Introduction

Metastatic tumors are the most prevalent cause of death in cancer patients. A major aim in studying metastasis is to understand the mechanism by which cancer cells acquire distinct genetic and epigenetic changes that result in their progression through metastatic states. Recent experiments using microarray studies have expanded our understanding of metastasis in various human tumor samples (1, 2). Although such studies have been powerful for producing gene expression fingerprints of metastatic tumor cells, it has been difficult to assess the contribution of individual genes to the metastasis progression. Breast cancer is the most common malignancy in women and it could be effectively cured if diagnosed at an early stage. The most commonly used predictive molecular markers for breast cancer include Ki-67, estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) (3). We searched for new prognostic markers that not only could be predictive of the more aggressive forms of breast cancers but also could further provide mechanistic insight into the molecular mechanism underlying metastasis. In this study, we describe the increased expression of TREX84, a subunit of a multiprotein complex involved in transcriptional elongation and mRNA export, in human breast cancer and its intimate association with breast cancer progression and metastasis.

Materials and Methods

Primary breast cancer specimens. Human breast tissue specimens used in this study were collected following NIH guidelines and using protocols approved by the Institutional Review Board at Fox Chase Cancer Center. These specimens were surgically obtained from breast cancer patients at Fox Chase from 1991 to 2002. A total 72 primary breast cancer were examined which included 69 invasive ductal carcinomas and 3 invasive lobular carcinomas. Seventy females and two males were included in the study. Ninety percent (65 of 72) of the patients were Caucasian (i.e., white non-Hispanic), 8% (6 of 72) were African American, and 1% (1 of 72) were Asian. The age range was 31 to 97 years with a median age of 56 years. Grading of histologic malignancy of each specimen was assessed according to the system as reported previously (4, 5). Lymphonodal metastatic status was determined by histopathologic examination in each case according to the pTNM classification as proposed by the American Joint Committee on Cancer. Thirty-seven paired normal breast tissues were also obtained from the above patients. All of the samples were snap frozen in liquid nitrogen and kept at −80°C until used. Tissue extracts were prepared as previously described (6).

Affinity purification of Flag-p84. Flag-p84 and a selectable marker for puromycin resistance were cotransfected into HeLa cells. Transfected cells were grown in the presence of 5 mg/mL puromycin, and individual colonies were isolated and analyzed for Flag-p84 expression. To purify the p84 complex, nuclear extract from the Flag-p84 cell line was incubated with anti-Flag M2 affinity gel (Sigma, St. Louis, MO), and after extensive washing with buffer A [20 mmol/L Tris-HCl (pH 7.9), 0.5 mol/L KCl, 10% glycerol, 1 mmol/L EDTA, 2 mmol/L MgCl2, 5 mmol/L DTT, and 0.5% NP40], the affinity column was eluted with buffer A containing Flag peptide (500 mg/mL) according to manufacturer's instructions (Sigma). p84-containing eluate were fractionated on a Superdex 200 (Pharmacia, Peapack, NJ) equilibrated in 0.5 mol/L KCl in buffer A containing 0.1% NP40 and 1 μg/mL aprotinin, leupeptin, and pepstatin. Analysis of nuclear extract on Superose 6 was as described previously (7).

Glutathione S-transferase pulldown with UAP56. Control glutathione S-transferase (GST, lanes 1) or GST-UAP56 (lane 2) was incubated with HeLa nuclear extract. After washing with BC500 buffer [20 mmol/L Tris-HCl (pH 8), 500 mmol/L KCl, 10% glycerol, 0.2 mmol/L EDTA, and 1 mmol/L phenylmethylsulfonyl fluoride] proteins bound to GST-UAP56 or GST were analyzed by Western blot with p84 antibodies.

