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Current strategies and opportunities to manufacture cells for modeling human lungs

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ABSTRACT

Chronic lung diseases remain major healthcare burdens, for which the only curative treatment is lung transplantation. In vitro human models are promising platforms for identifying and testing novel compounds to potentially decrease this burden. Directed differentiation of pluripotent stem cells is an important strategy to generate lung cells to create such models. Current lung directed differentiation protocols are limited as they do not 1) recapitulate the diversity of respiratory epithelium, 2) generate consistent or sufficient cell numbers for drug discovery platforms, and 3) establish the histologic tissue-level organization critical for modeling lung function. In this review, we describe how lung development has formed the basis for directed differentiation protocols, and discuss the utility of available protocols for lung epithelial cell generation and drug development. We further highlight tissue engineering strategies for manipulating biophysical signals during directed differentiation such that future protocols can recapitulate both chemical and physical cues present during lung development.

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1. Introduction

End-stage lung disease is the third leading cause of morbidity and mortality worldwide [1,2], and produces a significant burden on healthcare systems due to extensive resource expenditures for disease management and as lung transplantation is the only curative treatment option. Such diseases include acute respiratory distress syndrome, chronic obstructive pulmonary disease, cystic fibrosis, and pulmonary fibrosis. Chronic pulmonary diseases result in 3 million global deaths per year [3,4]. Patients who receive transplants face continued complications associated with chronic immunosuppression and graft rejection with the transplant survival rates at 5 and 10 years being 54% and 32%, respectively [5]. Furthermore, since lungs function as an important barrier between the internal and the external environments, they are a critical site for bacterial and viral infections and disease transmission; particularly relevant given the current COVID-19 pandemic. There is, therefore, a critical need to better elucidate the mechanisms of infection, disease progression, host response, and cellular repair in the lung to enable the development of novel targeted therapeutics for lung disease.

Tissue-engineered models have emerged as a technology to address this challenge and shown some success in drug identification and toxicology studies. For example, commercially available airway epithelial models such as EpAirway™ (MatTek Life Sciences) serve as convenient platforms, with air-liquid interface culture capabilities, for assessing the effect of chemical and physical stimuli [6–8]. Other examples include the Alveolus Lung-Chip and Airway Lung-Chip systems (Emulate Inc.), originally developed in the Ingber laboratory, which mimic the epithelial-endothelial interface of the airway and provide a more dynamic platform for testing new anti-inflammatory compounds in asthma [9] and new small molecule targets to decrease cancer-associated pulmonary edema [10]. More complex models have also been reported which involve self-assembly of heterogeneous progenitor cells into 3D structures, termed organoids [21]. These organoid models can recapitulate aspects of human lung development in terms of tissue structures and protein expression, and therefore present a promising opportunity for drug screening [11,12].

A challenge in developing such models is to recapitulate different airway and lung diseases. Finally, we discuss how tissue engineering and biophysical cues using biomaterials can be utilized during lung directed differentiation to mimic patterning cues present in development to augment current differentiation protocols.

2. Human embryology as a blueprint for lung directed differentiation

2.1. Overview of key developmental stages

Directed differentiation protocols have been designed to mimic in vivo human lung development [39]. Indeed, in vitro models of lung development have provided unique insight into human lung development [40]. As human lung development has been described at great length in earlier reviews, [41,42], we provide a brief overview as follows (schematically represented in Fig. 1). During early embryogenesis (at 14 days post fertilization), a process called gastrulation begins with the appearance of a structure called primitive streak, through which cells migrate to form the primary embryonic germ layers (definitive endoderm, mesoderm, and ectoderm) [43–45]. Definitive endoderm expands, thereby forming the primitive gut tube comprised of three endodermal regions: foregut, midgut, and hindgut [46,47]. This is when lung
development begins, at approximately four weeks into embryonic life, with the outgrowth of foregut endoderm [48,49] and continues through eight years of post-natal life [50]. There are five stages to lung development:

1. Embryonic (weeks 4–7): The future lung buds emerge from the ventral side of the primitive foregut endoderm into the surrounding mesenchyme and develop into embryonic lung buds with early trachea and bronchi [51–55].

2. Pseudoglandular (weeks 5–16): Branching of the airway continues, leading to formation of conducting and terminal bronchioles, while the proximal airway epithelium begins to develop [50,56].

3. Canalicular (weeks 16–25): Development of the respiratory, or gas-exchanging, airways is initiated, primitive alveoli form, and the future distal epithelium begins to thin as distal epithelial markers are expressed [41,57].

4. Saccular (weeks 24–40): Emergence of sac-shaped distal airways, which develop crests with muscle and elastin to create indentations. These distal airways extend to form alveoli by 29 weeks [58]. The developing epithelium and vasculature within the future alveolus continue to merge closer together to facilitate future gas exchange and further differentiation of alveolar epithelial cells (AEC I and II) takes place.

5. Alveolar periods (week 40–8 years): True alveoli are seen in week 36 and the majority of alveolarization takes place through saccule septation, a process by which the sac-shaped distal airways change their internal architecture and create thin walls intraluminally. Septation leads to an increase in surface area of the gas exchanging portion of the developing lung and prepares the fetus to breathe air during this stage [50,59].

2.2. Lung organogenesis: molecularly defining lung fate in the embryo

During the embryonic period, early lung is genetically defined by the expression of transcription factor NK2 Homeobox 1 (NKX2.1) and Sry-box 2 (SOX2) [60–62]. During human lung development, it has been found that the lung buds and branches given off during the pseudoglandular period are mostly SOX2+SOX9+ [17,62,63]. Both SOX2 and SOX9 are individual markers of the early proximal or distal lineage, respectively [60,64–66]. Over the course of the canalicular and saccular periods of development (weeks 16–40), these double positive populations downregulate one SOX protein and maintain expression of the other as these cells mature towards proximal or distal lineages [62]. The proximal airway (closer to the mouth) is comprised of a pseudostratified columnar epithelium that is responsible for the conducting airway function: debris and pathogen removal (ciliated cells), mucus production (goblet cells), prevention of airway inflammation (club cells), and humidification of air as it passes through to the distal lung compartment [67–69]. The squamous distal epithelium, composed of alveolar epithelial cells (AEC I and II), facilitates the respiratory function of the lung as air in the epithelial compartment is brought into close apposition to blood from the pulmonary vasculature; it also secretes surfactants, which play an immunologic role and decrease the surface tension present at the air-liquid interface, thereby preventing alveolar collapse [70]. In humans, a number of cell types are found in the proximal airway, each identified with specific markers (Table 1). This includes basal cells (tumor protein p63/P63, keratin/KRT5, nerve growth factor receptor/NGFR, integrin α6/ITGA6, integrin β4/ITGB4), ciliated cells (Forkhead BoxJ1/FOXJ1, acetylated tubulin/AcTUB), goblet cells (mucin SAC/MUC5AC, mucin 5B/MUC5B), club cells (club cell secretory protein/CCSP or SCGB1A1), and pulmonary neuroendocrine cells (PNECs; synaptophysin/SYP, chromogranin A/CHGA). On the other hand, homeodomain-only protein (HOPX) identifies the distal lung along with AEC I cells (T1α, podoplanin/PDPN, aquaporin 5/AQP5) while AEC II cells are recognized via surfactant protein B (SPC), pro-surfactant protein C (pro-SPC or SPC), and HT2-280 [71,72].

One mechanism by which lung epithelia begin to mature is based on chemokine secretions from the surrounding mesenchyme and the developing heart field which are well reviewed here [73]. Key players including fibroblast growth factors (FGFs) [64,74–80], WNTs [81–84], and bone morphogenetic proteins (BMPs) [85–90] are known to induce the differentiation of early lung progenitors in a controlled manner. For example, in mouse, it has been found that FGF10 plays a role in bud outgrowth [77] and drives lung progenitors towards a distal fate [78,79] through canonical WNT signaling [64,81,91]. Proximal epithelia develop because they are located further away from distally located FGF reservoirs in the mesenchyme, in a mechanism that appears dependent on concentration gradients [64]. BMP4 plays a key role in lung bud formation from foregut endoderm and establishment of both dorsoventral (back to front) and proximodistal (top to bottom) patterning in the nascent lung [88]. BMP4 is also present at high levels in distal bud tips and epithelia including AEC II cells [88,89], however, its inhibition promotes a proximal fate and, along with BMP2 inhibition, ciliated cell development [87,88,90].

