Nuclear Factor (Erythroid-Derived 2)–Like 2 Regulates Drug Resistance in Pancreatic Cancer Cells

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Objective: To investigate the molecular basis of drug resistance in pancreatic cancer.

Methods: The expression of nuclear factor (erythroid-derived 2)–like 2 (Nrf2) levels in pancreatic cancer tissues and cell lines was analyzed. Clinical relevance between Nrf2 activation and drug resistance was demonstrated by measuring cell viability after *Nrf2* and adenosine 5′-triphosphate–binding cassette, subfamily G member 2 (*ABCG2*) regulation by overexpression or knock-down of these genes. Activity of *ABCG2* was measured by Hoechst 33342 staining.

Results: Abnormally elevated Nrf2 protein levels were observed in pancreatic cancer tissues and cell lines relative to normal pancreatic tissues. Increasing Nrf2 protein levels either by overexpression of exogenous Nrf2 or by activating endogenous Nrf2 resulted in increased drug resistance. Conversely, a reduction in endogenous Nrf2 protein levels or inactivation of endogenous Nrf2 resulted in decreased drug resistance. These changes in drug resistance or sensitivity were also positively correlated to the expression levels of Nrf2 downstream genes. Similarly, the expression of *ABCG2* was correlated with drug resistance.

Conclusions: Because the intrinsic drug resistance of pancreatic cancers is, in part, due to abnormally elevated Nrf2 protein levels, further research on regulating Nrf2 activity may result in the development of novel pancreatic cancer therapies.

Key Words: Nrf2, drug resistance, pancreatic cancer

Abbreviations: Nrf2 - nuclear factor (erythroid-derived 2)–like 2, ABCG2 - adenosine 5′-triphosphate–binding cassette, subfamily G member 2, MRP - multidrug resistance protein

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Pancreatic cancer is an extremely life-threatening disease. In 2005, estimated deaths from pancreatic cancers (31,800) were approximately equal to its incidence (32,180) in the United States.[1] Among patients with a diagnosis of pancreatic cancer, only 15% to 20% can be treated with surgical resection, the only chance of a cure. The remaining 80% to 85% of the patients have locally advanced, unresectable, or metastatic disease.[2] Chemotherapy, radiation therapy, and their combination are used as treatment options for unresectable pancreatic cancers.[1] However, pancreatic cancer has been reported to be highly resistant to both radiation therapy and chemotherapy.[1,3] Because current therapeutic approaches are minimally effective, better understanding of the molecular basis of drug resistance in pancreatic cancers may lead to better treatments.

The innate drug resistance of many pancreatic cancers may have multiple causes. One known mechanism of drug resistance involves the adenosine 5′-triphosphate–binding cassette (ABC) transporter family of proteins (reviewed in Szakács et al[4]). The function of ABC transporters is to translocate solutes across cellular membranes.[5] Forty-eight ABC transporter genes, classified into 7 subfamilies, are encoded in the human genome.[5] The ABC subfamily B (ABCB) endows tumors with resistance against a wider variety of drug types than other ABC subfamilies.[4] For example, elevated ABCB1 expression is associated with doxorubicin and docetaxel resistance in mammary gland cancer in the conditional double (BRCA1 plus TP53) knockout mouse model.[6] In addition, transfection of ABCB4 and ABCB11 into hepatocyte and insect cells caused increased chemoresistance.[7,8]

Several ABC transporter genes are transcriptionally regulated by nuclear factor (erythroid-derived 2)–like 2 (Nrf2) protein.[9,10] The protein Nrf2, which associates with Kelch-like ECH (erythroid cell–derived protein with CNC homology (ECH)–associated protein 1 (Keap1) in the cytoplasm, is known to be a principal transcription factor of intracellular antioxidants and phase II detoxification enzymes, thus playing a key role in cellular responses to oxidative stress.[11–13] Under oxidative stress, Nrf2 is activated through hyperphosphorylation, then it translocates into nucleus, where it binds the antioxidant response element (ARE) creating the Keap1-Nrf2-ARE pathway.[14] A number of antioxidant genes (eg, *NQO1*, *HO-1*, *SOD1*, and *GCLC*) and drug resistance genes (eg, some of *ABCC* family and *ABCG2*) are reported to be controlled by Nrf2.[11]

In lung cancer, recent studies have demonstrated that the activation of Nrf2 results in enhanced resistance against chemotherapeutic agents and that the down-regulation of Nrf2 renders these cells more susceptible to therapy.[15,16] Furthermore, protein levels of *HO-1*, a Nrf2 target gene, was suppressed by Nrf2-specific small interfering RNA (siRNA), resulting in enhanced chemosensitivity in lung cancer.[17] Furthermore, elevated levels of *ABCC1*, 2, and 3 (multidrug resistance proteins [MRPs] 1, 2, and 3), which are downstream targets of Nrf2, were correlated with increased drug resistance in these studies.[18] In this study,

we analyzed Nrf2 expression levels in pancreatic cancer tissue and cell lines and identified the role of Nrf2 in drug resistance of these tissues.

MATERIALS AND METHODS

Cell Culture

PANC-1 was cultured in Dulbecco modified Eagle medium supplemented with 5% fetal bovine serum, 100-U/mL penicillin, and 100-μg/mL streptomycin. AsPC-1, Capan-1, and Colo-357 were cultured in Roswell Park Memorial Institute 1640 media supplemented with 100-U/mL penicillin, 100-μg/mL streptomycin, and fetal bovine serum (20% for AsPC-1, 15% for Capan-1, and 10% for Colo-357) with 1% sodium pyruvate. All cell culture reagents were purchased from BioWhittaker, Inc (Walkersville, Md). All pancreatic cancer cell lines were obtained from the Tissue Culture Shared Resource of Georgetown University Medical Center (GUMC; Washington, DC). Human pancreatic duct epithelial (HPDE) cells, HPDE6-C7 and HPDE6-C11 from Dr Tsao,[19] were cultured in keratinocyte serum-free medium supplemented by epidermal growth factor and bovine pituitary extract (LifeTechnologies, Inc, Grand Island, NY).

