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Performing Human Skeletal Muscle Xenografts in Immunodeficient Mice

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Corresponding Author:	Kyla Britson Johns Hopkins University School of Medicine Baltimore, MD UNITED STATES
Corresponding Author's Institution:	Johns Hopkins University School of Medicine
Corresponding Author E-Mail:	kbritso1@jhmi.edu
Order of Authors:	Kyla A Britson
	Aaron D. Black
	Kathryn R. Wagner
	Thomas E. Lloyd
Additional Information:	
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Cover Letter

Thomas Lloyd, M.D., Ph.D. **Department of Neurology**
Associate Professor of Neurology,
855 N. Wolfe Street, Room 248
Neuroscience, and Genetics
Baltimore, Maryland 21205
410-502-6851 Office
410-502-5459 Fax
tlloyd4@jhmi.edu

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MEDICINE

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Stephanie R. Weldon, PhD
Science Editor
JoVE
1 Alewife Center | Suite 200 | Cambridge | MA | 02140
Phone: 617-401-7677 Ext: 850 | Fax: 866.381.2236

RE: Submission to JOVE

Dear Dr. Weldon,

Please find attached our submission entitled "Performing Human Skeletal Muscle Xenografts in NOD-Rag1null IL2rγ null mice". This novel method allows researchers to study human muscle tissue in a mouse model for the first time, and we think will be broadly of interest to many scientists studying human muscle regeneration, inflammation, and disease.

Potential reviewers include:

Tahseen Mozaffar, MD
Professor and Interim Chair, Neurology
Professor of Orthopaedic Surgery and Pathology and Laboratory Medicine
University of California, Irvine
200 S. Manchester Avenue, Ste. 110, Orange, CA 92868.
Academic Issues: (714) 456-2238 (Michi Baylon); Fax: (714) 456-8805
Email: mozaffar@uci.edu

Olivier Benveniste, MD, PhD
Professor, Institute of Myology
Head the Department of Internal Medicine and Clinical Immunology, Pitié-Salpêtrière Hospital
Centre Director of UPMC/INSERM laboratory: Inflammatory Muscle and Targeted Innovative Therapies, UMR974, INSERM, Sorbonne Université
Pitié-Salpêtrière Hospital Centre, AP-HP, Paris, France
Secretary: 0142161088
olivier.benveniste@aphp.fr

Kanneboyina Nagaraju, DVM, PhD
Professor and Founding Chair
Department of Pharmaceutical sciences
School of Pharmacy and Pharmaceutical Sciences
Binghamton University
PO Box 6000

Binghamton, NY 13902-6000
Phone: 607-777-5814
Email: Nagaraju@binghamton.edu

Jason Pomerantz, M.D.
Associate Professor of Surgery
Division of Plastic and Reconstructive Surgery
Departments of Surgery and Orofacial Sciences,
Program in Craniofacial Biology,
Eli and Edythe Broad Center of Regeneration Medicine,
University of California, San Francisco
San Francisco, CA 94143, USA.
Email: jason.pomerantz@ucsf.edu

Sincerely,

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

TITLE:

Performing Human Skeletal Muscle Xenografts in Immunodeficient Mice

AUTHORS AND AFFILIATIONS:

Kyla A. Britson[1,2], Aaron D. Black[2,3], Kathryn R. Wagner[1,2,3,4], Thomas E. Lloyd[1,2,4]

[1]Graduate Program in Cellular and Molecular Medicine Johns Hopkins University School of Medicine, Baltimore, MD, USA
[2]Department of Neurology, Johns Hopkins University School of Medicine, Baltimore, MD, USA
[3]Kennedy Krieger Institute, Baltimore, MD, USA
[4]Department of Neuroscience, Johns Hopkins University School of Medicine, Baltimore, MD, USA

Corresponding author:

Thomas E. Lloyd (tlloyd4@jhmi.edu)

Email addresses of co-authors:

Kyla A. Britson (kbritso1@jhmi.edu)
Aaron D. Black (aaron.black@jhmi.edu)
Kathryn R. Wagner (wagnerk@kennedykrieger.org)

KEYWORDS:

xenograft, skeletal muscle, transplantation, model, NOD-*Rag1*^{null}*IL2r*γ^{null} mice, immunocompromised, myositis, muscle disease

SUMMARY:

Complex human diseases can be challenging to model in traditional laboratory model systems. Here, we describe a surgical approach to model human muscle disease through the transplantation of human skeletal muscle biopsies into immunodeficient mice.

ABSTRACT:

Treatment effects observed in animal studies often fail to be recapitulated in clinical trials. While this problem is multifaceted, one reason for this failure is the use of inadequate laboratory models. It is challenging to model complex human diseases in traditional laboratory organisms, but this issue can be circumvented through the study of human xenografts. The surgical method we describe here allows for the creation of human skeletal muscle xenografts, which can be used to model muscle disease and to carry out preclinical therapeutic testing. Under an Institutional Review Board (IRB)-approved protocol, skeletal muscle specimens are acquired from patients and then transplanted into NOD-*Rag1*^{null} *IL2r*γ^{null} (NRG) host mice. These mice are ideal hosts for transplantation studies due to their inability to make mature lymphocytes and are thus unable to develop cell-mediated and humoral adaptive immune responses. Host mice are anaesthetized with isoflurane, and the mouse tibialis anterior and extensor digitorum longus muscles are removed. A piece of human muscle is then placed in the empty tibial compartment and sutured to the proximal and distal tendons of the peroneus

longus muscle. The xenografted muscle is spontaneously vascularized and innervated by the mouse host, resulting in robustly regenerated human muscle that can serve as a model for preclinical studies.

INTRODUCTION:

It has been reported that only 13.8% of all drug development programs undergoing clinical trials are successful and lead to approved therapies[1]. While this success rate is higher than the 10.4% previously reported[2], there is still significant room for improvement. One approach to increase the success rate of clinical trials is to improve laboratory models used in preclinical research. The Food and Drug Administration (FDA) requires animal studies to show treatment efficacy and assess toxicity prior to Phase 1 clinical trials. However, there is often limited concordance in treatment outcomes between animal studies and clinical trials[3]. In addition, the need for preclinical animal studies can be an insurmountable barrier for therapeutic development in diseases that lack an accepted animal model, which is often the case for rare or sporadic diseases.

