ACVR1^{R206H} increases osteogenic/ECM gene expression and impairs myofiber formation in human skeletal muscle stem cells

Emilie Barruet^1,2,3*, Steven M. Garcia^2, Jake Wu^2, Blanca M. Morales^1, Stanley Tamaki^2, Tania Moody^1, Jason H. Pomerantz^2,3 and Edward C. Hsiao^1*

^1Division of Endocrinology and Metabolism, Department of Medicine, and the Institute for Human Genetics, University of California, San Francisco, CA 94143, U.S.A.

^2Departments of Surgery and Orofacial Sciences, Division of Plastic and Reconstructive Surgery, Program in Craniofacial Biology, Eli and Edythe Broad Center of Regeneration Medicine, University of California San Francisco, 94143, USA.

^3Department of Surgery, Division of Plastic and Reconstructive Surgery, University of California, San Francisco, 94143, USA.

Email addresses:

Emilie.Barruet@ucsf.edu
Edward.Hsiao@ucsf.edu

*Co-Corresponding Authors:

Emilie Barruet, PhD
Edward Hsiao, MD, PhD

Division of Endocrinology and Metabolism, Department of Medicine
Institute for Human Genetics
University of California at San Francisco
513 Parnassus Ave., HSE 901G
San Francisco, CA 94143-0794
Phone: 415-476-9732
Fax: 415-476-1356
**Abstract:**

Abnormalities in skeletal muscle repair lead to poor function and complications such as scarring or heterotopic ossification (HO). Here, we use fibrodysplasia ossificans progressiva (FOP), a disease of progressive HO caused by ACVR1\(^{R206H}\) (Activin receptor type-1 receptor) mutation, to elucidate how ACVR1 affects skeletal muscle repair. Rare and unique primary FOP human muscle stem cells (Hu-MuSCs) isolated from cadaveric skeletal muscle demonstrated increased ECM marker expression, and showed skeletal muscle-specific impaired engraftment and regeneration ability. Human induced pluripotent stem cell (iPSC)-derived muscle stem/progenitor cells (iMPCs) Single cell transcriptome analyses from FOP also revealed unusually increased ECM and osteogenic marker expression compared to control iMPCs. These results show that iMPCs can recapitulate many aspects of Hu-MuSCs for detailed in vitro study, that ACVR1 is a key regulator of Hu-MuSC function and skeletal muscle repair; and that ACVR1 activation in iMPCs or Hu-MuSCs contributes to HO by changing the local tissue environment.
Human diseases of skeletal muscle are major medical problems. Aberrant repair after muscle injury results in scarring or heterotopic ossification (HO; bone formation in an inappropriate site) which can be devastating. Disrupted signaling of bone morphogenetic proteins (BMPs), originally identified by their ability to induce bone formation when injected into muscle (Urist, 1965), changes muscle homeostasis (Ono et al., 2011) by controlling the proliferation and differentiation of satellite cells (SCs) (Ono et al., 2011; Stantzou et al., 2017). SCs marked by PAX7 (Paired Box 7) (Seale et al., 2000), are a prerequisite for skeletal muscle regeneration (Mauro, 1961) and are thought to be the main human muscle stem cells (Hu-MuSCs) of postnatal skeletal muscle. Upon injury, activated SCs give rise to myoblasts, which form new myofibers or fuse to existing muscle fibers to repair muscle damage (Kuang, Kuroda, Le Grand, & Rudnicki, 2007). A subset of SCs does not differentiate and serves to replenish the SC pool. However, how the BMP pathway regulates muscle repair or SC function in conditions of HO remains unclear and is a major knowledge gap.

Recent protocols to create Hu-MuSCs-like cells from human induced pluripotent stem cells (hiPSCs) (Takahashi et al., 2007) could generate skeletal myogenic lineage cells, but with limited muscle regenerative capacity (Borchin, Chen, & Barberi, 2013; Magli et al., 2017; Shelton et al., 2014; van der Wal et al., 2018; Xi et al., 2017). PAX7 cell engraftment capability was often not reported or showed limited success. Transgene-induced PAX7 or PAX3 expression in hiPSCs can increase engraftment of PAX7+ cells and contribution to the SC pool (Al Tanoury et al., 2020; Wu et al., 2018); however, these engineered cells may not reflect physiology due to the genetic manipulation of these master transcription factors.

BMP signaling is one pathway that could be manipulated to make Hu-MuSCs-like cells from hiPSCs (iMPCs) (Chal et al., 2016). Although inhibiting the BMP pathway is an important step that promotes iMPC formation, the BMP pathway is also critical for maintaining PAX7 expression...
in primary MuSCs and for preventing commitment to myogenic differentiation (Friedrichs et al., 2011). Abrogation of BMP signaling in SCs slowed myofiber growth (Stantzou et al., 2017), and increased BMP4 levels in Duchenne’s muscular dystrophy (DMD) can exacerbate the disease (Shi, de Gorter, Hoogaars, t Hoen, & ten Dijke, 2013).

Fibrodysplasia ossificans progressiva (FOP), a congenital disease of abnormal skeletal muscle regeneration and severe HO, provides a unique opportunity to understand how changes in BMP signaling affect Hu-MuSC and iMPC formation and function. The FOP iMPCs and Hu-MuSCs carry the classical ACVR1<sup>R206H</sup> (c.617>A) mutation (Shore et al., 2006) that causes hyperactivation of the BMP-SMAD signaling (Billings et al., 2008) and aberrant responses to Activin A (Hatsell et al., 2015). Primary FOP Hu-MuSCs can engraft and regenerate injured muscle of NSG mice, but at lower efficiency than Hu-MuSCs from non-FOP donors, and that the source of FOP Hu-MuSCs (biceps vs. diaphragm) appears to impact their engraftment efficiency.

A new non-transgenic strategy for creating human iMPCs was developed and applied to multiple iPSC lines from control subjects and subjects with FOP. These iMPCs shared transcriptional similarities with primary Hu-MuSCs, and abnormal activation of the BMP pathway by ACVR1<sup>R206H</sup> changed the transcription profiles of the FOP iMPCs compared to controls.
Results:

1- Lower efficiency engraftment of Primary Human FOP Hu-MuSCs

Muscle tissue samples were obtained from FOP cadavers which allowed the study of markers in situ and the isolation of primary human cells with endogenous activated ACVR1 signaling. Hematoxylin and eosin and alcin blue staining of FOP primary muscle samples from two deceased FOP subjects (Figure 1-Source Data 1) in a region without HO showed no gross defects. FOP muscle tissue near a HO lesion showed increased ECM proteoglycan components (alcian blue staining) in the interstitial space of the muscle fibers near the HO lesion (Figure 1A). Primary Hu-MuSCs carrying the ACVR1R206H activating mutation were isolated from unfixed muscle tissue from two FOP autopsies. Biceps muscle, commonly affected by HO, and diaphragm muscle, one of the rare skeletal muscle sites spared from HO in patients with FOP, were analyzed. PAX7 staining confirmed the presence of SCs in human FOP biceps (Figure 1B, top and middle). Interestingly, a small subregion showed Collagen Type 1 expression with embedded satellite cells (Figure 1B, bottom) suggesting possible early HO formation despite absence of gross HO.

Sufficient Hu-MuSCs (Figure 1-figure supplement 1A) were sorted for transplant and gene expression analysis (Figure 1-Source Data 2). FOP Hu-MuSCs showed lower PAX7 expression compared to control Hu-MuSCs. COL1A1 and ID3 were increased in affected FOP muscle (biceps) (Figure 1C). However, ID1 was increased in both the FOP diaphragm and biceps compared to control muscles (Figure 1C). Five weeks after transplantation, biceps and diaphragm FOP Hu-MuSCs had engrafted and formed human fibers (Figure 1D, E). However, the number of human DYSTROPHIN-positive fibers was significantly lower with FOP Hu-MuSCs vs. control Hu-MuSCs (Figure 1F). The number of engrafted PAX7+ cells was qualitatively lower with FOP vs. control Hu-MuSCs (Figure 1F and Figure 1-figure supplement 1B). Ten weeks after re-injury with bupivacaine at week 5 (Figure 1D), the number of human DYSTROPHIN fibers was significantly decreased when FOP biceps Hu-MuSCs were transplanted compared to control Hu-
MuSCs, but not when FOP diaphragm Hu-MuSCs were transplanted (Figure 1G, H). No differences in the number of human PAX7 cells were identified (Figure 1G, H). No radiologic
evidence of HO was found in any mice (Figure 1-figure supplement 1C).

Thus, primary FOP Hu-MuSCs can engraft and regenerate injured muscle of NSG mice, but at lower efficiency than unaffected MuSCs, and the source of FOP Hu-MuSCs (biceps or diaphragm) may impact MuSC engraftment efficiency.

2- Human FOP iPSCs can differentiate into skeletal muscle cells

The rarity of FOP disease and difficulty obtaining human tissue samples from patients with FOP makes it difficult to obtain a reliable source of muscle stem cells. Therefore, we used established and fully characterized control hiPSCs (Wtc11, 1323-2, and BJ2) and ACVR1^{R206H} hiPSCs (F1-1, F2-3, F3-2) lines previously derived from patients with FOP. The control hiPSCs yielded PAX7 and MYOGENIN-expressing cells (Figure 2A,B and Figure 2-figure supplement 1A,B) as early as day 25 (Figure 2-figure supplement 1A,B), expressed DYSTROPHIN (Figure 2B), and formed contractile myotubes (Figure 2C and Video1-2).

