Progesterone Receptor-B Regulation of Insulin-Like Growth Factor–Stimulated Cell Migration in Breast Cancer Cells via Insulin Receptor Substrate-2

Yasir H. Ibrahim,² Sara A. Byron,² Xiaojiang Cui,³ Adrian V. Lee,³ and Douglas Yee¹,²

Departments of ¹Medicine and ²Pharmacology, Masonic Cancer Center, University of Minnesota, Minneapolis, Minnesota; and ³Sue and Lester Smith Breast Center, Baylor College of Medicine, Houston, Texas

Abstract

Progesterone action contributes to the signaling of many growth factor pathways relevant to breast cancer tumor biology, including the insulin-like growth factor (IGF) system. Previous work has shown that insulin receptor substrate-2 (IRS-2) but not IRS-1 levels were regulated by progestin in progesterone receptor-B (PR-B) isoform expressing MCF-7 cells (C4-12 PR-B). Furthermore, type 1 IGF receptor (IGF1R) signaling via IRS-2 correlated with the increased cell migration observed in a number of breast cancer cell lines. Consequently, in this study, we examined whether the elevation of IRS-2 protein induced by progestin was sufficient to promote IGF-I–stimulated cell motility. Treatment of C4-12 PR-B cells with progestin shifted the balance of phosphorylation from IRS-1 to IRS-2 in response to IGF-I. This shift in IRS-2 activation was associated with enhanced migration in C4-12 PR-B cells pretreated with progestin, but had no effect on cell proliferation or survival. Treatment of C4-12 PR-B cells with RU486, an antiprogestin, inhibited IGF-induced cell migration. Attenuation of IRS-2 expression using small interfering RNA resulted in decreased IGF-stimulated motility. In addition, IRS-2 knockdown resulted in an abrogation of PKB/Akt phosphorylation but not mitogen-activated protein kinase. Consequently, LY294002, a phosphoinositide-3-kinase inhibitor, abolished IGF-induced cell motility in progestin-treated C4-12 PR-B cells. These data show a role for the PR in functionally promoting growth factor signaling, showing that levels of IRS proteins can determine IGF-mediated biology, PR-B signaling regulates IRS-2 expression, and that IRS-2 can mediate IGF-induced cell migration via phosphoinositide-3-kinase in breast cancer cells. (Mol Cancer Res 2008;6(9):1491–8)

Introduction

Approximately 70% to 80% of primary breast cancers are estrogen receptor (ER) and/or progesterone receptor (PR)–positive and the majority of these tumors are hormone responsive (1). The hormone dependence of ER-positive tumors is more often seen when both ER and PR are expressed. Clinical trial data underscore progesterone’s role in breast cancer; patients who took hormonal replacement therapy (HRT) preparations containing estrogen plus progesterone were at a decreased risk of developing later stage breast cancer compared with patients treated with placebo or estrogen alone (2). Indeed, the hormonal regulation of breast cancer is critical to cancer progression and patient survival.

In normal breast tissue, hormones act in concert with growth factors to maintain the milieu of this organ system. In addition to the role of steroids and growth factors in normal mammary gland biology, it is clear that alterations in the function of steroid receptors and/or growth factor pathways both contribute to tumorigenesis and cancer progression. In addition, steroid pathways encourage growth factor signaling to promote tumor biology by directly modulating the expression of ligands, receptors, and signaling intermediates (3). Although a substantial effort has been placed on understanding ER’s role in promoting growth factor signaling and function, there has been less effort focused on understanding PR’s role in this context.

