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-
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 interaction; therefore, NRF2-deleted lung cancer was regulated by BCRP contained AREs (Singh 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 1 (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 (Kim 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 Pittsburgh, PA, USA). Lentiviral expression plasmids for the human NRF2 short hairpin RNA (shRNA), lentiviral packaging mix, hexadimethrine bromide, puromycin, doxorubicin, daunorubicin, MK571, and 3-(4,4-dimethylthiazol-2-yl)-2,5-diphenyterazolium bromide (MTT) were obtained from Sigma-Aldrich (St 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% CO2 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 dT12-18 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 5×104 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. *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. *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.
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 × 10⁴ 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 (WEGENE 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
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 than those in monolayers. 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 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 \( \gamma \)-glutamate cysteine ligase catalytic subunit (GCLC), heme oxygenase-1 (HO-1), aldotriose 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 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 increased expression of drug efflux transporters is associated with NRF2 elevation.
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 oxidative 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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REFERENCES

Achuthan, S., Santhoshkumar, T. R., Prabhakar, J., Nair, S. A. and Pillai, M. R. (2011) Drug-induced senescence generates chemoresistant stemlike cells with low reactive oxygen species. J. Biol. Chem. 286, 37813-37829.

Al-Hajj, M. and Clarke, M. F. (2004) Self-renewal and solid tumor stem cells. Oncogene 23, 7274-7282.

Al-Hajj, M., Wicha, M. S., Benito-Hernandez, A., Morrison, S. J. and Clarke, M. F. (2003) Prospective identification of tumorigenic breast cancer cells. Proc. Natl. Acad. Sci. U.S.A. 100, 3983-3988.

Bonnet, D. and Dick, J. E. (1997) Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. Nat. Med. 3, 730-737.
Calkins, M. J., Johnson, D. A., Townsend, J. A., Vargas, M. R., Dowell, J. A., Williamson, T. P., Kraft, A. D., Lee, J. M., Li, J. and John-son, J. A. (2009) The Nrf2/ARE pathway as a potential therapeutic target in neurodegenerative disease. Antioxid. Redox Signal. 11, 497-508.

Chau, W., Ip, C., Mak, A., Lai, H. and Wong, A. (2013) c-Kit mediates chemoresistance and tumor-initiating capability of ovarian cancer cells through activation of Wnt/β-catenin-ATP-binding cassette G2 signaling. Oncogene 32, 2767-2781.

Cho, H. Y., Reddy, S. P. and Kleeberger, S. R. (2006) Nrf2 defends the lung from oxidative stress. Antioxid. Redox Signal. 8, 76-87.

Dean, M. (2009) ABC transporters, drug resistance, and cancer stem cells. J. Mammary Gland Biol. Neoplasia 14, 3-9.

Dean, M., Fojo, T. and Bates, S. (2005) Tumour stem cells and drug resistance. Nat. Rev. Cancer 5, 275-284.

Diehn, M., Cho, R. W., Lobo, N. A., Kalisky, T., Dorie, M. J., Kulp, A. N., Qian, D., Lam, J. S., Alles, L. E., Wong, M., Joshua, B., Kaplan, M. J., Wapnir, I., Dibas, F. M., Somlo, G., Garberoglio, C., Paz, B., Shen, J., Lau, S. K., Quake, S. R., Brown, J. M., Weissman, I. L. and Clarke, M. F. (2009) Association of reactive oxygen species levels and radioresistance in cancer stem cells. Nature 458, 780-783.

Frank, N. Y., Schatton, T. and Frank, M. H. (2010) The therapeutic promise of the cancer stem cell concept. J. Clin. Invest. 120, 41-50.

Hayes, J. D. and Dinkova-Kostova, A. T. (2014) The Nrf2 regulatory network provides an interface between redox and intermediary metabolism. Trends Biochem. Sci. 39, 199-218.

Hayes, J. D. and McMahan, M. (2009) Nrf2 and KEAP1 mutations: permanent activation of an adaptive response in cancer. Trends Biochem. Sci. 34, 176-188.

Jeong, H. S., Ryoo, I. G. and Kwak, M. K. (2015) Regulation of the expression of renal drug transporters in KEAP1-knockdown human tubular cells. Toxicol. In vitro 29, 884-892.

Kim, T. H., Hur, E. G., Kang, S. J., Kim, J. A., Thapa, D., Lee, Y. M., Ku, S. K., Jung, Y. and Kwak, M. K. (2011) Nrf2 blockade suppresses colon tumor angiogenesis by inhibiting hypoxia-induced activation of HIF-1α. Cancer Res. 71, 2260-2275.

Lau, A., Villeneuve, N. F., Sun, Z., Wong, P. K. and Zhang, D. D. (2008) Dual roles of Nrf2 in cancer. Pharmacol. Res. 58, 262-270.

McMahone, M., Itoh, K., Yamamoto, M. and Hayes, J. D. (2003) Keap1-dependent proteasomal degradation of transcription factor Nrf2 contributes to the negative regulation of antioxidant response element-driven gene expression. J. Biol. Chem. 278, 21592-21600.

Mizuno, T., Suzuki, N., Makino, H., Funui, T., Morii, E., Aoki, H., Kunisada, T., Yano, M., Kuni, S., Hirashima, Y., Arawaka, A., Nishio, S., Ushijima, K., Ito, K., Itani, Y. and Morishige, K. (2015) Cancer stem-like cells of ovarian clear cell carcinoma are enriched in the ALDH-high population associated with an accelerated scavenging system in reactive oxygen species. Gynecol. Oncol. 137, 299-305.

