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Tumorsphere derivation and treatment from primary tumor cells isolated from mouse rhabdomyosarcomas

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Cover Letter

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April 14th 2019

Dr. Stephanie Weldon
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Dear Stephanie,

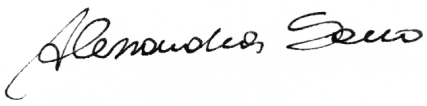
In response to your invitation, please find enclosed our resubmitted manuscript entitled "Tumorsphere derivation and treatment from primary tumor cells isolated from mouse rhabdomyosarcomas" for consideration for publication in JoVE.

We have now addressed all the editorial and reviewers' comments, as discussed in the "Detailed response to Editorial and Reviewers Comments".

Overall, the described method allows reliable identification and testing of rare RMS tumorigenic populations, that can be applied to RMS arising in different contexts. Finally, the protocol can be utilized as a platform for drug screening and the future development of therapeutics.

Thank you in advance for your consideration of our manuscript for publication. We greatly appreciate your time and effort on our behalf.

Sincerely,



TITLE:

Tumorsphere Derivation and Treatment from Primary Tumor Cells Isolated from Mouse Rhabdomyosarcomas

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KEYWORDS:

tumorspheres, rhabdomyosarcoma, skeletal muscle, primary cells, recombinant protein treatment, plasmid transfection

SUMMARY:

This protocol describes a reproducible method for isolation of mouse rhabdomyosarcoma primary cells, tumorsphere formation and treatment, and allograft transplantation starting from tumorspheres cultures.

ABSTRACT:

Rhabdomyosarcoma (RMS) is the most common soft tissue sarcoma in children. Although significant efforts have enabled the identification of common mutations associated with RMS and allowed discrimination of different RMS subtypes, major challenges still exist for the development of novel treatments to further improve prognosis. Although identified by the expression of myogenic markers, there is still significant controversy over whether RMS has myogenic or non-myogenic origins, as the cell of origin is still poorly understood. In the present study, a reliable method is provided for the tumorsphere assay for mouse RMS. The assay is based on functional properties of tumor cells and allows the identification of rare populations in the tumor with tumorigenic functions. Also described are procedures for testing recombinant proteins, integrating transfection protocols with the tumorsphere assay, and evaluating candidate genes involved in tumor development and growth. Described further is a procedure for allograft transplantation of tumorspheres into recipient mice to validate tumorigenic function *in vivo*. Overall, the described method allows reliable identification and testing of rare RMS tumorigenic populations that can be applied to RMS arising in different contexts. Finally, the

protocol can be utilized as a platform for drug screening and future development of therapeutics.

INTRODUCTION:

Cancer is a heterogeneous disease; furthermore, the same type of tumor can present different genetic mutations in different patients, and within a patient a tumor is composed by multiple populations of cells[1]. Heterogeneity presents a challenge in the identification of cells responsible for initiating and propagating cancer, but their characterization is essential for the development of efficient treatments. The notion of tumor propagating cells (TPC), a rare population of cells that contribute to tumor development, has been previously extensively reviewed[2]. Despite the fact that TPCs have been characterized in multiple types of cancer, the identification of markers for their reliable isolation remains a challenge for several tumor types[3-9]. Thus, a method that does not rely on molecular markers but rather on TPC functional properties (high self-renewal and the ability to grow in low-attachment conditions), known as the tumorsphere formation assay, can be widely applied for the identification of TPCs from most tumors. Importantly, this assay can also be employed for expansion of TPCs and thus directly applied to cancer drug screening and studies on cancer resistance[1,10].

Rhabdomyosarcoma (RMS) is a rare form of soft tissue sarcoma most common in young children[11]. Althoug RMS can be histologically identified through assessment of expression of myogenic markers, the RMS cell of origin has not been univocally characterized due to the multiple tumor subtypes and high heterogeneity of the tumor developmental stimuli. Indeed, recent studies have generated significant scientific discussion about whether RMS is of myogenic or non-myogenic origins, suggesting that RMS may derive from different cells types depending on the context[12-17]. Numerous studies on RMS cell lines have been performed employing the tumorsphere formation assay for the identification of pathways involved in tumor development and characterization of markers associated with highly self-renewal populations[18-21].

However, despite the tumorsphere formation assay's potential for identifying RMS cells of origin, a reliable method that can be employed on primary RMS cells has not yet been described. In this context, a recent study from our group employed an optimized tumorsphere formation assay for the identification of the RMS cells of origin in a Duchenne muscular dystrophy (DMD) mouse model[22]. Multiple pre-tumorigenic cell types, isolated from muscle tissues, are tested for their ability to grow in low-attachment conditions, allowing the identification of muscle stem cells as cells of origin for RMS in dystrophic contexts. Described here is a reproducible and reliable protocol for the tumorsphere formation assay (**Figure 1**), which has been successfully employed for the identification of extremely rare cell populations that are responsible for mouse RMS development.

PROTOCOL:

The housing, treatment, and sacrifice of mice were performed following the approved IACUC protocol of the Sanford Burnham Prebys Medical Discovery Institute.

1. Reagent preparation

1.1. Prepare 100 mL of cell isolation media: F10 medium supplemented with 10% horse serum (HS).

1.2. Prepare 50 mL of collagenase type II solution: dissolve 1 g of collagenase type II powder in 50 mL of cell isolation media (note the units of the enzyme per 1 mL of media, since the number of units changes depending on the lot). Aliquot the solution and store in a -20 °C freezer until ready for use.

NOTE: Every lot of collagenase should be tested before use, since the enzyme activity may change across different lots.

1.3. Prepare 10 mL per sample of second digestion solution: cells isolation media with 100 units/mL of collagenase type II solution and 2 units/mL of dispase II freshly weighted. Prepare immediately before use.

1.4. Prepare 500 mL of tumor cells media: DMEM high glucose supplemented with 20% FBS and 1% pen/strep.

1.5. Prepare 500 mL of FACS buffer: 1x PBS supplemented with 2.5% v/v normal goat serum and 1 mM EDTA.

1.6. Prepare 500 mL of tumorsphere media: DMEM/F12 supplemented with 1% pen/strep. Just before use, add the following growth factors: 1% N2 supplement, 10 ng/mL EGF, and 10 ng/mL β-FGF.

