The Adverse Outcome Pathway For Mucus Hypersecretion in Chronic Bronchitis

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Introduction

Chronic obstructive pulmonary disease (COPD) is one of the adverse outcomes resulting from cigarette smoking and manifests as obstruction of the small airways, chronic bronchitis, or emphysema. There is a need to establish a mechanistic understanding of COPD development in response to chronic exposure to inhaled toxicants for risk assessment and regulatory decision-making. The Adverse Outcome Pathway (AOP) framework provides a means to outline a knowledge-driven sequence of events from exposure to adverse outcome (AO). Here, we describe an AOP for EGFR-mediated mucus hypersecretion that leads to chronic bronchitis and COPD. The AOP is based on existing experimental findings with the activation of EGFR by its ligands in response to oxidative stress as the molecular initiating event (MIE). In response to EGFR activation, the key events (KEs) include decreased apoptosis of ciliated epithelial cells, mediated by PI3K/AKT signaling, transdifferentiation of ciliated cells into goblet cells by IL-4 and IL-13, and increased goblet cell proliferation. These KEs lead to goblet cell hyperplasia or metaplasia. Additionally, EGFR stimulation leads to the activation of SP-1 that upregulates MUC5AC, increasing mucin production by goblet cells. Together these processes ultimately result in mucus hypersecretion that, when chronic, results in declining lung function and allows for COPD diagnosis. We describe the biological plausibility of the AOP and the weight of each evidence supporting the KEs and the key even relationships (KERs). Finally, we have evaluated the mechanisms described in the AOP with transcriptomics data from respiratory tissue of human smokers and rodents exposed to cigarette smoke. These results can be further compared to experimental *in vitro* data in the context of the AOP for translational purposes to reduce animal experimentation in toxicological assessment.

Methods

The AOP for decreased lung function that arises from oxidative stress-mediated EGFR activation in the airway epithelium was developed based on the guidance provided by the Organisation for Economic Cooperation and Development (OECD) using currently available mechanistic evidence from *in vitro*, *in vivo* and clinical studies. The evidence has been summarized and evaluated using a modified weight-of-evidence approach and will be available for review and comment on the AOPwiki (www.aopwiki.org).

A computable network model that describes the biological signaling pathways regulating the increase in the number of airway goblet cells by increased proliferation or transdifferentiation, clinically known as goblet cell hyperplasia (GCH) was constructed using a semi-automated knowledge extraction workflow (BELIEF). BELIEF allows for the transformation of unstructured information available in the literature and published datasets into a structured, cause-effect, scientific representation in Biological Expression Language (BEL; Szostak *et al.*). To date, the network model contains causal relationships from over 60 scientific publications and focuses on EGFR-mediated goblet cell proliferation and mucin production.

Publicly available gene expression datasets were used to validate the network model using the NPA algorithm (Martin *et al.*). Dataset 1 (GSE22430), summarizes the effects of pyocyanin (PYO), a redox-active toxin, on gene expression in H292 lung epithelial cells. Dataset 2 (GSE5264) reflects gene expression changes in airway basal epithelial cells undergoing mucociliary differentiation at early, intermediate and late time points, respectively. Dataset 3 (GSE37693) was derived from bronchial epithelial cells treated with IL-13.

Additional datasets were then used to test the model, including gene expression data from nasal epithelia of *Apoe*-deficient mice exposed to cigarette smoke (dataset 4; Phillips *et al.*), large airway epithelial cells of smokers and never-smokers (dataset 7; GSE16008) and COPD patients and controls (dataset 5; DOI:10.1038/sdata.2014.9), and from small airway epithelial cells from smokers and never-smokers (dataset 8; GSE19667) and COPD patients and controls (dataset 6; GSE10006/GSE11906/GSE11952/GSE13933/GSE19407/GSE19667/GSE20257/GSE5058/GSE8545).

References:

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Adverse Outcome Pathway - Description and Evaluation

