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Tissue Engineering Human Skeletal Muscle for Clinical Applications

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1. INTRODUCTION

Skeletal muscle-derived cells isolated from a variety of mammalian as well as non-mammalian species can be tissue-engineered to generate three-dimensional muscle-like structures (alternately called bioartificial muscles (BAMs), organoids, or myoids) that, when cultured in vitro under passive or active tension, form parallel arrays of postmitotic myofibers and express sarcomeric contractile proteins [Strohman et al., 1990; Vandenburgh et al., 1991; Swasdison and Mayne, 1992; Okano et al., 1997; Dennis and Kosnik, 2000, reviewed in Kosnik et al., 2003]. BAMs formed from animal-derived muscle cell cultures have many applications for research, including performing muscle physiology studies, elucidating the mechanics of the development of organized, multinucleated, functional myofibers from proliferating myoblasts, and as a model for muscle wasting induced by disuse or decreased tension. For example, avian BAMs flown on several Space Shuttle missions have shown significant myofiber atrophy due to a pronounced decrease in protein synthesis, indicating that skeletal muscle fibers in BAMs are directly affected by microgravity in the absence of innervation or circulating hormones [Vandenburgh et al., 1999]. Tissue-engineered skeletal muscle BAMs can therefore provide a unique tool to develop countermeasures to muscle atrophy resulting from space travel, which may have applications to earth-based wasting muscle disorders.

BAMs formed from cells isolated from human skeletal muscle biopsy tissue have the potential to be used clinically for both structural and functional skeletal muscle repair or replacement, and for the treatment of a variety of diseases when used as a delivery vehicle for recombinant protein therapeutics from genetically engineered muscle cells. Human skeletal myoblasts have been transduced to secrete

recombinant proteins and tissue engineered ex vivo into human BAMs (HBAMs), which continue to secrete the foreign gene products in vitro [Powell et al., 1999] and in vivo [unpublished data]. Autologous, genetically modified HBAMs could potentially be implanted into patients for delivery of recombinant proteins for therapeutic treatment of diseases such as growth hormone deficiency (human growth hormone), hemophilia A (factor VIII) or B (factor IX), and heart disease (vascular endothelial growth factor or insulin-like growth factor-I). The implanted HBAMs would serve as in vivo protein factories capable of delivering predictable levels of therapeutic gene products, and would offer the advantage over other gene therapy protocols of reversible implantation of postfused, nonmigrating cells with high, long-term protein synthesis levels. With additional structural/functional HBAM engineering improvements to more closely resemble in vivo muscle (including higher myofiber density, larger diameter fibers, reduced extracellular matrix materials, and innervation), HBAMs offer the additional promise of structural repair or replacement of skeletal muscle in the future [Powell et al., 2002].

Following protocols for rodent muscle cells, Powell et al. [1999] engineered human BAMs, which had morphological characteristics similar to those of other mammalian BAMs [Okano et al., 1997]. With the goal of developing a tissue-engineered product that could be implanted for clinical applications, our laboratory has extended these initial human muscle cell isolation and tissue-engineering techniques by optimizing the muscle cell isolation procedure, cell expansion protocol, and engineering methods, using clinically approved matrix materials.

2. PREPARATION OF REAGENTS AND MEDIA

2.1. Culture Media

2.1.1. DMEM/pen/strep

Dulbecco's modified Eagle's medium, supplemented with penicillin, 50 U/ml, streptomycin, 50 μg/ml (1% v/v of stock pen/strep)

2.1.2. SKGM/15, SKGM/2

Skeletal muscle growth medium (SKGM) supplemented with 15% or 2% fetal bovine serum

2.1.3. CMF

Ca^{2+}- and Mg^{2+}-free Earle's balanced salt solution (EBSS)

2.1.4. Differentiation Medium

DMEM supplemented with:

Insulin, 10 μg/ml
Bovine serum albumin (BSA), 0.5 mg/ml
hEGF, 10 ng/ml
Gentamycin, 0.05 mg/ml

2.2. Digestive Enzyme Solutions

2.2.1. Collagenase-Dispase Solution

Collagenase type II, 0.1% (w/v), dispase, 4 mg/ml in DMEM. Sterilize by filtration.

2.2.2. Trypsin Stock

Trypsin, 0.5%, EDTA, 5.4 mM (0.2%). Dilute 1:10 in CMF for use.

2.3. Staining Solutions

2.3.1. Coomassie Blue Staining Solution

Coomassie Brilliant Blue, 1 g/l in 40% (v/v) methanol, 10% (v/v) acetic acid

2.3.2. Blocking Buffer

Bovine serum albumin, 1% (w/v), 0.2% (v/v) Triton-X in PBS

3. SKELETAL MUSCLE NEEDLE BIOPSY

A complete description of the biopsy procedure has been previously described [Hennessey et al., 1997]. Biopsies are taken from the vastus lateralis, a mixed fast/slow muscle fiber type. Briefly, the biopsy site is shaved, sterilized, and anesthetized, and an incision is made through the skin. A muscle sample is removed with a 6-mm biopsy needle apparatus by a suction-enhanced technique (Fig. 10.1), and immediately thereafter, a second muscle sample is removed from the same incision site. The muscle tissues are transferred to sterile tubes containing chilled DMEM supplemented with antibiotics (See materials list), and transported on ice to the cell culture facility.

