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# Exploring *In Vitro* Models: Advances and Challenges in Human Respiratory Tract Research

# Exploración de modelos *in vitro*: avances y desafíos en la investigación del tracto respiratorio humano

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**Abstract:**

In recent years, significant efforts have been made to develop *in vivo* and *ex vivo* models that replicate the histology, physiology, and pathology of human airways. However, translating these models to human applications has presented significant challenges, particularly concerning ethical concerns related to the number of animals required for efficacy and safety assessments. As an alternative, *in vitro* models have gained prominence, aiming to replicate human tissue characteristics. These models offer promising correlations with human tissue, providing a more ethical, cost-effective, and rapid approach for obtaining reproducible results. Standardizing patient-derived cell lines, spheroid and organoid culture models, scaffold and hydrogel cultures, tissue slices, tumor xenograft models, patient-derived xenografts, and circulating tumor cells *in vitro* models is a crucial challenge. This standardization would enhance the reproducibility and reliability of experimental outcomes, ultimately advancing our understanding of human biology and disease mechanisms, and reducing the reliance on animal testing. *In vitro* models also offer the advantage of reducing reliance on animal testing while providing a controlled environment that better elucidates cell-cell interactions compared to ex vivo models, however, standardizing patient-derived cell lines, spheroid and organoid culture models, scaffold and hydrogel cultures, tissue slices, tumour xenograft models, patient-derived xenograft and circulating tumour cell, is indeed a crucial challenge. The present manuscript aims to summarize the state of the art regarding these culture tools designed to mimic specific human organ structures and functions, with a focus on their application to regenerative pharmacology.

***Keywords:***

*Organoids, ex vivo models, ALI, hIPSC, human airways*

**Resumen:**

En los últimos años, se han realizado importantes esfuerzos para desarrollar modelos in vivo y ex vivo que repliquen la histología, fisiología y patología de las vías respiratorias humanas. Sin embargo, traducir estos modelos a aplicaciones humanas ha presentado desafíos importantes, particularmente en lo que respecta a preocupaciones éticas relacionadas con la cantidad de animales necesarios para las evaluaciones de eficacia y seguridad. Como alternativa, han ganado importancia los modelos *in vitro*, cuyo objetivo es replicar las características del tejido humano. Estos modelos ofrecen correlaciones prometedoras con el tejido humano, proporcionando un enfoque más ético, rentable y rápido para obtener resultados reproducibles. La estandarización de líneas celulares derivadas de pacientes, modelos de cultivo de esferoides y organoides, cultivos de andamios e hidrogeles, cortes de tejido, modelos de xenoinjertos tumorales, xenoinjertos derivados de pacientes y modelos *in vitro* de células tumorales circulantes es un desafío crucial. Esta estandarización mejoraría la reproducibilidad y confiabilidad de los resultados experimentales, lo que en última instancia mejoraría nuestra comprensión de la biología humana y los mecanismos de las enfermedades, y reduciría la dependencia de las pruebas con animales. Los modelos *in vitro* también ofrecen la ventaja de reducir la dependencia de las pruebas con animales al tiempo que proporcionan un entorno controlado que aclara mejor las interacciones entre células en comparación con los modelos ex vivo; sin embargo, estandarizan líneas celulares derivadas de pacientes, modelos de cultivo de esferoides y organoides, cultivos de andamios e hidrogeles. , cortes de tejido, modelos de xenoinjerto tumoral, xenoinjerto derivado de paciente y células tumorales circulantes, es de hecho un desafío crucial. El presente manuscrito tiene como objetivo resumir el estado del arte con respecto a estas herramientas de cultivo diseñadas para imitar estructuras y funciones de órganos humanos específicos, con un enfoque en su aplicación a la farmacología regenerativa.

***Palabras Clave****:*

*Organoides, modelos ex vivo, ALI, hIPSC, vías respiratorias humanas*

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## INTRODUCTION

Air pollution, comprising particulate matter (PM), gaseous pollutants, and traffic-related emissions, contributes to over two million global deaths annually, causing significant respiratory system damage.[1] Additionally, it accounts for 14% of childhood asthma cases.[2] The upper airway epithelium serves as the primary protective barrier against environmental pollutants.

