Generation of human muscle fibers and satellite-like cells from human pluripotent stem cells *in vitro*

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Progress toward finding a cure for muscle diseases has been slow because of the absence of relevant cellular models and the lack of a reliable source of muscle progenitors for biomedical investigation. Here we report an optimized serum-free differentiation protocol to efficiently produce striated, millimeter-long muscle fibers together with satellite-like cells from human pluripotent stem cells (hPSCs) *in vitro*. By mimicking key signaling events leading to muscle formation in the embryo, in particular the dual modulation of Wnt and bone morphogenetic protein (BMP) pathway signaling, this directed differentiation protocol avoids the requirement for genetic modifications or cell sorting. Robust myogenesis can be achieved *in vitro* within 1 month by personnel experienced in hPSC culture. The differentiating culture can be subcultured to produce large amounts of myogenic progenitors amenable to numerous downstream applications. Beyond the study of myogenesis, this differentiation method offers an attractive platform for the development of relevant *in vitro* models of muscle dystrophies and drug screening strategies, as well as providing a source of cells for tissue engineering and cell therapy approaches.

INTRODUCTION

Until recently, little progress has been made in differentiating hPSCs toward a myogenic fate. This in turn has hampered both the development of relevant human *in vitro* models of muscle diseases and the assessment of cell therapy approaches. Here we report an optimized directed differentiation protocol for *in vitro* production of mature muscle fibers and their associated progenitors from hPSCs. This is a detailed version of the protocol reported in Chal *et al.*[1], with a number of improvements, including the expansion and cryopreservation of the hPSC-derived myogenic progenitors. By mimicking the early signaling events that occur during paraxial mesoderm specification in the embryo—in particular the simultaneous activation of Wnt and inhibition of BMP signaling pathways—this protocol recapitulates the essential steps of skeletal myogenesis *in vitro* in <30 d. The resulting fibers show spontaneous contraction and provide a niche for associated Pax7-positive satellite-like cells. An additional subculturing step allows for the preparation of proliferative myogenic populations that can be further amplified, cryopreserved or differentiated into muscle fibers. This protocol is readily amenable to multiformat *in vitro* cell assays and various downstream applications. Overall, the recapitulation of myogenesis in a dish will be an invaluable tool for the muscle, stem cell, muscle physiology and pathology, and developmental biology communities for both basic and applied research.

Development of the protocol

Directed differentiation methods aim to expose PSCs to differentiation cues, thus allowing the sequential recapitulation of key stages of paraxial mesoderm development and their differentiation into skeletal muscle. Early attempts to generate skeletal muscles used spontaneous differentiation of embryoid bodies, resulting in heterogeneous differentiation and low efficiency[2,3]. Progress in 2D-culture systems was slow until recently, when manipulations of key signaling pathways involved in paraxial mesoderm specification were incorporated into differentiation protocols. In the embryo, Wnt signaling is required for paraxial mesoderm induction[4–7] (for a review, see Chal *et al.*[8]). In embryos that are mutant for Wnt3a or its targets T (Brachyury) or Tbx6, ectopic neural tubes form instead of the paraxial mesoderm in the posterior part of the embryo[7,9–11]. In PSC culture, Wnt signaling promotes mesodermal differentiation[12–15], and several recent myogenic induction protocols rely on early Wnt activation[1,16–19]. Wnt signaling also acts upstream of the fibroblast growth factor (FGF) pathway in paraxial mesoderm precursors of the tail bud, by triggering expression of the Fgf8 ligand[20]. Later on, at the somite level, secreted Wnts from surrounding tissues are also critical for dermomyotome specification (for review, see Geetha-Loganathan *et al.*[21]).

BMP signaling controls the medio-lateral identity of posterior mesoderm cells[22–26]. In amniote embryos, high BMP leads to the specification of more lateral tissues such as extraembryonic mesoderm, or lateral-plate mesoderm (LPM), which is a tissue that contributes to the limbs and body wall mesenchyme and to the long bones of the limbs but does not form skeletal muscle. Ectopic BMP signaling can divert cells fated to form paraxial mesoderm to a lateral-plate fate *in vivo*[22]. Conversely, BMP loss-of-function mutation leads to expanded paraxial mesoderm domain[27]. *In vivo*, the paraxial mesoderm is protected from LPM BMP signaling by the inhibitors noggin and chordin, which are expressed by the notochord, the intermediate mesoderm and the dorsal somite[24,25,28]. *In vitro*, in the absence of proper BMP signaling inhibition, the early paraxial mesoderm induced by Wnt signaling produces BMP4 and 'drifts' by an autocrine effect to generate LPM derivatives (J.C., Z.A.T. and O.P., unpublished data). At the heart of the protocol presented here lies the use of dual modulation of the Wnt and BMP pathways to efficiently induce paraxial mesoderm from hPSCs. This is a prerequisite for the production

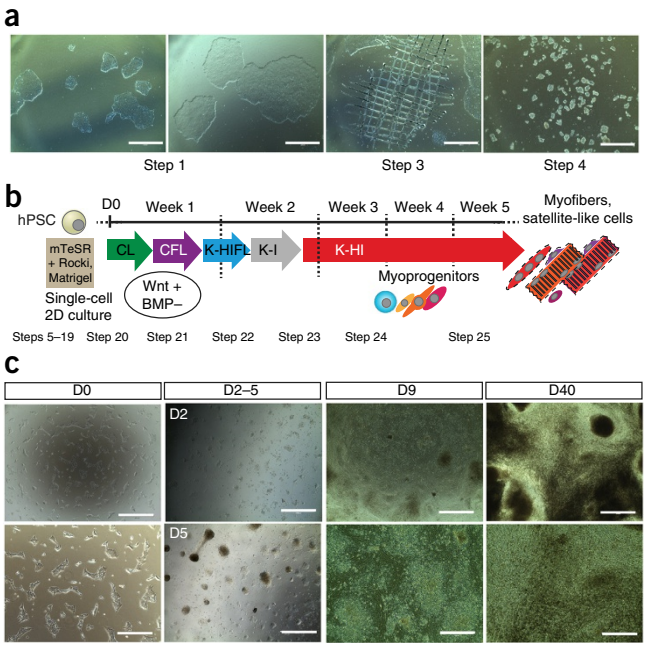

Figure 1 | Maintenance and stepwise protocol for myogenic differentiation *in vitro* and phase-contrast images of differentiating cultures (Steps 1–25). (**a**) Maintenance of undifferentiated human pluripotent stem cells (hPSC) culture. Representative NCRM1 hPSC colonies cultured on a Matrigel-coated plate in mTeSR medium. Small colonies (left) grow within a few days to larger colonies (second from left) ready for passaging or for differentiation setup. Note the well-defined, refringent colony borders. Note the tightly packed and smooth appearance of the colonies' centers. (second from right) Representative grid pattern for hPSC colonies using the cutting method[61,62] for undifferentiated hPSC colony passaging. The resulting colony pieces have been collected. (Right) hPSC colony pieces immediately after transfer to a new Matrigel-coated dish. Scale bars, 1 mm. Corresponding procedure steps are shown below. (**b**) Diagram of the myogenic differentiation protocol for hPSCs, highlighting key culturing steps (arrows) and their associated time scale. hPSCs were seeded as single cells on Matrigel-coated dishes (left) and differentiated over several weeks. Mature muscle fibers and their associated progenitors can be obtained directly from the primary culture. The sequence of media aims to modulate specific signaling pathways, notably the Wnt and BMP signaling pathways. C: CHIRON99021, L: LDN193189, F: FGF-2, K: KSR, H: HGF, I: IGF-1. Corresponding procedure steps are shown below. (**c**) Representative phase-contrast images of the differentiating hiPS11a cultures following the protocol depicted in **b**. Time course is shown in days (D*x*). Scale bars, 1 mm (top panels); 400 µm (bottom panels, except for D5, which is 1 mm).

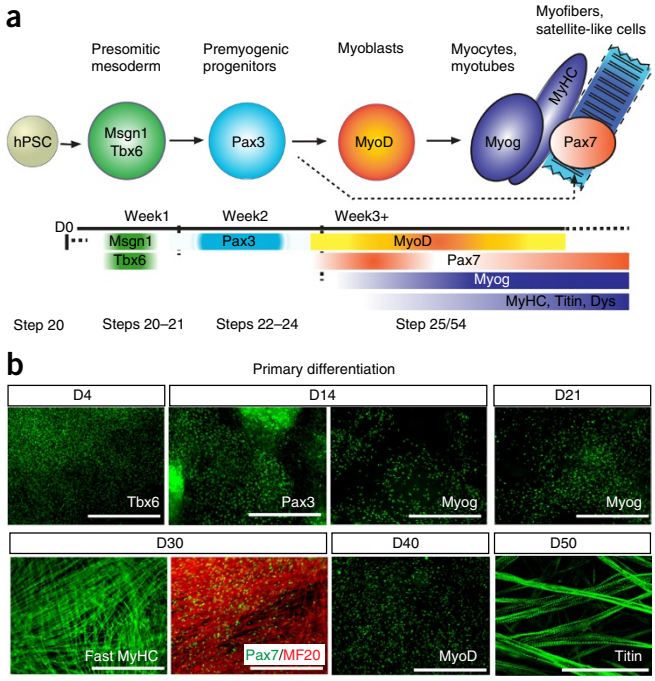

Figure 2 | Immunophenotyping of hPSC-derived myogenic cultures (Steps 26–36). (**a**) Diagram mapping the developmental stages of paraxial mesoderm differentiation and skeletal myogenesis from human pluripotent stem cells (hPSCs) highlighting key markers and a time line of their approximate expression. Dys, dystrophin. (**b**) Primary differentiation. Representative expression sequence of markers detected by immmunohistochemistry (**Table 2**) in hiPS11a culture differentiated according to this protocol. Paraxial mesoderm (Tbx6-positive) population peaks around day 4 of differentiation. Dermomyotomal progenitors (Pax3-positive) are detectable as early as 1 week after the start of differentiation and increase during week 2. Starting at 2 weeks, large fields of myogenin (Myog)-positive myocytes appear. By 4 weeks, cultures comprise large sheets of MyHC-positive myofibers (fast MyHC and MF20-positive) and Pax7-positive satellite-like cells. Scale bars, 400 µm. By days 40–50, the primary differentiation generates a large amount of skeletal (MyoD-positive) myoblasts (scale bar, 400 µm) and (titin-positive) striated myofibers (scale bar, 100 µm).

of skeletal myogenic progenitors in large numbers. Other studies have shown that Wnt activation and BMP inhibition can produce mesodermal fates from hPSCs, such as intermediate mesoderm[29] and chondrogenic mesoderm[30], which are closely related to the paraxial mesoderm. On the other hand, activation of BMP signaling has been used to produce anterior LPM derivatives such as cardiovascular cell types[31–34]. Although little is known about the culture of early embryonic paraxial mesoderm from primary cultures[35–38], myogenic progenitors and skeletal myoblast cultures have been extensively studied *in vitro*[39–43].

