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ARTICLE

An *in vitro* model of hepatic steatosis using lipid loaded induced pluripotent stem cell derived hepatocyte like cells

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Abbreviations used: AQP1, aquaporin 1; BMP4, bone morphogenetic protein 4; BSA, bovine serum albumin; CL, clearance; COL3A1, collagen type 3, alpha 1 chain; CPM, counts per minute; DMEM, Dulbecco's modified Eagle's medium; EB, embyroid bodies; EDTA, ethylene diamine tetra acidic acid; E8, essential medium 8; FAS, fatty acid synthesis; FGF2, fibroblast growth factor 2; HDFn, human dermal fibroblasts, neonatal; HepG2, hepatoma G2; HGF, hepatocyte growth factor; HLC, hepatocytes like cells; hoFH, homozygous familial hypercholesterolemia; IBSC, Institute Biosafety Committee; iPSC, induced pluripotent stem cells; JBL, Jubilant Biosys Ltd; LDL-C, low density lipoprotein-cholesterol; LN2, liquid nitrogen 2; MEF, murine embryonic feeder; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; NEAA, non-essential amino acids; PBS, phosphate buffered saline; RPMI, Roswell Park Memorial Institute; OA, oleic acid; PA, palmitic acid

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ABSTRACT

Hepatic steatosis is a metabolic disease, characterized by selective and progressive accumulation of lipids in liver, leading to progressive non-alcoholic fatty liver disease (NAFLD), non-alcoholic steatohepatitis (NASH), and cirrhosis. The existing *in vitro* models of hepatic steatosis to elucidate the molecular mechanisms behind the onset of hepatic steatosis and to profile small molecule modulators uses lipid loaded primary hepatocytes, and cell lines like HepG2. The limitation of these models includes high variability between the different donor samples, reproducibility, and translatability to physiological context. An *in vitro* human hepatocyte derived model that mimics the pathophysiological changes seen in hepatic steatosis may provide an alternative tool for pre-clinical drug discovery research. We report the development of an *in vitro* experimental model of hepatic steatosis using human induced pluripotent stem cell (iPSC) derived hepatocytes like cells (HLC), loaded with lipids. Our data suggests that HLC carry some of the functional characteristics of primary hepatocytes and are amenable for development of an *in vitro* steatosis model using lipid loading method. The *in vitro* experimental model of hepatic steatosis was further characterized using biomarker analysis and validated using telmisartan. With some refinement and additional validation, our *in vitro* steatosis model system may be useful for profiling small molecule inhibitors and studying the mechanism of action of new drugs.

Keywords: iPSC, hepatocyte-like cells, hepatic steatosis

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD), and non-alcoholic steatohepatitis (NASH) are devastating liver diseases characterized by steatosis (lipid accumulation). While the former lacks hepatocyte injury, the latter is prone for inflammation related liver dysfunction [1]. These pathological depositions of lipids in the liver are thought to be one of the main reasons for the observed progressive reduction in the function of liver in NAFLD/NASH subjects [2]. Reduction of the lipid content in liver is a highly reliable strategy for the development of novel therapeutic approach. Several advancements have been made to understand the pathogenesis of these liver diseases and this has been achieved using *in vitro* systems and *in vivo* animal models [3]. However, the translation from *in vitro* tests to *in vivo* models is poor and most of the small molecule compounds, peptides and/or biosimilars fail or respond differently in animal models as compared to *in vitro* experiments. The lack of correlation between *in vitro* to *in vivo* models may be due to improper selection of *in vitro* experimental systems.

The currently available *in vitro* models of human hepatic steatosis use either cancerous cell lines or transgenics, which do not resemble

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the mature liver cells either morphologically or functionally and may have different protein expression profile [4]. The immortalized cells lack the features that define true hepatocytes and do not represent the exact pathophysiology of disease. Further, these traditional transformed cell lines also lack the vital proteins that are important for the differentiation into mature hepatocytes and development of hepatic steatosis [5]. HepaRG cells, derived from human hepatocellular carcinoma, shares some features & properties of hepatocytes and has been proposed as a surrogate to human hepatocytes for drug metabolism and toxicity studies [6]. HepaRG cells have also been used for the development of vesicular steatosis model [7]. However, the physiological status of a tumor cell is very different from a normal cell and it does not reflect the physiological status of a normal cell *in vivo*. Hepatic cells derived from postnatal skin precursors (hSKP-HPCs), a stem cell population residing in human dermis have been reported in the investigation of steatosis-inducing effects of sodium valproate [8]. Primary human hepatocytes represent a better source for *in vitro* hepatocyte disease model [9]. The primary human hepatocytes based hepatic steatosis model expresses the genes and biomarkers that closely resemble the *in vivo* model system [10]. However, every batch of primary human hepatocyte comes from different donors and therefore they are prone for variability. The lack of availability of enough donors and expensive nature of these cells has hampered liver research and there is a need for an alternate source, which truly mimics the primary hepatocytes [11].