2. Cell isolation and culture

2.1. Prepare a 10 cm plate containing 5 mL of cells isolation media (one plate per tumor sample) and place it in an incubator at 37 °C until ready to harvest the tumor tissue.

2.2. RMS have been reported to spontaneously develop in both male and female mouse models for Duchenne muscular dystrophy, such as B10 mdx mice at around 18 months of age and in B6 mdx/mTR mice by 9 months[22,23]. Anesthetize the mouse that develops the RMS tumor using isoflurane, and sacrifice the animal through cervical dislocation or according to the IACUC guidelines of the institution. With scissors, make an incision on the skin in the area where the tumor is localized, and (using tweezers) pull the skin away from the area of interest. Employing a razor blade, excise the tumor from the animal.

2.3. Weigh 500–1,000 mg of the tumor tissue and place it in the plate prepared in step 2.1 (**Figure 1A**).

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 into parts and sample randomly

until the desired weight is reached. Random sampling is necessary for evaluating tissue heterogeneity.

2.4. Place the plate containing tumor tissues in the sterile cell culture hood and mince it with a razor blade. Tumor tissue from RMS is heterogeneous; thus, different areas may present different resistance to the cuts. Make sure that the sizes of the resulting minced pieces are uniform to ensure optimal digestion.

NOTE: RMS exists as mixtures of different tissue types (mainly fibrotic, vascularized, and fatty tissues) that can be identified in every tumor. To 1) isolate a heterogeneous cell population accurately recapitulating the composition of the original tumor and 2) not bias the isolation procedure, perform random sampling of the harvested tissue. Cells from different areas of the tumor should be digested and tested in parallel *in vitro* in the assay described in section 3.

2.5. Move the minced tissue and cell isolation media in a 15 mL centrifuge tube, wash the plate with 4 mL of cell isolation media and place it in the tube.

2.6. Add 700 units/mL of collagenase solution to digest the tissue and incubate in a shaking water bath at 37 °C for 1.5 h.

2.7. After incubation, spin down the tissue at 300 x *g* for 5 min at RT. Aspirate the supernatant without disturbing the pellet, resuspend the pellet in 10 mL of second digestion solution (dispase), and incubate in a shaking water bath at 37 °C for 30 min.

2.8. Once the second digestion is completed, pipet up and down and pass the cell suspension through a 70 µm nylon filter on a 50 mL centrifuge tube. Then add 10 mL of cell isolation media to wash the filter and dilute the digestion solution, and spin down the tissue at 300 x *g* and RT for 5 min.

2.9. Aspirate the supernatant and resuspend the pellet in 20 mL of tumor cells media. Then transfer the cell suspension in a 15 cm cell culture plate. Place the cells in the incubator at 37 °C overnight. This plate will be identified as P0.

2.10. The day after isolation, change the media. This step is necessary for ensuring removal of debris and dead cells that may negatively influence cell survival.

2.11. Assess cell confluency after media is changed, which ranges from 30%–60% depending on the amount of starting material and cell size. Leave the cells growing in the incubator until they reach 90% confluency. Monitor cells every day and change media every 2 days. The time necessary for tumor cells to become confluent varies depending on multiple parameters: tumor aggressiveness, genotype of the tumor, age of the mouse, heterogeneity of the tissue.

2.12. For cell passaging, do the following:

2.12.1. Pre-warm the cell detachment solution and tumor cells media in a water bath at 37 °C.

2.12.2. Wash the cells with 1x sterile PBS and incubate them at 37 °C in 10 mL of warm cell detachment solution for 5–10 min.

2.12.3. When all the cells are detached from the plate, add 10 mL of warm tumor cells media, move the solution into a 50 mL centrifuge tube and spin cells down at 300 x *g* for 5 min at RT.

2.12.4. Resuspend the cells in 5–10 mL of tumor cells media, depending on the pellet size, and count live cells using Trypan blue (1:5 dilution) to exclude dead cells.

2.12.5. Plate 10^5 cells in 10 cm plates or 3 x 10^5 cells in 15 cm plates. Cell doubling time varies depending on the factors detailed in step 2.10.

3. Tumorsphere derivation

3.1. Use tumor cells at passage P1 or P2 to avoid cell selection through multiple passages (**Figure 1B**). To detach cells from the plate, first wash the dish with 1x PBS, without disturbing the cells, then cover them using cell detachment solution (5 mL for a 10 cm plate or 15 mL for a 15 cm plate) and place them in the incubator for 5–10 min.

3.2. Confirm that cells are detached by looking at the plate under a bright-field microscope, add 1:1 volume of tumor cells media (cell detachment solution:tumor cells media), place the cell suspension in a centrifuge tube and spin the cells down at 300 x *g* for 5 min at RT.

3.3. Resuspend cells in either FACS buffer (section 3.4) or tumorspheres media (section 3.5), according to the method used for plating.

3.4. Plating cells through flow cytometer

3.4.1. Resuspend cells in FACS buffer (the amount depends on the pellet size) and manually count live cells using Trypan blue exclusion. Make sure that the final cell concentration is 10^7 cells/mL (100 µL of FACS buffer per 10^6 cells). Add 1 µL of Fx Cycle Violet stain per 10^6 cells to discriminate live from dead cells during cell sorting. Prepare an unstained control in which Fx Cycle Violet stain is not added to the cells.

NOTE: The concentration is optimized for efficient staining and speed during sorting. A lower cell concentration will result in a longer sorting time, while a higher concentration will affect staining.

3.4.2. Employing the unstained control, set up a FACS gate segregating alive (Fx Cycle Violet$^-$) from dead (Fx Cycle Violet$^+$) cells. Then, employ fluorescent-activated cell sorting (with 450/50 filter band pass) 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. Each well of the plate needs to be filled with 200 µL of tumorspheres media before starting the sorting.

NOTE: Given that TPCs are a rare subpopulation within the whole tumor, optimize the protocol by plating 100 cells/well from mouse RMS to observe the formation of tumorspheres in suspension culture. The cell number per well should be adjusted for the specific tumor tested.

