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Regulatory Role of KEAP1 and NRF2 in PPARγ Expression and Chemoresistance in Human Non-small Cell Lung Carcinoma Cells

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Abstract

The nuclear factor-E2-related factor 2 (NRF2) serves as a master regulator in cellular defense against oxidative stress and chemical detoxification. However, persistent activation of NRF2 resulting from mutations of *NRF2* and/or downregulation or mutations of its suppressor Kelch-like ECH-associated protein 1 (*KEAP1*) are associated with tumorigenicity and chemoresistance of non-small-cell lung carcinomas (NSCLCs). Thus, inhibiting NRF2-mediated adaptive antioxidant response is widely considered a promising strategy to prevent tumor growth and reverse chemoresistance in NSCLCs. Unexpectedly, stable knockdown of *KEAP1* by lentiviral shRNA sensitized three independent NSCLC cell lines (A549, HTB-178 and HTB-182) to multiple chemotherapeutic agents, including arsenic trioxide (As_2O_3), etoposide and doxorubicin, despite moderately increased NRF2 levels. In lung adenocarcinoma epithelial A549 cells, silencing of

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KEAP1 augmented the expression of peroxisome proliferator-activated receptor γ (PPARγ) and genes associated with cell differentiation, including E-Cadherin and Gelsolin. In addition, *KEAP1*-knockdown A549 cells displayed attenuated expression of proto-oncogene Cyclin D1 and markers for cancer stem cells (CSCs), and reduced non-adherent sphere formation. Moreover, deficiency of *KEAP1* led to elevated induction of PPARγ in response to As_2O_3. Pretreatment of A549 cells with PPARγ agonists activated PPARγ and augmented the cytotoxicity of As_2O_3. A mathematical model was formulated to advance a hypothesis that differential regulation of PPARγ and detoxification enzymes by KEAP1 and NRF2 may underpin the observed landscape changes in chemo-sensitivity. Collectively, suppression of KEAP1 expression in human NSCLC cells resulted in sensitization to chemotherapeutic agents, which may be attributed to activation of PPARγ and subsequent alterations in cell differentiation and CSC abundance.

Introduction

Nuclear factor-E2-related factor 2 (NRF2) is a master regulator of the transcription of many antioxidant and phase II detoxification enzymes [1]. Under normal homeostatic conditions, the low constitutive amount of NRF2 protein is mainly controlled by Kelch-like ECH-associated protein 1 (KEAP1)-mediated ubiquitination and the proteasomal degradation system [2]. Upon oxidative and/or electrophilic stress, the enzymatic activity of the KEAP1-Cullin3 E3 ubiquitin ligase is compromised, resulting in NRF2 stabilization and nuclear accumulation. Partnered with small Maf proteins, NRF2 binds to the antioxidant response elements (AREs) of target cytoprotective genes and augments their transcription [2, 3]. Thus, NRF2-mediated adaptive antioxidant response plays pivotal roles against oxidative/electrophilic stress and in chemical detoxification. As a result, activation of NRF2 has been demonstrated as an effective approach for cancer chemoprevention [4]. Paradoxically, a deleterious role of NRF2 activation in cancer progression has emerged, with evidence showing that the stress-response program is turned on in early tumour development and oncogene activity is coupled with NRF2 activation [5–7]. NRF2 and its downstream genes are overactivated/overexpressed in many cancer cells, thereby providing them a survival and growth advantage [8–10]. Most recently, DeNicola et al. reported that oncogene-induced NRF2 activation promotes reactive oxygen species (ROS) detoxification and tumorigenesis [6]. Since tumor cells may exploit the NRF2 pathway for their survival by deactivating chemotherapeutic agents [11], KEAP1 and NRF2 have been intensively investigated as a promising target to combat chemoresistance [2, 3, 12, 13].

Non-small-cell lung carcinoma (NSCLC) is the most common type of lung cancer, which is subdivided into squamous carcinoma, adenocarcinoma and large cell carcinoma. Currently, surgery, radiation and platinum-based chemotherapy are the standard treatment for NSCLCs. Compared to small cell carcinoma, NSCLCs are relatively insensitive to chemotherapy. Although the mechanism for the chemoresistance of NSCLC is poorly understood, low expression of KEAP1 and/or its inactivation due to mutations and attendant activation of NRF2 are common in NSCLC cells, suggesting persistent induction of cytoprotective and phase II enzymes by NRF2 underlie the enhanced resistance of NSCLC cells to chemotherapeutic agents [3, 11, 12, 14] and radiation [15]. Peroxisome proliferator-activated receptor γ (PPARγ) is a member of the nuclear hormone receptor superfamily of ligand-activated transcription factors [16–19]. The expression of PPARγ was shown to correlate with the degree of differentiation and survival rate in lung cancer patients [20, 21]. In addition to adipogenic and anti-inflammatory effects, PPARγ activation was shown to modulate various hallmarks of cancer through its pleiotropic effects on different cell types in the tumor microenvironment. An overwhelming number of preclinical studies demonstrate the efficacy of PPARγ agonists in the control of tumor progression through their effects on various cellular processes, including differentiation, proliferation, apoptosis, angiogenesis,

inflammation and metastasis [22]. Many PPARγ agonists, such as ciglitazone, troglitazone (Tro), pioglitazone and rosiglitazone (Rosi), were shown to inhibit tumor growth and progression in preclinical models of lung cancer, by influencing various signaling pathways in a PPARγ-dependent and independent manner [23–25]. We have recently demonstrated that NRF2 is an important nuclear factor regulating PPARγ expression in adipogenic differentiation [26]. It is intriguing to ascertain whether NRF2 is also involved in the regulation of PPARγ expression in other cell types, in particular NSCLC cells.

Arsenic trioxide (As_2O_3) is an anti-cancer drug approved by the U.S. Food and Drug Administration to specifically treat acute promyleocytic leukemia. As_2O_3 slows cancer cell growth by influencing multiple signaling pathways, including cell-cycle progression, differentiation and apoptosis [27]. As_2O_3 has also shown efficacy in treating other malignancies, particularly multiple myeloma and myelodysplastic syndromes [28]. Although As_2O_3 modulates DNA synthesis and apoptosis in lung carcinoma cells [29], NSCLCs are relatively resistant to As_2O_3 therapy, which may be associated with their enhanced NRF2 activity [30]. Inhibition of the NRF2-dependent antioxidant response through overexpression of KEAP1, knockdown of NRF2 or chemical inhibitors has been reported to render lung cancer cells more susceptible to chemotherapeutic agents [3, 13, 31, 32]. Our study found that silencing *NRF2* in three independent NSCLC-derived cell lines – adenocarcinoma epithelial cell line A549, adenosquamous carcinoma cell line HTB-178 and squamous cell carcinoma cell line HTB-182 – sensitized them to multiple chemotherapeutic agents, including As_2O_3, etoposide and doxorubicin. Surprisingly stable knockdown of *KEAP1* by lentiviral shRNA also sensitized the NSCLC cell lines to chemotherapeutic drugs, despite increased NRF2 activity attained. Suppression of KEAP1 in A549 cells resulted in increased expression of PPARγ, which was accompanied by induction of cell differentiation markers, attenuated expression of cancer stem cell (CSC) markers and reduced non-adherent sphere formation. A mathematical model was formulated to support the hypothesis that activation of PPARγ by *KEAP1* knockdown could overcome the increase in chemoresistance resulting from NRF2 activation in these cancer cells, leading to enhanced chemo-sensitivity. Our studies provide new information about the mechanisms for chemoresistance of NSCLC, and raise new questions regarding the distinct roles of the KEAP1-NRF2 pathway and PPARγ in the process.