Motohashi, H. and Yamamoto, M. (2004) Nrf2-Keap1 defines a physiologically important stress response mechanism. Trends Mol. Med. 10, 549-557.

Nakai, E., Park, K., Yawata, T., Chihara, T., Kumazawa, A., Nakabayashi, H. and Shimizu, K. (2009) Enhanced MDRI expression and chemoresistance of cancer stem cells derived from glioblastoma. Cancer Invest. 27, 901-908.

Ponti, D., Costa, A., Zaffaroni, N., Pratesi, G., Petrangolini, G., Corradini, D., Piliotti, S., Pierotti, M. A. and Daidone, M. G. (2005) Isolation and in vitro propagation of tumorigenic breast cancer cells with cancer stem/progenitor cell properties. Cancer Res. 65, 5506-5511.

Reynolds, B. A. and Weiss, S. (1992) Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system. Science 255, 1707-1710.

Ricci-Vitiani, L., Lombardi, D. G., Pilozzi, E., Biffoni, M., Todaro, M., Peschle, C. and De Maria, R. (2007) Identification and expansion of human colon cancer initiating cells. Nature 445, 111-115.

Ryoo, I., Choi, B. and Kwak, M. K. (2015a) Activation of Nrf2 by p62 and proteasome reduction in sphere-forming breast carcinoma cells. Oncotarget 6, 8167-8184.

Ryoo, I. G., Shin, D. H., Kang, K. S. and Kwak, M. K. (2015b) Involvement of Nrf2-GSH signaling in TGFβ1-stimulated epithelial-to-mesenchymal transition changes in rat renal tubular cells. Arch. Pharm. Res. 38, 272-281.

Saha, A., Padhi, S. S., Roy, S. and Banerjee, B. (2014) HCT116 colonospheres shows elevated expression of hTERT and β-catenin protein-a short report. J. Stem Cells 9, 243-251.

Shim, G. S., Manandhar, S., Shin, D. H., Kim, T. H. and Kwak, M. K. (2009) Acquisition of doxorubicin resistance in ovarian carcinoma cells accompanies activation of the Nrf2 pathway. Free Radic. Biol. Med. 47, 1619-1631.

Singh, A., Misra, V., Thimmulappa, R. K., Lee, H., Ames, S., Hoque, M. O., Herman, J. G., Baylin, S. B., Sidransky, D., Gabrielson, E., Brock, M. V. and Biswal, S. (2006) Dysfunctional KEAP1-NRF2 interaction in non-small-cell lung cancer. PLoS Med. 3, e420.

Singh, A., Wu, H., Zhang, P., Happei, C., Ma, J. and Biswal, S. (2010) Expression of ABCG2 (BCRP) is regulated by Nrf2 in cancer cells that confers side population and chemoresistance phenotype. Mol. Cancer Ther. 9, 2365-2376.

Singh, S. K., Clarke, I. D., Terasaki, M., Bonn, V. E., Hawkins, C., Squire, J. and Dirks, P. B. (2003) Identification of a cancer stem cell in human brain tumors. Cancer Res. 63, 5821-5828.

Sztok, P. P., Piretteri-Vanmarcke, R., Masaiako, P. T., Dinulescu, D. M., Connolly, D., Foster, R., Domtbkowski, D., Preffer, F., Mclaughlin, D. T. and Donahoe, P. K. (2006) Ovarian cancer side population defines cells with stem cell-like characteristics and Mullerian Inhibiting Substance responsiveness. Proc. Natl. Acad. Sci. U.S.A. 103, 11154-11159.

Taguchi, K., Motoshii, H. and Yamamoto, M. (2011) Molecular mechanisms of the Keap1-Nrf2 pathway in stress response and cancer evolution. Genes Cells 16, 123-140.

Trosko, J. E. (2009) Review paper: cancer stem cells and cancer non-stem cells: from adult stem cells or from reprogramming of differentiated somatic cells. Vet. Pathol. 46, 176-193.

Wang, X. J., Sun, Z., Villeneuve, N. F., Zhang, S., Zhao, F., Li, Y., Chen, W., Yi, X., Zheng, W., Wondrak, G. T., Wong, P. K. and Zhang, D. D. (2008) NRF2 enhances resistance of cancer cells to chemotherapeutic drugs, the dark side of NRF2. Carcinogenesis 29, 1235-1243.

Wang, X., Campos, C. R., Peart, J. C., Smith, L. K., Boni, J. L., Cannon, R. E. and Miller, D. S. (2014) NRF2 upregulates ATP binding cassette transporter expression and activity at the blood-brain and blood-spinal cord barriers. J. Neurosci. 34, 8585-8593.

Yang, B., Ma, Y. and Liu, Y. (2015) Elevated expression of Nrf2 and ABCG2 involved in multi-drug resistance of lung cancer SP cells. Drug. Res. (Stuttg) 65, 526-531.

Ye, X. Q., Li, Q., Wang, G. H., Sun, F. F., Huang, G. J., Bian, X. W., Yu, S. C. and Qian, G. S. (2011) Mitochondrial and energy metabolism-related properties as novel indicators of lung cancer stem cells. Int. J. Cancer 129, 820-831.

Zhu, J., Wang, H., Sun, Q., Ji, X., Zhu, L., Cong, Z., Zhou, Y., Liu, H. and Zhou, M. (2013) Nrf2 is required to maintain the self-renewal of glioma stem cells. BMC Cancer 13, 380.

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