

Insulin-Like Growth Factor-I Inhibits Progesterone Receptor Expression in Breast Cancer Cells via the Phosphatidylinositol 3-Kinase/Akt/Mammalian Target of Rapamycin Pathway: Progesterone Receptor as a Potential Indicator of Growth Factor Activity in Breast Cancer

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Although interactions between estrogen and growth factor signaling pathways have been studied extensively, how growth factors and progesterone regulate each other is less clear. In this study, we found that IGF-I sharply lowers progesterone receptor (PR) mRNA and protein levels in breast cancer cells. Other growth factors, such as epidermal growth factor, also showed the same effect. The decrease of PR levels was associated with reduced PR activity. Unlike progestins, IGF-I does not utilize the proteasome for down-regulating PR. Instead, the IGF-I-mediated decrease in PR levels is via an inhibition of PR gene transcription. In addition, the phosphatidylinositol 3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) pathway was found to be specifically involved in this IGF-I effect. Our data also suggest that the IGF-I down-regulation of PR is not mediated via a reduc-

tion of estrogen receptor (ER) levels or activity. First, IGF-I induced ligand-independent ER activity while reducing ER-dependent PR levels. Second, whereas PR and cyclin D1 are both ER up-regulated, IGF-I increased cyclin D1 levels while decreasing PR levels. Third, constitutively active PI3K or Akt induced ER activity but reduced PR levels and activity. Taken together, our data indicate that IGF-I inhibits PR expression in breast cancer cells via the PI3K/Akt/mTOR pathway. Because low or absent PR in primary breast cancer is associated with poor prognosis and response to hormone therapy, our results suggest that low PR status may serve as an indicator of activated growth factor signaling in breast tumor cells, and therefore of an aggressive tumor phenotype and resistance against hormonal therapy. (*Molecular Endocrinology* 17: 575-588, 2003)

THE IGF SYSTEM is composed of a complex interacting network of ligands, ligand binding proteins, and receptors (1). In different cell types, the IGFs can elicit distinct downstream signaling cascades, of which the phosphatidylinositol 3-kinase (PI3K)/Akt and Ras/MAPK pathways are the best studied (2). The IGFs can lead to cell proliferation, survival, and differentiation. As key regulators of cell cycle progression,

Abbreviations: DCS, Dextran-charcoal-treated serum; DRB, 5, 6-dichlorobenzimidazole riboside; EGF, epidermal growth factor; ER, estrogen receptor; ERE, estrogen response element; GAPDH, glyceraldehyde-3-phosphatedehydrogenase; HIMOC, 1L-6-hydroxymethyl-*chiro*-inositol-(R)-2-O-methyl-3-O-octadecylcarbonate; HRG, heregulin; ICI, ICI 182,780; IGF-IR, IGF-I receptor; IMEM, improved MEM zinc option; IRSs, insulin receptor substrates; luc, luciferase; mTOR, the mammalian target of rapamycin; PBST, PBS plus 0.05% Tween-20; PI3K, phosphatidylinositol 3-kinase; PKA, protein kinase A; PKC, protein kinase C; PR, progesterone receptor; PR-A and PR-B, two isoforms of human PR; PRE, progesterone response element; Q-PCR, real-time quantitative RT-PCR; SFM, serum-free medium; tk, thymidine kinase.

they also play an important role in malignant transformation and invasion (3, 4). Extensive study has demonstrated that the IGFs are mitogenic and antiapoptotic agents for breast epithelial cells *in vitro* (5) and are crucial for mammary gland development (6). Additionally, numerous lines of evidence have supported a role for the IGFs in breast cancer pathogenesis (7). First, breast cancer cell lines express all of the components required for eliciting a response to the IGFs, and this results in the IGFs being one of the most potent mitogens for breast cancer cells (8). Second, blockade of IGF action *in vitro* and *in vivo* can inhibit breast tumor growth (9, 10). Third, epidemiological study shows that circulating levels of IGF-I predict breast cancer risk (11). Finally, IGF components are expressed in primary breast tumors, and high expression of several of them are associated with poor prognosis (12).

Progesterone is also critically involved in the development of the mammary gland and breast cancer, and

its effects are mostly mediated via the progesterone receptor (PR; Refs. 13–15). Mice lacking PR display incomplete mammary ductal branching and failure of lobular-alveolar development (16). Although PR expression is estrogen receptor (ER) dependent (17, 18), some breast cancer cell lines constitutively express high levels of PR independent of estrogens (19). Human PR normally exists in two isoforms (PR-A and PR-B) of 94 and 116 kDa, originating from two PR promoters (20).

Progesterone is considered differentiative in the uterus but proliferative in the normal mammary gland (14). However, progestin inhibition of breast cancer cell growth in tissue culture has been well documented (21–23). High doses of progestins have been used to treat estrogen-mediated mammary carcinomas, even though their antitumor mechanisms are not clear (14). Interestingly, progestins have been found to exert a biphasic regulation of breast cancer cell growth—accelerating cells through the first mitotic cell cycle, then arresting them in G1 of the second cycle. At this stage, the cell cycle progression machinery is poised to restart, as expression of growth factors and their receptors is increased by progestins (21, 22). Thus, it is proposed that progestins are neither inherently growth proliferative or inhibitive, but rather sensitize cells for growth factor and cytokine signals (24, 25).

The presence of PR in breast tumors is an important indicator of likely responsiveness to endocrine agents (13, 26). Approximately two thirds of breast cancers express the ER, some of which are ER positive (ER+)/PR negative (PR–). Their likelihood of response to endocrine therapy drops significantly compared with those that are ER+/PR+. It has also been reported that absence of PR in primary breast tumors is associated with secondary breast cancer in postmenopausal women (27), and absence of PR correlated significantly with a less differentiated phenotype of breast tumors (G1/G2 grading) and the presence of ErbB2/HER2/neu (28). Abnormal expression of ErbB2 and other growth factor receptors is normally associated with more aggressive tumors and a poorer patient prognosis (29, 30). So is there an intrinsic correlation between PR and intensity of growth factor action? We hypothesize that PR status may reflect growth factor function: low or absent PR expression indicates high IGF, epidermal growth factor (EGF), and heregulin (HRG) activities, and this correlation is independent of ER status. This assumption, combined with the fact that progesterone may inhibit breast tumor invasion, might explain why absence of PR is a marker of an aggressive tumor phenotype (27).

Recently, much effort has been directed to the study of the cross-talk between growth factors and ER signaling pathways in breast cancer cells (31), but how growth factors may interact with PR is less well defined. In this study, we have investigated the mechanisms for growth factor regulation of PR in breast cancer using IGF-I and MCF-7 cells because these cells are sensitive to the IGFs, have considerable PR levels, and possess intact

PI3K, MAPK, and other common signaling pathways. We find that IGF-I dramatically down-regulates PR through a transcriptional mechanism involving the PI3K pathway, independent of ER activity. Our data provide the first evidence that activation of a growth factor signaling pathway can directly reduce PR levels, and may explain why PR-negative tumors, which possibly have highly active growth factor signaling, poorly respond to endocrine therapy.

RESULTS

IGF-I Inhibits PR Expression

As a first step to investigate the effect of IGF-I on PR in breast cancer cells, we treated MCF-7 cells with IGF-I and then performed Western blot analysis to determine how PR expression was affected. We found that IGF-I treatment caused a dramatic decrease of both PR-A and PR-B protein levels in MCF-7 cells. The dose-response assay using a 24-h time point of treatment showed that maximal reduction of PR protein levels occurred with 10 nM IGF-I (Fig. 1, A and B), which is physiologically relevant to the circulating concentrations of IGF-I in women (11). Much lower concentrations of IGF-I, 0.1 nM, also resulted in an apparent reduction of PR protein levels. From a time course experiment using 10 nM IGF-I, it was found that PR protein levels began to drop after 6 h of IGF-I treatment and continued to decrease with time, whereas the untreated control cells did not display visible down-regulation of PR (Fig. 1, C and D). After 24 h, the sharp fall in PR protein levels stopped, but levels remained suppressed for at least 72 h. We also observed similar results in other PR+ breast cancer cells such as T47D and ZR75 (data not shown). Hence, these data indicate that IGF-I lowers PR levels in breast cancer cells.

