Role of FoxO1 activation in MDR1 expression in adriamycin-resistant breast cancer cells

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Introduction
Cancer cells can acquire chemoresistance via the overexpression of drug efflux transporters such as multidrug resistance 1 (MDR1) and multidrug resistance-associated proteins which facilitate the efflux of diverse therapeutic agents out of cells and prevent the accumulation of cytotoxic drugs in tumor cells (1). MDR1 (or P-glycoprotein, ABCB1), the best characterized drug efflux pump, is a member of the ATP-binding cassette transporter family (2) and its gene expression levels are regulated by transcriptional and posttranscriptional processes (3). However, the molecular mechanisms on the regulation of MDR1 expression are not fully understood yet (4).

Breast cancer is the most common malignant disease in Western women (5). Increased MDR1 expression leads to chemoresistance in breast cancer cells, which tend to develop cross-resistance to other structurally and functionally diverse therapeutic agents out of cells and prevent the accumulation of cytotoxic drugs in tumor cells (1). MDR1 (or P-glycoprotein, ABCB1), the best characterized drug efflux pump, is a member of the ATP-binding cassette transporter family (2) and its gene expression levels are regulated by transcriptional and posttranscriptional processes (3). However, the molecular mechanisms on the regulation of MDR1 expression are not fully understood yet (4).

Materials
The anti-MDR1 antibody was supplied by Calbiochem (Darmstadt, Germany). The FoxO1 and FoxO3a antibodies were purchased from Cell Signaling Technology (Beverly, MA). The antibody against enhancer/enhancer-binding protein (CEBP) β was provided by Santa Cruz Biotechnology (Santa Cruz, CA). Mouse C-reactive protein and anti-rabbit IgG or anti-mouse IgG and alkaline phosphatase-conjugated antirabbit or antimouse IgG were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA). Most of the reagents used for molecular studies were obtained from Sigma (St Louis, MO). The small interfering RNA (siRNA) targeting human FOXO1 and C/EBPβ were acquired from Ambion (Austin, TX).

Preparation of nuclear extracts
Nuclear extracts were prepared essentially as described by Schreiber et al. (15). Briefly, cells in dishes were washed with ice-cold PBS, scraped, transferred to microtubes and allowed to swell after adding 100 µl of lysis buffer containing 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (pH 7.9), 0.5% Nonidet P-40, 10 mM KCl, 1 mM sodium orthovanadate, 0.1 mM sodium pyrophosphate, 1 mM phenylmethylsulfonyl fluoride and 1 µg/ml leupeptin. The cell lysates were incubated for 10 min on ice and centrifuged at 7200g for 5 min. Pellets containing crude nuclei were resuspended in 60 µl of extraction buffer containing 20 mM HEPES (pH 7.9), 400 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol and 1 mM phenylmethylsulfonyl fluoride and then incubated for 30 min on ice. The samples were then centrifuged at 15 800g for 10 min to obtain supernatants containing nuclear extracts, which were stored at –80°C until required.

Immunoblot analysis
After washing with sterile PBS, MCF-7 or MCF-7/ADR cells were lysed in EBC lysis buffer containing 20 mM Tris–HCl (pH 7.5), 1% Triton X-100, 137 mM sodium chloride, 10% glycerol, 2 mM EDTA, 1 mM sodium orthovanadate, 25 mM β-glycerophosphate, 2 mM sodium pyrophosphate, 1 mM phenylmethylsulfonyl fluoride and 1 µg/ml leupeptin. The cell lysates were centrifuged at 10 000g for 10 min to remove the debris, and the proteins were fractionated using a 10% separating gel. The fractionated proteins were then transferred electrophoretically to nitrocellulose paper, and the proteins were immunoblotted with specific antibodies. Horseradish peroxidase- or alkaline phosphatase-conjugated antirabbit or antimouse IgG were added, and the blots were incubated for 1 h at room temperature. The blots were washed with Tris-buffered saline, and the bands were visualized using a chemiluminescent detection system (ECL detection system; Amersham, UK). The proteins were quantified using a scanner and densitometric analysis (ImageJ, National Institutes of Health, Bethesda, MD). The results were presented as mean ± SD, and statistical comparisons were made using the Student’s t-test.
phosphatase-conjugated anti-IgG antibodies were used as the secondary antibodies. The nitrocellulose papers were developed using 5-bromo-4-chloro-3-indoly1 phosphate/4-nitroblue tetrazolium or an enhanced chemiluminescence system. For chemiluminescence detection, the LAS3000-mini (Fujifilm, Tokyo, Japan) was used.

