Lab on a Chip

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ARTICLE TYPE

Live Human Nasal Epithelial Cells (hNECs) On Chip for In Vitro Testing of Gaseous Formaldehyde Toxicity via Airway Delivery

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Cell models based on human nasal epithelial stem/progenitor cells (hNESPCs) are developed to obtain functional ciliated cells on modified transwell inserts. The live hNECs are
10 **integrated into microfluidic platforms to mimic the 3D features of human upper airway for *in vitro* testing of gaseous formaldehyde toxicity via airway delivery.**

In humans, nasal epithelium is always exposed first to all environmental agents, including infectious agents, allergens, and 15 air pollutants, and thus protects the lower airways [1]. Studies on the airway epithelium can illuminate the pathophysiology of most respiratory diseases, ranging from infectious, genetic, to most inflammatory diseases. Since the cilia movement or beating of epithelial cells is one of the intrinsic defence mechanisms of the 20 airway to external irritants, it can be utilized directly for testing the response of human airway to dust, bacteria, drug/toxicant and so on [2]. However, this has long been hampered by the challenges to obtain the *in vitro* 3D features of human nasal epithelium, controlled airway delivery of irritants, and clear imaging/videoing 25 of the beating sub-micron cilia. Microfluidic systems allow well control of microenvironment for cellular studies and offer highly sensitive molecule detections [3-5]. Here, we report a micro-environment closer to the *in vivo* situations to mimic the human upper airway on a microfluidic platform. Thanks to the small 30 footprint of the microfluidic platform, a very short distance can be established between the cilia and objective lens while maintaining an airway in between for the delivery of gaseous drugs/toxicants. Such microfluidic setup enables the clear real-time observation of cilia beating through high magnification lens.

35 Instead of utilizing cell lines or primary cell cultures, we have successfully developed an experimental model to obtain beating cilia by cultivation and differentiation of hNESPCs at an air-liquid interface (ALI) on a modified transwell insert. This eliminates the concerns that cell lines may not give rise to fully 40 differentiated phenotypes of the original tissue such as cilia formation & mucus secretion, and that primary cells have no passages which limits the cell source from the same donor. The functional epithelial cells on the modified transwell insert can be inserted into our microfluidic platform for conducting gaseous 45 drug/toxicant testing in an airway delivery manner, instead of the traditional solution-mediated drug/toxicant delivery. In this way, the real inhalation exposure process can be well recapitulated in

an *in vitro* experimental model.

As a demonstration of on-chip toxicity testing on the functional 50 epithelial cells, formaldehyde (FA) is chosen because it is widely used in a variety of industries, i.e. FA-based resins, and it is a well-known airway irritant and a potential occupational carcinogen as defined by the Occupational Safety and Health Administration (OSHA) carcinogen policy. Human nose is the 55 primary contact site of gaseous FA and 90% of FA is uptaken by nasal mucosa. To date, the short term effect of environmental and occupational exposure of FA on the human epithelium has not been verified in human cell models due to the challenging to establish live human upper airway *in vitro*. With our microfluidic 60 setup and the versatile cell model, we are able to investigate the human nasal cilia beating behaviours upon the exposure of gaseous FA at different hygiene and occupational limits (European Scientific Committee on Occupational Exposure Limits (ESCOEL): 0.4 ppm or 0.5 mg/m^3, OSHA short term 65 exposure limit: 2 ppm or 2.5 mg/m^3).
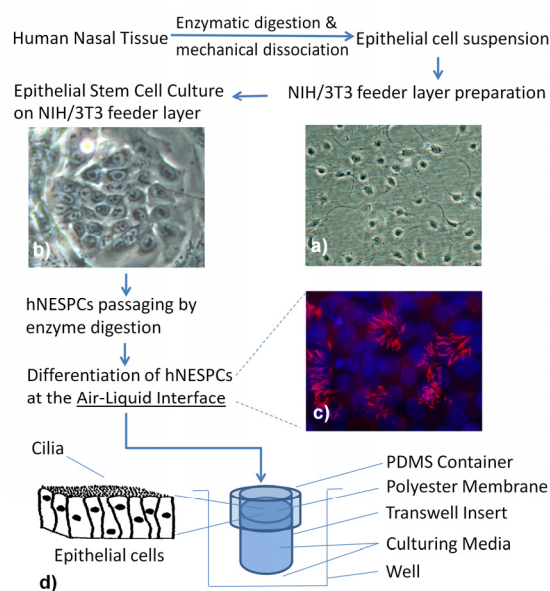

Figure 1. Obtaining functioning ciliated cells by a modified air-liquid interface culturing. a) NIH/3T3 cells are settled and flattened as feeder layer cells for seeding hNESPCs; b) Epithelial colonies are formed after 3 days of culture. c) Immunostaining of

motile cilia on transwell by acetylated-TUBULIN marker (red) and DAPI (blue); d) Modified air-liquid interface culture.

