

## Expression of ABCG2 (BCRP) Is Regulated by Nrf2 in Cancer Cells That Confers Side Population and Chemoresistance Phenotype

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### Abstract

ATP-binding cassette, subfamily G, member 2 (ABCG2) is expressed in both normal and cancer cells and plays a crucial role in side population (SP) formation and efflux of xenobiotics and drugs. Nrf2, a redox-sensing transcription factor, on constitutive activation in non-small-cell lung cancer cells upregulates a wide spectrum of genes involved in redox balance, glutathione metabolism, and drug detoxification, which contribute to chemoresistance and tumorigenicity. This study examined the mechanism underlying Nrf2-dependent expression of ABCG2 and its role in the multidrug resistance phenotype. *In silico* analysis of the 5'-promoter flanking region of ABCG2 identified an antioxidant response element (ARE) at -431 to -420 bp. A detailed promoter analysis using luciferase reporter assays showed that ARE at -431 to -420 bp is critical for the Nrf2-mediated expression in lung cancer cells. Electrophoretic mobility shift assays and chromatin immunoprecipitation assays revealed that Nrf2 interacts with the ABCG2 ARE element at -431 to -420 bp *in vitro* and *in vivo*. Disruption of Nrf2 expression in lung and prostate cancer cells, by short hairpin RNA, attenuated the expression of ABCG2 transcript and protein, and dramatically reduced the SP fraction in Nrf2-depleted cancer cells. Moreover, depleted levels of ABCG2 in these Nrf2 knockdown cells sensitized them to mitoxantrone and topotecan, two chemotherapy drugs detoxified mainly by ABCG2. As expected, overexpression of Nrf2 cDNA in lung epithelial cells led to an increase in ABCG2 expression and a 2-fold higher SP fraction. Thus, Nrf2-mediated regulation of ABCG2 expression maintains the SP fraction and confers chemoresistance. *Mol Cancer Ther*; 9(8); 2365-76. ©2010 AACR.

### Introduction

Lung cancer is the leading cause of cancer-related deaths in both men and women in the United States (1). The prognosis for lung cancer remains poor, with an overall 5-year survival of 14%. The death toll caused by lung cancer alone counts more than that of breast, colorectal, and prostate cancers combined. Non-small cell lung carcinoma (NSCLC) constitutes ~85% of all lung cancers (1). Chemotherapy is the standard treatment for advanced NSCLC patients, but chemotherapy resistance remains an obstacle and leads to mortality.

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Recent discoveries have provided clear evidence that cancers may develop from rare self-renewing stem cells, which are biologically distinct from differentiated cancer cells. The eradication of these cancer stem cells is likely a critical component of any successful anticancer strategy, and this may explain why conventional cancer therapies are often effective in reducing tumor burden but are rarely curative. Cancer stem cells have been identified in several cancerous tissues, such as acute myelogenous leukemia, neuroblastoma, lung, colon, and breast cancers (2-4). These cancer stem cells represent only a small percentage of total cell populations. They show distinct features such as resistance to irradiation and chemotherapy, and reconstitution of whole populations after irradiation (3, 5). Interestingly, cancer stem cells efficiently efflux Hoechst dye, resulting in the dye-negative phenotype, also known as the side population (SP) phenotype (3). Further investigations revealed that Hoechst dye efflux and the SP formation capacity of cancer stem cells are largely attributable to ATP-binding cassette, subfamily G, member 2 (ABCG2) molecule (6-8). ABCG2, also known as breast cancer resistance protein (BCRP), was originally cloned from multidrug-resistant breast cancer cells (9). Its upregulation has been linked to the chemoresistance phenotype in various cancer cells (3, 6). It was shown that ABCG2 is responsible for SP formation in lung cancer cells (10, 11).

Nrf2, a cap 'n' collar basic leucine zipper transcription factor, protects against environmental toxicants, oxidative injury, inflammation, and apoptosis through transcriptional induction of a broad spectrum of cytoprotective genes involved in electrophile/drug detoxification function, including several ATP-dependent drug efflux pumps (e.g., ATP-binding cassette, subfamily C, member 1, and ATP-binding cassette, subfamily C, member 2; refs. 12–14). Kelch-like ECH-associated protein (KEAP1) is a cytoplasmic anchor of Nrf2 and maintains steady-state levels of Nrf2 and Nrf2-dependent transcription by signaling Nrf2 for proteosomal degradation (15, 16). Somatic mutations in KEAP1 and loss of heterozygosity at KEAP1 locus result in loss of KEAP1 function in cancer cells and gain of Nrf2 function (17). Activating mutations in Nrf2 have been recently reported in squamous cell lung carcinomas (18). Gain of Nrf2 function in lung cancer cells upregulates the expression of genes involved in protection against oxidative stress and thereby promotes tumorigenicity and chemoresistance (17, 19–22).

The *ABCG2* gene is highly expressed in the plasma membrane of several drug-resistant cell lines, where it has been shown to transport antitumor drugs, including mitoxantrone, topotecan, doxorubicin, and daunorubicin (2, 9, 23). *ABCG2* has also been identified as a protective pump against endogenous and exogenous toxic agents. Oltipraz and *tert*-butylhydroquinone, which are known to activate Nrf2-dependent gene expression, upregulated *ABCG2* expression in primary human hepatocytes and human hepatocellular carcinoma cell lines, respectively (24, 25). Because Nrf2 is a stress-inducible transcription factor, which regulates the expression of several cytoprotective genes and drug detoxification enzymes through a common antioxidant response element (ARE) located in the promoter, we decided to investigate whether Nrf2 regulates the expression of *ABCG2* as well. A better understanding of the role of Nrf2 in the regulation of *ABCG2* expression in cancer cells will help elucidate its role in promoting the multidrug resistance phenotype in cancer cells. Here, we show that Nrf2 controls *ABCG2* expression at the transcriptional level and is required for maintaining SP in A549 and H460 lung cancer cells as well as in prostate cancer cells. Reduced Nrf2 expression results in enhanced sensitivity to mitoxantrone and topotecan in both A549 and H460 lung cancer cells.

## Materials and Methods

### Cell culture and reagents

A549, H460, H23, and Du145 cells were purchased from the American Type Culture Collection and were maintained in DMEM with 10% fetal bovine serum and penicillin/streptomycin. The cell lines were routinely tested for *Mycoplasma* contamination. However, the authors did not attempt to authenticate the cell lines again. Generation of A549 and H460 cells constitutively expressing short hairpin RNA (shRNA) against Nrf2 or

control luciferase shRNA cells with stable expression of Nrf2 shRNA or control nontargeting luciferase shRNA were maintained in DMEM containing 0.2 µg/mL of puromycin (Roche). The human airway epithelial cell line NuLi, derived from normal lung, was grown in a serum-free medium, bronchial epithelial growth medium (BEGM, serum-free), from Cambrex, made of BEBM basal medium and SingleQuot additives supplemented with 50 µg/mL G-418. Details on the generation of A549-LucshRNA, A549-Nrf2shRNA, H460-LucshRNA, H460-Nrf2shRNA, Du145-LucshRNA, and Du145RNA cells used in this article have been published (21, 22). The sequence of Keap1 shRNA used to downregulate Keap1 expression was 5'-CCGGCCCTTAATTCAGCTGAGTGTTCTCGAGAACACTCAGCTGAATTAAGGCTTTTTTG-3' (Sigma-Aldrich). Lentiviral Nrf2 expression vector (PLOHS\_100067113) was purchased from Open Biosystems. Mitoxantrone was obtained from Sigma-Aldrich, and topotecan was purchased from LKT Laboratories.

### Identification of ARE(s) in the promoter of *ABCG2*

To identify the presence and location of AREs in the *ABCG2* promoter, a 600-bp upstream region sequence from the transcription start site and exons 1 and 2 was downloaded from the National Center for Biotechnology Information database (Human Genome resources). This sequence was screened for ARE binding sites with the help of Genamics Expression 1.1 software using the primary core sequence of ARE (RTGABNNNGCR; ref. 26) as the probe.

