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Research Article

Proteomic and redox-proteomic study on the role of glutathione reductase in human lung cancer cells

Glutathione reductase (GR), a cytosolic protein, plays a vital role in maintaining a correct redox status in cells. However, comprehensive investigations of GR-modulated cellular responses, including protein level alteration and redox regulation, have yet to be performed. In this study, we cultured a human lung adenocarcinoma line transfected with empty pLKO.1 vector as a control, CL1-0shControl, and its GR-knockdown derivative, CL1-0shΔGR, to evaluate differential protein level alteration and redox regulation of these two cell lines. We identified 34 spots that exhibited marked changes in intensities, and 13 proteins showing significant changes in thiol reactivity, in response to GR depletion. Several proteins involved in redox regulation, calcium signaling, cytoskeleton regulation, and protein folding showed significant changes in expression, whereas proteins involved in redox regulation, protein folding, and glycolysis displayed changes in thiol reactivity. Interestingly, GR knockdown induces peroxiredoxin-1 overexpression in the air-exposed tissue and high oxygen consuming tissue such as cornea and liver, but not in the low oxygen consuming tissues such as breast and uterine. In summary, we used a comprehensive lung adenocarcinoma based proteomic approach for identifying GR-modulated protein expression alteration and redox modification. Based on our research, this is the first comprehensive proteomic and redox-proteomic analysis used to investigate the role of GR in a mammalian cell model.

Keywords:
DIGE / Glutathione reductase / MALDI-TOF MS / Proteomics / Redox-proteomics
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1 Introduction

Reactive oxygen species (ROS) are byproducts of aerobic respiration that can chemically modify various biomolecules and cause cell injury. Previous studies have shown that numerous human diseases are associated with the overproduction of ROS. Accordingly, cells have evolved several mechanisms to prevent oxidative damage by generating intracellular antioxidants, expressing redox-regulated enzymes, and maintaining high concentrations of ROS scavengers such as glutathione (GS). The sulfhydryl form of GS (GSH) can reduce oxidized thiol groups or hydrogen peroxide through GS peroxidase to form disulfide-linked GS (GSSG). GSH can be regenerated from GSSG through glutathione reductase (GR) using cellular nicotinamide adenine dinucleotide phosphate (NADPH) as the reducing source. Thus, GR plays a central role in maintaining the correct redox status in cells [1].

Recent reports showed that high levels of intracellular ROS can activate growth factor receptors, cytokine receptors as well as intracellular signaling molecules such as transcription factors [2–4]. On the other hand, the high concentrations of ROS are also mediators of damage to plasma membranes, nucleic acids, and lipid and play a critical role to promote the formation of cancers [5, 6]. In current understanding,

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Abbreviations: **CCB**, Colloidal Coomassie Blue; **DCFH-DA**, 2,7-dichlorofluorescin diacetate; **GS**, glutathione; **GSH**, sulfhydryl form of GS; **GAPDH**, glyceraldehyde 3-phosphate dehydrogenase; **GSSG**, disulfide-linked GS; **GR**, glutathione reductase; **ICy dyes**, iodoacetylated cyanine dyes; **PI**, propidium iodide; **ROS**, reactive oxygen species

Colour Online: See the article online to view Figs. 2–4 in colour.

oxidation and reduction of proteins are one of the major mechanisms to modulate the functions of these proteins. A number of chemical moieties have been found to be potential targets of ROS in cells. One of these, the free thiol group of cysteine residues is a potent nucleophilic target and can undertake numerous redox-induced modifications under physiological conditions. Oxidative modifications of free thiol groups can not only form the disulfide bond between cysteine residues, also modify the free thiol groups into the sulfenic acid, sulfinic acid, and sulfonic acid relying upon the oxidative capacity of the oxidant [7]. Notably, oxidation of free thiol groups to sulfinic and sulfonic acids is irreversible under physiologic conditions and induces loss of biological functions of proteins [8, 9].

We investigated the influence of GR depletion using shRNA knockdown on human lung cancer cells. To examine the levels of differential expression and redox regulation of lung cancer proteins associated with GR depletion, a quantitative proteomics-based approach was performed using cysteine- and lysine-labeling 2D-DIGE combined with MALDI-TOF MS analysis [10–14]. A panel of proteins was obtained and found to be differentially altered in both protein expression and thiol reactivity, both with and without GR depletion.

2 Materials and methods

2.1 Chemicals and reagents

Generic chemicals were purchased from Sigma-Aldrich (St. Louis, USA) and the reagents for 2D-DIGE were purchased from GE Healthcare (Uppsala, Sweden). The synthesis of the ICy3 and ICy5 dyes (where ICy dyes stand for iodoacetylated cyanine dyes) has been previously reported by our group [15]. All the chemicals and biochemicals used in this study were of analytical grade. All primary antibodies were purchased from Genetex (Hsinchu, Taiwan) and anti-rabbit secondary antibody was purchased from GE Healthcare.

2.2 Cell lines and cell cultures

CL1-0, human lung adenocarcinoma cells that were used in this study, was originally obtained from the American Type Culture Collection (Manassas, VA, USA) and were cultured in DMEM medium supplemented with 10% fetal bovine serum, L-glutamine (2 mM), streptomycin (100 μg/mL), penicillin (100 IU/mL; all from Gibco-Invitrogen, UK). The experimental control strain, CL1-0shControl, was selected from CL1-0 cells transfected with the empty pLKO.1 vector in the presence of puromycin (0.8 μg/mL). The GR-knockdown strain, CL1-0shΔGR, was selected from CL1-0 cells transfected with the pLKO.1-shGR vector in the presence of puromycin (0.8 μg/mL) as described previously [16]. All cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2.

2.3 Immunoblotting analysis

The detailed experimental procedures for immunofluorescence analysis were described in our previous reports [17]. All primary antibodies used for expression validation were purchased from Genetex (Hsinchu, Taiwan).

2.4 Assay for endogenous ROS using DCFH-DA

The detailed experimental procedures have been described in our previous study [13]. Briefly, CL1-0shControl and CL1-0shΔGR cells (10 000 cells/well) were incubated in culture medium for at least 24 h. After two washes with PBS, cells were treated with 10 μM of 2,7-dichlorofluorescin diacetate (DCFH-DA; molecular probes) at 37°C for 20 min, and subsequently washed with PBS. Fluorescence was recorded at an excitation wavelength 485 nm and emission wavelength at 530 nm.

