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Role of FoxO1 in *MDR1* gene expression: Novel target to overcome chemoresistance in Adriamycin-resistant breast cancer cells

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약학과

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(국문 초록)

FoxO1 에 의한 MDR1 발현 조절 및 Adriamycin 저항성 유방암세포에서의 항암제 저항성과의 관련성 규명

한 창 엽

지도 교수 : 강 건 욱

조선대학교 대학원 약학과

MDR1 의 발현 및 활성 증가는 Adriamycin 저항성 유방암세포(MCF-7/ADR)를 포함하는 다양한 암세포의 화학요법 저항성 획득에 있어 중요한 원인 중의 하나이다. 이 논문에서 본인은 FoxO1 전사인자가

MDR1 의 발현을 조절하고, MCF-7/ADR 세포의 MDR1 발현 증가와 관련되어 있음을 규명하였다. MCF-7/ADR 세포는 대조군 유방암세포(MCF-7)와 비교하여 MDR1 의 발현 및 활성이 확연히 증가되어 있었으며, 이는 doxorubicin 저항성과 관계되었다. 또한 MDR1 유전자의 proximal promoter 부위에서 FoxO 결합부위로 추정되는 서열 (5'-TGTTTCG-3', -150/-144)을 발견하였고, 이 부위가 결합 활성이 있음을 증명하였다. 예상 FoxO 결합부위는 결손 변이 promoter 를 이용한 실험에서 MCF-7/ADR 세포의 MDR1 유전자 활성에 중요하게 작용하는 것으로 나타났고, MDR1 유전자는 FoxO1 에 의해 전사활성화가 증가하였다. MCF-7/ADR 세포의 핵 내에서 FoxO1 이 선택적으로 활성화되어 있었으며, 이 세포에서의 MDR1 발현 유도 및 전사활성화 정도가 FoxO1 siRNA 에 의해 유의적으로 억제되었다. 또한 FoxO1 의 기능을 생리적으로 불활성화 시키는 신호 중 하나인 insulin 에 의해서도 MCF-7/ADR 세포의 MDR1 발현이 감소함을 확인하였다. 이러한 결과들은 FoxO1 이 MDR1 유전자를 조절하는 새로운 전사인자로서 작용함을 시사하며, MCF-7/ADR 세포의 MDR1 발현 증가 및 화학요법 저항성에 관련되어 있음을 보여준다.

ABSTRACT

Role of FoxO1 in *MDR1* gene expression: Novel target to overcome chemoresistance in Adriamycin-resistant breast cancer cells

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Increased expression of MDR1 is believed to be one of the major causes for the chemoresistance acquisition of various cancer cells, including adriamycin-resistant breast cancer cells (MCF-7/ADR). Forkhead bOX-containing protein, O subfamily (FoxO) transcription factors are involved in the diverse cellular responses, such as differentiation, proliferation and metabolism, but possible roles of FoxO in the expression of drug efflux pumps have not been studied. In this study, we found that the expression and activity of MDR1 are enhanced in MCF-7/ADR cells and these are essential for the doxorubicin resistance. A putative binding site to FoxO was identified

at the proximal promoter region of human *MDR1* gene and this site was partially overlapped with C/EBPβ binding region. Gel shift and immunoblot analysis of subcellular fractions revealed that nuclear levels of FoxO1 and its DNA binding activity were selectively enhanced in MCF-7/ADR cells, which was reversed by FoxO1 antibody. Reporter gene assays showed that transcription of *MDR1* gene is stimulated by FoxO1 overexpression. Moreover, the expression and transactivation of *MDR1* gene in MCF-7/ADR cells were completely inhibited by FoxO1 siRNA. The MDR1 expression in MCF-7/ADR cells was also inhibited by a functional FoxO1 inactivator, insulin. In conclusion, FoxO1 functions as a novel transcriptional activator of *MDR1* gene and is crutial for MDR1 induction in MCF-7/ADR cells, which might be a therapeutic target to overcome doxorubicin resistance in MCF-7/ADR cells.

Keywords: chemoresistance, adriamycin-resistant breast cancer, MDR1, FoxO1

1. Introduction

Chemotherapy is the widespread treatment for various cancers. However, it is a serious problem that cancer cells can acquire chemoresistance to interrupt the successful therapy. Multi-drug resistance (MDR) is one of the main causes of chemoresistance of cancer cells, which is explained the phenomenon of concurrent resistance to unrelated anti-cancer agents (1, 2). Overexpression of drug efflux transporters such as MDR1 and multidrug resistance-associated proteins (MRPs) is considered to attribute multi-drug resistance by pumping out diverse therapeutic agents, leading to preventing accumulation of cytotoxic drugs into tumor cells (1).

MDR1 (or P-glycoprotein, ABCB1), the best characterized drug efflux pump, is a member of the ATP-binding cassette (ABC) transporter family that more includes MRP1 (ABCC1), MRP2 (ABCC2) and breast cancer resistance protein (BCRP, ABCG2) (3). A number of endogenous and exogenous stimuli, which induce cellular response, regulate the expression of MDR1 via transcriptional and post-transcriptional processes (4). Many studies have revealed possible candidates that control the expression and/or activity of MDR1,

however, molecular mechanistic basis for the MDR1 modulation has not been fully understood (5).

Breast cancer is the most frequent malignant disease in Western women. Chemo-resistance caused by MDR is the common clinical obstacle in the treatment of breast cancer. Increased expression and the subsequent activation of MDR1 are believed to be one of the major reasons for the chemoresistance acquisition of breast cancer cells, including adriamycin-resistant breast cancer cells (MCF-7/ADR), which have tendency to develop cross-resistance to other structurally or mechanistically dissimilar anti-cancer agents (6). Thus, it is important to reveal how the cells secure the resistance by MDR1 overexpression for the improvement of chemotherapy effectiveness.

Forkhead bOX-containing protein, O subfamily (FoxO) transcription factors contain four mammalian isoform members; FoxO1 (FKHR), FoxO3a (FKHRL1), FoxO4 (AFX), and FoxO6 (7). The modulation mechanisms of FoxO function are phosphorylation, acetylation, and ubiquitination, which affect nuclear-cytoplasmic translocation, DNA binding and protein-protein interactions, consequently leading to positive or negative control of target genes expression (8, 9). FoxO proteins play an important role in regulating a

variety of cellular processes, such as differentiation, metabolism, proliferation, and protection against oxidative stress (10). It has been presented that many of signaling pathways to control FoxO factors seem to be mis-regulated in several cancers (9, 11). In addition, FoxO1 is supposed to participate in transcriptional regulation of the glucose transporter-4 isoform (*GLUT4*) gene (12, 13). However, possible roles of FoxO in the expression of drug efflux pumps and chemoresistance acquisition of cancer cells have not been studied. In the present study, we demonstrate for the first time that FoxO1 is potently activated in MCF-7/ADR cells and functions as a novel transcription factor to control *MDR1* gene expression, which might be a therapeutic target to overcome chemoresistance in MCF-7/ADR cells.

2. Materials and Methods

2-1. Materials

The anti-MDR1 antibody was supplied by Calbiochem (Darmastadt, Germany). The FoxO1 and FoxO3a specific antibodies, the horseradish peroxidase-conjugated anti-rabbit and anti-mouse IgGs were purchased from Cell Signaling Technology (Beverly, MA). The antibody against C/EBPβ and the alkaline phosphatase-conjugated donkey anti-mouse IgG were provided by Santa Cruz Biotechnology (Santa Cruz, CA) and Jackson Immunoresearch Laboratories (West Grove, PA), respectively. Most of the reagents used for molecular studies were obtained from Sigma (St. Louis, MO). The siRNA targeting human FoxO1 and C/EBPβ were acquired from Ambion (Austin, TX).

