Istituti Clinici Scientifici Maugeri

March 29th, 2021

To the Editor of *Journal of Visualized Experiments*

Dear Editor,

I propose, for your kind attention, the revised version of the manuscript entitled "Isolation of primary cancer-associated fibroblasts from a syngeneic murine breast cancer: a model to study targeted nanoparticles" by Mart Truffi, Leopoldo Sitia, Marta Sevieri, Arianna Bonizzi, Maria Antonietta Rizzuto, Serena Mazzucchelli and Fabio Corsi, to be considered for publication in *Journal of Visualized Experiments.*

Point-by point reply to reviewers and editor

Editorial comments:

Editorial Changes

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.

We thank for this suggestion. The manuscript has been proofread for grammar and spelling errors.

2. Please define all abbreviations before use.

Thank you for these corrections. The paper has been amended.

3. For in-text citations, use "…formation5,6,8." instead of "…formation.5,6,8"

The citations are now included in the article as requested.

4. Maintain a 0-inch left indent throughout the text and indicate new paragraphs using single-line spacing. Justify all text.

The paper has been amended as requested.

5. Please remove the formatting from the tables.

The tables have been amended; bold fonts and margins have been removed.

6. Please add details about the age, sex, breed of the mice used.

We thank for this suggestion. Required information has been included in the revised manuscript.

7. Line 146: Please clarify "..1/2 mice..".

The paper has been amended as requested.

8. Line 254: Could you add more details about column preparation?

We thank for this suggestion. For columns preparation it is sufficient that a magnetic stand is placed under the hood and that ferromagnetic separation columns (one column for up to 107 cells) are hung up on it with the tip pointing down. Then 0.5 mL of cold 1× Binding Buffer is placed on the column to equilibrate them. When the solution has passed through by gravity, the columns are ready for incubation with the bead/cell suspension for magnetic separation. The revised version of the manuscript now contains more details.

9. Line 560: Please check the value in Table 2.

We thank for this comment. We have checked values in Table 2, which are correct. Accordingly, we have decided to amend the text as follows:

"The depletion beads cocktail efficiently removes CD45+ cells (accounting for 67.35% and 0.69% of total cells pre- and post-depletion, respectively, Table 2)".

10. Add a single space between the quantity and its unit. E.g. use "2 mm" instead of "2mm". Please use decimal points instead of "," (line 506).

Thank you for these corrections. The paper has been amended.

11. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. 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. E.g. IVIS Lumina II, gentleMACS, etc.

The paper has been amended as requested.

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12. 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. Do not abbreviate journal names. Please include volume and issue numbers for all references.

The references have been formatted to better fit the journal requests

13. Please sort the Materials Table alphabetically by the name of the material.

The Materials Table is now alphabetically sorted

Reviewer #1:

Manuscript Summary:

Isolation of primary cancer-associated fibroblasts from a syngeneic murine breast cancer: a model to study targeted nanoparticles

This manuscript reports a method outlining the isolation of primary cancer-associated fibroblasts from a murine breast cancer cell line to enable ex vivo testing of nanoparticles. The present work is informative and well presented, it is recommended that the paper be accepted with minor revisions.

We thank the Reviewer for the overall positive judgement of our manuscript. A point-by-point response to the raised comments is available below.

The suggestions to improve the manuscript are presented below.

1. Introduction, page 1, Line 71. Replace "throughout" with "thorough".

Thank you for this correction. The paper has been amended.

2. Protocol, page 7, section 3.2.4. Replace "trice" with "thrice"

Thank you for this correction. The paper has been amended.

3. Representative results, page 12, line 554. "…dead cells is found..." replace "is" with "are"

Thank you for this suggestion. In the examined sentence the term “is” was referred to “a high percentage” instead of “dead cells”; therefore, the singular third person was used. We hope that the Reviewer agree with us to let it as “is”

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4. Figure 3. Cell Morphology. These images could benefit with altering the contrast to better show the shape/outline of the cells.

Thank you for this suggestion. The images have been adjusted for contrast.