One way to model human disease is by transplanting human tissue into immunodeficient mice to generate xenografts. There are three key advantages to xenograft models: First, they can recapitulate the complex genetic and epigenetic abnormalities that exist in human disease that may never be reproducible in other animal models. Second, xenografts can be used to model rare or sporadic diseases if patient samples are available. Third, xenografts model the disease within a complete in vivo system. For these reasons, we hypothesize that treatment efficacy results in xenograft models are more likely to translate to trials in patients. Human tumor xenografts have already been successfully utilized to develop treatments for common cancers, including multiple myeloma, as well as personalized therapies for individual patients[4-7].

Recently, xenografts have been used to develop a model of human muscle disease[8]. In this model, human muscle biopsy specimens are transplanted into the hindlimbs of immunodeficient NRG mice to form xenografts. The transplanted human myofibers die, but human muscle stem cells present in the xenograft subsequently expand and differentiate into new human myofibers which repopulate the engrafted human basal lamina. Therefore, the regenerated myofibers in these xenografts are entirely human and are spontaneously revascularized and innervated by the mouse host. Importantly, fascioscapulohumeral muscular dystrophy (FSHD) patient muscle tissue transplanted into mice recapitulates key features of the human disease, namely expression of the *DUX4* transcription factor[8]. FSHD is caused by overexpression of DUX4, which is epigenetically silenced in normal muscle tissue[9,10]. In the FSHD xenograft model, treatment with a DUX4-specific morpholino has been shown to successfully repress *DUX4* expression and function, and may be a potential therapeutic option for FSHD patients[11]. These results demonstrate that human muscle xenografts are a new approach to model human muscle disease and test potential therapies in mice. Here, we describe in detail the surgical method for creating human skeletal muscle xenografts in immunodeficient mice.

PROTOCOL:

All use of research specimens from human subjects was approved by the Johns Hopkins Institutional Review Board (IRB) to protect the rights and welfare of the participants. All animal experiments were approved by the Johns Hopkins University Institutional Animal Care and Use Committee (IACUC) in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals. Male NOD-*Rag1*^{null} *IL2rγ*^{null} (NRG) host mice (8-12 weeks old) are used to carry out xenograft experiments. These mice are housed in ventilated racks and are given HEPA-filtered, tempered, and humidified air as well as reverse osmosis filtered hyperchlorinated water. Mice are provided water and an irradiated antibiotic diet (**Table of Materials**) ad libitum, and the facility provides 14 hours of light to 10 hours of dark as controlled by central timer.

1. Equipment preparation

1.1. Acquire NOD-*Rag1*^{null} *IL2rγ*^{null} (NRG) mice, 8-12 weeks of age.

1.2. Autoclave surgical equipment: scissors, forceps, needle holder, surgical stapler (Table **of Materials**), wound clips, surgical wipes (**Table of Materials**), and beaker (**Figure 1A**).

1.3. Prepare 50 mL of muscle media (20% fetal bovine serum, 2% chick embryo extract, 1% antibiotic/antimycotic in Hams F10 Medium). Keep all chemicals/drugs/solutions used for surgery at room temperature unless stated differently in the protocol.

1.4. Prepare a 1 mL syringe with a 26 gauge needle that is 3/8 inches long containing 2 mg/mL analgesic (**Table of Materials**), and place on ice. The analgesic can be diluted to the proper concentration using sterile phosphate buffered saline (PBS).

2. Surgical preparation

2.1. Obtain a human muscle biopsy under an IRB-approved protocol from patients whose muscles display strength > 4-/5 on the MRC (Medical Research Council) scale[12]. Place the research specimen in a 100 mm x 15 mm Petri dish containing muscle media.

NOTE: The MRC scale is used in clinical practice as an assessment of muscle strength with 0 showing no contraction, 5 showing normal power, and 4 (4- to 4+) showing movement against resistance[12]. We have found that muscles with mild to moderate weakness (MRC > 4-/5) typically show disease pathology but are not extensively replaced by fatty tissue or fibrosis, both of which impede xenograft regeneration. In the case of autopsy tissue where a recent MRC score is not available, muscle quality can be accessed via gross observation. Muscle biopsies that are pale pink in appearance or have large areas of fatty tissue are not likely to xenograft successfully.

2.2. Remove any remaining fascia or fatty tissue from the specimen with surgical scissors using a stereo microscope and light source to assist visualization.

2.3. Dissect the muscle biopsy into approximately 7 mm x 3 mm x 3 mm pieces with surgical scissors using the stereo microscope and a light source. Ensure fibers are arranged longitudinally within the specimen.

2.4. Place the Petri dish containing dissected muscle on ice. On average, the xenografts are kept in media for 4 hours while surgeries are being performed. However, biopsies have been stored in media for 24 hours prior to xenografting, and this delay did not appear to negatively impact transplantation or regeneration.

2.5. Place synthetic, non-absorbable sutures (**Table of Materials**) in a 100 mm x 15 mm Petri dish containing 70% ethanol.

2.6. Set up a dual procedure anesthesia circuit: arrange the Mapleson E breathing circuit on the stereo microscope and place the induction chamber in a biosafety cabinet (**Figure 1A**,**B**).

2.7. Obtain the weight of the NRG mouse by placing in an autoclaved beaker on a scale, and transfer to the induction chamber. Induce anesthesia under 3% isoflurane. Once the appropriate anesthetic depth is achieved—as assessed by observation of respiratory rate, muscle relaxation, and lack of voluntary movement—reduce the vaporizer setting to 1.5% for the remainder of the surgery.

2.8. Transfer the mouse from the induction chamber to the Mapleson E breathing circuit and apply ophthalmic ointment to eyes.

2.9. Remove hair overlying the tibialis anterior (TA) from ankle to knee with a trimmer, followed by a 1 minute treatment with hair removal lotion (**Table of Materials**) (**Figure 2A**).

2.10. Disinfect the surgical site by swabbing the leg with povidone-iodine solution. Then wash away the remaining povidone-iodine with 70% ethanol.

2.11. Inject the mouse subcutaneously with analgesic (**Table of Materials**) at a dose of 5 mg/kg.

3. Xenograft surgery

3.1. Tape down the leg and make a straight incision over the tibilalis anterior (TA) muscle with scissors and iris forceps originating at the distal tendons and terminating below the knee (**Figure 2B**).

