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CD24 enhances DNA damage-induced apoptosis by modulating NF-κB signaling in CD44-expressing breast cancer cells

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Cluster of differentiation 24 (CD24) is a small glycosylphosphatidylinositol-linked cell surface molecule that is expressed in a variety of human carcinomas, including breast cancer. To determine the role of CD24 in breast cancer cells, we expressed CD24 in CD24-negative/low and cluster of differentiation 44 (CD44)-positive MDA-MB-231 metastatic breast cancer cells. Forced expression of CD24 resulted in a decrease in c-Raf/mitogen-activated protein/extracellular signal-regulated kinase kinase (MEK)/mitogen-activated protein kinase signaling and reduced cell proliferation. Apoptosis induced by DNA damage was greatly enhanced in MDA-MB-231 CD24 cells as compared with MDA-MB-231 vec cells. CD24 expression efficiently attenuated DNA damage-induced nuclear factor-kappaB (NF-κB) signaling in MDA-MB-231 cells. However, in CD24-positive and CD44-negative/low MCF-7 cells, knockdown of CD24 did not significantly affect DNA damage-induced apoptosis nor NF-κB signaling. Silencing of CD24 in CD24/CD44-double-positive MDA-MB-468 cells partially rescued DNA damage-induced apoptosis. Transient transfection studies with 293T cells also revealed that CD24 attenuated cell viability and NF-κB signaling only when CD44 was cotransfected. These data indicate that CD24 expression potentiated DNA-induced apoptosis by suppressing antiapoptotic NF-κB signaling in CD44-expressing cells.

Introduction

Cluster of differentiation 24 (CD24) is a glycosylphosphatidylinositol-anchored glycoprotein with N- and O-linked glycosylation sites that binds to P-selectin but not to E- or L-selectin (1). CD24 stimulates the adhesion of monocytes or neutrophils to P-selectin-expressing endothelial cells or platelets (2) and is expressed in many cancer types, including renal, ovarian, lung and pancreatic cancers (3–5). In particular, CD24 expression is a candidate for a new prognostic marker in breast cancer (6). A possible role for CD24 in the mediation of the progression and metastasis of breast cancer via the interaction between tumor cells and platelets or endothelial cells has been postulated (7). However, CD24-low/negative and cluster of differentiation 44 (CD44)-positive cells are considered as to be breast cancer-initiating cells, which have the ability to form new tumors in immunocompromized mice (8). These conflicting findings might be explained by the fact that CD24 is generally regarded as a marker for metastatic cancer cells. Although the signaling downstream of CD24 and/or CD44 has not yet been fully elucidated, the metastatic process might be mechanistically different from the growth of new tumors.

Nuclear factor-kappaB (NF-κB) is a ubiquitous transcription factor that forms protein dimers that bind kappaB sites (5′-GGGGYNNCCY-3′) (9). In normal cells, NF-κB interacts with inhibitors of NF-κB α (IκBα). The interaction between NF-κB and IκBα causes NF-κB to be retained in the cytoplasm. IκBα has two conserved serines that can be phosphorylated by the IκB kinases (IKKs). Phosphorylated IκBα is degraded by proteasomes (10). NF-κB plays a key role in stimulating proliferation of both normal and cancer cells by activating target genes such as cyclin D1 (11). In addition to its role in cell proliferation, NF-κB is an inhibitor of tumor necrosis factor α- induced apoptosis (12), and NF-κB is able to inhibit the apoptotic response of DNA-damaging anticancer drugs and ionizing radiation (13). Cancer cells with constitutively activated NF-κB are resistant to anticancer drugs and radiotherapy and inhibition of NF-κB activity in these cells increases their sensitivity to anticancer drugs (14,15). NF-κB inhibits apoptosis by inducing proapoptotic factors including cellular inhibitors of apoptosis, c-FLICE inhibitory protein and Bcl-2 family proteins such as A1 and Bcl-xl (16).

In this study, we used a retroviral vector to deliver CD24 complementary DNA into CD24-negative/low and CD44-positive MDA-MB-231 metastatic human breast cancer cells. Doxorubicin and irradiation were used to induce apoptosis (17,18) to comparatively analyze the effect of these DNA-damaging agents on MDA-MB-231 CD24 and MDA-MB-231 vec control cells. Also, we knocked down CD24 expression in CD24 positive, CD44 low/negative MCF-7 cells and CD24/CD44-double positive MDA-MB-468 cells (19) to check the possible involvement of CD44 in CD24-induced events inside the cells.

Abbreviations: CD24, cluster of differentiation 24; CD44, cluster of differentiation 44; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; IKK, IκB kinase; MAPK, mitogen-activated protein kinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NF-κB, nuclear factor-kappaB; PARP, poly adenosine diphosphate-ribose polymerase; PBS, phosphate-buffered saline.

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Materials and methods

Cell culture

Cell lines 293T (ATCC CRL-11268), MDA-MB-231 (ATCC HTB-26), MCF-7 (ATCC HTB-22) and MDA-MB-468 (ATCC HTB-132) cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 100 U/ml penicillin and 100 mg/ml streptomycin (Welgene, Daegu, Korea). All cell lines were incubated at 37°C with 5% CO_2 in a humidified atmosphere.

Antibodies and reagents

Antibodies to phospho-c-Raf (Ser338), phospho-mitogen-activated protein/extracellular signal-regulated kinase kinase (MEK)1/2 (Ser217/221), phospho-p44/42 mitogen-activated protein kinase (MAPK) (Thr202/Tyr204), phospho-p90 ribosomal S6 kinase (RSK) (Ser380), phospho-Elk-1 (Ser383), p90RSK, Elk-1, Bcl-xl, caspase-3, IKKα, IKKβ, phospho-IKKα/β (Ser176/180), IκBα, phospho-IκBα (Ser32), NF-κB p65, phospho-NF-κB p65 (Ser536) and phospho-epidermal growth factor receptor (EGFR) (Tyr1173) were from Cell Signaling Technology (Beverly, MA); phospho-44/42 MAPK, cyclin D1, CD24 (SN3), EGFR and p27 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Phycoerythrin mouse anti-human CD24 (ML5) and poly adenosine diphosphate-ribose polymerase (PARP) antibody were from BD Biosciences (San Jose, CA). Doxorubicin was from Calbiochem (Darnstadt, Germany). Epidermal growth factor (EGF) was purchased from Peprotech (Rocky Hill, NJ). CD24 siRNA and control siRNA were from Santa Cruz Biotechnology.

Plasmids and transfection

The retroviral vector encoding CD24 (MSCV-EGFP-CD24) and the empty vector (MSCV-EGFP-vec) were kindly provided by Dr K.Ozawa (Jichi Medical School, Japan). The 293T cells were seeded in 100-mm dishes (5×10^5 cells per dish) or 12-well plates (5×10^4 cells/per well) and transfected with calcium phosphate in *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid-buffered saline as described (20,21). MDA-MB-231 cells were seeded in 100-mm dishes (5×10^5 cells per dish) or 12-well plates (5×10^4 cells per well) and then transfected with FuGENE HD (Roche, Basel, Switzerland) according to the manufacturer's protocols. MDA-MB-468 cells (5×10^6 cells/100 mm dish) were transfected

with CD24 siRNA and control siRNA using Lipofectamine2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's protocols.