The hNESPCs were isolated from inferior turbinate (IT) biopsies of 3 healthy individuals according to the established protocol by our group[6]. Donors' medical background is summarized in Table S1 in ESI 1. As shown in the Figure 1.a, the NIH/3T3 cells (American Type Culture Collection, Manassas, VA) was cultured in Dulbecco's modified Eagle medium (DMEM, Sigma) supplemented with 10% FCS (fetal calf serum) and 1% Antibiotic-Antimycotic solution (Gibco-Invitrogen) as feeder layer. Epithelial colonies are then formed after 3 days of primary culture on the mitomycin c-treated NIH/3T3 feeder layer as shown in Figure 1b. After 2 passages (from P0 to P1) the hNESPCs were collected by Accutase enzyme dissociation (Invitrogen) and seeded onto the modified transwell insert. The beating cilia were then obtained by the cultivation and differentiation of hNESPCs by 5 weeks of ALI culture on the transwell inserts (Figure 1d). Instead of using normal transwell inserts, we removed the rim of transwell inserts by cutting and then placed them back to the basal chambers with the polyester membrane facing up. Customized poly(dimethylsiloxane) (PDMS) seals (cylindrical tubes, ID = 8.5mm, OD = 11mm, Height = 8 mm) are put around the top of the modified transwell inserts to form a PDMS chamber together with the transwell membrane. To prevent bubble formation under the membrane when putting the modified transwell insert into the basal chamber, 400 µl of B-ALI[TM] growth medium (Lonza, Walkersville, MD) are prefilled into the apical chamber of the transwell and the solution can be retained even when turning the opening of chamber down because of surface tension. Approximately 5×10^4 hNESPCs suspended in 200 µl B-ALI[TM] growth medium are pipetted into the PDMS chamber for seeding on the membrane. On day 3 after seeding, growth medium from the apical and basal chamber is replaced with B-ALI[TM] differentiation medium with inducer and the medium is refreshed every two days during the culturing period.

After 5 weeks culture functional ciliated cells were obtained on the membrane of the modified transwell insert. Immunostaining of acetylated-TUBULIN, a ciliated cell marker, confirmed the hNESPCs-derived motile ciliated cells on transwell as shown in Figure 1c. After the removal the PDMS seal, the insert can be integrated into the microfluidic chip for cilia observation and drug/toxicity testing. As shown in Figure 2, the microfluidic device is made from molded PDMS and cover glass (0.17 mm thickness). It consists of two straight channels (2 mm × 300 µm × 10 mm, width × depth × length) joint by a square chamber (10 mm × 10 mm × 300 µm, width × length × depth). One 100µm-thick Poly(methyl methacrylate) (PMMA) film (9 mm × 1.2 mm, length × width) is put under the transwell insert into the microfluidic chamber during plasma bonding of chip. This is to create an air chamber and prevent direct contact of insert membrane with the glass substrate when putting the transwell insert into the microfluidic chamber. The round opening (8.6 mm in diameter and 4 mm in depth) of the microluidic chamber will form a tight seal around the transwell insert. With 100 µm-thick PMMA film in the microfluidic chamber, the 120 µm working distance of a high magnification lens (Olympus UPLFLN60XOI)

is ensured. Together with a high speed camera, Basler acA2000-340kc (resolution 5.5 × 5.5µm, maximum frame rate 340 fps), the setup ensured a clear observation of cilia beating. For the refreshment of cilia culture medium in the apical chamber, another PDMS seal (7.8 mm in diameter, 3 mm in thickness) with both inlet and outlet (holes of 1 mm in diameter and connected to tubing through 19G metal tube) is placed into the apical chamber of the insert as shown in Figure 2.