2.3. Branching morphogenesis and other mechanical cues generated during lung development

While the cell fate of early proximal and distal lineages is directed through chemical signals, the lung epithelium itself undergoes marked
changes in architecture, a process known as branching morphogenesis [79,92]. From the simple tube of the anterior foregut endoderm to the complex tubular structure of the adult, a highly stereotyped mechanism of branching morphogenesis facilitates the outgrowth, division, placement, and structure of lung airway [42]. Branching morphogenesis of the lung is driven by three simple and iteratively used processes: domain branching, planar and orthogonal bifurcation [93]. The first form of branching is domain branching: along a primary branch, buds form in a linear and sequential fashion, from proximal to distal. The next form of branching is planar bifurcation, in which the tip of the forming tube bifurcates to create two new tips, which subsequently elongate and bifurcate again, creating four tips. The last process of branching is known as orthogonal bifurcation. In this process, the initial planar bifurcation is followed by a rotation around the planar axis which creates two new tips through bifurcation. A critical gene in this process, Sprouty, has been found to attenuate Erk1/2 signaling, thereby altering the orientation of cell division and future tube elongation [94]. Other critical genes and regulatory networks associated with FGF signaling also contribute to controlling the periodicity of the branched network [95]. Although elements such as domain specification, bifurcation, rotation and branch generation remain largely undetermined [93,96], new technologies involving high-resolution live imaging, tension sensing, and force-mapping are opening paths to further explore and explain the branching morphogenesis phenomenon [97].

The early structure of the lung gives rise to a striking architectural separation of future SOX2+ proximal lineages and SOX9+ distal lineages, at least in mice [98]. The diameter of tube generated during branching morphogenesis in the pseudoglandular and canalicular stages has a small degree of variance within each stage as measured from electron micrograph sources of fetal human tissue [99]. This suggests that the branching program is rigorous in its control of lung structure and that tubes themselves may have instructive potential on the fate of the lung progenitor. In utero, the fetal lung is a secretory organ that propels branching morphogenesis outwards into the developing thoracic cavity [100,101]. Lack of amniotic fluid in the developing lung alters the expression of distal epithelial markers and consequently results in the creation of smaller than normal lungs (pulmonary hypoplasia) [102], highlighting the importance of this mechanical pressure during lung development. In addition, cyclic strain is generated from fetal breathing movements (FBM) in utero that prime the airway for use after birth. FBM are detectable from the tenth week of pregnancy and begin as infrequent and erratic activity with long quiescent periods. As development continues, these quiescent periods decrease and sustained periods of fetal breathing occur. These breathing movements vary with the fetal sleep cycle and can be chemically tuned [57], and alter the volume of terminal sacs by around 5% [100,103], again highlighting the importance of mechanical signals influencing lung development. Finally, a novel FGF10/FGFR2-dependent tensional mechanism has been shown by which distal epithelial cells in the lung accumulate motor proteins at the apex of the cell, thereby becoming resistant to compression from increasing fluid pressure within the tube lumen. Cells under this tension are more likely to become AEC II cells, while those under compression become AEC I cells [102]. Interestingly, while the above examples highlight the importance of specific mechanical signals in the growth, development, and differentiation of the lung, PSC-directed differentiation protocols of the lung are primarily based on mimicking the sequential chemical changes that occur during lung development.

### 3. Directed differentiation of lung epithelia inspired by embryology

Early attempts to create lung epithelia from PSCs began in mouse and did not attempt to mimic the stepwise changes in chemical signaling that occur during development. Rather, groups focused on applying lung-like physical cues such as air-liquid interface [21,104]. These protocols, while successful in generating NKX2.1+ positive populations, also produced contaminating cells expressing pluripotency markers (OCT4, NANOG, SSEA4, TRA1-60, TRA1-81). These early attempts solidified that further optimization, particularly related to the chemical cues applied, was needed to reliably create lung progenitors from pluripotent sources without remnant pluripotent contaminating cells. More successful directed differentiation protocols were rationalized from the detailed understanding of the chemical changes during lung embryology. In this section, we describe in detail the different differentiation protocols currently available that evolved from this approach.

#### 3.1. Mouse embryonic stem cell derived lung epithelia

Although mouse models do not fully recapitulate human lung development, they have served as guides for earlier iterations of PSC directed differentiation protocols and have identified critical chemical cues for lung organogenesis. Broadly speaking, these protocols begin by driving stem cells towards a definitive endoderm fate (SOX17+ and FOXA2+; mimicking the pre-embryonic period of human lung development, weeks 0–2) through high doses of the nodal activating molecule, Activin A [105–108]. Foregut endoderm is then induced via transforming growth factor beta (TGFβ) inhibition either alone [24] or with BMP inhibition [22] for a short period; a process called anteriorization (as during the embryonic period of human lung development, weeks 0–6) through high doses of the nodal activating molecule, Activin A [105–108]. Foregut endoderm is then induced via transforming growth factor beta (TGFβ) inhibition either alone [24] or with BMP inhibition [22] for a short period; a process called anteriorization (as during the embryonic period of human lung development, weeks 0–6) through high doses of the nodal activating molecule, Activin A [105–108]. Foregut endoderm is then induced via transforming growth factor beta (TGFβ) inhibition either alone [24] or with BMP inhibition [22] for a short period; a process called anteriorization (as during the embryonic period of human lung development, weeks 0–6) through high doses of the nodal activating molecule, Activin A [105–108]. Foregut endoderm is then induced via transforming growth factor beta (TGFβ) inhibition either alone [24] or with BMP inhibition [22] for a short period; a process called anteriorization (as during the embryonic period of human lung development, weeks 0–6) through high doses of the nodal activating molecule, Activin A [105–108]. Foregut endoderm is then induced via transforming growth factor beta (TGFβ) inhibition either alone [24] or with BMP inhibition [22] for a short period; a process called anteriorization (as during the embryonic period of human lung development, weeks 0–6) through high doses of the nodal activating molecule, Activin A [105–108]. Foregut endoderm is then induced via transforming growth factor beta (TGFβ) inhibition either alone [24] or with BMP inhibition [22] for a short period; a process called anteriorization (as during the embryonic period of human lung development, weeks 0–6) through high doses of the nodal activating molecule, Activin A [105–108]. Foregut endoderm is then induced via transforming growth factor beta (TGFβ) inhibition either alone [24] or with BMP inhibition [22] for a short period; a process called anteriorization (as during the embryonic period of human lung development, weeks 0–6) through high doses of the nodal activating molecule, Activin A [105–108]. Foregut endoderm is then induced via transforming growth factor beta (TGFβ) inhibition either alone [24] or with BMP inhibition [22] for a short period; a process called anteriorization (as during the embryonic period of human lung development, weeks 0–6) through high doses of the nodal activating molecule, Activin A [105–108].
entire human conducting airway is comprised of a pseudostratified epithelium, even at diameters less than 0.5mm [112,113]. In contrast, conducting airways in mice only exhibit pseudostratified epithelium with accompanying submucosal glands and cartilage in the most proximal portion of the airway and transition directly into alveolar sacs [72]. This difference in histology affects the residing cell populations, as evidenced by the lack of basal cells in the lower portion of the proximal airways of mice [114,115]. Similarly, mouse models suggest that Sox2+ Sox9+ progenitors are quite rare, and their cell fate is ambiguous [116]. However, evidence from directed differentiation of human lung epithelia [26,63], which has been confirmed in vivo [17,26,62,63], reveals that Sox2+ Sox9+ progenitors are common in the developing lung buds and that branch tips of the pseudoglandular staged lung give rise to both proximal and distal epithelia [63]. Moreover, specific protein markers have been found to differ in both timing and location of expression between human and mouse models: pro-SPC in mouse is expressed early and throughout the developing mouse epithelium [117,118], while in human, pro-SPC is rarely detected early in development and is only robustly found later in distal epithelia [63]. These examples highlight that, while there are similarities, development and patterning of mouse and human lungs is different, and these differences require human models to be fully appreciated.