2.5 MTT cell viability assay

The detailed experimental procedures have been described in our previous study [18]. Briefly, CL1-0shControl and CL1-0shΔGR cells were trypsinized, counted using a haemocytometer and 5000 cells/well were seeded into 96-well plates. The culture was then incubated for 24 h followed by removal of the medium. A total of 50 μL of MTT (3-(4,5-cimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) working solution (1 mg/mL; Sigma) was added to the cells in each well, followed by a further incubation at 37°C for 4 h. The supernatant was carefully removed; 100 μL of DMSO was added to each well, and the plates were shaken for 20 min. The absorbance of samples was then measured at a wavelength of 540 nm in a multiwell plate reader. Values were normalized against the untreated samples and were averaged from eight independent measurements.

2.6 Flow cytometry analysis for apoptosis detection

The detailed experimental procedures have been described in our previous study [19]. Briefly, annexin V/propidium iodide (PI) double assay was performed using the annexin V, Alexa Fluor® 488 Conjugate Detection kit (Life technologies). CL1-0shControl and CL1-0shΔGR cells were typsinized from culture dish and washed twice with cold PBS. A total of 1×10^6 cells were resuspended in 500 μL binding buffer and stained with 5 μL Alexa Fluor 488 conjugated annexin V according to the manufacturer's instructions. One microliter PI (100 μg/mL) was added and mixed gently to incubate with cells for 15 min at room temperature in the dark. After incubation, samples were subjected to flow cytometry analysis for 1 h using BD Accuri C6 Flow Cytometry (BD Biosciences, San Jose, CA, USA) . The data were analyzed using Accuri CFlow@ and CFlow Plus analysis software (BD Biosciences).

2.7 Sample preparation for total cellular protein and thiol reactivity analysis

The detailed experimental procedures have been described in our previous study [20]. Briefly, 150 μg of protein sample was minimally labeled with 375 pmol of either Cy3 or Cy5 for comparison on the same 2DE. To facilitate image matching and cross-gel statistical comparison, a pool of all samples was also prepared and labeled with Cy2 at a molar ratio of 2.5 pmol Cy2 per μg of protein as an internal standard for all gels. Thus, the triplicate samples and the internal standard could be run and quantified on multiple 2DE. All samples were run in triplicate against the standard pool. For redox DIGE analysis, cells were lysed in 2DE buffer (4% w/v CHAPS, 8 M urea, 10 mM Tris-HCl pH 8.3, and 1 mM EDTA) in the presence of ICy3 or ICy5 (80 pmol/μg protein) on ice to limit postlysis thiol modification. Test samples were labeled with the ICy5 dye and mixed with an equal amount of a standard pool of both samples labeled with ICy3. Since ICy dyes interfered with the protein assay, protein concentrations were determined on replica lysates not containing dye. All samples were run in triplicate against the standard pool. Afterward, the fluorescence 2DE were scanned directly between the low fluorescent glass plates using an Ettan DIGE Imager and gel analysis was performed using DeCyder 2D Differential Analysis Software v7.0 (GE Healthcare) to codetect, normalize, and quantify the protein features in the images. Features detected from nonprotein sources (e.g., dust particles and dirty backgrounds) were filtered out. Spots displaying a $\geqq 1.5$ average-fold increase or decrease in abundance with a p-value < 0.05 were selected for protein identification.

2.8 Protein staining, in-gel digestion, and MALDI-TOF/TOF MS analysis

Colloidal Coomassie Blue (CCB) G-250 staining was used to visualize CyDye labeled protein features in 2DE followed by excising interesting poststained gel pieces for MALDI-TOF MS identification. The detailed procedures for protein staining, in-gel digestion, MALDI-TOF MS analysis, and the algorithm used for data processing have been described in our previous publication [21]. The spectrometer was also calibrated with a peptide calibration standard (Bruker Daltonics) and internal calibration was performed using trypsin autolysis peaks at m/z 842.51 and 2211.10. Peaks in the mass range of m/z 800–3000 were used to generate a peptide mass fingerprint that was searched against the Swiss-Prot/TrEMBL database (released on November 2011) with 53 3049 entries using MASCOT software v2.3.02 (Matrix Science, London, UK). The following parameters were used for the search: *Homo sapiens*—tryptic digest with a maximum of one missed cleavage; carbamidomethylation of cysteine, partial protein N-terminal acetylation, partial methionine oxidation, and partial modification of glutamine to pyroglutamate and a mass tolerance of 100 ppm. Identification was accepted based on significant MASCOT Mowse scores ($p < 0.05$), spectrum annotation, and observed versus expected molecular weight and pI on 2DE as well as at least five peptides in each identified protein. MALDI-TOF/TOF analysis was performed on the same instrument using the LIFT mode. MS/MS ion searches were performed using MASCOT with the same search parameters as above and using an MS/MS tolerance of ±0.2 Da.

2.9 Validation of thiol reactivity changes by immunoprecipitation coupled to immunoblotting

The detailed experimental procedures have been described in our previous study [12]. Briefly, CL1-0shControl and CL1-0shΔGR cells were lysed in the presence of ICy3 or ICy5 dyes to limit postlysis thiol modification. After labeling reaction and immunoprecipitation, ICy images were scanned directly between low-fluorescence glass plates using an Ettan DIGE Imager (GE Healthcare) followed by immunoblotting analysis with the same primary antibody to detect the specific protein. The immunoblotting procedure is described above.

3 Results

3.1 GR expression in CL1-0 cells, CL1-0shControl, and the GR shRNA knockdown strain, CL1-0shΔGR

To investigate the regulatory roles of GR in living cells, CL1-0shΔGR lung cancer cells were selected from CL1-0 cells that were transfected with GR shRNA in a puromycin-containing medium. The CL1-0shΔGR cells exhibited a distinct downregulation in GR levels compared to those of CL1-0 cells and CL1-0shControl. This indicates that CL1-0shΔGR and CL1-0shControl cells are appropriate for use as a model to study GR-modulated cellular protein alteration and redox regulation (Fig. 1A).