Since BMPs control skeletal muscle differentiation from hiPSCs (Chal et al., 2016; Xi et al., 2017), we investigated if genetic activation of the BMP pathway via ACVR1^{R206H} could alter the myogenic differentiation of hiPSCs (F1-1, F2-3, F3-2) derived from patients with FOP (Matsumoto et al., 2013). All three FOP iPSC lines formed contractile myotubes with cells expressing DYSTROPHIN and PAX7 (Figure 2C,D and Figure 2-figure supplement 1A,B).

PAX7, MYOGENIN, and DYSTROPHIN (Figure 2E and Figure 2-figure supplement 1C) gene
expression showed some heterogeneity among the different hiPSC lines but these differences were not statistically significant. Adding a SMAD inhibitor of the BMP pathway (LDN193189) into the
differentiation protocol (Figure 2F) improved the differentiation of the control hiPSC lines. LDN also improved differentiation of the FOP F3-2 (Figure 2G) line, which had shown lower PAX7 and MYOGENIN expression (Figure 2E). These findings show that individual hiPSC lines are heterogeneous in differentiation to skeletal muscle lineages, similar to other protocols (Volpato & Webber, 2020), that FOP hiPSCs can form skeletal muscle cells and Hu-MuSC-like cells expressing PAX7 despite upregulation of the BMP pathway by ACVR1R206H, and that chemical blockade of the BMP pathway can improve the formation of Hu-MuSC-like cells from the FOP iPSC line that showed the lowest efficiency.

3- **PAX7 expressing cells can be isolated from myogenic differentiation**

To test the regenerative properties of PAX7-expressing MuSCs, we used FACS to purify HNK1−CD45−CD31− cells co-expressing CD29, CXCR4, and CD56 markers present on human PAX7+ cells (Garcia et al., 2018) (Figure 3A and Figure 3-figure supplement 1A,B). FACS analysis identified intermediate CD56 cells expressing high PAX7 and low MYOGENIN (Figure 3B and Figure 3-figure supplement 1C), consistent with Hu-MuSC expression profiles. All HNK1−CD45−CD31−CXCR4+CD29+CD56dim cells formed myotubes expressing MHC (Figure 3-figure supplement 1D) when cultured in terminal differentiation media demonstrating isolation of functional iMPCs with satellite cell characteristics from the cultures.
Figure 3: Isolation and transplantation of PAX7-expressing cells from iPSC muscle differentiation culture. hiPSCs were differentiated into iMPCs until day 50 and sorted via flow cytometry. (A) Gating strategy. (B) Myogenic gene expression of CD56<sup>dim</sup> and CD56<sup>hi</sup> cells (n=3 biological and technical replicates, ***p < 0.001, ****p < 0.0001 by two-way ANOVA test). (C) Representative human DYSTROPHIN (top, 200 µm scale bar), and total LAMININ, human LAMIN A/C, human SPECTRIN, and PAX7 (bottom, 100 µm scale bar) immunohistochemistry of NSG mice anterior tibialis, where sorted iMPCs were transplanted. White arrows show engrafted hiPSC-derived muscle stem cells. (D) Quantification of human DYSTROPHIN fibers and human PAX7 cells at week 5 after transplant (n=3 biological replicates).
4- Isolated iMPCs can regenerate injured mouse muscle and form human fibers

To assess iMPC regenerative capacity in vivo, we injected 1,000-10,000 iMPCs derived from control or FOP hiPSCs (Figure 3-Source Data 1) into the tibialis anterior (TA) muscle of whole-body irradiated NSG immunocompromised mice (to hinder endogenous satellite cells) previously injured with bupivacaine (Garcia, Tamaki, Xu, & Pomerantz, 2017). The bupivacaine step induces myofiber injury and promotes engraftment of donor SCs. New fibers expressing human DYSTROPHIN and PAX7 cells were found after 5 weeks, showing that iMPCs could engraft and promote muscle regeneration (Figure 3C). However, the number of human fibers and human PAX7+ cells remained low (Figure 3D) compared to primary Hu-MuSCs. While some iMPC transplants yielded up to 60 new human fibers, some did not yield any human fibers. By comparison, 2,000 primary non-FOP Hu-MuSCs resulted in an average of 155 human fibers based on prior assessments using the same assay (Garcia et al., 2018). No significant differences between control and FOP iMPCs were identified, though some individual FOP samples showed higher engraftment (Figure 3D).

These results showed that iMPCs can engraft into a muscle injury site in mice, but engraftment efficiency may be lower than primary Hu-MuSCs or be the result of differences in experimental conditions. Also, ACVR1R206H did not significantly impact muscle fiber regeneration in this assay.

5- Transcriptional profiling of iMPCs

The lower engraftment of iMPCs compared to primary Hu-MuSCs suggested that the FACS-purified population was still heterogeneous or that iMPCs do not fully recapitulate adult primary Hu-MuSCs. Single cell RNA sequencing (scRNAseq) from control (1323-2) and FOP (F3-2) iMPCs (the lines were selected based on their lower intra-line variability, Figure 2) were analyzed (Figure 4A; Figure 4-Source Data 1). Cell populations for both samples were defined by the dimension reduction technique of uniform manifold approximation and projection (UMAP) (Becht...
et al., 2018) and unsupervised clustering with Seurat v3 package (Stuart et al., 2019) (Figure 4B). Both control (Figure 4-figure supplement 1A) and FOP (Figure 4-figure supplement 1B) samples had clusters expressing myogenic genes (PAX7 and MYOD); mesenchymal genes (PDGFRA); and neuronal genes (SOX2). Merged analysis to allow direct comparison identified 13 distinct clusters (Figure 4C and Figure 4-figure supplement 1C). Clusters 0-2, 4-9, and 12...
consisted of cells expressing neuronal stem/progenitor cell genes such as *EFNB3*, *SOX2*, and *ASCL1* (Figure 4D,E and Figure 4-figure supplement 1D,E). Mesenchymal genes (*PDGFRA*, *ASPN*, and *COL1A1*) were expressed in cluster 8 (Figure 4D,E and Figure 4-figure supplement 1D,E). Cells expressing muscle markers (*PAX7*, *MYF5*, *MYOD* and *MYOG* (*MYOGENIN*)) were found in clusters 3, 10, and 11 (Figure 4D,E and Figure 4-figure supplement 1D,E). The frequency of muscle cells (clusters 3, 10, 11) was higher in FOP (14%, 2.5%, and 1.7%) compared to controls (4.2%, 0.5%, and 0.5%) (Figure 4F) suggesting that FOP hiPSCs may be more efficient at making muscle progenitor cells. Thus, FACS-purified iMPC cultures contain muscle stem/progenitor cells, but other cell types such as mesenchymal, neuronal progenitor cells, and myoblasts persist in the culture. Furthermore, the frequency of iMPCs appears to be higher in FOP vs. control cell cultures.

6- **FACS- sorted iMPC transcriptome is heterogeneous**

Typical muscle progenitor cultures are expected to contain cells undergoing expansion, differentiation, and maturation. Sub-clustering (Figure 5A,B) identified 5 new myogenic subpopulations (Figure 5B). We ordered the myogenic cells into 3 major branches using the Monocle analysis package (Trapnell et al., 2014) and constructed pseudotime differentiation trajectories (Figure 5C). *PAX7* and *MYF5* were upregulated in branch B but downregulated in branches A and C (Figure 5C,D). *MYOD* and *MYOG* showed higher expression patterns early in branch A and late in branch C (Figure 5C,D). Quiescent iMPCs were at the intersection of the 3 branches (clusters 0, 1) while distal parts of the 3 branches, notably comprised of clusters 3 and 4, contained more mature cells with transcriptional profiles similar to activated MuSCs, progenitor cells, or myoblasts. Cluster 4 expressed higher levels of *MYOD*, *MYOG*, *SOX8*, and *MEF2C* (markers of differentiated MuSCs/myoblasts/myocytes), while clusters 0-3 expressed higher levels
Figure 5: Transcriptional profile of iMPCs. (A) Identified myogenic clusters (in red) were sub-clustered from the other clusters (neurogenic and mesenchymal) and re-analyzed. (B) UMAP of the new myogenic subclusters was generated. (C) Pseudotime trajectory plot generated via Monocle analysis depicting all myogenic clusters. (D) Gene plots displaying the expression of specific myogenic genes as a function of pseudotime. Arrows represent the direction and major branches of the pseudotime. Branches A-C are marked with letters along the trajectories. (E) Feature expression of cells expressing myogenic markers. (F) Genes expression associated with stemness, quiescence, and activation for the described Hu-MuSCs subsets. (G) Feature expression plots of additional top differentially expressed marker for each cluster. (H-I) Violin plots of myogenic (H) and chondro/osteogenic genes (I) that were significantly differentially expressed. Violin plot width depicts the larger probability density of cells expressing each particular gene at the indicated expression level. *, significantly different following differential expression testing using the Wilcoxon rank sum test per cluster. (J) SMAD and P38MAPK pathway markers significantly differentially expressed between control and FOP myogenic cells. p values for H-J are in Figure 5-Source Data 1.

