Deficiency in Nrf2-GSH Signaling Impairs Type II Cell Growth and Enhances Sensitivity to Oxidants

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Redox imbalance has been implicated in the pathogenesis of many acute and chronic lung diseases. The b-Zip transcription factor Nrf2 acts via an antioxidant/electrophilic response element to regulate antioxidants and maintain cellular redox homeostasis. Our previous studies have shown that Nrf2-deficient mice (Nrf2^{-/-}) show reduced pulmonary expression of several antioxidant enzymes, which renders them highly susceptible to hyperoxia-induced lung injury. To better understand the physiologic significance of Nrf2-induced redox signaling, we have used primary cells isolated from the lungs of Nrf2^{+/+} and Nrf2^{-/-} mice. Our studies were focused on type II cells because these cells are constantly exposed to the oxidant environment and play key roles in host defense, injury, and repair processes. Using this system, we now report that an Nrf2 deficiency leads to defects in type II cell proliferation and greatly enhances the cells' sensitivity to oxidant-induced cell death. These defects were closely associated with high levels of reactive oxygen species (ROS) and redox imbalance in Nrf2^{-/-} cells. Glutathione (GSH) supplementation rescued these phenotypic defects associated with the Nrf2 deficiency. Intriguingly, although the antioxidant N-acetylcysteine drastically squelched ROS levels, it was unable to counteract growth arrest in Nrf2^{-/-} cells. Moreover, despite their elevated levels of ROS, Nrf2^{-/-} type II cells were viable and, like their wild-type counterparts, exhibited normal differentiation characteristics. Our data suggest that dysfunctional Nrf2-regulated GSH-induced signaling is associated with deregulation of type II cell proliferation, which contributes to abnormal injury and repair and leads to respiratory impairment.

Keywords: oxidative stress; lung; antioxidants; cell proliferation

Exposure to a prooxidant load or incomplete reduction of inhaled oxygen generates superoxides, hydroxyl radicals, and H₂O₂ products, which are collectively known as reactive oxygen species (ROS). ROS are also endogenously released during inflammation. The endogenous cellular defense system consists of a number of antioxidant enzymes, such as superoxide dismutases (SODs), catalase, and glutathione (GSH) peroxidases (Gpxs), as well as endogenous small thiol-containing compounds, such as GSH. This system provides protection against cellular damage

CLINICAL RELEVANCE

Redox imbalance has been implicated in the pathogenesis of lung diseases. Our data suggest that a dysfunctional Nrf2-regulated glutathione-induced signaling may contribute to abnormal lung injury and repair leading to the development of respiratory pathogenesis.

by squelching of ROS levels and subsequent ROS-initiated reactions. However, an imbalance between the prooxidant load and the antioxidant defense system can contribute to or perpetuate the pathogenesis of many acute and chronic lung diseases, including malignancy (1).

Several *ex vivo* and *in vivo* studies have clearly shown that the rapid induction of antioxidant enzyme expression in response to oxidant and toxic insults is mainly mediated by the antioxidant/electrophilic response element (ARE or EpRE), which is commonly found in the regulatory regions (promoter and/or enhancers) of detoxifying and antioxidant enzymes (2, 3). Emerging evidence strongly supports a pivotal role for Nrf2, a b-Zip transcription factor, in mediating the induction of several antioxidant enzymes in response to a variety of stimuli (2, 3). We have previously shown that Nrf2-germ line mutant mice are notably more susceptible than wild-type mice to hyperoxia (4), bleomycin (5), cigarette smoke (6), and LPS (7), suggesting that this transcription factor plays a key role in regulating prooxidant-induced lung inflammation, injury, and repair processes. Moreover, gene expression profiling in the lungs of Nrf2^{+/+} and Nrf2^{-/-} mice exposed to these prooxidants has revealed that Nrf2 regulates at least several hundred genes, including several antioxidant enzymes. The most notable of these enzymes are GSH-biosynthesizing enzymes (2, 3).

