Journal of Visualized Experiments

Protocols to induce and characterize vesicular steatosis in differentiated HepaRG cells.

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE59843R2
Full Title:	Protocols to induce and characterize vesicular steatosis in differentiated HepaRG cells.
Keywords:	HepaRG cells, liver, differentiation, vesicular steatosis, NAFLD, lipid droplets, CARS microscopy
Corresponding Author:	Natalia Pediconi Istituto Italiano di Tecnologia ROME, RM ITALY
Corresponding Author's Institution:	Istituto Italiano di Tecnologia
Corresponding Author E-Mail:	natalia.pediconi@iit.it
Order of Authors:	Silvia Di Cocco
	Laura Belloni
	Abigail D. G. Nunn
	Debora Salerno
	Silvia Piconese
	Massimo Levrero
	Natalia Pediconi
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Rome, Italy

TITLE:

Inducing and Characterizing Vesicular Steatosis in Differentiated HepaRG Cells

AUTHORS AND AFFILIATIONS

Silvia Di Cocco[1,2], Laura Belloni[3], Abigail D. G. Nunn[3], Debora Salerno[3], Silvia Piconese[2,4], Massimo Levrero[2,3,5,6] and Natalia Pediconi[1,3]

[1] Department of Molecular Medicine, Sapienza University, Rome, Italy
[2] Department of Internal Medicine - DMISM, Sapienza University, Rome, Italy
[3] Center for Life Nano Science@Sapienza, Istituto Italiano di Tecnologia, Rome, Italy
[4] Pasteur Institute Italy-Fondazione Cenci Bolognetti, Rome, Italy
[5] INSERM U1052-Cancer Research Center of Lyon, France
[6] Department of Hepatology, Croix Rousse Hospital, Hospices Civils de Lyon, France

Corresponding Author:

Natalia Pediconi
natalia.pediconi@iit.it

Email Addresses of Co-authors:

Silvia Di Cocco (silvia.dicocco@gmail.com)
Laura Belloni (laura.belloni@iit.it)
Abigail D. G. Nunn (abi.nunn@gmail.com)
Debora Salerno (debora.salerno@iit.it)
Silvia Piconese (silvia.piconese@uniroma1.it)
Massimo Levrero (massimo.levrero@gmail.com)

KEYWORDS:

HepaRG, liver, vesicular steatosis, NAFLD, lipid droplets, CARS microscopy

SUMMARY

In this study, we describe a detailed protocol for inducing liver vesicular steatosis in differentiated HepaRG cells with the fatty acid salt sodium oleate and employ methods for detection and quantification of lipid accumulation, including coherent anti-Stokes Raman scattering (CARS) microscopy, cytofluorimetric analysis, Oil red O staining, and qPCR.

ABSTRACT

Hepatic steatosis represents a metabolic dysfunction that results from an accumulation of triglyceride-containing lipid droplets in hepatocytes. Excessive fat accumulation leads to non-alcoholic fatty liver disease (NAFLD), which is potentially reversible and may evolve into non-alcoholic steatohepatitis (NASH) and eventually cirrhosis and hepatocellular carcinoma (HCC). The molecular mechanisms linking lipid accumulation in hepatocytes with the progression to NASH, irreversible liver damage, fibrosis, cirrhosis, and even HCC still remains unclear. To this end, several in vitro and in vivo models have been developed to elucidate the pathological processes that cause NAFLD. In the present study, we describe a cellular model for the induction of liver vesicular steatosis that consists of DMSO-differentiated human hepatic HepaRG cells treated with the fatty acid salt sodium oleate. Indeed, sodium oleate-treated HepaRG cells accumulate lipid droplets in the cytoplasm and show typical features of steatosis. This in vitro human model represents a valuable alternative to in vivo mice models

as well as to the primary human hepatocytes. We also present a comparison of several methods for the quantification and evaluation of fat accumulation in HepaRG cells, including Oil Red O staining, cytofluorimetric Bodipy measurement, metabolic gene expression analysis by qPCR, and coherent anti-Stokes Raman scattering (CARS) microscopy. CARS imaging combines the chemical specificity of Raman spectroscopy, a chemical analysis technique well-known in materials science applications, with the benefits of high-speed, high-resolution non-linear optical microscopies to allow precise quantification of lipid accumulation and lipid droplet dynamics. The establishment of an efficient in vitro model for the induction of vesicular steatosis, alongside an accurate method for the quantification and characterization of lipid accumulation, could lead to the development of early stage diagnosis of NAFLD via the identification of molecular markers, and to the generation of new treatment strategies.

INTRODUCTION

Hepatic steatosis is defined as intrahepatic fat accumulation, within triglyceride-containing lipid droplets, of at least 5% of liver weight. Prolonged hepatic lipid storage is a potentially reversible process, however, it can lead to liver metabolic dysfunction, inflammation and advanced forms of nonalcoholic fatty liver disease (NAFLD), the predominant cause of chronic liver disease in many parts of the world[1,2]. NAFLD is a multifactorial disease that may evolve to the more aggressive non-alcoholic steatohepatitis (NASH), which in turn can progress to cirrhosis and, in a small percentage of patients, to hepatocellular carcinoma (HCC)[1,3]. No approved therapy is currently available as a specific treatment for NAFLD and the combination of diet and lifestyle modifications remains the pillar of NAFLD and NASH management[4-6].

The molecular mechanisms leading to the development of hepatic steatosis in the pathogenesis of NAFLD still remain to be elucidated[7]. In this context, mouse models have been developed to study human steatosis disease progression. A myriad of different models exists, and each one has its advantages and disadvantages, including genetic, nutritional and chemically induced models combining different approaches. Genetically modified (transgenic or knockout) mice spontaneously develop liver disease. However, it should be noted that these mutations are very rare in humans and deletion or over-expression of a single gene (e.g., ob/ob mouse) may not mimic the etiology of the multifactorial human disease at the molecular level[8,9]. Likewise, the disease acquired by mice after dietary or pharmacological manipulation may not mimic the effects of human diets associated with development of NAFLD in man[8]. Animal models have, however, facilitated developments in the understanding of NAFLD and this approach is currently the most frequently used strategy in laboratory research. Nevertheless, the replication in humans of results obtained in animal models has repeatedly failed, causing poor translation into the clinic[10].

Therefore, in vitro models of NAFLD may play a fundamental role in elucidating the molecular mechanisms of NAFLD progression, and they represent a valuable tool to screen a large number of compounds. Primary cell cultures, immortalized cell lines and liver biopsies have been extensively used for research purposes[11]. Primary human hepatocytes closely resemble human clinical conditions, but there is a limited number of donors, and primary cell cultures show poor reproducibility due to the variability of the cells. These observations, together with ethical and logistic issues, have resulted in the use of human primary hepatocytes being limited[12]. Thus, hepatic cell lines represent a convenient alternative, having several essential

advantages over primary culture, as hepatic cell lines grow steadily, have an almost unlimited life-span, and have a stable phenotype. Moreover, cell lines are easily accessible and the culture conditions of hepatic cell lines are simpler than those of primary hepatocytes and are standardized among different laboratories.

Here, we describe in detail an in vitro cell-based model of liver vesicular steatosis, represented by hepatic differentiated HepaRG cells treated with the fatty acid sodium oleate. The HepaRG cell line was established from a female patient affected by hepatitis C infection and an Edmondson grade I well-differentiated liver tumor[14]. The HepaRG cell line is a human bipotent progenitor cell line capable of differentiating upon exposure to 2% dimethyl sulfoxide (DMSO) toward two different cell phenotypes: biliary-like and hepatocyte-like cells. Differentiated HepaRG cells (dHepaRG) share some features and properties with adult hepatocytes and possess the ability to stably express liver-specific genes such as Albumin, AldolaseB, Cytochrome P450 2E1 (CYP2E1), and Cytochrome P450 3A4 (CYP3A4)[13] (step 3). Treatment of dHepaRG cells with the fatty acid salt sodium oleate (250 µM) for 5 days lead to the generation of cytoplasmic lipid droplets, mimicking the effects of fatty liver[14,15,17,18] (step 4). Accumulation of lipid droplets can be easily detected by Oil Red O staining (step 5), a lysochrome fat-soluble dye that stains neutral triglycerides and lipids red-orange. To efficiently quantify lipids in fatty dHepaRG, here we illustrate cytofluorimetric analysis after staining with 4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-s-indacene (Bodipy 505/515) (step 6), a lipophilic fluorescent probe that localizes to intracellular lipid bodies and has been used to label lipid droplets[19]. Moreover, here we show how to evaluate steatosis by quantitative polymerase chain reaction (qPCR) (step 7) gene expression deregulation of several metabolic genes in dHepaRG cells. To further characterize and quantify the accumulation of lipid droplets after sodium oleate treatment, we performed coherent anti-Stokes Raman scattering (CARS) microscopy (step 8), an innovative technique that enables the visualization and quantification of lipid droplets without labeling[20,21].

PROTOCOL

1. Preparation of culture media and reagents

1.1. Proliferating medium: supplement William's E medium with GlutaMAX, 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, 5 µg/mL insulin and 0.5 µM hydrocortisone hemisuccinate.

1.2. Differentiation medium: supplement William's E medium with GlutaMAX, 10% FBS, 1% penicillin/streptomycin, 5 µg/mL insulin, 50 µM hydrocortisone hemisuccinate and 2% DMSO.

1.3. Freezing medium: supplement proliferating medium with 10% DMSO.

1.4. Sodium oleate: dissolve in 99% methanol at a 100 mM concentration, stir O/N, and store at -20 °C.

1.5. Oil Red O: prepare a stock solution. Weigh 0.35 g of Oil Red O and dissolve in 100 mL of isopropanol. Stir O/N, filter (0.2 µm), and store at RT. Prepare a working solution: mix 6 mL

of Oil Red O Stock solution with 4 mL of ddH_2O. Let sit at room temperature (RT) for 20 min, and then filter with a 0.2 µm filter. Proper filtration is highly recommended for successful staining to avoid background.

1.6. Bodipy (505/515): dissolve Bodipy (505/515) dye in DMSO at a 100 µM stock concentration, store at -20 °C in dark, and use at a final 100 nM concentration.

1.7. Acquire transparent glass-bottomed dishes for CARS experiments.

2. Thawing, amplification and cryopreservation of HepaRG cells

2.1. Thaw nitrogen cryopreserved HepaRG cells by immersing a batch in a 37 °C bath until defrosted. Select a low passage batch (<20). HepaRG cells are commercially available.

2.2. Rapidly transfer the cells into a 15 mL tube containing 10 mL of proliferating medium and centrifuge for 5 min (200 × *g*, 4 °C).

2.3. Discard the supernatant and resuspend the cells with 5 mL of proliferating medium.

2.4. Count the cells and dilute in order to plate 2.5×10^4 cells/cm^2.

2.5. Renew the medium every 2 or 3 days. Cells proliferate with a doubling time of around 24 h.

2.6. Detach HepaRG cells when at 80% confluency by 3-5 min incubation with trypsin solution 0.05%. Collect the cells in proliferating medium and centrifuge for 5 min (200 × *g*, 4 °C).

