

Cell Cycle, Cell Death, and Senescence**FOXM1 Confers Acquired Cisplatin Resistance in Breast Cancer Cells**

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Abstract

The transcription factor Forkhead box M1 (FOXO1) is a key regulator of cell proliferation and is overexpressed in many forms of primary cancers, leading to uncontrolled cell division and genomic instability. To address the role of FOXO1 in chemoresistance, we generated a cisplatin-resistant breast cancer cell line (MCF-7-CIS^R), which had an elevated level of FOXO1 protein and mRNA expression relative to the parental MCF-7 cells. A close correlation was observed between FOXO1 and the expression of its proposed downstream targets that are involved in DNA repair; breast cancer-associated gene 2 (BRCA2) and X-ray cross-complementing group 1 (XRCC1) were expressed at higher levels in the resistant cell lines compared with the sensitive MCF-7 cells. Moreover, cisplatin treatment induced DNA damage repair in MCF-7-CIS^R and not in MCF-7 cells. Furthermore, the expression of a constitutively active FOXO1 (Δ N-FOXO1) in MCF-7 cells alone was sufficient to confer cisplatin resistance. Crucially, the impairment of DNA damage repair pathways through the small interfering RNA knockdown inhibition of either FOXO1 or BRCA2/XRCC1 showed that only the silencing of FOXO1 could significantly reduce the rate of proliferation in response to cisplatin treatment in the resistant cells. This suggests that the targeting of FOXO1 is a viable strategy in circumventing acquired cisplatin resistance. Consistently, the FOXO1 inhibitor thiothrepton also showed efficacy in causing cell death and proliferative arrest in the cisplatin-resistant cells through the downregulation of FOXO1 expression. Taken together, we have identified a novel mechanism of acquired cisplatin resistance in breast cancer cells through the induction of FOXO1. *Mol Cancer Res*; 8(1); 24–34. ©2010 AACR.

Introduction

Platinum-based chemotherapeutics, such as *cis*-diamminedichloro-platinum (cisplatin) have long been established in the routine treatment of ovarian, testicular, and non-small cell lung cancer patients under clinical settings (1). Cisplatin treatment results in the formation of intrastrand and interstrand DNA adducts (2), triggering the nucleotide excision repair and homologous recombination pathways (3–5). Failure to activate or execute appropriate DNA repair leads to the accumulation of DNA strand breaks, and ultimately to cell death (6). Recent clinical data suggest an emerging role for platinum-based chemotherapy for advanced breast cancer patients. For example, three independent phase II clinical trials involving HER2-positive

or advanced metastatic breast cancer patients treated with a combination of Herceptin with cisplatin and docetaxol showed clinically significant improvement of survival rates (7–9). Furthermore, recent clinical trial data also suggest that triple-negative breast cancer patients who are estrogen receptor negative, progesterone receptor negative, and with low HER2 expression levels show better survival rates in response to cisplatin chemotherapeutic treatment (10). However, acquired cisplatin resistance is a major clinical obstacle for patients that relapse after initial favorable responses. Cisplatin resistance is a complex and multifaceted problem that involves multiple pathways including increased cisplatin efflux, inactivation of intracellular cisplatin, evasion of apoptotic pathways, replication checkpoint bypass, increased cell proliferation, and increased DNA damage repair (1, 11). Several targets have been implicated to have a role in breast cancer cisplatin resistance including amphiregulin (12), BCL2 (13), BCL2L12 (14), cyclin D1 (13), Siva-1 (15), and the miRNA regulator Dicer (16). However, a better understanding of the molecular mechanism underlying chemotherapeutic resistance is needed for the development of effective platinum-based therapeutic strategies for treatment of breast cancer. Forkhead box M1 (FOXO1) is a transcription factor that belongs to the wider forkhead transcription factor family (17) and is required for normal cell cycle execution during G₁-S (18, 19), G₂ and M phase transitions (20), apoptosis

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(21, 22), angiogenesis (23), metastasis (24), and DNA damage repair (25, 26). The overexpression of FOXM1 in primary breast cancer tissues has also been associated with breast cancer tumorigenesis (27) and poor prognosis (26, 28). Intriguingly, several lines of studies have indicated that in breast cancer patients, the loss of functional breast cancer-associated gene 2 (BRCA2) and X-ray cross-complementing group 1 (XRCC1) expression contributes to breast cancer development (29, 30), although this is also hypothesized to render the cells more susceptible to DNA damage and cytotoxic chemotherapies. Increasing evidence has emerged to show that regaining the expression of DNA repair genes such as BRCA1, BRCA2, and XRCC1 could lead to enhance cisplatin resistance in these cells (29, 31, 32). In the present study, we investigated the role of FOXM1 and its proposed downstream DNA damage repair targets BRCA2 and XRCC1 might play in conferring cisplatin resistance.

Materials and Methods

Cell Lines

MCF-10A and MCF-7 cells originated from the American Type Culture Collection and were acquired from the Cell Culture Service, Cancer Research UK, where they were tested and authenticated. These procedures include cross-species checks, DNA authentication, and quarantine. Cell lines used in the present study were in culture for <6 mo. MCF10-A were cultured in DMEM HAM-F12/1:1 mix (Sigma UK), supplemented with 5% (v/v) horse serum, 10 mg/mL insulin, 5 mg/mL hydrocortisol, 100 ng/mL cholera toxin and 20 ng/mL of epidermal growth factor, 100 μ M penicillin, and 100 μ g/mL streptomycin (Sigma). MCF-7 cells were cultured in DMEM (Sigma UK) supplemented with 10% (v/v) FCS, 2 mmol/L glutamine, 100 μ M penicillin, and 100 μ g/mL streptomycin at 37°C. The MCF-7- Δ N-FOXM1 cell line was established by cotransfecting MCF-7 cells with a cytomegalovirus-driven expression vector of Δ N-FOXM1 and pBABEpuro (Addgene) that contains a puromycin selection marker. Cells were then selected at 1.5 μ g/mL of puromycin (Autogen Bioclear Ltd) and were maintained at 0.75 μ g/mL of puromycin (Invitrogen). The MCF-7-CIS^R cell line is a cisplatin-resistant cell line derived from parental MCF-7 cells. MCF-7 were subjected to increasing concentrations of cisplatin (Onco-tain DBL, Leamington Spa) until the MCF-7 cells acquire resistance to 0.112 mol/L of cisplatin. MCF-7-CIS^R cell line was then maintained in 0.1 mol/L of cisplatin.

