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FoxM1 Mediates Resistance to Herceptin and Paclitaxel

Janai R. Carr, **Hyun Jung Park**, **Zebin Wang**, **Megan M. Kiefer**, and **Pradip Raychaudhuri**[1]
Department of Biochemistry and Molecular Genetics, University of Illinois at Chicago, College of Medicine, Chicago, IL 60607

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 HER2 monoclonal antibody Herceptin and microtubule-stabilizing drug paclitaxel, both as single agents and in combination. FoxM1 altered microtubule dynamics in order 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 siRNA or an ARF-derived peptide inhibitor increased therapeutic sensitivity. Our findings indicate that targeting FoxM1 could relieve therapeutic resistance in breast cancer.

Keywords

FoxM1; Herceptin; Paclitaxel; Drug Resistance; Stathmin

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 G2/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 G1/S transition by downregulation of the Cdk inhibitor p27 through multiple mechanisms. Specifically, by increasing expression of Skp2 and Cks1, members of the E3 ubiquitin ligase complex responsible for the degradation of p27 (12). Also, by up-regulation of KIS kinase FoxM1 promotes localization of p27 to the cytoplasm (13). During G2/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 PI3K/Akt and Ras/Raf/MAPK 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, all of which results in phosphorylation and

[1]**Address correspondence to**: Pradip Raychaudhuri, Department of Biochemistry and Molecular Genetics, University of Illinois at Chicago, College of Medicine, 900 S. Ashland Ave M/C 669, Chicago, IL 60607-7170, Tel. (312) 413-0255; FAX: (312) 355-3847; pradip@uic.edu.

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activation of FoxM1 (15–17). Recently, it has been shown that 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 accumulation of the Cdk inhibitor p27 and subsequent G1/S cell cycle arrest. Unfortunately, the efficacy of Herceptin as a monotherapy is thought to be less than 30% and in combination with microtubule stabilizing drugs approximately 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, resistance does occur. Insensitivity to Taxol has been shown in cells that overexpress HER2. On average, cells with HER2 amplification require a 100-fold higher dose of Taxol to produce the same effect (25). Resistance to Taxol has been attributed to additional mechanisms including increased expression of multi-drug 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 Taxol 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 regulation of Stathmin, which renders cells resistant to Taxol treatment. Moreover, we show that inhibition of FoxM1 by an ARF-derived peptide sensitizes cells to Hercepin and Taxol therapy.

Materials and Methods

Cell Culture and Chemotherapeutic Agents

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

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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 (PI) solution (50ug/ml PI, 0.1mg/ml RNaseA, 0.05% Triton-X). All reagents were purchased from Sigma. After 40 minutes of incubation at 37° cells were analyzed using a flow cytometer. 10µM of 5-Bromo-2-Deoxyuridine (BrDU) from Sigma was added to culture media. Cells were fixed and stained with anti-BrdU antibody (1:250, Dako) followed by anti-mouse FITC (Dako) and DAPI (Molecular Probes). Cell viability was measured using CellTiter-Glo Luminescent assay (Promega), which measures the amount of oxygenated oxyluciferin that has a direct correlation to ATP present. For colony forming assay, 3–5 × 10^3 cells were plated in triplicate in a 24-well plate. 24 hours later, treatment was initiated. After 14–17 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.

Semi-Quantitative RT-PCR

RNA was extracted using Trizol (Invitrogen) and cDNA was synthesized using reverse transcriptase (Bio-Rad). Equal amounts of cDNA were used for all PCR reactions (Promega). PCR products were analyzed over a series of cycle numbers in order to ensure that data was produced during the PCR log-scale amplification. Samples were run on agarose gels, photographed, and quantified using Image J. The following primers were used: 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' Polo Like Kinase 1: 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' 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 1mM EDTA, 0.15M NaCl, 0.05M 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 (1:1000, 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 Image J software (NIH).

Chromatin Immunoprecipitation (ChIP)

ChIP assay was performed as described previously (30). Briefly, cells were fixed in 1% formaldehyde for 10 minutes to allow crosslinking and then were quenched with 125nM glycine. Cells were collected and lysed in SDS lysis buffer (1% SDS, 10mM EDTA, 50mM Tris pH8, protease and phosphate inhibitors). Lysate was sonicated, pre-cleared, 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 PCR a 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 non-specific control.