Retroviral infection

293T cells on 100-mm dishes were transfected with MSCV-EGFP-CD24, MSCV-EGFP-vec, pSuper-retro-mCD24 shRNA or pSuper-retro-hCD24 shRNA along with phCMV-VSVG and pCMV gag-pol (8:3:4 mass ratio). After 48 h, retroviral particles were collected from 293T cell media. MDA-MB-231 and MCF-7 cells were treated with retroviral particles containing media and 4 μg/ml of polybrene. Six hours later, the media was changed with fresh media. Enhanced green fluorescence protein (EGFP)-positive cells were sorted by flow cytometry (Becton & Dickinson Biosciences, Mountain View, CA). Expression of CD24 was confirmed by cell surface labeling assays.

Construction of the CD24 shRNA plasmid

Human CD24-directed shRNA and control mCD24 shRNA were inserted into pSuper.retro.puro. viral vector (OligoEngine, Seattle, WA). The hCD24 shRNA was produced by subcloning of CD24 shRNA oligonucleotides; 5′-gatcccccaactggaacttcaagtaattcaagagattacttgaagttccagttgtttttggaaa-3′ and 5′-agcttttccaaaaacaactggaacttcaagtaatctcttgaattacttgaagttccagttgggg-3′ were annealed and inserted into the BglII and HindIII site of pSuper.retro.puro. viral vector. As control oligonucleotides, 5′-gatccccctctacacctaccccaaattcaagagattttggggtaggtgtagaagtttttggaaa-3′ and 5′-agcttttccaaaaacttctacacctaccccaaatct cttgaatttggggtaggtgtagaagggg-3′ specific against mouse CD24 gene were inserted into the same vector. This mouse CD24 shRNA oligo does not interfere with human CD24 gene expression.

Cell-surface labeling

Cells were harvested with 0.25% trypsin and washed once with phosphate-buffered saline (PBS). After centrifugation, cells were incubated with phycoerythrin mouse anti-human CD24 (BD Biosciences, San Jose, CA). for 1 h, then washed three times with PBS. Cell surface labeling analysis was performed with a BD flow cytometer (Becton & Dickinson Biosciences).

Proliferation assays

Cells were seeded at 5×10^4 or 1×10^5 cells per well in 12-well dishes, trypsinized and counted with a hemocytometer in triplicate, every 24 h for 5 days. For 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays, cells were seeded in 96-well culture plates at 5×10^3 cells per well and exposed to indicated concentrations of doxorubicin. Cell viability was assessed by adding 20 μl of 10 mg/ml MTT (Sigma, St Louis, MO) to 100 μl of culture medium and incubating for 3 h at 37°C. After removing the medium, formazan was dissolved in dimethyl sulfoxide (Sigma) and the optical density was measured at 590 nm using a Multiskan EX (Thermo, Vantaa, Finland).

Dual luciferase assays

MDA-MB-231, MCF-7, MDA-MB-468 or 293T cells were transfected with 1 μg of total DNA containing reporter constructs (500 ng of NF-κB-Luc and 2 ng of pCMV-Rl). The cells were analyzed 48 h after transfection. Dual luciferase assays were performed using the Dual-luciferase Reporter Assay System (Promega, Madison, WI) according to the manufacturer's instructions.

Western blot analyses

Cells were washed once with PBS and lysed in a lysis buffer [20 mM Tris–HCl, pH 7.4, 0.1 mM ethylenediaminetetraacetic acid, 150 mM NaCl, 1% NP-40, 0.1% Triton X-100, 0.1% sodium dodecyl sulfate, 20 mM NaF, 1 mM Na_3VO_4, 1× protease inhibitor (Roche, Indianapolis, IN)]. Protein samples were resolved using 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis after boiling for 10 min in sodium dodecyl sulfate sample buffer. For western blot analysis, proteins were electrotransferred onto nitrocellulose membranes for 1 h. Membranes were blocked with 5% skim milk in Tris-buffered saline containing Tween-20 (TBST) for 1 h and incubated with appropriated dilutions of primary antibody in TBST containing 5% bovine serum albumin overnight. Membranes were washed three times with TBST and incubated with secondary antibody solution for 2 h. Blots were visualized with the WEST-ZOL-plus detection system (iNtRON Biotechnology, Seoul, Korea).

Cell cycle analysis by flow cytometry

Cells were harvested with 0.25% trypsin and washed once with PBS. After centrifugation, cells were fixed in 100% ice-cold methanol overnight at −20°C. Fixed cells were incubated with 50 μg/ml of propidium iodide in PBS and 1 mg/ml RNAase in PBS for 30 min. Cell cycle analysis were performed on a BD FACS (Becton & Dickinson Biosciences).

Irradiation Procedure

MDA-MB-231 cells were seeded in 12-well plates for dual luciferase assays or in 100-mm dishes for western blotting and cell cycle analysis. After 24 h, cells were irradiated with ^{137}Cs γ-rays (10 Gy) (Atomic Energy of Canada Limited, Ontario, Canada).

Results

CD24 expression downregulated MDA-MB-231 cell proliferation

Firstly, we compared the proliferation rate of MDA-MB-231 CD24 cells expressing CD24 by retroviral infection with that of MDA-MB-231 vec control cells. Flow cytometry analyses confirmed the surface expression of CD24 in MDA-MB-231 CD24 cells and Enhanced green fluorescence protein in the cytosol of MDA-MB-231 CD24 and MDA-MB-231 vec cells (Figure 1A). As seen in Figure 1B, the growth rate of MDA-MB-231 CD24 cells was downregulated compared with that of MDA-MB-231 vec cells (4.2×10^5 versus 5.9×10^5 cells 5 days after cell seeding), suggesting that CD24-induced cellular event(s) may decrease cell proliferation rate. We also checked the effect of CD24 on cell migration and invasion since it was previously known that CD24 is associated with cell migration and invasion (22,23). Our results suggest that forced expression of CD24 resulted in increase migration of MDA-MB-231 cells as revealed by both transwell migration and wound healing assays (supplementary Figure 1 is available at *Carcinogenesis* Online). The results indicate that the ectopic CD24 was functional in MDA-MB-231 cells.

The effects of CD24 expression on intracellular effector molecules

To investigate the mechanisms responsible for the CD24-mediated downregulation of MDA-MB-231 cell proliferation, we examined the statuses of intracellular signaling molecules. Signaling by c-Raf/MEK/MAPK was downregulated in MDA-MB-231 CD24 cells (Figure 1C). Moreover, when CD24 was overexpressed in MDA-MB-231 cells, cyclin D1 was downregulated and expression of p27 was upregulated. Activation of the c-Raf/MEK/MAPK signaling cascade is required for cell proliferation (24,25), and the cell cycle regulatory proteins cyclin D1 and p27 affect cell proliferation by modulating cell cycle progression (26,27). Our results suggest that forced CD24 expression might cause a decrease in cell proliferation by downregulating c-Raf/MEK/MAPK and by modulating cell cycle regulatory proteins in MDA-MB-231 cells.

CD24 increased doxorubicin and ionizing irradiation-induced apoptosis in MDA-MB-231 cells

To determine the effects of CD24 on drug sensitivity and radiation sensitivity in MDA-MB-231 cells, cells were treated with dimethyl sulfoxide (vehicle), doxorubicin (500 nM) or ionizing radiation (10 Gy of ^{137}Cs γ rays). Cells were counted every 24 h for 5 days (Figure 2A). Treatment with doxorubicin or radiation resulted in death of almost all MDA-MB-231 CD24 cells, whereas induction of cell death in MDA-MB-231 vec cells was significantly attenuated compared with that of MDA-MB-231 CD24 cells (Figure 2A). These results indicated that forced expression of CD24 might confers sensitivity to DNA damage-induced apoptosis in MDA-MB-231 cells.

