Metastatic Potential of 21T Human Breast Cancer Cells Depends on Akt/Protein Kinase B Activation

Meng Qiao, J. Dirk Iglehart, and Arthur B. Pardee

Abstract

Most cancer lethality is caused by metastasis. To gain insight into the molecular basis of tumor progression to metastasis, we used the 21T series of human mammary epithelial cells obtained by successive biopsies from one breast cancer patient. The c-erbB2 gene is amplified and overexpressed in each of three 21T tumor lines. The erbB2 receptor tyrosine kinase–activated phosphatidylinositol 3-kinase/Akt signaling cascade is crucial for the development and maintenance of epithelial cells, and dysregulation of this pathway is frequently associated with cellular transformation and cancer. For Akt to be fully activated, Ser473 on its COOH terminus needs to be phosphorylated. We detected more Ser473 Akt phosphorylation in MT cells, derived from a pleural effusion, compared with cells from the primary tumor. This phosphorylation has recently been shown to be catalyzed by mammalian target of rapamycin (mTOR)/riector kinase. By using genetic and pharmacologic activators and inhibitors, we showed that Ser473 Akt phosphorylation is more sensitive to mTOR/riector inhibition in metastatic tumor cells than normal mammary epithelial and primary tumor cells. The mTOR/riector kinase activity was indispensable for both Ser473 Akt phosphorylation and migration of metastatic MT2 cells. In addition, a large decrease of protein phosphatase PH domain leucine-rich repeat protein phosphatase (PHLPP) was found, which could be responsible for the overexpression of Ser473 Akt in MT cells. Our data indicate that these breast cancer cells acquire new vulnerabilities, rictor and PHLPP, which might provide an Achilles’ heel for therapeutic intervention of breast cancer metastasis. [Cancer Res 2007;67(11):5293–9]

Introduction

Metastasis is a critical step in progression from localized tumors to lethal cancers. Although sequential steps involved in the cascade of metastasis have been discerned, relatively little is known about the molecular biology of this event (1). The 21T series of cells developed in the laboratory of Dr. Ruth Sager represents a well-defined model to study tumor progression. In the order of increasing malignancy (16N, NT, MT1, and MT2), the 21T series are continuous lines of human mammary epithelial cells obtained by successive biopsies from one breast cancer patient diagnosed with infiltrating, erbB2-positive carcinoma (2–4). The erbB2 receptor tyrosine kinase belongs to the epidermal growth factor receptor (EGFR) family. Heterodimers containing erbB2 are potent activators of the phosphatidylinositol 3-kinase (PI3K)-Akt/protein kinase B (PKB) pathway, whose signaling plays essential roles in regulating cell proliferation, survival, and motility, and contribute to the development and maintenance of the mammary gland (5–7). Full activation of Akt requires phosphorylation on two residues, Ser473 on the hydrophobic COOH terminus and Thr308 on the T-loop kinase domain (8, 9). Inhibition of Ser473 activation leads to reduced phosphorylation of Akt on Thr308, confirming the important role of Ser473 in full activation of Akt (10–13). Hyperactivation of PI3K/Akt signaling pathway plays an essential role in tumor progression. Defective PTEN activates Akt signaling by preventing conversion of phosphatidylinositol 3,4,5-trisphosphate (PIP3) back to phosphatidylinositol 4,5-bisphosphate (PIP2) and contributes to retaining Akt phosphorylation. Nonetheless, there are many examples of elevated Akt phosphorylation in cancer cells that have intact PTEN expression. The PH domain leucine-rich repeat protein phosphatase (PHLPP) containing a PDZ-binding motif specifically dephosphorylates Ser473 of Akt1 to promote apoptosis and suppress tumor growth. PHLPP levels are markedly reduced in some cancer cell lines that have elevated Akt phosphorylation, and reintroduction of PHLPP reduced cell growth (14). More than 10 kinases have been proposed to phosphorylate Akt on Ser473, and the evidence supporting mammalian target of rapamycin (mTOR)/riector is among the most compelling (10, 15–17). This rapamycin-insensitive TORC2 complex, whose components include mTOR, rictor, mLST8, and Sin1 (18–20), can directly phosphorylate Akt Ser473 in Drosophila and in several human cell lines (10, 21). Rictor-null embryos and fibroblasts exhibit low proliferation rates and impaired Akt/PKB activity, and mTORC2 is essential during embryonic development (22). mTOR/riector mediates polarization of actin cytoskeleton and cell spreading (23, 24). Recent studies by Tan et al. (25) indicated that the mTOR/riector complex is activated by erbB2 and increases metastasis of human breast cancer cells. The TOR signaling pathway is a master controller of cell growth, and its dysregulation commonly occurs in many human diseases (26). Evidence implicates the involvement of mTOR in controlling not only cell proliferation but also migration (23, 24, 27). Inhibition of mTOR by rapamycin results in inactivation or hypophosphorylation of S6K1 and 4E-BP1, two major downstream effectors of TOR, and leads to reduction in cell growth (28–30). In addition to suppression of cell proliferation, rapamycin has recently been shown to inhibit cell motility in various types of cells and metastasis in vivo, which is partly attributed to its suppression of protein synthesis and partly to inhibition of the kinase activity of mTOR (28).

