2 MATERIALS AND METHODS

2.1 Genome Editing

2.1.1 Design

The guide RNA (sgRNA) sequences were designed using the Zhang Lab MIT CRISPR Design (http://crispr.mit.edu) and E-Crisp (http://www.e-crisp.org/E-CRISP/index.html) online design tools. The sgRNAs were designed to target exon 3 where the I148M variant resides. sgRNAs were chosen to maximize specificity and minimize off-target effects. The first set of sgRNAs were designed to target hiPSC lines that were homozygous for the risk allele. The second set of the sgRNAs were designed to target hiPSC lines that were heterozygous for the risk allele. Their sequences are as follows: sgRNA1 Forward: CACCGCTGTAGAAGGGGATGAAGC, sgRNA1 Reverse: AAACGCTTCATCCCCT TCTACAGC, sgRNA2 Forward: CACCGGGATAAGGCCACTGTAGAA, sgRNA2 Reverse: AAACTTCTACAGTGGCCTTATCCC The single-stranded donor oligonucleotides (ssODN) were then designed to contain the variant of interest as well as three silent mutations to prevent re-editing. The sequence of the ssODN in the sense direction was tctctcctttgctttcacagGCTTTGGTATGTTCTTGTTTCATGCCCTTATATA GTGGCCTTATCCCTCCTTCCTTCAGAGGCGTGgtaa. The sequence of the ssODN in the anti-sense direction was ttacCACGCCTCTGAAGGAAGGAGGGATAAGGCCAC TATATAAGGGCATGAAACAAGAACATACCAAAGCctgtgaaagcaaaggagaga. The sense ssODN was used with sgRNA1 which targeted the sense strand of the DNA while the anti-sense ssODN was used with sgRNA2 which targeted the anti-sense strand of the DNA.

2.1.2 Molecular Cloning

The sgRNA was then cloned into the pSpCas9(BB)-2A-Puro (PX459) (Addgene Plasmid #48139) expression vector [213]. To start, the forward and reverse oligos were annealed together to create a double stranded molecule with BbsI overhangs. A 10 µL reaction containing 100 µM of sgRNA top and bottom oligos and nuclease free water was annealed at 95°C for 5 minutes followed by a ramp down to 25°C at 5°C/min. The PX459 plasmid was then digested with BbsI. The 20 µL digestion reaction contained 2 µL of 10X digestion buffer (Thermo Fisher), 1 µL of the PX459 plasmid (stock concentration: 1

µg/µL), 1 µL of BbsI (Thermo Fisher), and 16 µL of nuclease free water. The digestion reaction was incubated at 37°C for 1 hour. The annealed oligonucleotides were then ligated into the expression vector. The ligation components (1.5 µL T4 DNA ligase (Promega), 2.5 µL 10X T4 ligase buffer (Promega), and 1 µL annealed sgRNA oligos) were added directly to the digest and incubated at 37°C for 1 hour. Following ligation, 2 µL of the ligation reaction was transformed into 25 µL of Alpha select Gold competent cells (Bioline) according to the manufacturers protocol. The transformed cells were plated onto LB Ampicillin agar plates (10 g LB powder (Formedium), 7.5 g agar (Sigma), 400 mL distilled water, and 40 mg Ampicillin (Sigma) and incubated at 37°C overnight. The following day, 2-4 colonies were picked and miniprepped according to the manufacturer's instructions. The miniprepped DNA was sent for sequencing using the U6-Forward primer, GAGGGCCTATTTCCCATGATCC. The sequenced plasmid was then purified from 35-50 mL of LB broth (10 g LB and 400 mL distilled water) culture using the high yield protocol of the Plasmid Plus Midiprep Kit (Qiagen). The purified plasmid was eluted using 50 µL of sterile water for embryo transfer and the DNA concentration was measured.