Protocol 10.1. Skeletal Muscle Needle Biopsy

Reagents and Materials

Sterile

- ❑ DMEM/pen/strep: Dulbecco's modified Eagle's medium, supplemented with penicillin, 50 U/ml, streptomycin, 50 μg/ml (See Section 2.1.1)
- ❑ Iodophor solution
- ❑ Novocain solution, 1%, without epinephrine
- ❑ Sterile saline
- ❑ Surgilube
- ❑ Tissue culture Petri dish, 10 cm
- ❑ Conical centrifuge tubes, 50 ml
- ❑ Biopsy needles (4- and 6-mm Bergstrom cutting trocars)
- ❑ Cutting cannula adapters with argyle female Luer lock connector
- ❑ Adapter to syringe tubing: Interlink catheter extension kit with male Luer lock adapter 15.2 cm long, 0.5-ml volume

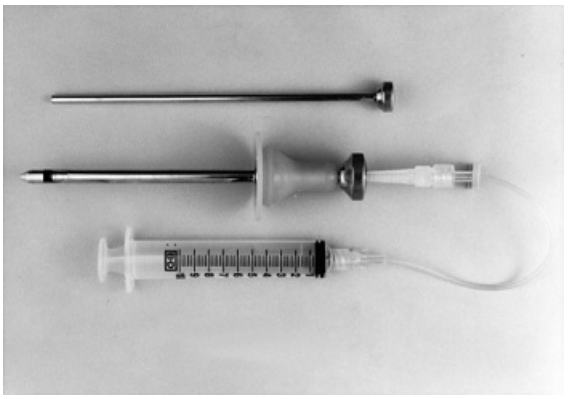

Figure 10.1. Human skeletal muscle biopsy instruments assembled and ready for use. From top to bottom: clearing rod used to expel samples; cutting trocar inserted through nipple (used to enhance suction) into outer needle with cutting chamber on left; syringe connected to adapter tubing, which attaches to the proximal end of the cutting cannula. The closed system generates enhanced suction, resulting in increased sample size compared to previous percutaneous muscle biopsy techniques [Hennessey et al., 1997]. Reprinted from Hennessey et al., 1997 by permission of the American Physiological Society.

- ❑ Suction-enhancing nipples: infant nipples, 4 mm
- ❑ Syringe, 10 ml
- ❑ No. 11 scalpel
- ❑ Fenestrated drape
- ❑ Sterile gauze sponges, 2 × 2 in. and 4 × 4 in.
- ❑ Steristrips, $\frac{1}{4}$ in.
- ❑ Foam or elastic tape
- ❑ Curved forceps, 12 cm

Nonsterile

- ❑ Ice bath

Protocol

(a) Set up sterile instruments on a sterile field.
(b) Assemble the Bergstrom biopsy needle apparatus:
  (i) Insert fully the clearing rod through the cutting cannula, easing the blunt end through the outside opening of the infant nipple.
  (ii) Place the assembly on the trocar so the nipple can slide firmly over the open end, creating a tight seal.
  (iii) Fully insert the cutting cannula into the trocar so that there is full closure of the cutting chamber, using surgical lubricant to maintain the seal.
  (iv) Insert the male end of the syringe-tubing adapter into the proximal end of the cutting cannula and attach to a 10-ml syringe (See Fig. 10.1).
(c) Withdraw syringe to 3-ml position to a create buffer of air.
(d) Soak a sterile 2 × 2-in. gauze sponge with sterile saline in 10-cm dish, replace lid, and place on ice.

(e) Place a 50-ml sterile conical centrifuge tube containing 25 ml DMEM/pen/strep on ice.
(f) Place patient in a comfortable reclining position and locate biopsy site 25 cm proximal from the tuberositas tibiae and 5 cm lateral from the midline of the femoral course.
(g) Shave the area, and sterilize with Iodophor solution.
(h) Anesthetize the skin and subcutaneous tissue with 10 ml 1% Novocain solution.
(i) Using the No.11 scalpel, make a 4- to 6-mm incision through the skin.
(j) Insert the trocar into the incision site, pushing through the muscle fascia, and advance to ensure that the cutting chamber opening lies fully within the muscle.
(k) Slide the nipple down against the patient's thigh to ensure a seal.
(l) Withdraw the trocar by 2.5 cm.
(m) Apply suction (an assistant is required) by withdrawing the plunger of the syringe, which will draw muscle tissue into the cutting chamber; advance the trocar forward, cutting off a sample as the assistant releases the suction.
(n) Rotate the trocar clockwise 90° to maximize the amount of muscle sample obtained.
(o) Withdraw the trocar, depress the syringe to expel the 3 ml of air, remove the muscle tissue sample from the chamber, and place on the 2 × 2-in. sterile gauze on ice.
(p) Repeat the procedure beginning at step (j) to obtain a second muscle sample.
(q) Place a clean, sterile 2 × 2-in. gauze sponge over the wound and fix into place with 3 Steristrips. Apply pressure on the wound for 10 min.
(r) Transfer the biopsy tissue with forceps to the chilled tube of DMEM/pen/strep.
(s) Transport the biopsy tissue on ice to the cell culture facility.

