Generation of craniofacial myogenic progenitor cells from human induced pluripotent stem cells for skeletal muscle tissue regeneration

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Abstract

Craniofacial skeletal muscle is composed of approximately 60 muscles, which have critical functions including food uptake, eye movements and facial expressions. Although craniofacial muscles have significantly different embryonic origin, most current skeletal muscle differentiation protocols using human induced pluripotent stem cells (iPSCs) are based on somite-derived limb and trunk muscle developmental pathways. Since the lack of a protocol for craniofacial muscles is a significant gap in the iPSC-derived muscle field, we have developed an optimized protocol to generate craniofacial myogenic precursor cells (cMPCs) from human iPSCs by mimicking key signaling pathways during craniofacial embryonic myogenesis. At each different stage, human iPSC-derived cMPCs mirror the transcription factor expression profiles seen in their counterparts during embryo development. After the bi-potential cranial pharyngeal mesoderm is established, cells are committed to cranial skeletal muscle lineages with inhibition of cardiac lineages and are purified by flow cytometry. Furthermore, identities of iPsc-derived cMPCs are verified with human primary myoblasts from craniofacial muscles using RNA sequencing. These data suggest that our new method could provide not only in vitro research tools to study muscle specificity of muscular dystrophy but also abundant and reliable cellular resources for tissue engineering to support craniofacial reconstruction surgery.

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Data availability

The authors declare that all data supporting the findings of this study are available within the paper and it’s Supplementary Information. Source data for the figures in this study are available from the authors upon request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biomaterials.2020.119995.
Keywords
Human induced pluripotent stem cells; Craniofacial myogenesis; Direct differentiation; Muscle tissue engineering; Craniofacial myogenic precursor cells

1. Introduction

Skeletal muscle has primary roles for movement and metabolism in the human body. While limb and trunk muscles regulate movement, posture and energy metabolism, craniofacial muscles control vision, mastication, swallowing and facial expression [1]. Diverse functions of skeletal muscles are influenced by embryonic origins, myogenic regulatory programs and functional/metabolic requirement from their location [2]. During vertebrate embryogenesis, skeletal muscles in the trunk and limb originate from precursor cells in segmented paraxial mesoderm referred to as somites [3,4]. The majority of craniofacial skeletal muscles, however, arise from cranial paraxial mesoderm of pharyngeal arches (or branchial arches). Pharyngeal arches are positioned along both sides of the neural tube and notochord [5,6] and give rise to cranial pharyngeal mesoderm (PM). These distinct embryologic origins of the craniofacial muscles, when compared to those of trunk or limb muscle, are accompanied by different genetic programs controlling their development. While somite-derived myogenesis is under the regulation of the transcription factors, PAX3 and, later, PAX7 [7], craniofacial myogenesis employ distinct combinations of transcription factors, TBX1, PITX2, TCF21 and LHX2 to induce the pharyngeal arches in early stage of myogenesis [8]. However, late myogenesis of both craniofacial and limb muscles converges with respect to the common myogenic regulatory factors (MRFs), such as MYF5, MYOD and Myogenin [9]. Governed by myogenin, myogenic progenitors are fused to each other to generate elongated muscle fiber [10].

Muscle fibers found in craniofacial muscles are unique compared to limb muscle fibers. Generally, muscle fibers are classified by fast or slow twitch and oxidative or glycolytic mechanism to generate the speed and force [11], which are determined by myosin heavy chain (MyHC) isoforms [12]. Adult craniofacial muscles express unique MyHC isoforms including embryonic (Myh3), neonatal (Myh8), cardiac isoforms (Myh6 and Myh13), and slow tonic (Myh14) and Myh15 [13–15] in addition to common MyHC isoforms, such as type I (Myh7), IIA (Myh2), IIB (Myh4), and IIX (Myh1), which are usually found in limb skeletal muscle tissues. In addition, multiple MyHC expression in single muscle fiber has been observed in extraocular muscles, which are responsible for eye ball movement [16,17]. Unique MyHC expressions of extraocular muscle are regulated by Pitx2, a critical transcription factor for craniofacial, particularly eye, muscle development [18]. Another distinctive aspect of craniofacial muscle relates to the cellular properties of adult muscle stem cells, called satellite cells. Satellite cells reside under lamina of muscle fibers [19] and are responsible for muscle regeneration [20–22]. While both limb and craniofacial muscles contain satellite cells, which express Pax7, a transcription factor to specify myogenic lineage [23], Pax7 is not involved in embryonic development of craniofacial muscle [24,25]. In addition, craniofacial satellite cells express a relatively low level of Pax7 compared to limb satellite cells and still express embryonic transcriptional factors like Pitx2 [26,27]. Satellite
cells of extraocular muscles, a well-studied craniofacial muscle group, are very unique since they have intact regenerative capacity of their satellite cells regardless of age and disease status, which may explain the sparing of extraocular muscle from age and neuromuscular diseases [28]. Therefore, limb/trunk muscle biology would not cover distinctive aspects of craniofacial muscle [2], which leads us to develop in vitro craniofacial muscle research tools.

