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The Road Less Traveled: Advancing SARS-CoV-2 Research with the Exploration of Non-Structural Proteins Using Organoid Models

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SUMMARY Until recently, the study of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) pathogenesis has primarily focused on surface structural proteins, with non-structural proteins (NSPs) regarded as less important. However, recent findings have shown the critical role of NSP6 in replication organelles, shedding light on these lesser-studied proteins. This paper proposes using organoids to explore NSP functions in SARS Cov-2. The study addresses two main questions: how can organoids be further applied to allow SARS-CoV-2 research advancement with NSPs, and what NSPs of SARS-CoV-2 need further exploration? A systems biology approach is suggested, involving the overexpression of NSPs in cells forming the organoid and analyses using transcription screening, proteomics, and microRNA profiling. Exploring all 16 NSPs using the proposed approach will provide a more robust understanding of SARS-CoV-2 mechanisms and could lead to the development of therapeutic agents. This research also has implications for "long COVID" research, in which various organoids can be used to model changes in different affected organs. Furthermore, it has implications for developing nanoparticles targeting viral or host chaperone proteins. By implementing the proposed approaches, we can move closer to understanding SARS Cov-2 mechanisms and developing a therapeutic that can be used against the virus.

INTRODUCTION

Despite nearly four years since the coronavirus disease (COVID-19) pandemic, research efforts persist in attempting to understand the pathogenesis of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and its potential long-term effects on the host (1–3)

SARS-CoV-2 has a relatively small genome compared to other human coronaviruses, with a nucleotide ranging from 26 to 32kb (4). It contains five major reading frames (5). Of these frames, 16 non-structural and four structural proteins are encoded (5). Structural proteins are classified as those incorporated into the virions, while non-structural proteins (NSPs) are only expressed in the host cell (5). Research efforts have extensively focused on understanding interactions of structural proteins and therapeutics that could target them, such as Spike protein (5). However, non-structural proteins have received less attention and investigation as therapeutic targets.

In SARS-CoV-2, Open Reading Frame (ORF) 1a and 1ab encode the non-structural polypeptides pp1a and pp1ab, respectively. These polypeptides are further processed to produce 11 and 16 non-structural proteins (NSPs), respectively (FIG.1). The pp1a has NSP 1-10 only, and pp1ab contains NSP 1-16.

The NSPs of SARS-Cov-2 generally exhibit multifunctionality. They have been shown to have critical roles in the viral life cycle, such as viral replication/transcription, inhibiting innate immunity defenses, degrading host mRNA etc. (6–9). Understanding the various functions of a single NSP is crucial for understanding viral pathogenesis and its interaction with the host cell and developing effective antiviral treatments targeting these viral proteins. Table 1 summarizes the state of knowledge on all the known roles of the 16 non-structural

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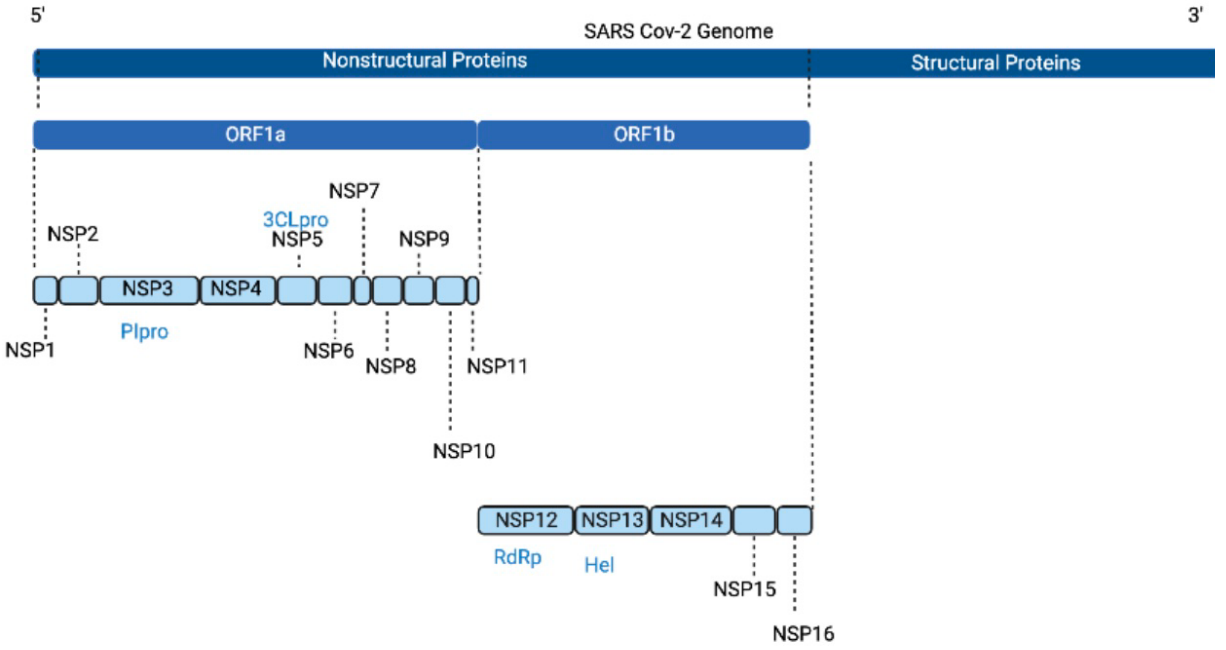