The insulin-like growth factor (IGF) system is critical in the function of normal and tumorigenic tissues. IGF has been shown to promote and maintain cancer progression (reviewed in ref. 4). In addition, IGF is mitogenic and promotes cancer metastasis (5, 6). Activation of the type 1 IGF receptor (IGF1R) results in the phosphorylation of adaptor proteins, IRS-1 and IRS-2, and the activation of pathways such as phosphoinositide-3-kinase (PI3K) and mitogen-activated protein kinase (MAPK). In breast cancer, the IRS proteins are the primary mediators of the IGF1R signal, making them a critical regulatory step in the IGF signaling cascade (7). Because different phenotypes are stimulated by the IGF1R in breast cancer cells, a differential role for IRSs has been suggested (8). Specific knockdown of IRS-2 in the mammary gland of polyoma virus middle-T–infected female mice displayed significantly impaired tumor metastasis (9). In contrast, specific knockdown of IRS-1 in the mammary gland of polyoma virus middle-T–infected animals elevated IRS-2...
function and promoted tumor metastasis. However, the over-expression of mouse mammary tumor virus IRS-1 or IRS-2 in the mammary gland resulted in tumorigenesis and mammary tumor metastases in both sets of animals (10). Although such differences in IRS function may exist in vivo, it is difficult to determine if these phenotypes are regulated by IGF1R activation or if other pathways are affected by the genetic manipulation of IRS expression in these mouse models. Cell line models indicate that IRS-1 and IRS-2 could mediate specific IGF-induced biology in breast cancer. We and others have found that cell lines expressing and signaling via IRS-1 proliferate in response to IGF-I, whereas cell lines expressing and signaling via IRS-2 migrate and invade in response to the same stimulus (8, 9, 11).

The presence of PR in growth factor-responsive breast cancer tissues and cell lines suggests a role for progesterone in functionally influencing growth factor pathways. In some breast cancers, growth factor pathways depend on hormonal influences to contribute to the cancer phenotype. The loss of ER in MCF-7 cells disrupts functional IGF signaling (12). In fact, it has been suggested that PR functions to prime cells for secondary factors such as growth factor signals (13). In response to progesterone, PR expressing cells up-regulate up to 94 genes, some of which are involved in cell adhesion, signal transduction, metabolism, cell cycling, and apoptosis (14). Two isoforms, PR-A and PR-B, are coexpressed in breast cancer tissues but can regulate different gene sets when expressed independently, with PR-B exhibiting enhanced recruitment of transcriptional coactivators (14). Gene regulation by the two PR isoforms is mediated by activation function (AF) domains with AF-3 in the B-upstream segment of PR-B dictating differential transcriptional regulation (15). Indeed, gene regulation by progesterone involves the expression of proteins important in signaling mediated by multiple pathways, including the IGF pathway because IRS-2 is a PR-B-regulated gene (16). To examine PR-B effects on the IGF system, we wanted to use a model in which a nonfunctional IGF system existed. To do this, MCF-7 cells were selected for a loss of ER and a concurrent loss of the PR (C4-12), and then PR-B was stably overexpressed (C4-12 PR-B). This model is ideal for evaluating the direct effects of PR-B on the IGF system because ER effects on the system are absent; C4-12 cells express IGF1R and IRS-1 in a non–PR-regulated manner, the cells treated with progestin have increased IRS-2 levels, and exhibit an enhancement in IGF signaling via PI3K and MAPK after progestin pretreatment (17). Therefore, we propose that PR expressing cells up-regulate up to 94 genes, some of which are involved in cell adhesion, signal transduction, metabolism, cell cycling, and apoptosis (14). Two isoforms, PR-A and PR-B, are coexpressed in breast cancer tissues but can regulate different gene sets when expressed independently, with PR-B exhibiting enhanced recruitment of transcriptional coactivators
of IRS protein activation in response to IGF from IRS-1 to IRS-2 in PR-B—expressing breast cancer cells. In the absence of progestin, IRS-2 phosphorylation remained at vector control levels (data not shown).

Alteration of IRS-2 Protein Levels by Progesterone Conferred Biological Function to IGF-I

We had previously shown that IRS-2 is critical in the regulation of cell motility, but plays little role in IGF-mediated proliferation (11). We hypothesized that by elevating IRS-2 levels, C4-12 cells would respond to IGF-I by exhibiting a migratory phenotype only, and not a survival or proliferative phenotype.

To examine cell proliferation, C4-12 cells were pretreated with progestin for 24 hours followed by IGF-I and cell number was estimated over a period of 6 days using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Figure 2A illustrates that IGF-I and R5020 did not affect monolayer growth of C4-12 PR-B cells. Furthermore, IGF-I did not alter C4-12 vector cell growth (data not shown; ref. 12). These data show that neither progestin nor IGF-I stimulated cell proliferation of C4-12 cells. In addition, progestin’s effects on IGF-induced survival from chemotherapy were examined. Under these conditions, IGF-I treatment resulted in a slight reduction in PARP cleavage in both the C4-12 vector and PR-B—transfected cells, and was not significantly altered by progestin pretreatment (data not shown).