**Rhodamine-123 retention assay**

The MCF-7 and MCF-7/ADR cells were seeded in 24-well plates. At 80% confluence, the cells were incubated in fetal bovine serum-free Dulbecco’s modified Eagle’s medium for 18 h. The culture medium was changed with Hanks’ balanced salt solution and the cells were incubated at 37°C for 30 min. After incubation of the cells with 20 μM rhodamine-123 (R-123) in the presence or absence of verapamil (100 μM) for 90 min, the medium was completely removed. The cells were then washed three times with an ice-cold phosphate buffer (pH 7.0) and lysed in EBC lysis buffer. The R-123 fluorescence in the cell lysates was measured using excitation and emission wavelengths of 480 and 540 nm, respectively. Fluorescence values were normalized to the total protein content of each sample and presented as the ratio to controls.

**Reporter gene assay**

Promoter activity was determined using a dual-luciferase reporter assay system (Promega). Briefly, cells (3 × 10⁴ cells per well) were plated in 12-well plates overnight and transiently transfected with the p-MDR1 Luc, hPXR reporter or PXR reporter plasmids/pTRI-SV plasmid (brenilla luciferase expression for normalization) (Promega) using Hilymax® reagent (Djoindo Molecular Tech., Gaithersburg, MD). The cells were then incubated in culture medium without serum for 18 h, and the firefly and brenilla luciferase activities in the cell lysates were measured using a luminometer (Berthold Tech.). The relative luciferase activities were calculated by normalizing the promoter-driven firefly luciferase activity to brenilla luciferase.

**Gel shift assay**

A double-stranded FoxO consensus oligonucleotide in the human MDR1 gene was used for gel shift analysis after end labeling the probe with [γ-32P] ATP using T4 polynucleotide kinase. The sequences of the FoxO1-binding site-containing oligonucleotide and C/EBP consensus oligonucleotide were (5′-TTCAACCCTTGTTTCG-3′) and (5′-TGCGAATGGCGAATCTGCA-3′), respectively. Reaction mixtures contained 4 μl of 5× binding buffer containing 20% glycerol, 5 mM MgCl₂, 250 mM NaCl, 2.5 mM EDTA, 2.5 mM dithiothreitol, 0.25 mg/ml poly dI-dC, 50 mM Tris–Cl (pH 7.5), 10 μg of nuclear extracts and sterile water to a total volume of 20 μl. The reaction mixtures were incubated for 10 min, and DNA-binding reactions were performed at room temperature for 20 min after adding 1 μl of probe (10⁶ c.p.m.). Binding specificity was determined with competition experiments, which were performed by adding a 10-fold excess of an unlabeled oligonucleotide to reaction mixtures before the DNA-binding reaction. For immunoinhibition assays, antibodies or normal rabbit serum (2 μg of each) were added to reaction mixtures after initial 20 min incubation, and then incubated for an additional 30 min at 25°C. Samples were loaded onto 4% polyacrylamide gels at 100 V, and gels were dried and autoradiographed using FLA-7000 (Fujifilm, Tokyo, Japan).

**Results**

**Overexpression and increased activity of MDR1 in MCF-7/ADR cells.** We performed a crystal violet staining assay to determine chemoresistance of MCF-7/ADR cells to doxorubicin (Dox). Dox treatment decreased MCF-7 cell viability in a concentration-dependent manner (3–100 μM), whereas Dox did not induce cell death in MCF-7/ADR cells over the drug concentration up to 10 μM (Figure 1a). Cotreatment of Dox with verapamil (an MDR1 inhibitor, 100 μM) (16) significantly potentiated Dox-mediated cell death in MCF-7/ADR cells (Figure 1a).