Immunohistochemical Staining of Nrf2 Protein

Immunohistochemistry was performed to detect the expression of Nrf2 using rabbit polyclonal anti-Nrf2 antibody (Santa Cruz Biotechnology Inc, Santa Cruz, Calif). Immunohistochemical staining for 5 normal and 20 pancreatic ductal adenocarcinoma tissues was carried out on 4-μm-sectioned, formalin-fixed, and paraffin-embedded human pancreas tissues. Ten of 20 pancreatic carcinomas were offered from the Histopathology and Tissue Shared Resource of GUMC and the others from Keimyung University Hospital (Daegu, Korea). The sections were deparaffinized routinely in xylene and rehydrated through a series of graded alcohols to distilled water. The slides were covered with a 10-mmol/L sodium citrate buffer, pH 6.0 and then heated at 95°C for 10 minutes for antigen retrieval. Endogenous peroxidase activity was quenched by applying 0.3% hydrogen peroxide for 10 minutes, followed by washing with a buffer. The sections were then incubated for 10 minutes in 10% goat serum to reduce nonspecific antigen-antibody reaction. Previously 1:100 diluted Nrf2 antibody was applied and incubated on the slides for 2 hours at room temperature. After washing with an appropriate buffer, the reaction was visualized using a biotinylated secondary antibody (Vector Laboratories, Burlingame, Calif) at a 1:200 dilution and then with the Vectastain ABC reagent (Vector Laboratories). The slides were counterstained with Meyer hematoxylin, dehydrated through graded alcohols, placed in xylene, and sealed with coverslips.

Scoring Nrf2 Expression

The immunoreactivity of Nrf2 was evaluated by nuclear and cytoplasmic expressions with a semiquantitative method: (1) the percentage of distinct nuclear staining and (2) the intensity of cytoplasmic staining. The nuclear staining was scored as 1 (<5%), 2 (5–50%), and 3 (>50%) at the percentage of positive tumor cells on 10 high-power fields at ×400 magnification. No staining or a nuclei staining of less than 5% was considered negative. The cytoplasmic staining of tumor cells was divided into 4 categories by degree of staining intensity: no expression, mild or faint, moderate, and strong. In addition, it was regarded that a degree of intensity of less than faint or an uncertain cytoplasmic intensity was negative.

Western Blotting

Western blots were prepared and analyzed essentially as described previously.[20] Briefly, cells were cultured for 24 hours, lysed, and centrifuged. Proteins in the supernatants were separated in sodium dodecyl sulfate–polyacrylamide gel electrophoresis gels, which were then used for Western blots, which were analyzed using various primary antibodies: anti–Nrf2 rabbit polyclonal, anti–GCLC mouse monoclonal, anti–Lamin B mouse monoclonal, anti–β-tubulin mouse monoclonal, anti–actin mouse monoclonal (Santa Cruz Biotechnology Inc), and anti–ABCG2 mouse monoclonal (Abcam, Inc, Cambridge, Mass). Then, the membranes were incubated with horseradish peroxidase–conjugated secondary antibodies (Sigma, St Louis, Mo). Membrane-bound secondary antibodies were visualized by enhanced chemiluminescence detection kits (Santa Cruz Biotechnology Inc).

DNA Binding Assay

For measuring DNA binding activity, the TransAM Nrf2 assay kit (Active Motif, Carlsbad, Calif) was used. Nuclear extracts (10 μg) from AsPC-1, PANC-1, Colo-357, and HPDE6-C7 and HPDE6-C11 cells were incubated for 1 hour at room temperature in 96-well plates, on which DNA oligonucleotides containing ARE consensus binding sequences (5′-GTCACAGT GACTCAGCA GAATCTG-3′) were immobilized. After binding and multiple washing steps, anti-Nrf2 antibody was added for 1 hour at room temperature. The horseradish peroxidase antibody was used as a secondary antibody for colorimetric detection of ARE-bound Nrf2 at 450 nm for 5 minutes with a reference wavelength at 655 nm.

Transfection With DNA and siRNA

For transient expression experiments, cells were transfected with expression vectors for Flag-Nrf2, a dominant-negative mutant of Nrf2 (DN-Nrf2), ABCG2, and pCDNA3 (Invitrogen, Carlsbad, Calif) as a control. For Nrf2 knock-down, exponentially proliferating cells were transfected with chemically synthesized control siRNA (5′-gacgagcggcacgugcacauu-3′) or Nrf2-specific siRNA (5′-gaguaugagcuggaaaaacuu-3′), both purchased from Dharmacon, Inc (Lafayette, Colo). DNA and siRNA transfections using Lipofectamine 2000 or Lipofectamine Plus (Invitrogen), respectively, were performed as described in Bae et al.[20]

Cell Viability Assays

Cell viability was measured by 3-(4,5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT) assays as previously described.[20] Briefly, cells were seeded in 96-well plates at densities of 3×10^4 cells per well, grown overnight, then treated with different concentrations of camptothecin, cisplatin, and phenethyl isothiocyanate (PEITC; Sigma) for 24 hours and then assayed for MTT dye reduction. Cell viability values were calculated relative to the control cells (100%) and expressed as mean ± SE of 3 independent experiments.

