Published in final edited form as:

Biomaterials. 2014 November ; 35(35): 9438–9446. doi:10.1016/j.biomaterials.2014.07.035.

Roles of Adherent Myogenic Cells and Dynamic Culture in Engineered Muscle Function and Maintenance of Satellite Cells

Mark Juhas¹ and Nenad Bursac^{1,*}

¹Department of Biomedical Engineering, Duke University, Durham, NC

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 hours. 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.

Keywords

Tissue engineering; skeletal muscle; contractile force; fusion; satellite cell

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,

© 2014 Elsevier Ltd. All rights reserved.

*Corresponding author: Nenad Bursac, PhD, Associate Professor of Biomedical Engineering, Faculty of Cardiology, Duke University, 3000 Science Drive, Hudson Hall, Room 136, Durham, NC 27708, phone: 919-660-5510, fax: 919-684-4488, nbursac@duke.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

undergo activation, proliferation, and differentiation to create a pool of myogenic precursors that fuse to form new or repair damaged myofibers; 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 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.

Materials and methods

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 $\sim 50,000$ cells/cm². Plated cells were washed in PBS after 24 hr and growth media was replenished. At 36 hr 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 hr of plating were also used to engineer muscle tissues.

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 (2mm x 4mm) were pinned at ends of the wells to anchor the hydrogel. The cell/hydrogel mixture (10 million cells/mL, 2x growth medium, 4mg/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 $+30^\circ$ 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].

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 20-40x 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 μ m 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 μ m bundle depth and 20x 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].

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, 3V/mm pulses) and isometric twitch contraction (response to a single pulse) and tetanic contraction (response to a 40 Hz, 1 sec 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.

Statistics

Results are presented as mean $\pm$ 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.

Results

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: $0.84 \pm 0.11 \text{ mm}^2$, AF: $0.63 \pm 0.05 \text{ mm}^2$) than static (FI: $0.23 \pm 0.01 \text{ mm}^2$, AF: $0.18 \pm 0.01 \text{ mm}^2$) 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: $15.56 \pm 0.65 \mu\text{m}$; dynamic+FI: $11.94 \pm 0.49 \mu\text{m}$; static+AF: $11.14 \pm 0.59 \mu\text{m}$; static+FI: $9.81 \pm 0.36 \mu\text{m}$, 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 figure 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.

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 fusion events as well as mature and/or hypertrophied myofiber phenotype [22]. As in our previous studies [15], MyoG⁺ nuclei in 2-week engineered muscle bundles were only found within multinucleated myofibers. Quantitative image analysis revealed that fraction of total bundle nuclei that were positive for MyoG⁺ (myonuclei fraction) was significantly larger in AF (static: $57.63 \pm 4.46\%$, dynamic: $61.73 \pm 4.57\%$) than in FI (static: $37.97 \pm 2.47\%$, dynamic: $42.02 \pm 2.26\%$) groups and was not different for static vs. dynamic culture. Furthermore, the amount of MyoG⁺ nuclei per myofiber area (myonuclear density) was also significantly higher in AF (static: 1870 ± 88 cells/mm², dynamic: 1881 ± 100 cells/mm²) than FI (static: 1333 ± 152 cells/mm², dynamic: 1267 ± 101 cells/mm²) groups (Fig. 3B). Simultaneously, the total number of MyoG⁺ nuclei in the bundle cross-section (calculated as the product of myonuclear density and total cross-sectional myofiber area) was increased ~4-fold in dynamic compared to static culture and was comparable between AF and FI groups (Fig. 3C).

Effect of dynamic culture and cell source on retention of Pax7⁺ cells

Since the existence of functional satellite cells (SCs) in skeletal muscle tissue is required for its growth [22] and regeneration [1, 10], we assessed the maintenance of SC pool in 2-week old engineered muscle bundles by quantifying the fraction and density of cells expressing Pax7, a common marker of satellite cells (Fig. 4A). The fraction of all cells expressing Pax7 (Pax7⁺ nuclei/total nuclei) depended on both cell source ($p = 0.045$) and culture ($p = 0.0004$) as well as their interaction ($p = 0.017$), with the greatest SC fraction found in dynamically cultured AF cells ($14.37 \pm 1.14\%$, Fig. 4B). The satellite cell density (Pax7⁺ cells per F-actin⁺ muscle area) was significantly impacted by cell culture ($p = 0.0018$) and the interaction of cell source and culture ($p = 0.013$). Under dynamic conditions, engineered muscle bundles made of AF cells were able to maintain significantly greater SC fraction and density (dynamic+AF: 539 ± 63 cells/mm²; dynamic+FI: 312 ± 29 cells/mm², Fig. 4C) compared to those made of FI cells.

