Cell Death and Survival

Molecular Cancer Research

STAT3-RANTES Autocrine Signaling Is Essential for Tamoxifen Resistance in Human Breast Cancer Cells

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Abstract

The acquisition of tamoxifen resistance is a major therapeutic problem in breast cancer. We developed a tamoxifen-resistant MCF-7 (TRM-7) cell line to elucidate the molecular mechanisms and factors associated with acquisition of such resistance. We showed that phosphorylation of STAT3 at tyrosine 705 (Y705) and RANTES expression are increased in response to tamoxifen in human breast cancer cells. On the basis of these results, we hypothesize that upregulated STAT3 phosphorylation and RANTES may be correlated with the development of drug resistance. Here, we showed that STAT3 and RANTES contribute to the maintenance of drug resistance. STAT3 phosphorylation is constitutively retained via a RANTES autocrine loop, which in turn upregulates anti-apoptotic signals in TRM-7 cells. STAT3–RANTES autocrine signaling affected expression of anti-apoptotic BCL-2 family genes and prevented TRM-7 cells from undergoing programmed cell death by inhibiting PARP and caspase-9 cleavage. Subsequently, blockade of STAT3 and RANTES in TRM-7 cells resulted in reduction of anti-apoptotic signals, which was rescued by exogenous RANTES treatment; drug resistance was also restored. Taken together, our results suggested that STAT3–RANTES autocrine signaling is essential for maintenance of drug resistance and inhibition of programmed cell death. These mechanisms of STAT3–RANTES autocrine signaling suggest a novel strategy for management of patients with tamoxifen-resistant tumors. *Mol Cancer Res; 11(1); 31–42.* ©*2012 AACR.*

Introduction

Breast cancer is the second most common cancer worldwide after lung cancer, the fifth most common cause of cancer death, and the leading cause of cancer death in women. The global burden of breast cancer exceeds that of all other cancers, and its incidence is increasing (1). In women younger than 50 years with breast cancer, chemotherapy increases the 15-year survival rate by 10%, whereas the increase is only 3% in older women. However, chemotherapy has a wide range of acute and long-term side effects that substantially affect the patient's quality of life (2).

Tamoxifen is one of the most widely prescribed drugs for the treatment of estrogen receptor α (ERα)-positive breast cancer. Tamoxifen has been shown to greatly prolong disease-free survival and induce remission in more than half of all patients with ERα-positive metastatic disease (3, 4). It is also a preventative agent for hormone-dependent breast cancer. The significant reduction in breast cancer mortality during the last decade is thought to be attributable to the widespread use of tamoxifen (5). Despite the indisputable benefits of tamoxifen treatment, its success is limited by the ability of cancer cells to acquire resistance. Tamoxifen resistance is the underlying cause of treatment failure in a significant number of patients with breast cancer (6). Furthermore, tamoxifen-resistant breast cancer cells can contribute to an enhanced aggressive tumor phenotype and transition toward a mesenchymal phenotype (7). Therefore, clarification of the mechanisms underlying tamoxifen resistance in breast cancer has important clinical implications.

The STAT proteins are a family of transcription factors that mediate responses to a variety of cytokines and growth factors (8, 9). There are 7 known mammalian STAT proteins, STAT1, 2, 3, 4, 5a, 5b, and 6, which are involved

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in cell proliferation, differentiation, and apoptosis (10, 11). STAT3 is constitutively activated and contributes to oncogenesis in many human cancers (12, 13), including 82% of prostate cancers (14), 70% of breast cancers (15), more than 82% of squamous cell carcinomas of the head and neck (16), and 71% of nasopharyngeal carcinomas (17). STAT3 participates in oncogenesis by upregulating genes encoding apoptosis inhibitors (BCL-2, BCL-xL, MCL-1, and survivin), cell-cycle regulators (cyclin D1 and c-Myc), and inducers of angiogenesis (VEGF; ref. 18). High levels of constitutive STAT3 activation are thought to arise from autocrine stimulation (18–21). Thus, interruption of constitutive STAT3 signaling in tumor cells will likely inhibit expression of several important classes of oncogenic proteins (22).

Regulated upon activation normal T-cell expressed and secreted (RANTES) is a member of the CC-chemokine family and plays a crucial role in the migration and metastasis of human cancer cells. Associations between RANTES expression and melanoma, lung, prostate, and pancreatic cancers have been reported (23–25). In terms of breast cancer, a number of studies have suggested that RANTES is associated with breast malignancy. In patients with breast cancer, advanced disease, early relapse, and poor prognosis are significantly correlated with elevated RANTES expression levels in biopsy specimens and serum (26–28). Therefore, the precise role of RANTES signaling in breast cancer should be clarified.

Here, we report a significant correlation between STAT3–RANTES autocrine signaling and acquisition of tamoxifen resistance. We found that STAT3 and RANTES in TRM-7 cells regulate each other via autocrine signaling, leading to induction of an anti-apoptotic signal, which facilitates maintenance of drug resistance. These findings may be relevant to the development of tamoxifen resistance.

Materials and Methods

Materials

Tamoxifen, MTT, and AG490 were purchased from Sigma-Aldrich. Recombinant human RANTES and anti-RANTES neutralizing antibody were purchased from PeproTech.

Cell culture conditions and establishment of tamoxifen-resistant cells (TRM-7)

MCF-7 cells were cultured at 37°C in 5% CO_2/95% air in Dulbeccos' Modified Eagle's Media (DMEM; Lonza) containing 10% FBS (Lonza), 100 units/mL penicillin, and 100 μg/mL streptomycin (Lonza). Tamoxifen-resistant MCF-7 cells (TRM-7) were established using the modification of the methodology reported elsewhere (3) MCF-7 cells were washed with PBS, and the culture medium was replaced with that containing 1 μmol/L tamoxifen. Cells were continuously exposed to this treatment regimen, during which the medium was replaced every 2 to 3 days. After 6 months of treatment, the tamoxifen concentration was gradually increased to 3 μmol/L over a 9-month period. Initially, the MCF-7 cell growth rates were reduced. However, after tamoxifen exposure, growth rates increased gradually and cells eventually became tamoxifen-resistant (29) T47D cells were cultured at 37°C in RPMI (Lonza) containing supplements, as mentioned above, and treated with 1 μmol/L tamoxifen for 2 weeks.

MTT assay

To determine viability, cells were plated at 0.3×10^4/well in 96-well plates or 2×10^4/well in 24-well plates. MCF-7 and TRM-7 cells were incubated in serum-free medium with or without tamoxifen for 24 hours. Viable adherent cells were stained with MTT (2 mg/mL) for 4 hours. The medium was then removed, and the formazan crystals produced were dissolved by addition of dimethyl sulfoxide. The absorbance at 540 nm was then measured. Viability is expressed as the ratio relative to untreated control cells.

Annexin V and propidium iodide staining

Early and late induction of apoptosis were evaluated by flow cytometry using a FACS Canto II (BD Biosciences). Cells were plated at 0.5×10^5/well in 12-well plates. After incubation with tamoxifen for 24 hours, the medium was collected and attached cells were harvested. Cells were washed by centrifugation at 1,800 rpm for 5 minutes, and early and late apoptosis were evaluated by FITC-Annexin V (BioLegend) and propidium iodide (PI; Sigma) staining, according to the manufacturer's protocol.

Western blotting analysis

Cells were washed twice with cold PBS and then lysed in ice-cold modified radioimmunoprecipitation assay (RIPA) buffer containing protease and phosphatase inhibitors. Lysates were centrifuged at 13,000 rpm for 20 minutes at 4°C, and supernatants were collected. Proteins in lysates were separated by SDS-PAGE and transferred onto nitrocellulose membranes (Millipore). Membranes were incubated with primary antibodies, washed, treated with peroxidase-conjugated secondary antibodies, rewashed, and then visualized using an enhanced chemiluminescence (ECL) system (Amersham). Primary antibodies were as follows: anti-tyrosine-phosphorylated STAT3, anti-total STAT3, and anti-caspase-9 (Cell Signaling Technology); anti-α-tubulin (NeoMarkers); and anti-PARP (Santa Cruz).

Culture medium treatment

Approximately 1×10^5 MCF-7 and TRM-7 cells were seeded. After culture for 24 hours, cells were starved of serum for 48 hours. Culture medium of TRM-7 cells was then collected without debris and applied to MCF-7 cells. MCF-7 cell culture medium was also applied to MCF-7 cells as a positive control.

Cytokine array

The concentrations of several chemokines and cytokines in culture supernatants were determined using the Proteome Profiler Array Human Cytokine Array Panel A (R&D Systems). Membranes were developed according to the manufacturer's protocol, and relative cytokine levels were

determined by comparing the density of each spot with the negative and positive controls.

