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Cardioprotective effects of statin in doxorubicin-induced cardiotoxicity by modulating survivin expression through inhibition of FOXO1 activity

Jaewon Oh

Department of Medicine

The Graduate School, Yonsei University

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Cardioprotective effects of statin in doxorubicin-induced cardiotoxicity by modulating survivin expression through inhibition of FoxO1 activity

Directed by Professor Seok-Min Kang

The Doctoral Dissertation submitted to
the Department of Medicine,
the Graduate School of Yonsei University
in partial fulfillment of the requirements
for the degree of Doctor of Philosophy

Jaewon Oh

December 2017

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This certifies that the Doctoral Dissertation of Jaewon Oh is approved.

_____

Thesis Supervisor: Seok-Min Kang

_____

Thesis Committee Member#1: Joo Hyuk Sohn

_____

Thesis Committee Member#2: Eun Seok Kang

_____

Thesis Committee Member#3: Ji Hyung Chung

_____

Thesis Committee Member#4: Jinu Lee

The Graduate School
Yonsei University

December 2017

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ACKNOWLEDGEMENTS

First of all, I am deeply indebted to my supervisor, Professor Seok-Min Kang, M.D., Ph.D., whose stimulating suggestions and encouragement helped me throughout my academic life.

I wish to express my warm and sincere thanks to Professor Joo Hyuk Sohn, M.D., Ph.D., Eun Seok Kang, M.D., Ph.D., Ji Hyung Chung, Ph.D., and Jinu Lee, M.D., Ph.D. who advising and encouraging me throughout my years at the graduate school.

I am grateful to Professor Namsik Chung, M.D., Ph.D., Yangsoo Jang, M.D., Ph.D., Donghoon Choi, M.D., Ph.D., Sang-Hak Lee, M.D., Ph.D., Sungha Park, M.D., Ph.D., and Beom Seob Lee, Ph.D. for their constructive advices.

I would like to thank my cardiology and laboratory colleagues for their assorted efforts and supports.

I would like to express my deep and sincere gratitude to my wife, daughter and family who has been with me and led me throughout my life.

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ABSTRACT

Cardioprotective effects of statin in doxorubicin-induced cardiotoxicity by modulating survivin expression through inhibition of FOXO1 activity

Jaewon Oh

Department of Medicine

The Graduate School, Yonsei University

(Directed by Professor Seok-Min Kang)

Survivin is an inhibitor of apoptosis protein and has an anti-apoptotic effect against doxorubicin-induced cardiotoxicity. Statin use is associated with a lower risk for incident heart failure in breast cancer patients with anthracycline chemotherapy. So, the purpose of this study is to investigate whether survivin mediates the protective effect of statin against doxorubicin-induced cardiotoxicity. Doxorubicin treatment suppressed survivin expression via activation of FOXO1 in H9c2 cardiomyocytes. Interestingly, statin inhibited FOXO1 by not only increasing phosphorylation, but also inhibiting nuclear localization, which replenish survivin expression in doxorubicin-induced

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cardiotoxicity. These effects were reversed by FOXO1 small interfering RNA. Doxorubicin treatment induced FOXO1 binding to STAT3 and prevented STAT3 from interacting with Sp1. However, statin significantly inhibited FOXO1 binding to STAT3 and restored STAT3 binding to Sp1 and stabilized transcription complex of STAT3/Sp1. Chromatin immunoprecipitation analysis demonstrated that doxorubicin decreased Sp1-STAT3 complex, whereas statin increased this complex binding to the promoter region of the survivin gene. In chronic doxorubicin-induced cardiomyopathy mouse model, oral administration of statin rescued decrease of survivin expression in myocardium and of left ventricular ejection fraction measured by cardiac MRI. The present study suggests that survivin mediates protective effect of statin against doxorubicin-induced cardiotoxicity via FOXO1/STAT3/Sp1 transcriptional network.

Key words: Survivin, FOXO1, statin, cardiotoxicity, heart failure

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I. INTRODUCTION

The development of myocardial dysfunction and heart failure (HF), described as cardiotoxicity, is the most important and concerning cardiovascular complication of anti-cancer treatments and cause an increase in morbidity and mortality in cancer patients. An interdisciplinary collaborative effort among specialists involved in the treatment of cancer patients is pivotal to prevent and manage cardiotoxicity to improve the patient's clinical overall outcomes.[1] One of the best-known cancer drugs related to cardiotoxicity is anthracycline (e.g. adriamycin, doxorubicin). Anthracyclines have high

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efficacy for treatment of solid tumors and hematological malignancies, and avoiding their administration due to concerns about cardiotoxicity could negatively impact prognosis. On the contrary, anthracyclines may cause irreversible cardiovascular dysfunction, which also affects prognosis in cancer patients. Therefore, the cardiotoxicity issue of anthracyclines is clinically crucial in decision making (e.g. to use or not to use) in real world practice. Numerous approaches have been tried to find the effective and safe methods for preventing chemotherapy-induced cardiotoxicity.[1,2]

The pathophysiologic mechanisms of anthracycline-induced cardiotoxicity have been known as cardiomyocyte apoptosis, oxidative stress (e.g. oxygen free radicals) and so on.[2] So regulating cardiomyocyte apoptosis could be one method for preventing anthracycline-induced cardiotoxicity.

Survivin (encoded by *BIRC5*), a member of inhibitor of apoptosis protein (IAP) family, plays a crucial role in regulating apoptosis and cell division.[3,4] The physiologic function of survivin in the heart has been elucidated in a few studies. Levkau et al. showed that cardiac-specific deletion of survivin caused reduction of cardiomyocyte number, resulting in premature cardiac death.[5] In previous study, survivin has a cytoprotective effect against doxorubicin-induced cardiomyocyte apoptosis.[6] Lee et al. also found that the survivin expression is largely regulated at the transcription level. The survivin gene

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promoter region contains binding sites for numerous transcription factors, including GATA-1, Stat3, E2F, c-myc, KLF5, Sp1 and p53.[4] Lee et al. also reported that Sp1 was a critical transcription factor in the transcriptional regulation of survivin in cardiomyocyte, especially for doxorubicin-induced cardiotoxicity.[7]

The FOXO transcription factor family (FOXO1, FOXO3, FOXO4, FOXO6) belongs to the winged helix or forkhead box class of transcription factors.[8] FOXO1 is expressed in various cell types and tissues during development, including endothelial cells, smooth muscle cells, neural crest cells and adipose tissue.[8-11] However, there have been not enough studies about the functional role of FOXO1 in cardiomyocytes, especially for doxorubicin-induced cardiotoxicity.

Statin has been one of the most widely used drugs for cholesterol-lowering and cardiovascular disease prevention. The pleiotropic effects (beyond lipid-lowering effect) of statins, including decreased inflammation and oxidative stress, may reduce chemotherapy-induced cardiotoxicity. Recent cohort study reported that statin use was associated with a lower risk for incident heart failure in breast cancer patients with anthracycline chemotherapy.[12] Several animal studies have shown that statin could reduce doxorubicin-induced cardiotoxicity.[9,13] However, the precise cardioprotective mechanism of statin

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has not fully uncovered, especially for reducing cardiomyocyte apoptosis.

The purpose of this study is to discover a new pathophysiologic mechanism of statin for reducing doxorubicin-induced cardiotoxicity in terms of transcriptionally modulating an anti-apoptotic protein, survivin.

II. MATERIALS AND METHODS

1. Reagents and antibodies

Doxorubicin, atorvastatin were obtained from Tocris (Bristol, U.K). Anti-survivin, anti-caspase-3 (cleaved form), anti-FOXO1, anti-phospho-FOXO1 (Ser 256), anti-phospho-Akt (Ser 473), anti-Akt antibodies were obtained from Cell Signaling (Beverly, MA, U.S.A). Anti-Sp1, anti-STAT3, anti-lamin B, anti-β-actin, anti-GAPDH, anti-Smac/DIABLO antibodies and p70S6K inhibitor PF4708671 were purchased from Santa Cruz Biotechnology (Dallas, TX, U.S.A). Anti-VDAC1, anti-Bcl-2, and anti-Bax antibodies were obtained from Abcam (Cambridge, UK). PI3K inhibitor LY294002 was purchased from Calbiochem (San Diego, CA, U.S.A). Anti-cytochrome C antibody was included in ApoAlert Cell Fractionation Kit (Clontech, Mountain View, CA, U.S.A).

