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Involvement of NRF2 Signaling in Doxorubicin Resistance of Cancer Stem Cell-Enriched Colonospheres

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Abstract

Cancer stem cells (CSCs) are a subset of tumor cells, which are characterized by resistance against chemotherapy and environmental stress, and are known to cause tumor relapse after therapy. A number of molecular mechanisms underlie the chemoresistance of CSCs, including high expression levels of drug efflux transporters. We investigated the role of the antioxidant transcription factor NF-E2-related factor 2 (NRF2) in chemoresistance development, using a CSC-enriched colonosphere system. HCT116 colonospheres were more resistant to doxorubicin-induced cell death and expressed higher levels of drug efflux transporters such as P-glycoprotein (Pgp) and breast cancer resistance protein (BCRP) compared to HCT116 monolayers. Notably, levels of NRF2 and expression of its target genes were substantially elevated in colonospheres, and these increases were linked to doxorubicin resistance. When NRF2 expression was silenced in colonospheres, Pgp and BCRP expression was downregulated, and doxorubicin resistance was diminished. Collectively, these results indicate that NRF2 activation contributes to chemoresistance acquisition in CSC-enriched colonospheres through the upregulation of drug efflux transporters.

Key Words: Cancer stem cells, NRF2, Colonospheres, Drug efflux transporters, Chemoresistance

INTRODUCTION

Cancer stem cells (CSCs), a small population of cancer cells within tumors, are known to have an infinite proliferation potential and self-renewing capacity (Al-Hajj and Clarke, 2004; Frank *et al.,* 2010). Since the initial identification of CSCs in hematopoietic cancers by Dick and colleagues (Bonnet and Dick, 1997), CSCs have been identified in and isolated from different types of cancers such as brain, breast, and colon tumors (Al-Hajj *et al.,* 2003; Singh *et al.,* 2003, Ricci-Vitiani *et al.,* 2007). The origin of CSCs still remains unclear; although it is hypothesized that CSCs can originate from normal stem cells or dedifferentiated cancer cells (Trosko, 2009). Recently, scrutiny of CSCs has increased, as they are believed to be associated with tumor relapse. According to previous studies, CSCs are more resistant to conventional anticancer therapies compared to differentiated cancer cells. CSC chemoresistance seems to be related to activated anti-stress and drug efflux systems (Diehn *et al.,* 2009; Nakai *et al.,* 2009; Ye *et al.,* 2011; Chau *et al.,* 2013).

Cancer cells acquire characteristics of CSCs in non-adherent sphere culture systems. Under serum-free conditions, anoikis-resistant cancer cells can be grown in spheres (Chen *et al.,* 2012). Non-adherent sphere culture systems were initially used to culture neurospheres, using neuronal cells (Reynolds and Weiss, 1992), and have since been applied for culturing different cell types such as breast cancer cells (Ponti *et al.,* 2005). Recent findings have revealed that CSC signaling pathways, such as the Wnt/β-catenin pathway, are activated in colonospheres, which are derived from colon cancer cells. Furthermore, cells positive for the CSC surface markers CD44 and aldehyde dehydrogenase-1 (ALDH1) were found to be enriched in colonospheres (Kanwar *et al.,* 2010; Saha *et al.,* 2014). However, evidence regarding chemoresistance mechanisms in colonospheres is limited.

Transcription factor NF-E2-related factor-2 (NRF2) plays a major role in maintaining cellular redox status and protecting cells from oxidative stress. The expression of NRF2-regulated genes, which include antioxidant genes and drug efflux transporters, can be induced by the binding of NRF2 to the antioxi-