2-2. Cell culture

The MCF-7 cells and the adriamycin-resistant MCF-7 (MCF-7/ADR) cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 100 units/ml penicillin, and 100 μg/ml streptomycin at 37°C in a 5 % CO_2

humidified atmosphere. The MCF-7/ADR cells were kindly supplied by Dr. Hoo Kyun Choi (Chosun University, Gwang-ju, Korea).

2-3. Plasmids

The p195-MDR1 and p131-MDR1 reporter plasmids were generated by ligating PCR-amplified MDR1 promoter regions with pGL3-enhancer vector (Promega, Madison, WI). The FoxO1 forkhead response element (FHRE) containing minimal reporter plasmid and pCMV5-FoxO1 overexpression plasmid was supplied from Addgene Inco. (Cambridge, MA). The PXR reporter plasmid containing three copies of the pregnane X receptor (PXR) responsive elements from the CYP3A23 gene and pGL3-MRP2-1 (rat MRP2 gene promoter) was a kind gift from Dr. Edward PA (University of California Los Angeles, Los Angeles, CA).

2-4. Crystal violet assay

Cell viability was determined by the crystal violet staining as previously described (14). In brief, cells were stained with 0.4% crystal violet in methanol for 30 min at room temperature (RT), and then washed with tap water. Stained cells were extracted with 50% methanol,

and dye extracts were measured at a 550 nm wavelength using a microtiter plate reader (Berthold Tech., Bad Wildbad, Germany).

2-5. 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 HEPES (pH 7.9), 0.5% Nonidet P-40, 10 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol and 0.5 mM phenylmethylsulfonylfluoride. Cell membranes were disrupted by vortexing, and the lysates were incubated for 10 min on ice and centrifuged at 7,200g 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 phenylmethylsulfonylfluoride, 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.

2-6. Immunoblot analysis

After washing with sterile PBS, the MCF-7 or MCF-7/ADR cells were lysed in EBC lysis buffer containing 20 mM Tris·Cl (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 phenylmethylsulfonylfluoride, and 1 μg/ml leupeptin. The cell lysates were centrifuged at 10,000*g* 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 the specific antibodies. Horseradish peroxidase- or alkaline phosphatase-conjugated anti-IgG antibodies were used as the secondary antibodies. The nitrocellulose papers were developed using 5-bromo-4-chloro-3-indolylphosphate (BCIP)/4-nitroblue tetrazolium (NBT) or an ECL chemiluminescence system. For chemiluminescence detection, the LAS3000-mini (Fujifilm, Tokyo, Japan) was used.

2-7. Rhodamine-123 retention assay

The MCF-7 and MCF-7/ADR cells were seeded in 24-well plates. After 80% confluency reached, the cells were incubated in FBS-free DMEM for 18 h. The culture medium was changed with Hanks'

balanced salt solution and the cells were preincubated at 37°C for 30 min. After incubation of the cells with 20 μM rhodamine-123 in the presence or absence of verapamil (100 μM) for 1 h 30 min, the medium was completely removed. Then, the cells were washed three times with an ice-cold phosphate buffer (pH 7.0) and lysed in EBC lysis buffer. The rhodamine-123 fluorescence in the cell lysates was measured using the excitation and emission wavelengths of 480 and 540 nm, respectively. After the fluorescence values were normalized by total protein contents of each sample, the divided values were represented by ratio to control.

2-8. Reporter gene assay

The promoter activity was determined using a dual-luciferase reporter assay system (Promega, Madison, WI). Briefly, the cells (3×10^5 cells/well) were replated in 12-well plates overnight and transiently transfected with the p-MDR1 Luc, FHRE reporter and PXR reporter plasmids/phRL-SV plasmid (*hRenilla* luciferase expression for normalization) (Promega, Madison, WI) using Hilymax® reagent (Dojindo Molecular Tech., MD). The cells were then incubated in the culture medium without serum for 18 h, and the firefly and hRenilla

luciferase activities in the cell lysates were measured using a luminometer (Berthold Tech., Bad Wildbad, Germany). The relative luciferase activities were calculated by normalizing the promoter-driven firefly luciferase activity versus hRenilla luciferase.

2-9. Electrophoretic mobility shift assay (Gel shift analysis)

A double stranded FoxO consensus oligonucleotide in human *MDR1* gene was used for gel shift analysis after end-labeling the probe with [γ-^{32}P] ATP using T4 polynucleotide kinase. The sequences of FoxO1 binding site-containing oligonucleotide and C/EBP consensus oligonucleotide were (5'-TTCAACCTGTTTCGCAGTTTC-3') and (5'-TGCAGATTGCGCAATCTGCA-3'), respectively. Reaction mixtures contained 4 μl of 5× binding buffer containing 20% glycerol, 5 mM $MgCl_2$, 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 or 15 μg of nuclear extracts, and sterile water to a total volume of 20 μl. The reaction mixtures were preincubated for 10 min. DNA-binding reactions were carried out at room temperature for 20 min after adding 1 μl of probe (10^6 cpm). Binding specificity was determined by using competition experiments, which were carried out by adding a 10-fold excess of an

unlabeled oligonucleotide to reaction mixtures before the DNA-binding reaction. For immuno-inhibition assays, antibodies or normal rabbit serum (2 μg of each) were added to reaction mixtures after an initial 20 min incubation, and then incubated for additional 1 h 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).

2-10. Data analysis

One way analysis of variance (ANOVA) was used to assess significant differences between the treatment groups. The Newman-Keuls test was used to compare multiple group means for each significant effect of treatment. Statistical significance was set at either $p<0.05$ or $p<0.01$.

3. Results

3-1. Overexpression and increased activity of MDR1 in MCF-7/ADR cells

Crystal violet staining assay was performed to determine chemoresistance of MCF-7/ADR cells to doxorubicin (Dox). The cell viability of MCF-7 cells was decreased by Dox treatment in a concentration-dependent manner (3-100 μM). In contrast, Dox up to 10 μM did not induce cell death in MCF-7/ADR cells (Figure 1A). Co-treatment of MCF-7/ADR cells with verapamil (a chemical MDR1 inhibitor, 100 μM)(16) significantly potentiated Dox-mediated cell death (Fig. 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 the MCF-7/ADR cells, while it was not detected in the wild-type MCF-7 cells (Fig. 1B). We further examined transport activity of MDR1 by Rhodamine-123 (R-123) retention assay to test the functional relevance of the MDR1 overexpression in MCF-7/ADR cells. The intracellular accumulation ratio of R-123, a substrate of MDR1, was 2-fold lower in the MCF-

7/ADR compared with the wild-type MCF-7 cells (Fig. 1C). These results indicate that the increased expression and the subsequent activation of MDR1 in the MCF-7/ADR cells contribute to the chemoresistance acquisition of the cells.

3-2. A putative binding site to FoxO in the proximal promoter region of human MDR1 gene

It has been implicated that the expression of human *MDR1* gene is regulated by a number of transcription factors mostly acting on the proximal region of MDR1 promoter (17). For example, Sp1, NF-Y, AP-1 (c-Fos and c-Jun), p53, HIF-1α, and YB-1 bind to their corresponding site(s) (Fig. 2A, upper) and modulate the MDR1 expression (5, 18). In addition, emerging evidence supports that C/EBPβ is one of key transcription factors to control the expression of *MDR1* gene (18, 19). It was suggested that C/EBPβ-binding like motif (-148 to -140) in the MDR1 promoter would be involved in transactivation of *MDR1* gene (19). However, Chen GK et al. (18) have presented that the inverted CCAAT box (Y-box, -82 to -73) is required for MDR1 transactivation induced by C/EBPβ.

