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JD Induced Pluripotent Stem Cell–Derived Hepatocytes Faithfully Recapitulate the Pathophysiology of Familial Hypercholesterolemia

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Elevated levels of low-density lipoprotein cholesterol (LDL-C) in plasma are a major contributor to cardiovascular disease, which is the leading cause of death worldwide. Genome-wide association studies (GWAS) have identified 95 loci that associate with control of lipid/cholesterol metabolism. Although GWAS results are highly provocative, direct analyses of the contribution of specific allelic variations in regulating LDL-C has been challenging due to the difficulty in accessing appropriate cells from affected patients. The primary cell type responsible for controlling cholesterol and lipid flux is the hepatocyte. Recently, we have shown that cells with hepatocyte characteristics can be generated from human induced pluripotent stem cells (iPSCs). This finding raises the possibility of using patient-specific iPSC-derived hepatocytes to study the functional contribution of GWAS loci in regulating lipid metabolism. To test the validity of this approach, we produced iPSCs from JD a patient with mutations in the low-density lipoprotein receptor (*LDLR*) gene that result in familial hypercholesterolemia (FH). We demonstrate that (1) hepatocytes can be efficiently generated from FH iPSCs; (2) in contrast to control cells, FH iPSC-derived hepatocytes are deficient in LDL-C uptake; (3) control but not FH iPSC-derived hepatocytes increase LDL uptake in response to lovastatin; and (4) FH iPSC-derived hepatocytes display a marked elevation in secretion of lipidated apolipoprotein B-100. *Conclusion:* Cumulatively, these findings demonstrate that FH iPSC-derived hepatocytes recapitulate the complex pathophysiology of FH in culture. These results also establish that patient-specific iPSC-derived hepatocytes could be used to definitively determine the functional contribution of allelic variation in regulating lipid and cholesterol metabolism and could potentially provide a platform for the identification of novel treatments of cardiovascular disease. (HEPATOLOGY 2012;56:2163-2171)

A study of cardiovascular disease in the United States revealed that approximately 1 in 3 (79 million) American adults suffer from heart disease, and approximately 16 million are specifically afflicted with coronary artery disease (CAD).[1] The level of circulating low-density lipoprotein cholesterol (LDL-C) within an individual has been functionally associated with CAD, and meta-analyses of patients that are aggressively treated with LDL-C–lowering drugs revealed that treatment reduces the incidence of heart attack and stroke.[2] Although environmental factors contribute to plasma LDL-C concentration, genome-wide association studies (GWAS) performed on >100,000 patients have identified genetic variants at 95 loci that are closely associated with cholesterol and lipid levels linked to CAD.[3] Some of these genetic

Abbreviations: apoB-100, apolipoprotein B-100; ANOVA, analysis of variance; ASGPR1, asialoglycoprotein receptor; CAD, coronary artery disease; FH, familial hypercholesterolemia; FL-LDL, fluorescently labeled LDL; GWAS, genome-wide association studies; hESC, human embryonic stem cell; hiPSC, human induced pluripotent stem cell; HMG-CoA, 3-hydroxy-3-methyl-glutaryl-coenzyme A; HNF4a, hepatocyte nuclear factor 4a; iPSC, induced pluripotent stem cell; LDL-C, low-density lipoprotein cholesterol; mRNA, messenger RNA; qRT-PCR, quantitative reverse-transcription polymerase chain reaction; SREBP, sterol regulatory element binding protein; VLDL, very low-density lipoprotein.

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variants are associated with genes encoding proteins with known roles in regulating cholesterol metabolism, including low-density lipoprotein receptor (*LDLR*), low-density lipoprotein receptor–associated protein 1 (*LDLRAP1*), and scavenger receptor class B, member 1 (*SCARB1*). In addition to identifying genes with known roles in controlling cholesterol flux, GWAS uncovered many novel loci whose contribution to CAD is not understood.

Historically, linking genetic findings to biological mechanisms has proven to be a challenge. In some cases, the mouse has provided a suitable system to relate genetic variation to the pathophysiology of disease; however, the usefulness of the mouse is tempered by differences from humans in terms of physiology, metabolism, and genetics, and this is exacerbated when complex traits are being analyzed. Cell culture models can also be useful; however, cholesterol metabolism is predominantly controlled by hepatocytes, and obtaining primary liver cells from patients would require a liver biopsy. Moreover, when primary hepatocytes are cultured, the cells quickly dedifferentiate and lose key liver functions, rendering them unsuitable for detailed metabolic studies. Recently, it has been shown that human induced pluripotent stem cells (hiPSCs) can differentiate into cells that are functionally similar to hepatocytes.[4-6] Because hiPSCs can be reprogrammed from easily accessible somatic cell types, such as skin fibroblasts, this raises the possibility of using hiPSCs from GWAS patients as a source of hepatocytes to study the role of specific allelic variants in regulating cholesterol metabolism. In addition, the availability of hepatocytes derived from patients with inborn errors in hepatic metabolism could provide a platform for drug discovery. Although the use of hiPSC-derived hepatocytes to recapitulate metabolic liver disease in culture is conceptually appealing, direct evidence demonstrating the validity of such an approach is scarce.[7] Importantly, although iPSC-derived hepatocyte-like cells can be generated with high efficiency, the resulting cells fail to express the complete repertoire of proteins found in adult primary hepatocytes and do not fully silence expression of fetal hepatocyte messenger RNAs (mRNAs) such as *AFP*.[4] These observations have raised questions over the credibility of using iPSCs to study hepatic dysfunction.[8] We therefore attempted to determine the feasibility of using hiPSCs to study genetic variants that could contribute to dysregulation of cholesterol flux by producing hepatocytes from hiPSCs that were generated from a familial hypercholesterolemia (FH) patient with defined mutations.

