RESEARCH LETTERS

Microfluidic Flow Promotes a Steatotic Phenotype in Induced Pluripotent Stem Cell–Derived Hepatocytes that is Influenced by Disease State of the Donor

Microfluidic technology has emerged as a powerful tool to recapitulate the dynamic cellular microenvironment under physiologic flow and to enable precise control over environmental (nutrients) and mechanical (shear stress) factors. In this study, we utilized a microfluidic perfusion system to model pathogenesis of metabolic dysfunction–associated steatohepatitis (MASH) and study the interplay between mechanical and environmental cues, such as free fatty acid supplementation, in driving steatosis in MASH.

The prevalence of MASH is increasing worldwide, and still very limited pharmacological treatment exists. The lack of human relevant preclinical model systems is a major barrier to developing new therapies. Patient-derived induced pluripotent stem cells (iPSCs) serve as a valuable tool to gain insight into human diseases, as they can recapitulate many of the disease pathologies observed in patients. It has recently been reported that iPSC-derived hepatocytes from metabolic dysfunction–associated fatty liver disease/MASH donors exhibit disease-specific features.[1] Extending that work, we utilized patient-derived iPSCs and perfusion-based microfluidic technology to investigate the effect of fluid flow on degree of steatosis in iPSC-derived hepatocytes. Microfluidics enables precise manipulation of the cellular microenvironment including flow rate and shear stress, which are important factors regulating cell viability, proliferation, and function, that cannot be achieved with conventional static-culture systems.[2]

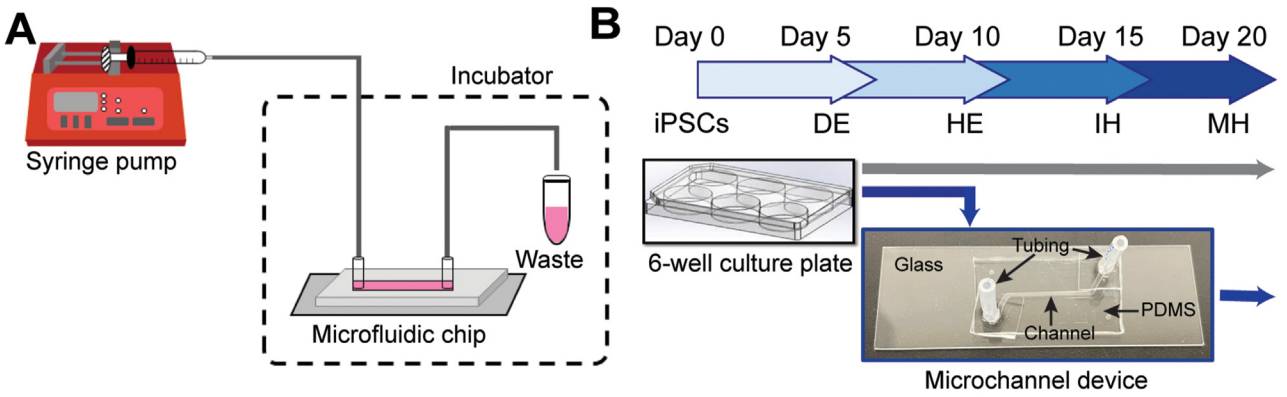
Most liver-based microphysiological systems utilized for modeling liver-related diseases, such as metabolic dysfunction–associated fatty liver disease, MASH, and drug-induced injury, rely mainly on primary hepatocytes; however, problems related to their limited availability and low proliferation potential present major drawbacks.[3–5] These challenges can be overcome with iPSC-derived hepatocytes, and iPSC-based microphysiological systems offer a promising approach to address patient-specific disease characteristics. To our knowledge, this is the first study in which patient-derived iPSC hepatocytes are cultured within microfluidic devices to demonstrate effect of fluid flow on steatosis.