To ascertain whether PR activity changed correspondingly with its protein levels after IGF-I treatment, we transiently transfected a progesterone response element (PRE)-luciferase construct into MCF-7 cells. Treatment with the synthetic progestin R5020 caused a more than 100-fold increase in reporter activity, which was almost completely blocked by the anti-progestin RU486 (Fig. 1E). IGF-I pretreatment for 6 h caused a 70% reduction in R5020-induced luciferase activity. This was most probably due to the decrease of PR protein levels elicited by IGF-I, which by itself, at the same time point, had no detectable activation of the reporter activity over the unstimulated control. Hence, these data suggest that IGF-I represses PR transcriptional activity through down-regulation of PR in MCF-7 cells.

Because MCF-7 cells also respond well to other growth factors like EGF and HRG (although response to EGF is not as sensitive as the response to HRG and IGF-I), and these growth factors share similar signaling pathways, we subsequently examined the effect of

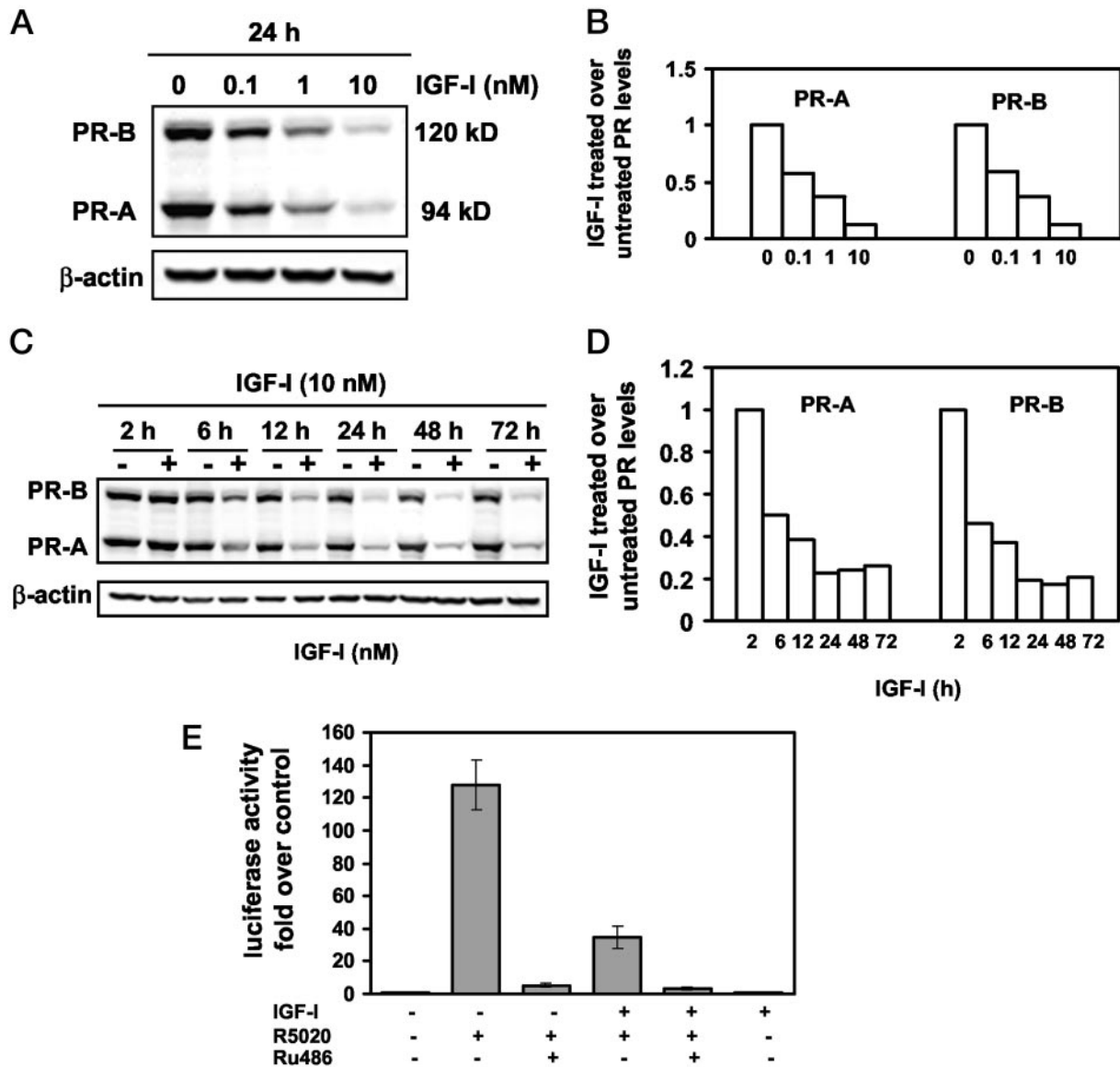


Fig. 1. IGF-I Down-Regulates PR in MCF-7 Cells

A, MCF-7 cells were stimulated for 24 h with increasing concentrations of IGF-I (0.1, 1, 10 nM). Cell lysate (40 μ g) was separated by 8% SDS-PAGE and immunoblotted with anti-PR antibody. β -Actin was used as a loading control. B, Graphical representation of data in panel A after densitometry and correction for β -actin expression. PR levels (represented by the densitometric readings) in IGF-I-treated samples were compared with those in the control sample. C, MCF-7 cells were stimulated with 10 nM IGF-I or vehicle for increasing periods of time. Cell lysates were immunoblotted with anti-PR antibody. D, Graphical representation of data in panel C after densitometry and correction for β -actin expression. PR levels (represented by the densitometric readings) in IGF-I-treated samples were compared with those in the control sample at each time point. E, MCF-7 cells in six-well dishes were transiently transfected with 0.2 μ g PRE-tk-luc vector. After treatment with 10 nM IGF-I for 6 h, cells were stimulated with 10 nM R5020, 100 nM RU486, or a combination for 12 h. Then, cells were lysed and luciferase assays were performed. The β -galactosidase expression vector pSV- β -Gal was used as an internal transfection control. Values are means \pm SE of three independent experiments, each in duplicate.

EGF, HRG, and insulin on PR expression using immunoblotting. As a control, we treated cells with R5020 and found the characteristic upward protein mobility shift (due to phosphorylation) and decrease in PR levels (due to proteasomal degradation—see Fig. 2B) as reported previously (32). Not surprisingly, HRG and EGF at similar concentrations to that of IGF-I also

markedly down-regulated PR (Fig. 2A), but insulin required 100-fold higher concentrations than IGF-I to achieve the same PR reduction. This suggests that the reduction of PR expression by IGF-I is a common effect shared by other growth factors in MCF-7 cells.

As reported previously, IGF-I can induce extensive phosphorylation of insulin receptor substrates (IRSs),

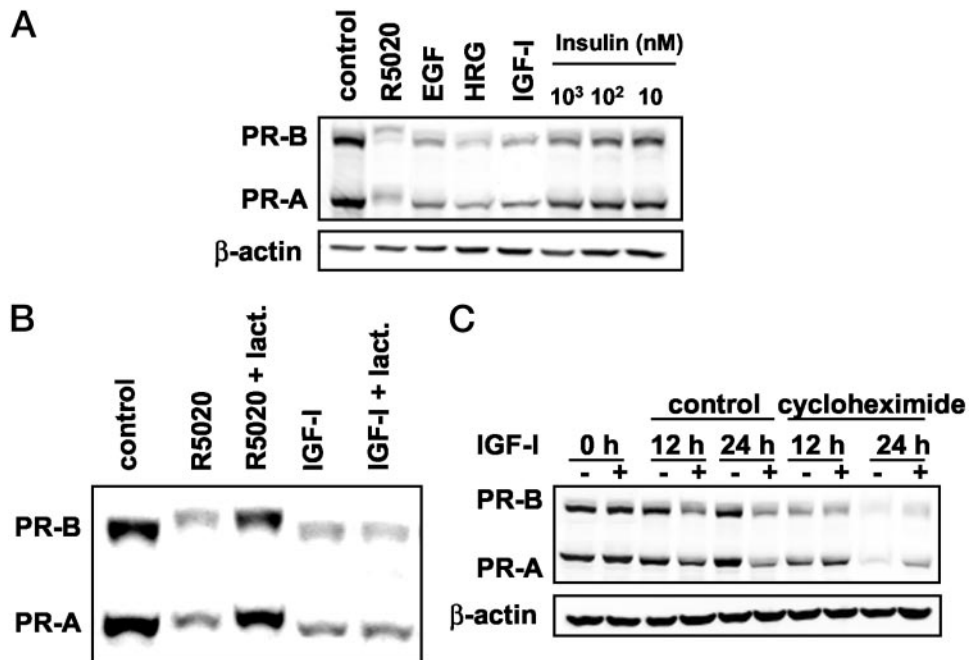


Fig. 2. IGF-I, EGF, HRG, and Insulin Down-Regulate PR by a Different Mechanism than R5020