2.1.3 Nucleofection

The sgRNA expression construct was then nucleofected into hiPSCs using the Lonza P3 Primary Cell 4D-Nucleofector X Kit. 24 hours prior to nucleofection, cell medium was changed to TeSR E8 (Stem Cell Technologies) without penicillin/streptomycin (Life Technologies) supplemented with 10 µM Y-27632 (Stem Cell Technologies). On the day of nucleofection, cells were dissociated into small colonies of 3-4 cells using Accutase (Thermo Fisher) for 5 minutes at 37°C and counted. Two million cells were then resuspended in P3 nucleofection solution with 10 µg plasmid DNA and 200 pmol ssODN before being transferred to a cuvette. The cuvette was then placed in the nucleofector and pulsed using the CA 137 program. Cells were allowed to recover for 5 minutes at 37°C before the addition of culture medium supplemented with Y-27632. The cells were allowed to recover for an additional 5 minutes before being plated onto two vitronectin coated p100 plates.

2.1.4 Selection

The cells were incubated for 24 hours before starting puromycin selection. 1 μg/mL of Puromycin (Sigma) was added to the cells and selection was allowed to continue for 48 hours. Following this selection, the cells were allowed to recover, and clonal colonies were picked approximately 14-21 days after selection.

2.1.5 Genotyping

DNA from the selected colonies was extracted using the Quickextract kit (CamBio). Cells were resuspended in 50 μL of Quickextract solution followed by vigorous pipetting. The solution was incubated at 65°C for 6 minutes, vortexed thoroughly, and incubated for an additional 2 minutes at 98°C. This DNA was used for PCR amplification. The PCR master mix included 1-4 μL genomic DNA, 2 μL 10X Titanium Taq buffer (Clontech), 0.4 μL of 10 mM dNTP's (Promega), 0.4 μL of Titanium Taq Polymerase (Clontech), 1 μL each of forward (ggagcaaggagaggaagttg) and reverse (cgggtagcctggaaatagg) genotyping primers (stock concentration: 10 μM), and nuclease-free water for a total volume of 20 μL. The mixture was heated for 2 minutes at 95°C before cycling 35 times (98°C for 30 seconds, melting temperature for 30 seconds, 68°C for 1 minute) and finally heated for 10 minutes at 68°C. The PCR product was checked using an agarose gel before being purified with the Exosap-it reagent (Affymetrix). 2 μL of Exosap-it reagent was added to 5 μL of PCR product and incubated at 37°C for 15 minutes followed by 80°C for 15 minutes. The samples were then sent to GATC Biotech for genotyping.

2.2 Cell Culture

2.2.1 hiPSC Culture

The hiPSC lines, FSPS13B and A1ATDR/R, were obtained from the hiPSC Core Facility, Cambridge Biomedical Research Centre [185, 190, 197]. Both hiPSC lines were derived from male donors. The FSPS13B line was homozygous for the reference PNPLA3 allele while the A1ATDR/R line was heterozygous for the risk PNPLA3 allele. Neither line harboured the risk allele for other genetic variants that play a role in NAFLD onset and progression including TM6SF2 and GCKR. These lines have been extensively characterized and do not contain any know genetic abnormalities. Both lines have been

shown to have a high propensity for differentiating into hepatocytes. The cells were maintained on vitronectin (Stem Cell Technologies) coated plates in Essential 8 medium (Gibco). A list of media and supplementation components for cell culture can be found in Table 2.1. Cells were maintained at 37°C and 5% CO_2 and split every 5-7 days using 0.5 mM EDTA (Quality Biological Inc.).

2.2.2 Differentiation Protocol

Tissue culture plastic was coated with 0.1% porcine gelatin (Sigma Aldrich) in water for embryo transfer (Sigma Aldrich) for 1 hour. The coating was then overlaid with MEF medium for 24 hours prior to cell seeding. For differentiation, hiPSCs were split into single cell suspension using accutase (Thermo Fisher) for 5 minutes at 37°C. The cells were then seeded at a concentration of 50,000 cells/cm^2 in Essential 8 medium supplemented with 10 μM Y-27632 (Stem Cell Technologies). Cells were allowed to recover for 48 hours before beginning the differentiation protocol. On day 1 of differentiation, cells were differentiated in CDM/PVA supplemented with 100 ng/mL Activin (Dr. Marko Hyvönen, University of Cambridge, 80 ng/mL FGF2 (Dr. Marko Hyvönen, University of Cambridge), 10 ng/mL BMP4 (R&D), 10 μM LY294002 (Promega), and 3 μM CHIR99021 (Tocris). On day 2, the CHIR99021 was removed. On day 3, the medium was replaced with RPMI supplemented with 100 ng/mL Activin, and 80 ng/mL FGF2. For days 4-8 FGF2 supplement was removed and the medium was replaced every 24 hours. Beginning on day 9, the medium was changed to Hepatozyme supplemented with 20 ng/mL Oncostatin M (Stem Cell Technologies), and 50 ng/mL HGF (Peprotech). For days 9-30 the medium was changed every 48 hours. For the duration of the differentiation protocol, cells were cultured at 37˚C, 5% CO_2, and 5% O_2. This differentiation protocol was adapted from Hannan et.al [184].