Effect of dynamic culture and cell source on force generation

To assess potential functional consequences of structural differences observed among the 4 bundle groups, we performed contractile force (twitch and tetanus) measurements (Fig. 5A) as previously described [10, 13]. Most notably, dynamic culture conditions (FI: 14.52 ± 1.55

mN (twitch) and 19.75 ± 2.01 mN (tetanus); AF: 17.83 ± 1.00 mN (twitch) and 28.80 ± 0.93 mN (tetanus)) yielded 6-7 fold higher twitch and tetanus force amplitudes compared to static culture conditions (FI: 2.04 ± 0.32 mN (twitch) and 2.95 ± 0.35 mN (tetanus); AF: 3.09 ± 0.26 mN (twitch) and 4.21 ± 0.42 mN (tetanus), Fig. 5B). Smaller but significant increase in generated contractile force also resulted from use of AF vs. FI cells. Consistent with our previous studies [10], the engineered muscle bundles made using AF cells and cultured in dynamic conditions generated tetanic forces nearing 30 mN. The dynamic culture and use of AF cells individually and synergistically increased the specific twitch and tetanus force amplitudes (force per cross-sectional muscle area) with the largest specific force (43.39 ± 3.82 mN/mm²) measured in dynamically cultured bundles made of AF cells (Fig. 5C). Based on the known average number of myofibers per bundle cross-section (Fig. 2C), we estimated the average force of an individual myofiber which was the highest (8.25 ± 0.99 N/fiber, tetanus) for myofibers from dynamically cultured AF group (Fig. 5C). In contrast to active (contractile) force generation, the passive tension generated by bundles was found to be comparable among different groups (Fig. S2A) while specific passive tension varied based on the differences in bundle diameter (Fig. S2B).

Morphological and myogenic changes in dynamically cultured bundles at early culture

While the observed differences in structure and function of statically and dynamically cultured bundles were anticipated based on the expected differences in their oxygen and nutrient transport rates, the origin of structural and functional differences between AF and FI groups required further investigation. We therefore decided to focus only on the dynamic culture and to compare early morphological and myogenic events during the formation of AF and FI bundles (culture days 2-4). By non-invasively tracking the bundle diameter, we observed considerable bundle compaction as early as culture day 2 (Fig. S3A) and found it to be significantly accelerated in AF vs. FI group (Fig. S3B). An important factor in cell-mediated gel compaction is the presence of fibroblasts [23], which, consistent with the changes in bundle diameter, were found to more rapidly form dense outer layer in AF compared to FI bundles (Fig. S4). To assess the time-course of early myogenesis in engineered bundles, we immunostained for key myogenic markers (Pax7, MyoD, and myogenin (MyoG)) and a marker of cell proliferation (Ki67) (Fig. 6A,B). At culture day 2, the Pax7⁺ cell fraction (Fig. 6A, C), a population consisting of quiescent or activated satellite cells, was higher in FI ($30.7 \pm 4.3\%$) than AF bundles ($23.0 \pm 5.6\%$). However, during early time of culture, the SC pool within FI bundles significantly decreased (day 3: $15.1 \pm 2.1\%$; day 4: $10.5 \pm 1.1\%$, Fig 6A, C) while it remained steady in the AF group (day 3: $24.1 \pm 3.4\%$; day 4: $21.5 \pm 2.7\%$, Fig 6A, C). Co-staining for proliferation marker Ki67 revealed a faster decrease in proliferating Pax7⁺ cells in AF vs. FI bundles, however by culture day 4 the vast majority of Pax7⁺ cells became quiescent in both bundle groups (Fig. S6A, C). Furthermore, by culture day 4, these cells were found to closely abut myofiber surface, express M-cadherin, and reside in laminin-rich matrix (Fig. S5), suggestive of early formation of native-like SC niches [24].