To gain insights into development toward metastasis, we analyzed the activation of PI3K signaling and phosphorylation of Akt in the 21T series. Importantly, we detected a change in Ser473 phosphorylation activity was indispensable for both Ser473 Akt phosphorylation and migration of metastatic MT2 cells. In addition, a large decrease of protein phosphatase PH domain leucine-rich repeat protein phosphatase (PHLPP) was found, which could be responsible for the overexpression of Ser473 Akt in MT cells. Our data indicate that these breast cancer cells acquire new vulnerabilities, rictor and PHLPP, which might provide an Achilles’ heel for therapeutic intervention of breast cancer metastasis. [Cancer Res 2007;67(11):5293–9]
Akt phosphorylation, with metastatic 21T cells exhibiting more Akt phosphorylation than cells derived from the primary tumor. Our studies showed that expression of erbB2, PI3K, and PTEN proteins was similar in all 21T cells. We identified decreased expression of Ser\textsuperscript{473} Akt phosphatase PHLP in the MT lines compared with NT cells. In addition, by using small interfering RNA (siRNA) and inhibitors of PI3K, mTOR, and rictor, we showed that Ser\textsuperscript{473} Akt hyperactivation is modulated by rictor in metastatic cells. Furthermore, our results indicated that rictor kinase activity is required for cell motility in the metastatic cell lines. Based on these findings, we propose that dysregulation of rictor together with PHLP contributes to the acquisition of an aggressive metastatic phenotype of these cancer cells.

**Materials and Methods**

**Cell culture and transfection.** 16N was derived from reduction mammoplasty (3); NT was isolated from the mastectomy samples from a patient with infiltrating ductal and intraductal carcinoma (4, 7). MT1 and MT2 cell lines were obtained from pleural effusions of the same patient after diagnosis of lung metastasis (31). BT474, SKBr3, and HMEC cells were purchased from the American Type Culture Collection. HMEC/16N cell line was obtained from Dr. Jean Zhao (Dana-Farber Cancer Institute); HMEC and 21T cells were maintained in mammary epithelial growth medium (MEGM) medium supplemented with 1% fetal bovine serum (FBS). BT474, SKBr3, and HEK293 cells were cultured in RPMI 1640 containing 10% FBS, 2 mmol/L l-glutamine, penicillin (100 units/mL), and streptomycin (100 μg/mL) at 37°C in a humidified atmosphere with 5% CO\textsubscript{2}. Transfection of cells was carried out in complete medium with LipofectAMINE (Invitrogen) for plasmids according to the manufacturer's protocols.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells were seeded into 96-well plates (2,500 per well) and cell viability was evaluated on days 1, 3, 5, and 7. Cells were washed with RPMI 1640 without phenol red before 100 μL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (5 mg/mL) were added into each well. After incubation at 37°C for 2 h, an equal volume (100 μL) of isopropanol containing 0.1 N HCl was added to dissolve the formazan dye. Absorbance was measured at 570 nm with background subtraction at 690 nm.

