Targeting p70S6K Prevented Lung Metastasis in a Breast Cancer Xenograft Model

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Abstract

Overexpression of p70S6K in breast cancer patients is associated with aggressive disease and poor prognosis. Recent studies showed that patients with breast cancer with increased p70S6K phosphorylation had poor survival and increased metastasis. The purpose of our study was to determine whether knockdown of p70S6K would inhibit cell growth, invasion, and metastasis in breast cancer. We therefore stably knocked down p70S6K expression in MDA-231, a highly metastatic breast cancer cell line, using a lentiviral short hairpin RNA (shRNA) based approach. Inhibition of p70S6K led to inhibition of cell growth, migration, and invasion in vitro. To determine the role of p70S6K in breast cancer tumorigenesis and metastasis, we used an MDA-231 orthotopic and metastatic animal model. In the orthotopic model, mice injected with MDA-231–p70S6K shRNA cells developed significantly smaller tumors than control mice injected with MDA-231 control shRNA cells (P < 0.01). No metastasis was observed in the p70S6K downregulated group, whereas lung metastasis was detected in all mice in the control group. To determine the role of p70S6K on growth and invasion, we tested downstream signaling targets by Western blot analysis. Knockdown of p70S6K inhibited phosphorylation of focal adhesion kinase, tissue transglutaminase 2, and cyclin D1 proteins, which promote cell growth, survival, and invasion. In addition, downregulation of p70S6K induced expression of PDCD4, a tumor-suppressor protein. In conclusion, we showed that p70S6K plays an important role in metastasis by regulating key proteins like cyclin D1, PDCD4, focal adhesion kinase, E-cadherin, β-catenin, and tissue transglutaminase 2, which are essential for cell attachment, survival, invasion, and metastasis in breast cancer.

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Introduction

The 70-kDa ribosomal protein S6 kinase (p70S6K) is a serine/threonine kinase that regulates protein translation by phosphorylating ribosomal protein S6 (1, 2). p70S6K is downstream of the phosphoinositide 3-kinase/AKT/mammalian target of rapamycin (mTOR) pathway, which is activated by HER2, insulin-like growth factor receptor, and estrogen receptor in breast cancer (3–6). mTOR/p70S6K can also be activated by extracellular signal-regulated kinase (7, 8). Phosphorylation of Thr389 by mTOR is critical for p70S6K activation and is a marker for mTOR activity (9, 10). Derepression of the mTOR/p70S6K pathway may play an important role in the development of cancer and many other diseases (11, 12).

Rapamycin, an inhibitor of mTOR, is currently being evaluated in clinical trials for the treatment of cancer. Rapamycin affects mTOR signaling in benign, premalignant, and malignant human breast epithelial cells (13). Phospho-p70S6K levels are twice as high in malignant MCF10CA1a cells as in benign MCF10A cells (14). Patients with tumors having increased p70S6K phosphorylation had worse disease-free survival and increased metastasis (15), which shows the importance of p70S6K in breast malignancy.

The chromosomal region 17q23 containing the p70S6K gene is frequently amplified in breast cancer cells, leading to p70S6K overexpression. Overexpression of p70S6K supports breast cancer cells with a proliferative advantage in low serum conditions. p70S6K also regulates estrogen receptor α by phosphorylating it on Ser167, leading to transcriptional activation of estrogen receptor α (16). In clinical studies, p70S6K1 gene amplification and increased p70S6K expression, determined by immunohistochemical analysis in breast cancer, were found to correlate with poor prognosis and survival (3).

Suppression of HER2 overexpression by the antidiabetic drug metformin seems to occur through direct (adenosine 5′-monophosphate-activated protein kinase independent) inhibition of p70S6K activity (17). D-glucosamine inhibits p70S6K activity and the proliferation...
of MDA-231 breast cancer cells (18). Apoptosis induced by the antioxidant resveratrol has been associated with the activation of p53 and inhibition of protein translation through p70S6K in T47D human breast cancer cells (19).

