Dicer-Mediated Upregulation of BCRP Confers Tamoxifen Resistance in Human Breast Cancer Cells

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Statement of Translational Relevance

Tamoxifen (Tam) is the most prescribed hormonal agent for the treatment of estrogen receptor (ER) α-positive breast cancer patients. However acquired Tam resistance frequently occurs, but very little is known about mechanisms of resistance. We discovered that Dicer overexpression leads to an increase in the breast cancer resistance protein (BCRP). We demonstrate that BCRP can function to exclude Tam from cells leading to a Tam-resistant (Tam\textsuperscript{R}) phenotype. We also show that BCRP inhibition or shRNA knockdown restored Tam sensitivity. Our results suggest that the combination of a BCRP inhibitor with Tam treatment may provide a strategy to circumvent Tam resistance and suggest that this approach should be studied further for clinical utility.
Abstract

Purpose: Tamoxifen (Tam) is the most prescribed hormonal agent for treatment of estrogen receptor α-positive breast cancer patients. Using microarray analysis, we observed that metastatic breast tumors resistant to Tam therapy had elevated levels of Dicer.

Experimental Design: We overexpressed Dicer in ERα-positive MCF-7 human breast cancer cells, and observed a concomitant increase in expression of the breast cancer resistance protein BCRP. We thus hypothesized that Tam resistance associated with Dicer overexpression in ERα-positive breast cancer cells may involve BCRP. We analyzed BCRP function in Dicer-overexpressing cells using growth in soft agar and mammosphere formation, and evaluated intracellular Tam efflux.

Results: In the presence of Tam, Dicer-overexpressing cells formed resistant colonies in soft agar, and treatment with BCRP inhibitors restored Tam sensitivity. Tumor xenograft studies confirmed that Dicer-overexpressing cells were resistant to Tam in vivo. Tumors and distant metastases could be initiated with as few as 5 mammosphere cells from both vector and Dicer-overexpressing cells, indicating that the mammosphere assay selected for cells with enhanced tumor initiating and metastatic capacity. Dicer-overexpressing cells with elevated levels of BCRP,
effluxed Tam more efficiently than control cells, and BCRP inhibitors were able to inhibit efflux.

**Conclusion:** Dicer-overexpressing breast cancer cells enriched for cells with enhanced BCRP function. We hypothesize that it is this population which may be involved in the emergence of Tam-resistant growth. BCRP may be a novel clinical target to restore Tam sensitivity.
Introduction

The antiestrogen tamoxifen (Tam), along with aromatase inhibitors (AIs), are the most frequently prescribed hormonal agents for the treatment of estrogen receptor α-positive breast cancer patients. However, despite the clinical success of these agents, treatment resistance in patients with ERα-positive breast tumors remains a major clinical problem (1).

It is possible that “targeted” therapeutics tailored to specific altered gene expression found in resistant tumors could restore hormone sensitivity. To search for targetable mechanisms of resistance in metastatic breast tumors, we performed a microarray analysis from clinical biopsies comparing Tam-sensitive (Tam\textsuperscript{S}) primary breast tumors to Tam\textsuperscript{R} metastatic tumors, and identified several potential gene candidates (2, 3). From this pilot screen, we identified Dicer as being overexpressed in metastatic, Tam\textsuperscript{R} tumors. Dicer expression has been shown to be important in maintaining the stem cell population, and in the transition of germ-line stem cells through the G1/S checkpoint (4-6). In addition to its role in stem cell proliferation, Dicer is an RNase III-containing enzyme which processes microRNA precursors into mature microRNA able to bind and repress translation, or induce degradation of complementary mRNAs (7), and microRNAs have been implicated in breast tumor invasion and metastasis (8, 9). Although a number of investigators have shown a role for breast cancer stem-like and progenitor cells in chemotherapeutic resistance (10-12), almost nothing is known about a role for these cells in hormonal therapy resistance.
Materials and Methods

Reagents, antibodies, and chemicals.

\(\beta\)-Estradiol 3-benzoate and Tam citrate for animal experiments, and 17\(\alpha\)-estradiol (E), 4-hydroxy-tamoxifen (T), and fumitremorgin C (FTC or F), Hoechst 33342, and propidium iodide (PI) were from Sigma (St. Louis, MO). MBLI-97 was synthesized as described (13). Antibodies against BCRP, IRS-1, and Cyclin D1 were from Millipore (Billerica, MA); PR A/B and p85 were from Cell Signaling (Danvers, MA); MDR-1, ER\(\alpha\), and \(\beta\)-actin were from Abcam (Cambridge, MA) Vector Labs (Burlingame, CA), and Sigma, respectively. Antibodies against ER\(\alpha\) (confocal), Dicer, Rho GDI\(\alpha\), CD24-FITC, CD44-PE, and MRP1 were from Santa Cruz Biotechnology (Santa Cruz, CA). Secondary antibodies goat anti-mouse or goat anti-rabbit were obtained from GE Healthcare (Piscataway, NJ). Alexa Fluor 546 goat anti-mouse and 488 goat anti-rabbit secondary antibodies were purchased from Invitrogen (Carlsbad, CA).

Tumor specimens and expression microarray analysis.

A cohort of frozen breast tumor specimens from nine patients who received adjuvant Tam was selected from the tumor bank of Breast Center, Baylor College of Medicine (Houston, TX) for use in the RNA analyses. This study was approved by the Baylor College of Medicine Institutional Review Board in accordance with federal human research study guidelines. Within this cohort, metastatic tumors from five patients who developed their recurrent lesions within 1 to 11 months while undergoing Tam treatment (Tam\(^R\)) and four primary tumors that were collected at the time of initial diagnosis from patients who remained disease-free for 93 to 123 months with a median follow-up of 106
months (Tam\textsuperscript{5}) were examined using Affymetrix expression microarray as described previously (2).