Interestingly, we found a putative binding site to FoxO (5'-

TGTTTCG-3', -150 to -144) (9), partly overlapped with C/EBPβ-binding like motif in the MDR1 proximal promoter (Fig. 2A). To examine a possibility that FoxO binding to its putative binding site has a role in the transcriptional regulation of *MDR1* gene, the basal reporter activities were determined in the MCF-7/ADR cells using p195-MDR1-Luc and p131-MDR1-Luc constructs. As shown in Figure 2A (lower), p195-MDR1-Luc reporter contains a putative FoxO binding site and C/EBPβ-binding like motif in the MDR1 promoter, whereas these sites are deleted in the p131-MDR1-Luc construct. In MCF-7/ADR cells, p195-MDR1-Luc reporter activity was 3.5-fold increased compared to p131-MDR1-Luc reporter activity (Fig. 2B, left). In contrast, the p195-MDR1-Luc reporter activity was rather lower than p131-MDR1-Luc reporter activity in control MCF-7 cells (Fig. 2B, right). These data indicate that -195 ~ -132 bp proximal promoter region containing FoxO (-150 to -144) and C/EBPβ-binding site (-148 to -140), might be selectively required for the transcriptional activation of *MDR1* gene in MCF-7/ADR cells.

3-3. Activation of FoxO1 in MCF-7/ADR cells

We then assessed if FoxO transcription factor(s) is activated in

MCF-7/ADR cells. FoxO factors can be translocated through nuclear-cytoplasmic shuttling, which is regulated by status of phosphorylation, acetylation and interactions with other proteins (8). Therefore, the basal nuclear levels of FoxO proteins (FoxO1 and FoxO3a) were measured in both the control MCF-7 and the MCF-7/ADR cells by subcellular fractionation and Western blot analyses. The nuclear FoxO1 level in MCF-7/ADR cells was higher than that in control MCF-7 cells; while, there was no difference in the nuclear levels of FoxO3a between the two cell types (Fig. 2C).

To further examine whether the FoxO1 putative binding site in human MDR1 promoter is functional, gel shift assay was performed using FoxO1 overexpressed cells. The nuclear extracts were isolated from the MCF-7 cells transfected with pCMV5-FoxO1 and incubated with the radiolabeled putative FoxO binding oligonucleotide. The intensity of slow migrating band was enhanced by the ectopic introduction of FoxO1 (10 or 30 ng pCMV5-FoxO1, Fig. 2D, left). Moreover, FoxO1 binding activity was completely reversed by a 10-fold excess unlabeled putative FoxO binding oligonucleotide in nuclear extracts, which confirmed the specificity of protein binding (Fig. 2D, left).

We then compared the basal FoxO binding activities between MCF-7 and MCF-7/ADR cells to assess whether the FoxO1 accumulation to the nucleus of MCF-7/ADR cells causes an increase in its binding to the FoxO putative binding site. As shown in Fig. 2D (right), FoxO binding activity was distinctly elevated in MCF-7/ADR cells compared to control MCF-7 cells. Immunodepletion experiment using specific FoxO1 antibody showed that the increased DNA binding activity was dependent on FoxO1 (Fig. 2D, right). These results imply that FoxO1 is consistently activated in MCF-7/ADR cells and raise a possibility that the activation of FoxO1 is linked with the transactivation of *MDR1* gene.

3-4. Transactivation of MDR1 gene by FoxO1

We next tested whether FoxO1 overexpression stimulates the *MDR1* gene transcription. In control MCF-7 cells, the p195-MDR1-Luc reporter activity was significantly elevated by FoxO1 overexpression in a concentration-dependent manner (3-30 ng, Fig. 3A left). We further determined the activity of forkhead-response element (FHRE) containing minimal reporter after transfection of MCF-7 cells with pCMV5-FoxO1. The FoxO1-inducible increase ratio of FHRE

promoter was almost similar to that of p195-MDR1 Luc reporter (Fig. 3A, right), which indicates that FoxO1 binding to its putative FoxO1 binding site in human *MDR1* gene result in transcriptional activation of the gene.

To clarify whether the consistently activated FoxO1 plays a key role in the MDR1 expression in MCF-7/ADR cells, Western blot analysis was performed in the cells introduced with specific FoxO1 siRNA. After transfection of the MCF-7/ADR and the control MCF-7 cells with FoxO1 siRNA, the enhanced expression of MDR1 in MCF-7/ADR cells was drastically reversed by FoxO1 siRNA (Fig. 3B). When we assess the level of FoxO1 in control and MCF-7/ADR cells, the FoxO1 expression was efficiently blocked by FoxO1 siRNA in both the cell types (Fig. 3B, lower band). We then performed reporter gene assays using p195-MDR1 Luc in MCF-7/ADR cells co-transfected with FoxO1 siRNA. The p195-MDR1 Luc reporter activity in MCF-7/ADR cells was significantly diminished by FoxO1 siRNA introduction (Fig. 3C, left). FHRE minimal promoter activity was also attenuated by FoxO1 siRNA, confirming the efficient blocking of FoxO1 (Fig. 3C, right). These data suggest that FoxO1 plays a critical role in the transcription of human *MDR1* gene as a positive regulator, leading to

up-regulation of MDR1.

3-5. Insulin-mediated MDR1 down-regulation through FoxO1 inactivation

Insulin signaling has been known to negatively regulate FoxO1's transcriptional function through phosphorylation and the subsequent nuclear exclusion of the protein (9, 20). To further investigate whether insulin-induced FoxO1 inactivation causes a reduction in the MDR1 expression, we carried out Western blot analyses using MCF-7/ADR cells. Treatment of MCF-7/ADR cells with insulin (0.01-1 μM) significantly decreased the MDR1 protein level in a concentration-dependent manner (Fig. 4A). Subcellular fractionation and immunoblotting with FoxO1 antibody also verified that FoxO1 was translocated from the nucleus to the cytoplasm in response to insulin (0.3 μM) (Fig. 4B). Gel shift assay was additionally performed using the nuclear extracts prepared from the insulin or vehicle-treated MCF-7/ADR cells in order to determine if the binding activity of FoxO1 to MDR1 promoter was altered by insulin treatment. Insulin (0.3 μM) treatment decreased the FoxO binding activity compared to untreated control (Fig. 4C). The data indicate that MDR1 overexpression in

MCF-7/ADR cells is dependent on FoxO1 activation and this response is reversed by physiological FoxO1 inactivator, insulin.

3-6. Activation of C/EBPβ in MCF-7/ADR cells

Since the putative FoxO1 binding site is partly overlapped with C/EBPβ-binding like motif and C/EBPβ is known as a positive regulator of *MDR1* gene transcription (19), the activation of C/EBPβ would be also cooperatively involved in MDR1 activation. In comparison to control MCF-7 cells, nuclear C/EBPβ and DNA binding activity to C/EBP consensus sequence were enhanced in MCF-7/ADR cells (Fig. 5A and 5B, left). Moreover, addition of specific antibody against C/EBPβ to nuclear extracts caused a complete reduction in C/EBP band intensity and a formation of super-shifted band (Fig. 5B, left), demonstrating C/EBPβ was consistently activated in adriamycin-resistant breast cancer cells. It has been recently reported that FoxO1 directly binds to C/EBPα via its forkhead domain and augments C/EBP-dependent transcriptional activity (21). Hence, we assessed whether the enhanced binding activity to putative FoxO binding oligonucleotide was associated with C/EBPβ activation in MCF-7/ADR cells. Although our FoxO binding oligonucleotide contains C/EBPβ-

binding like motif, anti-C/EBPβ antibody did not affect the FoxO1 band intensity (Fig. 5B, right). These results clearly demonstrate that the putative FoxO binding site, previously known as C/EBPβ-binding like motif, is solely dependent on FoxO1.