Human induced pluripotent stem cells (iPSCs) derived hepatocyte like cells (HLC) represents an alternative source for human primary hepatocytes [12]. Several studies have successfully used human iPSC derived HLC for functional studies, particularly in assays measuring CYP metabolism [13], hepatotoxicity [14] and infectious diseases such as hepatitis B [15]. iPSC derived HLC implanted in mice having acute liver failure has increased the survival rate of animals which suggests that these iPSC derived cells may represent HLC, not just in *in vitro* but *in vivo* as well [16]. Lipid profile of hepatocytes from patient derived iPSCs is reported as part of the studies on hepatocyte metabolism prior to the development of steatosis [17]. Previous studies have reported iPSC derived hepatocyte like cell models from homozygous familial hypercholesterolemia (hoFH) patients for profiling compounds that may lower serum LDL-C [18]. Recently, iPSC derived HLC have been used to screen small molecules [19].

Here, we report the development of an *in vitro* experimental model of hepatic steatosis using iPSC derived HLC loaded with lipids. The human iPSCs were differentiated into HLC, characterized using gene marker analysis & functional assays and hepatic steatosis model was developed *via* loading of palmitate & oleic acid. The steatosis model was characterized using biomarker and functional analysis. Finally, we tested telmisartan, a drug known to improve the overall histology of NASH patients [20] in our *in vitro* model. The HLC carry some of the functional characteristics and gene expression profile of primary hepatocytes and are amenable for development of an *in vitro* steatosis model using lipid loading method.

MATERIALS AND METHODS

Ethics statement

Normal human dermal fibroblasts, neonatal (HDFn) were used for reprogramming to generate iPSCs. The cells were purchased from Life Technologies (Gibco) and consent statement from the provider is: "The cells in this lot were derived from tissue obtained from accredited institutions. Consent was obtained by these institutions from the donor or the donor's legal next of kin, for use of the tissue and its derivatives for research purposes." The human Episomal Human iPSC line (A18944) was used as control lines to confirm iPSCs derived from HDFn cells. This study (proposal Cat. # JBL/IBSC/006) was approved by Institute Biosafety Committee (IBSC), Jubilant Biosys Ltd., Bengaluru, India.

Generation and characterization of undifferentiated iPSCs from fibroblasts

The detailed procedures on the generation and characterization of undifferentiated iPSCs from fibroblasts is given in the Supplementary information.

Differentiation of iPSCs into hepatocytes like cells

Prior to seeding MEF cells 0.3×10^6 cells/well, 6 well plates were coated with attachment factor for 1 h and incubated at 37°C for 24 h. iPSCs were incubated with 0.5 mM EDTA in DPBS at room temperature, monitored until the cells detach and neutralized with E8 medium. The cell suspension at a density of 0.3×10^5 cells/well were transferred to the MEF coated plate and incubate at 37°C with 5% CO_2. [Days 1–2]: The culture medium was replaced with RPMI that has been pre-warmed to 37°C and supplemented with 2% B27 (without insulin), 100 ng/ml activin A, 10 ng/ml BMP4, and 20 ng/ml FGF2. The cells were cultured with daily medium changes for 2 d at 37°C. [Days 3–5]: The culture medium was changed to RPMI/2% B27 (without insulin) containing 100 ng/ml activin A and the cells were continued in culture with daily media changes for an additional 3 d at 37°C. [Days 6–10]: Hepatic differentiation was induced by changing the medium to RPMI/2% B27 (with insulin) supplemented with 20 ng/ml BMP4 and 10 ng/ml FGF2 and the cells were continued to be cultured with daily medium changes for a total of 5 d at 37°C. [Days 11–15]: The hepatic progenitor cells were cultured for 5 d in RPMI/2% B27 (with insulin) supplemented with 20 ng/ml HGF, and with daily medium changes at 37°C. [Days 16–20]: The medium was replaced with hepatocyte maturation medium (DMEM, 10%FBS) supplemented with 1× nonessential amino acids, and 10^{-7} M dexamethasone. The medium was supplemented with 20 ng/ml of oncostatin M and the cells were cultured for at least 5 d with daily medium changes at 37°C. The cells were continued to be maintained in the same media till day 35.

Characterization of hepatocyte like cells using Q-PCR

The HLC were characterized using Q-PCR analysis of some of the critical liver genes. The list included the cytochrome p450 enzymes (Cyp3A4, Cyp1A2, Cyp2C9, Cyp2c19, Cyp2B6) and the transporters (Glut2, Glut4, FATP-4). Total RNA was extracted from iPSCs using Trizol™ (Invitrogen) according to the manufacturer's instructions. The concentration of RNA was determined spectrophotometrically and the integrity of the RNA was assessed using a NanoDrop® ND-1000 Spectrophotometer. Five hundred nanogram of total RNA was used to prepare the cDNA samples. iScript™ cDNA Synthesis Kit (BioRad: 1708890) was used for the cDNA synthesis and subsequent to synthesis, aliquots of cDNA were stored at −20°C. qPCR was performed using iTaq™ Universal SYBR® Green Supermix (BioRad: 172-5120) as per manufacturer's instructions. The cycling conditions included a denaturing step at 95°C for 2 min and 40 cycles of 95°C for 30 s, 60°C for

30 s and 45 s at 72°C. Bio-Rad® CFX96™ was used to determine the expression level of selected target genes. Human β-Actin was used as control for normalization. Primer sequences are detailed in **Table 1**.

