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Isolation, growth and differentiation of adult rabbit skeletal myoblasts in vitro

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Abstract. A simple and reliable method is described for the growth and differentiation of myoblasts isolated from adult New Zealand White rabbit fast-twitch (tibialis anterior) and slow-twitch (soleus) skeletal muscle. Cells were dissociated mechanically, and expanded in DMEM supplemented with 20% horse serum. The myoblasts were differentiated by switching to DMEM supplemented with 10% horse serum when the myoblasts were 80–90% confluent. The myoblasts fused into multinucleated myotubes that spontaneously contracted. At the light microscopic level, the myotubes exhibited a striated pattern as revealed by positive immunostaining for sarcomeric proteins such as α-actinin, myosin and titin. Electron microscopy demonstrated well ordered sarcomeres with localized mitochondria and sarcoplasmic reticulum. The only notable difference between the fiber types was in the initial response to isolation wherein the myoblast yield was four-fold greater from the soleus than the tibialis anterior, in agreement with satellite cell abundance in predominately slow-twitch vs fast-twitch muscles. These techniques repeatedly (n = 12) produced a population of healthy myoblasts isolated from either fast- or slow-twitch skeletal muscle which can be utilized for studies of skeletal muscle differentiation, assembly and signaling.

Key words: Fiber type, Myoblast, Myogenin, Satellite cell

1. Introduction

Skeletal muscle is composed of multinucleated myofibers that arise from the fusion of terminally differentiated myoblasts. These myofibers themselves have no regenerative capacity. However, a population of mononucleated muscle precursor cells, termed myoblasts or satellite cells, resides nestled between the sarcolemma and basal lamina of each fiber. This population of myoblasts proliferates in response to injury or other stimuli, and contributes to muscle regeneration in vivo. Expanding uses for skeletal myoblasts, including myoblast therapy and tissue engineering, have generated a renewed interest in techniques for the in vitro isolation and growth of myoblasts from adult muscle.

Techniques for the isolation, propagation, and differentiation of human, rodent and avian satellite cells have existed for some time [3, 8, 25]. However, we have found these protocols to be unsatisfactory for the isolation of myoblasts from skeletal muscles of adult rabbits. As the rabbit represents a popular model with which to examine skeletal muscle metabolism and function (i.e., 13, 17, 18), our goal was to characterize conditions under which skeletal myoblasts obtained from adult rabbits could be reproducibly harvested, propagated and differentiated in vitro.

Skeletal muscle myofibers exhibit a range of phenotypes that depend upon the functional requirements and activity patterns imposed on them. Tonically active fibers are slow-twitch (type I), which are fatigue resistant and primarily rely on oxidative metabolism. At the other end of the spectrum are phasically active, fast-twitch type II fibers that primarily rely on glycolytic metabolism, fatigue rapidly, and are called upon for fast, high force contractions.

Many muscles of rabbits, like other small mammals, tend to be composed predominately of one fiber type such as the slow-twitch soleus (93–99% type I) and the fast-twitch tibialis anterior (97–100% type II) [1, 7, 15]. Due to the differences in protein composition and function among fast and slow muscles, it is not known whether myoblasts derived from each fiber type differ in their requirements for growth and differentiation. One goal of the present study was to define a set of conditions that would prove optimal for the isolation, propagation and differentiation of myoblasts derived from both predominately fast- and slow-twitch muscles.

In order to explore skeletal muscle differentiation and plasticity, we also sought to determine medium conditions whereby differentiated myotubes could be maintained for periods of weeks to months, thus allowing access by light and electron microscopy to both early and late events in muscle differentiation

and assembly. Over this time the cultured myotubes can also form a small muscle organelle in vitro which can then be subjected to external stimuli similar to those experienced by skeletal muscles in vivo (e.g., electrical activity, mechanical deformation, growth factor variations, etc.) thereby allowing examination of signaling pathways that may control differentiation and fiber type characteristics.

The techniques detailed here have been designed to address all of these needs. They reproducibly result in a population of myoblasts derived from predominately fast-twitch or slow-twitch adult rabbit skeletal muscles. The myoblasts can be expanded, frozen, and differentiated into multinucleated contracting myotubes, that can be maintained for weeks to months, thus allowing detailed in vitro studies of muscle development, assembly, and signaling.