Since satellite cells have been implicated in the pathology of various muscular dystrophies, satellite cells have been proposed as a target of treatment as well as resources for cell therapies and disease modeling for muscular dystrophy [2,29]. Recently, induced human pluripotent stem cells (iPSCs) have been shown to provide myogenic progenitors for patient-derived muscular dystrophy models for drug screening as well as autologous cell-based therapies [30–33]. Muscular dystrophy is classified into 9 types according to mutations of responsible gene(s) and shows differential susceptibility among muscle groups [34]. For example, limb muscles are mainly influenced by Duchenne, Becker and limb-girdle muscular dystrophies. In contrast, facial muscles are severely affected in myotonic, facioscapulohumeral and oculopharyngeal muscular dystrophies. Therefore, the mechanism of muscle specificity could provide effective therapeutics for affected muscles for each type of muscular dystrophy. Although the given muscle-specific sensitivity of muscular dystrophies is obvious, an in vitro system to research the difference between limb and craniofacial human muscle is currently lacking because most current iPSC-derived skeletal muscle differentiation methods have been adapted from the somite-derived trunk/limb muscle development pathway [33,35–37]. Moreover, most of the muscle disease modeling studies using human iPSCs have focused only on somite-derived muscles [33,36,37]. Therefore, development of Ipse-derived craniofacial muscles could produce a valuable tool to investigate the mechanism of muscle-specific susceptibility in muscular dystrophy. In addition, iPSC-derived craniofacial muscles could serve as authentic cellular resources to generate craniofacial muscle tissues for craniofacial reconstruction surgery to treat patients with cleft lip/palate or craniofacial trauma.

Here, we describe a small molecule-based approach to induce cranial pharyngeal mesoderm and to efficiently differentiate craniofacial myogenic precursor cells (cMPCs) from human iPSCs. Our procedure mimics the regulation of the early signaling pathways during craniofacial muscle embryonic development. We validate the iPSC-derived cMPCs with several human craniofacial muscles by RNA-sequencing.

2. Materials and methods

2.1. Culture of human iPSCs

The healthy human iPSC lines (GM25256, GM23279 and GM23476) and an iPSC line from a patient with Duchenne muscular dystrophy (GM25313) were purchased from Coriell Institute (http://ccr.coriell.org/). iPSC lines were cultured in 6-well plates on Matrigel (Corning Life Sciences, New York, NY, Ca No. 354277), in mTeSR™1 media (Stem Cell Technologies, Vancouver, BC), and incubated at 37 °C under 5% CO₂. The media was changed daily and the cells were passaged every 3 or 4 days at a 1:4 or 1:6 seeding density for routine culture conditions. To passage human iPSCs, the ReLeSR™ (Stem Cell
Technologies) was used to detach the cells from the wells. Detached cells were re-suspended in fresh media and distributed to new Matrigel-coated plates.

2.2. Directed differentiation of craniofacial muscle progenitor cells from human iPSCs

We used a protocol to mimic on somite derived muscle differentiation pathway [37], referred to as somite method (Supplementary Fig. S1A). To develop a novel protocol to mimic pathways during craniofacial muscle differentiation, referred to as pharyngeal mesoderm-derived method (PM method), we used specific small molecules to induce cranial pharyngeal mesoderm (Supplementary Fig. S1B). Briefly, prior to dissociation of iPSCs, 80%-90% confluent iPSCs were treated ROCK inhibitor Y-27632 (10 μM, Stem Cell Technologies) for at least 2 h to reduce dissociation-induced apoptosis. To ensure homogeneous differentiation of human iPSCs, the dissociation of iPSC colonies into single cells is necessary while still maintaining their pluripotency. Accordingly, the dissociated cells were transferred into Matrigel coated dishes between 15,000 and 30,000 cells/cm² seeding densities in mTeSR1 media supplemented with the ROCK inhibitor (Y-27632, 10 μM). After 24 h of recovery, cells were switched to pharyngeal mesoderm induction media. During day 0 to day 2, the initial differentiation was induced using DMEM/F12 (Gibco) media containing 1% ITS (Stem Cell Technologies) supplemented with CHIR99021 (3 μM, Stem Cell Technologies) and BMP4 (25 ng/mL, R&D system, Minneapolis, MN) to simulate Wnt signaling during gastrulation. On day 3 of differentiation, the BMP4 was withdrawn and DAPT (N-[N-(3,5-difluorophenacetyl)-l-alanyl]-S-phenylglycine t-butyl ester, 10 μM, Stem Cell Technologies) was added with growth factor recombinant basic-FGF (20 ng/mL, PeproTech, Rocky Hill, NJ). On day 6 of differentiation, the media was changed to specification media which is new DMEM/F12 (Gibco) media containing knockout serum replacement (KSR) media supplemented with BMP inhibitor (0.5 μM LDN 193189, Stem Cell Technologies), recombinant basic-FGF (20 ng/mL), recombinant IGF-1 (2 ng/mL, Stem Cell Technologies) and recombinant HGF (10 ng/mL, Stem Cell Technologies). To increase the potential of myogenic lineage, media was changed daily until day 12 with or without IGF-1 or HGF as indicated.

2.3. Gene expression analysis by real-time PCR

The human iPSC derived MPCs or differentiated cells were analyzed for the expression of progenitor markers by comparative qRT-PCR. Total RNA from human iPSC-derived populations was extracted using Trizol reagent (Ambion/Life Technologies, Carlsbad, CA) according to the manufacturer’s instructions. Isolated RNA (250 ng) was reverse transcribed into complementary DNA (cDNA) using random hexamers and M-MLV reverse transcriptase (Invitrogen, www.thermofisher.com, Waltham, MA). Amplification of cDNA was performed using Power SYBR® Green Master Mix (Applied Biosystems, Waltham, MA) and 2.5 μM of each primer. All primer sequences are listed in Supplementary Table S1. PCR reactions were performed for 35 cycles under the following conditions: denaturation at 95 °C for 15 s and annealing + extension at 60 °C for 1 min. Quantitative levels for all genes were normalized to endogenous GAPDH expression. Fold change of gene expression was determined using the ΔΔCt method [38]. Experiments were repeated at least three times.
2.4. **Immunofluorescence**

