Chapter 3
Lung Development in a Dish: Models to Interrogate the Cellular Niche and the Role of Mechanical Forces in Development

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3.1 Introduction

Over the past decade, emphasis has been placed on recapitulating in vitro the architecture and multicellular interactions found in organs in vivo [1, 2]. Whereas traditional reductionist approaches to in vitro models enable teasing apart the precise signaling pathways, cellular interactions, and response to biochemical and biophysical cues, model systems that incorporate higher complexity are needed to ask questions about physiology and morphogenesis at the tissue scale. Significant advancements have been made in establishing in vitro models of lung development to understand cell-fate specification, gene regulatory networks, sexual dimorphism, three-dimensional organization, and how mechanical forces interact to drive lung organogenesis [3–5]. In this chapter, we highlight recent advances in the rapid development of various lung organoids, organ-on-a-chip models, and whole lung ex vivo explant models currently used to dissect the roles of these cellular signals and mechanical cues in lung development and potential avenues for future investigation (Fig. 3.1).

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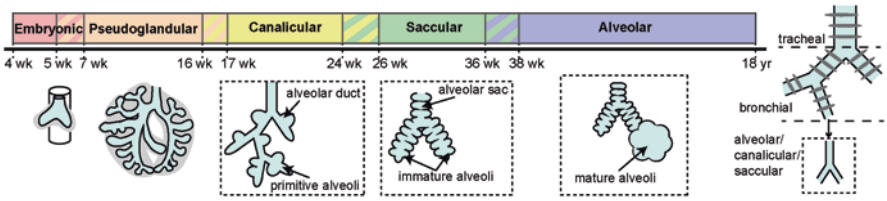

Fig. 3.1 Stages of human lung development starting at 4 weeks postconception and continuing through 18 years post birth. This process builds the entire complexity of the mammalian lung

3.2 Self-Assembled Organoid and Spheroid Models

Self-assembled differentiated organoids have become increasingly important and powerful models for studying the development and disease of many organs and tissues. In particular, numerous organoid models have been generated for the lung. Current models encompass varying levels of cellular complexity and regional specificity, from early developmental stages with multipotent progenitor cells to differentiated organoids that reflect the regional cellular heterogeneity of the large airways and the alveoli. Whereas traditionally organoids are derived from primary cells, lung organoids have also been generated from stem cell populations, such as induced pluripotent stem cells (iPSCs) or embryonic stem cells (ESCs) [2, 6–8] (Fig. 3.2a). This section will provide a high-level summary of the current state of the art in organoid modeling of the lung airway and a description of the recent advancements and opportunities these models may offer to study lung development.

3.2.1 Creating Lung Organoid Models That Represent Regional Composition and Heterogeneity

Since the initial 3D lung epithelial cultures were created [9–11], significant advancements have been made to determine cell source, culture medium formulation, and culture methods to generate region-specific organoid-like models, including tracheo- and tracheobronchospheres, bronchiolar organoids, and alveolospheres. As such, these models have enabled the dissection of cell-fate decision pathways, molecular mediators of morphogenesis, and intercellular and cell-extracellular matrix (ECM) interactions in the various lung niches. These models continue to advance our understanding of the importance and role of cellular ecology in development, homeostasis, and disease.

To model the tracheal niche, tracheospheres were initially generated from isolated mouse and, subsequently, human basal cells [12]. Adapting previous protocols for three-dimensional (3D) spheroid culture of other organ stem cells [13], isolated primary NGFR+ basal cells were FACS sorted and encapsulated as single cells in Matrigel and cultured over 2 weeks (Fig. 3.2a) [12]. These initial tracheospheres

