Forkhead box protein M1 (FOXM1) is a poor prognosis TF conferring FOXM1 mediates Dox resistance in breast cancer by enhancing DNA repair

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Materials and methods

Selection of genes significantly associated with overall survival of breast cancer patients

To avoid including too many potential false positives, we used two independent data sets of breast cancer patient (the Netherlands Cancer Institute (NKI) (9) and University of North Carolina (UNC) (10) cohorts containing both genome-wide expression data and patient survival data, and we analyzed each data set independently. We first dichotomized patients according to expression levels of individual genes (high/low) in microarray data by using the median as the cutoff. We estimated the prognostic significance of individual genes by applying Kaplan–Meier analysis and the log-rank test to dichotomized patients. The association of a gene with overall survival was considered significant if P < 0.01 by the log-rank test. When this criterion was applied to the two independent data sets, only 104 genes remained significant in both patient cohorts and were thus considered to be survival-associated genes (Figure 1A and Supplementary Figure 1 is available at Carcinogenesis Online).

Quantitative reverse transcription–polymerase chain reaction

Total RNA was extracted from the indicated cell lines by using a mirVana RNA isolation labeling kit (Ambion) according to the manufacturer’s instructions, and RT–PCR was assayed by using real-time quantitative reverse transcription–polymerase chain reaction (qRT–PCR) with TaqMan primers specific to each gene (Applied Biosystems). Real-time PCR was performed using the 7700HT real-time PCR system with a 96-well block module (Applied Biosystems). Cycling conditions were 45°C for 30 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 60 s. Relative amounts of mRNA were calculated from the threshold cycle number using expression of cyclophilin A as an endogenous control. All experiments were performed in triplicate and the values averaged.

Cell culture and chemotherapeutic agents

Breast cancer cell lines and U2OS cells were purchased from American Type Culture Collection. MDA-MB-231 cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS). MCF-7 cells were maintained in Dulbecco’s modified Eagle medium/nutrient mixture F-12 supplemented with 10% FBS. U2OS cells were maintained in McCoy’s 5A medium supplemented with 10% FBS with glutamine, penicillin and streptomycin. Cells were incubated at 37°C in a humidified incubator with 5% CO2. Paclitaxel, Dox, cisplatin, etoposide and mitoxantrone were dissolved in dimethyl sulfoxide (DMSO), 5-FU was dissolved in sterile water. All the chemotherapeutic agents were obtained from Sigma.

Plasmin and luciferase assay

pCDNA3-NFκB1 plasmid was purchased from Addgene. pGL3-PLK4 promoter was described previously (11). To generate pGL3-EXO1 and pGL3-RFC4, the promoter region from −2000 to +1 was cloned by PCR from human genomic DNA. Sequences were verified by automatic sequencer. For luciferase-based reporter assays, cells were transfected with indicated reporter genes and plasmids using Fugene6 (Roche) according to the manufacturer’s instructions. After 48 h, cells were harvested to measure luciferase activity, which was normalized with β-galactosidase.

Western blotting

Cells were maintained and western blotting was performed as described previously (18). Antibodies used were anti-FOXM1 (sc-500; Santa Cruz Biotechnology), NFκB1 (#3035; Cell Signaling Technology), β-actin (H-196; Santa Cruz) and α-tubulin (#3873; Cell Signaling).

Small interfering RNA

siLuc, siFOXM1, siEXO1, siCDCA45L, siRFC4 and siPLK4 were described previously (12–16). SiPOL2 and siNFκB1 were purchased from Ambion. Cells were transfected with indicated small interfering RNA (siRNA) using Oligofectamine (Invitrogen) for 48 or 72 h in the presence of indicated chemotherapeutic drugs.