2. Materials

A. Equipment
1. Laminar flow hood, model VBM600.[1]
2. Cell culture incubator, model 2710.[2]
3. Centrifuge, model J-B6, with rotor JS-4.2.[3]
4. Straight forceps, model 11002-12.[4]
5. Straight tough cut iris scissors, model 14058-11.[4]
6. Straight surgical scissors, model 14002-14.[4]
7. Curved hemostatic forceps, model 29-8343.[5]
8. Curved dissecting forceps, model 29-7405.[5]
9. Straight hemostatic forceps, model 29-8342.[5]
10. Inverted microscope, model TMS.[6]
11. Water bath.[7]
12. Dry block heater.[7]
13. Pipet-aid automatic pipettor, No. 53498-001.[7]
14. EM grids, 3.0 mm, Athene, thin bar, No. G2002.[8]
15. Silicone embedding molds, No. 70902.[9]
16. Reichert OMU3 ultramicrotome.[10]

B. Cultureware
1. Sterile 100 mm petri dish, No. 3003.[11]
2. Sterile 150 × 25 mm petri dish, No. 3025.[11]
3. Sterile 15 ml tubes, No. 62.554.205.[12]
4. Sterile 50 ml tubes, No. 21008-146.[7]
5. Sterile pipettes, 1 ml – No. 4011, 10 ml – No. 4101, 50 ml – No. 4501.[13]
6. Sterile pipettes, 5 ml – No. 27002-5, 25 ml – No. 27004-25.[14]
7. 1 ml plastic pipette tips, No. P1245BS.[15]
8. 0.22 μm bottle top filter, No. 7105.[11]
9. 1.2 ml polypropylene cryovials, No. 25703.[14]
10. Thermanox® coverslips, No. 9380B30.[16]

C. Reagents
1. Dulbecco's Modified Eagle Medium (DMEM), No. 11885-050.[17]
2. Horse Serum (HS), No. A-3311-L.[18]
3. Gentamicin (10 mg/μl), No. 15710-015.[17]
4. KH_2PO_4, No. 7100.[19]
5. NaCl, No. 7581.[19]
6. KCl, No. 6858.[19]
7. Na_2HPO_4, No. 7919.[19]
8. Dimethylsulfoxide (DMSO), No. 20684.[20]
9. 0.05% Trypsin-0.53 mM EDTA, No. 23500.[17]
10. Formaldehyde, No. 5016-8.[19]
11. Nonidet P-40, No. N-0896.[21]
12. Monoclonal antibody to α-actinin, No. A-7811.[21]
13. Monoclonal antibodies: titin (9D10), desmin (D3), sarcomeric myosin (MF20), embryonic myosin (F1.652).[22]
14. IgG secondary antibody and ABC reagent; Vectastain ABC Kit, No. PK 4002.[23]
15. Vector® VIP substrate kit, No. SK4600.[23]
16. Vector® SG substrate kit, No. SK-4700.[23]
17. AEC substrate kit, No. SK-4200.[23]
18. Growth Factor Reduced Matrigel, No. 40230A.[24]
19. Type B bovine skin gelatin, No. G-9391.[21]
20. Glutaraldehyde, 50%, Ultrapure EM, No. 1050A.[25]
21. Osmium tetroxide, No. 5323A.[25]
22. Araldite 506, No. 3013.[25]
23. Dodecenyl succinic anhydrite (DDSA), No. 3123.[25]
24. DER, No. 3111.[25]
25. Dimethylamino Methylphenol (DMP-30), No. 3103A.[25]
26. Tannic Acid, No. 21720.[9]
27. Beem Capsules, size 3, No. 69911-01.[9]
28. Glycerol, No. 16374.[26]
29. Collagenase, No. C-2674.[21]
30. Pancreatin, No. P-3292.[21]
31. Trypsin, No. 0152-13-1.[27]
32. Protease, No. P-3417.[21]
33. MEM, No. 12-611F.[28]
34. SkGM™, No. CC-3160.[29]
35. SkBM™, No. CC-3161.[29]
36. Hams F-12, No. 11059-011.[17]
37. Epidermal growth factor, No. E0890.[21]
38. Chick embryo extract, No. 16460-024.[17]
39. Insulin, No. I-5500.[21]
40. Bovine serum albumin, No. A-4503.[21]
41. Fetal bovine serum, No. A-1111-L.[18]

3. Procedures

A. Preparation of tissue culture solutions
1. Phosphate-buffered saline (PBS)
For 1 liter of 10× stock, dissolve 80 g NaCl, 2 g KCl, 6.09 g Na_2HPO_4 and 2 g KH_2PO_4 in distilled H_2O, pH to 7.4. To use, add 100 ml of 10× stock to 900 ml dH_2O. Filter sterilize (0.22 μm vacuum filter). Store at room temperature. The working solution is

137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4.

2. DMEM + 20% HS (Growth medium)
   To 500 ml DMEM add 126 ml horse serum (HS) and 3 ml gentamicin (10 mg/ml). Store at 4 °C.
3. DMEM + 10% HS (Differentiation medium)
   To 500 ml of DMEM add 56 ml HS and 2.5 ml gentamicin. Store at 4 °C.
4. 90% Growth medium, 10% DMSO (Freezing solution)
   To 9 ml of growth medium, add 1 ml of DMSO. Store at 4 °C.