Concomitant with the described changes in SC pool, expression of MyoD, a marker of activated satellite cells and committed precursors [1], was high at day 2 in both groups (FI: $42.0 \pm 5.3\%$, AF: $49.7 \pm 5.6\%$) (Fig. 6B, C) and steadily declined thereafter in the FI group

(day 3: $38.7 \pm 3.7\%$, day 4: $32.7 \pm 3.6\%$) while abruptly dropping in the AF group to the levels (day 3: 17.3 ± 1.0 ; day 4: $16.3 \pm 2.5\%$) significantly lower than those of the FI group (Fig. 6B, C). Co-expression of MyoD with MyoG marks more advanced stages of myogenesis including precursors primed for fusion or newly fused myonuclei. This cell population was less prevalent at day 2 in FI bundles ($24.5 \pm 3.4\%$) than AF bundles ($40.1 \pm 2.3\%$) and while it subsequently slightly increased in FI bundles, it abruptly dropped to significantly lower levels in AF bundles (Fig. 6B, C). Simultaneously, expression of myogenin, a marker of mature fusing precursors and already fused myofibers [1], was significantly lower at day 2 in FI ($29.6 \pm 3.9\%$) compared to AF ($50.0 \pm 4.3\%$) bundles (Fig. 6B, C). This MyoG⁺ myonuclei fraction underwent subsequent increase in both bundle groups but remained significantly higher in AF (e.g. day 4: $58.7 \pm 2.3\%$) than FI (day4: $40.8 \pm 4.6\%$) bundles (Fig. 6B, C). Overall, the assessment of myogenic markers revealed the presence of early myogenesis in bundles from both groups with faster differentiation observed for AF group (evident from abrupt decline in MyoD expression and higher MyoG expression).

To further analyze early dynamics of engineered muscle formation, we tracked changes in F-actin⁺ myofiber area between 2 and 4 days of culture (Fig 6A, B, D). In both FI and AF bundles, F-actin⁺ area steadily increased with time of culture, with more rapid myofiber formation resulting from the use of AF than FI cells (Fig. 6D). In addition to accelerated myofiber formation, the AF bundles supported rapid myofiber maturation evident from the appearance of robust cross-striations (Fig. S6).

Structure and function of engineered bundles made of non-adherent cell fraction

In this study, the AF cells represented a subset of FI cells that adhered to Matrigel-coated dish, while the cells that did not adhere (non-adherent fraction (NAF)) were usually discarded. To further understand differences in myogenic potential between AF and FI cells, we assessed the structural and functional properties of dynamically cultured 2-week engineered bundles made of NAF cells (Fig. 7). Compared to AF and FI bundles, NAF bundles had 1.2-fold larger diameter and contained a thicker outer layer of vimentin⁺ fibroblastic cells that were aligned along the long axis of the bundle (Fig. 7A). Furthermore, the NAF bundles exhibited considerably lower numbers and density of differentiated myofibers (Fig. 7B) and no evidence of Pax7⁺ cells. When quantitatively compared to the AF group, NAF bundles contained 2-fold thinner myofibers, 10-fold lower number of MyoG⁺ myonuclei, 2.5-fold lower density of myonuclei per myofiber, and negligible fraction of Pax7⁺ cells. As a result, the twitch and tetanus force of NAF bundles were ~20-fold lower than those measured in AF bundles.

Discussion

In this study we assessed the individual and combined roles of starting cell population and dynamic culture conditions in the satellite cell (SC) maintenance, myogenesis, and contractile function of engineered skeletal muscle. This allowed us to identify an optimized culturing procedure for engineering of relatively large muscle constructs with advanced functional properties and efficient retention of quiescent SCs. Previous reports have shown

that freshly isolated myogenic cells (including SCs) have more robust self-regenerative capacity upon implantation *in vivo* compared to cells that first underwent expansion in standard 2D culture [17-19]. This loss of regenerative capacity *in vitro* stems from disruption of native SC niche that leads to rapid and irreversible differentiation of initially quiescent and self-renewing SCs to committed myoblast progeny [25]. Our recent study has suggested that quiescent and functional SCs can be maintained *in vitro* within niche-like environments formed inside fibrin gel-based 3D engineered muscle [10]. However, freshly isolated (FI) myogenic cell population (expected to contain most intact/quiescent SCs) has not been used in that study. While the FI cells would be expected to optimally enhance the numbers of quiescent SCs in 3D engineered muscle, they may also contain significant fraction of non-myogenic cells that could impede robust myogenic process. We therefore compared FI with short-term (36 hr) cultured adherent fraction (AF) cells (containing a high percent of activated SCs [10]) for their abilities to yield highly functional engineered muscle and efficient formation of quiescent SC pool. Furthermore, dynamic culture, including use of spinner flasks, rotating wall vessels, shakers, and custom bioreactor systems, has been previously utilized to increase mass transport in various engineered tissues [20, 21], but has not been traditionally applied to engineering of functional skeletal muscle. Dynamic culture would be expected to improve survival and spatial distribution of metabolically active muscle cells within the engineered constructs [26] and potentially affect their behavior through introduction of shear forces on the construct surface [27, 28]. We therefore compared engineered muscles exposed to mild hydrodynamic conditions in rocking culture with those grown in traditional static culture.