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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 1nM Taxol for 24 hours. Cells were collected in hypotonic buffer (1mM $MgCl_2$, 2mM EGTA, 0.5% Nonidet P-40, 20mM Tris-HCl pH 6.8) and centrifuged for 10 minutes at room temperature (14,000 rpm). The supernatant was used as the soluble fraction while 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 10ug/ml of Herceptin for 14 days. As shown by quantification of the colony formation assay, FoxM1 overexpression resulted in a three to seven-fold increase in colony number as compared to pBabe expressing cells (Figure 1A), providing evidence that FoxM1 confers resistance to Herceptin.

The magnitude of the G1/S arrest induced by Herceptin was measured by propidium iodide staining followed by flow cytometry (FACS) analysis. Cells were treated in 10ug/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 G1, but the FoxM1 expressing cells did not exhibit any significant enrichment of the G1 population (Figure 1B). Herceptin alone does not induce apoptosis (21). Consistent with that, none of the cell lines showed an increase in the sub-G1 population. To further investigate resistance in FoxM1-expressing cells, we measured the ability to incorporate BrdU (Figure 1C). Upon treatment, SKBR3-pBabe showed a substantial (35%) reduction in the number of BrdU-positive cells. FoxM1 expressing cells did not show any significant decrease in BrdU-incorporation. Taken together, these results indicate that FoxM1 overcomes the G1/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

While 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 10ug/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 expected. Interestingly, in SKBR3-FoxM1 cell lines, the basal expression of p27 is lower and levels remained low even after a high-dose of Herceptin (Figure 2A and 2B). 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 G1/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 (Figure 2C).

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

In order to generate cell lines that have inherent resistance to Herceptin, we cultured parental SKBR3, MDA-MB-453, and BT474 lines continuously in 5ug/ml of Herceptin. At the end of six 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 (Figure 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, Polo Like Kinase 1 (1.5-fold) and Cyclin B1 (16.6-fold) were amplified in the resistant line as compared to the parental control line (Figure 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 re-sensitize lines with inherent resistance. Knockdown of FoxM1 by siRNA in SKBR3 resistant cells led to a more than 75% percent reduction in cell number when used in conjunction with Herceptin. This effect was also observed in MDA-MB-453 cells (Figure 3C). Collectively, these results indicate that FoxM1 is up-regulated 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). While microtubule-stabilizing agents such as Taxol 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 Taxol induced apoptosis.

We noted that after seven days of treatment in a low dose of Taxol (0.1μM), only 25% of SKBR3-pBabe cells survived, while nearly 50% of SKBR3-FoxM1 cells were still viable (Figure 4A). This effect was also observed in MDA-MB-453 and BT474 FoxM1 expressing lines (Figure 5C). Moreover, knockdown of FoxM1 by siRNA in SKBR3 cells was able to sensitize to Taxol as evidenced by a comparison of IC50 values between siRNA control and siRNA FoxM1 treated cells, 0.06uM vs. 0.01uM (Figure 4A). This data indicates that FoxM1 can protect cells from Taxol induced cell death.

Several mechanisms to combat Taxol induced apoptosis have been reported. Namely, up-regulation of MDR1 (multi-drug resistant protein 1) a P-Glycoprotein family member that can shuttle toxins out of cells, up-regulation of the CIAP (inhibitors of apoptosis) family members including Survivin, and altered microtuble dynamics (26). We sought to investigate the mechanism by which FoxM1 could prevent Taxol induced apoptosis. We did not detect any effect of FoxM1 on the levels of MDR1 (data not shown). FoxM1 is known to positively regulate the CIAP family member Survivin and increased expression is known to protect cells from Taxol. 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 Taxol 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 untreated or treated with Taxol. Without treatment,

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cells show similar tubulin ratios and nearly all detectable tublins were in the soluble form. Upon treatment with Taxol, SKBR3-pBabe cells show a dramatic shift towards the polymerized fraction. The FoxM1 expressing cells did show a shift towards the polymerized fraction but the ratio was considerably lower (0.56:1 FoxM1 vs. 3.76:1 pBabe (Figure 4B).

It has been previously established that increased expression and activity of the microtubule destabilizing protein Stathmin can confer resistance to Taxol 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 to the pBabe control cells. T his difference was also noted at the protein level (Figure 4C). In addition, chromatin immunoprecipitation (ChIP) 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 (Figure 4D). Together, these studies demonstrate that SKBR3-FoxM1 cell lines are resistant to Taxol induced apoptosis by directly targeting and up-regulating the microtubule destabilizing protein Stathmin.

FoxM1 overexpression protects from Herceptin and Paclitaxel in combination

While 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 Taxol. We were interested in determining the role of FoxM1 in resistance towards combination therapy.

