PMI RESEARCH & DEVELOPMENT

IIVS Workshop on "Assessment of In Vitro COPD Models For Tobacco Regulatory Science"

" Combining Systems Biology, a Computational Approach and a Human Organotypic In Vitro Model Exposed to Whole Cigarette Smoke: An Example of 21st Century Toxicology Assessment"

Carole Mathis, Philip Morris International, R&D

December 9th, 2014

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  - Example of NPA and BIF
- Network Models to Study Adverse Outcome Pathways
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Background

2007: Publication by the U.S. National Research Council of a new strategy plan for toxicology assessment to update and advance our knowledge on the toxicity and the Mode of Action of environmental agents.

Example of recommended approaches:

- Medium and high-throughput *in vitro* screening assays
- Computational toxicology
- Systems biology
- Pharmacokinetic modeling

21st Century Toxicology

2009: European Commission published a report on «Alternative Testing Strategies for «Replacing, Reducing, and Refining» («3R») the use of animals in research.

- Human organotypic tissues based on primary cells cultured in three dimensions, with proper cell-cell contact, recapitulating biological functions (e.g. mucus secretion, muco-ciliary clearance,..) and allowing whole cigarette smoke exposure at the air-liquid interface.

Background to Case Study

- Smoking causes serious diseases such as cardiovascular diseases, lung cancer and chronic obstructive pulmonary disease.
- Philip Morris International is therefore developing novel products that may have the potential to reduce smoking-related disease risk compared to conventional combustible cigarettes.
- To determine whether such potentially reduced-risk products (RRP) have the potential to reduce disease risk, among the other things, we compare their biological impact with that of a combustible reference cigarette (3R4F) on a mechanism-by-mechanism basis.

Quantitative Mechanism-Based Systems Impact Assessment

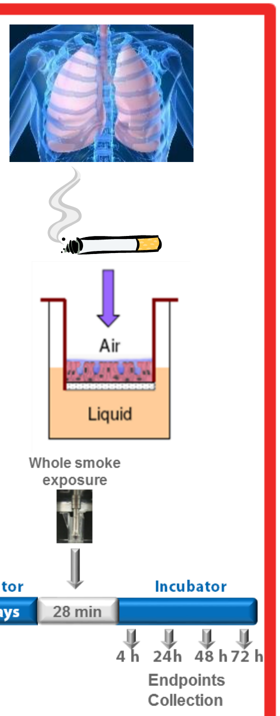
Experimental data production

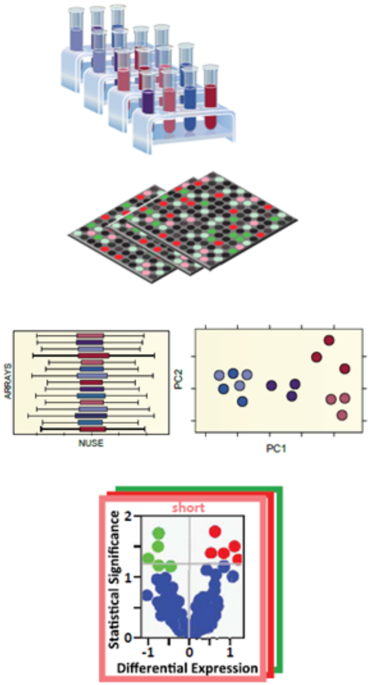
Compute systems response profiles

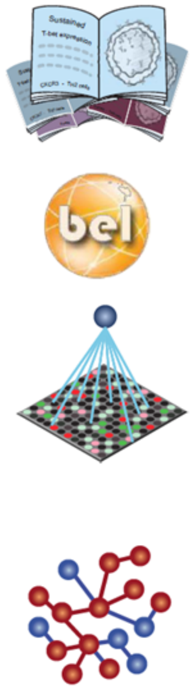
Identify perturbed biological networks

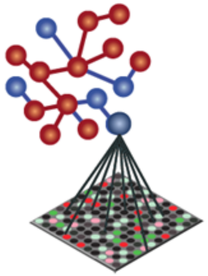
Compute network perturbation amplitudes

Compute product biological impact









Find x that minimizes

$$\sum_{\substack{\text{edges}\\(i,j)}} \omega(i,j)\cdot(x_i - \text{sign}(i,j)\cdot x_j)^2$$

subject to

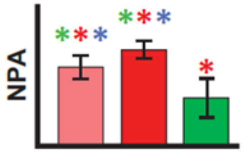
$$x|_{\text{transcript layer}} = \beta$$

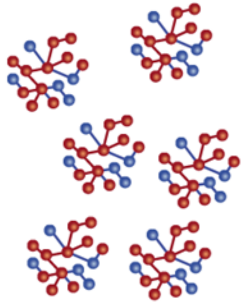
where $\omega(i,j)$ gives the edge weights.

Evaluate

$$\sum_{\substack{\text{edges}\\(i,j)}} (x_i + \text{sign}(i,j)\cdot x_j)^2$$

where the sum includes all the edges of the functional layer.

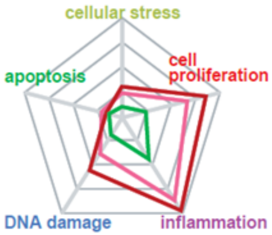




For each SRP c, evaluate

$$\text{BIF}(c) = \sum_{\text{networks } n} \omega(n,c)\cdot\text{NPA}(n,c)$$

where the weights $\omega(n,c)$ are non-zero for perturbed networks (***) only.



Step 1: Characterization of Both *In Vitro* Model/Exposure System

Exposure system characterization:

- **Characterization of the Vitrocell® 24/48 in vitro aerosol exposure system using mainstream cigarette smoke**

S Majeed, S Frentzel, S Wagner, D Kuehn, P Leroy, PA Guy, A Knorr, J Hoeng, MC Peitsch
Chemistry Central J. in press.

Organotypic tissue cultures: How close are they to *in vivo*?

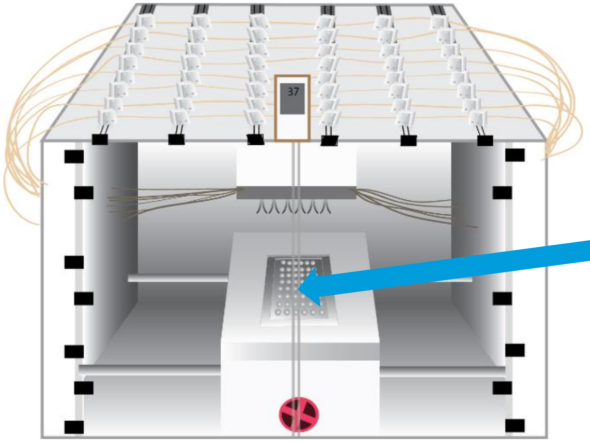
- **Human bronchial epithelial cells exposed in vitro to cigarette smoke at the air-liquid interface resemble bronchial epithelium from human smokers.**

Mathis C, Poussin C, Weisensee D, Gebel S, Hengstermann A, Sewer A, Belcastro V, Xiang Y, Ansari S, Wagner S, Hoeng J, Peitsch MC.
Am J Physiol Lung Cell Mol Physiol. 2013 Apr 1;304(7):L489-503.

