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

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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.

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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).

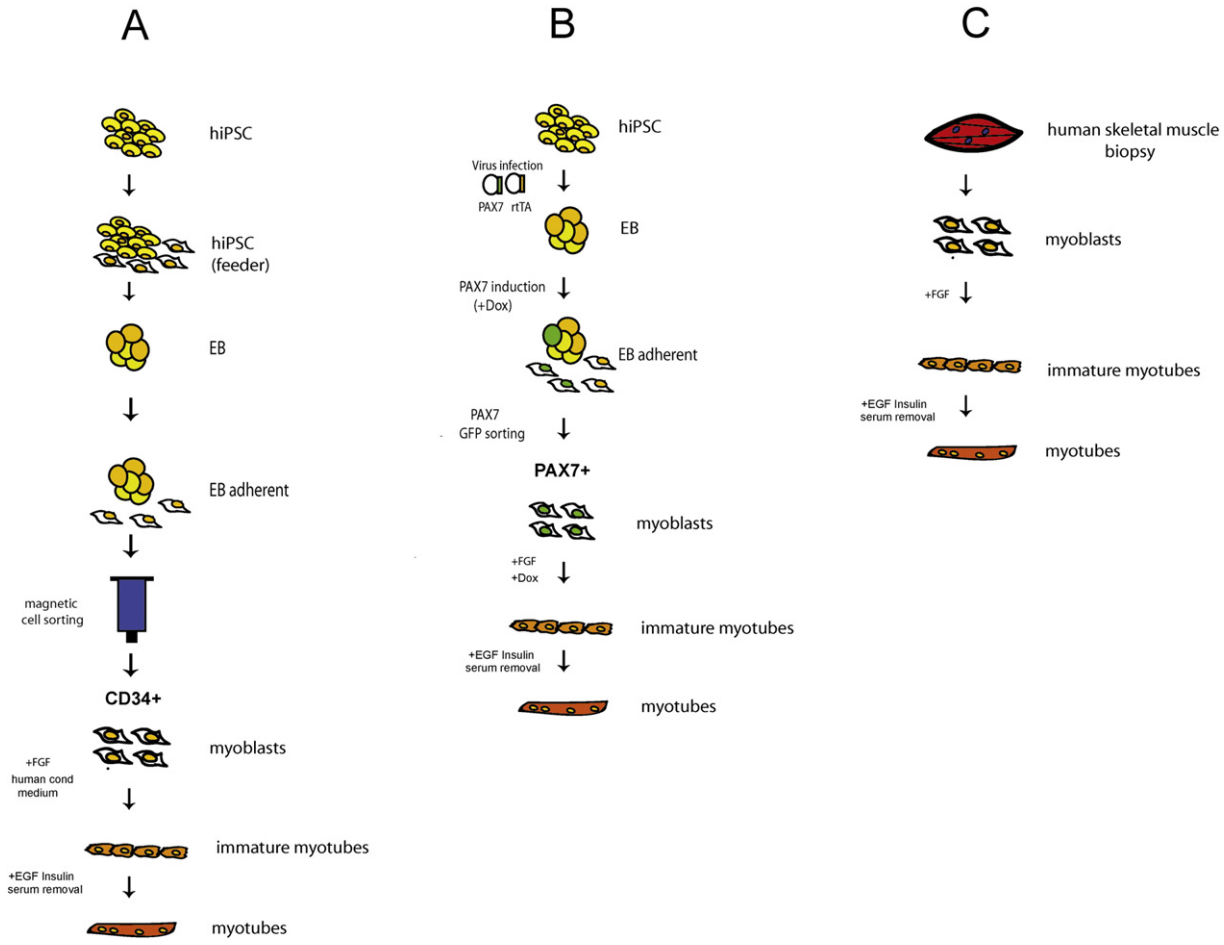

Fig. 1. A–C. Schematic diagram describing the 3 methods used to generate myoblasts A. CD34 + selected myoblasts derived from hiPSC. B. hiPSC derived myoblasts by PAX7 induction. C. Myoblast isolation from human skeletal muscle biopsies.

2.3. Generation of myoblast from human muscle biopsies

Primary skeletal muscle stem cells were isolated from a M. vastus lateralis muscle biopsy as described (Orth et al., 2003; Park et al., 2006) (Fig. 1c). Cells were expanded in myogenic medium supplemented with vitamin C and FGF-2 as described above. For passaging, human myoblasts were plated on gelatine coated flasks at a density of 25 cells per mm^2 in myogenic medium. Cultures were fed every 2 days by changing the whole medium. When cells were 60% confluent, medium was replaced to a commercial medium for 24 h (SKGM, Lonza Inc., USA) before differentiation.

2.4. Myotube motoneuron co-culture

Myoblasts generated from all three methods were differentiated into myotubes by changing the medium to DMEM supplemented with 10 μg/mL insulin (Sigma Aldrich, Germany), 500 μg/mL bovine serum albumin (Life technologies;) and 10 ng/mL epidermal growth factor (Prepotech). Cells were fed every 2 days by exchanging half of the medium.