Materials and Methods

Cell culture and reagents

Human lung carcinoma A549 (#CCL-185), HTB-178 (#NCI-H596) and HTB-182 (#NCI-H520) cell lines were purchased from American Type Culture Collection (ATCC, Manassas, VA). A549 cells were cultured with high-glucose DMEM (Invitrogen, Carlsbad, CA), whereas HTB-178 and HTB-182 cells were grown in the RPMI 1640 (ATCC) ; both media were supplemented with 10% fetal bovine serum (FBS), 100 U penicillin/ml, and 100 μg streptomycin/ml. Cultures were maintained at 37°C in a humidified 5% CO_2 atmosphere. Phosphate buffered saline (PBS, pH 7.4) and supplements for cell culture were purchased from Invitrogen. Rosiglitazone maleate (Rosi) was from SmithKline Beecham Pharmaceuticals (London, UK). All other reagents, including As_2O_3 and troglitazone (Tro), were purchased from Sigma-Aldrich (St. Louis, MO).

Lentiviral-based shRNA transduction

MISSION shRNA lentiviral particles were obtained from Sigma. Lentiviral transduction of A549, HTB-178 and HTB-182 lung cancer cell lines with particles for shRNAs targeting *NRF2* (SHVRS-NM_010902), *KEAP1* (SHVRS-NM_016679) or scrambled non-target negative control (sh-Scr/SCR, SHC002V) was performed as described previously [33]. The

selection media for A549, HTB-178 and HTB-182 cells contained 3.0, 2.0 and 1.5 μg/ml of puromycin (Invitrogen), respectively. Stable cell lines were continuously grown in the media containing the same concentration of puromycin.

Non-adherent sphere formation (NASF) assay

NASF assay was performed as described previously [34]. In brief, *KEAP1*-KD A549 and SCR cells were plated at density of 1 X 10^4 viable cells/cm^2 in 90-mm dishes. Following 48 h culture, the floating cells in medium were harvested by centrifuge. The resulted cell pellets were made into single-cell resuspensions with 4 ml complete medium with 10% FBS and seeded onto 30-mm Ultra low cluster plates (Corning Inc., Corning, NY). Cells were cultured for 14 days and 1 ml fresh complete medium was gently added every three days. On day 14, formed spheres were counted under a microscope and normalized by the number of attached cells.

Cell cycle analysis

Cell cycle analysis by flow cytometry was performed as described previously [25]. In brief, proliferating cells were trypsinized and washed two times with PBS and fixed in 70% ethanol-PBS. After 30 min of incubation at 4 °C, cells were centrifuged at 500 g for 10 min. The resulting cell pellet was resuspended and incubated at 37 °C for 30 min in 0.04 mg/ml propidium iodide (PI) and 1mg/ml RNAse A in PBS. The suspension was then analyzed on a flow cytometry (FACS CantoII with HTS option, BD Biosciences, San Jose, CA). The percentage of cells in the G_0/G_1, S and G_2/M phases of cell cycle were determined by their DNA content using the FCS Express 4 Plus (De Novo Software, Los Angeles, CA).

Real-time RT-PCR analysis

Total RNAs were isolated with TRIzol reagent (Invitrogen). Quantitative real-time RT-PCR was performed as described previously [33]. The primers described in Online Table S1 were designed by using Primer Express 4 (Applied Biosystems) and synthesized by Bioneer Inc. (Alameda, CA). Real-time fluorescence detection was carried out by using an ABI PRISM 7900 Sequence Detector (Applied Biosystems). 18S ribosomal RNA *(18S)* was used for normalization.

Western blot analysis

Isolation of cell fractions and Western blotting were performed as detailed previously [33, 35]. Antibodies recognizing NRF2 (sc-13032; 1:500), KEAP1 (sc-15246; 1:500), heme oxygenase 1 (HO-1, sc-136902; 1:500), E-Cadherin (E-CAD; sc-8426; 1:500), OCT-4 (sc-102051; 1:500) and WNT3 (sc-5213; 1:500) were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibodies against PPARγ (2435S; 1:500) and β-ACTIN (A1978; 1:2000) were purchased from Cell Signaling Technology (Danvers, MA) and Sigma, respectively.

Immunostaining

Fluorescence immunostaining was performed as described previously [35]. Briefly, cells were grown on glass coverslips in six-well plate for 48 h. Then the cells were washed with PBS and fixed for 10 min at room temperature in 2% (v/v) formaldehyde. After washing in PBS, cells were permeabilized in 1% (v/v) Triton X-100 in PBS, washed, and incubated with 10% goat serum (sc-2043, Santa Cruz) in PBS for 1 h at room temperature. The cells were first treated with monoclonal mouse anti-E-CAD (sc-8426; 1:50, Santa Cruz) for 16 h at 4°C and subsequently with Rhodamine-linked goat-anti-mouse IgG1 (sc-2092,1:50, Santa Cruz) for 45 min at room temperature. After PBS washing, coverslips were mounted with the Prolong Gold antifade reagent with DAPI (P36931, Invitrogen) on microscope slides and

examined using an Axio Observer Z1 fluorescence microscope (Carl Zeiss, Inc. Oberkochen, Germany).

Acute cytotoxicity assay

In vitro drug sensitivity to chemotherapeutic agents was assessed with 3-(4,5-dimethylthiazol-2-yl)-5-(3-Carboxymethoxyphenyl)-2-(sulfophenyl)-2H-tetrazolium (MTS) by using Cell Titer 96-Aqueous Assay Kit (Promega, Madison, WI) as detailed previously [36]. The cells were exposed to various concentrations of As_2O_3, etoposide or doxorubicin for 48 h. Measurements were expressed as percentage change from untreated control (Vehicle) of appropriate cells. Each data point represents a mean ± SD. The concentrations that were lethal to 50% of cells (LC_{50}) were determined from analysis of the log-linear phase of the curves.

Cell death assessment by flow cytometry

A549 cells were seeded in a six-well plate and grown to approximately 70% confluence. After the cells were treated with As_2O_3 for 48 h, the floating and attached cells were harvested for apoptosis analysis. Apoptotic and necrotic cells were analyzed by flow cytometry (FACS CantoII with HTS option, BD Biosciences) using the TACS Annexin V-FITC Apoptosis Detection Kit (Trevigen, Gaithersburg, MD) detailed previously [37]. For each sample, 10,000 cells were examined. The percent of apoptotic and necrotic cells was determined by statistical analysis of the various dot plots using CellQuest software (Diva 6.0, BD Biosciences).

Statistical analyses

All statistical analyses were performed by using Graphpad Prism 4 (GraphPad Software, San Diego, CA), with $p < 0.05$ taken as significant. For comparisons between two groups, t-test was performed. Statistical analyses to evaluate the effects of rosiglitazone or troglitazone on gene expression were carried out by using one-way ANOVA with Tukey's Multiple Comparison Test. Statistical analyses to evaluate the time- and concentration-dependent effects of As_2O_3, etoposide and doxorubicin on gene expression and cell viability were performed by using two-way ANOVA with Bonferroni post hoc test.

Mathematical modeling

The mathematical model was constructed and simulated in MatLab (The Mathworks, Inc., Natick, MA, USA). It provided a provisional description of how the chemo-sensitivity landscape changes with varying KEAP1 and NRF2 levels, which are assumed to regulate the canonical antioxidant/phase II gene pathway and PPARγ pathway with different sensitivities. The two pathways have opposite effects on chemo-sensitivity. See online supporting material for model details.

Results

Stable knockdown of either NRF2 or KEAP1 in NSCLC cells results in sensitization to chemotherapeutic drugs

To study the role of the KEAP1-NRF2 pathway in chemoresistance of NSCLC cells, we investigated the effect of stable knockdown of *NRF2* or *KEAP1* on the susceptibility of various NSCLC cell lines to chemotherapeutic agents-induced cytotoxicity. In A549 cells, transduction of lentiviral shRNA against human *NRF2* (termed *NRF2*-KD) significantly attenuated the mRNA level of *NRF2* (Fig. 1A). The effectiveness of knockdown was confirmed by notably diminished protein accumulation of NRF2 in cells challenged with tBHQ, a potent NRF2 activator [38], as well as in control cells not treated with tBHQ (Fig.

