Doctoral Thesis

Genetic modification of primary human airway epithelial cultures – a novel platform to study respiratory pathogens

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Genetic modification of primary human airway epithelial cultures – a novel platform to study respiratory pathogens

A thesis submitted to attain the degree of

DOCTOR OF SCIENCES of ETH ZURICH
(Dr. Sc. ETH Zurich)

presented by

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Summary

The human airways are large, complex and span a long anatomical distance. Their size results in regional differences in both epithelial type and composition. Furthermore, the polarized tissue has distinct functions based on anatomical location. Currently, different cell culture models are available to depict different areas of the human lung. Human lung organ cultures, where a piece of epithelium is surgically removed and placed in \textit{in vitro} culture, have been used for decades as a pulmonary tissue culture model. However, by isolating individual primary human epithelial cells from the lung tissue and re-differentiate them on porous inserts at air-liquid interface (ALI) a newer, more flexible model, is available. These ALI cultures have been successfully used to investigate epithelial morphology, drug delivery and pathogen – host interactions. The aim of this study was to use ALI cultures to study human coronavirus (HCoV) infection and to further develop the culture system to make it amenable to genetic modification. HCoVs are large RNA viruses with a relatively high incidence but a low burden in the human population. However, SARS-CoV and the recently emerging MERS-CoV have crossed the species barrier and caused epidemic respiratory tract infections in people. In 2012, we contributed to the first analysis of MERS-CoV replication in ALI cultures and assessed its zoonotic potential. To further characterize and elucidate important host factors and mechanism involved in HCoV infection we established transgenic human airway epithelial ALI cell cultures using lentiviruses and short hairpin (sh) RNAs for gene knockdown. However, using primary cells for genetic manipulation poses a number of challenges, most importantly, the finite life span of primary cells in culture. For successful mucociliary differentiation in ALI, primary human bronchial cells can only be passaged twice after isolation. Treatment of these cells with a Rho associated protein kinase inhibitor, Y-27632, induces a progenitor cell phenotype allowing for extended monolayer culture. Furthermore, cells treated with this inhibitor differentiate normally in ALI culture and can be transduced with lentiviral vectors to similar levels as naïve controls. This allows for the establishment of both hetero- and homogeneously transgenic ALI cultures from primary cells, which differentiate normally. In order to maximize the efficiency of the transgenic system we included an inducible component allowing for the induction of shRNA knockdown upon chemical treatment.

This inducible knockdown system will allow for detailed studies of virus – host interactions during respiratory tract infections with HCoVs or other pathogens.
Zusammenfassung

CHAPTER 1

GENERAL INTRODUCTION
Introduction

Human lung morphology

The human lungs are a complex, multifunctional organ and span a long anatomical distance. Due to their size, the structure and function of the lung epithelium varies depending on anatomical location. From the trachea and down to the large distal bronchioles the epithelium is columnar and pseudostratified. In this part of the airway, basal cells, nesting close to the basement membrane, are responsible for regenerating the epithelial layer, by self-maintenance and differentiation [1, 2]. Basal cells are distinguishable from luminal epithelial cells and can be fractionated from human airway cellular suspensions using various surface markers, including the nerve growth factor receptor (NGFR) [2]. The differentiated bronchial epithelium contains many different cell types with distinct roles in the functional tissue. Basal cells are the resident progenitor cells, goblet cells secrete protective mucus and ciliated cells are responsible for clearing the upper airway of mucus and debris. The proximal tracheobronchial epithelium also contains submucosal glands that consist of serous and mucus cells, producing airway lubrication, supporting mucociliary transport in the upper airway [3]. The further you descend into the lungs, the simpler the epithelial structure becomes. In the smaller bronchioles the epithelium is single and columnar. There, Clara cells replace basal cells as the primary regenerative cells. Furthermore, neuroendocrine cells organize into neuroendocrine bodies and have been speculated to support these Clara stem cells with purinergic signaling [4]. In the alveoli, where the actual air-exchange occurs, the epithelium is comprised of a single squamous layer. This single layer contains only two different cell types, alveolar type I and type II cells. It has long been speculated that alveolar type II cells serve as the progenitor cells in the distal airways, giving rise to type I cells [5-8]. The airway epithelium is separated from the pulmonary stroma by a basement membrane [4]. Since the exchange of oxygen and carbon dioxide happens in the alveoli, they are surrounded by blood vessels to facilitate air transport into the blood stream. Stromal cell signaling is very important for lung development and abnormal signaling from diseased or injured stroma can contribute to many chronic lung diseases [9-11].

Human lung organ cultures have been cultivated in vitro for decades [12-14]. These cultures are comprised of a piece of pulmonary epithelium surgically removed from the whole organ and cultured in a cell culture dish [15]. The basement membrane and a piece of stromal tissue can be excised along with the epithelium recapitulating the lung microenvironment. However, this makes it difficult to study the responses of individual cells or cell types within the tissue. Furthermore, these cultures are not viable for long.
Therefore, different cell culture models using isolated pulmonary epithelial cells instead of whole tissue are available to represent complex human pulmonary situations. For example, co-culture of human epithelial and endothelial cells results in recapitulation of branching morphogenesis [16]. Furthermore, the *in vitro* air-liquid interface (ALI) system represents the epithelial composition and structure of *in vivo* human epithelium [17, 18]. The composition of human airway epithelium and the different resident cell types are schematically represented in figure 1.

![Figure 1: Epithelial morphology and structure in the human lung.](image)

The upper conducting airways are pseudostratified and multicellular while the epithelium gradually gets thinner as you descend further into the bronchioles. Finally, the alveolar tissue is only comprised of a single squamous cell layer [4].
**Human airway epithelial cell cultures**

Advances in the isolation of primary human bronchial cells from human lung tissue have provided the possibility of establishing primary organotypic cell cultures of pulmonary origin *in vitro*.

Prior to cell isolation the lung tissue must be divided into roughly 2 cm pieces for optimal digestion. After washing and freeing the bronchial tubes of any excess mesenchyme, connective tissue, blood and fat, the pieces are digested with protease XIV and DNase I for 48 hours. After digestion, the bronchial tubes are cut open and the epithelium scraped off the cartilage into a petri dish. After a few more washing steps, to completely rid the epithelial cells of red blood cells, the remaining cells are collected in special medium and can be seeded into monolayer cell culture flasks coated with a mixture of collagens to mimic the *in vivo* basement membrane. After expansion in monolayer, the resulting de-differentiated pulmonary cells can be cryo-preserved or seeded directly on collagen-coated inserts for human airway epithelial (HAE) ALI cultures. Generally, only a few monolayer passages are possible prior to HAE ALI establishment [19]. Once the epithelial layer has reached confluency, the apical medium is aspirated, establishing ALI. This, along with a chemically defined medium, induces cellular differentiation. The medium contains various growth factors usually secreted by stromal cells, such as epidermal growth factor (EGF) and retinoic acid (RA), essential for differentiation [20]. Complete bronchial differentiation takes between 4 and 6 weeks but ciliary movement can often be observed as early as 2 weeks after the establishment of ALI. To validate the polarization of the epithelium, the transepithelial electrical resistance (TEER) can be measured. However, in the case of primary cells one can expect donor variations of TEER values and differentiation duration [21, 22].

This isolation method can also be adapted to isolate and culture cells from different species relevant to respiratory virus research, such as pigs [23, 24] and bats (Dijkman, R et al. unpublished data).
INTRODUCTION

Figure 2: Isolation of human bronchial epithelial cells with protease digestion from human lung tissue. Human bronchial tissue is cleaned of connective tissue and fat and divided into small pieces. These pieces are then digested in Protease XIV and DNase I for 48 hours and then epithelial cells are scraped from the cartilage and expanded in monolayer culture. Cells are then seeded on porous inserts or cryopreserved for later use [21].

Currently, both the upper and lower human airways can be represented by cell culture models. Collagen coated porous inserts serve as the basement membrane for the airway cells to attach to. Once the apical medium has been aspirated, cellular differentiation occurs and after differentiation the cultures contain the same mixture of cells found in in vivo epithelium and recapitulates its functionality [25].
Human bronchial ALI cultures: a tool for pathogen – host interaction studies

The airways serve as a barrier to the external environment; this includes exposure to pathogens, making human airway epithelial (HAE) ALI cultures quite suitable for studies involving respiratory pathogens that infect humans. Such pathogens, especially viruses, are traditionally studied in animal models, usually mice or ferrets. However, not all human viruses can replicate in animals without adaptation by serial passage. This might cause genotypic and/or phenotypic differences between the original human virus and the animal adapted virus, resulting in sub-optimal results. In virology, mice are often used to elucidate mechanisms of virus – host interactions, since various transgenic knockout strains are available to evaluate the roles of single or multiple genes in host responses to viral infections. However, results acquired in mouse models are often difficult to translate directly to human disease. Furthermore, the structure and regional epithelial cell distribution of the murine lung differs considerably compared to the human. For example, the pseudostratification of murine lungs is restricted to the upper tracheobronchial epithelium while it stretches much further down in the human. In turn, distribution of basal cells in human lung tissue spread, although sporadically, down to the bronchioles (reviewed in [26]).

Figure 3: Murine lung structure compared to the human. The human lungs are larger and contain ten times as many branches as murine lungs. Furthermore, regional epithelial cell distribution in the pulmonary tissue differs between the two. For example, pseudostratification in the murine lung is restricted to the tracheobronchial epithelium while the distribution of basal cells in the human airway stretches further down into the bronchioles (reviewed in [26]).
Prior to the establishment of ALI cultures for research purposes, lung organ cultures were commonly used as representatives of mammalian airways in vitro. To establish organ cultures of pulmonary epithelium, lung tissue has to be removed and segmented. The whole organ piece is then cultured, apical side up, in cell culture medium. These cultures can be established from all areas of the lung and in 1977, a novel method for ensuring the continuing inflation of the alveoli by filling them with agar, was described [12]. These cultures do not require any differentiation time allowing them to be used right after attachment, making them quicker to establish compared to HAE ALI cultures. However, in order to set up new cultures they must be established from a fresh donor. In contrast, isolated pulmonary epithelial cells can be cryo-preserved and the same donor cells used multiple times. Additionally, HAE ALI cultures can be maintained for up to one year while the shelf life of organ cultures is shorter, usually ranging from a few days to few weeks [27]. Both of these culture models have been successfully used for the study of respiratory pathogens but the organ cultures are much less flexible since terminal differentiation has already occurred. Furthermore, the study of respiratory virus – host interaction in human organotypic cultures should reflect the natural entry routes of these pathogens, through the apical side of the epithelium. HAE ALI cultures are cultivated in a closed system with only the apical side of the epithelium exposed, allowing for precise experimental infection.

Bronchial HAE cultures have already been used successfully to study various human respiratory viruses, including both human and avian influenza [28, 29], human bocavirus [30, 31], human rhinovirus [32, 33] respiratory syncytial virus [34] and all known human coronaviruses [35-37]. Additionally, alveolar HAE cultures are suitable for the propagation and study of viruses known to cause alveolar disease [38, 39].

Importantly, bronchial HAE cultures are responsive to recombinant interferon (rIFN) treatment as determined by the up-regulation of various IFN stimulated genes (ISGs) upon treatment with type I (α) or III (λ) IFN. However, the cultures do not mount a noticeable innate immune response upon infection with various coronaviruses [37]. This indicates intrinsic mechanisms of innate immune evasion employed by those viruses during infection, which could be further elucidated using the primary human airway epithelial cultures, not only for coronaviruses but also for other human respiratory pathogens that infect epithelial cells.

Furthermore, making the cultures amenable to genetic modification would enable careful and detailed studies of the epithelial cell host response to infection on the molecular level.
Coronaviruses

Coronaviruses (CoVs) are large RNA viruses that infect a wide variety of mammalian and avian hosts. They belong to the order of Nidovirales, organized into the family of Coronaviridae. Two sub-families belong to this family, the Coronavirinae and Torovirinae [40]. The Coronavirinae sub-family is further divided into four separate genera, alpha-, beta-, gamma- and deltacoronavirus [41]. CoVs cause mostly respiratory and enteric disease but can also have various other clinical manifestations, such as hepatitis and encephalitis [42]. The genome is the largest RNA genome discovered and within the enveloped virion it’s associated with nucleocapsid (N) proteins. The membrane (M) and spike (S) proteins are embedded in the viral envelope and the protrusion of the spike proteins gives the viruses their characteristic crown-like appearance, inspiring their name [43].

Genome organization

The CoVs share a general genome organization with an RNA genome of positive polarity around 27-30 kilobases (kb) in size. The largest part of it comprises the two overlapping reading frames (ORFs) 1a and 1b, extending from the 5’-end, encoding the viral replicase and proteins involved in the replicase complex. Depending on the CoV strain, this replicase gene can encode up to 16 non-structural proteins. The 3’-end of the genome, however, encodes for the various structural proteins required for virion assembly. The order of these proteins, from the 5’-end, is spike (S), envelope (E), membrane (M) and nucleocapsid (N) [44]. Furthermore, CoVs encode various accessory proteins distributed in between the structural protein genes [45]. The function, number and exact genome localization of these accessory genes varies depending on the CoV species. For example, the infectious bronchitis virus (IBV) accessory genes 3a and 3b are located between the S and E genes, while 5a and 5b are located between the M and N genes. IBV accessory protein 3a has been described to be involved in the resistance of the virus to IFN [46].

Viral life cycle

CoVs attach to their host cells using their S protein and upon attachment host proteases cleave and activate this protein causing membrane fusion and virus entry, resulting in the release of the N proteins and the genome into the host cell cytoplasm. Upon entry, the genome is immediately translated producing viral proteins essential for replication. CoVs replicate via replication-translation complexes (RTCs) that associate with virus-induced vesicle structures, termed double membrane vesicles (DMVs). These vesicles are associated with the CoV site of replication and might also serve as a hiding place for double stranded (ds) RNA during replication to avoid detection by cellular sensors. Almost all of the
components for the RTC are encoded by the ORF1a and ORF1b and translation results in two large polyproteins (pp1a and pp1ab), the second produced by a -1 ribosomal frameshift [47]. These polyproteins are then processed into the CoV non-structural proteins (nsp) that make up the RTC [48]. The structural and accessory genes are expressed from a nested set of subgenomic mRNAs. Translation of proteins follows replication. Progeny virions are assembled by budding of intracellular membranes and are released from the infected cell by cellular secretory mechanisms. During the viral cycle there are several steps where the viral RNA products can be detected by cellular sensors. However, many accessory proteins of CoVs have been found to interfere with innate immune sensing and signaling during infection.

Human Coronaviruses

The 6 human CoVs (HCoVs) discovered so far belong to the beta- and alphacoronavirus genera [49]. Four of those viruses are commonly circulating in the human population while the other two have caused epidemics of severe respiratory disease. One of the first HCoVs identified, in the late 1960s, were HCoV-229E and HCoV-OC43 [50]. Additionally, many other strains of HCoVs were characterized around the same time but it was unclear whether these were unique viruses or variations of the two already discovered. These strains were not studied further and all further HCoV research focused on these two distinct viruses. HCoV-229E circulates yearly in the human population with a low burden but can cause more severe disease in the immunocompromised and the elderly [51]. This virus was considered the HCoV prototype strain and grew readily in monolayer cell culture [52]. HCoV-229E also grows readily in HAE cultures after apical side inoculation [35]. The receptor for this virus was discovered to be CD13, also known as Aminopeptidase N (hAPN), and is expressed predominantly on epithelial cells, including those in the upper airway [53].

In 2002, an outbreak of severe acute respiratory disease surfaced in China. A novel HCoV, Severe Acute Respiratory Syndrome – CoV (SARS-CoV), was identified as the causative agent [54, 55]. This virus was later discovered to have originated in bats and entered the human population directly or through intermediate hosts in the Chinese animal wet markets [56-60]. In the aftermath of SARS-CoV, two new HCoVs were discovered. HCoV-NL63 in the Netherlands from an infant with bronchiolitis [61] and HCoV-HKU1 in Hong Kong from a patient with pneumonia [62]. HCoV-HKU1 was first cultured in bronchial HAE cultures in 2010 after attempts at culturing it in traditional cell lines had failed [63]. To date, only organotypic ALI culture systems support the propagation of this virus. Since it also has a strong tropism for alveolar cells, it causes epithelial disruption and syncytia formation in alveolar ALI cultures [38]. The cellular receptor for HCoV-HKU1 is currently
unknown but the receptor determinant, O-acetylated sialic acid, has been reported [64]. In contrast to SARS-CoV, these newly discovered HCoVs generally do not cause severe disease and can be found regularly circulating in humans. In 2012, another HCoV surfaced causing severe respiratory disease. This virus was isolated from a patient suffering from severe pneumonia in Saudi-Arabia and termed Middle East Respiratory Syndrome – CoV (MERS-CoV) [65]. Unlike SARS-CoV, this virus is still intermittently present in the human population and, most recently, caused a large outbreak in South Korea [66]. Phylogenetic evidence suggests a bat origin for MERS-CoV as well and evidence suggest that MERS-CoV infection of dromedary camels predates that in humans suggesting a possible role for camels as intermediate hosts or animal reservoir for MERS-CoV [67, 68]. Most of the currently discovered HCoVs are speculated to originate in bats (HCoV-229E, SARS-CoV, HCoV-NL63, MERS-CoV) and travel through an intermediate host prior to human transmission. The ability of CoVs to jump the species barrier highlights the importance of this virus family and its potential danger to the human population.

Therefore, it is necessary to study zoonotic respiratory viruses in a cell culture model that recapitulates the morphology, function and receptor distribution of the human airway. Furthermore, it is important to elucidate molecular determinants influencing both transmission and pathogenesis of these emerging viruses.

**RNA interference**

As described above, HAE cultures have various advantages compared to organ cultures or animal models. However, one major limitation is that there are currently no effective measures to genetically modify HAE cultures. For example, the knockdown of particular host genes or transgene expression within well differentiated HAE cultures would greatly enhance the value of this experimental model and would facilitate detailed studies on host-pathogen interactions on the molecular level. A promising experimental approach to make HAE cultures amenable to genetic modification is the delivery of shRNAs (RNA interference) or transgenes by lentiviral vectors.

RNA interference (RNAi) is a conserved biological reaction to double stranded RNA, mediating resistance to various pathogens [69] and it has become a vital part of the genetic toolkit for loss-of-function studies in eukaryotes [70]. The RNAi of endogenous genes is mediated by microRNAs (miRNAs), the largest class of small non-coding RNAs, which are transcribed as long-pri-miRNAs [71, 72]. The endonuclease Drosha then further processes these pri-miRNAs to shorter pre-miRNAs and they are transported from the nucleus to the cytoplasm and further cleaved into 22 nucleotide (nt) duplexes by another RNase, Dicer [73, 74]. One strand of the RNA duplex is then loaded onto the RNA induced silencing
complex (RISC), which mediates mRNA sequence recognition to guide either cleavage or translational inhibition of complementary mRNAs. To achieve binding one strand of the miRNA, termed the passenger strand, must be degraded. This is a crucial step in the RISC assembly [75]. Subsequently, Argonaute (Ago) proteins use one miRNA strand as a template for identification and Ago2, a component of RISC, cleaves the complementary mRNA, causing post-transcriptional knockdown [76]. However, mRNA degradation is not the only method of miRNA silencing in mammalian cells. The miRNA mediated binding of Ago proteins to mRNAs can also cause inhibition of translational initiation or elongation [77, 78].

Experimental RNAi
RNAi used experimentally in mammalian cells takes advantage of the conserved miRNA-processing pathway. Small interfering RNAs (siRNAs) are chemically synthesized and transfected directly into the cell cytoplasm where they identify homologous mRNAs for post-transcriptional knockdown (figure 4). However, the effects of siRNAs are short-lived and their delivery is limited to cell types that can be transfected. Many primary mammalian cells are not easily transfected, therefore other methods of gene delivery were explored. Lentiviral introduction of short hairpin RNAs (shRNAs) into target cells was found to result in a more stable knockdown of genes of interest (GOIs) since the viral genome containing the shRNA is integrated into the target cell genome. The shRNA is then transcribed from a Pol III promoter, a promoter traditionally used for the transcription of small RNAs, in the integrated viral sequence and processed in the cytoplasm by the Rnase Dicer, a component of the endogenous miRNA pathway. In contrast, siRNAs are not processed after transfection and can exert their effect immediately (figure 4).
miRNAs are partly processed in the nucleus before being transported to the cytoplasm and processed further. There, the RNA-induced-silencing complex sequesters mRNAs and their translation is repressed. Synthetic silencing RNAs (small interfering or short hairpin) take advantage of this pathway for gene knockdown [79].
**Lentiviral Vectors**

In order to establish long-term expression of shRNAs or transgenes in HAE cultures, lentiviral vectors must be used for stable transduction. Lentiviruses belong to a genus within the Retroviridae family. They are characterized by their long incubation period and their ability to infect non-dividing cells [80]. All retroviruses integrate their viral genome into the host cellular genome during infection, making them useful as gene delivery vectors. The ability to infect non-dividing cells, however, is unique for lentiviruses. Human immunodeficiency virus (HIV) derived viral vectors are commonly used in molecular biology to transduce cells with transgenes for over expression or shRNAs for gene knockdown. 

Virus particles used for cell transduction are replication deficient and only complete one round of infection and genome integration upon transduction. In order to produce lentiviral particles, a transfer vector containing the lentiviral pro-insert has to be co-transfected into packaging cells with other vectors encoding viral proteins required for viral particle production (figure 5). In the interest of safety the viral genes are distributed over several plasmids and only a fraction of the HIV genes is incorporated. In order to produce high titer lentiviral stocks, gag-pol, rev and an envelope glycoprotein must be expressed in the packaging cells. Gag-pol encodes for the HIV gag and pol polyproteins. Gag contains structural proteins required for virion assembly while pol contains the necessary enzymes, such as reverse transcriptase and integrase. Rev is a transactivating protein, essential to the regulation of HIV protein expression. Without rev the expression of gag-pol is low [81] resulting in less particle production. The vesicular stomatitis virus glycoprotein (VSV-g) is commonly used as an envelope protein due to its broad cellular tropism. Previous lentiviral packaging systems also included the HIV regulatory protein tat but it was discovered not to be required for high titer stock production. Lentiviral systems where four plasmids are required for particle packaging are called third generation systems [82].
Figure 5: Packaging of lentiviral particles and transduction and stable integration in target cells. Four separate plasmids are co-transfected into HEK 293T packaging cells. Two separate plasmids containing the HIV gag-pol and Rev genes are used for additional biosafety. Two other plasmids, encoding the desired envelope glycoprotein and transgenes/shRNA are also transfected simultaneously. Lentiviral containing supernatant is then harvested and applied to target cells where one round of replication and integration is completed. No infectious progeny is produced in target cells.

Lentiviral transduction of mammalian cells
siRNA transfection and retroviral transduction are not optimal methods for gene delivery into primary cells since various primary cell types are resistant to transfection with chemical agents and retroviral vectors can only transduce dividing cells. Therefore, lentiviruses are the optimal gene delivery system for primary mammalian cells [83]. Like other retroviruses, they integrate their genome into the host cell DNA and thereby allow for stable expression of shRNAs for knockdown.

Inducible lentiviral systems
To further increase the usefulness of lentiviral knockdown systems, an inducible component can be added. The addition of a chemical to the growth medium induces the expression of the shRNA allowing for a more controlled knockdown. This is ideal for the study of gene expression in complex systems that require development or differentiation. The most commonly used inducible knockdown systems are either tetracycline (tet) or lactose (lac) controlled. Both of these systems are adapted from bacteria and utilize transcriptional repression to keep the shRNA from being expressed. The addition of doxycycline (tet) or Isopropyl β-D-1-thiogalactopyranoside (IPTG, lac) to cell culture medium induces the expression of the shRNA since the binding of these substrates induces a conformational change in the repressors bound to the tet or lac operons within the shRNA promoter. The repressors are released and the shRNA is transcribed, processed by Dicer and incorporated into the RISC complex [84, 85]. Inducible lentiviral systems provide
a possibility to study genes important for cellular viability, differentiation and/or development where a constitutive knockdown would be detrimental to cell survival. The induction is controlled with chemical agents ensuring specific knockdown only upon treatment.

Implementing inducible lentiviral knockdown systems in HAE ALI cultures would allow modulation of host gene expression at different time points, providing new venues to study respiratory pathogen – host interactions in the authentic human airway epithelium, instead of the murine surrogate model.
References


INTRODUCTION


AIMS OF THE THESIS

Human coronaviruses (HCoVs) have a high incidence but a relatively low burden in the human population. They mainly cause respiratory tract infections in healthy individuals. This family of viruses, however, has a large zoonotic potential and in the last 15 years, two CoVs have jumped the species barrier and caused severe disease in people. Currently, all the known HCoVs can be propagated in organotypic cell cultures representing the upper conducting airways. The general aim of this work was to establish a system to study HCoV – host interactions at the molecular level in airway target cells.

The first aim of the thesis was to characterize the potential of the newly emerged MERS-CoV to replicate in the human airway and to determine the responsiveness of the interferon signaling system in HAE cultures. Lastly, we aimed to evaluate the sensitivity of this virus to recombinant interferon.

The second aim was to establish transgenic HAE cultures suitable for virus – host interaction studies, using lentiviral vectors.

The third and last aim was to further develop the transgenic HAE cultures to establish homogeneously transgenic cultures expressing inducible shRNAs for specific gene knockdown.
Data contribution

CHAPTER 2
Coronaviruses and the human airway: a universal system for virus-host interaction studies.
I performed the literature analysis, designed figures and tables and wrote the paper.

CHAPTER 3
Characterization of human coronaviruses on well-differentiated human airway epithelial cell cultures.
I wrote the book chapter in co-operation with Dr. Ronald Dijkman and participated in the establishment of the protocols described.

CHAPTER 4
Efficient replication of the novel human betacoronavirus EMC on primary human epithelium highlights its zoonotic potential.
I performed the immunofluorescence analysis of HAE cultures and contributed to the design of the figures.

CHAPTER 5
Successful lentiviral transduction and differentiation of primary human bronchial epithelial cells
I performed the experiments, designed figures and tables and wrote the paper.

CHAPTER 6
Inducible shRNA knockdown in homogeneously transgenic human airway epithelial cultures
I performed the experiments, designed figures and tables and wrote the paper.
CHAPTER 2
CORONAVIRUSES AND THE HUMAN AIRWAY: A UNIVERSAL SYSTEM FOR VIRUS – HOST INTERACTION STUDIES
Coronaviruses and the human airway: a universal system for virus-host interaction studies.

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Abstract

Human coronaviruses (HCoVs) are large RNA viruses that infect the human respiratory tract. The emergence of both Severe Acute Respiratory Syndrome and Middle East Respiratory Syndrome CoVs as well as the yearly circulation of four common CoVs highlights the importance of elucidating the different mechanisms employed by these viruses to evade the host immune response, determine their tropism and identify antiviral compounds. Various animal models have been established to investigate HCoV infection, including mice and non-human primates. To establish a link between the research conducted in animal models and humans, an organotypic human airway culture system, that recapitulates the human airway epithelium, has been developed. Currently, different cell culture systems are available to recapitulate the human airways, including the Air-Liquid Interface (ALI) human airway epithelium (HAE) model. Tracheobronchial HAE cultures recapitulate the primary entry point of human respiratory viruses while the alveolar models allows for elucidation of mechanisms involved in viral infection and pathogenesis in the alveoli. These organotypic human airway cultures represent a universal platform to study respiratory virus-host interaction by offering more detailed insights compared to cell lines. Additionally, the epidemic potential of this virus family highlights the need for both vaccines and antivirals. No commercial vaccine is available but various effective antivirals have been identified, some with potential for human treatment. These morphological airway cultures are also well suited for the identification of antivirals, evaluation of compound toxicity and viral inhibition.

Keywords

Human coronavirus, airway epithelium, ALI, antiviral compound, cell tropism.
Background

Respiratory diseases caused by human coronavirus infection are of both medical and socio-economic importance. Currently, they are studied in various model systems, ranging from cell lines to animal models. Originally, the importance of HCoVs in the burden of human disease was underestimated and as a result, no general therapy exists to treat coronavirus induced disease in humans. Furthermore, no commercial vaccine is available leaving the human population vulnerable to emerging coronavirus infections. Both the Severe Acute Respiratory Syndrome and Middle East Respiratory Syndrome coronaviruses have recently crossed the species barrier and entered the human population to cause severe disease. In this review, we summarize the current knowledge on human coronavirus infection emphasizing the usefulness of organotypic human airway cultures as a model system.

Coronaviruses

Coronaviruses (CoVs), a subfamily of the Coronaviridae family, are positive strand RNA viruses with the largest genome of all known RNA viruses (≥27 Kb). The genomic RNA is capped, polyadenylated and associated with nucleocapsid proteins within an enveloped virion. The envelope is covered by the characteristic surface glycoprotein that gives the virus particles their characteristic crown-like (Latin: corona) appearance [1]. All CoVs share a common genome organization where the replicase gene encompasses the 5'-two thirds of the genome and is comprised of two overlapping open reading frames (ORFs), ORF1a and ORF1b that encode for up to 16 non-structural proteins. The structural gene region, which covers the 3'-third of the genome, encodes the canonical set of structural protein genes in the order 5' - spike (S) - envelope (E) - membrane (M) and nucleocapsid (N) – 3'. The structural gene region also harbors several ORFs that are interspersed along the structural protein coding genes. The number and location of these accessory ORFs vary between the CoV species [2, 3].

In animals, CoV infections are mainly associated with respiratory and enteric disease and can have large economical impact on the veterinary industry, e.g Porcine Epidemic Diarrhea Virus (PEDV) causes gastrointestinal disease in pigs [4], Infectious Bronchitis Virus (IBV) causes severe kidney and respiratory disease in chicken [5] and Bovine Coronavirus (BCoV) causes both respiratory disease and diarrhea in cattle [6]. Additionally, CoV infections can have other disease manifestations, such as central nervous system (CNS) involvement, hepatitis and peritonitis [7-10].

In humans, CoV infections are mainly associated with respiratory diseases that are considered to have a large impact on the economy due to reduced productivity of the working population. Currently, 6 coronaviruses that cause disease in humans have been
discovered. Four of those are commonly circulating and two have caused epidemics of severe acute respiratory disease.

**Human coronaviruses**

The first human coronavirus (HCoV), B814, was described in 1965. In the following years, over 30 additional strains were characterized. Ten of those strains could only be isolated from primary embryonic tracheal organ culture. Others were readily isolated from monolayer cultures and were antigenically related to the prototype strain HCoV-229E. HCoV-OC43, for organ culture 43, was isolated and found to be distinct from the 229E prototype strain [11, 12]. In the subsequent decades, research on HCoVs would center on these two distinct viruses.

However, in 2002, an unknown respiratory illness, termed Severe Acute Respiratory Syndrome (SARS), surfaced in Asia. Research determined it to be caused by a novel coronavirus [13, 14]. At the end of the epidemic, this virus had infected over 8000 people, most in China, and caused 774 deaths [15].

Following the discovery of this virus, two additional CoVs causing human disease were identified. HCoV-NL63 was isolated in the Netherlands in 2004 from an infant with bronchiolitis [16] and HCoV-HKU1 in 2005 from a patient with pneumonia in Hong Kong [17]. In 2012, another respiratory HCoV, Middle East Respiratory (MERS)–CoV, was isolated from a patient with pneumonia in Saudi-Arabia [18]. Unlike SARS-CoV, this virus is still intermittently present in the human population and most recently caused a large outbreak in South-Korea [19]. To date, there have been over 1600 cases and almost 600 deaths related to MERS-CoV infection [20].

**Commonly circulating coronaviruses**

Out of the 6 known human coronaviruses, HCoV-229E, HCoV-OC43, HCoV-NL63 and HCoV-HKU1 are commonly circulating in the human population and usually cause general respiratory illness and cold symptoms in healthy individuals [21-23]. Like influenza, these viruses are capable of causing more severe disease in the immunocompromised and the elderly [24]. They infect the human airway from the luminal side and progeny viruses are released from the same side facilitating spread through coughing and sneezing [25, 26]. These coronaviruses are responsible for approximately 5-10% of all upper and lower respiratory tract infections [27-29] but the interactions between them and their natural host cells are poorly understood. Currently, it is hypothesized that most of the human coronaviruses may have originated from bats [30, 31]. For example, HCoV-229E is believed to originate from African hipposiderid bats possibly using camelids as intermediate hosts [32].
Emerging coronaviruses

In the last 15 years, two coronaviruses have crossed the species barrier and caused severe and fatal disease in humans. SARS-CoV surfaced in 2002 and MERS-CoV in 2012 [13, 14, 18]. As opposed to the commonly circulating viruses, which generally only cause mild respiratory symptoms, these viruses presented with higher case fatality ratios, around 10 and 20-50% respectively [33, 34].

Currently, there is abundant phylogenetic evidence for the bat origin of SARS-CoV, based on sequences of SARS-like viruses found among bats in the recent years [35-37]. The initial transmissions of SARS-CoV from animals to humans were traced back to the live animal wet markets and it was hypothesized that the virus made its way into the human population using the civet cat as an intermediate host. However, successful isolation of SARS-like viruses from bats [38] and the fact that a contemporary bat SARS-like virus can infect human airway cultures [39] suggest that an intermediate host between humans and bat might not have been needed for the transmission of SARS-CoV.