**Soft agar colony formation assay.** Cells (40,000 per well; six-well plates) were suspended in DMEM containing 10% FBS and 0.3% agarose and plated onto a base layer of solidified DMEM containing 5% agarose and 10% FBS. After 12 days of incubation at 37°C, colonies were photographed and the number of colonies per field was counted and expressed as the mean ± SD.

**Migration assay.** Cells cultured in serum-free MEGM medium for 16 h were trypsinized and resuspended in serum-free medium. Cells (2.0 × 10\textsuperscript{5}) were added to the top of a chamber well containing an 8-μm polycarbonate filter precoated with collagen I separating the top and bottom compartments. The bottom chamber was filled with MEGM containing 3% FBS as attractant. The cells were incubated for 6 h at 37°C. Filters were removed from the chambers and stained with 0.5% crystal violet for 30 min. Nonmigrating cells on the top of the filters were removed with cotton swabs, and the cells on the lower surface of the membrane were extracted with 0.1 mol/L citric acid in 50% ethanol. Samples were transferred to a 96-well plate and the absorbance at 595 nm was determined.

**RNA interference.** siRNAs targeting PIK3CA, PDK1, ILK1, mTOR, and rictor were purchased from Dharmacon. The nontargeting siRNA pool siControl was used as a negative control. The transfection was done according to SMARTPool reagent manufacturers’ instructions. The final concentration of each siRNA was 100 nmol/L. Cells were harvested at confluence from a 75-cm\textsuperscript{2} culture flask and plated at approximately 2 × 10\textsuperscript{5} per well into six-well plates. After 24 h, siRNA transfection was done in a total volume of 1 mL using Oligofectamine transfection reagent. Cells were harvested 48 h after transfection, and whole-cell lysates were prepared for immunoblotting assays.

**Western blotting.** Cells were washed twice with cold PBS and lysed with modified radioimmunoprecipitation assay (RIPA) buffer [50 mmol/L Tris-base (pH 7.4), 150 mmol/L NaCl, 1 mmol/L EDTA, 1% NP40, 0.25% deoxycholate, 1 mmol/L sodium orthovanadate, 1 mmol/L phenylmethylsulfonyl fluoride, 25 μg/mL leupeptin], Proteins (50 μg) were heat denatured and resolved by SDS-PAGE. Proteins were transferred to polyvinylidene difluoride membrane, blocked for 1 h with 5% nonfat dry milk in TBST [50 mmol/L Tris-base (pH 7.4), 150 mmol/L NaCl, and 0.1% Tween 20], and incubated with the primary antibodies in TBST containing 1% bovine serum albumin solution for 1 to 16 h. Membranes were washed several times in TBST solution and incubated with horseradish peroxidase–conjugated antimouse or anti-rabbit antibodies. Chemiluminescence kit from Pierce Biotechnology was used to detect immunoreactivity.

**Immunoprecipitation and in vitro kinase assay.** Whole-cell lysates (250 μg) were diluted into 0.5 mL of modified RIPA buffer containing protease inhibitors and precleared for 1 h at 4°C with 30 μL of protein A-Sepharose CL-4B (Amersham Biosciences). Anti-rictor antibody (4 μg) prebound to 50 μL of protein A-Sepharose was incubated with precleared protein extract at 4°C on an orbital shaker for 16 h. The mixture was centrifuged and the pellets were washed thrice with immunoprecipitation buffer and resuspended in 30 μL of kinase buffer [25 mmol/L HEPES (pH 7.5), 1 mmol/L MgCl\textsubscript{2}, 100 mmol/L KCl]. The immunoprecipitates were incubated with 500 ng of inactive Akt1/PKBα (Upstate Biotechnology) and 2 μCi of [γ\textsuperscript{32P}]ATP/sample for 45 min with modest agitation at 37°C. LDS sample buffer (4x; Invitrogen) was added to the mixture to terminate the kinase reaction. Samples were heated at 95°C for 5 min. After a quick spin, the supernatants containing the immunocomplex were resolved on 4% to 12% SDS-PAGE followed by autoradiography.

**Densitometry and statistical analysis.** Data shown are representative of at least three experiments with essentially similar results. The results are expressed as the mean ± SD from three independent experiments. The means are compared using Student’s t test with P values of <0.005 considered as statistically significant.