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dant-response element (ARE) in their promoter regions. Under homeostatic conditions, NRF2 is inactive and maintained at low levels through interaction with Kelch-like ECH-associated protein 1 (KEAP1), which can lead to proteasomal degradation of NRF2. However, when cells are exposed to oxidizing signals, NRF2 is liberated from the KEAP1 protein following modification of KEAP1 cysteine residues, and translocates into the nucleus, which consequently leads to transcriptional induction of ARE-bearing genes (McMahon *et al.,* 2003; Motohashi and Yamamoto, 2004). During the last few decades, extensive research has identified the cytoprotective role of NRF2 in normal cells and tissues (Cho *et al.,* 2006; Calkins *et al.,* 2009). Recent studies have drawn attention to NRF2 activation in cancer cells, which can render them more refractory to conventional anticancer therapies. These cancer cells utilize NRF2 for enhanced survival and drug resistance by elevating the expression of target genes such as antioxidant and glutathione (GSH) generating enzymes, detoxifying enzymes, and drug efflux transporters (Singh *et al.,* 2006; Lau *et al.,* 2008; Wang *et al.,* 2008). Above all, upregulation of drug efflux transporters, including P-glycoprotein (Pgp), breast cancer resistance protein (BCRP), and multidrug resistance proteins (MRPs), has an important role in the acquisition of resistance to chemotherapies. The expression of *MRP1* was regulated by NRF2 in small cell lung cancer (Ji *et al.,* 2013). It was shown that the proximal promoter region of *BCRP* contained AREs for NRF2 interaction; therefore, *NRF2*-deleted lung cancer cells could have increased sensitivity to the anticancer drugs mitoxantrone and topotecan (Singh *et al.,* 2010).

Previously, we observed that high levels of NRF2 elicited increased expression of antioxidant/detoxifying genes and drug efflux transporters in sphere-cultured breast cancer cells, termed mammospheres (Ryoo *et al.,* 2015a). This study indicated that NRF2 might be involved in CSC resistance to treatment. In the current study, we have investigated the potential association between NRF2 and CSC chemoresistance, using a HCT116-derived colonosphere system.

MATERIALS AND METHODS

Reagents

Antibodies recognizing SOX2, KLF4, Pgp, and BCRP were obtained from Cell Signaling Technology (Danvers, MA, USA). Antibodies against NRF2, NQO-1, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). A luciferase reporter plasmid containing the ARE was a gift from Dr. Wakabayashi (University of Pittsburg, PA, USA). Lentiviral expression plasmids for the human *NRF2* short hairpin RNA (shRNA), lentiviral packaging mix, hexadimethrine bromide, puromycin, doxorubicin, daunorubicin, MK571, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma-Aldrich (Saint Louis, MO, USA). A SYBR premix ExTaq system was obtained from Takara (Otsu, Japan).

Cell culture

The human colorectal carcinoma cell line HCT116 was purchased from the American Type Culture Collection (Rockville, MD, USA). Cells were maintained in RPMI1640 (HyClone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; HyClone) and penicillin/streptomycin (HyClone). The cells were grown at 37°C in a humidified 5% CO_2 atmosphere.

Colonosphere culture

HCT116 cells were plated at a density of 20,000 cells/mL in 100-mm ultralow attachment plates (Corning Costar Corp., Cambridge, MA, USA). Cells were grown in serum-free Dulbecco's modified Eagle's medium (HyClone) and Nutrient Mixture F-12 medium supplemented with B27 (Life Technologies, Carlsbad, CA, USA), 20 ng/mL epithelial growth factor, 20 ng/mL basic fibroblast growth factor (R&D Systems, Minneapolis, MN, USA), 5 μg/mL bovine insulin (Cell Applications Inc., San Diego, CA, USA), 0.5 μg/mL hydrocortisone (Sigma-Aldrich), and penicillin/streptomycin (HyClone). HCT116 cells were grown for 3 days under the sphere culture conditions. Colonospheres were dissociated by incubation with 0.05% trypsin/EDTA (WelGENE Inc., Daegu, Republic of Korea) on day 4. Dissociated HCT116 cells were cultured under sphere culture conditions for another 3 days and then harvested.

Production of shRNA lentiviral particles

Lentiviral particles were produced in HEK 293T cells following transfection with the relevant shRNA expression plasmid and Mission Lentiviral Packaging Mix (Sigma-Aldrich). Briefly, HEK 293T cells in Opti-MEM (Life Technologies) were transfected with 1.5 μg pLKO.1-*NRF2* shRNA (Kim *et al.,* 2011) with packaging mix, using Lipofectamine 2000 (Life Technologies). The control group was transfected with a nonspecific pLKO.1-scrambled (sc) RNA plasmid. The next day, the medium containing the transfection complex was removed and lentiviral particles were harvested after 4 days.