The evolutionary origin of MERS-CoV is less clear but it has been speculated to be bats as well. Characterization of an African bat virus closely related to MERS-CoV shows that both the human and camel strains belong to the same viral species and phylogenetic analysis suggests that MERS-CoV infection in camels predates that in humans, suggesting that camels infect humans and not the other way around. Furthermore, the bat virus roots the phylogenetic tree providing further evidence for the bat origin of MERS-CoV [40]. Additionally, human-to-human transmission, although not robust, seems to happen simultaneously as camel-to-human transmission. Therefore, any further adaptation of MERS-CoV to the human host must be monitored carefully and intermediate hosts identified [41].

Many bat coronaviruses have been identified in the recent years further highlighting the zoonotic potential of this family of viruses [30]. Given the documented history of coronaviruses overcoming the species barrier and causing severe disease in humans, it is important to investigate the zoonotic potential of close evolutionary relatives of common HCoVs in a culture model that recapitulates the aspects of the human airway, e.g. morphology and receptor distribution. It’s important to study the mechanisms of pathogenesis and the evolution of zoonotic viruses in detail in order to identify molecular determinants that affect either transmission or pathogenesis. It’s also important to elucidate whether coronaviruses currently circulating in animals are a potential danger to the human population.
Human coronavirus receptors and cell tropism

All of the known cellular receptors of HCoVs belong to the same protein family, the membrane ectopeptidases. Interestingly, the catalytic activity of these peptidases is not required for viral entry but rather the co-expression of other host peptidases activates the HCoV spike proteins [42, 43]. It has been established that the human transmembrane serine proteases TMPRSSII and HAT cleave and activate the HCoV-229E, SARS- and MERS-CoV spike proteins during viral entry [44, 45].

Out of the four commonly circulating coronaviruses, HCoV-229E is the only one that infects non-ciliated cells using the human Aminopeptidase N (hAPN) as its receptor [46]. This peptidase is predominantly expressed on non-ciliated cells in the human bronchus [47]. SARS-CoV and HCoV-NL63 both utilize the Angiotensin Converting Enzyme 2 (ACE2) for cellular binding [48, 49]. ACE2 is expressed on ciliated bronchial cells along with endothelial cells and both type I and II alveolar cells [50]. MERS-CoV was found to use a different receptor than SARS-CoV, namely the dipeptidyl-peptidase 4 (DPP4) [51]. DPP4 is widely expressed in endothelial cells and various epithelial tissues in the human body [52]. In ex vivo human lung organ cultures, different tropism of SARS- and MERS-CoVs was observed. MERS-CoV can actively replicate in both bronchial and alveolar tissue while SARS-CoV primarily replicates in alveolar tissue [53]. The wide cellular tropism of MERS-CoV might contribute to its associated disease severity and high mortality rate whereas the alveolar replication of SARS-CoV would explain why it generally presents with pneumonia.

The cellular surface receptors for HCoV-OC43 and HCoV-HKU1 are currently unknown but receptor determinants for these two viruses have been identified as N-acetyl-9-O-acetylneuraminic acid and O-Acetylated Sialic acid, respectively [54, 55].

All of these viruses can be successfully cultured and investigated in HAE cultures [56, 57]. The discovery of HCoVs, their receptor usage, cell tropism and receptor binding domain (RBD) is summarized in Table 1.

Furthermore, established reverse genetic systems for HCoV-229E [58], HCoV-OC43 [59] and HCoV-NL63 [60] allow for controlled virus mutation and fluorescent transgene insertion to better understand the interaction of these viruses with their pulmonary host cells.
Table 1: Human coronavirus overview.

<table>
<thead>
<tr>
<th>Name</th>
<th>Discovery</th>
<th>Protein Receptor</th>
<th>Tropism</th>
<th>Receptor Binding Domain (RBD)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCoV-229E</td>
<td>1966</td>
<td>Aminopep-tidase N (hAPN)</td>
<td>Non-ciliated cells</td>
<td>S407-547</td>
<td>[46, 56, 57, 103, 104]</td>
</tr>
<tr>
<td>HCoV-OC43</td>
<td>1967</td>
<td>Unknown (^1)</td>
<td>Ciliated cells</td>
<td>Unknown</td>
<td>[56, 105, 106]</td>
</tr>
<tr>
<td>SARS-CoV</td>
<td>2003</td>
<td>Angiotensin converting enzyme 2 (ACE2)</td>
<td>Ciliated cells</td>
<td>S303-537</td>
<td>[13, 14, 48, 57, 107]</td>
</tr>
<tr>
<td>HCoV-NL63</td>
<td>2004</td>
<td>Angiotensin converting enzyme 2 (ACE2)</td>
<td>Ciliated cells</td>
<td>S476-616</td>
<td>[16, 49, 56, 108]</td>
</tr>
<tr>
<td>HCoV-HKU1</td>
<td>2005</td>
<td>Unknown (^2)</td>
<td>Ciliated cells</td>
<td>Unknown</td>
<td>[17, 56]</td>
</tr>
<tr>
<td>MERS-CoV</td>
<td>2012</td>
<td>Dipeptyl-peptidase 4 (DPP4)</td>
<td>Non-ciliated cells</td>
<td>S358-588</td>
<td>[18, 34, 51, 109]</td>
</tr>
</tbody>
</table>

\(^1\) Receptor determinant identified as N-acetyl-9-O-acetylnuraminic acid.

\(^2\) Receptor determinant identified as O-acetylated sialic acid.

Animal models for human coronaviruses

Traditionally, respiratory viruses are studied in animal models, usually mice and ferrets [48, 61]. However, it is not always possible to correctly recapitulate human infection and disease in animal models. The establishment of transgenic animal models for human disease is attainable when either the virus receptor has been identified, which is not the case for all HCoVs, or when viruses can be adapted to a different host. An adapted human virus may not share the same properties as the original human virus. SARS-CoV was found to replicate naturally in various strains of inbred mice but to enhance clinical signs of disease the hACE2 was introduced into these mice. This resulted in murine models with varying degree of human disease similarity. Since SARS-CoV already replicated in mouse cells, adapting it to the murine host was quite successful. This resulted in three mouse adapted strains that caused disease in mice similar to severe SARS-CoV cases in humans [62].

In an effort to establish a mouse model for HCoV-229E infection transgenic hAPN mice were created. However, the insertion of the hAPN into mouse cells is not enough to
establish robust HCoV-229E infection \textit{in vivo}. Nevertheless, cells isolated from these transgenic animals could be infected \textit{in vitro} [63, 64]. The emergence of both SARS- and MERS-CoVs emphasized the importance of establishing animal models for human coronaviruses. Currently, a few animal models for MERS-CoV have been established. Mice carry their own variant of the viral receptor DDP4 that differs from the human in regions important for MERS-CoV spike interaction and by replacing this receptor with the human one, MERS-CoV can infect mouse cells but the method of hDPP4 insertion has an effect on the degree of pathogenesis observed in these mice [65, 66]. Various non-human primates (NHPs) can be naturally infected with both SARS- and MERS-CoVs. However, disease presentation and pathogenesis differs between the different subspecies and NHP models are expensive, although ideal to study human infection due to their genetic similarity [62].

To establish a link between the research conducted in animal models and humans, an organotypic airway culture system resembling the human airway epithelium has been developed. This model is a universal platform to study human respiratory viruses [67-70]. They have been used successfully for infection studies with all known human coronaviruses [56, 57]. Furthermore, the cultures can be inoculated with a low infectious dosage to mimic natural infection in the human airway. Whereas, animal models often require both high dosage and artificial inoculation routes.

**Human airway epithelial cell cultures**

Organotypic cell cultures are becoming increasingly common. Different cell culture models exist to depict different epithelial tissues [71]. These cultures closely resemble their tissue of origin and contain various different cell types with distinctive roles in the polarized tissue. Currently, various organotypic cell culture models exist to represent the different areas of the human airways. The human lungs span a long anatomical distance and carry out different functions depending on anatomical location [72, 73]. The structure of the epithelium also differs the further you descend into the airways. Tracheal and bronchial epithelium is columnar and pseudostratified, with every cell in contact with the basement membrane, while the epithelium in the alveoli is comprised of a single cell layer to facilitate air-exchange [74].

Tracheobronchial cells are one of the first targets of human respiratory viruses and can be cultured in air-liquid interface (ALI) where the apical side of the cell layer is exposed to air while the basolateral side is submerged in medium. Tracheobronchial cells cultured in that way form a pseudostratified epithelial layer that both morphologically and functionally resembles the human upper conducting airway (Figure 1A) [75, 76]. After differentiation, these cultures contain many different cell types such as basal, ciliated and goblet cells. They also produce protective mucus, much like \textit{in vivo} epithelium. When compared to primary
bronchial cells in submerged two-dimensional culture, the gene expression of primary ALI cultures differs significantly. However, the expression pattern of primary human bronchial ALI cultures is comparable to that of in vivo epithelium. The human bronchial cell line Calu-3 has been used as a culture model for respiratory epithelium but its gene expression in ALI cultures is more similar to submerged bronchial cell cultures than differentiated epithelium [77]. Additionally, Calu-3 cells respond differently to MERS-CoV infection compared to primary HAE cultures. During infection in Calu-3 cells, profound apoptosis was detected within 24 hours of infection [78] while infection of primary HAE cultures does not result in any disruption of the cell layer [57]. Therefore, the primary tracheobronchial ALI culture model is especially fitting for human respiratory virus research since it accurately recapitulates the primary entry point for these viruses. By using these cultures, virus replication and host interactions can be studied in natural target cells. Further establishing the usefulness of this system HCoV-HKU1 was propagated for the first time in ciliated cells of bronchial HAE cultures in 2010 after culturing it in conventional cell lines had failed [26]. Alveolar epithelial ALI cultures (figure 1B) can also be used for virus-host interaction studies and are especially applicable when a viral infection causes pneumonia and alveolar damage [79]. HCoV-HKU1 has also been propagated in alveolar HAE cultures and exhibits a strong tropism for alveolar type II cells and causes large syncytia formation upon infection [80].
When compared to traditional two dimensional cell cultures, the HAE cultures are more cumbersome and their preparation is time consuming but they do have an advantage over traditional monolayer cell cultures when it comes to virus-host interaction studies. Different types of ALI cultures used for virus research are summarized in table 2.

Table 2: Different types of ALI cultures used in coronavirus studies.

<table>
<thead>
<tr>
<th>Cell types</th>
<th>Origin</th>
<th>HCoV</th>
<th>Features</th>
<th>Refs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary bronchial</td>
<td>Trachea, Bronchus</td>
<td>All</td>
<td>Differentiated pseudostratified epithelium, many cell types</td>
<td>[56, 57]</td>
</tr>
<tr>
<td>Calu-3</td>
<td>Sub-mucosal glands (adenocarcinoma)</td>
<td>MERS-CoV, SARS-CoV</td>
<td>Single or polarized epithelium, one cell type</td>
<td>[110, 111]</td>
</tr>
<tr>
<td>Primary alveolar</td>
<td>Alveoli</td>
<td>MERS-CoV, SARS-CoV, HCoV-HKU1</td>
<td>Differentiated squamous epithelium, two cell types</td>
<td>[79, 80, 112]</td>
</tr>
</tbody>
</table>
Innate immunity

Within the respiratory epithelium the innate immune system has a major protective role as the first line of defense against respiratory pathogens. In particular, the interferon (IFN) system orchestrates hundreds of different cellular effector proteins that (i) protect the epithelial barrier by altering the physiological and cellular environment, (ii) impair virus propagation, spread and transmission, and (iii) shape the host’s adaptive immune response. Recent publications have demonstrated that the innate immune system is functional in the HAE cell culture system and that most pathogen recognition receptors are expressed and up-regulated upon treatment with exogenous stimuli [57, 81].

In general, HCoVs do not elicit a strong innate immune response in primary target cells of the human airway early during infection. Despite the presence of all major pathogen recognition receptors, no elevated expression of IFN beta, pro-inflammatory cytokines or interferon stimulated genes can be observed up to 12 hours post-infection in HAEs infected with HCoV-229E, MERS- or SARS-CoVs [57]. This is most likely due to the intrinsic CoV properties harbored in the replicative non-structural proteins that actively aid in avoiding recognition by the host innate immune system. For example, the 5’ termini of the viral mRNA are capped making them indistinguishable from the host cellular mRNAs and no longer detectable by cellular sensors. Furthermore, CoV replication is associated with the appearance of double membrane vesicles (DMVs) in the host cell cytoplasm, which might serve as a protective shield for viral RNA to prevent recognition by cytoplasmic RNA sensors [82-85].

In addition to the non-structural proteins, various CoV accessory proteins have been discovered to inhibit interferon signaling at different stages of the host innate immune response. For example, MERS-CoV accessory protein 4a inhibits innate antiviral signaling by suppressing the activation of MDAS and RIGI [86, 87] whereas 4b inhibits the induction of the IFN-beta promoter [88]. While ORF4a and 4b are IFN antagonists in the genome of MERS-CoV, SARS-CoV ORF3b antagonizes IFN signaling through MAVS/RIGI [89]. Whereas SARS-CoV ORF6 disrupts IFN signaling by blocking the nuclear translocation of STAT1 [89, 90]. These discoveries highlight that HCoVs employ similar yet different strategies to evade the innate immune response during infection in the respiratory epithelium.
**Therapy**

Despite that respiratory infections with HCoVs can result in severe respiratory disease there are currently no effective prophylactic or therapeutic treatment options available. However, the emergence of novel coronaviruses has emphasized the need to develop effective treatment options. For example, vaccines using the spike proteins of both SARS- and MERS-CoVs have proven protective in animal models [91, 92] suggesting that a vaccine against HCoVs for human use might be achievable.

Additionally, various drugs that inhibit HCoV infection at different stages of the replication cycle have been reported and some could potentially serve as treatment options for HCoV associated severe respiratory disease. For example, patients presenting with severe respiratory disease, caused by SARS- or MERS-CoVs, are generally treated with steroids and interferon, sometimes in combination with the antiviral drug Ribavirin [93-96]. This treatment, however, is not especially effective highlighting the need for HCoV specific antivirals. Many different compounds have been determined to have anti-HCoV activity. For example, protease inhibitors which suppress HCoV entry [97-99], Cyclosporin A (CsA) treatment blocks the replication of coronaviruses from all subgroups [100] and non-immunosuppressive derivatives of CsA represent a possible therapeutic option for both human and animal CoV infections.

HCoV infection can also be inhibited by pre-treating HAE cultures with either recombinant IFN alpha or lambda [57]. Similar effect has also been shown for recombinant IFN alpha and beta which could inhibit MERS-CoV in ex vivo lung cultures [53]. As previously described, IFN treatment of active HCoV infection is not particularly effective in vivo. Therefore, the use of IFN in humans might be limited to prophylactic treatment of exposed persons and/or health care workers treating infected patients.

Screenings of compound libraries have also resulted in the identification of some HCoV specific antivirals. For example, a novel small compound inhibitor (K22) has been identified, and showed to be effective against a broad spectrum of CoVs and could inhibit both HCoV-229E and MERS-CoV in HAE cultures [101]. Additionally, HCoV-NL63 has been inhibited in HAE cultures with polymer-based compounds [102].

To date, most treatment and inhibitor studies have been conducted in HCoV susceptible cell lines. However, the HAE cultures represent an ideal system to test the application and efficacy of those already identified, and new, antiviral compounds against HCoVs in cells that represent the primary site of replication. Furthermore, the HAE cultures are heterogenous, containing many different cellular sub-populations, and would allow for the evaluation of compound toxicity and effect in a differentiated layer similar to human airway epithelium. Compounds already shown to inhibit HCoVs in cell lines should be applied to HAE cultures as well before any animal or human trials.
Conclusions

HCoV induced respiratory diseases are of both medical and socio-economic importance. The emergence of SARS- and MERS-CoV and the yearly circulation of the four common HCoVs highlight the importance of elucidating the different mechanisms employed by HCoVs to evade the host immune system as well as identifying antiviral compounds and human vaccine candidates. The HAE culture system is based on primary human cells offering a unique platform to study respiratory viruses in cells representing the primary entry point of these viruses, bronchial epithelial cells, or investigate the interaction of HCoVs and the distal airways, in type I and II alveolar cells. Additionally, the inclusion of airway epithelial cultures for other species enables the study of zoonosis and animal-to-human transmission. Currently, many aspects of HCoV infection and pathogenesis remain to be determined. The HAE culture system, both tracheobronchial and alveolar, represents a unique platform to study virus-host interaction in natural target cells at the molecular level. These cultures are becoming more common and more relevant to HCoV research. Especially, for those viruses for which there is no animal model, as they provide an organotypic substitute for virus – host interaction studies.
List of abbreviations

CoV          Coronavirus
RNA         Ribonucleic Acid
ORF         Open Reading Frame
PEDV        Porcine Epidemic Diarrhea Virus
IBV         Infectious Bronchitis Virus
BCoV        Bovine Coronavirus
CNS         Central Nervous System
HCoV        Human Coronavirus
SARS        Severe Acute Respiratory Syndrome
MERS        Middle East Respiratory Syndrome
TMPRSSII    Transmembrane Protease, Serine 2
HAT         Human Airway Trypsin-like protease
hAPN        human Aminopeptidase N
hACE2       human Angiotensin Converting Enzyme 2
hDPP4       human Dipeptyl Peptidase 4
RBD         Receptor Binding Domain
HAE         Human Airway Epithelium
NHP         Non-Human Primate
ALI         Air-Liquid Interface
IFN         Interferon
MDA5        Melanoma Differentiation-Associated protein 5
DMV         Double Membrane Vesicles
CsA         Cyclosporin A
FDA         Food and Drug Administration
**Competing interests**

The authors declare no competing interests.

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**Authors’ contribution**

HR Jonsdottir wrote the review, designed tables and figures. R Dijkman revised the text, tables and figures. Both authors read and approved the final manuscript.

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HCoVs AND HAE CULTURES


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CHAPTER 3
CHARACTERIZATION OF HUMAN CORONAVIRUSES ON WELL-DIFFERENTIATED HUMAN AIRWAY EPITHELIAL CELL CULTURES
Characterization of human coronaviruses on well-differentiated human airway epithelial cell cultures

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Summary

The human airway serves as the entry point of human respiratory viruses, including human coronaviruses. In this chapter we outline the methods by which we establish fully differentiated airway epithelium and its use for human coronavirus propagation. Additionally, we outline methods for immunofluorescence staining of these cultures for virus detection, characterization of cell tropism and how to perform antiviral assays and quantify viral replication.

Key words

Human coronavirus, antivirals, cell tropism, human airway epithelial cells, virus detection

Running title: Characterization of HCoVs on HAEs
Introduction

The human airway serves as the entry point of human respiratory viruses, including human coronaviruses (HCoVs). In order to properly recapitulate the complex anatomy of the human lung specialized cell culture models have been developed to resemble both the upper and lower airways [1-3]. Primary human bronchial epithelial cells cultured in an air-liquid interface (ALI) system serve as a universal platform to study human respiratory viruses [4-6]. These human airway epithelial (HAE) cultures morphologically and functionally resemble the upper conducting airways in vivo. In these cultures, the epithelial layer is pseudostratified and after differentiation they contain many different cell types such as basal, ciliated and goblet cells and furthermore, generate protective mucus equivalent to that of in vivo epithelium [7]. Establishment of HAE cultures requires time and patience but the differentiated cultures allow for a number of advantageous analyses in respiratory virus research. We have adapted and optimized our methods based on previously published work [8-10]. Moreover, we have standardized methods for the propagation of human coronaviruses and evaluation of the effects of antiviral compounds on both viral replication and cell viability. We are able to propagate all known human coronaviruses in this system and can easily evaluate their tropism by immunohistochemistry [5, 11]. In this chapter we outline the methods by which we establish fully differentiated airway epithelium and use it for human coronavirus propagation. Additionally, we outline methods for immunofluorescence staining of these cultures for virus detection, characterization of cell tropism and how to perform antiviral assays and quantify viral replication.

Materials

**Human airway epithelial cell cultures**

1. Primary human tracheobronchial epithelial cells can be obtained in accordance with local ethical guidelines from patients willing to give informed consent, who are undergoing bronchoscopy and/or surgical lung resections. Alternatively isolated primary human airway epithelial cells can be obtained commercially from a number of distributors.
2. 10x digestion solution: Minimum Essential Medium (MEM), 1% m/v Protease from Streptomyces griseus Type XIV, 0.01% m/v Deoxyribonuclease I from bovine pancreas.
3. Isolation/washing solution: MEM, 100 U/mL penicillin, 100 µg/mL streptomycin, 0.25 µg/mL Amphotericin B Solution, 50 µg/ml Gentamicin, 100 U/mL Nystatin.
4. Bronchial epithelial cell serum-free growth medium (BEGM): LHC basal medium, supplemented with the required additives (Table 1).
5. Air liquid interface (ALI) medium: LHC basal medium and Dulbecco’s Modified MEM (DMEM) mixed in a 1:1 ratio, supplemented with the required additives (Table 1).
6. 12-well inserts, pore size 0.4 µM and 12-well cluster plates or 12-well deep well cluster plates.
7. 24-well inserts pore size 0.4 µM and 24 well cluster plates.
8. Human collagen Type I + III, Vitrocol 100.
9. Collagen Type IV from human placenta reconstituted in 5 mL filter sterilized water with 0.25% acetic acid. Dissolve for a few hours at 37°C, occasionally swirling. Once dissolved, increase volume to 20 mL and maintain acetic acid concentration at 0.25%, mix gently by pipetting. Filter sterilize the solution through a 0.22 µm filter, and store at -20°C in aliquots of 800 µL per eppendorf tube. The stock solution is stable for at least 1 year at –20°C.

**Human coronavirus propagation**

1. Apical wash solution: Hank’s Balanced Salt Solution (HBSS), without calcium and magnesium.
2. Virus transport medium (VTM): MEM, 25 mM HEPES-buffered, 0.5% Gelatin, 100 U/mL penicillin, 100 µg/mL streptomycin.
3. Aerosol barrier pipet tips and 1.5 ml Eppendorf Safe-lock tubes™.

**Immunofluorescence analysis**

1. Fixation solution: 4% formalin solution, neutral buffered (Formafix).
2. Confocal staining buffer (CB): 50 mM ammonium chloride (NH₄Cl), 0.1% saponin and 2% IgG and protease free BSA dissolved in 500 mL of phosphate buffered saline (PBS, pH 7.4). Filter-sterilize (0.2 µm filter) solution and prepare aliquots of 40 mL and store at -20°C.
3. Primary antibodies: see Table 2.
4. Fluorescent DNA dyes: DAPI or Hoechst 33528.
5. Wash solution: Phosphate buffered saline, pH 7.4, without calcium and magnesium.
6. Scalpel (No.10).
7. Rat-tooth forceps.
8. Fluorescence Mounting Medium.
**Antiviral assays**
1. Inhibitors: e.g. K22 [12], recombinant Interferon Alpha and Lambda proteins [13].

**Virus detection**

**Renilla luciferase assay**
1. Renilla Luciferase Assay System (Promega)
2. White, non-transparent 96 well plates.
4. Luminometer.

**Plaque assay**
1. Huh-7 cells.
2. Medium: DMEM, high glucose, 100 U/mL penicillin, 100 µg/mL streptomycin, 1 mM Sodium Pyruvate, 5% heat-inactivated FBS.
3. Overlay medium: 2.4 g of Avicel RC-581 (FMC biopolymer) dissolved 100 mL of distilled water and autoclaved for 20 minutes at 121 °C. 2.7 g of DMEM powder (high glucose) dissolved in 90 mL of distilled water and the pH adjusted to 7.4 with 1M NaOH. Fill volume up to 100 mL and filter-sterilize (0.2 µm filter). Freshly prepare a 1:1 mixture of Avicel (2.4%) and 2x DMEM solution, supplemented with 5% FBS and 100 U/mL penicillin, 100 µg/mL streptomycin.
4. Crystal-violet solution: 25 g of Crystal Violet, 40 g NaCl dissolved in 2500 mL of 99% Ethanol. Add 2250 mL of distilled water and 250 mL of 37% formaldehyde. Mix solution overnight at room temperature (see Note 6).

**Quantitative reverse transcriptase PCR**
1. Nucleospin RNA isolation kit (Machery Nagel).
4. RNase-free water.
5. FastStart Universal SYBR Green Master reaction mixture (Roche).
6. Positive control; *in vitro* transcribed RNA of target gene or plasmid DNA containing target gene.
Methods

Carry out all procedures in a biosafety cabinet according to local biosafety regulations.

Human airway epithelial cell cultures

Collagen Type I and III coating of cell culture flasks

Cell culture flasks are coated for 2 hours with a mixture of Type I and III collagen that is necessary to efficiently expand the number of primary airway epithelial cells.

1. Use filter sterilized dH₂O (0.22 μm) for preparing a 1:75 dilution of Vitrocol 100.
2. Use 4 mL per 75 cm², make sure that the entire surface is covered with the collagen solution.
3. Incubate for 2 hours at 37°C.
4. Aspirate remaining liquid and wash twice with 10 mL of PBS to remove traces of acetic acid.
5. Culture flasks can be directly used. Optional: Store coated flask at +4°C for a maximum of 6 weeks.

Collagen Type IV coating of inserts

The inserts need to be coated overnight with collagen type IV, necessary for development and long-term maintenance of differentiated primary airway epithelial cell cultures.

1. Mix 7.2 mL of filter sterile dH₂O with 800 μL of Collagen Type IV solution (0.5 mg/mL).
2. Apply 150 μL per 12-well inserts, or 50 μL per 24-well inserts. After completing one plate, make sure that the entire surface of each well is covered with the 1:10 collagen solution.
3. Air-dry the inserts overnight in a laminar flowhood, and afterwards expose them to UV-light (type C) for 30 minutes.
4. To remove traces of acetic acid wash inserts twice with at least 500 μL of PBS.
5. After these steps coated inserts can be used directly. Optional: Store at +4°C (wrapped in foil) for a maximum of 6 weeks. Repeat UV-exposure and washing steps before use.
Isolation of primary human tracheal and/or bronchial cells.
Primary epithelial cells can be isolated from whole lung tissue resections of tracheal and/or bronchial origin according to the following protocol. Smaller lung tissue resections can be processed with the same protocol. All procedures are performed at room temperature unless stated otherwise.

1. Trim the bronchial tissue free of connective tissue and fat using forceps and scissors or a scalpel. If needed, cut the bronchial tissue into 2 cm segments.
2. Wash the cleaned tissue three times in washing solution.
3. Fill the desired number of 50 mL tubes with 30 mL of wash solution and transfer as many tissue segments as possible into a single tube, until the volume reaches 36 mL. Then add 4 mL of 10x digestion solution to each tube, so the end volume will be 40 mL (40 mg Protease/0.4 mg DNase).
4. Place tubes on a rocking platform/tube roller at +4°C and incubate for 48 hours.
5. Place the 50 mL tube containing the digested tissue on ice and add 4 mL of heat-inactivated FBS to each tube (to a final concentration of 10% (v/v)), to neutralize protease activity. Invert tubes 3 times.
6. Pour solution along with the tissue onto a large petri dish, and gently scrape off the epithelium from the collagen-cartilage surface, using a scalpel in the reverted angle. Pool solutions containing dissociated cells into a 50 mL conical tube and wash the petri dish once with PBS.
7. Centrifuge for 5 minutes at 500 x g. Wash cells once with HBSS and resuspend cells in BEGM to a concentration of, approximately, 5 x 10^6 cells/mL.
8. Count cells using a hemocytometer and seed into collagen coated flasks with 20 mL of pre-warmed BEGM. An appropriate amount of cells for T75 flasks ranges between 0.5 – 1.0 x 10^6 cells.
9. Change medium the next day to remove blood cells and any unattached epithelial cells.
10. To prevent acidification of the medium change it every 2-3 days, until 80-90% confluence.
Establishment of fully differentiated HAE cultures

When primary cells have reached 80 – 90% confluence in the expansion phase one can dissociate and seed the de-differentiated primary cells on collagen type IV coated inserts, according to the following protocol. All procedures are performed at room temperature unless stated otherwise.

1. Remove BEGM and transfer it into a 50 mL tube and wash the cell monolayer twice with 12 mL of HBSS.
2. Dissociate the bronchial cells for 3 minutes at 37°C in a humidified 5% CO₂ incubator with the appropriate amount of trypsin (25 cm²: 1 mL, 75 cm²: 3 mL). If needed tap the flask to dissociate the cells (see Note 7).
3. Collect the cells in the previously collected BEGM and centrifuge for 5 minutes at 500 x g.
4. Carefully discard the supernatant and resuspend cells in HBSS and centrifuge the suspension for 5 minutes at 500 x g.
5. Discard the supernatant and resuspend cells in pre-warmed ALI medium and count using a hemocytometer.
6. For generation of differentiated HAE cultures the number of cells seeded should be 1.0 – 2.0 x 10⁵ cells per 12-well insert in 500 μL, or 0.3 – 0.6 x 10⁵ cells per 24-well insert in 200 μL of ALI medium. A single 75 cm² flask should provide enough cells for preparing 48 individual 12-well inserts or 96 individual 24-well inserts.
7. Fill the basolateral compartment of the plates with 1 mL of ALI medium (500 μL for 24-well inserts), and transfer 500 μL (200 μL for 24-well inserts) of diluted cell suspension to the upper chamber of the collagen coated inserts and incubate overnight at 37°C in a humidified 5% CO₂ incubator. Cells are now in liquid liquid-interface.
8. The next day, medium in the apical compartment must be changed to remove any unattached cells. Discard the old medium and wash the apical surface with 500 μL HBSS and apply 500 μL of pre-warmed ALI medium to the apical side. Adjust volume to 200 μL for 24 well inserts.
9. To prevent acidification of the medium it should be changed every 2-3 days until cells have reached complete confluence (see Note 8).
10. During media change in liquid liquid-interface change apical medium first (as described in step 8) followed by exchange of medium in the basolateral compartment.
11. To establish air-liquid interface aspirate apical side medium once cells have reached complete confluence and wash twice with HBSS (500 μL for 12-well inserts and 200 μL for 24-well inserts).
12. Incubate cultures for a few hours at 37°C in a humidified 5% CO₂ incubator and monitor if seeping of basolateral medium into the apical compartment occurs. If no seepage occurs cultures can be maintained at air-liquid interface. Otherwise cultures have to be cultured at liquid-liquid interface for another day.

13. Incubate cultures for 4-6 weeks to allow differentiation. Appearance of active ciliated cells can be used as an indicator of differentiation. During the extended culture time medium must be changed regularly (every 2-3 days). If desired inserts can be transferred to deep well plates that only require medium renewal every 7 days.

14. After differentiation HAE cultures are suitable for human coronavirus propagation.

**Human coronavirus propagation**

1. Wash the apical surface of the HAE culture twice with 500 μL of HBSS solution prior to inoculation with human coronavirus specimen to remove excess of mucus.

2. Dilute the clinical material or virus supernatant in HBSS and inoculate 200 μL drop wise to the apical surface and incubate for 2 hours at either 33°C or 37°C (see Note 9), in a humidified 5% CO₂-incubator. *Optional:* Centrifuge inoculum solution for 4 minutes at 1500 x g at room temperature to remove cell debris prior to inoculation.

3. Collect the inoculum and transfer it to a container and store at –80°C for later analysis, and wash the apical surface three times with 500 μL HBSS. *Optional:* Transfer the collected inoculum into an equal volume of VTM.

4. Incubate the infected cultures for the desired amount of time at the appropriate temperature in a humidified 5% CO₂-incubator, e.g. 48 hours at 33°C for HCoV-229E.

5. Apply 200 μL of HBSS drop wise to the apical surface 10 minutes prior to the desired collection time and incubate in the humidified 5% CO₂-incubator. Then collect progeny virus and transfer it to a container and store at –80°C for later analysis. *Optional:* Transfer the collected progeny virus into an equal volume of VTM.
Immunofluorescence analysis

All incubation steps are performed at room temperature on a gyro-rocker (20 – 30 rpm), unless stated otherwise

1. After the apical washing has been collected the apical surface is washed twice with 500 μL of PBS before cells are fixed with formalin-solution for later immunofluorescence analysis.
2. Apply 500 μL of 4% formalin-solution to the apical compartment and 1 mL to the basolateral. Incubate for 15 – 30 minutes.
3. After the fixation process remove the formalin-solution and wash both compartments three times with equal volumes of PBS.
4. Transfer the fixed HAE cultures to a new conventional 12-well plate.
5. Discard washing solution and apply 500 μL and 1 mL of confocal buffer (CB) solution to apical and basolateral compartments, respectively.
6. Incubate fixed cultures for 30 – 60 minutes to block non-specific binding of the antibodies (see Note 10)
6. Remove the CB solution from the upper and lower compartments.
7. From this stage one should only apply the CB solution in the apical compartment.
8. Wash the apical surface once with 500 μL of CB solution for 5 minutes.
9. Apply primary antibodies (see table 2) diluted in 250 μL CB solution drop wise to the apical surface and incubate for 120 minutes.
10. Wash the apical surface three times with 500 μL of CB solution for 5 minutes (see Note 11).
11. Apply the appropriately diluted conjugated secondary antibodies in 250 μL CB solution drop wise to the apical surface and incubate for 60 minutes.
12. Wash the apical surface twice with 500 μL of CB solution for 5 minutes.
13. Incubate cells with nucleic acid counter stain solution diluted in 250 μL of CB solution for 5 minutes.
14. Wash the apical surface once with 500 μL of CB solution for 5 minutes.
15. Lastly, wash the apical surface twice with 500 μL of PBS for 5 minutes to remove residual saponin and restore cell membrane integrity.
16. Before removing the washing solution, apply mounting medium on a glass slide (use 1 – 2 drops). Remove any air bubbles.
17. Excise the membrane from the plastic holder and carefully place the basolateral side of the membrane on top of the mounting medium, without generating air bubbles.
18. Then slowly add one drop of mounting medium on top of each membrane.
19. Slowly place the coverslip, in a tilted fashion, on top of the membrane without generating air bubbles.
20. Allow the mounting medium to polymerize for 30 minutes, after which the slide can directly be analysed.