5. Figure 3. Cell Morphology. The cells imaged do have fibroblastic morphology, but perhaps the readers would benefit here from an additional image of the native 4T1 for comparison to the isolated fibroblasts.

According to the Reviewer suggestion, a bright-field image of 4T1 tumor cells has been added as Figure 3 d.

6. Discussion, page 15, line 680. "…for long time." Insert "a" to make "for a long time".

The sentence has been amended as requested.

Reviewer #2:

Manuscript Summary:

The authors have elaborately described the methods for isolating cancer-associated fibroblasts from the murine breast cancer model. The study further employs the isolated CAFs to study the targeting capability of nanoparticles targeting the tumor microenvironment.

Minor Concerns:

1. The authors have described the methods very well which increases the reproducibility of the protocol. Furthermore, the authors have provided a list of chemicals, company names and catalogue numbers at the end which makes it easy to reproduce. However, the authors might need to check with the journal if their way of presenting the methods matches their manuscript requirements.

We thank the Reviewer for the overall positive comment to our manuscript. We have carefully followed the manuscript template from JoVE and we believe that the methods section now fits the journal requirements.

2. The authors have not mentioned the methods of statistical analysis used in this paper.

Thanks to the reviewer for pointing out this missing point. As suggested, in the revised version of the manuscript we included in the Protocol Section a new paragraph dedicated to the statistical analysis (6.3).

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3. I was wondering if the authors could organise the results in subsections. This will make it easy for the reader to focus on all points.

Many thanks for this precious suggestion. We revised the results section by creating three subsections entitled: In vivo model set-up for optimal CAF isolation;

Optimization of CAF isolation procedure, culture and characterization;

Use of isolated cells to evaluate CAF targeting potential of engineered nanodrugs.

4. I am not sure if the authors mention how many animals were used and how many times each experiment was performed in the text.

We thank the reviewer for this comment. We included the total number of animals used for the experiments in a new paragraph (6.1) of the Protocol Section.

5. I was wondering if the authors have performed any statistical tests for the flow cytometry.

Thanks for raising the topic. We did perform an analysis of the statistical differences among the cell populations found in the four quadrants after the three steps of cell isolation (reported here below). However, we did not include it in the main text, as this was mainly oriented to prove the efficacy of the protocol, while we did not want to make a comparison between ours and other methods.

6. It is not clear if the authors used any protein markers for the characterisation of CAFs.

We thank the reviewer for pointing out such an important point. Here, we first stained isolated cells with anti CD45 and CD90.2 antibodies. Then, we confirmed CAF characteristics by evaluating Fibroblast Activation Protein (FAP) expression by cytofluorimetry on isolated cells, as FAP is the most widely used and reliable marker for CAF characterization. In another study that we recently published, we further characterized isolated CAFs by evaluating FAP and α Smooth Muscle Actin (α SMA) expression by Western Blot, in comparison with 4T1 cells (Reference 32: Sitia L. et al. Cells. 2021 Feb 5;10(2):328), thus confirming the success of the isolation procedure.

7. The authors have not mentioned the number of times an experiment was performed for testing of Fbn on isolated cancer-associated fibroblasts.

Thanks for the comment. We added a paragraph in the protocol section (6.2) with the requested information.

8. In the discussion section, the authors need to mention more about nanomedicine and CAFs. Furthermore, if this method or any similar technique has been used for any other type of cancer in research.

We thank the reviewer for this suggestion that will improve our manuscript. We added two new paragraphs in the discussion, mentioning more methods of CAF isolation and some classes of nanomedicines that are being developed to specifically target CAFs.

9. Line 44 "reprogramming"

Thank you for this correction. The paper has been amended.

We have addressed to all issues raised by reviewers and we believe that this paper is now suitable for publication in *Journal of Visualized Experiments*.

All authors approve the submission of the present manuscript, which has been submitted exclusively to *Journal of Visualized Experiments.* None of the materials contained in this manuscript has been published or is under consideration elsewhere. There is no conflict of interest for this work.

Sincerely yours,
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