Withdrew
September 7, 2018

This article has been withdrawn by the authors. The corresponding author identified some issues and brought them to the attention of the Journal.

The following issues were discovered in the article. The GAPDH immunoblot in Fig. 5A was reused in Figs. 1H, 2F, 2I, 3B, 4A, and 5B. The ESR spectrum from BEAS-2B cells in Fig. 2A was previously published in Son et al. ((2014) J. Biol. Chem. 289, 28660-28675) without attribution. The BEAS-2B image in Fig. 2C was reused in Fig. 6A as control siRNA. The GAPDH immunoblot from Fig. 3A was previously published in Fig. 4A of Son et al. ((2014) J. Biol. Chem. 289, 28660-28675), representing different experimental conditions. The GAPDH immunoblot from Fig. 3D was reused in Fig. 3I. A portion of the same immunoblot was reused in Fig. 4A as actin. Lanes 9-12 of the β-actin gel in Fig. 4B was reused in lanes 9-10 of the Bcl-xl AREG F1 gel in Fig. 4E. Fig. 5B contained several undeclared splices. In Fig. 5C, the control images for BEAS-2B and AsT cells were reused.

Antioncogenic and Oncogenic Properties of Nrf2 in Arsenic-induced Carcinogenesis

Background: Arsenic induced cell transformation and carcinogenesis.
Results: Arsenic-transformed cells have the property of apoptosis/autophagy resistance.
Conclusion: The constitutive activation of Nrf2 in arsenic-transformed cells up-regulates antioxidants, decreases ROS generation, and causes apoptosis resistance and tumorigenesis.
Significance: The current study identifies a role for Nrf2 in the carcinogenic mechanism of arsenic exposure.

ArSENIC (As3/H11545) is a carcinogen with considerable environmental and occupational relevancy. The present study shows that As3 and occupational exposure to arsenic transformed cells) exhibit the property of apoptosis resistance. The level of basal generation, and causes apoptosis resistance and tumorigenesis.

Antioxidant and detoxification enzymes (1). The p62/sequestosome 1 protein is a multifunction, ubiquitin-binding adapter protein that binds to ubiquitinated proteins for degradation by proteasomes and autophagy. Nuclear factor erythroid 2-related factor (Nrf2) is very low in AsT cells in correlation with elevated expressions of both antioxidant enzymes and reactive oxygen species (ROS) is very low in AsT cells in correlation with elevated expressions of both antioxidant enzymes and reactive oxygen species (ROS).

These two proteins are constitutively expressed. Nuclear factor erythroid 2-related factor (Nrf2) and p62 are constitutively expressed. These two proteins exhibit the property of apoptosis resistance. The level of basal generation, and causes apoptosis resistance and tumorigenesis.

The GAPDH immunoblot from Fig. 3C-II, G, and D was previously published in Fig. 5B without attribution. In Fig. 3B, the control images for BEAS-2B and AsT cells were reused.

Collectively, this study demonstrates that a constitutively high level of Nrf2 protein levels and antiapoptotic and antioxidant enzyme function (11). Thus, p62 accumulates in unstressed conditions, Nrf2 is degraded by proteasomal pathways, and Nrf2 is able to activate antioxidant and detoxification enzymes (1). The p62/sequestosome 1 protein that binds to ubiquitinated proteins for degradation by proteasomes and autophagy.

Under physiological conditions, Nrf2 and its downstream target proteins function as a 1 protein that inhibits apoptosis, developmentally regulates to the cellular redox states, and accumulates (5, 6). Nrf2 is not an antiapoptotic protein but increases tumor growth by promoting tumor cells to the loss of apoptosis.

Nrf2, an antioxidant transcription factor, is involved in carcinogenesis, especially in its early stages (12–14). It has been shown that inducible Nrf2 activation is important in the anticancer effects of many natural compounds, including phenethyl isothiocyanate, curcumin, and resveratrol (12). However, Nrf2 (4) is a transcription factor that binds to antioxidant response elements (ARE) in the promoters of a wide variety of antioxidant genes (11).

This study demonstrates that arsenic exposure up-regulates the antioxidant proteins catalase and superoxide dismutase as well as the antiapoptotic proteins Bcl-2 and Bcl-xL. The final consequences are decreased ROS generation, and causes apoptosis resistance and tumorigenesis.