Materials and Methods

A detailed description of the Materials and Methods is provided in the Supporting Information. Procedures used for the generation of iPSCs and differentiation of pluripotent stem cells to hepatocytes have been described.[4,9] All culture of human embryonic stem cells (hESCs) and generation of iPSCs was approved by the MCW Human Stem Cell Research Oversight Committee (hSCRO approval #09-005), and all animal procedures were approved by the Medical College of Wisconsin's Institutional Animal Care and Use Committee.

Results

Generation of iPSCs From JD Fibroblasts. FH is an autosomal dominant dyslipidemia caused by mutations in the *LDLR* gene that result in severely elevated plasma LDL-C levels and premature cardiovascular disease.[10] The liver is central to the pathogenesis of FH, and homozygous FH patients are successfully treated with liver transplantation. Although hepatocytes are the key cells that control cholesterol flux, *LDLR* mutations have primarily been studied using fibroblasts.[10] Such studies revealed that LDLR-deficient fibroblasts had an impaired capacity to internalize LDL, which gave rise to the paradigm that the level of LDL-C in serum is determined by the rate of LDL clearance.[11] However, modifications to this model have recently been proposed based on evidence suggesting that FH patients often possess profoundly elevated hepatic very low-density lipoprotein (VLDL) production.[12] Given the extensive understanding of FH and the fact that single nucleotide polymorphisms have been identified in the vicinity of the *LDLR* gene, we felt that hepatocytes derived from FH hiPSCs would offer an ideal model to define the feasibility of using iPSCs to study genetic variations that could affect complex hepatic metabolism.

The generation of iPSCs from a patient with early onset atherosclerotic disease with hypercholesterolemia

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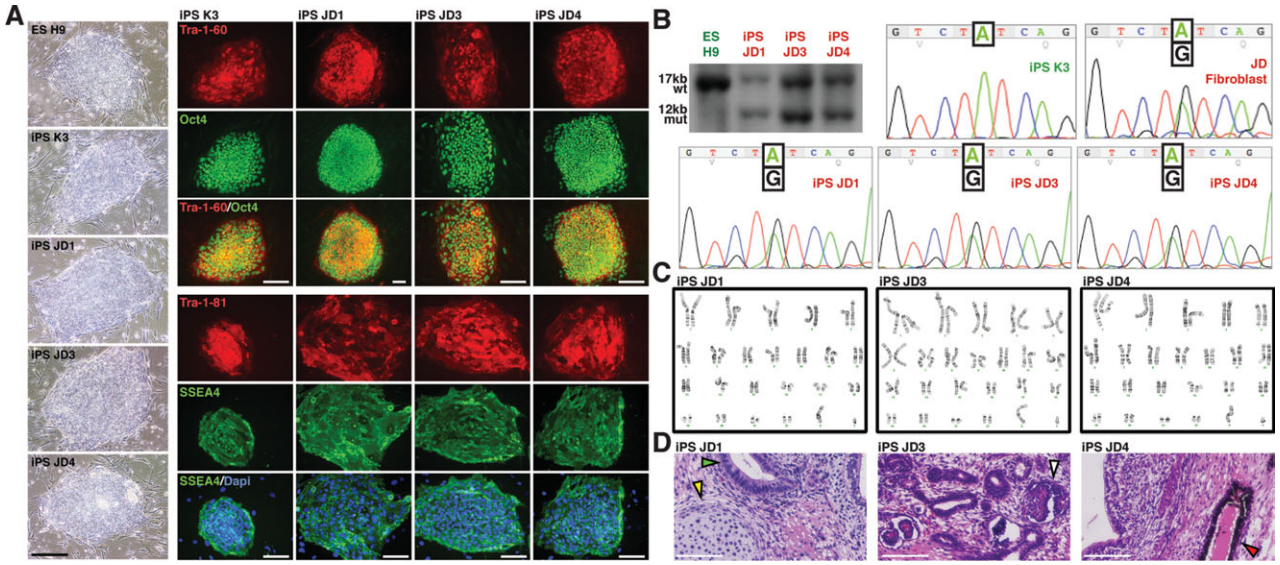