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2. Cell culture

The rat heart-derived myoblast cell line H9c2 (2–1) cardiac myocytes, were obtained from the American Type Culture Collection (ATCC CRL-1446). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplement with 10% fetal bovine serum (FBS) and 100 U/ml of penicillin and 100 μg/ml of streptomycin (Invitrogen, Carlsbad, CA, U.S.A) at 37℃ in a humidified atmosphere with 5% CO2. All experiments were performed using cells between 15 to 25 passage numbers. After adaptation in DMEM containing 10% FBS for 24 h, cells were starved in DMEM containing 0.5% FBS for 24 h. After starvation, cells were pretreated with atorvastatin (10 μM) in 0.5% FBS containing DMEM for 1 hour prior to doxorubicin treatment for 24 h.

3. Cell viability analysis

Cell viability was measured by 2-(4,5-dimethyltriazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay and cell apoptosis was determined by terminal deoxynucleotidyltransferase-mediated dUTP nick end-labeling (TUNEL) assay (Promega, Madison, WI, U.S.A), cytochrome c release and caspase-3 activity assay (ApoAlert CPP32/caspase-3 assay kit, BD Biosciences, San Jose, CA, U.S.A), which were performed as described previously.[7]

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4. Confocal immunofluorescence microscopy

Immunofluorescence microscopy was performed as previously reported.[7] Briefly, H9c2 cells cultured on Lab-Tek chamber slides (Nalgene Nunc, Penfiled, NY, U.S.A) were fixed with 3% paraformaldehyde and permeabilized with 0.5% Triton X-100. After blocking with PBS containing 0.3% goat serum and 5% bovine serum albumin, the slides were incubated with FOXO1 antibody and mounted with ProLongantifade reagent containing DAPI. The immunoreactive signals were visualized by confocal laser scanning microscope LSM700 (Carl Zeiss, Oberkochen, Germany).

5. Subcellular fractionation and immunoblot analysis

Mitochondrial and cytosolic fractions were obtained using Qproteome Cell Compartment Kit (Qiagen, Hiden, Gemany) according to the manufacturer's instructions, and protein sample preparation and immunoblot analysis were performed as described previously.[7]

6. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated using QIAzol-Regent (Qiagen, Hiden, Gemany) and reverse transcribed using Omniscript Reverse Transcriptase (Qiagen, Hiden, Gemany). The cDNAs were amplified using TaKaRa Ex Taq polymerase

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(Takara, Shiga, Japan). The sequences of the primers were as follows: survivin F, 5'-ATG GGT GCT ACG GCG CTG CCC-3'; survivin R, 5'-TCA GCG TAA GGC AGC CAG CTG-3'; GAPDH F, 5'-AAT GCA TCC TGC ACC ACC AAC TGC-3'; GAPDH R, 5'-GGA GGC CAT GTA GGC CAT GAG GTC-3'. PCR products were separated by electrophoresis in a 1% agarose gel containing Gel-red (Biotium, Fremont, CA. U.S.A).

7. RNA interference

H9c2 cells were transfected with scrambled RNA or small interfering RNA (siRNA) targeted to FOXO1 gene using Lipofectamine RNA iMAX (Invitrogen, Calsbard, CA, U.S.A) according to the manufacturer's protocol. Scrambled RNA was purchased from Santa Cruz Biotechnology (Dallas, TX, U.S.A).

8. Chromatin immunoprecipitation (ChIP)

ChIP assay was performed according to the protocol of previous study.[7] Briefly, formaldehyde-treated nuclear lysates were subjected to immunoprecipitation with anti-Sp1 antibodies. The cross-linked chromatin complex was reversed in the presence of proteinase K and DNA fragments were purified. The DNA fragment (257-bp) of survivin promoter region (between -265 and -9) was amplified by PCR using a pair of primers: Rat survivin

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promoter F, 5'-AGG ACA CAA CTC CCA GCA AG- 3'; Rat survivin promoter R, 5'-CGC CAC AAT CCC TAA TTC AA- 3'. PCR condition was as follows: at 95℃ for 30 sec; at 56℃ for 30 sec; and at 72℃ for 60 sec. After 36 cycles of PCR, products were analyzed by 2% agarose gel electrophoresis. For input data (5%), 25 μl aliquots of 500 μl samples were taken before immunoprecipitation.

9. Animal studies

Mice were maintained according to standard approved Institutional Animal Care Use Committee protocols at Yonsei University College of Medicine. The animals were maintained in pathogen-free cages and light, as well as temperature-controlled rooms provided with rodent chows and sterile water. Eight weeks old mouse were randomly divided into three groups (n=10 for each group); saline group, doxorubicin group & atorvastatin + doxorubicin group and they received a cumulative dose of 25 mg/kg of doxorubicin by intraperitoneal injection once a week with five treatments except for saline group. And they also received 20 mg/kg of atorvastatin (for atorvastatin+ doxorubicin group) or saline (for saline & doxorubicin group) every day by oral gavage. During experiment, some mice were died (one in saline group, two in doxorubicin group & one in atorvastatin + doxorubicin group). After 6 weeks,

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total 26 mice were sacrificed (n=9 for saline group, n=8 for doxorubicin group & n=9 for atorvastatin + doxorubicin group) after MRI measurement then mouse hearts were perfused with saline, removed, and fixed in 4% paraformaldehyde for 24 hours in 4℃. Then, they were embedded in paraffin and prepared in 4 μM cross sections. Mouse hearts were stained with conventional hematoxylin and eosin (H&E) and Masson's trichrome for analysis of histology and fibrosis. For immunohistochemical analyses, sections were deparaffinized in xylene, rehydrated in graded ethanol solutions and washed with distilled water as described from previous study.[7] Sections were blocked with 5% goat serum in antibody diluent for 30 minutes and incubated overnight at 4℃ with following antibodies: survivin, FOXO1 (1:150). After rinsing three times in PBS, RTU horseradish peroxidase streptavidin (Vector Laboratories, Burlingame, CA, U.S.A) was applied and the slides are incubated for 10 minutes.

10. MRI measurements of cardiac parameters

Mice were anesthetized and maintained under sedation with 3% isoflurane during the MRI procedure. MRI imaging was obtained using Biospec 94/20 USR (9.4T) MRI (Bruker, Billerica, MA, U.S.A). Cardiac functional parameters for left ventricular ejection fraction (LVEF) were calculated off-line

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with an in-house program on QMass (Medis medical imaging systems, Leiden, Netherlands). Endo- and epicardial contours were traced on short-axis images acquired at the end diastolic and end systolic phases.

11. Statistical analysis

Three experiments were performed for all *in vitro* studies. The results are presented as means ± standard deviation (SD). The data were subjected to a two-tailed Student's *t*-test. Statistical analyses were carried out using SPSS version 21.0 for Windows (SPSS/IBM Corporation, Chicago, IL, U.S.A). All *P* values less than 0.05 were considered statistically significant.

III. RESULTS

1. Statin decreased doxorubicin-induced apoptosis in H9c2 cardiomyocyte cell line

To investigate whether statin protects H9c2 cardiac myocyte against doxorubicin-induced cardiotoxicity, cardiomyocytes were pretreated with statin for 1 hour prior to doxorubicin treatment. Consistent with several previous studies, doxorubicin-induced cell death was significantly perturbed by pretreatment with 10 μM of atorvastatin (Fig. 1A). Consistently, doxorubicin

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treatment stimulated apoptosis in H9c2 cells, and statin pretreatment dramatically reduced doxorubicin-induced apoptosis, as determined by TUNEL assay (Fig. 1B&C). In addition, pretreatment with statin prevented doxorubicin-stimulated caspase-3 activation, decreased Bcl-2 protein levels and increased Bax protein levels (Fig. 1D), release of cytochrome c (Fig. 1E) and Smac/DIABLO (Fig. 1F) to cytosol. These results suggested that statin could protect H9c2 cardiac myocytes from doxorubicin-induced cardiotoxicity by inhibiting cardiomyocyte apoptosis.