The following issues were discovered in the article. The GAPDH immunoblot in Fig. 5A was reused in Figs. 1H, 2F, 2I, 3B, 3G, and 5B. The ESR spectrum from BEAS-2B cells in Fig. 2A was previously published in Son et al. ((2014) J. Biol. Chem. 289, 28660-28675) without attribution. The BEAS-2B image in Fig. 2C was reused in Fig. 6A as control siRNA. The GAPDH immunoblot from Fig. 3A was previously published in Fig. 4A of Son et al. ((2014) J. Biol. Chem. 289, 28660-28675), representing different experimental conditions. The GAPDH immunoblot from Fig. 3D was reused in Fig. 3I. A portion of the same immunoblot was reused in Fig. 4A as actin. Lanes 9-12 of the β-actin gel in Fig. 4B was reused in lanes 9-10 of the Bcl-xl AREG F1 gel in Fig. 4E. Fig. 5B contained several undeclared splices. In Fig. 5C, the control images for BEAS-2B and AsT cells were reused.
a constitutively high level of Nrf2 protects cancer cells against oxidative stress and chemotherapeutic agents (15). The antioncogenic or oncogenic role of Nrf2 may depend on the stage of carcinogenesis. Nrf2 activity may be desirable in early stages of carcinogenesis when the host is seeking to control premalignant carcinogenesis, but constitutive Nrf2 activation may be undesirable in later stages when it may induce resistance to apoptosis in malignant cancer cells. Despite recent advances in understanding the mechanism of As3+ induced Nrf2 activation, the role of Nrf2 in As3+-induced carcinogenesis (protection against or promotion of carcinogenesis) remains unexplored. The central hypothesis is that Nrf2 is antioncogenic in early stages of As3+-induced carcinogenesis (cell transformation) by an up-regulation of antioxidants to reduce As3+-induced ROS; however, once a cell is transformed, Nrf2 is oncogenic by inducing apoptosis resistance. This study also will examine how constitutive activation of Nrf2 contributes to apoptosis resistance as well as a decrease in ROS generation and tumorigenesis in transformed cells.

Materials and Methods

Cell Culture and Treatment—The human lung bronchial epithelial cell line BEAS-2B was obtained from the American Type Culture Collection (Manassas, VA). As3+-transformed cells were generated as described previously (16). The transformation ability and tumorigenicity of the transformed cells were confirmed by soft agar assay and xenograft assay.

Plasmids and Transfection—The overexpression plasmids for SOD1, and SOD2 in BEAS-2B cells have been described previously (17). The mCherry-EGFP fusion plasmid was obtained from Addgene (Cambridge, MA). Silencer predesigned siRNAs for Nrf2 and p62 were purchased from Ambion (Austin, TX). Human p62 (siRNA ID s16960) and Nrf2 (siRNA ID s9491) and control siRNA (AM4611) were obtained from Ambion (Austin, TX). The lipofection was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol.

Nrf2 and p62 Knockdown—Specific shRNAs for human p62 (siRNA ID s16960) and Nrf2 (siRNA ID s9491) and control siRNA (AM4611) were obtained from Ambion (Austin, TX). Four unique human 29-mer shRNA constructs in retroviral GFP vector for Nrf2 (TG311194) and p62 (TG309121) were purchased from OriGene Technologies, Inc.

Chromatin Immunoprecipitation (ChIP) Assay—A ChIP assay was performed using a Pierce Agarose ChIP kit (Thermo Scientific, Rockford, IL). Sheared chromatin was diluted and immunoprecipitated with 2 µg of anti-Nrf2 or control IgG antibody. DNA-protein complexes were eluted from the protein A/G-agarose beads using a spin column and reverse cross-linked by incubating with NaCl at 65 °C. The relative Nrf2 binding to the ARE regions of p62, Bcl-2, and Bcl-xL was analyzed by the MyiQ Single-Color Real-Time PCR Detection System (Bio-Rad) with SYBR Green PCR Master Mix. General PCR amplification was also performed using Mastercycler® thermal cyclers (Eppendorf, Foster City, CA).

Human Tissue Samples—Human lung adenocarcinoma tissue (stage Ia or IIA) was provided from the Biospecimen and Tissue Procurement Shared Resource Facility of the University of Kentucky Markey Cancer Center. Frozen lung cancer tissues were homogenized with MagNA Lyser Green Beads using a MagNA Lyser instrument (Roche Applied Science), and Western blotting analysis was performed.