The protocol described here stems from the application of a serum-free protocol developed for adherent mouse embryonic stem cell (mESC) culture to human pluripotent cell cultures[1] (**Fig. 1**). At its core lies a dual modulation of Wnt and BMP pathways. It is based on our understanding of paraxial mesoderm development *in vivo*, in which Wnt is activated to specify early paraxial mesoderm, whereas BMP is inhibited to prevent the newly specified paraxial mesoderm cells from drifting to a lateral-plate fate[22]. In the absence of BMP inhibition, Wnt-activated PSCs differentiate preferentially to LPM derivatives such as endothelial progenitors[44]. For the hPSC protocol, the first step of the mESC protocol aiming at producing epiblast stage cells was removed, as hPSCs are more related to mouse epiblast-derived stem cells[45]. Fine-tuning of factor concentrations and exposure time was also necessary to obtain optimal paraxial mesoderm induction and subsequent skeletal myogenesis (**Fig. 2**). Long-term (>1 month) differentiated hPSC cultures are very dense; they produce abundant extracellular matrix (ECM), but retain Pax7$^+$ myogenic progenitors. We noticed that dissociation and replating of these differentiated cultures led to a substantial enrichment in myogenic cells (**Fig. 3**). This step allows large-scale production of homogeneous myogenic cultures that are amenable to cryostorage or diverse culture setups (**Figs. 4** and **5**).

Key advantages of this differentiation protocol are the ease of implementation of a simple and robust 2D-culture system, high yields, the production of both myogenic progenitors and

mature fiber cell types, and a shorter experimental time line as compared with those of existing protocols[16,19,46–48]. Unlike other protocols, this method does not require genetic manipulation such as forced expression of transcription factors[49–53], nor does it rely on cell sorting strategies to purify the populations of interest[17,54–57]. Primary myoblast cultures obtained from biopsies and myogenic cell lines do not usually generate mature fibers but produce shorter, irregularly shaped myotubes that lack striation. By contrast, the protocol presented here describes how to generate striated millimeter-long skeletal muscle fibers *in vitro*, in 30 d, thus providing the relevant cell types for the study of both embryonic development and mature muscle diseases.

Overview of the procedure

The starting material can be standard human ESC or human induced pluripotent stem cell (hiPSC) culture[58–62] (**Fig. 1a**). Briefly, cells are dissociated to single cells and seeded at low density on Matrigel-coated wells. The cells are left to recover in maintenance conditions until they form small irregular aggregates, at which time (day (D)0) they are changed to a sequence of differentiation media (**Fig. 1b**). This sequence initially aims to generate induced paraxial mesoderm progenitors (iPAMs) that correspond to the presomitic mesoderm stage *in vivo*, and then it goes on to promote differentiation of iPAMs into skeletal muscles within 30 d (**Figs. 1** and **2**). In addition to myofibers, the hPSC-derived culture retains myogenic progenitors, including Pax7$^+$ satellite-like cells (**Fig. 2**). These progenitors can be expanded to generate myogenic subcultures that can in turn be cryostored, re-differentiated (**Figs. 3** and **4**) or used for various downstream applications (**Fig. 5**).

Applications of the protocol

This protocol allows for the production of myogenic progenitors, satellite-like cells and myofibers, which enable the use of novel hPSC-based models to study important fundamental and applied skeletal muscle biology questions (**Table 1**). The expansion of hPSC-myogenic cultures is compatible with various experimental designs (**Fig. 5**). Below we discuss the potential applications of the different cell types generated by the protocol.

Paraxial mesoderm. The protocol describes a novel, tractable, *in vitro* system for the study of paraxial mesoderm formation (**Fig. 2**). It is amenable for quantitative and real-time imaging and flow cytometry analysis. Important developmental biology questions related to paraxial mesoderm development can be investigated, including the specification of neuro-mesodermal progenitors, segmentation, somitogenesis and lineage specification[63,64]. Combined with genetic manipulations, it allows the assessment of the role of individual genes in normal and pathological conditions. Production of hPSC-derived myogenic progenitors may also be used as a more relevant and standardized source of cells for bioengineering approaches that have traditionally relied on cell lines and biopsies (**Fig. 5**).

Myotubes and fibers. One of the main advantages of this protocol is the production of both myogenic progenitors and millimeter-long, striated, often spontaneously contracting, skeletal myofibers from hPSCs (**Figs. 2** and **4**). Primary differentiation or secondary differentiation after subculturing allows the production of myofibers on demand. This culture system allows for

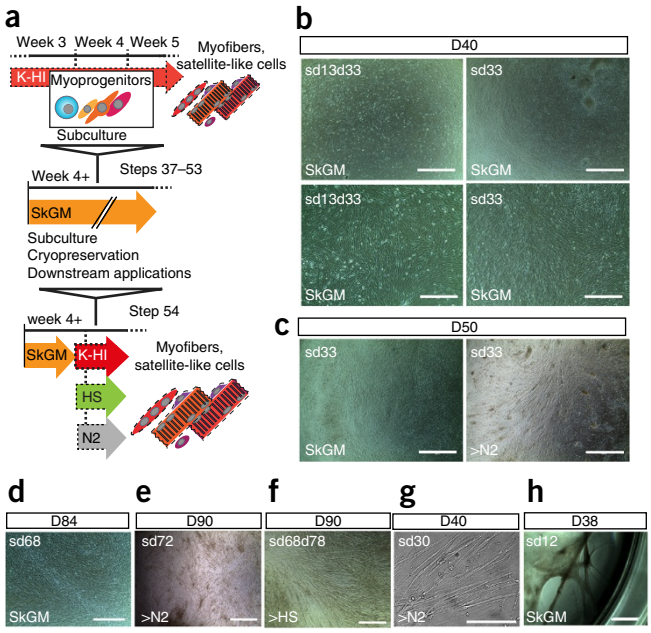

Figure 3 | Subculturing of hPSC-derived myogenic culture (Steps 37–54). (**a**) Diagram of the subculturing protocol, expansion and secondary myogenic differentiation. (Top) After 3 weeks of differentiation, the culture can be enzymatically dissociated for passaging. (Middle) Similar to a myogenic cell line, subcultures can be amplified in SkGM to generate frozen stocks and for downstream applications. (Bottom) As needed, the culture can be further subcultured at high density and differentiated to myofibers. H, HGF; HS, horse serum–based medium I, IGF-1; K, KSR; N2, N2-based medium; SkGM, skeletal muscle growth medium. (**b**–**h**) Representative phase-contrast images of hPSC-myogenic progenitor subculture according to the protocol depicted in **a**. Inset label sd*x*d*y* indicates passage at D*x* and D*y* of differentiation. Except where otherwise noted, re-differentiated subcultures were first maintained for 4 d in SkGM medium before being changed to differentiation medium. (**b**) 40-d-old hiPS11a cultures passaged once or twice as indicated and expanded in SkGM medium. Compare with primary differentiation at day 40 (**Fig. 1c**). (Top panels) Scale bars, 1 mm; (bottom panels) scale bars, 400 µm. (**c**) 50-d-old hiPS11a cultures passaged once and maintained in SkGM medium for 17 d (left) or further differentiated in N2 medium after 4 d in SkGM medium (right). Scale bars, 1 mm. Note the elongated refringent myofibers in N2 medium (>N2). (**d**) NCRM1-derived myogenic subculture that was subcultured once at D68, maintained in SkGM and imaged at D84. Scale bar, 400 µm. (**e**) Long-term H9 myogenic culture passaged and re-differentiated in N2 medium (>N2) with fully differentiated millimeter-scale skeletal muscle fibers. Scale bar, 1 mm. (**f**) Long-term NCRM1 myogenic culture passaged twice, maintained in SkGM medium for 4 d and re-differentiated in horse serum 2% (>HS) with differentiated millimeter-scale skeletal muscle fibers. Scale bar, 1 mm. (**g**) Detail of skeletal myofibers in NCRM1 subculture after 10 d of secondary differentiation in N2 medium (>N2) according to **a**. Scale bars, 100 µm. (**h**) Spontaneous bundle formation in 38-day-old hiPS11a myogenic cultures maintained at high density in SkGM. Scale bar, 1 mm. "N2" indicates culture that was first amplified with SkGM but was then shifted to another medium (e.g., N2 or K-HI).

the study of skeletal myogenesis, including important events that are difficult to visualize *in vivo*, such as myoblast fusion, satellite cell formation, myofibrillogenesis and fiber type specification. hPSC-derived myogenic cultures are a valuable alternative to primary myogenic cultures and immortalized cell lines. They are amenable to scaling up or miniaturization for drug screening[65], and they can be combined with 3D bioengineered substrates (**Fig. 5a**), tissue array/chip[66,67] (**Fig. 5b**) and complex electrophysiology or optogenetic time-lapse platforms[68–78]. Miniaturization

