Therapeutics, Targets, and Chemical Biology

FoxM1 Mediates Resistance to Herceptin and Paclitaxel

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Abstract

Inherent and acquired therapeutic resistance in breast cancer remains a major clinical challenge. In human breast cancer samples, overexpression of the oncogenic transcription factor FoxM1 has been suggested to be a marker of poor prognosis. In this study, we report that FoxM1 overexpression confers resistance to the human epidermal growth factor receptor 2 monoclonal antibody Herceptin and microtubule-stabilizing drug paclitaxel, both as single agents and in combination. FoxM1 altered microtubule dynamics to protect tumor cells from paclitaxel-induced apoptosis. Mechanistic investigations revealed that the tubulin-destabilizing protein Stathmin, whose expression also confers resistance to paclitaxel, is a direct transcriptional target of FoxM1. Significantly, attenuating FoxM1 expression by small interfering RNA or an alternate reading frame (ARF)–derived peptide inhibitor increased therapeutic sensitivity. Our findings indicate that targeting FoxM1 could relieve therapeutic resistance in breast cancer. *Cancer Res; 70(12); 5054–63. ©2010 AACR.*

Introduction

The proliferation-specific oncogenic transcription factor FoxM1 is overexpressed in a broad range of tumor types examined, including those of mammary, neural, gastrointestinal, and reproductive origin (1–4). This expression pattern is attributed to the ability of FoxM1 to transactivate genes required for cell cycle progression (5, 6). In dividing cells, FoxM1 begins to accumulate during S phase, peaks at the G_2-M transition, and is degraded by APC/C-Cdh1 immediately following M phase (7, 8). Ablation of FoxM1 leads to a failure to enter S phase and improper M-phase completion, resulting in mitotic catastrophe (8–12). FoxM1 promotes the G_1-S transition by downregulation of the cyclin-dependent kinase (Cdk) inhibitor p27 through multiple mechanisms. Specifically, FoxM1 can increase the proteolytic degradation of p27 through upregulation of Skp2 and Cks1, members of the E3 ubiquitin ligase complex responsible for the degradation of p27 (12). In addition, by upregulation of KIS kinase, FoxM1 promotes the localization of p27 to the cytoplasm (13). During G_2-M, FoxM1 increases levels of various factors such as Aurora B Kinase, Survivin, and Cdc25B to allow successful entry and completion of mitosis (5, 12).

FoxM1 is regulated throughout the cell cycle by phosphorylation. Growth factors activate surface receptors to initiate signaling pathways such as phosphoinositide 3-kinase (PI3K)/Akt and Ras/Raf/mitogen-activated protein kinase that promote cell division (14). The result of these signaling cascades is the activation of kinases such as Cyclin-Cdk and Polo-like kinase 1 (PLK1), all of which results in the phosphorylation and activation of FoxM1 (15–17). Recently, it has been shown that human epidermal growth factor receptor 2 (HER2)/ErbB2, a surface receptor, functions upstream of FoxM1. Overexpression or silencing of HER2 correlated directly with FoxM1 levels in mammary cell lines and in transgenic mouse models (18). In a study of human mammary tumors, FoxM1 expression was 8.7-fold higher in tumor cells versus normal tissue controls and showed increased nuclear staining. In addition, it was shown that FoxM1 expression correlated with that of HER2 and functioned as a predictor of poor patient outcome (1).

HER2 is a member of the epidermal growth factor (EGF) family of receptors. HER2 has no known ligand, but functions by forming heterodimers with other family members to promote intracellular signaling (14). Amplification of HER2 is a sign of a highly aggressive tumor type with few treatment options. Several therapies aimed at inhibiting HER2 signaling are in use, including the monoclonal antibody Herceptin (trastuzumab) that functions to disrupt the interaction between HER2 and its preferred binding partner HER3 (19). Treatment with Herceptin results in the accumulation of the Cdk inhibitor p27 and subsequent G_1-S cell cycle arrest. Unfortunately, the efficacy of Herceptin as a monotherapy is thought to be <30% and in combination with microtubule stabilizing drugs ~60% (20). Resistance to Herceptin develops quickly and is thought to stem from compensated signaling by other EGF family members or dysregulation of downstream pathways such as PI3K/Akt (21–23).

Herceptin is commonly used in conjunction with other therapies, including paclitaxel. The primary mechanism of action of paclitaxel is to bind β-tubulin and prevent dissociation of α/β tubulin dimers, resulting in mitotic failure and consequent apoptosis (24). Paclitaxel is used in the treatment of multiple tumor types and has shown particular success in treatment of metastatic breast cancer. Yet,

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resistance does occur. Insensitivity to paclitaxel has been shown in cells that overexpress HER2. On average, cells with HER2 amplification require a 100-fold higher dose of paclitaxel to produce the same effect (25). Resistance to paclitaxel has been attributed to additional mechanisms including increased expression of multidrug resistant 1 (MDR1), a protein that can pump toxins out of cells. Other commonly documented mechanisms of resistance include changes in microtubule stability or mutations in the tubulin proteins (26). In studies of human samples, Stathmin, a regulator of microtuble dynamics, has been shown to promote paclitaxel resistance (27).

We investigated the possibility that the high levels of FoxM1 in HER2-amplified mammary tumors could confer resistance to treatments and whether targeting FoxM1 could sensitize tumor cells to therapy. We found that FoxM1 mediates both inherent and acquired resistance to the HER2 targeting monoclonal antibody Herceptin. Additionally, we identified a novel function of FoxM1, to alter microtubule dynamics through the regulation of Stathmin, which renders cells resistant to paclitaxel treatment. Moreover, we show that inhibition of FoxM1 by an alternate reading frame (ARF)–derived peptide sensitizes cells to Hercepin and paclitaxel therapy.

Materials and Methods

Cell culture and chemotherapeutic agents

SKBR3, MDA-MB-453, and BT474 cell lines were obtained from the American Type Culture Collection. Cells were cultured in RPMI 1640 with 10% fetal bovine serum and penicillin/streptomycin. Stable cell lines were generated by transfection of pBabe or pBabe-FoxM1 retroviral constructs followed by selection in puromycin. Control small interfering RNA (siRNA) as well as siRNA specific to FoxM1 (Dharmacon) were transfected using Lipofectamine (Invitrogen). Mutant and ARF peptide have been previously described (28). Paclitaxel (Sigma) was dissolved in DMSO. Herceptin, a gift from Genentech, was dissolved in sterile water.