Fig. 1. Generation of induced pluripotent stem cells from JD fibroblasts. (A) Micrographs showing colony morphology and expression of characteristic markers of pluripotency (Tra-1-60, OCT4, Tra-1-81, SSEA4) identified by immunocytochemistry in hESCs (H9), control hiPSCs (K3), and three independently derived JD iPSC lines (JD1, JD3, JD4). (B) Southern blot analysis (upper left panel) using a probe from *LDLR* exon 18 to identify a 17-kb wild-type *Bam*H1 fragment (wt) in control cells and an additional 12-kb mutant fragment (mut) in JD hiPSCs that represents the maternal allele. DNA sequencing revealed the presence of the paternal A-G transition exclusively in JD fibroblasts and JD iPSCs. (C) JD hiPSCs retain a normal chromosomal arrangement as revealed by karyotyping. (D) Teratomas generated in immunocompromised mice from JD hiPSCs contained cell types representative of all germ layers (arrowheads). Scale bars = 100 μm.

has been described[7]; however, the genetic lesion was undefined. In addition, this study by Rashid et al. was designed only to test whether cells derived from LDLR-deficient iPSCs could internalize LDL. However, LDLR-mediated uptake of LDL is not a hepatocyte-specific process, and most cells use this pathway to internalize cholesterol. Therefore, the goal of the current study was to test the feasibility of using iPSC-derived hepatocytes to study complex metabolic disorders that specifically affect hepatocyte function. To develop a clearer understanding of the pathophysiology of FH iPSC–derived hepatocytes, we reprogrammed fibroblasts from JD, a 14-year-old boy with cutaneous xanthomatosis and advanced cardiovascular disease.[13] The choice to generate JD hiPSCs was considered historically relevant because Brown, Goldstein, and colleagues, in establishing the LDLR paradigm, studied JD fibroblasts extensively.[10,11] We produced several JD iPSC lines by transducing primary fibroblasts with lentiviral vectors encoding the transcription factors *OCT4*, *SOX2*, *NANOG*, and LIN28[14] and demonstrated that they expressed characteristic markers of pluripotency (Fig. 1A). In each hiPSC line, we confirmed the retention of the JD *LDLR* mutations (Fig. 1B, Supporting Fig. 1), established that each had a normal karyotype (Fig. 1C), and determined that each JD hiPSC line could differentiate into derivatives of all three germ layers using teratoma assays (Fig. 1D).

JD iPSCs Can be Induced to Form Hepatocytes With High Efficiency and Reproducibility. Using a previously described protocol (Fig. 2A), which we had shown could generate functional hepatocyte-like cells (referred to here as hepatocytes),[4,9] we demonstrated that each JD hiPSC clone was capable of directed differentiation toward a hepatic fate. On day 20 of differentiation, the morphology of both control hiPSC– and JD hiPSC–derived cells was indistinguishable and closely resembled that of hepatocytes, including the presence of lipid vesicles, a high cytoplasmic to nuclear ratio, granular cytoplasm, and prominent nucleoli (Fig. 2B). In addition, the differentiated cells expressed hepatocyte markers, including hepatocyte nuclear factor 4a (HNF4a) and albumin (Fig. 2C). Flow cytometric analyses of hepatocytes from both control and JD hiPSCs confirmed that the cells differentiated into asialoglycoprotein receptor (ASGPR1)-positive hepatocytes with comparable efficiency (Fig. 2D). Only cells expressing high levels of ASGPR1 were counted to avoid the possibility of counting false negatives. Finally, hepatocytes derived from control hESCs or hiPSCs as well as JD hiPSCs were found to express hepatic mRNAs at similar levels, whereas expression of each of these mRNAs was not detected in undifferentiated hESCs (Fig. 2E). Based on these data, we conclude that JD iPSCs could be directed to form cells with hepatocyte characteristics at efficiencies that were comparable to hESCs or control hiPSCs.

