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# Refining In Vitro Toxicity Models: Comparing Baseline Characteristics of Lung Cell Types

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# ABSTRACT

There is an ever-evolving need in the field of *in vitro* toxicology to improve the quality of experimental design, ie, from illdefined cell cultures to well-characterized cytotoxicological models. This evolution is especially important as environmental health scientists begin to rely more heavily on cell culture models in pulmonary toxicology studies. The research presented in this study analyzes the differences and similarities of cells derived from two different depths of the human lung with varying phenotypes. We compared cell cycle and antioxidant-related mRNA and protein concentrations of primary, transformed, and cancer-derived cell lines from the upper and lower airways. In all, six of the most commonly used cell lines reported in *in vitro* toxicology research papers were included in this study (ie, PTBE, BEAS-2B, A549, PSAE, Met-5A, and Calu-3). Comparison of cell characteristics was accomplished through molecular biology (q-PCR, ELISA, and flow cytometry) and microscopy (phase and fluorescence) techniques as well as cellular oxidative stress endpoint analyses. After comparing the responses of each cell type using statistical analyses, results confirmed significant differences in background levels of cell cycle regulators, inherent antioxidant capacity, pro-inflammatory status, and differential toxicological responses. The analyzed data improve our understanding of the cell characteristics, and in turn, aids in more accurate interpretation of toxicological results. Our conclusions suggest that *in vitro* toxicology studies should include a detailed cell characterization component in published papers.

Key words: in vitro toxicology; antioxidant capacity; cell cycle; lung cell types.

Over the past decade, *in vitro* toxicology testing has evolved from simply a cost-effective screening method to a viable alternative to animal testing (Cohen *et al.*, 2014; Fernandes *et al.*, 2009; Goldberg and Frazier, 1989). As with many other tools and techniques available to environmental health scientists, there are advantages and disadvantages to using cell culture-based models to gauge dose-response relationships, mechanistic analyses, and biotransformation profiles of xenobiotics exposed to mammalian systems (Blaauboer, 2008; Hartung and Daston, 2009; Kroll *et al.*, 2009; Phalen *et al.*, 2006). Some experts cite resistance of regulators to use data collected from *in vitro* studies to inform decisions about chemicals and other substances due to lack of representative three-dimensional anatomical structure of human airways (Hartung and Daston, 2009; Liebsch and Spielmann, 2002). Others have stated that in vitro models fail to identify indicators of disease. Even with these oppositional arguments, there is a clear need to reduce the reliance on test animals for both new and existing substances, and in vitro models can provide useful data for decision-making. In fact, because the speed, ease, and low cost of cellular tests can be combined with precise gene, protein, cytokine, metabolite, and enzyme analysis tools, research teams (within industry and academia) have invested substantially in cell and tissue culture (Carere et al., 2002; Godoy et al., 2013; Guillouzo and Guguen-Guillouzo,

© The Author(s) 2019. Published by Oxford University Press on behalf of the Society of Toxicology. All rights reserved. For permissions, please e-mail: journals.permissions@oup.com 2008; Lin and Chang, 2008; Nemmar et al., 2013). However, study designs must be created with scrutiny to ensure the utility of the data gleaned from the *in vitro* experiment; namely, the choice of cells used in the culture model. Choosing the best cell type for an *in vitro* toxicology study requires comprehensive cell characterization.

To improve the practicality of in vitro models, immortalized cells have been created by transforming certain intracellular pathways or characteristics (Hahn et al., 1999, 2002). These transformed cells are crafted by altering a selection of intracellular pathways to produce cells that will proliferate beyond primary cells while also avoiding the acquisition of a tumorigenic classification. Alterations to cellular genotypes include changes in mitogenic signaling, cell cycle checkpoint controls pRB and p53, telomerase maintenance, or signaling pathways controlled by PP2A (Hahn and Weinberg, 2002; Mooi and Peeper, 2006). Due to the dysregulation of intracellular pathways, transformed cells possess basal expression levels of "normal" cells with the immortalization of "cancerous" cells. The altered pathways resemble the pathways cancer cells are known to modify or hijack. These pathways are known as the "hallmarks of cancer" (Hanahan and Weinberg, 2000).

The hallmarks of cancer originally described by Hananhan et al. included sustained angiogenesis, ability to avoid apoptosis, self-induced growth signaling, and metastasis (Hanahan and Weinberg, 2000). An updated list also included dysregulation of cellular metabolism and tumor-promoting inflammation as essential pathways perturbed within cancer cells (Hanahan and Weinberg, 2011). These updated pathways are vital in *in vitro* toxicology studies and must be taken into account for proper cell line selection and subsequent interpretation of results.

There are a few common endpoints among studies in the fields of cell biology, toxicology, and cancer research. Two overarching endpoints have significant overlap when characterizing cells: antioxidant capacity and cell cycle deregulation. In cell biology, normal cellular functions such as metabolism can generate reactive oxygen species (ROS) causing oxidative DNA damage (Finkel and Holbrook, 2000; Murphy, 2009; Yu, 1994). Another outcome of this endogenous ROS generation is the adverse effect on the cell cycle and cytoskeleton (Boonstra and Post, 2004; Menon and Goswami, 2007; Sauer et al., 2001). In toxicology, increasing particle dose correlates with increasing amount of ROS generation (ROS) (Foldbjerg et al., 2011; Guo et al., 2009; Hussain et al., 2005; Sayes et al., 2014). New studies have shown particles to cause cell cycle arrest due to ROS generation increasing cell cycle regulators such as p53 and p21 (Eom and Choi, 2010; Wu et al., 2010). In recent years, cancer research has also investigated the impact that excess reactive species, inflammation, and cell cycle regulation can have on tumorigenesis (Gupta et al., 2012; Ishii et al., 2005; Kongara and Karantza, 2012). With these fields of research investigating oxidative stress, proinflammatory response, and cell cycle disruption, it is important to know the state of these characteristics within a selected cell culture model.