To better understand the Nrf2-regulated mechanisms that provide protection against prooxidants, we have used *in vitro* primary cultures of cells isolated from the lungs of Nrf2^{+/+} and Nrf2^{-/-} mice. We have focused on lung epithelial cells present in alveolar space because these cells are constantly exposed to an oxidant environment and they play key roles in host defense, injury, and repair (Ref. 8 and reference therein). Using this system, we now report that an Nrf2 deficiency leads to defects in lung type II cell proliferation and greatly enhances the cells' sensitivity to oxidant-induced cell death, while GSH supplementation rescues the cells from the deleterious effects of such defects. In particular, we found that although supplementation of cells with the antioxidant NAC appreciably reduced ROS levels, it failed to counteract the phenotypic defects associated with

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cell proliferation. These observations further underscore a role for Nrf2-dependent GSH-induced signaling in type II cell proliferation and cellular protection against oxidant stress.

MATERIALS AND METHODS

Isolation and Culture of Mouse Alveolar Type II Cells

The generation and characterization of mice with a disruption of the Nrf2 gene (Nrf2^{-/-} mice) have been described elsewhere (9). Both wild-type (Nrf2^{+/+}) and Nrf2^{-/-} mice were maintained under the guidelines of the institutional Animal Care Use Committee of the Johns Hopkins University Bloomberg School of Public Health. Cells were prepared from these mice by a modification of the method of Corti and coworkers (10): The lungs were perfused with 10 ml of 0.9% saline via the pulmonary artery and filled with 2 ml of dispase solution (0.8 U/ml) (Roche, Applied Science, Indianapolis, IN). The trachea was closed with a ligature, and the lungs were submerged in 1 ml dispase solution and incubated at 37°C for 45 min. Lung tissue was gently teased and minced in a 100-mm culture dish containing 15 ml of N-cyclohexyl-2-aminoethane sulfonic acid (HEPES)-buffered Dulbecco's modified Eagle's medium (DMEM) and DNase I (100 U/ml). The cell suspension was passed through 100- and 40- μ m cell strainers, centrifuged at 130 $\times$ g for 8 min at 4°C, and incubated at 37°C in a 100-mm plate coated with mouse IgG for 2 h. After incubation, the nonadherent cells were collected by centrifugation and resuspended in HEPES-buffered DMEM supplemented with 10% fetal bovine serum, 10 ng/ml keratinocyte growth factor (Sigma-Aldrich Co., St. Louis, MO), 100 U/ml penicillin, and 100 μ g/ml streptomycin. The cells were plated on culture dishes coated with fibronectin and collagen, and the medium was changed after the first day of culture and every 2 d thereafter. The cell viability of each preparation was determined by trypan blue (0.1%) dye exclusion. The identity of the type II epithelial cells was verified with Nile Red as previously described (11).

Immunostaining

Cells were grown on round coverslips, washed with PBS containing 1 mM sodium orthovanadate, and fixed in cold methanol for 10 min at -20°C. The cells were permeabilized with 0.1% Triton X-100 and blocked with 5% bovine serum albumin. They were then incubated with polyclonal anti-surfactant protein (SP)-C antibody (Seven Hills Bioreagents, Cincinnati, OH) followed by green-fluorescent Alexa Fluor 488 goat anti-rabbit IgG antibody (Invitrogen, Carlsbad, CA) for the appropriate time periods. The cells were washed, mounted using 4',6-diamidino-2-phenylindole (DAPI) (H-1500; Vector Laboratories, Burlingame, CA), and examined using a fluorescence microscope (Nikon Eclipse TE2000-S; Nikon Instruments, Melville, NY).

Western Blot Analysis

A comparable amount of whole cell lysate (40 μ g) was separated and membrane was probed with anti-GCLM (kindly provided by Dr. Terrance Kavanagh, University of Washington, Seattle, WA), anti-caspase 3 and anti- β -actin antibodies (Cell Signaling Technology, Inc., Danvers, MA).

Measurement of Cellular GSH Levels

GSH levels in whole cell extracts were determined spectrofluorometrically using *o*-phthalaldehyde (OPA), which reacts with GSH but not with oxidized glutathione (GSSG), as previously described (12). Cell lysate was mixed with equal volume of redox quenching buffer (20 mM HCl, 10 mM diethylenetriaminepentaacetic acid [DTPA] and 20 mM ascorbic acid) with 5% Trichloroacetic acid (TCA), and the supernatant was used for GSH estimation. The supernatant equivalent to 5 μ g of protein (10 μ l) was mixed with 100 μ l potassium phosphate (1 mM, pH 8.0) and followed by 15 μ l of OPA (5 mg/ml). After 30 min incubation at room temperature, the fluorescence of the sample was read at 360 nm excitation and 465 nm emission using a fluorescence plate reader (HT7000; Perkin-Elmer, Waltham, MA).