3.2. Separate skin from muscle using blunt dissection with surgical scissors.

3.3. Cut through the epimysium of the TA muscle with scissors starting at the tendon and ending at the knee.

NOTE: This is a very superficial cut (less than 0.5 mm; **Figure 2B**, **black dashed line**), and the underlying TA should not be damaged in the process as this would make removal more challenging. When performed correctly, the muscle fibers will visibly relax.

3.4. Cut the distal tendon of the TA with scissors, grab the tendon with iris forceps, and pull the TA up toward the knee (**Figure 2C**).

3.5. Cut the distal tendon of the extensor digitorum longus (EDL) with scissors and pull the EDL up toward the knee (**Figure 2D**). Once the proximal tendon of the peroneus longus (PL) muscle is visible, remove the EDL with scissors (**Figure 2D**, **green dashed line**).

3.6. Remove the TA with scissors (**Figure 2D**, **blue dashed line**) and use a surgical wipe wetted with PBS and slight pressure to achieve hemostasis (**Figure 2E**).

3.7. Thread a suture through proximal peroneus longus (PL) tendon and trim, leaving approximately 1.5 inch of thread on either side of the tendon (**Figure 2F**).

3.8. Perform the first half of a two-hand surgical square knot, but do not tighten: this will form a circle. Place a xenograft in this circle and tighten the loop to secure the xenograft. Complete the other half of the square knot (**Figure 2G**,**H**). This will suture the xenograft to the proximal tendon of the PL.

NOTE: The medial tarsal artery and vein can lie close to or on top of the distal tendon of the PL. Do not place sutures through or around these vessels. It is easy to tell if a suture has been improperly placed as vessels will blanch or bleed. If this occurs, remove the suture and place in a different location.

3.9. Thread suture through distal PL tendon and repeat the square knot technique from step 3.8 to tie the xenograft to the distal tendon (**Figure 2H**,**I**).

3.10. Pull skin over xenografted muscle, seal with surgical glue, and place 2-3 surgical staples over the incision (**Figure 2J**).

3.11. Place mouse in a clean cage on a heated pad to recover. Monitor mouse until fully conscious and periodically over the next few days for signs of local systemic infection and to ensure the surgical site is not reopened.

4. Xenograft collection

NOTE: Xenografts are typically collected between 4 to 6 months post-surgery. However, collections have been performed up to 12 months post-surgery.

4.1. Place a covered beaker containing 200 mL of 2-methylbutane in a box containing dry ice for a minimum of 30 minutes before xenograft collection.

4.2. Induce anesthesia under 3% isoflurane in induction chamber. Once the appropriate anesthetic depth is achieved, reduce the vaporizer setting to 1.5% for the remainder of the surgery.

4.3. Transfer the mouse from the induction chamber to the Mapleson E breathing circuit arranged on a stereo microscope.

4.4. Remove hair overlying the tibialis anterior from ankle to knee with a trimmer and hair removal lotion. The sutures holding the xenograft in place can be seen through the skin (**Figure 3A**).

4.5. Tape down the leg and use scissors and iris forceps to open skin over the xenograft until both sutures are visible (**Figure 3B**). Skin overlying the xenograft can be removed as shown to make removal of the xenograft easier.

4.6. Use a scalpel to cut between the xenograft and the tibia (**Figure 3B**, **arrow denotes initial site and direction of incision**). This will free one side of the xenograft.

4.7. Use a scalpel to cut between the PL muscle and the gastrocnemius muscle (**Figure 3C**, **incision along epimysium labeled with arrow**). The PL will be removed with the xenograft.

4.8. Cut below the distal suture and through the distal tendon of the PL (**Figure 3D**, **cut along dotted line**).

4.9. Remove the xenograft and PL by grabbing the suture with iris forceps and deflecting it toward the knee while using scissors to cut it away from the underlying muscle (**Figure 3E**).

4.10. Cut above the proximal suture with scissors to remove the xenograft and PL (**Figure 3F**, **cut along dotted line in 3E**).

4.11. Place the specimen on a small piece of cardboard or plastic, and pin as close to the sutures as possible. While pinning the specimen, gently stretch the muscle to ensure that the fiber orientation is maintained during the snap freezing process. After the pins are securely in place, slide the muscle up the pins so it rests just above the cardboard.

NOTE: Alternatively, one end of the xenograft can be mounted in tragacanth on a cork, or it can be submerged entirely in optimal cutting temperature (O.C.T.) compound in a cryomold. With care, muscle conformation can be retained with both methods.

4.12. Snap freeze the xenograft in pre-cooled 2-methylbutane.

4.13. Store xenograft at -80 °C.

4.14. Immediately following xenograft collection, euthanize mice in accordance with American Veterinary Medical Association guidelines:

4.14.1. Place mice in a sealed chamber with an appropriate waste gas scavenging system. Use isoflurane at a concentration of 3-4% to induce anesthesia.

4.14.2. Once the appropriate anesthetic depth is achieved—as assessed by observation of respiratory rate, muscle relaxation, and lack of voluntary movement—increase the vaporizer setting to 5% to induce death. Leave the mice in the chamber for an additional 2 minutes after breathing has ceased. Death is verified by observing that the mice fail to recover within 10 minutes after overdose of isoflurane.

4.14.3. Finally, perform cervical dislocation on the mice.

NOTE: In the case of bilaterally xenografted mice, the contralateral xenograft can be saved for a later collection. To perform a survival collection, open the skin overlying the xenograft with a single straight cut with surgical scissors, and remove the xenograft as described in steps 4.6 to 4.10. Then close the skin over the empty tibial compartment using surgical glue and staples. Treat the mouse with analgesic as described in step 2.11 and place the mouse in clean cage on heated pad to recover. Monitor the mouse until fully conscious and periodically over the next few days for signs of local systemic infection and to ensure the surgical site is not reopened.

5. Xenograft immunohistochemistry

5.1. Use a cryostat to cut 10 to 12 μm sections from the collected xenograft onto positively charged slides (**Table of Materials**).

5.2. Fill staining jar with methanol and pre-cool at -20 °C for 30 minutes.

5.3. Place slides in ice cold methanol for 10 minutes to fix and permeabilize the xenograft sections.

5.4. Place slides in staining jar and wash 3x with phosphate buffered saline (PBS) for 5 min.

5.5. Block with anti-mouse IgG (**Table of Materials**) for 2 h at 4 °C.

5.6. Blot with primary antibodies, such as spectrin, lamin A/C, and embryonic myosin (**Table of Materials**) in PBS supplemented with 2% goat serum overnight at 4 °C.