**Antiviral assays**

**Treatment**

1. Pre-warm ALI medium to 37°C.
2. Mix antiviral compounds (e.g. K22, recombinant interferons) in various concentrations or by serial dilution in ALI medium. Include non-treated controls. Also, to exclude viral inhibition by solvents (e.g. DMSO) include solvent controls.
3. For evaluation of either prophylactic or therapeutic effects of antivirals, the HAE cultures can be incubated with the compounds diluted in the basolateral medium prior to, during or after infection.
4. Infect cultures apically with human coronaviruses as described in section 3.2.
5. Collect apical washing in HBSS as described in section 3.2 for viral quantification by plaque assay and cells for viral quantification by Renilla Luciferase Assay or qRT-PCR.

**Cytotoxicity Assay**

1. Thaw CellTiter-Glo buffer and equilibrate both buffer and CellTiter-Glo substrate to room temperature.
2. Transfer the buffer to the amber bottle containing the substrate to reconstitute the enzyme. Mix by gently swirling the bottle.
3. Wash the apical side of the HAE cultures three times with 500 µL HBSS to remove excess mucus.
4. Apply 50 µL of HBSS to the apical side and mix with equal volume of reconstituted CellTiter-Glo enzyme solution (optimized for 24 well inserts, for other insert sizes adjust buffer amount accordingly) and incubate for 5 minutes at room temperature on a gyro-rocker to induce cell lysis.
5. Next incubate the plate for 10 minutes at room temperature to allow for stabilization of the luminescence signal.
6. Transfer 20 µL of cell lysate to a white, non-transparent 96-well plate for analysis.
7. Record luminescence (see Note 12). To account for background signal include empty wells in your analysis.
Virus Detection

Renilla Luciferase Assay (see Note 13)
1. Thaw Renilla Luciferase Assay buffer and dilute 1:5 in water.
2. Wash HAE inserts with HBSS three times prior to cell lysis.
3. Incubate inserts with 80µL of Renilla lysis buffer on a gyro-rocker for 30 minutes at room temperature (optimized for 24 well inserts, adjust lysis buffer amount accordingly for other insert sizes).
4. During incubation, thaw Renilla Assay buffer.
5. Transfer the cell lysate to a 96 well plate.
6. Transfer 20µL of the lysate to a white, non-transparent 96-well plate for analysis.
7. Add Renilla substrate at 1:200 dilution to the required amount of Renilla Assay buffer (100µL per sample). Protect from light (see Note 14).
8. Program your luminometer settings with 10 second measure time followed by a 2 second delay. 100µL of assay buffer should be dispensed into each well. If the luminometer is not equipped with an injector the assay buffer can be added manually using a multichannel pipette.
9. To adjust samples for background include empty wells in your analysis.
10. Plot your values as Log$_{10}$ RLU (Relative Light Units).

Plaque assay
The current protocol is optimized for HCoV-229E, but can easily be adapted to any other cell line and coronavirus strain.

1. Seed target cells in a 12-well cluster plates with 1 mL of complete medium per well and incubate overnight at 37 °C in a humidified 5% CO$_2$-incubator.
2. Make six 10-fold serial dilutions of the harvested virus supernatants in 1 mL and inoculate the cells.
3. Incubate inoculum for two hours at 33°C in a humidified 5% CO$_2$-incubator before removing the serial diluted virus inoculums from the cells and replace with 1 mL of overlay medium.
4. Incubate titration plates for 3 – 4 days at 33°C in a humidified 5% CO$_2$-incubator.
5. Remove overlay and wash wells twice with water to remove residual Avicel.
6. Subsequently add approx. 0.5 – 1 mL of crystal violet solution to each well and incubate for 10 minutes.
7. Remove crystal violet solution and wash the cells once with water and allow the plates to air dry before counting the number of plaques.
Quantitative reverse transcriptase PCR

1. Isolate viral RNA with NucleoSpin RNA kit according to the manufacturer's protocol and elute in the appropriate amount of RNase-free water.

2. For reverse transcription use M-MLV reverse transcriptase (100 units), M-MLV buffer and random primers and 10 μL of extracted total RNA in a total volume of 20 μL, at 37°C for 60 minutes. Optional: include serial dilutions of in vitro transcribed RNA of the target gene for virus yield quantification.

3. To quantify viral HCoV RNA yields from contemporary strains use the FastStart Universal SYBR Green Master reaction mixture. Amplify two microliters of cDNA according to the manufacturer's protocol, using the previously published sense and antisense strain-specific primers (see Note 15). Measurements and analysis can, for instance, be done on a LightCycler 480 II instrument, using the LightCycler 480 software package (Roche). Use the following cycle profile of 10 minutes at 95°C followed by 45 cycles of 10 seconds at 95°C, 20 seconds at 60°C, and 30 seconds at 72°C followed by a melting curve step to confirm product specificity.
Notes

**Note 1:** Dissolve 5 gram of BSA, globulin free, powder in 20 mL PBS in a 50 mL tube (Do not vortex). Place the tube on a shaker/roller-bank for 2 – 4 (max 24) hours at +4°C, until BSA is completely dissolved. Add the volume up to 34 mL, mix gently by inverting the tube 3 times. Filter sterilise the solution through a 0.22 μm filter, and store at -20°C in aliquots of 3.5 mL in 15 mL tubes. Invert the tube 3 times before usage.

**Note 2:** Dissolve 12 mg of Retinoic Acid (RA) in 40 mL absolute EtOH in a 50 mL tube wrapped in aluminium foil, the RA-EtOH stock (1 x 10^{-3} M) should be stored at -20°C. To prepare the 1000x stock, first confirm the RA concentration of the ethanol stock by diluting it 1:100 in absolute EtOH. Measure the absorbance at 350 nm using a spectrophotometer and a 1 cm light path quartz cuvette (or nanodrop with 0.1 cm light path), blanked on 100% EtOH. The absorbance of the diluted stock should equal 0.45 (0.045 on a nanodrop). RA absorbance readings below 0.18 should be discarded. If the absorbance equals 0.45, add 3 mL of RA-EtOH stock solution to 53 mL PBS and add 4 mL of BSA 150 mg/mL stock. For absorbance values less than 0.45, calculate the needed volume of RA-EtOH stock as 1.35/absorbance and adjust the PBS volume appropriately. The 1000x stock solution should be stored at -20°C in aliquots of 1 mL in eppendorf tubes.

**Note 3:** Dissolve 42 mg ferrous sulfate, 12.2 g magnesium chloride and 1.62 g calcium chloride-dihydrate in 80 mL H_{2}O, add 500 μL concentrated hydrochloric acid (HCl). Filter sterilise the solution through a 0.22 μm filter, and store at -20°C in aliquots of 1100 μL per eppendorf vial.

**Note 4:** Prepare seven separate 25 or 50 ml stock solutions (see Table 3a & b) in H_{2}O. Filter sterilize (0.22 μm) each component after preparation. Afterwards, transfer an aliquot of 50 μL from each separate component into 49600 μL filter sterilized water (0.22 μm) and add a volume of 50 μL concentrated HCl solution. Mix the solution well through gentle vortexing and filter sterilise the solution through a 0.22 μm filter, and store at -20°C in aliquots of 1100 μL per eppendorf vial.

**Note 5:** Gentamicin and Amphotericin B should be omitted from ALI medium. These antibiotics are only required in BEGM medium right after cell isolation to prevent contamination.

**Note 6:** For preparation of crystal violet solution safety glasses and protective clothing should be worn. Any spillage must be cleaned with 96% ethanol.

**Note 7:** Cells might take longer to dissociate from the bottom of the flask due to the collagen coating. If the cells are not dissociated after three minutes additional rounds of one-minute incubations can be performed until all cells have detached.
Note 8: The seeded primary cells should reach confluence on the inserts within one week. If this takes longer then the success rate of establishing well-differentiated HAE cultures declines exponentially.

Note 9: Human coronavirus NL63, 229E, HKU1 and OC43 are predominantly found in the upper respiratory tract and are therefore incubated at 33°C. Both MERS-CoV and SARS-CoV are predominantly found in the lower respiratory tract and are therefore incubated at 37°C.

Note 10: The fixed HAE cultures can be kept for 1 – 3 months at 4°C if the CB is filter sterilized (0.2 µM) and all the procedure were performed under sterile conditions. After cold storage it is preferential to acclimatize the fixed cultures for 15 minutes to room temperature on a gyroo-rocker (20 - 30 rpm) prior to continuation of the staining protocol.

Note 11: To prevent bleaching of the fluorophores one should cover the inserts from daylight exposure during each incubation step.

Note 12: Luminometer settings depend on the manufacturer. However, a measurement time of 1-2 seconds per well has proved effective.

Note 13: For this assay cultures must be infected with coronaviruses expressing a Renilla Luciferase reporter gene.

Note 14: If your luminometer is equipped with an injector you must remember to account for priming by increasing the volume of required Renilla Assay buffer by 2-3 mL.

Note 15: Primers targeting HCoV-NL63, HCoV-HKU1, HCoV-229E, and HCoV-OC43 have been characterized and described [4, 14, 15].

Acknowledgements

This work was supported by the 3R Research Foundation Switzerland (project 128-11).
Table 1: Preparation of stock additives for BEGM and ALI medium. All additives from Sigma Aldrich and should be aliquoted and stored at -20°C unless stated otherwise.

<table>
<thead>
<tr>
<th>Component</th>
<th>Cat.Number</th>
<th>Stock Concentration</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine Serum Albumin (BSA)</td>
<td>A3059</td>
<td>300x 150 mg/ml</td>
<td>See Note 1</td>
</tr>
<tr>
<td>Bovine pituitary extract (BPE)</td>
<td>P1476</td>
<td>1000x ±14 mg/ml</td>
<td></td>
</tr>
<tr>
<td>Insulin</td>
<td>I9278</td>
<td>2000x, 10 mg/ml</td>
<td>Store at +4°C</td>
</tr>
<tr>
<td>Transferrin (TF)</td>
<td>T0665</td>
<td>1000x, 10 mg/ml</td>
<td></td>
</tr>
<tr>
<td>Hydrocortisone (H)</td>
<td>H0396</td>
<td>1000x, 0.072 mg/ml</td>
<td></td>
</tr>
<tr>
<td>Triiodothyronine (T3)</td>
<td>T6397</td>
<td>1000x, 0.0067 mg/ml</td>
<td></td>
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<tr>
<td>Epinephrine (EP)</td>
<td>E4642</td>
<td>1000x, 0.6 mg/ml</td>
<td></td>
</tr>
<tr>
<td>Epidermal Growth Factor (EGF)</td>
<td>Repligen, 6082-253</td>
<td>1000x or 50000x, 25 µg/ml</td>
<td>1000x for BEGM, 50000X for ALI medium.</td>
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<tr>
<td>Retinoic acid (RA)</td>
<td>R2625</td>
<td>1000x, 5 x 10^{-5} M</td>
<td>Light sensitive. See Note 2</td>
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<td>Phosphorylethanolamine (PE)</td>
<td>P0503</td>
<td>1000x, 70 mg/ml</td>
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<tr>
<td>Ethanolamine (EA)</td>
<td>E0135</td>
<td>1000x, 30 µl/ml</td>
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<tr>
<td>Stock 11 (S11)</td>
<td>Z0251</td>
<td>1000x, 0.863 mg/ml</td>
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<tr>
<td>Stock 4 (S4)</td>
<td>F8048 M2393 C7902</td>
<td>1000x</td>
<td>See Note 3</td>
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<tr>
<td>Trace Elements (TR)</td>
<td>Various</td>
<td>1000x</td>
<td>See Note 4</td>
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<tr>
<td>Penicillin/Streptomycin (P/S)</td>
<td>Invitrogen, 15140-122</td>
<td>100x 10,000 units/mL of penicillin and 10,000 µg/mL of streptomycin</td>
<td>Store at +4°C</td>
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<tr>
<td>Gentamicin</td>
<td>G1397</td>
<td>1000x, 50 mg/ml</td>
<td>Store at +4°C. See Note 5</td>
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<tr>
<td>Amphotericin B</td>
<td>A2942</td>
<td>1000x, 50 mg/ml</td>
<td>See Note 5</td>
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### Table 2. Primary antibodies

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<tr>
<th>Antibody</th>
<th>Target</th>
<th>Dilution</th>
<th>Host</th>
<th>Cat. number</th>
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<tr>
<td>Anti-β-Tubulin IV</td>
<td>Cilia</td>
<td>1:400</td>
<td>Mouse, IgG1</td>
<td>Abcam, AB11315</td>
<td>Clone ONS.1A6</td>
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<tr>
<td>anti-ZO1</td>
<td>Tight junctions</td>
<td>1:200 - 400</td>
<td>Goat</td>
<td>Abcam, AB99462</td>
<td>Directed against C-terminal domain</td>
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<tr>
<td>anti-dsRNA</td>
<td>dsRNA</td>
<td>1:500 - 1000</td>
<td>Mouse, IgG2a</td>
<td>ES consulting, MAb J2</td>
<td>Clone J2</td>
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<td>Anti-CD13</td>
<td>CD13/APN</td>
<td>1:200</td>
<td>Sheep</td>
<td>R&amp;D Systems, AF3815</td>
<td>Receptor 229E</td>
</tr>
<tr>
<td>Anti-CD26</td>
<td>CD26/DPPIV</td>
<td>1:200</td>
<td>Goat</td>
<td>R&amp;D Systems, AF1180</td>
<td>Receptor MERS</td>
</tr>
<tr>
<td>Anti-ACE2</td>
<td>ACE2</td>
<td>1:200</td>
<td>Goat</td>
<td>R&amp;D Systems, AF933</td>
<td>Receptor SARS NL63</td>
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<tr>
<td>intravenous</td>
<td>Viral proteins</td>
<td>1:1000</td>
<td>Human</td>
<td>Sanquin B.V. Nanogam</td>
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<tr>
<td>immunoglobulin</td>
<td></td>
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<td>(IVIG)</td>
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<tr>
<td>Anti-β-Tubulin</td>
<td>Cilia</td>
<td>1:400</td>
<td>Mouse, IgG1</td>
<td>Abcam, AB11309</td>
<td>Clone Tub2.1, Cy3</td>
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### Table 3a. Stock solutions for Trace elements

<table>
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<tr>
<th>Component</th>
<th>Formula</th>
<th>Cat.No.</th>
<th>Amount/ 25 ml</th>
<th>Comment</th>
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<tbody>
<tr>
<td>Selenium</td>
<td>NaSeO₃</td>
<td>S5261</td>
<td>130.0 mg</td>
<td>Solution stable 30 days at +4°C</td>
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<tr>
<td>Silicone</td>
<td>Na₂SiO₃· 9H₂O</td>
<td>S4392</td>
<td>3.55 gram</td>
<td></td>
</tr>
<tr>
<td>Molybdenum</td>
<td>(NH₄)₆MoO₂₄· 4H₂O</td>
<td>M1019</td>
<td>31.0 mg</td>
<td></td>
</tr>
<tr>
<td>Vanadium</td>
<td>NH₄VO₃</td>
<td>A1183</td>
<td>14.75 mg</td>
<td>Heat &gt;100°C to dissolve</td>
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### Table 3b. Stock solutions for Trace elements

<table>
<thead>
<tr>
<th>Component</th>
<th>Formula</th>
<th>Cat.No.</th>
<th>Amount/ 50 ml</th>
<th>Comment</th>
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<tr>
<td>Nickel</td>
<td>NiSO₄· 6H₂O</td>
<td>N4882</td>
<td>13.0 mg</td>
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<tr>
<td>Tin</td>
<td>SnCl₂· 2H₂O</td>
<td>S9262</td>
<td>5.5 mg</td>
<td></td>
</tr>
<tr>
<td>Manganese</td>
<td>MnCl₂· 4H₂O</td>
<td>M5005</td>
<td>10.0 mg</td>
<td></td>
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</tbody>
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References


CHAPTER 4
EFFICIENT REPLICATION OF NOVEL HUMAN BETACORONAVIRUS EMC ON PRIMARY HUMAN EPITHELium HIGHLIGHTS ITS ZOONOTIC POTENTIAL
Efficient replication of the novel human betacoronavirus EMC on primary human epithelium highlights its zoonotic potential.

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Abstract

The recent emergence of a novel human coronavirus (HCoV-EMC) in the Middle East raised considerable concerns as it is associated with severe acute pneumonia, renal failure and fatal outcome, and thus resembles the clinical presentation of severe acute respiratory syndrome (SARS) observed in 2002/2003. Like SARS-CoV, HCoV-EMC is of zoonotic origin and closely related to bat coronaviruses. The human airway epithelium (HAE) represents the entry point and primary target tissue for respiratory viruses and is highly relevant for assessing the zoonotic potential of emerging respiratory viruses, such as HCoV-EMC. Here we show that pseudostratified HAE cultures derived from different donors are highly permissive to HCoV-EMC infection, and by using RT-PCR and RNAseq data we experimentally determined the identity of seven HCoV-EMC subgenomic mRNAs. Although, the HAE cells were readily responsive to type-I and type-III interferon (IFN), we observed neither a pronounced inflammatory cytokine nor any detectable IFN responses following HCoV-EMC, SARS-CoV, or HCoV-229E infection, suggesting that innate immune evasion mechanisms and putative IFN antagonists of HCoV-EMC are operational in the new host. Importantly, however, we demonstrate that both type-I and type-III IFN can efficiently reduce HCoV-EMC replication in HAE cultures, providing a possible treatment option in cases of suspected HCoV-EMC infection.
Importance

A novel human coronavirus, HCoV-EMC, has recently been described to be associated with severe respiratory tract infection and fatalities, similar to SARS observed during the epidemic 2002/2003. Closely related coronaviruses replicate in bats, suggesting that, like SARS-CoV, HCoV-EMC is of zoonotic origin. Since the animal reservoir and circumstances of zoonotic transmission are yet elusive, it is critically important to assess potential species barriers of HCoV-EMC infection. An important first barrier against invading respiratory pathogens is the epithelium, representing the entry point and primary target tissue of respiratory viruses. We show that human bronchial epithelia are highly susceptible to HCoV-EMC infection. Furthermore HCoV-EMC, like other coronaviruses, evades innate immune recognition, reflected by the lack of interferon and minimal inflammatory cytokine expression following infection. Importantly, type-I and type-III interferon treatment can efficiently reduce HCoV-EMC replication in the human airway epithelium, providing a possible avenue for treatment of emerging virus infections.

Coronaviruses are enveloped positive-stranded RNA viruses of veterinary and medical importance that are mainly associated with respiratory and enteric infections [1, 2]. Some animal coronaviruses have long been known to cause severe diseases. In humans, however, it was long believed that coronaviruses cause mainly less severe respiratory infections known as common cold. This changed with the appearance of the Severe Acute Respiratory Syndrome-associated Coronavirus (SARS-CoV) which caused for the first time a coronavirus-induced life-threatening disease in humans and was responsible for the 2002/2003 epidemic involving more than 8000 reported cases and almost 800 fatalities [1]. The emergence of a novel coronavirus, HCoV-EMC, raises concerns that we may again face an epidemic caused by a zoonotic coronavirus [3]. HCoV-EMC is associated with severe respiratory tract infection, renal failure and fatalities [4, 5] and is like SARS-CoV closely related to bat coronaviruses [3]. Since the HCoV-EMC animal reservoir and circumstances of zoonotic transmission are yet elusive, it is critically important to assess which barriers of HCoV-EMC host switching and human-to-human transmission are operational.

An important first barrier against invading respiratory pathogens is the respiratory epithelium, which represents the entry point and primary target tissue of respiratory viruses. To assess the zoonotic potential of HCoV-EMC it is therefore critically important to determine if the human respiratory epithelium is susceptible to HCoV-EMC infection. To address this question we used HAE cultures that morphologically and functionally resemble the upper conducting airways in vivo [6]. The HAE culture system is based on primary human bronchial epithelial cells obtained by biopsy, brushing, surgery, or lung transplant. Isolated bronchial epithelial cells are manipulated with chemical defined medium to initiate
their differentiation into a pseudo-stratified human airway epithelial culture. When differentiation is complete, the pseudo-stratified HAE cell layer (i) contains basal, secretory, columnar, and ciliated cell populations, and (ii) will generate mucus [6, 7]. Therefore, this *in vitro* system recapitulates many aspects of the human airway epithelium, namely the presence of well defined cell types of the human airway epithelium, and physical barriers, such as the mucus layer.

To this end we have infected fully differentiated HAE cultures derived from three different donors with HCoV-EMC [3, 5] or SARS-CoV (strain Frankfurt-1) at a multiplicity of infection (MOI) of 0.1 and assessed viral growth kinetics. As shown in Fig. 1A, HAE cells are highly susceptible to HCoV-EMC infection, with peak virus production already at 48 hours post infection (h.p.i.). In contrast, replication of SARS-CoV appeared slower and reached peak virus production later at 72 – 96 h.p.i. (Fig. 1A). For both viruses, the vast majority of progeny virus was released at the apical side of HAE cultures, i.e. towards the mucus layer (Fig. 1A, upper and lower panels), compared to the lower and not always detectable virus release at the basolateral side, i.e. towards the medium (Fig. 1A, middle panels). We further analyzed HCoV-EMC and SARS-CoV infection of HAE cultures by immunofluorescence microscopy and stained for ciliated cells (β-tubulin), double-stranded (ds) RNA (as a marker for infected cells), and replicase gene-encoded non-structural protein 3 (nsp3; as marker for coronavirus replicase/transcriptase complexes). As shown in Fig. 1B, HCoV-EMC-infected cells were readily identified with a cross-reacting polyclonal antiserum directed against SARS-CoV nsp3 or a monoclonal antibody directed against dsRNA, which both visualized the characteristic punctuated perinuclear staining pattern for coronavirus replicase/transcriptase complexes. HCoV-EMC infected predominantly non-ciliated cells, suggesting that the putative receptor for HCoV-EMC host cell entry is likely to be expressed on non-ciliated cells of the human bronchial epithelium. We also analyzed intracellular HCoV-EMC -derived mRNAs by using an RNAseq approach. Total RNA from HCoV-EMC-infected HAE cultures was isolated at 6 h.p.i. using Qiagen’s RNAeasy Kit followed by mRNA subtraction according to manufacturer’s protocols. RNA-Seq libraries for an indexed Illumina sequencing run were established using ScriptSeq™ mRNA-Seq Library Preparation Kit (Epicentre, WI, USA) started from 1ng mRNA. Quality proven RNA-Seq libraries were analysed using Illumina’s HiSeq2500 system according to Illumina’s TruSeq protocols for single reads (TruSeq SBS Kit v3-HS, 50 cycles). Data analysis was performed using CLC Genomics Workbench 5.5 (CLC bio, Denmark). Before single read mapping raw reads were trimmed to eliminate ambiguous or remaining adapter sequences. We used all reads collected from 3 donors in duplicate experiments (total of 6 datasets) that failed to map to the human genome (25.053.494 out of 195.541.919 reads) for an alignment against the published HCoV-EMC genome sequence (Genbank accession: JX869059.2). 1616 out of 25.053.494 (0.006%) reads could be assigned to the HCoV-EMC genome, and we observed
a genome coverage reflecting the characteristic mRNA replication and transcription pattern expected for the coronavirus nested set of viral mRNAs (Fig. 1C). Indeed we could identify several reads representing leader-body fusion sequences of predicted HCoV-EMC mRNAs 2, 4, 7, and 8 (Fig. 1D) [3]. In addition, we experimentally determined by RT-PCR using total RNA from HCoV-EMC-infected HAE cells the leader-body fusion sequences of predicted mRNAs 3, 5, and 6 that were not represented in the RNAseq data (Fig. 1D; Suppl. Table 1). Collectively, our data show that the human bronchial epithelium is highly permissive to HCoV-EMC infection, and accordingly, that all cellular factors required for cell entry (e.g. receptor), replication and transcription of viral mRNAs, virus assembly and release are available in the human host.

Next we assessed HAE host cell responses to HCoV-EMC infection on the transcriptional level and compared them to responses to SARS-CoV and HCoV-229E infection (MOI=1). We chose to analyze the expression of a set of 15 cellular mRNAs (Suppl. Table 2) representing type-I IFN, type-III IFN, endosomal and cytoplasmic RNA sensor molecules, IFN-stimulated genes (ISGs), chemokines and inflammatory cytokines, at 3, 6 and 12 hours following type-I IFN (100 IU of IFN-a) and type-III IFN (100 ng/ml of IFN-I3) [8] treatment or virus infection. As shown in Fig. 2A, HAE cultures respond swiftly to type-I and type-III IFN treatment with upregulation of ISG expression (i.e. Mx1, 2'-5'OAS, Stat1, Mda5, Rig-I; Fig. 2A). Notably, the response of HAE cultures to IFN-a treatment supports previous studies showing high expression of the IFN-I receptor a-subunit (IFNLR1) in lungs and in epithelial cells [9]. In contrast to IFN treatment, the HAE cultures displayed only limited early transcriptional response to coronavirus infection, and particularly, no induction of IFN-b was observed in HCoV-EMC, SARS-CoV and HCoV-229E-infected cells (Fig. 2B). Also the expression of pro-inflammatory cytokines was only marginally induced, mainly in the common cold virus (HCoV-229E) infected HAE cultures at 6 h.p.i. Thus, immediate host responses to HCoV-EMC infection of HAE cultures are very similar to those observed in SARS-CoV- and HCoV-229E-infected cells, suggesting that HCoV-EMC is already well adapted to replication in HAE cultures, and that the human bronchial epithelium is not capable to mount a strong innate immune response in the absence of professional cytokine producing cells, such as plasmacytoid dendritic cells, conventional dendritic cells and macrophages [2, 10].

Since HAE cultures responded well to type-I and type-III IFN treatment, we addressed if these cytokines can reduce replication of HCoV-EMC, SARS-CoV, and HCoV-229E. HAE cultures derived from three different donors were left untreated or pretreated with IFN-a (100 IU) or IFN-I3 (10 ng/ml and 100 ng/ml) [8] 16 hours prior to infection (MOI=0.1) with HCoV-EMC, SARS-CoV, or HCoV-229E, and apically released progeny virus genomes were determined by qRT-PCR at 48 h.p.i.. As shown in Fig. 2C, pretreatment of HAE cultures with IFN-a reduced replication of HCoV-EMC and HCoV-229E for all three
different donors and reduced replication of SARS-CoV for two of three donors. Accordingly, we observed a pronounced reduction of the number of dsRNA-positive cells in IFN-a-treated HAE cultures that had been infected with HCoV-EMC, SARS-CoV or HCoV-229E (Fig. 2D). Notably, pretreatment of HAE cultures with IFN-I3 also reduced replication of HCoV-EMC, SARS-CoV and HCoV-229E for all three donors at both concentrations used (10ng/ml and 100 ng/ml). Like for IFN-a treatment, we observed a pronounced reduction of the number of dsRNA-positive cells in IFN-I3-treated and virus infected HAE cultures, further corroborating the importance of type-III IFN in epithelial antiviral host defense [9, 11].

In summary, we provide here conclusive evidence that the novel coronavirus HCoV-EMC can productively infect human bronchial epithelia cultures, suggesting that all necessary host cell factors for virus entry, RNA synthesis, virus assembly and release are available in the human host. HCoV-EMC replication in HAE cultures was at least as efficient as replication of SARS-CoV and HCoV-229E (this study and Dijkman et al., in press). We conclude that HCoV-EMC is capable of infecting the primary target tissue, the human respiratory epithelium, which is in accordance to the reported clinical presentation of severe respiratory symptoms [4, 5]. HCoV-EMC has been suggested to have a zoonotic origin, since closely related coronaviruses are known to replicate in bats. Considering that there is not yet any study reporting the successful isolation of a bat coronavirus, HCoV-EMC differs compared to known bat coronaviruses because it displays broad replication capability in diverse mammalian cell lines [12]. Our data show that the highly pathogenic viruses HCoV-EMC and SARS-CoV can both replicate in HAE cultures similar as the common cold viruses HCoV-229E, HCoV-OC43, HCoV-NL63 and HCoV-HKU1 (Dijkman et al., in press). Also early host cell responses are very similar between high and low pathogenic human coronaviruses. Thus, although our data depict the zoonotic potential of HCoV-EMC by demonstrating efficient replication in the human respiratory epithelium, decisive factors that impact HCoV-EMC pathogenicity remain to be determined. Importantly, we could demonstrate that HCoV-EMC replication is equally vulnerable to the antiviral effects of type-I and type-III IFNs, suggesting a possibility to interfere with HCoV-EMC replication in the human respiratory tract. IFN-a treatment has indeed been explored as therapeutic strategy during the SARS epidemic and raised considerable promise [13]. The critical importance of type-III IFNs in epithelial host defense [11], recent reports that treatment of Hepatitis C Virus-infected patients with pegylated IFN-I achieved rapid virological response, while adverse side effects were minimal [14], and our data concerning efficient inhibition of HCoV-EMC replication, should encourage the further development IFN-I treatment options specifically for respiratory virus and emerging virus infections.
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Ethics statement

Human bronchial epithelial cells were isolated from patients (age > 18 years old) who underwent bronchoscopy and/or surgical lung resection in their diagnostic pathway for any pulmonary disease and that gave informed consent. This was done in accordance with local regulation of the Kanton St.Gallen, Switzerland, as part of the St. Gallen Lung Biopsy Biobank (SGLBB) of the Kantonal Hospital St.Gallen which received approval by the ethics committee of the Kanton St.Gallen (EKSG 11/044, EKSG 11/103).
CHARACTERIZATION OF MERS-CoV

A

HCoV-EMC

SARS-CoV

Log_{10} GE virus copies/mL

0 24 48 72 96

Log_{10} PFU/mL

0 2 4 6 8 10

hpi

B

β-Tubulin Nsp3 dsRNA DAPI

Mock

HCoV-EMC

SARS-CoV

C

D

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Figure 1. Replication of HCoV-EMC and SARS-CoV on HAE cultures. A: HAE cultures from three donors (0712, black; 1001, light grey; 1505, dark grey) were prepared as described previously [7], and infected with HCoV-EMC or SARS-CoV (MOI=0.1). Progeny virus release at the apical (upper and lower panels) and basolateral (middle panels) surface of HCoV-EMC or SARS-CoV infected HAE cultures was determined as genome equivalents (GE) or plaque-forming units (PFU) per ml at indicated h.p.i. using quantitative real-time reverse transcription-PCR (qRT-PCR) specific for HCoV-EMC [15] and SARS-CoV [16] or titration of infectious particles on Vero cells. Experiments were performed in triplicate for each donor. Data are depicted as mean value ± standard deviation (SD); nd, not detected. B: HCoV-EMC and SARS-CoV infected (MOI 0.1) or mock-treated HAE cell cultures were fixed 48 h.p.i. with 6% PFA and immunostained using the procedure as described [17]. Rabbit polyclonal antiserum directed against SARS-CoV nsp3 (green; anti-SARS-CoV, Rockland) and mouse monoclonal antibody directed against dsRNA (red; J2, English & Scientific Consulting Bt.) were used as primary antibodies. Dylight 488 labeled, anti-mouse IgG (H+L), Dylight 647 labeled, anti-rabbit IgG (H+L) (Jackson Immunoresearch) were applied as secondary antibodies, followed by two separate incubation steps with Cy3-conjugated mouse anti-β-tubulin (light blue; Sigma) for staining of ciliated cells and DAPI (4',6-diamidino-2-phenylindole; Invitrogen) for staining nuclei (dark blue). Images were acquired using EC Plan-Neofluor 63x/1.40 Oil DIC M27 objective on a Zeiss LSM 710 confocal microscope. Image capture, analysis and processing were performed using the ZEN 2010 (Zeiss) and Imaris (Bitplane Scientific Software) software packages. Representative images are shown from one (1505) of three donors. C: Schematic representation of sequence reads of an RNAseq analysis of polyA-containing RNA derived from HCoV-EMC-infected HAE cultures (MOI=1; 6 h.p.i.). Single reads are depicted in green (sense) and red (antisense). The density of reads exceeding 34 for particular regions are shown condensed in grey. Blue arrows depict HCoV-EMC genes and ORFs. D: Summary of detected HCoV-EMC mRNAs. Leader-body junctions of HCoV-EMC mRNAs are shown with 15 nucleotides upstream and downstream the transcription regulatory sequence (TRS; bold). Numbers depict corresponding nucleotide positions in the HCoV-EMC genome. For all 8 viral mRNAs the open reading frames (ORFs) residing in the unique region and the method used for identification (RT-PCR or RNAseq) are indicated.
Characterization of MERS-CoV