Utilizing cell culture models require characterization of the baseline cellular features and processes. Different cell types can express different levels of biomolecules (eg, mRNAs and proteins), thus influencing oxidative capacity, cellular adhesion, proliferation rate, metabolic activity, and sensitivity to exogenous materials (Diamond *et al.*, 2000; Liu, 2001; Thiery *et al.*, 2009). When assessing the available literature, 1000+ research papers between 2015 and 2017 have cited "in vitro toxicology." Within those papers, almost half used "lung" cells. Currently available primary cell types consist of primary tracheal/

bronchial epithelial (PTBE) or primary small airway epithelial (PSAE) cells. When conducting toxicological assessments, the four most common cell types used include human bronchial epithelial cells (BEAS-2B), which are transformed from the upper airway; human alveolar epithelial cells (A549), which are cancer-derived from the upper airway; human mesothelial cells (MeT-5A), which are transformed from the lower airway; and human mesothelial epithelial cells (Calu-3), which are cancer-derived from the lower airway.

The purpose of this manuscript is 2-fold: Our first objective was to compare the antioxidant capacity of each of these cell types. Our second objective was to compare the cell cycle population distribution of the same six cell types. It is anticipated that information regarding appropriate cell type use for mechanistic pulmonary toxicology studies will be acquired by examining the comparative baseline expressions of commonly utilized lung cells. Furthermore, statistical analysis allows for testing for differences in means of the cell types separated by phenotype and lung location.

# MATERIALS AND METHODS

General experimental design. The general approach to this study was to characterize cells in culture (Figure 1). Specifically, we conducted substantial cellular assessments (ie, whole cell, protein, cytokine, enzyme, and gene expression analyses) of six human lung cell types (ie, PTBE cells; BEAS-2B normal lung epithelial adenovirus 12-SV40 virus-transformed cells; A549 lung epithelial carcinoma cells; PSAE cells; Met-5A lung mesothelial pRSV-T plasmid-transfected cells; and Calu-3 lung epithelial adenocarcinoma cells from the pleural effusion) used in pulmonary toxicology studies. Figure 1 lists the types of cells, incubation time points, and endpoint analyses.

Maintaining cell culture. PTBE (PCS-300-010, American Type Culture Collection [ATCC], Manassas, Virginia) and PSAE (PCS-301-010, ATCC) were cultured in "Airway Epithelial Cell Basal Medium" supplemented with the "Bronchial Epithelial Cell Growth Kit" (PCS-300-040, ATCC), as described by ATCC. A549 cells (CCL-185, ATCC), BEAS-2B cells (CRL-9609, ATCC), and Calu-3 cells (HTB-55, ATCC) were cultured using the same media (Table 1). Medium consisted of a 1:1 mixture of Dulbecco's Modified Eagle's Medium and Ham's F-12 Nutrient Mixture (DMEM/F12; Gibco, Waltham, Massachusetts) supplemented with 10% fetal bovine serum (FBS; Equitech-Bio, Inc. Kerrville, Texas) and 1% antibiotic cocktail of penicillin-streptomycin (MP Biomedical, Solon, Ohio). Met-5A cells were cultured using Media 199 (Gibco, Adair, Oklahoma) supplemented with 10% FBS, 1% antibiotic cocktail, 3.3 nM epidermal growth factor (Invitrogen, Waltham, Massachusetts), 400 nM hydrocortisone (ACROS Organics, Geel, Belgium), 870 nM insulin (Cell Applications, San Diego, California), 20 mM HEPES (Gibco), and "Trace Elements B" (Corning, Pittsburgh, Pennsylvania). All cells were cultured at 37°C in an air-jacketed humidified incubator with 5% CO<sub>2</sub>. All cells were tested at a passage number 3 or 4 after receipt from the ATCC stock.

Cell proliferation. All cell types were collected at ~70% confluency using trypsin/EDTA 0.25% (Gibco) to detach cells, pelleted, and re-suspended in appropriate media. A trypan blue exclusion assay (Gibco) was performed using a Countess automated cell counter (Invitrogen). Cell seeding densities were carefully measured and recorded. After seeding approximately 30 000 cells/ml



Figure 1. The design of the *in vitro* experiments. Six different cell types were used (PTBE, BEAS-2B, A549, PSAE, MeT-5A, and Calu-3). Six incubation time points were used for the proliferation rate and metabolic activity endpoints (1, 2, 6, 12, 24, and 48 h). Antioxidant capacity and cell cycle regulation were analyzed using whole cell, protein expression, and gene expression at the 48 h incubation time point.

