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Human muscle production *in vitro* from pluripotent stem cells: basic and clinical applications

Lu Yan[#1,2,3], **Alejandra Rodríguez-delaRosa**[#1,2,3], **Olivier Pourquié**[1,2,3,*]

[1]Department of Pathology, Brigham and Women's Hospital, Boston, Massachusetts, USA.

[2]Department of Genetics, Harvard Medical School, Boston, Massachusetts, USA.

[3]Harvard Stem Cell Institute, Boston, Massachusetts, USA.

[#] These authors contributed equally to this work.

Abstract

Human pluripotent stem cells (PSCs), which have the capacity to self-renew and differentiate into multiple cell types, offer tremendous therapeutic potential and invaluable flexibility as research tools. Recently, remarkable progress has been made in directing myogenic differentiation of human PSCs. The differentiation strategies, which were inspired by our knowledge of myogenesis *in vivo*, have provided an important platform for the study of human muscle development and modelling of muscular diseases, as well as a promising source of cells for cell therapy to treat muscular dystrophies. In this review, we summarize the current state of skeletal muscle generation from human PSCs, including transgene-based and transgene-free differentiation protocols, and 3D muscle tissue production through bioengineering approaches. We also highlight their basic and clinical applications, which facilitate the study of human muscle biology and deliver new hope for muscular disease treatment.

Keywords

Human pluripotent stem cells (PSCs); Skeletal muscle; Tissue engineering; Muscular dystrophy; Myogenesis; Satellite cells

1. Introduction

Skeletal muscle, which makes up almost half of the human body mass, plays a critical role in movement and metabolism [1]. This tissue originates from the paraxial mesoderm, and forms from dermomyotome precursors through two main consecutive waves known as primary and secondary myogenesis (reviewed in [2,3]). Functional muscles are composed

[*]Corresponding author: pourquie@genetics.med.harvard.edu.

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of contractile multinucleated myofibers, satellite cells (SCs), connective tissue, blood vessels, motor and sensory axons, Schwann cells and immune cells. Crosstalk between these different cell types is essential not only for muscle contraction, but also for muscle development, homeostasis and regeneration. Moreover, skeletal muscle possesses the capacity to regenerate after injury caused by overuse, disease, trauma or toxins. This process is primarily accomplished by SCs, the muscle stem cells lying under the basal lamina of myofibers [4-7].

Effective methods to restore or improve muscle function in patients suffering from muscle disorders are required for the alleviation of symptoms and improvement in their quality of life. Embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), collectively referred to as pluripotent stem cells (PSCs), have almost unlimited proliferative potential and the ability to differentiate into multiple lineages, and thus they constitute a promising source for cell-based therapies and drug screening [8]. Moreover, iPSCs allow the creation of patient-derived cells, which enables disease modelling *in vitro*. The production of myogenic cells from PSCs was first observed after differentiation of embryoid bodies derived from mouse ESCs [9]. The recent development of transgene-based and transgene-free strategies has made it possible to generate skeletal muscle *in vitro* more efficiently [2,3,10]. While transgene-based protocols can achieve direct myogenic differentiation with overexpression of transcription factors, transgene-free protocols manipulate signaling pathways using small molecules and growth factors to recapitulate the muscle development process *in vitro* [10]. Therefore, the stepwise transgene-free approach not only has immediate therapeutic implications, but also provides a platform for understanding the basic developmental biology of human myogenic lineage differentiation. In this review, we summarize the remarkable progress in generating myogenic cells *in vitro* from human PSCs and discuss both basic and clinical applications.

2. Making muscle *in vitro*

The road towards creating muscle in a dish started 30 years ago, when transfection of the transcription factor MyoD1 into fibroblasts was shown to induce their myogenic conversion [11]. In the last 10 years, there has been a surge of protocols for myogenic differentiation from human PSCs [12]. The two main strategies include (1) myogenic induction by overexpression of transcription factors and (2) stepwise induction of skeletal muscle by mimicking signaling pathways deployed during development (Fig. 1).

2.1. Myogenic induction by overexpression of transcription factors

Selective overexpression of myogenic transcription factors, such as *MYOD1*, was the first reported approach to induce myogenic differentiation [12,13]. Such transgenic approaches traditionally require the transduction of the myogenic construct via adenovirus [14], lentivirus [15,16] or piggyback transposons [17-19]. Additionally, the timing of overexpression can be controlled by inducible systems such as tetracycline/doxycycline [15,17-19] or tamoxifen [16].

Even though *MYOD1* may seem the obvious candidate gene to induce the myogenic program in PSCs, it is expressed late in the differentiation of myogenic cells. Thus,

overexpression of this transcription factor gives rise to terminally differentiated muscle cells but no muscle progenitors [10]. Moreover, human PSCs (hPSCs) are resistant to myogenic induction mediated by *MYOD1* and require additional epigenetic cues to differentiate to skeletal muscle [13]. Protocols involving the use of *PAX3* or *PAX7*, which are transcription factors expressed by myogenic progenitor cells (MPCs) and SCs, can give rise to MPCs able to differentiate into myotubes *in vitro* and endowed with the capacity to engraft in mouse muscles [15].