Quantitative Real-Time Reverse Transcriptase Polymerase Chain Reaction

Pancreatic cell line knock-down was done with Nrf2-specific siRNA or with a control, nonspecific siRNA, for 3 days. Then, total messenger RNAs (mRNAs) were extracted with RNAzol B (Tel-Test, Friendswood, Tex) and used as the template for reverse transcriptase (RT) using the SuperScript II Reverse Transcriptase (LifeTechnologies, Inc, Rockville, Md) according to the manufacturer's instructions. The RT primer sequences
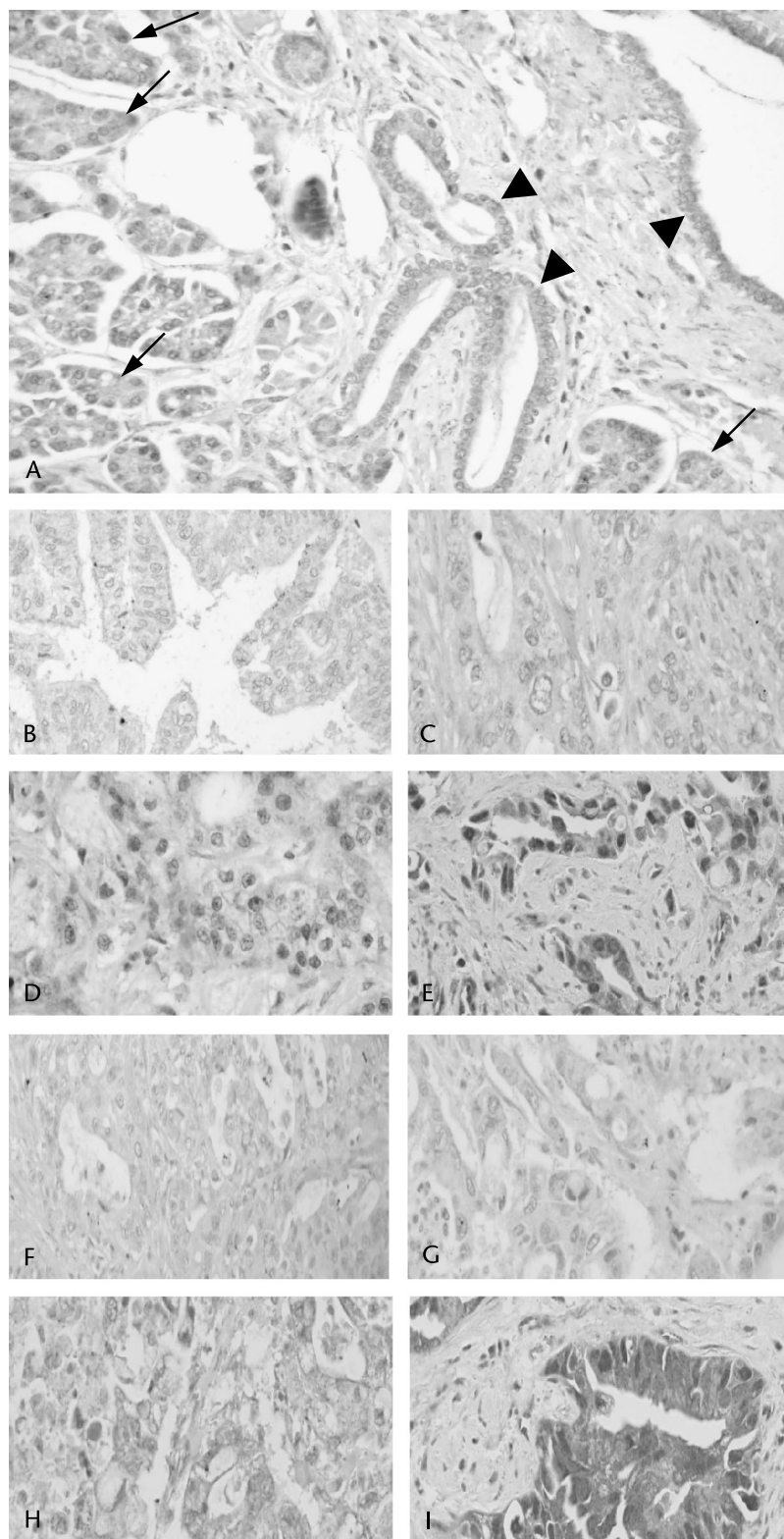
FIGURE 1. Differential expression of Nrf2 in human pancreatic tissues and cells. A, Expression of Nrf2 in normal pancreatic tissue, which is restricted to the cytoplasm of acini and ductal epithelium. Cytoplasmic expression of acini showing mild to moderate intensity (arrow). Cytoplasm of ductal cells revealing focal and faint expression (arrowhead). B–E, Nuclear immunoreactivity of Nrf2 in pancreatic carcinoma. Nuclear scoring is no expression (B), less than 5% (C), 5% to 50% (D), and more than 50% (E) of tumor cells. F–I, Cytoplasmic immunoreactivity of Nrf2 in pancreatic carcinoma. Cytoplasmic staining of tumor cells is divided into 4 categories by degree of intensity: no expression (F), mild or faint (G), moderate (H), and strong and diffuse intensity (I).

TABLE 1. Pancreatic Ductal Carcinomas Showing Nuclear and/or Cytoplasmic Immunoreactivity of Nrf2

Localization	Immunoreactivity	No. Cases
Nucleus	0	5
	<5% of tumor cells	1
	5–50%	8
	>50% of tumor cells	6
Cytoplasm	No expression	1
	Weak or faint	3
	Moderate	10
	Strong	6

were 5′-GCTATCCCTGTACGCCTCTG-3′ (forward) and 5′-ACATCTGCT GGAAGGTGGAC-3′ (reverse) for β-actin, 5′-AAACCACCCTGAAACGACAG-3′ (forward) and 5′-AGCGGCTTGAATGTTTGTCT-3′ (reverse) for Nrf2, 5′-CTGGGGAGTGATTTCTGC AT- 3′ (forward) and 5′-AGGAGGGGGCTTAAATCTCA-3′ (reverse) for GCLC, 5′-TTATCCGTGGTGTGTCTGGA-3′ (forward) and 5′-CCTGCTTGGAAG GCTCTATG-3′ (reverse) for ABCG2, 5′-ACCAAGACGTATCAGGTGGCC-3′ (forward) and 5′-CTGTCTGGGCATCCAGGAT-3′ (reverse) for MRP1, 5′-TGAA AGGCTACAAGCGTCCT-3′ (forward) and

5′-GAGCCGCAGTGAATAAGAGG-3′ (reverse) for MRP2, 5′-GAGGGATGAATTTGGCTTCA-3′ (forward) and 5′-CAGGG CTGCTGAGACACATA-3′ (reverse) for MRP4, and 5′-ACCC GTTGTTGCCATCTTAG-3′ (forward) and 5′-TCTGTCAAC AGCCACTGAGG-3′ (reverse) for MRP5. The real-time polymerase chain reactions (PCRs) were performed in 20-μL volumes that included 1 μL of RT products as template, 10 μL of SYBR Green PCR Master Mix (Applied Biosystems, Warrington, UK), and 20 pmol of each primer described previously. The PCR amplifications (40 cycles of 95°C for 30 seconds and 60°C for 60 seconds) were performed using the ABI 7900 (Applied Biosystems) in the Genomics and Epigenomics Shared Resource of GUMC. For each target gene, the mean Ct values calculated from triplicate PCR reactions were normalized to the mean Ct values for β-actin. These normalized values were then used to calculate a value expressing the extent of knock-down relative to the nonspecific control siRNA according to the formula $2^{-(mean \Delta\Delta Ct)}$.