Establishment of *NRF2* knockdown in HCT116 cells

HCT116 cells in 6-well plates were transduced with lentiviral particles containing either the nonspecific pLKO.1-scRNA (sc), or pLKO.1-*NRF2* shRNA (shNRF2) in the presence of 8 μg/mL hexadimethrine bromide. Transduction was continued for 48 h and followed by 24 h recovery in complete medium. For the selection of stable transgene-expressing cells, incubation with puromycin (2 μg/mL) was continued for up to 4 weeks (Kim *et al.,* 2011).

Total RNA extraction and RT-PCR analysis

Total RNA was isolated from the cells, using TRIzol (Life Technologies). cDNA was synthesized using RT reactions: 200 ng of total RNA was incubated with a reaction mixture containing 0.5 μg/μL oligo $dT_{12\text{-}18}$ and 200 U/μL moloney murine leukemia virus reverse transcriptase (Life Technologies). Real-time RT-PCR was carried out using a Roche Light Cycler (Mannheim, Germany) with the Takara SYBR Premix ExTaq System for relative quantification as described previously (Ryoo *et al.,* 2015b). Primers were synthesized by Bioneer (Daejeon, Republic of Korea), and primer sequences for the human genes are described in our previous studies (Jeong *et al.,* 2015).

MTT assay

Viable cell numbers were determined by MTT assay. Dissociated cells from HCT116 colonospheres were seeded at a density of 5×10^3 cells/well in 96-well plates and incubated with the relevant compounds (doxorubicin or daunorubicin) for the indicated times. MTT solution (2 mg/mL) was added to the

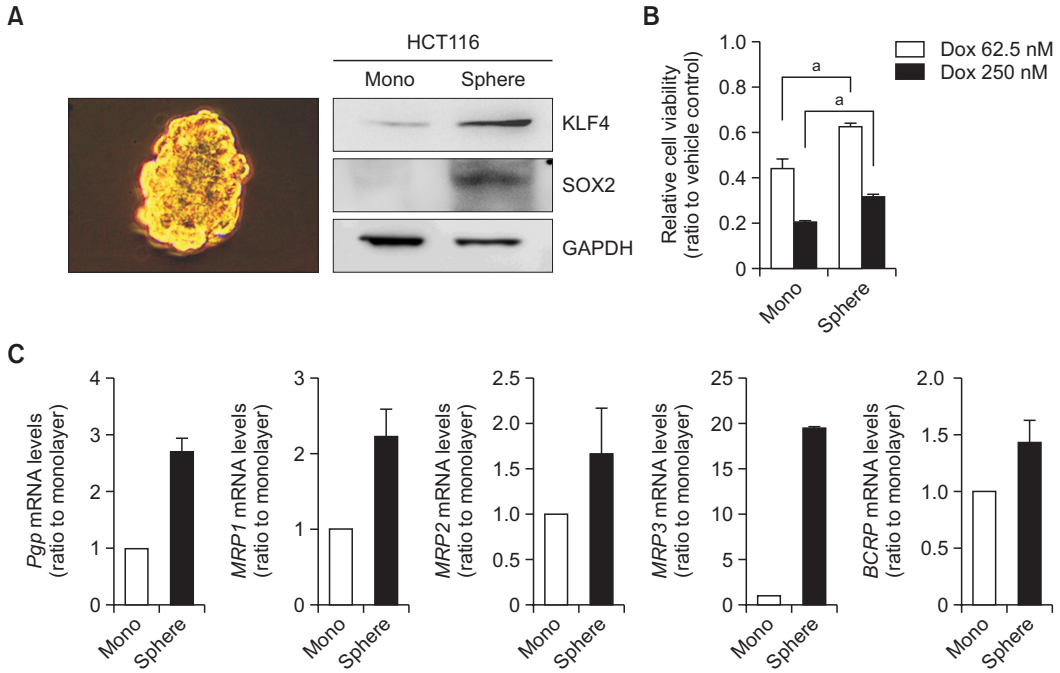

Fig. 1. Doxorubicin resistance and upregulation of drug efflux transporters in HCT116 colonospheres. (A) HCT116 cells were grown as colonospheres for 7 d under serum-free conditions. KLF4 and SOX2 protein levels were determined in monolayers and colonospheres. (B) Cell viability was monitored after doxorubicin (Dox) incubation for 72 h in monolayers and colonospheres. [a]$p<0.05$ compared with monolayer. (C) *Pgp*, *MRPs* (*MRP1~3*), and *BCRP* transcript levels were assessed in monolayers and colonospheres by real-time PCR for relative quantification.