Table 2.1 Cell Culture Media Composition

Medium	Use	Components	Concentration	Supplier
E8	hiPSC Maintenance	Essential 8 medium	-	Gibco
		Penicillin/Streptomycin	1%	Life Technologies
MEF	Plate Coating for Differentiation	Advanced DMEM/F12, GlutaMAX	-	Gibco
		FBS	10%	Sigma
		L-glutamine	1%	Life Technologies
		Penicillin/Streptomycin	1%	Life Technologies
		β-Mercaptoethanol	0.0007%	Sigma Aldrich
CDM/PVA	Differentiation of hiPSCs toward HLCs	F-12	-	Gibco
		IMDM	-	Gibco
		Chemically Defined Lipid Concentrate	1%	Thermo Fisher
		1-Thio Glycerol	0.004%	Sigma Aldrich
		Insulin	7 µg/mL	Sigma Aldrich
		Transferrin	15 µg/mL	Sigma Aldrich
		Penicillin/Streptomycin	1%	Life Technologies
		Polyvinyl Alcohol	0.5g	Sigma Aldrich
RPMI	Differentiation of hiPSCs toward HLCs	RPMI	-	Gibco
		B27 Serum-Free Supplement	2%	Life Technologies
		MEM Non-Essential Amino Acids Solution	1%	Gibco
		Penicillin/Streptomycin	1%	Life Technologies
Hepatozyme	Differentiation of hiPSCs toward HLCs	Hepatozyme	-	Life Technologies
		MEM Non-Essential Amino Acids Solution	2%	Gibco
		Chemically Defined Lipid Concentrate	2%	Thermo
		L-glutamine	1%	Life Technologies
		Insulin	14 µg/mL	Sigma Aldrich
		Transferrin	30 µg/mL	Sigma Aldrich
		Penicillin/Streptomycin	1%	Life Technologies
3D Culture Medium	Long term maintenance in 3D culture	William's E	-	Invitrogen
		Nicotinamide	10 mM	Sigma Aldrich
		Hepes	20 mM	Sigma Aldrich
		Sodium Carbonate	17 mM	Sigma Aldrich
		Sodium Pyruvate	6.3 mM	Invitrogen
		2-Phospho-L-Ascorbic Acid Trisodium Salt	0.2 mM	Sigma Aldrich
		Glucose	14 mM	Thermo
		ITS+ Premix Universal Culture Supplement	1%	Corning
		Dexamethasone	0.1 µM	R&D
		L-glutamine	1%	Life Technologies
		Penicillin/Streptomycin	1%	Life Technologies

2.2.3 3D Cell Culture

Cells designated for 3D culture followed the above hepatocyte differentiation protocol until day 23. On day 23, the cells were dissociated with cell dissociation buffer (Thermo Fisher) for 12 minutes at 37˚C, 5% CO_2, and 5% O_2. Following incubation, cells were removed from the plate and manually broken into small clumps using a serological pipette. Cell count was estimated using the Luna Automated Cell Counter. Cells were then resuspended at $2.0x10^6$ cells/mL in growth factor reduced matrigel basement membrane matrix (Corning). The matrigel was dispersed in 24 well plates at 50 µL/well and the plates were stored upside-down at 37˚C, 5% CO_2, and 5% O_2 until the matrigel had completely solidified. The matrigel was then overlaid for 24 hours with 3D culture medium supplemented with 20 ng/mL Oncostatin M, and 50 ng/mL HGF. Following the 24-hour recovery period, experimentation could begin including treatment with fatty acids. Cells could be maintained in 3D culture at 37˚C, 5% CO_2, and 5% O_2 for up to 2 weeks changing the medium every 48 hours.

