

Nrf2 Enhances Cell Proliferation and Resistance to Anticancer Drugs in Human Lung Cancer

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Abstract Purpose: NF-E2-related factor 2 (Nrf2), a key transcription regulator for antioxidant and detoxification enzymes, is abundantly expressed in cancer cells. In this study, therefore, the role of Nrf2 in cancer cell proliferation and resistance to anticancer drugs was investigated.

Experimental Design: We used three human lung cancer cell lines with different degrees of Nrf2 activation: Nrf2 was highly activated in A549 cells, slightly activated in NCI-H292 cells, and not activated in LC-AI cells under unstimulated conditions.

Result: A549 cells showed higher resistance to cisplatin compared with NCI-H292 and LC-AI cells. The resistance to cisplatin was significantly inhibited in A549 but not in NCI-H292 or LC-AI cells by knockdown of Nrf2 with its specific small interfering RNA (Nrf2-siRNA). The cell proliferation was also most prominently inhibited in A549 cells by treatment with Nrf2-siRNA. In A549 cells, the expression of self-defense genes, such as antioxidant enzymes, phase II detoxifying enzymes, and drug efflux pumps, was significantly reduced by Nrf2-siRNA concomitant with a reduction of the cellular glutathione level. The degree of DNA crosslink and apoptosis after treatment with cisplatin was significantly elevated in A549 cells by Nrf2-siRNA. Knockdown of Nrf2 arrested the cell cycle at G₁ phase with a reduction of the phosphorylated form of retinoblastoma protein in A549 and NCI-H292 cells but not in LC-AI cells.

Conclusion: These results indicate that the Nrf2 system is essential for both cancer cell proliferation and resistance to anticancer drugs. Thus, Nrf2 might be a potential target to enhance the effect of anticancer drugs.

Lung cancer is the leading cause of cancer-related death in developed countries (1). Despite decades of intensive efforts to combat this disease, the prognosis of lung cancer remains unfavorable and is especially miserable in advanced non-small cell lung cancer (NSCLC) cases. One of the main causes of the poor outcome in NSCLC treatment is the innate resistance of NSCLC patients to anticancer drugs. The response rates of most regimens used in first-line chemotherapies are ~30% even after the introduction of third-generation drugs such as taxanes,

gemcitabine, and irinotecan (2). In addition, the acquired resistance that develops in response to repeated therapies is a great obstacle to cancer treatments (3). Accordingly, the control of such resistance is considered an urgent issue for the treatment of NSCLC. Several studies have reported that NSCLC expresses several self-defense genes that are involved in the protection against anticancer drugs, including phase II detoxifying enzyme genes, antioxidant genes, and drug efflux protein genes (4–7).

The transcription factor NF-E2-related factor 2 (Nrf2) is a cap “n” collar basic-leucine-zipper transcription factor originally identified as a pivotal factor for cell protection from oxidative and electrophilic insults (8). Under unstimulated conditions, Nrf2 is retained in the cytoplasm by the anchor protein Kelch-like ECH-associated protein-1 (Keap1) and is maintained at a reduced level by the Keap1-dependent ubiquitination and proteasomal degradation systems (9, 10). On exposure to oxidative or xenobiotic stress, Keap1-dependent ubiquitin ligase activity is inhibited and Nrf2 can translocate to the nucleus, where it forms a heterodimer with small Maf proteins and binds to a consensus sequence called the antioxidant response element. Nrf2 effector genes bearing antioxidant response element include a majority of antioxidant and phase II detoxifying enzymes (11). In addition to these enzymes, recent studies have shown that Nrf2 transactivates a wide variety of genes, including several ATP-dependent drug efflux pumps (12, 13). It has

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Translational Relevance

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also been reported that Nrf2 regulates the proliferation of pulmonary epithelial cells via modulating cellular glutathione levels (14). These findings suggest that activation of Nrf2 in cancer cells provides advantages for the cell proliferation and the survival from the exposure to anticancer drugs.

Indeed, activation of Nrf2 has been observed in both patients with lung cancer and lung cancer cell lines (15, 16). In these previous studies, functional mutation of the *Keap1* gene led to constitutive activation of Nrf2 and thus provided advantages for cancer cell growth and anticancer drug resistance. However, the effects of Nrf2 inactivation in lung cancer cells have not been fully understood. In the present study, therefore, to clarify the benefits of Nrf2 inhibition in terms of cancer cell proliferation and drug resistance, we silenced the expression of the *Nrf2* gene in three human lung cancer cell lines, A549, LC-AI, and NCI-H292, with a RNA interference (RNAi) technique. We then evaluated the alterations in cell proliferative potencies and susceptibility to the anticancer drugs.

Materials and Methods

Reagents. DMEM, *cis*-diamminedichloroplatinum (cisplatin; CDDP), ethidium bromide, DMSO, 5-fluorouracil (5-FU), and *N*-ac-

tyl-L-cysteine (NAC) were purchased from Sigma. Bleomycin was purchased from Nippon Kayaku. RPMI 1640, antibiotics, and fetal bovine serum were obtained from Life Technologies. Stealth RNAi directed against human Nrf2, RNAi negative control, Lipofectamine RNAi Max, and horseradish peroxidase-conjugated secondary antibodies were purchased from Invitrogen. Primary antibodies against Nrf2, lamin B, GAPDH, retinoblastoma protein (pRb), and p21 were purchased from Santa Cruz Biotechnology. Phospho-pRb antibody was obtained from Epitomics.

Cell lines and cell culture. Human lung cancer cell lines A549 (originated from adenocarcinoma) and LC-AI (squamous cell carcinoma) were obtained from Riken BioResource Center. NCI-H292 (mucoepidermoid cancer) was obtained from the American Type Culture Collection. A549 and LC-AI cells were maintained in DMEM with 10% fetal bovine serum. H292 cells were cultivated in RPMI 1640 with 10% fetal bovine serum.

Transfection of small interfering RNA. The small interfering RNAs (siRNA) directed against human Nrf2 (Nrf2-siRNA) or nontargeting negative control siRNA (NC-siRNA) were purchased from Invitrogen. Cancer cells were transfected with the Nrf2-siRNA or NC-siRNA with Lipofectamine RNAi Max according to the manufacturer's protocol. The final concentration of the siRNAs was 20 nmol/L.

Immunohistochemistry. Cancer cells were plated in 10 cm² dishes in which collagen-coated glass slides were tiled. After 24 h of cultivation, the cells were fixed and stained immunohistochemically using anti-Nrf2 antibody. Diaminobenzidine was used as a chromogen.

Cell proliferation analysis. After transfection of the siRNAs, viable cells were quantified every 24 h using a Cell Counting Kit-8 (Dojindo) according to the manufacturer's protocol.