Western Blot Analysis and Antibodies

Cells were lysed and SDS-PAGE gel electrophoresis was done as previously described. The antibodies against FOXM1 (c-20), β -tubulin (H-235), CDC25B (C-20), and PLK-1 (F-8) were purchased from Santa Cruz Biotechnology (Autogen Bioclear), whereas FOXO3a (06-951) and BRCA2 (5.23) were from Upstate. Antibodies against phospho-Akt (Ser473), total Akt, and phospho-FOXO3a

(Thr32) were from Cell Signaling Technology, and XRCC1 (AHP832) was from AbD Serotec. Primary antibodies were detected using horseradish peroxidase-linked anti-mouse, anti-goat, or anti-rabbit conjugates as appropriate (DAKO), and visualized using the enhanced chemiluminescence detection system (Amersham Biosciences). Protein expression levels were quantified using the software ImageJ to detect intensity of the protein bands.

Real-time Quantitative PCR

Total RNA was extracted using the RNeasy Mini kit (Qiagen), and cDNA was prepared using the SuperScript III reverse transcriptase and random primers (Invitrogen). For real-time quantitative PCR (RTQ-PCR), 100 ng of cDNA were added to SYBER-Green Master Mix (Applied Biosystems) and run in 7900 HT Fast Real-time PCR System (Applied Biosystems). The cycling program was 95°C for 20 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Each sample was assayed in triplicates, and the results were normalized to the level of ribosomal protein L19 RNA. The forward and reverse primers used were as follows: FOXM1-F, 5'-TGCAGCTAGG-GATGTGAATCTTC-3', FOXM1-R, 3'-GGAGCC-CAGTCCATCAGAACT-5'; L19-F, 5'-GCGGAGAGGGTACAGCCAAT-3', L19-R, 3'-GCAGCCGGCGCAA-5'; BRCA2-F, 5'-GCTGGCTTCAACTCCAATAATATTC-3'; BRCA2-R GTCTACTATTGGGAACATTCCTTCTC; XRCC1-F, 5'AAAGGGAAGAGGAAGTTGGATTG; and XRCC1-R, 3'-GCAATTTAGGTCTCTTGGGAACA.

Sulforhodamine B Assay

Approximately 3,000 cells were seeded in each well of the 96-well plates. After culture, 100 μ L of trichloroacetic acid were added to each well and incubated for 1 h at 4°C. The plates were then washed with deionized water thrice, before incubation at room temperature for 1 h with 0.4% sulforhodamine B (SRB) in 1% acetic acid. The plates were then washed with deionized water and air dried. Tris base (10 mmol/L) was then added to the wells to solubilize the bound SRB dye, and the plates were then read at 492 nm using the Anthos 2001 plate read (Jencons Scientific Ltd).

Cell Cycle Analysis

Cell cycle analysis was done by propidium iodide staining, as previously described (33). The cell cycle profile was analyzed using Cell Diva software (Becton Dickinson UK Ltd).

Small Interfering RNA Transfection

MCF-7 and MCF-7-CIS^R cells were transfected with 100 nmol/L FOXM1 small interfering RNA (siRNA; Dharmacon) or 100 nmol/L XRCC1 or 100 nmol/L BRCA2 siRNA using oligofectamine (Invitrogen). Twenty-four hours after transfection, transfected cells were treated with 0.1 μ mol/L of cisplatin and viable cell counts were quantified by SRB assay.

Phospho- γ H2AX Immunofluorescent Staining and Quantification

MCF-7, MCF-7-CIS^R, and MCF-7- Δ N-FOXM1 cells were transfected with 100 nmol/L FOXM1 siRNA (Dharmacon), 100 nmol/L XRCC1, or 100 nmol/L BRCA2 siRNA by oligofectamine (Invitrogen). After 24 h, cells were then treated with 0.1 μ mol/L of cisplatin for 6 h (34, 35). Subsequently, cells were then subjected to anti- γ H2AX (Ser139) staining. Briefly, cells were fixed with 4% paraformaldehyde (Sigma), permeabilized with 0.1% Triton X-100 in 10% FCS for 10 min. Samples were then blocked with 5% goat serum in 10% FCS for 30 min and then incubated overnight with the primary rabbit anti- γ H2AX (Ser139; 1:120; Cell Signaling). Following washes with PBS, secondary goat anti-rabbit IgG-FITC (1:500; Invitrogen) was added to the samples for an hour. Cells were counterstained with 4',6'-diamidino-2-phenylindole (DAPI) before mounting. Images were captured and quantified using the Zeiss Axiovert 100 confocal laser scanning microscope and software Zeiss LSM 500 (Zeiss Ltd). DNA damage foci were selected based on the following parameters—DNA foci: min width, 0.05 μ m; max width, 8 μ m; intensity above background: 200 gray levels; nuclei: min width, 10 μ m; max width, 20 μ m; intensity above background, 400 gray levels.

Results

FOXM1 and Targets BRCA2 and XRCC1 Are Upregulated in Cisplatin-Resistant MCF-7 Cells

Previously, it has been shown that the overexpression of FOXM1 is indicative of poor prognosis in breast cancer patients (26, 28). FOXM1 has also been reported to regulate the expression of the DNA damage repair genes, *BRCA2* and *XRCC1* (25). Hitherto, the role of FOXM1 in cisplatin resistance through the repair of cisplatin DNA adducts resistance has not been established. In the first instance, we established a new cisplatin resistance cell line, MCF7CIS^R, through repeated exposures of MCF7 cells to successive rounds of cisplatin until resistance up to 1.2 μ mol/L was reached as indicated by SRB proliferation assay (Fig. 1A). Subsequent Western blot analysis reveals that MCF7 cells expressed a higher level of FOXM1 relative to the untransformed MCF-10A breast epithelial cells. Interestingly, MCF-7-CIS^R showed an even higher FOXM1 level compared with the parental MCF-7 cells (Fig. 1B). Furthermore, MCF-7-CIS^R also had higher levels of DNA repair proteins BRCA2 and XRCC1. Relative FOXM1 protein expression level was on average 2.5-fold higher in MCF-7-CIS^R cells compared with MCF-7 cells (Fig. 1C). The results were mirrored at mRNA level, where MCF-7-CIS^R had a 2-fold increase (Fig. 1D).