GR has been recognized to reduce intracellular ROS levels by catalyzing GSSG into GSH. The dysregulation of GR might lead to ROS accumulation and mediate modifications on biomolecules, such as DNA, lipids, and proteins, in cells. Our experimental results indicated that shRNA knockdown of GR in CL1-0 cells leads to an increased ROS level compared to the CL1-0shControl cells (Fig. 1B). However, there was no discernible change in cell viability and cell apoptosis between CL1-0shControl and CL1-0shΔGR. This suggests that, in the absence of environmental oxidative stress (Fig. 1C), GR contributes to a redox balance in CL1-0 cells, but does not modulate cell survival or death. Numerous redox-regulatory pathways may be activated to reduce ROS-induced cell damages, namely peroxiredoxin/thioredoxin pathways, GS peroxidase/GS *S*-transferase pathways, and protein disulfide isomerase/glutaredoxin/thioredoxin pathways.

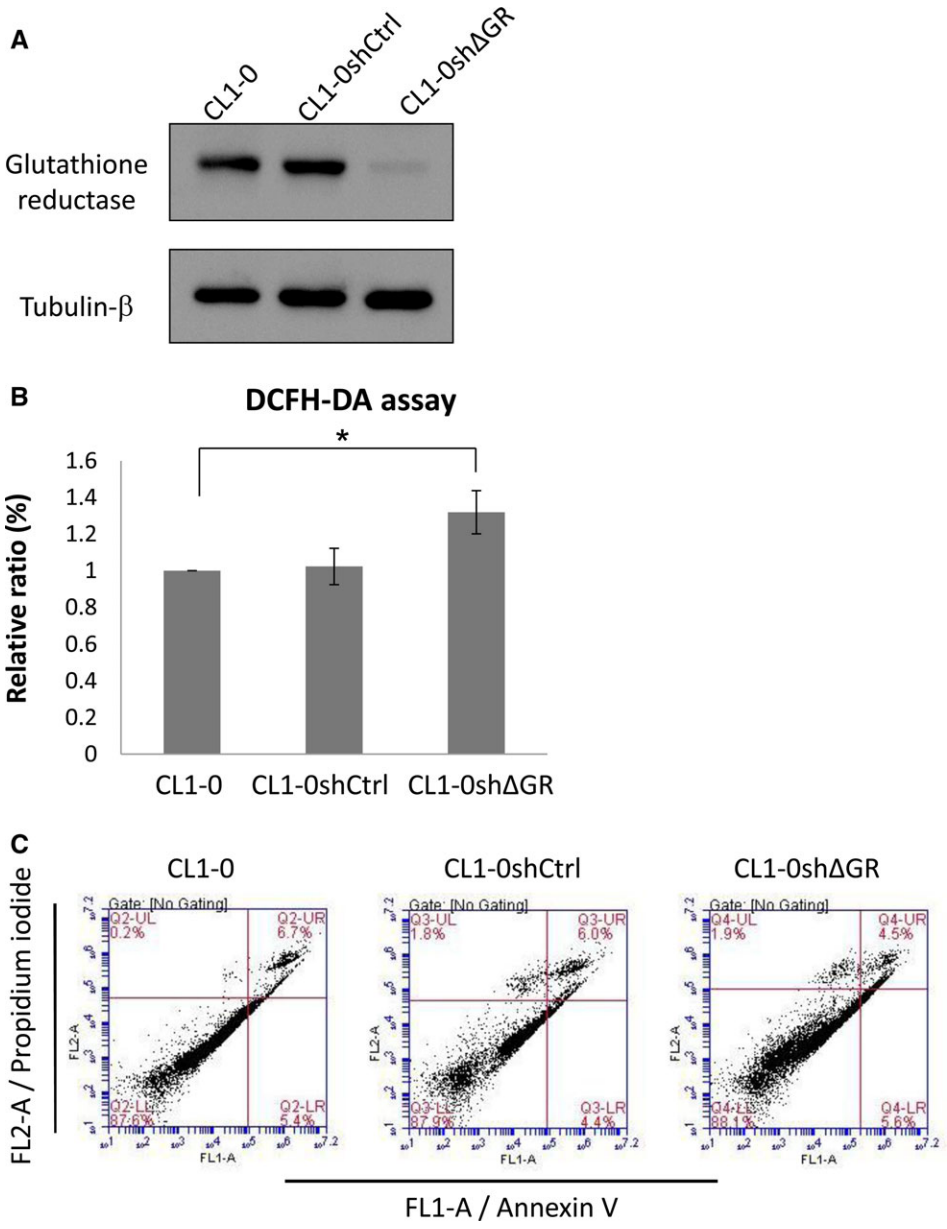
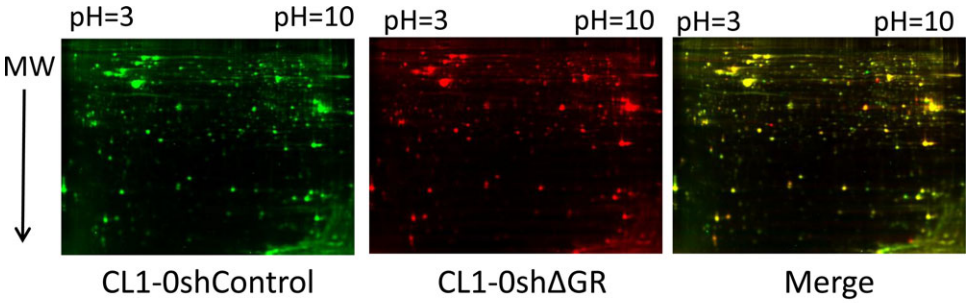


Figure 1. GR levels, ROS levels, and cell viabilities of CL1-0 cells, CL1-0shControl, and CL1-0shΔGR. (A) CL1-0 cells, CL1-0shControl, and CL1-0shΔGR cells grown overnight and the expression of GR in these three cell lines were monitored by immunoblotting. GAPDH was used as loading control in this study. (B) DCFH-based intracellular ROS production assays were performed where 10 000 CL1-0 cells, CL1-0shControl, and CL1-0shΔGR cells were plated into 96-well plates in medium containing 10% FBS. After overnight incubation, the cells were treated with 10 μM of DCFH-DA at 37°C for 20 min and the fluorescence images were recorded at excitation and emission wavelengths of 485 and 530 nm, respectively. (C) A total of 10^6 CL1-0 cells, CL1-0shControl, and CL1-0shΔGR cells were incubated with Alexa Fluor 488 and PI in 1× binding buffer at room temperature for 15 min, and then stained cells were analyzed by flow cytometry to examine the existence of GR on apoptosis in CL1-0, CL1-0shControl, and CL1-0shΔGR cells. Annexin V is presented in *x*-axis as FL1-H and PI is presented in *y*-axis as FL2-H. LR quadrant indicates the percentage of early apoptotic cells (annexin V positive cells), and UR quadrant indicates the percentage of late apoptotic cells (annexin V positive and PI positive cells).