5.7. Place slides in a staining jar and wash 3x with phosphate buffered saline (PBS) for 5 min.

5.8. Blot with fluorescent-dye conjugated secondary antibodies (**Table of Materials**) in PBS supplemented with 2% goat serum for 1 h at room temperature.

5.9. Place slides in staining jar and wash 3x with phosphate buffered saline (PBS) for 5 minutes.

5.10. Place mounting medium (**Table of Materials**) over xenografts sections, place coverslip on top, and use nail polish to seal the coverslip.

[Place **Figure 2** here]

[Place **Figure 3** here]

REPRESENTATIVE RESULTS:

As demonstrated by Yuanfan Zhang et al., this surgical protocol is a straightforward method to produce human skeletal muscle xenografts[8]. Regenerated xenografts become spontaneously innervated and display functional contractility. In addition, muscle xenografted from FSHD patients recapitulates changes in gene expression observed in FSHD patients[8].

In our experience, approximately 7 out of 8 xenografts performed from control patient specimens will show successful muscle engraftment. A successful xenograft shows robust regeneration of human myofibers as identified with human specific antibodies (**Figure 4**). Positive embryonic myosin staining within a proportion of myofibers indicates that the regeneration process is still ongoing. In contrast, poor surgical technique or an inadequate specimen may lead to poor regeneration of muscle fibers (**Figure 4**).

Xenografts performed from a patient diagnosed with an idiopathic inflammatory myopathy (IIM) show moderate numbers of regenerated human myofibers at 4- and 6-month collections, and embryonic myosin staining persists at 6 months (**Figure 5A**). Inflammatory cells are present in the xenograft as shown by H&E staining (**Figure 5A**), and have been confirmed with CD3, CD68, and other immunological markers (data not shown). Xenografts are stable within the mouse, and up to 12-month collections have been performed. Individual myofiber size is comparable between the 4- and 6-month IIM xenografts and the original IIM patient biopsy (**Figure 5B**). Rare fibers showing a cross sectional area (CSA) greater than 3500 μm^2 are observed in xenografts but not in the IIM biopsy, indicating that some myofibers in the xenografts can regenerate to a CSA comparable in size to healthy myofibers (**Figure 5B**).

FIGURE AND TABLE LEGENDS:

Figure 1: Surgical Set-up. A) Standard orientation of stereo microscope, Mapleson E breathing circuit, and surgical tools during xenograft surgery. **B**) Placement of induction chamber in biosafety cabinet.

Figure 2: Xenograft Surgery. A) Hair is removed from surgical site. **B**) An incision is made over the tibialis anterior (TA). The distal tendons of the TA and extensor digitorum longus (EDL) are marked with arrows. The black dashed line indicates where the epimysium will be cut in step 3.3. **C**) The distal tendon of the TA is cut and the muscle is pulled up to the knee. **D**) The tendon of the EDL is cut and the EDL is pulled up to the knee. This exposes the proximal tendon of the peroneus longus (PL) marked with an arrow. Dashed lines indicate where to cut with scissors to remove the EDL (green) and PL (blue). **E**) The EDL and TA are removed. **F**) A suture is placed through the proximal tendon of the PL. **G**) The xenograft is placed in the empty tibial compartment and sutured to the proximal PL tendon using a two-hand surgical square knot. **H**) A suture is placed through the distal tendon of the PL, marked with an arrow, and another two-hand surgical square knot is used to suture the xenograft to the distal tendon. **I**) The xenograft is fully transplanted and sutured to the PL. **J**) The skin is closed with surgical glue.

Figure 3: 4 Month Xenograft Collection. A) Hair is removed from surgical site. Sutures are visible under skin. **B**) The skin overlying the xenograft is removed. Then the xenograft is grabbed with the iris forceps at the distal suture and gently pulled upward. Starting at the ankle, a scalpel is used to cut along the tibia and free the xenograft. The arrow shows the beginning of the incision along the tibia. **C**) By pulling the gastrocnemius muscle to the side, a faint white line of epimysium separating the peroneus longus (PL) muscle and the gastrocnemius (shown by the arrow) becomes visible. Use the scalpel to cut along this line to separate the PL from the other leg muscles. **D**) The right side of the xenograft, and the PL are now free from the other muscles in the leg and are ready for removal. The dashed line indicates where to cut with surgical scissors to start removing the xenograft and PL. **E**) After cutting below the distal suture, deflect the xenograft toward the knee. The dashed line indicates where to cut with surgical scissors to remove the xenograft and PL from the tibial compartment. **F**) The empty tibial compartment with the xenograft and PL successfully removed.

Figure 4: Expected Positive and Negative Results. Xenografts collected 4-months post-surgery showing good or poor regeneration are stained with human-specific lamin A/C (1:50) and human-specific spectrin (1:20) and embryonic myosin (1:10) (**Table of Materials**). Regions indicated by the white dashed boxes are shown as higher magnification inserts. Scale bar: 200 µm.

Figure 5: Representative Xenograft regeneration. **A**) Xenografts (outlined with dashed lines) performed from a patient diagnosed with an idiopathic inflammatory myopathy (IIM) stained with Hematoxylin and Eosin (H&E), human specific Lamin A/C, and human specific spectrin, show myofiber formation within NRG mice at both 4- and 6-month time points. Embryonic myosin staining demonstrates that regeneration is still ongoing at both time points. Scale bar: 200 µm. **B**) Histograms depicting cross sectional area (CSA) of myofibers from 4- and 6-month xenografts and human biopsies from one patient diagnosed with an idiopathic inflammatory myopathy (IIM) and one healthy control patient.

DISCUSSION:

Patient-derived xenografts are an innovative way to model muscle disease and carry out

preclinical studies. The method described here to create skeletal muscle xenografts is rapid, straightforward, and reproducible. Unilateral surgeries can be performed in 15 to 25 minutes, or bilaterally in 30 to 40 minutes. Bilateral xenografts can provide additional experimental flexibility. For instance, researchers can perform localized treatment of one xenograft, with the other left as a control. The NRG mice are resistant to surgical site infection when housed in a pathogen-free facility; in our experience performing more than 200 xenografts, we have never had a mouse acquire a surgical infection. In addition, host mice tolerate the removal of the TA and EDL very well. Within an hour post-surgery, unilaterally and bilaterally xenografted mice will be active and walking around their cage, and even standing up on their hindlimbs. Occasionally we observe some foot drop in host mice, but usually only after a period of inactivity, such as if recently awoken, and within minutes of waking leg use will be normal.