201 of PAX7 and MYF5 (markers of more quiescent MuSCs) (Figure 5E,F). Quiescence (SPRY1,
and cell cycle (TOP2A or KI67) markers were increased in clusters 0-2 and cluster 3 respectively (Figure 5F,G and Figure 5-figure supplement 1A,B). Cluster 3 had a higher proportion of cells in G2M and S phase (Figure 5-figure supplement 1B). The cell cycle distribution was similar in control Figure 5: Transcriptional profile of iMPCs. (A) Identified myogenic clusters (in red) were sub-clustered from the other clusters (neurogenic and mesenchymal) and re-analyzed. (B) UMAP of the new myogenic subclusters was generated. (C) Pseudotime trajectory plot generated via Monocle analysis depicting all myogenic clusters. (D) Gene plots displaying the expression of specific myogenic genes as a function of pseudotime. Arrows represents the direction and major branches of the pseudotime. Branches A-C are marked with letters along the trajectories. (E) Feature expression of cells expressing myogenic markers. (F) Genes expression associated with stemness, quiescence, and activation for the described Hu-MuSCs subsets. (G) Feature expression plots of additional top differentially expressed marker for each cluster. (H-I) Violin plots of myogenic (H) and chondro/osteogenic genes (I) that were significantly differentially expressed. Violin plot width depicts the larger probability density of cells expressing each particular gene at the indicated expression level. *, significantly different following differential expression testing using the Wilcoxon rank sum test per cluster. (J) SMAD and P38MAPK pathway markers significantly differentially expressed between control and FOP myogenic cells. p values for H-J are in Figure 5-Source Data 1.

and FOP (Figure 5-figure supplement 1C). Cells expressing APOE and KRT17 defined cluster 0. CDH15, a niche regulator of SCs quiescence (Goel, Rieder, Arnold, Radice, & Krauss, 2017) was enriched in cluster 1. Cluster 2 consisted of cells expressing high levels of FOS (Figure 5F,G and Figure 5-figure supplement 1A). The proportion of cells in clusters 3 and 4 were similar in both control (10% and 12.7%) and FOP (14.3% and 9%) samples. The proportion of cells in clusters 0 and 3 was higher in FOP (31.4%
and 23.4%) compared to control (20% and 5.5%), while the proportion of cells in cluster 2 was increased in control (51.8% vs 21.9%) (Figure 5-figure supplement 1D).

Thus, hiPSC differentiation cultures contain subpopulations of iMPCs showing the expected spectrum of quiescence, activation, and differentiation with FOP cultures having a higher proportion of cells in the stem cell/progenitor and proliferating phases and fewer mature myoblasts.

7- **FOP iMPCs cells express increased chondro/osteogenic and ECM markers**

Differential expression analysis on the transcriptional profiles of the sub-clustered myogenic cells (Figure 5A,B and Figure 5-figure supplement 1E) was used to see if ACVR1^{R206H} altered transcriptional signatures. While PAX7 was significantly increased in control vs. FOP cells in cluster 3 only (Figure 5H), MYF5 was significantly increased in FOP cells from cluster 1 compared to control cells (Figure 5-Source Data 1). Since Hu-MuSCs show heterogeneous levels of PAX7 and MYF5 expression (Kuang et al., 2007), this suggests the ACVR1^{R206H} mutation may favor one sub-population over another. Interestingly, SPRY1, a known regulator of quiescence (Shea et al., 2010) which decreases with age (Bigot et al., 2015), was significantly downregulated in FOP cells of clusters 0-4, while DLK1, which act as a muscle regeneration inhibitor (Andersen et al., 2013) was significantly increased in FOP cells (Figure 5H and Figure 5-Source Data 1).

Since the ACVR1^{R206H} mutation increases BMP pathway activity and expression of chondrogenic and osteogenic markers in multiple lineages (Barruet et al., 2016; Culbert et al., 2014; Matsumoto et al., 2013), we examined the expression levels of extracellular matrix, fibrogenic, chondrogenic, and osteogenic genes. *ITM2A, COL1A1, DCN, CD15, SPARC,* and *TIMP1* were significantly increased in FOP cells (Figure 5I and Figure 1-Source Data 1). ECM proteoglycans known to be involved in inflammation, including *BGN* (Nastase, Young, & Schaefer, 2012) and *LUM* (Nikitovic, Papoutsidakis, Karamanos, & Tzanakakis, 2014), *TAGLN*...
[regulates osteogenic differentiation (Elsafadi et al., 2016)], and IGBP5 [increased in aged satellite cells (Soriano-Arroquia, McCormick, Molloy, McArdle, & Goljanek-Whysall, 2016)], were also increased in FOP cells (Figure 5-figure supplement 1F and Figure 1-Source Data 1).

Finally, gene expression of target genes of the BMP pathway (ID1, ID3, BMPs, and SMADs) and the p38MAPK pathway (Figure 5J and Figure 5-figure supplement 1G) was assessed to see if activated ACVR1 altered these pathways. ID1 was significantly higher (clusters 0, 1, 3, and 4) in control cells while ID3 was significantly higher in FOP cells (clusters 1, 2). In addition, the BMP/TGFβ pathway downstream target gene SMAD5 was significantly higher in cluster 2 of control cells. The p38 pathway components JUN (clusters 1, 3), JUNB (clusters 0-2), and FOS (clusters 1, 3) were significantly increased in FOP cells. Within the known ACVR1 co-receptors, BMPR2 (clusters 3, 4) and ACVR2B (clusters 0, 2, 3) expression were significantly higher in control cells while ACVR2A expression was significantly higher in FOP cells (cluster 1, 4). ACVR2B was significantly higher in control cells (cluster 0, 1, 4) (Figure 5J). Similar to the primary FOP Hu-MuSCs (Figure 1), these results suggest that FOP iMPCs have a chondrogenic/osteogenic signature, increased ID3 expression, and also showed higher p38 pathway activity and higher levels of the ACVR2A co-receptor at different stages of myogenic differentiation.

8- iMPC transcriptome shows similarities to primary Hu-MuSCs

Comparing the iMPC scRNAseq to primary Hu-MuSCs data of sorted satellite cells from a human vastus lateralis muscle (Barruet et al., 2020) was used to identify if their lower engraftment efficiency was due to transcriptional differences. The merged data UMAP (Figure 6A) showed that clusters 0, 1, 4, 6, and 7 contained myogenic cells (Figure 6B,C). PAX7+ cells were identified in clusters 0, 1, 4, and 7 (Figure 6C). Myocyte contaminants present in the primary sorted cells constituted cluster 6. Although iMPCs expressed PAX7 and MYF5, the gene expression levels were
higher in primary Hu-MuSCs. In contrast, MYOD was higher in iMPCs (Figure 6C and Figure 6-figure supplement 1).

Detailed analysis of the myogenic cell subset (cluster 0, 1, 4, 6 and 7, Figure 6D, cells labeled in red, and Figure 6E) was done using pseudotime trajectory analysis to elucidate the states of the iMPCs with respect to primary Hu-MuSCs. Cells from clusters 0, 1, and 4 were distributed along branches B and C. Myocytes ordered at the distal end of branch A. iMPCs ordered away from primary Hu-MuSCs (PAX7+) and primary myocytes (MYL1+) (Figure 6F-H). Branch expression analysis modeling (BEAM) allowed us to investigate significant gene that are branch-dependent in their expression (Figure 6-Source Data 1). Branch B, consisting of iMPCs and subset of primary Hu-MuSCs, expressed significantly higher levels of genes associated with mesenchymal, fibrogenic, chondrogenic, osteogenic lineages and extracellular matrix (Figure 6H,I). Thus, iMPCs retained strong bi-potency compared to primary Hu-MuSCs, suggesting that iMPCs may not be as committed to the muscle lineage as primary adult muscle stem cells (Xi et al., 2020).
Figure 6: iMPC transcriptional signature compared to human primary muscle stem cells. (A-B) UMAP of cells combined from human primary muscle stem cells (vastus muscle) and the control and FOP samples. (A) UMAP showing the distribution of cells per sample, and (B) with clusters labeled. (C) Feature expression plots of cells expressing myogenic markers. (D) Myogenic cells (in red) are comprised in cluster 0, 1, 4, 6, and 7. (E) UMAP of the myogenic clusters only used for the pseudotime analysis. (F-G) Pseudotime trajectory plot generated via Monocle analysis depicting all myogenic clusters (F) and samples (G). Branches are annotated A-C in (F). (H) Level of expression of ECM/osteogenic genes along the cell trajectories. (I) Heatmap representing genes that are significantly branch dependent using the BEAM analysis (Figure 6—Source Data 1) and also genes that have similar lineage-dependent expression patterns. Cell fate (branches) are shown in the lower right panel.
Developing optimal strategies for skeletal muscle regeneration and repair requires a detailed understanding of how these processes are regulated. Especially for diseases like FOP where muscle injury can trigger disease progression or severe complications, generating large numbers of human iMPCs using transgene-free protocols, holds promise for understanding pathological mechanisms and finding new therapeutic targets. This study revealed several novel findings, including deficiencies in the muscle repair capacity of FOP Hu-MuSCs, abnormal production of extracellular matrix components by iMSCs that likely contribute to FOP disease progression, and important differences between primary Hu-MuSCs and iMPCs that may impact other studies. In addition, our results suggest that there are muscle specific differences in Hu-MuSC repair capacity, potentially explaining why some skeletal muscles appear to be protected from developing HO.

The iMPC and primary Hu-MuSC single cell transcriptomes revealed clear subsets of cells at all stages of muscle differentiation (quiescent, activated, and differentiated). Satellite cell subtype markers [e.g. COLs, DLK1, ID3, and HES1, (Barruet et al., 2020)] were also highly expressed in iMPCs suggesting that in vitro differentiation may favor specific subtypes of satellite cells. iMPCs also expressed high levels of mesenchymal/ECM markers, similar to that in human fetal muscle progenitor cells (Xi et al., 2020). These results suggest that iMPCs may retain a more progenitor-like phenotype as compared to Hu-MuSCs. In addition, we showed that PAX7+ iMPCs can engraft in muscle injury models in mice, despite heterogeneity in the cultures and lower expression of typical satellite cells markers (e.g. PAX7, MYF5).