FOXM1 and DNA Repair Are Upregulated in the Resistant MCF-7-CIS^R Cells but not in MCF-7 Cells

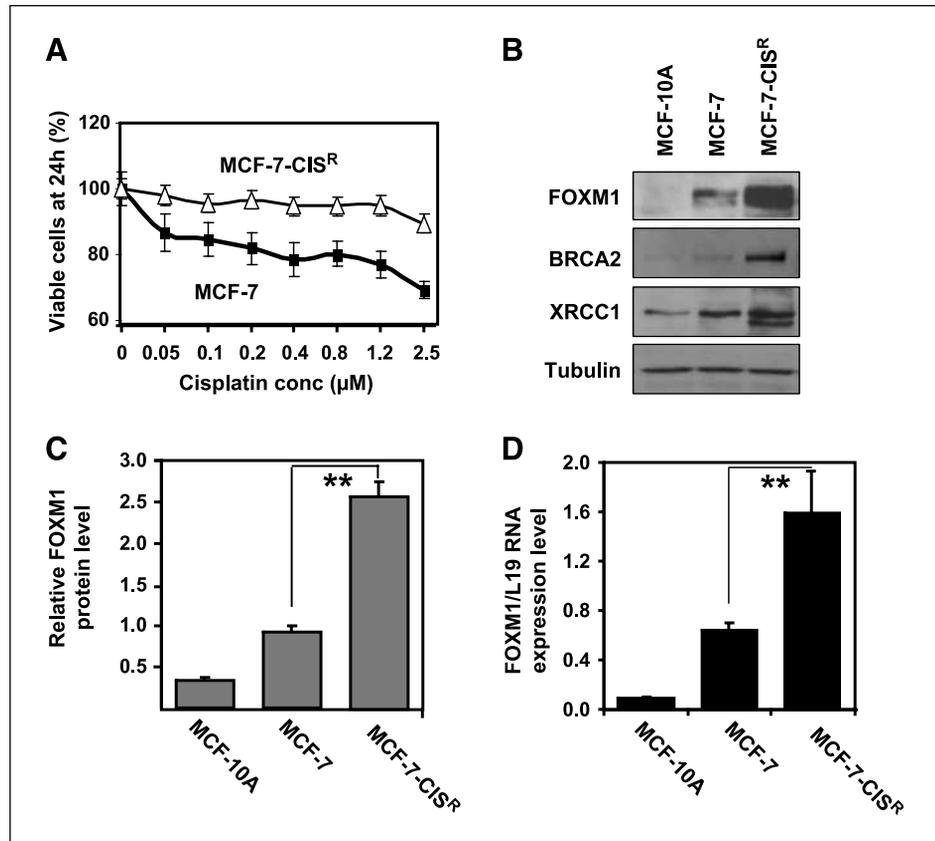
Next, we sought to determine molecular mechanism that confers acquired cisplatin resistance in breast cancer cell lines. Cell cycle analysis showed that following cisplatin

treatment (100 nmol/L; 0-72 hours) high numbers of MCF-7 cells contained sub-G₁ DNA content, indicative of DNA fragmentation and cell death, whereas no significant changes in sub-G₁ population were observed for MCF-7-CIS^R cells (Fig. 2A). A series of short time courses revealed that no significant changes in FOXM1, BRCA2, and XRCC1 levels occurred before 24 h of cisplatin treatment (Supplementary Fig. S1). However, MCF-7 cells treated with cisplatin (0-72 hours) showed a decrease in FOXM1 expression, and that of its downstream targets CDC25B and PLK, in addition to the DNA repair proteins XRCC1 and BRCA2 (Fig. 2B). In contrast, FOXM1 and BRCA2 expression levels were further increased following cisplatin treatment in MCF-7-CIS^R cells, whereas CDC25B, PLK, and XRCC1 levels remained relatively constant. Consistently, RTQ-PCR analysis revealed that in MCF-7 cells FOXM1 mRNA level decreased by 50% at 72 hours, whereas FOXM1 transcript level increased by 2-fold in MCF-7-CIS^R cells (Fig. 2C), suggesting that the ability to maintain elevated FOXM1 expression in acquired cisplatin-resistant breast cancer cell lines is mediated at least partially at transcriptional level. Interestingly, although BRCA2 mRNA levels closely mirrored FOXM1 mRNA levels, XRCC1 mRNA levels did not change significantly in both MCF-7 and MCF-7-CIS^R cells, this suggests that an increase in FOXM1 expression level could stabilize XRCC1 expression indirectly through its other downstream targets. We next performed the immunostaining of phosphorylated histone H2AX loci to assay for DNA damage in response to cisplatin in the drug-sensitive and drug-resistant MCF-7 cells. γ H2AX staining was examined at the earlier 6-hour time point to avoid cell loss due to cell cycle arrest, and cell death as a result of DNA damage induced by cisplatin (34, 35). Quantification of γ H2AX staining (Fig. 3A) showed that MCF-7 cells had significantly higher levels of DNA damage after cisplatin treatment compared with MCF-7-CIS^R cells, indicating that MCF-7-CIS^R are more efficient than MCF-7 cells in the repair of damaged DNA induced by cisplatin, which correlates with a much lower amount of apoptosis.

Overexpression of Δ N-FOXM1 Is Sufficient to Confer Cisplatin Resistance by Enhancing DNA Repair Pathways in Reducing DNA Damage

To prove definitively that FOXM1 is important in acquired cisplatin resistance, we used the MCF-7- Δ N-FOXM1 cell line previously described, which overexpresses a constitutively active form FOXM1 (33). Consistently, the expression of Δ N-FOXM1 was accompanied by slightly higher levels of BRCA2 and XRCC1 at both the protein and mRNA level (Fig. 3B and C). We next examined the level of cisplatin-induced (0.1 μ mol/L) DNA damage in MCF-7 and MCF-7- Δ N-FOXM1 cells. A 3.5-fold increase in DNA damage was observed in MCF-7 cells, whereas no significant increase in DNA damage was observed following cisplatin treatment in MCF-7- Δ N-FOXM1 cells (Fig. 3D). Therefore, MCF-7- Δ N-FOXM1 showed an enhanced ability for DNA repair. Significantly,

FIGURE 1. Cisplatin-resistant cell line shows elevated FOXM1 protein and mRNA expression levels. **A.** MCF-7 and MCF-7-CIS^R cells were treated with increasing concentrations of cisplatin and their rates of proliferation were measured by SRB assay. **B.** Western blot analysis determining the relative protein expression levels of FOXM1, BRCA2, and XRCC1 in MCF-10A, MCF-7, and MCF-7-CIS^R cells. **C.** FOXM1 protein expression level was quantified using ImageJ normalized against tubulin levels. **D.** FOXM1 mRNA transcript levels were determined by RTQ-PCR analysis. Columns, mean derived from at least three independent experiments; bars, SD. Statistical analysis was done using Student's *t* tests. **, *P* ≤ 0.01, significant.



SRB assay also revealed that the overexpression of Δ N-FOXM1 alone was sufficient to confer resistance to MCF-7 (Supplementary Fig. S2; Figs. 2 and 4A). Furthermore, the expression levels of FOXM1, BRCA2, and XRCC1 were maintained at both the protein and mRNA levels in the MCF-7- Δ N-FOXM1 cell line following cisplatin treatment (0.1 μ mol/L; 0-72 hours), whereas in MCF-7 cells, FOXM1 expression decreased rapidly after 24 hours of cisplatin treatment (Fig. 4B and C). These data suggest that the introduction of constitutive active Δ N-FOXM1 can protect breast cancer cells against cell death by enhancing cisplatin-induced DNA damage repair.