Pretreatment for 72 hours with Herceptin followed by Taxol treatment of both SKBR3-pBabe and FoxM1 cell lines revealed significant differences. The FoxM1-expressing cells exhibited resistance to killing. For example, seven days after Taxol treatment, only 10–12% of the pBabe cells survived, whereas the survival of the FoxM1-expressing cells was greater than 40% (Figure 5A). Knockdown of FoxM1 in SKBR3 sensitized the cells to combination treatment as evidenced by IC50 calculations, 0.097uM (siRNA Control) vs. 0.028uM (siRNA FoxM1) (Figure 5B).

Long-term combination treatment was also investigated by colony forming assay. Quantification of colony numbers show that approximately 55% of FoxM1-expressing cells survived after combination therapy, whereas only 26% of pBabe lines survived the treatment in SKBR3 cells (Figure 5C). The ability of FoxM1 to mediate resistance to combination therapy was observed also in a comparison of pBabe vs. FoxM1 expressing MDA-MB-453 (4.5 vs. 39.6%) and BT474 (2.3 vs. 31%) cell lines (Figure 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 lab 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 both SKBR3 and MDA-MB-453 resistant cell number as measured by colony forming assay, similar to parental lines treated with both (Figure 6A). As expected, treatment with a mutant peptide did not show a difference in colony number as compared to parental lines and therefore was used as a control.

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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, Taxol, or combination treatment showed a dramatic reduction in cell number as compared to 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 less than 3% of cells surviving combination treatment. This data reveals 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 in order 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 lab 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 re-sensitization. This data indicates 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 Taxol and some have implicated increased expression of Stathmin (27,33). Yet, upstream transcriptional regulators of Stathmin have not been reported. Not only do we demonstrate that FoxM1 directly increases expression of stathmin, but that microtubules in FoxM1 overexpressing lines fail to polymerize in response to Taxol 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, Taxol has significant and limiting side effects including a decrease in blood cells (neutropenia, anemia, 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. As a result, 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 EGFR inhibitor can target FoxM1 (36). In addition, proteasome inhibitors, a number 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

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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, Polo Like Kinase 1 (PLK1), and Cks1. Survivin was shown to induce resistance to Taxol, VEGF inhibitors, and radiation therapy (40). Knockdown of PLK1 could sensitize cells to Cisplatin, Herceptin, and Taxol while Cks1 is implicated in Taxol 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 re-sensitization 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.

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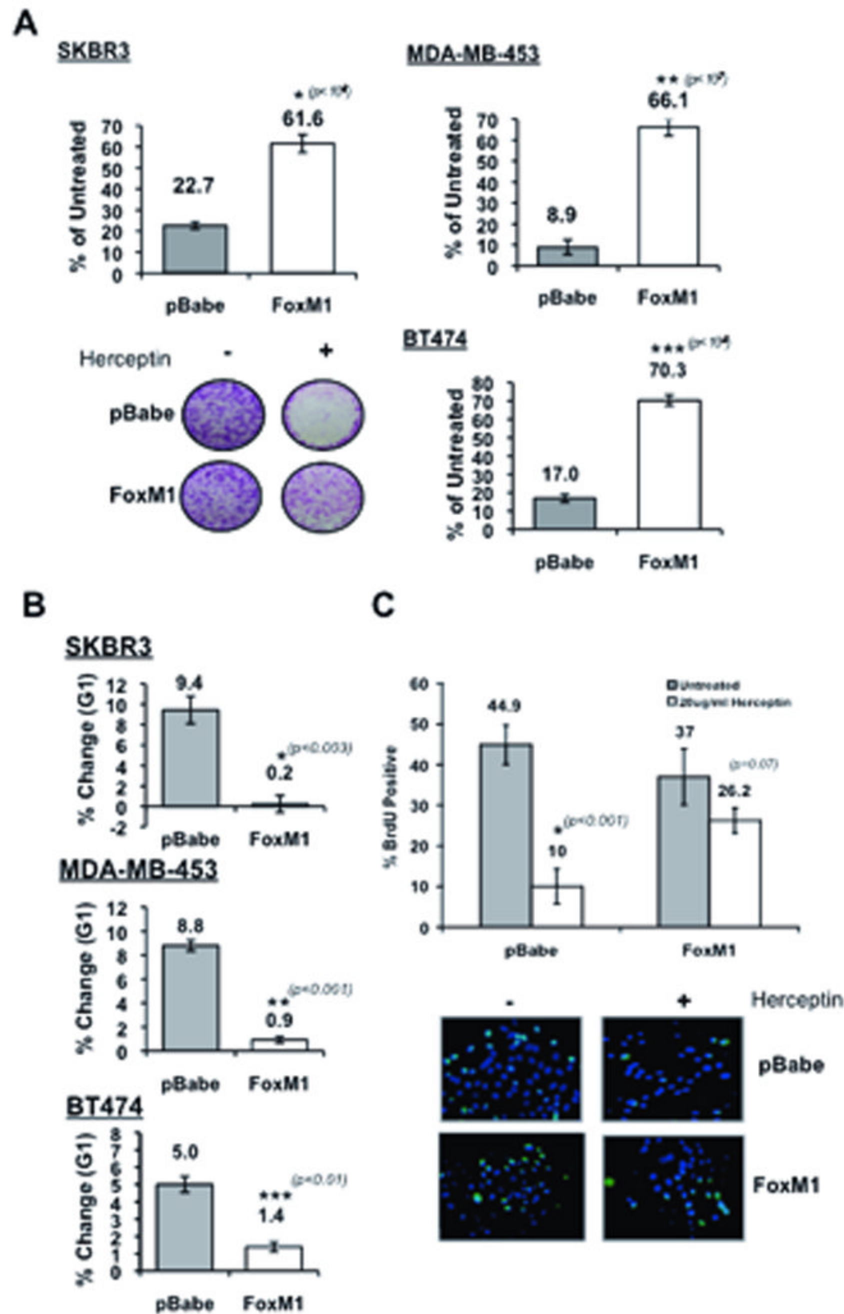

