ABSTRACT Human adenoviruses type 3 (HAdV-3) and type 55 (HAdV-55) are frequently encountered, highly contagious respiratory pathogens with high morbidity rate. In contrast to HAdV-3, one of the most predominant types in children, HAdV-55 is a reemergent pathogen associated with more severe community-acquired pneumonia (CAP) in adults, especially in military camps. However, the infectivity and pathogenicity differences between these viruses remain unknown as in vivo models are not available. Here, we report a novel system utilizing human embryonic stem cell-derived 3-dimensional airway organoids (hAWOs) and alveolar organoids (hALOs) to investigate these two viruses. Firstly, HAdV-55 replicated more robustly than HAdV-3. Secondly, cell tropism analysis in hAWOs and hALOs by immunofluorescence staining revealed that HAdV-55 infected more airway and alveolar stem cells (basal and AT2 cells) than HAdV-3, which may lead to impairment of self-renewal functions post-injury and the loss of cell differentiation in lungs. Additionally, the viral life cycles of HAdV-3 and -55 in organoids were also observed using Transmission Electron Microscopy. This study presents a useful pair of lung organoids for modeling infection and replication differences between respiratory pathogens, illustrating that HAdV-55 has relatively higher replication efficiency and more specific cell tropism in human lung organoids than HAdV-3, which may result in relatively higher pathogenicity and virulence of HAdV-55 in human lungs. The model system is also suitable for evaluating potential antiviral drugs, as demonstrated with cidofovir.

IMPORTANCE Human adenovirus (HAdV) infections are a major threat worldwide. HAdV-3 is one of the most predominant respiratory pathogen types found in children. Many clinical studies have reported that HAdV-3 causes less severe disease. In contrast, HAdV-55, a reemergent acute respiratory disease pathogen, is associated with severe community-acquired pneumonia in adults. Currently, no ideal in vivo models are available for studying HAdVs. Therefore, the mechanism of infectivity and pathogenicity differences between human adenoviruses remain unknown. In
this study, a useful pair of 3-dimensional (3D) airway organoids (hAWOs) and alveolar organoids (hALOs) were developed to serve as a model. The life cycles of HAdV-3 and HAdV-55 in these human lung organoids were documented for the first time. These 3D organoids harbor different cell types, which are similar to the ones found in humans. This allows for the study of the natural target cells for infection. The finding of differences in replication efficiency and cell tropism between HAdV-55 and -3 may provide insights into the mechanism of clinical pathogenicity differences between these two important HAdV types. Additionally, this study provides a viable and effective in vitro tool for evaluating potential anti-adenoviral treatments.

**KEYWORDS** adenovirus, human adenovirus type 55, human adenovirus type 3, community-acquired pneumonia, lung organoid, cell tropism, antiviral, viral replication

**H**uman adenovirus (HAdV) is a common pathogen responsible for a range of pathogenicity, including upper and lower respiratory tract infections (1, 2). Symptoms can manifest as pharyngitis, tonsillitis, cough, fever, or pneumonia (1). It is a highly contagious pathogen, and the epidemics of adenovirus infections may occur among people in closed or crowded settings, for example group homes, boarding schools, hospitals, and military settings (2–4). Adenovirus infections in general are relatively mild and self-limiting in immunocompetent individuals. The disease is more severe, and dissemination is more likely in patients with impaired immunity (e.g., organ transplant recipients and human immunodeficiency virus infected individuals) (5).

To date, 113 HAdV types, parsing into 7 species (A to G), have been identified and characterized (http://hadvwg.gmu.edu/). Of these, with 4 exceptions (HAdV-11, 34, 35, and 50), types within the HAdV-B species are associated with respiratory diseases (6, 7). This species is further subdivided into B1 (including HAdV-3, 7, 16, 21, and 50) and B2 (including HAdV-11, 14, 34, 35, and 55). Among these, HAdV-3 is implicated in endemic and epidemic HAdV infections in children and adults globally (8), including infections in young children and neonates (9, 10), leading to upper respiratory symptoms (11). HAdV-55, a relatively recent reemergent recombinant with parentals of types 11 and 14 (12), has been linked to severe and fatal pneumonia in immunocompetent adult, with outbreaks occurring in both the military and the civilian communities (4, 13–17). Moreover, compared with other genotypes linked with adenoviral infections, the symptoms of pneumonia caused by HAdV-55 infection are usually more severe (15, 17). Despite this, specific antiviral drugs and vaccines are still unavailable. The mechanisms underlying the pathogenicity of HAdV-55 have not been elucidated. One of the main constraints is the lack of ideal models to support adenoviral pathogenicity studies.

