Pre-print journal club review of BioRxiv article: Functional assessment of cell entry and receptor usage for lineage B β-coronaviruses, including 2019-nCoV

Craig McCormick[1]

[1]Dalhousie University

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We, the students of MICI5029/5049, a Graduate Level Molecular Pathogenesis Journal Club at Dalhousie University in Halifax, NS, Canada, hereby submit a review of the following BioRxiv preprint:

Functional assessment of cell entry and receptor usage for lineage B β-coronaviruses, including 2019-nCoV. (2020) Michael Letko and Vincent Munster. bioRxiv doi: https://doi.org/10.1101/2020.01.22.915660

We will adhere to the Universal Principled (UP) Review guidelines proposed in:

*Universal Principled Review: A Community-Driven Method to Improve Peer Review.*Krummel M, Blish C, Kuhns M, Cadwell K, Oberst A, Goldrath A, Ansel KM, Chi H, O'Connell R, Wherry EJ, Pepper M; Future Immunology Consortium.*Cell* . 2019 Dec 12;179(7):1441-1445. doi: 10.1016/j.**cell** .2019.11.029.

SUMMARY: Letko and Munster report a new functional viromics platform whereby receptor binding domains (RBDs) from different lineage B betacoronaviruses were cloned into a codon-optimized gene for SARS CoV spike protein which was then incorporated into pseudotyped VSV particles for functional assays. Entry was indicated by luciferase reporter activity. This screen facilitated rapid identification of RBD-receptor interactions, with less expense than previous methods. The authors confirmed previous findings that only RBDs belonging to clade-1 of the B-lineage of beta-coronaviruses use the ACE2 receptor. Furthermore, the authors showed that for a variety of B-lineage coronaviruses, protease treatment prior to infection enhances entry into different cell types from different species. They confirmed that protease treatment enhanced receptor-dependent viral entry. By introducing 14 amino acids known to contact the ACE2 receptor into clade-2 and clade-3 RBDs, the authors confirmed that these AAs are important for ACE2 recognition. They also determined that the surrounding AA sequence context is important for ACE2 recognition. Finally, they showed that the new 2019-nCoV coronavirus (now known as SARS-CoV-2 according to the ICTV) is related to Clade-1 betacoronaviruses, similar to SARS, and also utilizes the ACE2 entry receptor.

OVERALL ASSESSMENT:

STRENGTHS: Overall, we conclude that this is a scientifically sound and well-written article by Letko and Munster. The authors' conclusions are generally well-supported by the data. The authors report a screen that is rapid, effective, and cost-efficient compared to previous methods to screen coronavirus receptor usage. The authors were also the first to show that SARS-CoV-2 uses the ACE2 receptor similar to SARS. This demonstrates the effectiveness and rapidity of their screen. The authors confirmed results from previous studies that protease treatment aids viral entry but is not sufficient to promote viral entry into cells that lack cognate receptors. Overall this a strong manuscript that provides important information relevant to the current SARS-CoV-2 outbreak.

WEAKNESSES: Some improvements could be made to strengthen the manuscript and provide better support

for the authors' conclusions. This study relies heavily on a well-controlled luciferase reporter assay; the use of another well-established virus-receptor binding assay would strengthen the dataset surrounding the successful binding of synthesized RBDs and their receptors. No statistical tests were applied to the luciferase reporter data, and the replicates appear to be technical replicates only. Pursuing multiple biological replicates and performing appropriate statistical analysis would strengthen the authors' claims. Finally, the Methods require further exposition to allow these experiments to be replicated by others, in particular concerning the luciferase assays, protease treatments, and infections (MOI and titering) (see specific comments below). Further development of the methods is critical to describe this important advance in the field.

DETAILED U.P. ASSESSMENT:

OBJECTIVE CRITERIA (QUALITY)

Quality: Experiments (1–3 scale) SCORE = 1

> Figure by figure, do experiments, as performed, have the proper controls?

Yes, experiments as performed have the proper controls, consistent with other research in the field.

> Are specific analyses performed using methods that are consistent with answering the specific question?

- Yes, the methods are appropriate to address the research question.
- The authors should provide some rationale to support the choice of cell lines used in this study. Would other cell lines been appropriate as well (see cell lines used here:*https://doi.org/10.1371/journal.pone.0007870*)?