This iMPC system revealed that activation of the BMP pathway by the FOP ACVR1<sup>R206H</sup> mutation induced changes that could contribute to abnormal muscle healing. As expected, the classical SMAD pathways were active in FOP cells; however, it was unexpected to find that the p38 pathway via JUN/FOS was more active in the FOP iMPCs, considering that FOP cultures had a higher proportion of cells in the stem cell/progenitor and proliferating phases. While increased
SMAD activity (Billings et al., 2008) or misinterpretation of the Activin A ligand (Hatsell et al., 2015) occur with ACVR1^{R206H} mutation, abnormal p38 signaling may be critical in some cells like macrophages (Barruet et al., 2018). Since p38 is a major regulator of Hu-MuSC function (Segales, Perdiguero, & Munoz-Canoves, 2016) and has been associated with inflammation and ECM accumulation in aged muscle (Cosgrove et al., 2014), further studies are needed to determine how this p38 signaling contributes to satellite cell subtype specification (Barruet et al., 2020) and affect healing in FOP.

Importantly, our iMPC and Hu-MuSC transplant studies showed no HO in the recipient mice, consistent with prior FOP genetic studies (Dey et al., 2016; Lees-Shepard et al., 2018). However, FOP iMPCs showed increased chondrogenic/osteogenic and ECM gene expression, a feature seen in other bone-related cell types in FOP (Barruet et al., 2016; Culbert et al., 2014; Lees-Shepard et al., 2018). Thus, FOP Hu-MuSCs may contribute to HO formation indirectly by modulation of the osteogenic environment such as contributing to changes in muscle stiffness, as previously reported in mice (Stanley, Heo, Mauck, Mourkioti, & Shore, 2019). In addition, while our results suggest no major differences or possibly a slight increase in the ability to form skeletal muscle progenitors, activated ACVR1 by the R206H mutation decreased (but did not abrogate) in vitro formation of mature myoblasts and in vivo muscle repair after transplant. This impaired formation of mature skeletal muscle likely contributes to the abnormal skeletal muscle healing in FOP and may predispose patients with FOP to HO formation.

Comparing primary FOP Hu-MuSCs from muscles that develop HO (biceps) and non-affected muscle (diaphragm) suggests that the source of the Hu-MuSCs impacts engraftment efficiency. The re-injury model showed that engraftment of Hu-MuSCs from FOP biceps, but not from diaphragm, remained significantly decreased. Thus, it is intriguing to consider that the clinical sparing of the diaphragm from HO in patients with FOP may result from a less impaired or unimpaired muscle repair process in diaphragm satellite cells. Further delineation of muscle-
specific Hu-MuSC properties will be revealing to understand this observation. Our finding that primary FOP Hu-MuSCs have lower engraftment ability provides a potential explanation for the poor skeletal muscle repair observed in patients with FOP (Shore, 2012). However, the FOP and control iMPCs showed no major differences in engraftment, possibly due to decreased assay sensitivity from the lower engraftment efficiency of iMPCs in general, because iMPCs may be more immature than primary Hu-MuSCs, or that iMPCs represent a subtype of cells that may be more reflective of non-ossifying skeletal muscle like diaphragm.

This study has several limitations. Our transplant experiments used immunocompromised mice where mature B and T cells are absent and macrophages are defective (Shultz et al., 2005). This may dampen the engraftment/regenerative phenotypes, particularly as immune cells are important in muscle regeneration (Furrer & Handschin, 2017) and there is growing awareness of the role of the immune system in the pathogenesis of FOP (Barruet et al., 2018; Convente et al., 2018). Also, while our FACS strategy can isolate Hu-MuSCs and iMPCs without the need of transcription factors or markers, we noted that the engraftment efficiency still varied among the iMPC lines.

**Figure 7. Testing the role of activated ACVR1 signaling in muscle repair.** FOP iMPCs and FOP primary human muscle stem cells are used for modeling muscle stem cell engraftment and regeneration properties.
despite FACS purification. This heterogeneity is an ongoing problem among all published iMPC protocols to date. In our case, this was counter-balanced by our consistent findings across multiple lines and between iMPCs and the availability of primary Hu-MuSCs. Although our primary cell studies were limited by the rarity of FOP (estimated at 1 in 1.4 million people) and even rarer suitable cadaveric samples, the combination of iPSC-derived lineages with the rare primary samples provided multiple avenues for supporting our conclusions. Finally, the ACVR1$^{R206H}$ mutation increases BMP signaling but also introduces neofunction to Activin A. Our studies are not able to distinguish between these two contributing pathways. Our finding of increased p38 activity in the FOP iMPCs was also seen in FOP subject monocyte-derived macrophages (Barruet et al., 2018), suggesting that this alternate signaling pathway by ACVR1 should also be investigated further. Future studies will help elucidate the different factors that contribute to the hiPSC-line specific effects, including potential roles for disease modifier genes or BMP pathway modulators.

This study shows that human iPSC-derived muscle stem cells can be a valuable tool to model musculoskeletal diseases of skeletal muscle injury and repair. Correlating the findings in iMPCs with primary Hu-MuSCs revealed an indirect role for skeletal muscle progenitors in HO formation, as well as subtypes of Hu-MuSCs that may contribute to skeletal muscle specific regenerative capacity. These studies highlight the importance of skeletal muscle regeneration in disease pathogenesis and establish a foundation for understanding how skeletal muscle repair and osteogenesis are linked.
### Materials and Methods:

#### Key Resources Table

| Reagent type (species) or resource | Designation | Source or reference | Identifiers | Additional information |
|-----------------------------------|-------------|---------------------|-------------|------------------------|
| NSG mice                          | NOD.Cg-PrkdcscidIl2rgtm1Wj1/SzJ | https://www.jax.org/strain/005557 | 005557 | 8-12 week-old |
| Sequenced-based reagent           | Human RT-PCR Primers | Applied Biosystems Taqman Assays | B-ACTIN | Hs01060665_g1 |
|                                   | Human RT-PCR Primers | Applied Biosystems Taqman Assays | DYSTROPHIN | Hs007758098_m1 |
|                                   | Human RT-PCR Primers | Applied Biosystems Taqman Assays | PAX7 | Hs00242962_m1 |
|                                   | Human RT-PCR Primers | Applied Biosystems Taqman Assays | CD56 | HS00941830_m1 |
|                                   | Human RT-PCR Primers | Applied Biosystems Taqman Assays | MYOGENIN | Hs01072232_m1 |
|                                   | Human RT-PCR Primers | Applied Biosystems Taqman Assays | COL1A1 | Hs01076780_g1 |
|                                   | Human RT-PCR Primers | Applied Biosystems Taqman Assays | ID1 | Hs03676575_s1 |
|                                   | Human RT-PCR Primers | Applied Biosystems Taqman Assays | ID3 | Hs00954037_g1 |
| antibody                          | Mouse monoclonal anti-Human DYSTROPHIN | DSHB | MANDYS104(7F7) | IF(1:10) |
| antibody                          | Mouse monoclonal anti-Human/Mouse DYSTROPHIN | Thermofisher | PA5-16734 | IF(1:500) |
| antibody                          | Mouse monoclonal anti-Human/Mouse PAX7 | DSHB | PAX7 | IF(1:10) |
| antibody                          | Mouse monoclonal anti-Human/Mouse MHC | DSHB | MF20 | IF(1:100) |
| antibody | Rabbit polyclonal anti-LAMININ | Sigma-Aldrich | L9393 | IF(1:250) |
|----------|--------------------------------|---------------|-------|-----------|
| antibody | Mouse monoclonal anti-Human SPECTRIN | Leica Microsystems | NCL-SPEC1 | IF(1:100) |
| antibody | Mouse monoclonal anti-Human LAMIN A/C | Vector Laboratories | VP-L550 | IF(1:100) |
| antibody | Mouse monoclonal anti-Human CD31 (Beads) | Miltenyi Biotec | 130-091-935 | FACS |
| antibody | Mouse monoclonal anti-Human CD45 (Beads) | Miltenyi Biotec | 130-045-801 | FACS |
| antibody | Mouse monoclonal anti-Human CD31 AF450 (WM-59) | Ebioscience | 48-0319-42 | FACS |
| antibody | Mouse monoclonal anti-Human CD34 eFluor450 (4H11) | Ebioscience | 48-0349-42 | FACS |
| antibody | Mouse monoclonal anti-Human CD45 AF450 (30-F11) | Ebioscience | 48-0451-82 | FACS |
| antibody | Mouse monoclonal anti-Human CD29 FITC (TS2/16) | Ebioscience | 11-0299-41 | FACS |
| antibody | Recombinant human anti-CD56 APC-vio-770 (REA196) | Miltenyi Biotec | 130-114-548 | FACS |
| antibody | Mouse monoclonal anti-Human CXCR4 PE (12G5) | Ebioscience | 12-9999-41 | FACS |
| antibody | Mouse monoclonal anti-Human HNK1 PE (TB01) | Ebioscience | 12-057742 | FACS |
| antibody | Mouse monoclonal anti-Human CD45 PE (30-F11) | Ebioscience | 12-04551-82 | FACS |
| antibody | Mouse monoclonal anti-Human CD31 PE (390) | Ebioscience | 12-0311-82 | FACS |
Pluripotent hiPSC lines derived from control and FOP fibroblasts (Matsumoto et al., 2013; Spencer et al., 2014) were cultured in mTeSR1 medium (StemCell Technologies) on irradiated SNL feeder cells (McMahon & Bradley, 1990) as described previously. hiPSCs were passaged at least once on Matrigel (Corning)-coated plates (150-300µg/ml) to remove the SNLs before use in differentiation assays. ROCK inhibitor Y-27632 (10µM, StemCell Technologies) was added to mTeSR1 when cells were split and removed the following day.

hiPSC lines were differentiated into skeletal muscle cells using modifications based on prior protocols (Chal et al., 2016; Shelton et al., 2014). Our hiPSC lines differentiated better with a lower cell number seeding and a longer time of recovery between the seeding and the start of the differentiation.
differentiation (2 days, data not shown). Cells were seeded at 7.5x10^5 cells per well of a 12-well plate on Matrigel two days before the differentiation medium (E6 medium, supplemented with either 10µM CHIR99021 (Tocris) for 2 days or with 3µM CHIR99021 and 0.5µM LDN193189 for six days). Cells were then grown in un-supplemented E6 media until day 12, then changed to StemPro-34 media supplemented with 10ng/ml bFGF until day 20. The medium was then replaced by E6 medium until day 35, when DMEM/F12 supplemented with N2 (Gibco) and Insulin-Transferrin-Selenium (ITS-A, 100X Gibco) was added. Media was changed daily until harvest at day 50. After sorting, cells were plated in satellite cell media [DMEM/F12 (Gibco), 20% FBS (Hyclone), 1X ITS (Gibco), 1X Penicillin/ Streptomycin (Gibco)] for further functional assays (Figure 2A,F). Once cells reached confluence, cells were cultured in differentiation media (DMEM, 2% horse serum, 1X Penicillin/ Streptomycin; Gibco).