A, MCF-7 cells were stimulated for 24 h with 20 nM EGF, 10 nM HRG, 10 nM IGF-I, increasing concentrations of insulin (10, 10², 10³ nM), or 10 nM R5020. Cell lysates were immunoblotted with anti-PR antibody. β -Actin was used as a loading control. B, Cells were first treated with the 26S proteasome inhibitor lactacystin (10 μ M) for 30 min before IGF-I and R5020 stimulation in the presence of lactacystin for 12 h. Cell lysates were immunoblotted with anti-PR antibody. C, MCF-7 cells were stimulated with 10 nM IGF-I or vehicle for increasing periods of time in the presence of the translation inhibitor cycloheximide (10 μ g/ml) after preincubation with cycloheximide for 30 min. Cell lysates were immunoblotted with anti-PR antibody. β -Actin was used as a loading control. This figure is representative of three independent experiments.

which leads to an upward protein mobility shift on SDS-PAGE gels and decreased IRSs levels via proteasomal degradation (33). Similarly, progestins cause phosphorylation and a mobility up-shift of PR, and they decrease PR levels via proteasomal degradation (32). Interestingly, IGF stimulation did not lead to a mobility shift of PR on SDS-PAGE gels, unlike the progestin R5020 (Fig. 2, A and B). Thus, the mechanism by which IGF-I down-regulates PR in breast cancer cells may be different from the 26S proteasome degradation pathway. This was confirmed by the inability of lactacystin, a 26S proteasome inhibitor, to block the decrease of PR protein levels by IGF-I (Fig. 2B). To further investigate how IGF-I affects PR protein stability, we used the translation inhibitor cycloheximide. Western blot analysis demonstrated that, in contrast to noncycloheximide conditions, PR protein levels were not lowered by IGF-I treatment compared with non-IGF treatment under cycloheximide conditions (Fig. 2C, see 12-h and 24-h lanes). This indicates that IGF-I does not impede PR protein stability, which is consistent with the result from the proteasome inhibitor assay (Fig. 2B).

IGF-I Represses PR Transcription

To investigate whether IGF-I suppresses PR protein levels by inhibiting PR transcription, we conducted

real-time quantitative RT-PCR (Q-PCR) to detect changes of PR mRNA concentrations in MCF-7 cells after IGF-I treatment. Because more than five PR mRNA transcripts with a wide range of sizes have been found in breast cancer cells and tumors, and it has not been clarified which codes for PR-A, PR-B, or both (20, 34), we chose primers and the fluorescent probe located near the 3' end of the PR coding region so as to detect all PR transcripts. We found that total PR mRNA transcript levels gradually decreased during the 24-h time period (Fig. 3A), which can probably be attributed to the fact that PR is ER dependent and estradiol is depleted in the serum-free medium (SFM) that was used in our cell stimulation experiments. However, compared with controls, PR mRNA levels dropped dramatically after 2 h of IGF-I treatment and continued to decrease with time, which was consistent with the change of PR protein levels in the IGF-I time course experiment (Fig. 1B). Interestingly, PR protein levels did not show a detectable drop at the 2-h time point, suggesting that the repression of PR mRNA levels by IGF-I occurred earlier than the reduction of PR protein levels. At any selected time point, PR mRNA levels under IGF-I-treated conditions were clearly lower than untreated controls. The decrease of PR mRNA levels by IGF-I was confirmed by Northern blotting using a probe at the 3' end of the PR coding

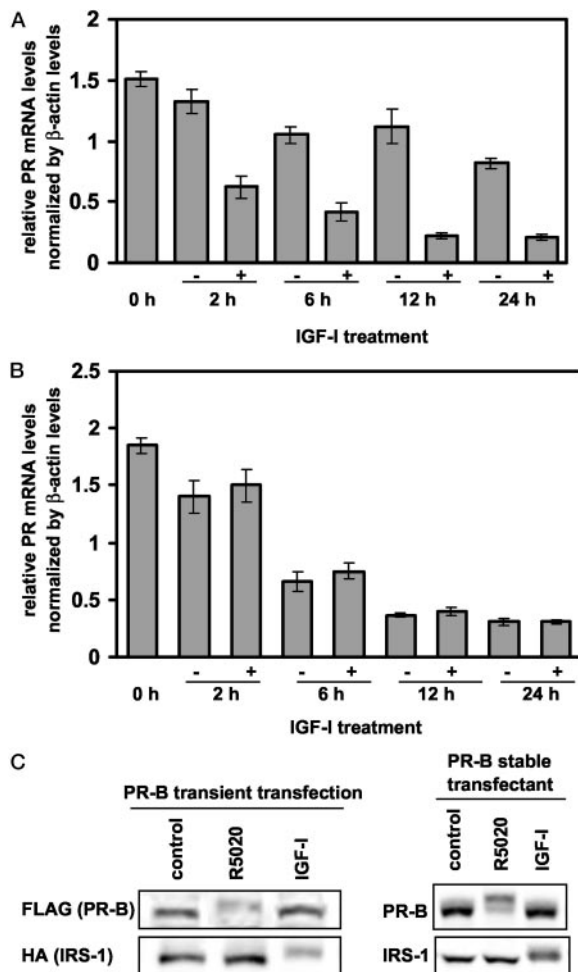


Fig. 3. IGF-I Down-Regulates PR mRNA Levels in MCF-7 Cells

A, MCF-7 cells were stimulated with 10 nM IGF-I or vehicle for increasing periods of time. Total RNA was isolated and PR mRNA levels measured by Q-PCR. PR mRNA levels in each sample were calculated from a standard curve and normalized using β -actin mRNA levels. B, MCF-7 cells were stimulated with 10 nM IGF-I or vehicle for increasing periods of time in the presence of the transcription inhibitor DRB (50 μ M) after preincubation with DRB for 30 min. Total RNA was isolated and Q-PCR was performed. C, FLAG-PR-B and HA-IRS-1 constructs were transiently cotransfected into MCF-7 cells. After 18 h, cells were stimulated with 10 nM IGF-I or 10 nM R5020 for 24 h. Similarly, stable transfectants of PR-B were stimulated with 10 nM IGF-I or 10 nM R5020 for 24 h. Cell lysates were immunoblotted with anti-FLAG, anti-HA, anti-IRS-1, and anti-PR antibodies. IRS-1 mobility shift and down-regulation was used as a marker for IGF-I responsiveness. This figure is representative of two independent experiments.

region (data not shown). These results indicate that IGF-I represses PR mRNA levels in breast cancer cells.

To determine whether posttranscriptional mechanisms are also involved in the IGF-I down-regulation of PR mRNA, we preincubated MCF-7 cells with the transcription inhibitor 5,6-dichlorobenzimidazole riboside (DRB), and then stimulated the cells with IGF-I in the

presence of DRB. Q-PCR showed that in the presence of DRB, PR mRNA levels dropped due to RNA degradation (Fig. 3B). However, in contrast to non-DRB conditions (Fig. 3A), PR mRNA levels under DRB conditions were not lowered by IGF-I compared with non-IGF-I treatment. The same result was confirmed by Northern blotting (data not shown). This suggests that IGF-I does not impair PR mRNA stability.

