Evaluation of reconstituted human bronchial epithelium (MucilAir™) suitability for repeated dose testing

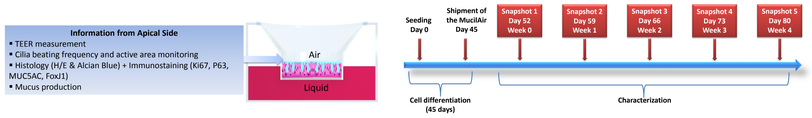
Samuel Constant[(1)], Stefan Frentzel[(2)], Marianna Gaca[(3)], Davide Grandolfo[(2)], Linsey Haswell[(3)], Julia Hoeng[(2)], Song Huang[(1)], Patrice Leroy[(2)], Karsta Luettich[(2)], Emmanuel Minet[(3)], Melany Monachino[(1)], Keyur Trivedi[(2)], Ludovic Wiszniewski[(1)]

[(1)] Epithelix, Geneva, Switzerland; [(2)] Philip Morris International R&D, Philip Morris Products S.A., Neuchâtel, Switzerland; [(3)] British American Tobacco R&D Centre, Southampton, UK

Introduction

The airway epithelium reconstituted *in vitro* is a powerful tool for studying respiratory diseases as well as for the assessment of inhaled toxicants. However, one of the problems in long term culture is the de-differentiation of the ciliated cells: After a certain period in culture, cells lose cilia and cilia beating becomes weak or absent. The morphology of the epithelia may also change with time, and the ratio between the different cell types could be altered.

In order to use MucilAir™, a 3D cell model of the human airway epithelium, for long-term or repeated dose toxicity testing, it is important to assess its stability in terms of morphology and function. The stability of key mucociliary characteristics over time can be easily monitored on a fully differentiated bronchial model. This study, designed to assess the stability of bronchial MucilAir™ tissues, was performed in three different laboratories and by different operators over 38 days of culture post-differentiation. The measurement of transepithelial electrical resistance (TEER), cilia beat frequency (CBF) and mucin secretion is non-destructive and allow for monitoring the same tissues over a long period of time



Methods

- ✓ *Cell culture:* Human bronchial MucilAir™ tissue cultures (Epithelix Sàrl, Switzerland) from a 66-year old non-smoker Caucasian male were used in this study. Pre-study cells were cultured until fully differentiated into mucociliary pseudostratified epithelia. Cultures were then shipped to PMI and BAT laboratories, the same shipping conditions were replicated at Epithelix. During the study, culture media (0.7 ml/well) were changed twice a week.
- ✓ *Study design*: To evaluate the stability of MucilAir™ cultures, endpoints were measured and samples taken over 38 days of culture post-differentiation in the three independent laboratories (Epithelix, PMI and BAT). At each time point, 6 culture inserts were used per measurement. Where possible endpoints were measured independently at the 3 laboratories.
- ✓ *CBF:* The beat frequency was recorded digitally at 37ºC using a high-speed video camera connected to an inverted microscope. Cultures were equilibrated for 5 minutes prior to measurement and video was recorded at 90 frames per second, capturing at least 256 frames. Automated software was used to determine the CBF from the whole image field.
- ✓ *TEER:* 200 µl pre-warmed saline solution or culture medium was added to apical surface of MucilAir™ cultures and TEER was measured using STX2 electrodes and an EVOM^2 epithelial voltohmeter (WPI).
- ✓ *Mucin secretion:* The amount of mucin in the apical rinse was determined by an Enzyme-Linked Lectin Assay (ELLA). The apical rinse proteins were bound to immunoassay plates and the amount of mucin was detected by HRP-conjugated wheat germ agglutinin.
- ✓ *Immunohistology:* MucilAir™ inserts were stained and image analysis using ImagePro Plus (version 6.2, MediaCybernetics) was conducted by Epithelix to quantify the 3 main types of cells in the cultures. The whole images of stained sections were scanned, and an average 40 images/section were analysed. The H/E Alcian Blue staining is presented as a ratio of the Alcian Blue-stained area to the total surface area of the epithelium on the section. The P63, Ki67, MUC5AC and FoxJ1 results are presented as the ratio between the positive cells and the total number of cells on the section.