RANTES ELISA

RANTES expression levels in serum-free supernatants after 48 hours of incubation were determined using a commercially available ELISA kit (PeproTech), according to the manufacturer's instructions.

RNA purification and cDNA preparation

RNA was isolated using the TRIzol reagent (RNAiso; TaKaRa), according to the manufacturer's instructions. RNA was extracted with phenol-chloroform, ethanol-precipitated, and resuspended in diethyl pyrocarbonate–treated H_2O. cDNA was prepared with a QuantiTect Reverse Transcription kit (Qiagen) and subjected to quantitative real-time PCR and reverse transcription PCR (RT-PCR).

Quantitative real-time PCR and RT-PCR

Primer pairs for RANTES (Cat. # QT00090083), BCL-2 (Cat. # QT00025011), BCL-xL (Cat. # QT00236712), and GAPDH (Cat. # QT00090083) were purchased from Qiagen. Quantitative real-time PCR was carried out using EvaGreen (ABM), according to the manufacturer's protocol. Each sample was processed in parallel with assays for the glyceraldehyde-3-phosphate dehydrogenase *(GAPDH)* housekeeping gene; the absolute levels of each mRNA were thus normalized relative to that of GAPDH. The same primers used for RT-PCR were applied to duplicate RNA samples. Polymerase activation was conducted at 94°C for 5 minutes, followed by 25 cycles of denaturation at 94°C for 30 seconds, annealing at 58°C for 30 seconds, and extension at 72°C for 30 seconds, with a final extension at 72°C for 5 minutes. All amplicons were resolved by gel electrophoresis, visualized under UV illumination, photographed, and scanned when necessary using a Gel Doc 2000 imaging system (Bio-Rad Laboratories, Ltd.).

RANTES neutralization

TRM-7 culture medium was collected over a 48-hour period. Twenty-four hours before collection of medium, anti-RANTES neutralizing antibody (1 μg/mL; PeproTech) was added to the cultures.

Transient transfection and luciferase assay

A RANTES promoter–reporter construct (pGL3-RANTES) was prepared by inserting a 1.3-kb region of the human RANTES promoter into the pGL3-basic luciferase reporter plasmid. MCF-7 cells were seeded onto 24-well plates at a density of 2×10^4/well in serum-free media. Cells were cotransfected with the promoter–luciferase reporter construct (250 ng), and various combinations of the following constructs (250 ng of each plasmid): MOCK, wild-type STAT3 (WT STAT3), point-mutation STAT3 (Y705F STAT3), and β-galactosidase construct (150 ng). Cells were transfected using Lipofectamine LTX reagent (Invitrogen), according to the manufacturer's instructions. After 48 hours, cells were harvested and luciferase activities were determined using the Luciferase Assay System (Promega).

siRNA

STAT3 and RANTES were knocked down by transfection with siSTAT3 (5′-CAG CCT CTC TGC AGA ATT CAA-3′; Qiagen) and siRANTES (5′-CAG-GAG-CTT-ACT-GGC-AAA-CAT-3′; Qiagen) RNAs, respectively. AllStars Negative Control siRNA (Qiagen) was used as a control. siRNA transfection was conducted using the HiPerfect Transfection Reagent (Qiagen), according to the manufacturer's protocol.

Breast cancer tissue immunohistochemical staining

The human ERα-positive breast cancer tissue microarray (TMA) was purchased from SuperBioChips (Cat#CBA4). Eight ERα-positive breast cancer tissue specimens were obtained from patients with breast cancer who had been treated with tamoxifen at Seoul National University Hospital (Seoul, Korea). Tissue sections were deparaffinized with xylene and washed with ethanol. For antigen unmasking, slides were incubated in 10 mmol/L sodium citrate buffer (pH 6.0) and maintained at a sub-boiling temperature for 10 minutes. Endogenous peroxidase activity was inhibited using 3% hydrogen peroxide for 10 minutes, and specimens were then washed with PBS. Slides were in blocked with 5% goat serum for 1 hour at room temperature and incubated at 4°C overnight with either anti-phospho STAT3 (1:200) or anti-RANTES (1:200) antibody diluted in Antibody Diluent (Invitrogen). After washing 3 times with PBS containing Tween-20 (PBST), slides were incubated with biotinylated secondary antibody, diluted in antibody diluent, for 30 minutes. Slides were then incubated with ABC reagent (Vector Labs) for 30 minutes at room temperature and treated with diaminobenzidine (DAB) as the chromogen (DakoCytomation) for 1 to 3 minutes. Immediately upon development, slides were immersed in distilled water and counterstained with hematoxylin, followed by mounting using Canada balsam reagent.

Statistical analysis

Data are presented as means ± SEM of n independent experiments. Differences between groups were analyzed using Student t test. In all analyses, $P < 0.01$ was taken to indicate statistical significance.

Results

Tamoxifen treatment induces STAT3 phosphorylation in breast cancer cells

Tamoxifen, a partial ERα antagonist, is part of the treatment modality for patients with ERα-positive breast cancer. Acquired resistance, however, is an emerging problem. Thus, we hypothesized that tamoxifen-induced signaling pathways are important for acquisition of resistance by breast cancer cells. To clarify the mechanisms associated with resistance, TRM-7 cells were established over a 9-month period. MCF-7 cells were cultured simultaneously as a

control. After 9 months, the phenotypes of the 2 cell lines were similar (Supplementary Fig. S1). Tamoxifen resistance was verified by MTT assay. Tamoxifen treatment decreased the viability of MCF-7 cells in a dose-dependent manner. However, TRM-7 cells showed significantly greater viability than MCF-7 cells under the same conditions (Fig. 1A). TRM-7 cells were more resistant to tamoxifen ($IC_{50} = 14.9$ μmol/L) than were MCF-7 cells ($IC_{50} = 7.5$ μmol/L). To confirm this observation, MCF-7 and TRM-7 cells were treated with 15 μmol/L tamoxifen for 24 hours. Tamoxifen-induced apoptosis was then detected by Annexin V and PI staining. Tamoxifen-induced apoptosis (Annexin V and PI double-positive) was markedly greater in MCF-7 (87.3%) than in TRM-7 cells (43%; Fig. 1B). These results suggest successful establishment of TRM-7 cells by long-term tamoxifen exposure.

In a variety of human cancers, constitutively activated STAT3 is sufficient to induce tumor formation (30, 31) and is frequently detected in specimens from patients with advanced breast cancer (32, 33). Phosphorylated STAT3 levels in MCF-7 cells are low. However, recent research has indicated enhanced STAT3 activation in tamoxifen-resistant MCF-7 cells (34). We also detected phosphorylated STAT3 in MCF-7 cells exposed to tamoxifen (Fig. 1C). Moreover, chronic induction of phosphorylated STAT3 was detected in TRM-7 cells (Fig. 1D). Thus, phosphorylated STAT3 is induced during the acquisition of tamoxifen resistance.

RANTES-mediated STAT3 activation

Many studies have suggested an association between STAT3 activation and breast tumorigenesis (20, 35). Moreover, factors released from cancer cells may activate STAT3 in an autocrine manner and maintain tumor STAT3 activity and oncogenic potential (19, 21,36, 37). These findings led us to assume that whether TRM-7 cells maintain STAT3 activation by an autocrine loop, and they will likely be more clinically aggressive. To test this hypothesis, we evaluated STAT3 phosphorylation in MCF-7 cells after treatment with TRM-7 culture medium. TRM-7 culture medium strongly induced STAT3 phosphorylation after treatment for 10 minutes; this phosphorylation was also noted in MCF-7 cells (Fig. 2A). Thus, TRM-7 cells can activate STAT3 in an autocrine manner.

Next, to identify the factors associated with STAT3 phosphorylation, we determined cytokine levels in MCF-7 and TRM-7 CM using a cytokine array. Serum-starved MCF-7 and TRM-7 culture medium were collected after 48 hours. Secreted RANTES levels were upregulated in TRM-7 culture medium (Fig. 2B). Other cytokines were detected at comparable levels in culture medium of the 2 cell lines (Fig. 2B, Supplementary Fig. S2). We determined RANTES mRNA and protein levels by real-time PCR (Fig. 2C) and ELISA (Fig. 2D), respectively. The results were correlated with RANTES cytokine array expression profiles.