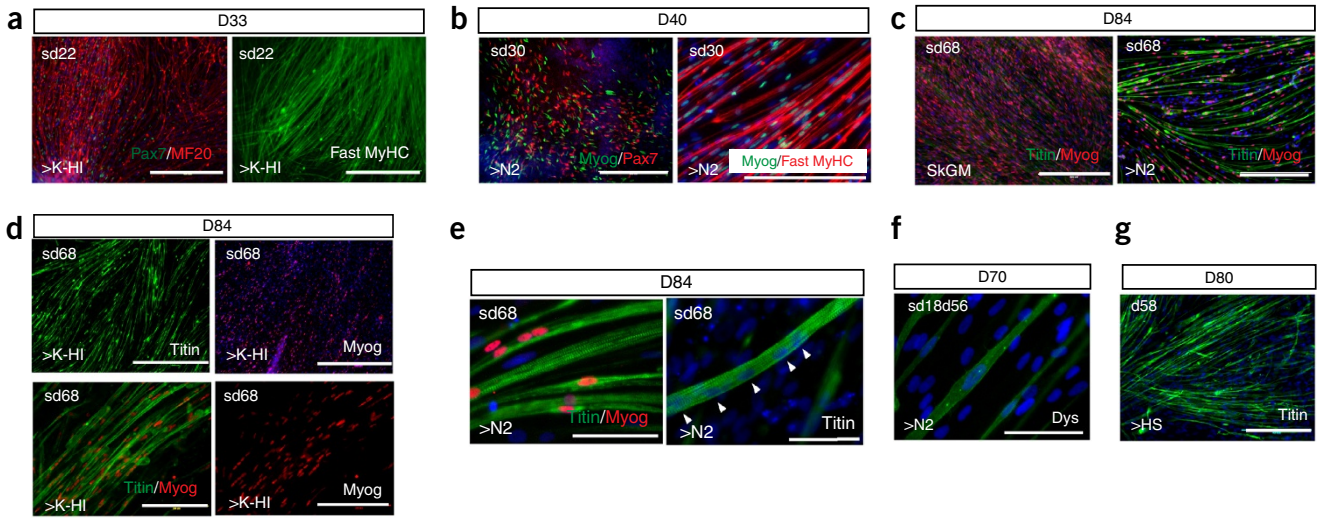

Figure 4 | Immunophenotyping of myogenic subcultures (Steps 26–36). Subcultures can be maintained in SkGM medium or re-differentiated to obtain myofibers. Except where otherwise noted, re-differentiated subcultures were first maintained for 4 d in SkGM medium before changing to differentiation medium. Inset label sdxdy: passage at Dx and Dy of differentiation. (**a**) hiPS11a culture passaged once and re-differentiated in DK-HI (>K-HI) medium. (Left) Large MyHC (MF20)-positive skeletal fibers and associated Pax7-positive progenitors are detected within 10 d. (Right) >10-µm-wide fibers are fast MyHC-positive. Scale bars, 400 µm. (**b**) 40-d-old NCRM1 cultures passaged once and re-differentiated in N2 (>N2) medium. (Left) Differentiated subcultures retained a Pax7-positive population interspersed with differentiated Myog-positive cells. (Right) Most of the Myog-positive myonuclei are incorporated in large millimeter-size fast MyHC-positive skeletal fibers. Scale bars, 400 µm. (**c**) Long-term NCRM1 culture passaged once, expanded in SkGM medium and re-differentiated (right) or not (left) in N2 medium. Passaging allows for a homogeneous amplification of progenitors that in turn give rise to myocytes (myogenin-positive) that can be differentiated to mature striated skeletal muscle fibers (titin-positive). Scale bars, 400 µm. (**d**) Long-term NCRM1 culture passaged once, maintained in SkGM for 4 d and re-differentiated in DK-HI medium. (Top) Note large yield of skeletal fibers evidenced by titin and Myog expression. (Bottom) Detail of the myofibers. Scale bars, 1 mm (top), 200 µm (bottom). (**e**) Sarcomeric organization and multinucleation of NCRM1-derived myofibers re-differentiated in N2 medium. Nuclei are counterstained with Hoechst (blue). Arrowheads indicate aligned myonuclei. Scale bars, 100 µm. (**f**) Example of hiPS11a-derived dystrophin (Dys)-positive myofibers (green) obtained after multiple passaging. Nuclei were counterstained with Hoechst (blue). Scale bar, 100 µm. (**g**) Cryopreservation and recovery. Dissociated hiPS11a culture was cryopreserved at day 58, thawed, expanded in SkGM and re-differentiated in horse-serum–based medium (>HS). Nuclei were counterstained with Hoechst (blue). Note the large fields of titin-positive myofibers. Scale bar, 400 µm.

is necessary for drug screening strategies that aim to identify compounds that are able to promote progenitor proliferation or fiber maturation[79,80]. Furthermore, coculture with other relevant cell types could allow the study of important aspects of muscle biology, namely, the neuromuscular junction (motoneurons)[81–89], the myotendinous junction (tenoblasts)[90,91], vascularization (endothelial cells)[92–95] and metabolic coupling (adipocytes)[96]. Healthy cells can also be directly compared with cells harboring various mutations. *In vitro* models of human muscular dystrophies involving forced expression of myogenic factors such as Pax3/7 or MyoD in patient iPSCs to induce myogenesis have been reported, but the physiological relevance of the induced fibers remains to be established[97–102]. The protocol presented here recapitulates the developmental sequence of myogenic differentiation from hPSCs without introduction of exogenous genetic material and thus may provide more physiologically relevant models of these diseases. This may eventually allow the development of *in vitro* strategies for therapeutic approaches[103–108]. Muscle tissue engineering is a very active field that has so far essentially relied on primary cultures derived from human or animal biopsies, and immortalized cell lines. Such applications will benefit from an unlimited and reproducible source of human myofibers and their progenitors, including satellite-like cells[68,109–113].

Satellite-like cells. In adult muscle, quiescent stem cells ($Pax7^+$ satellite cells) found in very small numbers in close association with muscle fibers allow for muscle repair during regeneration[114,115]. Elegant mouse genetics experiments have shown that satellite cells represent the key population allowing muscle regeneration *in vivo*[116–118]. The transplantation of human skeletal myoblasts was shown to be inefficient, as many grafted cells die and thus fail to engraft in the host[119–122]. By contrast, even minute numbers of satellite cells efficiently contribute to muscle regeneration in injured or dystrophic mouse muscles[123–128]. Thus, satellite cells represent the ideal candidate for cell therapy approaches that aim at reconstructing muscles. However, these cells are found in very limited numbers in adult muscles, and they cannot be amplified *in vitro* as they lose their regenerative properties in culture[123,129]. Thus, access to an unlimited source of satellite cells differentiated from pluripotent cell cultures could open the possibility of developing cell therapy protocols for muscular dystrophies. We have shown that the long-term $Pax7^+$ cells produced *in vitro* from mESCs become tightly associated with differentiating fibers, which are located under their basal lamina, as expected for satellite cells. These $Pax7^+$ cells can give rise to both muscle fibers and $Pax7^+$ satellite cells when grafted *in vivo*, suggesting that the $Pax7^+$ cells differentiated *in vitro* correspond to satellite cells[1]. The muscle fibers differentiated *in vitro* from mESCs exhibit characteristics of early postnatal fibers, suggesting that the associated $Pax7^+$ cells probably correspond to postnatal satellite cells. Remarkably, whereas adult satellite cells immediately become activated *in vitro*, losing the capacity to self-renew, immature satellite cells can still produce both $Pax7^+$ satellite cells and myoblasts when cultured *in vitro*[130]. This property

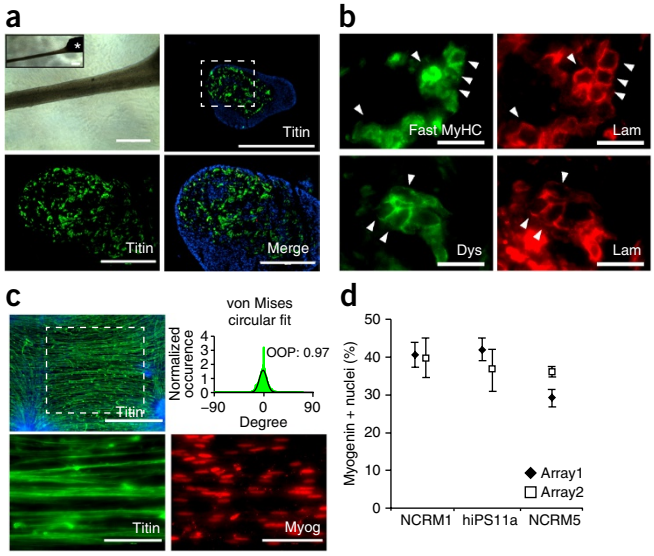

Figure 5 | Example of downstream applications for hPSC-derived myogenic cultures. (**a**) Muscle construct. hiPS11a-derived myogenic subculture was used to make muscle constructs according to Dennis *et al.*[70]. (Top left) The centimeter-long fascicle-like muscle construct spontaneously assembles around silk suture anchors (phase contrast; inset is a lower-magnification image showing the silk anchor (*; scale bar, 1 mm)). Scale bar, 400 µm. (Top right) The construct contains hundreds of aligned human skeletal fibers visualized in cross-section with titin staining (green) and nuclear counterstaining (blue). Scale bar, 1 mm. (Bottom) Details of the top right panel, Scale bars, 400 µm. (**b**) Distribution of fast MyHC and dystrophin (Dys) in transversal sections of hiPS11a-derived muscle constructs, as described in **a**. Arrowheads show individual fibers delimited by laminin (Lam). Scale bars, 25 µm. (**c**) Micropatterned skeletal muscle fiber array (muscle chip). NCRM1-derived myogenic subculture was seeded on micropatterned substrate composed of adhesion-permissive stripes, prepared according to McCain *et al.*[187]. (Top left) Overview of the array showing differentiated titin-positive skeletal myofibers. Note overall alignment from left to right. Scale bar, 1 mm. (Top right) Representative distribution diagram of myofiber alignment on an 1,800 × 1,500 µm array area (dotted white box in top left panel) relative to the orientation of the substrate. Structural analysis was done according to Pasqualini *et al.*[188]. The orientational order parameter (OOP) is close to 1. (Bottom) Detail of top left panel, striated skeletal fibers stained with titin and corresponding myogenin staining. Scale bars, 100 µm. (**d**) Proportion of myogenin-positive nuclei after 2 weeks of culture on muscle chips described in **c**. Quantifications for three hPSC lines are shown. For each cell line, duplicate chips (arrays) were generated and three fields of view (>4,000 nuclei per chip) were quantified by device. Errors bars, means ± standard deviation.