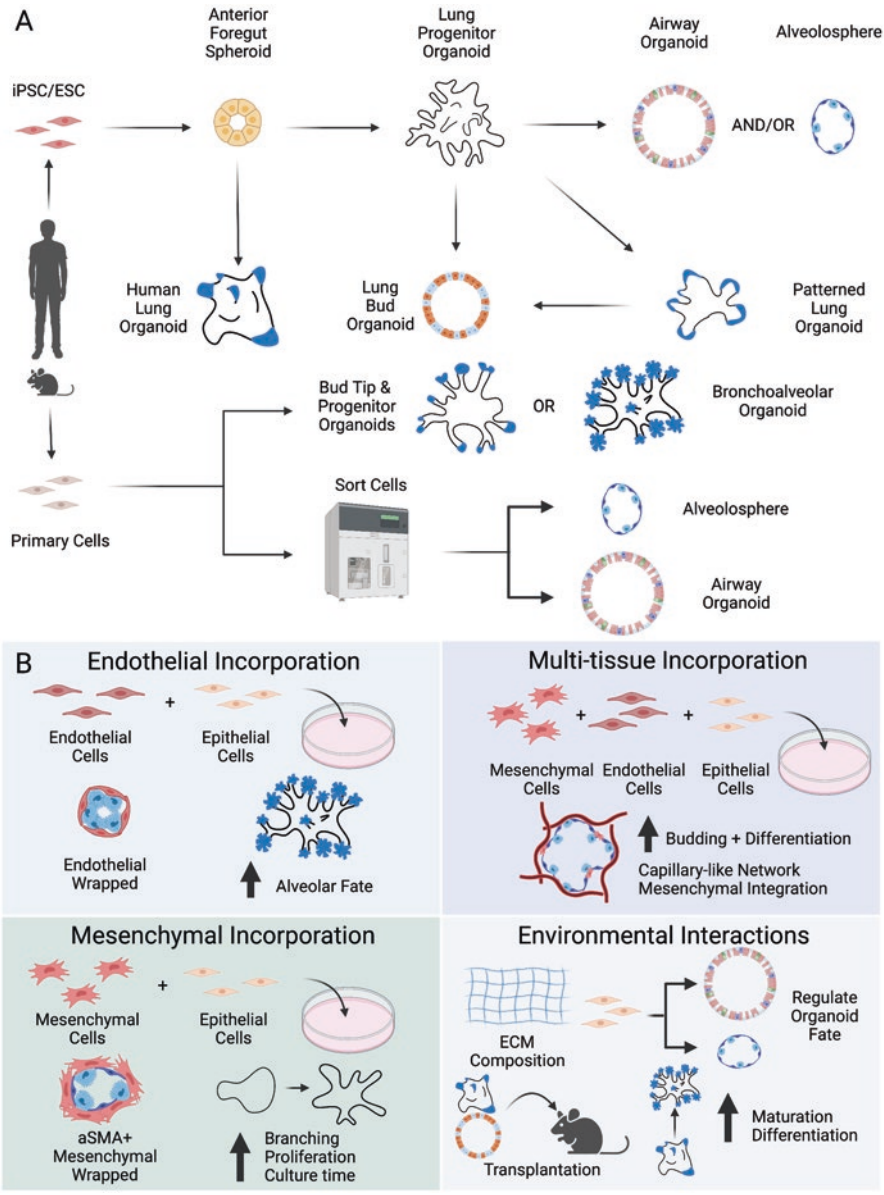

Fig. 3.2 Organoid models for studying lung development. (**a**) Stem and primary cells are used to develop lung spheroids and organoids. Methodologies vary by cell source and depend on the lung region or cell type of interest. (**b**) Current techniques for increasing spheroid/organoid complexity

showed the ability of basal cells to differentiate into multiple cell types, including KRT8+ luminal and acetylated tubulin+ ciliated cells, with spheroids maintaining internally oriented apical polarization and beating cilia. Following these methods, murine primary basal cell-derived tracheospheres were used to interrogate cell-fate decisions and lineage commitment [14–17]. Significant work has been done using murine cell sources, which has established the essential protocols for these models and elucidated the roles of signaling molecules, such as BMP4 [15] and Notch [16], in stem progenitor population maintenance and cell differentiation. Although human cell sources have been used less frequently to develop tracheo- or tracheobronchospheres, these models have made strides in increasing organoid cellular complexity and understanding the mediators of differentiation. Spheroids generated from p63;NGFR;ITGA6+ human tracheal/bronchial primary epithelial cells contained MUC5AC+ goblet cells with material consistent with secreted mucus inside the lumen, showing a step towards greater functional relevance [18]. Notch2 was required for goblet cell differentiation in these human tracheospheres, while proteins from the epidermal growth factor (EGF) family were found to inhibit basal cell differentiation [18]. A primary limitation of the reliance on basal cells to generate tracheospheres is the generation of only the surface epithelial layer in the trachea [19, 20]. The submucosal glands, which exist between the surface epithelium and cartilage rings, have a distinct cellular niche and give rise to most of the mucus and serous secretions [19]. Further efforts to isolate and culture submucosal gland duct cells have produced gland-like spheroids and revealed that high ALDH activity promotes sphere formation, suggesting an important role for ALDH in progenitor/stem-cell self-renewal [20]. Studies using tracheospheres have elucidated numerous critical regulatory factors for the proliferation and subsequent differentiation of basal and duct cells in the trachea. These spheroids are therefore valuable tools for decoding the regulators of cell-fate decisions in the upper respiratory tract and serve as models of late-stage airway development.