Table 1. Comparison of Culture Conditions

| Cell Name | Lung Location | Phenotype      | Culture Medium                      | Doubling Time (h) | Other Details                                       |
|-----------|---------------|----------------|-------------------------------------|-------------------|---|
| PTBE      | Upper airway  | Primary        | Airway epithelial cell basal medium | 72                | Not diseased, normal                                |
| BEAS-2B   | Upper airway  | Transformed    | DMEM/F12                            | 26                | Immortalized via adenovirus<br>12-SV40 virus hybrid |
| A549      | Upper airway  | Cancer derived | DMEM/F12                            | 22                | Diseased, carcinoma                                 |
| PSAE      | Lower airway  | Primary        | Airway epithelial cell basal medium | >72               | Not diseased, normal                                |
| MeT-5A    | Lower airway  | Cancer derived | Media 199                           | 30                | Immortalized via pRSV-T plasmic                     |
| Calu-3    | Lower airway  | Primary        | DMEM/F12                            | 72                | Diseased, carcinoma                                 |

Characteristics of the cells used in this study that are available from the source/vendor. All six types of cells are derived from Homo sapiens (human), have a morphological appearance as epithelial-like, are adherent, and were cultured with 5% CO<sub>2</sub>, 90% humidity at 37°C.

in each well of a 6-well plate, the exclusion assay was repeated after 1, 2, 6, 12, 24, and 48-h incubation time points.

In addition, a [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium assay (MTS; Promega, Fitchburg, Wisconsin) was used to determine growth rate and mitochondrial activity. Briefly, cells were collected, counted, and seeded at the same density as before. At 1, 2, 6, 12, 24, and 48-h incubation time points, the MTS solution was added, incubated for 2h, and spectroscopically measured at 490 nm on a Synergy H1 microplate reader (BioTek, Winooski, Vermont).

Cell morphology. All cells types were seeded and incubated into one of 4 wells of a chamber slide (Lab-Tek II, Rochester, New York) for 48 h to allow adhesion and acclimation. Subsequently, the cells were fixed and permeabilized as described in the Image-it Fix-Perm kit (Molecular Probes, Eugene, Oregon). Cells were then washed ( $3\times$ ) with wash buffer and incubated in the dark at room temperature with ActinGreen 488 ReadyProbes reagent (Invitrogen) for 15 min, followed by the addition of MitoTracker Red CM-H2XRos and NucBlue Live Cell Stain Ready Probes reagent (Molecular Probes). Samples were incubated, again, in the dark at room temperature for 15 min. Each well was then washed with PBS solution  $(3\times)$  and fixed with two drops of ProLong Diamond Anti-fade Mountant (Molecular Probes). A glass cover slip was then carefully placed on the slide and set for 24 h. Images of the cells were taken using fluorescence microscopy (CytoViva Inc., Auburn, Alabama) with the accompanying Ocular software (Advanced Scientific Camera Control Version 1.0), and the 3-colored probes were stitched together.

mRNA expression. Specific mRNA concentration was measured to determine the baseline level of gene expression within each cell culture. CDKN1A, TP53, IL-6, and GSR mRNA content was analyzed using Real-Time quantitative Polymerase Chain Reaction (RT-qPCR). All work areas, gloves, and pipettes were wiped down with RNaseZap to avoid contamination. RNA was harvested using a PureLink RNA mini kit (Invitrogen), followed by the generation of cDNA and DNase treatment using a SuperScript IV VILO master mix with ezDNase (Invitrogen). Concentrations of RNA and DNA were calculated and kept consistent across all cell types using a Qubit high sensitivity RNA assay (Invitrogen) and NanoDrop One/One Microvolume UV-Vis Spectrophotometer (Thermo Scientific, Waltham, Massachusetts) set at 260 nm wavelength, respectively. TaqMan

fast advanced master mix (Applied Biosciences, Beverly Hills, California) was substituted for the master mix in the SuperScript kit to allow for more rapid data acquisition. TaqMan gene expression assay were used to perform qPCR. A TaqMan gene expression assay of  $\beta$ -actin was also included and used as an endogenous control. The reactions were analyzed on a Step-One Real-Time PCR system (Applied Biosystems). Upper airway cell types BEAS-2B and A549 were compared with PTBE as a control, whereas lower airway cell types MeT-5A and Calu-3 cell types were compared with PSAE as a control. Data are presented as Expression Fold Change (2<sup>- $\Delta ACt$ </sup>).

Cell cycle regulation: protein expression. To collect the baseline level of p21 and p53 within the cells, the cells were collected and lysed using the same methodology, and then the protein levels were measured using either a p21 or p53 ELISA kit (Invitrogen). Cells were collected, pelleted, and re-suspended in PBS. The cells were again pelleted and re-suspended in cell extraction buffer (Invitrogen) containing protease inhibitor cocktail (Millipore-Sigma, St. Louis, Missouri) and phenylmethylsulfonyl fluoride (PMSF; Thermo Scientific). Samples were placed on ice and vortexed 3X over a 30-min period. Samples were centrifuged at 13 000 RPM for 10 min. The standards and samples were added to the well plate and read at an absorbance of 450 nm on a Synergy H1 (BioTek, Broadview, Illinois) microplate reader.

Antioxidant capacity: cytokine expression. The baseline levels of IL-6 within the cells were measured using a human IL-6 enzymelinked immunosorbent assay (ELISA; Invitrogen). Cells were washed with cold phosphate-buffered saline solution  $(2\times)$  before being covered with ice-cold radioimmunoprecipitation cell lysis buffer (RIPA; Pierce, Rockford, Illinois) for 10 min. A cell scrapper was used to detach the cells before the lysis solution was transferred into a microcentrifuge tube. The tube was spun at 18 705 RCF for 10 min and kept on ice until use. The plate layout, standards, controls, and samples were performed following the kit's protocol. Immediately after the addition of the stop solution, the plate was read at an absorbance of 450 nm on a Synergy H1 microplate reader.

