Stem Cell Research 30 (2018) 122–129

Contents lists available at ScienceDirect

Stem Cell Research

journal homepage: www.elsevier.com/locate/scr

Efficient and high yield isolation of myoblasts from skeletal muscle

Aref Shahini[a], Kalyan Vydiam[b], Debanik Choudhury[a], Nika Rajabian[a], Thy Nguyen[b], Pedro Lei[a], Stelios T. Andreadis[a,b,c,*]

[a] Bioengineering Laboratory, Department of Chemical and Biological Engineering, University at Buffalo, The State University of New York, Amherst, NY 14260-4200, USA
[b] Department of Biomedical Engineering, University at Buffalo, The State University of New York, Amherst, NY 14260-4200, USA
[c] Center of Excellence in Bioinformatics and Life Sciences, Buffalo, NY 14263, USA

ARTICLE INFO

Keywords:
Skeletal muscle progenitors
Myoblast isolation
Satellite cells
Myogenic differentiation

ABSTRACT

Skeletal muscle (SkM) regeneration relies on the activity of myogenic progenitors that reside beneath the basal lamina of myofibers. Here, we describe a protocol for the isolation of the SkM progenitors from young and old mice by exploiting their outgrowth potential from SkM explants on matrigel coated dishes in the presence of high serum, chicken embryo extract and basic fibroblast growth factor. Compared to other protocols, this method yields a higher number of myoblasts (10–20 million) by enabling the outgrowth of these cells from tissue fragments. The majority of outgrowth cells (~90%) were positive for myogenic markers such as α7-integrin, MyoD, and Desmin. The myogenic cell population could be purified to 98% with one round of pre-plating on collagen coated dishes, where differential attachment of fibroblasts and other non-myogenic progenitors separates them from myoblasts. Moreover, the combination of high serum medium and matrigel coating provided a proliferation advantage to myogenic cells, which expanded rapidly (~24 h population doubling), while non-myogenic cells diminished over time, thereby eliminating the need for further purification steps such as FACS sorting. Finally, myogenic progenitors gave rise to multinucleated myotubes that exhibited sarcomeres and spontaneous beating in the culture dish.

1. Introduction

Satellite cells are myogenic progenitors that are located between the basal lamina and the plasma lemma of myofibers. Regeneration of adult skeletal muscle relies on the activation, proliferation and fusion of these myogenic progenitors into degenerated myofibers (Yablonka-Reuveni, 2011; Yin et al., 2013). Isolation of myogenic progenitors from the skeletal muscle niche provides us an in vitro test bed to study muscle physiology and enables studies on the intrinsic and extrinsic factors affecting myogenic differentiation (Shahini et al., 2018) as well as cellular and molecular pathways that can lead to muscle atrophy or dystrophy (Bareja and Billin, 2013). In vivo assessments of cellular function can also be done by delivering cells to the skeletal muscle of animal models in order to test their contribution to muscle regeneration after injury or disease. Such studies may lead to development of cellular therapies to promote muscle regeneration (Bareja and Billin, 2013) and combat debilitating diseases such as muscular dystrophy.

Satellite cells comprise 30–35% of the total muscle fiber nuclei at birth but decrease dramatically to 2–5% of the nuclei in adult animals which further depletes with age (Allbrook et al., 1971; Shefer et al., 2006; Day et al., 2010). Although these cells can be isolated directly from the mononuclear cell population by enzymatic digestion and titration of skeletal muscle tissue,(Rando and Blau, 1994; Hindi et al., 2017) the efficiency of this method is low due to the limited number of satellite cells released from the skeletal muscle niche ($1–2 \times 10^5$ myoblasts from the hindlimb muscles of one adult mouse (Yi and Rossi, 2011, Motohashi et al., 2014)). Furthermore, these protocols require passing the muscle slurry through 40 or 70 μm cell strainer, eliminating progenitor cells that may remain bonded to the myofibers (Danoviz and Yablonka-Reuveni, 2012). The majority of cells isolated using these methods are fibroblasts and other non-myogenic cell types, necessitating further purification steps by applying several rounds of pre-plating and attachment of fibroblasts to collagen coated surface (Hindi et al., 2017), fluorescence-activated cell sorting (FACS) (Yi and Rossi, 2011) or magnetic-activated cell sorting (MACS) (Motohashi et al., 2014).

