Thomas Lloyd, M.D., Ph.D.
Associate Professor of Neurology
Neuroscience, and Genetics

Department of Neurology
855 N. Wolfe Street, Room 248
Baltimore, Maryland 21205
410-502-6851 Office
410-502-5459 Fax
tlloyd4@jhmi.edu

JOHNS HOPKINS
MEDICINE

May 6, 2019

Phillip Steindel, PhD
Review Editor
JoVE
1 Alewife Center | Suite 200 | Cambridge | MA | 02140
Phone: 617-401-7677 Ext: 850 | Fax: 866.381.2236

RE: Revisions for JoVE Manuscript JoVE59966

Dear Dr. Steindel,

We thank you and our reviewers for their comments on our submission, and please find attached our revised manuscript. We have addressed each of the editorial and peer review comments in the following ways:

Editorial comments:
1. The manuscript has been fully proofread by all authors.
2. Lines 98-101 have been revised to avoid previously published text.
3. Commercial language has been removed throughout the main body of the manuscript.
4. Personal pronouns have been removed from the Protocol text.
5. An approximate volume of 50 mL to prepare has been added to step 1.3.
6. The syringe size has been specified in step 1.4.
7. The petri dish size of 100 mm x 15 mm has been specified in step 2.1.
8. The sutures used the in the protocol have been more fully described in step 2.5.
9. A new section "5. Xenograft Immunohistochemistry" has been added to the protocol to describe how the data present in Figure 3 and Figure 4 can be generated.
10. Full journal titles are now used in the reference section.

Reviewers' comments:

Reviewer #1:
"In this manuscript, the authors describe the graft of a human muscle biopsy into immunodeficient mice as a new tool to model human muscle disease and to test potential therapies.
This is a very powerful and valuable tool for the scientific community, that can serve as a model for preclinical studies."

We appreciate the reviewer's enthusiasm for our work.

Major Concerns:
1. "In the equipment Preparation (1.3) the authors use a very rich muscle medium ((20% fetal bovine serum, 2% chick embryo extract, 1% antibiotic/antimycotic in Hams F10 Medium). Can

authors tell us if this medium is necessary or if a basic medium (eg F10) supplemented with antibiotic/antimycotic can be used as well?"

We have never used a basic medium supplemented with antibiotic/antimycotic, so we can not comment on whether this is necessary for the protocol. However, we do not have any reason to think that use of a more basic medium would impact the outcome of the xenograft surgeries.

2. "In the Surgical preparation (2.1): what do the authors mean by "muscles display strength > 4-/5". Is this a specific point that has to be observed for a successful muscle engraftment? The authors have already grafted FSHD muscles in two previous papers (Zhang et al, 2014, Chen JC, 2016): are those scores compatible with measures obtained in dystrophic muscles? The authors also propose to use muscles from autopsy: is this score still applicable in this case?"

This strength rating refers to the 0 to 5 numerical scale described by the Medical Research Council (MRC). This scale is used in clinical settings as a qualitative assessment of muscle strength with 0 showing no contraction and 5 showing normal power. We have found that muscles with a strength greater than 4- (meaning the muscle displays some resistance against the examiner) typically show disease pathology without extensive fatty replacement or fibrosis, in contrast to muscles that are MRC grade 3 or less. Since fatty replacement and/or fibrosis of muscle would be expected to impede xenograft regeneration, we avoid using these extremely weak muscles in the procedure. In the case of autopsy tissue where a recent MRC score is not available, muscle quality can be accessed via gross observation. We have revised the manuscript to include a brief description of the MRC scale to help readers determine what patient biopsies or autopsy tissue will likely engraft successfully.

3. "In the Surgical preparation (2.1): the authors write that muscle specimen is placed in petri dish containing muscle media. When muscles are put in medium, they generally rapidly absorb liquid and change architectural structure. Can authors specify how long the can keep muscles in the medium and if they have noticed some changes in the muscle structure after keeping them in the medium?"

We have not observed changes in the muscle structure during the time the biopsy is kept in the media, which is typically for 4 hours. Biopsies have been stored in media for 24 hours before xenografting, and this delay did not appear to negatively impact transplantation. Since the mature myofibers degenerate upon transplantation, we would not anticipate that an alteration in architecture would significantly impact the procedure. We have revised step 2.4 to include this information about the average length of time the biopsies remain in media.

4. "In the Xenograft Collection (4). Removal of the xenograft from the empty tibial compartment seems to be challenging. Could the authors add pictures in the text of these steps, at least to show how the graft looks like after 4-6 months?"

We have added a new figure (**Figure 3**) to show the xenograft collection in a stepwise fashion.

Minor Concerns:

5. "Line 41 abstract. IRB: please define." IRB is now defined in the abstract (line 41) and is also defined in the protocol (line 95).

6. "Line 76 introduction. NRG: please define." NRG is defined in the abstract (line 42), and refers to **N**OD-***R***$ag1^{null}$ $IL2r\boldsymbol{\gamma}^{null}$,(G = gamma ($\gamma$)).