Because C/EBPβ acts as one of main transcription factors in *MDR1* gene expression (18, 19), we then tested whether the enhanced nuclear accumulation of C/EBPβ in MCF-7/ADR cells is essential for the induction of MDR1. In the MCF-7 cells co-transfected with the p195-MDR1 Luc and the C/EBPβ-overexpressing plasmid (pC/EBPβ), the reporter activity was increased, compared to the Mock-transfected cells (3-30 ng, Fig. 5C, left). However, the similar elevation intensity was also observed by pC/EBPβ overexpression in the cells transfected with p131-MDR1 Luc reporter (Fig. 5C, right). These results suppose that human *MDR1* gene transactivation is also stimulated by C/EBPβ possibly through its interaction with Y-box region, but not with C/EBPβ-like motif overlapped with FoxO1 binding site.

We also determined the effect of C/EBPβ siRNA on the MDR1 expression in MCF-7/ADR cells. C/EBPβ inactivation by specific siRNA introduction partially decreased the MDR1 protein levels in MCF-7/ADR cells (Fig. 5D), which raise a possibility that C/EBPβ

activation would be also partly involved in the induction of MDR1 in the adriamycin-resistant breast cancer cells.

4. Discussion

MDR1-associated multi-drug resistance is involved in the intrinsic or acquired chemoresistance of various tumor cells to a number of anti-neoplastic agents (22). Breast cancer cells resistant to adriamycin which is one of the commonly used therapeutic drugs against the malignant disease, have the characteristic of MDR1 overexpression (6). In this study, we also showed the essential role of MDR1 in the doxorubicin-resistance of MCF-7/ADR cells, which was evidenced by the results showing that a P-glycoprotein inhibitor, verapamil potentiated the cytotoxicity of doxorubicin. Unfortunately, many MDR modulators have not been successful in clinical trials because they have severe side effects at the effective dose ranges for the inhibition of MDR1 and can affect the kinetics of other drugs (23). Discovery of compounds and strategies to decrease the expression of MDR1 has been considered to be more useful in altering the MDR phenotype and improving chemotherapy effectiveness (23). Thus, elucidating the mechanistic basis for the regulation of MDR1 expression could be important for the advance of therapeutics of chemotherapy-resistant malignancies. Here, we demonstrate for the

first time that FoxO1 is a novel transcriptional regulator of *MDR1* gene and may offer one of the solutions to overcome the chemoresistance of MCF-7/ADR cells.

Among the mammalian FoxO subgroup, FoxO1 is the most explored member. Many molecular and genetic approaches have shown the diverse functions of FoxO1, namely associated with normal development, metabolism, differentiation, tumor suppression, and angiogenesis (7, 9, 10, 24). A recent study has presented that FoxO3 is involved in the expression of ROS scavenging enzymes and is required for the modulation of oxidative stress in erythropoiesis (25). When we compared the nuclear levels of FoxO1 and FoxO3 between MCF-7 and MCF-7/ADR cells, FoxO1 was specifically up-regulated in MCF-7/ADR cells. Hence, we hypothesized that FoxO1 might be related with the phenotype of adriamycin-resistant MCF-7 cells.

In the present study, we identified a putative binding site to FoxO protein(s) at the proximal promoter region (5'-TGTTTCG-3', -150 to -144) in human *MDR1* gene. In addition, we confirmed that this site is indeed functional for the binding of FoxO1 factor and further transcription of *MDR1* gene is stimulated by FoxO1 overexpression. It has been presented that FoxO proteins can bind to insulin response

sequences (IRSs) and recruit other transcription factors to their corresponding binding sites (9). C/EBPβ can be gathered and binds to the decidual prolactin promoter by FoxO factors, thereby enhancing the activation (26). Moreover, the FoxO binding site is partially overlapped with the putative C/EBPβ binding site (-148 to -140) (19) and C/EBPβ has been known as a transcriptional activator of *MDR1* gene (18, 19). Therefore, we hypothesized that FoxO1 and C/EBPβ synergistically function as transactivators of the human *MDR1* gene through the FoxO binding site. However, super-shift analysis result verified that C/EBPβ did not interact with the putative binding site to FoxO which overlaps with the C/EBPβ binding-like motif. Considering our result that p131-MDR1 reporter activity was almost similarly increased by C/EBPβ overexpression compared with p195-MDR1 reporter activity, C/EBPβ-induced MDR1 transactivation may be mainly mediated through its interaction at the Y-box region (-82 to -73), not through the putative FoxO1 binding site overlapped C/EBPβ-like motif.

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, since FoxO1 and C/EBPβ stimulated the transcription of *MDR1* gene and both the factors

were activated in the MCF-7/ADR cells. In this study, the MDR1 expression in MCF-7/ADR cells were potently inhibited by FoxO1 siRNA, while C/EBPβ siRNA caused a marginal decrease in MDR1 protein levels, emphasizing the importance of FoxO1 for the *MDR1* gene regulation. Moreover, the role of FoxO1 in the regulation of MDR1 expression was also confirmed by insulin treatment experiments. We found that insulin acting as a functional inhibitor of FoxO1 (9), significantly reduced MDR1 expression in MCF-7/ADR cells. Finally, we revealed that the diminished responsiveness of doxorubicin cytotoxicity in MCF-7/ADR cells was recovered by FoxO1 inactivation. These results raise a possibility that exaggerated FoxO1 activity is a main cause of adriamycin resistance in breast cancer.

Although the majority of transcription factors to control the human *MDR1* gene act on the proximal region of the promoter, a functional binding site of Pregnane X Receptor (PXR) is located in the distal enhancer region (about -8 kb) of the human *MDR1* gene. It has been reported that PXR activation is required for the expression of the ABC transporters such as MDR1 and MRP2 (27, 28). Hence, we compared the activities of PXR in the wild-type and adriamycin-resistant MCF-7 cells using the PXR reporter plasmid (three copies of

the PXR response elements from *CYP3A23* gene) (28). The reporter activities were similar between the two cells (data not shown), implying that PXR activation might be not related with the MDR1 transactivation in the MCF-7/ADR cells.

It has been appeared that modulation mechanisms of MDR1 are partly shared with that of other transporters such as MRPs and some drug-metabolizing enzymes (e.g., CYP3A4, UDP-glucuronosyltransferase and glutathione S-transferase) and these have a tendency of similar physiologic control (27-30). FoxO function is integrated by multiple and complex regulatory mechanisms, which alter depending on context including cell/tissue type, differentiation status, and environment (11). Thus, our new findings about the regulation of *MDR1* gene by FoxO1 might be able to offer opportunities for understanding regulation mechanisms of these transporters and metabolizing enzymes, though there should be still large variations among the cell and the target protein types.

In summary, FoxO1 positively regulates *MDR1* gene transcription through its binding to putative binding site in the target gene promoter. FoxO1 is consistently activated in MCF-7/ADR cells, which is essential for the overexpression of MDR1 leading to

chemoresistance. Thereby, FoxO1 could be proposed as a novel therapeutic target to overcome the chemoresistance of adriamycin-resistant breast cancer cells.

5. References

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the receptor to the gene. Can J Physiol Pharmacol. 2006;84:713-24.