Abbreviations: FACS, fluorescence activate cell sorting; MACS, magnetic-activate cell sorting; EHS, Engelbreth-Holm-Swarm; TGF-β, transforming growth factor beta; EGF, epidermal growth factor; IGF1, insulin-like growth factor; PDGF, platelet derived growth factor; bFGF, basic fibroblast growth factor; CEE, Chicken Embryo Extract; PBS, phosphate buffer saline; PM, proliferation medium; DM, Differentiation Medium; FBS, fetal bovine serum; HS, Horse Serum

* Corresponding author at: Bioengineering Laboratory, 908 Furnas Hall, Department of Chemical and Biological Engineering, Department of Biomedical Engineering, Center of Excellence in Bioinformatics and Life Sciences, University at Buffalo, The State University of New York, Amherst, NY 14260-4200, USA.
E-mail address: sandread@buffalo.edu (S.T. Andreadis).

https://doi.org/10.1016/j.scr.2018.05.017
Received 20 January 2018; Received in revised form 18 May 2018; Accepted 22 May 2018
Available online 24 May 2018

Previous studies showed that satellite cells within the basal lamina of myofibers preserve their quiescent state in low serum media, while they proliferate and outgrow from their niche in high serum media (Pasut et al., 2013). The activated satellite cells (myoblasts) that migrate out of myofibers can be subcultured on the collagen coated (Calve et al., 2010; Goetsch et al., 2015) or matrigel coated dishes (Wang et al., 2014). Indeed, the combination of matrigel coating and high serum media promotes migration of myogenic progenitors from myofibers onto the tissue culture dish (Pasut et al., 2013), where they proliferate and maintain their myogenic differentiation capacity to fuse into myotubes in vitro (Shefer and Yablonka-Reuveni, 2005; Grefte et al., 2012; Pasut et al., 2013; Wang et al., 2014). This combination also provides growth advantage to myoblasts over other non-myogenic cells and maintains their differentiation capacity over prolonged culture times (Grefte et al., 2012; Wang et al., 2014).

Matrigel matrix is a protein mixture extracted from the Engelbreth-Holm-Swarm (EHS) mouse sarcoma, a mouse tumor rich in extracellular matrix proteins. Matrigel is composed of approximately 60% laminin, 30% collagen IV, and 8% entactin. Entactin interacts with laminin and collagen IV bridging these two extracellular matrix molecules and providing a structurally organized scaffold for cell attachment and proliferation. Matrigel also contains heparan sulfate proteoglycans that aid cell attachment in synergy with integrins and other adhesion receptors (Sarrazin et al., 2011), as well as growth factors such as transforming growth factor beta (TGF-β), epidermal growth factor (EGF), insulin-like growth factor 1 (IGF1), platelet derived growth factor (PDGF) and basic fibroblast growth factor (bFGF) (Hughes et al., 2010).

Here we describe a simple protocol that employs the combination of matrigel coating with culture medium containing high serum, chicken embryo extract (CEE) and bFGF to isolate myoblasts by promoting the proliferation and migration of satellite cells out of their niche. This protocol yields a high number of myoblasts (1–2 × 10^7 myoblasts from the hindlimb muscles of one adult mouse) and eliminates downstream purification steps such as FACS or MACS. To characterize the outgrowth population, we performed flow cytometry to quantify the myogenic fraction positive for α7-integrin and non-myogenic fraction positive for SCA-1/CD31/CD45. We also performed immunostaining for the myogenic markers MyoD and Desmin and the fibroblastic marker α-smooth muscle actin (αSMA). Our results showed that ~90% of outgrowth cells were myogenic progenitors and the small fraction of non-myogenic cells could be eliminated from the culture by pre-seeding the cells on collagen coated dishes prior to seeding them on matrigel coated dishes (pre-plating). Since the combination of matrigel coating and high serum medium supplemented with bFGF and CEE provides myoblasts with growth advantage (Wang et al., 2014), the non-myogenic cells cannot overgrow in culture, yielding highly purified myogenic progenitors. In addition to proliferation, isolated myoblasts exhibit high myogenic differentiation capacity as evidenced by formation of multinucleated myotubes capable of spontaneous beating.