Flow cytometry, proliferation measurements, and colony-forming assay

For cell cycle analysis by flow cytometry, cells were trypsinized, pelleted, and then resuspended in propidium iodide solution (50 µg/mL propidium iodide, 0.1 mg/mL RNaseA, and 0.05% Triton-X). All reagents were purchased from Sigma. After 40 minutes of incubation at 37°C cells were analyzed using a flow cytometer. 5-Bromo-2-Deoxyuridine (BrDUrd; 10 µmol/L) from Sigma was added to culture media. Cells were fixed and stained with anti-BrDUrd antibody (1:250, DAKO) followed by anti-mouse FITC (DAKO) and 4′,6-diamidino-2-phenylindole (DAPI) (Molecular Probes). Cell viability was measured using the CellTiter-Glo Luminescent assay (Promega), which measures the amount of oxygenated oxyluciferin that has a direct correlation to ATP present. For colony-forming assay, 3 to 5×10^3 cells were plated in triplicate in a 24-well plate. Twenty-four hours later, treatment was

initiated. After 14 to 17 days, cells were fixed and stained with crystal violet. Quantification was done using Adobe Photoshop, a method described elsewhere (29). All P values were calculated using the Student's t test.

Semiquantitative reverse transcription-PCR

RNA was extracted using Trizol (Invitrogen), and cDNA was synthesized using reverse transcriptase (Bio-Rad). Equal amounts of cDNA were used for all PCRs (Promega). PCR products were analyzed over a series of cycle numbers to ensure that data were produced during the PCR log-scale amplification. Samples were run on agarose gels, photographed, and quantified using Image J. The primers used were as follows: glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5′-ACA CCC ACT CCT CCA CCT TT-3′ and 5′-TTC CTC TTG TGC TCT TGC TG-3′; FoxM1, 5′-GCA GGC TGC ACT ATC AAC AA-3′ and 5′-TCG AAG GCT CCT CAA CCT TA-3′; CyclinB1, 5′-AAA GTC TAC CAC CGA ATC CCT A-3′ and 5′-CCA AAA CAC AAA ACC AAA ATG A-3′; Cks1, 5′-GAA TGG AGG AAT CTT GGC GTT C-3′ and 5′-TCT TTG GTT TCTT GGG TAG TGG G-3′; PLK1, 5′-TGT AGA GGA TGA GGC GTG TTG AG-3′ and 5′-AGC AAG TGG GTG GAC TAT TCG G-3′; Skp2, 5′-CAC GAA AAG GGC TGA AAT GTT C-3′ and 5′-GGT GTT TGT AAG AGG TGG TAT CGC-3′; and Stathmin, 5′-GCC AGT GTC CTT TAC TTT CCC TCC-3′ and 5′-TTC AGT TTC TCC CCT TAG GCC C-3′.

Western blot

Extracts were prepared in lysis buffer containing 1 mmol/L EDTA, 0.15 mol/L NaCl, 0.05 mol/L Tris-HCl (pH 7.5), and 0.5% Triton-X. Phosphate inhibitor cocktail set II (Calbiochem), and protease inhibitor (Roche) were added before each experiment. The rabbit polyclonal antibody against FoxM1 has been previously described (17). Anti-kip1/p27 (1:10,000, BD Biosciences), Stathmin (1:1,000, Cell Signaling), and Cdk2 (1:200, Santa Cruz) were also used. For tubulin fractionation, α-tubulin antibody (1:10,000, Sigma) and β-tubulin (1:10,000, NeoMarkers) were used for analysis. Quantification was performed using the Image J software (NIH).

Chromatin immunoprecipitation

Chromatin immunoprecipitation assay was performed as previously described (30). Briefly, cells were fixed in 1% formaldehyde for 10 minutes to allow cross-linking and then were quenched with 125 nmol/L glycine. Cells were collected and lysed in SDS lysis buffer [1% SDS, 10 mmol/L EDTA, 50 mmol/L Tris (pH 8), protease, and phosphate inhibitors]. Lysate was sonicated, precleared, and incubated with FoxM1 antibody followed by collection with Protein-A and Protein-G-Sepharose beads with salmon sperm (Upstate). Beads were washed and DNA was extracted using a PCR purification kit (Qiagen). The following primers were used for PCR: 5′-CAA ATG TGC TTG CCT TTT AGC C-3′ and 5′-TGG GAT TAC AGA TGT GAG CCA CC-3′ for −5397, and 5′-CAC GGT CAG ACC AAT TTC T-3′ and 5′-TGA TAG GGG AGG AAG AGC AA-3′ as a nonspecific control.

Tubulin assay

Separation of polymerized and soluble fractions was done in accordance with previously published assays (31). Cells were seeded at 80% confluency in 24-well plates. The following day, they were treated with 0 or 1 nmol/L paclitaxel for 24 hours. Cells were collected in hypotonic buffer [1 mmol/L MgCl$_2$, 2 mmol/L EGTA, 0.5% Nonidet P-40, and 20 mmol/L Tris-HCl (pH 6.8)] and centrifuged for 10 minutes at room temperature (14,000 rpm). The supernatant was used as the soluble fraction, whereas the pellet made up the polymerized fraction. Samples were analyzed by Western blot.