Antioxidant capacity: enzyme analyses. The glutathione reductase (GSR) activity within the cells was measured using the OxiSelect glutathione reductase assay kit (Cell Biolabs Inc., San Diego, California). Following the OxiSelect protocol, cells were collected, pelleted, and re-suspended in ice-cold assay buffer. Cell suspension was transferred and homogenized. After the addition of the glutathione disulfide (GSSG) solution, the absorbance of the plate was read using the kinetic assay reading at 405 nm every minute for 10 min on the Synergy H1 microplate reader.

Whole cell analysis: cell cycle distribution. The cell cycle population distribution was analyzed among the six cell types using a Vybrant DyeCycle stain (Invitrogen) analyses via FACSVerse flow cytometer (BD Biosciences). Cells were pelleted, resuspended in sheath fluid, stained, and briefly vortexed at room temperature. Samples were then incubated in the dark at  $37^{\circ}$ C for 20 min. Flow cytometer performance QC was run using 2 µm polystyrene research beads (BD Biosciences). All samples were vortexed prior to analysis. Analyses at 488 nm excitation and 670 nm emission corresponded to the APC-Cy7-A filter. The range was first optimized while previewing the cells, and then 10 000 cells/run were acquired. Each sample was run in triplicate before the data was saved and analyzed using FlowJo

software (FlowJo LLC, Ashland, Oregon). Histograms were created in FlowJo by comparing counts versus APC-Cy7-A, and then gates were placed over the two peaks or the valley to calculate the percent of cells in that range. The data from each triplicate were averaged.

Whole cell analysis: general oxidative stress. Endogenous antioxidants (ie, the capacity of the cell to neutralize free radicals using basal levels of antioxidants) in each of the six cell lines were analyzed using a general oxidative stress CellROX Deep Red Flow Cytometry Assay Kit (Invitrogen) and a FACSVerse flow cytometer (BD Biosciences, San Jose, California). Cells were collected, pelleted, and re-suspended in complete media. A prepared aliquot of CellROX dye was added; after 45 min, SYTOX dead cell stain was added and incubated for 15 min. Prior to testing samples, a flow cytometer performance QC was run using 2 µm polystyrene research beads (BD Biosciences). All samples were vortexed prior to analysis to reduce aggregation. The CellROX dye had an excitation at 644 nm and emission at 665 nm, which corresponded to the APC-Alexa Flour filter. The SYTOX dead cell stain had an excitation at 444 nm and emission at 480 nm, which corresponded to the SSC-A filter. The range was first optimized while previewing the cells and then 10 000 cells/run were acquired. These data were saved and analyzed using FlowJo software (FlowJo LLC, Ashland, Oregon). Flow cytometry graphs were generated by comparing the CellROX and SYTOX emissions. Gates were placed to discriminate dead cells without oxidative stress, dead cells with oxidative stress, alive cells with oxidative stress, and alive cells without oxidative stress.

Whole cell analysis: change in oxidative stress after tert-butyl hydroperoxide exposure. Change in the amount of detectable ROS in each of the six cell types were analyzed using a general oxidative stress as described previously (ie, CellROX Deep Red Flow Cytometry Assay Kit). Cells were collected, pelleted, and resuspended in complete media. Tert-butyl hydroperoxide was used as a positive ROS generating control and was added to each of the cell cultures and subsequently incubated at  $37^{\circ}$ C for 1 h at a concentration of  $250 \,\mu$ M. A prepared aliquot of CellROX dye was added and analyzed via flow cytometry.

Statistical analysis. For each cellular response, a 2-way analysis of variance (ANOVA) in lung location and phenotype was performed (Kuehl, 2000). When the interaction between lung location and phenotype is found to be highly significant (with *p*values less than .01), follow-up tests to identify those pairs of treatments that are significantly different from each other were done with Tukey's Honest Significant Differences (HSD). To confirm results for responses where the assumption of constant variance across treatments was not met, a nonparametric ANOVA was also implemented to confirm the parametric 2-way ANOVA results (Wobbrock *et al.*, 2011). All statistical analyses were performed in the open-source software package R (R Core Team, 2016).

## RESULTS

## Cell Culture Growth and Morphology

Figure 2 shows the differential cellular densities via microscopy. Each color in the fluorescence image represents a different component of an individual cell's structure. The blue shade is a nuclear stain (Hoechst), the red shade preferentially enters the mitochondria and only fluoresces upon oxidation (MitoTracker),



Figure 2. Fluorescent microscopy shows distinct growth patterns, degrees of contact inhibition, and respiratory capacity. Image of (A) PTBE cells, (B) BEAS-2B, (C) A549, (D) PSAE, (E) MeT-5A, and (F) Calu-3 cell types. Flourescent dyes highlight F-actin (ie. the cytoskeleton), mitochondria (and flouresces when oxidized), and DNA. The bottom figure suggests that these cell types lie on a spectrum of translatability and cost where primary cell types are the most translatable and most expensive. The cancer cell types are the least translatable and least expensive. The transformed cell lines represent a middle ground for translatability and cost.

and the green shade illuminates the F-actin in the cytoskeleton (ActinGreen). Each nucleus appears as a different shade of blue to purple to pink based on the differences in mitochondrial density/oxidative state among the cells. The upper airway primary and transformed cells have a more blue to purple shade of nuclei than the A549 cells, which have a higher density of mitochondria as indicated by the pink shade. BEAS-2B and MeT-5A cells have a lower density of mitochondria as indicated by the purple shade. The primary cells have large areas of red indicating large amounts of mitochondria when compared with Calu-3 cells, which have the least amount of mitochondria as indicated by the distinct and de-convoluted blue versus red-stained areas (nuclei vs mitochondria, respectfully).

