A novel microfluidic platform for pulmonary nanoparticle exposure

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Introduction

Nanoparticles (NPs) are a common byproduct of many modern technologies and industrial processes. Studying their toxicity, however, has been a challenge due to the lack of appropriate *in vitro* models. NPs are mainly taken up via inhalation and they deposit deep into the lung. The lung's barrier function determines the uptake of NPs into the system and their resulting toxicity on other organs. Furthermore, their acute toxicity on the lung tissue needs to be considered.

Aim

Our goal was to establish an *in vitro* alveolar lung and liver model for airborne NP testing using a cutting-edge organ-on-a-chip technology. This, in combination with the adapted P.R.I.T.® ExpoCube would enable elaborate toxicity studies, where lung equivalents are subjected to repetitive nanoparticle exposure and toxic effects on both lung and liver can be examined. In this work, we present the development of a hAELVi cell line-based lung model, mimicking the alveolar epithelial cell type I (AECI) and its successful co-cultivation with induced pluripotent stem cell (iPSC) derived liver organoids in a specifically adapted HUMIMIC Chip platform.

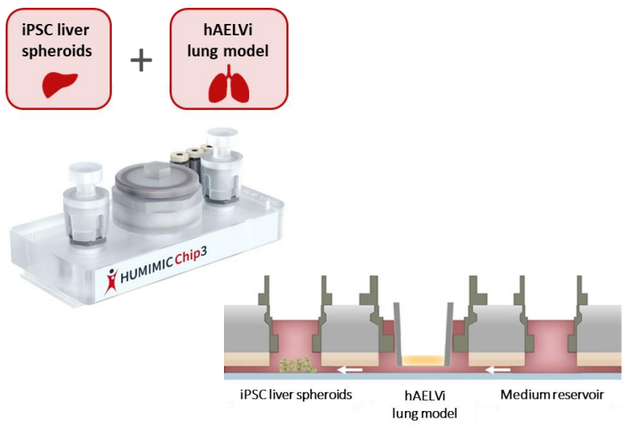


Fig. 1 ***Co-cultivation of iPSC liver spheroids with the hAELVi lung model in the adapted HUMIMIC Chip3.*** The HUMIMIC Chip3 has a microfluidic circuit consisting of three tissue culture compartments, two of 96-well size and one of 24-well size connected by microchannels. The liver spheroids were placed on the bottom of one 96-well compartment, while the hAELVi models were cultured on a Corning Transwell® insert placed in the 24-well compartment. In addition to the dynamic co-cultures in the chip, static single-cultures in cell culture plates were included as controls from each organ model.

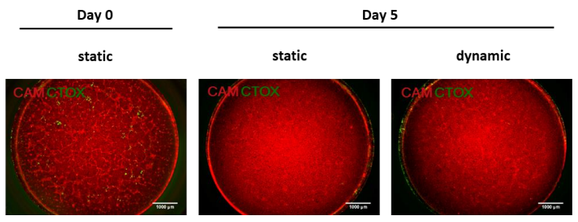


Fig. 2 ***Fluorescence viability staining of hAELVi lung models cultured statically and co-cultured dynamically for 5 days.*** Day 0 is defined by the start of dynamic cultivation. Calcein-AM (red-vital) and CellTox™ Green (green-dead) viability staining showed vital cells both on day 0 and 5 of cultivation with only minor presence of dead cells. Scale: 1000 µm.

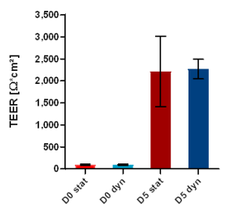


Fig. 3 ***Transepithelial electrical resistance (TEER) of static single- and dynamic co-cultures of hAELVi lung models cultured for 5 days.*** During the 5 days of cultivation the TEER value of the lung models reached an average of 2300 Ω·cm², demonstrating the development of an intact barrier, essential for the assessment of NP uptake and distribution.

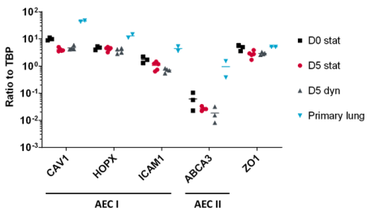


Fig. 4 ***Alveolar gene expression of hAELVi-based lung models under static single and dynamic co-culture conditions.*** The lung models showed strong expression of the AECI-specific genes CAV1, HOPX, and ICAM1, as well as the tight junction marker ZO1. However, no significant changes in expression levels were observed between different cultivation conditions or time points. Almost all markers showed approximately 10-fold greater expression in the primary lung samples compared with all other conditions, with the exception of ZO1, where expression levels were similar, underscoring the barrier function of the model. Interestingly, the AEC II marker ABCA3 was also expressed in hAELVi lung tissue. All data were normalized to the housekeeping gene, TBP.