Jiang et al. (20) showed that tissue factor–factor VIIa–factor Xa complex induces phosphorylation of mTOR and p70S6K in human breast cancer cell line. Inhibition of mTOR and p70S6K markedly decreased cell migration induced by formation of the tissue factor–factor VIIa–factor Xa complex. Zhang et al. (21) observed that most of the phosphoinositide 3-kinase catalytic subunit mutant could activate p-AKT and phospho-p70S6K in the absence of epidermal growth factor stimulation. p70S6K is defined as a specific biomarker for the biological effects of the dual HER1/HER2 inhibitor lapatinib (22).

Tissue transglutaminase 2 is a multifunctional enzyme, mainly known for its calcium-dependent posttranslational modification of proteins. Transamidation by tissue transglutaminase 2 can both facilitate and inhibit apoptosis, whereas the GTP-bound form of the enzyme generally protects cells against death (23). Under certain conditions, tissue transglutaminase 2 can also catalyze the protein disulfide isomerase and serine/threonine kinase activity (24–27). Tissue transglutaminase 2 expression on the cell membrane, as a result of its association with certain integrins, has been reported to promote the cell survival signaling pathway (28). Increased expression of tissue transglutaminase 2 in cancer cells has been implicated in conferring resistance to chemotherapeutic drugs and promoting the metastatic phenotype and cell survival functions in breast cancer, melanoma, and pancreatic cancer cells (29). Furthermore, previous reports have suggested that tissue transglutaminase 2 inhibits apoptotic and autophagic cell death in pancreatic cancer cell lines (30, 31). A recent study showed that tissue transglutaminase 2 expression can protect various cancer cell types from apoptosis or autophagy by constitutively activating focal adhesion kinase/phosphoinositide 3-kinase/AKT and NF-κB cell survival signaling pathways (32, 33). Indeed, failure of cells to die can render cells resistant to chemotherapeutic drugs and promote their survival in stressful environments of the foreign tissues (metastasis). Tissue transglutaminase 2 promotes cell attachment, survival, and invasion in breast cancer. MDA-231 is a triple-negative, highly metastatic breast cancer cell line that expresses a high level of tissue transglutaminase 2 (29).

PDCD4 is a downstream protein of p70S6K. p70S6K phosphorylation of PDCD4 results in rapid degradation by proteasomes, promoting tumor formation (34). PDCD4 is a tumor-suppressor protein that inhibits metastasis by regulating β-catenin and E-cadherin (35). PDCD4 also increases tissue inhibitor of metalloproteinase 2 expression to inhibit breast cancer cell invasion (36).

p70S6K has been associated with breast cancer, poor prognosis, and metastasis, but the mechanisms associated with the role of p70S6K in metastasis are not well understood. The aim of this study was to investigate if p70S6K is involved in breast cancer metastasis by knocking down p70S6K in in vivo animal experiments and to find downstream targets of p70S6K that may also induce metastasis in breast cancer.

Materials and Methods

Cell lines, culture conditions, and reagents. Human breast cancer cell lines MCF-7, MCF-Dox, MDA-231, Sum-149, BT-20, BT549, and MDA-MB-361 were cultured at 37°C in DMEM supplemented with 10% fetal bovine serum in a humid incubator with 5% CO₂. MCF10A cells were cultured in DMEM media supplemented with 15% fetal bovine serum, insulin (10 μg/mL), epidermal growth factor (20 ng/mL), and hydrocortisone (0.5 μg/mL). For the cell proliferation experiments, cells were seeded at a density of 1 to 2 × 10⁵ cells in 10-mm tissue culture dish. Adherent cells were collected by trypsinization, and cell numbers were determined by using a cell counter (Beckman Coulter). MDA-231 cells were stably transduced with lentiviral luciferase gene.

Cell transfections. p70S6K and control shRNA–expressing lentiviral particles were prepared in our laboratory. Transfection of MDA-231 was done at a 5 multiplicity of infection virus particle concentration. Five days after transfection, cells were treated with 5 μg of puromycin concentration to select stably expressed shRNA cells.