Figure 2. Human coronavirus-host interaction. A: Gene expression analysis of IFN-treated HAE cultures. HAE cultures derived from three different donors were used untreated or were stimulated from the basolateral side with recombinant IFN-α (100 IU/ml; IFN-α A/D human, Sigma) or recombinant IFN-β (10 ng/ml) for 3, 6, and 12 hours until total cellular RNA was extracted using RNAeasy (Qiagen). Reverse transcription was performed with Moloney murine leukemia virus reverse transcriptase according to the manufacturer protocol (Invitrogen) using 1 μg of DNase-treated total RNA. Two μl of diluted cDNA was amplified according to manufacturer protocol, using primers targeting 15 different mRNA transcripts (Suppl. Table 1). Measurements and analysis were performed using a LightCycler® 480 II instrument and software package (Roche). Cycle profile, 10 min 95 °C, 45 cycles of 10 s at 95 °C, 20 s at 55 °C and 20 s at 72 °C followed by a melting curve step to confirm product specificity. Relative gene expression was calculated using the 2^ΔΔCt-method [18] and is shown as fold induction of IFN-treated samples compared to untreated controls. B: Gene expression analysis of virus infected HAE cultures. HAE cell cultures were infected with HCoV-EMC, SARS-CoV or HCoV-229E (MOI=1) and total cellular RNA was isolated at 3, 6, and 12 h.p.i. Relative gene expression analysis was performed as described above. C: Analysis of virus replication following IFN pretreatment. HAE cell cultures were left untreated or were treated from the basolateral side for 16 hours with recombinant IFN-α (100 IU/ml; Sigma) or recombinant IFN-β (10 ng/ml or 100 ng/ml) [8]. The basolateral medium was replaced prior to infection with HCoV-EMC, SARS-CoV and HCoV-229E infected (MOI=0.1). Apical progeny virus release was determined at 48 h.p.i. by qRT-PCR and is given as GE per ml. Each bar represents the mean ± S.D. from independent experiments performed in duplicate using HAE cultures derived from three different donors. ns, not significant (P>0.05); * P<0.05; ** P<0.01 (paired t-test). D: Immunofluorescence analysis of IFN-treated and virus infected HAE cultures. HAE cultures were fixed with 6% PFA and immunostained using the procedure as described [17]. Mouse monoclonal antibody directed against dsRNA (J2, English & Scientific Consulting Bt.) was applied as primary antibody and Dylight 488 labeled, anti-mouse IgG (H+L) as secondary antibody (green; Jackson ImmunoResearch), followed by staining of cilia with Cy3-conjugated mouse anti-β-tubulin (red; Sigma). Images were acquired using EC Plan-Neofluor 63x/1.40 Oil DIC M27 objective on a Zeiss LSM 710 confocal microscope. Image capture, analysis and processing were performed using the ZEN 2010 (Zeiss) and Imaris (Bitplane Scientific Software) software packages. Representative images are shown from one (0401) of three donors.
References

CHAPTER 5
SUCCESSFUL LENTIVIRAL TRANSDUCTION
AND DIFFERENTIATION OF PRIMARY
HUMAN BRONCHIAL EPITHELIAL CELLS
Successful lentiviral transduction and differentiation of primary human bronchial epithelial cells

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Abstract

Human respiratory viruses enter the human body through the upper conducting airways. Some remain there and cause mild respiratory symptoms, the common cold or mild flu, others venture further down to the distal alveoli and cause pneumonia and alveolar disease. Either way, the primary entry point of those viruses and other respiratory pathogens is the tracheobronchial tissue. This section of the lung tissue can be recapitulated in vitro using primary bronchial cells and collagen-coated cell culture inserts. The cells are cultured at air-liquid interface (ALI) and contain, after differentiation, many different cell types that have distinct functions in the polarized tissue. This bronchial epithelial layer is pseudostratified and ciliated. This ALI culture system is a universal platform to propagate and study human respiratory viruses. Coronaviruses (CoVs) are large RNA viruses that infect a broad range of vertebrate species and cause mostly respiratory and enteric disease. Six CoVs infect humans and four of those are commonly circulating in the human population and cause mild respiratory symptoms in healthy persons. Human airway epithelial (HAE) cultures at ALI have been successfully used to culture all of the known human CoVs (HCoVs). Despite their relatively high incidence the detailed interactions of HCoVs and respiratory host cells are not well understood. To further elucidate the mechanisms of virus – host interaction we established a robust protocol to make HAE cultures amenable to genetic modification for the knockdown of viral and/or host factors relevant to HCoV and other respiratory infections.
Introduction

The human lungs are a large organ and span a relatively long anatomical distance. As a result, the pulmonary histology differs depending on anatomical location and tissue function. The upper airways are ciliated and pseudostratified and contain multiple cell types with varying roles in the differentiated tissue [1]. Basal cells serve as resident stem cells in the conducting airways [2], goblet cells produce protective mucus and ciliated cells are responsible for cleaning out both mucus and debris [3]. The distal alveolar region, however, contains only two different cell types organized into a single squamous epithelial layer. Alveolar type (AT) II cells maintain the epithelium by maintaining themselves and differentiating into AT I cells [4].

Various viruses infect the human airways and cause a range of respiratory illness. The most notorious are the influenza A viruses that not only cause yearly seasonal respiratory illness in humans but have also caused many pandemics in the human population [5, 6]. Another family of viruses, the coronaviruses, is also of medical importance. Four human coronaviruses (HCoVs) circulate in the human population, HCoV-229E, HCoV-OC43, HCoV-NL63 and HCoV-HKU1 and are thought to cause between 5 and 10% of all upper and lower respiratory tract infections [7, 8]. These commonly circulating viruses mostly cause general respiratory tract illness but can cause more severe disease in the immunocompromised and the elderly [9-11]. Additionally, two other HCoVs, SARS-CoV and MERS-CoV, have caused epidemic infections in humans and are capable of causing more severe disease compared to the other four [12, 13]. Despite their somewhat high incidence, many exact mechanisms of virus host interactions of HCoVs remain to be elucidated.

Human airway epithelial (HAE) cultures are organotypic cell cultures that morphologically and functionally resemble the human airway in vivo [14]. Currently, both the upper and lower airways can be recapitulated in vitro by using this system. The cells are cultured on porous inserts where only the basolateral side is in contact with growth medium while the apical side is exposed to air (air-liquid interface, ALI), like in vivo airway epithelium. To represent different areas of the pulmonary epithelium different cell types can be seeded into this system. Bronchial HAE cultures contain, after differentiation, many different cell types, such as basal, ciliated and goblet cells. Furthermore, these cultures are pseudostratified and generate protective mucus much like in vivo bronchial epithelium [15, 16]. Therefore, in vitro bronchial HAE cultures are ideal for virus - host interaction studies with human respiratory viruses since they represent the primary entry point of these pathogens. In vitro, alveolar cultures have only a single epithelial layer composed of only two cell types and are ideal for studies with viruses that have a tropism for the lower airways and are known to cause pneumonia and alveolar disease. Currently, all known
HCoVs can be cultured in the bronchial HAE system [17, 18]. Additionally, SARS-CoV, MERS-CoV and HCoV-HKU1 have been successfully cultured in the alveolar model [19-21].

Traditionally, virus host interactions are studied in animal models and human respiratory viruses are usually investigated in ferrets and transgenic mice [22, 23]. However, in order to infect these animals, viruses often have to be adapted by serial passage. This may cause both genotypic and phenotypic differences between the original human virus and the animal adapted virus being studied. Furthermore, it is often difficult to translate these results directly to human disease. Therefore, it’s important to study human viruses in authentic human target cells. The HAE cultures serve as a universal platform for the study and propagation of human respiratory viruses [17, 24-26]. However, in order to fully utilize the potential of this culture system it must be made amenable to genetic modification. Transgenesis would enable the study of viral and/or host factors important for respiratory virus infections and allow us to elucidate specific mechanism of virus-host interactions by specific knockdown. However, using primary cells for genetic modification can prove challenging since they have a finite life span in cell culture, for example primary human bronchial cells can only be passaged twice after isolation if they are supposed to differentiate normally in ALI.

RNA interference (RNAi) causes gene knockdown on the RNA level and has become a vital part of the genetic toolkit for loss-of-function studies in eukaryotes [27]. It is a conserved biological reaction to double stranded RNA, mediating resistance to various pathogens [28]. The RNAi of endogenous genes is mediated by processing microRNAs (miRNAs), the largest class of small non-coding RNAs. After initial processing [29, 30] they are transported from the nucleus to the cytoplasm and further cleaved into 22 nucleotide (nt) duplexes by Dicer [31, 32]. One strand of the RNA duplex is then loaded onto the RNA inducing silencing complex (RISC), which mediates mRNA sequence recognition. Argonaute (Ago) proteins use one miRNA strand as a template for identification and Ago2, a component of RISC, cleaves complementary mRNAs [33]. Post-transcriptional gene silencing with short RNAs can also be mediated through translational inhibition since the cellular translational machinery is unable to recruit mRNAs already bound to RISC. RNAi used for experimental purposes takes advantage of this cellular processing pathway [34]. Short-interfering RNAs (siRNAs) are chemically synthesized and directly transfected into mammalian cells and cause post-transcriptional knockdown of genes of interest (GOIs). However, the effect of siRNAs is transient and limited to cells that are transfectedable. For a more stable knockdown, lentiviral transduction of target cells and genome integration of short-hairpin RNAs (shRNAs) provide a more robust and long-term knockdown of GOIs [27]. Both sequence and structure of these hairpin RNAs can affect the quality of gene knockdown. Furthermore, off-target effects caused by sequence homology to transcripts not meant for knockdown always have to be considered during shRNA design and testing.
In the present study we use shRNA lentiviral vectors to establish transgenic organotypic human airway cultures. Specifically, we determined optimal timepoints and procedures for the transduction of primary human bronchial cells. We also demonstrated that HAE cultures constitutively expressing a non-targeting scrambled (Scr) control shRNA and green fluorescent protein (GFP) differentiate normally and transgene expression is readily observed in all cellular subgroups post-differentiation. In the long term, this will allow for the establishment of HAE cultures homogenously expressing shRNAs against GOIs involved in human respiratory virus infections, greatly facilitating further detailed studies of virus – host interactions in primary human airway epithelium.

Materials and Methods

Cell isolation
Primary human bronchial cells were isolated from patients (>18 years old) undergoing either bronchoscopy or pulmonary resection at the Cantonal Hospital in St. Gallen, Switzerland. Informed consent was acquired from all patients. This was done according to the regulations of the Canton St.Gallen, Switzerland as a part of the St. Gallen Lung Biopsy Biobank (SGLBB) of the Cantonal Hospital in St.Gallen. Approval was acquired by the ethics committee of the Canton of St.Gallen (EKSG 11/044, EKSG 11/103). Cell isolation was performed with protease and DNase digestion as previously described ([36], chapter 3). Cells were either cryopreserved at -150°C for later use or used directly for transduction.

Cell culture
All cell cultures were incubated at 37°C and 5% CO₂. Furthermore, all primary cells were cultured on collagen type I (col I) coated culture flasks.

Primary human bronchial cells in monolayer and ALI were cultured as previously described ([36], chapter 3), briefly summarized below.

Col I coating: Cell culture flasks (Techno Plastic Products, TPP, Switzerland) were coated with col I (VitroCol, Advanced Biomatrix, USA) diluted 1:75 in sterile filtered water and incubated for at least 2 hours at 37°C (5% CO₂) with enough col I solution to cover the bottom of the flask. After incubation, flasks were washed three times with corresponding volumes of phosphate buffered saline (PBS). Flasks were stored dry at 4°C for maximum 6 weeks.
Monolayer: Primary human bronchial cells were cultured on col I coated culture flasks in Bronchial Epithelial Growth Medium (BEGM), LHC basal medium (Gibco, Thermo Scientific Inc.) supplemented with growth factors (Sigma Aldrich, USA), penicillin (60mg/mL, Gibco, Thermo Scientific Inc.) and streptomycin (100mg/mL, Gibco, Thermo Scientific Inc.).

Air-liquid interface (ALI): Primary human bronchial cells were seeded in ALI medium, supplemented with growth factors and penicillin (60mg/mL, Gibco, Life Technologies) and streptomycin (100mg/mL, Gibco, Life Technologies) into 24-well transwell plates (Corning international, USA) at the density of 80.000 cells/well. Medium was changed every other day. Once confluent, the apical medium was removed to establish ALI. Cultures were allowed to differentiate for 4-6 weeks prior to any analyses.

Lentiviral vectors
Lentiviral packaging plasmids (pMDL (gagPol), pRev, pVSV-g) and a pLKO.1 vector expressing Scr shRNA (Mission™ SHC002, Sigma Aldrich) and enhanced GFP were generously provided by Prof. Dr. B. Berkhout and Dr. J. Eekels [37]. The transfer vector will be referred to in this study as pLKO_GFP_Scr.

Lentiviral vector production
On day 1, 2.2 million low passage (<30) HEK 293T cells were seeded in 2.2mL Dulbecco’s Modified Eagles Medium (DMEM Glutamax™, Gibco, Thermo Scientific Inc.) supplemented with 10% heat inactivated Fetal Bovine Serum (FBS) and penicillin (60mg/mL, Gibco, Thermo Scientific Inc.) and streptomycin (100mg/mL, Gibco, Thermo Scientific Inc.) in a T25 cell culture flask (TPP, Switzerland) 24 hours prior to transfection. On day 2 cells were transfected with Lipofectamine 2000® (Thermo Scientific Inc.) and Optimem (Gibco, Thermo Scientific Inc.) mixed with 2.4μg pLKO_GFP_Scr, 1.6μg pMDL, 0.6μg pRev and 0.8μg pVSV-g. 16μL Lipofectamine 2000® and 670μL Optimem were mixed in an eppendorf tube and incubated at room temperature (RT) for 5 minutes. In the meantime, plasmid DNA was mixed in another tube. Transfection mixture and DNA was then mixed together and incubated for 20 minutes at RT. Lastly, the mixture was added dropwise to the cells and incubated for at least 48 hours. Lentivirus containing supernatant was then collected on ice and spun down at 400g for 5 minutes at 4°C and stored at -80°C. Virus particles were either used directly for transduction of primary human bronchial cells or titrated on Huh7 cells in a 12 well cell culture plate (TPP, Switzerland) and analyzed for GFP positivity with FACS Canto (BD bioscience, USA). Lentiviral titer was calculated using the method described by Barde, Salmon and Trono [38].
Transduction
Well differentiated HAE cultures were incubated at a multiplicity of infection (MOI) of 5 in 200μL Hank’s balanced salt solution (HBSS, Gibco, Thermo Scientific Inc.) either apically or basolaterally for 24 hours, after which the cultures were washed three times with HBSS. GFP expression was evaluated daily for 7 days.

Undifferentiated primary human bronchial cells were transduced in suspension with either 500μL lentiviral supernatant or at indicated MOI for 4 hours at 37°C and 5% CO₂ in batches of 100.000 cells in 1mL total BEGM with gentle shaking every hour. Subsequently, cells were seeded into coll coated T25 flasks (TPP, Switzerland) for monolayer culture in 4mL total BEGM with lentiviral supernatant left on for 24 hours prior to washing with PBS and BEGM media change. Mock cells were incubated with the corresponding volume of DMEM Glutamax™ + 10% FBS to account for the effects of serum. Once confluent, cells were expanded into T75 flasks (TPP, Switzerland) to acquire enough cells for the establishment of HAE cultures.

Flow Cytometry
Cells were trypsinized with 0.05% Trypsin/EDTA (Gibco, Thermo Scientific Inc), resuspended and fixed with 1mL 4% buffered formaldehyde (FORMAFIX, Formafix Switzerland AG) at RT for 15 minutes and washed with PBS. Cells were stained with antibodies against tubulin (Cell Signaling, USA, 3624S, Alexa Fluor-488), Nerve growth factor receptor (NGFR, BD bioscience, USA, 562122, PE-Cy7) and Mucin 1 (Biolegend, USA, 355604, PE) in 100μL Cell Wash buffer (CB, BD Bioscience, USA) in batches of 200.000 cells on ice for 20 minutes and washed twice in 1mL CB (400g, 4°C, 5 minutes). Cells were then resuspended in 100μL of CB and analyzed with FACS Canto (BD Bioscience, USA).

Immunofluorescence
HAE cultured were fixed with 4% FORMAFIX (Formafix, Switzerland AG) and stained as previously described ([36], chapter 3).

Briefly, filters were washed with HBSS prior to fixation with 4% FORMAFIX (Formafix Switzerland AG) for 15 minutes at RT. Primary antibodies were incubated in confocal buffer (CB) for 2 hours followed by incubation of secondary antibodies for 1 hour. Finally cultures were counterstained with DAPI and washed with CB buffer and PBS. Lastly, the filter membrane was cut out of the insert and mounted on a glass slide for confocal analysis. In the current study, cultures were stained for β-tubulin (Abcam, USA, ONS.1A6) and tight junctions (ZO-1, Abcam, USA). Nuclei were counterstained with DAPI (Fisher Scientific Inc.). Samples were imaged on a Zeiss LSM7 confocal microscope (Carl Zeiss AG, Germany).
corresponding Zeiss LSM software along with IMARIS (Bitplane, Switzerland) was used for image analysis.

**Data presentation**
Data was plotted using GraphPad Prism 5 and figures were assembled using Corel Draw X3.

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This work was supported by the 3R research foundation Switzerland, project 128-11.

**Data contribution**
Hulda R. Jonsdottir performed the experiments, assembled data and figures and wrote the manuscript. Regulo Rodriguez provided human lung tissue. Ronald Dijkman provided technical assistance and supervision. Volker Thiel provided supervision and revised the manuscript.
Results

Vector design
The lentiviral vector used for the transduction of primary human bronchial cells expresses a constitutively active Scr shRNA (Mission™ SHC2, Sigma Aldrich) and GFP. shRNA expression is driven by the human U6 promoter, a polymerase III promoter, commonly used for the expression of small RNAs [39]. However, the GFP is expressed using a different promoter, the human phosphoglycerate kinase promoter (hPGK), a constitutive promoter, better suited for the expression of GFP in lentiviral constructs [40, 41]. The woodchuck hepatitis virus regulatory element (WPRE) both reduces transcriptional readthrough of the vector as well as enhancing gene expression [42, 43]. In this vector the shRNA and GFP are simultaneously and continuously expressed (figure 1). Flanking those elements are the HIV 5´ and 3´ long terminal repeats (LTRs) marking off the pro-viral insert. A packaging signal and a rev response element (RRE) are also required within the integrated sequence to ensure correct packaging of the viral insert and proper gene expression, respectively. Lentiviral particles are packaged using a third generation lentiviral system [44]. Three packaging plasmids are required for viral particle production, pMDL, encoding the gag-pol gene of HIV driven by the cytomegalovirus (CMV) promoter, pRSV-Rev for expression of the HIV rev gene from the Rous sarcoma virus (RSV) U3 promoter. Lastly, the viral particle production requires an envelope glycoprotein expressing plasmid. In this study, we used the vesicular stomatitis virus glycoprotein (VSV-g). This glycoprotein is often used for lentiviral pseudotyping due to its broad cellular tropism (reviewed in [45]).

![Figure 1: GFP lentiviral vector.](image)

Both shRNA and GFP are constitutively active but transcribed from separate promoters. WPRE promotes higher gene expression and less transcriptional readthrough. Flanking the elements displayed here are essential HIV components required for genome integration, viral packaging and proper gene expression during virus replication.

Well differentiated HAE cultures cannot be transduced
In an effort to establish transgenic HAE cultures we attempted to transduce well differentiated cultures with VSV-g pseudotyped lentiviral particles. However, intact cultures are refractory to both apical and basolateral transduction while damaged cultures can be transduced from the apical side when the basolateral side of the epithelium is exposed. In those cultures GFP expression is only observed around the damaged epithelium (figure 2, GFP). This is consistent with the observation that receptors for VSV-g are predominantly
located on the basolateral side of polarized epithelia [46]. Basolateral application of lentiviral particles does not seem to penetrate the porous inserts and the collagen coating and no GFP positivity is observed post-transduction.

Figure 2: Well differentiated HAE cultures cannot be transduced. Neither apical nor basolateral transduction of intact HAE cultures with VSV-g pseudotyped lentiviral vectors results in transduction. Disrupted cultures can be transduced around the edge of the damaged epithelium. Original magnification 10x.

Since the transduction of well differentiated cultures is not possible without the disruption of the epithelial layer another consistent method of primary cell transduction had to be developed. However, the finite life span of primary cells *in vitro* poses a unique challenge.

**Transduction of undifferentiated primary human bronchial cells**

Since well differentiated HAE cultures cannot be transduced with VSV-g pseudotyped lentiviral particles we assessed the efficacy of other transduction methods for primary bronchial cells. HAE differentiation takes at least four weeks; therefore, the cultures require stable incorporation of genes to be expressed. Stable integration can be achieved with lentiviral vectors where the proviral insert is integrated into the cellular genome. This way both transgenes and/or shRNAs can be integrated for either over expression or knockdown of host and/or viral factors. We assessed the optimal duration and method of transduction of human bronchial cells in the undifferentiated state with VSV-g pseudotyped pLKO_GFP lentiviral particles (table 1 and figure 1). Although GFP expression varied from experiment to experiment, suspension transduction of these cells resulted in the highest percentage of GFP positive cells. Addition of polybrene during transduction did not seem to increase the efficacy of transduction. Furthermore, spinoculation with polybrene was not particularly effective and resulted in a general loss of cell viability. Additionally, immediate washing of
cells post-suspension to remove lentiviral supernatant by centrifugation also resulted in a loss of viability. The final optimized protocol utilizes suspension transduction for 4 hours, followed by additional 24 hours incubation of lentivirus containing supernatant during cell attachment in monolayer (table 1).

### Table 1: Transduction of primary human bronchial cells

<table>
<thead>
<tr>
<th>Cells ($10^3$)</th>
<th>Method</th>
<th>Polybrene</th>
<th>Time (h)</th>
<th>MOI</th>
<th>$V_{LV-GFP}$</th>
<th>%GFP</th>
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<tr>
<td>100</td>
<td>Suspension</td>
<td>No</td>
<td>4</td>
<td>--</td>
<td>100μL</td>
<td>26</td>
</tr>
<tr>
<td>500</td>
<td>Suspension</td>
<td>No</td>
<td>4</td>
<td>--</td>
<td>500μL</td>
<td>11-25</td>
</tr>
<tr>
<td>100</td>
<td>Spinoculation</td>
<td>Yes</td>
<td>1.5</td>
<td>--</td>
<td>100μL</td>
<td>9-13</td>
</tr>
<tr>
<td>100</td>
<td>Suspension</td>
<td>Yes</td>
<td>4</td>
<td>--</td>
<td>100μL</td>
<td>15</td>
</tr>
<tr>
<td>100</td>
<td>Suspension</td>
<td>No</td>
<td>28</td>
<td>1</td>
<td>--</td>
<td>2-5</td>
</tr>
<tr>
<td>100</td>
<td>Suspension</td>
<td>No</td>
<td>28</td>
<td>5</td>
<td>--</td>
<td>27</td>
</tr>
<tr>
<td>100</td>
<td>Suspension</td>
<td>No</td>
<td>28</td>
<td>7</td>
<td>--</td>
<td>16-18</td>
</tr>
<tr>
<td>100</td>
<td>Suspension</td>
<td>No</td>
<td>28</td>
<td>1</td>
<td>--</td>
<td>29-33</td>
</tr>
<tr>
<td>100</td>
<td>Suspension</td>
<td>No</td>
<td>28</td>
<td>5</td>
<td>--</td>
<td>62-74</td>
</tr>
</tbody>
</table>

Specifically, cells were incubated in suspension at 37°C and 5% CO$_2$ with gentle shaking every hour for 4 hours, after which cells were seeded in T25 culture flasks with lentiviral containing supernatant in 4mL total BEGM. Medium was changed after 24 hours, bringing the total lentiviral incubation time to 28 hours.

Using this protocol we were able to transduce primary bronchial cells with an efficacy of 30-70%, depending on the MOI (table 1). GFP positivity in this range allows for the evaluation of the differentiation potential of GFP positive HAE cultures.
Establishment of transduction protocol
Suspension transduction of primary human bronchial cells requires at least two extra passages prior to the establishment of HAE cultures compared to naïve cells. After transduced cells have been expanded in monolayer the cells are seeded on porous inserts and liquid-liquid interface (LLI) established. Once confluent, the apical medium is aspirated, establishing ALI (figure 3). By incorporating the transduction and expansion steps, transgenic bronchial cells cannot be seeded in LLI before monolayer passage three. Unfortunately, differentiated HAE cultures cannot be reliably established after monolayer passage two due to cellular senescence and loss of differentiation potential. Therefore, we had to devise a method that would extend the life span of primary human cells in cell culture.

Figure 3: Establishment of transgenic HAE cultures. Post suspension transduction, a monolayer expansion step is required to acquire enough cells for LLI interface. Upon establishment of ALI, differentiation is induced.

Treatment of primary cells with Y-27632 prolongs basal cell phenotype.
Mammalian primary cells cultured in vitro have a short and finite lifespan and can only be continuously cultured for a brief period of time. Primary human bronchial cells meant for HAE cultures can only be passaged twice after isolation before the basal cell phenotype, along with their differentiation capabilities, is gradually lost. In order to establish transgenic HAE cultures, their life span must be extended to allow for lentiviral transduction, expansion and eventual cell sorting. To achieve this, a minimum of five passages is required. Treatment of both bronchial and cervical epithelial cells with a commercially available Rho associated protein kinase (ROCK) inhibitor, Y-27632, has been shown to induce a basal epithelial cell phenotype, thereby preserving the differentiation capabilities of human epithelial cells in vitro [47]. We therefore assessed the differentiation potential of Y-27632 treated primary human bronchial cells in ALI conditions. Indeed, when these cells were treated with 10μM of this inhibitor they showed an increase in the expression of alpha6-integrin and neural growth factor receptor (NGFR), common basal cell markers, compared to non-treated cells in later passage (figure 4A). The up-regulation of these basal cell markers indicates that the epithelial basal cell phenotype can be induced and prolonged in treated cells. Furthermore, HAE cultures established with treated cells showed the same cellular composition as non-treated cells up to passage (p) 4, as measured by the expression of tubulin (ciliated cells), NGFR (basal cells) and Mucin 1 (goblet cells). After p4, non-treated cells were unable to sustain growth in ALI while treated cells, independent of
donor, differentiated normally (figure 4B). The removal of the inhibitor from culture medium upon LLI culture seem to be sufficient for the cells to proceed through differentiation normally post-treatment. To confirm the proper structure and morphology, HAE cultures from both treated and non-treated cells were stained for cilia (β-tubulin) and tight junctions (ZO-1). In low passage (p2) the two groups share the same structure and morphology. However, in p4, only Y-27632 treated cells were able to maintain epithelial integrity post – differentiation. Non-treated cells in p4 seem to differentiate normally early on but eventually the epithelial layer gets disrupted (figure 4C).

Collectively, these results indicate that the treatment of primary human bronchial cells with Y-27632 induces a basal cell phenotype in primary human bronchial cells and allows for increased monolayer passage before establishment of ALI HAE cultures. These extra passages will allow for cellular expansion post lentiviral transduction to establish transgenic organotypic epithelial cultures using primary cells.
Y-27632 treated cells can be transduced to similar levels as non-treated cells

Next we assessed whether Y-27632 treated cells could be transduced to the same levels as naive cells. Suspension transduction of cells isolated from different donors with an MOI of 5 results in varying levels of transduction efficacy (40-70%) and thereby GFP positivity (figure 5A, top). Treatment of primary bronchial cells with Y-27632 results in slightly lower transduction efficacies under the same conditions (30-60%, figure 5A, bottom). Interestingly, although basal phenotype has been induced in all three donors the donor variability is still present and slightly exaggerated (figure 5A). Furthermore, Y-27632 treated cells grow faster and to a higher confluency, compared to non-treated cells (figure 5B, phase contrast). GFP expression is uniformly cytoplasmic and has varying brightness, most likely reflecting the number of integration events per cell (figure 5B, fluorescence).

Figure 5: Cells treated with 10 µM Y-27632 can be transduced with lentiviral vectors to similar levels as non-treated cells. **A:** Primary human bronchial cells treated with 0 and 10 µM Y-27632 were transduced with pLKO_GFP_Scr and analyzed for GFP expression. Treated cells can be transduced to similar levels as non-treated cells but donor variability is more evident. Data is shown for three donors; 1809, 1401 and 1904. **B:** Expression of GFP in non-treated (top) and Y-27632 treated (bottom) cells is of similar strength. However, treatment results in faster growth and a more confluent cell layer, resulting in higher total number of cells. Original magnification 10x. Data is shown for donor 1401.
Heterogeneously transgenic HAE cultures differentiate normally and transgene expression remains stable

The unique cellular composition of fully differentiated HAE cultures is essential to all virus host-interaction studies and the composition of transgenic HAE cultures must remain similar to naïve cultures to preserve the functionality of the system. As analyzed by flow cytometry, the cell composition of heterogeneously transgenic HAEs does not differ between pLKO_GFP_Scr and naïve cultures, measured by the division into ciliated (tubulin), goblet (Mucin1) and basal cells (NGFR) (figure 6A). More importantly, GFP expression can be observed in all cellular subgroups within the differentiated cultures (figure 6B). This data indicates that lentiviral transduction does not interfere with the differentiation potential of primary human bronchial cells. Also, transgene (GFP) expression can still be observed 6 weeks post-differentiation (figure 6C).

The maintenance of the cellular composition of the HAE cultures post-lentiviral transduction is of the utmost importance. Since no difference was observed in the composition of transgenic cultures compared to naïve controls and GFP transgene expression can be observed after 6 weeks of differentiation, in all cellular subgroups, we are able to successfully transduce progenitor basal cells in monolayer prior to LLI establishment. This suggests that FACS sorting of transgene positive cells would not deplete any cell population from the resulting differentiated cultures and allow for the establishment of transgenic cultures with uniform distribution.
Figure 6: Heterogeneously transgenic cultures share the culture composition of naïve cultures while transgenic expression can be observed in all cellular subgroups. Moreover, transgene expression remains stable post-differentiation. A: Culture composition of transgenic HAE cultures (MOI 1 and 5), compared by flow cytometry, resembles that of naïve ones. Cell transduction does not seem to interfere with differentiation potential. B: Transgene (GFP) expression can be observed by flow cytometry in all cellular subgroups post-differentiation; indicating that transduction of pre-differentiated progenitor cells in suspension has been successful. Eventual sorting of positive cells should therefore not effect culture composition. C: Transgene expression (GFP) is stable post-differentiation (6 weeks). Original magnification 10x. Data is shown for donor 0510.
Discussion

In this study we show that establishment of transgenic bronchial HAE cultures from primary cells is feasible, primary human bronchial cells can be transduced with lentiviral vectors and properly differentiated (figure 6A). Importantly, transgene expression (GFP) can be observed in all differentiated cellular subgroups (figure 6B) suggesting that cell sorting and the eventual establishment of homogenously transgenic cultures can be implemented. However, primary bronchial epithelial cells must be transduced in the undifferentiated state since differentiated HAE cultures are refractory to both apical and basolateral transduction (figure 2). Furthermore, we show that by treating the cells with a Rho associated protein kinase inhibitor (Y-27632), we can prolong their basal cell phenotype and induce proper HAE differentiation in later passages compared to naïve cells (figure 4).

Primary organotypic cell culture systems, such as the HAEs, are well suited for the study of virus-host interactions. By using these cultures, virus infection can be observed in natural target cells representing the human airway. So far, HAE cultures have been successfully used to propagate and study various respiratory viruses, including all known human coronaviruses (HCoV). Further emphasizing the usefulness of this culture system, there are no animal models available for most of the commonly circulating HCoVs and HCoV-HKU1 does not replicate in any two-dimensional monolayer culture and was first propagated in HAE cultures in 2010, after cultivation in cell lines had failed [48]. This further demonstrates the importance of these cultures in human respiratory virology.

By making HAE cultures amenable to genetic modification we can create various knockdown cultures and shed further light on the host response to HCoV infection. When naïve HAE cultures are infected with SARS-CoV, MERS-CoV and HCoV-229E, no notable innate immune response is observed. However, when the cultures are treated with recombinant type I or III interferon (rIFN) various interferon stimulated genes (ISGs) are up-regulated. This indicates that the HAE cultures have functioning interferon signaling pathways but during coronavirus infection these pathways are suppressed [18]. By using transgenic HAEs along with wild-type (wt) HCoVs and various available virus mutants, host or viral factors involved in innate immune sensing and signaling can be clarified. For example, the interaction between the viral non-structural protein (nsp) 16 and the host cellular sensor MDA5 were elucidated using a mouse hepatitis virus (MHV) Δnsp16 mutant and MDA5 knockout mice [49].