Flow cytometry

HNK1^−CD45^−CD31^−CXCR4^+CD29^+CD56^dim^ cells were sorted from skeletal muscle differentiation of control and FOP hiPSCs (described in detail in Supplemental Experimental Procedures). Human primary satellite cells were isolated and sorted as described (Garcia et al., 2018; Garcia et al., 2017). Human muscle was freshly harvested and stored in DMEM with 30% FBS at 4°C overnight or for 2 extra days (delay due to shipping). Muscle samples were digested, erythrocytes were lysed, and hematopoietic and endothelial cells were depleted with magnetic column depletion using CD31, CD34, and CD45 (eBioscience). Cells were further gated as described in Figure 1-figure supplement 1A and sorted for CXCR4^+/CD29^+/CD56^+^ and collected for subsequent experimentation.

Animal care and Transplantation studies
All mouse studies were performed using protocols approved by the UCSF Institutional Animal Care and Use Committee. Mice were either bred and housed in a pathogen-free facility at UCSF or purchased from The Jackson Laboratory. 8-12 week-old NSG (NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ) mice were randomized to all experimental groups by sex and littermates. The TA of each mice was irradiated with 18 Gy before transplantation. Isolated primary human satellite cells (Hu-MuSCs) or iMPCs were injected with 50 µl 0.5% bupivacaine directly into the TA muscle of one leg as described (Garcia et al., 2018; Garcia et al., 2017) and summarized in the Supplemental Experimental Procedures. The TA for each mouse was harvested at week 5 or week 10 after transplantation and frozen in O.C.T. compound in 2-methylbutane chilled in liquid nitrogen. Serial 6µm transverse frozen sections were analyzed or stored at -80ºC.

**Cell Immunostaining and NSG Tibialis Anterior Analysis**

iMPCs were fixed with 4%PFA/PBS for 10min at room temperature, permeabilized with 0.1%Triton-100X (Sigma-Aldrich), and blocked with 5% BSA (Sigma-Aldrich). Cells were stained overnight with primary antibodies for PAX7, MYOGENIN, DYSTROPHIN, and MHC. Cells were then incubated for 1 hr at room temperature in the dark with secondary antibodies Alexa488-conjugated goat anti-mouse IgG and Alexa546-conjugated goat anti-mouse IgG (Invitrogen). Nuclei were stained with DAPI (Sigma-Aldrich). Images were taken on a Nikon Eclipse E800 or Leica DMI 4000B.

**Immunohistochemistry and Immunofluorescence of Human Muscle Samples**

Human muscle samples were fixed in neutral buffered formalin for 24 h and then placed in 70% ethanol for at least 24 h. The sample with heterotopic bone was decalcified in 10% EDTA (pH 7.2-7.4) before paraffin embedding and sectioning. Sections were stained with hematoxylin and eosin.
(J. David Gladstone Institutes Histology Core) or for alcian blue (pH 1.0) for cartilage and nuclear red stain for nuclei.

Freshly harvested human muscle was stored in DMEM with 30% FBS at 4°C, or snap in frozen in O.C.T. compound in 2-methylbutane chilled in liquid nitrogen. Serial 6µm transverse frozen sections were analyzed or stored at -80°C and processed similarly to the mouse TA samples above. Sections were stained with PAX7 (DSHB) and mouse monoclonal anti-Collagen Type I (Millipore-Sigma). Details about specimens are in Figure 1-Data Source 1.

RT-PCR and Quantitative Analysis

Tissues were collected in TRI Reagent (Sigma-Aldrich) to isolate total RNA using the Arcturus™ PicoPure™ RNA kit (Applied Biosystems) as previously described for small samples (Scheper, Hsiao, Garg, Scott, & Passegue, 2012). 0.2 to 0.5 µg of RNA were transcribed into cDNA with VeriScript cDNA synthesis kit (Affymetrix). cDNA was then pre-amplified with GE PreAmp Master Mix (Fluidigm Inc). Real-time quantitative PCR was performed in triplicated with either VeriQuest Probe qPCR Master Mix (Affymetrix) or Taqman Universal PCR Master Mix (Life Technologies) on either a Viia7 thermocycler (Life Technologies) or on a BioMark 48.48 dynamic array nanofluidic chip (Fluidigm, Inc) according to manufacturers’ instructions. Beta actin was used for normalization as endogenous control.

Single cell RNA Sequencing and Analysis

scRNAseq was performed using the Chromium Single Cell 3' Reagent Version 2 Kit from 10X Genomics. 45,000 (FOP) and 30,000 (control) HNK1⁺CD45⁺CD31⁺CXCR4⁺CD29⁺CD56<sup>dim</sup> cells isolated from iMPC differentiations were analyzed, using the sequencing procedures and analytical strategies described in Supplemental Experimental Procedures.
**Statistical analysis**

The data were analyzed utilizing GraphPad Prism v.7 software (GraphPad) using one-way (transplant) and two-way ANOVA with post hoc Tukey’s or Sidak’s multiple comparison test (gene expression). The Sidak test was used when comparing means between control and FOP, and the Tukey test was used when means of both control and FOP were compared together with other groups for the gene expression data. For the transplantation studies, at least 3 mice were used per group. At least three biological replicates were performed for each experiment unless indicated otherwise. All error bars are depicted as standard deviation, p-values are (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001).

**Human Specimen Procurement**

Human samples were collected through the UCSF Biospecimens and Skeletal Tissues for Rare and Orphan Disease Genetics (BSTROnG) Biobank, using protocols approved by the UCSF Institutional Review Board. All participants provided written consent. For the transplantation studies (Figure 6D-H and Figure 6-figure supplement 1), biopsy of the control subject was obtained from a 44 yo female healthy individual undergoing surgery at UCSF, the muscle from the FOP patient was obtained at autopsy from a 55 yo. Written informed consent was obtain from all subjects or their families.

**Acknowledgments:**

The authors thank Kelly Wentworth and Samuel Kou for their assistance collecting the autopsy samples and Francesco Tedesco for his support on the AFM grant. This work was supported by a NIH/NIAMS R01AR066735 to ECH, a French Muscular Association (AFM-Telethon) Trampoline grant to ECH and EB, the Radiant Hope Foundation to ECH, and the UCSF Cohort.
Development Grant to ECH; the California Institute for Regenerative Medicine Fellowship Program to UCSF (TG2-01153) to EB, and the UCSF Program for Breakthrough Biomedical Research (PBBR) to EB; and the NIH R01AR072638-03 to JHP. Finally, the authors would like to thank the patients and their families for their generous specimen donations.

Author contributions:

Conceptualization, ECH, JHP, EB; Methodology, EB; Software Analyses, EB, TM; Validation, EB, SMG; Formal Analysis, EB; Investigation, EB, SMG, JW, BMM, ST; Resources, ECH, JHP; Data Curation, EB, TM; Writing – Original Draft, EB, ECH; Writing – Review and Editing, EB, ECH, JHP; Visualization, EB; Supervision, ECH, JHP; Project Administration, ECH, EB; Funding, ECH, EB, JHP; clinical samples, ECH, JHP.

Competing interests:

ECH receives clinical trial research funding from Clementia Pharmaceuticals, an Ipsen company, and Neurocrine Biosciences, Inc., through his institution. ECH received prior funding from Regeneron Pharmaceuticals, through his institution. ECH serves in an unpaid capacity on the international FOP Association Medical Registry Advisory Board, on the International Clinical Council on FOP, and on the Fibrous Dysplasia Foundation Medical Advisory Board. These activities pose no conflicts for the presented research.

