ATM and p53 Regulate FOXM1 Expression via E2F in Breast Cancer Epirubicin Treatment and Resistance

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Abstract

In this report, we investigated the role and regulation of forkhead box M1 (FOXM1) in breast cancer and epirubicin resistance. We generated epirubicin-resistant MCF-7 breast carcinoma (MCF-7-EPIR) cells and found FOXM1 protein levels to be higher in MCF-7-EPIR than in MCF-7 cells and that FOXM1 expression is downregulated by epirubicin in MCF-7 but not in MCF-7-EPIR cells. We also established that there is a loss of p53 function in MCF-7-EPIR cells and that epirubicin represses FOXM1 expression at transcription and gene promoter levels through activation of p53 and repression of E2F activity in MCF-7 cells. Using p53−/− mouse embryo fibroblasts, we showed that p53 is important for epirubicin sensitivity. Moreover, transient promoter transfection assays showed that epirubicin and its cellular effectors p53 and E2F1 modulate FOXM1 transcription through an E2F-binding site located within the proximal promoter region. Chromatin immunoprecipitation analysis also revealed that epirubicin treatment increases pRB (retinoblastoma protein) and decreases E2F1 recruitment to the FOXM1 promoter region containing the E2F site. We also found ataxia-telangiectasia mutated (ATM) protein and mRNA to be overexpressed in the resistant MCF-7-EPIR cells compared with MCF-7 cells and that epirubicin could activate ATM to promote E2F activity and FOXM1 expression. Furthermore, inhibition of ATM in U2OS cells with caffeine or depletion of ATM in MCF-7-EPIR with short interfering RNAs can resensitize these resistant cells to epirubicin, resulting in downregulation of E2F1 and FOXM1 expression and cell death. In summary, our data show that ATM and p53 coordinately regulate FOXM1 via E2F to modulate epirubicin response and resistance in breast cancer.

Introduction

Breast cancer is the most common cancer in women and one of the most prevalent causes of women cancer death worldwide (1, 2). Endocrine agents, including antiestrogens and aromatase inhibitor, have become the primary adjuvant treatment of breast cancer (3). However, in addition to endocrine agents, cytotoxic chemotherapeutic drugs taxanes and anthracyclines have been used more frequently in the neoadjuvant and adjuvant settings to reduce tumor size before surgery and to reduce the chance of cancer relapse or metastasis, respectively (4, 5). Moreover, cytotoxic chemotherapy is used to treat breast cancer patients who are resistant to or not suitable for hormonal therapy and it is particularly important in the treatment of advanced or metastatic solid cancers, as it is sometimes the sole treatment option (6, 7).

Anthracyclines, including doxorubicin (also called Adriamycin) and epirubicin (Supplementary Fig. S1), are a group of Streptomyces peucetius–derived antibiotics commonly used in cancer chemotherapy. These compounds have been shown to be effective in the treatment of a broad spectrum of cancers such as breast, lung, and ovary carcinomas as well as leukemia (8, 9). Despite being some of the most effective and widely used anticancer drugs in the clinic, anthracycline treatment will eventually fail and patients will relapse because of the development of acquired drug resistance (10–12). The exact mechanism of action of anthracyclines is still not completely understood but is likely to involve inducing DNA intercalation and damage (13, 14). For DNA-targeting anticancer drugs, such as anthracyclines, enhanced DNA repair can confer resistance and hamper the efficacy of the chemotherapeutic drugs. Consistently, DNA repair gene network signature has been found to be associated with anthracycline resistance.
response in triple-negative metastatic breast cancer (15). A better understanding of the molecular mechanisms of anthracycline action and resistance will be required both for the development of novel strategies for the treatment of advanced or metastatic breast cancer and for overcoming the resistance to anthracyclines.