For cell cycle analysis, propidium iodide-labeled nuclei were analyzed by using flow cytometry. We found that the sub-G_1 apoptotic fraction was significantly increased in MDA-MB-231 CD24 cells after doxorubicin treatment or irradiation, whereas MDA-MB-231 vec cells showed a less prominent increase in the apoptotic fraction (Figure 2B and C). These results suggest that overexpression of CD24 made MDA-MB-231 cells more susceptible to doxorubicin- or radiation-induced apoptosis. In irradiated cells, we observed massive increases in the G_2/M fraction and in the apoptotic fraction (Figure 2B and C). Radiation-induced DNA damage has been shown to inhibit cdc2/cyclin B activity and cause G_2/M arrest (28,29).

We also found efficient downregulation of the antiapoptotic protein Bcl-xl and enhanced proteolytic cleavage of caspase-3, an apoptosis marker, after doxorubicin treatment or irradiation of MDA-MB-231 CD24 cells compared with those of the MDA-MB-231 vec cells (Figure 2D). These findings suggest that the forced expression of

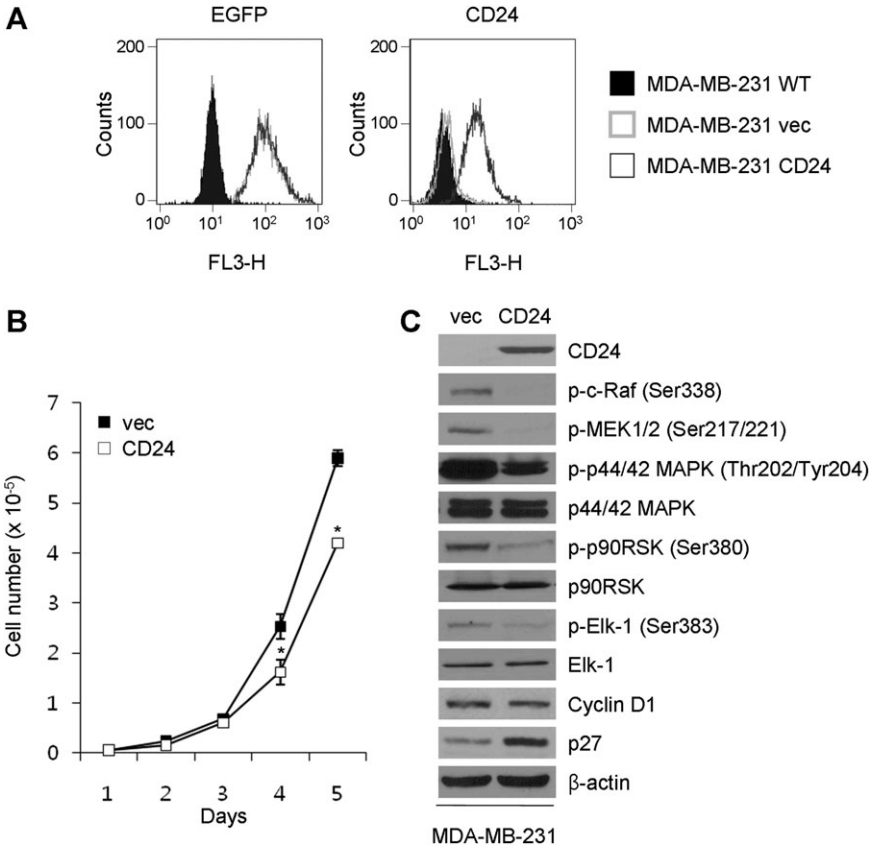

Fig. 1. Effect of CD24 overexpression on proliferation and migration of MDA-MB-231 cells. (**A**) Retroviral expression of CD24 in MDA-MB-231 breast cancer cells. MDA-MB-231 cells were infected with retroviruses encoding MSCV-EGFP-CD24 (CD24) and MSCV-EGFP vector (vec). The expression of GFP and CD24 was confirmed by surface labeling experiments with CD24 antibody using flow cytometry. (**B**) Activation of CD24 downregulated the growth of MDA-MB-231 cells. Cells were seeded at the density of 5×10^3 cells per well in 12-well dish and counted with hemocytometer for 5 days ($^*P < 0.05$). (**C**) Effects of CD24 on intracellular signaling molecules. MDA-MB-231 vec and MDA-MB-231 CD24 cells were seeded at the density of 1×10^6 cells per dish in 100-mm dishes. Twenty-four hours after, the cells were subjected to western blot analysis with each indicated antibodies.

CD24 may accelerate DNA damage-induced apoptotic cell death by regulating apoptosis-related intracellular effector molecules.

CD24 expression affected NF-κB signaling

To investigate the molecular mechanism of CD24-induced apoptosis in MDA-MB-231 cells, we compared NF-κB signaling in MDA-MB-231 vec and MDA-MB-231 CD24 cells. When the basal status of NF-κB signaling in the cell lines was examined, phosphorylations of IKKα/β, IκBα and NF-κB were significantly decreased in MDA-MB-231 CD24 cells compared with those in MDA-MB-231 vec cells, suggesting that overexpression of CD24 inhibited NF-κB signaling (lane 1 versus lane 2, Figure 3A). Furthermore, upon induction of apoptotic cell death by either doxorubicin (Figure 3A left panel) or ionizing irradiation (Figure 3A right panel), we observed increases in the phosphorylations of IKKα/β, IκBα and NF-κB in both MDA-MB-231 CD24 and MDA-MB-231 vec cells (lane 1 versus lane 3, lane 2 versus lane 4, Figure 3A). Interestingly, the increases in phosphorylation of these NF-κB signaling molecules due to DNA damage were significantly impaired in MDA-MB-231 CD24 cells compared with those in MDA-MB-231 vec cells (lane 3 versus lane 4, Figure 3A). The results in Figure 3A collectively suggest that CD24 attenuates both basal and DNA damage-induced NF-κB signaling in MDA-MB-231 cells.

Having confirmed that CD24 efficiently downregulated NF-κB signaling in MDA-MB-231 cells, we investigated the effect of CD24 expression on NF-κB-dependent transcription using NF-κB-dependent transcription reporter assays in MDA-MB-231 vec and MDA-MB-231 CD24 cells. Consistent with the results in Figure 3A, overexpression of CD24 inhibited NF-κB-dependent transcription in MDA-MB-231 cells (Figure 3B and C). Upon induction of apoptosis by either doxorubicin or ionizing radiation, NF-κB-dependent transcription increased in both MDA-MB-231 vec and MDA-MB-231 CD24 cells. DNA damage did not induce NF-κB transcriptional activity as efficiently in MDA-MB-231 CD24 cells as in MDA-MB-231 vec cells. These results collectively suggest that CD24 modulated NF-κB signaling, resulting in the downregulation of NF-κB-dependent transcription in MDA-MB-231 cells. Indeed, the level of Bcl-xl protein, a product of an NF-κB target gene, was significantly reduced in CD24-expressing MDA-MB-231 cells after DNA damage (Figure 2D).