Figure 1. Overexpression of FoxM1 renders multiple HER-2 amplified cell lines resistant to the effects of Herceptin treatment

(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 10ug/ml Herceptin for 14 days, media was changed every 3 days. 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 untreated wells. (B) Stable cell lines expressing either pBabe or FoxM1 were treated with 10ug/ml of Herceptin for 48 hours, stained with propidium iodide, and subjected to FACS analysis. Percentage change in G1 phase is shown. Inset shows relative protein expression in FoxM1 versus

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pBabe stable cell lines. (C) SKBR3-pBabe and FoxM1 lines were either untreated or treated for 72 hours with Herceptin followed by a pulse of BrdU for 2 hours. Percentage of BrdU positive compared to 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.

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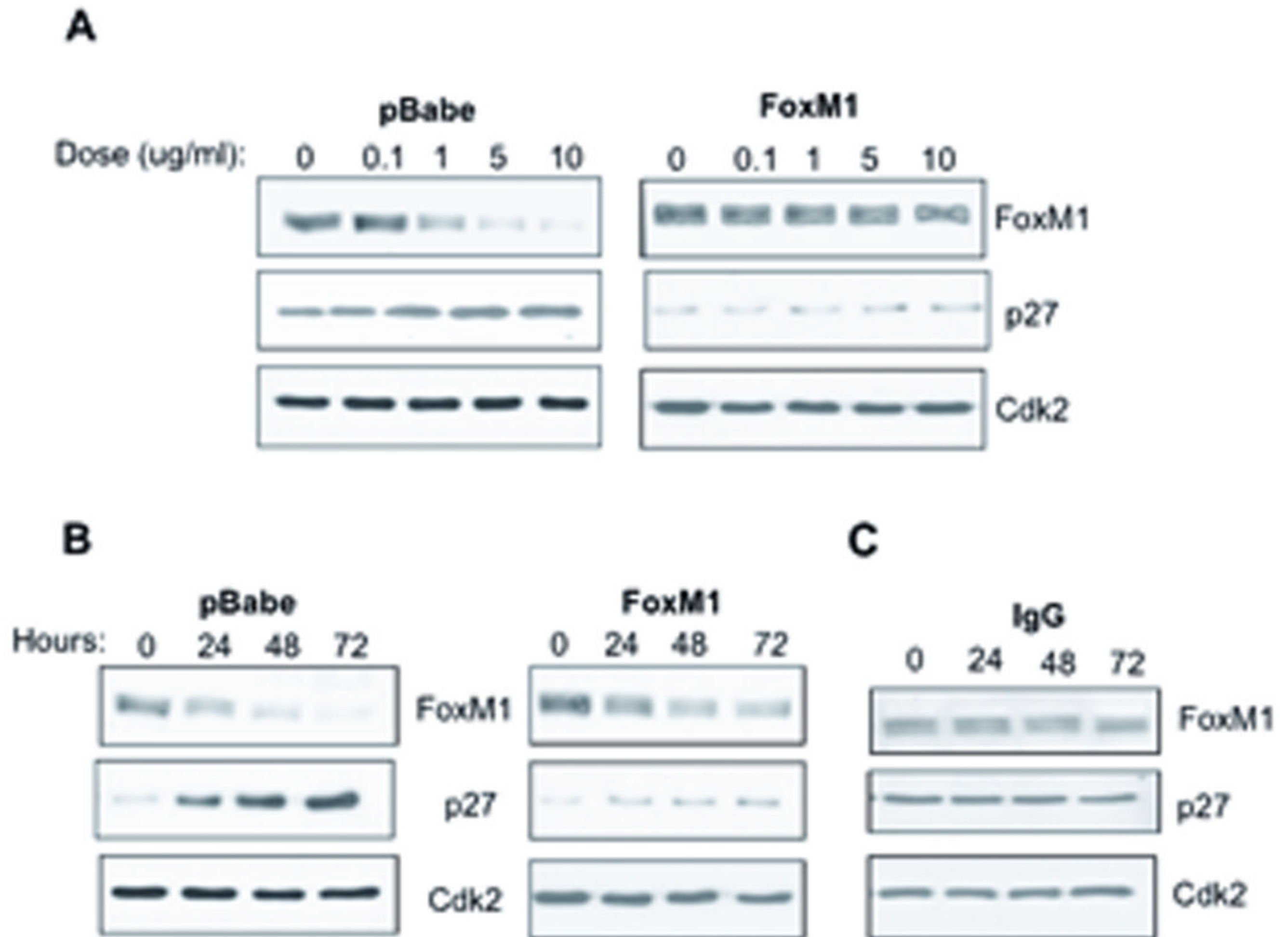