However, these protocols usually require a cell-sorting step in order to purify the precursor populations [20-22], thus complicating translational applications. Moreover, the required introduction of a transgene makes them less suitable for human cell-based therapies due to the risk of insertional mutagenesis [8,23]. This limitation was bypassed by Kim et al., who proposed an integration-free protocol using minicircle DNA vectors. While able to generate human iPSC (hiPSC)-derived $PAX7^+$ MPCs, multiple transfections were required to maintain PAX7 expression and these MPCs showed limited engraftment potential [21]. On a similar note, Akiyama et al. proposed an integration-free protocol by introducing mRNA encoding MYOD1 together with siRNA-mediated knockdown of *OCT4* [23]. This protocol reported efficient differentiation of PSCs into fetal-like myofibers, but no expression was reported for PAX7 or for MYH2, a marker of adult fast myofibers. Komatsu et al. proposed the use of an RNA virus-based episomal vector encoding MYOD1. This integration-free system induces persistent RNA transgene production and leads to the formation of Myosin Heavy Chain (MHC)-expressing myotubes. The novel advantage of this system is that the vector can be eliminated upon treatment with a small molecule, T-705, increasing the safety of this approach [24].

While still requiring integration, Kim et al. proposed using genomic safe harbor (GSH) loci for the integration of transgene components. The GSH-targeted *PAX7* integration led to efficient generation of MPCs with lower proliferative capacity but similar engraftment potential in mice than lentivirus-induced MPCs [25].

Alternatively, Kwon et al. proposed to induce endogenous PAX7 activation using a CRISPR/Cas9-based transcriptional activator. MPCs obtained through endogenous *PAX7* activation showed higher proliferation and engraftment capacity than by exogenous *PAX7* overexpression [22].

Although transgenic-based methods do not recapitulate physiological development, they have become increasingly popular in the field. Research towards methodologies with higher security (e.g. integration-free approaches) and higher engraftment capacity (e.g. *PAX7* overexpression) will progressively increase the suitability of these protocols towards human application.

2.2. Stepwise myogenic induction by mimicking development

Skeletal myogenesis can be recapitulated *in vitro* from PSCs by exposure to combinations of small molecules and growth factors, which activate the core signaling pathways deployed during development (reviewed in [2,3,10]). Most of these stepwise induction methods start with the formation of neuro-mesodermal progenitors (NMPs) which are characterized by

the expression of SOX2 and Brachyury/T. This first step is achieved by treating PSCs with a Wnt activator such as the GSK3β inhibitor CHIR99021 (CHIR) [26-28]. Treating human PSC cultures with CHIR (in combination with the BMP inhibitor LDN) is sufficient to trigger Nodal activation, which is known to play a key role in mesoderm induction and patterning [27]. Thus, while some protocols include treatment with activin to activate Nodal at these early stages [29-31], this does not appear necessary as endogenous Nodal is activated following Wnt activation [32]. The same is true with FGF signaling, which is also found in several protocols aiming at differentiating paraxial mesoderm *in vitro*, but is spontaneously activated in cells downstream of Wnt activation [26,28]. Remarkably, Wnt activation *in vitro* appears sufficient to trigger an epithelium-to-mesenchyme transition (EMT) evoking cell ingression during gastrulation and resulting in the conversion to presomitic mesoderm (PSM)-like cells, indicated by *MSGN1* and *TBX6* expression [26].

Cell signaling in the culture also shows tight endogenous control which can be independent of the signaling activators added to the culture medium. For instance, both Wnt and FGF signaling are downregulated *in vitro* exactly as observed during PSM differentiation, where gradients of these two pathways control the segmentation process [33]. *In vitro*, their downregulation is observed despite the constant presence of the Wnt activator CHIR and of FGF, suggesting that it reflects an intrinsic regulation of the differentiation of the cells. This regulation involves a crosstalk between cell metabolism and signalling with FGF acting upstream of glycolysis to control Wnt activity [34]. Glycolysis acts by increasing the intracellular pH, thus promoting non-enzymatic β-catenin acetylation triggering Wnt activation required for paraxial mesoderm specification [35].

PSCs treated with CHIR *in vitro* differentiate towards a PSM fate but start to express BMP4 [36]. As BMP signaling promotes the lateral plate mesoderm fate, the induced paraxial mesoderm cells expressing BMP4 eventually lose their paraxial mesoderm identity to acquire a lateral plate fate [37]. BMP inhibition mediated by LDN-193189 in parallel with or after Wnt activation decreases BMP4 activation and stabilizes the paraxial mesoderm fate *in vitro*, leading to the recapitulation of a myogenic sequence very similar to that described for mouse *in vivo* [26]. Together, these treatments recapitulate the early stages of paraxial mesoderm induction resulting in the production of MPCs of the dermomyotome expressing PAX3 [26]. By comparing the transcriptional profiles of human PSM and somites, Xi et al. observed that both TGFβ and BMP signaling pathways are downregulated during human somite specification. Thus, they introduced treatment with a TGFβ inhibitor (SB-431542) along with BMP inhibition at the somite specification stage [38]. When further cultured with media containing the myogenic growth factors (FGF, HGF and IGF), $PAX7^+$ MPCs and MHC^+ myofibers are obtained [26,39].

These directed differentiation methods do not involve genetic modifications and thus may represent a more promising approach for cell therapy [26,38]. Moreover, the entire myogenic differentiation process *in vitro* closely resembles the *in vivo* myogenic sequence [26,40,41]. Therefore, it also provides a powerful tool to model human muscle development, which has been poorly understood due to limited access to human embryos.