HAdV-3 and HAdV-55 are highly host-range species-specific pathogens. Specifically, they do not bind to mouse or hamster cells. Transgenic mice allow HAdV-3 and 55 infections, but do not appear to support viral replication (18, 19). Traditional human cell lines, such as primary human airway human epithelial cells, are difficult to obtain (20); other transformed or cancer cell lines, e.g., A549, are abnormal states that are unable to support mimicking virus-host interactions faithfully. They also poorly recapitulate the histology of the human airway epithelium (21). Thus, a biologically relevant, reproducible, and readily available in vitro model is urgently needed for studying the biology and pathology of these viral pathogens in the human respiratory tract.

Organoids are self-organizing three-dimensional (3D) structures derived from stem cells that recapitulate essential aspects of organ structure and function (22). Unlike cell lines, organoids present ideally all cellular components of a given organ, and are theoretically well-suited for infectious disease studies, particularly for pathogens that are restricted to humans and that are dependent on specialized cell types (23). IPS-derived lung organoids have already been used to study various respiratory viruses (24–26), and our previous study has demonstrated that SARS-CoV-2 infects and extensively replicates in human embryonic stem cells (hESCs)-derived lung organoids, including airway and alveolar organoids (27). Here, using a human lung organoids platform that
includes lung airway organoids (hAWOs) and alveolar organoids (hALOs), we explore and report on HAdV-3 and HAdV-55 cell tropism, viral replication efficiencies, and viral life cycles, as well as demonstrate an antiviral drug application.

RESULTS

Effective generation of hAWOs and hALOs derived from hESCs. Based on our previous studies (27), hESCs were first differentiated into definitive endoderm by the addition of Activin A and CHIR-99021, followed by a change to and incubation in AFE medium containing Noggin, SB431542, CHIR-99021, and FGF4 for 4 days. On day 7, the 2D culture was transformed into 3D culture by embedding with Matrigel and adding VAFE stage culture medium, which included ATRA, CHIR, and BMP4. Afterwards, during days 14 to 21, a combination of CHIR99021, FGF10, KGF, and DAPT (CFKD) was added as a precondition treatment for the optimal induction of NKX2.1+ lung progenitor cells. At day 21, lung progenitor cells differentiated into lung airway organoids (hAWOs) and alveolar organoids (hALOs) by regulating Wnt signaling (Fig. 1A). As expected, lung progenitor cells (E-cad1, NKX2.11) were effectively generated by day 21 of the differentiation period, as detected by immunohistochemistry and reverse transcription-quantitative PCR (qRT-PCR) (Fig. 1B). Analysis also showed that, compared to controls (day 0 hESCs), lung hAWOs (evidenced by robust mRNA and protein expression of basal cells, ciliated cells, goblet cells, and club cells) and hALOs (evidenced by high expression of alveolar type 1 (AT1) cell markers and alveolar type 2 (AT2) cell markers) were successfully generated (Fig. 1C and D).

Previous reports have identified Desmoglein 2 (DSG2) as the primary high-affinity cell receptor used by both HAdV-3 and HAdV-55 (19, 28). In addition, CD46 could also mediate the infection of HAdV-3 and HAdV-55, but the efficiency was much lower than observed for DSG2 (28, 29). In our platform, both CD46 and DSG2 can be detected in hAWO, as well as in hALO. CD46 is expressed in all nucleated cells (30) (Fig. 1C and D). In addition, the presence of many lamellar bodies (AT2 cell-specific intracellular organelles), ciliated cells, and club cells were confirmed via electron microscopy (Fig. 1E).

HAdV-55 shows more robust reproduction ability than HAdV-3 in lung organoids. Viral spread was observed in infected hAWOs and hALOs with either HAdV-3-GFP or HAdV-55-GFP (multiplicity of infection (MOI) = 0.05). With the prolongation of infection, the organoids showed a state of blackening, shrinking, and fragmentation (Fig. 2A), indicating that the adenovirus could replicate in the organoids and cause observable damage to the organoids.