is shared by mouse and human Pax7$^+$ cells differentiated *in vitro* with our protocols. Furthermore, immature satellite cells were recently shown to exhibit remarkable regenerative properties, which might be a substantial advantage in the context of cell therapy protocols aiming at regenerating muscle[130]. We observed that the differentiation of human Pax7$^+$ cells produced *in vitro* with our protocol is very similar to that of the mouse Pax7$^+$ satellite-like cells, and we anticipate that these cells also correspond to immature human satellite-like cells. Further analysis of the human Pax7$^+$ cells produced with this protocol will be necessary to compare them with recently characterized human muscle stem cells[128,131,132]. In particular, the assessment of their regenerative properties *in vivo* will be a key consideration in their evaluation as a potential source of cells for cell therapy approaches[133]. These cultures could also permit the study of the development of human satellite cells, a process about which little is currently known.

Alternative methodology

Distinct methodologies have been used to produce skeletal muscle cells from hPSCs. In most cases, differentiation strategies have been first optimized on mouse ESCs before transposition to hPSCs (reviewed in refs. 119,134–141). Direct conversion methods consist of overexpressing myogenic factors in stem cells to force them to differentiate into myogenic progenitors, bypassing paraxial mesoderm specification[142,143]. The general concept of cell reprogramming by introduction of a factor was first demonstrated with the ability of MyoD to convert fibroblasts into myoblasts[144]. The method was perfected over time by the use of different vectors, including inducible systems to overexpress transcription factors—chiefly MyoD, Pax3 or Pax7, which can all elicit myogenic differentiation with various degrees of efficiency[100,145–150]. However, these methods require introduction of exogenous DNA into the cells, often via viral vectors, which is an issue for potential biomedical applications. In addition, the molecular identity of the generated myogenic cell has not been investigated in great detail. For MyoD conversion, myofiber maturation *in vitro* is usually limited by incomplete myogenic activation and conversion[143]. The use of the upstream regulators, Pax3/7, although not restricted to the myogenic lineage[151–155], allows for the production of *in vitro* myogenic progenitors[146,150] that need to be further enriched by cell sorting. Recent work suggests that these induced myoblasts are able to generate striated fibers[156]. However, culture conditions are generally undefined, involving embryoid body formation and the use of serum. Other efforts have also aimed at generating other cell types found in the adult skeletal muscle, such as pericytes/mesoangioblasts, that exhibit myogenic properties[157–160].

Limitations of the protocol

Biological relevance. Very little is known about human paraxial mesoderm development because of the difficulty of accessing early human embryos. The protocol described here can provide a window into the development of this key lineage that not only gives rise to skeletal muscle but also to the axial skeleton and the dermis of the back. Access to an unlimited supply of genetically defined myogenic cells will allow the development of many biomedical applications to the study of muscle physiology and pathology. One major critique is the intrinsically artificial nature of the culture system, which is vastly different compared with an organism in its full complexity[161]. Another relates to the differentiated cell types obtained *in vitro*, whose maturation is incomplete, being closer to the early postnatal stage than to the adult stage, which would be more desirable for relevant physiological and disease modeling investigations[162]. This might be overcome with more integrated cell culture systems, such as cocultures or 'on-a-chip' design aiming at interconnecting tissues in a physiologically relevant manner[76,81,88].

Research applications. Dense cell culture can render the optical analysis difficult, if not infeasible. Furthermore, primary differentiation is typically done on plastic cell culture dishes, which might not be compatible with high-end imaging. However, we also successfully differentiated human muscle fibers from iPS cells on Thermanox coverslips. Subculturing can also circumvent both issues by allowing the control of seeding densities and cell culture format, thus allowing more flexible experimental setups (**Fig. 5**). It is also well known that various hPSCs exhibit different propensities to differentiate toward one specific germ layer and

TABLE 1 | Overview of the possible applications pertaining to hPSC-derived myogenic cultures.

	Paraxial mesoderm Myogenic progenitors	Skeletal muscle fibers Myotubes	Satellite-like cells
Developmental biology *in vitro* model	Cell fate decision	Myogenesis	Specification and niche
Cell biology Physiology	Bioengineering Tissue chips	Electrophysiology Coculture system	Homeostasis
Pathology	Disease modeling Musculoskeletal system, myopathies	Disease modeling Musculoskeletal system, myopathies	Gene correction
Regenerative medicine	Bioengineering, tissue repair, and drug screening	Bioengineering, tissue repair, and drug screening	Cell therapy Regeneration

Columns represent distinct developmental/differentiation stages. Rows represent biomedical disciplines.

tissue[163,164]. As such, it is expected that the nature of the starting cell lines will be a source of variability that can lead to a different level of myogenic differentiation. We believe we provide a robust conceptual framework based on developmental principles, that is amenable to most cell lines with limited adaptations. Using this protocol, we have routinely achieved efficient differentiation of ~8 independent lines, including hiPS11a (HSCI, Harvard University), NCRM1 and NCRM5 (RUCDR, Rutgers University), H9 (WiCell, Madison, WI) and HUES1 (HSCI, Harvard University).

Biomedical applications. All cell material and reagents will need to meet safety regulations and be approved by the relevant regulatory agencies if the cell populations are to be used in clinical trials and in a regenerative/cell therapy context[165]. However, we expect that the first biomedical applications of PSCs differentiated *in vitro* will be in the domain of high-throughput screening, *in vitro* disease modeling and cell toxicity assays[65,76,166], for which such approval is not required.

Experimental design

hPSC lines. Quality of the starting hPSC line is essential (**Fig. 1a**). Cell lines should be karyotypically normal, pathogen-free and pluripotent. hPSC cell lines should come from recognized cell repositories. hPSCs can be maintained by a variety of methods[58,59]. Regular monitoring and characterization of hPSC culture is essential. hPSCs from large scale and working banks should be obtained at low passage numbers.

Mycoplasma detection can be performed using a PCR-based detection method such as that of the Venor GeM Mycoplasma Detection Kit. Complementary ways to assess the hPSC status include evaluation of colony growth, immunohistochemistry or fluorescence-activated flow cytometry analysis for stem cell marker expression, embryoid body formation and histological analysis of hPSC-derived teratoma[60]. All cell lines used in this study came from recognized repositories and were mycoplasma-free.

Cell culture reagents. Key reagents, including the knockout serum replacement, chemical compounds and recombinant proteins should be systematically tested. In particular, reagent providers and production lots should be recorded. Because of variations in manufactured biologics, different lots should be tested in parallel and benchmarked against previous references in side-by-side differentiations to ensure an optimal experimental outcome.

Culture format. The protocol was originally developed for standard 2D adherent cell culture in 6- or 24-well plates. Although the step-by-step procedure provided below is optimized for a 12-well plate format, transposition to6- or 24-well plate formats is straightforward by respecting the corresponding surface ratio and volumes. It is possible also to further scale up or scale down by seeding on other types of plates (see, for example, Desbordes *et al.*[167]). We successfully differentiated cells in 96-well plate and in 10-cm dish formats.

Culture density. Both the cell seeding density and the culture's confluency at D0 are critical for the success of differentiation. Seeding density should be within 15,000 and 45,000 cells/cm^2 (it can be lower for a larger surface area). After 1–2 d of recovery in medium containing the antiapoptotic ROCK inhibitor[168], 10–25% confluent homogeneous cultures can be used for directed differentiation. Deviation from these guidelines usually leads to failure of differentiation, either because of cell death or because of overcrowding and culture detachment en masse.

Culture substrate. The default substrate is Matrigel (Corning; see also Kleinman *et al.*[169]), which is compatible with robust cell attachment and myogenic differentiation. More defined substrates such as gelatin or specific recombinant laminin and fibronectin coatings have been used in the past for myogenic progenitors[170–173]; however, we observed that maturing myogenic cultures were more susceptible to detach from those substrates.

Subculturing step. During differentiation, cultures become confluent within a week. After 30–40 d (**Fig. 1c**), they are very dense and tend to detach from the substrate as sheets of cells, possibly because of the myofibers' spontaneous contractions. We found that replating and splitting of these original cultures leads to a substantial enrichment in myogenic cells and to less dense cultures that are easier to image (**Figs. 3** and **5**). Passaging can be performed by combined mechanical and enzymatic dissociation of cultures that are 10 to 50+ d old, with 25–45 d being the optimal window (**Fig. 3**). Because of extensive ECM deposition, older cultures are harder to dissociate properly without causing substantial cell death. Optimal reseeding density corresponds to a splitting ratio of 1:4 to 1:8, or ~60–70,000 cells/cm^2. Subculturing results in selection of myogenic progenitors while depleting unwanted differentiated contaminants and accumulated culture debris. Although postmitotic

TABLE 2 | List of markers used to characterize the differentiating hPSC-derived myogenic cultures and corresponding antibodies for immunohistochemistry (**Figs. 2–5**).