Quantification of Hoechst-Positive Cells

The Hoechst staining method was used as described in Kim et al.[21] AsPC-1 cells transfected with DNA or siRNA were incubated in the presence of 10-μmol/L Hoechst 33342 (Sigma) for 90 minutes. After washing with phosphate-buffered saline, fluorescence-containing cells were counted under a microscope
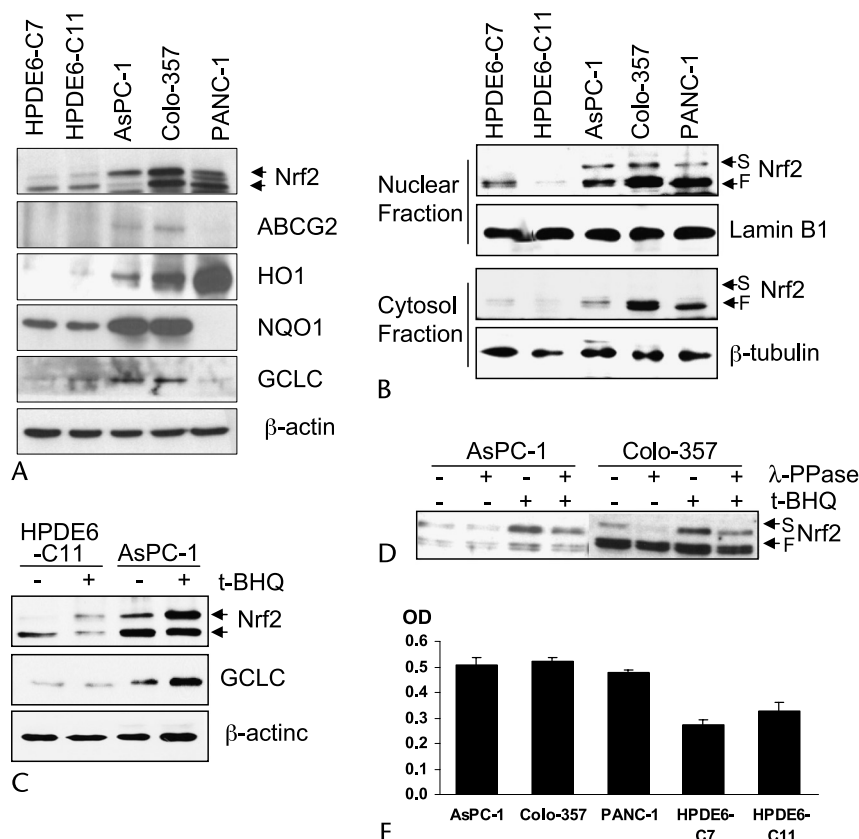

FIGURE 2. Human pancreatic cancer cell lines accumulate Nrf2. A, Western blot analysis of total lysates revealing that pancreatic tumor cell lines (AsPC-1, Colo-357, and PANC-1) have elevated level of Nrf2 and its target proteins when compared with normal pancreatic cell lines (HPDE6-C7 and HPDE6-C11). B, Western blot analysis of nuclear and cytosolic fractions for Nrf2 protein levels showing that 3 pancreatic cancer cell lines accumulate activated Nrf2 in the nuclei. C, *tert*-Butylhydroquinone treatments (100 μmol/L for 8 hours) increased the relative amounts of GCLC and the slower migrating Nrf2 in the HPDE6-C11 and AsPC-1 cell lines. D, λ-Phosphatase (λ-PPase) treatments (30 minutes) of whole cell lysates of t-BHQ–treated (100 μmol/L for 16 hours) cells selectively reduced amount of the slower migrating Nrf2 species. E, TransAM Nrf2 activity assay showing that DNA binding activities of nuclear Nrf2 are also elevated in pancreatic cancer cell lines.

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(Carl Zeiss, Berlin, Germany). At least 2000 Hoechst-positive (light blue) or Hoechst-negative (dark blue) cells were counted for each sample of 3 independent experiments.

RESULTS

Overexpression of Nrf2 in Human Pancreatic Cancer Tissue

The expression of Nrf2 in the normal pancreas samples revealed 2 types of immunoreactivity (Fig. 1A). The acini of normal pancreas tissue showed diffused cytoplasmic granular staining with mild or moderate intensity without nuclear expression, and normal pancreatic ductal epithelial cells demonstrated focal and weak cytoplasmic staining without nuclear expression in all of 5 cases of normal pancreas tissue (Fig. 1A). However, pancreatic ductal carcinoma showed either nuclear- or cytoplasmic-positive expression of Nrf2 (Figs. 1B–I). The scoring of Nrf2 immunoreactivity in nucleus (Figs. 1B–E) and cytoplasm (Figs. 1F–I) of the pancreatic ductal carcinomas was determined by 4 degrees of staining intensity. Seventy percent (14/20) of the tumor showed positive staining in nucleus, and 80% (16/20) revealed moderate or strong cytoplasmic intensity for Nrf2 (Table 1).

