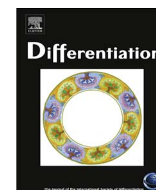
Differentiation 96 (2017) 70–81

Contents lists available at ScienceDirect

Differentiation

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



Differentiation and sarcomere formation in skeletal myocytes directly prepared from human induced pluripotent stem cells using a sphere-based culture

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ARTICLE INFO

Keywords:
Skeletal muscle
Human pluripotent stem cells
Induced pluripotent stem cells
Muscle differentiation
Sarcomere
Neuromuscular diseases

ABSTRACT

Human induced-pluripotent stem cells (iPSCs) are a promising resource for propagation of myogenic progenitors. Our group recently reported a unique protocol for the derivation of myogenic progenitors directly (without genetic modification) from human pluripotent cells using free-floating spherical culture. Here we expand our previous efforts and attempt to determine how differentiation duration, culture surface coatings, and nutrient supplements in the medium influence progenitor differentiation and formation of skeletal myotubes containing sarcomeric structures. A long differentiation period (over 6 weeks) promoted the differentiation of iPSC-derived myogenic progenitors and subsequent myotube formation. These iPSC-derived myotubes contained representative sarcomeric structures, consisting of organized myosin and actin filaments, and could spontaneously contract. We also found that a bioengineering approach using three-dimensional (3D) artificial muscle constructs could facilitate the formation of elongated myotubes. Lastly, we determined how culture surface coating matrices and different supplements would influence terminal differentiation. While both Matrigel and laminin coatings showed comparable effects on muscle differentiation, B27 serum-free supplement in the differentiation medium significantly enhanced myogenesis compared to horse serum. Our findings support the possibility to create an *in vitro* model of contractile sarcomeric myofibrils for disease modeling and drug screening to study neuromuscular diseases.

1. Introduction

Pluripotent stem cells, such as embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), have received much attention because of their potential use for cell-based therapies and *in vitro* modeling (Hosoyama et al., 2012; Rinaldi and Perlingeiro, 2014; Roca et al., 2015). Human iPSCs, established from somatic cells, represent a valuable source of tissue for generating human myogenic progenitors (Tedesco and Cossu, 2012). Furthermore, these progenitors are able to form myotubes in culture, which can provide a useful platform for understanding normal muscle development and disease mechanisms *in vitro* (Hosoyama et al., 2012; Rinaldi and Perlingeiro, 2014; Roca et al., 2015).

In recent years, several protocols have been reported to propagate human myogenic progenitors from pluripotent cell sources and to differentiate these progenitors into the skeletal muscle cell lineage as myoblasts or myotubes (Zhu et al., 2014). While many protocols require cell sorting and/or rely on exogenous expression of myogenic genes such as PAX3, PAX7, and MYOD (Abujarour et al., 2014; Darabi et al., 2012; Maffioletti et al., 2015; Skoglund et al., 2014; Tanaka et al., 2013), more recent advances have been made with the application of small molecules and growth factors to directly promote myogenic differentiation from human iPSCs (Barberi et al., 2007; Borchin et al., 2013; Caron et al., 2016; Chal et al., 2016, 2015; Choi et al., 2016; Hosoyama et al., 2014; Hwang et al., 2013; Shelton et al., 2014; Xu et al., 2013). Our group recently reported a unique method for the derivation of myogenic progenitors from human pluripotent cells using free-floating spherical culture (Hosoyama et al., 2014). Human ESC and iPSC colonies were expanded in medium supplemented with high concentrations of fibroblast growth factor-2 (FGF-2) and epidermal growth factor (EGF). The cells then formed sphere aggregates named EZ spheres. We could confirm Pax7-positive ($Pax7^{+}$) myogenic pro-

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http://dx.doi.org/10.1016/j.diff.2017.07.004
Received 25 February 2017; Received in revised form 1 July 2017; Accepted 31 July 2017
Available online 01 August 2017
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genitors (approximately 40–60% of total cells) in EZ spheres, and myosin heavy chain-positive (MHC^+) myotubes were identified following sphere dissociation and 2 weeks of terminal differentiation (Hosoyama et al., 2014). Importantly, our culture protocol is applicable to create myogenic progenitors and myotubes from human iPSCs generated from both healthy donors and patients with neuromuscular disorders (Hosoyama et al., 2014).

In the present study, we expand our previous efforts and attempt to create mature skeletal myotubes that contain organized sarcomeric structures from iPSCs. Sarcomere formation is critical for morphologically modeling the functional units of muscle contraction (Alter, 2004). The rationale of the present study is based on the previous observations using satellite cells and primary myoblasts, which showed that differentiation duration, culture surface coatings, and nutrient supplements in the medium can significantly influence muscle differentiation (Grefte et al., 2012; Hartley and Yablonka-Reuveni, 1990; Lawson and Purslow, 2000; Molnar et al., 2007). Here we determined the time course of muscle differentiation and sarcomere formation in EZ sphere-derived myogenic progenitors. We also used a bioengineering approach and tested three-dimensional (3D) cultures to create elongated and matured myotubes. Further, we evaluated the effects of different extracellular matrix coatings and serum-free or serum supplements for myotube differentiation.

2. Materials and methods

2.1. Human induced pluripotent stem cells

A human iPSC line (IMR90) was used in this study and maintained following feeder-independent protocols (Ludwig et al., 2006). This iPSC line was obtained from WiCell (Madison, WI, USA) and was originally created from human fibroblasts transduced with lentivirus to overexpress *OCT4*, *NANOG*, *SOX2*, and *LIN28* (Yu et al., 2007). iPSC colonies were cultured in mTeSR1 medium on a 6-well plate coated with Matrigel (BD Bioscience; San Jose, CA), and passaged using Versene (Life Technologies, Grans Island, NY, USA).