Organoid isolation, cell lines, and cell culture. Media and cell culture reagents were prepared by the Cell Culture Facility at Fox Chase Cancer Center. Eighteen cases of organoids were separated and prepared by using collagenase digestion as described previously (8, 9). Six primary cultures of human breast fibroblast cells were cultured in DMEM supplemented with 20% FBS and 1× antibiotic-antimycotic solution. Human breast cancer cell lines MDA-MB-231, MDA-MB-435, MDA-MB-468, MCF-7, BT-20, and ZR-75-1 were cultured in DMEM supplemented with 10% FBS and 1× antibiotic-antimycotic solution. T47D cells were maintained in RPMI supplemented with 10% FBS and 0.2% penicillin and streptomycin. SK-BR-3 cells were maintained in McCoy's 5a medium supplemented with 15% FBS.

Immunofluorescence. Cells grown in monolayer cultures were fixed with 4% paraformaldehyde in PBS, permeabilized with 0.2% Triton X-100,
and blocked with 10% FCS before antibody staining. Staining by anti-p84 antibodies was visualized with corresponding fluorescein-labeled secondary antibody. All images were acquired with a Bio-Rad MRC1000 confocal microscope.

**Western blotting assay.** After cell lysates were obtained from cell lines or tissues, 30 μg of total protein from each sample were analyzed by Western blotting. Protein extracts were electrophoresed on a 4% to 20% Tris-glycine gel, and the separated proteins were electrophoretically transferred to nitrocellulose for immunodetection. The membrane was blocked in 5% nonfat dry milk in TBST for 1 hour at room temperature and incubated with monoclonal antibody to human p84N5 at a dilution of 1:2000 in TBST + 2.5% nonfat dry milk, followed by horseradish peroxidase-conjugated antimouse secondary antibody (Amersham, Piscataway, NJ) at a dilution of 1:10,000. Immunoblots were reprobed with β-actin monoclonal antibody to confirm equal loading. MDA-MB-435 cell extracts were used as a control sample in each of the experiments. The expression levels of p84 and β-actin detected by immunoblotting were quantitated using the program IMAGE (NIH) for the integrated density of each band. Western blot assays were conducted in duplicate for each sample and the mean value was used for the calculation of protein expression levels.

![Western Blot](image)

**Figure 1.** p84N5 is aberrantly expressed in breast cancer. A, p84N5 protein expression in immortal breast epithelial cell lines (MCF-10A and MCF-10F), breast tumor cell lines, paired normal (N1-4), and breast cancer (T1-4) tissues. Protein samples were separated on a SDS-polyacrylamide gel and proteins were immunoblotted using anti-p84N5 or β-actin monoclonal antibodies. B, p84N5 protein expression in primary breast epithelial cell cultures (P1-P6) and purified organoids (O1-O6) by Western blotting. C, p84N5/β-actin ratio in breast cancer cell lines (TC), primary breast epithelial cell cultures (EP), fibroblast cell cultures (FB). D, immunohistochemical analysis of frozen sections of normal breast tissue and breast tumor specimens for the p84N5 protein. I, p84N5 is weakly expressed in the cytoplasm and nuclei of normal ductal epithelia and lobular epithelia. A few epithelial structures showed moderate immunostain. Inset, same region at lower magnification to show overall staining pattern with only few moderately stained ductal structures. II, p84N5 is intensively expressed in the cytoplasm and nuclei of a grade 1 invasive ductal carcinoma. III, p84N5 is expressed at high levels exclusively in the nuclei of a grade 3 invasive ductal carcinoma. IV, previous tumor section evaluated without the primary antibody to serve as a negative control. Magnification 200×.
**Immunohistochemistry.** p84N5 protein immunostaining was carried out with mouse monoclonal p84N5 antibody (Novus Biologicals, Littleton, CO), at a dilution of 1:100. Because the antibody available does not recognize p84N5 in formalin-fixed, frozen sections were used. For frozen section immunohistochemistry, the sections were fixed in cold acetone for 10 minutes and rinsed in cold PBS for 5 minutes. The sections were then incubated in methanol/0.3% hydrogen peroxide for 10 minutes, washed with PBS, and treated with 0.1% Triton X-100 in PBS for 5 minutes and washed with PBS again. The sections were then incubated at 4°C overnight with p84N5 antibody. Reaction products were visualized by immersing the glass slides in 3,3-diaminobenzidine tablet sets (Sigma Fast, Sigma) and counterstained with hematoxylin. A positive control was included in each experiment. As negative controls, either the p84N5 antibody was omitted or sections were washed in 1× PBS.

