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# Reply to "The Role of Aquaporin 9 in Modeling of Ornithine Transcarbamylase Deficiency"

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**Footnote page**

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Zhang et al question the novelty of our paper, claiming that Guan et al previously reported an hiPSC-Hep model of OTCD (1). However, that publication simply reported making hiPSCs from an OTCD patient's cells—analyses were limited to stainings of germ layer markers in embryoid bodies, i.e., the hiPSCs were not differentiated into hepatocytes and OTC expression/activity and urea secretion were not analyzed, all essential aspects of OTCD modeling. More importantly, by identifying impaired AQP9 expression as the reason for impaired urea secretion inherent to hiPSC-Heps generated with current protocols, our paper establishes adequate AQP9 expression as a prerequisite for faithful hiPSC-Hep-based modeling of OTCD and other urea cycle disorders.

Zhang et al ask us to use statistics to explain why we hypothesized that factors other than the observed lower expression levels of urea cycle enzymes contributed to impaired urea secretion by hiPSC-Heps. We are unsure how to answer this question since the relevant Figures 1B-D in our paper include statistical analysis. Moreover, we stated in the corresponding results section that we reasoned that other factors are at play because urea secretion was more impaired than what we expected from the levels of reduction of urea cycle enzyme expression.

Zhang et al further ask why we focused on AQP9 as the cause of impaired urea secretion in hiPSC-Heps and not on other genes highly differentially expressed between fetal and adult hepatocytes such as *AFP* and *CYP3A4*. The obvious reason—given in our paper's introduction, results and discussion sections—is that, unlike AFP and CYP3A4, AQP9 is logically connected to urea secretion because of its known function as a urea-permeable water channel (2).

Finally, Zhang et al suggest an additional experiment showing how much AQP9 expression improves urea secretion in hiPSC-Heps relative to PHHs. Our paper already contains that information: Figures 2D and E show that AQP9 expression causes a 100% increase in urea secretion by hiPSC-Heps, which is accompanied by 50% reduction in intracellular urea levels. Confirming these results obtained under standard conditions, Figure 2F shows a 100% increase in urea secretion by AQP9-expressing hiPSC-Heps after ammonium chloride challenge. Thus, the low level of urea secretion by hiPSC-Heps lacking AQP9—25% of PHHs in Figure 1B—can be expected to increase by 100%, i.e., to 50% of PHHs, which accords with the protein levels of urea cycle enzymes in Figure 1D and underscores the contribution of impaired AQP9 expression to the urea secretion defect that has hampered research using hiPSC-Heps.