Functional characterization of hepatocyte like cells using glucose uptake assay and its comparison against primary human hepatocytes

Radiometric glucose uptake assay was performed using 3H-2-deoxy-D-glucose. Human primary hepatocytes (Caucasian male, aged 71) were procured from Thermofisher scientific (Cat. # HMCPMS, Lot # HU1955). The cells were stored at vapor phase of liquid nitrogen. Post thawing, cell viability was found to be 91%. Cells were cultured in DMEM + 10% FBS + 1× antibiotic. 0.2% w/v gelatin solution was prepared in sterile tissue culture grade H_2O. Multi well plates were coated and maintained at 37°C prior to assay initiation. Fifty thousand cells (primary human hepatocytes or HLC) per well were seeded in a multi well plate (DMEM + 10% FBS + 1× antibiotic) and incubated overnight (O/N) at 37°C with 5% CO_2. After O/N incubation, the cells were washed with PBS twice and starved for 2 h in serum free DMEM media. After 2 h, the media was removed; cells were washed twice in PBS. Five hundred microlitre KRPH buffer containing 1% fatty acid free BSA was added and incubated for 30 min. Post 30 min, the spent buffer was removed; cells were washed once again with PBS. Cells were stimulated with and without insulin (10 µg/ml) in KRPH buffer containing 0.05% fatty acid free BSA. Stimulation buffer was removed and replaced with 300 µl KRPH buffer containing 0.5 µCi/ml of 3H-2-deoxy-D-glucose (hot) and 2.5 µM of 2-deoxy-D-glucose (cold) and incubated for 10 min. After 10 min, the radioisotope containing buffer was removed, and cells were washed thrice with ice cold PBS to attenuate glucose uptake. The reaction was stopped by the addition of 150 µl of 0.2 N NaOH. The cells containing plate was incubated in orbital shaker for 3 h at room temperature to homogenate samples. One hundred and fifty microliters of the sample was transferred into a pico-plate, 150 µl of microscint-40, was added, and incubated again for 3 h to O/N and counts read using Trilux micro beta plate reader. Counts per minute (CPM) per well was recorded and analyzed. Analysis was done using Dunnett's multiple comparison test.

Functional characterization of hepatocyte like cells using drug metabolism and clearance assays and its comparison against primary human hepatocytes

Hepatocytes reflect the heterogeneity of cytochrome P450 gene expression, and it is a suitable model for drug metabolism studies. The metabolic stability of chemical entities can be investigated by incubating the drugs with primary human hepatocytes. We undertook the metabolic stability analysis of the known chemical entity, naloxone in primary human hepatocytes and in HLC. The idea of this experiment was to study the functional drug metabolizing ability of the HLC as compared to the primary human hepatocytes. Human Primary hepatocytes (Caucasian male, aged 71) were procured from Thermofisher scientific (Cat. # HMCPMS, Lot # HU1955). The cells were stored at vapor phase of liquid nitrogen. Post thawing, cell viability was found to be 91%. Cells were cultured in DMEM + 10% FBS + 1× antibiotic.

Table 1. Primers for characterization of hepatocytes.

Gene	Forward primer	Reverse primer
CYP3A4	GTGGGGCCTTTGTCAGAACT	TGGGCAAAGTCACAGTGGAT
FATP-4	AGGGGCCAATAAACTCTGCC	CTGCGGCATCTTACCTGCTA
CYP1A2	CTTCGCTACCTGCCTAACCC	GTCCCGGACACTGTTCTTGT
CYP2C9	GGTGAGTGTTAGAGTTACTTGAGGA	TAGAGGTTAGAGCTGCCCCT
CYP2C19	CCAGGGTTTAATCTTTTTCAGCTTC	AAGACAGGAAGAGTGGTGAACA
CYP2B6	CTTCACGGTACACCTGGGAC	CGACCATGGCGATTTTTCCC
GLUT4	TCTCCAACTGGACGAGCAAC	CAGCAGGAGGACCGCAAATA
GLUT2	CACAATCTCATACTCAATGAACCCA	GGCCTGAAATTAGCCCTTCCAA
Actin	CATTCCAAATATGAGATGCGTTGT	TGTGGACTTGGGAGAGGACT
18sRNA	GTAACCCGTTGAACCCCATT	CCATCCAATCGGTAGTAGCG

The HLC's were re-plated after differentiation to perform the clearance assay. The viability of HLC was ~70% after re-plating. The primary human hepatocytes or the HLC were incubated with 3 µM of naloxone at 37°C for different (0, 30, 60, 120 and 180 min) time periods. Samples were collected at the end of each time points, and the reaction was terminated with a quenching solution. The samples were centrifuged and the supernatants were analyzed using LC-MS/MS. The clearance of naloxone over time was determined *via* changes of concentrations (chromatographic peak area ratios) relative to the control (time 0 samples) over a period of 180 min. The data was normalized to the cell number. Half-life of the drug was calculated based on the concentration data. Calculations for hepatic clearance were carried out with "well-stirred" model.