3. Bioengineering approaches

A significant caveat of myogenic differentiation protocols in 2D is the non-physiological stiffness (~1 GPa) of the substrates employed, which do not mimic the physiological environment of myofibers (12-18 kPa). Traditional tissue culture substrates, such as plastic, do not support long-term spontaneous muscle contraction, leading to progressive myotube detachment and preventing maturation [42]. The growing knowledge of skeletal muscle microenvironment *in vivo*, as well as improvements in biomaterials, have allowed the development of 3D hiPSC-derived skeletal muscle tissues with greater biomimetic structure and function. Importantly, 3D engineered muscle tissues permit longer-term culture, leading to enhanced maturation of myotubes [42].

The field of tissue engineering has developed several techniques to mimic native skeletal muscle tissue, such as the creation of cell sheets [43], cell aggregates [44-47], fibrous scaffolds [48], hydrogels [20,49-51], and 3D bioprinting [52]. While initially these techniques were tested with primary myoblasts or immortalized myoblast cell lines, such as C2C12, recent studies have started to describe the use of hiPSC-derived skeletal muscle cells [20,44-47,51].

The first 3D systems supporting myogenic differentiation of PSCs were based on self-organization principles as observed in embryoid bodies (EBs), in which myogenesis can be spontaneously observed [9]. Hosoyama et al. proposed a method for the derivation of MPCs using a free-floating spherical culture (EZ spheres). Spheres containing PSCs were cultured in a medium rich in EGF and FGF2 for 6 weeks. Upon dissociation and culture, a portion of the resulting cells expressed PAX7, MYOD, and Myogenin [47]. A refined version of this protocol was presented by Jiwlawat et al., in which EZ spheres were dissociated and plated for a longer time resulting in more mature myofibers. Importantly, they also generated 3D muscle constructs with sphere-derived myogenic progenitors by using a mixture of collagen/Matrigel plated between two Velcro anchoring points [46]. This approach, based on the work of Van der Schaft et al. [53], gave rise to myofibers surrounded by basal lamina [46]. Chal et al. presented a similar muscle construct as a potential downstream application of their protocol to induce stepwise differentiation of hiPSCs towards the myogenic lineage [39]. These constructs, initially termed myooids by Dennis et al., consisted of fascicle-like muscle constructs ensembled around silk suture anchors [39,54].

Rao et al. combined the induction of paraxial mesoderm by GSK3β inhibition with a PAX7 inducible system to obtain MPCs. Then, they embedded those cells in a fibrin-based hydrogel and generated 3D induced-skeletal muscle (iSKM) bundles. Both $PAX7^+$ cells and aligned contractile multinucleated myofibers were observed in the constructs. Although iSKM bundles still had a relatively immature expression pattern of MYH isoforms compared to adult muscle, they showed progressive maturation in their gene expression pattern, structure and function over time, while maintaining a $PAX7^+$ cell pool. Importantly, skeletal muscle maturation in their 3D system exceeded that of their 2D system in molecular, structural and functional aspects, supporting the necessity of establishing 3D systems for successful maturation of the differentiating muscle [20]. Using the same 3D system, Khodabukus et al. showed that intermittent electrical stimulation significantly increased

iSKM bundle growth, maturation and force generation independently of the frequency. Also, higher frequency stimulation resulted in greater myofiber hypertrophy and promoted a metabolic shift towards longer-chain fatty acid oxidation [55].

Recently, Maffioletti et al. used a *MYOD1* overexpression protocol [16] to obtain hiPSC-derived myogenic cells. They embedded them in fibrin hydrogels polymerized between two silicone posts, which provided the tension necessary for myofiber alignment [51]. Moreover, in order to increase the complexity of these artificial tissues, the authors used established protocols to differentiate hiPSCs into vascular endothelial cells, pericytes and motor neurons [56,57]. Incorporation of ECs and pericytes into the artificial muscle resulted in the appearance of $CD31^+$ vessel-like formations coexisting with myofibers. While no detailed characterization was made when all four cell types were added, the artificial constructs were stable and harbored cells with long axon-like processes resembling motor neurons. Hence, this supports the feasibility of more complex hiPSCs-based muscle scaffolds with the incorporation of supporting cell types [51].

Faustino Martins et al. described a protocol to derive hiPSC into NMPs and then in 3D organoids which contain both motoneurons and skeletal muscle fibers. NMP cells obtained from hiPSCs by activating Wnt and FGF signaling were induced to form 3D aggregates in the presence of FGF, HGF and IGF [44]. These structures progressively elongated and segregated into a neuroectodermal and a mesodermal part. Over time, maturation of these organoids gave rise to spinal cord motor neurons innervating skeletal muscle cells, with terminal Schwann cells capping the neuromuscular junction as observed *in vivo*. The authors reported the presence of $PAX7^+$ cells and myofibers in the mesodermal part and showed that while the myofibers present were not aligned, these contained highly organized sarcomeric units [44].

The recent incorporation of 3D models might hold the key towards long-term culture and improved myofiber maturation, a current limitation of these systems.