While numerous *in vitro* and *ex vivo* models have been developed to emulate respiratory diseases,[3] replicating complex cell-cell interactions and tissue remodeling processes remains a significant challenge in typical cell culture systems. An ideal model should establish robust correlations between *in vitro* and *in vivo* data, thereby expediting drug development, conserving resources, and enhancing product quality.[4] Recent improvements in *in vitro* models aim to establish better parallels with human tissue.[5] However, methodological variations, diverse cell sources across species, and differing drug delivery systems often complicate result interpretation and hinder cross-study comparisons. This review provides an overview of the most commonly employed strategies in attempting to simulate the *in vitro* development of airway models.

## THE AIRWAY SYSTEM

The respiratory system comprises a complex network, including the trachea, airways, and distal alveoli, designed primarily for gas exchange between blood and the external environment. Environmental factors such as temperature fluctuations, particulate matter, and allergens can pose risks to the overall functionality of this branched airway network. Distinct anatomical compartments within the lung's conducting airways are lined with highly specialized epithelial cells, each with specific functions that vary along the proximal-to-distal axis. These specialized cells establish comprehensive communication to facilitate their respective roles.[6]

The lung can be anatomically divided into two zones: the conducting zone and the respiratory zone. The conducting zone encompasses the trachea, bronchi, and conducting bronchioles, while the respiratory zone includes regions responsible for gas exchange—specifically, the terminal (respiratory) bronchioles and alveoli (Figure 1).[7-14] The lung presents a highly intricate network of airways, lined with diverse luminal epithelial cell types that manage airway hydration and safeguard against pathogens and debris. Histologically these airway lineages are sustained by basal cells capable of self-renewal and differentiation into various luminal airway epithelia, including goblet cells, club cells, multiciliated cells, neuroendocrine cells, tuft cells, and ionocytes (Figure 2)[7-10] arranged in a pseudostratified epithelium. This epithelium plays a pivotal role in protecting against inhaled toxins, particles, and pathogens. Its structure and composition gradually shift from a pseudostratified form with mainly ciliated cells to a simpler columnar or cuboidal epithelium, with fewer ciliated cells and an increased number of secretory cells, notably club cells. The respiratory zone comprises endothelial cells in conjunction with alveolar type 1 (AT1) and type 2 (AT2) cells. This intricate arrangement, including the interjacent basement membrane, facilitates the $O_2/CO_2$ exchange. This configuration characterizes the critical respiratory zone (Figure 2).[11,12] In humans, the airways gradually decrease in size, leading to the generation of distinct compartments, such as the respiratory bronchioles, ultimately culminating in the alveolar parenchyma.[13] The alveoli serve as sites where alveolar epithelial type 1 (AT1) cells interface with the capillary plexus to facilitate gas exchange between the external environment and the vascular system. Alongside AT1 cells, the function of alveolar epithelial type 2 (AT2) cells, which secrete pulmonary surfactant, is crucial to prevent alveolar collapse at this air–liquid interface. While the mature respiratory system maintains its homeostatic structure and function, it possesses regenerative capacities in response to injury. However, a deeper understanding of the molecular mechanisms and available tools governing the spatial and temporal guidance of cells during this intricate regenerative process is crucial.[14]

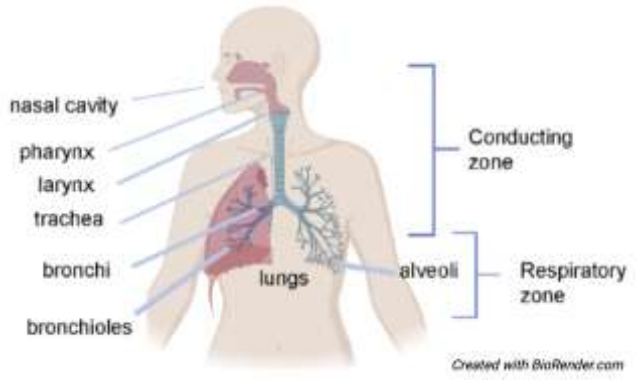


**Figure 1. Anatomical model of human airway[14]**

## AIRWAY DEVELOPMENT

The development of the branched network of airways and gas exchange surfaces is closely intertwined with the cardiovascular system, establishing a crosstalk between endodermal development and the lung mesoderm, influencing the various cell lineages within the lung, airways, and vascular smooth muscle and pericytes.[15-17] In human development, the formation of the right and left lung anlage arises from the primitive foregut endoderm around day 26 post-conception (p.c.). This process is guided by the hallmark transcription factor Nkx2.1. Subsequent to the separation of the trachea from the esophagus, the two lung buds emerge.[18] Both lung buds elongate, initiating repetitive growth into the surrounding mesenchyme through branching morphogenesis. These developmental events persist through the canalicular and saccular stages. The terminal branches progressively narrow, and the epithelial sacs cluster to form alveoli, maturing during the alveolarization stage for respiration at birth.[19]

