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The Role of Physical Microenvironmental Cues on Myogenesis: Implications for Tissue Engineering of Skeletal Muscle

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Abstract

Skeletal muscle tissue engineering holds promise for the treatment of a variety of degenerative and traumatic muscle conditions. The ultimate goal of skeletal muscle tissue engineering is very challenging, since it needs to closely reproduce a highly complex structure consisting of muscle cells, connective tissue, blood vessels, and nerves. One of the fundamental prerequisites is to obtain cells arranged in parallel to efficiently produce force by contraction. Several studies have demonstrated that structural cues, such as topography and stiffness of the extracellular microenvironment, are crucial to control the organization and behavior of the cells. In addition to these passive signals, dynamic electrical and mechanical signals are also important for the assembly and maturation of skeletal muscle cells. The aim of this review is to present the current *in vitro* approaches used to investigate the influence of physical microenvironmental cues in skeletal myogenesis. These experimental approaches have been very valuable to answer fundamental biological questions and may help developing novel platforms for the fabrication of tissue-engineered muscle.

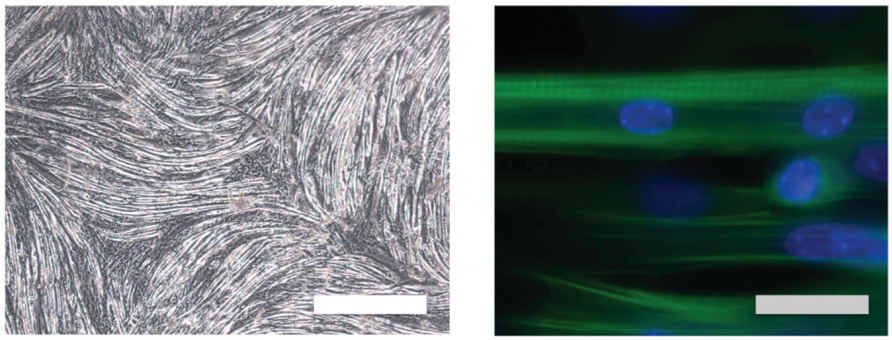
Keywords: Skeletal muscle, Tissue engineering, Microtopography, Skeletal myogenesis, Stiffness, Cyclic tensile strain, Uniaxial strain.

5.1 Introduction

Skeletal muscle comprises up to 40% of the adult human body weight, representing the largest tissue class in the body. Proper muscle function is fundamental for carrying out the voluntary movements of everyday life activities and for maintaining the metabolic homeostasis of the body [1]. Although adult muscle tissue possesses an exceptional capacity for regeneration, restoration to the original state is not possible in the case of large tissue losses. Thus, loss of functional skeletal muscle due to traumatic or degenerative conditions often results in deficits with poor treatment options [2]. Currently, tissue transplantation represents the only viable therapeutic alternative, which is associated with significant donor site morbidity [3]. *Ex vivo* engineered muscles, consisting of scaffolds containing differentiated muscle progenitor cells, may represent a viable alternative to replace or regenerate the damaged tissue. Although significant advances have been achieved in recent years, several practical challenges still remain. One major hurdle consists in procuring the appropriate amount of muscle progenitor cells [4]. Another important requirement is to establish the optimal conditions for cell proliferation, maturation, and assembly of the skeletal muscle fibers [5]. Finally, clinically relevant amounts of tissue will require means to provide for vascularization and innervation [6, 7]. In this chapter, we will focus on current approaches to efficiently organize and differentiate skeletal muscle fibers, which represents the primary step toward development of fully functional skeletal muscle tissue.