Whereas all tracheospheres to date have been made from isolated primary cells, bronchospheres have been generated from both primary cells and pluripotent stem cells. This combination of cell sources has enabled the investigation of early- and late-stage development, specifically uncovering cell-fate decisions related to functional and morphological changes. In combination with murine tracheospheres, Rock et al. created the first human bronchosphere from primary human bronchial epithelial (HBE) cells. This work confirmed that human bronchiolar basal cells function as a progenitor population that gives rise to secretory and ciliated cells. Mutating *Grhl2* or deleting the likely gene targets of GRHL2 in isolated HBEs with CRISPR/Cas9 genome editing technology showed critical roles for GRHL2 and ZNF750 were identified for basal cell proliferation, barrier function, and ciliogenesis [14]. This highlights the potential of organoid models to be used for rapid screening of genes related to the regulation of morphogenesis and differentiation. As these primary cell bronchosphere cultures were generated from adult cells, they do not capture the full multipotency of progenitors in development nor provide information on when these cell types arise in the developing lung. However, embryonic day (E)12.5–14.5 murine lung epithelial progenitors expressing *Nkx2.1* have

been used to generate spheroids that were positive for markers of basal and secretory cells and, to a lesser extent, ciliated and submucosal gland cell markers [21]. Spheroids showed expression of SCGB1A1, microvilli presence, and secretory granule production, indicative of club cell precursors. Additionally, there was diversity among these cells, with region-specific markers *Reg3g, Gabrp,* and *Upk3a* heterogeneously distributed within the spheroids. Interestingly, these club cells further indicated the presence of a distinct, multipotent cell type from mature secretory cells as they gave rise to basal and submucosal gland cells (KRT5;SMA+) in addition to club and ciliated cells [21].

The first airway spheroids generated from stem cells were mouse ESC-derived NKX2.1+ progenitors suspended in Matrigel and transplanted in vivo [22]. These spheroids differentiated to contain proximal airway epithelial cells, basal cells, Clara cells, ciliated cells, and secretory cells, thereby producing a greater range of mature cell types than in previous work. Building on this, human iPSCs were differentiated into NKX2.1+ progenitors with subpopulations of SOX2+ and SOX9+ cells, suggesting the presence of committed airway (NKX2.1;SOX2+) and multipotent progenitors (NKX2.1;SOX9+) [22]. This work provided a basis for generating stem cell-derived bronchospheres; therefore, subsequent work focused on uncovering the regulatory factors involved in cell differentiation, recapitulating cellular functions, and investigating the effects of environmental mechanics. iPSC-derived NKX2.1;SOX2+ proximal airway progenitor spheroids were able to grow and fuse into large amorphous structures but could not undergo further lineage commitment under normal culture conditions [23]. However, when subjected to primary bronchial epithelial media, spheroids differentiated into CHGA+ and SYP+ pulmonary neuroendocrine cells, SCGB1A1+ club cells, KRT5+ basal cells, and acetylated tubulin;FOXJ1+ ciliated cells, with acetylated tubulin+ cells closely aligned with mucus-secreting MUC5AC+ cells [23]. The localization of these various cell types resembled that of the human fetal lung, and beating cilia were visualized. Further interrogation of signaling pathways in proximalized airway organoids via Notch inhibition [23], Wnt up- or downregulation [24–26], or fibroblast growth factor (FGF) supplementation [24] has indicated the importance of these factors in epithelial differentiation and lineage commitment. Airway organoids were occasionally generated from hepatic- and gastric-like cells [26], underscoring the need for continued investigation of pathways responsible for cell-fate decisions.

To model the distal lung, alveolospheres were generated from primary human alveolar type 2 (AT2) cells cultured in Matrigel (Fig. 3.2a) [27, 28]. These methods created spheroids through migration and attachment rather than proliferation and apoptosis, as in previous organoid models. Additionally, they could differentiate into alveolar type 1 (AT1) cells, with human serum treatment inducing AGER+ cells and forskolin-induced swelling resulting in a shift toward a flattened morphology [27, 28]. This phenotypic shift was thought to be due to increased secretion and pressure within the spheroid, aligning with previous reports of increased mechanical tension in epithelial acini driving morphogenesis and homeostasis [29]. Using similar AT2 spheroids, it was found that depleting EGF led to differentiation of AT2s into the newly discovered alveolar type 0 (AT0) cell, an intermediate