Cytotoxicity analysis. Cells were plated in 24-well plates and incubated for a further 24 h. Then, cells were treated with CDDP, 5-FU, and bleomycin with or without simultaneous siRNA transfection. Viable cells were quantified 48 h after administration of anticancer drugs using Cell Counting Kit-8. Cell survival ratio was calculated by dividing absorbance at indicated drug concentration by that of 0 μmol/L after subtracting blank absorbance.

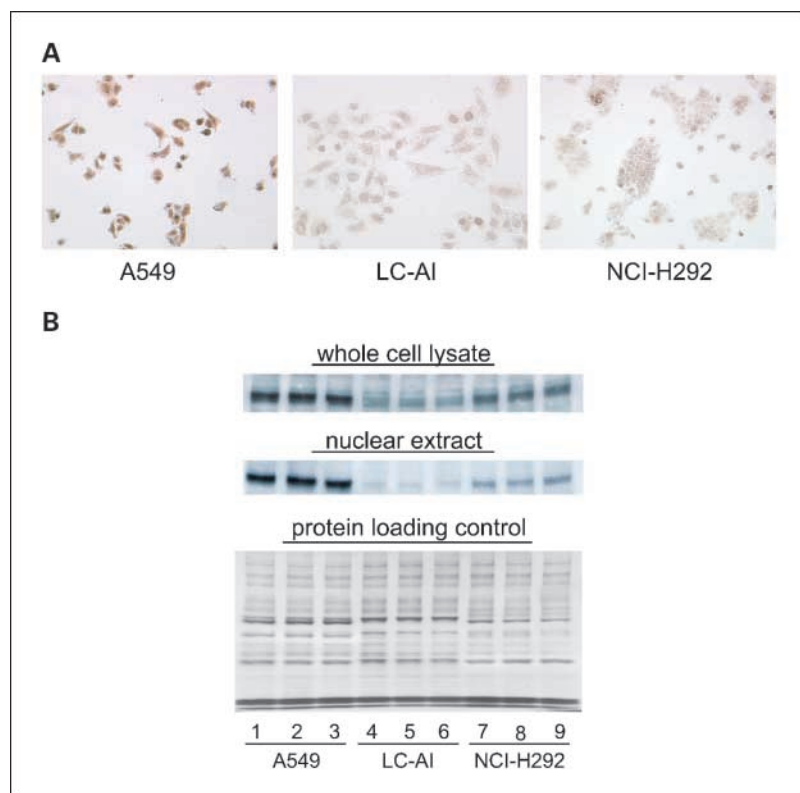


Fig. 1. Expression of Nrf2 in lung cancer cell lines. *A*, nuclear localization of Nrf2 in A549 cells (*left*), LC-AI cells (*middle*), and NCI-H292 cells (*right*) under unstimulated conditions. Immunohistochemistry was done using an antibody against Nrf2. *B*, Nrf2 protein levels in total cell lysates (*top*) or nuclear extracts (*middle*) of A549 cells (*lanes 1-3*), LC-AI cells (*lanes 4-6*), and NCI-H292 cells (*lanes 7-9*) under unstimulated conditions. An immunoblot analysis was done using an antibody against Nrf2. Coomassie blue-stained protein bands in each nuclear extract (*bottom*) were used as the protein loading control.