Western blot analysis. After treatment, the cells were trypsinized and collected by centrifugation, and whole-cell lysates were obtained by using a cell lysis buffer. Total protein concentration was determined by using a detergent-compatible protein assay kit (Bio-Rad). Aliquots containing 30 μg of total protein from each sample were subjected to SDS-PAGE with a 12% gradient and electrotransferred to nitrocellulose membranes. The membranes were blocked with 5% dry milk in TBS-Tween 20, probed with primary antibodies anti-p70S6K monoclonal antibody, cyclin D1 (Santa Cruz), pRS6Pser235/236 (Cell Signaling), phosphorylation of focal adhesion kinase, or tissue transglutaminase 2 (Alexis). The antibodies were diluted in TBS-Tween 20 containing 2.5% dry milk and incubated at 4°C overnight. After the membranes were washed with TBS-Tween 20, they were incubated with horseradish peroxidase–conjugated anti-rabbit or anti-mouse secondary antibody (Amersham Life Science). Mouse anti-β-actin and donkey anti-mouse secondary antibodies (Sigma Chemical) were used to monitor β-actin expression to ensure equal loading of proteins. Chemiluminescent detection was done with Chemi-glow detection reagents (Alpha Innotech). The blots were visualized with a FluorChem 8900 imager and quantified by densitometer software (Alpha Innotech).

Flow cytometric analysis of apoptosis. Cells were collected and double stained with Annexin V–Fluorescein isothiocyanate (FITC) and propidium iodide using an Annexin V–FITC apoptosis detection kit (BD Pharmingen) and evaluated by flow cytometer.
Cell cycle analysis. Cells were washed with PBS, fixed with 70% cold ethanol, incubated with 5 μg/mL RNase (Roche Applied Science) overnight at 4°C, and stained with propidium iodide (Sigma-Aldrich). The propidium iodide-stained cells were evaluated by flow cytometer.

Matrigel invasion assay. Cells (1 × 10⁵ cells per well) were seeded onto Matrigel-coated Transwell filters (8-μm pore size) in Matrigel invasion chambers (BD Biosciences), and the number of cells that invaded the lower side of the membrane was determined by counting cells in randomly selected areas.

MDA-231 orthotopic and metastatic tumor model. Athymic female nu/nu mice (4-wk old) were obtained from the Department of Experimental Radiation Oncology at M.D. Anderson Cancer Center. The mice were housed three per cage in standard acrylic glass.

Figure 1. Proteins extracted from normal epithelium and breast cancer cell lines. Equal amounts of protein from each cell line were transferred to nitrocellulose membrane. Total p70S6K, phosphor-ser235/236 S6RP, and β-actin protein expression levels were visualized by Western blotting as described in the Materials and Methods section.

Figure 2. MDA-231-Luc cell line stably transfected with nonsilencing control shRNA or p70S6K shRNA. Immunoblotting was done with p70S6K and pS6RP(Ser235/236) and actin antibodies (A). Pictures of cells grown on 100-mm plates were taken under a phase contrast microscope (B). Original magnification, ×100. Five-day growth rate analyzed by counting cells growing in culture dish (C). Matrigel invasion assays were done to compare invasiveness. Data from three independent experiments were compared using ANOVA statistical analysis (D).
cages in a room maintained at constant temperature and humidity with a 12-hours light-and-dark cycle; they were fed a regular autoclaved chow diet with water *ad libitum*. All studies were conducted according to an experimental protocol approved by the M.D. Anderson Institutional Animal Care and Use Committee.

To detect orthotopic tumor growth, we injected 3 million MDA-231-Luc cells into the right middle mammary fat pad of each mouse. To detect metastatic tumor growth, we injected 1 million MDA-231-Luc cells into the tail vein of each mouse. Imaging was done by a Xenogen (Caliper LS) device under anesthesia after 5 minutes of i.p. D-luciferin injection.

### Results

*p70S6K and its downstream pS6RP are higher in breast cancer cells than in normal breast tissue cells.* To compare p70S6K and pS6RP protein expression in breast cancer cells with expression in normal breast tissue, we first assayed cell lysates from several estrogen receptor–negative and estrogen receptor–positive breast cancer cell lines, including Sum-149, MDA-231, BT-20, MCF-7, MCF-7-Dox, BT-549, and MDA-MB-361. Cells from all breast cancer cell lines that we tested, except BT-20, showed higher expression of p70S6K and pS6RP than did benign MCF10A cells. Metastatic MDA-231 cells, Sum-149 cells, and drug-resistant MCF-7 (MCF-7-Dox) cells showed the highest levels of p70S6K and pS6RP activity (Fig. 1).