To further elucidate the functional connection between CD24 and NF-κB signaling in DNA damage-induced apoptosis, we observed the effect of EGF on doxorubicin-induced apoptosis in MDA-MB-231 cells. EGF was known to activate NF-κB signaling through EGFR kinase activity-dependent mechanism (30). When serum-starved MDA-MB-231 vec and MDA-MB-231 CD24 cells were treated with EGF, we observed ∼30% increase in cell proliferation as compared with controls (Figure 3D, first bar versus third bar and second bar versus fourth bar). When doxorubicin was added to EGF-pretreated cells, we observed that pretreatment of EGF rescued doxorubicin-induced decrease in cell proliferation (Figure 3D, fifth bar versus seventh bar and sixth bar versus eighth bar), suggesting that EGF could rescue doxorubicin-induced cell death possibly via modulation of NF-κB signaling. Indeed, the results in Figure 3E indicate that treatment with EGF could effectively increase phosphorylation of NF-κB. Of note, EGF and doxorubicin cotreatment-induced increase in NF-κB phosphorylation was significantly impaired in cells expressing CD24. These results further confirm that CD24 could modulate DNA damage-induced apoptosis via regulation of NF-κB signaling. Phosphorylation

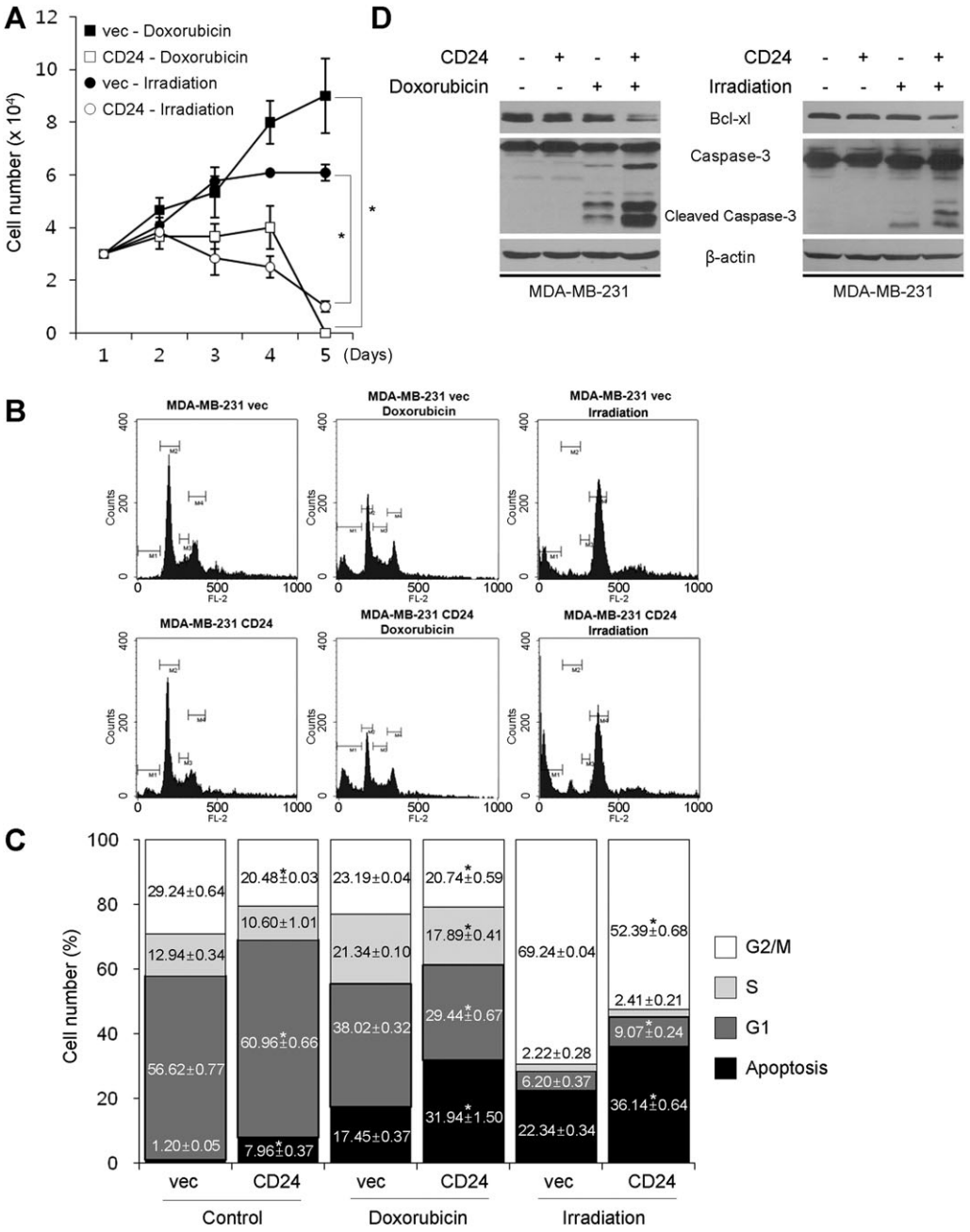

Fig. 2. CD24 expression sensitized MDA-MB-231 cells to DNA damage-induced apoptosis. (**A**) Proliferation of MDA-MB-231 vec and MDA-MB-231 CD24 upon treatment with doxorubicin and irradiation. Cells were seeded at the density of 3×10^4 cells per well in 12-well dishes and counted with hemocytometer for 5 days. Twenty-four hours after cell seeding, doxorubicin was treated at the concentration of 500 nM. Medium containing fresh drug was replenished every 2 days. For irradiation, the cells were exposed to ^{137}Cs γ rays at the dose of 10 Gy in 24 h after cell seeding. (**B**) Cell cycle profiles of MDA-MB-231 vec and MDA-MB-231 CD24 upon treatment with doxorubicin and irradiation. Cells were seeded at the density of 5×10^6 cells per dish in 100-mm dishes. Doxorubicin was treated at the concentration of 500 nM for 48 h. For irradiation, the cells were exposed to ^{137}Cs γ rays at the dose of 10 Gy and harvested 24 h after the exposure. The cells were then fixed in methanol and incubated in PBS containing 50 μg/ml propodium iodide and 1 mg/ml RNase. Propodium iodide-labeled nuclei were analyzed by flow cytometry. (**C**) The DNA histograms in Figure 2. (B) were represented as bar graphs from three independent experiments ($^*P < 0.05$). (**D**) Effects of DNA-damaging agents on caspase3 and Bcl-xl. MDA-MB-231 vec and MDA-MB-231 CD24 cells were seeded at the density of 1×10^6 cells per dish in 100-mm dishes. The cells were then treated with doxorubicin (500 nM) for 24 h. For irradiation, the cells were exposed to ^{137}Cs γ rays at the dose of 10 Gy 24 h before cell harvest. After treatment, the cell lysates were subjected to western blot analyses with each indicated antibodies.

of EGFR was effectively increased by EGF treatment, suggesting that EGF used in our study is biologically active.

Differential effect of CD24 on NF-κB signaling in MCF-7 cells

To further assess the effect of CD24 on apoptosis and NF-κB signaling, we knocked down CD24 in CD24-positive breast cancer cells, MCF-7 by using retroviral delivery of shRNA directed against human CD24 (Figure 4A). Interestingly, CD24 in MCF-7 cells exhibited slight increase in the electrophoretic mobility as compared with CD24 in MDA-MB-231 cells, possibly due to differences in glycosylation pattern between two cell lines (31). When the effect of CD24 ablation on NF-κB signaling was tested in MCF-7 cells, we found that doxorubicin decreased NF-κB signaling by inhibiting phosphorylation of IKKα/β, IκBα and NF-κB (Figure 4B). The