Figure 2. SKBR3-FoxM1 cell lines fail to accumulate p27 after treatment with Herceptin

(A) SKBR3-pBabe and FoxM1 expressing cell lines were treated with increasing doses of Herceptin for 48 hours. 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 10ug/ml of Herceptin for 24, 48, and 72 hours. FoxM1 and p27 levels are shown by western blot. (C) SKBR3-pBabe cell lines were treated with 10ug/ml of IgG for indicated periods of time. FoxM1 and p27 were assayed by western blot and Cdk2 was used as a loading control.

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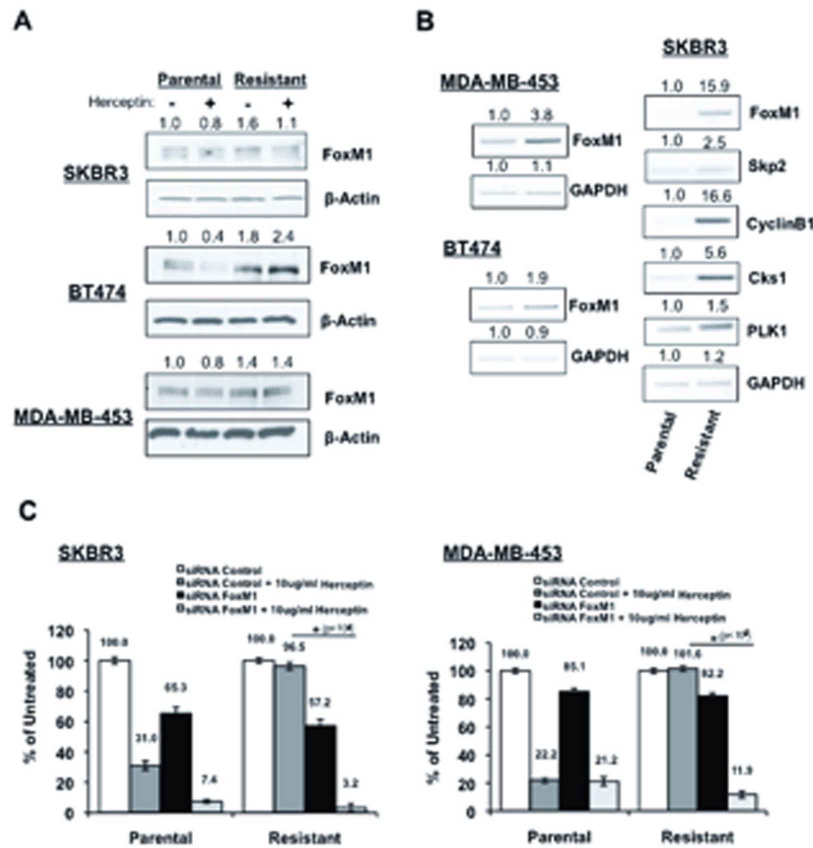

Figure 3. FoxM1 expression is higher in resistant lines and can be targeted to reintroduce sensitivity

(A) Western blot showing FoxM1 protein levels in SKBR3, BT474, and MDA-MB-453 parental and resistant lines. Lines were continuously cultured in 5ug/ml of Herceptin for six months. Cells were placed in drug free media for 7 days prior to treatment and lysates were collected 72 hours after treatment with 10ug/ml of Herceptin. Quantification of FoxM1 bands by Image J is shown, using untreated parental lines for normalization. (B) Semi-quantitative 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. 48 hours later, 3×10^3 cells were plated in each well or a 24-well plate and left untreated or treated with 10ug/ml Herceptin. Media was changed every 3–4 days. After 14 days, colonies were stained with crystal violet and quantified by photoshop.