Reviewer #2:
"In addition to the following points, authors need to add a succulent section on analyzing the xenograft by a robust examination pipeline as previously described in 2009 paper by Zhang et al."

We are unclear what "robust examination pipeline" this reviewer is referring to. I assume the reviewer means the 2014 paper by Zhang et al; however, further assessment of the xenograft are beyond the scope of this manuscript.

Major Concerns:

"1. Could authors provide a reference or brief section in protocol (as a brief description is provided only in the figures 3-4) on immunofluorescence assay and antibodies to test the success of xenograft."

A new section "5. Xenograft Immunohistochemistry" has been added to the protocol to describe how the data present in Figure 3 and Figure 4 can be generated, and the Table of Materials has been expanded to include primary and secondary antibodies as well as other materials used in the staining procedure.

"2. It will be helpful to add a couple of lines about pathology in IIM and main pathological features associated with IIM, then simple mentioning different things were checked. also could authors describe the progression of IIM in patients vs in xenograft conditions. As both extrinsic and intrinsic factors regulate muscle growth, one concern is that by providing a healthy external milieu in healthy mice, would patient muscle exhibit less severity in comparison to the situation if patient muscle had not been xenografted. It is not a practical to obtain a biopsy from the same patient again to compare two conditions but this point should be explained or discussed."

The reviewer raises several excellent points about the complexity of muscle regeneration and the potential impact the inflammatory milieu observed within IIM xenografts may have on xenograft regeneration. However, the goal of this manuscript is to provide an in-depth description of the xenograft procedure, and not to discuss the use of the model in IIM. In addition, the questions of how introducing inflammatory cells into xenografts impacts regeneration or how IIM xenografts compare to patient muscle is beyond the scope of this manuscript.

"3. Could authors provide a high magnification image for good regeneration muscle stained with laminA/C in a small panel? Lamin positive nuclei are difficult to visualize."

Higher magnification inserts have been provided for the lamin A/C and spectrin stains in Figure 3 (now Figure 4 in the revised manuscript).

"4. Figure 4: Are these images from a normal control or IIM patient? If these are from a control, could authors also provide similar images from the IIM patient for a fair comparison."

Figure 4 (now Figure 5 in the revised manuscript) contains images from a patient with IIM. This was stated in the representative results section, and in the revised manuscript, the Figure 5 figure legend also states that the xenograft images shown are from an IIM patient.

"5. Materials: Could authors provide details on diluent, concentration and storage of different chemicals used for surgery. Also it will be helpful to have details on antibodies (source and

concentrations)"

The table of materials now includes details on antibodies and recommended concentrations. The proper diluent (PBS) for the surgical analgesic (Rimadyl) is now stated in step 1.4. We now state that all chemicals/drugs/solutions used for surgery are kept at room temperature unless stated differently in the protocol.

"6. Comments: comments section looks scattered. Could authors provide source and model number for the induction chamber and Mapleson E breathing circuit? What do 8 mm cutting edge and 9 mm specify?"

The table of materials has been revised to show the Mapelson E breathing circuit and Induction chamber as separate items. They were previously listed in the table as the "dual procedure circuit" from VetEquip. The comments section of the table has also been revised for clarity.

<u>Minor Concerns:</u>

1. "1.1Please specify the source of these mice in main text"

JoVE cannot publish manuscripts containing commercial language, including company names. Therefore, the source of the mice (The Jackson Laboratory) is described in the Table of Materials, but not in the body of the manuscript.

2. "1.4 Provide size of the syringe and diluent used for Rimadyl in main text"

The size of the syringe and diluent in step 1.4 is now described in the main text.

3. "2.1 is there a reference for the muscle display strength? Also specify the time, temperature and buffer of biopsy samples from collection to engraftment."

A reference for the Medical Research Council muscle strength scale has been added to step 2.1. In addition, step 2.4 has been revised to specify the duration of time the biopsy is kept in the muscle media on ice (or approximately 4^{o}C).

4. "2.6 source and model number for Mapelson E breathing circuit"

The table of materials has been revised to show the Mapelson E breathing circuit and Induction chamber as separate items. They were previously listed in the table as the "dual procedure circuit" from VetEquip.

5. "3.3 could authors specify the approximate depth of the cut"

We haven't measured the depth, but estimate it is less than 0.5 mm. In the revised manuscript we state that "this is a very superficial cut (less than 0.5 mm; Figure 2B, black dashed line)"

6. "3.8 Could the same mice be used for xenograft successfully if there is any damage to vessels?"

The same mice can be used even if there is damage to the vessels. As outlined in step 3.8 of the

protocol, if there is damage to the vessels you can simply remove the suture, place it in a different location, and proceed with the surgery.

7. "4.1 The better way to describe would be to place the beaker with 2-methylbutane in a styrofoam box containing dry ice."

We thank the reviewer for this suggestion, and step 4.1 has been reworded for clarity.

Sincerely,

Thomas Lloyd, M.D., Ph.D.
tlloyd4@jhmi.edu