The basal expression levels of MDR1 in both MCF-7 and MCF-7/ADR cells were determined by western blot analysis. The MDR1 protein was highly expressed in MCF-7/ADR cells, but not detected in wild-type MCF-7 cells (Figure 1b). We further examined the transport activity of MDR1 in a R-123 retention assay. The intracellular accumulation ratio of R-123, a substrate of MDR1, was 2-fold lower in MCF-7/ADR cells than wild-type MCF-7 cells (Figure 1c). These results indicate that the increased activity of MDR1 in the MCF-7/ADR cells contributes to the chemoresistance acquisition of the cells.

A putative FoxO-binding site in the proximal promoter region of the human MDR1 gene

Human MDR1 gene expression is regulated by a number of transcription factors acting on the proximal region of the MDR1 promoter (17). For example, Sp1, nuclear factor-Y, activator protein-1 (c-Fos and c-Jun), p53, hypoxia-inducible factor-1α and Y-box-binding protein-l bind to corresponding sites (Figure 2a) and modulate MDR1 expression (4,18), as does C/EBPβ (18,19). The C/EBPβ-like binding motif (−148 to −140) in the MDR1 promoter may be important (19), as is the inverted enhancer box (Y-box, −82 to −73) (18). As shown in Figure 2a, we found a putative FoxO-binding site (5′-TGTTCG-3′, −150 to −144) (9), partly overlapped with the C/EBPβ-like motif in the MDR1 proximal promoter. We then determined basal reporter activity in MCF-7/ADR cells using p195-MDR1-Luc and p131-MDR1-Luc constructs. The p195-MDR1-Luc reporter contains a putative FoxO-binding site and C/EBPβ-like motif in the MDR1 promoter, whereas these sites are deleted in the p131-MDR1-Luc construct (Figure 2a, lower). p195-MDR1-Luc reporter activity was

**Figure 1.** Overexpression and increased activity of MDR1 in MCF-7/ADR cells. (a) Cell viability after Dox treatment. Cell viabilities were determined by crystal violet assays 24 h after exposure of MCF-7 and MCF-7/ADR cells with Dox (3, 10 and 100 μM) with or without verapamil (100 μM). Data represent means ± SDs of six separate samples. (b) Immunoblot analysis of MDR1. A representative immunoblot shows MDR1 protein in both MCF-7 and MCF-7/ADR cells deprived for 24 h. Equal loading of proteins was verified by actin immunoblot. (c) R-123 retention. After incubation of MCF-7 and MCF-7/ADR cells with 20 μM R-123 for 90 min, the R-123 fluorescence values in cell lysates were measured using the excitation and emission wavelengths of 480 and 540 nm, respectively. The values were divided by total protein content of each sample. Data represent means ± SDs of 10 separate samples (significant versus the control MCF-7 cells, ∗∗p < 0.01).
3.5-fold higher than p131-MDR1-Luc reporter activity in MCF-7/ADR cells (Figure 2b, left), but lower in control MCF-7 cells (Figure 2b, middle). Moreover, in comparison with MCF-7 cells, the basal p195-MDR1 reporter activity was significantly higher in MCF-7/ADR cells (Figure 2b, right). These data indicate that the −195 to −132 bp proximal promoter region containing FoxO (−150 to −144) and C/EBPβ-binding sites (−148 to −140) might be required for the enhanced MDR1 transcription in MCF-7/ADR cells.