### Plasmids and mutagenesis

The 5' flanking region of human *ABCG2* promoter region (−496 bp to +198 bp) was PCR amplified from human genomic DNA using high-fidelity Taq polymerase (Applied Biosystems). The primers used for amplification were as follows: forward, 5'-CACTTCTCAGAATCC-CATTACAC-3'; reverse, 5'-GAACCTTTGAGTGGGCACAG-3'. The isolated PCR product was ligated to pCR2.1 vector (Invitrogen), and a *KpnI-XhoI* fragment from this construct was cloned into the pGL3 basic vector (Promega). A deletion construct (−310 bp to +198 bp) was generated from the full-length promoter construct. To clone the ARE enhancer sequence in pTAL vector, the ARE binding site with minimal flanking region was amplified using the following primers: forward, 5'-AAAAAAGGTACCATCCCATCCAGAAACCA-3'; reverse primer, 5'-AAAAAACTCGAG CGAACGGAATGAACCAGAGT-3'. Mutant ARE sequences were generated by using a site-directed mutagenesis kit from Stratagene. Primers containing the mutant ARE sequences (GCAGCGCTTGgGcCTGGGCAACCTGTGCGTC) were used for PCR amplification of the mutant *ABCG2* ARE binding site in the promoter, and PCR products were digested with *DpnI* for 1 hour to cleave the wild-type promoter template. The sequence of each promoter construct was verified by sequencing.

### DNA transfection and luciferase activity

Cells were transfected at 75% to 85% confluency using Lipofectamine 2000 (Invitrogen). Briefly, cells were seeded in 24-well plates at a density of  $2 \times 10^5$  cells/mL and grown overnight. After ~12 hours, the medium was removed, and transfection complex containing 800 ng of plasmid DNA, 40 ng of pRL-TK plasmid (Promega) at a ratio of 20:1, and transfection reagent were added to each well in the presence of fetal bovine serum. Cells were incubated for another 36 hours, and were then lysed and assayed. *Renilla* and *Firefly* luciferase activities were measured using the dual luciferase assay kit (Promega) with a luminometer (EG&G). *Firefly* luciferase activity was normalized to *Renilla* luciferase activity for calculation of relative reporter activity for each construct. Results were plotted from three independent experiments, with each assay conducted in triplicate.

### Flow cytometry

Analysis for SP formation was carried out following the protocol of Goodell's laboratory with minor modifications (27). Briefly, cells ( $1 \times 10^6$ /mL) were incubated at 37°C for 60 minutes with 5 µg/mL Hoechst 33342 (Sigma-Aldrich), washed, and resuspended in ice-cold HBSS with 2% FCS and 2 µg/mL propidium iodide (Sigma-Aldrich). Fumitremorgin C (FTC, 10 µmol/L), a potent and specific inhibitor of ABCG2 activity, was used as a positive control for the assay. Side population was analyzed with fluorescence-activated cell sorting (FACS) Vantage (Becton Dickinson).

### Western blot

Western blot was carried out using the protocol published by Singh et al. (17). Primary antibody incubation was carried out at 4°C for 16 hours with a mouse anti-ABCG2 antibody (Sigma-Aldrich) diluted at 1:300 in 3% bovine serum albumin, followed by incubation with horseradish peroxidase-conjugated horse anti-mouse secondary antibody, and developed using an enhanced chemiluminescence detection system (GE Healthcare). Anti- $\alpha$ -tubulin and anti-glyceraldehyde-3-phosphate dehydrogenase antibodies were used as the loading control in immunoblot assays (Santa Cruz Biotechnology).

### Cell proliferation (MTT) assays

Chemotherapy drug treatments were done following protocols published by Singh et al. (17). The *in vitro* drug sensitivity experiments were carried out by using a cell proliferation assay kit (Roche) according to the manufacturer's instructions.

### Real-time reverse transcriptase-PCR

Real-time reverse transcriptase-PCR (RT-PCR) reactions were carried out using a protocol published by Singh et al. (17). Briefly, 500 ng of total RNA were reverse transcribed using a high-capacity cDNA synthesis kit from Applied Biosystems. An aliquot of diluted cDNA

was used to measure human ABCG2 (Hs01053790\_m1) and Nrf2 (Hs00232352\_m1), NQO1 (Hs00168547\_m1), and GCLm (Hs00157694\_m1) gene expression using Taqman primer and probe mixes from Applied Biosystems. The assays were done using the ABI 7000 TaqMan system (Applied Biosystems).  $\beta$ -Actin (Hs99999903\_m1) was used for normalization.

### Electrophoretic mobility shift assays

Double-stranded DNA oligonucleotides corresponding to the consensus ARE sequence (5'-GCGCTTGTGACTGGGCAACCTGTGC-3') in the ABCG2 gene promoter were synthesized commercially from Integrated DNA Technologies. The duplex was end-labeled using T4 kinase (Promega) in the presence of [ $\gamma$ - $^{32}$ P]dCTP (MP Biochemicals). The probe was purified once with NAP-5 column (GE Healthcare) according to the manufacturer's instruction. The binding reactions were carried out with 5 µg of nuclear protein isolated from A549-LucshRNA cells or A549-Nrf2shRNA cells and  $^{32}$ P-labeled probe ( $5 \times 10^5$  cpm/reaction) in a 20 µL reaction mixture containing 5 mmol/L HEPES (pH 7.9), 100 mmol/L NaCl, 0.25 mmol/L EDTA, and 0.25 mmol/L DTT. Poly (deoxyinosinic-deoxycytidylic) acid 50 µg/mL (Roche) was included in each reaction as nonspecific carrier DNA. The mixture was placed on ice for 30 minutes. Protein-DNA complexes were separated by electrophoresis on a nondenaturing 5% PAGE gel at 200 V with a running buffer consisting of 25 mmol/L Tris, 25 mmol/L borate acid, and 1 mmol/L EDTA. Gels were dried, and the radioactive bands were visualized by autoradiography. For competition reactions, either 25- or 50-fold excess of the unlabeled oligonucleotides harboring the wild-type ARE sequence of the ABCG2 gene or corresponding mutant ARE (5'-GCGCTTGGGCGCTGGGCAACCTGTGC-3') was added during the preincubation period. Unlabeled oligonucleotides containing AP1 or NF- $\kappa$ B binding sequence were used as nonspecific competitors.

### Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) assays were conducted using the ChIP assay kit from Upstate Cell Signaling according to the manufacturer's instructions. Cells ( $\sim 10^7$ ) were harvested and chromatin was cross-linked by adding formaldehyde to the cell culture medium to a final concentration of 1% and incubating the mixture for 10 minutes at 37°C. Cells were washed twice with ice-cold PBS containing a protease inhibitor cocktail (Roche) and were suspended in 0.2 mL of lysis buffer [50 mmol/L Tris-HCl (pH 8.1), with 1% SDS and 5 mmol/L EDTA]. Samples were sonicated on ice to an average length of 500 to 1,000 bp (four pulses of 10 seconds each) and were centrifuged at 10,000  $\times$  g. Solubilized chromatin was diluted 10-fold with ChIP dilution buffer [16.7 mmol/L Tris-HCl (pH 8.1), with 0.01% SDS, 1.1% Triton X-100, 1.2 mmol/L EDTA, and 167 mmol/L NaCl] and was used for ChIP assays. An aliquot of the soluble fraction was saved for total chromatin input.

Chromatin was precleared with salmon sperm DNA/protein A-agarose for 30 minutes and then incubated with either antibody to (Nrf2 sc-722X; Santa Cruz Biotechnology), and rabbit IgG for 18 hours at 4°C with rotation. Immunoprecipitation, washing, and elution were carried out according to the manufacturer's instructions. Cross-linked immunoprecipitates and total chromatin input were reverse cross-linked, and samples were treated with proteinase-K (Sigma) and extracted with phenol-chloroform-isoamyl alcohol. DNA was precipitated and resuspended in 100  $\mu$ L of TE (10 mmol/L Tris-Cl and 0.1 mmol/L EDTA). Then, 1  $\mu$ L of DNA was used for PCR (35 cycles) with primers specific for the ABCG2 promoter. For PCR amplification, forward primer 5'-ATCCCATTCACCAGAAACCA-3' and reverse primer 5'-ACTCTGGTTCATTCCGTTTCG-3' were used.

### Statistical analysis

Data are presented as the mean  $\pm$  SD. To assess statistical significance of differences, Student's *t* test or one-way ANOVA was conducted. *P* values <0.05 were considered statistically significant as indicated by asterisks. IC<sub>50</sub> values in drug sensitization studies were obtained by using GraphPad Software.