To determine the effects of RANTES secreted from TRM-7 cells on STAT3 phosphorylation, RANTES was

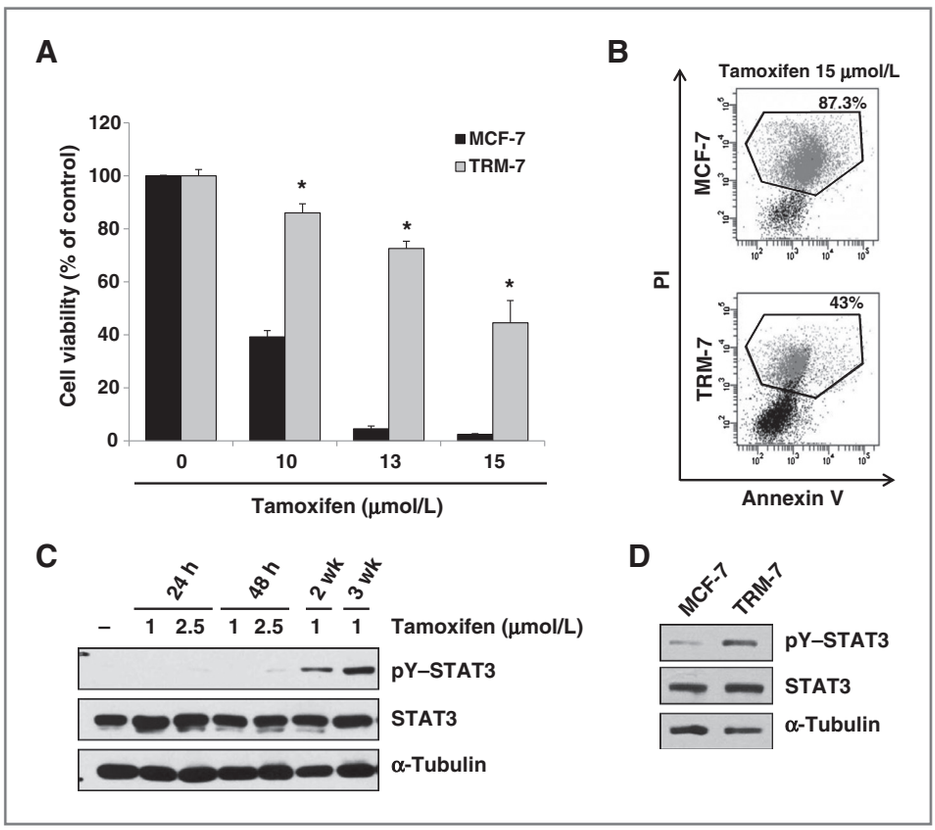

Figure 1. Establishment of TRM-7 cells and constitutive activation of STAT3. A, after tamoxifen treatment (0–15 μmol/L, 24 hours), the viabilities of MCF-7 and TRM-7 cells were determined by MTT assay. Columns, means of triplicate samples; bars, SD. *, $P < 0.01$, compared with control cells. B, the effects of acute tamoxifen treatment (15 μmol/L, 24 hours) on apoptosis were evaluated by FITC-Annexin V and PI staining. Early and late apoptosis were then evaluated by flow cytometry. C, MCF-7 cells were incubated with tamoxifen (1, 2.5 μmol/L) for 24 or 48 hours, or 2 or 3 weeks. Total cell lysates were immunoblotted. D, constitutively activated STAT3 status of TRM-7 cells. After culture with or without 1 μmol/L tamoxifen for 9 months, MCF-7 and TRM-7 cells were harvested and lysates were subjected to Western blotting.

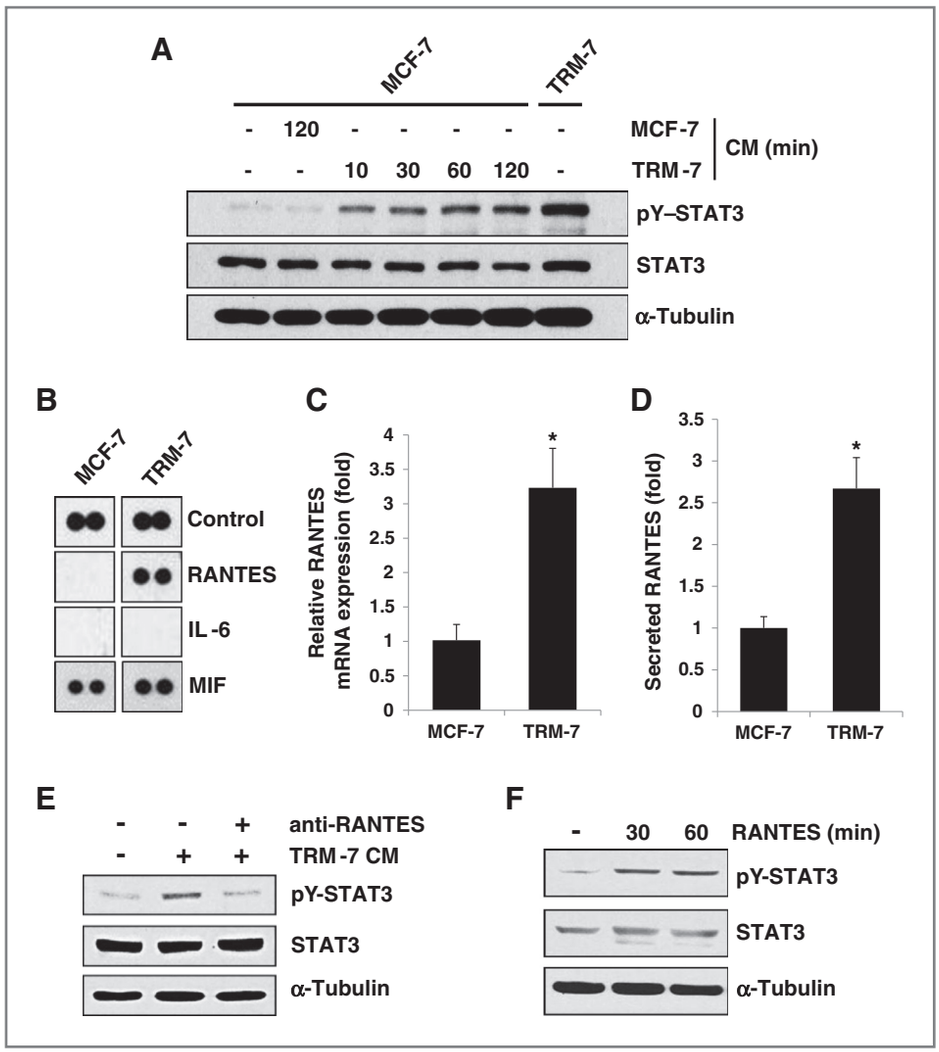

Figure 2. TRM-7 cell culture medium containing RANTES promotes STAT3 phosphorylation in MCF-7 cells. A, MCF-7 cells were incubated with TRM-7 culture medium (CM) for 10, 30, 60, or 120 minutes. Phosphorylated STAT3 levels were then evaluated by Western blotting. B, MCF-7 and TRM-7 CM was collected over a 48-hour period, and cytokine levels were determined using the human cytokine array. IL, interleukin; MIG, macrophage-inhibitory factor. C, RANTES mRNA levels were determined by real-time PCR. (D) RANTES secretion levels were evaluated by ELISA. Columns, means of triplicate samples; bars, SD. *, $P < 0.01$, compared with MCF-7 cells. E, MCF-7 cells were incubated in the absence or presence of secreted RANTES in TRM-7 culture medium for 30 minutes. Phosphorylated STAT3 levels were determined by Western blotting. F, MCF-7 cells were incubated with recombinant RANTES (25 ng/mL) for 30 or 60 minutes. Phosphorylated STAT3 levels were determined by Western blotting.

neutralized in TRM-7 culture medium using a neutralizing antibody (Supplementary Fig. S3). We collected TRM-7 culture medium in the absence and presence of secreted RANTES protein and applied these to MCF-7 cells for 30 minutes. In the presence of RANTES, TRM-7 culture medium strongly activated STAT3 in MCF-7 cells. In contrast, in the absence of RANTES, STAT3 phosphorylation levels were similar to those in control MCF-7 cells (Fig. 2E). In addition, exogenous RANTES activated STAT3 in MCF-7 cells (Fig. 2F). This observation was in agreement with previous reports that RANTES activates STAT3 signaling in breast cancer cells (38, 39). These results indicated that high STAT3 phosphorylation levels in TRM-7 cells are maintained by RANTES overexpression and autocrine signaling.