FIG. 1 Genomic arrangement of SARS-CoV-2, highlighting the location of non-structural proteins. ORF1a and ORF1b regions encode for non-structural proteins. Specifically, NSP1-16 are located within these two ORFs. The ORFs coding for structural proteins are not included in the figure. Figure created using BioRender.com.

proteins; however, there remain potential other roles that are undiscovered (multifunctionality).

Researchers should not prematurely limit their understanding of a protein function(s), as evidenced by a 2022 Nature paper on NSP6 by Ricciardi et al. (1). It was previously thought that NSP6 was involved in autophagy in 2020 and was primarily left unexplored until 2022 (10). Ricciardi et al. revealed that NSP6 played three significant roles in SARS Cov-2 pathogenesis: 1) mediating contact with lipid droplets, 2) filtering in communication between the replication organelle and endoplasmic reticulum, and 3) organizing double-membrane vesicles (DMVs) (1). This is a reminder that NSPs may possess additional, previously unknown features crucial for SARS-CoV-2 pathogenesis. This should serve as a lesson to further explore other SARS Cov-2 NSPs beyond the currently known functions. NSP6 will be further used as a case study for research on all other NSPs throughout this paper.

It should be noted that studying integral proteins experimentally is generally more difficult, as they are harder to crystalize (11). With NSP6 being an integral protein, this may have contributed to its roles not being thoroughly investigated until recently. Other integral membrane proteins include NSP3 and NSP4 (12).

TABLE. 1 Comprehensive Review of Proposed Functions for Non-Structural Proteins in SARS-CoV-2

Non-structural Protein (NSP)	Function(s)
1	Binds to ribosomes, inhibiting host translation and promoting the degradation of host mRNA. Enhances expression of mRNAs that contain the leader sequence of SARS-CoV-2 and inhibits the production of interferons (IFN)(6, 13)
2	Inhibits production of IFN and silences host mRNA's (7, 14)
3	Protease (PLpro), involved in DMV formation and disrupts interferon pathways (15)

4	Involved in DMV formation (8)
5	Protease suppresses IFN expression and attenuates antiviral stress granule (9)
6	Role in DMV formation, modulation of autophagy and suppresses IFN expression (1)
7	Forms viral replication complex (with NSP8) and synthesis of viral RNA (16)
8	Forms viral replication complex (with NSP7), blocks ribosomal membrane protein recognition signals (17)
9	Involved in viral replication and involved in evasion of the host immune responses (18)
10	Involved in viral replication and NSP14/NSP10 RNA repair complex (19)
11	Unknown (difficult protein to study due to its small size of 13 amino acids) (20)
12	RNA-dependent RNA polymerase and attenuates interferon production (21)
13	Helicase and involved in formation of the viral 5′ mRNA cap (22)
14	Serves as proofreading exoribonuclease and involved in formation of the viral 5′ mRNA cap (23)
15	Endoribonuclease that processes viral RNA to evade immune detection (24)
16	Involved in mRNA capping (25)

Organoid model systems are an increasingly popular model used to explore protein functionality that may be applied to SARS CoV-2 NSP research (26). Organoid models of human tissues better mimic in vivo conditions compared to 2D cell models (26). They may also be a cheaper and more accurate representation of human tissue than animal models. Organoids have been previously applied to SARS-CoV-2 (27–31). However, they have yet to be extensively used for NSP's research (see research question #2). Table 2 summarizes the major impactful organoid studies that have been done and what they revealed about SARS Cov-2 pathogenesis.