5.2 *In Vitro* Models of Adult Myogenesis

Adult skeletal muscle regeneration relies on a population of resident mononucleated stem cells known as the satellite cells. Quiescent satellite cells are located immediately under the basal lamina of myofibers and are readily activated by muscle damage. By undergoing asymmetric divisions, they contribute in the replenishment of the satellite cell compartment and formation of new, functional myofibers [8, 9]. Most of the current knowledge concerning skeletal muscle regeneration has been obtained through highly controllable and well-defined *in vitro* models, designed to investigate the role of microenvironmental cues in myogenic cell proliferation and differentiation. Although satellite cells isolated from healthy or diseased muscle tissue have been employed,



(a) (b)

Figure 5.1 Skeletal muscle myotubes obtained from the C2C12 cell line. (a) Phase contrast microscopy image displaying the arbitrary orientation of the myotubes. Scale bar denotes 300 μm. (b) Fluorescence microscopy image showing myofibrils in mature myotubes. The actin filaments are stained by phalloidin (green) and nuclei counterstained by Hoechst (blue). Scale bar denotes 50 μm.

primary myogenic cultures possess various limitations, including cellular heterogeneity and low replicative capacity [10]. Therefore, the most widely used cell line for *in vitro* studies has been the immortalized mouse myoblast cell line C2C12. These cells display a large proliferation rate in high-serum conditions, and readily differentiate and fuse into myotubes upon reduction of serum mitogens (Figure 5.1a). Mature myofibers displaying actin-myosin cross-striations and contractile capacity are usually found after 5 to 7 days of induction (Figure 5.1b). Additionally, mesenchymal stem cells (MSCs) have been investigated for application in skeletal muscle tissue engineering. However, their ability to undergo terminal myogenic differentiation remains limited. Currently, diverse strategies are being employed to enhance their myogenic potential, including cyclic mechanical stimulation, coculture with other myogenic precursors, and hypoxic preconditioning [11–13]. While various types of skeletal muscle progenitors can be cultured and differentiated *in vitro*, without appropriate engineering strategies these cells only differentiate into poorly organized and nonfunctional arrays of myotubes.

5.3 Effect of Soluble and Bound Biochemical Cues

The process of muscle regeneration is highly regulated and the factors that contribute in this process are, among others, muscle stretch, trauma, neural stimulation, and soluble growth factors. The interplay of inflammatory

cytokines, such as transforming growth factor-β (TGFβ), and soluble growth factors, such as fibroblast growth factor (FGF) and insulin-like growth factors (IGFs), defines muscle regeneration and repair outcome. Specialized components of the extracellular matrix (ECM), such as laminin and several forms of collagen, are also key mediators of regeneration process. The effects of soluble and bound factors regulating satellite cell activity are well studied and comprehensive reviews are readily available in the literature [8, 14, 15].

5.4 Regulation of Cell Fate by Passive Physical Cues

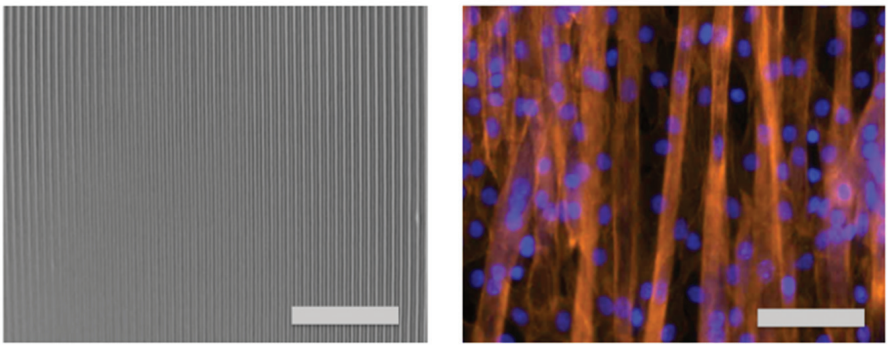
Apart from biochemical signals, several biophysical and structural cues are also part of the *in vivo* muscle microenvironment. In the following subsections, some of the approaches that have been exploited *in vitro* to control attachment, organization, and myogenic differentiation of myogenic precursors will be described.

5.4.1 Substrate Topography

Topographical features of the microenvironment influence migration and organization of the cells. This phenomenon has been termed contact guidance, in which physical shapes of the substrate induce alignment or directional growth of cells. Contact guidance has been investigated *in vitro* using culture substrates patterned with micrometer-sized structures such as fibers or grooves [16–19]. In particular, it has been found that microgrooved substrates can efficiently align skeletal myoblasts, supporting the formation of parallel arrays of muscle fibers [20–23]. As an example, Figure 5.2a displays a microgrooved substrate featuring tracks of 4 μm of width and 1 μm of depth. This kind of micropattern favors the parallel assembly of myoblasts, which upon fusion will result in the formation of a highly aligned layer of myotubes (Figure 5.2b). However, this approach has some limitations. For instance, alignment is limited to a single layer of cells, since cells that are not in direct contact with the substrate do not adopt the expected orientation [20]. Furthermore, topographical cues do not seem to provide any significant advantage for enhancement of the myogenic differentiation process in comparison to non-textured substrates [24, 25].