2.7. Discard the supernatant and resuspend the cells with 5 mL of proliferating medium.

2.8. Count the cells and dilute in order to plate 2.5×10^4 cells/cm^2.

2.9. At this stage, amplify the cells by repeating steps 2.5-2.8 in order to reach an appropriate number of cells to start experiments, or cryopreserve as in step 2.10.

2.10. To cryopreserve HepaRG cells, detach the cells 24 h after plating by 3-5 min incubation with trypsin solution 0.05%. Collect the cells in proliferating medium and centrifuge for 5 min (200 × *g*, 4 °C). Discard the supernatant, resuspend the cells in 1 mL/batch of freezing medium, 1.5×10^6 cells/batch and cryopreserve the batches in liquid nitrogen.

3. Differentiation of HepaRG cells (Day 0–21) (Figure 1A)

3.1. Day 0. Seed HepaRG cells at low density (2.5×10^4 cells/cm^2) into culture treated dishes with appropriate volume of proliferation medium (**Figure 1B**). Two dishes need to be plated for each assay (Oil Red O staining, FACS analysis, qPCR): one for control cells and one for oleate-treated cells. If performing CARS measurements (step 8), seed the cells in parallel into transparent glass-bottomed dishes. As a control, harvest one untreated dish (proliferating

cells Day 0) to perform gene expression analysis of differentiation marker genes (see step 3.6).

3.2. Day 2 and Day 4. Change the medium with appropriate volume of proliferation medium and let the cells grow until confluence.

3.3. Day 7. Cells must be 100% confluent (**Figure 1C**). Wash the cells once with 1x phosphate-buffered saline (PBS). Remove 1x PBS and add an appropriate volume of differentiation medium.

3.4. Day 8, 9, 12, 15, 18. Wash the cells once with 1x PBS. Remove 1x PBS and add an appropriate volume of differentiation medium.

3.5. Day 21. Observe HepaRG cells under a microscope and ensure that the cells are confluent differentiated cultures (**Figure 1D**).

3.6. As a control, harvest one control dish (differentiated cells Day 21) to perform gene expression analysis (step 7) of differentiation marker genes (**Figure 1E**).

3.7. At Day 21, cryopreserve HepaRG cells in liquid-nitrogen.

4. Induction of vesicular steatosis (Day 21–26)

4.1. Day 21. Dilute sodium oleate (100 mM) with an appropriate volume of complete differentiation medium to a final concentration of 250 µM (1:400). Add 99% methanol 1:400 (same volume as that of sodium oleate) into another aliquot of differentiation medium to make the vehicle-control treatment. (For instance: add 25 µL of sodium oleate to 10 mL of medium and in parallel add 25 µL of 99% methanol to 10 mL of medium). Wash the cells once with 1x PBS and add vehicle or sodium oleate medium.

4.2. Day 23 and Day 25. Change the medium with appropriate volume of freshly prepared medium as in step 4.1.

4.3. Day 26. Observe by optical microscope that lipid droplets accumulate in the sodium oleate-treated cells and are easily visible as translucent droplets in the cytoplasm as in **Figure 2A**.

5. Evaluation of lipid overloading and steatosis induction: Oil Red O staining

5.1. Wash he cells once with 1x PBS and remove 1x PBS completely.

5.2. Add 4% paraformaldehyde (diluted in 1x PBS) and incubate for 15 min at RT.

CAUTION: Paraformaldehyde is toxic, so this step must be performed in a fume hood, with protective equipment, including gloves, a lab coat, and a mask.

5.3. Remove paraformaldehyde and wash the cells twice with 1x PBS. Cells can be kept in

1x PBS at 4 °C for a couple of days before staining. Wrap with parafilm and cover with aluminum foil to prevent the cells from drying.

5.4. Remove 1x PBS. Incubate the cells with 60% isopropanol for 5 min at RT.

5.5. Remove isopropanol and let the cells dry completely at RT.

5.6. Add Oil Red O working solution and incubate at RT for 30 min. The volume of working solution required for each sample corresponds to the volume of media used for culturing the cells.

5.7. Remove Oil Red O solution and immediately add ddH_2O. Wash the cells 4 times with ddH_2O.

5.8. Acquire images under the microscope for analysis (**Figure 2B**).

5.9. To elute Oil Red O dye: remove all the water and allow to dry; add 1 mL of 100% isopropanol and incubate for 10 min with gentle shaking at RT.

5.10. Pipet the isopropanol with eluted Oil Red O dye up and down several times, ensuring that all the Oil Red O is in the solution. Transfer the solution to a cuvette. Measure OD at 500 nm by spectrophotometry and use 100% isopropanol as blank (**Figure 2C**).

6. Evaluation of lipid overloading and steatosis induction: Bodipy staining and cytofluorimetric analysis

6.1. Wash the cells once with 1x PBS. Incubate with 100 nM of Bodipy diluted in 1x PBS in the dark for 40 min at 37 °C. An unstained control should be included in the flow cytometry measurements. From this point, protect the samples from light as much as possible.

NOTE: The volume of staining solution required for each sample corresponds to the volume of media used for culturing cells.

6.2. Remove staining solution and wash the cells once with 1x PBS. Proceed to steps 6.3–6.6. for FACS (Fluorescence-activated cell sorting) analysis or to step 6.7 for microscope imaging.

6.3. Gently scrape the cells with 1x PBS and transfer it into a 15-mL tube. Centrifuge for 10 min (200 × *g*, 4 °C).

6.4. Gently remove the supernatants without disturbing the pellets and wash with 3 mL of 1x PBS. Centrifuge for 5 min (200 × *g*, 4 °C).

6.5. Remove the supernatants and resuspend in 300 µL of 1x PBS, then transfer into a FACS tube.

6.6. Immediately measure Bodipy fluorescence intensity by cytofluorimetric analysis with

excitation/emission wavelengths of 505/515 nm (**Figure 3A,B**).

6.7. Wash the cells once with 1x PBS and remove PBS completely.

6.8. Add 4% paraformaldehyde (diluted in 1x PBS) and incubate for 15 min at RT.

CAUTION: Paraformaldehyde is toxic, so this step must be performed in a fume hood, with protective equipment, including gloves, a lab coat, and a mask.

6.9. Remove paraformaldehyde and wash the samples 3× for 5 min in PBS. Cells can be kept in 1x PBS at 4 °C or imaged immediately (**Figure 3C**). For storage, wrap with parafilm and cover with aluminum foil to prevent the cells from drying.

7. Evaluation of lipid overloading and steatosis induction: qPCR

7.1. Wash the cells once with 1x PBS. Scrape the cells with 1x PBS, centrifuge at 200 × *g*, and discard the supernatant. At this step, cells can be harvested at -80 °C or processed as in step 7.2.

7.2. Perform total RNA isolation by standard methods using commercial reagents following manufacturer's instructions.

7.3. Assess RNA concentration by UV spectrophotometric measurement, ensuring that the RNA purity is high (close to 2.0) based on the A260/A280 reading.

7.4. Synthesize cDNA from 1 µg of total RNA with a standard cDNA synthesis kit.

7.5. Dilute cDNA to a final 50 µL volume with H_2O.

7.6. Analyze each cDNA sample in triplicate by qPCR: prepare one qPCR master mix (**Table 1**) that is sufficient for the needed reactions for each primer, including primers specific for the three housekeeping genes as controls: gliceraldeide-3-fosfato deidrogenasi (GAPDH), actinB and ribosomal 18S (see **Table of Materials** for primer sequences):

7.7. Dispense 18 µL of master mix per well in a PCR multiwall plate.

7.8. Add 2 µL of cDNA sample in each well. Seal the plate.

7.9. Run the samples according to the Thermal Cycler instruction.

8. Evaluation of lipid overloading and steatosis induction: CARS

8.1. Fix the cells previously prepared on glass-bottomed dishes, as described in steps 5.1–5.3.

8.2. Switch on a commercial tunable picosecond pulsed laser system, tuning it to obtain two outputs of different wavelengths. The frequency difference between the outputs must

be 2840 cm^{-1} to generate the intense CARS light signal corresponding to methylene symmetric stretching; for a fixed-wavelength output at 1064 nm ("Stokes" light), the other wavelength should be tuned to 817 nm ("pump" light).

8.3. Ensure the 817-nm output is spatially and temporally overlapped with the 1064-nm output: use an appropriate dichroic mirror (i.e., with a cut between 817 and 1064 nm) to spatially combine the beams and use an optical delay line to obtain temporal overlap of the laser pulses.

8.4. Ensure the two copropagating beams are both collimated and that their diameters have similar values that are appropriate for the optical system within the microscope that will focus them onto the sample; if necessary, separately collimate the beams before the dichroic mirror that combines them.

8.5. Switch on a commercial inverted microscope system, which should include an infrared laser scanning unit and a dual-channel red/green epi detection unit and align the copropagating laser beams into the scanning unit.

8.6. Opening the dual-channel epi detection unit, remove the filter cube and replace its red-wavelength detection filter with a bandpass filter that can select the 2840-cm^{-1} CARS signal that is centered at 663 nm for the 817/1064-nm pump/Stokes excitation scheme. A narrow-bandwidth (20 nm) filter is preferred to avoid the collection of high levels of fluorescent background signals.

8.7. Place a dish on the stage of the inverted CARS microscope, and using a 100X oil-immersion objective, shift the vertical position of the objective to focus on the cells.

8.8. Set up the microscope software to collect high-resolution (1024 × 1024 pixels) images over a field of view spanning 127 µm × 127 µm.

8.9. Set the microscope software to continuously acquire and display images of the 127 µm × 127 µm field of view and check the displayed images whilst optimizing the image collection parameters. Ensure that the laser powers are balanced for rapid image collection and minimal damage, inserting appropriate neutral-density filters in the beam paths as necessary, and select a pixel dwell time that allows the collection of images with a good signal-to-noise ratio under the selected laser power conditions.

8.10. Acquire and save images of several different fields of view within the dish under the optimized conditions. Optionally, multiphoton fluorescence images, generated by a green-wavelength emission (mostly from two-photon excitation at 817 nm), may be simultaneously collected via the other epi detector. Repeat steps 8.7–8.10 for each dish.

8.11. For the CARS image analysis, process images using the FIJI implementation of ImageJ. Operate the **Despeckle** function (or a similar denoising tool) on each image before further analysis to improve signal-to-noise ratios without loss of detail.

8.12. Use FIJI to select cells manually and then automatically count lipid droplets within

segmented individual cells to produce statistics on lipid droplet areas and numbers for each cell and for the entire image dataset for each dish.

9. MTT cell viability assay

9.1. Plate differentiated HepaRG cells into a 96-well plate with a density of 1×10^5 cells/well in a final 100 µL volume of differentiation culture medium.

9.2. 24 h after seeding, treat the cells with 99% methanol (vehicle) and with 100 µM, 250 µM and 500 µM sodium oleate and sodium palmitate diluted in 100 µL of differentiation culture medium/well in triplicate.