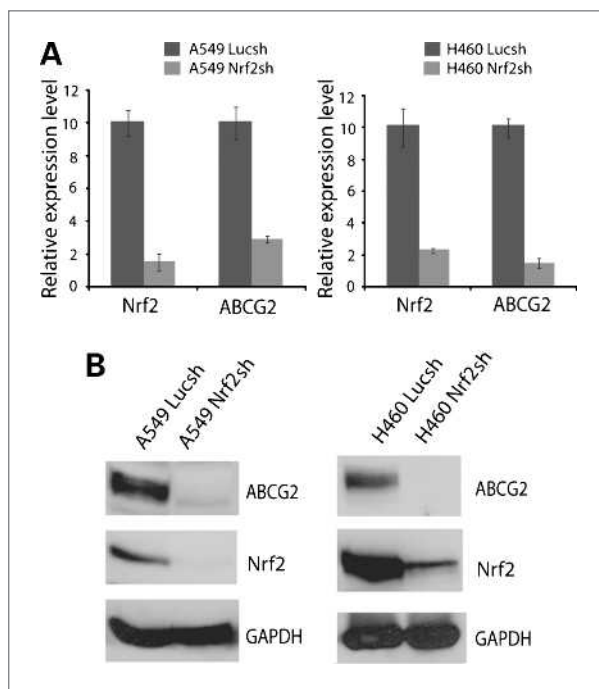
## Results

### Attenuation of Nrf2 expression in lung cancer cells leads to a decline in ABCG2 mRNA and protein levels

To study the Nrf2-dependent regulation of ABCG2 expression, we selected A549 and H460 lung cancer cells as a model system because they harbor the *KEAP1* gene mutation that leads to a high basal level of Nrf2 pathway activity and are drug resistant (17, 22). We have established and characterized A549 and H460 cells constitutively expressing shRNA targeting Nrf2 (17, 22). Short hairpin targeting luciferase shRNA-expressing cells were used as nontargeting control. A549 and H460 cells stably expressing Nrf2 shRNA (A549-Nrf2shRNA, H460-Nrf2shRNA) showed significant reduction in Nrf2 mRNA expression and a parallel decrease in ABCG2 transcript levels compared with cells expressing luciferase shRNA (A549 LucshRNA and H460 LucshRNA; Fig. 1A). Immunoblot analysis revealed diminished levels of ABCG2 protein in Nrf2 knockdown A549 and H460 cells compared with control cells (Fig. 1B). The expression of Nrf2 and ABCG2 did not change significantly between the control cells transfected with luciferase shRNA and the untransfected parent cancer cells (Supplementary Fig. S1).

### Nrf2 directly regulates transcriptional expression of ABCG2

To investigate whether ABCG2 is a direct transcriptional target of Nrf2, we used promoter reporter assays to study ABCG2 promoter regulation in Nrf2-proficient (A549 control cells or parental A549 cells) and Nrf2-



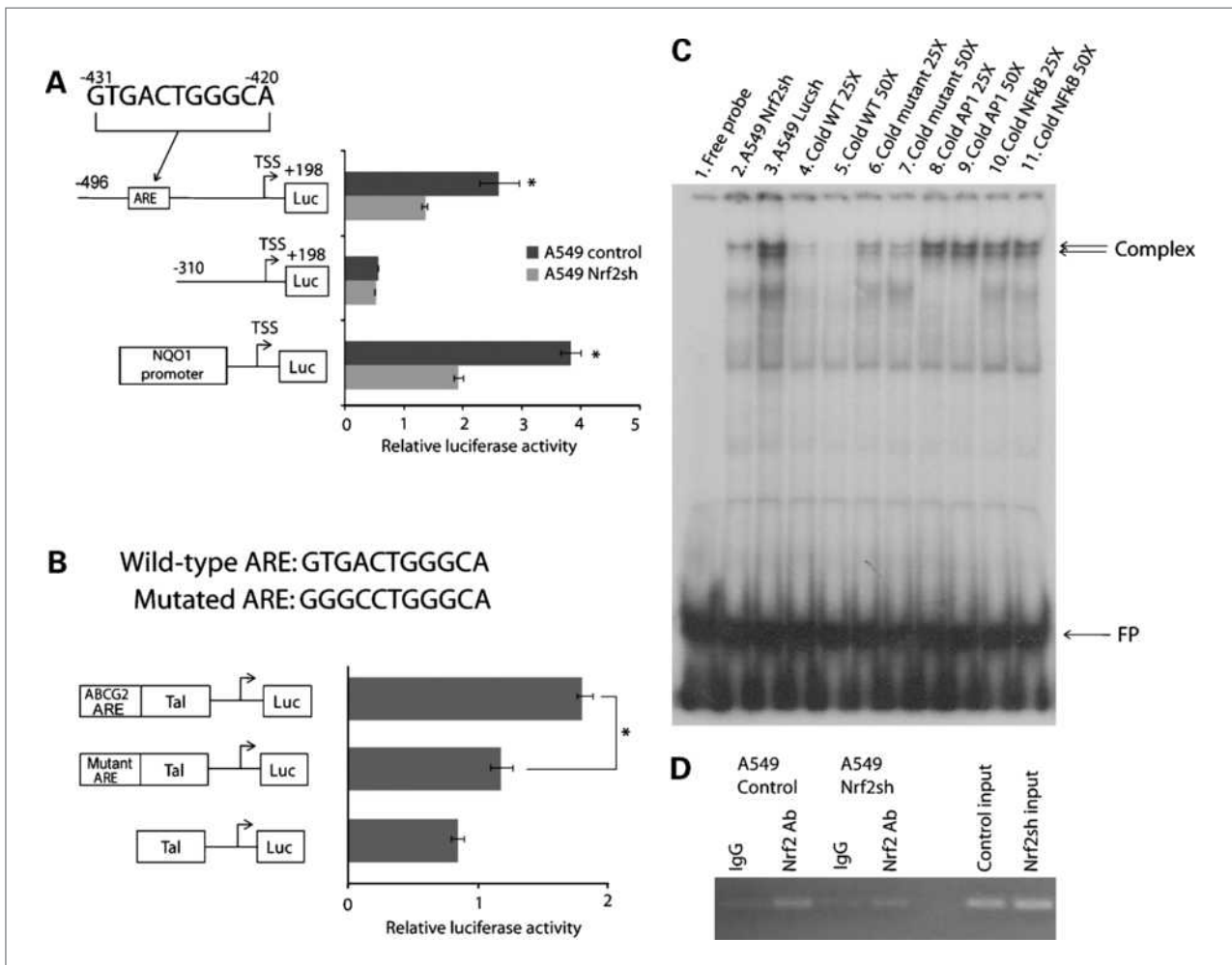
**Figure 1.** Nrf2-dependent ABCG2 expression in lung cancer cells. A, relative expression of ABCG2 in A549 and H460 cells constitutively expressing Nrf2shRNA analyzed by real-time RT-PCR.  $\beta$ -Actin was used for normalization. Data are presented as fold change calculated using gene expression levels in control luciferase shRNA cells as baseline. B, immunoblot analysis of ABCG2, Nrf2, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression in A549 and H460 cells expressing control luciferase shRNA and Nrf2 shRNA.

depleted (A549-Nrf2shRNA) lung cancer cells. Two ABCG2 promoter deletion constructs were made according to the *in silico* analysis of ABCG2 promoter that identified a putative ARE located at -431 to -420 bp upstream of the ABCG2 transcription start site (TSS). The full-length reporter construct contained the putative ARE (-496 bp to +198 bp), whereas truncated reporter construct did not (-310 to +198 bp; Fig. 2A, schematic). The two reporter constructs were transfected into A549 control and A549-Nrf2shRNA cells, and luciferase reporter activity was measured. As shown in Fig. 2A, promoter activity of the ABCG2 full-length construct (-496 to +198 bp) was significantly reduced in Nrf2-depleted A549-Nrf2shRNA cells compared with Nrf2-proficient A549 control cells. The shorter promoter deletion construct lacking the putative ARE (-310 to +198 bp) exhibited similar activity in both Nrf2-depleted and control cells. To further confirm that Nrf2-dependent luciferase reporter activity was suppressed in A549Nrf2shRNA cells compared with control cells, a Nrf2-dependent NQO1 basal promoter reporter construct (28) was included in the promoter reporter studies. The NQO1 reporter plasmid showed significantly reduced luciferase activity in A549 Nrf2 shRNA cells compared with control A549 cells (Fig. 2A). These data strongly suggest that the ARE

binding site in the ABCG2 promoter region is active and functions as a transcriptional enhancer. To further confirm that the ARE sequence in ABCG2 promoter functions as an enhancer, we cloned the ABCG2 promoter ARE sequence with sufficient flanking sequence into a pTAL-Luc reporter vector containing a minimal promoter to make a ABCG2 ARE construct, and cloned ABCG2 promoter fragment with mutant ARE into a pTAL-Luc construct to make a mutant ARE construct (Fig. 2B, sche-

matic). Reporter assays results showed that mutation of the ARE core sequence significantly inhibited the Nrf2-dependent luciferase reporter activity in A549 cells (Fig. 2B). These observations suggest that the ARE element located at -431 to -420 bp is involved in Nrf2-dependent regulation of ABCG2.