3.2. Human pluripotent stem cell-derived lung epithelia

Human PSC protocols have generally followed the same differentiation chronology as that of mouse directed differentiation, wherein definitive endoderm, anterior foregut endoderm, and NKX2.1+ lung progenitors are produced sequentially.

Different groups have adhered to their own methods of generating definitive endoderm, which primarily involves exposing PSCs to high concentrations of Activin A. Slight variations such as introducing WNT agonism (through WNT3a or CHIR99021) prior to [25] or alongside [11,30,32,33] Activin A, or additional exposure to BMP4 and FGF2 [23,25,29] during this stage exist across protocols for differentially inducing primitive streak and its anteriorization towards producing definitive endoderm. In addition, the use of embryoid bodies, which are limited by user experience and technique, has resulted in a wide range of production efficiencies for achieving this stage: from 45% CKI+ CCR4+ EPCAM+ cells [33] to >90% CKI+ CCR4+ cells [23,25]. Recent advances in commercial products have led to development of standardized 2D culture-based media (STEMdiff Definitive Endoderm Kit, STEMCELL Technologies) which allow reliable derivation of >95% of definitive endoderm [27,28,36,119].

Similarly, generation of both anterior foregut endoderm and ventralized lung progenitor populations has been subject to much investigation and modification. Earlier work suggested that Sox2+ FOXA2+ (92 ± 2% in their case) anterior foregut endoderm can only be induced by subjecting definitive endoderm to TGFβ and BMP inhibition [23]. Subsequent studies, however, attempted to anteriorize definitive endoderm to foregut endoderm through TGFβ inhibition alone (50-60% Sox2+), a combination of endogenous WNT, TGFβ and BMP inhibition (not quantified) [25], and via Sonic Hedgehog (SHH) and FGF2 signaling (78% FOXA2+ EPCAM+) [11]. A comparison of the latter two strategies demonstrated that SHH and FGF2 are insufficient in producing reliable NKX2.1+ lung progenitors [25], possibly because FGF2 is involved in promoting thyroid lineages [120]. In general, TGFβ and BMP inhibition [23] is the basis for currently applied endoderm anteriorization strategies [27,29–31,121].

Factors involved in early versions of ventralization in directed differentiation protocols included WNT3a, FGF7, FGF10, BMP4, epidermal growth factor (EGF), and RA have now been reduced based on elimination studies [25,120]. As such, CHIR99021 (ChIR; WNT agonist), BMP4, and RA are necessary and sufficient for producing lung progenitors from anterior foregut endoderm derived from both mouse and human PSCs [25,30,32,119]. Despite finding that FGF7 and FGF10 are non-essential for inducing NKX2.1 expression, they continue to be used for ventralization in some protocols [29]. Although each protocol differs in terms of the duration of each phase, NKX2.1+ lung progenitors are generally achieved by 15 days, with the exception of a study by de Carvalho et al., in which they maintained their cultures for an additional 10 days in FGF7, FGF10, and CHIR99021 to attain 90-98% NKX2.1+ FOXA2+ lung progenitors. In all cases, these lung progenitors are then either sorted or directly guided towards proximal or distal progeny in 2D or 3D culture systems. Ideally, products of directed differentiation protocols should mimic the cell proportions present in human airways and lungs (Table 1), however current protocols have not progressed that far. While these protocols continue to be refined, the percentage of select cell populations generated from these protocols have been summarized in Table 2.

3.3. Creation of human proximal lung epithelia

Protocols to create proximal lung epithelia have focused on the production of the four major cell types present: ciliated, goblet, club, and basal cells (see Table 1 for a summary of markers for each cell type). Motivation for creating proximal epithelia in the field has primarily been to develop patient-specific cystic fibrosis (CF) models [11,24,27] and/or to produce epithelia with multi-ciliated cell populations for protocol validation [30,33]. A shift towards human PSC-derived CF models has been critical as mouse models do not accurately represent CF disease progression and phenotypes seen in humans [128–130]. As such, the first evidence of human PSC proximalization using CF patient-derived PSCs was shown by Mou et al., who exposed anterior foregut endoderm to BMP4, GSK3iXV (WNT agonist), FGF2, and RA-supplemented B27 to generate 10-30% NKX2.1+ cells by Day 12. Although contaminating neuroectodermal and distal lung NKX2.1+ Sox9+ cells were present, day 12 populations included proximal NKX2.1+ Sox2+ progenitors. Subcutaneous implantation of this population in immunodeficient mice for 30 days resulted in emergence of NKX2.1+ P63+ cells, however no mature epithelial markers for ciliated, goblet, and club cells were found.

Wong et al. employed a longer, 2D differentiation approach to produce mature proximal airway epithelia in vitro. Through a process they called “proximal specification”, they generated day 15 lung progenitors via low levels of BMP4 (mimicking signaling gradients in the airway), FGF7, and FGF10 which began expressing proximal genes. Further culture with FGF7, FGF10, and FGF18 resulted in upregulated gene expression of KRT5, P63, FOXJ1, SOX17, cystic fibrosis transmembrane conductance regulator (CFTR), and SCGB1A1 to a lesser extent, along with low levels of distal SOX9 and SP by day 19. Protein expression amounted to 32.1% NKX2.1+ 32.2% panKRT+, 24.8% FOXJ1+ with 14.6% co-expressing CFTR, and 19% panKRT, 53.9% P63+, 36% FOXJ1+ cells. These cells were subsequently matured in air-liquid interface (ALI) culture for 5 weeks (1 week of submerged culture with FGF18, followed by 4 weeks of ALI culture) to generate 42.8% CFTR+ panKRT+, 24.8% FOXJ1+ with 14.6% co-expressing CFTR, and 19% CFTR+ LHS28+ cells. The resulting epithelium ranged from being squamous to cuboidal with sparse pseudostratified regions, implying that this protocol lacked specific maturation cues. Contaminating thyroid (thyroglobulin and PAX9), liver (HNF4 and AFP), and pancreatic (PDX1) lineages were detected through quantitative PCR, while percentages of goblet, club, and basal cell populations (barring gene expression analysis) were not evaluated.

