Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

CHAPTER

26 Modeling viral infection with tissue engineering: COVID-19 and the next outbreaks

Alexander M. Tatara

Division of Infectious Diseases, Massachusetts General Hospital, Boston, MA, United States

Introduction

Pandemics caused by respiratory viruses are an increasing threat to global stability and health. Since the turn of the 21st century, there have been a number of respiratory viral outbreaks including Severe Acute Respiratory Syndrome Coronavirus 1 (SARS-CoV-1) in 2003 [1], H1N1 influenza in 2009 [2], and Middle East Respiratory Syndrome Coronavirus (MERS-CoV) in 2012 [3]. Starting in 2019, the Coronavirus Disease 2019 (COVID-19) pandemic caused by the Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) has led to rapid unprecedented worldwide morbidity and mortality.

Tissue engineering is a convergence science that intersects biology, medicine, and engineering [4]. As leaders in a multidisciplinary field that works to innovate healthcare, tissue engineers can serve an important role in future viral outbreaks [5]. To study respiratory viruses successfully, it is necessary to generate reproducible and validated in vitro tissue models to understand fundamental host/pathogen relationships and to design new diagnostic and therapeutic strategies. One of the key areas in which tissue engineers have expertise is the design of ex vivo tissue constructs. The use of tissue engineering to build models for the study of respiratory viruses with emphasis on SARS-CoV-2 will be important as it is expected that respiratory virus pandemics will continue to plague society in the coming years.

In this chapter, the following topics will be reviewed: SARS-CoV-2 pathophysiology; traditional respiratory virus culture including cell lines used in two-dimensional culture and current state of high-throughput drug screening; tissue engineering-based principles and models for study of respiratory viruses, and future directions of the field.

SARS-CoV-2 pathophysiology

In order to discuss model development, it is important to have a basic understanding of SARS-CoV-2 pathophysiology. The respiratory virus is spread from human to

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human via airborne droplets dispelled when hosts talk, cough, sneeze, or otherwise have airway passages in communication with the environment [6]. The virus initially infects the airway epithelium of the nasopharynx or oropharynx. After a period of inoculation (approximately 5 days), viral particles are shed and the host becomes contagious and capable of transmitting the virus to subsequent new hosts [7]. From a healthcare systems standpoint, the virus is challenging to control due to the prevalence of asymptomatic carriers and the high rate of transmission.

Early in the pandemic, morbidity and mortality were very high. In one of the first series of patients from Wuhan, China, in December 2019, 7% of patients were critically ill and 28-day mortality was 61.5% among those admitted to the intensive care unit [8]. In general, mortality rates are age dependent and were as high as 16.6% in patients greater than 80 years old [9]. In one study from two hospitals in New York City, United States, the level of viral load significantly correlated with risk of intubation and mortality in multivariate models [10]. Being able to measure viral load accurately will be important for models. Epidemiologically, host factors play a role in disease prognosis and progression [11]. Age [9], obesity [12], heart disease [13], and diabetes [14] have all been associated with increased disease severity and mortality. Although challenging, it would be beneficial if a model could be built such that these systemic host factors could be incorporated.

Molecularly, the SARS-CoV-2 genome encodes four major genes related to structural proteins: the nucleocapsid (N), the viral membrane (M), the viral envelope (E), and the spike protein (S) [15]. The S protein is thought to be key to the ability of the virus to interact with host human cells. It interacts with the host angiotensin-converting enzyme 2 receptor (ACE2) to enter the cell and cause infection. Host proteases "prime" the S protein for ACE2 interaction, including transmembrane serine protease 2 (TMPRSS2) [16]. ACE2 and TMPRSS2 are expressed by human epithelium, including cells of the lungs and gut [16]. Another host factor that has been studied in human coronavirus infection is dipeptidyl peptidase 4 (DPP4). It is thought to be the primary binding site for MERS-CoV [17] and may also play a role in SARS-CoV-2 entry into host cells through S protein interaction [18]. Given the importance of these factors in host/pathogen interaction, it is important that tissue models include these host proteins and ensure that they are presented in a physiologic manner.

Traditional respiratory virus culture

Unlike bacteria, viruses require host cells for replication in vitro which creates an additional challenge for virologists. Traditionally, immortalized mammalian cell lines are used for culture. These cells are plated while submerged in cell media and form sheets that are one cell thick. They can then be inoculated with virus [19]. For clinical diagnostics, cytopathic changes can be observed in infected cells or immunostaining can be performed to determine if virus is present within cells after inoculation. For modeling to study viruses, a number of other techniques have been leveraged which include reverse transcriptase quantitative polymerase chain reaction (RTqPCR),

Table 1 Examples of common mammalian cell lines used in respiratory virus research.