To further verify that Nrf2 binds to the ARE binding site in the ABCG2 promoter *in vitro* and *in vivo*, we performed electrophoretic mobility shift assays. As shown in



**Figure 2.** Nrf2 regulates ABCG2 transcription through an ARE element in the ABCG2 promoter. **A**, a putative ARE at -431 to -420 bp in the proximal promoter region of the human *ABCG2* gene was identified by *in silico* analysis and promoter reporter assay. Left, schematic representation of the two constructs with and without putative ARE. The 5' end of each of the constructs relative to the transcription start site (arrows) is indicated. These constructs were transfected into A549 control and A549-Nrf2shRNA cells, and luciferase activity was measured. Luciferase activities were normalized relative to the *Renilla* luciferase activity. NQO1 basal promoter construct was included as a positive control for measuring Nrf2-dependent reporter expression. **B**, ABCG2 promoter fragment containing the ARE core sequence was cloned upstream of heterologous minimal TATA-like promoter driving the luciferase expression (pTAL vector). Mutations in the ARE core sequence (shown on top) were introduced by site-directed mutagenesis. These constructs were transfected into A549 cells, and luciferase activity was measured. \*,  $P < 0.05$ , significant when compared with wild-type ARE (analyzed by Student's *t* test). **C**, *in vitro* DNA binding activity of Nrf2 to ABCG2 ARE using electrophoretic mobility shift assays. Nuclear proteins from Nrf2-depleted cells (lane 2) and control A549 cells (lane 3) were incubated with  $^{32}$ P-labeled oligonucleotides harboring the ARE consensus sequence of *ABCG2* gene. The resulting complexes were resolved by nondenaturing PAGE and analyzed. For competition assays, a 25- and 50-fold excess of unlabeled oligonucleotides harboring the wild-type ARE (lanes 4 and 5) of *ABCG2* gene or a 25- to 50-fold excess of unlabeled oligonucleotides with the mutated ARE sequence was added (lanes 6 and 7) during the preincubation period. Nonspecific unlabeled oligonucleotides that contain AP1 and NF- $\kappa$ B binding sequences were used as a negative control (lanes 8–11). Nuclear extracts from A549 control cells were used in both specific and nonspecific competitions (lanes 4–11). Arrow, ARE binding complex; FP, free probe. **D**, ChIP assays were done in A549 cells stably expressing Nrf2shRNA and control cells expressing luciferase shRNA.

Fig. 2C, there was a marked Nrf2-dependent complex formation when  $^{32}\text{P}$ -labeled ABCG2 ARE oligonucleotide duplexes were incubated with nuclear extract from control A549-LucshRNA cells (double arrows). In contrast, the Nrf2-dependent complex formation was reduced when the  $^{32}\text{P}$ -labeled ARE probes were incubated with nuclear extracts from Nrf2-deficient A549 cells (Fig. 2C, compare lane 3 with lane 2). The specificity of Nrf2-dependent complex formation was tested using 25 $\times$  and 50 $\times$  excess of unlabeled ABCG2-ARE oligonucleotides as cold competitor. The competition results showed that the Nrf2-dependent complex formation was completely abolished by cold wild-type ARE oligonucleotides (Fig. 2C, lanes 4 and 5), but was competed to a less extent by mutant ARE oligonucleotides (Fig. 2C, lanes 6 and 7) and was poorly competed by nonspecific AP1 or NF- $\kappa$ B oligonucleotides (Fig. 2C, lanes 8–11). Next, we conducted ChIP assays to examine whether Nrf2 constitutively binds to the endogenous ABCG2 promoter in cancer cells. As shown in Fig. 2D, constitutive recruitment of Nrf2 to the ARE element located in ABCG2 promoter was observed in Nrf2-proficient A549 LucshRNA control cells. As expected, in A549-Nrf2shRNA cells, Nrf2 was present at low levels and reduced amplification was detected using ABCG2 promoter primers. Immunoprecipitations with control IgG failed to enrich the ARE-containing ABCG2 promoter region (Fig. 2D), suggesting that the sites were not enriched in a nonspecific fashion. Overall, these results indicate that Nrf2 specifically binds to the ARE element in the ABCG2 promoter. In conclusion, these promoter reporter studies and *in vitro* and *in vivo* binding assays revealed that Nrf2 regulates ABCG2 promoter activity through the ARE element located at –431 to –420 bp from the transcription start site.

#### Decreasing Nrf2-dependent ABCG2 expression by shRNA results in loss of the SP phenotype

ABCG2 activity is required for maintaining the SP phenotype in cancer cells. To investigate whether reduced ABCG2 expression affects SP formation in Nrf2-deficient A549 and H460 cells versus control cells, Hoechst dye exclusion assays were conducted. The results show that there are ~58% cells that belong to SP fraction in control A549-LucshRNA cells; on the contrary, the percentage of SP drops to <2% in Nrf2-knockdown A549 cells. Similarly, SP content in Nrf2-knockdown H460 cells are ~0.18% compared with ~1.3% in control H460-LucshRNA cells (Fig. 3). To confirm the specificity of the Hoechst dye efflux assay, we used fumitremorgin C (FTC), a specific inhibitor of ABCG2. The cells were incubated with Hoechst dye in the presence of FTC followed by propidium iodide staining and flow cytometric analysis. The SP phenotype was completely blocked by FTC treatment in both A549 and H460 cells (Fig. 3; Supplementary Fig. S2). To determine if antibiotic selection during generation of stable cell lines altered the percentage of cells in the SP, we compared the SP in A549-parent cells, A549-LucshRNA, and

A549-Nrf2shRNA cells (Supplementary Fig. S3A). We detected ~1.75- to 2-fold higher numbers of cells in the SP of A549-LucshRNA relative to A549-parent cells. Similar results were obtained with H460-parent, H460-LucshRNA, and H460-Nrf2shRNA cells (Supplementary Fig. S3B). However, Nrf2-depleted cells A549 and H460 had dramatically smaller fraction of cells in SP.

#### Inhibiting Nrf2-dependent ABCG2 expression by shRNA causes chemosensitization to its target anticancer drugs

ABCG2 is crucial for detoxification of several chemotherapeutic drugs; thus, its increased expression confers chemoresistance. It has been reported that efflux of mitoxantrone and topotecan depends on ABCG2 (9, 23). To determine whether decreased expression of ABCG2 could sensitize A549 and H460 cells to mitoxantrone and topotecan-induced cytotoxicity, we incubated both Nrf2-depleted and control A549 and H460 cells with mitoxantrone and topotecan for 2 to 4 days, and measured the percentage of viable cells using the MTT assay. As expected, the Nrf2-deficient A549 and H460 cells (A549-Nrf2shRNA and H460-Nrf2shRNA), with low endogenous levels of ABCG2, displayed enhanced sensitivity to mitoxantrone and topotecan *in vitro* (Fig. 4). The  $\text{IC}_{50}$  of mitoxantrone in A549 LucshRNA and A549-Nrf2shRNA cells was 31.38 and 23.09 nmol/L, respectively. The  $\text{IC}_{50}$  of mitoxantrone in H460-LucshRNA and H460-Nrf2shRNA cells was 22 and 6 nmol/L, respectively. Similar to mitoxantrone, Nrf2-depleted A549 and H460 cells showed lower  $\text{IC}_{50}$  values for topotecan (A549-LucshRNA, 35.68 nmol/L; A549-Nrf2shRNA, 11.07 nmol/L; H460-LucshRNA, 78.8 nmol/L; and H460-Nrf2shRNA, 30.1 nmol/L).