Results

FoxM1 overexpression confers Herceptin resistance

To investigate the hypothesis that increased FoxM1 is sufficient to induce resistance to Herceptin, we stably introduced FoxM1 expression cDNA in SKBR3, BT474, and MDA-MB-453. All cell lines have HER2 amplification and BT474 is estrogen receptor positive. Drug sensitivity was tested by colony formation assay. Cells were plated at low density and treated continuously with 10 μg/mL of Herceptin for 14 days. As shown by the quantification of the colony formation assay, FoxM1
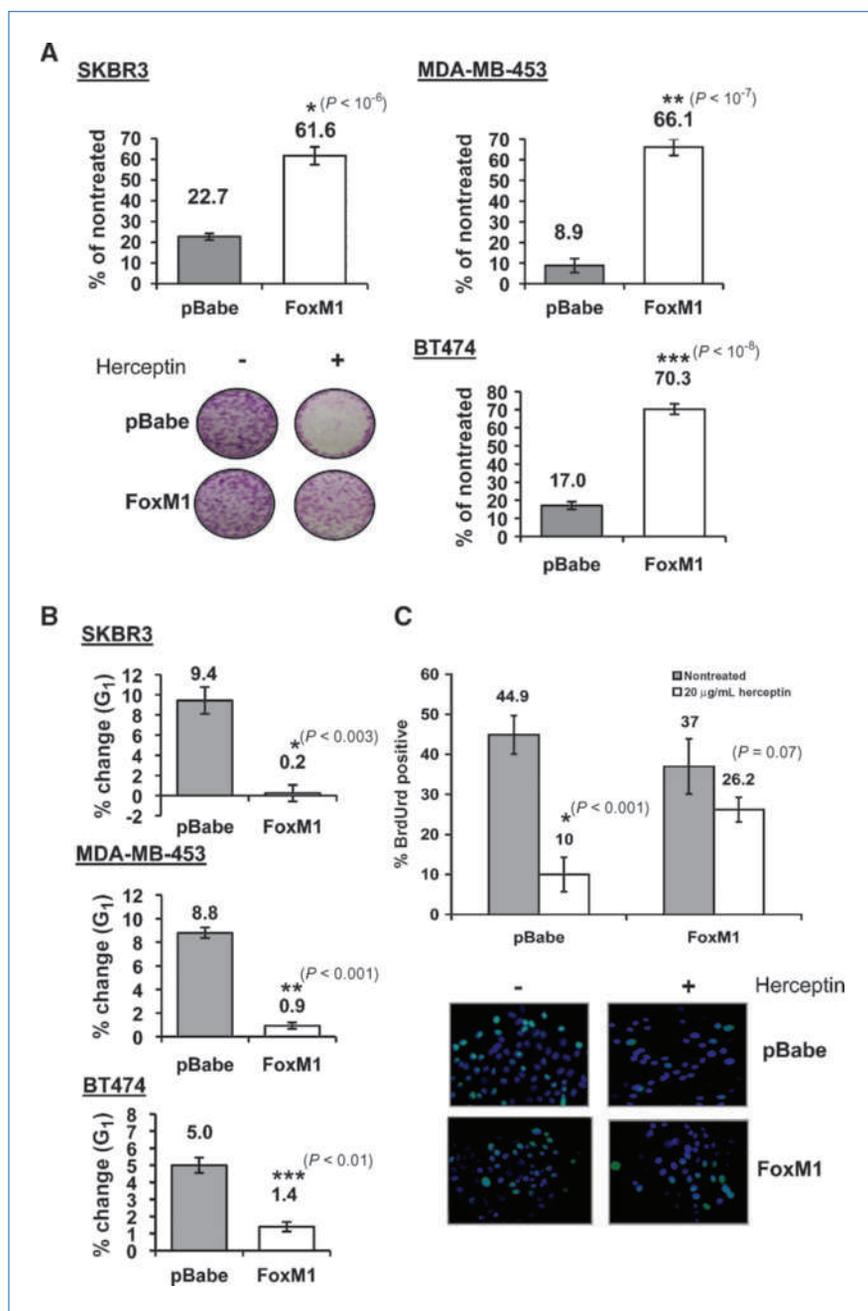

Figure 1. Overexpression of FoxM1 renders multiple HER-2–amplified cell lines resistant to the effects of Herceptin treatment. A, the response of SKBR3, MDA-MB-453, and BT474 stable cell lines to Herceptin was tested by colony-forming assay. Cell lines were treated continuously with either 0 or 10 μg/mL Herceptin for 14 d; media was** changed every 3 d. Cells were plated in triplicate, and the experiment was repeated three times. Representative wells for SKBR3 cells are shown. Graphs provide average quantification as a percentage of the nontreated wells. B, stable cell lines expressing either pBabe or FoxM1 were treated with 10 μg/mL of Herceptin for 48 h, stained with propidium iodide, and subjected to FACS analysis. Percentage change in G$_1$ phase is shown. Inset, relative protein expression in FoxM1 versus pBabe stable cell lines. C, SKBR3-pBabe and FoxM1 lines were either nontreated or treated for 72 h with Herceptin followed by a pulse of BrdUrd for 2 h. Percentage of BrdUrd-positive compared with DAPI-positive cells are shown for each group; 500 cells in each experiment were counted. Average values are shown above error bars, and representative pictures are shown below the graph.

Figure 2. SKBR3-FoxM1 cell lines fail to accumulate p27 after treatment with Herceptin. A, SKBR3-pBabe and FoxM1-expressing cell lines were treated with increasing doses of Herceptin for 48 h. Representative Western blots of FoxM1 and p27 levels are shown. Cdk2 was used as a loading control. B, SKBR3 stable cell lines were treated with 10 μg/mL of Herceptin for 24, 48, and 72 h. FoxM1 and p27 levels are shown by Western blot. C, SKBR3-pBabe cell lines were treated with 10 μg/mL of IgG for indicated periods of time. FoxM1 and p27 were assayed by Western blot and Cdk2 was used as a loading control.
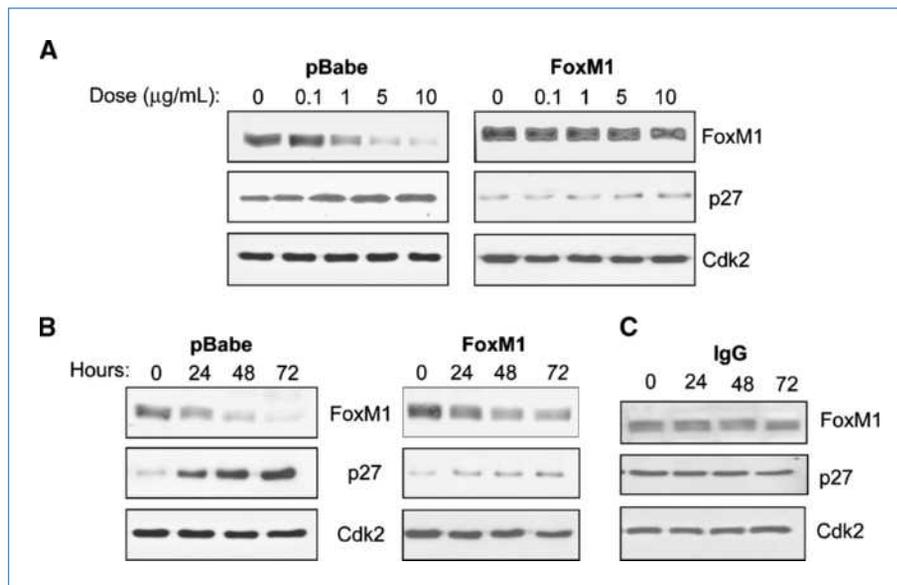
overexpression resulted in a 3- to 7-fold increase in colony number compared with pBabe-expressing cells (Fig. 1A), providing evidence that FoxM1 confers resistance to Herceptin.

