April 14th 2019

Point by point response to Editorial comments:

General:

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

RE. We have thoroughly checked the manuscript to eliminate spelling or grammar issues.

2. Please limit the use of personal pronouns (you, your, we).

RE. We have removed all the unnecessary use of personal pronouns.

3. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please limit the use of 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: Accutase, BD Biosciences FACSAria, Matrigel, Lipofectamine.

RE. We have removed from the manuscript text all the trademark and company names, which are now only listed in the Table of materials.

Protocol:

1. The protocol should largely be in the imperative. Please ensure that ever protocol step/substep has at least one instruction in the imperative tense.

RE. We have modified the protocol text to include the use of imperative tense.

2. For each step, please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. If revisions cause a step to have more than 2-3 actions and 4 sentences per step, please split into separate steps or substeps.

RE. In each step we addressed the "how" question, and provided clear details on how it is performed.

Specific Protocol steps:

1. 2.2: Please include a reference here for the development of RMS tumors in mice, and include age, sex, and strain information. Please also do not highlight the portion of the step including euthanasia.

RE. In step 2.2 we added references and information about the mice as suggested, and removed the highlighting from this section.

2. 2.3: If the tumor does not weight 500-1000 mg, how can you account for this?

RE. In step 2.3 we have addressed this point in the Note: "Larger amounts of tissue negatively affect the digestion steps, and decrease the overall yield. If the harvested tumor is larger than 1000 mg, divide it in parts and sample them randomly until the desired weight is reached. Random sampling is necessary for evaluating tissue heterogeneity.".

3. 3.4.2: Please include more information on the FACS procedure.

RE. We have now included more information on the FACS procedure: “Employing the unstained control set up a gate segregating alive (Fx Cycle Violet$^-$) from dead (Fx Cycle Violet$^+$) cells. Fluorescent activated cell sorting (with 450/50 filter band pass) will be then employed for separation and count of live/dead cells and for plating the desired number of live cells in each well of the 96 well low-attachment plates.”

4. 4.3.2: Please include a reference for such a literature search.

RE. We now state to utilize PubMed to perform literature search.

5. 6.7: Please mention how animals are anesthetized and how proper anesthetization is confirmed. Please specify the use of vet ointment on eyes to prevent dryness while under anesthesia.

RE. We now included details on the anesthesia procedure: “Turn on the flowmeter to 200 ml/min oxygen and the isoflurane vaporizer to 2.5 %. Anesthetize a 2 month old male NOD/SCID mouse placing it inside the induction chamber. Wait 2–3 min until the mouse appears asleep and the breeding has slowed down. Before starting the procedure first confirm, through foot pinch, that the mouse is asleep, and then apply vet ointment on the eyes.” The specific vet ointment utilized is indicated in the Table of Materials.

Figures*:*

1. Please remove ‘Figure 1’ etc. from the Figures themselves.
2. Figure 1A: Please consistently include a space between ‘)’ and ‘collagenase’.
3. Figure 1D: ‘Matrigel’ is a commercial term; please replace with a generic one.
4. Figure 2: What are the mRNA levels relative to?
5. Figure 3A: ‘Lipofectamine’ is a commercial term; please replace with a generic one (in the figure itself).

RE. All the above requested changes in the Figures have been made.

Discussion*:*

1. Discussion: As we are a methods journal, 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
c) Any limitations of the technique

RE. We have now modified the discussion to include the requested sections. We left the two initial paragraphs in this section as we believe they provide a broad perspective of this protocol within the context of the field.

References:

1. Please do not abbreviate journal titles.
RE. We have now modified the references and included full journal titles.

Table of Materials:

1. Please ensure the Table of Materials has information on all materials and equipment used, especially those mentioned in the Protocol.

RE. The Table of Materials includes a list of all the reagents and equipment used in the protocol.

Point by point response to Reviewers

Reviewer #1:

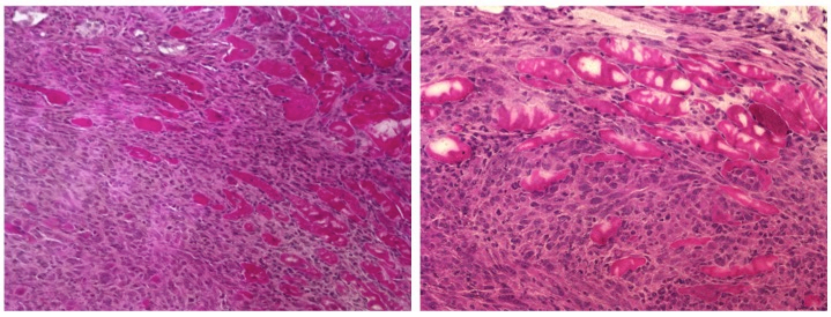
Manuscript Summary:
The authors provide a detailed hands-on protocol to isolate and culture cells from rhabdomyosarcomas (RMS) and to establish tumorspheres for allograft transplantation. This protocol will aid in developing drug screening methodologies for the identification of RMS therapeutics.

RE. Thank you.

Minor Concerns:

The authors should specify the RMS type from which cells were isolated from and confirm that tumors developing from tumorsphere transplantation maintain the original cell characteristics.

