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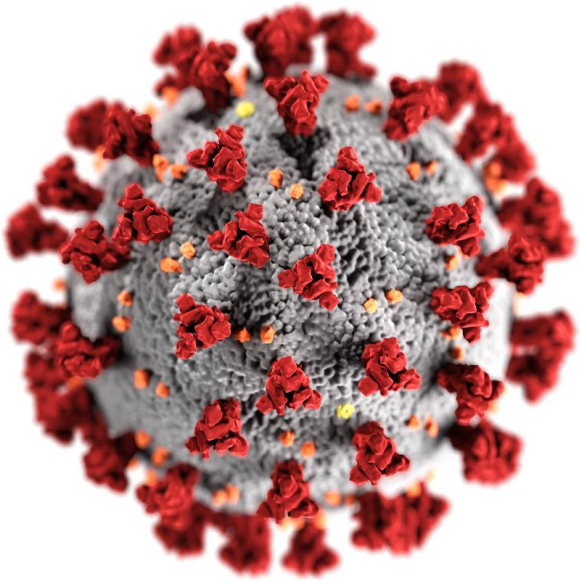
THESIS

The impact of SARS-CoV-2 on nasal respiratory epithelial cell differentiation



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PATHOLOGY AND MEDICAL BIOLOGY RESEARCH

The impact of SARS-CoV-2 on nasal respiratory epithelial cell differentiation

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Abstract

Since December 2019 there has been a global outbreak of the coronavirus severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). SARS-CoV-2 infection varies from patients being asymptomatic to experiencing severe respiratory destress and/or multi organ failure. Generally, the lower airways are marked as SARS-CoV-2' main target for infection, replication and transmission, leading to the nasal respiratory epithelium (NRE) to be overlooked. However, the NRE could also be a main target site since this area is in closest contact to the outside world and therefore has great potential for viral transmission. Hence, it is of interest to examine the viral tropism of SARS-CoV-2 within the NRE and its pathogenesis. Numerous studies showed high expression of entry-related host factors and the detection of the virus or its related content in the ciliated and secretory cells. Once SARS-CoV-2 infects these cells, it may exert several effects on cellular morphology and physiology. Research showed a significant increase in transcripts in clusters of basal- and secretory cells and dedifferentiation of ciliated cells. The dedifferentiation leads to loss and abnormal structures of cilia, resulting in reduced mucociliary clearance. Moreover, the cellular composition of the NRE showed significant differences of certain cell populations between healthy controls and Coronavirus Disease-2019 (COVID-19) patients. Arguably, indicating critical amounts of cell death of ciliated and secretory cells. In conclusion, SARS-CoV-2 specifically targets the ciliated and secretory cells of the NRE and is by means of different mechanisms able to alter their differentiation and cellular survival.

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Introduction

Two years since the first report of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) cases in the Wuhan province in China, over 300 million cases have been confirmed and almost 5.5 million people have died globally as of January 11, 2022 (*Situation by Region, Country, Territory & Area*, 2022). Worldwide, countries try to manage the viral spread by encouraging self-distancing and vaccination, promoting contact tracing and requiring people to wear a mask in public. The disease caused by SARS-CoV-2 is named Coronavirus Disease-2019 (COVID-19). People with COVID-19 show clinical features ranging from asymptomatic state to acute respiratory distress syndrome and multi organ dysfunction. The common clinical features include fever, cough, sore throat, olfactory dysfunction, fatigue, headache, myalgia and breathlessness (Raoult et al., 2020; Singhal, 2020). The virus is transmitted through large droplets generated during coughing and sneezing by symptomatic patients, but can also occur from asymptomatic people and before onset of symptoms (Rothe et al., 2020). Fortunately, there is an increasing number of promising results being published. The number of deaths due to COVID-19 is decreasing (*Global Situation*, 2022) and the fact that most people on the intensive care are unvaccinated, suggests that the used vaccines are indeed effective (*4 in 5 COVID-19 Patients in ICU Are Not Vaccinated*, 2021; *Wöchentlicher Lagebericht Zu COVID-19*, 2021)

Since the discovery of SARS-CoV-2, numerous studies have been done on its molecular biology and pathogenesis. These studies generally focussed on the lower respiratory tract since this is the site with high levels of viral entry, inflammation and tissue damage (Chu et al., 2020; Singhal, 2020). Most patients with COVID-19, however, only have mild to moderate symptoms. These pre-symptomatic or mildly symptomatic patients have a high viral load of SARS-CoV-2 in samples taken by nasal swab and show high transmissibility of the virus through respiratory droplets or aerosols (Gandhi et al., 2020; He et al., 2020). Nasal tissue mostly consists of pseudostratified ciliated epithelium, also called respiratory epithelium, that is interspersed with mucus-secreting goblet cells (Harkema et al., 2006). This epithelium covers the majority of the nasal cavity and forms a thin layer of mucus with active ciliary movement that ultimately traps and removes pathogens, also called mucociliary clearance (Bustamante-Marin & Ostrowski, 2017; Haschek et al., 2002).

Although the nasal respiratory epithelium (NRE) serves as a front line in respiratory defence against lower respiratory tract infections, it also could be a target for viral infection, replication and transmission (Fodoulian et al., 2020; Gengler et al., 2020; Hewitt & Lloyd, 2021). Subsequently, if the NRE get infected, then it is important to determine the effects of SARS-CoV-2 infection and replication on the epithelium. In general, only the cells that express the necessary entry-related host factors are able to get infected. The corresponding pathological effects, however, are most likely not restricted to the infected cells. These affects possibly include altered cell differentiation, which may lead to impaired tissue functioning and altered epithelium regeneration. All things considered, it is of high interest to investigate the viral tropism of SARS-CoV-2 within the NRE and to determine the associated impact on functionality, differentiation and cellular survival.

SARS-CoV-2

Coronaviruses (CoVs) consist of a highly diverse family that is not only able to infect humans, but also other mammals and avian species. The most recently discovered CoV was identified in December 2019 as SARS-CoV-2. Genetic analysis showed that SARS-CoV-2 is closest related to a β-coronavirus found in bats in China. Furthermore, it is the seventh CoV known to infect humans, and with the severe acute respiratory syndrome (SARS) and the Middle East respiratory syndrome (MERS), the only CoVs that can cause fatal respiratory tract infections (Gorbalenya et al., 2020; Munster et al., 2020). The origin of SARS-CoV-2 is still debated. The most closely related viruses originate from bats with 96.2% sequence homology at the whole-genome level to the bat CoV RaTG13 (Zhou et al., 2020). CoVs are enveloped viruses with a positive-sense single stranded RNA genomes ranging from 26–30 kb (Cui et al., 2019; Orhan & Senol Deniz, 2020). The SARS-CoV-2 genome contains 14 open reading frames (ORFs) encoding for 27 proteins. Nearly 70% codes for 15 non-structural proteins (nsps), the remaining ORFs encode for four structural proteins; membrane (M), envelope (E), spike (S) and nucleocapsid (N) proteins and eight accessories' proteins (Wu et al., 2020). The S protein comprises two subunits: S1 and S2. S1 holds the receptor-binding domain (RBD) while S2 is needed for fusion of the virus with the cellular membrane of the host (V'kovski et al., 2021). At the inner side of the virion, the N proteins are bound to the positive-sense single-stranded RNA genome to stabilize the RNA (Bakhiet & Taurin, 2021; Chang et al., 2014).

Variants

Viruses are microorganism that are known for their ability to continuously evolve due to mutations during replication. Mutations are an intrinsic characteristic of all viruses, which occurs in DNA as well as RNA viruses. Nevertheless, RNA viruses present higher mutation rates than DNA viruses. Replication of RNA viruses

involves an intrinsically error-prone RNA polymerase, resulting in the introduction of mutations in their genome in each step of the copying cycle. These replication cycles are able to occur within hours, ensuring the generation of a diverse virus population just within one infected host (Almubaid & Al-Mubaid, 2021). CoVs are in possession of enzymes that remove erroneous mutagenic nucleotides incorporated by RNA polymerases. Consequently, generating less mutations in their replication cycles than other RNA viruses, which leads to a relatively elevated replication accuracy preservation and virus transcription (Ferron et al., 2017). Usually, mutations that cause a disadvantage concerning transmission, viral replication or immunity escape, will escalate in frequency with the consequence of reduction of its efficiency. Virus development is extremely unpredictable, specifically because mutation frequency can randomly increase or decrease. Most RNA viruses show considerable potential to adapt to new habitats and hosts, and to withstand the impact of various selective pressures. The extensive progression of the SARS-CoV-2 pandemic gave viruses the opportunity to come across a diverse host genetic varieties and a wide variety of distinct cellular microenvironments. Especially these factors provided the right circumstances for increased mutation rates that will influences SARS-CoV-2 virulence, transmissibility and/or pathogenesis (Chakraborty, 2021; Janik et al., 2021). Currently, various SARS-CoV-2 variants have been documented worldwide during the COVID-19 pandemic. Five variants have rapidly become a great concern in many countries. They possess mutations of interest and provide evidence of international spreading: alpha (B.1.1.7 and Q lineages), beta (B.1.351 and descendent lineages), gamma (P.1 and descendent lineages), delta (B.1.617.2 AY lineages) and omicron (B.1.1.529 and BA lineages)(O'Toole et al., 2021; *SARS-CoV-2 Variant Classifications and Definitions*, 2021). All these variants share one specific mutation called D614G. Early on in the COVID-19 pandemic the D614G mutation became the global dominant variant. The D614G mutation results in a replacement of aspartic acid with glycine at position 614 of the S glycoprotein. Due to the role of the S protein in viral entry, mutations in this protein can drastically reduce or enhance infectivity and transmissibility (Hou, Chiba, et al., 2020; Volz et al., 2021).