9.3. Change the medium with 99% methanol and with 100 µM, 250 µM and 500 µM sodium oleate and sodium palmitate diluted in 100 µL of differentiation culture medium/well every 24 h.

9.4. 96 h after methanol/sodium oleate/sodium palmitate treatment, treat the cells with 2 µM doxorubicin diluted in 100 µL of differentiation culture medium/well in triplicate as a control.

9.5. 18 h after doxorubucin treatment, change the medium with 100 µL of differentiation culture medium.

9.6. Pipet 20 µL of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium reagent MTT (**Table of Materials**) into each well of the 96-well assay plate containing the cells in 100 µL of differentiation culture medium. Include a background control by pipetting 20 µL of reagent into a control well without cells in 100 µL of differentiation culture medium in triplicate.

9.7. Incubate the plate at 37 °C in a humidified, 5% CO_2 atmosphere.

9.8. After incubating for 30, 60, and 90 min with MTT tetrazolium reagent, record the absorbance at 490 nm using a 96-well plate reader. Subtract the background absorbance of the no-cell control wells from the absorbance values for the other samples.

REPRESENTATIVE RESULTS:

This protocol describes an efficient method to induce and characterize vesicular steatosis in DMSO-differentiated HepaRG cells by sodium oleate treatment (**Figure 1A**).

Differentiation of HepaRG cells.

To efficiently induce differentiation, proliferating cells must be seeded at a low density (2.5×10^4 cells/cm^2) in the proliferation medium. When seeded at low density, the cells actively divide and acquire an elongated undifferentiated morphology (**Figure 1B**). The cells should be left to grow in the proliferation medium for 7 days, until 100% confluency is reached (**Figure 1C**). After exposure to 2% DMSO, cells start to differentiate and to form typical hepatocyte-like colonies surrounded by biliary epithelial-like cells (**Figure 1D**). Differentiated HepaRG cells express hepato-specific markers, such as Albumin, Cyp3A4 and Aldolase B. To verify proper

differentiation, the expression levels of these hepatic marker genes in the differentiated cells (Day 21) and in the proliferating cells (Day 0) were analyzed by qPCR (**Figure 1E**). Albumin, Cyp3A4 and Aldolase B genes should be upregulated in the differentiated HepaRG cells as compared to the proliferating HepaRG cells, and we observed this trend, confirming the effectiveness of our differentiation protocol.

Induction of vesicular steatosis in HepaRG cells by sodium oleate treatment.

Sodium oleate treatment of dHepaRG cells induces fat accumulation, visible under an optical microscope as lipid droplets in the cytoplasms (**Figure 2A**). To verify efficient induction of steatosis, oleate-treated and control HepaRG cells were stained with Oil Red O dye. After staining, lipid droplets are easily visible as red droplets (**Figure 2B**) and can be quantified by spectrophotometric measurement of Oil Red O dye eluted with isopropanol (**Figure 2C**). Absorbance of eluted Oil Red O from the cells is directly proportional to cytoplasmic lipid droplet accumulation.

Sodium oleate concentration and exposure time were determined by MTT colorimetric cell viability assay (step 9). Sodium oleate treatment was compared to sodium palmitate treatment and the apoptotic drug doxorubucin (2 µM) was used as a control (**Figure 2D**). Cytoxicity of the compounds was evaluated by MTT, taking advantage of a commercial compound (**Table of Materials**), that contains the MTT tetrazolium. The water-soluble yellow MTT tetrazolium compound is bioreduced by metabolically active cells into a purple water-insoluble formazan product. The formazan product is quantified by its absorbance at 490 nm and the amount is directly proportional to the number of living cells in culture (**Figure 2D**).

Quantification of vesicular steatosis in sodium oleate-treated HepaRG cells

To quantify the increase of cellular lipid content after sodium oleate treatment, we performed staining with Bodipy dye, a probe that labels lipid droplets. Using cytofluorimetric analysis, it is possible to quantify the triglyceride content by Bodipy mean fluorescent intensity, which is higher in oleate-treated cells as compared to control cells (**Figure 3A,B**), indicating efficient fat accumulation after oleate treatment. Indeed, images of Bodipy-stained cells show bright green fluorescent lipid droplets in oleate-treated cells that are not visible in the control cells (**Figure 3C**).

Sodium oleate treatment of dHepaRG cells deregulates lipid metabolism and inflammatory gene expression. To evaluate efficient induction of vesicular steatosis, we analyzed by qPCR the expression levels of selected genes in sodium oleate-treated cells as compared to those of control cells (**Figure 4**). Acetyl-CoA carboxylase beta (ACACB), glycerol-3-phosphate acyltransferase mitochondrial (GPAM), perilipins (PLIN2, PLIN4), apolipoprotein B (APOB), pyruvate dehydrogenase kinase isozyme 4 (PDK4), carnitine palmitoyltransferase 1A (CPT1A) and interleukin 6 (IL6) were upregulated in oleate-treated dHepaRG cells, whereas solute carrier family 2 member 1 (SLC2A1), apolipoprotein C-III (APOC3), and stearoyl-CoA desaturase (SCD) were downregulated (**Figure 4**). To further characterize and quantify lipid storage in droplets upon addition of sodium oleate to differentiated HepaRG cells, we utilized an innovative microscopy technique, coherent anti-Stokes Raman scattering (CARS) microscopy, which enables visualization and quantification of lipid droplets without labeling (**Figure 5A**). Lipid droplets were statistically quantified in terms of numbers, distribution and morphology at the single-cell level using CARS images. Treatment with sodium oleate (250

µM) induced a significant increase in the number of lipid droplets (**Figure 5B**), which led to a higher total droplet area per cell (**Figure 5C**) and a higher percentage droplet area per cell (**Figure 5D**), as compared to control cells, indicating that dHepaRG cells efficiently accumulated fat after sodium oleate treatment.

FIGURE LEGENDS

Figure 1. Differentiation of HepaRG cells. (A) Representative diagram showing HepaRG cell differentiation/treatment protocol as described in steps 3 and 4. **(B-D)** Images showing unstained proliferating HepaRG cells at Day 0 after seeding (**B**), at confluence Day 7 after seeding (**C**), and differentiated HepaRG (dHepaRG) at Day 21 after seeding (**D**). (**E)** Total RNA was extracted from proliferating and dHepaRG cells, cDNA was synthesized and analyzed by qPCR using primers specific for the indicated genes (**Table of Materials**). Samples were normalized to the mean of GAPDH, actinB and ribosomal 18S housekeeping genes. Histograms show fold induction of proliferating (Day 0) versus differentiated cells (Day 21) (bars indicate S.D.; p-values were computed by Student's t-tests).

Figure 2. Sodium oleate treatment of dHepaRG cells induced lipid droplet accumulation. (A) Images of unstained differentiated HepaRG (dHepaRG) cells treated for 5 days with vehicle (control) (left image) or with 250-µM sodium oleate (right image). (**B)** After treatment, the cells were stained with Oil Red O dye; lipid droplets are visible in red. (**C)** Oil Red O dye was eluted and OD was measured at 570 nm. Results are expressed as means of three independent experiments (bars indicate S.D.; p-value by Student's t-test). (**D)** Cell viability evaluation of dHepaRG cells vehicle (99% methanol) treated (Ctrl) or treated for 5 days with sodium oleate and sodium palmitate (100 µM, 250 µM, or 500 µM), or treated 18 h with doxorubucin (2 µM). Assessment of cytotoxicity was performed using an MTT assay kit (**Table of Materials**), recording absorbance at 490 nm, according to the manufacturer's instruction. Results are expressed as means of three independent experiments (bars indicate S.D.; p-values were determined using Student's t-test: *$0.01 \leq p < 0.05$; **$0.001 \leq p < 0.01$; ***$p < 0.001$).

Figure 3. Quantification of fat accumulation after sodium oleate treatment by Bodipy staining. Differentiated HepaRG (dHepaRG) cells were treated with vehicle (control) or with 250-µM sodium oleate for 5 days. After treatment, dHepaRG cells were stained with Bodipy dye and analyzed by flow cytometry. (**A)** Representative overlay profiles (% of max: percentage of maximum staining intensity). (**B)** Histograms show mean fluorescence intensity (MFI) as a percentage of treated cells over control from three independent experiments (bars indicate S.D.; p-values were determined using Student's t-test: *$0.01 \leq p < 0.05$; **$0.001 \leq p < 0.01$; ***$p < 0.001$). (**C)** Cells were fixed with 4% paraformaldehyde. Images show Bodipy-stained lipid droplets in green.

Figure 4. Sodium oleate treatment of dHepaRG cells induces deregulation of lipid metabolism and inflammatory gene expression. Differentiated HepaRG (dHepaRG) cells were treated with vehicle (control) or with 250-µM sodium oleate for 5 days. cDNAs were analyzed by qPCR with primers specific for the indicated genes and results were normalized to the mean of GAPDH, actinB and ribosomal 18S housekeeping genes; the primers are given in the **Table of Materials**. The histogram shows expression levels of indicated genes as fold

inductions of treated cells over control (bars indicate S.D.; p-values were computed by Student's t-test).

Figure 5. Characterization of lipid droplet accumulation after sodium oleate treatment by CARS microscopy. Differentiated HepaRG (dHepaRG) cells were treated with vehicle (control) or with 250-µM sodium oleate for 5 days and were analyzed by CARS microscopy. (**A)** Representative images showing lipid droplet CARS contrast in red. (**B)** Histogram showing the numbers of lipid droplets per cell. (**C)** Histograms showing total image area covered by droplets per cell. (**D)** Histogram showing % droplet area covered by droplets per cell. All results are expressed as means of three independent experiments (bars indicate S.E.; p-values were determined by Student's t-test: *$0.01 \leq p < 0.05$; **$0.001 \leq p < 0.01$; ***$p < 0.001$).

Table 1. qPCR master mix.

DISCUSSION

This protocol describes how to differentiate HepaRG cells and how to induce vesicular steatosis by sodium oleate treatment (**Figure 1A**). Indeed, compared to other human hepatocellular carcinoma (HCC) cell lines, the HepaRG cell line exhibits features of adult human hepatocytes, representing a valuable alternative to ex vivo cultivated primary human hepatocytes[13-15]. The HepaRG cell line has been widely used for liver cytotoxicity studies, drug metabolism, and virology studies[15,16,22].

In comparison with HepaRG cells, other HCC cell lines such as HepG2, HUH7, HUH6 and Hep3B display lower metabolic capacities, lacking a substantial set of liver-specific functions[23,24], and exhibiting a higher basal level of cytosolic fat accumulation stored in lipid droplets. Thus, these HCC cell lines are less useful as models for the induction of vesicular steatosis after lipid overloading than the HepaRG cell line.

Critical steps within the protocol

When seeded at low density (2.5×10^4 cells/cm^2), HepaRG cells acquire an elongated undifferentiated morphology, actively dividing; after having reached 100% confluency, they are capable of differentiating upon exposure to 2% DMSO and they form typical hepatocyte-like colonies surrounded by biliary epithelial-like cells (**Figure 1D**). This mixed biliary/hepatocyte cell culture recapitulates features of liver tissue, resembling a physiological condition, despite lacking other liver cell types (sinusoidal or Kupffer).