In all studied engineered muscle bundles, we observed rapid formation of a fibroblast layer at the bundle surface that resembled fibroblast distribution present in native muscle fascicles [29]. In general, fibroblast accumulation at the bundle surface could be a result of cell durotaxis towards the tissue region with higher stiffness [30], migration towards more favorable oxygen and nutrient concentration, and/or enhanced cell proliferation. Given the reported effects of shear stress on enhancing fibroblast migration [31] and proliferation [32], higher fibroblast density after 2 weeks of dynamic vs. static culture could be expected. Contraction of fibroblasts and secretion of proteolytic enzymes have been previously shown to contribute to hydrogel remodeling and compaction [23, 33] and thus increased compaction of dynamically cultured bundles at 2 weeks of culture could be at least in part attributed to their higher fibroblast density. Interestingly, we have also noticed a more rapid accumulation of fibroblasts at the periphery of AF bundles (starting as early as culture day 2) along with their accelerated initial compaction and smaller diameter at 2 weeks compared to those of FI bundles (Fig 1, S1). While 2D culture could have increased initial fibroblast content of AF cells, potentially different types of non-myogenic cells and specific kinetics of early myogenesis in AF vs. FI cells may have all contributed to the observed differences in bundle compaction between the two groups. Consistent with this reasoning, bundles made of non-adherent cells (a subset of FI but not AF cells) underwent significantly less compaction and myogenesis compared to AF bundles (Fig. 7).

In addition to increased bundle compaction, dynamic culture resulted in a significant increase in viable muscle cross-sectional area and number of myofibers compared to static

culture which, consistent with prior reports in relatively large engineered muscles [13, 34], yielded only a thin layer of musculature at the bundle exterior (Fig. 1). Interestingly, the use of FI compared to AF cells also tended to generate higher number of myofibers and larger muscle area. Increased muscle area in dynamically cultured bundles yielded higher absolute twitch and tetanic forces as more myofibers in parallel contributed to contraction. In addition, dynamic culture significantly increased specific contractile force, a parameter that characterizes the differentiation state of myofibers, regardless of their size and number (Fig. 5). Despite lower myofiber number and muscle area in cross-section, the AF bundles generated significantly higher absolute, specific, and per-myofiber contractile forces compared to the FI bundles, thus revealing a beneficial effect of initial short-term 2D culture of myogenic cells on the maturation and contractility of 3D formed myofibers. Additively, dynamic culture and the use of AF cells for engineering of muscle bundles resulted in absolute contractile forces approaching 30 mN and specific forces comparable to those of native neonatal muscle [35].

A key indicator of muscle differentiation during early development is the myofiber hypertrophy that is usually associated with increased myonuclear density, nutrient uptake, protein synthesis, and activation of specific growth pathways [22, 36, 37]. In this study, both dynamic culture and the use of AF cells increased myofiber size (diameter) after 2 weeks of culture (Fig. 2), while only AF cells yielded significant increase in myonuclear density suggestive of increased fusion efficiency of pre-cultured compared to freshly isolated cells (Fig. 3). Taken together, it could be postulated that the observed stimulating effects of dynamic culture on myofiber hypertrophy stemmed from enhanced transport of oxygen and nutrients, previously shown to support cell survival and hypertrophy [38]. Further, as shear stress is known to enhance insulin-like growth factor 1 (IGF-1) signaling in various cell types [39, 40], dynamic culture could also stimulate common myogenic hypertrophic pathways downstream of IGF-1 [36, 37]. Additional increase in nuclear density of hypertrophied myofibers in AF bundles may be a result of enhanced expression of adhesion proteins in AF cells during 2D culture, which made these cells more primed for fusion compared to FI cells.