progenitor cell [30]. AT0s could then be differentiated into AT1s with the addition of serum or into terminal and respiratory bronchial secretory cells by continued depletion of EGF [30]. To further confirm the differentiation capability of AT2 to AT1 cells, alveolar epithelial progenitor cells were directly differentiated from human pluripotent stem cells (hPSCs) into spheroids positive for AT1 and AT2 cell markers and distal lung lineage markers [31]. Adapting a 2D lung cell differentiation protocol, human iPSC alveolar organoids were generated containing functional lamellar bodies and clustered transcriptomically with primary fetal lung cells without the need for a feeder cell population [32–34]. These organoids were used to investigate the differentiation program of AT2 and AT1 cells, showing the importance of Wnt downregulation and the existence of an intermediate IGFBP2− cell, confirming in vivo lineage tracing results [34, 35]. Looking upstream of AT2s, a newly identified SCGB3A2+ respiratory airway secretory (RAS) cell was found to localize between alveoli [36]. Human ESC-derived RAS cell organoids supported Wnt and Notch regulation of AT2 differentiation [33, 36]. Further culture of RAS-derived AT2-committed organoids in airway media did not rescue *Scgb3a2* expression, demonstrating this is a unidirectional differentiation process [36]. While most alveolosphere culture methods use Matrigel, initial steps have been taken to use other hydrogel formulations with known compositions and tunable biophysical or biochemical properties [37]. For example, the culture of iPSC-derived AT2 alveolospheres in various 3D hyaluronic acid hydrogels showed that chemical and mechanical properties influence spheroid formation [38]. The incorporation of hyaluronic acid microwells further enabled spatial control of alveolospheres and maintained a larger proportion of cells with AT2 markers than Matrigel culture [38].

3.2.2 Advancing the Complexity of Organoids to Investigate Tissue Crosstalk

Cell-cell interactions across tissue types are known to be essential to lung development [39–42]. More complex organoids, incorporating different tissue compartments, including immune, endothelial, and other stromal cell populations, have been created for investigating and elucidating reciprocal tissue signaling mechanisms necessary for lung development. Adding mesenchymal cells, either as a feeder layer or as part of the organoid, was one of the first steps toward organoid models for intertissue interactions (Fig. 3.2b). Recent studies have begun investigating the effects of stromal cell populations and other hydrogel compositions on tracheal and basal cell spheroids. Tracheospheres cultured with a mitotically inactivated 3T3 fibroblast feeder layer and Rho-associated protein kinase (ROCK) inhibitor Y-27632 increased proliferation and maintained mucosecretory and ciliated cell fate [43]. Conditionally reprogrammed isolated human primary basal cells cultured with 3T3 fibroblast feeder cells and ROCK inhibition developed tracheobronchospheres with externally oriented apical polarization, providing the ability to test therapeutic delivery [44]. Additionally, investigation into matrix composition and signaling

pathways found that a mixture of collagen I, collagen III, and Matrigel produced significantly more organoids than Matrigel alone, while supplementation of R-spondin 2 (Wnt agonist) and Noggin (BMP antagonist) further stimulated growth and differentiation [44]. Coculture of human lung-derived primary EPCAM+ epithelial cells and EPCAM-;SCA-1+ mesenchymal cells in Matrigel generated epithelial organoids that were wrapped in aSMA+ mesenchymal cells, mimicking in vivo interactions in the large airways [45]. Investigation of mesenchymal factors responsible for epithelial organoid formation revealed FGF10 and HGF supplementation could facilitate organoid formation without coculture of mesenchymal cells, suggesting the EPCAM-;SCA-1+ mesenchymal cells secrete these factors to direct epithelial proliferation and differentiation [45].

Coculture of VA10 human bronchial cells with endothelial cells led to branching organoids with alveolar-like buds that included tertiary level branches (Fig. 3.2b) [46]. Branching was endothelial mediated and relied on FGF signaling [46]. In other studies, upregulation of FGFR1 and VEGFR2 led to murine pulmonary capillary endothelial cell-mediated generation of alveolar saclike organoids [47]. Treatment with various inhibitors and agonists revealed that VEGFR2 and FGFR1 signaling triggered pulmonary capillary endothelial cells to produce MMP14, which was necessary for lung progenitor activation and alveolar-sac organoid formation [47]. Further investigation with epithelial-endothelial coculture organoids revealed a BMP4-controlled NFATC1-TSP1 axis in endothelial cells, which directs bronchoalveolar stem cell differentiation toward an alveolar fate [48]. Whereas the incorporation of multiple cell types within a single 3D culture model has been achieved with some cell types, a persistent challenge to the widespread incorporation of multiple cell types, particularly epithelial and endothelial cells, is the lack of an optimized culture medium to support both populations, even though relevant morphological structures can be generated in vitro with each cell type individually [49, 50].