Development of *in vitro* steatosis model

iPSCs derived HLC were seeded onto a 96-well plate in Hepatocyte culture medium at 37°C, 5% CO_2 and allowed to grow to 75% confluence. iPSC derived hepatocytes were allowed to attach for at least 24 h before initiating lipid overloading. Lipid over-loading was carried out for 24 h in the presence of oleate: palmitate in the ratio of 3:1 (75 µM:25 µM). The lipid mixture in the absence and presence of test compound was incubated for 48h at 37°C and 5% CO_2. The culture medium was gently removed on the following day and 100 µl of Nile red working solution was added to each well and incubated for 60 min at 37°C. After incubation, the Nile red solution was washed with 100 µl of PBS to remove the debris. The lipid droplets stained with Nile red was quantified using fluorescence plate reader with excitation at

488 nm and emission at 550 nm.

Statistics

GraphPad Prism 5.04 (GraphPad software Inc. La Jolla, CA, USA) was used for statistical analysis and graphical presentation. Dunnett's multiple comparison test was used to study the statistical significance.

RESULTS

The details regarding the generation and characterization of iPSCs are provided in the Supplementary information. Briefly, the iPSCs were generated from fibroblasts and characterized using a variety of techniques including staining, qPCR analysis and western blot analysis with pluripotency markers; embryoid body (EB) formation and differentiation into germ layers; and karyotyping (**Fig. S1**-S7; **Table S1** and **S2**). The various analyses confirmed the pluripotency and genome stability of the iPSC colonies 4 and 8. iPSC colony number 4 was used in all further experiments.

Differentiation of iPSCs to hepatocytes like cells

The iPSCs were expanded and differentiated into HLC. The iPSCs were exposed to differentiation media supplemented with factors N2B27 and beg that are required for the transition to definitive endoderm to specific hepatocyte endoderm (day 10) and finally to HLC (Day 20+). The HLC displayed the typical morphology seen with hepatocytes that are polygonal in shape (**Fig. 1**).

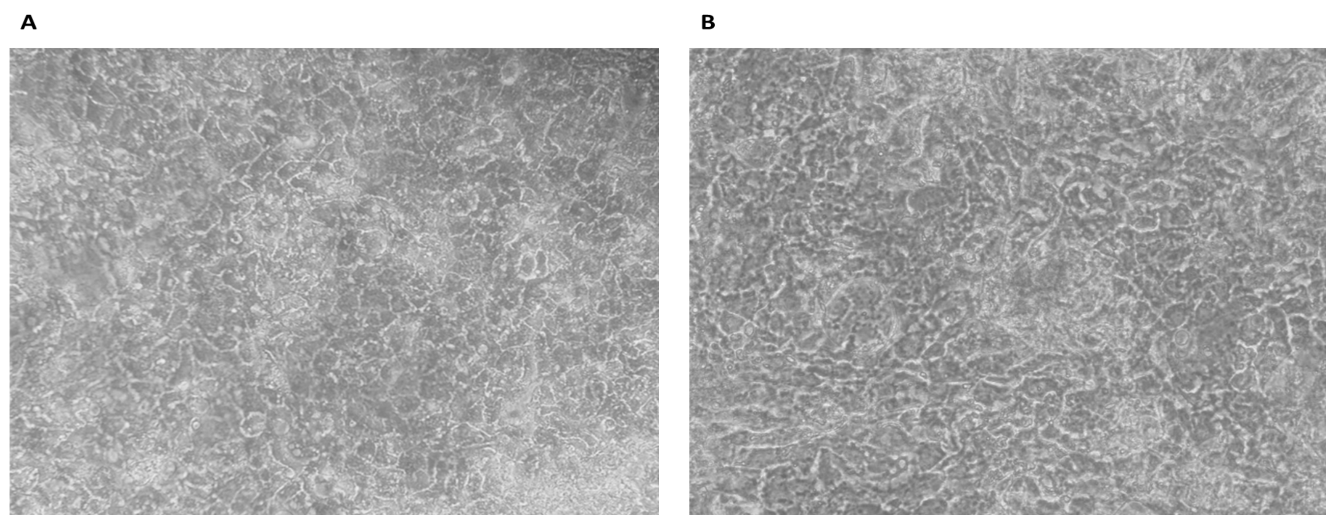

Figure 1. Differentiation of iPSCs to hepatocyte like cells. A. Specific hepatic endoderm (day 10). **B.** HLC (day 20). iPSCs were directed to differentiate into HLCs. The differentiated cells displayed the typical morphology seen with hepatocytes that are polygonal in shape. These cells were used for further characterization using gene expression studies and functional analysis.

Characterization of hepatocytes like cells using gene markers specific for hepatocytes

Q-PCR analysis of some of the hepatocyte specific genes, cytochrome p450 enzymes and transporters genes [21-24] were studied at the end of differentiation period to confirm the differentiation to HLC. The list of investigated hepatocyte specific genes include Fibrinogen alpha chain (FGA), fibrinogen gamma chain (FGG), alpha-fetoprotein (AFP), albumin (ALB), and SOX1. These genes are highly expressed in the HLC (**Fig. S8**). The list of cytochrome p450 enzymes analyzed include Cyp3A4, Cyp1A2, Cyp2C9, Cyp2c19, Cyp2B6 and the transporters include Glut2, Glut4, FATP-4 (**Fig. 2**). The hepatocyte specific gene expression of cytochrome p450 enzymes and transporters in HLC were studied in comparison with primary human hepatocytes. The gene expression in primary human hepatocytes were normalized to a scale of 1 (one), and the relative gene expression in iPSC derived HLC were plotted (**Fig. 2**).