Life cycle

SARS-CoV-2 enters the host cell by direct fusion of the viral envelope with the host cell membrane, or through membrane fusion after endocytosis. Both pathways are initiated by binding of the RBD of the S protein to the human host cell receptors at the cell surface (W. Li et al., 2003; Wan et al., 2020). The major host receptor that binds to the RBD domain of the S protein is angiotensin-converting enzyme 2 (ACE2)(W. Li et al., 2003). Consequently, expression and tissue distribution of entry receptors influence viral tropism and pathogenicity (V'kovski et al., 2021). The RDB-receptor interaction triggers the S protein to undergo proteolytic cleavage. If this is initiated, then one or several of the multiple ways for the S protein to be cleaved will get activated. Certain cellular proteases, predominantly furin, are able to cleave at the S1/S2 site and other proteases, mainly TMPRSS2, at the S2' site of the S protein on the cell surface of host cells (Bestle et al., 2020). This cleavage modifies the conformation of the cleaved S proteins irreversibly and allows the S2 subunit to insert into the host membrane and guide the fusion of the viral and cellular membranes (Peng et al., 2021). Once fused, viral RNA gets released into the host cytoplasm where it utilizes the host and its own machinery to replicate its genetic material and assemble new viral particles (Hoffmann et al., 2020). First, the RNA replicase-transcriptase complex (RTC) gets formed to produce both genomic and sub-genomic RNAs, the latter which serves are mRNA for structural proteins such as the E, N, M, and S proteins that are produced through discontinuous transcription. Both RNAs are produced through negative-sense intermediates via the RNA-dependent RNA polymerase (RdRp). Subsequently, the viral nucleocapsids get assembled with N-protein encapsidated genomic RNA in the cytoplasm. The assembled viral nucleocapsid buds into the lumen of the endoplasmic reticulum-Golgi intermediate compartment and the completed, mature virion is released from the infected cell through exocytosis (Alturki et al., 2020; Fehr & Perlman, 2015) (Fig. 1).

Viral entry of SARS-CoV-2 through direct fusion, the primary pathway, has some differences compared to membrane fusion after endocytosis, the alternative pathway. The primary pathway uses proteolytic enzymes, mainly TMPRSS2, which is present at the cell surface; meaning that TMPRSS2-mediated S protein activation occurs at the plasma membrane, whereas activation of the alternative pathway occurs in the endolysosome, in the cytoplasm after endocytosis (Jackson et al., 2022; Zhang et al., 2021). Some of the most important proteases of the alternative pathway are the endosomal cysteine proteases Cathepsin B (Cat B) and Cathepsin L (Cat L) (Cat B/L) (Simmons et al., 2005). Another study investigated whether there is a difference between Cat B' and Cat L' efficacy in priming the S protein in the lysosome. Here they showed no marked effects on viral entry in cells that received Cat B inhibitor treatment, but they did detect a significant decrease of viral entry after Cat L inhibition treatment. Indicating, the importance of Cat L on S protein priming in the lysosome (Ou et al., 2020). Notably, SARS-CoV-2 entry relies rather on TMPRSS2 than Cat B/L co-expression. *In vitro* testing even showed that TMPRSS2 expression dictates the used entry pathway of SARS-CoV-2 to infect host cells (Koch et al., 2021). Moreover, TMPRSS2 expression proved to be essential

in SARS-CoV-2 entry in lung cell lines and primary lung cells (Hoffmann et al., 2020; Shang et al., 2020). Thus, based on these results it can be assumed that ACE2 and TMPRSS2 are the most crucial factors in the viral tropism of SARS-CoV-2, and are possible host targets for blocking the viral entry.

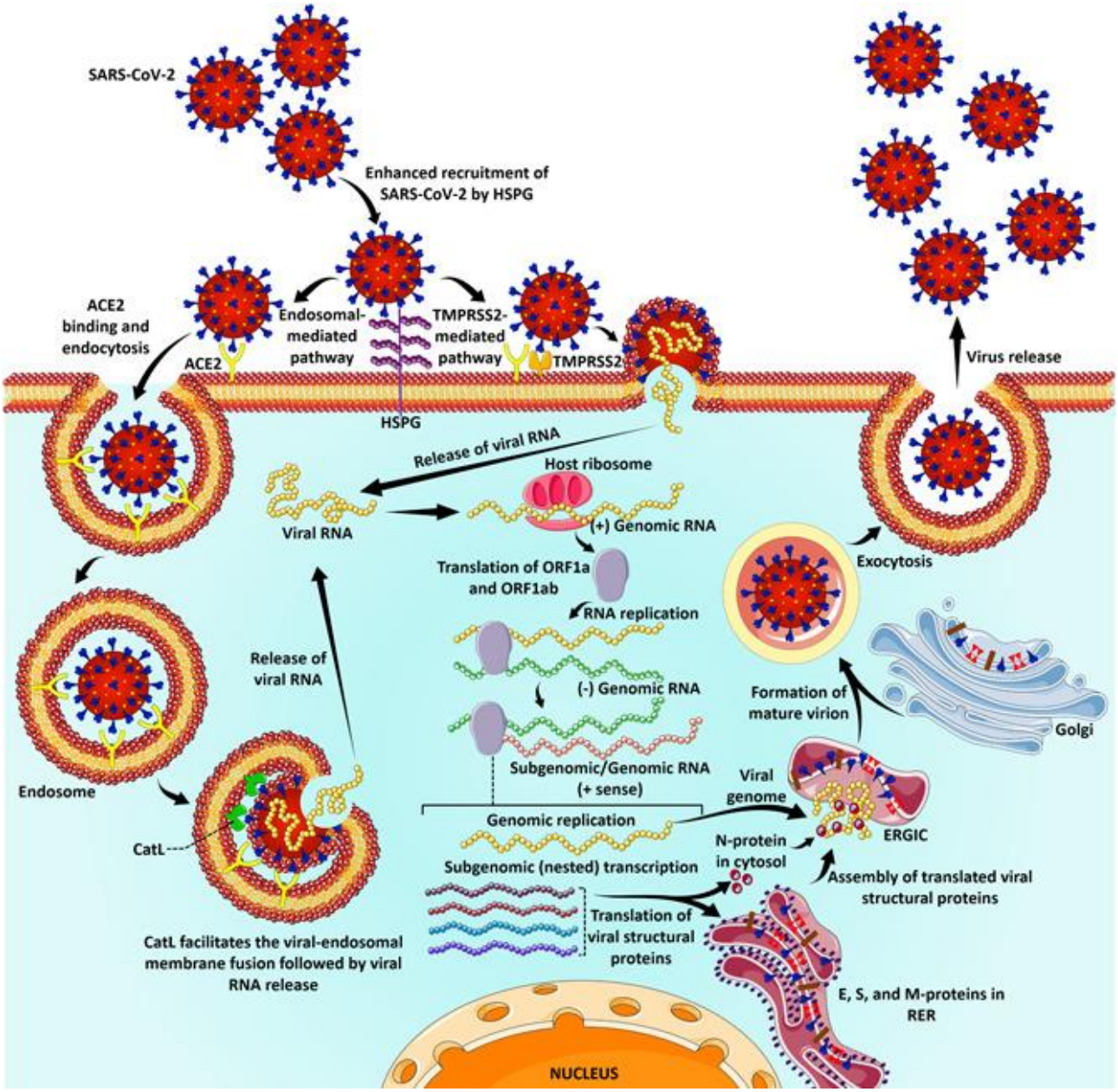

Figure 1. SARS-CoV-2 life cycle. *SARS-CoV-2 S protein binding to the ACE2 receptor initiates viral entry of the host cell through direct fusion or membrane fusion after endocytosis. Both TMPRSS2-mediated and Cat L-mediated S protein activation leads to the release of viral ssRNA. By its own machinery, the RNA is transcribed and replicated forming genomic and sub-genomic RNAs. The sub-genomic RNAs gets translated and the resulting proteins assemble with the genomic RNA to form a new virion. These virions get released by exocytosis* (Muralidar et al., 2021).