**Cells and tissue culture conditions.**

MCF-7 parental breast cancer cells were cultured as described (14). MCF-7 cells stably overexpressing Dicer or vector control plasmid (pcDNA3.1, Invitrogen, Carlsbad, CA) were generated using stable transfection, and supplemented with 800μg/mL Geneticin (Invitrogen). The human Dicer expression vector has been previously described (15). To generate Tam\textsuperscript{R} cells, we cultured our parental MCF-7 cells in phenol red-free MEM medium supplemented with 5% charcoal-stripped FBS, antibiotics, and 10\textsuperscript{-7} 4-hydroxy-tamoxifen. Cells were continuously exposed for 6 months during which time the medium was replaced every 4-5 days (these cells are designated MCF-7 TR2). Initially cell growth was slow, but gradually increased, and cells have been maintained in Tam for greater than 1 year.

**Immunoblot analysis.**

After specified treatments, cells were lysed in warm 5% SDS in water or in RIPA buffer containing protease inhibitor cocktail (Calbiochem, San Diego, CA) and sodium orthovanadate (Sigma). Protein concentrations were measured using BCA protein assay (Thermo Scientific, Rockford, IL). Equal amounts of protein extracts were subjected to SDS-PAGE as described (14).

**MTT assay.**
Cells were starved in 5% charcoal-stripped FBS phenol red-free (PRF) MEM media (Invitrogen) for 48hrs prior to plating 500 cells per well in a 96 well plate. Cells were treated with vehicle, 1nM estradiol, or 100nM Tam in 5% stripped PRF MEM for 4, 8, and 12 days. After treatment, 100μl MTT (Sigma) was added to each well and incubated at 37°C for 2hrs. Medium was aspirated and 100μl DMSO added to each well. OD570 was read subtracting OD650 on a microplate reader (Biorad, Hercules, CA).

Quantitative RT-PCR assay.

cDNA was synthesized from 250ng of total RNA using Superscript III Reverse Transcriptase from Invitrogen. 20pmoles of each specific primer were used for cDNA synthesis in 20μl reactions. cDNA was analyzed by REAL TIME PCR using an Applied Biosystem7500 system using TaqMan Universal PCR Master Mix and primer-probe sets for β-actin, human BCRP (400nM forward primer TGCAACAGGAAACAATCCTTGT, 400nM reverse primer AGATCGATGCCCTGTTCACC, and 100nM 6FAM-CAACATGTACTGGCGAAGA-MGBNFQ), and human Dicer by the TaqMan Gene Expression Assay (Applied Biosystems, Foster City, CA). RNA expression was quantified using ABI Prism sequence detection software. qRT-PCR results are represented as relative copy number normalized to β-actin.

Anchorage-independent growth assay.

Cells (5X10^3 cells/well) were plated in 3ml of 0.3% SeaPlaque agarose on top a base of 0.6% agarose in six-well plates. The cells were treated with vehicle, 1nM estradiol, 10nM or 100nM Tam, and/or 5μM FTC in full medium containing 10% serum. On day
14, the colonies (>50μm) were counted using the Gelcount machine and software (Oxford Optronix, Oxford, UK). Experiments with shRNA to Dicer and BCRP were performed in 5% charcoal-stripped FBS in PRF-MEM.

**Flow cytometry.**

Secondary mammosphere cells were dissociated with Versene (Invitrogen), and passed through a 40μm sieve (BD Falcon, San Diego, CA) to ensure single cell suspension prior to antibody staining. Cells were resuspended in PBS with CD24-FITC and/or CD44-PE antibodies for 15min at 4°C. The cells were then resuspended in 2% paraformaldehyde at a concentration of 1X10^7 cells/ml, and analyzed on a LSRII flow cytometer (BD Biosciences). Isotype controls were used to set the gate for the analysis of the CD44^+/CD24^-/low population.

**Hoechst 33342 efflux assays.**

Cells were resuspended in MEM, with 2% fetal calf serum, and 10mM HEPES buffer at a concentration of 1X10^6 cells/ml. Cells were incubated in Hoechst 33342 dye (5μg/ml) for 2hrs at 37°C, then resuspended in HBSS with 2% fetal calf serum, 10mM HEPES buffer containing 2μg/ml PI, and analyzed on a LSRII.

**Mammosphere culture.**

Mammospheres were grown as described (16). Mammospheres were allowed to grow for approximately 14 days and then they were dissociated enzymatically, and replated to grow secondary mammospheres.
**Tumor xenografts.**

All animal studies were carried out according to the guidelines and with the approval of the Baylor College of Medicine Animal Care and Use Committee. Female nude ovariectomized athymic mice, 5-6 weeks old, were obtained from Harlan (Indianapolis, IN). Our preliminary data showed that we could use much fewer cells if cells grown in mammosphere culture were injected, rather than cells grown on plastic (data not shown). Thus, secondary mammospheres were dissociated and counted on a hemocytometer for injection. Cells in conditioned media were mixed 1:1 with growth factor-reduced PRF matrigel (BD Biosciences), and inoculated s.c. into the 4th mammary gland as described (2). Animals were supplemented subcutaneously with silastic tubing releasing 80pg/ml of β-estradiol,3-benzoate. When the tumors grew to ~200mm³, estrogen treatment was continued, or the tubing was removed and daily subcutaneous Tam injections were begun. Tumor volumes were measured three times weekly for 4-6 weeks.