B. Preparation and gross removal of muscles

1. Add 5 ml of growth medium to a 100 mm uncoated plate (2 plates per muscle) and place in incubator (37 °C, 5% CO_2, 100% humidity) for 1 hour to equilibrate. Place bottle of growth medium in 37 °C water bath to warm.
2. Sterilize all instruments. Pipette 30 ml of PBS into 50 ml tubes (one tube per muscle) and place on ice.
3. Excise the soleus and tibialis anterior muscles by clamping the tendons with the hemostatic forceps and cutting distally to the clamps with surgical scissors to avoid any damage to the muscle. Muscles from adult (3–5 kg) New Zealand White rabbits typically weigh 1.5–2 g (soleus) to 4–5 g (tibialis anterior).
4. Store the excised muscles immersed in PBS (50 ml tube) on ice until fine dissection (the samples can be stored for 1–2 hours, but do not store them overnight).

C. Isolation of Cells (sterile)

1. Under flow hood, place muscle on 150 mm plate in PBS.
2. Grossly dissect away attached connective tissue (tendons and visible connective tissue) with surgical scissors and forceps.
3. With the surgical scissors and straight forceps, cut out pieces from the deep belly region of the muscle that are approximately 5 mm sq. Take care not to include any residual connective tissue. Transfer these pieces to a fresh 150 mm plate, which has 2–3 ml of fresh PBS in it. There should be approximately 5–15 pieces (about 300–900 mg, depending on the initial size of the muscle excised).
4. Hold a piece of tissue with the curved forceps, and finely mince with the iris scissors into 1 mm^3 pieces. Place pieces in clean 150 mm dish that contains 2–3 ml of fresh PBS. The minced pieces should resemble very large eraser shavings.
5. Trim a sterile 1 ml plastic pipette tip about 6–8 mm up from the tip with a sterile razor blade to increase the size of the opening to 3–4 mm (these can be cut ahead of time and autoclaved in bulk).
6. Using the tip with the widened opening and a 1 ml pipette, transfer the muscle pieces into a 15 ml tube. If necessary, add more PBS to facilitate pipetting the pieces.
7. Spin the 15 ml tube at $130 \times g$ (800 rpm) in a low speed centrifuge for 5 min to pellet the pieces.
8. Aspirate and remove the PBS, and resuspend the pieces in 10 ml fresh PBS to wash. Invert or vortex to mix well. Spin again at $130 \times g$ for 5 min.
9. Aspirate and remove the PBS again, resuspend the pieces in 10 ml fresh PBS to wash. Invert or vortex to mix well. Spin a third time at $130 \times g$ for 5 min.
10. Aspirate and remove the PBS and resuspend the pieces in 10 ml of prewarmed growth medium per muscle.
11. Vortex the 15 ml tube, flame the lip, and pour half of the pieces suspended in growth medium onto each of the 2 prewarmed uncoated 100 mm plates.
12. Leave the plates in the incubator undisturbed for 72 hours. During this time the cells will migrate from the tissue, adhere to the plate, and start to divide.
13. 72 hours after plating, add 3 ml of growth medium (warmed to 37 °C) to each plate. Gently rotate plate to mix.
14. 2 days later refeed the plates by aspirating off 5 ml of the medium and adding 5 ml of fresh warmed medium.
15. Within the next 1–2 days, after extensive colonies have formed, the cells can be trypsinized to expand or freeze. Almost all of the tissue pieces are easily removed by washing. The number of cells at this point typically ranges from $1–5 \times 10^6$ cells per starting material from each muscle. The cells should be passaged whenever they reach 50–60% confluence, unless proceeding with differentiation. When replating, they should be kept at a minimum of 20–25% confluence or their viability is adversely affected. For trypsinization procedures, see Cell Freezing section below.

D. Differentiation

1. When the cells are 80–90% confluent, aspirate off the growth medium. Do not wash the plates. Refeed with prewarmed differentiation medium (use 10 ml for a 100 mm plate).
2. Refeed the plates every 24 hours. Myotube formation will be evident by 1 week.
   As long as the plates are refed regularly, the cells will stay viable for up to 9–10 week.

E. Cell freezing and thawing
   1. The cells may be frozen when they are 50–80% confluent. Wash the plates twice with sterile PBS.
   2. Pipette 3 ml of 0.05% trypsin-0.53 mM EDTA solution onto each plate. Allow the plates to stand for a few min while the cells round up. Premature agitation of the plate will cause the cells to tear up in sheets, and will greatly reduce the number of viable cells.
   3. Once the majority of the cells have rounded up and detached, add 7 ml of growth medium and gently rock the plate.
   4. Pipette the cells into a 15 ml tube. Save an aliquot to count.
   5. Spin the cells at 130 × g for 5 min.
   6. Aspirate and remove the trypsin-medium mixture. Thoroughly mix and resuspend the cells in freezing solution (90% growth medium, 10% DMSO) at a density of 1×10^6 cells/ml.
   7. Aliquot 0.5 ml of the mixture (5×10^5 cells) into cryovials.
   8. Place cryovials into isopropyl alcohol bath at –80 °C overnight to freeze, then transfer to liquid nitrogen storage facility.
   9. Before thawing, pipette 10 ml of growth medium onto a 100 mm plate and place in incubator to equilibrate for 1 hour.
   10. Thaw cryovial of cells by placing in a 37 °C heat block for 1–2 min. Pipette the cells onto the plates. Flush cryovial with medium to maximize transfer.
   11. The next day refeed the cells with fresh growth medium to remove residual DMSO.

