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 In this paper, we show new and unpublished data and images from experiments performed as in other papers.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.