**Immunofluorescence and confocal microscopy.**

Secondary mammospheres were harvested, washed in PBS, and seeded in matrigel:PBS (1:1) solution on 8-well chamber slides. Matrigel was allowed to solidify at 37°C for 30min. Mammospheres were fixed in 2% PFA/PBS (Sigma) for 20min. Acini were permeabilized with 0.5% TritonX-100/PBS for 10min and then wash three times with 100mM Glycine/PBS. Mammospheres were then blocked with 10% BSA/IF buffer (7.7mM Sodium Azide, 0.1% BSA, 0.2% TritonX-100, 0.05% Tween-20 in PBS) for 1hr. Primary antibody was diluted in 10% BSA/PBS and slides incubated at RT
overnight. Slides were washed three times with IF buffer. Mammospheres were incubated with 1:500 secondary antibodies (Alexa Fluor 546 goat anti-mouse and 488 goat anti-rabbit, Invitrogen) in 10% BSA/PBS for 1hr. Slides were washed three times in IF buffer and mounted with Vectashield with DAPI (Vector). Immunofluorescence was imaged with a Leica Confocal microscope.

**shRNA.**

Cells were plated in regular growth medium to 60–70% confluence. Then the medium was changed with serum free medium (SFM) without P/S and cells were transfected with 5μg of shRNA empty vectors and shRNA plasmids using Lipofectamine™ LTX with PLUS™ (Invitrogen) as recommended by the manufacturer. Dicer shRNA plasmids and empty vector pRS were purchased from ORIGENE (Rockville, MD). BCRP shRNA plasmid and empty vector pLKO.1 were purchased from Thermo Scientific. 24hrs post transfection the cells were split into 15 cm plates with regular media. Puromycin (1μg/ml) was added and cells were cultured until colonies were picked and assayed for Dicer and BCRP expression. The colonies with low expression of Dicer and BCRP were chosen for further experiments.

**Efflux assay.**

Cells (0.16x10^6) were plated in MEM+P/S+1.5% BSA in 96-well plate and incubated at 37°C overnight. Cells were loaded with 56nM ³¹H-Mitoxantrone (Moravek Biochemicals, Brea, CA) or 5nM ³¹H-4-OH-Tamoxifen (American Radiolabeled Chemicals, St. Louis, MO) in SFM+1.5% BSA for 24hrs. Cells were washed in pre-
warmed serum/BSA free media, and then incubated in 1.5% BSA/media +/- FTC or MBLI97 for the time course indicated. $^3$H-Mitozantrone and $^3$H-4-OH-Tam efflux was performed at 37°C and 25°C, respectively. Efflux media was collected and cells were lysed in 0.1N NaOH. All samples were mixed with Opti Fluor liquid scintillation cocktail (PerkinElmer, Waltham, MA). Radioactivity of all samples was measured on a Beckman LS6500. Fraction of $^3$H-compound efflux was calculated as radioactivity of efflux media/(media + cells).

Statistical analyses.

All data are expressed as the mean (+/-SD) of at least three independent experiments unless otherwise stated. Unpaired two-sample t-test analysis was used to analyze the differences between the treatment and control groups. Some data was compared using two-way ANOVA testing for a significant interaction between the treatment and cell types. Statistical analysis was performed using R (www.r-project.org) or Red-R (www.red-r.org). P-values were adjusted using the Bonferroni method. L-calc software was not used to model tumor initiation because our data did not fit the model, so Poisson regression was utilized to model tumor initiation rates in the Dicer-overexpressing (MCF/D15) and vector-control cell groups (MCF/VC1) adjusting for the effect of different serial dilutions.

Results

Dicer overexpression and resistance to Tam.
To identify genes whose expression was associated with the development of Tam resistance, we compared primary tumors from patients that did not relapse after Tam treatment, with metastatic tumors from patients that progressed during adjuvant Tam treatment using expression microarray analysis (2, 3). These gene expression analyses identified Dicer as being more highly expressed in the TamR metastatic tumor group compared to the TamS group (Fig. 1A).

To evaluate the potential relationship between Dicer overexpression and Tam resistance, TamS MCF-7 parental breast cancer cells were stably transfected with a Dicer overexpression vector (clones MCF/D12 and D15), or stable Dicer pooled transfectants were generated (MCF/DP1). Parental cells were also stably transfected with a vector-alone plasmid (MCF/VC1), or vector-alone pools were generated (VP1 and VP2) (Fig. 1B). All of the Dicer-transfected clones expressed higher Dicer protein levels (Fig. 1C).

To rule out the possibility that loss of ER expression could account for Tam resistance, we performed immunoblot analysis for ERα. ERα expression was maintained in both vector control and Dicer-overexpressing clones, although variable levels of ERα were observed, probably due to clonal variability. As an additional control, we also transiently transfected MCF-7 cells with a Dicer expression vector along with an ERE-luciferase reporter, but did not see alterations in activity with estradiol (E), or Tam (T) treatments (Supplemental Information [SI] 1). In addition we performed immunoblot analysis for ERα target genes including IRS-1, progesterone receptors (PR) A and B, and cyclin D1 (Figure 1D). These proteins were similarly regulated by estradiol and Tam in
Dicer clones demonstrating that ERα activity was not altered with Dicer overexpression in MCF-7 cells.

In *Drosophila*, germ-line stem cell division is regulated by Dicer, and in mammals, proliferation is compromised in Dicer-deficient murine embryonic stem cells (6, 17). To determine whether Dicer overexpression affects cellular response to Tam resistance, we conducted MTT (Fig. 1E) and soft agar (Figure 1F) growth assays in the presence or absence of Tam. Tam treatment of vector cells significantly reduced proliferation by 81% compared to a reduction of 35% for Dicer-overexpressing cells. Tam treatment of vector cells reduced the number of soft agar colonies by 50%. In contrast, Tam treatment of Dicer-overexpressing cells decreased colony number by only 21-33% (MCF/DP1 and D15, respectively). These data indicate that Dicer overexpression rendered cells less responsive to Tam.