In order to establish HAE cultures with homogenously transgenic cell layers, FACS sorting of positive cells has to be applied. Homogenously transgenic cultures are required to ensure the overlap between virus infection and transgenic cells. Homogenously transgenic HAE cultures can be applied to various research fields. Firstly, all human respiratory pathogens, including viruses, can be studied in this system and knockdown of
innate immune components could also shed further light on the zoonotic potential of animal viruses. Second, genetic respiratory diseases where gene expression is abnormal or absent, can be mimicked in the transgenic system and studied \textit{in vitro}. However, to further increase the usefulness of this system it should be made inducible since a delicate gene expression pattern is required for normal differentiation and pseudostratification of HAE cultures. Constitutive knockdown of host genes might interfere with this process resulting in compromised cultures. By employing an inducible system, knockdown can be chemically induced only around time of infection and/or analysis ensuring the proper differentiation and cellular composition of transgenic HAE cultures.
References


CHAPTER 6
INDUCIBLE shRNA KNOCKDOWN IN HOMOGENEOUSLY TRANSGENIC HUMAN AIRWAY EPITHELIAL CELLS
Inducible shRNA knockdown in homogeneously transgenic human airway epithelial cultures

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Abstract

Human airway epithelial (HAE) cultures are a universal platform to study respiratory virus–host interactions in natural target cells. These cultures represent the primary entry point of human respiratory viruses and so far many different types of viruses have been propagated in these cultures. That includes human coronaviruses (HCoVs). Six HCoVs have currently been identified; four of those circulate yearly in the human population and generally cause the common cold, while two others have caused epidemics of more severe disease. HCoV-299E is one of the common HCoVs that circulate in the human population. To further elucidate the interactions between HCoVs and important host factors in the human airway epithelium we have utilized lentiviral transduction and short hairpin RNAs (shRNAs) to establish transgenic HAE cultures to knockdown specific host or viral genes. We have previously shown that heterogeneously transgenic HAE cultures differentiate normally after lentiviral transduction and transgene expression is present in all cellular subgroups post-differentiation. In this study, we have adapted this system further, establishing homogenously transgenic HAE cultures. Simultaneously, we developed an inducible lentiviral system for drug induced shRNA knockdown. By using various homogenously transgenic HAE cultures with inducible knockdown and HCoVs we aim to shed further light on the molecular interactions between important viral and host factors in the human respiratory epithelium.
Introduction

The primary entry point of human respiratory viruses is the human upper conducting airway. Organotypic cell cultures representing the tracheobronchial epithelium of the human lungs are especially fitting for virus-host interaction studies since they accurately recapitulate the human in vivo epithelium. After differentiation, these cultures contain many different cell types with distinct functions in the polarized tissue. Basal cells reconstitute the epithelium, goblet cells secrete productive mucus and ciliated cells clear away mucus and debris [1-3]. These human airway epithelial (HAE) cultures have been used successfully in various infection studies with human respiratory viruses [4-6]. Coronaviruses (CoVs) are RNA viruses that infect a broad range of mammalian and avian hosts and mainly cause respiratory and enteric disease [7]. Six human coronaviruses (HCoVs) have been identified; four circulate regularly in the human population while two others have caused epidemics of severe respiratory disease [8-14]. All of the currently known HCoVs can be successfully cultured in HAE cultures [15, 16]. HCoV strain 229E is one of the commonly circulating CoVs and generally causes mild upper respiratory tract symptoms in healthy individuals. However, HCoV-229E infection can cause severe respiratory disease in frail elderly persons and the immunocompromised [17, 18]. The commonly circulating HCoVs are believed to be responsible for 5-10% of all upper respiratory tract infections but despite their high incidence their interactions with host cells in the human respiratory epithelium are not well understood [19-21]. In order to shed further light on host-virus interactions during HCoV infection, we established a transgenic model of HAE cultures using lentiviral transduction and short hairpin RNAs (shRNAs) for stable knockdown of host genes (Jonsdottir HR et al. unpublished data, chapter 5). In this study, we further explored this system and established an inducible transgenic HAE system. The possibility of inducing gene silencing post-differentiation ensures that the knockdown does not interfere with the differentiation potential of the primary bronchial cells [22]. Furthermore, constitutive knockdown of genes can also affect the growth and/or viability of these cells making ALI establishment unfeasible or impossible [23]. The expression of the shRNAs in our lentiviral vector is regulated by the Lactose operon (LacO), located within the shRNA human U6 promoter, and treatment of transduced cells with Isopropyl β-D-1-thiogalactopyranoside (IPTG), an allolactose mimic, induces shRNA expression by binding the operon bound Lactose repressor (Lacl), relieving the transcriptional repression of the shRNA [24]. The transcribed shRNA is then processed by the endogenous RNAi pathway creating a small RNA duplex which binds the RNA induced silencing complex (RISC) and sequesters complementary mRNAs and either blocks their translation by physically preventing it or causes mRNA degradation, resulting in gene knockdown on the RNA level [25]. Furthermore, this vector contains an mCherry fluorescent gene which is expressed in
combination with the Lacl as a 2A-linked polyprotein [26]. Unlike the shRNA, the mCherry is continuously expressed in transduced cells allowing for fluorescence-based cell sorting and monitoring of transduced cells during differentiation. Using this lentiviral vector we were able to establish homogenously transgenic HAE cultures expressing mCherry in the nuclei. Furthermore, the IPTG induction of shRNAs against GFP and Aminopeptidase N (CD13, receptor for HCoV-229E) inserted into this vector is functional in both Huh7 cells and fully differentiated transgenic HAE cultures.

**Materials and Methods**

**Cell isolation**

Primary human bronchial cells were isolated from patients (>18 years old) undergoing either bronchoscopy or pulmonary resection at the Cantonal Hospital in St. Gallen, Switzerland. Informed consent was acquired from all patients. This was done according to the regulations of the Canton St.Gallen, Switzerland as a part of the St. Gallen Lung Biopsy Biobank (SGLBB) of the Cantonal Hospital in St.Gallen. Approval was acquired by the ethics committee of the Canton of St.Gallen (EKSG 11/044, EKSG 11/103). Cell isolation was performed with protease and DNase digestion as previously described ([27], chapter 3). Cells were either cryopreserved at -150°C for later use or used directly for transduction.

**Cell Culture**

*All cell cultures were incubated at 37°C and 5% CO₂ unless otherwise stated. All primary monolayer cultures were cultured on collagen type I (col I) coated cell culture plastics.*

Primary human bronchial cells in monolayer and ALI were cultured as previously described ([27], chapter 3), briefly summarized below.

*Primary monolayer:* Primary bronchial cells were cultured on col I coated flasks (TPP, Switzerland) in Bronchial Epithelial Growth Medium (BEGM), LHC basal medium (Gibco, Fisher Scientific Inc.) supplemented with growth factors (Sigma Aldrich), penicillin (60mg/mL, Gibco, Fisher Scientific Inc.) and streptomycin (100mg/mL, Gibco, Fisher Scientific Inc.). After FACS sorting, 10μM Rho associated protein kinase inhibitor (Y-27632, Sigma Aldrich) was added to the culture medium. Medium was changed every other day.

*Primary air-liquid interface (ALI):* Primary cells in air-liquid interface (ALI) were cultured on porous Transwell™ inserts (0.4μm, Corning International, USA) in 1:1 Dulbecco’s Modified Eagle’s Medium F12 nutrient mix combination (Gibco, Fisher Scientific Inc.) supplemented
with 2% UltraG (UG, Pall Biosepra, France), Retinoic Acid (RA, Sigma Aldrich), penicillin (60mg/mL, Gibco, Fisher Scientific Inc.) and streptomycin (100mg/mL, Gibco, Fisher Scientific Inc.) 80,000 cells were seeded per 24 well insert in 200μL of medium. Basolateral compartment was filled with 500μL. Once confluent, the apical side medium was aspirated, establishing ALI. The epithelial layer was allowed to differentiate for at least four weeks prior to any analyses. Basolateral medium was changed every other day during the differentiation period. Additionally, the cell layer was washed with 200μL Hank’s Balanced Salt Solution (HBSS, Gibco, Fisher Scientific Inc.) once a week.

**MRC-5 monolayer:** MRC-5 cells were cultured in Minimum Essential Medium (MEM, Gibco, Fisher Scientific Inc.) supplemented with penicillin (60mg/mL, Gibco, Fisher Scientific Inc) and streptomycin (100mg/mL, Gibco, Fisher Scientific Inc.) and 10% Fetal Bovine Serum (FBS). Medium was changed every other day. For Interferon alpha treatment, 70,000 cells were seeded in a 12 well plate (TPP, Switzerland) and treated with 100 IU of type I IFN (alpha, I4401 SIGMA, Sigma Aldrich) for 16 hours at 37°C (5% CO₂). Whole cell RNA was then isolated for qRT-PCR analysis.

**pLKO_mCherry_Scr lentiviral vector preparation**

pLKO_3x_LacO lentiviral vector containing a scrambled (Scr) shRNA and a puromycin resistance gene was purchased from Sigma Aldrich (MISSION™SHC332 SIGMA, pLKO-puro-IPTG-3xLacO). Monomeric Cherry (mCherry) was amplified from a pLKO donor vector using polymerase chain reaction (PCR) incorporating overhangs with restriction sites compatible with the pLKO_3x_LacO vector. The PCR product was digested with DpnI (NEB, USA) for 1 hour to digest away any template plasmid DNA. It was then purified using a PCR clean-up kit (Machery-Nagel, Switzerland) according to the manufacturers instructions. The accepting LacO vector was digested, removing the puromycin resistance gene, and purified from a 1% agarose gel using a Gel extraction kit (Machery-Nagel, Switzerland) according to the manufacturers instruction. mCherry was then ligated into the donor vector with T4 DNA ligase (Promega, USA) overnight at 16°C. 10% of the ligation reaction was used for the transformation of chemically competent Stbl3 E.Coli and plated on ampicillin containing agar plates and incubated at 37°C overnight. Positive colonies were assessed by colony PCR and the resulting clones further cultured and sequenced.

**shRNA ligation**

shRNA sequences for the knockdown of specific genes were extracted from the RNAi consortium (https://www.broadinstitute.org/rnai/public/) and ordered as separate sense and antisense strands from Microsynth Switzerland, incorporating specific restriction sites. shRNAs were annealed in New England Biolabs CutSmart buffer (NEB, USA) by incubating
the sense and antisense strands at 94°C for 10 minutes and cooling them to room temperature (RT).

For cloning of specific shRNAs into the constitutive vector, an empty pLKO_GFP control vector (Mission™ SHC001, Sigma Aldrich) was acquired from Dr. B. Berkhout and Dr. J. Eekels [28].

Specific shRNAs were inserted into pLKO_GFP_SHC001 and pLKO_mCherry_Scr using an adaptation of the RNAi consortium pLKO cloning protocol (http://www.broadinstitute.org/rnai/public/resources/protocols). Several colonies were further cultured and sequenced to assess ligation. Positive clones were cultured and sequenced for further use.

Table 1: Overview of shRNA sequences used in the current study.

<table>
<thead>
<tr>
<th>ID no.</th>
<th>Gene</th>
<th>Match Location</th>
<th>Target sequence</th>
<th>Hairpin Sequence</th>
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<tr>
<td>TRCN0000231 750</td>
<td>clontechGF P_1</td>
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<td>5'-CCGG-CAACAGCCACAAGCTCTATA-3'</td>
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<td>TRCN0000230 213</td>
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<td>Position 4326 (3UTR)</td>
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<td>5'-CCGG-CCAAAAGAAGCAGTGTTAT-3'</td>
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</tbody>
</table>

*sequence of MxA_1 was provided by Prof. Dr. M. Schwemmle.

All cloning strategies were designed using Geneious 7 [29].

Lentiviral particle production

VSV-g pseudotyped lentiviral particles were produced as previously described (Jonsdottir HR. et.al. unpublished data, chapter 5) using various different transfer vectors.

Briefly, 2.4μg of transfer vectors (pLKO_GFP_Scr, pLKO_GFP-MxA-1/2, pLKO_GFP-Mda5-1/2, pLKO_GFP-RigI-1/2, pLKO_mCherry_Scr, pLKO_mCherry_GFP-1/2, pLKO-mCherry_CD13-1/2) were transfected along with 1.6μg pMDL (gagPol), 0.6μg pRev and 0.8μg pVSV-glycoprotein per flask. Supernatant was collected on ice after 48 - 60 hours and minimal titer estimated with GoStix rapid lentiviral titer detection kit (Takara Clontech,
Lentiviral transduction

Lentiviral transduction of primary human bronchial cells was conducted in suspension for 4 hours at 37°C as previously described (Jonsdottir, HR et. al. unpublished data, chapter 5).

FACS sorting

After monolayer expansion, transduced cells were sorted for moderate mCherry expression at 4°C using FACS Aria III and the corresponding FACS Diva software (BD Bioscience, USA). Cells were sorted from HBSS into BEGM with FACS flow (BD Bioscience, USA) and washed with phosphate buffered saline (PBS, 400g, 5 min, 4°C) prior to further culturing. Cells were resuspended in complete BEGM supplemented with Y-27632 (Sigma Aldrich), amphotericin B (Sigma Aldrich) and gentamicin (Sigma Aldrich). Medium was changed to complete BEGM the next day and every other day thereafter until 90% confluent. Cells were then expanded to larger culture flasks to acquire enough cells for HAE cultures. Cells were sorted at the FACS core facility, Institute of Pathology, University of Bern, Bern, Switzerland.

Immunofluorescence

HAE cultures were fixed and stained for immunofluorescence as previously described ([27], chapter 3 and Jonsdottir, HR. et. al. unpublished data, chapter 5).

Briefly, filters were washed with HBSS prior to fixation with 4% buffered formaldehyde (FORMAFIX, Formafix Switzerland AG) for 15 minutes at RT. Prior to blocking with confocal buffer (CB) the filters were washed once with PBS. Primary antibodies were incubated in CB for 2 hours followed by incubation of secondary antibodies for 1 hour. Finally cultures were stained with DAPI and washed with CB and PBS. Lastly, the filter membrane was cut out of the insert and mounted on a glass slide for confocal analysis.

In the current study, cultures were stained for β-tubulin (Abcam, USA, ONS.1A6) and tight junctions (ZO-1, Abcam, USA). Nuclei were counterstained with DAPI (Fisher Scientific Inc.). Confocal analysis was performed on a Nikon confocal microscope (Nikon Europe, Switzerland). IMARIS (Bitplane, Zürich, Switzerland) was used for image processing.

Isopropyl β-D-1-thiogalactopyranoside induction

Cells were treated with various concentrations of Isopropyl β-D-1-thiogalactopyranoside (IPTG, Sigma Aldrich) diluted from a stock solution of 200mM in medium. IPTG containing medium was changed every 24 hours.
Huh7 cells were cultured in Dulbecco’s modified eagles medium (DMEM Glutamax™) supplemented with 5% heat inactivated fetal bovine serum (FBS), penicillin (60mg/mL, Gibco, Life Technologies) and streptomycin (100mg/mL, Gibco, Life Technologies). Naïve or GFP positive (pLKO_GFP_Scr) Huh7 cells were transduced with lentiviral vectors (pLKO_mCherry_Scr, pLKO_mCherry_GFP-1/2, pLKO_mCherry_CD13-1/2) and FACS sorted. For the analysis of GFP knockdown, double positive mCherry_Scr and mCherry_GFP-1/2 cells were seeded onto T25 flasks and treated with 0 or 1mM IPTG for 72 hours. GFP expression was assessed with microscopy and flow cytometry analysis (FACS Canto, BD Bioscience, USA).

For the analysis of CD13 knockdown, cells were seeded into T25 flasks and treated with 0, 1 or 2mM ITPG for 2 and 5 days. At day 2 and 5 18,000 cells were seeded per well into a 96-well plate and incubated with IPTG for another 24 hours. Subsequently, cells were infected in triplicate with two different multiplicity of infection (MOI), 0.1 and 0.01, of HCoV-229E expressing the Renilla Luciferase gene [30]. Virus replication was evaluated using the Renilla Luciferase Assay kit (Promega, USA) according to the manufactures instructions using half the recommended substrate concentration and a luminometer. Additionally, the expression of CD13 was assessed by flow cytometry (FACS Canto, BD Bioscience, USA).

Transgenic HAE cultures were established from primary bronchial cells that had been sorted with FACS. For assessment of GFP knockdown, cells were transduced with pLKO_GFP_Scr in addition to pLKO_mCherry_GFP-1/2. After differentiation HAE cultures were treated with 0, 1, 2 and 4mM of IPTG in basolateral medium for 6 days. GFP expression was monitored every 24 hours using microscopy. Additionally, GFP expression was evaluated by flow cytometry (FACS Canto, BD Bioscience, USA) at the end of the treatment period (6 days).

For evaluation of CD13 knockdown, transgenic HAE cultures (mCherry_Scr and mCherry_CD13-1/2) were treated with 0, 1 or 2mM IPTG in basolateral medium for 3 and 6 days and then infected with HCoV-229E at 4000 pfu per insert. Viral replication was assessed with quantitative real time PCR (qRT-PCR).

**Flow Cytometry**

Cells were analyzed by flow cytometry for GFP directly or stained for CD13 (mlgG1, 347830, PE-conjugated, BD Bioscience, USA). Prior to analysis cells were fixed with 4% FORMAFIX (Formafix Switzerland AG) for 15 minutes at RT and subsequently washed with PBS. Cells were then resuspended in Cell Wash buffer (CWB, BD Bioscience) and either analyzed directly (GFP) or stained for CD13 on ice in CWB for 20 minutes and then washed once (400g, 5 min, 4°C) and resuspended in the same buffer. Cells were analyzed with FACS Canto (BD bioscience, USA) using unstained cells as control.
**Cell viability**

Cell viability was assessed with AlamarBlue (Fisher Scientific, Inc.). AlamarBlue was added to growth medium at 10% (v/v) end concentration and incubated at 37°C and 5% CO₂ for at least 4 hours, depending on cell density. After incubation fluorescence was measured in a luminometer at 595nm.

**HCoV-229E infection**

Huh7 cells were infected with a multiplicity of infection (MOI) 0.1 and 0.01 in 100μL DMEM+5%FBS, 18,000 cells per well in a 96 well plate in triplicate. HAE cultures were infected with 4000 plaque forming units (pfu) per insert in duplicate. During infection, cultures were incubated with inoculum solution at 33°C (5% CO₂) for 1 (Huh7 cells) or 2 hours (HAE cultures). Progeny was collected for Huh7 cells at 48 hours by harvesting the supernatant. Progeny virus was collected from the apical side of HAE cultures at 24 and 48 hours by incubating them with 100μL of HBSS for 10 minutes.

**Quantitative Real-time PCR (qRT-PCR)**

*Host genes:* RNA was isolated from MRC-5 cells expressing shRNAs against MxA, Mda5 and Rigl (pLKO_GFP_MxA/Mda5/Rigl) as previously described (chapter 4). RNA was also isolated from IPTG treated and non-treated Huh7 cells expressing two different shRNAs against CD13 (mCherry_CD13-1/2), using the Nucleospin™ RNA isolation kit by Machery-Nagel (Switzerland) according to the manufacturers instructions. cDNA was then prepared from 300ng of RNA using random primers as previously described ([16], chapter 4). Gene expression of CD13 was evaluated using SYBR Green™ dye (Roche) and AB 7500 light cycler and the corresponding analysis software (Applied Biosystems, USA). Primer sequences: CD13: fwd primer: 5’-TTCAACATCAGCGTACCACC-3’, rev primer: 5’-AGTCGAACTCAGCAGAATAAG-3’. Primer sequences for MxA, Mda5 and Rigl have been previously published ([16], chapter 4). Cycle parameters: 94°C for 15 seconds and 45 cycles of 94°C for 10 seconds, 55°C for 15 seconds and 72°C for 30 seconds, followed by a melting curve to confirm product specificity.

*HCoV-229E:* Viral RNA was quantitatively determined directly from virus containing supernatant with qPCR directed against the HCoV-229E membrane (M) gene using Qiagen Quantitect™ Probe RT-PCR kit (Qiagen, Germany) and AB 7500 light cycler and the corresponding analysis software (Applied Biosystems, USA). Primer sequences: Fwd primer: 5’-TTCCAGCTGACGACTTT-3’, Rev primer: 5’-CCAACACGTTGACAGTA-3’, probe: 5’-FAM-TTGGGCGATGCTGCTTGAGGT TAATGCA-TAMRA-3’. Cycle parameters: 50°C for 30 minutes, 95°C for 15 minutes and 45 cycles of 94°C for 15 seconds and 60°C for 1 minute.
Plasmid standard expressing the HCoV-229E M gene was used for the quantification of genome equivalents (GE) per mL.

Data presentation
Data was plotted using GraphPad Prism 5 and figures were assembled in Corel Draw X3.

Data Contribution
Hulda R. Jonsdottir performed the experiments, assembled data and figures and wrote the manuscript. Sabrina Marti contributed to the cloning of ISG specific shRNAs. Dirk Geerts provided the IPTG inducible 3xLacO vector. Regulo Rodriguez provided human bronchial tissue for cell isolation. Ronald Dijkman provided technical assistance and supervision. Volker Thiel provided supervision and revised the manuscript.

Funding
This work was supported by the 3R research foundation Switzerland, project 128-11.

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We thank Dr. Stefan Müller for his assistance with FACS sorting and Dr. Pavlos Englezou for his assistance on confocal microscopy. We also thank Prof. Dr. Martin Schwemmle for providing us with shRNA sequences for MxA.
Results

Non-inducible knockdown of interferon stimulated genes results in varying cell viability in different cell types

To elucidate innate immune mechanisms involved in virus–host interactions during respiratory virus infection we assessed the potential knockdown of three different interferon stimulated genes (ISGs), MxA, Mda5 and RigI, two different shRNAs targeted at every gene using the constitutively active shRNA lentiviral vector pLKO_GFP. The transduction efficacy in MRC-5 cells ranges from 60-90%, measured by the percentage of GFP positive cells (figure 1A). Cellular viability differs both between the different genes and the different shRNAs. Naïve and scrambled (Scr) transduced cells have the highest viability. MxA_1 has higher viability than MxA_2, Mda5_2 higher than Mda5_1 and RigI_1 than RigI_2 (figure 1B). To assess gene knockdown on the RNA level, transduced MRC-5 cells were treated with 100 U of type I IFN (alpha) and mRNA levels of endogenous MxA, Mda5 and RigI evaluated for every shRNA construct 16 hours post IFN treatment. The expression of all three genes was analyzed in every knockdown subgroup. The results showed that only the desired ISG was knocked down, while the other two were up-regulated compared to Scr, as expected upon IFN induction. Knockdown seemed similar for each shRNA within the knockdown subgroups (figure 1C). One shRNA, combining high transduction efficacy, good viability and proper knockdown, was selected for every gene knockdown for further analysis in primary human bronchial cells (MxA_1, Mda5_2 and RigI_1). To achieve knockdown, undifferentiated primary human bronchial cells were transduced with lentiviral vectors encoding the same shRNAs targeting MxA, Mda5 and RigI (pLKO_GFP_MxA/Mda5/RigI) and subsequently sorted to achieve a homogeneous population of transduced primary airway cells. Based on cell proliferation of transduced primary cells after FACS sorting of GFP positive cells, MxA and RigI knockdown results in low viability. MxA knockdown cells are large and irregularly shaped while the RigI knockdown cells are very small, round and attach poorly. Scr control and Mda5 knockdown cells have similar viability in monolayer although cellular morphology is different (figure 1D). However, when seeded on porous inserts, only Scr cells are capable of proliferation (data not shown). This indicates that constitutive knockdown in primary cells; even of ISGs unlikely to be involved in bronchial differentiation, has both proliferation and culture limitations. These limitations restrict the use of constitutively expressed shRNAs for cultivation of the cells in monolayer and the establishment of differentiated ALI HAE cultures.
Figure 1: Knockdown of interferon stimulated genes (ISGs) results in reduced gene expression for all constructs but cellular viability differs. 

A: The transduction efficacy of MRC-5 cells varies between the different pLKO_GFP shRNA constructs tested but remains over 60%. B: Cellular viability of MRC-5 cells decreases after transduction with specific shRNAs against ISGs. Only MxA_1 and Mda5_2 have similar viability as control cells. C: Specific knockdown assessed by qRT-PCR in MRC-5 cells after IFN induction reveals knockdown of desired ISGs. D: Three shRNA constructs (MxA_1, Mda5_2 and RigI_1) were selected for primary cell transduction along with a Scr control. Interestingly, MxA transduced cells exhibit reduced growth and viability in monolayer even though transduced MRC-5 cells showed a viability level similar to non-transduced control. Mda5 shows high viability both in primary cells and MRC-5 while RigI_1 shows reduced viability in both cell systems. Original magnification 10x.

Data is shown in duplicate for one independent experiment. Primary data is shown for donor 1505.
Establishment of inducible transgenic human airway epithelial cultures

Non-inducible heterogeneously transgenic HAE cultures constitutively expressing Scr shRNA have already been established (Jonsdottir, HR et. al. unpublished data, chapter 5). However, since the differentiation of HAE cultures is a complicated process constitutive knockdown of certain genes could interfere with differentiation resulting in unusable HAE cultures. For example, the knockdown of p63, a basal cell transcription factor, results in the loss of epithelial integrity in HAE cultures established from a cell line. However, p63 knockdown in primary cells results in accelerated cellular senescence and primary human bronchial cells do not survive long enough to be seeded on inserts for ALI [23]. Furthermore, using the established transduction protocol (Jonsdottir, HR et. al. unpublished data, chapter 5) for ISG knockdown in primary cells, results in non-viable cells (figure 1D). To circumvent these limitations, we aimed to establish an inducible lentiviral system for gene knockdown in HAE cultures. The inducible lentiviral vector constitutively expresses the mCherry fluorescence gene for cell sorting and microscopic evaluation of transduced cells along with an inducible shRNA controlled by the Lactose operon (LacO). Treatment of transduced cells with Isopropyl β-D-1-thiogalactopyranoside (IPTG), a lactose mimic, induces the expression of the shRNA (figure 2A).

**Figure 2: Inducible lentiviral construct (pLKO_mCherry_Scr) can be used to establish homogeneously transgenic HAE cultures.**

**A:** An inducible lentiviral vector with mCherry fluorescent marker for cell sorting and IPTG inducible shRNA. **B:** Homogeneously transgenic HAE culture establishment involves two expansion steps and FACS sorting.

In order to establish homogeneously transgenic cultures, positive cells have to be sorted by flow cytometry. This requires the incorporation of extra expansion steps to ensure enough cells for 1) expansion and sorting and 2) HAE cultures (figure 2B). This is made possible by treating the epithelial cells with a Rho associated protein kinase inhibitor (Y-27632) during
monolayer culture. This inhibitor prolongs the progenitor cell phenotype of these cells allowing them to retain their differentiation potential for longer (Jonsdottir, HR et. al. unpublished data, chapter 5). This inhibitor allows for the establishment of both hetero- and homogeneously transgenic HAE cultures that differentiate normally. Comparison of naive and mCherry_Scr HAE cultures revealed the same morphology (figure 3), a pseudostratified layer with distinct ciliated cells (β-tubulin, green) and tight junctions (ZO-1, cyan). Furthermore, nuclear expression of mCherry was observed in all nuclei in the epithelial layer (purple). This indicates that transduction and sorting of primary bronchial cells prior to HAE establishment does not affect their differentiation potential or morphology.

Figure 3: Scr homogenously transgenic HAE cultures retain the proper morphology. Sorted primary bronchial cells differentiate normally and exhibit the characteristic pseudostratification of naive HAE cultures, along with ciliated cells (β-tubulin, green) and proper tight junctions (ZO-1, cyan).
**Chemical induction of shRNAs**

**shRNA-mediated knockdown of GFP expression in Huh7 cells**

Treatment of transduced cells with IPTG releases the translational repression of the shRNA and induces its expression. To test the induction cassette in our lentiviral vector (figure 1A) we transduced and sorted Huh7 cells with a lentiviral vector expressing GFP (pLKO_GFP_Scr) and pLKO_mCherry vectors encoding two different shRNAs against GFP (mCherry_GFP-1 or mCherry_GFP-2) along with a scrambled control (mCherry_Scr), resulting in three different subgroups of GFP/mCherry double positive cells. When these cells were treated with 1mM IPTG for 72 hours, a reduction in the number of GFP positive cells could be observed in cells transduced with lentiviral vectors expressing shRNAs targeting GFP, but not in the scrambled control (figure 4A, top). However, the reduction of GFP-positive cells does not exceed 30%. When taking a closer look at GFP brightness by comparing the median fluorescent intensity (MFI), there is 60-70% reduction of GFP fluorescence in knockdown cells while Scr cells have a MFI similar to non-treated controls (figure 4A, bottom). Fluorescent microscopy also shows a striking reduction of fluorescence intensity in treated cells while still showing a high number of positive cells (figure 4B). The successful knockdown of GFP upon induction demonstrates the functionality of the IPTG induction cassette within the pLKO_mCherry lentiviral vector.

**shRNA-mediated knockdown of human Aminopeptidase N (CD13) expression in Huh7 cells**

To further test the knockdown potential of the inducible IPTG system, Huh7 cells were transduced and sorted with pLKO_mCherry vectors for two different shRNAs against human Aminopeptidase N (hAPN, CD13, mCherry_CD13-1 or mCherry_CD13-2), the receptor for HCoV-229E, and Scr control (mCherry_Scr). Upon treatment with 1mM IPTG for 3 and 6 days, a reduction in the gene expression of CD13, assessed by qRT-PCR, compared to Scr control, can be observed. Longer IPTG treatment results in less CD13 expression (Figure 4C, top). However, generally CD13 expression is higher in mCherry_Scr cells compared to naïve Huh7 cells (figure 3C, top, dashed line). Interestingly, based on the MFI, assessed by flow cytometry, the level of CD13 in cells treated for either 3 or 6 days with 1mM IPTG indicates that the pattern of knockdown is reversed. That is, the MFI of CD13 is lower at day 3 compared to day 6. However, the MFI of mCherry_Scr after 6 days of treatment is higher compared to mCherry_Scr at 3 days of treatment resulting in similar percentage reduction (figure 4C, bottom). Although, reduction of CD13 levels was less pronounced compared to knockdown of GFP (Figure 4A and 4B), HCoV-229E replication was clearly reduced. Infection of naïve, Scr and CD13 knockdown Huh7 cells with HCoV-229E encoding the Renilla luciferase gene, after 3 and 6 days of IPTG treatment, shows a reduction in luminescence signal for both knockdown subgroups, slightly more for mCherry_CD13-2. Furthermore, the
difference in viral replication became more pronounced when cells were infected with an MOI of 0.01 (approx. 2 log reduction), while the level of replication between the naïve and scrambled subgroups are comparable (figure 4D).

Based on these results, mCherry_GFP-1 and mCherry_CD13-2 were selected for further analysis in HAE cultures.

Figure 4: IPTG induction of specific shRNAs is functional in Huh7 cells post-transduction and sorting. A: 72-hour IPTG treatment of double mCherry/GFP positive Huh7 cells expressing two different shRNAs against GFP results in 20-30% reduction of GFP positive cells. The MFI of GFP is less than half compared to non-treated cells. B: Treatment with IPTG results in less GFP brightness as assessed by fluorescence microscopy. Original magnification 10x. C: CD13 expression in Huh7 cells transduced with two shRNAs against CD13. mRNA expression, upper graphs, shows a reduction in the amount of CD13 mRNA after 3 and 6 day treatment with IPTG. The number of CD13 positive cells is the same when analyzed by flow cytometry (data not shown). However, the reduction of MFI in treated cells indicates that there are fewer receptors per cell after treatment with 1mM IPTG. Dashed line: level of expression/fluorescence in naïve Huh7 cells. D: Infection of Huh7 cells with HCoV-229E with two different MOIs results in lower viral titer as measured by luciferase content. After 6 days of 1mM IPTG treatment and 48 hours of infection, the two knockdown groups show reduced viral infection (grey and green columns) for both MOIs. However, the effect is more pronounced when cells are infected with a lower MOI (0.01, lower graph). Dashed line: mock levels of luminescence. Data is shown in triplicate for one independent experiment.
shRNA-mediated knockdown of GFP expression in HAE cultures
IPTG uptake in Huh7 cells is straightforward as the drug-containing medium covers the monolayer cell culture. However, since HAE cultures are only in contact with medium from the basolateral side, IPTG induction of HAE cultures might need higher concentrations to be as effective as in Huh7 cells. To test this, we treated well differentiated GFP/mCherry double positive HAE cultures with 1, 2, and 4mM of IPTG for 6 days. These cultures were transduced with plko_GFP_Scr and pLKO_mCherry_GFP-1, previously demonstrated to be effective in Huh7 cells (figure 4A). Treatment of HAE cultures with 2mM of IPTG results in a GFP MFI reduction of approximately 50%. Increasing the concentration to 4mM does not seem to have any additional effect and treatment with the standard 1mM results in slightly lower reduction of signal compared to 2mM (figure 5A and B). Although MFI reduction was less apparent in HAE cells, these results demonstrate that GFP-expression in HAE cultures can be reduced by IPTG-induced expression of shRNAs directed against GFP.

shRNA-mediated knockdown of human Aminopeptidase N (CD13) expression in HAE cultures
Next we assessed the shRNA-mediated knockdown of CD13 expression in HAE cultures. Naïve HAE cultures and HAE cultures transduced with pLKO_mCherry_Scr, pLKO_mCherry_CD13-2, were treated with 1mM or 2mM IPTG for 3 or 6 days prior to HCoV-229E infection. Release of viral RNA at the apical side was assessed by qRT-PCR. As shown in Figure 5C, viral RNA was only slightly reduced in CD13-2 shRNA expressing cells at both IPTG concentrations, and compared to the Scr control, likely not significant.
Figure 5: IPTG induction of transgenic primary cells in differentiated HAE cultures is functional. A: Treatment of GFP/mCherry double transduced HAE cultures expressing an shRNA against GFP with IPTG results in reduced GFP fluorescence as evaluated by microscopy. Original magnification 10x. B: After 6 days of treatment the number of GFP positive cells in these cultures remain the same (data not shown) but the MFI of GFP is reduced. C: HCoV-229E infection (4000 pfu) of transgenic HAE cultures expressing an inducible shRNA for CD13 results in slightly lower viral titer for treated cultures as measured by qRT-PCR. Dashed line: inoculum titer. Data is shown in duplicate for donor 1305.
Discussion

We have previously shown that the establishment of transgenic HAE cultures for respiratory virus research is possible and transduction does not interfere with the differentiation potential of primary human bronchial cells. Additional expansion steps and FACS sorting of primary bronchial cells needed for the establishment of homogenously transgenic cultures, require the cells be treated with a Rho kinase inhibitor (Y-27632) to prolong their basal cell phenotype, extending both the in vitro culture time and allowing them to be seeded into the ALI system at a later passage (Jonsdottir HR et.al. unpublished data, chapter 5) [31, 32]. The morphology of homogeneously transgenic HAE cultures is comparable to naïve controls, indicating that the transduction itself does not effect the cellular composition of the cultures. This is consistent with the observations that embryonic stem cells transfected with an IPTG inducible vector exhibit transgene expression after differentiation and their differentiation capabilities are not impaired [22]. In GFP positive Huh7 cells, transduced with shRNAs against GFP or naïve cells expressing shRNAs against CD13, the HCoV-229E receptor, treatment with 1mM IPTG is sufficient to see a reduction of median fluorescence intensity (MFI) for both GFP and CD13. However, the number of GFP positive cells is only reduced by 20-30\% after 72h of IPTG induction whereas the MFI reduction is closer to 70\%, indicating that the inducible knockdown is indeed functional, with pLKO_mCherry_GFP-1 performing better than GFP-2. Treatment with a higher concentration of IPTG or 1mM over a longer period might have resulted in a larger percentage decrease of GFP positive cells. Nonetheless, IPTG induction of the integrated lentiviral insert is functional in mammalian cells (Huh7). A functional analysis of HCoV-299E replication in both naïve and knockdown cells revealed, after 6 days of treatment, about 50\% reduction in the expression of CD13 mRNA, whereas the MFI is only reduced by 20-30\%. However, during infection of cells treated with IPTG for 6 days, with a low multiplicity of infection (MOI 0.01) viral titers are almost reduced to mock levels for mCherry_CD13-2 while CD13-1 shows less reduction.

