P16-13 Systems biology approach reveals a dose-dependent recovery of primary human airway epithelium culture after exposure to cigarette smoke

C. Mathis*, S. Gebel*, C. Poussin, D. Weisensee, A. Sewer, V. Belcastro, S.Ansari, M. Cabanski, S. Wagner, J. Hoeng , M.C. Peitsch,
Philip Morris International R&D, CH-2000 Neuchâtel, Switzerland

INTRODUCTION

The use of more reliable and relevant human *in vitro* models as well as Systems Biology-based approaches to investigate the impact of toxicants such as cigarette smoke (CS) is in line with the recent shift of toxicological assessment strategy to find alternatives for animal testing and to tackle systems level questions in an integrative way. We recently published a comparison study which demonstrated that primary organotypic culture of normal human bronchial epithelial (NHBE) cells (Fig. 1) exposed to CS at the air-liquid interface (Fig. 2) can recapitulate the biology observed in the bronchial epithelium of smokers (1). In this study, data from large scale mRNA, miRNA, protein analysis, and from immunohistological investigations were captured at different post-exposure time points (0.5h, 2h, 4h, 24h, 48h). Here, we will give the example of the time-dependent and dose-dependent analysis performed using Reverse Causal Reasoning (RCR) approach (4) on the perturbations of pathways related to cell proliferation (Fig. 3 and 4), focusing on two exposure periods (14 and 28 min) and on all post-exposure time points. We will also substantiate this analysis with immunohistological observations (Fig. 5) and suggest dose-dependent regenerative processes in the exposed tissue.

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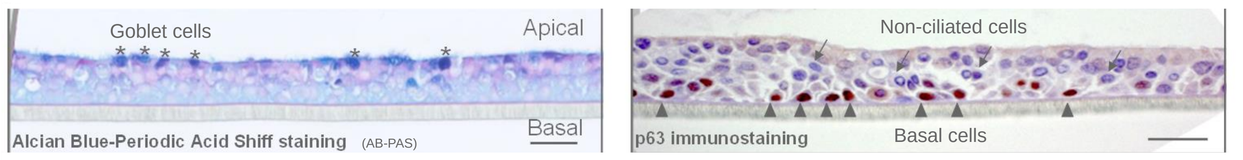
MATERIALS & METHODS



AIR-100 tissue cross-section

Figure 1: Morphology of organotypic culture of differentiated NHBE cells. (Scale bar = 30 μm).

Unperturbed human organotypic bronchial epithelial cell culture (= AIR-100) resembles closely to human lung epithelium both at the morphological level (1) and at the molecular level (2).

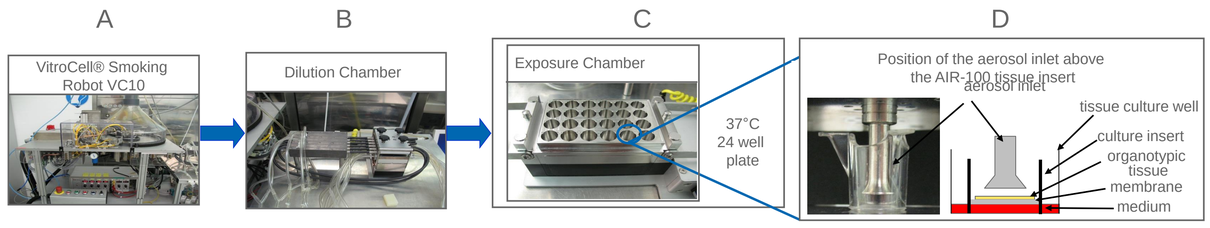
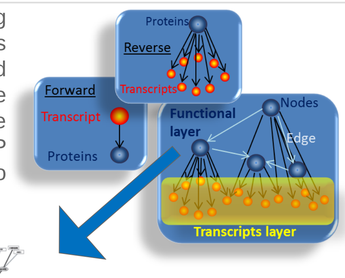
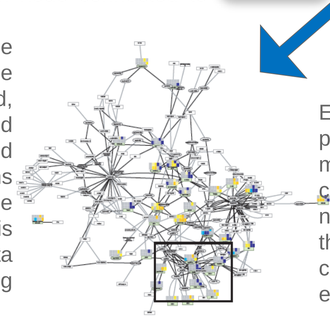