There are several critical steps in the protocol. First, during removal of the EDL and TA, it is very important to not injure the adjacent PL muscle or its tendons. This can be avoided by carefully and correctly identifying the placement of all distal tendons after the initial incision over the TA is performed. In addition, the proximal tendon of the PL should be identified and clearly visible before removal of the EDL (**Figure 2D**). Second, sutures must be placed through tendons and tightened fully in a proper two-hand surgical square knot. Xenografts regenerate more robustly under tension, and this is only obtainable if the xenograft is tethered to the PL tendons and if the sutures do not loosen post-operatively. Finally, it is important to not damage or sever any major blood vessels supplying the foot. In particular, the medial tarsal artery and vein can lie close to or on top of the distal tendon of the PL. Do not place sutures through or around these vessels. It is easy to tell if a suture has been improperly placed as vessels will blanch or bleed. If this occurs, remove the suture and place in a different location.

This method does have several limitations. It is not amenable to standard functional assays used in mouse models of muscle disease, such as grip strength or treadmill endurance. However, electrophysiological assessments of xenograft function can still be performed. Evoked force measurements can be recorded from xenograft explants, and single enzymatically isolated myofibers from xenografts loaded with ratiometric calcium dyes and electrically stimulated can be used to study calcium dynamics[8]. Another inherent challenge in this model is that acquiring and working with human tissue can be difficult. Not all laboratories will have easy access to fresh muscle biopsies, but it has been shown that xenografts performed from autopsy tissue approximately 48 hours *post mortem* can successfully engraft, and this tissue may be easier to obtain for some laboratories[8]. It is also challenging to manipulate gene expression in human tissue, whereas researchers using standard mouse models of disease can readily use the plethora of mouse genetic tools available.

A strength of this xenograft model is that it allows researchers to study human muscle in vivo. Tissue culture has been used extensively to study the cell and molecular biology of human muscle. Yet, these short-term, ex vivo studies do not always approximate functional muscle in vivo. However, one caveat is that is it challenging to determine how closely xenograft biology and function approximates human muscle due to the contribution of host mouse components during the regenerative process. For instance, human and mouse neuromuscular junctions

(NMJs) are morphologically distinct, and there is significant divergence between the synaptic proteome of human and mouse NMJs[13]. As xenografts are innervated by the mouse host, this may result in biological changes unique to the human xenografts.

In future studies, this skeletal muscle xenograft method could be used to better understand human muscle cell biology and to develop novel models for rare or acquired muscle diseases that currently lack animal models. We anticipate that this will have a significant beneficial impact on therapeutic development for these diseases.

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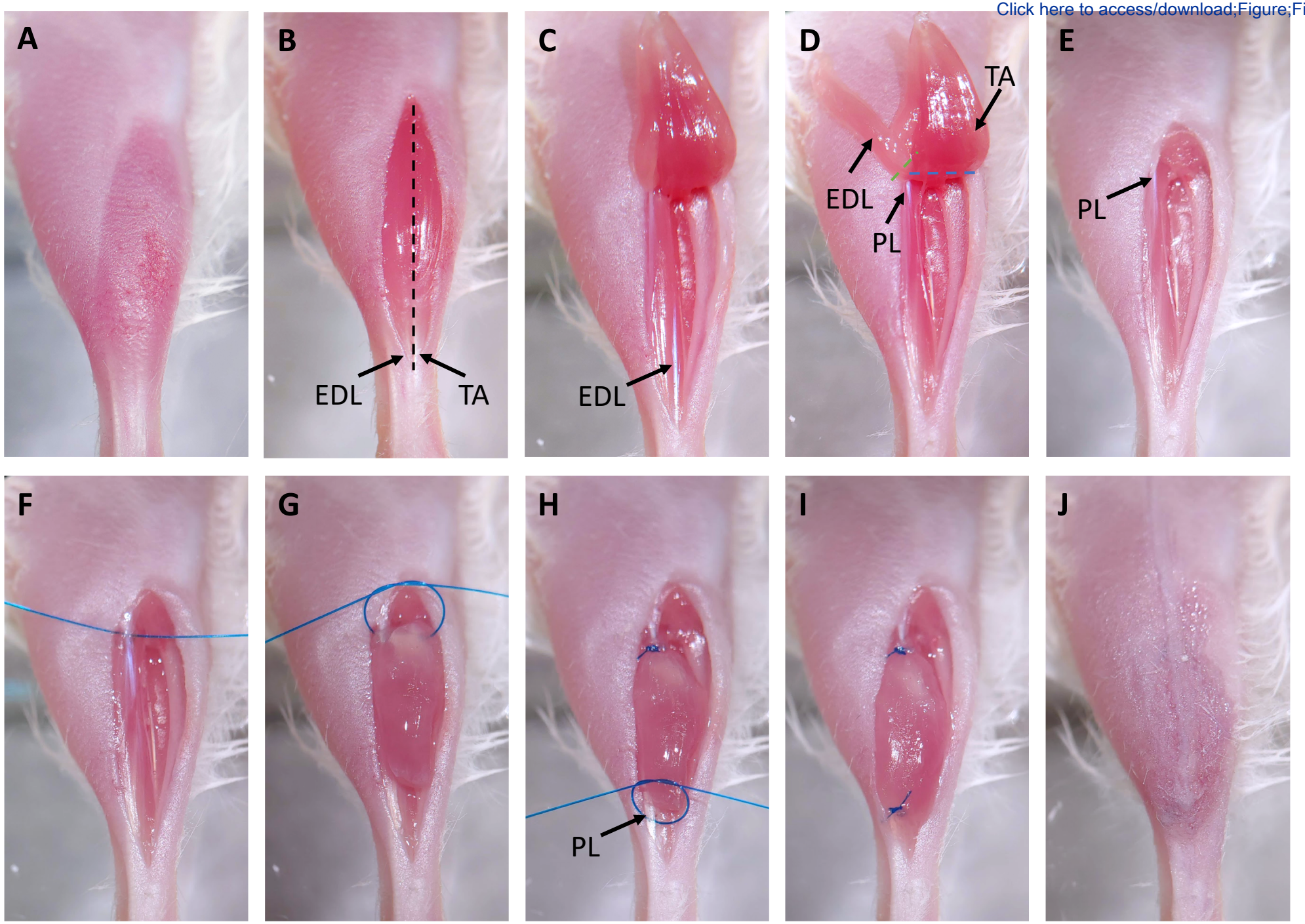
DISCLOSURES:

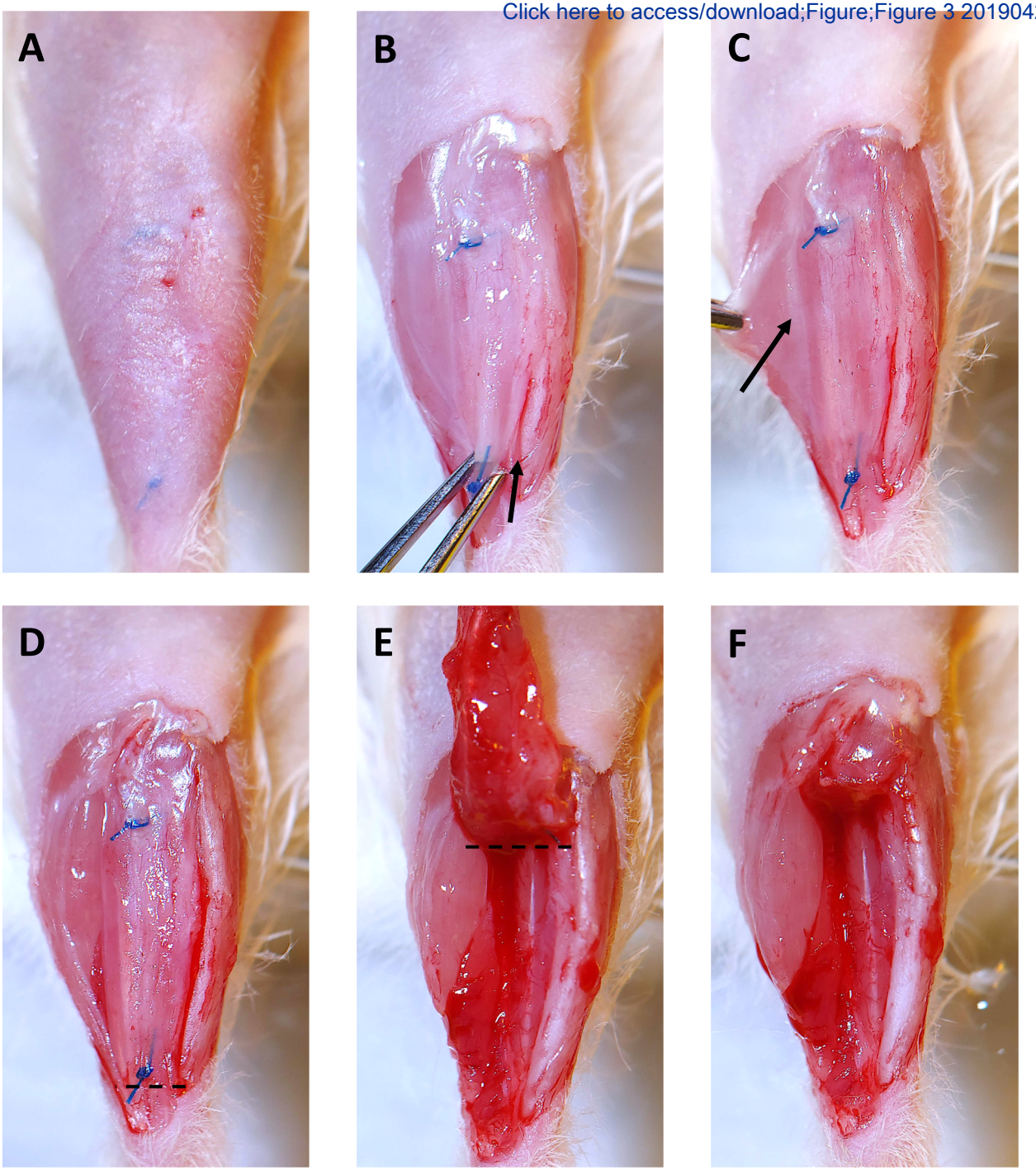
The authors declare that they have no competing financial interests.

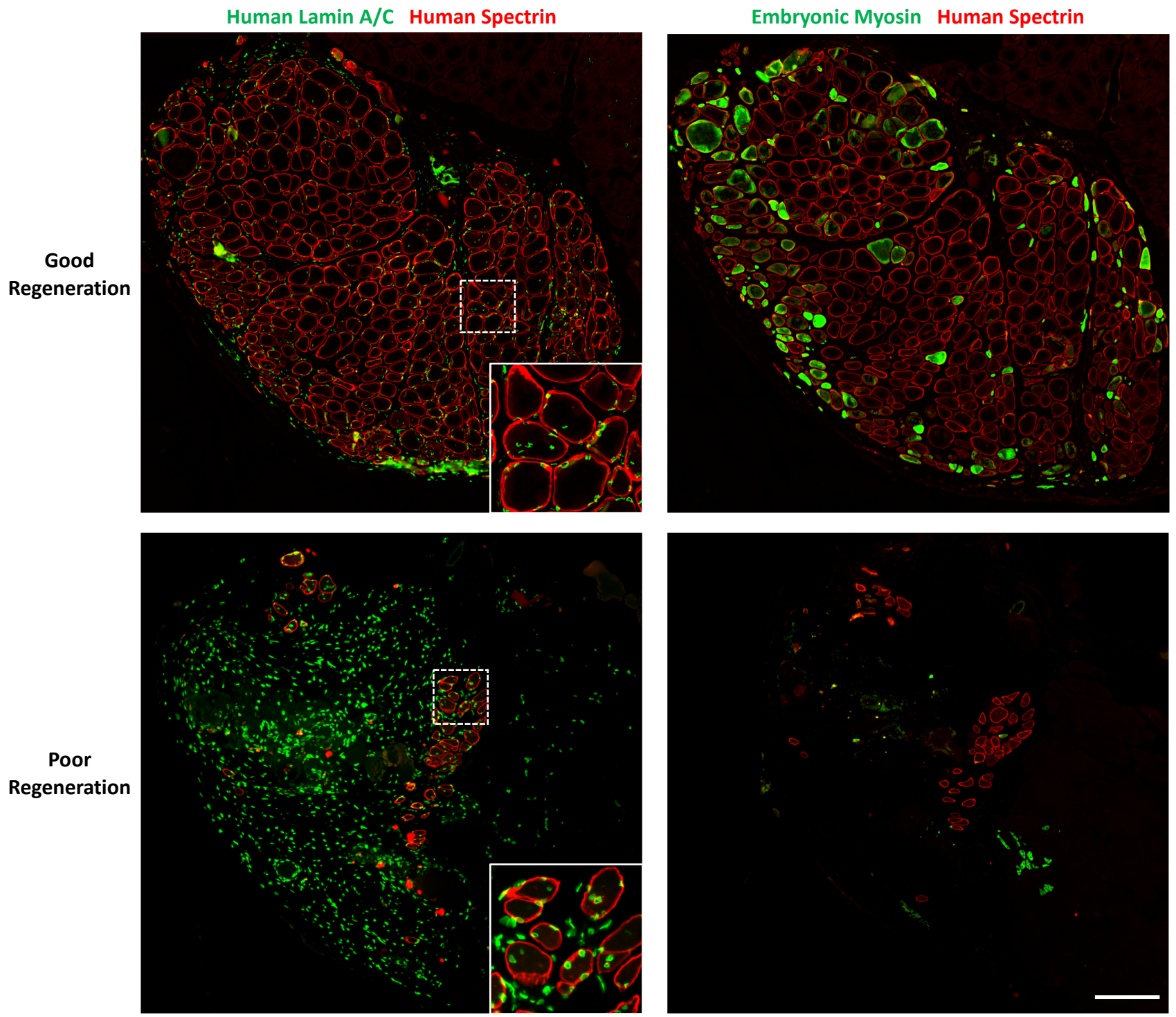
REFERENCES:

1. Wong, C. H., Siah, K. W., Lo, A. W. Estimation of clinical trial success rates and related parameters. *Biostatistics* **0**, 1–14 (2018).
2. Hay, M., Thomas, D. W., Craighead, J. L., Economides, C., Rosenthal, J. Clinical development success rates for investigational drugs. *Nature Biotechnology.* **32**, 40–51 (2014).
3. Perel, P. et al. Comparison of treatment effects between animal experiments and clinical trials: systematic review. *BMJ.* **334**, 1–6 (2007).
4. Rubio-Viqueira, B., Hidalgo, M. Direct in vivo xenograft tumor model for predicting chemotherapeutic drug response in cancer patients. *Clinical Pharmacology Therapeutics*. **85**, 217–221 (2009).
5. Roberts, K. G. et al. Targetable Kinase-Activating Lesions in Ph-like Acute Lymphoblastic Leukemia. *New England Journal of Medicine.* **371**, 1005–15 (2014).
6. Kim, J. et al. GDF11 Controls the Timing of Progenitor Cell Competence in Developing Retina. *Science.* **308**, 1927–1930 (2005).
7. Sako, D. et al. Characterization of the ligand binding functionality of the extracellular domain of activin receptor type IIB. *Journal of Biological Chemisty.* **285**, 21037–21048 (2010).
8. Zhang, Y. et al. Human skeletal muscle xenograft as a new preclinical model for muscle disorders. *Human Molecular Genetics.* **23**, 3180–3188 (2014).
9. Gabellini, D., Green, M. R., Tupler, R. Inappropriate Gene Activation in FSHD : A Repressor Complex Binds a Chromosomal Repeat Deleted in Dystrophic Muscle. *Cell.* **110**, 339–348 (2002).
10. Lemmers, R. J. L. F. et al. A Unifying Genetic Model for Facioscapulohumeral Muscular Dystrophy. *Science.* **329**, 1650–1654 (2010).
11. Chen, J. C. J. et al. Morpholino-mediated Knockdown of DUX4 Toward Facioscapulohumeral Muscular Dystrophy Therapeutics. *Molecular Therapy.* **24**, 1405–1411 (2016).

12. Medical Research Council. Aids to the investigation of the peripheral nervous system. London: Her Majesty's Stationary Office (1943).
13. Jones, R. A. et al. Cellular and Molecular Anatomy of the Human Neuromuscular Junction. *Cell Reports.* **21**, 2348–2356 (2017).