Perhaps the most complex organoids have combined epithelial, mesenchymal, and endothelial interactions (Fig. 3.2b). Embryonic day 17.5 murine fetal pulmonary cells were cultured in collagen to generate lung organoids containing epithelial, endothelial, and mesenchymal cells [51]. Distinct roles of FGFs were found, with FGF2 supplementation leading to increased mesenchymal proliferation and endothelial network formation, whereas FGF7 and 10 increased epithelial and mesenchymal proliferation, with FGF10 specifically increasing bud formation [51]. Comparing various supplementation schemes suggested that epithelial proliferation and budding positively affect vascular development, likely through increased proangiogenic paracrine signaling [51]. Utilizing the same model, crosstalk between the FGF, SHH, and VEGF-A signaling pathways was observed [52]. Specifically, it was suggested that exogenous FGFs begin a cascade of many endogenous mediators, including SHH and VEGF-A, which affect epithelial and endothelial development in the lung [52]. Further, these signaling pathways regulated extracellular matrix ligand tenascin-C deposition, and tenascin-C patterns were important for endothelial network morphology and epithelial saccularization in the organoids [52]. In other studies, human bronchial epithelial cells were cultured with human lung microvascular endothelial and mesenchymal cells in soluble ECM-supplemented

media atop a Matrigel layer to form branched and budding airway organoids [53]. The multiple cell types self-organized to create tubular structures within the organoid that recapitulated the basic structure observed in lung development, although mature airway and alveolar cells were not observed [53]. Generation of these tubular structures was dependent on myosin 2 contraction in fibroblasts and YAP signaling, respectively shown by attenuation of tubules with blebbistatin treatment or fibroblast-free culture and YAP localization in tubule tips with knockdown. Resulting in reduced tubular formation and disorganization of the epithelium, mesenchyme, and fibronectin deposition [53]. Multicell coculture organoids, therefore, provide a robust model for lung development and enable the investigation of various signaling pathways involved in cell differentiation and organ morphogenesis.

3.2.3 Induction of Lung Organoids to Create Multiple Tissue Compartments

hPSCs have been used to develop multi-tissue compartment organoids with different differentiation schemes and medium supplements directing organoid composition (Fig. 3.2a). hPSCs treated with activin A and a combination of Noggin, SB431542, FGF4, smoothened agonist (SAG), and CHIR99201 generated medium-suspended organoids that were subsequently embedded in Matrigel and treated with FGF10 to form human lung organoids (HLOs), which mimicked early fetal murine lung development [54]. These HLOs included epithelial cell populations with proximal and distal airway-like structures surrounded by mesenchymal cells. Seeding of HLOs onto a decellularized lung matrix (Fig. 3.2b) resulted in the differentiation of multiciliated cells, reinforcing that extracellular matrix cues can regulate lung cell fate [54]. Other studies had demonstrated that when HLOs were seeded onto rigid microporous poly(lactide-co-glycolide) scaffolds and implanted for 4 weeks into the epididymal fat pad of mice, differentiated airway structures with beating multiciliated cells, a vasculature, the scattered presence of goblet and club cells, and surrounding smooth muscle, myofibroblasts, and cartilage were formed [55]. Using a slightly modified differentiation scheme of CHIR99021 followed by Noggin, FGF4, SB431542, and CHIR99021, before implantation of these HLOs into the kidney capsule of immunodeficient NSG mice, resulted in differentiation of bipotent alveolar progenitors, AT2, AT1, ciliated, basal, goblet, and club cells after 120 days [56]. These organoids had developed ACTA2+ vasculature and PGP9.5+ neuroendocrine cells, with transmission electron microscopy imaging revealing blood vessel and myelin sheath structures [56]. Although these HLOs achieved more mature cell fates, they never developed tubular structures. As such, HLOs have enabled the investigation of proximal and distal airway maturation and the mesenchyme's role in development. Using the same differentiation protocols, HLOs have been generated from iPSCs derived from human fetuses and neonates with normal lungs or congenital diaphragmatic hernia (CDH) [57]. This work highlights the

potential of lung organoids to be used in the investigation of mechanisms underlying developmental diseases, such as CDH, and to study the saccularization and alveolarization stages of lung development [58].

Like HLOs, hPSC-derived lung bud organoids (LBOs) with mesoderm and endoderm develop structures upon transplantation and Matrigel culture [59]. Development of LBOs slightly differed from HLOs, with BMP4, FGF10, FGF7, retinoic acid, and CHIR99201 supplementation of the media. The resulting LBOs had no markers of maturity present other than p63, indicating their progenitor state [59]. Implantation of these organoids under the kidney capsule of immunodeficient NSG mice led to the development of proximal airway epithelium, branched structures with surrounding mesenchymal cells, cells positive for markers of later stage proximal (FOXJ1, CC10, and mucins) and distal (SFTPC and SFTPB) cell types, the presence of submucosal gland structures, neuroepithelial body-like structures, and networks of thin cell layers with AT1 and AT2 markers [59]. Interestingly, LBOs did not require an implantation scaffold for maturation and could develop into patterned lung-like structures, while HLOs only developed into airway-like structures. LBOs could also be cultured in Matrigel in vitro to generate branched structures with dilated tips, and RNAseq analysis of these organoids was found to match late second-trimester fetal lungs. However, they were distally biased and did not achieve mature AT1 cells [59]. Further development and improvement of organoid models that combine multiple tissue compartments is therefore an exciting opportunity space for understanding signaling mechanisms that underlie lung development.