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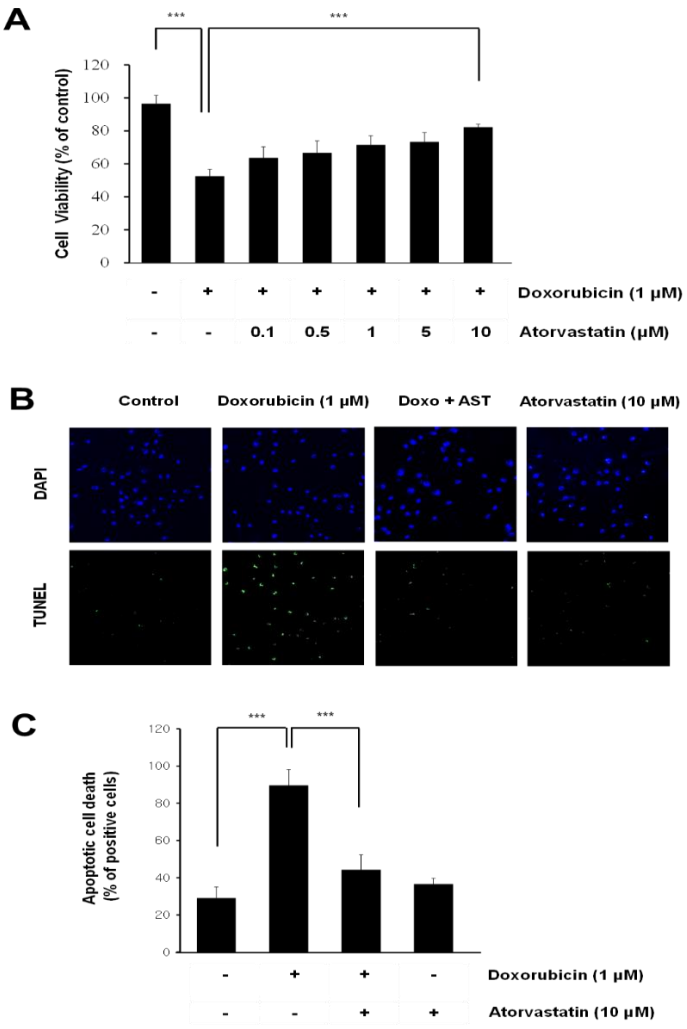

Figure 1 A-C legend (following pages)

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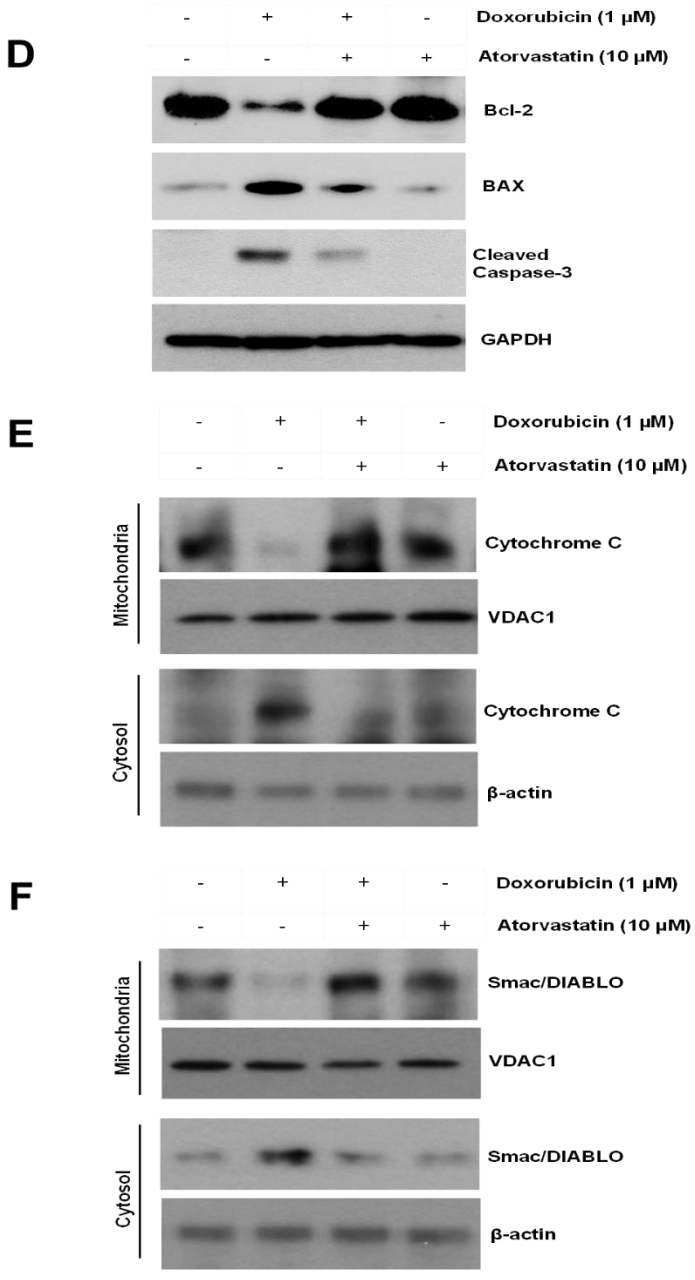

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Figure 1. Effect of statin on doxorubicin-induced apoptosis in H9c2 cardiomyocyte cell line.

The H9c2 cardiomyocytes were treated with various concentration of statin pretreatment in doxorubicin-induced cardiac injury cell model. (A) Cell viability was assessed by the MTT assay. (B) Apoptotic cells were visualized with DAPI & TUNEL staining. (C) Apoptotic cells were measured and counted by TUNEL assay (x200). (D) Immunoblot analysis for anti-apoptotic protein, Bcl-2 and apoptotic protein, Bax. (E and F) Mitochondrial and cytoplasm fractions were prepared, and equal amounts of protein were separated by SDS-PAGE gel. The release of Smac/DIABLO or cytochrome c from mitochondria to cytosol were detected using anti-Smac/DIABLO or anti-cytochrome c antibody. VDAC1/β-actin bands show that equal amounts of sample for mitochondria/cytosol fraction were loaded. Note that blots represent one of three independent experiments. The results present the means of three independent experiments. Values are mean ± S.D. ***$P < 0.05$.

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2. Statin recovered doxorubicin-induced decreased expression of survivin at transcription level

Lee et al. found that doxorubicin treatment decreased survivin expression in H9c2 cardiac myocytes.[6] Considering previous findings which show statin could reduce doxorubicin-induced cardiotoxicity, it was hypothesized that statin could recover doxorubicin-induced decrease of survivin expression in cardiomyocyte. In addition, to elucidate whether survivin expression was regulated by transcription or translation level, RT-PCR in addition to immunoblot analysis for demonstrating the change of survivin mRNA and protein expression was done. Interestingly, doxorubicin treatment reduced both levels of survivin protein (Fig. 2A&C) and mRNA (Fig. 2B), and these reductions were attenuated with statin pretreatment. Therefore, these results suggested that survivin expression was transcriptionally regulated in doxorubicin-induced cardiotoxicity model.

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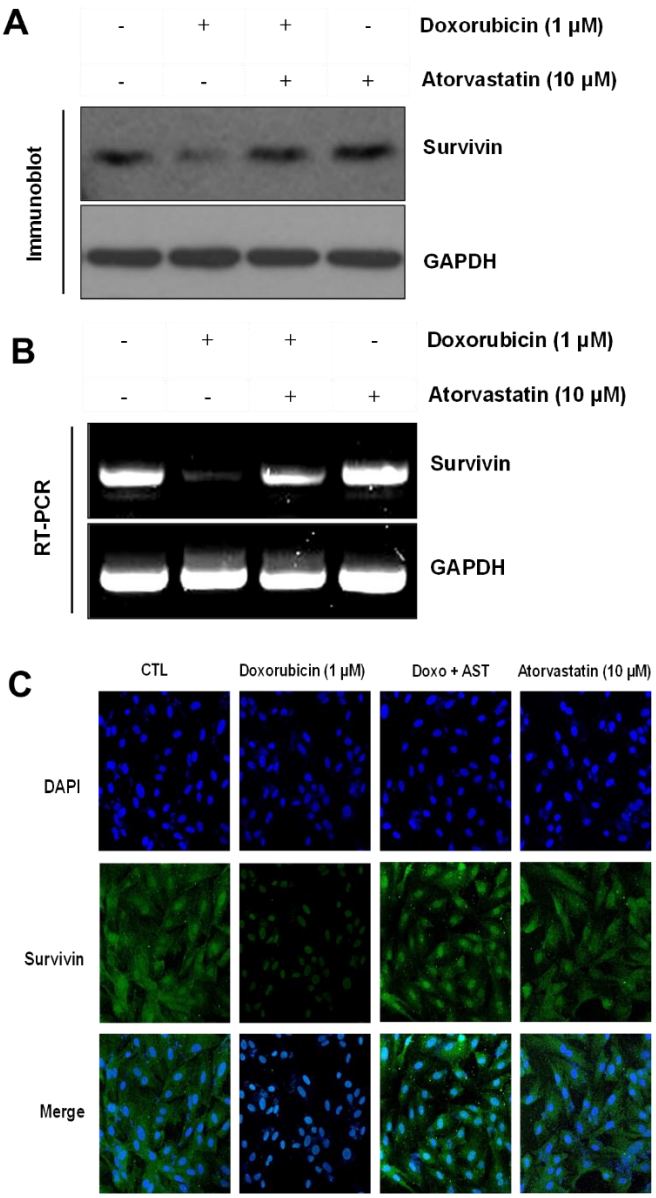

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Figure 2. Effect of statin on doxorubicin-induced decrease of survivin expression in H9c2 cardiomyocyte cell line.