*Dicer overexpression affects mammosphere formation efficiency and Tam response in vivo.*

It has been shown that culturing cells as nonadherent mammospheres enriches for stem-like cells from normal epithelium (16), and for tumorigenic cells from breast cancer cell lines (18). Changes in mammosphere formation efficiency in consecutive passages is used as an *in vitro* assay for anchorage-independent growth (16). There were no detectable differences in primary mammosphere formation efficiency in untreated Dicer-overexpressing versus vector cells, however in the presence of Tam Dicer-overexpressing cells had a 3.75-fold higher sphere forming efficiency (%SFE) than vector cells (data not
shown). Upon passage, secondary mammospheres were either left untreated, or were treated as indicated, and the %SFE calculated. Again, there were no significant differences in mammosphere-forming potential in either vector only or Dicer-overexpressing cells treated with vehicle. Cells treated with estrogen showed variable %SFE, probably due to the presence of epidermal growth factor in the culture medium (19). In contrast, mammosphere formation in Dicer-overexpressing clones was significantly elevated with Tam treatment, but significantly decreased secondary mammospheres in vector cells (Fig. 2A). The ~2.5-fold enhancement in relative %SFE of the Dicer-overexpressing cells suggests that Tam treatment eliminated mammosphere-initiating cells in the vector only population, but that Dicer overexpression stimulated the relative frequency of TamR mammosphere-initiating cells.

MCF-7 cells cultured for long periods in the presence of 100nM Tam develop acquired Tam resistance (20). Immunoblot analysis of MCF-7 parental and acquired TamR MCF-7 cells generated in our laboratory (MCF/TR2) showed that endogenous Dicer levels were elevated 6-fold (Fig. 2B); these cells also expressed elevated levels of ERα, and exhibited enhanced proliferation in the presence of Tam (Fig. 2C). Secondary mammosphere formation of MCF-7 cells was similar when treated with vehicle or Tam (Fig. 2D). In contrast, Tam treatment enhanced %SFE 2-fold in MCF/TR2 cells, suggesting that Tam might be acting as an agonist. Together these data suggest that Dicer overexpression may be associated with acquired Tam resistance.
Previous studies in xenografts and breast cancer cell lines, including MCF-7, have shown that cells flow-selected for the CD44+/CD24low-/ESA+ population efficiently initiated tumors in nude mice (21). In addition, several studies suggest that the CD44+/CD24low- tumor-initiating population represents a basal, ER-negative subtype (22). Given the observed increase in %SFE of Dicer-overexpressing cells in response to Tam, we hypothesized that these mammospheres might be enriched for CD44+/CD24low- cells. We found that mammospheres from vector cells contained a 0.85% CD44+/CD24low- compartment (Fig. 2E and SI 2). Treatment with estrogen expanded this compartment approximately 2-fold, whereas Tam treatment significantly reduced it. Mammospheres derived from an untreated Dicer-overexpressing clone were enriched for CD44+/CD24low- cells relative to vector (2.2% and 0.85%, respectively); neither estrogen nor Tam treatments significantly affected this subpopulation. These results suggest that Dicer overexpression enhanced the CD44+/CD24low- compartment which is TamR.

We next asked whether the observed enhancement in anchorage-independent growth detected in the mammosphere cultures, and the increased CD44+/CD24low- population could similarly affect Tam tumor initiation and growth in vivo. First, we confirmed that extracts from Dicer-overexpressing mammospheres continued to express ERα, and as expected, ERα levels were increased with Tam treatment (Fig. 2F). Estrogen-induced progesterone receptor (PR-A and B isoform) levels which were also decreased with Tam, demonstrating that hormonal regulation of total ERα and PR levels were not globally altered with mammosphere culture.
To evaluate the effect of Dicer overexpression on the tumor-initiating population in mammospheres, we performed a limiting dilution transplantation experiment using mammosphere-cultured cells implanted as xenografts into athymic mice. Because mammary tumor initiation in athymic nude mice requires estrogen supplementation, secondary mammospheres from the estrogen supplemented groups were dissociated, and serial dilutions from 10,000 to 2 cells were used for tumor initiation (Supplement Table 1). The tumor initiation rate in the MCF/VC1 vector group was 0.40 (95% CI: 0.28 – 0.57) compared to 0.19 (95% CI: 0.12 – 0.32) in the Dicer-overexpressing group. This two-fold decrease in tumor initiation was statistically significant (95% CI: 1.17 – 3.67, p = 0.012, Poisson regression modeling). Tumor initiation from mammosphere culture was observed with as few as 5 cells (4/17 in the vector, and 1/10 in the Dicer group), confirming that mammosphere culture enhances tumor initiation. In addition, tumor initiation from mammospheres in this experiment represents a higher frequency than that reported for CD44+/CD24low− flow-sorted cells obtained from pleural effusions of breast cancer patients (21). We also detected metastatic lesions in the lungs of the animals injected with both groups, although there was no significant difference in metastasis incidence between the two groups (representative H&E of the metastases is shown in Fig. 2G, left panels). To date, there are few models of ERα-positive breast cancer cells which efficiently metastasize to distant organs. The lung metastases were positive for the estrogen-induced PR, possibly indicating an intact downstream ERα signaling network in the metastases (Fig. 2G, right panels). These data demonstrate that mammosphere growth selects for a population of cells with enhanced tumor initiation and metastatic potential. Because mammosphere formation assays and limiting-dilution tumor initiation
experiments results may negatively correlate if progenitor-like cells are increased at the expense of regenerative stem cells (23), Dicer overexpression may be preferentially influencing the survival of division-competent cells during Tam treatment, but not enhancing tumor initiation.