Immunofluorescence (IF) was performed as follows: cultured cells were fixed in freshly prepared 2% paraformaldehyde (Electron Microscopy Sciences, http://www.emsdiasum.com, Hatfield, PA) for 15 min and incubated with blocking buffer (5% goat serum, 5% donkey serum, 0.5% BSA, 0.25% Triton-X 100 in PBS) for 1 h. Cells were then labeled with primary antibodies (Supplementary Table S2) or isotype controls overnight at 4 °C in blocking buffer. The following day, cells were washed three times with washing buffer (0.2% Tween-20 and PBS) and incubated with fluorescence probe-conjugated secondary antibodies for 1 h at room temperature. For eMyHC imaging, after 1 h incubation with biotinylated goat-anti-mouse F(ab’)2 IgG fragments (2.5 μg/ml), a TSA Green kit (Tyramide Signal Amplification; Perkin Elmer, www.perkinelmer.com, Waltham, MA) was used to enhance the immunostaining signal. Nuclei were then stained with 4',6-diamidino-2-phenylindole (DAPI) and mounted on Vectashield (Vector Labs, www.vectorlabs.com, Burlingame, CA).

2.5. **Flow cytometry and cell sorting**

Human iPSC-derived MPCs from day 30 cultures were dissociated using TrypLE Express (Invitrogen, cat. no. 12605010) after the pretreatment of 10 μM ROCK inhibitor for at least 2 h. For specific cell marker analyses, cells were stained with indicated antibodies (Supplementary Table S2) for 30 min at 4 °C in fluorescence-activated cell sorting (FACS) buffer consisting of PBS with 2 mM EDTA and 0.5% BSA. Stained cells were analyzed using the BD FACS Aria II cell sorter (Becton-Dickinson, http://www.bd.com, Franklin Lakes, NJ). Analyses of flow cytometry data were performed using FACS Diva (BD version 8.0.1). Differentiated cells at D30 were purified using surface markers, HNK1, ERBB3, and NGFR [33]. Isolated HNK1<sup>-</sup>ERBB3<sup>+</sup>NGFR<sup>+</sup> cells were cultured in SkGM on Matrigel-coated plates for 3 or 5 days before cryopreservation.

2.6. **Maturation of human iPSC derived craniofacial MPCs and fusion assays**

To examine the effects of TGF-β signaling on human iPSC-craniofacial MPCs maturation, small molecule inhibitors of TGF-β signaling (SB-431542, 10 μM, Stem Cell Technologies) were evaluated and the effect of recombinant IGF (2 ng/mL) supplement was also evaluated. The human iPSC-craniofacial MPCs were differentiated for 6–18 days in N2 media or skeletal muscle growth media-2 (SkGM, Lonza, Allendale, NJ), with or without SB-431542 and IGF-1 treatment. For fusion assay, cells were fixed in 2% formaldehyde in PBS for 20 min at room temperature and immunostained with anti-MyHC antibody (1:100, A4.1025, Developmental Studies Hybridoma Bank) after 6 or 18 days of maturation. Myoblast fusion was quantified by counting myonuclei in MyHC-positive myotubes after 18 days of SB-431542 and IGF-1 treatment.

2.7. **Isolation of human craniofacial muscles and culture of human primary myoblasts**

Human primary muscles (tibialis anterior, extraocular, cricopharyngeus, masseter and zygomaticus muscles) were isolated by experienced otolaryngology surgeons from a donated subject (89 year old, Caucasian female) to Emory Body Donor Program. Isolated muscle chunks were minced by blades and incubated with 0.25% Trypsin for 20 min and filtered.
to isolate mononucleated cells including satellite cells [39]. Cells were cultured on gelatin-coated plates to expand and then sorted by surface markers (CD31−/CD45−/CD56+) using flow cytometry.

2.8. RNA sequencing

Total RNA was isolated using QIAamp-RNA-Blood Mini kit (Quigen) and was validated by Agilent Technologies 2100 bioanalyzer. mRNA molecules were purified from total RNA using oligo(dT)-attached magnetic beads and were fragmented into small pieces using divalent cations under elevated temperature. First-strand cDNA was generated using random hexamer-primed reverse transcription, followed by a second-strand cDNA synthesis using DNA Polymerase I and RNase H. The synthesized cDNA was subjected to end-repair and then was 3′ adenylated. Adapters were ligated to the ends of these 3′ adenylated cDNA fragments. The cDNA fragments with adapters were amplified by PCR. PCR products were purified with Ampure XP Beads (AGENCOURT) and dissolved in elution buffer solution. Library was validated on the Agilent Technologies 2100 bioanalyzer. The double stranded PCR products were heat denatured and circularized by the splint oligo sequence. The single strand circle DNA (ssCir DNA) was formatted as the final library. The library was amplified with phi29 to make DNA nanoball (DNB) with more than 300 copies of one molecule. The DNBs were load into the patterned nanoarray and single end 50 (pair end 100/150) bases reads were generated using Combinatorial Probe-Anchor Synthesis (cPAS). After sequencing, low quality reads, reads with adaptors, and reads with unknown bases were removed to obtain clean reads. Then the clean reads were mapped with reference genome to detect novel gene prediction, SNP and INDEL calling and gene splicing. Finally, differentially expressed genes between samples were identified and analyzed by clustering analysis and functional annotations.

2.9. Statistical analysis

Statistical analysis was performed using Prism 8.0. Results are expressed as the means ± SEM. Experiments were repeated at least three times unless a different number of repeats is stated in the legend. Statistical testing was performed using the unpaired two-tailed Student t-test or ANOVA analysis as stated in the figure legends. P < 0.05 was considered statistically significant. Methods used, P values, and sample numbers are indicated in the figure legends.