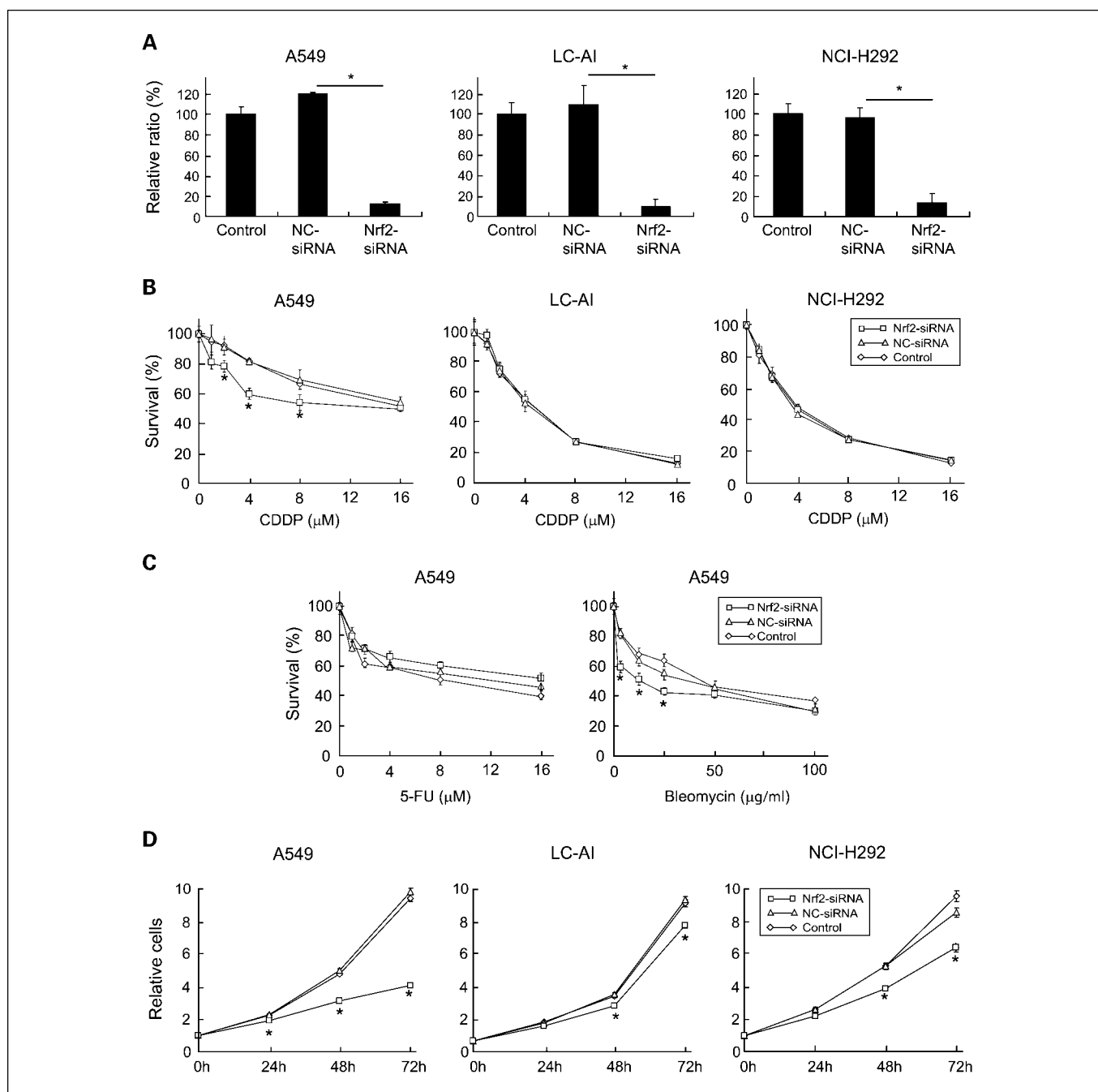


Fig. 2. Effects of Nrf2 knockdown on cancer cell proliferation and the resistance to CDDP. **A**, efficacy of siRNA against human Nrf2. Nrf2-siRNA or NC-siRNA was transfected into A549 cells (*left*), LC-AI cells (*middle*), and NCI-H292 cells (*right*). Expression of Nrf2 mRNA was analyzed by real-time PCR 24 h after the transfection. Untransfected cells were used as a control. *, $P < 0.001$, significantly different from NC-siRNA-transfected cells. **B**, cell survival following treatment with CDDP. A549 cells (*left*), LC-AI cells (*middle*), and NCI-H292 cells (*right*) were transfected with Nrf2 siRNA (*open square*) or NC siRNA (*open triangle*) and treated with CDDP. Cell survival was monitored by a Cell Counting Kit-8 48 h after the treatment. *, $P < 0.05$, significantly different from NC-siRNA-transfected cells. **C**, cell survival following treatment with 5-FU or bleomycin. A549 cells were transfected with Nrf2 siRNA (*open square*) or NC siRNA (*open triangle*) and treated with 5-FU (*left*) or bleomycin (*right*). Cell survival was monitored by a Cell Counting Kit-8 48 h after the treatment. *, $P < 0.05$, significantly different from NC-siRNA-transfected cells. **D**, effect of Nrf2 knockdown on cell proliferation. A549 cells (*left*), LC-AI cells (*middle*), and NCI-H292 cells (*right*) were transfected with Nrf2-siRNA (*open square*) or NC-siRNA (*open triangle*). Cell proliferation was monitored for 24 h by a Cell Counting Kit-8. *, $P < 0.05$, significantly different from NC-siRNA-transfected cells.

DNA interstrand crosslink analysis. DNA interstrand crosslink formed by CDDP was quantified 48 h after administration of CDDP using fluorescence assay with ethidium bromide as described previously (17).

Protein extraction and Western blotting. Nuclear fractions were prepared using a Nuclear Extraction Kit (Panomics) according to the manufacturer's protocol. To prepare whole-cell lysates, cells were lysed

with radioimmunoprecipitation assay buffer (10 mmol/L Tris, 1% Triton, 1% sodium deoxycholate, 0.1% SDS, 150 mmol/L NaCl, 5 mmol/L EDTA, 1 μg/mL aprotinin, 1 μg/mL leupeptin, 2 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L Na_2VO_4 , 1 mmol/L NaF). The protein concentration was determined by a BCA protein assay kit (Bio-Rad). Proteins in nuclear fractions and cell lysate were separated

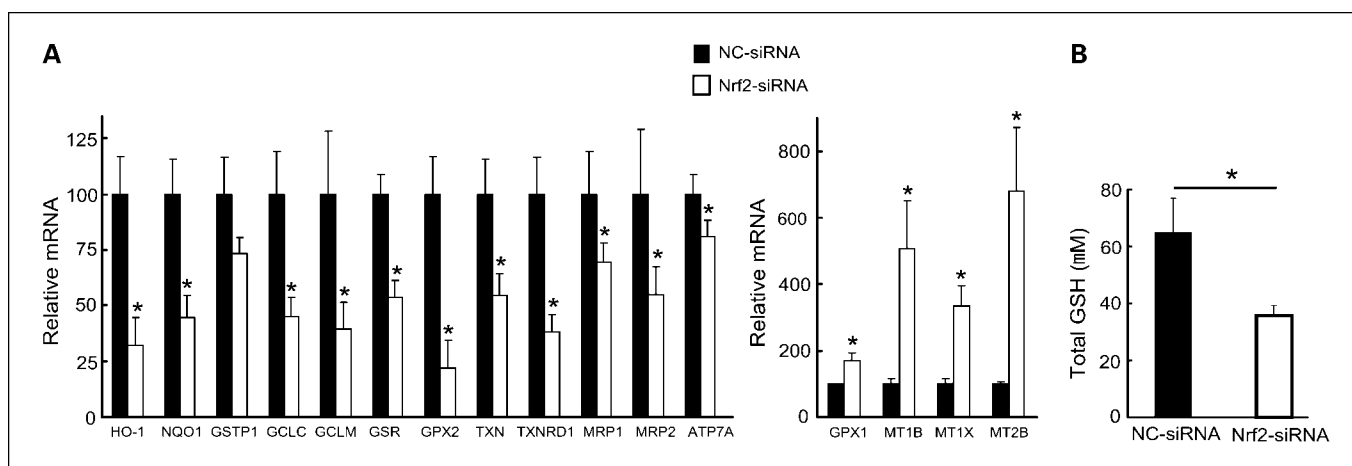


Fig. 3. Effects of Nrf2 knockdown on the expression of self-defense genes. **A**, Nrf2 knockdown in A549 cells altered the expression of various antioxidant enzymes, xenobiotic metabolism enzymes, and drug efflux pumps. A549 cells were transfected with Nrf2-siRNA (*open columns*) or NC-siRNA (*closed columns*). Expression of these mRNAs was analyzed by real-time PCR. *, $P < 0.05$, significantly different from NC-siRNA-transfected cells. **B**, total glutathione levels in A549 cells transfected with Nrf2-siRNA or NC-siRNA. *, $P < 0.05$, significantly different from NC-siRNA-transfected cells.

on 5% to 15% gradient SDS-polyacrylamide gels and transferred onto a polyvinylidene difluoride membrane. The membrane was stained immunochemically using anti-Nrf2, anti-phospho-pRb, and anti-p21 antibodies. Immunoreactive bands were detected using enhanced chemiluminescence Western blotting detection reagents (Amersham).

Total RNA extraction and real-time quantitative reverse transcription-PCR. Reagents and instruments not noted individually were obtained from Applied Biosystems. Total RNA was extracted using a RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. Reverse transcription-PCR was done using a SYBR Green PCR kit (Applied Biosystems), gene-specific primers, and an ABI 7700 thermal cycler. Primer sequences are available in the online supplement. The gene expression levels for each amplicon were calculated using the $\Delta\Delta C_T$ method (18) and normalized against GAPDH mRNA.

Total glutathione quantification. Intracellular total glutathione was quantified using a Total Glutathione Quantification Kit (Dojindo) according to the manufacturer's protocol.

Flow cytometry. All instruments and reagents were from Becton Dickinson Biosciences. In cell cycle analysis, cells stained with propidium iodide were counted and classified according to their DNA contents using Mod Fit Lt software. Apoptosis analysis was conducted with an Annexin V-FITC Apoptosis Detection Kit (Becton Dickinson) according to the manufacturer's protocol. A FACScalibur flow cytometer was used in both analyses.

Statistical analyses. All experiments were done at least three times. Data are expressed as mean \pm SE. Data were evaluated by ANOVA and Tukey's multiple comparison test. P values < 0.05 were considered to be significant.