Overexpression of Nrf2 in Pancreatic Cancer Cell Line

We used Western blots to examine Nrf2 levels in various pancreatic cancer and normal cell lines. At least 2 types of Nrf2 (slower and faster migrating forms) were identified. In 2 normal pancreatic cell lines, HPDE6-C7 and HPDE6-C11, there is no detectable slower migrating form of Nrf2, whereas in all the pancreatic cancer cell lines, AsPC-1, Capan-1 (data not shown), Colo-357, and PANC-1, and in ABCG2 and HO-1, the slower migrating form of Nrf2 is present (Fig. 2A). To characterize the slower migrating band, we fractioned cells into nuclear and cytosolic. The slower migrating Nrf2 protein was detected only in the nuclear fractions of all the pancreatic cancer cell lines but not in the 2 normal cell lines (Fig. 2B). In our unpublished data, we observed increased accumulation of the slower migrating Nrf2 protein after treatments with an oxidizing compound, *tert*-butylhydroquinone (t-BHQ), which promotes nuclear localization and activation of Nrf2.[22] Both HPDE6-C11 and AsPC-1 cells showed increased amount of the slower migrating form of Nrf2 in accordance with t-BHQ induction, and this caused increased accumulation of Nrf2 target gene, *GCLC* (Fig. 2C). To determine whether the slower migrating nuclear Nrf2 is phosphorylated, we treated nuclear fractions of t-BHQ–treated cells with λ-phosphatase. The relative amount of the slower
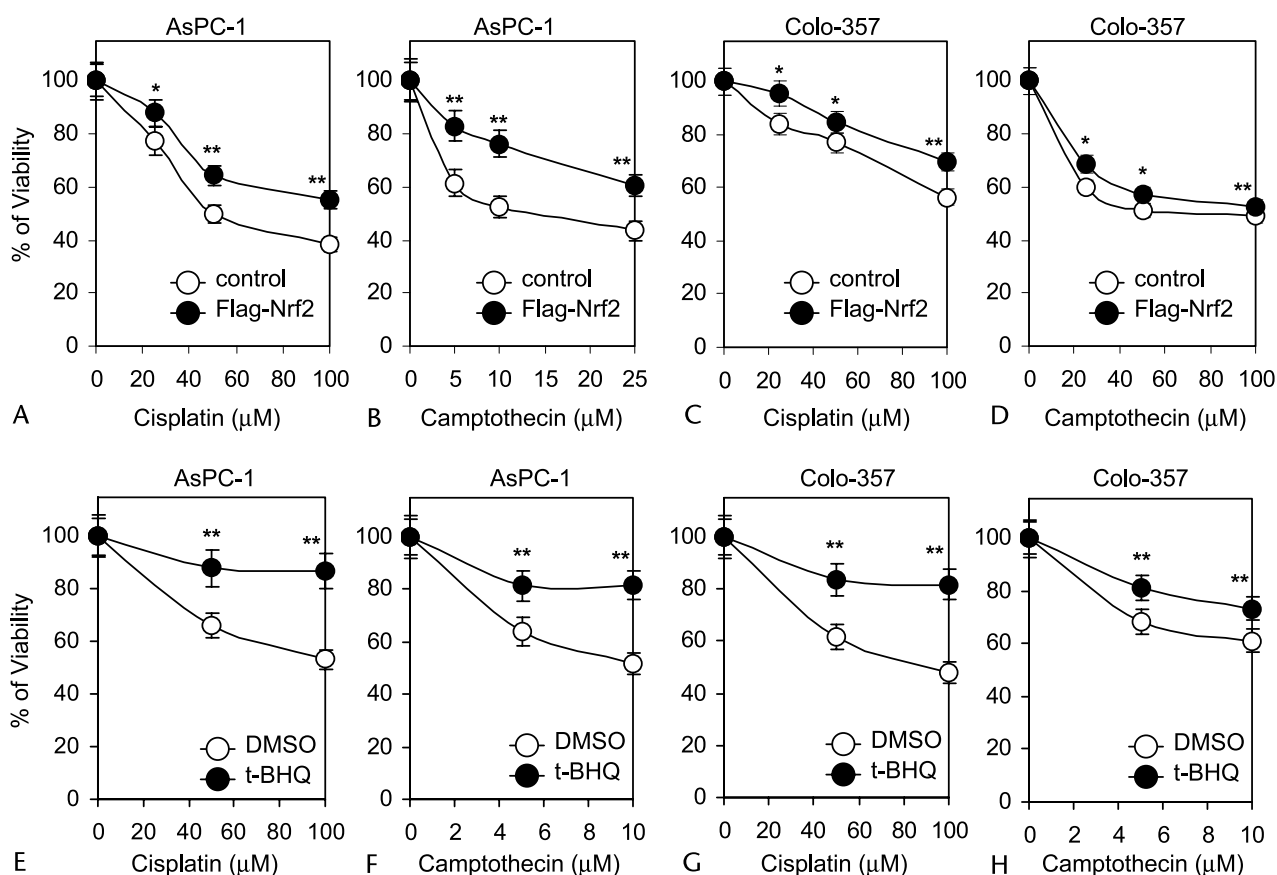

FIGURE 3. Overexpression of Nrf2 increased drug resistance in pancreatic cancer cells. A–D, AsPC-1 and Colo-357 transiently transfected with an Nrf2 expression vector (Flag-Nrf2) for 24 hours showing increased resistance to cisplatin (A and C) and camptothecin (B and D) as compared with those transfected with control vector. E–H, Preincubation with 100-μmol/L t-BHQ for 24 hours resulted in drug resistance of AsPC-1 and Colo-357 to cisplatin (E and G) and camptothecin (F and H). Indicated concentrations of cisplatin or camptothecin were treated for 24 hours. Cell survival relative to untreated cells (set at 100%) was measured by a standard MTT assay. *$P < 0.05$; **$P < 0.01$.

migrating form of Nrf2 in both AsPC-1 and Colo-357 cells was decreased by a 30-minute incubation with λ-phosphatase, although this did not result in a corresponding increase in the faster migrating Nrf2 species (Fig. 2D). The DNA binding assay showed that nuclear Nrf2 of pancreatic cancer cell lines has a higher ARE-binding activity than that of normal cell lines (Fig. 2E). Therefore, Nrf2 is overexpressed and transcriptionally active, which might cause overexpression of downstream genes in pancreatic cancer cell lines.

Increased Amount of Nrf2 Confers Drug Resistance to Cancer Cell Lines

To investigate the possible role of Nrf2 in drug resistance, we determined the changes of drug resistance levels after over-expression of Nrf2 in AsPC-1 and Colo-357 cells. Transfection of Flag-Nrf2 significantly increased the percentage of AsPC-1 and Colo-357 cells that remained viable after cisplatin and camptothecin treatments (Figs. 3A–D). The viability of Flag-Nrf2–transfected AsPC-1 cells increased after both 50-μmol/L (50%–64%) and 100-μmol/L (39%–55%) cisplatin treatments (Fig. 3A). The viability of Flag-Nrf2–transfected AsPC-1 cells increased in 10-μmol/L (52%–76%) and 25-μmol/L (43%–60%) camptothecin treatments (Fig. 3B). Similarly, overexpression of

Nrf2 in Colo-357 also significantly increased viability against cisplatin and camptothecin exposures (Figs. 3C, D). Transfection efficiencies, which were measured using green fluorescent protein–Nrf2 vector, were 34.4% in AsPC-1 and 16.5% in Colo-357 cells (data not shown).