F. Immunostaining
   1. The protocol for cell staining is based on published methods [19]. All steps are performed at room temperature unless otherwise indicated.
   2. Wash the cells twice with ice-cold PBS.
   3. Incubate the cells in 2% formaldehyde (in PBS) for 15 min to fix, and then wash the cells twice in PBS.
   4. Incubate the cells with 0.25% nonidet P-40 (NP-40) in PBS for 15 min to permeabilize, and then wash the cells twice in PBS.
   5. Incubate the cells overnight at 4 °C in rinse solution (PBS with 2% horse serum and 0.1% NP-40) to block nonspecific binding. Use this solution for all subsequent rinses.
   6. The following day, incubate the cells with primary antibodies (i.e., titin, sarcomeric and embryonic myosin, α-actinin, and myogenin) for 1 hour, and then rinse twice for 10 min each. (The antibody to myogenin used here was a kind gift from W. E. Wright.)
   7. Incubate the cells for 30 min in biotinylated anti-mouse IgG secondary antibody (dilute according to the manufacturers directions) for 30 min, and then rinse twice for 10 min each.
   8. Incubate in ABC reagent (make per the manufacturers directions) for 30 min, and then rinse twice for 10 min each.
   9. To visualize the staining, incubate in a peroxide substrate (Vector VIP, SG, or AEC) until the desired level of staining develops (usually 1–10 min). Rinse the dishes with PBS.

G. Electron microscopy
   1. Coat Thermanox coverslips with either 2% type B bovine skin gelatin or 100 μg/ml Reduced Growth Factor Matrigel by covering with the substrate and incubating at room temperature for 1 hour. Rinse the coverslips with DMEM.
   2. Passage the myoblasts, and plate 5×10^3 cells per coverslip. Grow the myoblasts to 80–90% confluence, and then differentiate for 10 days.
   3. Fix the myotube cultures by flooding in situ with 3% glutaraldehyde, 0.2% tannic acid in 160 mM NaCl, 5 mM $MgCl_2$, 10 mM MOPS, pH 7.0 (Ringers buffer), for 30 min.
   4. Rinse the coverslips in Ringers buffer, and then rinse in a buffer of 100 mM PO_4, 10 mM $MgCl_2$, pH 6.0.
   5. Postfix the cells for 30 min in ice cold 1% osmium tetroxide in 10 mM $MgCl_2$, 100 mM PO_4, pH 6.0.
   6. Rinse the coverslips with water, and then block stain the specimens for 30 min in 2% uranyl acetate in water.
   7. Rinse in water and dehydrate in a graded 50–100% ethanol series as described [9].
   8. Pour off the ethanol and instantly flood the petri dishes containing the cover slips with accelerated Araldite 506 mix (50 g Araldite 506, 75 g dodecenyl succinic anhydrite, and 10 g DER, accelerated by thorough, gentle stirring with 0.2% dimethylamino methylphenol). Incubate at 65 °C for 20 min.
   9. Remove the coverslips, drain briefly, and place on polyethylene sheets or on the backside of silicon-embedding molds. Add a thin layer of fresh, accelerated Araldite mix dropwise, and then invert beem capsules filled with the accelerated Araldite 506 mix over the coverslips.
   10. Cure the blocks for two days at 65 °C. Grasp the beem capsules and break them from the coverslips. The tissue will be at the surface of the beem capsule block.
   11. Trim blocks as desired for cross or longitudinal sections. Cut sections with diamond knife on ultramicrotome, pick up on carbon

coated grids, and stain with 2% $KMnO_4$ followed by Sato Lead as previously described [20].

4. Results and discussion

We describe a simple technique for the isolation, expansion and differentiation of satellite cells (myoblasts) obtained from adult rabbit skeletal muscle. Most currently available protocols for the isolation of myoblasts have employed proteolytic enzymes such as trypsin, papain, ficin, pronase and collagenase to facilitate dissociation and release of satellite cells from the muscle via dissolution of the basal lamina [3, 6, 10]. We initially tested a wide range of enzymatic conditions to generate a population of viable myoblasts. These included 0.05–0.25% trypsin, 0.1–0.4% collagenase, 0.07% protease, and 0.4% pancreatin. Unfortunately, these protocols either resulted in a very small cell population (less than one-half of the cell number obtained with the present technique), or in a cell population that consisted primarily of fibroblasts (data not shown).