To confirm that IGF-I down-regulates PR through regulation of PR promoter activity, we transiently transfected a FLAG epitope-tagged PR-B cDNA and an HA epitope-tagged IRS-1 cDNA, both of which were driven by the cytomegalovirus (CMV) promoter, into MCF-7 cells. As expected, immunoblotting showed that IGF-I treatment did not reduce the exogenous PR protein levels that were generated from a heterologous promoter (CMV), whereas it did lower the exogenous IRS-1 levels and cause its mobility up-shift (Fig. 3C). As a control, the exogenous PR protein could still be regulated normally by R5020, *i.e.* PR protein levels were decreased and PR protein mobility was shifted upward. Furthermore, we stably transfected the PR-B cDNA into a specifically selected MCF-7 cell subline, which does not have detectable ER and PR but still responds to IGF-I (35). Similar to the transient transfection, IGF-I did not decrease PR-B levels in the stable transfectants but altered the endogenous IRS-1 levels and mobility (Fig. 3C). Then we transiently transfected a PRE-luciferase construct into these PR-B stable transfectants. Opposite to the data in regular MCF-7 cells, IGF-I could not inhibit the R5020-induced luciferase activity (data not shown).

In summary, these data show that IGF-I down-regulates PR mRNA transcription, and this is dependent upon the original PR promoter context.

IGF-I Regulates PR Expression through the PI3K/Akt/Mammalian Target of Rapamycin (mTOR) Pathway

The transmission of IGF signals in breast cancer cells involves several well-characterized signaling cascades downstream of IGF-I receptor (IGF-IR), including the PI3K/Akt and Ras/MAPK pathways (2). To identify the signaling pathway implicated in the IGF-I down-regulation of PR, we used a series of potent signaling inhibitors against Akt, MEK, mTOR, p38 MAPK, PI3K, protein kinase A (PKA), and protein kinase C (PKC). These inhibitors have been widely used in signal transduction research including our own previous studies (33). MCF-7 cells were first preincubated with these inhibitors and then stimulated with IGF-I in the presence of the inhibitors. Western blot analysis showed that blockade of Akt with 10 μ M 1L-6-hydroxymethyl-*chiro*-inositol- (R)-2-O-methyl-3-O-octadecylcarbonate (HIMOC), of mTOR with 40 nM rapamycin, or of PI3K with 20 μ M LY294002, rescued IGF-I down-regulated PR to the control level (Fig. 4A). In contrast, the MEK inhibitor U0126, the p38 MAPK inhibitor SB203580, the PKA inhibitor H-89, and the PKC inhibitor GF109203X did not prevent IGF-I re-

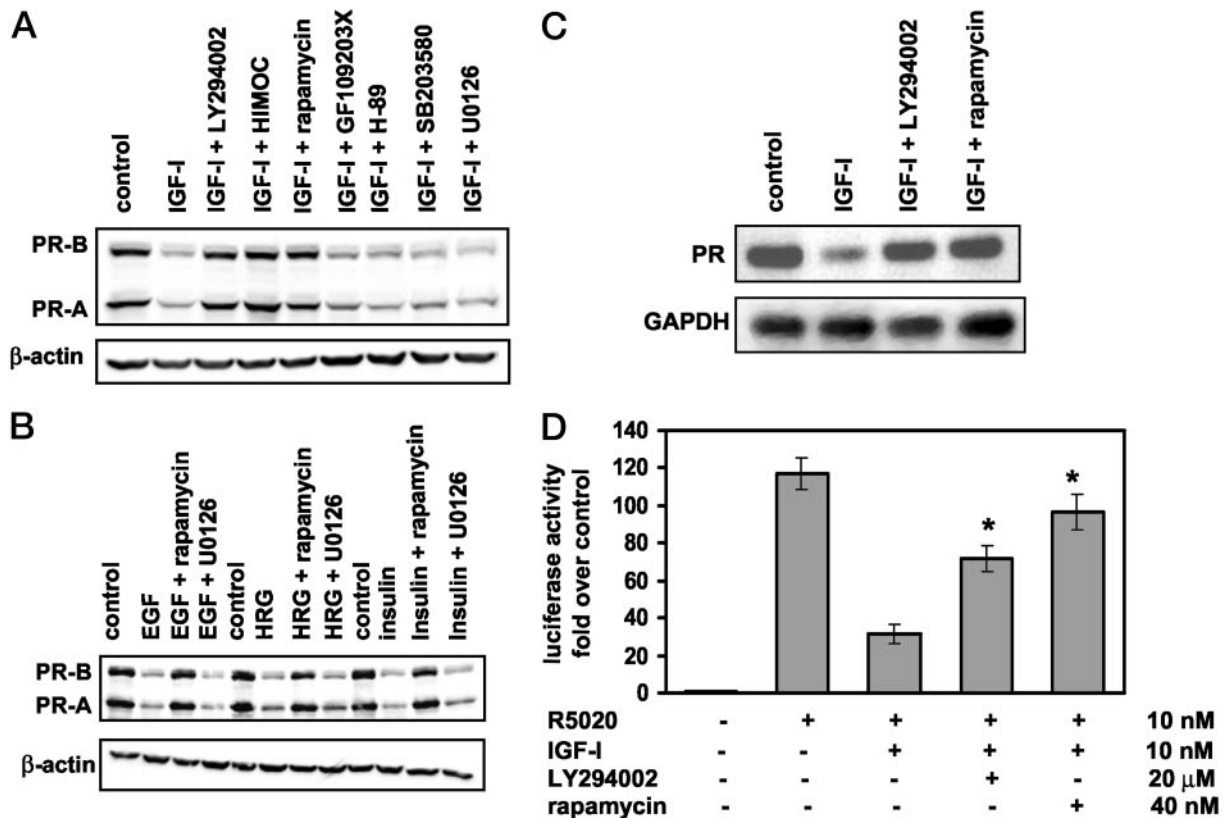


Fig. 4. The PI3K Inhibitor LY294002 and the mTOR Inhibitor Rapamycin Prevent IGF-I Down-Regulation of PR

A, MCF-7 cells were stimulated with 10 nM IGF-I for 24 h in the presence of the inhibitors after preincubation with 20 μ M LY294002 (PI3K), 10 μ M HIMOC (Akt), 40 nM rapamycin (mTOR), 5 μ M GF109203X (PKC), 10 μ M H-89 (PKA), 10 μ M SB203580 (p38 MAPK), or 5 μ M U0126 (MEK) for 30 min. Cell lysates were immunoblotted with anti-PR antibody. β -Actin was used as a loading control. B, MCF-7 cells were stimulated with 20 nM EGF, 10 nM HRG, or 1 μ M insulin for 24 h in the presence of the inhibitors of mTOR and MEK. Cell lysates were immunoblotted with anti-PR antibody. β -Actin was used as a loading control. C, MCF-7 cells were stimulated with 10 nM IGF-I in the presence of the inhibitors for 12 h after preincubation with 20 μ M LY294002 or 40 nM rapamycin for 30 min. Total RNA was isolated and RT-PCR was conducted. PCR product was visualized with ethidium bromide staining under UV light. GAPDH was used as an internal control for RT-PCR. D, MCF-7 cells in six-well dishes were transiently transfected with 0.2 μ g PRE-tk-luc vector. The cells were preincubated with LY294002 and rapamycin for 30 min before IGF-I treatment for 6 h followed by R5020 stimulation for 12 h. Then, cells were lysed and luciferase assays were performed. pSV- β -Gal was used as an internal transfection control. Values are means \pm SE of three independent experiments, each in duplicate. *, $P < 0.005$ as compared with IGF-I plus R5020 treatment.

duction of PR in MCF-7 cells. These results were confirmed using other common inhibitors against the same target kinases such as the MEK inhibitor PD98059, the PI3K inhibitor wortmannin, and the PKC inhibitor GO6983 (data not shown). As PI3K, Akt, and mTOR form a sequential signaling cascade activated by the IGF-IR (36), the IGF-I effect on PR appears to be specifically elicited via the PI3K/Akt/mTOR pathway. Not surprisingly, the same signaling pathway was found to be involved in the EGF, HRG, and insulin down-regulation of PR (Fig. 4B). Because IGF-I represses PR at the transcription level, we confirmed by RT-PCR that inhibitors of PI3K or mTOR abolished the decrease of PR mRNA by IGF-I (Fig. 4C).