4. Applications

4.1. Basic application: understanding human muscle development

The study of human muscle development has been a formidable task due to the limited access to human embryos and tissues [58]. However, the recent development of *in vitro* myogenic cell production strategies provides new tools for deciphering human myogenesis and the biology of skeletal muscle progenitors, SCs, and myofibers. These differentiating cells can be compared to mouse myogenic cells whose developmental programme has been well characterized. Thanks to the development of new imaging techniques such as light sheet fluorescence microscopy, which uses a plane of light to optically section the sample with high resolution [58], and to single cell omics [40,59], an increasing amount of data on human embryonic muscle is being generated. This data can be used to efficiently benchmark the data generated from human PSCs differentiated *in vitro*.

Stepwise myogenic induction protocols attempt to recapitulate skeletal muscle development *in vivo* including NMP induction, PSM and paraxial mesoderm formation, and primary

and secondary myogenesis [2,3]. They can therefore be used to analyze in detail the roadmap of human myogenesis and to perform functional explorations of the differentiation process that would be impossible to carry out *in vivo*. Muscles of the trunk and neck are segmented structures periodically arrayed along the body axis. This repeated pattern is established during embryogenesis when the embryonic segments, the somites, are rhythmically produced from the PSM [60]. The rhythm of somite production is controlled by a molecular oscillator called segmentation clock, which has so far only been characterized in model organisms [61]. During the first two days of induction, following Wnt activator and BMP inhibitor treatment, hiPSCs acquire a posterior PSM fate [26,33]. Tracking the expression of oscillating genes of the segmentation clock such as *HES7* in these hiPSC-derived PSM cells has allowed to identify the human segmentation clock, which ticks with a 5-hour period [33,62]. Further induction by manipulating core signaling pathways using small molecules and growth factors leads to the generation of skeletal muscle containing myofibers and $PAX7^+$ myogenic precursors [26,38] which include the precursors of adult SCs [63,64]. Thus, hiPSC-derived $PAX7^+$ cells provide an opportunity to investigate the development of the human SC lineage on which virtually nothing is known. Using iPSC reporter lines and single-cell RNA sequencing (scRNA-seq), the developmental trajectory of $PAX7^+$ skeletal muscle progenitors has been characterized [40,41]. This also revealed their heterogeneity, which resembles that of mouse muscle progenitors and SCs *in vivo* [41].

In addition, this myogenic differentiation platform allows functional dissection of gene regulatory networks and signaling pathways during myogenesis. For instance, by comparing transcriptome differences between cells at different myogenic states, Choi et al. identified key factors and signaling pathways involved in myogenesis and discovered the role of the transcription factor TWIST1 in $PAX7^+$ muscle progenitor cell maintenance [65]. Moreover, hiPSC-derived muscle production may permit the study of some important structures such as the neuromuscular (NMJ) and myotendinous junctions (MTJ) [44,66-68]. NMJs can be generated by co-culture of motor neurons and skeletal myotubes both in 2D and 3D culture systems [44,51,67,69].

Analyses of myogenic cells derived from mouse and human PSCs show that although they recapitulate muscle differentiation, these cells only reach the embryonic/fetal/perinatal stage [26,36,40,70,71]. Such is the case for most other lineages differentiated *in vitro* from PSCs, including cardiomyocytes, neurons or hepatocytes, for instance [72]. Using scRNA-seq, single-cell transcriptomic atlases were established for the hiPSC-derived skeletal muscle cells produced by three different transgene-free methods [26,38,73] and compared to human limb skeletal muscle cells at embryonic (week 5-8), fetal (week 9-18), juvenile (year 7-11) and adult (year 34-42) stages. By comparing these transcriptomes, Xi et al. concluded that the hiPSC-derived muscle progenitors produced in these conditions are at an embryonic-to-fetal transition stage [40].

Several studies have been conducted to promote the maturation of PSC-derived myotubes generated *in vitro* [70,74,75]. By comparing transcriptomic differences between myogenic progenitors and myotubes derived from both hiPSC and human fetal-stage muscles, TGFβ signaling was found to be reduced in differentiated myotubes. TGFβ inhibition of late stage PSC cultures with inhibitors of activin receptor like kinases ALK4/5/7 such as SB-431542

or A83-01, increases myotube fusion efficiency and leads to a cellular morphology that resembles late-stage fetal myotubes [70]. Specific myosin isoforms are expressed during skeletal muscle development [76]. Without TGFβ inhibition, hiPSC-derived myotubes mainly express embryonic MHC (MYH3) and fetal/neonatal MHC (MYH8) instead of late fetal/adult isoforms such as MYH1 or MYH2, indicating a fetal-like state. However, with TGFβ inhibition, expression of all myosin isoforms was elevated, including MYH1 [70,74]. Better-organized sarcomeres were also formed after TGFβ inhibition, as evidenced by transmission electron microscopy [70]. Another improvement of skeletal myofiber maturation was achieved by the addition of prednisolone, a synthetic glucocorticoid. The combination of prednisolone and TGFβ inhibition leads to the formation of better organized myofibrils and to an upregulated expression of the fast myosins *MYH1/2/4* [74]. Moreover, a small molecule library screen identified the TGFβ inhibitor SB-431542, the Notch inhibitor DAPT, Dexamethasone and Forskolin as effective molecules regulating myotube maturation [75]. Notch inhibition with DAPT after the pervasive initial Wnt activation gave rise to robust myogenic induction, indicated by a large fraction of $MYOG^+$ cell population [77]. However, treatment with DAPT was also shown to induce the differentiation of all $PAX7^+$ cells, thus resulting in cultures depleted of SCs [41].