#### Nrf2-dependent ABCG2 expression in prostate cancer cells

We have established and characterized Du145 cells constitutively expressing shRNA targeting Nrf2 transcript (Du145-Nrf2shRNA) and the control DU145 cells expressing shRNA against luciferase gene (Du145-LucshRNA; ref. 21). Du145 cells stably expressing Nrf2shRNA (Du145-Nrf2shRNA) showed >85% reduction in Nrf2 mRNA expression and a parallel decrease in ABCG2 transcript levels compared with cells expressing luciferase shRNA (Du145-LucshRNA; Fig. 5A). Immunoblot analysis revealed diminished levels of Nrf2 and ABCG2 protein in Nrf2 knockdown Du145 cells compared with parent Du145 cells and Du145-LucshRNA cells (Fig. 5B and C). The expression of Nrf2 and ABCG2 in Du145-LucshRNA cells was similar to that of the parent Du145 cells. To determine whether Nrf2-dependent ABCG2 expression in prostate cancer cells affects SP formation, we used the Hoechst dye exclusion assay. The immunoblotting data presented above show that Du145 cells express low levels of ABCG2 protein. Analysis of SP in Du145-parent cells, Du145-LucshRNA, and Du145-Nrf2shRNA cells revealed very low SP fraction

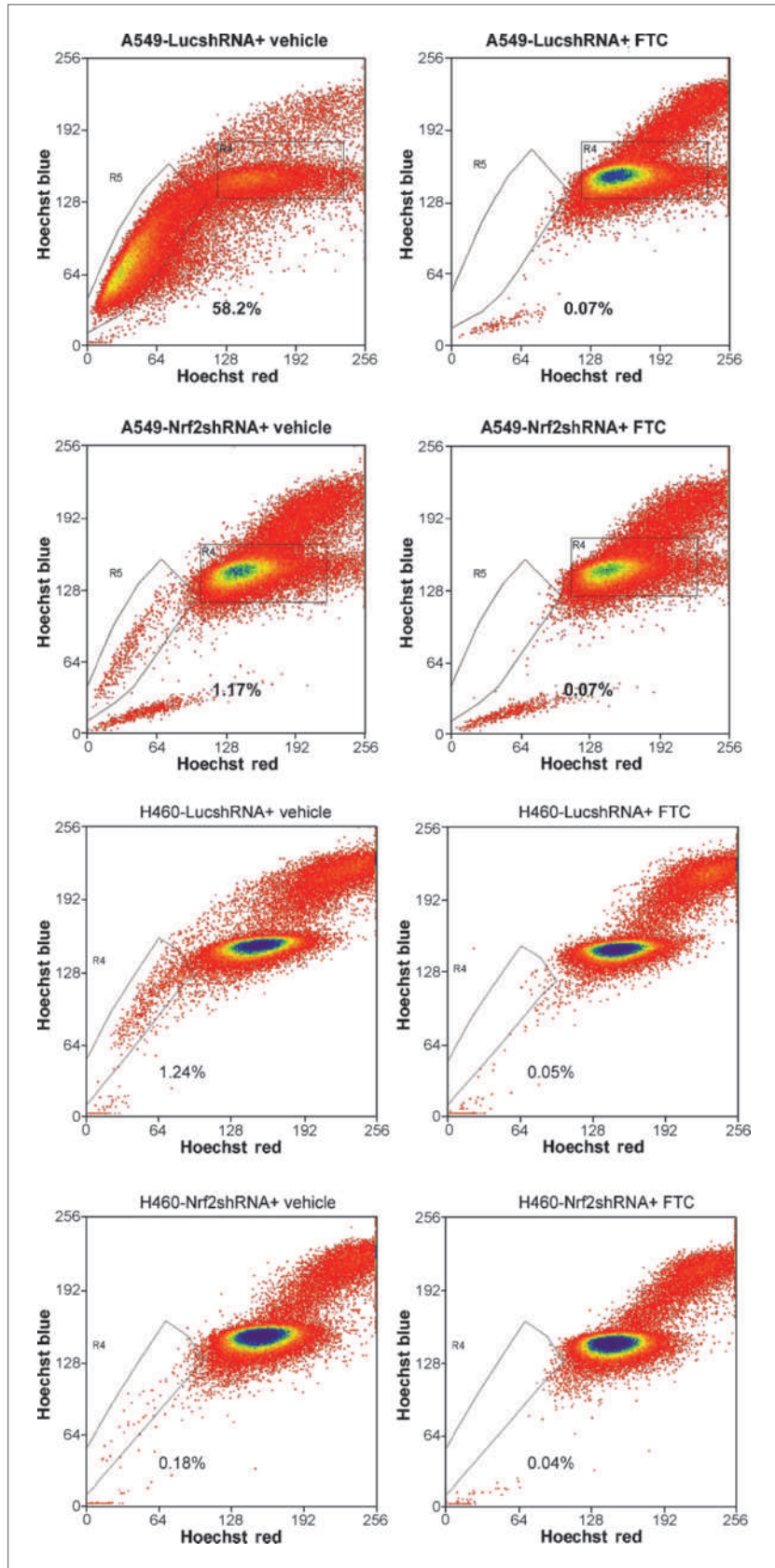
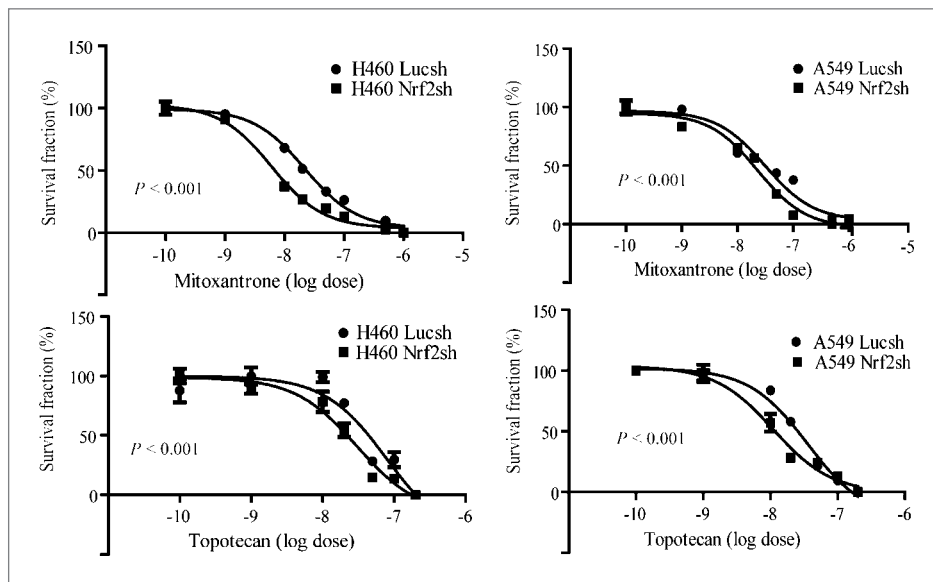


Figure 3. Alteration of the SP phenotype in Nrf2-depleted lung cancer cells. A549 and H460 cells were incubated with Hoechst 33342 dye and propidium iodide, and were analyzed by flow cytometry. To show assay specificity, cells were incubated with Hoechst dye in the presence of FTC. The SP is shown as a percentage of the whole viable cell population. Reactions were done in triplicates and were repeated three times.



**Figure 4.** Reversal of chemoresistance in Nrf2-depleted lung cancer cells. A549 and H460 cells were treated with mitoxantrone and topotecan for 2 to 4 days, and cell viability was analyzed by MTT assays. Results shown are median values obtained from three independent experiments with eight replicates for each dose. Results were analyzed by one-way ANOVA analysis. \*,  $P < 0.001$  in all panels.

in Du145 cells. We detected no cells in the SP fraction of Du145-Nrf2shRNA cells (Supplementary Fig. S4). Thus, similar to Nrf2-depleted lung cancer cells, Du145-Nrf2shRNA cells showed reduced ABCG2 mRNA, protein expression, and SP compared with Nrf2 proficient cells. Collectively, these results suggest that transcriptional regulation of ABCG2 in lung and prostate cancer cells is mediated by Nrf2.

#### Nrf2 activation induces ABCG2 expression in lung epithelial cells

Next, we examined whether activation of Nrf2 induces ABCG2 expression in nontumorigenic airway epithelial cells (i.e., NuLi cells). We used a shRNA targeting Keap1 to inhibit Keap1 expression and activate Nrf2-dependent gene expression. Luciferase shRNA was used as nontargeting control shRNA. As shown in Fig. 6A, reduction in Keap1 transcript levels by Keap1 shRNA resulted in a parallel increase in NQO1, GCLm, and ABCG2 transcript levels. We observed an inverse correlation between Keap1 expression and Nrf2-dependent ABCG2 expression in lung epithelial cells.

To show that commonly used oxidative stress-inducing agents like cigarette smoke condensate and *tert*-butyl hydroquinone, known to upregulate Nrf2-dependent signaling, also upregulate ABCG2 expression, we treated NuLi cells with these agents and measured gene expression. The cells were exposed to cigarette smoke condensate (100  $\mu$ g/mL) and *tert*-butyl hydroquinone (20  $\mu$ mol/L) for 24 hours, and induction of ABCG2 in response to these treatments was quantified by real-time RT-PCR. Pretreatment with Nrf2-activating agents upregulated the expression of ABCG2 and other classic Nrf2 target genes, suggesting that Nrf2 mediates inducible expression of ABCG2 (Fig. 6B).