The magnitude of the G_1-S arrest induced by Herceptin was measured by propidium iodide staining followed by flow cytometry (fluorescence-activated cell sorting, FACS) analysis. Cells were treated in 10 μg/mL of Herceptin for 72 hours, and cell cycle profiles were examined. The control pBabe lines showed a statistically significant increase in the number of cells in G_1, but the FoxM1-expressing cells did not exhibit any significant enrichment of the G_1 population (Fig. 1B). Herceptin alone does not induce apoptosis (21). Consistent with that, none of the cell lines showed an increase in the sub-G_1 population. To further investigate resistance in FoxM1-expressing cells, we measured the ability to incorporate BrdUrd (Fig. 1C). Upon treatment, SKBR3-pBabe showed a substantial (35%) reduction in the number of BrdUrd-positive cells. FoxM1-expressing cells did not show any significant decrease in BrdUrd incorporation. Taken together, these results indicate that FoxM1 overcomes the G_1-S arrest and proliferation defect caused by Herceptin, allowing cells to continue to grow in the presence of the drug.

FoxM1 prevents Herceptin-induced accumulation of p27

Although multiple mechanisms of resistance exist, previous reports indicated that low levels of p27 could contribute to Herceptin insensitivity (21). FoxM1 functions as a negative regulator of p27 by increasing proteolysis. We hypothesized that the resistance observed in FoxM1-overexpressing cells could be due to a failure to accumulate p27. To test that possibility, SKBR3-pBabe or FoxM1-expressing lines were treated with 10 μg/mL of Herceptin for 0, 24, 48, or 72 hours or with increasing doses. Western blot of FoxM1 and p27 levels showed that in control SKBR3 cells, the levels of FoxM1 decreased with treatment and the p27 levels accumulate as ex-

pected. Interestingly, in SKBR3-FoxM1 cell lines, the basal expression of p27 is lower and levels remained low even after a high-dose of Herceptin (Fig. 2A and B). These results show that the likely mechanism by which FoxM1 confers resistance is by preventing the accumulation of p27 that is required for Herceptin-induced G_1-S arrest. Treatment with IgG did not cause changes in FoxM1 or p27; therefore, these effects are specific to inhibition of the HER2 pathway and not a general antibody-induced response (Fig. 2C).

Targeting FoxM1 in mammary tumor cells with inherent Herceptin resistance increases sensitivity

To generate cell lines that have inherent resistance to Herceptin, we cultured parental SKBR3, MDA-MB-453, and BT474 lines continuously in 5 μg/mL of Herceptin. At the end of 6 months, the lines grew at the same rate in the presence or absence of Herceptin and had regained original cell morphology. The source of resistance in these lines is not uniform as we observed an increase in phosphorylated Akt in only SKBR3 (data not shown). FoxM1 levels in parental and resistant lines were assayed by Western blot. Interestingly, levels of FoxM1 were higher in all resistant lines (Fig. 3A). This increase was reflected at the RNA level. To confirm a higher activity of FoxM1, we assayed the RNA levels of the known FoxM1 target genes. As shown in the SKBR3-resistant line, FoxM1 RNA levels were significantly increased (15-fold) as well as the levels of the p27 ubiquitin ligase components Skp2 (2.5-fold) and Cks1 (5.6-fold). Additionally, levels of the cell cycle regulators, PLK1 (1.5-fold) and Cyclin B1 (16.6-fold) were amplified in the resistant line compared with the parental control line (Fig. 3B).

FoxM1 levels are elevated in resistant lines and we observed that overexpression of FoxM1 could confer acquired resistance to Herceptin. We wanted to determine whether targeting FoxM1 could resensitize lines with inherent resistance. Knockdown of FoxM1 by siRNA in SKBR3-resistant cells led to a >75% reduction in cell number when used in

conjunction with Herceptin. This effect was also observed in MDA-MB-453 cells (Fig. 3C). Collectively, these results indicate that FoxM1 is upregulated in resistant lines and that targeting FoxM1 provides a method of sensitizing resistant cells to Herceptin treatment.

FoxM1 induces expression of Stathmin to confer resistance to paclitaxel

It has been previously reported that cells that overexpress HER2 display decreased sensitivity to apoptosis caused by paclitaxel (25, 32). Although microtubule-stabilizing agents such as paclitaxel induce mitotic arrest and consequent apoptosis, some patients fail to respond to this drug. We were curious to determine whether FoxM1, which is downstream of HER2, could protect from paclitaxel-induced apoptosis.

We noted that after 7 days of treatment in a low dose of paclitaxel (0.1 μmol/L), only 25% of SKBR3-pBabe cells survived, whereas ~50% of SKBR3-FoxM1 cells were still viable (Fig. 4A). This effect was also observed in MDA-MB-453 and BT474 FoxM1–expressing lines (Fig. 5C). Moreover, knockdown of FoxM1 by siRNA in SKBR3 cells was able to sensitize to Taxol as evidenced by a comparison of IC_{50} values between siRNA control and siRNA FoxM1–treated cells, 0.06 μmol/L versus 0.01 μmol/L

(Fig. 4A). These data indicate that FoxM1 can protect cells from paclitaxel-induced cell death.