3.4.3. Place cells in the incubator until the end of the experiment. Try not to disturb the plate unless necessary. For a 30 day experiment, each well should be replenished with media and an appropriate proportion of growth factors every week (media tend to evaporate and growth factors are not effective after 1 week).

3.4.4. After completion of the experiment, manually screen plates under a bright-field microscope to identify tumorspheres (see step 3.6).

NOTE: A timepoint of 30 days was optimized to easily detect mouse RMS tumorspheres of sizes ranging from 50–300 µm of diameter. The timepoint should be adjusted depending on the aggressiveness of the tumor tested and its proliferative rate.

3.5. Plating cells manually

3.5.1. Resuspend cells in tumorsphere media (the amount depends on the pellet) and manually count live cells using trypan blue (1:10 dilution). Calculate the cell concentration in the tube and plate the proper number of cells in a 96 well low attachment plate. Place cells in the incubator until the end of the experiment. Try not to disturb the plate unless necessary for replenishing media and growth factors, as described in step 3.4.3.

3.5.2. After completion of the experiment, screen plates manually under a bright-field microscope to identify tumorspheres or using the Celigo software, as previously described in Kessel et al.[24] See step 3.6 below.

3.6. Note that two separate readouts can be evaluated as result of this assay: number and size of formed tumorspheres.

NOTE: When more than one cell is plated in a well, either tumorspheres or cell clusters can form (**Figure 1C**, third and fourth panels). Cell clusters are small cellular aggregates that form in suspension culture that enhance cell survival and are characterized by an irregular shape. Tumorspheres are large and have a more compact structure with a spheroid shape. They derive from a single cell that has the ability to survive in an anchorage-independent manner and proliferate at a high rate[25]. 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 sized different than 96 well plates is possible and dependent on the required outcome. Indeed, assessment of tumorsphere frequency should be done employing 96 well low attachment plates, whereas initial screening for assessment of cells tumorigenic potential will yield faster and reliable results on 6 well low-attachment plates.

4. Tumorsphere treatment with recombinant proteins

4.1. Repeat steps 3.1 and 3.2.

4.2. If setting up a treatment with recombinant proteins, first determine the best concentration to use following section 4.3, or if the optimal concentration has been previously determined, skip to section 4.4.

4.3. Determine the recombinant protein treatment concentration.

4.3.1. Resuspend the cells in tumorsphere media (the volume depends on pellet size) and manually count live cells using Trypan blue exclusion. Calculate cell concentration in the tube and plate 100,000 cells in a 6 well low attachment plate. Plate two wells per each concentration tested and two wells for untreated controls (**Figure 2**). Protein concentrations to be tested are based on literature search.

4.3.2. Treat each well of suspension cells with a different protein concentration and place the cells in the incubator for 48 h (time necessary to be able to evaluate the effect of the treatment on both cell viability and on the expression of downstream target genes). Then, assess the following parameters:

4.3.2.1. Cell survival: using a bright-field microscope as well as comparison with untreated controls, check cell morphology. Healthy cells appear bright and reflective under the microscope, while excessive cell death will induce the accumulation of debris in the media. For a quantifiable determination of cell death, Trypan blue exclusion, crystal violet staining, MTT, or TUNEL assay can be employed (in this case, add one well to the original plating).

4.3.2.2. Effect of recombinant proteins on downstream pathways: perform a literature search using PubMed for identification of the downstream genes known to be affected by the tested protein. Design qRT-PCR primers for the target genes, and perform qRT-PCR analysis on the RNA isolated from the treated cells (**Figure 2**).

4.4. Treat with recombinant protein.

4.4.1. Resuspend the cells in tumorsphere media (the volume depends on pellet size) and manually count live cells using Trypan blue exclusion. Determine the total number of cells needed for the desired experiment (100 cells per each well of a 96 well low attachment plate) and dilute them in the appropriate tumorsphere media volume. If performing more than one treatment, prepare separate cell tubes.

4.4.2. Treat each tube with the appropriate concentration of recombinant protein and plate the cells in a separate well of 96 well low-attachment plate. The treatment will be repeated on each well depending on the half-life of the recombinant protein until the 30 day endpoint of the experiment.

4.4.3. At the end of the experiment, follow steps 3.4.4 and 3.6 to analyze the data.

5. Tumorsphere treatment with overexpression plasmids

5.1. Repeat steps 3.1 and 3.2.

5.2. If setting up the treatment on a new tumor type, first determine the best concentration of plasmid to use following section 5.3, or if the optimal DNA concentration has been previously determined, skip to section 5.4.

5.3. Determine optimal plasmid concentration.

5.3.1. Resuspend cells in tumor cells media (the volume depends on pellet size) and manually count live cells using Trypan blue exclusion. Plate the counted cells to achieve a confluency from 70%–90% (cell number is highly dependent in cell size and morphology). GFP-plasmid will be employed to test transfection efficiency following the manufacturer protocol of the transfection reagent for adherent cells. In parallel, also test an untreated control (**Figure 3A**). Perform an efficiency test in a 24 well plate.

NOTE: Adherent cells are used to enhance the transfection efficiency, as transfection performed on suspension cells is not efficient and negatively affects cell viability.

5.3.2. 48 h after transfection assess cells for the following parameters:

5.3.2.1. Cell survival: using a bright-field microscope, compare the number of cells present in each-well and compare it to the untreated cells well.

5.3.2.2. GFP expression: count the percentage of cells that are GFP positive over the total number of cells in each well (**Figure 3B**).

5.4. Overexpression plasmid treatment

5.4.1. Resuspend the cells in tumor cell media (the volume depends on pellet size) and manually count live cells using Trypan blue exclusion. Plate 200,000 cells per well of a 6 well plate. Each well will be used for an independent transfection event. Each well will be transfected using the set-up developed for the specific tumor type (**Figure 3A**).

5.4.2. 24 h after transfection, wash each well with 1x PBS and incubate the cells in warm cell detachment solution (enough to cover the well). Place the plate in the incubator at 37 °C for 5–10 min, depending on factors detailed in section 2.15.

