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CELLULAR AND MOLECULAR GASTROENTEROLOGY AND HEPATOLOGY

RESEARCH LETTER

Undifferentiated Induced Pluripotent Stem Cells as a Genetic Model for Nonalcoholic Fatty Liver Disease

Patient-derived induced pluripotent stem cells (iPSCs) have been transformational in biomedical research for their ability to differentiate into any cell type while retaining the genetic information of the donor individual, for example iPSC-derived hepatocyte-like cells (iPSC-Heps) for studies of nonalcoholic fatty liver disease (NAFLD).[1] However, differentiation protocols are time-intensive, use costly reagents, require highly specialized training, and can result in heterogeneous cultures that are limited in number.[2] Thus, iPSC-Heps are poorly suited for studies of genetic variation that require scalability and reproducibility. In contrast, iPSCs exhibit self-renewal, can be cryopreserved, have standardized and robust protocols available for their generation and culturing, and are substantially less expensive to produce. We tested whether iPSCs in their undifferentiated state may be informative to model genetic factors underlying NAFLD. NAFLD is initiated by hepatic steatosis, often attributed to excess synthesis, retention, or uptake of fatty acids by the liver, where they are stored as triglycerides within lipid droplets. As nearly all cells can take up fatty acids, synthesize triglycerides, and create lipid droplets,[3] we sought to determine whether iPSCs could model fatty acid-induced lipid accumulation.

Authenicated iPSCs (Supplementary Table 1) were previously described.[4] We confirmed that a representative iPSC accumulates intracellular lipids in response to 24-hour oleate challenge in a dose dependent manner, with lipids detected by 2 neutral lipid stains (Nile Red and LipidTox Red) and through Simulated Raman Spectroscopy, a highly specific detection method for unlabeled triglycerides[5] (Figure 1, *A*–*B*). To improve quantitation accuracy, we developed a flow cytometry-based assay (Figure 1, *C*), resulting in highly reproducible measures (Figure 1, *D*), which confirmed that oleate treatment increased intracellular lipids in cell lines from 30 donors (2.0 ± 0.11 fold mean ± standard error; $P = 4.0^{e\text{-}10}$ (Figure 1, *E*; Supplementary Table 2).

We next compared the degree of oleate-induced lipid accumulation in iPSCs from 8 donors both in their