**Results**

**Characterization of 21T series.** The 21T lines represent a progression series correlated with increasing malignancy in vivo. We verified by single nucleotide polymorphism (SNP) genotyping that our 21T cell lines were derived from the same individual (data not shown). 16N normal mammary epithelial cells derived from normal breast tissue from the affected patient and subsequently immortalized with human papillomavirus (HPV)-16 were used as a control (3). To determine if the immortalization of 16N cells with HPV dramatically influenced cell proliferation, MT1 cell growth assays were done. In these studies, the growth rate of 16N cells was similar to other 21T cells (data not shown).

We measured 21T cell anchorage-independent growth by soft agar colony formation assay (Fig. 1A; ref. 32). HMEC cells transformed with ras were used as positive control. Consistent with their in vivo tumorigenic potential, the order of increasing colony formation capability was 16N, NT, MT1, MT2, and HMEC/ ras. We also examined their migration capability with a modified Boyden chamber assay. A linear increase from low to high degrees of migration was observed with normal (16N), primary (NT), and then metastatic (MT) cells (Fig. 1B). In addition, active matrix metalloproteinase-2 (MMP-2) and CXCR4 (33, 34) are two markers associated with metastasis; they were up-regulated in the two MT cells of metastatic origin (Fig. 1C). Thus, MT lines maintained their in vivo metastatic behavior as shown by these assays and can be used to correlate changes in cell signaling with effects on cell motility.

We analyzed expression profiles of several proteins, confirming the high expression of erbB2 proteins in all the 21T tumor lines.

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and detected no differences between primary and metastatic cell lines (Fig. 1C). BT474, an erbB2-positive and estrogen receptor (ER)-positive cell line, was used as positive control. All the 21T cells were ER and p53 negative, which is consistent with previous reports (2, 35). Because dysregulated erbB2 signaling activates the PI3K/Akt pathway (14–18), we examined the activation of phosphorylated Ser473 Akt (p-Ser473 Akt) in 21T cell lines. NT expressed p-Ser473 Akt at a lower level than the two MT cells, and MT2 expressed the highest level of Akt phosphorylation (Fig. 1C). Given the opposing roles of different Akt isoforms in cell proliferation and migration (36, 37), we measured the protein expression of Akt1, Akt2, and Akt3 with isoform-specific antibodies. Akt1, whose expression level was similar to total Akt, was the only detectable isoform in the 21T series (data not shown). Because Akt Ser473 phosphorylation differed among normal, primary, and invasive cells despite similar rates of proliferation, and because PI3K/Akt signaling may be involved in metastasis as well as in proliferation and apoptosis, we explored Akt activation in more depth.

erbB2 stimulated by heregulin regulates Ser473 Akt phosphorylation in 21T cells. All the 21T cells showed increased erbB2 mRNA (2) as well as protein expression and decreased EGFR compared with normal 16N cells (Fig. 1C). All expressed c-erbB3 but not c-erbB4 (38). erbB2, with no known ligand, forms a heterodimer with erbB3 and the latter can be potently stimulated with growth factors, such as heregulin (39). On heregulin binding, the erbB2/erbB3 dimers recruit the 85-kDa regulatory subunit of PI3K to initiate downstream cascades. All 21T cells require serum to grow in culture (2, 4). To determine if Ser473 Akt phosphorylation is also dependent on serum, we compared 16N and MT2 treated with or without serum (Fig. 2A). The FBS withdrawal for 16 h had

![Figure 1. Evaluation of anchorage-independent cell growth, migration, and protein expression profiles of 21T cells. A, measurement of anchorage-independent growth of 16N, NT, and MT2 cells by colony formation on soft agar. Bars, SD. *, P < 0.01, compared with 16N; **, P < 0.01, compared with NT. B, migration in modified Boyden chambers. Cells were cultured for 6 h on collagen-coated filters, and cell migration in response to 3% FBS in the bottom chamber was quantitated. SKBr3 was used as a positive control for migration. Results are representative of three different experiments. Columns, mean of triplicate samples; bars, SD. *, P < 0.01, compared with 16N; **, P < 0.01, compared with NT. C, Western blotting analysis of protein expression. EGFR, erbB2, ER, p53, MMP-2, CXCR4, p-Ser473 Akt, and total Akt (T-Akt) were determined with specific antibodies, respectively. Protein loading was monitored by immunoblotting for β-actin.