Forkhead box M1 (FOXM1), also previously called HNF-3, HFH-11, WIN, MPP2, or Trident, is a transcription factor of the forkhead box (FOX) protein superfamily characterized by a conserved winged helix DNA–binding domain (16). FOXM1 is required for normal G1–S, G2, and M phase cell-cycle transitions. Besides its involvement in cell-cycle transitions, FOXM1 is also a key regulator of mitotic spindle integrity (17), angiogenesis (18), metastasis (18, 19), apoptosis (16, 19), DNA damage repair (20, 21), and tissue regeneration (22). FOXM1 is frequently overexpressed in a diversity of human cancers, including colorectal (23), lung (24), prostate (25), liver (26), and breast (27) carcinomas. In agreement, a microarray study also found FOXM1 expression to be elevated in carcinomas of the prostate, lung, ovary, colon, pancreas, stomach, bladder, liver, kidney, and breast, compared with their normal counterparts. Besides its potential involvement in tumorigenesis, FOXM1 dysregulation has also been implicated in drug resistance in breast cancer. For example, FOXM1 dysregulation has been shown to be involved in the development of cisplatin resistance in breast cancer (20). Accordingly, FOXM1 overexpression has been shown to confer resistance to the humanized anti-HER2 monoclonal antibody herceptin (also called trastuzumab) and microtubule-stabilizing drug taxane paclitaxel (taxol; ref. 28). In addition, FOXM1 has been found to be a transcriptional target of ERα (estrogen receptor alpha) and play a key role in breast cancer endocrine therapy resistance (29). In this report, we investigated the expression of FOXM1 and its regulation in response to epirubicin treatment in drug-sensitive and -resistant MCF-7 breast carcinoma cell lines.

Materials and Methods

Cell culture and transfections

The human breast carcinoma cell lines MCF-7 and U2OS cell lines originated from the American Type Culture Collection and were acquired from Cancer Research UK, where they were tested and authenticated. Knockout mouse embryo fibroblasts (MEF) for p21Cip1 and p53 have previously been described (refs. 30, 31; Supplementary Fig. S0).

Western blot analysis

Cells were lysed and SDS-PAGE was done as described (32).

Antibodies

See Supplementary Fig. S0.

Quantitative real-time PCR

See Supplementary Fig. S0.

Transfection and luciferase assay

The human FOXM1 promoter constructs have previously been described (29). Cells were transfected with the human FOXM1 promoter and Renilla (pRL-TK, Promega) as an internal transfection control using Fugene-6 (Qiagen) as described (20) alone or in combination with pCMV-E2F1 or pDNA3-Flag-p53. The FoxM1 promoter-reporter constructs have previously been described (29). Putative forkhead site mutagenesis was done using a Stratagene QuikChange site-directed mutagenesis kit and oligonucleotides mE2F1F (5′-GGAGATCCGGAGCCGGATCCGATGTTCGACGTTC-3′), mE2F1R (5′-GGAGTCGACATGCGCTGACGCAAGGCCGGTCTGGCCCTT-3′), mE2F2F (5′-CGTGAACCTTACGCACGCGGACCTCAAAACAGCAGACACGC-3′), mE2F2R (5′-GGATCCGGCGTTTGAATTGAGATCCGGCGTGTTCCAAGGC-3′). The human breast carcinoma cell lines MCF-7 and MDA-MB-231 were used as a control. The luciferase assay was performed using a dual-luciferase reporter assay system (Promega) as described previously (29). Supershift assays were performed using the Proteintech (Rosemont, IL) p53 antibody.

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) assay was conducted as described previously (33) by using MCF-7 cells grown to 70% confluence. DNA fragments were purified using the QIAquick Spin Kit (Qiagen). Antibody for E2F1 (KH95) was purchased from Abcam, and retinoblastoma family of proteins (pRB) antibody (C-15) was purchased from Santa Cruz Biotechnology (Autogen Bioclear). For PCR one-twentieth of the extracted DNA was used and amplified in 33 PCR cycles by using FOXM1 primers: FOXM1-F 5′-CCGGAGCTTTCAGTTTGTTC-3′, FOXM1-R 5′-CCGGACGTCCGTGTTCCAAGGATCCGCGTGTTCCAAGGACGTTCAG-3′.

Sulforhodamine B assay and cell-cycle analysis

Sulforhodamine B (SRB) assays and cell-cycle analysis were conducted and read as described (31).

Phospho-γH2AX immunofluorescent staining and quantification

Phospho-γH2AX (p-γH2AX) immunofluorescent staining was done as described (19) and the images were acquired using a confocal or ImageXpress device (Molecular Devices).