Introduction

Transcription factors (TFs) are direct effectors of altered signaling pathways in cancer and frequently determine clinical outcomes in cancer patients. To uncover new transcription factors that would determine clinical outcomes in breast cancer, we systematically analyzed gene expression data from breast cancer patients. Our results revealed that Forkhead box protein M1 (FOXM1) is the top-ranked survival-associated transcription factor in patients with triple-negative breast cancer. Surprisingly, silencing FOXM1 expression led breast cancer cells to become more sensitive to doxorubicin (Dox). We found that FOXM1-dependent resistance to Dox is mediated by regulating DNA repair genes. We further demonstrated that NFκB1 interacts with FOXM1 in the presence of Dox to protect breast cancer cells from DNA damage. Finally, silencing FOXM1 expression in breast cancer cells in a mouse xenograft model significantly sensitized the cells to Dox. Our systematic approaches identified an unexpected role of FOXM1 in Dox resistance by regulating DNA repair genes, and our findings provide mechanistic insights into how FOXM1 mediates resistance to Dox and evidence that FOXM1 may be a promising therapeutic target for sensitizing breast cancer cells to Dox.

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Abbreviations: Dox, Doxorubicin; ER, estrogen receptor; FOXM1, Forkhead box protein M1; HER2, human epidermal growth factor receptor 2; NFκB, nuclear factor kappa-light-chain-enhancer of activated B cells; PR, progesterone receptor; qRT–PCR, quantitative reverse transcription–polymerase chain reaction; TF, transcription factors.
The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to assess cell proliferation after drug treatment with or without specific siRNA. Cells were plated into 96-well plates, transfected with specific siRNA and incubated for 36 h. Cells were then exposed to indicated chemotherapy drugs at indicated concentrations for 36 h. After treatment, 20 µl of MTT was added to each well, the wells were incubated for 3 h and 100 µl of DMSO was then added. The absorbance of individual wells was read at 570 nm.

Chromatin immunoprecipitation assay
Chromatin immunoprecipitation (ChIP) experiments were performed as described previously (18). Antibodies used were anti-FOXM1 (K-19) and Y.-Y. Park et al.
rabbit normal IgG (SC-2027), both from Santa Cruz Biotechnology. Primer sequences used are listed in Supplementary Table 4 is available at Carcinogenesis Online.

Microarray
Total RNA was extracted from MDA-MB-231 cells by using a mirVana RNA isolation labeling kit (Ambion). We used 500 ng of total RNA for labeling and hybridization, according to the manufacturer’s protocols (Illumina). After the bead chips were scanned with a BeadArray Reader (Illumina), the microarray data were normalized using the quantile normalization method in the Linear Models for Microarray Data (LIMMA) package in the R language environment (20). The expression level of each gene was log2-transformed before further analysis. Primary microarray data are available in the National Center for Biotechnology Information Gene Expression Omnibus public database (microarray platform, GPL6947; microarray data, GSE25741).

Homologous recombination assay
The homologous recombination (HR) assay was described previously (17,18). Briefly, U2OS cells stably expressing DR (tandem repeated) GFP reporter were transfected with siFOXM1 or siLuc. After I-ScrI endonuclease was delivered to the cells by transient transfection, GFP-positive cells were sorted and analyzed by flow cytometry to indicate the efficiency of HR repair.

Stable isotope labeling by amino acids in cell culture method
Stable isotope labeling by amino acids in cell culture (SILAC) kits were obtained from Invitrogen. As described previously (19), MDA-MB-231 cells were grown in RPMI 1640 medium lacking arginine and lysine supplemented with dialyzed FBS. The medium was supplemented with heavy 13C6-l-arginine and 13C6-l-lysine or with 12C6-l-arginine and 12C6-l-lysine. Cells were passaged two times in the ‘heavy’ or ‘light’ SILAC medium to ensure complete labeling. Then, MDA-MB-231 cells labeled with light medium were exposed to 5 µM Dox for 36 h. Lysates from MDA-MB-231 cells labeled with the heavy and light medium were mixed together, purified and immunoprecipitated with FOXM1 antibody. The immunoprecipitates were washed thrice with NETN buffer (20 mM Tris–HCl [pH 8.0], 100 mM NaCl, 1 mM EDTA, 0.5% NP40). The washed beads were boiled with Laemmli buffer and subjected to SDS-PAGE (4–20% Tris/glycine Novex gel; Invitrogen). The Coomassie brilliant blue-stained protein bands were excised and destained with 50 mM ammonium bicarbonate solution in 50% methanol. Gel pieces were then washed in HPLC buffer (20 mM Tris–HCl [pH 8.0], 100 mM NaCl, 1 mM EDTA, 0.5% NP40) for 20 min. The washed beads were boiled with 200 µl of acetonitrile. The supernatants were dried in a SpeedVac dryer.