3.3 Microfluidic and Organ-on-a-Chip Models to Study Lung Development

Microfluidic organ-on-a-chip systems are advantageous in their ability to incorporate well-defined mechanical stimuli, such as fluid flow, pressure, stretch, substratum stiffness, and shear stress, into in vitro human cell culture systems. Traditionally, microfluidic devices consist of micron-sized cellular channels perfused with cell culture medium or other fluids (Fig. 3.3). The perfusion provides nutrient replenishment from medium circulation and, if desired, the application of fluid forces for long-term studies. Microfluidic platforms have advanced dramatically from the investigation of single cells on a glass substratum within a channel to entire tissue networks within 3D hydrogel scaffolds [60–62]. With the advancements in device design and fabrication, the field has increasingly incorporated the ability to build and test tissue and organ physiology. Much of the work developing microfluidic platforms to investigate lung biology, signaling, and physiology has been used for understanding adult homeostasis and disease. However, there is tremendous opportunity to use these platforms for investigating lung development. In particular, the embryonic and fetal lungs are fluid filled [63], which can easily be captured by the

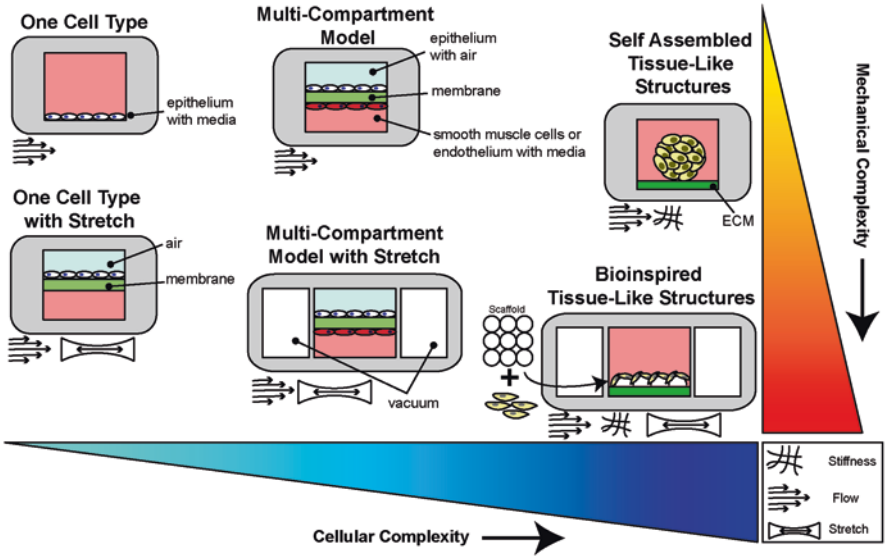

Fig. 3.3 Diagram of organ-on-a-chip device designs illustrated based on cell complexity and the complexity of mechanical stimuli that can be achieved

small size and fluid control of these devices to answer questions of early lung development at a relevant length scale [64]. Similarly, scaling geometry and mechanical stimuli relevant to neonates provides significant opportunities for these models to advance our knowledge of late-stage lung development, which is directly translatable to the clinical management of preterm infants.

3.3.1 Moving Toward More Complex Physiology with Multiple Channels

Beyond numerous studies using single fluidic channels, the introduction of multichannel devices for lung modeling improved the physiological relevance of the design by enabling controlled interrogation of one or more mechanical stimuli (Fig. 3.3). Takayama's group was among the first to use microfluidic platforms for modeling the lung and developed a compartmentalized microfluidic model of the airway epithelium [65, 66]. The structure and dimensions were designed to recapitulate in vivo airways and respiratory bronchioles. A two-chamber design created apical and basal compartments of the airway epithelium, separated by a thin "basement" membrane. Human primary small airway epithelial cells seeded in the top channel were subject to tunable airflows, with the bottom channel serving as a media reservoir. These mechanical forces led to cellular differentiation into

secretory phenotypes [66]. Additional iterations of this device have been used to elucidate the protective role of surfactants from the damaging mechanical stresses caused by liquid-plug airway obstructions formed in premature infants or lungs with insufficient production of surfactants [67–70]. Since the initial development of these epithelial models, numerous designs have been used to study mechanical stimuli on airway epithelial function in various regions along the airway tree. In one such design, a two-channel microfluidic device was developed to understand how breathing-induced stretch affects AT2 cell phenotype and surfactant production [71]. A deformable membrane separated a medium-perfused channel from an air-filled pneumatic channel, and the epithelial cell monolayer on the membrane was deformed by pressurizing the pneumatic channel inducing stretch within the tissue in vitro. Loading via cyclic stretch to mimic breathing and ventilator-induced lung injury could be achieved, and by tuning the frequency and magnitude of cyclic stretch, healthy and pathological forces can be incorporated into the microfluidic model [71]. Importantly, although these models have been used to investigate adult airway mechanics, they have broad applicability. They specifically could be used to investigate airway development in utero and postnatally to model developmental effects and ventilator-induced lung injury in neonates.