Subsequently, to evaluate the viral growth kinetics of HAdV-3-GFP and HAdV-55-GFP, the organoids were harvested at specific time points postinfection (0 h, 2 h, 24 h, 48 h, 72 h, and 96 h). The viral DNA copies in the organoids were detected by qPCR, and the infectious viral titers were determined by the fluorescent focus units (FFU) assay by scoring of GFP expression of HAdV in A549 cells with fluorescence photograhy at 48 h postinfection (hpi) (31). The results of the qPCR assay revealed that the virus initiated DNA replication robustly beginning 2 hpi. DNA replication of HAdV-3-GFP and HAdV-55-GFP in both hALOs and hAWOs presented statistical differences at 96 hpi, with the higher DNA copies observed for HAdV-55-GFP versus HAdV-3-GFP (Fig. 2B). HAdV-3 and -55 had robust replication activity during the 2 to 96 hpi. At 96 hpi, the infectious virus titers (FFU) of HAdV-55-GFP in hALOs was nearly 1.5 log10 higher than HAdV-3-GFP, but there was no statistical difference between the 2 types in hAWOs (Fig. 2C). In summary, compared to HAdV-3-GFP, HAdV-55-GFP replicated more robustly both in hAWOs and hALOs, with the infectious virus titer of HAdV-55-GFP in hALOs at 96 hpi significantly higher than for HAdV-3-GFP.

HAdV-55 optimally infects basal cells, whereas HAdV-3 mainly infects luminal cells in hAWOs. Cell tropism for HAdV-3-GFP and HAdV-55-GFP were explored in infected hAWOs (MOI = 0.05) by an immunofluorescence staining assay at 48 hpi. Markers of each cell type were co-stained with GFP expressed by HAdVs. This experiment showed HAdV-3-GFP targeted mainly goblet cells (MUCSAC+) and ciliated cells...
Ac-Tub+), accounting for around 30% of infected cells, respectively. Simultaneously, approximately 20% of the club cells (CC10+) were infected. However, HAdV-3-GFP apparently rarely infected the basal cells (P63+) (Fig. 3A and B). Compared to HAdV3-GFP, HAdV-55-GFP infected more basal cells, which accounted for 80% of the infected cells (Fig. 3B). Approximately 30% of the club cells and ciliated cells were infected by HAdV-55-GFP, but the goblet cells were rarely infected (Fig. 3A and B). The proximal airways of the human lung are lined with a pseudostratified columnar epithelium comprising basal stem cells (BSCs) and luminal airway cells. The luminal airway cells contain secretory (or club, formerly Clara) cells, multi-ciliated cells, and goblet cells. Basal cells are the principal stem cells, with the ability to self-renew after injury and to differentiate into many other cell types, including goblet, club, and ciliated cells (32). Clinically, symptoms of upper respiratory tract infection with HAdV-3 are usually mild and self-limiting (9, 33). In contrast, HAdV-55 usually presents as a significant pneumonia-associated pathogen in adults, with a significant number of patients exhibiting rapid
progression to acute respiratory distress syndrome (ARDS) with severe morbidity (34) and including mortality (4, 35, 36). Data presented here suggest that HAdV-55 mainly infects the basal cells to cause massive airway stem cells damage (Fig. 2A), which may lead to the airway epithelium not being repaired quickly and presumably associated with more severe clinical outcomes.

FIG 2 HAdV-55 shows more robust replication ability than HAdV-3 in lung organoids. (A) Expression of GFP as observed under the Inverted Fluorescence Microscope at different time points after HAdV-3-GFP and HAdV-55-GFP infection of organoids (multiplicity of infection [MOI] = 0.05), hAWOs (left) and hALOs (right). GFP staining represents viral expression. Scale bar: 100 μm. (B) HAdV-3-GFP and HAdV-55-GFP replication kinetics in hAWOs and hALOs were determined by quantitative PCR assays (error bars represent standard deviations of biological triplicates), MOI = 0.05. (C) Virus titers in infected hAWOs and hALOs were detected by FFU method (error bars represent standard deviations of biological triplicates), MOI = 0.05.
HAdV-55 and HAdV-3 mainly infect AT2 cells in hALOs. In hALOs, cell types comprise mainly AT2 cells and AT1 cells. Infected hALOs by HAdV-3-GFP or HAdV-55-GFP (MOI = 0.05) were harvested at 48 hpi. AT2 cells were identified as the major target cells for both HAdV-3-GFP and HAdV-55-GFP. According to the data, HAdV-55-GFP infects more AT2 cells than HAdV-3-GFP (Fig. 4A and B). AT1 cells are infected by both HAdV3-GFP and HAdV55-GFP, and account for only 10% of infected cells. AT2 cells are a key structure of the distal lung epithelium, where they exert their innate immune response and serve as progenitors of AT1 cells, thereby contributing to alveolar epithelial repair and regeneration (37). In the healthy lung, AT2 cells secrete surfactant proteins, which are important for lung protection against pathogen exposure. Moreover, surfactant proteins help to maintain homeostasis in the distal lung and reduce surface tension at the pulmonary air–liquid interface. Once an adenovirus enters the alveolar area, a large number of AT2 cells are likely infected, resulting in impaired or lost AT2 cell functions. This, in turn, may result in severe clinical symptoms, for example pneumonia and acute respiratory distress syndrome. As noted earlier, HAdV-3 is associated with upper respiratory tract infections (8, 38), which differs from HAdV-55. The symptoms of pneumonia caused by HAdV-55 infection are usually more serious than those from HAdV-3 (4, 15, 39–41). The cell tropism difference between HAdV-3 and -55 may
be an important factor for the pathogenicity difference of these 2 adenovirus types. HAdV-3-GFP and HAdV-55-GFP tend to infect cells with high DSG2 expression in hAWOs and hALOs, which is consistent with earlier studies (28, 29, 42) (Fig. 3A and Fig. 4A). Confocal immunofluorescence micrographs showed that DSG2 and CD46 are widely expressed in lung organoids.

