# Establishment of a fully ciliated and functional *in vitro* cell model of the rat airway epithelia

**C. Mathis*[1], S. Huang[2], S. Frentzel[1], D. Kuehn[1], C. Merg[1], M. Guertz[1], K. Trivedi[1], E. Guedj[1], S. Boue[1], A. W. Hayes[3], S. Constant[2], M.C. Peitsch[1], J. Hoeng[1]**

*[1]Philip Morris International, Philip Morris Products S.A., Neuchâtel, Switzerland; 2 Epithelix Sarl, Plan-les-Ouates, Switzerland; [3] Spherix Consulting, Rockville, MD, USA and member of Philip Morris International Scientific Advisory Board*

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## INTRODUCTION

Animal models have been widely used in inhalation studies (acute or chronic) to assess the biological effect of new drugs or environmental agent exposure on the human respiratory system.

By using *in vitro* systems, it may be possible to get further insight into the mode of action of specific compound by testing larger range of doses and/or by investigating multiple time points. The use of *in vitro* models is less expensive, time consuming and poses less ethical issues when compared to animal studies.

To be as close as possible to the biological processes occurring *in vivo*, the use of primary airway epithelial cells cultured at the air-liquid interface (ALI) have been shown to be the way to go. Various studies have demonstrated how this *in vitro* model recapitulates well the key functionalities (e.g. mucus secretion, cilia beating, release of cytokines upon challenge, etc..) of the *in vivo* airway epithelia[1,2]. It has also been shown to be a powerful system to investigate normal biological processes[3], disease mechanisms[4] and responses to toxic exposures of the respiratory system[5].

As presented in figure 1, a big challenge in toxicology assessment is to assess which part of the biology can be translated across species or can be extrapolated from *in vitro* study to the *in vivo* situation.

Up to now, the production of a well differentiated rodent bronchial organotypic model was challenging, did not address species translational questions, did not help to reduce the use of animals in research.

In the following poster, we are presenting the results of a collaborative work between Epithelix, Philip Morris International (R&D) and the Nickel Producers Environmental Research Association (NiPERA) to establish and characterize a new rat organotypic bronchial epithelial model, the rat Mucilair™.

The results of the first series of tests to assess the functionality of this new model are promising:

- Fully differentiated epithelia with ciliated cells, basal cells and mucus secreting cells
- Ability to release pro-inflammatory markers upon challenge
- Functional ion channels activity

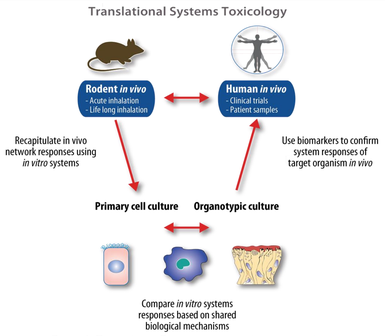


Figure 1: Translational Framework concept including both translation across species and from *in vitro* to *in vivo* models

## RESULTS

After exposure to TNF-a/Il-1b, the rat Mucilair™ tissue cocultured with fibroblasts is releasing multiple cytokines (Vegf, Rantes, MCP-1, MIP1-a, GRO/KC, IP-10, Il-6) suggesting a good functionality of the tissue culture upon challenge with pro-inflammatory inducers.

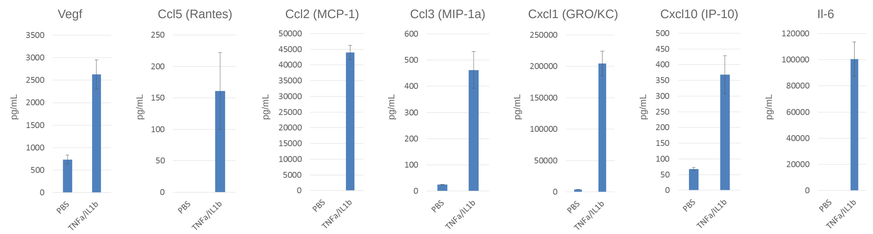


Figure 4: Measurement (using Luminex multiplex based technology) of pro-inflammatory markers release in the medium of rat Mucilair™ tissue cocultured with fibroblasts after 24h basolateral exposure to PBS or rat TNFa/Il1b (10ng/mL). Mean (N=3 inserts/treatment) ± SD. The following kit was used: Miliplex MAP Rat cytokine/chemokine magnetic bead panel, RECYTMAG-65K, Milipore.

