

Reviewer 2:

The authors demonstrated that the human pluripotent stem cell-hepatocyte-like cells generated using their protocol, could support efficient hepatitis B virus infection and have the potential to be used as an *in vitro* HB infection model for anti-viral drug studies. Please see below for some comments:

1. In figure 1, only the mRNA expression of HNF1a, PROX1, and FOXA3 were presented. What about the protein level expression of those genes or the protein expression of hepatocyte markers?

We added mRNA expression data of control HLCs (without 3 transcription factor overexpression) and primary human hepatocytes to Figure 1B to demonstrate that the mRNA expression is increased upon doxycycline-induced overexpression of the 3 transcription factors during the HLC differentiation process. The expression levels of *HNF1α*, *PROX1*, and *FOXA3* in HC3x-AA-HLCs are similar those observed in PHHs. We included some additional explanation in the main text (lines 240-241).

We also performed western blot analysis on control HLCs and HC3x-AA-HLCs for HNF1α and PROX1 (see Figure A below), demonstrating the presence of HNF1α and PROX1 in HC3x-AA-HLCs, but not in control HLCs. We did not perform a western blot for FOXA3, due to the lack of a functional antibody in our laboratory. However, as the exogenous HNF1α, PROX1 and FOXA3 construct encodes for a polycistronic mRNA and both HNF1α and PROX1 are present in HC3x-AA-HLCs, we believe FOXA3 is present as well. At the moment, we did not include the western blot data in the main manuscript due to its poor quality, however, we can add the data to the manuscript upon request of the reviewer.

Altogether, these data, combined with immunofluorescent staining for NTCP and HNF4α (shown in Figure 1D) should provide sufficient complementary evidence that demonstrates the presence and expression of the 3 overexpressed transcription factors and hepatocyte markers in HC3x-AA-HLCs.

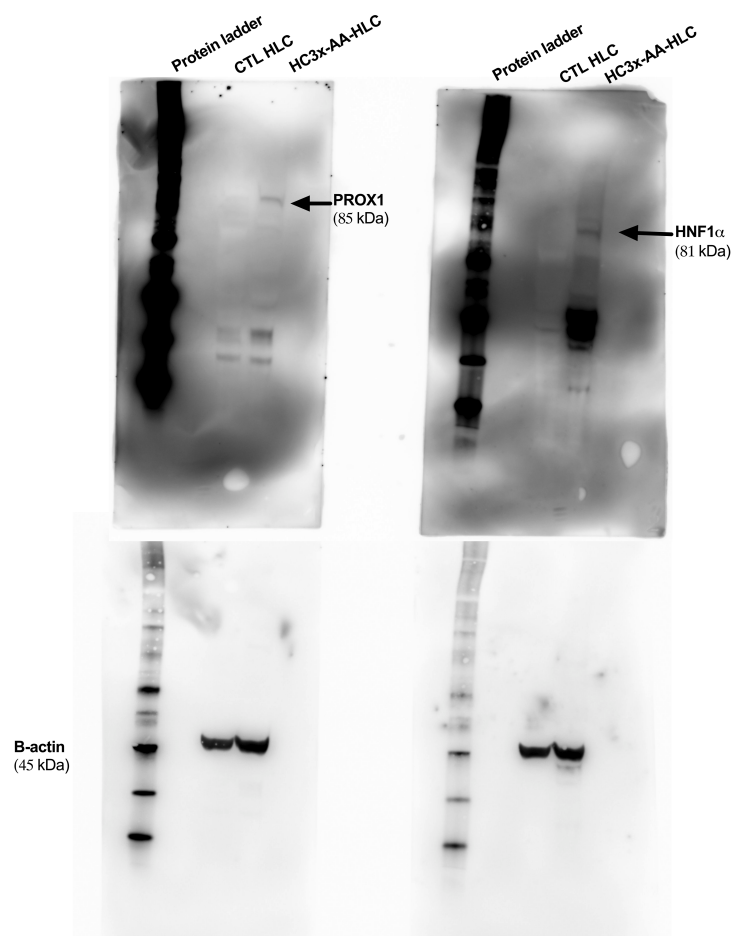


Figure A. Western blotting for PROX1 and HNF1 α in control HLCs and HC3x-AA-HLCs.

Western blot for PROX1 (left upper panel) and HNF1 α (right upper panel) in HLCs without the overexpression of the 3 TFs HNF1 α , PROX1, FOXA3 (CTL HLC) or with the overexpression of the 3 TFs (HC3x-AA-HLC). Lower panel represents the corresponding β -actin expression.

2. What is the maturity of those hPSC-HLCs when compared to other published hPSC-derived hepatocytes and primary human hepatocytes?