Immunohistochemical Staining—Tumor tissues were fixed with 4% paraformaldehyde at room temperature for 24 h, embedded in paraffin, and sectioned (3–4 µm thickness). The slides were deparaffinized, rehydrated, and processed for immunohistochemical staining according to the VectaStain ABC kit protocol (Vector Laboratories, Burlingame, CA).

Statistical Analysis—All the data are expressed as means ± S.E. One-way analysis of variance (ANOVA) using IBM SPSS Statistics 21 was used for the multiple comparisons. A value of p < 0.05 was considered statistically significant.

Results

As3+-transformed BEAS-2B Cells Have the Property of Cell Death Resistance—Arsenic-induced transformed BEAS-2B cells (AsT cells) show reduced cell death when these cells were exposed to As3+ when compared with non-transformed cells (Fig. 1, A–C). The AsT cells had a higher proliferative potential and colony formation ability than the non-transformed BEAS-2B cells (Fig. 1, D). The AsT cells had a higher proliferative potential and colony formation ability than the non-transformed BEAS-2B cells (Fig. 1, E). The AsT cells had a higher proliferative potential and colony formation ability than the non-transformed BEAS-2B cells (Fig. 1, F). The AsT cells had a higher proliferative potential and colony formation ability than the non-transformed BEAS-2B cells (Fig. 1, G). The AsT cells had a higher proliferative potential and colony formation ability than the non-transformed BEAS-2B cells (Fig. 1, H). Further study indicated that the induced cell death occurred mainly through caspase-dependent apoptosis (Fig. 1).

A Low Level of ROS Is Caused by High Expressions of Antioxidant Enzymes, and High Expressions of Bcl-2 and Bcl-xL Are Involved in Cell Death Resistance Mechanisms of AsT Cells—Electron spin resonance (ESR) measurements show that the normal BEAS-2B cells generated a high 1:2:2:1 quartet signal indicative of ROS generation and that the signal was very low in the AsT cells (Fig. 2A). Furthermore, we stained the cells with 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate to measure intracellular ROS levels and analyzed fluorescence intensity using flow cytometry (Fig. 2B), fluorescence microscopy (Fig. 2C), and a fluorescence microplate reader (Fig. 2D). The expressions of catalase and superoxide dismutase 2 were much higher in AsT cells than in non-transformed parent cells (Fig. 2E). When the cells were exposed to As3+, the expressions of antioxidant enzymes were reduced in a dose-dependent manner in normal BEAS-2B cells, whereas the levels of antioxidant enzymes were only slightly decreased upon exposure to As3+ at 20 µM in the AsT cells (Fig. 2F). The knockdown of these antioxidant enzymes by shRNA transfection in the AsT cells restored the levels of ROS (Fig. 2G). The basal levels of Bcl-2 and Bcl-xL were higher in the AsT cells than in the non-transformed cells (Fig. 2H). The decrease in the levels of Bcl-2 and Bcl-xL by As3+ was much lesser in AsT cells in a dose- and time course-dependent manner (Fig. 2, I and J). Depletions of Bcl-2 and Bcl-xL expressions by adding ABT-263, a Bcl-2 family inhibitor, resulted in complete inhibition of Bcl-2 and Bcl-xL expressions (Fig. 2K). The viability of As3+-exposed...
transformed cells is similar to that of non-transformed cells (Fig. 2L).

Nrf2 and p62 Play a Critical Role for Cell Survival and Apoptosis Resistance of the AsT Cells—The basal levels of Nrf2 and p62 were higher in AsT cells than in non-transformed cells (Fig. 3, A and F). When normal cells were exposed to As\(^{3+}\), the expression levels of Nrf2 and p62 were slightly increased. In AsT cells, the increased expression level was dramatic (Fig. 3, B, C, G, and H). The Nrf2 downstream target proteins NAD(P)H dehydrogenase, quinone 1 (NQO1) and HO-1 were elevated in the AsT cells (Fig. 3C). The Nrf2 negative regulator Keap1 was less expressed in AsT cells than in normal BEAS-2B cells (Fig. 3, B and C). Moreover, the Keap1 expression level decreased by As\(^{3+}\) was restored after 12 h and started to increase in the BEAS-2B cells but not in the AsT cells (Fig. 3C). To further study the roles of Nrf2 and p62 in cell death resistance, we inhibited Nrf2 and p62 expressions by siRNA transfection (Fig. 3, D and I). The As\(^{3+}\)-mediated apoptosis was greatly accelerated by the blockage of Nrf2 or p62 expression in AsT cells (Fig. 3, E and J).