In addition to color saturation differences, each cell type exhibit differences in confluency. PTBE, BEAS-2B, and A549 cells create uniform monolayers with rounded cytoskeletons; however, A549 cells can continue to proliferate past plate saturation, demonstrating the absence of contact inhibition. In contrast, the PTBE cells will grow isolated from one another and become quiescent if 100% confluency is reached. PSAE and MeT-5A cells also create uniform monolayers but depict an elongated cytoskeleton. These cells proliferate more slowly compared with upper airway cell types. Results also indicate that Calu-3 cells grow in colony formations (rather than uniform monolayers) as indicated by the clustering of multiple nuclei co-located within a dense cytoskeleton. Together, these cell adhesion images demonstrate that integrin cell signaling pathways are likely activated soon after initial seeding and create connections to extracellular matrix for either monolayer or colony formations. Calu-3 cells have a metastatic phenotype and may have overactive integrin signaling as demonstrated by both the bright green cytoskeleton and colony structure.

Figure 3 shows the differential proliferation rate and metabolic activity of the six cell types used in this study (PTBE, BEAS-2B, A549, PSAE, MeT-5A, and Calu-3). Line graphs include mean value over multiple observations with standard deviations. The cell proliferation data showed that the bronchial epithelial cells (BEAS-2B and A549) proliferate faster than mesothelial cells (MeT-5A and Calu-3) while transformed and cancerous phenotypes grow more rapidly than their primary cell counterparts (PTBE and PSAE). It is important to note that all cells were seeded at the same density and normalized to the 1h incubation time point. The mitochondrial activity data showed that the primary cells and cancer-derived cell types are much more metabolically active when compared with transformed cells. The primary cells are under stress while growing in culture plates and may have overactive mitochondria to counteract this stress. The cancer cells have altered metabolic pathways (ie, the "Warburg effect"), which may be indicative of a higher MTS assay reading (Vander Heiden et al., 2009; Warburg, 1956). This phenomenon occurs because the MTS is cleaved via aerobic glycolysis; the same pathway commonly perturbed in cancer cells. Together, these results imply that proliferation rates may be dependent on location of the cell type in the lung, whereas mitochondrial activity assays can be utilized as a multifaceted approach to determining cell growth and mitochondrial health.



Figure 3. Cellular proliferation and mitochondrial activity are different among the six cells. A, PTBE, BEAS-2B, A549, PSAE, MeT-5A, and Calu-3 cells were collected and counted via a cell counter at 1, 2, 6, 12, 24, and 48 h time points. All cells were normalized to their first cell count at the 1 h time point. B, The six cell types were seeded at the same density, and at the designated time point, MTS was added to the well, incubated for 2 h, and then the absorbance of the solution was collected.

| Normalized to Up               | per Primary (PTBE)             |        | Normalized to Lower Primary (PSAE) |                                  |  |  |  |
|--------------------------------|--------------------------------|--------|------------------------------------|----------------------------------|--|--|--|
| Upper Transformed<br>(BEAS-2B) | Upper Cancer-derived<br>(A549) | GENE   | Lower Transformed<br>(Met-5A)      | Lower Cancer-derived<br>(Calu-3) |  |  |  |
| 0.0033                         | 0.0055                         | CDKN1A | 0.1175                             | 0.0704                           |  |  |  |
| 0.0776                         | 0.1027                         | TP53   | 1.0467                             | 0.7870                           |  |  |  |
| 0.0300                         | 0.1250                         | IL-6   | 1.0626                             | 6.8446                           |  |  |  |
| 0.5685                         | 0.3611                         | GSR    | 0.9922                             | 1.3365                           |  |  |  |

Figure 4. Heatmap of mRNA expressions. The upper airway cells BEAS-2B and A549 are normalized to PTBE, and the lower airway cells MeT-5A and Calu-3 are normalized to PSAE. Values ranging 0 to 0.99 indicate a decreased expression of the corresponding mRNA target, while values ranging 1.1 to 7 indicate an increased expression. Values between 0.99 to 1.1 indicate a expression similar to the primary cell mRNA expression (ie. 1.0).

## Differential mRNA Expressions

Figure 4 shows a heatmap of the transformed and cancer cell lines normalized to the primary cell line from their respective area of the lung. In the upper airway cell types (BEAS-2B and A549), the probed mRNAs CDKN1A, TP53, and IL-6 are drastically under expressed when compared with the primary cell line. The GSR mRNA is also under expressed, but less so than the others. In the lower airway cells, CDKN1A is downregulated in both cell types (MeT-5A and Calu-3). MeT-5A cells expressed relatively the same amount of mRNA for TP53, IL-6, and GSR. Calu-3 cells had downregulated TP53, upregulated IL-6, and slightly upregulated levels of GSR.

## Protein/Cytokine Expression and Enzyme Activity

Figure 5A shows the concentration of cyclin-dependent kinase inhibitor 1 (p21) and tumor (suppressor) protein (p53). The primary cells have larger concentrations of p53 than the other cells with MeT-5A expressing this protein the least. PTBE cells also express more p21 protein than the other upper airway cell lines.



Figure 5. Cells produce similar amounts of p53 but differing amounts of p21, IL-6, and GSR. A, Comparison of p21 and p53 protein expression in each cell type determined using an ELISA. B, Comparison of IL-6 protein concentration and GSR enzyme activity in each cell type determined using ELISA and enzyme activity kits.