Alternatively, we overexpressed Nrf2 cDNA in a lung adenocarcinoma cell line, H23, harboring wild-type Keap1 and Nrf2 activity. H23 cells were transduced with lentiviral particles containing Nrf2 cDNA or the control empty vector. Four to 6 days posttransduction, cells were harvested and expression of Nrf2 and its target genes, including ABCG2, was measured by real-time RT-PCR and immunoblotting. As expected, Nrf2 overexpression resulted in upregulation of ABCG2 expression at the mRNA and protein levels (Fig. 6C). Analysis of SP in H23 cells revealed that cells overexpressing Nrf2 (H23-Nrf2 cDNA) had a 2-fold higher SP fraction compared with H23 empty vector control cells (Fig. 6D). In summary, we have convincingly shown that Nrf2 regulates ABCG2 gene expression and SP fraction in cancer cells.

#### Discussion

In the present study, we show that Nrf2 regulates the transcriptional expression of human ABCG2 gene through an ARE element located at the -431 to -420 bp region of the promoter, and Nrf2 is essential for maintaining ABCG2 expression and function in lung cancer and prostate cancer cells. Nrf2 depletion resulted in dramatic reduction of SP formation and reversal of chemoresistance in non-small-cell lung cancer cell lines. Similarly, upregulation of Nrf2 activity induced the expression of ABCG2 and increased the SP fraction in lung epithelial cells.

One of the defining characteristics of cancer stem cells is their ability to transport Hoechst dye, leading to the SP phenotype, which is attributable to the expression of ABCG2 gene (3, 6). A study by Scharenberg et al. (11) showed that A549 cells contain an increased fraction of SP-positive cells, and the high Hoechst dye efflux capacity of A549 cells correlates very strongly with ABCG2

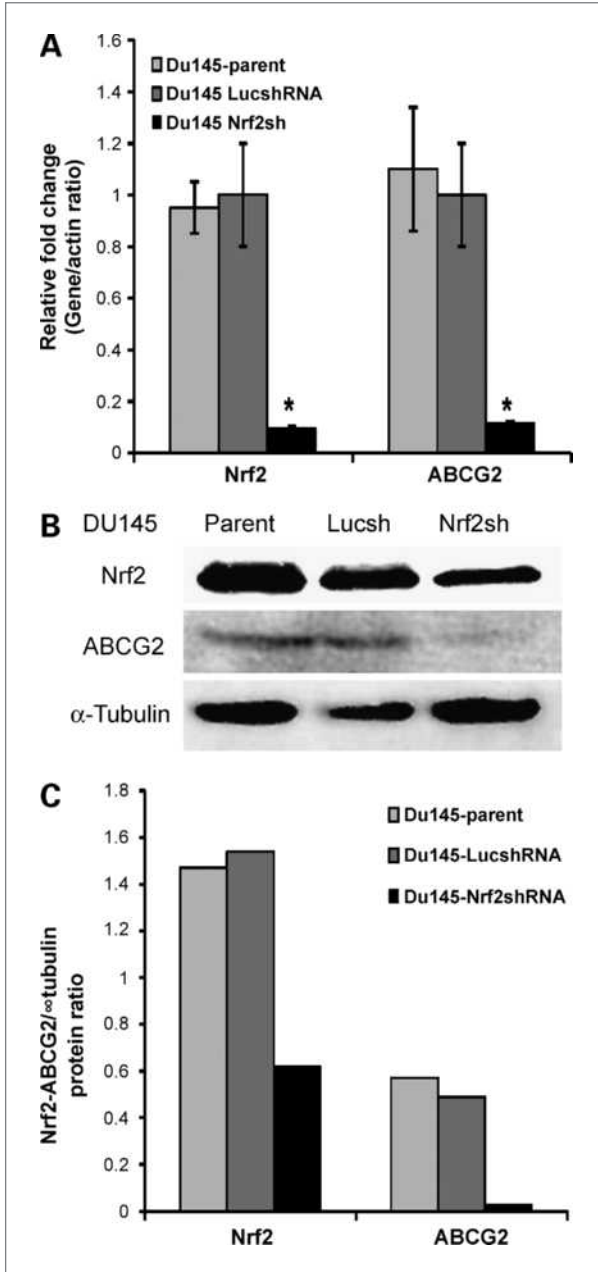


activity. Transcriptome analyses of embryonic, hematopoietic, and neural stem cells revealed a common signature of gene expression, which includes transcripts that function as a cytoprotective factor against environmental

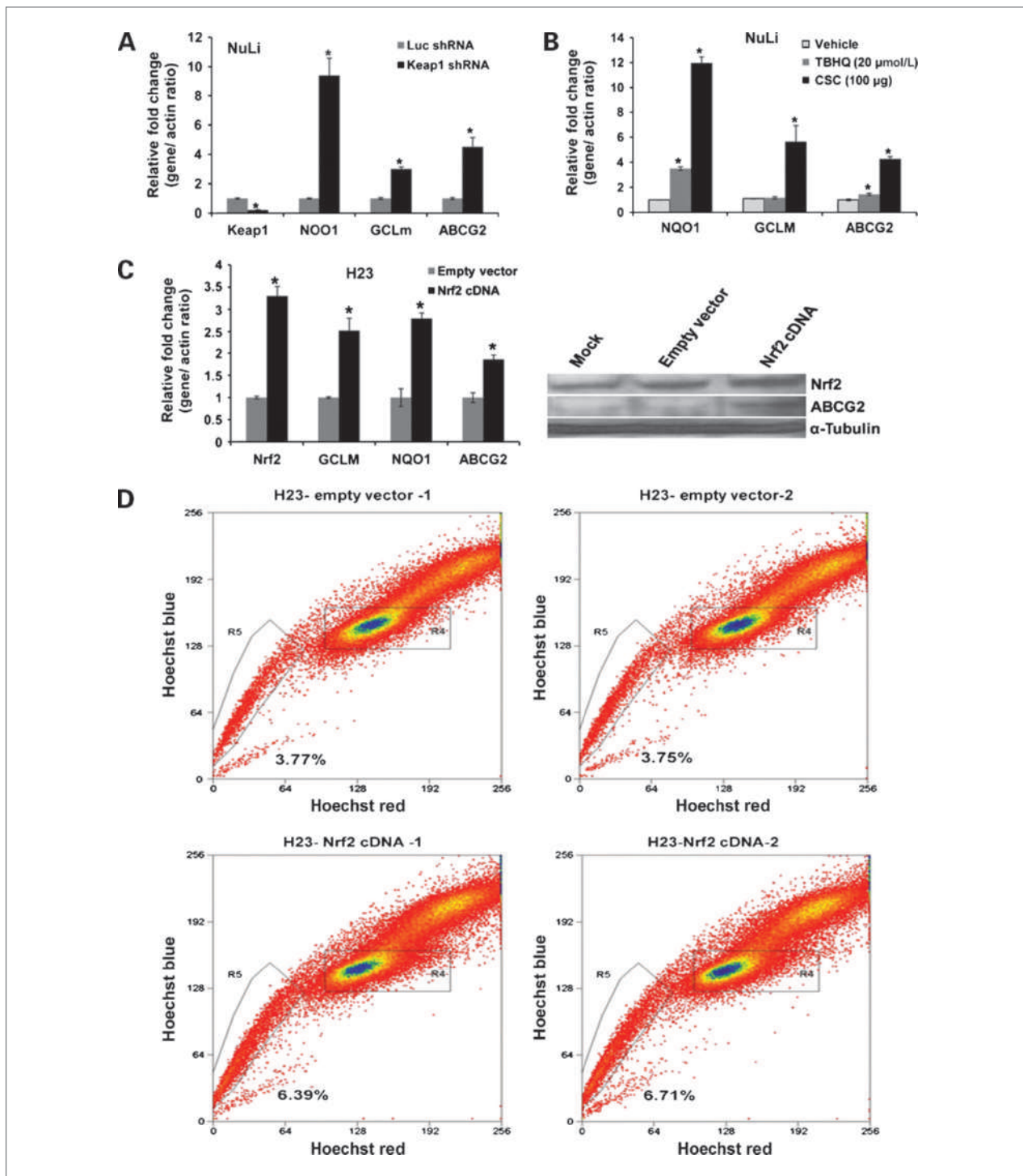
and xenobiotic stress (29). Studies showed that stem cells express high levels of drug detoxification enzymes such as P-glycoprotein, BCRP (ABCG2), glutathione S-transferases, and glutathione peroxidase (6, 30, 31). Interestingly, several of these cytoprotective and drug detoxification genes are classic Nrf2-dependent genes.

