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Expanding Donor Muscle-Derived Cells for Transplantation

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Abstract

Studies in mice showed tremendous promise for the eventual clinical utility of myoblast transplantation to treat human muscular dystrophies. Initial attempts to translate the murine studies to humans, however, were not successful, due in part to limited engraftability of expanded donor myoblasts. Conventionally, muscle cells have been cultured on collagen-coated tissue culture treated polystyrene. However, this promotes lineage progression and differentiation of cells, which limits engraftment potential. This unit describes the isolation of canine muscle-derived cells, *ex vivo* expansion of cells on plates coated with a modified Notch ligand, and the xenotransplant method used to evaluate engraftment potential. Activation of Notch signaling in freshly isolated canine muscle-derived cells with Delta-1^{ext}-IgG inhibits myogenic differentiation, and maintains cells earlier in myogenic lineage progression. Delta-1^{ext}-IgG-expanded cells engraft into the regenerating muscle of NOD/SCID mice more effectively than control cells expanded on human IgG, as evidenced by a significant increase in the number of muscle fibers expressing canine dystrophin in recipient murine muscle. Therefore, this protocol provides the basis for further developing culture conditions for *ex vivo* expansion of donor muscle cells for transplant.

Keywords

Notch signaling; skeletal muscle; satellite cells; transplantation

INTRODUCTION

Basic Protocol 1 describes the protocol for isolating the mixed population of mononuclear cells from canine skeletal muscle. We use canine skeletal muscle because our objective is to expand a population of donor cells that will effectively engraft into the muscle of *cxmd* canines, a large animal model of Duchenne muscular dystrophy. We chose this model because canine models of cell and organ transplantation have accurately predicted clinical outcomes in humans for more than 40 years. The xenotransplantation model described in Basic Protocol 2 involves transplanting canine cells into the regenerating muscle of NOD/SCID mice. NOD/SCID mice are immunocompromised, and do not reject the xenograft. This provides a pre-screening system to quantitatively assess engraftment potential of canine cell populations, and prioritize experiments in *cxmd* canines.

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Basic Protocol 1 involves enzymatic digestion of the muscle, filtration and centrifugation steps that separate the mononuclear cells from the muscle fiber debris, and set up of cultures for *ex vivo* expansion. The alternate protocol uses a 2-step digestion, to remove interstitial cells and slightly increase the proportion of myogenic cells. The cells are expanded on plates coated with Delta-1^{ext}-IgG, a modified Notch receptor ligand. Activating Notch signaling maintains the cells earlier in lineage progression than control cells, and improves engraftment potential (Parker et al., 2012a). *Ex vivo* expansion increases the number of cell injections possible from a single donor biopsy, which has important implications for establishing a protocol for human muscle cell transplantation to treat muscular dystrophies.

Together, these protocols outline the basic steps involved in further developing a protocol for *ex vivo* expansion of donor muscle cells for transplantation.

NOTE: Aseptic technique is required for handling of all solutions, materials, and equipment in contact with living cells

BASIC PROTOCOL 1: ISOLATION OF CANINE MUSCLE-DERIVED MONONUCLEAR CELLS FOR CULTURE AND TRANSPLANT

This protocol describes the method used to isolate the mixed population of mononuclear cells from a canine skeletal muscle biopsy. This protocol can be used for muscle samples from other species, such as mouse and human. This protocol describes the one-step method for isolating cells. A two-step protocol that slightly increases the proportion of myogenic cells is described below (Alternate Protocol 1). The procedures involved in obtaining the muscle biopsy are not described.

Materials

Dulbecco's phosphate buffered saline (D-PBS) – Ca²⁺ and Mg²⁺ free

Sterilized 10-cm glass petri dishes

Sterilized paper towel

2 scalpel blade holders with #11 blades

Dulbecco's modified eagle's medium (DMEM) – high glucose

Collagenase, Type 4 (Worthington Biochemical; catalog #LS004186)

It is important to test different lots of collagenase, as there is lot-to-lot variation in the specific activity of collagenase, and contaminating enzymes, such as trypsin and clostripain. Worthington Biochemical has a collagenase sampling program to determine which lot is most appropriate for your needs, and then permits selection of specific lots based on enzyme activity.

15-ml conical polystyrene tubes

Parafilm®

Water bath set at 37°C

Plastic disposable Pasteur pipettes

Pipette-aid

5-ml plastic pipettes

20-ml syringes

16½ and 18½ gauge needles

Cell strainers – 75µm and 40µm (BD Biosciences; catalog # 352340 (40 µm) and cat# 352350 (70µm))

50-ml conical polystyrene tubes

Centrifuge that can spin 50-ml and 15-ml tubes at 1000 and 1500 rpm

MACS® pre-separation filters (Miltenyi Biotec; catalog #130-041-407)

Plates coated with Delta-1^{ext}-IgG, human IgG (see Support Protocol 1)

Growth medium (see recipe)

37°C humidified incubator with 5% CO₂

Inverted phase-contrast microscope

Enzymatic Digestion of the Muscle

- 1 Obtain a skeletal muscle sample that is stored in sterile D-PBS and kept on wet ice. All remaining steps should be performed in a laminar flow hood, taking precautions to maintain sterility.

Muscle samples should be processed as soon as possible after the biopsy procedure. Although we have not quantitatively compared engraftment of cells from biopsies stored for various time points after isolation, we have determined that overnight storage of the biopsy almost completely abolishes engraftment potential.