- **Systems approaches evaluating the perturbation of xenobiotic metabolism in response to cigarette smoke exposure in nasal and bronchial tissues.**

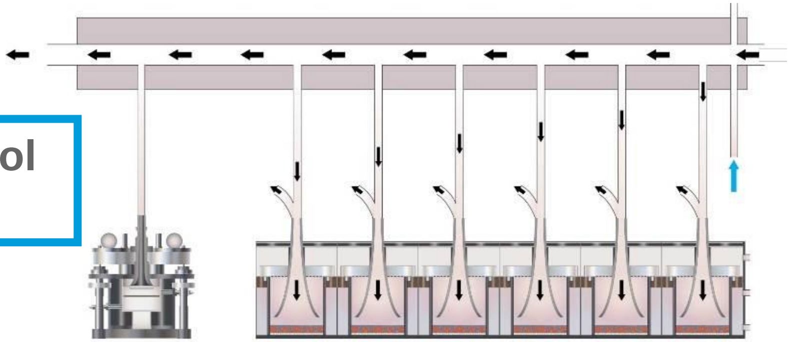
Iskandar AR, Martin F, Talikka M, Schlage WK, Kostadinova R, Mathis C, Hoeng J, Peitsch MC.
Biomed Res Int. 2013;2013:512086.

VITROCELL® Whole Smoke Exposure System

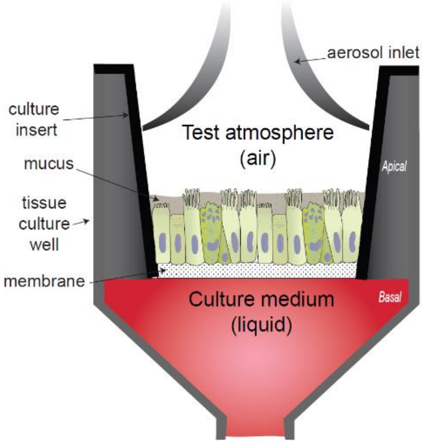


Climatic chamber with an exposure module

Up to 48 wells can be exposed simultaneously. The base module has a format of 8 rows x 6 columns.



The smoking machine produces aerosol from research cigarettes and RRPs



Human organotypic bronchial tissue cultures can be exposed at the air-liquid interface to whole mainstream smoke of the 3R4F or aerosol generated from RRPs

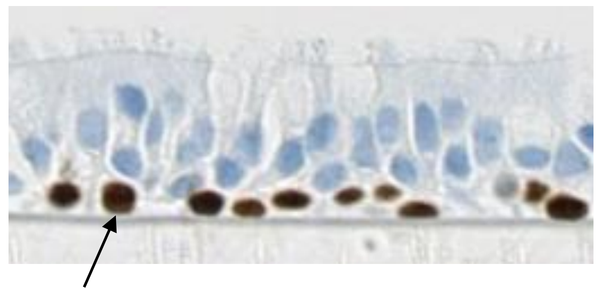
Whole Cigarette Smoke/Aerosol Exposure System (Vitrocell®)

VITROCELL® EXPOSURE SYSTEM

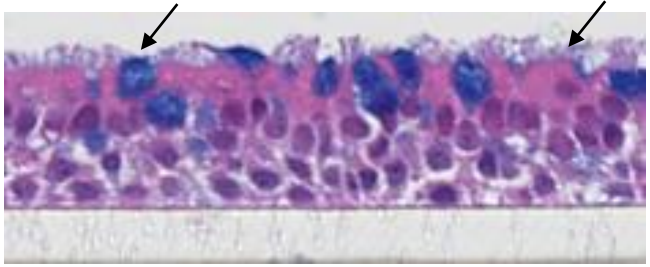
VITROCELL® DEPOSITION SENSOR

MOVIES

Human Organotypic Bronchial Epithelial Cells Resemble *In Vivo* Bronchial Epithelium



P63 immunostaining



Hematoxylin/Eosin/Alcian Blue

Unperturbed human organotypic bronchial epithelial cell culture closely resembles to human lung epithelium both at the morphological level (Karp et al. 2002) and at the molecular level (Pezzulo et al. 2011).

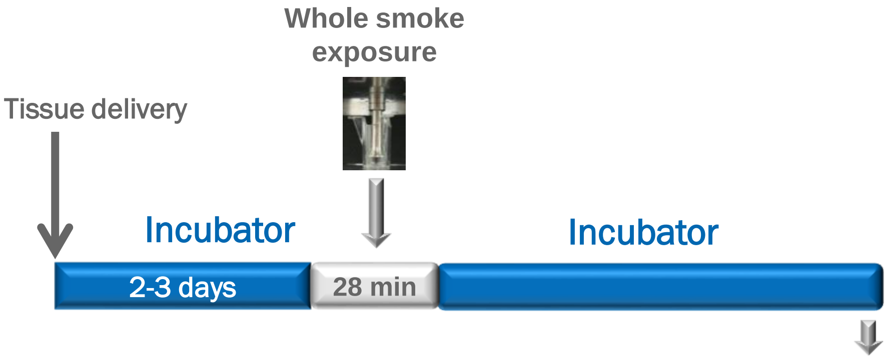
Goblet Cells Metaplasia Can Be Induced in Human Bronchial Organotypic Tissue Cultures

- **HBEC cultured in ALI system in the presence of IL-13 (10ng/mL, from day 2) and a combination of IL-13 and IL-4 (10ng/mL, days 6-12) developed mucous cell metaplasia**. – *Eicosanoid biosynthesis during mucociliary and mucous metaplastic differentiation of bronchial epithelial cells* - Jakiela B. et al. Prostaglandins and other Lipid Mediators; 106 (2013) 116-123.

- **Mucous metaplasia is induced in primary human airway epithelial cells cultured at ALI and treated with IL-13 for 5 days.** – *SAM-pointed domain ETS factor mediates epithelial cell-intrinsic innate immune signaling during airway mucous metaplasia* – Korfhagen, TR. et al. Proc. Natl. Acad. Sci. USA (2012) Oct 9;109(41):16630-5.

