Assessment of different strategies for scalable production and proliferation of human myoblasts

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Abstract

Objectives: Myoblast transfer therapy (MTT) is a technique to replace muscle satellite cells with genetically repaired or healthy myoblasts, to treat muscular dystrophies. However, clinical trials with human myoblasts were ineffective, showing almost no benefit with MTT. One important obstacle is the rapid senescence of human myoblasts. The main purpose of our study was to compare the various methods for scalable generation of proliferative human myoblasts.

Methods: We compared the immortalization of primary myoblasts with hTERT, cyclin D1 and CDK4R24C, two chemically defined methods for deriving myoblasts from pluripotent human embryonic stem cells (hESCs), and introduction of viral MyoD into hESC-myoblasts.

Results: Our results show that, while all the strategies above are suboptimal at generating bona fide human myoblasts that can both proliferate and differentiate robustly, chemically defined hESC-monolayer-myoblasts show the most promise in differentiation potential.

Conclusions: Further efforts to optimize the chemically defined differentiation of hESC-monolayer-myoblasts would be the most promising strategy for the scalable generation of human myoblasts, for applications in MTT and high-throughput drug screening.
1 | INTRODUCTION

Skeletal muscle stem cells exist as satellite cells in adult mammalian skeletal muscles. These stem cells are located on the periphery of myofibers’ plasma membrane, beneath the muscle basal lamina or endomysium. Postnatal growth, maintenance and regeneration of skeletal muscles in vivo rely on muscle satellite cells that proliferate as myoblasts, which are marked by expression of PAX7 and MyoD but not myogenin (MYOG). In the early stages of myoblast differentiation, MyoD+/MYOG+ myocytes begin to accumulate muscle-specific α-actin (ACTA1) and other myofilament components such as embryonic myosin heavy chain (MYH3). Subsequently, myocytes can fuse together to form α-actinin+/MYOG+ multinucleated myotubes and finally myofibers, which are marked by very high levels of mature myofilament components such as adult myosin heavy chain isoforms. Phenotypic analyses of genetic mouse models strongly suggest that the loss of muscle satellite cells abolishes the regenerative capacity of adult skeletal muscles. Dysfunction in the proliferative muscle satellite cells, or myoblasts, leads to a decrease in regenerative capacity of muscles, resulting in muscle dysfunction during both normal ageing and the progression of muscle degenerative diseases, such as muscular dystrophies.

Amongst the large variety of heritable muscular dystrophies, Duchenne muscular dystrophy (DMD) is the most common, affecting one in 3600 boys due to a mutation in the dystrophin gene. Mouse models bearing mutations similar to those described in human muscular dystrophies, such as the dystrophin mutation in DMD, have been employed to develop myoblast transfer therapy (MTT) against muscular dystrophies. This is a technique to replace muscle satellite cells with genetically repaired or healthy myoblasts, to treat the muscular dystrophy. However, clinical trials with human myoblasts were ineffective, showing almost no benefit with MTT. In addition to obstacles such as the limited migration capabilities of human myoblasts, and the immune response during MTT, another important obstacle is the rapid senescence of human myoblasts. Unlike primary rodent myoblasts, primary human myoblasts rapidly show senescence in vitro. This limit is manifested as progressively compromised differentiation and proliferation potential, during in vitro culture. This limitation not only prevents us from achieving MTT for muscular dystrophy patients, but also limits our ability to conduct high-throughput drug screening and carry out molecular characterization in human myoblasts with high reproducibility.

To overcome this limitation, several approaches have been used, such as expression of the simian virus 40 large T (SV40-LT) antigen and human telomerase reverse transcriptase (hTERT). SV40-LT is an oncogenic protein that forcibly promotes cell cycle turnover, but its expression can cause genomic instability and disrupt myogenesis. By combining lentiviral hTERT, with cyclin D1, and/or oncogenic CDK4R24C, human myoblasts could proliferate indefinitely while maintaining a normal karyotype. However, the immortalized human myoblasts could also undergo osteogenesis and adipogenesis under appropriate conditions, a phenomenon that is never seen in primary human myoblasts, suggesting that immortalization had deranged their differentiation potential.