- 2 Remove the muscle from D-PBS and place in a 10-cm sterilized glass dish. Remove as much connective tissue and fat as possible. These will appear white against the pink or red of the muscle fibers. Cut the muscle along the length of the fibers, such that new pieces of muscle are approximately 0.5 cm wide. Place muscle pieces in a new 10-cm glass dish containing enough D-PBS to cover the muscle, and gently swirl.

For mouse muscle, or small muscle biopsies, the later part of this step is not necessary. In all cases, remove connective tissue and fat.

- 3 Cut the muscle pieces along the fiber length into even smaller pieces, approximately 0.2 cm wide, and place into a second sterile glass dish containing enough D-PBS to cover the muscle, and gently swirl.

For mouse muscle, or small muscle biopsies, this step is not necessary.

- 4 Remove the muscle pieces and dab dry on sterilized paper towel. Transfer pieces to a sterile 10-cm glass dish.

- 5 Using the 2 scalpels, chop the muscle using opposing strokes across the muscle pieces. Continue until the muscle adopts a smooth, thick mushy appearance, which generally requires at least 5 minutes of continuous chopping.

This step is particularly important, as insufficient chopping decreases cell yield.

- 6 Prepare two 15-ml conical polystyrene tubes containing 400 units/ml of collagenase in DMEM. Divide the muscle between the two tubes. Shake the tubes vigorously, and wrap the caps securely with Parafilm®. Incubate tubes in a 37° water bath for 60 minutes. Vigorously shake the tubes every 10 minutes.

The tubes can be placed upright in the water bath, but the digestion is more efficient if the tubes are laid on their side. Alternatively, the tubes can be placed upright in a shaking water bath. To prevent contamination, ensure the tube caps are securely fastened and tightly wrapped in Parafilm®. Check the muscle every 10 minutes, and vigorously shake the tube. The digest is complete when the muscle pieces are very small and the medium looks cloudy.

- 7 Using a plastic Pasteur pipette, triturate the muscle by pipetting up and down 20 times. Repeat using a 5-ml plastic pipette, being careful not to breach the cotton plug barrier.

If it is difficult to triturate the muscle with the 5 ml pipette, return the muscle to the 37° water bath for an additional 10–15 minutes, and repeat the trituration process.

Separation of Mononuclear Cells from Debris

- 8 Transfer the contents of the two 15-ml conical tubes to a single 50-ml conical polystyrene tube. Triturate the sample by passing the sample 20 times through a 16½ gauge needle attached to a 20-ml syringe, and repeat using an 18½ gauge needle attached to a 20-ml syringe.

- 9 Place a 70-µm cell strainer in a 50-ml conical polystyrene tube and wet the membrane with 5 ml of D-PBS. Add 10 ml of D-PBS to the muscle slurry and apply the mixture to the membrane using a 5-ml pipette or a plastic Pasteur pipette. After the sample has passed through completely, wash the membrane with at least 5 ml of D-PBS.

If the muscle biopsy was large or fibrous, it can be difficult to filter the slurry. A plastic Pasteur pipette can be used to pipette the slurry up and down on top of the membrane to encourage filtration. Alternatively, the samples can be split between 2 separate cell strainers and the filtrates combined at the end of step 10.

- 10 Place a 40-µm cell strainer in a 50-ml conical polystyrene tube and wet the membrane with 5 ml of D-PBS. Apply the flow-through from step 9 to the membrane. After sample has passed through completely, wash the membrane with at least 5 ml of D-PBS.

- 11 Remove the filter, and add enough D-PBS to bring the total volume to 50 ml. Cap the tube and centrifuge the sample at 1500 rpm for 10 minutes at room temperature.

- 12 Carefully remove the supernatant by aspiration, and flick the tube to dislodge the cell pellet. Resuspend the cell pellet in 7.5 ml of D-PBS.

The cell pellet can be loosely packed, and care should be taken while aspirating the supernatant so as not to lose any of the cells. Flicking the tube to dislodge the pellet is a gentle means of facilitating cell suspension in D-PBS.

- 13 Place a MACS® 30-µm pre-separation filter in a 15-ml conical polystyrene tube, and wet the filter with 2.5 ml of D-PBS. Apply the cell suspension from step 12 to the filter, and wash the filter with 2.5 ml of D-PBS.

Similar to step 9, pipetting the cell suspension up and down gently can encourage filtration.

- 14 Remove the filter and cap the tube. Centrifuge the flow-through at 1000 rpm [$\times$ g] for 5 minutes at room temperature. Remove the supernatant by aspiration, and flick the tube to dislodge the cell pellet. Resuspend the pellet in 10 ml of D-PBS. Centrifuge a last time at 1000 rpm [$\times$ g] for 5 minutes at room temperature. Remove the supernatant, and flick the tube to dislodge the cell pellet. Resuspend the pellet in 2 ml of D-PBS.

Carefully remove the supernatant, to prevent cell loss. If the muscle biopsy is smaller than 1 cm³, the cell pellet can be resuspended in a smaller volume of D-PBS.