FOXM1 Can Promote Cisplatin Resistance through DNA Damage Repair Independent of BRCA2 and XRCC1 in MCF-7-CIS^R Cells

Having identified FOXM1 as a mediator of cisplatin resistance in breast cancer cells, we next examined whether BRCA2 and XRCC1 are the downstream targets of FOXM1 required in conferring cisplatin resistance in breast cancer cells. Surprisingly, the siRNA-mediated knockdown of FOXM1 did not result in a downregulation of BRCA2 and XRCC1 expression in either the MCF-7 or MCF-7-CIS^R cell lines at the protein or mRNA level (Fig. 5A and B), suggesting that FOXM1 is not the primary or sole regulator of BRCA2 and XRCC1 expression in these cells. The requirement of FOXM1, BRCA2, and XRCC1 expression

for MCF-7-CIS^R cisplatin resistance was examined using siRNA-mediated knockdown of these genes. The knockdown of FOXM1, BRCA2, and BRCA2/XRCC1 in MCF-7-CIS^R cells increased the amount of DNA damage sustained by 1.5- to 2 to-fold (Fig. 5C). Interestingly, following knockdown of FOXM1, the expression levels of BRCA2 and XRCC1 were maintained, and yet an increase in DNA damage was observed, suggesting that other FOXM1 downstream targets are involved. Moreover, SRB proliferation assay revealed that only the knockdown of FOXM1 was potent at resensitizing MCF-7-CIS^R cells to cisplatin treatment and not the knockdown of BRCA2, XRCC1, or in combination (Fig. 5D). This suggests that other unknown DNA repair targets or proliferative targets of FOXM1 could overcome the loss of one or two individual DNA repair. Thus, the inactivation of FOXM1 is essential for reversing cisplatin resistance, and targeting FOXM1 could potentially be a better therapeutic strategy for overcoming cisplatin resistance, rather than just through the inactivation of DNA repair pathways.

Thiostrepton Can Overcome Cisplatin Resistance in Breast Cancer Cells through the Downregulation of FOXM1

To test if FOXM1 inactivation is a viable strategy for overcoming cisplatin resistance, we studied the effects of MCF-7-CIS^R cells treated with the thiazole antibiotic

thiostrepton, which has previously been showed to inhibit FOXM1 expression (33), alone (10 $\mu\text{mol/L}$), and in combination with cisplatin (100 nmol/L). SRB proliferative assays indicated that MCF-7-CIS^R cells treated with thiostrepton or in combination with cisplatin showed a significant decrease in rate of cell proliferation (Fig. 6A). Cell cycle analysis revealed that MCF-7-CIS^R cells treated with thiostrepton alone showed an 18.7% cell death rate by 72 hours, whereas in combination, cisplatin and thiostrepton showed synergy, exhibiting a cell death rate of 64.1% at 72 hours in this experiment (Fig. 6B). In MCF-7-CIS^R cells treated with thiostrepton, or thiostrepton and cisplatin, the downregulation of FOXM1 and its downstream targets

CDC25B and PLK occurred at 48 and 24 hours following treatment, respectively (Fig. 6C). The shorter time needed for FOXM1 downregulation in the cotreated cells may reflect the higher levels of cell death observed when both drugs were administered together. In conclusion, inhibition of FOXM1 is able to circumvent cisplatin resistance in breast cancer cells.

Discussion

In the present study, we have shown for the first time that FOXM1 possesses a crucial role in cisplatin resistance in breast cancer cells through enhancing DNA damage repair

