prolactin receptor were not affected by the addition of IGF-I (Fig. 6E). Thus, the interaction we observe seems to be a result of a significant increase in the activation of IGF-IR. Hyperactivation of IGF-IR can contribute to the important biological consequences associated with neoplastic progression, such as those observed in Figs. 4 and 5 (13).

## Discussion

Although epidemiologic and experimental studies are resolving the controversy surrounding a possible role for prolactin in breast cancer, the mechanism(s) by which this

hormone increases breast cancer risk is not well understood (4). To move forward in developing preventatives and therapeutics targeting prolactin and/or prolactin receptor, we must understand the differential actions of prolactin on normal versus tumor cells as well as its interactions with other oncogenic growth factors. In this study, we showed that prolactin does not require an IGF intermediate to induce cyclin D1 and subsequent proliferation in breast cancer cells, indicating that prolactin can directly elicit these effects, contrary to reports in normal murine models. Furthermore, prolactin cooperated with IGF-I in breast cancer cells to increase ERK1/2, AKT, and IGF-IR $\beta$  phosphorylation. This led to increased AP-1 activation, deactivation of GSK-3 $\beta$ , and up-regulation of cyclin D1 protein and MMP-2 transcripts. Most importantly, this interaction ultimately resulted in dramatic increases in cell number and invasive behavior.

ERK1/2 are activated in response to a plethora of growth factors, cytokines, and other extracellular stimuli, and elicit a myriad of downstream effects associated with malignancy including breast cancer. ERK1/2 is hyperexpressed and hyperactivated in malignant neoplasias of the breast compared with surrounding tissue (31, 32). Activated ERK1/2 phosphorylates numerous substrates, many of which are transcription factors. One family is AP-1 proteins, which regulate many genes involved in proliferation, survival, and metastasis, implicating this transcription factor complex in transformation (24, 33). Among the AP-1 targets are cyclin D1 and MMPs, both of which are highly expressed in primary breast tumors and contribute to disease progression (34-37). Thus, the ability of IGF-I and prolactin to cooperatively activate ERK1/2 and downstream targets would greatly enhance the proliferation and invasive behavior of breast tumors.

Like ERK1/2, phosphorylated AKT and its downstream target, GSK-3 $\beta$ , are associated with oncogenesis. AKT augments levels of cyclin D1 via multiple mechanisms including regulation of Forkhead transcription factors (38) and stabilization of cyclin D1 mRNA (39). Downstream of AKT, phosphorylated GSK-3 $\beta$  can stabilize cyclin D1 protein (23) and free  $\beta$ -catenin to translocate to the nucleus and associate with T-cell factor/lymphoid enhancer factor transcription factors, whose targets include cyclin D1 (25). This pathway also plays a major role in cell survival via downstream targets including BAD, procaspase-9, Forkhead transcription factors, and MCL-1, a Bcl-2 family member (26, 38, 40). Furthermore, tumor invasion in response to IGF-I has been shown to be regulated by this pathway (29, 41). Finally, AKT has been implicated in the resistance of tumors to antiestrogen therapies, and its activation was correlated with increased relapse and decreased survival (42). Thus, the interaction between IGF-I and prolactin at this signaling junction would also contribute to breast cancer progression, in addition to potentially mediating resistance to endocrine therapy.

Whereas both IGF-I and prolactin alone can modestly stimulate migration and invasion in some systems (13, 22, 43), our data show a particularly potent interaction at this activity. The ability of tumor cells to degrade extracellular matrix components, enter blood vessels, and metastasize to distant sites, such as the lungs, is a major determinant of mortality.