1B). In addition, the expression of ARE-dependent genes, including NAD(P)H quinone oxidoreductase 1 (*NQO1*), *HO-1,* glutamate-cysteine ligase catalytic (*GCLC*) and regulatory subunit (*GCLM*), was significantly attenuated indicating that NRF2-mediated transcription was suppressed in *NRF2*-KD cells (Fig. 1C). Importantly, *NRF2*-KD cells displayed enhanced cytotoxicity in response to various chemotherapeutic agents, including As_2O_3, etoposide and doxorubicin (Fig. 1D, E and F).

A549 cells transduced with lentiviral shRNA against human *KEAP1* (termed *KEAP1*-KD) showed efficient knockdown of *KEAP1* expression (Fig. 2A). Supporting the silencing effect of *KEAP1,* markedly reduced protein levels of KEAP1 (Fig. 2B), moderately increased protein levels of NRF2 (Fig. 2B), and induction of ARE-dependent genes (Online Figure S1) were observed in *KEAP1*-KD cells. Surprisingly, silencing *KEAP1* rendered the cells somewhat more susceptible, rather than resistant, to cytotoxicity induced by As_2O_3, doxorubicin or etoposide (Fig. 2C, D and E). This unexpected sensitization to cytotoxicity was further confirmed by measurement of apoptosis and necrosis by using flow cytometry with Annexin V-FITC and PI double staining (Fig. 2F). To ascertain whether the finding is a common phenomenon in NSCLC cells, we investigated the effects of silencing *NRF2* or *KEAP1* on As_2O_3-induced cytotoxicity in lung adenosquamous carcinoma cell line HTB-178 cells and squamous cell carcinoma cell line HTB-182 cells. As with A549 cells, knockdown of either *NRF2* or *KEAP1* in both cell lines resulted in significantly increased sensitivity to As_2O_3 toxicity (Fig. 3).

Effects of silencing NRF2 or KEAP1 on As_2O_3-induced antioxidant response in A549 cells

To define the role of NRF2-mediated antioxidant response in the chemoresistance of NSCLC cells, the expression of NRF2 and some ARE-dependent genes, including *NQO1, GCLM and HO-1*, were determined in SCR, *NRF2*-KD, and *KEAP1*-KD A549 cells following As_2O_3 exposure. In SCR cells NRF2 protein accumulation and ARE-dependent gene expression increased in a time- and concentration-dependent manner (Fig. 4 and 5), confirming our previous findings that arsenite is a potent NRF2 activator [35, 38]. In contrast, *NRF2*-KD cells exhibited dramatically attenuated expression of NRF2 and ARE-dependent genes under both basal and As_2O_3-treated conditions (Fig. 4). In addition, deficiency of NRF2 resulted in attenuated mRNA (not shown) and protein expression of KEAP1 under basal and As_2O_3-treated conditions (Fig. 4), which is consistent with the finding by Lee et al. [39] who reported that NRF2 regulates the transcription of KEAP1 through a specific ARE. As expected, in *KEAP1*-KD cells, enhanced NRF2 activity was observed, as measured by its protein accumulation and downstream gene and protein expression under both basal and As_2O_3-treated conditions (Fig. 5). The immunoblotting of HO-1 confirmed its gene expression in SCR, *NRF2*-KD and *KEAP1*-KD cells (Fig. 4 and 5).

Effects of silencing NRF2 or KEAP1 in A549 cells on expression of PPARγ and markers associated with cell differentiation and CSC attributes

NRF2 is a key transcription factor regulating PPARγ gene expression [40, 41]. In A549 cells, we found that knockdown of *NRF2* reduced the expression of PPARγ at mRNA and protein levels (Online Figure S2). In contrast, *KEAP1* silencing resulted in a significantly increased expression of PPARγ at mRNA and protein level (Fig. 6A-C). Since NRF2 level is only moderately increased in *KEAP1*-KD cells (Fig. 2B and Fig. 5A and B), it is highly likely that the significantly increased PPARγ expression by *KEAP1* silencing requires an NRF2-independent mechanism as well. Consistent with the pleiotropic roles of PPARγ in cell differentiation and proliferation, *KEAP1*-KD cells displayed significantly increased expression of *E-CAD* and *GELSOLIN* (*GSN*) and attenuated expression of proto-oncogene *CYCLIN D1* (*CCND1*) (Fig. 6A, B and D). In addition, *KEAP1*-KD cells expressed reduced

levels of several CSC markers, including OCT-4, ABCG2 and WNT3 (Fig. 6A and B), followed by decreased non-adherent sphere formation (Fig. 6E), suggesting silencing KEAP1 in A549 cells promotes differentiation of CSC cells. However, cell cycle analysis revealed that knockdown of *KEAP1* did not significantly affect cell cycle, even under high concentrations of As_2O_3-treated conditions (Fig. 6F)

As_2O_3 increases PPARγ expression in A549 cells

As shown in Online Figure S3 and Fig. 7A, As_2O_3 treatment caused a concentration- and time-dependent increase in the mRNA expression of *PPARγ* in SCR A549 cells. Knockdown of *NRF2* significantly lowered As_2O_3-induced *PPARγ* induction (Online Figure S3). In contrast, deficiency of *KEAP1* led to elevated mRNA expression of PPARγ, under both basal and As_2O_3-treated conditions (Fig. 7A). In addition, As_2O_3 concentration-dependently increased the protein level of PPARγ in *KEAP1*-KD A549 cells (Fig. 7C and D). Furthermore, *KEAP1*-KD cells exhibited a dramatic reduction in *CCND1* expression under both basal and As_2O_3-treated conditions (Fig. 7B).

PPARγ agonists potentiate the toxic effect of As_2O_3 in A549 cells

To verify that activation of PPARγ resulting from *KEAP1* silencing is involved in the sensitization to chemotheraputic agents, we investigated the effect of PPARγ agonists on As_2O_3-induced cytotoxicity in A549 cells. As shown in Fig. 8A, non-cytotoxic levels of PPARγ agonists Rosi (5 μM) and Tro (5 μM) (Online Figure S4) time-dependently increased PPARγ mRNA expression in A549 cells. Consistent with the regulatory role of PPARγ in cell differentiation, increased mRNA expression of fatty acid synthsse (FASN), *E-CAD* and *GSN* and reduced expression of *CCND1* were observed in both Rosi- and Tro-treated cells. In *NRF2*-KD cells, a substantially reduced expression of PPARγ was observed under Rosi-treated conditions (Fig. 8B). Knockdown of *KEAP1* enhanced the basal expression of PPARγ, however, no additional induction by Rosi was observed in *KEAP1*-KD A549 cells. Interestingly, pretreatment of SCR A549 cells with 5 μM Rosi or Tro for 48 h significantly sensitized the cells to As_2O_3-induced cytotoxicity (Fig. 8C and D). However, the same pretreatments with Rosi or Tro exhibited no tangible sensitization to As_2O_3-induced cytotoxicity in *NRF2*-KD and *KEAP1*-KD cells (Fig. 8C and D).