The present technique is based on an explant procedure wherein the muscle is finely minced. As shown in Figures 1a and 1b, within the first week after plating, myoblasts migrate from the pieces of tissue, and divide. Chemical dissolution of the basal lamina is not required for satellite cell release, as the myoblasts are able to cross the basal lamina that surrounds the mature myofiber, and to migrate substantial distances [4, 16].

Satellite cells in adult muscle are quiescent, in cell cycle arrest. Proliferation in vivo is induced by the presence of various concentrations of growth factors, such as fibroblast growth factor and insulin-like growth factor. We tested a variety of medium conditions for the ability to stimulate myoblast proliferation. These included SkGM + 5% fetal bovine serum (FBS), SkGM + 20% HS, Hams F-12 + 15% HS and 2% chick embryo extract (CEE), as well as DMEM + 20% HS [14, 23]. The DMEM + 20% HS consistently yielded a population of healthy, dividing myoblasts, whereas the other growth medium conditions resulted in inconsistent or suboptimal myoblast proliferation (data not shown). The resultant myoblasts could be readily propagated for storage or used immediately in subsequent experiments.

In response to growth factor deprivation in vitro, proliferating myoblasts withdraw from the cell cycle and become terminally differentiated. We tested a variety of medium conditions for the ability to induce myoblast differentiation as demonstrated by myotube formation. These included SkBM, SkGM, MEM and DMEM, with and without epidermal growth factor (10 ng/ml), insulin (10 μg/ml), bovine serum albumin (0.5 mg/ml), FBS (10%), CEE (1–5%), and HS (1–10%) [6, 10, 14]. While quite a number of the conditions induced myoblast fusion to some extent (data not shown), only the DMEM + 10% HS reproducibly (n = 12 separate cell isolations) produced a large population of multinucleated myotubes that spontaneously contracted and that stained positive for muscle-specific proteins (e.g., striated myosin, α-actinin, titin, myogenin). The DMEM + 10% HS is much richer than most differentiation media, which often contain less than 5% serum [6, 14]. We have found that with DMEM + 10% HS the cells stay viable for 2 months as long as they are fed regularly. Subsequent testing of different lots of horse serum from several companies demonstrated a noticable difference in the extent of growth and fusion of the myoblasts (data not shown), thus lot testing is highly recommended.

DMSO is typically used for freezing cells, although it has been reported to inhibit terminal differentiation of myoblasts [5]. We previously found that it adversely affected fusion of commercially available human myoblasts (unpublished data) when they were stored in its presence. We therefore tested a number of freezing solutions that included (1) 90% FBS, 10% glycerol, (2) 90% HS, 10% glycerol, (3) 90% growth medium, 10% DMSO, and (4) 90% growth medium, 10% glycerol. The optimal combination under these conditions was found to be 90% growth medium and 10% DMSO, followed closely by 90% FBS and 10% glycerol. Cells frozen in these solutions retained their ability to proliferate after thawing, and to differentiate into multinucleated, contracting myotubes that stained for a battery of muscle-specific proteins.

The access provided by myoblasts developing in vitro facilitated coordination of light and electron microscopy (EM). Immunostaining at the light microscopic level was used to identify the overall organization of component proteins while EM was used to resolve fine subcellular structure. This coordination allowed insight into development of the myotubes, with emphasis on the assembly of sarcomeric myofibrils, from the immature myoblast cytoplasm to the contractile arrays of mature fibers.

At the light microscopy level, the developing myotubes exhibited numerous central nuclei (i.e., Figure 1c), which over time shifted to a subsarcolemmal position, indicative of maturing myofibers (Figure 1g). The nuclei stained for myogenin, a member of the family of myogenic determination factors [12]. Myogenin staining was initially evident in myoblast nuclei just prior to their fusion, and was abundant in myotubes (Figure 1d), in concordance with similar findings in rat satellite cells [22].

The myotubes formed during differentiation displayed characteristics typical of cultured skeletal muscle. Like mature skeletal muscle fibers, over time the myotubes exhibited highly-ordered myofibrils which consisted of interdigitating thick myosin-containing and thin actin-containing filaments. The