Taking advantage of the hiPSC-derived myogenic induction system, basic studies on human muscle specification, maturation and homeostasis can be conducted with an unlimited source of cells. However, as mentioned above, these myogenic cells generated from hiPSCs exhibit mainly fetal or embryonic features. Additionally, although the stepwise induction protocol mimics the *in vivo* muscle development, further studies are required to provide more detailed comparison between these cells and their *in vivo* counterparts. Nevertheless, the improved understanding of basic muscle biology and protocols of muscle generation will further contribute to the optimization of *in vitro* muscle generation and benefit the clinical applications.

4.2. Disease modelling

Human primary myogenic cells can be obtained from invasive biopsies, but their availability is very limited. Hence, the virtually infinite expansion capacity and controllable differentiation of PSCs into myogenic precursors hold great potential for muscle disease modelling and drug development [51]. Within musculoskeletal pathologies, muscular dystrophies are a group of heterogeneous genetic diseases characterized by progressive degeneration of skeletal muscle. The most common form of muscular dystrophy is Duchenne Muscular Dystrophy (DMD), a lethal X-linked muscular disease (affecting approximately 1 in 5000 live male births) caused by mutations in the dystrophin (*DMD*) gene [78,79]. This disease starts to manifest around 3 years of age, progressively leading to muscle weakness, respiratory insufficiency and cardiac failure that causes premature death in young adulthood [80]. While our knowledge of DMD etiology and pathogenesis has progressed, to date there is no cure, and current treatment is based on the chronic administration of corticosteroids, cardioprotective treatment, ventilatory support, physical therapy, and antisense-mediated exon-skipping treatment in a subset of patients [78,81].

The difficulty of accessing muscle material from human patients has resulted in much of the research on DMD to be carried out in the *mdx* mouse, a spontaneous dystrophin mutant [82]. However, a significant problem with the *mdx* mouse is that the pathology is much less severe and only partly phenocopies the human DMD [83]. Thus, progress in understanding the onset of the disease has suffered from the lack of an appropriate model, in which early stages of the pathology can be analyzed to identify the primary causes of DMD. This has led to considerable research efforts directed to model DMD using patient-derived hiPSCs. A summary of the recent publications modelling DMD with hiPSCs can be found in Table 1. Previous studies on biopsies from DMD patients and *mdx* mice have reported several specific phenotypes, such as branching/fusion defects [84], calcium signaling hyperactivation [85] and defective contractions of the myofibers [86]. These phenotypes are variably recapitulated in hiPSC-derived DMD myofibers depending on the differentiation protocol [19,74,77,87,88]. Interestingly, some studies have shown partial or complete defective myotube formation from DMD hiPSCs [77,89], while others document that DMD hiPSCs generate myotubes comparably to those of control hiPSCs [14,19,51,87,88,90] (See Table 1). This discrepancy may result from the use of different protocols for the differentiation of myotubes (e.g., transgene-free vs transgene-based). Alternatively, it might also result from the comparison of iPSC lines from unaffected and affected patients with different genetic backgrounds. Indeed, it is well known that different iPSC lines often exhibit different differentiation potential even with the same protocol. This problem has been circumvented in a recent study using isogenic lines in which common DMD mutations were introduced in a wild-type parental iPSC line or where a patient iPSC line was corrected using CRISPR-Cas9 to restore the dystrophin coding frame [74]. Isogenic mutant lines were able to differentiate to a myogenic fate, while showing reduced myogenic potential, and recapitulated the hallmarks of the DMD myofiber phenotype including branching, intrinsic defective contraction and calcium handling defects.

Choi et al. also reported increased branching and fusion defects together with increased levels of BMP4 and TGFβ signaling in hiPSC-derived myofibers [77]. Treatment with dual SMAD inhibitors partially rescued the fusion defects, implying that aberrant TGFβ signaling might be responsible for the defective fusion. Similarly, Caputo et al. described that DMD hiPSC-derived myotubes exhibit a constitutive activation of TGFβ-SMAD2/3 signaling [88]. In contrast, Al Tanoury et al. reported the presence of increased branching accompanied by higher fusion of hiPSC-derived DMD myofibers, supporting the hypothesis that aberrant branching is due to increased fusion required for sustained regeneration in DMD patients [74,84].

Current studies have not been limited to the analysis of molecular mechanisms of DMD, but have also explored potential new treatments for DMD such as exon skipping [19], human artificial chromosome transfer [77], CRISPR/Cas9-mediated gene editing [87,89], and cell-based therapies [91].

Another potential application of hiPSC-derived DMD myofibers is the possibility to perform screenings of chemical libraries to identify drug candidates for skeletal muscle diseases. Sun et al. recently published the results of a screen performed on hiPSC-derived DMD myoblasts [92]. Using a high-content imaging approach, they evaluated fusion in hiPSC-derived

DMD myoblasts after treatment with 1,524 compounds from the Johns Hopkins Clinical Compound Library. Two potential hits, ginsenoside Rd and fenofibrate, were identified as enhancers of myogenic fusion and were later validated *in vivo* in *mdx* mice. While ginsenoside Rd appears to interfere with FLT3 signaling, fenofibrate is a suppressor of TGFβ signaling [92]. The results of this study support the feasibility of using hiPSC-derived myogenic cultures for drug screening to identify new treatments for muscular dystrophies.