Fig. 2. Activation of FoxO1 in MCF-7/ADR cells. (a) A putative binding site of FoxO in the proximal promoter region of human MDR1 gene. Upper: putative binding sites to transcription factors in the proximal promoter region of human MDR1 gene. Lower: structures of the p195-MDR1-Luc and p131-MDR1-Luc constructs. p195-MDR1-Luc (195 bp human MDR1 promoter) contains the overlapped putative binding site to FoxO and C/EBPβ in the MDR1 promoter. This site is deleted in the p131-MDR1-Luc construct (131 bp human MDR1 promoter). (b) Reporter activities of deletion mutant MDR1 promoters in MCF-7 (left panel) and MCF-7/ADR (middle panel) cells. Both the cells were transiently transfected with p195-MDR1-Luc or p131-MDR1-Luc plasmid. The basal p195-MDR1-Luc reporter activities were compared between MCF-7 and MCF-7/ADR cells (right panel). Dual-luciferase reporter assays were performed on the lysed cells cotransfected with pMDR1-Luc plasmid (firefly luciferase) and phRL-SV (hRenilla luciferase) (a ratio of 200:1) 18 h after transfection. Reporter gene activity was calculated as a relative ratio of firefly luciferase to hRenilla luciferase activity. Data represent means ± SDs with three different samples (significant versus the control, ***P < 0.001; control level = 1). (c) Nuclear levels of FoxO1 and Fox3a in the control and adriamycin-resistant MCF-7 cells. Western blot analysis was performed using nuclear extracts obtained from both the cells serum starved for 24 h, and the levels of FoxO1 and Fox3a in the nuclear fractions were immunochemically detected using specific antibodies. (d) Increase in FoxO1-binding activity in MCF-7/ADR cells. Nuclear fractions were isolated from MCF-7 and MCF-7/ADR cells serum deprived for 24 h. All lanes contained 10 μg of nuclear extracts and radiolabeled putative FoxO consensus sequence. Competition studies were carried out by adding a 10-fold excess of unlabeled FoxO oligonucleotide or FoxO1 antibody to the nuclear extracts. The arrowheads indicate FoxO bands.
Activation of FoxO1 in MCF-7/ADR cells
FoxO factors can be translocated through nuclear–cytoplasmic shuttling, which is regulated by phosphorylation, acetylation and interactions with other proteins (8). Therefore, the basal nuclear levels of FoxO proteins (FoxO1 and FoxO3a) were measured in both control MCF-7 and MCF-7/ADR cells by subcellular fractionation and western blot analyses. Nuclear FoxO1 levels were higher in MCF-7/ADR cells than in control MCF-7 cells, but there was no difference in nuclear levels of FoxO3a (Figure 2c).

To further examine whether the putative FoxO1-binding site in the human MDR1 promoter is functional, we performed a gel shift assay using FoxO1-overexpressing cells. Nuclear extracts were isolated from MCF-7 cells transfected with pCMV5-FoxO1 and incubated with a radiolabeled putative FoxO-binding oligonucleotide. The intensity of the slow migrating band was enhanced by the ectopic introduction of FoxO1 (data not shown).

We then compared basal FoxO-binding activities to assess whether FoxO1 accumulation in the nucleus of MCF-7/ADR cells increases its binding to the FoxO putative binding site. FoxO-binding activity was distinctly elevated in MCF-7/ADR cells compared with control MCF-7 cells (Figure 2d). Immunodepletion with a specific FoxO1 antibody showed that the increased DNA-binding activity was dependent on FoxO1 (Figure 2d). These results imply that FoxO1 is consistently activated in MCF-7/ADR cells and linked to the transactivation of MDR1.

Transactivation of MDR1 by FoxO1
We next tested whether FoxO1 overexpression stimulates MDR1 transcription. In control MCF-7 cells, FoxO1 overexpression dose dependently increased p195-MDR1-Luc reporter activity (3–30 ng, Figure 3a, left). We further determined the activity of a minimal reporter containing a FHRE after transfection of MCF-7 cells with pCMV5-FoxO1. FoxO1 induced a similar increase in FHRE promoter levels as the p195-MDR1-Luc reporter (Figure 3a, right), indicating that FoxO1 binding to its putative FoxO1-binding site stimulates MDR1 transcription.

We next measured MDR1 levels in MCF-7/ADR cells transfected with FoxO1 siRNA. FoxO1 siRNA distinctly reduced MDR1 expression in MCF-7/ADR cells (Figure 3b). FoxO1 siRNA also reduced p195-MDR1-Luc reporter activity (Figure 3c, left) and FHRE reporter activity (Figure 3c, right).
minimal promoter activity, confirming efficient blocking of FoxO1 (Figure 3c, right). These data suggest that FoxO1 upregulates MDR1 transcription.

**Insulin-mediated downregulation of MDR1 through FoxO1 inactivation**

Insulin signaling inhibits FoxO1 function through phosphorylation and subsequent nuclear exclusion of the protein (9,20). Treatment of MCF-7/ADR cells with insulin (0.01–1 μM) significantly decreased MDR1 protein levels in a concentration-dependent manner (Figure 4a). Subcellular fractionation and immunoblotting with a FoxO1 antibody also verified that FoxO1 translocated from the nucleus to the cytoplasm in response to insulin (0.3 μM) (Figure 4b). Insulin (0.3 μM) treatment also decreased FoxO-binding activity to the MDR1 promoter compared with untreated controls (Figure 4c). These data indicate that MDR1 overexpression in MCF-7/ADR cells is dependent on FoxO1 activation and can be inhibited by insulin, a FoxO1 inactivator.