Nrf2 and p62 Cross-talk and As\(^{3+}\) Increase the Binding of Nrf2 to ARE Region of the p62/Bcl-2/Bcl-xL Promoter—After Nrf2 was knocked down, the basal level and increased level of Nrf2 by As\(^{3+}\) were abolished along with the p62 level (Fig.
Furthermore, depletion of p62 reduced the basal level and As$^{3+}$-exposed normal and AsT cell p62 and Nrf2 levels (Fig. 4A). These results reveal that Nrf2 and p62 cross-talk with each other in the As$^{3+}$-exposed cells. The results of the ChIP assay demonstrate that Nrf2 binding to the ARE F5 (−493 to −481) in the p62 gene promoter was slightly increased in response to As$^{3+}$ treatment in the normal BEAS-2B cells, whereas the binding was dramatically enhanced in AsT cells (Fig. 4, B and C). The decreased levels of Bcl-2/Bcl-xL expressions by As$^{3+}$ and basal levels of Bcl-2/Bcl-xL were further accelerated by siRNA Nrf2 or p62 transfection in AsT cells (Fig. 4D, lower panel).

ChIP analysis revealed that Nrf2 binds to the ARE region of the Bcl-2 promoter (F1, −2991 to −2980) and that binding to the Bcl-xL promoter (F1, −2992 to −2984) increased in As$^{3+}$-exposed transformed cells (Fig. 4, E and F).

As$^{3+}$-transformed BEAS-2B Cells Have the Property of Autophagy Deficiency—When compared with normal cells, the expression levels of autophagy-related proteins such as ATG3, ATG5, and ATG7 were low in AsT cells without (Fig. 5A) and with As$^{3+}$ treatment (Fig. 5B). As$^{3+}$ treatment dramatically increased the LC3-II level in the normal cells, and LC3-II did not accumulate significantly in the AsT cells (Fig. 5B). Fluores-

**FIGURE 2.** AsT cells have a low level of ROS and highly express antioxidant enzymes and Bcl-2/Bcl-xL proteins. The cell suspensions were prepared from AsT or normal cells, and then ESR spectra were recorded. The generation of a 1:2:2:1 quartet ESR signal is shown (A). ROS levels of the AsT cells and their parent BEAS-2B cells also were measured using flow cytometry (B), fluorescence microscopy (C), and a fluorescence microplate reader (D) after staining with 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (DCF) (5 μM) for 30 min. The basal cellular levels of catalase (CAT), SOD2, Bcl-2, and Bcl-xL were measured (E and H). The ROS levels were measured after knockdown of catalase or SOD by shRNA transfection in the AsT cells (G). The effects of As$^{3+}$ (0–20 μM) on the antioxidant enzymes and antiapoptotic proteins in the AsT cells or normal cells also were analyzed by Western blotting (F, I, and J). In addition, the quantification of Bcl-2 levels is represented (J, lower panel). For the inhibition assay, cells were incubated with As$^{3+}$ (20 μM) for 24 h in the presence or absence of ABT-263 (ABT; 10 μM). Thereafter, the expression levels of Bcl-2 and Bcl-xL were detected (K), and cell viability was determined (L). The results are shown as the mean ± S.E. (error bars) of three separate experiments. *, p < 0.05 and **, p < 0.01 versus the normal BEAS-2B cells and ***, p < 0.001 represent a significant difference between the experiments (ANOVA and Scheffé’s test). ABT-263 is a Bcl-2 family inhibitor. Scale bars in C, 100 μm. Cont, control.

**Dual Role of Nrf2 in Arsenic Carcinogenesis**

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cent puncta increased in both normal and transformed BEAS-2B cells when treated with As3+, but significantly fewer puncta were observed in AsT cells (Fig. 5, C and D). Increased LC3-II levels were observed with bafilomycin A1 (an inhibitor of autophagosome and lysosome fusion) treatment in both BEAS-2B cells and transformed cells. LC3-II levels were further increased when treated with a combination of As3+/H11001 and bafilomycin A1, suggesting that As3+/H11001 increased autophagy flux rather than blocking the fusion of autophagosomes with lysosomes in BEAS-2B cells (Fig. 5, E and F). When the BEAS-2B cells were treated with As3+ in the presence of wortmannin, an inhibitor of autophagosome initiation, the As3+-induced up-regulation of LC3-II was attenuated (Fig. 5, E, bottom, and F). To further study autophagy flux in the normal or transformed cells, we used tandem fluorescence-tagged LC3. When the transfected cells were exposed to As3+, both yellow (mCherry+/GFP+) (autophagosome) and red (mCherry+/GFP−) (autolysosome) puncta were increased in normal BEAS-2B cells, whereas only yellow puncta with low intensity were increased in AsT cells (Fig. 5, H and I). Each image of