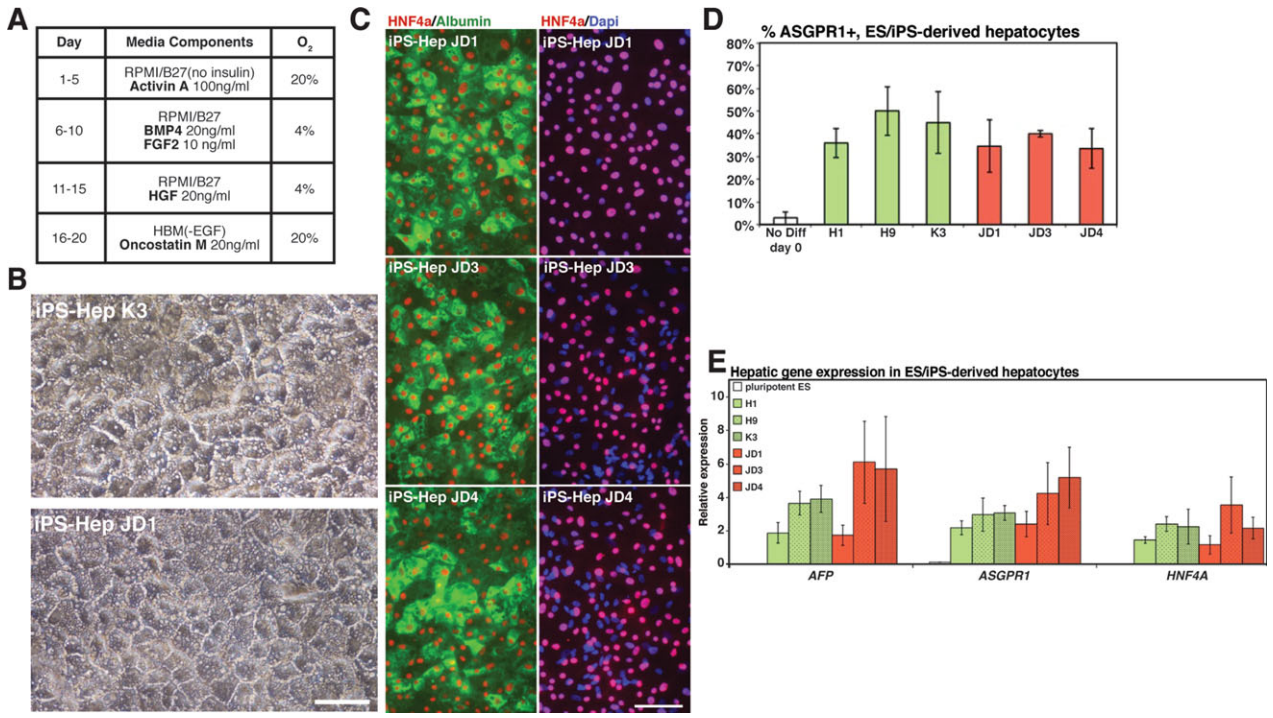

Fig. 2. Hepatocyte-like cells can be efficiently derived from JD hiPSCs. (A) Schematic showing procedure used to generate hepatocytes from hiPSCs. (B) Micrographs showing morphology of hepatocytes derived from wild-type and JD hiPSCs. (C) Immunocytochemistry revealing the presence of HNF4a in the nucleus (red) and albumin in the cytoplasm (green) of JD hiPSC–derived hepatocytes. Nuclei are identified by DAPI staining (blue). (D) Bar graph illustrating the efficiency and reproducibility (n = 3 independent experiments) of generating ASGPR1-positive hepatocytes from control (H1, H9, K3) and JD (JD1, JD3, JD4) pluripotent stem cells. No significant difference was observed between control and JD cells (analysis of variance [ANOVA], $P > 0.05$). (F) Bar graph showing the result of real-time qRT-PCR analyses of characteristic hepatocyte mRNAs (*AFP*, *ASGPR1*, *HNF4A*) in undifferentiated pluripotent stem cells and in hepatocytes generated from control (H1, H9, K3) and JD (JD1, JD3, JD4) pluripotent stem cells. No significant difference was observed between control and JD cells (ANOVA, $P > 0.05$). Scale bars = 100 μm.

JD iPSC–Derived Hepatocytes Show Deficiencies in LDL Uptake and Response to Lovastatin Treatment. The FH associated with JD is a consequence of compound heterozygosity at the *LDLR* locus. JD inherited a maternal allele containing a 5-kb deletion spanning part of exon 13 and all of exons 14 and 15 that results in the absence of functional protein.[13] The inherited paternal allele contains an A>G transition within exon 17, which encodes a tyrosine>cysteine substitution at residue 807 in the LDLR cytoplasmic domain resulting in a mutant protein that can still bind LDL, but is inefficiently internalized.[13] The combination of these two alleles encoded by the JD genome therefore effectively results in homozygous loss of LDLR function. We first measured whether JD hiPSC–derived hepatocytes exhibited the expected deficiencies in LDL uptake. After 3.5 hours incubation with fluorescently labeled LDL particles (FL-LDL), control hiPSC-derived hepatocytes contained intense fluorescence staining extending from a perinuclear location throughout the cytoplasm (Fig. 3A). In contrast, cytoplasmic fluorescence within JD hiPSC–derived hepatocytes was reduced (Fig. 3A; Supporting Fig. 2), and we observed intense clusters of staining at the cell surface, which is consistent with trapping of FL-LDL by the paternally encoded mutant LDLR. These results therefore confirm that JD-encoded LDLR alleles are defective, as has been described in the studies of JD fibroblasts.

In addition to probing GWAS phenotypes, patient-specific hiPSC-derived hepatocytes could provide a platform to identify cholesterol lowering pharmaceuticals; however, again proof-of-feasibility experiments have not been described. Lovastatin is a hepatoselective lipid-lowering drug whose activity is conferred by oxidation of the lactone prodrug to its β-hydroxy acid form, which then inhibits 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG-CoA) reductase. Because activation of the prodrug is hepatocyte-specific, *in vitro* studies using lovastatin ubiquitously employ biochemically activated lovastatin β-hydroxy acid rather than the lactone prodrug. Under normal circumstances, the response of the hepatocyte to HMG-CoA reductase inhibition is to increase expression of the *LDLR* gene