Establishing and Maintaining Ex Vivo Expansion Cultures

- 15 Remove 10 μ l of the cell suspension for counting (see Support Protocol 1).
- 16 Dilute the cells in growth medium (see recipe) at 1×10^4 cells/ml. Add 10 ml (1×10^5 cells) to each coated plate (see Support Protocol 2) and place in a 37°C humidified incubator with 5% CO₂.
- 17 Two days after plating, add 10 ml of growth medium without removing any medium. Four days after plating, entirely replace the medium with 10 ml of fresh growth medium. Replace the medium every 2 days with fresh growth medium.
- 18 Look at the cells daily using an inverted phase-contrast microscope. Proliferating myogenic cells appear as round, refractile cells on plates coated with human IgG, and small, but slightly flattened cells, on plates coated with Delta-1^{ext}-IgG. Cell confluence should not exceed 80% of the plate; otherwise the cells will enter differentiation. Cells that have initiated differentiation appear elongated.
- 19 Once the cells have reached 80% confluence, they are ready for transplantation. See Protocol 2.

Cells proliferate more extensively on plates coated with human IgG as compared to cells on plates coated with Delta-1^{ext}-IgG. As such, they will not reach 80% confluence on the same day. For *in vitro* assays, simply process the cells on different days. For transplantation, harvest both sets of cells when the human IgG plates have reached 80% confluence.

ALTERNATE PROTOCOL 1 TWO-STEP DIGESTION METHOD TO INCREASE THE PROPORTION OF MYOGENIC CELLS

Materials

Dulbecco's phosphate buffered saline (D-PBS) – Ca²⁺ and Mg²⁺ free
 Sterilized 10-cm glass petri dishes
 Sterilized paper towel
 2 scalpel blade holders with #11 blades
 Dulbecco's modified eagle's medium (DMEM) – high glucose
 Collagenase, Type 4 (Worthington Biochemical)

It is important to test different lots of collagenase, as there is lot-to-lot variation in the specific activity of collagenase, and contaminating enzymes,

such as trypsin and clostripain. Worthington Biochemical has a collagenase sampling program to determine which lot is most appropriate for your needs, and then permits selection of specific lots based on enzyme activity. For the first digestion, you will want a lot with low clostripain activity, to prevent removal of the basal lamina from the fibers. For the second digestion, you will use the lot from the one-step digestion method.

15-ml conical polystyrene tubes

Parafilm®

Water bath set at 37°C

Plastic disposable Pasteur pipettes

5-ml plastic pipettes

10-ml syringes

16½ and 18½ gauge needles

Cell strainers – 75µm and 40µm (BD Biosciences; catalog # 352340 (40 µm) and cat# 352350 (70µm))

50-ml conical polystyrene tubes

Centrifuge that can spin 50-ml and 15-ml tubes at 1500 rpm

MACS® pre-separation filters (Miltenyi Biotec; catalog #130-041-407)

Plates coated with Delta-1^{ext}-IgG, human IgG (see Support Protocol 1)

Growth medium (see recipe)

37°C humidified incubator with 5% CO₂

Inverted phase contrast microscope

1. Complete steps 1 – 4 in Basic Protocol 1.
2. Prepare two 15-ml conical polystyrene tubes containing 400 units/ml of the first lot of collagenase in DMEM. Divide the muscle pieces between the two tubes. Shake the tubes gently back and forth along the length of the tube, and wrap the caps securely with Parafilm®. Incubate tubes in a 37° water bath for 30 minutes. Gently shake the tubes every 10 minutes.

The tubes can be placed upright in the water bath, but the digestion is more efficient if the tubes are laid on their side. Alternatively, the tubes can be placed upright in a shaking water bath. To prevent contamination, ensure the tube caps are securely fastened and tightly wrapped in Parafilm®. Check the muscle every 10 minutes, and gently shake the tube. Muscle fibers should still be visible. The purpose of this shorter, gentle digestion is to release interstitial cells and blood cells without losing satellite cells from the fibers.

3. Allow the contents of the tubes to settle, and carefully remove 7–8 mls of medium with a plastic Pasteur pipette, without disturbing the muscle pieces on the bottom of the tube. Add 8 mls of D-PBS, rock the tube gently, and allow the muscle pieces to settle to the bottom of the tube. Carefully remove 7–8 mls of the D-PBS wash with a plastic Pasteur pipette, without disturbing the muscle pieces on the bottom of the tube.

4. Remove the muscle pieces and dab dry on sterilized paper towel. Transfer pieces to a sterile 10-cm glass dish.
5. Using the 2 scalpel blades, chop the muscle using opposing strokes across the muscle pieces. Continue until the muscle adopts a smooth, thick mushy appearance, which generally requires at least 5 minutes of continuous chopping.

This step is particularly important, as insufficient chopping decreases cell yield.

6. Prepare two 15-ml conical polystyrene tubes containing 400 units/ml of the second lot of collagenase in DMEM. Transfer the muscle to these two tubes. Shake the tubes vigorously, and wrap the caps securely with Parafilm®. Incubate tubes in a 37° water bath for 60 minutes. Vigorously shake the tubes every 10 minutes.

The tubes can be placed upright in the water bath, but the digestion is more efficient if the tubes are laid on their side. Alternatively, the tubes can be placed upright in a shaking water bath. To prevent contamination, ensure the tube caps are securely fastened and tightly wrapped in Parafilm®. Check the muscle every 10 minutes, and vigorously shake the tube. The digest is complete when the muscle pieces are very small and the medium looks cloudy.