Mathematical model of modulation of chemo-sensitivity by KEAP1 and NRF2

Based on the above observations, we propose that KEAP1 and NRF2 regulate two opposing pathways with differential sensitivities to modulate the susceptibility of NSCLC cells to the killing effect of chemotherapeutic agents (Fig. 9A). With the canonical ARE pathway, an increase in NRF2 upregulates endogenous antioxidant and phase II enzyme expression. By enhancing detoxification of ROS and xenobiotics, elevated antioxidant and phase II enzyme levels provide cells survival advantage against cytotoxicity of ROS derived from immune cells and against chemotherapeutic agents (Fig. 9A, pink blocks). The current study suggests KEAP1 and NRF2 could also regulate a second, novel pathway involved in cancer sensitivity to chemotherapeutic agents. PPARγ can be directly activated by NRF2 and indirectly repressed by KEAP1 in an NRF2-dependent manner as well as in a yet-to-be-characterized NRF2-independent manner (Fig. 9A dashed line). By inhibiting cell proliferation and promoting differentiation, PPARγ reduces the self-renewing potential of cancer cells, leading to enhanced sensitivity to the killing effect of chemotherapeutic agents (Fig. 9A, green blocks). However, activation of this PPARγ-mediated pathway requires lower KEAP1 and higher NRF2 levels than would be required to activate the antioxidant/phase II enzyme pathway. Therefore a moderate decrease in KEAP1 and increase in NRF2 would first upregulate phase II enzymes, decreasing sensitivity to the toxicity of therapeutic agents. Only upon further decrease in KEAP1 and increase in NRF2 levels would PPARγ

be significantly activated to promote cell differentiation and inhibit proliferation, leading to increases in the sensitivity to chemotherapeutics.

To investigate whether the above proposed mechanism is plausible, we formulated a simple mathematical model to capture the differential regulation of the antioxidant/phase II enzyme and PPARγ pathways by KEAP1 and NRF2 as illustrated in Fig. 9A (see online supporting material for model details). The model (Fig. 9B) produced a nonmonotonic landscape that describes how chemo-sensitivity might be altered as KEAP1 and NRF2 levels vary. Here chemo-sensitivity is defined as a function that correlates positively to PPARγ and negatively to antioxidant/phase II enzymes. A normal cell situated at location X on the landscape (blue dot) has high KEAP1 and low NRF2 levels. From this point, overexpression of *NRF2* (*NRF2*-OE) decreases chemo-sensitivity by upregulating antioxidant/phase II enzymes (Online Figure S5A). Knockdown of *NRF2* has an opposite effect on chemo-sensitivity by further decreasing the basal antioxidant/phase II enzyme level. *KEAP1*-KD cells, which acquire increased NRF2 activity, follow a trajectory delineated by the pink curve and exhibit decreased chemo-sensitivity due to increased expression of antioxidant/phase II enzymes (Online Figure S5A). Thus, the model recapitulates the effects of genetically manipulating *NRF2* and *KEAP1* in normal cells. In certain cells, including NSCLC cells, mutations in *KEAP1* alone move the cellular state from location X to a state of less chemo-sensitivity (Location Y; Fig. 9B, red dot) with lower KEAP1 and higher NRF2. This state confers cells higher resistance (lower chemo-sensitivity) to chemotherapeutic agents through upregulation of antioxidant/phase II enzymes, yet the PPARγ level is only marginally increased due to its lower sensitivity to changes in KEAP1 and NRF2 levels (Online Figure S5). As expected, knockdown of *NRF2* in these cells still leads to increased chemo-sensitivity. But more importantly, knockdown of *KEAP1* also increases the sensitivity to chemotherapeutics (Fig. 9B). The latter occurs because knockdown of *KEAP1* – which reduces KEAP1 to further lower levels – would lift its inhibition on PPARγ expression and allow the activation of PPARγ by NRF2 fully manifested (Online Figure S5B). The resulting significant upregulation of PPARγ would lead to an increase in chemo-sensitivity. In the absence of *KEAP1* knockdown, overexpression of *NRF2* alone either has no effect or only marginally increases chemo-sensitivity.

Discussion

Despite advances in developing effective therapeutic agents, chemoresistance remains the top obstacle in the treatment of NSCLCs. In keeping with the literature our current study supports the notion that inhibiting NRF2 sensitizes NSCLC cells to cytotoxicity induced by a variety of chemotherapeutic drugs, including As_2O_3, etoposide and doxorubicin. Unexpectedly, stable knockdown of *KEAP1* in three independent NSCLC cell lines - A549, HTB-178 and HTB-182 cells - also resulted in sensitization to these drugs, despite moderately increased NRF2 activity attained with *KEAP1* silencing. Induction of PPARγ and subsequent alterations in cell differentiation and CSC markers and/or abundance in A549 cells are associated with the phenotype of *KEAP1*-KD cells in response to chemotherapeutic drugs. The new finding that *KEAP1*-KD NSCLC cells exhibited enhanced sensitivity to multiple chemotherapeutic agents is different from that seen in normal human cells, such as HaCaT keratinocytes, where stable knockdown of *KEAP1* resulted in *NRF2* activation and resistance to As_2O_3 toxicity [42]. The distinctive phenotype of knockdown of *KEAP1* in NSCLC cells indicates that the landscape of chemoresistance with respect to KEAP1 and NRF2 in these cells is altered.

Persistent activation of NRF2 resulting from missense mutations, insertions or deletions in *KEAP1* and/or *NRF2* genes or downregulation of KEAP1 are associated with enhanced

resistance of NSCLCs to chemotherapeutic agents and radiation therapy [2, 5, 15]. The A549 cells have a mutation in the Kelch domain of KEAP1 (Gly333 to Cys) [11]. In these cells, reduced mRNA expression of *KEAP1* has been attributed to hypermethylation of CpG sites in the *KEAP1* (47) and these cells express high basal levels of NRF2, and many ARE-dependent antioxidant and detoxification enzymes. Stable knockdown of the mutated *KEAP1* in A549 cells resulted in an additional increase in the basal expression level of NRF2 and its target genes, suggesting the mutated *KEAP1* retains some residual activity to suppress NRF2. This interpretation is supported by the work of Tirumalai et al. [43] who demonstrates that acrolein stabilizes NRF2, increases ARE-DNA binding activity, induces ARE-mediated reporter activity, and induces *GCLC* and *NQO1* in A549 cells. In addition, As_2O_3 caused an enhanced NRF2-mediated antioxidant response in *KEAP1*-KD cells. Thus, changes in expression of detoxification enzymes and antioxidants, all of which increase in *KEAP1*-KD cells, cannot explain the increased sensitivity to chemotherapeutic agents by *KEAP1* silencing.

The frequent expression of PPARγ in various cancer tissues and cells, including NSCLC cells and tumor samples [25], and the ability of PPARγ agonists to inhibit cell proliferation and angiogenesis, promote differentiation and induce apoptosis suggest that PPARγ may play an important role in chemotherapy. Indeed, PPARγ agonists are efficacious in control of tumor progression in a PPARγ-dependent manner [22–25, 44, 45]. NRF2 is an important nuclear factor regulating PPARγ expression during adipogenesis [26]. Stable knockdown of *Keap1* in 3T3-L1 cells results in increased expression of PPARγ and accelerated adipogenic differentiation [26]. Our studies here show that the sensitization to chemotherapeutic agents observed in *KEAP1*-KD cells is associated with induction of PPARγ expression and altered cell differentiation. Of note, NRF2 activation alone, such as As_2O_3 treatment in SCR A549 cells (Fig. 7A and C), had no significant effect on PPARγ expression. In addition, both Rosi and Tro had no effect on NRF2 protein accumulation in A549 cells (data not shown). These findings suggest that NRF2 is necessary, but not sufficient, for PPARγ induction in A549 cells.

As expected, *KEAP1*-KD A549 cells had augmented expression of PPARγ, and enhanced expression of genes associated with cell differentiation, including *E-CAD* and *GSN,* and downregulation of proto-oncogene *CCND1*. E-CAD is a marker of cell–cell junctions, which is important in the control of invasion and migration of cancer cells. GSN is a general differentiation marker induced during PPARγ-mediated differentiation [20]. PPARγ agonists promote cell cycle arrest by downregulating CCND1 in several tumour cell lines, including NSCLC cells [20, 46–48]. In the current study, a reduced expression of *CCND1* accompanied with PPARγ upregulation occurred in *KEAP1*-KD cells. However, *KEAP1* silencing did not significantly affect cell cycle, even under high concentrations of As_2O_3-treated conditions, suggesting that the alteration in cell differentiation is the major contributor to their increased sensitivity to chemotherapeutic agents.