iPSCs derived from a normal control or MASH patient were obtained deidentified from California Institute of Regenerative Medicine's iPSC repository and differentiated into iPSC hepatocytes following a modified protocol.[6] Stepwise hepatic differentiation and characterization at different stages are schematically shown in Figure A1. To evaluate the effect of micro-perfusion flow, differentiation of iPSCs into hepatocyte-like cells was carried out in both microfluidic devices, under steady perfusion flow, and treated plastic plates under conventional static culture conditions. On day 10, hepatic endoderm cells were transferred into microfluidic devices allowing continuous differentiation through the immature-hepatocyte to mature-like hepatocyte stage (day 20) under flow conditions. The average shear stress exerted on iPSC hepatocytes at a flow rate of 30 μl/hr is estimated to be 1.65×10^{-3} dyne/cm^2, thus providing a steady shear stress at a level closely replicating the physiological environment for hepatocytes.[7] For static control, the complete differentiation was accomplished in culture dishes. Schematic illustrations of the microfluidic experimental setup and culture plan under static and flow conditions are depicted in Figure 1A and B.

Under both flow and static conditions, hepatocyte-like cells on day 20 exhibited typical polygonal morphology with well-defined cell boundaries, and binucleated cells were observed in some microscopic fields. Moreover, hepatocyte-like cells from a MASH donor differentiated under flow conditions exhibited hepatocellular ballooning and macrosteatosis (Figure A2). Oil Red-O staining was performed to visualize the intracellular lipid-droplet accumulation which, in general, demonstrated that microfluidic flow significantly increased lipid production in iPSC hepatocytes. Furthermore, when compared to cells from a healthy control, MASH iPSC hepatocytes displayed more pronounced lipid accumulation under flow (Figure 1C).

We further evaluated the effect of flow on the maturation status of iPSC hepatocytes. We found that expression of the gene for albumin and its protein in the culture supernatant were both significantly increased under microfluidic flow, whereas expression of the gene for alpha fetoprotein was comparable across all groups (Figures 1D, and 2G and H). The increased albumin production under flow suggests that fluid flow may promote maturation of iPSC hepatocytes. These results are consistent with previous work where albumin secretion by iPSC hepatocytes was reported to be significantly higher in a perfusion-based microbioreactor compared to static culture dishes[8]; albeit, the concentration of albumin production in the bioreactor system on day 21 was much lower than the level detected in our current system.