Line	Cell type	Species	Example virus
A549	Lung carcinoma	Human	Adenovirus
B95-8	Lymphoid tumor	Primate	Measles
HeLa	Cervical carcinoma	Human	Rhinovirus
HEp-2	Laryngeal carcinoma	Human	RSV
LLC-MK2	Kidney epithelium	Primate	Rubella
MDCK	Kidney epithelium	Canine	Influenza
NCI-H292	Lung carcinoma	Human	Parainfluenza
Vero E6	Kidney epithelium	Primate	SARS-CoV

enzyme-linked immunosorbent assay (ELISA), and genetic manipulation of virus and/or host. The cells that are often used for the study of respiratory viruses need to be capable of propagation for multiple passages. Therefore they are often restricted to immortal cell lines [19]. Examples of common mammalian cell lines used in the culture respiratory viruses with representative examples of viruses studied in each line can be found in Table 1 [1,20–26]. Note that these cell lines feature mutations that lend themselves to in vitro culture as normal host cells cannot be passaged indefinitely. Therefore some of these mutations do not reflect normal cell physiology and the subsequent effects of these mutations on viral pathogenesis are not necessarily clear. Out of available mammalian cell lines, Vero cells have rose to prominence in the study of SARS-CoV-2.

Vero cells

Vero cells were first isolated from an African Green Monkey kidney in 1962 by Yasumura and Kawakita [27]. They intrinsically contain a deletion in type I interferon genes so cannot produce interferon alpha or beta, making them more susceptible to viral infection. Subsequently, they have been used to study of a number of viruses, including simian polyoma virus SV-40, measles virus, rubella virus, arboviruses, adenoviruses, influenza virus, Ebola hemorrhagic fever virus, and MERS-CoV [27].

During the 2003 SARS outbreak, investigators from the SARS Working Group attempted to isolate the virus from infected human specimens [1]. They attempted to culture the virus using a variety of cell lines including Vero E6, NCI-H292, MDCK, LLC-MK2, and B95-8 cells (see Table 1 for description). They monitored cell lines for 14 days for cytopathic effects suggesting viral infection—only Vero E6 cells were able to be inoculated with cytopathic effects observed by day 5. From this point on, Vero cells became one of the most common mammalian cell lines used for the study of SARS-CoV. During the SARS-CoV-2 pandemic, these efforts were repeated and once again, Vero cells compared to other mammalian cell lines demonstrated increased viral replication [28]. Therefore Vero cells have continued to be the primary

cell line for laboratory culture of SARS-CoV. Two-dimensional monolayers of Vero cells are used to study SARS-CoV host/pathogen interactions, evaluate diagnostic strategies, and test and screen for new therapeutic compounds to treat COVID-19.

High-throughput drug screens

Because of ease of culture, passage, and infection, Vero cells and other cell lines have been leveraged to develop high-throughput drug screens (HTDSs). Two-dimensional monolayers can be grown and expanded into thousands of wells on incubator plates. Each well represents an experiment. There are commercially available libraries of chemical compounds as well as specific libraries of drugs that have already received FDA approval for other indications that are attractive candidates for repurposing. Vero cells in monolayer can be exposed to these compounds at different concentrations, one drug at a time. If compounds are being tested as prophylactic (preventative) agents, they can be added to Vero cells prior to viral inoculation. If compounds are being tested as therapeutic agents, they can be added to Vero cells after viral inoculation.

There are two major types of output from a HTDS—the effect of the compound on the host cell (compound cytotoxicity) and the effect of the compound on the virus/viral replication (compound efficacy) [29]. The output of these screens can be measured in different ways, but the key to a high-throughput system is that the assay can be measured quickly and efficiently. For example, using manual bright field microscopy to measure cytoplasmic response to infection would not be feasible when screening thousands of compounds with replicate wells. Assay output is typically measured using biomarkers that are bioluminescent/fluorescent or by measuring gene expression surrogates, such as by RTqPCR.

Vero cells have been used in a number of HTDSs, including drug discovery for emergent viruses such as Ebola hemorrhagic fever virus [30], dengue [31], Lassa virus [32], Zika virus [33], and West Nile virus [34]. Severson and colleagues used Vero E6 cells to screen 100,000 compounds for efficacy against SARS-CoV-1 [35]. After incubation in compounds, a commercial luminescent viability assay was performed to evaluate cytotoxicity. By measuring the cytotoxicity of the agent alone versus cytotoxicity of the viral infection itself, a calculation was made to measure total "cytopathic effect." While this method may be more challenging to differentiate compound cytotoxicity from viral cytoplasmic response, it does simplify assay output to essentially a single variable. Using this HTDS, the authors had a hit rate of 0.8% (compounds with cytopathic effect > 50% without compromising cell viability in the absence of viral infection). Three compounds in particular had a favorable ratio of reducing cytoplasmic viral response while not inhibiting host cell Vero cell growth [35].

