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Formation and characterisation of neuromuscular junctions between hiPSC derived motoneurons and myotubes

M. Demestre [a,*], M. Orth [b], K.J. Föhr [c], K. Achberger [d], A.C. Ludolph [b], S. Liebau [d], T.M. Boeckers [a,*]

[a] Institute of Anatomy and Cell Biology, Ulm University, Germany
[b] Department of Neurology, Ulm University, Germany
[c] Clinic for Anaesthesiology, Ulm University, Germany
[d] Institute of Neuroanatomy, Eberhard Karls University, Tübingen, Germany

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ABSTRACT

Striated skeletal muscle cells from humans represent a valuable source for *in vitro* studies of the motoric system as well as for pathophysiological investigations in the clinical settings. Myoblasts can readily be grown from human muscle tissue. However, if muscle tissue is unavailable, myogenic cells can be generated from human induced pluripotent stem cells (hiPSCs) preferably without genetic engineering. Our study aimed to optimize the generation of hiPSCs derived myogenic cells by employing selection of CD34 positive cells and followed by distinct, stepwise culture conditions. Following the expansion of CD34 positive single cells under myogenic cell culture conditions, serum deprived myoblast-like cells finally fused and formed multinucleated striated myotubes that expressed a set of key markers for muscle differentiation. In addition, these myotubes contracted upon electrical stimulation, responded to acetylcholine (Ach) and were able to generate action potentials. Finally, we co-cultured motoneurons and myotubes generated from identical hiPSCs cell lines. We could observe the early aggregation of acetylcholine receptors in muscle cells of immature co-cultures. At later stages, we identified and characterised mature neuromuscular junctions (NMJs). In summary, we describe here the successful generation of an iPS cell derived functional cellular system consisting of two distinct communicating cells types. This *in vitro* co-culture system could therefore contribute to research on diseases in which the motoneurons and the NMJ are predominantly affected, such as in amyotrophic lateral sclerosis or spinal muscular atrophy.

1. Introduction

Motoneurons make contact with the skeletal muscle to form the neuromuscular junction (NMJ), which is required for signal transmission. Disorders of the NMJ such as myasthenia gravis lead to varying degrees of muscle weakness (Gomez et al., 2010). Muscle wasting and weakness in motoneuron diseases arise predominantly from the loss of motoneurons. Hence, research has focused on investigating the mechanisms of motoneuron cell death (de Carvalho et al., 2014). In addition, there is growing evidence indicating that alterations of NMJs and/or the skeletal muscle itself affect the pathogenic process in amyotrophic lateral sclerosis (ALS) (Miller et al., 2006; Murray et al., 2010; Wong and Martin, 2010).

In vitro models could contribute to the analysis of the pathophysiology of motoneuron diseases and might help to screen for therapeutic options. Human induced pluripotent stem cell (hiPSC) technology has opened new avenues for elucidating underlying pathomechanisms and even putative treatment options. Cell culture systems generated from patient specific somatic tissue harbour the genetic defects giving the opportunity to analyse the functional impairment *in vitro*. Furthermore, the ability to repair underlying gene mutations in patient specific cells with modern gene editing systems provides the possibility of autologous cell transplantations. Recently, different methods have been published describing the generation of myoblasts from hiPSCs by introducing lentiviral systems forcing myogenic differentiation (Darabi et al., 2012; Goudenege et al., 2012; Tanaka et al., 2013; Abujarour et al., 2014). In addition, a sphere based culture system to generate hiPSC derived myoblast has recently been described but in these myogenic cultures a high degree of neuronal contamination was observed (Hosoyama et al., 2014). However, protocols giving rise to significant numbers of skeletal muscle cells without genetic engineering are therefore more physiological methods and are still in need to be developed.

Abbreviations: Ach, acetylcholine; AchR, acetylcholine receptor; ALS, amyotrophic lateral sclerosis; BDNF, brain derived neurotrophic factor; BT, bungarotoxin; EB, embryoid body; GDNF, glial derived neurotrophic factor; FGF, fibroblast growth factor; hiPSC, human induced pluripotent stem cell; IGF, insulin derived growth factor; MEFS, mouse embryonic fibroblasts; NMJ, neuromuscular junction; PBS, phosphate buffered saline.

