UNIVERSITÉ DU QUÉBEC À TROIS-RIVIÈRES

CONSTRUCTION D'UN SYSTÈME DE VECTEUR VIRAL DÉRIVÉ DU SARS-CoV-2

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A perfect thesis is never finished
A finished thesis is never perfect

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To my parents, family and all my friends,
without whom none of my success would be possible

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RÉSUMÉ EN FRANÇAIS

L'émergence du Syndrome Respiratoire Aigu Sévère Coronavirus 2 (SARS-CoV-2), le virus responsable de la COVID-19, a suscité des efforts considérables dans le développement de vaccins et de médicaments antiviraux. Bien que les vaccins actuels aient montré leur efficacité, ils présentent également certaines limitations. Le développement de médicaments antiviraux pour la COVID-19 est davantage complexifié par la classification du SARS-CoV-2 comme pathogène de niveau de biosécurité 3 et l'absence d'un système robuste permettant de mesurer précisément l'infection virale. Afin de relever ces défis, le programme de recherche présenté dans cette thèse vise à développer des outils alternatifs en utilisant des particules pseudo-virales (Virus-Like Particles, VLPs) et des systèmes d'ARN réplicons, avec l'objectif ultime de créer un vecteur viral entièrement basé sur le SARS-CoV-2.

Des VLPs du SARS-CoV-2 ont été générées avec succès grâce à la co-expression des quatre protéines structurales essentielles – membrane (M), petite enveloppe (E), spicule (S) et nucléocapside (N) – dans des cellules de mammifères. La formation et la libération réussies de ces VLPs ont validé le rôle essentiel de ces protéines dans la production de VLPs, puisque l'élimination d'une ou deux de ces protéines structurales a entraîné une diminution de l'efficacité. En s'appuyant sur cette base, des vecteurs lentiviraux ont été utilisés pour établir des lignées cellulaires humaines stables capables de produire des VLPs. Ces VLPs, en se liant aux récepteurs naturels du virus et en facilitant l'entrée cellulaire et la présentation d'antigènes, présentent un potentiel significatif pour le développement de candidats vaccins intranasaux innovants.

Parallèlement, cette étude a exploré l'expression d'ARN réplicon du SARS-CoV-2 à partir de l'ADN et le rôle de la protéine N dans l'amélioration de la réplication de l'ARN. En introduisant un chromosome artificiel bactérien (BAC) codant un réplicon du SARS-CoV-2 sous un promoteur T7 dans des cellules HEK293T modifiées pour exprimer l'ARN polymérase T7, une expression faible mais constante de la protéine rapporteuse a été observée. De manière inattendue, cette expression dépendait de la linéarisation préalable de l'ADN BAC avant la transfection, mais elle était indépendante de l'ARN polymérase T7 et résistante au remdésivir, révélant l'absence d'autoréplication de l'ARN réplicon. En résumé, nous avons pas réussi à établir un système

d’expression de réplicon ADN initié par une polymérase T7, mais nous avons fait des découvertes intéressantes sur l’expression atypique des gènes du réplicon.

Malgré la disponibilité de divers systèmes vecteurs pour étudier l’infection et la réplication du SARS-CoV-2, beaucoup sont limités par leur incapacité à simuler complètement les étapes postérieures à l’entrée ou par la complexité des protocoles de transfection multi-plasmidiques. Pour surmonter ces limitations, nous avons développé un nouveau vecteur viral du SARS-CoV-2. Ce vecteur inclut les quatre protéines structurales exprimées de manière stable par transduction lentivirale, associées à l’électroporation d’un ARN réplicon codant une protéine fusion luciférase-GFP. Concentré par ultracentrifugation, ce vecteur a infecté avec succès des lignées cellulaires humaines exprimant les récepteurs ACE2 et TMPRSS2, comme l’ont démontré l’activité détectable de la luciférase et la fluorescence GFP. La sensibilité du vecteur au remdésivir et aux interférons de type I souligne son potentiel pour le criblage de médicaments antiviraux. Bien qu'une amélioration des titres viraux soit nécessaire, ce système représente un outil prometteur pour les études de virologie moléculaire du SARS-CoV-2.

En conclusion, ces études apportent des contributions substantielles à la recherche sur le SARS-CoV-2 en établissant une plateforme innovante basée sur des cellules de mammifères pour la production de VLPs, qui présentent un potentiel significatif pour le développement de vaccins et les études d’expression génique. De plus, le système de vecteurs viraux introduit ici offre des opportunités précieuses pour faire progresser le développement de vaccins, améliorer le criblage de médicaments antiviraux et approfondir notre compréhension des mécanismes moléculaires sous-jacents à l’infection par le SARS-CoV-2.

ABSTRACT

The emergence of Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2), the virus responsible for COVID-19, has sparked extensive efforts in vaccine and drug development. While current vaccines have shown effectiveness, they also have certain limitations. The development of antiviral drugs for COVID-19 is further complicated by the classification of SARS-CoV-2 as a biosafety level 3 pathogen and the lack of a robust system for accurately measuring viral infection. To address these challenges, the research program detailed in this thesis aims to develop alternative tools by utilizing Virus-Like Particles (VLPs) and RNA replicon systems, ultimately striving to create a fully SARS-CoV-2-based viral vector.

SARS-CoV-2 VLPs were successfully generated through the co-expression of the four essential structural proteins – membrane (M), small envelope (E), spike (S), and nucleocapsid (N) – in mammalian cells. The successful formation and release of these VLPs validated the essential role of all four proteins in VLP production, as removal of one or two structural proteins resulted in reduced efficiency. Building on this foundation, lentiviral vectors were employed to establish stable human cell lines capable of producing VLPs. As these VLPs can bind to the virus's natural receptors, facilitating cellular uptake and antigen display, they hold significant promise for the development of innovative intranasal vaccine candidates.

In parallel, this study investigated the expression of SARS-CoV-2 replicon RNA from DNA and the role of the N protein in enhancing RNA replication. By introducing a bacterial artificial chromosome (BAC) encoding a SARS-CoV-2 replicon under a T7 promoter into HEK293T cells engineered to express T7 RNA polymerase, weak but consistent reporter protein expression was achieved. Unexpectedly, reporter expression was dependent on BAC DNA linearization before transfection but was unaffected by T7 RNA polymerase and resistant to remdesivir, revealing an absence of replicon RNA self-replication. In summary, we were not successful in establishing a T7 polymerase-dependent DNA-launched replicon expression system, but we made interesting discoveries on the atypical expression of replicon genes.

Despite the availability of various vector systems for studying SARS-CoV-2 infection and replication, many are constrained by their inability to efficiently achieve post-entry replication stages or the complexity of multi-plasmid transfection protocols. To overcome these limitations, we developed a novel SARS-CoV-2 viral vector. This vector includes all four structural proteins

stably expressed through lentiviral transduction, together with the electroporation of a replicon RNA encoding a luciferase-GFP fusion protein. Concentrated by ultracentrifugation, this vector successfully infected human cell lines expressing ACE2 and TMPRSS2 receptors, as evidenced by detectable luciferase activity and GFP fluorescence. The vector's responsiveness to remdesivir and type I interferons further underscores its potential for antiviral drug screening. Although improvement of viral titers is needed, this system represents a promising tool for molecular virology studies of SARS-CoV-2.

In conclusion, these studies make substantial contributions to SARS-CoV-2 studies by establishing an innovative mammalian cell-based platform for producing VLPs, which demonstrate significant potential for vaccine development and gene expression studies. Additionally, the viral vector system introduced here offers valuable opportunities for advancing vaccine development, enhancing antiviral drug screening, and deepening our understanding of the molecular mechanisms underlying SARS-CoV-2 infection.

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LIST OF ABBREVIATIONS

AAV	Adeno-associated virus
ACE2	Angiotensin-converting enzyme 2
ARDS	Acute respiratory distress syndrome
BAC	Bacterial artificial chromosome
BSL-2	Biosafety level 2
BSL-3	Biosafety level 3
CFR	Case fatality rate
CoV	Coronavirus
CT	cytoplasmic tail
CTL	Cytotoxic T lymphocyte
E	Envelope protein
ER	Endoplasmic reticulum
ERGIC	Endoplasmic reticulum-Golgi intermediate compartment
EUA	Emergency use authorization
FP	Fusion peptide
GFP	Green fluorescent protein
HR1	Heptad repeat 1
HR2	Heptad repeat 2
IFN	Interferon
IL-1β	Interleukin-1 beta
IL-6	Interleukin-6
LTR	Long terminal repeat
LV	Lentiviral vector
M	membrane protein
mAb	Monoclonal antibody
MHC	Major histocompatibility complex
MLV	Murine leukemia virus

Mpro	Main protease
N	Nucleocapsid protein
Nsp	Non-structural protein
NTD	N-terminal domain
ORF	Open reading frame
Ori	Origine of replication
PTM	Post-translational modification
R0	Reproduction number
RBD	Receptor-binding domain
RdRp	RNA-dependent RNA polymerase
RNP	Ribonucleoprotein
RTC	Replicase-transcriptase complex
S	spike protein
saRNA	Self-amplifying RNA
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
SR	Serine/arginine-rich
T7 RNAP	T7 RNA polymerase
TM	transmembrane domain
TMPRSS2	Transmembrane serine protease 2
TNF-α	Tumor necrosis factor-alpha
TRS	Transcription-regulating sequences
UTR	Untranslated region
VEEV	Venezuelan equine encephalitis virus
VLP	Virus-like particle
VOC	Variant of concern
Vpr	Viral protein R
VSV-G	Vesicular Stomatitis Virus G
WHO	World Health Organization

CHAPTER I

INTRODUCTION

1.1 An introduction to SARS-CoV-2/COVID-19

Throughout history, infectious diseases have presented significant challenges, resulting in widespread outbreaks and substantial losses in human lives (Morens and Fauci 2013). COVID-19 is a respiratory illness caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) (Zhu, Zhang et al. 2020). The first cases of this pathogen were reported in Wuhan, China in December 2019, where several hospitals documented cases of pneumonia of unknown origin (Huang, Wang et al. 2020). Common symptoms included fever, cough, fatigue, and muscle pain (Guan, Ni et al. 2020). The novel coronavirus was officially named 'SARS-CoV-2' by the International Committee on Taxonomy of Viruses on February 11, 2020, with the associated disease named 'COVID-19' (2020). On March 11, 2020, the WHO officially declared the global COVID-19 outbreak as a pandemic ((WHO) 2020)

1.1.1 SARS-CoV-2 Origin and Zoonotic Transmission

The term *coronavirus* is derived from the crown-like (corona) shape observed in these viruses under the electron microscope, first identified in the 1960s (Tyrrell and Bynoe 1966). Coronaviruses (CoVs) are a diverse family of enveloped, single-stranded positive-sense RNA viruses belonging to the family *Coronaviridae* and the order *Nidovirales* (Masters 2006). They are categorized into four genera: alpha-, beta-, gamma-, and deltacoronaviruses, based on genetic and antigenic characteristics. Of these, only alphacoronaviruses and betacoronaviruses are known to infect humans (Perlman and Netland 2009).

To date, seven human coronaviruses (HCoVs) have been identified. Four of them—HCoV-229E and HCoV-NL63 (alphacoronaviruses), and HCoV-OC43 and HCoV-HKU1 (betacoronaviruses)—are endemic worldwide and typically cause mild upper respiratory

tract infections, often referred to as the “common cold” (Susan, Weiss et al. 2005). In contrast, three highly pathogenic betacoronaviruses have emerged in the past two decades, causing severe epidemics and pandemics. SARS-CoV, identified in 2002 in Guangdong, China, led to the severe acute respiratory syndrome (SARS) outbreak, infecting more than 8,000 people with a mortality rate of ~10% (Thomas, Ksiazek et al. 2003). A decade later, MERS-CoV emerged in the Middle East in 2012, causing Middle East respiratory syndrome (MERS) with a case fatality rate of ~35% (Ali, Zaki et al. 2012; de Wit, Van Doremalen et al. 2016). Most recently, SARS-CoV-2, the causative agent of COVID-19, was identified in Wuhan, China, in late 2019 and rapidly escalated into a global pandemic with profound health, social, and economic impacts (Zhou, Yang et al. 2020).

SARS-CoV-2 is genetically linked to a diverse group of bat coronaviruses, with its closest known relative being BatCoV RaTG13, a virus found in the horseshoe bat (*Rhinolophus affinis*), sharing approximately 96% genomic similarity with SARS-CoV-2 (Zhou, Yang et al. 2020). This high level of sequence homology strongly suggests that bats are the primary reservoir hosts for SARS-CoV-2. However, direct transmission from bats to humans is considered unlikely due to ecological and behavioral barriers (Andersen, Rambaut et al. 2020). It is theorized that SARS-CoV-2 may have been transmitted to humans via an intermediate host, with pangolins (*Manis javanica*) being the main suspects. Pangolins were found to host a coronavirus with amino acid identities of 100%, 98.6%, 97.8%, and 90.7% to SARS-CoV-2 in the envelope (E), matrix (M), nucleocapsid (N), and spike (S) structural proteins, respectively (Zhang, Wu et al. 2020). The exact pathway of zoonotic transmission remains the subject of ongoing research, and SARS-CoV-2 may have multiple host species, with pangolins potentially acting as an intermediary (Zhao, Cui et al. 2020). Alternatively, the virus could have emerged through a recombination event between a virus like pangolin-CoV and another resembling RaTG13 (Andersen, Rambaut et al. 2020).

1.1.2 Structural Biology of SARS-CoV-2

SARS-CoV-2 is an enveloped virus approximately 60-140 nm in diameter (Zhu, Zhang et al. 2020). The viral genome encodes four major structural proteins: Spike (S), Envelope (E), Membrane (M), and Nucleocapsid (N), each playing a vital role in the virus's life cycle (Astuti 2020).

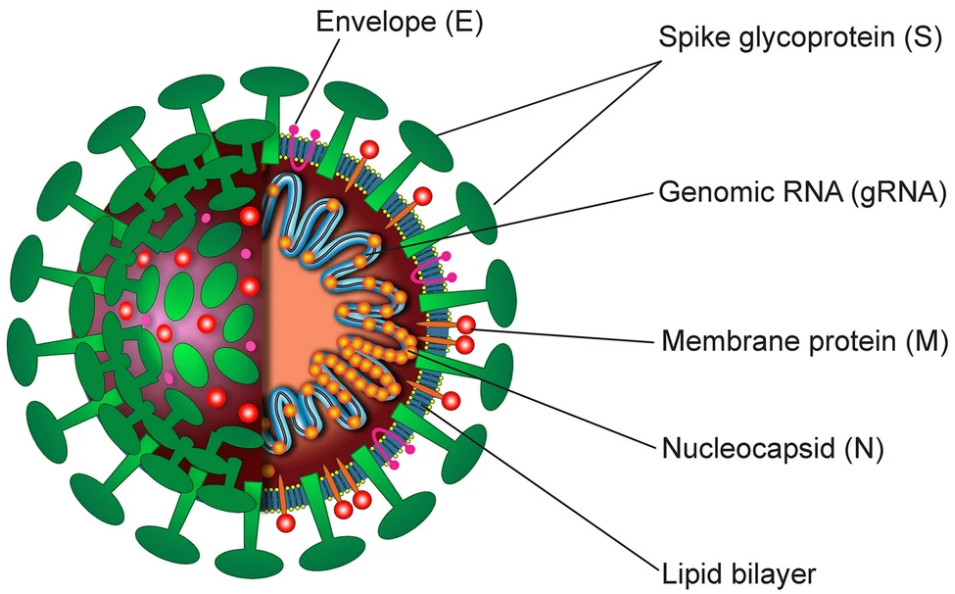

Figure 1 **Schematic structure of SARS-CoV-2 and its structural proteins**
The spike, envelope, and membrane glycoproteins are embedded in the lipid bilayer, and nucleocapsid protein binds to genomic RNA (Beig Parikhani, Bazaz et al. 2021)

The spike (S) protein of SARS-CoV-2, a crucial component of the viral envelope, plays a vital role in the virus's entry into host cells by interacting with the angiotensin-converting enzyme 2 (ACE2) receptor (Hoffmann, Kleine-Weber et al. 2020). Structurally, the spike protein is a large, homotrimer class I fusion protein, with each monomer consisting of approximately 1,273 amino acids (Walls, Park et al. 2020). The S protein comprises two functional subunits: S1 and S2. The S1 subunit, about 690 amino acids in length, is responsible for receptor binding and includes the N-terminal domain (NTD) and the receptor-binding domain (RBD) (Lan, Ge et al. 2020). The RBD specifically interacts with the ACE2 receptor on host cells, facilitating viral entry (Shang, Wan et al. 2020). The S2 subunit, which comprises approximately 600 amino acids, is responsible for mediating membrane fusion and contains several crucial regions, such as the fusion peptide (FP), heptad repeat 1 (HR1), heptad repeat 2 (HR2), the transmembrane domain (Kemp, Collier et al.), and the cytoplasmic tail (CT) (Walls, Park et al. 2020). Upon binding to ACE2, the S1 subunit undergoes significant conformational changes that prompt the S2 subunit to transition from a pre-fusion to a post-fusion state. This transition leads to the insertion of the fusion peptide into the host

cell membrane, followed by the formation of a six-helix bundle through interactions between HR1 and HR2, ultimately facilitating the fusion of the viral and host membranes (Cai, Zhang et al. 2020). The SARS-CoV-2 spike protein alternates between a closed state, where the receptor-binding domains (RBDs) are tucked down, shielding the ACE2 binding sites and helping the virus evade immune recognition. In the open state, at least one RBD is lifted up, exposing the binding interface to engage ACE2 receptors on host cells and initiate viral entry (Wang, Zhao et al. 2020)

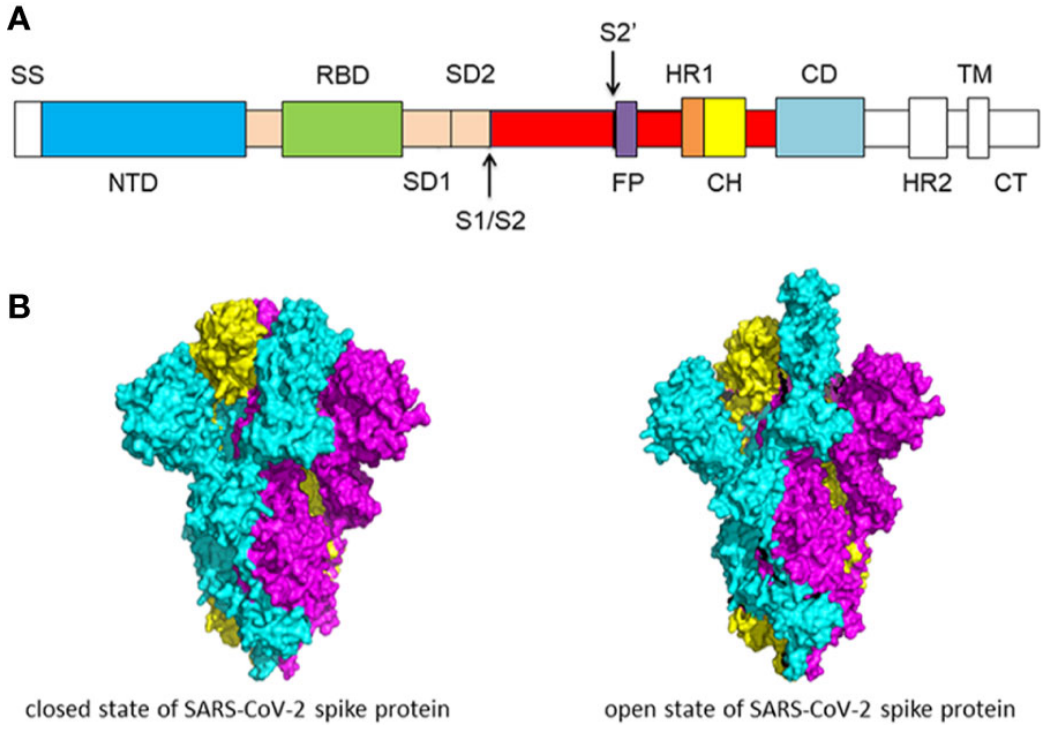

Figure 2 **Schematic of SARS-CoV-2 spike protein primary structure**
Different domains are shown by different colors. SS, single sequence; NTD, N-terminal domain; RBD, receptor-binding domain; SD1, subdomain 1; SD2, subdomain 2; S1/S2, S1/S2 protease cleavage site; S2', S2' protease cleavage site; FP, fusion peptide; HR1, heptad repeat 1; CH, central helix; CD, connector domain; HR2, heptad repeat 2; TM, transmembrane domain; CT, cytoplasmic tail. The protease cleavage site is indicated by arrows. (B) Cryo-EM structure of the SARS-CoV-2 spike protein. The closed state (PDB: 6VXX) of the SARS-CoV-2 S glycoprotein (left) the open state (PDB: 6VYB) of the SARS-CoV-2 S glycoprotein (right) (Wang, Zhao et al. 2020)

The spike protein is extensively glycosylated, with approximately 22 N-linked glycosylation sites per monomer, which serve to shield the virus from immune recognition and assist in proper protein folding (Watanabe, Allen et al. 2020). The trimeric spike protein is found

in a metastable prefusion state and undergoes cleavage at the S1/S2 boundary, a process initiated by host proteases such as furin, and a subsequent cleavage at the S2' site by proteases like TMPRSS2. These cleavages are essential for activating the spike protein for membrane fusion (Hoffmann, Kleine-Weber et al. 2020). The dynamic nature and functional significance of the spike protein make it a key target for vaccines, therapeutic antibodies, and antiviral drugs (Tortorici and Veesler 2019).

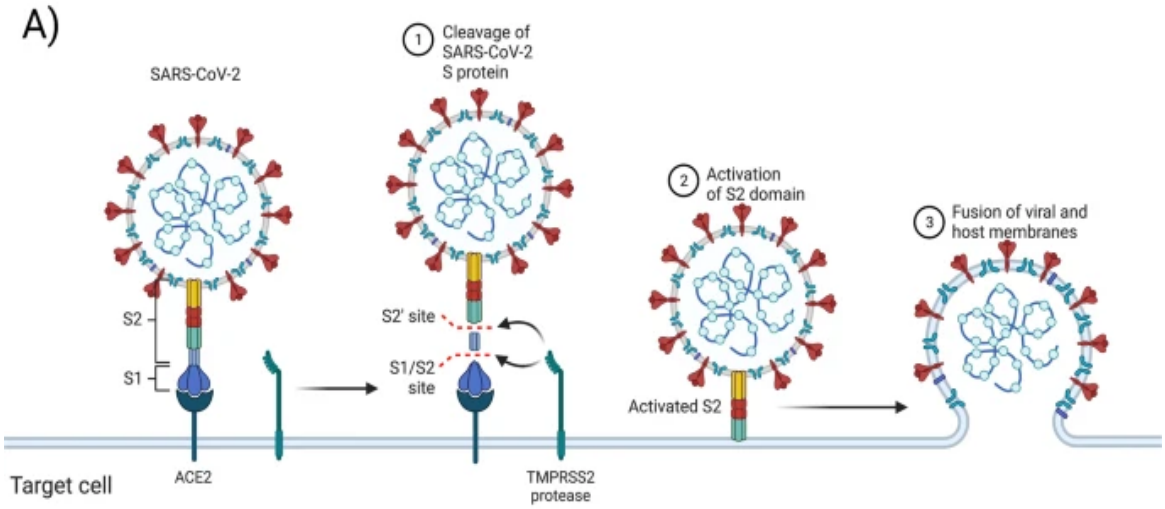

Figure 3 **Structural interaction between SARS-CoV-2 and target cell**
Including the viral spike protein, ACE2-receptor, TMPRSS2 reaction to cleave and begin the viral intracellular internalization (Jamison Jr, Anand Narayanan et al. 2022)

The nucleocapsid (N) protein of SARS-CoV-2 is a versatile phosphoprotein that plays a crucial role in the viral life cycle, particularly in RNA genome packaging, replication, and modulation of host cell responses (McBride, Van Zyl et al. 2014). Structurally, the N protein consists of two main domains: the N-terminal domain (NTD) and the C-terminal domain (CTD), connected by an intrinsically disordered central linker region (Chang, Hou et al. 2014). The NTD primarily binds to the viral RNA genome through specific interactions with the conserved packaging signal (Bar-On, Flamholz et al.), facilitating the encapsidation of the RNA into ribonucleoprotein (RNP) complexes (Cubuk, Alston et al. 2021). The CTD is involved in oligomerization, enabling the N protein to form dimers and higher-order structures necessary for

the assembly of the nucleocapsid (Ye, West et al. 2020). Additionally, the N protein interacts with the viral RNA-dependent RNA polymerase (RdRp) complex, enhancing the efficiency of viral RNA transcription and replication (Wu, Zhong et al. 2022). Furthermore, the N protein modulates the host cell environment to favour viral replication by interacting with host cell proteins involved in RNA processing, transport, and immune responses (Zeng, Liu et al. 2020).

One of the key features of the N protein is its phosphorylation, particularly within the central serine/arginine-rich (Hafiz, Illian et al.) region. This post-translational modification modulates the protein's RNA-binding affinity, oligomerization state, and interactions with other viral and host proteins (Surjit, Kumar et al. 2005). Phosphorylation is believed to play a crucial role in the dynamic reorganization of the nucleocapsid during different stages of the viral life cycle, especially during the transition from RNA replication to virion assembly (Carlson, Asfaha et al. 2020). Apart from its structural and regulatory functions, the N protein acts as a major immunogen, triggering a strong immune response during SARS-CoV-2 infection (Dutta, Mazumdar et al. 2020). The N protein's capacity to interact with a wide range of host proteins also highlights its role in immune evasion. For example, it can neutralize the host's interferon response by disrupting the RNA sensing pathways, thereby reducing the antiviral immune response (Liu, Bai et al. 2022).

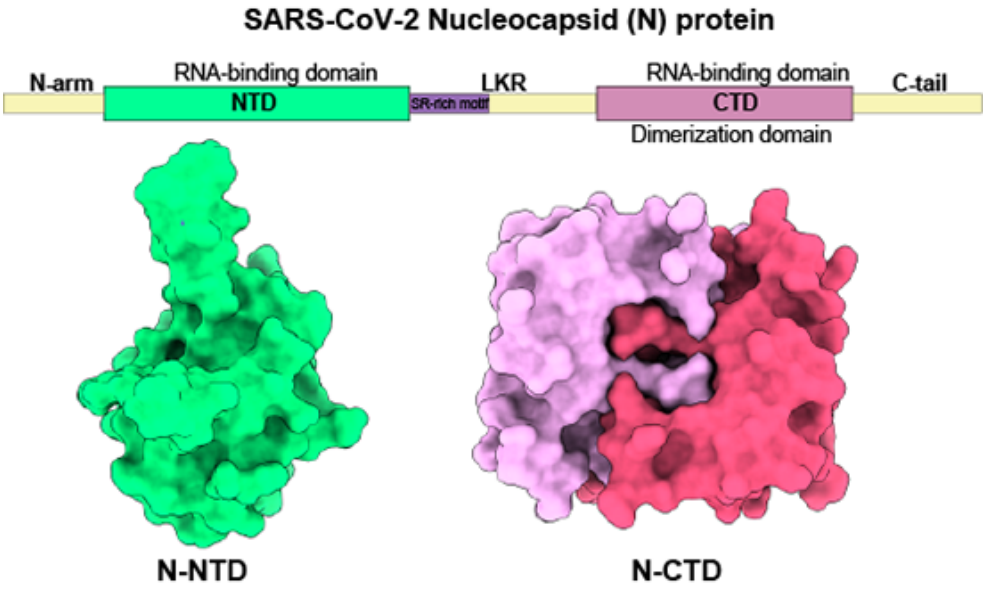

Figure 4 **Structures of the N- and C-terminal domains of SARS-CoV-2 N** at 1.8 and 1.5 Å resolution, respectively (Peng, Du et al. 2020)

The membrane (M) protein of SARS-CoV-2 plays a crucial role in the virus's structure, assembly, and budding (Neuman, Kiss et al. 2011). As an integral membrane protein, it is the most abundant protein in the viral envelope and is characterized by triple-spanning transmembrane domains that anchor it within the lipid bilayer of the viral membrane (Corse and Machamer 2003). The M protein consists of a short N-terminal ectodomain, three transmembrane helices, and a long C-terminal endodomain that extends into the interior of the virion (Thomas 2020). This protein is essential for maintaining the virion's shape and stability, providing the necessary backbone for the assembly of the viral envelope (Neuman, Kiss et al. 2011).

The M protein plays a critical role in coordinating the assembly of viral particles by interacting with other structural proteins such as the spike (S), envelope (E), and nucleocapsid (M) proteins (Tseng, Wang et al. 2014). Its interaction with the N protein facilitates the encapsidation of the viral RNA genome and the formation of the ribonucleoprotein (RNP) complex (He, Leeson et al. 2004). Additionally, the M protein is essential for virion morphogenesis as it interacts with the E protein, which is crucial for the final stages of viral budding from the host cell's membrane. This interaction is vital for membrane curvature, leading to the formation of mature virions (Boson, Legros et al. 2021). Moreover, the M protein influences the incorporation of the S protein into the virion, which is indispensable for the virus's infectivity (Ujike and Taguchi 2015).

The envelope (E) protein of SARS-CoV-2 is a small yet important protein with multiple functions. It plays a crucial role in the virus's life cycle, particularly in viral assembly, budding, and pathogenesis (Schoeman and Fielding 2019). Structurally, the E protein is an integral membrane protein consisting of approximately 75 amino acids (Nieto-Torres, DeDiego et al. 2011). It comprises a short hydrophilic N-terminal domain, a single hydrophobic transmembrane domain, and a long hydrophilic C-terminal domain (Cohen, Lin et al. 2011). Despite its small size, the E protein is highly conserved among coronaviruses and is vital for efficient virus assembly and release (Schoeman and Fielding 2019).

The E protein plays a crucial role in the assembly and release of viral particles by interacting with other structural proteins, particularly the membrane (M) protein. This interaction coordinates the assembly of new virions in the host cell's endoplasmic reticulum-Golgi intermediate compartment (ERGIC) (Corse and Machamer 2003). Furthermore, the E protein is essential in the budding process, as it induces membrane curvature and scission, facilitating the formation of mature virions (J Alsaadi and Jones 2019). In addition to its structural role, the E

protein acts as an ion channel, which is vital for the virus's ability to modify the host cell environment to favour viral replication (Schoeman and Fielding 2019). This ion channel activity is linked to the viroporin class of proteins and is believed to modulate the host cell's ion homeostasis, contributing to processes such as viral release and apoptosis of infected cells (Pervushin, Tan et al. 2009). The ion channel function of the E protein is also implicated in the virus's pathogenesis, as it can contribute to the inflammatory response observed in severe COVID-19 cases. By altering ion concentrations within the host cell, the E protein can activate inflammatory pathways, including the NF-κB pathway, leading to the secretion of pro-inflammatory cytokines (Zhou, Lv et al. 2023).

1.1.3 Genomic Organization and Variants of Concerns

The genome of SARS-CoV-2 consists of a positive-sense, single-stranded RNA molecule that is approximately 29.9 kilobases long (Kim, Lee et al. 2020). It is composed of multiple open reading frames (ORFs) that encode both structural and non-structural proteins crucial for the virus's replication, assembly, and evasion of the immune system (Wu, Zhao et al. 2020). The ORF1a and ORF1b regions are the largest and encode two polyproteins that are cleaved by viral proteases into 16 non-structural proteins (nsps), which are vital components of the replicase-transcriptase complex (RTC) (Fehr and Perlman 2015). This complex is responsible for replicating the viral RNA genome and transcribing subgenomic RNAs that act as templates for synthesizing viral proteins (V'kovski, Kratzel et al. 2021). The nsps include important enzymes such as RNA-dependent RNA polymerase (nsp12), helicase (nsp13), and exonuclease (nsp14), which play crucial roles in maintaining the accuracy of viral replication and proofreading newly synthesized RNA (Subissi, Posthuma et al. 2014). The SARS-CoV-2 genome also encodes four structural proteins—Spike (S), Membrane (M), Envelope (E), and Nucleocapsid (N)—as well as several accessory proteins. Although not essential for viral replication in vitro, these accessory proteins, such as ORF3a, ORF6, and ORF7a, contribute to the virus's ability to manipulate the host immune response and increase its pathogenicity (Cao, Xia et al. 2021). These accessory proteins have been found to interfere with various aspects of the host's immune defense, including the interferon response, apoptosis, and antigen presentation (Yuen, Lam et al. 2020).

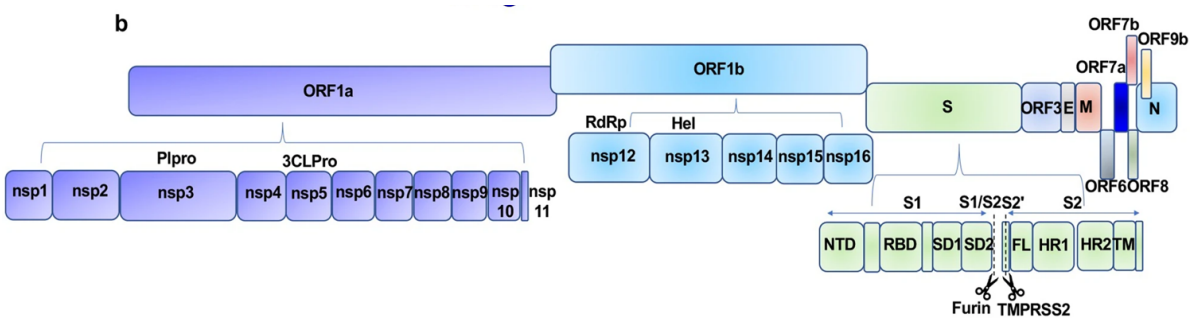

Figure 5 **Schematic diagram of the SARS-CoV-2 genome**

The genome includes ORF1a-ORF1b-S-ORF3-E-M-ORF6-ORF7 (7a and 7b)-ORF8-ORF9b-N in order. Sixteen nonstructural proteins (nsp1–11, 12–16) are encoded by ORF1a and ORF1b, respectively, and six accessory proteins were delineated. Plpro, papain like protease; 3CLPro, 3C-like proteinase; RdRp, RNA-dependent RNA polymerase; Hel, Helicase; S encodes NTD N-terminal domain; RBD, receptor-binding domain; SD1, subdomain 1; SD2, subdomain 2; FL, fusion loop; HR1, heptad repeat 1; HR2, heptad repeat 2; TM, transmembrane domain. Dotted line indicates S1/S2 and S2′ site cleavage by Furin and TMPRSS2(Zhang, Xiang et al. 2021)

The rapid mutation rate of SARS-CoV-2, particularly in the Spike protein, has resulted in the emergence of numerous variants, some of which have been designated as Variants of Concern (VOCs) due to their impact on transmissibility, disease severity, and ability to evade the immune system (Harvey, Carabelli et al. 2021). Notable VOCs include Alpha (B.1.1.7), Beta (B.1.351), Gamma (P.1), Delta (B.1.617.2), and Omicron (B.1.1.529). Each variant has accumulated mutations that provide specific advantages in terms of viral fitness (Kemp, Collier et al. 2021). For instance, the Alpha variant, first identified in the United Kingdom, is characterized by the N501Y mutation in the Spike protein, which enhances the virus's binding affinity to the ACE2 receptor and increases transmissibility (Davies, Abbott et al. 2021). Both the Beta and Gamma variants, discovered in South Africa and Brazil, carry the E484K mutation, which reduces the neutralizing activity of antibodies produced by prior infection or vaccination, thereby increasing the potential for immune evasion (Wang, Casner et al. 2021). The Delta variant, initially found in India, is linked to multiple mutations in the Spike protein, including L452R and T478K, which intensify viral transmission and are associated with more severe disease outcomes (Planas, Veyer et al. 2021). The emerged Omicron variant carries over 30 mutations in the Spike protein, including

several in the RBD that significantly alter its antigenic properties, showing reduced vaccine effectiveness and the necessity for updated vaccines or booster doses (Cao, Wang et al. 2022).

1.1.4 Life Cycle of SARS-CoV-2

The life cycle of SARS-CoV-2 begins with the viral Spike protein binding to the ACE2 receptor on the host cell's surface, a crucial step for viral entry (Hoffmann, Kleine-Weber et al. 2020). This interaction is further facilitated by the cleavage of the Spike protein by the host cell proteases TMPRSS2 and furin, which promote the fusion of the viral and cellular membranes (Bestle, Heindl et al. 2020). In an alternative 'late pathway,' the virus can enter an intracellular vesicle through endosomal uptake (Bayati, Kumar et al. 2021). Following membrane fusion, the viral RNA is released into the host cell cytoplasm, where it acts as a template for translation by host ribosomes (V'kovski, Kratzel et al. 2021). Initially, the viral RNA is translated into two large polyproteins, pp1a and pp1ab, which are later cleaved by viral proteases (nsp3 and nsp5) into 16 non-structural proteins (nsps) that form the replicase-transcriptase complex (RTC) (Fehr and Perlman 2015). The RTC is responsible for synthesizing a full-length negative-sense RNA template, which is then utilized to generate new positive-sense genomic RNA and a set of subgenomic RNAs (Romano, Ruggiero et al. 2020). The subgenomic RNAs are translated into both structural and accessory proteins, which play a crucial role in the assembly of new virions. The structural proteins (Spike, Envelope, Membrane, and Nucleocapsid) are produced in the host cell's endoplasmic reticulum (ER) and then transported to the Golgi apparatus, where they undergo assembly into new virions (Klein, Cortese et al. 2020). The Nucleocapsid protein binds to the newly synthesized genomic RNA, forming the ribonucleoprotein complex that is packaged into the virion (McBride, Van Zyl et al. 2014). The assembled virions are carried to the cell surface in vesicles and released from the host cell through exocytosis, ready to infect new cells (Ghosh, Dellibovi-Ragheb et al. 2020). The efficiency of this viral life cycle is influenced by various host factors, including the expression levels of ACE2 and TMPRSS2, as well as the presence of viral mutations that can enhance or diminish the virus's ability to replicate and evade the host's immune response (Daniloski, Jordan et al. 2021).

