Methods xxx (2015) xxx–xxx

Contents lists available at ScienceDirect

Methods

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

Design, evaluation, and application of engineered skeletal muscle

Mark Juhas, Jean Ye, Nenad Bursac *

Department of Biomedical Engineering, Duke University, Durham, NC, United States

ARTICLE INFO

Article history:
Received 4 May 2015
Received in revised form 3 September 2015
Accepted 4 October 2015
Available online xxxx

Keywords:
Tissue engineering
Skeletal muscle
Satellite cells
Animal implantation

ABSTRACT

For over two decades, research groups have been developing methods to engineer three-dimensional skeletal muscle tissues. These tissues hold great promise for use in disease modeling and pre-clinical drug development, and have potential to serve as therapeutic grafts for functional muscle repair. Recent advances in the field have resulted in the engineering of regenerative muscle constructs capable of survival, vascularization, and functional maturation *in vivo* as well as the first-time creation of functional human engineered muscles for screening of therapeutics *in vitro*. In this review, we will discuss the methodologies that have progressed work in the muscle tissue engineering field to its current state. The emphasis will be placed on the existing procedures to generate myogenic cell sources and form highly functional muscle tissues *in vitro*, techniques to monitor and evaluate muscle maturation and performance *in vitro* and *in vivo*, and surgical strategies to both create diseased environments and ensure implant survival and rapid integration into the host. Finally, we will suggest the most promising methodologies that will enable continued progress in the field.

1. Introduction

Skeletal muscle is a highly complex organ with robust contractile and regenerative capacity contributed to a variety of cell types and intra- and extracellular mechanisms. Muscle contractions, the source of voluntary movement of our body, are produced by myofibers; aligned, multi-nucleated cells with registered sarcomeric structures powering force generation. The myofibers are embedded in a specific extracellular matrix (ECM), metabolically supported by a dense capillary network, and temporally coordinated by specialized neuronal inputs. A unique feature of the muscle tissue is its ability to regenerate in response to acute injuries over the course of a lifetime [1,2]. Responsible for this feature are the resident muscle stem cells, or satellite cells (SCs). Upon injury, SCs will activate, proliferate, and differentiate to form new, or repair damaged, muscle. After completion of the repair process, SCs will reestablish their numbers and quiescent state by homing back to highly specialized niches, thus allowing future rounds of regeneration [1].

Although a robust trait in healthy conditions, the self-repair capacity of skeletal muscle can be seriously impaired in different types of congenital myopathies or large muscle loss following trauma [3,4]. Furthermore, myofiber atrophy and reduced functionality and regenerative capacity are inevitable consequences of aging [5]. Over the years, extensive research on skeletal muscle biology and regeneration has been carried out to develop cellular and genetic therapies aimed to recover or rejuvenate damaged, diseased, or aged muscle. One solution that combines engineering and biological concepts is the construction of whole muscle tissues *in vitro* or *in vivo*. The ability to recreate the cellular complexity, function, and regenerative properties of muscle offer potential for on-site tissue reconstruction as well as the development of physiologically accurate *in vitro* models of human muscle disorders.

Over the last 25 years, researchers in the field have developed a variety of methods for engineering of functional three-dimensional (3D) skeletal muscle tissues. In particular, recent reports have demonstrated construction of rodent muscle tissues that recreate the functional [6–8] and regenerative [6] properties of native muscle *in vitro* and survive and contribute to contractile muscle function *in vivo* [9–12]. Most recently, Madden et al., reported engineering of the first functional human muscle tissues able to contract in response to electrical and chemical stimuli [13]. Here we will review procedures used by multiple groups in the field of skeletal muscle tissue engineering that involve generation of a myogenic cell pool, fabrication of 3D tissue constructs, structural and functional characterization *in vitro*, and implantation of tissue grafts *in vivo*.

* Corresponding author at: Faculty of Cardiology, Duke University, 3000 Science Drive, Hudson Hall, Room 136, Durham, NC 27708, United States.
E-mail address: nbursac@duke.edu (N. Bursac).

http://dx.doi.org/10.1016/j.ymeth.2015.10.002
1046-2023/

2. Preparation of myogenic cell source

In vitro generation of tissues for use as therapeutic implants or screening models requires a large numbers of cells. Considering the myonuclei within myofibers are post-mitotic, the only suitable source of expandable primary myogenic cells are the satellite cells (SCs), a putative muscle stem cell source capable of myogenesis [1]. Myogenesis, the process of forming new muscle, begins with activation of the quiescent SCs, marked by transcription factor Pax7, and the ensuing expression of myogenic regulatory factor MyoD. Activated SCs will proliferate and either commit to a myogenic fate yielding a population of precursor cells termed myoblasts or self-renew and return to the quiescent state. Myoblasts will continue to proliferate and later differentiate, losing Pax7 and gaining expression of myogenin, a mature muscle marker found in myonuclei. At this point, the cells will either undergo primary fusion, in which they will generate new multi-nucleated myofibers, or secondary fusion, in which they will fuse to existing, damaged ones [1]. Unfortunately, the percentage of SCs in adult muscle is relatively low, amounting to roughly 4% of the total muscle nuclei [14]. Therefore, the common paradigm in muscle tissue engineering is to isolate the resident muscle stem cells, stimulate their continuous proliferation while maintaining their purity and differentiation potential, and utilize them in engineered tissue formation. In this section we will review various methods to generate large quantities of myogenic cells for use in skeletal muscle tissue engineering (reviewed in Fig. 1).