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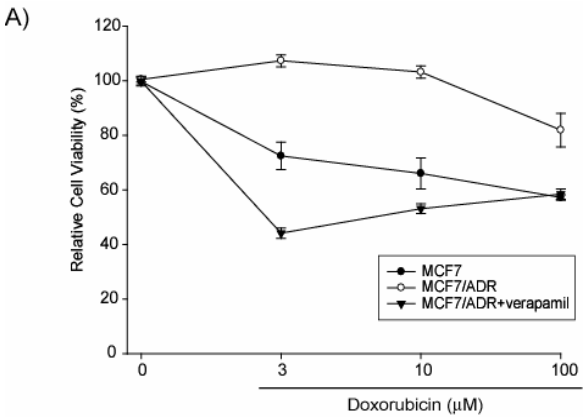
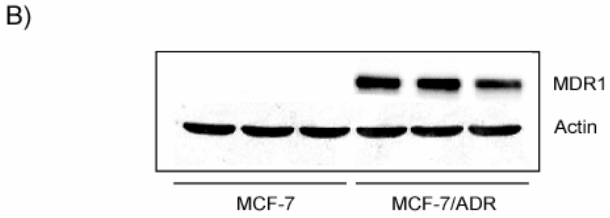
6. Figure Legends





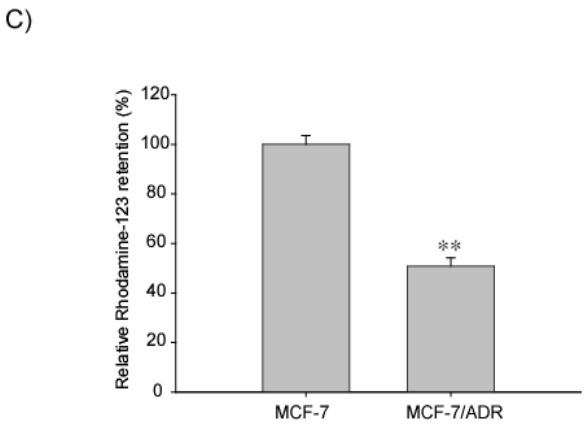
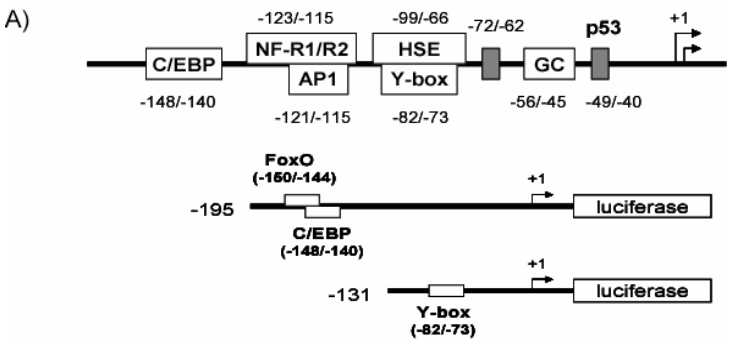
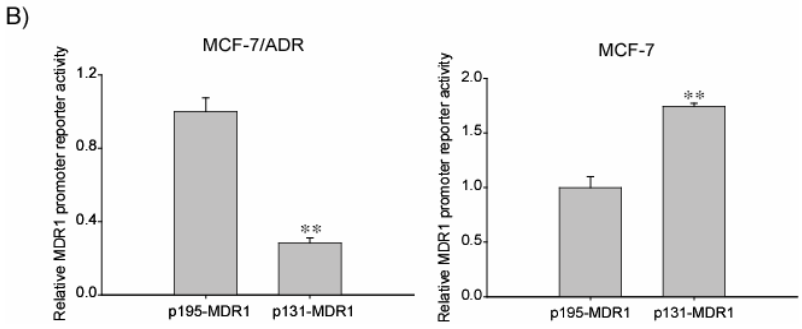
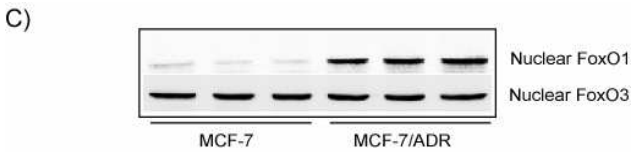


Figure 1. Overexpression and increased activity of MDR1 in MCF-7/ADR cells. (A) Cell viability after treating with doxorubicin. After treating MCF-7 and MCF-7/ADR cells with doxorubicin (3, 10, 100 μM) in the presence or absence of verapamil (100 μM) for 24 h, cell viabilities were determined using crystal violet assays. Data represents means ± SD of 6 separate samples (B) Immunoblot analysis of MDR1. A representative immunoblot shows MDR1 protein in both MCF-7 and MCF-7/ADR cells serum-deprived for 24 h. Equal loading of proteins was verified by actin immunoblot. (C) Rhodamine-123 retention. After incubation of MCF-7 and MCF-7/ADR cells with 20 μM R-123 for 1 h 30 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 contents of each sample. Data represents means ± SD of 10 separate samples (significant versus the control MCF-7 cells, **$p<0.01$).


A)
-123/-115
-99/-66
-72/-62
p53
+1
C/EBP
NF-R1/R2
HSE
GC
AP1
Y-box
-148/-140
-121/-115
-82/-73
-56/-45
-49/-40
FoxO
(-150/-144)
+1
-195
luciferase
C/EBP
(-148/-140)
+1
-131
luciferase
Y-box
(-82/-73)


B)
MCF-7/ADR
Relative MDR1 promoter reporter activity
1.2
0.8
0.4
0.0
**
p195-MDR1
p131-MDR1
MCF-7
Relative MDR1 promoter reporter activity
2.0
1.5
1.0
0.5
0.0
**
p195-MDR1
p131-MDR1


C)
Nuclear FoxO1
Nuclear FoxO3
MCF-7
MCF-7/ADR

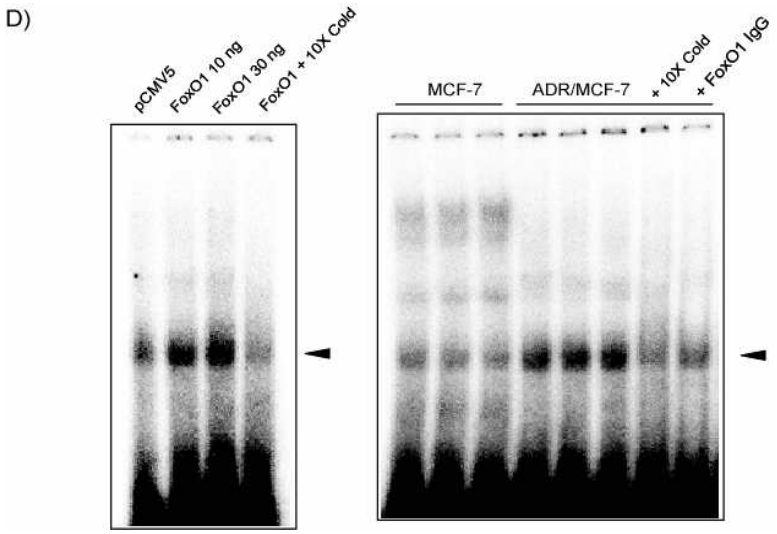
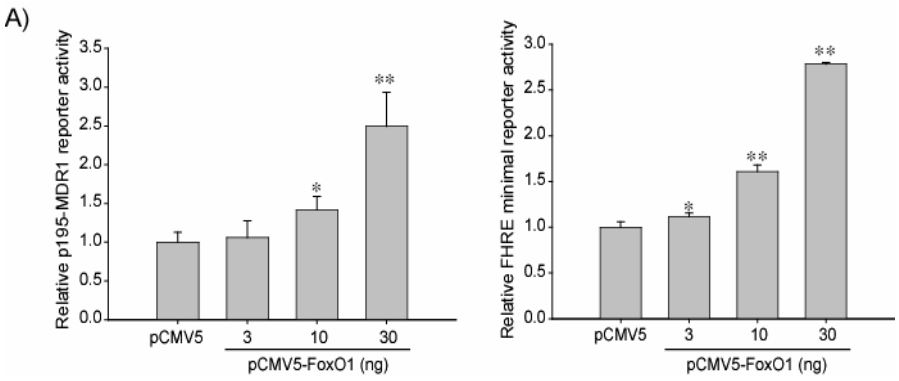
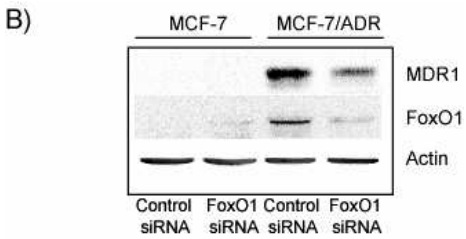

