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Monday November the 30th 2020,

To the Editor,

We would like to thank you and the Reviewers for the valuable feedback. Our protocol manuscript has now been significantly strengthened by the thorough review and constructive suggestions we received. We have included new experiments and clarified the text as requested by the reviewers. You will find a point-by-point response to each reviewer's concerns below (in blue) and our changes to the text are marked with red font.

We hope that with all these changes, our manuscript will be now considered for publication in *JoVE.*

Please, do not hesitate to contact me if any further information is required.

Best regards,

Dr. Jennifer Jager

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Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.
Answer: We went through the manuscript and did our best to check the spelling and the grammar.

2. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section. Please use Calibri 12 points
Answer: We made these changes.

3. Please provide an email address for each author.
Answer: We added the email address for each author.

4. Please rephrase the Short Abstract/Summary to clearly describe the protocol and its applications in complete sentences between 10-50 words: "Presented here is a protocol …"
Answer: We made these changes.

5. Please ensure that the long Abstract is within 150-300-word limit and clearly states the goal of the protocol.
Answer: We made these changes.

6. Please ensure the Introduction include all of the following with citation:
a) A clear statement of the overall goal of this method
b) The rationale behind the development and/or use of this technique
c) The advantages over alternative techniques with applicable references to previous studies
d) A description of the context of the technique in the wider body of literature
e) Information to help readers to determine whether the method is appropriate for their application
Answer: We made these changes.

7. JoVE cannot publish manuscripts containing commercial language. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.
For example: INTERFERin, Opti-MEM, etc.
Answer: We have removed all the commercial language from the manuscript and added all the commercial products to the Table of Materials and Reagents.

8. JoVE policy states that the video narrative is objective and not biased towards a particular product featured in the video. The goal of this policy is to focus on the science rather than to present a technique as an advertisement for a specific item. To this end we ask you to use a generic term for INTEFERin.
Answer: We have removed all the commercial language from the manuscript and replaced the generic term "INTERFERin" by "transfection reagent".

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9. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note."
Answer: We made the changes when needed.

10. The Protocol should contain only action items that direct the reader to do something.
Answer: We made the changes when needed.

11. Please ensure that individual steps of the protocol should only contain 2-3 actions sentences per step.
Answer: We made the changes when needed.

12. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed?
Answer: We made the changes when needed.

13. 1.1: How many cells are seeded.
Answer: We have added the number of cells seeded per cm^2.

14. 1.2: So the cells are not divided after 100% confluency?
Answer: It is right, at confluency the fibroblasts stop to proliferate (divide) and after two days of confluency the fibroblasts are ready to differentiate into adipocytes.

15. Only one note can follow one step. In the JoVE Protocol format, "Notes" should be concise and used sparingly. They should only be used to provide extraneous details, optional steps, or recommendations that are not critical to a step. Any text that provides details about how to perform a particular step should either be included in the step itself or added as a sub-step. Other details can be moved to the discussion.
Answer: We made the changes when needed.

16. There is a 10-page limit for the Protocol, but there is a 3-page limit for filmable content. Please highlight 3 pages or less of the Protocol (including headings and spacing) 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.
Answer: We made the changes when needed. The Protocol's length is 3 pages.

17. 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]."
Answer: There are no reuse of any figures.

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18. As we are a methods journal, please ensure that the Discussion 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
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique

19. Figure: For all images with microscope please include a scale bar.
Answer: We made the changes when needed.

20. Please upload each Figure individually to your Editorial Manager account. Please combine all panels of one figure into a single image file.
Answer: We made the changes.

21. Please sort the materials table in alphabetical order.
Answer: We made the changes.

Reviewers' comments:

Reviewer #1:
Manuscript Summary:
Jager et al describe a method to reverse transfect mature 3T3-L1 with siRNAs and miRNA mimics, a protocol that is notably challenging. To overcome the challenges, they used a transfection reagent called INTERFERin and seeded differentiated 3T3-L1 onto collagen-coated plates containing the transfection complex. These settings appeared to successfully deliver small RNAs to the cells as evidenced by expression of target genes/proteins and functional assays. Although it is clear that the method works, I have some concerns that should be address.