*p70S6K shRNA inhibits p70S6K and pS6RP protein expression, in vitro growth, cell motility, and invasion in MDA-231 cell line.* Because p70S6K and pS6RP protein expression was highest in the MDA-231 cell line, we decided to target p70S6K in MDA-231 cells. We used a lentiviral shRNA transduction approach to inhibit p70S6K stably in the MDA-231 cell line (MDA-231 sh p70S6K). We also transfected the MDA-231 cells with nonsilencing shRNA lentiviral particles to use as a negative control (MDA-231 sh cont). Stably transfecting MDA-231 cells with p70S6K inhibited p70S6K and phospho-Ser235/236 S6RP expression (Fig. 2A). After p70S6K downregulation, cells started to grow slower and to make colonies, unlike the control or parental cell lines (Fig. 2B). The growth of control shRNA–transfected cells was morphologically similar to the growth in untransfected parental cells, which shows that shRNA itself has

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**Figure 3.** MDA-231-Luc cell line stably transfected with nonsilencing control shRNA or p70S6K shRNA. After 4 days, cell culture cells are collected to detect apoptosis and to measure cell cycle. A, cells are stained with Annexin V and analyzed by flow cytometer to detect apoptosis. B, cells are stained with propidium iodide and analyzed by flow cytometer to measure cell cycle distribution.
no effect on morphology in culture conditions. We cultured p70S6K downregulated and control cells for 5 days in the same conditions to compare growth rates. MDA-231 sh p70S6K cells grew 50% slower than did control cells (Fig. 2C). Because microscopic images showed less motility and colony type growth, we further tested the invasion ability of p70S6K-downregulated MDA-231 cells.

A Matrigel in vitro invasion assay revealed that p70S6K downregulation inhibited 65% of the invasion ability in MDA-231 cells (Fig. 2D). Because growth inhibition occurred in p70S6K knockdown cells, we tested apoptosis and cell cycle. We did not observe apoptosis by doing Annexin V analysis, but there was a decline in S phase and a significant increase in G1 phase in p70S6K knockdown cells according to cell cycle analysis (G1 phase, 43.7% in control shRNA cells and 54.3% in p70S6K shRNA cells; Fig. 3).

**p70S6K shRNA inhibits orthotopic tumor growth and lung metastasis in animal model.** To investigate the role of p70S6K in breast cancer tumorigenesis and metastasis, we used an MDA-231 orthotopic (mammary fat pad) and metastatic (i.v.) animal model. In the orthotopic model, mice injected with MDA-231–p70S6K shRNA cells (0.95 ± 0.02 × 10⁸ photons) developed significantly smaller tumors than did the control mice injected with MDA-231 control shRNA cells (0.33 ± 0.15 × 10⁸ photons) as measured by in vivo luciferase activity (P < 0.01; Fig. 4A). To determine metastatic activity, we injected 1 million cells into the tail vein of each mouse. After 4 weeks, we used whole-body luciferase imaging to detect metastatic growth; we observed no metastasis in the p70S6K downregulated group but detected lung metastasis in all of the control mice (0.34 ± 0.07 × 10⁶; P < 0.001; Fig. 4B).

Targeting p70S6K inhibits transglutaminase 2, phosphorylation of focal adhesion kinase, β-catenin, and cyclin D1 and induces PDCD4 and E-cadherin. To determine the downstream targets and mechanisms that may play a role in metastasis, we used Western blot analysis to detect proteins that may be critical in cell attachment, motility, invasion, and metastasis. We observed that knockdown of p70S6K inhibited phosphorylation of focal adhesion kinase, a nonreceptor tyrosine kinase that transmits adhesion-dependent signals to promote cell growth, survival, and invasive functions. We also observed the downregulation of cyclin D1, a cell cycle progression protein. We previously showed that tissue transglutaminase 2 promotes cell attachment, survival,
and invasion and that the MDA-231 cell line expresses a high level of tissue transglutaminase 2 (TG2). Downregulation of p70S6K also inhibited tissue transglutaminase 2 and β-catenin expression in the MDA-231 cell line. In contrast, downregulation of p70S6K induced expression of PDCD4 and E-cadherin tumor suppressor proteins (Fig. 5).