The viral life cycles of HAdV-3 and -55 in hAWOs. The viral life cycles of HAdV-3 and HAdV-55 in hAWOs and hALOs are essentially the same, so the data for hAWOs are presented as a representative example. After 48 hpi, stages of the replication cycle of HAdV-3 (MOI = 0.05) (Fig. 5A to J) and HAdV-55 (MOI = 0.05) (Fig. 5K to R) could be visualized in hAWOs by Transmission Electron Microscopy (TEM). The diameter of HAdV virions was determined to be between 60 and 90 nm. As observed, the extracellular HAdV-3 virus particles attach to the plasma membrane to initiate the infection process (Fig. 5A, B, and K for HAdV-55), and clusters of virions in membrane-bound vesicles or free virion are observed in the cytoplasm after cell entry (Fig. 5C and L for
AdV-55). Some HAdV-55 virions are concentrated around the nucleus (Fig. 5M and N). Vallee and Strunze reported that a microtubule translocation factor exported from the nucleus enables the virion to decouple from the filaments in the perinuclear region, to allow the virion to reach the nuclear pore complex (NPC) and result in the viral genome entry into the nucleus (43). Upon entry, the virus genome begins to replicate. At this initial stage, no complete virion is generally observed in the nucleus but may be noted exterior to the nuclear membrane. Simultaneously, there are many round or irregular dense inclusions contained in the nucleus (Fig. 5D, O, and P for HAdV-55). The next stage finds the nucleus filled with multiple virion particles in a parallel-arranged formation (Fig. 5E). At the later stages of viral DNA replication and the resultant production of progeny viruses, the nuclear envelope begins to rupture, and the dense crystalline adenovirus particles can be observed as a matrix, aggregating near the nuclear envelope (Fig. 5F). During the late replication cycle, autophagic vacuoles were numerous, and covered a greater part of the cytoplasm, accompanied by a decrease in the number of organelles (such as few visible mitochondria) (Fig. 5G to I, and Q for HAdV-55). At the final stage, as the number of virions increases, the nuclear membrane disrupts, and virions are observed throughout the cytoplasm (Fig. 5H). Finally, cell death,
occurring late in the infection cycle, results in the degradation of the cytoplasmic structure and cellular membranes, which may facilitate the release of virions from the cells (Fig. 5J and R for HAdV-55).

**Apoptosis analysis of hAWOs and hALOs infected by HAdV-3-GFP and HAdV-55-GFP.** Within the data captured by TEM, the entire viral life cycles have been clearly delineated: adenovirus-cell attachment and entry, replication in nucleus, cell lysis, and virus particles release. As adenoviruses have been reported to induce cell death pathways (44, 45), HAdV-3-GFP and HAdV-55-GFP infection-induced apoptosis was examined. The expression of the apoptosis marker Cleaved Caspase-3 was analyzed at 96 hpi by either HAdV-3-GFP or HAdV-55-GFP (MOI = 0.05) using immunofluorescence staining. Apoptotic cells in both HAdV3-GFP and HAdV55-GFP infected organoids were evident (Fig. 6A), which supports earlier reports that adenovirus infection could induce cell apoptosis (44, 45). However, there is no statistical difference in the percentages of apoptotic cells induced between HAdV-3 or HAdV-55 in both hAWOs and hALOs (Fig. 6B).