To generate primary motoneurons, time-mated adult pregnant Sprague–Dawley rats at E14 were anaesthetized and sacrificed by CO_2 overdose, the embryos were removed from the uterus, brains/spinal cords were dissected out and placed into ice-cold Hank's balanced salt solution (HBSS) supplemented with 1% Antibiotic–Antimytotic and 1% glucose (Sigma Aldrich).

Primary spinal cord cultures were established adapting previously published methods (Martinou et al., 1989; Stockmann et al., 2013). For co-culture experiments rat primary motoneurons were plated on top of differentiated myotubes at a density of 10×10^4 cells per well in neuronal medium containing 32% DMEM-high glucose, 62% knockout-DMEM/F12, 1% glutamate, 2% B27 supplement (Life technologies), 1% Antibiotic–Antimycotic, 2% knockout-serum replacement and 1% non-essential amino acids. Cells were cultured in a humidified incubator at 37 °C and 5% CO_2 for two weeks.

For co-culture experiments with hiPCS-derived motoneurons, motoneuron-enriched neurospheres (after 14 days of pumorphamine (50 ng/mL, R&D Systems, USA)-treatment) were seeded onto hiPSC-differentiated myotubes and cultured in DMEM/F12-Glutamax medium supplemented with 1:50 hormone mix (1,2 μM sodium selenite, 0,8 μM progesterone, 4 mg/mL apotransferrin, 1 mg/mL insulin and 386 μg/mL putrescin (all from Sigma)) together with brain derived neurotrophic factor (BDNF), glial derived neurotrophic factor (GDNF), insulin growth factor (IGF)-1 (all 10 ng/mL, Prepotech) in the presence of 2% B-27, 2% knockout serum replacement and 1% Antibiotic–Antimycotic. Co-cultures were kept in culture for up to 3–4 weeks.

2.5. Immunocytochemistry and α-bungarotoxin labelling

For immunocytochemistry and labelling of AchR with α-bungarotoxin (BT) previously established methods were used (Demestre et al., 2005). The primary antibodies used were: mouse anti human actinin (Sigma Aldrich, 1:200), mouse anti-human desmin (DAKO, Denmark, 1:500), rabbit anti-human myosin heavy chain (MHC) (abcam, UK, 1:500), goat anti-human AChR (Serotec, Germany, 1:500) mouse anti-human Bassoon (Synaptic System, Germany, 1:1000). Fluorescence labelled secondary antibodies were: Alexa Fluor® 488, Alexa Fluor® 568, and Alexa Fluor®

647 (from Life technologies) diluted 1:1000. Conjugated α-BT-488 or 568 (Life technologies) was used at 5 ng/mL.

2.6. Quantitative one-step real time PCR

RT-PCR (qPCR) analysis was performed as previously described (Stockmann et al., 2011) Briefly, one-step real-time qPCR was carried out using the Rotor-Gene Q (Qiagen, Germany) and the QuantiTect SYBR Green RT-PCR kit (Qiagen). Primers for *PAX3*, *PAX7*, *MYF5*, *MYH6*, *MYOD1*, *MYOGENIN*, *DESMIN*, and *CHRNA1* were purchased from Qiagen as validated primers without sequence information (www.qiagen.com). Relative transcript expression was expressed as the ratio of target gene concentration to the housekeeping gene hydroxymethylbilane synthase from Qiagen (Illing et al., 2013).

2.7. Electrophysiology of myotubes

Electrophysiological experiments were performed as previously described (Stockmann et al., 2011). In brief, hiPSC derived myotubes and myotubes from a biopsy from a healthy donor were differentiated with Neurobasal A medium (Life technologies) supplemented with G5 (1×, Life technologies) GDNF, BDNF, IGF-1 (all 10 ng/mL), retinoic acid (0.1 μM, Sigma Aldrich) pumorphamin (50 ng/mL), cAMP (1 μM, Sigma Aldrich), neurotrophin 3, 4 (20 ng/mL, Prepotech), vitronectin (100 ng/mL, Sigma Aldrich), laminin (4 μg/mL), agrin (100 ng/mL) for 3 to 4 weeks, as described elsewhere (Guo et al., 2011, 2013).

In order to facilitate myotube contraction bath temperature was elevated to 37 °C (Guo et al., 2011). Electrical field stimulation of the cells was performed with an ELM-2 stimulator. The stimuli, typically set to 2 V and a duration of 1 ms, were applied at a frequency of 0.5 Hz. Changes of membrane current or voltage were recorded in the whole-cell recording mode using an EPC-9 amplifier and Patchmaster software (HEKA, Lambrecht, Germany; (Hamill et al., 1981)). Before recording, the cells were rinsed twice with an extracellular standard solution containing (in mM): 140 NaCl, 5 KCl, 1.5 $CaCl_2$, 10 glucose and 12 HEPES; pH 7.3. Patch pipettes were drawn from borosilicate glass with tip resistances between 3 and 6 MΩ when filled with the internal standard solution (in mM): 140 KCl, 2 $MgCl_2$, 4 ATPx2Na, 4 EGTA, 10 HEPES; pH 7.2. To improve sealing, tips were briefly dipped into 2% dimethylsilane dissolved in dichloromethane. All patch clamp recordings were performed at room temperature.