2.2.4 Lipid Treatment

Oleic acid (Sigma Aldrich) and palmitic acid (sigma Aldrich) were reconstituted to 100 mM in 95% Ethanol. To prepare fatty acid medium, fatty acids are first conjugated to BSA (Sigma Aldrich) in order to facilitate uptake into the cells. Fatty acids were warmed at 60˚C for 30 minutes. Fatty acids were then mixed with 12.5% BSA and heated at 60˚C for an additional 10 minutes. Control medium consisted of 8% heated BSA in 3D culture medium while OA and PA medium consisted of 3D culture medium supplemented with 250 µM of BSA-conjugated oleic acid and palmitic acid respectively. It is important to note that ethanol was not added to the control medium as a vehicle control. This was a mistake in the experimental design that should be rectified in any future experiments. However, I believe that the effect of this oversight will be minor as the OA and PA medium contain only 0.25% ethanol. The cells were maintained in their respective medium for 7 days at 37˚C, 5% CO_2, and 5% O_2, changing the medium every 48 hours for the duration.

2.2.5 Triglyceride Blocking Assay

Cells were differentiated and placed into 3D cell culture as described above. The cells were then treated for 1 week with PA medium or PA medium supplemented with 0.5µM

Triacsin C (R&D) or 10 μM T863 (Sigma Aldrich) to block triglyceride formation. Following 1 week of treatment, cells were assessed for viability and lipid accumulation.

2.2.6 Ethanol Toxicity Assay

Cells were differentiated and placed into 3D cell culture as described above. The cells were then treated for 48 hours with either control or OA medium to induce steatosis. Following this treatment, "untreated cells" continued to be treated with their respective medium for the duration of the experiment. The cells treated with the toxic insult were cultured in their respective medium supplemented with 100 mM ethanol for 48 hours. For the final 24 hours of the experiment, the ethanol treated cells were treated with their respective medium supplemented with 100 mM ethanol and 20 ng/mL of TNFα. TNFα was used to enhance the ethanol toxicity.

2.2.7 Acetaminophen Toxicity Assay

Cells were differentiated and placed into 3D cell culture as described above. The cells were then treated for 48 hours with either control or OA medium to induce steatosis. Following this treatment, the cells were cultured in their respective medium supplemented with 25 mM acetaminophen (Sigma) for 48 hours.

2.2.8 Iron Toxicity Assay

Cells were differentiated and placed into 3D cell culture as described above. The cells were then treated for 48 hours with either control or OA medium to induce steatosis. Following this treatment, the cells were cultured in their respective medium supplemented with 200 μM ferric ammonium citrate (Sigma) for 24 hours.

2.2.9 Cytochrome P450 Assay

CYP3A4 activity was measured using the P450-Glo CYP3A4 Assay System (Promega). This assay was performed in Hepatozyme medium because the 3D culture medium exhibited a high background luminescence. The Matrigel-embedded cells were incubated with 3 μM luciferin-IPA in Hepatozyme for 4 hours at 37˚C, 5% CO_2, and 5% O_2. The medium was then collected and mixed 1:1 with luciferin detection reagent and allowed to equilibrate for 20 minutes at room temperature. The luminescence was measured using

the SpectraMax I3x monochromator with an integration time of 5 seconds/well. Each assay was performed in technical triplicate. The luminescence measurements of the technical replicates were averaged and then normalized to cell number. Since hepatocytes did not replicate in 3D culture, the number of cells seeded in the Matrigel at the beginning of the 3D culture was used for normalization purposes (100,000 cells/well).

2.2.10 Presto Blue Viability Assay

Cell viability was measured using the presto blue viability reagent (Thermo Fisher). Matrigel-embedded cells were incubated for 4 hours at 37˚C, 5% CO_2, and 5% O_2 in a solution of 10% Presto Blue/3D culture medium. Following incubation, the medium was collected and the fluorescence in the excitation/emission range of 560/590 nm was measured. Each assay was performed in technical triplicate. The fluorescence measurements of the technical replicates were averaged and then normalized to cell number. Since hepatocytes did not replicate in 3D culture, the number of cells seeded in the Matrigel at the beginning of the 3D culture was used for normalization purposes (100,000 cells/well).