The data suggests that the genes expressed in primary human hepatocytes were also expressed in iPSC derived HLC, albeit to different levels. In order to further ascertain the functionality of iPSC derived HLC, the cells were subjected to glucose uptake experiments in comparison with primary human hepatocytes (**Fig. 3**). Insulin dependent glucose uptake at 100 µg/ml was similar in both the cell types (~30 CPM over the control) lending support to the functionality of the HLC similar to primary human hepatocytes (**Fig. 3**).

Additionally, we carried out studies to assess the potential of HLC to metabolize the known drugs, similar to primary hepatocytes. This experiment was carried out in comparison with primary human hepatocytes (**Table 2**). Drugs are known to be metabolized by the cytochrome P450 (CYP450) enzyme system in hepatocytes and historical data is available for some standard drugs including Naloxone [25,26]. In our experiment, the calculated half-life of naloxone was 139 min in primary human hepatocytes as compared to 368 min in HLC (**Table 2**). Calculations for hepatic clearance were carried out with "well-stirred" model [25]. The ratio of the hepatic clearance of Naloxone to the hepatic blood flow (scaled CL fraction blood flow, **Table 1**) was found to be 0.82 for primary human hepatocytes and 0.39 for HLC. HLC were able to metabolize Naloxone, albeit to a lower level as compared to human primary hepatocytes. This could be explained by the relative lower expression levels of the CYP450 family of enzymes (including Cyp3A4, **Fig. 2**) in HLC.

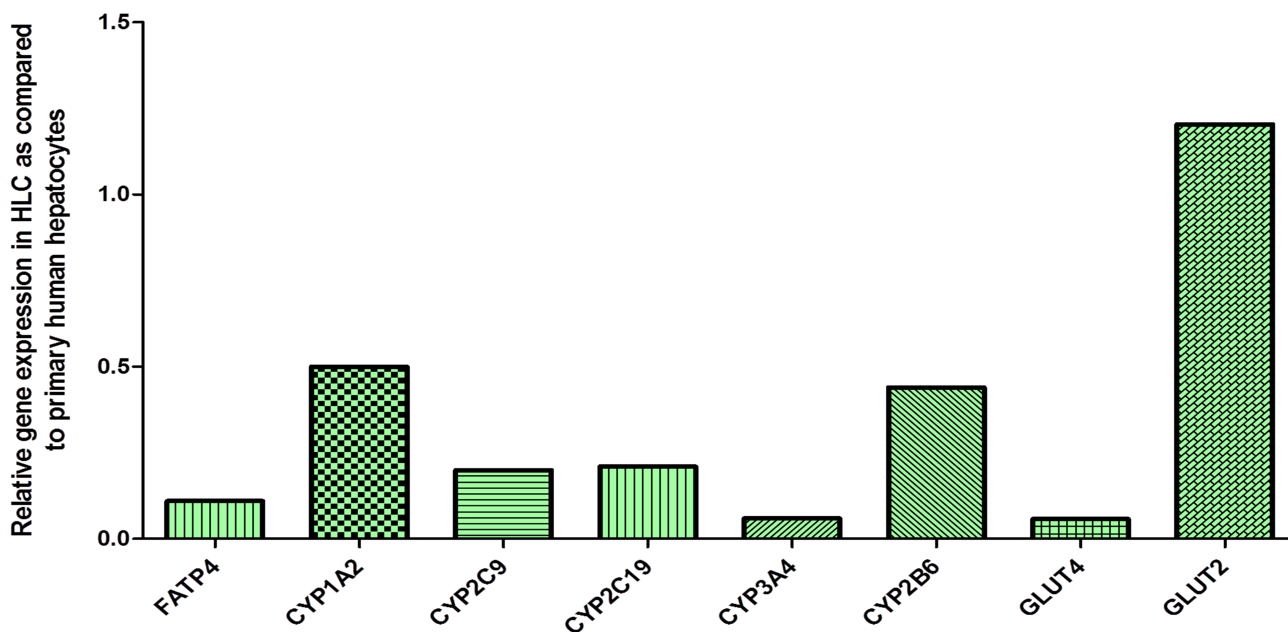

Figure 2. Characterization of hepatocyte specific gene expression profile in HLC derived from iPSCs. QPCR analyses of cytochrome p450 and transporter genes were performed using RNA samples from primary human hepatocyte and HLC. The gene expression levels in primary human hepatocytes were normalized to a scale of 1 (one) and the comparative expression profile of the respective genes in HLC is shown. The genes from cytochrome p450 family are expressed in lower levels in the HLC as compared to hepatocytes and GLUT2 is expressed in higher levels as compared to primary hepatocytes.