As shown previously (Fig. 1E), IGF-I down-regulates R5020-induced PR transcriptional activity. To assess the role of PI3K and mTOR in this event, we transiently transfected a PRE-luciferase construct into MCF-7

cells. Subsequent stimulation experiments using R5020, IGF-I, and the PI3K and mTOR inhibitors showed that the inhibitors impaired IGF-I reduction of R5020-induced luciferase activity (Fig. 4D). Noticeably, luciferase activity did not fully recover, unlike the PR protein levels (Fig. 4A). We speculate that the toxicity of these inhibitors to transfected cells and to the transcription machinery on the PRE promoter may contribute to their partial effect.

To confirm that the PI3K/Akt/mTOR pathway is involved in IGF-I down-regulation of PR, we used MCF-7 cells stably transfected with either a kinase-defective Akt (KD-Akt) containing a K179M substitution or a constitutively active Akt (myr-Akt) containing a myristoylation membrane-targeting sequence. Western blot analysis showed that overexpression of KD-Akt moderately elevated PR levels in MCF-7 cells in SFM, whereas overexpression of myr-Akt dramatically de-

creased PR levels (Fig. 5A). Interestingly, ER α protein levels were not different in the parental and transfected MCF-7 cells. As expected, mTOR phosphorylation was attenuated in KD-Akt cells but enhanced in myr-Akt cells (Fig. 5A). These data are consistent with Akt having a role in IGF-I down-regulation of PR in breast cancer cells.

To examine how ER regulates PR in both stable transfectants, we treated the cells with estradiol. As shown in Fig. 5B, 10 nM estradiol induced PR expression over the basal control levels in both KD-Akt and myr-Akt cells, suggesting that ER functions properly in both cell lines and that the effect of mutant Akt on PR

is not mediated by influencing ER. Interestingly, LY294002 pretreatment displayed a stronger effect in blocking the IGF-I effect in KD-Akt transfectants than in myr-Akt transfectants, although rapamycin had a similar effect in both cells (Fig. 5B). This can be explained by the fact that Akt is downstream of PI3K and upstream of mTOR, and that myr-Akt activity is largely independent of growth factor and PI3K activation. Taken together, our data demonstrate that the PI3K/Akt/mTOR pathway uniquely mediates the alteration of PR expression elicited by IGF-I in breast cancer cells.

IGF-I Down-Regulation of PR Is Independent of ER Activity

It has been reported previously that IGF-I down-regulates ER levels in breast cancer cells (37, 38), which we also found (data not shown). Accordingly, this raised a question as to whether the IGF-I effect on PR was actually caused by the IGF-I effect on ER. This intriguing enigma prompted us to explore if there is a correlation between ER and PR expression regulated by IGF-I.

Initially, we had three pieces of evidence to suggest that the down-regulations of PR and ER are two independent events triggered by IGF-I. First, PR expression in T47D breast cancer cells, which is independent of ER (19), was also significantly reduced by IGF-I (data not shown). Second, as shown in Fig. 5, PR levels were markedly lower in myr-Akt stable transfectants than in KD-Akt cells, even though ER levels, and ER's ability to induce PR, were similar in both cells. Third, PR levels were remarkably lower when MCF-7 cells were grown in dextran-charcoal-treated serum (DCS: estradiol and other steroid hormones removed, but growth factors maintained) medium than in SFM, whereas ER levels were constant in both media (data not shown). Thus, to further test this separation of effects, we performed the following experiments.

Previous reports have shown that IGF-I can cause ligand-independent activation of ER via alteration of ER phosphorylation status in breast cancer and other cells, and even *in vivo* (39–41). To test this in our experimental system, we transiently transfected an estrogen response element (ERE)-luciferase vector into MCF-7 cells, which were then stimulated with estradiol, IGF-I, or the antiestrogen ICI 182,780 (ICI). As shown in Fig. 6A, luciferase activity was significantly increased by estradiol or IGF-I and was further elevated by the two together, whereas ICI blocked the effect of both estradiol and IGF-I, suggesting that the IGF-I-induced luciferase activity is mediated by ER and accordingly that IGF-I can directly induce the transcriptional activity of ER. Surprisingly, Western blot analysis showed that estradiol and IGF-I cotreatment severely attenuated estradiol-induced PR levels, which dropped even lower than that of the unstimulated control (Fig. 6B). This suggests that IGF-I may directly repress PR expression, even while it activates ER, and that this repression is dominant over ER ac-

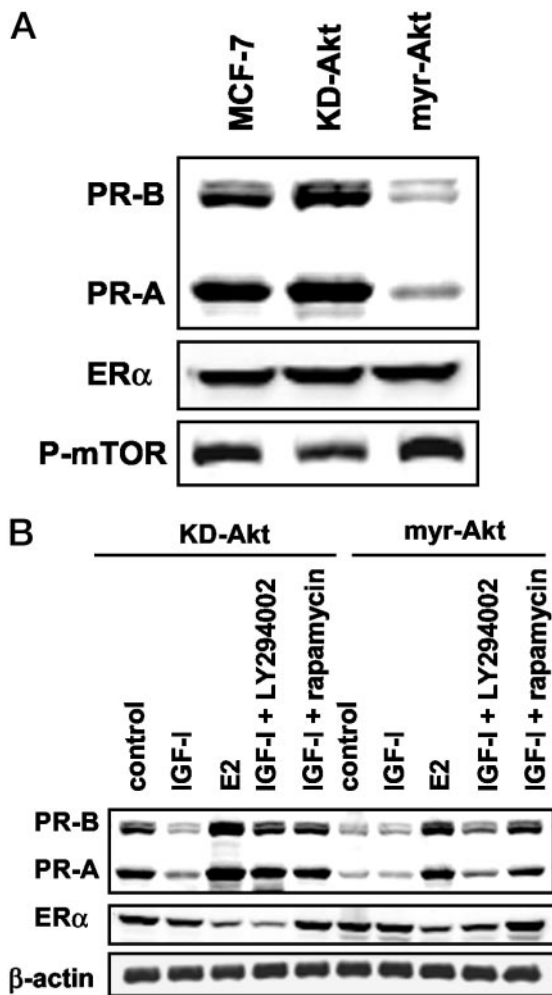


Fig. 5. Expression of KD-Akt and myr-Akt Alters PR Levels in MCF-7 Cells

A, Cell lysates from stable transfectants of KD-Akt and myr-Akt, and parental MCF-7 cells grown in SFM for 48 h were immunoblotted with anti-PR, anti-ER α , and anti-phospho-mTOR antibodies. **B**, KD-Akt and myr-Akt transfectants were stimulated for 24 h with 10 nM estradiol or 10 nM IGF-I in the presence of the inhibitors after preincubation with 20 μ M LY294002 or 40 nM rapamycin for 30 min. Cell lysates were immunoblotted with anti-PR and anti-ER α antibodies. β -Actin was used as a loading control. This figure is representative of three independent experiments.

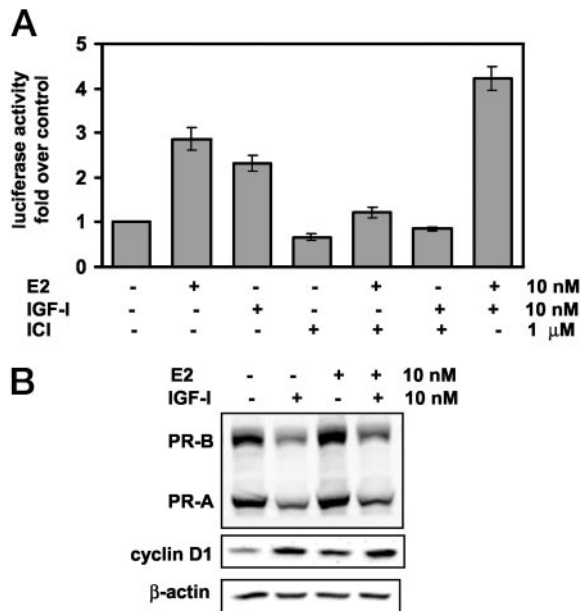


Fig. 6. IGF-I Induces Ligand-Independent ER Activity While Reducing Estradiol-Induced PR Expression

A, MCF-7 cells in six-well dishes were transiently transfected with 0.2 μ g ERE-tk-luc vector. After stimulation with 10 nM IGF-I, 10 nM estradiol, 1 μ M ICI, or their combination for 12 h, cells were lysed and luciferase assays were performed. pSV- β -Gal was used as an internal transfection control. Values are means \pm SE of three independent experiments, each in duplicate. **B**, MCF-7 cells were stimulated with 10 nM IGF-I, 10 nM estradiol, or their combination for 12 h. Cell lysates were prepared and immunoblotted with anti-PR and anti-cyclin D1 antibodies. β -Actin was used as a loading control.

tivity on PR. In contrast to PR, expression of cyclin D1, which is also ER dependent (42), was increased by estradiol, IGF-I, and the two together (43, 44). Thus, IGF-I has opposite effects on ER-inducible PR and ER-inducible cyclin D1 expression, even though it activates ER transcriptional activity.