Phosphorylated STAT3 upregulates RANTES expression

RANTES is a STAT3 target gene (40, 41). To investigate the role of STAT3 activation in RANTES expression, we used the JAK2 inhibitor, AG490, which suppresses constitutive STAT3 activation. In TRM-7 cells, STAT3 phosphorylation levels were markedly decreased by AG490 (Fig. 3A). Interestingly, RANTES secretion was decreased in concert with STAT3 phosphorylation (Fig. 3A), as was RANTES mRNA level, as determined by real-time PCR (Fig. 3B). These observations were supported by the changes in RANTES promoter activity. MCF-7 and TRM-7 cells were transfected with a RANTES promoter–luciferase reporter construct. Endogenously activated STAT3-dependent RANTES promoter activity was then determined (Fig. 3C). To further investigate STAT3-dependent RANTES regulation, MCF-7 cells were cotransfected with the RANTES promoter–luciferase reporter construct or various combinations of the STAT3 constructs and a β-galactosidase construct. The cells were harvested and assayed 48 hours after transfection. Wild-type STAT3 enhanced RANTES promoter activity, but this effect was reduced by Y705F STAT3. Taken together, these observations suggest that STAT3 activates RANTES expression (Fig. 3D).

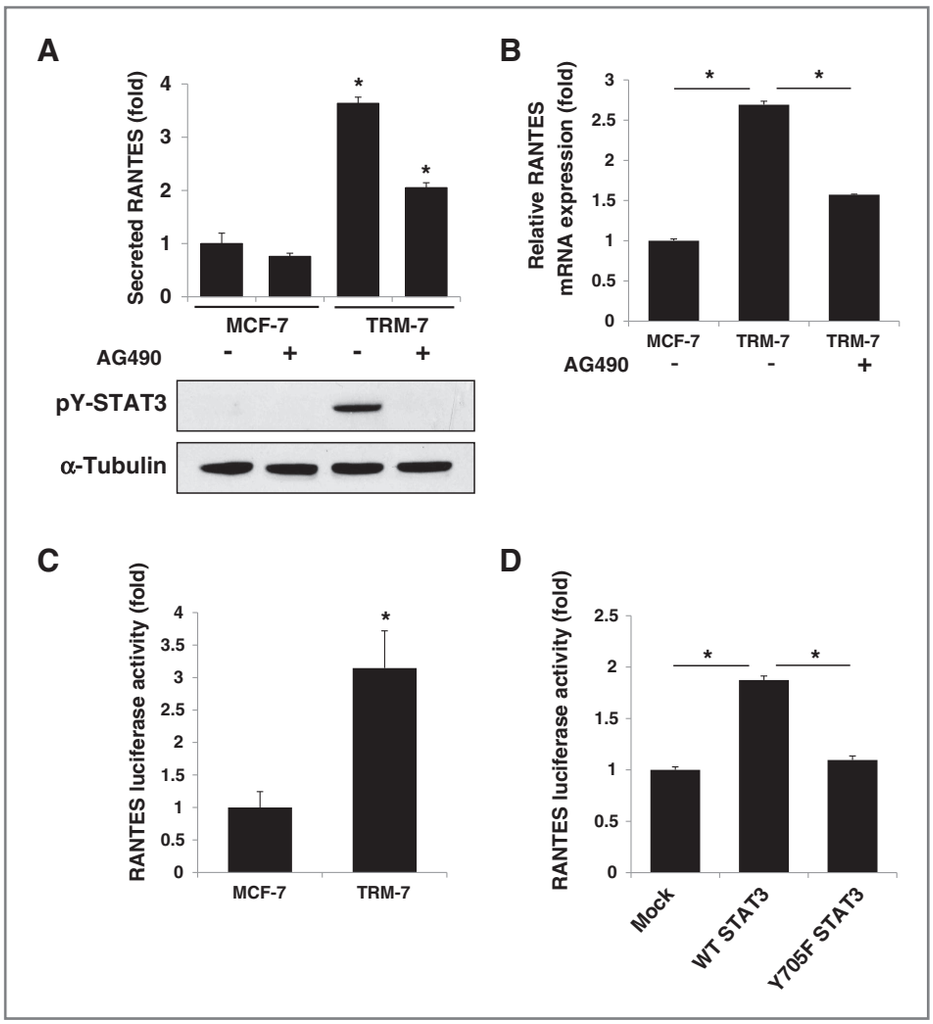

Figure 3. Phosphorylated STAT3 contributes to RANTES expression. A, MCF-7 and TRM-7 cells were treated with AG490 (40 μmol/L) for 24 hours. Proteins were then extracted and subjected to Western blotting. B, extracted total RNA was reverse-transcribed into cDNA, and real-time PCR was carried out. C, after transfection of MCF-7 and TRM-7 cells with a RANTES promoter–reporter construct (pGL3-RANTES), RANTES luciferase activity was measured and normalized relative to that of β-galactosidase. D, MCF-7 cells were cotransfected with a RANTES promoter–reporter construct (pGL3-RANTES) in various combinations with mock, wild-type STAT3 (WT STAT3), or point-mutant STAT3 (Y705F STAT3). After 48 hours in culture, luciferase activity was measured and normalized relative to that of β-galactosidase. Columns, means of triplicate samples; bars, SD. *, $P < 0.01$.

Moreover, these results suggest positive feedback regulation between STAT3 and RANTES in TRM-7 cells.

RANTES and STAT3 blockade inhibits drug resistance in TRM-7 cells

RANTES has been detected in samples from patients with breast cancer, and its expression was correlated with disease progression (23, 42, 43). Positive feedback between STAT3 and RANTES may increase the aggressiveness of TRM-7 cells. Decreased drug sensitivity is associated with tumor aggressiveness and poor clinical prognosis in many cancers (44, 45). We hypothesized that knockdown of RANTES and STAT3 in TRM-7 cells would have serious physiologic effects. To test this hypothesis, we knocked down RANTES and STAT3 expression in TRM-7 cells. First, TRM-7 cells were incubated with anti-RANTES neutralizing antibody, followed by tamoxifen treatment for 24 hours. RANTES neutralization led to increased tamoxifen sensitivity and cell death. In the absence of RANTES, TRM-7 cells lost their tamoxifen resistance, suggesting that RANTES is important for its maintenance (Fig. 4A). We next investigated the effects of STAT3 knockdown. The efficacies of STAT3-knockdown siRNAs in TRM-7 cells were evaluated, and the best functional clone was selected (Supplementary Fig. S4). TRM-7 cells were transfected with siSTAT3, treated with tamoxifen, and the level of apoptotic cell death was determined by fluorescence-activated cell-sorting (FACS) analysis. Tamoxifen-induced apoptotic cell death was enhanced by STAT3 knockdown (Fig. 4B). Taken together, these data suggest that RANTES upregulation and highly activated STAT3 play key roles in drug sensitivity. STAT3 activation has been reported to inhibit apoptosis and promote proliferation by regulating the BCL-2 family of anti-apoptotic proteins (46, 47). Therefore, we compared the expression levels of BCL-2 family members in MCF-7 and TRM-7 cells (Fig. 4C). In TRM-7 cells, BCL-2 and BCL-xL levels were higher than those in MCF-7 cells. To determine whether upregulation of these BCL-2 family members is linked to STAT3–RANTES positive feedback activation, we knocked down RANTES and STAT3 in MCF-7 and TRM-7 cells by