2.8. Data analysis and statistics

For counting cells in each coverslip ($n = 3$) 5 representative images were taken randomly. To quantify myoblasts desmin positive cells and the total number of cells labelled with DAPI were counted by using Image J Software (www.imageJ.nih.gov). Then the mean of the desmin positive/total number of cells ratio was calculated for each experiment ($n = 3$). To quantity myotubes the same procedure was used but in this case actinin positive cells with 2 or more nuclei and showing some degree of striation were counted.

Expression levels were compared using an un-paired *t-test*. All statistical analysis was performed using GraphPad Prism (Version 3.0) software. Results are represented as mean values ± standard error of the mean (SEM). Statistical significance levels were set to $p = 0.05$.

2.9. Ethics statement

All animal experiments were performed in compliance with the guidelines for the welfare of experimental animals issued by the Federal Government of Germany, the National Institutes of Health and the Max Planck Society. The experiments in this study were approved by the review board of the Land Baden-Württemberg, permit Nr. O.103. All human material used in this study has been approved by the ethical committee of Ulm University (Nr. 0148/2009, or 265/12) in compliance with the guidelines of the federal government of Germany and the Declaration of Helsinki concerning Ethical Principles for Medical Research Involving Human Subjects.

3. Results

3.1. Protocol for the selection of myogenic progenitors from hiPSC

Our first aim was to develop a method to generate hiPSC-derived myoblasts. In this respect, we established a method to separate myogenic progenitors from hiPSCs by CD34 positive selection. In a second step, we compared myogenic differentiation following the published protocol using PAX7 overexpression in hiPSCs (Darabi et al., 2012) and with myoblasts generated from a muscle biopsy from a healthy volunteer. A diagram of all 3 procedures is presented in (Fig. 1a–c). For the CD34-method, EBs were grown adherent. After one week in myogenic medium, cells migrated out of the EBs and formed a mixed population containing mesodermal cells (actinin positive cells) but also ectodermal (tubulin positive) and endodermal cells (β-catenin positive) (Supplemental Fig. S1). Thereafter, in order to specifically select myogenic progenitors, cells were immuno-purified for CD34 and kept in myogenic medium supplemented with human conditioned myogenic medium and FGF-2. In addition, to further purify myogenic precursors, small cells were gated by size using FACS analysis, plated as single cells and then further expanded. The mean average of myogenic clones derived from one single cell was 27.5 ± 7.5%, as assessed by morphology and further skeletal muscle characterisation described below. In addition, cultures generated from non-sorted cells were cultivated as cultures generated from sorted populations. The number of desmin positive cells was 3.157 ± 0.75% (in hiPSC 1) and therefore further myogenic expansion was not performed.

3.2. Comparison of myoblast and myotube differentiation protocols

We compared the myogenic cells derived by the three different methods according to morphological features and markers known to be characteristic for myoblasts. Myoblasts that developed after CD34 immuno-purification were spindle shaped (Fig. 2a) as shown in the phase contrast images, and were immunopositive against desmin (Fig. 2a), a marker specific for early myoblast differentiation (Paulin and Li, 2004). About 40% of cells were desmin positive from the two hiPSC cell lines that we originally differentiated into myoblasts (hiPSC 1: 37 ± 7.2%; hiPSC 2: 39 ± 6.3%). These results were quite similar to myoblasts derived from the primary human myoblast culture, which also displayed spindle shaped morphology (Fig. 2a). Here, 48 ± 6.3% of the cells were desmin positive (Fig. 2a). In contrast, myoblasts that have been differentiated according to the PAX7 method became desmin positive after 4 or more passages (Fig. 2a). An exact quantification was not conducted. PAX7 myogenic progenitors, however, also showed typical myoblast morphology (Fig. 2a). Myoblasts derived by all 3 methods were also actinin positive (Fig. 2a). We then differentiated myoblasts from all 3 protocols into myotubes. Following serum deprivation myoblasts started to fuse after approximately 7 days (Fig. 2b), and after 2 weeks in differentiation medium multinucleated syncytia formed (Fig. 2b). Actinin was also strongly expressed in early and mature myotubes from all 3 methods and showed the typical striatal pattern in mature fibrers that were differentiated for two weeks (Fig. 2b). Myotubes containing more than one nucleus, showing some degree of striation and strongly positive for actinin were also quantified relative to the total number of cells. Differentiated cultures form the human primary cultures and from the CD34 contained a similar percentage of myotubes (human primary: 26.96 ± 6.989, hiPSC1: 23.96 ± 6.396). Myoblast from the PAX7 method differentiated more efficiently and presented a higher number of actinin positive-myotubes (58.66 ± 7.499). Next, we analysed a selected set of mRNAs important for the myogenic lineage (Fig. 2b). Myoblasts generated by the CD34 selection