A similar 2D culture approach was employed by Firth et al. to generate proximal lung progenitors which were subsequently matured into multi-ciliated epithelia. They optimized lower concentrations of BMP4 required during the ventralization phase (day 9–17) to promote higher gene expression of FOXA2, SOX17, MUC1, MUC5AC, KRT5, P63, CFTR, and NGFR by the end of their differentiation protocol (day 45). Proximal lung progenitor efficiency was not reported at day 17, however earlier assessments at day 9 and 10 identified contaminating thyroid and neuroectodermal cells, with approximately 28% of total cells portraying...
mesenchymal characteristics based on CD90 protein expression. This is plausible given the low definitive endoderm efficiency (45% CKIT+CXCR4+ EPCAM+). Regardless, day 17 cells underwent further maturation in ALI culture for 28 days, resulting in a cuboidal epithelial layer with an underlying mesenchymal layer; only 45-50% of these cells were EPCAM+. The resulting epithelium was devoid of multi-ciliated cells but comprised 27.3±3.7% CCSP+ club cells, 1–2% MUC5AC+ goblet cells, and some functional CFTR+ cells. Multi-ciliated cells were only achieved through NOTCH inhibition (DAPT) during ALI culture.
Konishi et al. were the first to introduce a 3D culture-based protocol to generate proximal epithelia from human PSCs. Post-ventralization, they sorted day 14 cells based on expression of carboxypeptidase M (CPM), an AEC I-specific marker which was used as a surrogate marker for identifying lung progenitors based on the finding that 92% of CPM+ cells reliably co-expressed NKX2.1 [32]. Inspired by the formation of tracheospheres from primary tracheal epithelia in earlier studies [113,132], they embedded CPM+ cells in Matrigel and cultured them for 14 days in the presence of FGF10 and CHIR. Resulting spheroids were NKX2.1+ SOX2+ but devoid of multi-ciliated cells, therefore an additional 14 days of culture in PneumacultTM−ALI media (Stem Cell Technologies) was added for further maturation. Day 42 spheroids depicted AEC IIB and MUC5AC+ cells in the lumen with smaller numbers of KRT5+ SCGB1A1 +, CHGA + and SVP+ cells; no AQP5+ and SPC+ cells were detected. NOTCH inhibition between day 28 and 42 resulted in significant upregulation of FOXJ1, SVP, and CHGA genes. Meanwhile, SCGB1A1 was undetected and MUC5AC expression was 100-fold less than levels found in adult trachea. Spheroid cells were dissociated and cultured under ALI conditions (with continued NOTCH inhibition) for 14 days to generate multi-ciliated cells (72±6.6% to 87.06±0.43% FOXJ1+ across cell lines) with ciliary beating frequency similar to that of primary bronchial epithelial cells; other proximal markers were not evaluated at this point. Overall, the use of 3D culture resulted in trilineage (ciliated, goblet, and club cell) differentiation of proximal airway epithelia, which was not observed in their 2D ALI cultures.

McCauley et al. developed a protocol [119] based on their findings that withdrawal of WNT signaling post lung progenitor specification at day 15 promotes a proximal fate (upregulated SOX2, P63, SCGB2A2) both in 2D and 3D culture [27]. They sorted day 15 lung progenitors, through an NKX2.1-GFP reporter or by selecting a CD47− ciliated cell marker which was used as a surrogate marker for obtaining mature airway progenitors, such as NGFR+ cells, comes at the cost of elongated protocol lengths, heterogenous maturation levels of resulting populations, and missed opportunities for understanding why these populations do not result in a histologically appropriate epithelium [29].

It is apparent that the timing of signaling molecule delivery as well as the competence of subjected cell populations to respond to a given signaling molecule are of extreme importance. The spatiotemporal dynamics of cell signaling are non-linear, are more complex in vivo, and are not fully appreciated in the latter stages of current directed differentiation protocols. This may explain the incongruence amongst different protocols, primarily those assessing the effects of GSK3β- and WNT signaling [27,29,30], all of which targeted populations at non-comparable protocol stages. Therefore, a deeper analysis is required to appropriately explain and mimic these dynamics in vitro. Furthermore, recreating these spatiotemporal signaling patterns during directed differentiation protocols may potentially require repurposing molecular delivery tools from other fields such as drug delivery and tissue engineering [134–136].

Interestingly, most existing protocols have been skewed towards generating multi-ciliated cells at the expense of goblet and club cells by subjecting airway progenitors to NOTCH inhibition, which is known to decrease goblet cell populations [137,138]. Goblet cells, in addition to club cells, have recently been discovered as a source for generating multi-ciliated cells in primary airway epithelia [139]. Club cells play a key role in epithelial injury, wherein they de-differentiate into basal cells in the absence of basal cells such that they can give rise to ciliated and club cell populations to repair a denuded epithelium [140]. Therefore, in the future it will be critical to identify protocols to create PSC-derived cultures containing these cell types, and not just multi-ciliated cells, in order to fully capture the dynamics of airway injury and repair for drug screening. Overall, based on current progress, the Konishi et al. and McCauley et al. protocols are considered the most relevant for generating functional airway epithelia.

### 3.5. Creation of human distal lung epithelia

The alveolar space in the distal lung is comprised of two epithelial cell types: AEC I and AEC II (see Table 1 for specific markers of each cell type). Distal lung directed differentiation protocols have progressed drastically in the last 5 years, with most of them employing a 3D organoid culture phase for maturation to acquire these progenies. Earlier work by Huang et al. focused on efficiently inducing lung progenitors in 2D culture by streamlining the ventralization phase of the
protocol. Day 15 lung progenitors (86.4±1.7% FOXA2+ NKX2.1+) were multipotent, leading to proximal and distal lineages both in vitro and in vivo, albeit with contaminating non-lung and mesenchymal cells, respectively. Aggregate culture of these cells in the presence of CHIR, FG7, FG10, and DCI for 10 days resulted in proximal NKX2.1+ SOX2+ and NKX2.1+ P63+ cells by day 25. Interestingly, subsequent culture in the presence of DCI alone resulted in distal SPB+ cells (50%) by day 48, of which 52% could metabolize surfactant, a characteristic of AEC II cells.

A study by Gotoh et al. demonstrated that 3D co-culture with fetal lung fibroblasts significantly augmented distal epithelial maturation. In this investigation, day 14 lung progenitors (57–77% FOXA2+ NKX2.1+) were sorted based on CPM+ expression and cultured in 2D in the presence of FG7 and DCI for 14 days. Resulting cells expressed SPB but not SPC (indicating a lack of AEC II cells), which remained unchanged even when CPM+ cells were extracted on day 23 instead of day 14; this implies that prolonged 2D culture does not promote AEC maturity, necessitating transition into a 3D culture phase. As such, when day 14 CPM+ cells were embedded with fetal lung fibroblasts in Matrigel for 3D culture, spheroids expressing distal epithelial markers formed by day 24, of which 3.82±0.5% of extracted cells were SPC+. Therefore, 3D culture of lung progenitors rapidly induced a distal population in contrast to 2D culture of lung progenitor aggregates, by Huang et al., which required prolonged culture in different medium conditions to achieve distal maturation.

Furthering this work, Yamamoto et al. developed a modified protocol, wherein day 14 lung progenitors were "preconditioned" for 7 days in CHIR, FG7, FG10, and DAPT. This additional culture phase enhanced the lung progenitor population, as compared to the older Gotoh et al. protocol, by resulting in 85.2±5.6% NKX2.1+ by day 21 [12]. This preconditioning also positively influenced the 3D co-culture phase, wherein day 35 alveolar organoids expressed SPC (51.2±1.2%), SPB and PDPN (AEC I marker) and depicted lamellar-body-like organelles reminiscent of AEC II cells. With an ability to self-renew across multiple passages, SPC+ populations gained maturity, while SPC-PDPN+AQP5+ cells, respectively. Aggregate culture of these cells in the presence of CHIR, FGF7, FGF10, and DCI for 10 days resulted in proximal MUC5AC+, HOPX+, SPB+, and SPC+ cells after 120 days. However, when seeded into naphthalene-injured mouse airways, they gave rise to ActUB+ and MUC5AC+ cells. In general, this protocol diverged to produced lung organoids with heterogeneous populations of either predominantly proximal or distal epithelia, which required prolonged culture or in vivo implantation for maturation (limited in this case). A key aspect of the "human lung" organoids was their inclusion of a mesenchymal population to study epithelial-mesenchymal crosstalk during lung development.

Chen and colleagues described a similar approach wherein 3D branching projections of anterior foregut endoderm (89.07±3.36% FOXA2+ and 92.08±1.88% EPCAM+) were cultured in CHIR, BMP4, FG7 and FG10 for 25 days [26]. Resulting lung bud organoids (LBOs) were NKX2.1+ (51.26±4.37%), were surrounded by mesodermal cells, and did not express any mature lung or airway markers other than P63 (18.59±1.49%). When embedded in Matrigel and grown in the same media conditions, they rapidly branched, forming tubular structures. Day 70 LBOs expressed FOXA2 (95.17±1.54%), NKX2.1 (74.97±4.37%), and SOX9 (92.42±3.81%), along with MUC1, SP, and SPB; proximal markers were absent, except for minimal MUC5AC+ cells. Further growth of LBOs up to 170 days revealed sacculle-like structures, depicting NKX2.1+ (84.86±5.21%), SOX9+ (76.75±6.88%), and SOX2+ (23.78±5.21%) cells. The presence of AEC II-like cells with lamellar bodies was evident, which were able to process surfactant. Meanwhile, except for HOPX, markers of AEC I were not present and proximal markers were limited to MUC5AC and SCGB3A2. Other mature airway and lung markers did not appear until after 7 months of in vivo implantation, with those of AEC I cells still absent. This work further confirmed that lung organoids produce immature, heterogeneous populations of proximal and distal epithelia which require extended 3D culture or in vivo implantation to promote maturation (incomplete in this case).