We recently demonstrated the importance of maintaining Pax7⁺ SCs within engineered muscle as they can contribute muscle regeneration following injury [10]. Since use of FI cells avoids potential myogenic differentiation during 2D culture, we expected to observe greater SC density in 2-week bundles made of FI compared to AF cells. Surprisingly, after 2 weeks of dynamic culture, engineered muscle bundles made of AF cells retained significantly more Pax7⁺ cells compared to those made of FI cells (Fig. 4). From assessment of early fusion events, we found that, as expected, FI bundles contained a greater percentage of Pax7⁺ cells at culture day 2 compared to AF bundles (Fig. 6A, C). However, by culture day 4, the Pax7⁺ fraction in FI bundles was rapidly reduced, while it remained steady and twice as abundant in AF bundles, a trend that persisted to 2 weeks of culture. Furthermore, as AF cells underwent short-term differentiation in 2D culture, they expectedly contained a greater population of MyoD⁺ and MyoG⁺ cells within 2-day bundles. However, by day 3, the AF cells underwent accelerated differentiation evidenced by a significant drop in MyoD⁺ cell density as compared to FI cells which experienced a slower transition from MyoD⁺ to MyoG⁺ population (Fig 6C). Overall, the AF bundles supported a more rapid formation and

structural maturation of myofibers in early culture, a process that may have been further enhanced by their accelerated compaction.

Our observations also suggest that efficient retention of quiescent SC pool in engineered muscle may rely on its ability to support the rapid formation of differentiated muscle fibers, a requisite part of the native SC niche [24]. Key adhesion proteins associated with the SC niche *in vivo* are $\alpha 7 \beta 1$ integrin, which enables attachment of the satellite cells to laminin [24], and M-cadherin, which is the link between the satellite cells and myofibers [24, 41]. Even though FI cells provided a greater initial percentage of Pax7⁺ cells, the AF cells consisted of activated myogenic population conducive to rapid formation of native-like SC niches within engineered muscle bundles. Specifically, short time culture on laminin-containing Matrigel may have enhanced the $\alpha 7 \beta 1$ integrin expression of AF cells in addition to increasing the fraction of mature MyoD⁺ and MyoG⁺ precursors capable of rapid fusion. Following gentle disassociation with dispase in attempt to preserve cell surface proteins, the AF cells within engineered bundles quickly formed multi-nucleated myotubes, known to express M-cadherin in developing muscle [42, 43]. Rapid myofiber formation and bundle compaction provided favorable conditions for undifferentiated Pax7⁺ cells to dock to myofiber surface and by culture day 4 form niche-like environment rich in laminin and M-cadherin expression (Fig. S5). Less myogenic FI cells, on the other hand, experienced a delay in myofiber formation, resulting in enhanced differentiation and loss of quiescence of SCs caused by the lack of homing locations [44].

5. Conclusions

Our study revealed significant and synergistic benefits of the use of an adherent myogenic cell fraction and dynamic culture conditions toward the development of engineered skeletal muscle capable of replicating the cellular heterogeneity, structure, and contractile function of natural muscle tissues. Importantly, our results suggest that differentiated myofibers, as the requisite constituents of natural SC niche, have critical roles in the creation and maintenance of robust satellite cell pool in engineered muscle *in vitro*. The methods and findings described in this study can be applied to create more accurate models of skeletal muscle tissue for use in studies of muscle function and repair and potential regenerative therapies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We acknowledge the Duke University Light Microscopy Core Facility for the use of their equipment as well as R. Kirkton, W. Bian, S. Hinds, L. Madden, and E. Krol for their technical support. This study was supported by the National Science Foundation's Graduate Research Fellowship to M. Juhas and grant AR055226 from National Institute of Arthritis and Musculoskeletal and Skin Diseases to N. Bursac.