5.4.2 Substrate Stiffness

Aside from responding to structural cues, cells are able to sense the elasticity of their microenvironment. The dynamic tensional balance between the cell



(a) (b)

Figure 5.2 Organization of skeletal myotubes on micropatterned substrates. (a) Microgrooved silicone substrate used for cell patterning. Scale bar denotes 60 μm. (b) Fluorescence microscopy image showing C2C12 myotubes aligned on a micropatterned silicone substrate. The actin filaments are stained by phalloidin (orange) and nuclei counterstained by Hoechst (blue). Scale bar denotes 100 μm.

cytoskeleton and its environment determines the strain state of the cell at a given time. Cell migration is, for instance, activated by changes in the environmental stiffness that occur after diverse pathologic conditions. This property has been termed durotaxis or durokinesis, which has been exploited to efficiently control *in vitro* cell location and phenotype [26, 27]. In skeletal muscle, external stiffness is a key regulator of myogenic repair process. In particular, differentiation of skeletal myocytes is optimal in substrates matching the stiffness of muscles [28]. As shown by Monge et al., microstructured substrates for guiding cell adhesion and differentiation can be combined with thin films with tunable mechanical properties to devise optimal conditions for alignment and maturation of myotubes *in vitro* [29, 30].

5.5 Active Stimulation

The use of substrates with physical features that mimic the native environment has proven a valuable approach to control cell arrangement. However, in most of the cases, cues provided by the substrate are static in time and fail reproducing the dynamic characteristics of the *in vivo* environment. In the following subsections, some of the *in vitro* approaches used to provide dynamically changing cues will be described.

5.5.1 Electrical Stimulation

Electrical activity is part of the muscle cell niche *in vivo* and the effects of electric stimulation on skeletal muscles are well described. Application of electric fields of appropriate amplitude and frequency depolarize the membrane of muscle fibers and trigger a contractile response. Following this principle, electrical stimulation has been applied to enhance maturation of skeletal muscle cells *in vitro*. Diverse protocols have been proposed, leading to advanced myogenic differentiation, mainly in terms of increased synthesis of myosin heavy chain [31, 32]. However, the optimal stimulation parameters (duration, amplitude, frequency, etc.) remain largely unknown.

5.5.2 Mechanical Loading

Externally applied mechanical forces are also determinants of cell fate. Endothelial cells in blood vessels, for instance, are constantly exposed to a spectrum of hemodynamic forces caused by the pulsatile blood flow [33]. These forces include hydrostatic pressure, shear stress from the vessel wall, and cyclic strain, which all together determine the function and location of the cells. The link between the sensing of mechanical cues and the activation of cellular responses has been defined as mechanotransduction, which is fundamental for the maintenance of normal structure and function of various tissues [34]. In skeletal muscles, mechanotransduction is crucial, as mechanical forces control the development, maintenance, and repair of the tissue [35]. The sensitivity of muscle cells toward external mechanical loading has been demonstrated in diverse *in vitro* settings [36–38]. However, cultured muscle cells have been shown to respond differently to different types of mechanical stimulation paradigms. Cell responses depend, among others, on the rate, amplitude, and direction of the applied loading. Uniaxial tensile strain applied at a rate of few micrometers per minute has been shown to favor the alignment of muscle cells in the direction of the imposed strain [39]. On the other hand, the application of alternating phases of extension/relaxation, known as cyclic tensile strain (CTS), promotes a significant effect on mammalian myoblastic precursors, which respond by G-protein activation and increased protein synthesis [40]. In addition, cells are able to rearrange themselves on the culture substrate with the major axis aligned perpendicularly to the axis of the strain, following the principle of actin fiber reorganization (Figure 5.3). Remarkably, cell maturation is enhanced in response to uniaxial CTS, as evidenced by the presence of large numbers of myosin-positive myofibers [41].