Marker	Temporal expression	Antibody Reference	Working dilution	Differentiation stage specificity (and other tissues marked)
T/Brachyury	~D2	R&D, cat. no. AF2085	1:250	Early mesoderm (notochord)
Tbx6	~D4	Abcam, cat. no. ab38883	1:200	Early mesoderm, PSM
Pax3	~D5–15	DSHB cat. no. Pax3-c	1:250	Anterior PSM, dermomyotome (neural tube, neural crest)
MyoD	~D15+	Santa Cruz Clone 5.8A, cat. no. sc-32758	1:200	Myoblasts, myotubes
Pax7	~D25+	DSHB, cat. no. Pax7-c	1:250	Pre-myoblasts, satellite-like cells (neural tube, neural crest)
Myogenin (Myog)	~D25+	DSHB, cat. no. F5-c	1:250	Myocytes, myotubes and myofibers
Myogenin (Myog)	~D25+	Santa Cruz, cat. no. sc-576x	1:800	Myocytes, myotubes and myofibers (leaky staining in sarcoplasm, use at 1:800+)
Myosin heavy chain (MyHC)	~D25+	DSHB, cat. no. MF20-c	1:300	Myocytes, myotubes and skeletal myofibers (cardiomyocytes)
Myosin heavy chain fast perinatal (fast MyHC)	~D25+	Sigma Clone MY-32, cat. no M4276	1:300	Striated myotubes and fibers
Titin (Ttn)	~D25+	DSHB, cat. no. 9 D10	1:300	Striated muscles (cardiomyocytes)
Dystrophin (Dys)	~D25+	Zeiss/Novocastra Clone Dy4/6D3, cat. no. NCL-DYS1	1:200	Skeletal myofibers (possibly other dystrophin expressing tissues)

cells (myocytes and myofibers) are essentially lost by the subculture step, myogenic precursors are preserved and can be amplified with commercially available growth media used for primary muscle cultures and cell lines (compare **Figs. 1c** and **3b**). For this purpose, we used the Skeletal Muscle Cell Growth Medium-2 (Clonetics, Lonza; see Media composition). *De novo* myofibers can be produced again by differentiation of the subculture (**Figs. 3–5**). Furthermore, confluent cultures left in proliferative medium are fairly stable and retain a pool of Pax7-myogenic progenitors for months, which can be used later for downstream applications (**Figs. 3–5**). The subculture is thus amenable to further passaging, cryostorage and/or secondary differentiation (**Fig. 3**).

Myogenic differentiation medium. Secondary differentiation can be achieved by transferring to either 'terminal differentiation' medium such as horse serum–containing medium[40] or serum-free medium containing insulin and the N2 supplement[57,174–176] (**Figs. 3–5**).

Differentiation yield. Both the total number and the percentage of the target cell type should be quantified to assess protocol efficacy and reproducibility, and for possible troubleshooting. The protocol was designed as a multistep protocol with a sequence of defined media and developmental milestones. Proper differentiation should be assessed regularly. This is particularly important when working with novel hPSC lines or several lines for side-by-side comparison. We favor immunohistochemistry for specific validated markers to evaluate differentiation efficiency (**Figs. 2** and **4**; **Table 2**). Paraxial mesoderm specifications can be monitored with antibodies against T (Brachyury) or Tbx6 (refs. 10,177,178). It should be kept in mind that most markers are not entirely specific to the target tissue along the whole developmental spectrum (**Table 2**). We found that Msgn1 (refs. 179,180) is the most reliable and specific marker of paraxial mesoderm. Although, unfortunately, no validated Msgn1 antibody is currently available, Msgn1 expression can be monitored by RT–qPCR or by using reporter cell lines (data not shown). Similar to that of Tbx6, Msgn1 expression is expected to peak around D4–5 of differentiation with this protocol, accounting for 70–90% of the cells. Myogenesis should be monitored starting at week 2 by following MyoD and myogenin expression[181–183], whereas myotube formation and fiber maturation can be assessed using skeletal muscle proteins or isoforms, such as myosin heavy chains[184] (**Fig. 2** and **Table 2**). The presence of satellite-like cells in long-term culture can be evaluated with the expression of Pax7 (refs. 185,186; **Figs. 2b** and **4a**,**b**). By 4 weeks (~D30) of differentiation, ~22% of nuclei are myogenin-positive, whereas another 23% are Pax7-positive[1]. Upon subculturing and two more weeks of differentiation of three independent cell lines, up to 40% of total nuclei were myogenin-positive (**Fig. 5d**). Mature skeletal myofiber density ranged from 9,000 to 20,000 fibers per cm^2 (**Fig. 5c**, and data not shown).

Level of expertise to implement the protocol. Prior experience with routine hPSC culture and cellular phenotyping is valuable, especially for scaling the culture up or down, and for adapting the protocol to specific applications. Familiarity with the concept of directed differentiation can be advantageous for protocol troubleshooting.

MATERIALS

REAGENTS

Cells

- Human pluripotent stem cells; we have successfully used hiPSCs (hiPS11a (HSCI, Harvard University), NCRM1 and NCRM5 (RUCDR, Rutgers University)) and hESCs (H9 (WiCell, Madison, WI), HUES1 (HSCI, Cambridge, MA)) ! CAUTION Cell line identity should be regularly verified and cultures should be tested for mycoplasma contamination. Experiments involving human pluripotent stem cells must conform to relevant institutional and national regulations; we gained ethical approval from Institutional Animial Care and Use Committee/Embryonic Stem Cell Research Oversight Committee 2014-06-02. ! CAUTION Different cell lines may respond to directed differentiation with variable levels of myogenic differentiation.

Media

- mTeSR1 (Stemcell Technologies, cat. no. 05851)
- DMEM/F12 1:1 (Life Technologies, cat. no. 11320-082)
- Knockout serum replacement (KSR; Life Technologies, cat. no. 10828-028) ! CAUTION Lot-to-lot variability may impair the differentiation protocol.
- Penicillin–streptomycin-glutamine, 200 mM (Thermo Fisher Scientific, cat. no. 25030081)
- 2-mercaptoethanol, 55 mM (βME; Life Technologies, cat. no. 21985-023)
- Nonessential amino-acid solution (NEAA; Thermo Fisher Scientific, cat. no. 11140-050)
- Insulin–transferrin–selenium (ITS; Life Technologies, cat. no. 41400-045)
- BSA, 0.1% (wt/vol) (Sigma-Aldrich, cat. no. A7906)
- N-2 supplement (Life Technologies, cat. no. 17502-048)
- Horse serum (HS; Invitrogen, cat. no. 16050-130)
- Skeletal Muscle Growth Medium-2 (SkGM; in SkGM BulletKit; Lonza, cat. no. CC-3245)
- CryoStem freezing medium (Stemgent, cat. no. 01-0013-50)

Cell culture reagents

- Dulbecco's PBS (DPBS), calcium/magnesium free (Gibco, cat. no. 14190-144)
- Tris-buffered saline (TBS, 10×; Sigma-Aldrich, cat. no. T5912-1L)
- Sterile cell-culture-grade water (Invitrogen, cat. no. 10977-015)
- Matrigel, hESC-qualified (MG; Corning, cat. no. 35277)
- EmbryoMax 0.1% (wt/vol) gelatin solution (EMD Millipore, cat. no. es-006-b)
- Trypsin EDTA, 0.25 M, pH 8.0 (Life Technologies, cat. no. 25200-056)
- TrypLE Express (Invitrogen, cat. no. 12605010)
- Dispase (1U/ml; Stem Cell Technologies, cat. no. 07923)
- Collagenase type I (Thermo Fisher Scientific, cat. no. 17100-017)
- DMSO, cell culture grade (Sigma-Aldrich, cat. no. D2650)
- Trypan blue solution 0.4% (wt/vol), cell culture grade (Sigma-Aldrich, cat. no. T8154-20ML)

Factors

- CHIR99021 (Tocris Bioscience, cat. no. 4423) ▲ CRITICAL Other GSK3 beta inhibitors may not be as efficient as CHIR.
- LDN-193189 (Stemgent, cat. no. 04-0074) ▲ CRITICAL Other BMP type I receptor inhibitors may not be as efficient as LDN.
- Y-27362 dihydrochloride (Rocki; Tocris Bioscience, cat. no. 1254)
- Hepatocyte growth factor (HGF), recombinant murine (PeproTech, cat. no. 315-23)
- Fibroblast growth factor 2 (FGF2), recombinant murine (PeproTech, cat. no. 450-33)
- Insulin-like growth factor 1 (IGF1), recombinant murine (Peprotech, cat. no. 250-19)
- Paraformaldehyde 2–4% (vol/vol) (Electron Microscopy Sciences, cat. no. 15710) ! CAUTION Paraformaldehyde must be handled in a chemical cabinet.
- Triton X-100 (Sigma-Aldrich, cat. no. T8787-250ML)
- Tween-20 (Sigma-Aldrich, cat. no. P7949-500ML)
- FBS (Hyclone/GE, cat. no. SH30070.03)
- Hoechst 33342 (Life Technologies, cat. no. H3570)
- Primary antibodies (**Table 2**)
- Secondary antibodies, species-specific anti-IgG(H+L)—Alexa-Fluor-conjugated (Molecular Probes)
- Isopropanol, 70% (VWR, cat. no. 89499-420)
- Venor GeM Mycoplasma Detection Kit, PCR based (Minerva Biolabs, Sigma-Aldrich, cat. no. MP0025-1KT)