Intercellular and intertissue signaling is essential to physiological function in homeostasis and disease. Likewise, complex signaling networks driven by soluble and mechanical cues exist in the developing lung that drive proliferation, differentiation, and ultimately morphogenesis. As such, multichannel microfluidic systems have sought to construct complex tissue environments to dissect the mechanisms of cellular crosstalk and understand the functional impacts on tissue physiology. Using similar approaches to the previously described multichannel devices, multi-tissue lung models have been developed (Fig. 3.3). Using a simple two-channel device, interactions can be studied between airway epithelium and smooth muscle cells separated by a thin hydrogel to mimic chronic lung disease [72]. Similar devices have also been used to investigate epithelial-endothelial crosstalk in the lung [73–75]. In one such device, the application of dynamic mechanical forces in a combined epithelial-endothelial device was introduced in a similar two-channel design with two lateral channels [73]. Air was applied in the epithelial channel and culture medium in the endothelial channel. With a dynamic vacuum force applied to the lateral channels, in-plane stretch is achieved on a porous elastic membrane that separates the epithelial and endothelial compartments. Further iterations of the device have enabled the application of breathing-like forces without the need for parallel chambers [75]. Seeding the epithelial channel with distal or proximal epithelial cells created an alveolar or airway model, respectively, which could be exposed to air to mimic adult and neonatal in vivo conditions. This model enables the investigation of epithelial-endothelial interactions in response to breathing-like forces. Additional variations on these device designs have enabled the study of signaling between tissue compartments [76–78] to decipher mechanisms of pathogenesis in adult lung models. However, these would be powerful models to investigate the relative contributions of tissue crosstalk and mechanical forces, including

changes in ECM stiffness [79], during lung development. In addition to mechanistic insight, another important use of these in vitro models is for drug screening and understanding physiological transport across these barrier tissues [75, 78, 80–83], which would be directly translatable to developing new treatments for diseases associated with the neonatal lung and patient-specific therapeutic testing [84].

3.3.2 Integration of Dimensionality and Biomaterials into Organ-on-a-Chip Platforms

It is well established that three-dimensionality, composition, and stiffness regulate gene expression, phenotype, proliferation, differentiation, and function of cells. As but one example, varying tissue structures were formed by human bronchiolar epithelial cells when cultured in 3D depending on whether they were cultured at the air-liquid interface on collagen, on Matrigel, or encapsulated within Matrigel [85]. Whereas air liquid interface (ALI) on collagen generated a monolayer, culture on top of Matrigel resulted in branched and budding tubule structures reminiscent of patterned lung organoids, and encapsulation in Matrigel resulted in spheroid formation. As such, in vitro lung models have sought to incorporate relevant geometric scales and material properties into microfluidic organ-on-a-chip devices. Strategies have ranged from biomimetic geometries to advanced materials, including hydrogels [86], microbeads [87], 3D printed plastics [88], and membranes [89]. Generally, the scaffolds are designed in the shape of the desired tissues to guide and direct cell growth, alignment, and differentiation. Circular or spherical geometries are often used to mimic alveolar structures, which are subsequently seeded with human primary alveolar epithelial cells to develop 3D alveolar-like constructs (Fig. 3.3). For example, a gold membrane with large hexagonal pores supported an elastin-collagen gel seeded with a mixture of AT1 and AT2 cells on the top and endothelial cells on the underside [89]. The membrane was stretched via negative pressure on the underside to mimic the distal airways during breathing. Similar strategies to model the distal airway include dome-shaped polycarbonate membranes [88] and porous gelatin methacryloyl hydrogel scaffolds [87]. Apart from these material approaches to generate relevant geometries and biophysical niches, methods have been developed to directly incorporate organoids within microfluidic systems [90] (Fig. 3.3). This latter approach may yield a powerful system wherein an organoid generates a complex cellular community that can be dissociated to serve as a cell source for these organ-on-a-chip systems. Subsequently, controlled biophysical cues, biochemical gradients, and defined geometric length scales not achievable in organoids can be used to investigate complex human cell populations within these microfluidic platforms. Combining these technologies is an exciting possibility as it will further develop the understanding of many mechanistic questions about the developing lung.