3. Results

3.1. Control of BMP signaling with notch inhibition induces pharyngeal mesoderm from human PSCs

To induce cranial paraxial mesoderm from iPSCs (Fig. 1A), we treated CHIR99021, glycogen synthase kinase 3 (GSK3) inhibitor, to simulate Wnt signaling during gastrulation. In cranial muscle differentiation method group, referred to as pharyngeal mesoderm (PM) method, we briefly treated BMP4 during the initial induction steps for more rapid and efficient pharyngeal mesoderm induction [40] contrast by using LDN193189. BMP inhibitor to induce paraxial mesoderm for trunk/limb muscle differentiation method, referred to as somite method [37]. After mesoderm induction, we confirmed the loss of the
human pluripotent marker \textit{POU5F1} (known as \textit{OCT4}) in both of methods (Fig. 1B) and observed that cells were flattened and generated aggregates (white dotted circle) 2 days post-differentiation using both methods (Fig. 1C). We validated induction of paraxial mesoderm to derive myogenic lineage, by increased mRNA expression of \textit{MSGN1}, a paraxial mesoderm marker, and \textit{MESP1}, a pharyngeal mesoderm marker, in PM method at day 3 (Fig. 1B). We also examined protein expression of intracellular \textit{MESP1}, a transcription factor for pharyngeal mesoderm, at day 3 (Fig. 1D). From day 3 to day 6, we used gamma-secretase inhibitor II, DAPT, to inhibit Notch signaling for PM method to induce bi-potential pharyngeal mesoderm, which is able to differentiate into myogenic or cardiogenic progenitors [41]. In addition, FGF2 was used to not only promote progenitor cell proliferation, but also to suppress premature expression of myogenic regulatory factors (MRFs) [42]. The key transcriptional factor for somite-derived myogenesis, \textit{PAX3}, was upregulated in somite method at day 6, but not in PM method, which implies PM method is generating non-somite derived myogenic cells. Indeed, we discovered significantly upregulated expressions of key upstream genes at cranial skeletal muscle formation, such as \textit{PITX2} and \textit{TCF21}, only in PM method at day 3 or 6 (Fig. 1B). In addition, the expressions of \textit{NKX 2.5}, \textit{LHX2}, and \textit{ISL1}, co-transcriptional regulators for craniofacial myogenic lineage and second heart field related cardiogenic lineage, were also significantly observed only in the PM method, consistent with process of pharyngeal mesoderm formation during embryogenesis [43]. We confirmed the protein expression of PDGFR\alpha, a surface marker of pharyngeal mesoderm, at day 6 in cells using PM method (Fig. 1D). These results suggest that our protocol induced cranial pharyngeal mesoderm.

### 3.2. Specification of cMPCs fate depressing cardiac lineage and purification

Pharyngeal mesoderm has bipotential to generate craniofacial muscles as well as cardiac progenitors for second heart field. We designed specification media, which not only enhances skeletal myogenic lineage by adding IGF-1 and HGF but also inhibits cardiogenic lineage from day 6 to day 12 (Fig. 2A). Since inhibition of BMP signaling or ROCK signaling has been known to suppress cardiac lineage from PM [44], we tested BMP inhibitor, LDN193189 or ROCK inhibitor, Y-27632 or combination of both inhibitors for suppression of cardiac lineage. Comparable with dual inhibition, the treatment of LDN193189 alone resulted in significantly lower mRNA expression of cardiac muscle development related genes, such as \textit{GATA4} and \textit{TBX5}, the first heart field (FHF) markers, and \textit{ISL1}, the second heart field (SHF) marker (Fig. 2B). The treatment of Y-27632 alone showed no significant effect on heart development-related genes. In contrast, the mRNA expression of \textit{TBX1}, a craniofacial myogenic marker, was significantly higher in LDN193189 alone-treated group compared to control and dual inhibited group. Thus, the inhibition of BMP pathway appears to be sufficient to block the FHF cardiac lineage (Fig. 2C).

To select and enrich cranial skeletal muscle progenitor cells with high myogenic potential from bipotent cranial/cardiac pharyngeal MPCs, we applied a previously reported sorting strategy that removed HNK1\(^+\) cells (a neuroectodermal marker) [45,46] and selected for skeletal muscle-specific receptors ERBB3 and nerve growth factor receptor (NGFR; also known as CD271) [33]. The FACS analysis showed that HNK1 expressing cells were rarely
detected in both somite and PM methods (< 3.8 ± 1.3% and < 1.1 ± 0.1%, respectively), indicating that the majority of the cells were not neuroectodermal progenies (Supplementary Table S3). In HNK1− cell populations, the majority of the cells generated by both methods expressed ERBB3 and NGFR double positive (> 84.7 ± 0.9% and 79.7 ± 1.2% for somite and PM methods, respectively), reflecting the efficiency of both differentiation protocols to generate highly myogenic progenitor cells. To validate our approach, we confirmed that HNK1−ERBB3+NGFR+ cell population highly expressed (> 99%) myogenic factor 5 (Myf5), a key marker for early skeletal myogenic precursors, in somite and cranial derived muscle differentiation methods (Fig. 2D). However, the HNK1−ERBB3+NGFR− population did not contain Myf5+ cells. This result clearly indicates that HNK1−ERBB3+NGFR+ populations represent Myf5 positive MPCs which have skeletal myogenic potential. To assess the reproducibility of this protocol across human iPSC lines, another cell line (GM23279) was used. This cell line has also been shown to efficiently and homogeneously generate ERBB3+NGFR+ cells (96.2 ± 0.7%) in HNK1− cell populations using PM method (Supplementary Table S3). These experiments also attest that the effect of our novel PM method is cell line-independent. Taken together, the cell sorting strategy using surface marker proteins HNK1−ERBB3+NGFR+ was efficient to isolate human PSC-cMPCs with high myogenic potential.