Assay	Laboratory		
	PMI	Epithelix	BAT
CBF* (n=6)	✓	✓	✓
Microscope	Leica DMi8	Leica DMi8	Zeiss AX 10
Lens objective	4x	4x	10x
Temperature control	Heated stage	Heated stage	Heated chamber
Analysis software	CiliaFA	CiliaFA	SAVA
TEER* (n=6)	✓	✓	✓
Mucin ELLA*(n=6)	✓	✓	✓
Immunohistology** (n=6)			
Fixation	✓	✓	✓
Sectioning	✓	Performed at Epithelix	
Staining	✓		
Image analysis	Performed at Epithelix		

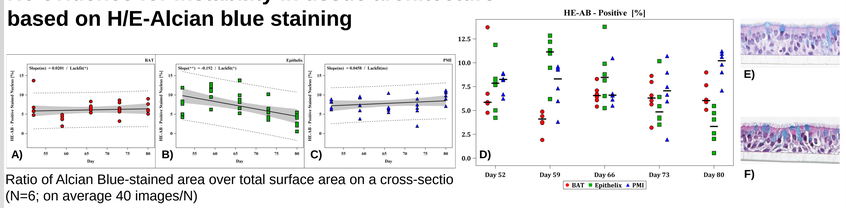
** Destructive endpoint
✓ Performed separately at each laboratory using the same methodology

Statistical analysis

All data from all time points across the 3 test sites were included in the analysis. Missing data were not replaced. Data are presented as scatter plots of individual measurements for each endpoint (N=6, unless otherwise indicated), with different colors/symbols representing data from each individual test site.

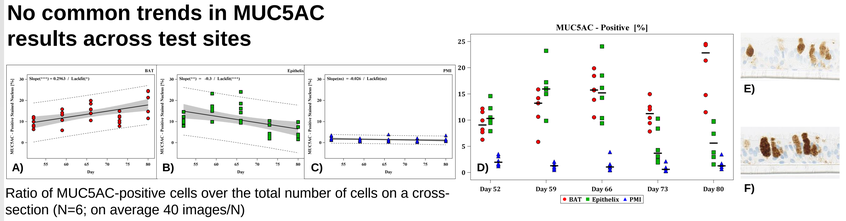
To examine if there is a trend for each endpoint over time, a linear regression model was employed. Additionally, a lack-of-fit test was performed to confirm that the use of the linear model was appropriate. The resulting significance for slope and lack-of-fit inform about the true fit and appropriateness of the linear regression model. Hence, if the linear regression is significant (i.e., slope (***)), but the lack-of-fit test is not significant (i.e., LackFit(ns)), then the regression line is a true fit. Further, if the lack-of-fit test was not significant (i.e., LackFit(ns)) and the linear regression was not significant (i.e., slope (ns)), the endpoint in question cannot be considered unstable over time.

No evidence for instability in tissue architecture based on H/E-Alcian blue staining



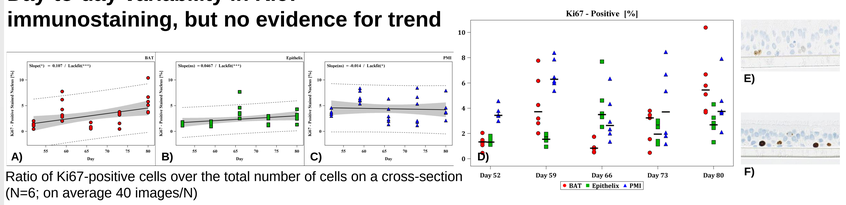
Ratio of Alcian Blue-stained area over total surface area on a cross-sectio (N=6; on average 40 images/N)

No common trends in MUC5AC results across test sites



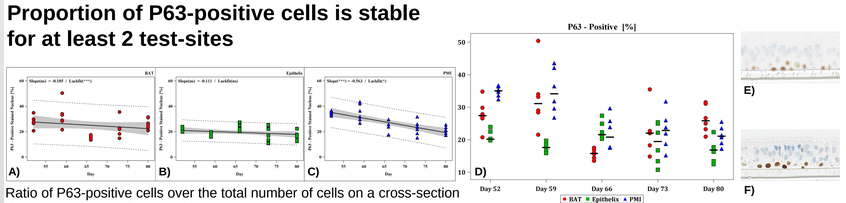
Ratio of MUC5AC-positive cells over the total number of cells on a cross-section (N=6; on average 40 images/N)

Day-to-day variability in Ki67 immunostaining, but no evidence for trend



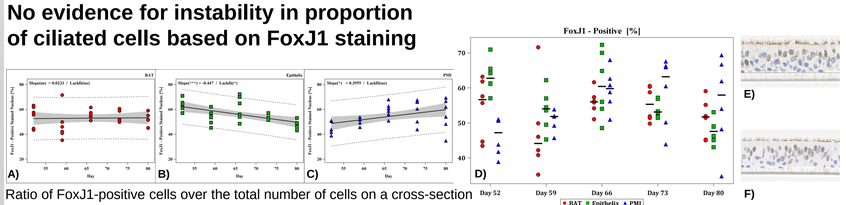
Ratio of Ki67-positive cells over the total number of cells on a cross-section (N=6; on average 40 images/N)