5.4.3. When cells are detached, count the cells derived from each single well independently using Trypan blue exclusion. Place 100,000 cells per well of a 6 well low attachment plate, making sure

not to mix cells derived from different wells. Place the plate in the incubator at 37 °C and leave undisturbed for a week.

NOTE: The duration of this assay is 7 days, a sufficient time to allow tumorsphere formation while preventing tumorsphere fusion. Tumorsphere fusion is a phenomenon that becomes evident when 100,000 or more cells are plated together in suspension for over 1 week, which can bias the assessment of tumorsphere formation ability. In the event that the experiment requires longer incubation time, cell density should be adjusted or polymeric scaffolds used to avoid tumorsphere fusion[26].

5.4.4. At the end of the experiment, follow steps 3.5.2 and 3.6 to analyze the data.

6. Tumorsphere preparation for allograft transplantation

6.1. Place extracellular matrix (ECM) solution (50 µL per allograft) and tumor cells media (50 µL per allograft) on ice.

6.2. Tumorspheres can be used for allograft transplantations. Pull together all the tumorspheres obtained from a specific cell type or treatment into a 15 mL or 50 mL tube (according to the total volume of media) (**Figure 1D**). Spin the tumorspheres down at 300 x *g* for 5 min at RT. Remove the supernatant over the tumorspheres and wash them with 10 mL of sterile 1x PBS.

6.3. Spin the tumorspheres down again at 300 x *g* for 5 min at RT, aspirate the 1x PBS, and add 500 µL to 1 mL of cell detachment solution on top of the cell pellet, which starts the dissociation process. Incubate tumorspheres in digestive solution into a shaking water bath at 37 °C and check the progression of the digestion every 10 min. To help tumorspheres dissociation into a single cell solution, pipette the cells up and down for mechanical disruption. The digestion process might take up to 30 min.

NOTE: Despite the fact that incubation time varies considerably when tumorspheres are derived from different primary RMS cells, a significant decrease in cell viability in relationship to digestion time was never observed.

6.4. When a single cell solution is obtained, add a volume of tumor cells media (1:1, cell detachment solution: tumor cells media) and spin it down at 300 x *g* for 5 min at 4 °C.

NOTE: From this time on, all the steps need to be performed on ice. After spinning, tumorspheres do not form a stable pellet as the single cells solutions do. To make sure not to dislodge or aspirate the tumorspheres, use a 1 mL pipette and gently aspirate the liquid. Move to a 200 µL pipette when only 1 mL remains.

6.5. Resuspend cells in cold tumor cells media (the volume will depend on pellet size), place the cells on ice and count live cells using Trypan blue exclusion. After determining the proper amount of cells to use for the allograft, resuspend them in a total volume of 50 µL of cold tumor cells

media. Cool down a pipette tip in cold tumor cells media. When the tip is cold, use it to take 50 µL of ECM solution and add it to the tube containing the cells. Do not remove the tubes from the ice during this process.

NOTE: The number of cells to use for the transplant should be determined according to the tumor tested and experimental goal: a larger number of transplanted cells will decrease the time of tumor development (20,000 cells from mouse RMS tumorspheres have been shown to develop into a tumor 6 weeks after injection). To be able to compare different cell lines or different treatments, it is important to start from the same number of cells.

6.6. As cells are now ready for transplantation, maintain on ice until injection. Place a capped 0.5 mL insulin syringe with a 29 G needle on ice, to prevent the cell solution from becoming solid upon aspiration.

6.7. 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 by 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 a foot pinch that the mouse is asleep, then apply vet ointment on the eyes. Shave the right side of the animal, aspirate the cell solution in the pre-cooled syringe, and inject them subcutaneously into the shaved area. A visible bump under the skin will form if the injection is performed correctly.

NOTE: Cell allografts can be performed in the same mouse strain as that of the transplanted cells. For example, if the RMS cells were originally isolated from a C57BL/6 mouse, the allograft can be performed in C57BL/6 mice. If the strain is different, then immunodeficient recipient mice should be utilized to avoid rejection. The ages of recipient mice can also be adjusted depending on the experimental goal.

6.8. Monitor the mice for tumor formation once per week.

NOTE: 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 and expression of myogenic markers as well as more comprehensive RNAseq can be performed.

REPRESENTATIVE RESULTS:

Tumorspheres detection

Cell isolation was optimized to obtain the maximum heterogeneity of cell populations present in the tumor tissue. First, since isolated tissues presented morphologically dissimilar areas, to enhance the chances of isolating uniform rare cell populations, sampling was performed from multiple areas of the tumor (**Figure 1A**, first panel on the left). Second, mechanical dissociation of the harvested samples was performed while maintaining homogeneity in the minced tissue size, despite different resistances that may have been present across the samples (**Figure 1A**, third and fourth panel from the left). Depending on the starting material (tumor aggressiveness,

age and genotype of mouse, tumor location), recovery of the cells from the mechanical stress of the isolation process may vary, ranging from 3–7 days (**Figure 1A**, last panel on the right). To enhance cell survival and growth, media should be changed the day after isolation and then every 2 days. This will remove the debris and dead cells accumulated during the isolation process that might affect to cell viability. Tumorsphere formation assay should start after the cells have been passaged at least once to ensure optimal viability when placed in suspension cultures (**Figure 1B**, first panel on the left). In our hands, optimal results were obtained starting with cells from passage 2 (P2) (**Figure 1C**, first and second panel on the left). This specific passage was chosen after multiple testing. When P0 cells were plated in low-attachment conditions, they formed a low number of tumorspheres compared to later passages, possibly due to cellular debris still present after isolation. In later passages, different patterns of tumorsphere formation were observed and attributed to the selection that occurs after numerous passages in culture. When different cell lines are compared, it is suggested to start from the same passage.

Discrimination between tumorspheres and cell clusters is of fundamental importance for quantification of the assay. **Figure 1C** (last two panels on the right) clearly shows the morphological differences between a tumorsphere (left) and a cell cluster (right). Tumorspheres derive from a single cell that has a high ability to self-renew and grow in low-attachment conditions (both features of TPCs). Indeed, tumorsphere development indicates tumorigenicity potential. However, this assay is performed *in vitro* independently from the cues derived from the whole organism; thus, to validate cells' tumorigenic potential *in vivo*, allografts transplantation experiments should be performed (**Figure 1D**).