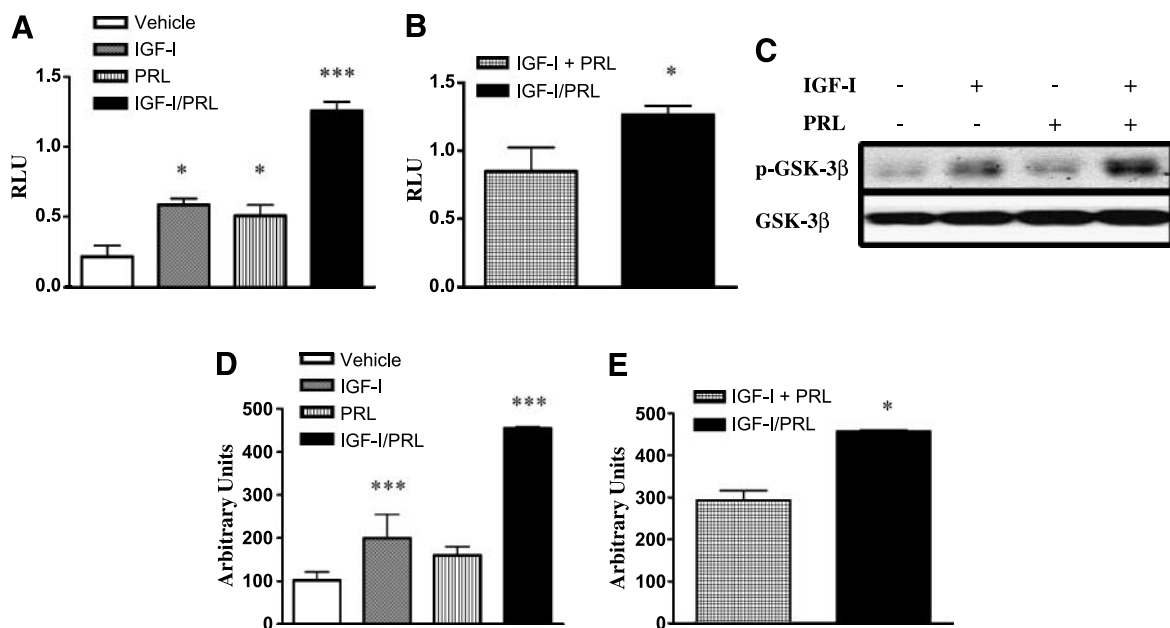
Increased expression and activity of MMPs, including MMP-2, are critical for this action and correlate with the shortened survival of patients with most cancers (29). Expression of these genes is controlled by a variety of factors including AP-1 and phosphatidylinositol 3-kinase/AKT (28, 41); the marked synergistic invasive behavior elicited by IGF-I and prolactin is consistent with the cross talk that we observed at these signaling junctions.

The mechanism by which IGF-I and prolactin cooperate is not completely understood. We show that prolactin augmented IGF-I-induced phosphorylation of Tyr<sup>1135</sup> and Tyr<sup>1136</sup>, two of the three tyrosines present in the activation loop of IGF-IR (12), suggesting that prolactin augments IGF-IR activity. Treatment with prolactin alone had no effect, suggesting that IGF-I is necessary to trigger activation, which then permits prolactin action. In contrast, IGF-I did not enhance prolactin receptor tyrosine phosphorylation, consistent with data showing that STAT5 activation was not enhanced on cotreatment. However, examination of total rather than specific tyrosine phosphorylation events may have obscured effects at specific residues. This phenomenon seems to be distinct from that described for the interaction between prolactin and EGF, in which no discernable increase in EGF receptor tyrosine phosphorylation was observed (44), suggesting that prolactin can augment oncogenic signals from tyrosine kinase receptors in a variety of ways. The mechanism by which prolactin enhances IGF-IR activation remains to be elucidated, but possible explanations include effects of downstream pathways

on the trafficking of IGF-IR and/or phosphatases regulating IGF-IR.

Hyperactivation of the IGF-IR promotes tumorigenesis and contributes to tumor progression (13, 17, 45), and thus IGF-IR signals have become attractive drug targets for a variety of malignancies (17, 46, 47). Moreover, enhanced activation of IGF-IR has been shown to mediate resistance to molecularly targeted therapies, such as those directed at the EGF receptor and estrogen receptor (48-50). IGF-IR signals confer resistance to traditional chemotherapies; IGF-I rescues breast cancer cells from doxorubicin-induced apoptosis, and down-regulation of IGF-IR expression increases the susceptibility of tumor cells to established chemotherapeutic agents (46, 51). These reports point to the clinical importance of the prolactin-enhanced phosphorylation of IGF-IR.

To better understand the contributions of prolactin to breast cancer, one must study not only the direct actions of prolactin but also its indirect activities, including interactions with other growth factors or cytokines, which can potentially accelerate tumorigenesis. Our data show that prolactin can enhance IGF-I-initiated signaling, augmenting critical processes associated with neoplastic progression, including invasive behavior. Prolactin can also promote signals of other mammary mitogens such as estrogens and EGF family ligands *in vitro* and *in vivo* (8, 39, 40). Better understanding of the mechanism(s) underlying this cross talk will further delineate the actions of prolactin contributing to breast carcinogenesis as it occurs *in vivo*, potentially leading to novel therapeutic interventions.