In contrast, MeT-5A cells express the most p21 out of all the different cell types. This could be attributed to the method of transformation used in MeT-5A cells, which is hypothesized to increase p21 protein expression. Figure 5B shows the concentration of interleukin 6 (IL-6) and enzyme activity of GSR. There is an inverse relation seen with the enzyme activity of GSR and the subsequent expression of IL-6. In all cell types, the level of GSR activity and IL-6 are either clustered in the same area, or the reduction of GSR activity correlates to the level of IL-6 expression. Both primary cell types have higher levels of IL-6 than the other cell types and also exhibit lower GSR activity. In contrast, the BEAS-2B cell type has highly active GSR activity and a low IL-6 expression. A549, MeT-5A, and Calu-3 cell types exhibit a GSR activity level that correlates to a reduced IL-6 expression compared with the primary cells.

#### Whole Cell Analysis

Figure 6A shows the whole cell analyses data of the six cell types, demonstrating varying oxidative states. Primary cells PTBE and PSAE as well as MeT-5A cells have a percentage of their population that is experiencing high enough levels of endogenous ROS to be detected by flow cytometry. In contrast, BEAS-2B, A549, and Calu-3 cell populations are not experiencing high enough levels of endogenous ROS to be detected via flow cytometry. These results support the premise that primary cell types and lower airway cells (MeT-5A) are more sensitive to exogenous exposures, such as ambient light, change in temperature/humidity, and cleavage processes. All cell types have a low percentage of dead cells (<5%). Induced oxidative stress could be related to the sample preparation procedure; however,

sample preparation used in this study is identical among all cell types.

Figure 6B demonstrates the cell cycle distributions of the six cell types. The upper airway primary and transformed cell types (PTBE and BEAS-2B) show a similar cell cycle distribution, whereas the lower airway primary and transformed cell lines (PSAE and MeT-5A) also exhibit the same pattern. The cancer phenotypes, A549 and Calu-3, cells have similar cell cycle distributions that are different from that of the primary or transformed cells. The upper airway primary and transformed cell populations have slightly more than 50% of the cells in the G2/ M phase, with the transformed cell line having less cells in the G2/M phase and more cells in the G0/G1 phase. The same pattern is seen in the lower airway primary and transformed cells, with the only difference being the larger ratio of cells in the G0/ G1 phase (about 75%). The cancer phenotypes, A549 and Calu-3, are mostly in the G2/M phase. As expected, none of the populations have a large number of cells in the S phase.

Figure 7 shows the change in the cell cultures' oxidative stress. Overall, the lower airway cell types showed an increase in the amount of ROS as compared with their un-exposed counterparts. Furthermore, the lower primary cells demonstrated the highest amount of oxidative stress, as compared with the lower transformed and lower cancer derived cells. Similarly, the upper primary cell type also demonstrated the highest amount of oxidative stress, as compared with the upper transformed and upper cancer-derived cells. However, the upper primary cells showed a significantly elevated oxidative stress level as compared with the increased observed in the lower primary cells  $(1.4 \times)$ .



A CellROX & SYTOX whole cell analyses

Figure 6. Whole cell analysis of cell cycle distributions and general oxidative stress. FACS flow cytometry data comparing (A) the florescence of dead cells (SYTOX), cells undergoing oxidative stress (CellROX), and healthy cells. Florescence-activated cell sorting (FACS) flow cytometry data comparing (B) cell cycle distributions.



Comparative change in oxidative stress after exposure to tert-butyl hydroperoxide

Figure 7. Comparative change in oxidative stress after exposure to tert-butyl hydroperoxide. Florescence-activated cell sorting (FACS) flow cytometry data comparing the ROS detected after incubation with a positive oxidative stress control (tert-butyl hydroperoxide) among the 6 cell types used in the study. Change in oxidative stress was normalized against each cell's baseline ROS concentration.

#### **Statistical Analysis**

Boxplots are useful in visualizing variation as part of preliminary exploratory data analysis. Figure 8 shows that the measured cell-type markers have different ranges of values, including both their centers and spreads. The distributions of expression vary within each marker expression panel. However, in each cell-type marker, the majority of the distributions appear to be symmetric around the median. Each marker is scalewise comparable, as measured by original experimental parameters. Reading across the biomarker expressions, it is clear that some cells do not express some proteins. For example, lower cancer-derived cells (A549) do not express p21; upper primary cells (PTBE) do not express GSR; and upper transformed cells (Met-5A) do not express IL-6 when cultures are maintained in normal, healthy conditions.

Visual comparisons can be made in 3 ways: first, upper airway cells (PTBE, BEAS-2B, and A549) can be compared with corresponding lower airway cells (PSAE, Met-5A, and Calu-3, respectively). Second, primary cells can be compared against transformed or cancer-derived cells (eg, PTBE against BEAS-2B or A549); and third, cell type can be compared across the biomarkers (eg, compare p21, p53, GSR, and IL-6 expressions for PTBE). In p21 protein expression, lower transformed (Met-5A) have the largest distribution while lower cancer (Calu-3) have



Figure 8. Boxplots for the protein, cytokine, and enzyme concentrations measured in experimental datasets. A, p21 protein, B, GSR enzyme, C, p53 protein, and D, IL-6 cytokine. The cell types include PTBE (upper primary), BEAS-2B (upper transformed), A549 (upper cancer derived), PSAE (lower primary), Met-5A (lower transformed), and Calu-3 (lower cancer derived).

the smallest distribution. Upper cancer cells (A549) express p21 protein most similarly to upper primary cells (PTBE), whereas upper transformed cells (BEAS-2B) express p21 at lower concentrations than PTBE. Lower transformed cells (Met-5A) express p21 protein higher than lower primary cells (PSAE), whereas lower cancer cells (Calu-3) express p21 at lower concentrations than PSAE. When comparing CDKN1A mRNA data against p21 protein expression data (results not shown), the upper airway cells follow a similar pattern of primary cells expressing higher concentrations of p21 gene and protein as compared with transformed or cancer derived. When reading across the protein expression data, PTBE cells express a large amount of p53, moderate amount of p21 and IL-6, and low amount of GSR as compared with the other cell types.