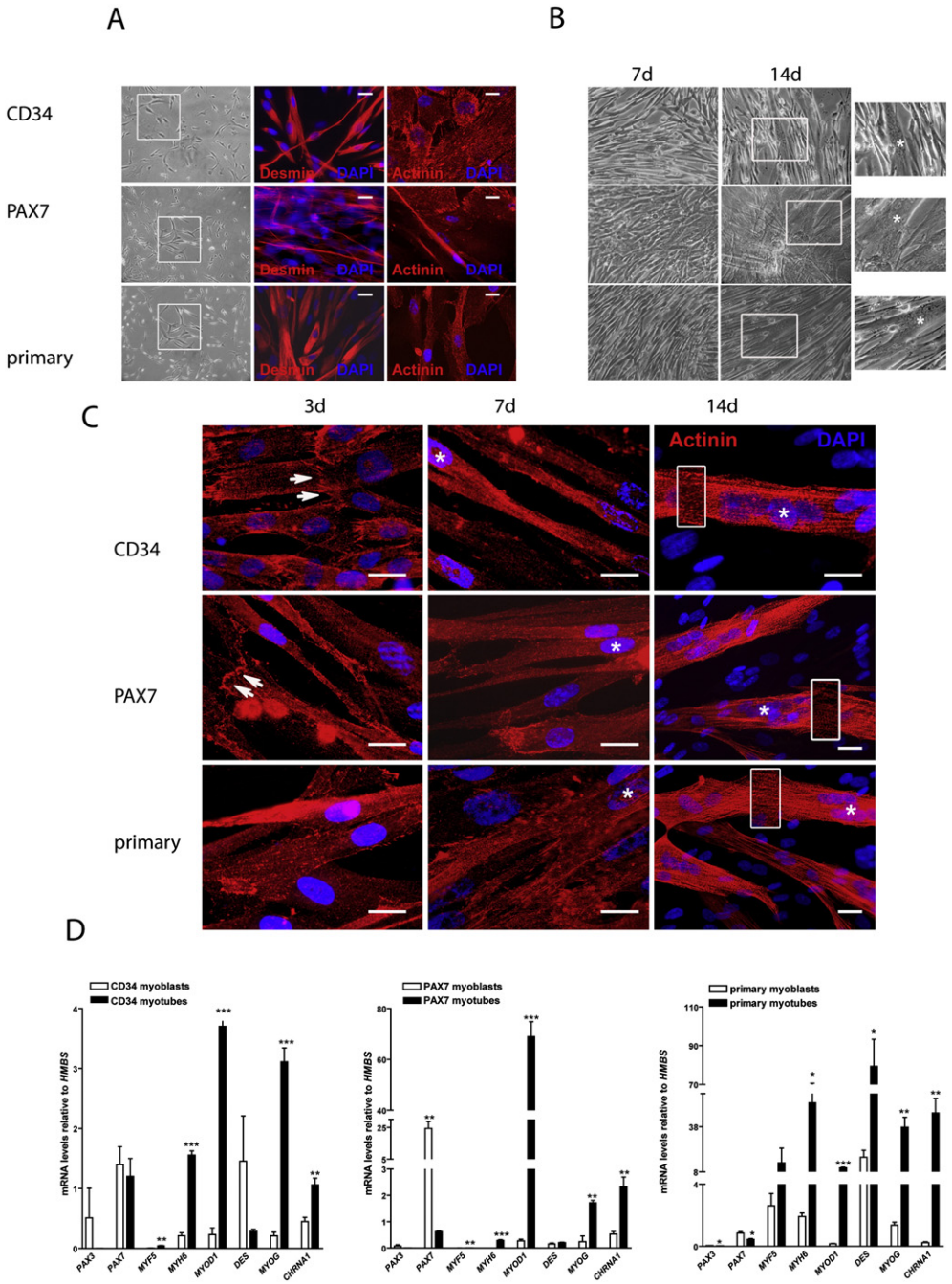

Fig. 2. A. Myoblast proliferation. Phase contrast micrographs representing proliferating myoblasts generated using the three methods. Myoblasts were round, small cells, proliferated very rapidly and were desmin and actinin positive. Representative myoblasts are magnified in the inserts. B. Myotube formation. Phase contrast micrographs representing myoblasts from all three methods fusing and forming short myotubes after 7 days in differentiation medium. At 21 days multinucleated fibres formed (*asterisks*). Myoblasts generated from the three methods were immunostained fort actinin at different stages of differentiation (3 days, 5 days, and 14 days). During differentiation cells fused and formed bi/tri nucleated myotubes (*asterisks*) and costameres (*arrows*). 14 days after differentiation typical striated multinucleated (*asterisks*) and fused fibres were seen. A typical striated pattern labelled with actinin is shown in the magnified inserts. Scale bar: 20 μm. C. Expression markers of myogenic differentiation. Myogenic markers were up-regulated at the mRNA level during myoblast proliferation and differentiation into myotubes. Unpaired *t-test* analysis comparing the expression of one gene during proliferation *versus* differentiation revealed statistical significance on the following genes: *MYF5*, *CHRNA1* ($p < 0.001$), *MYH6*, *MYOD1*, *MYOG* (all $p < 0.001$) using the CD34 protocol. For the PAX7 protocol: *PAX7*, *MYF5*, *MYOG* (all $p < 0.01$), *MYH6*, *MYOD1* (all $p < 0.001$). The following genes were up-regulated in myotubes derived from a human biopsy: *PAX3*, *PAX7*, *MYH6*, *DES* ($p < 0.05$), *MYOG*, *CHRNA1* ($p < 0.01$) *MYOD1* ($p < 0.001$). $^{*}p < 0.05$, $^{**}p < 0.001$, $^{***}p < 0.001$. *DES* = *DESMIN*, *MYOG* = *MYOGENIN*.

expressed low levels of *PAX3*, a paired box transcription factor, which contributes to early striated muscle development by playing a critical role in embryonic muscle formation. The paired box transcription factor *PAX7*, an early myogenic marker important for the maintenance of *satellite cells* and *DESMIN*, also an early myogenic marker, were slightly up-regulated. Following differentiation to myotubes, *PAX7* mRNA expression remained similar. However, *MYH6*, *MYOD1* and *MYOGENIN*, markers specific for early and late myotube differentiation, were significantly up-regulated (Fig. 2d). Myoblasts generated by the PAX7 method expressed – as expected – high levels of *PAX7*, while *DESMIN* was expressed at low levels. When doxycyclin and serum were removed from the cultures to stop the induction of *PAX7* and to force the cells to fuse and differentiate *PAX7* mRNA dropped to very low levels and markers of final differentiation were expressed (*MYH6*, *MYOD1* and *MYOGENIN*) (Fig. 2d). In comparison, biopsy-derived myoblasts expressed low levels of *PAX3*, slight *PAX7* up-regulation and high