> Is there the appropriate technical expertise in the collection and analysis of data presented?

- Additional clear rationale for some experiments would strengthen the study. What is the rationale for the choice of protease and for the use of protease treatment *prior* to infection? What is the rationale for the different cell lines used?
- It is unclear why certain modifications in the RBD would result in reduced spike protein accumulation and impair pseudotype incorporation. The reader would benefit from additional information in the Discussion that addresses this issue.

> Do analyses use the best-possible (most unambiguous) available methods quantified via appropriate statistical comparisons?

Statistical analysis was not performed on luciferase experiments.

> Are controls or experimental foundations consistent with established findings in the field? A review that raises concerns regarding inconsistency with widely reproduced observations should list at least two examples in the literature of such results. Addressing this question may occasionally require a supplemental figure that, for example, re-graphs multi-axis data from the primary figure using established axes or gating strategies to demonstrate how results in this paper line up with established understandings. It should not be necessary to defend exactly why these may be different from established truths, although doing so may increase the impact of the study and discussion of discrepancies is an important aspect of scholarship.

- Yes, it is well known at SARS-CoV uses the ACE2 receptor, and those results were recapitulated here as well as the finding that MERS-CoV uses the DPP4 receptor.
- There are now also several BioRxiv pre-prints that show, through different methods, that SARS-CoV-2 uses the ACE2 entry receptor.

Quality: Completeness (1–3 scale) SCORE = 1.5

Does the collection of experiments and associated analysis of data support the proposed title- and abstract-level conclusions? Typically, the major (title- or abstract-level) conclusions are expected to be supported by at least two experimental systems.

- The authors' conclusions rely heavily on the luciferase reporter system. Could these findings be validated using an alternative method?
- Much of the Results and Discussion focus on protease treatment and proteolytic cleavage of the chimeric spike constructs, but the authors do not show what trypsin treatment does to their chimeric constructs besides enhancing entry into cells in a receptor-dependent manner. Perhaps they could have used protease inhibitors to provide further support for their claim that proteases can be a barrier to viral entry (cathepsin L, for example, cleaves the SARS spike and cathepsin L inhibitors are readily available). Alternatively, they could have used a western blotting approach to demonstrate proteolytic processing of Spike (trypsin treatment vs. untreated; the chimeric RBD-Spike constructs are already FLAG-tagged to facilitate western blotting).

Are there experiments or analyses that have not been performed but if "true" would disprove the conclusion (sometimes considered a fatal flaw in the study)? In some cases, a reviewer may propose an alternative conclusion and abstract that is clearly defensible with the experiments as presented, and one solution to "completeness" here should always be to temper an abstract or remove a conclusion and to discuss this alternative in the discussion section.

We don't see a fatal flaw in the study. Although there are a variety of techniques to investigate viral entry besides luciferase assays and pseudotyped particles, we think that it is unlikely that they would provide conflicting data.

Quality: Reproducibility (1–3 scale) SCORE = 2

Figure by figure, were experiments repeated per a standard of 3 repeats or 5 mice per cohort, etc.?

- Most figures contain panels with multiple replicates (n=3), although they appear to be technical replicates rather than biological replicates. Having biological replicates would strengthen the data.
- Statistics could be performed on experiments with multiple biological replicates. Pursuing statistical analysis would enhance the strength of the claims in all figures.

Is there sufficient raw data presented to assess rigor of the analysis?

Yes. Raw luciferase assay data is not typically presented in the field.

Are methods for experimentation and analysis adequately outlined to permit reproducibility?

- We struggled to fully understand the methods for infection of target cells with pseudotyped VSV. Generally, the use of protease before the infection was unclear and we were unsure how that could reasonably be expected to increase infectivity (see this paper which describes what we understand to be the current consensus on proteolytic cleavage in coronavirus*https://www.pnas.org/content/114/42/11157*). Some literature even suggested that pretreatment with trypsin would decrease infectivity (ex. PMID: 19924243). Overall, we suggest that the "*Luciferase-based cell entry assay"* method be clarified to include enough information for it to be reasonably reproduced, as well as to explain the rationale for protease treatment before infection and for using trypsin instead of another cellular protease. If these methods are well-established in the literature, that literature should be cited here.
- The authors should describe how pseudoparticle titration was performed and the MOI used for each experiment.