4. ISOLATION AND CULTURE OF HUMAN SKELETAL MYOBLASTS

Biopsies are performed on volunteers according to procedures approved by the Institutional Clinical Review Board. Skeletal muscle-derived cells have been isolated successfully from healthy volunteers as well as frail elderly, growth hormone-deficient, and heart failure patients. No significant differences in myoblast purity and cell growth characteristics were found among any of the patient populations when compared with healthy volunteers [Powell et al., 1999]. Wet weight of muscle tissue samples ranged from 140 to 1070 mg, with a mean weight of 398 ± 54 mg in 25 muscle biopsies from healthy volunteers.

Skeletal muscle biopsy tissue from each volunteer or patient is digested separately. The current procedure used for enzymatic digestion of the muscle tissue is modified from published protocols [Powell et al., 2002, Kosnik et al., 2001]. All muscle samples are handled with universal safety precautions because volunteers are not pretested for viral/bacterial disorders. Biopsies are given an identifying ID number at the time of biopsy to maintain privacy of the volunteer.

Protocol 10.2. Isolation and Culture of Human Skeletal Myoblasts

Reagents and Materials

Sterile

- ❑ DMEM/pen/strep (See Section 2.1.1)
- ❑ Collagenase-dispase solution (See Section 2.2.1)
- ❑ SKGM/15 (See Section 2.1.2)
- ❑ CMF: Ca^{2+}- and Mg^{2+}-free EBSS (See Section 2.1.3)
- ❑ Trypsin-EDTA solution: dilute stock 1:10 in CMF to give 0.05% trypsin in 5 mM EDTA (See Section 2.2.2)
- ❑ Fetal bovine serum at 4 °C
- ❑ Conical centrifuge tubes, 15 ml, 50 ml
- ❑ Dissecting forceps
- ❑ Dissecting scissors
- ❑ Petri dishes, 6 cm
- ❑ Tissue culture Petri dishes, 10 cm
- ❑ Polypropylene jar, 30 ml, autoclaved

Nonsterile

- ❑ Orbital platform shaker
- ❑ Balance
- ❑ Water bath at 37 °C
- ❑ Table top centrifuge

Protocol

A. Isolation of human skeletal myoblasts

(a) Obtain isolated muscle biopsy tissue in chilled DMEM/pen/strep solution.
(b) Working under sterile conditions, transfer muscle tissue to a 6-cm Petri dish containing 2 ml DMEM/pen/strep.
(c) Using sterile forceps, carefully remove excess connective tissue and fat from biopsy sample.
(d) Weigh tissue in a covered, sterile 6-cm Petri dish.
(e) Add 1–2 ml sterile DMEM/pen/strep to cover tissue.
(f) Using sterile scissors, cut tissue into strips approximately 1 mm × 10 mm.
(g) Place strips into sterile polypropylene jar containing 10 ml DMEM/pen/strep.
(h) Cap jar loosely and place in 37 °C CO_2 incubator.
(i) After 48 h of tissue incubation, prepare collagenase-dispase solution.
(j) Remove incubation medium from tissue strips and add 10 ml collagenase-dispase solution.
(k) Cap jar tightly, secure to orbital shaker in 37 °C incubator, and incubate for 60 min at approximately 60 rpm.
(l) Remove digested tissue along with enzyme solution and transfer to sterile centrifuge tube.
(m) Add 10% (v/v) chilled FBS to digested tissue solution.

(n) Pellet cells at 200 *g* for 5 min.
(o) Suspend cell pellet in 10.5 ml SKGM/15 and plate 10 ml tissue suspension into a 10-cm tissue culture dish.
(p) Plate a second 10-m tissue culture dish with 0.1 ml cell suspension in 10 ml SKGM/15 for cell yield determination (See Section 5).

B. Maintenance of human skeletal muscle cultures

(a) Feed muscle cultures 4 days after plating cells with 10 ml fresh prewarmed SKGM/15 for each 10-cm dish.
(b) Repeat feedings every 2–3 days until cultures are approximately 75–80% confluent.
(c) Subculture cells at 75–80% confluence.
(d) Aspirate medium from cells.
(e) Rinse plates with CMF, leaving rinse on dish for 2–5 min.
(f) Remove rinse and add 4 ml 1 × trypsin-EDTA solution to each 10-cm dish.
(g) Place cells in 37 °C incubator for 5 min to enhance detachment, monitoring process every 2–3 min under a microscope.
(h) Once cells are detached, remove to sterile 15-ml centrifuge tube.
(i) Rinse plate with 4 ml SKGM/15 and add rinse to tube.
(j) Centrifuge at 200 *g* for 5 min.
(k) Carefully aspirate supernate.
(l) Suspend cell pellet in SKGM/15, and count an aliquot on a hemocytometer.
(m) At this step, cells can be either expanded further, plated for characterization, plated for retroviral transduction, cryopreserved, or tissue engineered.
(n) For further expansion, plate cells at a density of not less than 1×10^5/10-cm dish and subculture after reaching 80% confluence.