Nrf2, a bZIP transcription factor, protects cells from oxidative stress and xenobiotics by induction of a transcriptional program that includes major antioxidants such as enzymes in the glutathione and thioredoxin pathways, as well as xenobiotic detoxification enzymes that include the glutathione S-transferase and UDP glycosyltransferase families, aldehyde dehydrogenase, and various drug efflux pumps—members of the multidrug resistance protein family (32–34). KEAP1 negatively regulates Nrf2 activity by targeting it for proteasomal degradation (15, 16). Nrf2-KEAP1 interactions are frequently dysfunctional in NSCLC, leading to constitutive activation of Nrf2 (17). Our previous studies have shown that point mutations in the *KEAP1* gene lead to nonconservative amino acid substitutions and nonsense mutations, resulting in loss of KEAP1 function in a high percentage of the NSCLC cell lines and primary tumors (17). Recently, two Nrf2 mutation “hotspots” were identified in ~10% of patients with squamous lung carcinoma, enabling the transcription factor for the evasion of KEAP1-mediated repression (18, 35). Furthermore, mutations in KEAP1 have been reported in breast, gall bladder, and prostate cancers (21, 36, 37). High Nrf2 activity in cancer cells results in upregulation of phase II drug detoxification enzymes and several ATP-dependent multidrug-resistant drug efflux pumps (e.g., ABCC1 and ABCC2) and promotes drug resistance (12, 38–42). ABCG2 transcript and protein levels were dramatically reduced in Nrf2-depleted lung cancer cells, suggesting that ABCG2 gene expression is Nrf2 dependent in lung cancer cells. Attenuated ABCG2 promoter reporter activity in the absence of Nrf2 activity implies that Nrf2 regulates ABCG2 transcription by modulating ABCG2 promoter activity. Loss of Nrf2 expression and a corresponding decrease in ABCG2 expression reduced SP formation in lung cancer cells. Similar Nrf2-dependent ABCG2 expression was observed in Du145 cells, a prostate cancer cell line.

Recently, it has been reported that the expression of ABCG2 along with some other efflux pumps was decreased in hepatocytes of Nrf2 null mice compared with that of wild-type mice, which indicates that Nrf2 is involved in physiologic regulation of ABCG2 (43). *Tertiary-butylhydroquinone*, an agent known to activate Nrf2 by inducing oxidative stress, upregulated the expression of ABCG2 in hepatocellular carcinoma cells (25). In another study, Jigorel et al. (24) reported that Oltipraz, a small-molecule activator of Nrf2, upregulated the expression of ABCG2 in primary human hepatocytes. Studies focused on regulation of the expression of ABCG2 revealed that estrogen receptor-, hypoxia-, and peroxisome proliferator-activated receptor  $\gamma$ -inducible



**Figure 5.** Attenuated expression and activity of ABCG2 in Nrf2-deficient prostate cancer cells. **A**, relative expression of ABCG2 and Nrf2 in Du145-parent cells and Du145-LucshRNA and Du145-Nrf2shRNA cells analyzed by real-time RT-PCR. Data are presented as fold change calculated using gene expression levels in control luciferase shRNA cells as 1. **B**, immunoblot analysis of Nrf2 and ABCG2, expression in Du145 cells. **C**, densitometric quantitation of Nrf2 and ABCG2 relative protein expression in Du145-parent cells, Du145-LucshRNA, and Du145-Nrf2shRNA cells (arbitrary units).



**Figure 6.** Nrf2 activation induces ABCG2 expression in nontumorigenic lung epithelial cells and tumorigenic lung epithelial cells. **A**, upregulation of ABCG2 expression in cells expressing Keap1 shRNA. Total RNA from NuLi cells expressing Keap1 shRNA and luciferase shRNA was isolated, and expression of Keap1 and ABCG2 was determined by real-time RT-PCR. Downregulation of Keap1 expression led to an increase in Nrf2-dependent NQO1, GCLM, and ABCG2 expression. \*,  $P < 0.05$ , significant when compared with Luc-shRNA group. **B**, induction of ABCG2 expression in response to treatment with multiple oxidative stress-inducing agents. NuLi cells were treated with *tert*-butylhydroquinone (TBHQ; 20 nmol/L) and cigarette smoke condensate (CSC; 100 μg/mL) for 24 hours, and expression of NQO1, GCLM, and ABCG2 was measured by real-time RT-PCR. \*,  $P < 0.05$ , significant when compared with vehicle-treated sample. **C**, ectopic expression of Nrf2 in lung cancer cells upregulates Nrf2-dependent ABCG2 expression. Overexpression of Nrf2 cDNA in H23 lung cancer cells resulted in activation of Nrf2 target gene expression including ABCG2. \*,  $P < 0.05$ , significant when compared with the empty vector group. **D**, enforced expression of Nrf2 cDNA in H23 cells increases the proportion of SP cells.

*cis*-elements are located at the 5' promoter region of ABCG2 gene in normal SP-positive cells (44–46).

Decreasing the expression of the redox master regulator Nrf2 potentially affects cellular protection against xenobiotics and drugs (17). The attenuation in ABCG2 expression in Nrf2-deficient lung cancer cells leads to chemosensitization (22). Cancer cells with reduced Nrf2 activity and a parallel decline in ABCG2 expression displayed enhanced sensitivity to mitoxantrone and topotecan. However, besides reduced ABCG2 levels, alternate mechanisms such as decrease in glutathione level or/and reduced drug detoxification may also be involved in mitoxantrone and topotecan sensitization of Nrf2-abrogated lung cancer cells. We recently reported that Nrf2 inhibition in lung cancer cells results in enhanced intracellular accumulation of carboplatin and etoposide, and thus, enhanced carboplatin- and etoposide-induced cell death (21, 22). Interestingly, no significant difference in Nrf2-dependent sensitivity to another drug, methotrexate, was observed (data not shown). These findings imply that reversal of drug resistance by mitigating Nrf2-dependent drug detoxification genes is specific and relies on different Nrf2 downstream executors in the context of different chemotherapeutic agents. One seemingly obvious advantage of targeting the Nrf2 molecule, to increase sensitivity to chemotherapeutic drugs under different scenarios, is that electrophile drug detoxification pathways involved in detoxification of a wide spectrum of chemotherapy drugs are tackled at the same time, thus reducing the possibility of alternative or redundant detoxification pathway activation in targeting only one or few detoxification and/or drug pump pathways/molecules alone.

## References

- Jemal A, Siegel R, Ward E, et al. Cancer statistics, 2008. *CA Cancer J Clin* 2008;58:71–96.
- Wulf GG, Wang RY, Kuehne I, et al. A leukemic stem cell with intrinsic drug efflux capacity in acute myeloid leukemia. *Blood* 2001;98:1166–73.
- Hirschmann-Jax C, Foster AE, Wulf GG, et al. A distinct "side population" of cells with high drug efflux capacity in human tumor cells. *Proc Natl Acad Sci U S A* 2004;101:14228–33.
- O'Brien CA, Pollett A, Gallinger S, Dick JE. A human colon cancer cell capable of initiating tumour growth in immunodeficient mice. *Nature* 2007;445:106–10.
- Dylla SJ, Bevilgia L, Park IK, et al. Colorectal cancer stem cells are enriched in xenogeneic tumors following chemotherapy. *PLoS ONE* 2008;3:e2428.
- Zhou S, Schuetz JD, Bunting KD, et al. The ABC transporter Bcrp1/ABCG2 is expressed in a wide variety of stem cells and is a molecular determinant of the side-population phenotype. *Nat Med* 2001;7:1028–34.
- Feuring-Buske M, Hogge DE. Hoechst 33342 efflux identifies a subpopulation of cytogenetically normal CD34(+)CD38(–) progenitor cells from patients with acute myeloid leukemia. *Blood* 2001;97:3882–9.
- Kondo T, Setoguchi T, Taga T. Persistence of a small subpopulation of cancer stem-like cells in the C6 glioma cell line. *Proc Natl Acad Sci U S A* 2004;101:781–6.
- Doyle LA, Yang W, Abruzzo LV, et al. A multidrug resistance trans-

porter from human MCF-7 breast cancer cells. *Proc Natl Acad Sci U S A* 1998;95:15665–70.

In conclusion, the loss of KEAP1 function and aberrant activation of Nrf2-dependent pathways are frequently detected in lung cancer and other solid tumors (17, 21, 36, 37). Upregulated Nrf2 activity in cancer cells/tissues potentially stimulates ABCG2 expression and promotes the multidrug resistance phenotype. Because ABCG2 along with other cytoprotective genes are upregulated in cancer stem cells and protect against stress, it remains to be determined whether Nrf2 and its downstream signaling is amplified in cancer stem cells and whether targeting Nrf2 activity in cancer stem cells can be an effective strategy for overcoming multidrug resistance.