Airway delivery of drug/toxicant is through the chamber formed by the cell membrane and the PDMS/glass microfluidic cavity. FA at concentrations of 0.5, 1.0 and 3.0 mg/m^3 are chosen as 0.5 mg/m^3 (0.4 ppm) is the occupational exposure limit from ESCOEL, 3.0 mg/m^3 is close to OSHA short term exposure limit and 1.0 mg/m^3 is as an intermediate concentration for verifying the irritant effect of FA exposure [7].
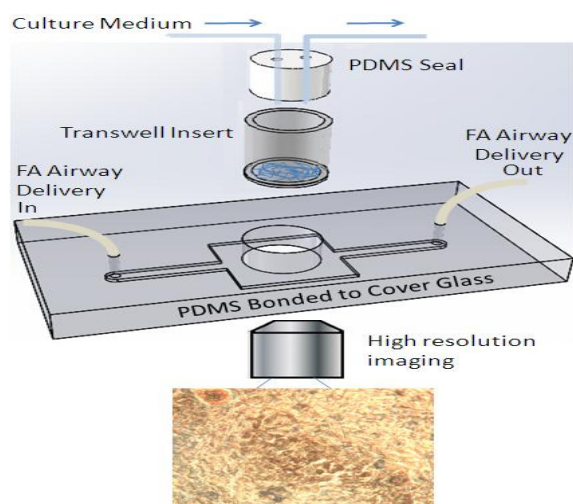

Figure 2. *In-vitro* FA toxicity testing via airway delivery on a microfluidic platform integrated with the functional ciliated cells on transwell insert membrane.

Gaseous FA was prepared from 16% formalin (Dako, Denmark) by placing required molar mass of FA solution into an enclosed 2-liter glass bottle (Schott, Germany) and equilibrated into gaseous phase and stable for 72 h with gentle agitation in a fume hood. The final FA concentrations were confirmed by an FA electrochemical sensor (Interscan, model 4160-2, USA). FA was exerted on ciliated cell on microfluidic platform by connecting the FA bottle to the inlet of microchannel and the outlet of channel to a 20-ml syringe which was driven by a syringe pump (Pump 33, Harvard Apparatus, Holliston, MA). For both FA delivery and fresh air delivery, the pump was set to run at withdrawing mode at 200 µl/min.

Cilia beating frequency (CBF) was monitored by placing the cilia embedded microchip on an inverted phase contrast microscope (Olympus IX51, Melville, NY) equipped with the high speed/resolution camera and the high magnification lens mentioned earlier. A view of beating cilia on membrane was carefully chosen and monitored throughout the toxicity testing process by video recording (as shown in ESI 2, video of cilia beating) at 2-minute interval. Then the dominant CBF was

obtained by the whole field analysis through a commercialized program – SAVA system [8].
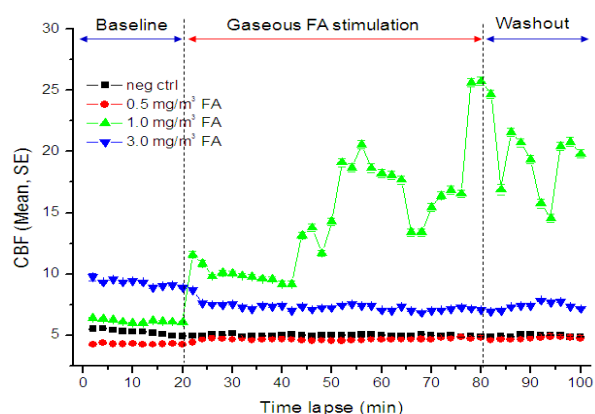
Figure 3. A typical CBF measurement of hNESPCs-derived cilia (No.62IT) exposed to different concentrations of gaseous FA.

Figure 3 shows a typical CBF measurement over time course for gaseous FA exposure. Totally four transwells were used, one for monitoring under fresh air exposure, three for monitoring under FA exposure at concentrations of 0.5, 1.0 and 3.0 mg/m^3. FA exposures were conducted in such a sequence, 20 min control air exposure to determine the baseline CBF, 60 min FA exposure to measure the cilia response and 20 min control air exposure to monitor the potential CBF recovery after washout of FA.