5. CHARACTERIZATION OF HUMAN SKELETAL MYOBLASTS

Muscle-derived cells from each biopsy are characterized for total cell yield (number of cells isolated/100 mg of biopsy tissue), myoblast purity (percent desmin-positive cells) and cell population doubling time. Cell yield is determined by counting colonies in the cell yield plate (Protocol 10.2A, Step (p)) and extrapolating to the number of colonies that would be present in a 100-mg tissue sample. The cell isolation method currently in use, in which the muscle tissue is incubated for 48 h before being digested, consistently yields more cells/mg wet weight when compared to tissue samples digested immediately after biopsy [Powell et al., 1999, but also using a different enzyme solution]. Although the current protocol adds 2 days of incubation to the procedure outlined previously [Powell et al., 1999], significantly more cells are isolated. As a result, a clinical procedure requiring 1.0×10^8 myogenic cells would be achieved an average of 4 days earlier by this method (14 days) than when the tissue is enzymatically digested immediately after the biopsy (18 days).

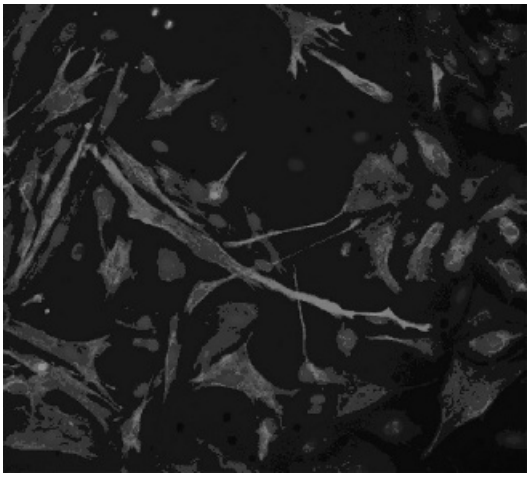

Figure 10.2. Characterization of myoblast purity of skeletal muscle cultures. Human skeletal muscle-derived cells are immunostained with an antibody to desmin [Powell et al., 1999] and developed with AlexaFluor 488-conjugated goat anti-mouse IgG. Nuclei are visualized by DAPI staining. (See Color Plate 5A.)

Myoblast purity is determined for cells isolated from each volunteer by immunostaining with an antibody to desmin, an intermediate filament protein located in proliferating skeletal myoblasts [Powell et al., 1999]. Cultures are counterstained with DAPI (4′,6-diamidino-2-phenylindole) to identify all nuclei, and random fields were counted to determine average myoblast percentage (Fig. 10.2, See Color Plate 5A). Myoblast purity is routinely determined from cells plated after the first harvest from the tissue digest (P_1), although the percentage of myoblasts in a particular biopsy sample remains constant with time in culture and can therefore be assayed at any passage number (data not shown). As a minimum tissue-engineering requirement for use of a cell population isolated from a particular biopsy, we selected those with greater than 50% myoblasts. Approximately 80% of skeletal muscle tissue samples exceeded this requirement.

Typical characteristics of skeletal muscle derived cultures are as follows: yield of 1700–20,000 cells/100 mg wet weight; myoblast purity of $77.5 \pm 4.9\%$; and doubling time of 24.5 ± 0.8 h (Table 10.1).

Table 10.1. Characteristics of skeletal muscle biopsies from 9 representative donors.

Biopsy #	Tissue wet weight (mg)	Cell yield (no. of cells isolated/100 mg tissue)	Myoblast purity (% desmin-positive cells)	Cell doubling time (h)
1B	333	20090	64.6	28
2B	413	9010	65.3	22
3B	1070	5400	71.7	24
4B	1013	1710	91.8	24
5B	444	9275	89.6	24
6B	210	17765	82.0	25
7	285	4600	67.9	26
*8	443	4970	20.8	23
9	377	3310	79.5	26

Characteristics are determined as described in text. *Biopsy 8 was rejected for further expansion for tissue engineering, as the myoblast purity was <50%.

Protocol 10.3. Characterization of Human Skeletal Myoblast Cultures

Reagents and Materials

Sterile

- ❑ SKGM/15 (See Section 2.1.2)
- ❑ PBS (phosphate-buffered saline), containing calcium and magnesium
- ❑ SonicSeal slides, 4 well

Nonsterile

- ❑ Coomassie Blue staining solution (See Section 2.3.1)
- ❑ Methanol-acetone, 1:1 (v/v)
- ❑ Blocking buffer (See Section 2.3.2)
- ❑ Mouse anti-desmin
- ❑ Alexa Fluor 488 goat anti-mouse IgG
- ❑ Microscope with fluorescent light source; fluorescein and DAPI filters
- ❑ Vectashield® Mounting Medium with DAPI
- ❑ Circular coverslips, 12 mm

Protocol

A. Cell yield

Cell yield plate is ready for counting when macroscopic colonies are visible.