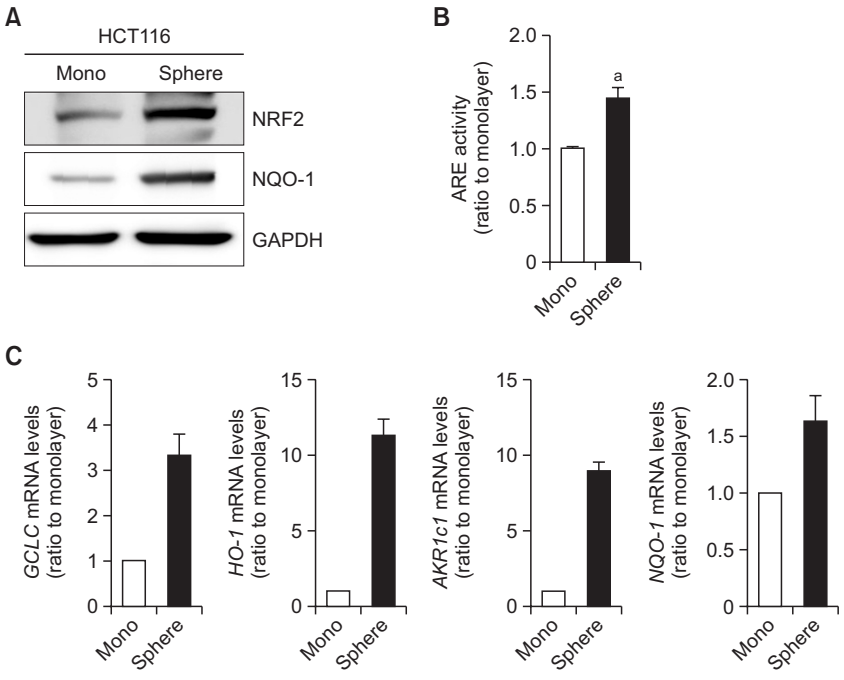

Fig. 2. Activation of NRF2 signaling in HCT116 colonospheres. (A) NRF2 and NQO-1 protein levels were determined in monolayers and colonospheres by western blot analysis. (B) NRF2 transcription activity was monitored using a *NQO-1* ARE-driven luciferase reporter. [a]$p<0.05$ compared with monolayer. (C) *GCLC*, *HO-1*, *AKR1c1*, and *NQO-1* transcript levels were assessed in monolayers and colonospheres by real-time PCR for relative quantification.

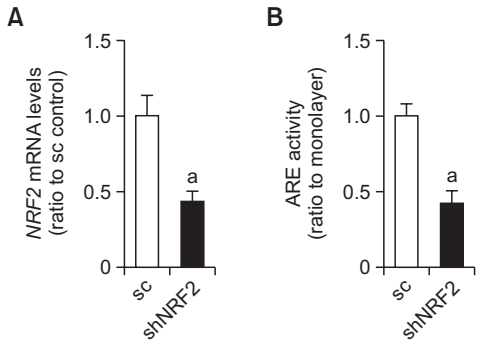

Fig. 3. Establishment of *NRF2* knockdown HCT116 cells. (A) *NRF2* transcript levels were assessed in shNRF2 HCT116 cells by real-time PCR for relative quantification. (B) NRF2 transcription activity was monitored using a *NQO-1* ARE-driven luciferase reporter. [a]$p<0.05$ compared with sc control HCT116 cells.

cells and further incubated for 4 h. The MTT solution was removed, 100 μL/well of DMSO was added, and the absorbance was measured at 540 nm, using a SPECTRO Star[Nano] (BMG LABTECH GmbH, Allmendgruen, Ortenberg, Germany).