Figure 2: Schematic view of the whole CS exposure system and the exposure chamber.
(A) 3R4F reference cigarettes (www.ca.uky.edu/refcig) smoked on the VC10 smoking robot (Vitrocell®) in basic conformity with the International Organization for Standardization smoking regimen (ISO 2000) produce discontinuously whole CS at the rate of 1 puff/min/cigarette. CS enters the dilution chamber (B) (35ml/2sec) where it is mixed continuously with synthetic air (0.2 to 0.5 l/min depending of the CS concentration to apply). The flow of diluted CS passes into the exposure chamber (C) where the cell culture inserts are placed under the aerosol inlet (D) during the defined length of exposure. Inside the exposure chamber, the Total Particulate Matter was estimated to 7.74 μg per well per min of exposure and the CO dose to 8.3 μg per well per min of exposure. In a cell culture insert, NHBE cells are grown and differentiated on a porous membrane support allowing an air-liquid interface with warmed culture medium below the cells and a gaseous test atmosphere above the cells.

Figure 3: Reverse Causal Reasoning (4) is a knowledge-driven backward reasoning technique for identifying potential upstream regulators or controllers, also called hypothesis (HYPs), which could explain the observed differential gene expression profiles. HYPs and significantly regulated genes (absolute fold change≥1.3; FDR≤0.05 and abundance level≥$\log_2$(100)) selected from each control-treatment pairwise gene expression profile (different doses and post-exposure times) was used as input for RCR analysis. A HYP corresponds to a simple causal network constituted by an upstream node connected to downstream nodes (e.g., expression of target gene) by a causal edge.

Each edge represents the causal relationship between “HYPs” and the target gene derived from an evidence line extracted from literature. The notion of causal relationship directionality is important in RCR. Indeed, HYPs can be evaluated as potential explanations for the observed differences in measurement sets by calculating a statistic called “concordance”, which measures the consistency of the directions between the observed state changes (corresponding to the pairwise differentially expressed genes) and the expected direction. A p-value is computed for each concordance statistic. The RCR analysis of our data was carried out on the Gene Technology Platform (4) currently holding more than two thousand HYPs.





Example of the cell proliferation network model (2) which is composed by 848 nodes and 1597 edges that are supported by curated literature evidences.

RESULTS

Figure 4: Predicted HYPs within the Cell Proliferation Network model selected as an example to substantiate the RCR approach. Colour code: yellow = HYPs predicted increase in abundance or activity, blue = HYP predicted decrease in abundance or activity; light yellow/blue = 0.01 <= conc. < 0.05; yellow/blue = 0.001 <= conc. < 0.01; dark yellow/blue = conc. < 0.001; grey = no significant changes. taof(X): transcriptional activity of X; kaof(X): kinase activity of X; gtpof(X): GTP-bound activity of X; catof(X): catalytic activity of X. RCR analyses were performed using differential gene expression (CS-exposed vs sham control) derived from AIR-100 tissues exposed to whole CS for 14 min or 28 min followed by different post-exposure times (0.5h, 2h, 4h, 24h, and 48h). Mapping the significant HYPs to the Cell Proliferation Network model, a coordinated downregulation of HYPs related to cell cycle inhibitors and an activation of HYPs for cell cycle inducers was observed 2h after exposure at low dose (14 min). This response fully recovered after 48h after exposure. For example we could identify HYPs describing proteins and/or biological processes that are downregulated in proliferating cell such as “taof (p53)”, “RB1”, “taof(E2F4)”, “CDKN1A”, as well as HYPs describing increased protein abundance or upregulated transcriptional activation involved in cell proliferation e.g., “E2F1”, “E2F2”, “E2F3”, CDK4”, “CCND1” and “taof(Myc)”. In addition, we observed significant upregulation of specific subnetwork of the Cell Proliferation Network model that is related to Growth Factor Response. Especially nodes that are in relation to epidermal growth factor receptor (EGFR) family (e.g., “NRG1”, “EGFR:ERBB3”, “ERBB3”), and related ligands for EGFR activation (e.g., ”EGF”, “AREG”). were significantly upregulated. Beside that, activation of MET (= hepatocyte growth factor receptor) (“kaof(MET)”, “HGF”), an alternative pathway leading into lung cell proliferation was also detectable. An additional pathway that was identified is related to fibroblast growth factors (“FGF2”, “FGF6”, and “FGF7”). Moreover HYPs for mediator of cell migration and tissue differentiation are strongly upregulated (e.g., “gtpof(RHOC)”, “IHH” “taof(GLI2)”). In contrast to the low dose, at high dose (28 min) a considerable stronger response was already visible 0.5h after exposure and a sustained perturbation of the system was visible by a large number of significant HYPs after 2h, 4h, 24h and even after 48h post-exposure times.