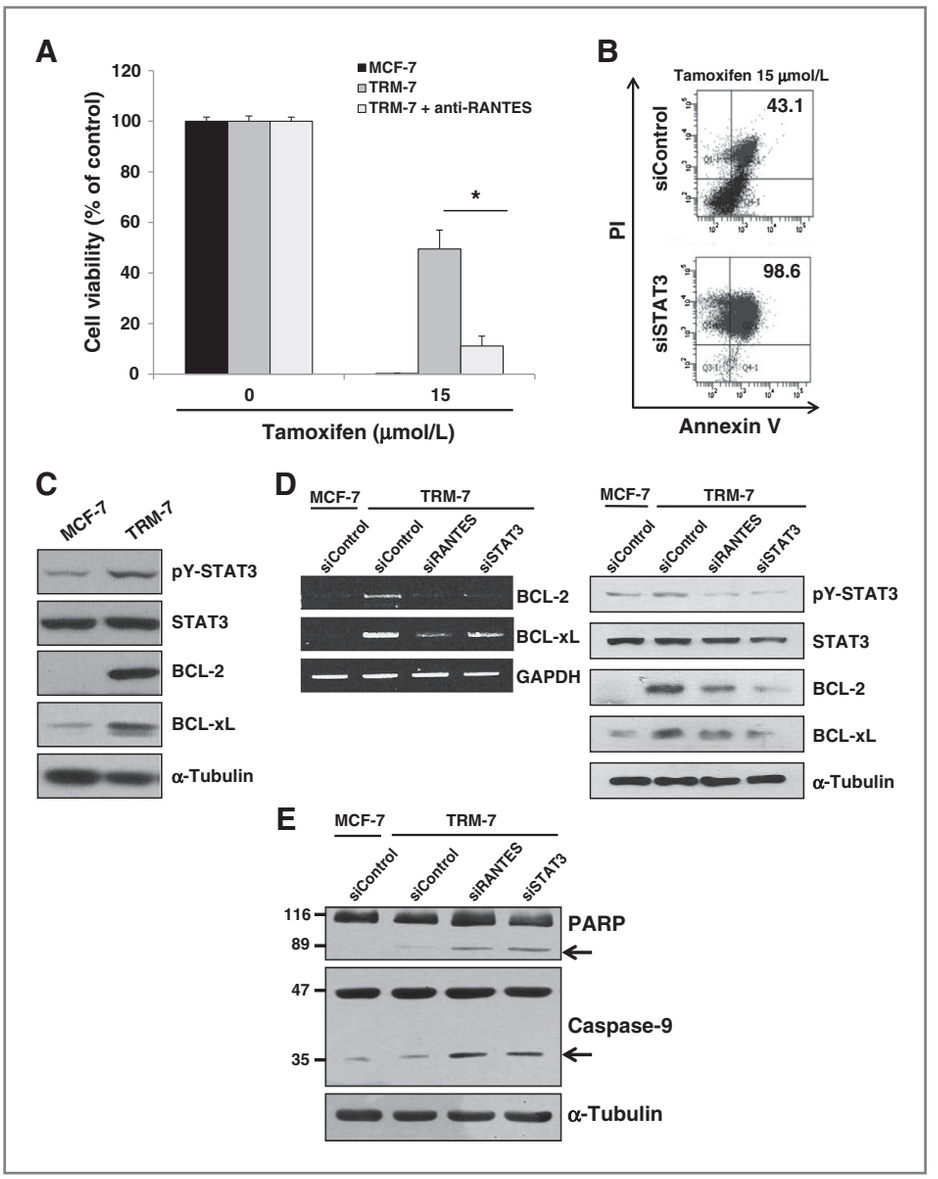

Figure 4. RANTES and STAT3 knockdown increased drug sensitivity of TRM-7 cells by downregulation of anti-apoptotic signaling. A, to evaluate the effects of RANTES neutralization, TRM-7 cells were incubated with anti-RANTES neutralizing antibody (1 μg/mL) for 24 hours and then treated with tamoxifen (0 or 15 μmol/L) for 24 hours. Viability was evaluated by MTT assay. Columns, means of triplicate samples; bars, SD. *, $P < 0.01$, compared with control cells. B, to evaluate the effects of STAT3 knockdown, TRM-7 cells were transfected with siControl and siSTAT3 (50 nmol/L). Thereafter, cells were treated with tamoxifen (15 μmol/L) for 24 hours, and apoptosis was evaluated by FITC-Annexin V and PI staining. C, equal amounts of proteins from MCF-7 and TRM-7 cells were subjected to Western blotting using the indicated antibodies. D, total RNA was purified from siRNA-transfected MCF-7 and TRM-7 cells, and then subjected to RT-PCR analysis using primers specific for the BCL-2 family. PCR products were resolved by electrophoresis on 2% agarose gels. Cell lysates were subjected to Western blotting using the indicated antibodies. E, cleaved PARP and caspase-9 levels in siRNA-transfected MCF-7 and TRM-7 cells were determined by Western blotting.

siRNA transfection (Supplementary Fig. S4C). In accordance with the upregulation of RANTES and STAT3, the levels of BCL-2 family gene expression were increased in TRM-7 cells. Knockdown of RANTES and STAT3 resulted in downregulation of BCL-2 family proteins in TRM-7 cells (Fig. 4D). To investigate the roles of other apoptotic signals, we next determined the changes in caspase-dependent apoptotic signals by Western blotting. PARP and caspase-9 activation were defined by the appearance of cleaved forms (arrow) under apoptotic conditions. Cleaved PARP and caspase-9 were detected in RANTES and STAT3 siRNA-treated TRM-7 cells (Fig. 4E). We were unable to detect caspase-3 in MCF-7 cells because they do not express this factor.

We concluded from these results that STAT3–RANTES positive feedback provides a survival advantage to TRM-7 cells in the presence of tamoxifen by regulating anti-apoptotic signals.

Exogenous RANTES recovers drug resistance in TRM-7 cells

The STAT3–RANTES positive feedback suggested RANTES autocrine signaling. If this is the case, treatment of TRM-7 cells lacking STAT3 and RANTES with exogenous RANTES should induce drug resistance. After ablation of endogenous STAT3 and RANTES in TRM-7 cells, we evaluated the effects of exogenous RANTES on recovery of drug resistance (Fig. 5A). siRANTES- and siSTAT3-transfected TRM-7 cells exhibited enhanced tamoxifen-induced cell death (#2 and #4). In contrast, exogenous RANTES treatment ameliorated cell death and caused recovery of tamoxifen resistance (#3 and #5).