3.2 2D-DIGE and MALDI-TOF MS analysis of GR depletion induced proteomic alterations in CL1-0 cells

To comprehensively analyze GR depletion induced proteomic alterations, the biological triplicates for CL1-0shControl and CL1-0shΔGR cells were compared using 2D-DIGE. Image analysis revealed more than 1902 well-defined protein features (Fig. 2). Those appearing in all replicates and showing a change in average abundance of >1.5-fold ($p < 0.05$) between CL1-0shControl and CL1-0shΔGR cells were excised from gels for protein identification. MALDI-TOF MS identification revealed 34 proteins that were differentially expressed between CL1-0shControl and CL1-0shΔGR cells (Fig. 2, Supporting Information Fig. 1, and Supporting Information Table 1). Using functional information from the KEGG and Swiss-Prot pathway databases, numerous biological functions were ascribed to the identified proteins with possible roles in GR depletion induced proteome alterations. Supporting Information Fig. 2 shows a comparison of the spot intensity profiles of the differential protein features. Proteins known to regulate redox regulation, calcium signaling, cytoskeleton regulation, and protein folding were found to be altered in abundance between CL1-0shControl and CL1-0shΔGR cells. Nearly two-thirds of the differentially identified protein spots were cytosolic proteins (Supporting Information Fig. 2).

To further verify the upregulation or downregulation of the identified proteins, we performed an immunoblot analysis of proteins regulated by the depletion of GR and



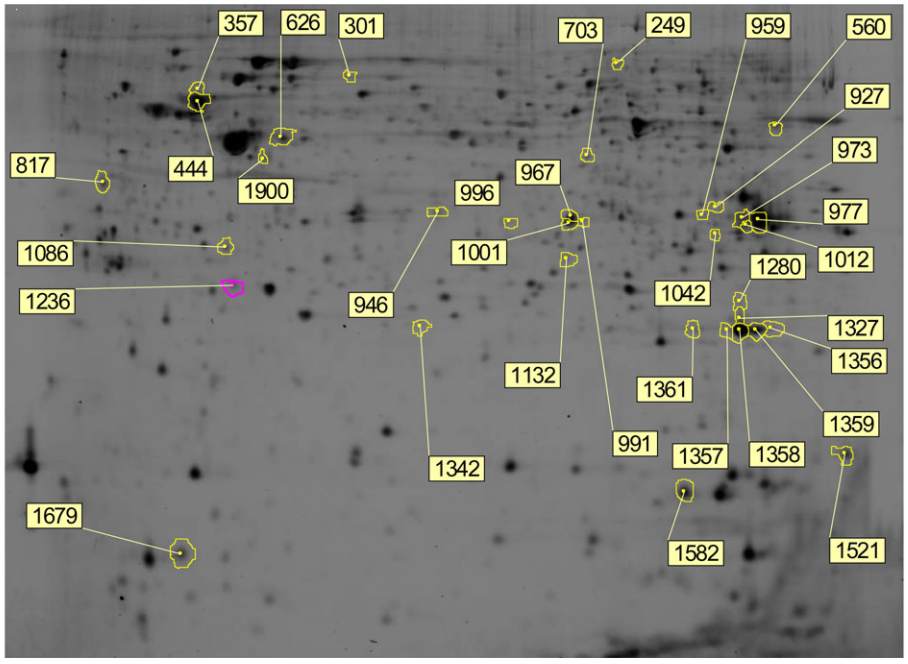


Figure 2. 2D-DIGE analysis of GR knockdown induced proteome alterations in CL1-0 cells. CL1-0shControl and CL1-0shΔGR cells were lysed and arranged for a triplicate 2D-DIGE experiment. Protein samples (150 μg each) were labeled with Cy-dyes and separated using 24 cm, pH 3-10 nonlinear IPG strips. 2D-DIGE images of CL1-0 cells and CL1-0ΔGR cells at appropriate excitation and emission wavelengths were pseudocolored and overlaid with ImageQuant Tool (GE Healthcare) (upper images). The differentially expressed identified protein features are annotated with spot numbers (bottom image).

compared it with proteins in CL1-0shControl cells (Fig. 3). We used specific antibodies against annexin A1, peroxiredoxin 1, GAPDH, galectin-1, and cyclophilin A. In general, there was a positive correlation between changes observed in 2D-DIGE analysis and the immunoblot analysis. Validation confirmed the upregulation of annexin A1, peroxiredoxin 1, GAPDH, and galectin-1 in GR-depleted CL1-0shΔGR cells compared to the levels in CL1-0shControl cells (Fig. 3A–D). By contrast, cyclophilin A was confirmed to be downregulated in response to the depletion of GR (Fig. 3E).