Figure 2. Growth factor requirements for 21T cells. A, serum deprivation reduced Ser473 Akt phosphorylation in both normal and metastatic tumor cells. 16N and MT2 cells were cultured in MEGM medium containing 1% FBS for 2 d until 80% confluence. Cells were replated in the presence (lanes 1 and 3) or absence (lanes 2 and 4) of 1% FBS for 24 h before extraction of whole-cell lysates. B, Ser473 Akt phosphorylation was regulated by erbB2. PT, NT, and MT2 cells were cultured in MEGM medium in the presence or absence of 1% FBS for 24 h. Cells were then stimulated with or without 20 ng/mL heregulin (Hrg) for an additional 24 h before cell lysates were isolated. Protein expression was determined by immunoblots.
no effect on erbB2 receptor expression but completely eliminated p-Ser473 Akt in NT and MT2 cells. The levels of p110α of PI3K, phosphorylated PTEN, or phosphorylated PDK1 were not affected by serum depletion (data not shown).

To investigate the possible role in Ser473 Akt activation of heregulin in comparison with serum, 21T lines with or without overnight serum starvation were treated with 1% FBS or 20 ng/mL heregulin for 24 h. Heregulin restored Ser473 Akt phosphorylation to levels similar to those without serum depletion within 24 h (Fig. 2A). Thus, stimulation by heregulin alone was enough to achieve maximum activation of Akt, as adding 1% FBS did not lead to further increase of p-Ser473 Akt. All these results confirmed that the erbB2 pathway is a key regulator of Ser473 Akt phosphorylation in the 21T series regardless of their metastatic capability. Because no difference at receptor level was detected, we pursued our search for changes in the downstream PI3K pathway.

PI3K is required for Akt activation in all 21T cells. PI3K, activated by receptor tyrosine kinases, such as erbB, converts the plasma membrane lipidPIP2 to PIP3. PIP3 recruits inactive Akt activated by receptor tyrosine kinases, such as erbB, converts the plasma membrane lipidPIP2 to PIP3. PIP3 recruits inactive Akt

![Figure 3. PI3K is required for Ser473 Akt phosphorylation in 21T cells.](image)

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Figure 3. PI3K is required for Ser473 Akt phosphorylation in 21T cells. A, inhibition of PI3K reduced p-Ser473 Akt in all 21T cells. Cells were treated with or without 20 μmol/L of PI3K inhibitor LY294002 (LY) for 16 h before cell lysates were extracted. B, siRNA targeting PI3K p110α reduced p-Ser473 Akt in 16N, NT, and MT2 cells. Cells were untransfected (−) or transfected with the control (C) siRNA or p110α siRNA (100 nmol/L).

Similarly reduced p-Ser473 Akt in a dose-dependent manner, and cell viability was not affected (data not shown).

To further substantiate the importance of PI3K in Ser473 Akt phosphorylation in the 21T series, we achieved specific inhibition of PI3K activity by exploiting siRNA targeting of the p110α catalytic subunit of PI3K, which reduced protein expression of p110α by 80% to 100% in all the 21T lines. Ser473 Akt phosphorylation was also decreased by >80% in those cells (Fig. 3B). Control siRNA inhibited neither p110α nor p-Ser473 Akt levels. By both pharmacologic and genetic approaches, we firmly established the necessity of PI3K in mediating erbB2-stimulated Ser473 Akt phosphorylation in all 21T cell lines.

**PHLPP phosphatase expression is inversely related to Ser473 Akt activation in 21T series.** Cells maintain a dynamic steady state of phosphorylated Akt, balanced by the activity of kinases and phosphatases. To investigate the possible role of phosphatase in Ser473 Akt activation, we did a time course study by blocking de novo Akt phosphorylation with 10 μmol/L LY294002 and measured the level of p-Ser473 Akt for up to 60 min. Ser473 Akt dephosphorylation was time related in NT and MT2 cells, falling within 5 and 10 min, respectively, after partial and indirect inhibition by LY294002 and then settled at a lower level (Fig. 4A). The more rapid decrease of p-Ser473 Akt in NT cells after PI3K inhibition suggested a higher phosphatase activity in NT cells than in MT2 cells. PHLPP inactivates Akt by directly dephosphorylating the hydrophobic motif of Akt, where Ser473 resides (14). We analyzed protein expression of PHLPP in 21T lines and observed a much decreased expression of PHLPP in the two metastatic MT1 and MT2 cells compared with the primary tumor NT cells (Fig. 4B). This result is in concordance with the increased Ser473 Akt phosphorylation in the 21T series. Therefore, dysregulation of the Ser473 phosphatase PHLPP could be one of the mechanisms accountable for higher level of p-Ser473 in the most malignant cells in the 21T series.