A similar induction of the release of pro-inflammatory markers (VEGF, RANTES, MCP-1, IL-8, IP-10, IL-6) was measured in the basal culture medium of human bronchial epithelial organotypic tissues cocultured with fibroblasts after 24h exposure to human TNF-a/IL-1b.

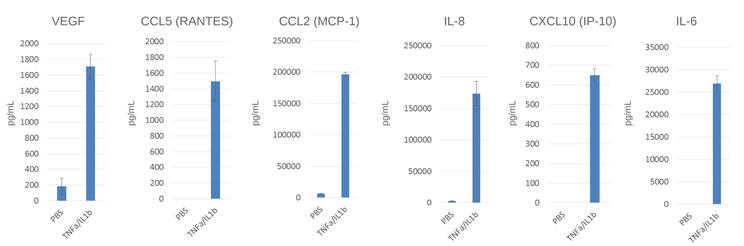


Figure 5: Measurement (using Luminex multiplex based technology) of pro-inflammatory markers release in the medium of human bronchial epithelial organotypic tissue cocultured with fibroblasts after 24h basolateral exposure to PBS or human TNF-a/IL1-b (10ng/mL). Mean (N=3 inserts/treatment) ± SD. The following kit was used: Miliplex MAP human cytokine/chemokine magnetic bead panel, HCY-TOMAG-60K, Millipore)

## MATERIALS and METHODS

Rat MucilAir™ model has been developed from a joint effort among of Epithelix, Philip Morris International R&D and the Nickel Producers Environmental Research Association (NiPERA) .

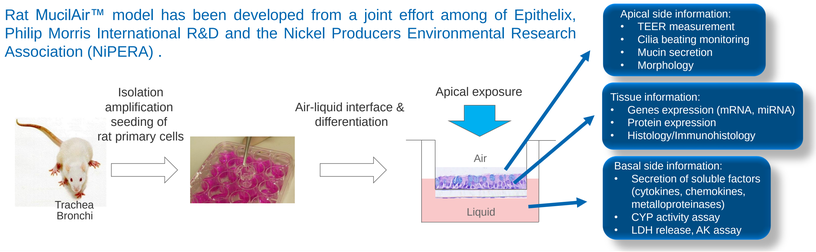


Figure 2: Tracheo-bronchial epithelial primary cells and fibroblasts were isolated from Wistar rats airway tissues. After an amplification step, epithelial cells were seeded at a minimum cell density of 125 000 cells three days after fibroblasts were put in culture. Tracheo-bronchial epithelia are then reconstituted in separate transwells of 6.5 mm of diameter on the polyester membrane with pore size of 0.4 µm (Costar 3470, Corning incorporated) allowing an ALI. Cell differentiation occurs when the culture has an ALI and is considered fully differentiated and ready to use after two weeks in culture.

Tissue integrity is assessed by measuring Trans Epithelial Electrical Resistance (TEER).

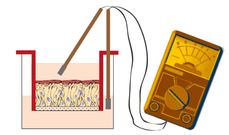

Figure 3: TEER is measured using chopsticks electrodes connecting the apical surface of the tissue culture (immersed with culture medium) and the latero-basal medium.

## RESULTS

At the beginning of the culture, TEER values varied greatly due to difference in cell density. After two weeks, when differentiation is completed, TEER values stabilized between 300 and 400 Ω.cm². A similar evaluation can be done with human Mucilair™ tissue culture (data not shown).