Excessive lipid accumulation is a hallmark of MASH. Therefore, we analyzed the effect of fatty acid supplementation on steatosis in MASH and control iPSC hepatocytes. To induce lipidosis on day 20, iPSC hepatocytes were exposed to either 25-μM oleic acid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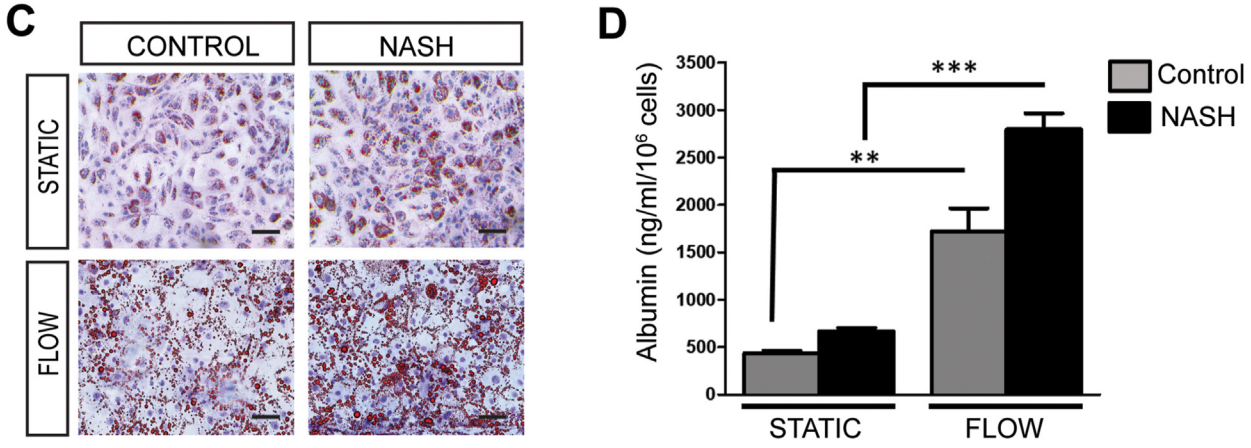
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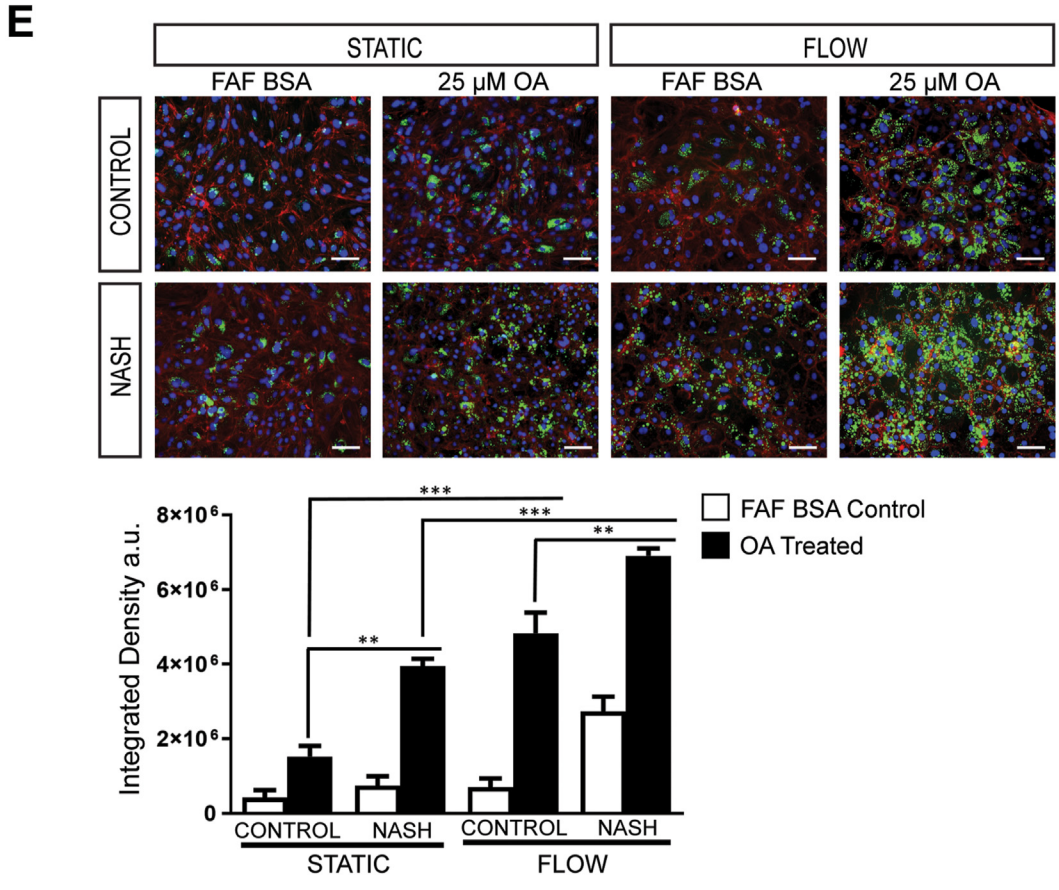
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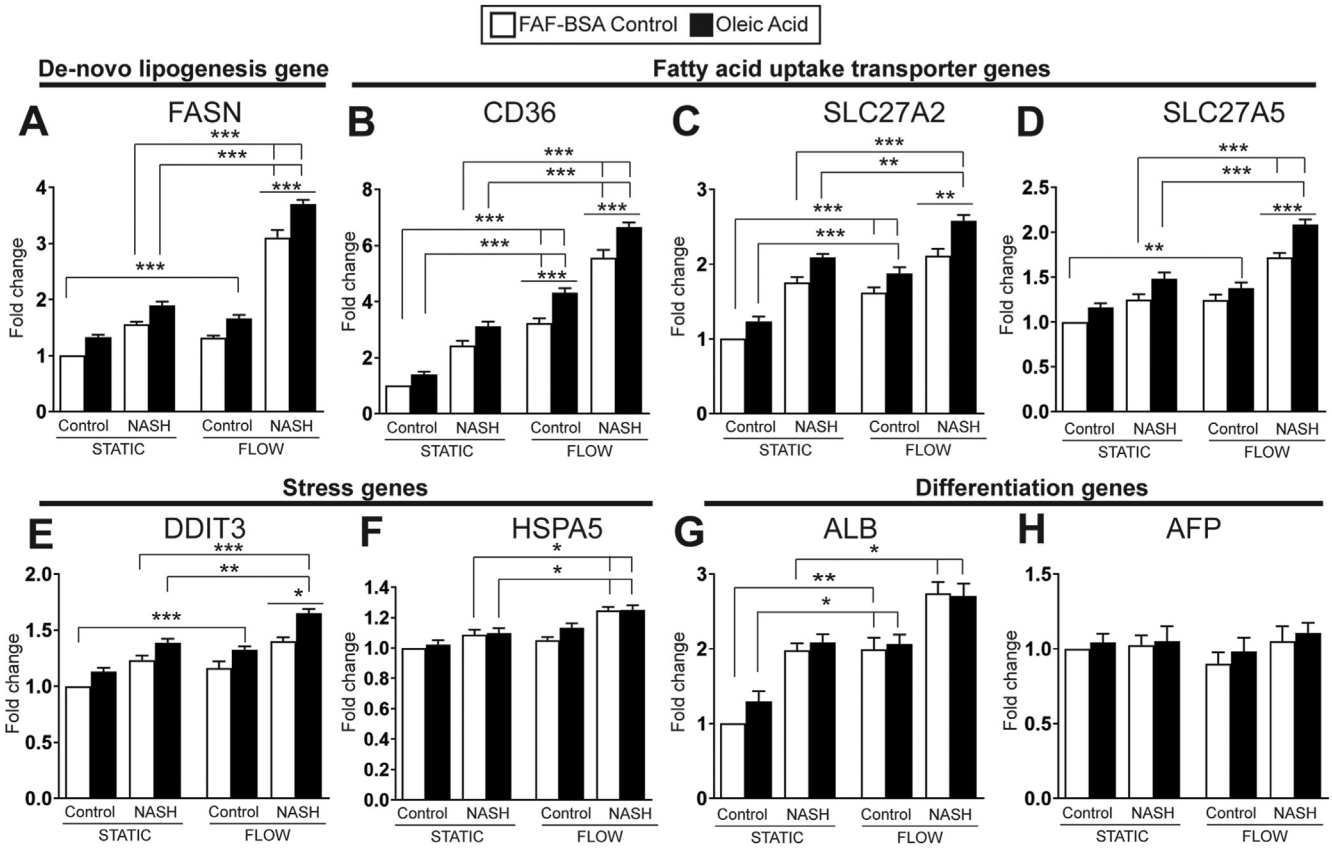