**Rictor is required for hyperactivation of Ser473 Akt in metastatic MT2 cells.** Akt belongs to the AGC protein kinase family whose members are activated in an analogous manner. As...
PDK1 phosphorylates every AGC enzyme at its T-loop kinase domain, it was predicted that another kinase may phosphorylate their hydrophobic domains on the COOH terminus. mTOR, a well-known hydrophobic motif kinase in a complex with raptor (TORC1), phosphorylates one AGC member, S6K. mTOR in a complex with rictor (TORC2) is involved in rapamycin-insensitive phosphorylation of Ser473 Akt. Both mTOR and rictor knockdown reduced p-Ser473 Akt in all the 21T lines as shown on Fig. 5A. But the degree of p-Ser473 Akt decrease differed among the normal, primary tumor, and metastatic tumor cells. As rictor is essential for Ser473 phosphorylation under normal conditions, we quantified the densitometry of p-Ser473 Akt signal and the value was shown underneath the band. siRNAs targeting mTOR or rictor reduced p-Ser473 Akt protein expression in MT2 by 76% and 92%, respectively, and they had much milder effects on Ser473 phosphorylation in 16N and NT cells (Fig. 5A). We observed similar effect of rictor siRNA on Ser473 Akt phosphorylation in SKBr3 cells, another well-characterized ER/erbB2+ breast cancer cell line derived from human metastases (data not shown). mTOR/rictor thus seems to be important for the kinase activity that phosphorylates Akt in these cancer cells, derived from a metastatic deposit. To exclude the possibility of off-target effects with SMARTPool siRNA, which is composed of four individual siRNAs targeting different regions of rictor mRNA, MT2 cells were transfected with each of the four siRNAs individually. Each siRNA decreased Ser473 Akt expression by >70%, although combination of the four showed the greatest inhibition, siRNA-resistant rictor vectors (wobble mutant) introduced to cells were able to rescue rictor knockdown, further confirming the specificity of the rictor siRNAs (data not shown).

The amount of rictor cannot account for differences of Ser473 Akt phosphorylation because we observed similar levels of rictor protein in all 21T lines (Fig. 5A). To determine whether a difference in kinase activity contributes to Ser473 Akt hyper-phosphorylation in MT cells, we did *in vitro* mTOR-rictor kinase assays (Fig. 5B). MT2 cell exhibited the highest TORC2 kinase activity that was followed by MT1, NT, and 16N cells. HEK293 cell lysates containing high mTOR-rictor kinase activity were used as a positive control.

The increase in rictor kinase activity, combined with the decreased PHLPP, offers a reasonable explanation of the higher Ser473 Akt in MT2 cells. ILK phosphorylated Akt on Ser473 in several cell types. However, we did not observe any significant decrease in p-Ser473 Akt levels in 21T cells through...
introduction of ILK siRNA into 16N, NT, and MT2 cells, although almost complete knockdown of ILK protein expression was achieved (data not shown).

**Rictor is involved in mediating cell migration.** The PI3K pathway has been implicated in regulating cell motility required for metastasis (41, 42). Wortmannin (20 nmol/L), a potent PI3K inhibitor, decreased migration 50% to 70% in NT, MT2, and SKBr3 cells as analyzed by a modified Boyden chamber assay (Fig. 6A). This inhibition was not due to a decrease in cell number because cell viability was not affected by 6-h wortmannin treatment in the three cell lines (data not shown). To determine if inhibition of rictor expression affects cell migration, we exploited rictor siRNA compared with control and p110β siRNA (Fig. 6B). Rictor siRNA reduced rictor protein expression in NT, MT2, and SKBr3 cells equally well. Motility of MT2 cells was reduced ~50% by siRNAs for rictor or p110β. Interference with rictor and p110β expression reduced migration of SKBr3 cells, derived from metastatic sites, by 90% and 75%, respectively. In NT cells, the siRNAs did not exert a significant influence. Cell viability was not decreased by these siRNAs compared with control siRNA (data not shown). Thus, migration was greatly reduced only in metastatic cells, in which higher Ser\(^{473}\) Akt phosphorylation was detected. Rictor is thus a key regulator of Akt phosphorylation and a crucial mediator of cell migration in MT2 and SKBr3 cells.