Discussion

The results of our study show that p70S6K is involved in lung metastasis in breast cancer cells. p70S6K plays an important role in metastasis by regulating key proteins such as cyclin D1, PDCD4, focal adhesion kinase, E-Cadherin, β-catenin, and tissue transglutaminase 2 that are essential for cell attachment, survival, and invasion, as well as metastasis in breast cancer (Fig. 6). To study the role of p70S6K in lung metastasis, we generated stable p70S6K shRNA expression in the MDA-231 human breast cancer cell line. MDA-231 cell line metastasizes to the lung when it is injected i.v. into nu/nu mice (37). We showed p70S6K shRNA expression in MDA-231 cells inhibits lung metastasis in mice (Fig. 4B).

Because p70S6K shRNA transfectant MDA-231 cells showed no metastasis, we decided to evaluate the downstream signaling events. Previously, p70S6K had been linked to the upregulation of VEGF in breast cancer cell lines in animal experiments and patient samples.
Recent findings proved that p70S6K inhibits PDCD4 by phosphorylating it. PDCD4 inhibits eIF4A, which catalyzes the unwinding of secondary structure at the 5’ untranslated region of mRNAs. Phosphorylation of PDCD4 causes its degradation by proteasomes, which activate the translation of proteins necessary for tumor growth (34). Nieves-Alicea et al. (36) also showed that PDCD4 inhibits invasion in *in vitro* breast cancer cell lines. We found in our study that PDCD4 was upregulated by targeting p70S6K, which supports previously published data.

The metastasis process has three major steps. First is the separation of the cells from original tissue; second is immune surveillance in circulation; and third is the homing of cells on other tissues. In our animal model, we focused on the third step of metastasis: the homing cells on lung tissue. We injected MDA-231 cells into the tail vein of each mouse and observed lung metastasis. Because we focused on the homing process of metastasis, we also wanted to determine the link between p70S6K and cell attachment proteins such as tissue transglutaminase 2 and focal adhesion kinase, which were previously reported to be involved in metastasis (32). Transglutaminase 2 has been linked to focal adhesion kinase phosphorylation and phosphatase and tensin homolog degradation (38).

Our study is the first, to our knowledge, to report that p70S6K regulates tissue transglutaminase 2 and focal adhesion kinase expression. We found that p70S6K inhibition led to tissue transglutaminase 2 and phosphorylation of focal adhesion kinase inhibition, consistent with a previous findings that phosphorylation of focal adhesion kinase was downregulated in tissue transglutaminase 2–silenced cell lines (32). The strong correlation of phospho-p70S6K–Thr389 expression and increased metastasis with worse disease-free survival in patient samples supported the notion that p70S6K can exert its metastatic function through upregulation of protein translation. Our data indicated that knockdown of p70S6K in MDA-231 cells resulted in low levels of cyclin D1, tissue transglutaminase 2, β-catenin, and phosphorylation of focal adhesion kinase in oncogenes and high levels of PDCD4 and E-Cadherin in tumor suppressor genes (Fig. 5).

The strategy of blocking protein synthesis in cancer treatment is currently being investigated in clinical trials. The mTOR inhibitors, such as CCI-779, RAD001, and AP23573, all at various stages of clinical trials, are also known to inhibit p70S6K (39). Rapamycin resistance has been reported in human breast epithelial tumor cells (13). Targeting p70S6K may also be an alternative strategy in rapamycin-resistant cases. Clearly, the role of p70S6K, tissue transglutaminase 2, and phosphorylation of focal adhesion kinase in the regulation and targeting of p70S6K in metastatic breast cancer cells is an important area for future investigation.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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**References**

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