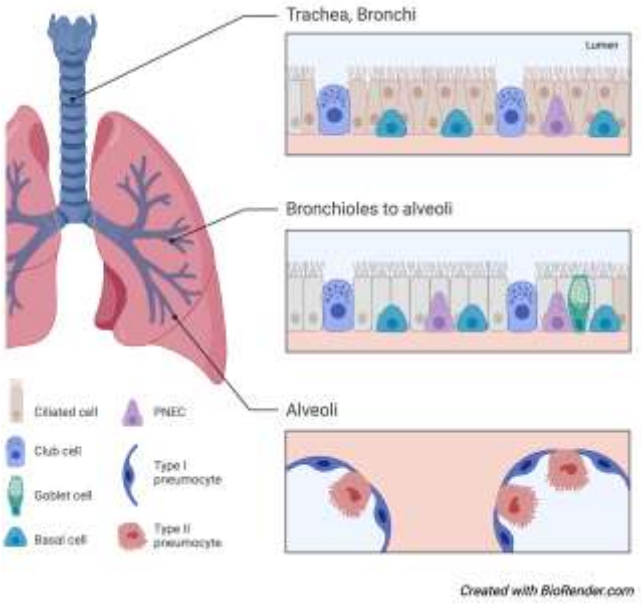


**Figure 2. Cell types found in the proximal airways (trachea, bronchi, bronchioles and alveoli). Basal cells in the airway pseudostratified epithelium function as the primary progenitors and could differentiate into virtually every airway epithelium cell population.[12]**

Histologically, lung development and maturation are categorized into four stages: pseudoglandular, canalicular, terminal saccular, and alveolar. During the pseudoglandular stage (5–17 weeks of human pregnancy), the epithelial tubes resembling an exocrine gland undergo branching morphogenesis.[14,20,21] However, the immature fluid-filled primitive respiratory tree at this stage cannot efficiently support gas exchange. In the canalicular stage (16 to 25 weeks of human pregnancy), the respiratory tree expands in diameter and length, accompanied by vascularization and angiogenesis. The airway undergoes segmentation into respiratory bronchioles and alveolar ducts, with differentiation of airway epithelial cells into peripheral squamous cells and proximal cuboidal cells.[14,20,21]

From 24 weeks to the late fetal period in humans, the terminal saccular stage is characterized by significant thinning of the interstitium and clearer differentiation of alveolar epithelial cells into mature squamous type I pneumocytes and secretory rounded type II pneumocytes.[20,21] This stage is crucial for the development of surfactant synthesis and secretion, ensuring the lung can support air exchange in prematurely born neonates without collapsing.

The alveolar stage (late fetal period to childhood in humans) marks the culmination of lung development, showcasing extensive surface area for efficient gas exchange, approximately 70 $m^2$ in the human lung (Figure 3).[19,22-24]

## *IN VITRO* MODELS

Chronic respiratory diseases, ranked as the third leading cause of death globally, prominently include chronic obstructive pulmonary disease (COPD) and asthma, while conditions like cystic fibrosis (CF) and primary ciliary dyskinesia (PCD) have lower prevalence.[25] The demand for effective treatments to address the diverse clinical cases continues to rise. However, many drug candidates, although proven safe and effective in animal models, encounter setbacks during clinical trials.[26,27] These limitations have underscored disparities between animal models and human physiology[28], emphasizing the need for *in vitro* models of differentiated airway epithelium that offer practical and ethical advantages.[29]