Results

Dysregulated FOXM1 expression is associated with epirubicin resistance in breast cancer

The involvement of FOXM1 in DNA damage response and chemotherapeutic drug resistance led us to hypothesize that FOXM1 has a role in anthracycline sensitivity and resistance in breast cancer. To test this conjecture, we established an epirubicin-resistant breast cell line MCF-7-EPIR by chronic exposure of the parental drug-sensitive MCF-7 to stepwise increases in epirubicin concentration, until a concentration of resistance up to 10 μmol/L was achieved. SRB proliferation assays showed that MCF-7-EPIR displayed strong resistance to epirubicin compared with the parental MCF-7 cells (Fig. 1A). We next examined the effect of epirubicin on the proliferation of the MCF-7
and the MCF-7-EPIR cells at 1µmol/L, a concentration generally used in cancer therapy, and the SRB assay revealed that the proliferation of the MCF-7 cells was significantly inhibited following epirubicin treatment whereas the growth of the MCF-7-EPIR cells was relatively unaffected in the presence of epirubicin (Fig. 1A). There was also a notable significant difference in the rates of proliferation between the epirubicin-treated MCF-7 and MCF-7-EPIR cells at both 24 and 48 hours. Cell-cycle analysis showed that epirubicin exposure (1 µmol/L) induced an accumulation of MCF-7 cells at G2–M and sub-G1 phases, indicative of G2–M delay and cell death whereas no significant changes in cell-cycle profile are observed for the MCF-7-EPIR cells (Fig. 1B). Subsequent Western blot analysis revealed no significant changes in the levels of FOXM1 and FOXM1 protein targets cyclin B1 and Polo-like kinase (PLK), following 48 hours of treatment with epirubicin at 1 µmol/L in MCF-7-EPIR cells. In contrast, FOXM1 protein expression decreased within 24 hours and was completely abrogated at 48 hours in MCF-7 cells (Fig. 1C). Consistently, Quantitative real-time PCR (qRT-PCR) analysis revealed no significant decrease in FOXM1 transcript level in the MCF-7-EPIR cells whereas epirubicin induced a drastic reduction in FOXM1 mRNA level in MCF-7 cells (Fig. 1C). Collectively, these results show that FOXM1 is downregulated at mRNA and protein levels in response to epirubicin in the sensitive MCF-7 cells whereas FOXM1 expression is deregulated in epirubicin-resistant cells (Supplementary Fig. S2), suggesting that FOXM1 has a role in epirubicin sensitivity and resistance.

**The loss of FOXM1 repression by p53 contributes to epirubicin resistance**

The recent observation that p53 represses FOXM1 expression following daunorubicin treatment (34) led...
us to predict that epirubicin also activates p53 to repress FOXM1 expression in breast cancer cells. To assess the role of p53 in mediating the epirubicin response in breast cancer cells, we first examined the expression of p53 and its target \( p21^{Cip1} \) in the sensitive and resistant MCF-7 cell lines in response to epirubicin treatment. Western blot analysis revealed that epirubicin treatment strongly induced the expression of the p53 protein and its target, the cyclin-dependent kinase inhibitor \( p21^{Cip1} \), in the MCF-7 cells. In contrast, the expression of p53 and \( p21^{Cip1} \) was undetectable in the MCF-7-EPIR cells before and after epirubicin treatment (Fig. 2A). qRT-PCR analysis also showed an induction in \( p21^{Cip1} \) transcript level in the MCF-7, but not in the MCF-7-EPIR cells, in response to epirubicin treatment (data not shown). To test whether p53 is responsible for the downregulation of FOXM1 expression in MCF-7 cells following epirubicin treatment, MCF-7 cells were transiently transfected with nontargeting or p53-targeting short interfering RNA (siRNA), treated with epirubicin, and then examined for FOXM1 expression. Western blot and qRT-PCR analysis showed that silencing of p53 attenuated FOXM1 downregulation at both protein and mRNA levels in response to epirubicin (Fig. 2B). The inability of p53 depletion to completely abolish the downregulation of FOXM1 also suggests that p53 might not be the sole regulator of FOXM1 expression.
expression in response to epirubicin. A previous study showed that p53 represses FOXM1 expression via pRB following daunorubicin treatment (34). Thus, one mechanism by which p53 can repress FOXM1 expression is through its ability to induce p21Cip1, which can, in turn, represses cyclin-dependent kinase (CDK)-mediated pRB hyperphosphorylation, resulting in the repression of E2F transcriptional activity. Surprisingly, although p53 knockdown abrogated the induction of p21Cip1 and the downregulation of FOXM1 by epirubicin, silencing of p21Cip1 had little effects on the epirubicin-induced FOXM1 downregulation, suggesting that epirubicin can also repress FOXM1 expression via p21Cip1-independent mechanisms (Fig. 2B). To investigate further the role of p53 and p21Cip1 in regulating FOXM1 expression in response to epirubicin, wild-type, p53-deficient (p53~−/−), and p21-deficient (p21Cip1−/−) MEFs were subjected to epirubicin treatment and the expression of FOXM1 was investigated. Treatment of the wild-type, p21Cip1−/− MEFS with epirubicin resulted in a reduction in FOXM1 expression within 16 hours, further confirming that p21Cip1 is not essential for the repression of FOXM1 expression by epirubicin. In contrast, epirubicin did not cause a downregulation of FOXM1 expression in the p53-deficient MEFs. Together, these data support the idea that epirubicin represses FOXM1 expression at the transcriptional level through p53.