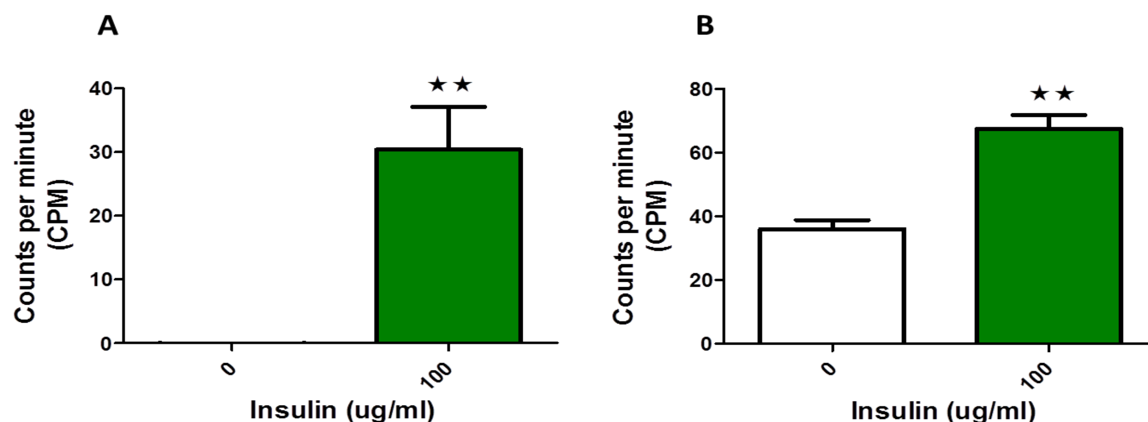

Figure 3. Radiometric glucose uptake assay using primary human hepatocytes (A) and iPSC derived HLC (B). Glucose uptake was undetectable in the control primary hepatocytes whereas the iPSC derived HLC had detectable levels of glucose uptake even in the control cells. Insulin dependent glucose uptake at 100 µg/ml was similar in both primary human hepatocytes and iPSC derived HLC (~30 CPM over the control). Analysis was done using Dunnett's multiple comparison test. Compared with control, $^{**}P < 0.01$.

Table 2. Assessment of the ability of iPSC derived hepatocyte like cells to metabolize naloxone.

	Half-life (min)	ln^2 (min^{-1})	Cells (million)	hClint (µl/min/10^6 cells)	CL hepatic (ml/min/kg)	Scaled CL fraction blood flow
Primary human hepatocytes	139	0.69	0.15	33.2	17.2	0.82
Hepatocyte like cells	368.3	0.69	0.4	4.7	8.2	0.39

In essence, the iPSC derived, HLCs were characterized using their morphology, gene expression analysis (**Table S3**), glucose uptake assays and their ability to metabolize the known drug (naloxone). Subsequently, we proceeded with the development of *in vitro* steatosis model using these cells.

Development of *in vitro* steatosis model and its validation using telmisartan

We used the lipid over-loading method in the presence of oleate: palmitate for the development of *in vitro* steatosis model. Treatment of iPSC derived hepatocytes like cells with lipid mixture (oleic acid: palmitate) in the ratio of 3:1 (75 μM:25 μM) resulted in a significant increase in lipid load (**Fig. 4**, please refer red bar *vs*. blue bar). The concentration and the ratio of oleic acid: palmitate used in this experiment was chosen based on prior information available in our lab using HepG2 cells (data not shown).

The lipid loaded iPSC derived hepatocyte model (steatosis model) was further tested using telmisartan. Telmisartan is an angiotensin receptor blocker (ARB) that functions by modulating PPAR-γ activity that leads to decreased hepatic fat accumulation. Telmisartan also blocks angiotensin II receptor resulting in the inhibition of hepatic stellate cell activation and suppression of hepatic fibrogenesis [27]. Due to the above properties, telmisartan was selected and tested as a protecting agent in the steatosis model. Telmisartan (100 μM, 30 μM and 10 μM) was added to the iPSC derived hepatocytes like cells and incubated for 48 h along with the lipid mixture. There was a significant reduction (~80%) in lipid droplet formation even in the presence of the lowest tested concentration of telmisartan as compared to the disease state (steatosis), measured by Nile red fluorescence (**Fig. 4**).

Lastly, some of the gene markers associated with NAFLD such as aquaporin 1 (AQP1), collagen type 3, alpha 1 chain (COL3A1), collagen type 1 alpha 1 chain (COL1A1), and fatty acid synthase (FAS) were studied for their expression levels to validate the steatosis disease model and its reversal by Telmisartan. There was an increase in the expression of these genes upon lipid loading, and upon treatment with telmisartan, the gene expression was reduced (**Fig. 5**).