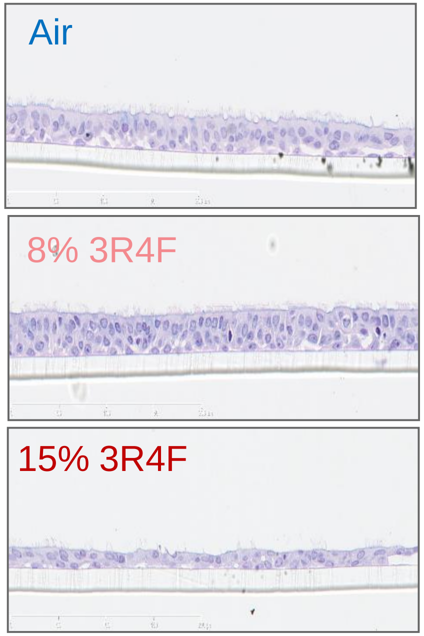
- **IL-4 induces mucous cell metaplasia in primary human bronchial epithelial cells cultured at ALI.** – *Association of TMEM16A chloride channel overexpression with airway goblet cell metaplasia* – Scudieri P. et al. J. Physiol. (2012) 590-23: 6141-6155.

Example of CS Acute Exposure Impact on Human Organotypic Bronchial Epithelial Cells



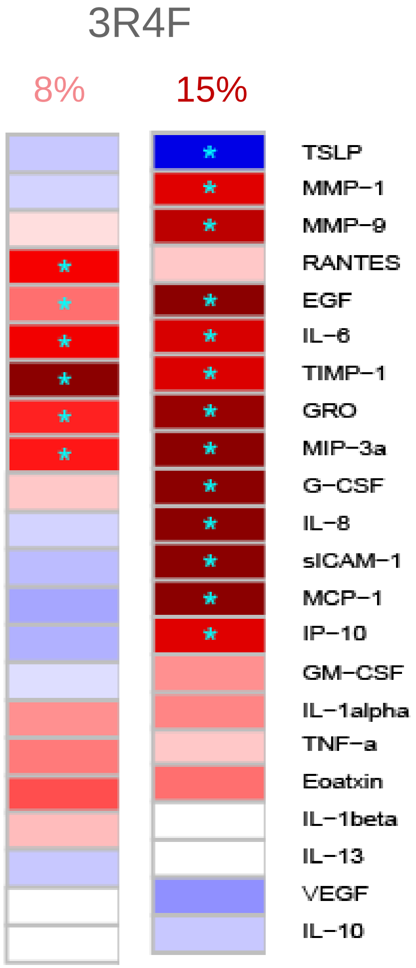
48 h

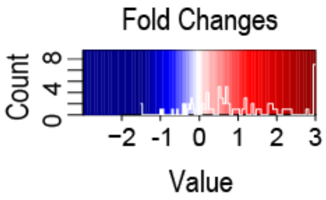
Histology



Hematoxylin/Eosin & Alcian Blue

Inflammatory markers release





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Organotypic Cultures of Human Primary Bronchial Epithelial Cells Exposed to Whole Smoke

Experimental Data Production

Experimental Design

TEST SUBSTANCE	SHAM				CIGARETTE SMOKE				ENDPOINTS
Exposure Time (Min)	7	14	21	28	7	14	21	28	
Post-Exposure (p-e) Time (Hrs)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	**Gene Expression** **MicroRNA** **MMP-1 Release** **Differential Cell Counts** **Survival**
	2	2	2	2	2	2	2	2	
	4	4	4	4	4	4	4	4	
	24	24	24	24	24	24	24	24	
	48	48	48	48	48	48	48	48	

Human Organotypic Bronchial Epithelial Cells Exposed to CS Resemble Bronchial Epithelium From Human Smokers

- For all four *in vivo* smoking gene signatures used in the GSEA, a similar pattern of enrichment score was found in CS-exposed AIR-100 up-regulated gene regulation profile (Fig. A) and in down-regulated gene regulation profile (Fig. B).
- Com o and *in*
  vitro
- Gene le genes
  after gulated
  gene

Functional Analysis

In both
in vivo / in vitro
context

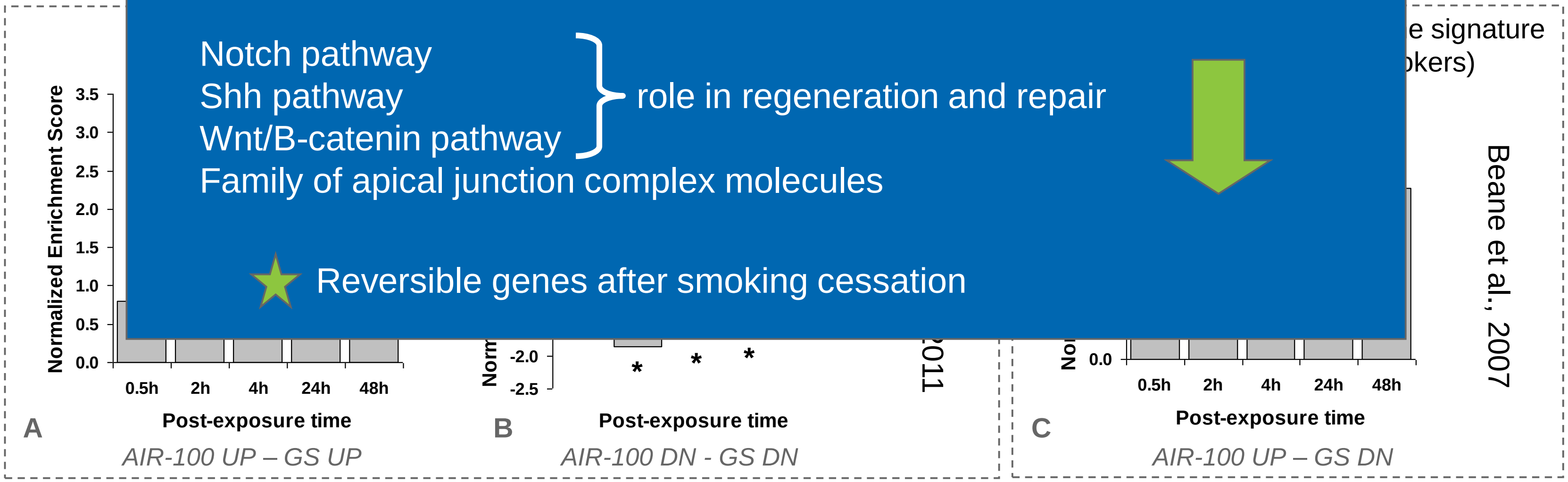
↑

Metabolism of xenobiotics ★
Redox balance ★
Glutathione metabolism
Pentose Phosphate pathway ★
Many direct target genes of Nrf2 transcription factor

↓

Notch pathway
Shh pathway
Wnt/B-catenin pathway
} role in regeneration and repair
Family of apical junction complex molecules