(a) Remove medium from dish, and rinse with 10 ml PBS.
(b) Remove rinse, and add 5 ml Coomassie Blue staining solution.
(c) Incubate 15 min.
(d) Remove staining solution.
(e) Count all colonies in plate.
(f) Calculate yield:

Cells/100 mg of biopsy tissue = (Number of colonies × 100)÷ (wt of biopsy in mg/100)

B. Myoblast purity

(a) Plate 2 wells of a 4-well chamber slide with 3000 cells/well in 1 ml SKGM/15 and place in 37°C incubator for 3 days.
(b) Aspirate medium, rinse wells 2 × quickly with PBS at room temperature.
(c) Fix for 10 min with 1:1 (v/v) methanol-acetone.
(d) Remove fixative, and rinse 3 × 5 min with PBS. Fixed cultures may be stored at 4° in PBS for several weeks before immunostaining.
(e) Incubate cultures with blocking buffer for 30 min at room temperature.
(f) Incubate with anti-desmin (1:200 dilution in blocking buffer) for 2 h at room temperature.
(g) Wash with 3 × 5-min rinses of PBS at room temperature.
(h) Incubate with Alexa Fluor 488 goat anti-mouse IgG (1:200 in blocking buffer) 30 min at room temperature.

(i) Wash with 3 × 5-min rinses of PBS at room temperature.
(j) Mount coverslip with Vectashield® DAPI.
(k) Count desmin-positive cells and total nuclei in 10 random fields for each isolated cell population under fluorescence microscope.
(l) Quantify myoblast percentage for each field, and calculate mean percentage of myoblasts.

6. TISSUE ENGINEERING OF HUMAN SKELETAL MYOBLASTS WITH CLINICALLY APPROVED EXTRACELLULAR MATRIX MATERIALS

Skeletal myoblasts can be tissue-engineered into 3-dimensional organ-like HBAM structures containing parallel arrays of postmitotic myofibers [Powell et al., 1999]. For casting BAMs, silicone rubber molds with internal dimensions of 6.5 mm wide × 30 mm long × 6 mm deep are formed in preconstructed aluminum molds (Fig. 10.3). Wells are washed and glued into six-well tissue culture dishes, with one rubber mold/well. Attachment points are constructed from syringe needles and inserted at each end of the well, approximately 20 mm apart (Fig. 10.4) (See *note (i)*.)

Myoblasts are suspended in an ice-cold extracellular matrix solution, and the cell suspension is pipetted into the silicone rubber molds. Within 2–3 days after casting, the cell-gel mixture contracts, detaching from the mold but remaining secured in place by the end attachment sites (See Fig. 10.4). BAM morphology is assessed by immunostaining with an antibody to sarcomeric tropomyosin, a contractile protein found only in fused muscle fibers. Cross sections of 10-day in vitro HBAMs show arrays of muscle fibers aligned primarily perpendicular to the long axis of the BAM and distributed uniformly throughout the entire cross-sectional area (Fig. 10.5, See Color Plate 5B) when the BAM diameter is small (<250 μm).

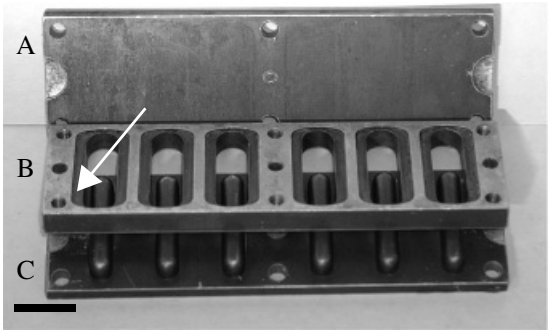

Figure 10.3. Aluminum casting mold before assembly. The aluminum mold used to cast silicone rubber wells for BAM formation is assembled from the three parts shown. Part B, containing a lip (See arrow) at the top of the cut-out region, provides a base for the cast silicone rubber well, and is placed on top of part C. Screws are inserted up through the holes in part C and part B to secure the assembly in position. The liquid silicone solution is poured into each well of the mold, and part A is set in place over the screws. Wing nuts are tightened on the screws to secure the assembly. Bar = 15 mm.

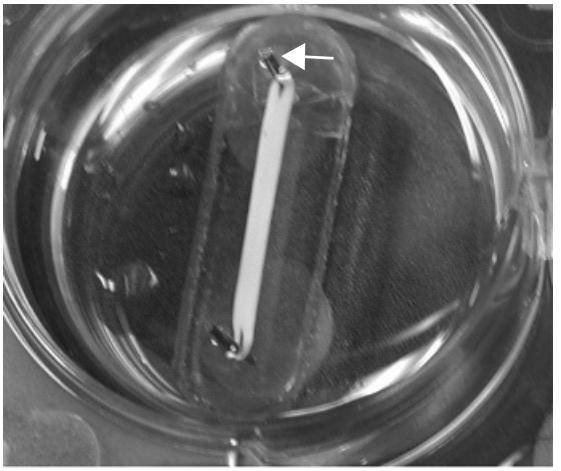

Figure 10.4. Fourteen day in vitro HBAM cast in a silicone rubber mold. Human skeletal muscle-derived cells are suspended in a 2 mg/ml collagen solution, and 2×10^6 cells in 1 ml collagen solution are poured into the silicone rubber mold. Arrow points to a pin attachment site. The contracted HBAM has detached from the mold sides and bottom and is held in place only by the pins. The HBAM is under passive tension because detachment from one pin results in a 60–70% retraction in length within 12 h. The passive tension in the construct aligns the fusing myogenic cells into parallel arrays of postmitotic muscle fibers [Powell et al., 1999]. Bar = 10 mm.