To elucidate the difference between the IGF-I effects on ER activity and PR levels, we transiently co-transfected ERE-luciferase with myr-Akt or a constitutively active PI3K catalytic subunit p110 (myr-PI3K) into MCF-7 cells. As shown in Fig. 7A, estradiol or IGF-I treatment caused a 2- to 3-fold increase of the reporter activity over the nontreated control, whereas overexpression of myr-Akt or myr-PI3K led to a similar or higher increase of the luciferase activity in the absence of estradiol. This was due to the ligand-independent activation of ER by Akt and PI3K (PI3K utilizes both Akt-dependent and Akt-independent pathways) (45, 46). On the other hand, when we transiently co-transfected PRE-luciferase with myr-Akt or myr-PI3K into MCF-7 cells, either IGF-I or overexpression of myr-Akt or myr-PI3K inhibited the R5020-induced luciferase activity (Fig. 7B). Thus, myr-Akt and myr-PI3K can mimic the effect of IGF-I in suppressing PR activity through down-regulation of PR. Taken together, these data indicate that IGF-I regulates ER activity and ER-

dependent PR levels in opposite ways, and that IGF-I down-regulation of PR expression is independent of ER (Fig. 8).

DISCUSSION

PR is an important factor in mammary gland and breast cancer development. Because PR is a highly ER-dependent gene, regulation of PR expression by other proteins and growth factors has been naturally regarded as indirect and attributed to changes of ER status. Here, we provide evidence showing that IGF-I down-regulates PR in breast cancer cells irrespective of ER activity through a transcriptional mechanism involving the PI3K/Akt/mTOR signaling pathway (Fig. 8).

In this study, we found that EGF, HRG, and insulin can also sharply reduce PR levels. An obvious common feature between IGF-I and these growth factors is that they all can activate the PI3K/Akt signaling cascade in breast cancer cells, which explains why they all display the same effect on PR expression. One could speculate that other growth factors like PDGF, which also activates the PI3K/Akt pathway through its receptors, may likewise down-regulate PR in breast cancer cells that express PDGF receptors (47). But why does insulin require much higher doses to achieve the same effect as IGF-I even though MCF-7 cells have relatively high levels of insulin receptor (48)? We found that IGF-I is more potent than insulin at the same concentration (10 nM) to induce phosphorylation of IRS-1 and Akt, an indicator of activated PI3K/Akt signaling (Ref. 33 and data not shown). Thus, high concentrations of insulin may be necessary for insulin to act through IGF-IR to decrease PR levels in MCF-7 cells because insulin has a relatively low affinity to IGF-IR (33, 49). Alternatively, high doses of insulin may simply enhance the insulin receptor-mediated PI3K/Akt signaling pathway.

It is a long-held rule of thumb that optimal estradiol stimulation of PR occurs in medium supplemented with 5% DCS (50). This may be attributed to the fact that DCS medium contains large amount of growth factors and consequently suppresses PR to barely detectable levels, whereas the basal level of PR is much higher in SFM. Hence, the fold-induction of PR with estradiol is seemingly more dramatic in DCS medium. This is perhaps also the reason behind the previous conflicting reports in which PR was used as a reporter gene monitoring ER activity in the study of IGF-I regulation of ER. Clayton *et al.* (37) showed that insulin and IGF-I considerably impaired the estradiol induction of PR mRNA in SFM, which is in line with our result. In another study that showed that IGF-I had a potentiating effect on the estradiol induction of PR mRNA (38), the cells were grown in DCS medium for 2 d. In this system, because PR transcription was already suppressed by growth factors, the observed IGF-I effect might actually indicate a change in PR

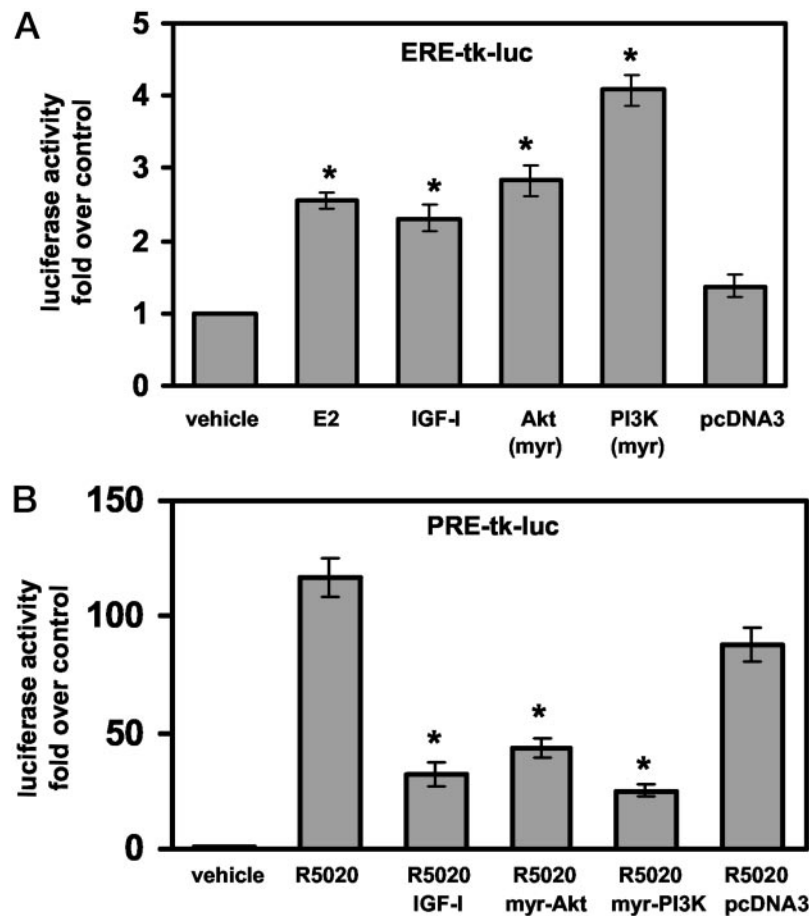


Fig. 7. Myr-Akt and myr-PI3K Mimic the IGF-I Effect on Ligand-Independent ER Activity and R5020-Induced PR Activity

A, MCF-7 cells in six-well dishes were transiently cotransfected with 0.2 μ g ERE-tk-luc and 0.5 μ g myr-Akt, myr-PI3K, or empty pcDNA3 vector. ERE-tk-luc-only transfected cells were stimulated with 10 nM estradiol or 10 nM IGF-I and were compared with nonstimulated ERE-tk-luc plus myr-Akt or myr-PI3K cotransfected cells in luciferase assay. Values are means \pm SE of three independent experiments, each in duplicate. *, $P < 0.005$ as compared with pcDNA3 transfection. B, MCF-7 cells in six-well dishes were transiently cotransfected with 0.2 μ g PRE-tk-luc and 0.5 μ g myr-Akt, myr-PI3K or pcDNA3.1 vectors. Cells were treated with R5020 to induce PR activity. PRE-tk-luc-only transfected cells were stimulated with 10 nM R5020 or 10 nM IGF-I plus 10 nM R5020. Values are means \pm SE of three independent experiments, each in duplicate. *, $P < 0.005$ as compared with pcDNA3 transfection.

mRNA and/or protein stability. Previously, Cho *et al.* (50) reported that IGF-I does not affect PR transcription but elevates PR protein levels in MCF-7 cells under SFM conditions. The discrepancy between their and our results may be due to the different cell culture conditions before IGF-I stimulation. In our study, before IGF-I treatment, cells were starved for 16 h after being switched to SFM from regular culture medium, whereas there was no delay of IGF-I treatment after the change to SFM from DCS medium in their studies.