★ Reversible genes after smoking cessation



AIR-100 UP – GS UP

AIR-100 DN - GS DN

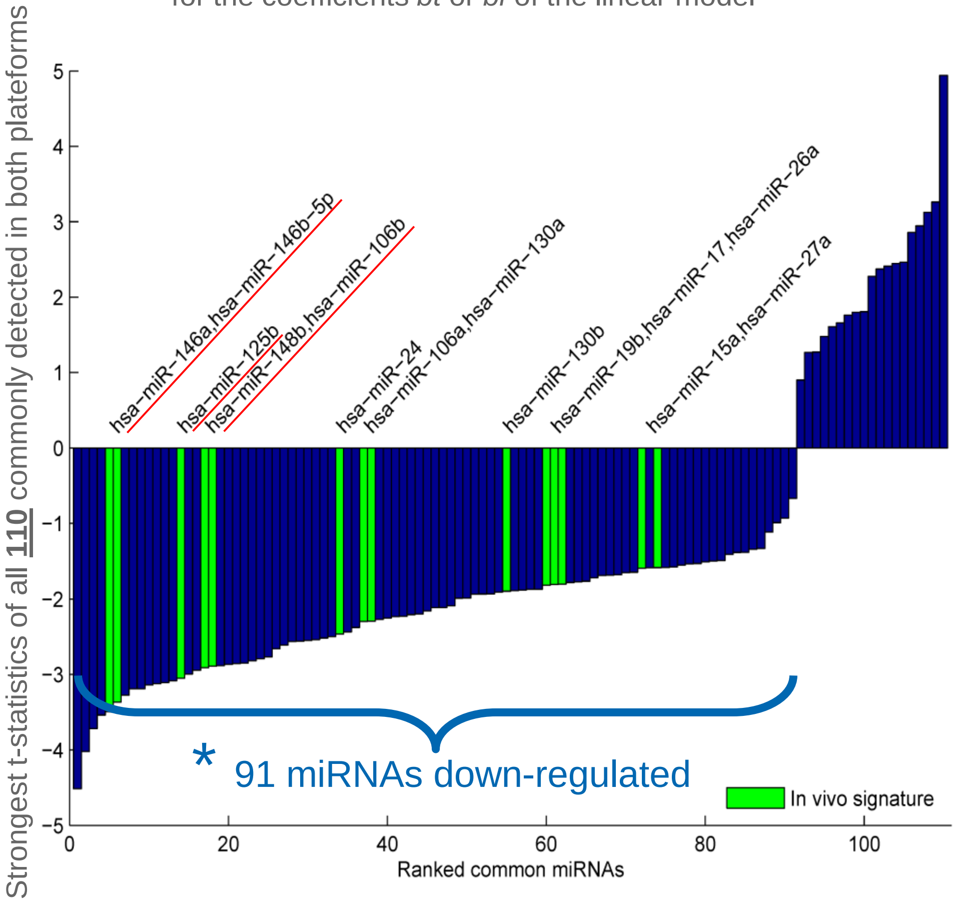
AIR-100 UP – GS DN

Human Organotypic Bronchial Epithelial Cells Exposed to CS Resemble Bronchial Epithelium From Human Smokers

- Only one human *in vivo* miRNA dataset from bronchial epithelial cells published so far (Schembri et al. 2009).
- Out of ~ 230 miRNAs detectable in this tissue context, half of them are commonly detected in both *in vivo* and our *in vitro* studies. Only 14 miRNAs differentially expressed are common between both *in vivo* and *in vitro* datasets (GREEN tag).
- CS down-regulates a large majority of miRNA expression (*: 91 miRNAs out of 110) in both *in vivo* and *in vitro* situation.
- The biological functions associated with some of the highly "translatable" miRNAs are related to inflammation (miR-146b and miR-125b) and cell cycle processes (miR-106a and miR-106b) that are also known to be perturbed by CS in lung tissue context.

Comparison between *in vivo* human smoker miRNA signature and CS-exposed AIR-100 *in vitro* miRNA dataset.

The vertical axis of the bar plot represents the best t-statistics (i.e. lowest negative or highest positive t-scores) obtained for the coefficients *bt* or *bi* of the linear model



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Advantages/Limitations of the Air-Lifted Bronchial Epithelial Culture Model

Advantages

- Human primary cells (No species translatability issue - 3 Rs)
- Different donors available (e.g., smokers, non smokers, COPD patients)
- Long-term culture possible
- Like *in vivo*:
  - Direct exposure to whole smoke at the air-liquid interface
  - Similar morphology/structure (Goblet cells, basal cells, ciliated cells, tight junctions, pseudostratified epithelium)
  - Similar gene expression pattern (normal untreated conditions)
- Various endpoints can be collected:
  - From tissue insert: RNA/miRNA/DNA/Proteins/Metabolites, morphological changes (histo/IHC), Tissue integrity (TEER), ion channels activity measurement
  - From basal side medium: Release of inflammatory markers, cytotoxicity (AK assay, LDH release,..), CYPs activity
  - From the apical side: Mucin secretion, cilia beating frequency, Mucociliary clearance

Limitations

Limited number of inserts from one donor

Risk of contamination when performing chronic/long-term exposure

Donor-to-donor variability

Missing other key players (immune cells, fibroblasts, smooth muscle cells)

Difficult to find the right exposure design (appropriate dose, how many exposure per day/per week, reproducibility…) -> Large experiment -> High cost/Time consuming

Step 2: Mono- or Co-culture Organotypic Bronchial Tissue Culture?

An Impact Assessment of Cigarette Smoke on Organotypic Models of Bronchial Epithelial Mono-culture and Bronchial Epithelial/Fibroblast Co-culture

A Iskandar, X Yang,S Frentzel, C Mathis, P Leroy, D Kuehn, S Majeed, C Merg, A Elamin, E Guedj, R Dulize, F Martin, M Talikka, MC Peitsch and J Hoeng

POSTER

Step 3: Assessment of Whole Smoke Exposure Impact on Organotypic Bronchial Tissue Culture