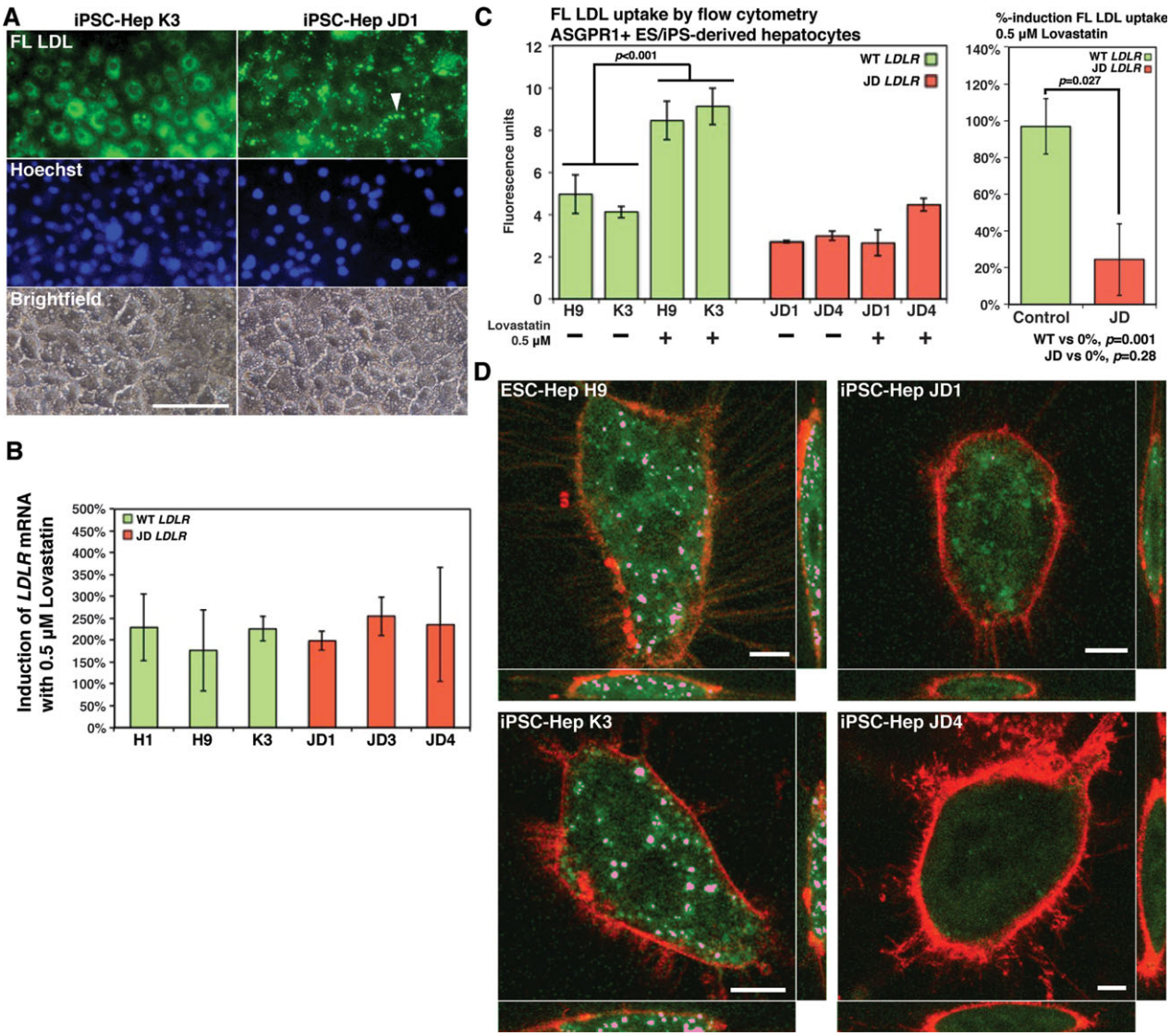

Fig. 3. JD hiPSC–derived hepatocytes exhibit deficiencies in uptake of LDL and in their response to lovastatin. (A) Micrographs showing localization of FL-LDL (green) after incubation of control and JD iPSC–derived hepatocytes with BODIPY-labeled LDL for 3.5 hours. Note the clustering of FL-LDL specifically on the surface of JD cells (arrowhead). Staining with Hoechst dye identified nuclei (blue), and cell distribution is shown by bright field images. Scale bar = 100 μm. (B) Bar graph shows percent increase in *LDLR* mRNA following lovastatin treatment of control (green bars) and JD (red bars) hepatocytes. No statistically significance difference in the level of induction was observed between control and JD cells (ANOVA, $P > 0.05$). (C) Bar graph (left panel) showing a significant (Student *t* test, $P < 0.05$) increase in FL-LDL uptake was observed in response to lovastatin treatment by control hepatocytes (green bars) but not by JD hepatocytes (red bars). The right bar graph shows that the differential response to lovastatin treatment between control (green bar) and JD (red bar) hepatocytes is significant (Student *t* test, $P < 0.05$). (D) Volume render images obtained by confocal microscopy showing that uptake of FL-LDL (pink) could be identified in endosomes of lovastatin-treated control (ES-Hep H9, iPS-Hep K3) hepatocytes but not in JD hepatocytes. Plasma membranes were identified using FM 4-64 (Molecular Probes) (red). Scale bars = 20 μm.