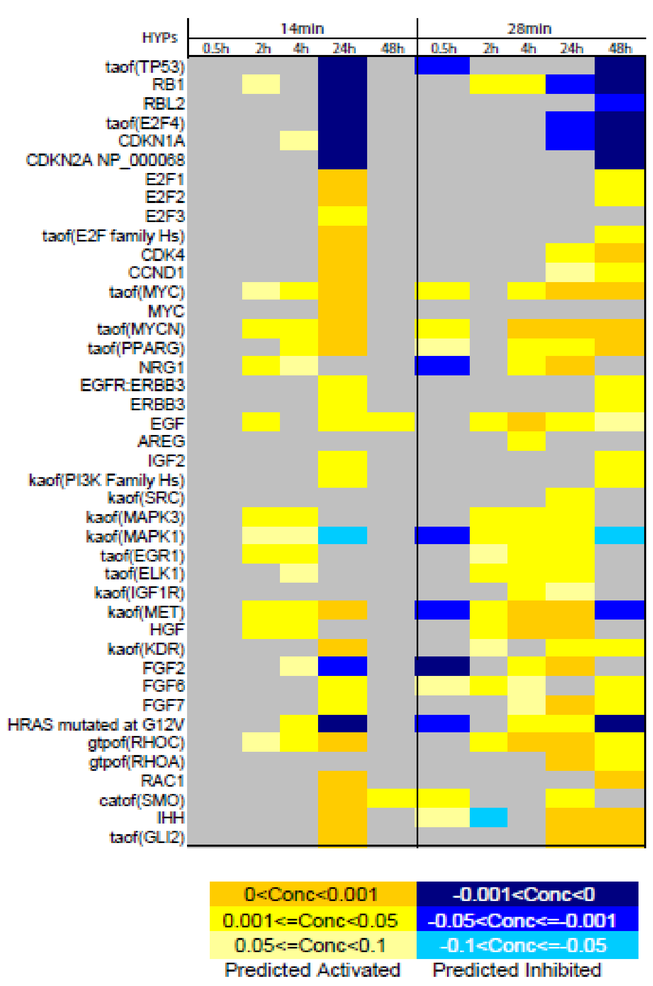
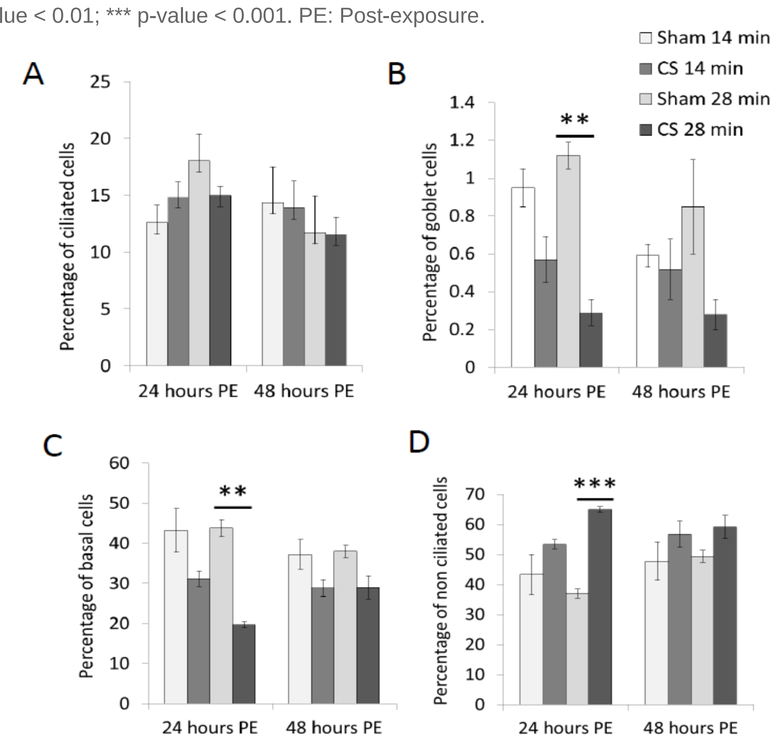