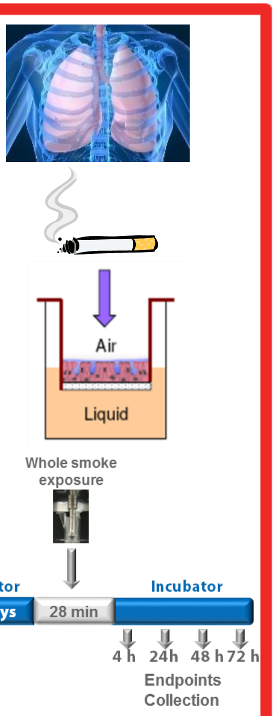
Experimental data production

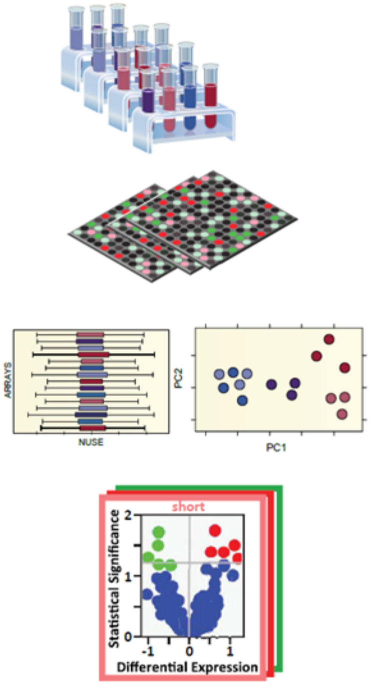
Compute systems response profiles

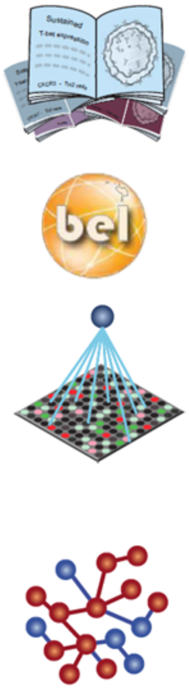
Identify perturbed biological networks

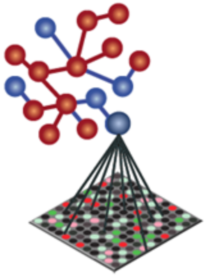
Compute network perturbation amplitudes

Compute product biological impact









Find x that minimizes

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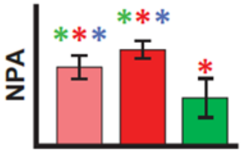

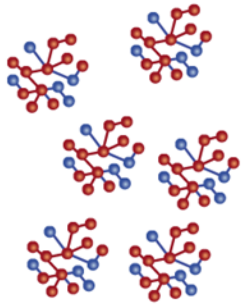

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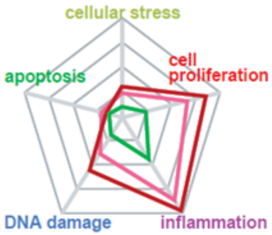

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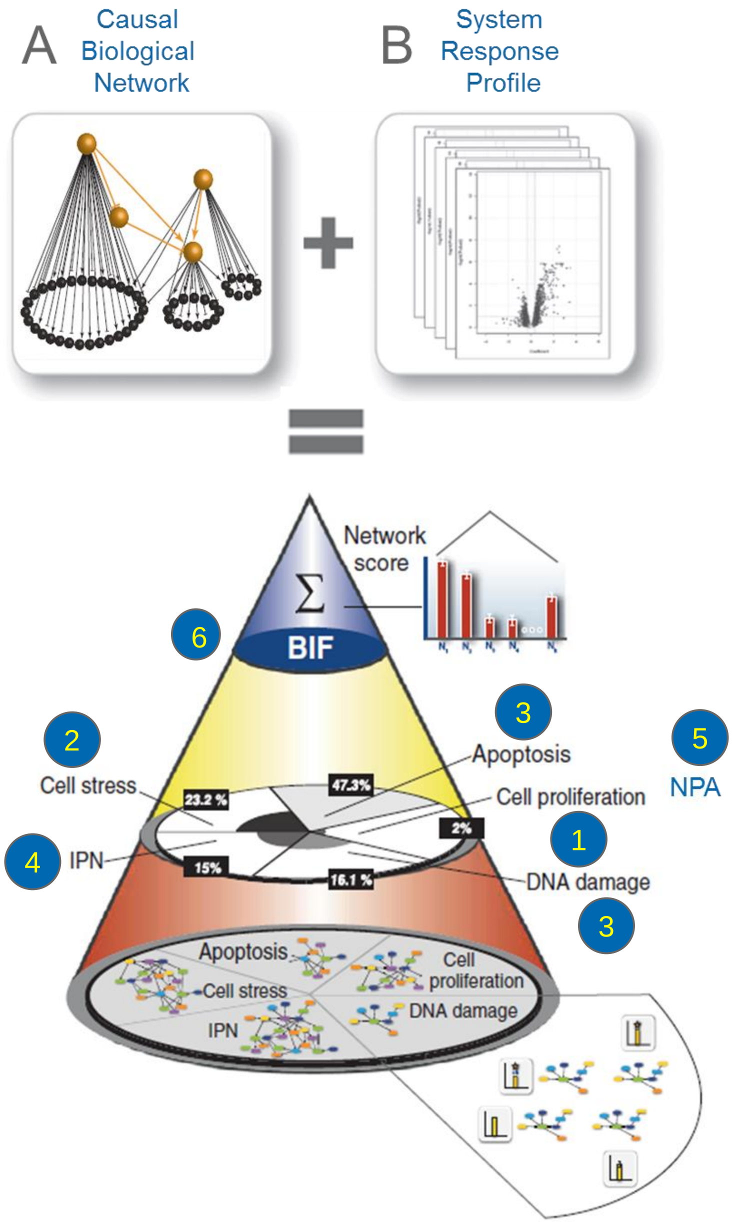
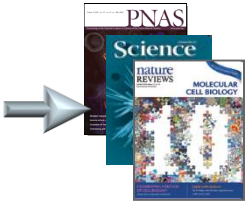
Systems Toxicology Assessment of Whole Smoke Exposure



1. **Construction of a computable cell proliferation network for non-diseased lung tissue.** *J. Westra, et al.* BMC Systems Biology, 2011 Jul 5:105.
2. **Construction of a computable cellular stress network for non-diseased lung and cardiovascular tissue.** *W.K. Schlage, et al.* BMC Systems Biology, 2011 Oct 5:168.
3. **Construction of a Computable Network Model for DNA Damage, Cell Death, Autophagy, and Senescence.** *S. Gebel, et al.* Bioinformatics and Biology Insights 2013 7:97-117.
4. **A modular cell-type focused inflammatory process network model for non-diseased pulmonary tissue.** *J. Westra, et al.* Bioinformatics and Biology Insights 2013 Jun 20; 7:167-92.
5. **Assessment of network perturbation amplitude by applying high-throughput data to causal biological networks.** *F. Martin, et al.* BMC Systems Biology 2012, 6:54.
6. **Quantification of biological network perturbations for mechanistic insight and diagnostics using two-layer causal models assessment of biological impact using transcriptomic data and mechanistic network models.** *F. Martin, et al.* BMC Bioinformatics. 2014 Jul 11;15(1):238.