Pathophysiology

SARS-CoV-2 is a cytopathic virus that kills the cells and damages the host tissue as part of its replication cycle. Cell death following viral infection has been associated with pyroptosis, apoptosis, necroptosis (all three combined is called PANoptosis) and autophagy (S. Li et al., 2020; Yap et al., 2020). Some of these programmed cell deaths can trigger the release of pro-inflammatory cytokines. Previous studies have reported this release in patients infected with SARS-CoV-2. The secretion of cytokines and chemokines induces inflammation and recruits monocytes and macrophages, which release cytokines to prime the adaptive immune response. In most patients, immune cells' recruitment will eventually clear the virus, and inflammation will recede. However, in some patients with a weak immune system and/or other pre-existing

conditions, an uncontrolled inflammatory response linked to a cytokine storm has been associated with high severity of the pulmonary inflammation (Li et al., 2020).

ACE2 is widely expressed in cells of the respiratory system, mainly alveolar epithelial type II cells (AEC2), which are critical for the gas exchange function by preventing the alveoli from collapsing (Chu et al., 2020; Silverthorn, 2019) and the respiratory epithelium (Sungnak et al., 2020), but also in extrapulmonary tissues including intestines, liver, heart, vascular endothelium, kidneys, urinary bladder and testis (Cheng et al., 2007; Zou et al., 2020). Therefore, it is likely that the pathological effects will not only occur in the respiratory tract, but also in several other organs of the body. Increasing SARS-CoV-2' pathogenicity.

Viral tropism of SARS-CoV-2 in the nasal epithelium

One of the main targets of the upper respiratory tract of SARS-CoV-2 infection is nasal tissue. This is supported by the study of Nakayama and co-workers, where they demonstrated that in individuals with COVID-19, SARS-CoV-2 infects and propagates within multiple regions of the proximal respiratory tract, but the highest signals could be detected in nasal and tracheal epithelia (Nakayama et al., 2021). The nasal epithelium is located in the nasal cavity and can be divided into five distinct surface populations. These include the squamous, respiratory, olfactory, transitional and lymphoepithelial epithelium. Each type of epithelium covers a different area within the nasal cavity to perform its specialised and required functions for proper tissue functioning (Harkema et al., 2006; Herbert et al., 2018).

The majority of the nasal cavity is covered by respiratory- and olfactory epithelium, of which the respiratory epithelium is most prominent. The olfactory epithelium, the sheet of neurons and supporting cells that line approximately half of the nasal cavities, is the main site of transduction of olfactory information. The olfactory epithelium contains mucus-secreting glands and three distinct cell types: olfactory receptor neurons, sustentacular (supporting) cells and basal cells (stem cells) (Purves et al., 2001). The respiratory epithelium is a pseudostratified epithelium that is the first-line of defence against environmental stimuli such as cigarette smoke, allergens and microbes. It consists of six morphologically distinct cell types: ciliated columnar, non-ciliated columnar, goblet (secretory), brush, cuboidal, and basal cells (Fig.2) (Monteiro-Riviere & Popp, 1984; Uraih&Maronpot, 1990). Fully differentiated ciliated cells each possess over 200 motile cilia that beat in a coordinated, directional manner to propel inhaled contaminants trapped by the mucus layer out of the respiratory tract. This makes the mucociliary clearance process essential to maintain respiratory health and prevent infection (Bustamante-Marin & Ostrowski, 2017; Haschek et al., 2002). The basal cells are present in both respiratory and olfactory epithelia and function as the stem/progenitor cells, responding to cell decrepitude and injury by generating precursor/intermediate undifferentiated cells. These undifferentiated precursor cells are able to differentiate into epithelium cells, including ciliated and secretory cells. Therefore, strongly contribute to airway homeostasis (Rock et al., 2010; Tilley et al., 2015).

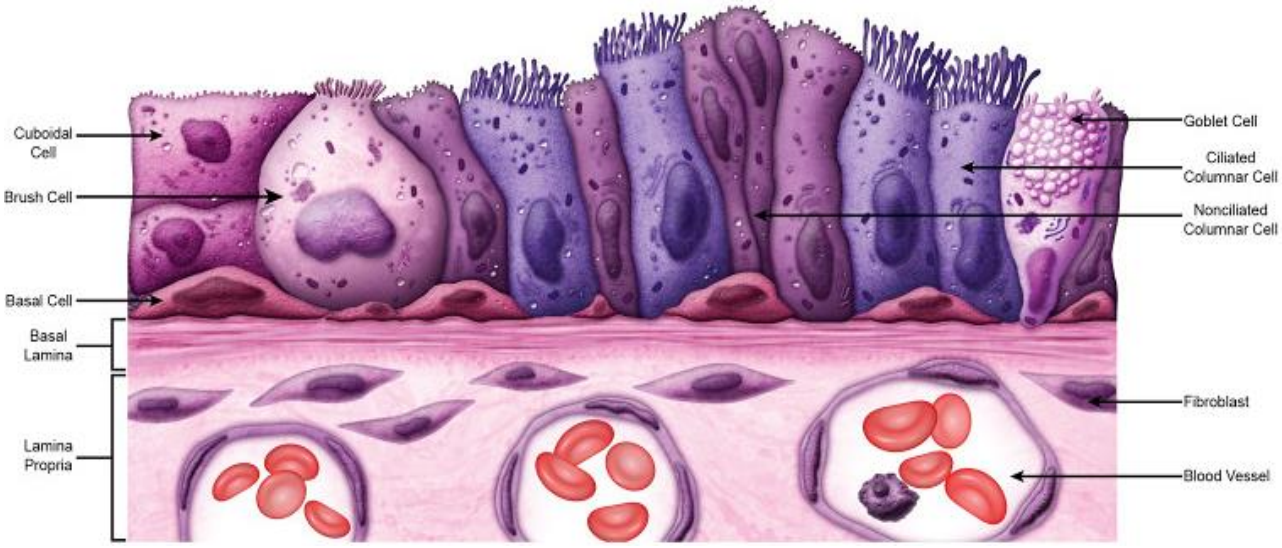

Figure 2. Illustration of the nasal respiratory mucosa. *The six different cell types of the NRE presented on top of the basal lamina and the lamina propria* (Diambra et al., 2021; Herbert et al., 2018).

Expression of SARS-CoV-2 entry–related host factors in the NRE

Viral entry of SARS-CoV-2 mostly relies on the affinity of the S protein and the ACE2 receptor, TMPRSS2 protease activity and their expression levels on the surface of the host cell (Hoffmann et al., 2020). This makes them essential indicators of infection susceptibility. Gene expression of *ACE2* and *TMPRSS2* has been

reported to occur largely in AEC2 cells (Chu et al., 2020; Zou et al., 2020). Nevertheless, other cells of the respiratory tract also express these proteins. In 2004, the study of Hamming and colleagues did not detect *ACE2* expression on the surface of epithelial cells from the upper respiratory tract (Hamming et al., 2004). However, in 2012 another study was published that had detected ACE2 and TMPRSS2 positive cells in the nasal respiratory- and transitional epithelium by means of immunohistochemistry (Bertram et al., 2012). Other studies confirmed this, but also reported that none of the analysed cells expressed high levels of *ACE2* (Zou et al., 2020). To clarify the expression patterns, the study of Sungnak and colleagues analysed their expression and the expression of other genes potentially associated with SARS-CoV-2 pathogenesis at cellular resolution, using single-cell RNA sequencing (scRNA-seq) with datasets from healthy donors. Here they determined that *ACE2* and *TMPRSS2* were expressed across multiple tissues, e.g., lung, ileum, colon, gallbladder, kidney and prostate. Overall, the expression of *TMPRSS2* was higher and broader distributed. Suggesting that for initial infection, ACE2 rather than TMPRSS2, may be a limiting factor for viral entry. Some of the ACE2 and/or TMPRSS2 positive cells are part of the lung and airway epithelium. Despite low level of overall expression, *ACE2* was expressed in multiple epithelial cell types across the airway, as well as in AEC2 cells in the parenchyma. Notably, the ciliated and secretory nasal epithelial cells show the highest *ACE2* expression among all examined cells of the respiratory system, and are complemented with relatively high expression levels of *TMPRSS2* (Fig.3). To validate their findings, they performed the same analysis with datasets from two different studies (Deprez et al., 2019; Vieira Braga et al., 2019). The following results were consistent with the previously found enriched *ACE2* expression in nasal ciliated and secretory cells. Subsequently, all *ACE2* positive cells were analysed on co-expression. Only a small subset of *ACE2* positive cells showed co-expression of *TMPRSS2*, wherefrom most were fully differentiated ciliated cells. These results imply that the virus might use an alternative pathway, such as membrane fusion after endocytosis by Cat L, to enter the cells (Sungnak et al., 2020). Remarkably, the study of Ziegler and colleagues presented results that contradict the findings by Sungnak and co-workers. They obtained samples from the ethmoid sinus and inferior turbinate and found low levels of *ACE2* and *TMPRSS2* in ciliated cells and high levels in the secretory cells, specifically the goblet cells (Ziegler et al., 2020).