We further assessed Dox-induced apoptosis in MCF-7/ADR cells after FoxO1 inactivation by siRNA introduction. Representative terminal dUTP nick-end labeling (TUNEL) assay photographs showed that exposure of control MCF-7 cells to Dox (30 μM) for 24 h caused severe apoptosis, but neither in MCF-7/ADR cells nor in control siRNA-treated cells (Figure 4d). However, TUNEL-positive cells were found in MCF-7/ADR cells preincubated with FoxO1 siRNA for 24 h (Figure 4d), suggesting that FoxO1-dependent MDR1 activation is associated with Dox resistance in MCF-7/ADR cells.

**Activation of C/EBPβ in MCF-7/ADR cells**

Since the putative FoxO1-binding site partially overlaps with the C/EBPβ-like motif, and C/EBPβ can regulate MDR1 transcription (19), C/EBPβ activation could also involve in MDR1 activation. In

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**Fig. 4.** Insulin-mediated MDR1 downregulation through FoxO1 inactivation. (a) Insulin-mediated MDR1 deregulation in MCF-7/ADR cells. Western blot analysis was performed in MCF-7/ADR cells treated with insulin (0.01–1 μM) for 24 h. (b) Effect of insulin on FoxO1 localization. Nuclear and cytoplasmic FoxO1 levels were measured by subcellular fractionation and immunoblotting in MCF-7/ADR cells incubated with insulin (0.3 μM) for 1–24 h. (C) FoxO1-binding activity change by insulin. Gel shift analysis was carried out using nuclear extracts prepared from MCF-7/ADR cells treated or untreated with insulin for 1–6 h. All lanes contained 10 μg of nuclear extracts and the labeled putative FoxO-binding sequence. Competition studies were performed by adding a 10-fold excess of unlabeled FoxO oligonucleotide to the nuclear extracts of control (insulin-untreated) cells. (d) Representative photographs of TUNEL assays on cells cultured with or without 30 μM Dox for 24 h. MCF-7/ADR cells were pretransfected with control siRNA or FoxO1 siRNA (10 pmole) 24 h before Dox exposure.
fact, nuclear C/EBPβ levels and DNA-binding activity to C/EBP consensus sequence were enhanced in MCF-7/ADR cells (Figure 5a and b, left). Moreover, addition of a specific C/EBPβ antibody to nuclear extracts reduced C/EBP band intensity and formed a supershifted band (Figure 5b, left). FoxO1 can directly bind to C/EBPβ via its forkhead domain and augment C/EBP transcriptional activity (21). However, although our FoxO-binding oligonucleotide contained a C/EBPβ-like binding motif, the anti-C/EBPβ antibody did not affect the FoxO1 band intensity (Figure 5b, right). These results demonstrate that the putative FoxO-binding site, previously known as a C/EBPβ-like binding motif, is solely regulated by FoxO1.

Because C/EBPβ affects MDR1 expression (18,19), we also tested whether the enhanced nuclear accumulation of C/EBPβ is essential for the induction of MDR1. Cells cotransfected with the p195-MDR1 Luc and a C/EBPβ-overexpressing plasmid (pC/EBPβ) had higher reporter activity compared with mock-transfected cells (3–30 ng, Figure 5c, left). However, almost a similar change also occurred following pC/EBPβ overexpression in cells transfecte

![Figure 5. Activation of C/EBPβ in MCF-7/ADR cells.](attachment:figure5.png)
Role of FoxO1 in MDR1 expression

p131-MDR1 Luc reporter (Figure 5c, right), indicating that C/EBPβ stimulates MDR1 transcription through the Y-box region rather than the C/EBPβ-like motif overlapped with the FoxO1-binding site. In addition, C/EBPβ inactivation by siRNA partially decreased MDR1 levels (Figure 5d), suggesting that a more complex regulatory pathway may involve in the MDR1 expression.