An alternative strategy to generate human myoblasts in large scale is by directed differentiation of human embryonic stem cells (hESCs), to recapitulate development to form cell lineages that are similar to their in vivo counterparts. Directed differentiation of hESCs to specific lineages for cell therapies is showing promise in clinical settings and in preclinical animal models for various diseases. However, for many cell lineages, directed differentiation results in progeny that are heterogeneous and functionally immature compared to primary in vivo cells. Protocols that are used to differentiate skeletal muscle cells from hESCs often require virus-mediated overexpression of transcription factor transgenes. Although many transgene-free, chemically defined protocols for generating myoblasts from hESCs have also been described, their heterogeneity and differentiation potential remain poorly characterized in comparison with other methods.

Here, we compared the various methods for generating human myoblasts at large scale, including immortalization of primary myoblasts with hTERT, CDK4R24C, cyclin D1, two chemically defined methods for hESC-myoblasts, and introduction of viral MyoD into hESC-myoblasts. Our results show that all the methods above are suboptimal at generating bona fide human myoblasts that can both proliferate and differentiate robustly. Our results further suggest that hESC-myoblasts show more promise in differentiation potential, and that further efforts to optimize the directed differentiation of hESC-myoblasts would be useful.

2 | METHODS

2.1 | Cell culture and virus production

The female (WA07) hESC line and the male (WA01) hESC line from WiCell, certified to be mycoplasma-free and bona fide human pluripotent stem cells, were propagated in mTeSR1 (Stem Cell Technologies) supplemented with 1% penicillin-streptomycin (Gibco) and were maintained feeder-free on hESC-qualified Matrigel (BD Biosciences) in a humidified atmosphere (5% CO₂, 37°C). The medium was changed daily. Both hESC lines were passaged using collagenase Type IV (Gibco) at a 1:4-1:6 split ratio every 4-6 days, and routinely checked every 2 months to prevent any mycoplasma contamination.

Overtly differentiated hESC colonies were mechanically removed prior to induction of differentiation. When the hESC colony density on the plate was approximately 30%-40%, differentiation of hESCs was induced. For EB differentiation into myoblasts and myotubes, we exactly followed the protocol of Xu et al. For monolayer differentiation into myoblasts and myotubes, we exactly followed the protocol of Shelton et al. All culture media were refreshed daily throughout the protocols.

Commercial primary adult HSKM myoblasts were derived from healthy adult patient donors (Gibco), and cultured in growth medium composed of DMEM/F12 supplemented with 10% heat-inactivated foetal bovine serum (FBS) (GE), 1% penicillin-streptomycin (Gibco) and
### Table 1