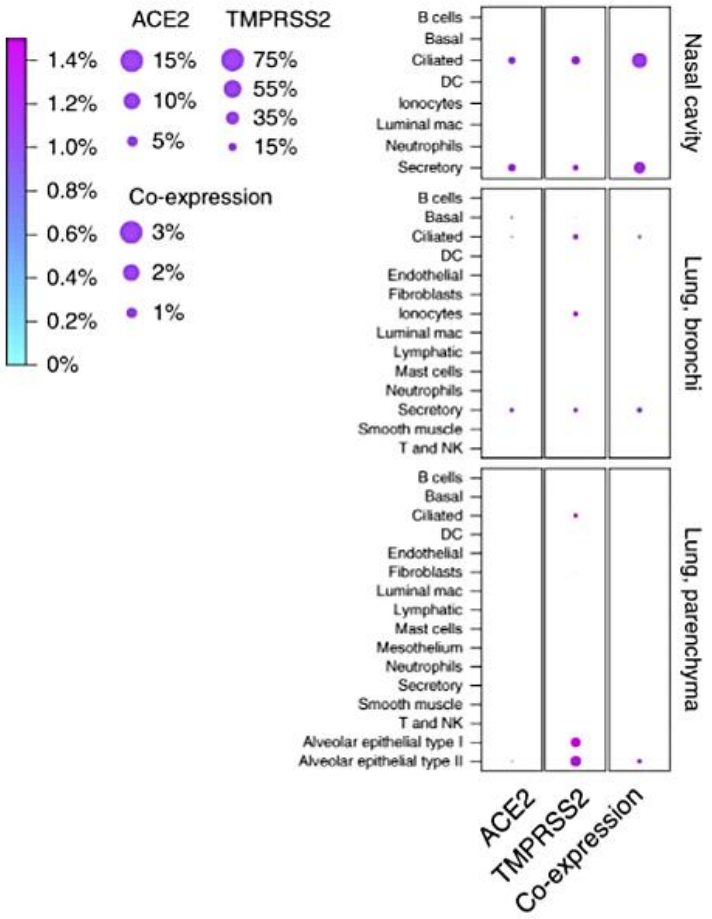

Figure 3. Expression of *ACE2* and *TMPRSS2* across tissues from the respiratory tract. *Enrichment of ACE2 and TMPRSS2 expression in nasal ciliated and secretory cells. These cells have relatively high levels of co-expression in comparison with the rest of the cells of the respiratory tract* (Sungnak et al., 2020).

Besides the cells of the respiratory epithelium, there are multiple cells in the olfactory epithelium that also express the entry factors ACE2 and TMPRSS2. These cells are non-neuronal cells, including sustentacular, basal and perivascular cells. No expression has been detected in the olfactory sensory neurons (Brann et al., 2020; Fodoulian et al., 2020). Viral entry in the olfactory epithelium is confirmed by Meinhardt and colleagues, they showed the presence of intact CoV particles and a relatively high amount of viral RNA of SARS-CoV-2 in the olfactory mucosa (Meinhardt et al., 2021). Interestingly, another study used a mouse model to obtain more information about *ACE2* and *TMPRSS2* expression. Here they confirmed the high levels of *ACE2* and *TMPRSS2* expression in sustentacular cells and showed that in mice, with older age, the amount of ACE2 protein increases in the olfactory epithelium, as does the amount of TMPRSS2 (Bilinska et al., 2020).

Although cellular tropism of SARS-CoV-2 has been described based on detection of mRNA using scRNA-Seq or *in situ* mapping, transcript levels in an isolated single cell do not fully reflect the real-world expression and cellular localization of the protein of interest in a tissue (Liu et al., 2016). Likewise, in host-virus interactions, the presence of some viral mRNA in a cell does not necessarily equal viral replication. Therefore, it is of interest to not only examine the mRNA- but also the protein levels. In 2021, the study of Ahn and colleagues delineated the localization of SARS-CoV-2 entry–related host factors and their relative expression in the NRE by combining immunofluorescence staining (IFS) and scRNA-Seq. In addition, in patients with COVID-19 the same methods were used to define the cellular tropism of SARS-CoV-2 in the NRE. First, they examined the localization and relative levels of ACE2, TMPRSS2, and furin in nasal respiratory epithelia obtained from 6 patients with pituitary adenoma during transnasal dissection surgery by means of IFS. The signal intensity of ACE2, TMPRSS2 and furin in the ciliated cells was relatively high and most notably, the signal was higher at the apical versus the basal side of the epithelium, whereas it was not present at motile cilia themselves (Fig. 4A-C). This confirms previous findings where they also detected a relatively abundant presence of ACE2 on the apical side of the membrane of the cells of the respiratory epithelium (Jia et al., 2005). Unlike the results of the ciliated cells, there was no distinct detection of ACE2, TMPRSS2 and furin in the goblet cells (Fig. 4D-F). Secondly, to validate these findings they also executed IFS in respiratory epithelia and lung tissues collected from two macaque monkeys. Consistent with previous reports, ACE2 and TMPRSS2 were highly and selectively present in the ciliated cells of the nasal mucosa, bronchus and bronchioles, but rarely or never detected in goblet cells. Next, they also validated whether the protein expression levels matched previously published detectable levels of mRNA since several studies already analysed tissue infection susceptibility by means of scRNA-Seq analysis and *in situ* mapping (Sungnak et al., 2020; Ziegler et al., 2020; Zou et al., 2020). However, the reported mRNA data did not match their IFS results. To clarify this mismatch, they performed scRNA-Seq analysis of the NRE from the patients whose tissues were used for the IFS study. Only 1.8% of the respiratory epithelium had a detectable level of *ACE2* mRNA transcript, whereas only a small number of the *ACE2* positive cells showed detectable levels of *TMPRSS2* and *FURIN*. Finally, they compared the mRNA expression levels among the respiratory epithelial cell clusters: basal, suprabasal, club, goblet and ciliated cells (Fig. 5). The overall expression of *ACE2* was low in all epithelial cell types, but showed relatively high expression in ciliated cells. *TMPRSS2* expression was relatively high in both ciliated and goblet cells, and *FURIN* expression was relatively high in secretory cells, but remarkedly low in ciliated cells. To verify these results another similar analysis was performed with a public scRNA-Seq dataset of human nasal tissues. Only 2.7% of the NRE had detectable levels of *ACE2* mRNA, and its expression was relatively low in all cell types, which was consistent with their own results. Moreover, similar patterns were identified for *TMPRSS2* mRNA and *FURIN* mRNA in the respiratory epithelial cell clusters. Thus, based on the findings of this research, it can be concluded that the mRNA levels do not correlate with the protein levels or cellular localization of the SARS-CoV-2 entry–related host proteins in the NRE (Ahn et al., 2021).