Additional studies have used hiPSCs to model other genetic diseases affecting muscles, such as carnitine palmitoyltransferase II deficiency [18], Pompe disease [93,94] and Miyoshi myopathy [17]. Recently, Maffioletti et al. generated fibrin-based artificial muscles using hiPSCs from healthy donors and patients diagnosed with several different muscular dystrophies such as Duchenne, limb-girdle type 2D, and LAMIN A/C (LMNA)-related muscular dystrophies. LMNA-mutant artificial muscles successfully recapitulated the nuclear abnormalities characteristic of this laminopathy, opening the door to more 3D-based muscle disease modelling studies [51].

Overall, there is a growing body of studies that are shifting towards the usage of PSC-derived muscle cultures. Further research is needed to understand the basis of the discrepancies found between differentiation protocols and genetic backgrounds. Moreover, the immature status of the myogenic populations generated *in vitro* poses a limitation for the study of adult stages of myogenic diseases. However, usage of isogenic lines, more standardized protocols and 3D bioengineered approaches will progressively increase the suitability of these systems. Nonetheless, their potential ability to recapitulate human pathophysiology without the limitation of sample obtainment provides a significant advantage for studying rare genetic disorders such as DMD.

4.3. Cell-based therapies

In recent years, stem cell-based technologies that can serve as the basis for cell-based therapies have been rapidly developing. The main purpose of cell therapy is to replenish lost cells or replace dysfunctional cells with new cells generated from transplanted progenitors. Several cell therapy clinical trials involving transplantation of primary myoblasts or pericytes into skeletal muscles have been carried out but they failed to show significant engraftment and restoration of muscle function [95-98]. However, research from the last two decades suggests that the ideal material for regenerating muscle by transplantation are the $PAX7^+$ SCs [99-101]. This has led to the development of protocols aiming at differentiating human PSCs toward the PAX7-expressing SC fate with the long-term goal of developing cell therapy protocols for muscle repair.

The production of human SC generated *in vitro* has been optimized using PSC reporter lines in which GFP is driven from the PAX7 or MYF5 locus. Their engraftment potential and regenerative capacity *in vivo* has been demonstrated in mice [26,41,91,102]. Purification of SCs before transplantation is required for safety to avoid the risk of teratoma from residual PSCs and to increase efficiency (Fig. 2). This requires identification of surface markers to purify the cell populations exhibiting the highest regeneration potential. ERBB3 and NGFR were identified as surface markers allowing enrichment of hiPSC-derived SCs, exhibiting *in vivo* myogenic regenerative capacity after engraftment [70]. Sorted cells have

been used for transplantation experiments in rodent models to evaluate their myogenic potential [70,77,102-105]. Using a PAX7/MYF5 double-reporter line, a surface marker screen led to the identification of CD10 as a positive marker and CD24 as a negative marker for MPC purification. Successful engraftment was detected after $CD10^{+}CD24^{-}$ cells injection [102,106]. Moreover, by comparing transcriptomic differences between early-stage (6 weeks) and late-stage (10 and 11 weeks) $MYF5^{+}$ cells, a recent study revealed that late-stage $MYF5^{+}$ cells resemble fetal SCs and possess high regeneration potential after injection in mice [91].

Although significant progress has been made with the production of SC for cell therapy, some limitations still exist. Cell survival after transplantation needs to be improved, as the injected cells undergo necrosis due to the acute inflammatory response, which decreases the engraftment efficiency [107]. While immune rejection of the transplanted cells may be avoided by using autologous patient-specific hiPSCs, this approach is difficult to implement due to the cost and time associated with current good manufacturing practice (cGMP) requirements [108]. Alternative solutions involving allogeneic donor cells are more realistic and involve human leukocyte antigen (HLA)-matched donor cells or “universal” donor cells [109-111].

5. Conclusions and perspectives

Our understanding of the development and physiopathology of human skeletal muscle has been limited until recently due to the limited access to human biopsies. The protocols to differentiate PSCs towards the myogenic lineage, while still new to the field of tissue engineering, are becoming progressively more efficient, generating increasingly mature cultures. So far, the two main strategies used are the overexpression of transcription factors and the stepwise induction of skeletal muscle by small molecules or growth factors treatments [8,12]. The combination of these two approaches with bioengineering techniques allows to mimic more faithfully the skeletal muscle 3D environment, hence promoting its maturation [42] (Fig. 3). The co-culture of PSC-derived muscle with other cell types present in physiological muscle, such as motor neurons or tenogenic cells, has started to be explored as a potential enhancer of maturation. During muscle development, innervation plays an important role in myofiber maturation [112], hence, the addition of this element into PSC-derived myogenic cultures might be a requirement for further development. Mechanical tension is another important element in the development and maturation of myofibers [113]. Tendon cells are key regulators of muscle fiber attachment, so their co-culture could also promote myofiber maturation. Another interesting approach is the electrical stimulation of hiPSC-derived muscles, which increases myofiber size, force generation and changes their metabolic profile [55]. Moreover, additional treatments including drugs such as prednisolone should be considered to promote maturation [74]. An improvement of the maturation of myofibers generated *in vitro* could contribute to recreate a more physiological muscle stem cell niche and lead to more mature SCs, which may improve cell-based therapies. The discovery of methods to improve maturation of these cultures towards an adult-like phenotype is therefore an important goal. While several challenges still lay ahead towards fully mimicking human muscle, hiPSC-derived myogenesis holds promise of becoming a

gold standard methodology for developmental and disease modelling in muscle biology and for drug screening and cell-based therapies in pre-clinical studies.