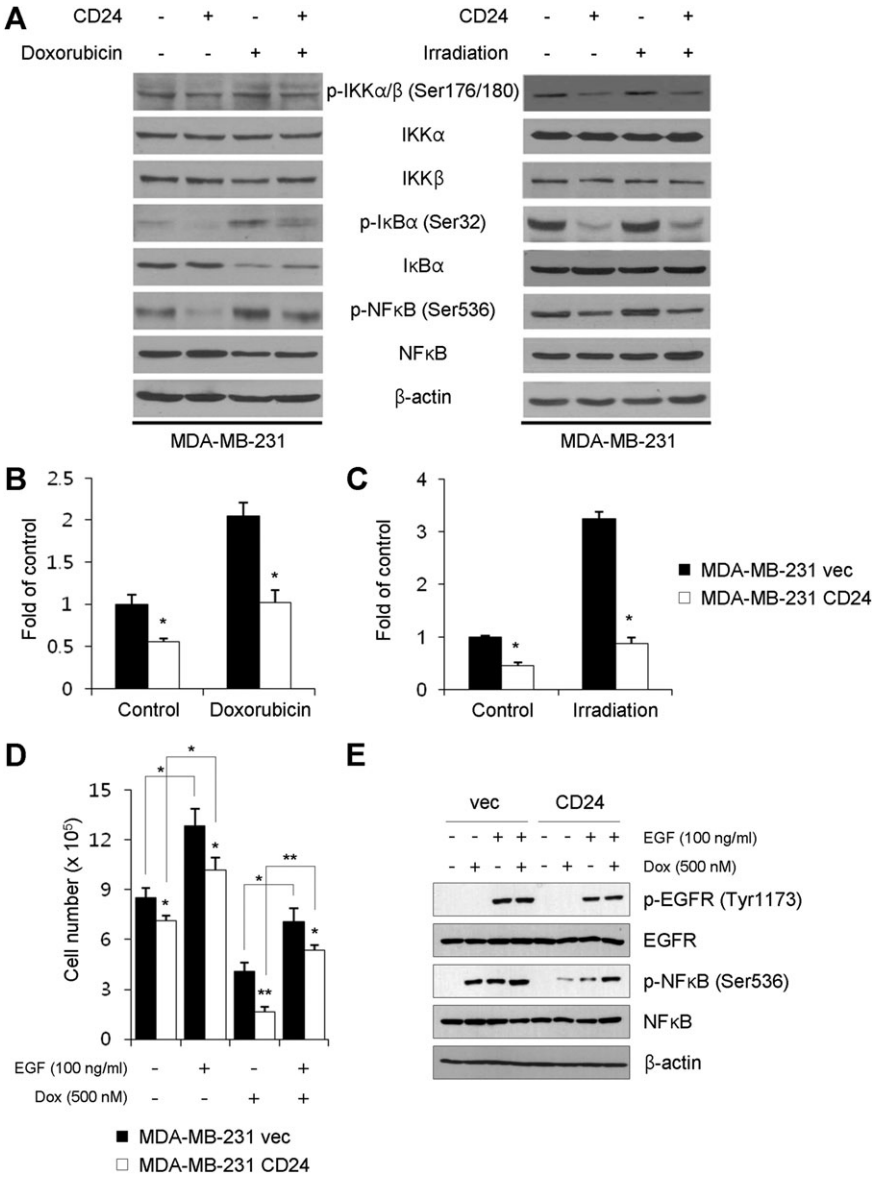

Fig. 3. Effects of CD24, doxorubicin and irradiation on NF-κB signaling. (**A**) Effects of CD24, doxorubicin and irradiation on NF-κB signaling molecules. MDA-MB-231 vec and MDA-MB-231 CD24 cells were prepared as in Figure 2D. The cell lysates were subjected to western blot analyses with each indicated antibodies. (**B** and **C**) NF-κB-dependent transcriptional activity of MDA-MB-231 vec and MDA-MB-231 CD24 cells upon treatment with doxorubicin and irradiation. Cells were transfected with 500 ng of reporter constructs and analyzed by dual luciferase assays 48 h after transfection. Doxorubicin was treated at the concentration of 500 nM for 24 h. For irradiation, the cells were exposed to ^{137}Cs γ rays at the dose of 10 Gy 24 h before assays ($^{*}P < 0.05$). (**D**) Protective effect of EGF on doxorubicin-induced cell death. After serum starvation for 24 h, cells were pretreated with 100 ng/ml EGF for 24 h and treated with 500 nM doxorubicin for additional 24 h. The cells were then counted with hemocytometer ($^{*}P < 0.05$, $^{**}P < 0.005$). (**E**) Effect of EGF on doxorubicin-induced NF-κB signaling. After treatments as in Figure 3D, the cells were subjected to western blot analyses with each indicated antibodies.

results are contradictory to what we observed in MDA-MB-231 cells (Figure 3B).

When the effect of doxorubicin on the proliferation of MCF-7 cells was tested, it was found that CD24 did not significantly affect the inhibitory effect of doxorubicin on MCF-7 cell proliferation (Figure 4C and D). Interestingly, knockdown of CD24 in MCF-7 cells resulted in a decrease of MCF-7 cell proliferation in the absence of doxorubicin. Furthermore, within a various range of doxorubicin concentration tested, we could not observe sensitizing effect of CD24 on cytotoxicity of doxorubicin in MCF-7 cells (Figure 4E). Rather, CD24-expressing MCF-7 cells (mCD24sh) were shown to be more resistant to doxorubicin than CD24 knockout MCF-7 cells (hCD24sh) at lower doxorubicin doses. The results rule out the possibility that doxorubicin concentration used in Figure 4C was too high to see the differential effect on two cell lines used. Furthermore, treatment with doxorubicin rather decreased NF-κB transcriptional activity in both MCF-7 mCD24sh and MCF-7 hCD24sh cells (Figure 4F). Induction of PARP cleavage, a hallmark of apoptosis, by doxorubicin was not significantly different between two cell lines (Figure 4B). The results in Figure 4B–D and F are different from what we observed with MDA-MB-231 cells in which CD24 expression decreased cell proliferation and sensitized the cells to DNA damage-induced apoptosis via attenuation of NF-κB signaling (Figures 1B, 2A and 3A–C).

To explain the differential effect of CD24 on NF-κB signaling between MDA-MB-231 and MCF-7 cells, we checked a possible involvement of another cell surface molecule, CD44 in modulating CD24-dependent regulation of NF-κB signaling. It has been previously reported that MDA-MB-231 cells are CD24-negative/low and CD44-positive, whereas MCF-7 cells are CD24-positive and CD44-negative/low (19). To test if co-existence of CD44 with CD24 is