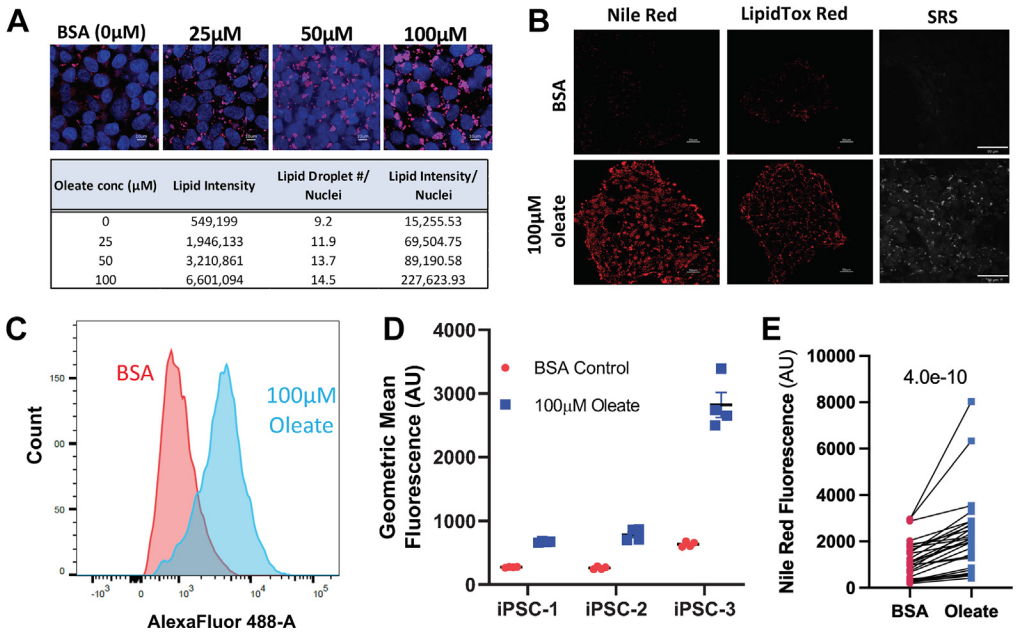

Figure 1. iPSCs accumulate intracellular lipids when challenged with oleate. (*A*) Images taken at 100× magnification of an iPSC line challenged for 24 hours with (0–100μM) sodium oleate conjugated to bovine serum albumin (BSA). Cells were stained with 100 μg/mL of Nile Red (*pink*) to visualize lipid droplets and Hoescht (*blue*) to stain nuclei. 10-μm size bars shown. (*B*) Oleate- vs BSA-treated iPSCs were stained with Nile Red or LipidTox Red and visualized via fluorescence microscopy. A separate aliquot of cells was left unstained and subjected to SRS microscopy, in which unstained triglycerides are visualized as *white* areas. 50-μm size bars shown. (*C*) Representative histogram of Nile Red fluorescence values of BSA- and 100 μM oleate-treated iPSCs. Cells were stained with Nile Red prior to quantitation by flow cytometry. (*D*) Biological replicate measures of intracellular lipid levels in iPSCs from 3 donors (n = 4). (*E*) Geometric means of the Nile Red fluorescence values indicative of intracellular lipids in 30 iPSC lines treated with BSA and 100 μM oleate.

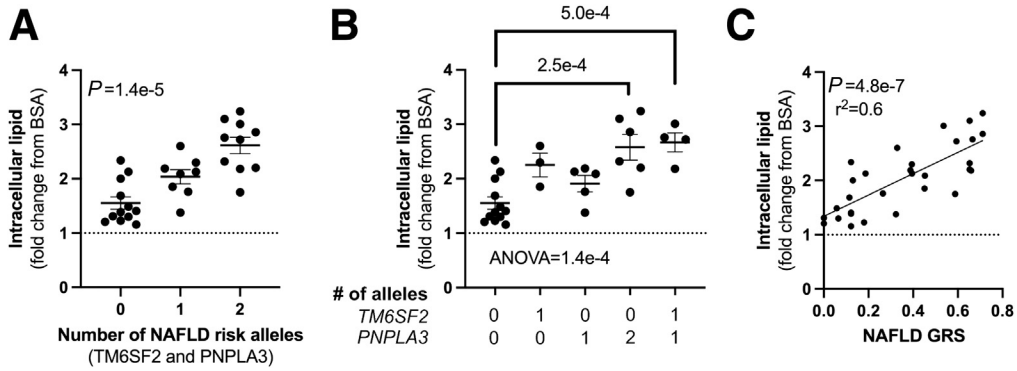

Figure 2. The magnitude of oleate-induced intracellular lipid accumulation in undifferentiated iPSCs is correlated with NAFLD genetic risk. Oleate-induced intracellular lipid accumulation was quantified in iPSCs from 30 donors as described in Figure 1, and the fold change in lipid accumulation was plotted separated by the number of NAFLD risk alleles for *TM6SF2* and/or *PNPLA3* together (*A*) or separately (*B*). Linear regression (*panel A*) and analysis of variance with posthoc multiple comparisons against the 0 allele carrier group was performed with adjusted *P*-values (*panel B*) shown. (*C*) Correlation of intracellular lipid accumulation with 4-SNP NAFLD genetic risk score.

undifferentiated state and after differentiation into iPSC-Heps through a 23-day protocol as we previously described.[6] iPSC-Heps were authenticated by expression of hepatocyte markers and secretion of albumin into the culture media (Supplemental Figure 1, *A*–*B*). There were no differences in the levels of intracellular lipids in the isogenic iPSCs and iPSC-Heps, either with values expressed as absolute levels or the magnitude of change between oleate vs. BSA treated cells (Supplemental Figure 1, *C*–*E*).