D)
pCMV5
FoxO1 10 ng
FoxO1 30 ng
FoxO1 + 10X Cold
MCF-7
ADR/MCF-7
+ 10X Cold
+ FoxO1 IgG

Figure 2. Activation of FoxO1 in MCF-7/ADR cells. (A) A putative binding site to FoxO in the proximal promoter region of human *MDR1* gene. Upper panel; Putative binding sites to transcription factors in the proximal promoter region of human *MDR1* gene. Lower panel; Structures of the p195-MDR1-Luc and p131-MDR1-Luc constructs. p195-MDR1-Luc (195 bp human MDR1 promoter) contains the putative binding site to FoxO and the C/EBPβ binding-like motif in the MDR1 promoter. These sites are deleted in the p131-MDR1-Luc construct (131 bp human MDR1 promoter). (B) Reporter activities of deletion mutant MDR1 promoters in MCF-7 (right panel) and MCF-7/ADR (left panel) cells. Each cell type was transiently transfected with p195-MDR1-Luc or p131-MDR1-Luc plasmid. Dual luciferase reporter assays were performed on the lysed cells co-transfected with pMDR1-Luc plasmid (firefly luciferase) and phRL-SV (*hRenilla* luciferase) (a ratio of 100:1) 18 h after transfection. Reporter gene activation was calculated as a relative ratio of firefly luciferase to h*Renilla* luciferase activity. Data represent means ± SD with 3 different samples (significant versus the control, **$p<0.01$; control level = 1). (C) Nuclear levels of FoxO1 and FoxO3a in the control and adriamycin-resistant MCF-7 cells. Western blot analysis was performed using nuclear

extracts obtained from the both cells serum-starved for 24 h, and the proteins in each fraction were detected immunochemically with specific antibody. (D) Left panel; FoxO1 binding to the putative binding site in human MDR1 promoter. Nuclear extracts were prepared from MCF-7 cells transfected with pCMV5-FoxO1 (10, 30 ng) or pCMV5. Right panel; 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. DNA-binding reactions were performed by gel shift analysis.


A)
Relative p195-MDR1 reporter activity
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
pCMV5
3
10
30
pCMV5-FoxO1 (ng)
Relative FHRE minimal reporter activity
3.0
2.5
2.0
1.5
1.0
0.5
0.0
pCMV5
3
10
30
pCMV5-FoxO1 (ng)


B)
MCF-7
MCF-7/ADR
MDR1
FoxO1
Actin
Control siRNA
FoxO1 siRNA
Control siRNA
FoxO1 siRNA

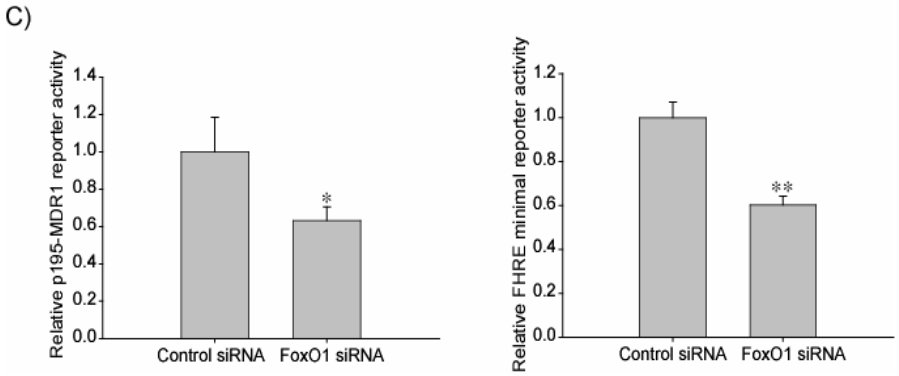
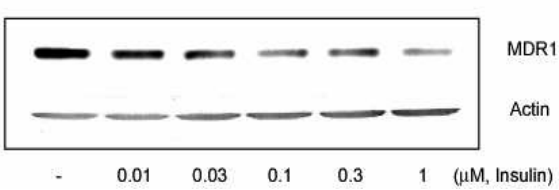
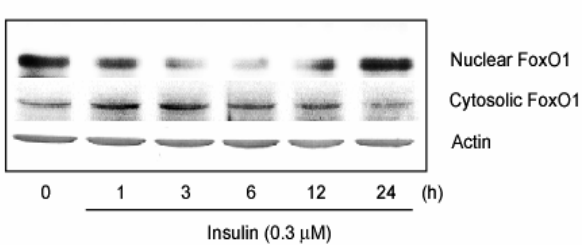

C)
Relative p195-MDR1 reporter activity
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Control siRNA
FoxO1 siRNA
Relative FHRE minimal reporter activity
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Control siRNA
FoxO1 siRNA

Figure 3. Role of FoxO1 in MDR1 induction. (A) Transactivation of *MDR1* gene by FoxO1. Reporter activities of p195-MDR1-Luc (left panel) and FHRE promoter (right panel) by FoxO1 in MCF-7 cells transiently co-transfected with p195-MDR1-Luc or FHRE minimal reporter plasmid in combination with pCMV5-FoxO1 (3-30 ng) or pCMV5 vector. Data represents means ± SD with 3 different samples (significant versus the control, **p<0.01; control level = 1). (B) Inhibition of MDR1 expression by FoxO1 suppressions. MDR1 and FoxO1 levels were determined by immunoblotting in MCF-7ADR cells transfected with FoxO1 siRNA (60 pmole) or control siRNA. (C) Inhibition of MDR1 transactivation by FoxO1 siRNA. MCF-7/ADR cells were co-transfected with p195-MDR1-Luc (left panel) or FHRE reporter plasmid (right panel) in combination with FoxO1 siRNA (20 pmole) or control siRNA. Data represents means ± SD with 4 different samples (significant versus the control, **p<0.01; control level = 1).

A)


MDR1
Actin
-
0.01
0.03
0.1
0.3
1
(μM, Insulin)

B)


Nuclear FoxO1
Cytosolic FoxO1
Actin
0
1
3
6
12
24
(h)
Insulin (0.3 μM)

C)

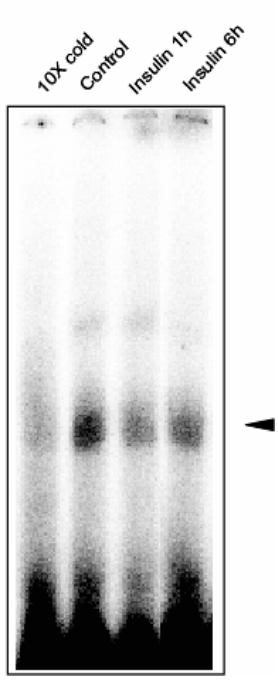

10X cold
Control
Insulin 1h
Insulin 6h

Figure 4. Insulin-mediated MDR1 down-regulation through FoxO1 inactivation. (A) Reduction of MDR1 expression by insulin in MCF-7/ADR cells. Western blot analysis was performed in MCF-7ADR cells treated with insulin (0.01-1 μM) for 24 h. (B) Nuclear exclusion of FoxO1 by insulin. 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) Decrease in FoxO1 binding activity 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 insulin-untreated cells. DNA-binding reactions were done by Gel shift analysis methods.