(A) The H9c2 cardiomyocyte cells were treated with or without pretreatment with 10 μM of atorvastatin in addition to doxorubicin treatment for 24 hours. Equal amounts of protein were separated by SDS-PAGE gel, and immunoblot analysis was performed using anti-survivin antibody. (B) Total RNA was analyzed by RT-PCR (28 cycles) using primers specific to survivin and GAPDH gene, which shows that equal amounts of sample were loaded. (C) The cells were treated with 1 μM of doxorubicin treatment for 24 hours. Survivin protein expression was also observed with confocal immunofluorescence microscopy using primary anti-survivin antibody and FITC-conjugated secondary antibody (x200).

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3. Statin reduced doxorubicin-induced nuclear translocation of FOXO1 in H9c2 cardiomyocyte cell line

According to previous study, Sp1 was a critical transcription factor in the transcriptional regulation of survivin expression in doxorubicin-induced cardiac injury model.[7] In cancer cell, FOXO is another important transcription factor for regulating survivin transcription. In general, FOXO signaling is temporally regulated by nuclear import and export. In case of FOXO1, unphosphorylated FOXO1 undergoes nuclear export, whereas phosphorylated FOXO1 is sequestered in nucleus. So it was hypothesized that FOXO1 could be another transcriptional regulator for survivin expression in cardiomyocyte via nuclear-cytoplasmic translocation.

Doxorubicin treatment increased the expression of FOXO1 time-dependently in nuclear fraction samples (Fig. 3A). This increase of nuclear FOXO1 expression was attenuated by statin pretreatment, as determined by immunoblot using nuclear fraction samples (Fig. 3B) and confocal imaging analysis with DAPI and FOXO1 (Fig. 3C).

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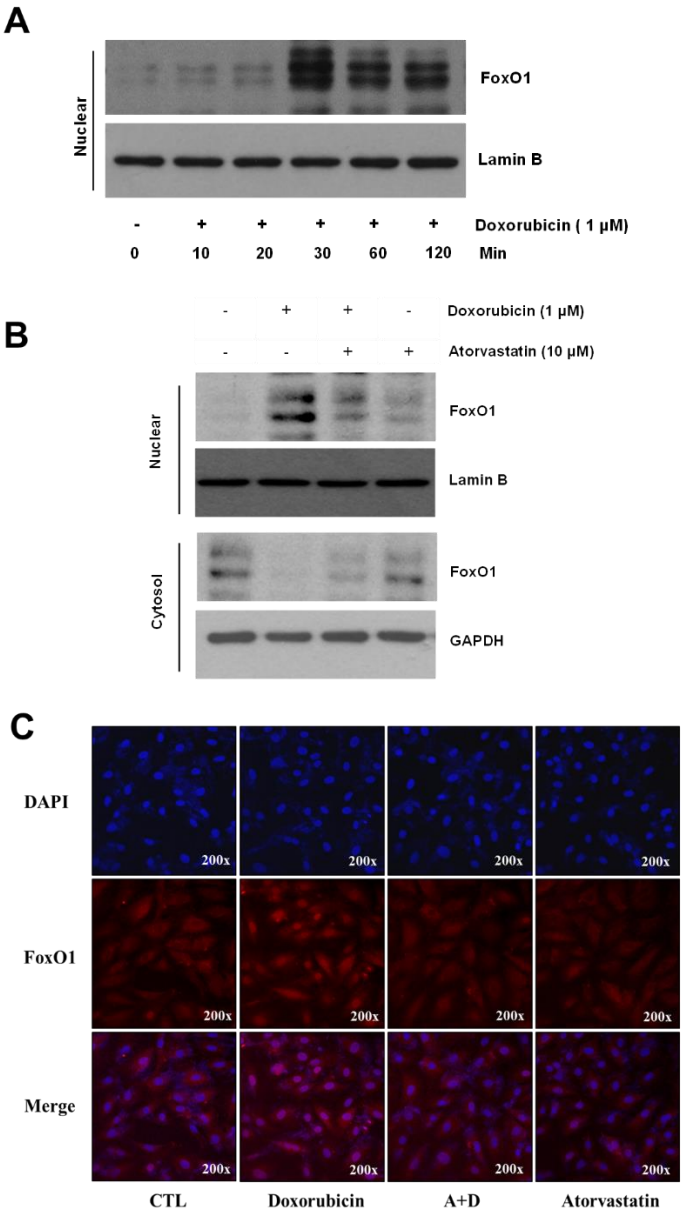

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Figure 3. Effects of statin on doxorubicin-induced nuclear translocation of FOXO1 in H9c2 cardiomyocyte cell line.

(A) Nuclear and cytoplasm fractions were prepared, and equal amounts of protein were separated by SDS-PAGE gel. The FOXO1 from nuclear fraction was immunoblotted and lamin B bands show that equal amounts of nuclear sample were loaded. (B) Nuclear and cytoplasm fractions were prepared, and FOXO1 from nuclear and cytoplasmic fraction was immunoblotted and GAPDH bands show that equal amounts of sample for cytoplasmic fraction were loaded. (C) Confocal immunofluorescence imaging analysis with DAPI and FOXO1 for showing nuclear-translocated FOXO1 (x200).

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4. Statin activated doxorubicin-induced depressed PI3K-Akt-FOXO1 signaling in H9c2 cardiomyocyte cell line

PI3K-Akt is a well-known upstream stimuli for FOXO signaling pathway so it was tested whether PI3K-Akt signaling pathway was involved in the present cardiotoxicity model. Consistent with the results of previous studies, the activation of PI3k-Akt signaling which assessed by the expression of phospho-Akt (Ser-473) was reduced by doxorubicin treatment but this inhibition was blocked by statin pretreatment (Fig. 4A). In addition, the downstream target of Akt kinase, phospho-FOXO1 (Ser 256) was also decreased by doxorubicin treatment but this decrease was also attenuated by statin pretreatment (Fig. 4A). Then, the decrease of doxorubicin-induced nuclear FOXO1 import (a downstream target of PI3k-Akt signaling) by statin was attenuated by PI3K-Akt signaling inhibitor, LY294002 (Fig. 4B lane 4). These results suggested that statin could activate the doxorubicin-induced deactivation of PI3k-Akt-FOXO1 signaling in H9c2 cardiomyocyte.

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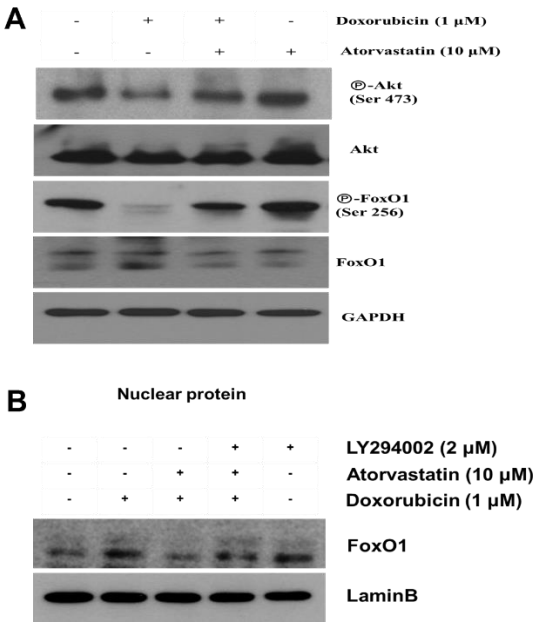

Figure 4. Effect of statin in doxorubicin-induced PI3K-Akt-FOXO1 signaling in H9c2 cardiomyocyte cell line.

(A) The H9c2 cardiomyocytes were treated with or without pretreatment with 10 μM of atorvastatin in addition to doxorubicin treatment for 24 hours. Equal amounts of protein were separated by SDS-PAGE gel, and immunoblot analysis was performed using anti-phospho-Akt (Ser 473), Akt, phospho-FOXO1 (Ser 256), FOXO1 antibody. (B) Nuclear fractions were prepared with or without PI3k-Akt signaling inhibitor, LY294002. The FOXO1 from nuclear fraction was immunoblotted and lamin B bands show that equal amounts of nuclear sample were loaded.