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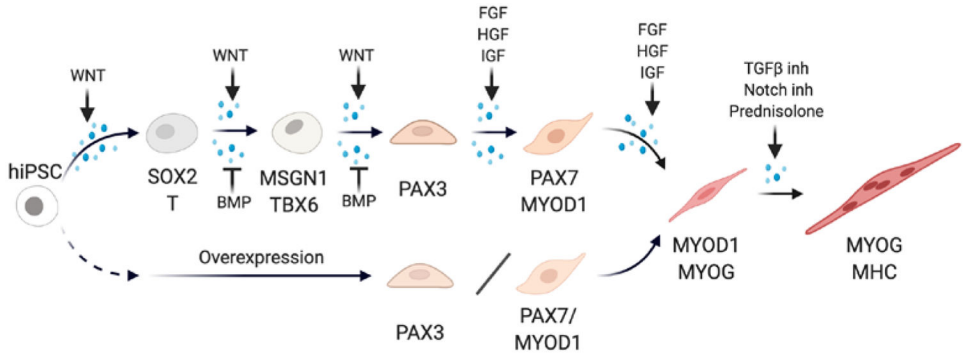

Fig. 1. Strategies of skeletal muscle production from hiPSCs.

The process of *in vitro* muscle generation by the stepwise differentiation protocol (top) or transgenic induction (bottom). The stepwise strategy uses small molecules and growth factors to manipulate critical signaling pathways in myogenesis while the transgenic approach induces myogenic differentiation by overexpression of core transcription factors. Key marker genes at different differentiation stages and key pathways manipulated in the stepwise protocol are indicated.

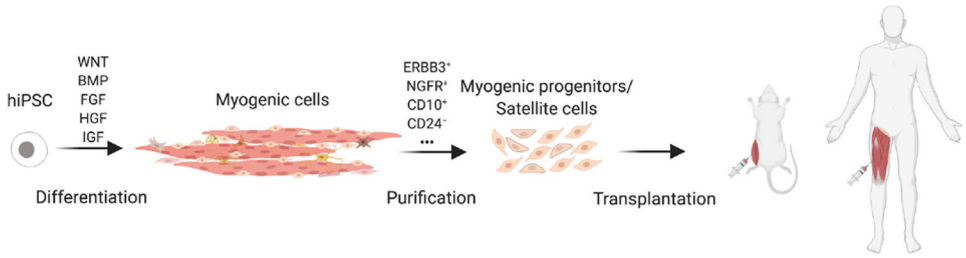

Fig. 2. Schematic model for cell therapy using hiPSC-derived myogenic cells.

Successful muscle cell therapy requires transplantation of sufficient myogenic progenitors with regenerating capacity. After myogenic differentiation of hiPSCs using the aforementioned protocols, the heterogeneous cell population obtained needs to undergo purification and enrichment through cell sorting based on specific surface markers.

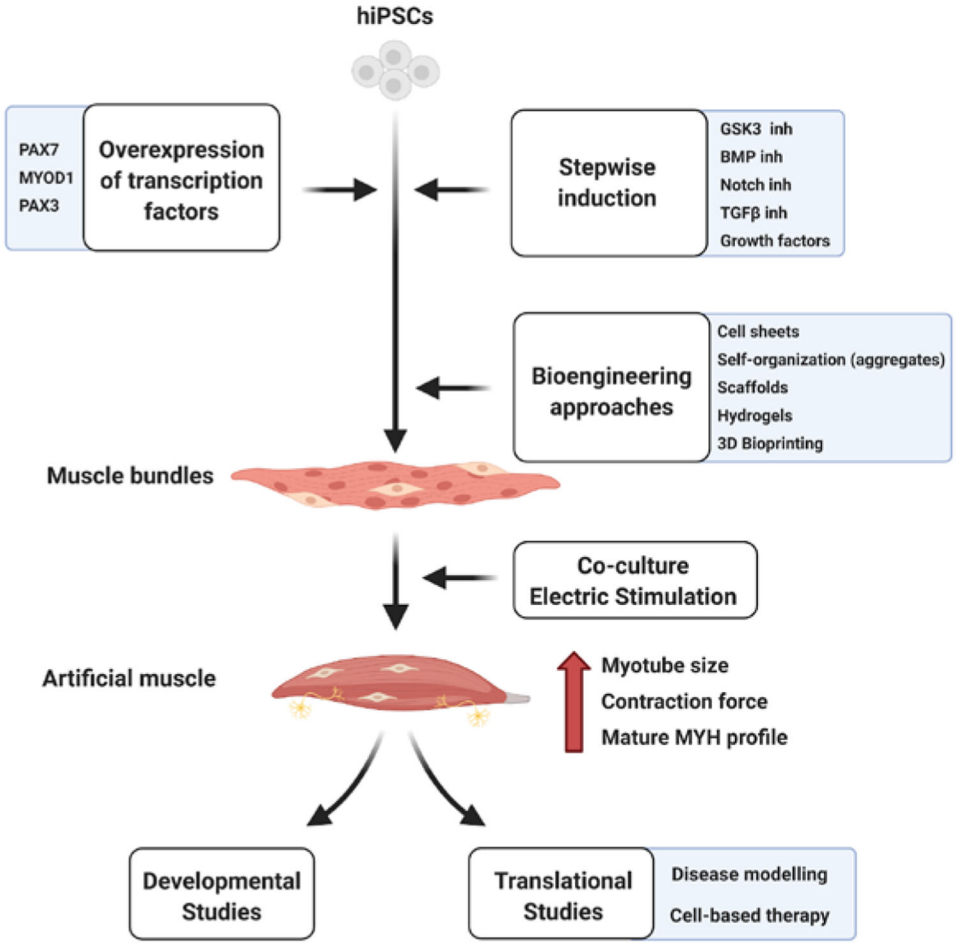

Fig. 3. Overview of different techniques used in hiPSC-derived myogenesis.