3.3 Redox-proteomic analysis of GR depletion induced cysteine modifications in CL1-0 proteins

To investigate how GR modulates the redox regulation of cellular proteins, we applied a recently developed redox-2D-DIGE strategy using ICy dyes to evaluate alterations in protein thiol reactivity induced by GR depletion. First, CL1-0shControl and CL1-0shΔGR cells were lysed in the presence of ICy5 in triplicate. Next, individual ICy5-labeled samples were run on 2DE against an equal load of an ICy3-labeled standard pool, which comprised an equal mixture of two sample types to aid in spot matching and improve the accuracy of quantification (Fig. 4). The ICy5-labeled samples were subsequently labeled with lysine-labeling Cy2 dye as an internal protein level control used to normalize the corresponding ICy5/ICy3 signals (Supporting Information Table 2). We detected 2207 protein features, of which 116 displayed statistically significant changes in labeling in response to GR depletion. Finally, 13 of these features were identified as unique gene products using MALDI-TOF peptide mass fingerprinting (Supporting Information Table 2). All the identified proteins contained at least one cysteine according to the Swiss-Prot database. Because the target of the ICy dyes are reduced cysteinyl thiols, these results suggest that GR depletion modified the oxidative status of some of these thiol groups. We classified these identified proteins according to their

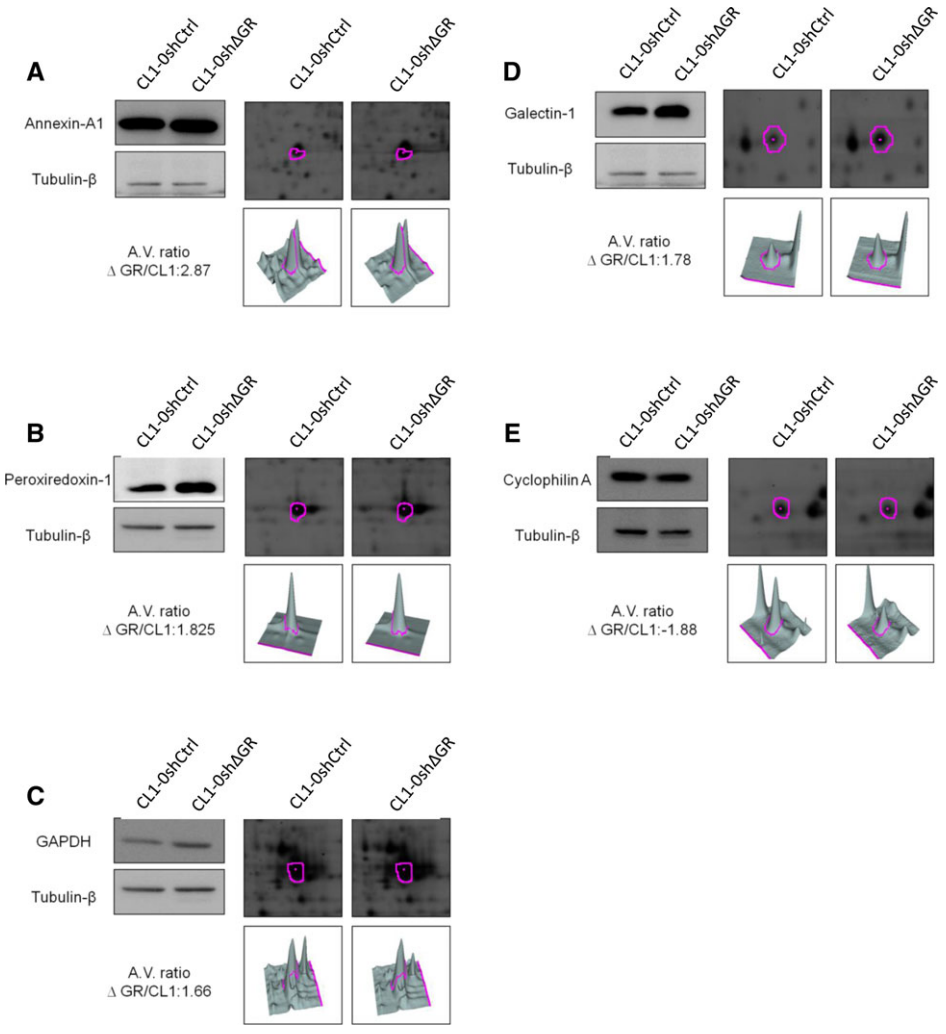


Figure 3. Representative immunoblot analyses and 2D-DIGE images for selected differentially expressed proteins [(A) annexin A1, (B) peroxiredoxin 1, (C) GAPDH, (D) galectin-1, and (E) cyclophilin A] identified by 2D-DIGE and MALDI-TOF MS between CL1-0shControl and CL1-0shΔGR cells. The levels of identified proteins, annexin A1, peroxiredoxin 1, GAPDH, galectin-1, and cyclophilin A in CL1-0shControl and CL1-0shΔGR cells were confirmed by immunoblot (left images). Beta-tubulin was used as loading control in this study. The levels of these intracellular proteins were also visualized by fluorescence 2DE images (top right images) and 3D spot images (bottom right images).

biological functions and subcellular locations: 25% of the proteins were predominantly involved in redox regulation, 12% were involved in protein folding, and 12% were involved in glycolysis. In addition, 59% of the proteins were cytosolic, 17% were nuclear proteins, 12% were plasma membrane proteins, 6% were mitochondrial proteins, and 6% were located in the Golgi apparatus (Supporting Information Fig. 3).

To validate alterations in thiol reactivity of the identified proteins, Stress-70 protein and PHB, immunoprecipitation, and immunoblotting were performed to compare differences in free thiol group levels and protein level alterations between CL1-0shControl and CL1-0shΔGR cells. In brief, the thiol reactivities of the Stress-70 protein and PHB were monitored by immunoprecipitation of ICy dye labeled CL1-0shControl and CL1-0shΔGR cells. The fluorescent images were then scanned using an Ettan DIGE Imager. Subsequently, immunoblot analysis with the same primary antibodies was used to measure the expression levels of the Stress-70 protein and PHB. The observed thiol reactivities of the Stress-70 protein and PHB were normalized with their respective expression levels using immunoblotting to accurately measure the GR knockdown induced alteration in thiol reactivities. Validation confirmed the increase of free thiol content per molecule of the Stress-70 protein and the increase of free thiol content per molecule of PHB in CL1-0shΔGR cells compared to CL1-0shControl cells (Fig. 5).