| Gene   | Orientation | Sequence 5′-3′ |
|--------|-------------|---------------|
| CHUA et al. | Gene Orientation | Sequence 5′-3′ |
| PAX3   | FORWARD    | CTC CAC CCT CCG GAT AGT TC |
|        | REVERSE    | ATC TTG TGG CGG ATG TGG TT |
| PAX7   | FORWARD    | CGT CTT CAT AAT CAA GGT TGT CG |
|        | REVERSE    | GCT AAG TCC GGA CAT CAG AT  |
| ALX4   | FORWARD    | ATG AAT GCT GAG ACT TCC GTC |
|        | REVERSE    | GGG AAA TGC CCT AAA AGG CG |
| SOX2   | FORWARD    | TGG TCG GAG AGG GAG AAG CG |
|        | REVERSE    | TGA CCA CGG AAC CCA TGG AG |
| PAX6   | FORWARD    | TCT AAT CGA AGG GCC AAA TG |
|        | REVERSE    | TGT GAG GGC TGT TGG TGT TC |
| TWIST1 | FORWARD    | CTG CAG CAC CGG CAC GT T |
|        | REVERSE    | CCC AAC AAT TGG ACG CAC AC |
| FLK1   | FORWARD    | AGT GAT CGG AAA TGA CAC TGG A |
|        | REVERSE    | GCA CAA AGT GAC AGG ATG A |
| GFP    | FORWARD    | AGC TTG GTG GTG GAT GAA AC |
|        | REVERSE    | CCC TCT TCA GCA AAG CAC |
| GATA4  | FORWARD    | CTA GAC CGT GGG TTG TAT |
|        | REVERSE    | TGG GTG AAG TGC CCC TGT AG |
| VECAD  | FORWARD    | TGT GAT GGT GCC CGT GTT AT |
|        | REVERSE    | CAG CCC AAA GTG TGT GAG |
| MYOD1  | FORWARD    | CGG CAT GAT GGA CTA CAG C |
|        | REVERSE    | CAG GCA GTC TAG GCT CAG C |
| MYOG   | FORWARD    | GGG GAA AAC TAC CCT G  |
|        | REVERSE    | AGG CGC TGG ATG TAC TGG A |
| EN1    | FORWARD    | GTGTTGACGTTGACTGCAAGC |
|        | REVERSE    | GCTTTGCTCTCTCTCTCTCTC |
| NCAM1  | FORWARD    | ATG GAA ACT CTA TTA AAG TGA ACC TG |
|        | REVERSE    | TAG ACC TCA TAC TCA GCA TTA CAG T |
| ACTA1  | FORWARD    | CGA CAT CAG GAA GGA CCT GTA TGC C |
|        | REVERSE    | GGC CTC GTC GTA CTC TGG CTG TT |
| MYHC   | FORWARD    | TTC ATT GGG GTC TGG GAC AT |
|        | REVERSE    | AAC GTC CAC TCA ATG CCT TC |
| MYH3   | FORWARD    | ATT GCT TCG TGG TGG ACT CA |
|        | REVERSE    | GGC CAT GTC TTC GAT CCT GTC |
| MYH8   | FORWARD    | TAA ACA CAC CTG CCT GAG |
|        | REVERSE    | TCA GCT TTA ACA GGA AAA TAA ACG |
| MYH7   | FORWARD    | TGC CAC ATC TTG ATC TGC |
|        | REVERSE    | CTC GGC TTT AAG GAA AAT TG |
| MYH2   | FORWARD    | CTG ATG CCA TGG AAT GAC G |
|        | REVERSE    | CCC TAT GCT TTA TTT CCT TGG C |
| OCT4   | FORWARD    | GAC AGG GGG AGG GGA GGA GCT AGG |
|        | REVERSE    | CTT CCC TCC AAC CAG TGG CCC CAA AC |
| GAPDH  | FORWARD    | TGG TAT CGT GGA AGG ACT CA |
|        | REVERSE    | TCC AGC TCA GGG ATG ACC TT |

1% l-glutamine (Gibco) in a humidified atmosphere (5% CO2, 37°C) for <5 passages. About 100% confluent HSKM myoblasts were induced to differentiate into myotubes under growth factor withdrawal conditions with 2% horse serum in DMEM supplemented with 1% penicillin-streptomycin (Gibco) and 1% l-glutamine (Gibco) for 7 days.

GP2-293 cells (Clontech) were seeded at 10% confluency and transfected with a 12 µL: 3.33 µg: 0.66 µg mix of PEI (1 mg/mL): retroviral plasmids (Addgene plasmid #1773, #26357): VSV-G envelope plasmid (Addgene #8454). 293FT HEK cells (Clontech) were seeded at 10% confluency and transfected with a 42 µL:7 µg:6.3 µg:0.7 µg mix of PEI (1 mg/mL): lentiviral plasmid (Addgene #19119): dR8.2 packaging plasmid (Addgene #8455): VSV-G envelope plasmid (Addgene #8454). 293FT and GP2 cells were initially cultured in DMEM (Gibco) with 10% FBS (GE Healthcare), 1% l-glutamine (Gibco) and 1% penicillin-streptomycin (Gibco). 24-hour post-transfection, growth medium was replaced with DMEM (Gibco) with 20% FBS (GE), 1% l-glutamine (Gibco) and 1% penicillin-streptomycin (Gibco). The following plasmids were used to make the viruses: pBABE-MDER (gift from Stephen Tapscott; Addgene plasmid #13494), pBABE-neo-hTERT (gift from Bob Weinberg; Addgene plasmid #1774), pBABE-hygro CDN4 R24C (gift from Bob Weinberg; Addgene plasmid #11254) and pBABE puro cyclinD1 HA (gift from William Hahn; Addgene plasmid #9050).