A primary mechanism for As_2O_3's effectiveness in treating cancers, in particular acute promyelocytic leukemia, is induction of apoptosis and differentiation and inhibition of proliferation [27]. In A549 cells, As_2O_3 dose-dependently enhanced the expression of PPARγ, suggesting As_2O_3 may stimulate NSCLC cell differentiation through induction of PPARγ. We saw a dramatic induction of PPARγ in response to As_2O_3 treatment followed by induction of E-CAD in *KEAP1*-KD A549 cells. Interestingly, non-cytotoxic levels of PPARγ agonists Rosi and Tro significantly enhanced the inhibitory effect of As_2O_3 on cell viability in SCR A549 cells. In contrast, no further inhibition was observed by Rosi and Tro pretreatment in *KEAP1*-KD cells. In these cells, PPARγ may already be close to maximal activation and non-responsive to further alteration by the agonists. Rosi and Tro pretreatment did not potentiate the toxicity of As_2O_3 in *NRF2*-KD cells, likely because

silencing *NRF2* already diminished the cellular detoxification capacity and substantially enhanced the sensitivity to toxicity of As_2O_3. These findings suggest that combination of PPARγ agoinsts with classical anti-tumor drugs may be a novel approach for combating chemoresistance in NSCLC treatment. Despite the fact that the induction of PPARγ expression resulting from *KEAP1* silencing, might require NRF2, activation of NRF2 alone is not sufficient to significantly induce PPARγ. For instance, chemical activators of NRF2, such as As_2O_3 and tBHQ, have no significant effect on PPARγ expression in SCR A549 cells even though they both markedly increase NRF2. These results suggest that downregulation of KEAP1 is also necessary for maximal induction of PPARγ in NSCLC cells, potentially through an NRF2-independent mechanism.

CSCs are a small reservoir of self-sustaining cells with exclusive ability of self-renewal and tumor maintenance [49]. Cancer is most likely a disease of stem cells. More and more studies suggest that CSCs play a role in the formation and progression of tumours, such as chemoresistance, metastasis, and recurrence [50]. It is known that there is a differential distribution of progenitor cell markers among different histological types of lung cancer and that poorly differentiated tumors have the highest expression of stem cell markers [51]. Given that stem-like cells often display higher tolerance to cytotoxins [52], the sensitization of NSCLC cells by *KEAP1* silencing to chemotherapeutic agents is likely to decrease the CSC population. We did find decreased expression of CSC markers, including ABCG2, WNT3 and OCT-4, and reduced NASF along with increased PPARγ expression in *KEAP1*-KD A549 cells. ABCG2 is molecular determinant of the side population (SP) phenotype [53, 54], and its expression is high in SP from A549 cells. Canonical WNT signaling plays an important role in lung CSCs [52]. OCT-4 is a marker of embryonic stem cells and a biological marker of lung CSCs. Thus, reduction of CSCs or cells with CSC attributes may explain some of the sensitization of *KEAP1*-KD cells to chemotherapeutic drugs.

In summary, our study highlights that the KEAP1-NRF2 pathway may be a double-edged sword in combating chemoresistance in NSCLC treatment. When KEAP1 and NRF2 are considered as a target to enhance chemo-sensitivity, the effect on differentiation and proliferation of CSCs may prove a serious liability for using this mode of action in chemotherapeutics. Combined mutations of *KEAP1* and *NRF2* may situate cancer cells in various locations on the landscape of chemo-sensitivity, leading to non-monotonic responses to manipulation of KEAP1 or NRF2 activities. If this hypothesis is true, an effective approach to increase cancer cell sensitivity to chemotherapeutic drugs would be simultaneous to silence both NRF2 and KEAP1, a proposal that sounds counter-intuitive. PPAR3 may become a new target to overcome chemoresistance in NSCLC treatment. Clearly, the physiological and pathophysiological role(s) of KEAP1, NRF2 and PPARγ will benefit from further investigations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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List of Abbreviation

ARE antioxidant response element

As_2O_3	arsenic trioxide
CCND1	CYCLIN D1
CSC	cancer stem cell
E-CAD	E-CADHERIN
FBS	fetal bovine serum
GCLC	glutamate-cysteine ligase catalytic subunit
GCLM	glutamate-cysteine ligase regulatory subunit
GSN	GELSOLIN
HO1	heme oxygenase 1
KD	knockdown
KEAP1	Kelch-like ECH-associated protein 1
NASF	non-adherent sphere formation
NQO1	NAD(P)H quinone oxidoreductase 1
NRF2	nuclear factor-E2-related factor 2
NSCLC	non-small-cell lung carcinoma
OE	overexpression
PPARγ	peroxisome proliferator-activated receptor γ
Rosi	rosiglitazone
SCR	scramble
SP	side population
tBHQ	tert-Butylhydroquinone
Tro	troglitazone

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Highlights

- Silencing *KEAP1* in NSCLC cells results in sensitization to chemotherapeutic agents.
- Knockdown of *KEAP1* augments PPARγ expression and cell differentiation-related genes.
- Deficiency of *KEAP1* leads to elevated induction of PPARγ in response to As_2O_3.
- PPARγ agonists augment As_2O_3–induced cytotoxicity in NSCLC cells.
- NRF2 and KEAP1 are involved in the regulation of PPARγ expression.

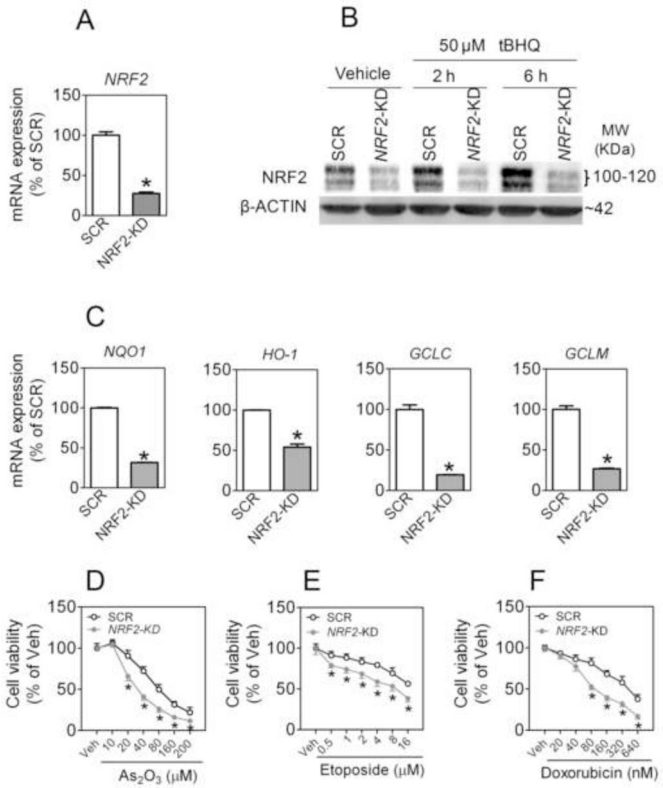

Fig. 1.