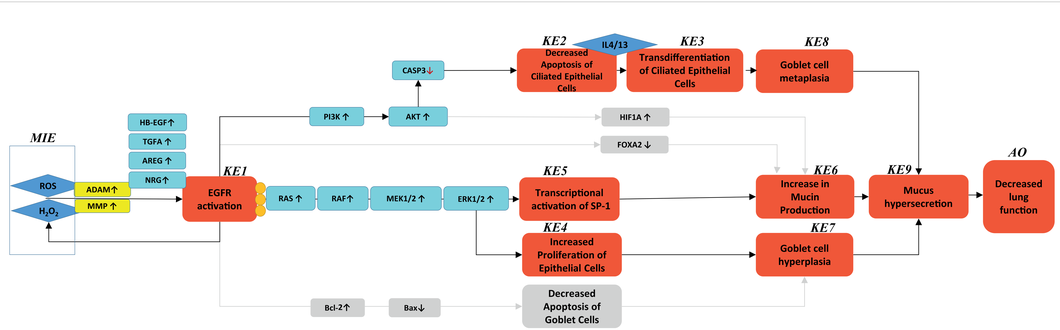


Figure 1. Schematic presentation of the decreased lung function AOP. MIE; molecular initiating event, KE; key event, AO; adverse outcome, ROS; reactive oxygen species, H_2O_2; hydrogen peroxide, ADAM; a disintegrin and metalloproteinase, MMP; matrix metalloproteinase, HB-EGF; heparin-binding EGF-like growth factor, TGFA; transforming growth factor alpha, AREG; amphiregulin, NRG; neuregulin, EGFR; epidermal growth factor receptor, PI3K; phosphoinositide 3-kinase, AKT; protein kinase B, CASP3; caspase 3, RAS; p21/Ras GTPase, RAF; RAF proto-oncogene serine/threonine-protein kinase, MEK1/2; mitogen-activated protein kinase kinase 1/2, ERK1/2; extracellular signal–regulated kinases 1/2, HIF1A; hypoxia-inducible factor 1 alpha, Bcl-2; B-cell lymphoma 2, Bax; bcl-2-like protein 4, IL4; interleukin 4, IL13; interleukin 13, HIF1A; hypoxia-inducible factor 1-alpha, FOXA2; forkhead box A2

Table 1. Summary overview of biological plausibility and empirical support of KERs, essentiality of KEs and applicability of AOP.

Biological Plausibility of KERs Is there a mechanistic relationship between KEs consistent with established biological knowledge?		Empirical support for KERs Does the empirical evidence support that a change in KE_{up} leads to an appropriate change in KE_{down}? Temporality? Inconsistencies?		Essentiality of KEs Are KES_{down} and/or the AO prevented if a KE_{up} is blocked?	
MIE→ KE1: Oxidative stress directly leading to EGFR activation	Strong	Strong		**KE1**	High: Considered critical for mucus hypersecretion and GCH/GCM
KE1→ KE2: EGFR activation indirectly leading to decreased epithelial cell apoptosis	Moderate	Moderate		**KE2**	Moderate: While the evidence supports essentiality of EGFR activation for decreased ciliated cell apoptosis, there is also evidence supporting decreased apoptosis in airway goblet cells in vitro.
KE2→ KE3: Decreased epithelial cell apoptosis directly leading to transdifferentiation into goblet cells	Moderate	Weak	There is only correlative evidence for this KER.	**KE3**	High: Blocking EGFR signaling suppresses GCH/GCM.
KE1→ KE4: EGFR activation directly leading to increased epithelial cell proliferation	Moderate	Moderate		**KE4**	Moderate: The evidence suggests that other transcription factors could contribute to increased MUC5AC expression.
KE1→ KE5: EGFR activation directly leading to Sp-1 activation	Moderate	Weak	There is little direct evidence for this KER.	**KE5**	High
KE1→ KE6: EGFR activation indirectly leads to increased mucin production	Moderate	Strong		**KE6**	Moderate
KE4→ KE7: Increased epithelial cell proliferation directly leading to GCH	Moderate	Weak	Inferred: The term 'hyperplasia' refers to an increase in a tissue or organ that is linked to an increase in cell number or cell size. Therefore, increased proliferation can be considered a root cause of GCH.	**KE7**	Moderate
KE3→ KE8: Transdifferentiation into goblet cells directly leading to GCM	Moderate	Weak	Inferred: Following injury, airway epithelial repair is accomplished by (transient) remodeling processes. In the absence of cell proliferation, this remodeling is thought to be facilitated by transdifferentiation.	**KE8**	Moderate
KE5→ KE6: Sp-1 activation directly leading to increased mucin production	Moderate	Moderate		**KE9**	Moderate: It is currently unclear whether chronic mucus hypersecretion alone is sufficient to affect a decrease in lung function
KE6→ KE9: Increased mucin production directly leading to mucus hypersecretion	Moderate	Weak	Inferred: Increased mucin production is a requirement in states of mucus hypersecretion to restore depleted mucin stores (Rose et al.).	**Applicability**	
KE7→ KE9 and **KE8→ KE9**: GCH/GCM directly leading to mucus hypersecretion	Moderate	Weak	Inferred: According to Rose et al., "Secretory cell hyperplasia is a prerequisite for sustained mucus hypersecretion/mucin overproduction".	The majority of KEs are preserved across small rodents and humans. However, the link between mucus hypersecretion and airflow obstruction is much less supported by studies in laboratory animals where the human disease phenotype cannot be modelled in its entirety.	
KE9→ AO: Chronic mucus hypersecretion directly leads to decreased lung function	Moderate	Moderate		In general, the exposures resulting in oxidative stress to subsequently induce EGFR activation apply to adults who are more likely to be exposed to these stressors.	
				The available in vivo and clinical evidence in support of the proposed AOP suggest that there is no remarkable gender difference.	