Another example of a HTDS for SARS-CoV-1 was performed using a 2000 compound library of drugs that had previously been approved by the FDA for other indications [36]. Compound cytotoxicity against host cells was measured by methylthiazolyl-tetrazolium assay. To determine the effect of compounds on viral infection,

a SARS-CoV-1 strain was engineered to express luciferase and luminescence was measured as a reporter for viral replication. BHK-21 cells were used rather than Vero cells. The group had 56 hits in this screen and then validated those hits using wild-type SARS-CoV-1 with viral replication measured using RTqPCR in which 36 of 54 prior hits were effective against wild-type SARS-CoV-1. The group further screened those 36 compounds against four other strains of human coronavirus and found seven of the compounds to be effective against all strains. They demonstrated that two of those seven compounds were able to increase survival rates in a murine model of coronavirus infection [36].

With the emergence of SARS-CoV-2, there has been significant interest in discovering new therapeutics to treat or prevent COVID-19. In a Vero cell screen, SARS-CoV-2 was added after host cells were exposed to 10 μM of drug from a library of 1520 compounds [37]. Three days after infection, plates were incubated in CellTiter-Blue reagent for a fluorescent live/dead assay to calculate cell viability and RTqPCR was performed to determine viral replication. There were 90 positive hits—11 had favorable antiviral potency while minimizing host cell cytotoxicity. Of note, hydroxychloroquine and chloroquine were the third and fourth most potent candidates, respectively.

By using HTDSs, tens of thousands of compounds can be examined to rapidly identify a handful of candidates that mitigate viral infection in 2D cell culture. However, a screen is only useful if its targets have physiologic relevance. As discussed earlier, there are significant mutations in 2D cell culture lines. For example, Vero cells do not produce interferon (a key host mechanism against viral infection) and originate from primate kidney epithelium. This raises questions regarding whether or not it is a physiologically representative system to be screening compounds against a respiratory virus.

Challenges and limitations of 2D viral culture

The techniques discussed previously were harnessed in response to the Ebola hemorrhagic fever virus outbreak in 2014. A Vero-based HTDS was used to search for candidate compounds. One of the top hits that arose from the screen was sertraline [30]. Sertraline is a selective serotonin reuptake inhibitor that has been approved by the FDA for treatment of depression and other psychiatric illnesses. The study authors proposed that the mechanism for viral efficacy may be through interference with endosomes and fusion based on subsequent Vero E6 cell studies. They demonstrated that there was a partial protective effect of sertraline in a murine model [30]. Next, a preclinical study was performed to determine if sertraline had any effect in preventing or treating Ebola hemorrhagic fever virus infection in nonhuman primates. Unfortunately, sertraline was not effective in improving survival or reducing viral load in the nonhuman primate model despite its promising effects in the protection of Vero cells [38].

Relevant to respiratory virus pandemics, recall that hydroxychloroquine and chloroquine were two of the top four drug candidates in the Vero cell HTDS for

SARS-CoV-2 discussed previously [37]. The two are closely related molecules but hydroxychloroquine is considered less cytotoxic and results of its efficacy in protecting Vero cells from SARS-CoV-2 were replicated by other groups [39]. However, in a nonhuman primate preclinical model of SARS-CoV-2 infection, hydroxychloroquine was not effective [40].

During the initial phases of the COVID-19 pandemic, a small nonrandomized clinical study was performed using hydroxychloroquine based on in vitro 2D culture data [17]. The study suggested that hydroxychloroquine (with and without azithromycin) had therapeutic benefits in the treatment of COVID-19 [41]. This led to widespread treatment of COVID-19 patients with hydroxychloroquine. Subsequent studies with larger data sets have suggested that hydroxychloroquine is not an effective agent against SARS-CoV-2 in humans and in fact may be associated with greater side effects without benefit [42].

These are two examples of failure of current 2D viral culture techniques to translate to human pathophysiology. In the latter case, these results may have even misguided clinical practice during a pandemic. These illustrate some of the limitations of 2D culture. Vero cell culture results in a monolayer of mutated nonhuman primate kidney epithelium that cannot produce interferon. SARS-CoV-2 infects the nasopharynx airway epithelium, a complex anatomic structure with multiple cell types as depicted in the cartoon in Fig. 1. Lung epithelium is comprised of multiple cell types, including ciliated cells for moving particles out of the airways, goblet cells for secretion of mucins and other defense molecules, and a basal layer with adult stem cells. Native lung tissue features polarity, where host cells in the top "apical" layer have different features than those against the basement membrane in the "basolateral" layer. The apical cells are exposed to the air while the basolateral cells are against the basement membrane and interstitial fluid. With deficiencies in anatomic cues and native cell types, it is possible that current monolayer cell culture systems lack relevant physiologic information to model viral infection.