### 2.2 Virus transduction and selection

HSKM myoblasts were seeded in six-well plates (Falcon) in growth medium comprising of DMEM/F-12 (Gibco) with 20% heat-inactivated FBS (GE), 1% l-glutamine (Gibco) and 1% penicillin-streptomycin (Gibco). Cells were then transduced with 0.1-1 mL of concentrated viral supernatant in the presence of polybrene (Sigma), and incubated for 16-24 hours. Transduced cells were selected with growth media containing either hygromycin (0.5 mg/mL) for 6-8 days, puromycin (1 µg/mL) for 3 days, or G418 (2 mg/mL) for 5-7 days (InvivoGen).

### 2.3 Population doubling curve

1.5 × 10^4 cells were seeded in one gelatin-coated well of a six-well plate (Falcon) with growth medium comprising of DMEM/F-12 (Gibco) with 20% heat-inactivated (FBS; Gibco), 1% l-glutamine (Gibco) and 1% penicillin-streptomycin (Gibco). Upon reaching a confluency of 80%-100%, cells were lifted with 0.25% trypsin (Gibco) and counted, and 1.5 × 10^4 cells were then subcultured. This process was repeated until cells could no longer achieve 80% confluency, or until a period of 100 days. Recorded cell counts were calculated as cumulative population doubling levels and plotted over the number of days in culture.

### 2.4 Immunofluorescence

Cells were first washed with PBS (Thermo Fisher) and fixed with 4% PFA (MS). Cells were then stained with the following primary antibodies and concentrations, Desmin (ab6322; Abcam; 1:250); PAX7 (Pax7-c; DSHB; 5 µg/mL), MYOD1 (sc-760; Santa Cruz; 1:50), MHC-Alexa...
Fluor 488 (53-6503-82 [MF-20]; Thermo Fisher; 1:100), α-actinin (A7811; Sigma; 1:500) and myogenin (sc-576; Santa Cruz; 1:200). The following secondary antibodies were also used together with non-conjugated primary antibodies, Goat-anti-mouse Alexa Fluor 488 (A11001; Thermo Fisher; 1:500), Goat-anti-rabbit Alexa Fluor 594 (A11012; Thermo Fisher; 1:500) and Goat-anti-mouse Alexa Fluor 647 (A21235; Thermo Fisher; 1:500). DAPI (d9542; Sigma) was used as a nuclear counter stain according to manufacturer’s recommendations. Stained cells were imaged with a Zeiss fluorescence microscope.

2.5 | Quantitative PCR

RNA was extracted by TRIzol (Thermo Fisher) and reverse transcribed with Superscript III (Thermo Fisher) according to manufacturer’s instructions. The resulting cDNA was diluted 5× before performing qPCR with KAPA SYBR FAST on ABI Prism 7900HT (Applied Biosystems) according to manufacturers’ instructions. For primer sequences, see Table 1.

3 | RESULTS

3.1 | Immortalization of human myoblasts with hTERT, CDK4R24C and cyclin D1

First, we turned to methods for immortalization of primary human myoblasts. While previous studies have shown that telomerase hTERT alone is insufficient to immortalize primary human myoblasts, some success has been obtained with the combination of hTERT and oncogenic
CDK4R24C, or cyclin D1, or both.\textsuperscript{12,15} We overexpressed lentiviral hTERT, CDK4R24C, and/or cyclin D1 in adult primary human skeletal muscle (hskm) myoblasts, and indeed observed that each of the combinations could effectively immortalize primary hskm myoblasts with continuously linear population doubling curves for over 100 days, whereas adult primary hskm myoblasts became senescent within 30 days and about five population doublings (Figure 1A). To assess whether the immortalized myoblasts are bona fide myoblasts, we allowed them to fuse and differentiate into multinucleated myotubes (Figure 1B). When the immortalized myoblasts were allowed to differentiate into myotubes under standard culture conditions, we found that only a small fraction of cells formed multinucleated myotubes, unlike primary myoblasts (Figure 1C-D).
Combination of hTERT, CDK4R24C and cyclin D1 showed the worst differentiation potential, as determined by α-actinin (Figure 1C) and myosin heavy chain (MHC) immunofluorescence (Figure 1D), despite manifesting the highest proliferation rate (Figure 1A). When the differentiated cells were subjected to miRNA profiling, we found that the immortalization factors severely compromised their expression of a large variety of myogenic markers, compared to primary cells (Figure 2A-I). It can be inferred that these cells are no longer bona fide human myoblasts after immortalization by ectopic factors.