* Corresponding authors at: Institute of Anatomy and Cell Biology, Albert Einstein Allee 11, 89081 Ulm, Germany.
E-mail addresses: maria.demestre@uni-ulm.de (M. Demestre), tobias.boeckers@uni-ulm.de (T.M. Boeckers).

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Promising candidate markers for the positive or negative selection of myogenic progenitors to enhance cell populations with myogenic potential are membrane bound surface proteins of the large family of CD factors, involved in a variety of cellular functions. One interesting candidate marker to select for myogenic precursors is CD34, expressed in quiescent satellite cells and proliferating myogenic precursors (Beauchamp et al., 2000; Jankowski et al., 2002; Zammit et al., 2006). Additionally, factors involved in promoting the myogenic potential of stem cells are produced and secreted by muscle stem or progenitor cells that in turn enhance *in vitro* differentiation of stem cells into the myogenic lineage (Stern-Straeter et al., 2014).

We and others have shown that hiPSC have the ability to differentiate into different cell types belonging to the motor system, especially to lower motoneurons (Stockmann et al., 2011). In this context, a co-culture system using hiPSC derived motoneurons and differentiated primary myotubes represents the *bona fide* model to investigate motoneuron and muscle cell degeneration. This model system is able to generate functional NMJs displaying all features of functional connections (Stockmann et al., 2011). In another study, disease specific motoneurons derived from a dystrophy type 1-hiPSC cell line were co-cultured with myotubes generated from a 'non-diseased' human muscle cell line, Mu2b3 (Marteyn et al., 2011). In the present study, we successfully established co-cultures for the first time combining motoneurons and myotubes generated from the same donor hiPSC cell line. In these co-cultures, the aggregation of acetylcholine receptors (AchR)s and the formation of intact NMJs could be detected. This *in vitro* culture system can be used to further our understanding of the pathogenesis of diseases in which NMJs are affected.

2. Materials and methods

2.1. Generation of hiPSCs

Keratinocytes from plucked human (hiPSC1: female 40 years old, hiPSC2: male 35 years old) hair were generated as described previously (Linta et al., 2011; Stockmann et al., 2011; Illing et al., 2013). Keratinocytes were grown on 20 μg/mL collagen IV-coated dishes and cultured in EpiLife medium supplemented with HKGS (both Life technologies, USA). After reaching 75% confluency keratinocytes were infected on two consecutive days with proviral genome copies of a lentivirus containing a polycistronic expression cassette encoding *OCT4*, *SOX2*, *Klf4*, and *cMYC* (Sommer et al., 2009), diluted in EpiLife medium containing 8 μg/mL polybrene. Lentiviral particles were produced as described previously (Linta et al., 2011). More details are described in Supplemental methods. On the third day, keratinocytes were transferred onto irradiated rat embryonic fibroblasts used as feeder cells, which had been isolated from E14 Sprague Dawley rat embryos as described previously (Linta et al., 2011) and previously cultured in DMEM supplemented with 15% foetal bovine serum, 2 mM GlutaMAX, 100 mM non-essential amino acids and 1% Antibiotic–Antimycotic (all from Life technologies). Keratinocytes growing on rat embryonic fibroblasts were cultured in hiPSC medium consisting of knockout/DMEM supplemented with 20% knockout serum replacement, 2 mM GlutaMAX, 100 mM non-essential amino acids (all from Life technologies), 1% Antibiotic–Antimycotic, 100 mM β-mercaptoethanol (Millipore, USA), 50 mg/mL vitamin C, and 10 ng/mL fibroblast growth factor-2 (FGF-2) (both from PeproTech, USA) in a 5% O_2 incubator. Medium was changed daily. After 3–5 days small colonies appeared with typical hiPSCs morphology. Around 14 days later, hiPSC colonies had the appropriate size for mechanical passaging and were transferred onto irradiated mouse embryonic fibroblasts (MEFs) (Stem Cell Technologies, France) and further cultivated with hiPSC medium. After one passage hiPSC colonies were mechanically picked and transferred to plates free of feeder cells and maintained with mTReSR1 medium (Stem Cell Technologies). For splitting, hiPSCs colonies were incubated with dispase (StemCell Technologies) for 5–7 min at 37°C and subsequently detached using a cell scraper.