Data and materials availability:

Single cell gene expression data have been deposited (GSE151918). The dataset used for the primary Hu-MuSCs can be found here,
https://datadryad.org/stash/landing/show?id=doi%3A10.7272%2FQ65X273X. Detailed scripts can be found here, https://github.com/EmilieB12/FOP_muscle/tree/main.
REFERENCES

Al Tanoury, Z., Rao, J., Tassy, O., Gobert, B., Gapon, S., Garnier, J. M., . . . Pourquie, O. (2020). Differentiation of the human PAX7-positive myogenic precursors/satellite cell lineage in vitro. Development, 147(12). doi:10.1242/dev.187344

Andersen, D. C., Laborda, J., Baladron, V., Kassem, M., Sheikh, S. P., & Jensen, C. H. (2013). Dual role of delta-like 1 homolog (DLK1) in skeletal muscle development and adult muscle regeneration. Development, 140(18), 3743-3753. doi:10.1242/dev.095810

Barruet, E., Garcia, S. M., Striedinger, K., Wu, J., Lee, S., Byrnes, L., . . . Pomerantz, J. H. (2020). Functionally heterogeneous human satellite cells identified by single cell RNA sequencing. Elife, 9. doi:10.7554/eLife.51576

Barruet, E., Morales, B. M., Cain, C. J., Ton, A. N., Wentworth, K. L., Chan, T. V., . . . Hsiao, E. C. (2018). NF-kappaB/MAPK activation underlies ACVR1-mediated inflammation in human heterotopic ossification. JCI Insight, 3(22). doi:10.1172/jci.insight.122958

Barruet, E., Morales, B. M., Lwin, W., White, M. P., Theodoris, C. V., Kim, H., . . . Hsiao, E. C. (2016). The ACVR1 R206H mutation found in fibrodysplasia ossificans progressiva increases human induced pluripotent stem cell-derived endothelial cell formation and collagen production through BMP-mediated SMAD1/5/8 signaling. Stem Cell Res Ther, 7(1), 115. doi:10.1186/s13287-016-0372-6

Becht, E., McInnes, L., Healy, J., Dutertre, C. A., Kwok, I. W. H., Ng, L. G., . . . Newell, E. W. (2018). Dimensionality reduction for visualizing single-cell data using UMAP. Nat Biotechnol. doi:10.1038/nbt.4314

Bigot, A., Duddy, W. J., Ouandaogo, Z. G., Negroni, E., Mariot, V., Ghimbovschi, S., . . . Duguez, S. (2015). Age-Associated Methylation Suppresses SPRY1, Leading to a Failure of Re-
quiescence and Loss of the Reserve Stem Cell Pool in Elderly Muscle. *Cell Rep, 13*(6), 1172-1182. doi:10.1016/j.celrep.2015.09.067

Billings, P. C., Fiori, J. L., Bentwood, J. L., O'Connell, M. P., Jiao, X., Nussbaum, B., . . . Kaplan, F. S. (2008). Dysregulated BMP signaling and enhanced osteogenic differentiation of connective tissue progenitor cells from patients with fibrodysplasia ossificans progressiva (FOP). *J Bone Miner Res, 23*(3), 305-313. doi:10.1359/jbmr.071030

Borchin, B., Chen, J., & Barberi, T. (2013). Derivation and FACS-mediated purification of PAX3+/PAX7+ skeletal muscle precursors from human pluripotent stem cells. *Stem Cell Reports, 1*(6), 620-631. doi:10.1016/j.stemcr.2013.10.007

Chal, J., Al Tanoury, Z., Hestin, M., Gobert, B., Aivio, S., Hick, A., . . . Pourquie, O. (2016). Generation of human muscle fibers and satellite-like cells from human pluripotent stem cells in vitro. *Nat Protoc, 11*(10), 1833-1850. doi:10.1038/nprot.2016.110

Choi, I. Y., Lim, H., Estrellas, K., Mula, J., Cohen, T. V., Zhang, Y., . . . Lee, G. (2016). Concordant but Varied Phenotypes among Duchenne Muscular Dystrophy Patient-Specific Myoblasts Derived using a Human iPSC-Based Model. *Cell Rep, 15*(10), 2301-2312. doi:10.1016/j.celrep.2016.05.016

Convente, M. R., Chakkalakal, S. A., Yang, E., Caron, R. J., Zhang, D., Kambayashi, T., . . . Shore, E. M. (2018). Depletion of Mast Cells and Macrophages Impairs Heterotopic Ossification in an Acvr1(R206H) Mouse Model of Fibrodysplasia Ossificans Progressiva. *J Bone Miner Res, 33*(2), 269-282. doi:10.1002/jbmr.3304

Cosgrove, B. D., Gilbert, P. M., Porpiglia, E., Mourkioti, F., Lee, S. P., Corbel, S. Y., . . . Blau, H. M. (2014). Rejuvenation of the muscle stem cell population restores strength to injured aged muscles. *Nat Med, 20*(3), 255-264. doi:10.1038/nm.3464

Culbert, A. L., Chakkalakal, S. A., Theosmy, E. G., Brennan, T. A., Kaplan, F. S., & Shore, E. M. (2014). Alk2 regulates early chondrogenic fate in fibrodysplasia ossificans progressiva
heterotopic endochondral ossification. Stem Cells, 32(5), 1289-1300. doi:10.1002/stem.1633

Dey, D., Bagarova, J., Hatsell, S. J., Armstrong, K. A., Huang, L., Ermann, J., . . . Yu, P. B. (2016). Two tissue-resident progenitor lineages drive distinct phenotypes of heterotopic ossification. Sci Transl Med, 8(366), 366ra163. doi:10.1126/scitranslmed.aaf1090

Elsafadi, M., Manikandan, M., Dawud, R. A., Alajez, N. M., Hamam, R., Alfayez, M., . . . Mahmood, A. (2016). Transgelin is a TGFbeta-inducible gene that regulates osteoblastic and adipogenic differentiation of human skeletal stem cells through actin cytoskeleton organization. Cell Death Dis, 7(8), e2321. doi:10.1038/cddis.2016.196

Friedrichs, M., Wirsdoerfer, F., Flohe, S. B., Schneider, S., Wuelling, M., & Vortkamp, A. (2011). BMP signaling balances proliferation and differentiation of muscle satellite cell descendants. BMC Cell Biol, 12, 26. doi:10.1186/1471-2121-12-26

Furrer, R., & Handschin, C. (2017). Optimized Engagement of Macrophages and Satellite Cells in the Repair and Regeneration of Exercised Muscle. In B. Spiegelman (Ed.), Hormones, Metabolism and the Benefits of Exercise (pp. 57-66). Chamcham.

Garcia, S. M., Tamaki, S., Lee, S., Wong, A., Jose, A., Dreux, J., . . . Pomerantz, J. H. (2018). High-Yield Purification, Preservation, and Serial Transplantation of Human Satellite Cells. Stem Cell Reports, 10(3), 1160-1174. doi:10.1016/j.stemcr.2018.01.022

Garcia, S. M., Tamaki, S., Xu, X., & Pomerantz, J. H. (2017). Human Satellite Cell Isolation and Xenotransplantation. Methods Mol Biol, 1668, 105-123. doi:10.1007/978-1-4939-7283-8_8

Goel, A. J., Rieder, M. K., Arnold, H. H., Radice, G. L., & Krauss, R. S. (2017). Niche Cadherins Control the Quiescence-to-Activation Transition in Muscle Stem Cells. Cell Rep, 21(8), 2236-2250. doi:10.1016/j.celrep.2017.10.102
Hatsell, S. J., Idone, V., Wolken, D. M., Huang, L., Kim, H. J., Wang, L., . . . Economides, A. N. (2015). ACVR1R206H receptor mutation causes fibrodysplasia ossificans progressiva by imparting responsiveness to activin A. *Sci Transl Med*, 7(303), 303ra137. doi:10.1126/scitranslmed.aac4358

Hicks, M. R., Hiserodt, J., Paras, K., Fujiwara, W., Eskin, A., Jan, M., . . . Pyle, A. D. (2018). ERBB3 and NGFR mark a distinct skeletal muscle progenitor cell in human development and hPSCs. *Nat Cell Biol*, 20(1), 46-57. doi:10.1038/s41556-017-0010-2

Kuang, S., Kuroda, K., Le Grand, F., & Rudnicki, M. A. (2007). Asymmetric self-renewal and commitment of satellite stem cells in muscle. *Cell*, 129(5), 999-1010. doi:10.1016/j.cell.2007.03.044

Lees-Shepard, J. B., Yamamoto, M., Biswas, A. A., Stoessel, S. J., Nicholas, S. E., Cogswell, C. A., . . . Goldhamer, D. J. (2018). Activin-dependent signaling in fibro/adipogenic progenitors causes fibrodysplasia ossificans progressiva. *Nat Commun*, 9(1), 471. doi:10.1038/s41467-018-02872-2

Magli, A., Incitti, T., Kiley, J., Swanson, S. A., Darabi, R., Rinaldi, F., . . . Perlingiero, R. C. R. (2017). PAX7 Targets, CD54, Integrin alpha9beta1, and SDC2, Allow Isolation of Human ESC/iPSC-Derived Myogenic Progenitors. *Cell Rep*, 19(13), 2867-2877. doi:10.1016/j.celrep.2017.06.005

Matsumoto, Y., Hayashi, Y., Schlieve, C. R., Ikeya, M., Kim, H., Nguyen, T. D., . . . Hsiao, E. C. (2013). Induced pluripotent stem cells from patients with human fibrodysplasia ossificans progressiva show increased mineralization and cartilage formation. *Orphanet J Rare Dis*, 8, 190. doi:10.1186/1750-1172-8-190

Mauro, A. (1961). Satellite cell of skeletal muscle fibers. *J Biophys Biochem Cytol*, 9, 493-495. doi:10.1083/jcb.9.2.493
McMahon, A. P., & Bradley, A. (1990). The Wnt-1 (int-1) proto-oncogene is required for development of a large region of the mouse brain. *Cell, 62*(6), 1073-1085.