Diagram depicting the different techniques and bioengineering approaches used towards the induction of skeletal muscle from hiPSCs. Further electrical stimulation or co-culture with other non-myogenic cell types present in adult muscle, such as motor neurons, might contribute towards increasing the maturation and function of the artificial muscles obtained, optimizing their suitability for basic and clinical applications.

Table 1.

Overview of the current publications using hiPSC-derived DMD myogenic cultures.

Reference	Cell line	Differentiation protocol	Myogenic markers detected in DMD-hiPSC myogenic cultures (protein)	Myogenic differentiation	DMD Phenotype *in vitro*	Engraftment
Goudenege et al., 2012	DMD patient hiPSC and healthy controls	MYOD1 overexpression (Adenovirus)	MYOD1, MHC, Spectrin, Lamin A/C	Similar to control	Larger myotubes compared to healthy controls	Performed in rag/*mdx* mice with/without cardiotoxin. Documented presence of hybrid myofibers
Abujarour et al., 2014	DMD patient hiPSCs and healthy controls	Doxycycline-dependent MYOD1 overexpression (Lentivirus)	MHC, MYOD1, MYOG, NCAM, CD44, CD29	Similar to control		No
Shoji et al., 2015	DMD patient hiPSCs and healthy controls treated or not with exon-skipping oligonucle otides	Tetracycline-dependent *MYOD1* overexpression (PiggyBac vector)	CKM, MHC, Skeletal Muscle Actin	Similar to control	Excess of calcium flux (Fluo-8 dye) upon electrical stimulation	No
Young et al., 2016	DMD patient hiPSCs and CRISPR edited version rescued by deletion of exon 45-55	Tamoxifen-dependent *MYOD1* overexpression (Lentivirus) or Shelton et al. (2014) directed differentiation protocol	MHC, NCAM, Spectrin, Lamin A/C	Similar to control	β-dystroglycan downregulation and mislocalization	Performed in NOD-SCID-IL2rg^{null} (NSG)-mdx mice with cardiotoxin. Documented presence of engrafted myofibers by human Spectrin and Lamin A/C expression
Choi et al., 2016	DMD patient hiPSCs, healthy controls and correction with human artificial chromoso me expressing DMD	CHIR/DAPT treatment in N2 medium, replating and FACS sorting of NCAM^{+}/HNK1^{-} cells	Desmin, Lamin A/C, Laminin	Defective	Decreased fusion and myogenic marker expression. Absence of spontaneous contractions. Increased branching. Increased levels of BMP4 and TGFβ signaling. Increased expression of interleukins 6 and 8 and collagen 3	Performed in NOD-Rag1^{nu11} IL2rg^{nu11} (NRG) mice and NSG-mdx mice after cardiotoxin. Comparable levels of human myofiber formation in both models
Maffioletti et al., 2018	DMD Patient hiPSC lines and healthy controls	Tamoxifen-dependent *MYOD1* overexpression (Lentivirus) or directed differentiation (Caron et al., 2016) and 3D constructs	Laminin, Lamin A/C, MHC, Sarcomeric actin	Similar to control		Performed in NSG mice previously injured. Documented presence of engrafted myofibers by human Lamin A/C and embryonic MHC expression

Reference	Cell line	Differentiation protocol	Myogenic markers detected in DMD-hiPSC myogenic cultures (protein)	Myogenic differentiation	DMD Phenotype *in vitro*	Engraftment
Caputo et al., 2020	DMD patient hiPSC lines and healthy controls	Doxycycline-dependent *MYOD1* and *BAF60C* overexpression (PiggyBac vector)	MYOD1, Desmin, MHC	Similar to control	DMD hiPSC-derived myotubes exhibit constitutive activation of TGFβ-SMAD2/3 signaling. Electrically paced DMD hiPSC-derived myotubes exhibit greater and persistent increase in the expression of pro-fibrotic genes (TGFβ1, TGFβ2, IL6, and CTGF)	No
Moretti et al., 2020	DMD patient hiPSC line, CRISPR edited version rescued by deletion of exon 51 and healthy control	Commercial kit Amsbio; SKM-KITM	Not detected	Defective	No multinucleated myotubes	No
Al Tanoury et al., 2020	DMD patient hiPSC, CRISPR edited version rescued, CRISPR-engineered DMD mutants and isogenic healthy parental control	Directed differentiation (Chal et al., 2015, 2016)	MYOG, Desmin, Titin, α-actinin, NCAM, nNOS, DAG1 and delta-Sarcoglycan	Defective	Increased branching and fusion. Mislocalization of proteins of the Dystrophin-associated Glycoprotein Complex. Force contraction defects and Ca^{2+} hyper-excitability	No