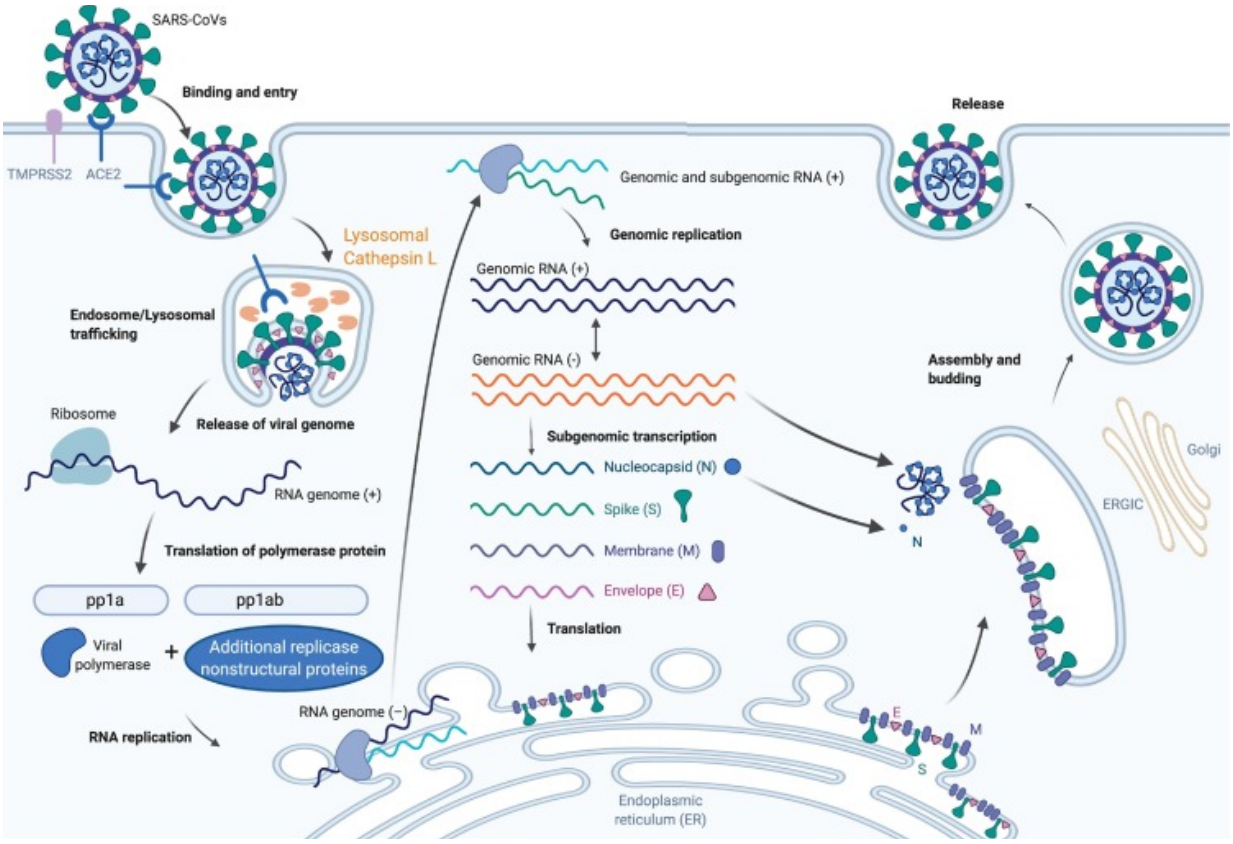

Figure 6 **Schematic of SARS-CoV-2 lifecycle**

The SARS-related coronavirus (SARS-CoV and SARS-CoV-2) lifecycle commences by binding of the envelope Spike protein to its cognate receptor, angiotensin-converting enzyme 2 (ACE2). Efficient host cell entry then depends on: (i) cleavage of the S1/S2 site by the surface transmembrane protease serine 2 (TMPRSS2); and/or (ii) endolysosomal cathepsin L, which mediate virus–cell membrane fusion at the cell surface and endosomal compartments, respectively. Through either entry mechanism, the RNA genome is released into the cytosol, where it is translated into the replicase proteins (open reading frame 1a/b: ORF1a/b). The polyproteins (pp1a and pp1b) are cleaved by a virus-encoded protease into individual replicase complex nonstructural proteins (nsps) (including the RNA-dependent RNA polymerase: RdRp). Replication begins in virus-induced double-membrane vesicles (DMVs) derived from the endoplasmic reticulum (ER), which ultimately integrate to form elaborate webs of convoluted membranes. Here, the incoming positive-strand genome then serves as a template for full-length negative-strand RNA and subgenomic (sg)RNA. sgRNA translation results in both structural proteins and accessory proteins (simplified here as N, S, M, and E) that are inserted into the ER–Golgi intermediate compartment (ERGIC) for virion assembly. Finally, subsequent positive-sense

RNA genomes are incorporated into newly synthesized virions, which are secreted from the plasma membrane (Harrison, Lin et al. 2020)

1.1.5 Pathophysiology of SARS-CoV-2

SARS-CoV-2 triggers a multifaceted pathophysiological response characterized by extensive respiratory, immune, and systemic implications (V'kovski, Kratzel et al. 2021). The virus primarily targets the respiratory system by binding to the angiotensin-converting enzyme 2 (ACE2) receptor, which is expressed in the alveolar epithelial cells of the lungs, as well as in other tissues such as the heart, kidneys, and gastrointestinal tract (Hoffmann, Kleine-Weber et al. 2020). Upon viral entry, the innate immune system is rapidly activated, with pattern recognition receptors such as RIG-I, MDA5, and TLRs detecting viral RNA and triggering downstream signaling cascades that culminate in the production of type I and type III interferons (IFNs) (Park and Iwasaki 2020). These IFNs induce the expression of interferon-stimulated genes (ISGs) that restrict viral replication and shape adaptive immunity (Ana, Ortega-Prieto et al. 2024)

However, SARS-CoV-2 has evolved multiple mechanisms to antagonize IFN responses. Accessory protein ORF6 impedes nuclear translocation of STAT1 and STAT2 by interacting with the nuclear pore complex, effectively blocking IFN-stimulated gene expression (Lisa, Miorin et al. 2020). Similarly, the nonstructural protein NSP1 suppresses host protein synthesis by binding to the 40S ribosomal subunit and promoting host mRNA degradation, thereby preventing the translation of IFN transcripts and antiviral effectors (Katharina, Schubert et al. 2020). In addition to ORF6 and NSP1, other viral proteins target RNA sensing and downstream signaling, leading to reduced IRF3 activation and blunted IFN induction (Daniel, Beyer et al. 2022). The net effect is an imbalanced immune response, with delayed or suppressed IFN production that permits robust viral replication, followed by dysregulated hyperinflammation (Blanco-Melo, Nilsson-Payant et al. 2020).

As viral replication progresses, dendritic cells and macrophages present antigens to T cells and amplify inflammatory cascades. In severe cases, this leads to excessive immune activation, resulting in a "cytokine storm" (Fajgenbaum and June 2020). This state is characterized by markedly elevated levels of pro-inflammatory mediators, including interleukin-6 (IL-6), interleukin-1 beta (IL-1β), and tumor necrosis factor-alpha (TNF-α), which drive widespread

tissue damage and contribute to systemic symptoms such as fever, fatigue, and malaise (Luka, Hiti et al. 2025). In the lungs, this uncontrolled inflammation may culminate in acute respiratory distress syndrome (ARDS), defined by diffuse alveolar damage, increased pulmonary vascular permeability, and impaired oxygen exchange (Matthay, Aldrich et al. 2020; Giuliano, Ramadori. 2022).

Beyond the acute phase, SARS-CoV-2 infection can also result in persistent post-viral complications known as long COVID or post-acute sequelae of SARS-CoV-2 infection (PASC) (Nalbandian, Sehgal et al. 2021). Patients with long COVID frequently report symptoms such as chronic fatigue, dyspnea, cough, chest pain, palpitations, and neurocognitive dysfunction ("brain fog"), which may last for months after viral clearance (Sudre, Murray et al. 2021). Proposed mechanisms include low-level viral persistence in immune-privileged sites, chronic immune activation with aberrant cytokine production, endothelial dysfunction leading to microvascular injury, autonomic nervous system dysregulation, and sustained tissue damage from the initial infection (Xiaoming, Wu et al. 2024; Zaw, Hein et al. 2025). Importantly, impaired or dysregulated IFN responses during the acute phase, in part due to viral antagonists such as ORF6 and NSP1, have been linked to inadequate viral clearance and may predispose patients to long-term sequelae (Maximilian, Hirschenberger et al. 2021).

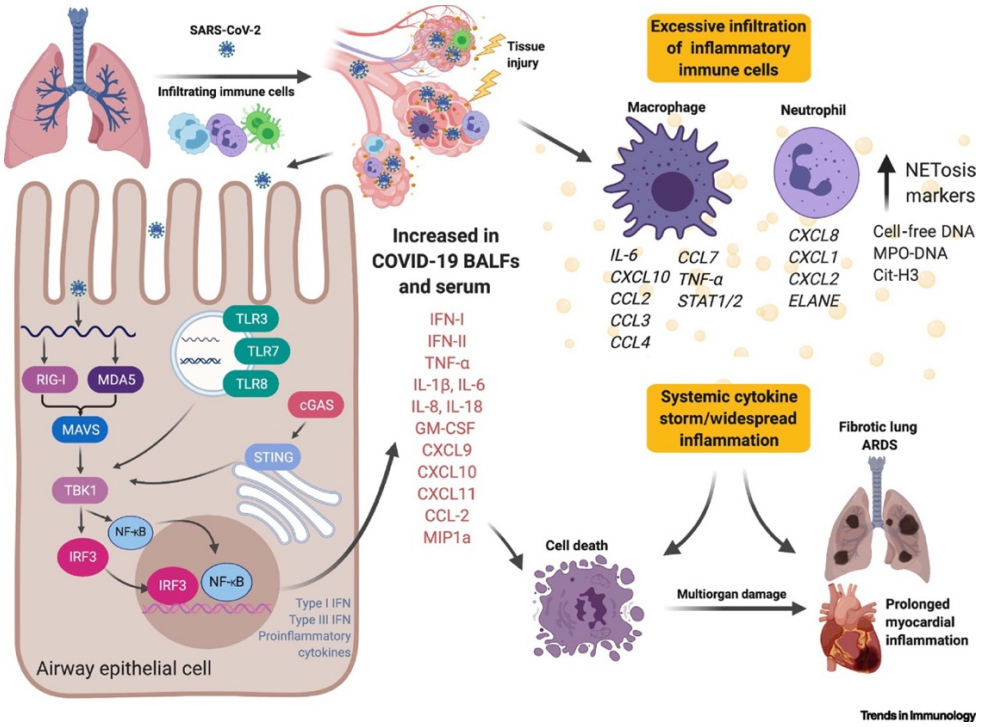

Figure 7 **A brief review of lung pathology in patients with COVID-19**

Following inhalation of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) into the respiratory tract, the virus traverses deep into the lower lung, where it infects a range of cells, including alveolar airway epithelial cells, vascular endothelial cells, and alveolar macrophages. Upon entry, SARS-CoV-2 is likely detected by cytosolic innate immune sensors, as well as endosomal toll-like receptors (TLRs) that signal downstream to produce type-I/III interferons (IFNs) and proinflammatory mediators. The high concentration of inflammatory cytokines/chemokines amplifies the destructive tissue damage via endothelial dysfunction and vasodilation, allowing the recruitment of immune cells, in this case, macrophages and neutrophils. Vascular leakage and compromised barrier function promote endotheliitis and lung edema, limiting gas exchange that then facilitates a hypoxic environment, leading to respiratory/organ failure. The inflammatory milieu induces endothelial cells to upregulate leukocyte adhesion molecules, thereby promoting the accumulation of immune cells that may also contribute to the rapid progression of respiratory failure. Hyperinflammation in the lung further induces transcriptional changes in macrophages and neutrophils that perpetuate tissue damage that ultimately leads to irreversible lung damage. Recent evidence suggests that systemic inflammation induces long-term sequela in heart tissues. Abbreviations: BALF, bronchoalveolar lavage fluid; IRF3, interferon regulatory factor 3; NF-κB, nuclear factor-κB; RIG-I, retinoic acid-inducible gene I; STAT1/2, signal transducer and activator of

transcription 1/2; STING, Stimulator of interferon genes (Harrison, Lin et al. 2020)

1.1.6 Transmission of SARS-CoV-2

The spread of SARS-CoV-2 is influenced by a complex interplay of biological, environmental, and social factors, which collectively determine the virus's transmission within and between populations (Li, Guan et al. 2020) (Du, Wang et al. 2020). SARS-CoV-2 primarily spreads through respiratory droplets and aerosols released by infected individuals during activities such as talking, coughing, and sneezing (Jayaweera, Perera et al. 2020). The estimated basic reproduction number (R0) is approximately 2.2, based on early case tracking at the onset of the pandemic (Liu, Gayle et al. 2020), with a doubling time of 5 days (Li, Guan et al. 2020). While transmission through contaminated surfaces (fomites) is possible, it is considered a less common route of transmission (Kampf, Todt et al. 2020). The viral load in the upper respiratory tract, which is highest during the early stages of infection, is closely linked to the efficiency of transmission, often occurring before symptoms appear, making pre-symptomatic and asymptomatic individuals significant contributors to the virus's spread (Zou, Ruan et al. 2020); (Johansson, Quandelacy et al. 2021). Environmental factors such as temperature, humidity, and ventilation play also a crucial role in the transmission dynamics of diseases. Poor ventilation and crowded indoor settings are associated with a higher risk of airborne transmission (Allen and Marr 2020) (Moriyama, Hugentobler et al. 2020). The emergence of new SARS-CoV-2 variants with increased transmissibility, such as the Delta and Omicron variants, has further complicated efforts to control the spread of the virus (Campbell, Archer et al. 2021), necessitating the adoption of more stringent public health measures and the acceleration of vaccination campaigns (Bar-On, Flamholz et al. 2020). Public health interventions, including widespread testing, contact tracing, quarantine, and isolation, have been critical in controlling the spread of SARS-CoV-2, particularly in the absence of effective vaccines during the early stages of the pandemic (Hellewell, Abbott et al. 2020).

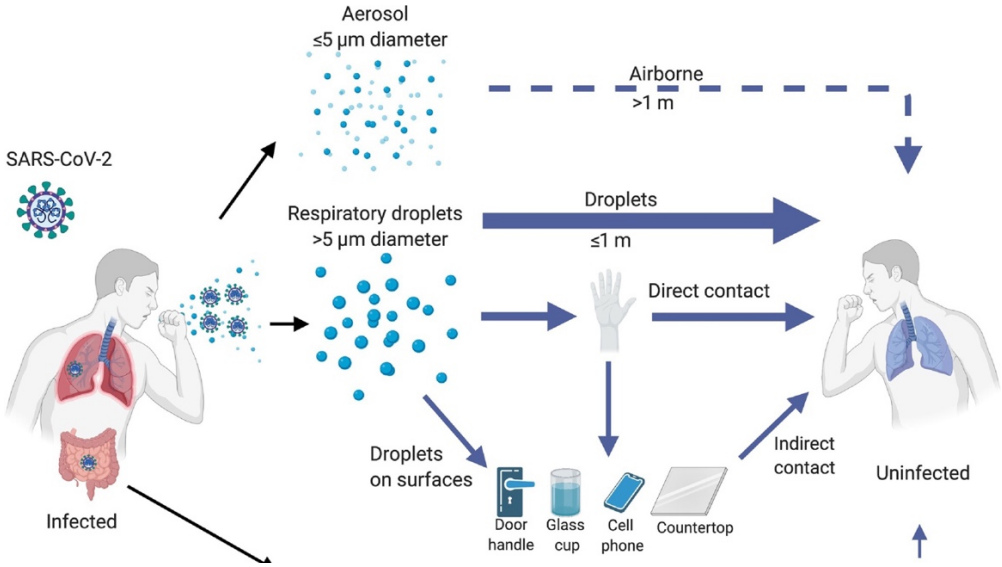

Figure 8 **Proposed SARS-CoV-2 transmission routes**
The ongoing COVID-19 pandemic has resulted in numerous accounts of different transmission routes between humans. Droplet transmission (>5 μm) has been identified as the predominant and most strongly implicated mode of spread during the pandemic. Direct contact spread from one infected individual to a second, naïve person has also been considered a driver of human-to-human transmission, especially in households with close interactions between family members. Solid arrows show confirmed viral transfer from one infected person to another, with a declining gradient in arrow width denoting the relative contributions of each transmission route. Dashed lines show the plausibility of transmission types that have yet to be confirmed. SARS-CoV-2 symbol in 'infected patient' indicates where RNA/infectious virus has been detected (Harrison, Lin et al. 2020)

1.1.7 SARS-CoV-2 pandemic

The global impact of the SARS-CoV-2 pandemic, which originated in late 2019, has been significant in terms of both public health and socio-economic stability ((WHO) 2020). As of July 2024, the worldwide confirmed cases of COVID-19 have exceeded 770 million, leading to over 7 million deaths, as reported by the World Health Organization ((WHO) 2024). This indicates a global case fatality rate (CFR) of approximately 0.9% (University 2024). The United States has documented the highest number of infections and fatalities globally, with over 104 million

confirmed cases, constituting around 13.5% of global cases. The U.S. has reported more than 1.1 million deaths, accounting for roughly 16% of the global death toll ((CDC) 2024). In Canada, there have been approximately 4.9 million confirmed cases of COVID-19, accounting for roughly 0.64% of global cases. The country has reported over 53,000 deaths, representing about 0.77% of the global fatalities, resulting in a CFR of approximately 1.08% (Canada 2024) Canada's extensive vaccination campaign has been a crucial part of its public health response. By mid-2023, over 100 million vaccine doses had been administered ((PHAC) 2023). Around 85% of Canada's total population had received at least one dose of a COVID-19 vaccine, with approximately 83% being fully vaccinated with two doses (Canada 2024). Globally, vaccination campaigns have resulted in the administration of over 13 billion vaccine doses by October 2023 ((WHO) 2023). Approximately 69% of the world's population has received at least one dose of a COVID-19 vaccine, and about 65% are fully vaccinated (Data 2023). However, vaccine distribution has been uneven; in low-income countries, only around 24% of people have received at least one dose ((WHO) 2023). This inequality raises concerns about the ongoing risk of virus transmission and the potential emergence of new variants in under-vaccinated regions (Pitzer, Chitwood et al. 2021).

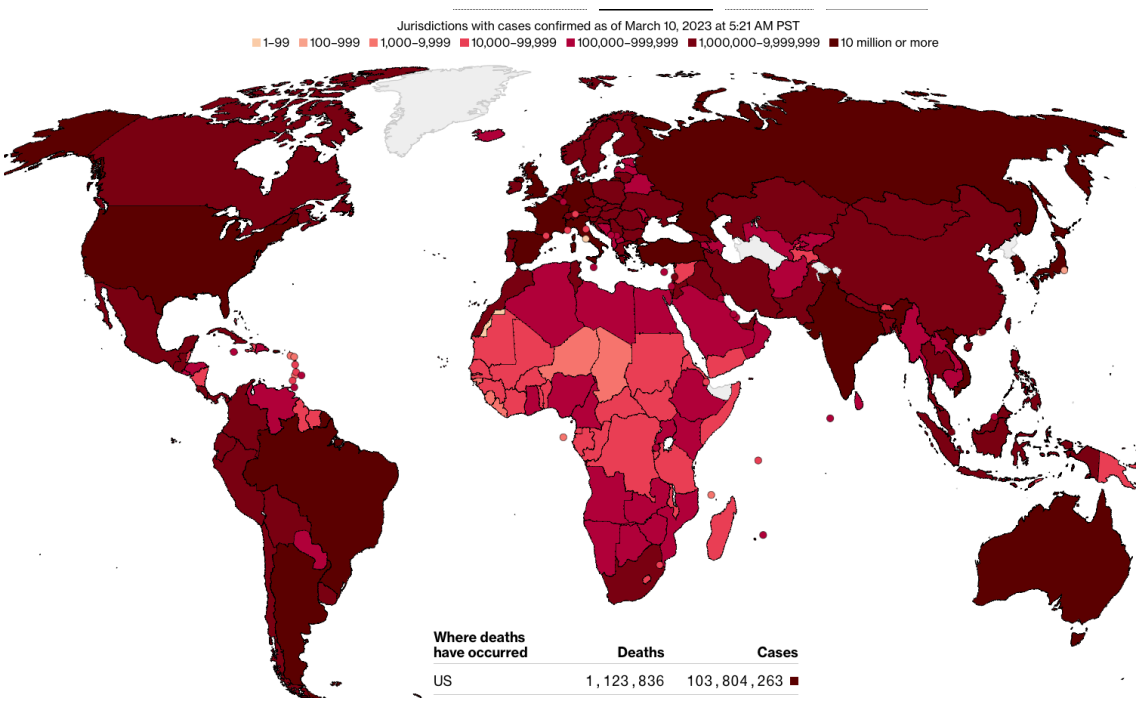

Figure 9 **Mapping the coronavirus outbreak across the world**
Image source Bloomberg

1.1.8 Overview of Current SARS-CoV-2 Treatments and Preventive Measures: Effectiveness and Challenges

The approach to treating COVID-19 has progressed rapidly since the start of the pandemic. Initially, treatment options were limited, leading medical professionals to repurpose existing drugs with antiviral effects, such as remdesivir (Beigel, Tomashek et al. 2020). Originally developed to combat the Ebola virus, remdesivir acts as an RNA polymerase inhibitor (Wang, Zhang et al. 2020). It was administered early in the pandemic and granted emergency use authorization (EUA), but the World Health Organization (WHO) later recommended against its use due to insufficient evidence of a significant impact on mortality (Consortium 2021). Additionally, Paxlovid, a newly developed antiviral drug by Pfizer, is designed to treat mild to moderate cases of COVID-19 (Niraj, Mahajan et al. 2022). The treatment consists of two components: nirmatrelvir and ritonavir (Hammond, Leister-Tebbe et al. 2022). Nirmatrelvir is a protease inhibitor that targets the main protease (Mpro), also known as 3CL protease, essential for SARS-CoV-2 viral replication (Owen, Allerton et al. 2021). Ritonavir, a well-known protease inhibitor used in HIV treatment, is included not for its antiviral effects but to inhibit the CYP3A4 enzyme, which metabolizes nirmatrelvir (Hoffmann, Arora et al. 2021). Furthermore, the use of corticosteroids, particularly dexamethasone, has been shown to reduce mortality in critically ill patients by reducing the hyperinflammatory response commonly seen in severe cases of COVID-19, known as the cytokine storm (Group 2021). Moreover, monoclonal antibodies like bamlanivimab, casirivimab, and imdevimab target the Spike protein to block SARS-CoV-2 from binding to ACE2, reducing viral load and improving outcomes in non-hospitalized COVID-19 patients (Baum, Ajithdoss et al. 2020) (Weinreich, Sivapalasingam et al. 2021). Bamlanivimab inhibits viral entry, while the casirivimab-imdevimab cocktail enhances neutralization by binding to different Spike protein sites (Gottlieb, Nirula et al. 2021) (Chen, Nirula et al. 2021).

The development of effective COVID-19 vaccines has transformed the fight against the pandemic (Krammer 2020). mRNA vaccines, such as Pfizer-BioNTech's BNT162b2 and Moderna's mRNA-1273, demonstrated high efficacy in preventing symptomatic COVID-19 in large Phase III trials (Polack, Thomas et al. 2020) (Baden, El Sahly et al. 2021). These vaccines deliver synthetic mRNA encoding the SARS-CoV-2 spike (S) protein, encapsulated in lipid nanoparticles for cellular delivery, inducing strong immune responses with neutralizing antibodies

and T-cell activation (Walsh, Frenck Jr et al. 2020) (Sahin, Muik et al. 2020). Adenoviral vector vaccines like AstraZeneca's ChAdOx1-S and Johnson & Johnson's Ad26.COV2.S use modified adenoviruses to transport the S protein gene into host cells (Sadoff, Gray et al. 2021). AstraZeneca's vaccine showed varying efficacy based on dosing intervals, while Johnson & Johnson's single-dose vaccine demonstrated efficacy against moderate to severe COVID-19, with higher efficacy for severe cases (Voysey, Clemens et al. 2021) (Sadoff, Gray et al. 2021). Protein subunit vaccines, such as Novavax's NVX-CoV2373, directly introduce the S protein with an adjuvant to boost immune response and have shown effectiveness against different variants (Vohra-Miller and Schwartz 2022) (Heath, Galiza et al. 2021). Inactivated virus vaccines like Sinovac's CoronaVac and Sinopharm's BBIBP-CorV employ chemically inactivated whole viruses to elicit an immune response, with varying efficacy rates in trials (Gao, Bao et al. 2020) (Palacios, Batista et al. 2021) (Al Kaabi, Zhang et al. 2021).

Current drugs and vaccines for SARS-CoV-2 encounter significant limitations, with one major challenge being the virus's high mutation rate (McLean, Kamil et al. 2022). Variants with spike mutations, such as Delta and Omicron, can partially evade immune recognition, which diminishes the neutralizing effect of antibodies and reduces the efficacy of both vaccines and therapeutic monoclonal antibodies. This adaptability necessitates frequent updates to vaccines and antibody treatments, complicating the effort to maintain protection as new variants emerge (Ramesh, Govindarajulu et al. 2021, Fernandes, Inchakalody et al. 2022). While vaccine-induced immunity is initially robust, studies indicate that protection against infection may wane significantly within a few months (Townsend, Hassler et al. 2022). This decline in immunity has resulted in the need for booster doses to sustain efficacy, adding logistical complexities, particularly in lower-income regions where vaccine distribution and access are already limited (Wald 2022). Antiviral drugs, including remdesivir and Paxlovid, exhibit limited effectiveness and can lead to viral rebound when administered later in the course of infection, thus reducing their utility in treating severe COVID-19 cases or those progressing to critical stages (Parums 2022). These treatments are most effective when given early, requiring timely diagnosis and access to care, which is not always feasible in resource-limited settings (Wang, Zhao et al. 2023). Additionally, widespread use of antiviral medications may lead to the development of resistance, as observed in other viral infections, posing a future risk to their effectiveness (Vitiello 2022). Moreover, current vaccines and treatments do not adequately address long COVID, a complex

condition characterized by persistent symptoms such as fatigue, cognitive impairment, and respiratory issues that can linger for months (Sivan, Greenhalgh et al. 2022). The mechanisms underlying long COVID remain poorly understood, and existing preventive measures do not fully mitigate its risks (Greenhalgh, Sivan et al. 2024). This highlights the urgent need for further research into therapeutics that target long-term effects, as well as the development of broader-spectrum vaccines that can induce immunity beyond the spike protein, potentially providing more durable and variant-resistant protection (Forni and Mantovani 2021).

1.2 General Introduction to Viral Vectors

Viral vectors are engineered viruses designed to deliver genetic material into cells for applications such as drug screening, gene therapy, and vaccine development. These vectors are modified to eliminate their pathogenic components, enabling them to function as vehicles for transporting specific genes into host cells (Zhang and Godbey 2006). Common types of viral vectors include adenoviruses, lentiviruses, and vesicular stomatitis viruses, each offering unique advantages regarding transduction efficiency, tissue targeting, and immune response (Warnock, Daigre et al. 2011).

1.2.1 Lentiviral Vectors

Lentiviral vectors (LVs), which are derived from the human immunodeficiency virus (HIV), have a unique ability to integrate stably into the host genome, allowing for long-term expression of genes (Milone and O'Doherty 2018). The construction of lentiviral vectors typically involves three key plasmids (Dull, Zufferey et al. 1998): The transfer vector plasmid, contains the gene of interest (transgene) as well as essential regulatory elements such as promoters, enhancers, and polyadenylation signals (Escors and Breckpot 2010). The transgene is flanked by long terminal repeats (LTRs) derived from the original lentivirus, which are crucial for the vector's integration into the host genome (Zufferey, Dull et al. 1998). The packaging plasmid encodes the viral proteins necessary for packaging the RNA genome into viral particles, including gag (structural proteins), pol (reverse transcriptase, integrase, and protease), and rev (regulator of viral RNA export) (Dull, Zufferey et al. 1998). These genes are expressed in the producer cell line but are not included in

the final viral particle, ensuring that the vector is replication-incompetent (Naldini, Blömer et al. 1996). The envelope plasmid encodes a viral glycoprotein that determines the tropism of the lentiviral vector. The most used envelope protein is the Vesicular Stomatitis Virus G (VSV-G) protein, which confers a broad tropism, enabling the lentiviral vector to infect a wide range of cell types across different species. The VSV-G protein is also stable, enhancing the durability of the viral particles during production and purification (Burns, Friedmann et al. 1993).

The generation of lentiviral vectors is mostly conducted in HEK293T cells, a human embryonic kidney cell line commonly utilized for efficient transfection (Naldini, Blömer et al. 1996). The process includes co-transfecting the transfer, packaging, and envelope plasmids into these cells (Sakuma, Barry et al. 2012). Following transfection, the HEK293T cells initiate the production of viral particles by encapsulating the RNA copy of the transfer vector into viral capsids, which are then enveloped by the VSV-G protein (Tiscornia, Singer et al. 2006). The newly formed lentiviral particles are released into the culture medium, from which they are collected and concentrated using methods such as ultracentrifugation or filtration (Elegheert, Behiels et al. 2018).

Additionally, lentiviral vectors effortlessly integrate into the host genome via the viral integrase enzyme, ensuring stable and long-term transgene expression (Coffin, Hughes et al. 1997). This integration allows the genetic material to be maintained and passed on during cell division, making lentiviral vectors particularly useful for applications that require sustained gene expression, such as the development of stable cell lines (Naldini, Blömer et al. 1996). Furthermore, lentiviral vectors possess broad tropism, allowing them to infect a wide range of cell types, including difficult-to-transduce cells like neurons and hematopoietic stem cells (Cronin, Zhang et al. 2005). Moreover, lentiviral vectors offer a relatively large packaging capacity, capable of accommodating transgenes up to 8-10 kilobases, which surpasses other viral vectors like adeno-associated viruses (AAV) (Kumar, Keller et al. 2001). This large capacity makes lentiviral vectors particularly well-suited for delivering complex or multi-gene constructs (Kang, Stein et al. 2002).

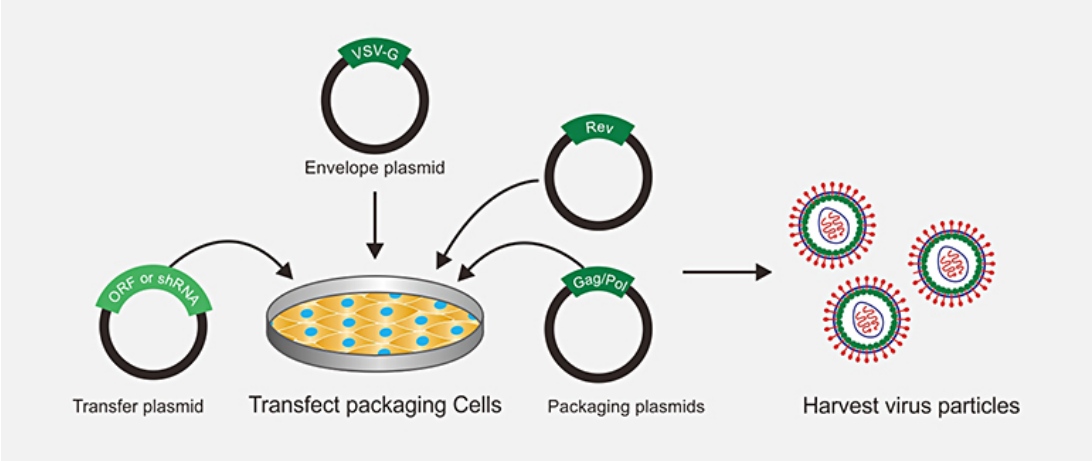

Figure 10 **Schematic of lentiviral vector production**
(Image source Cyagen)

1.2.2 Other Viral Vectors

In addition to lentiviral vectors, various other viral vectors, such as vesicular stomatitis virus (VSV), pseudotyped vectors, and adenoviral vectors, are widely utilized in drug screening and vaccine development due to their unique characteristics (Bouard, Alazard-Dany et al. 2009). Vesicular stomatitis virus (VSV), an enveloped RNA virus, is particularly valuable in vaccine design due to its strong tropism and capacity to elicit a robust immune response (Roberts, Buonocore et al. 1999). Pseudotyped viral vectors involve modifying the envelope proteins of a primary virus with those from another virus, which broadens the host range or targets specific cell types. This approach is frequently employed with retroviral and lentiviral vectors to enhance their entry into particular cells (Joglekar and Sandoval 2017). Adenoviral vectors, which originate from adenoviruses, are favored for their high transduction efficiency and capability to deliver genes to both dividing and non-dividing cells. However, they often trigger a strong immune response, which, while advantageous for vaccine development, limits their suitability for long-term gene expression in therapeutic applications (Volpers and Kochanek 2004).

1.3 Overview of Viral Vectors Used in SARS-CoV-2 Research

In the context of SARS-CoV-2, viral vectors can be engineered to deliver genetic material into host cells and can be tailored to express components of the virus for drug screening and vaccine development (Chen, Keiser et al. 2018). This can be achieved through complementation, wherein structural proteins are expressed in trans to compensate for deleted genes in the viral genome. For instance, a modified SARS-CoV-2 RNA genome may be engineered to lack specific structural protein-coding sequences, depending on a co-expressed plasmid to produce these critical proteins (Zhang, Liu et al. 2021). This trans expression facilitates the independent production of viral components while preserving the integrity of the modified RNA genome. Such strategies enhance both the safety and efficacy of viral vectors in applications related to drug screening and vaccine development (Signor and Nuzhdin 2018).

1.3.1 Applications of Viral Vectors Used in SARS-CoV-2 Research

In the context of SARS-CoV-2, viral vectors that express the spike protein initiate their action by interacting with the ACE2 receptor, simulating the viral entry pathway (Hoffmann, Kleine-Weber et al. 2020). Host proteases such as TMPRSS2 further enhance this process by cleaving the spike protein, enabling membrane fusion or endocytosis. These mechanisms enable researchers to explore entry inhibitors that can block the spike-ACE2 interaction (Matsuyama, Nao et al. 2020); (Jackson, Farzan et al. 2022). Following internalization, the viral vector delivers its genetic content often encoding viral proteins or reporter genes for easy detection (Howarth, Lee et al. 2010) (Kang and Chung 2008). Several systems utilize viral vectors that transport non-infectious RNA replicon systems. These replicons enable long-term expression of viral RNA and proteins without generating infectious particles (Xie, Muruato et al. 2020). This allows researchers to evaluate compounds that target RNA synthesis, protein translation, or replication complexes, offering crucial insights into the efficacy of antivirals at different stages of the viral life cycle (Riva, Yuan et al. 2020).

In drug screening, viral vectors commonly express a reporter gene to quantify infected cell in real-time. Fluorescent proteins or luminescent markers are used to track viral entry, replication, and protein synthesis, making them extremely valuable for high-throughput screening assays (Ellinger, Pohlmann et al. 2020). The intensity of fluorescence or luminescence is directly related

to viral activity, allowing researchers to evaluate how effectively a compound inhibits viral processes (Liu, Pan et al. 2023). Additionally, viral vectors can be modified to include mutations from emerging SARS-CoV-2 variants, enabling rapid evaluation of treatments targeting variant-specific proteins (Harvey, Carabelli et al. 2021).

1.3.2 Pseudotyped Lentivirus Systems for Investigating SARS-CoV-2 Entry

Pseudotyped lentivirus systems have emerged as a crucial tool for investigating SARS-CoV-2 (Hyseni, Molesti et al. 2020). These systems incorporate the SARS-CoV-2 spike (S) protein into the replication-incompetent lentivirus envelope to create a 'pseudotyped' virus. This model allows for the safe study of SARS-CoV-2 entry mechanisms in biosafety level 2 (BSL-2) conditions, reducing the risks of handling the live virus (Condor Capcha, Lambert et al. 2021). Furthermore, this system offers significant advantages for studying the functional effects of spike protein mutations, on viral entry efficiency, receptor binding affinity, and susceptibility to neutralizing antibodies (Shukla, Roelle et al. 2024). Moreover, the pseudovirus system can be easily adapted to express spike proteins from emerging variants of concern (VOCs), making it an essential tool for tracking viral evolution, changes in transmissibility, and immune evasion (Zhu, Yang et al. 2022). However, this system has its limitations. Since these pseudoviruses lack all viral proteins except for S and replication machinery, they do not completely the full lifecycle of SARS-CoV-2. This limitation restricts their utility in studying downstream events following viral entry, such as replication, transcription, and immune evasion (Hu, Gao et al. 2020). Additionally, lentiviral pseudotyping may not accurately mimic the conformational dynamics of the SARS-CoV-2 spike protein as it appears in the complete virus, which could impact the reliability of results in studies centred on the spike-ACE2 interaction (Millet, Tang et al. 2019). Furthermore, these pseudoviruses do not account for other SARS-CoV-2-specific factors that could influence cell entry and the immune response, potentially leading to discrepancies when translating findings to actual SARS-CoV-2 infections (Tan, Wang et al. 2023).