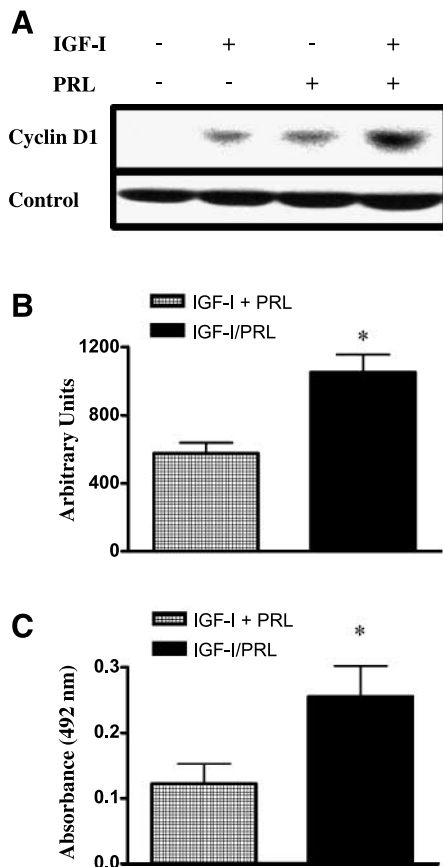


**FIGURE 3.** IGF-I/prolactin cotreatment cooperatively activates AP-1 enhancer elements and phosphorylates GSK-3 $\beta$ . **A.** Serum-starved MCF-7 cells were transfected with a 4xAP-1-luciferase construct and treated with vehicle, IGF-I, prolactin, or IGF-I/prolactin for 24 h. Histogram represents data from independent experiments in relative light units (RLU). Columns, mean ( $n = 3$ ); bars, SE. \*,  $P < 0.05$ ; \*\*\*,  $P < 0.001$ , compared with vehicle (one-way ANOVA, Neuman-Keuls post-test). **B.** Comparison of the sum of individual effects of IGF-I and prolactin treatments to IGF-I/prolactin cotreatment. Columns, mean ( $n = 3$ ); bars, SE. \*,  $P < 0.05$ , compared with the sum of IGF-I and prolactin single hormone treatments (paired  $t$  test). **C.** Serum-starved MCF-7 cells were treated with vehicle, IGF-I, prolactin, or IGF-I/prolactin for 15 min. Immunoblots were done with the indicated antibodies (representative experiment shown). **D.** Signals from independent experiments were quantified by densitometry. Columns, mean ( $n = 3$ ); bars, SE. \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ , compared with vehicle (one-way ANOVA, Neuman-Keuls post-test). **E.** Comparison of the sum of individual IGF-I and prolactin treatments to IGF-I/prolactin cotreatment calculated as described in Materials and Methods. Columns, mean ( $n = 3$ ); bars, SE. \*,  $P < 0.05$ , compared with the sum of IGF-I and prolactin single hormone treatments (paired  $t$  test).

## Materials and Methods

### Cell Culture

Prolactin-deficient MCF-7 cells were maintained in phenol red-free RPMI 1640 containing 10% horse serum and 50  $\mu\text{mol/L}$  ganciclovir as previously described (11). T47D cells were maintained in phenol red-free RPMI 1640 containing 10% fetal bovine serum. Before all experiments, cells were grown in phenol red-free RPMI 1640 containing 5% charcoal-stripped fetal bovine serum for 4 to 5 d, then serum starved for 24 or 48 h in phenol red-free RPMI 1640. All hormone treatments, with the exception of the invasion assay, were done in this medium at the following concentrations: IGF-I, 20 ng/mL; prolactin, 100 ng/mL; EGF, 30 ng/mL.



**FIGURE 4.** IGF-I/prolactin cotreatment increases cyclin D1 and cell number. Serum-starved MCF-7 cells were treated with vehicle, IGF-I, prolactin, or IGF-I/prolactin for 6 h (**A** and **B**) or 48 h (**C**). **A**. Immunoblots were done with the indicated antibodies (representative experiment shown). **B**. Signals from independent experiments were quantified by densitometry, and comparison of the sum of individual IGF-I and prolactin treatments to IGF-I/prolactin cotreatment was calculated as described in Materials and Methods. Columns, mean ( $n = 3$ ); bars, SE. \*,  $P < 0.05$ , compared with the sum of IGF-I and prolactin single hormone treatments (paired  $t$  test). **C**. Absorbance values from independent experiments were measured at 492 nm. The histogram compares the sum of individual IGF-I and prolactin treatments to IGF-I/prolactin cotreatment, quantified as described in Materials and Methods. Columns, mean ( $n = 5$ ); bars, SE. \*,  $P < 0.05$ , compared with the sum of IGF-I and prolactin single hormone treatments (Student's  $t$  test).