No activation of PXR in MCF-7/ADR cells

Although most transcription factors that control the MDR1 gene act on the proximal region of the promoter, a functional binding site of the PXR is located in the distal enhancer region (about −8 kb). PXR activation is required for the expression of the ATP-binding cassette transporters such as MDR1 and multidrug resistance-associated protein 2 (22,23). Hence, we measured PXR activity in MCF-7 and MCF-7/ADR cells using PXR reporter plasmid (three copies of the PXR response elements from CYP3A23) (23). The reporter activities were similar in the two cell lines (Figure 6), implying that PXR activation is not related to MDR1 transactivation in the MCF-7/ADR cells.

Discussion

MDR1-associated multidrug resistance has often been linked to the intrinsic or acquired chemoresistance to antineoplastic agents (24). Therefore, over the past decades, extensive work has been done to identify the effective MDR1 inhibitors to improve the effectiveness of anticancer drugs; however, the modulators of MDR1 transport activity often have severe side effects or affect the kinetics of other drugs (25). Considering that downregulation of MDR1 gene expression may be an alternative way for improving chemotherapy (25), elucidating the mechanistic basis of MDR1 expression should be important to identify the potential therapeutic target to overcome chemoresistance. Therefore, the present study investigated the role of FoxO1 in the regulation of MDR1 gene expression.

In this study, FoxO1 was specifically upregulated in MCF-7/ADR cells, suggesting that FoxO1 might impart the phenotype of drug resistance. Here, we identified a putative FoxO-binding site in the proximal promoter region (5′-TGTTCG-3′, −150 to −144) of the human MDR1 gene. In addition, we confirmed that FoxO1 binds there to stimulate MDR1 transcription. FoxO proteins can bind to insulin response sequences and recruit other transcription factors to their corresponding binding sites (9), including C/EBPβ binding to the decidual prolactin promoter (26). Moreover, the FoxO-binding site partially overlaps with the putative C/EBPβ-binding site (−148 to −140) (19), and C/EBPβ can activate MDR1 transcription (18,19). Therefore, we hypothesized that FoxO1 and C/EBPβ synergistically functioned as MDR1 transactivators through the FoxO-binding site. However, supershift analysis and promoter experiments verified that C/EBPβ did not interact with the FoxO-binding site and further suggested that C/EBPβ-induced MDR1 transactivation may be primarily mediated through its interaction at the Y-box region. In addition, when we determined MDR1 promoter activity in MCF-7 cells cotransfected with pC/EBPβ and pCMV5-FoxO1 plasmids, we could not find the synergistic increase of MDR1 promoter activity (data not shown). Hence, FoxO1 and C/EBPβ may not synergistically stimulate MDR1 gene transcription.

We further introduced the specific siRNA targeting FoxO1 or C/EBPβ to elucidate the potential role of these factors in MDR1 induction in the adriamycin-resistant MCF-7 cells. MDR1 expression in MCF-7/ADR cells was potently inhibited by FoxO1 siRNA, whereas C/EBPβ siRNA caused a marginal decrease in MDR1 protein levels, emphasizing the importance of FoxO1 for the MDR1 gene regulation. Moreover, insulin treatment which inhibits FoxO1 activity significantly reduced MDR1 expression, confirming the role of FoxO1 in the regulation of MDR1 expression. Finally, FoxO1 inactivation restored sensitivity to Dox. These results suggest that exaggerated FoxO1 activity is a main cause of adriamycin resistance in breast cancer.

Modulation of MDR1 shares mechanisms with other transporters such as multidrug resistance-associated proteins and drug-metabolizing enzymes (e.g. CYP3A4, UDP-glucuronosyltransferase and glutathione S-transferase) (22,23,27,28). FoxO function also shows complex regulation, depending on cell/tissue type, differentiation status and environment (11). Thus, these insights into FoxO1-mediated regulation of MDR1 may be applicable to other regulatory mechanisms, but may show variability depending on cell types and target proteins.

In summary, FoxO1 stimulates MDR1 transcription through binding to putative binding sites in the target gene promoter. FoxO1 is activated in MCF-7/ADR cells and required for MDR1 overexpression, which leads to chemoresistance. Thus, FoxO1 could be a novel therapeutic target for overcoming chemoresistance in breast cancer cells.

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References


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