3.4 Whole Organ Models to Understand the Mechanics of Lung Development

Whereas much work has been done across a range of small and large animal models of lung development and diseases, including bronchopulmonary dysplasia (BPD) and CDH [91–95], the mouse has provided critical insights into the gene regulatory networks and cellular differentiation events that underpin mammalian lung morphogenesis. For decades the workhorse models used in lung developmental biology have been mesenchyme-free murine epithelial lung tips embedded in Matrigel [96] and ex vivo whole rodent lung culture on floating membranes at the air-liquid interface [97]. These approaches offer the opportunity to have the inherent complexity of the organ to recapitulate morphogenic processes in vitro. However, applying controlled mechanical forces to whole organs ex vivo remains a persistent challenge. In recent years, methods have been developed for lung culture models to determine how mechanical forces, including transmural fluid pressure, breathing movements, and airway smooth muscle contractions, are coupled to developmental programs to guide cell proliferation, differentiation, and airway morphogenesis [93, 98–100].

Indeed, work using whole mouse lung explants has revealed the critical roles of transmural fluid pressure in early embryonic airway branching in a microfluidic chest cavity model [101, 102]. Similarly, the trachea was intubated and pulmonary vasculature was perfused in E16.5–18.5 mouse embryos to investigate the role of fetal breathing movements and FGF10 signaling on distal airway development during saccularization [103]. These whole organ perfusion approaches are analogous to whole organ culture models in adult rodents, large animals, and human lung explants, which are often used for disease modeling and drug inhalation studies [104–108]. The adaptation of these techniques to mouse embryonic, fetal, and neonatal lung explants offers a powerful tool to interrogate how mechanical forces guide lung development in a genetically tractable lung model. Continued adaptation of these systems to murine models of late-stage development, encompassing the end of saccularization through alveolarization, is an important avenue for continued mechanistic investigation of normal development and disease.

3.5 Conclusion

The model systems reviewed herein represent significant and exciting advancements in our ability to add and interrogate complex cellular niches and physiological functions in the developing lung. These approaches have unique advantages, but the collective synergy of these models offers the ability to decouple tissue and organ-scale biochemical and biophysical signaling networks that drive and regulate lung organogenesis (Fig. 3.4). Organoids can generate cellular heterogeneity

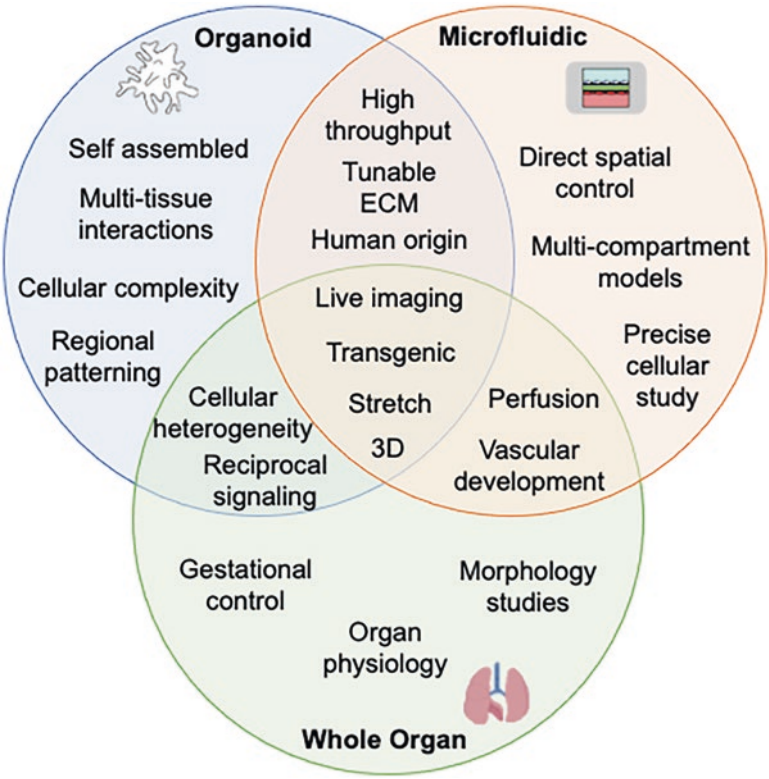

Fig. 3.4 Capabilities and benefits of the three central model systems for building complexity into lung development studies.

and structural complexity that mimic native lung niches more closely. Moreover, given the ability to genetically modify the cells being used, organoids provide a powerful platform for screening signaling pathways and their impacts on morphology and function that are often intractable in humans. Organ-on-a-chip systems allow for the direct spatial control of cellular positioning and biochemical and biophysical cues. These systems provide geometric control with the ability to successfully integrate multiple tissue compartments to provide quantitative physiological and functional outputs from human cell sources. Whole organ culture captures the full complexity of the mammalian lung and gestational-stage control. Current efforts to advance these models and integrate them, leveraging the unique advantages of each, will enable the mechanistic understanding of lung development and provide insight into congenital disease mechanisms and translational therapies for newborns.

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