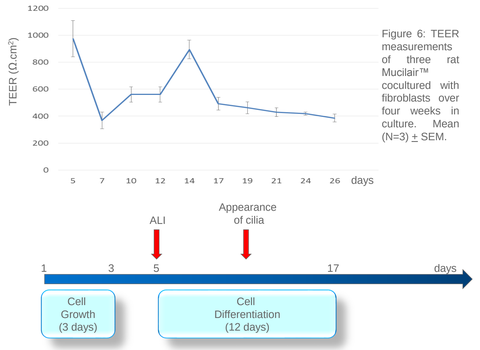


Figure 6: TEER measurements of three rat Mucilair™ cocultured with fibroblasts over four weeks in culture. Mean (N=3) ± SEM.

Similar morphology (presence of cilia, basal cells, mucus expressing cells) and expression of cell-type specific markers (p63, Muc5AC) between human and rat Mucilair™.

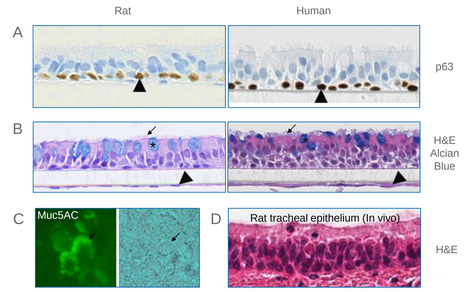


Figure 7: A and B: Morphological comparison between rat and human Mucilair™ inserts. Both tissue cultures are expressing p63, a marker of basal cells (arrowheads, A). Cilia are present on the apical side (arrows, B) of both in vitro models as well as goblet cells (asterix, B) and a thin layer of fibroblasts is observable on the bottom of the membrane (arrowheads, B). H&E: Hematoxylin & Eosin. C: Whole mount immunostaining of Muc5AC positive cells of rat Mucilair™ (GX40) also expressed by goblet cells in human Mucilair™ tissue culture (Data not shown). D: Cross section of an adult rat trachea stained with H&E.

Rat Mucilair™ tissues show (similar to human bronchial epithelial organotypic tissues) functional ion channels activity.

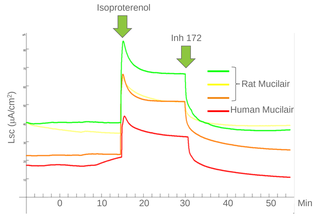


Figure 8: The ion channel activities of three rat Mucilair™ inserts and one human bronchial organotypic insert were monitored in a modified Ussing Chamber after treatment with Isoproterenol (a non selective beta adrenergic agonist that increases cAMP and activates ion channels such as CFTR) or with Inh 172 (a specific inhibitor of CFTR).

## CONCLUSION

Unlike the human bronchial epithelial organotypic culture, the *in vitro* reconstitution of rat airway epithelial tissue is challenging. Primary rat tracheo-bronchial epithelial cells are difficult to grow and handle. In this collaborative work, the protocol to produce a fully ciliated and functional rat airway epithelial model has been established overcoming some of the major difficulties previously encountered (like the absence of ciliated cells or the limited number of inserts produced).

In this study, two key factors were identified which are essential for the quality of the epithelia :

- the seeding of an appropriate epithelial cell density on the membrane of the Transwell insert
- the coculture with primary rat fibroblasts.

The characterization of the new rat Mucilair™ model demonstrated:

- The presence of functional ciliated cells with a beating frequency equal to 12.9 ± 1.63 Hz (similar to human bronchial epithelial organotypic model: 7-12Hz)
- A typical pseudo-stratified airway epithelial tissue structure, with basal cells (p63-positive), ciliated cells and mucus secreting cells (expressing Muc5AC)
- A functional ion channels activity similar to the one observed in the human *in vitro* model counterpart.
- Its ability to respond to pro-inflammatory inducers (e.g. TNF-a/Il-1b) by producing and releasing various cytokines (e.g. vegf, MCP-1..) like human bronchial epithelial organotypic culture.

To conclude, the data presented here suggest that the new rat Mucilair™ model is a promising *in vitro* rat model that can be used for translational toxicology studies from rodent to human and for toxicological assessment of a variety of products including novel Modified Risk Tobacco Products.

## REFERENCES

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2014 Phoenix, Arizona

**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