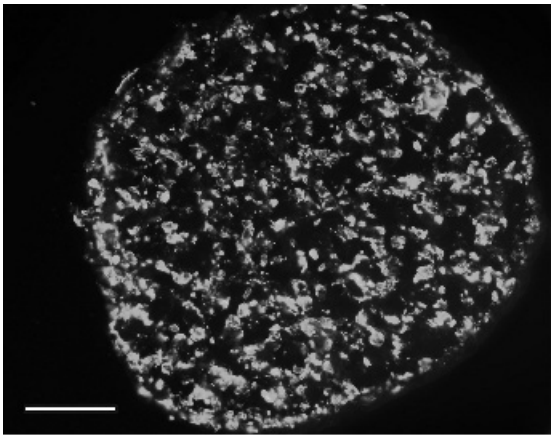

Figure 10.5. Cross section of 10-day in vitro HBAM. After 10 days in vitro, an HBAM is fixed for 1 h in 2% formaldehyde, and 10-μm cryostat cross sections are cut. Sections are stained with an antibody to sarcomeric tropomyosin, followed by incubation with AlexaFluor 488-conjugated goat anti-mouse IgG to identify muscle fibers. Bar = 50 μm. (See Color Plate 5B.)

Protocol 10.4. Formation of Silicone Rubber Casting Molds

Reagents and Materials

Nonsterile

- ❑ Ultra-pure water (UPW)

- ❑ Lemon Joy liquid dishwashing detergent (nontoxic mold release agent), 50% in ultra-pure water
- ❑ Tissue culture plates, 6 well, 35 mm diameter
- ❑ Centrifuge tubes, 50 ml
- ❑ Silicone rubber (Med6015 Silicone Elastomer)
- ❑ Preformed aluminum casting molds, machined to user specifications (See Fig. 10.3)
- ❑ Silicone rubber adhesive sealant (RTV108)
- ❑ Syringe needles, 22 g, cut into 8- to 10-mm lengths with beveled end removed
- ❑ Vacuum pump
- ❑ Vacuum chambers
- ❑ Standard bench-top lab heating oven, capable of 150 °C
- ❑ Air brush
- ❑ Ethylene oxide source

Protocol

(a) Mix silicone elastomer components, following the manufacturer's instructions.
(b) Pour mixture into 50-ml centrifuge tubes, place open tubes in vacuum chamber, and run vacuum until solution is clear (i.e., bubble free, approximately 25 min).
(c) Heat clean aluminum casting mold plates at 150 °C for 20 min.
(d) Using an air brush and working in a fume hood, spray hot disassembled mold plates with 50% Lemon Joy, being sure to cover all corners and angles (rotate mold as necessary).
(e) Assemble mold plates when soap has dried (See Fig. 10.3).
(f) Pour silicone mixture into assembled aluminum casting mold plates.
(g) Place mold plates containing silicone in 150 °C oven for 30 min.
(h) Remove from oven and rinse in cold running tap water for 1 min.
(i) Disassemble aluminum casting mold plates, and remove hardened silicone rubber molds.
(j) Rinse molds well with tap water to remove soap, and soak in UPW overnight.
(k) Assemble silicone molds in 6-well plates, gluing 1 BAM mold/well with a small amount of RTV sealant.
(l) Insert pins vertically at ends of each BAM mold at desired separation distance.
(m) Once the glue has dried, wash entire plate 3 times with distilled water, and then fill wells again with UPW and soak for 6 h minimum.
(n) Remove water and air-dry plates in a laminar flow hood.
(o) Sterilize with ethylene oxide.

Protocol 10.5. Tissue-Engineering and Maintenance of Human Skeletal Muscle

Outline

HBAMs are engineered by suspending isolated muscle-derived cells in an ice-cold collagen solution and casting the cell-gel solution into the silicone rubber molds. Each

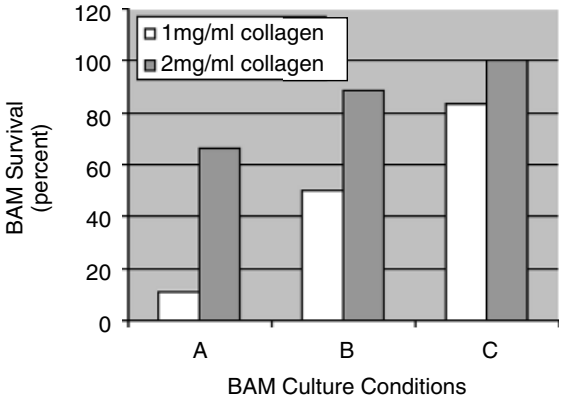

Figure 10.6. Survival of HBAMs engineered with varying collagen concentrations and cell numbers. HBAMs are engineered (See Protocol 10.5) with either 1 mg/ml or 2 mg/ml collagen, and with 1×10^6 cells in 500 μl of cell-matrix suspension (A), 1.5×10^6 cells in 750 μl (B), or 2.0×10^6cells in 1000 μl (C). BAMs are evaluated for survival (18 BAMs/group) on days 11–12 after casting. Surviving BAMs are identified as being intact and secured to both end attachment sites.