Measurement of intracellular doxorubicin

HCT116 colonospheres were dissociated to single cells and incubated with 100 nM doxorubicin for 24 h. Then, cells were washed twice with PBS and trypsinized. Cells stained with doxorubicin were analyzed using a Becton-Dickinson FACS Canto flow cytometer (San Jose, CA, USA) and data were analyzed with FACSDiva software (Becton-Dickinson).

Measurement of ARE-luciferase activity

HCT116 cells were seeded in 24-well plates at a density of 2.0×10^4 cells/well and grown overnight. The next day, the transfection complex, containing 0.5 μg of the ARE-luciferase plasmid along with 0.05 μg of pRLtk control plasmid (Promega, Madison, WI, USA) and the transfection reagent (WelGENE Inc.), was added to each well. After 18 h, the transfection complex was removed and the cells were incubated in a complete medium for 24 h. The cells were then lysed. Renilla and firefly luciferase levels were measured using the Dual Luciferase Assay System (Promega) with a luminometer (Turner Designs, Sunnyvale, CA, USA).

Statistical analysis

Statistical significance was analyzed by Student's *t*-test or analysis of variance (ANOVA) followed by the Student Newman-Keuls test for multiple comparisons, using Prism software (GraphPad Prism, La Jolla, CA, USA).

RESULTS

Doxorubicin resistance in HCT116 colonospheres

For colonosphere formation, the HCT116 single cell suspension was seeded into an ultralow attachment plate and cultured in serum-free medium with growth factors for a week (Fig. 1A). Expression of the CSC markers Kruppel-like factor 4 (KLF4) and sex determining region Y-box 2 (SOX2) was increased compared to that in the monolayer control (Fig. 1A). Next, we compared the sensitivity of colonospheres to the anticancer drug doxorubicin with that of monolayers by MTT test. After treatment of cells with doxorubicin (62.5 and 250 nM) for 72 h, colonospheres showed a higher cell viability than