Several mechanisms to combat Taxol-induced apoptosis have been reported, namely, upregulation of multidrug resistant 1, a P-glycoprotein family member that can shuttle toxins out of cells; upregulation of the IAP (inhibitor of apoptosis) family members including Survivin; and altered microtuble dynamics (26). We sought to investigate the mechanism by which FoxM1 could prevent paclitaxel-induced apoptosis. We did not detect any effect of FoxM1 on the levels of multidrug resistant 1 (data not shown). FoxM1 is known to positively regulate the CIAP family member Survivin, and increased expression is known to protect cells from paclitaxel. However, in the mammary tumor cells, we did not observe increased expression of Survivin (data not shown). We went on to examine the possibility of altered microtubule dynamics induced by FoxM1. As paclitaxel is known to stabilize tubulin, we compared the ratio of polymerized to soluble microtubule fractions. We fractionated cell lysates to obtain polymerized and soluble tubulin fractions in SKBR3-pBabe and SKBR3-FoxM1-expressing lines that were left nontreated or treated with paclitaxel. Without treatment, cells show similar tubulin ratios and nearly all detectable tubulins
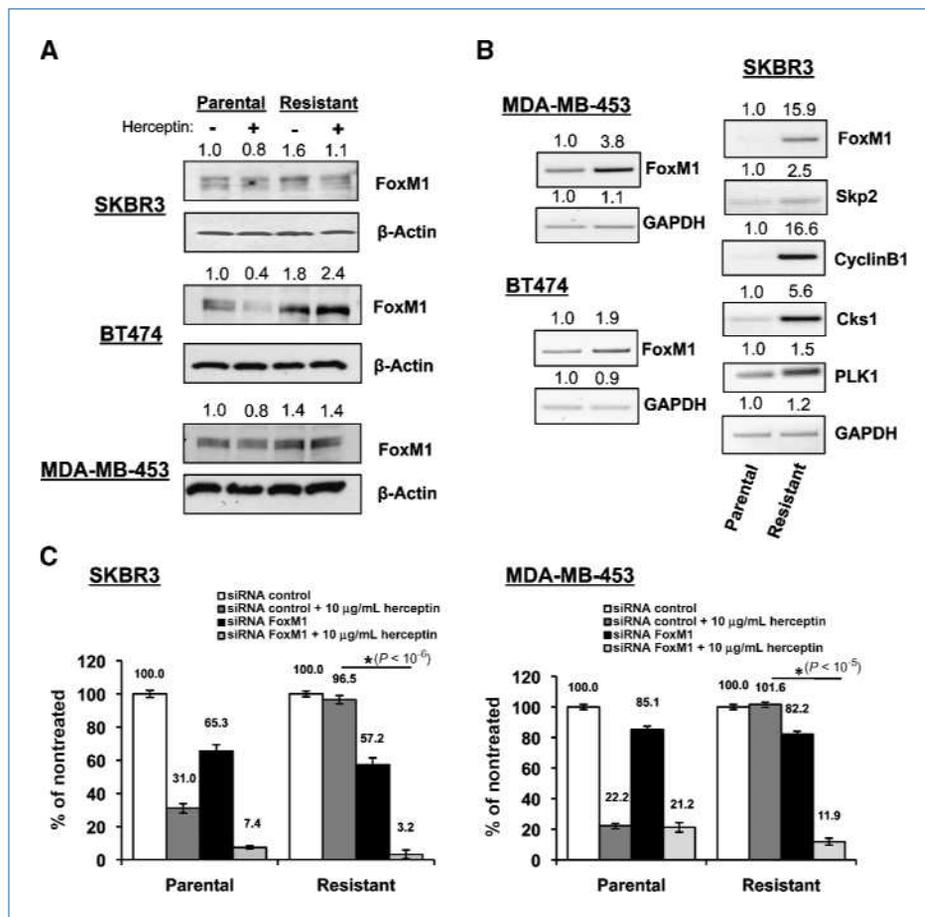

Figure 3. FoxM1 expression is higher in resistant lines and can be targeted to reintroduce sensitivity. A, Western blot showing FoxM1 protein levels in SKBR3, BT474, and MDA-MB-453 parental and resistant lines. Lines were continuously cultured in 5 μg/mL of Herceptin for 6 mo. Cells were placed in drug-free media for 7 d before treatment, and lysates were collected 72 h after treatment with 10 μg/mL of Herceptin. Quantification of FoxM1 bands by Image J is shown, using nontreated parental lines for normalization. B, semiquantitative reverse transcription-PCR (RT-PCR) using cDNA from either parental or resistant SKBR3 cells was used to analyze target gene expression. Representative gel pictures are shown and quantification values normalized to GAPDH are shown above. C, parental and resistant SKBR3 and MDA-MB-453 cells were transfected with control or FoxM1 specific siRNA. Forty-eight hours later, 3×10^{3} cells were plated in each well or a 24-well plate and left nontreated or treated with 10 μg/mL Herceptin. Media were changed every 3 to 4 d. After 14 d, colonies were stained with crystal violet and quantified by Photoshop.
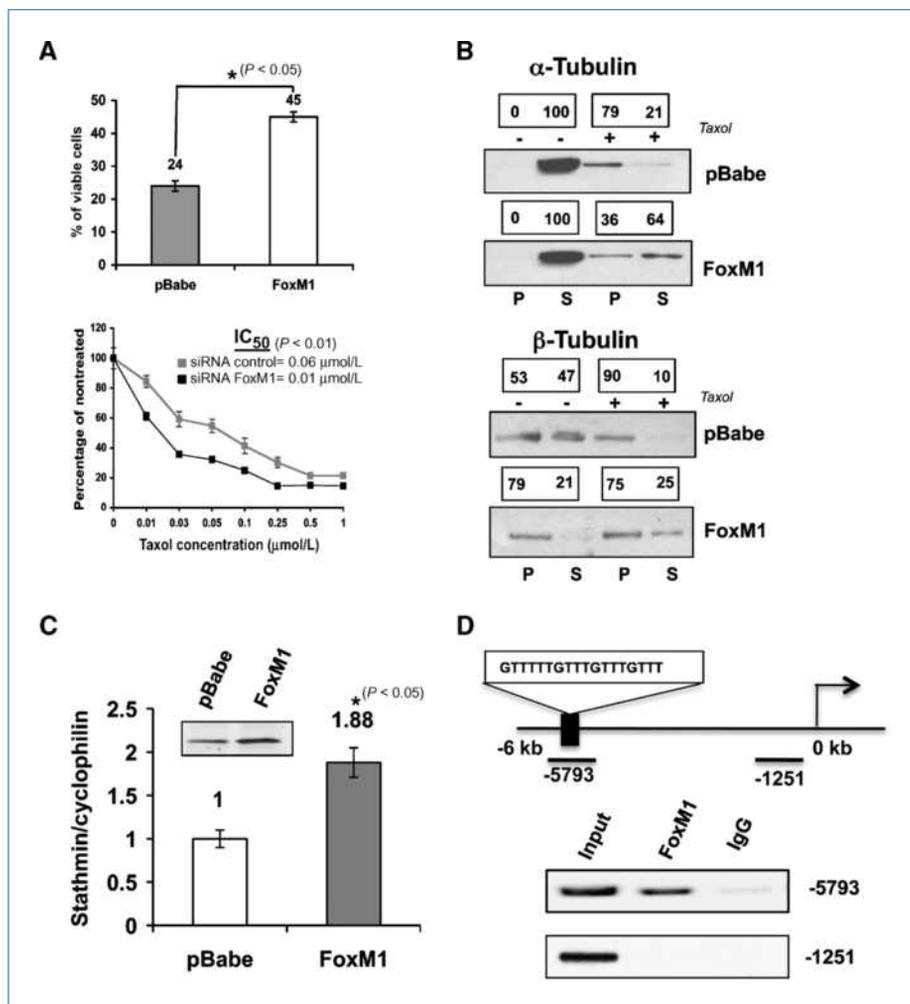
Figure 4. FoxM1 expression induces resistance to paclitaxel alone by increasing Stathmin expression and activity. A, top, SKBR3-pBabe and FoxM1-expressing lines were treated continuously with 0.1 μmol/L of paclitaxel for 7 d, and viable cell numbers were determined by luminescent measurement of ATP. Bottom, SKBR3 parental cells were treated with control siRNA or siRNA specific to FoxM1 for 72 h then treated with indicated doses of paclitaxel for 24 h. CellTiter Glo (Promega), a luminescence assay, was used to measure cell viability. B, polymerized and soluble tubulin fractions from nontreated and treated SKBR3-pBabe and FoxM1 cell lines was generated by centrifugation. Western blot was used to assay α-tubulin and β-tubulin ratios in polymerized and soluble fractions. Relative percentages are shown above the Western blot. C, RNA from SKBR3 pBabe and FoxM1 lines was collected, and RT-PCR was used to measure stathmin. Values were normalized against cyclophilin. Inset, relative protein expression by Western blot. D, chromatin immunoprecipitation assay was performed in SKBR3 cells using an antibody specific to FoxM1 or IgG as a control. PCR was used to amplify the region surrounding the putative FoxM1 binding site at −5,793 upstream of the transcriptional start site and the region surrounding −1,371 as a nonspecific control. Representative PCR results are shown.