### Reagents

Antibodies used were as follows:  $\alpha\text{IR3}$  (GR11L; Calbiochem); p-IGF-IR Tyr<sup>1135/1136</sup>, p-ERK1/2, ERK1/2, p-AKT Ser<sup>473</sup>, AKT, p-GSK-3 $\beta$  Ser<sup>9</sup>, and GSK-3 $\beta$  (Cell Signaling Technology); cyclin D1 (MS-210-P1; NeoMarkers); IGF-IR $\beta$ , prolactin receptor, STAT5, mouse IgG, and protein A/G-Plus agarose (Santa Cruz Biotechnology, Inc.); p-tyrosine 4G10 (Upstate Biotechnology); and p-STAT5a/b Tyr<sup>694</sup> (Zymed). Recombinant human prolactin was obtained from Dr. A.F. Parlow (National Hormone and Pituitary Program, National Institutes of Diabetes, Digestive and Kidney Diseases, Torrance, CA). Recombinant human IGF-I was purchased from Cell Sciences. All other reagents were obtained from Sigma unless otherwise noted.

### Western Blot Analysis and Immunoprecipitation

Western blot analysis was done as previously described (11). All primary antibodies were used at 1:1,000 dilution except for cyclin D1 (1:500) and STAT5 (1:50,000). Signals were visualized by enhanced chemiluminescence and some were quantified by densitometry (ImageQuant software, version 4.2a, Amersham Biosciences). Representative immunoblots from at least three independent experiments are shown. To compute the predicted sum of IGF-I and prolactin treatments, we added the value observed for each alone, but then subtracted the value of one vehicle treatment because the sum of individual treatments included basal activity twice. Cooperative interactions were defined as those that were significantly greater than this value. For immunoprecipitations, cells were lysed with IPA buffer [10 mmol/L Tris (pH 8), 150 mmol/L NaCl, 1 mmol/L EDTA (pH 8), 1% Triton, 0.5% sodium deoxycholate, 1 mmol/L Na<sub>3</sub>VO<sub>4</sub>, 1 mmol/L phenylmethylsulfonyl fluoride, 1  $\mu\text{g/mL}$  leupeptin, 1  $\mu\text{g/mL}$  aprotinin]. One milligram of protein was cleared for 1 h and immunoprecipitated with 1  $\mu\text{g}$  of antibody for 2 to 3 h at 4°C. Bead slurry (30  $\mu\text{L}$ ) was added and the mixture was allowed to tumble overnight at 4°C. Beads were subsequently washed thrice with IPA buffer and prepared for electrophoresis.

### Real-time PCR

Total cellular RNA was isolated with RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. cDNA was synthesized from 1- $\mu\text{g}$  RNA using random hexamers (Amersham Biosciences) and Moloney murine leukemia virus reverse transcriptase (Promega). Quantitative real time-PCR reactions were done as described (22).

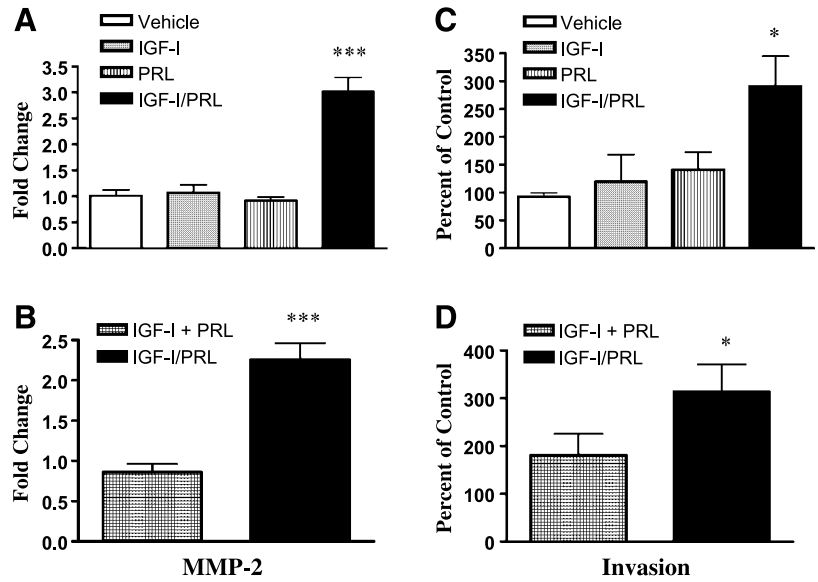
### Transfection and Reporter Gene Assays

The AP-1-luciferase construct contains four GCN4 consensus AP-1-responsive elements upstream of a luciferase reporter (52). Transient transfections were done as previously described (53) using SuperFect (Qiagen). Luciferase values were normalized to  $\beta$ -galactosidase activity to correct for transfection efficiency, yielding relative luciferase units.