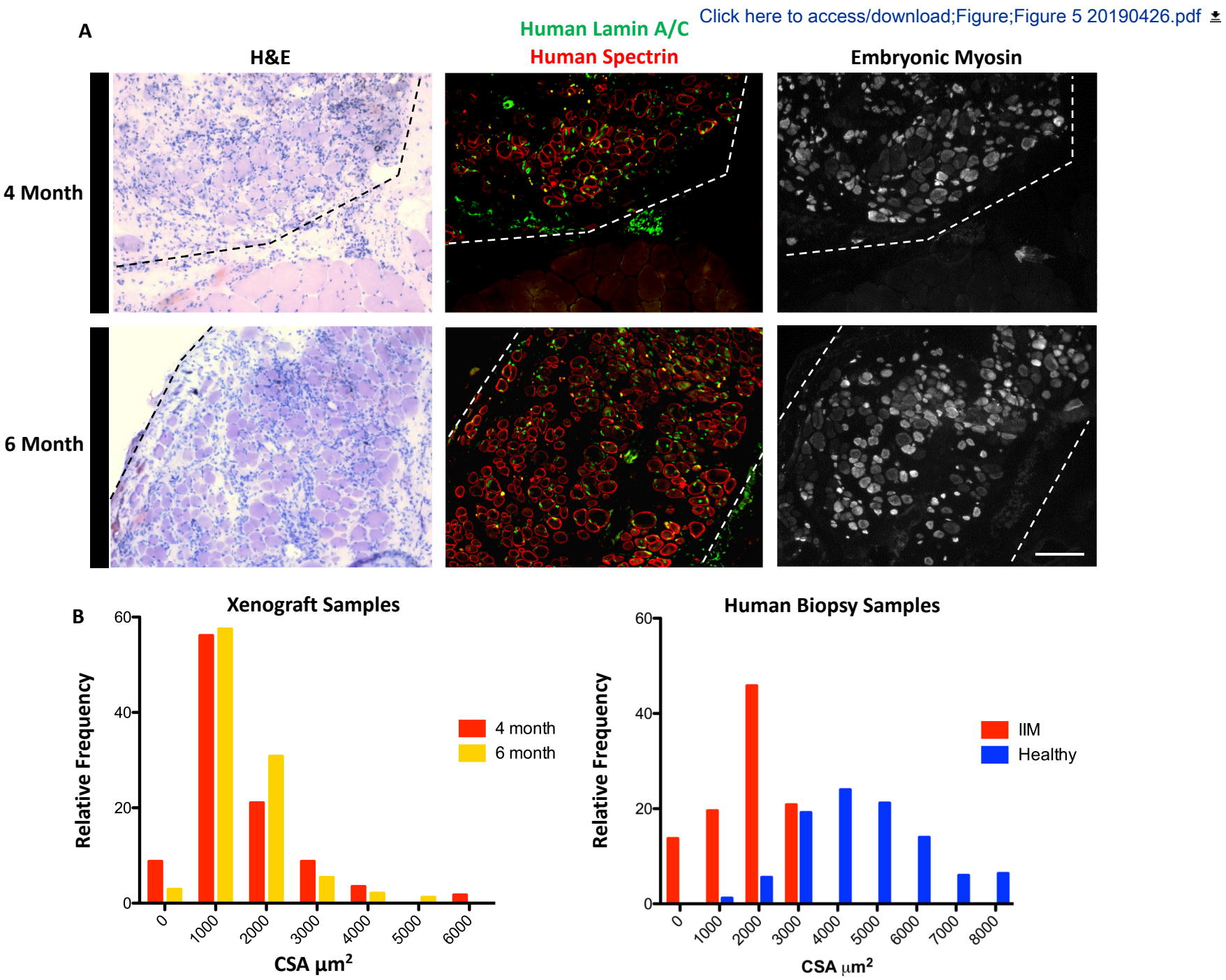


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Table of Materials

Name of Material/ Equipment	Company	Catalog Number
100 mm x 15 mm Petri dish	Fisher Scientific	FB0875712
2-Methylbutane	Fisher	O3551-4
20 x 30 mm micro cover glass	VWR	48393-151
Animal Weighing Scale	Kent Scientific	SCL- 1015
Antibiotic-Antimycotic Solution	Corning, Cellgro	30-004-CI
AutoClip System	F.S.T	12020-00
Castroviejo Needle Holder	F.S.T	12565-14
Chick embryo extract	Accurate	CE650TL
CM1860 UV cryostat	Leica Biosystems	CM1860UV
Coplin staining jar	Thermo Scientific	19-4
Dissection Pins	Fisher Scientific	S13976
Dry Ice - pellet	Fisher Scientific	NC9584462
Embryonic Myosin antibody	DSHB	F1.652
Ethanol	Fisher Scientific	459836
Fetal Bovine Serum	GE Healthcare Life Sciences	SH30071.01
Fiber-Lite MI-150	Dolan-Jenner	Mi-150
Forceps	F.S.T	11295-20
Goat anti-mouse IgG1, Alexa Fluor 488	Invitrogen	A-21121
Goat anti-mouse IgG2b, AlexaFluor 594	Invitrogen	A-21145
Gum tragacanth	Sigma	G1128
Hams F-10 Medium	Corning	10-070-CV
Histoacryl Blue Topical Skin Adhesive	Tissue seal	TS1050044FP
Human specific lamin A/C antibody	Abcam	ab40567
Human specific spectrin antibody	Leica Biosystems	NCLSPEC1
Induction Chamber	VetEquip	941444
Iris Forceps	F.S.T	11066-07

Irradiated Global 2018 (Uniprim 4100 ppm)	Envigo	TD.06596
Isoflurane	MWI Veterinary Supply	502017
Kimwipes	Kimberly-Clark	34155
Mapleson E Breathing Circuit	VetEquip	921412
Methanol	Fisher Scientific	A412
Mobile Anesthesia Machine	VetEquip	901805
Mouse on Mouse Basic Kit	Vector Laboratories	BMK-2202
Nail Polish	Electron Microscopy Sciences	72180
NAIR Hair remover lotion/oil	Fisher Scientific	NC0132811
NOD-*Rag1* ^{null} *IL2rg* ^{null} (NRG) mice	The Jackson Laboratory	007799
O.C.T. Compound	Fisher Scientific	23-730-571
Oxygen	Airgas	OX USPEA
PBS (phosphate buffered saline) buffer	Fisher Scientific	4870500
Povidone Iodine Prep Solution	Dynarex	1415
ProLong™ Gold Antifade Mountant	Fisher Scientific	P10144 (no DAPI); P36935 (with DAPI)
Puralube Ophthalmic Ointment	Dechra	17033-211-38
Rimadyl (carprofen) injectable	Patterson Veterinary	10000319
Scalpel Blades - #11	F.S.T	10011-00
Scalpel Handle - #3	F.S.T	10003-12
Stereo Microscope	Accu-scope	3075
Superfrost Plus Microscope Slides	Fisher Scientific	12-550-15
Suture, Synthetic, Non-Absorbable, 30 inches long, CV-11 needle	Covidien	VP-706-X
1ml Syringe (26 gauge, 3/8 inch needle)	BD Biosciences	329412
Trimmer	Kent Scientific	CL9990-KIT
Vannas Spring Scissors, 8.0 mm cutting edge	F.S.T	15009-08
VaporGaurd Activated Charcoal Filter	VetEquip	931401

Wound clips, 9 mm	F.S.T	12022-09

Comments/Description
recommended concentration 1:10
recommended concentration 1:500
recommended concentration 1:500
recommended concentration 1:50-1:100
recommended concentration 1:20-1:100

Antibiotic rodent diet to protect again respiratory infections

surgical wipes

mouse IgG blocking reagent

2 to 3 months old

surgical analgesic, administered subcutaneously at a dose of 5mg/kg

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Title of Article:	Performing Human Skeletal Muscle Xenografts in NOD-RAG1null IL2rynull mice
Author(s):	Kyla A. Britson, Aaron D. Black, Kathryn R. Wagner, and Thomas E. Lloyd

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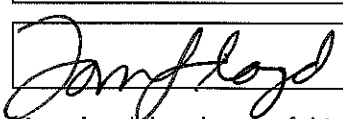
CORRESPONDING AUTHOR

Name:	Thomas E. Lloyd
Department:	Departments of Neuroscience and Neurology
Institution:	Johns Hopkins University School of Medicine
Title:	Assistant Professor
Signature:	
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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

May 6, 2019

Phillip Steindel, PhD
Review Editor
JoVE
1 Alewife Center | Suite 200 | Cambridge | MA | 02140
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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