Protein identification (LC-MS/MS analysis)
Each dried sample was dissolved in 20 µl of 5% methanol/95% water/0.1% formic acid solution and injected into the Surveyor HPLC system (Thermo Finnigan) using an autosampler. A 100 mm × 75 µm I. C18 column (5 µm, 300 Å pore diameter, Picofrit, New Objective) with mobile phases of A (0.1% formic acid in water) and B (0.1% formic acid in methanol) was used, with a gradient of 5–95% of phase B over 15 min followed by 95% of phase B for 5 min at 200 nl/min. Peptides were directly electrosprayed into the mass spectrometer (Finnigan LTQ, Thermo Finnigan) using a nanospray source. The LTQ was operated in the data-dependent mode to acquire fragmentation spectra of the 20 strongest ions.

Statistical analysis of microarray data and survival analysis
The random-variance t-test was used to identify genes differentially expressed between the two classes that were compared using BRB-ArrayTools (20). The random-variance t-test is an improvement over the standard separate t-test as it allows information to be shared among genes about within-class variation without assuming that all genes have the same variance. Gene expression differences were considered significant if P < 0.001. Kaplan–Meier analysis and the log-rank test were used to estimate patient prognosis.

Xenograft experiments
Female athymic nude mice (NC-nu) were purchased from the NCI-Frederick Cancer Research and Development Center (Frederick, MD) and maintained as described previously (21). All mice studies were approved by the Institutional Animal Care and Use Committee. Mice were cared for in accordance with guidelines set forth by the American Association for Accreditation of Laboratory Animal Care and the US Public Health Service Policy on Humane Care and Use of Laboratory Animals. Tumor implantation, siRNA incorporation into dioleoylphosphatidylcholine (DOPC) nanoliposomes and delivery operated in the data-dependent mode to acquire fragmentation spectra of the (Finnigan LTQ, Thermo Finnigan) using a nanospray source. The LTQ was...
of the change in sensitivity was less than in MDA-MB-231 cells, suggesting that FOXM1 might play a greater role in Dox resistance in basal-like ER-negative breast cancer cells (MDA-MB-231) than in ER-positive breast cancer cells (Figure 2C). We next investigated whether the increase in sensitivity on silencing FOXM1 expression is universal to TOP2 inhibitors, which can be subdivided into intercalating (Dox, mitoxantrone and mAMSA) and nonintercalating (etoposide, teniposide and fluoroquinolones) poisons (24). Interestingly, although silencing FOXM1 expression significantly increased the sensitivity of MDA-MB-231 cells to mitoxantrone, it did not change their sensitivity to etoposide, indicating the presence of a specific mechanism of resistance to intercalating TOP2 inhibitors by FOXM1 in breast cancer (Figure 2D).

**FOXM1 govern DNA repair involved in Dox resistance**

To gain mechanistic insights into how FOXM1-depleted cells become sensitized to Dox, we carried out microarray experiments to identify genes whose expression is directly altered by silencing FOXM1 expression and Dox treatment. We first identified genes whose expression is significantly altered when cells were treated with siFOXM1 + Dox by comparing the siFOXM1 + Dox group with the other treatment groups (siLuc, siFOXM1 and siLuc + Dox) as controls (gene list A) (Figure 3A). We next identified genes whose expression was altered on Dox treatment by comparing the siFOXM1 group with the siFOXM1 + Dox group (gene list B) and the siLuc group with the siLuc + Dox group (gene list C). To remove genes whose altered expression is due to the general toxicity of Dox treatment, we excluded genes in gene list B and C from gene list A. A series of analysis yielded 65 genes whose expression is only specific to Dox treatment in FOXM1-depleted breast cancer cells. Genes were selected according to statistical significance ($P < 0.001$, by Student’s $t$-test) and fold difference (2-fold). We identified 65 genes as significantly associated with Dox sensitization in FOXM1-depleted cells (Figure 3B and Supplementary Table 2 is available at Carcinogenesis Online (65 gene list). Interestingly, many of these 65 genes are known to be involved in DNA repair (e.g. *EXO1*, *CDC45L*, *RFC4*, *POLE2* and *PLK4*). The altered expression of these five genes in treated cells was also validated by qRT–PCR (Figure 3C).
We next investigated whether the DNA repair genes regulated by FOXM1 participate directly in Dox sensitization. When expression of these DNA repair genes was silenced by specific siRNAs (Supplementary Figure 5 is available at Carcinogenesis Online), MDA-MB-231 cells became more sensitive to Dox treatment (Figure 3D), suggesting that downregulation of these genes might account for sensitization of MDA-MB-231 cells to Dox on silencing of FOXM1 expression. Not surprisingly, expression of these DNA repair genes is significantly associated with prognosis in breast cancer patients (Supplementary Figure 6 is available at Carcinogenesis Online).