2.3 Molecular Biology Techniques

2.3.1 qPCR

Total RNA was extracted using the RNeasy Micro Kit (Qiagen). Depending on the total RNA yield, 50ng-500ng of total RNA was reverse-transcribed using Superscript II Reverse Transcriptase (Invitrogen). For reverse transcription, RNA for each sample was first diluted to a total volume of 11.8 µL using nuclease free water. This RNA mixture was then denatured by adding 0.5 µL of Random Primers and 1 µL of dNTPs and incubating the mixture at 65°C for 5 minutes. The samples were snap cooled on ice before beginning the reverse transcription reaction. The reverse transcription master mix contained 4 µL of 5X 1st strand buffer, 2 µL of 0.1M DTT, 0.5 µL of RNase OUT, and 0.2 µL of Superscript II. The master mix (6.7 µL) was added to each denatured RNA sample and mixed thoroughly by pipetting. The solutions was then incubated at 25°C for 10 minutes, 42°C for 50 minutes, and 70°C for 15 minutes. The resulting cDNA was then diluted to a final concentration of 0.83 ng/µL with nuclease free water for use in qPCR. The qPCR reaction mixture for each well was prepared using 5 µL of 2X Kapa Sybr Fast

Low Rox (Kapa Biosystems), 0.125 µL each of forward and reverse primers, 2.05 µL of nuclease free water, and 2.5 µL of cDNA for a total rection volume of 10 µL. Each reaction was performed in technical duplicate. The mixture was denatured at 95°C for 10 minutes. The mixture was then heated for 30 seconds at 95°C, cooled to 60°C for 30 seconds, and reheated to 72°C for 30 seconds. This cycle was repeated 40 times before the mixture was maintained at 72°C for 10 minutes. Following amplification, the threshold for all genes was set to 0.1. The genes were normalized to the geometric mean of the housekeeping genes ubiquitin (UBC) and porphobilinogen deaminase (PBGD). Primer sequences can be found in Table 2.2.

Table 2.2 qPCR Primer Sequences

Gene Name	Forward Sequence	Reverse Sequence
PNPLA3	AGTCGTGGATGCCTTGGTATG	CGGTGATGGTTGTTTTGGCA
A1AT	CCACCGCCATCTTCTTCCTGCCTGA	GAGCTTCAGGGGTGCCTCCTCTG
ALB	CCTTTGGCACAATGAAGTGGGTAACC	CAGCAGTCAGCCATTTCACCATAG
HNF4a	CATGGCCAAGATTGACAACCT	TTCCCATATGTTCCTGCATCAG
TTR	ATGGCTTCTCATCGTCTGCT	TGTCATCAGCAGCCTTTCTG
BIP	TGTTCAACCAATTATCAGCAA	TTCTGCTGTATCCTCTTCACCAGT
GADD34	CGCCCAGAAACCCCTACTCAT	TCGGAGAAGCGCACCTTTCT
CHOP	GGAACCTGAGGAGAGAGTGTTC	GCTTTCAGGTGTGGTGATGT
PERK	TCCAGAGATTGAGACTGCGTG	TAATGACCTTTTCTTCCCTGCTCC
UBC	ATTTGGGTCGCGGTTCTTG	TGCCTTGACATTCTCGATGGT
PBGD	GGAGCCATGTCTGGTAACGG	CCACGCGAATCACTCTCATCT

2.3.2 Immunofluorescence Microscopy

Cells grown in 2D were rinsed with dPBS with calcium chloride and magnesium chloride (Gibco) before being fixed for 20 minutes in a cold 4% paraformaldehyde (ChemCruz) solution. Following fixation, cells were washed 3 times for 5 minutes in dPBS and stored at 4°C until staining. Cells were blocked and permeabilized in a 10% Donkey Serum (Jackson ImmunoResearch)/0.1% Triton-X (Sigma) for 30 minutes at room temperature. The cells were then incubated overnight at 4°C in primary antibody (1:100 in 1% Donkey Serum/0.1% Triton-X). Following incubation, the cells were rinsed 3 times for 5 minutes

in dPBS. The cells were then incubated with the secondary antibody (1:1000 in 1%Donkey Serum/0.1% Triton-X) for 1 hour at room temperature. Following incubation, the cells were washed 3 times for 5 minutes in dPBS. In order to stain the nucleus, cells were incubated with Hoeschst (1:5,000 in dPBS; Thermo Scientific) for 5 minutes at room temperature. The samples were rinsed twice with dPBS and stored at 4°C until imaging. A list of antibodies used can be found in Table 2.3.

