DefiniGEN
DISEASE MODEL INNOVATION

Development of human iPSC-derived hepatocytes for the study of Urea Cycle Disorders

Urea Cycle Disorders encompass a group of metabolic disorders characterised by defects in any of the enzymes involved in urea cycle, which is responsible for the removal of toxic ammonia from the blood stream. Urea Cycle Disorders have a prevalence of 1:35,000 births, and are associated with increased ammonia and other precursor metabolite accumulation within human body, downstream leading to the development of life-threatening hepatic and neurological symptoms.

Background

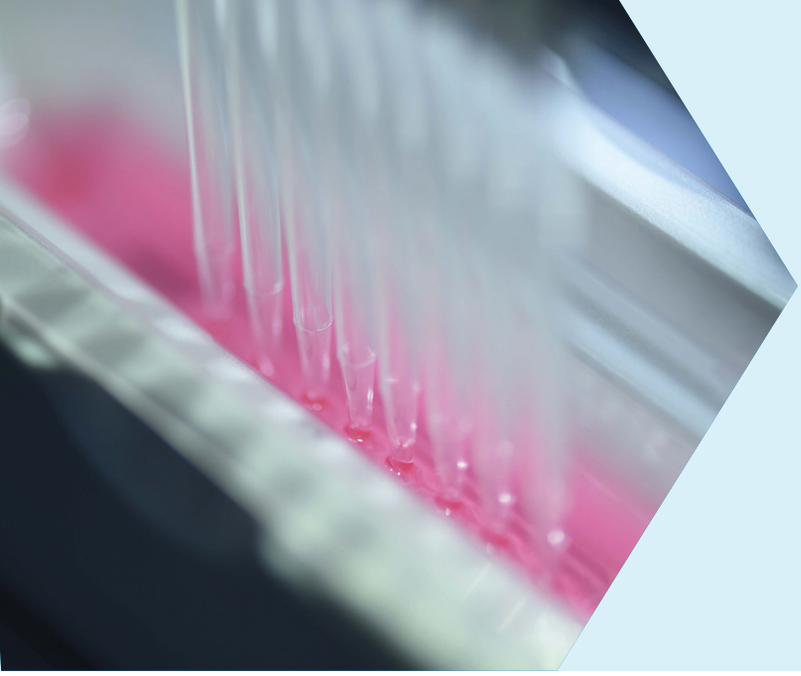
Urea cycle represents a series of biochemical reactions comprising of five enzymes that work together to detoxify nitrogen waste (i.e., ammonia) towards urea formation. The cycle is fully functional in the liver, and all enzymes are expressed in periportal hepatocytes following a metabolic-zonation pattern. Deficiency in any of the enzymes within the urea cycle results in accumulation of ammonia as well as other nitrogenous products, downstream leading to a range of symptoms, including developmental delay, cerebral edema, coma, and eventually death. This group of metabolic disorders is known as urea cycle disorders (UCDs) and, despite their severity, there are still no licensed treatments.

Current Challenges

One of the main challenges in identifying effective treatments for UCDs is the lack of reliable *in vitro* hepatocyte models that can mimic disease phenotype. Although human primary hepatocytes are considered the gold standard, there are significant challenges in their use, such as donor variability, limited proliferative capacity, rapid loss on hepatic function when in culture, and short life span. A variety of human hepatoma cell lines are, instead, often used to investigate human hepatocyte metabolism and function, including the HepG2, Hep3b, and Huh7.0 cell lines.

However, due to their malignant origin, they tend to present an abnormal metabolic phenotype and lack of expression of key metabolic enzymes compared to human primary cells.

Human induced pluripotent stem cells (iPSCs) can be differentiated into hepatocytes and recapitulate disease-related phenotypes, therefore providing an alternative strategy to model liver metabolic disorders, such as UCDs.