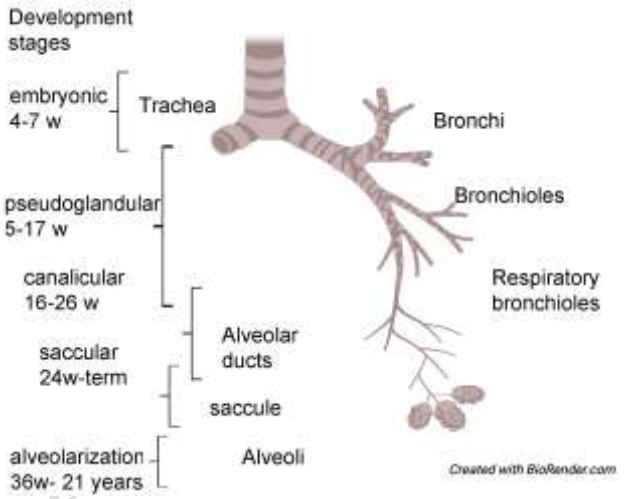


**Figure 3. Development of the airways. On average, an airway of a human lung ends in an alveolar saccule after 23 generations; however, due to the shape of the lung, a range of 18–30 generations.[24]**

## *IN VITRO* MODELS

Chronic respiratory diseases, ranked as the third leading cause of death globally, prominently include chronic obstructive pulmonary disease (COPD) and asthma, while conditions like cystic fibrosis (CF) and primary ciliary dyskinesia (PCD) have lower prevalence.[25] The demand for effective treatments to address the diverse clinical cases continues to rise. However, many drug candidates, although proven safe and effective in animal models, encounter setbacks during clinical trials.[26,27] These limitations have underscored disparities between animal models and human physiology[28], emphasizing the need for *in vitro* models of differentiated airway epithelium that offer practical and ethical advantages.[29]

Recognizing the physiological relevance of 3-D biomimetic human tissue models, the United States Food and Drug Administration (FDA) waived the prerequisite for drugs in development to undergo animal testing before human trial participation in 2023.[30] This shift highlights the imperative to develop diverse *in vitro* models of airway epithelium. Presently, these models vary in complexity and degree of differentiation, encompassing two-dimensional (2-D) cell cultures that grow cells as a monolayer on a flat plastic surface, offering a cost-effective option but neglecting crucial cellular aspects such as

polarity.[31-33] In response, more intricate models have emerged, including air-liquid interface (ALI), organoid, lung-on-a-chip, and three-dimensional (3-D) aggregate models utilizing rotating wall vessel (RWV) bioreactors (Figure 4).

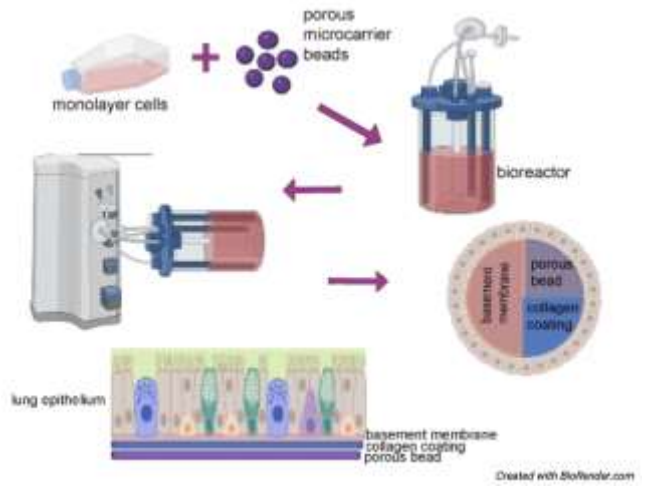


**Figure 4. Trypsinized confluent monolayers get in contact to porous microcarrier beads coated with extracellular matrix are introduced into the slow-turning lateral vessel. the lateral vessel is rotated along a horizontal axis offsets sedimentation, causing gentle falling of the beads and cells through the culture medium, resulting in the establishment of three-dimensional tissue models.[32]**

Air-liquid interface (ALI) cultures involve cells initially submerged in cell culture medium using cell culture inserts on microporous membranes. Once confluent, these cells undergo differentiation upon exposure to air at the apical surface.[34-37] However, this model requires a 21-day incubation period and involves expensive, labor-intensive maintenance, with limitations in throughput. Organoids offer an alternative to maintain in vivo cell characteristics, emerging as well-organized 3-D structures derived from lung stem cells obtained from bronchial brushing and embedded in an extracellular matrix (ECM) gel.[38,39] While efficient and physiologically relevant, they pose challenges for infection studies.[40,41] Addressing this limitation, a recent innovation called "apical-out" lungs has been introduced, specifically designed for studying viral infections.[42,43]