TABLE. 2 Review of Organoid Model Systems that have been used in SARS-CoV-2 research

Type of Organoid Tissue	Research Findings
Kidney	There are proteomic signatures of COVID-19 in urine (27) and induces fibrosis (32)
Cardiac	There is some level of reversibility of COVID-19 cardiac injuries (28)
Lung	Large number of studies. Among these studies, one investigation found alveolar organoids produce higher level of particles in B.1.1.7 variant compared to ancestral strain (33)
Brain	Damage to nervous system may be due to neuroinflammation and neuroinflammation (29)
Gut	Stomach may play a role in fecal-oral transmission of virus (34)
Liver	There may be liver-mediated activation of macrophages (30)
Retinal	Virus can infect retinal cells using ACE2 (31)

PROPOSED RESEARCH QUESTIONS

Non-enzymatic NSPs of SARS Cov-2 have not been thoroughly investigated until recently (1, 35). Even four years into the pandemic, many NSP's still require further examination. Furthermore, despite organoids having been used in some SARS Cov-2 research, they have yet to be applied to exploring NSPs. To address these gaps, we propose a detailed systems biology approach using organoids with inducible plasmids containing the NSPs of interest. By investigating NSPs, we will better understand SARS-Cov-2 pathogenesis and have the potential to find new therapeutic targets to combat COVID-19. This paper uses NSP6 as a case study to emphasize the importance of studying NSPs. This paper will achieve these aims by answering two research questions:

1. How can organoids be further applied to allow for SARS-CoV-2 research advancement with NSPs?
2. What non-structural proteins of SARS-CoV-2 need further exploration

PROPOSED PROJECT NARRATIVE

How can organoids be further applied to allow for SARS-CoV-2 research advancement with NSPs? As organoid models become more sophisticated, they have become an increasingly popular model selection. In December 2022, the FDA waived the 50-year-old requirement that drugs be tested on animal models before human clinical trials (26). Now, if successful, drugs tested on either animals or organs-on-a-chip, organoids, and computer models can proceed to human trials (26). This marks a new era of research promising more swift drug and vaccine approval in the event of a new pandemic or the pandemic we are currently in (as of March 2023). While organoids have been extensively used in SARS-Cov-2 research, no published investigations have used them to study NSPs.

Organoids can be of induced pluripotent stem cell (IPS) or patient-derived origin. Both of which have their independent benefits and drawbacks. IPS organoids present more homogeneity and eliminate some variables of patient variability (36). However, they are less clinically relevant. Patient-derived organoids from biobanks contain patients of diverse backgrounds (male, female, children, the elderly, polymorphisms etc.). While this is less generalizable to the entire population, it is more clinically relevant and may give us information on how certain patients are affected. For example, using a cardiovascular organoid with the PCSK9 polymorphism (proprotein convertase subtilisin/kexin type 9) will provide insight into the interaction between cardiovascular diseases and COVID-19 (37). Generally, studies are advised to use IPS organoids as a preliminary tool before moving to patient-derived organoids.

This paper recommends a four-step systems biology approach using organoids to study NSP's. This approach involves the following:

1. DNA level: Over-expression of NSPs using inducible plasmids. This should be tightly regulated and tested as overexpression could be lethal to the cell (ex. NSP6 regulates inflammasomes which can kill the cell) (38). There should be parental organoids as a baseline to compare with the organoids that contain the inducible plasmids.
2. mRNA level: Cells within the organoid will have a transcription screening with next-generation sequencing for the pathways associated with the gene. This will allow for the creation of a heat map.
3. Protein Level: Mass spectrometry to quantify the levels of proteins involved in these same pathways
4. miRNA Level: miRNA profiling should be completed on these same pathways.

This four-step approach is depicted graphically in FIG.2. This approach can be applied in more advanced ways by using multiple types of organoids or overexpressing a combination of NSPs simultaneously. The exact study design will depend on the topic being explored. Furthermore, whether IPS or patient-derived organoids are selected depends on what property of the NSP is being studied in the specific investigation.