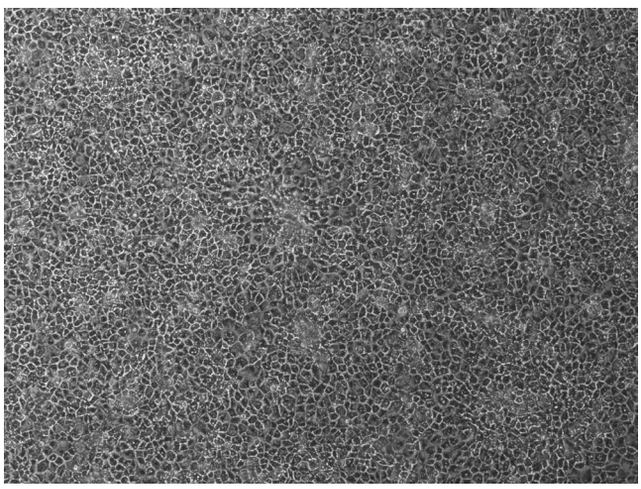


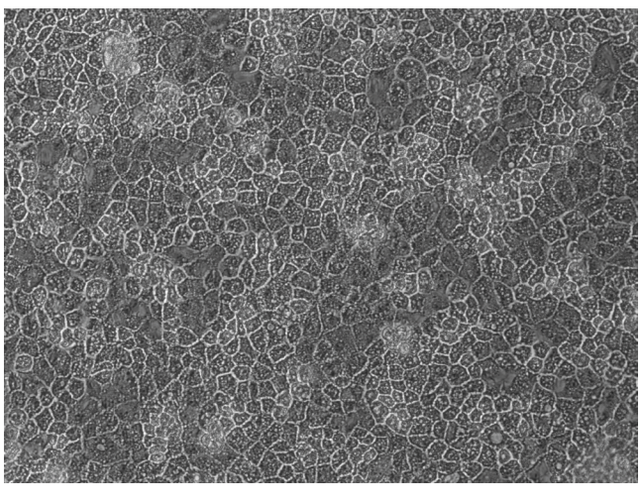
Phenotypic Characterization

Through our proprietary differentiation protocol, which derives a pure definitive endoderm population early in the cell generation process, DefiniGEN's terminally differentiated hepatocytes have comparable levels of key markers (albumin, A1AT) to primary human hepatocytes (PHH) (Figure 1).

Given the metabolic-zonation pattern human hepatocytes demonstrate (which is dependent on their distribution across the periportal-to-perivenous region), our cells were cultured in different oxygen gradients to investigate urea cycle functionality.

A)





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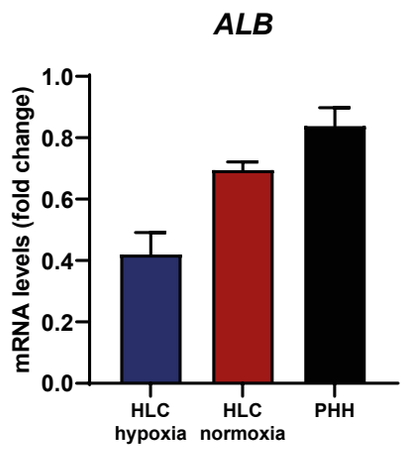


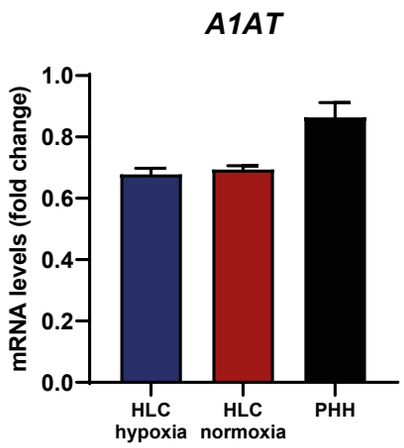


Figure 1. **(A)** Microscopy pictures demonstrating the characteristic cobblestone-like morphology of hepatocyte-like cells (HLCs) and the presence of a uniformed monolayer, following 3 weeks of iPSC differentiation. **(B)** mRNA levels of hepatocyte maturity markers albumin and alpha-1-antitrypsin (ALB, A1AT) in hypoxia-cultured HLCs, normoxia-cultured HLCs, and primary human hepatocytes (PHH), indicative of successful differentiation of iPSCs towards HLCs. mRNA data were normalised to 18SrRNA, and are presented as mean±SEM of n=4 biological replicates.

Our results revealed that DefiniGEN's iPSC-derived hepatocytes expressed significantly higher mRNA expression levels of the five key genes involved in the urea cycle pathway when cultured in normoxic conditions compared to hypoxic. Importantly, mRNA expression levels were also significantly higher compared to HepG2 hepatocellular carcinoma cells, a cell line commonly used in *in vitro* drug screening studies (Figure 2).