Recently, evidence is emerging to support our conclusion that IGF-I and other growth factors inhibit PR expression. First, it was found that overexpression of ER in a specifically selected ER⁻/PR⁻ MCF-7 breast cancer cell subline did not restore PR expression, although ER did restore cyclin D1, IRS-1, and IGF-IR levels (35). These cells were grown in DCS medium, which might completely suppress PR expression after

long-term cell culture. Similarly, it was reported that in antiestrogen-resistant MCF-7 cells generated by continuous culture of the PR⁺ parental cells in antiestrogen-supplemented DCS medium, EGF receptor signaling was enhanced, whereas PR levels diminished (51). Interestingly, replacement of antiestrogen by estradiol failed to induce PR, whereas expression of other estrogen-responsive genes was significantly elevated. Conceivably, DCS medium plus enhanced EGF receptor signaling may permanently silence PR expression. In another interesting report, the mammary epithelial cells that retain stem/progenitor cell characteristics were found to lack PR (52). These cells rely on growth factors to continuously proliferate. Recently, in a study that confirmed that ER⁺/PR⁺ breast cancer patients respond better to hormonal therapy than ER⁺/PR⁻ patients, Dowsett *et al.* (53) found that among ER⁺/PR⁻ samples 25% are HER2⁺, com-

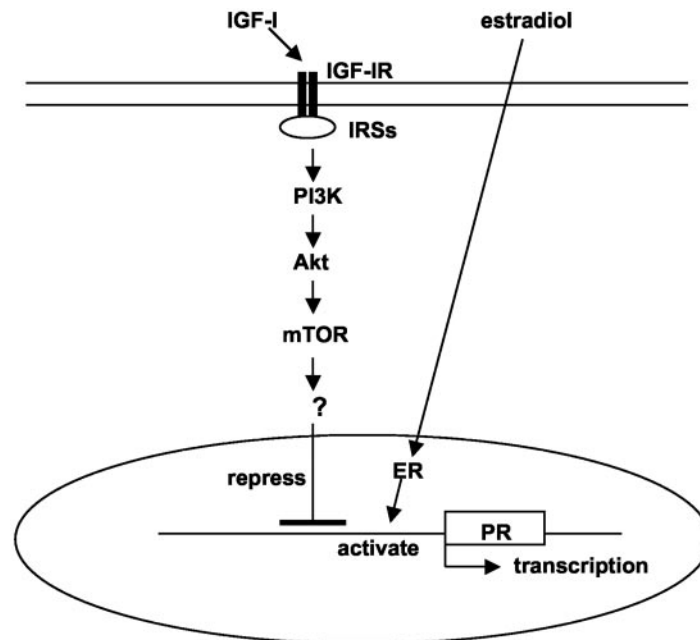


Fig. 8. Illustration of the IGF-I Signaling Pathway Repressing PR Expression

pared with 10% HER2+ among ER+/PR+. Moreover, HER2+/PR- patients responded much worse than HER2-/PR+ in hormonal therapy, again suggesting that PR status may reflect HER2 signaling.

Our result indicates that the PI3K/Akt/mTOR pathway is responsible for the IGF-I down-regulation of PR. In a relevant report, Shen *et al.* (54) showed that, although overexpression of MEKK1, through p42 and p44 MAPK, increased R5020-induced PR activity, it alone could not down-regulate PR. Thus, PI3K/Akt and Ras/MAPK pathways may play distinct roles in regulating PR activity in breast cancer cells. However, we only observed IGF-I down-regulation of PR despite IGF-I's ability to trigger both PI3K/Akt and Ras/MAPK pathways in MCF-7 and other breast cancer cells. This may be due to the finding that Ras can also bind to and activate PI3K (55), and the PI3K/Akt pathway is very potent in eliciting IGF-I signals in MCF-7 cells. Hence, the PI3K/Akt pathway is dominant in IGF-I regulation of PR. Interestingly, the PI3K/Akt/mTOR pathway is also involved in the IGF-I reduction of IRSs (33). The former event is mediated by a transcriptional mechanism, whereas the latter is via the 26S proteasome pathway for protein degradation. Different downstream proteins of mTOR may account for this seeming dilemma—although p70 S6 kinase is a typical target for mTOR, other kinases or signaling proteins may also function downstream of mTOR. For example, it has been shown that insulin regulation of IGFBP-1 gene expression is dependent on mTOR but independent of p70 S6 kinase activity (56). We hypothesize that mTOR may directly or indirectly activate a PR transcription repressor or inactivate a transcription activator to mediate the IGF-I effect on PR expression (Fig. 8).

The ER and PR status in breast cancer is highly correlated with the response to endocrine therapy. Key areas of study in breast cancer are those mechanisms that regulate ER and PR expression. The loss of PR gene expression has been attributed to loss of heterozygosity, loss of ER function, and methylation of a CpG island in the PR promoter (26). Our data provide implications for another theory, in which potent growth factor signaling, especially PI3K/Akt, may contribute to the PR down-regulation. This is reflected clinically with reduced PR levels in breast tumors with HER2 amplification (53). Because the IGF-I effect involves a transcriptional mechanism, whether IGF-I increases methylation of PR promoter and accordingly silences PR expression remains an intriguing question.

Low or absent PR in primary breast cancer is associated with faster disease progression and poor response to hormonal therapy. Our results suggest that low PR may be serving as an indicator of activated growth factor signaling in breast tumor cells, and therefore of an aggressive tumor phenotype and resistance against hormonal therapy.

MATERIALS AND METHODS

Materials

All general materials and chemicals were purchased from Sigma (St. Louis, MO) unless otherwise noted. IGF-I was purchased from GroPep Pty. Ltd. (Adelaide, Australia). ICI was a kind gift from Zeneca Pharmaceuticals (Macclesfield, UK). The inhibitors GF109203X, H-89, lactacystin, LY294002, HIMOC, rapamycin, SB203580, and U0126 were from Calbiochem (La Jolla, CA). All tissue culture reagents were purchased from Invitrogen (Carlsbad, CA) unless otherwise

stated. [³²P]deoxy-CTP (3000 Ci/mmol) was purchased from NEN Life Science Products (Boston, MA).

Cell Culture and Plasmids

MCF-7 cells have been maintained in our laboratory for many years (57). Cells were routinely maintained in improved MEM zinc option (IMEM) supplemented with 5% fetal bovine serum, 2 mM glutamine, 50 IU/ml of penicillin, 50 μg/ml of streptomycin, and 10 μg/ml insulin. SFM consists of IMEM without phenol red plus 10 mM HEPES (pH 7.4), 1 μg/ml of transferrin, 1 μg/ml of fibronectin, 2 mM glutamine, 50 IU/ml of penicillin, 50 μg/ml of streptomycin, and trace elements (BioSource Technologies, Inc., Camarillo, CA). Cells were kept at 37 C in a humidified incubator with 5% CO₂. The estrogen-responsive reporter plasmid ERE-tk-luc contains a single consensus ERE upstream of a minimal thymidine kinase (tk) promoter and the luciferase (luc) gene (40). The PRE-tk-luc was constructed in the same way. The expression vectors for constitutively active myr-Akt and myr-PI3K were described elsewhere (58), and were gifts from Dr. Thomas Franke and Dr. Anke Klippel. The murine myr-Akt and myr-PI3K (p110α) each have a myristoylation signal at the N terminus to target the protein to the membrane.

