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Roles of adherent myogenic cells and dynamic culture in engineered muscle function and maintenance of satellite cells

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ABSTRACT

Highly functional engineered skeletal muscle constructs could serve as physiological models of muscle function and regeneration and have utility in therapeutic replacement of damaged or diseased muscle tissue. In this study, we examined the roles of different myogenic cell fractions and culturing conditions in the generation of highly functional engineered muscle. Fibrin-based muscle bundles were fabricated using either freshly-isolated myogenic cells or their adherent fraction pre-cultured for 36 h. Muscle bundles made of these cells were cultured in both static and dynamic conditions and systematically characterized with respect to early myogenic events and contractile function. Following 2 weeks of culture, we observed both individual and synergistic benefits of using the adherent cell fraction and dynamic culture on muscle formation and function. In particular, optimal culture conditions resulted in significant increase in the total cross-sectional muscle area (~3-fold), myofiber size (~1.6-fold), myonuclei density (~1.2-fold), and force generation (~9-fold) compared to traditional use of freshly-isolated cells and static culture. Curiously, we observed that only a simultaneous use of the adherent cell fraction and dynamic culture resulted in accelerated formation of differentiated myofibers which were critical for providing a niche-like environment for maintenance of a satellite cell pool early during culture. Our study identifies key parameters for engineering large-size, highly functional skeletal muscle tissues with improved ability for retention of functional satellite cells.

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1. Introduction

Skeletal muscle has a remarkable capacity for regeneration that is attributed to a population of resident muscle stem cells, known as *satellite cells* (SCs). During development, as well as in acute injuries or muscle tears, SCs, commonly marked by the transcription factor Pax7, undergo activation, proliferation, and differentiation to create a pool of myogenic precursors that fuse to form new myofibers or repair damaged ones; a process referred to as *myogenesis* [1]. Although a robust process in minor injury, myogenesis in severe trauma and muscle loss is often inadequate as fibrosis proceeds at a more rapid rate [2], leaving the muscle denervated and non-functional. For nearly 2 decades, bioengineering efforts, reviewed elsewhere [3,4], have focused on developing functional replicates of skeletal muscle tissue capable of replacing or repairing large portions of lost or damage muscle [5–9]. Additionally, engineered muscle tissues have been utilized as *in vitro* models to study skeletal muscle function and regeneration [10] and more recently to conduct toxicological and drug screening studies [11,12].

We have previously investigated various aspects of tissue fabrication including cell density, hydrogel composition [13], tissue geometry [14,15], and biochemical supplementation [16] to optimize myofibril alignment, contractile function, and expression of acetylcholine receptors in engineered skeletal muscle. Most recently, we have fabricated highly functional engineered muscle tissues [10] with the ability to maintain a pool of $Pax7^{+}$ satellite cells that supported myofiber formation, growth, and self-repair *in vitro* and further survived, vascularized, and underwent continued myogenesis *in vivo* [10]. In the current study, we set to define the roles that specific fractions of neonatal rat myogenic cells and dynamic culture conditions play in early myogenesis and function of engineered muscle. We compared a freshly-isolated (FI) cell population with the adherent fraction (AF) of these cells that attached to a Matrigel-coated flask since it has been known that FI cells better retain self-regenerative capacity compared to cultured cells which usually undergo rapid myogenic commitment upon plating [17–19]. We hypothesized that the use of undifferentiated

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FI cells will result in better retention of SCs within engineered muscle, while more committed AF cells will undergo robust early fusion events. Furthermore, we hypothesized that compared to static culture, dynamic culture conditions (not traditionally utilized for engineering of skeletal muscle) would lead to an increase in the survival of muscle cells within the interior of the relatively large muscle constructs due to enhanced transfer of oxygen and nutrients [20,21]. To test these hypotheses, we systematically investigated individual and combined effects of the cell source and dynamic culture on engineered muscle morphology, maintenance of satellite cells, early fusion events, myogenic maturation, and contractile function.