were in the soluble form. Upon treatment with paclitaxel, SKBR3-pBabe cells show a dramatic shift toward the polymerized fraction. The FoxM1-expressing cells did show a shift toward the polymerized fraction but the ratio was considerably lower (0.56:1 FoxM1 versus 3.76:1 pBabe; Fig. 4B).

It has been previously established that increased expression and activity of the microtubule-destabilizing protein Stathmin can confer resistance to paclitaxel-induced apoptosis both in patient and cell culture samples (27, 33). The hallmark of increased activity is a low ratio of polymerized to soluble tubulin as we observed in FoxM1-expressing cells (31). Therefore, we compared Stathmin

RNA expression in pBabe and FoxM1-expressing cell lines, and observed that FoxM1 cells express 2-fold more Stathmin compared with the pBabe control cells. This difference was also noted at the protein level (Fig. 4C). In addition, chromatin immunoprecipitation using SKBR3 cells with FoxM1 antibody showed enrichment of the Stathmin promoter region, indicating that the RNA and subsequent protein increase in FoxM1-expressing lines is likely due to a direct interaction of FoxM1 with the Stathmin gene promoter (Fig. 4D). Together, these studies show that SKBR3-FoxM1 cell lines are resistant to paclitaxel-induced apoptosis by directly targeting and upregulating the microtubule-destabilizing protein Stathmin.

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FoxM1 overexpression protects from Herceptin and paclitaxel in combination

Although the success of Herceptin as a single agent is significant, the best therapeutic response is seen when Herceptin is used in conjunction with other chemotherapeutic agents such as paclitaxel. We were interested in determining the role of FoxM1 in resistance toward combination therapy.

Pretreatment for 72 hours with Herceptin followed by paclitaxel treatment of both SKBR3-pBabe and FoxM1 cell lines revealed significant differences. The FoxM1-expressing cells exhibited resistance to killing. For example, 7 days after

paclitaxel treatment, only 10% to 12% of the pBabe cells survived, whereas the survival of the FoxM1-expressing cells was >40% (Fig. 5A). Knockdown of FoxM1 in SKBR3 sensitized the cells to combination treatment as evidenced by IC_{50} calculations, 0.097 μmol/L (siRNA Control) versus 0.028 μmol/L (siRNA FoxM1; Fig. 5B).

Long-term combination treatment was also investigated by colony-forming assay. Quantification of colony numbers show that ~55% of FoxM1-expressing cells survived after combination therapy, whereas only 26% of pBabe lines survived the treatment in SKBR3 cells (Fig. 5C). The ability of FoxM1 to mediate resistance to combination
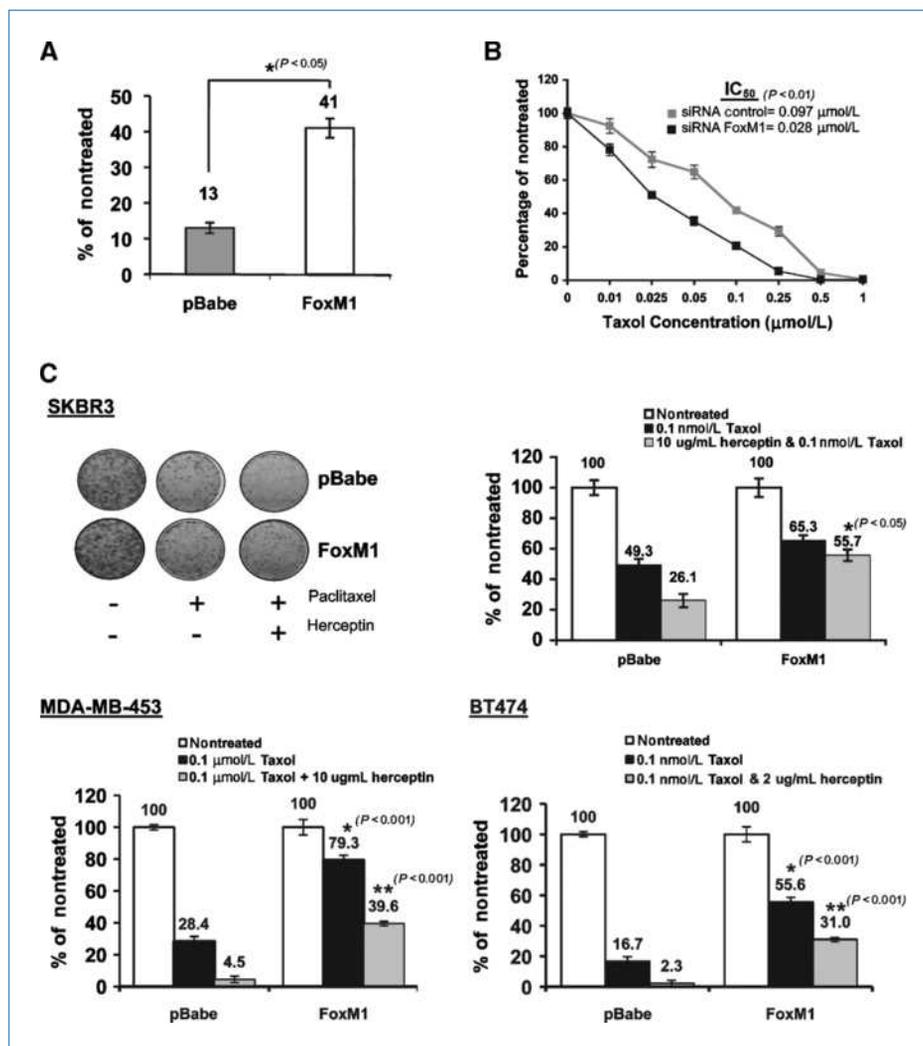