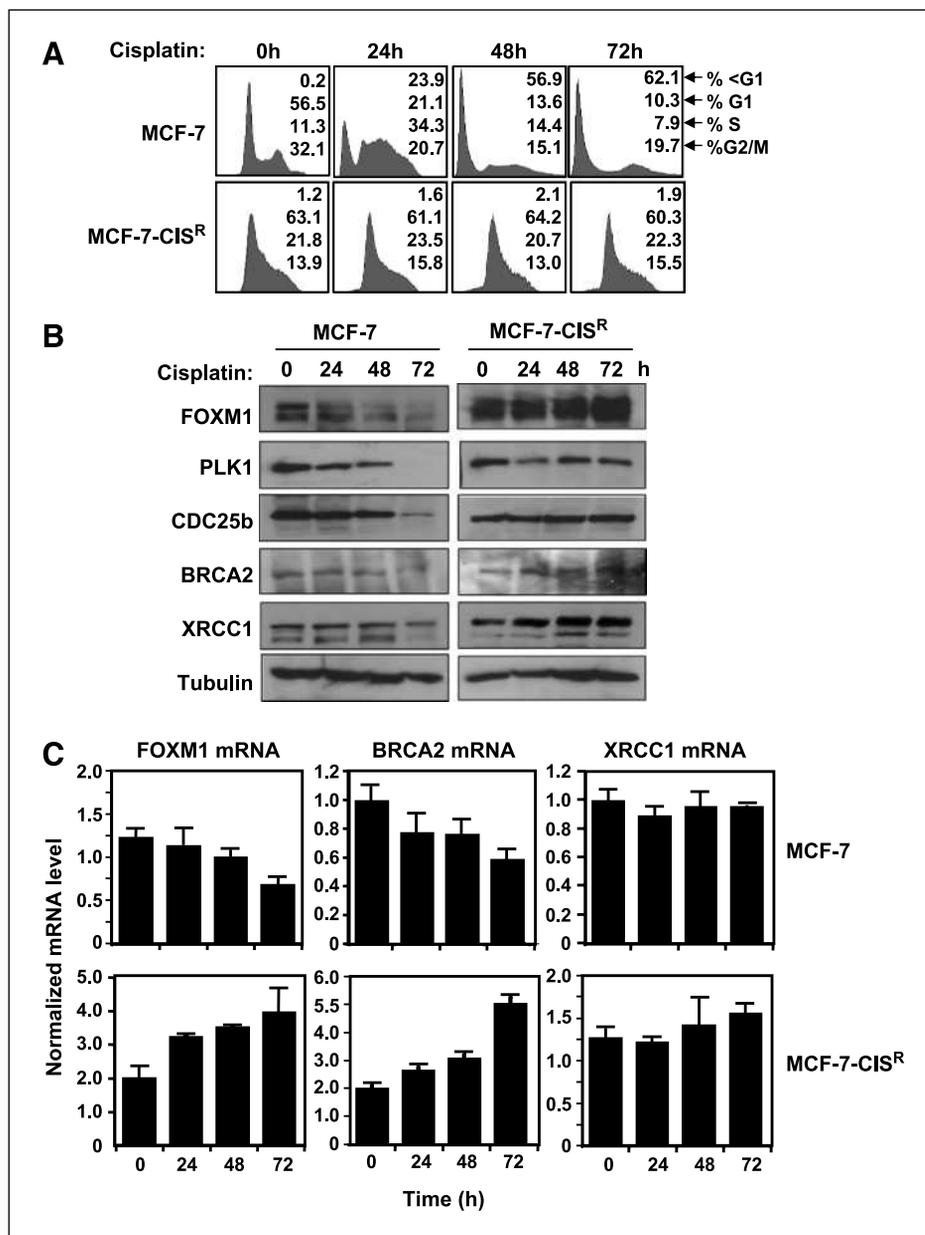


FIGURE 2. Elevated levels of FOXM1 correlate with enhanced DNA damage repair in MCF-7-CIS^R cells. **A.** MCF-7 and MCF-7-CIS^R cells were treated with 0.1 $\mu\text{mol/L}$ of cisplatin for 0 to 72 h, and fluorescence-activated cell sorting analysis was done after propidium iodide staining. The percentage of cells in each phase of the cell cycle (sub-G₁, G₁, S, and G₂-M) is indicated. Representative data from three independent experiments are shown. **B.** MCF-7 and MCF-7-CIS^R cells were treated with 0.1 $\mu\text{mol/L}$ of cisplatin and Western blot analysis was done to determine the protein expression levels of FOXM1, CDC25B, PLK1, BRCA2, XRCC1, and β -tubulin. **C.** FOXM1, BRCA2, and XRCC1 mRNA transcript levels were determined by RTQ-PCR and normalized to L19 RNA expression. Columns, mean of three independent experiments in triplicate; bars, SD.

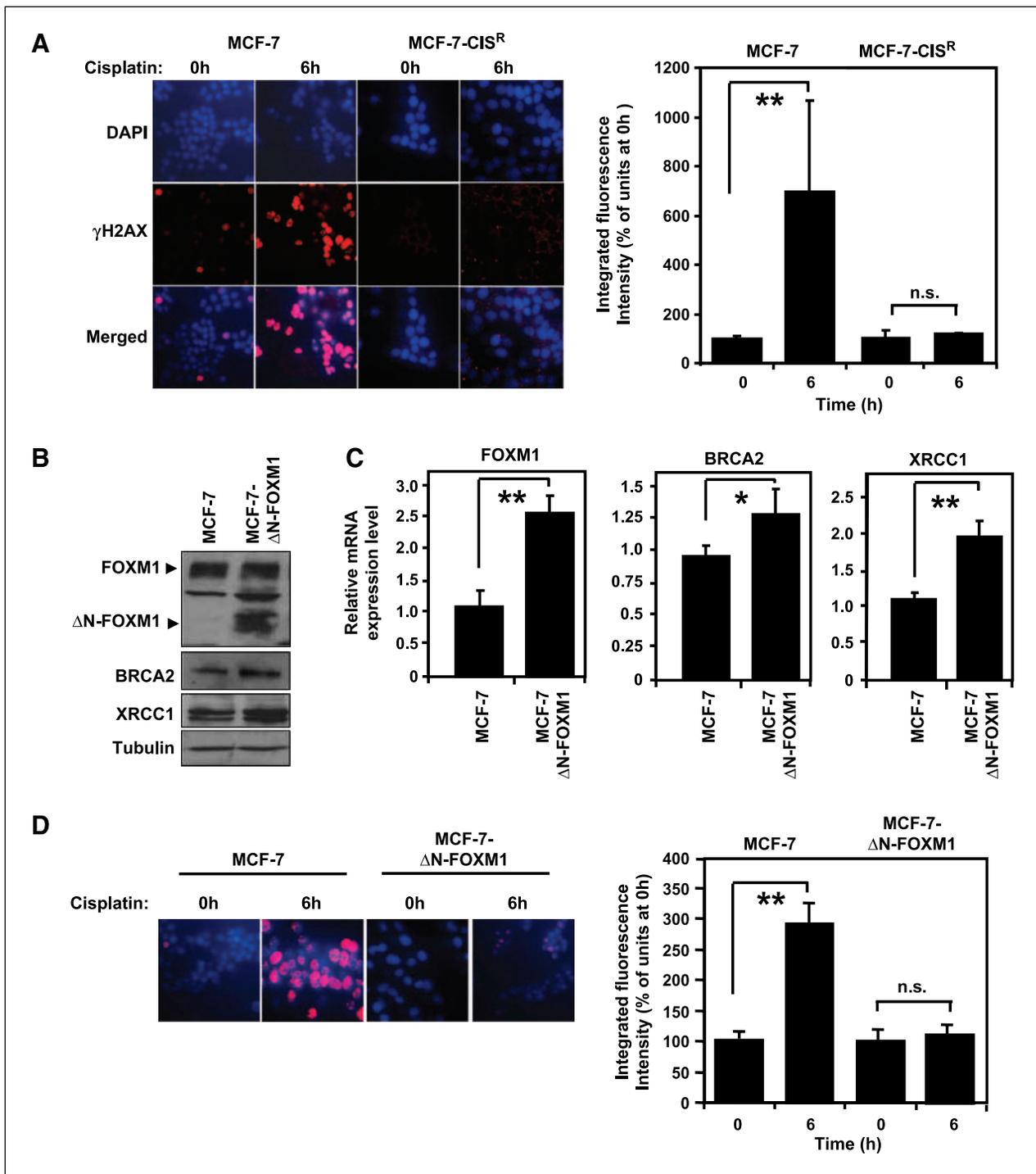


FIGURE 3. Overexpression of Δ N-FOXM1 is sufficient to confer cisplatin resistance by enhancing DNA repair pathways in reducing DNA damage. **A.** MCF-7 and MCF-7-CIS^R cells were treated with 0.1 μ mol/L of cisplatin for 0 or 6 h and stained with γ H2AX antibodies and DAPI. Images were visualized by confocal microscopy, and the average integrated fluorescence intensity was quantified by Zeiss Axiovert 100 confocal laser scanning microscope using Zeiss LSM 500 software. Original magnification, $\times 40$. The relative expression levels of FOXM1, BRCA2, and XRCC1 in MCF-7 and MCF-7- Δ N-FOXM1 were determined by Western blotting (**B**) and RTQ-PCR analysis (**C**), respectively. **D.** MCF-7 and MCF-7- Δ N-FOXM1 cells were treated with 0.1 μ mol/L of cisplatin for 0 or 6 h and were stained with γ H2AX antibodies and DAPI. Images were visualized by confocal microscopy; the average integrated fluorescence intensities are shown. Original magnification, $\times 40$. Columns, mean of three independent experiments in triplicates; bars, SD. Statistical analyses were done using Student's *t* tests. *, $P \leq 0.05$; **, $P \leq 0.01$ significant.