**p53 can repress FOXM1 expression through an E2F site in its promoter**

The pRB-E2F transcription factors are principal regulators of the cell cycle and function downstream of the p53 canonical pathway. To assess whether the E2F transcription factors are involved in the p53-dependent FOXM1 repression, we analyzed the expression pattern of E2F1, a well-characterized E2F-responsive gene product and a subunit of the E2F transcription factor dimers. Treatment of the MCF-7 cells with epirubicin markedly reduced E2F1 mRNA levels within 16 hours (Fig. 3A), confirming that epirubicin represses E2F1 transcriptional activity. Surprisingly, although p53 knockdown abrogated the induction of p21Cip1 and the downregulation of FOXM1 by epirubicin, silencing of p21Cip1 had little effects on the epirubicin-induced FOXM1 downregulation, suggesting that epirubicin can also repress FOXM1 expression via p21Cip1-independent mechanisms (Fig. 2B). To investigate further the role of p53 and p21Cip1 in regulating FOXM1 expression in response to epirubicin, wild-type, p53-deficient (p53~−/−), and p21-deficient (p21Cip1−/−) MEFs were subjected to epirubicin treatment and the expression of FOXM1 was investigated. Treatment of the wild-type, p21Cip1−/− MEFs with epirubicin resulted in a reduction in FOXM1 expression within 16 hours, further confirming that p21Cip1 is not essential for the repression of FOXM1 expression by epirubicin. In contrast, epirubicin did not cause a downregulation of FOXM1 expression in the p53-deficient MEFs. Together, these data support the idea that epirubicin represses FOXM1 expression at the transcriptional level through p53.

**p53 is involved in the repression of FOXM1 expression and induction of cell death in response to epirubicin**

Much evidence has indicated that p53 is activated through phosphorylation on serine 15 by ATM upon DNA damage (35). Consistent with this, Western blot analysis also showed that concomitant with an increase in its expression level, p53 was phosphorylated at serine 15 in the MCF-7 cells after epirubicin treatment (Fig. 4A). To investigate the mechanism by which FOXM1 expression is repressed by epirubicin, p53~−/− MEFs were transfected with an empty vector or the wild-type p53 and FOXM1 promoter (Fig. 3B; Supplementary Fig. S3). The ChIP assay showed that the putative E2F-binding sites (site 1: 58 bp; site 2: 24 bp) locate inside all 3 FOXM1 promoter constructs (Fig. 3B). We next examined whether p53 exerts its repression on the FOXM1 promoter activity through these putative E2F-binding sites. To this end, we cotransfected into MCF-7 cells increasing amounts of p53 together with either the wild-type Apal FOXM1 promoter-reporter (WT-luc) or the Apal FOXM1 promoter lacking one (mE2F1-luc or mE2F2-luc) or both (mE2F1/2-luc) putative E2F sites. The results showed that p53 caused a drastic (12.7-fold) reduction in mE2F1-luc activity comparable with that (11.5-fold) observed for WT-luc (Fig. 3C). In contrast, the repression by p53 was considerably reduced in both the mE2F2-luc and the mE2F1/2-luc, suggesting that the second putative E2F-binding site (site 2) mediates the repression of the FOXM1 promoter by p53. Next, the activity of both the wild-type (WT-luc) and the mutated Apal-luc constructs (mE2F1-, mE2F2-, and mE2F1/2-luc) was examined by cotransfection assays in MCF-7 cells with different amounts of E2F1 expression vector. The results showed that the mE2F1-luc construct showed similar responsiveness to E2F1 as the WT-luc. In contrast, both the mE2F2-luc and the mE2F1/2-luc mutants lost the majority of their responsiveness to E2F1. Together these cotransfection results provide strong evidence that the E2F-binding element located at −24-bp confers the responsiveness to p53 and E2F, further confirming that p53 represses FOXM1 expression through E2F activity.
FOXM1 mRNA expression and promoter activity, suggesting that epirubicin induces p53 to repress FOXM1 expression at transcription and gene promoter levels (Fig. 4C). Notably, the moderate repression of FOXM1 expression observed in these transfection studies probably reflects the low transfection efficiencies. To circumvent this problem, immunofluorescence staining was done on the p53−/− MEFs transfected with p53 in the