Figure 5. FoxM1 protects cells against treatment with Herceptin and paclitaxel in combination. A, cell lines were pretreated with 10 μg/mL of Herceptin for 3 d then treated with 0.1 μmol/L of paclitaxel for 7 d. Relative cell number was determined by Cell Titer-Glo (Promega) measurement every other day for 7 d. B, SKBR3 parental cells were treated with control or FoxM1 siRNA for 72 h followed by 10 μg/mL of Herceptin for 3 d. Equal numbers of cells were treated for 24 h with increasing amounts of paclitaxel, and ATP was measured by luminescence. C, 3 to 5 × 10³ SKBR3, MDA-MB-453, or BT474 cells were seeded in each well in triplicate. Cells were either left nontreated or pretreated in 10 μg/mL Herceptin for 72 h then pulsed in 0.1 μmol/L paclitaxel for 4 h. Wells that received Herceptin were continuously cultured in 10 μg/mL for the duration of the experiment. Media were changed every 3 d. Cells were stained after a total of 17 d in crystal violet and representative wells are shown (left). Graph shows quantification of triplicates from three separate experiments. Top left, representative wells of SKBR3-pBabe and FoxM1 cells.
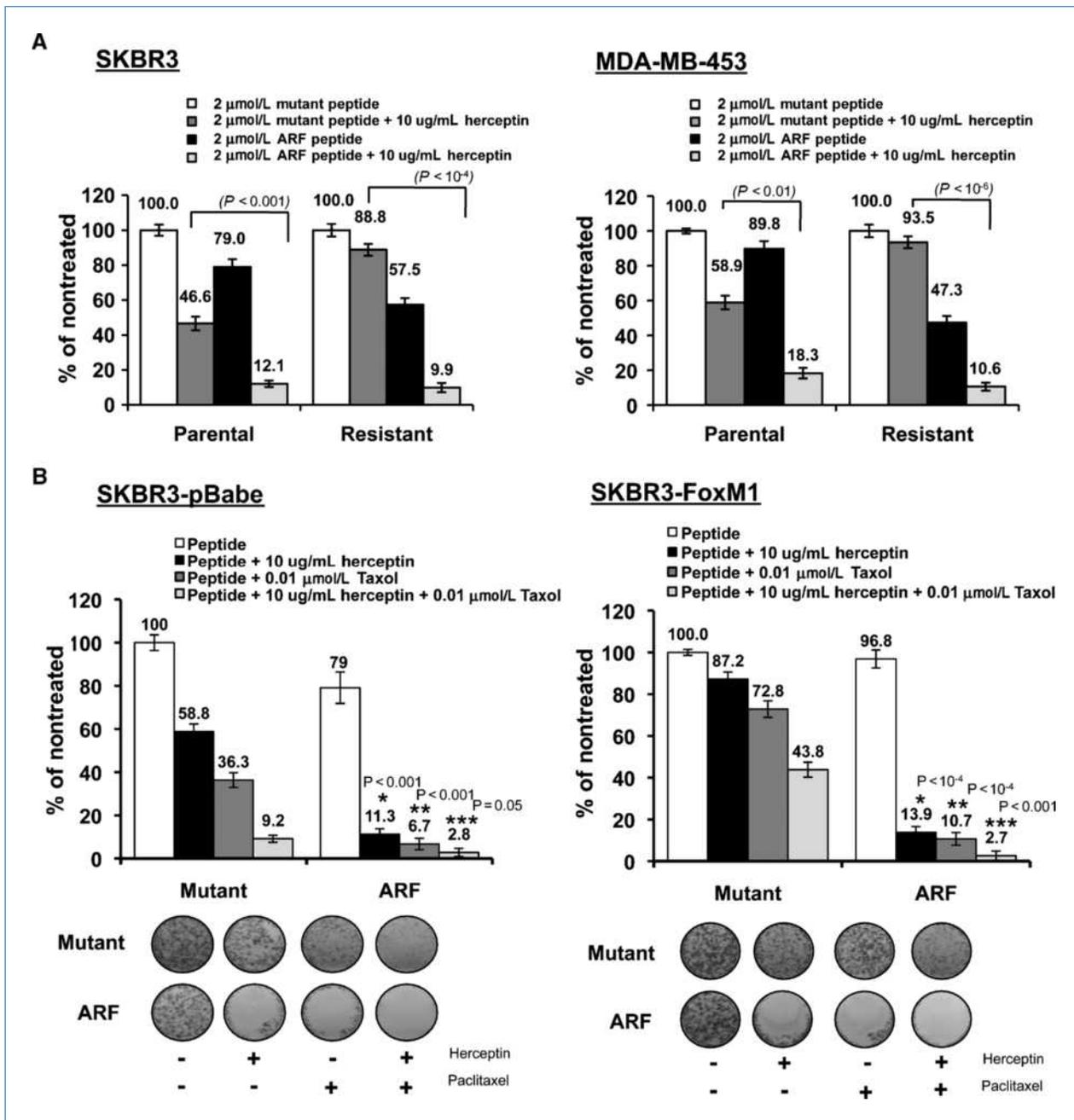
Figure 6. Targeting FoxM1 with ARF peptide is sufficient to overcome inherent Herceptin resistance and to sensitize pBabe or FoxM1-overexpressing cells to treatment. A, ARF peptide or mutant peptide (2 μmol/L) was used to treat either parental or resistant SKBR3 and MDA-MB-453 cells. Cells were pretreated with peptide for 3 d then treated with both 10 μg/mL Herceptin and peptide. Media were changed daily, and cells were stained with crystal violet after 17 total days. Graph shows quantification of colony-forming assay by Photoshop. B, SKBR3-pBabe and FoxM1 cell lines were treated with either mutant or ARF peptide for 3 d. Wells receiving both Herceptin and paclitaxel were pretreated for 72 h with Herceptin before receiving indicated doses of paclitaxel. Quantification of triplicates is shown in the graph with representative images below.