Variants in *TM6SF2* (rs58542926), *PNPLA3* (rs738409), *GCKR* (rs1260326), and *MBOAT7* (rs641738) are associated with NAFLD in multiple independent cohorts, and have published effect sizes for their association with hepatic fat.[7] All 4 genes had detectable expression in undifferentiated iPSCs, unlike lymphoblastoid cell lines, another patient-derived cell line (Supplemental Figure 2). Importantly, iPSCs carrying increasing numbers of rs58542926 and rs738409 NAFLD risk alleles had greater intracellular lipid accumulation with an additive relationship observed ($P = 1.4^{e-5}$) (Figure 2, *A*). The magnitude of this effect was nearly identical between the 2 risk alleles, consistent with their reported effect sizes[7] (Figure 2, *B*). Moreover, we found a significant positive correlation ($r^2 = 0.60$; $P = 4.8^{e-7}$) between oleate-induced intracellular lipid accumulation and a weighted genetic risk score based on the reported associations of *TM6SF2* rs58542926, *PNPLA3* rs738409, *GCKR* rs1260326, and *MBOAT7* rs641738 alleles with hepatic fat[7] (Figure 2, *C*).

Here, we show that patient-derived iPSCs in their undifferentiated state can be used to model genetic factors that influence individual-level variation in fatty acid-induced lipid accumulation, critical in NAFLD pathobiology. Compared with iPSC-Heps or liver organoids, iPSCs are significantly more scalable, enabling their use for genetic discovery. This could support future use of iPSCs for identifying high-risk individuals, testing variation in response to treatment, and informing the development of precision medicine guidelines for NAFLD prevention and management. Our results also raise the possibility of using iPSCs for investigating genetic influences on other diseases characterized by excess lipid storage. Notably, both the *TM6SF2* rs5854296 and *PNPLA3* rs738409 risk variants are thought to cause lipid accumulation in hepatocytes by impairing intracellular lipid transport and reducing triglyceride secretion in APOB-containing lipoprotein particles,[8,9] processes that have not been identified in iPSCs. Additional study is needed to assess the mechanisms underlying these relationships and to determine the extent to which NAFLD relevant pathways can be modeled in the iPSC. Lastly, these findings challenge the current paradigm of iPSC use, which assumes that cells must be differentiated to be informative, highlighting the potential utility of undifferentiated patient-derived iPSCs as a cellular model of individual level disease risk.

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***Abbreviations used in this paper*: iPSCs, induced pluripotent stem cells; iPSC-Hep, iPSC-derived hepatocyte-like cells; NAFLD, nonalcoholic fatty liver disease.**

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Conflicts of interest
This author discloses the following: Aras N. Mattis is a consultant for Hepatx, Ambys Medicines, and BioMarin. The remaining authors disclose no conflicts.

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Supplementary Materials and Methods

Post Induced Pluripotent Stem Cell (iPSC) Donor Demographics

Cell line donors were genotyped on Illumina Infinium OmniExpressExome bead chips. Thirty-five iPSC lines were selected for this study based on their sex, ancestry, and genetic information (Supplementary Table 1). Because most of the nonalcoholic fatty liver disease (NAFLD) genetic studies have been performed in individuals of European ancestry, we used cell lines from donors of European descent so the effect sizes and genetic risk score would be most accurate.

iPSC and iPSC-derived Hepatocyte-like (iPSC-Hep) Cell Culture

iPSCs were cultured in mTESR1 media at 37 °C at 5% CO_2. iPSCs were passaged using accutase (Stemcell Technologies, Cat. # 07920) and media supplemented with Y-27632 2HCl inhibitor (Selleckchem, Cat. # S1049). iPSC-Heps were cultured at 37 °C and 5% CO_2 in Lonza Hepatocyte Culture Medium (HCM; Cat. # CC-3198). iPSCs were differentiated into hepatoblasts as previously published.[1] Expression of hepatocyte-specific markers albumin and hepatic nuclear factor 4 alpha (HNF4A) were confirmed by fluorescence-activated cell sorting at a threshold of >90% dual-positive cells.

Intracellular Lipid Accumulation

iPSCs and iPSC-Heps were grown to 70% to 75% confluency in 6-well plates. Cell lines were challenged with HCM containing 0 to 100 μM oleate conjugated to fatty acid-free (FAF) bovine serum albumin (BSA), and all BSA-containing supplements were removed. A volume of FAF-BSA equivalent to the oleate condition was used as a negative control. After 24 hours, cells were fixed with 10% paraformaldehyde.