Tissue engineers have been studying 2D versus 3D tissue constructs for many years, most typically in the context of modeling tumor behavior. Compared to 2D models, 3D tissue models allow for more physiologic cell morphology, polarization, cell-to-cell contact, and gene/protein expression than 2D counterparts [43]. Tissue engineering may be leveraged to produce modeling systems that more accurately represent host/pathogen interactions in vitro than current 2D viral culture.

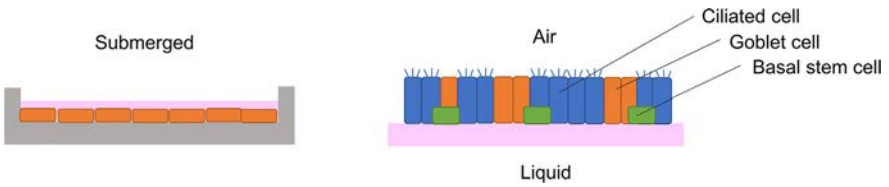

FIG. 1

Cartoon comparing current ex vivo 2D cell culture (left) to native lung epithelium (right).

No Permission Required.

Tissue engineering-based models

In order to discuss 3D tissue models for viral culture, the basic principles of tissue engineering will be reviewed in the context of human respiratory tissues. The tissue engineering paradigm consists of three components—cells, scaffold, and signals (Fig. 2).

Different combinations of elements from the tissue engineering paradigm have been studied. To engineer human 3D lung tissue constructs, labs have used adult pulmonary epithelial stem cells harvested from donors during biopsy, reprogrammed adult cells to create induced pluripotent stem cells, or embryonic stem cell lines [44–46]. The most common biomaterials used as scaffold include collagen membranes, gelatin scaffolds, and Matrigel, a commercially available extracellular matrix produced from mouse sarcoma cells [45–47]. Synthetically derived biomaterials such as biodegradable polymers can also be used but are less common in lung models for viral infection [48]. Signals nearly always include a cocktail of growth factors to induce differentiation into the different lung cell types as well as different mechanical signals, such as exposing the apical surface to air instead of liquid media and treating the cells with physiologic shear stress to activate specific biological pathways [46,47].

Using the tissue engineering paradigm, three major 3D models associated with respiratory viral infection have emerged in the field. These are the human airway epithelial model, the organoid model, and the rotating wall vessel bioreactor model.

Human airway epithelium model

In the human airway epithelium (HAE) model, a protocol has been developed to create pseudostratified pulmonary epithelium in vitro from adult stem cells isolated

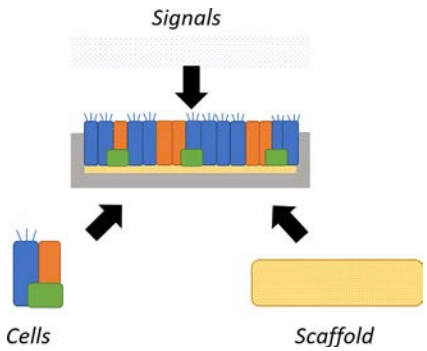

FIG. 2

The tissue engineering paradigm as applied for pulmonary tissue engineering states that combinations of cells relevant to lung tissue, scaffolds with properties similar to tracheobronchial tissue, and specific pulmonary biochemical and mechanical signals will result in generation of lung-like tissue.

from the basal layer of biopsies from human donors [49]. They are then cultured on a collagen membrane while submerged under cell media. Growth factors are used to promote differentiation into other respiratory epithelial cell types, including goblet cells, club cells, and ciliated cells, while maintaining basal stem cells. As the tissue matures, the mechanical signals are changed as media is removed and the apical surface of the tissue is exposed to the air. This facilitates the maturation of the ciliated cells and results in further polarization [49,50]. The cell types in the HAE express some features of native innate immunity, including secretion of mucous by goblet cells and beating of cilia to clear particles by ciliated cells. The resulting 3D tissue construct, featuring multiple respiratory epithelial cell types and cell polarization, has been harnessed to study respiratory viruses.