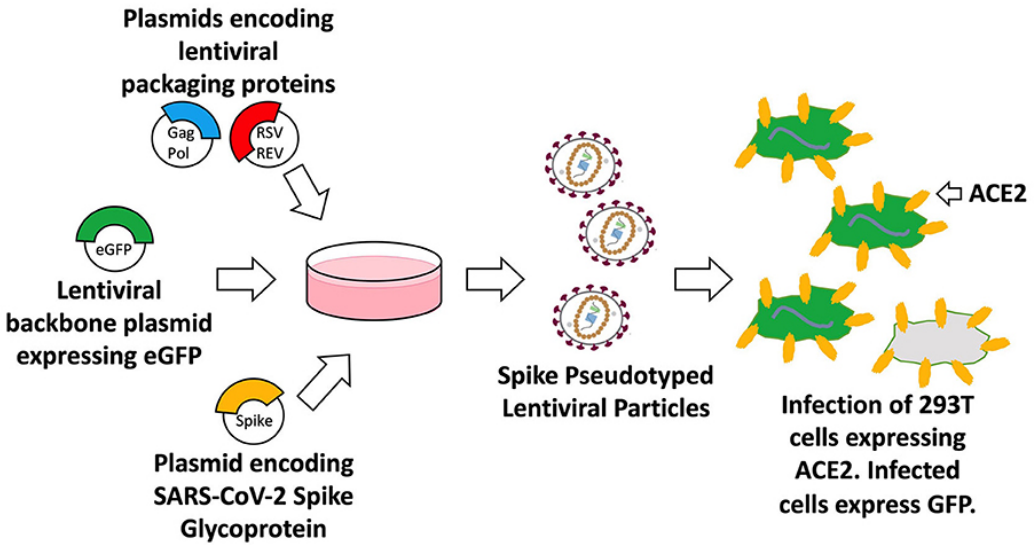

Figure 11 **General approach for SARS-CoV-2 lentiviral pseudotyping** The spike (S) glycoprotein variants were packaged in PKR-/- HEK-293T cells alongside plasmids encoding for a 3rd generation HIV-1 lentivirus and a plasmid encoding for an eGFP transgene (Ayoub, Purkayastha et al. 2021)

1.3.3 Pseudotyped Vesicular Stomatitis Virus (VSV) Systems: Tools for Exploring SARS-CoV-2 Entry

SARS-CoV-2 S-pseudotyped VSV vectors are widely used in drug screening against SARS-CoV-2 (Salazar-García, Acosta-Contreras et al. 2022). These vectors are modified to express the SARS-CoV-2 spike (S) protein on their surface, replacing the native VSV glycoprotein (G), thus mimicking the entry of SARS-CoV-2 into host cells via the ACE2 receptor (Fukushi, Watanabe et al. 2008). One notable study utilizing these systems aimed to screen small molecule inhibitors for their ability to block SARS-CoV-2 spike-mediated entry into host cells (Khan, Partuk et al. 2024). Moreover, the SARS-CoV-2 S-pseudotyped VSV vectors are commonly used in neutralization assays, allowing for the quantification of antibody-mediated inhibition of viral entry (Case, Rothlauf et al. 2020) (Avila-Herrera, Kimbrel et al. 2024). Furthermore, these vectors can be utilized as a vaccine platform (Case, Rothlauf et al. 2020) (Todesco, Gafuik et al. 2024). Despite their advantages, SARS-CoV-2 S-pseudotyped VSV vectors present notable limitations. One major concern is the potential for pre-existing immunity within certain populations, stemming

from prior exposure to VSV or related viruses (Tober, Banki et al. 2014). Furthermore, while SARS-CoV-2 S-pseudotyped VSV-based vectors are effective at inducing immune responses, they may not faithfully mimic the complex mechanisms of SARS-CoV-2 infection, which can limit their ability to generate immune protection specific to SARS-CoV-2 (Liniger, Zuniga et al. 2007). Lastly, the risk of recombination with wild-type VSV strains during co-infection poses a theoretical biosafety concern, highlighting the need for careful evaluation during vaccine development and deployment (Condit, Williamson et al. 2016)

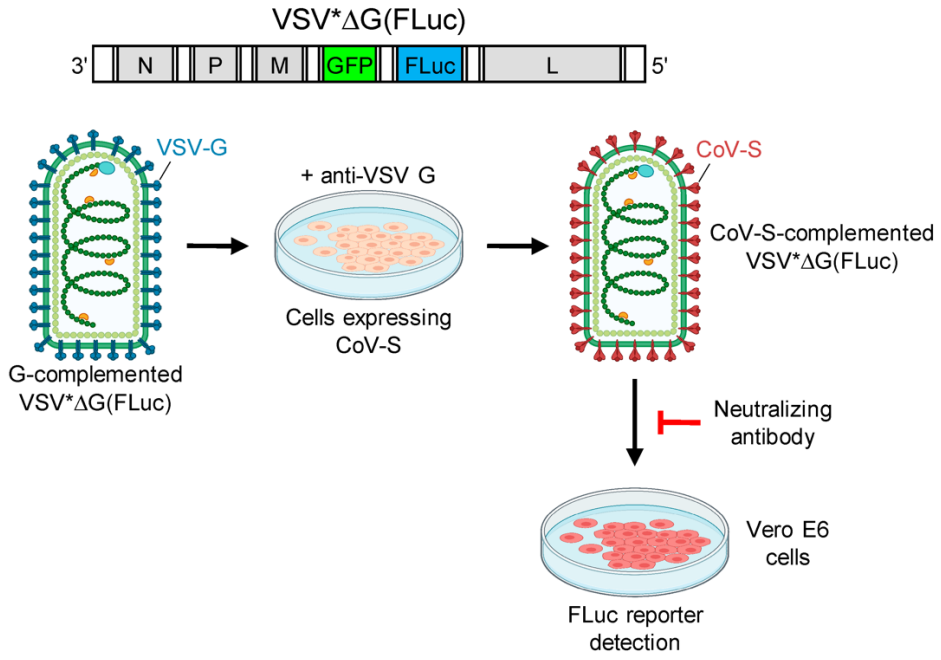

Figure 12 **Pseudotyping of the vesicular stomatitis virus reporter virus with the SARS-CoV-2 spike protein**

The RNA genome of this virus encodes for the nucleoprotein (N), the phosphoprotein (P), the matrix protein (M), the green fluorescent protein (GFP), the firefly luciferase (FLuc), and the large RNA polymerase (L), but lacks the viral glycoprotein (G) gene. Propagation of the G-deficient VSV*ΔG(FLuc) on cells expressing the VSV G protein results in trans-complemented viruses (G-complemented VSV*ΔG(FLuc), depicted in blue), which are used to infect cells transiently expressing either the SARS-CoV-1 or the SARS-CoV-2 spike protein. In the presence of the VSV-neutralizing antibody Mab I1 (anti-VSV G), pseudotype VSV*ΔG(FLuc) particles bearing the corresponding CoV spike protein in the envelope (depicted in purple) are released. Infection of Vero E6 cells by these pseudotype particles is inhibited by spike-specific neutralizing antibodies. The pseudotype virus infection rate is

determined by measuring the FLuc reporter activity in the cell lysates 18 h post-infection (Zettl, Meister et al. 2020)

1.3.4 Adenoviral Vectors as Platforms for SARS-CoV-2 Vaccine Development

Adenovirus vectors have been broadly utilized in developing vaccines, including those against SARS-CoV-2, due to their ability to deliver genetic material into host cells and stimulate a strong immune response (Mendonça, Lorincz et al. 2021). These vectors are engineered to carry the gene encoding the spike (S) protein of SARS-CoV-2, which is the primary target of neutralizing antibodies (Chavda, Bezbaruah et al. 2023). One key advantage of adenoviral vectors is their ability to induce robust and long-lasting immune responses, engaging both humoral and cellular responses (S. Ahi, S. Bangari et al. 2011). Furthermore, adenovirus vectors can be rapidly designed and produced, making them particularly attractive for developing vaccines during the COVID-19 pandemic (Sallard, Zhang et al. 2023) (Joe, Chopra et al. 2023). However, the adenovirus vector system presents several limitations. A significant concern is the pre-existing immunity within the human population; many individuals have previously been exposed to adenoviruses, leading to the presence of neutralizing antibodies that can diminish the vaccine's efficacy (Zaiss, Machado et al. 2009). Additionally, the size of the adenoviral vector restricts the volume of genetic material that can be delivered, potentially limiting the inclusion of other viral antigens necessary for a comprehensive immune response (Sun, Li et al. 2000). Furthermore, adenoviral vectors may trigger strong innate immune responses, which can result in adverse effects or rapid clearance of the vector from the body, thereby reducing the vaccine's overall effectiveness (Fausther-Bovendo and Kobinger 2014). Lastly, while adenovirus vectors can elicit robust immune responses, they do not offer the same level of durability as some other vaccination platforms, such as mRNA vaccines, which may impact long-term immunity against variants of SARS-CoV-2 (Bangari and Mittal 2006).

1.3.5 SARS-CoV-2 Viral Vectors for Drug Screening

Research on drugs targeting SARS-CoV-2 is confronted with significant challenges due to the virus's airborne transmission and the potential for severe illness (Kaufer, Theis et al. 2020). A primary objective in this area is to develop SARS-CoV-2-based vectors that carry reduced

biosafety risks (Ghosh, Brown et al. 2020). These vectors should also integrate marker genes to facilitate easy, cost-effective, and sensitive quantification of infections (Jiang, Xing et al. 2008). Various research teams have created SARS-CoV-2-based vectors that include the virus's structural proteins, thereby providing a more accurate representation of coronaviral infections. Typically, these systems utilize a complementation strategy, where a subgenomic RNA that lacks certain structural proteins is introduced into mammalian cells, accompanied by the expression of the missing proteins from alternative sources. The resulting vector particles can complete a single round of infection and are then harvested from the culture supernatants (Thiel, Karl et al. 2003). One of the more straightforward iterations of this model was developed by Syed et al., who utilized plasmids for the four primary structural proteins of SARS-CoV-2 (S, E, M, N), in conjunction with mRNA encoding reporter proteins and signals for virion encapsidation (Syed, Taha et al. 2021). While this system offers flexibility, it also presents certain limitations, such as the short duration of marker protein expression and the inability to study replication post-viral entry, as it lacks the presence of viral RNA, preventing replication. Recently, another research team enhanced this model by incorporating amplification elements from the Venezuelan equine encephalitis virus (VEEV), which extended the duration of marker expression (Liu and Liu 2023). Some research groups have integrated the viral replication machinery into their systems, enabling the exploration of viral replication processes following entry. For instance, one team created a simplified model using a SARS-CoV-2 replicon RNA that substitutes the N protein with GFP, allowing replication in cells that express N, and facilitating the production of SARS-CoV-2 vectors capable of single-cycle infections in N-deficient cells (Ju, Zhu et al. 2021). Another group established a vector system based on an S-deleted SARS-CoV-2 replicon RNA, complemented by an S-expressing plasmid (Ricardo-Lax, Luna et al. 2021). Despite the advancement of various viral vectors designed to enhance the study of SARS-CoV-2, there remains a significant gap in the availability of vectors that effectively incorporate both the complete genome and the protein components of the virus. Moreover, the use of these vectors remains complicated, often necessitating repeated transfection of replicon RNA along with one or more plasmids to express the complementary structural proteins (Liu and Liu 2023).

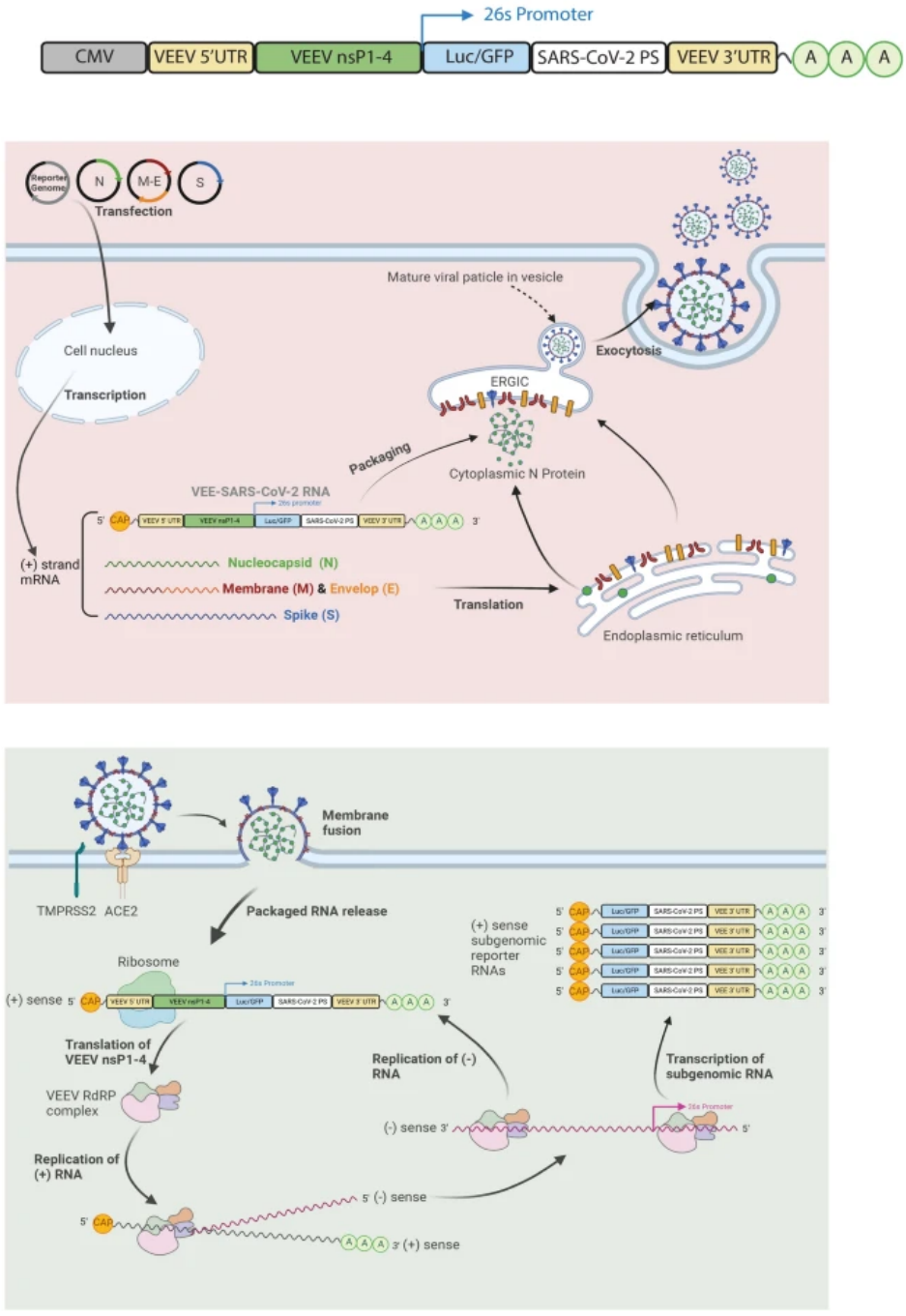

Figure 13 **Design of VEE-SARS-CoV-2 viral vectors**

Schematic diagram of the core elements in VEE-SARS-CoV-2 plasmid including a CMV promoter, 5' UTR and nsP1-4 gene of VEEV, 26 subgenomic VEEV promoter (26S), reporter genes (Luc or GFP), the SARS-CoV-2 packaging signal (PS9 sequence), 3' UTR of VEEV genome, and a poly(A) signal. (**B**) Mechanistic illustration of production of VEE-SARS-CoV-2 viral vectors.

Components of viral vectors were produced in 293T cells by co-transfection with plasmids carrying structural protein genes (S, M-E, N) and reporter gene with regulatory elements for amplification and expression. The viral vectors are assembled and released from 293T cells. (**C**) Schematic presentation of transduction of viral vectors. RNA amplification and reporter gene expression are accomplished in target cells via ACE2-mediated internalization, translation of VEEV nsP1-4 genes, formation of VEEV nsP1-4 protein complex, and synthesis of the negative-strand RNA. The newly synthesized negative-strand RNA serves as the template for synthesis of positive strand RNA and production of sub-genomic RNA encoding reporter gene (Liu and Liu 2023)

1.3.6 Constraints of SARS-CoV-2 Viral Vectors

The use of viral vectors for drug screening against SARS-CoV-2 is associated with several limitations that can impact the efficacy and interpretation of results (Liu and Liu 2023). Viral vectors does not faithfully replicate the complete viral lifecycle or the host-cell interactions complex observed in natural infections (Robert-Guroff 2007). For instance, vectors carrying non-replicating viral genomes may not fully mimic all aspects of viral replication, overlooking drugs that target specific stages of the viral lifecycle (Spunde, Korotkaja et al. 2022). Moreover, the selection of viral vectors and the method of pseudotyping can influence the specificity and efficiency of receptor binding and entry, leading to variability in experimental outcomes (Tomanin and Scarpa 2004).

Another limitation is the immune response triggered by viral vectors may not accurately represent the response triggered by an actual SARS-CoV-2 infection. This could impact the evaluation of antiviral agents (Nayak and Herzog 2010). Viral vectors may not fully mimic the host immune responses or cytokine profiles observed during a natural infection, influencing the effectiveness of immune-modulating drugs (Tse, Moller-Tank et al. 2015). Additionally, the expression levels of viral proteins from vectors may not precisely mirror the expression levels during natural infection, potentially impacting the sensitivity of screening assays (Bouard, Alazard-Dany et al. 2009).

Another obstacle is the complexity and expense involved in producing high-titer viral vector batches. This process demands specialized facilities and expertise, which restricts access to

these vectors to research programs with substantial funding (Van Der Loo and Wright 2016). Furthermore, technical challenges such as vector stability, and reproducibility, can restrict the use of viral vectors in high-throughput drug screening (Srivastava, Mallela et al. 2021). Inconsistencies may arise from variations in vector production, transduction efficiency, and reporter gene expression, impacting result accuracy (Logan, Nightingale et al. 2004). Moreover, the transduction efficiency of viral vectors varies across different cell types (Ellis, Hirsch et al. 2013). For instance, some vectors may not efficiently transduce certain cell types relevant for studying SARS-CoV-2 infection, such as primary human lung cells or specific immune cell subsets This limitation can hinder the exact modelling of SARS-CoV-2 infection in relevant cellular conditions (Fu, Tao et al. 2021).

1.4 Introduction to Virus-Like Particles (VLPs)

Virus-like particles (VLPs) are macromolecular structures that resemble authentic viruses but do not contain the viral genome (Roldão, Mellado et al. 2010) (Grgacic and Anderson 2006). These particles consist of viral structural proteins, which naturally self-assemble into nanoparticle structures replicating the external morphology of the original virus (Zeltins 2013) (Chroboczek, Szurgot et al. 2014). The key advantage of VLPs lies in their capacity to present viral antigens in a highly organized and repetitive format, essential for eliciting robust and specific immune responses (Bachmann and Jennings 2010).

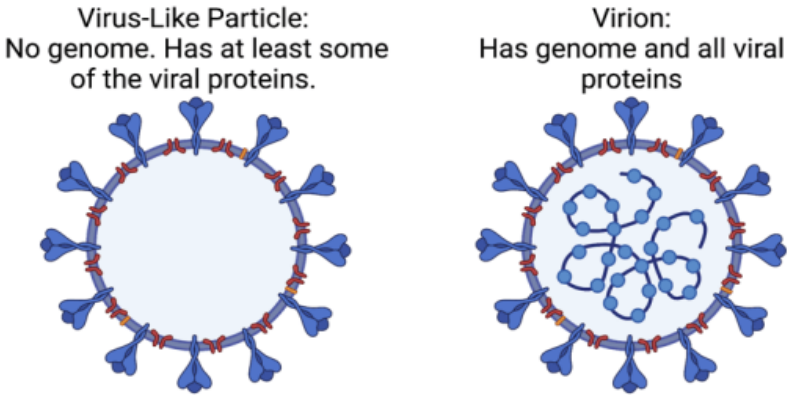

Figure 14 **Virus-like particule vs virion**
(Image source Biorender)

1.4.1 Structural Morphology and assembly of SARS-CoV-2 VLPs

The morphology and assembly of SARS-CoV-2 virus-like particles (VLPs) are designed to mimic the virus's structure without containing infectious genetic material (Swann, Sharma et al. 2020). Early investigations into VLPs, based on research on hepatitis B and influenza viruses, provided the framework for understanding how viral proteins can self-assemble into particles that closely resemble the native virus, without containing its genetic material. This offers a safer platform for vaccine development (Pumpens and Grens 1999). The process of assembling SARS-CoV-2 VLPs begins with the co-expression of the S, E, and M proteins in various expression systems such as insect cells, plant, or mammalian cells (Moon, Jeon et al. 2022) (Mi, Xie et al. 2021). These proteins are produced and then transported to the endoplasmic reticulum and Golgi apparatus, where they assemble to form VLPs (Kumar, Hawkins et al. 2021). The E and M proteins aid in the release of these particles from the host cell membrane, while the S protein becomes part of the budding VLPs, orienting itself externally to mimic its natural expression on SARS-CoV-2 (Xu, Shi et al. 2020). The N protein, which typically encloses the viral RNA genome, plays a role in stabilizing the VLPs structure (Chen, Yan et al. 2021). The lipid bilayer of VLPs is derived from the host cell membrane, incorporating the viral proteins into a structure that closely resembles the native virus’s envelope (Naskalska and Pyrć 2015). The yielding Virus-Like Particles (VLPs) have a spherical shape with a diameter ranging from 100 to 150 nanometers. They display an outer layer of spike proteins arranged in a crown-like structure (Kumar, Singh et al. 2022). The defined organization of the spike proteins is essential for replicating the receptor-binding domain (RBD) and other antigenic sites of the native virus (Syed, Taha et al. 2021).

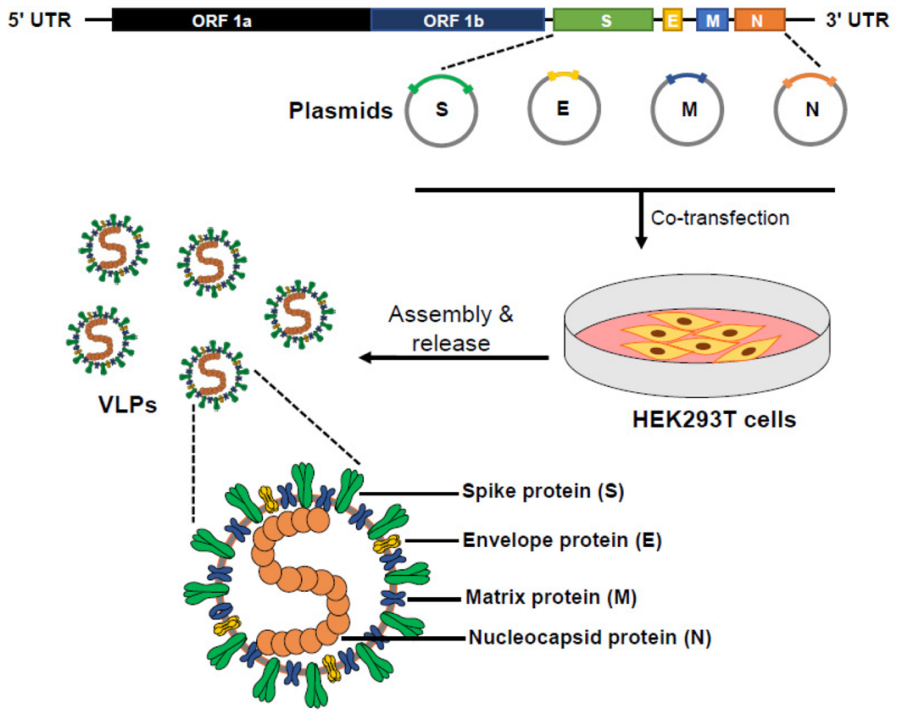

Figure 15 **Schematic outline of SARS-CoV-2 virus-like particle production**
Plasmids encoding severe acute respiratory syndrome coronavirus 2 (SARS-CoV2) structural proteins E, M, N and S are co-transfected in HEK293T cells. VLPs are collected from culture media between 24 and 72 h post-transfection (Kumar, Hawkins et al. 2021)

1.4.2 Immunogenic Properties of VLPs

Virus-like particles (VLPs) are designed to mimic the structural and antigenic properties of the natural virus (Tagliamonte, Tornesello et al. 2017). They display viral antigens in a repetitive and organized manner, crucial for stimulating strong immune responses (Tariq, Batool et al. 2022). This repetitive presentation enhances the cross-linking of B-cell receptors, leading to robust B-cell activation and high-affinity antibody production (Zhang, Cubas et al. 2009). The structural integrity of VLPs also activates Toll-like receptors and other pattern recognition receptors on antigen-presenting cells like dendritic cells and macrophages, triggering innate immune responses and promoting antigen presentation to T cells (Zepeda-Cervantes, Ramírez-Jarquín et al. 2020). This leads to a comprehensive adaptive immune response and the generation of neutralizing antibodies that block pathogen interactions and provide protection against future infection (Fraser, Tomassini et al. 2007). In addition to eliciting strong systemic antibody responses, VLPs can also

stimulate cellular immunity (Nooraei, Bahrulolum et al. 2021). They can be engineered to contain epitopes that are processed and presented by major histocompatibility complex (MHC) class I and class II molecules, resulting in the activation of cytotoxic T lymphocytes (CTLs) and helper T cells, respectively (McFall-Boegeman and Huang 2022). This activation is crucial for eliminating infected cells and establishing long-lasting immune memory (Braun, Jandus et al. 2012). The capacity of VLPs to stimulate both $CD4^+$ T helper cells and $CD8^+$ cytotoxic T cells enhances their potential as vaccines for viruses with high mutation rates (Fuenmayor, Gòdia et al. 2017).

Moreover, when VLPs are administered intranasally, they have the unique ability to trigger mucosal immunity, which is particularly important for respiratory pathogens like SARS-CoV-2 (Monrat, Chulanetra et al. 2024). Intranasal delivery allows VLPs to interact directly with the nasal-associated lymphoid tissue (NALT), where specialized antigen-presenting cells induce robust local immune responses. This results in the production of secretory IgA (sIgA) at mucosal surfaces, which provides a first line of defense by neutralizing pathogens at their entry site (Eleni, Kehagia et al. 2023). In parallel, mucosal vaccination also recruits resident memory T cells (T_RM) to the respiratory mucosa, offering rapid and localized immune protection upon re-exposure to the virus (Henry, Sutanto et al. 2025). Importantly, mucosal immunity complements systemic immunity by both reducing infection at the portal of entry and limiting viral shedding, thereby lowering transmission (Zhihao, Zhang et al. 2025).

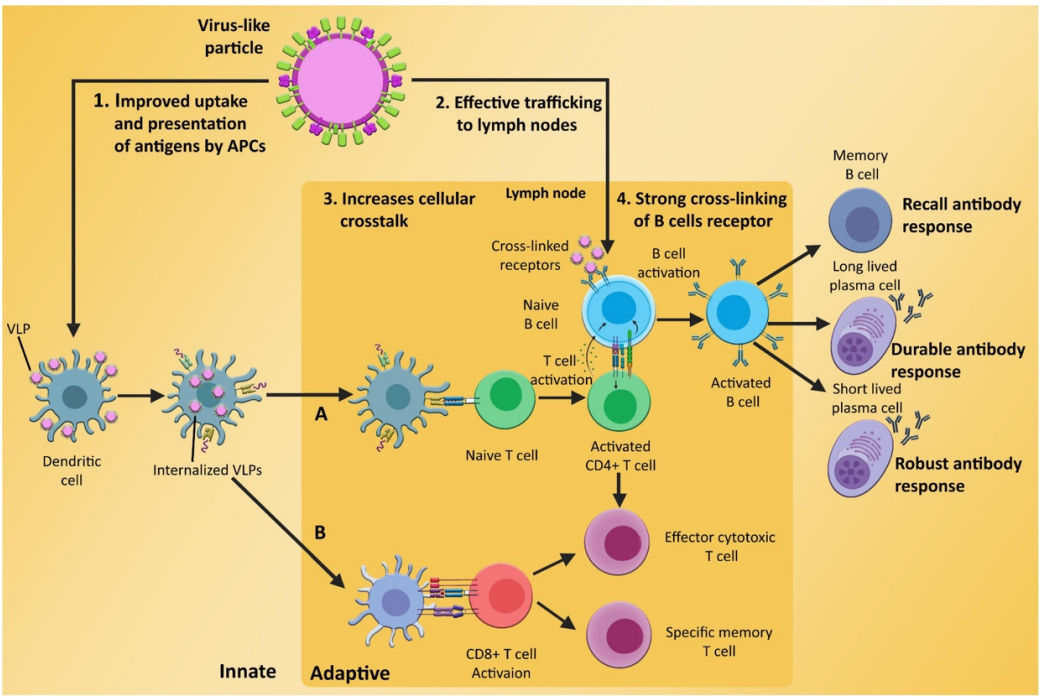

Figure 16 **Induction of innate and adaptive immunological responses** **(A)** humoral immunity; **(B)**cell-mediated immunity) by VLPs, (1) enhanced absorption and presentation of antigens based on VLP by APCs such as dendritic cells, which inform T cells about potential risks, (2) efficient VLP trafficking to lymph nodes, a crucial site for adaptive immunological responses, (3) improved cellular communication between B cells, T cells, and APCs, and (4) the ability of VLP-based antigen to effectively cross-link and activate B cells receptors, which develop into memory cells and long and short lived plasma cells after antigen exposure (Tariq, Batool et al. 2022)

1.4.3 Applications of Virus-Like Particles in SARS-CoV-2 Research

SARS-CoV-2 virus-like particles (VLPs) have emerged as a promising platform for vaccine development due to their capability to mimic the structure of the virus without carrying its infectious genome (Tariq, Batool et al. 2022). When used as a vaccine, VLPs can effectively stimulate both humoral and cellular immune responses by encouraging the immune system to recognize and react to the spike protein (Yong, Liew et al. 2022). This mechanism closely resembles natural infection, enabling the immune system to prepare defenses against future exposures to the actual virus (Gao, Xia et al. 2023). One notable example of a SARS-CoV-2 VLP vaccine is the one developed by the Technological Research Council of Turkey (TUBITAK) in

collaboration with the Middle East Technical University. This VLP platform incorporates the N, M, E and S proteins of SARS-CoV-2 (Yilmaz, Ipekoglu et al. 2022). In contrast to many other vaccine platforms currently in clinical use, this SARS-CoV-2 VLP-based vaccine candidate is administered via subcutaneous injection. By integrating multiple structural components, it aims to provide a more comprehensive mimicry of the SARS-CoV-2 virus, which could elicit a broader immune response targeting various aspects of the viral structure rather than concentrating solely on the spike protein (Mandal 2021). The inclusion of multiple viral proteins may enhance the vaccine's potential to induce both humoral and cellular immune responses, potentially increasing its efficacy against different variants of SARS-CoV-2 (Motamedi, Ari et al. 2021). Despite its promising potential, challenges remain in scaling up production and optimizing formulations to ensure effectiveness across diverse populations (Dhawan, Saied et al. 2023).

Another property of VLPs is their ability to be engineered into 'mosaic' vaccines. This innovative approach requires combining antigens from multiple viral strains or different viruses onto a single VLP (Liu, Zhao et al. 2023). In the case of SARS-CoV-2, this could entail creating VLPs that present spike proteins from various SARS-CoV-2 variants, such as Alpha, Beta, Delta, and Omicron (Boix-Besora, Gòdia et al. 2023) (Kaabi, Yang et al. 2022). Mosaic VLPs can elicit a broad immune response against multiple viral variants simultaneously, thus enhancing the vaccine's effectiveness and longevity. This could result in better protection against a diverse range of viral strains and potentially reduce the need for frequent booster doses (Chang, Zeltins et al. 2021). Additionally, mosaic VLPs could be utilized to combine antigens from different pathogens, potentially leading to the development of universal vaccines (Naskalska and Pyrć 2015). For example, VLPs could be engineered to include not only SARS-CoV-2 spike proteins but also antigens from influenza viruses or other respiratory pathogens. This approach has the potential to yield vaccines that protect against multiple infectious diseases with a single administration (Denis, Acosta-Ramirez et al. 2008) (Jaishwal, Jha et al. 2024).

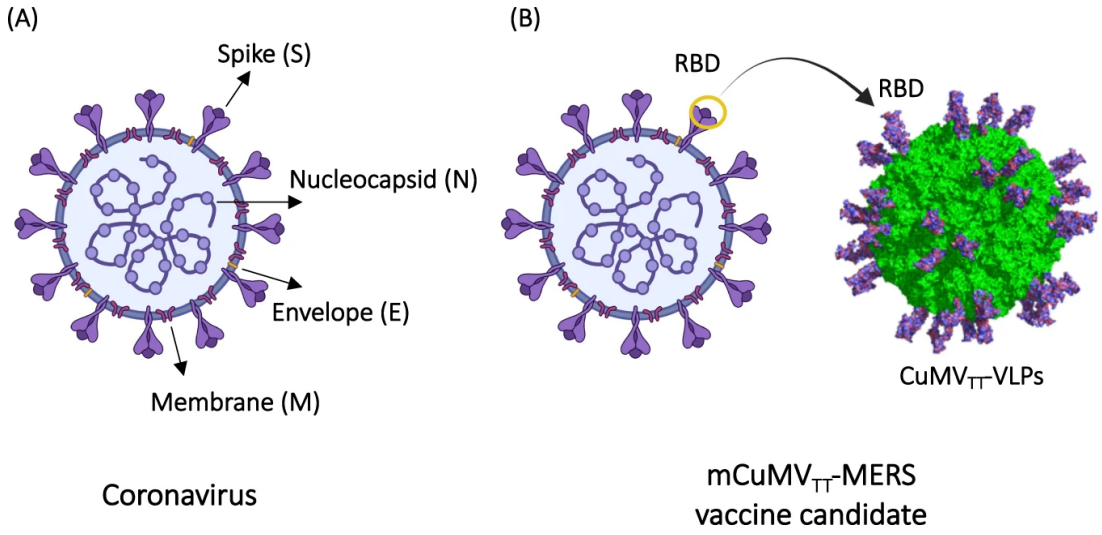

Figure 17 **Strategy of developing a mosaic VLP-based vaccine** by genetically fusing the RBD of MERS-CoV into the optimized CuMVtt -VLPs which incorporate a universal TT epitope and TLR7/8 ligand (Mohsen and Bachmann 2022)

Another potential application of VLPs lies in their capacity to serve as candidates for intranasal vaccines against SARS-CoV-2 (Moreno-Fierros, García-Silva et al. 2020). Intranasal delivery of VLP-based vaccines targets the mucosal immune system, the first line of defence in respiratory infections like COVID-19. This route of administration induces both systemic and mucosal immunity, generating localized IgA responses in the nasal passages, which are crucial for neutralizing the virus at the entry site (Rothen, Krenger et al. 2022). Additionally, intranasal VLP vaccines can stimulate robust cellular immunity, including T-cell responses, essential for long-term protection (Diallo, Ní Chasaide et al. 2023). One example is a study that developed a VLP-RBD vaccine by utilizing a virus-like particle antigen consisting of a Hepatitis B surface antigen (HbsAg) with SARS-CoV-2 WA-1 receptor binding domain (RBD) proteins bound to its surface using the irreversible SpyTag/SpyCatcher conjugation system. When administered intranasally in a murine model, the vaccine elicited a strong neutralizing antibody response and robust mucosal IgA production, preventing viral replication in the upper respiratory tract. Furthermore, it induced a significant T-cell response, protecting against subsequent viral challenge (Lee, Rader et al. 2023). The study highlights the potential of VLPs in intranasal vaccines as a needle-free delivery system

that enhances patient compliance and reduces logistical challenges associated with vaccine administration (Kar, Devnath et al. 2022).

1.4.4 Challenges and Limitations in the Development and Application of SARS-CoV-2 VLPs

The utility of VLPs is restricted by several fundamental limitations. Although VLPs mimic the structural proteins of the virus they do not contain a viral genome. This limitation prevents VLPs from fully mimicking the viral replication cycle or the associated pathogenesis observed in live SARS-CoV-2 infections (Mohsen, Zha et al. 2017). Another limitation is the challenge of correctly assembling VLPs with structurally accurate SARS-CoV-2 proteins, particularly the spike (S) protein, which must be presented in its functional trimeric form for optimal immunogenicity (Duan, Zheng et al. 2020). This is critical because only correctly folded spike proteins with an intact receptor-binding domain (RBD) will effectively elicit neutralizing antibodies (He, Zhou et al. 2004). Furthermore, VLPs are often generated in heterologous expression systems, such as yeast or insect cells. These systems do not replicate the post-translational modifications (PTMs) that occur in the natural human cellular environment (Dai, Wang et al. 2018). These variations can impact the antigenicity of the VLPs and may lead to differences in the immune response triggered by the actual SARS-CoV-2 virus, potentially impacting the efficacy and safety of vaccines developed using VLPs (Cervera, Gòdia et al. 2019). Moreover, to enhance the immunogenicity of VLPs, adjuvants are often required yet finding an adjuvant that is both effective and safe can complicate regulatory approval processes (Cimica and Galarza 2017).

Another challenge in the production of virus-like particles is scalability, especially for complex or large-scale applications. Ensuring consistent quality and purity of VLPs across production batches is crucial for their successful commercialization and clinical use (Gupta, Arora et al. 2023). Achieving this consistency is particularly challenging when structural proteins must be transfected during each production cycle (Mittal, Banerjee et al. 2022). The transient transfection methods commonly employed in VLP production can introduce variability in protein expression levels and folding, resulting in inconsistencies in VLP assembly, quality, and potency from batch to batch (Vicente, Roldão et al. 2011). As a result, optimization of purification techniques is vital for addressing these challenges and enhancing the efficiency of VLP production

(Pattenden, Middelberg et al. 2005). Another obstacle is the possibility of pre-existing immunity to the viral capsid proteins used in VLPs. Pre-existing antibodies in the population may reduce the effectiveness of VLP-based vaccines by neutralizing the particles before they can trigger a protective immune response (Chuan, Rivera-Hernandez et al. 2013).

1.5 Introduction to Replicons

Replicons are distinct nucleic acid molecules, either DNA or RNA, capable of independent replication within a host cell. This ability is attributed to an origin of replication (Ori), a specific sequence of nucleotides that initiates the replication process (Hass, Gölnitz et al. 2004). Moreover, replicons often harbour genes encoding proteins or enzymes essential for their replication, enabling them to replicate and persist within the host (Kato, Date et al. 2003). Due to their self-sustaining nature, replicons are invaluable tools in molecular biology, genetic engineering, and therapeutic applications, facilitating controlled gene expression and the development of sophisticated genetic systems (Bartenschlager 2005).

1.5.1 General Overview of SARS-CoV-2 Replicon Systems

SARS-CoV-2 replicons are sophisticated molecular tools derived from the viral RNA genome. They are created by removing the genes responsible for encoding structural proteins such as the spike (S), envelope (E), and membrane (M) (Ricardo-Lax, Luna et al. 2021). These proteins are crucial for assembling infectious virions but not for viral RNA replication. This deletion renders the replicon non-infectious, making it safer for laboratory use while still retaining the ability to replicate autonomously within host cells (He, Quan et al. 2021). Moreover, the replicon contains essential non-structural proteins (nsps) encoded within the open reading frame 1a/1b (ORF1a/ORF1b) region, which are vital for RNA replication and transcription (He, Quan et al. 2021). Of these, the RNA-dependent RNA polymerase (RdRp, also known as nsp12) is crucial for synthesizing new viral RNA molecules. It collaborates with nsp7 and nsp8 as cofactors to ensure efficient RNA synthesis (Kotaki, Xie et al. 2021). Other nsps, such as nsp13 (helicase), nsp14 (exoribonuclease), and nsp16 (2'-O-methyltransferase), play critical roles in viral replication fidelity, capping, and evasion of the host immune system (Yuen, Lam et al. 2020).

In the context of a replicon system, researchers typically insert reporter genes, such as luciferase or GFP, into the viral genome to monitor real-time replication (Zhang, Deng et al. 2021). The level of reporter expression directly corresponds to the replicon's activity, enabling researchers to quantitatively measure RNA replication and evaluate the impact of potential antiviral drugs by assessing the reduction in reporter signal (Nguyen, Falzarano et al. 2021). This method offers a secure and effective platform for high-throughput screening of compounds that inhibit viral replication (Tanaka, Saito et al. 2022).