To address whether the elevation in IRS-2 levels altered IGF-induced cell motility, C4-12 cells were pretreated with progestin overnight and then placed in a modified Boyden chamber in the presence of 5 nmol/L of IGF-I for 6 hours (Fig. 2B). In this experiment, we used the migratory MDA-231BO cell line as a positive control for IGF-I—stimulated motility (11). The C4-12 vector cells were unresponsive to IGF-I stimulation in this assay. Furthermore, progestin alone did not enhance the migration of either the C4-12 vector or PR-B cells. However, C4-12 PR-B cells pretreated with progestin responded to IGF-I with increased migration, whereas IGF-I had no effect in R5020-treated vector cells. The Boyden chamber assay is dependent on cell adhesion. We showed that IGF-I and R5020 did not differentially affect cell adhesion in these cells (data not shown). However, to further confirm that these effects were due to the ability of IGF-I and R5020 to stimulate cell motility, we further confirmed these findings in a wound healing response of adherent monolayer cells. Results were consistent with data shown in Fig. 3 (data not shown). Therefore, ligand-dependent functions of PR-B enhanced IGF-I biology by promoting IGF-induced cell migration.

Progestosterone Receptor Function Is Required for Progestin to Promote IGF-Induced Cell Migration

Because progestin was pivotal in promoting the IGF-induced cell migration of C4-12 PR-B cells, we decided to evaluate whether this effect was dependent on PR-B function. C4-12 cell migration was examined after treatment with the antiprogestin, RU486. To distinguish between IGF effects on adhesion versus migration on fibronectin, we examined time-lapse images of adherent cells pretreated with progestin/antiprogestin. It has been reported that integrin receptors are PR-regulated genes,
and the IGF system has been linked to regulating migration specifically via the fibronectin binding receptor, α5β1 integrin, in breast cancer cells (14, 19). Neither integrin expression nor function were altered by progestin in C4-12 cells, however, IGF promoted adhesion to fibronectin (data not shown). Therefore, following adherence of untreated C4-12 cells to fibronectin, cells were pretreated with 1 nmol/L of R5020 in the presence or absence of 1 μmol/L of the antiprogestin RU486 overnight, and then stimulated with 5 nmol/L of IGF-I. Neither progestin, antiprogestin, nor IGF-I altered the adherence of preadherent cells as observed by time-lapse imaging (data not shown). As previously reported, IGF treatment of C4-12 PR-B cells activated the IGF pathway (Fig. 4A). Transient transfection with IRS-2 siRNA specifically reduced IRS-2 protein levels, but not IRS-1. A parallel reduction in IRS-2 phosphorylation was also observed, but IRS-1 phosphorylation was not changed by IRS-2 siRNA. Interestingly, IRS-2 siRNA-treated cells exhibited a reduction in PKB/Akt activation in response to IGF-I as compared with mock-transfected controls, whereas MAPK activation was unaltered. Therefore, IGF-mediated signaling via IRS-2 is preferentially linked to PI3K signaling in C4-12 PR-B cells.

To test whether IRS-2 function was required for IGF-induced cell motility, cells were transiently transfected with IRS-2 siRNA as described above. Following progestin pretreatment, cells were detached and examined for migration via other pathways not dependent on IRS-2 expression, an IRS-2–specific knockdown approach was employed. Cells were grown to 70% confluence and serum-starved for 24 hours prior to transient transfection with small interfering RNA (siRNA) specifically targeting the IRS-2 sequence for an additional 24 hours in serum-free medium (SFM). Progestin was added posttransfection for another overnight period, at which time cells were treated with 5 nmol/L of IGF-I for 10 minutes and lysates were collected. As previously reported, IGF treatment of C4-12 PR-B cells activated the IGF pathway (Fig. 4A). Transient transfection with IRS-2 siRNA specifically reduced IRS-2 protein levels, but not IRS-1. A parallel reduction in IRS-2 phosphorylation was also observed, but IRS-1 phosphorylation was not changed by IRS-2 siRNA. Interestingly, IRS-2 siRNA-treated cells exhibited a reduction in PKB/Akt activation in response to IGF-I as compared with mock-transfected controls, whereas MAPK activation was unaltered. Therefore, IGF-mediated signaling via IRS-2 is preferentially linked to PI3K signaling in C4-12 PR-B cells.