2.1. Satellite cell isolation

Isolation of SCs from skeletal muscle has been achieved via two main routes, cellular outgrowth and whole tissue digestion. The outgrowth method involves gentle separation of myofibers from the extracellular matrix, physical trituration to separate individual muscle fibers, and subsequent seeding of the fibers onto protein-coated dishes. This method relies on maintaining SCs within their niche on the myofiber periphery and their activation following plating. SCs will initiate the process of myogenesis and 'outgrow' from the myofiber onto the dish. Depending on the micro-environment, the cells will continue to proliferate and commit down the myogenic pathway, yielding a population of myoblasts capable of generating myofibers [15]. Alternatively, whole muscle tissue can be extensively minced and fully digested to release the SCs from their home on the myofiber [16]. The digested solution can be strained to filter out the mixture of fragmented myofibers, resulting in a single cell suspension that can be plated and expanded.

A number of methods may be used to purify the myogenic, and specifically SC, population following extraction. Pre-plating is a simple strategy used to remove the population of fast-adhering cells, primarily fibroblasts, from the culture. Myogenic cells are re-suspended in a growth media following digestion and plated on non-coated tissue culture plastic; a surface in which fibroblasts, but not SCs, will attach [17]. After roughly 2 h, the supernatant, consisting of a purified cell mixture, can be transferred to a different flask to encourage myogenic cell attachment [6,7]. Myogenic cells with a higher SC fraction can be obtained using fluorescence-activated cell sorting (FACS) with antibodies against the extracellular domains of SC-specific membrane proteins [18], including SM/C-2.6 [19,20], α7-integrin [21,22], β1-integrin [21], CD34 [22,23], CXC chemokine receptor 4 (CXCR4) [21], neural cell adhesion molecule (NCAM/CD56) [24], Syndecan-3 and -4 [25], and vascular cell adhesion protein 1 (VCAM-1) [26]. For further purification, negative markers have also been identified to remove non-myogenic cells, including CD31 [19,20,22], CD45 [21–23], CD11b [22], Sca-1 [19–21,23], and Mac-1 [21]. Multiple antibodies can be used to sort for a distinct combination of positive and negative markers to ensure a proliferative muscle stem cell pool. For example, the population of $CD45^-/CD31^-/CD11b^-/Sca1^-/CD34^+/integrin\text{-}\alpha7^+$ cells from mouse muscle purified through two rounds of FACS was shown to contain 100% pure $Pax7^+/Myf5^+$ SCs that were capable of self-renewal and expansion following transplantation [22]. Similarly, in a recent report [27], $Pax7^+$ SCs were enriched from human fetal and adult muscle isolates based on positive and negative expression of the aforementioned marker proteins.

2.2. Myoblast cell culture

As commonly acknowledged [22,28], traditional passaging of isolated SCs results in their rapid differentiation towards a myoblast phenotype. Myoblasts, typically expressing transcription factor MyoD, are at a proliferating state and, under particular

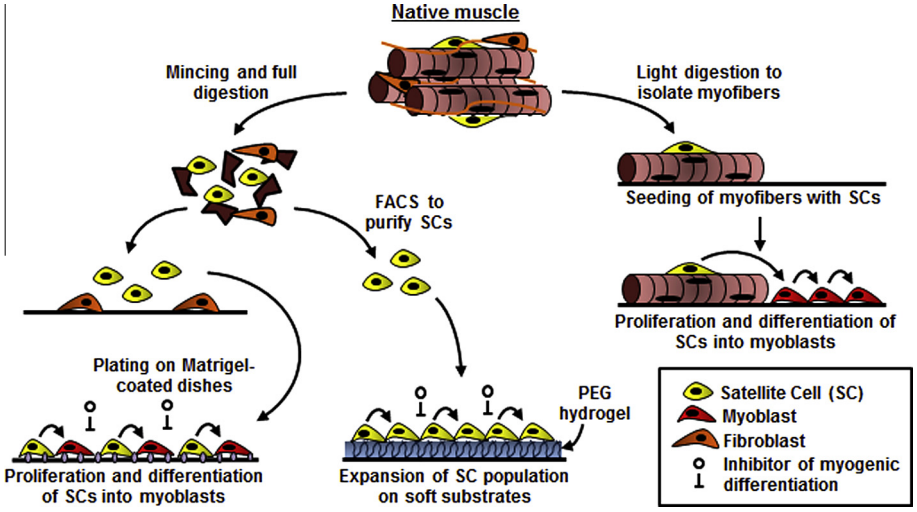

Fig. 1. Methods to isolate and expand myogenic cells. Starting from whole native muscle, the tissue can be lightly enzymatically digested (right) to isolate and plate intact myofibers from which satellite cells (SCs) will outgrow, proliferate, and differentiate into myoblasts. Alternatively (left), native muscle can be fully digested to yield a suspension of mononuclear cells. These cells can be FACS sorted for extracellular markers or preplated to purify the SC population. The purified SCs can be either plated on Matrigel-coated dishes or on a soft, laminin-coated hydrogel substrate. Supplementation of culture media with differentiation inhibitors prevents cell fusion and allows SC expansion with preserved regenerative capacity.

Please cite this article in press as: M. Juhas et al., Methods (2015), http://dx.doi.org/10.1016/j.ymeth.2015.10.002