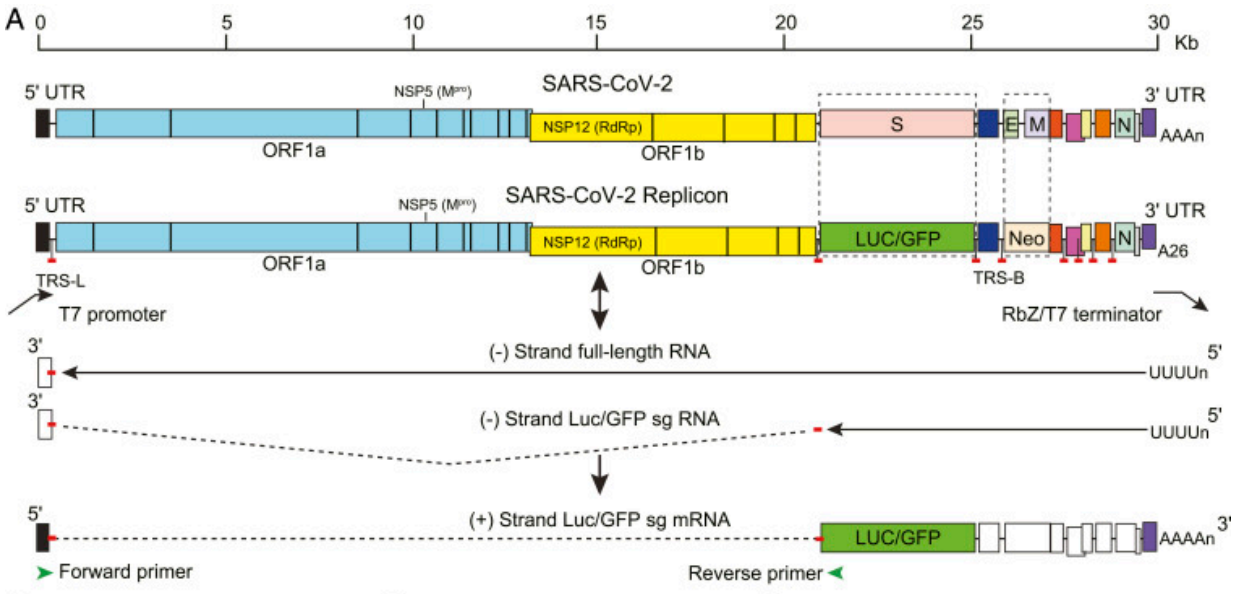

Figure 18 **Construction and transcriptional analysis of a SARS-CoV-2 replicon** Schematic diagram of reporter replicon genome structure, replication, and sg mRNA production. The S and E/M ORFs in the wt viral genome were deleted and replaced with Luc/GFP and Neo genes, respectively (He, Quan et al. 2021)

1.5.2 Mechanisms of Replication of SARS-CoV-2 Replicons

The replication of SARS-CoV-2 replicons mimics the mechanisms utilized by the full viral genome, leveraging the host cell machinery and exploiting the unique characteristics of its single-stranded, positive-sense RNA genome (Wang, Zhang et al. 2021). Upon entering the host cell cytoplasm, the replicon RNA, which typically contains the viral elements necessary for replication but lacks structural genes, acts as a direct template for translation by host ribosomes (Liu, Chou et al. 2022). The initial translation of open reading frame 1a (ORF1a) and ORF1b results in the

production of two large polyproteins, pp1a and pp1ab, which are subsequently cleaved by viral proteases (such as 3CLpro and PLpro) into nonstructural proteins (NSPs) (V'kovski, Kratzel et al. 2021). These NSPs, particularly NSP12 (RNA-dependent RNA polymerase, RdRp), NSP7, and NSP8, constitute the core of the replication-transcription complex (RTC), which drives RNA synthesis (Gao, Yan et al. 2020). NSP12 synthesizes a negative-sense RNA intermediate, which then acts as a template to produce new positive-sense RNA molecules. These positive-sense RNAs can serve as templates for further replication or as mRNA for the synthesis of accessary proteins (Romano, Ruggiero et al. 2020). In SARS-CoV-2 replicons, the absence of structural genes prevents the formation of infectious virions; however, the replicon still maintains the ability to replicate and transcribe subgenomic RNAs (Jin, Lin et al. 2021). Furthermore, the viral genome contains crucial regulatory elements, such as the 5′ untranslated region (UTR) with a leader sequence, transcription-regulating sequences (TRS), and the 3′ UTR. These elements form a complex regulatory network for replication and transcription (Yang and Leibowitz 2015). The TRS facilitates discontinuous transcription, enabling the production of subgenomic RNAs that encode accessory and nonstructural proteins, thus supporting the replicative cycle (Kim, Lee et al. 2020). SARS-CoV-2 replicons can serve as a model for understanding replication mechanisms without the biosafety risks associated with working with the complete infectious virus (He, Quan et al. 2021).

1.5.3 Application of SARS-CoV-2 Replicons systems in Vaccine Development and Antiviral Drug Screening

In the field of vaccine research, SARS-CoV-2 replicons are engineered to incorporate reporter genes (Nakamura, Kotaki et al. 2022). These replicons retain the essential replication machinery but lack structural genes, allowing protein synthesis in host cells without producing infectious virions. This makes them excellent platforms for eliciting a strong immune response without the risk of infection (McKay, Hu et al. 2020). One notable example is the self-amplifying RNA (saRNA) vaccine platform (Keikha, Hashemi-Shahri et al. 2021). These replicon vaccines encode the SARS-CoV-2 spike protein and utilize the viral RNA replication machinery to boost the production of spike protein within the host cell, enhancing the immunogenicity with smaller doses of RNA compared to traditional mRNA vaccines (Myatt, Wharram et al. 2023). These self-replicating RNA vaccines have exhibited promising results in preclinical and clinical studies,

presenting a novel approach to vaccine development (Pollock, Cheeseman et al. 2022); (Szubert, Pollock et al. 2023).

Furthermore, SARS-CoV-2 replicons have played a crucial role in the creation of viral vectors for gene delivery. Viral vectors, such as those based on adenoviruses or lentiviruses, can be engineered to carry SARS-CoV-2 replicons (Pamukcu, Celik et al. 2020). These vectors are designed to transport the replicon RNA into host cells, where it undergoes replication and expresses key viral antigens, thereby triggering immune responses while maintaining the safety profile (Liu and Liu 2023). Furthermore, replicon-based vectors have been utilized to study immune responses, enabling researchers to gain deeper insights into how SARS-CoV-2 antigens interact with the host immune system (Lundstrom 2016). These insights are critical for designing next-generation vaccines that not only stimulate robust antibody responses but also enhance T-cell immunity, which is crucial for long-lasting protection (Aliahmad, Miyake-Stoner et al. 2023).

The use of replicon-based viral vectors for drug screening offers a robust platform for evaluating antiviral compounds. By integrating reporter genes such as luciferase or GFP into the replicon, these vectors can be employed in high-throughput drug screening assays (Liu and Liu 2023). These assays enable researchers to monitor replicon replication and gene expression in real time, generating quantifiable data on the efficacy of potential antiviral drugs (Tanaka, Saito et al. 2022). Due to the autonomous replication and absence of the structural components necessary to form infectious virions, replicon systems provide a safer and more controlled environment for screening large compound libraries (Malicoat, Manivasagam et al. 2022). This approach can advance the discovery of inhibitors targeting the SARS-CoV-2 RNA-dependent RNA polymerase (RdRp), as well as assess the effectiveness of inhibitors against emerging variants (He, Quan et al. 2021).

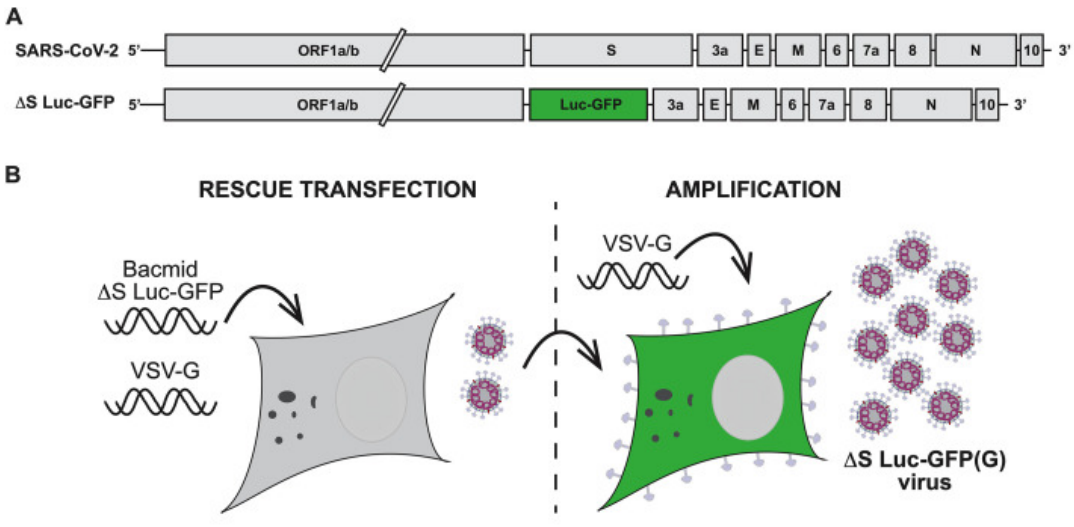

Figure 19 **Development of single-cycle infectious SARS-CoV-2 replicon system with a dual reporter**

Schematic representation of SARS-CoV-2 and ΔS Luc-GFP SARS-CoV-2 genomes. The S gene in the SARS-CoV-2 genome was replaced with a luciferase and GFP dual reporter. (B) Generation and amplification of ΔS virus replicon particles. (Left) 293T/Huh7.5 cell mixture was transfected with ΔS Luc-GFP bacmid and VSV-G plasmid. (Right) Supernatants from ΔS-VRP(G) rescue transfections were amplified in Huh7.5 cells transfected with VSV-G plasmid (Malicoat, Manivasagam et al. 2022)

1.5.4 Challenges and Key Considerations in the Utilization of SARS-CoV-2 Replicons

The utilization of SARS-CoV-2 replicons in scientific research, vaccine development, and drug screening presents several unique challenges. One major challenge is to ensure the safety and biosafety of SARS-CoV-2 replicon systems (Malicoat, Manivasagam et al. 2022). These replicons are designed to lack essential structural genes, which are necessary for generating infectious virions. However, incomplete deletion of these genes or recombination events with viral or cellular components could potentially restore the ability to produce infectious particles (van der Meulen, Smets et al. 2023). This risk highlights the need for validation protocols to confirm the non-infectious nature of the replicons, ensuring safe laboratory practices and minimizing biosafety concerns (Wang, Wang et al. 2008).

Another crucial consideration is the accuracy of SARS-CoV-2 replicons in mimicking the complete viral replication cycle (Li, Teague et al. 2019). While replicons can independently replicate the viral RNA, they may not fully recreate the interactions between structural proteins and host cell factors, which are vital in processes such as viral assembly, immune evasion, and pathogenesis (Bartenschlager 2005). This limitation could impact the relevance of results in understanding viral behaviour, particularly when studying immune responses or the effectiveness of antiviral drugs (Lohmann, Korner et al. 1999).

An additional limitation to consider is that the efficiency of SARS-CoV-2 replicon replication and protein expression is highly dependent on the precise design and integration of different system components. Variations in elements such as the viral replication machinery, regulatory sequences, and plasmid constructs utilized for expression can result in significant variability in replicon performance. These discrepancies can greatly influence the reproducibility and sensitivity of experimental assays, particularly in high-throughput drug screening platforms (He, Quan et al. 2021). Consistently achieving high-level expression of viral components, such as the viral RNA-dependent RNA polymerase (RdRp), is a significant challenge. Any inefficiency in this process has the potential to affect the findings from drug effectiveness studies or immune response evaluations (Xie, Muruato et al. 2020) (Wu, Chen et al. 2023).

1.6 DNA-Launched Systems for SARS-CoV-2 Viral Genomes

1.6.1 Introduction

Utilizing DNA to express viral genomes, rather than RNA, presents an innovative approach in virology that overcomes the challenges associated with RNA manipulation (Usme-Ciro, Lopera et al. 2017). This method requires incorporating the complete viral/vector genome into a DNA vector, which is then introduced into host cells. Once inside the host cells, the DNA is transcribed into RNA, initiating the viral replication process (Ljungberg, Whitmore et al. 2007). Due to the stability and ease of manipulation of DNA, this technique serves as a reliable alternative to traditional RNA-based methods for studying RNA viruses (Leitner, Ying et al. 2000). Furthermore, it plays a critical role in vaccine development and therapeutic research, offering a more efficient and practical platform for these applications (Öhlund, García-Arriaza et al. 2018).

1.6.2 Mechanisms of Action of DNA-Launched Systems for SARS-CoV-2 Viral Genomes

The development of DNA-launched systems for SARS-CoV-2 viral genomes requires the insertion of key viral genes or the entire viral genome into a DNA plasmid vector. This vector is specifically produced to include essential regulatory elements, such as promoters that stimulate high levels of transcription in mammalian cells (Zhang, Fischer et al. 2022). This arrangement guarantees that when the DNA is introduced into host cells, it is transcribed into viral RNA (Feng, Zhang et al. 2022).

Following entry into the host cell, the DNA-launched system triggers the host's transcription machinery to transcribe the viral genome from DNA into mRNA. Afterwards, the mRNA is translated into viral proteins by the host's ribosomes (Nguyen, Falzarano et al. 2021). For example, the expression of the SARS-CoV-2 spike protein facilitates its display on the cell surface, making it a valuable tool for vaccine development or antibody testing (Szurgot, Hanke et al. 2021). In certain systems, the viral RNA transcribed from DNA-launched construct can also initiate self-replication in the presence of necessary viral nonstructural proteins, offering a comprehensive model of viral replication and protein synthesis (Zhang, Song et al. 2021).

DNA-launched systems offer several advantages over RNA manipulation. One key advantage is the stability of DNA constructs, which are less susceptible to degradation compared to RNA, allowing for more reliable and sustained expression in various experimental conditions. DNA-based systems also offer greater flexibility in terms of cloning and modification, enabling easier integration of regulatory sequences, promoters, and other elements critical for optimal expression (Varnavski, Young et al. 2000). Moreover, the use of DNA simplifies the delivery method, as plasmid DNA can be directly introduced into cells through standard transfection protocols, bypassing the need for complex RNA delivery systems. This reduces the risk of RNA degradation and improves the reproducibility and sensitivity of experimental assays (Fan, Hu et al. 2023). Additionally, DNA-launched systems are generally more cost-effective and easier to scale for high-throughput applications, making them advantageous for large-scale screening and vaccine development efforts (Pushko, Lukashevich et al. 2016).

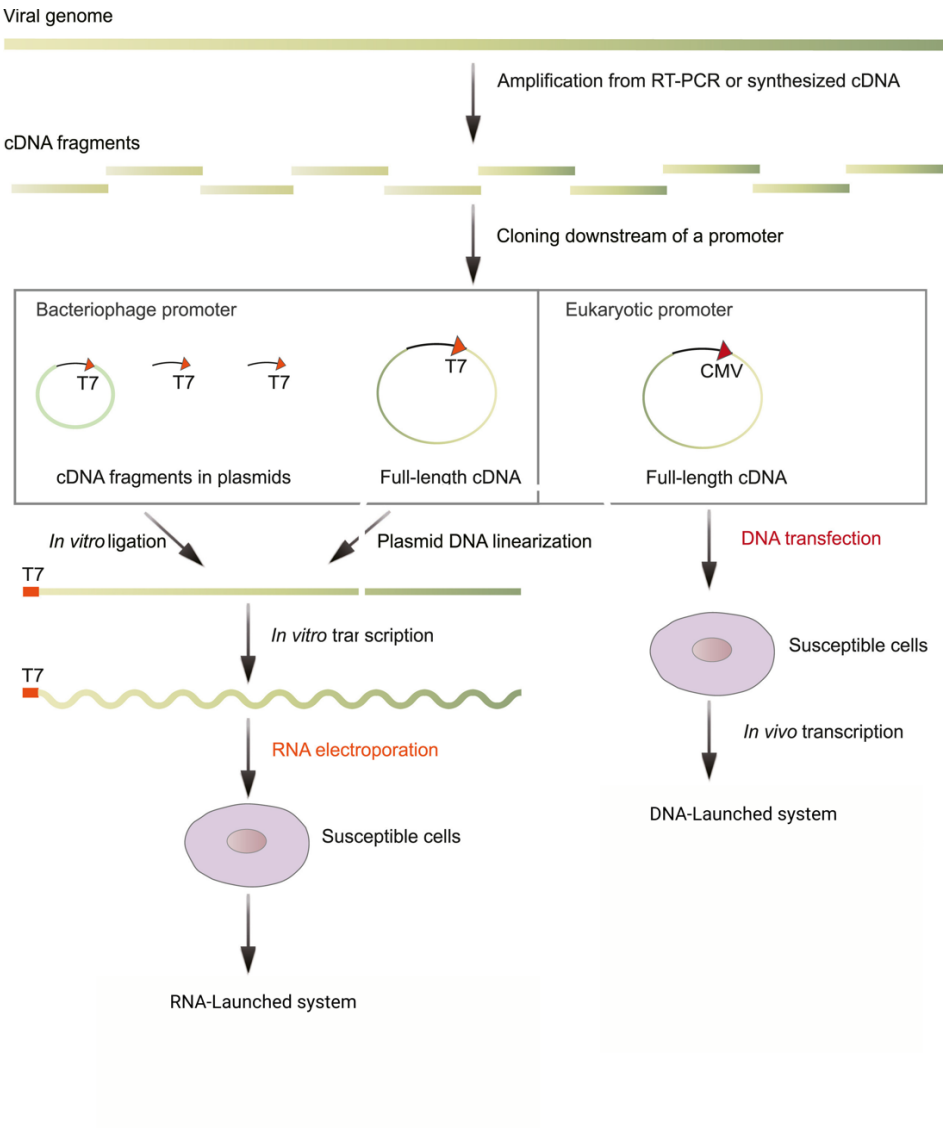

Figure 20 **Genetic procedure for the construction of DNA-launched systems** The viral genome is divided into several fragments that are amplified from RT-PCR or chemically synthesized DNA. These cDNA fragments are stably incorporated into vectors with a bacteriophage promoter or eukaryotic promoter. In RNA-launched systems, cDNA fragments are cloned into plasmids with T7 promoters and used to assemble the full-length cDNA by *in vitro* ligation, or directly achieved by one-step assembly in plasmids. Then, the ligated products or linearized DNA serve as templates for *in vitro* transcription to obtain capped infectious genomic RNA, which will generate infectious viruses by electroporation of RNA transcripts into susceptible cells. In DNA-launched systems, direct transfection of cytomegalovirus (CMV)-driven plasmids containing the full-length cDNA allows initiation of RNA polymerase II-dependent *in vivo* transcription in host cells, leading to the successful rescue of the virus (Cai and Huang 2023)

1.6.3 Applications of DNA-Launched Systems for SARS-CoV-2 Viral Genomes

Several DNA-launched replicon systems have been developed to facilitate safe, high-throughput research on SARS-CoV-2, particularly in antiviral drug discovery and vaccine evaluation (Szurgot, Hanke et al. 2021). One notable example is the SARS-CoV-2 replicon created by Nguyen et al. In their system, the viral nonstructural proteins encoded by ORF1a and ORF1b—essential for RNA replication—are incorporated into a plasmid that lacks structural genes (spike, envelope, and membrane). This plasmid is transfected into mammalian cells, allowing for RNA replication without generating infectious virions, thereby providing a secure model for studying viral replication (Nguyen, Falzarano et al. 2021). Additionally, this replicon features a luciferase reporter gene, which enables quantitative real-time monitoring of viral RNA synthesis through luminescence measurements, thereby offering a straightforward method for assessing the efficacy of antiviral compounds (Nguyen, Falzarano et al. 2021).

Another example is the replicon system developed by Feng et al., which incorporates a green fluorescent protein (GFP) reporter gene to facilitate cell imaging of SARS-CoV-2 replication. This system has proven valuable for evaluating the efficacy of antiviral drugs, such as remdesivir and chloroquine, by monitoring reductions in GFP fluorescence following treatment (Feng, Zhang et al. 2022). Furthermore, the transfection of SARS-CoV-2 DNA replicon systems with plasmids expressing the viral structural proteins has led to the production of single-round infectious particles that mimic viral entry and replication for a single cycle while not generating infectious progeny (Ricardo-Lax, Luna et al. 2021). When combined, the replicon RNA is encapsulated within the VLPs, enabling their entry into target cells and initiating a single replication cycle. These single-round infectious particles are non-propagative because they are missing the complete genomic elements required for subsequent rounds of infection, providing a safer platform for investigating viral entry, replication, and immune responses at lower biosafety levels (Su, Chen et al. 2023). Additionally, some DNA replicon systems are engineered with specific mutations within the replicase genes, allowing researchers to explore drug resistance mechanisms or immune escape variants without the need for live virus (Zhang and Guo 2024). These DNA-launched replicon systems have quickly established themselves as essential tools in SARS-CoV-2 research, offering a robust, safe, and flexible platform for studying viral replication,

screening antiviral drugs, and investigating the immune response to viral proteins in a controlled, non-infectious environment (Tanaka, Saito et al. 2022).

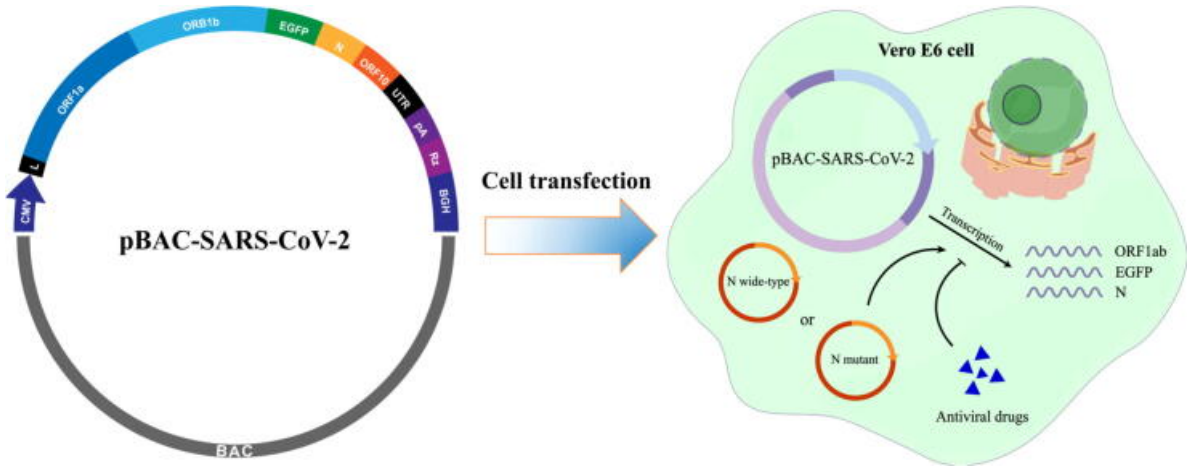

Figure 21 **A DNA-based non -infectious replicon system to study SAR-CoV-2 RNA synthesis** (Feng, Zhang et al. 2022)

1.6.4 Key Challenges and Considerations in the Use of DNA-Launched Systems

The use of DNA-launched systems for SARS-CoV-2 viral genomes presents technical and biological challenges. A major obstacle is the large genome of SARS-CoV-2, inserting such genome into bacterial plasmids is problematic due to the instability of large inserts during bacterial replication (Thi Nhu Thao, Labroussaa et al. 2020). This instability can result in recombination, deletions, or mutations in regions such as the spike protein gene or ORF1a and ORF1b, which are vital for viral replication and immune evasion (Dos Santos 2021). Furthermore, some viral sequences are toxic to bacterial hosts, adding to the complexity of creating stable DNA constructs (Willemsen and Zwart 2019). Another challenge is the accurate and efficient transcription of SARS-CoV-2 RNA from DNA templates. High-fidelity transcription is crucial for producing viral RNA that closely resembles the native viral genome, ensuring proper translation of viral proteins and RNA-dependent RNA polymerase (RdRP) activity (Malone, Urakova et al. 2022). Errors in RNA synthesis can significantly impact the stability and function of the viral transcripts (Petushkov, Esyunina et al. 2023).

A significant challenge associated with DNA-launched systems is the capacity to generate replication-competent viruses (RCVs) (Wrzesinski, Tesfay et al. 2003). Recombination between viral and host sequences could lead to the generation of a replication-competent virus, presenting major biosafety risks. Therefore, safety protocols must be in place when working with full-length SARS-CoV-2 genomes or subgenomic fragments in DNA-based systems (van der Meulen, Smets et al. 2023). Moreover, the possibility of DNA integrating into the host genome is a concern that needs further investigation (van der Meulen, Smets et al. 2023).

For large-scale applications, such as vaccine production or antiviral drug screening, it's crucial to optimize DNA-launched systems for scalability. Achieving highly efficient transcription of large viral genomes at industrial levels while maintaining fidelity and yield presents a significant challenge (Lu, Lim et al. 2024). Additionally, ensuring robust expression without causing cytotoxic effects when delivering these DNA constructs into human or mammalian cells through viral vectors, electroporation, or lipid nanoparticles is another obstacle that needs to be addressed (Patil, Rhodes et al. 2005).

The next section briefly introduces the importance and rationale for conducting this research. It also outlines the hypotheses and specific objectives of the studies, along with a summary of the results obtained

1.7 Importance, hypotheses and objectives

Vaccines play a crucial role in preventing SARS-CoV-2 infections, providing immunity and reducing the severity of the disease. Although current vaccines have significantly lessened the pandemic's impact, they mainly generate systemic immunity through intramuscular administration, which results in limited mucosal immunity in the respiratory tract—the primary entry point for the virus (Baden, El Sahly et al. 2021). Furthermore, the emergence of viral variants with mutations in the spike protein raises concerns about diminished vaccine efficacy and the necessity for frequent updates. Virus-like particles (VLPs) present an encouraging alternative as intranasal vaccines, as they mimic the virus's structure without causing infection. This approach has the potential to stimulate both mucosal and systemic immunity, thereby enhancing overall protection. Intranasal delivery, which resembles the natural route of viral entry, offers a convenient and effective means of bolstering respiratory immunity (Kar, Devnath et al. 2022). Here, we hypothesize that by utilizing a lentiviral transduction approach, a mammalian cell-based platform can be developed to enable the continuous production of SARS-CoV-2 virus-like particles (VLPs), providing a potential tool for large-scale vaccine production.

Moreover, the development of antiviral drugs targeting SARS-CoV-2 faces several significant challenges. One of the primary obstacles is the virus's rapid ability to mutate, particularly within the spike protein. These mutations can lead to the emergence of variants that may be less susceptible to existing treatments, necessitating continuous updates to the drug formulations (Harvey, Carabelli et al. 2021). Moreover, antiviral drugs are most effective when administered early in the infection. Unfortunately, many patients tend to delay seeking treatment until the disease has progressed, thereby limiting the drug’s effectiveness (Beigel, Tomashek et al. 2020). Another critical issue is the narrow specificity of many antiviral drugs, which typically target only one step in the viral life cycle. This limited approach can result in incomplete viral clearance and the development of drug resistance as the virus evolves to escape treatment

(Alshanqeeti and Bhargava 2022). Furthermore, delivering antiviral drugs to the lungs—where the virus causes the most damage—presents a significant challenge, especially in severe cases characterized by inflammation and dominant immune responses. These limitations underscore the urgent need for broad-spectrum antivirals that can target multiple stages of the viral life cycle while remaining effective against various viral variants (Niraj, Mahajan et al. 2022). The development of effective SARS-CoV-2 antiviral drugs is hampered by the absence of robust viral vector systems that can effectively model the virus's entire life cycle. While viral vectors—like lentiviruses and adenoviruses—are commonly employed in virology for the investigation of viral gene expression and replication, these systems are not fully optimized for SARS-CoV-2 (Condor Capcha, et al. 2021). Many current viral vectors fail to replicate the full spectrum of SARS-CoV-2 replication processes, including viral entry, replication, assembly, and release, as well as their interactions with host cells (Liu and Liu 2023). Here, we hypothesized that employing the complementation approach, involving the transfection of a SARS-CoV-2 replicon system lacking structural protein genes but retaining the nucleocapsid (N) gene, combined with a cell-based system capable of continuously producing the structural proteins, could enable the development of a propagation-incompetent SARS-CoV-2 infectious vector. This system would eliminate the need for repeated transfection of viral structural proteins, thereby simplifying the study of SARS-CoV-2 replication and accelerating antiviral drug development.

1.7.1 Objective I: To develop mammalian cell-based platforms for producing SARS-CoV-2 virus-like particles

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has spurred the development of a variety of vaccines. However, most existing vaccines are administered via intramuscular injection, which results in relatively weak mucosal immunity at the site of natural infection. Virus-like particles (VLPs) are nanostructures that self-assemble from key viral structural proteins, resembling the native virus in form but lacking the ability to infect or replicate due to the absence of viral genetic material. Importantly, VLP-based vaccines can also be administered intranasally, thereby mimicking the natural route of viral entry and enhancing mucosal immune responses at the primary site of infection. Based on this, we hypothesized that

the formulation of VLPs utilizing the structural proteins of SARS-CoV-2—specifically spike (S), membrane (M), nucleocapsid (N), and envelope (E)—would significantly enhance immunogenicity compared to conventional injectable vaccine approaches. This enhancement is anticipated due to the unique structural properties of VLPs, which closely mimic the architecture of the native virion, leading to improved recognition by the immune system. As a result, this VLP-based strategy may induce stronger B cell responses and result in higher levels of mucosal immunity through increased local secretory IgA production. In the study presented in Chapter II, we systematically developed and characterized SARS-CoV-2 VLPs using both transient transfection and stable transduction methods. Specifically, we investigated the necessity and sufficiency of the four essential structural proteins (S, M, N, and E) to effectively generate and assemble VLPs. We thoroughly characterized the produced VLPs using advanced techniques such as electron microscopy, which provided valuable insights into their structural integrity and morphology. Additionally, we established stable cell lines capable of producing SARS-CoV-2 VLPs, ensuring consistent generation of these particles for further research. Furthermore, we isolated clones that consistently produce VLPs, exhibiting significant variability in spike (S) protein production. Ultimately, the findings from this study could pave the way for innovative mucosal vaccination strategies that leverage VLP technology to enhance the effectiveness and accessibility of vaccines against SARS-CoV-2 and potentially other emerging viral pathogens.

1.7.2 Objective II: To investigate the expression of SARS-CoV-2 replicon RNA from DNA and explore the role of the N protein in enhancing RNA replication

RNA virus-based replicons are genetically engineered systems that retain essential viral enzyme-coding genes while omitting the genes responsible for structural proteins. When introduced into host cells, these replicons replicate autonomously and express the encoded genes without generating viral particles. Typically, replicon RNAs are synthesized externally and delivered into cells via electroporation. A more advantageous approach would involve generating replicons directly within the cells through DNA transfection, thus eliminating the need for RNA introduction. Therefore, we hypothesized that a DNA-based SARS-CoV-2 replicon system driven by a T7/CMV promoter could effectively produce replicon RNA in human cells, resulting in the expression of non-viral markers. This would offer a more cost-effective and efficient alternative

for antiviral compound screening and reverse genetics studies compared to traditional RNA-based replicon systems. In the study outlined in Chapter III, a bacterial artificial chromosome (BAC) DNA containing a SARS-CoV-2 replicon under the control of a T7 promoter was transfected into HEK293T cells engineered to express T7 RNA polymerase (T7 RNAP). Following the transfection of the BAC DNA, we noted low but reproducible expression of reporter proteins GFP and luciferase, which are encoded by the replicon. Significantly, this expression required the linearization of the BAC DNA prior to transfection and occurred independently of T7 RNAP activity. Furthermore, the expression of these genes remained unaffected by remdesivir treatment, suggesting that the self-replication of replicon RNA did not occur. Similar results were observed in Calu-3 cells, known for their high permissiveness to SARS-CoV-2 infection. Interestingly, the prior expression of the SARS-CoV-2 N protein enhanced expression from the RNA replicon, although this enhancement did not occur with the BAC DNA replicon. In summary, the transfection of large DNA constructs encoding a coronavirus replicon consistently resulted in gene expression through a yet unknown mechanism. These findings offer a novel approach for achieving replicon gene expression from transfected cDNA and provide valuable insights for advancing DNA-based RNA replicon applications.

1.7.3 Objective III: To develop a SARS-CoV-2 propagation-incompetent infectious vector system

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the virus responsible for COVID-19, poses significant challenges for antiviral drug development due to its classification as a BSL3-level pathogen and the lack of straightforward, accurate infection quantification systems. While various vector systems for SARS-CoV-2 have been developed to investigate viral infection and replication, many exhibits considerable limitations. For example, some models struggle to effectively simulate the post-entry stages of viral replication, while others necessitate complex and repetitive transfections of multiple plasmids, complicating their implementation in experimental settings. Therefore, we hypothesized that creating a SARS-CoV-2 viral vector would facilitate the efficient expression of replication-dependent marker genes, thus supporting productive infection. This approach could streamline the generation of infectious particles and enhance our understanding of viral replication dynamics, potentially advancing antiviral screening methods and

deepening insights into SARS-CoV-2 pathogenesis. In the study presented in Chapter IV, we developed a SARS-CoV-2 viral vector in HEK293T cells, which were stably transduced to constitutively express the complete set of viral structural proteins (nucleocapsid (N), spike (S), envelope (E), and membrane (M)), alongside the transfection of a replicon RNA that omits S, M, and E proteins and expresses luciferase and GFP. Following concentration by ultracentrifugation, this viral vector effectively infected two human cell lines expressing the ACE2 and TMPRSS2 receptors, demonstrating both luciferase activity and GFP fluorescence. As anticipated, vector transductions were sensitive to remdesivir and three type I interferons (IFN-I) subtypes. While there is room for improvement in viral titers, this system holds promise for antiviral drug screening and molecular virology research on SARS-CoV-2.

Chapter II is based on the following publication:

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This study describes the development of mammalian cell–based platforms for the generation of SARS-CoV-2 virus-like particles

CHAPTER II

Mammalian Cells-based Platforms For The Generation Of SARS-CoV-2 Virus-like Particle

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Highlights

- Identification of protein requirements for SARS-CoV-2 VLP production by transient transfection
- Lentiviral transduction to create cells stably producing SARS-CoV-2 VLPs
- Isolation of cell clones for the production of SARS-CoV-2 VLPs
- New putative platforms for vaccine development

2.1 Abstract

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is the causative agent of COVID-19. Though many COVID-19 vaccines have been developed, most of them are delivered via intramuscular injection and thus confer relatively weak mucosal immunity against the natural infection. Virus-Like Particles (VLPs) are self-assembled nanostructures composed of key viral structural proteins, that mimic the wild-type virus structure but are non-infectious and non-replicating due to the lack of viral genetic material. In this study, we efficiently generated SARS-CoV-2 VLPs by co-expressing the four SARS-CoV-2 structural proteins, specifically the membrane (M), small envelope (E), spike (S) and nucleocapsid (N) proteins. We show that these proteins are essential and sufficient for the efficient formation and release of SARS-CoV-2 VLPs. Moreover, we used lentiviral vectors to generate human cell lines that stably produce VLPs. Because VLPs can bind to the virus natural receptors, hence leading to entry into cells and viral antigen presentation, this platform could be used to develop novel vaccine candidates that are delivered intranasally.

keywords: SARS-CoV-2; COVID-19; virus-like particles; lentiviral vector.

2.2 Introduction

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), a recently emerged member of the *Coronaviridae* family, is responsible for the ongoing pandemic declared in March 2020 by the World Health Organization (WHO). Previous outbreaks of pathogenic coronaviruses occurred in 2002 and 2012 (SARS-CoV-1 and MERS-CoV, respectively). SARS-CoV-2 is relatively close to SARS-CoV-1 phylogenetically [1], yet is less harmful but more infectious with a higher spreading rate [2]. As a result of the rapidly spreading virus, novel vaccines were quickly developed [3]. Just a year after the pandemic emergency declaration by WHO, more than 100 COVID-19 vaccines candidates were in clinical stages of development, and close to 20 were approved [4]. Among the approaches undertaken are mRNAs and adenoviral vectors expressing the SARS-CoV-2 surface (S) protein [5]. These vaccines initially reported high effectiveness, *e.g.* 95% effectiveness for the BNT162b2 mRNA COVID-19 vaccine (Pfizer-BioNTech) [6], 94.1% for the mRNA-1273 vaccine (Moderna) [7], and 70.4% for the adenoviral vector ChAdOx1 vaccine (AZD1222; Oxford-AstraZeneca) [8]. However, the rapid evolution of the virus leading

to the emergence of new variants of concern contributed to significant losses in vaccine effectiveness [9]. Regardless of virus evolution, injectable SARS-CoV-2 vaccines quickly lose effectiveness, consistent with the rapid reduction in vaccine-induced antibodies months after vaccination [10]. In particular, injectable vaccines elicit weak mucosal immunity in the airways. This is partly explained by low levels of local secretory IgA antibodies production, which is also linked to SARS-CoV-2 transmissions from vaccinated people [11]. Recently, progress has been made toward developing mucosal vaccines, delivered through intranasal and oral routes [12]. In addition to potentially improving effectiveness, mucosal delivery may facilitate large scale campaigns, due to the non-invasive route of administration.

As a relatively novel type of vaccine, virus-like particles (VLPs) represent an attractive solution to the challenges outlined above [13]. VLPs are formed through the natural self-assembly of viral proteins, hence mimicking native virions or subviral structures [14, 15]. Thus, VLPs present conformational epitopes, which are arranged repeatedly on the surface. Consequently, VLPs are particularly apt at inducing strong B cell responses in the absence of adjuvants [16]. In addition, the size of VLPs is favorable to uptake by antigen-presenting cells, leading to the induction of robust innate and adaptive immune responses, similar to the immunogenic properties of the natural virus [17-19]. The lack of genetic material makes VLPs non-infectious, and thus they can be manipulated in low-level biosafety laboratory settings [20]. Finally, VLP vaccines can rapidly cope with epidemic viral diseases because of the relatively short time required for proceeding from design to expression [21]. Several expression systems have been described to generate VLPs, such as mammalian cell lines, bacteria, insect cell lines, yeast, and plant cells [22-24]. One important advantage of mammalian cells resides in the correct protein glycosylation pattern for VLPs produced in the mammalian cells [25]. Several teams have reported on the production of SARS-CoV-2 VLPs by transient transfection in mammalian cells or expression in insect cells, though there is no absolute consensus on which viral proteins are required to achieve efficient VLP assembly [26-30].

In this study, we report on the development of SARS-CoV-2 VLPs using transient transfection and stable transduction approaches. We demonstrate that the four structural proteins S, M, N and E are necessary and sufficient for efficient generation of VLPs. We use electron microscopy to further characterize those VLPs. Moreover, we successfully establish stable cell clones producing SARS-CoV-2 VLPs.

2.3 Materials and methods

Cell culture

HEK293T human embryonic kidney cells were cultured in Dulbecco's modified essential medium (DMEM) (Fisher Scientific) supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin (Hyclone). Cells were maintained in a 5% CO2 incubator at 37°C.

Plasmid construction and molecular cloning

The following SARS-CoV-2 structural protein-encoding plasmids were obtained from the Roth laboratory (University of Toronto): pDONR223_SARS-CoV-2-N, -S, -ORF3b, -ORF6, -ORF7a, -ORF8, as well as pDONR207_SARS-CoV-2-M, -E, -ORF3a, -ORF7a [31]. All SARS-CoV-2 protein coding sequences were cloned into the mammalian expression vector pEZY3, which was a gift from Yu-Zhu Zhang (Addgene plasmid #18672) [32] using the Gateway LR Clonase II Enzyme Mix kit (Invitrogen) as instructed by the manufacturer. 1 µl of each LR reaction was transformed into 50 µl of Library Efficiency DH5α competent cells (Invitrogen) using the heat-shock method. Transformed bacteria were plated on LB-agar dishes containing 50 µg/ml ampicillin and plates were incubated at 30°C overnight. Individual colonies were amplified and analyzed by restriction enzyme digestion and Sanger DNA sequencing of the inserts. To generate lentiviral vector constructs, SARS-CoV-2 S and M coding sequences were cloned into pLentiCMVPuroDEST (pLCPD), whereas E and N were cloned into pLentiCMVHygroDEST (pLCHD). pLentiCMVPuroDEST (w118-1) and pLentiCMVHygroDEST (w117-1) were gifts from Eric Campeau & Paul Kaufman (Addgene plasmids #17452 and #17454) [33]. SARS-CoV-2 coding sequences were inserted using the Gateway LR Clonase II Enzyme Mix kit (Invitrogen, CA), yielding plasmids pLCPD-S, pLCPD-M, pLCHD-E and pLCHD-N, respectively. Clonase reaction products were electroporated into competent *E. coli* (JM109 strain) and grown at 30°C. Plasmids were verified by restriction enzyme digestions and Sanger sequencing. Expression of SARS-CoV-2 S and N was verified by transfection into HEK293T cells followed by Western blotting of protein lysates (not shown). Expression of SARS-CoV-2 E and M proteins could not be verified due to the unavailability of commercial antibodies.