3.6. Comparisons of distal lung directed differentiation protocols

All described directed differentiation protocols for distal epithelia utilized a 3D culture approach in some format, however only those that established lung specification in 2D culture prior to a 3D transition demonstrated promising results. These protocols do not completely depend on spontaneous organoid assembly, are highly responsive to fine tuning with morphogens, and can therefore provide better insight into the cellular responses in lung development to generate therapeutic strategies accordingly.

Although the "human lung" organoid and "lung bud progenitor" organoid-based protocols may be useful for studying complex cellular interactions during disease progression or repair, they are currently under-developed, do not recapitulate proximodistal patterning adequately in vitro, and are limited in terms of protocol lengths and incomplete set of relevant cell types produced. Especially in the Miller et al. study, both organoid models stochastically led to production of proximal cells ex vivo or in vivo, thereby diluting the need for developing two approaches for generating either proximal ("human lung" organoids) or distal ("lung bud progenitor" organoids) epithelia.
potential factor contributing to complications in the Miller protocol was their method for determining signaling cues: they eliminated FGFI7, FGFI0, BMP4, CHIR, and RA one at a time while culturing bud tips isolated from mice [62]. In contrast to analysis done using mouse and human PSCs during directed differentiation in previous studies [25,120], they found that removal of BMP4 enhanced SOX9 gene expression. This was perhaps due to the differences in complexity of the afore-mentioned models studied, especially in relation to the mouse lung buds being equipped with a heterogeneous, potentially mesenchymal, cell population and its associated paracrine signaling that may compensate for presence or absence of growth factors. Therefore, it is important to elucidate essential cues in simpler models prior to progressing towards 3D models that involve complex signaling patterns.

The Yamamoto et al. and Jacob et al. protocols presented the strongest results for generating AEC II-like cells that expanded rapidly and matured with serial passaging. While the surfactant processing ability of differentiated AEC II cells was only exhibited by Jacob and co-authors, both protocols generated alveolar organoids (simple spheroids containing alveolar cells) that can mimic functional properties of AEC II cells by responding appropriately to pharmaceutical and molecular stimuli.

The presence of mature AEC I cell markers was only seen in the Yamamoto protocol, with a major difference being its dependence on a mesenchymal feeder population. Although feeder-free generation of alveolar organoids was possible, only 23.1% ± 1.4% of collected cells expressed SPC. Evidently, the mesenchymal niche provided key matura-
sion signals that require further investigation for advancing feeder-free protocols. While there is general consensus regarding the involvement of WNT signaling for distal patterning, RNA sequencing–associated gene ontology, in this study, suggested the involvement of Hippo and MAPK pathways as well. Furthermore, the employment of NOTCH inhibition during the “preconditioning” phase may have played a role in promoting both AEC I and AEC II populations, and therefore the aforementioned signaling pathways need to be collectively assessed. Inspiration can be sought from a recent study which described a computational modeling approach, based on single cell RNA sequencing, that predicted the optimal time point for CHIR withdrawal for maintaining a NKX2.1+SPC+ approach, based on single cell RNA sequencing, that predicted the optimal time point for CHIR withdrawal for maintaining a NKX2.1+SPC+ signaling.

In the context of airway models, ALI culture has been the gold-standard for studying primary airway epithelia derived from CF patients. In concordance with this method, Wong et al. attempted to differentiate CF patient-derived PSCs, albeit with great heterogeneity with regards to CFTR expression and activity across PSC lines and trials. Regardless, they used the models to explore mechanisms of drug action and provided evidence of CFTR activity in response to cyclic-AMP agonism through forskolin, isobutylmethyl xanthine, and genistein in select PSC lines. Additionally, upon treatment of a CF patient-derived PSC line with an analog of VX-809 (an FDA-approved drug for CF), partial restoration of CFTR localization in the plasma membrane was observed [11]. Similarly, Firth et al. demonstrated characteristic apical expression of CFTR in their differentiated PSC-derived ALI cultures along with appropriate CFTR activity upon exposure to forskolin and/or an CFTR inhibitor [33].

Organoid-based culture systems have also been used for drug exploration in CF. Based on a previously developed CFTR assay [151], McCauley et al. used an organoid-based approach to assess the application of PSC-derived airway epithelial cultures for CF disease modeling. Their protocol was able to reproducibly generate airway spheroids from normal PSC lines that were responsive to forskolin. In contrast, CF patient-derived spheroids were non-responsive, thereby replicating the expected CF disease phenotype. This effect was confirmed to be CFTR-dependent as CFTR-corrected PSC spheroids behaved similarly to normal spheroids [155]. While CFTR function can therefore be modeled in both 2D and 3D culture systems, the function of mucociliary clearance may only be appropriately modeled using the ALI culture system. Assessment of this specific airway function, of course, is dependent on the ability of the chosen directed differentiation protocol to produce all the relevant cell types involved (ciliated and goblet cells) using ALI culture.

Distal lung organoid models have been applied to drug development in a broader range of diseases. For example, pulmonary fibrosis is a fatal disease which involves progressive and irreversible alveolar fibrosis, leading to lung failure [156,157]. A handful of reports have emerged using human PSC-derived epithelium to model pulmonary fibrosis. In one study, Chen et al. developed LBOs (complex organoids with branching structures) to model pulmonary fibrosis associated with an autosomal recessive disorder called Hermansky-Pudlak syndrome (HPS) by deleting the HPS1 gene in their PSC line [158,159]. These LBOs demonstrated diminished branching, contained a high proportion of proliferative mesenchymal cells, and showed increased deposition of ECM proteins (collagen I, collagen III, and fibronectin), consistent with a fibrotic state [26]. Similarly, using the Yamamoto et al. protocol, Korogi and colleagues modeled pulmonary fibrosis in alveolar organoids (simple spheroids containing alveolar cells) with an HPS2 mutation [160] and reported deviant size and distribution of lysosome-like organelles of lamellar bodies, as well as abnormal lamellar body enlargement and secretion. At the same time, a wider range of PSC lines was generated by Strikoudis et al., who mutated individual HPS genes (HPS1, HPS2, HPS4, and HPS8) and comprehensively corroborated the LBO fibrotic phenotype of each mutation with its clinical presentation [161]. This study implicated interleukin 11 (IL-11) in inducing fibrotic phenotypes (increased mesenchymal populations and ECM deposition), which was validated by IL-11 expression pulmonary fibrosis and HPS clinical patient samples. Deletion of IL-11 in fibrotic HPS4⁻⁴ LBOs rescued the wildtype phenotype, thereby identifying IL-11 as a therapeutic target for lung fibrotic disease [161]. In a concurrent study, Ng et al. confirmed the pro-fibrotic role of IL-11 and developed an IL-11 antibody which reversed late-stage lung fibrosis by significantly decreasing ECM deposition in an animal model [162]. Evaluation of this IL-11 antibody using PSC-derived lung organoid models can provide better insight into their applicability for human disease.