Nastase, M. V., Young, M. F., & Schaefer, L. (2012). Biglycan: a multivalent proteoglycan providing structure and signals. *J Histochem Cytochem, 60*(12), 963-975. doi:10.1369/0022155412456380

Nikitovic, D., Papoutsidakis, A., Karamanos, N. K., & Tzanakakis, G. N. (2014). Lumican affects tumor cell functions, tumor-ECM interactions, angiogenesis and inflammatory response. *Matrix Biol, 35*, 206-214. doi:10.1016/j.matbio.2013.09.003

Ono, Y., Calhabeu, F., Morgan, J. E., Katagiri, T., Amthor, H., & Zammit, P. S. (2011). BMP signalling permits population expansion by preventing premature myogenic differentiation in muscle satellite cells. *Cell Death Differ, 18*(2), 222-234. doi:10.1038/cdd.2010.95

Regev, A., Teichmann, S. A., Lander, E. S., Amit, I., Benoist, C., Birney, E., . . . Human Cell Atlas Meeting, P. (2017). The Human Cell Atlas. *Elife, 6*. doi:10.7554/eLife.27041

Satija, R., Farrell, J. A., Gennert, D., Schier, A. F., & Regev, A. (2015). Spatial reconstruction of single-cell gene expression data. *Nat Biotechnol, 33*(5), 495-502. doi:10.1038/nbt.3192

Schepers, K., Hsiao, E. C., Garg, T., Scott, M. J., & Passegue, E. (2012). Activated Gs signaling in osteoblastic cells alters the hematopoietic stem cell niche in mice. *Blood, 120*(17), 3425-3435. doi:10.1182/blood-2011-11-395418

Seale, P., Sabourin, L. A., Girgis-Gabardo, A., Mansouri, A., Gruss, P., & Rudnicki, M. A. (2000). Pax7 is required for the specification of myogenic satellite cells. *Cell, 102*(6), 777-786.

Segales, J., Perdiguer, E., & Munoz-Canoves, P. (2016). Regulation of Muscle Stem Cell Functions: A Focus on the p38 MAPK Signaling Pathway. *Front Cell Dev Biol, 4*, 91. doi:10.3389/fcell.2016.00091
Shea, K. L., Xiang, W., LaPorta, V. S., Licht, J. D., Keller, C., Basson, M. A., & Brack, A. S. (2010). Sprouty1 regulates reversible quiescence of a self-renewing adult muscle stem cell pool during regeneration. *Cell Stem Cell, 6*(2), 117-129. doi:10.1016/j.stem.2009.12.015

Shelton, M., Metz, J., Liu, J., Carpenedo, R. L., Demers, S. P., Stanford, W. L., & Skerjanc, I. S. (2014). Derivation and expansion of PAX7-positive muscle progenitors from human and mouse embryonic stem cells. *Stem Cell Reports, 3*(3), 516-529. doi:10.1016/j.stemcr.2014.07.001

Shi, S., de Gorter, D. J., Hoogaars, W. M., t Hoen, P. A., & ten Dijke, P. (2013). Overactive bone morphogenetic protein signaling in heterotopic ossification and Duchenne muscular dystrophy. *Cell Mol Life Sci, 70*(3), 407-423. doi:10.1007/s00018-012-1054-x

Shore, E. M. (2012). Fibrodysplasia ossificans progressiva: a human genetic disorder of extraskeletal bone formation, or--how does one tissue become another? *Wiley Interdiscip Rev Dev Biol, 1*(1), 153-165. doi:10.1002/wdev.9

Shore, E. M., Xu, M., Feldman, G. J., Fenstermacher, D. A., Cho, T. J., Choi, I. H., . . . Kaplan, F. S. (2006). A recurrent mutation in the BMP type I receptor ACVR1 causes inherited and sporadic fibrodysplasia ossificans progressiva. *Nat Genet, 38*(5), 525-527. doi:10.1038/ng1783

Shultz, L. D., Lyons, B. L., Burzenski, L. M., Gott, B., Chen, X., Chaleff, S., . . . Handgretinger, R. (2005). Human lymphoid and myeloid cell development in NOD/LtSz-scid IL2R gamma null mice engrafted with mobilized human hemopoietic stem cells. *J Immunol, 174*(10), 6477-6489. doi:10.4049/jimmunol.174.10.6477

Soriano-Arroquia, A., McCormick, R., Molloy, A. P., Mc Ardle, A., & Goljanek-Whysall, K. (2016). Age-related changes in miR-143-3p:lgfbp5 interactions affect muscle regeneration. *Aging Cell, 15*(2), 361-369. doi:10.1111/acel.12442
Spencer, C. I., Baba, S., Nakamura, K., Hua, E. A., Sears, M. A., Fu, C. C., . . . Conklin, B. R. (2014). Calcium transients closely reflect prolonged action potentials in iPSC models of inherited cardiac arrhythmia. Stem Cell Reports, 3(2), 269-281. doi:10.1016/j.stemcr.2014.06.003

Stanley, A., Heo, S. J., Mauck, R. L., Mourkioti, F., & Shore, E. M. (2019). Elevated BMP and Mechanical Signaling Through YAP1/RhoA Poises FOP Mesenchymal Progenitors for Osteogenesis. J Bone Miner Res, 34(10), 1894-1909. doi:10.1002/jbmr.3760

Stantzou, A., Schirwis, E., Swist, S., Alonso-Martin, S., Polydorou, I., Zarrouki, F., . . . Amthor, H. (2017). BMP signaling regulates satellite cell-dependent postnatal muscle growth. Development, 144(15), 2737-2747. doi:10.1242/dev.144089

Stuart, T., Butler, A., Hoffman, P., Hafemeister, C., Papalexi, E., Mauck, W. M., 3rd, . . . Satija, R. (2019). Comprehensive Integration of Single-Cell Data. Cell, 177(7), 1888-1902 e1821. doi:10.1016/j.cell.2019.05.031

Takahashi, K., Tanabe, K., Ohnuki, M., Narita, M., Ichisaka, T., Tomoda, K., & Yamanaka, S. (2007). Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell, 131(5), 861-872. doi:10.1016/j.cell.2007.11.019

Team, R. C. (2014). R: A language and environment for statistical computing.

Trapnell, C., Cacchiarelli, D., Grimsby, J., Pokharel, P., Li, S., Morse, M., . . . Rinn, J. L. (2014). The dynamics and regulators of cell fate decisions are revealed by pseudotemporal ordering of single cells. Nat Biotechnol, 32(4), 381-386. doi:10.1038/nbt.2859

Urist, M. R. (1965). Bone: formation by autoinduction. Science, 150(3698), 893-899. doi:10.1126/science.150.3698.893

van der Wal, E., Herrero-Hernandez, P., Wan, R., Broeders, M., In ’t Groen, S. L. M., van Gestel, T. J. M., . . . Pijnappel, W. (2018). Large-Scale Expansion of Human iPSC-Derived Stem Cell Models of Inherited Arrhythmia. Stem Cell Reports, 11(4), 335-349. doi:10.1016/j.stemcr.2018.03.003
Skeletal Muscle Cells for Disease Modeling and Cell-Based Therapeutic Strategies. Stem Cell Reports, 10(6), 1975-1990. doi:10.1016/j.stemcr.2018.04.002

Volpato, V., & Webber, C. (2020). Addressing variability in iPSC-derived models of human disease: guidelines to promote reproducibility. Dis Model Mech, 13(1). doi:10.1242/dmm.042317

Wu, J., Matthias, N., Lo, J., Ortiz-Vitali, J. L., Shieh, A. W., Wang, S. H., & Darabi, R. (2018). A Myogenic Double-Reporter Human Pluripotent Stem Cell Line Allows Prospective Isolation of Skeletal Muscle Progenitors. Cell Rep, 25(7), 1966-1981 e1964. doi:10.1016/j.celrep.2018.10.067

Xi, H., Fujiwara, W., Gonzalez, K., Jan, M., Liebscher, S., Van Handel, B., . . . Pyle, A. D. (2017). In Vivo Human Somitogenesis Guides Somite Development from hPSCs. Cell Rep, 18(6), 1573-1585. doi:10.1016/j.celrep.2017.01.040

Xi, H., Langerman, J., Sabri, S., Chien, P., Young, C. S., Younesi, S., . . . Pyle, A. D. (2020). A Human Skeletal Muscle Atlas Identifies the Trajectories of Stem and Progenitor Cells across Development and from Human Pluripotent Stem Cells. Cell Stem Cell. doi:10.1016/j.stem.2020.04.017

Xu, X., Wilschut, K. J., Kouklis, G., Tian, H., Hesse, R., Garland, C., . . . Pomerantz, J. H. (2015). Human Satellite Cell Transplantation and Regeneration from Diverse Skeletal Muscles. Stem Cell Reports, 5(3), 419-434. doi:10.1016/j.stemcr.2015.07.016

Zheng, G. X., Terry, J. M., Belgrader, P., Ryvkin, P., Bent, Z. W., Wilson, R., . . . Bielas, J. H. (2017). Massively parallel digital transcriptional profiling of single cells. Nat Commun, 8, 14049. doi:10.1038/ncomms14049
Abbreviations:

- PAX7: Paired Box 7
- hiPSCs: Human induced pluripotent stem cells
- BMP: bone morphogenetic protein
- ACVR1/2A: activin receptor type 1/2A
- MYOD1: Myogenic Differentiation 1
- HNK1: Human Natural Killer-1
- CXCR4: C-X-C chemokine receptor type 4
- MYF5: Myogenic Factor 5
- COL1A1: Collagen Type I Alpha 1 Chain
- PDGFRA: Platelet Derived Growth Factor Receptor Alpha
- SOX2: SRY-Box 2
- EFNB3: Ephrin B3
- MAP2: Microtubule Associated Protein 2
- ASCL1: Achaete-Scute Family BHLH Transcription Factor 1
- ASPN: Asporin
- APOE: Apolipoprotein E
- OGN: Osteoglycin
- TOP2A: DNA Topoisomerase II Alpha
- FABP7: Fatty Acid Binding Protein 7
- SPRY1: Sprouty 1
- MYL1: Myosin Light Chain 1
- DLK1: Delta Like Non-Canonical Notch Ligand 1
- ITM2A: Integral Membrane Protein 2A
- DCN: Decorin
SPARC: Secreted Protein Acidic and Cysteine Rich
TIMP1: Tissue Inhibitor of Metalloproteinase 1
BGN: Biglycan
LUM: Lumican
TAGLN: Transgelin
IGBP5: Insulin Like Growth Factor Binding 5
ID1/3: Inhibitor of DNA Binding 1/3
TGFb: Transforming Growth Factor b
BMPR2: Bone Morphogenetic Protein Receptor 2
CAV1: Caveolin 1
ECM: Extracellular Matrix