DESMIN levels. Further differentiation of these cells resulted in high levels of *MYH6* and *MYOGENIN* (Fig. 2d). The mRNA for *CHRNA1*, the gene for the muscle specific AchR-α, was expressed at low levels in proliferating myoblasts and was up-regulated in differentiating myotubes from all three protocols (Fig. 2d).

3.3. Functional properties of myotubes

The main characteristic of muscles is the ability to contract in response to electrical stimulation induced by the motoneuron. To address this, we cultivated myotubes in a serum free medium containing growth factors necessary for myotube maturation and contraction (Guo et al., 2011). Then, we analysed the functionality of myotubes derived from the three different myogenic cell lines by maintaining the cultures in a bath at 37 °C. None of the myotubes contracted spontaneously, although myotubes derived from the human biopsy contracted spontaneously when removed from the incubator, this only lasted a few seconds. However, by maintaining the cultures at 37 °C and following electrical field stimulation we were able to detect contractions in all 3 lines.

In CD34-hiPSC derived-myotubes 3 weeks after differentiation, about 4 small long multinucleated fibres per dish contracted rhythmically upon electrical stimulation (supplemental movie). These cells displayed resting membrane potentials (mP) from −50 to −70 mV, were able to generate action potentials and responded to acetylcholine (Ach) (Fig. 3a,b). In PAX7 hiPSC-generated myotubes 1–2 long multinucleated and striated myotubes per dish were contracting when electrically stimulated. Fibres had a resting membrane potential of −60 to −64 mV and also responded to Ach. However, a clear action potential could only be recorded in more mature fibres that had been cultured for more than 4 weeks in the differentiation medium (supplemental movie, Fig. 3a,b). Following electrical stimulation, myotubes derived from a human muscle biopsy presented contractions. This was observed in about 5 fibres per dish (supplemental movie). These fibres exhibited a resting membrane potential from −55 to −73 mV, showed action potentials and responded to Ach (Fig. 3a,b). This data provides further indications of the high level of maturation of our cultured hiPSC derived myotubes at the functional level.