**FOXM1 directly regulates DNA repair genes**  
Because HR is one of the mechanisms for repairing DNA double-strand breaks (DSBs) generated by genotoxic reagents, including Dox, and because inhibition of HR leads to increased sensitivity to chemotherapy drugs in human cancers (25,26), we next examined the contribution of FOXM1 to HR by measuring the repair rate of defined genomic DSBs induced at an I-SceI restriction site in a defective GFP reporter gene (17,18). HR repair was significantly impaired in FOXM1-depleted cells, as evidenced by a significant decrease (50%) in HR-repaired GFP-positive cells (Figure 4A).

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**Fig. 3.** 65 genes confer Dox resistant by FOXM1 in breast cancer. (A) Gene expression profiles of MDA-MB-231 cells were collected after treating cells with siLuc alone, siFOXM1 alone, siLuc + Dox or siFOXM1 + Dox. (B) Expression patterns of 65 genes identified as Dox-sensitizing genes from analysis of gene expression profile data from MDA-MB-231 cells. Data are presented in matrix format; each row represents an individual gene, and each column represents a tissue. Each cell in the matrix represents the expression level of a gene feature in treated cells. In the cells, red and green reflect relatively high and low expression levels of genes, respectively, as indicated in the scale bar (a log2-transformed scale). (C) MDA-MB-231 breast cancer cells were transiently transfected with siFOXM1 or control siLuciferase (siLuc.) After 36h, cells were treated with Dox. We analyzed 30ng of total RNA from transfected cells by qRT-PCR using gene-specific primers as indicated. Student’s t-test (two-tailed) was used to estimate the significance of gene expression changes. All expression levels changed significantly compared with siLuc (P < 0.01). Results are shown as mean ± SD from three independent replicates. (D) MDA-MB-231 cells were transiently transfected with indicated gene-specific siRNA or siLuc as control. After 36h, cells were treated with Dox (1 or 10 µM) for 36h, and proliferation rates were measured by MTT assay. Values shown were normalized to control cells and represent mean ± SD. Student’s t-test was used for comparisons.
Fig. 4. FOXM1 regulates DNA repair genes. (A and B) Cells were transfected with siRNA or siLuc, and the HR repair rate of I-SceI-induced DSBs was measured in U2OS cells. Each value is relative to the percentage of GFP-positive cells in I-SceI-transfected cells without siRNA transfection. Results are shown as mean ± SD from three independent experiments; Student’s t-test was used for comparisons. Western blotting demonstrated effective FOXM1 knockdown. –I-SceI: absence of I-SceI endonuclease; +I-SceI: presence of I-SceI endonuclease. (C and D) MDA-MB-231 cells were transiently transfected with indicated reporters, cDNA (FOXM1) and siRNAs. Cells were used to measure luciferase activity. (E) Schematic representation of promoter region of DNA repair genes (EXO1, PLK4 and RFC4). (F) ChIP assay was performed in MDA-MB-231 cells with FOXM1 antibody. Recruitment of FOXM1 to the indicated gene promoters was analyzed using primers specific to the indicated promoter region. IgG was used as an internal control. All results are shown as mean ± SD from three independent replicates (*P < 0.05, **P < 0.01, ***P < 0.005).
Fig. 5. Proteomic screen identifies NFκB1 as a FOXM1 binding protein. (A) Schematic diagram of the proteomic screen for FOXM1 binding proteins responding to Dox using SILAC method. MDA-MB-231 cells were labeled with 13C-arginine, 13C-lysine (heavy medium) or 12C-arginine, 12C-lysine (light medium), respectively. Cells are treated with DMSO or Dox (5 µM) for 36 h, and cell lysates were used for mass spectrometry analysis. (B) Immunoprecipitation of cultured cells with anti-FOXM1 antibody. FOXM1 binding proteins. (C) Screen results using commercially available software showing the SILAC ratios (heavy/light) for the proteins identified by LC-MS/MS. Differentially bound proteins identified by LC-MS/MS are indicated on a log2 scale. Circles at the top represent proteins with increased interaction with FOXM1 in the presence of Dox, and those at the bottom represent proteins with decreased interaction with FOXM1 in the presence of Dox, is highlighted in red. (D) Coimmunoprecipitation (IP) of NFκB1 with FOXM1 analyzed by western blotting of MDA-MB-231 cells treated with DMSO or Dox. β-actin was used as a negative control. (E) MDA-MB-231 cells were transfected with indicated siRNAs in the presence of Dox or DMSO, and cell lysates were used for western blotting with indicated antibodies.
NFκB1 is a binding partner of FOXM1 conferring Dox resistance