Figure 3. p53 represses FOXM1 expression through an E2F-binding site located in the proximal FOXM1 promoter region. A, MCF-7 and MCF-7-EPIR cells were treated with 1 μmol/L of epirubicin for 0, 16, 24, and 48 hours, and qRT-PCR was done to determine E2F1 transcript levels. Columns, means derived from 3 independent experiments; bars, SD. B, schematic representation of the full-length Trident, HindIII, and Apal FOXM1-luc reporter constructs and the E2F-binding sites 1 and 2 (top). MCF-7 cells were transiently transfected with 20 ng of the empty pGL3-basic, pGL3-Trident, pGL3-HindIII, or the pGL3-Apal, and cells were treated with 1 μmol/L of epirubicin. Cells were then harvested at 0, 24, and 48 hours after treatment and assayed for luciferase activity. All relative luciferase activity values are corrected for cotransfected Renilla activity. The folds of repression were calculated between 0 and 48 hours of epirubicin treatment. Columns, means derived from 3 independent experiments; bars, SD. C, MCF-7 cells were transiently transfected with 20 ng of the pGL3-Apal (WT), pGL3-Apal-mE2F1, pGL3-Apal-mE2F2, or pGL3-Apal-mE2F1/2 together with increasing amounts (0, 10, and 30 ng) of p53 expression vector in the left panel and E2F1 in the right panel. Cells were harvested after 24 hours of transfection and assayed for luciferase activity. All relative luciferase activity values are corrected for cotransfected Renilla activity. The folds of repression and activation were calculated indicated between 0 and 48 hours of epirubicin treatment. Columns, means derived from 3 independent experiments; bars, SD. Statistical analyses were done using Student’s t test. **, P ≤ 0.01; ***, P ≤ 0.001, significant; n.s., nonsignificant. D, MCF-7 cells untreated or treated with 1 μmol/L epirubicin for 24 hours were used for ChIP assays by using IgG negative control, anti-E2F1, and anti-pRb antibodies as indicated. After reversal of cross-linking, the coimmunoprecipitated DNA was amplified by PCR, using primers amplifying the FOXM1 E2F-binding sites containing region (−184/+4) and a control region (−1,157/−1,257), and resolved in 2% agarose gel. Inverted images are shown.
presence or absence of epirubicin treatment (Fig. 4D). The staining results showed an inverse correlation between p53 and FOXM1 expression, with p53−/− MEFs having high levels of p53 showing low FOXM1 expression, and vice versa. The results also showed that p53 was capable of repressing FOXM1 expression and inducing apoptosis in response to epirubicin treatment, as only the cells expressing ectopic p53 displayed apoptotic morphologies in response to epirubicin. Collectively, these results suggest that p53 is required for the repression of FOXM1 expression and the induction of cell death on epirubicin treatment.
Increased DNA repair in epirubicin-resistant cells

Next, we sought to determine the molecular mechanism that confers epirubicin resistance in the MCF-7-EPIR cells. It has been previously shown that FOXM1 expression is associated with the cisplatin-induced DNA damage response and drug resistance (20). We therefore examined the formation of DNA damage foci by p-γ H2AX staining in MCF-7 and MCF-7-EPIR cells in response to epirubicin treatment. The results showed an increase in the mean number of γH2AX foci per cell over time after epirubicin treatment in the MCF-7 cells, whereas the level of p-γ H2AX foci per cell remained relatively constant in the MCF-7-EPIR cells, suggesting an increase in DNA repair activities in these cells (Fig. 5A and B). To investigate this further, we evaluated the expression level of the DNA repair protein ATM in the MCF-7 and MCF-7-EPIR cells. The Western blot and qRT-PCR analysis showed that the ATM protein and mRNA levels were strongly upregulated in the MCF-7-EPIR cells compared with MCF-7 cells (Fig. 5C), thus suggesting a role of ATM in mediating the increase in DNA repair activity in the resistant cells. Notably, no measurable changes in ATR expression levels were detected in MCF-7-EPIR cells (data not shown).