**FIGURE 3.** Nrf2/p62 plays a critical role for cell survival and cell death resistance in the AsT cells. The basal expression levels of Nrf2 and p62 were measured in the AsT cells and non-transformed BEAS-2B cells (A and F). The normal and AsT cells were exposed to various concentrations of As3+ (0–20 μM) for 24 h (B and G) or various times (0–24 h) with 20 μM As3+ (C and H), and then the levels of Nrf2, Keap1, p62, heme oxygenase-1 (HO-1), and NQO1 were detected. To diminish Nrf2 or p62 levels, cells were transfected with siRNA specific to Nrf2 or p62. After overnight transfection, cells were exposed to 20 μM As3+ for an additional 24 h (D and I), and an apoptosis assay was performed (E and J). The expression levels of Nrf2 or p62 were quantified and are represented in the lower panels. The results represent the mean ± S.E. (error bars) of three independent experiments. *, p < 0.05; **, p < 0.01; and ***, p < 0.001 represent a significant difference between the experiments (ANOVA and Scheffé’s test). S, short exposure; L, long exposure; Cont, control.
and mCherry/H11001 is provided (Fig. 5, top and middle). These results together with those in Fig. 5, A–G, indicate that autophagosomes were fused with lysosomes to generate autolysosomes in BEAS-2B cells but not in As3/H11001-transformed BEAS-2B cells. The relatively strong autophagy flux was induced by rapamycin alone, and As3/H11001 exhibited some inhibition as shown by decreased levels of autophagosomes and autolysosomes (Fig. 5, H and I). These results show that As3/H11001 is able to decrease stress-activated autophagy flux in BEAS-2B cells. In transformed BEAS-2B cells, rapamycin alone induced autophagy flux at a lower level than that of normal BEAS-2B cells, showing that the transformed BEAS-2B cells indeed have some autophagy deficiency compared with their parent non-transformed BEAS-2B cells (Fig. 5, H and I). Similar to the results obtained using non-transformed cells, As3 was able to decrease stress-induced autophagy flux in transformed BEAS-2B cells. Taken together, the above autophagy flux results indicate that the autophagy process was uncompleted in the AsT cells. The expression levels of LC3-II in response to rapamycin and starvation condition (Fig. 5, G) and lower level of ubiquitinated p62 (Fig. 5, J) further confirmed autophagy deficiency in the AsT cells compared with the normal BEAS-2B cells. The results of the immunoprecipitation assay reveal that the high expression of Bcl-2 in the AsT cells may alter Beclin1 activity even though the Beclin1 expression levels were not different between these two types of cells (Fig. 5, K). The results of the cell viability assay reveal that As3/H11001-induced autophagy was involved in the cell death mechanism rather than the cell survival mechanism because the autophagy activator rapamycin accelerated As3+-induced cell death, whereas the autophagy inhibitor wortmannin attenuated it (Fig. 5L). Moreover, As3+-induced apoptosis was significantly attenuated in autophagy-
Defective Beclin1 knockdown cells when compared with the vehicle control (Fig. 5M). This result corresponded to the GFP-LC3 punctum-positive cell population (Fig. 5N).

Nrf2 and p62 Regulate Intracellular ROS Levels, and ROS Is a Primary Initiator of Nrf2 and p62 Induction—The low basal levels of ROS were up-regulated in the siRNA p62- or in the

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Moreover, As₃⁺-exposed BEAS-2B cells used as a control (Fig. 7F). The number of colonies was also attenuated by shRNA Nrf2- or p62-transfected AsT cells in the clonal assay (Fig. 7F).