Validation of recombinant proteins treatment

Before setting up tumorspheres treatment, the optimal concentration at which the protein of interest triggers an effect on tumor cells should be determined. To do so, assessment of the level of expression of the protein's downstream target genes is necessary (**Figure 2**). A literature search on PubMed was performed before determining the target genes to test through qRT-PCR. In the event that the protein of interest has been shown to modulate different downstream pathways, selection of multiple genes associated with each of these pathways should be performed. For instance, Flt3l (Fms-like tyrosine kinase 3) has been shown to modulate STAT5 signaling in acute myeloid leukemia, inducing the downstream expression of p21, c-Myc, and CyclinD1 (**Figure 2**)[27]. Moreover, expression of Flt3l is required for dendritic cells differentiation mediating activation of STAT3 signaling pathway[28]. To assess STAT3 activity, Socs3 and CyclinD1 expression were checked (**Figure 2**). Analysis of the results showed a dose dependent effect of recombinant protein treatment on some of the tested genes (p21, CyclinD1, and Socs3), whereas others were not affected (c-Myc). It is important to determine the downstream genes responsive to the protein of interest in the specific tumor tested for reliable assessment of treatment effectiveness.

Optimization of the protocol for plasmid transfection

To establish an efficient protocol for plasmid transfection and further tumorsphere formation assay, the effects of transfection on adherent tumor cells were tested (**Figure 3A**). Transfection reagent treatment was performed following the manufacturer's protocol, and two different

amounts of the reagent were tested. Efficiency was assessed employing a GFP reporter plasmid. In our hands, we observed a higher transfection efficiency using lower amounts of the reagent (**Figure 3A**). Indeed higher concentrations lead to increased cell death, starting at 48 h and becoming more evident after 72 h from the time of transfection. The same transfection protocol was not efficient in cells in suspension, as accumulation of dead cells and cellular debris became evident 24 h after the beginning of the treatment, indicating decreased cellular viability. To overcome this technical challenge, a two steps protocol was employed: perform transfection on adherent cells, detach them 24 h after the treatment, and plate them in suspension for 7 more days. Control tumorspheres were indeed expressing GFP at the end of the experiment (**Figure 3B**).

FIGURE AND TABLE LEGENDS:

Figure 1: Tumor cell isolation, tumorsphere derivation, and transplantation. (A) Schematic representation of section 2 of the protocol (tumor cell isolation). Each key step of the protocol is summarized. **(B)** Schematic representation of section 3 of the protocol (tumorspheres derivation). Each key step of the protocol is summarized. **(C)** From left: bright-field image of isolated tumor cells at passage 2 (P2) at low magnification (scale bar = 50 µm) and high magnification (scale bar = 50 µm); bright-field image of a tumorsphere derived from tumor cells after 30 days in suspension culture (scale bar = 250 µm), and bright-field image of a cell cluster formed from tumor cells after 30 days in suspension culture (scale bar = 50 µm). **(D)** Schematic representation of section 6 of the protocol (tumorsphere allograft transplantation). Each key step of the protocol is summarized.

Figure 2: Validation of downstream target genes for determination of recombinant protein concentration. qRT-PCR results for assessment of Flt3l treatment concentration. Two-way ANOVA was performed. Significance is shown for the comparison with non-treated control. (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; $n = 3$).

Figure 3: Set up of tumorspheres transfection protocol. (A) Representative bright-field and fluorescent images of tumor cells treated with low (top) or high (bottom) concentration of transfection reagent (0.75 µL or 1.5 µL in 24 well plates) (scale bar = 50 µm). **(B)** Representative image of tumorspheres formed from GFP plasmid-treated cells, 7 days after plating the cells in suspension (scale bar = 50 µm).

DISCUSSION:

Multiple methods have been employed for isolation and characterization of TPCs from tumor heterogeneous cell populations: tumor clonogenic assays, FACS isolation, and tumorsphere formation assay. The tumor clonogenic assay was first described in 1971, used for stem cell studies, and only subsequently applied to cancer biology[29,30]. This method is based on the cancer stem cells intrinsic property to expand without constraints in soft gels cultures[31]. Since its development, this method has been widely used in cancer research for multiple purposes, including tumor cell heterogeneity studies, effect of hormonal treatment on cell growth, and

tumor resistance studies[31]. To date, this assay is still employed for the identification of tumor initiating cells for multiple types of cancers[32].

FACS isolation is based on prior knowledge of molecular markers present on the surface of cells on interest. It has been widely utilized for the isolation of TPCs from both liquid and solid tumors. For example, the first identification of human acute myeloid leukemia (AML) initiating cells was performed by Dr. Dick's group by utilizing FACS fractionation and transplantation assays based on the knowledge of the markers present on bulk AML cells[9]. Utilizing a similar approach, Dr. Clarke's group isolated breast cancer initiating cells[3]. Tumorsphere formation assay is a different approach used for the identification and study of TPCs. This method was first developed to identify cancer stem cells from brain tumors[33]. Interestingly, it was initially tested using the same conditions known to favor neural stem cells growth, thus favoring the self-renewal property also associated with TPCs[33]. Moreover, tumorsphere formation relies on the capacity of TPCs to growth in an anchorage-independent manner.

The three above described approaches can be used in parallel to enhance the probability to isolate and molecularly characterize TPCs populations, overcoming the limitations of each method. For instance, FACS, despite bringing the great advantage of isolating pure cell populations, strongly relies on the use of surface markers that are not yet known for all cancer types. Thus, its use is limited to the isolation of TPCs expressing known markers. Tumor clonogenic and tumorsphere formation assays are both based on cellular properties, known to be associated with TPCs. Both these methods can be employed as the first line of studies on new or not yet studied cancers. Moreover, these two methods can ensure the expansion and enrichment of the cell population of interest, facilitating the identification of molecular markers, and allowing for both cancer resistance and drug screening studies. In this context, tumorsphere formation assay is more advantageous, since these spheroid structures better recreate the environment present in tumor tissues (hypoxic areas in the center of the spheres)[34]. Indeed, it has been previously shown that 3D cultures are more reliable for predicting drug treatments outcome[34]. Tumorspheres can be recovered after culture, and used for allograft experiments: digestion of tumorspheres into single cells solutions and transplantation into recipient mice will allow for *in vivo* assessment of tumorigenic capacity of newly identified TPCs, compared to bulk tumor cells.