**Laser capture microdissection.** Laser capture microdissection (LCM) was done as previously described with minor modification (7). In brief, frozen normal and tumor breast tissue samples were embedded in ornithine carbamyl transferase medium, sectioned in a cryostat at 8-µm thickness, and mounted on nonadhesive glass slides. Fixation was done in 70% ethanol for 60 seconds. Breast epithelial cells were visualized by H&E staining. H&E-stained frozen sections were dehydrated for 30 seconds in 70%, 95%, and 100% ethanol with a final 2-minute dehydration step in xylene. Air-dried sections were then laser captured and microdissected by a PixCell II LCM system (Arcturus Engineering, Mountain View, CA). The normal or malignant mammary epithelial cells to be selectively microdissected away from stroma were identified and targeted through a 15-µm laser beam pulse activated the film on a CapSure LCM cap (Arcturus Engineering). Approximately 5 x 10² cells were captured for each specimen. Based on careful review of the histologic sections, each microdissection is estimated to contain ~90% of the desired cells. After microdissection, 100 µL of guanidinium isothiocyanate-containing lysis buffer with 0.7 µL mercaptoethanol were applied directly to the microdissected cells adhered on the CapSure LCM cap, samples were placed into a 0.5-mL microfuge tube, and vortexed vigorously. Total RNAs were extracted using the Strata Prep Total RNA Microprep Kit (Stratagene, La Jolla, CA). A DNease treatment was done according to the manufacturer’s recommendations. The RNA was resuspended in 20 µL RNA elution buffer. After being reconstituted by vacuum without heat, total RNA from each LCM sample was reverse transcribed in a 20-µL reaction as described above.

**Quantitative real-time PCR analysis.** cDNA mixture (0.63 µL) above was used in a real-time PCR reaction (25 µL total volume) done with Smart Cycle TD (Cepheid, Sunnyvale, CA) following methods recommended by the manufacturer. Optimal conditions were defined as step 1, 95°C for 10 minutes; step 2, 95°C for 15 seconds and 60°C for 60 seconds with Optics, repeated for 50 cycles. The relative mRNA expressions of p84N5 were adjusted with ACTB. The primer and probe sets used for real-time PCR were as follows: p84N5, forward primer 5’-GGACCCCTTGCAATGCTATG-3’ and reverse primer 5’-ACATGTCTCTCTGTTTTCAATT-3’Taqman probe, (FAM) 5’-ATAAATTATGATGACTCTCGGCTCAGGCTCAAGAAAAAGATGG-3’ (BH2Q); ACTB: forward primer 5’-GCCAGGTACACCACTGG-3’ and reverse primer 5’-GCCGCAGGTCTCTTTGCGGAT-3’Taqman probe, (Cal red) 5’-CGGTTCCCCTCTGCTGAGCC-3’ (BH2Q).

**Small interfering RNA transfection and cell proliferation.** The small interfering RNA (siRNA) sequences targeting p84N5 corresponded to the coding region 1652 to 1672 (5’-AATGATGCTCTACTGAGAA-3’) relative to

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Table 1. Relationship between p84 protein expression and clinicopathologic variables

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the start codon. The corresponding siRNA duplexes with the following sense and antisense sequences were used: 5'-UGAUGCUCUACUGAAGGAAdTdT (sense) and dTdTACUACGAGAUGACUUCCUU-5' (antisense). A nonspecific control XI siRNA duplex had the following sequences: 5'-AUAGAUAAGCACAAGCCUUACUU (sense) and UUUAUCUAUUCGUUCGGAAUGP-5' (antisense). All of the siRNA duplexes were synthesized by Dharmacon Research, Inc. (Lafayette, CO) using 2'-ACE protection chemistry.