References

- [1]. Charge SB, Rudnicki MA. Cellular and molecular regulation of muscle regeneration. *Physiol Rev.* 2004; 84:209–38. [PubMed: 14715915]
- [2]. Turner NJ, Badylak SF. Regeneration of skeletal muscle. *Cell Tissue Res.* 2012; 347:759–74. [PubMed: 21667167]
- [3]. Juhas M, Bursac N. Engineering skeletal muscle repair. *Curr Opin Biotechnol.* 2013; 24:880–6. [PubMed: 23711735]
- [4]. Cheng CS, Davis BN, Madden L, Bursac N, Truskey GA. Physiology and metabolism of tissue-engineered skeletal muscle. *Exp Biol Med* (Maywood). 2014
- [5]. Shansky J, Del Tatto M, Chromiak J, Vandeburgh H. A simplified method for tissue engineering skeletal muscle organoids in vitro. *In Vitro Cell Dev Biol Anim.* 1997; 33:659–61. [PubMed: 9358276]
- [6]. Dennis RG, Kosnik PE 2nd. Excitability and isometric contractile properties of mammalian skeletal muscle constructs engineered in vitro. *In Vitro Cell Dev Biol Anim.* 2000; 36:327–35. [PubMed: 10937836]
- [7]. Levenberg S, Rouwkema J, Macdonald M, Garfein ES, Kohane DS, Darland DC, et al. Engineering vascularized skeletal muscle tissue. *Nat Biotechnol.* 2005; 23:879–84. [PubMed: 15965465]
- [8]. Koffler J, Kaufman-Francis K, Shandalov Y, Egozi D, Pavlov DA, Landesberg A, et al. Improved vascular organization enhances functional integration of engineered skeletal muscle grafts. *Proc Natl Acad Sci U S A.* 2011; 108:14789–94. [PubMed: 21878567]
- [9]. Corona BT, Machingal MA, Criswell T, Vadavkar M, Dannahower AC, Bergman C, et al. Further development of a tissue engineered muscle repair construct in vitro for enhanced functional recovery following implantation in vivo in a murine model of volumetric muscle loss injury. *Tissue Eng Part A.* 2012; 18:1213–28. [PubMed: 22439962]
- [10]. Juhas M, Engelmayer GC Jr, Fontanella AN, Palmer GM, Bursac N. Biomimetic engineered muscle with capacity for vascular integration and functional maturation in vivo. *Proc Natl Acad Sci U S A.* 2014; 111:5508–13. [PubMed: 24706792]
- [11]. Vandeburgh H. High-content drug screening with engineered musculoskeletal tissues. *Tissue Eng Part B Rev.* 2010; 16:55–64. [PubMed: 19728786]
- [12]. Truskey GA, Achneck HE, Bursac N, Chan H, Cheng CS, Fernandez C, et al. Design considerations for an integrated microphysiological muscle tissue for drug and tissue toxicity testing. *Stem Cell Res Ther.* 2013; 4(Suppl 1):S10. [PubMed: 24565225]
- [13]. Hinds S, Bian W, Dennis RG, Bursac N. The role of extracellular matrix composition in structure and function of bioengineered skeletal muscle. *Biomaterials.* 2011; 32:3575–83. [PubMed: 21324402]
- [14]. Bian W, Bursac N. Engineered skeletal muscle tissue networks with controllable architecture. *Biomaterials.* 2009; 30:1401–12. [PubMed: 19070360]
- [15]. Bian W, Juhas M, Pfeiler TW, Bursac N. Local tissue geometry determines contractile force generation of engineered muscle networks. *Tissue Eng Part A.* 2012; 18:957–67. [PubMed: 22115339]
- [16]. Bian W, Bursac N. Soluble miniagrin enhances contractile function of engineered skeletal muscle. *FASEB J.* 2012; 26:955–65. [PubMed: 22075647]
- [17]. Montarras D, Morgan J, Collins C, Relaix F, Zaffran S, Cumano A, et al. Direct isolation of satellite cells for skeletal muscle regeneration. *Science.* 2005; 309:2064–7. [PubMed: 16141372]
- [18]. Rossi CA, Flaibani M, Blaauw B, Pozzobon M, Figallo E, Reggiani C, et al. In vivo tissue engineering of functional skeletal muscle by freshly isolated satellite cells embedded in a photopolymerizable hydrogel. *FASEB J.* 2011; 25:2296–304. [PubMed: 21450908]
- [19]. Sacco A, Doyonnas R, Kraft P, Vitorovic S, Blau HM. Self-renewal and expansion of single transplanted muscle stem cells. *Nature.* 2008; 456:502–6. [PubMed: 18806774]
- [20]. Freed LE, Guilak F, Guo XE, Gray ML, Tranquillo R, Holmes JW, et al. Advanced tools for tissue engineering: scaffolds, bioreactors, and signaling. *Tissue Eng.* 2006; 12:3285–305. [PubMed: 17518670]