What non-structural proteins of SARS-CoV-2 need further exploration? Insights gained from the study of NSP6 can be applied to identify and investigate the functions of other NSPs of SARS-CoV-2 that are yet to be fully explored. NSP6 was not initially an attractive research candidate, and focus was put on enzymatic NSPs such as 3CLpro (NSP5) and PLpro (NSP3) (10). NSP6 of SARS-Cov-2 was initially explored in silico by making a 3D molecular model (based on topology) in 2021 (39). The structure was determined, and a virtual screening of FDA-tested drugs that may target the drug was identified (4). In 2022, three of its essential roles were elucidated through overexpressing the NSP6 protein and observation under the microscope (1). In 2023, it was found that NSP6 was essential to the attenuated phenotype of Omicron BA.1) (1). NSP6 can teach that even non-enzymatic NSPs should be

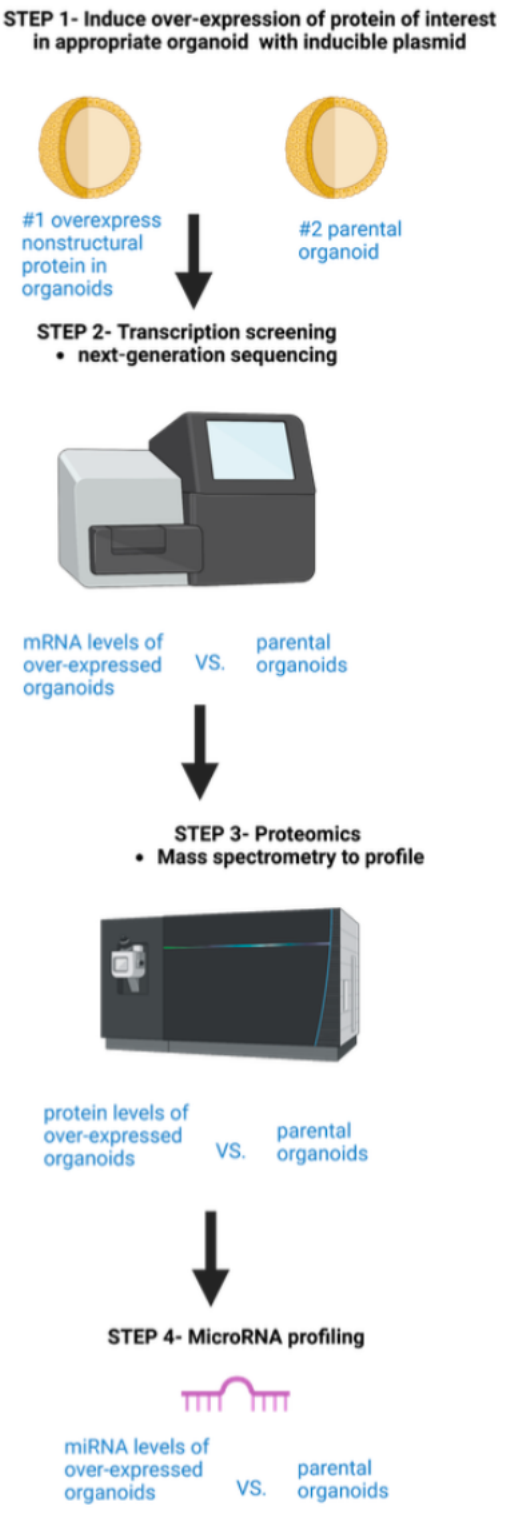

FIG. 2 Graphical illustration of proposed systems biology approach workflow to study NSPs of SARS-Cov-2. The four-step approach involves a comprehensive analysis of NSP at the transcript, protein and miRNA levels. Figure created using BioRender.com.

studied, as they can serve essential roles in viral pathogenesis. This should be remembered in the case of future pandemics and how research approaches what they explore to understand viral pathogenesis.

This paper highlights the potential of applying a four-step systems biology approach to study NSPs (see research question 1). Specifically, we propose that it could be used to further NSP6 research and explore NSP7. It is to be noted that this approach is not exclusive to NSP6 or NSP7. Other SARS CoV-2 NSPs still should be explored and could have this approach applied.

1) Further current NSP6 research

After four years of the pandemic, NSP6 is now one of the more explored NSPs, yet its functions still need to be fully elucidated. In the 2022 Nature paper, after overexpression of NSP6 in cell lines, cells were only observed cells underneath a microscope (1). In-depth transcriptional profiling and analysis were not performed. By conducting an overexpression of NSP6 in organoids and conducting the systems biology approach, we could reveal whether NSP6 is a master regulator of lipid droplets and DMV formation, which is not currently known.