To successfully obtain a starting cellular material representative of tumor heterogeneity, it is critical to first perform random sampling of the tissue. RMS are characterized by fibrotic, fatty, or highly vascularized areas which are clearly distinguishable in isolated tumors, thus, to maintain this cellular diversity, collection of each area of the tissue will be required. Moreover, to enhance the chances of cells survival during the digestion process, mincing of the collected tissue needs to result in evenly sized pieces: smaller fragments are more likely to be overdigested inducing reduction of cells viability. This may be particularly tedious due to the diverse morphology and stiffness of RMS.

For quantification of the result of tumorsphere formation assay, it is of critical importance to distinguish between real tumorspheres and cellular clusters (**Figure 1C**, last two panels on the

right). A tumorsphere is solid spheroid structure in which it is not possible to discriminate the cell composiiton; in contrast, in a cell cluster, single cells can be easily discriminated. Cell clusters may not assume a rounded shape and are significantly smaller when compared to tumorspheres, which range between 50–250 μm[25].

To achieve tumorspheres transplantation, obtaining a uniform single cells solution is a key step. Indeed, given the tight structure and large size of a tumorsphere, digestion of the cells becomes a major limiting step in the preparation of a single cell suspension that is further employed for transplantation. Multiple cycles of enzymatic digestion combined with mechanical dissociation are thus necessary for complete dissociation of tumorspheres. To confirm the progress of dissociation and a successful outcome, the solution must be monitored under a bright-field microscope.

Despite the multiple advantages provided by the tumorsphere formation assay, tumorspheres have been shown not to originate from every type of tumor or from all commercially available cell lines. In these cases, the assay cannot be employed as the standard for determining cell tumorigenicity and quantification of TPCs within a heterogeneous population. Another limitation of this assay is associated with the fact that different tumor types require different growing and dissociation conditions; thus, it requires time-consuming optimization and troubleshooting of both protocols for each tumor type or cell line. Moreover, fusion of multiple tumorspheres may occur in culture, making assessment of their sizes and numbers unclear.

Despite the fact that the tumorsphere formation assay has been previously utilized in RMS studies, it has been mainly applied to commercially available RMS cell lines to identify the molecular pathways involved in tumor formation and development[18,20,21]. Given the heterogeneous tissue composition, the numerous subtypes of tumors and diverse developmental contexts from which RMS originates, employment of RMS cell lines limits application of this assay for the identification of both cells of origin and developmental cues that lead to tumor development *in vivo*.

An attempt to develop an efficient protocol for tumorsphere derivation, starting from human sarcoma samples, has previously shown poor results. Indeed, tumorspheres were developed from only 10% of the samples[35]. Thus, there is a need for a reproducible and reliable assay for isolation of primary RMS cells and tumorsphere development. In response to this need, the described tumorsphere formation assay was optimized to be employed in primary cell cultures. The development of this protocol is the first step towards answering major questions in the field (i.e., how RMS cells of origin differ depending on environmental context). In more detail, described here is a reproducible protocol for the isolation of primary tumor cells from RMS tissues, formation of tumorspheres, tumorsphere treatment (both performed with recombinant proteins or overexpression plasmids), and allograft transplantation experiments.

In conclusion, the tumorsphere formation assay is a well-established and versatile method for the identification of TPCs in different types of tumors, enriching the cells with increased self-renewal capacity and the ability to grow in an anchorage-independent manner. This assay is

based on functional characteristics of the cell populations being studied and not on the previous knowledge of molecular markers; thus, it can be applied as an exploratory tool for a wide range of tumors types. Moreover, the isolation of rare cell populations achieved with tumorsphere cultures makes this *in vitro* assay an ideal platform for cancer drug testing.

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The authors have nothing to disclose.