**Discussion**

Breast tumor development and progression are complicated multistep processes driven by accumulated genetic alterations (43). Hyperactivation of Akt has been correlated with advanced cancer (14, 44). In a quest for metastasis-related changes in signal transduction pathways, we used the 21T cell lines, a progressive mammary series from one patient (2). We uncovered an increase in p-Ser\(^{473}\) Akt levels, coincident with progression to metastasis. Therefore, the lethality of erbB2-positive breast cancers may in part be mediated by a subsequent mutation that activates Akt and thereby increasing metastatic capability, in addition to positive effects on cell growth and survival.

Akt is activated in cancer by several mechanisms, including receptor overexpression, loss of PTEN, and PI3K mutation. With growth factors, kinase inhibitors, and siRNAs to manipulate the erbB/PI3K pathway and the phosphorylation status of Ser\(^{473}\) Akt, we identified changes in the control of Akt activation in the 21T series. In particular, rictor-dependent kinase activity, but not the amount of rictor protein, was elevated in the metastatic cells.

Compared with the plethora of information about mTOR, the understanding of rictor and the TORC2 complex is just emerging, and rictor attracts attention due to its role in Ser\(^{473}\) phosphorylation. Disruption of TORC2 (mTOR/rictor) inhibits cell spreading and filamensous actin polymerization of NIH3T3 cells (23), suggesting that TORC2 may be crucial for cell motility. Rictor regulates cytoskeleton organization through PKC1 (24, 27, 45). TORC2 in Dictyostelium signals to Akt to regulate chemotaxis and cell polarity (46). Rictor homologue pianissimo in Dictyostelium discoideum is implicated in cyclic AMP–induced cell migration (47). In a human cancer model, we find that rictor becomes a principle activator of Akt during tumor progression and its inhibition by siRNA retarded MT2 cell migration. In particular, the kinase activity of TORC2, but not the quantity of rictor, is elevated in the metastatic cells. These results implicate rictor as a tumor progression factor and a potential therapeutic target.

Phosphatases together with kinases dynamically set levels of phosphoproteins. We determined the amounts of Ser\(^{473}\) Akt phosphatase PHLPP protein in 21T series cells and observed its greatly decreased expression in the metastatic MT lines (Fig. 4B). Therefore, the elevated Ser\(^{473}\) Akt in the most malignant MT cells can be explained by both increased TORC2 kinase activity and also decreased PHLPP expression. Their interactions are possibly within the TORC2 complex (14). Although we knocked down PHLPP expression with siRNAs in 16N and NT cells, we did not notice significant increase in Akt phosphorylation. One possible reason is that the nonmetastatic cells develop certain regulatory mechanism to maintain the balance between the kinases and phosphatases that will prevent Akt overactivation. In addition, other PHLPP-like phosphatases may compensate for the loss of PHLPP. The role of PHLPP in breast cancer progression is currently under investigation.

Cancer cells may develop preferential dependence on certain genes during tumor progression and develop new patterns of “oncogene addiction” (48–50). Our data suggest that metastatic cells develop an increased reliance on rictor for activation of Ser\(^{473}\) Akt.
and so exhibit greater susceptibility to rictor inhibition. This study questions the assumption that events in the primary tumor predict behavior of metastatic sites that develop later in the natural history of a single tumor. The apparent rictor addiction of metastatic cells relative to their normal counterparts provides the basis for designing therapeutic agents with specificity for tumor metastasis and suggests the need for reassessment of signaling targets during tumor progression. Modification of Akt phosphorylation control has potential as a therapeutic target for breast cancer.

References

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