Generation of VLPs by transient transfection

HEK293T cells were seeded into 10-cm plates 24 h prior to transfection with pEZY3 plasmids expressing SARS-CoV-2 proteins using polyethyleneimine (PEI; PolyScience). For this, plasmid DNA (10 µg each) was diluted in 1 ml of serum-free, antibiotics-free cell culture medium. 45 µl of a 1 mg/ml aqueous PEI solution were added, followed by vortexing. Following a 10 min incubation on ice, the PEI-DNA solution was spread onto HEK293T cells plated the day before in a 10-cm tissue culture dish at approximately 50% confluence. Supernatants were replaced with complete medium the following day, and were collected 48 h post transfection by centrifugation at 3,000 rpm for 10 min at 4°C. Supernatants were filtered through 0.45 µm PVDF filters (Millipore), loaded on top of 20% sucrose-TNE cushions and ultracentrifuged for 90 min at 30,000 rpm at 4°C. The pelleted particles were recovered in TNE buffer [50 mM Tris-HCl, 100 mM NaCl, 0.5 mM EDTA (pH 7.4)]. Meanwhile, cells were washed with cold PBS, and then harvested and lysed with prechilled lysis buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1% Triton X-100, 0.1% Sodium Deoxycholate, 140 mM NaCl) supplemented with protease inhibitor cocktail (Pierce). Pelleted supernatants and cell lysates were immediately processed for Western blotting or frozen at -80°C.

Lentiviral vectors production and transductions

To produce lentiviral vectors encoding S, M, E, or N, HEK293T cells seeded in 10-cm plates at 80% confluency were PEI-transfected with 10 µg of pLCPD-S, pLCPD-M, pLCHD-E or pLCHD-N, 10 µg of the packaging construct pΔR8.9 and 5 µg of the VSV G-expressing construct pMD2G, as described previous [34]. The next day, supernatants were removed and replaced with fresh medium. Supernatants containing lentiviral vectors were harvested 2 days post-transfection and clarified by low-speed centrifugation.

HEK293T cells seeded in 6-well plates at 60% confluency were challenged with lentiviral vectors expressing combinations of S, M, E and/or N in the presence of 5 µg/ml polybrene (Millipore). 1 ml of each undiluted vector preparation was used for transduction of cells plated in one well, except for the simultaneous transduction with all 4 viral vectors, in which case we tried two conditions: 1 ml of each vector S/M/N/E (“SMNE1”), and 1 ml of each vector M/N/E mixed with 2 ml of the S vector (“SMNE2”). Supernatants were replaced with fresh medium the next day. One day later,

cells were submitted to selection with 2 µg/ml puromycin (Gibco) and/or 100 µg/ml hygromycin (Enzo Life Sciences). Antibiotic selection was done for two days, which killed control untransduced cells.

To establish clonal cultures, transduced and selected SMNE2 cells were seeded in 96-well plates at 0.5 cells/well in 100 µl, using medium supplemented with 10% conditioned medium. The conditioned medium was obtained by collecting the supernatant of confluent HEK293T cells, then purifying it by centrifugation for 10 min at 3000 rpm followed by filtration through 0.45 µm PVDF filters (Millipore). Cell growth was monitored for one week. Wells that contained only one colony were selected and transferred to 10-cm plates. Purification of SARS-CoV-2 VLPs in supernatants was performed as before.

Western blotting

20 µl of VLPs or cell lysates were denatured using Laemmli sample buffer (0.125 M Tris pH 7.0, 4% SDS, 20% glycerol, 0.004% bromophenol blue,) and boiled at 95°C for 5 min. Proteins were separated by SDS-PAGE and then transferred to nitrocellulose membranes (Millipore). Membranes were probed with a 1:1000 dilution of anti-SARS-CoV-2 S mouse monoclonal primary antibody (1A9, GenTex) or a 1:1000 dilution of anti-N rabbit polyclonal primary antibody (GenTex). HRP-conjugated secondary anti-mouse and anti-rabbit IgG (Cell Signaling Technology) were used diluted at 1:10,000. Membranes were scanned using the multiplex scanning method on the Azure Biosystems scanner (software version 1.2.1228.0) according to the manufacturer's manual.

Electron microscopy

Supernatants were purified using low-speed centrifugation for 10 min at 3,000 rpm, followed by filtration through 0.45 µm PVDF filters (Millipore). Filtrates were fixed with 1:10 volume of 37% formaldehyde for 10 min at room temperature, and the samples were then loaded on top of 20% sucrose-TNE cushions and ultracentrifuged for 3 h at 25,000 rpm at 4°C in a Sorval WX Ultra 100 ultracentrifuge. The final pelleted particles were recovered in 100 µl of 0.2 M sodium cacodylate buffer, pH7.4 (Electronic Microscopy Science). 10-20 µl of this sedimented material were incubated on carbon and formvar-coated grids for 30 sec. The grids were negatively stained with

4% Uranyl Acetate for 30 sec then air dried for a few minutes before observation with a transmission electron microscope (Philips EM208S Transmission Electron Microscope at 80 kV).

2.4 Results

Generation of SARS-CoV-2 VLPs in human cells using transient transfection

To define the minimal SARS-CoV-2 structural proteins required to generate SARS-CoV-2 VLPs, we first transfected HEK293T cells with plasmid vectors expressing each of the four structural proteins, *i.e.* S, M, E, or N, either alone or in combination. VLPs were purified using low-speed centrifugation followed by filtration. The particles were concentrated using 20% sucrose cushions followed by immunoblotting (Fig. 1A). We detected the expression of S and N proteins in whole cell lysates (WCLs) and the ultracentrifuged fraction (UCF). At the time of this work, no antibodies were available for M and E. As shown in Fig. 1B, S and N were detected in WCLs in the presence or absence of other structural proteins. The anti-S antibody detected two bands at around 200 kDa in cells, similar to what was observed by other authors [35], as well as the S2 furin cleavage product [36] as expected for this antibody. High amounts of both S and N were detected in the UCF pellets upon co-transfection of SARS-CoV-2 N, S, E and M. In contrast, S was found in smaller amounts in UCF fractions of cells transfected with S alone or in combination with one or two other structural proteins (Fig. 1B). Similarly, N was found in smaller amounts in the UCF pellets of cells transfected with N+S, N+S+E or N+S+M, and was not seen at all when transfected alone. Altogether, this result suggests that all four structural proteins S, M, N and E are required for the efficient assembly and egress of SARS-CoV-2 VLPs. However, both S and N seemed to be present in particulate form in the supernatants of cells in conditions in which the formation of true SARS-CoV-2 VLPs is not expected to occur, for instance upon transfection of S alone. This underlines the challenges of segregating true VLP assemblies from protein aggregates or other forms of particulate materials.

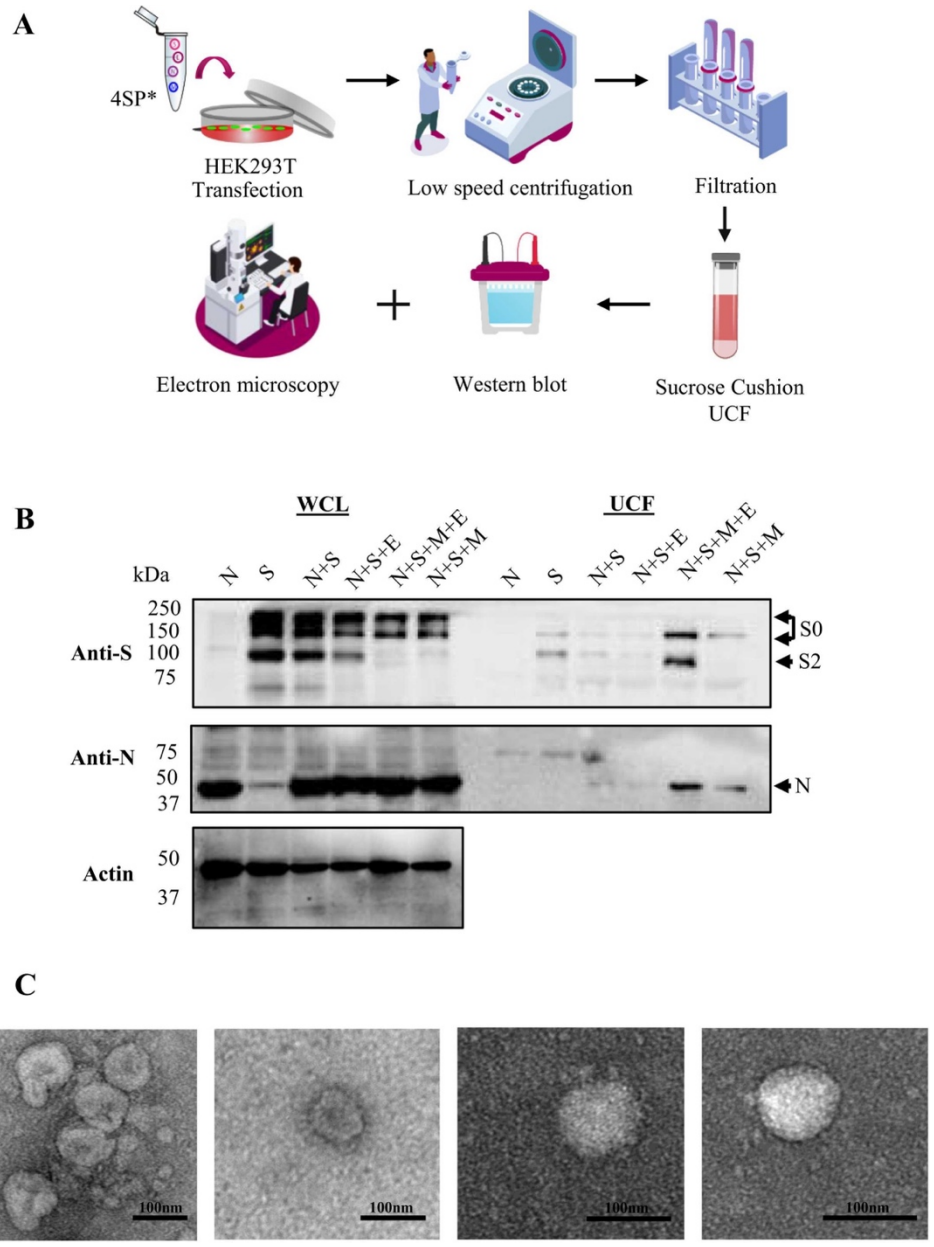

Fig. 1. Production of SARS-CoV-2 VLPs by transient transfection. (A) Schematic outline of SARS-CoV-2 VLPs generation in HEK293T cells transfected with SARS-CoV-2 structural protein-expressing plasmids. (B) Western blot analysis of VLPs in supernatants of transfected cells. SARS-CoV-2 proteins present in whole cell lysates (WCLs) and ultracentrifuged supernatants (UCF) 48 h after transfection with the indicated plasmids, and detected using anti-S and anti-N antibodies. Protein size markers are shown. This experiment is representative of two independent replicates. (C) Electron microscopy detection of SARS-CoV-2 VLPs. Supernatants of transfected HEK293T cells were collected 48 h after transfections, purified, fixed, concentrated by ultracentrifugation and processed for EM analysis. Scale bar = 100 nm.

We used transmission electron microscopy (EM) to confirm the presence of virion-like VLPs in the supernatant of cells transfected with the four structural proteins. Several protocols were tested for the fixation, concentration and staining of samples putatively containing VLPs. We settled on a protocol in which supernatants are clarified by low-speed centrifugation and filtration, then fixed with formaldehyde prior to concentration by ultracentrifugation, and UCF pellets are deposited on grids and negatively stained. As shown in Fig. 1C, particles with a SARS-CoV-2 virion-like morphology were found that displayed typical corona-like spikes. These particles were about 100 nm in diameter, spikes included (Fig. 1C), which is consistent with SARS-CoV-2 particles. These observations strongly suggest that SARS-CoV-2 VLPs were generated through this transient transfection approach.

Role of SARS-CoV-2 accessory proteins in VLP production

The SARS-CoV-2 ~30 kb genome encodes accessory proteins, the exact number of which is still under debate [37]. These accessory proteins have important roles in virus infectivity and pathogenicity [38]. For instance, ORF3a and ORF7 promote immune escapes by downregulating MHC expression at the surface of infected cells [39]. It is conceivable that some accessory proteins would indirectly increase SARS-CoV-2 VLP production. For instance, ORF8 is known to increase viral replication through the modulation of endoplasmic reticulum functions [40]. To investigate the possibility that SARS-CoV-2 accessory proteins increase VLP production, we transfected HEK293T cells with the four structural proteins alone or in combination with accessory proteins. As shown in Fig. 2, none of the accessory proteins tested increased S and N expression in transfected cells. In fact, ORF3a/b, ORF6 and ORF7a/b seemed to decrease N protein expression. Analysis of the ultracentrifuged supernatants showed that the cells transfected with the four structural proteins and no accessory proteins produced higher levels of VLPs than in the other conditions tested. These data suggest that the four SARS-CoV-2 structural proteins are sufficient for the efficient formation of VLPs.

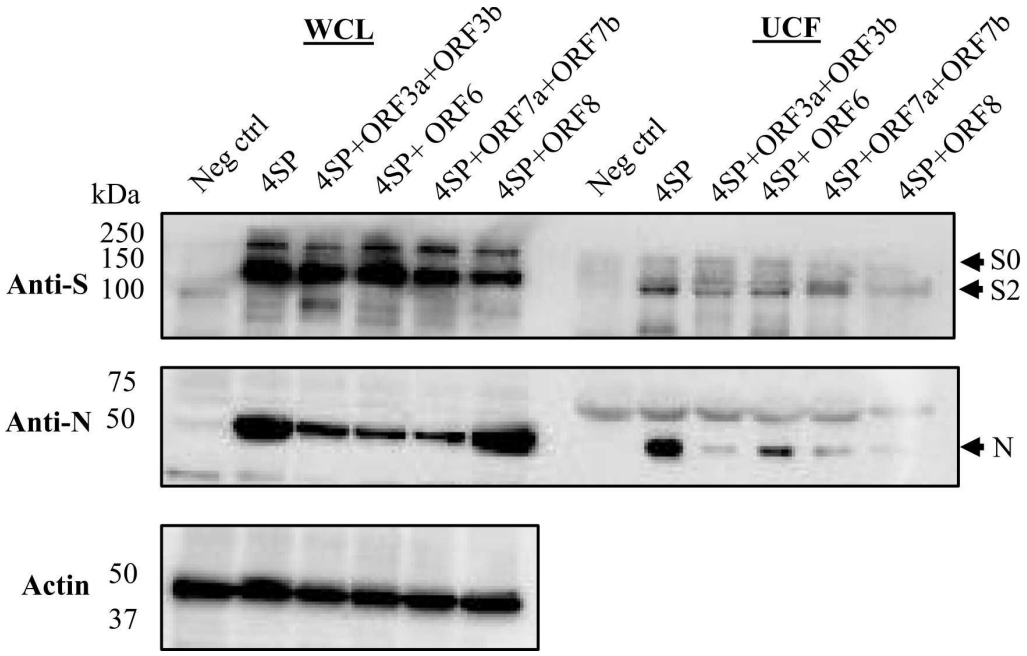

Fig. 2. Characterization of minimal protein requirements for efficient SARS-CoV-2 VLPs production. Western blot analysis of SARS-CoV-2 S and N proteins in WCLs and in ultracentrifuged supernatants (UCF) 48 h after transfection of the plasmids expressing the indicated proteins. 4SP ("4 structural proteins") stands for a mixture of S, M, E and N-expressing plasmids.

Efficient production of SARS-CoV-2 VLPs using lentiviral vectors

As described above, we were able to generate SARS-CoV-2 VLPs by transient transfection. To facilitate VLP production, we explored the possibility of creating HEK293T cell lines stably expressing VLPs through lentiviral transduction. This approach would allow cells to continuously release VLPs into the cell culture supernatant, eliminating the need for the transient transfection of plasmids. As summarized in Fig. 3A, we first generated S, M, E and N lentiviral vectors by transfecting cells with appropriate plasmid combinations. The vectors encoding SARS-CoV-2 S and M also carried puromycin resistance, whereas the vectors encoding N and E carried hygromycin resistance. HEK293T cells were transduced with the four lentiviral vectors simultaneously, or with single vectors. We were concerned that the transduction of S would be less efficient owing to the large size of its coding sequence. Thus, we tried two different transduction conditions for the quadruple transduction: either an equivalent volume of all four lentiviral vectors (SMNE1) or twice as much of the S vector, relative to the four other vectors (SMNE2). Whole cell lysates were prepared, and supernatants were purified and concentrated as

previously, yielding UCF fractions that contained particulate materials that sedimented through the sucrose cushion. Both WCLs and UCF samples were then analyzed by immunoblotting. As shown in Fig. 3B, S and N were detected in the WCLs of cells transduced with the four structural proteins or with the relevant individual proteins, as expected. Of note, we did not see any difference in S expression between SMNE1 and SMNE2. Surprisingly, both S and N were readily detected in UCF pellets of cells transduced with a single lentiviral vector, in addition to being present in the supernatants of cells transduced with the four structural proteins. Presumably, the particulate materials purified from cells transduced with N or S alone are either aggregates and/or exosomes containing the proteins. But because the signals in cells transduced with one or 4 vectors were of similar intensities, this Western blotting analysis did not allow us to demonstrate the presence of "true" VLPs.

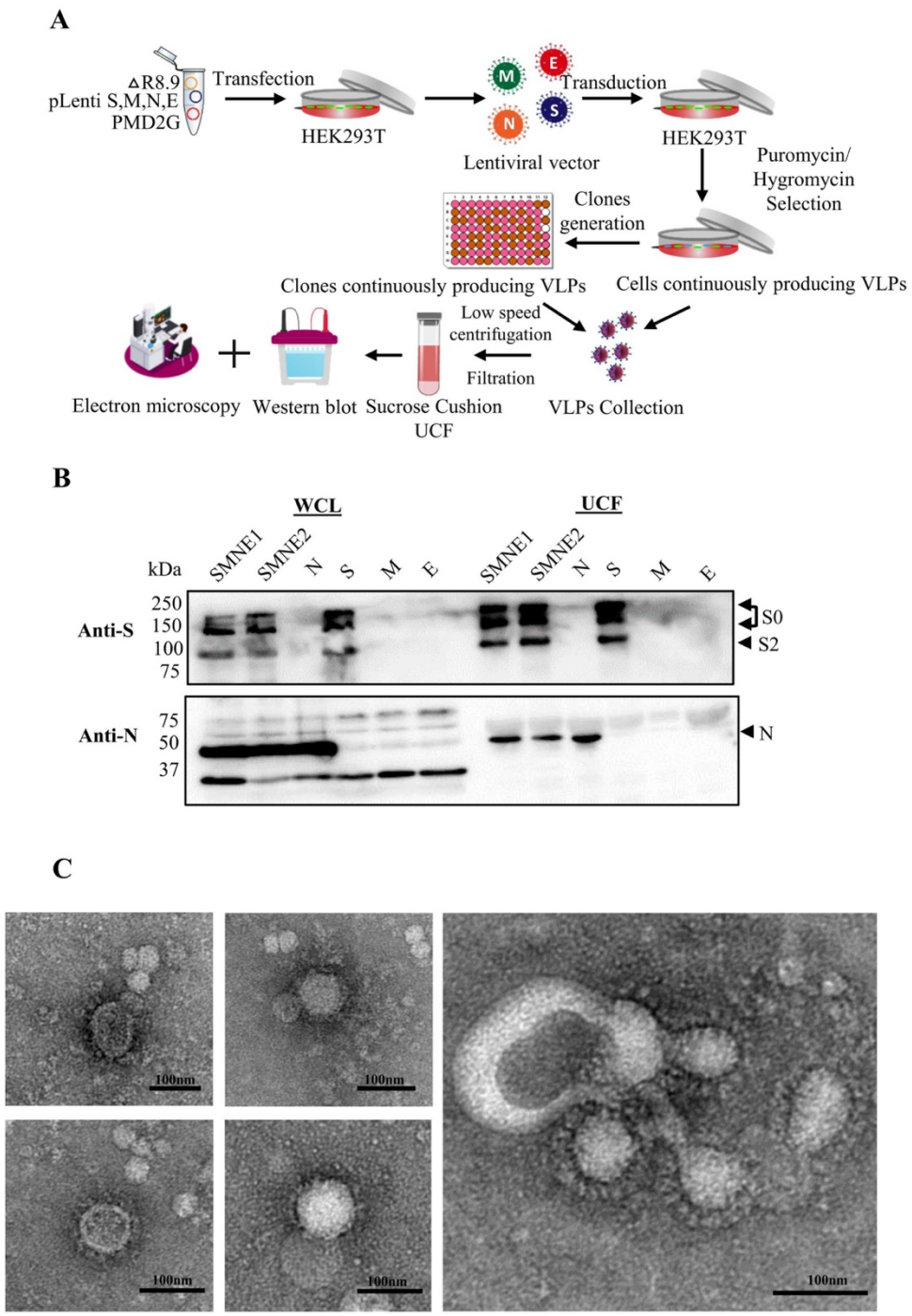

Fig. 3. Generation of SARS-CoV-2 VLPs using lentiviral transduction. (A) Schematic outline for the generation of cell populations and cell clones producing SARS-CoV-2 VLPs. (B) Western blot analysis of SARS-CoV-2 proteins S and N in WCLs and ultracentrifugated supernatants (UCF) from cells transduced with lentiviral vectors expressing the indicated SARS-CoV-2 proteins. SMNE1 and SMNE2 refer to differences in the amount of the S vector relative to the other vectors (see 2.4). Protein size markers are shown. This experiment is representative of two independent replicates. (C) Electron

microscopy detection of SARS-CoV-2 VLPs. Shown are EM images of putative SARS-CoV-2 VLPs produced by HEK293T cells transduced with S, M, N and E. The two pictures at the top-left, and the one at the bottom-left, show particles from "SMNE1″ cells, whereas the two other images are from "SMNE2″ cells. Scale bar = 100 nm.

To confirm the formation of VLPs in cells transduced with the four SARS-CoV-2 structural proteins, we analyzed the UCF fractions using EM. As shown in Fig. 3C, we found particles that resembled SARS-CoV-2 virions in shape and size with the presence of distinctive spikes. We did not find similar structures in cells transduced with single vectors, though the observations were too infrequent for us to perform a quantitative analysis. Therefore, these data suggest that we successfully generated mammalian cell lines stably producing SARS-CoV-2 VLPs.

Generation of cell clones stably producing SARS-CoV-2 VLPs

For industry-scale applications of transduced mammalian cell lines continuously producing SARS-CoV-2 VLPs, it is desirable to isolate cell clones that produce high amounts of VLPs. For this, single cell cultures of HEK293T cells transduced with the four structural proteins were initiated by limiting dilution of the cells transduced with all four lentiviral vectors ("SMNE2"). Surviving cell populations were amplified, and we analyzed the presence of N and S in purified, ultracentrifuged supernatants by immunoblotting followed by EM analysis. As shown in Fig. 4A, all the analyzed clones expressed S and N, but significant variations were observed, especially for S. EM analyses (Fig. 4B) showed the presence of VLPs similar in shape and size to those found before. Thus, these data suggest that most or all cells transduced with the four structural proteins produce SARS-CoV-2 VLPs, but that analysis of individual cell clones is useful to identify the most promising ones for downstream applications.

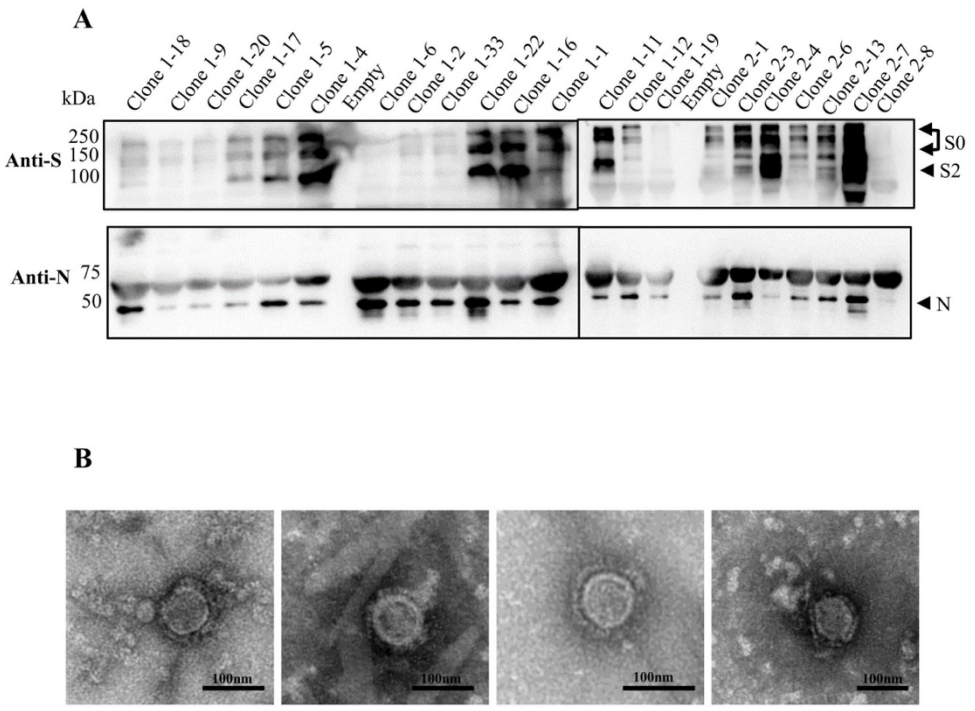

Fig. 4. Generation of cell clones stably producing SARS-CoV-2 VLPs. (A) Western blot analysis of SARS-CoV-2 proteins S and N present in ultracentrifugated supernatants of 22 cell clones derived from HEK293T cells transduced with S, M, N and E ("SMNE2″ cells) as shown in Fig. 3. Protein size markers are shown. (B) Morphological analysis of SARS-CoV-2 VLPs. Shown are EM images of putative SARS-CoV-2 VLPs found in the supernatants of HEK293T clones (from left to right: 1–22, 1–16, 2–13, 2–7). Scale bar = 100 nm.

2.5 Discussion

Virus-like particles (VLPs) are nanoscale structures made up of self-assembled viral proteins, mimicking the native virus but lacking the genetic material so they are not capable of productively infecting the host cell [41]. VLPs have gained increasing interest as immunogens capable of eliciting both mucosal and cellular immune responses [42]. Their repetitive antigenic structure enables them to induce strong T-helper response [43]. VLPs are also prone to activate dendritic cells (DCs) which are essential players in the initiation of an immune response through the capture and processing of antigens, delivering them to secondary lymphoid organs and providing co-stimulatory signals [44]. VLPs produced in mammalian cells offer additional benefits, including the possibility of VLP surface proteins glycosylation [45]. In the present study, we generated SARS-CoV-2 VLPs by co-expressing four SARS-CoV-2 structural proteins in HEK293T, a

mammalian cell line commonly used to produce biological reagents such as monoclonal antibodies. We first transfected HEK293T cells with vectors expressing S, M, E, or N, respectively, as well as combinations of these proteins. Other teams that had done similar work detected the presence of VLPs or viral proteins in supernatants even when just 1, 2 or 3 proteins were transfected. For instance, Alpuche-Lazcano *et al.* found that the transfection of S alone in Chinese Hamster Ovary cells was sufficient to generate vesicles displaying the S protein [46]. Xu et al. [26] produced VLPs by transiently transfecting HEK293T and VeroE5 cells with a variety of plasmid combinations expressing S, M, N and E, or even with single plasmid transfections. Other researchers produced VLPs in insect cells through the expression of S, M and E [28-30]. Swann *et al.* as well as Chang *et al.* used this same combination of S, M and E in HEK293T cells [27, 47]. In contrast, in our hands all four structural proteins were required for the efficient release of VLPs from HEK293T cells. Of note, Boson *et al.* as well as Syed *et al.* arrived to a similar conclusion, and in addition, they performed a detailed analysis of S maturation, showing that the presence of M and E was necessary to this process [48, 49]. Roessler *et al.* as well as Resch *et al.* used transient transfections of the four structural proteins and obtained VLPs as seen for instance by cryoelectron microscopy [50, 51], but they did not investigate other combinations. It is likely that the apparent discrepancy between those various studies could be explained by differences in purification procedures or in other experimental conditions. The role of RNA in the assembly of VLPs containing all four structural proteins remains unresolved. Although the presence of a genomic RNA is clearly not indispensable to VLP production, the Doudna team reported that VLP production was more efficient in the presence of a reporter RNA containing the SARS-CoV-2 RNA encapsidation signal T20 [48]. This raises the possibility that in the absence of SARS-CoV-2 genomic RNA, VLP assembly involves non-specific binding of N to cellular RNAs, similar to what is observed in HIV-1 particles devoid of viral RNA due to mutations in the RNA encapsidation domain Ψ [52].

Our results suggest that we created HEK293T cell populations stably expressing VLPs through lentiviral transduction. This approach may allow cells to continuously release VLPs into the cell culture supernatant, facilitating large-scale VLP production. Future experiments should include a more detailed characterization of VLPs produced in this way, such as time-dependent analysis of VLP production over extended periods of time. We further isolated cell clones that seemingly produce high amounts of VLPs. Such clonal populations might prove useful for downstream

industrial applications. However, in the context of these VLPs produced through lentiviral transduction, Western blotting was insufficient to prove true VLP assembly, since we were able to sediment SARS-CoV-2 proteins from the supernatants of cells transduced with S or N alone. Why was this problem encountered in the context of transduced but not transfected cells, is presently unclear. It will be important to develop methods that allow for more efficient VLP purification. In this context, it is worth noting that other teams have recently contributed novel UCF-free methods based on diafiltration and PEG precipitation [53, 54] or affinity chromatography [46]. Using electron microscopy, we however observed the presence of viral particles displaying typical S protein projections that are a hallmark of coronaviruses, in the supernatant of both transfected and transduced cells, as well as in clonal populations of transduced cells. Those particles were about 100 nm diameter, which is comparable to wild-type virions.

Previous studies had shown that some accessory proteins such as ORF3b, ORF6, ORF7a and ORF8 are important IFN-I antagonists, leading to an impairment in the host immune response [37]. It is conceivable that some of these proteins would also be important for VLP egress, perhaps by inhibiting innate immune responses targeting these stages of viral replication. To test this hypothesis, we co-transfected HEK293T cells with the four structural proteins in combination with selected accessory proteins. Our data showed that no additional proteins other than the four structural proteins are required for SARS-CoV-2 VLPs assembly and release.

The term "VLPs" is the subject of confusion, as it is used to describe not only assemblies of proteins from the virus of interest, as in this study, but also the incorporation of a single viral protein (typically a surface protein) in lipid droplets or heterologous viral particles [55], and even assemblies of a single type of viral protein that may not have a true viral structure at all. For instance, the company Medicago has developed a plant-based vaccine for SARS-CoV-2 by expressing the S protein in *Nicotiana benthamiana* plants; though the resulting immunogens are called "VLPs" in the literature, they may simply be S aggregates [56]. In the context of SARS-CoV-2 VLPs used as vaccines, the presence of multiple SARS-CoV-2 viral proteins (*i.e.* multiple antigens) as in the current study might result in increased effectiveness, compared to current vaccines that typically include only one, and might also be less susceptible to loss of effectiveness due to virus evolution. It should also be pointed out that the VLPs containing all four SARS-CoV-2 structural proteins are expected to fuse into target cells in a manner similar to actual viruses. This feature might translate into increased immunogenicity compared to antigens that must be actively

internalized by antigen-presenting cells. From the perspective of fundamental virology research, the systems described here could constitute useful tools for the study of not only assembly and egress but also virus entry.

Note added in proof. During the revision phase of this article, the Hirschberg group similarly reported the efficient production of SARS-CoV-2 VLPs from suspension-adapted 293 cells stably expressing S, M, N and E [53]. In this study, M, N and E were integrated into the DNA through transposition rather than using lentiviral vectors. Upon microscopy analysis, VLPs were very similar to what we observed; importantly, they were immunogenic in mice as evidenced by the production of antibodies against S and N.

2.6 Conclusions

In this study, we successfully generated SARS-CoV-2 VLPs from mammalian cells, either transiently or through stable transduction. These VLPs presumably contained all four structural proteins and thus closely resemble SARS-CoV-2 virions, which may translate into higher mucosal immunogenicity compared with vaccines composed of only one viral protein. However, VLPs immunogenicity remains to be tested, and VLP purification needs to be improved. In addition to the development of nasally-administered vaccines for mucosal immunity against coronaviruses, the tools presented here could also be useful to the study of virus assembly, egress and entry.

Data availability

Data pertaining to this manuscript have been uploaded on FigShare:
https://figshare.com/projects/Elfayres_et_al_2023/180877

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This study investigates DNA linearization-dependent expression of reporter genes from a SARS-CoV-2 replicon in human cells, occurring independently of replicon RNA amplification

CHAPTER III

RNA replication-independent, DNA linearization-dependent expression of reporter genes from a SARS-CoV-2 replicon-encoding DNA in human cells

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Short title: Cryptic gene expression from SARS-CoV-2 cDNA

3.1 Abstract

Replicons, derived from RNA viruses, are genetic constructs retaining essential viral enzyme genes while lacking key structural protein genes. Upon introduction into cells, the genes carried by the replicon RNA are expressed, and the RNA self-replicates, yet viral particle production does not take place. Typically, RNA replicons are transcribed *in vitro* and are then electroporated in cells. However, it would be advantageous for the replicon to be generated in cells following DNA transfection instead of RNA. In this study, a bacterial artificial chromosome (BAC) DNA encoding a SARS-CoV-2 replicon under control of a T7 promoter was transfected into HEK293T cells engineered to functionally express the T7 RNA polymerase (T7 RNAP). Upon transfection of the BAC DNA, we observed low, but reproducible expression of reporter proteins GFP and luciferase carried by this replicon. Expression of the reporter proteins required linearization of the BAC DNA prior to transfection. Moreover, expression occurred independently of T7 RNAP. Gene expression was also insensitive to remdesivir treatment, suggesting that it did not involve self-replication of replicon RNA. Similar results were obtained in highly SARS-CoV-2 infection-permissive Calu-3 cells. Strikingly, prior expression of the SARS-CoV-2 N protein boosted expression from transfected SARS-CoV-2 RNA replicon but not from the replicon BAC DNA. In conclusion, transfection of a large DNA encoding a coronaviral replicon led to reproducible replicon gene expression through an unidentified mechanism. These findings highlight a novel pathway toward replicon gene expression from transfected replicon cDNA, offering valuable insights for the development of methods for DNA-based RNA replicon applications.

3.2 Introduction

In late 2019, the severe acute respiratory syndrome related coronavirus 2 (SARS-CoV-2) emerged in Wuhan, China, causing the global COVID-19 pandemic. COVID-19 is associated with mild cold symptoms to severe pneumonia which can lead to hospitalization and death (1). The disease can also transform into a chronic disease known as long COVID, which can involve increased fatigue, organ injury and an increased risk for type 2 diabetes (2). As of February 2024, there had been nearly 775 million confirmed cases of SARS-CoV-2 infections worldwide with a death toll of over 7 million (source: World Health Organization). The first vaccines against SARS-CoV-2 were made available in a matter of months after the beginning of the pandemic. COVID-19 vaccine

campaigns quickly proved to be especially effective in preventing severe infection (3). The search for antiviral compounds to prevent infection or complications in high-risk individuals continues to be of high importance (4). Currently, several antiviral therapies have been approved, with the SARS-CoV-2 protease inhibitor nirmatrelvir (Paxlovid) probably being the most frequently prescribed. However, the global use of antiviral compounds to target SARS-CoV-2 has been low, which is explained by a combination of disappointing effectiveness and high cost (5).

The discovery and characterization of novel putative antiviral drugs for SARS-CoV-2 has been complicated by two factors. First, SARS-CoV-2 has long been a biosafety level 3 pathogen, though an increasing number of countries are now classifying it as a level 2 pathogen (6). Secondly, SARS-CoV-2-derived viral vector systems encoding marker proteins that facilitate screening have been slow to develop, and to this day have very low infectivity (7). To circumvent those roadblocks, viral replicons have often been used instead of the wild-type coronavirus. Replicons are non-infectious subgenomic viral RNAs in which key structural genes are eliminated, making them unable to form progeny virions (8). Self-copying of the replicon RNA is still possible because all non-structural genes required for genomic replication and transcription are present. Transfection of the replicon RNA into different cell lines thus leads to expression of viral genes as well as any marker inserted in the replicon. This potentially allows the screening for antiviral compounds that target viral replication *in cellulo* (8). Prior to SARS-CoV-2, replicon systems had been created for other coronaviruses, such as SARS-CoV (sometimes called SARS-CoV-1) as well as the Middle East respiratory syndrome-related coronavirus (MERS-CoV) (9, 10).

Traditionally, replicons are introduced into mammalian cells through *in vitro* transcription of replicon cDNA into RNA, followed by transfection by electroporation. Replicon-encoded proteins are then translated by the host cell's ribosomes, leading to RNA replication. This method, while widely employed, is plagued with drawbacks such as high cost, time-intensive procedures, and inefficiency in achieving successful electroporation of replicons into cells. Establishing cell lines supporting the stable self-replication of replicon RNAs is also not trivial. A transformative alternative is the direct expression of replicon RNA from a DNA construct within cells, offering several advantages for reverse genetics as applied to RNA viruses: (i) amplifying and purifying DNA is inexpensive compared to RNA; (ii) DNA transfection is less expensive and more efficient, routinely reaching >90% in some cellular models; (iii) stably maintaining a DNA construct is a reachable goal, through its integration into the cellular genome. Prior attempts at such an objective

have been made, for instance by using a CMV promoter to govern replicon expression (11). In this study, we explored the possibility to produce SARS-CoV-2 replicon RNA from a dual T7/CMV promoter-driven BAC construct introduced into human cells by standard transfection protocols. Here, we specifically report on the observed expression of two non-viral markers that are part of the replicon. Our findings contribute to advancing DNA-based RNA replicon applications with potential implications for efficient and cost-effective reverse genetics studies in RNA virus research.

3.3 Materials and methods

Plasmids

The SARS-CoV-2 N protein-expressing plasmid pEZY3-N was described before (12). T7-CMVtrans-FFLuc-polyA (13), which expresses the firefly luciferase under control of the T7 promoter, was a gift from Marcel Bruchez (Addgene plasmid #101156). The codon-optimized T7RNAP coding sequence was amplified from T7 opt in pCAGGS (14) (Addgene #65974; a kind gift from Benhur Lee) by PCR. PCR reactions were done in 50 µl total volume, using 0.5 µl of Q5 HF DNA polymerase (New England Biolabs), 10 µM of dNTPs (New England Biolabs) and 10 µM of primer XhoI-T7-5' [AGCTCTCGAGACCATGAACACCATCAATATTGCC] and EcoRI-§-T7-3' [GCATGAATTCTCAGGCAAATGCGAAATCGGA]. Amplification conditions were as follows: 20 sec denaturation at 98°C, 25 cycles (10 sec at 98°C, 20 sec at 58°C, 2 min at 72°C) and 5 min at 72°C to complete synthesis. PCR products were purified using the QIAquick purification kit (QIAGEN), then cut with XhoI and EcoRI, and finally gel-purified using the QIAEX II kit (QIAGEN). The purified inserts were ligated in separate reactions into pLPCX(AB) and pMIP (15) that were linearized by digestion with XhoI and EcoRI. pLPCX(AB) is a version of pLPCX (Clontech Laboratories) in which the EcoRI-ClaI region of the multi-cloning site was removed and replaced by a duplex created by annealing oligodeoxynucleotides Linker-EcoBamNotCla-s (5'-AATTCACGGATCCTTGCGGCCGCAT) and Linker-EcoBamNotCla-as (5'-CGATGCGGCCGCAAGGATCCGTG). Ligation reactions were performed in 20 µL total volume, with 2 µg vector, half of a purified reaction product, and 1 µL of T4 DNA ligase (New England Biolabs). Following ligation, 1:10 vol. of the ligated product was transformed into DH5α *E. coli* by electroporation. Clones were analyzed by restriction enzyme digestions followed by Sanger sequencing.