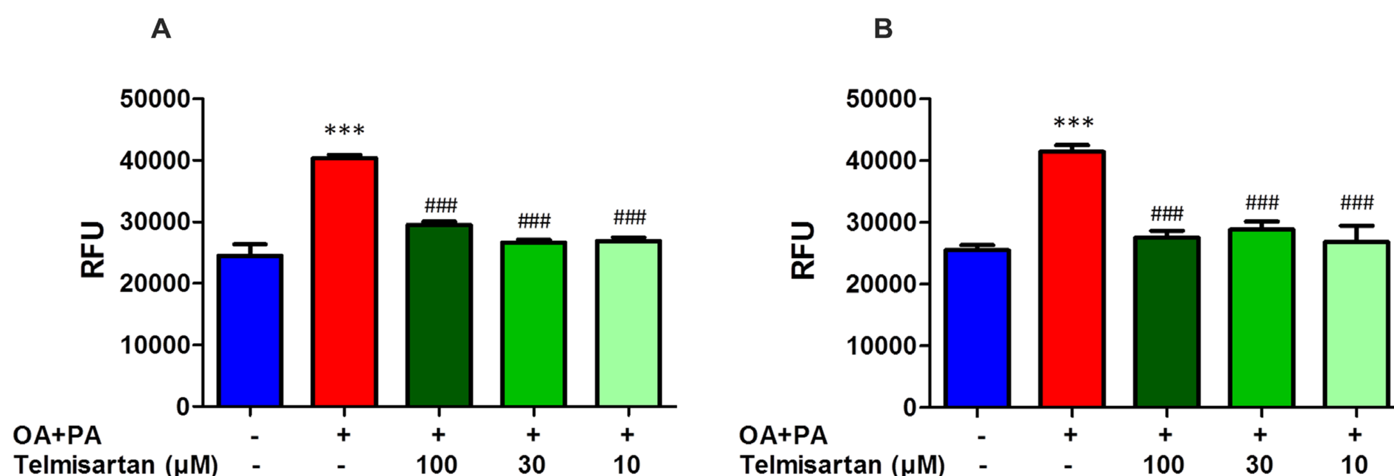

Figure 4. *In vitro* model of hepatic steatosis. Treatment of iPSC derived hepatocytes like cells with lipid mixture [oleic acid (OA) + palmitic acid (PA)] in the ratio of 3:1 resulted in a significant increase in lipid load. The cells were treated with telmisartan (100 μM, 30 μM, 10 μM) for 48 h. There was a significant reduction (~80%) in lipid droplet formation as compared to the disease state (steatosis) in the presence of telmisartan as measured by Nile red fluorescence. Two independent experiments (*N* = 1, **A**; *N* = 2, **B**) were conducted with similar outcome. Analysis was done using Dunnett's multiple comparison test. Compared with control, ****P* < 0.001, and when compared with OA + PA group, $^{###}$*P* < 0.001.

DISCUSSION

The difficulties faced during procurement and handling of rodent and human hepatocytes are accompanied with complications [28]. The immortalized cell lines (HepG2) which continue to be an important source for hepatocyte related studies, doesn't provide an appealing solution to mimic *in vivo* physiological conditions [29]. Although the transformed cell lines can serve as surrogate models, the gene array results from these cells suggests that the profiles are completely different from that of primary hepatocytes. Further, these cell lines have little or no hepatic metabolizing activities, which make them irrelevant for drug discovery [30].

Overall, the limitations with human primary hepatocyte based steatosis model and the intrinsic differences between the steatosis models from hepatocytes of rodent species (rat and mouse) make the human iPSC derived HLC based steatosis model an attractive tool for pharmaceutical research. Human iPSC derived HLC can be a good alternative as they have high resemblance to hepatocytes.

In the current study, we have generated the iPSCs from human fibroblasts, and demonstrated that the iPSCs can be differentiated into hepatocytes like cells, while retaining the morphology, gene expression profile, and functionality of hepatocytes. These highly specific, differentiated cells have the morphological and functional features of the primary hepatocytes. One of the most distinctive features of hepatocytes is the process of fatty acid metabolism [44]. The glucose uptake data and the drug metabolism data provided in this study with the iPSC derived HLC suggests that these cells are metabolically active and resemble the primary hepatocytes.

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Subsequently, we used these differentiated HLC to develop an *in vitro* experimental model of Hepatic Steatosis *via* loading of palmitate and oleic acid. The disease model was characterized using biomarker profiling, functional analysis and was validated using telmisartan. The accumulation of lipid droplets is the hallmark of steatosis and one of the key pathology in the progression of NAFLD, to NASH and ultimately to non-cancerous inflammation driven cirrhosis [28]. Several genes have been identified as biomarkers of NALFD disease progression [29]. We have selected a few of these genes (AQP1, FAS, COL3A1, and COL1A1) and tested them in our model. The data suggests that the expression pattern of these genes are in line with expression pattern seen with NAFLD [30]. Further, we have also tested telmisartan, a drug that is known to mediate improvement in hepatic inflammation and fibrosis in hypertensive patients with NAFLD or NASH in our model. The data from telmisartan treatment suggests that telmisartan was able to inhibit the expression of these genes associated with NAFLD.