2. Materials, solutions, and methods

2.1. Animals

Transgenic mice with backgrounds of C57BL/6-DBA2 (Fischedick et al., 2014), C57BL/6-129Sv (Piazzolla et al., 2014), and C57BL/6-129Ola (Osorio et al., 2011) were used in this study. Mice from other backgrounds including C57BL/6 (a gift from Dr. Kenneth L. Seldeen, University at Buffalo, NY), and FOXn1nu nude (a gift from Dr. Natesh Prashurama, Universtity at Buffalo, NY) were also used to demonstrate broader applicability of this protocol. All animals were maintained in University at Buffalo laboratory animal facility according to the guidelines of Institutional Animal Care and Use Committee (IACUC) at the University at Buffalo. Animals at different ages (4 weeks to 2 years old) were euthanized in standard CO2 chambers and hindlimb muscles were dissected up to 4 h post sacrifice.

2.2. Matrigel coating

Cell culture dishes or flasks were cooled down to 4 °C and matrigel coating was performed on ice. Matrigel at 8–9 mg/ml (depending on the lot number, CORNING, Corning, NY) was diluted in phosphate buffer saline (PBS) to a working concentration of 0.9 mg/ml or 0.09 mg/ml (Note 4). Diluted matrigel solutions were added to the plate at 0.05 ml/cm^2 corresponding to 45 μg/cm^2 or 4.5 μg/cm^2, respectively and kept at 4 °C for 10 min. Subsequently, the solution was removed and the flasks were incubated at 37 °C for 1 h before seeding cells.

2.3. Collagen coating

To coat the tissue culture dishes or flasks with collagen, the dishes were incubated overnight at 4 °C with 0.1 mg/ml solution of type I rat tail collagen (CORNING) in sterile water at 0.05 ml/cm^2 or 5 μg/cm^2. The solution was aspirated and the plates were allowed to dry before seeding cells.

2.4. Stock solutions for enzymatic digestion

Stock solutions for enzymes were reconstituted in sterile PBS to the following concentrations: 5000 U/ml collagenase type II (Sigma-Aldrich, St. Louis, MO), 150 U/ml collagenase D (Sigma-Aldrich), 250 U/ml dispase II (Sigma-Aldrich). A stock of 250 mM $CaCl_2$ in water was also prepared. The stock solutions were stored in −20 °C. The final enzymatic solution mix was PBS containing collagenase type II (500 U/ml), collagenase D (1.5 U/ml), dispase II (2.5 U/ml), and $CaCl_2$ (2.5 mM).

2.5. Cell culture medium

The medium that was employed for the isolation and proliferation of myogenic progenitors (proliferation medium, PM) was composed of high glucose Dulbecco's Modified Eagle Medium (DMEM, Gibco, Grand Island, NY), 20% fetal bovine serum (FBS, Atlanta Biologicals, Flowery Branch, GA), 10% horse serum (HS, Gibco), 0.5% chicken embryo extract (CEE, Accurate Chemical and Scientific, Westbury, NY), 2.5 ng/ml bFGF (ORF Genetics, Iceland), 10 μg/ml gentamycin (Gibco), and 1% Antibiotic-Antimitotic (AA, Gibco), and 2.5 μg/ml plasmocin prophylactic (Invivogen, San Diego, CA). Differentiation medium (DM) containing DMEM with high glucose, 5% HS and 1% AA was used to promote formation of multinucleated myotubes.

2.6. Isolation protocol

The video and schematic of myoblast isolation process are shown in Supplementary Video 1 and Fig. 1A, respectively, and described below:

1- Sacrifice the mice using CO_2 asphyxiation.
2- Spray the mice with 70% ethanol and transfer the mice to a sterile hood.
3- Cut the skin in the back region and peel it to completely expose the hindlimb muscles.
4- Isolate skeletal muscle from the hindlimbs and carefully discard pieces of fat.
5- Mince the muscle tissues to small pieces and transfer them to a 50 ml conical tube.
6- Digest the small pieces of tissue in 1 ml enzymatic solution and incubate for 60 min in 37 °C water bath while agitating the tube every 5 min (Note#1).
7- Centrifuge at 300 ×*g* for 5 min and resuspend the pellet in proliferation medium (PM).
8- Seed the suspension containing small pieces of muscle tissue on