Results

Degree of Nrf2 activation differed among the human lung cancer cell lines. We first evaluated the baseline activation of Nrf2 in three human cancer cell lines. To assess the activation of Nrf2, the nuclear accumulation of Nrf2 was detected by immunohistochemistry and Western blot analysis. Among the cell lines examined, the nuclei of A549 cells showed strong immunostaining for Nrf2 (Fig. 1A, *left*). On the other hand, the immunoreactivity to Nrf2 was faint in the nuclei of LC-AI and NCI-H292 cells (Fig. 1A, *middle and right*). Western blot analysis also revealed that Nrf2 was accumulated abundantly in the nuclear fraction of A549 cells (Fig. 1B). Although Nrf2 was expressed in the cytoplasm of NCI-H292

cells, only a few Nrf2 were translocated to the nuclei of these cells under the unstimulated conditions (Fig. 1B). Nrf2 immunoreactivity was not observed in LC-AI cells (Fig. 1B).

Nrf2 knockdown reduced cancer cell proliferation and anti-cancer drug resistance. To assess the effects of Nrf2 on cancer cell proliferation and anticancer drug resistance, the expression of Nrf2 was knocked down using a RNAi technique. We first evaluated the efficacy of siRNA against human Nrf2 (Nrf2-siRNA); the expression of Nrf2 mRNA was detected by real-time quantitative reverse transcription-PCR 24 h after transfection of either Nrf2-siRNA or NC-siRNA in A549, LC-AI, and NCI-H292 cells. The Nrf2 mRNA level was diminished by $\sim 10\%$ in all cell lines compared with the level in untransfected control cells (Fig. 2A). The Nrf2 mRNA level was not altered in any of the cell lines transfected with NC-siRNA (Fig. 2A). These results suggest that the Nrf2-siRNA used in the present study successfully knocks down Nrf2 mRNA in lung cancer cell lines.

We then examined the effects of Nrf2 on resistance to CDDP in the cancer cell lines. Although the cell viability decreased dose-dependently in all cell lines by treatment with CDDP, A549 cells were more resistant to CDDP compared with LC-AI and NCI-H292 cells (Fig. 2B). Nrf2 knockdown with Nrf2-siRNA significantly enhanced the susceptibility to CDDP in A549 cells (Fig. 2B). However, Nrf2-siRNA did not increase the sensitivity to CDDP in LC-AI and NCI-H292 cells (Fig. 2B). Treatment with NC-siRNA did not affect the sensitivity to CDDP in any of the cancer cell lines (Fig. 2B).

To clarify whether Nrf2 knockdown enhances the susceptibility to other type of anticancer drugs, A549 cells transfected with Nrf2-siRNA or NC-siRNA were treated with 5-FU and bleomycin. Treatment with Nrf2-siRNA did not increase the sensitivity to 5-FU (Fig. 2C, *left*). On the other hand, Nrf2 knockdown with Nrf2-siRNA significantly enhanced the susceptibility to bleomycin (Fig. 2C, *right*). Treatment with NC-siRNA did not affect the sensitivity to either 5-FU or bleomycin (Fig. 2C).

We further assessed the effects of Nrf2 on cancer cell proliferation. Knockdown of Nrf2 with Nrf2-siRNA potently inhibited the proliferation of A549 cells (Fig. 2D, *left*). However, the inhibitory effect of Nrf2-siRNA on the cell

proliferation was much lower in LC-AI and NCI-H292 cells than in A549 cells (Fig. 2D). The results indicated that the inhibitory effects of Nrf2 knockdown on cell proliferation and CDDP resistance paralleled the degree of constitutive activation of Nrf2 among those cells. Thus, Nrf2 may contribute to both cancer cell proliferation and anticancer drug resistance.

Nrf2 transactivated the expression of self-defense genes. Several studies have shown that the development of anticancer drug resistance is related to the expression of self-defense genes such as xenobiotic metabolism enzymes, antioxidants, and drug efflux pumps. We therefore assessed the effects of Nrf2 on the expression of various drug resistance-related genes in A549 cells. Nrf2 knockdown with Nrf2-siRNA significantly reduced the expression of antioxidant genes [heavy and light subunits of γ -glutamyl cysteine synthetase (GCLC and GCLM), heme oxygenase 1, glutathione reductase, and glutathione peroxidase 2], xenobiotic metabolism enzymes [NAD(P)H-quinone oxidoreductase 1, thioredoxin, and its related genes, including thioredoxin reductase 1], and ATP-dependent drug efflux pumps (multidrug resistance-associated proteins 1 and 2 and ATP7A; Fig. 3A). Interestingly, the expression of glutathione peroxidase 1 and metallothioneins, MT1B, MT1X, and MT2B, was significantly enhanced in A549 cells transfected with Nrf2-siRNA (Fig. 3A).

Because GCLC and GCLM are rate-limiting enzymes for glutathione biosynthesis, we then quantified total glutathione levels in A549 cells transfected with Nrf2-siRNA or NC-siRNA. In accordance with the down-regulation of synthetic enzymes, total glutathione levels were significantly lower in Nrf2-siRNA-transfected A549 cells than in NC-siRNA-transfected A549 cells (Fig. 3B).

Nrf2 knockdown augmented CDDP-induced DNA interstrand crosslink formation and apoptosis in A549 cells. Because Nrf2 knockdown with Nrf2 siRNA enhanced the susceptibility to CDDP, we next assessed the degree of CDDP-induced DNA crosslink formation and apoptosis in A549 cells transfected with Nrf2-siRNA or NC-siRNA. The degree of DNA crosslink after treatment with CDDP was much higher in A549 cells

transfected with Nrf2-siRNA than in those transfected with NC-siRNA (Fig. 4A). We also assessed the proportion of apoptosis after treatment with CDDP in A549 cells transfected with Nrf2-siRNA or NC-siRNA. The proportion of both early apoptotic cells that were positive for only Annexin V and late apoptotic cells that were positive for both Annexin V and propidium iodide was significantly increased in Nrf2-siRNA-transfected A549 cells compared with NC-siRNA-transfected A549 cells (Fig. 4B). These results indicate that Nrf2 knockdown enhanced CDDP-induced DNA interstrand crosslink formation and apoptosis in A549 cells.

Nrf2 regulated the cell cycles. Because Nrf2 knockdown with Nrf2-siRNA inhibited cancer cell proliferation, we assessed how Nrf2 affects the cell cycles in A549, LC-AI, and NCI-H292 cells. In NC-siRNA-transfected control A549 cells, the proportions of G₀-G₁, S, and G₂-M phase cells were $39.2 \pm 0.6\%$, $44.9 \pm 0.5\%$, and $15.9 \pm 0.2\%$, respectively, at 48 h after the culture (Fig. 5A, top). The proportion of G₀-G₁ phase cells, however, increased to $51.6 \pm 0.6\%$ in A549 cells transfected with Nrf2-siRNA at the same time (Fig. 5A, top). Similarly, the proportion of G₀-G₁ phase cells was increased to $27.7 \pm 3.8\%$ in NCI-H292 cells transfected with Nrf2-siRNA compared with $7.9 \pm 1.6\%$ in NC-siRNA-transfected control cells 48 h after the culture (Fig. 5A, bottom). The proportion of G₀-G₁ phase cells was not different between Nrf2-siRNA-transfected LC-AI cells and NC-siRNA-transfected LC-AI cells 48 h after the culture (Fig. 5A, middle). The results indicate that Nrf2 knockdown arrests the cell cycle in G₁ phase in A549 and NCI-H292 cells.