For example, respiratory syncytial virus (RSV) is a pathogen which can cause severe disease in infants, the elderly, and the immunosuppressed. It has been demonstrated in the HAE model that RSV preferentially infects apical cells rather than basolateral cells using virus that has been engineered to express green fluorescent protein [46]. Furthermore, the virus specifically infected ciliated cells on the apical surface, suggesting that cilia may be critical in RSV pathogenesis. Similar results regarding selective infection of apical ciliated cells were found in an HAE model using cells sourced from pediatric patients. This study also demonstrated that cells in the HAE model upregulated the inflammatory cytokines CXCL10, CCL5, IL-6, and CXCL8, similar to the upregulation pattern seen in vivo during infant RSV infection [51].

In addition to modeling host/virus relationships and the host inflammatory response in RSV infection, the HAE model has also been used to explore therapeutics for RSV. Palivizumab is a monoclonal antibody that targets the RSV fusion protein and is given as prophylaxis against RSV in high-risk infants. Nirsevimab is a newer monoclonal antibody that has been engineered to have longer half-life for easier delivery. It was tested against RSV in the HAE model and compared to palivizumab where it was shown to have similar efficacy at lower concentrations [52]. A clinical trial also demonstrated that a single dose of nirsevimab for prophylaxis in infants statistically significantly reduced RSV incidence by 70% compared to placebo [53], suggesting that the HAE model may be helpful in evaluating therapeutics in vitro for respiratory viruses. The same platform was used to evaluate a neutralizing antibody against human metapneumovirus [52].

In addition to seasonal respiratory viruses, the HAE model has been leveraged to study pandemic strains of respiratory viruses. For example, the H1N1 pandemic in 2009 resulted in over 12,000 deaths in the United States alone [2]. Using the HAE model, the seasonal H1N1 strain was compared to the 2009 H1N1 pandemic strain to better understand its infection kinetics. It was demonstrated that viral replication in the pandemic strain occurred more rapidly in HAE models generated from bronchial biopsies than the seasonal strain. This difference was not observed in HAE models generated from nasopharyngeal biopsies and the difference was even more pronounced in in vitro models of conjunctival tissue [54]. Host cell cytokine expression in response to viral infection was also measured but there were no significant differences between seasonal and pandemic strains.

In addition to seasonal and pandemic respiratory viruses, novel/emerging respiratory viruses have been studied using the HAE model. Some respiratory viruses are unable to be cultured by traditional 2D methods. For example, human rhinovirus C (HRV-C) was found to be a major contributor to pediatric rhinovirus infections but 2D monoculture could only be infected by human rhinovirus strains A and B. Bochokoy et al. demonstrated that HRV-C could be cultured using the HAE model with cells from pediatric nasal biopsies. This work suggested that HRV-C has a different mechanism of host cell entry compared to other human rhinoviruses [55]. Human bocavirus, another pathogen responsible for pediatric respiratory illness, was also first grown in vitro using the HAE model where its messenger RNA transcription was studied [56]. The HAE model has been used to study the host/pathogen interface of seasonal, pandemic, and novel respiratory viruses as well as to evaluate the promise of new therapeutics against these infections.

Given its success, the model has also been applied to the study of human coronavirus. HAE models have shown that different strains of human coronavirus (hCoV) have different replication kinetics in host cells, regardless of donor [57]. However, all strains preferentially exhibit tropism toward apical cells rather than basolateral cells. Unlike RSV, some strains of hCoV infect ciliated cells while others selectively infect nonciliated apical cells [57]. Specifically in a strain that preferentially infects nonciliated cells, it was shown that DPP4 (discussed earlier in this chapter) was associated with the viral S protein. Anti-DPP4 antibody decreased hCoV infection in the HAE model [58]. This suggests firstly that DPP4 may be a significant host factor in hCoV strains that target nonciliated apical cells and secondly that anti-DPP4 may have a therapeutic role. While both ideas need further validation, these are examples of hypothesis generation that can occur using a 3D tissue culture model of hCoV infection.

As discussed previously, ACE2 is known to be a significant host factor involved in SARS-CoV infection through interactions with viral S protein [16]. However, ACE2 is not strongly expressed in 2D culture—work by Jia et al. demonstrated that the 3D HAE model expresses significantly more ACE2 specifically on the apical surface and that this expression further increased when the HAE model matured after transfer to the air-liquid interface [50]. SARS-CoV-1 was used to infect both traditional 2D culture and the 3D HAE model and it was shown that the 3D HAE model resulted in significantly upregulated viral S protein and N protein. Further work demonstrated that apical ciliated cells specifically expressed ACE2 rather than nonciliated cells, suggesting a mechanism for SARS-CoV-1 tropism toward apical ciliated cells. Polyclonal antibodies against ACE2 decreased SARS-CoV-1 infection in the HAE model [59]. Expression of ACE2 is upregulated by interferon on human respiratory epithelium, and as discussed previously, Vero cells do not produce type I interferon [16]. In the HAE model, it was demonstrated that interferon alpha and lambda upregulate expression of CXCL10, a chemoattractant for different immune cells [60].