Detection of ROS

ROS were detected in type II cell cultures using 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate acetyl ester (CM-H₂DCFDA) and 10-acetyl-3,7-dihydroxyphenoxazine (ampleread)

according to the manufacturer's recommendations (Invitrogen). In brief, the cell cultures on Day 3 were washed with PBS and incubated with CM-H₂DCFDA (2 μ M) and ampleread (2 μ M) for 10 min. Dimethyl sulfoxide was used as a vehicle control. After incubation, the cultures were washed with PBS, and the images were captured using a fluorescent microscope (NIKON Eclipse, TE2000-S) with Spot software (Diagnostic Instruments, Sterling Heights, MI).

Statistical Analysis

Values are shown as means $\pm$ SD, with $n \geq 4$ for each experimental condition. Data were analyzed by a one-way ANOVA with Bonferroni's correction. Significance in all cases was defined as $P \leq 0.05$.

RESULTS

Nrf2 Is Essential for the Proliferation, but Not the Differentiation, of Lung Epithelial Cells

Type II cells isolated from the Nrf2^{+/+} mice proliferated well and reached confluence within 7 d after the initial plating (Figure 1A). In contrast, Nrf2^{-/-} cells did not proliferate (Figure 1A, *bottom panel*). The characteristics of type II cells were confirmed by multiple approaches: We first analyzed the expression of cytokeratin 8 (Krt8) and aquaporin 5 (Aqp 5), which is specific for type II and type I cells, respectively. Semiquantitative RT-PCR analysis revealed the expression of Krt8 in Nrf2^{+/+} and Nrf2^{-/-} cells (Figure 1B). As anticipated, the expression of Aqp 5 was not detected in either cell type. We next looked for the presence of lamellar bodies, which are an important and specific characteristic of type II cells. For this purpose, we incubated the cells with Nile Red, which specifically stains lamellar bodies (13); as expected, Nile Red staining was observed in both cell types (Figure 1C). To further confirm the RT-PCR and Nile Red results, we immunostained both wild-type and knockout cells with antibodies specific for SP-C (Figure 1D), which is specifically expressed by type II cells but not type I cells. SP-C antigen was present both in Nrf2^{+/+} and Nrf2^{-/-} cells, and we found no significant difference in the levels of immunostaining of SP-C antigen in the two cell types. Collectively, these data suggest that Nrf2 deficiency leads to defects in type II cell proliferation without having any significant effect on their differentiation.

Nrf2 Deficiency Leads to Oxidative Stress and Redox Imbalance in Lung Epithelial Cells

Several *in vivo* studies have shown that an Nrf2 deficiency results in oxidative stress and redox imbalance (2, 3). To further characterize this effect, we determined the levels of ROS in type II cells using DCF-DA dye, which emits green fluorescence in the presence of ROS. To confirm this result, cells were stained with ampleread reagent, which reacts with H₂O₂ in the presence of peroxidases and emits red fluorescence (Figure 2B). A greater level of green fluorescence (intracellular ROS) was apparent in Nrf2^{-/-} cells, while negligible amount of ROS were found in Nrf2^{+/+} cells (Figure 2C). To further correlate these results with those related to cellular redox status, we determined the GSH levels in both types of cells (Figure 2D). We found diminished levels of GSH (Figure 2D) levels in Nrf2^{-/-} cells (*bar 2*) compared with Nrf2^{+/+} cells (*bar 1*). Immunoblot analysis revealed diminished levels of GCLM expression in Nrf2^{-/-} cells compared with wild-type cells (Figure 2E). Taken together, these data indicate that the Nrf2 deficiency enhances ROS levels and redox imbalance due to diminished levels of GSH in type II cells.