We therefore assessed the phosphorylation of pRb, a regulator of G₁ phase, in A549, LC-AI, and NCI-H292 cells transfected with Nrf2-siRNA or NC-siRNA. Under a serum-free condition in which the cell proliferation was stopped, only a very small amount of phosphorylated pRb was detected in all cells (Fig. 5B, top, 0h). Forty-eight hours after culture with serum, the phosphorylated form of pRb was elevated in all cancer cell lines transfected with NC-siRNA (Fig. 5B, top, NC). The phosphorylation of pRb was much inhibited in A549 and NCI-H292 cells transfected with Nrf2-siRNA at that time (Fig. 5B, top left and top right, Nrf2). However, Nrf2

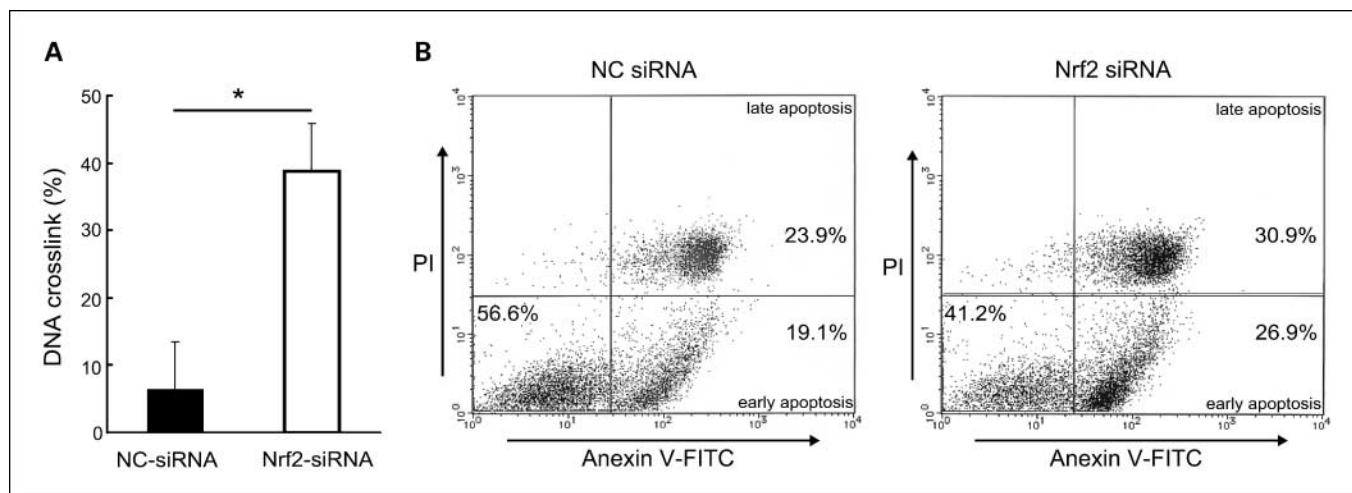


Fig. 4. Effects of Nrf2 knockdown on CDDP-induced cytotoxicity. **A**, degree of CDDP-induced DNA crosslinking in A549 cells transfected with Nrf2 siRNA or NC siRNA. *, $P < 0.05$, significantly different from NC-siRNA-transfected cells. **B**, degree of CDDP-induced apoptosis in A549 cells transfected with Nrf2 siRNA or NC siRNA. Apoptosed cells were detected by fluorescence-activated cell sorting using Annexin V and propidium iodide.