After 6 days of 2mM IPTG treatment of fully differentiated HAE cultures the MFI of GFP is reduced to about 50\% compared to non-treated controls. Treatment with 1 and 4mM, however, results in similar levels of knockdown, around 30\%. Interestingly, increasing the IPTG concentration does not seem to enhance the reduction of the MFI of GFP, indicating a possible saturation of the inducible system. The microscopic evaluation of the GFP knockdown confirms the data acquired by flow cytometry. However, the assessment of GFP expression in HAE cultures is hindered by the green auto fluorescence of the multilayered cells and the collagen coat on the inserts.
After induction of CD13 knockdown, HCoV-229E titers exhibit the same decrease after 6 days of 1mM treatment and 3 days of 2mM treatment, indicating that 2mM might be an ideal concentration for IPTG induction in HAE cultures. However, the knockdown of CD13 might not be a good target in these cultures due to the stability of the system. The rate of cellular turnover in the lungs is slow [33] and extracellular receptors are likely not recycled quickly. Therefore, the knockdown of CD13 on the RNA level might not be reflected in the concentration of protein receptor in the cell membrane. Taken together, this data indicates that the establishment of inducible transgenic HAE cultures with an mCherry reporter gene is achievable and the Lac induction cassette within the lentiviral vector is functional. However, the knockdown of GFP and CD13 in HAE cultures is less compelling than in Huh7 cells. Possibly, longer induction times are required to see the knockdown at the protein level. However, it is also possible that other targets than CD13 are required for proper testing of the induction in HAE cultures since the cultures are quite stable. Alternatively, the use of more potent shRNAs targeting CD13 may circumvent this problem. Importantly, however, our data convincingly demonstrate that robust transgene expression (e.g. GFP, mCherry) can be achieved in fully differentiated HAE cultures. Therefore, a different option to evaluate the optimal conditions for IPTG induction in HAE cultures would be the expression of transgenes instead of shRNAs. A second fluorophore, for example, would facilitate monitoring the induction with microscopy.

Collectively, our data demonstrates that transgenic HAE cultures with uniform fluorescent transgene expression are morphologically and functionally similar to naive cultures and can be used as a research tool in various fields of respiratory biology, including pathogen – host interaction studies.
References


CHAPTER 7
GENERAL DISCUSSION
Discussion

Well-differentiated organotypic human airway cultures represent a universal platform to study respiratory pathogens. This model recapitulates the human airway epithelial morphology and function by culturing pulmonary epithelial cells at air-liquid interface. However, this system requires special handling and training before use, making it more demanding and time consuming compared to traditional monolayer cell culture. Furthermore, both ethical permissions and informed consent have to be acquired from the proper authorities and patients, respectively.

Well differentiated HAE cultures for HCoV propagation and study

Using primary human bronchial cells isolated from surgical patients, we established fully differentiated HAE ALI cultures and used them successfully for the propagation of all known HCoVs, including the epidemic viruses SARS- and MERS-CoVs (chapters 3 and 4, [1, 2]). We also demonstrated that when HAE cultures were treated with recombinant interferon, various ISGs were up-regulated, indicating that the interferon signaling pathways are functional in the differentiated cultures. However, during infection with HCoV-229E, SARS-CoV and MERS-CoV, well differentiated HAE cultures did not mount a noticeable innate immune response at 3, 6 or 12 hours post-infection (chapter 4). This is consistent with reports of the CoV accessory proteins acting as interferon antagonists [3-7]. Furthermore, the CoV non-structural proteins may also play a role in their innate immune evasion [8, 9], further adding to the complexity of CoV – host interactions in human airway cells during infection.

In addition, we determined that treatment with IFN lambda could serve as a potential prophylactic treatment for HCoV infection but this needs further optimization and testing in HAE cultures (chapter 4). IFN alpha has been used for the treatment of respiratory virus infections but has severe side effects [10]. Treatment with IFN lambda is milder, presenting a viable treatment option for HCoV infections in vivo [11].

Moreover, using the HAE ALI model we could assess the zoonotic potential of MERS-CoV after its sudden emergence in the human population (chapter 4). This work was also instrumental in identifying the cellular receptor used by MERS-CoV [12]. Additionally, we have attempted to study a camel variant of the HCoV-229E virus in this model but there seems to be a species barrier in place halting viral replication, either on the host or virus side, which could be elucidated using the transgenic human system (Corman et al. in press).

In order to elucidate host and/or viral factors involved in shaping the species barrier, innate immune responses and immune evasion we developed a protocol to make
HAE cultures amenable to genetic modification. Additionally, we have access to a robust system, based on vaccinia virus recombination, to introduce mutations into the coronavirus genome [13]. Combining the use of HCoV mutants and genetically modified HAE cultures will be instrumental to further characterize virus – host interactions on the molecular level.

**Establishment of genetically modified HAE cultures for gene knockdown**

To establish genetically modified HAE cultures, a reliable method of gene vector delivery into primary bronchial cells had to be determined. Due to the long differentiation time, extended shelf life of HAE cultures and limited transfectability, transient transfection of siRNAs is not feasible for gene knockdown in this system. However, since lentiviruses integrate their viral genome into the host cell genome upon infection allowing for stable gene expression or knockdown, even in non-dividing cells, they are ideal to use for genetic manipulation in the HAE ALI system. However, the transduction, expansion and sorting of primary human bronchial cells prior to HAE ALI culture establishment poses a number of unique challenges and pitfalls. Most prominently, the limited passage capacity of primary cells in vitro severely restricts the possibilities of cell manipulation. Treatment with Rho associated protein kinase inhibitor, Y-27632, induced a progenitor cell phenotype in treated cells and allowed for the establishment of HAE cultures that differentiated normally at a later passage. To establish fluorescent transgenic HAE cultures a minimum of 5 passages is required (chapter 5) and to establish homogenously transgenic cultures, FACS sorting of positive cells was implemented using an mCherry expressing lentiviral vector. Furthermore, the shRNA expression in this vector was chemically inducible allowing for controlled knockdown of particular host genes (chapter 6). This further increased the flexibility and usefulness of the transgenic system since constitutive knockdown of certain genes can interfere with cellular viability, growth and/or differentiation (chapter 6, [14]). Since the aim was to develop a consistent and reliable protocol for primary cell transduction and HAE ALI establishment, it is counter-productive to create lentiviral vectors for gene knockdown only to find out that ALI culturing is not possible. Therefore, the inducible shRNA system is a better choice for any further analysis since neither growth nor differentiation will be affected by the expression of specific shRNAs or their off target effects.

**Selection of lentiviral transduced cells**

In this study, we sorted transduced cells based on fluorescence signal (GFP or mCherry) using flow cytometry. Fluorescence markers allow for both cell sorting and close monitoring of cells during mucociliary differentiation by microscopy and flow cytometry. For HAE cultures, mCherry is better suited since the detection of GFP in these multilayered
cultures is complicated by the autofluorescence of the collagen coating as well as the cells themselves. Alternatively, cells can be selected with antibiotics like puromycin by incorporating a resistance gene into the lentiviral insert. Previously, it has been shown that primary human airway cells can be transduced with a lentiviral vector encoding puromycin resistance and seeded directly on porous inserts. Puromycin selection is then applied during the initial growth phase [15]. Depending on the transduction efficacy, an excess of cells need to be seeded to ensure confluency prior to ALI establishment. Therefore, antibiotic selection is most applicable where researchers have access to whole lung donors and large quantities of low passage primary human bronchial cells. However, combining both fluorescence and drug selection would result in a robust system where transduced cells could be selected based on fluorescence before HAE seeding, and further cultured under constant drug selection to ensure homogeneity.

**Possible genetic modifications of HAE cultures**

Two types of genetic modifications are currently achievable in the transgenic HAE ALI model, 1) knockdown and 2) transgene expression.

**Gene knockdown in HAE cultures**

In this study, we have shown that gene knockdown in HAE cultures using lentiviral vectors is feasible since they provide stable integration of transgenes (GFP and mCherry, chapters 5 and 6), visible throughout and post-differentiation. Furthermore, we demonstrated the potential of inducible shRNA-mediated gene knockdown using GFP positive HAE cultures and IPTG inducible lentiviral vector expressing shRNA against GFP (chapter 6). However, shRNAs are not capable of complete gene knockout as they exert their function on the mRNA level and the degree of knockdown depends on the half-life of the eventual protein product. Additionally, optimal shRNA design results in the highest possible knockdown and fewer off-target effects. Furthermore, expressing multiple shRNAs targeting different regions of the targeted mRNA in the same lentiviral cassette could increase the knockdown potential [16, 17]. However, some genes might require a complete knockout before any effect on cellular phenotype can be seen. Therefore, it might be interesting to assess the potential of the CRISPR/Cas9 knockout system in primary human airway cells. The CRISPR/Cas system is a prokaryotic immune system that confers resistance to foreign genetic elements, such as plasmids and phages, and provides a form of acquired immunity upon pathogen interaction. This system is analogous to the RNA interference system in eukaryotes and can also be used for the manipulation of gene expression in an experimental setting. CRISPR/Cas9 lentiviral delivery vectors are available, both inducible and non-inducible [18-20].
The experimental knockout system is based on clustered regularly interspaced short palindromic repeats (CRISPR) that are followed by a short spacer sequence, and the CRISPR associated protein 9 (Cas9). In bacteria, the exposure to invading genetic material induces the transcription of spacer and palindromic DNA sequences into a long RNA molecule. Following transcription, Cas9, a nuclease, cuts this molecule into smaller pieces of crRNAs. These small crRNAs are then used to target foreign genetic elements and mark them for inactivation. In 2012, a synthetic single guide-RNA molecule was designed combining the uses of crRNAs and spacer RNAs, which can, when mixed with Cas9, find a specific DNA sequence and cleave it. The synthetic design of the guide-RNA allows the system to be used for gene editing [21]. The CRISPR/Cas9 system causes complete gene knockout by cleaving DNA and destroying protein-coding ORFs at the genome level. However, in order to confirm proper cleavage and knockout of GOIs, cells need to be clonally expanded and sequenced for confirmation. This is not optimal for primary human bronchial cells meant for ALI HAE cultures since clonal expansion of the heterogeneous population isolated from the lung tissue might result in problems with differentiation. Furthermore, accurate knockout of both alleles in diploid cells provides an additional hurdle limiting the likelihood of obtaining homogeneously modified HAE cultures, since the DNA cleavage and repair may not be the same in all transduced cells, causing phenotypic differences. Despite these limitations, the CRISPR/Cas9 system has already been used in primary airway cultures although without clonal expansion resulting only in knockdown, not knockout, of the selected gene [22]. Future development of this system might enable complete knockout of genes in diploid cells without the need for clonal expansion of cells.

Limitations of shRNA expression in HAE cultures

The use of lentiviral vectors encoding shRNAs is necessary in HAE cultures due to their extended culture time. However, compared to siRNAs the effect of shRNAs is much slower. They must first be transcribed from the integrated viral insert and processed before they can sequester mRNAs and post-transcriptionally regulate gene expression. siRNAs, however, are functional immediately upon transfection into target cells. Since siRNAs are only provided through transfection and not synthesized, they are eventually degraded and their effect abolished. To use shRNAs for gene knockdown requires more patience. Furthermore, good shRNA design is imperative to effective knockdown [23]. The appearance of knockdown in shRNA-transduced cells can take a while to appear on the protein level since already expressed proteins have to be degraded before knockdown can be observed. This represents a hindrance in HAE cultures, due to the stability of the system. Cellular turnover in the lung is slow and therefore it might take longer for proteins to be degraded after the induction of shRNA expression. However, since the cultures have a long shelf life it is possible to chemically induce shRNA expression until sufficient level of
knockdown has been achieved. Alternatively, systems where chemical treatment inhibits shRNA expression rather than inducing it (Tet-off) might also be an option. Even though the inducible shRNA system has its limitations we believe it is the best suited for knockdown in HAE ALI cultures.

Transgene expression in HAE cultures
The expression of fluorescent transgenes in the HAE ALI culture model remains stable throughout the differentiation process. This data (chapters 5 and 6) indicates that the expression of other transgenes in this system could be another useful application of the transgenic HAE ALI cultures. The expression of transgenes in this system has many different applications, for example different viral receptors could be expressed in HAE cultures facilitating viral infection with zoonotic or animal viruses that don’t normally replicate in human epithelium. Furthermore, it has recently been demonstrated that expressing a host protein from chicken, ANP32A, can facilitate the replication of avian influenza A in human cells [24] representing interesting possibilities for the use of transgenic HAE cultures to study the parameters of zoonotic transmission. For the expression of transgenes in the HAE ALI culture system, inducible lentiviral vectors might also be required to circumvent cellular toxicity due to overexpression as was observed for shRNA knockdown (chapter 6). However, the expression and effects of transgenes in the HAE system should work faster than shRNA knockdown, since GFP and mCherry transgene expression can be observed as early as 24 hours post transduction. Therefore, the expression of other non-fluorescent transgenes in HAE ALI cultures represents a promising approach to study pathogen – host interaction in these cultures. The current study has actually already provided evidence that stable transgene expression can be achieved and translation of this data and optimization towards an inducible transgenic expression system should be straightforward.

Conclusions and future perspectives
The presented data demonstrates that the establishment of transgenic HAE cultures is possible, since transduction of primary human bronchial cells with lentiviral vectors expressing fluorescent transgenes and Scr shRNA does not interfere with their differentiation potential. However, constitutive expression of shRNAs targeting MxA, Mda5 and RigI resulted in decreased viability in monolayer culture, which did not allow for HAE ALI establishment. Therefore, we utilized an inducible lentiviral vector where shRNA expression is induced upon IPTG treatment. We chose the lactose operon system over the Tet-on (doxycycline) system since it has less transcriptional readthrough. Furthermore, a system based on an antibiotic would make any studies on pathogen – host interactions with bacteria impossible. Furthermore, treatment of HAE cultures with the antibiotic
azithromycin has a marked effect on the epithelial integrity and development of trans-epithelial electrical resistance (TEER) and other antibiotics might have a similar effect, making the lactose system a more practical choice for this culture system [25, 26].

Theoretically, all respiratory pathogens, including viruses, bacteria and fungi can be propagated in both the naïve and transgenic HAE culture system. Furthermore, airway epithelial cell isolation, transduction and sorting protocols could be applied to any other mammalian or avian species to study animal respiratory diseases and zoonosis. Additionally, transcriptome and proteome analysis of naïve and transgenic HAE cultures during steady state, activation of innate signaling or infection with various pathogens could aid in the elucidation of pathways important to virus – host interactions.

Moreover, we are currently developing a reporter system for use in HAE cultures to study cellular responses to viral infection. This system harbors different promoter elements known to be induced early (e.g ISG54 [27]), intermediate (e.g IFNbeta1 [28]) or late (e.g MxA [29]) during the innate immune response. These promoters are then fused to fluorescent reporters or secreted luciferases for analysis. Incorporating this lentiviral cassette into bronchial cells prior HAE ALI culture will allow for detailed analysis of the innate immune response during virus infection both in single cells within the epithelium and the epithelium as a whole.

Various other questions can be addressed using the transgenic system. For example, fate of virus infected cells can be determined by infecting transgenic HAE cultures expressing Cre reporter and a virus expressing Cre recombinase. The emergence or disappearance of a fluorescent signal can then be monitored on a single cell level and the fate of infected cells determined [30].

Furthermore, this system can also be used to study genetic diseases of the pulmonary system. Those diseases where gene expression is low or absent can be mimicked in this system using shRNA knockdown while abnormal up-regulation of genes could be studied by introducing transgenes into the epithelium. Additionally, development and differentiation of the epithelium can be examined with loss of function studies.

In conclusion, we established a robust protocol to make HAE ALI cultures amenable to genetic modification. The resulting transgenic system is versatile and provides a plethora of research possibilities, not only for pathogen – host interactions but also for developmental lung biology.
DISCUSSION

References


CHAPTER 8
APPENDICES
## List of abbreviations

<table>
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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>Ago</td>
<td>Argonaute</td>
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<tr>
<td>ALI</td>
<td>Air liquid interface</td>
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<tr>
<td>ATI</td>
<td>Alveolar type I</td>
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<tr>
<td>ATII</td>
<td>Alveolar type II</td>
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<tr>
<td>BCoV</td>
<td>Bovine Coronavirus</td>
</tr>
<tr>
<td>BEGM</td>
<td>Bronchial epithelial growth medium</td>
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<tr>
<td>CB</td>
<td>Confocal buffer</td>
</tr>
<tr>
<td>CD13</td>
<td>Cluster of differentiation 13, Aminopeptidase N</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>CNS</td>
<td>Central Nervous System</td>
</tr>
<tr>
<td>CO₂</td>
<td>Carbon dioxide</td>
</tr>
<tr>
<td>Col I</td>
<td>Collagen type I</td>
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<tr>
<td>CoV</td>
<td>Coronavirus</td>
</tr>
<tr>
<td>CsA</td>
<td>Cyclosporin A</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco’s modified eagles medium</td>
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<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
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<tr>
<td>DMV</td>
<td>Double membrane vesicle</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<tr>
<td>Ds</td>
<td>Double stranded</td>
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<tr>
<td>E.coli</td>
<td>Escherichia Coli</td>
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<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
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<tr>
<td>EtOH</td>
<td>Ethyl alcohol</td>
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<tr>
<td>FACS</td>
<td>Fluorescence activated cell sorting</td>
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<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
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<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
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<tr>
<td>Fwd</td>
<td>Forward</td>
</tr>
<tr>
<td>GE</td>
<td>Genome equivalents</td>
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<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>GOI</td>
<td>Gene of interest</td>
</tr>
<tr>
<td>hACE2</td>
<td>human Angiotensin Converting Enzyme 2</td>
</tr>
<tr>
<td>HAE</td>
<td>Human airway epithelium</td>
</tr>
<tr>
<td>hAPN</td>
<td>human Aminopeptidase N</td>
</tr>
<tr>
<td>HAT</td>
<td>Human Airway Trypsin-like protease</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hank’s balanced salt solution</td>
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<tr>
<td>HCl</td>
<td>Hydrochloric acid</td>
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<tr>
<td>HCoV</td>
<td>Human coronavirus</td>
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<td></td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>HCoV-EMC</td>
<td>MERS-CoV</td>
</tr>
<tr>
<td>hDPP4</td>
<td>human Dipeptidyl Peptidase 4</td>
</tr>
<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
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<tr>
<td>hPGK</td>
<td>Human phosphoglycerate kinase promoter</td>
</tr>
<tr>
<td>hpi</td>
<td>Hours post infection</td>
</tr>
<tr>
<td>hU6</td>
<td>Human U6 promoter</td>
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<tr>
<td>IBV</td>
<td>Infectious bronchitis virus</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl β-D-1-thiogalactopyranoside</td>
</tr>
<tr>
<td>ISG</td>
<td>Interferon stimulated genes</td>
</tr>
<tr>
<td>Kb</td>
<td>Kilobases</td>
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<tr>
<td>Lac</td>
<td>Lactose</td>
</tr>
<tr>
<td>LacI</td>
<td>Lac repressor</td>
</tr>
<tr>
<td>LacO</td>
<td>Lactose operon</td>
</tr>
<tr>
<td>LLI</td>
<td>Liquid-liquid interface</td>
</tr>
<tr>
<td>LTR</td>
<td>Long terminal repeats</td>
</tr>
<tr>
<td>M-MLV RT</td>
<td>Moloney murine leukemia virus reverse transcriptase</td>
</tr>
<tr>
<td>mCherry</td>
<td>Monomeric Cherry</td>
</tr>
<tr>
<td>Mda5</td>
<td>Melanoma differentiation associated protein 5</td>
</tr>
<tr>
<td>MEM</td>
<td>Minimal essential medium</td>
</tr>
<tr>
<td>MERS</td>
<td>Middle east respiratory syndrome</td>
</tr>
<tr>
<td>MFI</td>
<td>Median fluorescence intensity</td>
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<tr>
<td>mg</td>
<td>Milligram</td>
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<tr>
<td>MHV</td>
<td>Mouse hepatitis virus</td>
</tr>
<tr>
<td>miRNA</td>
<td>micro RNA</td>
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<tr>
<td>mL</td>
<td>Milliliter</td>
</tr>
<tr>
<td>mM</td>
<td>Millimolar</td>
</tr>
<tr>
<td>MOI</td>
<td>Multiplicity of infection</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>MxA</td>
<td>Myxovirus resistance protein 1</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium chloride</td>
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<td>NaOH</td>
<td>Sodium hydroxide</td>
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<tr>
<td>NEB</td>
<td>New England Biolabs</td>
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<tr>
<td>ng</td>
<td>Nanogram</td>
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<tr>
<td>NGFR</td>
<td>Nerve growth factor receptor</td>
</tr>
<tr>
<td>NHP</td>
<td>Non-Human Primate</td>
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<tr>
<td>NSP</td>
<td>Non-structural protein</td>
</tr>
<tr>
<td>NT</td>
<td>Nucleotide</td>
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<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>ORF</td>
<td>Open reading frame</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<tr>
<td>PEDV</td>
<td>Porcine Epidemic Diarrhea Virus</td>
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<tr>
<td>PFU</td>
<td>Plaque forming units</td>
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<tr>
<td>qRT-PCR</td>
<td>Quantitative Real Time PCR</td>
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<tr>
<td>RA</td>
<td>Retinoic Acid</td>
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<tr>
<td>RBD</td>
<td>Receptor Binding Domain</td>
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<tr>
<td>Rev</td>
<td>Reverse</td>
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<td>RIG-I</td>
<td>Retinoic acid inducible protein 1</td>
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<td>RISC</td>
<td>RNA induced silencing complex</td>
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<td>RNA</td>
<td>Ribonucleic acid</td>
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<tr>
<td>RNAi</td>
<td>RNA interference</td>
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<tr>
<td>ROCK</td>
<td>Rho associated protein kinase</td>
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<tr>
<td>RRE</td>
<td>Rev response element</td>
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<tr>
<td>RSV</td>
<td>Respiratory syncytial virus</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>RTC</td>
<td>Replication – translation complex</td>
</tr>
<tr>
<td>SARS</td>
<td>Severe acute respiratory syndrome</td>
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<tr>
<td>Scr</td>
<td>Scrambled</td>
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<td>SGLBB</td>
<td>St. Gallen Lung Biopsy Biobank</td>
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<td>shRNA</td>
<td>short hairpin RNA</td>
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<tr>
<td>siRNA</td>
<td>short interfering RNA</td>
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<td>TEER</td>
<td>Transepithelial electrical resistance</td>
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<tr>
<td>Tet</td>
<td>Tetracycline</td>
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<tr>
<td>TMPRSSII</td>
<td>Transmembrane Protease, Serine 2</td>
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<td>UG</td>
<td>Ultroser G</td>
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<tr>
<td>UV</td>
<td>Ultra violet</td>
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<td>VSV</td>
<td>Vesicular stomatitis virus</td>
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<td>VTM</td>
<td>Virus transport medium</td>
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<tr>
<td>WPRE</td>
<td>Woodchuck hepatitis virus posttranscriptional regulatory element</td>
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<td>WT</td>
<td>Wild type</td>
</tr>
<tr>
<td>ZO-1</td>
<td>Tight junction protein 1</td>
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</table>
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CHAPTER 9

ORIGINAL PUBLICATIONS
Coronaviruses and the human airway: a universal system for virus-host interaction studies

Hulda R. Jonsdottir1,2 and Ronald Dijkman1,2*

Abstract

Human coronaviruses (HCoVs) are large RNA viruses that infect the human respiratory tract. The emergence of both Severe Acute Respiratory Syndrome and Middle East Respiratory syndrome CoVs as well as the yearly circulation of four common CoVs highlights the importance of elucidating the different mechanisms employed by these viruses to evade the host immune response, determine their tropism and identify antiviral compounds. Various animal models have been established to investigate HCoV infection, including mice and non-human primates. To establish a link between the research conducted in animal models and humans, an organotypic human airway culture system, that recapitulates the human airway epithelium, has been developed. Currently, different cell culture systems are available to recapitulate the human airways, including the Air-Liquid Interface (ALI) human airway epithelium (HAE) model. Tracheobronchial HAE cultures recapitulate the primary entry point of human respiratory viruses while the alveolar model allows for elucidation of mechanisms involved in viral infection and pathogenesis in the alveoli. These organotypic human airway cultures represent a universal platform to study respiratory virus-host interaction by offering more detailed insights compared to cell lines. Additionally, the epidemic potential of this virus family highlights the need for both vaccines and antivirals. No commercial vaccine is available but various effective antivirals have been identified, some with potential for human treatment. These morphological airway cultures are also well suited for the identification of antivirals, evaluation of compound toxicity and viral inhibition.

Keywords: Human coronavirus, Airway epithelium, ALI, Antiviral compound, Cell tropism

Background

Respiratory diseases caused by human coronavirus infection are of both medical and socio-economic importance. Currently, they are studied in various model systems, ranging from cell lines to animal models. Originally, the importance of HCoVs in the burden of human disease was underestimated and as a result, no general therapy exists to treat coronavirus induced disease in humans. Furthermore, no commercial vaccine is available leaving the human population vulnerable to emerging coronavirus infections. Both the Severe Acute Respiratory Syndrome and Middle East Respiratory Syndrome coronaviruses have recently crossed the species barrier and entered the human population to cause severe disease. In this review, we summarize the current knowledge on human coronavirus infection emphasizing the usefulness of organotypic human airway cultures as a model system.

Coronaviruses

Coronaviruses (CoVs), a subfamily of the Coronaviridae family, are positive strand RNA viruses with the largest genome of all known RNA viruses (≥27 Kb). The genomic RNA is capped, polyadenylated and associated with nucleocapsid proteins within an enveloped virion. The envelope is covered by the characteristic surface glycoprotein that gives the virus particles their characteristic crown-like (latin: corona) appearance [1].

All CoVs share a common genome organization where the replicase gene encompasses the 5'-two thirds of the genome and is comprised of two overlapping open reading frames (ORFs), ORF1a and ORF1b that encode for...
up to 16 non-structural proteins. The structural gene region, which covers the 3′-third of the genome, encodes the canonical set of structural protein genes in the order 5′ - spike (S) - envelope (E) - membrane (M) and nucleocapsid (N) – 3′. The structural gene region also harbors several ORFs that are interspersed along the structural protein coding genes. The number and location of these accessory ORFs vary between the CoV species [2, 3].

In animals, CoV infections are mainly associated with respiratory and enteric disease and can have large economic impact on the veterinary industry, e.g. Porcine Epidemic Diarrhoea Virus (PEDV) causes gastrointestinal disease in pigs [4], Infectious Bronchitis Virus (IBV) causes severe kidney and respiratory disease in chicken [5] and Bovine Coronavirus (BCoV) causes both respiratory disease and diarrhea in cattle [6]. Additionally, CoV infections can have other disease manifestations, such as central nervous system (CNS) involvement, hepatitis and peritonitis [7–10].

In humans, CoV infections are mainly associated with respiratory diseases that are considered to have a large impact on the economy due to reduced productivity of the working population. Currently, 6 coronaviruses that cause disease in humans have been discovered. Four of those are commonly circulating and two have caused epidemics of severe acute respiratory disease.

**Human coronaviruses**

The first human coronavirus (HCoV), B814, was described in 1965. In the following years, over 30 additional strains were characterized. Ten of those strains could only be isolated from primary embryonic tracheal organ culture. Others were readily isolated from monolayer cultures and were antigenically related to the prototype strain HCoV-229E. HCoV-OC43, for organ culture 43, was isolated and found to be distinct from the 229E prototype strain [11, 12]. In the subsequent decades, research on HCoVs would center on these two distinct viruses.

However, in 2002, an unknown respiratory illness, termed Severe Acute Respiratory Syndrome (SARS), surfaced in Asia. Research determined it to be caused by a novel coronavirus [13, 14]. At the end of the epidemic, this virus had infected over 8000 people, most in China, and caused 774 deaths [15].

Following the discovery of this virus, two additional CoVs causing human disease were identified. HCoV-NL63 was isolated in the Netherlands in 2004 from an infant with bronchiolitis [16] and HCoV-HKU1 in 2005 from a patient with pneumonia in Hong Kong [17]. In 2012, another respiratory HCoV, Middle East Respiratory (MERS)–CoV, was isolated from a patient with pneumonia in Saudi-Arabia [18]. Unlike SARS-CoV, this virus is still intermittently present in the human population and most recently caused a large outbreak in South-Korea [19]. To date, there have been over 1600 cases and almost 600 deaths related to MERS-CoV infection [20].

**Commonly circulating coronaviruses**

Out of the 6 known human coronaviruses, HCoV-229E, HCoV-OC43, HCoV-NL63 and HCoV-HKU1 are commonly circulating in the human population and usually cause general respiratory illness and cold symptoms in healthy individuals [21–23]. Like influenza, these viruses are capable of causing more severe disease in the immunocompromised and the elderly [24]. They infect the human airway from the luminal side and progeny viruses are released from the same side facilitating spread through coughing and sneezing [25, 26]. These coronaviruses are responsible for approximately 5–10% of all upper and lower respiratory tract infections [27–29] but the interactions between them and their natural host cells are poorly understood. Currently, it is hypothesized that most of the human coronaviruses may have originated from bats [30, 31]. For example, HCoV-229E is believed to originate from African hipposiderid bats possibly using camelds as intermediate hosts [32].

**Emerging coronaviruses**

In the last 15 years, two coronaviruses have crossed the species barrier and caused severe and fatal disease in humans. SARS-CoV surfaced in 2002 and MERS-CoV in 2012 [13, 14, 18]. As opposed to the commonly circulating viruses, which generally only cause mild respiratory symptoms, these viruses presented with higher case fatality ratios, around 10 and 20–50% respectively [33, 34].

Currently, there is abundant phylogenetic evidence for the bat origin of SARS-CoV, based on sequences of SARS-like viruses found among bats in the recent years [35–37]. The initial transmissions of SARS-CoV from animals to humans were traced back to the live animal wet markets and it was hypothesized that the virus made its way into the human population using the civet cat as an intermediate host. However, successful isolation of SARS-like viruses from bats [38] and the fact that a contemporary bat SARS-like virus can infect human airway cultures [39] suggest that an intermediate host between humans and bat might not have been needed for the transmission of SARS-CoV.

The evolutionary origin of MERS-CoV is less clear but it has been speculated to be bats as well. Characterization of an African bat virus closely related to MERS-CoV shows that both the human and camel strains belong to the same viral species and phylogenetic analysis suggests that MERS-CoV infection in camels predates that in humans, suggesting that camels infect humans and not the other way around. Furthermore, the bat virus roots
the phylogenetic tree providing further evidence for the bat origin of MERS-CoV [40]. Additionally, human-to-human transmission, although not robust, seems to happen simultaneously as camel-to-human transmission. Therefore, any further adaptation of MERS-CoV to the human host must be monitored carefully and intermediate hosts identified [41].