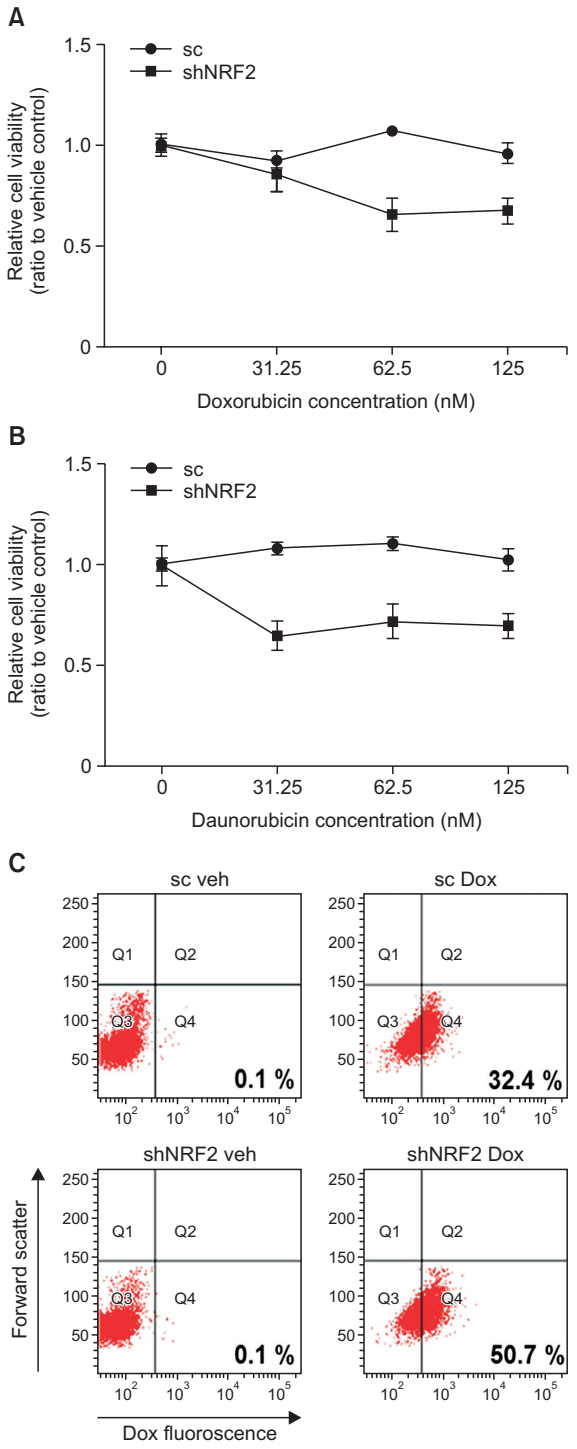

Fig. 4. Doxorubicin resistance in shNRF2 colonospheres. (A-B) Cell viability was monitored after doxorubicin (A) or daunorubicin (B) incubation for 24 h in sc control and shNRF2 colonospheres. (C) Intracellular doxorubicin (100 nM) was monitored for 24 h in sc control and shNRF2 colonospheres by flow cytometry.

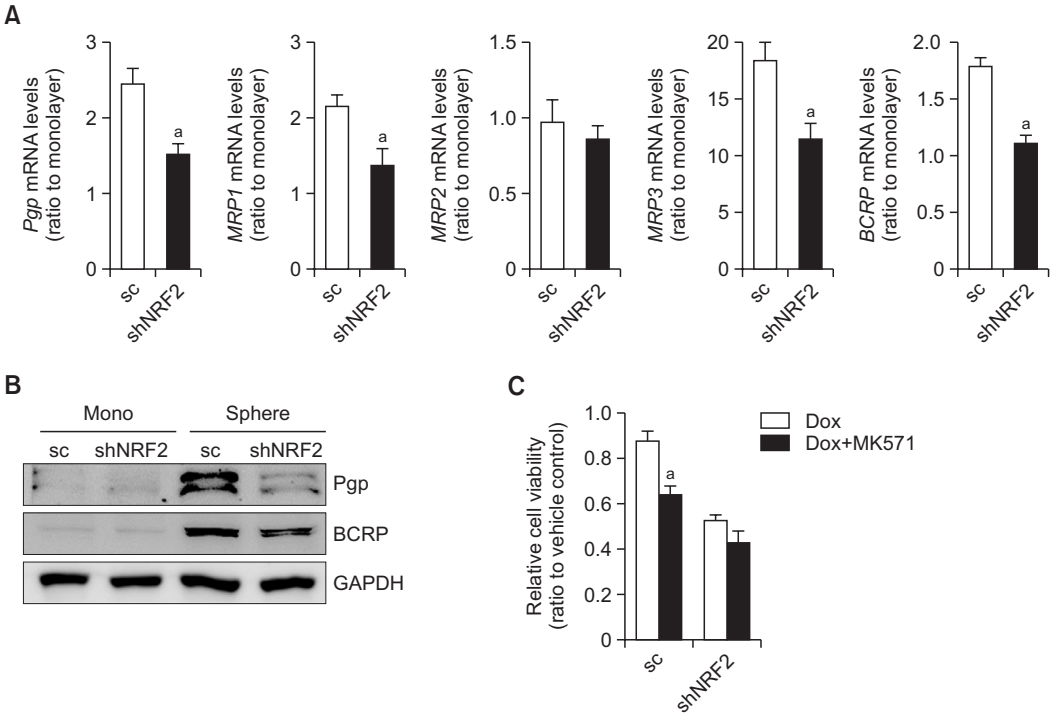

Fig. 5. Downregulation of drug efflux transporters in shNRF2 colonospheres. (A) *Pgp*, *MRPs* (*MRP1~3*) and *BCRP* transcript levels were assessed in sc control and shNRF2 colonospheres by real-time PCR for relative quantification. [a]$p<0.05$ compared with sc control colonospheres. (B) Pgp and BCRP protein levels were determined in sc control and shNRF2 colonospheres. (C) Cell viability was monitored after incubation with doxorubicin only (500 nM) or doxorubicin plus MK571 (50 μM) for 24 h in sc control and shNRF2 colonospheres. [a]$p<0.05$ compared with doxorubicin-treated sc control colonospheres.

monolayer-cultured cells. Approximately 40% of monolayer cells survived following incubation with 62.5 nM doxorubicin, whereas more than 60% of cells in colonospheres survived (Fig. 1B). Based on these data, transcript levels of drug efflux transporters were analyzed by real-time PCR. The expression levels of *Pgp*, *MRP1*, *MRP2*, *MRP3*, and *BCRP* were higher in colonospheres than those in monolayers. In particular, the expression level of *MRP3* was 19-fold higher in colonospheres (Fig. 1C). These results indicate that HCT116-derived colonospheres exhibit CSC properties such as CSC marker expression and drug resistance.