Table 2.3 Antibodies

Antibody Name	Primary Antibody	Secondary Antibody
PNPLA3	R&D AF5208	Thermo Scientific A-11015
ALB	Bethyl A80-229A	Thermo Scientific A-11055
HNF4a	Abcam ab92378	Thermo Scientific A-10042

In order to visualize the lipid droplets within the cells, 3D Matrigel-embedded cells were stained with Bodipy (Thermo Scientific). Cells in the 3D matrigel system were first rinsed with dPBS before being fixed for 20 minutes at room temperature in a warm 4% paraformaldehyde solution to reduce the loss of the Matrigel. Following fixation, cells were washed 3 times for 5 minutes in dPBS and stored at 4˚C until staining. The cells were incubated in a 1 µg/mL solution of Bodipy for 30 minutes at room temperature. Following the incubation, the cells were washed twice with dPBS. Nuclei were stained using Hoescht (1:5000 in dPBS) for 30 minutes at room temperature. Following staining, the cells were washed twice with dPBS and stored at 4˚C until imaging.

2.3.3 Flow Cytometry

Flow cytometry was used to quantitatively measure the amount of lipid accumulation within fatty acid-treated cells. Due to technical difficulties dissociating cells grown in 3D, all flow cytometry experiments were performed on cells grown in 2D. Cells were differentiated into hepatocyte-like cells in 2D as described above. Beginning on day 23, the cells were treated with Control, OA, or PA medium for 1 week, changing the medium every 48 hours. Following this treatment, the cells were dissociated into single cells using

TryplE Express (Gibco) for 20 minutes and 37˚C. The dissociated cells were then collected and fixed in 4% paraformaldehyde at 4˚C for 15 minutes. Cells were rinsed and stored at 4˚C in dPBS/1% BSA until staining. Cells were incubated for 30 minutes at room temperature with 1 µg Bodipy in dPBS/1%BSA. Following staining, cells were rinsed twice with dPBS/1% BSA before being analysed by flow cytometry.

2.4 RNA Sequencing

2.4.1 Library Preparation

Total RNA was extracted using the RNeasy Micro Kit. The quantity of RNA was measured using the Quant-iT RiboGreen RNA Assay Kit (Thermo Scientific). The quality of RNA was then measured using the Bioanalyzer 2100 (Agilent). Library preparation was performed using the NEBNext Ultra II Directional RNA Library Prep Kit for Illumina (New England Biosystems) according to the manufacturer's protocol. Twelve libraries were multiplexed per lane and single end, 50 base pair sequencing was performed on the HiSeq 2500 (Illumina).

2.4.2 Bioinformatics Analysis

The initial quality control, alignment to the human reference genome (GRCh37/hg19), and read counting was performed using the CCBR RNA-Seq Pipeliner. Differential expression analysis was performed using DESeq2 1.26.0. An adjusted P value of less than 0.05 was considered as significantly differentially expressed. Principal component analysis was performed on normalized, vsd transformed, and batch corrected counts. Heatmaps were acquired using the ClustVis web tool and gene ontology analysis was performed using the DAVID bioinformatics resources 6.8 functional annotation tool.

2.5 Lipidomics

Cells were removed from Matrigel using Cell Recovery Solution (Corning) for 30 minutes at 4˚C. The cells were then washed twice with dPBS to remove any remaining Matrigel before lipid extraction. A Folch lipid extraction was performed to extract total lipids from the cells. The cells were homogenized in a 2:1 chorloform:methanol solution. The solution was centrifuged for 5 minutes at 20,000 g. The organic phase was recovered

for analysis. The extraction was performed twice on each sample and the organic phase was from each extraction was combined and sent for analysis via direct injection liquid chromatography mass spectrometry. Each experiment was performed in technical quintuplicate. The lipidomic data was then analysed using the MetaboAnalyst web-based analytical pipeline for high-throughput metabolomic studies.

2.6 Statistical Analysis

All graphical values are shown as mean ± standard deviation. Statistical analyses were performed using GraphPad Prism 8 and the R statistical environment. Ordinary one-way ANOVAs with Dunnett's multiple comparisons tests were performed to test statistical significance between means. P-values less than 0.05 were considered to be statistically significant.