Characterization of the Cellular and Molecular Dynamics of Inhaled Chemical Exposure Effects and Susceptibility Using Organotypic In Vitro Models

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US Environmental Protection Agency
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Conflict of Interest and Disclaimer

• No conflicts of interest

• The information presented here does not necessarily reflect the views or policies of the U.S. Environmental Protection Agency. Mention of trade names or commercial products does not constitute endorsement or recommendation for use.
Key Considerations for the Extrapolation of *In Vitro* Data

- Differentiated primary human bronchial epithelial cell (dpHBEC) model
  - Improved physiological relevance compared to cell lines
  - What about inter-individual variability from donors?

- Extrapolation of exposure scenarios
  - What is the relationship between single and repeated exposure outcomes?

- Dosing method
  - Air-liquid interface exposures are physiologically relevant, but a significant technical limitation
  - How does the dosing method affect exposure outcomes and subsequent interpretation?

- Complex *in vitro* tissue models
  - The lung is a complex organ with over 40 cell types, but nearly all *in vitro* models are monocultures
  - How can we model these aspects of the tissue, and do we even need to?
Building confidence in the ability of *in vitro* systems and data to recapitulate/predict *in vivo* responses is a complex issue that is critical for acceptance.

- Part 1: How do inter-individual variability and repeated exposures affect exposure outcomes?
- Part 2: How are *in vitro* exposure outcomes and subsequent extrapolations affected by test agent dose delivery?
- Part 3: Do commonly used *in vitro* systems represent the roles of different cell types that exist *in vivo* as targets and mediators of inhaled chemical exposures?
Part #1
Inter-individual Variability and Repeated Exposures
Background

- Nearly all *in vitro* studies on inhaled chemicals are conducted using a single exposure with a small number (e.g., *n* = 3-5) donors.
- Ozone (O$_3$) is a model oxidant and ubiquitous air pollutant:
  - Decades of existing *in vivo* human and animal exposure data.
- Inter-individual variation in the pro-inflammatory/inflammatory response to ozone occurs *in vivo* and is reproducible over time.
- Individuals are frequently subject to repeated ozone exposures:
  - Controlled *in vivo* exposure studies indicate attenuation of effects.
ALI-Differentiated dpHBEC System
Experimental Design

Exposures: 2 hr/day
FA: filtered air (control)
O₃: 0.5 ppm
Harvest: 2 hr post Day 4 exposure

n = 25 donors (20M, 5F)
Mean age: 29.0 ± 5.1
“Healthy” non-smokers
Inter-Individual Variability in Ozone-Responsive Gene Expression

## Estimated versus Observed Toxicodynamic Variability

**Intra-human Uncertainty Factor (UF<sub>H</sub>)**

\[
UF_H = TK \times TD = 10\text{-fold}
\]

\[
UF_H = (\sqrt{10}) \times (\sqrt{10})
\]

\[
UF_H = (3.16) \times (3.16)
\]

<table>
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<tr>
<th></th>
<th>Range of 1X O&lt;sub&gt;3&lt;/sub&gt; Induction</th>
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Repeated Exposure Results in Attenuation of Target Gene Expression

### Implications on *In Vitro* Sample Size

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Calculated based on identifying a statistically significant 2.0-fold change

Part #1 Conclusions

- Inter-individual variability exists at the cellular level in the dpHBEC-ALI cell model.
- Pro-inflammatory gene attenuation occurs in dpHBEC in response to repeated exposures.
  - Induction and attenuation outcomes align with *in vivo* data.
- The default toxicodynamic (TD) component of the intra-human uncertainty factor may be underestimating the range of response within the “normal healthy” population.
- Sample size should be considered when studies need to reflect inter-individual variability and/or *in vitro* uncertainty factors may be required.
Part #2
Can the dosing method make or break the poison?
Part 2: Background

How does the dosing method affect IVIVE?
• Is the exposure conducted in a manner that is comparable to *in vivo* conditions?
• Air-liquid interface (ALI) conditions complicate test agent delivery
  • Especially for methodologically challenging chemicals
• Liquid dosing of ALI cultures is commonly used
• Does the application of liquid alone to differentiated ALI cultures affect their physiology?
Study Design

Liquid application for 6 and 24 hr

Maintained at ALI ("PRE")

TEER FITC-Dextran

Separate Cell Layers

ELISA

RNA-Sequencing

RNA-Sequencing

Mallek NM, et al. In Preparation
Liquid Application Alters Global pHBEC Gene Expression

- **6 hr**: 18% of 4169 genes are significantly alternatively regulated.
- **24 hr**: 43% of 10,268 genes are significantly alternatively regulated.
Liquid Application Alters pHBEC Gene Expression

Alternatively Regulated Genes in pHBEC at 6 hr vs. 24 hr

Mallek NM, et al. In Preparation
**Liquid Application Alters Cell Physiology**

### Canonical Pathways

**pHBEC 6 hour**

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<th>Pathway</th>
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![Graph showing canonical pathways and their fold changes](image)

**Categories**

- Inflammation
- Signal Transduction
- Transcription Factor
- Chromatin
- Transcription
- Translation
- Cell Cycle
- Structural
- Redox

**Target Fold Change**

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Mallek NM, et al. In Preparation
**Liquid Application Alters Cell Physiology**

**Canonical Pathways**

**pHBECS 24 hour**

### Tumor Microenvironment Pathway
- P53/TP53 ( activation)

### Hepatic Fibrosis/Stellate Cell Activation Pathway
- HIF1α signaling

### Regulation Of The Epithelial Mesenchymal Transition By Growth Factors Pathway
- Airway Pathology in Chronic Obstructive Pulmonary Disease
- Osteoarthritis Pathway