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5. Effect of statin on doxorubicin-induced transcriptional factor interaction (Sp1, STAT3, FOXO1) in H9c2 cardiomyocyte cell line

To investigate how FOXO1 regulates survivin transcription, especially through the interaction with Sp1, the transcription network complex which involved both FOXO1 and Sp1 was reviewed. According to the previous study by Yang et al., FOXO1 could inhibit the pro-opiomelanocortin transcription regulation of leptin signaling in HEK293 cells by blocking the binding of STAT3 with Sp1.[11] As it were, FOXO1 competitively binds STAT3 by inhibiting the interaction between STAT3 and Sp1. Therefore, it was tested whether this transcription network could work in doxorubicin-induced cardiomyocyte injury model, using immunoprecipitation and ChIP experiments.

The immunoprecipitation experiments revealed that doxorubicin decreased the binding between STAT3 and Sp1 (Fig. 5A), whereas increased the binding between STAT3 and FOXO1 (Fig. 5B). Then, ChIP experiments were done to show whether decreased interaction between STAT3 and Sp1 results in decreased transcription of survivin via inhibiting the binding of Sp1 on survivin promoter (Fig. 5C). This ChIP data showed that doxorubicin treatment decreased Sp1-mediated survivin transcription, and it was restored by statin treatment.

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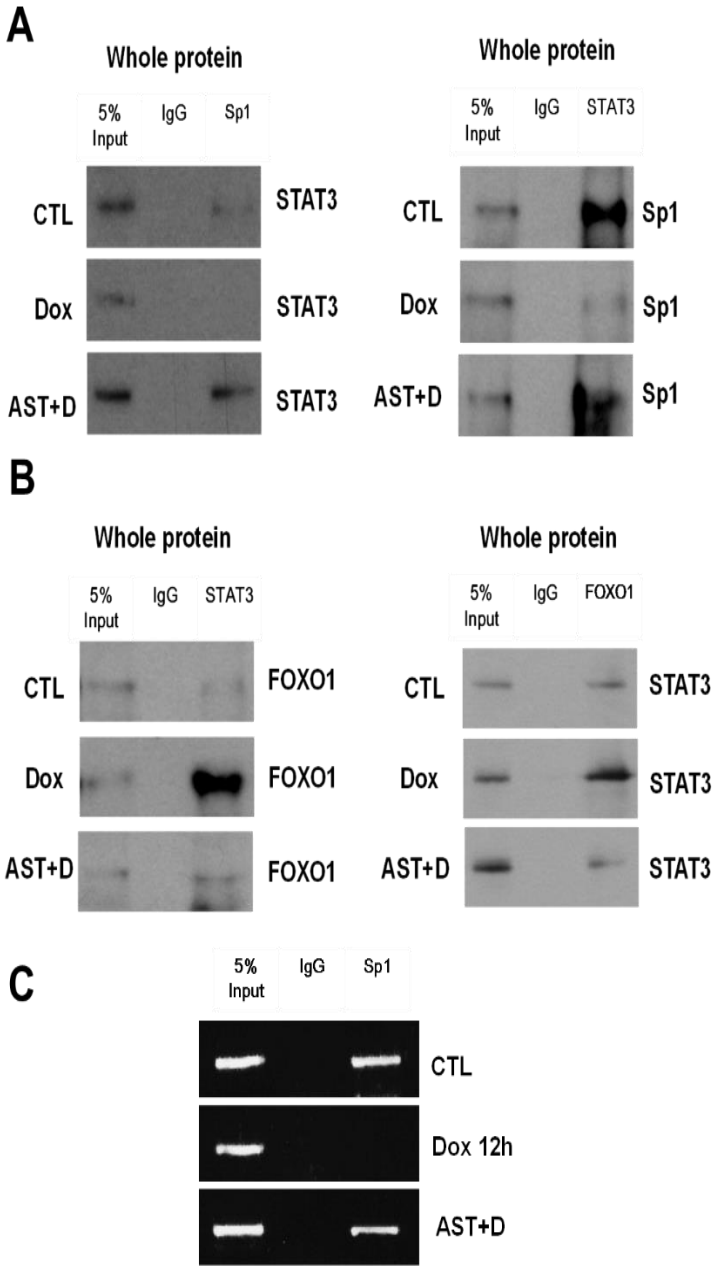

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Figure 5. Effect of statin on doxorubicin-induced transcriptional factor interaction (Sp1, STAT3, FOXO1) in H9c2 cardiomyocyte cell line.

(A, B) Whole cell lysates were immunoprecipitated by antibody (e.g. Sp1, STAT3, FOXO1) then separated by SDS-PAGE gel and analyzed by immunoblot analysis with antibodies against Sp1, STAT3, FOXO1. (C) Cross-linked cell lysates were subjected to ChIP analysis with anti-Sp1 antibody. RT-PCR (36 cycles) was performed with ChIP primer as listed in Materials and Methods.

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6. FOXO1 as a negative regulator for Sp1-dependent transactivation of survivin in H9c2 cardiomyocyte cell line

To investigate whether FOXO1 plays a pivotal role in Sp1-dependent transactivation of survivin in cardiomyocyte, the experiments using FOXO1 siRNA knockdown method were done. The selected FOXO1 siRNA could decrease FOXO1 mRNA and protein expression. Interestingly, FOXO1 siRNA could increase both mRNA and protein expression of survivin, suggesting the transcription regulation of FOXO1 on survivin at basal condition in cardiomyocyte (without doxorubicin or statin treatments) (Fig. 6A).

Then immunoblotting for survivin (Fig. 6B) revealed that doxorubicin-induced decrease of survivin expression was attenuated by FOXO1 siRNA pretreatment (lane 3), similarly to statin pretreatment (lane 4). In addition, the co-pretreatment of FOXO1 siRNA and statin increased the survivin expression synergistically (lane 5), which suggests that the mechanisms of statin on survivin transactivation could involve other signaling pathways than FOXO1 signaling.

These findings suggested that FOXO1 played a pivotal role in the cytoprotection of statin by blocking the doxorubicin-induced decrease of survivin expression in cardiomyocyte.

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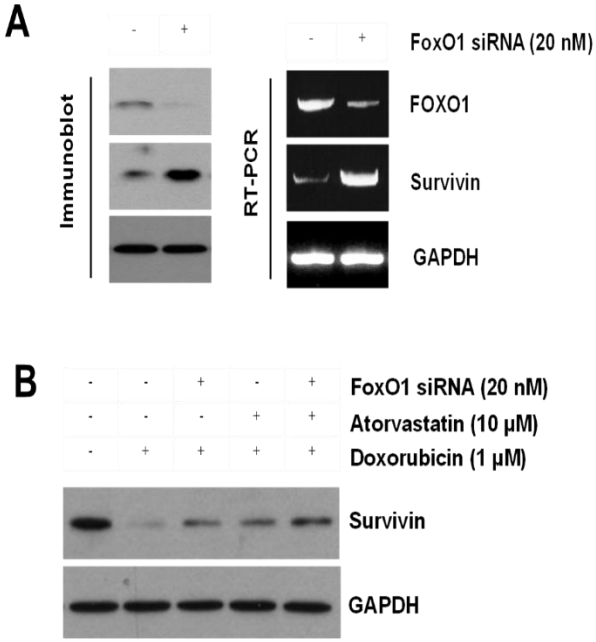

Figure 6. FOXO1 as a negative regulator for Sp1-dependent transactivation of survivin in H9c2 cardiomyocyte cell line.

(A) The H9c2 cardiomyocytes were transfected with FOXO1 siRNA. Equal amounts of protein were separated by SDS-PAGE gel, and immunoblot analysis was performed using anti-survivin antibody and total RNA was analyzed by RT-PCR (28 cycles). (B) After FOXO1 siRNA transfection, whole lysates were immunoblotted for survivin protein expression.

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7. Statin decreased doxorubicin-induced cardiac injury in mice

The doxorubicin-induced cardiac injury mouse model was developed by treatment with intraperitoneal injection of doxorubicin (5 mg/kg) once a week for 1 month in C57BL/6 male mice. To investigate the effect of statin on doxorubicin-induced cardiotoxicity, these mice were treated with 20 mg/kg of atorvastatin or saline once daily by oral gavage for 6 weeks (Fig. 7A). After sacrifice, mouse heart was isolated and paraffin embedded for H & E staining, Masson's trichrome staining, and was also prepared for electron microscopic examination. The results of H & E staining clearly showed the multifocal areas of patchy and scattered cardiomyocyte with vacuolation, suggesting doxorubicin-induced cardiac injury and it was reduced in atorvastatin + doxorubicin group (Fig. 7B). The Masson's trichrome staining revealed that fibrotic areas, demonstrated by blue staining area were increased in doxorubicin treatment group, but it was attenuated in statin co-treatment group (Fig. 7C). The electron microscopic findings showed that myocardial disarray and mitochondrial destruction were demonstrated in doxorubicin group, but it was decreased in atorvastatin + doxorubicin group (Fig. 7D).