Among the various mechanisms of mechanotransduction involved in this process, integrin mediated focal adhesions are believed to play a key role in transforming the externally applied forces in intracellular biochemical signals [42]. Integrin-mediated signaling initiates downstream activation of adaptor proteins such as the Rho family of GTPases and focal adhesion kinases (FAK). These signaling cascades are key activators of various transcription factors from the myogenic regulatory factor (MRF) family, involved in proliferation and differentiation of muscle precursors [43].

Although the application of mechanical stimulation in the field of skeletal tissue engineering is very promising, its full potential remains to be realized. Given that the outcomes of different stimulation paradigms have been contradicting, stimulation protocols have to be optimized in terms of frequency, amplitude, and duration to maximize the growth and maturation of skeletal muscle precursors. One of the fundamental questions is whether the effects observed in two-dimensional systems are also valid for the three-dimensional settings.

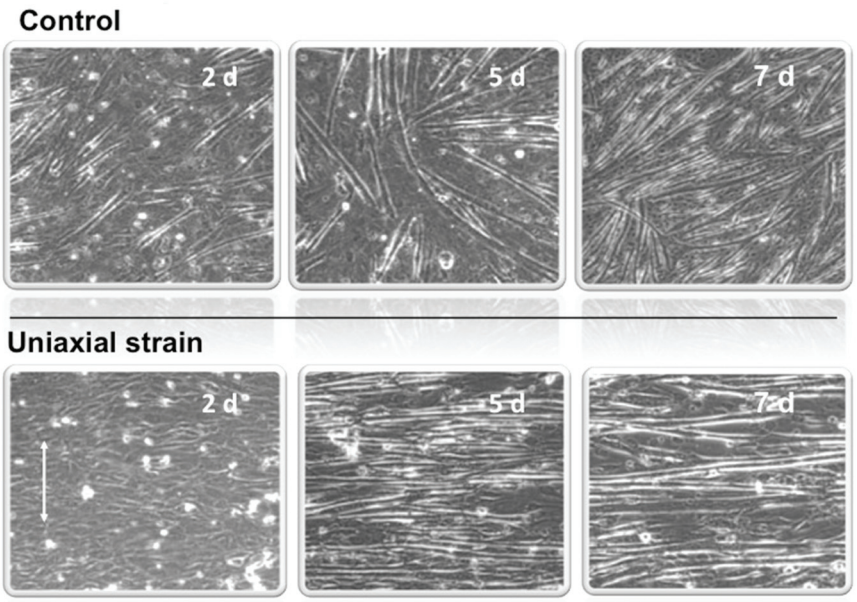


Figure 5.3 Muscle precursors after 2, 5 and 7 days of differentiation. The top row displays static C2C12 cultures. The bottom row shows the cells that were subjected to uniaxial CTS. The white arrow indicates the direction of the principal strain.

5.6 Summary and Perspectives

This review has attempted to provide a brief overview of *in vitro* studies useful to describe some fundamental aspects of the responses of skeletal muscle cells to environmental cues. These studies demonstrated that myoblasts differentiate optimally when the physical signals resemble the cues encountered by the cells in their natural environment. It is worth noting that the different physical signals cannot be completely separated from each other, since the extent of mechanical loading of the cells at a given point in time is determined by the balance between the external and the intrinsically generated forces. It is reasonable to assume that substrate stiffness, topography, applied strain, and even electrical stimulation trigger mechanotransduction mechanisms that activate downstream signaling cascades subserving identical functions. Thus, skeletal muscle engineering approaches aiming to develop highly organized cellular assemblies that mimic the natural skeletal muscle morphology necessarily need to take into account the contributions from all these physical cues.

Significant challenges remain along the way to establish tissue-engineered muscle as a viable therapeutic option. Future work should be focused on the development of novel skeletal muscle scaffolds providing appropriate mechanical and structural cues supporting muscle maturation. In addition, there is a need to investigate alternative cellular models that would overcome the limitations of using immortalized cell lines. Furthermore, efforts should be focused in investigating responses of skeletal muscle cells in more realistic, three-dimensional environments, since the principles established in two-dimensional cultures might not be applicable.

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