resulting in enhanced LDL uptake. Importantly, because this drug manifests its activity primarily through increasing LDLR, lovastatin is ineffective in FH patients that encode defective *LDLR* alleles. We therefore examined the response of both control- and JD-derived hepatocytes to lovastatin treatment (Figs. 3B-D). When either control or JD hepatocytes were treated for 24 hours with 0.5 μM lovastatin lactone, we observed a significant induction of *LDLR* mRNA (control, $P = 0.003$; JD, $P = 0.011$) (Fig. 3B), and the extent of induction was similar regardless of genotype (Fig. 3B). In addition, both control and JD hepatocytes expressed similar levels of enzymes involved in oxidative metabolism of lovastatin lactone (CYP 3A4, CES1, CES2, PON2, and PON3; Supporting Fig. 3). Induction of *LDLR* gene expression is predominantly regulated through proteolytic activation of sterol regulatory element binding protein (SREBP) 2 (encoded

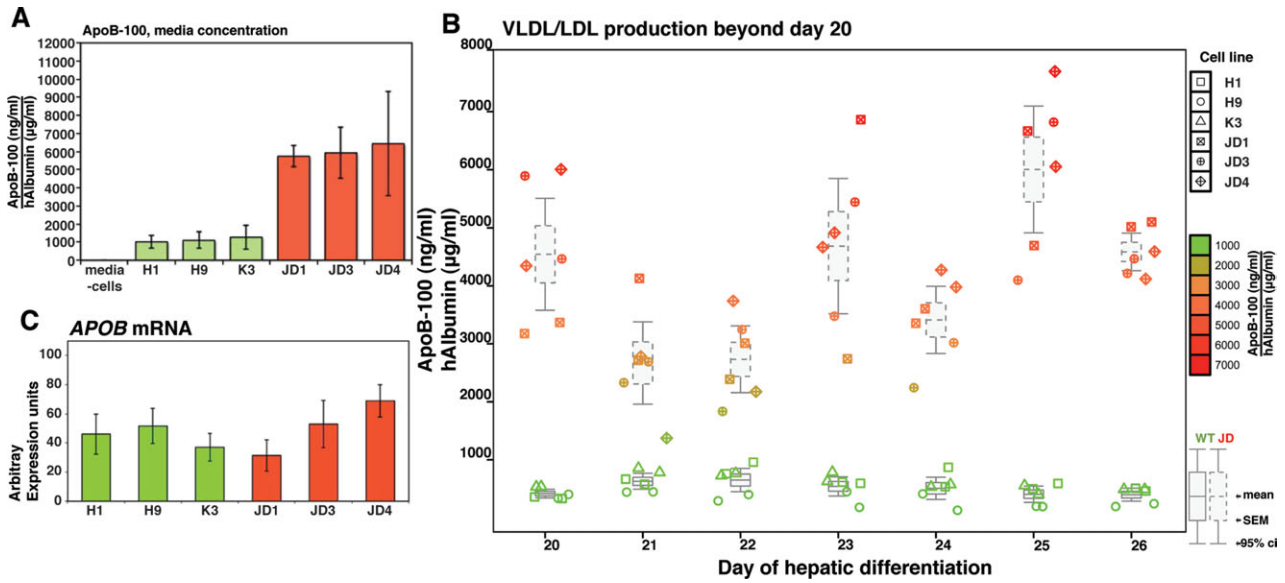

Fig. 4. apoB-100 secretion is elevated in JD iPSC–derived hepatocytes. (A) Bar graphs showing concentration of lipidated apoB secreted into the medium by control (H1, H9, and K3) (green boxes) or JD (JD1, JD3, JD4) (red boxes) iPSC-derived hepatocytes as determined by enzyme-linked immunosorbent assay ($n = 3$ independent differentiations). No significant difference was observed within control or mutant groups; however, secreted apoB levels were significantly elevated in JD compared with control hepatocytes (Tukey-Kramer *post hoc* multiple comparison test, $P < 0.05$). (B) Box and whisker plot showing that elevated apoB production is maintained in hepatocytes derived from JD iPSCs compared with control hepatocytes after extended culture (days 20-26) of the differentiated cells. (C) Bar graph showing levels of *APOB* mRNA in hepatocytes derived from control (H1, H9, and K3) (green boxes) or JD (JD1, JD3, JD4) (red boxes) pluripotent stem cells as determined by qRT-PCR analyses. *APOB* mRNA levels were not significantly different between cell lines (ANOVA, $P > 0.05$).