Distal lung organoids have also been applied to model respiratory viral infections caused by respiratory syncytial virus (RSV), human parainfluenza virus type 3 (HPIV3) [163,164], as well as the measles
3.8. Limitations of current directed differentiation protocols

While there has been great progress in the establishment and maturation of lung epithelia from PSC populations, a number of limitations have emerged that will require optimization and augmentation of current protocols to create better developmental and disease models, and specific cell populations:

a. Lack of control over which populations are produced - understanding or recapitulation of signaling pathways beyond proximodistal patterning is currently limited, as the ratio of AEC II versus AEC I cells; or club cells versus goblet cells cannot be reliably predicted. Furthermore, while development of reporter lines and identification of surface markers for sorting have [32,34] helped the advancement of distal lung protocols (for AEC II cells specifically) in the last few years [28,36], such techniques are limited in current proximal airway protocols [168].

b. Lack of maturity and characterization - there is inconsistent maturation of resulting cell populations within and across available protocols with results ranging from first trimester to adulthood-like cells [25,26,28,29,35,37].

c. Variability in cell production - the same protocol does not consistently and reliably produce the same types of cells in similar ratios. Further, inherent differences amongst PSC lines limits development of standardized protocols.

d. Cell are not correctly patterned and organized - with respect to lung epithelial directed differentiation, proximal and distal populations are interspersed amongst each other and do not mimic the patterning seen in vivo. In organoid cultures, while separate populations of both proximal and distal epithelia have been created, these organoids do not accurately reproduce the patterning of the in vivo lung.

e. Contamination from other organ cell types - lung protocols suffer from contamination of thyroid, gastrointestinal, and neural tissues. Protocols that did not employ a lung progenitor sorting step were often contaminated with thyroid, pancreas and liver contaminants. While thyroid contamination was decreased via streaming of signaling cues, specifically elimination of FGF2 [120], liver contaminants (12.1%) continue to remain despite NXX2.1 cell sorting [37]. Beyond the usual suspects, Huang et al. found undefined contaminants that cannot be categorized as any specific tissue type [25]. This issue is partially due to the stochastic nature of PSC-based directed differentiation and a lack of early definitive lung markers for selection.

f. Minimal recapitulation of physiological conditions - current alveolar organoids are beginning to represent relevant cell types, however, they are embedded in Matrigel and grown in subcultured state. They, therefore, fail to provide an ALI environment, which is critical to in vivo functionality. As in proximal protocols, cell products often need to be dissociated and regrown on transwells, which allow ALI culture by exposing cells to media basally and air apically, for further assessment.

g. High cost - associated with the growth factor and small molecules required for chemically directed stem cell differentiation, and the expertise required to reliably create lung epithelia with these protocols. The use of commercially available 2D based endoderm differentiation kits have greatly decreased the level of expertise required to achieve this early stage of differentiation.

Based on current advances, we have made recommendations for directed differentiation protocols that generate proximal and distal epithelia in Fig. 2.

4. Opportunities to exploit mechanical cues for improving directed differentiation protocols in the future

The timing of chemical signals present during lung development has been well mimicked in current differentiation protocols. The lung, however, develops in response to chemical signals within a highly dynamic mechanical environment of cyclic strain, pressure and a complex branching tubular architecture [169]. Indeed, it is well established that mechanical cues, specifi cally elimination of FGF2 [120], liver contaminants (12.1%) continue to remain despite NXX2.1 cell sorting [37]. Beyond the usual suspects, Huang et al. found undefined contaminants that cannot be categorized as any specific tissue type [25]. This issue is partially due to the stochastic nature of PSC-based directed differentiation and a lack of early definitive lung markers for selection.

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above limitations [170,185–187]. Organoid cultures, for instance, allow self-assembly of tissue-like structures and enable further maturation of proximal and distal epithelia [12,26,28,119]. In this section of the review, we highlight tools from tissue engineering that have been used to manipulate tissue structure and the resulting biophysical signals experienced during stem cell differentiation in both 2D and 3D (Fig. 3 and Table 3). Furthermore, we explore the opportunity to utilize such tools to engineer mechanical signaling as a strategy to augment and refine existing chemical differentiation protocols. Note, we do not consider the use of simply culturing differentiating cells on substrates or in 3D hydrogels with variable mechanical stiffness but point the reader to excellent reviews on this subject [188,189].

4.1. Micropatterning in 2D

Micropattering of the culture surface is one strategy that has been used to manipulate the physical organization of 2D stem cell colonies and the resulting mechanical environment individual cells experience within the cell sheet. Micropatterning entails deposition of extracellular matrix (ECM) protein islands with highly specific shapes and sizes on non-adhesive surfaces, via micro-contact printing (μCP) [190–194] or soft lithography [195–197]. Individual cells or cell populations are thereby restricted to the area of the surface where the adhesive protein islands are present. The shape of the island, therefore, geometrically constrains the shape of single and groups of cells in 2D, which determines the pattern of adhesive attachments between the cells and the underlying surface, and hence the mechanical state of the cells [198].

Chen and colleagues presented early evidence that geometric constraint affects cell fate by demonstrating that cell growth and apoptosis are directly related to ECM pattern size through its control of cell spreading [194,199]. Not only is the size of the ECM pattern important, but also the shape it holds, specifically in relation to its aspect ratio and subcellular curvature. As shown by Kilian et al., despite the presence of equipotential differentiation signals, osteogenic differentiation of heterogeneous mesenchymal stem cells (MSCs) was promoted by increasing the ECM pattern aspect ratio at the single cell level [181]. Further, in a pentagon-shaped design, the curvature of the lines connecting vertices was varied from convex to concave and was shown to guide cell differentiation choice from adipogenic to osteogenic, respectively, by manipulating subcellular myosin II polarization, tension, and integrin localization. Evidently, such micropatterned islands not only exert control over the growth and survival of cells, but also enable manipulation of cell differentiation through changes in intracellular tension. For example, by probing tension at the cell-boundary interface through confined 2D geometries, Lee et al. found that patterned melanoma cancer cells occupying larger arc angles, or smaller magnitudes of curvature, expressed higher cancer stem cell markers [195]. Furthermore, these markers were preferentially found at the edge of the micropattern, a consequence of perimeter tension acting through the p38-MAP kinase pathway. Interestingly, once removed from the defined geometric environment, the cells lost their activated cancer phenotype. These tools could provide an excellent platform for subtly manipulating self-organization of PSC populations to understand and influence their differentiation.

While not in the context of augmenting directed differentiation specifically, the use of micropatterning has been applied to explore pluripotency and fate choice during early development. Based on a previous finding that human PSC differentiation is dependent on colony size [200], Nazareth et al. developed a high throughput μCP platform, with optimized colony size, and probed early cell fates (pluripotent, neuroectoderm, primitive streak, and extraembryonic) in response to different media conditions and developmental factors [190]. Such micropatterned surfaces were also used by Warmflash and colleagues to investigate embryonic germ layer patterning of human PSCs [201]. They found that BMP4 treatment results in spatially segregated regions that delineate ectoderm, primitive streak and trophoblast-like tissues within the patterned colonies. This pattern was shown to be mediated by the colony edge, as opposed to colony size, with BMP4 signalling progressively being restricted to the edge of the colony. These findings were further confirmed by Tewary et al., who explained this effect to

![Fig. 2. Organization of airway and lung epithelium with recommended directed differentiation protocols to generate specific populations.](https://example.com/figure2.png)
be caused by the emergence of a phosphorylated SMAD1 gradient. The establishment of this gradient is initially controlled via inhibition by Noggin, followed by a restriction of BMP4 sensitivity to the edge of the colony due to re-localization of BMP receptors throughout the rest of the colony [202]. Such micropatterned platforms have also been used to map fate choices made during mouse [203] and human [204] gastrulation events, and therefore are a powerful tool for elucidating fate choice during lung development. It is not clear however, how the mechanical state of the cells within these micropatterned islands impacts chemical cue secretion, and hence the local gradients of chemical signals that result in patterning of cell fate in these studies.

4.2. Substrate texture

Another method to manipulate the physical organization and biophysical cues in a differentiating cell sheet is through substrate texture [205]. Cellular behaviours including proliferation, adhesion, and differentiation have been linked to underlying substrate topographical cues [205–210]. These cues are recognized by cellular protrusions called filopodia and lamellipodia, through integrin receptors and focal adhesions [211–214], which in turn dynamically modify their shape and exert protrusive forces [215–219]. In this section, we will provide key examples of substrate topographies, as well as grooves, based on their relevance for lung epithelial organization. These topographies can be microfabricated using various techniques, such as etching, photolithography, soft lithography, and stereolithography, that are scalable, precise, and provide high fidelity [220].