7. Complete steps 7 – 19 from Basic Protocol 1.

SUPPORT PROTOCOL 1 CELL COUNTING USING THE HEMOCYTOMETER AND SETTING UP DILUTIONS FOR CULTURE

Materials

Hemocytometer

Inverted microscope to count cells on hemocytometer

P20 pipettor and appropriate tips

1. Place the special coverslip on the surface of the hemocytometer so that the two cell counting areas are covered.
2. Carefully pipette 10 µl of cells into one of the V-shaped wells under the coverslip. The hemocytometer should fill by capillary action.
3. Look at the cells using an inverted microscope. The counting area should look like Figure 1. Count the cells in the 2 sets of 16 squares outlined in Figure 1. Be careful to count cells and not debris. Cells are normally round and refractile. Make sure cells are not overlapping and are evenly distributed over the counting surface. If you have more than 100 – 150 cells in one set of 16 squares, dilute the sample and repeat the counts.
4. Calculations: Divide the number of cells counted in the 2 sets of squares by 2. Multiple this value by 1×10^4 to give the cells per ml. If you diluted the sample for counting, this value is the cells per ml in the dilution. Multiply by your dilution factor to give the number of cells per ml in the original cell suspension.
5. Go to step 16 of Basic Protocol 1.

SUPPORT PROTOCOL 2 GENERATING DELTA-1^{EXT}-IgG AND HUMAN IgG COATED PLATES

This protocol describes the preparation of plates coated with modified Notch ligand, Delta-1^{ext}-IgG, or human IgG control. The Delta-1^{ext}-IgG molecule is a kind gift of Dr. Irwin Bernstein. It is produced in NS0 cells and purified at the Fred Hutchinson Cancer Research Center. Preparation of the plates must begin the day before the cell isolation.

Materials

Delta-1^{ext}-IgG – prepared as a 1.2 mg/ml solution and frozen in 500 μ l aliquots at -80°C

Human IgG – prepared as a 1 mg/ml solution and frozen in 500 μ l aliquots at -80°C (Sigma Aldrich; catalog #I-4506)

Dulbecco's phosphate buffered saline (D-PBS) – Ca^{2+} and Mg^{2+} free

Tissue culture-treated polystyrene plates

Parafilm®

4°C refrigerator or cold room

Growth medium (see recipe)

2% bovine serum albumin (BSA) in D-PBS (Sigma Aldrich; catalog #A-3294)

37°C humidified incubator with 5% CO_2

1. Thaw Delta-1^{ext}-IgG and human IgG on ice. Dilute Delta-1^{ext}-IgG and human IgG in D-PBS to give 10 $\mu\text{g/ml}$ solutions of each.
Keep stock solutions and dilutions on ice.
2. Apply 5 mls of either Delta-1^{ext}-IgG or human IgG to 10-cm polystyrene plates and swirl to ensure that the entire surface is covered with solution.
Use a proportionately smaller volume for smaller plates or wells. For example, a 10-cm plate has a surface area of $25\pi \text{ cm}^2$, and a 6-cm plate has a surface area of $9\pi \text{ cm}^2$. Therefore, a 6-cm plate would require $(9/25)*5$ mls, or 1.8 mls of each solution for coating. Each 10-cm plate generates approximately $5\text{--}7.5 \times 10^5$ cells in 8 days.
3. Wrap plates in Parafilm® to prevent evaporation, and place at 4°C overnight.
Overnight is equivalent to a minimum of 16 hours.
4. The following day, remove the plates and aspirate the solution from the plates. Immediately add 5–10 mls of D-PBS and gently swirl the plate. Wash the plates at least 3 times with D-PBS.
Do not let the plates dry. Work in small batches of plates if necessary.
5. After the last D-PBS wash, add 10 mls of growth medium (see recipe) or 2% bovine serum albumin dissolved in D-PBS. Place in 37°C humidified incubator with 5% CO_2 for 1 hour.
6. Aspirate medium or BSA, and wash plate once with D-PBS. Immediately add cells according to step 16 of Protocol 1. If cells are not ready, add D-PBS to ensure plates do not dry.

SUPPORT PROTOCOL 3 DETERMINING THE PERCENTAGE OF MYOGENIC CELLS IN THE FRESHLY ISOLATED CELL SUSPENSION BY COUNTING COLONIES

On average, 1.5% of the population of freshly isolated muscle cells from a canine muscle biopsy is myogenic. Accurately determining the percent of myogenic cells using flow cytometry has proven difficult, and therefore, we use a method of counting colonies to determine the percentage of myogenic cells.

Materials

Cells from Protocol 1 or Alternate Protocol 1

Growth medium (see recipe)

10-cm tissue culture treated polystyrene plates

Inverted phase-contrast microscope

Object marker for microscope objective or Sharpie marker

1. After step 15 of Basic Protocol 1 or Alternate Protocol 1, and completion of Support Protocol 1, dilute the cells to 1×10^3 cells/ml in growth medium. Add 10 ml of the cell dilution to each of five 10-cm polystyrene plates.
2. Two days after plating, add 10 ml of medium to each plate without removing any medium.
3. After an additional two days (four days after plating), entirely replace medium with 10 ml of fresh growth medium. Replace medium every two days.
4. When cells have generated colonies that have an average of 10 cells per colony or more, count the colonies on each plate that have round, refractile cells. Divide this by 10,000 to calculate the fraction of cells that are myogenic. Calculate the average and the standard deviation of the counts from the 5 plates.