### 3.2 | hESC-myoblasts via embryoid body differentiation

Human embryonic stem cells derived from the early human embryo intrinsically possess indefinite self-renewal capabilities, thus allowing for expansion at any scale as desired. As demonstrated before, hESCs can proliferate rapidly while preserving a stable karyotype for extended periods of time without any problems with replicative senescence. The second biggest advantage with using hESCs is that their pluripotency allows us to direct their differentiation into a variety of lineage progenitor cells. By following a previous protocol of inducing mesoderm formation via 3D cultures of embryoid bodies (EBs), we derived mesodermal progenitors from hESCs (Figure 3A). These EB-derived mesodermal progenitor cells can also proliferate very rapidly, similar to their in vivo counterparts in the gastrulating embryo, thus providing yet another level of scalability in expansion and proliferation prior to the derivation of myoblasts. The problem with EB-derived mesodermal progenitors (Figure 3B), however, is that they are highly heterogeneous (Figure 3C) and highly stochastic in their differentiation efficiencies.

**FIGURE 3** Derivation of human myoblasts and myotubes from highly proliferative human embryonic stem cells (hESCs) via embryoid bodies. A, Schematic of directed differentiation protocol for embryoid body (EB)-derived myoblasts. B, Phase contrast micrographs of EBs derived from hESCs. Scale bar 200 μm. C, Phase contrast micrographs of mesodermal progenitors cultured from the hESC-EBs. Scale bar 50 μm. D, Phase contrast micrographs, and (E) desmin immunofluorescence staining of heterogeneous myogenic cells derived from the hESC-EB-mesodermal progenitors. Scale bar 50 μm. F, Phase contrast micrograph of myotubes and myocytes derived from hESC-EB-myoblasts that were subjected to myogenic differentiation culture conditions for 2 wk. Scale bar 50 μm. G, Phase contrast micrograph of myotubes and myocytes derived from hESC-EB-myoblasts that were subjected to myogenic differentiation culture conditions for 3 mo. Scale bar 50 μm.

**FIGURE 4** Quantitative RT-PCR for myogenic markers in human embryonic stem cell (hESC)-EB-myoblasts. A, Paired box 3, PAX3; B, Paired box 7, PAX7; C, Myogenic differentiation 1, MYOD1; D, Engrailed 1, EN1; E, Neural cell adhesion molecule 1, NCAM1; F, Myogenin, MYOG; G, Skeletal muscle actin alpha 1, ACTA1; H, Myosin heavy chain, MYHC; I, Embryonic myosin heavy chain 3, MYH3; J, Perinatal myosin heavy chain 8, MYH8; K, Adult slow myosin heavy chain 7, MYH7; L, Adult fast myosin heavy chain 2, MYH2. *P < 0.05, **P < 0.01, EB-myocytes vs EB.
The resultant human myogenic cells only constitute 48.3 ± 3.6% of the final population, according to desmin immunofluorescence (Figure 3E). To assess whether the resultant myoblasts are bona fide myoblasts, we allowed them to fuse and differentiate into multinucleated myotubes. When the EB-derived myoblasts were allowed to differentiate into myotubes in the presence of horse serum, we found that only a small fraction of cells formed multinucleated myotubes that emanated from a dense cluster of EB-derived cells.

*(Figure 3D-E)*. The resultant human myogenic cells only constitute 48.3 ± 3.6% of the final population, according to desmin immunofluorescence (Figure 3E). To assess whether the resultant myoblasts are bona fide myoblasts, we allowed them to fuse and differentiate into multinucleated myotubes. When the EB-derived myoblasts were allowed to differentiate into myotubes in the presence of horse serum, we found that only a small fraction of cells formed multinucleated myotubes that emanated from a dense cluster of EB-derived cells.
**FIGURE 7**  Quantitative RT-PCR for myogenic markers in human embryonic stem cell (hESC)-monolayer-myotubes. A, Paired box 3, PAX3; B, Paired box 7, PAX7; C, Myogenic differentiation 1, MYOD1; D, Myogenin, MYOG; E, Neural cell adhesion molecule 1, NCAM1; F, Engrailed 1, EN1; G, Skeletal muscle actin alpha 1, ACTA1; H, Myosin heavy chain, MYHC; I, Embryonic myosin heavy chain 3, MYH3; J, Perinatal myosin heavy chain 8, MYH8; K, Adult slow myosin heavy chain 7, MYH7. **P < 0.01, Primary myotubes vs Monolayer-myotubes**
myoblasts and myocytes (Figure 3F). Most of the cells could not fuse or differentiate into myotubes even after three more months of culture (Figure 3G).