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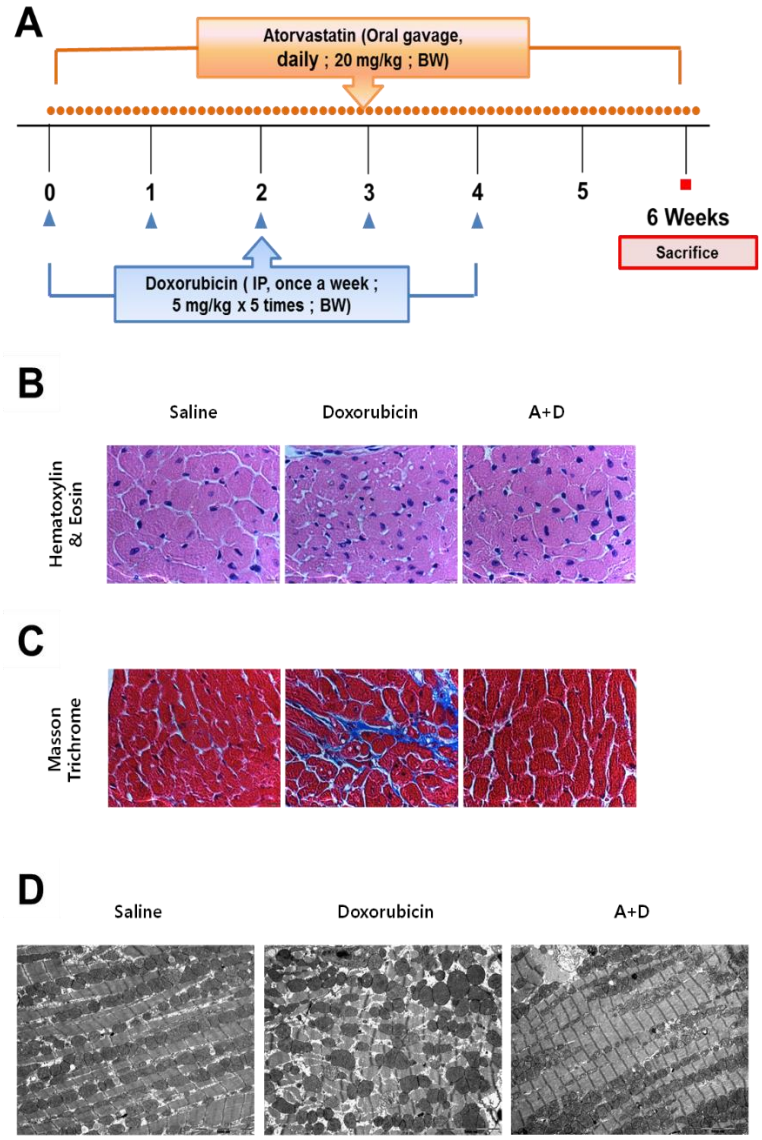

Figure 7 A-D legend (following pages)

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Figure 7. Effect of statin on histology in doxorubicin-induced myocardial injury mouse model.

(A) Diagram of animal experimental protocol. Mice were treated with 5 mg/kg of doxorubicin by intraperitoneal (IP) injection for 4 weeks and treated with 20 mg/kg of atorvastatin or saline once daily by oral gavage for 6 weeks (n=9 for saline group, n=8 for doxorubicin group & n=9 for atorvastatin + doxorubicin (A+D) group). Representative images for myocardial section 7 days after the doxorubicin injection, stained with (B) Hematoxylin and Eosin (x1000), (C) Masson's trichrome staining (x1000) and (D) electron microscopic findings (x6000). Each figure shows the representative image of each group.

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8. Statin reduced doxorubicin-induced cardiomyocyte apoptosis in mice

As shown in Fig. 8A, cardiomyocyte apoptosis, assessed by TUNEL assay was increased in doxorubicin group and this increase was reduced in atorvastatin + doxorubicin group. Then, the protein expression of survivin in the same mouse model was further investigated. The immunohistochemistry microscopy image showed that survivin expression was down-regulated by doxorubicin treatment and recovered by statin co-treatment and this change was also observed in immunoblot assay using the whole lysate of mouse heart of each group (Fig. 8B).

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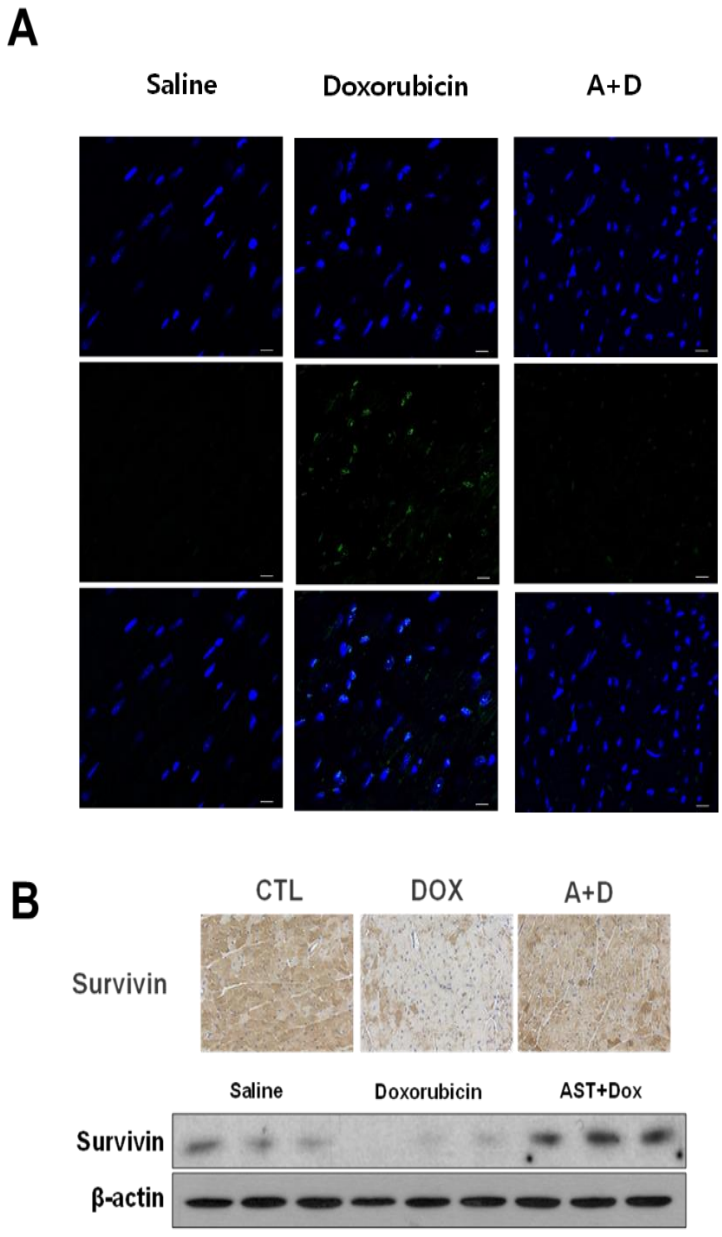

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Figure 8. Effect of statin on cardiomyocyte apoptosis in doxorubicin-induced myocardial mouse model.

After sacrifice, mouse heart was isolated and (A) cardiomyocyte apoptosis was visualized with DAPI & TUNEL staining assay (x400). (B) Survivin protein expression was measured by immunohistochemistry analysis (x400) and immunoblotting for survivin for each group (saline, doxorubicin & atorvastatin + doxorubicin (A+D) group)**.**

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9. Statin decreased doxorubicin-induced FOXO1 nuclear translocation in mice

To confirm the novel signaling pathway in vivo which found in vitro experiment, it was investigated whether FOXO1 translocated to nucleus in doxorubicin-induced cardiac injury mice model. The confocal immunofluorescence analysis revealed that the merged areas between DAPI and FOXO1, which mean nuclear-translocated FOXO1 were increased in doxorubicin group but it was decreased in atorvastatin + doxorubicin group (Fig. 9), consistent with in vitro findings.