### 3.3. Characterization of cMPCs

We have demonstrated that our PM method generates Myf5 positive myogenic progenitor cells from human iPSCs (Fig. 2D). To investigate further, we next tested whether these HNK1−ERBB3+NGFR+ sorted cMPCs could keep their identity and expand properly during differentiation (Fig. 3A). After sorting, the cultures were grown in N2 or Skgm media, which supports both the proliferation and expansion of cMPCs (Supplementary Fig. S2A). The mRNA expression of TBX1, ISL1, PITX2 TCF21 and MYOR, which are the hallmarks of pharyngeal and craniofacial muscles, were significantly higher in PM method compared to somite method at day 28 after differentiation (Fig. 3B). However, the mRNA expression of PAX7 was significantly higher in somite method than that in PM method at this stage. Supporting our previous analysis, immunostaining further showed that all of the PM method-derived cells from normal (GM25256, GM23476) and muscular dystrophy patient (GM23513)-derived human iPSCs expressed embryonic MyHC (eMyHC) and TBX1 (Fig. 3C) at day 28 after differentiation, which shows its identity as the progenitor of craniofacial muscles. We have also observed expressions of Myogenin, PAX7 and ISL1 at day 28 after differentiation (Supplementary Fig. S2B). At day 44 post differentiation, the cMPCs also showed the weak expression of adult MyHC (MyHC IIA and MyHC IIB) (Supplementary Fig. S2C). Taken together, these results demonstrate that the human iPSC-cMPCs induced by our PM method showed representative characteristics of craniofacial MPCs.

### 3.4. Maturation of cMPCs in vitro

To promote terminal differentiation and maturation of the craniofacial myogenic cultures (Fig. 4A), we examined whether IGF-1 and TGF-β receptor type 1 kinase inhibitor (TGF-βi or SB431542) combined treatment boosts myotube formation of HNK1−ERBB3+NGFR+cells synergistically. After 6 days of treatment, the use of Skgm supplemented with IGF-1 and TGF-βi (SB431542) induced more mature myotubes.
compared to the Skgm alone or single IGF-1 or TGF-β treatment (Supplementary Fig. S3A). These data demonstrate that inhibition of TGF-β with supplement of IGF during cMPC differentiation can produce mature myotubes in vitro. After 18 days of double treatment, cells were fused to each other to generate long-multinucleated myotubes (Supplementary Fig. S3B) expressing h-Dystrophin and embryonic and late fetal MyHCs (Fig. 4B), which is consistent with the previous report of trunk/limb muscle generations by Hicks et al. [33]. TGF-βi and IGF-1 treated myotubes displayed clearly organized sarcomeres and Z-disk patterning (Fig. 4C, white arrow in MYH1 staining with white pseudo-color for better presentation of the striation of matured myotubes). By contrast, untreated control myotubes showed limited organization. As another measure of maturation, we quantified muscle cell fusion with double treatment of IGF-1 and SB431542 (IGF + SB) by the number of nuclei within MyHC-positive myotubes (Fig. 4D). Nuclear number analysis revealed that the mature myotubes containing 5–14 nuclei were significantly increased in IGF + SB treated cells compared with control cells, suggesting that IGF and TGF-β signaling is involved in muscle cell fusion to promote matured myotube formation. We confirmed that IGF + SB treatments consistently induced mature myotubes with striation in several lines of iPSC-derived muscle cells, which were differentiated using PM methods (Fig. 4E). These data suggest that TGF-βi and IGF-1 promotes human PSC craniofacial myotube maturation.

3.5. Verification of human iPSC-PM-derived MPC and muscles

Since RNA sequencing analysis have been used a powerful tool to verify cell identity including iPSC-derived muscle cells [33], we performed RNA sequencing of iPSC-derived MPCs compared to human primary myoblasts. Human primary myoblasts were isolated from several craniofacial muscles, expanded and then sorted by surface proteins (CD31−/CD45−/CD56+). Pearson correlation map by RNA-seq analysis revealed that iPSC-PM-derived MPCs showed close correlations (Spearman ρ = 0.86–0.9) to myoblasts isolated from 4 different craniofacial muscles. Interestingly, iPSC-PM MPCs showed the lowest correlation with primary myoblasts from somite-derived limb muscles (tibialis anterior, TA). In contrast, iPSC-somite-derived MPCs showed the lowest correlation with extraocular myoblasts (Fig. 5A). Using principal component analysis (PCA), we observed the cluster of cricopharyngeus, masseter, and zygomaticus myoblasts, which showed distance to TA (limb muscle) and extraocular myoblasts. Although iPSC-somite-MPC and iPSC-PM-MPC presented differences in the PCA map, those differences were smaller than the distance between the cluster of craniofacial myoblasts and TA myoblasts. Also, we observed major differences between the iPSC-derived MPCs and primary myoblasts (Fig. 5B).