Fibroblasts will appear flattened on uncoated polystyrene plates, whereas myogenic cells are round and highly refractile. Some cells within myogenic colonies will appear elongated and somewhat flattened if differentiation has begun. Don't let cells go too long, or colonies will begin to overlap. If your microscope is fitted with an object marker on the objective, you can simply mark the myogenic colonies with an "O" on the bottom of the plate, and count the number of "O"s on the plate. Otherwise, you can tape the lid to the plate, and mark the top of the plate with a Sharpie marker to spot the colonies.

BASIC PROTOCOL 2 TRANSPLANTATION OF EXPANDED CELLS

Once the desired confluence is reached, cells are detached from the plates, washed and prepared for transplantation into mice. See Support Protocol 4 for the method for preparing the mice for transplant. Steps 1 – 7 outlined below should be completed in a laminar flow hood.

Materials

Dulbecco's phosphate buffered saline (D-PBS) – Ca^{2+} and Mg^{2+} free

Cell Dissociation Buffer (Invitrogen – catalog #13151-014)

37°C humidified incubator with 5% CO₂
Inverted phase-contrast microscope
15-ml conical polystyrene tubes
5 ml pipettes
Pipette-aid
Centrifuge that can spin 50-ml and 15-ml tubes at 1500 rpm
P200 pipettor and appropriate tips
Sterile microfuge tubes
0.5cc insulin syringe, or similar small volume syringe with ½ inch needle
70% ethanol in a spray bottle

Cell Preparation

- 1 Wash the plates of cells once with D-PBS, and aspirate the D-PBS. Add 5 ml of Cell Dissociation Buffer and incubate at 37°C for 5 minutes.
- 2 Bang the side of the plates against the inside of your wrist 10 times, turning the plate after each hit. The impact must be enough to disrupt the cells, but not allow the buffer to slosh outside of the plate.

This step helps to detach the cells from the plate, and is gentler than repeated trituration with a pipette.
- 3 Look at the plate using an inverted phase-contrast microscope to determine if cells are detached. If cells remain attached, repeat step 2 until cells are detached.
- 4 Transfer cells from the plate to a 15-ml conical polystyrene tube using a 5-ml pipette. Wash the plate with D-PBS and transfer the D-PBS wash to the same tube. Centrifuge the capped tube at 1000 rpm for 5 minutes at room temperature.
- 5 Aspirate the supernatant, being careful not to disturb the cell pellet. Flick the tube several times to gently dislodge the cell pellet. Add 10 ml of D-PBS, and gently triturate by pipetting up and down 10 times. Centrifuge at 1000 rpm for 5 minutes at room temperature.
- 6 Repeat step 5, and resuspend the cells in 1–2 mls of D-PBS. Count the cells using the method described in Support Protocol 1 and determine the concentration of the cells (cell/ml).

Keep the cells on ice after this step to help maintain engraftment potential.
- 7 Dilute the cells to 1×10^6 cell/ml in D-PBS. Transfer 50 µl to each sterile microfuge tube for a cell dose of 50,000 cells. Keep microfuge tubes on ice.

Cell Transplant into Mice

- 8 Anesthetize mice (see Support Protocol 4 for preparation of mice). We normally use isoflurane for this step, since the mice recover most quickly with this form of anesthesia. Using an insulin syringe, or similar syringe, pull the 50 µl volume of cells into a single syringe.
- 9 Spray lower hindlimb with 70% ethanol, and mat down fur to expose skin. Insert the needle into the skin near the foot, and parallel to the length of the tibialis

anterior muscle, and push the needle gently into the muscle, so that the tip is close to the distal portion of the muscle. Inject the cells slowly as you pull out the needle.

The tibialis anterior (TA) is used for transplant because it is easily accessible without exposing the muscle with an incision. When the mouse is resting on its back, the TA muscle is positioned laterally to the tibia. By feeling for the tibia, you can easily locate the adjacent muscle.

- 10 Monitor mice until fully recovered from anesthesia, and mobile.
Mice will favor the injected leg for a short period of time after recovery from anesthesia. This normally resolves within an hour.
- 11 Monitor mice daily for 4 weeks. Remove the injected muscle, and freeze in OCT, using an aluminum block submerged in liquid nitrogen. Store the muscle at -80°C .

SUPPORT PROTOCOL 4 PREPARING MICE FOR TRANSPLANTATION

For donor muscle cells to engraft, the host muscle is irradiated and induced to regenerate. The irradiation prevents host satellite cell contribution to regeneration, giving the donor cells a competitive advantage. In this protocol, mice are irradiated and induced to regenerate the day before cell transplant. Other timelines are possible, and should be explored to determine the best regimen.

Materials

NOD/SCID mice (or other appropriate strain)
 Injectable anesthesia, e.g. avertin, ketamine/xylazine
 Cesium-137 source for irradiation
 Holder for mice – to keep mice in one position and only expose hindlimbs to irradiation
 0.5cc insulin syringe, or similar small volume syringe with $\frac{1}{2}$ inch needle
 1.2% barium chloride (Sigma-Aldrich; catalog #B0750)
 70% ethanol in a spray bottle

1. Anesthetize mice with an appropriate dose of injectable anesthesia. Once mice do not respond to stimulation (e.g. foot pinching), place the mice in the holder. Place the holder in the irradiator, so that only the hindlimbs are exposed to the beam of irradiation.

The mice will need to be immobile for at least 20 minutes, so use an appropriate anesthetic and dose of anesthesia. If mice awaken during the procedure, there is a risk of total body exposure, which is lethal.