The interaction plots in Figure 9 show that the measured cell-type markers appear to be dependent upon both lung location and type of cell and are useful in visualizing the effect of one factor in conjunction with another. Generally, lines with differing slopes indicate the possible presence of an interaction, indicating that the effect of lung location on the protein expression differs depending on the cell type. Conversely, parallel lines indicate that the effect of lung location does not depend on the cell type.

Based on the interaction plots and the test for significance of the interaction in a 2-way ANOVA (both parametric and nonparametric versions), a strongly significant interaction effect is present for each protein (all *p*-values less than .0001), implying that conclusions must be made that are specific to combination of lung location and cell type (Table 2). For example, when comparing the primary cells to the transformed cells in the p21 protein panel, lung location does have a strong effect on p21 protein expression, but its effect depends on the cell type. Cell type also has a strong effect on p21 protein expression, but its effect depends on the lung location. Follow-up analysis to test the null hypothesis that the means of each possible pair of treatments (15 total pairs of means to compare) are the same indicate that only the upper transformed and lower primary cells do not have significantly different average p21 protein expression (*p*-value .8711). All other p21 treatments have significantly different mean protein expression (*p*-values less than .001).

Table 3 lists a subset of 6 of the 15 possible pairwise comparisons for each biomarker expression (ie, p21 protein, p53 protein, IL-6 cytokine, and GSR enzyme). Comparisons across (i) lung locations with the same phenotype and (ii) between transformed and cancer-derived with primary for the same lung location are listed. The first set of comparisons is chosen to draw inference across lung locations with the same phenotypes, and only three of these comparisons (ie, Lower: Primary vs Upper: Primary for p53 protein and GSR enzyme and Lower: Cancer derived and Upper: Cancer-derived for the p53 protein) have p-values that are larger than .01. The remainder of the comparisons in mean biomarker expression across lung location have p-values less than .01, indicating that the mean biomarker expression differs significantly depending on the location from which the cells were extracted from the lung. The "Diff" column in Table 3 gives difference in the means between the two treatments and gives a sense of the scale of the differences detected and which combination of phenotype and lung location results in stronger gene expression. Note, a positive (negative)



Figure 9. Interaction plots for the protein, cytokine, and enzyme concentrations measured in experimental datasets showing the sample mean for each treatment connected by a line. A, p21 protein, B, GSR enzyme, C, p53 protein, and D, IL-6 cytokine. The cell types include PTBE (upper primary), BEAS-2B (upper transformed), A549 (upper cancer derived), PSAE (lower primary), Met-5A (lower transformed), and Calu-3 (lower cancer derived).

|                            | -           |             | -            |            |             |           |          |          |
|----------------------------|-------------|-------------|--------------|------------|-------------|-----------|----------|----------|
| Effect                     | p21 Protein | p53 Protein | IL6 Cytokine | GSR Enzyme | CDKN1A mRNA | TP53 mRNA | IL6 mRNA | GSR mRNA |
| LL                         | <.0001      | <.0001      | <.0001       | <.0001     | <.0001      | <.0001    | <.0001   | <.0001   |
| PT                         | <.0001      | <.0001      | <.0001       | <.0001     | <.0001      | <.0001    | <.0001   | .0109    |
| $\text{LL}\times\text{PT}$ | <.0001      | <.0001      | <.0001       | <.0001     | <.0001      | <.0001    | <.0001   | <.0001   |

Table 2. Two-Way ANOVA Results for Each Response

"LL" stands for "lung location," and "PT" stands for "phenotype."

difference in means indicates that the mean in column T1 (T2) is larger than the mean in column T2 (T1).

The second set of comparisons in Table 3 controls for location in the lung and compares the transformed and cancerderived phenotypes to the "gold standard" primary phenotype. If indeed the transformed and cancer derived phenotypes are equivalent to the primary cell type, then there should be no difference in the mean expression of each of these four biomarkers; however, the opposite conclusion is reached with this data. Every comparison indicates a significant difference between the mean expression for the transformed or cancer-derived cells and the primary cells, evidence that the transformed and cancer-derived cells cannot be substituted for primary cells with the expectation that the gene expressions will remain, on average, the same.

# DISCUSSION

Spontaneous generation of ROS produced during normal cell culture and proliferation can create low levels of oxidative

stress within cells. These endogenous sources of ROS generation include cellular respiration (ie, mitochondrial activity) and integrin-signaled adhesion (Murphy, 2009; Nohl *et al.*, 2005; Ravuri *et al.*, 2011; Sauer *et al.*, 2001). Endogenous ROS has the potential to trigger multiple adverse cellular effects through oxidative stress pathways and cell cycle arrest if the cell does not have the proper antioxidant compensation mechanisms.