Progestin Promoted IGF-Induced Migration by Increasing IRS-2 Levels and Enhancing IGF-Induced PI3K Function

Elevated IRS-2 levels induced by progestin in C4-12 PR-B cells were associated with enhanced IGF-induced cell migration. To rule out the possibility that progestin enhanced IGF-induced cell migration via other pathways not dependent on IRS-2 expression, an IRS-2–specific knockdown approach was employed. Cells were grown to 70% confluence and serum-starved for 24 hours prior to transient transfection with small interfering RNA (siRNA) specifically targeting the IRS-2 sequence for an additional 24 hours in serum-free medium (SFM). Progestin was added posttransfection for another overnight period, at which time cells were treated with 5 nmol/L of IGF-I for 10 minutes and lysates were collected. As previously reported, IGF treatment of C4-12 PR-B cells activated the IGF pathway (Fig. 4A). Transient transfection with IRS-2 siRNA specifically reduced IRS-2 protein levels, but not IRS-1. A parallel reduction in IRS-2 phosphorylation was also observed, but IRS-1 phosphorylation was not changed by IRS-2 siRNA. Interestingly, IRS-2 siRNA-treated cells exhibited a reduction in PKB/Akt activation in response to IGF-I as compared with mock-transfected controls, whereas MAPK activation was unaltered. Therefore, IGF-mediated signaling via IRS-2 is preferentially linked to PI3K signaling in C4-12 PR-B cells.
using a modified Boyden chamber assay (Fig. 4B). Similar to the data in Fig. 2B, IGF did not stimulate the migration of progestin-pretreated C4-12 vector cells. Furthermore, IRS-2 siRNA did not alter the biological response of the C4-12 vector cells. C4-12 PR-B cells treated with progestin responded to IGF-I with increased migration, whereas pretreatment of these cells with IRS-2 siRNA abrogated this response. In contrast, increasing concentrations of IRS-1 siRNA in non–progestin-treated C4-12 cells resulted in a migratory response to IGF-I, however, the magnitude of this effect was less than that achieved by IGF-I in non-siRNA, progestin-treated C4-12 PR-B cells (data not shown). IRS-1 siRNA did not alter IRS-2 protein levels in the C4-12 vector cells (data not shown). Therefore, the enhanced migration induced by progestin and IGF-I was dependent on IRS-2 expression.

Because IRS-2 knockdown abrogated the IGF-induced phosphorylation of PKB/Akt and not MAPK, the role of PI3K in progestin-promoted IGF-induced motility was examined. Following progestin pretreatment, cells were detached and pretreated with PI3K inhibitor (10 μmol/L LY294002) or MAPK inhibitor (5 μmol/L U0126) for 30 minutes, and then placed in a modified Boyden chamber in the presence of 5 μmol/L of IGF-I (Fig. 4C). As observed previously, progestin-pretreated cells migrated in response to IGF-I. Inhibition of MAPK did not alter IGF-I–induced motility of C4-12 PR-B cells. In contrast, inhibition of PI3K abrogated IGF-induced motility to basal levels. LY294002-mediated disruption of IGF-induced motility was not a result of changes in IRS protein expression or tyrosine phosphorylation (data not shown). Thus, PI3K functions mediate IGF-induced motility of progestin-treated cells.

Discussion

A number of studies have established IRS proteins as the primary mediators of IGF signaling in breast cancer. Here, we show how PR can modulate IGF responsiveness by altering IRS adapter protein expression. Because IGF signaling is mediated through both IRS-1 and IRS-2, and IGF action leads to multiple biological phenotypes, it has been hypothesized that IRS-1 and IRS-2 have distinct roles in IGF action. Our data show that the progestin-induced expression of IRS-2 led to enhanced IGF signaling and an increase in cell migration. To show that IRS proteins could in fact regulate these distinct IGF-induced phenotypes, IRS-1 and IRS-2 were overexpressed in T47D-YA breast cancer cells which lack endogenous IRS-1 or IRS-2 expression (8). In this model, IRS-1 coupled IGF signaling to cell proliferation and colony formation, whereas IRS-2 coupled IGF signaling to cell migration.