The estrogen treated mammospheres (shown in Fig. 2A) were disassociated, and 1000 cells injected into mice supplemented with estradiol. Once the tumors grew to 200mm³, they were randomized to continue estrogen, or the estrogen was withdrawn and Tam injections begun. We compared tumor volumes for each group over time. Growth rates for Dicer and control-expressing groups were not statistically different when the groups were treated with E2. We also observed no difference in growth between the E2-treated and Tam-treated Dicer expressing tumors, indicating that the effects of E2 and Tam were similar when Dicer is overexpressed, and that Tam may be acting as an agonist. As expected, treatment of control cells with Tam significantly reduced tumor growth rates, indicating that these cells are sensitive to Tam treatment. These data suggest that Dicer confers Tam resistance in vivo, and that Tam treatment stimulates growth of Dicer-overexpressing cells similar to estrogen treatment.

**Dicer increases BCRP expression.**

We performed immunoblot analysis for proteins associated with drug resistance in Dicer-overexpressing cells (24, 25) (26). Dicer-overexpressing mammospheres expressed elevated levels of the breast cancer resistance protein BCRP, which was independent of hormone treatment in one-dimensional cultures (Fig. 3A). Since elevated
levels of the BCRP “pump” are known to be associated with resistance to a number of chemotherapeutics (27), we next focused on whether elevated levels may mediate the TamR phenotype of Dicer-overexpressing cells.

Levels of BCRP protein were also found to be elevated in Dicer-overexpressing cells grown in mammosphere cultures; the MCF/D12 clone exhibited a 4-fold increase, MCF/D15 clone a 4.5-fold, and the MCF/DP1 pool a 3.5-fold increase in BCRP levels (Fig. 3B and quantified in 3C). Thus, increases in BCRP expression were not just a consequence of cell culture conditions. The effects of Dicer overexpression on BCRP levels may be at the transcriptional level, since higher levels of BCRP mRNA were also seen in the MCF/D15 clone (Fig. 3D). Importantly, we found that 3/5 of the TamR breast tumors initially screened in our microarray analysis (Fig. 1A) also expressed elevated levels of BCRP mRNA, whereas none of the TamS tumors expressed measurable levels using qRT-PCR assay (Fig. 3E).

BCRP, along with MDR1 and MRP1, are three of the most studied multidrug resistance proteins. Clinically, a Phase III trial in 99 breast cancer patients found that the combination of the MDR1 inhibitor Verapamil with a chemotherapeutic yielded significantly longer overall survival and a higher response rates than those treated with chemotherapy alone (28). MDR1 was expressed at comparable levels in both cells, but MRP1 was expressed at slightly higher levels in Dicer-overexpressing pools (Fig. 3F). We have focused on BCRP in this study, but we have not yet eliminated a role for MRP1 in the phenotype of Dicer-overexpressing cells.
**Dicer overexpression increases the side population.**

It has been demonstrated that the BCRP-expressing side population (SP) in MCF-7 cells represents a small proportion of the total population, but can exhibit enhanced stem/progenitor cell properties compared to non-SP (NSP) cells (25), although this has been disputed by others (29). SP cells are also known to be resistant to chemotherapeutic agents (30). Cells treated with vehicle or Tam were subjected to flow separation to isolate the SP, and results presented as the %SP (Fig. 3G and SI 3). The %SP of both Dicer-expressing clones was significantly elevated compared to vector control cells treated with vehicle (C). Tam treatment did not alter the %SP in control cells, however a significant increase in the %SP was observed in MCF/D15 (the Dicer clone with the highest levels of BCRP protein compared to its control). The SP fraction of MCF/DP1 (1.6%) was 8-fold higher than the control cells (0.2%), however the NSP fraction of both control cells and the Dicer pool were similar, 29.8% and 29.3% respectively. Immunoblot analysis was performed on the sorted NSP and the SP fractions (Fig. 3H). The SP of the vector cells contained very few cells (0.2%) and thus BCRP protein levels were undetectable by immunoblot. The SP fraction in the Dicer-overexpressing pool (MCF/DP1) expressed elevated levels of BCRP protein while maintaining Dicer and ERα expression (Fig. 3I). The SP fraction of MCF/VC1, though low (0.2%, Fig. 3I), contained high levels of ERα consistent with our data demonstrating that tumor-initiating cells can be ERα-positive. To further demonstrate that the cells grown as mammospheres expressing BCRP also expressed ERα, we performed immunofluorescence followed by confocal microscopy (Fig. 4). As expected, MCF/VP1
mammospheres expressed ERα in the nucleus, but no BCRP was seen under control conditions. ERα expression was maintained in mammosphere growth conditions, and BCRP was detected in Tam treated control cells. In MCF/DP1 mammospheres, ERα was similarly expressed in the nucleus with both treatments. However, elevated levels of BCRP were detected in the plasma membrane of Tam-treated Dicer-overexpressing mammospheres. The merge of ERα and BCRP demonstrated co-expression of ERα and BCRP within the same cell, with ERα being nuclear and BCRP in the membrane. These data suggest that the TamR phenotype of Dicer-overexpression is not due to a simple loss of ERα during mammosphere culture.

**Modulation of Tam response by BCRP knockdown and inhibitors.**

To confirm the role of BCRP in response to Tam, we first used a specific BCRP inhibitor, MBL197 (M,(13)) in soft agar assays (Fig. 5A). Tam treatment reduced growth by 82%, but the combination of Tam plus M did not further reduce soft agar growth of vector cells. In contrast, the combination of Tam plus M significantly enhanced Tam’s effects in Dicer-overexpressing cells, 63 to 73% (D15 and D12 clones, respectively).