ATM is involved in FOXM1 regulation and drug resistance in the epirubicin-resistant cells

To determine whether the ATM pathway is involved in FOXM1 regulation in response to epirubicin, we treated the U2OS p53−positive osteosarcoma cells with
Epirubicin in the absence or presence of caffeine, a known ATM inhibitor. Western blot analysis showed that epirubicin induced p53 activation but did not affect FOXM1 protein level, suggesting that FOXM1 expression is not sensitive to p53 induction in these cells (Fig. 6A). However, when the U2OS cells were pretreated with caffeine, which inhibits ATM as well as p53 activity, epirubicin strongly represses FOXM1 expression. Epirubicin-resistant MCF-7-EPI\textsuperscript{R} cells express higher levels of ATM in response to epirubicin to promote E2F1 and FOXM1 expression and cell survival. A, U2OS cells were treated for 0 to 24 hours with 1 mmol/L of epirubicin in the presence or absence of 5 mmol/L caffeine. At indicated time, cells were collected for Western blot analysis to determine the protein expression levels of FOXM1, E2F1, phospho-p53 (p-p53; Ser 15), p53, cleaved caspase 7, and \( \beta \)-tubulin. B, MCF-7 and MCF-7-EPI\textsuperscript{R} cells were treated with 1 mmol/L of epirubicin and the protein expression levels of phospho-ATM (p-ATM), ATM, E2F1, phospho-CHK2 (p-CHK2), CHK2, p-p53 (Ser 15), p53, PARP, and \( \beta \)-tubulin were analyzed by Western blot analysis. C, MCF-7 and MCF-7-EPI\textsuperscript{R} cells were either transfected with nonspecific (NS) siRNA (100 nmol/L) or siRNA smart pool against ATM (100 nmol/L). Twenty-four hours after transfection, cells were treated with 1 mmol/L of epirubicin and harvested for Western blot analysis at 0, 24, and 48 hours. The protein expression levels were determined for FOXM1, ATM, E2F1, PARP, and \( \beta \)-tubulin.
expression. Paradoxically, it was noted that in the caffeine-treated cells, p53 expression was significantly downregulated, suggesting that FOXM1 repression by epirubicin is independent of p53 in these caffeine-treated cells. Our results also suggested that the ATM DNA damage response pathway could be involved in FOXM1 regulation in a p53 independent–manner. Consequently, ATM expression and activity were investigated in the MCF-7 and MCF-7-EPIR cells by Western blot analysis (Fig. 6B). Treatment with epirubicin induced the phosphorylation (serine 1981) and expression of ATM in the MCF-7-EPI R within 24 hours, whereas this induction was not detectable in the MCF-7 cells (Fig. 6B). Phosphorylation of the ATM downstream target checkpoint kinase 2 (CHK2) was strongly increased in the MCF-7-EPI R and to a much lesser extent in the MCF-7 cells. Accumulation of E2F1, another ATM target (36, 37), was detected in the MCF-7-EPI R cells, whereas E2F1 level decreased in the MCF-7 cells in response to epirubicin treatment. It was also noticeable that epirubicin induced apoptosis as revealed by PARP cleavage in MCF-7 but not in the MCF-7-EPI R cells.

These data indicate a pathway linking ATM with E2F1 and FOXM1 expression independent of p53 in MCF-7-EPI R cells. To determine whether ATM is involved in E2F1 and FOXM1 regulation in MCF-7-EPI R cells, we silenced ATM expression by using siRNA in both MCF-7 and MCF-7-EPI R cells and studied E2F1 and FOXM1 expression in response to epirubicin treatment (Fig. 6C). Western blot analysis showed that the knockdown of ATM had little effects on E2F1 and FOXM1 expression in the MCF-7 cells. In contrast, while the expression level of E2F1 and FOXM1 increased in the control MCF-7-EPI R cells on epirubicin treatment, epirubicin caused a decrease in E2F1 and FOXM1 protein expression in the MCF-7-EPI R cells with ATM silencing, suggesting that the induction of ATM in the MCF-7-EPI R is responsible for the induction of E2F1 and FOXM1 in the MCF-7-EPI R cells. Moreover, silencing of ATM by using siRNA abrogated the induction of FOXM1 mRNA by epirubicin, suggesting that ATM regulates FOXM1 at the transcriptional level (Supplementary Fig. S4). Consistent with this, inhibition of ATM by Ku-55933 repressed E2F1 and FOXM1 induction and resestituted the resistant MCF-7-EPI R cells to epirubicin (Supplementary Fig. S5). Moreover, the role of FOXM1 in epirubicin sensitivity and resistance is further supported by the observations that overexpression of FOXM1 in MCF-7 cells can decrease the sensitivity of MCF-7 cells to epirubicin (Supplementary Fig. S6) and that FOXM1 knockdown in MCF-7-EPI R cells mimics the antiproliferative effects of epirubicin on MCF-7 cells (Supplementary Fig. S7).