3.4. Neuromuscular junction formation between hiPSC-derived motoneurons and hiPSC derived myotubes

A key characteristic of myotubes and muscle fibres is the ability to connect with motoneurons *via* neuromuscular endplates. Since we had demonstrated that our cultures respond to Ach, a key molecule that binds to the AchR present in the muscle opening ligand gated sodium channels; and sequentially initiating different steps to produce muscle contraction *in vivo*. We first examined myotubes using antibodies directed against the AChR-α that is solely localised in skeletal muscle cells. Myotubes generated using the CD34- or PAX7 methods as well as from the human biopsy showed AChR-α staining in clusters along the fibres (Fig. 4a). These cells were also positive for two specific myotube markers, myosin heavy chain (Fig. 4a) and actinin (Fig. 4a). In co-cultures of hiPSC derived motoneurons and hiPSC CD34-derived myotubes after 1 day neurites deriving from the motoneuron-spheres started to grow out from the motoneuron-spheres seeking contact with the myotubes (Fig. 4b). Two weeks later, outgrowing neurites made contacts with myotubes (Fig. 4b) as shown in the phase contrast images. At this stage α-BT AchR-α formed big clusters (Fig. 4c) but fully

A

	resting MP (mV)	Ach (100μM) (mA)	Capacitance (pF)	AP
CD34 21 days n=3	-56±4.41	-12,92±4.107	228±31.43	Yes
PAX7 28 days n=3	-53±6.58	-1.135±0.088	119.3±33.08	Yes
primary 21 days n=3	-65.33±5.36	-16.50±6.882	147±41.48	Yes

B

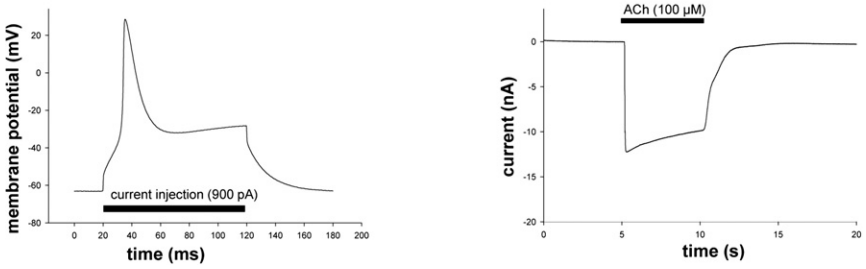

Fig. 3. A. Table. Electrophysiological properties of myotubes in culture after differentiation. B. Representative traces of current-clamp measurements and the generation of and action potential following acetylcholine treatment. Abbreviations: mP = resting membrane potential, AP = action potential.