In these studies, antibodies and interferon were explored to understand pathophysiology rather than as specific therapeutics, although these agents are being

actively explored for treatment of SARS-CoV-2 [61]. The host/virus interface that is recapitulated in 3D tissue culture models may allow for discovery and evaluation of novel drugs. In one study, tracheobronchial epithelial cells from surgical specimens were grown in the HAE model to study host TMPRSS2 and SARS-CoV-1 [62]. It was demonstrated that camostat, an inhibitor of TMPRSS2, caused significantly less viral replication in the HAE model of SARS-CoV-1 infection. In traditional 2D culture, there was no effect of camostat on SARS-CoV-1 infection. These results were validated in a murine model [63] and camostat is currently in a phase II trial for SARS-CoV-2 [64].

In addition to suggesting efficacy of different therapeutics in situations where 2D modeling does not replicate key host/pathogen interactions, the HAE model has been leveraged to rule out therapeutic candidates. As discussed earlier, there was significant controversy about the efficacy of hydroxychloroquine for treatment of SARS-CoV-2 [65]. In one comprehensive study, the effect of hydroxychloroquine on SARS-CoV-2 infection was examined in the 2D Vero cell model, the 3D HAE model, and a nonhuman primate model. Hydroxychloroquine was effective in traditional 2D culture but not in the HAE model nor nonhuman primate model [40]. This suggests that 3D tissue models, at least in this specific instance, may be more physiologically relevant for therapeutic evaluation than traditional 2D culture prior to animal or clinical trials for drug candidates in COVID-19.

With the success of the HAE model, it has become widely used in the field to study lung physiology in general. There are even commercial products available to purchase kits or entire HAE tissue constructs for laboratory study [66]. The advantages of HAE as a 3D model include inclusion of multiple epithelial cell types, physiologically relevant air-liquid interface, polarization, and the ability to generate the model from specific donor biopsies, allowing for a "personalized" approach and customization of cell sourcing. Disadvantages include the exclusion of other pulmonary cell types (no cells of mesenchymal or endothelial origin) and the time it takes for the system to mature (6–8 weeks).

Organoid models

Organoids are tissue constructs that are grown from clusters of embryonic or adult stem cells grouped in embryoid clusters. After exposure to different growth factor cocktails meant to simulate developmental stages, they form their own extracellular matrix and differentiate into spheroids that possess native architectural elements observed in human tissues. One of the first examples of using organoids for viral culture was when human norovirus was grown in intestinal organoids, a virus that previously could not be reproducibly cultured in vitro using traditional methods [67]. Given this landmark work, most organoid work in virology has focused on the intestinal model [68]. However, there has been some preliminary work to assemble pulmonary organoids for respiratory viral modeling.

For example, Porotto et al. grew organoids to study parainfluenza infection. They created organoids from an embryonic stem cell line embedded in Matrigel and

demonstrated that these cells could be differentiated to produce a tissue with ciliated cells, club cells, goblet cells, type II alveolar epithelial cells, and mesenchymal cells with polarization and architecture resembling native lung tissue [45]. They were able to infect these organoids with parainfluenza and demonstrate that the viral genome was stable for at least 28 days within these cultures. In 2D culture for comparison, the viral genome had a higher mutation rate which suggested that 3D models have a selection pressure similar to native lung. RSV was also studied using this model and resulted in increased epithelial sloughing similar to human RSV infection. Likewise, the model was used to study measles virus which resulted in increased host cell syncytia [45]. Another group created respiratory organoids using the A549 line of alveolar epithelial type II cells and methylcellulose as a thickening agent to form 3D spheroids. The organoids were capable of being inoculated by RSV and could sustain viral replication for at least 7 days in vitro [69].

While not a respiratory virus, one of the earliest coronavirus work in organoids was performed to study porcine epidemic diarrhea virus (PEDV), an alphacoronavirus that is contagious among swine. Porcine intestinal crypt stem cells were used to generate spheroids in a Matrigel matrix [70]. As the apical layer was innermost in the 3D sphere, organoids were broken down into 2D enteroid sheets for infection. PEDV was able to infect multiple cell types in the model including enterocytes, goblet cells, and crypt stem cells. PEDV preferentially infected ileal-derived cells over colonic-derived cells.