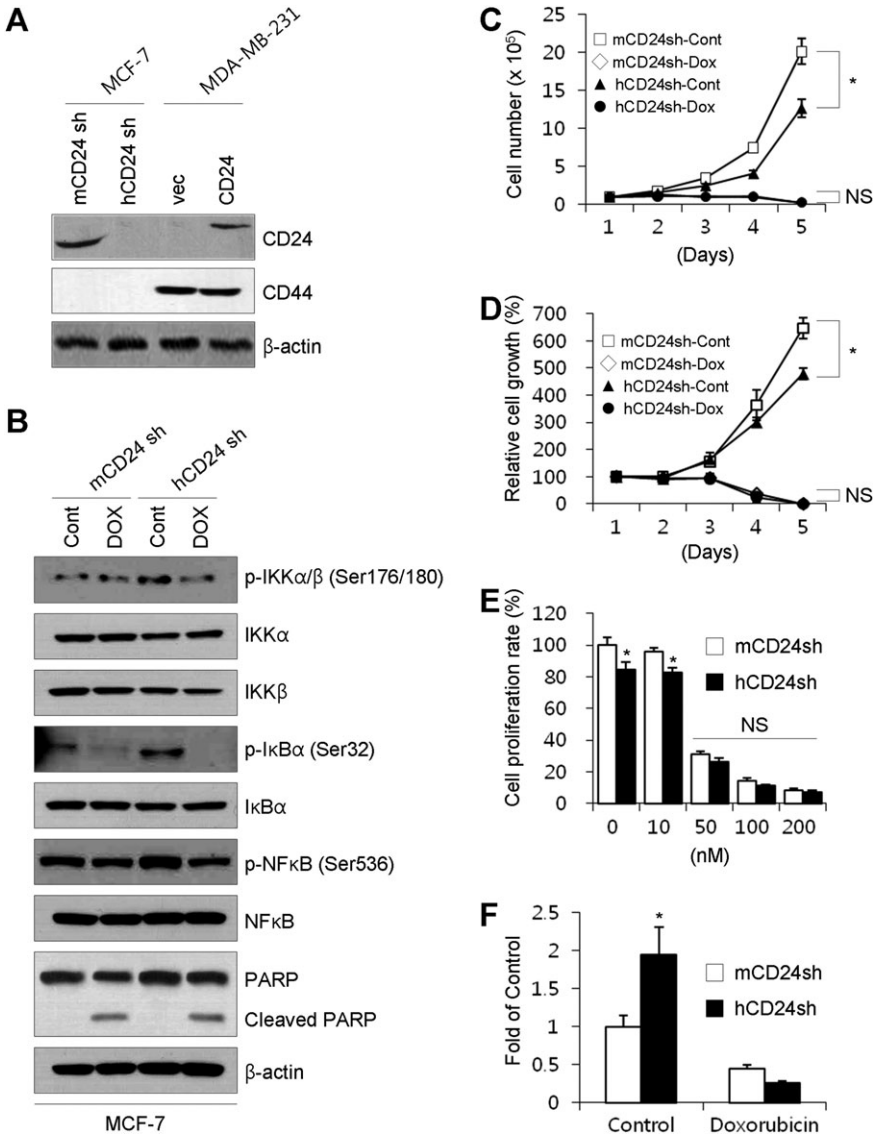

Fig. 4. Effect of CD24 on cell proliferation and NF-κB signaling in MCF-7 cells upon doxorubicin treatment. (**A**) Establishment of CD24 knockout MCF-7 cells. MCF-7 human breast cancer cells were infected with retroviruses pSuper-retro-control shRNA (mouse CD24 shRNA, mCD24sh) and human CD24 shRNA (hCD24sh). The inhibition of CD24 expression was confirmed by western blot analyses. (**B**) Effect of doxorubicin on NF-κB signaling in MCF-7 mCD24sh and MCF-7 hCD24sh cells. MCF-7 mCD24sh and hCD24sh cells were seeded at the density of 1×10^6 cells per dish in 100-mm dishes. The cells were then treated with doxorubicin (500 nM) for 24 h. After treatment, the cells were subjected to western blot analyses with each indicated NF-κB signaling antibodies and PARP antibody. (**C** and **D**) Effect of doxorubicin on MCF-7 cell proliferation. MCF-7 mCD24sh and hCD24sh cells were treated with 500 nM doxorubicin and subjected to cell counting (C) and MTT assays (D) at each indicated time points ($^*P < 0.05$, NS, non-significant). (**E**) Differential effect of various doses of doxorubicin on MCF-7 cell proliferation. MCF-7 mCD24sh and hCD24sh cells were treated with various concentrations of doxorubicin for 48 h and subjected to MTT assays ($^*P < 0.05$, NS, non-significant). (E) NF-κB-dependent transcriptional activity of MCF-7 mCD24sh and hCD24sh cells upon treatment with doxorubicin. Cells were transfected with 500 ng of reporter constructs and analyzed by dual luciferase assays 48 h after transfection. Doxorubicin was treated at the concentration of 500 nM for 24 h ($^*P < 0.05$).

required for CD24-induced downregulation of NF-κB signaling and apoptosis, we employed MDA-MB-468 cells expressing both CD24 and CD44. When CD24 expression was reduced by CD24 siRNA transfection (Figure 5A), we observed increases in both basal levels (Figure 5B lane 1 versus lane 3) and doxorubicin-induced levels (Figure 5B lane 2 versus lane 4) of p-IKKα/β, p-IκBα and p-NF-κB, suggesting that CD24 also downregulated NF-κB signaling in CD44-positive MDA-MB-468 cells as well as in CD44-positive MDA-MB-231 cells. Cleavage of PARP was also significantly reduced by CD24 silencing (Figure 5B). When the effect of CD24 siRNA on proliferation of MDA-MB-468 cells both in the absence and presence of doxorubicin was tested, we observed that CD24 siRNA treatment could effectively increase MDA-MB-468 cell proliferation regardless of doxorubicin treatment (Figure 5C). Furthermore, we found that CD24 siRNA treatment could rescue doxorubicin-induced decrease in cell proliferation at various doses of doxorubicin tested (Figure 5D). The results show interesting comparison with the results with MCF-7 in Figure 4E in which CD24 silencing rather potentiated doxorubicin effect at lower doxorubicin doses and had no effect at higher doses. In accordance with the results in Figure 5B, both basal levels and doxorubicin-induced NF-κB transcriptional activities were significantly increased by CD24 siRNA (Figure 5E). The data in Figure 5 together indicate that CD24 potentiated cytotoxic effect of DNA-damaging agents via downregulation of NF-κB signaling in CD44-positive MDA-MB-468 cells.

To further confirm whether co-expression of CD44 with CD24 may affect CD24-induced modulation in NF-κB signaling, 293T cells were transfected with various combinations of CD24 and CD44 as