Additionally, a systems biology approach of NSP6 can have implications in "long COVID" research. Long COVID is characterized by patients with viral symptoms more than 12 weeks after their initial illness (40). It is possible that some of the symptoms associated with long COVID are due to the virus causing irreparable damage to the host cells. By inducing overexpression of NSP6 in organoids, followed by halting the NSP6 induction and applying the previously highlighted systems biology approach, we can observe whether changing the ER's reticulum affects the long-term functioning of the cell. This approach would reveal if there is irreparable damage or if it causes epigenetic dysregulation. In order to explore a systematic effect, this can be done for multiple relevant organoids (heart, lung and gut). This would reveal whether some NSPs could be associated with long COVID.

2) NSP7 Research

NSP7 is a conserved NSP among the SARS Cov-2 variants and shows potential to be an important component of SARS Cov-2 viral replication yet remains understudied (41). While NSP6 was initially overlooked due to its non-enzymatic activity, we should not make the same mistake with NSP7.

Currently, it is known that NSP7 forms a complex with NSP8 to boost its RNA-dependent RNA polymerase activity (42). Computational studies have also shown that it is also α-helical in structure (43). Recently, it has been highlighted that NSP6-NSP7 may be a putative precursor (1). NSP7 is covalently bound to and flanks NSP6 before being processed by 3CLpro (NSP5). The putative NSP6-NSP7 precursor was explored in the 2022 paper by Ricciardi et al. that elucidated the major roles of NSP6. The NSP6–NSP7 from the ancestral SARS Cov-2 strain (Wuhan-Hu-1) was found to go to ER and partially to Golgi (1). However, six SARS-CoV-2 variants of concern (Alpha, Beta, Gamma, Eta, Iota and Lambda) showed NSP6 with a deletion that gave it a selective advantage (NSP6(ΔGF)) (1). This NSP6(ΔGF)–NSP7 mainly went to the ER before cleavage (1). This raises the question of why NSP7 is cleaved and if it plays a role in regulating NSP6-NSP7-containing viruses. This can be explored by using a systems biology approach where NSP6 and NSP7 are expressed separately then together and the outputs are compared. This investigation would be impactful as if it is found that NSP6 is a master regulator of DMV's and NSP7 regulates it, NSP7 could be the ultimate master regulator.

POTENTIAL IMPACT/CONCLUSIONS

While all NSPs of SARS Cov-2 have been somewhat explored, a complete understanding has yet to be achieved. This is especially true for the integral membrane proteins that are harder to explore experimentally, such as NSP6 (12). A comprehensive understanding of the NSPs is needed to comprehend SARS Cov-2 pathogenesis. This paper proposed a systems biology approach with the overexpression of NSPs of SARS Cov-2 to achieve this goal. However, this approach comes with some challenges, including time and cost. Organoid growth can take up to a couple of months. For example, one study using brain organoids to study "COVID-19 Brain fog" took 150 days to grow (44). The systems biology approach

proposed in this paper will also require additional time, leading to slow project development. This is contrary to animal research, which can generally be done immediately after the animals are acquired. Furthermore, organoid culture can be relatively expensive, and a systems biology approach using transcriptomics, a mass spectrometry machine and miRNA analysis requires additional technology and workforce (45).

It is recommended that computational analyses be completed for the NSP to be studied and existing FDA-approved drugs that can target be identified. Prioritizing the NSP proteins with drug applications may be a way to ensure that the cost is focused on the more promising proteins.

This proposed organoid systems biology approach offers a promising avenue for advancing "long COVID" research and the development of nanoparticle antivirals targeting viral or host chaperone proteins. It is possible to use various organoids and express the NSP(s), then halt it to explore the long-term impact. This may explain some of the long-term effects of infection in "long COVID" patients, which will be a growing issue post-pandemic. Furthermore, by better understanding the NSP proteins with the high throughput analysis, developing nanoparticles or repurposing existing antivirals that may target it will be possible. Alternatively, antivirals targeting the host chaperone protein that folds it could be explored. This would have to be done carefully, and a cocktail drug approach would be advised, given its toxicity to the host cell (46).

NSPs were overlooked and considered unimportant. This held especially true for non-enzymatic NSPs, such as NSP6. After over two years in the pandemic, two major papers brought attention to the significance of NSP6 (35, 47). In the future, we can avoid overlooking important non-enzymatic proteins and not make the same mistake. The systems biology approach proposed in this paper has implications for the COVID-19 pandemic and any future pandemic that may arise.

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