To investigate whether PM and somite methods induce the expression of craniofacial and limb specific genes respectively, we identified 1480 and 397 genes that were exclusively upregulated in iPSC-PM-derived MPCs and iPSC-somite-derived MPCs, respectively, by differentially expressed gene (DEG) analysis (Fig. 5C). The iPSC-PM-MPCs showed significantly up-regulated expression of PITX2, TBX1, and ISL1, which are strongly enriched for pharyngeal mesoderm development and craniofacial muscle differentiation, compared to somite method-derived MPCs. On the other hand, the iPSC-PM-MPCs showed significantly down-regulated expression in PAX3, which is a key early developmental
regulator for somite-derived cell fates and has been shown to be suppressed during craniofacial differentiation process (Fig. 5D). These differences reflect the different regulatory programs during myogenesis of each MPCs. To study whether PM and somite method induced iPSC-MPC contain specific genes of adult myoblasts of craniofacial and limb muscles, respectively, we identified exclusively up-regulated genes from RNA-seq data. Fig. 5E shows the strategy to select the enriched gene sets of primary TA myoblasts (118 genes) compared to the primary craniofacial myoblasts. Fig. 5F presents commonly up-regulated genes (157 genes) in all primary craniofacial myoblasts compared to primary TA myoblasts. We then analyzed whether those limb and craniofacial myoblast specific genes are found in exclusive gene set of PM and somite method induced iPSC-MPC (Fig. 5G). However, iPSC-PM-MPCs and iPSC-somite-MPCs exclusive transcriptome did not show the preference of craniofacial and limb myoblast specific gene expression, respectively. Taken together, though iPSC-PM-MPCs express critical transcription factors for craniofacial myogenic development, these cells are still premature to express adult craniofacial myoblasts exclusive genes.

4. Discussion

Human PSCs can self-renew and possess the potential to differentiate into skeletal muscles [33,36,37], and therefore represent a theoretically unlimited source of healthy myogenic progenitors and mature skeletal muscles. As such, they could provide valuable resources for regenerative medicine. To date, however, the major approach to differentiate skeletal muscle progenitors from PSCs has been only focused on the developmental pathways of somite-derived trunk and limb muscle rather than those of craniofacial muscles. This study demonstrates a novel and robust method for the generation of craniofacial skeletal myogenic progenitors from human iPSCs via small molecule modulations during embryo development (Fig. 6).

In our method, bone morphogenetic protein (BMP) signaling affects cells in two phases conversely: activation of BMP signaling is initially required in the earliest phase during induction step; while at later stages, inhibition of BMP signaling is required for specification. To induce the paraxial mesoderm from iPSCs for the differentiation of somite-derived muscles, several research groups have controlled two signaling pathways; activation of WNT signaling pathway by GSK3β inhibitor, CHIR99021, to promote mesodermal differentiation [47–50], and inhibition of BMP by BMP inhibitor, LDN193189, to prevent drifting to non-muscle lineage [37,51–53]. To facilitate the robust formation of cranial paraxial mesoderm, we also used GSK3β inhibitor (CHIR99021) to activate Wnt signaling to induce mesodermal differentiation and adapted BMP4 to drive lineage to cardiac/cranial pharyngeal mesoderm [54,55]. This early step for the contrast regulation of BMP signaling represents a major difference between the somite- and the PM-derived muscle differentiation protocols. However, in the later step, we inhibited BMP signaling in specification step to ‘lock’ these cells into the skeletal muscle lineage and to ‘block’ cardiogenic commitments from bipotent cardiac/cranial PM (CPM). In vertebrates, the progenitors of CPM are transcriptionally primed to activate two distinct fates; specific cardiac muscle precursors, referred to as the first heart field (FHF) and pharyngeal MPCs. The pharyngeal MPCs can committed to craniofacial skeletal muscle progenitors and second heart field (SHF) which
contributes to the outflow tract, right ventricle, and a majority of the atria [56]. These common progenitors activate overlapping transcription factors, such as Pituitary homeobox 2 (PITX2) and T-box gene (TBX1), in early developmental signaling cascades before lineage decision [6,57]. Divergent fates of CPM are affected by BMP4 or BMP7 [6,58]. Specifically, Chan et al. demonstrated that BMPs promotes cardiac lineage, while Rho kinase inhibition enhances myogenic lineage [44]. Thus, our protocol uses differential BMP regulation to mimic PM-derived muscle cell development, such as BMP4 to induce PM at an early step and LDN 193189 (BMP inhibitor) to inhibit cardiac commitment from CPM at a later step. However, Rho kinase inhibition (Y-27632) did not efficiently induce skeletal myogenic lineage in our protocol, which may imply different cell sources (mouse embryonic stem cell lines vs. human iPSCs) as well as differentiation methods (cells from embryoid body vs. cells from monolayered culture) between laboratories.

One of the key questions in mammalian developmental study is the proper activation timing of gene regulatory program during organogenesis when the progenitor becomes specified to differentiate into their commitment lineages. Compared to the somite-derived muscles, activation of myogenesis in the head depends on different upstream factors and also responds differently to signaling pathways. In our PM method, we found that PAX3, a major upstream regulator of somite-derived myogenesis [7], is not expressed during early stage of CPM formation. Instead, our PM-method is capable of producing the colonies expressing MESPI, which acts as an essential early upstream regulator for the bipotent CPM formation during craniofacial skeletal myogenesis [59,60] and SHF development [61]. Also, we found that our PM-method showed the involvement of PITX2 and TBX1 genes which cross-regulate each other and activate the same target genes during the specification of progenitor cells that give rise to craniofacial muscles [8,57]. PITX2 plays a critical role in specifying the first pharyngeal arch muscles and EOM [62]. PITX is not only required but is also sufficient to activate the T-box gene, TBX1, which is a transcription factor expressed in the pre-myoblast in the first and second branchial arches [8]. In TBX1 mutants, pharyngeal muscles are frequently hypoplastic and asymmetric, whereas EOMs are spared due to presence of PITX2 [63]. The expression of the basic helix-loop-helix repressors TCF21 (Capsulin) or MYOR (Msc) are also observed in our PM-method. In TCF21/MYOR double mutants, the masseter, pterygoid, and temporalis muscles are missing by the failure of activation of MYF5, which is the first MRFs for initiating early skeletal myogenic fate, in the progenitors that gives rise to facial muscles [64]. Our sorting strategy using the HNK1-ERBB3*NGFR+ subpopulations enriched for MYF5 positive progenitor cells from human iPSC-cMPCs. The majority (at least 85%) of generated cells by our PM-method are double positive subsets (ERBB3*NGFR+) regardless of PSC lines we tested. This result is consistent with a previous study that reported skeletal myogenic potential could be measured by antibody against ERBB and NGFR in the somite-derived myogenic progenitors from human PSC differentiation cultures [33].