Supplementation of Nrf2-Deficient Cells with GSH Restores Cell Proliferation

Since type II cells can take up intact GSH (14), we asked whether exogenous supplementation of Nrf2^{-/-} type II cells with GSH

Figure 1. Lung alveolar type II cell proliferation is impaired with the disruption of the Nrf2 gene. Type II cells from the lungs of Nrf2^{+/+} and Nrf2^{-/-} mice were isolated and cultured as described in MATERIALS AND METHODS. (A) Inverted microscope images of the Nrf2^{+/+} (upper panel) and Nrf2^{-/-} (lower panel) cell cultures on Days 3, 5, and 7 after initial plating. (B) Total RNA was isolated from Nrf2^{+/+} and Nrf2^{-/-} cells, and RT-PCR analysis was performed using primers specific for Krt8 and for Aqp5. β -actin was used as a reference. (C and D) Type II cells on Day 5 were characterized by staining lamellar bodies with Nile Red (C) and immunostaining with SP-C antibody (D).

would restore their intracellular redox status and cell proliferation. When Nrf2^{-/-} cell cultures were supplemented on Day 1 with 5 mM GSH, *N*-acetyl-L-cysteine (NAC), or vehicle (PBS), we found that supplementation with GSH, but not NAC, restored the GSH levels (Figure 3A). GSH also restored cell proliferation in the Nrf2^{-/-} cells to a rate similar to that of Nrf2^{+/+} cells, and the treated cells reached confluence within 7 d (Figure 3B). Interestingly, we found that supplementation of Nrf2^{-/-} cells with NAC, a precursor of GSH, did not induce cell proliferation to the same extent as was seen in the wild-type or

Nrf2^{-/-} cells supplemented with GSH, although it completely eliminated the high levels of ROS that were otherwise present in Nrf2^{-/-} cells (Figure 3C). These results collectively suggest that, in addition to scavenging ROS, GSH-induced signaling is critical for type II cell proliferation.

Nrf2 Deficiency Enhances Oxidant-Induced Protection Against Oxidants

To ensure that the Nrf2^{-/-} cells were viable, we used trypan blue dye (Figure 4A), which is taken up by dead but not intact

Figure 2. Type II cells deficient in Nrf2 undergo oxidative stress as a result of decreased GSH levels. (A) ROS were detected in type II cells on Day 3 after initial plating by using DCF-DA, which emits green fluorescence in presence of ROS. ROS were not detected in Nrf2^{+/+} cells (upper panel) but were readily detected in Nrf2^{-/-} cells (lower panel). (B) Detection of ROS in Nrf2^{+/+} and Nrf2^{-/-} cells with amplex red. (C) Relative fluorescent intensity of DCF and amplex red staining in Nrf2^{+/+} and Nrf2^{-/-} cells. The intensity of Nrf2^{-/-} cells was set as 100%. (D) GSH levels in Nrf2^{+/+} and Nrf2^{-/-} cells on Day 5 were determined. *P < 0.001 when compared with Nrf2^{+/+} cells. (E) Immunoblot analysis of cellular extracts isolated from Nrf2^{+/+} and Nrf2^{-/-} cells on Day 5 was performed with anti-GCLM and anti- β -actin antibodies.

Figure 3. GSH restores Nrf2 deficiency-related phenotypic defects such as cell proliferation. (A) Supplementation of Nrf2^{-/-} cells with GSH restores the normal GSH levels. Nrf2^{-/-} cells were grown in the absence (Nrf2^{-/-}) or presence of 5 mM GSH (Nrf2^{-/-}-GSH) or 10 mM NAC (Nrf2^{-/-}-NAC). On Day 5 the cells were washed with PBS, lysed, and GSH levels were determined. * $P \leq 0.001$ as compared with PBS (vehicle)-treated wild-type controls. (B) Supplementation of Nrf2^{-/-} cells with GSH restores cell proliferation. Inverted microscope images of the Nrf2^{-/-} (upper panels) and Nrf2^{-/-}-GSH (lower panels) cell cultures on Days 3, 5, and 7 after initial plating. Insets show the staining of the cells with Nile Red. Nrf2^{+/+} cell cultures (right panels) on Day 7 after initial plating were shown as control (see Figure 1C for Nile red staining). (C) The effects of N-acetyl-L-cysteine (NAC) on Nrf2^{-/-} type II cell proliferation. Cells were grown in the presence of GSH (5 mM) and NAC (10 mM) for 3 d and the images were captured.