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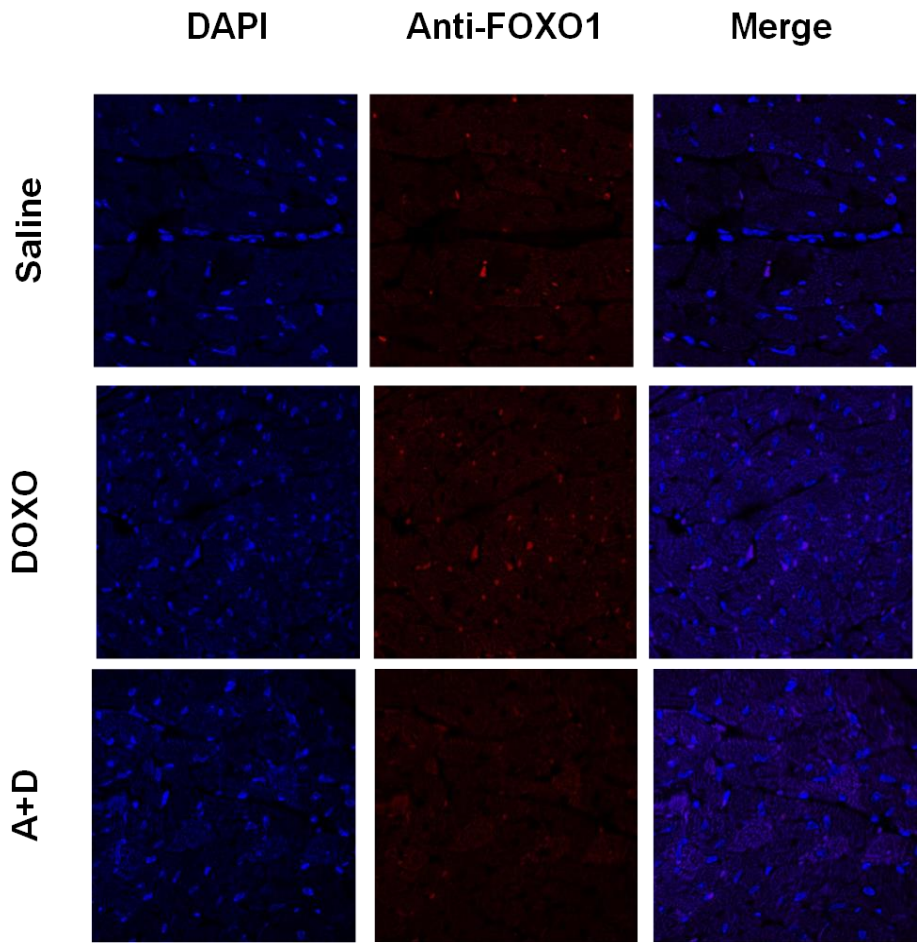

Figure 9. Effect of statin on FOXO1 nuclear translocation in doxorubicin-induced myocardial mouse model.

Confocal immunofluorescence microscopic imaging analysis with DAPI, FOXO1 and merge imaging in the section from mouse heart for saline, doxorubicin (DOX) & atorvastatin + doxorubicin (A+D) group (x400).

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10. Statin recovered doxorubicin-induced depression of cardiac function in mice

To further confirm the cardio-protective effect of statin in vivo, the cardiac function was measured in doxorubicin-induced cardiac injury model. Using cardiac MRI imaging analysis, LVEF was measured. The LVEF was reduced in doxorubicin group (46.0 ± 5.1%) but, it was also significantly recovered in atorvastatin + doxorubicin group (53.8 ± 5.8%, $p<0.001$) (Fig. 10B).

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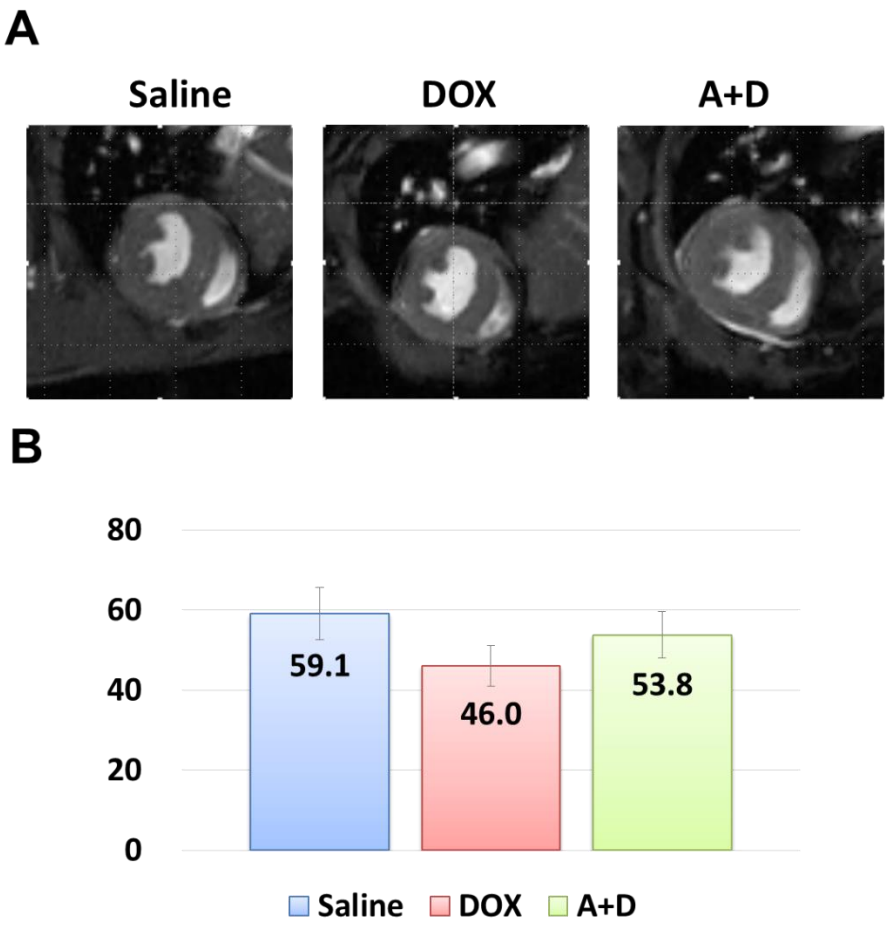

Figure 10. Effect of statin on cardiac function in doxorubicin-induced myocardial mouse model.

(A) Representative cardiac MRI image in end systolic phase and (B) measured left ventricular ejection fraction of mice for saline (n=9), doxorubicin (DOX, n=8) & atorvastatin + doxorubicin (A+D, n=9) group.

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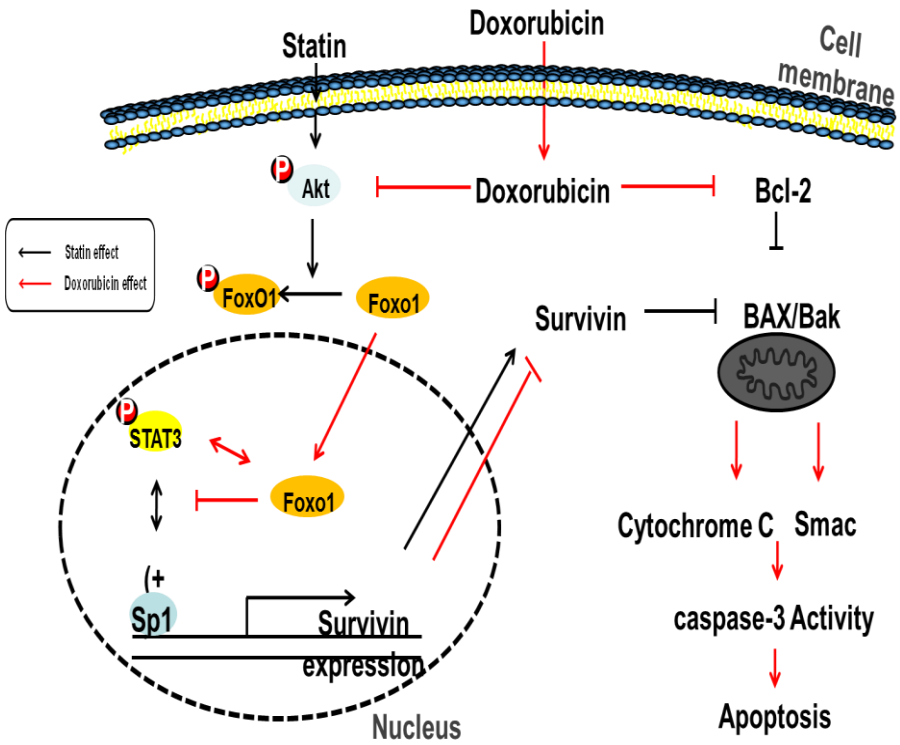

Figure 11. Suggested cardioprotective mechanisms of statin in doxorubicin-induced cardiotoxicity.