Major Concerns:
1) Since fully differentiated adipocytes are expected to float, I am concerned that during the process of trypsin treatment and seeding, some of the adipocytes might be lost, thus creating a bias. Did the authors compare the number of cells before and after seeding? Did they notice cell loss? Did they notice any change in lipid accumulation in the cells after seeding?
Answer: It is true that mature adipocytes isolated from adipose tissue are floating cells due to high amount of lipid content. However, this is not the case for differentiated 3T3-L1 adipocytes that contains multilocular lipid droplets with lower amount of lipids compared to primary adipocytes. When we change the media 24 hours after the transfection, the cells are seeded and well attached. It can happen sometimes that the adipocytes accumulate at the center of the well and are not well attached to the plate, but this happens when the trypsin treatment is not well performed and, the cells are not well separated and homogenized before seeding.
We did compare the number of cells before and 24 hours after seeding, and did not notice any cell loss, most of the cells were still there and attached.

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We showed that the lipid content was not different between the transfected and non-transfected adipocytes (Figure 1D-E).

2) The authors should try to determine the proportion of cells that are infected with the small RNAs. Although there is a clear effect, that could be due to transfection of just a subpopulation of cells. One possibility to address this concern is by transfecting cells with a fluorescence-labelled siRNA and looking at the microscope for fluorescence distribution.
Answer: We thank the Reviewer for this comment and the suggestion. We have performed transfection with a fluorescent-labelled si-RNA (Figure 1G-H) and showed that 70-80% of the cells were transfected. We also performed experiments to knock-down perilipin-1 and analyzed the expression of perilipin using fluorescent microscopy. We showed 92% of knockdown and that most of the adipocytes were almost completely depleted of perilipin-1 and only a few cells were still expressing perilipin-1 (Figure 2D-F).

3) The conclusion regarding cell viability is based on cell function, however, cell viability may be significantly compromised despite the fact that some remaining cells continue to function. I would measure cell number and markers of cell viability.
Answer: Dying adipocytes detach and float. We did compare the number of cells before and 24 hours after seeding, and did not notice any cell loss, most of the cells were still there and attached. Moreover, we think that it is important to check adipocyte function such as lipolysis, glucose uptake, and insulin signaling because it reflects the health of the cells.

Minor Concerns:
1) Correct "adipocytes function" for "adipocyte function".
Answer: We thank the Reviewer for this comment. We have made the correction when needed.

2) In item 3.5, I assume they are referring to the collagen-coated plates. It seems obvious but I believe they should add it for the sake of accuracy.
Answer: We thank the Reviewer for this comment. We have made the correction.

3) One limitation of the assay which was pointed out by the authors is the necessity of cells reaching close to 100% differentiation prior to transfection. However, my experience working with 3T3-L1 and seeing many people work with these cells is that rarely one reaches almost 100% differentiation.
Answer: Indeed, in the discussion part of the manuscript we mentioned that one limitation of the assay is the necessity to reach a really high level of differentiation for 2 reasons:

- It is crucial to have a good differentiation into adipocyte to study adipocyte function
- If the differentiation is not good (less than 80%), after the trypsin treatment and the seeding of the cells, the remaining fibroblasts would proliferate and lead to a mixed population of cells which would have an impact on the experiments.

When using our protocol of differentiation, most of the fibroblast differentiate into adipocytes but we do use rosiglitazone though. I guess the confluency and the duration of confluency before inducing the differentiation are important points as well as the composition of the differentiation cocktail, especially the addition of rosiglitazone. We have changed the “almost 100% differentiation” by the “a high level of differentiation (>80%)”.