Flow Cytometry for Quantification of Intracellular Lipids

Cells were stained with Nile Red (Sigma, Cat. # 72485) diluted to 100 μg/mL in Dulbecco's phosphate-buffered saline for 30 minutes, and fluorescence was quantified using the BD LSRFortessa. Data was analyzed using FloJo v10.7.1. Oleate-induced increases in cellular lipids were quantified as the fold change of the oleate-treated/BSA-treated cells. Two outliers were identified using the ROUT test. Because they were from the same batch of samples, all 5 samples in the batch were excluded from the analyses, resulting in a sample size of n = 30. Paired Student *t* tests were used to identify statistically significant differences between BSA- and oleate-treated cells. Linear regression was used to evaluate the correlation between variation in the magnitude of oleate-induced increase in intracellular lipid accumulation and the number of *TM6SF2* rs58542926 and/or *PNPLA3* rs738409 risk alleles. All statistical analyses were performed using JMP Pro 16.0.0 and GraphPad Prism version 9.1.0.

Calculation of a Weighted NAFLD Genetic Risk Score

A 4 single nucleotide polymorphism (SNP)-weighted genetic risk score (GRS) was calculated for each iPSC line using the following variants: *PNPLA3* rs738409, *TM6SF2* rs58542926, *GCKR* rs1260326, and *MBOAT7* rs641738 using previously estimated effect sizes for their relationships with hepatic fat.[2] The DHS coefficients used were 0.2653 for each rs738409 G allele, 0.2711 for each rs58542926 T allele, 0.0649 for each rs1260326 T allele, and 0.0575 for each rs641738 T allele. The GRS was calculated as the sum of the product of the weights for each SNP and the numbers of each risk allele present.

Fluorescence Microscopy

Cells were stained with Nile Red (100 μg/mL) and Hoescht 33342 (5 μg/mL) for 30 minutes (ThermoFisher, Cat. # H3570). Images were captured on a Keyence BZX-700 microscope at 100× and 20× magnification using phase contrast and widefield fluorescence microscopy. Fiji was used to quantify both nuclei and lipid droplet counts as well as the integrated intensity of lipid droplets in 100× images.

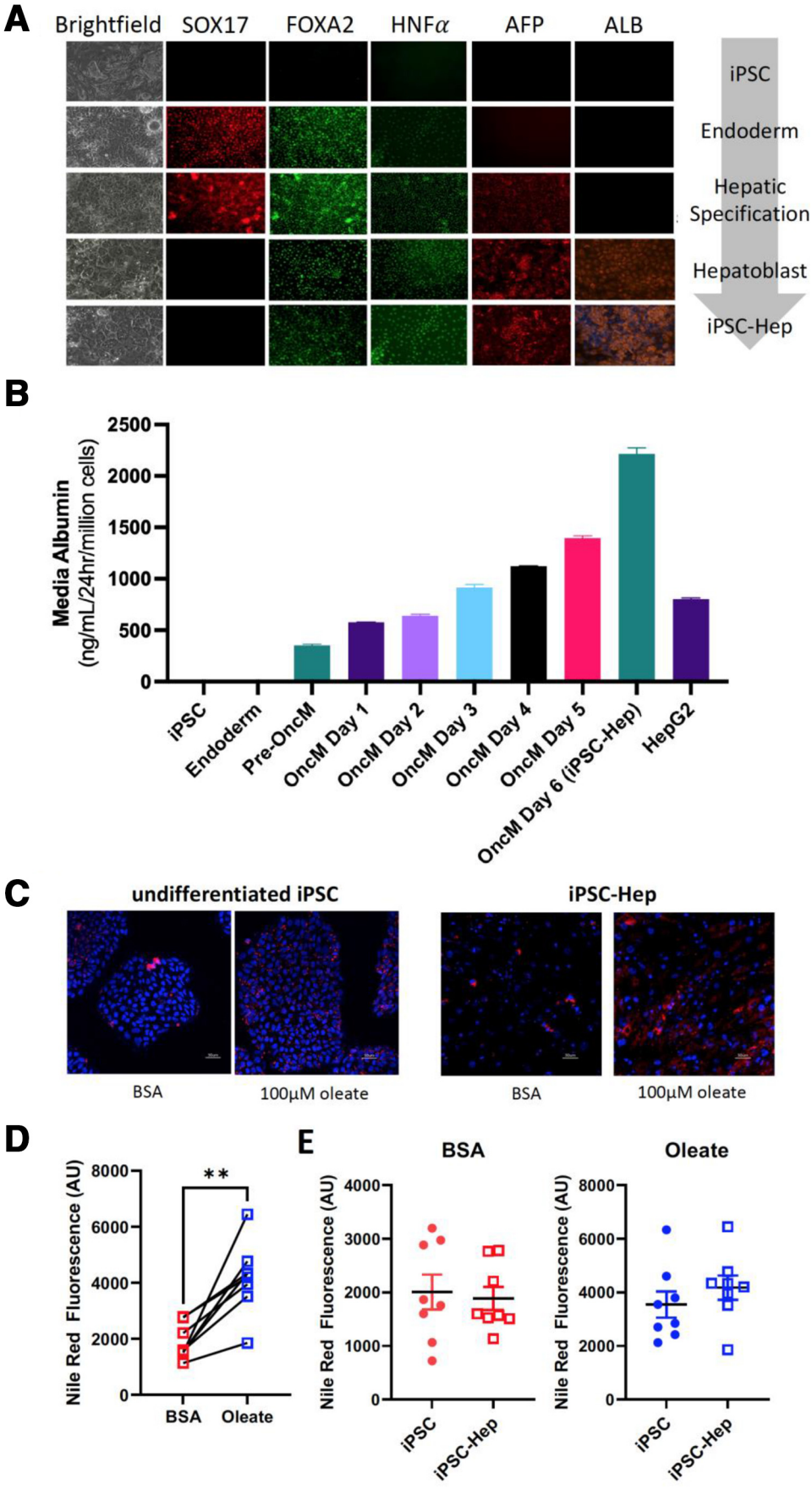
Stimulated Raman Spectroscopy (SRS) Microscopy