HBAM contains 2×10^6 cells suspended in 1 ml of a 2 mg/ml collagen solution. Cell number and collagen concentration may be varied depending on the application, but these conditions are optimal for cell and HBAM survival (Fig. 10.6).

Reagents and Materials

Sterile

- ❑ Zyderm
- ❑ Sterile silicone rubber BAM casting molds
- ❑ SKGM/15 (See Section 2.1.2)
- ❑ Acetic acid, 0.1 N
- ❑ NaOH, 1.0 N
- ❑ Tisseel stock solution, 7.5 mg/ml
- ❑ Differentiation medium (See Section 2.1.4)
- ❑ SKGM/2 maintenance medium (See Section 2.1.2)

Protocol

(a) Calculate the total volume of cell/Zyderm solution required.

(b) Prepare a working solution of Zyderm (3.25 mg/ml) by diluting stock Zyderm (65 mg/ml) 1:20 with 0.1 N sterile acetic acid.

(c) Suspend the Zyderm working solution well by vortexing gently, and incubate for at least 1 h at room temperature to solubilize. The working solution can be stored for at least several weeks at 4 °C.

(d) Calculate the volume of each component required for HBAM formation. (See *note (ii)*.)

i) Final concentration of Zyderm = 2 mg/ml. Calculate volume of working solution needed: final volume of cell-gel solution × 2 ÷ 3.25.
ii) Final concentration of tisseel = 0.125 mg/ml. Volume of stock tisseel required = 0.125 mg/ml × volume of Zyderm working solution ÷ 7.5 mg/ml
iii) 1 N NaOH volume: 0.065 × volume of Zyderm working solution
iv) SKGM/15 volume: final volume of Zyderm/cell solution−(Zyderm working solution volume + Tisseel volume + NaOH volume).

(e) Chill SKGM/15, Zyderm working solution, 1 N NaOH, and Tisseel on ice.
(f) Prepare collagen solution by adding Zyderm to SKGM/15, suspending slowly with sterile pipette and taking care not to create bubbles.
(g) Add Tisseel and NaOH, and suspend very well with sterile pipette, keeping solution chilled on ice. (See *note (iii)*.)
(h) Harvest and pellet required number of cells.
(i) Suspend cells in Zyderm solution at a concentration of 2×10^6 cells/ml.
(j) Cast Zyderm-cell mixture into molds with a sterile, chilled pipette tip, adding 1 ml/silicone rubber mold. Distribute solution evenly along length of mold and around pins with the pipette tip.
(k) Carefully transfer the mold plate to a 37 °C incubator for 1 h.
(l) Flood wells with SKGM/15, pipetting slowly so as not to disturb the partially solidified cell-gel mix, adding 8–9 ml medium/well so that entire mold and HBAMs are covered.
(m) Three days after casting, change medium to differentiation medium (8–9 ml/well).
(n) After HBAMs have been in differentiation medium for 3 days, change to SKGM/2 maintenance medium (8–9 ml/well).
(o) Continue to change SKGM/2 medium every 2–3 days for duration of experiment.

Notes:

1. Molds can also be formed from silicone rubber tubing (0.187-in. ID × 0.312-in. OD) cut into appropriate lengths. A wedge of 1/3 of the circumference of the tubing is removed longitudinally. Tubing ends are blocked by either gluing a small piece of a silicone rubber sheet, 0.01 in. thick, at each end or, alternatively, gluing Velcro (loop side only; available at fabric stores) pieces 2 mm × 2 mm as attachment points in place of pins. The cell solution will integrate well into the Velcro because of its high surface tension and will therefore not flow out through the ends of the tubing [Vandenburgh et al., 1996].
2. It is essential to make up enough cell-matrix solution (Steps (d)–(i)) for several extra BAMs as volume is lost while pipetting and casting BAMs.
3. Once Tisseel and NaOH have been added to the Zyderm-SKGM/15 mixture, the solution must be kept chilled on ice and used within 1 hour of preparation.

7. APPLICATIONS OF TISSUE-ENGINEERED HUMAN SKELETAL MUSCLE

7.1. Gene Therapy

BAMs made from nonhuman myoblasts and implanted subcutaneously have been shown to deliver biologically active, therapeutic proteins long-term in several animal models [Vandenburgh et al., 1996, 1998; Lu et al., 2001, 2002]. Human growth hormone (hGH) constantly secreted from implanted mouse BAMs attenuated skeletal muscle wasting in a muscle atrophy model in mice more effectively than daily hGH injections [Vandenburgh et al., 1998]. In a large-animal model, BAMs engineered from ovine skeletal muscle genetically modified to secrete recombinant vascular endothelial growth factor (rhVEGF) were implanted onto the surface of the sheep heart, resulting in increased capillary ingrowth in the neighboring host myocardium [Lu et al., 2002]. These studies suggest the potential of tissue-engineered HBAMs formed from human skeletal muscle-derived cells genetically modified to secrete therapeutic proteins as an implantable protein delivery device.