A critical step in the differentiation process is the confluency of cells. The cells must be seeded at low density (2.5×10^4 cells/cm^2) (**Figure 1B**) and allowed to actively grow in the proliferation medium for at least 1 week (Day 0–7). At Day 7, before adding 2% DMSO to start the differentiation process, the cells must be at 100% confluence (**Figure 1C**). If at Day 7 (step 2.2) complete confluency has not been reached, it is highly recommended that the cells are allowed to continue growing in the proliferation medium for some additional days, until 100% confluence is achieved.

Limitations of the protocol

It has been observed that after adding differentiation medium, some cells suffer, and typically 10% of cells die during the subsequent 2–3 days. At this stage, daily washing and medium

changing (step 2.4) must be continued to discard floating dead cells. The usage of a specific FBS (**Table of Materials**) to supplement both the differentiation and proliferation media, should increase differentiation efficiency and lower cell death during Days 7–21 (step 2.4). It is highly recommended to maintain cells up to passage 20, and to choose lower passages (<20) for differentiating cells: less cells death occurs for the youngest HepaRG cells. Moreover, HepaRG differentiation seems to be more efficacious in 35-mm and 60-mm dishes, whereas cells tend to suffer more when seeded in the 100-mm and 150-mm dishes.

Modifications and troubleshooting

Exposure to different ratios of both palmitic and oleic fatty acids has been shown to result in the formation of intracytoplasmatic lipid droplets[25,26]. However, we observed that sodium oleate treatment of differentiated HepaRG cells exhibited better results than palmitic acid exposure, in terms of efficient induction of lipid accumulation and cell viability (**Figure 2D**). Indeed, palmitic acid treatment proved to be toxic for differentiated HepaRG cells (**Figure 2D**), in agreement with literature data on hepatoma cell lines and on human and rat hepatocyte primary cultures that describe palmitic acid as a considerably cytotoxic agent[25-29]. Moreover, the utilization of palmitate in cell-based assays is challenging due to its low solubility. Nevertheless, due to the intrinsic variability of cell lines, it is recommended to verify the sodium oleate working concentration and treatment time length, testing different concentrations in a time-course experiment with a cell viability assay.

Significance and comparison of lipid detection techniques

Accurate determination of lipid amounts in cells was established via a number of different approaches in this study. Qualitative and also approximately quantitative agreement was observed between the results obtained via Oil Red O staining, Bodipy staining, and CARS imaging. For a rapid estimation of lipid quantities in cell populations, the use of Bodipy-flow cytometry or a stain such as Oil Red O is ideal. For a more detailed examination of the lipid droplet content of cells, an imaging modality is preferred. Furthermore, specificity problems have been reported for lipid stains such as Oil Red O[20,30], and we have observed that in some cases, the Bodipy stain has a lower capacity than CARS to exclusively label lipid droplets among other cell organelles (data not shown). Therefore, the use of a label-free microscopic imaging technique such as CARS provides significant advantages for steatosis quantification and characterization. The avoidance of the use of a large fluorescent label makes CARS imaging favorable due to the small size of lipid molecules compared to typical fluorophores; hence, for the detection of lipids, label-free methods are desirable, even more so than for observing large protein molecules. The lipid CARS signal is an optical emission that is generated only when a nonlinear interaction occurs in the sample. This interaction can only be detected when the difference between the frequencies of the two excitation lasers focused onto the sample matches the methylene (CH_2) stretching vibrational frequency. The abundance of methylene groups in lipids results in a very intense signals with high signal-to-noise ratios, and the nonlinearity of the optical interaction also means that high spatial resolution is possible in CARS microscopy. The excellent quality of CARS images in general allows the collection of statistics on the sizes and numbers of lipid droplets, as demonstrated in this study. In addition, other studies have demonstrated the use of CARS microscopy to correlate different subcellular localizations and sizes of lipid droplets with different treatments[18], and by using supplementary CARS measurements at different wavelengths, or a broadband CARS or similar stimulated Raman approach, researchers have shown that it is

possible to characterize different types of fatty acids within lipid droplets[31,32]. Furthermore, the rapidity of CARS image collection has enabled in-situ imaging of live cells to examine the temporal evolution of lipid droplet growth and aggregation[33].

Future applications

In recent decades, views on the roles and functions of lipid droplets in cell biology have evolved. Previously thought to be basically inert storage vesicles, they are now understood to be highly dynamic cellular organelles, and their role in disease is increasingly being recognized[34-36]. Although in the case of liver disease, lipid accumulation (steatosis) has long been known to be a critical aspect, the precise mechanism of the involvement of lipid droplets in disease progression is not completely clear. Therefore, methods such as CARS that can characterize the dynamic behavior of lipid droplets are of critical importance to the development of a molecular understanding of diseases including NAFLD. Metabolic gene expression analysis by qPCR is highly complementary to molecular imaging of lipids via CARS, as demonstrated in previous studies[17,18], allowing deeper insights into disease mechanisms. In the present study, we observe fatty acid accumulation with deregulation of lipid metabolism and inflammatory gene expression, which may contribute to the construction of a panel of bio-markers for early disease diagnosis.

The sodium oleate-treated dHepaRG cell-based model may increase knowledge of the molecular mechanisms involved not only in the onset but also in the progression of NAFLD, providing a basis for the development of better therapeutic approaches to the disease.

ACKNOWLEDGMENTS:

We thank prof. Christian Trepo (INSERM U871, Lyon, France), who kindly provided HepaRG cells. We are grateful to Rita Appodia for administrative help. This work was supported by MIUR- Ministero dell'istruzione, dell'università e della ricerca (FIRB 2011–2016 RBAP10XKNC) and Sapienza University of Rome (prot. C26A13T8PS; prot. C26A142MCH; prot. C26A15LSXL).

DISCLOSURES:

The authors declare that they have no competing financial interests.

REFERENCES:

1. Starley, B. Q., Calcagno, C. J. & Harrison, S. A. Nonalcoholic fatty liver disease and hepatocellular carcinoma: a weighty connection. *Hepatology*. **51**(5), 1820–1832 (2010).
2. Byrne, C. D. & Targher, G. NAFLD: a multisystem disease. *Journal of Hepatology*. **62**(1 Suppl), S47–S64 (2015).
3. Marra, F., Gastaldelli, A., Svegliati Baroni, G., Tell, G. & Tiribelli, C. Molecular basis and mechanisms of progression of non- alcoholic steatohepatitis. *Trends in Molecular Medicine*. **14**, 72–81 (2008).
4. Chalasani, N., et al. The diagnosis and management of nonalcoholic fatty liver disease: Practice guidance from the American Association for the Study of Liver Diseases. *Hepatology.* **67**(1), 328–357 (2018).
5. Banini, B. A. & Sanyal, A. J. Current and future pharmacologic treatment of nonalcoholic steatohepatitis. *Current Opinion in Gastroenterology.* **33**(3), 134–141 (2017).

6. Takahashi, Y., Sugimoto, K., Inui, H. & Fukusato, T. Current pharmacological therapies for nonalcoholic fatty liver disease/nonalcoholic steatohepatitis. *World Journal of Gastroenterology*. **21**(13), 3777–3785 (2015).
7. Younossi, Z., et al. Global burden of NAFLD and NASH: trends, predictions, risk factors and prevention. *Nature Reviews Gastroenterology & Hepatology*. **15**(1), 11–20 (2018).
8. Santhekadur, P. K., Kumar, D. P. & Sanyal, A. J. Preclinical models of non-alcoholic fatty liver disease. *Journal of Hepatology.* **68**(2), 230–237 (2018).
9. Nagarajan, P., Mahesh Kumar, M. J., Venkatesan, R., Majundar, S. S. & Juyal, R. C. Genetically modified mouse models for the study of nonalcoholic fatty liver disease. *World Journal of. Gastroenterology*. **18**(11), 1141–1153 (2012).
10. Oseini, A. M., Cole, B. K., Issa, D., Feaver, R. E., & Sanyal, A. J. Translating scientific discovery: the need for preclinical models of nonalcoholic steatohepatitis. *Hepatology International*. **12**(1), 6–16 (2018).
11. Ramboer, E., Vanhaecke, T., Rogiers, V. & Vinken. M. Immortalized human hepatic cell lines for in vitro testing and research purposes. *Methods in Molecular Biology*. **1250**, 53–76 (2015).
12. Gómez-Lechón, M.J., Donato, M.T., Castell, J.V. & Jover R. Human hepatocytes in primary culture: the choice to investigate drug metabolism in man. *Current Drug Metabolism* **5**(5), 443–462 (2004).
13. Marion, M. J., Hantz, O. & Durantel, D. The HepaRG cell line: biological properties and relevance as a tool for cell biology, drug metabolism, and virology studies. *Methods in Molecular Biology*. **640**, 261–272 (2010).
14. Gripon P, et al. Infection of a human hepatoma cell line by hepatitis B virus. *Proceedings of the National Academy of Sciences.* **99**(24), 15655-60 (2002).
15. Anthérieu S, Rogue A, Fromenty B, Guillouzo A, Robin MA. Induction of vesicular steatosis by amiodarone and tetracycline is associated with up-regulation of lipogenic genes in HepaRG cells. *Hepatology.* **53**(6), 1895-905 (2011).
16. Rouge A., et al., PPAR agonists reduce steatosis in oleic acid-overloaded HepaRG cells. *Toxicology and Applied Pharmacology*. **276**(1), 73-81 (2014).
17. Belloni, L., et al. Targeting a phospho-STAT3-miRNAs pathway improves vesicular hepatic steatosis in an in vitro and in vivo model. *Scientific Reports*. **8**, 13638 (2018).
18. Nunn, A. D. G., et al. The histone deacetylase inhibiting drug Entinostat induces lipid accumulation in differentiated HepaRG cells. *Scientific Reports.* **6**, 28025 (2016).
19. Donato, M.T., et al. Cytometric analysis for drug-induced steatosis in HepG2 cells. *Chemico-Biological Interactions*. **181**(3), 417-23 (2009).
20. Hellerer, T., Axäng, C., Brackmann, C., Hillertz, P., Pilon, M. & Enejder, A. Monitoring of lipid storage in Caenorhabditis elegans using coherent anti-Stokes Raman scattering (CARS) microscopy. *Proceedings of the National Academy of Sciences*. **104**(37), 14658–14663 (2007).
21. Le, T. T., Ziemba, A., Urasaki, Y., Brotman, S. & Pizzorno, G. Label-free evaluation of hepatic microvesicular steatosis with multimodal coherent anti-Stokes Raman scattering microscopy. *PLoS One*. **7**(11), e51092 (2012).
22. Guillouzo, A., Corlu, A., Aninat, C., Glaise, D., Morel, F., Guguen-Guillouzo, C. The human hepatoma HepaRG cells: a highly differentiated model for studies of liver metabolism and toxicity of xenobiotics. *Chemico-Biological Interactions*. **168**(1), 66–73 (2007).
23. Nelson, L. J., et al. Human hepatic HepaRG cells maintain an organotypic phenotype with high intrinsic CYP450 Activity/metabolism and significantly outperform standard