The dual output of a commercial oscillator/optical parametric oscillator (Insight DS+, Spectra-Physics) was used for SRS imaging. The output of the optical parametric oscillator was set to 802 nm corresponding to a wavenumber of ~2850 cm^{-1} with the fundamental at 1040 nm used as the Stokes field. The fundamental was amplitude modulated at 10.28 MHz using a resonant EOM (EO-AM-R-C2, Thorlabs) and a Glan-laser polarizer (Thorlabs). The 802 and 1040 nm beams were combined on a 1000 nm short-pass dichroic mirror (Thorlabs) and fed into a commercial inverted scanning microscope (Olympus IX83-FV1200). Temporal coincidence of the pulses was controlled using a variable delay stage placed on the 802 nm arm (FCL200, Newport). A 60× water-immersion objective (1.2 NA) was used for imaging (UPLSAPO60XWIR, Olympus), with a 1.4 NA oil-immersion condenser (CSC1003, Thorlabs) used to collect the light sent to the detector. The Stokes beam was blocked using a 1000 nm shortpass filter (Thorlabs), and the 802 nm pump was detected on a photodiode reverse biased at 61.425 V. The output of the photodiode was demodulated by a lock-in amplifier (H2FLI, Zurich Instruments) for image formation. All images were acquired at 512 × 512 pixels per field of view, using a pixel dwell time of 10 μs, and a lock-in time constant of 3 μs. The average power of both the 802 and 1040 nm lines was 10 mW. Intracellular lipid content was measured as the