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Figure 1

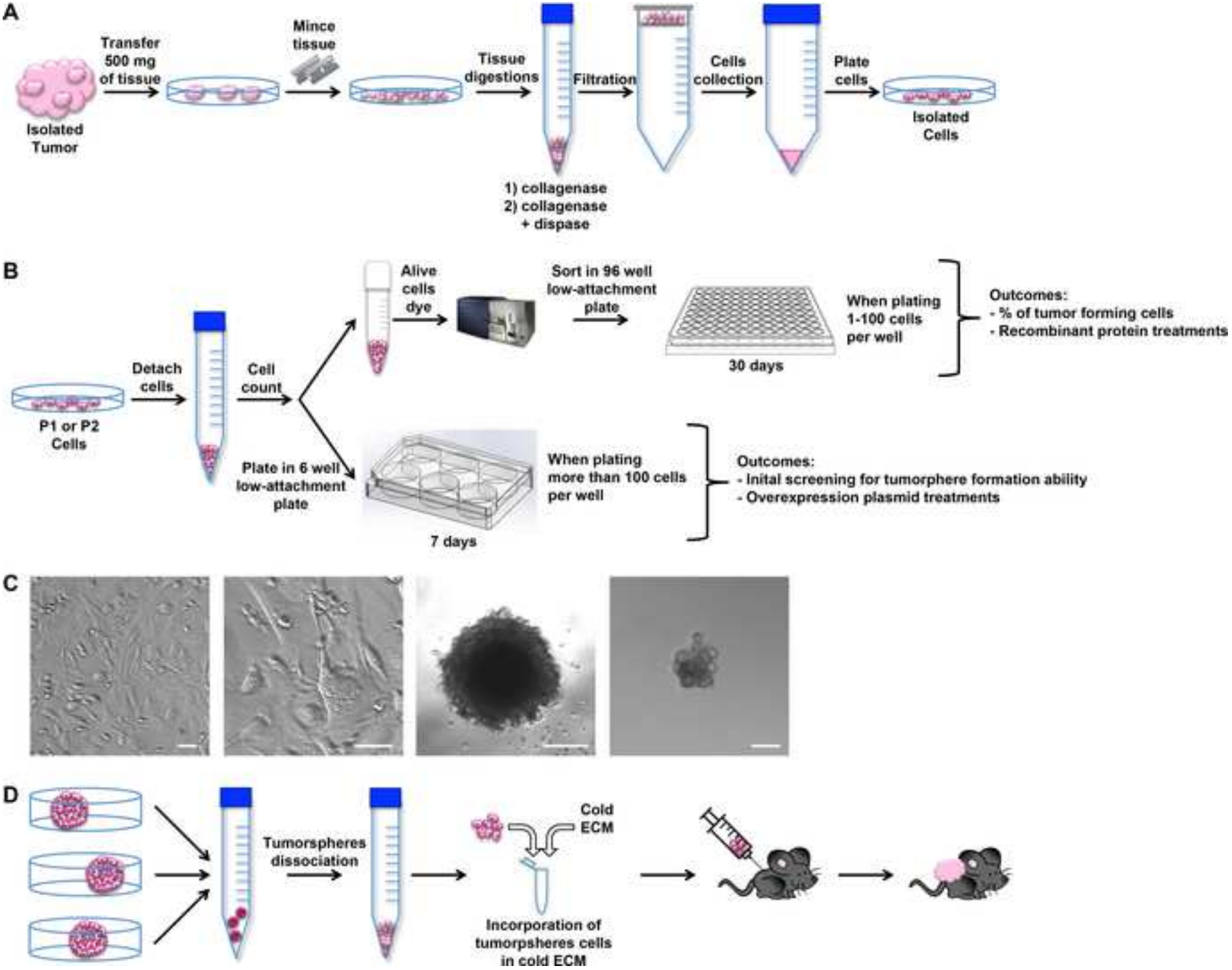

Figure 2

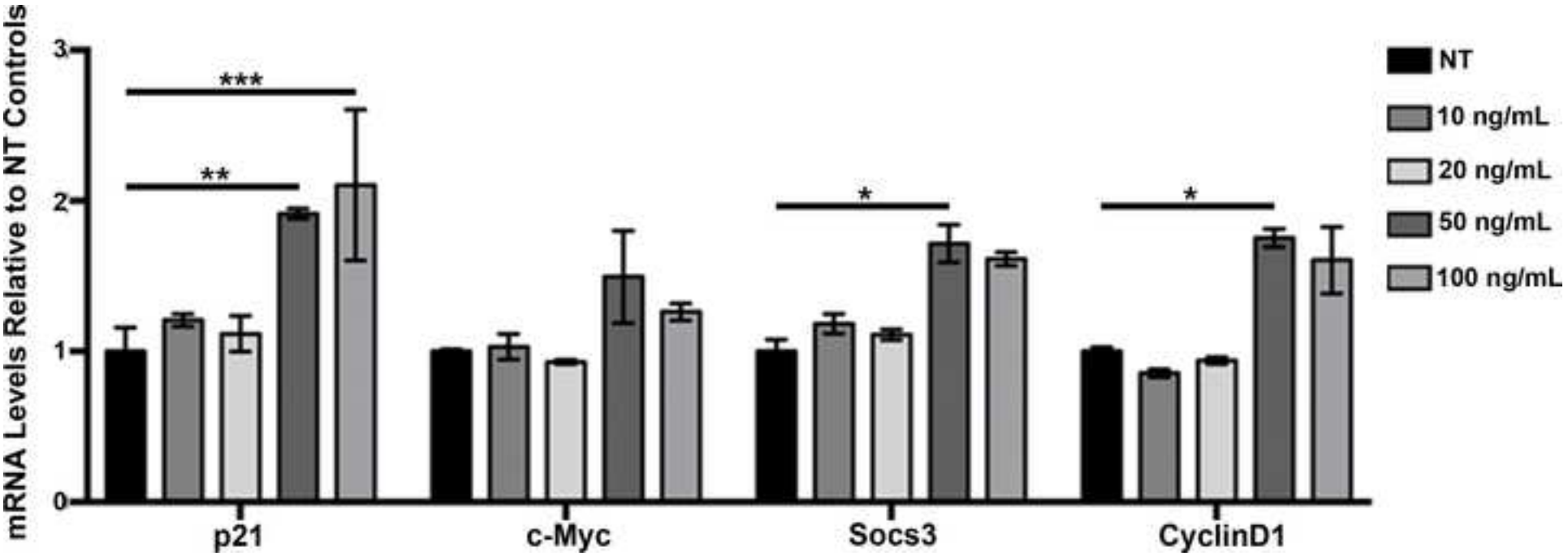

Figure 3

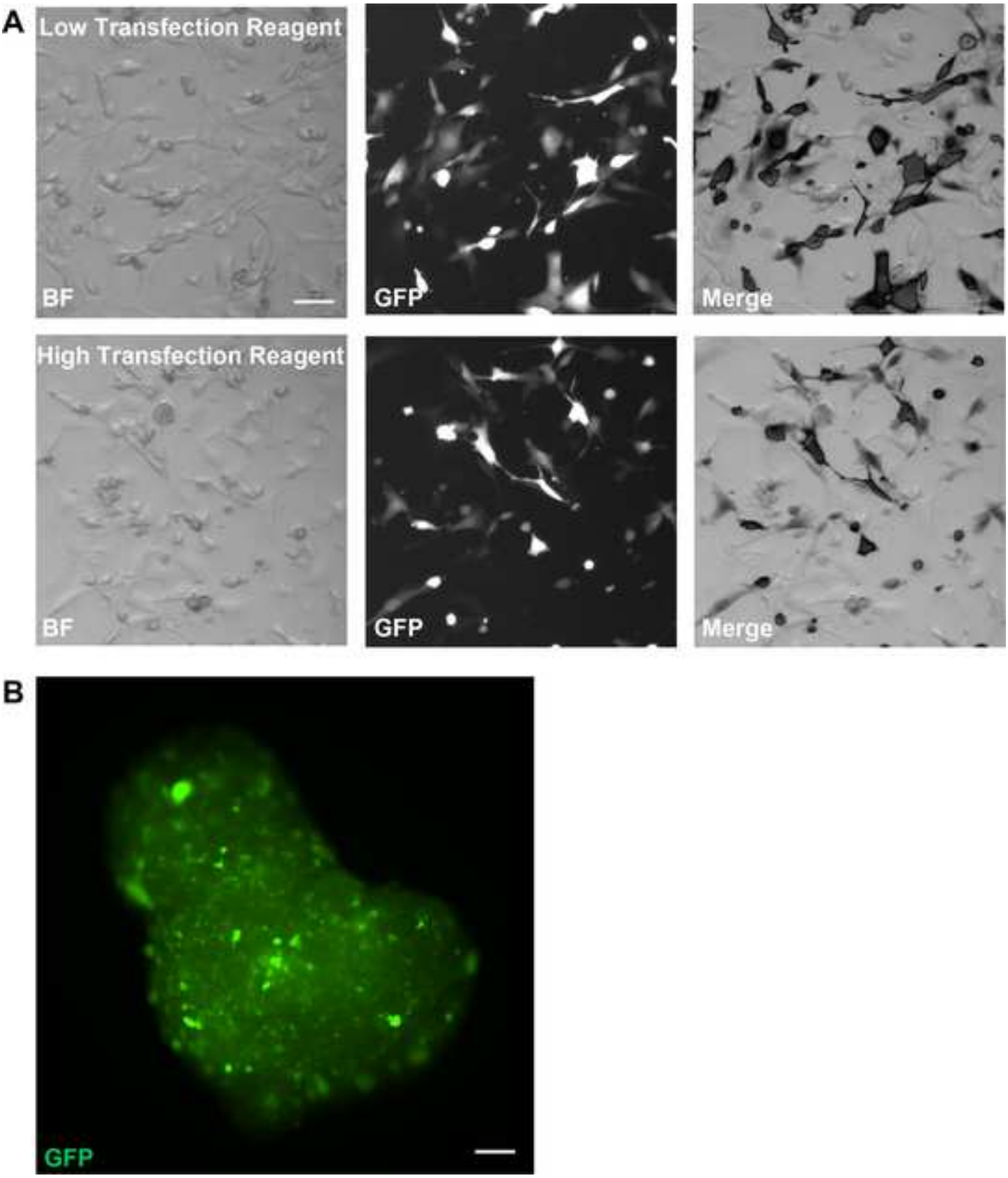

Table of Materials

Name of Material/ Equipment	Company	Catalog Number
Accutase cell dissociation reagent	Gibco	A1110501
Celigo	Nexcelom	Celigo
Collagenase, Type II	Life Technologies	17101015
Dispase II, protease	Life Technologies	17105041
DMEM high glucose media	Gibco	11965092
DMEM/F12 Media	Gibco	11320033
EDTA	ThermoFisher	S312500
EGF recombinant mouse protein	Gibco	PMG8041
FACSAria II Flow Cytometry	BD Biosciences	650033
Fetal Bovine Serum	Omega Scientific	FB-11
Fluriso (Isofluornae) anesthetic agent	MWI Vet Supply	502017
FxCycle Violet Stain	Life Technologies	F10347
Goat Serum	Life Technologies	16210072
Ham's F10 Media	Life Technologies	11550043
Horse Serum	Life Technologies	16050114
Lipofectamine 3000 transfection reagent	ThermoFisher	L3000015
Matrigel membrane matrix	Corning	CB40234
N-2 Supplemtns (100X)	Gibco	17502048
Neomycin-Polymyxin B Sulfates-Bacitra	MWI Vet Supply	701008
PBS	Gibco	10010023
pEGFP-C1	Addgene	6084-1

Penicillin - Streptomyocin	Life Technologies	15140163
Recombinant Human βFGF-basic	Peprotech	10018B
Recombinant mouse Flt-3 Ligand Protein	R&D Systems	427-FL-005
Trypan blue	ThermoFisher	15250061

Comments/Description
Detach adherent cells and dissociate tumorspheres