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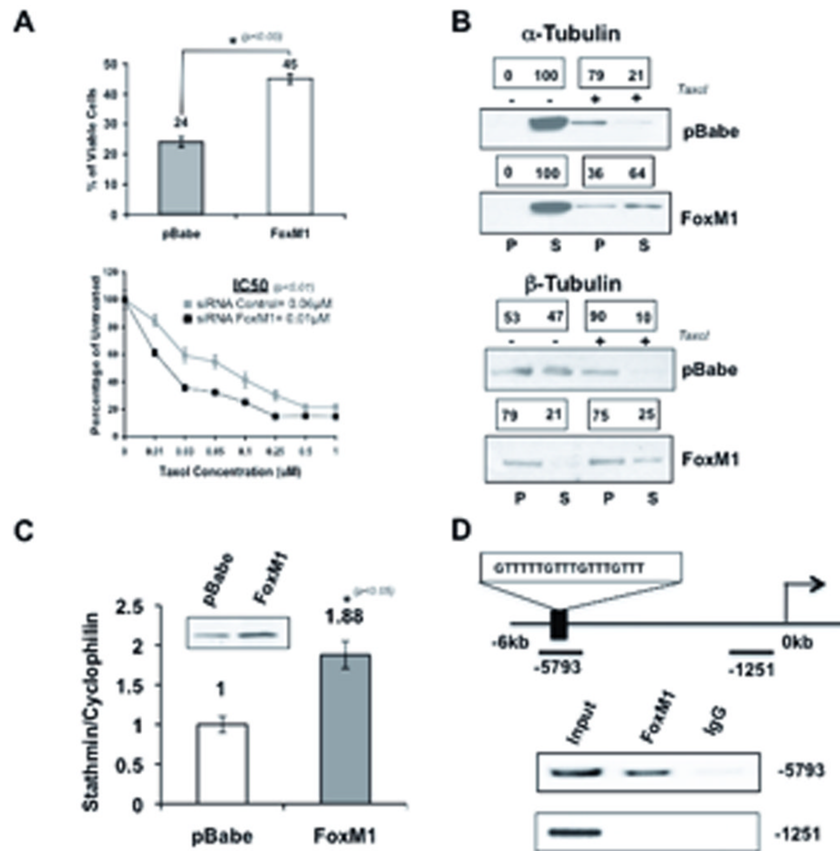

Figure 4. FoxM1 expression induces resistance to Taxol alone by increasing Stathmin expression and activity

(A) Top Panel: SKBR3-pBabe and FoxM1 expressing lines were treated continuously with 0.1 uM of Taxol for 7 days and viable cell numbers were determined by luminescent measurement of ATP. Bottom Panel: SKBR3 parental cells were treated with control siRNA or siRNA specific to FoxM1 for 72 hours then treated with indicated doses of Taxol for 24 hours. CellTiter Glo, a luminescence assay was used to measure cell viability. (B) Polymerized and soluble tubulin fractions from untreated and treated SKBR3-pBabe and FoxM1 cell lines were 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 western blot. (C) RNA from SKBR3 pBabe and FoxM1 lines were collected and RT-PCR was used to measure stathmin. Values were normalized against cyclophilin. Inset shows relative protein expression by western blot. (D) Chromatin immunoprecipitation assay (ChIP) 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 −5793 upstream of the transcriptional start site and the region surrounding −1371 as a non-specific control. Representative PCR results are shown.