The Expressions of Nrf2 and Its Related Proteins in Lung Adenocarcinoma—The expression levels of Nrf2, its downstream target protein NQO1, and p62 were dramatically increased in lung tumor tissues when compared with normal tissues from the same patients (Fig. 7D). However, the Keap1 expression levels were lower in all lung tumor tissues than in normal tissues (Fig. 7D). These results suggest that high expression of p62 along with down-regulation of Keap1 confers Nrf2 activation in lung adenocarcinoma. The antioxidant enzymes (catalase and SOD2) and antiapoptotic proteins (Bcl-2 and Bcl-xl) were highly expressed in lung tumor tissues (Fig. 7D). The high levels of expressions of Nrf2 and p62 in patient samples were further confirmed by immunohistochemistry (Fig. 7C). The malignance of tumor tissue was confirmed by ki67 and hematoxylin and eosin (H&E) staining (Fig. 7C).

Discussion

As₃⁺ is a group I human carcinogen and ranks first on the Agency for Toxic Substance and Disease Registry 2013 Priority List. The xenograft assay also demonstrated that the tumor growth increased in the 2-month As₃⁺-exposed Nrf2 knockdown BEAS-2B cells. The tumor growth was attenuated for 6-month As₃⁺-exposed Nrf2 knockdown cells compared with As₃⁺-exposed BEAS-2B cells used as a control (Fig. 7B). The growth of transformed cells on agar was dramatically inhibited by either Nrf2 shRNA or p62 shRNA transfection (Fig. 7E). The number of colonies was also attenuated by shRNA Nrf2- or p62-transfected AsT cells in the clonal assay (Fig. 7F).
List of Hazardous Substances (19). Occupational or environmental exposure to As$^{3+}$ induces lung cancer (20). Occupational As$^{3+}$ exposure mainly occurs in mining, manufacturing, wood preservation, and agriculture (21). Cigarette smoke, As$^{3+}$-contaminated food, and As$^{3+}$-contaminated water are non-occupational sources of human exposure to As$^{3+}$ (22). Our previous study has shown that chronic exposure of human bronchial epithelial cells to As$^{3+}$ generates ROS and that ROS are responsible for As$^{3+}$-induced transformation of these cells (16). In our present study, we have shown that As$^{3+}$-transformed cells have a low level of ROS along with highly expressed antiapoptotic proteins (Bcl-2 and Bcl-xL) and antioxidant enzymes (catalase and SOD2) as well as Nrf2 and p62. We have also shown that As$^{3+}$-transformed cells have the properties of apoptosis resistance and autophagy dysfunction.

Most types of cancer cells show an apoptosis resistance and have a high proliferative potential (23). Our present study shows that AsT cells exhibit apoptosis resistance. The cell death resistance of AsT cells might be due to high expression of Bcl-2 and Bcl-xL based on the following results: 1) the basal levels of Bcl-2 and Bcl-xL are higher in AsT cells and 2) AsT cells show higher expression of Bcl-2/Bcl-xL than non-transformed cells when exposed to As$^{3+}$ (Fig. 2, H–L). Another factor contributing to the apoptosis resistance is the low level of ROS in AsT cells. These cells carry a low level of ROS, making the cells less sensitive to As$^{3+}$ toxicity (Fig. 2, A–D). Furthermore, expressions of antioxidant enzymes such as catalase help keep a low ROS concentration and contribute to apoptosis resistance (Fig. 2, G). Nrf2 acts as an antioxidant regulator of antiapoptotic proteins in AsT cells. Nrf2 contributes to maintaining the regulation of catalase and SOD2. Moreover, Nrf2 also activates the antiapoptotic function of Bcl-2/Bcl-xL in As$^{3+}$-exposed AsT cells (Fig. 4). This up-regulation of antiapoptotic proteins by Nrf2 may be a major role in apoptosis resistance in the As$^{3+}$-transformed cells.

Autophagy involves a cell death pathway, not a survival function, in our experimental setting because cell viability was enhanced by the addition of pharmacological inhibitors or the genetic depletion of autophagy in the As$^{3+}$-exposed normal BEAS-2B cells (Fig. 5, L and M). Moreover, cell viability is decreased by the addition of rapamycin, an autophagy activator (Fig. 5L). These results suggest that defective autophagy in the transformed cells has a beneficial effect for survival mechanisms. It has been reported that dysfunction of autophagy or defective autophagy causes up-regulation of p62 (24, 25). Our data show that AsT cells are characterized by autophagy deficiency (Fig. 5), which may result in the high expression of p62 in transformed cells (Figs. 3F and 5). Notably, up-regulation of Bcl-2/Bcl-xL may further contribute to dysregulation of autophagy in the transformed cells as Bcl-2/Bcl-xL inhibits the autophagy function through the binding of the BH-3 domain of Beclin1, which is necessary for the induction of autophagy (26). Our findings also demonstrate that the binding of Bcl-2 with Beclin1 was higher in the AsT cells than in the normal BEAS-2B cells (Fig. 5K). Accumulation of p62 in transformed cells may contribute to apoptosis resistance and tumorigenesis (Figs. 3, F–J, and 7) through down-regulation of ROS generation (Fig. 6, A–E). Depletion of p62 by siRNA transfection resulted in a decrease of As$^{3+}$-induced Nrf2 expression in transformed cells (Fig. 4A). Knockdown of Nrf2 by siRNA transfection also attenuated As$^{3+}$-induced p62 expression in the AsT cells (Fig. 4A).