2.2. Differentiation of iPSCs to myotubes

Human iPSC-derived myogenic progenitors and myotubes were prepared using our protocol as recently described (Fig. 1) (Hosoyama et al., 2014). Briefly, iPSC colonies were lifted by 0.1% collagenase (Life Technologies) and transferred into expansion medium [Stemline medium (S-3194, Sigma-Aldrich, St. Louis, MO, USA) supplemented with 100 ng/ml recombinant human FGF-2 (WiCell), 100 ng/ml human EGF (Millipore, Billerica, MA, USA), 5 ng/ml heparin sulfate (Sigma-Aldrich), and penicillin/streptomycin/amphotericin B (PSA, 1%v/v; Life Technologies)]. After 1 week, the colonies formed spherical aggregates (EZ spheres). The spheres were passaged every week by mechanical chopping using a McIlwain tissue chopper (Mickle Laboratory Engineering, Surrey, UK).

After 6 weeks, EZ spheres were dissociated using trypsin (TrypLE, Life Technologies), plated on coverslips coated with poly-L-ornithine (0.1 mg/ml) and laminin (50 μg/ml) (both Sigma-Aldrich) (Hosoyama et al., 2014). Alternatively, in order to determine the effect of Matrigel on myogenic differentiation, coverslips were coated with Matrigel (83.3 μg/ml; Corning Incorporated, Corning NY, USA) instead of poly-L-ornithine and laminin. Unless otherwise specified, the plated cells were then maintained in terminal differentiation medium [Dulbecco's Modified Eagle's Medium (DMEM, Sigma-Aldrich) containing 2% B27 supplement (Life Technologies) and 1% PSA] for differentiation into myotubes. To evaluate the effect of horse serum on myogenic differentiation, 2% horse serum (Life Technologies) was used instead of B27 supplement.

2.3. Immunocytochemistry

Immunocytochemistry was performed as described previously (Hosoyama et al., 2014). Briefly, cells were fixed with 4% paraformaldehyde (PFA), permeabilized in 0.2% Triton-X 100, and blocked with 5% normal donkey serum in phosphate-buffered saline (PBS). The fixed cells were stained with primary antibodies against Pax3, Pax7, MyoD, or Myogenin (MyoG). For labeling myosin heavy chain (MHC), MYH isoforms, and titin, cells were fixed in ice-cold methanol. To identify co-localization of multiple markers for myogenic cells, rabbit polyclonal anti-MyoG antibody was co-labeled with mouse monoclonal antibodies against Pax7. After incubating with primary antibody overnight, the cells were stained with secondary antibody conjugated to Alexa Fluor 488 or Cy3 (anti-IgG, 1:1000, Jackson Immunotech Research Laboratories, West Grove, PA, USA). To identify the expression of Pax3, Pax7, and MyoG in myotubes, anti-MHC antibodies directly conjugated with Alexa Fluor 488 or Alexa Fluor 660 were additionally used. The details of the primary antibodies used in this study are described in Table 1. Hoechst 33258 (0.5 μg/ml in PBS, Sigma-Aldrich) was used to label cell nuclei. The fluorescence images were captured at 20x objective using a Nikon Eclipse 80i fluorescence microscope with a DS-QilMC CCD camera (Nikon, Tokyo, Japan) or Leica TCS SP8 confocal microscope (Leica, St. Gallen, Switzerland). For each staining, we prepared technical replicates (2–3 coverslips), and the positive cells were counted from 6 randomly selected fields per coverslip. We repeated three independent experiments for cell counting.

2.4. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted and purified from cell lysates using an RNeasy kit (Qiagen, Germantown, MD, USA). RT-PCR was run using RT

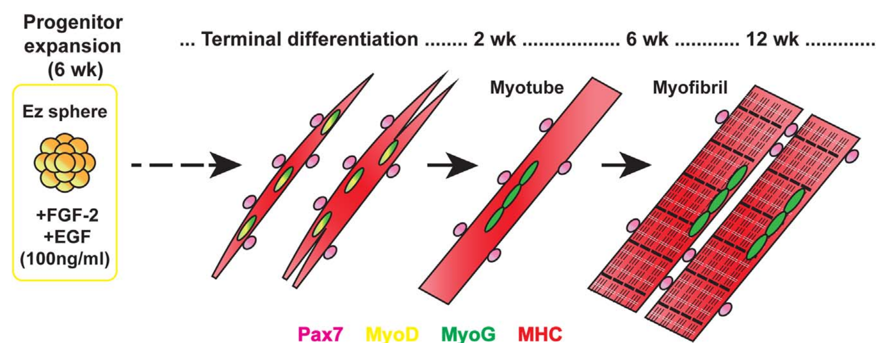

Fig. 1. Muscle differentiation of iPSC-derived myogenic progenitors. Human iPSCs were maintained as spherical aggregates (termed as EZ spheres) in suspension medium containing high concentration (100 ng/ml) of FGF-2 and EGF for 6 weeks. Myogenic progenitors in EZ spheres were plated on coverslips and terminally differentiated for 2–12 weeks. In this study, we determined the time course of muscle differentiation and sarcomere formation in EZ sphere-derived myogenic progenitors. Further, we evaluated the effects of different extracellular matrix coatings and serum-free or serum supplements for myotube differentiation. The stage of muscle cells was determined by the expression of Pax7 (myogenic progenitors), MyoD (myoblasts), Myogenin (MyoG; committed myocytes), and myosin heavy chain (MHC).

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