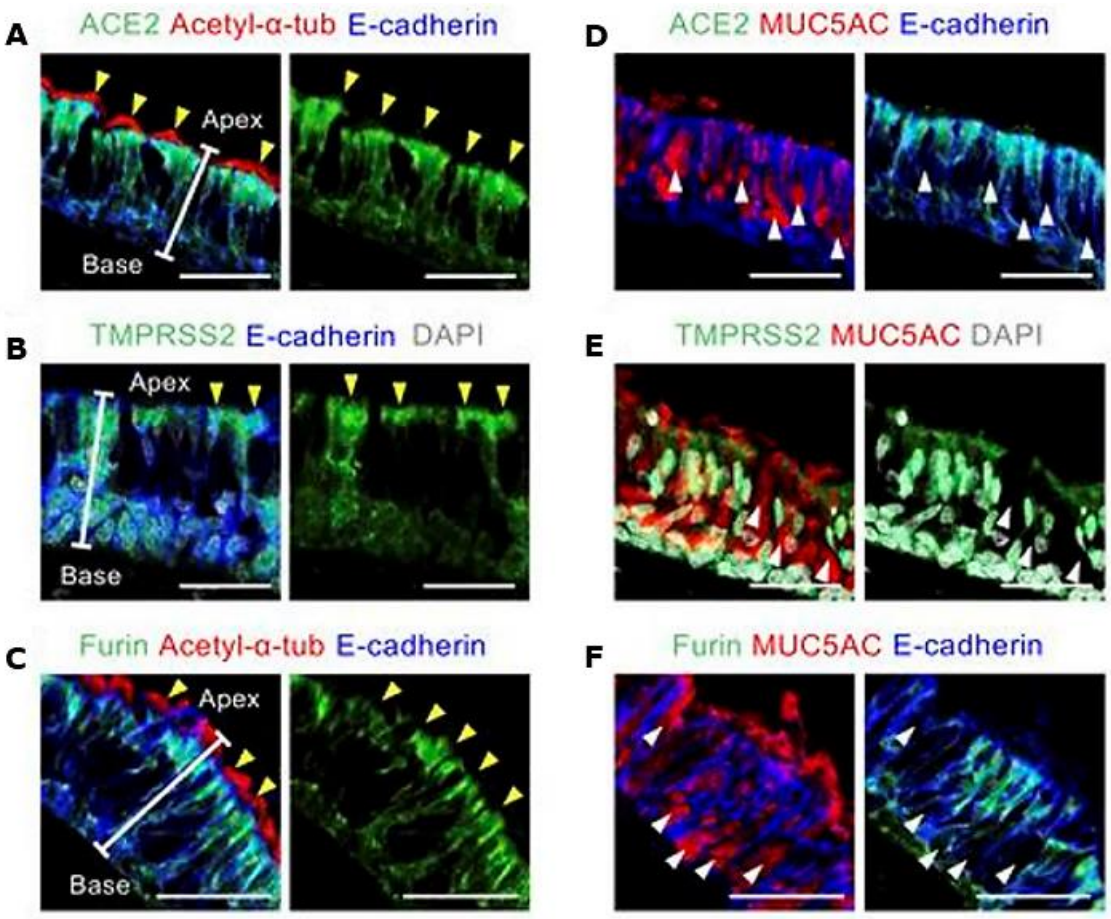

Figure 4. Protein expression pattern of SARS-CoV-2 entry molecules in the NRE by means of IFS. *A-C) Representative images of cross-sectional view of nasal epithelium showing ACE2, TMPRSS2, and furin protein expression in acetylated α-tubulin⁺ ciliated epithelium (yellow arrowheads). Ciliated cells show high protein expression of all three entry molecules. D-F) Representative images of cross-sectional views of nasal epithelium showing ACE2, TMPRSS2, and furin protein expression in MUC5AC⁺ goblet cells (white arrowheads). The entry molecules were not distinctly detected in the goblet cells. Scale bars: 50 μm* (Ahn et al., 2021).

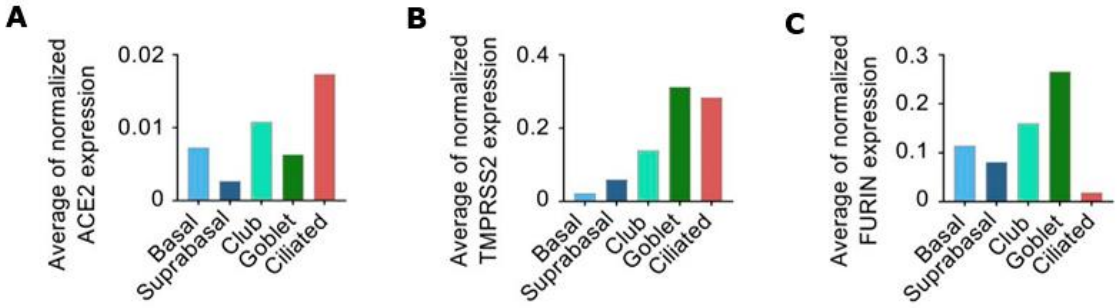

Figure 5. Normalised expression levels of each SARS-CoV-2 entry–related host molecule gene. *Comparison of the average normalized mRNA expression level of ACE2 (A), TMPRSS2 (B) and FURIN (C), per each indicated NRE cell cluster* (Ahn et al., 2021).

Ciliated and secretory cells as the main targets for SARS-CoV-2 infection

By analysing which cell types of the NRE express the highest levels of SARS-CoV-2 entry-related host factors, a substantiated indication of the main target cells can be obtained. This is, however, an indication and to verify these findings more direct analysis of SARS-CoV-2–infected cells is required. Numerous studies investigated the presence of SARS-CoV-2 viruses or its related content, e.g., viral RNA, S protein and N protein in cells of the NRE by using scRNA-Seq, IFS and even transmission electron microscopy (TEM) and/or laser scanning confocal microscopy. Multiple reports showed relatively high quantities of SARS-CoV-2 or its related particles inside both ciliated and secretory cells and stated them both as main target cells for SARS-

CoV-2 infection (Fiege et al., 2021; Pizzorno et al., 2020; Zhu, Wang, et al., 2020). Nevertheless, other reports stated that even though both cell types get infected, SARS-CoV-2 showed preferential targeting of ciliated cells and low viral levels in the secretory cells (Ahn et al., 2021; Robinot et al., 2021). Two reports could not find any evidence that secretory cells were infected (Hou, Okuda, et al., 2020; Zhu, Zhang, et al., 2020). Overall, most reports used the same analytical techniques, suggesting there is a variation in results due to the use of different samples or difference in interpretation of the relative quantities of the virus in both cell types. In summary, these findings arguably state that both ciliated and secretory cells are the main target cells of SARS-CoV-2 of the NRE.

The impact of SARS-CoV-2 on the NRE

When cells get infected by a cytocidal virus, such as SARS-CoV-2, then the production of new infectious viruses kills the cell. This type of infection is usually associated with alterations in cell morphology, physiology and sequential biosynthetic events. Examples are the rounding of the infected cell, fusion with adjacent cells, DNA damage and changes in transcriptional activity, cellular metabolism and antigenic or immune properties. Many of these changes are necessary for efficient virus replication (Albrecht et al., 1996; Pant et al., 2021). After enormous quantities of virions are produced, host cell death will be induced. In addition, an excessive release of cytokines and chemokines, known as a COVID-19-related cytokine storm, can also cause host cell death (S. Li et al., 2020; Yapasert et al., 2021).

As shown earlier, both ciliated and secretory cells are the main targets of SARS-CoV-2. Together these cells perform the mucociliary clearance process which is essential to maintain homeostasis and remove pathogens (Harkema et al., 2006; Herbert et al., 2018). Consequently, excessive cell death of one or both of these cells may not only have a significant impact on tissue composition, but also on tissue function. The fact that breakdown of airway clearance can directly precipitate and/or aggravate acute infections and chronic inflammatory conditions such as chronic rhinosinusitis (CRS), cystic fibrosis (CF) and primary ciliary dyskinesia (PCD) proves this theory (Tilley et al., 2015).

Cytopathic effects of SARS-CoV-2 on ciliated cells

Changes in cell morphology that are caused by viral infection are called cytopathic effects (CPE) (Albrecht et al., 1996). Unfortunately, there are currently no studies specifically on the CPE of SARS-CoV-2 on the NRE. However, several studies have been done on the CPE on other respiratory epithelia, i.e., nasopharyngeal- and bronchial respiratory epithelium. These epithelia are similar to the NRE and all have the same prominent group of cells: ciliated cells (Widdicombe, 2019). One of the observed CPE includes shedding of the ciliary axonemes (Pinto et al., 2021), which disables mucociliary clearance and likely enables disease progression. Another study showed the lack of cilium beating of the infected cells by means of light microscopy (Zhu, Zhang, et al., 2020b). Then later in 2020, by means of laser scanning confocal microscopy, scanning electron microscopy (SEM) and IFS further details of CPE were detected. First, they observed plaque formation that was consistently present in different propagations of infected cells. A viral plaque is defined as a physical entity: "a clear area on a lawn of bacteria or a monolayer of cells, where viruses have destroyed the cells" or functionally as "the progeny of one virus" (Shors, 2008). Secondly, analysis showed that different infected cells, located from the plaque region to the far plaque periphery, consistently experienced plaque-like CPE. These cells showed cilium shrinking, beaded changes, cilia disordering and multinucleation. Lastly, IFS with a specific SARS-CoV-2 N protein antibody and cell tight junction antibody showed giant syncytial cell formation and destruction of cell tight junctions (Zhu, Wang, et al., 2020). This has been confirmed by the study of Robinot and co-workers. Here they showed impairment of epithelial barrier function after SARS-CoV-2 infection because of reduced tight junctions within the respiratory epithelium (Robinot et al., 2021).