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Reviewer#2:

Manuscript Summary:

In this manuscript, Jennifer et al. detail a transfection protocol to manipulate the expression of proteins and non-coding RNAs in maturely differentiated adipocytes. They first use a standard procedure to differentiate 3T3-L1 fibroblasts into adipocytes and then perform a reverse transfection using INTERFERin to manipulate the levels of protein and non-coding genes. Growing evidence support the critical role of non-coding RNAs in adipose biology so this protocol covers an area of significant interest. However, there are a number of issues that need to be addressed before further consideration.

Major Concerns:

1. The authors need to confirm that their transfection protocol does not substantially alter the differentiation. They have made some attempts by examining the morphology of the adipocytes before and after transfection, but morphology is a very crude approach to evaluate cell's identity and integrity. Instead, they should use known protein, miRNA and lncRNA markers associated with adipogenesis to carefully ascertain the differentiation status after transfection.

Answer: We agree on the Reviewer's comment. We have performed additional analysis presented in Figure 1D, 1E and, 1F to confirm that our transfection protocol does not substantially alter the differentiation. Figure 1D and 1E showed that the transfection does not alter the lipid content of the adipocytes. Figure 1F showed that the mRNA expression of key proteins associated with adipogenesis and/or adipocyte function is not changed by the transfection.

2. The authors need to provide a detailed introduction of INTERFERin's chemical characteristics which is a useful information for user to understand if this reagent will interfere with their functional study after the transfection.

Answer: Since we had to remove all the commercial language from the manuscript, we have replaced "INTERFERin" by "transfection reagent". We have added some information on INTERFERin's chemical characteristics in the discussion section.

3. Reduced toxicity and enhanced efficacy are often the primary reasons for a lab to switch to a new transfection protocol. The authors need to make a comparison between INTERFERin and other popular transfection approaches such as Lipofectamine to confirm that their approach is superior in both or either category.

Answer: The main point of our assay is the reverse transfection not the transfection reagent. We have added some information on INTERFERin's chemical characteristics in the discussion section and discussed the possibility to use our protocol with other transfection reagents.

4. There are substantially more lncRNAs than miRNAs in mammalian genomes. If the authors want to claim that their approach is suitable for non-coding RNAs, they should test the knockdown efficacy of a lncRNA.

Answer: We do agree that we shouldn't claim that our approach is suitable for all non-coding RNAs since we have presented data only for micro-RNA. One should have also performed experiments with lncRNA, eRNA, Y-RNA… to make general conclusion about the efficacy of the protocol to manipulate non-coding RNA expression. We have not performed additional experiments by lack of tools and good non-coding RNA candidates (that we know and have previously manipulated) and their targets to validate their manipulation. In the revised manuscript we have replaced non-coding RNA by micro-

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RNA, and we have discussed the possibility to use this protocol for modulating other non-coding RNA such as lncRNA.

5. Most experiments were performed in duplicate which is not sufficient. The authors need to repeat their experiment in triplicate, perform statistical analyses between samples and add error bars to their figures.
Answer: We thank the Reviewer for this comment. In the revised manuscript all the presented data in the figures are the mean ± SEM of at least three independent experiments, and we have included the error bars and the statistical analysis.

Minor Concerns:
1. Is the DMEM in step 1.1 with or without pyruvate? Also, the DMEM w/o pyruvate is not in the table of materials.
Answer: We thank the Reviewer for pointing out this lack of information. The DMEM in step 1.1 is without pyruvate, we have now added this information in the text and in the table of materials.

2. The description from 3.1 to 3.5 is not clear. In section 3.3, the authors include the reagent amount used in both the 12 and 24-well plates. However, in section 3.5, the adding amount (200ul) seems to be applied only to the 12-well-plate. I recommend the authors draw a table to specify the reagent amount for 12/24-well-plate settings.
Answer: we thank the Reviewer for this comment. We have made changes to clarify the description from 3.1 to 3.5. and we have also included a table describing all the reagent amount for 12 and 24-well-plate settings.