As an independent way to test whether activation of Nrf2 is related to drug resistance, we pretreated AsPC-1 cells with 100-μmol/L t-BHQ, which increased the slower migrating Nrf2 protein (Fig. 2C). Cells pretreated with t-BHQ for 16 hours had higher drug resistance than dimethylsulfoxide (DMSO)-treated cells (Figs. 3E–H). Eighty-seven percent of AsPC-1 cells pretreated with t-BHQ survived after a 24-hour treatment with 100-μmol/L cisplatin, whereas only 53% of the control cells survived (Fig. 3E). Cells resistant to camptothecin are also increased when pretreated with t-BHQ. More than 80% of AsPC-1 cells survived after a 24-hour incubation with 10-μmol/L camptothecin, whereas less than 50% of DMSO-pretreated cells survived (Fig. 3F). Preincubation with 100-μmol/L t-BHQ showed similar effects on Colo-357 cells. Resistance of Colo-357 cells to cisplatin is also significantly elevated when pretreated with t-BHQ (Fig. 3G). Likewise, 73% of Colo-357 cells pretreated with t-BHQ survived after a 24-hour incubation with 10-μmol/L camptothecin, whereas only 61% survived after DMSO pretreatment (Fig. 3H).
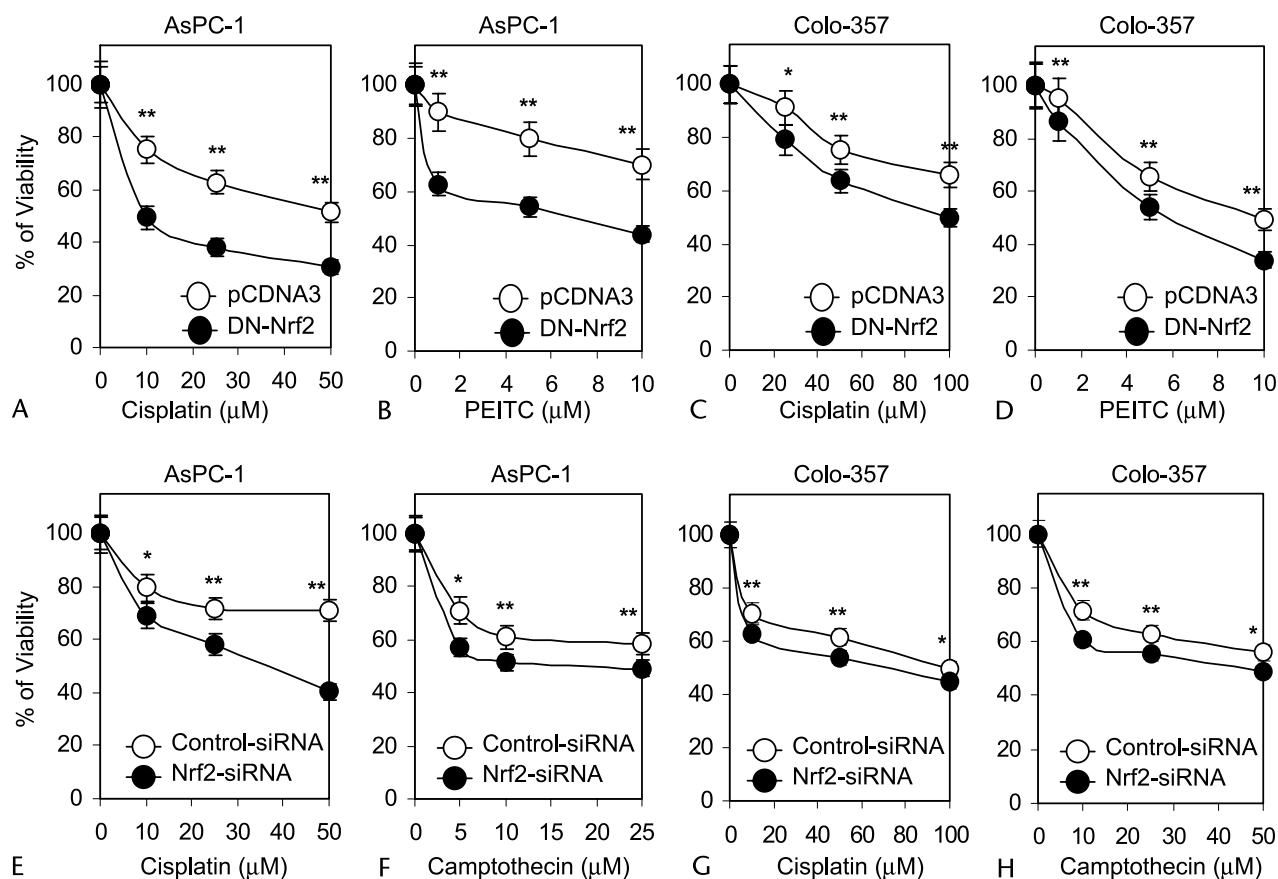

FIGURE 4. Inhibition of Nrf2 activity with DN-Nrf2 or knock-down with Nrf2 siRNA decreased drug resistance. A–D, Transfection with DN-Nrf2 increased sensitivity of AsPC-1 (A and B) and Colo-357 (C and D) to cisplatin (A and C) and camptothecin (B and D). E–H, Knock-down of Nrf2 with Nrf2-specific siRNA increased sensitivity of AsPC-1 (E and F) and Colo-357 (G and H) to various concentrations of both cisplatin (E and G) and camptothecin (F and H). Indicated concentrations of cisplatin or camptothecin were incubated for 24 hours. Cell survival relative to untreated cells (set at 100%) was measured by a standard MTT assay as described in Materials and Methods. *$P < 0.05$; **$P < 0.01$.

Reducing Nrf2 Level Renders Pancreatic Cell Lines More Sensitive to Drugs

Because elevated Nrf2 levels increased drug resistance in pancreatic cancer cell lines, we further investigated whether either reducing Nrf2 amounts or inhibiting Nrf2 activity would sensitize cells to chemotherapeutic drugs. First, we inhibited Nrf2's transcription regulation activity by transfection with DN-Nrf2. Transfection with DN-Nrf2 reduced the expression level of Nrf2 target proteins (HO-1 and GCLC) in AsPC-1 and Colo-357 (Supplementary Figure 1, Supplemental Digital Content 1, which shows the inhibition of Nrf2 activity by DN-Nrf2 vector, http://links.lww.com/MPA/A17). In AsPC-1 cells, Nrf2 inactivation renders cells more vulnerable to cisplatin and PEITC (Figs. 4A, B). The viability of AsPC-1 cells treated with 50-μmol/L cisplatin was significantly reduced from 51% to 30% in cells transfected with DN-Nrf2 compared with that with the control vector (Fig. 4A). The viability of AsPC-1 cells against 10-μmol/L PEITC was also reduced from 70% to 44% (Fig. 4B). Transfection with DN-Nrf2 reduced viability of Colo-357 against cisplatin and PEITC (Figs. 4C, D). To confirm whether reduced viability measured by MTT assay resulted from cell growth arrest or cell death, we directly counted cell numbers during cisplatin incubation for up to 72 hours (Supplementary Figure 2, Supplemental Digital Content 2, which shows the inhibition of Nrf2 activity with DN-Nrf2 that reduced cell numbers in AsPC-1 and Colo-357, http://links.lww.com/MPA/A18). The cell numbers of AsPC-1 and Colo-357 transfected with DN-Nrf2, respectively, reduced to 23% and 20% after incubation with 100-μmol/L cisplatin for 72 hours, whereas control DMSO-treated cells increased to 195% and 170%. There was less than 5% difference between the control vector and the DN-Nrf2–transfected cells when incubated with DMSO. However, incubation with 100-μmol/L cisplatin increased the gap of cell numbers up to 2-fold in both cells.

Reducing Nrf2 protein levels showed the same results. Knock-down of Nrf2 with Nrf2-specific siRNA reduced cisplatin and camptothecin resistance in AsPC-1 and Colo-357 (Figs. 4E–H). In AsPC-1 cells, transfection with Nrf2-specific siRNA dramatically reduced the viability from 71% to 40% in treatments with 50-μmol/L cisplatin (Fig. 4E). Similarly, the viability of Colo-357 against cisplatin and camptothecin was significantly decreased when Nrf2 levels were reduced (Figs. 4G, H). Therefore, we confirmed that reducing the total amount or the transcriptionally active form of Nrf2 rendered pancreatic cancer cells more vulnerable to chemotherapeutic drugs.