- [21]. Martin I, Wendt D, Heberer M. The role of bioreactors in tissue engineering. *Trends Biotechnol.* 2004; 22:80–6. [PubMed: 14757042]
- [22]. Davis TA, Fiorotto ML. Regulation of muscle growth in neonates. *Current Opinion in Clinical Nutrition and Metabolic Care.* 2009; 12:78–85. [PubMed: 19057192]
- [23]. De Jesus AM, Sander EA. Observing and quantifying fibroblast-mediated fibrin gel compaction. *J Vis Exp.* 2014:e50918. [PubMed: 24458198]
- [24]. Kuang S, Gillespie MA, Rudnicki MA. Niche regulation of muscle satellite cell self-renewal and differentiation. *Cell Stem Cell.* 2008; 2:22–31. [PubMed: 18371418]
- [25]. Radisic M, Euloth M, Yang L, Langer R, Freed LE, Vunjak-Novakovic G. High-density seeding of myocyte cells for cardiac tissue engineering. *Biotechnol Bioeng.* 2003; 82:403–14. [PubMed: 12632397]
- [26]. Stoltz JF, Dumas D, Wang X, Payan E, Mainard D, Paulus F, et al. Influence of mechanical forces on cells and tissues. *Biorheology.* 2000; 37:3–14. [PubMed: 10912174]
- [27]. Yeatts AB, Choquette DT, Fisher JP. Bioreactors to influence stem cell fate: augmentation of mesenchymal stem cell signaling pathways via dynamic culture systems. *Biochim Biophys Acta.* 2013; 1830:2470–80. [PubMed: 22705676]
- [28]. Borg TK, Caulfield JB. Morphology of Connective-Tissue in Skeletal-Muscle. *Tissue & Cell.* 1980; 12:197–207. [PubMed: 7361300]
- [29]. Lo CM, Wang HB, Dembo M, Wang YL. Cell movement is guided by the rigidity of the substrate. *Biophys J.* 2000; 79:144–52. [PubMed: 10866943]
- [30]. Song S, Han H, Ko UH, Kim J, Shin JH. Collaborative effects of electric field and fluid shear stress on fibroblast migration. *Lab Chip.* 2013; 13:1602–11. [PubMed: 23450300]
- [31]. Park JY, Yoo SJ, Patel L, Lee SH, Lee SH. Cell morphological response to low shear stress in a two-dimensional culture microsystem with magnitudes comparable to interstitial shear stress. *Biorheology.* 2010; 47:165–78. [PubMed: 21084742]
- [32]. Duong H, Wu B, Tawil B. Modulation of 3D Fibrin Matrix Stiffness by Intrinsic Fibrinogen-Thrombin Compositions and by Extrinsic Cellular Activity. *Tissue Engineering Part A.* 2009; 15:1865–76. [PubMed: 19309239]
- [33]. Rhim C, Lowell DA, Reedy MC, Slentz DH, Zhang SJ, Kraus WE, et al. Morphology and ultrastructure of differentiating three-dimensional mammalian skeletal muscle in a collagen gel. *Muscle Nerve.* 2007; 36:71–80. [PubMed: 17455272]
- [34]. Close R. Dynamic Properties of Fast + Slow Skeletal Muscles of Rat during Development. *Journal of Physiology-London.* 1964; 173:74. &
- [35]. Glass DJ. Signalling pathways that mediate skeletal muscle hypertrophy and atrophy. *Nat Cell Biol.* 2003; 5:87–90. [PubMed: 12563267]
- [36]. Brooks NE, Myburgh KH. Skeletal muscle wasting with disuse atrophy is multi-dimensional: the response and interaction of myonuclei, satellite cells and signaling pathways. *Front Physiol.* 2014; 5:99. [PubMed: 24672488]
- [37]. Rennie MJ, Edwards RH, Halliday D, Matthews DE, Wolman SL, Millward DJ. Muscle protein synthesis measured by stable isotope techniques in man: the effects of feeding and fasting. *Clin Sci (Lond).* 1982; 63:519–23. [PubMed: 6181926]
- [38]. Triplett JW, O'Riley R, Tekulve K, Norvell SM, Pavalko FM. Mechanical loading by fluid shear stress enhances IGF-1 receptor signaling in osteoblasts in a PKCzeta-dependent manner. *Mol Cell Biomech.* 2007; 4:13–25. [PubMed: 17879768]
- [39]. Passerini AG, Milsted A, Rittgers SE. Shear stress magnitude and directionality modulate growth factor gene expression in preconditioned vascular endothelial cells. *J Vasc Surg.* 2003; 37:182–90. [PubMed: 12514598]
- [40]. Cornelison DD, Wold BJ. Single-cell analysis of regulatory gene expression in quiescent and activated mouse skeletal muscle satellite cells. *Dev Biol.* 1997; 191:270–83. [PubMed: 9398440]
- [41]. Moore R, Walsh FS. The cell adhesion molecule M-cadherin is specifically expressed in developing and regenerating, but not denervated skeletal muscle. *Development.* 1993; 117:1409–20. [PubMed: 8404540]
- [42]. Pouliot Y, Gravel M, Holland PC. Developmental regulation of M-cadherin in the terminal differentiation of skeletal myoblasts. *Dev Dyn.* 1994; 200:305–12. [PubMed: 7994077]