The impact of SARS-CoV-2 on respiratory epithelium differentiation

As mentioned earlier, it is important that cells of the NRE maintain proper functioning to preserve homeostasis and to avoid infection and development of inflammatory conditions. A tight balance between self-renewal and generation of physiologically appropriate proportions of required cells is essential to keep balance within the respiratory epithelium (Rock et al., 2011). For this reason, it is not only important that the fully differentiated ciliated cells and secretory cells maintain proper functioning, but also that there is fast regeneration in an optimum ratio (Tilley et al., 2015).

Basal cells have self-renewal capacities and are closely located to the epithelial basement membrane to respond to injury by proliferating and differentiating into other epithelial cell types (Rock et al., 2010). Research showed that basal cells can get infected by SARS-CoV-2, but they themselves practically never get infected. Indicating that basal cells are no direct target of SARS-CoV-2 (Ahn et al., 2021; Robinot et al., 2021; Sungnak et al., 2020). Nevertheless, their cellular behaviour, e.g., replication rate and differentiation,

still can be affected due to environmental signalling. In 1977, it was already confirmed that basal cell replication and differentiation is accelerated in response to injury (Breeze & Wheeldon, 1977). Basal cells, characterized by p63, NGFR and Podoplanin (Pdpn) expression, are able to differentiate into suprabasal $CK8^{+}$ $p63^{-}$ progenitor cells that can further segregate into both ciliated and secretory cells (Rock et al., 2011). In the study of Pardo-Saganta and co-workers they identified exclusive populations of basal cells that expressed low levels of c-myb and N2ICD. These cells were rapidly proliferating after injury and eventually they were split into N2ICD positive and c-myb positive cells. The N2ICD positive cells eventually differentiated into mature secretory cells, while the other subset of basal cells that express c-myb transformed into mature ciliated cells. *In vitro* analysis showed that with not virally induced damage of basal cell cultures from mice, the replication and differentiation of c-myb positive cells was rapidly initiated and stayed relatively similar over time, whereas that of N2ICD positive cells peaked after 12 hours post infection and then quickly dropped (Pardo-Saganta et al., 2015). Subsequently, the study of Chua and colleagues specifically investigated basal cell replication and differentiation upon SARS-CoV-2 infection. They observed differentiation of basal cells through secretory cells to terminally differentiated ciliated cells by mediation of FOXN4 positive cells, making secretory cells intermediates of mature ciliated cells. The FOXN4 positive cells seemed to be in a differentiation state close to that of mature ciliated cells, but still had relatively high expression levels of genes that are expressed in secretory cells. *FOXN4* encodes for a TF that is strongly upregulated during ciliated cell differentiation and has proven to be involved in basal body docking and cilia extension (Campell et al., 2016). Interestingly, the results indicated that this differentiation pathway is driven by genes that code for the key TF for ciliated cells, FOXJ1, and a component for mature cilia, TCTEX1D2 (Chua et al., 2020). Although these results were observed during SARS-CoV-2 infection, the fact that goblet cells can be precursor cells of mature ciliated cells and that a precursor subgroup of mature ciliated cells can be identified by specific markers, such as FOXN4, had already been observed in another tissue differentiation dynamics study (Ruiz García et al., 2019). The fact that this differentiation process has been observed before makes the results much more reliable.

In the NRE, SARS-CoV-2 mainly infects ciliated and secretory cells. Once these cells are infected, they experience cytopathic effects, however, it is not completely understood why these effects arise. A possible explanation could be that SARS-CoV-2 infection has an effect on the differentiation state of the infected cell, therefore changing its morphology and physiology. In a recent article by Robinot and colleagues they analysed the effects of SARS-CoV-2 on ciliated cells by examining mucociliary clearance and differentiation. First, they visualised ciliated cells infected by SARS-CoV-2 by means of Scanning electron microscopy (SEM) imaging. Some infected cells showed a lack of cilia and a massive accumulation of virions at the cell surface and on the membrane ruffles (Fig. 6a). Indicating a highly productive SARS-CoV-2 infection. Notably, viral particles were only occasionally observed along the ciliary sheaths (Fig. 6b). Once the magnification was increased, the ultrastructural abnormalities were revealed (Fig. 7). The cilia appeared shortened and misshapen. To explain these observations, quantitative RT-PCR was performed to analyse "key gene transcription involved in the regulation of ciliogenesis". These key genes generally code for transcription factors (TF). The TF's FOXJ1, FOXN4, MYB, RFX2, RFX3 and p73 all influence the expression of genes involved in ciliogenesis, but also cytoskeletal dynamics, planar cell polarity pathway and basal body docking (Lewis & Stracker, 2021). Quantitative RT-PCR analysis showed a significant decrease in transcripts encoding the ciliary component DNAH7 and the ciliogenesis regulators FOXJ1 and RFX3 after SARS-CoV-2 infection. These results are consistent with the loss of fully differentiated cells due to dedifferentiation: "a process by which cells grow in reverse from a partially or terminally differentiated stage to a less differentiated stage within their own lineage" (Yao & Wang, 2020). The regulator FOXJ1 is a master regulator of motile cilia function and ciliogenesis (Yu et al., 2008). Notably, dedifferentiation of the ciliated was observed before *FOXJ1* expression significantly decreased. To explain this phenomenon, they used IFS to measure the FOXJ1 protein levels. In contrast to *FOXJ1* expression, FOXJ1 protein expression was significantly reduced after two days instead of four days, confirming an earlier indication of post-transcriptional regulation of FOXJ1 (Abdi et al., 2018). Notably, the IFS results showed that the decreased expression of FOXJ1 significantly occurred in infected areas that expressed the S protein. To confirm whether decreased FOXJ1 protein expression preceded cilia loss, a control analysis was performed with β-tubulin IV, a widely used cilium marker (Mollet et al., 2005; Tyner et al., 2006). All areas showed high levels of β-tubulin IV, even S protein areas with decreased FOXJ1 expression. These findings strongly indicate that SARS-CoV-2 infection first reduces FOXJ1 protein expression and then *FOXJ1* gene expression. In addition to the ciliated cells, they analysed the effects of SARS-CoV-2 on the goblet- and basal cells. According to their results both goblet- and basal cells do not get infected by the SARS-CoV-2. Still the virus was added to the respiratory epithelia to analyse possible effects it could have on goblet- and basal cell behaviour. Four days after viral introduction, they observed a significant increase in transcripts in clusters of both cells. Suggesting compensatory properties of both cells upon ciliated cell damage caused by SARS-CoV-2. No dedifferentiation of the goblet cells was reported (Robinot et al., 2021).