2. Materials and methods

2.1. Myogenic cell preparation

Skeletal muscle tissue was isolated from the lower hind limbs of 2-3-d-old Sprague–Dawley rats and all connective tissue and fat were carefully removed. The tissue was digested in 1 mg/mL collagenase (Worthington) and 2% dispase ((v/v) BD) dissolved in Wyles solution (137 mM NaCl, 5 mM KCl, 21 mM HEPES, 0.7 mM Na2HPO4, 100 mM glucose, and 0.1 mg/mL BSA) for 1 h at 37 °C on a rocker. The isolated cells were resuspended in growth medium (Dulbecco's modified Eagle's medium (DMEM), 10% (v/v) fetal bovine serum, 50 unit/mL penicillin G, 50 ug/mL streptomycin, 5 ug/mL gentamicin) and preplated for 2 h at 37 °C to reduce fraction of fast-adhering fibroblasts. These freshly-isolated cells (*Freshly-Isolated* (FI)) where either used directly to create engineered muscle, or were plated onto Matrigel-coated (1% (v/v), BD) flasks in growth media at ~50,000 cells/cm^2. Plated cells were washed in PBS after 24 h and growth media was replenished. At 36 h following initial plating, the adherent cells (*Adherent Fraction* (AF)) were detached by 2% dispase (v/v, BD) and used for generation of engineered muscle [10]. Non-adherent cells (*Non-adherent Fraction* (NAF)) that did not attach during the initial 24 h of platting were also used to engineer muscle tissues.

2.2. Fabrication of engineered muscle

Engineered muscle bundles were formed within polydimethylsiloxane (PDMS) molds containing semi-cylindrical wells (1.25 cm long, 3 mm diameter) cast from 3D-machined Teflon masters. PDMS molds were coated with 0.2% (w/v) pluronic (P3000MP, Invitrogen) to prevent hydrogel adhesion and two Velcro felts (2 mm × 4 mm) were pinned at ends of the wells to anchor the hydrogel. The cell/hydrogel mixture (10 million cells/mL, 2× growth medium, 4 mg/mL bovine fibrinogen (F8630, Sigma), Matrigel (20% (v/v)), and thrombin (0.2 unit/mg fibrinogen, T4648, Sigma)) was injected into the PDMS wells and polymerized at 37 °C for 45 min before addition of growth medium. The formed bundles were either cultured at static or dynamic (rocked at 24 Hz, −30° to +30° tilt) conditions for 2 weeks. After 4 days of culture, growth medium was replaced by differentiation medium (DMEM, 3% (v/v) horse serum, 50 unit/mL penicillin G, 50 ug/mL streptomycin, 5 ug/mL gentamicin) to promote differentiation of the myogenic cells into myofibers. Degradation of fibrin was inhibited by 1 mg/mL aminocaproic acid (A2504, Sigma) added to culture media. Cell-mediated hydrogel compaction generated passive tension between anchored hydrogel ends resulting in uniaxial cell alignment [13].

2.3. Immunostaining analysis

Engineered muscle bundles were fixed in 2% formaldehyde overnight on a rocker at 4 °C. Samples were treated using a blocking solution (0.5% Triton-X, 5% chicken serum in Ca^{+}/Mg^{+} PBS) overnight on a rocker at 4 °C. Primary antibody solutions (listed in Table S1) at 1:15 – 1:200 dilution were applied in blocking solution overnight on a rocker at 4 °C. Samples were then washed 3 times in 0.1% Triton-X and incubated in secondary antibody (1:200 dilution in blocking solution) with DAPI and/or Alexa Fluor® 488-conjugated phalloidin (Invitrogen) overnight on a rocker at 4 °C. Fluorescence images were acquired on an inverted confocal microscope (Zeiss LSM 510) at 20–40× magnification. For staining of transverse cross-sections, the samples were emerged in optimal cutting temperature (OCT) compound (Electron Microscopy Sciences), snap-frozen in liquid nitrogen, sliced (10–50 μm thick) perpendicular to bundle's long axis, and mounted on glass slides, followed by blocking and application of antibodies. Images were acquired at different magnifications either parallel or perpendicular to the bundle's long axis. For quantitative analyses of nuclear stains, we utilized a custom ImageJ (Fiji) program that identifies areas stained for DAPI, transcription factor (Pax7, MyoD, myogenin), or proliferation marker (Ki67), and, based on the median nucleus size for a given magnification, designates and automatically counts identified nuclei. Nuclear count is then manually verified by user. Myofiber area density in the engineered muscle bundles was quantified from longitudinal confocal sections acquired at 10–40 μm bundle depth and 20× magnification by calculating the percentage of bundle area positively stained for F-actin [10]. These images were also used to manually measure myofiber diameter by LSM Image Browser (Zeiss) [10].