## Disclosure of Potential Conflicts of Interest

S. Biswal: a pending Patent Cooperation Treaty application.

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- porter from human MCF-7 breast cancer cells. *Proc Natl Acad Sci U S A* 1998;95:15665–70.
- Sung JM, Cho HJ, Yi H, et al. Characterization of a stem cell population in lung cancer A549 cells. *Biochem Biophys Res Commun* 2008;371:163–7.
- Scharenberg CW, Harkey MA, Torok-Storb B. The ABCG2 transporter is an efficient Hoechst 33342 efflux pump and is preferentially expressed by immature human hematopoietic progenitors. *Blood* 2002;99:507–12.
- Kensler TW, Wakabayashi N, Biswal S. Cell survival responses to environmental stresses via the Keap1-2-ARE pathway. *Annu Rev Pharmacol Toxicol* 2007;47:89–116.
- Rangasamy T, Cho CY, Thimmulappa RK, et al. Genetic ablation of Nrf2 enhances susceptibility to cigarette smoke-induced emphysema in mice. *J Clin Invest* 2004;114:1248–59.
- Morito N, Yoh K, Itoh K, et al. Nrf2 regulates the sensitivity of death receptor signals by affecting intracellular glutathione levels. *Oncogene* 2003;22:9275–81.
- Kobayashi A, Kang MI, Okawa H, et al. Oxidative stress sensor Keap1 functions as an adaptor for Cul3-based E3 ligase to regulate proteasomal degradation of Nrf2. *Mol Cell Biol* 2004;24:7130–9.
- Zhang DD, Lo SC, Cross JV, Templeton DJ, Hannink M. Keap1 is a redox-regulated substrate adaptor protein for a Cul3-dependent ubiquitin ligase complex. *Mol Cell Biol* 2004;24:10941–53.
- Singh A, Misra V, Thimmulappa RK, et al. Dysfunctional KEAP1-2 interaction in non-small-cell lung cancer. *PLoS Med* 2006;3:e420.

18. Shibata T, Ohta T, Tong KI, et al. Cancer related mutations in NRF2 impair its recognition by Keap1-3 E3 ligase and promote malignancy. *Proc Natl Acad Sci U S A* 2008;105:13568–73.
19. Padmanabhan B, Tong KI, Ohta T, et al. Structural basis for defects of Keap1 activity provoked by its point mutations in lung cancer. *Mol Cell* 2006;21:689–700.
20. Ohta T, Iijima K, Miyamoto M, et al. Loss of Keap1 function activates Nrf2 and provides advantages for lung cancer cell growth. *Cancer Res* 2008;68:1303–9.
21. Zhang P, Singh A, Yegnasubramanian, et al. Loss of Keap1 function in prostate cancer cells causes chemoresistance and radioresistance and promotes tumor growth. *Mol Cancer Ther* 2010;9:336–46.
22. Singh A, Boldin-Adamsky S, Thimmulappa RK, et al. RNAi-mediated silencing of nuclear factor erythroid-2-related factor 2 gene expression in non-small cell lung cancer inhibits tumor growth and increases efficacy of chemotherapy. *Cancer Res* 2008;68:7975–84.
23. Maliepaard M, van Gastelen MA, de Jong LA, et al. Overexpression of the BCRP/MXR/ABCP gene in a topotecan-selected ovarian tumor cell line. *Cancer Res* 1999;59:4559–63.
24. Jigorel E, Le Vee M, Boursier-Neyret C, Parmentier Y, Fardel O. Differential regulation of sinusoidal and canalicular hepatic drug transporter expression by xenobiotics activating drug-sensing receptors in primary human hepatocytes. *Drug Metab Dispos* 2006;34:1756–63.
25. Adachi T, Nakagawa H, Chung I, et al. Nrf2-dependent and -independent induction of ABC transporters ABCC1, ABCC2, and ABCG2 in HepG2 cells under oxidative stress. *J Exp Ther Oncol* 2007;6:335–48.
26. Baker AF, Landowski T, Dorr R, et al. The antitumor agent imexon activates antioxidant gene expression: evidence for an oxidative stress response. *Clin Cancer Res* 2007;13:3388–94.
27. Goodell MA, Brose K, Paradis G, Conner AS, Mulligan RC. Isolation and functional properties of murine hematopoietic stem cells that are replicating *in vivo*. *J Exp Med* 1996;183:1797–806.
28. Harvey CJ, Thimmulappa RK, Singh A, et al. Nrf2-regulated glutathione recycling independent of biosynthesis is critical for cell survival during oxidative stress. *Free Radic Biol Med* 2009;46:443–53.
29. Ivanova NB, Dimos JT, Schaniel C, et al. A stem cell molecular signature. *Science* 2002;298:601–4.
30. Chaudhary PM, Roninson IB. Expression and activity of P-glycoprotein, a multidrug efflux pump, in human hematopoietic stem cells. *Cell* 1991;66:85–94.
31. Madhavan L, Ourednik V, Ourednik J. Increased “vigilance” of antioxidant mechanisms in neural stem cells potentiates their capability to resist oxidative stress. *Stem Cells* 2006;24:2110–9.
32. Itoh K, Chiba T, Takahashi S, et al. An Nrf2/small Maf heterodimer mediates the induction of phase II detoxifying enzyme genes through antioxidant response elements. *Biochem Biophys Res Commun* 1997;236:313–22.
33. Ramos-Gomez M, Kwak MK, Dolan PM, et al. Sensitivity to carcinogenesis is increased and chemoprotective efficacy of enzyme inducers is lost in nrf2 transcription factor-deficient mice. *Proc Natl Acad Sci U S A* 2001;98:3410–5.
34. Kwak MK, Kensler TW, Casero RA, Jr. Induction of phase 2 enzymes by serum oxidized polyamines through activation of Nrf2: effect of the polyamine metabolite acrolein. *Biochem Biophys Res Commun* 2003;305:662–70.
35. Hayes JD, McMahon M. NRF2 and KEAP1 mutations: permanent activation of an adaptive response in cancer. *Trends Biochem Sci* 2009;34:176–88.
36. Sjoblom T, Jones S, Wood LD, et al. The consensus coding sequences of human breast and colorectal cancers. *Science* 2006;314:268–74.
37. Shibata T, Kokubu A, Gotoh M, et al. Genetic alteration of Keap1 confers constitutive Nrf2 activation and resistance to chemotherapy in gallbladder cancer. *Gastroenterology* 2008;135:1358–68, 68 e1–4.
38. Hayashi A, Suzuki H, Itoh K, Yamamoto M, Sugiyama Y. Transcription factor Nrf2 is required for the constitutive and inducible expression of multidrug resistance-associated protein 1 in mouse embryo fibroblasts. *Biochem Biophys Res Commun* 2003;310:824–9.
39. Kim YJ, Ahn JY, Liang P, et al. Human prx1 gene is a target of Nrf2 and is up-regulated by hypoxia/reoxygenation: implication to tumor biology. *Cancer Res* 2007;67:546–54.
40. Lee TD, Yang H, Whang J, Lu SC. Cloning and characterization of the human glutathione synthetase 5'-flanking region. *Biochem J* 2005;390:521–8.
41. Nguyen T, Sherratt PJ, Pickett CB. Regulatory mechanisms controlling gene expression mediated by the antioxidant response element. *Annu Rev Pharmacol Toxicol* 2003;43:233–60.
42. Vollrath V, Wielandt AM, Iruretagoyena M, Chianale J. Role of Nrf2 in the regulation of the Mrp2 (ABCC2) gene. *Biochem J* 2006;395:599–609.
43. Reisman SA, Yeager RL, Yamamoto M, Klaassen CD. Increased Nrf2 activation in livers from Keap1-knockdown mice increases expression of cytoprotective genes that detoxify electrophiles more than those that detoxify reactive oxygen species. *Toxicol Sci* 2009;108:35–47.
44. Ee PL, Kamalakaran S, Tonetti D, et al. Identification of a novel estrogen response element in the breast cancer resistance protein (ABCG2) gene. *Cancer Res* 2004;64:1247–51.
45. Krishnamurthy P, Ross DD, Nakanishi T, et al. The stem cell marker Bcrp/ABCG2 enhances hypoxic cell survival through interactions with heme. *J Biol Chem* 2004;279:24218–25.
46. Szatmari I, Vamosi G, Brazda P, et al. Peroxisome proliferator-activated receptor gamma-regulated ABCG2 expression confers cytoprotection to human dendritic cells. *J Biol Chem* 2006;281:23812–23.