HepG2/C3A cells for pharmaceutical and therapeutic applications. *Basic & Clinical Pharmacology & Toxicology*. **120**(1), 30–37 (2017).
24. Gerets, H. H. J., Tilmant, K., Gerin, B., Chanteux, H., Depelchin, B. O., Dhalluin, S. & Atienzar, F. A. Characterization of primary human hepatocytes, HepG2 cells, and HepaRG cells at the mRNA level and CYP activity in response to inducers and their predictivity for the detection of human hepatotoxins. *Cell Biology and Toxicology*. **28**(2), 69–87 (2012).
25. Pusl, T., et al. Free fatty acids sensitize hepatocytes to bile acid-induced apoptosis. *Biochemical and Biophysical Research Communications*. **371**(3), 441–445 (2008).
26. Gentile, C. L. & Pagliassotti, M. J. The role of fatty acids in the development and progression of nonalcoholic fatty liver disease. *The Journal of Nutritional Biochemistry*. **19**(9), 567–576 (2008).
27. Mei, S. et al. Differential roles of unsaturated and saturated fatty acids on autophagy and apoptosis in hepatocytes. *Journal of Pharmacological Experimental Therapy.* **339**, 487-498 (2011).
28. Malhi, H., Bronk, S.F., Werneburg, N.W., Gores, G.J. Free fatty acids induce JNK-dependent hepatocyte lipoapoptosis*. Journal of Biological Chemistry*. **281**, 12093-12101 (2006).
29. Moravcová, A., Červinková, Z., Kučera, O., Mezera, V., Rychtrmoc, D., Lotková, H. The effect of oleic and palmitic acid on induction of steatosis and cytotoxicity on rat hepatocytes in primary culture. *Physiological Research.* **64** Suppl 5, S627-36 (2015).
30. Ohsaki, Y., Shinohara, Y., Suzuki, M., & Fujimoto, T. A pitfall in using BODIPY dyes to label lipid droplets for fluorescence microscopy. *Histochemistry and Cell Biology*. **133**(4), 477–480 (2010).
31. Rinia, H. A., Burger, K. N., Bonn, M., & Müller, M. Quantitative label-free imaging of lipid composition and packing of individual cellular lipid droplets using multiplex CARS microscopy. *Biophysical Journal*. **95**(10), 4908–4914 (2008).
32. Waschatko, G., Billecke, N., Schwendy, S., Jaurich, H., Bonn, M., Vilgis, & T. A., Parekh, S. H. Label-free in situ imaging of oil body dynamics and chemistry in germination. *Journal of The Royal Society Interface*. **13**(123), (2016).
33. Jüngst, C., Winterhalder, M. J., & Zumbusch, A. Fast and long term lipid droplet tracking with CARS microscopy. *Journal of Biophotonics*. **4**(6), 435–441 (2011).
34. Welte, M. A. & Gould, A. P. Lipid droplet functions beyond energy storage. *Biochimica et Biophysica Acta - Molecular and Cell Biology of Lipids*. **1862**(10 Pt B), 1260–1272 (2017).
35. Cohen, S. Lipid droplets as organelles. *International Review of Cell and Molecular Biology*. **337**, 83-110 (2018)
36. Farese, R. V., Jr & Walther, T. C. Lipid droplets finally get a little R-E-S-P-E-C-T. *Cell* **139**(5), 855–860 (2009).