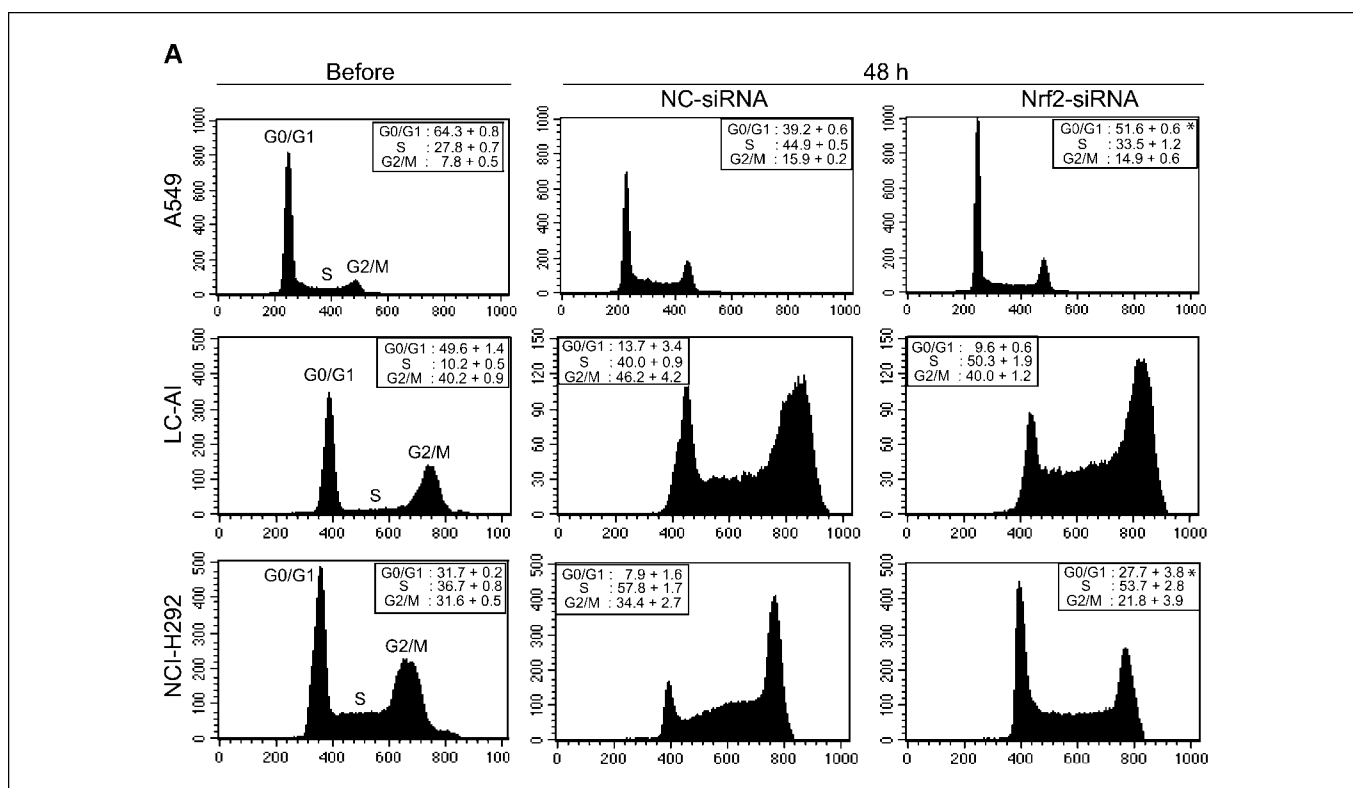


Fig. 5. Effects of Nrf2 knockdown on cell cycles. *A*, changes in cell cycle distribution in A549 cells (*top*), LC-AI cells (*middle*), and NCI-H292 cells (*bottom*) transfected with Nrf2-siRNA (*right*) or NC-siRNA (*middle*). Cell cycle distribution was monitored before (*left*) and 48 h after the transfection by fluorescence-activated cell sorting using propidium iodide. *, $P < 0.05$, significantly different from NC-siRNA-transfected cells.

knockdown did not affect the phosphorylation of pRb in LC-AI cells (Fig. 5B, *top middle*, Nrf2).

We further assessed the expression of p21, a downstream molecule of p53 that negatively regulates the phosphorylation of pRb, in A549, LC-AI, and NCI-H292 cells. Under the serum-free condition, a high level of p21 expression was observed in all cells (Fig. 5B, *bottom, 0h*). Forty-eight hours after culture with serum, the expression of p21 was reduced along with the proliferation in all NC-siRNA-transfected cells (Fig. 5B, *bottom, NC*). The expression of p21 was increased in A549 and NCI-H292 cells transfected with Nrf2-siRNA (Fig. 5B, *bottom left* and *bottom right*, Nrf2). The expression of p21 was not altered in LC-AI cells by transfection with Nrf2-siRNA (Fig. 5B, *bottom middle*, Nrf2). These results suggest that the expression of p21 protein is enhanced by inactivation of Nrf2 in A549 and NCI-H292 cells. Thus, Nrf2 might regulate the G₁-S transition by modulating the expression of p21 and the phosphorylation of pRb in these cells.

To clarify whether the alterations in the cell cycle by Nrf2 knockdown is due to the changes in cellular redox status, we treated Nrf2-siRNA-transfected A549 cells with NAC, an antioxidant and the precursor of glutathione. Although treatment with NAC reversed the expression of p21 in Nrf2-siRNA-transfected A549 cells to the level of NC-siRNA-transfected control (Fig. 5C, *bottom*), NAC did not change the phosphorylation of pRb in A549 cells transfected with Nrf2-siRNA (Fig. 5C, *top*). Treatment with NAC did not reverse Nrf2-siRNA-induced inhibition of cell proliferation in A549 cells (Fig. 5D).

Discussion

In the present study, we showed that the suppression of Nrf2 activity by Nrf2-siRNA inhibited the cellular proliferation and reduced the resistance to the anticancer drug CDDP in human lung cancer cells. Moreover, the inhibitory effects of Nrf2-siRNA were observed most prominently in A549 cells, in which Nrf2 was strongly activated, whereas the inhibitory effects were scarcely observed in LC-AI cells, which do not express Nrf2. These findings clearly indicate that Nrf2 contributes to both cancer cell proliferation and anticancer drug resistance.

The activation of Nrf2 is regulated by its cytoplasmic inhibitor Keap1. The *Keap1* gene is located at 19p13.2 and is divided into four domains: broad complex-tramtrack-bric-a-brac, intervening region, double glycine repeat (also called the Kelch domain owing to its homology with the *Drosophila* Kelch protein), and COOH-terminal region. The double glycine repeat/Kelch domain is required to bind the Neh2 domain of Nrf2, whereas the intervening region and broad complex-tramtrack-bric-a-brac domains are required for the redox-sensitive regulation of Nrf2 and for binding to Cul3-dependent E3 ubiquitin ligase. Several genetic alterations of the *Keap1* gene have been reported in both lung cancer patients and lung cancer cell lines (15, 16). Almost all reported mutations were located within the intervening region and double glycine repeat/Kelch domains. In A549 cells, a nonsynonymous somatic mutation (G333C) was detected in the first repeat of the double glycine repeat/Kelch domain that could not repress the activity of Nrf2 (15). A549 cells also

showed a loss of heterozygosity at 19p13.2 where the *Keap1* gene is located (15). On the other hand, sequencing of the *Keap1* gene in LC-AI and NCI-H292 cells revealed that these cells possess wild-type *Keap1* (data not shown). Taken together, these results indicate that constitutive activation of Nrf2 may be strongly related to dysfunctional mutation of *Keap1* in lung cancer cells. Singh et al. have also recently shown that constitutive activation of Nrf2 promotes tumorigenicity and contributes to chemoresistance by up-regulating its target genes in lung cancer cell lines (A549 and H460) with complete loss of functional Keap1 activity (19). However, activation of Nrf2 was observed in some lung cancer samples with wild-type *Keap1* because of the lower expression level of the *Keap1* gene. H1437 and H18 cells, the lung cancer cell lines expressing low level of *Keap1* gene, showed same degree of CDDP resistance as A549 cells with the constitutive activation of Nrf2 (16). Reduced expression of *Keap1* gene may occur frequently in lung cancer as is the case for the somatic mutation. Thus, more intensive studies are required to unveil the regulatory mechanisms of *Keap1* expression in cancer cells.

CDDP is a platinum-based anticancer drug used to treat various types of cancers, including lung, ovarian, cervical,

and testicular cancers (20, 21). CDDP attacks DNA by forming interstrand and intrastrand DNA crosslinks that ultimately trigger apoptosis (22). However, CDDP resistance is now an important clinical problem in the treatment of NSCLC. Studies have shown that CDDP resistance can be acquired by several different mechanisms. Increase in drug efflux systems may be important, because up-regulation of multidrug resistance-associated proteins has been found in various tumor tissues (23). Increased DNA repair capacity and the suppression of apoptosis pathways in response to DNA damage have also been observed in many types of cancer (24). Moreover, increased detoxification ability and cellular GSH levels have been observed in response to the induction of various detoxification enzymes and glutathione-related enzymes (25, 26).