2. Set the irradiator to provide a dose of 12 Gy of irradiation, at a rate of 0.7 Gy/minute. Ensure that the irradiator has a source of fresh air for the mice.

NOD/SCID mice require a minimum of 12Gy; however, immune competent mice require 18Gy for adequate inhibition of endogenous regeneration.

3. When the irradiation is complete, remove the mice from the holder, and spray the lower hindlimb(s) with 70% ethanol. As described in step 9 of Protocol 2, inject the tibialis anterior muscle with 50 μ l of 1.2% barium chloride.
4. Monitor mice until fully recovered from anesthesia, and mobile.

Mice will favor the injected leg for a short period of time after recovery from anesthesia. This normally resolves within an hour.

SUPPORT PROTOCOL 5 ANALYZING INJECTED MUSCLE BY IMMUNOSTAINING OF CRYOSECTIONS

To determine if donor cells have engrafted, cryosections are cut from the frozen muscle (from Protocol 2, Step 11) and immunostained for proteins found only in donor nuclei (canine lamin A/C), or muscle fibers derived from donor cells (canine dystrophin). The antibodies used for detecting canine lamin A/C and canine dystrophin cross-react with the human isoforms, and therefore, can be used to evaluate human donor cell engraftment. Sections throughout the muscle are stained to determine the region of highest engraftment. A minimum of 3 slides from the region of highest engraftment are used for quantitating engraftment.

Materials

Cryostat

Superfrost Plus slides (Fisher Scientific; catalog #12-550-15)

Acetone, pre-cooled to -20°C

Glass staining jars (e.g. Coplin jars)

Dulbecco's phosphate buffered saline (D-PBS) – Ca^{2+} and Mg^{2+} free

Staining chamber

Hydrophobic marker (e.g. ImmEdge Pen – Vector Laboratories; catalog #H-4000)

Paper towels

Blocking Buffer (see Reagents and Solutions)

1° Antibody Dilution Buffer (see Reagents and Solutions)

Lamin A/C antibody (Vector Laboratories; catalog#VP-L550)

Canine-specific dystrophin antibody (MANDYS102 or MANDYS107; Developmental Studies Hybridoma Bank)

Pan dystrophin antibody (MANEX1A; Developmental Studies Hybridoma Bank)

Secondary antibody – AlexaFluor488-labeled goat anti-mouse IgG (Invitrogen; catalog #A11001)

ProLong Gold Antifade with DAPI (Invitrogen; catalog # P-36931)

Glass coverslips

Fluorescence microscope

1. Warm up frozen muscles for 30 minutes in a cryostat set to -18°C . Cut cryosections from the distal to the proximal end of the muscle and adhere 4 serial sections to each Superfrost slide. Discard 16 sections between each slide, so that

the corresponding sections between slides will represent a separation of approximately 200 μm .

Label each slide carefully using a solvent resistant marker. The average tibialis anterior muscle generates 24 slides using this protocol.

2. Fix sections in acetone in a glass staining jar at -20°C for 10 minutes. Remove the slides from the acetone and lay flat in a staining chamber. Dry the slides at room temperature for 30 minutes. Encircle each section with a hydrophobic marker, and dry for 10 minutes. Rehydrate tissue sections by submerging slides in D-PBS for 10 minutes using a glass staining jar. Replace the D-PBS and incubate for an additional 10 minutes.
3. Remove the slides from the D-PBS, and lay flat in a staining chamber. Carefully add approximately 75 μl of Blocking Buffer to cover each section, and incubate for 1 hour at room temperature.
4. Remove Blocking Buffer by tapping the long edge of the slides on paper towels on the benchtop. Carefully add approximately 75 μl of primary antibodies diluted in 1 $^{\circ}$ Antibody Dilution Buffer to cover each section, and incubate for 1 hour at room temperature.

Stain the 4 sections on each slide as follows: section #1 – isotype control; section #2 –MANEX1A; section #3 – lamin A/C; section #4 – MANDYS102/MANDYS107. Initially stain slide # 6, 12, and 18.

Evaluate staining using a fluorescence microscope. Subsequently, stain even numbered slides surrounding the slide with the highest engraftment. Use cryosections from murine and canine muscle as negative and positive controls, respectively. Lamin A/C antibody is normally used at 1:100 and dystrophin antibodies (hybridoma supernatants) at 1:40.

5. Remove primary antibodies by tapping the long edge of the slides on paper towels on the benchtop. Wash tissue sections by submerging slides in D-PBS for 5 minutes using a glass staining jar. Replace the D-PBS and incubate for an additional 5 minutes. Repeat.
6. Remove the slides from the D-PBS, and lay flat in a staining chamber. Carefully add approximately 75 μl of secondary antibody diluted in D-PBS to cover each section, and incubate for 1 hour at room temperature.

The secondary antibody is normally used at a 1:200 dilution.

7. Remove secondary antibodies by tapping the long edge of the slides on paper towels on the benchtop. Wash tissue sections by submerging slides in D-PBS for 5 minutes using a glass staining jar. Replace the D-PBS and incubate for an additional 5 minutes. Repeat.
8. Remove the slides from the D-PBS, and mount and coverslip sections with ProLong Gold with DAPI.

Follow the manufacturer's protocol for using ProLong Gold Antifade. The D-PBS needs to be completely removed before mounting with ProLong Gold. Use an aspirator with a glass Pasteur pipette and pipette tip attached to carefully remove the D-PBS without disturbing the tissue section.