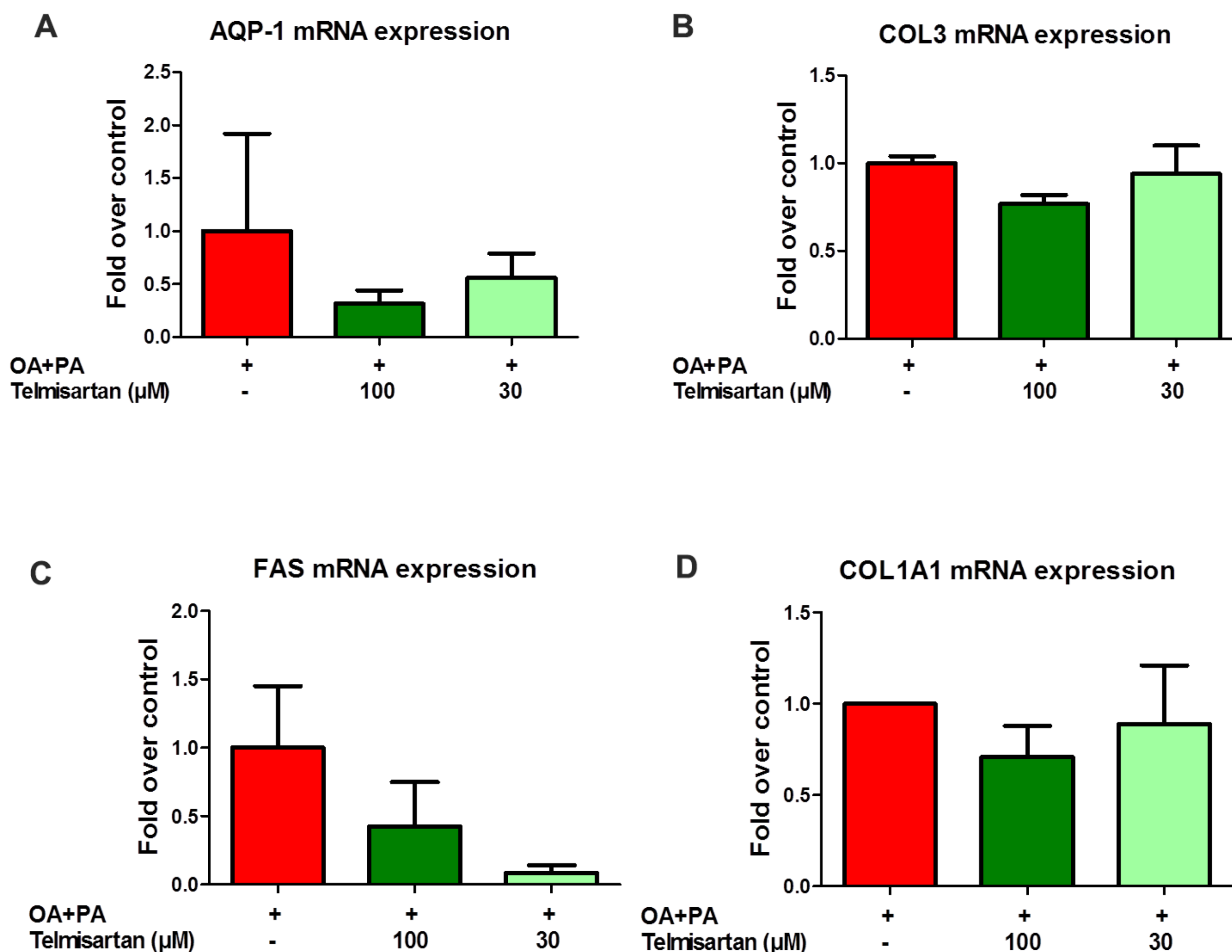

Figure 5. Gene expression analysis of genes associated with NAFLD and its modulation by telmisartan treatment. A. AOP-1 mRNA expression. **B.** Col3 mRNA expression. **C.** FAS mRNA expression. **D.** COL1A1 mRNA expression. The expression of the respective genes upon lipid loading [oleic acid (OA) + palmitic acid (PA)] was normalized against the control and set as 1 (one). There was an increase in the expression of these genes upon lipid loading which were reduced following the treatment with telmisartan (30 & 100 μM).

In conclusion, our data suggests that HLC carry some of the functional characteristics of primary hepatocytes and are amenable for development of an *in vitro* hepatic steatosis model that mimics the development, progression and pathogenesis of liver disease. The *in vitro* experimental model of hepatic steatosis was further characterized using biomarker analysis and validated using telmisartan. With some refinement and additional validation, our *in vitro* steatosis model system may be useful for profiling small molecule inhibitors, and studying the mechanism of action of new drugs and hepatotoxicity.

Data availability

The data used to support the findings of this study are available from the authors upon request.

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Supplementary information

Figure S1. Scheme on generation of iPSC colonies from fibroblasts cells.

Figure S2. Live cell imaging and staining of iPSCs.

Figure S3. Morphology of iPSCs was retained upon freeze/thawing.

Figure S4. Gene expression analysis of the pluripotency markers from the iPSC colonies using RT-PCR.

Figure S5. Characterization of iPSC lines via karyotyping.

Figure S6. Western blot analysis for pluripotency marker.

Figure S7. Embryoid body formation and differentiation into germ layers.

Figure S8. Gene expression analysis of the hepatic markers from HLC using Q-PCR.

Table S1. Primers for characterization of iPSCs.

Table S2. Pluripotency marker analysis using RT-PCR.

Table S3. Primers for the characterization of HLC.

Supplementary information of this article can be found online at http://www.jbmethods.org/jbm/rt/suppFiles/330.

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