In the present study, we showed that both the degree of CDDP-induced DNA crosslinking and the number of apoptosed cells were increased significantly in A549 cells transfected with Nrf2-siRNA. The expression of multidrug resistance-associated proteins, the drug efflux proteins, was also significantly reduced in Nrf2-silenced A549 cells. Furthermore, the expression of detoxification enzymes and glutathione-related enzymes, such as GCLC, GCLM, glutathione peroxidase 2, GST, glutathione

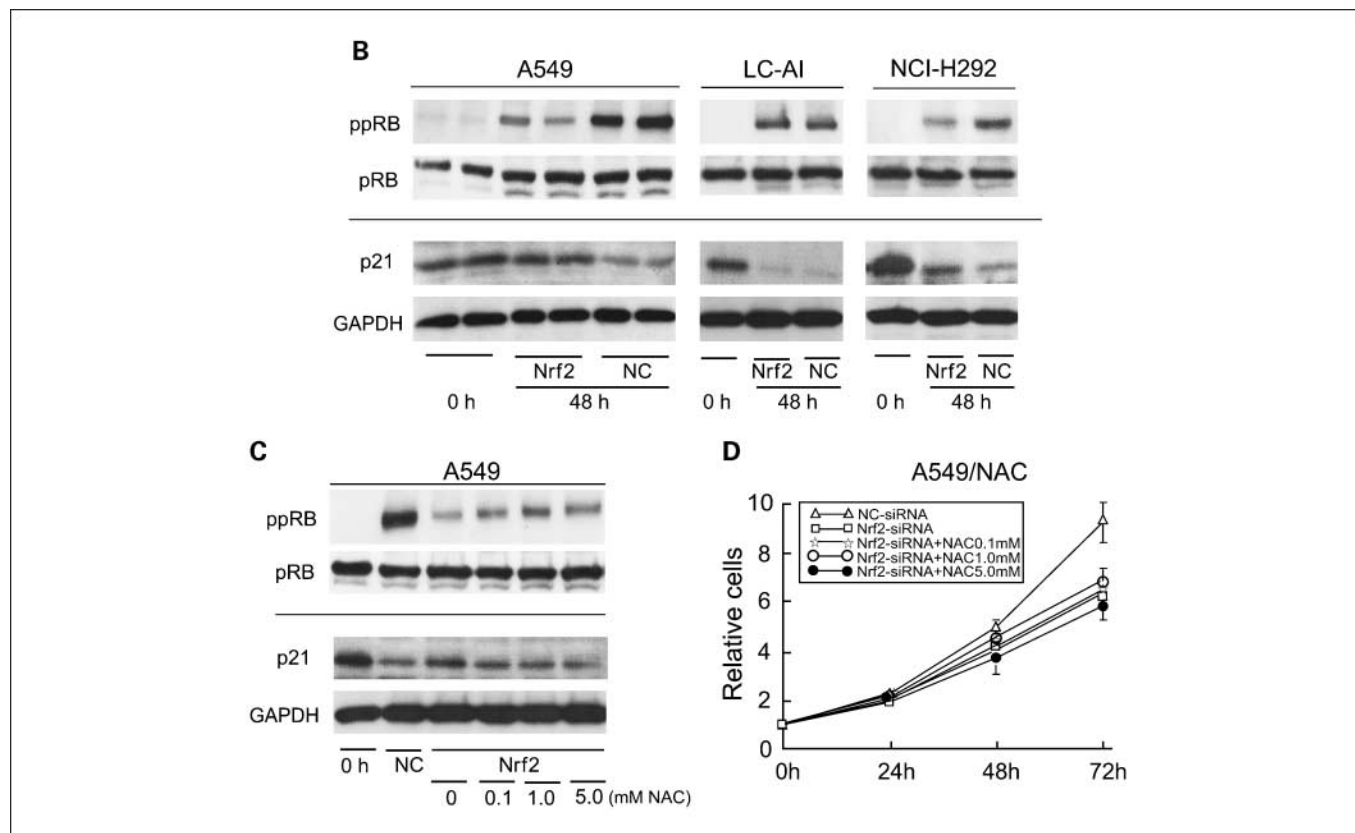


Fig. 5. Continued. B, protein level of phosphorylated pRb (ppRb; top) and p21 (bottom) in A549 cells (left), LC-AI cells (middle), and NCI-H292 cells (right) transfected with Nrf2-siRNA or NC-siRNA. Each protein level was determined by immunoblot analysis using antibodies against ppRb and p21. pRb (top) and GAPDH (bottom) were used as the internal control of ppRb and p21, respectively. C, effect of NAC on the expression of ppRb (top) and p21 (bottom) in A549 cells transfected with Nrf2-siRNA. A549 cells were transfected with Nrf2-siRNA and incubated with vehicle (0 mmol/L) or with 0.1, 1.0, and 5.0 mmol/L NAC. Protein level of ppRb and p21 was determined by immunoblot analysis using antibodies against ppRb and p21 48 h after incubation with NAC. pRb (top) and GAPDH (bottom) were used as the internal control of ppRb and p21, respectively. D, effect of NAC on the proliferation of Nrf2-siRNA-transfected A549 cells. A549 cells were transfected with Nrf2-siRNA and incubated with vehicle (open square) or with 0.1 mmol/L (open star), 1.0 mmol/L (open circle), and 5.0 mmol/L (closed circle) NAC. Cell proliferation was monitored for 24 h by a Cell Counting Kit-8.

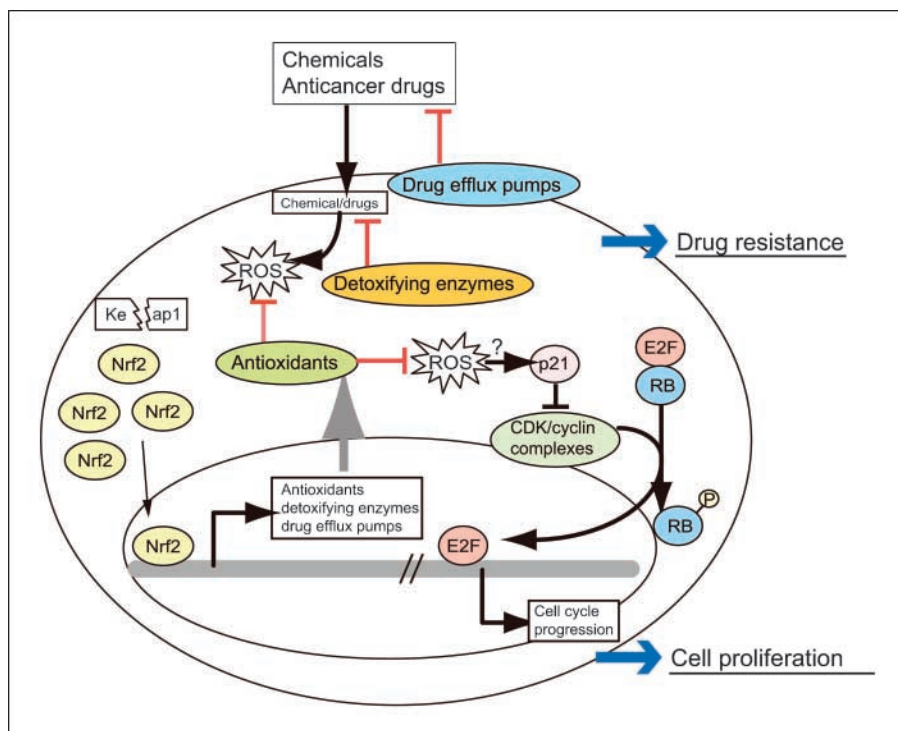


Fig. 6. Schematic presentation of the roles that Nrf2 plays in cancer cell proliferation and cancer cell resistance to anticancer drugs.

reductase, and NAD(P)H-quinone oxidoreductase 1, was significantly decreased along with a reduction of the glutathione level in A549 cells by suppression of Nrf2 activity with Nrf2-siRNA. These findings indicate that a wide variety of genes that are involved in the resistance to CDDP are under the regulatory influence of Nrf2 in the lung cancer cell line. These findings are consistent with a recent report by Cho et al. that inhibition of Nrf2 function restored CDDP sensitivity in human ovarian cancer SK-OV cells (27).