Intracellular oxidation is connected to the cell's cycle. Cell cycle regulation requires a balance of different cyclins at different phases. For instance, G0/G1 is dependent on cyclin D, G1/S is dependent on cyclin E, S/G2 is dependent on cyclin A, and G2/ M is dependent on cyclin B. However, research has shown that each of these cyclins are vulnerable, and oxidation of complementary proteins and genes impair the cell cycle progress from one phase to another. Increases in certain proteins (such as p21 and p53) and genes (such as TP53 and CDKN1A) contribute to cell cycle arrest as demonstrated in this and other studies (Agarwal et al., 1995; Brugarolas et al., 1995; Bunz et al., 1998; Vousden and Lu, 2002). However, oxidation of cyclins and/or their associated cofactors also causes cell cycle dysregulation.

| Table 3. Tukey's Honestly Significant Difference (HSD) for Each Pair of Treatments With the Difference in the Means Between the Two | Groups |
|---|--------|
| Listed Along With the Associated p-Value for the Biomarker Expression (ie, Protein, Cytokine, and Enzyme)                           |        |

| Comparable                 | Pair                     |                          | p21 Protein |         | p53 Protein |         | IL-6 Cytokine |         | GSR Enzyme |         |
|----------------------------|--------------------------|--------------------------|-------------|---------|-------------|---------|---------------|---------|------------|---------|
|                            | T1                       | T2                       | Diff        | p-Value | Diff        | p-value | Diff          | p-Value | Diff       | p-Value |
| Across same phenotype;     | Lower: Primary           | Upper: Primary           | -124.58     | .00     | -5.06       | .02     | 1.08          | .00     | 3.29       | .03     |
| different lung location    | Lower: Transformed       | Upper:<br>Transformed    | 208.62      | .00     | -18.94      | .00     | 1.08          | .00     | -34.33     | .00     |
|                            | Lower: Cancer<br>derived | Upper: Cancer<br>derived | -186.59     | .00     | -4.37       | .06     | -0.69         | .00     | -10.80     | .00     |
| Across same lung location; | Upper: Transformed       | Upper: Primary           | -117.20     | .00     | -12.24      | .00     | -2.07         | .00     | 51.28      | .00     |
| different phenotype        | Upper: Cancer Derived    | Upper: Primary           | -32.04      | .00     | -15.85      | .00     | -0.54         | .00     | 26.30      | .00     |
|                            | Lower: Transformed       | Lower: Primary           | 216.01      | .00     | -26.12      | .00     | -2.06         | .00     | 13.65      | .00     |
|                            | Lower: Cancer Derived    | Lower: Primary           | -94.04      | .00     | -15.17      | .00     | -2.31         | .00     | 12.21      | .00     |

The *p*-values of those pairs whose means are not significantly different at the .01 level are in bold.



Figure 10. Proposed pathway linking ROS generation to induction of inflammatory cascades, decreased viability, and cell cycle disruption. The flowchart lists the descriptions, steps, and the associated proteins and genes involved along the pathway.

For instance, the MAPK family members JNK, ERK, and p38 respond to ROS and can further affect the cell cycle (Burhans and Heintz, 2009; Menon and Goswami, 2007; Zhang and Liu, 2002).

Two of the most commonly reported causes of subcellular molecular oxidation are direct (through mitochondrial respiration) or indirect (through metabolism of an engulfed xenobiotic) (Oberdörster *et al.*, 2005). In either direct or indirect mechanism, multiple ROS species can be involved, including  $H_2O_2$ , °OH<sub>2</sub>, °OH, or °O<sub>2</sub><sup>-</sup>. Once ROS generation overcomes the antioxidant capacity of the cell, oxidative stress is induced. Some of the adverse effects that are especially responsive to this induction are DNA damage (at the molecular level), cell cycle arrest (at the cell

level), inflammation (at the tissue level), and cancer (at the organism level) (Figure 10).

To date, the six cell types presented in this study are cited as the most commonly utilized cell lines in *in vitro* toxicology, which include reports on pulmonary hazards, nanomaterial exposures, workplace scenarios, and particulate matter health effects. Each of these cell lines have major differences. PTBE, BEAS-2B, A549, PSAE, MeT-5A, and Calu-3 are not just limited to differing phenotypes, but also are unalike in their morphologies, proliferation rates, mRNA and protein expressions, antioxidant capacities, pro-inflammatory states, and cell cycle distributions. Furthermore, the elevated oxidative stress of each cell type is not equally perturbed after exposure to the same concentration of the oxidative stress control used in this study. In general, the results of the oxidative stress challenge can be analyzed in two different ways. First, the primary cells responded to the oxidative stress more pronouncedly than either the transformed or cancer-derived counterparts. As a group, the lower airway cells responded to the oxidative stress ubiquitously as compared with the group of upper airway cells. Comparing adverse health effects is not feasible for read-across efforts without the proper baseline characterization among cell types; put simply, these cells are not interchangeable.

When designing an in vitro toxicological study, it is important to select cell cultures with unaltered cell signaling pathways relevant to the expected adverse cellular effect as well as being derived from the relevant site of injury. For the lung, studies have shown that the effects of cells and tissues in the upper airways respond differently than cells and tissues in the alveolar space and in the pleural space (Berg et al., 2013; Hatch and Gross, 2013; Oberdörster, 2010; Pedley, 1977). These differential responses can be attributed to xenobiotic dose or physicochemical properties, as well as the characteristics of the cell culture. Just as the properties of the xenobiotic agent require careful characterization in any in vitro toxicology study, similar rigor must extend to collecting and reporting data on the antioxidant capacity and cell cycle population distribution. These are key indices gauging the relative health of the cell culture before toxicant exposure and will allow for reading across various studies of similar design or will warn when the cellular data are incongruous (Figure 8). With this new understanding, in vitro toxicology datasets have the potential to be exponentially more translatable to other areas of science and, eventually, policy.

# DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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