When the embryoid bodies and EB-derived myocytes were subjected to mRNA profiling, we found that the EBs indeed significantly upregulated their expression of myoblast markers such as Pax3, Pax7, Myod1 and En1 (Figure 4A-D). Myotube markers were only specifically upregulated in EB-derived myocytes, not EBs (Figure 4E-L). However, the EB-derived myocytes only showed 2-20-fold higher expression of myotube markers than hESCs (Figure 4E-L). These levels of expression were significantly lower than primary human myotubes (Figure 7). Moreover, the EB-derived myocytes also showed aberrant expression of the neuroectoderm marker Pax6, the cardiogenic mesoderm marker Gata4 and persistent expression of the dermomyotome markers Alx4 and Twist1, despite several months of myogenic differentiation culture (Figure 5).
3.3 | hESC-myoblasts via mesodermal monolayer differentiation

Based on the results above, the mesodermal monolayer method might produce purer myogenic cells by comparison (Figure 6A), as there are no 3D structures with stochastic sizes and variable local gradients. This proved to be true (90.6 ± 7.2% purity), according to desmin immunofluorescence (Figure 6B-C). However, this advantage is offset by the problem of human myoblast purity, as the method produces both myoblasts and differentiating myocytes at the same time (Figure 6D-G). Quantification by PAX7, MYOD1 and MYOG (myogenin) immunofluorescence shows that both PAX7+ myoblasts and MYOD1+ myoblasts typically only constitute a minor fraction of the population (Figure 6D-F). The majority of the remaining cells are often MYO+ myocytes or myotubes, although the variance can be large (Figure 6G).

Thus, a significant but highly variable proportion of the final population is made up of non-proliferative myocytes, instead of myoblasts.

When the monolayer-derived hESC-myoblasts were allowed to differentiate into myotubes under standard myogenic differentiation conditions, we found that most of the cells adopted an elongated morphology typical of myocytes (Figure 6H), but only a minor fraction (21.3 ± 5.3%) of these myocytes fused and differentiated into myotubes (Figure 6H-K). These monolayer-derived myotubes stained positively for α-actinin, myosin heavy chain (MHC) and nuclear myogenin (MyoG), indicating that they are terminally differentiated myotubes (Figure 6H-K).

When subjected to mRNA profiling, these hESC-monolayer-myotubes were still expressing high levels of the paraxial mesoderm myoblast markers PAX3 and PAX7 (Figure 7A-B), while many myogenic markers were expressed at significantly lower levels than primary human myotubes (Figure 7C-K). This is consistent with the immunofluorescence staining, which indicates that most of the hESC-myoblasts were still not differentiating into myotubes. One reason could be the relatively low levels of MYOD1 expression in the hESC-monolayer myocytes (Figure 7C).

3.4 | hESC-myoblasts via MyoD overexpression

In an attempt to further improve the purity of the hESC-myoblasts, and further enhance their myogenicity, we overexpressed human MYOD1 in the hESC-mesodermal monolayer, since mouse MyoD overexpression has been widely touted to increase the myogenicity of mouse fibroblasts and stem cells. The first thing we noticed upon tamoxifen-induced overexpression of hMYOD1-ER-GFP in hESC-mesodermal progenitors was that they rapidly adopted an elongated myocyte-like morphology within 1 day and gradually became more homogeneous in morphology (Figure 8A). During differentiation in myotube culture conditions, the hMYOD1-hESC-myocytes became even more elongated over time, but they never fused into multinucleated myotubes even after 21 days of differentiation (Figure 8A-B).

We performed mRNA profiling of the cells to ascertain their myogenic status, and found that both MYOD1 and the downstream transcription factor MYOG were significantly upregulated compared to controls (Figure 8C). Downstream myogenic biomarkers such as ACTA1, NCAM, MYH3, MYH7 and MYH8 were also significantly upregulated, suggesting that the human MYOD1 overexpression did increase myogenicity (Figure 8C). However, with the exception of MYOD1 itself, these levels of myogenic biomarker expression were still significantly lower than that of primary human myotubes (Figure 8D). Our results suggest that, unlike mouse cells, other cofactors besides MyoD are necessary to induce complete myogenesis in human mesodermal progenitor cells. It is also possible that constitutive MyoD overexpression actually inhibits downstream myogenesis in the later stages.