by *SREBF2*); however, it has also been reported that hepatocyte expression of *SREBF2* mRNA is increased in response to lovastatin treatment. Quantitative reverse-transcription polymerase chain reaction (qRT-PCR) analyses revealed modest increases in expression of *SREBF2* mRNA following lovastatin treatment of both control and JD hepatocytes (Supporting Fig. 4). Although SREBP1 has also been implicated in regulating cholesterol metabolism, no significant difference in *SREBF1* mRNA levels was detected. Cumulatively, these data demonstrate that both control and JD hepatocytes responded appropriately to statin treatment and that the pluripotent stem cell–derived hepatocytes were capable of converting the prodrug to an active form. We next measured the impact of lovastatin on LDL uptake. To ensure that flow cytometery could quantitatively measure LDL uptake, we first measured the level of FL-LDL uptake in control hESC-derived hepatocytes over time. We found that FL-LDL uptake tripled over a period of 1 hour, increasing linearly through 30 minutes (Supporting Fig. 5). We therefore used a 30-minute incubation with FL-LDL in all further analyses, which ensured that all measurements were in the linear range. In control stem cell–derived hepatocytes, flow cytometry revealed that the increase in *LDLR* mRNA levels in response to lovastatin treatment translated to a 99.1% increase in FL-LDL uptake compared with untreated cells ($P < 0.001$) (Fig. 3C). In contrast to control cells, no significant change in FL-LDL uptake was observed between treated and untreated JD hiPSC-derived hepatocytes (Fig. 3C). LDL uptake by hepatocytes is divided into a high-affinity, low-volume mechanism mediated by the LDLR, and a low-affinity, high-volume mechanism controlled independently. We therefore also examined the distribution of FL-LDL internalized by control and JD hiPSC-derived hepatocytes after lovastatin treatment using confocal microscopy (Fig. 3D, Supporting Fig. 2). In control cells, FL-LDL was identified within distinct subcellular foci consistent with transport of the FL-LDL to endosomes via clathrin-mediated endocytosis. In contrast, JD hiPSC-derived hepatocytes exhibited no endosomal localization of FL-LDL, although relatively low levels of fluorescence were uniformly distributed throughout the JD cell cytoplasm. Cumulatively, these data demonstrate that hiPSC-derived hepatocytes can be used effectively to identify lipid-lowering pharmaceuticals and that the JD hiPSC-derived hepatocytes accurately reflect the pathophysiology of FH.

Hepatocytes Derived From JD iPSCs Show Highly Elevated Secretion of Lipidated Apolipoprotein B-100. Several studies have supported a view that loss of LDLR function not only results in reduced LDL-C

uptake, but also significantly increases production of VLDL/LDL by hepatocytes, and it has been argued that enhanced VLDL/LDL secretion may be the predominant etiology of hypercholesterolemia.[12] The proposal that LDLR deficiency results in enhanced LDL production remains controversial because of conflicting results obtained from multiple patient and animal studies.[15-18] One problem is that direct study of LDL production in FH patients has been somewhat limited because of the difficulty in obtaining primary LDLR-deficient human hepatocytes. Additionally, studies using human hepatocellular carcinoma cells (e.g., HepG2) are confounded because these cells are deficient in lipoprotein production, requiring investigators to use transient incubation with exogenous lipids or drugs to coax them toward a more physiologically normal state. Using enzyme-linked immunosorbent assay to detect lipidated apolipoprotein B-100 (apoB-100), we confirmed that hepatocytes derived from both control and JD hESCs/hiPSCs actively secrete VLDL/LDL (Fig. 4A). Strikingly, JD iPSC-derived hepatocytes displayed an approximate eight-fold increase in the level of secreted apoB-100 compared with hepatocytes derived from three genetically independent control pluripotent stem cell lines across three independent differentiation experiments (JD, 1,484 ng/mL; control, 173 ng/mL; $P < 0.001$). When we controlled for the efficiency of hepatocyte differentiation by normalizing secreted lipidated apoB-100 concentration to human albumin concentration, similar results were obtained (JD, 6,034 ng/mL; control, 1,123 ng/mL; $P < 0.001$). Continued sampling from hESC/iPSC-derived hepatocyte cultures beyond day 20 of differentiation revealed that secretion of lipidated apoB-100 is maintained for at least 7 days and that the elevated apoB-100 concentration associated with the JD background is preserved throughout this period (Fig. 4B). Previous reports studying rodent hepatocytes have documented that increases in VLDL/LDL secretion in *Ldlr*$^{-/-}$ hepatocytes is determined by the amount of apoB that circumvents posttranslation degradation rather than by changes in gene expression.[17] Consistent with this finding, no significant difference in *APOB* mRNA levels was observed between control and JD hepatocytes ($P = 0.54$) (Fig. 4C).

Discussion

The idea of using hiPSCs to model diseases in culture is not novel.[19-21] Rashid et al.[7] made a significant advance in generating iPSCs from patients with several liver disorders, including alpha-1 anti-trypsin deficiency, glycogen storage disease type 1a, FH, Crigler-Najjar syndrome type 1, and hereditary tyrosinemia. However, due to the large number of disease-specific lines that were generated, a detailed characterization of each was beyond the scope of that study. With regard to FH, Rashid et al. limited their analysis to the ability of differentiated FH iPSCs to internalize LDL. The LDLR is ubiquitously expressed, and so determining LDL uptake, while important, does not address the pathophysiology of FH, which is primarily a consequence of defective production and metabolism of cholesterol specifically by the hepatocyte. Whether patient-specific iPSCs could be used to faithfully recapitulate complex metabolic disorders associated with hepatocyte function therefore remained unaddressed.[8] Several caveats that can affect efficiency of using iPSCs to study complex metabolic disorders need to be considered. For example, although the generation of hiPSCs from somatic cells can be relied upon, the procedure yields iPSC populations that are heterogeneous in nature.[22] In addition, the generation of iPSCs has been found to result in the presence of somatic mutations within the iPSC population,[23,24] and some iPSCs retain aspects of the epigenetic profile of donor cells,[25,26] both of which could affect the interpretation of data generated from iPSC-derived hepatocytes. Perhaps most importantly, however, hepatocytes derived from iPSCs fail to express the full repertoire of genes encoding proteins associated with mature hepatocyte function. The fact that not all hepatocyte mRNAs are expressed is especially concerning given that lipid and cholesterol homeostasis is strictly dependent upon a multitude of interactions that involve metabolic enzymatic activity, gene expression, and protein trafficking. To determine the feasibility of using iPSCs to model metabolic liver disease, we therefore chose to focus on a well-defined mutation that was inherited in Mendelian fashion. To control for variations associated with reprogramming, we performed our analyses on multiple independent JD iPSC clones and compared our data to genetically distinct hESC and iPSC lines. We believe our data convincingly show that key features of FH in cultures of JD iPSC–derived hepatocytes can be recapitulated and therefore conclude that it will be feasible to use patient-specific iPSCs to elucidate the functional contribution of allelic variations that potentially affect control of cholesterol and lipid flux. Although some genetic variations may manifest through hepatocyte-independent processes, given the central role of the liver in control of serum lipid and cholesterol levels, it seems likely that the majority of functional polymorphisms will affect hepatocyte metabolism. Although all of this is encouraging, in other