2.4. Assessment of contractile function

Force generating capacity of engineered muscle was assessed at 2 weeks of culture as previously described [10,13]. Engineered muscle bundles were loaded into a custom-made force measurement setup containing a sensitive optical force transducer and a computer-controlled linear actuator (ThorLabs). Samples were stimulated (10 ms, 3 V/mm pulses) and isometric twitch contraction (response to a single pulse) and tetanic contraction (response to a 40 Hz, 1 s duration pulse train) were recorded in bundles that were stretched to 110% of their culture length. Specific contractile force of a muscle bundle was determined by dividing its force of contraction with the cross-sectional muscle area measured at the center of the bundle.

2.5. Statistics

Results are presented as mean ± SEM. Statistically significant effects of culture type (static vs. dynamic) or cell source (AF vs. FI) were evaluated by two-way ANOVA with post hoc Tukey's tests to determine significant differences ($p < 0.05$) among individual groups using GraphPad Prism (GraphPad Software, Inc.). Levels of significance are noted in text, figures, or figure captions.

3. Results

3.1. Effect of dynamic culture and cell source on the engineered muscle morphology

After 2 weeks of culture, engineered muscle bundles made of both freshly-isolated (FI) and adherent fraction (AF) cells contained myofibers that were preferentially located at the bundle periphery (Fig. 1A) and, as previously described [10], were surrounded by an outer layer of vimentin^{+} fibroblasts (Fig. S1A). During 2 week culture, bundles compacted significantly more when cultured dynamically than statically as well as when made of AF vs. FI cells (Fig. 1B). Consistent with this observation, fibroblast density and coverage area at the bundle surface were increased in dynamic culture (Fig. S1) suggestive of increased fibroblast proliferation and/or outward migration. The total F-actin positive muscle area in bundle cross-sections was also higher in dynamic (FI: 0.84 ± 0.11 mm^2, AF: 0.63 ± 0.05 mm^2) than static (FI: 0.23 ± 0.01 mm^2, AF: 0.18 ± 0.01 mm^2) culture and was less affected by the cell source used (FI vs. AF, $p = 0.087$, Fig. 1C). As a result, the fraction of bundle cross-sectional area occupied by myofibers (muscle area fraction, Fig. 1D) was increased 3–4 fold in dynamic (FI: 0.44 ± 0.04, AF: 0.40 ± 0.05) compared to static cultures (FI: 0.066 ± 0.002, AF: 0.089 ± 0.01).

Along with the changes in the total muscle cross-sectional area, we observed that the diameter of myofibers, a marker of muscle maturity, was significantly increased by dynamic culture ($p = 0.0005$) as well as by use of AF cells ($p < 0.0001$). Combination of dynamic culture and AF cells yielded the largest myofiber diameter (dynamic + AF: 15.56 ± 0.65 μm; dynamic + FI: 11.94 ± 0.49 μm; static + AF: 11.14 ± 0.59 μm; static + FI: 9.81 ± 0.36 μm, Fig. 2B). By assuming circular cross-section of individual myofibers, we estimated the average number of myofibers in the bundle cross-section. As shown in Fig. 2C, the average myofiber #/bundle cross-section was higher in dynamic vs. static culture and in FI vs. AF bundles. Overall, the dynamic culture had stimulating effects on both the number and diameter of the formed myofibers, while using AF cells resulted in the formation of a smaller number of thicker myofibers relative to the use of FI cells that yielded a larger number of thinner myofibers.

3.2. Effect of dynamic culture and cell source on myogenesis

To further characterize the effects of dynamic culture and cell source on the myogenesis within engineered muscle, the 2-week bundles were stained for the transcription factor myogenin (MyoG), a marker of myonuclei and muscle differentiation (Fig. 3A). The greater presence of MyoG^{+} nuclei is an indicator of enhanced

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