The lung-on-a-chip model is another in vivo-like approach, utilizing a microfluidic device that provides continuous perfusion through porous membranes seeded with cells (Figure 5). This model simulates breathing motions under vacuum, integrating phenotypic, structural, and biomechanical properties akin to in vivo tissue, enhancing therapy responses.[44-46] However, the model's extensive hands-on manipulation and limited throughput constrain its widespread utilization.[47]

Another alternative developed is the low fluid-shear culture condition, where cells grow in an optimized suspension culture on the surface of porous ECM-coated microcarrier beads. This culture is facilitated within a rotating wall vessel (RWV) bioreactor, promoting cell suspension (Figure 5).[48-53] This method generates differentiated cell aggregates that are accessible to external stimuli.[31,32] Moreover, cells from the bioreactor can be differentiated and distributed across multi-well plates for simultaneous multi-testing under diverse conditions.[48-52]

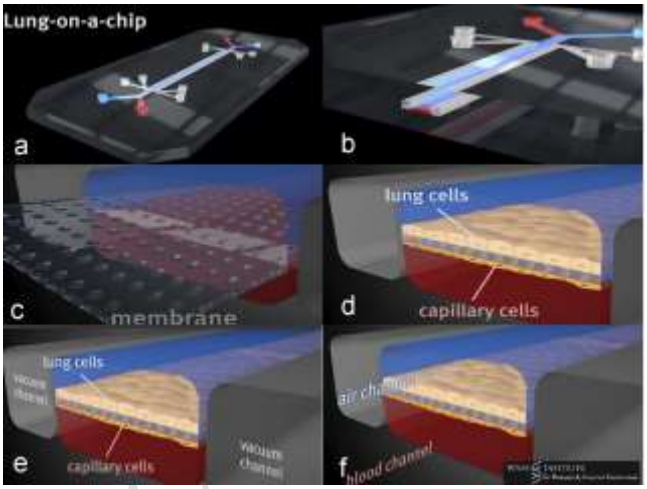


**Figure 5. Lung in a chip model. This model includes epithelial and lung-specific endothelial cells on either side of a porous, ECM-coated membrane. Mechanical cues and soluble factors critical to cell function and differentiation in vivo are included.[53]**

The RWV-derived lung model predominantly utilizes the adenocarcinoma A549 cell line, representing type II alveolar epithelial cells. This 3-D A549 model displays barrier function, apical-basolateral polarity, and multicellular complexity. It demonstrates a better replication of in vivo host responses to infection compared to 2-D monolayers[49,50] and accurately mirrors the pathogen biofilm phenotype observed in chronic lung diseases.[54,55]

Furthermore, there are attempts to create engineered organ replacements utilizing decellularized and recellularized lung scaffolds.[56] Although less probable, there's exploration into the de novo generation of complex three-dimensional lung-like tissues in culture, aiming for potential translational applications.

## 3D CULTURE SYSTEMS

3D culture systems, often referred to as organoids, have significantly advanced the understanding of epithelial cell behavior ex vivo, effectively mimicking the behaviors of their in vivo counterparts.[57,58] They have provided insights into the role of the microenvironment that governs cell behavior.[59,60] However, visualization of these 3D models can pose challenges, often relying on imaging technologies for analysis. Advancements in static and live imaging approaches, including advanced organoid-clearing techniques and improved automation in light-sheet fluorescence microscopy, have facilitated high-throughput screening of organoid experiments.[61]

The development of highly organized 3D tissues, known as organoid culture systems, combining multiple lung cell types, represents an optimal approach for regenerative strategies. They replicate in vivo organogenesis processes by manipulating critical aspects of the cell culture environment to mimic organ structure and function.[62,63] From airway and alveolar epithelial progenitors, various organoids such as tracheospheres[9], alveospheres[64], and other models have been developed. Although significant progress has been made in delineating lung development mechanisms and directing induced pluripotent stem (iPS) cells towards mature lung lineages[65-67], their application in tissue repair and translation to human studies, especially for modeling severe infections[68], is still in its nascent stage.