3.4 Immunoblot analysis of GR depletion induced peroxiredoxin-1 expression in different cell types

In order to globally investigate the cell response under GR knockdown, we examined the antioxidant protein, peroxiredoxin-1, levels in response to GR knockdown in other cell types including HCE-2 (corneal cells), HepG2 (liver cancer), MCF-7 (breast cancer), and MES-SA (uterine cancer). We found that GR knockdown induces the overexpression of peroxiredoxin-1 in HCE-2 and HepG2. In contrast, GR knockdown induces insignificant change of peroxiredoxin-1 in either MCF-7 or MES-SA cells (Fig. 6). The results suggest that GR knockdown induces peroxiredoxin-1 overexpression in the air-exposed tissue and high oxygen consuming tis-

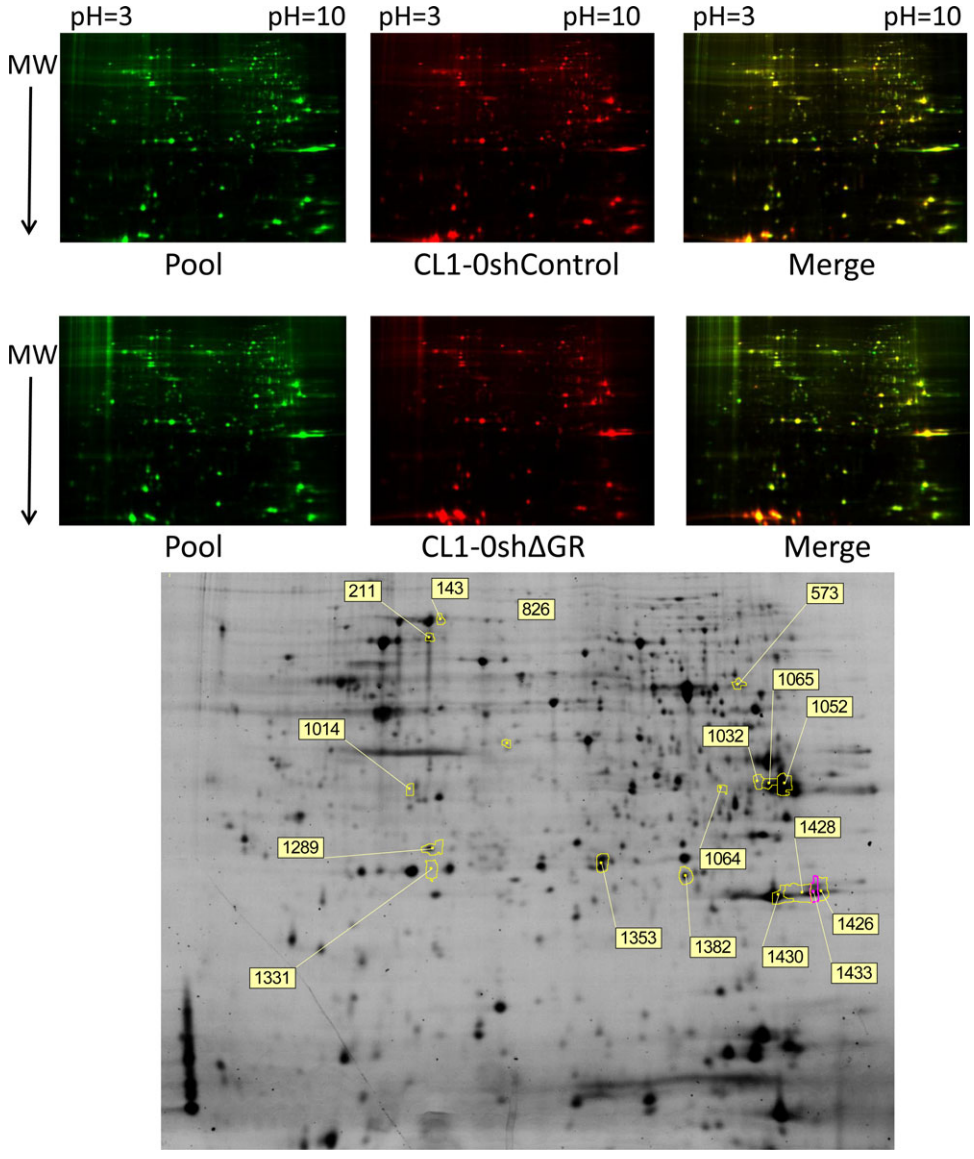


Figure 4. Redox 2D-DIGE analysis of GR-dependent differential cysteine-modification in CL1-0shControl and CL1-0shΔGR cells. Lysates from CL1-0shControl and CL1-0shΔGR cells were subjected to redox 2D-DIGE analysis as described in Section 2. 2DE-proteome map of CL1-0shControl and CL1-0shΔGR cells are displayed. The differentially labeled protein features are annotated with spot numbers.

sue such as cornea and liver, respectively, to overcome GR depletion induced ROS accumulation. Interestingly, the low oxygen consuming tissues such as breast and uterine have insignificant alterations in peroxiredoxin-1 levels in response to GR knockdown.

4 Discussion

By performing both lysine- and cysteine-based proteomic analyses, the goal of our experimental design was to monitor the differential levels of both protein translation and protein posttranslation in response to GR depletion. The lysine-based proteomic analysis examined the total cellular protein changes caused by GR depletion. In contrast, the determination of thiol reactivity changes investigated the quality of cellular proteins with or without redox alteration resulting from GR depletion because redox alteration is closely correlated with protein function. The redox-proteomic results were unaffected by changes in the level of proteins because a normalization of protein levels was performed before the calculation of thiol reactivity changes in our study. Thus, this study can simultaneously monitor the changes of cellular protein levels and cellular protein functions in both CL1-0shControl and CL1-0shΔGR cells.

The 2D-DIGE and MALDI-TOF MS analyses identified 34 GR depletion induced alterations in protein spot in CL1-0 cells. The results demonstrate that this experimental design has the ability to identify a broad range of GR depletion induced proteome alterations. The altered proteins had stimulation roles in calcium regulation, cytoskeleton regulation, redox regulation, cell–cell interaction, signal transduction,