Proportion of P63-positive cells is stable for at least 2 test-sites



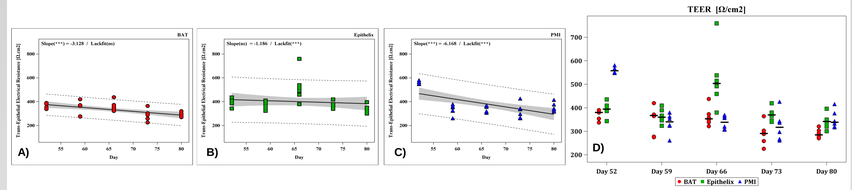
Ratio of P63-positive cells over the total number of cells on a cross-section (N=6; on average 40 images/N)

No evidence for instability in proportion of ciliated cells based on FoxJ1 staining

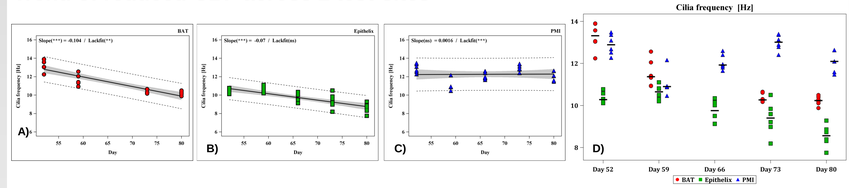


Ratio of FoxJ1-positive cells over the total number of cells on a cross-section (N=6; on average 40 images/N)

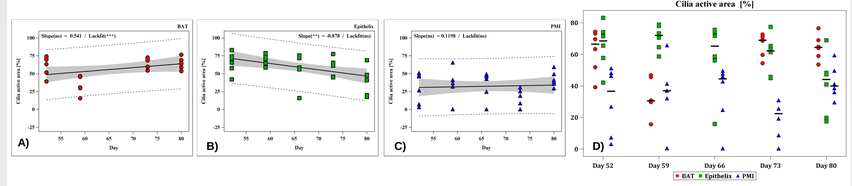
Clear trend of reduced TEER values for at least 1 test-site



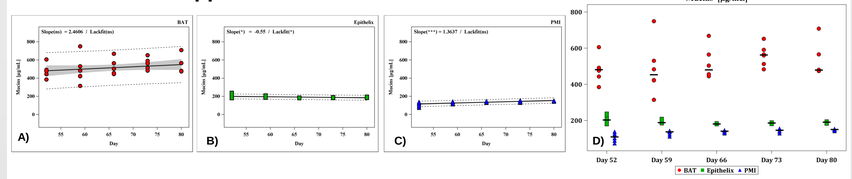
Trend of reduced CBF across 2 test-sites



Cilia active area values are highly variable



Mucin secretion appears to be stable over time



Legend

A-C) Regression analysis ("trend plots") of data points from test site 1 (BAT = A), test site 2 (Epithelix = B) and test site 3 (PMI = C). Individual data points from each test site are presented as red, blue and green symbols, respectively; the bold line in A-C) indicates the regression line, while broken lines delineate the 95% confidence interval of the individual measurements. The grey, plain area depicts the 95% confidence limit of the mean. (*) $p \leq 0.05$; (**) $p \leq 0.01$; (***) $p \leq 0.001$; (ns) non-significant. D) Scatter plot showing the end-points determined across all three test sites and over all time points. Horizontal bars indicate the respective mean value. E-F) Representative image of cell type-specific staining (Epithelix; 20X) at day 52 (E) and day 80 (F).

Conclusions

Despite some differences in the selected endpoints, the weight of evidence from this study supports the conclusion that MucilAir™ appears stable for at least 38 days post-differentiation. Notably:

- ✓ Markers for the **goblet cell population** (Alcian blue, Mucins) were stable over the test period at all test sites.
- ✓ The **ciliated cell population** marker FoxJ1 showed no difference over time, whilst there was evidence of a slight reduction in cilia beat frequency over time.
- ✓ The level of **basal cell population** markers Ki67 and P63 did not show any major trend in at least two test sites.

Variability was observed for some measured endpoints between the sites which is likely due to differences in the methods and should be considered to design future inter-laboratory testing. Cross-laboratory studies to investigate the reproducibility of the MucilAir™ response following exposure to inhaled chemicals should be a key step in the validation of this model for toxicological testing.