NRF2 activation in HCT116 colonospheres

Considering the elevated expression of drug efflux transporters in colonospheres, it was hypothesized that NRF2 activation is involved in colonosphere resistance. Hence, we examined whether NRF2 and expression of its target genes were activated in HCT116 colonospheres. Immunoblot analysis showed that there were substantial increases in protein levels of NRF2 and its target NAD(P)H:quinone oxidoreductase-1 (NQO1) in colonospheres when compared that in the monolayers (Fig. 2A). In line with this, ARE-driven luciferase activity was elevated in colonospheres (Fig. 2B), and transcript levels for NRF2-target genes such as γ-glutamate cysteine ligase catalytic subunit (*GCLC*), heme oxygenase-1 (*HO-1*), aldo-keto reductase 1c1 (*AKR1c1*), and *NQO-1* were enhanced by 3.3-, 11.3-, 8.9-, and 1.6-fold, respectively, in colonospheres compared to that in monolayers (Fig. 2C). These data indicate that the expression of NRF2 and downstream antioxidant genes is increased in colonospheres. They also imply that increased expression of drug efflux transporters is associated with NRF2 elevation.

Enhanced doxorubicin sensitivity in *NRF2*-knockdown colonospheres

To elucidate the relationship between NRF2 and drug efflux transporters and chemoresistance of colonospheres, we established an *NRF2*-knockdown stable cell line (shNRF2). Established knockdown cells showed significant reductions in both *NRF2* transcript levels (57%) and ARE-driven luciferase activity (58%) compared to the sc control cells (Fig. 3). With this established shNRF2 cell line, we monitored colonosphere viability in the presence of doxorubicin or daunorubicin for 24 h. Unlike sc control spheres, shNRF2 spheres were vulnerable to doxorubicin incubation: 96% of cells in control spheres survived following treatment with 125 nM doxorubicin, whereas 67% of shNRF2 sphere cells survived (Fig. 4A). Similar results were obtained following daunorubicin incubation (Fig. 4B). Additionally, in an attempt to estimate drug efflux capacity, cellular accumulation levels of doxorubicin were analyzed by flow cytometry. After incubation with 100 nM doxorubicin (24 h), intracellular levels of doxorubicin were relatively higher in *NRF2*-knockdown colonospheres: cell populations with doxorubicin-derived fluorescence were 32.4% and 50.7% in sc control and shNRF2 spheres, respectively (Fig. 4C). These data indicate that NRF2 activation is an important molecular event in the acquisition of chemoresistance in colonospheres.

Reduced drug efflux transporter expression in *NRF2*-knockdown colonospheres

Considering the relationship between NRF2 and anticancer drug resistance, we attempted to analyze the expression of drug efflux transporters in *NRF2*-knockdown colonospheres. The increase in *Pgp*, *MRP1*, *MRP3*, and *BCRP* mRNAs in colonospheres was suppressed by *NRF2* knockdown (Fig. 5A). Immunoblot analysis showed that protein levels of Pgp and BCRP were lower in knockdown spheres than in the control spheres (Fig. 5B).

Next, to confirm the role of NRF2 in efflux transporter-mediated drug resistance in colonospheres, we applied an MRP inhibitor, MK571. When spheres were treated with 50 μM MK571 and 500 nM doxorubicin for 24 h, doxorubicin resistance was significantly attenuated in the sc control group; however, MK571 incubation did not have an inhibitory effect on doxorubicin resistance in the *NRF2*-knockdown colonospheres (Fig. 5C). These results indicate that NRF2 activation is associated with chemoresistance of colonospheres by elevating the expression of drug efflux transporters.