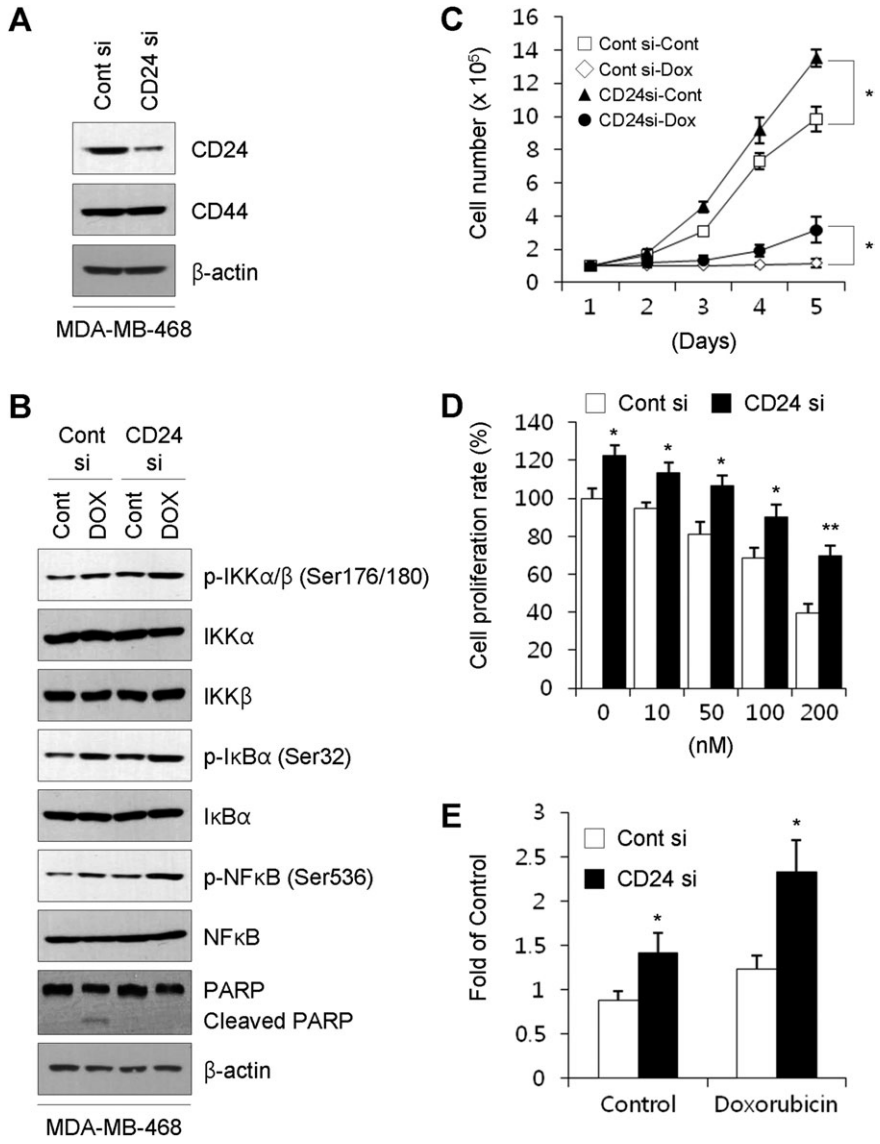

Fig. 5. Effect of CD24 siRNA on cell proliferation and NF-κB signaling in MDA-MB-468 cells upon doxorubicin treatment. (**A**) Inhibition of CD24 expression by siRNA in MDA-MB-458 cells. MDA-MB-468 human breast cancer cells were transfected with CD24 siRNA and control siRNA. The inhibition of CD24 expression was confirmed by western blot analyses. (**B**) Effect of doxorubicin on NF-κB signaling in MDA-MB-468 cells. MDA-MB-468 CD24 siRNA and control siRNA cells were seeded at the density of 1×10^6 cells per dish in 100-mm dishes. The cells were then treated with doxorubicin (500 nM) for 24 h. After treatment, the cells were subjected to western blot analyses with each indicated NF-κB signaling antibodies. (**C**) Effect of doxorubicin on proliferation of MDA-MB-468 cells. MDA-MB-468 CD24 siRNA and control siRNA cells were treated with 500 nM doxorubicin and subjected to cell counting with hemocytometer at each indicated time points ($^*P < 0.05$). (**D**) Effect of various doses of doxorubicin on MDA-MB-468 cell proliferation. MDA-MB-468 CD24 siRNA and control siRNA cells were treated with various concentrations of doxorubicin for 48 h and subjected to MTT assays ($^*P < 0.05$). (**E**) NF-κB-dependent transcriptional activity of MDA-MB-468 CD24 siRNA and control siRNA cells upon treatment with doxorubicin. Cells were transfected with 500 ng of reporter constructs and analyzed by dual luciferase assays 48 h after transfection. Doxorubicin was treated at the concentration of 500 nM for 24 h ($^*P < 0.05$).

indicated in Figure 6A. When CD44 was not expressed (lanes 1–4), doxorubicin treatment decreased NF-κB-dependent transcription and phosphorylation of NF-κB (lane 1 versus lane 2, lane 3 versus lane 4). In contrast, when CD44 was expressed (lanes 5–8), doxorubicin treatment increased NF-κB-dependent transcription as well as phosphorylation of NF-κB (lane 5 versus lane 6, lane 7 versus lane 8). When CD24 was expressed alone, the basal NF-κB transcriptional activity and phosphorylation of NF-κB were decreased as compared with control (lane 1 versus lane 3) by CD24 expression and doxorubicin treatment rather decreased NF-κB transcriptional activity (lane 1 versus lane 2). Similarly, when CD24 was co-expressed with CD44 (lanes 7 and 8), both basal and doxorubicin-induced transcriptional activities of NF-κB and phosphorylation of NF-κB were decreased compared with cells expressing CD44 alone (lanes 5 and 6). PARP cleavage was also significantly increased by doxorubicin in CD24/CD44 coexpressing cells (lane 8) as compared with cells expressing CD44 alone (lane 6). When the parallel sets of transfected cells were tested for their proliferation rates by MTT assays and cell counting (Figure 5B and C, respectively), we found that CD24 expression could only sensitize the cells to doxorubicin when CD44 was co-expressed (lane 2 versus lane 4 and lane 6 versus lane 8). So, the differential effect of CD24 on MDA-MB-231, MDA-MB-468 and MCF-7 cells might be explained by the presence (MDA-MB-231 and MDA-MB-468) and absence (MCF-7) of CD44.

Discussion

To investigate the role of CD24 in breast cancer cells, we expressed CD24 via retroviral infection in CD24-low/negative MDA-MB-231 cells. Ectopic CD24 expression was confirmed using surface labeling