### Effects on Cell Number

Cells were seeded at  $5 \times 10^3$  per well in a 96-well plate. After 24-h serum withdrawal, cells were treated with vehicle or

**FIGURE 5.** IGF-I/prolactin cotreatment increases MMP-2 transcripts and invasive behavior. Serum-starved MCF-7 cells were treated with vehicle, IGF-I, prolactin, or IGF-I/prolactin for 18 h (A and B) or 30 h (C and D). **A.** MMP-2 mRNA was extracted and quantified by real time-PCR as described in Materials and Methods. Columns, mean ( $n = 3$ ); bars, SE. **\*\*\***,  $P < 0.001$ , compared with vehicle (one-way ANOVA, Neuman-Keuls post-test; representative experiment). **B.** Comparison of the sum of IGF-I and prolactin treatments to that of IGF-I/prolactin from independent experiments. Columns, mean ( $n = 3$ ); bars, SE. **\*\*\***,  $P < 0.001$ , compared with sum of IGF-I and prolactin treatments (paired  $t$  test). **C.** Invasive capacity was determined as described in Materials and Methods. Columns, mean ( $n = 3$ ); bars, SE. **\***,  $P < 0.05$ , compared with vehicle-treated cells (one-way ANOVA, Dunnett's post-test; representative of three similar experiments). **D.** Comparison of the sum of IGF-I and prolactin treatments to that of IGF-I/prolactin cotreatment. Columns, mean ( $n = 3$ ); bars, SE. **\***,  $P < 0.05$ , compared with sum of IGF-I and prolactin treatments (one-tailed paired  $t$  test; representative of three similar experiments).

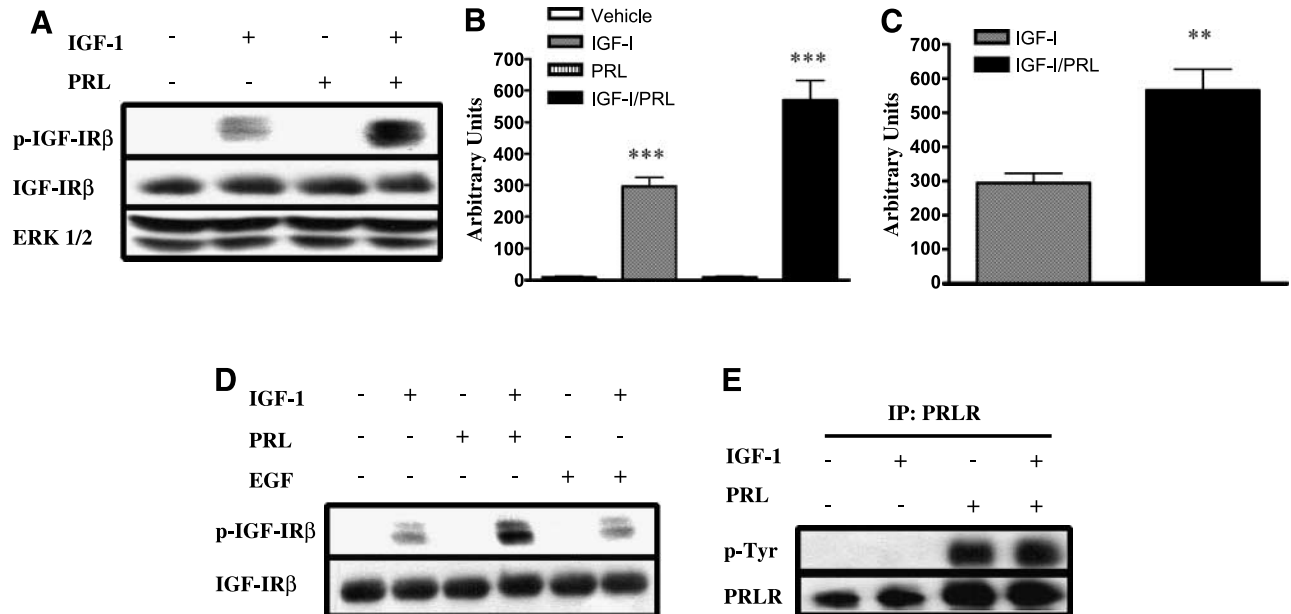


hormone for 48 h. Assays were done using CellTiter 96 AQueous Non-Radioactive Cell Proliferation Assay Kit (MTS; Promega) according to the manufacturer's protocol with modifications as previously described (54).