**Cidofovir effectively inhibits the replication of HAdV-3 and -55 in lung organoids.** Cidofovir is a nucleotide analogue that is reported to inhibit DNA polymerase from a variety of DNA viruses, including HAdV and CMV, thereby acting as an antiviral agent (46). The cidofovir used in this study is hydrated cidofovir (Fig. 7A). To test for the optimal time of efficacy, hAWOs and hALOs were treated with 10 μg/mL (35.8 μM) cidofovir and infected with either HAdV-3 or HAdV-55 (MOI = 0.05). Different time exposures were used, and the titers of wild type AdVs were determined by TCID50 method. The data demonstrated that, in both hAWOs and hALOs, the inhibition efficiency of cidofovir on HAdV-3 and HAdV-55 was highest at 48 hpi and 72 hpi, respectively (Fig. 7B).

**DISCUSSION**

Here, we report the first study of adenovirus infections using a hESCs-derived lung organoid platform that included models for the hAWOs and hALOs. This biologically relevant, reproducible, and readily available in vitro lung organoid platform is envisioned as an adequate model to study (i) cell tropism and receptors; (ii) viral life cycles; and (iii) antiviral drug identification and efficacies.

In this study, HAdV-3 and HAdV-55 growth kinetics assays were performed in hAWOs and hALOs. HAdV-55 has higher replication efficiency than HAdV-3 in hALOs, which implies that HAdV-55 may result in relatively higher virulence in human lungs. Moreover, this study reveals for the first time the differences in cellular tropism between HAdV-3 and HAdV-55. Compared to HAdV-3, HAdV-55 had a more robust infectivity of basal stem cells in the airway organoids and of distal stem cells-AT2 cells in alveolar organoids; both cells are implicated in the damage repair pathways in lung organoids (47). These data from the viral growth kinetics and the cell tropism differences may provide insights into how HAdV-55 is a highly pathogenic and possibly more virulent strain than HAdV-3, which is consistent with the clinical observations and outcomes, i.e., the symptoms of pneumonia caused by HAdV-55 infections are usually more severe than those of HAdV-3 (15, 48).

The organoid models have shown that HAdV-3 and -55 tend to infect cells with high DSG2 expression in hAWOs and hALOs, which is consistent with previous reports (28, 29, 42).
As previous studies reported, adenoviruses enter the cell through endocytosis and accumulate on the nuclear membrane surface, following which the genome is delivered into the nucleus. Subsequently, the viral genome replicates, and virus particles assemble into complete virions in the nucleus, followed by cell lysis and progeny virus release (49). Our research found, for the first time, the life cycles of HAdV-3 and HAdV-55 in human lung organoids. As noted, in these organoids, different virus-related inclusions were observed in the HAdV-3 or HAdV-55-infected cell nucleus: round dense inclusions and irregularly loose or dense inclusions. Some of these inclusions are believed to represent viral structural antigens or accumulations of viral nucleic acid.

**FIG 6** Apoptosis analysis of hAWOs and hALOs infected by HAdV-3-GFP and HAdV-55-GFP. (A) Immunofluorescence staining to detect the expression of cleaved-Caspase-3 in hAWOs and hALOs without or with adenovirus infection (MOI = 0.5) at 96 hpi, scale bar: 50 μm. (B) The statistical analysis of the percentage of apoptotic cells in hAWOs and hALOs infected with human adenovirus. Percentage of cleaved caspase-3-positive cells among GFP-positive cells were calculated for airway organoid infected with HAdV-3 (n = 7) or HAdV-55 (n = 6), as well as for alveolar organoid infected with HAdV-3 (n = 8) or HAdV-55 (n = 8).