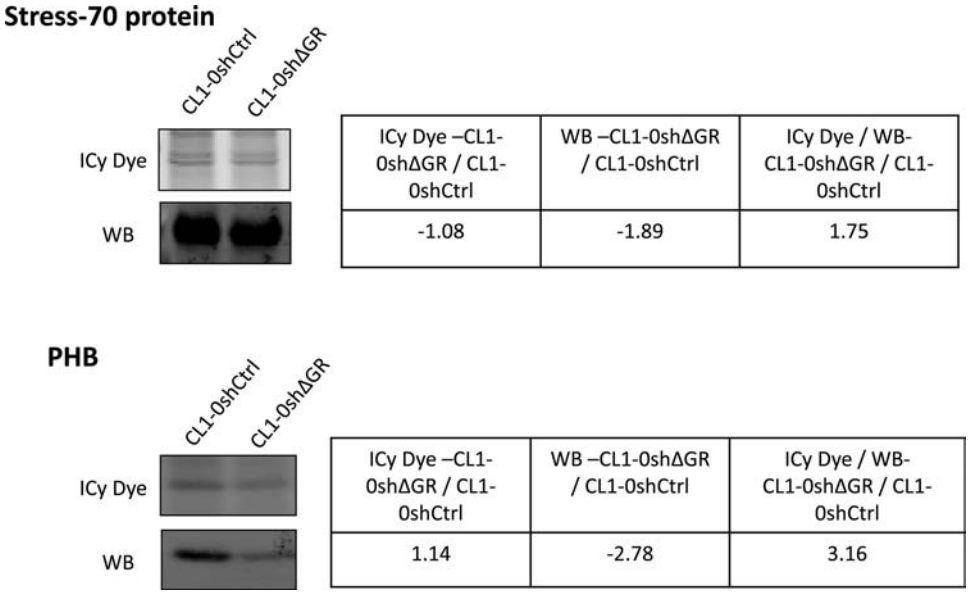


Figure 5. IP-WB validation of the thiol reactive proteins, Stress-70 protein, and PHB, identified through redox-proteomic study in CL1-0shControl and CL1-0shΔGR cells. ICy dye labeled protein samples from CL1-0shControl and CL1-0shΔGR cells were immunoprecipitatied with Stress-70 protein and PHB antibodies to confirm the alterations of thiol reactivity in Stress-70 protein and PHB. The image was visualized by Ettan DIGE imager. Immunoblotting against the corresponding antibodies was performed to gain the protein level.

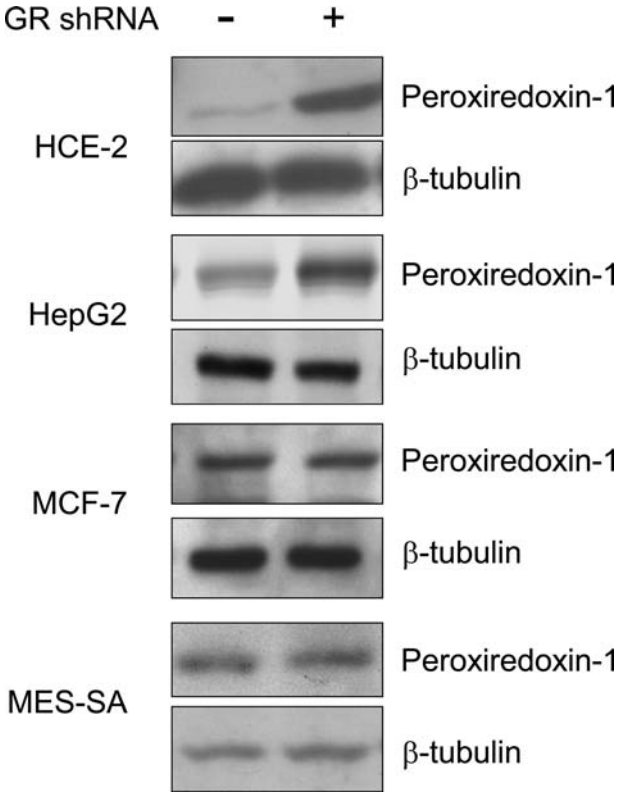


Figure 6. Immunoblot analysis of GR depletion induced peroxiredoxin-1 expression in HCE-2, HepG2, MCF-7, and MES-SA cells. Peroxiredoxin-1 expression was monitored by immunoblotting in pLKO.1 vector transfected and pLKO.1-shΔGR transfected HCE-2, HepG2, MCF-7, and MES-SA cells.

glycolysis, nuclear assembly, ion transport, protein folding, gene regulation, and protein regulation. In contrast, proteins involved in lipid metabolism, energy transduction, and nucleotide biosynthesis were downregulated (Supporting Information Table 1 and Supporting Information Fig. 4). Although the overall coverage of protein mixtures identified by LC-MS-based analysis is commonly recognized to be higher than that of 2DE-based analysis, 2DE-based analysis offers the advantage of direct protein quantification at the protein isoform level with reduced analytical variation [10]. Our study also demonstrated that GR depletion increases the intracellular ROS level. Because ROS can stimulate signaling pathways, regulate a range of cellular activities, and modulate disease progression, it is important to study the molecular events associated with ROS effects. Accordingly, our previously established experimental method was used to determine whether protein thiol reactivity is altered in cells following depletion of GR caused by ROS generation. The results indicated that proteins involving calcium homeostasis, protein folding, hydrolysis, protein degradation, gene regulation, redox regulation, glycolysis, calcium signaling, cytoskeleton regulation, and DNA biosynthesis are redox-modified on the cysteine residues of the identified proteins (Supporting Information Table 2 and Supporting Information Fig. 4). This finding implied that GR depletion induced oxidative stress disturbed the normal redox balance, causing redox modulation of specific proteins in CL1-0shControl cells. The ICy labeling results supported the hypothesis that GR depletion induces the formation of free thiols in certain proteins by breaking disulfide bonds, increasing ICy dye labeling. In addition, GR depletion induced, protein-derived peroxides or ROS may directly oxidize thiol groups to form the sulfenic, sulfinic, or sulfonic acid forms of cysteine, decreasing ICy dye labeling. These thiol modifications on cysteine residues have been reported to interfere with the normal functions of the proteins [22]. Accordingly, these results imply that GR depletion induced regulation of protein level alteration and redox modification considerably regulate cell physiology.