We thank the reviewer for this question. We would like to refer to Figure 3 and 4 of the publication of Boon *et al.* [1] to answer this question. Our lab performed transcriptomics analysis on control hPSC-HLCs, HC3x-AA-HLCs and PHHs among other conditions. PCA analysis showed that HC3x-AA-HLCs cluster more closely to PHHs than control hPSC-HLCs. Expression levels of markers for gluconeogenesis (*PEPCK* and *G6PC*) are significantly increased compared to control HLCs and similar to the levels seen in PHHs. Furthermore, glucose production and secretion were observed in HC3x-AA-HLCs, while not in control HLCs, and HC3x-AA-HLCs secreted more urea compared to control HLCs. Finally, *CYP3A4* expression was significantly higher in HC3x-AA-HLCs than control HLCs, however, these levels remained significantly lower compared to PHHs. As this has been described in Boon *et al.*, we believe that extra experiments may not be needed and a reference to this publication is sufficient, as was done in the introduction and at the beginning of the result section 3.1. However, we leave it as an editorial decision.

3. What is the purity of hPSC-HLCs used in those experiments and will the purity affect the results of the study?

Immunofluorescent staining for HNF4 α demonstrates that the percentage of HNF4 α ⁺ cells in the hESC and hiPSC HC3x-AA-HLCs is >90%, indicating a very high

differentiation efficiency and purity of the culture. Therefore, we do not expect the purity to affect the results of the study. We have added a graph of the quantification of HNF4 α ⁺ cells in Supplementary Figure 1 and included some additional explanation in the main text (lines 251-252).

4. In Figure 1C, what is the expression level of those maturation markers in primary human hepatocyte and hPSCs used for HLC differentiation?

We added the expression levels of the maturation markers *ALB*, *AAT*, *HNF4 α* and *NTCP* in PHHs to the graph of Figure 1C. No significant differences were observed in the expression levels of these markers between HC3x-AA-HLCs and PHHs. The onset of differentiation (Day 0, D0) on the x-axis are hPSCs. We changed the label D0 to hPSCs. At hPSC stage, no expression of the markers *ALB*, *AAT*, *HNF4 α* and *NTCP* was observed. We added a section on these data to the main text (lines 245-247).

5. The quality of immunofluorescence staining images in the manuscript needs to be improved. The images are blurry and it's hard to see positive staining in merged images. High magnification images or enlarged positive staining areas are needed.

We would like to thank the reviewer for pointing out this problem. As requested, we have improved the quality of the immunofluorescence staining images in the TIF files of the figures. However, we would also like to emphasize that these images have been acquired using a high content imaging device without a confocal module. Therefore, images are mainly used for accurate data quantification and less for high quality data visualization. Therefore, the images provided in Figure 2B primarily serve to visually supplement the quantification data provided in Figure 2C.

6. The scale bars are missing in Figure 2B.

Scale bars have been added to the images of Figure 2B.

7. Only lamivudine was tested in this study. Why not other anti-HBV antiviral compounds such as tenofovir alafenamide, entecavir, and telbivudine? Will HC3x-AA-HLCs have a similar effect?

We would like to thank the reviewer for this question. Lamivudine is generally considered to be a reference anti-HBV nucleoside reverse transcriptase inhibitor and the compounds the reviewer suggested (tenofovir alafenamide, entecavir, and telbivudine) are -alike lamivudine- nucleos(t)ide reverse transcriptase inhibitors as well [2]. Hence, we believe that our data on lamivudine treatment can be considered as proof-of-concept evidence that HBV replication can be blocked in these cultures and that therefore testing other NRTIs is not required. However, we have added data on the antiviral activity of a compound with another mechanism of action, namely myrcludex B, an inhibitor of HBV entry; we demonstrate a significant reduction in the percentage of HBc-positive cells when myrcludex B was added to the cultures at the time of infection. These data have now been added to Figure 3. In addition, we have inserted a section to the discussion mentioning that the antiviral activity achieved with both a HBV RT inhibitor (lamivudine) and an

entry inhibitor (myrcludex B) can be considered as proof of concept that these HC3x-AA-HLCs can be used for antiviral drug testing (lines 310-315 and 405-408).

References

1. Boon, R.; Kumar, M.; Tricot, T.; Elia, I.; Ordovas, L.; Jacobs, F.; One, J.; De Smedt, J.; Eelen, G.; Bird, M.; et al. Amino acid levels determine metabolism and CYP450 function of stem cell derived hepatocytes and hepatoma cell lines. *Nat. Commun.* **2020**, *11*, 1–16.
2. Tang, L.S.Y.; Covert, E.; Wilson, E.; Kottlil, S. Chronic Hepatitis B infection a review. *JAMA - J. Am. Med. Assoc.* **2018**, *319*, 1802–1813.