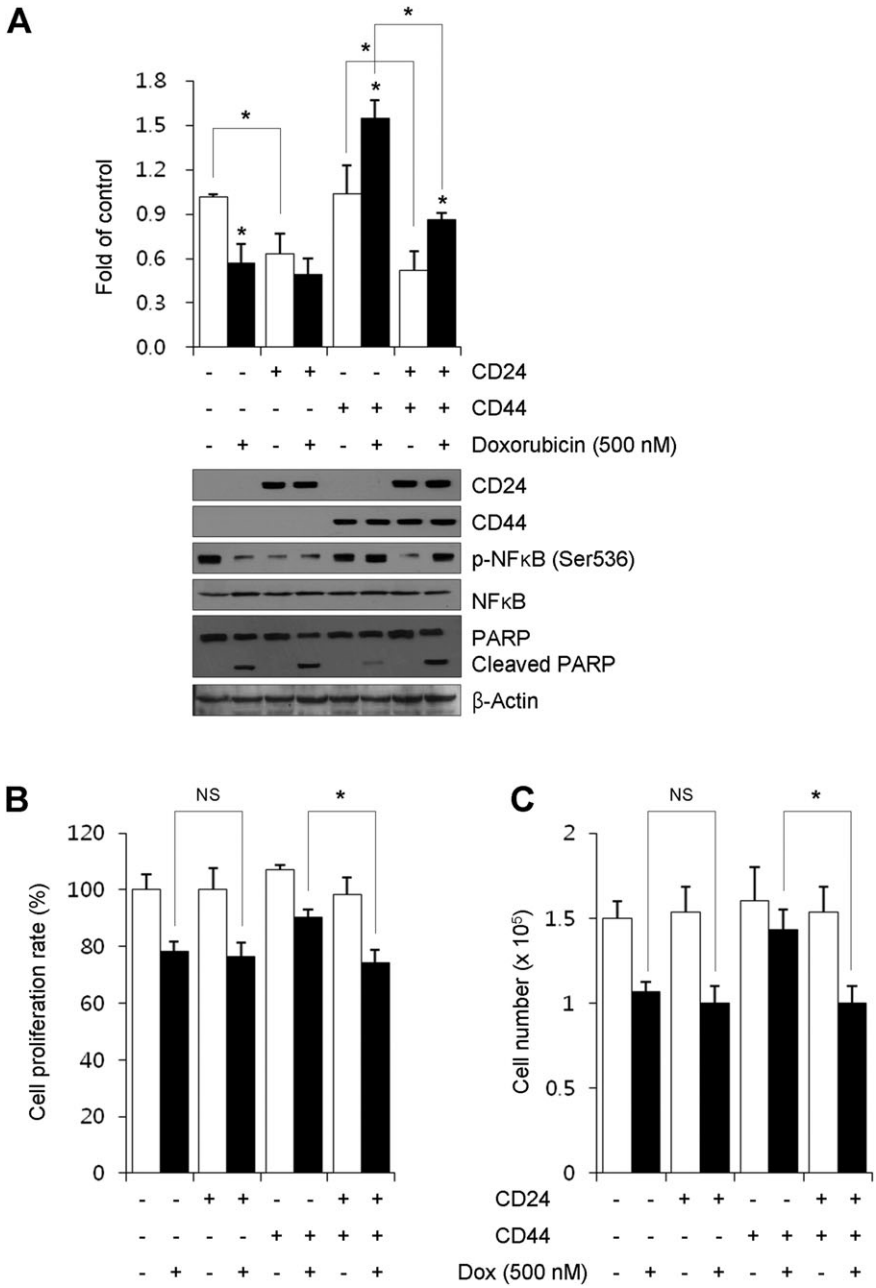

Fig. 6. Effect of CD24 and CD44 transient expression on NF-κB transcriptional activity and cell proliferation in 293T cells treated with doxorubicin. (**A**) Effect of CD24 and CD44 on NF-κB signaling in 293T cells. 293T cells were transfected with 1 μg of total DNA containing 500 ng of reporter constructs and analyzed for dual luciferase assays at 48 h posttransfection. Where indicated, 500 nM of doxorubicin was treated for 24 h. Parallel experimental sets were subjected to western blot analyses with each indicated antibodies. (**B** and **C**) Effect of CD24 and CD44 transient expression on cell proliferation in 293T cells treated with doxorubicin. 293T cells were transfected with 1 μg of total DNA and analyzed for (B) MTT assays and (C) cell counting with hemocytometer at 48 h posttransfection. Where indicated, 500 nM of doxorubicin was treated for 24 h ($^*P < 0.05$, NS, non-significant).

(Figure 1), which indicated that the exogenously expressed CD24 was functional and likely to be glycosylated properly. The results in Figure 1 indicate that ectopic expression of CD24 inhibited the growth of MDA-MB-231 metastatic breast cancer cells, suggesting that antiproliferative signaling was initiated by CD24 expression. MDA-MB-231 cells express phosphatase and tensin homolog, which inhibits phosphoinositide-3 kinase/Akt signaling. Accordingly, MDA-MB-231 cells have very low basal Akt activity (32). The phosphoinositide-3 kinase/Akt and MAPK pathways are two main intracellular signaling cascades that lead to proliferation in animal cells (33,34), so we investigated the possible modulation of MAPK signaling by CD24 expression. As indicated in Figure 1C, CD24 expression resulted in downregulation of Raf/MEK/MAPK phosphorylation and inhibition of MAPK downstream effectors such as p90RSK (35) and p-Elk1 (36). Downregulation of the MAPK pathway promotes modulation of cell cycle regulation proteins (37,38) and forced expression of CD24 resulted in the modulation of the cell cycle regulatory proteins p27 and cyclin D1 (Figure 1C). These results suggest that CD24-induced modulations in MAPK signaling resulted in downregulation of MDA-MB-231 cell proliferation. Interestingly, CD24 could still increase migration of MDA-MB-231 cells (supplementary Figure 1 is available at *Carcinogenesis* Online), which is in accordance with a previous report indicating that CD24 is involved in cell adhesion and metastatic tumor spread (39).

The increase in the sub-G_1 apoptotic population in MDA-MB-231 CD24 cells (Figure 2B) prompted us to investigate the possible function of CD24 in apoptosis induction. To promote apoptosis, we induced DNA damage with doxorubicin treatment (40) or ionizing radiation (29) in MDA-MB-231 vec and MDA-MB-231 CD24 cells. As expected, MDA-MB-231 CD24 cells exhibited robustly increased sub G_1 fractions upon induction of DNA damage. NF-κB signaling was significantly attenuated by CD24 overexpression (Figure 3); however, after induction of DNA damage, a marked upregulation of NF-κB signaling due to DNA damage was observed. Thus, the DNA damage-induced upregulation of NF-κB signaling was apparently attenuated in CD24-expressing cells (Figure 3). Consistent with our findings, doxorubicin and irradiation have been reported to induce NF-κB signaling (17,41).

The role of NF-κB signaling in apoptosis remains controversial (16). NF-κB is generally considered to be an antiapoptotic transcription factor (42). However, accumulating evidence also suggests that NF-κB acts as a proapoptotic factor (43). For example, daunorubicin, a doxorubicin analog, induces IκB degradation and subsequent nuclear translocation and activation of NF-κB (44). Whether NF-κB activity induced by doxorubicin plays an antiapoptotic or proapoptotic role is a matter of extensive debate. For example, in IKK1/2 knockdown mouse embryonic fibroblasts, which lack NF-κB activity, increased apoptosis is observed after doxorubicin treatment (45). Forced expression of a IκBα super-repressor or treatment with an NF-κB inhibitor strongly enhances doxorubicin-induced apoptosis (46). In contrast, Ashikawa *et al.* (47) reported that, in myeloid and lymphoid cells, apoptosis induced by doxorubicin or a similar anthracycline analog requires NF-κB activity. They also showed that functional receptor-interacting proteins may mediate doxorubicin-induced NF-κB activation and NF-κB suppression by pyrrolidine dithiocarbamate, and an IKKγ-binding domain peptide decreases the cytotoxic effects of doxorubicin and its analogs. In neuroblastoma cells, an NF-κB inhibitor protects against doxorubicin-induced apoptosis, suggesting that NF-κB mediates doxorubicin toxicity (48).

A report using tetracycline-inducible p53 in Saos-2 cells showed that the tumor suppressor p53 is a requirement for the activation of NF-κB by DNA-damaging agents including doxorubicin (49). The human metastatic breast cancer cell line MDA-MB-231 expresses mutant p53 (50). Therefore, MDA-MB-231 cells devoid of functional p53 might exhibit resistance toward doxorubicin-induced apoptosis because of a lack of p53-mediated apoptosis. When NF-κB was activated, an antiapoptotic signal was attenuated by CD24 expression and treatment with doxorubicin-induced apoptosis in MDA-MB-231 CD24 cells. This brings into question the molecular link between CD24 and the NF-κB signaling cascade. NF-κB-inducing kinase stimulates NF-κB activity (51) and has sequence similarity to MAPKinase kinase kinase. Our data (Figure 1) indicate that forced expression of CD24 decreased phosphorylation of the c-Raf/MEK/MAPK cascade. This suggests the possibility that CD24 modulates NF-κB signaling via inhibition of the MAPK signaling pathway in which NF-κB-inducing kinase might be involved. Elucidation of the detailed cross talk mechanism between c-Raf/MEK/MAPK and NF-κB warrants further experiments.

We also have presented some evidence that the interaction between CD44 and CD24 may also be required to modulate NF-κB signaling in response to doxorubicin treatment. Expression of CD24 in MDA-MB-231 positive for CD44 attenuated NF-κB signaling and inhibition of CD24 expression by siRNA in MDA-MD-468 cells double positive for CD24 and CD44 upregulated NF-κB signaling. In contrast, silencing of CD24 in CD44-negative MCF-7 cells had no apparent effect on NF-κB signaling. Furthermore, 293T sets transfected with CD44 ± CD24 behaved like MDA-MB-231 vec and MDA-MB-231 CD24 and 293T sets transfected with empty vector for CD44 ± CD24 behaved like MCF-7 mCD24sh and MCF-7 hCD24sh after doxorubicin treatment. Currently, we do not know whether the direct interaction between CD24 and CD44 is required for the interplay between intracellular signaling elicited by these cell surface molecule or other downstream modulators of these molecules are involved.

Supplementary material

Supplementary Figure 1 can be found at http://carcin.oxfordjournals.org/

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