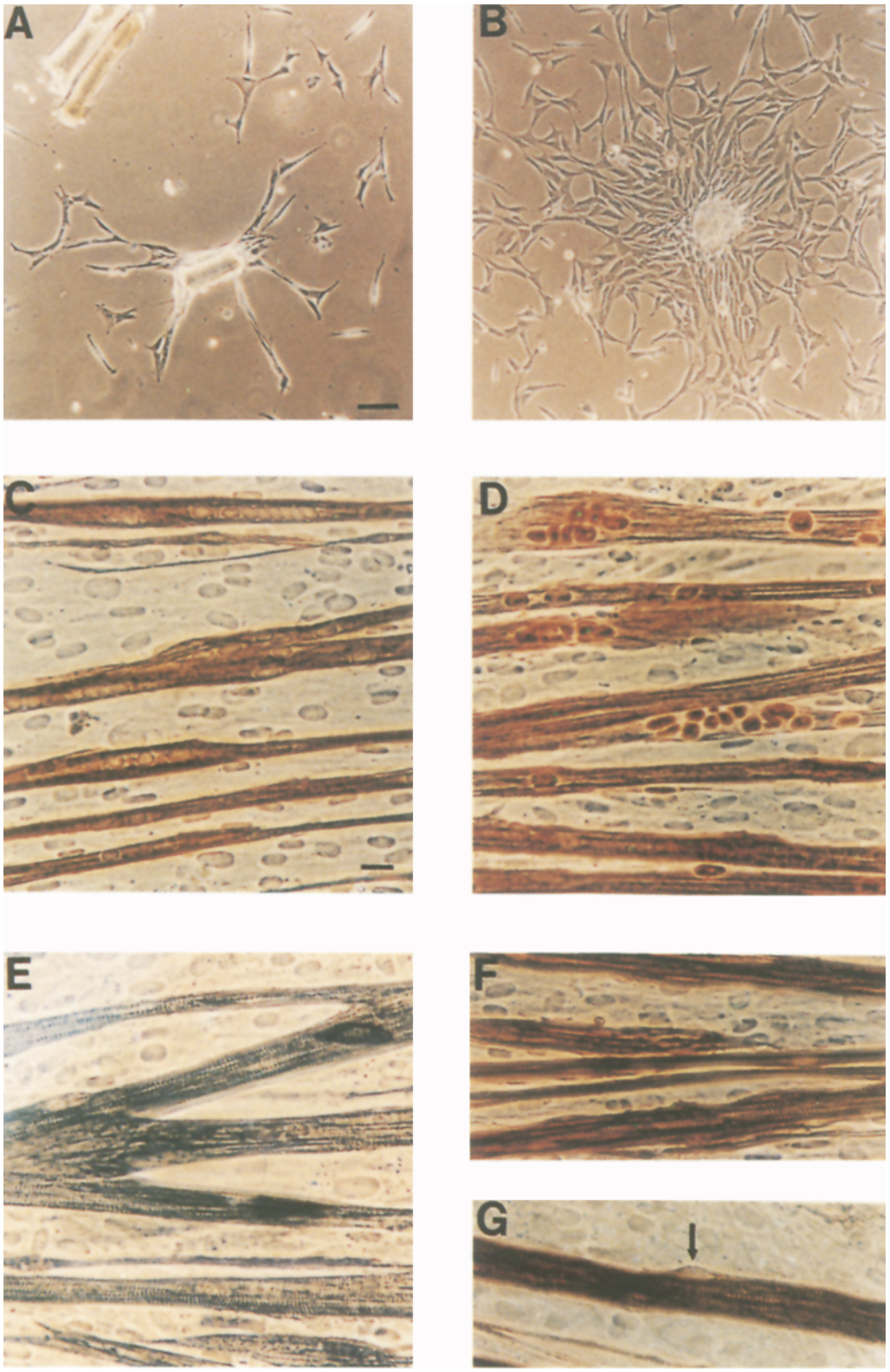

Figure 1. Myoblasts isolated from adult New Zealand White rabbit. **A**: cells from the fast-twitch tibialis anterior muscle 8 days after isolation, shown migrating from a piece of tissue. **B**: Cells from the slow-twitch soleus 5 days after isolation, also shown migrating from a piece of tissue. **C, D**: myotubes from the soleus after being grown in differentiation media for 9–12 days, stained for embryonic myosin, or for sarcomeric myosin (purple) and myogenin (red), respectively. **E, F**: myotubes grown in differentiation medium for 17–21 days, stained for α-actinin or titin, respectively. **G**: a myotube from isolated soleus muscle after being grown in differentiation medium for 21 days, and stained for α-actinin. A peripheral nucleus is marked by the arrow. Scale bars: A-B, bar = 100 μm; C-G, bar = 20 μm.

myotubes stained for both embryonic and general sarcomeric forms of myosin (Figures 1c, d). The filaments were organized into sarcomeres, which showed periodic staining for α-actinin, a primary component of Z-bands (Figure 1e). The myotubes also stained for titin (Figure 1f), which is associated with myosin and integrates the filaments within the sarcomere.