Cells in the exponential phase of growth were plated at 30% confluence in 6-cm plates, grown for 24 hours, and transfected with siRNA (p84N5 siRNA: 200 nmol/L) using oligofectamine and OPTI-MEM I reduced serum medium (Invitrogen Life Technologies, Inc., Carlsbad, CA), according to the manufacturer's protocol. The concentrations of siRNAs were chosen based on dose-response studies. Silencing was examined 24, 48, and 72 hours after transfection. Control cells were treated with oligofectamine (mock) or transfected using a control siRNA. Cell proliferation and apoptosis was examined using Guava ViaCount and Nexin assays, respectively as previously described (10). All studies were done in triplicates.

Statistical methods. Statistical analyses, including $\chi^2$ and $t$ test, were done using Microsoft Excel software. All statistical tests were two sided, and $P < 0.05$ were considered to be statistically significant. Error bars represent 95% confidence intervals.

Results and Discussion

To identify novel genes whose aberrant regulation may result in sporadic breast cancer, we analyzed the expression profiles of genes in breast tumors using public databases. We focused on p84N5, a nuclear protein containing a DEATH-domain previously reported to associate with Rb (11, 12), as one of the genes that displayed increased expression in breast cancers. To directly analyze the expression of p84N5 in breast cancers, we compared the p84N5 protein levels in the breast cancer tissues and the surrounding normal tissues using Western blot analysis. As Fig. 1A indicates, whereas cancerous tissues displayed high levels of p84N5 expression, the levels of p84N5 in normal tissues were nearly undetectable (compare N1 through N4 and T1 through T4). Similar increased expression of p84N5 is evident comparing breast cancer cell lines and normal primary epithelial cells or breast organoids (Fig. 1A and 1B). We substantiated these results by examining the expression of p84N5 using real-time PCR and immunohistochemistry. Using frozen sections, we detected by immunohistochemistry that normal breast tissue displayed a heterogeneous expression...
pattern with a few ductal and lobular epithelial structures exhibiting moderate expression of p84N5, whereas most of the normal breast showed mild or negative expression of the protein (Fig. 1D, I). Conversely, ductal carcinomas showed an intense and homogeneous expression of p84N5, which is consistent with the Western blot analysis (Fig. 1C and D, II-IV).

We next asked whether p84N5 expression levels were indicative of the aggressive nature of the breast cancers. Comparison of early-stage tumors (grade 2) and those of later stages (grade 3) revealed a marked elevation of p84N5 RNA and protein levels in late-stage tumors (Fig. 2A and data not shown). Importantly, analysis of p84N5 levels in a large number of tumors revealed a strong relationship between p84N5 expression levels and lymph node metastasis ($P = 0.002$) and tumor size ($P = 0.015$; Table 1). Other prognostic indicators, including ER positivity ($P = 0.063$) and histologic grade ($P = 0.033$) were also found to be associated with increased p84N5 protein levels. To further confirm these results, lobular epithelial cells from normal breast tissues and malignant epithelial cells from grade 3 tumors were captured by laser capture microdissection and p84N5 levels were analyzed by quantitative real-time PCR (Fig. 2B). As Fig. 2B attests, the expression levels of p84N5 transcripts are elevated in all but one of the tumors as compared with histologically normal epithelial cells. When these tumors were subdivided based on clinical staging [combined T (tumor size), N (nodal involvement), M (metastatic) classification], p84N5 levels correlated with more aggressive tumors (stage I-II versus III-IV). Taken together, these data indicate that p84N5 is highly expressed in breast cancers and its expression is strongly associated with an aggressive phenotype of human breast tumors.