2.2. Differentiation of hiPSC into myoblasts or motoneurons

HiPSCs were differentiated into myoblast in two ways (Fig. 1a, b). First, we generated myoblast from hiPSC using differentiation towards mesoderm and CD34+ selection. To favour mesoderm differentiation, hiPSCs cultivated on mTReSR1 were expanded on MEFs feeder layers and cultivated with hiPSCs medium supplemented with vitamin C and FGF-2 as described above. To split cells dispase was used, but additionally, in order to remove MEFs from the cultures detached cells were incubated in flasks for 2 h at 37 °C. During this time MEFs attached gradually onto the bottom of the flask and hiPSC cells remained in solution. Afterwards, cells remaining in solution were transferred into non-adherent flasks to generate embryoid bodies (EBs). EBs were grown in hiPSC medium for 2 days with 10 μM Rock-inhibitor Y-276342 (Ascent, UK) for the first 24 h. Then, medium was replaced to myogenic medium containing 15% horse serum, 10% foetal bovine serum (both from Life technologies), 10% chicken embryo extract (Biomol, Germany), 100 μM β-mercaptoethanol (Millipore), 1% Antibiotic–Antimycotic, for 14 days. Afterwards, EBs were carefully attached on laminin (25 μg/mL, Roche, Switzerland) coated plates and grown for 2 weeks with one half of myogenic medium supplemented with vitamin C (50 μg/mL) and FGF-2 (2 ng/mL) and the other half with conditioned medium generated from the cultivation of human primary myoblasts (described below).

To avoid the contamination of human primary myoblasts into our cultures the medium obtained from human primary myoblast cultures was centrifuged, filtered through a 30 μm filter and stored at −20 °C. To select specific CD34 positive cells by cell sorting, cells were digested with Accutase (Stem Cell Technologies) for 5 min at 37 °C and mechanically dissociated with a fire-polished glass pipette. Afterwards the cell suspension was passed through a 70 μm filter to remove cell aggregates. Cells were re-suspended in phosphate buffered saline (PBS) containing 0.5% bovine serum albumin and 2 mM EDTA. Cells were labelled with anti-CD34 microbeads using Miltenyi Biotec kits following the manufacturer's instructions. Magnetic separation was carried out with MACS separation columns (Miltenyi Biotec, Germany). Positive and negative fractions were eluted three times to increase the purity of the samples.

CD34 positive cells were then plated on laminin coated plates and fed with myogenic medium supplemented with FGF-2 and vitamin C and conditioned medium generated from human primary myoblasts cultures, for 2 weeks. Cells were split using TrypLE (Life technologies). To enrich myogenic cells the pre-plating method described by Park and collaborators (Park et al., 2006) with some modifications was used. Briefly, cells were detached and then pre-plated in a gelatin-coated flask. After 45 min of incubation, mainly fibroblasts are known to adhere. The supernatant containing the non-attached cells was collected and re-plated into a fresh gelatine coated flask. After 2 serial platings, the culture was enriched with small, round myogenic cells. To generate specific myogenic clones after one passage cells were sorted by size using live cell-FACS analysis (Beckton Dickinson/FACSARIA III). Only small cells, as assessed by forward scatter size, were sorted and plated as single cells. Subsequently single clones were further expanded in conditioned myogenic medium.

Second, the generation of hiPSC-derived myoblasts by PAX7 over expression was done as previously described by Darabi and colleagues (Darabi et al., 2012; Skoglund et al., 2014). Details are described in supplemental methods.

HiPSCs were differentiated into motoneurons essentially as previously described (Hu and Zhang, 2009) with some modifications and extensively characterised as outlined elsewhere (Stockmann et al., 2011).