As for other type of anticancer drugs, Nrf2 knockdown enhanced the sensitivity to bleomycin but not to 5-FU in A549 cells. Bleomycin is a glycopeptide antibiotic and acts as an anticancer drug by induction of DNA strand breaks. Bleomycin is known to strongly generate free radicals and lipid peroxides that induce apoptosis of tumor cells (28). 5-FU is an analogue of uracil and its active intracellular metabolites disrupt RNA synthesis and the action of thymidylate synthase (29). Nrf2 may contribute to enhance the resistance of cancer cells to bleomycin by increase of cellular antioxidant capacity. Thus, inhibition of Nrf2 in cancer cells might be a useful strategy to enhance the sensitivity to anticancer drugs that induce oxidative damages.

It is of interest that the expression of metallothioneins, MT1B, MT1X, and MT2B, was significantly increased by Nrf2 knockdown. Metallothioneins are low molecular weight cysteine-rich proteins and act to protect against oxidative and xenobiotic stress by capturing oxidant radicals and electrophiles with their cysteine residues (30). Metallothioneins may play a role in the resistance of anticancer drugs in NSCLC, because it has been reported that the expression of metallothioneins was significantly enhanced in NSCLC following chemotherapy with CDDP (31). Expression of metallothioneins is induced by a high variety of stimuli, such as metal exposure, oxidative stress, glucocorticoids, and hydric stress. The level of expression of *MT* genes by these

stimuli is regulated by the *cis*-acting element in their promoter regions, such as antioxidant response element, metal response elements, and glucocorticoid response elements (32). A recent study has shown that antioxidant response element-mediated expression of metallothioneins is preferentially activated by Nrf1 (33). It is also likely that CDDP can induce *MT* genes by interaction of its metal component and metal response elements independently with Nrf2/antioxidant response element transcription system. It is therefore possible to say that, in cancer cells, some self-defense genes are compensatively induced against cytotoxic stimuli when Nrf2 is inactivated. Although Nrf2 is a pivotal factor for cell protection from oxidative and electrophilic insults, cellular compensation mechanisms for Nrf2 function should be considered when Nrf2 is to be used as a therapeutic target for cancer treatment.

In the present study, we found that Nrf2 knockdown by Nrf2-siRNA inhibited the proliferation of lung cancer cells. The growth-inhibitory properties of Nrf2 suppression are likely attributable, at least in part, to its induction of cell cycle arrest at G₁ phase in A549 and NCI-H292 cells as indicated in Fig. 5. Our study also showed that the cell cycle arrest was associated with the repression of pRb phosphorylation in these cells. pRb belongs to the pocket protein family first identified as a tumor suppressor gene in retinoblastoma (34, 35). pRb is a nuclear phosphoprotein and is regulated in a cell cycle-dependent manner by phosphorylation. Underphosphorylated pRb in the G₀ and early G₁ phases can bind to E2F family transcription factors and represses E2F-mediated transcription of the genes necessary for entry to S phase and DNA replication. The protein becomes phosphorylated in late G₁ and liberates E2F to induce transcription of S-phase genes (34, 35). pRb functions to prevent the cell from replicating damaged DNA by preventing its progression along the cell cycle through G₁ into S phase. Dysfunction of pRb, therefore,

may be essential for cancer cell formation and growth. Actually, dysfunctional mutation or deletion of the *RB* gene has been reported in various types of cancer including lung cancer (36). Our study showed that Nrf2-mediated pRb hyperphosphorylation induces cancer cell proliferation without the dysfunctional mutation or deletion of the *RB* gene.

Because the *RB* gene itself is a wild-type gene in A549 cells (37), Nrf2 may affect the regulatory pathway of pRb phosphorylation. The enzymes that phosphorylate pRb are the cyclin-dependent kinase (CDK) complexes (34–36). The CDK complexes are composed of two components: the activating kinase component and the cyclin component. The specific CDK complexes that phosphorylate Rb are cyclin D1/CDK4/6 and cyclin E/CDK2. The cell has inhibitory pathways that block CDK activity during cell cycle progression as well as a cyclin-dependent CDK activation pathway. The p21 protein, a member of the Cip/Kip family, is a potent inhibitor of the CDK complexes (38, 39). Transcription of the *p21* gene is induced following DNA damage as a consequence of the accumulation of wild-type p53, and the elevated concentration of p21 protein mediates p53-induced growth arrest in response to DNA damage to avoid the replication and subsequent propagation of potentially hazardous mutations (38, 39).

In the present study, interestingly, we found that the expression of p21 was up-regulated by suppression of Nrf2 activity in A549 and NCI-H292 cells. These results suggest that Nrf2 enhances pRb hyperphosphorylation at least in part via inhibition of p21. Although A549 cells possess wild-type p53, the inhibitory effects of Nrf2 may be p53 independent because the expression of p53 was not altered by Nrf2 silencing (data

not shown). Previous studies have shown that p21 is induced through a p53-independent mechanism in response to oxidative stress (38). However, treatment with NAC, a thiol antioxidant, did not reverse the proliferation of Nrf2-silenced A549 cells, whereas it reversed the expression of p21 in A549 cells. It has been shown that GSH is an important component for stress-mediated cell cycle regulation in G₁-S phase (40). As Singh et al. (19) suggested, treatment with NAC might not restore GSH levels to normal in Nrf2 knockdown cells because the genes involved in GSH biosynthesis are regulated by Nrf2. However, Reddy et al. have recently shown that disruption of Nrf2 induces cell cycle arrest, mainly G₂-M phase, by impairing GSH-induced redox signaling in primary epithelial culture cells (41). The mechanisms of Nrf2-mediated cell cycle regulation are thought to be complicated and more intensive studies are required.

In summary, we have shown that functional inhibition of Nrf2 suppresses both cell proliferation and the resistance to the anticancer drugs in lung cancer cell lines with high Nrf2 activity. Up-regulation of Nrf2 in cancer cells may increase cellular antioxidant and detoxification abilities by inducing a wide variety of self-defense gene. The putative mechanisms of the Nrf2-dependent cell protection and proliferation are summarized in the Fig. 6. Thus, inhibition of Nrf2 activity, in combination with antineoplastic agents, may constitute a new therapeutic approach against lung cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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