DISCUSSION

Aberrant activation of NRF2 can be advantageous to cancers by rendering enhanced antioxidative capacity that reduces stress-induced apoptotic cell death (Hayes and McMahon, 2009; Taguchi *et al.,* 2011). One of the anti-tumor effects of chemotherapeutic agents is oxidative stress generation, which means that high levels of NRF2 can shelter cancer cells from chemotherapy-induced insults through induction of antioxidant genes and drug detoxifying enzymes. Clinically, patients carrying tumors with high NRF2 expression have poor prognosis and survival rates. (Singh *et al.,* 2006; Lau *et al.,* 2008; Wang *et al.,* 2008; Shim *et al.,* 2009). Multiple molecular mechanisms causing constitutive NRF2 activation have been identified: somatic mutations in *KEAP1* and *NRF2* genes, silencing of KEAP1 expression, activation of oncogenes such as *KRAS*, elevation of p62, and accumulation of the abnormal metabolite fumarate (Hayes and McMahon, 2009; Hayes and Dinkova-Kostova, 2014). In addition to its role in the acquisition of chemoresistance, NRF2 has been associated with CSCs. In glioblastoma stem cells, NRF2 was involved in maintenance of self-renewal capacity (Zhu *et al.,* 2013). The side population (SP) of lung cancer cells displayed elevated expression of NRF2 and BCRP, which was associated with multi-drug resistance of the SP (Yang *et al.,* 2015). In ovarian clear cell carcinoma cells that express high levels of ALDH1, the intracellular reactive oxygen species (ROS) levels were low and NRF2 expression was high (Mizuno *et al.,* 2015). Notably, our recent study demonstrated NRF2 activation in MCF7 mammospheres, and this in turn affected sphere cell growth, survival, and resistance against anti-cancer drug treatment (Ryoo *et al.,* 2015a). In the present study, we showed that expression of NRF2 and its target genes is increased in HCT116 colonospheres, and inhibition of NRF2 downregulated expression of drug transporters, including Pgp, MRPs, and BCRP, which led to increased sensitization to doxorubicin.

Elevated expression of drug efflux transporters is one of the characteristic features of CSCs (Dean *et al.,* 2005; Dean, 2009). Therefore, the expression level of transporters has been applied for CSC isolation. For instance, SP cells, which extrude BCRP substrate Hoechst 33342, are isolated from cancer cell lines or tumors by flow cytometry, and are used for studies on CSCs. Szotek *et al.* (2006) demonstrated that SP cells from ovarian cancer were more resistant to doxorubicin and displayed higher tumorigenic and stem-like characteristics than non-SP cells. Cancer cells could develop acquired resistance against anticancer drugs after repeated exposure whilst exhibiting CSC features. In a study by Achuthan *et al.* (2011) chemoresistant breast cancer cells exhibited higher levels of CSC markers such as octamer-binding transcription factor 4 (OCT4) and CD133, and an increased SP cell population compared with the parental cells. Our current study demonstrates the correlation between NRF2 and drug efflux transporters in HCT116 colonospheres. As NRF2 positively regulates efflux transporter expression, increased NRF2 induces colonosphere drug resistance. In particular, this correlation was confirmed by the effect of MK571 treatment, an inhibitor of MRPs. Treatment with MK571 showed a synergistic effect with doxorubicin in sc control colonospheres, but not in *NRF2*-silenced colonospheres. Clearly, these observations are explained by downregulation of ABC transporter expression by NRF2 inhibition. The role of NRF2 in drug efflux transporter expression has been identified in several *in vitro* and *in vivo* models. Treatment with sulforaphane (SFN), a pharmacological inducer of NRF2, increased protein levels of Pgp, Mrp2, and BCRP in the rat brain (Wang *et al.,* 2014). Genetic activation of NRF2 through *KEAP1* knockdown resulted in an upregulation of Pgp, MRP2/3, and BCRP in human renal tubular cells (Jeong *et al.,* 2015). In our previous work on mammospheres, the expression of MRP2 and MRP3 was enhanced by NRF2 activation, resulting in drug resistance in breast CSCs (Ryoo *et al.,* 2015a).

In summary, our results show that NRF2 activation is associated with doxorubicin resistance in CSC-enriched colonospheres, through the upregulation of antioxidant proteins and drug efflux transporters. Therefore, the development of NRF2 inhibitors might be an effective therapeutic approach to target chemoresistant colon CSCs.

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