4 | DISCUSSION

Rodent muscle cell lines, such as C2 or L6, have an unlimited proliferative potential and have been useful tools for the study of the cellular and molecular mechanisms involved in myogenesis. Mouse models have also been used to assess various therapeutic strategies, including MTT. However, the encouraging results obtained by grafting mouse myoblasts into the mdx mouse model, translated into several clinical trial failures with DMD patients. These clinical failures are ultimately due to intrinsic differences between mouse and human myoblasts in their proliferative capacities, and thus the scalability of human myoblasts.

While we successfully immortalized primary human myoblasts with the combined expression of CDK4R24C, cyclin D1 and hTERT, resulting in rapid proliferation rates, the immortalization process severely compromised the cells’ differentiation potential. Cyclin D1 has a crucial role as a limiting factor of CDK4 kinase activity. Overexpression of cyclin D1 increases CDK4R24C kinase activity to promote Rb phosphorylation, which then resulted in rapid proliferation and prevented senescence (Figure 1A). The slower proliferation of human myogenic cells immortalized by either CDK4R24C or cyclin D1 alone, with hTERT, also implies that higher CDK4 activity is required for rapid proliferation. However, although the rapid proliferation program mediated by CDK4R24C/cyclin D1 did overcome the senescence program, it also severely compromised the differentiation potential of the human myoblasts, likely because cell cycle inhibition is a prerequisite for proper myoblast differentiation. Moreover, these immortalized myoblasts also manifested some aberrant osteogenic and adipogenic potential. And even if the genes were switched off inducibly, the cells would still undergo senescence and cell death immediately (data not shown), before they can differentiate. Finally, even the hyper-proliferative mouse muscle cell lines suffer from progressive dysfunction in survival and differentiation over time. Taken together, these results suggest that immortalization is not likely to be a viable route for the scalable expansion of human myoblasts for clinical uses.

Inspired by the classic transdifferentiation work in mouse fibroblasts, several groups have reported the utility of using MYOD1 overexpression to obtain myogenic cells from the highly scalable hESCs and...
induced myogenesis due to the absence of BAF60C. However, even with the addition of BAF60C, the resultant hMYOD1-iPSC-myoblasts were still far from pure, and FACS for NCAM1+ staining was necessary to further purify the hPSC-myoblasts. This is consistent with our conclusion that other cofactors, besides MYOD1, are necessary to completely activate myogenesis in human hPSC-myoblasts.

For clinical applications and, by inference, preclinical studies, viral transgene-free approaches for scalable production of human myoblasts would be preferred out of safety concerns. This necessarily means that the chemically defined hPSC-myoblasts would still be the most promising strategy. And indeed, if one judges based on the maturity of the myotubes that were obtained after myogenic differentiation, instead of overall myotube efficiency, one can also conclude that the human myoblasts derived from hPSCs are still the most promising in recapitulating their in vivo counterparts. In this regard, several groups have recently made progress in improving modern large-scale microcarrier suspension cultures in bioreactors, have been applied recently with some limited success on non-human myoblasts. Moreover, most of the extant work has been based on 2D culture on plates. Future work should also shift onto modern large-scale microcarrier suspension cultures in bioreactors, which have been applied recently with some limited success on non-human myoblasts. Another dimension that deserves further exploration is the control of oxygen tension, which has been shown to exert varying effects on the proliferative capacity of myoblasts. Further work would still be needed to fully optimize the chemically defined approach to produce highly pure human myoblasts and highly mature human myotubes from hPSCs in large scale.

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CONFLICT OF INTEREST

The authors declare no competing interests.

AUTHOR CONTRIBUTIONS

M.-W. JC, EDY, J.-H. ET, ZJ, TBT, KY and NS-C. analysed the data and wrote the manuscript. M.-W. JC, EDY, J.-H. ET, Y.-JBC, SMCL, SLSD, ZJ, and CL performed the experiments and analysed the data.

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