Given the history of intestinal organoids, it is not surprising that some of the first organoid work for SARS-CoV-2 was in a gastrointestinal model to better understand hCoV gut infection and replication. Organoids were constructed from adult stem cells isolated from ileal and colonic biopsies collected from noninfected patients. Embryoid bodies were formed in Matrigel and were able to grow multiple cell types in a 3D structure with an apical and basolateral polarity [71]. SARS-CoV-2 preferentially infected the apical surface of the organoids rather than the basolateral surface. Virus preferentially associated with areas of ACE2 expression. This organoid model was also used to demonstrate that TMPRSS2 and TMPRSS4 serine proteases cleaved SARS-CoV-2 S protein and facilitated membrane fusion and infection in intestinal organoids. Virus preferentially infected ileal-derived organoids compared to colonic-derived organoids [71]. The 2D colonic-derived organoids have also been generated to explore the effect of interferon on SARS-CoV-2 gastrointestinal infection. It was demonstrated that interferon beta and interferon lamba both mitigated SARS-CoV-2 infection in 2D intestinal organoids [72]. Similar techniques were used to create human 3D kidney organoids with subsequent inoculation with SARS-CoV-2. The organoids were capable of being infected and it was shown that soluble ACE2 receptor inhibited viral infection [73]. These platforms may be used to better understand nonpulmonary COVID-19 pathology.

There are several groups working on lung organoids for SARS-CoV-2 and it is expected to be a very active research area in the future [68,74]. Some of the advantages of the organoid model are that organoids are relatively simple to generate, can be grown from patient biopsies, and similar culture techniques can easily be

modified to generate different types of tissues, including gastrointestinal and renal models. Disadvantages to the organoid platform include that it is less established for pulmonary models, may require manipulation to access the apical layer which is important in respiratory viral infection, and often depends on Matrigel as a scaffold system which is a heterogenous extracellular matrix created by a murine sarcoma line. Organoids are another tissue engineering-based tool available for the study of respiratory viral infection.

Rotating vessel wall bioreactor models

Another 3D tissue model that has been utilized for the study of respiratory virus infections is the rotating wall vessel (RWV) bioreactor model. This model involves a bioreactor which rotates and creates a shear force on cells that simulates physiologic conditions. The scaffold used for these models is typically a collagen or gelatin-based microparticle system [75]. Cells can be seeded onto the scaffold prior to placement in the bioreactor or seeded at different time points during shear conditions. This system has been used to generate a number of 3D tissue constructs, including genitourinary, neuronal, and pulmonary tissues [75]. Lung models have been generated to study RSV, parainfluenza, and SARS-CoV-1.

In one example, cyclodextran microparticles coated in collagen were seeded with human mesenchymal bronchial-tracheal cells (which contain endothelial and mesenchymal cell types) from patient biopsies and cultured in the RWV system for 4 days [76]. At that time, immortalized human bronchial epithelial cells (BEAS-2B) were added to the culture system to create tissue constructs with three cell lineages (mesenchymal, endothelial, and epithelial lines) in a layered approach. These tissues expressed cell-cell adhesion markers, produced mucin, and created extracellular matrix. The constructs were then inoculated with RSV and assayed at different time points for viral infection and replication. Immunostaining demonstrated expression of viral glycoproteins and viral titers confirmed successful infection and replication—compared to an analogous 2D system, RSV titers were persistently elevated in this 3D RWV model [76]. This same system was also compared to Vero and LLC-MK2 lines (Table 1) with parainfluenza and RSV infection and found to have comparable replication efficiency [77]. The cytokines secreted by these 3D systems were also compared to infected patient nasal washings as well as traditional 2D monolayer culture where it was demonstrated that the 3D RWV model resulted in cytokine release more similar to patient samples than 2D models [77].

Given its success in modeling other respiratory viruses, the RWV bioreactor platform was applied to SARS-CoV-1 using microporous gelatin beads as scaffold [47]. The layered coculture system was inoculated with SARS-CoV-1 and studied over 10 days. However, viral titers were negative when assayed. Under electron microscopy, disruption of the endoplasmic reticulum was observed following inoculation. In addition, immunostaining showed components of the virus within cells including spike antibody at the envelope. This suggested that the virus may be able to infect cells in this model but was unable to replicate. The authors

hypothesized that the BEAS-2B cell line may not be a suitable epithelial host cell for SARS-CoV-1 replication in this in vitro model.

Overall, the RWV model appears promising for some respiratory viruses but not others, such as SARS-CoV-1. Given that immortalized epithelial line used can be modified, experiments in the future may better be able to pair epithelial layer to specific virus to enable more favorable host/virus interactions. Some of the advantages of the model include the ability to incorporate mesenchymal, epithelial, and endothelial cell lines and the incorporation of physiologic shear stress mimicking fluid flow during native tissue development. Disadvantages include the need for specialized equipment (such as the rotating wall vessel apparatus) and the inability to demonstrate SARS-CoV-1 replication in the model.

