**Microstructured hydrogels to guide self-assembly and scalable growth of lung alveolar epithelial organoids**

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**Statement of Purpose:** The adult lung responds to injury (e.g., viral infections) by activating progenitor cells within the gas-exchanging alveoli; yet, the regenerative capacity of the lung may be insufficient for repair, resulting in high morbidity in patients around the world[1]. Alveolar epithelial organoids are emerging as a source of functional cells to both restore respiratory function in acute lung diseases and as model systems of lung injury[2]. Despite their potential, current protocols to grow these organoids almost exclusively depend on 3D Matrigel cultures, which are limited for transplantation as Matrigel introduces animal components into the cultures and the local differences in biophysical and biochemical signals result in heterogeneous (shape and size) organoids. Thus, we designed a microwell hydrogel platform to improve the formation and culture of human induced pluripotent stem cell (iPSC)-derived alveolar type 2 (iAT2) organoids for therapeutic and disease modeling applications (Fig. 1).

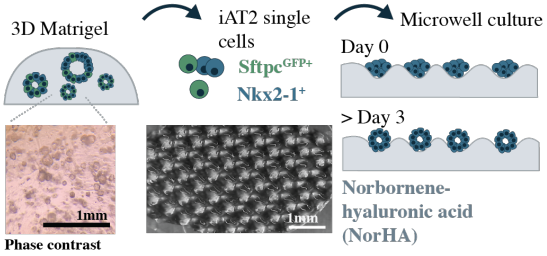


**Figure 1** Schematic illustrating the culture of human iPSC-derived iAT2 cells with early (Nkx2-1$^{+}$) and late (Sftpc$^{GFP+}$) markers of lung progenitors in Matrigel and subsequent seeding into microwells.

**Methods:** Hydrogels were fabricated through norbornene-modified hyaluronic acid crosslinked with matrix metalloproteinase-degradable peptide crosslinkers via a light-mediated thiol-ene reaction[3] and used to create microwells with controlled topographies by molding from patterned polydimethylsiloxane substrates. Expanded iAT2s (Sftpc$^{GFP+}$) were seeded atop the hydrogels with ~20-250 cells per microwell, and cultured in CHIR99021, keratinocyte growth factor, dexamethasone, 3',5'-cyclic adenosine monophosphate and 3-isobutyl-1-methylxanthine containing media[4]. iAT2 organoid formation efficiency, heterogeneity, proliferation, and organoid growth were quantified by transmitted light microscopy and progenitor fate was determined by immunofluorescence for GFP.

**Results:** Microwells promoted the formation of iAT2 organoids of uniform size (Fig. 2A), which depended on the seeding density and microwell size (not shown). iAT2 organoids increased as a function of time and initial cell seeding density and could be maintained for at least 12 days (Fig. 2B). Organoid formation efficiency at 12 days depended on the initial cell seeding density (Fig. 2C), indicating this as a critical parameter in generating robust microwell organoid cultures. When compared to Matrigel, microwell formation resulted in increased numbers of lung progenitors (GFP+) (Fig. 1D), which could further be used for 3D encapsulation (Fig. 1E), suggesting iAT2 fate maintenance throughout microwell culture.

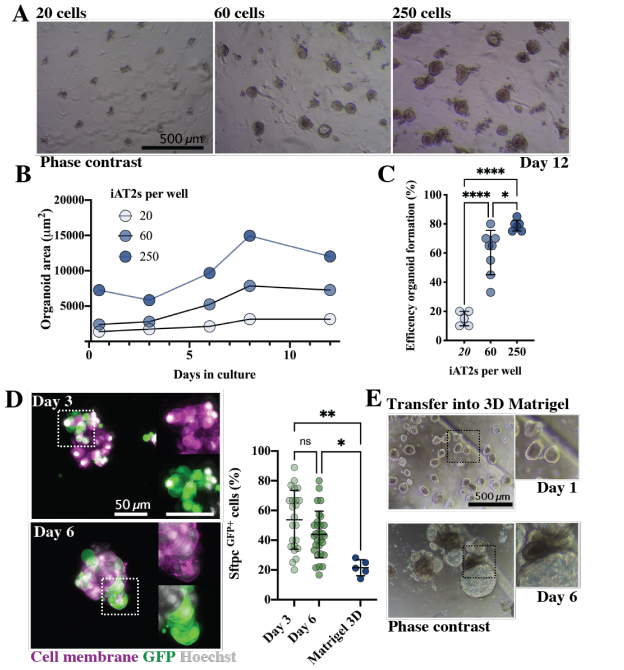


**Figure 2** **A** Images of iAT2 organoids in microwells formed with different cell numbers and quantification of **B** organoid area over 12 days and **C** efficiency of organoid formation as a function of iAT2 seeding density at day 12 (mean ±SEM, n > 5-8 regions per group, * p≤0.05, **** p≤0.0001). **D** Images and quantification of Sftpc$^{GFP+}$ expression of iAT2s cultured for 3 and 6 days in microwells (200/100 μm in width/depth) in comparison to iAT2 cultured within 3D Matrigel for 6 days (mean ±SD, n = 30 organoids/group, 5 regions for Matrigel, * p≤0.05, ** p≤0.01, ns = not significant). **E** Images of iAT2 organoids formed in microwells for 6 days and cultured for additional 1 and 6 days upon transfer into Matrigel.

**Conclusions:** Microstructured hydrogels enabled the generation of alveolar type 2 epithelial organoids with controlled size and fate. Moreover, Matrigel-free culture conditions hold promise as a means to expand functional epithelial cells for therapeutic and disease modeling applications. Ongoing work is to extend the platform to other lung epithelial cells, including with primary cells.

**References:** [1](Kotton D.N. & Morrisey E.E. Nat. Med. 2014; 20, 822-832), [2](Vaughan A. et al Nature 2015;621-25), [3](Gramlich WM. et al Biomaterials 2013;34, 9803−11), [4](Jacob A. et al Nat. Prot. 2019;3303-3332).