IV. DISCUSSION

The major findings of this study are as follows. First, survivin may be an important mediator in the cardioprotective effect of statin in doxorubicin-induced cardiotoxicity in H9c2 cell line and mouse model. Second, survivin

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mediates protective effect of statin via FOXO1/STAT3/Sp1 transcriptional network (summarized in Fig. 11).

Survivin, an inhibitor of apoptosis protein, plays a crucial role in regulating apoptosis and cell division.[3,4] The physiologic function of survivin in the heart has been elucidated in a few studies. Levkau et al. showed that cardiac-specific deletion of survivin caused reduction of cardiomyocyte number, resulting in premature cardiac death.[5] In previous study, survivin has a cytoprotective effect against doxorubicin-induced cardiomyocyte apoptosis.[6] It is recently known that survivin expression is largely regulated at the transcription level and Sp1 is a critical transcription factor in the transcriptional regulation of survivin in cardiomyocyte, especially for doxorubicin-induced cardiotoxicity.[7] However, other mechanisms on survivin transcriptional regulation other than Sp1 have been largely unknown in cardiomyocytes.

The FOXO transcription factor family belongs to the winged helix or forkhead box class of transcription factors. Mice and humans possess 4 FOXO genes; FOXO1, FOXO3, FOXO4, and FOXO6. Among them, FOXO1, FOXO3, and FOXO4 are highly related homologs with overlapping patterns of expression and transcriptional activity. The FOXO protein family is primarily regulated by post-translational modifications, including phosphorylation, acetylation, and ubiquitination. These various modifications control subcellular

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localization and protein expression levels, as well as DNA binding and transcriptional activity. Most notably, FOXO1, FOXO3, and FOXO4 have 3 conserved amino acids that are phosphorylation targets by Akt or serum/glucocorticoid regulated kinase (SGK). Phosphorylation at these sites leads to nuclear exclusion of FOXO.

FOXO1 has a specific role in vascular development, which cannot be compensated for by other FOXO family members. FOXO1 is expressed in various cell types and tissues during development, including endothelial cells, smooth muscle cells, neural crest cells and adipose tissue. However, there have been not enough studies about the functional role of FOXO1 in cardiomycoytes. Sengupta et al. reported a critical role for FOXO1 and FOXO3 in promoting cardiomyocyte survival during oxidative stress through induction of anti-oxidants and cell survival pathway-related proteins.[8] And Battiprolu et al. showed that activation of FOXO1 was an important mediator of diabetic cardiomyopathy via down-regulation of IRS1.[14] The present study showed that FOXO1 was an important regulator for doxorubicin-induced cardiomyocyte apoptosis. These results could be consistent with previous studies, considering oxidative stress as one of key mechanisms of doxorubicin-induced cardiotoxicity.

There have been few studies about the role of FOXO in survivin

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transcription regulation. Most studies were conducted in cancer cell lines. In human neuroblastoma cell, PI3K-AKT-FOXO3 pathway was reported to play a central role in chemotherapy resistance by regulating survivin expression.[10] In addition, HER2-PI3K-FOXO1/3-survivin axis was reported to be important in trastuzumab-resistant breast cancer cell lines.[15] There have been only one study to demonstrate the pathologic change of PI3K-AKT-FOXO1-survivin pathway in heart.[13] However, in this study, they just showed the change in expression of PI3K-AKT-FOXO1-survivin pathway after statin treatment in old-aged spontaneously hypertensive rats and did not experiment the causal-relationship between FOXO1 and survivin. Therefore, the present study is the first to demonstrate that PI3K-AKT-FOXO1 signaling pathway is important for transcriptional regulation of survivin in cardiomyocyte.

According to recent review, Sp1 and p53 were known to be key transcription regulators for survivin. Lee et al. reported that Sp1 and p53 was an important transcriptional regulator for survivin expression in cardiomyocyte.[7] So it was hypothesized for adding FOXO1 to these well-known transcriptional network for survivin regulation. Yang et al. showed that FOXO1 could inhibit pro-opiomelanocortin transcription regulation of leptin signaling in HEK293 cells by blocking STAT3 interaction with Sp1.[11] So, this transcriptional regulation model was tested in doxorubicin-induced cardiac

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injury model. Finally, the present study revealed that FOXO1 competitively bound STAT3 by inhibiting the interaction between STAT3 and Sp1 in cardiomyocyte for the first time.

The cardioprotective role of statin have been demonstrated in various cardiovascular disease including myocardial infarction, stroke, so statin has been one of the most-widely prescribed drugs. In consistent with these lines of clinical background, Lee et al. showed that high-glucose-induced, FOXO1-mediated KLF2 suppression could be reversed by statin treatment.[9] As it were, FOXO1 could be a therapeutic target of statin in endothelial cell. However, in CORONA study,[16] statin treatment was not beneficial for reducing mortality in patients with systolic heart failure, even though many basic research studies reported cardioprotective effect of statin in vitro and in vivo. However, recent clinical study showed that statin use was associated with a lower risk for incident HF in patients with breast cancer treated with anthracycline chemotherapy.[12] Following this report, several prospective randomize controlled studies (ClinicalTrials.gov identifier; NCT01988571, NCT02096588, NCT02674204, NCT02943590) are going on to show the cardioprotective effect of statin. The present study was the first to show FOXO1 as a therapeutic target of statin in cardiomyocyte.

In this study, a new imaging technique, cardiac MRI was used to prove the

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cardioprotective role of statin in doxorubicin-induced myocardial injury mouse model. In general, cardiac MRI is superior to echocardiogram in terms of objective, quantitative measurements of cardiac function. However, there have been few studies to show the cardioprotective effect of statin, especially in doxorubicin-induced cardiotoxicity animal model. The present study has superiority to show the cardioprotective role of statin in terms of molecular biologic, histologic and functional measurements in vivo.

V. CONCLUSION

The present study showed that survivin mediates the cardioprotective effect of statin against doxorubicin-induced cardiotoxicity in vitro and in vivo. In addition, doxorubicin induced FOXO1 bound to STAT3 and prevented STAT3 from interacting with Sp1 and statin significantly inhibited FOXO1 binding to STAT3 and restored STAT3 binding to Sp1 and stabilized transcription complex of STAT3/Sp1. The transcriptional regulation of survivin was mediated via this FOXO1/STAT3/Sp1 transcriptional network. The present study discovers a new pathophysiologic mechanism of statin for reducing doxorubicin-induced cardiotoxicity in terms of transcriptionally modulating anti-apoptotic protein, survivin.

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ABSTRACT (in Korean)

독소루비신 유도 심근 손상 모델에서 FOXO1 활성 저하와 survivin 전사 조절에 의한 스타틴의 심근보호효과

<지도교수 강 석 민>

연세대학교 대학원 의학과

오 재 원

Survivin은 세포사멸 저해 단백질의 하나로, 독소루비신 유도 심장독성모델에서 세포사멸 저해 효과가 있다. 스타틴은 항암치료를 받는 유방암 환자에서 심부전의 발생을 줄일 수 있는 약제이다. 따라서 독소루비신 유도 심장독성모델에서 스타틴의 심근보호효과에 survivin이 관여하는 지에 대해 연구하였다. H9c2 심근세포에 독소루비신을 처리하게 되면 FOXO1 활성화에 의해 survivin 발현이 감소하였다. 그러나, 스타틴을 전처리하게 되면 FOXO1의 인산화를 증가시키고 핵 내 발현을 감소시켜 독소루비신에 의해 감소하는 survivin

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의 발현 감소 정도를 억제하였다. 또한 독소루비신에 의해 심근세포 내의 FOXO1와 STAT3와의 결합이 유도되고, STAT3와 Sp1의 결합은 저해하게 되는데, 이러한 변화는 스타틴 전처리에 의해 회복되었다. Chromatin immunoprecipitation 분석을 통해 이러한 전사인자들 사이의 결합의 변화가 survivin promoter 부분의 결합과 연관이 있음을 확인하였다. 또한 독소루비신을 투여한 심근손상 동물 모델에서, 스타틴 경구 투여를 통해 심근 세포사멸이 감소하고, 심근 내 survivin 발현 감소가 저해되며, 심장 기능이 회복되는 것을 심장 자기공명 영상을 통해 확인하였다. 이러한 연구 결과를 통해 독소루비신 유도 심근손상 모델에서 스타틴은 FOXO1/STAT3/Sp1 전사인자 네트워크를 조절함으로써, survivin 발현을 조절하고, 이를 통해 심근보호효과를 나타낼 수 있음을 확인하였다.

핵심되는 말: Survivin, FOXO1, 스타틴, 심장독성, 심부전