cells. The Nrf2^{-/-} type II cells showed no appreciable trypan blue staining, suggesting that these cells were viable and possessed intact cell membranes. We also used Western blot analysis to measure the activation of caspase-3, a key downstream effector of apoptosis, in whole-cell lysates. Western blotting with anti-caspase-3 antibody, which detects both pro- and active forms of caspase-3, revealed a lack of caspase-3 activation in wild-type and Nrf2^{-/-} type II cells (Figure 4B). We next examined the susceptibility of the Nrf2^{+/+} cells and Nrf2^{-/-} cells supplemented without or with GSH or NAC to prooxidant stimuli (Figure 4C). Treatment of type II cells with H₂O₂ at 150 μ M revealed that the Nrf2^{-/-} cells were more sensitive to an oxidative stimulus, which resulted in greater cell death than in the case of Nrf2^{+/+} cells (43% versus 12%, $P \leq 0.001$). However, Nrf2^{-/-} cells supplemented with GSH showed a reduction in cell death to 41%, which was comparable to that seen for wild-type (Nrf2^{+/+}) cells (43%). NAC partially reduced H₂O₂ induced cell death in Nrf2^{-/-} cells (24% versus 12%), but displayed significantly greater cell death than Nrf2^{+/+} and Nrf2^{-/-}-GSH cells. These results indicate that GSH plays key roles in regulating type II cell proliferation and in providing cellular protection against oxidant stimuli.

DISCUSSION

Our findings demonstrate for the first time a critical role for Nrf2-dependent GSH-induced signaling in lung epithelial cell proliferation and cellular protection against oxidative stress. Interestingly, we found that, despite constant production of ROS, Nrf2-deficient alveolar epithelial cells were viable and expressed normal differentiation characteristics that resembled those of their wild-type counterparts (Figure 1). These results suggest that Nrf2-dependent GSH-mediated signaling, although essential for type II cell proliferation, may not be critical for maintaining the differentiation of the cells. Although previous studies using GSH-depleting chemical agents have demonstrated a role for

intracellular redox status in various biological processes (15–19), our study using freshly isolated primary cultures provides for the first time both genetic and pharmacologic evidence for a critical requirement for Nrf2-dependent GSH-induced signaling in lung type II alveolar cell proliferation.

Of particular interest is our observation that supplementation of Nrf2^{-/-} cells with NAC, a precursor of GSH, drastically lowered ROS levels but failed to induce cell proliferation to the extent seen in wild-type cells and Nrf2^{-/-} cells supplemented with GSH. Several studies, including ours, have shown that NAC attenuates or provides protection against prooxidant stimuli both *in vitro* and *in vivo* (7, 20). The inability of NAC to rescue the growth arrest of Nrf2^{-/-} cells is not surprising, however, because these cells express diminished levels of the γ -glutamyl cysteinyl ligase (GCL) components, GCLC and GCLM (Figure 2E), that are critical for *de novo* GSH biosynthesis and provide protection against prooxidants (21). Thus, it is likely that Nrf2^{-/-} cells may only inefficiently convert NAC to GSH, a process that would otherwise cause the intracellular GSH to rise to levels that are adequate for cell proliferation (16, 17, 22). Thus, it appears that in addition to its drastically reducing the high levels of ROS, signaling induced by GSH is critically required for normal proliferation of lung epithelial cells. Although the sources contributing to high levels of ROS in Nrf2^{-/-} cells remains to be investigated, it is likely that diminished levels of GSH may lead to an inefficient cellular detoxification of endogenous ROS levels generated by mitochondria constitutively in Nrf2^{-/-} cells. In addition, it is possible that Nrf2 deficiency may result in dysregulation of ROS-generating enzymes, such as Nox/Duox, Rac, or RAS, thereby contributing to elevated levels of ROS.

Unlike primary Nrf2^{-/-} type II epithelial cells, immortalized embryonic fibroblast cells (MEFs) lacking the Nrf2^{-/-} gene, established according to an NIH3T3 protocol, grow normally and do not display elevated levels of ROS or redox imbalance when compared with Nrf2^{+/+} MEFs. In fact, Nrf2^{-/-} MEFs