We next sought to identify FOXM1 binding partners in Dox-treated cells by using a proteomic approach with the SILAC strategy. Whole-cell lysates from MDA-MB-231 cells grown in indicated culture conditions (Figure 5A) were immunoprecipitated with anti-FOXM1 antibody (Figure 5B). We identified 415 proteins as FOXM1 binding partners in conditions (Figure 5C and Supplementary Table 3 is available at https://academic.oup.com/carcin/article-abstract/33/10/1843/2463412). There were many DNA repair genes.

NFκB1 expression did not change (Figure 5E). Previous reports demonstrated that NFκB1 expression increased significantly on Dox treatment, whereas FOXM1 expression decreased (Figure 5D). In addition, NFκB1 in Dox-treated cells co-operatively interacts with FOXM1 to regulate expression of DNA repair genes and protect cancer cells from DSBs induced by Dox. This idea is further supported by our finding that NFκB1-depleted cells were also defective in HR (Figure 6A). Moreover, NFκB1-depleted cells became more sensitive to Dox treatment (Figure 6B) (27). Taken together, these results clearly indicate that NFκB1 in Dox-treated cells co-operatively interacts with FOXM1 to regulate expression of DNA repair genes and protect cancer cells from DSBs induced by Dox. This idea is further supported by our finding that NFκB1-depleted cells were also defective in HR (Figure 6C). Reporter assays with 6xFOXM1 responsive promoter (28) clearly demonstrated that FOXM1 is a bona fide coactivator of FOXM1 (Figure 6D). FOXM1 transcriptional activity was significantly decreased by silencing endogenous NFκB1 expression, but overexpression of exogenous NFκB1 rescued this decrease (Figure 6E).

Therapeutic efficacy of FOXM1 silencing with Dox treatment

Next, we further determined the effect of silencing FOXM1 expression on xenograft tumor growth with Dox treatment. As expected, treatment with control siRNA + Dox reduced tumor weight significantly compared with treatment with control siRNA alone (Figure 7A). Treatment with FOXM1 siRNA + Dox resulted in an even greater reduction in tumor weight compared with treatment with control siRNA + Dox or FOXM1 siRNA alone, recapitulating the effects of combination treatment in vitro. Efficient silencing of FOXM1 by siRNA was confirmed by immunostaining of FOXM1 combination...
treatment significantly activated caspase-3 and decreased cell proliferation, as evidenced by immunostaining of cleaved caspase-3 and Ki-67 (Figure 7B). These xenograft experiments clearly demonstrated that silencing FOXM1 can greatly improve the therapeutic efficacy of Dox in breast cancer.