studies we have found that variations in differentiation efficiency exist among hESCs and hiPSCs, which add a significant complication to experimental interpretation. It is, therefore, important to note that all of the pluripotent stem cells used in the current study were chosen because they displayed a similar efficiency in their capacity to generate hepatocytes, and we believe that this is an important variable to consider if patient-specific iPSCs are to be used to probe disease mechanisms.

As expected, the JD hepatocytes exhibited reduced LDL uptake; however, the most striking change was a reproducible increase in apoB-100/VLDL secretion, which is consistent with several studies suggesting that plasma LDL-C concentrations may be significantly impacted by the VLDL production rate in FH patients.[15,27] The evidence describing the relationship between *LDLR* mutations and LDL-C production by hepatocytes has in some cases been contradictory. Loss of functional LDLR in primary mouse hepatocytes can result in elevated hepatic secretion of apoB-100,[17] which is exacerbated in $Ldlr^{-/-}$ hepatocytes that overexpress SREBP1a.[16] However, in other studies, apoB-100 production was unaffected in $Ldlr^{-/-}$ mice,[18] and similar results were obtained in the LDLR-defective WHHL rabbit.[28] The reasons for such discrepancies remain obscure, but they may reflect variations in animal models being studied, inherent differences in response of cultured hepatocytes compared with liver cells in a more complex *in vivo* environment, and perhaps most importantly, the nature of the *LDLR* mutations present in each model and whether or not these mutations encode proteins that are functionally null or inert. The mechanisms relating LDLR function to apo-B100/VLDL secretion are complex; however, it has been proposed that variances in intracellular pools of cholesterol may affect apoB-100 presecretory degradation.[17,29] Although our data are consistent with this view, preliminary analyses of changes in mRNA levels (data not shown) in JD hepatocytes have revealed that diverse aspects of cholesterol metabolism, secretion, and transport may be coordinately regulated at the level of gene expression and appear tightly linked to cholesterol flux. We believe that future analyses of iPSC-derived hepatocytes from FH patients with distinct *LDLR* alleles will likely enhance our understanding of the molecular mechanisms that link LDLR function to LDL production.

Finally, treatment of elevated cholesterol levels has relied heavily on the use of statins that inhibit HMG-CoA reductase activity. Statins act both by reducing cholesterol synthesis and elevating cholesterol uptake by increasing expression of the LDLR in hepatocytes. Although statins can be highly efficacious, there is a surprisingly wide variation of effectiveness between individuals, with >20% of patients showing a poor response to statin treatment.[30] The pharmacogenetics of statin action are highly complex and involve a large repertoire of regulators, and not surprisingly, several polymorphisms have been described that are associated with poor responders.[31] We propose that the generation of hepatocytes from hiPSCs from individuals that exhibit a differential statin response and display elevated lipid/cholesterol levels could be valuable in the search for novel cholesterol-lowering drugs. In this regard, our finding that control hiPSC-derived hepatocytes could respond to lovastatin treatment by effectively increasing LDL uptake is extremely encouraging if one is to consider using iPSC-derived hepatocytes as a platform for drug discovery. As an alternative to drug screens, it has been proposed that gene therapy could be applied to iPSCs, thereby providing an exogenous supply of "repaired" hepatocytes that could potentially be used to reverse at least a subset of metabolic liver disorders.[32] Although there are many significant hurdles that need to be overcome before iPSC-derived hepatocytes could be used as a therapeutic cell source, precise genome editing through zinc finger or TALEN technologies[32,33] could be valuable in confirming whether a given single nucleotide polymorphism is associated with a specific functional consequence in iPSC-derived hepatocytes.

In conclusion, our data show that if appropriate controls are included, hepatocytes derived from JD iPSCs recapitulate key aspects of FH in culture, which supports the proposal that patient-specific iPSC-derived hepatocytes will become an important tool to dissect the contribution of GWAS lipid loci in controlling hepatic lipid and cholesterol metabolism. In addition, our finding that the hiPSC-derived hepatocytes are competent to respond to hepatoselective pharmaceuticals implies that patient-specific iPSC-derived hepatocytes will facilitate the identification of drugs that can treat inborn errors of liver metabolism.

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