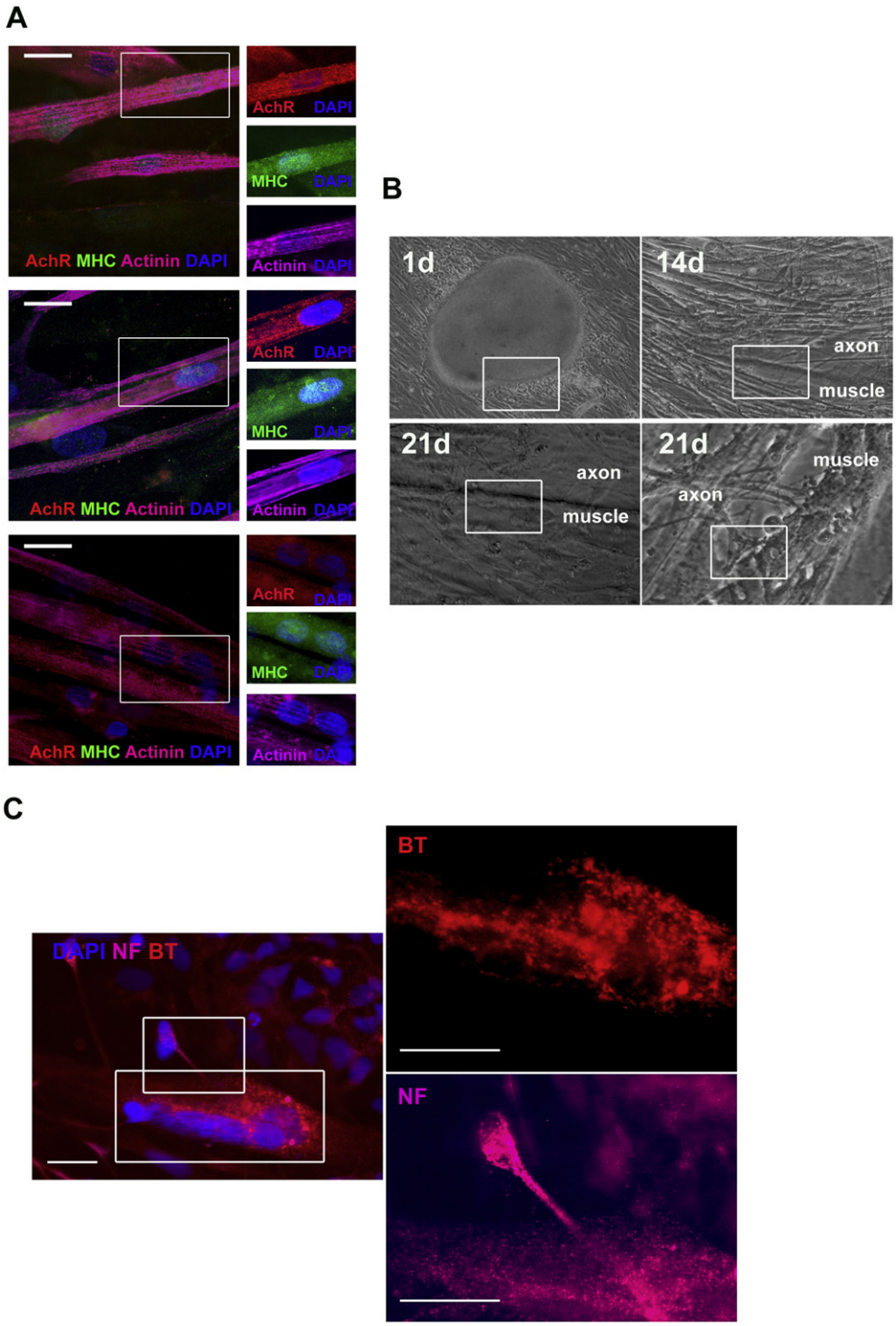

Fig. 4. A. Myotube characterisation. Myotubes differentiated for 21 days derived from all three methods (CD34, PAX7 and human muscle biopsy) were triple immunolabelled for MHC, actinin and AchR-α. B. Neurite extension during co-cultivation. Representative phase contrast images of a hiPSC derived moto-neurosphere 1 day after plating. Small neurites were out growing from the moto-neurosphere. After 14 and 21 days neurites made contacts with myotubes. Representative areas are shown in the inserts. C. Aggregation of AchR clusters. hiPSC-derived motoneurons were co-cultured with CD34-enrichment derived myotubes. 14 days after co-cultivation AchR clusters labelled with α-BT were detected. Scale bar: 20 μm, MHC = myosin heavy chain, AchR = acetylcholine receptor, BT = bungarotoxin. NF = neurofilament heavy chain.

mature NMJ were still not fully developed. After 21 days numerous nerve–muscle contacts were established (Fig. 5a) and AchR formed mature NMJs as shown by the typical curvy morphology labelled with α-BT, and tubulin positive neurons could be shown to make contacts with the end plate (Fig. 5a). Similarly, co-cultures were labelled with the pre-synaptic marker Bassoon that labels synaptic contacts along axons labelled with neurofilament (NF) (Fig. 5b, top). At the end plate area NF positive axons were in close apposition with α-BT labelled NMJ. In the same area small punctae stained with Bassoon were also detected, indicating pre and post-synaptic contacts (Fig. 5b, bottom).

Similarly, myotubes generated with the PAX7 method were co-cultured with hiPSC derived motoneurons for one and 3 weeks. Again, AchR formed typical clusters labelled with α-BT in early motor end plate formation and displaying a typical NMJ morphology in mature cultures, and tubulin positive neurons established contacts with mature end plates (Supplemental Fig. S2a,b).