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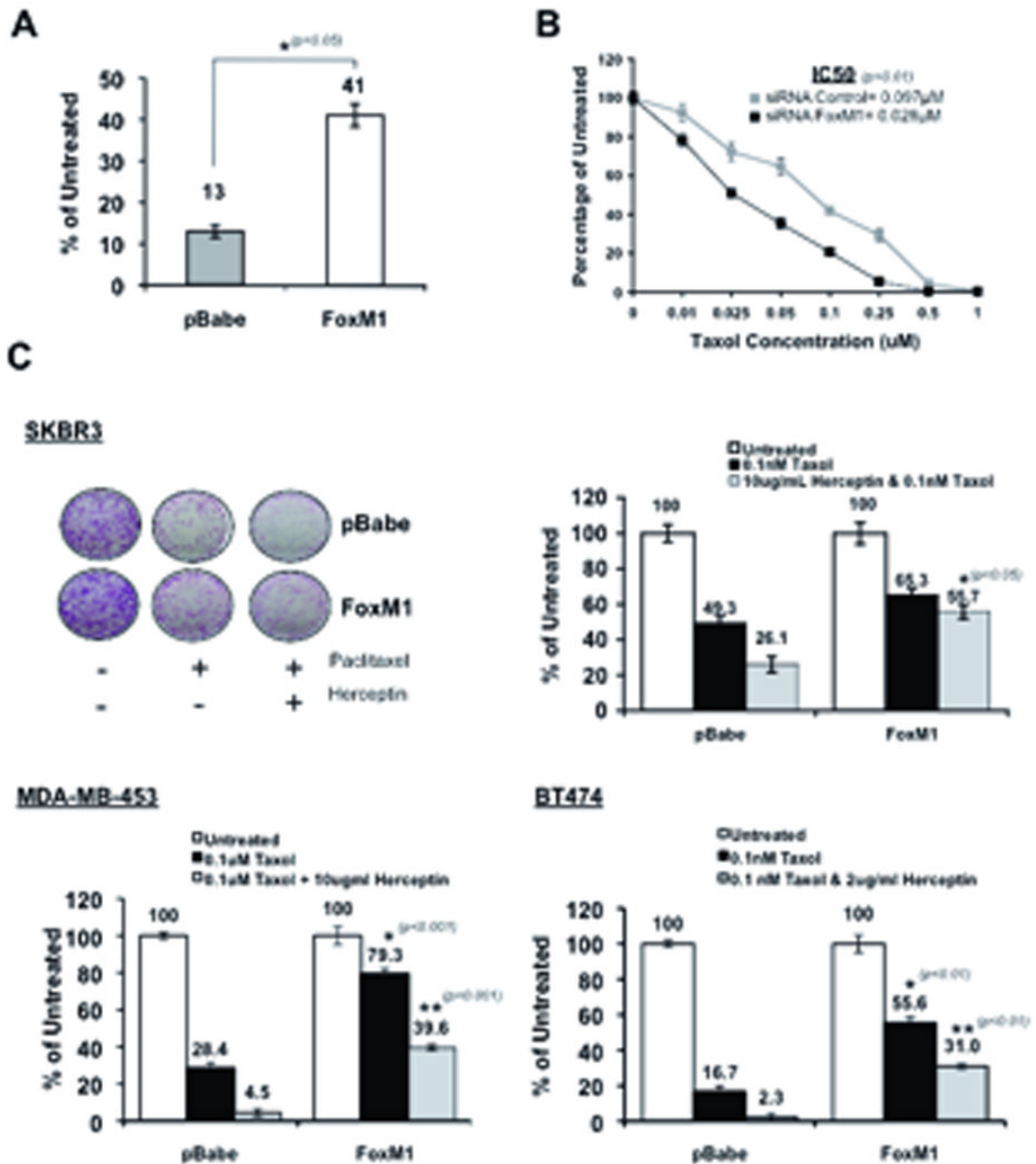

Figure 5. FoxM1 protects cells against treatment with Herceptin and Taxol in combination

(A) Cell lines were pretreated with 10ug/ml of Herceptin for 3 days then treated with 0.1uM of taxol for 7 days. Relative cell number was determined by cell titer glo measurement every other day for seven days. (B) SKBR3 parental cells were treated with control or FoxM1 siRNA for 72 hours followed by 10ug/ml of Herceptin for 3 days. Equal numbers of cells were treated for 24 hours with increasing amounts of taxol and ATP was measured by luminescence. (C) 3–5 $\times 10^3$ SKBR3, MDA-MB-453, or BT474 cells were seeded in each well in triplicate. Cells were either left untreated or pre-treated in 10ug/ml Herceptin for 72 hours then pulsed in 0.1μM Taxol for 4 hours. Wells that received Herceptin were continuously cultured in 10ug/ml for the duration of the experiment. Media was changed

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every 3 days. Cells were stained after a total of 17 days in crystal violet and representative wells are shown in the left panel. Graph shows quantification of triplicates from three separate experiments. Representative wells of SKBR3-pBabe and FoxM1 cells are shown in upper left.