Recently, the implementation of pluripotent stem cells (PSCs) in organoid assay development has shown promising correlations between mouse models and human diseases. This integration of PSCs in organoids has opened avenues for modeling genetic diseases, enabling drug screening, and the potential development of cell therapies.[69,70]

Previous studies have highlighted the regenerative potential of cell lineages within the mammalian respiratory system, demonstrating the capacity for regeneration following injury through the activation of stem/progenitor populations or proliferation-induced cellular expansion. Furthermore, significant progress has been made in generating lung epithelial cells from embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), providing an additional cell source for disease modeling and potential cell-based therapies.[71,72]

In the realm of basic and applied research, significant advancements have been made in tissue engineering. To establish human airway models, researchers have often used cancer-derived epithelial cell lines.[73-75] Conversely, primary airway epithelial cells possess the capacity to fully differentiate into mature epithelium, yet challenges persist due to limited cell duplications, donor tissue availability, and high donor variability.[73-75]

Efforts to induce epithelial differentiation have incorporated air-liquid-interface (ALI) culture methods[76,77], which have involved co-cultivation with fibroblasts to enhance the differentiation potential of epithelial cells.[78,79] Alternative approaches have utilized collagen-coated transwell inserts[80] or multilayered cultures with more than one scaffold, although maintaining cell-cell contacts poses challenges in these methods (Figure 6).[81-83] Key factors influencing the success of 3D human airway models include the cell culture medium and the growth factors added for enrichment.[81,82]

The ultimate goal of many endeavors is to generate a human 3D airway tissue model that develops a pseudostratified epithelium comprising actively beating ciliated cells, mucin-secreting goblet cells, and basal cells. These cell populations would be complemented by stromal cells to form a biological scaffold.

Differentiated respiratory epithelium showcases distinct characteristics including the formation of tight junctions, apical and basolateral polarity, mucin secretion, and diverse cellular complexity, encompassing ciliated cells, goblet cells, basal cells, and club cells.[84] Tight junctions, situated at cell apices, play a crucial role in regulating paracellular permeability and defining the boundary between the apical and basolateral surfaces, thereby establishing cell polarity.[85] Cell polarity, crucial for vesicle, ion, and solute transportation, as well as the localization of proteins and lipids, is delineated by the orientation of the apical cell surface toward the lumen and the basolateral surface away from it.[86]

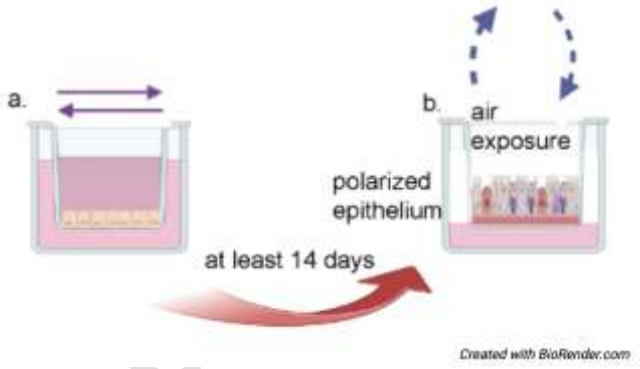


**Figure 6. Schematic representation of the differentiation protocol using culture of the air–liquid interface (ALI). This method enables airway epithelial cells from diverse sources to differentiate into a pseudostratified cell layer, consisting of ciliated cells, goblet/secretory cells, and basal cells. a. Key factors that must be considered during the process. b. featuring a horizontal flow with exposure of culture media. b. optimized fluid dynamics and an effective cell to gas contact.[83]**

ALI models of bronchial epithelial cells have emerged as invaluale tools, particularly in studying conditions like COPD and CF.[35,87,88] These models allow for the evaluation of cell differentiation, tight junction formation, polarity expression, mucin production, and the presence of diverse epithelial cell types using immunostaining and confocal microscopy, often considered the gold standard by many researchers.

## CONCLUSIONS

The generation of organoids is indeed a complex process, and while primary cells derived from patients or induced pluripotent stem cells (iPSCs) have facilitated the prediction of human responses, several challenges remain. One such challenge is ensuring the use of high-quality primary human cells and validating their functionality *in vitro*. Additionally, the poor functional fidelity of iPSCs derived from differentiated adult tissues presents another hurdle. Addressing these challenges is essential to improve the reliability and applicability of 3D technologies to ease the economic burden of drug screening and to provide highly predictive screening systems. However, extensive evaluation and standardization of these systems is required prior to their implementation in pre-clinical drug screening.

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