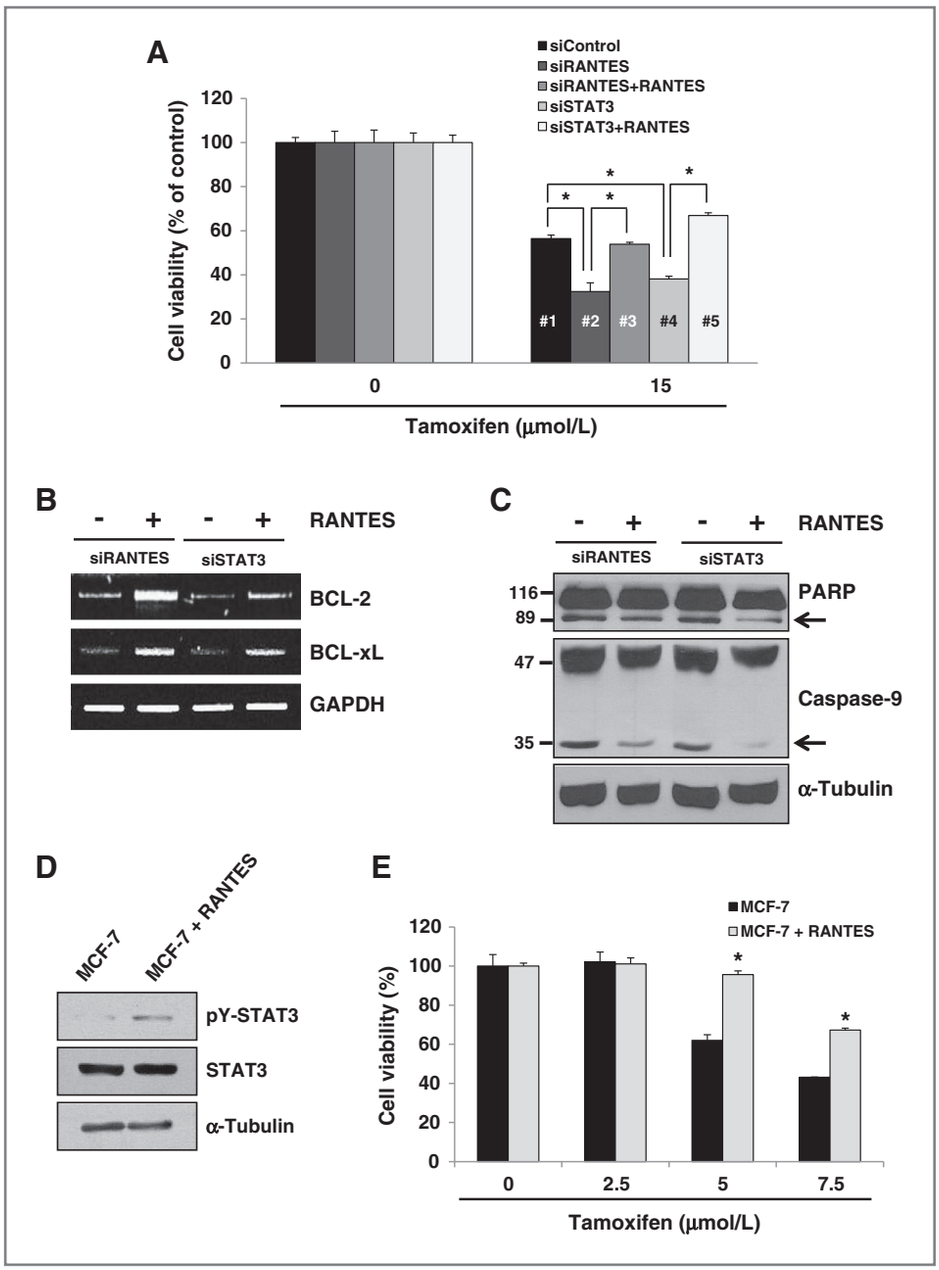

Figure 5. Decreased drug resistance and anti-apoptotic signals in TRM-7 cells were rescued by exogenous RANTES treatment. A, after siRNA transfection for 24 hours, recombinant RANTES protein (50 ng/mL) was added and cells were cultured for an additional 24 hours. Thereafter, cells were treated with tamoxifen (0 or 15 μmol/L) for 24 hours, and drug resistance levels were determined by MTT assay. Columns, means of triplicate samples; bars, SD. *, $P < 0.01$, compared with control cells. B, likewise, TRM-7 cells underwent siRNA transfection and treatment with recombinant RANTES (50 ng/mL). Total RNA was then extracted and subjected to RT-PCR analysis using primers specific for the BCL-2 family. PCR products were resolved by electrophoresis on 2% agarose gels. C, siRNA-transfected TRM-7 cells were treated with recombinant RANTES (50 ng/mL) and cultured for an additional 24 hours. Cleaved PARP and caspase-9 levels in cell lysates were determined by Western blotting. D, lysates of MCF-7 and RANTES-treated MCF-7 cells were subjected to Western blotting using the indicated antibodies. E, viability of RANTES-treated MCF-7 cells after exposure to tamoxifen was determined by MTT assay. Columns, means of triplicate samples; bars, SD. *, $P < 0.01$, compared with control cells.

Next, we examined whether RANTES treatment caused recovery of anti-apoptotic signaling in siRANTES- and siSTAT3-transfected TRM-7 cells. Downregulated BCL-2 family mRNA levels were rescued by exogenous RANTES treatment (Fig. 5B). Moreover, the increased PARP and caspase-9 cleavage were reversed by exogenous RANTES treatment (Fig. 5C). Therefore, autocrine RANTES signaling is essential for tamoxifen resistance.

As RANTES was shown to be critical for tamoxifen resistance in TRM-7 cells, we determined whether exogenous RANTES treatment rendered parental MCF-7 cells resistant to tamoxifen. MCF-7 cells were treated with recombinant RANTES for 2 weeks (50 ng/mL), during which the medium was changed every day. Data suggested that STAT3 phosphorylation was increased in RANTES-treated MCF-7 cells (Fig. 5D). Furthermore, RANTES-treated MCF-7 cells became more resistant to tamoxifen (Fig. 5E). Consistent with these data, phosphorylated STAT3 levels and expression levels of BCL-2 family genes in MCF-7 cells were upregulated by transient RANTES treatment (Supplementary Fig. S5A). These data suggested that RANTES plays a critical role in acquisition of tamoxifen resistance. To determine whether RANTES induces resistance to other chemotherapeutic agents, we evaluated its

effect on actinomycin D and cisplatin resistance (Supplementary Fig. S5B and S5C); no significant effects were identified.

Association of STAT3 phosphorylation and RANTES expression levels with tamoxifen resistance

To further explore the association between STAT3–RANTES positive feedback and tamoxifen resistance, we conducted parallel experiments using ERα-positive T47D breast cancer cells. When T47D cells were treated with 1 μmol/L tamoxifen for 2 weeks, phosphorylated STAT3, BCL-2 family, and secreted RANTES levels were elevated, as assessed by Western blotting and ELISA (Fig. 6A), consistent with the results in TRM-7 cells. To determine whether RANTES alone confers tamoxifen resistance, parental T47D cells were treated with recombinant RANTES (50 ng/mL) for 2 weeks, followed by incubation with tamoxifen for 24 hours. The viability of T47D cells was decreased in a tamoxifen dose-dependent manner. However, RANTES pretreatment attenuated tamoxifen-induced cell death (Fig. 6B). We also examined the effects of RANTES on resistance to other chemotherapeutic agents (Supplementary Fig. S6A and S6B). RANTES had no effect on actinomycin D or cisplatin-induced cell death.

We next examined whether STAT3 phosphorylation and RANTES were upregulated in the primary tissues of patients

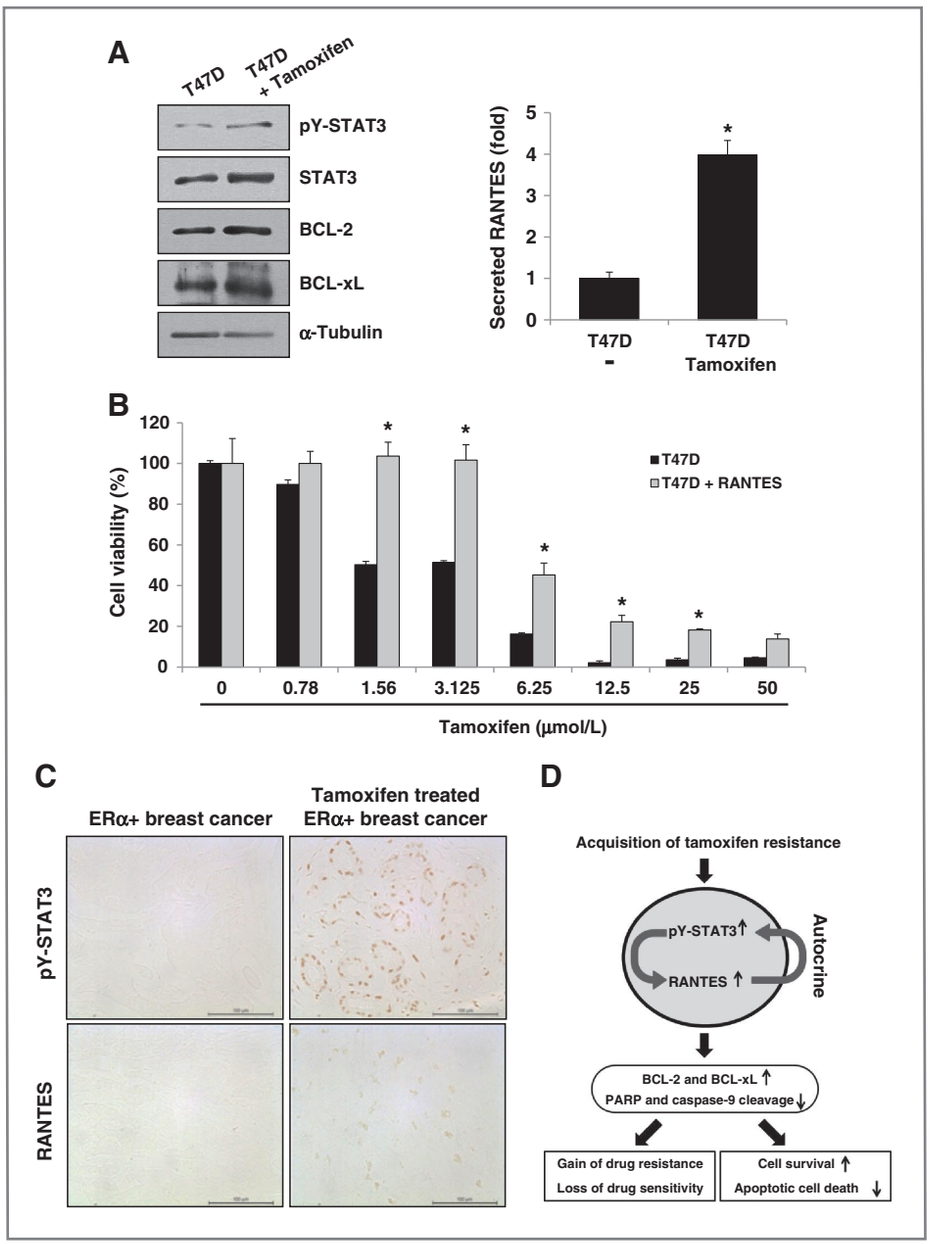

Figure 6. Elevated STAT3 phosphorylation and RANTES levels in tamoxifen-treated breast cancer cells. A, activated STAT3 status of tamoxifen-treated T47D cells was examined. After treatment with 1 μmol/L tamoxifen for 2 weeks, T47D- and tamoxifen-treated T47D cells were harvested and lysates were subjected to Western blotting. RANTES secretion levels were then determined by ELISA. Columns, means of triplicate samples; bars, SD. *, $P < 0.01$. B, after RANTES treatment (50 ng/mL) for 2 weeks, cells were exposed to tamoxifen for 24 hours. Drug resistance levels were then determined by MTT assay. Columns, means of triplicate samples; bars, SD. *, $P < 0.01$, compared with control cells. C, ERα-positive primary breast cancer tissue slides were subjected to immunohistochemical staining for detection of pY-STAT3 and RANTES. D, the mechanism of maintenance of tamoxifen resistance is described.

with tamoxifen-treated ERα-positive breast cancer ($n = 8$) by immunohistochemistry (Fig. 6C, Supplementary Fig. S7). As expected, phosphorylated STAT3 and RANTES levels were elevated in tamoxifen-treated primary breast cancer.