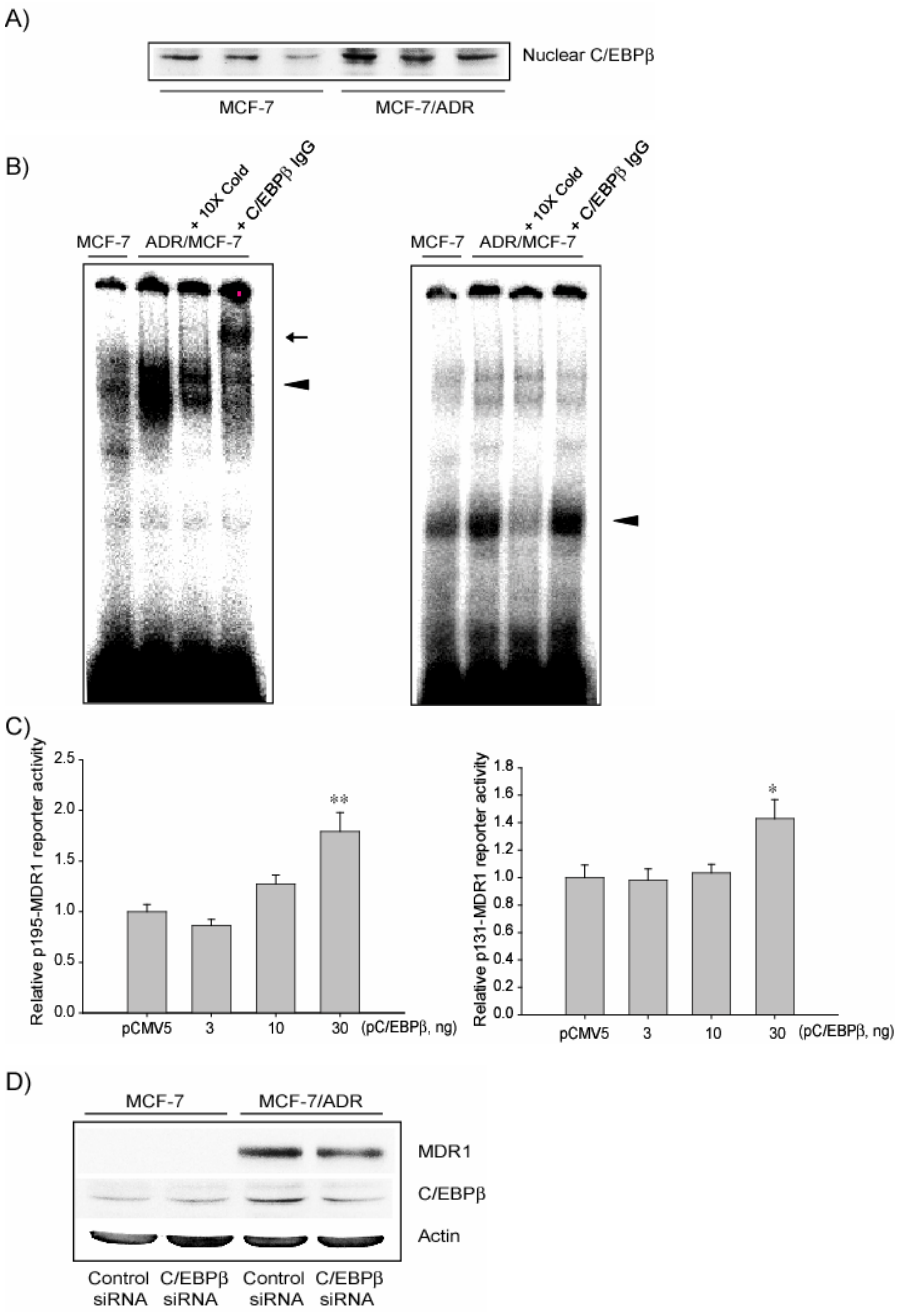

A)
Nuclear C/EBPβ
MCF-7
MCF-7/ADR
B)
+ 10X Cold
+ C/EBPβ IgG
MCF-7
ADR/MCF-7
+ 10X Cold
+ C/EBPβ IgG
MCF-7
ADR/MCF-7
C)
Relative p195-MDR1 reporter activity
2.5
2.0
1.5
1.0
0.5
0.0
**
pCMV5
3
10
30
(pC/EBPβ, ng)
Relative p131-MDR1 reporter activity
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
pCMV5
3
10
30
(pC/EBPβ, ng)
D)
MCF-7
MCF-7/ADR
MDR1
C/EBPβ
Actin
Control siRNA
C/EBPβ siRNA
Control siRNA
C/EBPβ siRNA

Figure 5. Activation of C/EBPβ in MCF-7/ADR cells. (A) Nuclear level of C/EBPβ in MCF-7 and MCF-7/ADR cells. Level of C/EBPβ was assessed immunochemically in nuclear fractions of the both cells serum-starved for 24 h. (B) Left panel; Increase in C/EBPβ binding activity in MCF-7/ADR cells. Gel shift analysis of the C/EBP transcription complex. Nuclear extracts were obtained from both MCF-7 and MCF-7/ADR cells serum-deprived for 24 h. Light panel; No role of C/EBPβ in binding to putative FoxO binding site in MDR1 promoter. Gel shift assay was performed using nuclear extracts prepared from the both cell types. All lanes contained 10 μg of nuclear extracts and the labeled C/EBP consensus sequence (left) or putative FoxO binding sequence (right). Competition experiments were carried out by adding a 10-fold excess of unlabeled C/EBP (left) or FoxO (right) oligonucleotide or C/EBPβ antibody. DNA-binding reactions were performed by gel shift analysis. (C) Transactivation of *MDR1* gene by C/EBPβ. Reporter activities of p195-MDR1-Luc (left panel) and p131-MDR1-Luc (right panel) by C/EBPβ in MCF-7 cells transiently co-transfected with p195-MDR1-Luc or p131-MDR1-Luc in combination with C/EBPβ-overexpressing vector (3-30 ng) or pCMV5 vector. Data represents means ± SD with 3 different samples (significant versus the

control, **p<0.01; control level = 1). (D) MDR1 level in MCF-7/ADR cells after introduction of C/EBPβ siRNA. Levels of MDR1 and C/EBPβ were determined by immunoblotting in MCF-7ADR cells transfected with C/EBPβ siRNA (60 pmole) or control siRNA.

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주 소	광주광역시 남구 방림동 명지맨션 1105 호				
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논문제목	한글 : FoxO1 에 의한 MDR1 유전자 발현 조절 및 Adriamycin 저항성 유방암세포에서의 항암제 내성과의 관련성 연구 영어 : Role of FoxO1 in *MDR1* gene expression: Novel target to overcome chemoresistance of adriamycin-resistant breast cancer cells				

본인이 저작한 위의 저작물에 대하여 다음과 같은 조건아래 -조선대학교가 저작물을 이용할 수 있도록 허락하고 동의합니다.