As previous studies reported, adenoviruses enter the cell through endocytosis and accumulate on the nuclear membrane surface, following which the genome is delivered into the nucleus. Subsequently, the viral genome replicates, and virus particles assemble into complete virions in the nucleus, followed by cell lysis and progeny virus release (49). Our research found, for the first time, the life cycles of HAdV-3 and HAdV-55 in human lung organoids. As noted, in these organoids, different virus-related inclusions were observed in the HAdV-3 or HAdV-55-infected cell nucleus: round dense inclusions and irregularly loose or dense inclusions. Some of these inclusions are believed to represent viral structural antigens or accumulations of viral nucleic acid.
Cidofovir effectively inhibits the replication of HAdV-3 and HAdV-55 isolates in lung organoid models. (A) Schematic diagram of the structural formula of cidofovir. (B) Time course of the inhibition effects of cidofovir on HAdV-3 and HAdV-55 production in hAWOs and hALOs. (C) The inhibition (Continued on next page)
The matrix structure formed by the crystalline adenovirus particles were simultaneously observed; these intranuclear virus inclusions manifest a pathognomonic pattern of adenovirus formation. Previous studies have also shown that HAdV-infected cells respond to virus-induced membrane rupture and activate local autophagy to clear damaged organelles (45). Some internalized viruses failed to break from endosomes into the cytoplasm and remained in lysosomes, which might affect the potential immune activation initiated by the virus and hence cause more serious infections (49). In our study, during the process of HAdV-3 and HAdV-55 infection, numerous autophagic vacuoles in the cytoplasm and some autophagosomes containing adenovirus particles were also observed (Fig. 5I and Q).

Currently, 2D cell cultures are used in Drug Discovery and Development Research (DDDR) protocols and play roles in preclinical drug testing. However, it is common that the data generated from them are not consistent with data from in vivo studies due to unnatural growth kinetics and cell attachment. Cells act differently in 3D environments compared to 2D ones, especially when it comes to drug discovery, for example, many prospective cancer therapeutics look favorable in the 2D cell culture dish, but fail subsequently in clinical development (52). Studies have shown that cellular responses to drug treatment in 3D cell culture are more similar to what occurs in vivo compared to 2D cell culture (53). Recently, 3D organoids have received more attention and interest as they provide more physiologically relevant information and more predictive data for in vivo tests when compared to classic monolayer cultures. The 3D organoid culture technologies have been widely used in different stages of drug discovery, including diseases modeling (54), target identification and validation (55), screening (56), target selection (57), and toxicity assessment (58), as well as representing potential tools for predicting therapeutic responses and screening effective treatments for individual patients (59). In this study, a pair of human lung organoids was used as a model system for studying antiviral drug efficacy of cidofovir, which presented low cytotoxicity to organoids and good antiviral effects against HAdV-3 and HAdV-55. Given that the in vivo infection and pathogenicity models for common human adenovirus types are currently not available, our study provides an ideal model for drug screening in vitro, which may be used for more anti-adenoviral drug screening beyond cidofovir.

In conclusion, the lung organoids platform described and presented in this report is useful for studying pathogenic respiratory adenoviral infections. The data suggest that HAdV-55 possesses distinct biological characteristics in terms of their cell tropism and viral replication efficacy, although it utilizes similar cell entry receptors as HAdV-3. These results are useful for understanding the acute respiratory pathogenicity of HAdV-55. The 2 lung organoid platforms provide a viable and effective tool for screening and evaluating potential antiviral treatments.

MATERIALS AND METHODS

Cells, viruses, and antibodies. A549 cells were purchased from ATCC and tested negative for mycoplasma contamination. Cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM), supplemented with 10% (vol/vol) FBS (Gibco, C11995008T) and 1% (vol/vol) penicillin-streptomycin (100 U/mL) (Gibco, 15140122). For culturing, cells were incubated at 37°C and 5% CO2 with 0.25% Trypsin-EDTA medium (Gibco, 25200072) used for digestion. HAdV-3-GFP and HAdV-55-GFP were constructed and reported by our laboratory previously (60, 61). Both adenovirus strains noted above are replication-competent, with the insertion of CMV-GFP-SV40 expression cassette in the E3 region. HAdV-3 and HAdV-55 isolates have been isolated, characterized, studied, reported, and archived in our laboratory (4, 40, 41, 62, 63); they were used in the EM analysis and antiviral drug tests. Cidofovir [(S)-1-[3-hydroxy-2-(phosphonylmethoxy) propyl]cytosine] was purchased from Sigma Chemical Company (113852-37-2), prepared with deionized water, and stored at −20°C as a 1 mg/mL stock.