EQUIPMENT

- Cell culture multiwell plates (VWR, cat. nos. 353046, 353043 and 353047)
- Cell culture dishes (Corning, cat. nos. 353001 and 353002)
- Cryovials (Nalgene, cat. no. 5000.0020)
- Needles, 25 gauge (Becton Dickinson, cat. no. 305125)
- Sterile cell scrapers (Celltreat, cat. no. 229306)
- Sterile plastic tubes, 15 ml and 50 ml (VWR, cat. nos. 89039-66 and 8939-658)
- Test tubes, 5ml round bottom, snap cap (Corning, cat. no. 352063)
- Celltrics cell filter, 30 µl sterile and nonsterile (Partec, cat. nos. 04-004-2326 and 04-004-2316)
- Sterile plastic pipettes (Corning, cat. nos. 356543, 356551 and 356525)
- Filter, 0.22 µm (Pall, cat. no. 4652)
- Cell strainer, 70 µm (Thermo Fisher Scientific, cat. no. 22363548)
- Pipettes with filtered tips (VWR, cat. nos. 89003-060, 89003-056, 89003-048 and 89368-974)
- Freezing container (Biocision, cat. no. BCS-405G)
- Filter for vacuum system, 0.2 µm hydrophobic (Pall, cat. no. 4251)
- Pasteur pipettes (VWR, cat. no. 14673-010)
- Water purification system (EMD Millipore, cat. no. SYNSVR0WW)
- Cell counter (Nexcelom Bioscience, cat. no. Auto2000)
- Pipette-aid Easypet 3 (Eppendorf, cat. no. 4430000018)
- Pipettes Research plus 4-pack (Eppendorf, cat. no. 022575442)
- Centrifuge 5810 with rotor A-4-62 (Eppendorf, cat. no. 022627007)
- Biosafety cabinet HERAsafe KS Type KS12 (Thermo Fisher Scientific, cat. no. 51022482)
- Vacuum filtering units, M-Vac (Argos Technologies; cat. no. VWR 89129-568)
- Incubator, CO_2 incubator (Panasonic, cat. no. KM-CC17RUI)
- Digital Microscope EVOS XL Imaging System (Life Technologies, cat. no. AME3300)
- Digital Fluorescence Microscope EVOS FL Imaging System (Life Technologies, cat. no. AMF4300)
- Picking station with Lynx Stereo Dynascopic Microscope (Vision Engineering)
- Freezer, −80 °C, ultra-low-temperature freezer (Panasonic, cat. no. MDF-U53VA)
- Liquid nitrogen storage tank (Cryosafe, cat. no. T CAT 7PS)
- Water bath (VWR, cat. no. 89032-200)

REAGENT SETUP

Recombinant factor stock solutions (FGF2, HGF and IGF1) Resuspend the compound in sterile cell-culture-grade PBS supplemented with 0.1% BSA to the desired stock concentration. Prepare 50-µl aliquots and store them at −20 °C for up to 6 months. The following stock solutions are used: FGF2 stock solution, 10 µg/ml—use at 20 ng/ml; HGF stock solution, 100 µg/ml—use at 10 ng/ml; and IGF stock solution, 10 µg/ml—use at 2 ng/ml.

Chemical compound stock solutions (CHIRON99021, LDN-193189 and Y27362) Resuspend the compound in cell-culture-grade DMSO (CHIRON and LDN-193189) or H_2O (Y27362) to the desired concentration. Prepare 50-µl aliquots and store them at −20 °C for up to 6 months. The following stock solutions are used: CHIR99021 stock solution, 10 mM—use at 3 µM; LDN-193189 stock solution, 1 mM—use at 500 nM; and Y27362 (Rocki) stock solution, 10 mM—use at 10 µM.

Matrigel and Matrigel-coated plates Thaw the stock solution overnight on ice at 4 °C, and once it is in liquid form, keep it on ice and divide the solution into aliquots (lot-specific volume by manufacturer's recommendation) using cold tips and cold tubes. Store the aliquots at –80 °C for up to 6 months. When needed, thaw the aliquots on ice, and dilute them with 12 ml of ice-cold DMEM/F12, resulting in a 3 mg/ml protein concentration; mix the solution by pipetting. Add 1 ml of diluted Matrigel solution per 10 cm^2 of multiwell plates or dishes to cover the surface evenly. Keep the plates on ice for at least 4 h, and before use, incubate them at 37 °C for 30 min. Store the Matrigel-coated plates at 4 °C for up to 2 weeks. ▲ CRITICAL Matrigel solidifies above 4 °C, so ensure that all of the cell culture plastic is ice-cold during handling.

mTeSR medium Thaw a 100-ml bottle of 5× supplement overnight at 4 °C and add it to the mTeSR medium. Mix and divide the medium into 40-ml aliquots. Store the aliquots at −20 °C for up to 3 months.

When needed for use, thaw an aliquot at room temperature (RT, 15–25 °C) and keep it at 4 °C for up to 2 weeks.

SkGM-2 medium Supplement SkGM basal medium by adding the components of the SkGM BulletKit. Mix the medium and prepare aliquots in 50-ml tubes. Store the medium at −20 °C for up to 3 months. When needed for use, thaw an aliquot and keep it at 4 °C for up to 2 weeks.

Paraformaldehyde Dilute the 16% glass-sealed electronic microscopy (EM)-grade paraformaldehyde stock in DPBS. Leftover 16% stock solution can be frozen for later use. 2–4% working solution should be freshly prepared. **! CAUTION** Paraformaldehyde is toxic, and it must be manipulated in a chemical cabinet. Used solution should be disposed of according to local institutional guidelines.

TBST buffer Dilute the TBS 10× buffer with ultrapure water, and supplement the solution with 0.1% (vol/vol) Tween-20. Store the buffer at RT for up to 3 weeks.

Blocking reagent Dilute the TBS 10× buffer with ultrapure water, and supplement the solution with 1% (vol/vol) FBS and 0.1% (vol/vol) Triton-X100. Keep the reagent at 4 °C for up to 2 weeks.

Differentiation media Prepare the cell media according to the tables below and filter them with a 0.22-µm mesh. Small compounds and recombinant proteins should be added immediately before use, but the base media can be stored at 4 °C for up to 2 weeks. **▲ CRITICAL** Small compounds and recombinant proteins should be added immediately before use.

Di-CL/Di-CLF medium

Composition	Volume (250 ml)	Final concentration
DMEM/F12	244.5 ml	
ITS	2.5 ml	1% (vol/vol)
Nonessential amino acids	2.5 ml	1% (vol/vol)
Penicillin/streptomycin	0.5 ml	0.2% (vol/vol) (20 IU + 0.02 mg)/ml
CHIR-99021	75 µl	3 µM
LDN-193189	125 µl	0.5 µM
Recombinant FGF-2	500 µl	20 ng/ml

DK-HIFL medium

Composition	Volume (250 ml)	Final concentration
DMEM/F12	209.5 ml	
KSR	37.5 ml	15% (vol/vol)
Nonessential amino acids	2.5 ml	1% (vol/vol)
Penicillin/streptomycin	0.5 ml	0.2% (vol/vol)
2-Mercaptoethanol	454 µl	0.1 mM
Recombinant HGF	25 µl	10 ng/ml
Recombinant IGF-1	50 µl	2 ng/ml
Recombinant FGF-2	500 µl	20 ng/ml
LDN-193189	125 µl	0.5 µM

DK-I/DK-HI medium

Composition	Volume (250 ml)	Final concentration
DMEM/F12	209.5 ml	
KSR	37.5 ml	15% (vol/vol)
Nonessential amino acids	2.5 ml	1% (vol/vol)
Penicillin/streptomycin	0.5 ml	0.2% (vol/vol)
2-Mercaptoethanol	454 µl	0.1 mM
Recombinant HGF	25 µl	10 ng/mL
Recombinant IGF-1	50 µl	2 ng/mL

N2 medium

Composition	Volume (250 ml)	Final concentration
DMEM/F12	242 ml	
ITS	2.5 ml	1% (vol/vol)
N2 supplement	2.5 ml	1% (vol/vol)
Penicillin/streptomycin	0.5 ml	0.2% (vol/vol)
L-Glutamine	2.5 ml	1% (vol/vol)

PROCEDURE

Pluripotent cell culture preparation ● TIMING 5–7 d

1| *Pluripotent cell culture maintenance and amplification.* Culture hPSC colonies on 35-mm Matrigel-coated dishes. Passage them when the cultures reach ~70% confluency (**Fig. 1a**).

2| Prepare new 35-mm dishes to passage colonies: preincubate Matrigel-coated dishes at 37 °C for 30 min. Just before passaging, replace Matrigel with 2 ml of RT mTeSR.

3| Passage the cells using either the cutting method (option A) or the dispase method (option B).

▲ CRITICAL STEP Although any hPSC lines can be passaged using the cutting method, allowing the selection of the optimal colonies, the dispase method allows for easier bulk passaging; however, the dispase method should be performed only on cultures with low overall spontaneous differentiation. Some hPSC lines are more prone to culture failure (cell death, differentiation) with the dispase method.

(A) Cutting method

(i) Use a 25-gauge needle tip to cut healthy hPSC colonies into small pieces using an 'eyepiece-less' stereomicroscope (Lynx). **▲ CRITICAL STEP** Colony pieces should not be too small or too big—they should be ~200 × 200 µm; use the width of the needle as a size proxy (**Fig. 1a**).

(ii) Scrape and collect colony pieces using a 20-µl pipette. Gently aspirate while detaching the pieces from the cell culture dish with the pipette tip.

(B) Dispase method

(i) Discard the medium, rinse the dish with PBS, add 1 ml of dispase (1 U/ml) to the dish and incubate the mixture at 37 °C, 5% CO_2, for 5–7 min.
▲ **CRITICAL STEP** Differentiated colonies should be scraped away beforehand.

(ii) Remove the dispase solution, rinse the dish twice with PBS, add 1 ml of mTeSR and detach the colonies using a cell scraper.

(iii) Triturate detached colonies to fragment them to a suitable size.
▲ **CRITICAL STEP** Colony pieces should not be too small or too big; they should be 200 × 200 µm, which is usually achieved by pipetting up and down two to three times using a 1,000-µl pipette.