Figure 5: CS effect on the proportion of cells composing the organotypic bronchial tissue. Goblet cells were identified by AB-PAS staining which stained mucus secreting cells. Basal cells were labelled with an anti-p63 antibody and ciliated cells with an anti-β-Tubulin IV antibody. The number of non-ciliated cells was determined by taking the number of all cells minus the number of basal, ciliated, and goblet cells. Bar plots representing the percentage to the total cell number of (A) ciliated cells, (B) goblet cells, (C) basal cells and (D) non ciliated cells estimated after 24 or 48h post-exposure in AIR-100 tissues exposed for 14 or 28 min to CS or sham. Numbers represent mean ± SEM. ** p-value < 0.01; *** p-value < 0.001. PE: Post-exposure.



CONCLUSIONS

With this study, we described the application of a System Biology-based approach to get a comprehensive view on the impact of whole CS exposure to human organotypic bronchial epithelial cell culture combining molecular endpoints with histological measurements. Investigating different doses and post-exposure periods up to 48h, we showed a recovery of the cellular response at lower CS concentrations, whereas at higher concentrations a sustained perturbation of cellular networks was visible. By applying RCR approach on different network models (e.g. Cell Proliferation (2), Cellular Stress (8), Inflammatory Response (9)), we investigated the different cellular pathways perturbed by CS exposure which were related to the cellular stress response, the inflammation and the proliferation/differentiation mechanisms. Results from this new approach were confirmed and further substantiated by additional analyses, i.e., Gene Set Enrichment Analysis (GSEA) (3) of gene expression data, measurement of miRNA expression and protein abundance/modification (data not shown). Further histological investigation confirmed a dose- and time-dependent recovery processes of the AIR-100 tissue after exposure. To ensure tissue integrity and enable defence mechanism, we observed by GSEA an increase in the mitochondrial electron respiratory chain activity and in energy metabolism suggesting an activation of the ciliary clearance mechanism to eliminate particles deposition. Based on the results from differential cells count, it seems that the most impacted cells from the stress induced by CS are the goblet cells. Their proportion diminished significantly 24h after exposure compared to sham and with a higher decrease observed for the higher dose (Fig. 5B). The number of ciliated cells which are the other cell type directly in contact with the smoke is on the other hand unchanged compared to sham (Fig. 5A). Interestingly, the proportion of basal cells (Fig. 5C) decreases inversely in the same proportion and at the same time that the non ciliated cells (Fig. 5D). This observation could suggest a regeneration process where the damaged cells from the apical side are replaced by non ciliated cells that will later on differentiate and compensate for the loss of goblet cells (Fig. 5B). In parallel to the increase of non ciliated cells proportion, the activation of HYPs for cell cycle inducers or related to the Growth Factor Response (Fig. 4) was observed. The whole scenario of the tissue integrity regeneration implies processes such as spreading, migration, differentiation, and proliferation that were described in the epithelial wound repair in lung (5), although one important cell type, the fibroblasts were missing in the AIR-100 tissue. Based on the HYPs analysis it seems that EGR-related processes are involved in the proliferation signalling. The EGFR pathway is activated in many human lung adenocarcinoma either CS-dependently via K-Ras activation or CS-independently by EGR amplification (6). Sauer described a positive feedback loop involving EGFR signalling via MEK, ERK, ELK-1, EGR1 induction of EGFR ligands EREG, AREG and HBEGF (7) that may also be present in the CS-exposed AIR-100 tissue. Collectively our results suggest a dose-dependent recovery of the irritated tissue involving probably mechanisms known from epithelial repair in human lung. To further characterize the fate of the AIR-100 tissue in our experimental set-up, longer post-exposure period would be worth to investigate.

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Philip Morris International Research & Development, Quai Jeanrenaud 5, 2000 Neuchâtel, Switzerland
T: +41 58 242 21 13, **F**: +41 58 242 28 11, **W**: www.pmi.com