The role of IRS proteins in IGF-induced biology is also supported by studies in animal models of breast cancer. Death et al. used a mouse mammary tumor virus targeting model to overexpress IRS-1 or IRS-2 in the mammary gland of female mice to show that IRS proteins could promote tumor growth and metastasis (10). Although both animal groups developed tumors and metastases, the kinetics of these studies are suggestive of differences in biological function of IRS-1 and IRS-2. Nagle et al. also showed the importance of IRS-2 in mediating tumor metastasis of mouse mammary tumor virus/polyoma virus middle-T–induced tumors. In these studies, IRS-2–null animals displayed significantly impaired tumor metastasis to the lungs, whereas primary tumor growth was unchanged (9). Furthermore, tumors from both wild-type and IRS-2–null animals displayed similar mitotic indices, suggesting that IRS-2 does not play a role in tumor growth. In a similar manner, IRS-2 overexpression in C4-12 PR-B cells did not enhance cell proliferation in the presence or absence of IGF-I. In contrast, IGF-induced cell growth of these cells is regulated by IRS-1 and requires ER (12). Because Nagle et al.’s studies involved tumor induction by polyoma virus middle-T, future studies using this model will help decipher IRS-2’s contribution to the later stages of cancer progression that lead to tumor metastasis. Taken together, these studies examine the function of IRS proteins in animal models and support a role for both IRS-1 and IRS-2 in breast cancer progression.

Because IRS-1 and IRS-2 are often both expressed in breast cancer, there must be a mechanism for regulating which IRS protein is mediating IGF action. To address this issue, some studies have suggested that the ratio of IRS proteins can dictate IGF-induced biology (8, 9). Ablation of IRS-1 in animals infected with the mouse mammary tumor virus/polyoma virus middle-T vector did not alter tumor onset but resulted in an enhanced metastatic potential (8). These IRS-1–null tumors also exhibited an elevation in IRS-2 signaling, demonstrating that IRS-2 is important in promoting the metastatic phenotype. In addition, we have also observed that the loss of IRS-1 in vivo by targeted knockdown results in enhanced IRS-2 phosphorylation in the mammary gland after mice are given a bolus of insulin (20). Our studies also show that IGF-I predominantly stimulated IRS-2 signaling in progestin-pretreated C4-12 PR-B cells, whereas IRS-1 signaling was reduced. These data show that the available IRS pool can determine downstream signaling from the IGF1R, and potentially dictate IGF-mediated cancer phenotypes.

Phosphorylation of both IRS-1 and IRS-2 resulted in the activation of the PI3K and MAPK pathways. However, the specificity of downstream signaling may be dependent on distinct signaling pathways. Byron et al. showed that MAPK signaling by IRS-1 is crucial for IGF-I’s mitogenic effect in T47D-YA breast cancer cells (8). The absence of a proliferative response to IGF-I in C4-12 cells is due to a dependence of the IGF system on ER expression, despite the activation of PI3K and MAPK pathways by IRS-1 in C4-12 cells (21). In a similar manner, IRS-1 expression in ER-negative MDA-MB-468, MDA-MB-435A, and MDA-231BO cells does not link IGF1R signaling to a mitogenic response (6, 22). These cells are unable to proliferate in response to IGF-I, yet have IGF-stimulated motility. Furthermore, it seems that multiple signaling pathways are involved in IRS-2–mediated cell migration (23).

In support of this, IRS-2 signals via the PI3K pathway to mediate cell migration in both the C4-12 PR-B and T47D-YA IRS-2 cells. Additionally, we observed that LY294002 treatment did not reduce IRS-1 or IRS-2 protein expression in the C4-12 cells (data not shown). If anything, pretreatment with the PI3K inhibitor maintained a slightly elevated level of IRS protein, indicating that PI3K effects on migration were not at the level of IRS-2 expression but downstream of IRS-2. However, both PI3K and MAPK can mediate the mitogenic effects of IGF-I in ER-positive breast cancer cells (24). Although the above studies indicate that distinct functions by IRS-1 and IRS-2 seem to be elucidated, the mechanisms by
which IRS’s link the same pathways to different biology are still being examined. At the biochemical level, it will be important to elucidate which downstream pathways are preferentially linked to specific phenotypes stimulated by the IGF system, despite the activation of multiple pathways by both IRS proteins. Furthermore, an understanding of how IRS-1 or IRS-2 limit the function of signaling pathways that are capable of promoting multiple phenotypes to only promoting distinct phenotypes will need to be addressed. Potential molecular regulation may be at the level of differential IRS serine/threonine and tyrosine phosphorylation and subsequent recruitment of different positive and negative regulators, as well as distinct compartmental cross-talk with biologically relevant pathways (25, 26). In the case of IRS-1, compartmental cross-talk with ER is suggested, whereas IRS-2 may be linked to integrins at the plasma membrane (27, 28).