Stable knockdown of *NRF2* increases the susceptibility of A549 lung carcinoma cells to chemotherapeutic agents. (A) mRNA expression of *NRF2* in A549 cells transduced with shRNA lentivirus targeted against human *NRF2* or Scrambled non-target negative control (SCR). n = 3; *$p < 0.05$ vs. SCR. (B) Reduced protein expression of NRF2 in *NRF2*-KD cells under basal and tBHQ-treated conditions. Whole cell lysates were used for analysis and β-ACTIN was used as a loading control. Vehicle, medium. (C) Lack of *NRF2* significantly reduces the expression of ARE-dependent genes. The expression of *NQO1, HO-1, GCLC* and *CLCM* was measured by real-time RT-PCR. (D–F) *NRF2*-KD cells are more sensitive to chemotherapeutic agents–induced cytotoxicity. Cell viability was assessed by MTT assay following 48 h treatment with the indicated concentrations of As_2O_3 (D), etoposide (E) or doxorubicin (F). Values are means ± SEM from 4 independent experiments, *$p < 0.05$ vs. SCR with the same treatment.

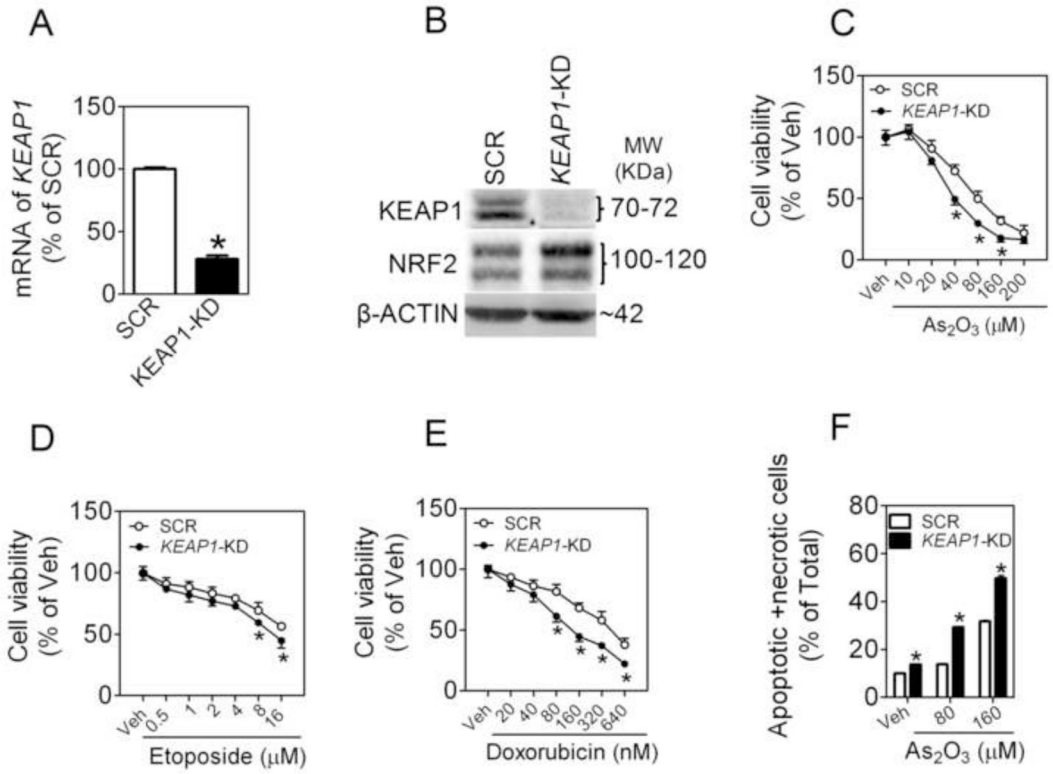

Fig. 2.

Stable knockdown of *KEAP1* expression by lentiviral shRNA sensitizes A549 cells to chemotherapeutic agents-induced cytotoxicity. (A) Reduced mRNA expression of *KEAP1* in *KEAP1*-KD cells. SCR, Scrambled non-target negative control; *KEAP1*-KD, *KEAP1*-knockdown. n = 3; **p* < 0.05 vs. SCR. (B) Protein levels of KEAP1 and NRF2 in SCR and *KEAP1*-KD cells. (C–E) *KEAP1*-KD cells are more sensitive to chemotherapeutic agents–induced cytotoxicity. Cell viability was assessed by MTT assay following 48 h treatment with the indicated doses of As_2O_3 (C), etoposide (D), or doxorubicin (E). (F) Apoptotic and necrotic cell death in response to As_2O_3 treatment was measured using flow cytometry. Cells were treated with As_2O_3 for 48 h. n = 4. **p* <0.05 vs. SCR with the same treatment.

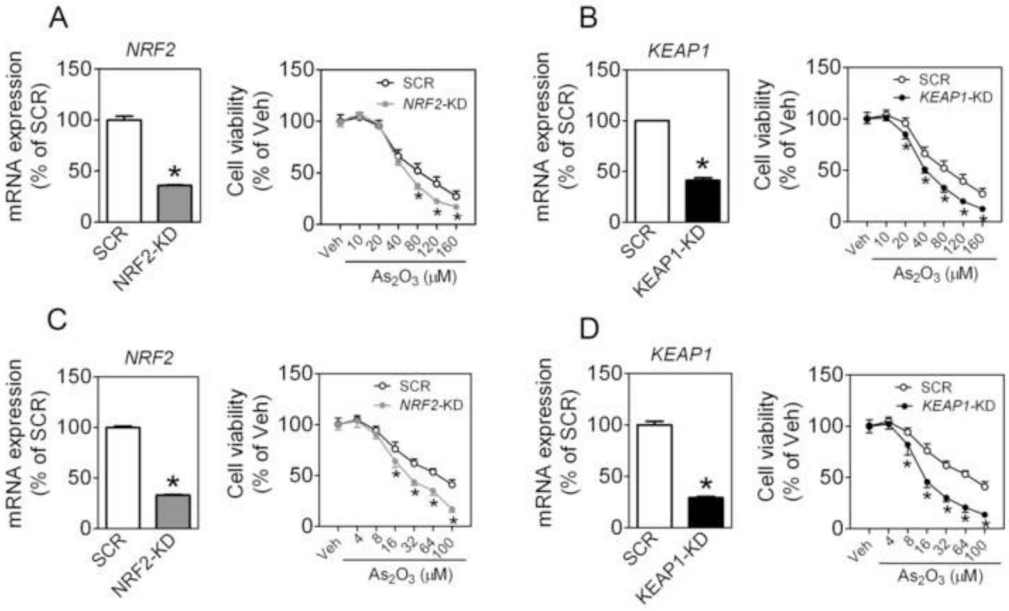

Fig. 3.

Effect of stable knockdown of *NRF2* or *KEAP1* on As_2O_3-induced cytotoxicity in HTB-178 and HTB-182 lung carcinoma cells. (A and C) Knockdown of *NRF2* sensitizes HTB-178 (A) and HTB-182 cells (C) cells to As_2O_3-induced cytotoxicity. Left panel, mRNA expression of *NRF2*; Right panel, Cell viability was assessed by MTT assay following 48 h treatment with the indicated concentrations of As_2O_3. n = 3. $*p < 0.05$ vs. SCR with the same treatment. (B and D) Silence of *KEAP1* in HTB-178 (B) and HTB-182 cells (D) increases their sensitivity to As_2O_3-induced cytotoxicity. Left panel, mRNA expression of *KEAP1*; Right panel, Cell viability was assessed by the MTT assay following 48 h treatment with the indicated concentrations of As_2O_3.