Many bat coronaviruses have been identified in the recent years further highlighting the zoonotic potential of this family of viruses [30]. Given the documented history of coronaviruses overcoming the species barrier and causing severe disease in humans, it is important to investigate the zoonotic potential of close evolutionary relatives of hCoVs in a culture model that recapitulates the aspects of the human airway, e.g. morphology and receptor distribution. It’s important to study the mechanisms of pathogenesis and the evolution of zoonotic viruses in detail in order to identify molecular determinants that affect either transmission or pathogenesis. It’s also important to elucidate whether coronaviruses currently circulating in animals are a potential danger to the human population.

**Human coronavirus receptors and cell tropism**

All of the known cellular receptors of HCoVs belong to the same protein family, the membrane ectopeptidases. Interestingly, the catalytic activity of these peptidases is not required for viral entry but rather the co-expression of other host peptidases activates the HCoV spike proteins [42, 43]. It has been established that the human transmembrane serine proteases TMPRSSII and HAT cleave and activate the HCoV-229E, SARS- and MERS-CoV spike proteins during viral entry [44, 45].

Out of the four commonly circulating coronaviruses, HCoV-229E is the only one that infects non-ciliated cells using the human Aminopeptidase N (hAPN) as its receptor [46]. This peptidase is predominantly expressed on non-ciliated cells in the human bronchus [47]. SARS-CoV and HCoV-NL63 both utilize the Angiotensin Converting Enzyme 2 (ACE2) for cellular binding [48, 49]. ACE2 is expressed on ciliated bronchial cells along with endothelial cells and both type I and II alveolar cells [50]. MERS-CoV was found to use a different receptor than SARS-CoV, namely the dipeptyl-peptidase 4 (DPP4) [51]. DPP4 is widely expressed in endothelial cells and various epithelial tissues in the human body [52]. In ex vivo human lung organ cultures, different tropism of SARS- and MERS-CoVs was observed. MERS-CoV can actively replicate in both bronchial and alveolar tissue while SARS-CoV primarily replicates in alveolar tissue [53]. The wide cellular tropism of MERS-CoV might contribute to its associated disease severity and high mortality rate whereas the alveolar replication of SARS-CoV would explain why it generally presents with pneumonia.

The cellular surface receptors for HCoV-OC43 and HCoV-HKU1 are currently unknown but receptor determinants for these two viruses have been identified as N-acetyl-9-O-acetylneuraminic acid and O-Acetylated Sialic acid, respectively [54, 55].

All of these viruses can be successfully cultured and investigated in HAE cultures [56, 57]. The discovery of HCoVs, their receptor usage, cell tropism and receptor binding domain (RBD) is summarized in Table 1.

Furthermore, established reverse genetic systems for HCoV-229E [58], HCoV-OC43 [59] and HCoV-NL63 [60] allow for controlled virus mutation and fluorescent transgene insertion to better understand the interaction of these viruses with their pulmonary host cells.

**Animal models for human coronaviruses**

Traditionally, respiratory viruses are studied in animal models, usually mice and ferrets [48, 61]. However, it is not always possible to correctly recapitulate human infection and disease in animal models. The establishment of transgenic animal models for human disease is attainable when either the virus receptor has been identified, which is not the case for all HCoVs, or when viruses can be adapted to a different host. An adapted human virus may not share the same properties as the original human virus. SARS-CoV was found to replicate naturally in various strains of inbred mice but to enhance clinical signs of disease the hACE2 was introduced into these mice. This resulted in murine models with varying degree of human disease similarity. Since SARS-CoV already replicated in mouse cells, adapting it to the murine host was quite successful. This resulted in three mouse adapted strains that caused disease in mice similar to severe SARS-CoV cases in humans [62].

In an effort to establish a mouse model for HCoV-229E infection transgenic hAPN mice were created. However, the insertion of the hAPN into mouse cells is not enough to establish robust HCoV-229E infection in vivo. Nevertheless, cells isolated from these transgenic animals could be infected in vitro [63, 64].

The emergence of both SARS- and MERS-CoVs emphasized the importance of establishing animal models for human coronaviruses. Currently, a few animal models for MERS-CoV have been established. Mice carry their own variant of the viral receptor DDP4 that differs from the human in regions important for MERS-CoV spike interaction and by replacing this receptor with the human one, MERS-CoV can infect mouse cells but the method of hDP4 insertion has an effect on the degree of pathogenesis observed in these mice [65, 66]. Various non-human primates (NHPs) can be naturally infected with both SARS- and MERS-CoVs. However, disease presentation and pathogenesis differs between the different subspecies.
and NHP models are expensive, although ideal to study human infection due to their genetic similarity [62].

To establish a link between the research conducted in animal models and humans, an organotypic airway culture system resembling the human airway epithelium has been developed. This model is a universal platform to study human respiratory viruses [67–70]. They have been used successfully for infection studies with all known human coronaviruses [56, 57]. Furthermore, the cultures can be inoculated with a low infectious dosage to mimic natural infection in the human airway. Whereas, animal models often require both high dosage and artificial inoculation routes.

Human airway epithelial cell cultures
Organotypic cell cultures are becoming increasingly common. Different cell culture models exist to depict different epithelial tissues [71]. These cultures closely resemble their tissue of origin and contain various different cell types with distinctive roles in the polarized tissue. Currently, various organotypic cell culture models exist to represent the different areas of the human airways. The human lungs span a long anatomical distance and carry out different functions depending on anatomical location [72, 73]. The structure of the epithelium also differs the further you descend into the airways. Tracheal and bronchial epithelium is columnar and pseudostratified, with every cell in contact with the basement membrane, while the epithelium in the alveoli is comprised of a single cell layer to facilitate air-exchange [74].

Tracheobronchial cells are one of the first targets of human respiratory viruses and can be cultured in air-liquid interface (ALI) where the apical side of the cell layer is exposed to air while the basolateral side is submerged in medium. Tracheobronchial cells cultured in that way form a pseudostratified epithelial layer that both morphologically and functionally resembles the human upper conducting airway (Fig. 1a) [75, 76]. After differentiation, these cultures contain many different cell types such as basal, ciliated and goblet cells. They also produce protective mucus, much like in vivo epithelium. When compared to primary bronchial cells in submerged two-dimensional culture, the gene expression of primary ALI cultures differs significantly. However, the expression pattern of primary human bronchial ALI cultures is comparable to that of in vivo epithelium. The human bronchial cell line Calu-3 has been used as a culture model for respiratory epithelium but its gene expression in ALI cultures is more similar to submerged bronchial cell cultures than differentiated epithelium [77]. Additionally, Calu-3 cells respond differently to MERS-CoV infection compared to primary HAE cultures. During infection in Calu-3 cells, profound apoptosis was detected within 24 h of infection [78] while infection of primary HAE cultures does not result in any disruption of the cell layer [57]. Therefore, the primary tracheobronchial ALI culture model is especially fitting for human respiratory virus research since it accurately recapitulates the primary entry point for these viruses. By using these cultures, virus replication and host interactions can be studied in natural target cells. Further establishing the usefulness of this system HCoV-HKU1 was propagated for the first time in ciliated cells of bronchial HAE cultures in 2010 after culturing it in conventional cell lines had failed [26].

Alveolar epithelial ALI cultures (Fig. 1b) can also be used for virus-host interaction studies and are especially applicable when a viral infection causes pneumonia and alveolar damage [79]. HCoV-HKU1 has also been propagated in alveolar HAE cultures and exhibits a strong tropism for alveolar type II cells and causes large syncytia formation upon infection [80].

When compared to traditional two dimensional cell cultures, the HAE cultures are more cumbersome and their preparation is time consuming but they do have an advantage over traditional monolayer cell cultures when it comes to virus-host interaction studies. Different types of ALI cultures used for virus research are summarized in Table 2.

**Table 1 Human coronavirus overview**

<table>
<thead>
<tr>
<th>Name</th>
<th>Discovery</th>
<th>Protein Receptor</th>
<th>Tropism</th>
<th>Receptor Binding Domain (RBD)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCoV-229E</td>
<td>1966</td>
<td>Aminopeptidase N (hAPN)</td>
<td>Non-ciliated cells</td>
<td>S407-547</td>
<td>[46, 56, 57, 103, 104]</td>
</tr>
<tr>
<td>HCoV-OC43</td>
<td>1967</td>
<td>Unknown²</td>
<td>Ciliated cells</td>
<td>Unknown</td>
<td>[56, 105]</td>
</tr>
<tr>
<td>SARS-CoV</td>
<td>2003</td>
<td>Angiotensin Converting Enzyme 2 (ACE2)</td>
<td>Ciliated cells</td>
<td>S303-537</td>
<td>[13, 14, 48, 57, 106]</td>
</tr>
<tr>
<td>HCoV-NL63</td>
<td>2004</td>
<td>Angiotensin Converting Enzyme 2 (ACE2)</td>
<td>Ciliated cells</td>
<td>S476-616</td>
<td>[16, 49, 56, 107]</td>
</tr>
<tr>
<td>HCoV-HKU1</td>
<td>2005</td>
<td>Unknown³</td>
<td>Ciliated cells</td>
<td>Unknown</td>
<td>[17, 56]</td>
</tr>
<tr>
<td>MERS-CoV</td>
<td>2012</td>
<td>Dipeptyl-peptidase 4 (DPP4)</td>
<td>Non-ciliated cells</td>
<td>S358-588</td>
<td>[18, 34, 51, 108]</td>
</tr>
</tbody>
</table>

-a Receptor determinant identified as N-acetyl-9-O-acetyleneuraminic acid
-²Receptor determinant identified as O-Acetylated Sialic acid
interferon (IFN) system orchestrates hundreds of different cellular effector proteins that (i) protect the epithelial barrier by altering the physiological and cellular environment, (ii) impair virus propagation, spread and transmission, and (iii) shape the host’s adaptive immune response. Recent publications have demonstrated that the innate immune system is functional in the HAE cell culture system and that most pathogen recognition receptors are expressed and up-regulated upon treatment with exogenous stimuli [57, 81].

In general, HCoVs do not elicit a strong innate immune response in primary target cells of the human airway early during infection. Despite the presence of all major pathogen recognition receptors, no elevated expression of IFN beta, pro-inflammatory cytokines or interferon stimulated genes can be observed up to 12 h post-infection in HAEs infected with HCoV-229E, MERS- or SARS-CoVs [57]. This is most likely due to the intrinsic CoV properties harbored in the replicative non-structural proteins that actively aid in avoiding recognition by the host innate immune system. For example, the 5′ termini of the viral mRNA are capped making them indistinguishable from the host cellular mRNAs and no longer detectable by cellular sensors. Furthermore, CoV replication is associated with the appearance of double membrane vesicles (DMVs) in the host cell cytoplasm, which might serve as a protective shield for viral RNA to prevent recognition by cytoplasmic RNA sensors [82–85].

In addition to the non-structural proteins, various CoV accessory proteins have been discovered to inhibit interferon signaling at different stages of the host innate immune response. For example, MERS-CoV accessory protein 4a inhibits innate antiviral signaling by suppressing the activation of MDA5 and RIGI [86, 87] whereas 4b inhibits the induction of the IFN-beta promoter [88]. While ORF 4a and 4b are IFN antagonists in the genome of MERS-CoV, SARS-CoV ORF3b antagonizes IFN signaling through MAVS/RIGI [89]. Whereas SARS-CoV ORF6 disrupts IFN signaling by blocking the nuclear translocation of STAT1 [89, 90]. These discoveries highlight that HCoVs employ similar yet different strategies

Table 2: Different types of ALI cultures used in coronavirus studies

<table>
<thead>
<tr>
<th>Cell types</th>
<th>Origin</th>
<th>HCoVs</th>
<th>Features</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary bronchial</td>
<td>Trachea, Bronchus</td>
<td>All HCoVs</td>
<td>Differentiated pseudostratified epithelium, many cell types</td>
<td>[56, 57]</td>
</tr>
<tr>
<td>Calu-3</td>
<td>Sub-mucosal glands (adenocarcinoma)</td>
<td>MERS-CoV, SARS-CoV</td>
<td>Single or polarized epithelium, one cell type</td>
<td>[109, 110]</td>
</tr>
<tr>
<td>Primary alveolar</td>
<td>Alveoli</td>
<td>MERS-CoV, SARS-CoV, HCoV-HKU1</td>
<td>Differentiated squamous epithelium, two cell types</td>
<td>[79, 80, 111]</td>
</tr>
</tbody>
</table>
to evade the innate immune response during infection in the respiratory epithelium.

**Therapy**

Despite that respiratory infections with HCoVs can result in severe respiratory disease there are currently no effective prophylactic or therapeutic treatment options available. However, the emergence of novel coronaviruses has emphasized the need to develop effective treatment options. For example, vaccines using the spike proteins of both SARS- and MERS-CoVs have proven protective in animal models [91, 92] suggesting that a vaccine against HCoVs for human use might be achievable.

Additionally, various drugs that inhibit HCoV infection at different stages of the replication cycle have been reported and some could potentially serve as treatment options for HCoV associated severe respiratory disease. For example, patients presenting with severe respiratory disease, caused by SARS- or MERS-CoVs, are generally treated with steroids and interferon, sometimes in combination with the antiviral drug Ribavirin [93–96]. This treatment, however, is not especially effective highlighting the need for HCoV specific antivirals. Many different compounds have been determined to have anti-HCoV activity. For example, protease inhibitors which suppress HCoV entry [97–99], Cyclosporin A (CsA) treatment blocks the replication of coronaviruses from all subgroups [100] and non-immunosuppressive derivatives of CsA represent a possible therapeutic option for both human and animal CoV infections.

HCoV infection can also be inhibited by pre-treating HAE cultures with either recombinant IFN alpha or lambda [57]. Similar effect has also been shown for recombinant IFN alpha and beta which could inhibit MERS-CoV in ex vivo lung cultures [53]. As previously described, IFN treatment of active HCoV infection is not particularly effective in vivo. Therefore, the use of IFN in humans might be limited to prophylactic treatment of exposed persons and/or health care workers treating infected patients.

Screenings of compound libraries have also resulted in the identification of some HCoV specific antivirals. For example, a novel small compound inhibitor (K22) has been identified, and showed to be effective against a broad spectrum of CoVs and could inhibit both HCoV-229E and MERS-CoV in HAE cultures [101]. Additionally, HCoV-NL63 has been inhibited in HAE cultures with polymer-based compounds [102].

To date, most treatment and inhibitor studies have been conducted in HCoV susceptible cell lines. However, the HAE cultures represent an ideal system to test the application and efficacy of those already identified, and new, antiviral compounds against HCoVs in cells that represent the primary site of replication. Furthermore, the HAE cultures are heterogenous, containing many different cellular sub-populations, and would allow for the evaluation of compound toxicity and effect in a differentiated layer similar to human airway epithelium. Compounds already shown to inhibit HCoVs in cell lines should be applied to HAE cultures as well before any animal or human trials.

**Conclusions**

HCoV induced respiratory diseases are of both medical and socio-economic importance. The emergence of SARS- and MERS-CoV and the yearly circulation of the four common HCoVs highlight the importance of elucidating the different mechanisms employed by HCoVs to evade the host immune system as well as identifying antiviral compounds and human vaccine candidates. The HAE culture system is based on primary human cells offering a unique platform to study respiratory viruses in cells representing the primary entry point of these viruses, bronchial epithelial cells, or investigate the interaction of HCoVs and the distal airways, in type I and II alveolar cells. Additionally, the inclusion of airway epithelial cultures for other species enables the study of zoonosis and animal-to-human transmission. Currently, many aspects of HCoV infection and pathogenesis remain to be determined. The HAE culture system, both tracheobronchial and alveolar, represents a unique platform to study virus-host interaction in natural target cells at the molecular level. These cultures are becoming more common and more relevant to HCoV research. Especially, for those viruses for which there is no animal model, as they provide an organotypic substitute for virus – host interaction studies.

**Abbreviations**

ATL: Air-Liquid Interface; BCoV: Bovine Coronavirus; CNS: Central Nervous System; CoV: Coronavirus; CsA: Cyclosporin A; DMV: Double Membrane Vesicles; FDA: Food and Drug Administration; HAE2: Human Angiotensin Converting Enzyme 2; HAE: Human Airway Epithelium; hAPN: human Aminopeptidase N; HAT: Human Airway Trypsin-like protease; HCoV: Human Coronavirus; hDPP4: human Dipeptyl Peptidase 4; IBV: Infectious Bronchitis Virus; IFN: Interferon; IL10: Interleukin 10; MDA5: Melanoma Differentiation-Associated protein 5; MERS: Middle East Respiratory Syndrome; NHP: Non-Human Primate; ORF: Open Reading Frame; PEDV: Porcine Epidemic Diarrhea Virus; RBD: Receptor Binding Domain; RNA: Ribonucleic Acid; SARS: Severe Acute Respiratory Syndrome; TMPRSS2: Transmembrane Protease, Serine 2.
References


Chapter 8

Characterization of Human Coronaviruses on Well-Differentiated Human Airway Epithelial Cell Cultures

Hulda R. Jonsdottir and Ronald Dijkman

Abstract

The human airway serves as the entry point of human respiratory viruses, including human coronaviruses. In this chapter we outline the methods by which we establish fully differentiated airway epithelium and its use for human coronavirus propagation. Additionally, we outline methods for immunofluorescence staining of these cultures for virus detection, characterization of cell tropism, and how to perform antiviral assays and quantify viral replication.

Key words Human coronavirus, Antivirals, Cell tropism, Human airway epithelial cells, Virus detection

1 Introduction

The human airway serves as the entry point of human respiratory viruses, including human coronaviruses (HCoVs). In order to properly recapitulate the complex anatomy of the human lung specialized cell culture models have been developed to resemble both the upper and lower airways [1–3]. Primary human bronchial epithelial cells cultured in an air–liquid interface (ALI) system serve as a universal platform to study human respiratory viruses [4–6]. These human airway epithelial (HAE) cultures morphologically and functionally resemble the upper conducting airways in vivo. In these cultures, the epithelial layer is pseudostratified and after differentiation they contain many different cell types such as basal, ciliated, and goblet cells and furthermore, generate protective mucus equivalent to that of in vivo epithelium [7].

Establishment of HAE cultures requires time and patience but the differentiated cultures allow for a number of advantageous analyses in respiratory virus research. We have adapted and optimized our methods based on previously published work [8–10]. Moreover, we have standardized methods for the propagation of human coronaviruses and evaluation of the effects of antiviral
compounds on both viral replication and cell viability. We are able to propagate all known human coronaviruses in this system and can easily evaluate their tropism by immunohistochemistry [5, 11]. In this chapter we outline the methods by which we establish fully differentiated airway epithelium and use it for human coronavirus propagation. Additionally, we outline methods for immunofluorescence staining of these cultures for virus detection, characterization of cell tropism and how to perform antiviral assays and quantify viral replication.

2 Materials

2.1 Human Airway Epithelial Cell Cultures

1. Primary human tracheobronchial epithelial cells can be obtained in accordance with local ethical guidelines from patients willing to give informed consent, who are undergoing bronchoscopy and/or surgical lung resections. Alternatively isolated primary human airway epithelial cells can be obtained commercially from a number of distributors.

2. 10× digestion solution: Minimum Essential Medium (MEM), 1 % m/v Protease from Streptomyces griseus Type XIV, 0.01 % m/v Deoxyribonuclease I from bovine pancreas.

3. Isolation/washing solution: MEM, 100 U/ml penicillin, 100 µg/ml streptomycin, 0.25 µg/ml Amphotericin B Solution, 50 µg/ml gentamicin, 100 U/ml nystatin.

4. Bronchial epithelial cell serum-free growth medium (BEGM): LHC basal medium, supplemented with the required additives (Table 1).

5. Air–liquid interface (ALI) medium: LHC basal medium and Dulbecco’s Modified MEM (DMEM) mixed in a 1:1 ratio, supplemented with the required additives (Table 1).

6. 12-Well inserts, pore size 0.4 µM and 12-well cluster plates or 12-well deep well cluster plates.

7. 24-Well inserts pore size 0.4 µM and 24-well cluster plates.

8. Human collagen Type I + III, Vitrocol 100.

9. Collagen Type IV from human placenta reconstituted in 5 ml filter-sterilized water with 0.25 % acetic acid. Dissolve for a few hours at 37 °C, occasionally swirling. Once dissolved, increase volume to 20 ml and maintain acetic acid concentration at 0.25 %, mix gently by pipetting. Filter-sterilize the solution through a 0.22 µm filter, and store at −20 °C in aliquots of 800 µl per eppendorf tube. The stock solution is stable for at least 1 year at −20 °C.
### Table 1
Preparation of stock additives for BEGM and ALI medium

<table>
<thead>
<tr>
<th>Component</th>
<th>Stock concentration</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine Serum Albumin (BSA)</td>
<td>300× 150 mg/ml</td>
<td>See Note 1</td>
</tr>
<tr>
<td>Bovine pituitary extract (BPE)</td>
<td>1,000× ±14 mg/ml</td>
<td></td>
</tr>
<tr>
<td>Insulin</td>
<td>2,000×, 10 mg/ml</td>
<td>Store at +4 °C</td>
</tr>
<tr>
<td>Transferrin (TF)</td>
<td>1,000×, 10 mg/ml</td>
<td></td>
</tr>
<tr>
<td>Hydrocortisone (H)</td>
<td>1,000×, 0.072 mg/ml</td>
<td></td>
</tr>
<tr>
<td>Triiodothyronine (T3)</td>
<td>1,000×, 0,067 mg/ml</td>
<td></td>
</tr>
<tr>
<td>Epinephrine (EP)</td>
<td>1,000×, 0.6 mg/ml</td>
<td></td>
</tr>
<tr>
<td>Epidermal Growth Factor (EGF)</td>
<td>1,000× or 50,000×, 25 µg/ml</td>
<td>1,000× for BEGM, 50,000× for ALI medium.</td>
</tr>
<tr>
<td>Retinoic acid (RA)</td>
<td>1,000×, 5×10^{-5} M</td>
<td>Light sensitive. See Note 2</td>
</tr>
<tr>
<td>Phosphorylethanolamine (PE)</td>
<td>1,000×, 70 mg/ml</td>
<td></td>
</tr>
<tr>
<td>Ethanolamine (EA)</td>
<td>1,000×, 30 µl/ml</td>
<td></td>
</tr>
<tr>
<td>Stock 11 (S11)</td>
<td>1,000×, 0,863 mg/ml</td>
<td></td>
</tr>
<tr>
<td>Stock 4 (S4)</td>
<td>1,000×</td>
<td>See Note 3</td>
</tr>
<tr>
<td>Trace Elements (TR)</td>
<td>1,000×</td>
<td>See Note 4</td>
</tr>
<tr>
<td>Penicillin/Streptomycin (P/S)</td>
<td>100× 10,000 U/ml of penicillin and 10,000 µg/ml of streptomycin</td>
<td>Store at +4 °C</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>1,000×, 50 mg/ml</td>
<td>Store at +4 °C. See Note 5</td>
</tr>
<tr>
<td>Amphotericin B</td>
<td>1,000×, 50 mg/ml</td>
<td>See Note 5</td>
</tr>
</tbody>
</table>

All additives should be aliquoted and stored at −20 °C unless stated otherwise.

### 2.2 Human Coronavirus Propagation
1. Apical wash solution: Hank’s Balanced Salt Solution (HBSS), without calcium and magnesium.
2. Virus transport medium (VTM): MEM, 25 mM HEPES-buffered, 0.5 % gelatin, 100 U/ml penicillin, 100 µg/ml streptomycin.
3. Aerosol barrier pipette tips and 1.5 ml Eppendorf Safe-Lock Tubes™.

### 2.3 Immuno-fluorescence Analysis
1. Fixation solution: 4 % formalin solution, neutral buffered (Formafix).
2. Confocal staining buffer (CB): 50 mM ammonium chloride (NH₄Cl), 0.1 % saponin, and 2 % IgG and protease-free BSA dissolved in 500 ml of phosphate buffered saline (PBS,
pH 7.4). Filter-sterilize (0.2 µm filter) solution and prepare aliquots of 40 ml and store at −20 °C.

3. Primary antibodies: see Table 2.

4. Fluorescent DNA dyes: DAPI or Hoechst 33528.

5. Wash solution: Phosphate buffered saline, pH 7.4, without calcium and magnesium.

6. Scalpel (No.10).

7. Rat-tooth forceps.

8. Fluorescence Mounting Medium.


### Table 2
**Primary antibodies**

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Target</th>
<th>Dilution</th>
<th>Host</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-β-Tubulin IV</td>
<td>Cilia</td>
<td>1:400</td>
<td>Mouse, IgG1</td>
<td>Clone ONS.1A6</td>
</tr>
<tr>
<td>anti-ZO1</td>
<td>Tight junctions</td>
<td>1:200–400</td>
<td>Goat</td>
<td>Directed against C-terminal domain</td>
</tr>
<tr>
<td>anti-dsRNA</td>
<td>dsRNA</td>
<td>1:500–1,000</td>
<td>Mouse, IgG2a</td>
<td>Clone J2</td>
</tr>
<tr>
<td>Anti-CD13</td>
<td>CD13/APN</td>
<td>1:200</td>
<td>Sheep</td>
<td>Receptor 229E</td>
</tr>
<tr>
<td>Anti-CD26</td>
<td>CD26/DPIV</td>
<td>1:200</td>
<td>Goat</td>
<td>Receptor MERS</td>
</tr>
<tr>
<td>Anti-ACE2</td>
<td>ACE2</td>
<td>1:200</td>
<td>Goat</td>
<td>Receptor SARS and NL63</td>
</tr>
<tr>
<td>intravenous immunoglobulin (IVIG)</td>
<td>Viral proteins</td>
<td>1:1,000</td>
<td>Human</td>
<td></td>
</tr>
<tr>
<td>Anti-β-Tubulin</td>
<td>Cilia</td>
<td>1:400</td>
<td>Mouse, IgG1</td>
<td>Clone Tub2.1, Cy3 conjugate</td>
</tr>
</tbody>
</table>

2.4 **Antiviral Assays**

1. Inhibitors: e.g. K22 [12], recombinant Interferon Alpha and Lambda proteins [13].


2.5 **Virus Detection**

2.5.1 **Renilla Luciferase Assay**

1. Renilla Luciferase Assay System (Promega).

2. White, non-transparent 96-well plates.


4. Luminometer.

2.5.2 **Plaque Assay**

1. Huh-7 cells.

2. Medium: DMEM, high glucose, 100 U/ml penicillin, 100 µg/ml streptomycin, 1 mM Sodium Pyruvate, 5 % heat-inactivated FBS.
3. Overlay medium: 2.4 g of Avicel RC-581 (FMC biopolymer) dissolved in 100 ml of distilled water and autoclaved for 20 min at 121 °C. 2.7 g of DMEM powder (high glucose) dissolved in 90 ml of distilled water and the pH adjusted to 7.4 with 1 M NaOH. Fill volume up to 100 ml and filter-sterilize (0.2 µm filter). Freshly prepare a 1:1 mixture of Avicel (2.4 %) and 2× DMEM solution, supplemented with 10 % FBS and 100 U/ml penicillin, 100 µg/ml streptomycin.

4. Crystal-violet solution: 25 g of Crystal Violet, 40 g NaCl dissolved in 2,500 ml of 99 % Ethanol. Add 2,250 ml of distilled water and 250 ml of 37 % formaldehyde. Mix solution overnight at room temperature (see Note 6).

2.5.3 Quantitative Reverse Transcriptase PCR

1. Nucleospin RNA isolation kit (Machery Nagel).
4. RNAse-free water.
5. FastStart Universal SYBR Green Master reaction mixture (Roche).
6. Positive control; in vitro transcribed RNA of target gene or plasmid DNA containing target gene.

3 Methods

Carry out all procedures in a biosafety cabinet according to local biosafety regulations.

3.1 Human Airway Epithelial Cell Cultures

3.1.1 Collagen Type I and III Coating of Cell Culture Flasks

Cell culture flasks are coated for 2 h with a mixture of Type I and III collagen that is necessary to efficiently expand the number of primary airway epithelial cells.

1. Use filter-sterilized dH₂O (0.22 µm) to prepare a 1:75 dilution of Vitrocol 100.
2. Use 4 ml per 75 cm², make sure the entire surface is covered with the collagen solution.
3. Incubate for 2 h at 37 °C.
4. Aspirate remaining liquid and wash twice with 10 ml of PBS to remove traces of acetic acid.
5. Culture flasks can be directly used. Optional: Store coated flasks at +4 °C for a maximum of 6 weeks.
3.1.2 Collagen Type IV Coating of Inserts

The inserts need to be coated overnight with collagen type IV, necessary for development and long-term maintenance of differentiated primary airway epithelial cell cultures.

1. Mix 7.2 ml of filter-sterile dH₂O with 800 µl of Collagen Type IV solution (0.5 mg/ml).
2. Apply 150 µl per 12-well inserts, or 50 µl per 24-well inserts. After completing one plate, make sure that the entire surface of each well is covered with the 1:10 collagen solution.
3. Air-dry the inserts overnight in a laminar flowhood, and afterwards expose them to UV-light (type C) for 30 min.
4. To remove traces of acetic acid wash inserts twice with at least 500 µl of PBS.
5. After these steps, coated inserts can be used directly. Optional: Store at +4 °C (wrapped in foil) for a maximum of 6 weeks. Repeat UV-exposure and washing steps before use.

3.1.3 Isolation of Primary Human Tracheal and/or Bronchial Cells

Primary epithelial cells can be isolated from whole lung tissue resections of tracheal and/or bronchial origin according to the following protocol. Smaller lung tissue resections can be processed with the same protocol. All procedures are performed at room temperature unless stated otherwise.

1. Trim the bronchial tissue free of connective tissue and fat using forceps and scissors or a scalpel. If needed, cut the bronchial tissue into 2 cm segments.
2. Wash the cleaned tissue three times in washing solution.
3. Fill the desired number of 50 ml tubes with 30 ml of wash solution and transfer as many tissue segments as possible into a single tube, until the volume reaches 36 ml. Then add 4 ml of 10× digestion solution to each tube, to end volume 40 ml (40 mg Protease/0.4 mg DNase).
4. Place tubes on a rocking platform/tube roller at +4 °C and incubate for 48 h.
5. Place the 50 ml tube containing the digested tissue on ice and add 4 ml of heat-inactivated FBS to each tube (to a final concentration of 10 % (v/v)), to neutralize protease activity. Invert tubes three times.
6. Pour solution along with the tissue onto a large petri dish, and gently scrape off the epithelium from the collagen-cartilage surface, using a scalpel in the reverted angle. Pool solutions containing dissociated cells into a 50 ml conical tube and wash the petri dish once with PBS.
7. Centrifuge for 5 min at 500×g. Wash cells once with HBSS and resuspend cells in BEGM to a concentration of, approximately, 5×10⁶ cells/ml.
8. Count cells using a hemocytometer and seed into collagen coated flasks with 20 ml of pre-warmed BEGM. An appropriate amount of cells for T75 flasks ranges between 0.5 and 1.0 × 10^6 cells.

9. Change medium the next day to remove red blood cells and any unattached epithelial cells.

10. To prevent acidification of the medium change it every 2–3 days, until 80–90% confluence.

When the primary cells have reached 80–90% confluence in the expansion phase one can dissociate and seed the dedifferentiated primary cells on collagen type IV coated inserts, according to the following protocol. All procedures are performed at room temperature unless stated otherwise.

1. Remove BEGM and transfer it into a 50 ml tube and wash the cell monolayer twice with 12 ml of HBSS.

2. Dissociate the bronchial cells for 3 min at 37 °C in a humidified 5% CO_2 incubator with the appropriate amount of trypsin (25 cm^2: 1 ml, 75 cm^2: 3 ml). If needed tap the flask to dissociate the cells (see Note 7).

3. Collect the cells in the previously collected BEGM and centrifuge for 5 min at 500×g.

4. Carefully discard the supernatant and resuspend cells in HBSS and centrifuge the suspension for 5 min at 500×g.

5. Discard the supernatant and resuspend cells in pre-warmed ALI medium and count using a hemocytometer.

6. For generation of differentiated HAE cultures the number of cells seeded should be 1.0–2.0 × 10^5 cells per 12-well insert in 500 µl, or 0.3–0.6 × 10^5 cells per 24-well insert in 200 µl of ALI medium. A single 75 cm^2 flask should provide enough cells for preparing 48 individual 12-well inserts or 96 individual 24-well inserts.

7. Fill the basolateral compartment of the plates with 1 ml of ALI medium (500 µl for 24-well inserts), and transfer 500 µl (200 µl for 24-well inserts) of diluted cell suspension to the upper chamber of the collagen coated inserts and incubate overnight at 37 °C in a humidified 5% CO_2 incubator. Cells are now in liquid–liquid interface.

8. The next day, medium in the apical compartment must be changed to remove any unattached cells. Discard the old medium and wash the apical surface with 500 µl HBSS and apply 500 µl of pre-warmed ALI medium to the apical side. Adjust volume to 200 µl for 24-well inserts.
9. To prevent acidification of the medium it should be changed every 2–3 days until cells have reached complete confluence (see Note 8).