*Invasion Assays*

Invasion assays were done exactly as described (55). Briefly, cells were allowed to invade collagen I (Upstate Biotechnology)

in transwell permeable supports with polycarbonate membranes containing 12- $\mu$ m pores (Corning, Inc.). Horse serum (10%) was placed in the lower chamber, and cells were seeded in the presence of serum-free RPMI 1640, 200 ng/mL prolactin, and/or 40 ng/mL IGF-I in the upper chamber. After 30 h of hormone stimulation, membranes were excised and stained using Hema 3 StatPak (Fisher Scientific), and the number of cells that traversed each membrane was counted. Invasive capacity was



**FIGURE 6.** Prolactin augments activation of IGF-IR. **A.** Serum-starved MCF-7 cells were treated with vehicle, IGF-I, prolactin, or IGF-I/prolactin for 15 min. Immunoblots were done with the indicated antibodies (representative experiment shown). **B.** Signals were quantified by densitometry. Columns, mean ( $n = 5$ ); bars, SE. **\*\*\***,  $P < 0.001$ , compared with vehicle (one-way ANOVA, Neuman-Keuls post-test). **C.** Signals from IGF-I/prolactin cotreatment were compared with those from IGF-I alone. Columns, mean ( $n = 5$ ); bars, SE. **\*\***,  $P < 0.01$ , compared with IGF-I treatment (paired  $t$  test). **D.** Serum-starved MCF-7 cells were treated with vehicle, IGF-I, prolactin, EGF, IGF-I/prolactin, or IGF-I/EGF. Immunoblots were done with the indicated antibodies (representative experiment shown). **E.** Serum-starved MCF-7 cells were treated with vehicle, IGF-I, prolactin, or IGF-I/prolactin for 15 min. One-milligram total cell lysates were immunoprecipitated as described in Materials and Methods and were subjected to Western blot analysis with the indicated antibodies (representative experiment shown).



determined as percentage of vehicle-treated cells. The average number of vehicle-treated cells that traversed the membrane was considered 100%.

### Statistical Analyses

Statistical analyses were done using Prism version 4.00 (GraphPad Software, Inc.).