Human skeletal myoblasts can be genetically engineered to secrete sustained levels of therapeutic proteins [Powell et al., 1999]. When tissue engineered into HBAMs they continue to secrete biologically active foreign gene products. Implantation of genetically engineered HBAMs for patients with a variety of disorders would thus present an attractive alternative to daily injections, which often result in serious side effects. Further improvements to this model will require scaling up of protein output from the HBAMs to reach efficacious levels of the recombinant protein. Modifications to the cell packing density, size of implant, and genetic engineering methods will help to increase delivery dosage levels.

7.2. Muscle Repair/Replacement

In addition to their value as a platform for gene therapy, HBAMs also have the potential to be used for structural and functional skeletal muscle repair or replacement. To achieve this goal, HBAMs will need to be engineered to more closely resemble in vivo human skeletal muscle and generate meaningful force. Our laboratory has designed a novel computer-controlled electromechanical tissue stimulator device for this purpose, which mechanically loads HBAMs during both formation and later development, while recording real-time passive and active forces. To date these active forces are only 1–2% of the force generated by adult muscle [unpublished data] and thus are of little clinical relevance.

By mechanically stimulating BAMs, we have measured structural and functional improvements, including increased elasticity, mean myofiber diameter, and myofiber area percent [Powell et al., 2002]. The mechanically loaded BAMs, although closer to in vivo muscle, will need to be improved further before they can be used as functional human muscle analogs. Improved design of mechanical conditioning programs may allow for advanced dynamic engineering techniques that will enable the engineering of more in vivo-like BAMs that will be more applicable for structural repair.

Finally, in addition to use as a platform for gene therapeutics and muscle repair, tissue-engineered human skeletal muscle offers other potential applications, including in vitro drug screening, and as a model system for the study of muscle wasting in the microgravity of space [Vandenburgh et al., 1999]. We anticipate that additional improvements in tissue engineering of HBAMs will expand research and clinical opportunities in the future.

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SOURCES OF MATERIALS

Item	*Catalog No.*	*Supplier*
Air brush	Model 200, Deluxe Set	Badger Air Brush Co.
Alexa Fluor 488 goat anti-mouse IgG	11029	Molecular Probes (See also Invitrogen)
Argyle female Luer lock connector	8888-275008	Sherwood Medical
Biopsy needles (4- and 6-mm Bergstrom cutting trocars)	n/a	Stille
Bovine serum albumin (blocking buffer)	A4161	Sigma
Bovine serum albumin (differentiation medium)	CC4160	Clonetics (See Cambrex)
CMF: Ca^{2+}- and Mg^{2+}-free EBSS (Earle's balanced salt solution)	14155-063	GIBCO
Collagenase type II	C6885	Sigma
Conical centrifuge tubes, 15 ml	430052	Corning
Conical centrifuge tubes, 50 ml	430290	Corning
Coomassie Blue R	B0149	Sigma
Curved forceps 12 cm	11002-12	Fine Scientific Tools
Cutting cannula adapters (B-D interlink vial access)	303367	Becton Dickinson
Dispase	165859	Roche
DMEM/pen/strep (Dulbecco's modified Eagle's medium)	11995-040	GIBCO (See Invitrogen)
Fetal bovine serum (FBS)	F2442	Sigma
Gentamycin	G1272	Sigma
Human epidermal growth factor (hEGF)	CC4017	Clonetics (See Cambrex)
Insulin	CC4025	Clonetics (See Cambrex)
Interlink catheter extension kit	2N3374	Baxter HealthCare
Iodophor	n/a	Hospital pharmacy
Mouse anti-desmin	D1033	Sigma
Novocain solution, 1%, without epinephrine	n/a	Hospital pharmacy
PBS with Ca^{2+} and Mg^{2+}	14040	GIBCO (See Invitrogen)
Penicillin/streptomycin	15140-122	GIBCO (See Invitrogen)

Item	*Catalog No.*	*Supplier*
Polypropylene jar, 30 ml	2118-0001	Nalge Nunc
Silicone elastomer, silicone rubber	Med6015	Nusil Silicone Technology
Silicone rubber adhesive sealant	RTV108	GE Silicones
Silicone rubber sheet (0.01 in. thick)	Custom order	Silicone Specialty Fabricators
Silicone rubber tubing	8060-0040	Nalgene
Skeletal muscle growth medium (SKGM)	CC3160	Clonetics (See Cambrex)
SonicSeal slides, 4 well	138121	Nalge Nunc
Steristrips, $\frac{1}{4}$ in.	3 M #R-1542	Seaway
Suction-enhancing nipples: infant nipples, 4 mm	00079	Ross Laboratories
Surgilube	0168-0205-06	Fougera
Syringe needles, 22 g	305155 BD	BD Biosciences
Tisseel VH fibrin sealant	921029	Baxter
Triton X-100	T9284	Sigma
Trypsin-EDTA solution	T4174	Sigma
Vectashield® Mounting Medium with DAPI	H1200	Vector Labs
Zyderm® 2 Collagen Implant	5024A	McGhan Medical

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