Figure 1

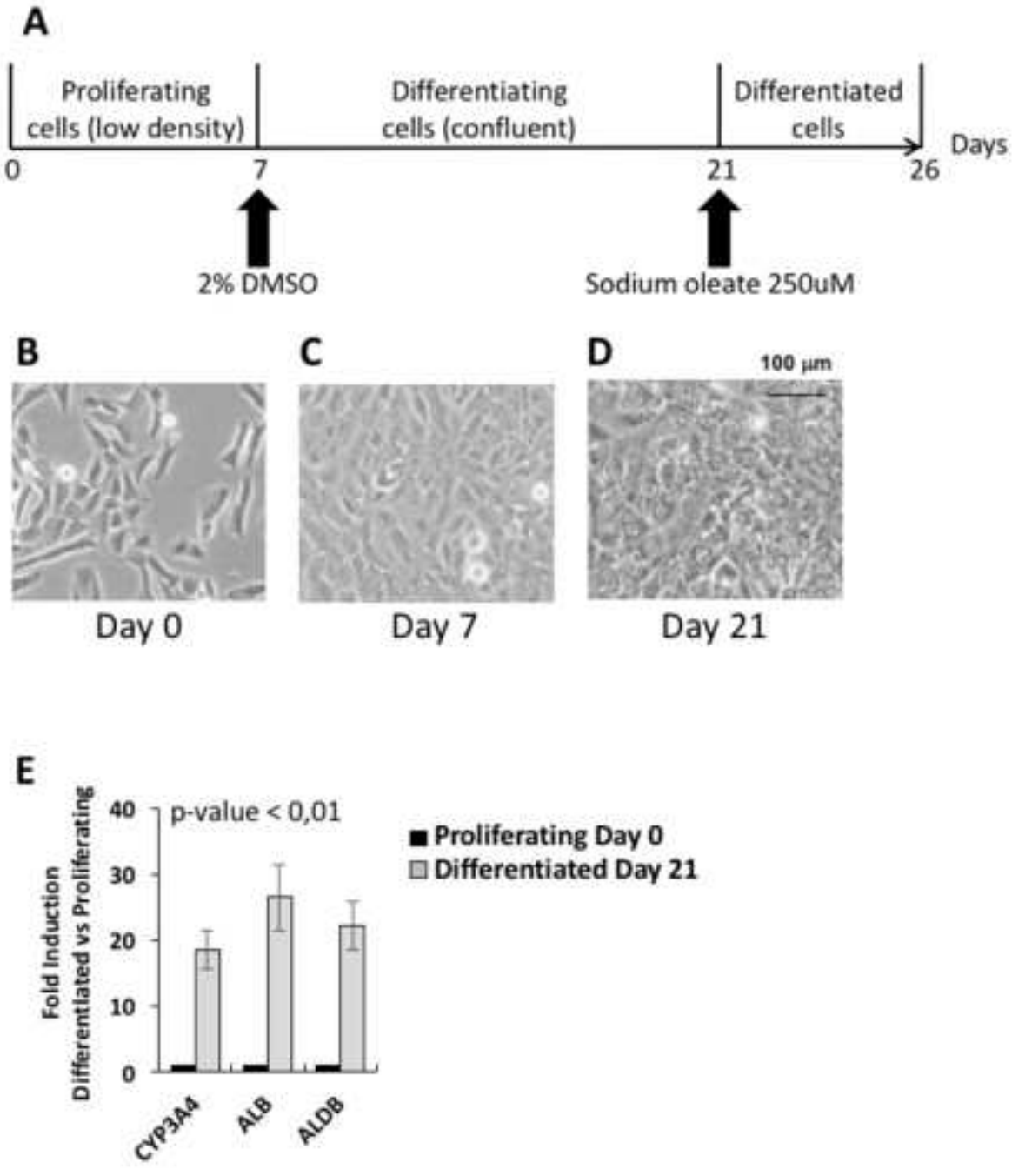


Figure 2

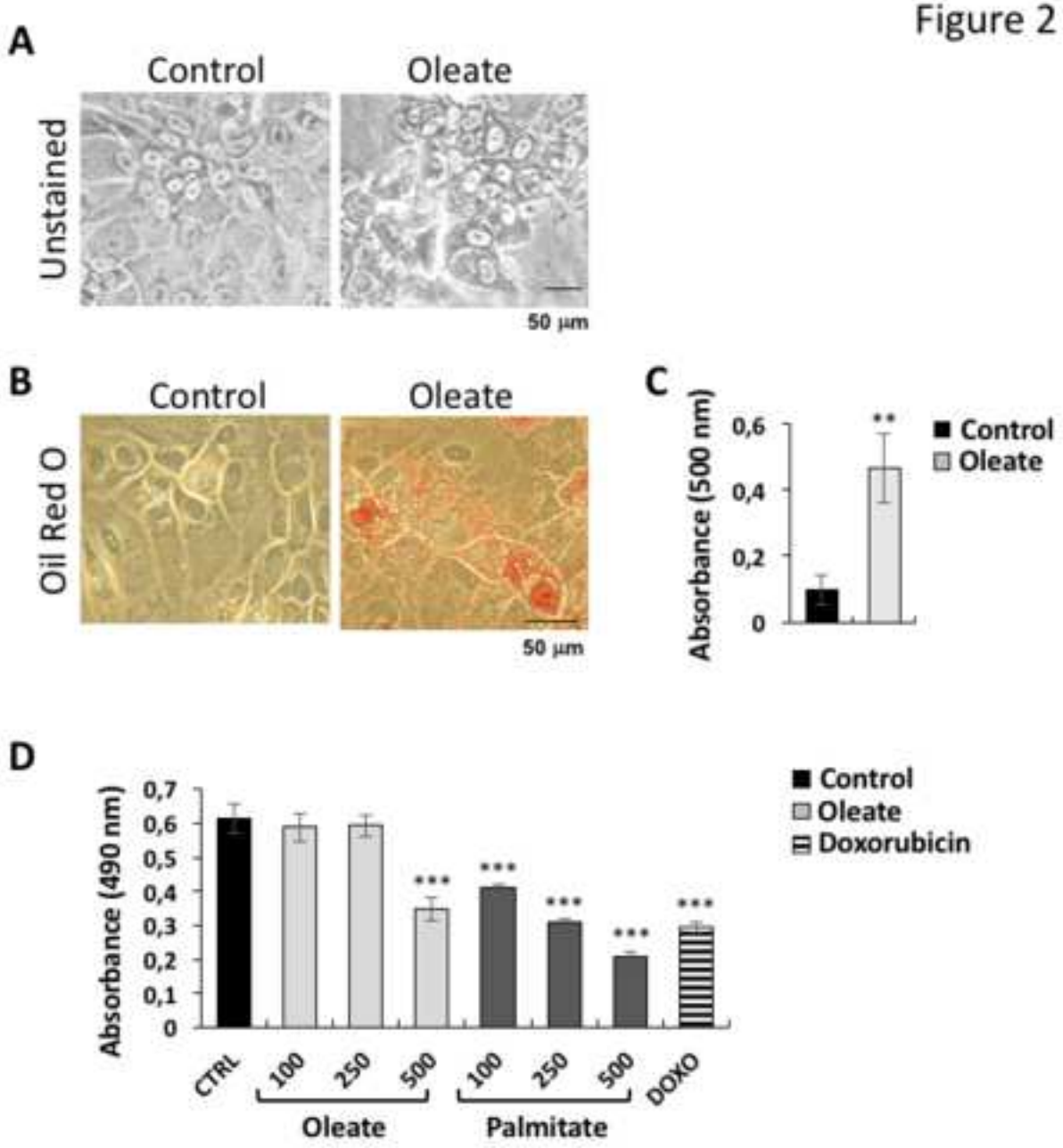


Figure 3

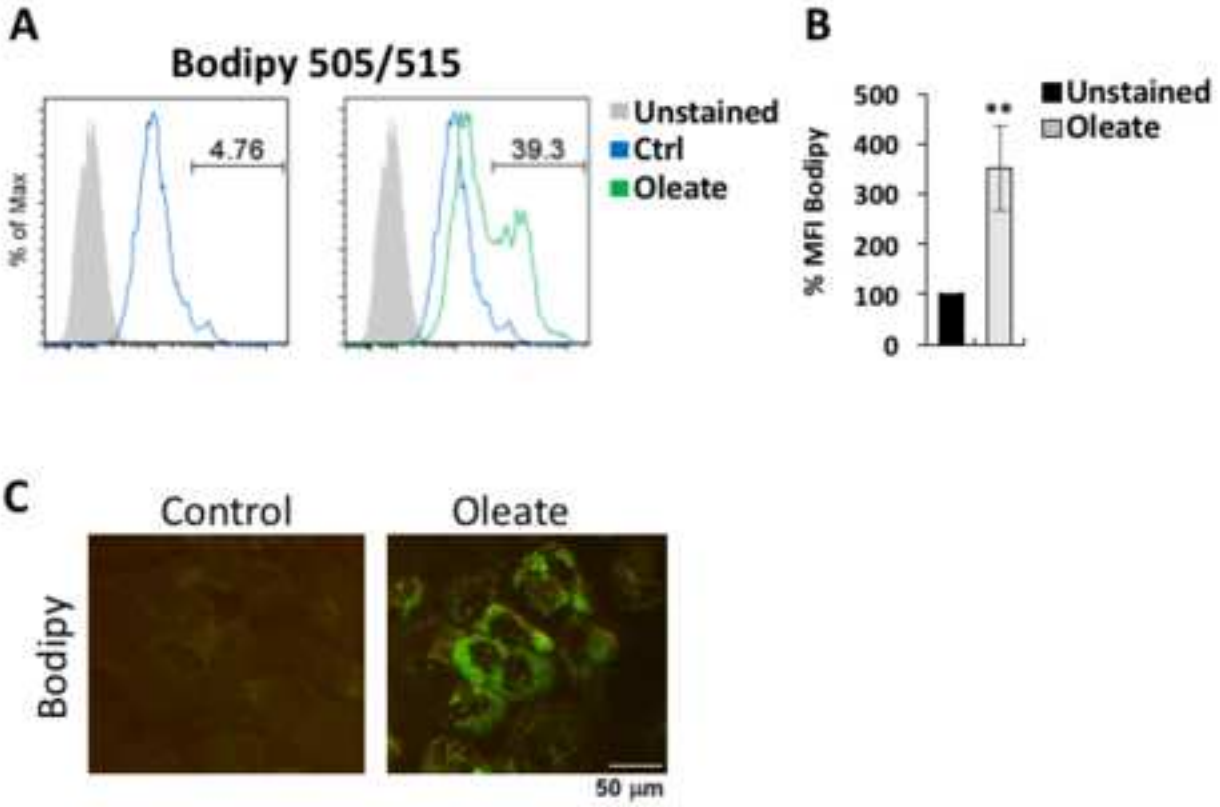


Figure 4

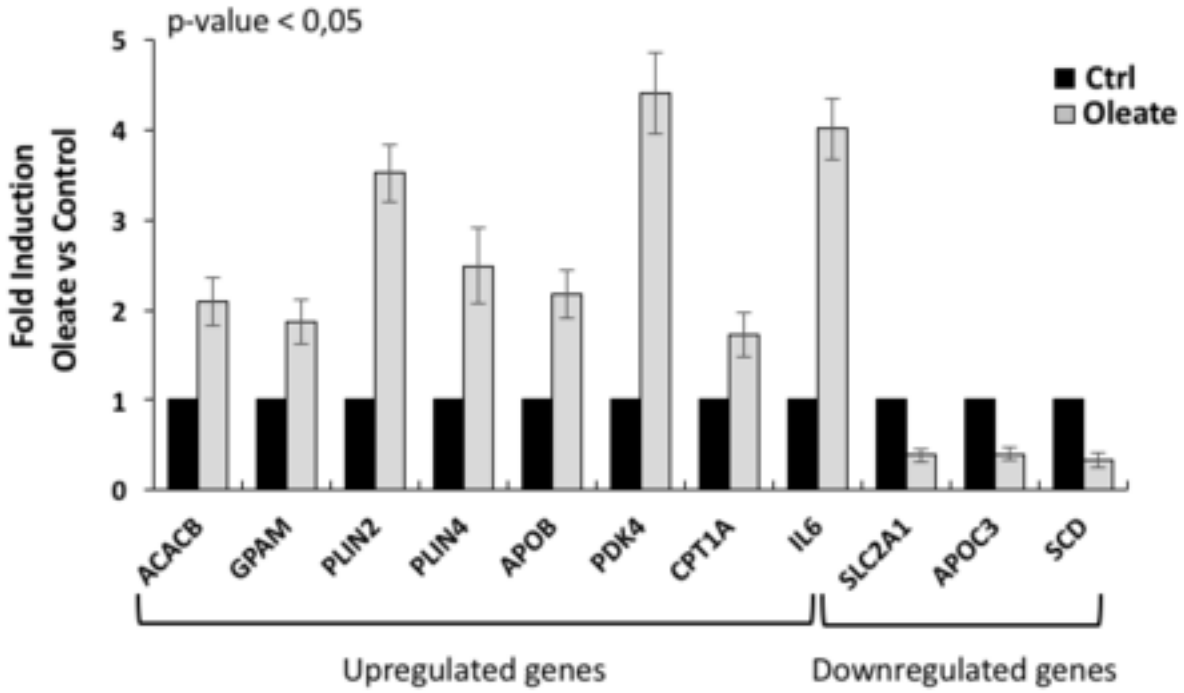
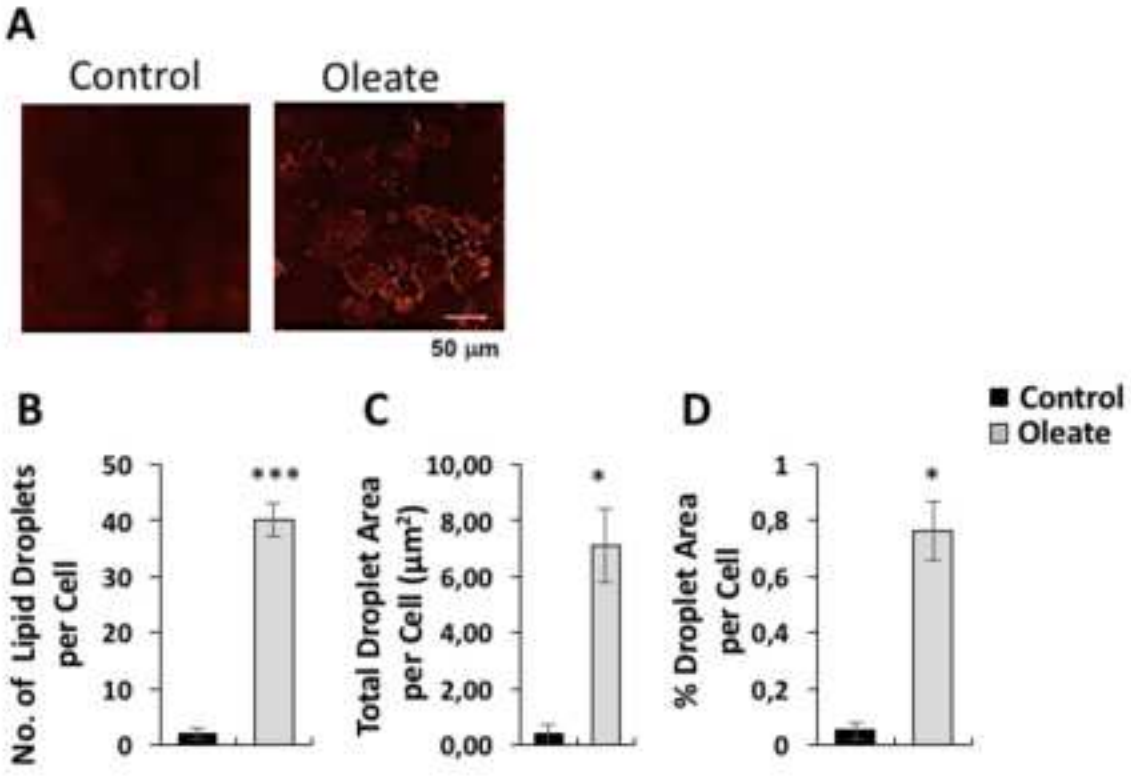


Figure 5



Table

Reagents	Volume (μL) for a single reaction
2×SYBR Green fluorescent dye	10
PCR grade H_2O	6
Forward primer (μM)	1
Reverse primer (μM)	1

Name of Reagent/ Equipment	Company	Catalog Number
Hyclone HyClone Fetal Clone II	GE Healthcare	SH30066
William's E medium with GlutaMAX	Thermofisher	32551087
Penicillin/streptomycin	SIGMA	P4333
Insulin	SIGMA	I9278
hydrocortisone hemisuccinate	SIGMA	H2270
DMSO, dimethyl sulfoxide	SIGMA	D2438
Sodium Oleate	SIGMA	O7501
Methanol	SIGMA	179337
Isopropanol	SIGMA	278475
BODIPY 505/515	Thermofisher	D3921
PBS	Thermofisher	14190-250
Formaldehyde solution	SIGMA	252549
RNAse free DNAseI	Promega	M198A
Glass-bottomed dishes	Willco Wells	GWST-5040
Oil Red solution	SIGMA	O625
CellTiter 96 AQueous One Solution	Promega	G3582

q-PCR oligo name	Sequence
ACACB FOR	CAAGCCGATCACCAAGAGTAAA
ACACB REV	CCCTGAGTTATCAGAGGCTGG
b-actin FOR	GCACTCTTCCAGCCTTCCT
b-actin REV	AGGTCTTTGCGGATGTCCAC
ALBUMIN FOR	TGCTTGAATGTGCTGATGACAGG
ALBUMIN REV	AAGGCAAGTCAGCAGGCATCTCATC
ALDOB FOR	GCATCTGTCAGCAGAATGGA
ALDOB REV	TAGACAGCAGCCAGGACCTT
APOB FOR	CCTCCGTTTTGGTGGTAGAG
APOB REV	CCTAAAAGCTGGGAAGCTGA
APOC3 FOR	CTCAGCTTCATGCAGGGTTA
APOC3 REV	GGTGCTCCAGTAGTCTTTCAG