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### References

- Goffin V, Binart N, Touraine P, Kelly PA. Prolactin: the new biology of an old hormone. *Annu Rev Physiol* 2002;64:47–67.
- Ben-Jonathan N, Liby K, McFarland M, Zinger M. Prolactin as an autocrine/paracrine growth factor in human cancer. *Trends Endocrinol Metab* 2002;13:245–50.
- Vonderhaar BK. Prolactin in human breast cancer development. In: *Either SP, editor. Endocrine oncology*. Totowa (NJ): Humana Press; 2000. p. 101–20.
- Clevenger CV, Furth PA, Hankinson SE, Schuler LA. Role of prolactin in mammary carcinoma. *Endocr Rev* 2003;24:1–27.
- Tworoger SS, Hankinson SE. Prolactin and breast cancer risk. *Cancer Lett* 2006;243:160–9.
- Meng JP, Tsai-Morris CH, Dufau ML. Human PRLR variants in breast cancer: low ratio of short forms to the long-form hPRLR associated with mammary carcinoma. *Cancer Res* 2004;64:5677–82.
- Rose-Hellekant TA, Arendt LM, Schroeder MD, Gilchrist K, Sandgren EP, Schuler LA. Prolactin induces ER $\alpha$ -positive and ER $\alpha$ -negative mammary cancer in transgenic mice. *Oncogene* 2003;22:4664–74.
- Arendt LM, Rose-Hellekant TA, Sandgren EP, Schuler LA. Prolactin potentiates TGF- $\alpha$  induction of mammary neoplasia in transgenic mice. *Am J Pathol* 2006;168:1365–74.
- Brisken C, Ayyannan A, Nguyen C, et al. IGF-2 is a mediator of prolactin-induced morphogenesis in the breast. *Dev Cell* 2002;3:877–87.
- Hovey RC, Harris J, Hadsell DL, Lee AV, Ormandy CJ, Vonderhaar BK. Local IGF-II mediates prolactin-induced mammary gland development. *Mol Endocrinol* 2003;17:460–71.
- Schroeder MD, Symowicz J, Schuler LA. Prolactin modulates cell cycle regulators in mammary tumor epithelial cells. *Mol Endocrinol* 2002;16:45–57.
- Adams TE, Epa VC, Garrett TP, Ward CW. Structure and function of the type I IGF-IR. *Cell Mol Life Sci* 2000;57:1050–93.
- Samani AA, Yakar S, LeRoith D, Brodt P. The role of the IGF system in cancer growth and metastasis: overview and recent insights. *Endocr Rev* 2007;28:20–47.
- Stull MA, Wood TL. Expression of the IGFs, IGF-IR and IGFIBPs in the normal mammary gland and breast. *Breast Dis* 2003;17:15–26.
- Hadsell DL, Bonnette SG, Lee AV. Genetic manipulation of the IGF-I axis to regulate mammary gland development and function. *J Dairy Sci* 2002;85:365–77.
- Marshman E, Streuli CH. IGFs and IGFIBPs in mammary gland function. *Breast Cancer Res* 2002;4:231–9.
- Pollak MN, Schernhammer ES, Hankinson SE. Insulin-like growth factors and neoplasia. *Nat Rev Cancer* 2004;4:505–18.
- Baserga R. The contradictions of the IGF-IR. *Oncogene* 2000;19:5574–81.
- Jammes H, Peyrat JP, Ban E, et al. IGF-IR in human breast tumour: localisation and quantification by histo-autoradiographic analysis. *Br J Cancer* 1992;66:248–53.
- Kull FC, Jr., Jacobs S, Su YF, Svoboda ME, Van Wyk JJ, Cuatrecasas P. Monoclonal antibodies to receptors for insulin and somatomedin-C. *J Biol Chem* 1983;258:6561–6.
- Kato H, Faria TN, Stannard B, Roberts CT, Jr., LeRoith D. Role of tyrosine kinase activity in signal transduction by IGF-IRs: characterization of kinase-deficient IGF-IR and the action of an IGF-I-mimetic antibody ( $\alpha$ IR3). *J Biol Chem* 1993;268:2655–61.
- Gutzman JH, Rugowski DE, Nikolai SE, Schuler LA. Stat5 activation inhibits prolactin-induced AP-1 activity: distinct prolactin initiated signals in tumorigenesis dependent on cell context. *Oncogene* 2007;26:6341–8.
- Diehl JA, Cheng M, Roussel MF, Sherr CJ. GSK-3 $\beta$  regulates cyclin D1 proteolysis and subcellular localization. *Genes Dev* 1998;12:3499–511.
- Shaulian E, Karin M. AP-1 as a regulator of cell life and death. *Nat Cell Biol* 2002;4:E131–6.
- Pestell RG, Albanese C, Reutens AT, Segall JE, Lee RJ, Arnold A. The cyclins and cdk inhibitors in hormonal regulation of proliferation and differentiation. *Endocr Rev* 1999;20:501–34.
- Vivanco I, Sawyers CL. The phosphatidylinositol 3-kinase-AKT pathway in human cancer. *Nat Rev Cancer* 2002;2:489–501.
- Schmelzle T, Hall MN. TOR, a central controller of cell growth. *Cell* 2000;103:253–62.
- Vincenzi MP. The MMP and TIMP genes: transcriptional and posttranscriptional regulation, signal transduction and cell-type-specific expression. *Methods Mol Biol* 2001;151:121–48.
- Egeblad M, Werb Z. New functions for the MMPs in cancer progression. *Nat Rev Cancer* 2002;2:161–74.
- Faisal G, Qureshi, Marcin T, Tchorzewski, Mark D, Duncan, John W, Harmon. EGF and IGF-I synergistically stimulate proliferation of human esophageal epithelial cells. *J Surg Res* 1997;69:354–8.
- Sivaraman VS, Wang H, Nuovo GJ, Malbon CC. Hyperexpression of MAPK in human breast cancer. *J Clin Invest* 1997;99:1478–83.
- Santen RJ, Song RX, McPherson R, et al. The role of MAPK in breast cancer. *J Steroid Biochem Mol Biol* 2002;80:239–56.
- Eferl R, Wagner EF. AP-1: a double-edged sword in tumorigenesis. *Nat Rev Cancer* 2003;3:859–68.
- Gillett C, Fantl V, Smith R, et al. Amplification and overexpression of cyclin D1 in breast cancer detected by immunohistochemical staining. *Cancer Res* 1994;54:1812–7.
- Alle KM, Henshall SM, Field AS, Sutherland RL. Cyclin D1 protein is overexpressed in hyperplasia and intraductal carcinoma of the breast. *Clin Cancer Res* 1998;4:847–54.
- Rudolph-Owen LA, Matrisian LM. Matrix metalloproteinases in remodeling of the normal and neoplastic mammary gland. *J Mammary Gland Biol Neoplasia* 1998;3:177–89.
- Polette M, Birembaut P. Matrix metalloproteinases in breast carcinoma. *Breast J* 1996;2:209–20.
- Liang J, Slingerland JM. Multiple roles of PI3K/PKB (AKT) pathway in cell cycle progression. *Cell Cycle* 2003;2:339–45.
- Dufourny B, van Teeffelen HAAM, Hamelers IHL, Sussenbach JS, Steenbergh PH. Stabilization of cyclin D1 mRNA via the PI3K pathway in MCF-7 human breast cancer cells. *Endocrinology* 2000;166:329–38.
- Maurer U, Charvet C, Wagman AS, DeJardin E, Green DR. GSK-3 regulates mitochondrial outer membrane permeabilization and apoptosis by destabilization of MCL-1. *Mol Cell* 2006;21:749–60.
- Zhang D, Brodt P. IGF-I regulates MT1-MMP synthesis and tumor invasion via PI3K/AKT signaling. *Oncogene* 2003;22:974–82.
- Kirkegaard T, Witton CJ, McGlynn LM, et al. AKT activation predicts outcome in breast cancer patients treated with tamoxifen. *J Pathol* 2005;207:139–46.
- Maus MV, Reilly SC, Clevenger CV. Prolactin as a chemoattractant for human breast carcinoma. *Endocrinology* 1999;140:5447–50.
- Huang Y, Li X, Jiang J, Frank SJ. Prolactin modulates phosphorylation, signaling and trafficking of EGFR in human T47D breast cancer cells. *Oncogene* 2006;25:7565–76.
- Carboni JM, Lee AV, Hadsell DL, et al. Tumor development by transgenic expression of a constitutively active insulin-like growth factor-I receptor. *Cancer Res* 2005;65:3781–87.
- Surmacz E. Growth factor receptors as therapeutic targets: strategies to inhibit the insulin-like growth factor-I receptor. *Oncogene* 2003;22:6589–97.
- Sachdev D, Yee D. Disrupting insulin-like growth factor signaling as a potential cancer therapy. *Mol Cancer Ther* 2007;6:1–12.
- Lu Y, Zi X, Zhao Y, Mascarenhas D, Pollak M. Insulin-like growth factor-I receptor signaling and resistance to trastuzumab (Herceptin). *J Natl Cancer Inst* 2001;93:1852–7.
- Jones HE, Goddard L, Gee JM, et al. Insulin-like growth factor-I signaling and acquired resistance to gefitinib (ZD1839; Iressa) in human breast and prostate cancer cells. *Endocr Relat Cancer* 2004;11:793–814.