Establishment of H9-Derived hAWOs and hALOs. The H9 hESC cell line was obtained from WiCell Research Institute (Madison [WI], USA) with no mycoplasma contamination. Stem cells were maintained in mTeSR1 medium (STEMCELL Technologies, 05850) on plates coated with Matrigel (BD Biosciences,
TABLE 1 Primers used in this study

| Gene    | Forward primer (5’ to 3’)                  | Reserve primer (5’ to 3’)                 |
|---------|--------------------------------------------|------------------------------------------|
| GAPDH   | ACAACCTTGTTGATCTGTTGAAAGG                  | GCCATCACGCCACAGTTTC                     |
| SOX9    | AGCGAAGCCACATCAGAC                       | CTGTAGGGATCGTGGGGG                      |
| SOX2    | TACAGCAGTCCACTGGCAAG                      | GAGGAAGGTTAACCACAGGG                    |
| NKX2.1  | CTCATGTTCATCCGCTC                        | GACACCATAGGAACAGCG                      |
| P63     | CCACCTGAGCTATTCCTCACT                     | TGCTAGATCAATGACTAGGAGGAGGG              |
| MUC5AC  | ACCAATGTCTGTATCTGCTCC                     | GTTTTGCTGGTGAAGCAAACCA                  |
| SFTPC   | AGCAGAGGAGGTCCTGTAGGGA                    | CGTAAGAAGGCGTTCAGAG                   |
| SCGB1A1 | TTCACGGTCTCATGAAACCC                     | ACAGTGACGTCCCCGCTATTTT                  |
| HEXON   | GCCCCARTGGGGCRTCAGTGCACATC                | AGACCCSCSGRATGCAAAG                     |

354(27), with the culture medium changed daily. Cells were passaged by digestion with TrypLE Express (Gibco, 12604013) and reseeded at a concentration of 1:10-1:15 into mTeSR1medium. Both hAWOs and hALOs were derived from hESCs and generated as previously reported by us (27).

**qRT-PCR and Quantitative HAdV-PCR.** Total RNA was extracted using TRIzol reagent (MRC, TR1187), followed by reverse transcription to cDNA from 1 μg total RNA using the RevertAAce Kit (TOYOBO, 34520B1). Adenovirus DNA was extracted using a Viral DNA Kit (Omega, D3892), according to the protocol supplied. The qPCR reactions were performed on a Roche LightCycler 96 PCR system with the SYBR Green Premix Ex Taq Kit (TaKaRa, RR420A). PCR DNA copy numbers were determined according to the standard curve method (64), and the gene expression levels were normalized to GAPDH and compared to gene expression levels in hESCs using the 2\(^{-\Delta\Delta Ct}\) method. Three or more replicates were performed for each assay, and the data bars represent mean ± SD. Primers used in this study are listed in Table 1.

**Immunocytochemical staining.** For immunofluorescence staining, samples were collected at 72 hpi, and treated with 4% paraformaldehyde overnight to fix at 4°C. Once fixation was completed, the organoids were rinsed three times with PBS. The samples were then covered with Optimal Cutting Temperature compound (Thermo Fisher Scientific, 23-730-571) and frozen in liquid nitrogen. The sections were cryosectioned into 6 μm sections, attached to the slide, washed with PBS three times, and subsequently permeabilized with 0.2% Triton X-100 (Sigma, T9284) in PBS. After 20 min at RT, these were rinsed again with PBS and then blocked with 5% BSA (Sigma-Aldrich, A1470) at RT for 1 h. The samples were then incubated with primary antibodies overnight at 4°C, and subsequently stained with secondary antibodies at RT for 40 min. Nuclei were counterstained with DAPI (Sigma, D9542) for 3 min, then covered with glass microscope slides and imaged with a Nikon A1 confocal microscope. NIS-Elements software was used to render the Z-stack 3D images.

**Viral propagation and titer determination.** Before infecting the A594 cells, the cells were pelleted, and the supernatant was discarded. The virus was diluted with 100 μL serum-free medium and placed into the incubator for further culturing. After 2 h of incubation, the virus dilution was replaced with 2% FBS culture medium and the infected cells were allowed to continue growth at 37°C in 5% CO₂. Daily observations of cytopathic effect (CPE) were performed. When the CPE reached 90 to 100%, the cultured cells were subjected to freezing and thawing cycles for three rounds so that the cells were fully lysed in order to release the virus. The supernatant was harvested after centrifugation at 15 min/14,000 g/4°C. Two methods were used in order to determine the viral titers: A fluorescence focus units assay (FFU) (31, 65) and the TCID₅₀ assay. The TCID₅₀ assay was performed as follows: A549 cells were distributed onto 96-well plates at an amount of 2 × 10⁴ cells/well and placed into a cell incubator for 12 to 16 h. Afterwards, 100 μL of 10-fold diluted viral solution was added to each well, with eight replicate wells per dilution, and incubated for 2 h. The viral dilution solution was then discarded and 200 μL DMEM with 2% FBS was added per well. Incubation was continued for 5 to 7 days and observed daily for CPE, which provided for the calculation of TCID₅₀/0.1 mL, as per the Reed-Muench method (66).