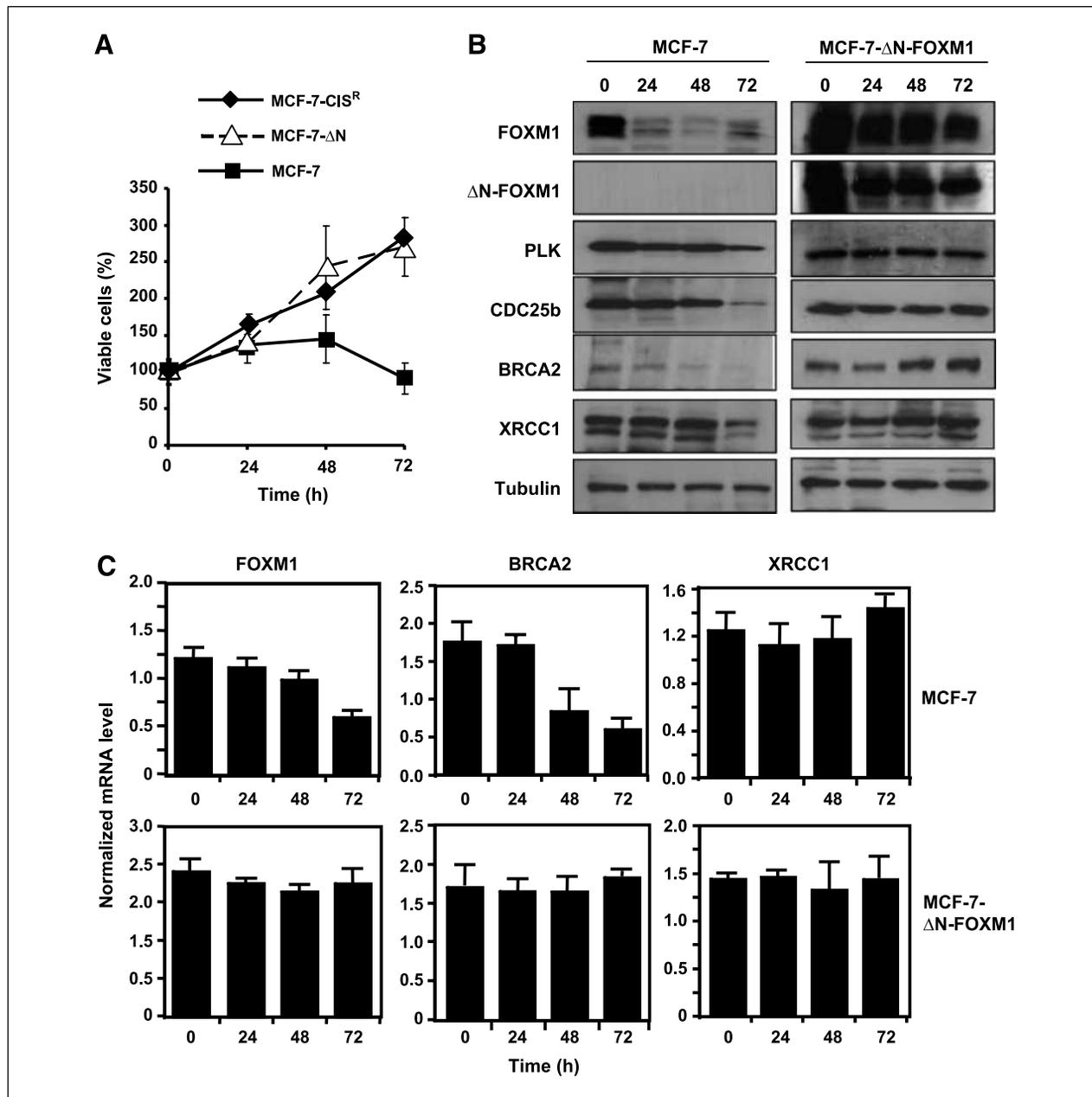


FIGURE 4. Overexpression of ΔN -FOXM1 is sufficient to confer cisplatin resistance by enhancing DNA repair pathways in reducing DNA damage. MCF-7 and MCF-7- ΔN -FOXM1 cells were treated with 0.1 $\mu\text{mol/L}$ of cisplatin for 0, 24, 48, and 72 h. **A**, Cell proliferation rates were determined by SRB assays, and MCF-7-CIS^R cells were also included as a control for cisplatin-resistant cells. The relative expression levels of FOXM1, BRCA2, and XRCC1 in MCF-7 and MCF-7- ΔN -FOXM1 cells were determined by Western blotting (**B**) and RTQ-PCR analysis (**C**). The expression levels of CDC25B and PLK1 proteins were also studied for comparison. RTQ-PCR results are shown are from three independent experiments in triplicates. Columns, mean; bars, SD.

pathways. Several observations suggest that FOXM1 expression is an important determinant of cisplatin sensitivity and resistance. First, the basal levels of FOXM1 protein and mRNA were higher in the cisplatin-resistant MCF-7-CIS^R cells relative to the parental MCF-7 cells. Following cisplatin treatment, FOXM1 was downregulated in the sensitive MCF-7 cells, whereas in the resistant MCF-7-CIS^R cells,

there was an upregulating of both FOXM1 mRNA and the protein expression levels. Moreover, expression of the constitutively active ΔN -FOXM1 was sufficient to confer resistance to the cisplatin-sensitive MCF-7 breast cancer cells, whereas the depletion of FOXM1 through siRNA knockdown reversed cisplatin resistance in MCF-7-CIS^R breast cancer cells.