Cell Stimulation and Lysis

Cells were plated at a density of 1.5×10^6 per 6-cm-diameter dish (Becton Dickinson and Co., Lincoln Park, NJ) and allowed to grow for 48 h. Then the medium was changed to SFM, and 16 h later, the cells were stimulated with 10 nM IGF-I, 20 nM EGF, 10 nM HRG, or insulin at 10, 100, or 10^3 nM for different periods of time. For experiments using inhibitors, cells were first preincubated separately with GF109203X (5 μM), H-89 (10 μM), lactacystin (10 μM), LY294002 (20 μM), a HIMO (10 μM), rapamycin (40 nM), SB203580 (10 μM), and U0126 (5 μM) for 30 min before stimulation with EGF, HRG, IGF-I, and insulin in the presence of an inhibitor as described previously (33). For the translation inhibitor cycloheximide (10 μg/ml), the same procedure was followed. Control cells were incubated with a similar concentration of the vehicle dimethylsulfoxide alone. After stimulation, cells were washed twice with ice-cold PBS and then lysed in 200 μl of lysis buffer, which contained 50 mM Tris-HCl (pH 7.4), 1% Nonidet P-40, 2 mM EDTA, 100 mM NaCl, 10% glycerol, and a fresh protease inhibitor cocktail (Roche Applied Sciences, Indianapolis, IN). Cells were left on ice for 30 min, and then the cell lysate was clarified by centrifugation at $14,000 \times g$ for 15 min at 4 C and stored at -20 C. Protein concentration of the supernatant was measured by bicinchoninic acid assay in accordance with the manufacturer's instruction manual (Pierce Chemical Co., Rockford, IL).

Immunoblotting

Total protein (40 μg) was resuspended in denaturing sample loading buffer (3% dithiothreitol; 0.1 M Tris-HCl, pH 6.8; 4% sodium dodecyl sulfate; 0.2% bromophenol blue; 20% glycerol), separated by 8% SDS-PAGE, and electrophoretically transferred to a nitrocellulose membrane overnight at 4 C. The remaining steps were all performed at room temperature. The membrane was blocked with PBS plus 0.05% Tween-20 (PBST) containing 5% nonfat milk for 1 h and followed by incubation with a 1:1000 dilution of anti-ER (Novocastra Laboratories, Newcastle upon Tyne, UK), anti-FLAG (Sigma), anti-HA (Covance Laboratories, Inc., Richmond, CA), anti-IRS-1 (Upstate Biotechnology, Lake Placid, NY), anti-PR (DAKO Corp., Carpinteria, CA), anti-β-actin (Upstate Biotechnology), and anti-cyclin D1 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) antibodies in blocking solution for 1–2 h. For phospho-mTOR detection, the membrane was first washed

three times for 5 min each with PBST and then incubated with a 1:1000 dilution of anti-phospho-mTOR antibody (Cell Signaling, Beverly, MA) in PBST. Subsequently, the membrane was again washed three times for 5 min each with PBST and then incubated with a horseradish peroxidase-linked secondary antibody (Amersham Pharmacia Biotech, Piscataway, NJ) at a dilution of 1:4000 in blocking solution. After the membrane was washed three times for 5 min each with PBST, bands were visualized by enhanced chemiluminescence according to the manufacturer's protocols (Pierce Chemical Co.).

RNA Blotting and RT-PCR

Cells were plated at a density of 5×10^6 per 10-cm-diameter dish and allowed to grow for 48 h. Then the medium was changed to SFM. After 16 h starvation, the cells were stimulated with IGF-I for an indicated time period. When the transcription inhibitor DRB (50 μM) and the signaling inhibitors LY294002 and rapamycin were used, cells were first preincubated with them for 30 min before IGF-I stimulation. Total RNA was prepared with RNeasy Midi kit (QIAGEN, Valencia, CA) according to the instruction manual. RNA integrity was checked by separation on a 1% agarose gel. For PCR of the 430-bp PR gene fragment, a pair of primers (5'-CAGTGGGCAGATGCTGTATTTTGC-3', 5'-GTGCAGCAATAACTTCAGACATC-3') was designed toward the 3' end of the PR coding region. Another pair of primers (5'-GGCTTC-CAGAACATCATCCCTGC-3', 5'-GGGTGTCGCTGTTGAAGTCAGAGG-3') was used in PCR of the 299 bp glyceraldehyde-3-phosphatedehydrogenase (GAPDH) gene fragment (59). In the RT-PCR experiment, total RNA (2 μg) was used to produce cDNA with Superscript II reverse transcriptase (Invitrogen) in a 20-μl volume. Then, 1 μl from the cDNA synthesis reaction was added to PCR mixture, and PCR amplification was performed with PR and GAPDH primers with an annealing temperature at 60 C and 30 cycles. Products were revealed by ethidium bromide staining under UV after agarose gel electrophoresis.

Q-PCR

Basically, reverse transcriptions of PR mRNA were performed in 96-well optical plates (PE Applied Biosystems, Foster City, CA) using Superscript II reverse transcriptase. All RNA samples were first treated with deoxyribonuclease I to eliminate residual genomic DNA. The reverse primer (5'-GGCT-TAGGGCTTGCTTTC-3') is at the 3' end of the PR coding region. Total RNA of 100 ng in a 10-μl reaction volume was added to each well. The plates were incubated at 50 C for 30 min followed by 10 min at 72 C. Then real-time quantitative TaqMan PCR of PR cDNAs was conducted using a PR-specific double fluorescence-labeled probe (5'-TCCCA-CAGCCATTGGGCGTTC-3') in an ABI PRISM 7700 Sequence Detector (PE Applied Biosystems). ROX was used as a reference dye. The PCR mixture also contains 300 nM each of the forward primer (5'-GAGCACTGGATGCTGTTGCT-3') and the reverse primer. The plates were incubated at 94 C for 1 min, followed by 40 cycles at 94 C for 12 sec and 60 C for 1 min. FAM was used as the fluorescent reporter coupled at the 5' end of the probe, whereas Block Hole Quencher was conjugated to the 3' end. Each experiment included three nontemplate controls to detect any template contamination. In addition, a control lacking reverse transcriptase was included for each sample to detect any residual genomic DNA. Standard curves for the quantification of PR and β-actin mRNAs were generated using serial 10-fold dilutions from 10^8 to 10^2 copies of synthesized templates. Q-PCR was performed in triplicate of each sample. The obtained PR mRNA concentration was normalized by the β-actin mRNA value.

Transfections

MCF-7 cells were grown for 48 h in IMEM + 5% FBS till 80% confluence before transfection. Stable transfectants of myr-Akt and KD-Akt, which contains K179M substitution, were gifts from Dr. Adriana Stoica (58). Using Lipofectamine (Invitrogen), a FLAG-PR-B expression vector pSG5-hPR1, which was a generous gift from Dr. K. Horwitz, was cotransfected with the neomycin resistant gene vector pcDNA3.1 (Invitrogen) in a 20:1 ratio (PR vector: neomycin vector). G418 of 600 $\mu\text{g/ml}$ was used to select stable clones that were later verified by Western blot analysis with the PR antibody. All transient transfections were performed using Polyfect reagents (QIAGEN) according to the instruction protocols. For cotransfections with FLAG-PR-B and HA-IRS-1 constructs, 0.1 μg DNA of each plasmid was added to one well in six-well dishes. After 18 h, the culture medium was changed to SFM, and cells were incubated for 12 h before stimulation with 10 nM IGF-I and 10 nM R5020 for 24 h. For transfections with only ERE-tk-luc and PRE-tk-luc constructs, cells in six-well dishes were transfected with 0.2 μg DNA. After 5 h, the serum medium was switched to SFM, and cells were incubated for 6–8 h in the presence or absence of IGF-I. Then the cells were stimulated with 10 nM R5020 10 nM or 10 nM estradiol for 10–12 h. For the study of the inhibitors, cells were preincubated with LY294002 and rapamycin for 30 min before IGF-I treatment in the presence of the inhibitors. To examine the effect of myr-Akt and myr-PI3K on ER and PR activity, cells in six-well dishes were cotransfected with 0.2 μg reporter plasmids and 0.5 μg myr-Akt, myr-PI3K or empty pcDNA3 vector. Transfection time was extended to 16 h to allow sufficient time for exogenous Akt and PI3K expression. Then the serum medium was switched to SFM, and cells were stimulated with estradiol or R5020 for 12 h. Twenty nanograms of a β -galactosidase expression vector pSV- β -Gal (Promega Corp., Madison, WI) were cotransfected as an internal control. Luciferase and β -galactosidase assays were performed using the Promega Corp. assay system.

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