**Discussion**

The FOXM1 transcription factor plays a crucial role in the regulation of a diversity of cellular functions, including cell proliferation, cell survival, and immortalization, which are essential for tumorigenesis. Consistent with this notion, FOXM1 has been found to be frequently upregulated in a host of human cancers, including colorectal (23), lung (24), prostate (25), liver (26), stomach (38), breast (27), and basal cell carcinomas (39) as well as glioblastoma (40). Recently emerging evidence reveals that FOXM1 has a role in cancer drug resistance as well. In concordance, latest studies show that FOXM1 expression level is an important determinant of sensitivity to breast cancer chemotherapeutic drugs such as herceptin (28), gefitinib (41), lapatinib (27), paclitaxel (28), and cisplatin (20). Consistent with these findings, we established in this study that FOXM1 is a crucial cellular target of the anthracycline epirubicin in breast cancer cells. Moreover, FOXM1 protein levels are higher in the epirubicin-resistant MCF-7-EPI R cells than in the sensitive MCF-7 cells and FOXM1 expression is downregulated by epirubicin in the sensitive MCF-7 cells but not in the resistant MCF-7-EPI R cells, suggesting further that FOXM1 also has a role in epirubicin resistance. In agreement, a recent study revealed that the anthracyclin daunorubicin could repress FOXM1 expression through the sequential activation of p53, p21Cip1, and pRB(34). We confirmed and extended these findings in breast cancer cell lines and showed that the MCF-7-EPI R cells failed to induce p53 expression and activity in response to epirubicin treatment. We established using p53−/− MEFs that FOXM1 expression is negatively regulated by p53. In contrast, epirubicin can effectively repress FOXM1 expression in the p21Cip1−/− MEFs. This finding indicates that p53 can repress E2F activity and FOXM1 expression through mechanisms independent of the cyclin-dependent kinase inhibitor p21Cip1 despite previous studies showing that the activation of pRB by the anthracyclin daunorubicin is mediated at least partially through p21Cip1(34). Consistently, anthracyclines have been shown to activate the forkhead transcription factor FOXO3a so as to induce another cyclin-dependent kinase inhibitor (CKI) p27Kip1, which can, in turn, inhibit CDKs and activate pRB proteins to repress E2F activity (16, 42). It is notable that E2F1 is an E2F-regulated gene and therefore its expression level reflects the cellular E2F activity. Transient promoter-reporter transfection assays indicate that the effects of epirubicin and its cellular targets p53 and E2F1 are mediated through a proximal E2F-binding site on the FOXM1 promoter. In agreement, a recent study revealed that a great majority of genes repressed by p53 and p73 contain E2F-binding sites, suggesting that p53 proteins repress gene expression through inhibiting E2F activity (43). The direct binding of pRB and E2F1 to the FOXM1 promoter was confirmed in vivo by ChIP analysis. ChIP assays also revealed that on epirubicin treatment there were increased levels of pRB and decreased levels of E2F1 recruited to the FOXM1 promoter region containing the E2F-binding site. Collectively, these findings indicate that epirubicin...
can repress FOXM1 expression through induction of p53, which, in turn, represses E2F activity through activating pRB and downregulating E2F1 expression. Transient transfection experiments in which p53 was reintroduced into deficient cells also showed that p53 activity is required for the cytotoxic function of epirubicin, confirming that the loss of p53 contributes toward the development of epirubicin resistance.