4.2.1. Stem cell behaviour on substrate topographies

Stem cell fate choices have been shown to respond to topographical features [221,222]. For example, Viswanathan et al. assessed the ability of different topographies to mimic sinusoidal epidermal undulation to induce in vivo-like biophysical cues. Their screened undulating topography created β1 integrin patterning that is reminiscent of the human dermis and more differentiated cells were found localized to the troughs of their pattern in a highly repeatable fashion. These findings suggest that replicating the physical organization of the dermal microenvironment promotes tissue-level organization and alters the positioning of the epidermal stem cells towards the in vivo state [223]. This group further applied a screening platform called TopoChip [224], that incorporated features of varying sizes, roundedness, and distribution density, to assess human PSC proliferation and pluripotency in the absence of ECM coatings [225]. Topographies that ranked high in the screen not only supported PSC proliferation, but also allowed maintenance of
OCT4 and SOX2–expressing pluripotent colonies. In conjunction with computational modeling, this platform was able to predict topographical features conducive to maintaining PSC pluripotency, thus demonstrating great promise for exploring how to use tissue organization patterning to control cell fate. Application of this platform for probing keratinocyte differentiation revealed that differentiation is linked to changes in cell morphology, which is influenced by substrate topographies, and mediated by Rho kinase activity [226].

Based on these studies, it is evident that application of biophysical cues alone can impact differentiation. High-throughput technologies like TopoChip could be used in the future to understand and mimic cell fate of proximal or distal lung epithelia. Towards this goal of recapitulating the physiological morphology of distal lung alveoli, we recently developed larger-sized topographical features, specifically hemispherical cavities, that enabled seeding of multiple cells and further allowed maintenance of primary AEC I and AEC II cells [227]. The ability of this platform to promote PSC differentiation towards these cell types has yet to be explored, however.

4.2.1.1. Stem cell behaviour on grooves. Grooved topographical cues specifically, through their ability to modulate cytoskeletal alignment and cellular shape, have also demonstrated great promise in guiding cell fate [228,229]. In the context of neural differentiation, Ankam and colleagues generated a multi-architectural chip (MARC), that incorporated a range of isotropic and anisotropic topographies at both micro and nano scales, to differentiate PSCs towards neural progeny without the use of embryoid bodies. Anisotropic nanoscale grooves (250 nm) promoted neuronal differentiation with cell alignment and elongation, and isotropic pillars enhanced astrocyte differentiation with cellular extension [228]. In the context of bone differentiation, various groups have utilized topographical cues to direct osteogenic and adipogenic differentiation, Mediated by colony boundary, not size, human PSCs organize into radially segregated germ layer regions in response of BMP4. Median by colony boundary, not size, human PSCs organize into radially segregated germ layer regions in response of BMP4. 

Table 3

| Physical Manipulation Technique | Cellularity Level | Dimension | Physical Cue Modulated | Effect of Physical Cue Modulation | Throughput | Reference |
|--------------------------------|-------------------|-----------|------------------------|-----------------------------------|------------|-----------|
| Micropatterning                | Single Cells      | 2D        | Cell size              | Endothelial cell apoptosis is inversely related to cell spreading area. | High*      | Chen et al. 1997, Killian et al. 2010 |
|                                |                   |           | Cell shape             | Constraining MSC culture area within pentagons with concave lines or in shapes with high aspect ratios promotes osteogenic differentiation due to increased actomyosin contractility. | High*      | Lee et al. 2017 |
| Cell Colonies                  |                   | 2D        | Perimeter topology     | Melanoma cells occupying larger arc angles at the tumour periphery demonstrate greater tumorigenicity due to hypoxia-induced mechanotransduction. | High*      | Lee et al. 2017 |
|                                |                   |           | Colony size            | Colonies of larger diameters allow maintenance of human PSC pluripotency and can further be used as a platform to study early PSC fate in response to specific chemical cues. | High       | Nazareth et al. 2013 |
| Organoids                      | Multiple cells    | 3D        | Organoid shape (surface area: volume ratio) using microfibres Cell position via micromoulding | Increased patterning and organization of cerebral organoids grown around microfibres lead to mature neuronal features. | Low        | Nelson et al. 2006, 2008 Mori et al. 2009 Gjorevski and Nelson 2010 Soles et al. 2020 Viswanathan et al. 2016 |
| Substrate Topography           |                   | 2D        | Cell curvature through micromoulding Cell position in response to undulation | Tubular diameters exert differential cellular tension and thereby dictate cell fate of bipotent progenitors. Epidermis-inspired topography induces human keratinocytes to pattern into distinct regions of progenitor, differentiating, and proliferating cells, as seen in vivo. | Low-Medium | Reimer et al. 2016 |
|                                | Single Cells/ Cell Patches | 2D | Cell shape via “TopoChip” topographies | Smaller feature size is most essential for maintaining human PSC pluripotency. Geometry of hollow tubules reliably predict branching patterns of mammary epithelia through mechanical stress gradients, and further reveal mechanisms of cellular rearrangement. | Low        | Unadkat et al. 2011 |
|                                | Single Cells/ Cell Clusters/ Monolayer | 2D | Cell shape via grooves (nano to micro scale) | Due to actomyosin contractility, neuronal differentiation is promoted on anisotropic nanoscale grooves in a highly expedited manner compared to standard protocols. | High       | Reimer et al. 2016 |
|                                | Single Cells/ Cell Patches | 2D | Cell shape via grooves (micro scale) | Associated with cell morphology and focal adhesion formation, wider groove ridges promote adipogenic differentiation, while thinner ridges promote osteogenic differentiation of MSCs. | High*      | Abagnale et al. 2015 |
|                                | Single Cells/ Cell Colonies | 2D | Colony shape via grooves (nano scale) | Linked to differential YAP and TAZ activity, grooves elongate human PSC colonies which maintain pluripotency and are highly responsive to morphogenic differentiation cues. | High*      | Abagnale et al. 2017 |
osteogenic differentiation with calcium phosphate precipitation [233]. Interestingly, groove width had minimal impact on favouring differentiation towards either lineage. The ridge-mediated differentiation effect was associated with cell morphology and focal adhesion formation wherein wider ridges resulted in rounder cell morphology with many large focal adhesions, as compared to thinner ridges leading to cellular elongation with fewer and smaller sized focal adhesions. Nano-scale groove topography was also shown to alter the spatial configuration of PSC colonies by elongating them, consequently affecting cell fate [234]. This effect was particularly potent at the colony edges and controlled by separate and differential localization of YAP and TAZ during PSC maintenance and differentiation.

In general, grooved topography can provide great insights into PSC fate choice and potentially expedite differentiation protocols. The MARC platform illustrates the importance of combining biophysical and biochemical cues, especially as exposure to topography induced a higher yield of functional and mature progeny within a short time frame as compared to flat substrates or standard directed differentiation protocols [230,235]. This is of extreme importance as current directed differentiation protocols for airway and lung epithelia require longer than 60 days of culture to achieve functional cell types [12,27,28,30,36,119]. Although the inclusion of topography has not been investigated for promoting lung differentiation, we have explored grooved substrates for aligning airway epithelia during differentiation to achieve coordinated unidirectional ciliary beating [236]. Our unpublished data demonstrates that while primary human basal-derived epithelia lose their alignment on grooved topography over time, epithelia generated from human PSC-derived airway progenitors maintain their alignment throughout differentiation in ALI culture.