Although iPSC-derived cells enable scientists to study the molecular mechanisms of disease in relevant human cell types including those that are inaccessible as primary tissue samples, a lack of maturity in the iPSC-derived cells is a well-recognized problem. We analyzed the maturation capacity of our in vitro PM-method derived cMPCs using TGF-β inhibitor with IGF-1 treatment. In mouse development, it is well known that the myoblasts at different
developmental stages respond differently to TGF-β signaling [65]. In fact, embryonic myogenesis is accelerated in the presence of TGF-β, but fetal myogenesis is strongly inhibited by TGF-β. The iPSC-derived myogenic progenitor cells have the phenotype of late fetal myoblasts and TGF-βi might be a major driver of human PSC maturation towards secondary or tertiary myogenesis [33,66].

Despite the iPSC-PM-MPCs retain a distinct set of upstream transcriptomes, particularly TBX1, PITX2 and ISL1 compared to the iPSC-somite-MPCs, the iPSC-derived MPCs are distinguished from primary myoblasts as shown by principal component analysis (PCA) plots and profiling of craniofacial and limb myoblast exclusive genes comparing DEG of iPSC-PM-MPCs and iPSC-somite-MPCs (Fig. 5). This results are in line with other reports of other iPSC-derived progenitor cells, such as iPSC-derived skeletal muscles [33], neurons [67], cardiomyocytes [68], and hepatocytes [69] which are immature and resemble fetal cells, not adult cells. However, the higher similarity of iPSC-cMPCs to cricopharyngeus (gene expression correlation Spearman \( \rho = 0.89 \)) and masseter (\( \rho = 0.90 \)) myoblasts may reflect a priming of our PM protocol to generate pharyngeal arches-derived fetal myoblasts from CPM. Given slightly different embryonic origins of each craniofacial muscles, for example, masseter muscles from 1st and 2nd pharyngeal arches and pharyngeal muscles from 3rd and 4th pharyngeal arches, our protocol has limitation in generating specific craniofacial muscles. Since our protocol produces PM-derived muscle without focusing of each pharyngeal arch developmental program, our current protocol might produce a mixture of several craniofacial muscle subgroups given heterogeneous embryonic origins within pharyngeal mesoderm. For more indepth comparative research and clinical application in the future, single cell RNA sequencing of iPSC-PM-MPC is highly desired to confirm whether iPSC-PM-MPC is heterogenous and, if heterogenous, whether it is mapped with slightly different origins of pharyngeal arches-derived as craniofacial myoblasts.

Though recent attempts to generate of bi-potential cranial cardio/pharyngeal mesoderm-derived muscles from human embryonic stem cells (ESCs) have been reported, bioengineering applications of those muscles are still limited due to MESP1 transgene injection to drive CPM [44] and lack of terminal muscle differentiation results [70]. Thus, our PM method overcomes current limitations to produce PM-derived muscles by utilizing small molecules to mimic developmental pathways and by inducing fully differentiated multinucleated myotubes with mature contracting units. To our knowledge, we are the first group to report comparative RNA-sequencing transcriptional profiling of human iPSC-derived craniofacial muscle cells with various human primary craniofacial muscle cells.

5. Conclusions

This work describes an in vitro method for generating craniofacial muscle lineage from human PSCs to produce multinucleated skeletal muscle fibers recapitulating the embryo development in the head. Our results show that combined small-molecule regulation of endogenous signals provides an efficient and non-genetic induction to control the craniofacial myogenic fate from human PSCs. This new generation of iPSC-cMPCs can be considered a promising source for cell-based transplantation therapy and may lead to study for drug discovery to combat skeletal muscle wasting disease in the future.
Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Induction of cranial pharyngeal mesoderm (CPM) from human iPSCs using BMP activation and Notch inhibition within 6 days. (A) Protocol day 0 to day 6 for mesoderm induction. (B) Relative mRNA expression levels of cranial mesoderm marker genes at day 3 and day 6. Mean ± SEM; n = 3 for each group. Data were analyzed by 1-way ANOVA. Asterisks indicate statistical significance (*P < 0.05, **P < 0.01, and ***P < 0.001). (C) Morphological changes during CPM induction from human iPSCs. Scale bars = 330 μm. (D). MESP1+ and PDGFRα+ protein expressed colonies at day 3 and day 6, respectively. White dotted boxes indicate a higher magnification. Scale bars = 330 μm.
Fig. 2. Specification and enrichment of craniofacial myogenic progenitor cells (cMPCs) using dual inhibition of BMP and Rho kinase (ROCK) signaling and sorting strategy.
(A) Protocol day 6 to day 12 for myogenic progenitor cell (MPC) specification. (B) Treatment of BMP inhibitor and ROCK inhibitors. LDN (LDN193189, a BMP inhibitor) suppresses cardiac muscle marker genes (GATA4 and TBX5 for first heart field and ISL1 for second heart field) and enhances a craniofacial muscle marker gene (TBX1) at day 8. Y indicates Y-27632, a Rho kinase inhibitor. Data represent the mean ± SEM; n = 3 for each group. Data were analyzed by 1-way ANOVA. Asterisks indicate statistical significance (*P < 0.05, **P < 0.01, and ***P < 0.001). (C) BMP signaling determines lineage fate from bipotent cardiac/cranial pharyngeal mesoderm (PM). (D) Sorting of MPC at day
12. Representative flow cytometry plots show gating strategy HNK1<sup>-</sup>ERBB3<sup>+</sup>NGFR<sup>+</sup> for sorting of MYF5<sup>+</sup> MPCs.
Fig. 3. Characterization of HNK1−ERBB3+NGFR+ sorted craniofacial myogenic progenitor cells (cMPCs).