Knock-Down of Nrf2 Reduces mRNA Levels of MRPs

As reduced or inactivated Nrf2 increased drug sensitivity, we analyzed the effect of Nrf2 knock-down on the expression level of drug resistance genes. After knock-down of Nrf2 using Nrf2-specific siRNA, we measured mRNA levels of *GCLC*, *ABCG2*, and several *ABCC* family and *Nrf2* in AsPC-1, Colo-357, and PANC-1 cells using real-time PCR (Fig. 5A). The ratios of change (Nrf2 siRNA versus control siRNA) in these genes showed that all the mRNA expression levels of multidrug resistance genes and Nrf2 were significantly reduced after knock-down of Nrf2 in 3 pancreatic cell lines. The reduced mRNA levels of *Nrf2* and *GCLC* were also correlated with reduced protein levels (Fig. 5B).

ABCG2 Expression Correlates With Camptothecin Resistance

As ABCG2 is reported to have a function in camptothecin resistance[23] and we found that it is regulated by Nrf2 in tran-
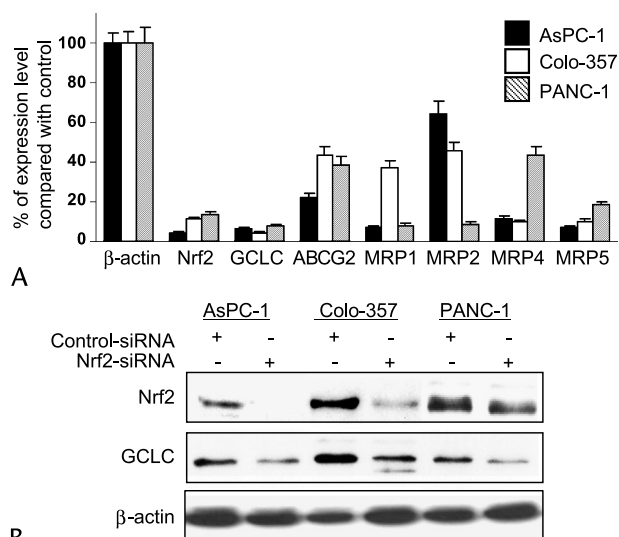

FIGURE 5. Knock-down of Nrf2 reduces expression of several multidrug resistance genes. A, The y-axis showing the percentage of mRNA expression levels of the Nrf2 and its target genes in the AsPC-1, Colo-357, and PANC-1 cells treated with Nrf2-siRNA, compared with those in the cells treated with control siRNA. Messenger RNA was quantified by real-time PCR described in Materials and Methods. B, Western blotting showing that Nrf2 knock-down reduces Nrf2 and its target gene product GCLC protein levels.

scription level (Fig. 5A), we analyzed the effect of Nrf2 and ABCG2 protein levels on Hoechst dye (a known substrate of ABCG2) efflux pumping using fluorescence microscopy (Figs. 6A–C). Transfection with expression vectors for either *Nrf2* (35%) or *ABCG2* (28%) gene significantly reduced the percentage of Hoechst-positive AsPC-1 cells compared with transfection of control DNA (60%; Fig. 6A). In contrast, Nrf2 knock-down increased the percentage of Hoechst-positive cell to 92% to 99% (Fig. 6B). The increased Hoechst-positive cells by knock-down of Nrf2 in turn decreased after overexpression of ABCG2 (Fig. 6C). As ABCG2 protein levels showed a positive correlation with Hoechst efflux activity, we analyzed the cell viability against camptothecin with changing expression levels of ABCG2. We reduced ABCG2 level through knock-down with Nrf2-specific siRNA then restored ABCG2 with transfection of ABCG2 DNA. In 50-μmol/L camptothecin treatment, AsPC-1 cells pretreated with control siRNA and ABCG2 DNA showed highest viability (67%) and cells pretreated with Nrf2 siRNA and control DNA showed lowest viability (43%; Fig. 6D). Changes of protein expression levels after knock-down of Nrf2 and overexpression of ABCG2 were confirmed by Western blotting (Fig. 6E).