To gain insight into the biological role of p84N5, we isolated a p84N5-containing multiprotein complex from mammalian cells. This was accomplished by developing a 293-derived stable cell line expressing Flag-tagged p84N5. Figure 3A depicts the purification of Flag-p84N5 using anti-Flag antibodies followed by the analysis of the Flag-p84N5 eluate using gel filtration chromatography. This analysis revealed the specific association of p84N5 with polypeptides of 125, 120, 90, 45, 40, and 30K (Fig. 3B and C). Interestingly, mass spectrometric sequencing of p84N5-associated polypeptides revealed the identity of p84N5 associated proteins as the human counterpart parts of the yeast TREX complex reported to couple transcriptional elongation and mRNA export (Fig. 3D; refs. 13, 14). Therefore, we have termed this complex human TREX and p84N5 as hTREX84. Importantly, in contrast to the yeast TREX complex, the human complex was devoid of the RNA export and splicing factors ALY and UAP56 (13). We therefore asked whether endogenous ALY and hTREX84 form a stable complex which is reflected by coelution of the two proteins on gel filtration. Analysis of HeLa nuclear extract by Superose 6 sizing fractionation showed distinct chromatographic elution profiles for hTREX84 and ALY proteins indicating that the two proteins are not stably associated (Fig. 3E). However, consistent with a previous report (13), we observed the association of hTREX and ALY through the UAP56 protein (Fig. 3F), and that hTREX and ALY colocalize in breast tumor cells as determined by immunofluorescence assays (data not shown). These results indicate that whereas hTREX and ALY may not be stably associated, their interaction is promoted by the UAP56 protein.

The yeast TREX complex was shown to be intimately involved in the export of mRNA to the cytoplasm (13, 14). We therefore, asked whether human TREX also plays a role in mRNA export.

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**Figure 4.** Knock down of TREX84 leads to defects in mRNA export and cellular proliferation. **A.** Analysis of TREX84 and GAPDH mRNA levels following treatment of HeLa cells with siRNA against TREX84 or control siRNA. **B.** Treatment of HeLa cells with siRNA against TREX84 or control siRNA; treatment of cells with siRNA against TREX84 results in accumulation of mRNA in the nucleus. **C.** Analysis of TREX84 expression following siRNA treatment for 72 hrs by immunofluorescence staining in MDA-MB-231 tumor cells (left, cells transfected with control siRNA; right, cells treated with TREX84-siRNA). **D.** Photomicrographs show the morphology of the MDA-MB-231 cells following abrogation of TREX84 expression (left, tumor cells transfected with control siRNA; right, cells treated with TREX84-siRNA). **E.** Cell proliferation and apoptosis (data not shown) were examined using Guava ViaCount and Nexin assays, respectively. Plotted is the number of viable cells ($\times 10^3$) at 24, 48, and 72 hrs after treatment with control siRNA or with TREX84-siRNA. Three independent experiments.
mRNA was visualized using immunofluorescent analysis using oligo-dt as probes. To address the role of human TREX in mRNA export, hTREX84 protein was depleted using siRNA against hTREX84 following which mRNA levels were analyzed (Fig. 4A). Whereas the mRNA in cells treated with control siRNA could be visualized in both the cytoplasmic and the nuclear domains, treatment of cells with siRNA against hTREX84 resulted in the accumulation of mRNA in the nucleus and the loss of cytoplasmic mRNA (Fig. 4B). These results indicate that similar to the role for yeast TREX complex, hTREX plays a pivotal function in mRNA export.

Because hTREX84 is highly expressed in aggressive forms of breast cancer, we asked whether reduction of hTREX84 concentrations may slow the proliferative capacity of breast cancer cells. Human breast cancer cell lines express high levels of hTREX84 compared with that of primary breast epithelial cells and organoids (Fig. 1A and B). To address the proliferative potential of hTREX84, we treated MDA-MB-231 breast cancer cell line with siRNA against hTREX84 (Fig. 4C). Treatment of breast cancer cells with siRNA against hTREX84 potently and specifically reduced the proliferative potential of these cells (Fig. 4D and E). Analyses of these cells using a GuavaNexin assay found no statistically difference for Annexin V-PE and 7-AAD positive cells in siRNA treated cells, indicating the absence of induction of apoptosis (data not shown). Taken together, our finding suggest a role for the hTREX complex in cellular proliferation and following confirmation by other studies conducted among different populations in the future, hTREX84 may serve as a prognostic marker for aggressive forms of human breast cancer. Furthermore, therapeutic interventions that target human TREX should be of tremendous value in the fight against breast cancer.

Acknowledgments


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References

Linking Transcriptional Elongation and Messenger RNA Export to Metastatic Breast Cancers

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