In this study, we sought to evaluate how the PR could functionally influence the IGF system. Several reports have shown ER’s role on the IGF system, however, none have shown a role for the PR in promoting IGF-mediated biology (3, 21). We have previously shown that progestin treatment of C4-12 PR-B cells could elevate IRS-2 levels, and promote IGF signaling (17). Because C4-12 cells do not have an IGF-driven phenotype, the C4-12 PR-B cell line is an ideal model to study the role of the PR-B, independent of the ER, on the IGF system. These cells also allow us to examine the effect of overexpressing IRS-2 to levels that would be similar to progesterone-responsive breast cancers. Indeed, progestin promoted IGF-I responsiveness through the PR. In addition, progestin did not alter integrin expression or function in the context of fibronectin, indicating that its effects on IGF action solely promoted cell migration.

The regulation of adapter protein expression to alter growth factor signaling is not unique to progesterone and IRS-2; estrogen also does this. Estrogen regulates IRS-1 levels, promoting IGF-proliferation (21). Not only is estrogen synergistic with IGF-I on cell proliferation, but ER is required for IGF1R and IRS-1 to stimulate proliferation (12). Therefore, PR function is linked to IRS levels, similar to the link between ER and IRS proteins. In this study, we show that PR elevates IRS-2 levels to alter IGF signaling from IRS-1 to IRS-2 and promote cell migration.

This work has several important implications about the therapeutic targeting of breast cancer. First, the IGF system is a bona fide breast cancer target (29). It is clear that with the development of new targeted therapies, patient selection will be critical for the success of such agents (30). Indeed, our studies have shown that the IGF system is not exclusively linked to mitogenic signaling by IRS-1, but is also linked to migratory signaling by IRS-2. Because response to cancer therapy is typically measured by primary tumor response, clinical response to therapies that target the IGF system in breast cancers signaling via IRS-2 would not be easily detected in clinical trials designed to measure response rates. Second, the PR can functionally promote the IGF system, leading cells towards an aggressive phenotype. PR action promotes the function of several growth factor pathways (13, 31). Our work suggests that PR function in breast cancer is needed for functional IGF1R signaling, similar to the role ER plays. However, whereas ER’s relationship with the IGF system is to promote cell proliferation, our data show that PR’s relationship with the IGF system is to promote cell migration. Therefore, PR and IRS-2 expression might identify tumors with high metastatic potential. Together, this work implies that IGF-driven breast cancers can exhibit a malignant phenotype, and that the selection of appropriate patient cohorts according to ER, PR, IRS-1, and IRS-2 expression should dictate anti-IGF study design. By providing a better understanding of how PR can influence IGF action, these pathways may offer more opportunities for therapeutic targeting of aggressive breast cancers.

**Materials and Methods**

**Cells and Reagents**

Generation of C4-12 vector and C4-12 PR-B cells was previously described (12, 17). These cells were routinely maintained in αMEM without phenol red (Invitrogen) + 5% charcoal/dextran-treated fetal bovine serum (HyClone) + 2 mmol/L of glucose + 50 IU/mL penicillin, 50 mg/mL of streptomycin. MDA-231BO is a bone-seeking metastatic variant of MDA-231, which was provided by Toshiyuki Yoneda (University of Texas Health Science Center, San Antonio, TX; ref. 32). These cells were maintained in DMEM with 10% FCS. The culture medium and fibronectin were purchased from Invitrogen Corporation. IGF-I was purchased from Gro Pep. Cells were serum-starved (SFM) in phenol red-free IMEM (improved MEM Zinc Option, supplemented with 20 mmol/L HEPES, 1 × trace elements, 2 μg/mL transferrin, and for IGF-I stimulation, 2 μg/mL fibronectin). All cells were grown at 37°C in a humidified atmosphere containing 5% CO2. Boyden chamber apparatus was purchased from NeuroProbe, Inc. HEMAs (Fisher). Hitachi camera, Leica microscope, SimplePCI software were purchased from North Central Instruments, Inc. Glass-chambered slides were purchased from NUNC. Protein A agarose was purchased from Sigma.