These results indicate a possible positive feedback loop between Nrf2 and p62 in the transformed cells. Furthermore, Nrf2 binding activities on p62 promoter were dramatically enhanced in the transformed cells upon exposure to As$^{3+}$ (Fig. 4, B and C). This might be the main reason for the increase of p62 levels during premalignant periods in the cells (Fig. 3, G and H). It is notable that high level of p62 in the transformed cells may be because of high expression of Nrf2 in these cells. Moreover, high expression of p62 contributes to apoptosis resistance and tumorigenesis (Figs. 3, F, and H). Depletion of p62 by siRNA transfection resulted in a decrease of As$^{3+}$-induced Nrf2 expression in transformed cells (Fig. 4A). Knockdown of Nrf2 by siRNA transfection also attenuated As$^{3+}$-induced p62 expression in the AsT cells (Fig. 4A).

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Further, our findings also demonstrate that binding of Bcl-2 with Beclin1 may further contribute to dysregulation of autophagy in the transformed cells as Bcl-2/Bcl-xL inhibits the autophagy function through the binding of the BH-3 domain of Beclin1, which is necessary for the induction of autophagy (26). Our findings also demonstrate that the binding of Bcl-2 with Beclin1 was higher in the AsT cells than in the normal BEAS-2B cells (Fig. 5K). Accumulation of p62 in transformed cells may contribute to apoptosis resistance and tumorigenesis (Figs. 3, F–J, and 7) through down-regulation of ROS generation (Fig. 6, A–E). Depletion of p62 by siRNA transfection resulted in a decrease of As$^{3+}$-induced Nrf2 expression in transformed cells (Fig. 4A). Knockdown of Nrf2 by siRNA transfection also attenuated As$^{3+}$-induced p62 expression in the AsT cells (Fig. 4A).

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Dual Role of Nrf2 in Arsenic Carcinogenesis

stratifies the integration of Nrf2, p62, ROS, autophagy, apoptosis, and cell survival in $\text{As}^{3+}$-induced carcinogenesis (Fig. 7G). $\text{As}^{3+}$-induced ROS have been shown to have oncogenic properties in the premalignant stage; exposure of human lung bronchial epithelial cells to $\text{As}^{3+}$ generates ROS, which are responsible for malignant transformation of these cells (16). In this stage, the Nrf2 is inducible and is regulated by Keap1. By up-regulating antioxidant enzymes to reduce ROS, this very well regulated protein has an antioncogenic property. Notably, the up-regulation of antiapoptotic proteins by Nrf2 is negligible in the premalignant stage. After transformation (e.g. the postmalignant stage), ROS play an oncogenic role. A low level of ROS increases survival and proliferation of transformed cells and tumorigenesis. In this stage, constitutive overexpression of Nrf2 has an oncogenic property. This protein is mainly regulated by p62 rather than Keap1. Its inducible property is lost. The constitutive overexpression of Nrf2 further up-regulates antioxidant enzymes and antiapoptotic proteins in the transformed cells. These phenomena cause low cellular ROS levels and the acquisition of apoptosis resistance. Furthermore, high expression of p62 due to defective autophagy leads to accelerated Nrf2 activation. This process promotes cell survival, proliferation, and carcinogenesis of transformed cells. Our findings provide a potential chemoprevention and chemotherapy strategy for metal-induced carcinogenesis through manipulation of Nrf2 expression and activity.

Author Contributions—Y.-O. S. designed the experiments and wrote the paper. P. P. performed apoptosis assay shown in Figs. 1, 2, and 6. R. V. R. performed the ChIP assay shown in Figs. 1 and 7. M. X., J. L., K. L., and G. C. provided technical assistance and contributed to the preparation of the Figs. 1 and 7.

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