9. Visualize staining with a fluorescence microscope. Engraftment of canine cells will be quantitatively assessed in two ways: (1) determining the average number of nuclei expressing canine lamin A/C per cross-section, indicative of overall donor cell survival, and (2) the average number of myofibers expressing canine

dystrophin per cross-section, indicative of donor cell differentiation. The average for each injected muscle will be determined using a minimum of three cross-sections from each injected muscle, from within the region of highest engraftment and covering a distance of approximately 800 to 1200 μm . The averages from all of the muscles injected will be used to calculate the overall average and standard deviation, and will be used in the t-test to calculate p-values.

REAGENTS AND SOLUTIONS

Growth medium:

DMEM or DMEM/F12

20% fetal bovine serum (we use HyClone defined bovine serum from ThermoScientific; catalog #SH30531.02)

2.5 ng/ml bFGF (Invitrogen; catalog #13256-029)

2 mM L-glutamine (Invitrogen; catalog #25030-081)

100 U/ml penicillin and 100 $\mu\text{g/ml}$ streptomycin (Invitrogen; catalog #15070-063)

Blocking Buffer:

2% goat serum

1% BSA

0.1% cold fish skin gelatin

0.05% sodium azide

1X D-PBS

1° Antibody Dilution Buffer:

1% BSA

0.1% cold fish skin gelatin

0.05% sodium azide

1X D-PBS

COMMENTARY

Background Information

Satellite cells are the stem cell of skeletal muscle. Satellite cells are positioned on the periphery of the muscle fiber, underneath the basal lamina. The basal lamina is required to keep the cells associated with the muscle fiber (Bischoff, 1986). The first enzymatic isolation of skeletal muscle stem cells was performed more than 50 years ago to study skeletal muscle differentiation *in vitro* (Konigsberg, 1960; Konigsberg et al., 1960; Rinaldini, 1959). A detailed description of the isolation method from chick embryo muscle is outlined by Konigsberg, and describes the basis of our method, including the preference for crude collagenase (Konigsberg, 1979). This method has been modified over the years, including optimization of the culture media to ensure proliferation and prevent differentiation. The addition of medium during the first 2 days after plating, without removing non-adherent cells ensures that all myogenic cells, especially those with a more stem cell-like phenotype, are maintained (Qu et al., 1998). The alternate protocol is based on single muscle fiber isolation for transplantation, and can slightly reduce the number of interstitial cells (Collins et al., 2005). Both protocols yield a mixed population of

mononuclear cells that comprises approximately 1–3 % myogenic cells. These cells can be cultured, or immediately transplanted into recipient muscle.

Skeletal muscle injury in mice results in increased expression of Delta-like-1 (Dll-1) within the niche, and activation of Notch signaling prevents differentiation and increases the number of proliferating myogenic cells after injury (Conboy and Rando, 2002). The extracellular domain of Dll-1 fused to the Fc portion of human IgG (Delta-1^{ext}-IgG) is sufficient for inhibition of differentiation in cultured C2C12 myoblasts; however, immobilization is required for effective signaling (Varnum-Finney et al., 2000). Importantly, expansion of hematopoietic progenitor cells with Delta-1^{ext}-IgG maintains their engraftment potential (Delaney et al., 2010; Varnum-Finney et al., 2003). Therefore, we adopted the use of Delta-1^{ext}-IgG to activate Notch signaling during *ex vivo* expansion of muscle stem cells (Parker et al., 2012a). Activating Notch signaling during *ex vivo* expansion maintains cells earlier in lineage progression and significantly improves engraftment potential of cultured cells.

Implantation of minced donor muscle effectively contributes to host skeletal muscle regeneration in murine-to-murine transplant experiments (Carlson, 1968; Partridge et al., 1978; Partridge and Sloper, 1977). Mononuclear cells isolated from minced muscle also participate in regeneration and restore dystrophin expression to the muscle of *mdx* mice, the mouse model of Duchenne muscular dystrophy (Partridge et al., 1989). Pre-irradiation of the host muscle with 18Gy 3–4 days before cell injection significantly enhances donor cell contribution by preventing host contribution to regeneration (Morgan et al., 1990). These pioneering experiments provided the platform for more than 2 decades of research into muscle cell transplantation. Moreover, these studies have provided the basis for the transplantation regimen described above, including pre-irradiation of host murine muscle before cell transplant. However, in our protocol, the host muscle is pre-irradiated only 1 day before cell transplant. Moreover, we inject barium chloride to induce degeneration, which is not required in *mdx* muscle since dystrophic muscle is already in a state of degeneration. Further evolution of these protocols is the beginning step towards realizing the tremendous promise for the eventual clinical utility of muscle cell transplantation to treat human muscular dystrophies.

Critical Parameters

The most critical parameter in the isolation of muscle cells is the collagenase used for enzymatic digestion of the muscle. We use a partially purified preparation of collagenase from *Clostridium histolyticum*, which exhibits lot-to-lot variation. Type 4 collagenase from Worthington Biochemical is low in tryptic activity, which reduces cleavage of important cell surface receptors and enhances muscle cell survival. For Protocol 1, the level of clostripain is generally higher, as this enzyme digests the non-collagen components of the basal lamina, releasing muscle stem cells, also known as satellite cells. However, the first enzymatic digest of the Alternate Protocol requires low clostripain activity, since the purpose of this initial digest is to release interstitial cells, but keep satellite cells attached to the muscle fibers. It is essential that lots of collagenase are tested and compared to choose the best lot. Once the best lots are identified, you can purchase lots with similar activities from Worthington Biochemical.

