Journal of Biotechnology xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

Journal of Biotechnology

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

Short communication

A multiplexed chip-based assay system for investigating the functional development of human skeletal myotubes *in vitro*

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ARTICLE INFO

Article history:
Received 27 March 2014
Received in revised form 19 May 2014
Accepted 23 May 2014
Available online xxx

Keywords:
Human
Skeletal muscle
Cantilevers
Functional assay
Body-on-a-chip

ABSTRACT

This report details the development of a non-invasive *in vitro* assay system for investigating the functional maturation and performance of human skeletal myotubes. Data is presented demonstrating the survival and differentiation of human myotubes on microscale silicon cantilevers in a defined, serum-free system. These cultures can be stimulated electrically and the resulting contraction quantified using modified atomic force microscopy technology. This system provides a higher degree of sensitivity for investigating contractile waveforms than video-based analysis, and represents the first system capable of measuring the contractile activity of individual human muscle myotubes in a reliable, high-throughput and non-invasive manner. The development of such a technique is critical for the advancement of body-on-a-chip platforms toward application in future pre-clinical drug development screens.

Development of novel body-on-a-chip technologies for eventual application in improved pre-clinical screening protocols has the potential to revolutionize the drug development process (Sung et al., 2013). Of particular interest, is the establishment of defined and controllable human *in vitro* systems as a means to curtail or even eliminate animal testing from toxicity/efficacy studies of novel therapeutics.

Skeletal muscle cells from mammalian sources have been studied *in vitro* for over 30 years (Bischoff, 1974; Yasin et al., 1977). However, an ability to maintain such cells in configurations which facilitate assessment of their contractile output has proved more difficult. While the seeding of myocytes within three-dimensional scaffolds has alleviated this issue to a degree (Dennis and Kosnik, 2000, 2001; Langelaan et al., 2011; Rhim et al., 2007, 2010; Sakar et al., 2012; Smith et al., 2012; van der Schaft et al., 2013; Vandenburgh, 2010; Weist et al., 2013), the ability to investigate skeletal muscle contraction at the single myotube level in a reliable and reproducible manner remains problematic. Moreover, many 3D models rely on visual interrogation in order to measure contractile activity, which can make accurate assessment of contraction profiles challenging (Agarwal et al., 2013; Sakar et al., 2012; Vandenburgh et al., 2008). Those systems which perform direct measurement of functional output (Rhim et al., 2010), do so with the use of a force transducer coupled to the muscle model which is invasive and difficult to integrate with more complex multi-organ platforms. Three dimensional systems are also difficult to integrate effectively with supporting and interacting cell types, limiting their applicability to body-on-a-chip platforms.

Here we present the development of a culture system for maintaining functional human myotubes on microscale silicon cantilever arrays *in vitro*. Individual cantilevers were interrogated using a non-invasive laser and photo-detector system which recorded substrate deflection in response to myotube contraction (Wilson et al., 2007). Although cantilever technology has been studied previously with rat tissue as a means to investigate muscle contractile behavior *in vitro* (Pirozzi et al., 2013; Wilson et al., 2007, 2010), adaptation of these systems to promote the long-term survival and functional maturation of human myotubes is critical in the development of such technology toward pre-clinical drug development applications.

Primary human myoblasts were isolated as described previously (Guo et al., 2014; Hennessey et al., 1997; Powell et al., 1999), and seeded at a density of 75 cells/mm^2 on microscale silicon cantilever chips. Cantilevers were first coated with a defined self-assembled monolayer, DETA, which is a spermidine analog and has been shown to aid the long-term attachment and survival of cells in culture (Eisenberg et al., 2009; Kaeberlein, 2009).

Abbreviation: DETA, (3-trimethoxysilyl propyl) diethylenetriamine.