4.3. Micropatterning in 3D: organoid systems

While 2D cues have enabled the community to clearly demonstrate the capability of biophysical cues to manipulate cell fate, 2D approaches are limited in their biological applicability as most physiologic physical and mechanical cues occur in 3D environments [237]. This necessitates development of 3D platforms that can better recapitulate the developmental structures and biophysical environments observed during tissue development and specification. Some efforts have emerged to control the 3D structure of differentiating cells and assess the resulting fate patterning. For example, to dissect fate choice events during embryonic patterning and subsequent cardiac development, Ma et al. created highly reproducible, confined microchambers of varying diameters (200, 400 and 600 μm) that stimulated PSCs towards a mesodermal fate via WNT agonism. This platform enabled mimicry of mesenchymal condensation during epithelial-mesenchymal transition as cells at the periphery of larger geometrically constrained patterns (400 and 600 μm) expressed OCT4 and E-cadherin. As such, cells in the middle of the patterns differentiated into beating cardiomyocytes with structures mimicking the linear heart tube, while cells at the periphery expressed myofibroblast markers [186]. Further, these microchambers were validated for use in developmental drug toxicity screening, through which they were able to represent Thalidomide embryopathy by exhibiting diminished contractility, beating frequency, and size of cardiac chambers.

Geometric constraint was also applied to cerebral organoids by Lancaster and colleagues to minimize variability associated with neural induction efficiency. This entailed addition of a physical cue in the form of polymer microfibres, around which the organoid self-organized [170]. In conjunction with an established chemical protocol, these microfibres served to pattern developing organoids leading to recognizable neuronal features including a cortical plate, radial units, along with organized radial neuronal migration in a reproducible manner. This addition of a simple physical cue substantially increased the patterning and organization of brain organoids compared to those derived from protocols only relying on biochemical cues.

Another strategy to control cell and tissue geometry in 3D is the use of micromoulding to create defined mechanical microenvironments which in turn alter cell and tissue level organization and differentiation. This approach was first developed by Nelson et al. using 3D collagen moulds to study branching morphogenesis of mammary epithelial cells [238,239]. Seeded mammary epithelia conformed to the 3D architectures, forming hollow tubules, and demonstrated predictable branching patterns according to mould geometry and presence of inhibitory morphogens. This technique was further used to understand the mechanism of cellular rearrangement in mammary ducts [240] and exhibit that mechanical stress gradients control the pattern of branching morphogenesis [241]. Inspired by this method and its applicability for studying lung branching morphogenesis, we developed tubular constructs of physiologically relevant diameters to guide self-assembly of lung progenitors [242]. Using this approach, we demonstrated that specification of these bipotent SOX2"SOX9" lung progenitors was dependent on geometry, wherein tubes of 100 μm diameter led to a distal SOX9⁺ fate, while 400 μm diameter tubes remained in a SOX2"SOX9⁺ lung progenitor state. The mechanism of this effect was dependent on canonical WNT signalling, and due to differences in cellular tension induced by patterning the progenitor cells into a 3D tube structure.

While the addition of mechanical cues influences fate choice, its role in inducing cell functionality, especially at the organoid level, needs to be elucidated. Currently, there is scant evidence of lung directed differentiation being manipulated in a 3D context [183,242,243]. Beyond our exploration of patterning early lung fates through micromoulding, Dye et al. have recently applied tissue engineering techniques during lung organoid formation. In their case, foregut endoderm spheroids cultured on highly degradable synthetic polymers demonstrated enhanced ability to differentiate into proximal airway epithelia after in vivo implantation [183]. Evidently, the field of lung directed differentiation is in its nascent stages for using biophysical manipulation to enhance differentiation during in vitro culture. The addition of simple mechanical cues such as polymer microfibres to already established lung directed differentiation protocols, as well as delving deeper into understanding cell fate in tubular constructs are excellent starting points for potentially enhancing 3D lung models.

5. Future outlook

In the future, human PSC-derived lung tissue models have the potential to enable exploration of infection, disease and regeneration mechanisms of action to impact drug discovery and drug development, and further inform patient-specific drug selection. While lung models remain in their infancy, the investment necessary to translate such models into practical use is worthwhile given that they offer a number of key advantages over primary cells or mouse models. Firstly, PSC-derived platforms enable modeling of human disease. Another major advantage of PSC-derived cells, specifically in the context of lung, is the potential to directly associate specific patient genetics and cell phenotypes with clinical conditions, as is underway in the field of lung cancer [244,245]. This will avoid complications associated with prior exposure of primary cell donors to a plethora of environmental (such as smoking) and pharmaceutical stimuli. Furthermore, establishing models specifically from PSC sources potentially enables generation of the large number of cells and cell types necessary for personalized disease modeling.

A number of challenges exist however, to translate these models into widespread use for drug discovery and development. One major challenge in the field, highlighted in this review, is the standardization of robust cell manufacturing protocols. Lung epithelial models require not only large cell numbers, but also the correct proportion of cell types. Additionally, for a variety of functional read-outs, these cell types must be appropriately spatially organized. Therefore, standardized protocols are needed to both manufacture lung cells and assemble these cells into reproducible and clinically representative “lung tissues” at the scales
required for screening. This will be essential to enable the generation of in vitro lung test tissues with sufficiently low batch-to-batch and within-batch variation for screening with high reproducibility.

Towards this challenge, methods will continue to emerge to control PSC differentiation into the different proximal airway and distal lung cell types. For example, in this review, we have highlighted the emerging evidence that an opportunity exists to further improve differentiation control and disease modeling by mimicking mechanical cues experienced during development. Beyond the strategies described in previous sections, this concept could be expanded further in the future to mimic additional aspects of lung development. For example, the developing lung is exposed to variations in oxygenation [246,247], which is known to impact cell fate choices [248–250], therefore the use of optimized oxygenation levels could be an attractive and easily scalable strategy to further refine directed differentiation culture protocols. The developing lung is also subject to various other physical cues at the organ-scale including 1) pressure from amniotic fluid, which serves to expand the nascent alveolar compartment; 2) fetal breathing movements that provide a stretch-based physical cue which serves as a maturation signal; and 3) pulsatile flow from the extensive vascular network present throughout the organ. Techniques that mimic these forces to control lung cell fate are emerging. These include the use of shear to produce relatively homogenous populations of AEC I and II [251], the use of cyclic mechanical stretch [252], and the use of patterned hydrogels to enable perfusion of lung-type structures [253]. Scoring some of these complex mechanical setups to enable large scale efficient manufacturing, however, could be a challenge.

The challenge of assembling lung cells into reproducible arrays of “lung tissues” is starting to be addressed by emerging high-throughput techniques that seek to purify specific cell populations [254–256] that could be later mixed in controlled ratios to generate precise tissue compositions. Bioprinting [257–259] and cellular assembly efforts, including organoid fabrication through DNA programming [260], could also potentially prove useful to enabling complex tissue assembly in a manner that is adequately scalable and reproducible for screening. Many of these techniques exploit biomaterials as carriers to pattern cells into 3D structures. An opportunity, therefore, exists to design novel materials that both pattern cells and augment directed differentiation culture protocols. Such materials should consider both approaches to enable timed and patterned delivery of chemical cues and the design of appropriate mechanical properties and structures (such as topography) to mimic any biophysical cues that can augment chemical signaling in the system.

Beyond the manufacturing challenge, another significant challenge limiting translation of tissue models for drug discovery and development is the inadequate availability of human validation data sets to demonstrate the accuracy of the models in predicting drug response in corresponding clinical populations. Based on single-cell RNA sequencing, data sets pertaining to airway [139,261] and lung [35] cellular compositions have emerged, that can serve as references for identifying and validating [262] differentiation products. Similarly, some evidence profiling the inflammatory response associated with CF [263] and epithelial dysfunction during pulmonary fibrosis [264,265] exists, however, establishment of a comprehensive repository informing the identity of diseased cells and their response to specific drugs is important. Data sets correlating genetic profiles with drug responses are severely limited in the lung field. Establishing such “ground-truth” benchmarking data sets to validate the ability of in vitro models to distinguish both positive and negative hits will be absolutely critical to establish confidence in lung culture models and to ensure wider spread community adoption and impact.

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