4| Transfer 50–100 colony pieces onto the new Matrigel-coated dishes (Step 2), and distribute the pieces evenly by gently rocking the plate back and forth, and side to side (**Fig. 1a**). Place the dishes in the incubator at 37 °C, 5% CO_2.
! CAUTION Some hPSC lines are better maintained under controlled 4% CO_2.

Predifferentiation setup (D2) ● TIMING 2 d

5| *Single-cell dissociation of PSC culture.* Change the mTeSR medium every day until hPSC cultures reach ~70% confluency.
▲ **CRITICAL STEP** Starting hPSC culture must consist of homogeneous and healthy-looking colonies.

6| Pretreat cells with 10 µM Rocki in mTeSR for at least 2 h.

7| Prepare 12-well plates coated with Matrigel.
▲ **CRITICAL STEP** This procedure can be easily performed with 6- and 24-well plates by adapting volume and cell numbers to the surface ratio.

8| Rinse the hPSC culture with DPBS without calcium and magnesium.

9| Add TrypLE Express at a volume of 100 µl/cm^2 and incubate the cells at 37 °C, 5% CO_2, for 5–7 min.

10| Observe cell dissociation with a light microscope, and aid the mechanical dissociation by tapping the plate. Most of the colonies will dissociate to a suspension of single cells or small, loose, irregular aggregates.

11| Collect the cell suspension in a 15-ml sterile tube and gently further triturate with a 1,000-µl pipette.

12| Add 10 ml of DMEM/F12 to inactivate the TrypLE Express.

13 | Centrifuge the cells at 300*g* for 5 min at RT.

14| Remove the supernatant and resuspend the pellet gently in 1 ml of mTeSR + 10 µM Rocki.

15| Count the cells and use trypan blue or a similar stain to assess cell viability.

16| Rinse the Matrigel-coated differentiation plates with PBS.

17| Dilute the cells in mTeSR + 10 µM Rocki, and plate the cells at 3 × 10^4 cells per cm^2 (i.e. 1.2 × 10^5 cells in 1.5 ml of medium for 1 well of a 12-well plate). One 35-mm dish can typically produce enough cells for a whole 12-well plate. Distribute the cells by rocking the plate gently four times back and forth, side to side.
▲ **CRITICAL STEP** Even distribution of the cells is critical to creating homogeneous differentiation conditions. Cells plated on a 24-well or smaller plate tend to clump on the edges and in the center of the well if not properly distributed. Cell density and plating efficiency are crucial to the survival of the culture. Too-sparse plating leads to detachment of the colony within the first 5 d, and too-dense culture will quickly become overconfluent and result in heterogeneous differentiation of the culture.

18| Incubate the plate at 37 °C, 5% CO_2, overnight.

19| Observe the cells and change mTeSR daily until hPSC cultures reach a confluency of ~15–20%.
During Rocki treatment, cells at a low density adopt a spiky morphology. Cultures will have many small (<30 cells) colonies, which is optimal for beginning differentiation (usually after 1–2 d; **Fig. 1c**).
▲ **CRITICAL STEP** Small colonies should be evenly distributed on the dish surface for optimal differentiation.
? TROUBLESHOOTING

Directed differentiation (D0–40) ● TIMING 30–40 d

20| *D0*. Once cultures have reached 15–20% confluency, change the medium to DiCL (Dmem-ITS-Chir-Ldn) medium to initiate differentiation. This is D0 of differentiation. Refresh the medium daily until D3 (**Fig. 1b**,**c**).
? TROUBLESHOOTING

21| *D3*. Change the medium to DiCLF (Dmem-ITS-Chir-Ldn-Fgf) medium and refresh it daily until D6.
Ideally, the medium should be changed daily until D12 of differentiation. However, 1 d (weekend) can be omitted while respecting the different media sequence.
? TROUBLESHOOTING

22| *D6*. Change the medium to DK-HIFL (Dmem-KSR-Hgf-Igf-Fgf-Ldn) and refresh it daily until D8.
▲ CRITICAL STEP Substantial cell death will be observed in the first week; this is the normal selective action of the sequence of media.
? TROUBLESHOOTING

23| *D8*. Change the medium to DK-I (Dmem-KSR-Igf) and refresh it daily until D12.
? TROUBLESHOOTING

24| *D12*. Change the medium to DK-HI (Dmem-KSR-Hgf-Igf) and refresh it every other day thereafter (**Fig. 1b**).
▲ CRITICAL STEP Medium change over the weekend can be avoided by doubling the culture medium volume beforehand.

25| Observe the cultures for myotube formation, and change the differentiation medium every other day. Myotubes are usually visible after 30 d of differentiation (**Figs. 1c** and **2b**). Proceed to Step 37 to continue to expand the culture; we recommend that you also characterize a subset of the culture by immunofluorescence, as described in Steps 26–36.
▲ CRITICAL STEP Differentiated cultures are delicate, as myotubes detach easily. Medium change should be done slowly to avoid cell detachment.
? TROUBLESHOOTING

Culture characterization ● TIMING 2–4 d

26| Wash the cultures with DPBS 2×, obtained either through primary differentiation (Steps 20–25) or through differentiation of subcultures (Steps 37–54).

27| Fix the culture plates with 2% (vol/vol) paraformaldehyde at a volume of 250 µl/cm^2 for 10 min at RT or overnight at 4 °C.
▲ CRITICAL STEP Paraformaldehyde must be handled in a chemical cabinet.

28| Rinse the cells with DPBS.
■ PAUSE POINT Fixed cells can be kept in DPBS at 4 °C for at least 2 weeks before proceeding with the staining.

29| Permeabilize the cells by adding TBST buffer at a volume of 250 µl/cm^2, at RT, for 3 min. Repeat this three times.

30| Incubate the cells in blocking solution at a volume of 250 µl/cm^2 for 30 min at RT.

31| Incubate the cells in primary antibodies diluted in Blocking solution overnight at 4 °C on a shaker. See **Table 2** for a list of antibodies that we have successfully used.
▲ CRITICAL STEP When you are working with novel/not validated antibodies, specificity and optimal dilution of the antibody should be evaluated separately.

32| Wash the cells three times with TBST for 5 min.

33| Incubate the cells in secondary antibodies conjugated with a fluorochrome and Hoechst diluted in blocking solution overnight at 4 °C on a shaker.
▲ CRITICAL STEP Protect cells from light from this step onward.

34| Wash the cells three times with TBST for 5 min.

35| Add DPBS at a volume of 250 µl/cm^2 to the cells.
■ PAUSE POINT Store this mixture for up to 3 weeks protected from light at 4 °C, for later analysis.

36| Image the cells with a fluorescence microscope equipped with the appropriate fluorescent filter cubes.
▲ **CRITICAL STEP** Despite being fairly stable, staining analysis should be performed within a few weeks to avoid signal fading and diffusion (**Figs. 2** and **4**).

Culture expansion (D10+) ● **TIMING 3–4 d**
▲ **CRITICAL** The culture can be split any time between D12 and D100+ of culture. Cultures older than 30 d are a mix of myogenic progenitors (PAX7$^+$, MYOD$^+$) and postmitotic myocytes, myotubes and fully differentiated myofibers (MYOG$^+$, MyHC$^+$, Titin$^+$), as well as other cell types, including fibroblasts and neuronal contaminants (**Fig. 1c**, D40). For best results, the initial differentiated hPSC culture can be prescreened for its myogenic phenotype, consisting of elongated bipolar cells organized in stream-like sheets of tissue (**Fig. 1c**).
37| *Passaging differentiating cells and myogenic progenitor culture expansion* (**Fig. 3**). Pretreat cultures to be dissociated with 10 µM Rocki in DK-HI medium for at least 2 h.

38 | Prepare multiwell plates or dishes coated with Matrigel for subculture.

39 | Rinse the cell culture to be dissociated with DPBS without calcium and magnesium.

40| Add TrypLE Express at a volume of 250 µl/cm^2 and incubate the culture at 37 °C, 5% CO_2, for 5–7 min.

41| Mechanically fragment the dissociating culture using two 25-gauge needles, to promote further enzymatic dissociation.

42| Add an additional 250 µl/cm^2 of TrypLE Express and incubate the culture again at 37 °C, 5% CO_2, for 5–7 min.

43| Collect the dissociated cells and transfer them into a 50-ml tube containing 20 ml of DMEM with 10% (vol/vol) FBS.

44| Repeat Steps 40–43 until all of the culture detaches from the dish and the tube contains dissociated cells and larger aggregates. Complete enzymatic incubation should not exceed 45 min.
! CAUTION Note that older cultures (those that are 50+ d old) cannot usually be fully dissociated, and pieces of culture will remain undissociated.
▲ **CRITICAL STEP** Dissociation needs to be thorough enough to result in a single-cell suspension, but it should be gentle enough not to reduce viability. Typically, you should observe >80% viability immediately after dissociation.
▲ **CRITICAL STEP** If two rounds of TrypLE Express incubation and mechanical fragmentation appear insufficient to dissociate the culture, TrypLE Express can be replaced by 0.25% trypsin on the next round. Other enzyme mixes and concentrations have also been used successfully, including dispase/collagenase and collagenase IV/trypsin–EDTA. Incubation time and trituration may have to be adjusted.

45| After dissociation of the cultures, further triturate the suspension with a 10-ml pipette to break cell clumps.
? TROUBLESHOOTING

46| Filter the cell suspension on a 70-µm cell strainer placed on a 50-ml collection tube.

47| Centrifuge the cells at 300*g* for 5 min at RT.

48| Remove the supernatant and resuspend the pellet gently in SkGM supplemented with 10 µM Rocki.

49| Count the cells and use trypan blue or a similar stain to assess cell viability.
▲ **CRITICAL STEP** Routinely, after dissociation with TrypLE/trypsin, cell viability decreases with the age of the dissociated culture. From <30-d-old culture, ~95% of cells are viable, whereas 85% from D30–D80 cultures and 80% from D80–120 cultures are viable.