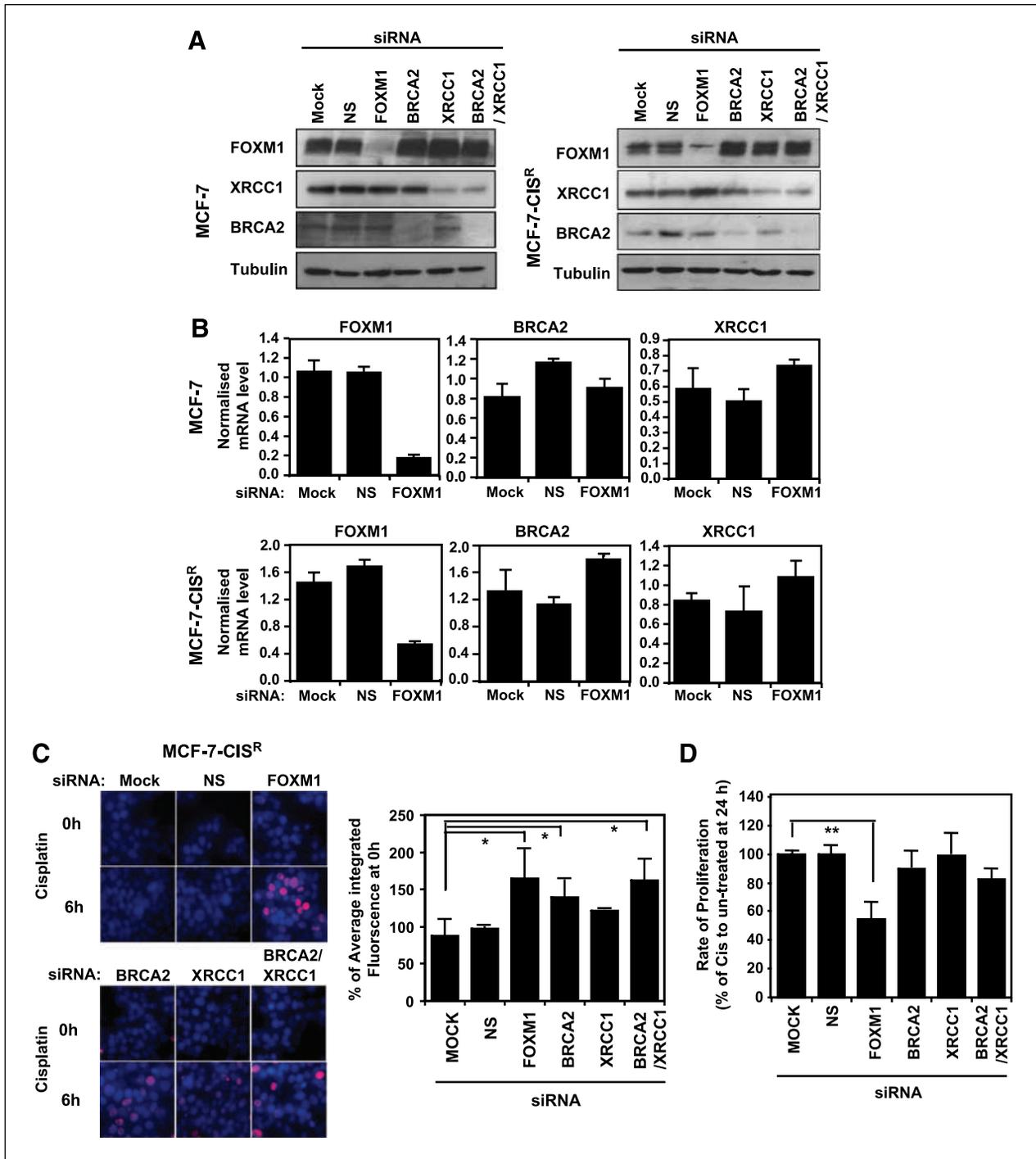


FIGURE 5. BRCA2 and XRCC1 are not the sole downstream targets of FOXM1 involved in cisplatin resistance. MCF-7 and MCF-7-CIS^R cells were either untransfected (*mock*), or transfected with nonspecific (*NS*) siRNA (100 nmol/L) or siRNA smart pool against FOXM1 (100 nmol/L), BRCA2 (100 nmol/L), XRCC1 (100 nmol/L), or BRCA2 plus XRCC1 (100 nmol/L) for 24 h. **A.** The expression levels of FOXM1, BRCA2, and XRCC1 in MCF-7 and MCF-7-CIS^R cells were determined by Western blotting. **B.** RTQ-PCR analysis was done to determine the relative FOXM1, BRCA2, and XRCC1 mRNA transcript levels. **C.** MCF-7-CIS^R cells were then treated with 0.1 μ mol/L of cisplatin for either 0 or 6 h, and stained with γ H2AX antibodies and DAPI. Images were visualized by confocal microscopy and the average integrated fluorescence intensity is shown. Original magnification, $\times 40$. **D.** SRB assay was done to gauge the changes in percentage in cell proliferation in the presence and absence of cisplatin treatment in MCF-7-CIS^R cells with different siRNA knockdown conditions. The cell proliferation results shown compared the proliferative rates of cisplatin-treated cells with the untreated cells of a given siRNA transfection. Data shown were derived from at least three independent experiments. Columns, mean; bars, SD). Statistical analysis was done using Student's *t* tests. *, $P \leq 0.05$; **, $P \leq 0.01$, significant.

FOXM1 has previously been reported to regulate the expression of the DNA repair genes *BRCA2* and *XRCC1* (25). However, despite the fact that *BRCA2* and *XRCC1* levels are elevated in the MCF-7-CIS^R- and MCF-7- Δ N-FOXM1-expressing cells, evidence suggests that FOXM1 is not the sole regulator of *BRCA2* and *XRCC1*. For instance, both *BRCA2* and *XRCC1* expression was not downregulated on FOXM1 silencing in both MCF-7 and MCF-7-CIS^R breast cancer cells. Additionally, we have cloned a *BRCA2* gene promoter whose activity is repressible by cisplatin but is not responsive to FOXM1 transactivation (Supplementary Fig. S3). This promoter is different from a previously published *BRCA2* promoter

(25), which locates the *BRCA2* gene further downstream (Supplementary Fig. S3). Furthermore, despite the fact the expression levels of *BRCA2* and *XRCC1* were higher in the MCF-7- Δ N-FOXM1 cells compared with the parental cells, transient transfection of MCF-7 with Δ N-FOXM1 failed to upregulate either *BRCA2* or *XRCC1* expression (Supplementary Fig. S4). All the supplementary evidence implies that additional regulators are needed, although FOXM1 plays a part in their activation.

Furthermore, it was also interesting to observe that although both *BRCA2* and *XRCC1* knockdown sensitized MCF-7-CIS^R cells, the elevated amount of cisplatin-induced DNA damage and the SRB proliferation assay also