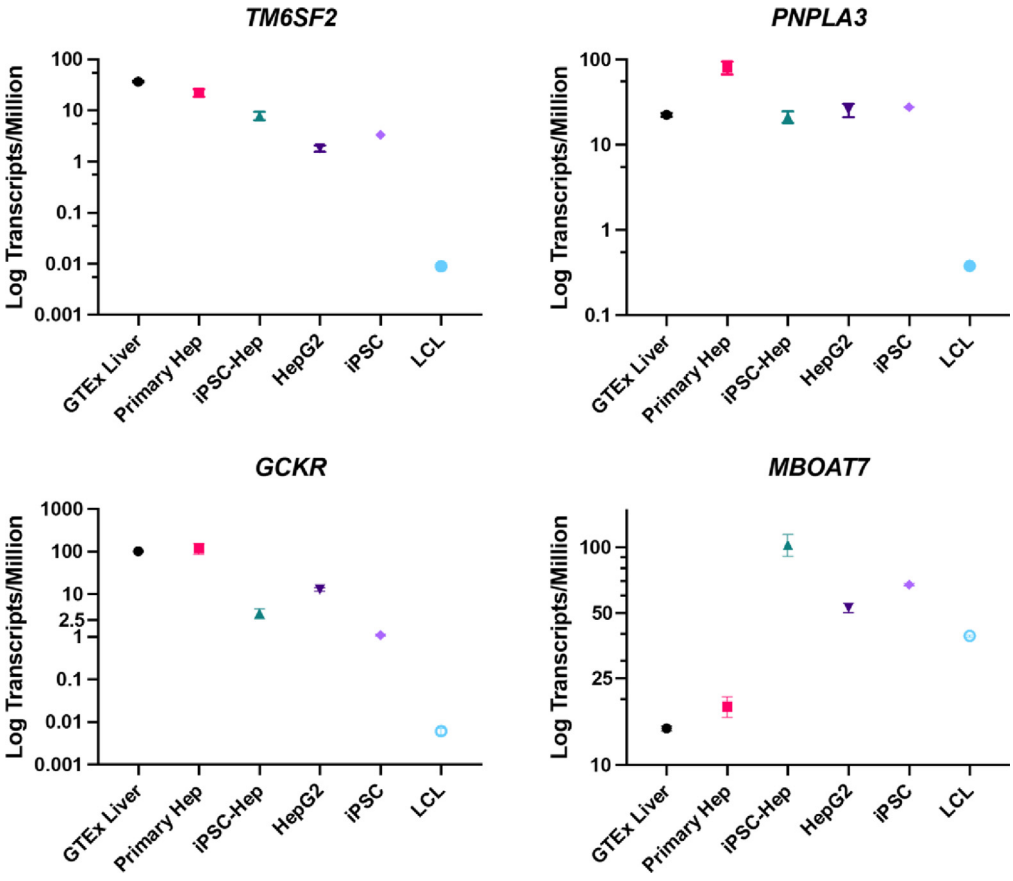
integrated SRS signal at 2850 cm^{-1}, which primarily corresponds to CH_2 stretching in lipid molecules. To calculate average cellular lipid content, the images were pseudo-flatfield corrected using a Gaussian convolved version of the image as the flatfield (with radius equal to 150 pixels). A thresholded cellular image for each field of view was then produced by first lowpass filtering the image, and then performing an adaptive local histogram equalization (with radius of 15 pixels).

RNA Sequencing Analysis

Isolated RNA was prepared into polyA-selected, strand-specific sequencing libraries for 100 bp paired-end sequencing at the Northwest Genomics Center. Gene expression levels in iPSCs were compared with previously generated RNAseq data, including 426 lymphoblastoid cell lines,[3,4] primary human hepatocytes from 4 donors (Supplementary Table 3), and 10 HepG2 biological replicates. GTEx V8 liver TPM expression levels were downloaded via the GTEx portal for comparison. Sequence transcript counts per million (TPM) were calculated by dividing the number of sequence fragments aligning to the gene by the gene length in kilobases (FPK). The sum of the FPK for each gene across all samples was then divided by one million to create a scaling factor (FPK/million). The FPK for each sample and gene were then divided by the scaling factor for that gene to create the final TPM value. These values were graphed using Graphpad prism 9.1.0 and shown as Log_{10} TPM.