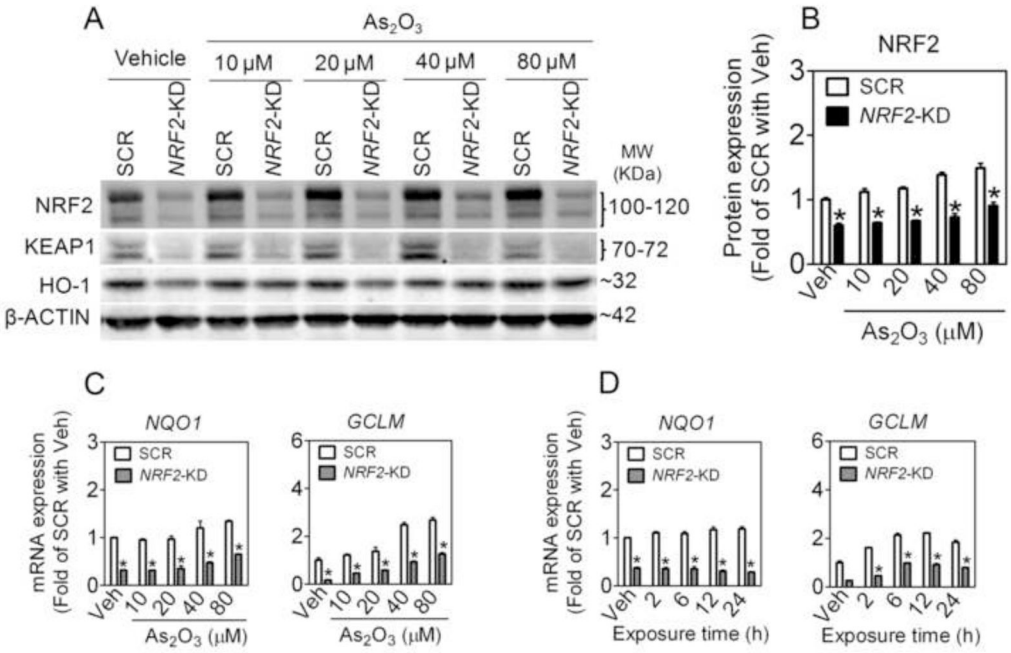

Fig. 4.

Lack of *NRF2* reduces *NRF2*-mediated antioxidant response induced by As_2O_3 in A549 cells. (A) Representative images of immunoblottings of NRF2, KEAP1 and HO-1. 80% confluent cells were treated with Vehicle (medium) or indicated concentrations of As_2O_3 for 6 h. Whole cell lysates were used for analysis and β-ACTIN was used as a loading control. (B) Quantification of NRF2 bands of three independent immunoblottings. Veh, Vehicle. n = 3; $*p < 0.05$ vs. SCR with the same treatment. (C) Concentration-response of As_2O_3-induced ARE-dependent gene expression. Cells were treated with As_2O_3 for 6 h. n = 3; $*p < 0.05$ vs. SCR with the same treatment. (D) Time-course of ARE-dependent gene expression induced by As_2O_3. Cells were treated with 40 μM of As_2O_3 for indicated time.

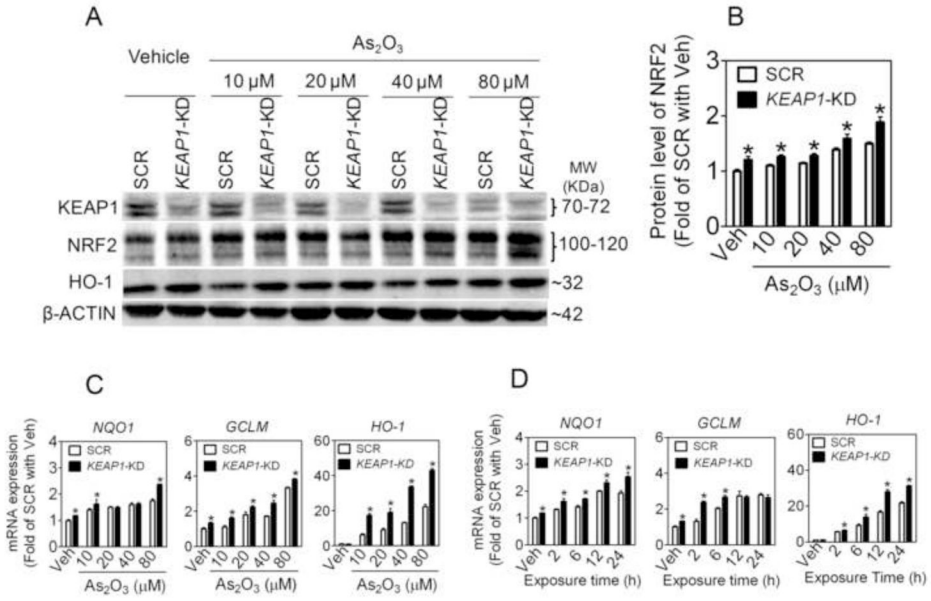

Fig. 5.

Silencing of *KEAP1* in A549 cells augments NRF2-mediated antioxidant response in response to As_2O_3 treatment. (A) Representative images of immunoblottings of KEAP1, NRF2 and HO-1. 80% confluent cells were treated with Vehicle (Veh, medium) or indicated concentrations of As_2O_3 for 6 h. Whole cell lysates were used for immunoblotting. (B) Quantification of NRF2 bands of three independent immunoblottings. n = 3; $*p < 0.05$ vs. SCR with the same treatment. (C) Concentration-response of As_2O_3-induced ARE-dependent gene expression. Cells were treated with As_2O_3 for 6 h. n = 3; $*p < 0.05$ vs. SCR with the same treatment. (D) Time-course of ARE-dependent gene expression induced by As_2O_3. Cells were treated with 40 μM of As_2O_3 for indicated time.

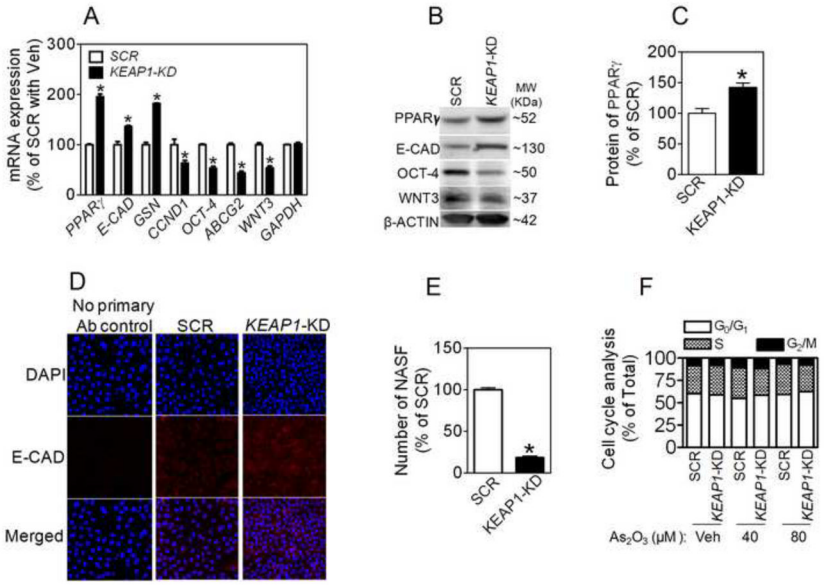

Fig. 6.

Effect of stable knockdown of *KEAP1* on expression of PPARγ and markers of differentiation and CSC features at mRNA (A) and protein (B) levels in A549 cells. n = 3; *$p < 0.05$ vs. SCR. (C) Quantification of PPARγ expression in (B). n = 3; *$p < 0.05$ vs. SCR. (D) Immunostaining of E-CAD in SCR and *KEAP1*-KD cells. (E) Quantification of NASF formed in SCR and *KEAP1*-KD cells. (F) Cell cycle analysis of SCR and *KEAP1*-KD cells. Cells were treated with As_2O_3 or Vehicle (Veh, medium) for 24 hours.