DISCUSSION

In this report, we showed for the first time that Nrf2 protein levels are elevated in human pancreatic cancer tissue and cell lines and that it accumulates in pancreatic cell nuclei and/or cytoplasm of some patient samples. We also observed nuclear localized Nrf2, which is regarded to be the active form and directly induces downstream genes in pancreatic cancer cell lines. Although we did not show the mechanism of elevated Nrf2 expression in pancreatic cancer, we can postulate the relevance of oxidative stress. During oxidative stress, Nrf2 activity is increased and expressions of antioxidant genes are elevated as well.[14] If some pancreatic cancers are derived from chronic
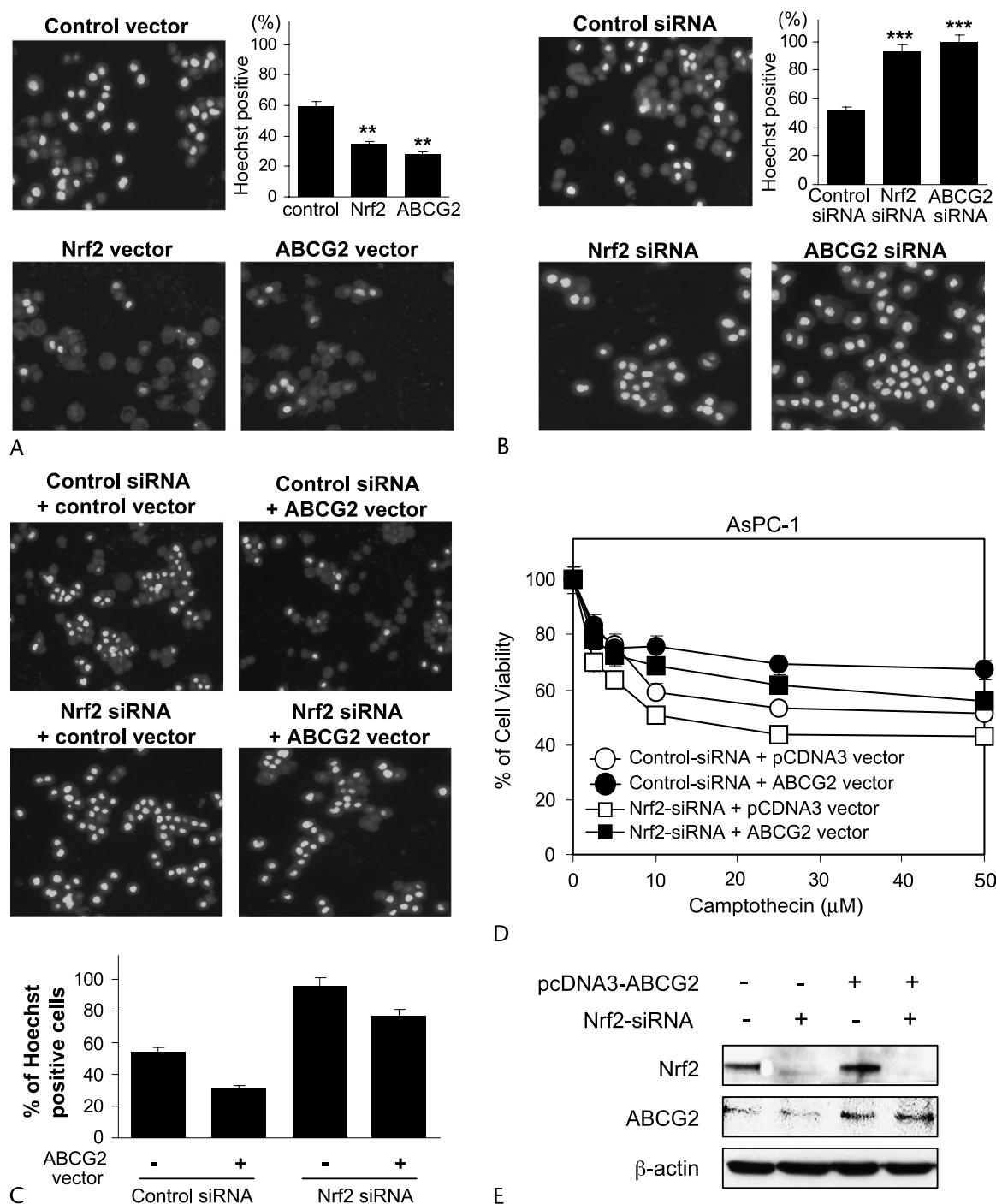
FIGURE 6. Knock-down of Nrf2 affects Hoechst 33342 efflux. A, Cell numbers of Hoechst 33342 positive decreased after overexpression of Nrf2 or ABCG2. B, Cell numbers of Hoechst 33342 positive increased after knock-down of Nrf2 or ABCG2. C, Restoring ABCG2 decreased the numbers of Hoechst 33342–positive cells in Nrf2 knock-down cells. D, Reduced cell viability on camptothecin by knock-down of Nrf2 is recovered by overexpression of ABCG2. E, Western blot showing changes of Nrf2 and ABCG2 protein levels after knock-down of Nrf2 and transient overexpression of ABCG2. **$P < 0.01$; ***$P < 0.001$.

pancreatitis, the tumor cells may have obtained their defense systems during survival of oxidative stress.[24]

Pancreatic cancer is classified as a lifestyle disease.[25] Pancreatic cancer risk is significantly associated with high energy, alcohol intake, and exposure to cigarette smoke.[26] Chronic pancreatitis is also associated with an increased risk of developing pancreatic cancer.[25,27] When protective mechanisms against tumorigenesis such as DNA repair, apoptosis, and immune-mediated destruction fail, chronic pancreatitis can develop into pancreatic cancer.[28] Furthermore, the pathogenesis of chronic pancreatitis shares characteristics with pancreatic cancer, as both have an elevated level of cytokines.[29] In acute and chronic

pancreatitis, oxidative stress is considered to play a key role in pathogenesis of inflammation. Oxidative stress triggers the activation of mitogen-activated protein kinase, nuclear factor-κB, and signal transducer and activator of transcription 3, resulting in the activation of oxidant-sensitive response signaling pathways in the acute pancreatitis model.[30] Likewise, reactive oxygen species (ROS) formation in chronic pancreatitis is directly linked to inflammatory pathogenesis.[31] In pancreatic cancer cells, oxidative stress also generates various ROS, including superoxide radicals, hydrogen peroxide, and hydroxyl radical.[32] These ROS along with cytokines and mediators of the inflammatory pathway have been linked to tumorigenesis by increasing cell cycling caused by loss of tumor suppressor function and by stimulating oncogene expression.[33]

We showed that drug resistance in pancreatic cancer results, in part, from elevated levels of Nrf2 and its target proteins. On the other hand, we also report that repression of Nrf2 activity sensitizes pancreatic cancer cells to chemotherapeutic drugs. In accordance with our data, Nrf2 knockout mouse embryonic fibroblasts were more sensitive to cisplatin treatment.[34] Therefore, reducing Nrf2 activity should arise as an important potential treatment of pancreatic cancer. Keap1 has been well characterized as a negative regulator of Nrf2. Keap1, as a component of E3 ubiquitin ligase, ubiquitinates Nrf2 and degrades it via a proteasome-mediated protein degradation pathway.[35] In lung cancer, the Nrf2-Keap1 regulation system has been reported to be impaired in many tissues and carcinoma cell lines.[36,37] Several protein kinases such as protein kinase C,[38] extracellular signal-regulated kinase,[39] p38 mitogen-activated protein kinase,[40] and phosphatidylinositol 3-kinase[41] were reported to be involved in signaling pathways during the activation of Nrf2-related antioxidant system. As a preliminary study, we observed that blocking several protein kinases using chemical inhibitors reduces Nrf2-related drug resistance (data not shown).

Regarding drug resistance in breast cancer, *ABCG2* (*BCRP*) has been well characterized as a mitoxantrone resistant (*MXR*) gene.[42] In this study, we targeted ABCG2 transporter as a direct regulator of camptothecin resistance. Reducing ABCG2 expression level sensitized pancreatic cells to camptothecin, and transient overexpression of ABCG2 conferred drug resistance. Therefore, regulation of ABCG2 should also be considered in treating pancreatic cancer. Recently, the importance of ABCG2 has been reported along with the identification of "side population" cells in many cancers.[43] The side population is distinguished by their low Hoechst 33342 dye staining and their potency as stem cells.[44] As expression level of ABCG2 is elevated in side population cells, expression level of Nrf2 can also be elevated in some cancer stem cells. As Nrf2 by itself is involved in cell proliferation,[45] overexpression of Nrf2 may serve to benefit cancer stem cell. Therefore, further research on regulating Nrf2 activity may provide a novel therapeutic strategy to overcome drug resistance in pancreatic cancer.

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