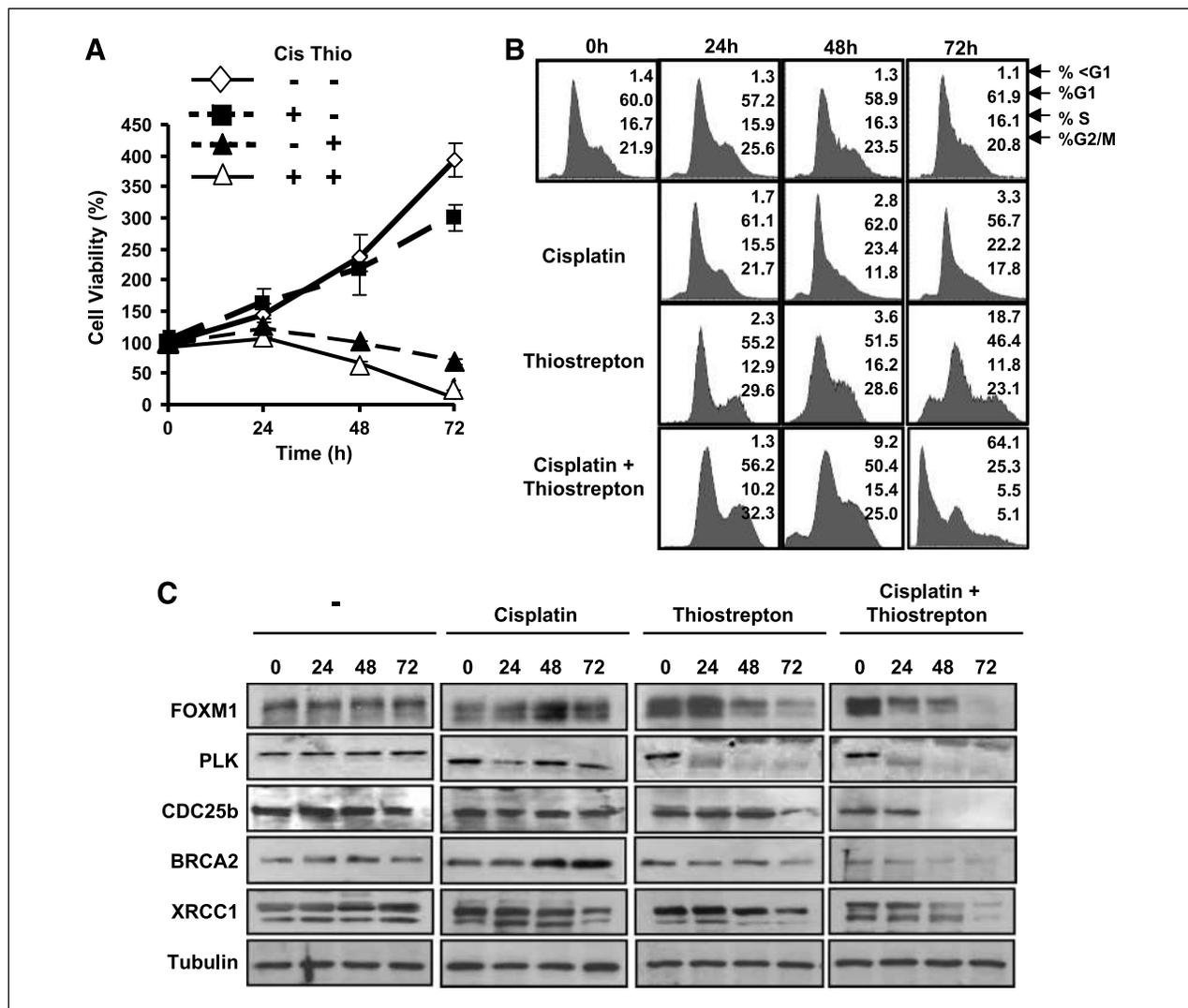


FIGURE 6. Thiostrepton can overcome cisplatin resistance in MCF-7-CIS^R breast cancer cells. MCF-7-CIS^R cells were treated with DMSO (vehicle control), 0.1 μ M cisplatin, 10 μ M thiostrepton, or a combination of 0.1 μ M cisplatin and 10 μ M thiostrepton for 72 h. **A.** SRB proliferation assays were performed on these cells and the percentage of viable cells at each time point is shown. **B.** Cells were fixed at 0, 24, 48, and 72 h after treatment, and cell cycle phase distribution was analyzed by flow cytometry after propidium iodide staining. Percentage of cells in each phase of the cell cycle (sub-G₁, G₁, S, and G₂-M) is indicated. Representative data from three independent experiments are shown. **C.** Cell lysates were prepared at the times indicated, and the expression of FOXM1, CDC25B, XRCC1, BRCA2, and β -tubulin were analyzed by Western blotting.

revealed that although BRCA2 and XRCC1 knockout did not show an appreciable antiproliferative effect, the knock-down FOXM1 significantly reduced the proliferative rate of MCF-7-CIS^R cells in response to cisplatin. These findings also suggest that besides DNA repair, other possible roles for FOXM1, such as the promotion of cell cycle progression or inhibition of cell cycle checkpoints and apoptosis, may also contribute to cisplatin resistance.

These observations may have implications in the development of a treatment regimen for cisplatin-resistant patients, suggesting it would be more efficient to target a “master” oncogene such as FOXM1, rather than targeting a subset of DNA repair machinery, where potential compensatory mechanisms could be present to hamper the treatment. Moreover, the inhibition of FOXM1 and co-treatment with DNA-damaging agents may be hypothesized to enhance therapeutic response. To test this hypothesis, we used the specific FOXM1 inhibitor thiostrepton (33). Our results indicate that thiostrepton synergized with cisplatin to reverse acquired cisplatin resistance in breast cancer cells, and caused a substantial increase in the amount of cisplatin-induced cell death. This probably reflects the role of FOXM1 in several aspects of physiologic processes including proliferation, cell cycle transition, and DNA repair, and thus, a reduction in the ability of cisplatin-resistant cells evade the cytotoxic effects of cisplatin. As a consequence, we have also shown for the first time that the use of a FOXM1 inhibitor such as thiostrepton, in conjunction with chemotherapy, could be provide a mechanism for reversing the phenomenon of wide-spread chemoresistance in breast cancer patients. Moreover, the fact that thiostrepton exclusively targets cancer cells and not nonmalignant cells can further enhance the specificity of cisplatin in combinatorial treatments (33).

The mechanism by which FOXM1 activity and/or expression is upregulated in MCF-7-CIS^R cells requires further investigation. Treatment with DNA-damaging agents, including γ -irradiation, etoposide, and UV, has been reported to increase CHK2-induced phosphorylation of FOXM1, potentially stabilizing the protein and leading to the transcriptional upregulation of downstream DNA repair genes (25). However, several previous studies suggest that it is unlikely that CHK2 phosphorylation of FOXM1 is relevant in acquired cisplatin-resistant breast cancer cells. For example, clinical assessment of a selective CHK2 inhibitor VRX0466617 has shown that it can sensitize can-

cer cells to apoptosis following exposure to ionizing radiation but not cisplatin (36). Moreover, loss or down-regulation of CHK2 expression has been shown to contribute to cisplatin resistance in ovarian cancer and lung cancer (37, 38). Thus, it is possible that additional mechanisms are responsible for the enhanced levels of FOXM1 in MCF7CIS^R cells. Indeed, our studies have shown that resistant cells upregulated FOXM1 expression at the mRNA level in response to cisplatin. This adds a new dimension to the FOXM1 signaling network whereby following DNA damage FOXM1 activity and expression may be modulated differentially depending on cellular background. In consequence, it will also be interesting to further unravel the exact molecular mechanisms by which FOXM1 mRNA expression levels are regulated in response to cisplatin.

In summary, FOXM1 is a critical mediator of cisplatin sensitivity and resistance in breast cancer cells. Therefore, FOXM1 can be a useful marker for predicting and monitoring cisplatin response. Through the inhibition of FOXM1, it is possible that acquired cisplatin resistance can be reversed, and FOXM1 could be a new therapeutic target in acquired cisplatin-resistant breast cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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