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http://dx.doi.org/10.1016/j.jbiotec.2014.05.029
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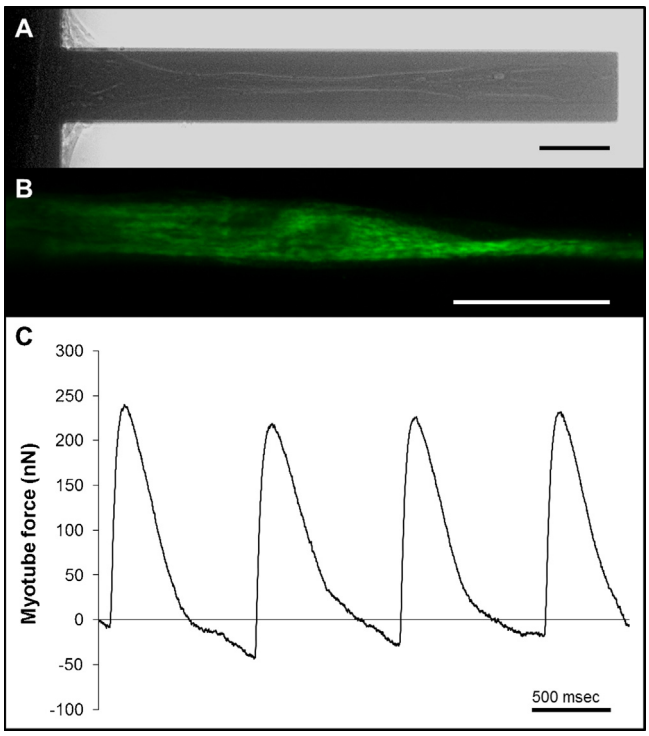

Fig. 1. Human myotubes survive on silicon cantilevers and contract, generating quantifiable substrate deflections which can be used to calculate force production *in vitro*. (A) Low magnification bright field image of a human myotube cultured on a silicon microscale cantilever. Scale bar = 50 μm. (B) High magnification image of a myotube cultured on a silicon cantilever and immunostained for myosin heavy chain presence. Scale bar = 50 μm. (C) Representative recording of myotube force production in response to broad-field electrical pulses at a 1 Hz frequency, calculated from real time measurement of cantilever deflection using our laser and photo-detector system. Raw data (in Volts) was converted to a measurement of myotube force (in nano-Newtons), using a modified Stoney's equation, and re-plotted.

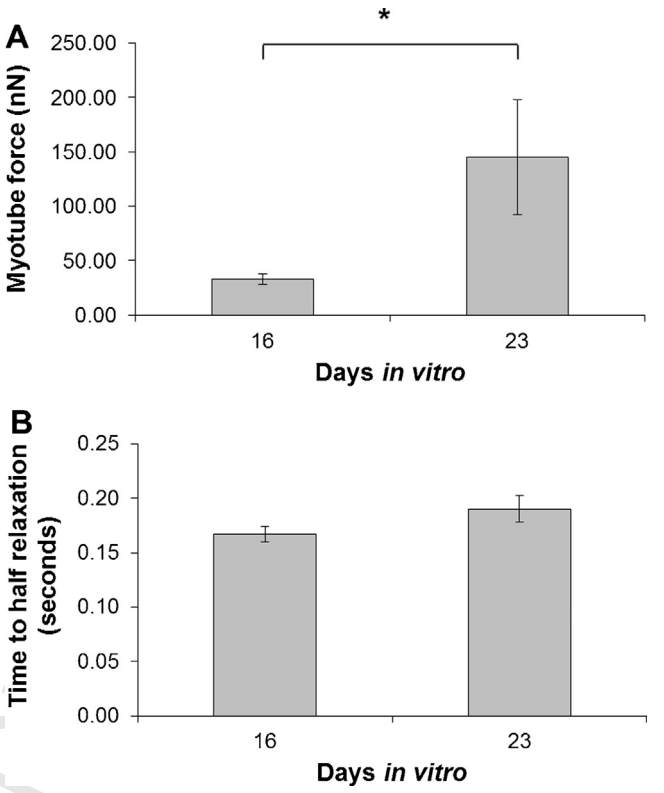

Fig. 2. Myotube force production increases over time in culture while force profiles remain constant. (A) Average myotube force produced following 16 and 23 days *in vitro*. $n = 3$, $^*p = 0.045$. (B) Average time to half relaxation in contracting myotubes analyzed following 16 ($n = 19$) and 23 ($n = 6$) days *in vitro*, $p = 0.11$.