Discussion

FOXM1 is the proliferation-specific oncogenic TF and overexpressed in a wide range of tumor types examined, including those of mammary, neural, gastrointestinal and reproductive origin (29). This expression pattern is attributed to the ability of FOXM1 to transactivate genes (SKP2, CDC25B, Aurora B Kinase and Survivin) required for cell cycle progression, suggesting that FOXM1 is a good therapeutic target for human cancer (29,30). Our systematic analysis in breast cancer suggests an oncogenic function for FOXM1, which is significantly higher in the basal-like subtype that comprise 75% of triple-negative tumors and shows poor prognosis (Figure 1) (31). Although many patients with ER-positive and human epidermal growth factor receptor 2 (HER2)-positive breast cancer have received great benefit from treatments targeting ER and HER2, chemotherapy is the only available systemic treatment for patients with triple-negative breast cancer, and for these patients, the prognosis remains very poor because of high recurrence rates after treatment and development of resistance during the course of treatment (32,33). Because chemotherapy is currently the sole option for basal-like patients, we explored whether FOXM1 expression had any effect on chemosensitivity in vitro. We observed a statistically significant increase in sensitivity to two TOP2A inhibitors, Dox and mitoxantrone but not etoposide, in FOXM1-depleted MDA-MB-231 (Figure 2). Dox treatment to cancer cell created double-stranded DNA breaks, and DNA repair genes were induced to rescue the cell from the DNA damage. Our gene expression data clearly demonstrated that DNA repair genes were

![Figure 7. Effect of silencing FOXM1 expression with or without Dox treatment on growth and apoptosis in an orthotopic breast cancer model. (A) Growth of MDA-MB-231 tumors in mice treated with FOXM1 siRNA-DOPC or control siRNA-DOPC + Dox. Tumor volume was measured at day 37. (B) Ki-67 and cleaved caspase-3 staining of MDA-MB-231 tumor tissues treated with FOXM1 siRNA, control siRNA or control siRNA + Dox. The positive cells per high-power field (HPF) were counted and are shown in the graphs. All results are shown as mean + SD. DOPC, dioleoyl phosphatidylcholine nanoliposomes.](https://academic.oup.com/carcin/article-abstract/33/10/1843/2463412)
directly regulated by FOXM1 (Figure 3). Thus, silencing FOXM1 expression significantly sensitizes basal-like breast cancer cells to Dox. Other investigators have also shown that FOXM1 might be involved in resistance to other chemotherapy drugs (cisplatin, paclitaxel and epirubicin) (34,35). As shown in Figure 2A, when compared with cisplatin, paclitaxel and Dox, depletion of FOXM1 expression greatly sensitized to Dox-resistant MDA-MB-231 cells to Dox. Furthermore, previous study also demonstrated that FOXM1 regulates DNA repair genes (XRCC1 and BRC2A2) and contributes to DNA damage response although they suggested different mechanism of action regarding FOXM1 function on DNA repair systems, suggesting FOXM1 clearly involved in DNA repair pathway via various repair mechanism (36). We further demonstrated that FOXM1-dependent resistance to Dox is mediated by FOXM1 interaction with NfxB1 to regulate multiple DNA repair genes and enhance HR (Figures 5 and 6). NfxB1 has been shown to be one of the principal mediators of resistance to chemotherapy (27). As a result, a number of pharmacological strategies to inhibit NfxB1 activation have been developed. Previous study suggested that suppression of NfxB1 overcomes Dox-resistance in vitro and in vivo (27).

Dox, which is a frequently used chemotherapeutic agent against breast cancer, has been reported to induce acute and chronic toxicities and immunosuppression (37,38). Thus, novel treatment options are needed to reduce dosages of Dox and consequently unwanted toxicities while enhancing its therapeutic efficacy in treating breast cancer (37). Recent study identified the thiazole antibiotics siomycin A and thiostraxen as potential inhibitors of FOXM1 (39). Thus, it will be interesting to test that combination of thiazole antibiotics with Dox would overcome resistance of breast cancer cells to Dox or reduce dosage of Dox in routine chemotherapies.

In this study, we have evaluated the potential therapeutic benefit of FOXM1 silencing with Dox in vitro cell culture systems and tested its potential clinical relevance in animal models of breast cancer. Thus, our study opens up new opportunities to test these new drugs as chemotherapy drugs in combination with Dox in future preclinical studies. Together, our results clearly demonstrate that FOXM1 is a promising therapeutic target to improve current systemic chemotherapy to treat breast cancer patients, especially those with triple-negative tumors.

Supplementary material
Supplementary Tables 1–4 and Figures 1–6 can be found at http://carcin.oxfordjournals.org/

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Conflict of Interest Statement: None declared.

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