The baseline CBF of human airway ciliated cells may vary from sample to sample in a difference of a few Hz as reported[9]. Here, we are interested in the response of CBF after FA stimulation. The results showed that the CBF is a sensitive index of human nasal epithelial cilia to environmental toxicants, such as FA. As indicated by the 1.0 mg/m^3 FA stimulation, the CBF increased immediately after the FA exposure and kept on increasing throughout the 60 min stimulation period, and peaked at the end of stimulation. The 0.5 mg/m^3 FA (Occupational Exposure Limits, SCOEL) was tested to be safe, in terms of FA-induced response of cilia beating. Interestingly, the CBF did not increase but slightly decreased and then remained unchanged throughout the process, upon exposure of relatively high concentration of FA (3.0 mg/m^3). This is probably due to the toxic and/or inflammation effects on the epithelial cells including cilia at the high concentration of FA[7]. The severe toxicity induced by 3.0 mg/m^3 FA exposure was confirmed by a quantitative PrestoBlue^{TM} cell viability assay (Invitrogen, USA. Refer to Figure S1 in ESI 1). The 20 min washout after FA stimulation didn't show significant effect in terms of CBF. More specifically for the 1.0 mg/m^3 FA exposure, the CBF increment did stop since the start of washout. However, there was no sign of CBF recovery to its baseline throughout the short period (20 min in this study) of control air washout process. Besides FA exposure, the group of cilia under fresh air exposure for 100 min showed no much change of CBF throughout the process. This indicated a good negative control was achieved by the experiment setup and there was no detectable effect of microscope light (potential heating) on the CBF measured.

The statistical analysis of CBF data from 3 samples was summarized in Table 1. A significant irritant effect (increased mucociliary function) of FA was seen at 1.0 mg/m^3 level, while inhibition of this physical response was seen at higher level of FA. Similar CBF profiles from different donors were observed, upon 3 levels of FA stimulation. This again indicated the sensitivity and reliability of our dynamic device and real-time monitoring system.

Table 1. Cilia beating response to gaseous FA stimulation

Exposure level	Baseline (20 min)	Response to FA (60 min)	Washout (20 min)	FA vs. Baseline % in change	Washout vs. Baseline
Neg ctrl	6.11±0.20	5.93±0.12	5.85±.215	↓ 3.0%	↓ 3.6%
0.5 mg/m^3 FA	5.80±0.35	6.23±0.21	6.40±0.38	↑ 7.4%	↑ 10.4%
1.0 mg/m^3 FA	6.16±0.04	14.56±0.86	19.46±0.95	↑ 136.4%	↑ 215.9%
3.0 mg/m^3 FA	9.22±0.09	7.31±0.06	7.397±0.10	↓ 20.7%	↓ 19.8%

Note: CBF (Hz) of three IT samples were shown in Mean±SE.

In summary, for the first time, the *in vitro* differentiated hNECs were integrated into a microfluidic chip for the real-time monitoring of cilia beating function. With such a live human upper airway on chip, *in vitro* testing of toxicity via airway delivery was demonstrated. Reactivity of the ciliary cells to dynamic gaseous FA exposure was monitored and analyzed simultaneously. CBF was shown to be a sensitive index of human ciliary cells to FA. The developed system and experimental model will greatly facilitate drug screening and be more realistic for clinical applications.

Notes and references

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1. W. J. Fokkens, V. J. Lund, J. Mullol, C. Bachert, et al, *Rhinol Suppl*, 2012, 3 p preceding table of contents, 1-298.
2. Y. Yan, W. M. Gordon and D. Y. Wang, *Curr Opin Otolaryngol Head Neck Surg*, 2013, **21**, 263-270.
3. L. Yu, S. R. Ng, Y. Xu, H. Dong, Y. J. Wang and C. M. Li, *Lab on a Chip*, 2013, **13**, 3163-3182.
4. Z. W. Ge, W. Wang and C. Yang, *Lab on a Chip*, 2011, **11**, 1396-1402.
5. W. Wang, C. Yang, X. Q. Cui, Q. L. Bao and C. M. Li, *Microfluidics and Nanofluidics*, 2010, **9**, 1175-1183.
6. X. N. Zhao, F. G. Yu, C. W. Li, Y. Y. Li, S. S. Chao, W. S. Loh, X. L. Pan, L. Shi and D. Wang, Y., *Am J Rhinol Allergy*, 2012 **26**, 345-350.
7. Y. Qiao, B. Li, G. Yang, H. Yao, J. Yang, D. Liu, Y. Yan, T. Sigsgaard and X. Yang, *Inhal Toxicol*, 2009, **21**, 1200-1207.
8. J. H. Sisson, J. A. Stoner, B. A. Ammons and T. A. Wyatt, *J Microsc*, 2003, **211**, 103-111.
9. T. F. Palmberger, P. Augustijns, A. Vetter and A. Bernkop-Schnurch, *Drug Dev Ind Pharm*, 2011, **37**, 1455-1462