The use of silicon based substrates in the development of these chips allowed for a high degree of repeatability and normalization of fabrication parameters. This feature is of vital importance when developing assays for integration with complex, multi-organ systems, giving this model a significant advantage over the use of others utilizing less highly regulated fabrication procedures. The cultured cells were maintained in a commercially available growth medium (Lonza, Allendale, NJ, USA, Cat # CC-3160) until confluent. They were then switched to a serum-free, differentiation medium for a further 4 days to induce myotube formation (Fig. 1). Following differentiation, the medium was slowly replaced with a neuronal base medium, NBActiv 4 (Brain Bits LLC, Springfield, IL, USA), by replacing half the medium every 2 days. Using this protocol, cells were routinely maintained on cantilevers for at least 3 weeks ($n = 6$), and were assessed following 14–21 days *in vitro*.

The contractile activity of human myotubes cultured on silicon cantilever chips was assessed as described previously (Pirozzi et al., 2013; Wilson et al., 2010). Briefly, cantilever chips were transferred to a transparent culture well mounted on the stage of a modified electrophysiology microscope. The culture well was filled with NBActiv 4 medium (+10 mM HEPES) to maintain the cells during the analysis. A Helium Neon laser beam was scanned through the underside of the culture well and across the tips of each cantilever. A quadrant photo-detector module was moved simultaneously to detect the reflected beam. Software written in LabView (National Instruments) facilitated automatic scanning from one cantilever to the next, effectively enabling simultaneous interrogation of entire cantilever arrays in real-time. Stainless steel electrodes were mounted inside the stage dish and connected to a pulse generator (A-M systems, Sequim, WA, USA), capable of producing field stimulation pulses of varying intensity, frequency, and waveform, which allowed the system to produce field stimulation of myotubes to induce controlled contraction events.

The photo-detector was connected through an Axon Instruments 1440 digitizer (Molecular Devices, Union City, CA) to a computer running AxoScope 10.0 and the change in position of the reflected laser beam on the photo-detector was recorded using this software. Recorded cantilever deflections (in Volts) were then converted to measurements of force (in nN) using Stoney's equations as described previously (Pirozzi et al., 2013) (Fig. 1c).

Data collected from human myotubes using this system suggests that human skeletal muscle myotubes undergo functional maturation in culture. A significant increase in contractile force was observed between cultures examined following 14 days *in vitro* ($n = 19$), and those assessed after 21 days in culture ($n = 6$, $p = 0.045$, one-tailed student t test) (Fig. 2a). No significant change was observed in time to half relaxation measurements between the 2 time-points examined ($p = 0.11$, two-tailed student t test) (Fig. 2b). Time to half relaxation is a well-established measure of muscle fiber functional properties (Itoh et al., 2013), and changes in fiber phenotypes are known to correlate with alterations in such characteristics (Irintchev et al., 2002). The fact that no change in half relaxation time was recorded following longer culture periods indicated that the added time did not alter the functional phenotype of the cultured cells with regard to fast and slow isoform switching, but rather allowed time for greater development of cellular contractile machinery, leading to an improved functional output *in vitro*.

This model facilitates the functional assessment of human muscle fibers at the single cell level, providing a greater degree of clarity with regard to culture variability and consistency than is

Please cite this article in press as: Smith, A.S.T., et al., A multiplexed chip-based assay system for investigating the functional development of human skeletal myotubes *in vitro*. J. Biotechnol. (2014), http://dx.doi.org/10.1016/j.jbiotec.2014.05.029