Next, MCF/DP1 cells were stably transfected with a shDicer expression vector or a control shRNA vector (Fig. 5B). All the shDicer clones exhibited a reduction in Dicer expression; BCRP expression was concomitantly reduced in the knockdown clones (Pearson’s correlation p=0.00038). Two shDicer clones were used in a soft agar growth assay to test for response to Tam and/or a BCRP inhibitor (Fig. 5C). Fumitremorgin C (FTC or F), a BCRP inhibitor which inhibits BCRP-related ATPase activity was used
(26). Tam treatment reduced the number of colonies of vector transfected cells by 35%. FTC alone had no significant effect, but the combination of Tam plus F significantly enhanced Tam’s growth inhibitory effects. In contrast, Tam reduced soft agar growth >50% in the shDicer knockdown clones, but no further differences were detected with the combination of Tam+F.

To confirm these findings, we stably transfected MCF/DP1 cells with shBCRP or control vectors. BCRP expression was reduced >50% in knockdown clones 5, 18, 20, and 22, but Dicer levels remained unchanged (Fig. 5D). These data suggest that Dicer can modulate BCRP levels, but BCRP does not appear to affect Dicer levels. Knockdown of BCRP significantly restored sensitivity to Tam in all the shBCRP clones (Fig. 5E). These collective data implicate a role for BCRP via modulation of Dicer in response to Tam in breast cancer cells.

To examine mechanisms associated with BCRP-mediated Tam resistance, we conducted drug efflux assays. Vector control and Dicer-overexpressing cells were preincubated with tritiated-Mitoxantrone (\(^{3}H\)-MTX), a known substrate for the BCRP efflux pump, then treated with the BCRP inhibitor MBLI97, and extracellular drug accumulation measured. Vector cells were able to efflux \(^{3}H\)-MTX at comparable rates in the presence or absence of MBLI97 (Fig. 6A). Dicer-overexpressing cells effluxed more MTX compared to control cells, and MBLI97 reduced efflux to levels seen in control cells. To determine if Tam was a substrate in our cells, we next performed efflux using tritiated-Tam (\(^{3}H\)-4-OH Tam). All cells effluxed Tam rapidly (Fig. 6B and C). FTC and
M treatments did not affect efflux of Tam in control cells. In contrast, Tam efflux in Dicer-overexpressing cells was reduced with FTC and M treatments. We conclude that Dicer overexpression enhanced for a subpopulation of ERα-positive progenitor cells expressing BCRP, and that targeting of BCRP may restore Tam sensitivity in breast cancer cells.

Discussion

In the present study, we investigated a unique molecular mechanism underlying a TamR phenotype in ERα-positive breast cancer cells. Dicer RNA was found to be relatively increased in TamR patient tumors, and Dicer via modulation of BCRP levels conferred Tam resistance in ERα-positive breast cancer cells. Dicer and BCRP were similarly found to be elevated in an acquired model of Tam resistance.

Mammosphere potential has been used as both a stem cell renewal and anchorage-independent growth assay (16). Mammosphere growth of Dicer-overexpressing MCF-7 cells in the presence of Tam was significantly enhanced. Tam treatment also enhanced the %SFE, and a potential marker of breast cancer stem cells, ALDHI, was enhanced in Dicer-overexpressing cells. Furthermore, Tam increased the SP in these cells. ERα expression was maintained in Dicer-overexpressing cells, and was thus not lost as has been reported in some models of TamR. These data are supportive of some reports suggesting that ERα-positive progenitor cells can express stem cell-like markers and contain an elevated SP (31). However, accumulating evidence suggests that breast cancer stem cells are ERα-negative, and that estrogen expands these cells via paracrine-acting
protein factors (10). It is possible that paracrine mechanisms may be operating in our models of TamR.

Hormone resistance is most probably multifactorial; growth factor signaling and altered ERα regulation are central to the problem of resistance (28). Although it is emerging that chemotherapeutic resistance may be due to expression and function of multidrug resistance proteins in cancer cells (32), it is not known if hormone resistance is associated with multidrug resistance proteins. To study Tam resistance in vivo, different tumor xenograft experiments were employed. To date, 500 mammosphere cells cultured from breast cancer patient pleural effusions (33) or 100 CD44+/CD24low/- lineage− cells from pleural effusions (21) have been shown to initiate tumors in immunocompromised mice. Dicer-overexpressing mammosphere-selected cells injected into the mammary fat pad of athymic mice required estradiol supplementation for efficient tumor take (data not shown), and tumors could be initiated with as few as 5 cells, demonstrating that mammosphere culture greatly enhanced for tumorigenic cells. Dicer-overexpressing mammosphere-selected cells treated with Tam also exhibited tumor growth rates comparable to estrogen-treated tumors, suggesting that they were not simply hormone-independent as several other models of resistance (34). These data highlight the uniqueness of Tam resistance uncovered with Dicer and BCRP overexpression.

Immunoblot analysis revealed a potential novel molecular mechanism for this resistance—upregulation of BCRP. Others have shown that BCRP functions to “pump” drugs from cancer cells (27, 35). The SP is a functional readout for BCRP function (25), and elevated BCRP is evident in the SP of many cancers, including breast cancer (30).
BCRP can also transport androgens, sulfated estrogens, and some metabolites of Tam (36-38). We determined that Dicer-overexpressing cells expressed an elevated SP when treated with Tam. Acquired resistance to long-term treatment with Tam in MCF-7 xenografts has been associated with markedly reduced cellular concentrations of Tam (39). Tumor Tam levels can vary widely, and in one study the majority of non-responding breast cancer patients exhibited low tumor Tam levels (40). We determined that BCRP could efflux Tam from Dicer-overexpressing TamR cells. Our in vitro results also show that BCRP inhibition could restore Tam sensitivity, therefore identifying a novel and clinically relevant model for TamR breast cancer.

In summary, our data provide a novel mechanism underlying Tam resistance in breast cancer, and provides preliminary preclinical evidence that BCRP may be a new biological target in combination with endocrine therapy for the treatment of TamR tumors.