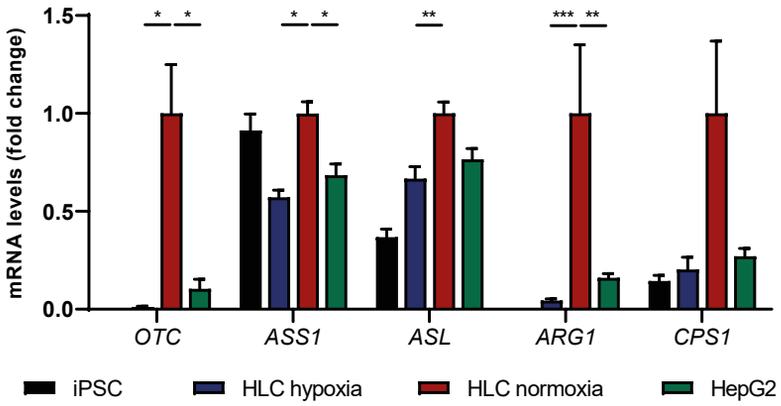


Figure 2. mRNA expression levels of key genes involved in urea cycle in iPSC, hypoxia-cultured hepatocyte-like cells (HLCs), normoxia-cultured HLCs, and HepG2 cells. mRNA data were normalised to 18SrRNA, and are presented as mean±SEM of n=3 biological replicates. *p<0.05, **p<0.01, ***p<0.001

In line with the mRNA expression data, western blotting analysis revealed significantly higher protein expression levels of all five enzymes when cells were cultured in normoxic conditions, as well as higher expression levels when compared to HepG2 cells. (Figure 3).

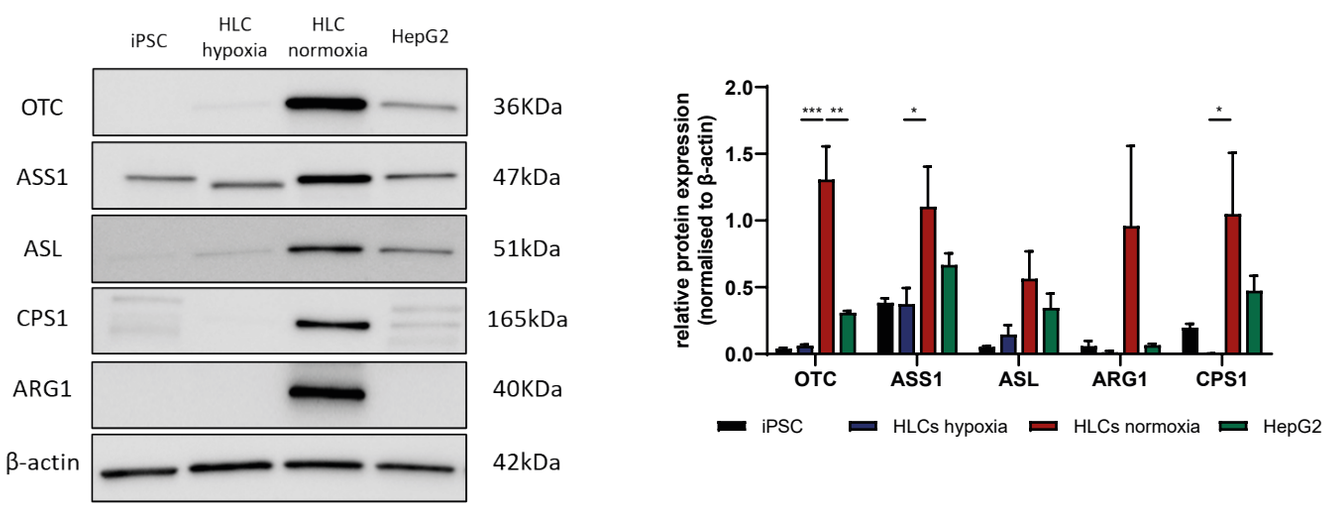


Figure 3: Protein expression levels of key enzymes involved in urea cycle in iPSC, hypoxia-cultured HLCs, normoxia-cultured HLCs, and HepG2 cells. Data were normalised to β-actin, and are presented as mean±SEM of n=3 biological replicates. *p<0.05, **p<0.01, ***p<0.001

Finally, and confirming the mRNA and protein data, we identified increasing levels of medium-secreted urea in DefiniGEN's iPSC-derived hepatocytes in a time-dependent manner, with concentrations higher than 2-fold compared to those observed in HepG2 cells (Figure 4).