Figure 4. GSH confers protection against oxidant-induced death in the absence of Nrf2. (A) Staining of Nrf2^{-/-} type II cells with trypan blue. (B) Whole cell lysates (~40 µg) isolated from Nrf2^{-/-} and Nrf2^{+/+} cells were separated and immunoblotted with anti-caspase-3 antibody. Membrane was stripped and probed with anti-β-actin antibodies. (C) Nrf2^{-/-} cells on Day 0 supplemented without (Nrf2^{-/-}) or with 5 mM GSH (Nrf2^{-/-}-GSH) or 10 mM NAC (Nrf2^{-/-}-NAC). On Day 5, cells were washed with PBS and replaced the medium with 150 µM H₂O₂ for 5 h. Cell viability was determined using a Cell Titer-Glo Live and Death Assay kit. The percent cell viability was calculated by setting the respective untreated control value to 100 percent. **P* ≤ 0.001 as compared with PBS (vehicle)-treated respective controls. #*P* ≤ 0.01 as compared with H₂O₂-treated Nrf2^{+/+} and Nrf2^{-/-}-GSH cells. \$*P* ≤ 0.01 as compared with H₂O₂-treated Nrf2^{-/-} cells.

proliferate more rapidly than do their isogenic wild-type counterparts (unpublished data). However, Nrf2^{-/-} MEFs are more susceptible than Nrf2^{+/+} cells to various stressors. There are several possible reasons for this discrepancy. One possibility is that during immortalization, the MEFs that survive selection may have undergone genetic changes that could drive cell proliferation in the absence of Nrf2. This type of discordant behavior has been reported for several genes. For example, primary MEFs lacking the Jun-D/AP1 transcription factor proliferate poorly and display senescent characteristics (23); in contrast, established MEFs lacking Jun-D showed an increased rate of cell proliferation when compared with isogenic wild-type cells. Another likely possibility to explain our results is that the effects of Nrf2 on cell proliferation are context-dependent. However, we can probably rule out this possibility because we found that primary lung fibroblasts lacking Nrf2 also proliferate poorly and display elevated levels of ROS (data not shown).

The decreased rate of cell proliferation that we observed for wild-type cells was clearly not caused by apoptosis of Nrf2^{-/-} cells, since we observed no cellular or nuclear staining of Nrf2^{-/-} cells with trypan blue (Figure 4A). Consistent with this result, we found no significant differences in the activation of caspase 3, the pro-apoptotic factor, in wild-type and Nrf2^{-/-} cells (Figure 4B). Although somewhat surprising, the lack of apoptosis or activation of caspase-3 that we observed in Nrf2^{-/-} type II cells, despite elevated levels of ROS, suggests that high levels of ROS

per se do not initiate death-activating signals in type II cells, at least under our experimental conditions. Further studies are needed to determine whether this response pattern is an intrinsic property of type II cells, which are constantly exposed to an oxidant environment in alveolar space (24), or whether they require additional cues, such as activation of Fas or TNF-α-activated signals. Consistent with this possible need for additional cues, exposure to H₂O₂ induced greater levels of death in Nrf2^{-/-} type II cells than in wild-type cells (Figure 4C). Moreover, we have previously reported that Nrf2 deficiency enhances the sensitivity of T-lymphocytes to Fas-mediated apoptosis (25).

Although the exact mechanisms by which GSH controls the proliferation of lung type II cells remain to be investigated, recent studies have shown that an oxidized intracellular environment causes protein glutathionylation, while reducing conditions favor protein deglutathionylation (19, 26–28). Both protein glutathionylation and deglutathionylation affect the function of various proteins, including transcription factors such as NF-κB and c-Jun (see review elsewhere; 19). We believe that decreased GSH levels in Nrf2^{-/-} cells may cause dysregulation of signal transduction pathways and effector transcription factor activation, which are required for gene expression. Delineating the glutathionylation and deglutathionylation mechanisms regulated by Nrf2-GSH-induced signaling may provide further insight into the roles of redox signaling in type II cell proliferation and differentiation during lung injury and repair. Preliminary expression profiling analysis has revealed that Nrf2, acting via GSH, regulates the expression of several antioxidant enzymes and genes involved in cell proliferation, including receptors, growth factors, kinases, and transcription regulators (data not shown).

In summary, the present study using freshly isolated primary cultures has provided genetic and pharmacologic evidence that Nrf2-dependent GSH-induced signaling plays a key role in lung type II cell proliferation and cellular protection against oxidant-induced death. Our findings suggest that in addition to quenching high levels of ROS, signaling induced by GSH is critically required for proper cell proliferation but may not be essential for maintaining differentiation. Since redox imbalance has been implicated in the pathogenesis of many acute and chronic lung diseases, this cell culture model (system) may be useful in further elucidating the precise mechanisms by which redox signaling regulates type II cell proliferation and cellular protection against oxidants during lung injury and repair.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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