- [43]. Brohl D, Vasyutina E, Czajkowski MT, Griger J, Rassek C, Rahn HP, et al. Colonization of the satellite cell niche by skeletal muscle progenitor cells depends on Notch signals. *Dev Cell*. 2012; 23:469–81. [PubMed: 22940113]

Fig. 1. Morphometric analysis of engineered bundle cross-sections

A) Representative transverse cross-sections of 2-week muscle bundles made from freshly-isolated (FI) or adherent fraction (AF) cells cultured in static or dynamic (Dyn) conditions. F-actin⁺ myofibers are preferentially located at the bundle periphery. B-D) Quantification of (B) total bundle area, (C) muscle area (F-actin⁺ area), and (D) muscle area fraction (muscle area per total area) from transverse cross-sections. *, $p < 0.05$; **, $p < 0.001$ ($n = 6-9$ bundles per group).

Fig. 2. Assessment of myofiber diameter and number

A) Representative confocal images of myofibers in 2-week engineered muscle bundles made from freshly-isolated (FI) or adherent fraction (AF) cells cultured in static or dynamic (Dyn) conditions. B-C) Quantification of myofiber diameter and (C) total number of myofibers per bundle cross-section. *, $p < 0.05$; **, $p < 0.001$ ($n = 4-6$ bundles per group, 3-5 locations per bundle, 5-20 myofibers per location).

Fig. 3. Characterization of myonuclei in engineered muscle bundles

A) Representative confocal images within the myofiber region in 2-week bundles made from freshly-isolated (FI) or adherent fraction (AF) cells cultured in static or dynamic (Dyn) conditions and stained for myonuclei marker myogenin (MyoG). B-C) Quantification of (B) MyoG⁺ myonuclei density (per muscle area) and (C) total number of MyoG⁺ myonuclei within a cross-section. *, $p < 0.05$; **, $p < 0.001$ ($n = 4-6$ bundles per group, 3-5 locations per bundle).

Fig. 4. Maintenance of satellite cell pool in engineered muscle bundles

A) Representative confocal images within the myofiber region in 2-week bundles made from freshly-isolated (FI) and adherent fraction (AF) cells cultured in static and dynamic (Dyn) conditions stained for satellite marker Pax7. B-C) Quantification of (B) Pax7⁺ cell fraction (in %) and (C) density (per muscle area). *, $p < 0.05$; **, $p < 0.001$ ($n = 4-6$ bundles per group, 3-5 locations per bundle).

Fig. 5. Contractile force generation of engineered muscle bundles

A) Representative contractile force traces of 2-week muscle bundles made from freshly-isolated (FI) or adherent fraction (AF) cells cultured in static or dynamic (Dyn) conditions. Twitch contraction was induced by a single 10 ms electrical pulse, while tetanus was induced by a 40Hz pulse train. B-C) Quantification of (B) absolute and (C) specific force (force per cross-sectional muscle area). Quantification of maximum (tetanic) force per myofiber using mean myofiber diameter for each group. *, $p < 0.05$; **, $p < 0.001$ ($n = 6-9$ bundles per group).

Fig. 6. Early myogenesis within dynamically cultured engineered muscle bundles

A-B) Representative confocal images within the myofiber region in bundles made from freshly-isolated (FI) and adherent fraction (AF) cells cultured dynamically and stained for (A) SC marker (Pax7) and proliferation marker (Ki67), and (B) myogenic markers (MyoD, and MyoG) at culture days 2, 3, and 4. C) Quantification of cell fractions (relative to total DAPI labeled nuclei) positive for Pax7, Pax7&Ki67, MyoD, MyoG, and MyoD&MyoG ($n = 5$ bundles per group, 4-8 locations per bundle). D) F-actin⁺ myofiber area fraction at culture days 2, 3, and 4. *, $p < 0.05$; **, $p < 0.001$. ($n = 10$ bundles per group, 4-8 locations per bundle).

Fig. 7. Characterization of dynamically cultured engineered muscle bundles made from non-adherent cells

(A) Representative confocal images of 2-week bundles made of non-adherent cell fraction and stained for Vimentin (Vim, non-muscle cells) and F-actin (myofibers) at different bundle depths (0 μm, bundle periphery). B-C) Representative confocal images within the myofiber region stained for (B) Myogenin (MyoG)⁺ myonuclei, (C) Pax7⁺ satellite cells, and F-actin⁺ myofibers. D) Structural and functional parameters of dynamically cultured bundles made from non-adherent cells shown relative to those of dynamically cultured bundles made from AF cells. *, $p < 0.05$; **, $p < 0.001$ compared to AF group ($n = 3$ bundles, 3-5 locations per bundle).