CAV1 - Caveolin-1, **HOPX** - Homeodomain-only protein, **ICAM1** - Intercellular adhesion molecule 1, **ABCA3** - ATP binding cassette subfamily A member 3, **ZO1** - Tight junction protein 1

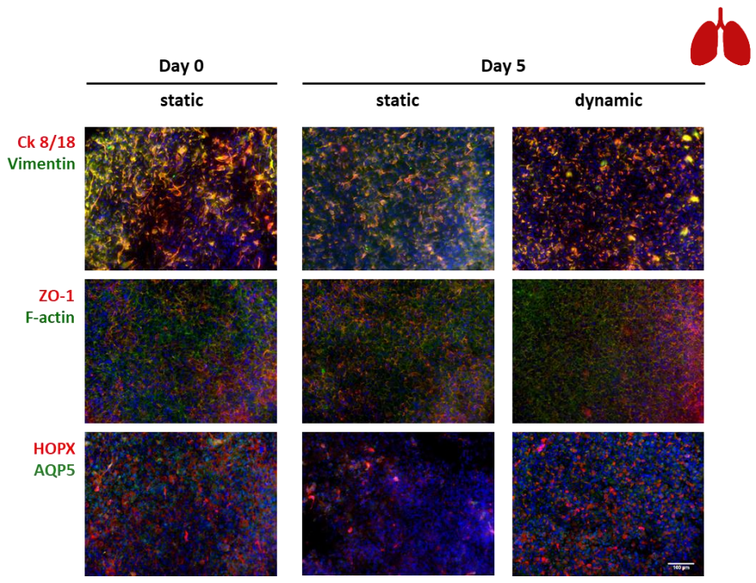


Fig. 5 ***Whole mount immunohistochemical staining of hAELVi models cultured for 5 days under static and dynamic conditions.*** Cytokeratin 8/18 (Ck 8/18) and vimentin are involved in the formation of the cytoskeleton. The staining showed the restriction of both proteins to the cytoplasm and confirmed that the cells grew in a confluent layer. Staining of the tight junction protein ZO1 and F-actin, an essential element of the eukaryotic cytoskeleton, showed strong expression of both proteins in hAELVi cells. ZO1 was visualized as thin lines between adjacent cells, showing high specificity. Aquaporin 5 (AQP5), an integral membrane protein of the water channel protein family and an AECI marker, was also localized in between the cells. Homeodomain-only protein (HOPX), a transcription factor present in AECI but not in AECII cells, was also detected in all conditions. Scale: 100 µm.

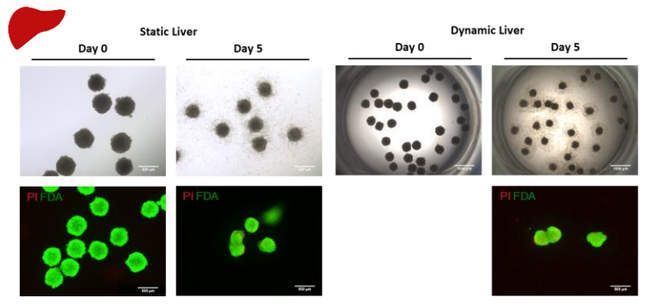
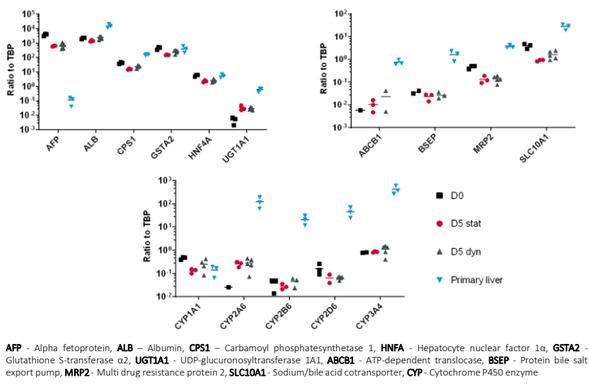


Fig. 6 ***Morphology and viability staining of iPSC-derived liver spheroids cultured statically and co-cultured dynamically.*** During the 5-day cultivation, both static and dynamic organoid cultures adhered to the bottom of the culture vessel with visible outgrowth of single cells. Despite the significant changes in morphology, whole spheroids could still be obtained by carefully scraping them off the surface. Their viability was assessed by staining the outer spheroid layer with fluorescein diacetate (FDA - green) for vital and propidium iodide (PI - red) for dead cells. At day 5, both conditions showed mostly viable cells on the spheroid surface. Scale: 100 µm.