Biological Statements from Scientific Literature/Original Research Are Coded into Network Models Using Biological Expression Language (BEL)

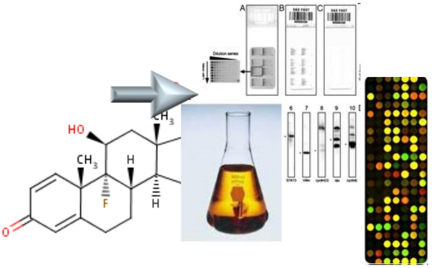


Scientific Literature

"RNA expression of RBL2 is directly mediated via activation of the FOXO3 transcription factor"

`tscript(p(HGNC:FOXO3)) => r(HGNC:RBL2)`

J Biol Chem 2002 Nov 22 277(47) 45276-84



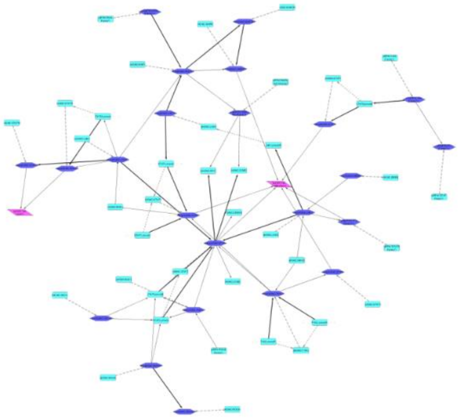
Original Research

"LY294002 inhibits the activity of the PI3K alpha catalytic subunit"

`a(CHEBI:LY294002) -| kin(p(HGNC:PIK3CA))`

XYZ Corp Document 12345

Network Models



Repeated Whole Smoke Exposure of Organotypic Cultures Derived from Human Primary Epithelial Cells

Experimental data production

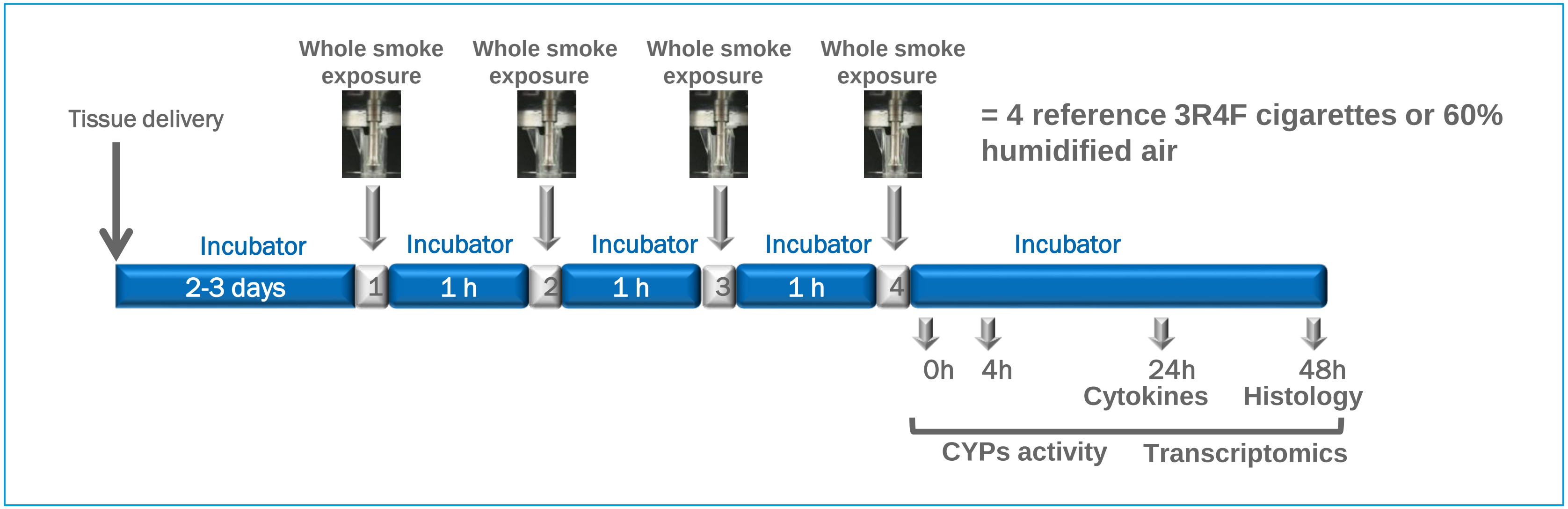
Compute systems response profiles

Identify perturbed biological networks

Compute network perturbation amplitudes

Compute product biological impact

EXPERIMENTAL DESIGN



Tissue delivery

Whole smoke exposure

Whole smoke exposure

Whole smoke exposure

Whole smoke exposure

= 4 reference 3R4F cigarettes or 60% humidified air

Incubator 2-3 days

1

Incubator 1 h

2

Incubator 1 h

3

Incubator 1 h

4

Incubator

0h

4h

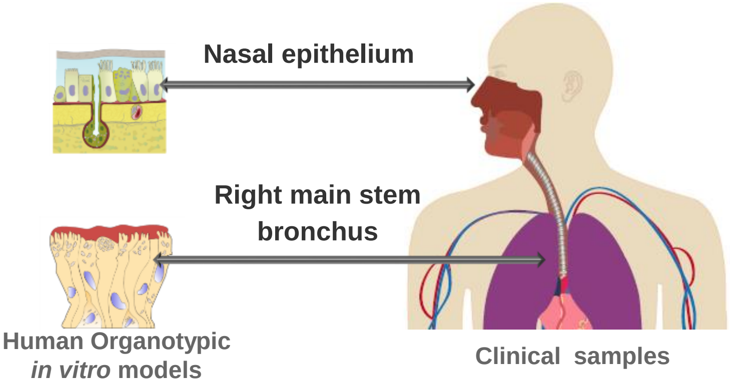
24h Cytokines

48h Histology

CYPs activity

Transcriptomics

Comparison of Clinical Samples from Smokers to CS Exposed Nasal and Bronchial Organotypic Cultures



Nasal epithelium

Right main stem bronchus

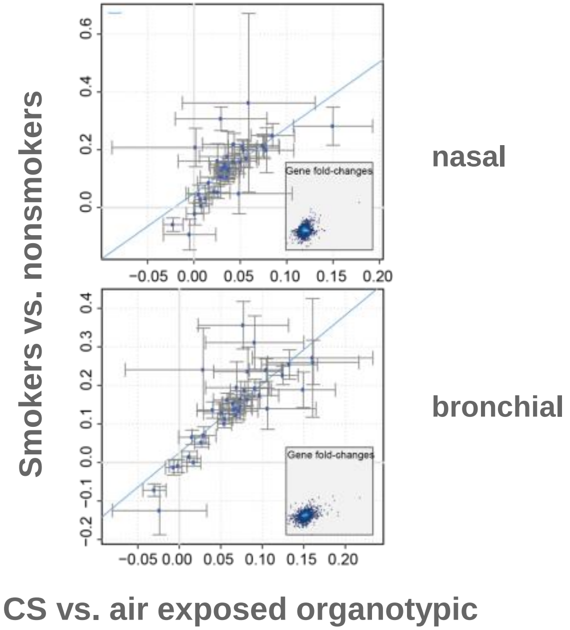
Human Organotypic
***in vitro* models**

Clinical samples

Healthy Non-smoker (n=14) Age	Healthy Smoker (n=13) Age	Smoker Pack-years
31.6 ± 10.8	35.4 ± 9.9	10.77 ± 9.3

(Zhang X. et al., Physiol. Genomics 2010)