50. Wiseman LR, Johnson MD, Wakeling AE, Lykkesfeldt AE, May FE, Westley BR. Type I IGF receptor and acquired tamoxifen resistance in oestrogen-responsive human breast cancer cells. *Eur J Cancer* 1993;29A: 2256–64.
51. Gooch JL, Van Den Berg CL, Yee D. Insulin-like growth factor (IGF)-I rescues breast cancer cells from chemotherapy-induced cell death-proliferative and anti-apoptotic effects. *Breast Cancer Res Treat* 1999;56:1–10.
52. Dong Z, Xu RH, Kim J, et al. AP-1/jun is required for early *Xenopus* development and mediates mesoderm induction by FGF but not by activin. *J Biol Chem* 1996;271:9942–6.
53. Gutzman JH, Nikolai SE, Rugowski DE, Watters JJ, Schuler LA. Prolactin and estrogen enhance the activity of AP-1 in breast cancer cells: role of ERK1/2-mediated signals to c-fos. *Mol Endocrinol* 2005;19: 1765–78.
54. Huang KT, Chen YH, Walker AM. Inaccuracies in MTS assays: major distorting effects of medium, serum albumin, and fatty acids. *Biotechniques* 2004; 37:406–12.
55. Keely PJ. Ras and Rho protein induction of motility and invasion in T47D breast adenocarcinoma cells. *Methods Enzymol* 2001;333: 256–66.