therapy was observed also in a comparison of pBabe versus FoxM1-expressing MDA-MB-453 (4.5% versus 39.6%) and BT474 (2.3% versus 31%) cell lines (Fig. 5C). These data clearly indicate that FoxM1 can protect breast cancer cells from treatment with Herceptin and paclitaxel in combination.

An ARF-derived peptide inhibitor of FoxM1 is sufficient to sensitize mammary tumor cells to treatment

Studies in our laboratory have shown that FoxM1 is inhibited by a small peptide that contains an 18-AA region of the p19ARF protein (residues between 26 and 44). This peptide has been shown to reduce proliferation and induce

apoptosis of hepatocellular carcinoma cells *in vivo* (28). Treatment with the ARF-derived peptide and Herceptin led to a staggering 90% reduction in SKBR3 and MDA-MB-453–resistant cell number as measured by colony-forming assay, similar to parental lines treated with both (Fig. 6A). As expected, treatment with a mutant peptide did not show a difference in colony number compared with parental lines and therefore was used as a control.

We went on to test the ability of the ARF peptide to sensitize the FoxM1-expressing cells to treatment. Addition of the ARF peptide to Herceptin, paclitaxel, or combination treatment showed a dramatic reduction in cell number compared with mutant peptide. In pBabe-expressing lines, the ARF peptide was able to sensitize cells to all treatments, resulting in a greater effect from the same dosage. Most notably, addition of ARF peptide had a significant effect in FoxM1 lines with <3% of cells surviving combination treatment. These data reveal that the use of ARF peptide in chemotherapeutic regimens could have great clinical promise.

Discussion

Drug resistance, either inherent or acquired, poses significant clinical challenges. The mechanisms by which cells acquire resistance are multiple and complex, and our understanding will be important to create better therapeutic options. The work presented here is the first report that high levels of FoxM1, commonly seen in tumors, offer mammary tumor cells an additional growth advantage, protection against Herceptin and paclitaxel both alone and in combination.

Previous reports from our laboratory have shown that FoxM1 can regulate p27 degradation and localization to allow cell cycle progression (5, 13). The work presented here shows that this ability of FoxM1 to keep basal levels of p27 low and prevent p27 accumulation in response to Herceptin treatment is mediating a resistant phenotype in FoxM1-overexpressing cell lines. Yet, it is likely that FoxM1 can mediate resistance by other mechanisms. This is evident in cells harboring inherent resistance to Herceptin. The basal levels of p27 in BT474 and MDA-MB-453 are higher in resistant lines than in parental (data not shown), indicating dysregulation. In a pooled resistant cell line, it is feasible that the mechanisms by which cells evade therapy are heterogeneous; yet, the result, as we observed, is increased FoxM1 expression and activity. These findings are significant because regardless of p27 or p-Akt status, inhibition of FoxM1 induces resensitization. These data indicate that FoxM1 is likely a downstream mediator of resistance caused by multiple mechanisms and therefore a valuable therapeutic target.

Several studies have reported alterations in microtubules as a source of resistance to paclitaxel, and some have implicated increased expression of Stathmin (27, 33). Yet, upstream transcriptional regulators of Stathmin have not been reported. Not only do we show that FoxM1 directly increases expression of stathmin, but that microtubules in FoxM1-overexpressing lines fail to polymerize in response to paclitaxel treatment, an indicator that the Stathmin activity is high in these cells. The implications of this finding spread past breast

cancer. As mentioned, FoxM1 expression is elevated in all tumor types examined to date and paclitaxel is a commonly used chemotherapeutic agent. It is likely that FoxM1 inhibition could be a successful tool to sensitize various tumor types to treatment. Therapeutically, paclitaxel has significant and limiting side effects including a decrease in blood cells (neutropenia, anemia, and leukopenia) and chemotherapy-induced neuropathy (34). The addition of a FoxM1 inhibitor to a chemotherapeutic regimen could result in lower effective doses and a potential reduction in side effects for patients.

In the past several years, the ability of FoxM1 to promote tumorigenesis and tumor growth has become apparent. Consequently, several groups have been working to develop FoxM1 inhibitors. In addition to the ARF-derived peptide inhibitor of FoxM1, it has been shown that the antibiotics siomycin A and thiostrepton could inhibit FoxM1 (28, 35). Gefitinib, an EGF receptor inhibitor, can target FoxM1 (36). In addition, proteasome inhibitors, several of which are in use clinically, can downregulate FoxM1 levels (37). Several studies have shown that FoxM1 functions to promote proliferation, inhibit apoptosis, evade senescence, and promote angiogenesis (30, 38, 39). Our studies also implicate that FoxM1 can promote a drug-resistant phenotype in breast tumors and could be targeted, perhaps by the ARF peptide, in sensitization therapy. Notably, previous *in vivo* studies using ARF peptide did not show toxicity in other organ systems, one important factor in choosing therapies (28).

Interestingly, several FoxM1 target genes have been implicated in resistance including *Survivin, PLK1*, and *Cks1*. Survivin was shown to induce resistance to paclitaxel, vascular endothelial growth factor inhibitors, and radiation therapy (40). Knockdown of PLK1 could sensitize cells to cisplatin, Herceptin, and paclitaxel, whereas Cks1 is implicated in paclitaxel resistance as well (41, 42). As these factors are downstream targets of FoxM1, it is likely that therapies aimed at reducing FoxM1 also will serve as a method of sensitizing tumor cells to other therapies. In line with this, it was recently shown that knockdown of FoxM1 in cells that have resistance to cisplatin could induce apoptosis (43). The ability of FoxM1 to induce resensitization could be applicable in a variety of tumor types and therapies. Our study shows that FoxM1 is a valid target in drug-resistant tumors, and inhibitors of FoxM1 should be considered in future therapeutic trials.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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