Microwell plate based image cytometer for adherent and suspension cells
Tissue digestion enzyme
Tissue digestion enzyme
Component of tumor cells media
Component of tumosphere media
Component of FACS buffer
Component of tumosphere media
Fluorescent activated cell sorter
Component of tumor cells media
Anesthetic reagent for animals
Discriminate live and dead cells
Component of FACS buffer
Component of FACS buffer
Component of cell isolation media
Transfection Reagent
Provides support to trasplanted cells
Component of tumosphere media
Eyes ointment
Component of FACS buffer and used for washing cells
GFP plasmid

Component of tumosphere and tumor cells media
Component of tumosphere media

Recombinant protein
Discriminate live and dead cells

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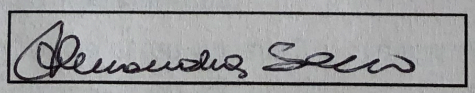
Signature:	
Date:	02/22/2019

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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:

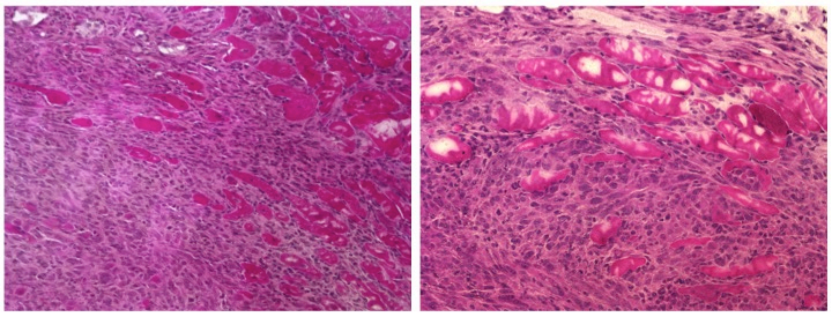
Manuscript Summary:
The authors provide a detailed hands-on protocol to isolate and culture cells from rhabdomyosarcomas (RMS) and to establish tumorspheres for allograft transplantation. This protocol will aid in developing drug screening methodologies for the identification of RMS therapeutics.

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.