(A) Protocol day 12 to day 35 for MPC differentiation. (B) Relative mRNA expression of craniofacial muscle specific marker genes in sorted craniofacial MPCs. Mean ± SEM; n = 3 for each group. Data were analyzed by t-tests. Asterisks indicate statistical significance (*p < 0.05 and **P < 0.01). (C) Immunostaining of craniofacial muscle differentiation marker proteins (embryonic MyHC and TBX1) in sorted craniofacial MPCs derived from normal human iPSCs (GM25256 and GM23476) and an iPSC line from a Duchenne muscular dystrophy (DMD) patient (GM25313). Scale bars = 70 μm.
Fig. 4. Inhibition of TGF-β signaling with IGF treatment for enhancing the maturation of craniofacial myogenic progenitor cells (cMPCs) in vitro.

(A) Protocol after day 35 for MPC maturation. (B) Immunostaining of myosin heavy chain (MYH3 and MYH1) and human dystrophin (H-Dystrophin) with or without treatment of IGF-1 and TGF-β inhibitors (SB431542) in matured iPSC-cMPCs (HNK1−ERBB3+NGFR+ cells) for 18 days. Green fluorescence indicates proteins by immunostaining and blue fluorescence is DAPI staining. Scale bars = 130 μm. (C) Pseudo-color images represent striation (white arrow) in matured iPSC-cMPCs (HNK1−ERBB3+NGFR+ cells). White pseudo-color indicates late fetal MyHC (MYH1) and green pseudo-color indicates DAPI staining. Scale bars = 130 μm. (D) Quantified percentage of nuclei present in MyHC-positive myotubes with the indicated number of nuclei after 18 days of maturation in HNK1−ERBB3+NGFR+ sorted craniofacial MPCs with or without IGF-1 and SB431542 treatment. Data represent the mean ± SEM; n = 3 for each groups. Data were analyzed by 2-way ANOVA. Asterisks indicate statistical significance (**P < 0.01 and ***P < 0.001). (E) Immunostaining of myosin heavy chain (MYH1) in matured craniofacial...
MPCs (HNK1−ERBB3+NGFR+ cells) derived from normal human iPSCs (GM23476 and GM23279) and an iPSC line from Duchenne muscular dystrophy (DMD) patient (GM25313). Bottom panel showed represent striations (white arrow) in matured iPSC-cMPCs. Scale bars = 130 μm.
Fig. 5. RNA-seq analyses of iPSC-derived myogenic progenitor cells (MPCs) compared to primary myoblasts.

(A) Pearson correlation analysis between transcriptome of myoblasts from extraocular (EO), zygomaticus (Zygo), masseter (Mas), circropharyngeus (CP) and tibialis anterior (TA) muscles. Numbers in the box indicates the correlation number, \( \rho \) between samples. (B) PCA plot of variant genes in iPSC-derived MPCs and primary myoblast groups. (C) Venn diagram showing the number of commonly or differentially expressed genes in somite and PM method-derived MPCs from human iPSCs. (D) Volcano plot showing differentially expressed genes (DEGs, cut off > 2 fold) in the iPSCs-derived MPCs using somite or PM method. X axis represents \( \log_2 \) transformed fold change and Y axis represents negative \( \log_{10} \) false discovery rate. Red points indicate the upregulated craniofacial muscle development related genes (PITX2, TBX1, ILS1) and a blue point represent the downregulated limb muscle development related gene (PAX3) in PM method-derived MPCs. (E) Venn diagram showing the number of exclusively expressed genes in somite-derived primary myoblast (TA) compared to PM-derived primary craniofacial muscles (EO, Zygo, Mas, and CP myoblasts). (F) Venn diagram showing the number of commonly up-regulated genes in primary craniofacial myoblasts (EO, Zygo, Mas, and CP myoblasts) compared to limb myoblasts (TA). (G) Total Venn diagram indicating the overlap of DEGs across four comparisons. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)
Fig. 6. Scheme of differentiation and enrichment of craniofacial skeletal myotubes from human iPSCs.

Our PM method mimicked the critical signaling pathways to induce cranial/cardiac pharyngeal mesoderm (CPM) from iPSCs by using small molecules. After the bi-potential CPM was established, cells were committed to cranial skeletal muscle lineages with inhibition of cardiac lineages. We purified HNK1−ERBB3+NGFR+ cMPCs using flow cytometry and confirmed that sorted cells expressed myogenic factor 5 (MYF5), a key marker for early skeletal myogenic precursors. To facilitate differentiation into mature myotubes, cells were treated with transforming growth factor-β (TGF-β) inhibitor and IGF.