Overall, our findings suggested that activated STAT3 upregulates RANTES expression and that secreted RANTES activates STAT3 via an autocrine pathway. This STAT3–RANTES positive feedback induces anti-apoptotic signals in breast cancer cells, leading to drug resistance and enhanced survival in the presence of tamoxifen (Fig. 6D).

Discussion

The results of this study indicated that tamoxifen resistance is activated via an autocrine STAT3–RANTES positive feedback loop.

First, we found that STAT3 is highly phosphorylated in TRM-7 cells and that a factor released from TRM-7 cells activates STAT3. STAT3 is constitutively activated in primary breast tumors, and levels of phosphorylated STAT3 in patients with advanced breast cancer are correlated with an imperfect response to chemotherapy (48). The correlation between STAT3 and the aggressiveness of many types of tumor suggests that TRM-7 cells maintain elevated activated STAT3 levels to facilitate their survival.

Next, we examined the factor responsible for activation of STAT3. Our findings suggested that RANTES is overexpressed and that it activates STAT3 in TRM-7 cells in an autocrine manner. A high incidence and elevated expression of RANTES are directly correlated with more advanced disease, suggesting its involvement in breast cancer progression (24, 25).

STAT3 activation by RANTES secreted from TRM-7 cells was supported by the observation that TRM-7 culture medium without RANTES did not induce STAT3 phosphorylation in MCF-7 cells. Moreover, exogenous RANTES protein treatment activated STAT3 phosphorylation in MCF-7 cells. These results were consistent with other reports of activation of STAT3 phosphorylation by RANTES (38). In addition, STAT3 can be activated through the actions of many autocrine and paracrine growth factors (9, 21, 49). Accordingly, we suggest a novel function of RANTES as an autoregulator of tamoxifen resistance.

Interestingly, RANTES is a STAT3 target gene. Our data also indicated that STAT3 contributed to RANTES overexpression in TRM-7 cells. Inhibition of STAT3 phosphorylation led to downregulation of RANTES mRNA levels (40). This was supported by the observation that STAT3 regulated RANTES promoter activity in MCF-7 cells. Therefore, STAT3 and RANTES positively regulate each other in TRM-7 cells.

We next determined the signaling pathways that are activated in TRM-7 cells. We found that ERα levels were identical in TRM-7 and MCF-7 cells (Supplementary Fig. S8A). There were little differences in activated extracellular signal–regulated kinase (ERK) and p38 levels in TRM-7 and MCF-7 cells (Supplementary Fig. S8B). Some studies have shown that activation of ERK and p38 regulates RANTES production (49, 50). As we detected only weak ERK and p38 activation, we cannot exclude the possibility that ERK and p38 are associated with tamoxifen resistance in TRM-7 cells. Nonetheless, STAT3 activation is probably the main regulator of RANTES because STAT3 knockdown resulted in marked downregulation of RANTES expression (Supplementary Fig. S4C).

Furthermore, we determined the receptor associated with RANTES autocrine signaling (Supplementary Fig. S8C). Expression levels of all RANTES receptors (CCR1, 3, and 5) were increased in TRM-7 cells due to the increased RANTES levels in the culture medium. Of these, CCR5 is the major RANTES receptor (CCL5), and RANTES–CCR5 activates STAT3 (51). Thus, CCR5 is likely the primary route of autocrine RANTES activation of STAT3.

Next, we investigated the role of STAT3–RANTES positive feedback in TRM-7 cells. Persistently activated STAT3 has been implicated in resistance to apoptosis, possibly through the expression of the anti-apoptotic BCL-2 family. The fundamental role of the BCL-2 family in the survival and proliferation of drug-resistant cells was described recently. Several recent reports showed that blockade of STAT3 expression in human cancers suppresses expression of BCL-2 family proteins and induces apoptosis *in vitro* (47).

Our data also suggest that blockade of RANTES and STAT3 in TRM-7 cells resulted in decreased drug resistance and BCL-2 family expression. In addition, induction of apoptosis was detected, as indicated by the PARP and caspase-9 cleavage product. In contrast, treatment with exogenous RANTES rescued (i) decreased drug resistance, (ii) downregulated BCL-2 family expression, and (iii) PARP induction and caspase-9 cleavage.

In summary, our findings suggest a novel mechanism of tamoxifen resistance in breast cancer cells. RANTES autocrine signaling increased the aggressiveness of tamoxifen-resistant breast cancer cells and perpetuated their resistance to tamoxifen by activating STAT3 and anti-apoptotic signals. Thus, STAT3 and RANTES cooperatively influence drug sensitivity. Therefore, disruption of STAT3 activation or blockade of secreted RANTES in combination with tamoxifen treatment may represent a new therapeutic approach for patients with breast cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Writing, review, and/or revision of the manuscript: E.H. Yi
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References