However, despite our finding that the loss of p53 in the drug-resistant MCF-7-EPIR cells has a role in epirubicin resistance, it is improbable that loss of functional p53 is the primary or sole cause for the development of epirubicin resistance, considering loss of p53 function is highly prevalent in cancer. In accordance with this idea, we obtained evidence that DNA damage-sensing kinase ATM also has a role in regulating FOXM1 expression and epirubicin sensitivity, independent of p53. For instance, in the osteosarcoma cell line U2OS with wild-type and functional p53 and pRB, epirubicin triggered the accumulation both of p53 and of ATM but failed to induce cell death. Furthermore, caffeine treatment attenuated p53 and ATM induction and yet sensitized the U2OS cells to epirubicin-induced cell death. It is also notable that E2F1 and FOXM1 levels were maintained, if not increased, after epirubicin treatment in the U2OS cells but decreased in the presence of the ATM inhibitor caffeine, suggesting that ATM also has a role in regulating E2F1 and FOXM1 expression as well as in epirubicin sensitivity. Like FOXM1, ATM is overexpressed in the drug-resistant MCF-7-EPIR cells compared with the MCF-7 cells and its expression is upregulated in response to epirubicin treatment. This FOXM1 induction is antagonized by the effects of p53 activation in the epirubicin-sensitive cells with functional p53 but is unopposed in the p53-deficient cells. In consequence, low levels of epirubicin will cause an induction of FOXM1 expression in the resistant cells. Together, these findings suggest that ATM is activated in response to epirubicin to enhance E2F activity and consequently FOXM1 expression to promote cell survival in drug-resistant cancer cells (Supplementary Fig. S8). Consistently, ample evidence has shown that ATM regulates E2F1 expression in response to DNA damage, although the mechanism involved is not completely understood (36, 44). For example, genotoxic stress has been reported to upregulate E2F1 expression at the transcriptional level through the activation of ATM (36). On the contrary, a previous study has also shown that E2F1 expression is upregulated in response to DNA damage because of an increase in protein stability and not because of an increase in E2F1 expression at the transcriptional level (44). Current evidence indicates that E2F1 expression can be involved in proliferation and tumorigenesis as well as apoptosis and tumor suppression (45, 46). However, in the context of cancer chemotherapy, the current observations evidently suggest that E2F1 is linked to cell survival by promoting FOXM1 expression. In a previous microarray study, E2F1–3 have been shown to promote the expression of genes involved in DNA replication, DNA repair, and mitosis (47), and interestingly, some of these E2F-regulated genes identified, such as cdc2, cyclin B1, and MCM members, are also transcriptional targets of FOXM1 (16, 48). Consistently, a number of recent studies have shown that E2F1 expression is induced by a variety of DNA-damaging agents and genotoxic chemotherapeutic drugs and mirrors that of p53, further supporting a possible involvement of E2F and FOXM1 in the DNA damage response and drug resistance (44, 49, 50).

On the basis of our current findings that ATM induces E2F activity and FOXM1 expression in response to DNA damage and that E2F can promote FOXM1 transcription, we propose that ATM enhances E2F1 expression and activates E2F-dependent FOXM1 expression at transcriptional level in response to DNA-damaging agents such as epirubicin. In addition, it has previously been shown that FOXM1 protein is phosphorylated by CHK2 on serine 361 in response to DNA damage and this phosphorylation has been proposed to increase the stability of the FOXM1 protein to promote expression of DNA repair genes (21). Given that CHK2 functions directly downstream of ATM in DNA damage response, it is predicted that the induction of FOXM1 expression by ATM may therefore also occur through posttranslational mechanisms in response to DNA damage (21). Irrespective of the mechanism by which ATM regulates FOXM1 expression, these observations also indicate that in the MCF-7-EPIR cells the increased ATM expression may promote DNA repair to counteract the DNA damage–induced cell death triggered by genotoxic chemotherapeutic drugs. Consistent with this idea, the levels of DNA damage sustained by the MCF-7-EPIR cells after epirubicin treatment is significantly reduced when compared with the drug-sensitive MCF-7 cells, as revealed by the γH2AX staining. Moreover, this idea is further supported by our finding that depletion of ATM activity by siRNA or the specific inhibitor Ku-55933 sensitized the resistant MCF-7-EPIR cells to epirubicin-induced cell death and abolished the accumulation of FOXM1, which has a role in DNA damage repair.

In summary, our data suggest that genotoxic chemotherapeutic agents, such as epirubicin, trigger the accumulation and activation of p53 and ATM and it is the antagonistic signals of activated ATM and p53 that converge on E2F to control FOXM1 expression, DNA damage repair, and cell survival (Supplementary Fig. S8). Specifically, p53 represses whereas ATM enhances E2F activity, FOXM1 expression, DNA repair, and cell survival in response to genotoxic drugs. In consequence, the development of epirubicin resistance can be due to the loss of p53 function and/or an increase in ATM expression and activity. The finding that ATM, as well as p53, modulates FOXM1 expression may have important implications for the diagnosis and treatment of drug-resistant cancers, particularly those lacking functional p53. For example, ATM and FOXM1 inhibitors can be important cancer therapeutics, as they can cause cell
death independent of p53 status. These ATM and FOXM1 inhibitors can also be used in combination with conventional genotoxic therapeutics to enhance the drug efficacy and for overcoming resistance. Furthermore, p53, ATM, and FOXM1 could be useful biomarkers for the prediction of epirubicin sensitivity in cancer patients.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References


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ATM and p53 Regulate FOXM1 Expression via E2F in Breast Cancer Epirubicin Treatment and Resistance

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