CPT1A FOR	TCATCAAGAAATGTCGCACG
CPT1A REV	GCCTCGTATGTGAGGCAAAA
CYP2E1 FOR	TTGAAGCCTCTCGTTGACCC
CYP2E1 REV	CGTGGTGGGATACAGCCAA
CYP3A4 FOR	CTTCATCCAATGGACTGCATAAAT
CYP3A4 REV	TCCCAAGTATAACACTCTACACAGACAA
GAPDH FOR	TGACAACTTTGGTATCGTGGAAGG
GAPDH REV	AGGGATGATGTTCTGGAGAGCC
GPAM FOR	TCTTTGGGTTTGCGGAATGTT
GPAM REV	ATGCACATCTCGCTCTTGAATAA
IL6 FOR	CCTGAACCTTCCAAAGATGGC
IL6 REV	ACCTCAAACTCCAAAAGACCAGTG
PDK4 FOR	ACAGACAGGAAACCCAAGCCAC
PDK4 REV	TGGAGGTGAGAAGGAACATACACG
PLIN2 FOR	TTGCAGTTGCCAATACCTATGC
PLIN2 REV	CCAGTCACAGTAGTCGTCACA
PLIN4 FOR	AATGAGTTGGAGGGGCTGGGGGACATC
PLIN4 REV	GGTCACCTAAACGAACGAAGTAGC
SCD FOR	TCTAGCTCCTATACCACCACCA
SCD REV	TCGTCTCCAACTTATCTCCTCC
SLC2A1 FOR	TGCTCATCAACCGCAACGAG
SLC2A1 REV	CCGACTCTCTTCCTTCATCTCCTG
18S FOR	CGCCGCTAGAGGTGAAATTC
18S REV	TTGGCAAATGCTTTCGCTC

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article: INDUCTION AND EVALUATION OF VESICULAR HEPATIC STEATOSIS IN LIVER DMSO-DIFFERENTIATED HEPANG CELLS.

Author(s): DI COCCO S, BELLONI L, NANNI ADC, SALENNO D, PICONESE S, LEUNENO M AND PEDICONI N

Item 1: The Author elects to have the Materials be made available (as described at http://www.jove.com/publish) via:

☒ Standard Access ☐ Open Access

Item 2: Please select one of the following items:

☒ The Author is **NOT** a United States government employee.

☐ The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.

☐ The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

ARTICLE AND VIDEO LICENSE AGREEMENT

1. **Defined Terms.** As used in this Article and Video License Agreement, the following terms shall have the following meanings: "**Agreement**" means this Article and Video License Agreement; "**Article**" means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; "**Author**" means the author who is a signatory to this Agreement; "**Collective Work**" means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; "**CRC License**" means the Creative Commons Attribution-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at: http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode; "**Derivative Work**" means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; "**Institution**" means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; "**JoVE**" means MyJove Corporation, a Massachusetts corporation and the publisher of The Journal of Visualized Experiments; "**Materials**" means the Article and / or the Video; "**Parties**" means the Author and JoVE; "**Video**" means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2. **Background.** The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. **Grant of Rights in Article.** In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4** and **7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and(c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the "Open Access" box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

4. **Retention of Rights in Article.** Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. **Grant of Rights in Video – Standard Access.** This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, Subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. **Grant of Rights in Video – Open Access.** This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. For any Video to which this **Section 6** is applicable, JoVE and the Author hereby grant to the public all such rights in the Video as provided in, but subject to all limitations and requirements set forth in, the CRC License.

7. **Government Employees.** If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum rights permitted under such statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. **Protection of the Work.** The Author(s) authorize JoVE to take steps in the Author(s) name and on their behalf if JoVE believes some third party could be infringing or might infringe the copyright of either the Author's Article and/or Video.

9. **Likeness, Privacy, Personality.** The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

10. **Author Warranties.** The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

11. **JoVE Discretion.** If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

12. **Indemnification.** The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in JoVE or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

13. **Fees.** To cover the cost incurred for publication, JoVE must receive payment before production and publication of the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

14. **Transfer, Governing Law.** This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to me one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement is required per submission.

CORRESPONDING AUTHOR

Name:	NATALIA PEDICONI
Department:	CENTER FOR LIFE NANO SCIENCE@SAPIENZA
Institution:	ISTITUTO ITALIANO DI TECNOLOGIA
Title:	INDUCTION AND EVALUATION OF VESICULAR HEPATIC STEATOSIS IN LIVER DMSO-DIFFERENTIATED HEPARG CELLS
Signature:	Natalia Pediconi
Date:	14/2/2019

Please submit a **signed** and **dated** copy of this license by one of the following three methods:

1. Upload an electronic version on the JoVE submission site
2. Fax the document to +1.866.381.2236
3. Mail the document to JoVE / Attn: JoVE Editorial / 1 Alewife Center #200 / Cambridge, MA 02140

Rebuttal letter
Manuscript: JoVE59843
Title: "Induction and evaluation of vesicular hepatic steatosis in liver DMSO-differentiated HepaRG cells"

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
- We proofread the manuscript and have corrected the grammar and spelling.

2. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."
- None of the figures presented in this paper have been included in any previous publications. In this paper, we show new and unpublished data and images from experiments performed as in other papers.

3. Unfortunately, there are a few sections of the manuscript that show significant overlap with previously published work. Though there may be a limited number of ways to describe a technique, please use original language throughout the manuscript. Please revise lines 73-76, 78-88.
- We have changed sentences in lines 73–76 and 78–88.

4. For in-text referencing, please superscript the reference number and remove the ( ) before and after the reference number.
5. Please use 12 pt font and single-spaced text throughout the manuscript.
6. Please add a one-line space between each of your protocol steps.
7. Please define all abbreviations before use, e.g., DMSO, PBS, etc.
8. Step 3.3: Please write this step in the imperative tense.
- We have formatted the manuscript and inserted all the corrections requested in points 4–8.

9. There is a 2.75 page limit for filmable content. Please highlight 2.75 pages or less of the Protocol steps (including headings and spacing) in yellow that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.
- We have highlighted the filmable steps of the protocol.

10. Please ensure that the references appear as the following:
Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage, (YEAR).
For more than 6 authors, list only the first author then et al.
11. Please do not abbreviate journal titles for references.
- We have now formatted the references as requested in points 10 and 11.

12. Please upload each Figure individually to your Editorial Manager account as a .png or a .tiff file.
- We have uploaded the figures individually as TIFF files.

13. Please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique. Passaggi giovani
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique. Live imaging cars/drug
- The Discussion has been revised and divided into paragraphs.

Reviewers' comments:

Reviewer #1:
Manuscript Summary:
Cocco et al. describe an in vitro protocol for hepatocyte cytoplasmic vacuolation in differentiated HepaRG cells as a model for liver macrosteatosis acquired in fatty liver diseases.
-Abstract and Introduction:
Authors present a manuscript with a clear and concise introduction of the proposed methodology/protocol and how it may help to improve current caveats. In particular, they introduce the current lack of approved treatments for fatty liver disease (including NAFLD, NASH, and their eventual progression) and comment on the failure of the plethora of current research models to fulfill human disease as one of the multiple possible causes of poor translation into the Clinic. Further, differentiated HepaRG cells and the described protocol are introduced and reasoned how in vitro models may pose a fundamental role in elucidating the molecular mechanisms and become valuable tools for screening of new compounds. However, there are some potential points to improve the manuscript by consolidating introduction and discussion. For instance, some sentences are repeated in both sections. Additionally, in some cases concepts are partially introduced and resumed in the next section as at the end of introduction in page 4 where authors comment on deregulated genes in dHepaRG which may be easily quantified by PCR -without even using a cite-, just to find additional comments in "Results" section, page 8, and in "Discussion" section, page 12. Reading would be clearer whether the authors try to consolidate as much as possible.
- We thank the reviewer for his/her comments and we have tried to improve and consolidate the Introduction and Discussion following the reviewer's suggestions.

-Protocol section.
Proposed protocols are clear and well detailed in general. In the case of protocol #6: "Evaluation of lipid overloading and steatosis induction: qPCR", since author mention any commercial methods, and hence do not detail the one they follow, step 6.4 may be considered futile since most of the commercially available kits already include DNase treatment and isolated RNA would not need to be treated. As well, there is no need to do special mention to random primers for the same reason in 6.5. I would suggest author use at least another two housekeeping genes as control besides GAPDH and recalculate their results on gene expression.
In protocol #7, section 7.8 references to points 6.7 and 6.8 must be corrected.
A detailed protocol for MTT colorimetric assay should be included in the manuscript, in protocol section. Expressions as "small amount of the reagent" in page 9 should be avoided.
- As rightly suggested by the reviewer, we added another two housekeeping genes, beta-actin and ribosomal 18S genes, as qPCR controls, and we recalculated the gene expression results. We have

also described a detailed protocol for an MTT assay as section 9, and we have made modifications in line with all the other suggested corrections.

-Results: Author present plausible results, supported for figures in adequate number, size and quality, with appropriate and descriptive figure legends. As mentioned before, some parts of the text regarding dHepaRG are more appropriate for an introduction or a discussion. Authors use the terms optical depth and absorbance as if they were the same, in figure 2, page 9.

- We have made changes in line with these correctly identified terminology errors, and furthermore, in Figure 2D we have added MTT assay data on oleate vs palmitate cytotoxicity in HepaRG cells.

-Discussion.

Discussion briefly deal with the comparison between the chosen dHepaRG cells vs. other cell types/lines and it seems to be shorter than needed. In a similar way, authors barely comment on the reasons to choose oleate vs. palmitate, without showing data that, if available, would be of great interest. Also, author should use this section to further comment of the issue of cell differentiation, and how they circumvent or tackle the differentiation of biliary-like cells surrounding the hepatocyte-like cell in order to avoid this potentially cofounding factor, for instance in the ORO recovery and colorimetric measurement. Additionally, it would be of great interest whether authors may comment on the three different techniques they use for detecting and characterizing (namely ORO, Bodipy and CARS), to compare advantages/disadvantages, potential applicability, limitations, etc. Also, author propose in vitro dHepaRG as a bona fide model for histopathology -and pathophysiology- of human NAFLD. They should further elaborate to justify such statement at least regarding histopathology concept since they propose a pure culture (except for the biliary-like cell although they do not comment on these) with no other liver cell type of those present in the liver parenchyma, namely sinusoidal or Kupffer, or remove such assertion.

- We agree with the reviewer that it is far-fetched to assert that an *in vitro* cell-based model can recapitulate the histopathology—and pathophysiology—of human NAFLD and we removed that sentence. In addition, as suggested, we added MTT assay data on oleate vs palmitate cytotoxicity in HepaRG cells. Moreover, we comment in the Discussion on the significance of the detection techniques we used, and we have added a detailed comparison of ORO, Bodipy, and CARS methods.

Major Concerns:

-Authors should further discuss how to deal with differentiated biliary-like cell, as mentioned in the summary.