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Conflict of Interest
None of the authors have any competing financial interests in relation to the work described.

Acknowledgements

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References


Figure Legends

Figure 1. Dicer expression in metastatic breast cancer and MCF-7 cell line. (A) Microarray analysis of a cohort of Tam\(^S\) (n=4) and Tam\(^R\) metastatic breast cancer tumors (n=5) (**\(P= 0.0024\)). (B) Immunoblot analysis of whole-cell lysates for Dicer and ER-\(\alpha\). \(\beta\)-Actin was used as the loading control. (C) Quantitative analysis shown is the fold change in Dicer/\(\beta\)-Actin protein expression ratio (from panel B) relative to MCF/VC1 for MCF/D12 and MCF/D15 and MCF/VP1 for MCF/DP1. (D) Whole-cell lysates were treated with vehicle, 1nM estrogen (E), or 100nM tamoxifen (T) for 48 hours and immunoblotted for estrogen responsive proteins IRS-1, PR-A/B, Cyclin D1, and \(\beta\)-actin. (E) MTT growth assay was performed on cells treated with vehicle and 100nM T for 12 days. Tam treatment of vector cells reduced the proliferation rate by 81% (\(P=3.83\times 10^{-6}\)) compared to only 34.5% for MCF/D15 (*\(P=0.014\)) and 34.9% for MCF/DP1 (**\(P=0.0006\)). The data represented the fold change of the mean and standard deviation of four independent experiments. ***\(P=1.04\times 10^{-6}\) for Tam-treated MCF/D15 clone and 8.38\times 10^{-8}\) for MCF/DP1 pool compared to Tam-treated vector cells based on an ANOVA analysis. (F) Cells were plated in soft agar and then treated with vehicle (C) or 100nM T. Cells were allowed to grow for 14 days and the number of colonies >50mm were quantified and results were graphed. The data represent the fold change in the mean of three independent experiments with 95% CI. ***\(P=1.32\times 10^{-6}\) (MCF/D15) and 1.32\times 10^{-6}\) (MCF/DP1) compared to Tam-treated vector cells based on an ANOVA analysis.

Figure 2. Tam resistance and tumor initiation. (A) Dissociated primary mammospheres from cells were plated as described in material and methods and treated
with vehicle (C), 1nM E, or 100nM T and allowed to propagate as mammospheres for 14 days, the number of mammospheres were quantified, and the results were graphed. The results shown are means and 95% CI of three independent experiments. *P= 0.015 MCF/VP1, P=0.019 MCF/D12, and P=0.036 MCF/D15 for vehicle compared to T. (B) Immunoblot analysis for Dicer, ERα, and p85 (loading control) was performed on parental and tamoxifen-resistant MCF-7 cells. MCF/TR2 cells were cultured in PRF-MEM + 5% charcoal stripped serum + 100nM T. (C) MTT growth assay was performed on cells treated with vehicle and 100nM T for 6 days. The data represented the mean and standard deviation of four independent experiments. ***P=8.88x10⁻⁷ compared to Tam-treated parental cells based on an ANOVA analysis. (D) Dissociated primary mammospheres from parental (MCF) or MCF/TR2 cells were plated as described in material and methods and treated with vehicle (C) or 100nM T and allowed to propagate as mammospheres for 14 days, the number of mammospheres were quantified, and the results were graphed. ** p= 0.0002 compared to vehicle. (E) Cells were plated as secondary mammospheres and were treated with vehicle (C), 1nM E, or 100nM T for 14 days. Flow cytometric analysis was performed for CD44-PE and CD24-FITC on an LSRII. Results shown are the mean and 95% CI of the percent CD44⁺/CD24low⁻ of two independent experiments. *P=0.0037 Tam-treated vector compared to vehicle. The difference between vehicle and Tam-treated Dicer-overexpressing cells was not significant (P=0.07). *P=0.01 vehicle treated MCF/D15 compared to MCF/VC1. Data was highly reproducible for Tam-treated groups. (F) Immunoblot analysis for Dicer, ER-α, PR-A, PR-B, and β-actin protein expression in secondary mammosphere cells cultured for 14 days in 1nM E or 100nM T. (G) Histological analysis and PR
immunohistochemistry of spontaneous lung metastases generated from the tumor xenograft experiment. Arrows point to metastatic lesions in H&E and positive PR-stained metastatic lesions. Photographs are representative, and were taken at 20X magnification. (H) Dissociated secondary mammosphere cells from the E and T-treated groups were injected into the mammary fat pad of athymic nude mice (n=5/group). Animals were supplemented with silastic tubing containing E (80pg/ml). Tumors were measured twice weekly; and when the tumor burden reached 200mm³, animals were randomized to remain on E or withdraw E and begin daily subcutaneous injections of Tam. Tumors were measured twice weekly and harvested when their size reached 600-800mm³. MCF/VC1 (E) (solid circles), MCF/D15 (E) (open squares), MCF/VC1 (E to T) (open squares), or MCF/D15 (E to T) (open diamonds). P=3.592e-7 for MCF/VC1 E compared with E to T group, P=0.1529 for MCF/D15 E compared with E to T. **P=1.217e-10 for MCF/D15 E to T group compared to MCF/VC1 E to T.