AFP - Alpha fetoprotein, **ALB** – Albumin, **CPS1** – Carbamoyl phosphatesynthetase 1, **HNFA** - Hepatocyte nuclear factor 1α, **GSTA2** - Glutathione S-transferase α2, **UGT1A1** - UDP-glucuronosyltransferase 1A1, **ABCB1** - ATP-dependent translocase, **BSEP** - Protein bile salt export pump, **MRP2** - Multi drug resistance protein 2, **SLC10A1** - Sodium/bile acid cotransporter, **CYP** - Cytochrome P450 enzyme

Fig. 7 ***Liver-specific gene expression of iPSC liver spheroids cultured statically and co-cultured dynamically.*** The expression of hepatocyte markers was comparable between conditions and showed a slight downregulation over the cultivation time. Only the phase 2 gene UGTA1 showed an approximately 10-fold increase in expression at day 5, yet remained below the primary tissue. The fetal liver marker, AFP, showed more than a 1000-fold increase in expression under all conditions, attributed to the fetal phenotype of the iPSC-based model. The hepatic transporter proteins were all detectable, yet expression was reduced compared to the primary tissue. Only the transporter protein ABCB1 showed an increased expression level over time, all others were slightly downregulated. A small increase in expression was also observed for the sodium/bile acid co-transporter gene SLC10A1 in favor of dynamic culturing. From the cytochrome P450s, only CYP1A1 gene expression levels were similar to those in the primary liver, all others showed reduced gene expression. Upregulation over time was observed for CYP2A5. All other P450s remained unchanged or were downregulated by day 5. Data were normalized to the housekeeping gene TBP.

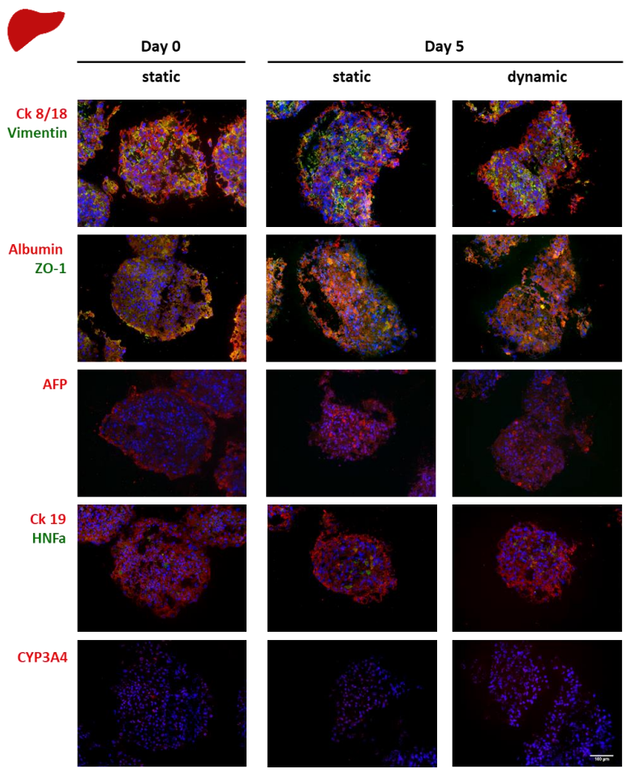


Fig. 8 ***Immunohistochemical staining of iPSC liver spheroid sections cultured under static and dynamic conditions.*** Staining of cytokeratin 8/18 (Ck 8/18) and vimentin, both of which are involved in cytoskeleton formation, showed uniform distribution in the organoids. Albumin, the most abundant liver-produced protein in human blood, and ZO1 were expressed in the entire spheroid under all conditions, but the typical polygonal shapes of the tight junctions were not detectable. Alpha-fetoprotein (AFP) expression was confirmed in all samples, with stronger expression at the edges on day 0. Cytokeratin 19 (Ck 19) was evenly distributed throughout the cell layer in all conditions, whereas hepatocyte nuclear factor 1 alpha (HNFα), a transcription factor responsible for the expression of liver-specific genes, was predominantly localized in the spheroid core. The most abundant P450, CYP3A4, was weakly stained in all sections. Cell nuclei were counterstained with DAPI (blue). Scale: 100 µm