10. During media change in liquid–liquid interface change apical medium first (as described in step 8) followed by exchange of medium in the basolateral compartment.

11. To establish air–liquid interface, aspirate apical side medium, once cells have reached complete confluence, and wash twice with HBSS (500 µl for 12-well inserts and 200 µl for 24-well inserts).

12. Incubate cultures for a few hours at 37 °C in a humidified 5 % CO₂ incubator and monitor if seeping of basolateral medium into the apical compartment occurs. If no seepage occurs cultures can be maintained at air–liquid interface. Otherwise cultures have to be cultured at liquid–liquid interface for another day.

13. Incubate cultures for 4–6 weeks to allow differentiation. Appearance of active ciliated cells can be used as an indicator of differentiation. During the extended culture time medium must be changed regularly (every 2–3 days). If desired, inserts can be transferred to deep well plates that only require medium renewal every 7 days.

14. After differentiation HAE cultures are suitable for human coronavirus propagation.

1. Wash the apical surface of the HAE culture twice with 500 µl of HBSS solution prior to inoculation with human coronavirus specimen to remove excess of mucus.

2. Dilute the clinical material or virus supernatant in HBSS and inoculate 200 µl dropwise to the apical surface and incubate for 2 h at either 33 °C or 37 °C (see Note 9), in a humidified 5 % CO₂ incubator. Optional: Centrifuge inoculum solution for 4 min at 1,500 × g at room temperature to remove cell debris prior to inoculation.

3. Collect the inoculum and transfer it to a container and store at −80 °C for later analysis, and wash the apical surface three times with 500 µl HBSS. Optional: Transfer the collected inoculum into an equal volume of VTM.

4. Incubate the infected cultures for the desired amount of time at the appropriate temperature in a humidified 5 % CO₂-incubator, e.g. 48 h at 33 °C for HCoV-229E.

5. Apply 200 µl of HBSS dropwise to the apical surface 10 min prior to the desired collection time and incubate in the humidified 5 % CO₂-incubator. Then collect progeny virus and transfer it to a container and store at −80 °C for later analysis. Optional: Transfer the collected progeny virus into an equal volume of VTM.
All incubation steps are performed at room temperature on a gyro-rocker (20–30 rpm), unless stated otherwise

1. After the apical washing has been collected the apical surface is washed twice with 500 µl of PBS before cells are fixed with formalin-solution for later immunofluorescence analysis.

2. Apply 500 µl of 4 % formalin-solution to the apical compartment and 1 ml to the basolateral. Incubate for 15–30 min.

3. Remove the formalin-solution and wash both compartments three times with equal volumes of PBS.

4. Transfer the fixed HAE cultures to a new conventional 12-well plate.

5. Discard washing solution and apply 500 µl and 1 ml of confocal buffer (CB) solution to apical and basolateral compartments, respectively.

6. Incubate fixed cultures for 30–60 min to block non-specific binding of antibodies (see Note 10).

7. Remove the CB solution from the apical and basolateral compartments.

8. From this stage one should only apply CB solution to the apical compartment.

9. Wash the apical surface once with 500 µl of CB solution for 5 min.

10. Apply primary antibodies (see Table 2) diluted in 250 µl CB solution dropwise to the apical surface and incubate for 120 min.

11. Wash the apical surface three times with 500 µl of CB solution for 5 min (see Note 11).

12. Apply the appropriately diluted conjugated secondary antibodies in 250 µl CB solution dropwise to the apical surface and incubate for 60 min.

13. Wash the apical surface twice with 500 µl of CB solution for 5 min.

14. Incubate cells with nucleic acid counter stain solution diluted in 250 µl of CB solution for 5 min.

15. Wash the apical surface once with 500 µl of CB solution for 5 min.

16. Lastly, wash the apical surface twice with 500 µl of PBS for 5 min to remove residual saponin and restore cell membrane integrity.

17. Before removing the washing solution, apply mounting medium on a glass slide (use 1–2 drops). Remove any air bubbles.
18. Excise the membrane from the plastic holder and carefully place the basolateral side of the membrane on top of the mounting medium, without generating air bubbles.

19. Then slowly add one drop of mounting medium on top of each membrane.

20. Slowly place the coverslip, in a tilted fashion, on top of the membrane without generating air bubbles.

21. Allow the mounting medium to polymerize for 30 min, after which the slide can directly be analyzed.

3.4 Antiviral Assays

3.4.1 Treatment

1. Pre-warm ALI medium to 37 °C.

2. Mix antiviral compounds (e.g. K22, recombinant interferons) in various concentrations or by serial dilution in ALI medium. Include non-treated controls. Also, to exclude viral inhibition by solvents (e.g. DMSO) include solvent controls.

3. For evaluation of either prophylactic or therapeutic effects of antivirals, the HAE cultures can be incubated with the compounds diluted in the basolateral medium prior to, during or after infection.

4. Infect cultures apically with human coronaviruses as described in Subheading 3.2.

5. Collect apical washings in HBSS as described in Subheading 3.2 for viral quantification by plaque assay and cells for viral quantification by Renilla Luciferase Assay or qRT-PCR.

3.4.2 Cytotoxicity Assay

1. Thaw CellTiter-Glo buffer and equilibrate both buffer and CellTiter-Glo substrate to room temperature.

2. Transfer the buffer to the amber bottle containing the substrate to reconstitute the enzyme. Mix by gently swirling the bottle.

3. Wash the apical side of the HAE cultures three times with 500 µl HBSS to remove excess mucus.

4. Apply 50 µl of HBSS to the apical side and mix with equal volume of reconstituted CellTiter-Glo enzyme solution (optimized for 24-well inserts, for other insert sizes adjust buffer amount accordingly) and incubate for 5 min at room temperature on a gyro-rocker to induce cell lysis.

5. Next incubate the plate for 10 min at room temperature to allow for stabilization of the luminescence signal.

6. Transfer 20 µl of cell lysate to a white, non-transparent 96-well plate for analysis.

7. Record luminescence (see Note 12). To account for background signal include empty wells in your analysis.
3.5 Virus Detection

3.5.1 Renilla Luciferase Assay (See Note 13)

1. Thaw Renilla Luciferase Assay buffer and dilute 1:5 in water.
2. Wash HAE inserts with HBSS three times prior to cell lysis.
3. Incubate inserts with 80 µl of Renilla lysis buffer on a gyro-rocker for 30 min at room temperature (optimized for 24-well inserts, adjust lysis buffer amount accordingly for other insert sizes).
4. During incubation, thaw Renilla Assay buffer.
5. Transfer the cell lysate to a 96-well plate.
6. Transfer 20 µl of the lysate to a white, non-transparent 96-well plate for analysis.
7. Add Renilla substrate at 1:200 dilution to the required amount of Renilla Assay buffer (100 µl per sample). Protect from light (see Note 14).
8. Program your luminometer settings with 10 s measure time followed by a 2 s delay. 100 µl of assay buffer should be dispensed into each well. If the luminometer is not equipped with an injector the assay buffer can be added manually using a multichannel pipette.
9. To adjust samples for background include empty wells in your analysis.
10. Plot your values as Log_{10} RLU (Relative Light Units).

3.5.2 Plaque Assay

The current protocol is optimized for HCoV-229E, but can easily be adapted to any other cell line and coronavirus strain.

1. Seed 150,000 target cells in a 12-well cluster plates with 1 ml of complete medium per well and incubate overnight at 37 °C in a humidified 5 % CO₂-incubator.
2. Make 6 tenfold serial dilutions of the harvested virus supernatants in 1 ml and inoculate the cells.
3. Incubate inoculum for 2 h at 33 °C in a humidified 5 % CO₂-incubator before removing the serial diluted virus inoculums from the cells and replace with 1 ml of overlay medium.
4. Incubate titration plates for 3–4 days at 33 °C in a humidified 5 % CO₂-incubator.
5. Remove overlay and wash wells twice with water to remove residual Avicel.
6. Subsequently add approximately 0.5–1 ml of crystal violet solution to each well and incubate for 10 min.
7. Remove crystal violet solution and wash the cells once with water and allow the plates to air-dry before counting the number of plaques.
1. Isolate viral RNA with NucleoSpin RNA kit according to the manufacturer’s protocol and elute in the appropriate amount of RNase-free water.

2. For reverse transcription use M-MLV reverse transcriptase (100 U), M-MLV buffer, and random primers and 10 µl of extracted total RNA in a total volume of 20 µl, at 37 °C for 60 min. Optional: include serial dilutions of in vitro transcribed RNA of the target gene for virus yield quantification.

3. To quantify viral HCoV RNA yields from contemporary strains use the FastStart Universal SYBR Green Master reaction mixture. Amplify 2 µl of cDNA according to the manufacturer’s protocol, using the previously published sense and antisense strain-specific primers (see Note 15). Measurements and analysis can, for instance, be done on a LightCycler 480 II instrument, using the LightCycler 480 software package (Roche). Use the following cycle profile of 10 min at 95 °C followed by 45 cycles of 10 s at 95 °C, 20 s at 60 °C, and 30 s at 72 °C followed by a melting curve step to confirm product specificity.

4. Notes

1. Dissolve 5 g of BSA, globulin free, powder in 20 ml PBS in a 50 ml tube (do not vortex). Place the tube on a shaker/roller-bank for 2–4 (max 24) hours at +4 °C, until the BSA is completely dissolved. Add the volume up to 34 ml, mix gently by inverting the tube three times. Filter-sterilize the solution through a 0.22 µm filter, and store at −20 °C in aliquots of 3.5 ml in 15 ml tubes. Invert the tube three times before usage.

2. Dissolve 12 mg of Retinoic Acid (RA) in 40 ml absolute EtOH in a 50 ml tube wrapped in aluminum foil, the RA–EtOH stock (1 × 10⁻³ M) should be stored at −20 °C. To prepare the 1,000× stock, first confirm the RA concentration of the ethanol stock by diluting it 1:100 in absolute EtOH. Measure the absorbance at 350 nm using a spectrophotometer and a 1 cm light path quartz cuvette (or NanoDrop with 0.1 cm light path), blanked on 100 % EtOH. The absorbance of the diluted stock should equal 0.45 (0.045 on a NanoDrop). RA absorbance readings below 0.18 should be discarded. If the absorbance equals 0.45, add 3 ml of RA–EtOH stock solution to 53 ml PBS and add 4 ml of BSA 150 mg/ml stock. For absorbance values less than 0.45, calculate the needed volume of RA–EtOH stock as 1.35/absorbance and adjust the PBS volume appropriately. The 1,000× stock solution should be stored at −20 °C in aliquots of 1 ml per eppendorf tube.

3. Dissolve 42 mg ferrous sulfate, 12.2 g magnesium chloride, and 1.62 g calcium chloride-dihydrate in 80 ml H₂O, add
500 µl concentrated hydrochloric acid (HCl). Filter-sterilize the solution through a 0.22 µm filter, and store at −20 °C in aliquots of 1,100 µl per eppendorf tube.

4. Prepare seven separate 25 or 50 ml stock solutions (see Table 3a and b) in H₂O. Filter-sterilize (0.22 µm) each component after preparation. Afterwards, transfer an aliquot of 50 µl from each separate component into 49,600 µl filter-sterilized water (0.22 µm) and add a volume of 50 µl concentrated HCl solution. Mix the solution well through gentle vortexing and filter-sterilize the solution through a 0.22 µm filter, and store at −20 °C in aliquots of 1,100 µl per eppendorf tube.

5. Gentamicin and Amphotericin B should be omitted from ALI medium. These antibiotics are only required in BEGM medium right after cell isolation to prevent contamination.

6. For preparation of crystal violet solution safety glasses and protective clothing should be worn. Any spillage must be cleaned with 96 % ethanol.

7. Cells might take longer to dissociate from the bottom of the flask due to the collagen coating. If the cells are not dissociated after 3 min additional rounds of 1-min incubations can be performed until all cells have detached.

8. The seeded primary cells should reach confluence on the inserts within 1 week. If this takes longer the success rate of establishing well differentiated HAE cultures declines exponentially.

### Table 3
Stock solutions for trace elements

<table>
<thead>
<tr>
<th>Component</th>
<th>Formula</th>
<th>Amount/25 ml</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Selenium</td>
<td>NaSeO₃</td>
<td>130.0 mg</td>
<td>Solution stable for 30 days at +4 °C</td>
</tr>
<tr>
<td>Silicone</td>
<td>Na₂SiO₃·9H₂O</td>
<td>3.55 g</td>
<td></td>
</tr>
<tr>
<td>Molybdenum</td>
<td>(NH₄)₆Mo₇O₂₄·4H₂O</td>
<td>31.0 mg</td>
<td></td>
</tr>
<tr>
<td>Vanadium</td>
<td>NH₄VO₃</td>
<td>14.75 mg</td>
<td>Heat &gt;100 °C to dissolve</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Component</th>
<th>Formula</th>
<th>Amount/50 ml</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nickel</td>
<td>NiSO₄·6H₂O</td>
<td>13.0 mg</td>
<td></td>
</tr>
<tr>
<td>Tin</td>
<td>SnCl₂·2H₂O</td>
<td>5.5 mg</td>
<td></td>
</tr>
<tr>
<td>Manganese</td>
<td>MnCl₂·4H₂O</td>
<td>10.0 mg</td>
<td></td>
</tr>
</tbody>
</table>
9. Human coronavirus NL63, 229E, HKU1, and OC43 are predominantly found in the upper respiratory tract and are therefore incubated at 33 °C. Both MERS-CoV and SARS-CoV are predominantly found in the lower respiratory tract and are therefore incubated at 37 °C.

10. The fixed HAE cultures can be kept for 1–3 months at 4 °C if the CB is filter-sterilized (0.2 µM) and all the procedure were performed under sterile conditions. After cold storage it is preferential to acclimatize the fixed cultures for 15 min to room temperature on a gyro-rocker (20–30 rpm) prior to continuation of the staining protocol.

11. To prevent bleaching of the fluorophores one should cover the inserts from daylight exposure during each incubation step.

12. Luminometer settings depend on the manufacturer. However, a measurement time of 1–2 s per well has proved effective.

13. For this assay cultures must be infected with coronaviruses expressing a Renilla Luciferase reporter gene.

14. If your luminometer is equipped with an injector you must remember to account for priming by increasing the volume of required Renilla Assay buffer by 2–3 ml.

15. Primers targeting HCoV-NL63, HCoV-HKU1, HCoV-229E, and HCoV-OC43 have been characterized and described [4, 14, 15].

Acknowledgement

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References


Efficient Replication of the Novel Human Betacoronavirus EMC on Primary Human Epithelium Highlights Its Zoonotic Potential

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ABSTRACT The recent emergence of a novel human coronavirus (HCoV-EMC) in the Middle East raised considerable concerns, as it is associated with severe acute pneumonia, renal failure, and fatal outcome and thus resembles the clinical presentation of severe acute respiratory syndrome (SARS) observed in 2002 and 2003. Like SARS-CoV, HCoV-EMC is of zoonotic origin and closely related to bat coronaviruses. The human airway epithelium (HAE) represents the entry point and primary target tissue for respiratory viruses and is highly relevant for assessing the zoonotic potential of emerging respiratory viruses, such as HCoV-EMC. Here, we show that pseudostratified HAE cultures derived from different donors are highly permissive to HCoV-EMC infection, and by using reverse transcription (RT)-PCR and RNAseq data, we experimentally determined the identity of seven HCoV-EMC subgenomic mRNAs. Although the HAE cells were readily responsive to type I and type III interferon (IFN), we observed neither a pronounced inflammatory cytokine nor any detectable IFN responses following HCoV-EMC, SARS-CoV, or HCoV-229E infection, suggesting that innate immune evasion mechanisms and putative IFN antagonists of HCoV-EMC are operational in the new host. Importantly, however, we demonstrate that both type I and type III IFN can efficiently reduce HCoV-EMC replication in HAE cultures, providing a possible treatment option in cases of suspected HCoV-EMC infection.

IMPORTANCE A novel human coronavirus, HCoV-EMC, has recently been described to be associated with severe respiratory tract infection and fatalities, similar to severe acute respiratory syndrome (SARS) observed during the 2002–2003 epidemic. Closely related coronaviruses replicate in bats, suggesting that, like SARS-CoV, HCoV-EMC is of zoonotic origin. Since the animal reservoir and circumstances of zoonotic transmission are yet elusive, it is critically important to assess potential species barriers of HCoV-EMC infection. An important first barrier against invading respiratory pathogens is the epithelium, representing the entry point and primary target tissue of respiratory viruses. We show that human bronchial epithelia are highly susceptible to HCoV-EMC infection. Furthermore, HCoV-EMC, like other coronaviruses, evades innate immune recognition, reflected by the lack of interferon and minimal inflammatory cytokine expression following infection. Importantly, type I and type III interferon treatment can efficiently reduce HCoV-EMC replication in the human airway epithelium, providing a possible avenue for treatment of emerging virus infections.

Coronaviruses are enveloped positive-stranded RNA viruses of veterinary and medical importance that are associated mainly with respiratory and enteric infections (1, 2). Some animal coronaviruses have long been known to cause severe diseases. In humans, however, it was long believed that coronaviruses cause mainly less severe respiratory infections known as the common cold. This changed with the appearance of the severe acute respiratory syndrome-associated coronavirus (SARS-CoV) which caused for the first time a coronavirus-induced life-threatening disease in humans and was responsible for the 2002–2003 epidemic involving more than 8,000 reported cases and almost 800 fatalities (1). The emergence of a novel coronavirus, HCoV-EMC, raises concerns that we may again face an epidemic caused by a zoonotic coronavirus (3). HCoV-EMC is associated with severe respiratory tract infection, renal failure, and fatalities (4, 5) and is, like SARS-CoV, closely related to bat coronaviruses (3). Since the HCoV-EMC animal reservoir and circumstances of zoonotic transmission are yet elusive, it is critically important to assess which barriers of HCoV-EMC host switching and human-to-human transmission are operational.

An important first barrier against invading respiratory pathogens is the respiratory epithelium, which represents the entry
FIG 1  Replication of HCoV-EMC and SARS-CoV on HAE cultures. (A) HAE cultures from three donors (0712, black; 1001, light gray; 1505, dark gray) were prepared as described previously (7) and infected with HCoV-EMC or SARS-CoV (MOI = 0.1). Progeny virus release at the apical (top and bottom) and basolateral (middle) surfaces of HCoV-EMC- or SARS-CoV-infected HAE cultures was determined as genome equivalents (GE) or plaque-forming units (PFU) per ml at the indicated hpi by using quantitative real-time reverse transcription-PCR (qRT-PCR) specific for HCoV-EMC (16) and SARS-CoV (17) or titration of infectious particles on Vero cells. Experiments were performed in triplicate for each donor. Data are depicted as mean values ± standard deviations (SD); nd, not detected. (B) HCoV-EMC- and SARS-CoV-infected (MOI = 0.1) or mock-treated HAE cell cultures were fixed 48 hpi with 6% PFA and immunostained using the procedure as described (18). Rabbit polyclonal antiserum directed against SARS-CoV Nsp3 (green; anti-SARS-CoV antibody; Rockland) and mouse monoclonal antibody directed against dsRNA (red; J2; English & Scientific Consulting Bt.) were used as primary antibodies. Dylight 488-labeled anti-mouse IgG (H+L) and Dylight 647-labeled anti-rabbit IgG (H+L) (Jackson Immunoresearch) were applied as secondary antibodies, followed by two separate incubation steps with Cy3-conjugated mouse anti-β-tubulin antibody (light blue; Sigma) for staining of ciliated cells and DAPI (4′,6-diamidino-2-phenylindole; Invitrogen) for staining nuclei (dark blue). Images were acquired using an EC, Plan-Neofluor 63 ×1.40 oil differential inference contrast (DIC) M27 objective on a Zeiss LSM 710 confocal microscope. Image capture, analysis, and processing were performed using the ZEN 2010 (Zeiss) and Imaris (Bitplane Scientific Software) software packages. Representative images are shown from one (1505) of three donors. (C) Schematic representation of sequence reads of an RNAseq analysis of poly(A)-containing RNA derived from HCoV-EMC-infected HAE cultures (MOI = 1; 6 hpi). Single reads are depicted in green (sense) and red (antisense). The (Continued)
point and primary target tissue of respiratory viruses. To assess the zoonotic potential of HCoV-EMC, it is therefore critically important to determine if the human respiratory epithelium is susceptible to HCoV-EMC infection. To address this question, we used human airway epithelium (HAE) cultures that morphologically and functionally resemble the upper conducting airways in vivo (6). The HAE culture system is based on primary human bronchial epithelial cells obtained by biopsy, brushing, surgery, or lung transplant. Isolated bronchial epithelial cells are manipulated with chemically defined medium to initiate their differentiation into a pseudostratified human airway epithelial cell. When differentiation is complete, the pseudostratified HAE cell layer (i) contains basal, secretory, columnar, and ciliated cell populations and (ii) will generate mucus (6, 7). Therefore, this in vitro system recapitulates many aspects of the human airway epithelium, namely, the presence of well-defined cell types of the human airway epithelium, and physical barriers, such as the mucous layer.

To this end, we have infected fully differentiated HAE cultures derived from three different donors with HCoV-EMC (3, 5) or SARS-CoV (strain Frankfurt-1) at a multiplicity of infection (MOI) of 0.1 and assessed viral growth kinetics. As shown in Fig. 1A, HAE cultures are highly susceptible to HCoV-EMC infection, with peak virus production already at 48 h postinfection (hpi). In contrast, replication of SARS-CoV appeared slower and reached peak virus production later at 72 to 96 hpi (Fig. 1A). For both viruses, the vast majority of progeny virus was released at the apical side of HAE cultures, i.e., toward the mucous layer (Fig. 1A, top and bottom), compared to the lower and not always detectable virus release at the basolateral side, i.e., toward the medium (Fig. 1A, middle). We further analyzed HCoV-EMC and SARS-CoV infection of HAE cultures by immunofluorescence microscopy and stained for ciliated cells (β-tubulin), double-stranded (ds) RNA (as a marker for infected cells), and replicase gene-encoded nonstructural protein 3 (Nsp3; as a marker for coronavirus replicase-transcriptase complexes). As shown in Fig. 1B, HCoV-EMC-infected cells were readily identified with a cross-reacting polyclonal antiserum directed against SARS-CoV Nsp3 or a monoclonal antibody directed against dsRNA, which both visualized the characteristic punctuated perinuclear staining pattern for coronavirus replicase-transcriptase complexes. HCoV-EMC-infected predominantly nonciliated cells, suggesting that the putative receptor for HCoV-EMC host cell entry is likely to be expressed on nonciliated cells of the human bronchial epithelium. We also analyzed intracellular HCoV-EMC-derived mRNAs by using an RNaseq approach. Total RNA from HCoV-EMC-infected HAE cultures was isolated at 6 hpi using Qiagen’s RNeasy kit followed by mRNA subtraction according to the manufacturer’s protocols. RNA-Seq libraries for an indexed Illumina sequencing run were established using ScriptSeq mRNA-Seq library preparation kit (Epiconcept, WI) started from 1 ng mRNA. Quality-verified RNA-Seq libraries were analyzed using Illumina’s HiSeq2500 system according to Illumina’s TrueSeq protocols for single reads (TrueSeq SBS kit version 3-HS; 50 cycles). Data analysis was performed using CLC Genomics workbench 5.5 (CLC bio, Denmark). Before single-read mapping, raw reads were trimmed to eliminate ambiguous or remaining adapter sequences. We used all reads collected from 3 donors in duplicate experiments (total of 6 datasets) that failed to map to the human genome (25,053,494 out of 195,541,919 reads) for an alignment against the published HCoV-EMC genome sequence (GenBank accession no. JX869059.2). A total of 1,616 out of 25,053,494 (0.006%) reads could be assigned to the HCoV-EMC genome, and we observed a genome coverage reflecting the characteristic mRNA replication and transcription pattern expected for the coronavirus nested set of viral mRNAs (Fig. 1C). Indeed, we could identify several reads representing leader-body fusion sequences of predicted HCoV-EMC mRNAs 2, 4, 7, and 8 (Fig. 1D) (3). In addition, we experimentally determined by reverse transcription (RT)-PCR using total RNA from HCoV-EMC-infected HAE cells the leader-body fusion sequences of predicted mRNAs 3, 5, and 6 that were not represented in the RNAseq data (Fig. 1D; see also Table S1 in the supplemental material). Collectively, our data show that the human bronchial epithelium is highly permissive to HCoV-EMC infection and, accordingly, that all cellular factors required for cell entry (e.g., receptor), replication, and transcription of viral mRNAs, virus assembly, and release are available in the human host.

Next we assessed HAE host cell responses to HCoV-EMC infection on the transcriptional level and compared them to responses to SARS-CoV and HCoV-229E infection (MOI = 1). We chose to analyze the expression of a set of 15 cellular mRNAs (see Table S2 in the supplemental material) representing type I IFN, type III IFN, endosomal and cytoplasmic RNA sensor molecules, IFN-stimulated genes (ISGs), chemokines, and inflammatory cytokines, at 3, 6, and 12 h following type I IFN (100 IU of IFN-α) and type III IFN (10 ng/ml of IFN-λ3) (8) treatment or virus infection. As shown in Fig. 2A, HAE cultures respond swiftly to type I and type III IFN treatment with upregulation of ISG expression (i.e., Mx1, 2’,5’-OAS, Stat1, Mda5, Rig-I; Fig. 2A). Notably, the response of HAE cultures to IFN-λ treatment supports previous studies showing high expression of the IFN-λ receptor α-subunit (IFNLR1) in lungs and in epithelial cells (9). In contrast to IFN treatment, the HAE cultures displayed only limited early transcriptional response to coronavirus infection, and particularly, no induction of IFN-β was observed in HCoV-EMC-, SARS-CoV-, and HCoV-229E-infected cells (Fig. 2B). Also the expression of proinflammatory cytokines was only marginally induced, mainly in the common cold virus (HCoV-229E)-infected HAE cultures at 6 hpi. Thus, immediate host responses to HCoV-EMC infection of HAE cultures are very similar to those observed in SARS-CoV- and HCoV-229E-infected cells, suggesting that HCoV-EMC is already well adapted to replication in HAE cultures and that the human bronchial epithelium is not capable to mount a strong innate immune response in the absence of professional cytokine-producing cells, such as plasmacytoid dendritic cells, conventional dendritic cells, and macrophages (2, 10).

Since HAE cultures responded well to type I and type III IFN...
FIG 2. Human coronavirus-host interaction. (A) Gene expression analysis of IFN-treated HAE cultures. HAE cultures derived from three different donors were used untreated or were stimulated from the basolateral side with recombinant IFN-α (100 IU/ml; IFN-na/D human; Sigma) or recombinant IFN-λ3 (10 ng/ml) (8) for 3, 6, and 12 h until total cellular RNA was extracted using RNeasy (Qiagen). Reverse transcription was performed with Moloney murine leukemia virus reverse transcriptase according to the manufacturer’s protocol (Invitrogen) using 1 μg of DNase-treated total RNA. Two microliters of diluted cDNA was amplified according to the manufacturer’s protocol, using primers targeting 15 different mRNA transcripts (see Table S1 in the supplemental material). Measurements and analysis were performed using a LightCycler 480 II instrument and software package (Roche). Cycle profile, 10 min at 95°C; 45 cycles of 10 s at 95°C, 20 s at 55°C, and 20 s at 72°C; followed by a melting curve step to confirm product specificity. Relative gene expression was calculated using the \( \Delta \Delta CT \) method (19) and is shown as fold induction of IFN-treated samples compared to that of untreated controls. (B) Gene expression analysis of virus-infected HAE cultures. HAE cell cultures were infected with HCoV-EMC, SARS-CoV, or HCoV-229E (MOI = 1), and total cellular RNA was isolated at 3, 6, and 12 hpi. Relative gene expression analysis was performed as described above. (C) Analysis of virus replication following IFN pretreatment. HAE cell cultures were left untreated or were treated from the basolateral side for 16 h with recombinant IFN-α (100 IU/ml; Sigma) or recombinant IFN-λ3 (10 ng/ml or 100 ng/ml) (8). The basolateral medium was replaced prior to infection with HCoV-EMC, SARS-CoV, and HCoV-229E (MOI = 0.1). Apical progeny virus release was determined at 48 hpi by qRT-PCR and is given as GE per ml. Each bar represents the mean ± SD from independent experiments performed in duplicate using HAE cultures derived from three different donors. ns, not significant (\( P > 0.05 \)); *, \( P < 0.05 \); **, \( P < 0.01 \) (paired t-test). (D) Immunofluorescence analysis of IFN-treated and virus-infected HAE cultures. HAE cultures were fixed with 6% PFA and immunostained using the procedure as described (18). Mouse monoclonal antibody directed against dsRNA (J2; English & Scientific Consulting Bt.) was applied as primary antibody and Dylight 488-labeled anti-mouse IgG (H+L) as secondary antibody (green; Jackson ImmunoResearch), followed by staining of cilia with Cy3-conjugated mouse anti-β-tubulin antibody (red; Sigma). Images were acquired using an EC, Plan-Neofluor 63×/1.40 oil DIC M27 objective on a Zeiss 710 confocal laser scanning microscope. Image capture, analysis, and processing were performed using the ZEN 2010 (Zeiss) and Imaris (Bitplane Scientific Software) software packages. Representative images are shown from one (0401) of three donors.
treatment, we addressed if these cytokines can reduce replication of HCoV-EMC, SARS-CoV, and HCoV-229E. HAE cultures derived from three different donors were left untreated or pretreated with IFN-α (100 IU) or IFN-α-3 (10 ng/ml and 100 ng/ml) (8) 16 h prior to infection (MOI = 0.1) with HCoV-EMC, SARS-CoV, or HCoV-229E, and acipically released progeny virus genomes were determined by quantitative RT-PCR (qRT-PCR) at 48 hpi. As shown in Fig. 2C, pretreatment of HAE cultures with IFN-α reduced replication of HCoV-EMC and HCoV-229E for all three different donors and reduced replication of SARS-CoV for two of these donors. Accordingly, we observed a pronounced reduction of the number of dsRNA-positive cells in IFN-α-treated HAE cultures that had been infected with HCoV-EMC, SARS-CoV, or HCoV-229E (Fig. 2D). Notably, pretreatment of HAE cultures with IFN-α also reduced replication of HCoV-EMC, SARS-CoV, and HCoV-229E for all three donors at both concentrations used (10 ng/ml and 100 ng/ml). Like for IFN-α treatment, we observed a pronounced reduction of the number of dsRNA-positive cells in IFN-α-treated and virus-infected HAE cultures, further corroborating the importance of type III IFN in epithelial antiviral host defense (9, 11).

In summary, we provide here conclusive evidence that the novel coronavirus HCoV-EMC can productively infect human bronchial epithelia cultures, suggesting that all necessary host cell factors for virus entry, RNA synthesis, and virus assembly and release are available in the human host. HCoV-EMC replication in HAE cultures was at least as efficient as replication of SARS-CoV (this study) and HCoV-229E (12). We conclude that HCoV-EMC is capable of infecting the primary target tissue, the human respiratory epithelium, which is in accordance to the reported clinical presentation of severe respiratory symptoms (4, 5). HCoV-EMC has been suggested to have a zoonotic origin, since closely related coronaviruses are known to replicate in bats. Considering that there is not yet any study reporting the successful isolation of a bat coronavirus, HCoV-EMC differs compared to known bat coronaviruses because it displays broad replication capability in diverse mammalian cell lines (13). Our data show that the highly pathogenic viruses HCoV-EMC and SARS-CoV can both replicate in HAE cultures similar to the common cold viruses HCoV-229E, HCoV-OC43, HCoV-NL63, and HCoV-HKU1 (12). Also, early host cell responses are very similar between high- and low-pathogenic human coronaviruses. Thus, although our data depict the zoonotic potential of HCoV-EMC by demonstrating efficient replication in the human respiratory epithelium, decisive factors that impact HCoV-EMC pathogenicity remain to be determined. Importantly, we could demonstrate that HCoV-EMC replication is equally vulnerable to the antiviral effects of type I and type III IFNs, suggesting a possibility to interfere with HCoV-EMC replication in the human respiratory tract. IFN-α treatment has indeed been explored as therapeutic strategy during the SARS epidemic and raised considerable promise (14). The critical importance of type III IFNs in epithelial host defense (11), recent reports that treatment of hepatitis C virus-infected patients with pegylated IFN-α achieved rapid virological response, while adverse side effects were minimal (15), and our data concerning efficient inhibition of HCoV-EMC replication should encourage the further development of IFN-α treatment options specifically for respiratory virus and emerging virus infections.

SUPPLEMENTAL MATERIAL
Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00611-12/-/DCSupplemental.

Table S1, DOCX file, 0.1 MB.

Table S2, DOCX file, 0.1 MB.

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Human bronchial epithelial cells were isolated from patients (>18 years old) who underwent bronchoscopy and/or surgical lung resection in their diagnostic pathway for any pulmonary disease and that gave informed consent. This was done in accordance with local regulation of the Kanton St. Gallen, Switzerland, as part of the St. Gallen Lung Biopsy Biobank (SGLBB) of the Kantonal Hospital, St. Gallen, which received approval by the ethics committee of the Kanton St. Gallen (EKSG 11/044, EKSG 11/103).

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