Electron microscopy of parallel cultures enabled resolution of the fine subcellular structure of the organization of the myotubes. As shown in Figure 2,

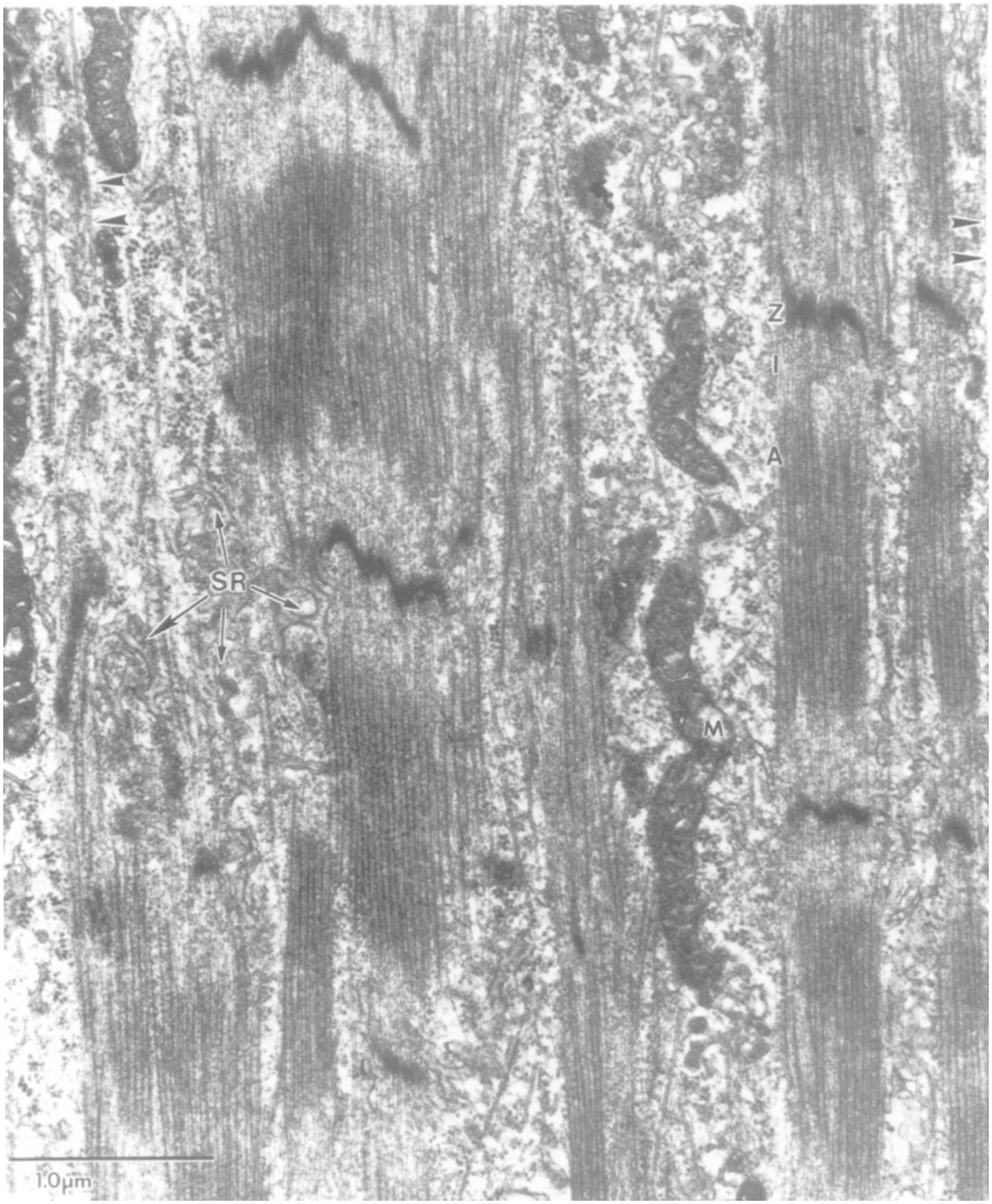

Figure 2. Electron micrograph of a longitudinal thin section of a myotube after culturing adult rabbit skeletal muscle in differentiation medium for 10 days in vitro. The development of sarcomeric myofibrils is shown. Thin actin-containing filaments have aligned laterally to form I-bands (I), crosslinked by Z-lines (Z), while regions within which thin filaments overlap with arrays of thick myosin-containing filaments form the A-bands (A). The sarcoplasmic reticulum (SR) has become localized around the I-Z-I regions. Small double arrowheads indicate microtubules. Mitochondria (M) are evenly spaced between the myofibrils.

thick myosin-containing and thin actin-containing filaments became organized into well-ordered sarcomeres. The thin filaments aligned laterally to form I-bands, which were cross-linked by the Z-lines. Regions within which thin filaments overlapped with arrays of thick filaments formed the A-bands. The sarcoplasmic reticulum became localized around the I-Z-I regions of the sarcomeres, and mitochondria became evenly spaced between the myofibrils.