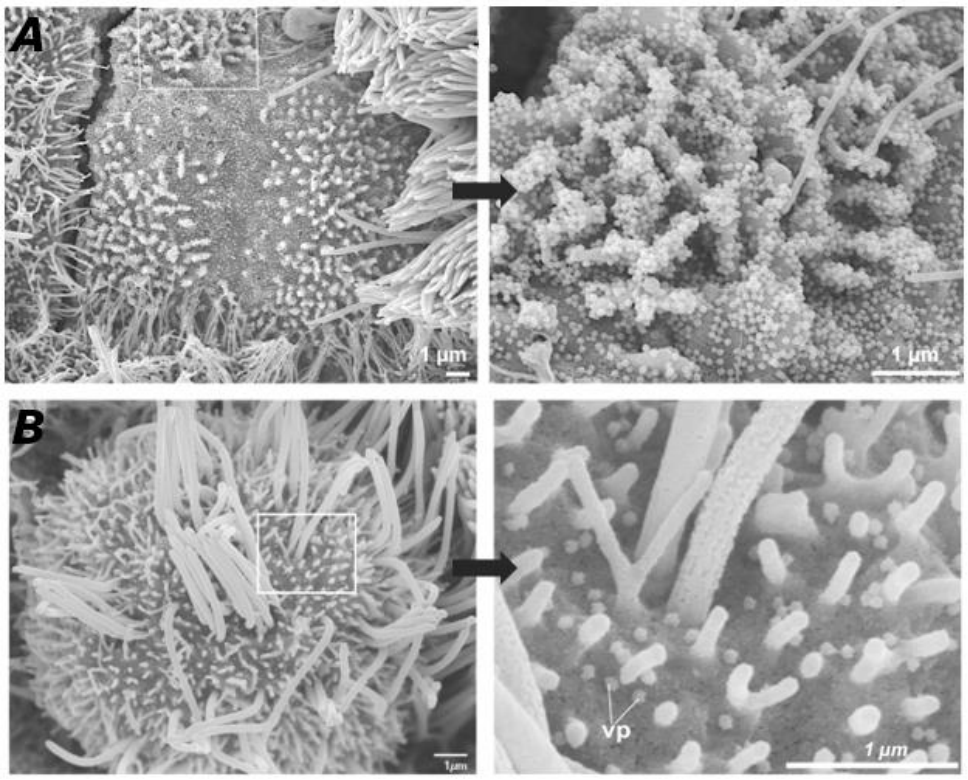

Figure 6. SEM image of a SARS-CoV-2 infected fully differentiated ciliated cell of the respiratory epithelium. *SEM images of ciliated cells two days after SARS-CoV-2 infection. A) Presenting lack of cilia and an accumulation of viral particles at the surface of membrane ruffles (enlarged in right panel). B) Presenting a few remaining cilia and scattered viral particles (vp) at the plasma membrane (enlarged in right panel)* (Robinot et al., 2021).

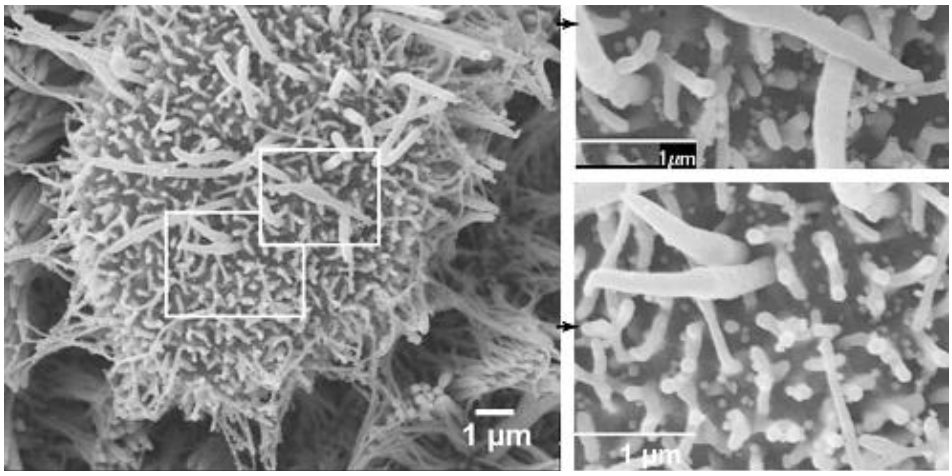

Figure 7. Magnified SEM image of ciliated cells 2 days after SARS-CoV-2 infection presenting cilia abnormalities. *Infected ciliated cell presenting shortened misshapen cilia and/or crescent shaped cytoskeletal core of the cilia (enlarged in right panels)* (Robinot et al., 2021).

One previous report also investigated the potential of viral infection to induce dedifferentiation. Although they used virus-like material, a synthetic double-stranded RNA that mimics a by-product of viral replication, instead of an actual virus and pancreatic β cells instead of respiratory epithelium cells, their findings could still provide an indication of the possibly induced dedifferentiation of ciliated cells by SARS-CoV-2. In this report they discovered that virus-like infection induces a decrease in β cell-specific gene expression, resulting in the dedifferentiation of β cells. These results support the findings of Robinot and colleagues on account of their similarity. Likewise, previous studies reported their findings concerning the association between FOXJ1

expression and ciliogenesis. In these reports they stated that in respiratory epithelium virus-induced damage, the cilia reduction is associated with decreased *FOXJ1* expression, and that during damage repair *FOXJ1* expression is associated with the formation of ultrastructural components of ciliogenesis (Look et al., 2001; You et al., 2004). To analyse the effects of virus-induced damage they used a virus from the *Paramyxoviridae* family, paramyxovirus, instead of SARS-CoV-2. Nevertheless, this are both RNA viruses and their effects on the respiratory epithelium seem to be highly similar. Justifying a comparison between their induced damage.

Altered cellular composition of the NRE upon SARS-CoV-2 infection

SARS-CoV-2 infection will often lead to host cell death (S. Li et al., 2020). As a result, because SARS-CoV-2' main target cells of the NRE are also the most abundant cells of the NRE, this will probably drastically alter the cellular composition of the NRE. The cellular composition of tissues is important to maintain proper organ function and avoid disruption of homeostasis. Hence, changes in the cellular composition will lead to a decrease in efficiency of the tissues' functions and possibly result in disease.

Some studies analysed the changes in cellular composition following SARS-CoV-2 infection extensively. The study of Ahn and colleagues used scRNA-sequencing and IFS to analyse cellular composition of the NRE in healthy controls and COVID-19 patients. After uncontrolled clustering, 13 different epithelial cell clusters could be distinguished and visualized with uniform manifold approximation and projection (UMAP) (Deprez et al., 2019) (Fig. 8). Of the total NRE cells of healthy controls, about 60% were fully differentiated cells, also multiciliated, and goblet cells while about 30% were suprabasal cells and club cells. In contrast to the healthy controls, the proportions of these cells were largely reduced, while those of differentiating secretory- and ciliated precursor cells, bestrophin-4hi (BEST4hi) cells and interferon gamma (IFN)-γ–responsive ciliated cells were considerably increased in COVID-19 patients. These results indicate that the damaged or dead cells are dynamically replaced with differentiating epithelial cells derived from their stem, basal cells, and precursor cells (Ahn et al., 2021). Furthermore, the study of Chua and co-workers also analysed the difference in cell types and their proportions within the NRE between healthy controls and moderate- and critical COVID-19 patients. Here they identified 8 different cell types of the respiratory epithelium. Interestingly, there was no significant difference in the proportion of ciliated between the three groups. Although this was not significant, there was a strong reduction in ciliated cells. Just as there was a, not significant, relatively high increase in ciliated-diff differentiating cells. The secretory and basal cells did show a significant reduction and the secretory-diff differentiating, the FOXN4+ positive and IFN-γ-responsive ciliated cells showed a significant increase. Indicating that NRE is actively trying to regenerate (Chua et al., 2020).

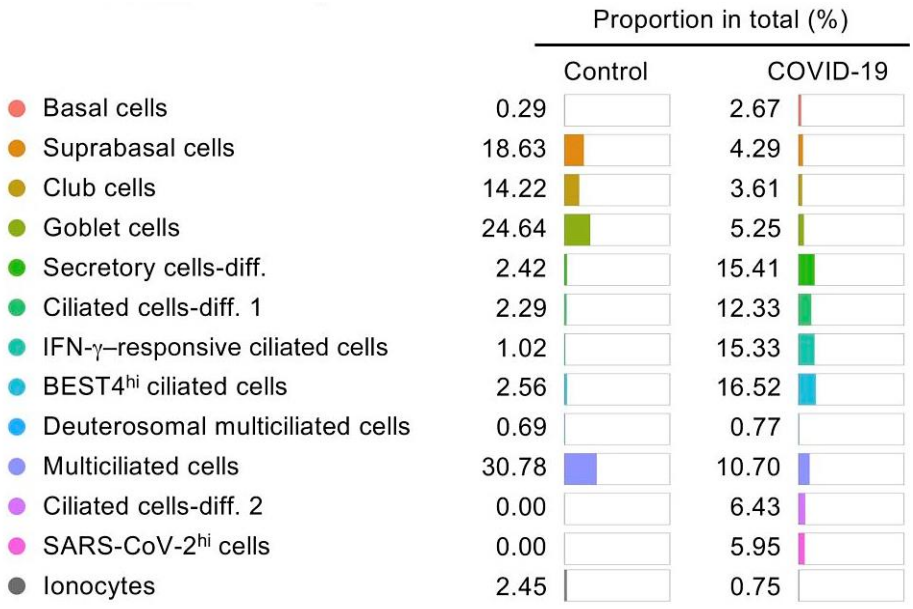

Figure 8. Comparison of cellular tropism of SARS-CoV-2 in NRE cells between healthy controls and COVID-19 patients. *List and proportion plots comparing proportion of each epithelial cell subset in total clustered epithelial cells between healthy controls and COVID-19 patients* (Ahn et al., 2021).