Materials and Methods

**Cells and Reagents**

Generation of C4-12 vector and C4-12 PR-B cells was previously described (12, 17). These cells were routinely maintained in αMEM without phenol red (Invitrogen) + 5% charcoal/dextran-treated fetal bovine serum (HyClone) + 2 mmol/L of glucose + 50 IU/mL penicillin, 50 mg/mL of streptomycin. MDA-231BO is a bone-seeking metastatic variant of MDA-231, which was provided by Toshiyuki Yoneda (University of Texas Health Science Center, San Antonio, TX; ref. 32). These cells were maintained in DMEM with 10% FCS. The culture medium and fibronectin were purchased from Invitrogen Corporation. IGF-I was purchased from Gro Pep. Cells were serum-starved (SFM) in phenol red–free IMEM (improved MEM Zinc Option, supplemented with 20 mmol/L HEPES, 1× trace elements, 2 μg/mL transferrin, and for IGF-I stimulation, 2 μg/mL fibronectin). All cells were grown at 37°C in a humidified atmosphere containing 5% CO2. Boyden chamber apparatus was purchased from NeuroProbe, Inc. HEMAs (Fisher). Hitachi camera, Leica microscope, SimplePCI software were purchased from North Central Instruments, Inc. Glass-chambered slides were purchased from NUNC. Protein A agarose was purchased from Sigma.

Materials and Methods

**Lysate Preparation**

Cells were grown to 70% confluency, incubated in SFM overnight, pretreated with 1 mmol/L of R5020 or an ethanol vector control for 24 h, then treated with 5 nmol/L of IGF-I for
10 min at 37°C. siRNA-treated samples were transiently trans- 
icted 24 h after serum starvation, before progestin treatment. 
The IRS-1 and IRS-2 siRNA transfection was done for a 
24-h period using Effectene transfection reagent, and medium 
was unchanged for progestin treatment. Cells were washed 
twice with ice-cold PBS on ice and lysed with 500 μL of TNEV 
lysis buffer (50 nmol/L Tris-Cl (pH 7.4), 1% NP40, 2 mmol/L 
EDTA (pH 8.0), 100 mmol/L NaCl, 10 mmol/L sodium ortho-
vanadate, 1 mmol/L phenylmethylsulfonyl fluoride, 20 μg/mL 
leupeptin, and 20 μg/mL aprotinin). Lysates were cleared by 
centrifugation at 12,000 × g at 4°C for 25 min. Protein concen-
trations were determined using the bicinchoninic acid kit.

**Immunoblot**

Total cell lysates (40 μg) in 1 × Laemmlili sample buffer were 
resolved on an 8% SDS-PAGE gel and transferred to a 
nitrocellosulose membrane. Membranes were blocked with 5% 
nonfat dry milk in TBST (0.05% Tween in TBS w/v) for 1 h at 
room temperature. Primary antibodies were used according to 
the manufacturer’s direction, followed by six washes with TBST 
at 5-min intervals. Secondary antibodies were used according 
to the manufacturer’s direction. Membranes were washed six 
times for 5 min each and chemiluminesence was detected.

**Immunoprecipitation**

Total cellular lysates (1,000 μg) were precleared with 50 μL 
of protein A agarose for 30 min at 4°C. Lysates were incubated 
overnight with IRS-1 or IRS-2 antibody at 4°C followed by 
incubation with protein A agarose for 4 h at 4°C. Samples were 
ashed thrice with TNEV. Laemmili sample buffer (1 ×) was 
added to an immunoprecipitated sample and run on an 8% 
SDS-PAGE gel.