### Wound Healing Signaling Pathway
- IL-17 Signaling
- PPAR Signaling

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<thead>
<tr>
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*Mallek NM, et al. In Preparation*
Liquid Application Disrupts Barrier Integrity

**TEER**

**FITC-dextran Permeability Assay**

Mallek NM, et al. In Preparation
Liquid Application Causes a Pro-Inflammatory Response

Mallek NM, et al. In Preparation
Part #2 Conclusions

• Liquid application causes an EMT/pre-cancerous phenotype in dpHBEC cultures

• The magnitude of the effect of liquid application on these common endpoints increased over time and was greater than that often observed with many known toxicants

• Liquid application up-regulated several stress-responsive cellular pathways, which could result in a “two-hit” or synergistic interaction when combined with a test agent

• Overall, these observations suggest that the effects of liquid application of dpHBEC ALI cultures alone on these endpoints has the potential to confound the interpretation of data collected using this method for *in vitro* inhaled chemical testing
Part #3
Are we looking in the right place at the right time?
Lung Structure and Function in *In Vitro* Testing

- **Trachea**
- **Bronchi**
- **Bronchioles**
  - Large conducting airways
  - “large airways”
- **Terminal bronchioles**
  - Small conducting airways
  - “small airways”
- **Respiratory bronchioles**
- **Alveoli**
  - Pulmonary airways
Consider the Biology

Epithelial barrier

Stroma

DIRECT

TRANS-EPITHELIAL
Trans-Epithelial Exposure Model (TEEM)

Bronchial epithelium

Bronchial stroma

Cilia
Lymphocyte
Goblet cells
Basement membrane
Connective tissue
Fibroblasts
Collagen fibers
Mucous acinus
Serous acinus
Basal cells
Blood vessels

Direct Exposure
Trans-Epithelial Exposure
TE-DEP Exposure Causes Redox Imbalance

Intracellular H$_2$O$_2$

Glutathione Oxidation

Addgene plasmid ID: 137170
Faber et. al., (2020) Toxicological Sciences 177(1):140-155
DEP Activates NRF2 in HBEC and HLF

Faber et al., (2020) Toxicological Sciences 177(1):140-155
DEP Induces Oxidative Stress Genes in Epithelial Cells and Fibroblasts

HMOX1

NQO1

COX2

SQSTM1

Fold Change (Relative to Vehicle)

Exposure Duration (hr)

Fold Change (Relative to Vehicle)

Exposure Duration (hr)

Fold Change (Relative to Vehicle)

Exposure Duration (hr)

Fold Change (Relative to Vehicle)

Exposure Duration (hr)

Fold Change (Relative to Vehicle)

Exposure Duration (hr)

Duox2
Gclm
Slc7a11
Ptgs2
Sod2
Txnrd1
Hmox1
Mpo
Txnrd2
Sqstm1
Nqo1
Sftpd
Srxd1

Fold Change (Relative to Vehicle)
DEP-Induced Oxidative Stress-Responsive Proteins

Faber et. al., (2020) Toxicological Sciences 177(1):140-155
Lung Structure and Function in *In Vitro* Testing

- **Trachea**
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- **Alveoli**
  - Pulmonary airways
Alveolar-Capillary Region Exposure (ACRE) Model

- Alveolar epithelium
- Alveolar interstitium
- Microvascular endothelium

Vitucci ECM, et al. In Preparation
ACRE Model Recapitulates Tight and Adherens Junctions

Vitucci ECM, et al. In Preparation
DEP Exposure Does Not Alter Alveolar Barrier Function

TEER

<1 kDa

4 kDa

20 kDa

n=3 independent experiments. Error bars represent ±SD.

Vitucci ECM, et al. In Preparation
Multi-Cellularity is Important for Identifying Cell Type-Specific Effects

6hr DEP

- HAEC: 55
- HLMVEC: 22
- Total: 146

24hr DEP

- HAEC: 15
- LMVEC: 7
- Total: 946

Source: Vitucci ECM, et al. In Preparation
Part #3 Conclusions

- Inhaled chemical exposures cause trans-epithelial effects
- Trans-epithelial effects can be greater than those observed in directly exposed epithelial cells
- Underlying cell types (i.e., fibroblasts and microvascular endothelial cells) appear to be highly sensitive to oxidative insult
- Cell types with different functions respond to insults differently
  - Epithelial cells alone are not a reliable surrogate for adjacent cell types
- Temporal resolution is important for the identification of different (or any) key events
- These low-cost readily transferrable models can be used to improve in vivo relevance and testing capabilities in low- and medium-throughput testing of inhaled chemical
  - ~$10 per culture when using primary cells and ~$5 with cell lines
  - Constructed from all commercially available materials
  - TEEM version 1.0 methods are publicly available
  - TEEM version 2.0 methods will be published soon
  - ACRE version 1.0 methods will be published soon
  - Feel free to contact me if you are interested in advanced drafts and support
Take Home Message(s)

• Building confidence in the ability of *in vitro* systems and data to recapitulate/predict *in vivo* responses is complex issue that is critical for acceptance.
  
  • Inter-individual variability exists *in vitro* and requires consideration for sample sizes and uncertainty factors
  
  • Single exposure outcomes are not a reliable surrogate for repeated exposure outcomes
  
  • The effect of dosing method alone needs to be included in studies to facilitate the proper context for data interpretation
  
  • Exposures cause trans-epithelial effects and impact cell types differently
  
  • Time course data are critical to reliable identification of exposure effects
Acknowledgements

McCullough Lab
• Present
  • Eva Vitucci
  • Nick Mallek
  • Emily Aungst
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UNC
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NIEHS
• Elizabeth Martin, PhD

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