In this study, 17 proteins with differential thiol reactivities were identified by MALDI-TOF MS. Alpha-enolase,

GAPDH the Stress-70 protein, peroxiredoxin-1, and peroxiredoxin-6 are reported to maintain glycolysis, protein folding, and redox regulation, and all displayed an increase in ICy dye labeling following GR depletion (Supporting Information Table 2). Thus, their cysteine residues must be reduced to generate new thiol groups for ICy labeling, suggesting possible oxidative damage and deregulation of these proteins. In support of this, the modulation of the glycolytic enzymes alpha-enolase and GAPDH showed that redox modifications can redirect carbohydrate fluxes to generate increased reducing power in the form of nicotinamide adenine dinucleotide phosphate (NADPH) at the expense of glycolysis through a pentose phosphate pathway. In contrast, several of the identified proteins, such as brain-specific angiogenesis inhibitor 1 associated protein 2 and prohibitin, displayed a decrease in ICy dye labeling following GR depletion (Supporting Information Table 2). Thus, their free thiol groups must be oxidized in response to GR depletion to block ICy labeling, implying possible oxidative damage and deregulation of these proteins. For example, prohibitin is a repressor of E2F-mediated transcription and is associated with cell cycle regulation, senescence, tumorigenesis, and immortalization [23]. Therefore, its oxidative modification may have profound effects on cellular proliferation.

In Supporting Information Table 2, a number of proteins showed an increase in labeling, suggesting the formation of new free thiols. This may occur through scission of disulfides or through labeling of isoforms generated by redox-dependent shifts in p*I*, as reported in the case of the peroxiredoxins [24–26]. The peroxiredoxins are antioxidant enzymes that function by reducing ROS and have been associated with redox regulation of cell signaling [27]. ICy dye labeling of 2DE isoforms corresponding to peroxiredoxin 1 and 6 was increased during GR knockdown (Supporting Information Table 2). The 1D immunoblotting showed that overall peroxiredoxin 1 level was correlated with ICy labeling, but the increased level of protein intensity was generally less than the increased level of ICy dye labeling (Fig. 3). Peroxiredoxin 1 contains an active-site cysteine that is oxidized to an oxidized cysteine (such as sulfenic acid) by the increased ROS level during GR knockdown. It is then regenerated back to a free thiol through various mechanisms, including intramolecular and intermolecular disulfide bond formation and reduction by the thioredoxin system. Previous studies have also shown that cysteines on peroxiredoxins can be overoxidized to sulfinic or sulfonic acid, resulting in an acidic shift on 2D gels [24–26]. Although it is expected that increased ROS level dependent oxidation of peroxiredoxin active-site cysteines would inhibit ICy labeling, we suppose that the increased labeling observed may occur because of the labeling of other free thiols on a p*I*-shifted pool of oxidized peroxiredoxin.

In current studies, both lysine-labeling and cysteine-labeling 2D-DIGE experiments are based on the quantification of the lysine groups and thiol groups of proteins and can detect the picogram level of Cy/ICy dye labeled proteins. In contrast, our poststaining experiment is based on modified CCB staining with sensitivity at a 10–50 ng range [11]. Thus, a number of differentially Cy/ICy dye labeled, low-abundant proteins can be detected using a fluorescent scanner, but cannot be visualized with CCB staining. Consequently, only 70% of the labeling features identified by lysine-labeling 2D-DIGE and 15% of the labeling features identified by cysteine-labeling 2D-DIGE can be taken from CCB stained gels and successfully identified by MALDI-TOF MS identification. Our unreported data also demonstrate that the sensitivity of Sypro Ruby staining is still significantly lower than that of ICy dyes because ICy dye labeling is based on saturation labeling with a sensitivity of 30-fold more than either lysine-labeling dyes or Sypro Ruby staining. Consequently, the fluorescent images of ICy dyes can be used for thiol group quantification and comparison, but the ICy dye labeled low-intensity spots are difficult to pick for protein identification. In addition, we used an automatic spot picker to directly pick ICy dye labeled spots without poststaining for MS/MS-based analysis. The spectral signals were generally low, implying that the abundance of these ICy dye labeled spots might be below the MS/MS detection level. Moreover, we also propose that the ICy dyes may disturb the ionization of ICy dye labeled proteins and peptides, leading to a low identification rate.

Garcia-Leiro et al. used knockdown experiment to investigate the role of GR on yeast strain, *Kluyveromyces lactis* [28]. Their result demonstrated that 42 proteins showed significant changes under GR knockdown. Most of the differentially expressed proteins belong to proteins with the functions in glycolysis, tricarboxylic acid cycle, and oxidative pentose phosphate pathways. However, our current proteomic analysis revealed proteins known to regulate redox regulation, calcium signaling, cytoskeleton regulation, and protein folding were found to be altered in abundance between CL1-0shControl and CL1-0shΔGR cells. The differential protein expression patterns of these two studies might be caused by the differential metabolic rate, antioxidant ability, and protein folding ability between human lung cells and yeast.

Protein species concept is proposed to be the smallest unit of proteome and consider the formation of different forms (such as posttranslational modifications) of proteins, each of which might have specific function as well as specific chemical structure. The initial synthesis product of translation represents a prototype protein species, the initial protein species. This initial protein species is possibly further proteolytically or biochemically processed, modified, and/or transported to intracellular organelles or secreted out of the cell, spliced or degraded. Each of these covalent modifications or processed of a protein leads to a new protein species [29, 30]. In current study, some of identified proteins including annexin A1, GAPDH, peroxiredoxin-1, vimentin, and Stress-70 protein have been identified in multiple spots implying these proteins encounter differential modifications/processes and form new protein species against initial translational products.

Although 2DE is a key technique used in the profiling of thousands of proteins in biological samples, some limitations of 2DE have been reported. One of the major limitations is the problem of gel-to-gel variation, which can be largely

overcome by 2D-DIGE technique described in this study. In addition, dye-stained gel quantitative comparisons show another problem. Thiede et al. demonstrated that only approximately half of the 2DE spots contained one dominant protein while the other half of 2DE spots reveal one to five proteins implying the protein abundant changes based on the spot intensity are incorrect for almost 50% of 2DE spots [31]. This problem has been improved by the same group through using stable isotope labeling by amino acids in cell culture (SILAC) in combination with 2DE and nano-LC/MS analysis.

In conclusion, we used a comprehensive lung adenocarcinoma-based proteomic approach for the identification of GR-modulated protein intensity alteration and redox modification. In addition to the expected induction of a set of redox-regulatory proteins in GR-depleted CL1-0 cells, this study also found that a number of redox-regulatory proteins were redox-modified. Further analysis demonstrated that cellular proteins involved in protein folding were also modulated by GR depletion either in protein expression alteration or in redox modification. To our knowledge, this is the first comprehensive proteomic and redox-proteomic analysis to investigate the role of GR in a mammalian cell model.

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