50| Replate the cells on Matrigel-coated wells at a density of 60–70,000 cells/cm^2 in SkGM + 10 µM Rocki, corresponding approximately to a subculturing ratio of 1:4–1:8 per surface area (i.e., ~2.5 × 10^5 cells in 1.5 ml of medium for 1 well of a 12-well plate). One 35-mm dish can typically produce enough differentiated cells to cover half of a 12-well plate.
▲ **CRITICAL STEP** Cell density is critical, and plating too few cells will prevent proliferation of progenitor cells. Replated cells should reach confluency within 3 d in SkGM medium for optimal culture of progenitor cells.

Box 1 | Culture cryopreservation and thawing ● TIMING 1–2 d Procedure

1. Dissociate primary culture or subcultures as in Steps 37–44.
2. Resuspend the pellet in 1 ml of DMEM/F12.
▲ CRITICAL STEP Cultures older than 30 d will have persisting cell clumps after dissociation that can be frozen together with single cells and when replated will give rise to myogenic cells.
3. Centrifuge the cells at 300*g* for 5 min at RT, remove the supernatant and resuspend the pellet in CryoStem freezing medium. Optimal freezing density is approximated by eye because of the existence of small clumps after dissociation.
▲ CRITICAL STEP As a guideline, one well of a six-well plate cultured for 30+ d can generate five 1-ml cryovials. Place the cryovials in a freezing container at –80 °C overnight.
4. On the next day, transfer the cryovials to a liquid nitrogen storage tank for long-term storage.
■ PAUSE POINT Frozen cryovials of cells can be kept indefinitely in liquid nitrogen.
5. To thaw: promptly place the frozen vials partially immersed in a water bath set at 37 °C; leave in the water bath until the vials are partially thawed.
6. Resuspend the vials' contents and then gently add the contents to 4 ml of DMEM/F12 in a 15-ml collection tube.
7. Centrifuge the cells at 300*g* for 5 min at RT, remove the supernatant and resuspend the pellet in SkGM. As a guideline for optimal seeding density, one vial as prepared in steps 1–4 of **Box 1** should be seeded to one well of a 24-well Matrigel-coated plate. After 3 d, the culture can be expanded again according to the main PROCEDURE Steps 37–53.

51| Incubate the plate at 37 °C with 5% CO_2 overnight.

52| Observe cell attachment and recovery, and change the SkGM medium every second day, until the desired confluency is reached. Passage the culture, which will now contain a high percentage of myogenic progenitors (**Figs. 3d–j** and **4**).

53| Expand the subculture (**Fig. 3**). We recommend that stocks be cryopreserved at the earliest passages, ideally at every passage (**Box 1** and **Fig. 4e**).
▲ CRITICAL STEP Cells can be further subcultured over 100 d for downstream applications, or they can be kept in SkGM at high density without losing myogenic competency.
? TROUBLESHOOTING

54| To further differentiate the cells to myofibers, change the medium to HS 2% medium after 3–4 d in SkGM. As a serum-free alternative, cultures can be differentiated in N2 medium (**Fig. 3c**,**e**,**g–j**). Cultures can be characterized by immunofluorescence, as described in Steps 26–36.
! CAUTION Although both HS- and N2-based media promote myofiber formation, N2 medium, being less rich, may not be able to support differentiated cultures over an extended time period (>2 weeks) as compared with HS-based medium.

? TROUBLESHOOTING
Troubleshooting advice can be found in **Table 3**.

TABLE 3 | Troubleshooting table.

Step	Problem	Possible reason	Solution
19	Poor cell survival	Single-cell dissociation was done by pipetting too forcefully or for too long	Start with a healthy undifferentiated hPSC culture with optimal colony size (before necrotic zones form at the colony centers; at ~70% confluence). Cultures will be more resistant to cell death if treated with the antiapoptotic Rocki and if dissociated cells are seeded in Rocki-containing mTeSR
	Poor cell attachment	Plates were not properly coated with Matrigel	Test Matrigel lot for routine hPSC culture maintenance
		Starting hPSCs were grown on feeders	Follow coating procedure properly, ensuring that Matrigel is manipulated at 4 °C at all times
			Adapt hPSC culture to Matrigel + mTeSR maintenance conditions. It may take several passages and manual selection of undifferentiated colonies
			Increase the seeding cell number density

(continued)

TABLE 3 | Troubleshooting table (continued).

Step	Problem	Possible reason	Solution
20–23	Cell cultures detach en masse	Cells were plated at too high a density The differentiation protocol was started on a too-dense culture	Optimize the seeding cell density and the recovery time before starting the differentiation (variable from cell line to cell line)
25	Poor myogenicity	Cells do not respond to signals Signaling pathway modulators are not prepared correctly or are not active	Start with a healthy undifferentiated hPSC culture Compare differentiation of several cell lines side-by-side, as differentiation efficiency varies from cell line to cell line Prepare chemical compounds and recombinant proteins according to the procedure and reagent setup to keep their activity
45	Poor cell dissociation Cell culture is resistant to enzymatic dissociation	Culture is very dense and deposits of extracellular matrix block the activity of the enzyme	Preincubate in DPBS for 10 min to weaken cell adhesion Triturate the culture mechanically with needles before and during enzymatic dissociation Extend the length and intensity of enzymatic incubation while pretreating the culture with Rocki
53	Poor myogenicity in subcultures	Proliferative contaminant cell populations have invaded the culture	Dissociate more mature cultures that are less likely to contain proliferative contaminants

● TIMING

Steps 1–4, pluripotent cell culture preparation: 5–7 d
Steps 5–19, predifferentiation setup (D–2): 2 d
Steps 20–25, directed differentiation (D0–40): 30–40 d
Steps 26–36, culture characterization: 2–4 d
Steps 37–54, culture expansion (D10+): 3–4 d
Box 1, culture cryopreservation and thawing: 1–2 d

ANTICIPATED RESULTS

This protocol allows for the efficient production of expandable muscle progenitors from hPSCs that can be amplified, subcultured, cryopreserved, further differentiated into skeletal myofibers and characterized. The derivation of the muscle progenitors is achieved by a sequence of differentiation media with defined compositions that directs hPSCs to form paraxial mesoderm and further differentiate into myoblasts.

Primary differentiation

The predifferentiation reagent setup and the first week of differentiation are the most critical steps for successful myogenic differentiation. After cell seeding, cells recover by reforming evenly distributed adherent small and irregular cell aggregates with up to 15–20% confluence within 1–2 d in mTeSR + Rocki (**Fig. 1c**). During the first week, the culture goes through several crisis phases associated with substantial cell death, possibly due to the selective action of the sequence of differentiation media (**Fig. 1c**). We usually observe that the addition of bFGF at D3 leads to important cellular aggregate rearrangements in the culture. Surviving mesodermal progenitors with a mesenchymal phenotype proliferate and become confluent within a week (**Fig. 1c**). Differentiation into paraxial mesoderm can be monitored by the expression of Tbx6 around D4 and D5 of differentiation, and the Tbx6-positive population should account for ~80% of the total culture at D4 (**Fig. 2**). Proper progression to an anterior presomitic mesoderm (PSM) fate and dermomyotomal specification can be assessed by the activation of Pax3 expression starting at ~1 week of differentiation and lasting through 3 weeks of differentiation (**Fig. 2**). Further myogenic commitment can be assessed by expression of the muscle regulatory factors, in particular MyoD and Myogenin, as well as by the presence of $Pax7^+$ progenitors. At 4 weeks of differentiation, both the $Myog^+$ and the $Pax7^+$ populations represent a combined 40–50% of the total nuclei. Myotubes/myofibers will be also clearly visible by phase contrast as early as 4 weeks (28 d+), and they mature over an additional 5 weeks+ of culture (**Fig. 3c**,**e**–**g**). At this stage, fibers can be a millimeter long and exhibit striations, best evidenced by immunohistochemistry for sarcomeric proteins such as titin and myosin heavy chain (**Fig. 2b**). Maturity of the contractile apparatus is evidenced by spontaneous twitching. Although the myogenic culture becomes very dense and tends to detach after 4 weeks of culture, it still contains myogenic progenitors (**Fig. 1c**). It is then advisable to proceed

to analysis or to passage the culture for expansion (**Fig. 3**). The expected fast MyHC+ fiber yield with this primary differentiation protocol is ~60–90% of hPSC cells seeded initially[1].

Subculture and secondary differentiation

The dissociation of the dense primary culture will eliminate dead cells, debris, ECM deposits and unwanted derivatives but also existing (postmitotic) myofibers (**Fig. 3b**). ~80% of the single mononucleated cells isolated are viable. The replating in SkGM selectively promotes myogenic progenitor expansion. This leads to more homogeneous, less dense cultures of proliferating myogenic progenitors and eliminates a lot of cell debris and unwanted derivatives that are byproducts of the differentiation (compare **Figs. 1c** and **3b**). With seeding at a density of 60–70,000 cells/cm^2, corresponding approximately to a subculture ratio of 1:4 to 1:8, culture confluence is achieved again within a few days with the formation of a sheet of elongated bipolar myoblasts. We found that confluent subcultures can be maintained in SkGM medium for an extended period of time without losing their myogenic potential when further passaged. Switching to K-HI, N2 media or, alternatively, HS-based media will promote further myogenic differentiation and fusion of progenitors (**Fig. 3a**). This can be achieved within a week, although muscle fibers continue to mature for 2 weeks, as evidenced by fiber size and development of myofibrils. Mature fibers from subcultures measure up to several millimeters in length, with a width ranging from 5 to 20+ µm (**Figs. 2b**, **4** and **5**). As for the primary culture, myogenic differentiation can be monitored and cellular phenotype can be assessed by immunohistochemistry (**Fig. 4**). After replating, up to 40% of total nuclei (from three independent cell lines) were Myog+, and mature skeletal myofiber density ranged from 9,000 to 20,000 fibers per cm^2 (**Fig. 5c–d**).

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