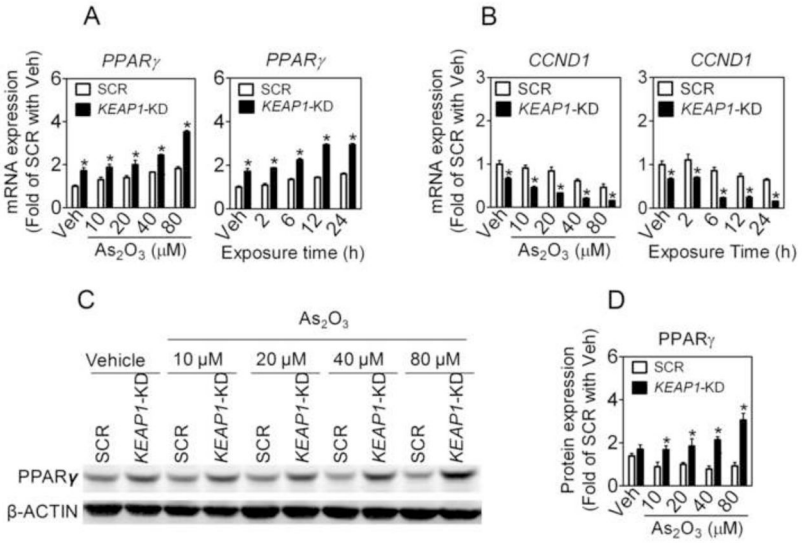

Fig. 7.

Knockdown of *KEAP1* in A549 cells augments the expression of PPARγ under basal and As_2O_3-treated conditions. (A) mRNA expression of *PPARγ*. Cells were treated with As_2O_3 for 6 h at indicated concentrations (left panel) or 40 μM of As_2O_3 for indicated time (right panel). *, $p < 0.05$ vs SCR with the same treatment. (B) *KEAP1-KD* cells show reduced mRNA expression of *CCND1 in response to* As_2O_3 treatment. Cells were treated as (A). (C) Protein level of *PPARγ*. Cells were treated with As_2O_3 for 6 h at indicated concentrations. (D) Quantification of (C). n = 3; *$p < 0.05$ vs. SCR.

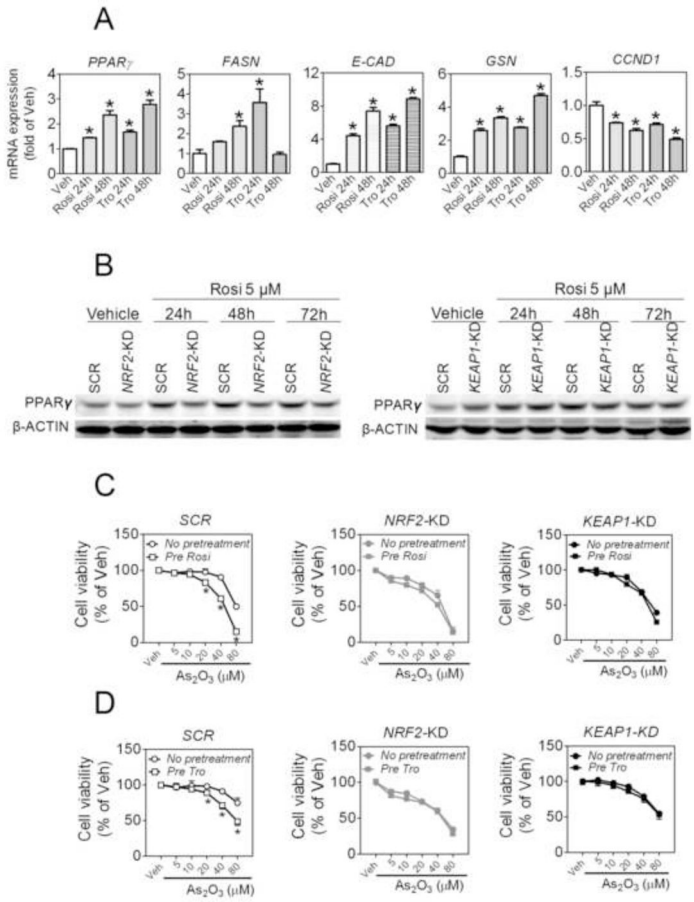

Fig. 8.

PPARγ agonists activate PPARγ in A549 cells and sensitize the cells to As_2O_3–induced cytotoxicity in a NRF2-dependent fashion. (A) Effects of rosiglitazone and troglitazone on mRNA expression of *PPARγ, FASN, E-CAD, GSN* and *CCND1* in A549 cells. A549 cells were treated with rosiglitazone (5 μM), troglitazone (10 μM) or Vehicle (Veh, medium) for 24 h and 48 h. *$p < 0.05$ vs. Veh. (B) Effects of rosiglitazone on protein expression of PPARγ in *NRF2*-KD (left panel) and *KEAP1*-KD cells (right panel). PPARγ expression was determined using immunoblotting. (C and D) Effects of rosiglitazone (C) and troglitazone (D) on As_2O_3–induced cytotoxicity in SCR (left), NRF2-KD (middle) and KEAP1-KD (right) A549 cells. Cells were pretreated with rosiglitazone (5 μM) or troglitazone (10 μM) for 48 h followed by subsequent 48 h As_2O_3 treatment. *NRF2*-KD, *NRF2*-knockdown; *KEAP1*-KD, *KEAP1*-knockdown; SCR, Scramble. *$p < 0.05$ vs. SCR with the same treatment.

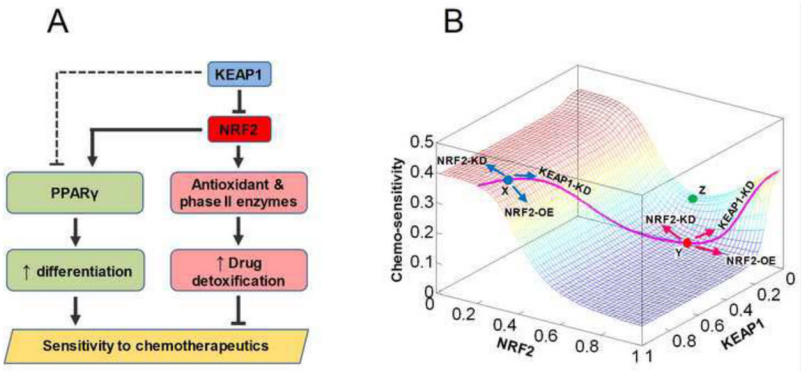

Fig. 9.

KEAP1 and NRF2 may modulate sensitivity of cells to chemotherapeutic agents through differentially regulating two opposing pathways. (A) In NSCLC cells, moderate downregulation of *KEAP1* and activation of the canonical NRF2-ARE pathway due to mutations upregulate antioxidant and phase II enzymes without appreciably activating the PPARγ pathway. Augmented drug detoxification capacity reduces the sensitivity of cancer cells to chemotherapeutics. Further knockdown of *KEAP1*, which is already lower than in normal cells, would lift its inhibition on PPAR through an unknown mechanism (dashed line), permitting activation of PPARγ by NRF2. Increased PPARγ promotes cancer cell differentiation and inhibits proliferation, thus enhancing the killing effect of chemotherapeutic agents. (B) The mathematical model recapitulated the differential responses of normal cells and cancer cells to knockdown of *KEAP1*. The landscape describes how chemo-sensitivity changes as KEAP1 and NRF2 levels are independently varied. In normal cells (represented by location X, blue dot) containing high KEAP1 and low NRF2, knockdown of *KEAP1* results in reduced sensitivity of cells to the toxicity of chemotherapeutic agents. In some cancer cells (represented by location Y, red dot) containing relatively lower KEAP1 and higher NRF2, further knockdown of *KEAP1* would result in increased sensitivity to chemotherapeutics. In other cases, combined mutations in *KEAP1* and *NRF2* may push cells to a location such as Z (green dot), where both increasing and decreasing NRF2 would increase chemo-sensitivity. The pink curve delineates the changes in NRF2 and chemo-sensitivity as the KEAP1 level is independently varied in the model.