**Virus DNA replication kinetics of HAdV-3 and -55 in hAWOs and hALOs.** Human airway and alveolar organoids were harvested, sheared, and resuspended in Ham’s F12 medium (Gibco, 21127022) and infected with virus at multiplicity of infection (MOI) of 0.05 (virus titer referred to infectious viral titer). After 2 h of virus adsorption at 37°C in the incubator, cultures were washed twice with Ham’s F12 medium to remove unbound viruses. hAWOs and hALOs were re-embedded into Matrigel (BD Biosciences, 356237) in 48-well tissue plates, and cultured in 500 μL corresponding organoid media, respectively. Samples were harvested at 2 h, 24 h, 48 h, 72 h, and 96 hpi. Fluorescence micrographs were captured, and the samples were transferred into 1.5 mL EP tubes. Organoids were harvested by incubation with 0.25% Trypsin-EDTA (Gibco, 25200072) for 30 min at 37°C until single cell suspension were achieved. The cells were then collected for both a qPCR assay and a determination of the virus titer.

**Antiviral drug tests in hAWOs and hALOs.** To determine the dose-response relationship between cidofovir and adenovirus, hAWOs and hALOs were infected with HAdV-3 and HAdV-55 (MOI = 0.05) using the method described earlier. At 2 h after infection, a series of drug concentrations (0.1, 1.0, 10, 50, and 100 μM) was introduced. Samples were collected at the 48 hpi, and TCID₅₀ assays were used to determine whether viral replication and proliferation were inhibited. These yielded dose-response curves. The cytotoxicity of organoids to cidofovir was determined by viable cell counts. Organoids were harvested by incubating with 0.25% Trypsin-EDTA for 30 min at 37°C until single cell suspension was achieved, 20 μL samples were mixed with 20 μL 0.4% trypan blue (Sigma, T8154) by gentle pipetting.
Subsequently, 20 μL of the mixtures were loaded into the chambers of a hemocytometer. Counts were performed in triplicate. Following this, hAWOs and hALOs were treated with 10 μg/mL cidofovir 2 h after virus infection. Samples were harvested at indicated time points (0 h, 24 h, 48 h, and 72 h), viral titers (TCID₅₀ equivalents per mL) were used to calculate the inhibition rate.

Whole-mount immunofluorescence. For whole-mount immunofluorescence staining, Cell Recovery Solution (Corning, 354253) was used for isolating the organoids from the Matrigel in the 96-well plates. These were fixed with 4% paraformaldehyde either overnight at 4°C or 2 h at RT, then washed with PBS three times, permeabilized, and blocked with 0.2% Triton X-100 and 5% BSA in PBS at RT for 1 h. Primary antibodies were incubated overnight at 4°C, and the nuclei counterstained with DAPI for 20 min at RT. The primary antibodies used in this study were listed in Table 2. Organoids were imaged using a Nikon A1 confocal microscope. These images were processed using NIS-Elements software for the 3D reconstruction.

Transmission electron microscopy. Organoids were collected and fixed in 2.5% glutaraldehyde for 24 h; washed with 0.1 M Phosphate buffer (19 mL 0.2 M NaH₂PO₄, 81 mL 0.2 M Na₂HPO₄) three times; and further fixed with 1% Osmium tetroxide for 2 h at RT. The fixed organoids were then washed with phosphate buffer and dehydrated with 30%, 50%, 70%, 80%, 85%, 90%, 95%, and 100% alcohol, sequentially. After a step of infiltration with different mixtures of acetone-Epon (2:1, 1:1, vol/vol), the samples were embedded in pure Epon. Polymerization proceeded by incubation at 60°C for 48 h. Ultra-thin sections (80 to 100 nm) were cut using an ultramicrotome (Leica EM UC7), placed on grids, and stained with uranyl acetate and lead citrate. After washing and drying, images were acquired by the digital camera by TEM (FEI, Tecnai G2 20 TWIN, 200kv), with identical magnification.

Statistical analysis. The package of SPSS Statistics 20.0 was used for analysis of the experimental data, and GraphPad 8.0 was used to record the results. Two independent samples were compared using the 2 independent samples t test, and multiple samples were compared using one-way ANOVA. Data from 3 independent experiments were obtained, with mean ± SD noted, and *, P < 0.05, **, P < 0.01, ***, P < 0.001, and ****, P < 0.0001.

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