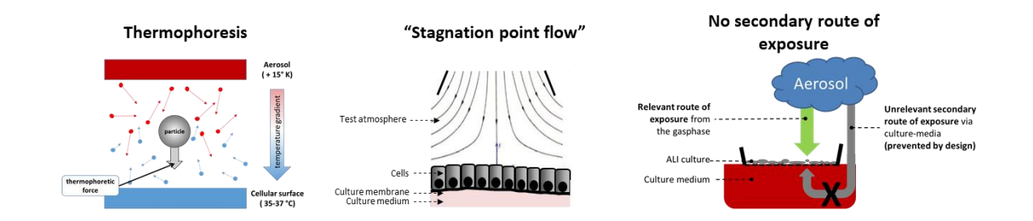


Fig. 9 ***Bases for a safe and efficient nanoparticle exposure as applied in the P.R.I.T.® ExpoCube.*** Thermophoresis in a stagnation flow alignment allows efficient deposition of small particles from aerosols, while also preventing irrelevant secondary route of exposure.

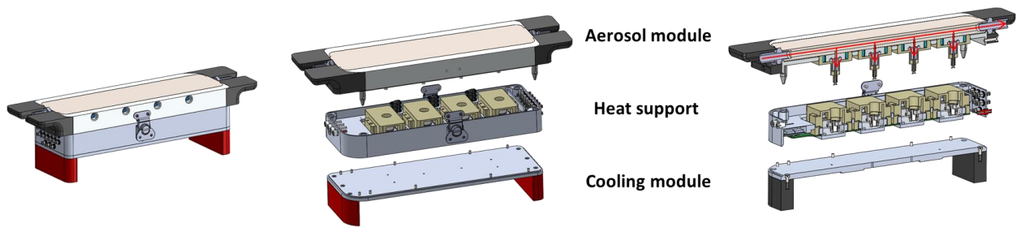


Fig. 10 ***Representation of the novel exposure system prototype, the NanoCube in assembled and exploded view.*** The cell exposure system, P.R.I.T.® ExpoCube (Fraunhofer ITEM) combined with the HUMIMIC Chips (TissUse GmbH) evolved into a novel exposure system, the NanoCube. This enables the investigation of direct effects of NPs on human respiratory models, but can also assess the potential secondary effects on the co-cultured organs. The NanoCube consists of 3 modules, the aerosol, the heat support and the cooling module. To achieve high exposure efficiency through thermophoresis a temperature gradient of about 15°K is desirable. In the aerosol module, the aerosol is heated to 52°C directed in a horizontal main flow and vertically as a secondary flow down to the HUMIMIC Chip3s. The middle segment, the heat support, houses the chips, which are heated independently to a target temperature of 37°C. The cooling module is located at the bottom of the NanoCube. It is used to create a controlled drop in temperature between the aerosol and the surface of the lung models using several Peltier elements. The waste heat is conducted to an external radiator by means of a water cooling system. Connections to the microfluidic pumps of the chips as well as to the chip heaters, the aerosol heater and the temperature sensors can be found on each side of the NanoCube.

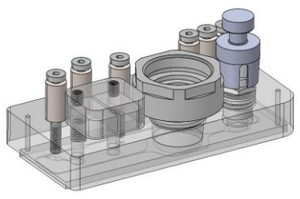


Fig. 11 ***Concept of the INHALChip.*** Based on the HUMIMIC Chip3, our goal was to develop a chip for automated or partially automated medium change for a minimum of 5 days in order to reduce manual effort during the use of the NanoCube. One 96-well compartment has been exchanged to two reservoirs, one for storing fresh, the other for used medium. The medium is changed by flushing the fresh medium through the microfluidic channels, while the used medium is pressed into the reservoir. The medium direction and flow is controlled by additional valves and pneumatic pumps, allowing either the medium circulation or replacement from the medium reservoir.

Conclusions

During the 5-day static single and dynamic co-cultivation, the hAELVi cells formed a tight barrier, demonstrated by their transepithelial electrical resistance values of up to 3000 Ω·cm². Strong expression of the AECI specific and tight junction genes further confirmed their suitability. Although the liver spheroids experienced major morphological changes during the co-cultivation, they remained viable and expressed key hepatic markers. Our results demonstrate an *in vitro* test system compatible with the modified P.R.I.T.® ExpoCube to mimic nanoparticle exposure-prone environments for safety assessments, generating high-quality *in vitro* data predictive of nanoparticle safety in humans.

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TISSUSE Emulating Human Biology
Fraunhofer SCAI
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SPONSORED BY THE
Federal Ministry of Education and Research
NanoINHAL
Grant No. 03XP0226