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Nanotopography-responsive myotube alignment and orientation as a sensitive phenotypic biomarker for Duchenne Muscular Dystrophy

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HIGHLIGHTS

- Non-diseased myotubes align perpendicular to nanogrooves on substrates having laminin.
- Nanogrooved, laminin-modified substrates distinguish non-diseased and DMD myotubes.
- This phenotypic biomarker may facilitate DMD drug development and early diagnosis.
- All myotubes align parallel to grooves when grooves are larger or laminin is missing.
- The DAPC and its interaction with laminin regulate perpendicular myotube alignment.

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ABSTRACT

Duchenne Muscular Dystrophy (DMD) is a fatal genetic disorder currently having no cure. Here we report that culture substrates patterned with nanogrooves and functionalized with Matrigel (or laminin) present an engineered cell microenvironment to allow myotubes derived from non-diseased, less-affected DMD, and severely-affected DMD human induced pluripotent stem cells (hiPSCs) to exhibit prominent differences in alignment and orientation, providing a sensitive phenotypic biomarker to potentially facilitate DMD drug development and early diagnosis. We discovered that myotubes differentiated from myogenic progenitors derived from non-diseased hiPSCs align nearly perpendicular to nanogrooves, a phenomenon not reported previously. We further found that myotubes derived from hiPSCs of a dystrophin-null DMD patient orient randomly, and those from hiPSCs of a patient carrying partially functional dystrophin align approximately 14° off the alignment direction of non-diseased myotubes. Substrates engineered with micron-scale grooves and/or cell adhesion molecules only interacting with integrins all guide parallel myotube alignment to grooves and lose the ability to distinguish different cell types. Disruption of the interaction between the Dystrophin-Associated-Protein-Complex (DAPC) and laminin by heparin or anti-α-dystroglycan antibody IIH6 disenables myotubes to align perpendicular to nanogrooves, suggesting that this phenotype is controlled by the DAPC-mediated cytoskeleton-extracellular matrix linkage.

1. Introduction

Duchenne Muscular Dystrophy (DMD) is a genetic disorder that causes progressive muscle weakness and wasting, and the patients often die from cardiac and respiratory failure at an age of 20 − 30s [1]. The disease affects one in 3500 male births worldwide [2]. It is known that DMD is caused by mutations in dystrophin, a cytoplasmic protein essential for muscle function [3,4]. In healthy skeletal muscle, dystrophin

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links intracellular cytoskeleton to the extracellular matrix (ECM) through the sarcolemma (muscle cell membrane)-associated Dystrophin-Associated-Protein-Complex (DAPC): the actin-binding domains in dystrophin associate with actin filaments and the C-terminal domain of dystrophin associates with the transmembrane α/β dystroglycan complex, which further binds to laminin in the surrounding basement membrane [5–8]. This cytoskeleton-ECM linkage stabilizes myofibers during muscle contraction and relaxation and is essential for muscle function [4,5,9,10]. When dystrophin is missing or defective, myofibers are less stable and susceptible to chronic injury, causing DMD symptoms and eventually depleting muscle regenerative ability. Given the severe consequences of DMD and its high incidence among genetic disorders, massive efforts have been made to search for DMD treatments [4,11]. However, there is still no cure for the disease; currently used corticosteroid treatment only slows down disease progression. In addition, despite the advances in genetic testing technologies, there is an average delay of about 2.5 years between onset of DMD symptoms and definitive diagnosis [12,13], because the dystrophin gene is very large and there are many possible types of pathogenic mutations and some of which require expensive full gene sequencing and even RNA analysis to identify [14]. Typical clinical diagnosis of DMD starts after symptoms become manifest, and genetic testing is conducted only after its necessity is suggested by the results of a series of other examinations such as physical examination, measurement of the serum creatinine phosphokinase level, electromyography, and muscle biopsy [14,15]. The well-documented negative clinical impact of delayed diagnosis and treatment, together with the ongoing efforts to develop new therapies that potentially can be initiated earlier than corticosteroid treatment, makes early, accurate, and cost-effective diagnosis of DMD increasingly important [12,13,16].

The advances in stem cell technologies, particularly those of human pluripotent stem cells (hPSCs), including human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs), have provided unprecedented opportunities to engineer human tissues and disease models *in vitro* for screening and validating drugs, facilitating diagnosis, and elucidating pathological mechanisms [17–23]. Human PSCs can be expanded extensively and induced for differentiation towards all somatic cell types, ensuring unlimited sources of healthy and disease-specific human cells, and studies using these cells eliminate the issue of interspecies differences associated with animal models. Patient-derived and disease-specific hiPSCs retain genetic characteristics of donors, and therefore are particularly valuable for studying genetic disorders such as muscular dystrophies. We have previously developed a method to efficiently produce myogenic progenitors from hPSCs by inducible expression of the paired box PAX 7 transcription factor (iPAX7), which are endowed with robust *in vitro* and *in vivo* muscle differentiation potential, representing a valuable tool for disease modeling and regenerative medicine [24].