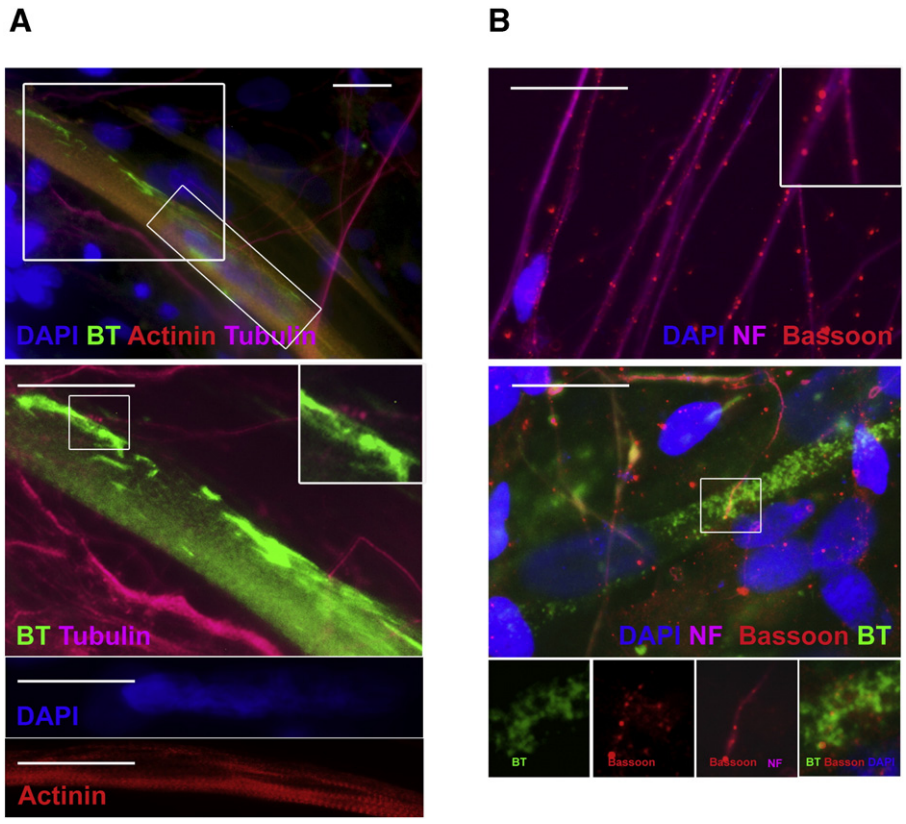

Fig. 5. A. Maturation of AchR clusters and NMJ formation. hiPSC-derived motoneurons were co-cultured with CD34-enrichment derived myotubes. After 21 days of co-cultivation the formation of mature end plates was observed by α-BT labelling. B. Formation of synaptic boutons and pre and post-synaptic appositions at the end plate. After 21 days in co-culture pre-synaptic terminals labelled with Bassoon were strongly detected along the axons (top figure). At the end plate region a close apposition between pre-synaptic and post-synaptic markers could be detected, as shown by Bassoon and α-BT stainings (bottom figure). Scale bar: 20 μm, AchR = acetylcholine receptor, BT = bungarotoxin. NF = neurofilament heavy chain. Inserts represent magnified areas at ×200.

In addition, CD34 derived myotubes were tested for their capacity to form end plates by using a human-rat hybrid co-culturing system with hiPSC myotubes and primary motoneurons isolated from rat embryonic tissue. After two weeks end-plates stained for α-BT were formed that have been induced by neurons positive for tubulin. Muscle cells were stained with antibodies directed against actinin (Supplemental Fig. S2c).

4. Discussion

In the present study, we show that a NMJ model can be established using different methods to derive myotubes from myogenic lineage precursor cells including CD34+ based enrichment. Moreover, we established a protocol to create a motor unit (motoneuron and contacting muscle cells) both derived from the same hiPSC line as a novel *in vitro* read out system.

Generating myoblast cultures from primary skeletal muscle tissue after a muscle biopsy is a well-established technique (Malatesta et al., 2013). However, access to biopsy-derived skeletal muscle to set up myoblast cultures is often restricted and the analysis of NMJs limited. In contrast, access to tissues from which hiPSc can be set up, such as hair-root cells, is simple, since this is a non-invasive procedure and ethical permission is more easily to be obtained. In addition, hiPSCs have successfully been differentiated into many different cell types, such as motoneurons, pancreatic cells and Schwann cells (Bilic and Izpisua Belmonte, 2012). In particular, we have previously shown that motoneurons can be derived reliably (Stockmann et al., 2011). For the formation of NMJs motoneurons need to liaise with myofibers. To establish a model for the NMJ from hiPSc generated from the same individual we therefore next derived skeletal muscle cells in two ways. First, we used a CD34+ enrichment method in which cells outgrowing from EBs were selected for CD34, a marker expressed by satellite cells (Jankowski et al., 2002). Secondly, we used a protocol over-expressing the myogenic factor PAX7 to force myogenic differentiation (Darabi et al., 2012; Tanaka et al., 2013; Abujarour et al., 2014).

After CD34+ enrichment myoblast generation was achieved by cell selection and consecutive culture under specific myogenic conditions. This resulted in cultures that closely reflected the morphology and expression profile of cells of the early myo-lineage of primary cultures with low expression of *PAX7*, and desmin and actinin localisation in myoblasts. Finally, we differentiated myoblasts into myotubes and assessed their morphological and functional features. Using either method (CD34 or PAX7), or primary myoblasts, we could identify multinucleated striated myotubes. Myotubes expressed actinin and MHC together with *MYOD1* and *MYOGENIN* mRNA, had a resting membrane potential and contracted following electrical stimulation. Myotubes derived by the three methods expressed the muscle specific AchR and generated action potentials in response to Ach indicating the binding of Ach to the specific AchR and the depolarisation of myotube membranes. However, myotubes generated by PAX7 induction took longer to mature and be able to generate an action potential, and only a few contracting fibres were observed.

Since the analysis of a complete motor unit is essential for the understanding of pathophysiological mechanisms of motoneuron diseases (Moloney et al., 2014), or conditions involving the NMJ, we established an *in vitro* model of motor units. To that end, we cultivated hiPSC cell lines from healthy control subjects and differentiated hiPSCs from the same cell line into motoneurons and myotubes. Subsequently, we co-cultivated these two cell types together *in vitro*. In the first week clusters of AchRs appeared, and early outgrowing neurites from motoneurons

became visible in close vicinity of future end plate regions. At 3 weeks of co-cultivation motoneurons seemed to actively induce the maturation of muscle cells and strong AChR aggregation. Finally, NMJs could readily be detected.

5. Conclusions

We report on a model of the NMJ derived from the same parental hiPS cell line. This model should be very useful in motor unit research in health and disease. For instance, the model will help to investigate the effects of specific neurodegenerative mutations on the morphology and physiology of NMJs. The co-culturing of affected motoneurons with control muscle cells and *vice versa* will for example help to decipher the role of both cell types for the motoneuron disease pathogenesis.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scr.2015.07.005.

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