Discussion

SARS-CoV-2 is a new CoV's that brings several challenges upon personal and global health. The impact of infection varies between individuals experiencing mild symptoms to developing fatal conditions. The most prominent reason for developing a fatal condition is infection of the respiratory system, especially the lungs, resulting in extreme inflammation and respiratory distress (Gorbalenya et al., 2020; Munster et al., 2020). Before reaching the lungs, the virus has to pass through the airways. It is possible that this just serves as a first line defence, however, the fact that most symptoms are related to airway distress and that the virus is highly transmissibly (Rothe et al., 2020) indicates that SARS-CoV-2 may also specifically target the airways. The airways start in the nasal cavity, which is generally lined by respiratory epithelium and olfactory epithelium. Numerous studies have been done on the effects of SARS-CoV-2 on the olfactory epithelium, but studies on the respiratory epithelium are lagging behind. Fortunately, there are currently more studies being performed on the viral tropism and the associated effects of infection on cellular behaviour.

Viral entry of SARS-CoV-2 mostly depends on the expression of entry-related factors, such as the ACE2 receptor and the protease TMPRSS2, on the surface of host cells (Hoffmann et al., 2020). The majority of reports confirmed *ACE2* and *TMPRSS2* expression in cells of the NRE (Bertram et al., 2012) (Zou et al., 2020). The study of Sungnak and colleagues further investigated this phenomenon by performing the same expression analysis on three different datasets. Here they observed the highest expression of *ACE2* in the ciliated and secretory cells of the NRE. The co-expression results were less conclusive, but generally point towards ciliated cells as the cells with high levels of *TMPRSS2* and secretory cells with lower levels of *TMPRSS2* (Sungnak et al., 2020). In 2020, another study was published that completely contradicted these results, here the secretory cells expressed relatively high levels of *ACE2* and *TMPRSS2* while the ciliated cells expressed almost no signal of both (Ziegler et al., 2020). The*se* differences could be due to the use of different techniques or samples. Not all samples were obtained from the same area of the upper respiratory tract and with the same technique, both of these factors may have contributed to the differences in results. To obtain clear answers, the study Ahn and colleagues analysed the protein expression instead of the gene expression because transcript levels in an isolated cell do not fully reflect their actual expression or provides information about their cellular location (Liu et al., 2016). Interestingly, the signal intensity of ACE2, TMPRSS2 and furin in the ciliated cells was relatively high while there was no distinct detection of them in the secretory cells. Consequently, the transcript levels did indeed not match up with the actual protein levels. These conclusions are based on the results of several tests performed with different datasets, which only validates the final conclusion more (Ahn et al., 2021). However, to form a definitive conclusion it is necessary to determine the actual presence and quantities of viruses inside the cells of the NRE. Most studies detected significant amounts in both ciliated and secretory cells, with ciliated cells showing the highest levels of infection (Fiege et al., 2021; Pizzorno et al., 2020; Zhu, Wang, et al., 2020). The change of this being accurate is relatively high since all reports used different samples and several of them used different techniques, decreasing the change of the results being coincidental.

SARS-CoV-2 infection may have a significant impact on cells of the NRE, suggesting that once epithelium gets infected, a wide variety of effects will arise. For this review it was chosen to focus on the impact SARS-CoV-2 may have on cellular morphology and differentiation and NRE cellular composition. Different reports analysed the cytopathic effects of SARS-CoV-2 on ciliated cells. All infected ciliated cells experienced certain cytopathic effects which ultimately disordered their morphology and therefore most of its functions (Pinto et al., 2021; Zhu, Wang, et al., 2020; Zhu, Zhang, et al., 2020b). Notably, no data was available of cytopathic effects on infected secretory cells. Possibly because there are no significant cytopathic effects on the secretory cells or it still has to be researched.

In addition to cytopathic effects, the virus may also affect the differentiation of ciliated cells or other cells within the affected area of the epithelium. Epithelial damage generally accelerates basal cell replication and differentiation (Breeze & Wheeldon, 1977; Rock et al., 2010, 2011). During SARS-CoV-2 infection there are progenitor cells originating from basal cells that still express markers of both ciliated and secretory cells. Interestingly, the derived secretory precursor cells are able to differentiate into ciliated cells through mediation of FOXN4 positive cells (Chua et al., 2020). Although this reaction is observed during SARS-CoV-2 infection, it was also observed in another NRE differentiation study (Ruiz García et al., 2019). The fact that this is a known process in the NRE supports it reliability of occurring in an accelerated rate in infected and/or damaged RNE. Just as important to evaluate is the differentiation of ciliated and secretory cells upon of SARS-CoV-2 infection. Currently, there is only the study of Robinot and colleagues, in this study they analysed the effects of SARS-CoV-2 on ciliated cells by examining mucociliary clearance and differentiation. Analysis showed a significant decrease in gene expression of genes involved in ciliary structure and ciliogenesis, eventually resulting in dedifferentiation of the ciliated cells. Furthermore, the results suggest that the dedifferentiation is post-transcriptionally regulated and specifically occurs in the areas that express the S protein. This could be beneficial for the virus since it decreases mucociliary clearance and possibly

prepares the cell surface for forming another virion. Additionally, they analysed the differentiation status of the secretory- and basal cells upon infection. Clusters of both cells showed significant increased expression. Suggesting that they have compensatory properties. However, no effects were seen involving their differentiation status. Unfortunately, these results are unreliable because according to their analysis SARS-CoV-2 was not able to enter the secretory and basal cells; therefore, it is not possible to examine the effects of infection on differentiation of these cells (Robinot et al., 2021).

SARS-CoV-2 infection will often lead to host cell death (S. Li et al., 2020), indicating an altered cellular composition of the NRE in COVID-19 patients. Two studies observed several cell populations being increased or decreased upon SARS-CoV-2 infection. As expected, the amount of fully differentiated ciliated and secretory cells decreased and the amount of precursor cells increased in COVID-19 patients (Ahn et al., 2021). Of note, in one of the studies the decrease of fully differentiated ciliated cells was not significant. Although this was not significant, there was a strong reduction in ciliated cells. Just as there was a, not significant, relatively high increase in ciliated-diff differentiating cells (Chua et al., 2020). Remarkably, there was a cell population that only occurred in COVID-19 patients, the SARS-CoV-2^{hi} cells. This are infected cells that produce mRNAs of SARS-CoV-2 at a high rate and have a highly effected morphology, making it difficult to determine the original cell type. The study of Ahn and colleagues tried to identify the cellular origin by means of pseudo-time trajectory analysis (Cannoodt et al., 2016). They obtained results that implied that the SARS-CoV-2^{hi} cells are most likely ciliated cells. This is not unlikely since this are the most targeted cells of the NRE.

Concerning viral tropism of SARS-CoV-2 in the NRE, several factors have not been taken in consideration. Research has shown that the differentiation state and the cell cycle phase the cell are in influences their infection susceptibility (Coffin et al., 1977; Fan et al., 2018; Legros et al., 2020; O'Sullivan & Killen, 1994). Although this has not been proven to be relevant for SARS-CoV-2 infection of cells of the NRE, it could be relevant and therefore should be looked into. Another important infection susceptibility factor is the presence of inflammation. Once SARS-CoV-2 infects the epithelium, it causes severe inflammation and barrier dysfunction (Deinhardt-Emmer et al., 2021). Inflammation has been proven to influence SARS-CoV-2 entry factor expression in the respiratory epithelium (Kimura et al., 2020; Sajuthi et al., 2020; Ziegler et al., 2020). Perhaps when the influence of these factors is better understood, they can be taken in consideration and explain and/or resolve the variation between studies. Besides influencing viral tropism, inflammation may also exert an effect on the differentiation of cells of the NRE upon infection. Nevertheless, inflammation also seems to be beneficial since certain inflammatory signals have shown to stimulate specific cells to differentiate into ciliated cells (Chua et al., 2020), probably to restore homeostasis.

Conclusion

To this day, SARS-CoV-2 is still a great threat to personal and global health. Often the NRE gets overlooked as just an air passage and first defence line, but not as a target of infection. High expression of entry-related host factors and the detection of the virus or its related content in the ciliated and secretory cells confirms them to be the main target cells of the NRE of SARS-CoV-2 infection. Arguably, even of the entire respiratory system. Subsequently, once the virus infects the NRE it will exert several effects on cellular morphology and behaviour. Research showed that infection alters the differentiation rate of secretory and basal cells and dedifferentiates ciliated cells. Dedifferentiation of ciliated cells leads to loss and abnormal structures of cilia, resulting in reduced mucociliary clearance and discomfort. Even without extreme inflammation, this could be a life-threatening situation where there is a reduced protection of the lower airways and lungs.

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