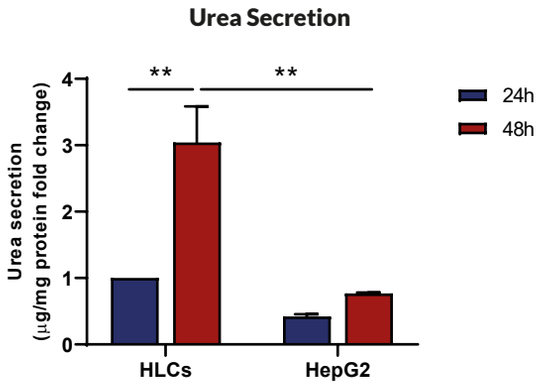


Figure 4: Media urea levels in normoxia-cultured HLCs and HepG2 cells for 24h and 48h. Data were normalised to total protein levels, and are presented as mean±SEM fold change of n=3 biological replicates. **p<0.01

These data demonstrate, in total, the presence of functional urea cycle in DefiniGEN's iPSC-derived hepatocytes and highlight their superiority as a suitable *in vitro* model for the study of UCDs.

Ornithine transcarbamylase (OTC) deficiency

Ornithine transcarbamylase (OTC) deficiency is an inherited, X-linked, recessive metabolic disorder and, currently, the most common UCD with a prevalence of one in 60-70,000 in humans. It is mainly caused by mutations on the OTC gene, which encodes the mitochondrial enzyme ornithine transcarbamylase. There are no prevalent mutations in the human population, and most of them are distributed throughout the gene. Due to difficulties in developing iPSC-derived hepatocytes with a functional urea cycle pathway, no *in vitro* disease models for OTC deficiency are available to date.

Here, we demonstrate, for the first time, functional OTC activity in DefiniGEN's wild-type iPSC-derived hepatocytes, with >30% increase in urea secretion when cells are stimulated with 1 mM NH4Cl and 1 mM ornithine for 24 hours (Figure 5). Informed by these data, and by applying CRISPR gene editing on our wild-type iPSCs, we have successfully generated an OTC mutated iPS cell line carrying the pathogenic, missense mutation D175V (Figure 6), which can serve as a platform for primary screening activities.

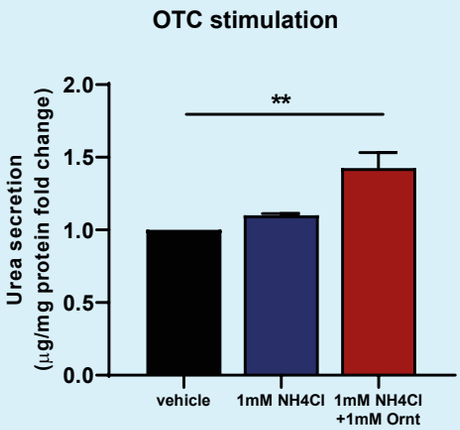


Figure 5: Media urea levels in normoxia-cultured HLCs cultured in the presence of either vehicle, 1mM NH4Cl, or 1mM NH4Cl +1mM Ornithine for 24h, suggestive of functional OTC activity. Data were normalised to total protein levels, and are presented as mean±SEM fold change of n=3 biological replicates. **p<0.01

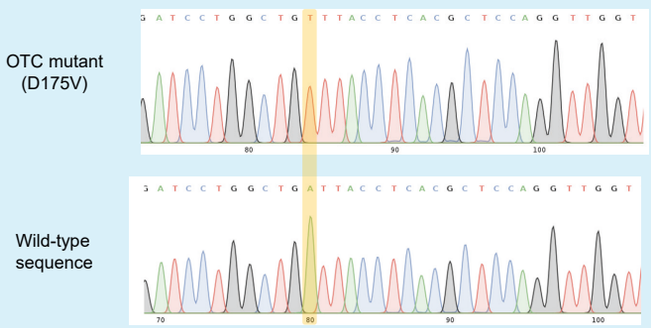
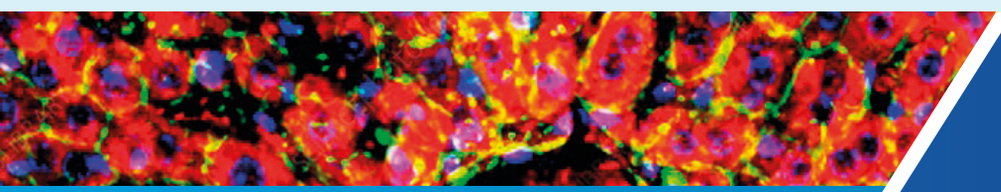


Figure 6: Sanger sequencing showing healthy wild-type (WT) as well as mutated iPSCs carrying the D175V mutation (GAT>GTT) on the OTC gene. The codon change is highlighted with yellow.



If you would like to speak with an expert about your discovery program, please contact one of our experts at info@definigen.com

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