Figure 2. Gene expression analysis: Relative gene expression levels of de-novo lipogenesis gene (A) FASN, fatty acid uptake transporter genes (B) CD36, (C) SLC27A2 (D) SLC27A5, stress genes (E) DDIT3, (F) HSPA5, and differentiation genes (G) ALB and (H) AFP in iPSC-derived hepatocytes from control or MASH background cultured under static or flow conditions in the presence of oleic acid (treatment; black bar) or fatty acid–free bovine serum albumin (Control; white bar). Values expressed as mean ± standard error from 3 independent experiments (*$P < .05$, **$P < .01$, and ***$P < .001$). AFP, alpha fetoprotein; ALB, albumin; FASN, fatty acid synthase.

(treatment group) or fatty acid–free bovine serum albumin (vehicle control group) for 24 hours under static or flow conditions. BODIPY staining was performed to determine the degree of steatosis. In the presence of oleic acid, MASH iPSC hepatocytes displayed an innate proclivity towards lipid production and storage, and microfluidic flow further enhanced the steatotic phenotype (Figure 1E). Gene expression analysis (Figure 2) further revealed that MASH iPSC hepatocytes treated with oleic acid under flow have increased de-novo lipogenesis (increased *FASN* expression), fatty acid transport (increased *CD36, SLC27A2, SLC27A5* expression), and cellular stress (increased *DDIT3* and *HSPA5*). These results suggest that disease state of the donor may lead to iPSC hepatocytes that are more susceptible to accumulation of intracellular lipid droplets exhibiting higher lipidosis in the presence of oleic acid when maintained under flow.

In summary, this study demonstrates that microfluidic flow promotes maturation and accentuates the steatotic phenotype. It also confirms that patient-derived iPSC hepatocytes can serve as a good alternative to primary hepatocytes since they reduce the

Figure 1. Effect of flow on steatosis in iPSC-derived hepatocytes from a control or MASH background: (A) Schematic representation of the microfluidic experimental set-up for continuous perfusion of media and waste collection. (B) Schematic illustration of the culture plan for iPSC-hepatocytes under static (gray arrow) and flow (blue arrow) conditions. (C) Oil Red O staining for visualization of intracellular lipid droplets (Scale bar: 50 μm) (D) Albumin secretion assessed by ELISA on day 21 in 24 hour-culture supernatant. (E) Representative immunoflorescent photomicrographs showing BODIPY staining of control or MASH iPSC-derived hepatocytes treated with oleic acid (treatment group) or fatty acid–free bovine serum albumin (vehicle-control group) under static and flow conditions. (BODIPY: green; Actin Red 555: red; 4′,6-diamidino-2-phenylindole: blue) (Scale bar: 50 μm). Quantification of fluorescent integrated density for BODIPY. Values expressed as mean ± standard error (*$P < .05$, **$P < .01$, and ***$P < .001$) from 3 independent experiments. ELISA, enzyme-linked immunosorbent assay.

genetic variability, by preserving the patient's genetic background, while faithfully recapitulating patient-specific clinical phenotypes in-vitro. We utilized a single iPSC cell line per group in this pilot study. However, subsequent research will incorporate more iPSC cell lines per group to examine line-to-line/intrabatch variability. Importantly, integration of patient-derived iPSCs with microfluidic technology can open new strategies to better understand disease progression as well as establishing a patient-specific in-vitro platform for personalized medicine and drug-screening. Extension of this work will include incorporation of other cell-types generated from a single patient donor iPSC, ie, kupffer, stellate, and liver sinusoidal endothelial cells, whose interactions with hepatocytes may influence MASH disease progression.[9] Successful implementation of coculturing these cells in microfluidic devices is critically needed for the development of a more comprehensive in-vitro human model for MASH treatment.

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Supplementary Materials

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***Authors share co-first authorship. **Authors share corresponding authorship.**

Abbreviations used in this paper: **CIRM, California Institute of Regenerative Medicine; DE, definitive endoderm; iPSC, induced pluripotent stem cells; MASH, metabolic dysfunction–associated steatohepatitis**

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Ethical Statement:
This study does not involve the use of vertebrate animals or human subjects. iPSC cell lines used in this study were received deidentified and obtained from the California Institute of Regenerative Medicine stem cell repository.

Data Transparency Statement:
The data, analytic methods, and study materials underlying this study will be made available from the corresponding authors upon reasonable request.

Reporting Guidelines:
Not applicable for this article type.