Perturbation of the Xenobiotic metabolism network model

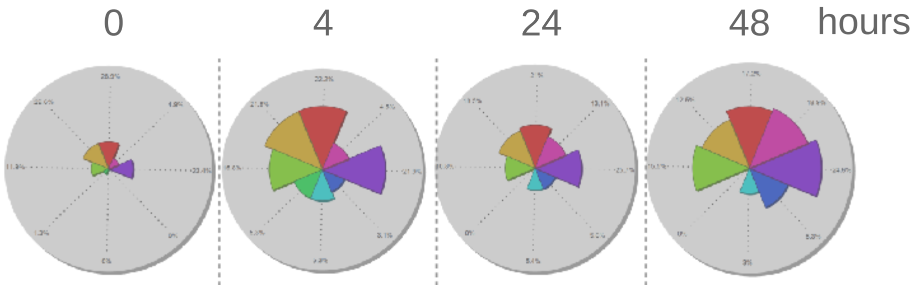


Systems Approaches Evaluating the Perturbation of Xenobiotic Metabolism in Response to Cigarette Smoke Exposure in Nasal and Bronchial Tissues
Iskandar AR, Martin F, Talikka M, Schlage WK, Kostadinova R, Mathis C, Hoeng J, Peitsch MC.
Biomed Res Int. 2013 Oct 3

Example of 3R4F Repeated Exposure Impact Quantification

- Experimental data production
- Compute systems response profiles
- Identify perturbed biological networks
- Compute network perturbation amplitudes
- Compute product biological impact

Network Perturbation Amplitude



Cell Proliferation

Cell Stress

IPN (Inflammatory Process Network)

Senescense

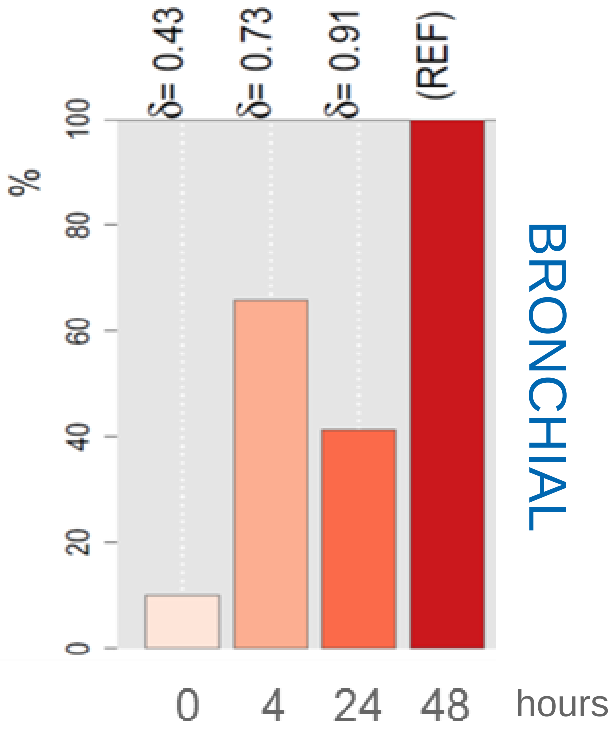
Necroptosis

DNA Damage

Autophagy

Apoptosis

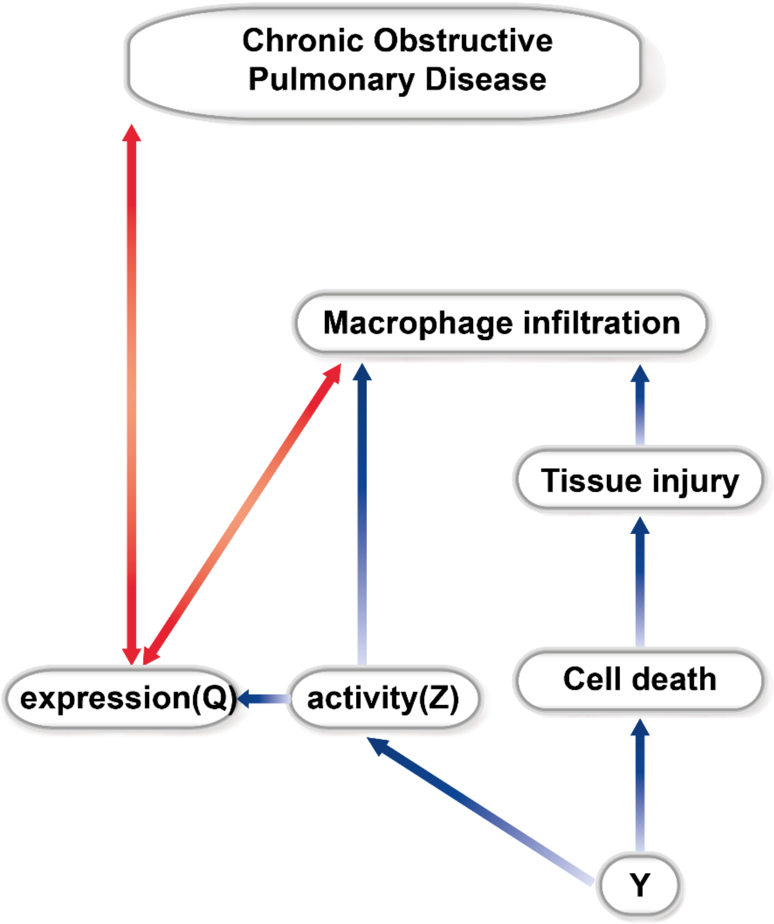
Relative BIF

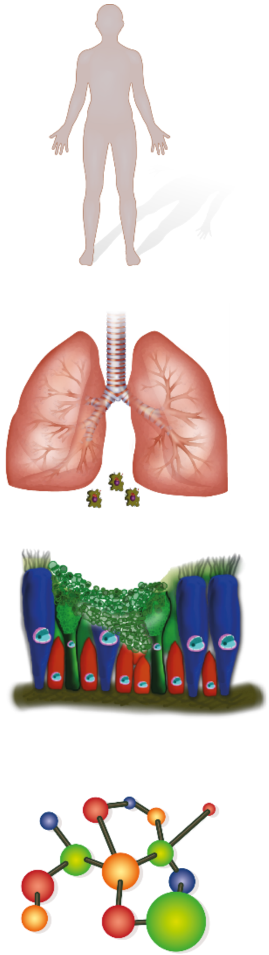


17% 3R4F (Nicotine 0.24 mg/L)