Biological Network Analysis as Potential Tool for Quantitative AOP Evaluation

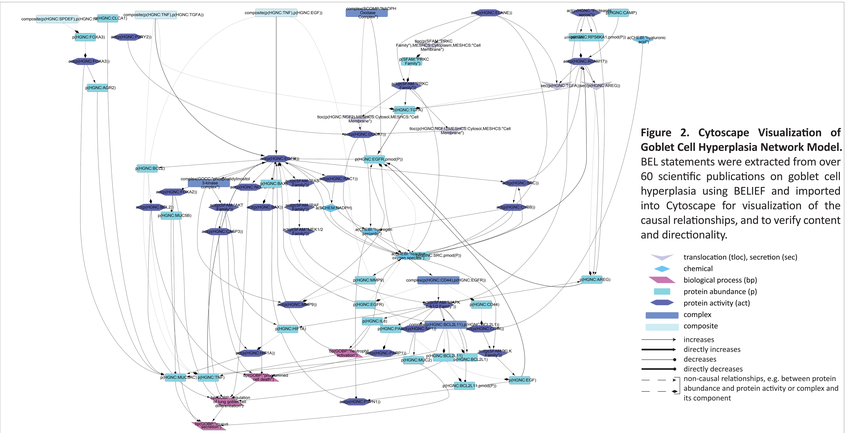
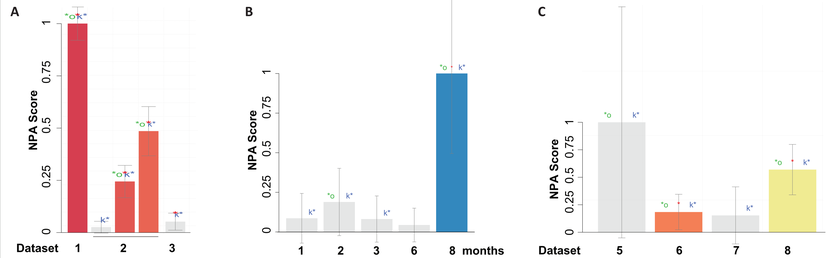


Figure 2. Cytoscape Visualization of Goblet Cell Hyperplasia Network Model. BEL statements were extracted from over 60 scientific publications on goblet cell hyperplasia using BELIEF and imported into Cytoscape for visualization of the causal relationships, and to verify content and directionality.



1: PYO Treated vs Untreated (GSE22430)
2: Epithelial cell differentiation, early (left bar) , intermediate (middle bar) and late time points (right bar; GSE5264)
3: IL13 Treated vs Untreated (GSE37693)

Dataset 4: Nasal epithelium, *Apoe*-/- mouse, exposed to mainstream smoke from 3R4F reference cigarettes

5: Large airways, COPD vs control (DOI: 10.1038/sdata.2014.9)
6: Small airways, COPD vs control (GSE10006/GSE11906/GSE11952/GSE13933/GSE19407/ GSE19667/GSE20257/GSE5058/GSE8545)
7: Large airways CS vs NS (GSE16008)
8: Small airways, CS vs NS (GSE19667)

Figure 3. NPA analysis confirmed that the model contains appropriate biological information, is specific for EGFR signaling and sensitive to an increase in goblet cell numbers. A) **Network Validation:** Pyocyanin treatment of H292 cells results in significant and maximum network perturbation, while IL-13 treatment of bronchial epithelial cells does not, confirming that the network is specific for EGFR-mediated signaling that results in GCH. The model is also sensitive toward the physiological increase in goblet cell numbers occurring during mucociliary differentiation of bronchial epithelial cells. B) **Network Testing:** Exposure of *Apoe*-deficient mice to mainstream cigarette smoke results in remodeling of the nasal epithelium which is reflected by significant perturbation of the GCH model, indicating the utility of the model in systems toxicological assessment. C) **Network Testing:** GCH is a common feature in the airways of asymptomatic smokers and COPD patients. Small airway epithelial cell transcriptomic profiles can be used to score the extent of GCH network perturbations in those populations.
Abbreviations: PYO, pyocyanin; IL13, interleukin 13; CS, current smoker; NS, never-smoker.

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