Quality: Scholarship (1–4 scale but generally not the basis for acceptance or rejection) SCORE = 1

Has the author cited and discussed the merits of the relevant data that would argue against their conclusion?

Yes.

Has the author cited and/or discussed the important works that are consistent with their conclusion and that a reader should be especially familiar when considering the work?

Yes, with the exception of the protease literature.

Specific (helpful) comments on grammar, diction, paper structure, or data presentation (e.g., change a graph style or color scheme) go in this section, but scores in this area should not be significant bases for decisions.

- Generally, the use of colour in the figures was quite helpful throughout the manuscript. However, in Figure 3A, the colour codes on the represented spike proteins are not identified. For clarity, we suggest that the authors identify what their orange and blue boxes represent.
- In Figure 5, the graphs (panels C and D) are in a different orientation than all the previous iterations of similar data in previous figures. We suggest that the authors redraw this graph in a vertical orientation for consistency. As well, the titles on panels C and D should be in a consistent format.
- Figure S4 could be moved into the main text to better support the following claim: "*However, the 2019-nCoV RBD contains most of the contact points with human ACE2 that are found in clade 1 as well as some amino acid variations that are unique to clade 2 and 3 (figure s4b). Taken together with our receptor assay results, it may be possible that 2019-nCoV arose from recombination between clade 1 and the other clades* ." This is a strong (and very interesting) claim, which the authors provide some evidence for and can be found in other recent pre-prints on this topic, but they do not mention it again until the Discussion. This should be discussed in more detail in the Results.
- Figure S1C shows something similar, that 2019-nCoV/SARS-CoV-2 clusters separately. Then Fig S4B shows that can be the result of a recombination. Although the paper's focus is not 2019-ncov in particular, due to the outbreak of SARS-CoV-2, we think it's important to include this in the main Results section.

MORE SUBJECTIVE CRITERIA (IMPACT)

Impact: Novelty/Fundamental and Broad Interest (1–4 scale) SCORE = 1

A score here should be accompanied by a statement delineating the most interesting and/or important conceptual finding(s), as they stand right now with the current scope of the paper. A "1" would be expected to be understood for the importance by a layperson but would also be of top interest (have lasting impact) on the field.

- The authors report a new functional viromics screen that is inexpensive, rapid and accurate, which stands to be the most important contribution. However, it will also be of broad interest to the research community that their screen quickly identified the receptor used by the SARS-CoV-2 virus causing current outbreaks in Wuhan, China.
- This screen is also valuable because it can be conducted at reduced biosafety levels (BSL2).

How big of an advance would you consider the findings to be if fully supported but not extended? It would be appropriate to cite literature to provide context for evaluating the advance. However, great care must be taken to avoid exaggerating what is known comparing these findings to the current dogma (see Box 2). Citations (figure by figure) are essential here.

- There is consensus that the creation of a new screen to identify coronavirus receptor binding domain specificity is an important advance for the field. This new method provides an efficient and cost-effective way to investigate receptor-binding specificity of any newly discovered betacoronavirus.
- One potential limitation of this system is that it can be used to determine which known receptor the coronavirus uses to enter the cells but cannot be used to identify unknown receptors. This doesn't diminish the importance of the system, but this limitation could be explored briefly in the Discussion.

Impact: Extensibility (1–4 or N/A scale) SCORE = N/A

Has an initial result (e.g., of a paradigm in a cell line) been extended to be shown (or implicated) to be important in a bigger scheme (e.g., in animals or in a human cohort)? This criterion is only valuable as a scoring parameter if it is present, indicated by the N/A option if it simply doesn't apply. The extent to which this is necessary for a result to be considered of value is important. It should be explicitly discussed by a reviewer why it would be required. What work (scope and expected time) and/or discussion would improve this score, and what would this improvement add to the conclusions of the study? Care should be taken to avoid casually suggesting experiments of great cost (e.g., "repeat a mouse-based experiment in humans") and difficulty that merely confirm but do not extend (see Bad Behaviors, Box 2).