Supplementary Figure 1. Authentication of iPSC-Heps and comparison with iPSCs. (*A*) Brightfield and fluorescence microscopy of iPSCs during differentiation into iPSC-Heps with immunohistochemical evaluation of endoderm (SOX17, FOX2A), and hepatocyte markers (HNFa, alpha fetal protein [AFP], albumin [ALB]) during various stages of differentiation. (*B*) Albumin in the culture media of iPSCs during differentiation into iHeps and compared with the human hepatoma cell line HepG2. Oncostatin M day 1 to day 5 represent the stage of hepatoblast formation and differentiation into iPSC-Heps at day 6 after addition of oncostatin M (or day 23 after initiating the differentiation protocol). Values shown are mean ± standard error of the mean. (*C*) Oleate-induced intracellular lipids were imaged at 20× magnification in undifferentiated iPSCs and iPSC-Heps as described in Figure 1. 50-μm size bars shown. (*D*) iPSC-Heps were treated with BSA or 100 μM oleate (n = 8), and Nile Red fluorescence was quantified by fluorescence-activated cell sorting. $^{**}P = .0018$, paired *t* test. (*E*) Intracellular lipid accumulation was quantified in iPSCs from 8 unique donors before and after differentiation into iPSC-Heps, and after treatment with 100 μM oleate or bovine serum albumen control.



Supplementary Figure 2. Undifferentiated iPSCs express genes identified by NAFLD genetic association analyses. PolyA-selected whole transcriptome sequencing was performed in GTEx liver (n = 226), primary human hepatocytes (n = 4), human iPSCs (n = 48), the human hepatoma HepG2 cell line (n = 10), and human lymphoblastoid cell lines (n = 426), and *TM6SF2, PNPLA3*, *GCKR*, and *MBOAT7* transcript levels were quantified as transcripts per million. The y-axis is scaled as Log_{10}. Primary hepatocytes were obtained from 3 female and 1 male donor between the ages of 49 and 75 years with body mass index ranging from 22.5 to 24.3 kg/m^2.

Supplementary Table 1. Demographic and Genetic Characteristics of iPSC Donors

iPSC line	Sex	Ancestry	*PNPLA3* rs738409 # of G alleles	*TM6SF2* rs58542926 # of T alleles	4 SNP-weighted GRS
1	F	European	0	1	0.451
2	M	European	0	1	0.329
3	F	European	0	1	0.394
4	M	European	0	0	0.058
5	M	European	0	0	0.122
6	M	European	0	0	0.187
7	F	European	0	0	0.122
8	F	European	0	0	0.065
9	F	European	0	0	0.000
10	F	European	0	0	0.122
11	F	European	0	0	0.180
12	M	European	0	0	0.115
13	F	European	0	0	0.000
14	F	European	0	0	0.122
15	M	European	0	0	0.130
16	F	European	1	0	0.395
17	F	European	1	0	0.388
18	F	European	1	0	0.323
19	M	European	1	0	0.453
20	M	European	1	0	0.265
21	F	European	1	1	0.659
22	M	European	1	1	0.594
23	M	European	1	1	0.666
24	F	European	1	1	0.536
25	F	European	2	0	0.653
26	M	European	2	0	0.711
27	F	European	2	0	0.653
28	M	European	2	0	0.653
29	M	European	2	0	0.711
30	M	European	2	0	0.588

Note: Informed consent was obtained from all study subjects for the creation of induced pluripotent stem cells, and studies were performed with institutional review board approval of both Kaiser Permanente Northern California and the University of California San Francisco Benioff Children's Hospitals. Donor individuals were genotyped using Illumina Infinium OmniExpressExome bead chips. A 4 SNP-weighted GRS was calculated for each iPSC line using the following variants: *PNPLA3* rs738409, *TM6SF2* rs58542926, *GCKR* rs1260326, and *MBOAT7* rs641738.
F, Female; GRS, genetic risk score; iPSCs, induced pluripotent stem cells; M, male; SNP, single nucleotide polymorphism.

Supplementary Table 2. Flow Cytometry Values of iPSCs Post Oleate Challenge and Nile Red Staining

iPSC line	BSA geometric mean	100 μM oleate geometric mean
1	1556	2880
2	661	1718
3	185	425
4	388	579
5	241	563
6	245	521
7	2038	2874
8	1487	1940
9	981	1285
10	1887	2185
11	2881	3549
12	1601	2698
13	1754	2123
14	974	1356
15	1059	2118
16	2971	6331
17	1502	3293
18	1814	2510
19	1121	2345
20	1141	2004
21	1266	2758
22	2952	8028
23	717	1982
24	255	767
25	965	2120
26	790	2559
27	274	637
28	469	1456
29	302	864
30	349	611

BSA, Bovine serum albumin; iPSCs, induced pluripotent stem cells.