**References**

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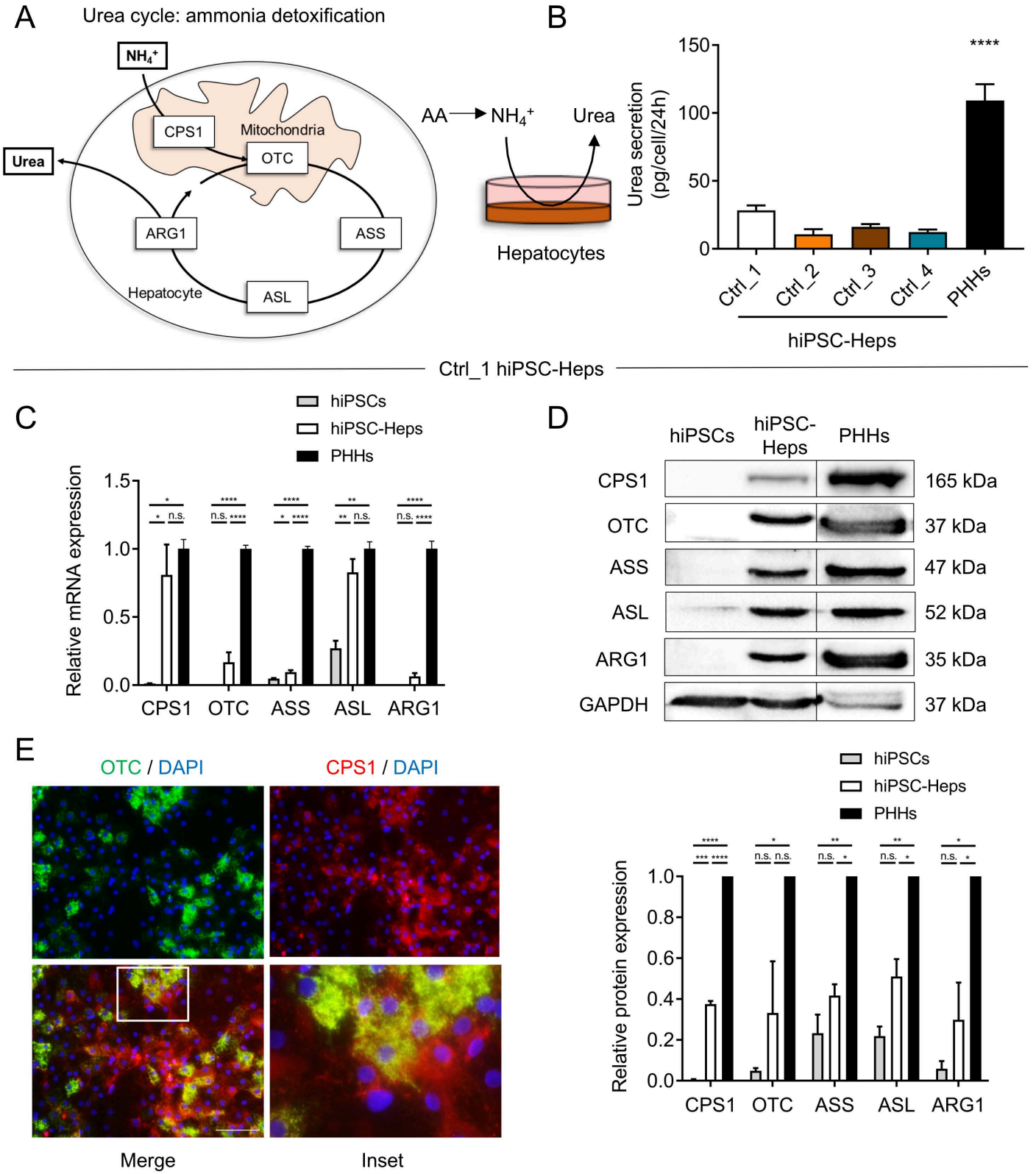
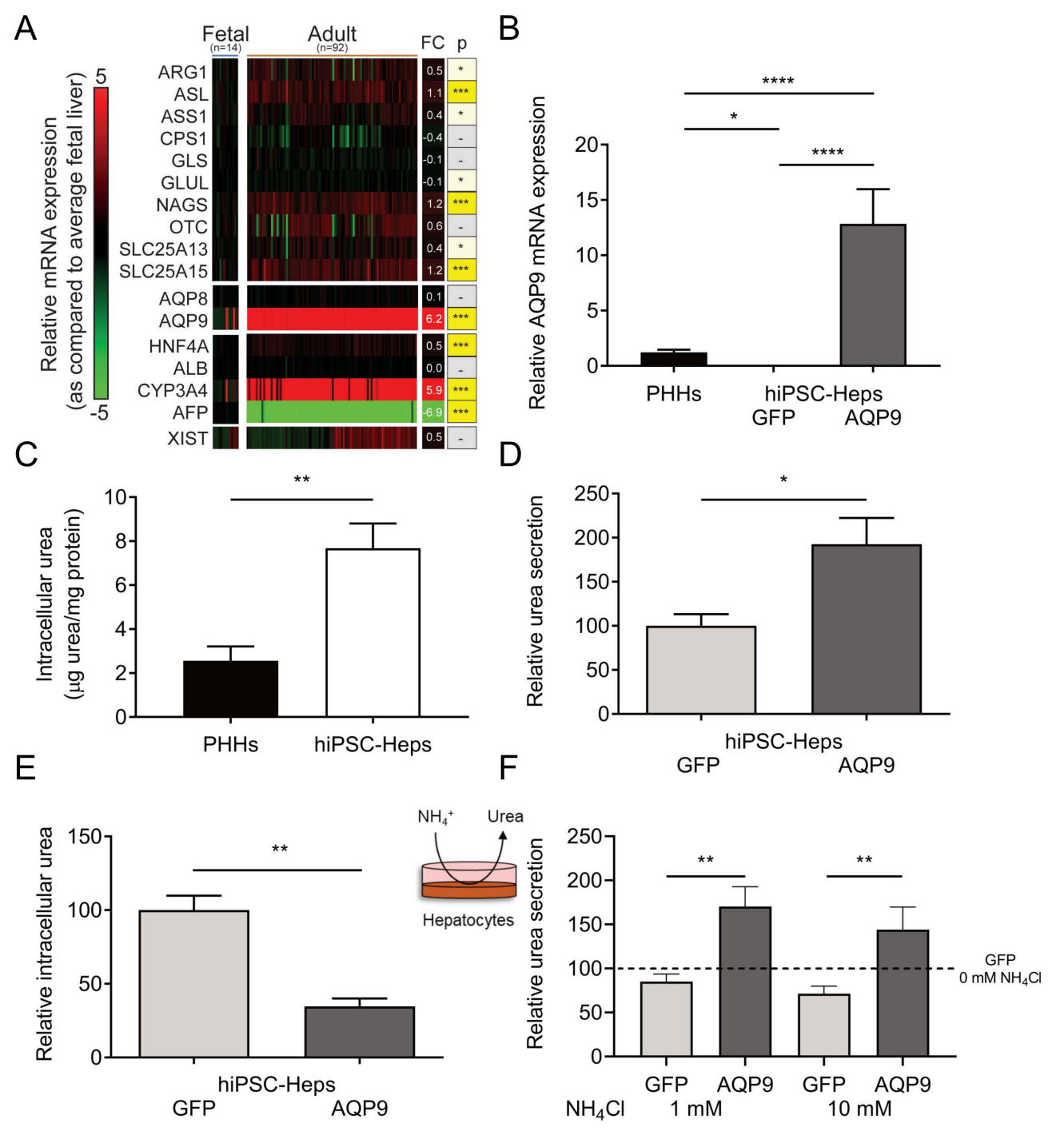