- We commented in the Discussion section that HepaRG mixed biliary/hepatocyte cell culture recapitulates features of liver tissue, resembling a physiological condition, despite the lack of other liver cell types (sinusoidal or Kupffer). For this reason, we did not further discuss how to deal with biliary-like cell. If a pure hepatocyte cell culture is needed, other cell-lines are more appropriate or the possibility of isolating hepatocytes from differentiated HepaRG cells, by FACS sorting, mild trypsinization, or centrifugation on an OptiPrep gradient, should be considered.

-Authors should include a detailed protocol for MTT colorimetry assay, even if brief, since the main goal of the publication is to present full protocols to the scientific community.

- We included, as rightly suggested, a detailed MTT assay protocol, in section 9.

Minor Concerns:
-Author should review typos and remove the redundant steps in the protocols as above mentioned
-As commented, discussion length is not adequate: Authors may include further comments on their choice for oleate over palmitate. Also, it will be of interest from a methodological point of view whether they compare the three presented techniques.
- We edited the manuscript as correctly suggested by reviewer, removing typos and trying to eliminate redundant steps. Moreover, we expanded the Discussion, including a comparison of the three presented lipid accumulation quantification techniques.

Reviewer #2:
Manuscript Summary:
This manuscript contains two parts
-The description of a human liver cell model , i.e. differentiated HepaRG cells, for obtaining steatotic hepatocytes with oleate overloading
-The description of protocols to detect and quantify lipid accumulation in intracellular vesicles

Major Concerns:
Surprisingly, although the model has already been well characterized the summary (lines 24 and 25) is focused only on the first part.
The origin and characterization of the HepaRG cell line have been first descripted by Gripon et al in 2002 (PNAS); this paper should be cited in 13 (instead of Marion et al.)
The description that vesicular steatosis induced by steatotic drugs and oleate can be observed in differentiated HepaRG cells was first described in 2011 (Antherieu S et al . in Hepatology). The title is very close to that of the present one : "Induction of Vesicular Steatosis by Amiodarone and Tetracycline Is Associated with Up-regulation of Lipogenic Genes in HepaRG Cells". This paper should be discussed and cited. Several other similar papers have been more recently published on the subject.
The second part on protocols is more original and interesting, in particular the method to quantify intracellular lipids using anti-Stokes 104 Raman scattering (CARS) microscopy.
The title of the manuscript should be changed and focused on Protocols to detect and quantify lipids in differentiated HepaRG cells exposed to oleate
- We agree with the reviewer on all the major concerns and we expanded and focused on the second part of the protocol, by adding more description and discussion of CARS microscopy as steatosis quantification and characterization method. In addition, we have changed the manuscript title and summary to place greater emphasis on steatosis quantification techniques alongside the liver vesicular steatosis cell-based HepaRG model, and we have cited papers that report previous characterization of the *in vitro* HepaRG cellular model

Minor Concerns:
1. Differentiated HepaRG cell cultures can also be obtained by seeding cryopreserved previously differentiated HepaRG cells; in such conditions they can be used a few days after seeding.
- The reviewer is correct and a cryopreservation step has now been added as point 2.6.

2. Please clarify the following point: was 10% serum maintained in the medium during exposure to oleate? Did 10% serum influence accumulation of lipids?

- As mentioned in point 4.1, the oleate was diluted in the complete differentiation medium, which contains 10% serum. Unfortunately, we have never tried sodium oleate exposure in the absence of serum.

3. Could you indicate cell numbers and type of dish/well appropriate for each protocol?
- As described in point 3.1, the cells have to be seeded at a density of 2.5×10^4 cells/cm^2. The type and number of dishes varies with the experimental conditions; the exceptions are for CARS microscopy, which requires the use of transparent glass-bottomed dishes (point 3.1), and the MTT assay, which requires 96-well plates (point 9.1).

4. Line 161 and others: Did you use 4% formaldehyde or 4% paraformaldehyde.
- We thank the reviewer for pointing this out. We have corrected 4% formaldehyde to 4% paraformaldehyde, which was used in the experiments.

5. Oleate treatment deregulated many genes. Were the major findings in agreement with previous works?
- In this paper we selected a panel of representative genes that are deregulated in lipid overloaded differentiated HepaRG cells, as already described by us ond others, and data presented confirmed previous work [Anthérieu et al., Hepatology 53(6), 1895-905 (2011); Rouge et al., Toxicology and Applied Pharmacology 276(1), 73-81 (2014); Belloni et al., Scientific Reports 8, 13638 (2018); Nunn et al., Scientific Reports 6, 28025 (2016)].

6. Discussion section : comparisons with other studies is usually missing. It is difficult to determine what is new in the present study
- In this manuscript we describe a point-by-point protocol for the differentiation of HepaRG cells and methods for quantification of lipid accumulation in differentiated HepaRG cells after sodium oleate treatment. Although the HepaRG cell line model has already been characterized, as indicated in the manuscript by many citations, we believe that in this manuscript we give useful information on critical steps within the differentiation process and vesicular steatosis induction protocol. In addition, we describe troubleshooting, limitations and future applications of the new CARS technique with respect to described existing methods for lipid quantification, such as Oil Red O and Bodipy staining.

7. In several figures replace comma by point , e.g. 2C and 2D (ordonnates)

Reviewer #3:
Manuscript Summary:
Di Cocco and colleagues describe a protocol for the differentiation of HepaRG cells and exposure of the obtained cells to sodium oleate to mimic hepatic steatosis, as well as an essay to evaluate intracellular triglyceride accumulation.

Major Concerns:
The originality of the paper is very limited as the differentiation protocol of HepaRG cells have been widely documented (often in the materials & methods sections) in multiple papers.
Additionally, very little information is provided regarding the used cell line. E.g. how were undifferentiate cells obtained? What were the passage numbers used during the experiments? Were cells from different batches compared? Were the cells previously cryopreserved? How were they thawed? Also, baseed on the figures presented, the HepaRG culture seems to be in a

transition stage, rather than fully differentiated. Little attention, has been put on biliary cell-like cell fraction of the culture.
Furthermore, some important references are missing, e.g. among others characterization of the HepaRG cell line "Gripon et al. "Infection of a human hepatoma cell line by hepatitis B virus." Proceedings of the National Academy of Sciences 99.24 (2002): 15655-15660." as well as application of HepaRG in oleate-induced steatosis "Rogue et al. "PPAR agonists reduce steatosis in oleic acid-overloaded HepaRG cells." Toxicology and applied pharmacology 276.1 (2014): 73-81."
- We agree with the reviewer on the limited originality of the *in vitro* model. The HepaRG cell line model has already been characterized, as we have indicated in the manuscript, citing many references including Gripon et al. and Rogue et al. (as correctly suggested by the reviewer). However, we believe that here we give useful information on critical steps within the differentiation process and on the vesicular steatosis induction protocol. In addition, we describe troubleshooting, limitations, and future applications of the new CARS technique with respect to described existing methods for lipid quantification, such as Oil Red O and Bodipy staining.
As rightly recommended by the reviewer, we inserted more information on the cell line, adding a new protocol section “Thawing, amplification, and cryopreservation of HepaRG cells” (see section 2), and mentioning prof. Christian Trepo (INSERM U871, Lyon, France), who kindly provided cells, in the acknowledgment section.

Minor Concerns:
-From the title it is unclear why the authors put emphasis on "liver DMSO-differentiated" HepaRG cells. Besides a handful of specific studies, HepaRG are always differentiated using DMSO and they were isolated from a 'liver' tumor.
- We agree with the reviewer and we have changed the title accordingly.

-The use of multiple reference genes gives a more reliable normalization of the targeted genes of interest. Expression of reference genes may differ between cell types and experimental conditions. Since the authors included only one reference gene (GADPH) for normalization, it is recommended to consider more reference genes for the normalization of the gene expression data. The use of at least 2-3 stable reference genes is required unless the authors can show the stability of GADPH in their experiments.
- As rightly suggested by reviewer, we added another two housekeeping genes, the beta-actin and ribosomal 18S genes, as qPCR controls, and we have recalculated the gene expression results.

- Page 2 Line 40: "This in vitro human model represents a valuable alternative to the in vivo mice models as well as to the primary human hepatocytes cells, which are not easily available."
A simple cellular model cannot completely replace all in vivo mouse models. In vitro systems can reduce the number of animals needed, and perhaps partially replace some animal experiments in early drug development. Please place this statement in the correct context.
- The reviewer is correct, and we agree that an *in vitro* model cannot completely replace *in vivo* models. Indeed, we suggest the HepaRG cellular model as a valuable alternative/option/possibility to study liver steatosis and not as a substitute of an *in vivo* model. A comparison between the advantages and disadvantages of *in vitro* and *in vivo* liver steatosis models is described in the Introduction, and we did not expand on these concepts within the Abstract only because of space limits.

-Page 4 Line 123: for the flow-cytometric analysis please refer to: "M. T. Donato et al., "Cytometric

analysis for drug-induced steatosis in HepG2 cells," Chem. Biol. Interact., vol. 181, pp. 417-423, 2009." Also, different BODIPY-labeled dyes exist. Specify.
- We added the suggested reference and specified that we used BODIPY 505/515.

-Page 5 Line 160: What is the difference between PBS1 and PBS?
- We corrected this misprint.

-Page 6 Line 188: Please convert rpm to x g, since the centrifugal force depends as well on the radius of the centrifuge.
- We converted rpm to x g.

-Page 7 Line 204: Please specify "Dishes can be harvested at -80 °C".
- We corrected point 6.1 (point 7.1 in the revised manuscript) as rightly suggested by the reviewer.

-Page 9 Line 283: What is "the optimal sodium oleate concentration"? Please specify.
The MTT assay is not described in the methods section.
- We corrected the sentence, as rightly suggested by the reviewer, and we added an MTT protocol as section 9.

-Figure 2D: since the control condition is compared to more than one condition in a single experiment, an ANOVA test should be used instead of a t-test.
- We performed an ANOVA test as suggested by the reviewer, comparing each condition to the control point, and it produced the same results as the t-test.

- Figure 4: No statistical analysis on this figure?
- We performed a t-test as indicated in figure legend, we decided not to insert asterisks on the histogram plot for aesthetic reasons, instead including “p-value < 0.05” in the figure legend.

-Page 12 Line 362: HepaRG cells are also derived from HCC!!!!!? Cfr Guillouzo et al. "The human hepatoma HepaRG cells : A highly differentiated model for studies of liver metabolism and toxicity of xenobiotics," Chem. Biol. Interact. (2007), 168: 66-73.
- The reviewer is correct, this sentence was not clear, and we have now corrected it.

-Page 13 Line 392 "The sodium oleate-treated dHepaRG cell-based model reflects the histopathology and pathophysiology of human NAFLD, and thereby may increase knowledge of the molecular mechanisms involved not only in the onset but also in the progression of NAFLD, providing a basis for the development of better therapeutic approaches to the disease."
It is not incorrect to state that HepaRG cells exposed to sodium oleate reflect 'the histopathology' of NAFLD based on the presence of intracellular lipid droplets.
- We agree with the reviewer and we have corrected this sentence.