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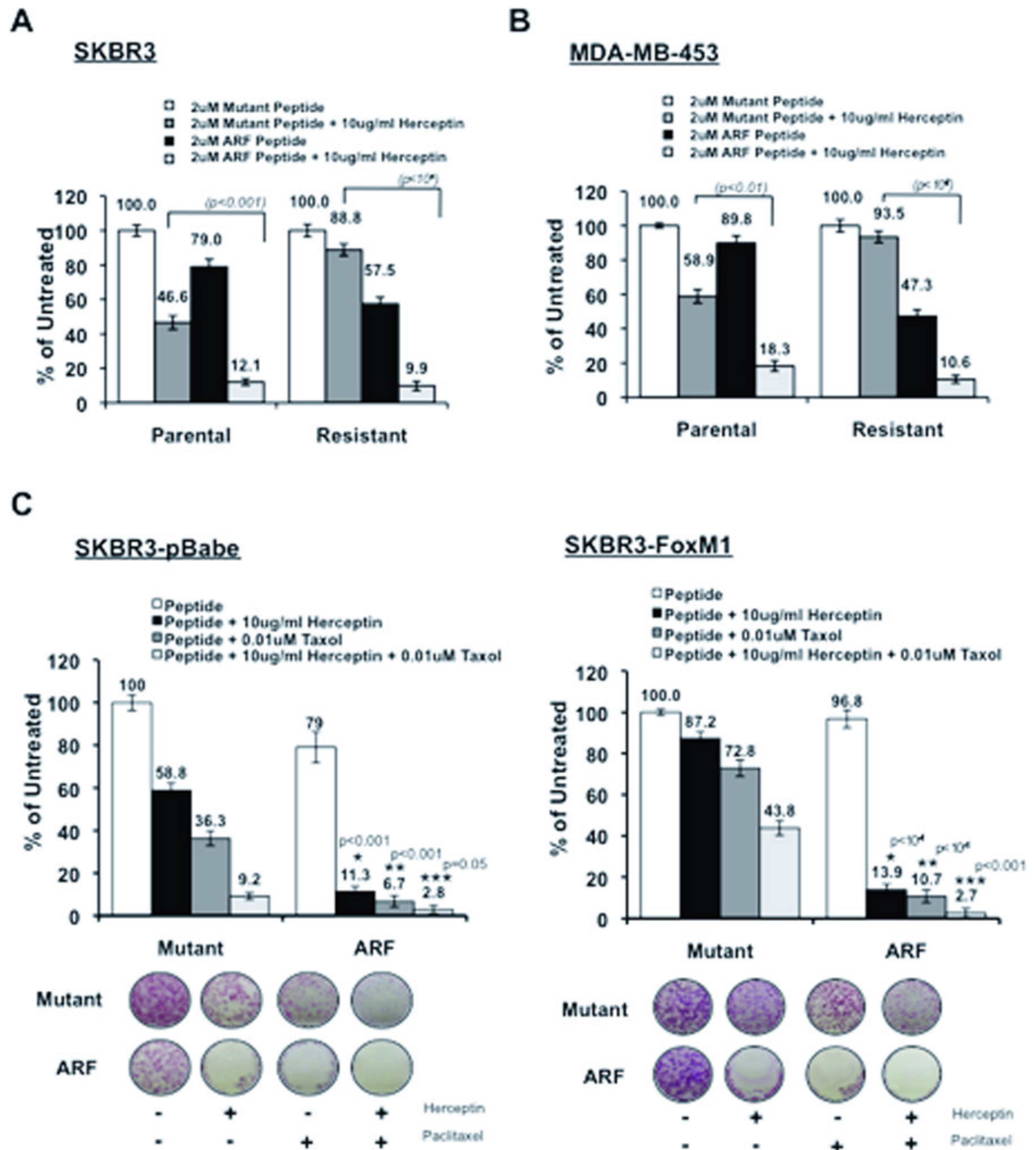

Figure 6. Targeting FoxM1 with ARF-peptide is sufficient to overcome inherent Herceptin resistance and to sensitize pBabe or FoxM1 overexpressing cells to treatment

(A) ARF-peptide or mutant peptide (2μM) was used to treat either parental or resistant SKBR3 and MDA-MB-453 cells. Cells were pretreated with peptide for 3 days then treated with both 10ug/ml Herceptin and peptide. Media was 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 three days. Wells receiving both Herceptin and Taxol were pre-treated for 72 hours with Herceptin before receiving indicated doses of Taxol. Quantification of triplicates is shown in the graph with representative images below.