A
Urea cycle: ammonia detoxification
NH4+
CPS1
Mitochondria
OTC
Urea
ARG1
ASS
Hepatocyte
ASL
AA
NH4+
Urea
Hepatocytes
B
Urea secretion (pg/cell/24h)
150
100
50
0
****
Ctrl_1
Ctrl_2
Ctrl_3
Ctrl_4
PHHs
hiPSC-Heps
Ctrl_1 hiPSC-Heps
C
hiPSCs
hiPSC-Heps
PHHs
Relative mRNA expression
1.5
1.0
0.5
0.0
CPS1
OTC
ASS
ASL
ARG1
D
hiPSCs
hiPSC-Heps
PHHs
CPS1
OTC
ASS
ASL
ARG1
GAPDH
165 kDa
37 kDa
47 kDa
52 kDa
35 kDa
37 kDa
E
OTC / DAPI
CPS1 / DAPI
Merge
Inset
hiPSCs
hiPSC-Heps
PHHs
Relative protein expression
1.0
0.8
0.6
0.4
0.2
0.0
CPS1
OTC
ASS
ASL
ARG1


A
Fetal
(n=14)
Adult
(n=92)
FC
p
Relative mRNA expression
(as compared to average fetal liver)
5
-5
ARG1
ASL
ASS1
CPS1
GLS
GLUL
NAGS
OTC
SLC25A13
SLC25A15
AQP8
AQP9
HNF4A
ALB
CYP3A4
AFP
XIST
0.5
1.1
0.4
-0.4
-0.1
-0.1
1.2
0.6
0.4
1.2
0.1
6.2
0.5
0.0
5.9
-6.9
0.5
B
Relative AQP9 mRNA expression
PHHs
hiPSC-Heps
GFP
AQP9
C
Intracellular urea
(µg urea/mg protein)
PHHs
hiPSC-Heps
D
Relative urea secretion
hiPSC-Heps
GFP
AQP9
E
Relative intracellular urea
NH4+
Urea
Hepatocytes
hiPSC-Heps
GFP
AQP9
F
Relative urea secretion
GFP
0 mM NH4Cl
GFP
AQP9
GFP
AQP9
NH4Cl
1 mM
10 mM