1. Jemal A, Siegel R, Xu J, Ward E. Cancer statistics, 2010. CA Cancer J Clin 2010;60:277–300.
2. Early Breast Cancer Trialists' Collaborative Group (EBCTCG). Effects of chemotherapy and hormonal therapy for early breast cancer on recurrence and 15-year survival: an overview of the randomised trials. Lancet 2005;365:1687–717.
3. Knowlden JM, Hutcheson IR, Jones HE, Madden T, Gee JM, Harper ME, et al. Elevated levels of epidermal growth factor receptor/c-erbB2 heterodimers mediate an autocrine growth regulatory pathway in tamoxifen-resistant MCF-7 cells. Endocrinology 2003;144:1032–44.
4. Gutierrez MC, Detre S, Johnston S, Mohsin SK, Shou J, Allred DC, et al. Molecular changes in tamoxifen-resistant breast cancer: relationship between estrogen receptor, HER-2, and p38 mitogen-activated protein kinase. J Clin Oncol 2005;23:2469–76.
5. Peto R, Boreham J, Clarke M, Davies C, Beral V. UK and USA breast cancer deaths down 25% in year 2000 at ages 20–69 years. Lancet 2000;355:1822.
6. DeGraffenried LA, Friedrichs WE, Fulcher L, Fernandes G, Silva JM, Peralba JM, et al. Eicosapentaenoic acid restores tamoxifen sensitivity in breast cancer cells with high Akt activity. Ann Oncol 2003;14:1051–6.
7. Hiscox S, Jiang WG, Obermeier K, Taylor K, Morgan L, Burmi R, et al. Tamoxifen resistance in MCF7 cells promotes EMT-like behaviour and involves modulation of beta-catenin phosphorylation. Int J Cancer 2006;118:290–301.
8. Bromberg J, Darnell JE Jr. The role of STATs in transcriptional control and their impact on cellular function. Oncogene 2000;19:2468–73.
9. Diaz N, Minton S, Cox C, Bowman T, Gritsko T, Garcia R, et al. Activation of stat3 in primary tumors from high-risk breast cancer patients is associated with elevated levels of activated SRC and survivin expression. Clin Cancer Res 2006;12:20–8.
10. Zhong Z, Wen Z, Darnell JE Jr. Stat3: a STAT family member activated by tyrosine phosphorylation in response to epidermal growth factor and interleukin-6. Science 1994;264:95–8.
11. Fukada T, Hibi M, Yamanaka Y, Takahashi-Tezuka M, Fujitani Y, Yamaguchi T, et al. Two signals are necessary for cell proliferation induced by a cytokine receptor gp130: involvement of STAT3 in anti-apoptosis. Immunity 1996;5:449–60.
12. Bowman T, Garcia R, Turkson J, Jove R. STATs in oncogenesis. Oncogene 2000;19:2474–88.
13. Kube D, Holtick U, Vockerodt M, Ahmadi T, Haier B, Behrmann I, et al. STAT3 is constitutively activated in Hodgkin cell lines. Blood 2001;98:762–70.
14. Mora LB, Buettner R, Seigne J, Diaz J, Ahmad N, Garcia R, et al. Constitutive activation of Stat3 in human prostate tumors and cell lines: direct inhibition of Stat3 signaling induces apoptosis of prostate cancer cells. Cancer Res 2002;62:6659–66.
15. Dolled-Filhart M, Camp RL, Kowalski DP, Smith BL, Rimm DL. Tissue microarray analysis of signal transducers and activators of transcription 3 (Stat3) and phospho-Stat3 (Tyr705) in node-negative breast cancer shows nuclear localization is associated with a better prognosis. Clin Cancer Res 2003;9:594–600.
16. Nagpal JK, Mishra R, Das BR. Activation of Stat-3 as one of the early events in tobacco chewing - Mediated oral carcinogenesis. Cancer 2002;94:2393–400.
17. Hsiao JR, Jin YT, Tsai ST, Shiau AL, Wu CL, Su WC. Constitutive activation of STAT3 and STAT5 is present in the majority of nasopharyngeal carcinoma and correlates with better prognosis. Br J Cancer 2003;89:344–9.
18. Buettner R, Mora LB, Jove R. Activated STAT signaling in human tumors provides novel molecular targets for therapeutic intervention. Clin Cancer Res 2002;8:945–54.
19. Song JI, Grandis JR. STAT signaling in head and neck cancer. Oncogene 2000;19:2489–95.
20. Berclaz G, Altermatt HJ, Rohrbach V, Siragusa A, Dreher E, Smith PD. EGFR dependent expression of STAT3 (but not STAT1) in breast cancer. Int J Oncol 2001;19:1155–60.
21. Li L, Shaw PE. Autocrine-mediated activation of STAT3 correlates with cell proliferation in breast carcinoma lines. J Biol Chem 2002;277:17397–405.
22. Jing N, Tweardy DJ. Targeting Stat3 in cancer therapy. Anti Cancer Drug 2005;16:601–7.
23. Huang CY, Fong YC, Lee CY, Chen MY, Tsai HC, Hsu HC, et al. CCL5 increases lung cancer migration via PI3K Akt and NF-kappa B pathways. Biochem Pharmacol 2009;77:794–803.
24. Luboshits G, Shina S, Kaplan O, Engelberg S, Nass D, Lifshitz-Mercer B, et al. Elevated expression of the CC chemokine regulated on activation, normal T cell expressed and secreted (RANTES) in advanced breast carcinoma. Cancer Res 1999;59:4681–7.
25. Niwa Y, Akamatsu H, Niwa H, Sumi H, Ozaki Y, Abe A. Correlation of tissue and plasma RANTES levels with disease course in patients with breast or cervical cancer. Clin Cancer Res 2001;7:285–9.
26. Bieche I, Lerebours F, Tozlu S, Espie M, Marty M, Lidereau R. Molecular profiling of inflammatory breast cancer: identification of a poor-prognosis gene expression signature. Clin Cancer Res 2004;10:6789–95.
27. Soria G, Yaal-Hahoshen N, Azenshtein E, Shina S, Leider-Trejo L, Ryvo L, et al. Concomitant expression of the chemokines RANTES and MCP-1 in human breast cancer: a basis for tumor-promoting interactions. Cytokine 2008;44:191–200.
28. Mrowietz U, Schwenk U, Maune S, Bartels J, Kupper M, Fichtner I, et al. The chemokine RANTES is secreted by human melanoma cells and is associated with enhanced tumour formation in nude mice. Br J Cancer 1999;79:1025–31.
29. Choi HK, Yang JW, Roh SH, Han CY, Kang KW. Induction of multidrug resistance associated protein 2 in tamoxifen-resistant breast cancer cells. Endocr Relat Cancer 2007;14:293–303.
30. Garcia R, Jove R. Activation of STAT transcription factors in oncogenic tyrosine kinase signaling. J Biomed Sci 1998;5:79–85.
31. Bromberg JF, Wrzeszczynska MH, Devgan G, Zhao YX, Pestell RG, Albanese C, et al. Stat3 as an oncogene. Cell 1999;98:295–303.
32. Watson CJ, Miller WR. Elevated levels of members of the Stat family of transcription factors in breast-carcinoma nuclear extracts. Br J Cancer 1995;71:840–4.
33. Hsieh FC, Cheng G, Lin J. Evaluation of potential Stat3-regulated genes in human breast cancer. Biochem Biophys Res Commun 2005;335:292–9.
34. Blanquart C, Karouri SE, Issad T. Implication of protein tyrosine phosphatase 1B in MCF-7 cell proliferation and resistance to 4-OH tamoxifen. Biochem Biophys Res Commun 2009;387:748–53.
35. Page C, Huang M, Jin XH, Cho K, Lilja J, Reynolds RK, et al. Elevated phosphorylation of AKT and Stat3 in prostate, breast, and cervical cancer cells. Int J Oncol 2000;17:23–8.
36. Takeda K, Noguchi K, Shi W, Tanaka T, Matsumoto M, Yoshida N, et al. Targeted disruption of the mouse Stat3 gene leads to early embryonic lethality. Proc Natl Acad Sci U S A 1997;94:3801–4.

37. DeArmond D, Brattain MG, Jessup JM, Kreisberg J, Malik S, Zhao SJ, et al. Autocrine-mediated ErbB-2 kinase activation of STAT3 is required for growth factor independence of pancreatic cancer cell lines. Oncogene 2003;22:7781–95.
38. Kim JE, Kim HS, Shin YJ, Lee CS, Won C, Lee SA, et al. LYR71, a derivative of trimeric resveratrol, inhibits tumorigenesis by blocking STAT3-mediated matrix metalloproteinase 9 expression. Exp Mol Med 2008;40:514–22.
39. Karnoub AE, Dash AB, Vo AP, Sullivan A, Brooks MW, Bell GW, et al. Mesenchymal stem cells within tumour stroma promote breast cancer metastasis. Nature 2007;449:557–63.
40. Kovacic JC, Gupta R, Lee AC, Ma MC, Fang F, Tolbert CN, et al. Stat3-dependent acute Rantes production in vascular smooth muscle cells modulates inflammation following arterial injury in mice. J Clin Invest 2010;120:303–14.
41. Yang JB, Liao XD, Agarwal MK, Barnes L, Auron PE, Stark GR. Unphosphorylated STAT3 accumulates in response to IL-6 and activates transcription by binding to NF kappa B. Gene Dev 2007; 21:1396–408.
42. Moran CJ, Arenberg DA, Huang CC, Giordano TJ, Thomas DG, Misek DE, et al. RANTES expression is a predictor of survival in stage I lung adenocarcinoma. Clin Cancer Res 2002;8:3803–12.
43. Mi ZY, Bhattacharya SD, Kim VM, Guo HT, Talbot LJ, Kuo PC. Osteopontin promotes CCL5-mesenchymal stromal cell-mediated breast cancer metastasis. Carcinogenesis 2011;32:477–87.
44. Kim HJ, Hwang JY, Kim HJ, Choi WS, Lee JH, Kim HJ, et al. Expression of a peroxisome proliferator-activated receptor gamma 1 splice variant that was identified in human lung cancers suppresses cell death induced by cisplatin and oxidative stress. Clin Cancer Res 2007; 13:2577–83.
45. McCarroll JA, Gan PP, Liu M, Kavallaris M. beta III-tubulin is a multifunctional protein involved in drug sensitivity and tumorigenesis in non-small cell lung cancer. Cancer Res 2010;70:4995–5003.
46. Aoki Y, Feldman GM, Tosato G. Inhibition of STAT3 signaling induces apoptosis and decreases survivin expression in primary effusion lymphoma. Blood 2003;101:1535–42.
47. Real PJ, Sierra A, de Juan A, Segovia JC, Lopez-Vega JM, Fernandez-Luna JL. Resistance to chemotherapy via Stat3-dependent overexpression of Bcl-2 in metastatic breast cancer cells. Oncogene 2002; 21:7611–8.
48. Gritsko T, Williams A, Turkson J, Kaneko S, Bowman T, Huang M, et al. Persistent activation of Stat3 signaling induces survivin gene expression and confers resistance to apoptosis in human breast cancer cells. Clin Cancer Res 2006;12:11–9.
49. Pazdrak K, Olszewska-Pazdrak B, Liu T, Takizawa R, Brasier AR, Garofalo RP, et al. MAPK activation is involved in posttranscriptional regulation of RSV-induced RANTES gene expression. Am J Physiol Lung Cell Mol Physiol 2002;283:L364–72.
50. Hashimoto S, Gon Y, Asai Y, Machino T, Jibiki I, Takeshita I, et al. p38 MAP kinase regulates RANTES production by TNF-alpha-stimulated human pulmonary vascular endothelial cells. Allergy 1999;54: 1168–72.
51. Wong M, Uddin S, Majchrzak B, Huynh T, Proudfoot AE, Platanias LC, et al. Rantes activates Jak2 and Jak3 to regulate engagement of multiple signaling pathways in T cells. J Biol Chem 2001;276: 11427–31.