Conclusions and future directions

In this chapter, the motivation to study respiratory viruses in vitro was introduced, the pathophysiology of SARS-CoV-2 was reviewed, current 2D viral techniques including HTDSs were evaluated, and the use of tissue engineering principles to generate 3D tissue constructs for respiratory viral culture and study was highlighted. Three specific models were discussed at length (Table 2). All three of these platforms feature 3D tissue constructs generated from the combination of specific human cells, biomaterial scaffolds, and mechanical and biochemical signals. Each model has also been adapted to be generated from patient-derived tissues from biopsies and other sources, allowing for customization and personalization of the system to study specific host physiology.

Table 2 Examples of advantages and disadvantages of three tissue engineering-based models for the study of respiratory viruses.

Model	Advantages	Disadvantages
Human epithelial airway	- Well established in the literature with many types of viruses - Features physiologic air-liquid interface	- Limited to epithelial cells - Takes 6–8 weeks for tissues to mature
Organoid	- Can be modified to generate different types of tissues - Demonstrated extended stability of replicating viral genome	- Limited data available on lung organoids and viral infection - Often dependent on Matrigel
Rotating wall vessel	- Able to incorporate different cell lineages - Incorporates low shear stress similar to developmental states	- Unable to model SARS-CoV-1 replication - Requires specialized equipment

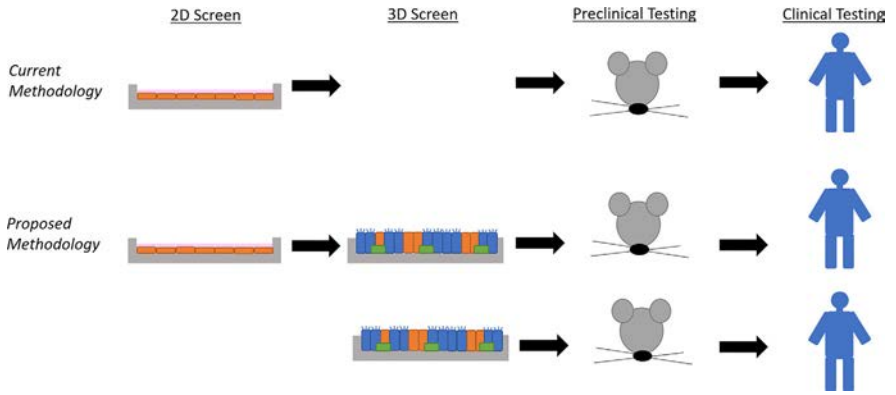

FIG. 3

Current and envisioned paradigms in drug discovery for viral pandemics.

No Permission Required.

Depicted in Fig. 3 is the current paradigm for using tissue culture for drug discovery. The 2D cell culture models are exposed to thousands of compounds and candidates are then tested in preclinical animal models prior to clinical trials. Using tissue engineering, one could envision a second model as shown in the center of Fig. 3, where candidates from the 2D screens could be further validated in 3D tissue engineered models which more faithfully recapitulate host/pathogen relationships. Candidates from the more stringent second screen could then go on to preclinical studies. As tissue engineering matures as a field, we may be able to more rapidly generate and evaluate these 3D tissue constructs, possibly even bypassing 2D culture entirely for drug discovery as shown in the bottom row of Fig. 3.

Technological breakthroughs may facilitate this vision. For example, work has been done to develop pharmacologic agents that may quicken the maturation of the HAE model [78], which could enable its ability to be used for HTDSs. Likewise, the process of generating organoids has recently been automated which could further lend the model to HTDSs [79]. While three tissue engineering platforms were discussed in this chapter, there are other examples of in vitro pulmonary systems and more will continue to emerge given the importance of this field. One exciting area of rapid development is "organ-on-a-chip," where microfluidics and cell biology are harnessed to create tissues with precise control over spatial alignment and fluid transfer [44–46]. These models can contain multiple compartments, such as vascular (endothelial-derived) space and connective tissue (mesenchymal-derived) component [80,81]. In addition, systems such as the microfluidic approach or organoid model may allow for coculture of different organ system representatives, such as culturing lung tissue, gastrointestinal tissue, and renal tissue in series. This approach may facilitate better understanding of how the virus propagates across tissues as well as capture endocrine interactions and cross-talk occurring between organ systems during respiratory viral infection.

Pandemics from respiratory viruses have affected nearly every facet of society in the 21st century. Biomedical science is working to overcome these emerging threats. Most recently, there have been tremendous efforts across many fields to improve diagnostics, therapeutics, and healthcare operations in response to the disease COVID-19 caused by the virus SARS-CoV-2. Tissue engineering is a promising area to create models which more faithfully recapitulate host/virus interactions for the study of human coronavirus and development of platforms to screen for therapeutics.

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