We hypothesized that engineered cell microenvironments would regulate the behavior of hPSC-derived myogenic progenitors and that the phenotypic disparity between non-diseased and DMD hiPSC-derived myotubes would be enhanced in response to certain microenvironmental cues to yield a DMD biomarker for facilitating drug development and diagnosis. It has been extensively reported that cell phenotypes and functions can be profoundly impacted by biochemical ligands and topographical features engineered on cell culture substrates [25–30]. In particular, micro- and nano-scale anisotropic topographical cues have been extensively used in designing biomimetic microenvironments to engineer skeletal muscle [31–49]. These topographical cues include parallel grooves, waves, wrinkles, and aligned fibers; in most studies, the substrates were functionalized with cell adhesion molecules such as collagen, gelatin, fibronectin, the RGD peptide, laminin, and Matrigel. All these studies reported enhanced myotube alignment along the topographical direction and subsequently improved tissue maturation, highlighting the importance of using anisotropic topographical cues to advance skeletal muscle tissue engineering. However, the myoblasts used in most of these studies are C2C12 cells (a mouse cell line) and primary murine or human myoblasts. To our knowledge, only one study reported the effects of parallel microgrooves on formation and alignment of hESC-derived myotubes [45], and no study has been reported on using topographical cues to enhance the phenotypic disparity between non-diseased and DMD hiPSC-derived myotubes [32], though it has been reported that parallel nanogrooves could stratify the normal and disease phenotypes of hiPSC-derived cardiomyocytes [50]. Therefore, we examined the behavior of non-diseased and DMD patient-specific myogenic progenitors derived from iPAX7 hPSCs in response to topographically patterned parallel grooves and substrate-bound cell adhesion molecules during myogenic differentiation.

2. Results and discussion

2.1. Myotubes differentiated from hESC-derived myogenic progenitors align nearly perpendicular to topographical nanogrooves patterned on Matrigel-functionalized substrates

We set out to examine the behavior of hESC-derived myogenic progenitors undergoing myogenic differentiation on polydimethylsiloxane (PDMS) substrates patterned with parallel nanogrooves (800 nm groove/ridge widths and 400 nm depth; Supplementary Fig. 1) and functionalized with Matrigel. This topographical feature was chosen because muscle cells are anatomically aligned in native tissue and parallel nanogrooves were expected to promote myotube alignment. Matrigel (reconstituted basement membrane extracted from Engelbreth-Holm-Swarm (EHS) mouse sarcoma) was chosen because the basement membrane is the ECM immediately adjacent to muscle cells in native tissue [6,51]. Myogenic progenitors were prepared from iPAX7 hESC by inducing PAX7 expression with doxycycline (dox) as previously reported [24], seeded on the substrates at a density of 2.5×10^5 cells/cm^2, cultured in a dox-containing expansion medium for one day, and subsequently induced for differentiation in a dox-free myogenic differentiation medium (medium components are listed in Supplementary Information). Noticeable cell elongation was observed on day 3 upon differentiation induction, and formation of long, multinucleated myotubes was obvious after 2 weeks. Immunofluorescent staining of the myotubes for sarcomeric α-actinin revealed that they possessed sarcomeres giving the striated appearance and aligned in a consistent direction throughout the entire area of each substrate (Fig. 1a). We then used bright-field optical microscopy to determine the nanogroove direction on each substrate. Remarkably, we found all the myotubes aligned nearly perpendicular to nanogrooves (Fig. 1b). In contrast, myotubes differentiated from the same cells on flat, Matrigel-functionalized control substrates oriented randomly (Fig. 1c).

We further quantitatively determined myotube orientation distribution using the Directionality plugin in ImageJ/Fiji [52,53]. The orientation angle is defined as 90° when a myotube aligns perpendicular to grooves and as 0°/180° when a myotube aligns parallel to grooves. An arbitrary direction is used as the 0° reference for samples cultured on flat controls. Fluorescent images acquired at a low magnification (with a 5× objective) were analyzed. The probability density function (p.d.f.) of myotube orientations has a single, narrow, and high peak near 90° for samples cultured on nanogrooved substrates (Fig. 1d), confirming that the myotubes aligned nearly perpendicular to nanogrooves throughout each entire substrate. Each p.d.f. curve was fitted to a Gaussian function (equation (1)), and the resulting peak position (mean myotube orientation angle) is highly reproducible for myogenic progenitors prepared in different batches. Analysis of 9 p.d.f. curves revealed a myotube orientation angle of 86.4° ± 2.1° (mean ± S.D.). In contrast, the orientation p.d.f. of myotubes cultured on the flat control does not have a narrow and high peak near 90° (Fig. 1d). Instead, low values of probability density spread throughout the whole angle range (Fig. 1d), suggesting random orientations of these myotubes (the wide