**Cell Proliferation Assay**

Cells were plated in 24-well plates with 10,000 cells/well in 
charcoal-stripped serum-containing medium. Cells were 
switched to SFM for 24 h and then treated with 1 nmol/L of 
R5020 for another 24 h. Cells were then treated with or without 
5 nmol/L of IGF-I for 6 days. All treatments were done in 
tiplicate. Growth was measured 1 to 6 days after treatment. 
Growth was assayed by the 3-(4,5-dimethylthiazol-2-yl)-2,5- 
diphenyltetrazolium bromide assay as described previously (33). 
Sixty microliters of 5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5- 
diphenyltetrazolium bromide solution in SFM was added to each 
well. After incubation for 4 h at 37°C, wells were aspirated and 
formazan crystals were lysed with 500 μL of solubilization 
solution (95% DMSO + 5% IMEM). Absorbance was measured 
with a plate reader at 570 nm using a 650 nm differential filter.

**Boyden Chamber Cell Motility Assay**

Cell motility was measured by a modified Boyden 
chamber assay as described previously (11). Cells were serum-
starved for 24 h, then pretreated with 1 nmol/L of R5020 for 
another 24-h period. Cells were briefly detached 
by trypsin-EDTA, washed twice with SFM, then resuspended 
in SFM. Cells (150,000) were placed in the upper chamber 
of a 10-well Boyden chamber apparatus. Upper and lower 
chambers were separated by a polycarbonate polycylin/pyrroli-
done–free filter with 12-μm pores. SFM (0.4 mL) with 
or without IGF (5 nmol/L) was placed in the bottom wells 
of the chamber. After 6 h of incubation at 37°C in a humidified 
atmosphere containing 5% CO2, cells remaining on the topside 
of the filter were removed with cotton swabs. The filter was 
than removed from the chamber and the cells that had migrated 
to the underside of the filter were fixed and stained in HEMA3. 
The filter was then mounted onto a glass microscope slide and 
cells were counted (in duplicate) in five different areas using a 
light microscope. For siRNA experiments, cells were serum-
starved overnight, transiently transfected for 24 h, then 
pretreated with 1 nmol/L of R5020 for another 24 h.

**Adhesion Assay**

Integrin function was assessed using an adhesion assay 
to fibronectin as described previously (28). Briefly, 24-well 
plates were precoated with 8 μg/mL of fibronectin overnight. 
Serum-starved cells pretreated with 1 nmol/L of progestin 
(24 h) were placed in suspension for 30 min (kept in 
suspension by vortexing every 5 min), and then treated with 
5 nmol/L of IGF-I for 10 min. Cells were then plated on 
fibronectin for 5, 10, 20, 25, or 30 min. Nonadherent 
cells were washed off and the remaining adherent cells stained and 
quantified.

**Time-lapse Microscopy of Single Cell Migration**

Transfection or progestin/antiprogestin treatments were 
conducted as explained in Results. Cells were plated at a 
density of 1 × 105 cells/mL (375 μL) on precoated (2 μg/mL 
fibronectin) chamber slides for 2 h, then serum-starved 
overnight. Cells were placed in a humidified CO2 chamber 
designed for microscope imaging and images were taken using 
a Hamamatsu camera attached to an inverted Leica microscope at 
20× bright-field every 10 min for 24 h (150 frames/field; five 
fields in duplicate). Distance clearance was analyzed using 
SimplePCI software. Images were analyzed manually for the 
migration of single cells coinciding with 20-min intervals for a 
total of 12 h.

**Apoptosis**

Cells were grown to 70% confluence, incubated in SFM 
overnight, and then either untreated (SFM) or treated with 
5 nmol/L of IGF-I for 24 h. Cells were then treated with 
1,500 ng/mL of doxorubicin with or without IGF-I for 48 h. 
Cell lysates were collected and analyzed using Western 
immunoblot methods for PARP and MAPK.

**Spot Densitometry**

Integrated densitometry values of immunoblot bands were 
generated using Fluorochem V.3.04SA by Alpha Innotech 
Corporation.

**Statistical Analysis**

Two-way ANOVA with Bonferroni post-test and Student’s 
t test were done using GraphPad Prism version 3.02 for 
Windows (GraphPad Software). Error bars represent the SE. All 
experiments were repeated three to five times.
Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Acknowledgments

We thank Drs. Carol Lange and Deepali Sachdev for critical review of this manuscript.

References

Progesterone Receptor-B Regulation of Insulin-Like Growth Factor–Stimulated Cell Migration in Breast Cancer Cells via Insulin Receptor Substrate-2

Yasir H. Ibrahim, Sara A. Byron, Xiaojiang Cui, et al.