The conditions outlined here worked equally well for the isolation of cells from either a predominately fast-twitch (tibialis anterior) or slow-twitch (soleus) muscle. There were no gross morphological differences in the myoblasts isolated from fast or slow muscles. The only notable difference was that the cell yield was four-fold greater from the soleus than from the tibialis anterior. Typically, we obtained 1×10^6 cells/g of muscle from the tibialis anterior, and 4×10^6 cells/g muscle from the soleus one week after cell isolation. This agrees with the observation that satellite cells are much more abundant in oxidative muscles, regardless of the species [21], and also agrees with the work of Düsterhöft et al. [11] who isolated five-fold more satellite cells from the soleus than from the tibialis anterior muscles of adult rats.

It should be noted that the nuclei of satellite cells account for approximately 30% of total muscle nuclei of newborn animals. However, as an animal ages, there is a continual decline in the number of satellite cells, such that they may account for less than 5% of the nuclei in older animals [21]. Thus, the absolute number of myoblasts obtained via isolation from adult tissue is much less than that obtained from fetal or newborn animals. In addition, satellite cells isolated from adult tissue samples have been found to exhibit a lag time of 1–4 days prior to initiation of proliferation [2, 6]. Thus, despite these age-related limitations, we have been able to generate a sizable population of myoblasts from the cultures of adult rabbit myoblasts. This population of cells can be easily expanded. We have observed that differentiation of the myoblasts is not adversely affected until after 25 population doublings. This limited life span agrees with that found for myoblasts isolated from humans [6].

The protocol presented here results in a population of cells which includes fibroblasts, in addition to the myoblasts. Currently, we have not quantitated the relative abundance of the different cell populations. However, due to the high percentage of fusion exhibited (number of nuclei incorporated into multinucleated syncytia), the myoblasts appear to make up the bulk of the isolated cells. For the purpose of supporting the generation of a highly differentiated muscle organelle in vitro, the presence of fibroblasts is desirable, as they are needed to form a basal lamina [24]. Therefore a mixed cell population represents a suitable model for the generation of a muscle organelle structure derived from isolated primary myoblasts, and may be preferred for experiments that require an extracellular matrix. If a pure myoblast population is desired, we recommend that the myoblasts be clonally isolated, as we have had little success with eliminating fibroblasts via preplating or with differential plating on various substrates. Uncoated plastic dishes were utilized in the outlined methodology for cell isolation, expansion and differentiation. For EM, the cells were passaged onto coverslips coated with either gelatin or Reduced Growth Factor Matrigel to facilitate attachment. We have occasionally noted very subtle differences in morphology associated with various substrates, which are the subject of future experiments.

In conclusion, we present a simple and reliable method for the isolation and expansion of myoblasts derived from fast-twitch and slow-twitch adult rabbit skeletal muscle. The resultant myoblasts could be induced to differentiate into multinucleated myotubes that contained well ordered sarcomeres, expressed muscle-specific proteins such as α-actinin, myogenin, and titin, and spontaneously contracted. The described techniques resulted in a cell population that can be used for studies of molecular and cellular events in skeletal muscle differentiation, including sarcomere assembly and molecular signaling.

Acknowledgments

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Notes on suppliers

1. Sterigard Hood, Baker Company Inc., Sanford, ME, USA
2. Queue Systems, Parkersburg, WV, USA
3. Beckman, Fullerton, CA, USA
4. Fine Science Tools, Inc., Foster City, CA, USA
5. PGC Scientific, Gaithersburg, MD, USA
6. Nikon, Garden City, NY, USA
7. VWR Scientific, West Chester, PA, USA
8. Ted Pella, Redding CA, USA

9. Electron Microscopy Sciences (EMS), Fort Washington, PA, USA
10. American Optical, Buffalo, NY, USA
11. Beckon Dickinson (Falcon), Lincoln Park, NJ, USA
12. Sarstedt Inc., Newton, NC, USA
13. Costar, Cambridge, MA, USA
14. Corning, Corning, NY, USA
15. Port City Diagnostics Inc., Wilmington, NC, USA
16. Thomas Scientific (Nunc Inc.), Swedesboro, NJ, USA
17. Gibco BRL, Grand Island, NY, USA
18. HyClone, Logan, UT, USA
19. Mallinckrodt, Paris, KY, USA
20. Pierce, Rockford, IL, USA
21. Sigma Chemical Co., St. Louis, MO, USA
22. Developmental Studies Hybridoma Bank, Iowa City, IA, USA
23. Vector Laboratories, Burlingame, CA, USA
24. Becton Dickinson Labware, Bedford, MA, USA
25. Tousimis, Rockville, MD, USA
26. United States Biochemical Corp., Cleveland, OH, USA
27. Difco Laboratories, Detroit, MI, USA
28. BioWhittaker, Walkersville, MD, USA
29. Clonetics Corp., San Diego, CA, USA

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