Network Models to Study Adverse Outcome Pathways

Network model gives “*a mechanistic representation of critical toxicological effects that span over different layers of biological organization*”





adverse outcome

series of intermediate steps and key events

molecular initiating event

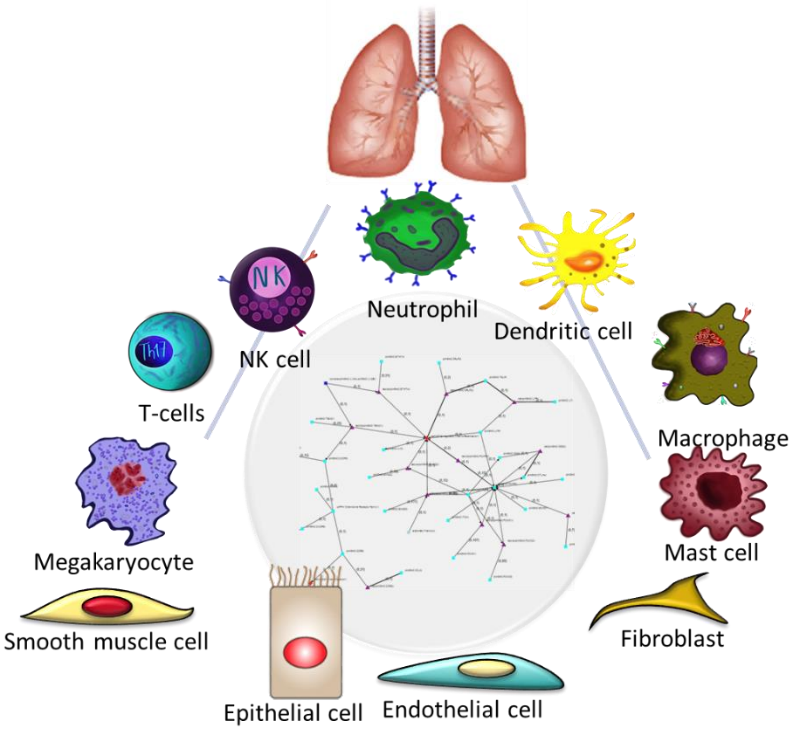
Vinken M (2013) The adverse outcome pathway concept: a pragmatic tool in toxicology. Toxicology 312:158-165
Vinken M, Whelan M, Rogiers V (2014) Adverse outcome pathways: hype or hope? Archives of toxicology 88:1-2

www.sbvimprover.com

NETWORK VERIFICATION CHALLENGE

sbv IMPROVER
SYSTEMS BIOLOGY VERIFICATION

The purpose of the Network Verification Challenge is to engage the scientific community to review, challenge, and make corrections to the conventional wisdom.



Mucus hypersecretion
Epithelial cell proinflammatory signaling
Epithelial barrier defense

Microvascular endothelium activation

Macrophage activation
Macrophage differentiation
Macrophage mediated neutrophil recruitment

Neutrophil response
Neutrophil chemotaxis

Dendritic cell activation
Dendritic cell migration to tissue
Dendritic cell migration to lymph

Th1 differentiation	Th1 response
Th2 differentiation	Th2 response
Th17 differentiation	Th17 response
Tc response	Treg response

Mast cell activation

NK cell activation

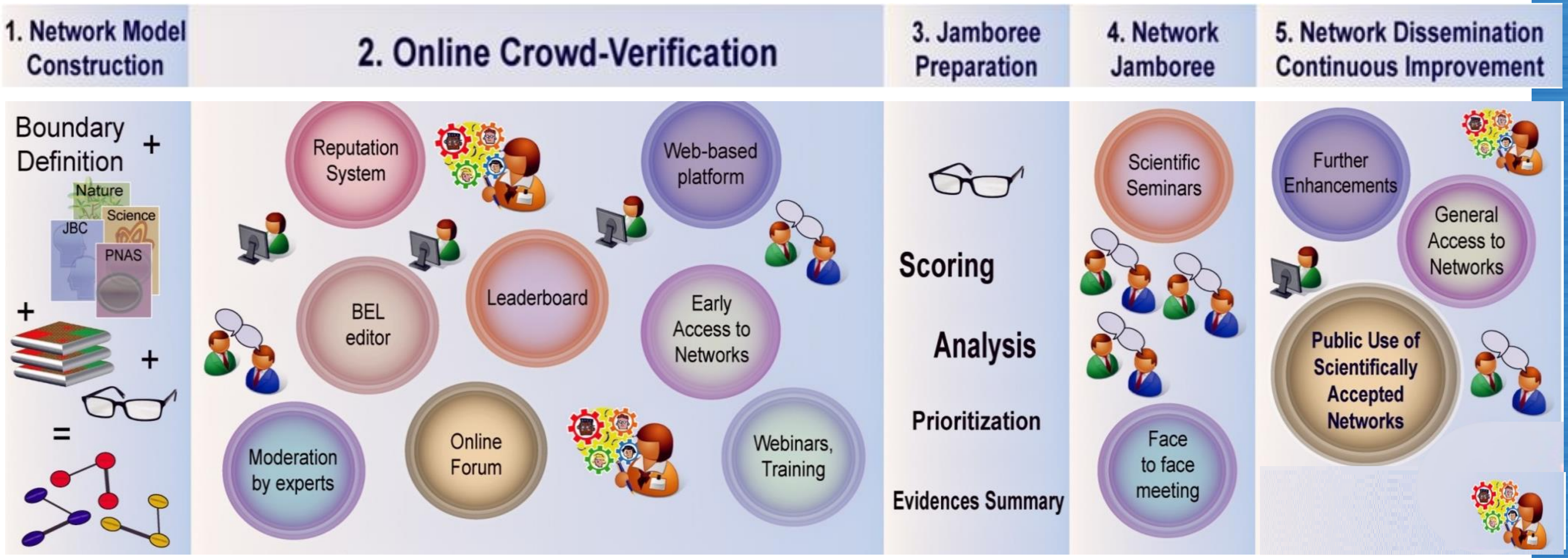
Megakaryocyte differentiation

Tissue damage

The sbv IMPROVER project, the website and the Symposia are part of a collaborative project designed to enable scientists to learn about and contribute to the development of a new crowd sourcing method for verification of scientific data and results. The current challenges, website and biological network models were developed and are maintained as part of a collaboration with Selventa, OrangeBus and ADS. The project is funded by Philip Morris International.

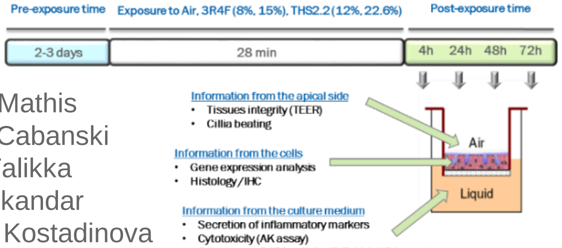
PMI RESEARCH & DEVELOPMENT

Network Verification Challenge in a Nutshell



The sbv IMPROVER project team (2013). On Crowd-verification of Biological Networks. **Bioinformatics and Biology Insights 2013:7 307-325.**

Acknowledgments



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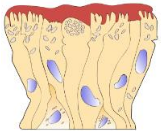

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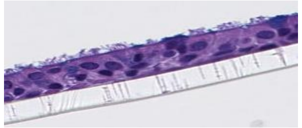

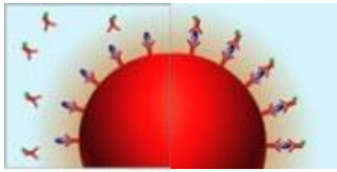

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