Cell culture

Human embryonic kidney (HEK)293T cells were maintained in DMEM (Hyclone) supplemented with 10% fetal bovine serum (Hyclone) and penicillin-streptomycin (Hyclone). HEK293T cells lentivirally transduced with SARS-CoV-2 N have been described before (12). The lung adenocarcinoma epithelial cells Calu-3 were maintained in EMEM (Hyclone) supplemented with 10% fetal bovine serum.

Retroviral vector production and HEK293T transductions

To create HEK293T cells stably expressing T7 RNAP, two retroviral vectors were used. HEK293T cells plated at approximately 80% confluence in 10-cm plates were polyethylenimine (PEI) transfected with pLPCX(AB)-T7RNAP (10 μg) along with psPAX2 (10 μg) and pMDG (5 μg) as described before (16). To generate the control vector, cells were transfected with the empty vector pLPCX(AB) instead of pLPCX(AB)-T7RNAP. We also generated MIP-based vectors (15) by transfecting HEK293T cells with pMIP-T7RNAP (10 μg), pCL-Eco (10 μg) and pMDG (5 μg) as described before (17). Again, a control vector was produced by transfecting with the empty pMIP instead of pMIP-T7RNAP. The following day, supernatants were removed and replaced with complete medium. Two days post transfection, supernatants were harvested, clarified by low-speed centrifugation and aliquoted. HEK293T cells plated at 70% confluency in 10-cm plates were transduced by adding 5 mL of vector-containing supernatant. The next day, supernatants were replaced with complete medium. Two days post transduction, all transduced cells were placed in 1 μM puromycin (Gibco). Selection was allowed to proceed for 4 to 9 days; control untransduced cells were killed by the treatment.

Luciferase-expressing plasmid DNA transfection and luciferase assays

Cells plated in 6-well plates at 70 % confluency were transfected with 2 μg per well of T7-CMVtrans-FFLuc-polyA using PEI. Cell supernatants were replaced with fresh medium six hours later. Cells were processed for luciferase assay approximately 36 h later. The Steady-Glo Luciferase Assay System kit from Promega was used for all luciferase assays. Cells grown in 6-well plates were washed with PBS, trypsinized and pelleted by centrifugation. Post-centrifugation pellets were resuspended in 300 μL of complete medium and plated in three wells of a 96-well plate (black wall, clear bottom) at 100 μL per well. 100 μL of Steady-Glo Reagent were

immediately added to each well. Luciferase activity was measured using the Biotek Synergy HT microplate reader according to the manufacturer's instructions.

Preparation of SARS-CoV-2 replicon-encoding bacterial artificial chromosome (BAC) DNA and *in vitro* transcription of replicon RNA

The pSMART BAC v2.0 vector containing the SARS-CoV-2 (Wuhan-Hu-1) non-infectious replicon (18) was obtained from BEI resources (#NR-54972). To transform Stbl3 *E coli* cells (Invitrogen) with the BAC construct, one vial of One Shot Stbl3 chemically competent cells was thawed on ice and mixed with 5 µL of DNA. The mixture was incubated on ice for 30 min followed by heat shock treatment at 42 °C for 45 sec without shaking. The vial was placed back on ice for 2 min, then 250 µL of pre-warmed SOC medium was added followed by incubation at 37°C for 1 h. The cells were plated onto LB plates containing 20 µg/mL chloramphenicol, and then cultured overnight at 37 °C. Individual colonies were isolated and grown, and then the BAC DNA was purified using the PhasePrep BAC DNA Kit (Sigma Aldrich) according to the manufacturer's instructions, and analyzed by restriction enzyme digestion.

For *in vitro* transcription, the BAC replicon-encoding DNA was first linearized with SwaI (New England Biolabs), and then purified by phenol/chloroform extraction and ethanol precipitation. The fragments were dissolved in nuclease-free water. The mMESSAGE mMACHINE T7 transcription kit (Invitrogen) was used to generate the replicon RNAs in the correct orientation from the linearized vector according to manufacturer's instruction. Briefly, 180 µL of T7 transcription reaction, containing 7.2 µg of linearized BAC, T7 RNAP as well as GTP, was incubated at 37 °C for 2.5 h. After incubation, 9 µL of TURBO DNase was added and the reaction was incubated at 37 °C for 15 min to digest DNA. The resulting RNA was purified using the Monarch RNA cleanup kit (New England Biolabs) and analyzed by agarose gel electrophoresis.

SARS-CoV-2 replicon BAC DNA transfections

Cells plated in 6-well plates were transfected at 50 % confluency with various amounts of BAC DNA, either intact or linearized with SwaI immediately prior to transfections. Following SwaI digestions (2 h at 25 °C), BAC DNA was heated for 20 min at 65 °C to denaturate the enzyme. Transfections were done using either Lipofectamine 3000 (Invitrogen), PEI or electroporation. For Lipofectamine 3000 transfections, 2 µg of BAC DNA in 125 µl Opti-MEM (Gibco) containing 3 µl of P3000 reagent were mixed with 125 µl of Opti-MEM containing 3 µl of Lipofectamine 3000.

After incubation at room temperature for 15 min, the lipofectamine:DNA mixture was added onto the cells. PEI transfections were performed as detailed previously (19), whereas electroporations were performed exactly as for the replicon RNA (see below). In some instances, remdesivir (Cayman Chemical) was added at a concentration of 100 nM or 1 μM, immediately after transfection and treatment was repeated with the media change the next day. Control cells were mock-transfected using identical conditions.

SARS-CoV-2 replicon RNA transfections

HEK293T cells were harvested using Trypsin/EDTA (Thermo Fisher Scientific), washed with PBS, and resuspended in Neon Resuspension Buffer R (Invitrogen) to a final density of 1×10^7 cells/mL. 10 μg of replicon RNA were added onto 1×10^6 resuspended cells. The mixture was immediately transferred to Neon tips (100 μL, Invitrogen), and electroporation was carried out in the Neon Device (Invitrogen) using the cell type-specific preloaded parameters. Transfected cells were transferred immediately into 6-well plates containing prewarmed DMEM medium supplemented with 10% FBS and without antibiotics, and placed in antibiotics-containing medium the next day. In some cases, cells were electroporated with 5 μg of pEZY3-N simultaneously to replicon RNA transfections.

Analysis of reporter proteins expression

To analyze GFP expression by flow cytometry, cells in a 6-well plate were trypsinized with 500 μl trypsin and the reaction was stopped with 500 μl of complete media. 800 μl of each cell suspension were then fixed using 4% formaldehyde diluted in PBS. The percentage of GFP-positive cells was determined by analyzing 20,000 cells on a FC500 MPL cytometer (Beckman Coulter) using the CXP Software (Beckman Coulter), or CytoFlex S (Beckman Coulter). Flow cytometry data were analyzed using FlowJo (Becton Dickinson) or FCS Express (De Novo Software). To analyze luciferase expression, 2 x 100 μL of the cell suspension were processed for firefly luciferase quantification assay, as described above.

Fluorescence microscopy

GFP expression in live cells was examined using an Axio Observer inverted fluorescence microscope (Zeiss) with a 20X objective, two days post transfection with SARS-CoV-2 replicon BAC DNA or with replicon RNA. Images were recorded using the ZEN software.

3.4 Results

Transduction of functional T7 RNAP into HEK293T cells

We generated two retroviral vector constructs, LPCX(AB)-T7RNAP and MIP-T7RNAP, and used them to transduce HEK293T cells followed by elimination of non-transduced cells by puromycin treatment. "Empty" LPCX(AB) and MIP vectors were used as controls. Validating T7 RNAP expression by Western blotting was not possible due to the unavailability of antibodies. However, we were able to perform a functional assay for T7 RNAP, by transfecting the transduced cell populations generated with a construct expressing the firefly luciferase under control of the T7 promoter (Fig 1). We observed increased luciferase activity in cells transduced with LPCX(AB)-T7RNAP or MIP-T7RNAP compared to the background levels of luminescence activity in cells transduced with the empty vector controls (Fig 1). These results demonstrate the functionality of T7 RNAP in the HEK293T cells generated.

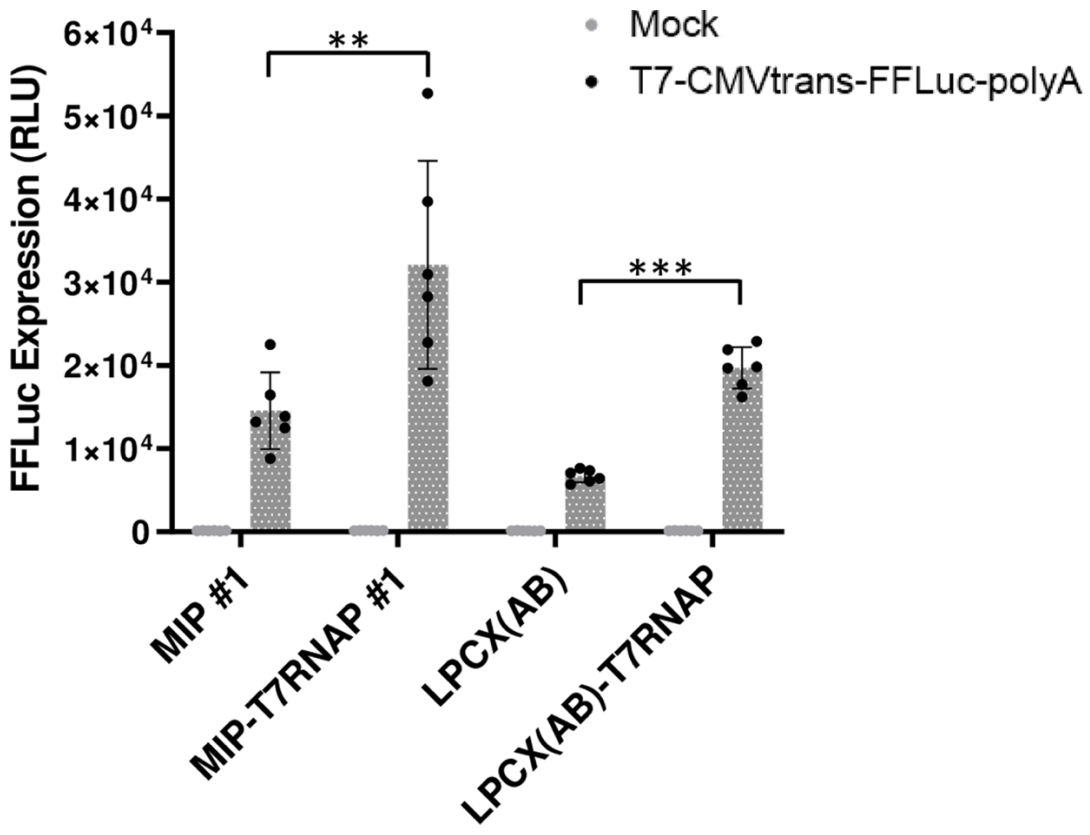

Fig 1. HEK293T cells transduced with T7 RNAP support T7 promoter-dependent expression. HEK293T cells were transduced with MIP-T7RNAP or LPCX(AB)-T7RNAP or with the empty vectors as controls. Cells were treated with antibiotics to kill

untransduced cells and then were transfected in triplicates with a plasmid expressing luciferase under control of the T7 promoter. Luciferase activity was quantified the next day as described in the Methods section. RLU, relative lights units. A Student's t-test was used to assess statistical significance; **, $P < 0.01$; ***, $P < 0.001$.

DNA linearization-dependent, T7 RNAP-independent, remdesivir-insensitive expression of replicon-encoded GFP following BAC DNA transfection in HEK293T cells

The SARS-CoV-2 replicon-encoding BAC construct used in this project has deletions in the viral structural proteins S, E and M, and the coding sequences for the firefly luciferase and GFP are expressed as a fusion protein in place of S (18). A CMV promoter is present in addition to the T7 promoter, which in theory may allow for the transcription of replicon RNA in mammalian cells. However, it is not clear whether CMV-driven expression was possible with this BAC construct (18), and thus, we aimed at attempting expression both in cells expressing T7 RNAP and in control cells. Transcription in mammalian cells using the T7 RNA polymerase is theoretically possible without linearization of the DNA (20), but it may be improved by it. Whether the BAC DNA is linearized or not, production of a replication-competent replicon RNA necessitates the generation of a correct 3' terminus. To achieve this, the BAC construct used here contains the hepatitis D virus (HDV) self-cutting ribozyme RNA (21) immediately downstream to the 3' terminus of the replicon RNA, which should allow for the generation of the correct replicon RNA 3' extremity in mammalian cells. However, whether this ribozyme is functional in cells in the context of this specific BAC construct or not is also unclear (18). In order to explore conditions permitting the *in cellulo* transcription and replication of the SARS-CoV-2 replicon, we used a PEI-based protocol to introduce the BAC DNA into the HEK293T-LPCX(AB) control cells (Fig 2A) and HEK293T-LPCX(AB)-T7RNAP-transduced cells (Fig 2B). Furthermore, we transfected both linearized and non-linearized SARS-CoV-2 replicon BAC DNA. Finally, transfection of the linearized BAC DNA in the HEK293T-LPCX(AB)-T7RNAP was also done in presence of remdesivir, a nucleoside analog that abrogates replicon RNA replication (22). GFP expression was measured 2 days later by flow cytometry. The data obtained show expression of replicon-encoded GFP in both empty vector-transduced cells (Fig 2A) and T7RNAP-expressing cells (Fig 2B) upon transfection with linearized BAC DNA. The observed percentages of GFP expression were low but non-negligible (in the 0.4-0.8% range) in both cell populations. In contrast, little to no GFP signal was detected in cells transfected with non-linearized SARS-CoV-2 replicon, showing that linearization

was essential for GFP expression from this DNA. Finally, treatment with 100 nM remdesivir did not prevent GFP expression (Fig 2B, bottom left panel).

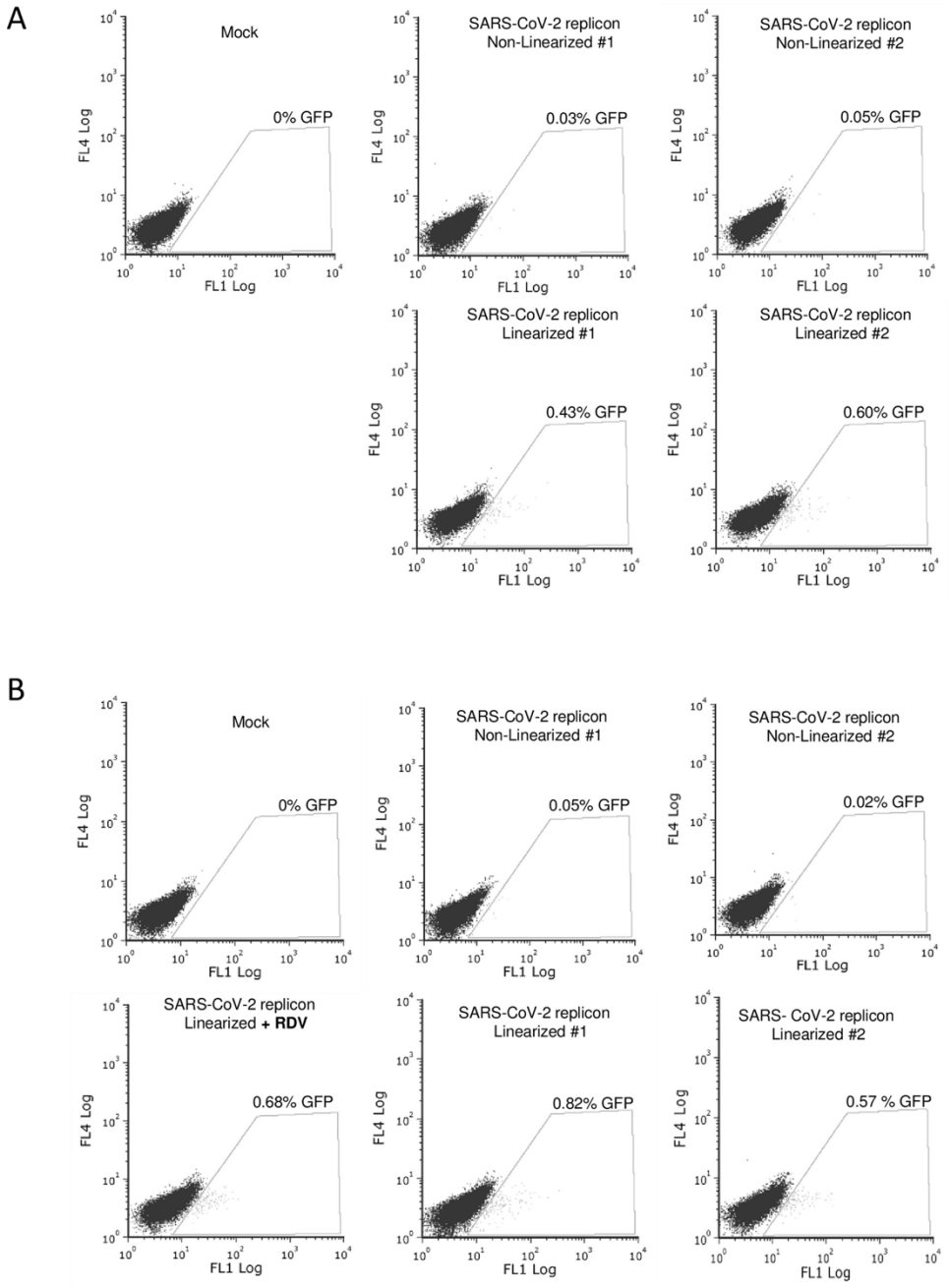

Fig 2. PEI transfection of SARS-CoV-2 replicon DNA leads to GFP expression that is dependent on DNA linearization but not T7 RNAP and is remdesivir-insensitive. HEK293T cells transduced with the empty retroviral vector LPCX(AB) (A) or with LPCX(AB)-T7RNAP (B) were PEI-transfected with the pSMART-BAC-T7-scv2 replicon or were mock-transfected as a control. BAC DNA was linearized or not with SwaI immediately prior to transfections. Two independent replicon DNA transfections were done

for both non-linearized and linearized replicon DNA. For the linearized replicon, an additional transfection was performed in the presence of 100 nM remdesivir (bottom left dot plot). GFP expression was analyzed two days post-transfection by flow cytometry.

The BAC DNA utilized in this study, with a substantial length of approximately 36 Kbp, poses a challenge for transfection, given its size. To address this, we repeated the experiment using Lipofectamine 3000 to introduce linearized or non-linearized replicon BAC DNA into HEK293T-LPCX(AB) (Fig 3A) and HEK293T-LPCX(AB)-T7RNAP (Fig 3B). Remarkably, GFP expression was consistently observed in both cell populations following transfection of linearized BAC DNA, but not non-linearized DNA. GFP was detected in 0.4-0.5% cells in both cell populations, and once again, remdesivir treatment had no effect. We used fluorescence microscopy to record images of transfected HEK293T-LPCX(AB)-T7RNAP cells in the absence (S1 Fig) or the presence (S2 Fig) of remdesivir. Fluorescence was relatively weak, as expected from the flow cytometry results, but fluorescent cells were adherent and appeared not to be at an advanced apoptotic state that may induce unspecific autofluorescence. The absence of autofluorescence artifacts is also evidenced by the fact that fluorescence was recorded in the FL1 channel of the cytometer, but not other channels like FL4 (Fig 2, Fig 3), proving that fluorescence was associated with GFP expression. Altogether, the data presented in Fig 2 and Fig 3 show that GFP expression following replicon BAC DNA is linearization-dependent, but T7 RNAP-independent and remdesivir-insensitive. These observations point to GFP expression in the absence of full-length replicon RNA expression or replication.

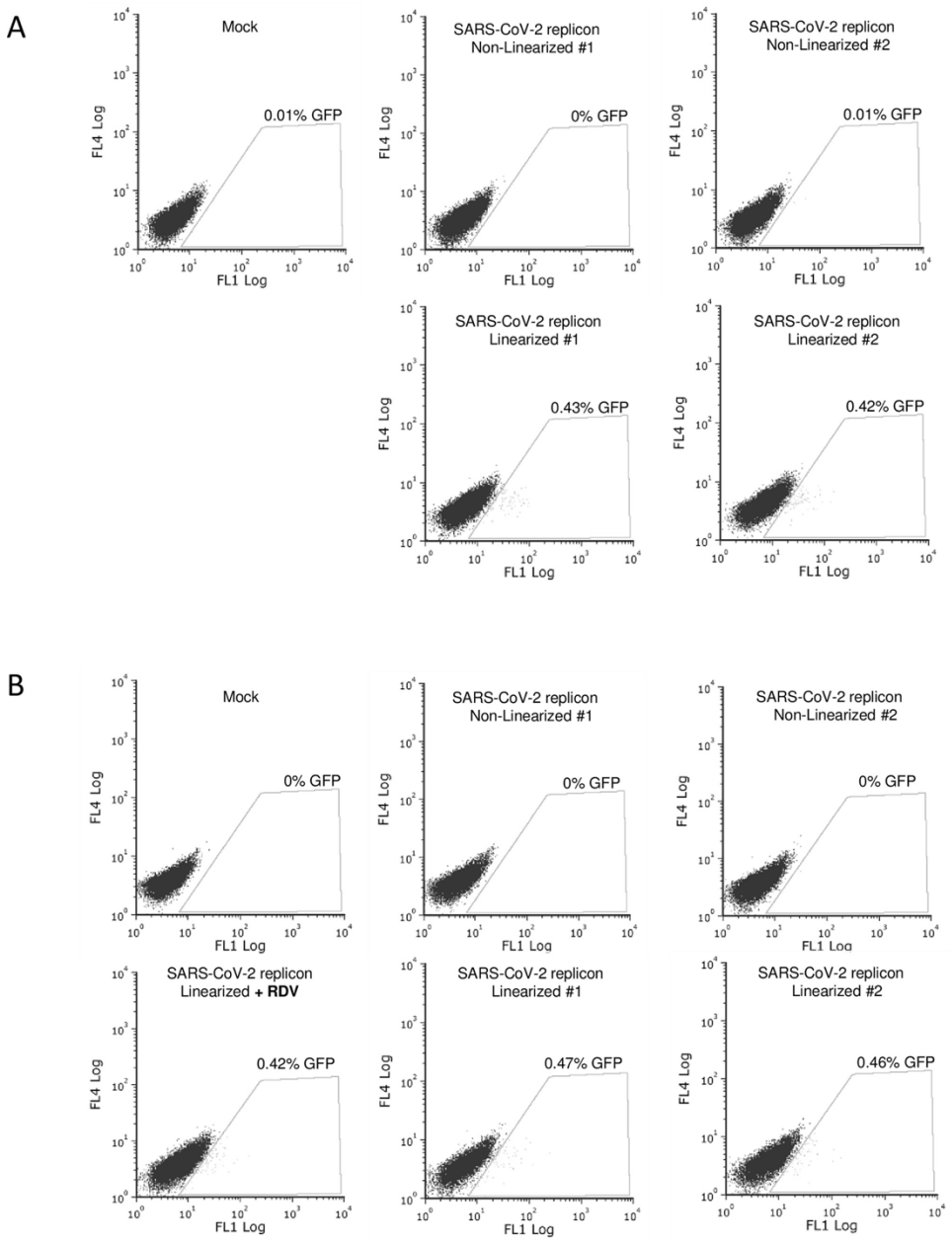

Fig 3. Lipofectamine transfection of SARS-CoV-2 replicon DNA leads to GFP expression that is dependent on DNA linearization but not T7 RNAP and is remdesivir-insensitive. HEK293T transduced with LPCX(AB) (A) or with LPCX(AB)-T7RNAP (B) were then transfected with pSMART-BAC-T7-scv2 DNA, linearized or not, and in the absence or presence of remdesivir, exactly as in Fig. 2, except that transfections were performed using Lipofectamine 3000. Cells were analyzed by flow cytometry two days later.

Luciferase activity following SARS-CoV-2 replicon-encoding DNA transfection

The replicon used in this study encodes a firefly luciferase-GFP fusion protein. Thus, GFP expression is expected to correlate with luciferase activity. HEK293T cells transduced with LPCX(AB) or LPCX(AB)-T7RNAP were transfected with SwaI-linearized or non-linearized replicon BAC DNA, and luciferase activity was measured in cellular lysates (Fig 4). The data obtained show an absence of luciferase activity upon transfection of non-linearized DNA, since RLU values were similar to background levels observed in mock-transfected cells. Luciferase activity was approximately 1.8-fold over background levels upon transfection of linearized DNA, and luciferase activity levels were similar in both LPCX(AB) and LPCX(AB)-T7RNAP cells. Furthermore, remdesivir treatment did not affect luciferase activity levels (Fig 4). Thus, these results recapitulate the GFP expression data: linearization of the SARS-CoV-2 replicon DNA is required for luciferase expression, but T7 RNAP is not, and expression is remdesivir-insensitive.

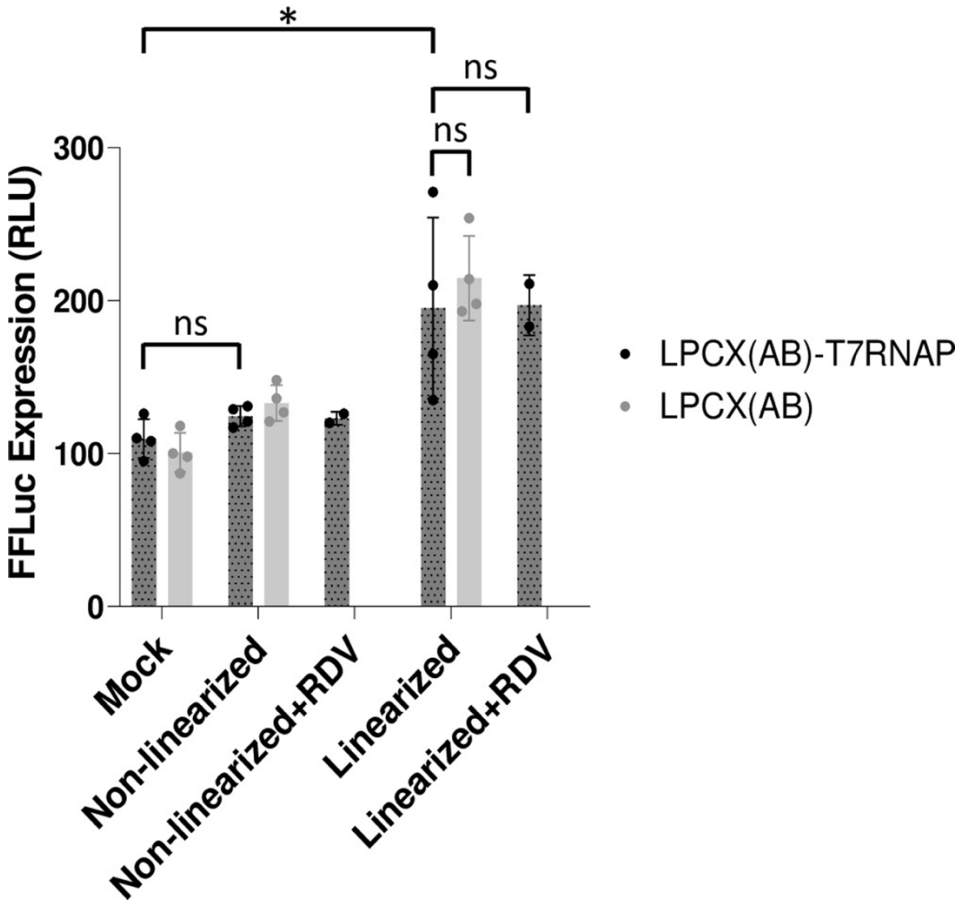

Fig 4. SARS-CoV-2 replicon DNA-encoded luciferase expression is dependent upon DNA linearization but occurs in the absence of T7 RNAP and is insensitive to

remdesivir. HEK293T cells transduced with LPCX(AB) or with LPCX(AB)-T7RNAP were PEI-transfected with pSMART-BAC-T7-scv2, linearized or not as indicated, and treated or not with remdesivir (RDV). Luciferase activity was quantified two days later as detailed in the Methods section, and is expressed in relative light units (RLU). Shown are averaged values from duplicate transfections with two luciferase assays done for each transfection. A Student's t-test was used to assess statistical significance; *, $P < 0.05$; ns, non-significant.

Characterization of SARS-CoV-2 replicon in HEK293T cells following replicon DNA or RNA transfection in presence of SARS-CoV-2 N

Prior presence of the SARS-CoV-2 N structural protein has been shown to improve replicon RNA survival and expression (11). To uncover a possible role for SARS-CoV-2 N in stimulating the T7 RNAP-independent expression of marker genes from the replicon BAC DNA, we used previously generated HEK293T cells lentivirally transduced to express the N protein (12), and we also transiently transfected a mammalian expression plasmid encoding the same protein. As shown in Fig 5A, expression of N in both transiently transfected and stably transduced cells was confirmed by Western blot. We then investigated whether GFP expression from the SARS-CoV-2 replicon RNA would be stimulated by the presence of N. We were not able to investigate the effect of transiently transfecting N prior to electroporation of the SARS-CoV-2 RNA, due to high toxicity associated with the dual transfection. Thus, we focused on N expression through lentivirally transduced cells (Fig 5B). SARS-CoV-2 RNA replicon electroporation was low (less than 1%), as expected, but GFP intensity was significantly higher than what was seen upon replicon DNA transfection. Fluorescence microscopy observations confirmed that GFP-expressing cells following RNA electroporation appeared intact and that GFP intensity was relatively high compared with BAC DNA transfections (S3 Fig). Stable N transduction greatly increased the efficiency of electroporated replicon RNA expression, as 8.1% cells were GFP-positive, a nearly 20-fold increase (Fig 5B). This was also reflected in fluorescence microscopy observations (S3 Fig). Moreover, remdesivir treatment strongly reduced replicon RNA expression (a 33-fold decrease) in these N-transduced cells (Fig 5B), indicating that replicon RNA self-replication was necessary for efficient expression.

Next, we tested what effect would N transduction have on T7 RNAP-independent GFP expression following replicon DNA transfection (Fig 5C). As in previous experiments, we observed a small but significant population (around 0.5%) of GFP-positive cells following the PEI transfection of

linearized replicon BAC DNA into control HEK293T cells. The proportion of GFP-expressing cells was not significantly modulated by the presence of transduced N, or upon treatment with remdesivir.

Collectively, the results shown in Fig 5 demonstrate that T7 RNAP-independent expression of GFP from the SARS-CoV-2 replicon BAC DNA is insensitive to the presence of the viral protein N, unlike gene expression from replicon RNA. In addition, remdesivir inhibits GFP expression from transfected replicon RNA but not replicon DNA, showing that the former, but not the latter, requires replicon RNA replication.

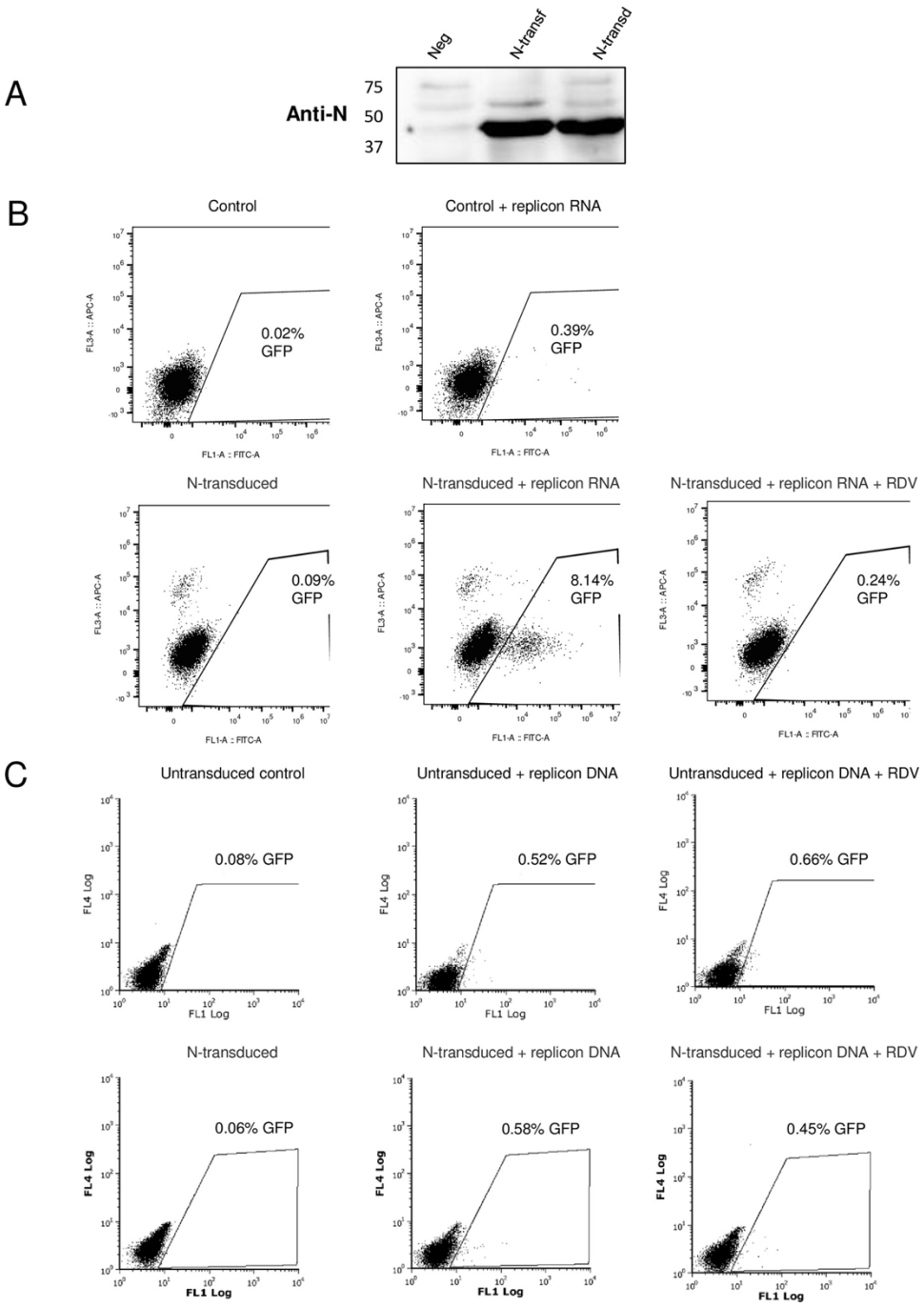

Fig 5. SARS-CoV-2 N promotes GFP expression following replicon RNA but not BAC DNA transfection. (A) Western blot showing N expression in HEK293T cells two days following PEI transfection of pEZY3-N, and in HEK293T cells lentivirally transduced with N. "Neg" are non-transfected, non-transduced HEK293T cells. (B) Effect of SARS-CoV-2 N transduction on SARS-CoV-2 replicon RNA expression. Control untransduced HEK293T cells were untransfected (top left dot plot), or were electroporated with SARS-CoV-2 replicon RNA (top right plot). Cells stably transduced with SARS-CoV-2 N were left untransfected (bottom left plot) or were electroporated with SARS-CoV-2 replicon RNA in the absence (bottom center plot) or the presence (bottom right plot) of 1 μM

remdesivir. GFP expression was analyzed by flow cytometry two days later. (C) Effect of SARS-CoV-2 N transduction on GFP expression from BAC replicon DNA. Control untransduced cells (top three plots) and N-transduced cells (bottom three plots) were left untransfected (left plots) or were electroporated with SwaI-linearized pSMART-BAC-T7-scv2 in the absence (center plots) or the presence (right plots) of 1 μM remdesivir. GFP expression was analyzed by flow cytometry two days later.

GFP expression from SARS-CoV-2 replicon-encoding DNA in Calu-3 cells

To test whether a reporter gene carried by a DNA encoding a SARS-CoV-2 replicon could be detected in a cell line relevant to SARS-CoV-2 replication, we used the pulmonary cell line Calu-3. Linearized replicon BAC DNA was electroporated (as PEI was found to be inefficient in this cell line) alone or along with a plasmid expressing T7 RNAP. As shown Fig 6, less than 1% cells were GFP-positive, similar to HEK293T cells, and the frequency of GFP-expressing cells was not improved in the presence of T7 RNAP. As seen by fluorescence microscopy, the GFP-expressing cells appeared intact, suggesting that the fluorescence was not nonspecific autofluorescence (S4 Fig). Like before, treatment with remdesivir did not inhibit GFP expression from transfected replicon BAC DNA. Thus, the results obtained in Calu-3 cells corroborate the conclusions reached in HEK293T cells.

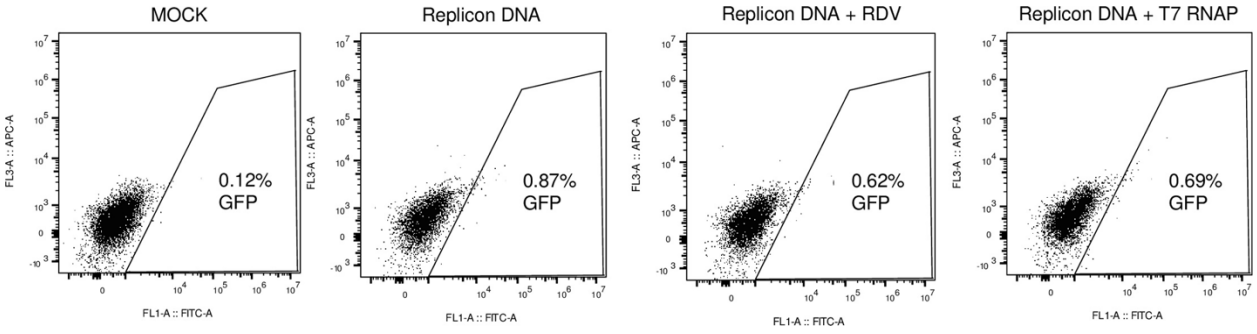

Fig 6. **Transfection of replicon BAC DNA in Calu-3 cells yields T7 RNAP-independent and remdesivir-insensitive GFP expression.** Calu-3 cells were electroporated with SwaI-linearized pSMART-BAC-T7-scv2 and co-transfected or not with T7 RNAP or in the presence of 1 μM remdesivir (RDV) as indicated. Cells were analyzed by flow cytometry two days later.