**Figure 3.** (A) Immunoblot analysis of secondary mammospheres for the expression of progenitor-like proteins BCRP, ERα, and β-Actin. Mammospheres were treated with vehicle (C), 1nM E or 100nM T for 14 days prior to immunoblot. (B) Immunoblot analysis on cells for BCRP and β-actin protein expression. (C) Quantitative analysis shown is the fold difference in BCRP/β-Actin protein expression ratio (from panel B) relative to MCF/VC1 for MCF/DP1 and MCF/D15, and MCF/VP1 for MCF/D12. (D) qRT-PCR for BCRP was performed on cells. (E) qRT-PCR for BCRP was performed on the cohort of TamS (n=4) and TamR (n=5) tumors from the microarray in figure 1a. (F) Immunoblot analysis for Dicer, BCRP, MDR1, MRP1, and β-actin. (G) SP analysis of
cells treated with vehicle (C) or 100nM T for 48hr. SP was performed on the LSRII. *P=0.0257 for MCF/D12 Tam-treated cells compared to vector Tam-treated cells. **P=0.0002 for MCF/D15 Tam-treated cells compared to vector Tam-treated cells. The mean and 95% CI of three independent experiments is represented. (H) Graphic representation of % gated singlets that were non-SP and SP fractions. (I) Immunoblot analysis on SP and non-SP (NSP) sorted cells (from panel h) for Dicer, BCRP, ERα, and RhoGDIα.

Figure 4. Immunofluorescence for ER and BCRP in vehicle (C) and 100nM T-treated secondary mammospheres. DAPI was used to stain nuclei of cells.

Figure 5. (A) Cells were plated in soft agar and treated with vehicle (C), 750nM MBLI97 (M) and/or 100nM T for 10 days. *P=0.0038 and **P=0.00025 for Tam-treated compared to T+M-treated MCF/D12 and MCF/D15, respectively. (B) MCF/DP1 cells were stably transfected with shRNA to Dicer. shDicer stable transfectants were analyzed for Dicer, BCRP, and GAPDH (loading control) protein expression. * indicates clones used in subsequent experiments. (C) Soft agar assay for anchorage-independent growth was performed on cells treated with vehicle, 100nM T, and/or 10μM Fumitremorgin C (F) for 14 days. Colony number was determined using GelCount machine and software. *P=0.008 for V6 (control plasmid transfected into MCF/DP1) tam-treated compared to T+F. (D) MCF/DP1 cells were stably transfected with shRNA to BCRP. shDicer stable transfectants were analyzed for Dicer, BCRP, and GAPDH protein expression. * indicates clones used in subsequent experiments. (E) Soft agar assay for anchorage-
independent growth was performed on cells treated with vehicle or 100nM T for 14 days. Colony number was determined using GelCount machine and software. **P=0.0008, *p=0.002, *p=0.032, and *p=0.027 compared to vehicle for shBCRP5, 18, 20, and 22, respectively.

**Figure 6.** (A) Cells were plated in 96-well dish and loaded for 24hrs with 56nM $^3$H-Mitoxantrone ($^3$H-MTX). Efflux was performed in the presence or absence of MBLI97 (M) for the time course indicated at 37°C. **P=0.00058 for MCF/D15+M compared to MCF/D15 utilizing an ANOVA test. (B) Cells were plated in 96-well dish and loaded for 24hrs with 5nM $^3$H-4-hydroxytamoxifen ($^3$H-4-OH-Tam). Efflux was performed in the presence or absence of FTC for the time course indicated at 25°C. P=0.082 (not significant) for MCF/D15+FTC compared to MCF/D15 based on an ANOVA test. (C) Cells were plated in 96-well dish and loaded for 24hrs with 5nM $^3$H-4-OH-Tam. Efflux was performed in the presence or absence of MBLI97 (M) for the time course indicated at 25°C. *P=0.024 for MCF/D15+M compared to MCF/D15 using ANOVA.
**Figure 1,** Selever

**A**

![Graph showing Dicer RNA levels](image)

**B**

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Figure 2. Selever

A. SFE (%) values for MCF/VP1, MCF/D12, and MCF/D15 with C, E, and T treatments.

B. Western blot analysis showing expression levels of Dicer, ERα, and p85 for MCF, MCF/TR2, MCF/VC1, and MCF/D15.

C. Graph showing time-course changes in normalized absorbance for MCF and MCF/TR2.

D. SFE (%) for C, T, and E treatments with MCF/D12 and MCF/D15.

E. % CD44+/CD24− expression for MCF, MCF/TR2, MCF/VC1, and MCF/D15.

F. Tumor volume (mm³) for MCF/VC1, MCF/D15, with E and T treatments.

G. H&E and PR staining images for MCF/VC1 and MCF/D15.

H. Graph showing normalized absorbance changes for MCF and MCF/TR2.

Legend:
- **: p < 0.01
- ***: p < 0.001

Note:
- All graphs and images are referenced and labeled appropriately with statistical significance.
- The graphs and images are from a research study on cancer.
Figure 4, Selever

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Figure 5, Selever

A

# of Colonies

C T M T+M
MCF/VC1

C T M T+M
MCF/D12

C T M T+M
MCF/D15

B

MCF/DP1

shDicer

V6 V7 V8 21 22 23 24

Dicer

0.89 0.94 1.17 0.25 0.08 0.29 0.3

BCRP

1.02 0.98 0.99 0.30 0.11 0.37 0.38

GAPDH

C

# of Colonies

C T F T+F
V6

C T F T+F
shDicer21

C T F T+F
shDicer22

D

MCF/DP1

shBCRP

V5 V7 5 6 10 11 18 19 20 22

BCRP

Dicer

GAPDH

E

# of Colonies

C T C T C T C T C T

V7 shBCRP5 shBCRP18 shBCRP20 shBCRP22

0.89 0.94 1.17 0.25 0.08 0.29 0.3
Figure 6, Selever

A

B

C

\[ \text{MCF/VC1} \quad \text{MCF/VC1 + M} \quad \text{MCF/D15} \quad \text{MCF/D15 + M} \]

\[ \text{MCF/VC1} \quad \text{MCF/VC1 + FTC} \quad \text{MCF/D15} \quad \text{MCF/D15 + FTC} \]