Characterization of 3D human pulmonary epithelial models under inflammatory hypoxia

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Summary
Infection generates localized hypoxia in affected tissue, inducing cellular survival responses and modulating inflammatory processes. Consideration of oxygen status as a parameter in *in vitro* infection research is therefore vital to the generation of physiologically relevant data within the 3R context. In this study, we characterize classical air-liquid interface (cALI) and liquid-liquid interface (LLI) permanent bronchial epithelial (Calu-3) models under hypoxia to assess the response of airway epithelial cells to inflammation-induced oxygen deprivation. We compare the hypoxic state of these models to their normoxic state within standard incubators, commonly employed in *in vitro* work, as well as to hypoxic and normoxic human primary bronchial epithelial cell (hBEC) cALI cultures. Additional juxtapositions are drawn between Calu-3 cALI and LLI models and Calu-3 conventional monolayer (CM) and inverted air-liquid interface (iALI) models, due to their relevance for basic and specialized research, respectively. Histological analysis showed each 3D model to possess a distinctive set of morphological features, with the hBEC cALI model displaying the greatest *in vivo* similarity. All models were found to exhibit characteristic changes in extracellular oxygen content under normoxia, resulting in diverse culture oxygenation statuses following incubation. Further variation in the rate of extracellular oxygen depletion observed between normoxic Calu-3 LLI, Calu-3 cALI, and hBEC cALI models suggests that cellular oxygen consumption may depend on both cell type and culture format. Differential stabilization of HIF-1α, HIF-2α, and/or HIF-3α revealed hypoxia stress responses to be model-specific, perhaps due to disparities in the nature and severity of hypoxia experienced. However, despite varying HIF profiles, all Calu-3 models showed a reduction in ACE2 protein level, suggesting that permanent lung epithelial cells react similarly to reduced oxygen availability, irrespective of culture format. This stands in contrast to the more complex hBEC model, in which no such decrease in ACE2 level under hypoxia could be detected, despite HIF-1α stabilization. Therefore, while all models examined provide valuable opportunities for *in vitro* exploration, variation in their morphological and molecular characteristics necessitates careful consideration during experimental design.

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