3.5 Discussion

Biological studies of human viruses and the search for antiviral compounds require convenient methods to manipulate and mutate viral genomes and then to introduce them into human cells. For coronaviruses as well as many other RNA viruses, this can be achieved through reverse genetics (23), whereby a cDNA copy of the RNA genome is introduced into a DNA vector such as the BAC used in this study, and put under control of a microbial promoter such as the T7 RNAP-dependent promoter also used in the present study. The constructed DNA is amplified, typically in bacteria, yeast or insect cells, then purified and *in vitro* transcribed to yield a viral genomic RNA that is then introduced into mammalian cells. This viral RNA can self-replicate, thus acting as a replicon, and it can also lead to the production of novel, infectious viral particles. SARS-CoV-2 has presented additional challenges as it long was a biosafety level 3 pathogen, greatly decreasing the number of laboratories in which the wild-type virus could be studied. Thus, several groups have created reverse genetics systems to express subgenomic rather than full-length SARS-CoV-2 RNAs, as these subgenomic replicons may be used in level 2 confinement laboratories. In four different SARS-CoV-2 replicons created in 2021, the deleted viral genes included S (24), N (25), E and ORF3 (26), or the replicon used here, which is truncated for S, M and E (18). In all cases, the deleted open reading frames were replaced with marker genes such as GFP, luciferase or mCherry, allowing for the convenient measurement of replicon RNA replication and expression.

Most replicon systems rely on the *in vitro* generation of the replicon RNA followed by its introduction into cells by transfection (electroporation being the most common method used). This creates a bottleneck to upscaling, as both *in vitro* transcription and electroporation are expensive and RNA is notoriously unstable and more difficult to transfect into cells, compared with DNA. To bypass these limitations, we explored here the possibility of introducing replicon DNA, rather than RNA, into human HEK293T cells. In this scenario, expression of the replicon would occur through transcription of the replicon cDNA *in cellulo* rather than *in vitro*. Toward this aim, we constructed cells stably expressing T7 RNAP, and verified that the polymerase was indeed functional. Several teams have shown that T7-specific transcription could take place in eucaryotic cells (27), using for instance a T7 RNAP expression construct stably integrated into a mammalian cell's genome (28). The T7 coding sequence that was used here was codon-optimized precisely to allow for higher expression levels in mammalian cells (14). However, transfection of the T7-dependent replicon-encoding BAC did not lead to T7-specific replicon expression, since we

observed low levels of replicon-encoded GFP-positive cells both in the presence and absence of T7 RNAP. GFP detection in ~0.5% of the cells was reproducible and independent of the method used for transfecting cells. However, it was not due to background fluorescence nor was it an artifact of cytotoxicity, since fluorescence was not observed when the BAC DNA was not linearized, and fluorescent cells did not show obvious signs of cytotoxicity when observed by microscopy. Moreover, luciferase expression was similarly detected in a T7 RNAP-independent, linearization-dependent fashion. At the moment, it is not clear whether the BAC DNA T7 promoter is functional or not in HEK293T cells expressing T7 RNAP, as the absence of detectable replicon RNA might be due to degradation rather than a lack of synthesis. It is also possible that T7 RNAP was not expressed at levels sufficient to support expression from the BAC T7 promoter. Thus, introduction of the replicon-encoded BAC DNA into HEK293T cells can lead to expression of replicon genes through a mechanism that remains to be investigated at the molecular level.

What mammalian polymerase could direct the expression of GFP/luciferase from the transfected BAC DNA? In addition to the T7 promoter, the replicon BAC DNA contains a CMV promoter; however, DNA linearization was required for marker gene expression, which at first glance is not consistent with the known characteristics of the CMV promoter. BAC DNA is supercoiled (29), similar to common plasmids. Thus, perhaps the RNA polymerase synthesizing the Luc-GFP mRNA here does not accommodate supercoiled DNA of bacterial origin. However, a simpler explanation would be that gene expression requires RNA transcription termination which is allowed by the linearization step, similar to T7 RNAP-led transcription (which in turn may imply that the HDV ribozyme is in fact non-functional). Another hypothesis is that BAC DNA linearization influences the nature and activity of histone proteins binding the newly introduced DNA. Yet another possible explanation for the importance of linearization might be that it facilitates integration of BAC DNA into human DNA. Indeed, linearization was shown to increase the frequency of plasmid DNA into mammalian cell's DNA (30-32). In part due to the low intensity of expression for the two marker genes, as well as low percentages of BAC-transfected cells, we did not attempt to analyze the RNA or RNAs expressed from the BAC DNA, and thus it is not clear whether the mRNA for GFP and luciferase is identical to the SARS-CoV-2 replicon RNA synthesized by T7 RNAP *in vitro* (full-length), or whether shorter mRNAs are synthesized. Interestingly, GFP expression was insensitive to remdesivir, and was not improved by the concomitant presence of SARS-CoV-2 N, a protein known to promote replication of the replicon

RNA (33, 34), as also observed in this study. These two observations suggest that the mRNA encoding GFP and luciferase is not self-replicating. In turn, this suggests that it is in fact a subgenomic RNA.

While this study was being conducted and this manuscript written, other groups contributed valuable advances to the goal of producing DNA-launched SARS-CoV-2 replicon systems. These constructs are all carried on BAC plasmids, similar to the one used here, but they are more minimalistic, typically including ORF1a, ORF1b and N, but excluding all or most of the structural and accessory proteins (11, 35, 36). Expression of the replicon RNA from these various BAC constructs was achieved with a CMV promoter and in the absence of linearization. It is likely that the presence of all the ORFs in the replicon used in the present study made it more challenging to be expressed from a DNA in mammalian cells, either using the CMV or T7 promoters present in the BAC construct. An interesting observation reported in these other studies is the relative insensitivity to antiviral drugs such as remdesivir upon replicon DNA transfection, similar to what we have observed here. In a recent paper, sensitivity to antiviral drugs was restored by using the RNA splicing inhibitor isoginkgetin, suggesting that replication-independent expression of replicon-associated genes stems from high levels of RNA splicing (37). Expression of a subgenomic RNA by splicing of the larger replicon RNA provides a possible explanation to the remdesivir insensitivity of GFP/luciferase expression in our own study, but it does not elucidate the T7 RNAP-independent expression, nor the linearization-dependent expression, which we observed. We also cannot discard the possible presence of a cryptic promoter upstream from GFP-Luc in the replicon-encoding BAC DNA. Thus, producing an RNA replicon containing most SARS-CoV-2 ORFs in a DNA-based fashion remains a highly challenging goal.

3.6 Conclusions

In vitro RNA transcription-free reverse genetics systems are desirable for coronaviruses and other RNA viruses. In the process of developing protocols for *in cellulo* replicon RNA expression from a large cDNA plasmid, we came across the unexpected observation that reporter genes were expressed in the apparent absence of replicon RNA replication. Despite low levels of reporter protein expression, it was found to be highly reproducible, required linearization of the plasmid, and occurred through an undetermined transcription mechanism. This observation is relevant to the development of novel DNA-launched tools for RNA virus research, including replicons and

viral vectors, that mimic as closely as possible the wild-type viral genome and viral replication steps.

3.7 Acknowledgements

We are grateful to Marcel Bruchez (Carnegie Mellon University, Pittsburgh, PA) and Benhur Lee (Icahn School of Medicine, New York City, NY) for sharing plasmid DNAs through Addgene, as well as Dai Wang and Merck & Co (West Point, PA) for sharing the SARS-CoV-2 replicon-encoding BAC construct through BEI Resources, NIAID, NIH. We also thank Andres Finzi (Université de Montréal) for providing the Calu-3 cells.

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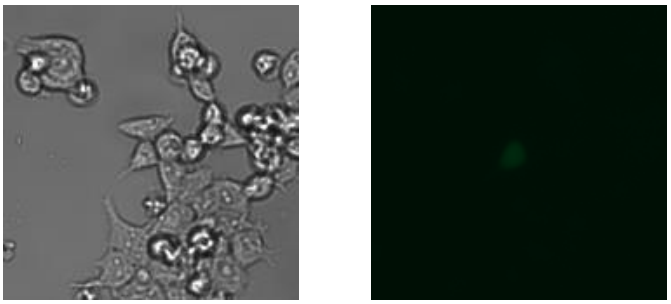
3.9 Supporting information

Fig S1. Fluorescence microscopy analysis of BAC DNA-transfected HEK293T cells

LPCX(AB)-T7RNAP, linearized BAC DNA, Lipo3000 transfection



LPCX(AB)-T7RNAP, linearized BAC DNA, PEI transfection

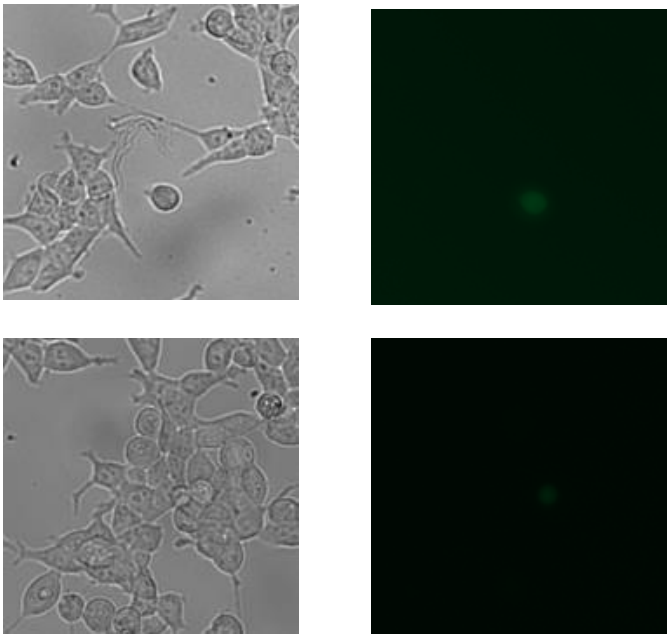


Fig S2. GFP expression in BAC DNA-transfected HEK293T cells in presence of remdesivir

LPCX(AB)-T7RNAP, linearized BAC DNA, PEI transfection, +RDV

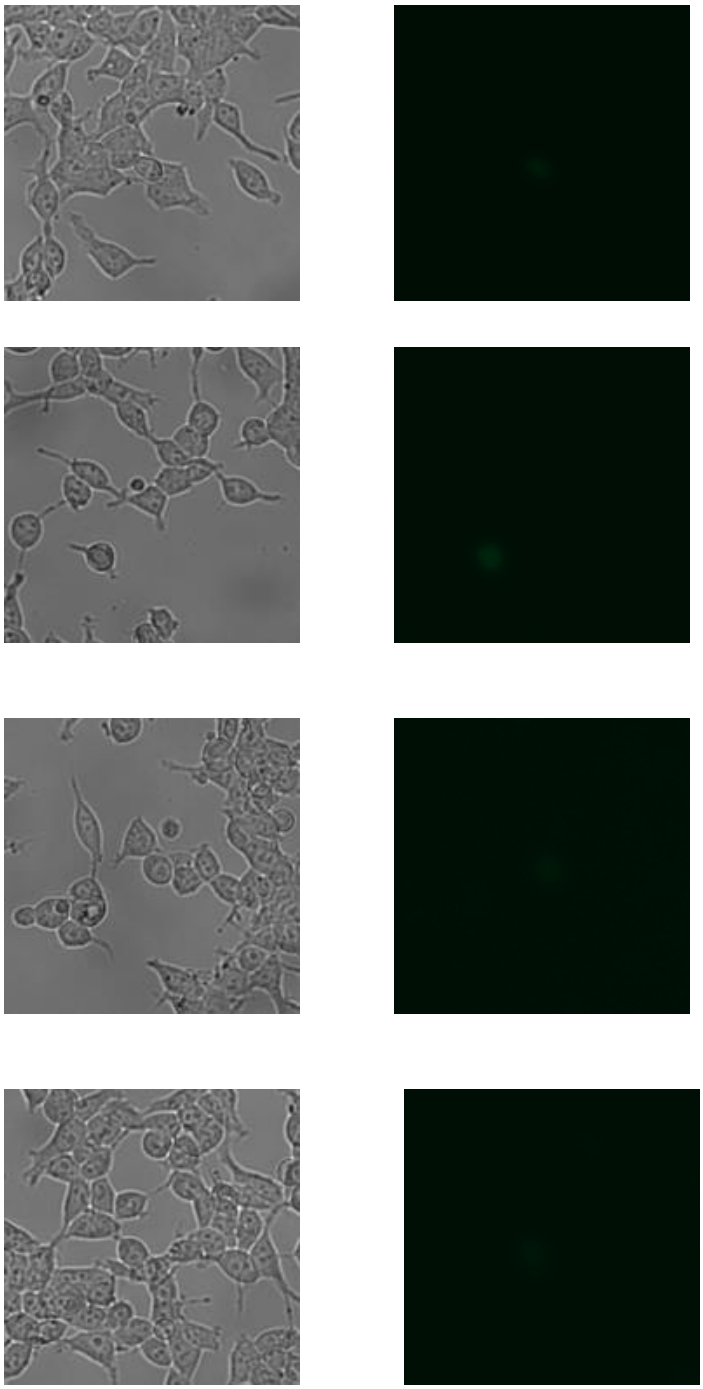
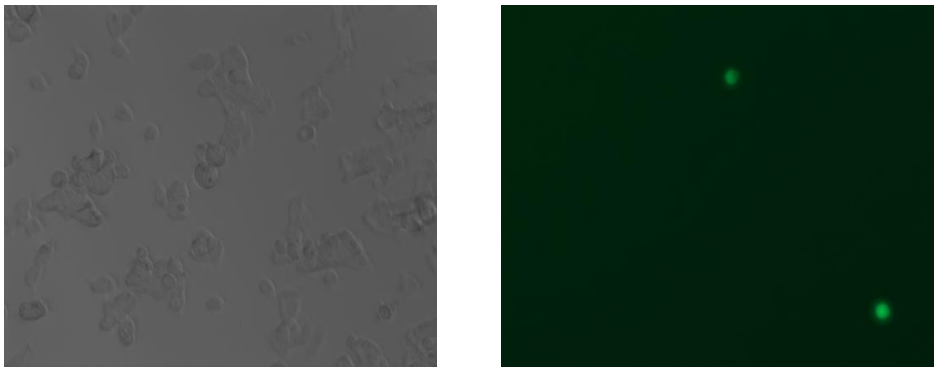


Fig S3. Fluorescence microscopy analysis of replicon RNA-transfected HEK293T cells transduced or not with SARS-CoV-2 N

HEK293T, replicon RNA electroporation



N-transduced HEK293T, replicon RNA electroporation

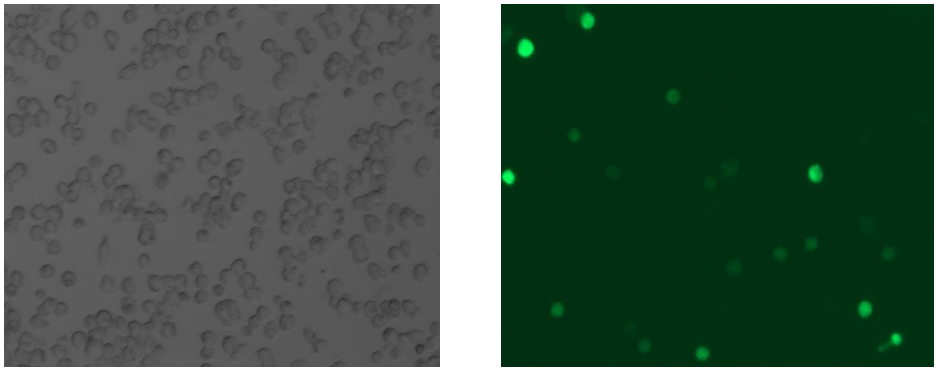
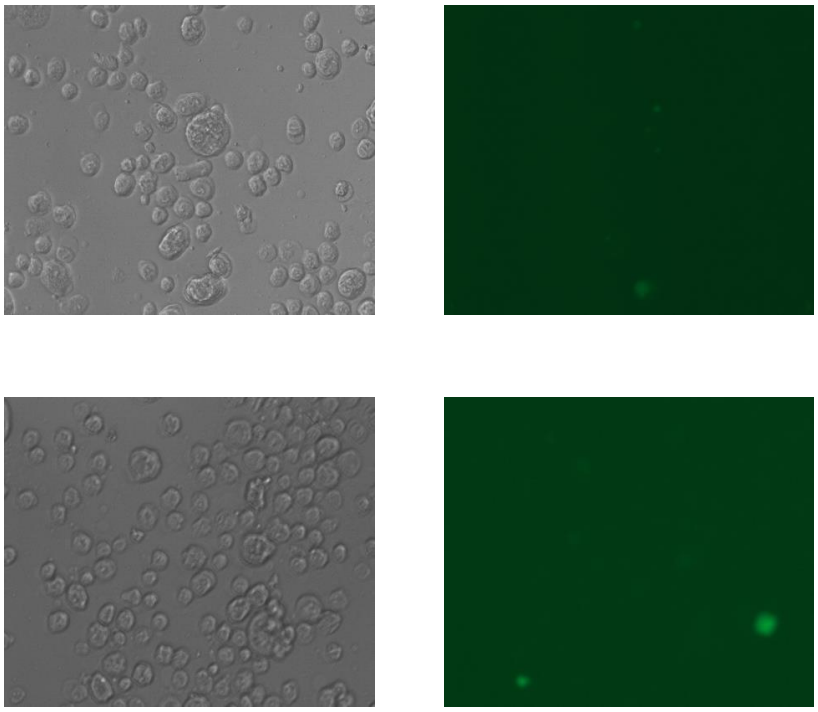


Fig S4. GFP expression in BAC DNA-transfected Calu-3 cells



Chapter IV is based on the following study:

Elfayres, G.; Xiao, Y.; Pan, Q.; Liang, C.; Barbeau, B.; Berthoux, L. A Novel SARS-CoV-2-Derived Infectious Vector System. *Microbiol. Res.* **2025**, *16*, 125. https://doi.org/10.3390/microbiolres16060125

This study describes the establishment of a SARS-CoV-2-derived vector system capable of producing single-round infectious particles for research applications

CHAPTER IV

A novel SARS-CoV-2-derived infectious vector system

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4.1 Abstract

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is the causative agent of COVID-19. The development of antiviral drugs for COVID-19 has been hampered by the requirement of a biosafety level 3 (BSL3) laboratory for experiments related to SARS-CoV-2, and by the lack of easy and precise methods for quantification of infection. Here, we developed a SARS-CoV-2 viral vector composed of all four SARS-CoV-2 structural proteins constitutively expressed in lentivirally transduced cells, combined with an RNA replicon deleted for SARS-CoV-2 structural protein genes S, M, and E, and expressing a luciferase–GFP fusion protein. We show that, after concentrating viral stocks by ultracentrifugation, the SARS-CoV-2 viral vector is able to infect two human cell lines expressing receptors ACE2 and TMPRSS2. Both luciferase activity and GFP fluorescence were detected, and transduction was remdesivir-sensitive. We also show that this vector is inhibited by three type I interferon (IFN-I) subtypes. Although improvements are needed

to increase infectious titers, this vector system may prove useful for antiviral drug screening and SARS-CoV-2-related investigations.

Key words: SARS-Cov-2; viral vectors; RNA replicon; drug discovery

4.2 Introduction

The emergence of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) in late 2019 marked the onset of a global health crisis unparalleled in recent history [1]. SARS-CoV-2 possesses a single-stranded positive-sense RNA genome of approximately 30 kilobases in length, encoding for a repertoire of proteins essential for viral replication, transcription, assembly, and immune evasion [2]. SARS-CoV-2 structural proteins include the spike (S), envelope (E), membrane (M), and nucleocapsid (N) proteins, which play pivotal roles in viral entry and assembly [3]. Notably, the S protein assumes a crucial role in the virus life cycle, enabling entry into human cells through interaction with the ACE2 receptor. Activation of the S protein is triggered by cellular proteases, including transmembrane protease serine 2 (TMPRSS2), further facilitating viral entry into host cells [4]. Advancement in vaccines and pharmacological agents against SARS-CoV-2 is still needed to address not only the COVID-19 pandemic but also future coronaviral outbreaks. Diverse vaccine formulations have emerged and received emergency authorization, leveraging platforms encompassing mRNA, viral vector, and protein subunit technologies [5-7]. However, the protection offered by these injectable vaccines has been consistently short-lived, and there is consensus that novel vaccines that can be delivered to the airways represent a more promising avenue [8, 9]. Antiviral drugs such as remdesivir and Paxlovid have been used to treat SARS-CoV-2. Remdesivir, an RNA polymerase inhibitor, was among the first antivirals authorized for emergency use [10]. However, its clinical effectiveness was controversial, with studies showing mild reductions in recovery time and little to no impact on overall mortality [11]. Paxlovid, a combination of nirmatrelvir and ritonavir, has shown more promising results in the early treatment of COVID-19, especially in high-risk patients, by reducing the severity of symptoms and the incidence of hospitalization [12]. However, no antiviral has been widely adopted due to limitations in their effectiveness, high costs, and logistical challenges of administration [13]. Additionally, Paxlovid has raised concerns over post-treatment viral rebound, where patients experience a

renewal of viral replication and symptoms after completing the treatment course, further limiting the utility of these antivirals as effective treatments [14].

SARS-CoV-2 antiviral research presents several challenges. Due to airborne transmissions and its potential for severe disease, many countries classified SARS-CoV-2 as a Biosafety Level 3 (BSL-3) pathogen. BSL-3 facilities are not universally accessible and greatly increase costs [15]. In addition, the large size of coronaviral genomes (≈30 kb) complicates reverse genetics manipulations [16]. Developing SARS-CoV-2-based vector systems with reduced biosafety risks has been an important goal [17]. Furthermore, such vectors should ideally encode marker genes to allow for the sensitive, easy, and inexpensive quantification of infection yields [18]. Several SARS-CoV-2 model systems have been devised as substitutes for the wild-type virus and contain a varying number of SARS-CoV-2 genes [19]. Lentiviral vectors pseudotyped with the SARS-CoV-2 spike protein were created, but they can only be applied to the study of the cellular entry step, such as the evaluation of neutralizing antibodies [20]. Other teams constructed SARS-CoV-2-based vector systems that rely on its structural proteins and thus reflect a more truthful coronaviral infection. All these systems are based on the complementation approach: they involve the introduction of a subgenomic RNA devoid of some of the structural proteins into mammalian cells, and the concomitant expression of the deleted structural proteins from other nucleic acids; vector particles that can perform a single cycle of infection are collected from the supernatants [21]. In what is perhaps the most minimalistic approach for such a model, Syed et al. created a system in which plasmids encoding the four SARS-CoV-2 structural proteins (S, E, M, and N) are co-transfected, along with a messenger RNA (mRNA) expressing reporter proteins that also contains virion encapsidation signals [22]. Although this system presents distinct advantages, such as its versatility, it is limited by the inherently short duration of marker protein expression; in addition, since no viral RNA is present and no RNA replication takes place, it cannot be used to study post-entry replication steps. More recently, another team developed a similar system, again involving the co-transfection of the four SARS-CoV-2 structural proteins as well as a reporter protein-expressing mRNA coupled with the SARS-CoV-2 packaging signal sequence, but this RNA also included amplification elements derived from the Venezuelan equine encephalitis virus (VEEV), increasing the duration of marker expression [23]. Other teams have created SARS-CoV-2 vector systems that include the viral replication machinery, allowing for the study of post-entry viral replication steps. Ju et al. have reported a simple system whereby a SARS-CoV-2 replicon

RNA, in which the N protein is replaced by GFP, is able to propagate in cells stably expressing N; SARS-CoV-2 vectors produced by these cells can achieve a single-cycle infection of cells not expressing N [24]. Ricardo-Lax et al. described a vector system combining an S-deleted SARS-CoV-2 replicon RNA complemented with an S-expressing plasmid [25]. Both this team and Malicoat et al. [26] successfully pseudotyped their vectors with the G protein of the vesicular stomatitis virus (VSV-G) instead of S. Zhang et al. developed a similar system with ORF3 and E being the transcomplemented proteins, but their system did not include a marker gene [27]. Several studies showed that, as expected, known pharmacological and biological inhibitors of SARS-CoV-2, such as remdesivir (RDV), type I interferons (IFN-I), and neutralizing antibodies, also efficiently reduced infection by SARS-CoV-2 vectors produced through complementation [24-27]. Thus, SARS-CoV-2 vector systems show great promise for use in antiviral screens. Yet, they still remain cumbersome to use, necessitating the repeated transfection of replicon RNA and of one or more plasmids for the expression of the complementing structural protein.

In this study, we successfully generated SARS-CoV-2 viral vectors in HEK293T cells constitutively producing SARS-CoV-2 VLPs made of all four structural proteins N, S, E, and M through lentiviral transduction. To produce a vector able to transduce marker genes, these cells were electroporated with a SARS-CoV-2 replicon RNA deleted for S, M, and E and expressing a luciferase–GFP fusion protein. We show that the expression of the marker genes following infection depends on replicon RNA replication and expression.

4.3 Materials and methods

Cell culture

SARS-CoV-2 N/S/E/M lentivirally transduced, virus-like particle (VLP)-producing HEK293T cells have been described before [28]. To obtain human embryonic kidney HEK293T cells expressing ACE2 and TMPRSS2, HEK293T cells were stably transfected with plasmids expressing each of the human proteins along with a selectable marker. First, human TMPRSS2 cDNA was excised from the TMPRSS2 plasmid (Addgene #53887) [29] cut with BglII and Not1, and cloned into pcDNA 3.1(+) Hygro cut with BamHI and NotI. HEK293T cells were co-transfected with linearized pcDNA 3.1(+) ACE-2 Flag (Genscript, Piscataway, NJ, USA) and pLVX puro (Takara Bio, Ann Arbor, MI, USA) using polyethylene imine [22] followed by

selection with puromycin (1 ug/mL). One selected cell clone confirmed for ACE2 expression by Western blotting was transfected with linealized pcDNA 3.1(+) Hygro TMPRSS2 using PEI followed with hygromycin B selection (100 ug/mL). The HEK293T-ACE2/TMPRSS2 cell line was established from an isolated clone tested for TMPRSS2 expression. A549 adenocarcinomic human alveolar basal epithelial cells stably expressing human ACE2 and TMPRSS2 were generated as described before [30]. Briefly, A549 cells (ATCC, CCL-185) were first transduced with lentiviral particles expressing human TMPRSS2. G418 (1 mg/mL) was added 40 h after transduction to select stably transduced cells. The selected cell population was further transduced with lentiviral particles expressing human ACE2, followed by selection with hygromycin B (1 mg/mL). Expression of ACE2 and TMPRSS2 was verified by Western blotting. Vero E6 African green monkey *kidney epithelial cells* (ATCC CRL-1586) were maintained in Eagle's minimal essential medium (EMEM) supplemented with 10% fetal bovine serum and penicillin–streptomycin. All other cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and penicillin–streptomycin. All cell culture media and supplements were from Cytiva Hyclone (Vancouver, BC, Canada).

Preparation of SARS-CoV-2 replicon-encoding bacterial artificial chromosome (BAC) DNA and *in vitro* replicon RNA transcription

The pSMART BAC v2.0 vector containing the SARS-CoV-2 (Wuhan-Hu-1) deleted in S, E, and M and encoding a luciferase–GFP fusion [31] was obtained from the Biodefense and Emerging Infections Research Resources Repository (BEI resources; #NR-54972). To transform Stbl3 *E. coli* cells (Invitrogen, Burlington, ON, Canada) with the BAC construct, one vial of One Shot Stbl3 chemically competent cells was thawed on ice and mixed with 5 µL of DNA. The mixture was incubated on ice for 30 min followed by heat shock treatment at 42 °C for 45 s without shaking. The vial was placed back on ice for 2 min, then 250 µL of pre-warmed SOC medium was added followed by incubation at 37 °C for 1 h. The cells were plated onto LB plates containing 20 µg/mL chloramphenicol and cultured overnight at 37 °C. Individual colonies were isolated and grown, and then the BAC DNA was purified using the PhasePrep BAC DNA Kit (MilliporeSigma, Oakville, ON, Canada) according to the manufacturer's instructions and analyzed by restriction enzyme digestion.

For *in vitro* transcription, the BAC replicon-encoding DNA was first linearized with SwaI (New England Biolabs, Whitby, ON, Canada), and then purified by phenol/chloroform extraction followed by ethanol precipitation and resuspension in nuclease-free water. The mMESSAGE mMACHINE T7 transcription kit (Invitrogen) was used to generate replicon RNA from the linearized vector according to manufacturer's instruction. Briefly, 180 µL of T7 transcription reaction, containing 7.2 µg of linearized BAC, T7 RNA polymerase, as well as GTP, was incubated at 37 °C for 2.5 h. A 9 µL measurement of TURBO DNase was then added and the reaction was incubated at 37 °C for 15 min to degrade DNA. The resulting RNA was purified using the Monarch RNA cleanup kit (New England Biolabs) and analyzed by agarose gel electrophoresis.

SARS-CoV-2 vector preparation

HEK293T cells continuously producing SARS-CoV-2 VLPs following stable transduction of S, N, E, and M [28] were harvested using Trypsin/EDTA (Thermo Fisher Scientific, Mississauga, ON, Canada), washed with PBS, and resuspended in Neon Resuspension Buffer R (Invitrogen) to a final density of 1×10^7 cells/mL. A 30 µg measurement of replicon RNA was added onto 2×10^6 resuspended cells. The mixture (100 µL) was immediately transferred to Neon tips (Invitrogen), and electroporation was carried out in the Neon Device (Invitrogen) using the cell type-specific preloaded parameters (1350 V, 20 ms, 1 pulse). Transfected cells were transferred immediately into 10 cm plates containing prewarmed DMEM supplemented with 10% FBS and without antibiotics and placed in antibiotic-containing medium the next day. Viral vector-containing supernatants were collected 48 h post-transfection by centrifugation at 3000 rpm for 10 min at 4 °C. Supernatants were filtered through 0.45 µm PVDF filters (Millipore Sigma, Oakville, ON, Canada), loaded on top of 20% sucrose–PBS cushions and ultracentrifuged for 90 min at 30,000 rpm at 4 °C. The pelleted particles were recovered in 50 µL of PBS solution and frozen at −80 °C.

Infections and flow cytometry

HEK293T-ACE2/TMPRSS2, A549-ACE2/TMPRSS2, and Vero E6 cells were plated in 96-well plates (3×10^4 cells/well in 100 µL) in complete DMEM. After 24 h, cells were infected with 50 µL of concentrated SARS-CoV-2 viral vector preparation. After 48 h, cells were washed with 1× PBS and detached using 100 µL trypsin. The reaction was stopped by adding 150 µL of 4% formaldehyde diluted in PBS. The percentage of GFP-positive cells was determined by analyzing

20,000 cells on a CytoFLEX S flow cytometer (Beckman Coulter, Mississauga, ON, Canada). Flow cytometry data were analyzed using FlowJo (Becton Dickinson, Mississauga, ON, Canada). In some instances, remdesivir (Cayman Chemical, Ann Arbor, MI, USA) was added immediately after infection at a concentration of 100 nM. Virus titer (FACS infectious units/mL) was assessed by flow cytometry using the formula titer = ((F × Cn)/V) × DF, where F is the frequency of GFP-positive cells determined by flow cytometry, Cn is the total number of cells exposed to the vector preparation, V is the volume of inoculum (mL), and DF the dilution factor.

Fluorescence microscopy

GFP expression in live cells was examined using an Axio Observer inverted fluorescence microscope (Zeiss) with a 20× objective, two days post-transfection with SARS-CoV-2 replicon RNA or two days post-infection with SARS-CoV-2 viral vectors. Images were recorded using the ZEN software (v3.3).

Luciferase assays

HEK293T-ACE2/TMPRSS2 cells were infected with the SARS-CoV-2 viral vector preparation as described above. Cells were processed for luciferase assay 48 h later. The Steady-Glo Luciferase Assay System kit from Promega (Madison, WI, USA) was used for all luciferase assays. Cells grown in 96-well plates were washed with PBS, trypsinized, and plated in wells of a 96-well plate (black wall, clear bottom) at 100 µL per well. A 100 µL measurement of Steady-Glo Reagent was immediately added to each well. Luciferase activity was measured using the Biotek Synergy HT microplate reader according to the manufacturer's instructions.

SARS-CoV-2 vector infections in presence of interferons

Recombinant human IFN-α was obtained from Shenandoah Biotechnology (Warwick, PA, USA). Recombinant human IFN-β and IFN-ω were obtained from PeproTech (Rocky Hill, NJ, USA). HEK293T-ACE2/TMPRSS2 cells (3×10^4 cells/well) were seeded in a 96-well plate in 100 µL complete DMEM. IFN-I was added to cell cultures 16 h prior to infection and at a final concentration of 20 ng/mL. Cells were next infected with 50 µL of SARS-CoV-2 viral vectors, as described above. Luciferase assays were performed 48 h post-infection as described above.

4.4 Results

Design and production of infectious SARS-CoV-2 viral vectors

The vector system described in this study was developed using HEK293T cells, which were modified by the simultaneous transduction of all four SARS-CoV-2 structural proteins (S, M, E, and N), as described in a previous report [28]. As illustrated in Figure 1A, we used both HEK293T cell populations continuously producing SARS-CoV-2 VLPs, as well as clones derived from these populations by limiting dilution [28]. Cells were subjected to electroporation with an in vitro-produced SARS-CoV-2 replicon RNA [31]. Following this, supernatants were harvested, and SARS-CoV-2 vector particles were purified and concentrated by filtration and ultracentrifugation. Subsequently, cells expressing ACE2 and TMPRSS2 were exposed to the vector preparations, and the resulting signals from GFP/luciferase activity were measured for quantification. As a preliminary test for SARS-CoV-2 vector production, HEK293T cells constitutively producing SARS-CoV-2 VLPs underwent electroporation with replicon RNA. Supernatants from the transfected cells were collected, concentrated, and used to infect HEK293T-ACE2/TMPRSS2 cells. As illustrated Figure 1B, GFP expression was detected by fluorescent microscopy both in the cells electroporated with the replicon RNA, as expected, and following infection of HEK293T-ACE2/TMPRSS2 with the supernatant. The GFP-positive transduced cells appeared healthy and dividing, suggesting that fluorescence was not an artifact from toxicity (Figure 1B).

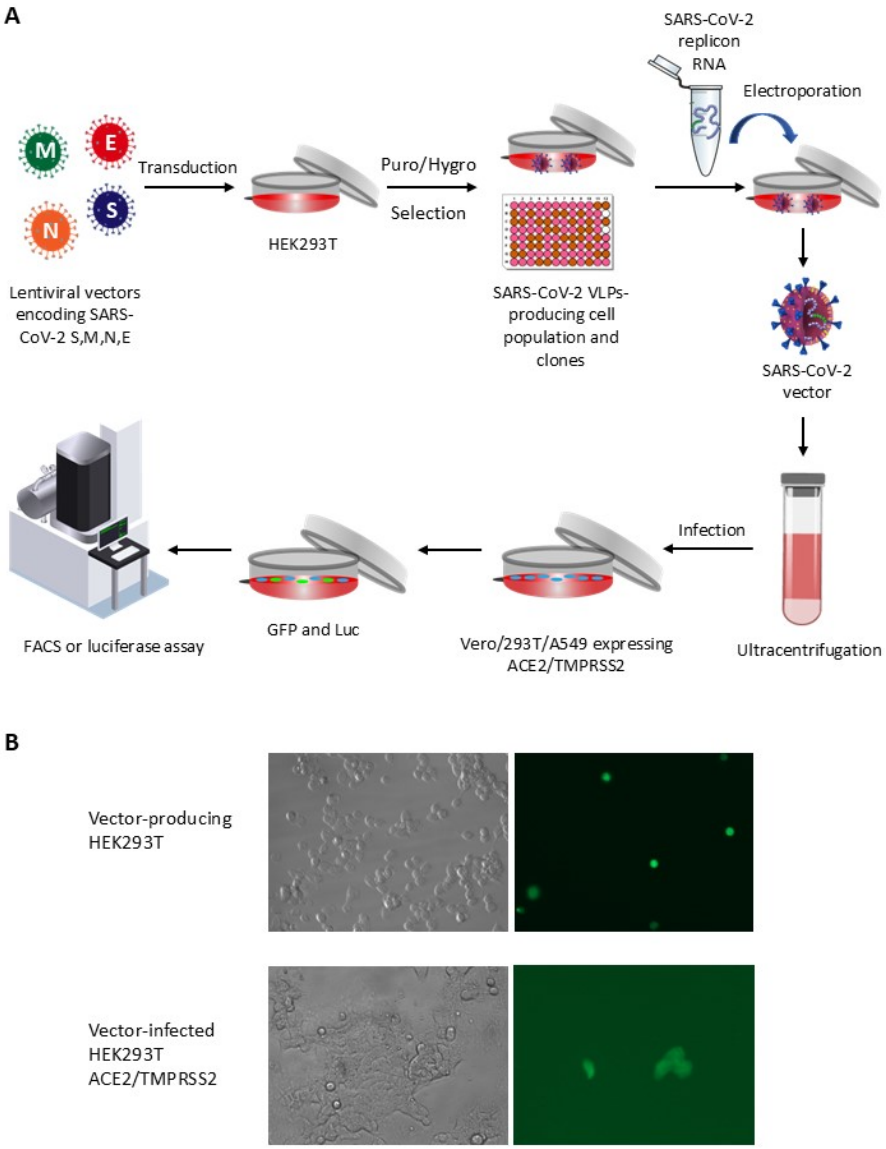
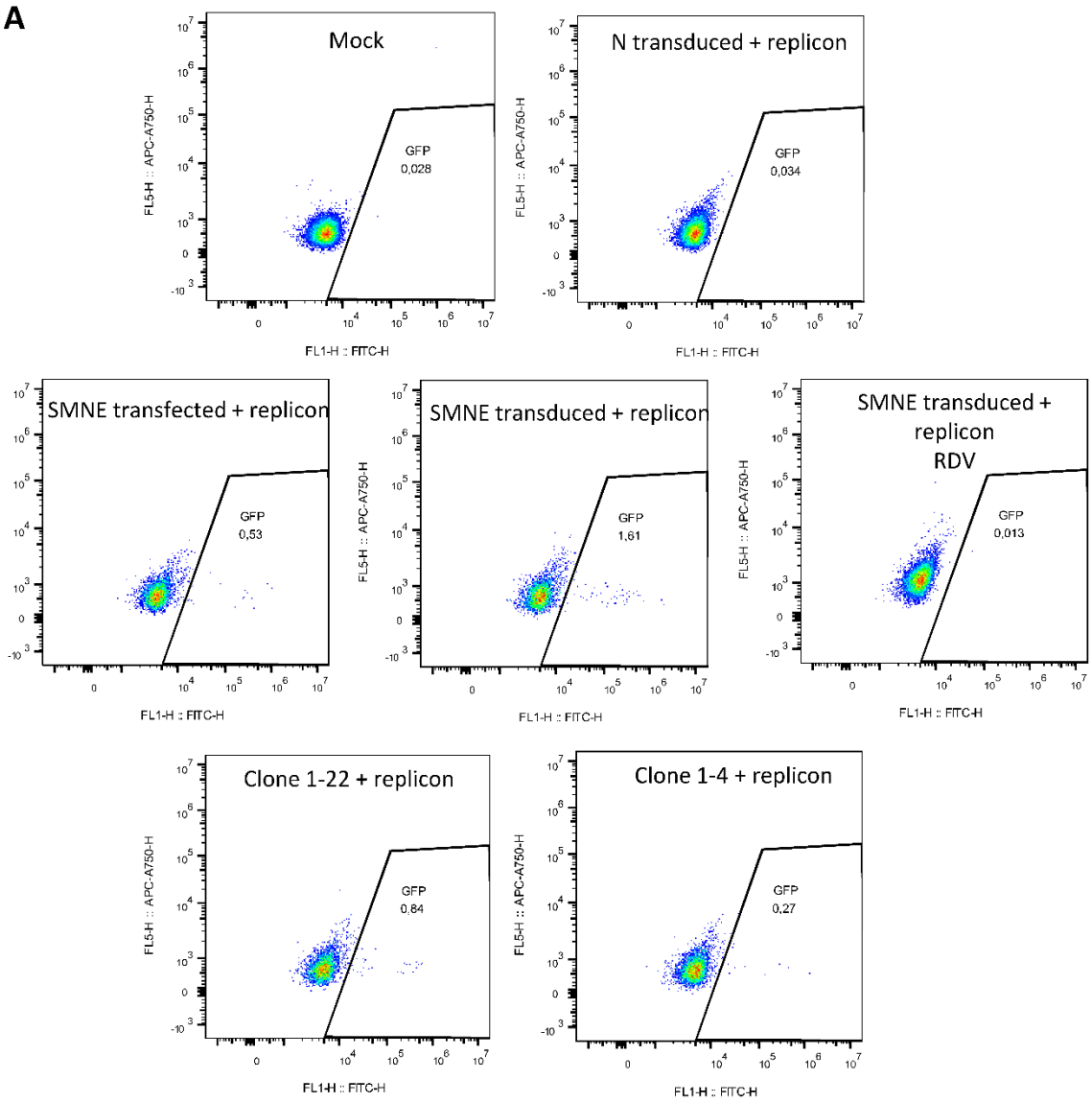


Figure 1. Design of a SARS-CoV-2 viral vector system through lentiviral transduction. (**A**) HEK-293T cells are transduced with lentiviral vectors encoding SARS-CoV-2 structural proteins to stably produce SARS-CoV-2 virus-like particles (VLPs). SARS-CoV-2 replicon RNA is subsequently electroporated into these cells. Vector particles are collected using low-speed centrifugation, followed by filtration and ultracentrifugation. The vectors are then used to infect SARS-CoV-2-permissive cell lines. Reporter gene expression (GFP and luciferase) is quantified using flow cytometry and luciferase assay, respectively. (**B**) Fluorescence microscopy analysis of HEK293T cells producing or infected with SARS-CoV-2 vectors. GFP detection in SARS-CoV-2 VLP-producing HEK293T cells following replicon RNA electroporation (top row), and in HEK293T cells expressing ACE2/TMPRSS2 following infection with SARS-CoV-2 vectors (bottom row).