RE. Thank you for the comment. When we compared morphologically the tumors spontaneously developed in mice to the one developed in nod/scid animals after allograft transplantation, we observed similar phenotypic features. As shown in the Figure below, in both cases, the isolated tumors show muscle fibers embedded in undifferentiated areas. Moreover, in both cases we observed the presence of elongated cellular structures, identified as spindle-like cells, as well as myogenic marker expression (Boscolo et al, Cell Reports 2019). We have now included a Note in Protocol 5 section 6.8 to detail evaluation of tumor identity after allograft transplantation: “To validate identity of the tumor derived from allograft transplantation, it should be compared to the original tumor from which the cells were isolated. To this aim, histological analysis for morphological features, expression of myogenic markers and more comprehensive RNAseq can be performed.”.



H&E for Isolated RMS

H&E for tumor developed after allograft transplantation of tumorpheres

Reviewer #2:

Manuscript Summary:

In this manuscript, the authors describe a new and reproducible method for the functional identification and characterization of the tumorigenic properties of rare cell populations present in rhabdomyosarcoma, the most common soft tissue sarcoma in children. This study could help to improve the knowledge about the cell of origin of these cells as well as to validate promising treatments against this disease. Protocol section is precisely described and well-organized and graphs and images associated to the protocols are self-explained. Likewise, Discussion section contains a detailed

explanation comparing the different methods to assess the tumorigenic properties of these cell populations which have been described in the field. Below I include a list of specific points which, in my opinion, should be clarified by the authors together with a few suggestions of modifications in the text that may facilitate the understanding of some of the sections of the manuscript.

RE. Thank you.

Minor concerns:

1. Why do the authors use a 6-well low attachment plate when they manually seed the cells instead of using a 96-well low attachment plate used in the FACS experiments? Please explain. In principle (one would expect that) the results would be more reproducible by using the same protocol in both cases.

RE. Thank you for the comment. We have now modified the protocol and state to utilize 96-well low attachment plates for both manual and FACS-based plating in Protocol 2. However, we added a note at the end of Protocol 2 to explain that depending on the purpose of the assay other types of plate sizes can be used and that FACS plating or manual plating are interchangeable: “Plating cells through flow cytometer or manually can be used interchangeably, depending on the capabilities available in the laboratory. Moreover, employing low-attachment plates of sizes different than 96-well plates is possible, and dependent on the required outcome. Indeed, assessment of tumorspheres frequency should be done employing 96-well low attachment plates, whereas initial screening for assessment of cells tumorigenic potential will yield faster and still reliable results on 6-well low attachment plates.”.

2. In the section 3.6, I suggest including a brief explanation (or at least a reference) about the bases of the Celigo software used to analyze the experiments. This would be useful, especially for those readers who are not familiar with this software.

RE. We added a reference in section 3.5.2 on the use of the Celigo for analysis of spheroid strictures.

3. In the standard protocols to assess self-renewal capacity, cells are subjected to the different treatments for 2 or 3 days before being seeded (in a medium not containing the treatment) in 96-well plates. This does not seem to be the procedure that has been followed in this case to analyze the effect of the treatment with recombinant proteins on tumorsphere formation. Please discuss or modify accordingly the protocol.

RE. Thank you for the comment. The reason why in this protocol we treat suspension cells is due to the short half-life of the proteins employed and the extended length of our experiment. For short term experiments, like the treatment we described for plasmid transfection, treating the cells before induction of tumorspheres formation is a viable option.

4. When describing the effect of the treatment with recombinant proteins on cell viability (protocols 3 and 4), the authors might want to discuss the possibility of using other techniques to estimate cell viability in addition to trypan blue such as crystal violet staining or MTT.

RE. We have now implemented the protocol and included these other methods to assess cells viability.

5. In allograft transplantation protocols, in my opinion it is highly recommendable the use of tumorspheres medium instead of PBS to mix the cells with Matrigel as this frequently facilitates obtaining higher rates of tumor formation/efficacy and/or a shorter latency periods to tumor onset. The authors should discuss this possibility in the text. Likewise, authors may want to discuss the possible advantage of using lower ratios of Matrigel:PBS/tumorsphere medium.

RE. We apologize for the error. We actually used tumor cells media: matrigel 50:50 dilution for cells transplant, as described in step 6.4 of protocol 5. We corrected the statement in step 6.1 of protocol 5.

6. The authors might want to consider the possibility of including a paragraph in the discussion section to elaborate on whether plating the cells in tumorsphere medium before seeding them in a 96 well-plate may facilitate the identification of/the obtention of cultures enriched on rare populations of rhabdomyosarcoma cells.

RE. The early passages have the goal of eliminating debris and dead cells that might affect cell viability in the experiment. Culturing the primary cells isolated from mouse RMS for longer passages did not result in the enrichment of tumor initiating cells. Indeed, some of the cell lines we derived from the mice had higher tumorspheres' formation frequencies when tested at lower passages rather then higher passages.

Reviewer #3:

Manuscript Summary:

It is a good manuscript well written and discussed, describing the protocol to obtain tumorspheres from primary cultures of rhabdomyosarcoma tumors from mouse. The techniques are well described and can be very useful for people working in the field.

RE. Thank you.

Minor Concerns:
Some little mistakes have to be corrected:
- accutase: should be better described the first time that appears on the text: page 5, line 165.

RE. The editor suggested to identify the reagents with general names rather than commercial names, thus we changed accutase to 'cell detachment solution' which should address this concern.

-Legend to Figure 1, panel (E) does no t exits on the figure, it should be (D).

RE. We corrected the Figure 1 legend.