The next most important parameter is the mincing in Step 5 of Basic Protocol 1 and Alternate Protocol 1. Incomplete mincing of the tissue prevents adequate collagenase digestion, and a reduced yield of myogenic cells from the muscle. As such, it is important to fully mince the tissue to reduce the amount of time in digestion.

The critical parameters in transplantation are the dose of irradiation, and the injection technique. The muscle of immune competent mice requires 18 Gy of focal irradiation to prevent host contribution to regeneration; however, NOD/SCID mouse muscle requires only 12 Gy. Importantly, the most effective rate of delivery of irradiation is 0.7 Gy/minute (Gross et al., 1999). It is important to determine the lowest dose of irradiation required for your particular strain of mice. This can be accomplished by irradiating mice with doses ranging from 8–20 Gy, using 2 Gy increments. After irradiation, inject the muscles with barium chloride as outlined in Support Protocol 4. Remove the irradiated muscle 7 days after injection, and freeze in OCT for cryosectioning. Stain cryosections from the middle of the muscle for expression of developmental myosin heavy chain (devMyHC), a marker of regeneration in adult skeletal muscle. Expression of devMyHC indicates that endogenous host cells were able to participate in regeneration, and that the dose of irradiation was inadequate. The lowest dose that prevents expression of devMyHC is the dose that should be used for transplantation of donor cells.

The technique used for injection of barium chloride and cells is also a critical parameter. Injecting along the length of the tibialis anterior (TA) muscle is necessary to properly distribute donor cells. Moreover, having a reproducible technique will be important for using the method to quantitatively compare engraftment potential. Review the muscle anatomy of the mouse hindlimb. Practice injections with an inert dye, such as methyl blue, to show where the liquid was injected. Ensure that the dye is spread throughout the entire TA muscle. Alternatively, an incision can be made in the skin overlying the muscle, and cells injected directly into the muscle. This method is more time consuming, and increases the risk for complications, yet allows you to precisely inject the muscle.

Anticipated Results and Troubleshooting

A normal canine biopsy is approximately 1cm³, and results in an average yield of 7.5×10⁶ cells. This is a mixed population of mononuclear cells, which normally contains 1.5% myogenic cells. A low percentage of myogenic cells may indicate the need for a different lot of collagenase, a longer digestion time, or more extensive mincing.

Cells cultured on Delta-1^{ext}-IgG appear to proliferate more slowly than cells on cultured on human IgG. Moreover, the cells appear slightly flattened, yet do not appear elongated as would be expected if the cells had initiated differentiation. Initiation of differentiation indicates the starting cell number was too high, or the cells were cultured too long. Currently, we have not assessed the effects of passaging cells on engraftment potential; however, this is a reasonable approach to circumventing the problem of over-confluence.

Analyzing expression of a small subset of genes is essential to ensure that Notch signaling is activated and differentiation has not been initiated. Isolate RNA from cells expanded on Delta-1^{ext}-IgG- and human IgG-coated plates, and convert to cDNA using reverse transcriptase. The cDNA provides the template for quantitative PCR reactions, comparing expression of Hey1, HeyL, and Dtx4 as a measure of Notch activity, and MyoD and myogenin as a measure of myogenic differentiation (Parker et al., 2012a). Expression of Pax7 and Myf5 should be monitored to confirm the presence of myogenic cells.

Injection of canine cells should result in muscle fibers expressing canine dystrophin and nuclei expressing canine lamin A/C in recipient mouse muscle 4 weeks after cell injection. An absence of staining suggests that the cell dose was not adequate, the irradiation was insufficient, or the injection technique was poor. It is important to note that the level of engraftment varies between donor cell populations (Parker et al., 2012b). Each donor cell population can be titrated by varying the cell dose from 2×10³ to 5×10⁴ cells, and measuring engraftment at each cell dose. The level of engraftment for a particular donor cell population

is consistent between experiments, as long as proper injection technique is employed (Parker et al., 2012b).

Time Considerations

Support Protocol 2 must be completed the day before cell isolation and plating (Basic Protocol 1 or Alternate Protocol 1). Do not complete Support Protocol 2 earlier than the day before and store the plates – make fresh plates for each experiment. Expect 6–8 hours will be required to complete cell isolation and plating, including Support Protocol 1, and set up of plates for Support Protocol 3. Normally, 7–8 days in culture is necessary to reach confluence on a 10-cm plate starting with 7.5×10^4 – 1.0×10^5 cells per plate. The colonies in Support Protocol 3 should be ready for counting within 5–6 days. To use the cells for transplant (Basic Protocol 2), NOD/SCID mice that are 7–12 weeks of age must be ready within 7–8 days of cell isolation. While learning the procedure, only perform injections into 12 mice; once you have become more proficient, 24 mice are a reasonable number for each transplant experiment.

In our experiments, the mice undergo Support Protocol 4 one day before cell transplant. Furthermore, the injected muscle is removed 4 weeks after cell injection. However, other time scenarios are possible and should be explored. For example, the muscle can be irradiated on Day 0, and injected with cells on Day 3, as described above. Moreover, the cells and barium chloride can be co-injected the day after irradiation, which will ensure that the cells are injected into the region of regeneration. However, this will alter engraftment, and any changes to the timing of the protocol must be maintained.

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Figure 1.
The cell counting area of a hemocytometer.