We repeated this experiment, this time using a flow cytometer to quantify the proportion of infected cells (Figure 2). In order to investigate the requirements for efficient vector production, we also tested the electroporation of the replicon RNA in cells co-transfected with all four SARS-CoV-2 structural proteins instead of being lentivirally transduced. We also electroporated the replicon RNA in two HEK293T cellular clones, 1-22 and 1-4, which were derived from the SARS-CoV-2 S/M/N/E transduced population [28]. Finally, as an additional control, we also electroporated the SARS-CoV-2 replicon in parental HEK293T cells expressing no SARS-CoV-2 protein, or in cells expressing N only. GFP levels were measured by flow cytometry two days post-infection. The data show expression of GFP in 1.6% HEK293T-ACE2/TMPRSS2 cells upon infection with SARS-CoV-2 vectors prepared by electroporation of N/S/E/M-transduced cells with the replicon RNA (Figure 2A). No GFP-positive cells were seen following infection with vector prepared by electroporation of the replicon RNA in untransduced cells or in cells transduced with N only, as expected. We did observe infection of HEK293T-ACE2/TMPRSS2 cells with SARS-CoV-2 vector produced in cells co-transfected with all four SARS-CoV-2 proteins along with the replicon RNA, but the frequency of transduced cells was lower (0.53%) compared to the vector produced from the N/S/E/M-transduced cells (1.61%). Thus, and at least based on these limited initial trials, the lentiviral transduction approach to express the SARS-CoV-2 structural proteins leads to higher vector yields. In addition, little to no GFP signal was detected in cells infected with SARS-CoV-2 vector in presence of remdesivir (0.01%), a nucleoside analog that abrogates replicon RNA replication [10], showing that GFP expression was dependent upon replicon RNA replication and expression in the target cells. Based on the flow cytometry data obtained, and assuming no infectivity loss upon vector cleaning and concentration, we calculated a vector titer of 800 IU/mL for the unconcentrated supernatant from the N/S/E/M-transduced cells compared to 265 IU/mL from N/S/E/M-transfected cells. SARS-CoV-2 vectors produced in clone 1-22 and clone 1-4 yielded 0.84% and 0.27% GFP-expressing cells, respectively. The infectious titers for these two vector preparations were calculated at 420 IU/mL and 135 IU/mL, respectively. The results obtained with these two clones confirm that VLPs are being stably produced from the transduced cells, since establishment of those clonal populations required several weeks. However, the vector yields obtained were lower compared to the lentivirally transduced parental population.


A
Mock
GFP
0,028
N transduced + replicon
GFP
0,034
SMNE transfected + replicon
GFP
0,53
SMNE transduced + replicon
GFP
1,61
SMNE transduced + replicon RDV
GFP
0,013
Clone 1-22 + replicon
GFP
0,84
Clone 1-4 + replicon
GFP
0,27
FL5-H :: APC-A750-H
FL1-H :: FITC-H

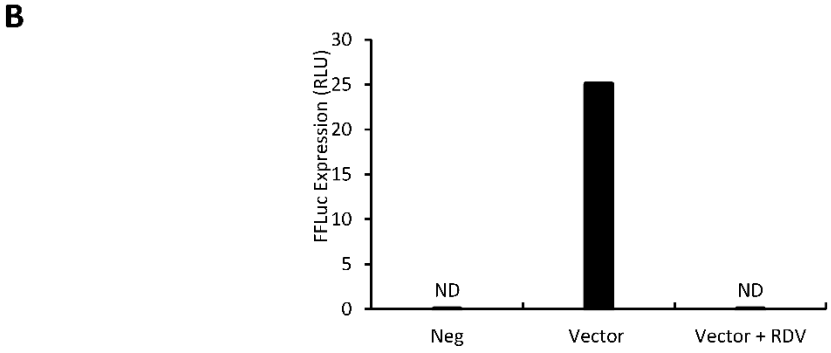

B
FFLuc Expression (RLU)
30
25
20
15
10
5
0
ND
ND
Neg
Vector
Vector + RDV

Figure 2. SARS-CoV-2 vectors transduce GFP and luciferase genes in ACE2/TMPRSS2-expressing HEK293T cells and exhibit remdesivir sensitivity. (**A**) HEK293T cells were exposed to SARS-CoV-2 vectors produced by electroporation of SARS-CoV-2 replicon RNA in a cell population stably transduced with S/M/N/E, or in selected clones (1-22, 1-4) derived from this population. As controls, (i) cells were exposed to the vector produced in S/M/N/E-transduced cells in the presence of 100 nM remdesivir (RDV); (ii) SARS-CoV-2 replicon RNA was electroporated in parental HEK293T cells (Mock) or in cells transduced with N only. GFP expression was quantified by flow cytometry 48 h post-infection (shown are density plots). (**B**) HEK293T-ACE2/TMPRSS2 cells were exposed to SARS-CoV-2 vectors and treated or not with remdesivir. Luciferase activity was measured 48 h post-infection and is reported in relative light units (RLU), as described in the Materials and Methods section.

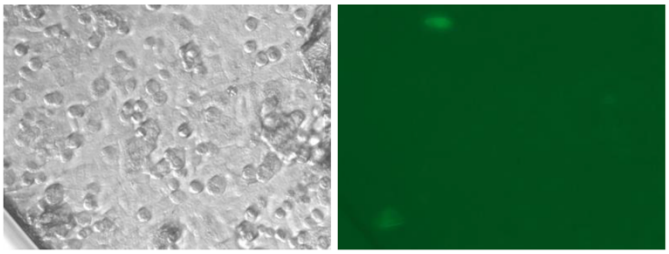
The SARS-CoV-2 replicon used in this study encodes a firefly luciferase–GFP fusion protein. Consequently, we anticipated a correlation between GFP expression and luciferase activity. HEK293T cells expressing ACE2 and TMPRSS2 were exposed to SARS-CoV-2 vector prepared in HEK293T cells lentivirally transduced with N/S/E/M, and luciferase activity was next quantified in cellular lysates. As shown in Figure 2B, luciferase activity was detected post-infection, as evidenced by measurement of high RLU values compared to uninfected cells. Moreover, treatment with remdesivir resulted in a strong reduction in luciferase activity levels. Thus, these results recapitulate the GFP expression data, confirming the production of a viral vector expressing GFP and luciferase reporter proteins.

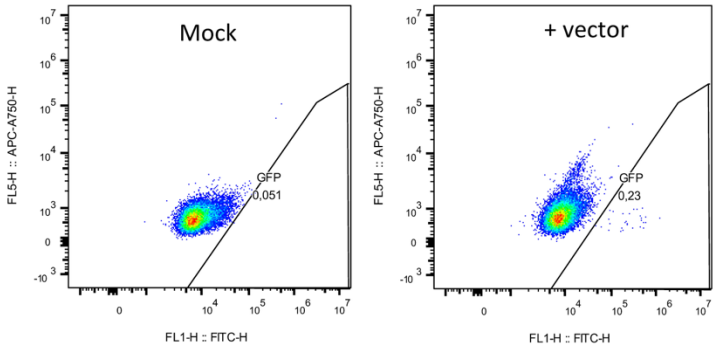
SARS-CoV-2 viral vector challenges in different cell lines

To investigate the cellular tropism of the SARS-CoV-2 vector, A549 cells expressing ACE2/TMPRSS2 (Figure 3A) and Vero E6 cells (Figure 3B) were subjected to vector infection. Both cell lines were found to be permissive to infection (0.23% and 0.37%, respectively). The virus titer was 115 IU/mL and 185 IU/mL, as determined in these two cell lines. Fluorescence microscopy images showed healthy GFP-expressing cells, instead of signals attributable to autofluorescence induced by toxicity. Altogether, results from Figures 2 and 4 suggest that this SARS-CoV-2 vector exhibits cellular tropism comparable to the authentic SARS-CoV-2 virus.

A

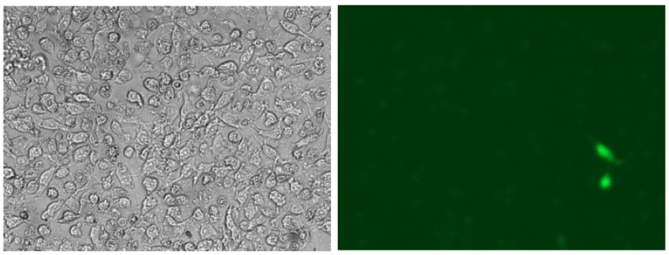
Vector-infected
A549 ACE2/TMPRSS2





B

Vector-infected
Vero E6



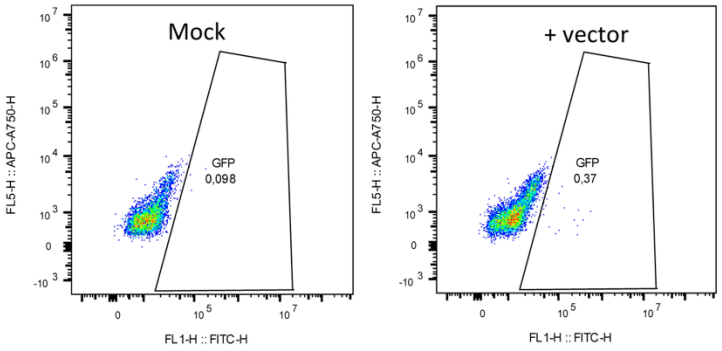


Figure 3. Detection of SARS-CoV-2 viral vector-mediated GFP expression in A549 ACE2/TMPRSS2 and Vero E6 cells. GFP detection in A549-ACE2/TMPRSS2 cells (**A**) or Vero E6 cells (**B**) infected with the SARS-CoV-2 vector, as analyzed by fluorescence microscopy and flow cytometry 48 h after infection. Density plots are shown.

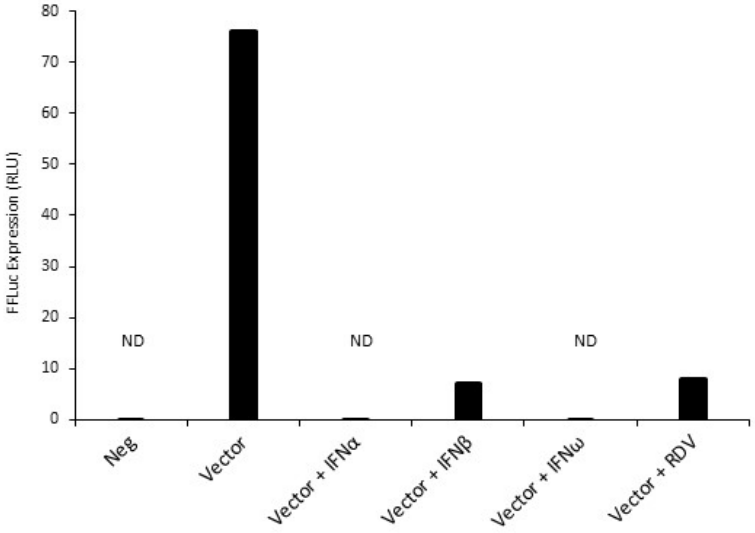


Figure 4. Inhibition of SARS-CoV-2 vector-mediated infection of HEK293T-ACE2/TMPRSS2 cells by type I interferons (IFN-I). HEK293T cells were pre-treated with IFN-α, IFN-β, or IFN-ω for 16 h prior to infection with the SARS-CoV-2 vector. As a control, infection was also performed in the presence of 100 nM remdesivir. Luciferase activity was measured 48 h post-infection. ND, not detected.

Inhibition of SARS-CoV-2-mediated infection by IFN-I

We employed the SARS-CoV-2 vector system to assess the antiviral efficacy of type I interferons (IFN-I), specifically human IFN-α and IFN-β, which are known for their capability to hinder SARS-CoV-2 infection, and IFN-ω, which, to the best of our knowledge, had not been tested yet. All three IFN-I subtypes efficiently decreased SARS-CoV-2 vector infectivity, as evidenced by luciferase assay (Figure 4), without inducing visible cytotoxicity to target cells at the tested concentration. Moreover, we again noted SARS-CoV-2 vector inhibition by remdesivir. This result suggests that the SARS-CoV-2 vector described here has a potential use in SARS-CoV-2 antiviral drug screening.

4.5 Discussion

Working with SARS-CoV-2 often requires strict containment measures in BSL-3 laboratories, which restricts research on the virus and the development of antiviral therapies. In this study, we developed a SARS-CoV-2 viral vector that offers a safer option for use in BSL-2 settings. The vector is based on SARS-CoV-2 replicon RNA lacking the structural protein genes S, M, and E, allowing for amplification of a reporter gene without generating infectious SARS-CoV-2 particles. Instead, the viral structural proteins are continuously expressed as VLPs in transduced HEK293T cells (Figure 1). Notably, the SARS-CoV-2 viral vector is capable of infecting two human cell lines that were modified to express the virus receptors ACE2 and TMPRSS2, replicating essential characteristics of the original virus, such as viral particle assembly, entry into ACE2-expressing cells, viral RNA replication, and viral gene expression. To provide evidence that replicon RNA replication was required for efficient GFP/luciferase expression, remdesivir sensitivity was repeatedly tested and demonstrated to abrogate these signals. Additionally, in contrast to other SARS-CoV-2 vectors that have been previously reported, the one described here uses stable lentiviral transduction to express the four SARS-CoV-2 structural proteins, facilitating subsequent vector production. It should be noted, moreover, that this system probably constitutes a safer option compared to vectors in which the replicon has only one deleted structural protein [24].

A key use of viral vectors is in screening potential drugs. The SARS-CoV-2 viral vector described here recapitulates all the different steps of the natural infection, making it a more accurate model for antiviral testing compared with previous systems [22]. To illustrate this point, we show for the first time that SARS-CoV-2 infection is sensitive to IFN-ω (Figure 4). Future research should focus on expanding the use of this vector system to high-throughput screening of novel therapeutic candidates targeting the structural proteins and replication machinery of SARS-CoV-2. However, a major limitation of this vector system as a drug screening tool is the low infectious titers. In this study, the detected vector titers ranged from 115 IU/mL to 800 IU/mL, which is significantly lower compared to the viral titers reported in other SARS-CoV-2 vector studies. One possible improvement could come from optimizing relative expression levels for the four structural proteins. For instance, vectors that use lower amounts of spike protein relative to other structural proteins were found to have higher titers [23]. Another possible explanation is that the large size of the SARS-CoV-2 replicon RNA creates a bottleneck to replicon stability, electroporation, and vector production. A potential solution to this problem could be to introduce mutations in N that enhance RNA expression, thereby promoting increased viral particle assembly [22]. Another possible approach would be

to test alternative SARS-CoV-2 replicons, particularly focusing on DNA-launched replicons [23, 26, 32]. These replicons offer a promising route for enhancing efficiency and consistency in viral production. By integrating the replicon directly into the host cellular genome, cells could be engineered to stably express the replicon continuously, bypassing the need for repeated RNA electroporations. This would not only improve efficiency but also reduce variability, as stable expression systems tend to generate more consistent viral titers over time [33]. Establishing stable cell lines would also open up opportunities for scalable production, essential for drug screening, vaccine development, or other therapeutic applications where high and consistent viral titers are critical. Higher vector titers will also be necessary to explore infections in animal models. A promising approach would involve testing this vector in SARS-CoV-2-permissive mouse models to study viral tropism and the capacity of the vector to infect airway cells following delivery in aerosolized form [34]. This, in turn, would open the door to using a SARS-CoV-2-derived vector in vaccine development.

Conclusions

This study contributes advances toward the ultimate goal of developing DNA-based, stable, scalable coronaviral vector production systems, with applications in anticoronaviral drug discovery as well as vaccine development.

Author contributions: Conceptualization, L.B.; Methodology, G.E. and L.B.; Formal Analysis, G.E. and L.B.; Investigation, G.E.; Resources, Y.X., Q.P., C.L. and B.B.; Writing—Original Draft Preparation, G.E. and L.B.; Writing—Review and Editing, L.B.; Visualization, G.E.; Supervision, L.B., C.L. and B.B.; Project Administration, L.B.; Funding Acquisition, L.B., C.L. and B.B. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: not applicable

Informed Consent Statement: not applicable

Data Availability Statement: Data are available on FigShare: https://figshare.com/projects/Elfayres_et_al_2024-2025/231452 (accessed 2025-06-10)

Conflicts of Interest: The authors declare no conflicts of interest.

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CHAPTER V

CONCLUSIONS

5.1.1 Generation and Characterization of SARS-CoV-2 Virus-Like Particles in HEK293T Cells

The study presented in Chapter II illustrates the successful generation of SARS-CoV-2 virus-like particles (VLPs) in HEK293T cells by co-expressing four essential structural proteins: spike (S), membrane (M), envelope (E), and nucleocapsid (N). Our findings underscore the significance of all four proteins for the efficient assembly and release of VLPs. The use of lentiviral transduction to create stable HEK293T cell lines and clones capable of continuous VLPs production offers considerable advantages for industrial-scale applications, particularly in vaccine development (Vicente, Roldão et al. 2011). This approach could streamline the production process, diminish the necessity for repeated transfections, and enhance the yield of VLPs over prolonged culture periods. However, further optimization is necessary, especially in developing robust purification techniques. Current purification challenges, such as distinguishing between VLPs and other viral protein aggregates, must be addressed, and future work should investigate advanced methods like affinity chromatography or novel filtration techniques (Koho, Mäntylä et al. 2012). Our data also indicate that accessory proteins do not play a critical role in VLPs assembly and release, reinforcing the sufficiency of the four structural proteins. Nonetheless, a more comprehensive exploration of how these proteins influence VLPs egress and their potential interactions with host immune responses remains a compelling area for future research (Hassan, Choudhury et al. 2022). From a vaccine development perspective, these VLPs, incorporating multiple SARS-CoV-2 antigens, may provide enhanced immunogenicity compared to existing vaccines that typically target only the spike protein. Their ability to mimic natural viral entry mechanisms and potentially elicit a more robust immune response—both cellular and humoral—positions them as promising candidates for next-generation intranasal COVID-19 vaccines. Additionally, by featuring multiple viral proteins, these VLPs may offer broader protection against

emerging variants, addressing a critical need in the ongoing battle against SARS-CoV-2 (Gao, Xia et al. 2023).

In the broader context of virology research, the VLPs production system outlined here serves as a valuable tool for investigating viral assembly, release, and entry mechanisms. Future studies should prioritize enhancing the scalability of VLPs production, refining purification processes, and further characterizing the immunogenic potential of these particles. Ultimately, this work could pave the way for more effective vaccines and therapeutics.

5.1.2 Future experiments

Several future experiments could be designed to build on the findings of this study and further optimize the use of SARS-CoV-2 virus-like particles (VLPs) in both research and vaccine development. A pivotal experiment would involve conducting a time-course analysis of VLPs production in lentivirally transduced HEK293T cells. The objective would be to determine the kinetics of VLPs release over time, thereby identifying the optimal timing for VLPs harvesting and assessing the stability of VLPs production over extended periods—an essential factor for scaling up industrial vaccine production (Boix-Besora, Lorenzo et al. 2022). Additionally, optimizing the purification of VLPs through techniques such as affinity chromatography, diafiltration, and PEG precipitation could reduce protein contaminants and significantly enhance the yield of high-purity VLPs. This is critical for both research applications and the production of vaccines (Effio and Hubbuch 2015). Further studies could focus on the immunogenicity of these VLPs, particularly by comparing VLPs incorporating all four SARS-CoV-2 structural proteins with other expression systems that use only the spike protein, as in most current vaccines. These experiments would be instrumental in determining whether a multivalent VLP-based approach could evoke a broader and more durable immune response by engaging both humoral and cellular immunity (Hirschberg, Ghazaani et al. 2023). Another area of research could explore how accessory proteins (e.g., ORF3b, ORF6, ORF7a, ORF8) interact with the immune system. By co-transfecting these proteins alongside structural proteins, researchers may evaluate their impact on immune response activation, aiding in the identification of new antiviral therapy targets (Abramovich 2024). Furthermore, identifying and isolating high-producing clones is critical for optimizing VLPs production for downstream applications such as vaccine development. While

traditional methods like limiting dilution have been employed to isolate these clones, future studies could focus on alternative approaches such as flow cytometry. This technique enables the rapid screening and sorting of individual cells based on fluorescence markers linked to VLPs production or S protein expression. By staining cells with antibodies specific to the spike protein and using fluorescent probes, clones with the highest S protein expression could be identified and isolated efficiently. This method not only enhances the precision and throughput of clone selection but also reduces the time required to establish high-yielding producer cell lines, accelerating the development of scalable and reproducible VLPs production platforms

Future work could focus on scaling up the production of SARS-CoV-2 VLPs to enable comprehensive preclinical evaluation. This would require optimization of culture conditions and the implementation of bioreactor-based systems to increase protein yields, together with refined purification workflows to obtain highly pure particles (Antonio, Roldão et al. 2010). Characterization of the scaled-up material should include quantitative measurement of VLPs production, such as total protein content, Western blot analysis of structural proteins, and particle quantification using nanoparticle tracking analysis or transmission electron microscopy (Linda, Lua et al. 2014). Advanced structural analysis of VLPs using cryo-electron tomography (cryo-ET) can further provide valuable insights into their architecture, particularly the arrangement of the spike protein and other structural components, confirming their integrity and identifying structural features that enhance immunogenicity (Wu, Fujioka et al. 2024). From a vaccine development perspective, comparing VLPs production across different cell systems, such as Vero, CHO, and insect cells, will deepen our understanding of how cellular backgrounds influence VLPs assembly, glycosylation patterns, and overall immunogenicity, thereby guiding the selection of the most suitable platform for large-scale vaccine development (Lu, Zhao et al. 2022). In addition, the immunogenicity of these VLPs should be assessed in vivo through intranasal administration in mice, with evaluation of both mucosal and systemic responses by measuring secretory IgA in bronchoalveolar lavage fluid, serum IgG titers, and T cell responses (Blaise, Corthésy. 2013; Ed, Lavelle & Ward. 2021). Testing various adjuvants in combination with VLPs could further help identify formulations that maximize protective immunity (Chulanetra, Punnakitikashem et al. 2024). Together, these directions hold great promise for optimizing VLPs technology for both fundamental research and vaccine development, potentially resulting in more effective and scalable solutions to combat COVID-19 and emerging variants.

5.2.1 Challenges in optimizing DNA-Launched SARS-CoV-2 Replicon Systems for Viral Genome Manipulation

The study presented in Chapter III addresses the technical challenges faced in optimizing DNA-launched SARS-CoV-2 replicon systems, particularly focusing on the difficulties of expressing a full viral replicon DNA with the correct 5' and 3' termini capable of self-replication after transcription. While we observed limited expression of marker genes like GFP and luciferase, this expression was unexpectedly independent of T7 RNA polymerase (RNAP) and required the linearization of the replicon. The need for DNA linearization suggests that factors such as supercoiling, RNA transcription termination, or even integration into the host genome could influence gene expression (Dorman and Dorman 2016). Despite that previous research has shown that T7-specific transcription can occur in eukaryotic cells (Yan, Li et al. 2021), using constructs expressing T7 RNAP integrated into the mammalian cell genome (Buchholz, Finke et al. 1999). The length of the SARS-CoV-2 replicon used in the study may explain the challenges associated with the absence of T7 replicon replication. Additionally, the T7 promoter's failure to function as anticipated in HEK293T cells, along with the observation that remdesivir did not inhibit GFP expression, indicates that the expressed RNA may not be self-replicating. Instead, it might represent subgenomic fragments of the viral RNA. This scenario opens up intriguing possibilities regarding alternative RNA splicing mechanisms (Zhang and Guo 2024), or the presence of cryptic promoters within the reporter genes might be triggering gene expression, thereby complicating the design of effective replicon systems (Vopálenský, Mašek et al. 2008). While other groups have reported greater success with simplified BAC constructs that exclude structural proteins (Feng, Zhang et al. 2022), our use of a more complicated SARS-CoV-2 ORF-based system may have contributed to the challenges encountered. Ultimately, achieving a fully functional DNA-launched SARS-CoV-2 replicon that accurately reflects the virus's natural replication cycle remains a significant challenge. Future efforts should concentrate on optimizing promoter selection, understanding the molecular requirements for RNA synthesis and replication, and exploring more simplified replicon designs to address the limitations identified in this study.

5.2.2 Future experiments

Several innovative experiments could address the current challenges associated with replicon-based systems and advance the application of DNA-launched SARS-CoV-2 replicons in virology research, vaccine development, and drug discovery. First and foremost, optimizing the choice of promoters is crucial for enhancing replicon expression in mammalian cells. By exploring alternative mammalian-specific promoters such as EF1α, SV40, and CAG, researchers may identify more effective drivers of replicon expression compared to the commonly utilized T7 and CMV promoters (Toktay, Dayanc et al. 2022). Streamlining the replicon design by removing structural and accessory genes could significantly enhance both replicon expression and stability. Comparing the performance of these simplified replicons with the more complex versions currently in use would provide valuable insights regarding their efficiency, replication, and cytotoxicity, ultimately aiding in the development of applications in HEK293T cells (Tanaka, Saito et al. 2022). A critical area of investigation lies in the role of RNA splicing and subgenomic RNA within replicon systems. By utilizing RNA splicing inhibitors such as isoginkgetin, researchers can evaluate whether splicing contributes to the expression of subgenomic RNA from BAC replicon constructs. RNA sequencing or RT-PCR could confirm the presence or absence of spliced subgenomic RNAs (Zhang and Guo 2024).

Improving the efficiency of DNA expression is another vital step. Linearizing BAC DNA and conducting RT-qPCR can help determine whether this linearization boosts replicon expression. Furthermore, exploring DNA modification strategies—such as DNA methylation, histone modification, or chromatin remodeling agents—could increase accessibility for transcription and facilitate the recruitment of RNA polymerase, thereby enhancing replicon efficiency (Cedar and Bergman 2009). These modifications may improve the expression of replicons by increasing the binding affinity of transcription factors and aiding RNA polymerase recruitment, particularly in mammalian cells. To further advance replicon-based systems, it is essential to identify alternative RNA polymerases capable of driving replicon expression in the absence of T7 RNA polymerase. Performing RNA polymerase profiling through chromatin immunoprecipitation sequencing (ChIP-seq) in HEK293T cells could reveal other polymerases involved in transcribing BAC DNA. Knocking down or overexpressing these candidate polymerases would help clarify their roles in replicon gene expression (Shao, Bi et al. 2022).

Additionally, exploring other expression systems that utilize Pol II-dependent transcription instead of T7 RNA polymerase could enhance replicon efficiency while minimizing the need for DNA linearization, thus streamlining the replicon production process (Ternette, Stefanou et al. 2007). Moreover, detecting cryptic promoters in DNA constructs is crucial for accurate gene expression analysis. To identify these, researchers use techniques like 5' RACE to locate transcription start sites and ChIP-seq to map RNA polymerase binding. Certain reporter genes, like luciferase, have exhibited the presence of cryptic promoters, which could lead to inaccurate results. Further investigation is required to explore this issue (Vopálenský, Mašek et al. 2008).

In the context of drug discovery and antiviral screening, the utility of DNA-launched SARS-CoV-2 replicons could be further strengthened through specific modifications. These include enhancing replication efficiency (e.g., by optimizing regulatory elements such as the 5′ and 3′ UTRs), improving reporter stability, or engineering versions of the replicon that contain known resistance mutations. While resistance mutations would not increase sensitivity, they could serve as important controls to validate whether a candidate compound targets the viral polymerase specifically or acts through off-target effects. Together, these modifications would help establish DNA-launched replicons as robust and versatile tools for both identifying novel antivirals and dissecting mechanisms of drug resistance (Zhang, Deng et al. 2021).

5.3.1 Developing a SARS-CoV-2 Viral Vector System for Biological Studies, Vaccine and Drug Research in BSL-2 Laboratories

The study presented in Chapter IV introduces a novel SARS-CoV-2 viral vector system that offers a safer yet highly effective model for studying SARS-CoV-2 in BSL-2 laboratories, addressing the stringent limitations associated with BSL-3 containment often required for live virus research (Kaufer, Theis et al. 2020). By incorporating all four structural proteins (S, M, E, and N) alongside a replicon that lacks the structural proteins except for N, the vector closely mimics the native viral structure, enabling detailed exploration of viral processes such as entry, viral genome replication and budding without the risk of generating infectious particles. A significant advantage of this vector is its ability to infect human cells that express the ACE2 and TMPRSS2 receptors, making it a reliable tool for investigating virus-host interactions. Additionally, it exhibits sensitivity to antivirals such as remdesivir and Types I inteferons.

Moreover, the inclusion of a reporter gene facilitates straightforward monitoring of viral transduction and replication, which is particularly valuable in antiviral drug screening (He, Quan et al. 2021). Compared to other systems (Malicoat, Manivasagam et al. 2022), this vector's utilization of stably expressed viral proteins from lentiviral vectors enhances safety and provides a simpler representation of the real virus, allowing for the simultaneous study of all structural components. Despite its many strengths, the system does face certain limitations, particularly in achieving higher viral titers, which may hinder its scalability for broader applications such as high-throughput drug screening and vaccine development. By tackling challenges related to transduction efficiency in cells with low ACE2 expression and optimizing the vector's titers, this platform could be adapted for various research applications. It could serve as a crucial tool for investigating SARS-CoV-2-related pathogenesis and studying immune responses. Future efforts should concentrate on enhancing viral titers and exploring more simplified replicon designs to address the limitations identified in this study.

5.3.2 Future experiments

Building on the findings of this study, future experiments could further optimize and expand the utility of the developed SARS-CoV-2 viral vector system for both fundamental research and practical applications. A critical area of focus would be the enhancement of viral titers. One promising approach involves the use of DNA-launched replicons, which offer several advantages over traditional RNA-based systems. DNA-launched replicons enable stable and efficient delivery of genetic material into host cells, facilitating enhanced transcription and replication. This approach not only mitigates issues related to RNA instability and degradation but also streamlines production by reducing reliance on in vitro transcription steps. Moreover, integrating optimized regulatory elements and codon usage into the DNA construct could further boost expression levels, leading to more robust amplification of replicons and, consequently, higher viral titers. Furthermore, researchers could systematically explore the ratios of plasmids encoding the structural proteins (S, M, E, and N) to identify the most effective balance for maximizing viral particle production (Liu and Liu 2023). Additionally, introducing specific mutations into the nucleocapsid (N) protein could yield deeper insights into its role in viral assembly and its overall impact on viral titers, potentially revealing mutations that either enhance

or hinder viral particle formation (Syed, Taha et al. 2021). Moreover, the production of viral vectors could be quantitatively assessed through a combination of molecular, biochemical, and functional assays. Viral RNA levels in producer cells and supernatants could be measured by quantitative RT-PCR to estimate replicon amplification (Jose, Malaga et al. 2023). Protein expression of structural components could be evaluated by Western blotting and ELISA, while total particle numbers could be quantified using nanoparticle tracking analysis (NTA) (Rebecca, Dragovic et al. 2011) or transmission electron microscopy. To specifically evaluate the proportion of particles that have successfully packaged replicon RNA, RNase-protection assays combined with RT-qPCR could be employed (Mireia, Ferrer et al. 2016; YJ, Ma et al. 1996). Functional particle output could then be determined using assays that do not rely on multiple rounds of infection, such as focus-forming assays based on early antigen staining, or reporter-based readouts in target cells measured by flow cytometry or luciferase assays (Seyyed, Elahi et al. 2021). These approaches allow distinction between total particles and single-round infectious particles, enabling accurate assessment of vector quality and systematic optimization of production conditions. In addition, evaluating the transduction efficiency of the viral vector across a range of human cell lines—particularly those with varying levels of ACE2 expression—would help illuminate how receptor density affects vector entry. Testing alternative spike protein variants, especially those with enhanced cell entry capabilities, could further refine the system for use in cells with low ACE2 expression, thereby broadening the vector's applicability (Peacock, Brown et al. 2022). In vivo studies are a crucial next step, particularly through the use of SARS-CoV-2-permissive animal models, such as genetically engineered mice. These studies would aim to evaluate the vector's infectivity, tissue tropism, and the immune responses it generates (Knight, Montgomery et al. 2021). By monitoring the biodistribution of the vector within these models, researchers can determine whether the vector shows a preference for particular tissues and investigate its potential for systemic spread. Such investigations will offer essential insights into the vector's safety and efficacy beyond controlled laboratory settings. Additionally, utilizing this viral vector for high-throughput antiviral screening could help drug discovery by enabling rapid testing of large libraries of small molecules and biologics against SARS-CoV-2 (Zhang, Deng et al. 2021). Researchers may also investigate combination drug therapies to uncover any synergistic effects that could enhance antiviral activity. This approach would not only aid in the identification of new therapeutic

candidates but also help optimize existing drug regimens for treating COVID-19 (Bobrowski, Chen et al. 2021).

In the field of vaccine development, viral vectors present a versatile platform for integrating various vaccine antigens, allowing researchers to assess immunogenicity in animal models (Su, Chen et al. 2023). Beyond evaluating antigen expression and vector stability, a critical step will be to test the immunogenicity of this viral vector system. Innate immune activation can be monitored through cytokine and chemokine profiling (e.g., IFN-α, IL-6, TNF-α) using multiplex bead-based assays or ELISA, as well as by assessing dendritic cell activation markers through flow cytometry (Estefanio, Alvarez et al. 2024). Humoral responses can be quantified by measuring antigen-specific antibody titers using ELISA, with further characterization of antibody functionality through neutralization assays or avidity testing (Henning, Gruell et al. 2022). Cellular immunity can be assessed by IFN-γ ELISPOT or intracellular cytokine staining to quantify antigen-specific T cell responses, as well as by multiparametric flow cytometry to profile $CD4^+$ and $CD8^+$ T cell subsets and memory phenotypes (Anne, Letsch et al. 2023). Investigations may also focus on the efficacy of the viral vector in prime-boost vaccination strategies, where it could enhance immune responses when administered alongside existing vaccines (Kim, Choi et al. 2021). Long-term stability studies of the vector will be essential to determine how well it retains its potency under varying storage conditions and to evaluate the durability of immune responses in vivo (Khan, Wagner et al. 2024). Comparative analyses with other SARS-CoV-2 viral vector systems, such as pseudoviruses or adenoviral vectors, will provide additional validation and highlight the relative strengths and weaknesses of the new system (Tan, Wang et al. 2023). By exploring not only entry mechanisms, replication kinetics, and transduction efficiency, but also innate and adaptive immune responses, researchers can gain comprehensive insights into the benefits of this viral vector system compared to existing models. Ultimately, pursuing these experimental pathways will enhance our understanding of the SARS-CoV-2 viral vector system, unlocking its full potential for advancing virology, therapeutics, and vaccination strategies.

5.4 Perspectives

As a perspective of this thesis, a pilot study using enhanced SARS-CoV-2 viral vectors with higher viral titers could be conducted for high-throughput antiviral drug screening. These vectors would be used to infect human cell lines, such as HEK293T or Calu-3 cells, expressing ACE2 and TMPRSS2 receptors to mimic physiological conditions. The HTS assay would employ 96- or 384-well plate formats to enable parallel testing of a wide range of compounds, including FDA-approved drugs, experimental molecules, and natural products. Infection efficiency would be quantified by measuring reporter gene activity, while cytotoxicity assays would ensure the compounds do not harm host cells. The screening process should also incorporate controls for uninfected cells, untreated infected cells, and known antiviral agents such as remdesivir to validate the assay's sensitivity and specificity.

To identify the specific phase of the viral replication cycle affected, time-of-addition assays could be performed. These assays involve introducing the drug at various time intervals post-transduction (e.g., during early entry, RNA replication, or late assembly stages) to ascertain when viral replication is most effectively inhibited. Compounds that diminish GFP/luciferase signal only when administered early may indicate an impact on viral entry or uncoating, while those that show efficacy at later stages likely target processes such as RNA synthesis or protein processing. This systematic approach would enable us to characterize the mechanisms of action for each antiviral candidate, thus identifying the most promising agents for further development against SARS-CoV-2. This pilot study could serve as a rapid, cost-effective method to screen and characterize potential SARS-CoV-2 inhibitors, providing valuable insights for therapeutic development.

5.6 Final Conclusions

In conclusion, the development of SARS-CoV-2 viral vectors and virus-like particles presented in this study, provide essential resources for exploring the virus's biology and its interactions with host cells. They also facilitate safer research practices in BSL-2 laboratories and provide a more physiologically relevant model for drug screening and vaccine development. By incorporating all four structural proteins of the virus and utilizing replicon technology, the study

enhances our understanding of viral entry, genome packaging, and replication while assisting in the identification of effective antiviral agents. The versatility of these systems offers substantial potential for future applications beyond SARS-CoV-2, enabling researchers to adapt these methodologies for future coronaviral outbreaks.

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