- 다 음 -

1. 저작물의 DB 구축 및 인터넷을 포함한 정보통신망에의 공개를 위한 저작물의 복제, 기억장치에의 저장, 전송 등을 허락함
2. 위의 목적을 위하여 필요한 범위 내에서의 편집·형식상의 변경을 허락함. 다만, 저작물의 내용변경은 금지함.
3. 배포·전송된 저작물의 영리적 목적을 위한 복제, 저장, 전송 등은 금지함.
4. 저작물에 대한 이용기간은 5 년으로 하고, 기간종료 3 개월 이내에 별도의 의사표시가 없을 경우에는 저작물의 이용기간을 계속 연장함.
5. 해당 저작물의 저작권을 타인에게 양도하거나 또는 출판을 허락을 하였을 경우에는 1 개월 이내에 대학에 이를 통보함.
6. 조선대학교는 저작물의 이용허락 이후 해당 저작물로 인하여 발생하는 타인에 의한 권리 침해에 대하여 일체의 법적 책임을 지지 않음
7. 소속대학의 협정기관에 저작물의 제공 및 인터넷 등 정보통신망을 이용한 저작물의 전송·출력을 허락함.

동의여부 : 동의(○) 반대( )

2008 년 2 월 25 일

저작자: 한 창 엽 (서명 또는 인)

조선대학교 총장 귀하

감사의 글

어느덧 시간이 흘러 졸업할 때가 다가와 그 동안을 되돌아보며 고마우신 분들에게 감사의 편지 글을 올리게 되었습니다. 하지만 이말 저 말이 어지럽게 머릿속을 맴돌기만 할 뿐, 쉽사리 첫머리가 잡히질 않습니다. 감사하다는 말 한마디 하는 것이 그리 어렵지 않은 일임에 불구하고 평소에 선뜻 하지 못하고 지나치기 쉬운 것이 또한 감사 인사 같습니다. 살아가는데 있어 자신을 둘러싼 어느 것 하나 주위 분들과 연관되지 않은 것이 없고, 때로는 도움을 받으며 때로는 베풀기도 하며 더불어 함께 살아가는 것이라고 생각하고는 있지만, 부끄럽게도 그것을 진정으로 느끼고 실천하며 사는 것은 정말 어려운 일 같습니다. 이런 죄송스러운 마음을 조금이나마 덜어보고자 단순히 편지 글을 쓰는 것 만으로 그치는 것이 아니라, 이 글을 쓰는 동안만이라도 진실되게 한 분 한 분에게 감사의 마음을 갖도록 하겠습니다.

감사의 마음에 있어 무엇이 더 고맙고 무엇이 덜 고맙다는 경중이 있겠습니까 마는 이 분들만큼은 더 감사하고, 그만큼 더 미안하고 죄송스러울 수 밖에 없을 것 같습니다. 바로, 가족입니다. 가장 고마우신 분들임에도 불구하고, 이 '가족'이라는 이름으로 오히려 감사의 표현에 더 인색해져 버리기 일쑤였습니다. 공부한답시고 평소에 더욱 잘해드리지 못하는 것 같아 죄송스러움이 앞서지만, 고마움에 보답하는 길은 앞으로 더욱 열심히 노력하는 것이라 생각합니다. 아버지, 어머니, 누나. 모두 사랑하고 고맙습니다.

다음으로 지도교수님이신 강건욱 교수님, 정말 고맙습니다. 학생들에게 친근하게 다가서시는 모습이나, 연구에 대한 열정을 지니신 모습들이 특히 보기 좋았습니다. 교수님으로부터 '꿈을 가지고 항상 변화하고 발전하라'는 말씀이나 삶에 도움이 될만한 여

러 조언들을 들을 때 뿐만 아니라, 교수님께서 스스로 그렇게 살아가시려고 노력하시는 것이 느껴질 때면 큰 가르침이 되었고, 긍정적인 자극이 되었습니다. 그리고 학부 수업시간이나 그 외에도 좋은 말씀을 많이 해주셨던 한효경 교수님, 최후균 교수님, 평소에 마주칠 때면 열심히 하라는 격려를 해주셨던 정혜광 교수님, 만나 뵙게 된지는 얼마 안 되었지만 잘 가르쳐주시고 이것 저것 많이 도와주셨던 최홍석 교수님, 또한 수업시간에 열심히 가르쳐 주셨던 다른 많은 교수님들께도 감사의 말씀 드립니다.

같이 대학원에 들어온 동기 친구들인 상희와 기수에게도 고맙습니다. 착하고 책임감 강하고 멋진 이 친구들과 함께 할 수 있었다는 것에 감사하고, 이들이 있어 대학원 생활이 더욱 편할 수 있었습니다. 지금은 졸업했지만 실험도 잘 가르쳐 주었고, 조언도 해주며 여러모로 선배다웠던 진원형, 또한 먼 길임에도 가끔씩 찾아와서 격려해주었던 정용형, 승식이형, 잘 챙겨주고 신경 써주었던 현택형, 모두 감사합니다.

실험실 후배로 들어왔지만 이것 저것 많이 챙겨주었고 같이 편하게 지내고 있는 상은형, 열심히 실험하고 있는 착한 후배들인 경빈이와 미라, 요즘 실험실에서 같이 지내고 계신 옥이씨, 모두에게 감사드리고, 평소에 좀더 잘해주지 못해 미안합니다.

외국인과도 소통하고 더불어 함께 할 수 있음을 몸소 느끼게 해주었던 실험실 동료인 유바와 그의 가족인 신두와 귀여운 꼬마 숴리아에게도 고맙습니다.

여러 기계를 사용하거나 시약을 빌리기 위해 자주 찾아 뵈었을 때 친절하게 대해주신 위생약학 실험실 선생님들, 다른 실험실 후배인 나연이, 영빈이, 졸업한 선배 은파누나, 또한 물리약학 실험실의 선자 선생님, 지영이 누나, 명학이, 지금은 없지만 찬호형에게도 고맙다는 인사를 하고 싶습니다.

실험실에 납품해 주시는 여러 회사 담당자분들, 기계 수리해 주시는 분들, 그리고 웃으며 인사 받아주시고 친절하게 대해주셨던 수위 아저씨들과 청소하시는 아주머니들

께도 고맙습니다.

학교 다니는 동안 잘 챙겨주셨던 형님들, 누님들, 다른 동기 친구들에게도 감사 드리며, 그 동안 무심하게 연락 드리지 못해 죄송합니다.

또한 오랜만에 만나도 힘이 되고 반가운 오랜 친구들에게도 고맙게 생각하고, 역시 자주 연락하지 못해 미안합니다.

시상식 같은 데서 수상한 사람들이 이름을 나열해가며 감사 수상소감을 발표하는 것을 들을 때면 그다지 별로라고 생각되곤 했었는데, 저 역시 그렇게 되었습니다. 그냥 짧게 감사 드린다는 말과 함께 앞으로 더욱 열심히 하겠다는 말을 하는 것이 좀더 제 성격에는 맞습니다만, 왠지 이름 한 번 더 불러보며 감사인사를 드려보고 싶은 마음에 시작한 것이 이렇게 길어졌습니다. 여기에 소개되진 않았지만 고마우신 다른 분들 모두에게도 감사하다는 말씀 드립니다.

물론 이 글을 보지 못하실 분들이 더욱 많겠지만, 진실되게 감사하는 마음을 갖는다면 그 마음만은 전해질 것이라 생각합니다. 이렇게 감사의 인사를 드리는 동안 저 역시 그 동안의 잊혀졌던 일들이 추억으로 되살아나 좋은 시간이 된 것 같습니다.

고마우신 모든 분들에게 조금이나마 보답할 수 있는 길은 그 고마운 마음을 잊지 않는 것, 그리고 저 스스로 더욱 성숙하여 그들 주변에 한 사람의 '고마운 사람'으로 남을 수 있도록 노력하는 것 이라 생각합니다. 마지막으로 앞으로 이를 위해 노력하고, 더불어 그 노력의 과정을 즐길 수 있도록 성숙할 것임을 스스로 다짐해 봅니다.

그럼 모두 행복하시길 바라겠습니다.

- 한 창 엽 올림