Supplementary Materials

Figure 1-figure supplement 1. Transplanted satellite cells from biceps and diaphragm of FOP patient do not form bone.
Figure 2-figure supplement 1. Early expression of muscle markers during the myogenic differentiation.
Figure 3-figure supplement 1. Differentiated cells types in the myogenic differentiation varies between cell lines, sorted cells express PAX7 and can differentiate into myotubes.
Figure 4-figure supplement 1. Markers expressed in sorted control and FOP HNK1-CD45-CD31-CXCR4+CD29+CD56dim cells.
Figure 5-figure supplement 1. Analysis of the myogenic sub-cluster.
Figure 6-figure supplement 1. Expression of myogenic markers across primary and hiPS-derived myogenic cells.

Figure 1-Source Data 1. Muscle specimen information.

Figure 1-Source Data 2. Hu-MuSCs transplantation details.

Figure 3-Source Data 1: hiPSC-derived HNK1−CD45−CD31− CXCR4+CD29+CD56dim cell transplants

Figure 4-Source Data 1: Quality control information for each sample.

Figure 5-Source Data 1. Differential expression analysis of control and FOP myogenic cells.

Figure 6-Source Data 1. BEAM analysis.

Video 1. Myotube contraction of differentiated Control (1323-2) hiPSCs.

Video 2. Myotube contraction of differentiated FOP (F1-1) hiPSCs.

Data File S1- hiPSCs samples integration.

Data File S2- Myogenic sub-clustering.

Data File S3- Pseudotime analyses.

Data File S4- Primary Hu-MuSCs/hiPSCs integration.
Figure 1-figure supplement 1. Transplanted satellite cells from biceps and diaphragm of FOP patient do not form bone. (A) Flow cytometry analysis sorting strategy to isolate human satellite cells. (B) Representative immunostaining of human satellite cells. White arrows indicate PAX7+ satellite cells (scale bar, 50 µm). (C) X-ray of transplanted mice at 5 and 10 weeks after initial transplantation (scale bars, 4mm (week 5), 7mm (week 10)) showing no heterotopic ossification. Details about muscle specimens used in this figure are in Figure 2-Source Data1.
Figure 2-figure supplement 1. Early expression of muscle markers during the myogenic differentiation. (A) MYOGENIN (scale bar, 200 µm) and (B) PAX7 immunofluorescence staining showing that MYOGENIN and PAX7 are expressed as early as day 25 during the myogenic differentiation in control and BMP impaired hiPSCs (scale bar, 200 µm). (C) DYSTROPHIN gene expression at day 25 and day 50 of differentiation (n=3 biological replicates and n≥3 technical replicates). Error bar represent mean and SD.
Figure 3-figure supplement 1. Differentiated cell types in the myogenic differentiation varies between cell lines, sorted cells express PAX7 and can differentiate into myotubes. Related to Figure 3. (A) Representative flow cytometry profiles of negative (CD45 and CD31) and positive (CD29 and CD56) markers used to purify muscle stem cells. (B) Quantification of the percentage of cells positive for CD45, CD31, CD29, and CD56 at day 20. (n=3 biological replicates and n=3 technical replicates). Error bar represent mean and SD. (C) FACS analysis of the myogenic differentiation at day 50. Muscle stem cells stained for PAX7, CD29, and CD56. (D) Representative immunofluorescence staining for MHC showing sorted muscle stem cells after being cultured in differentiation media for 7 days (scale bar, 200µm).
Figure 4-figure supplement 1. Markers expressed in sorted control and FOP HNK1-CD45-CD31-816 CXCR4+CD29+CD56dim cells. (A-B) Feature expression plots showing the localization of cells expressing myogenic markers (PAX7, MYF5) mesenchymal marker (PDGFRA) and neuronal progenitor marker (SOX2) in the control (A) and FOP (B) sorted cells. (C) UMAP visualization plots of merged samples with cells colored by samples. (D) Feature expression plots showing the localization of cells expressing myogenic (MYOG), mesenchymal (ASPN) and neuronal markers (MAP2, ASCL1) in the merged samples. (E) Heat map of the top 5 differentially expressed genes in each cluster of the merged sample analysis.
Figure 5-figure supplement 1. Analysis of the myogenic sub-cluster. (A) Heat map of the top 5 differentially expressed genes in each cluster. (B) Cycle genes were scored for each cluster. Bar plot depicting the proportion of cells in G1/G0, G2/M and S phase for each cluster. (C) Proportion of cells in G1/G0, G2/M and S phase for each sample. (D) Proportion bar graph of cells per cluster for the control and FOP samples. (E) UMAP showing the distribution of the two merged samples. (F) Additional genes significantly differentially expressed by more than 1-fold. (G) Dot plot displaying the expression of genes associated with BMP and P38MAPK pathways for each cluster.
Figure 6-figure supplement 1. Expression of myogenic markers across primary and hiPS-derived myogenic cells. Dot plot displaying the average expression and the percent of cells expressing myogenic genes across clusters for the 3 merged samples.
**Figure 1-Source Data 1. Muscle specimen information.**

| Figures              | Muscle          | Age | Sex | Subjects |
|----------------------|-----------------|-----|-----|----------|
| Figure 1A            | Psoas           | 52  | F   | FOP      |
| Figure 1A            | Intercostal     | 46  | F   | FOP      |
| Figure 1B (top)      | Vastus Lateralis| 59  | M   | Control  |
| Figure 1B (middle)   | Biceps Brachii  | 21  | F   | FOP      |
| Figure 1B (bottom)   | Biceps Brachii  | 32  | F   | FOP      |
| Figure 1C-H and S1   | Biceps Brachii  | 44  | F   | Control  |
| Figure 1C-H and S1   | Biceps Brachii  | 32  | F   | FOP      |
| Figure 1C-H and S1   | Diaphragm       | 32  | F   | FOP      |

**Figure 1-Source Data 2. Hu-MuSCs transplantation details.**

| Week 5 |
|------------------|-----------------|-----------------|-----------------|
| Satellite Cells isolated from | # of cells transplanted/mouse | Human Dystrophin fibers | Human PAX7+ cells |
| Ctrl Biceps      | 6000            | 38              | 4               |
| Ctrl Biceps      | 6000            | 30              | 7               |
| Ctrl Biceps      | 6000            | 20              | 1               |
| FOP Diaphragm    | 5000            | 13              | 1               |
| FOP Diaphragm    | 5000            | 8               | 1               |
| FOP Diaphragm    | 5000            | 6               | 0               |
| FOP Biceps       | 2800            | 5               | 3               |
| FOP Biceps       | 2800            | 5               | 2               |
| FOP Biceps       | 2800            | 0               | 2               |

**Week10**

| Ctrl Biceps      | 6000            | 133             | 24              |
| Ctrl Biceps      | 6000            | 112             | 13              |
| Ctrl Biceps      | 6000            | 82              | 19              |
| FOP Diaphragm    | 5000            | 98              | 23              |
| FOP Diaphragm    | 5000            | 120             | 22              |
| FOP Diaphragm    | 5000            | 57              | 10              |
| FOP Diaphragm    | 5000            | 42              | 12              |
| FOP Biceps       | 2800            | 0               | 0               |
| FOP Biceps       | 2800            | 58              | 15              |
| FOP Biceps       | 2800            | 12              | 5               |
Figure 3-Source Data 1: hiPSC-derived HNK1-CD45-CD31- CXCR4+CD29+CD56dim cell transplants.

| Cell line | # of cells transplanted | Human Dystrophin fibers | Human PAX7+ cells |
|-----------|-------------------------|-------------------------|------------------|
| WTC11     | 8000                    | 6                       | 0                |
| WTC11     | 8261                    | 0                       | 0                |
| WTC11     | 12341                   | 0                       | 0                |
| 1323-2    | 8011                    | 0                       | 0                |
| 1323-2    | 7873                    | 0                       | 0                |
| BJ2       | 15000                   | 2                       | 0                |
| BJ2       | 887                     | 0                       | 0                |
| BJ2       | 9767                    | 1                       | 0                |
| F1-1      | 5508                    | 0                       | 0                |
| F1-1      | 6084                    | 0                       | 0                |
| F1-1      | 15221                   | 0                       | 0                |
| F2-3      | 17638                   | 15                      | 2                |
| F3-2      | 30000                   | 51                      | 0                |
| F3-2      | 1300                    | 0                       | 0                |
| F3-2      | 40000                   | 10                      | 0                |

Figure 4-Source Data 1: Quality control information for each sample.

|                                | Control (1323-2) | FOP (F3-2) |
|--------------------------------|------------------|------------|
| Estimated Number of Cells      | 2,054            | 5,306      |
| Mean Reads per Cell            | 76,178           | 32,870     |
| Median Genes per Cell          | 2,689            | 2,501      |
| Number of Reads                | 156,470,954      | 174,413,305|
| Valid Barcodes                 | 97.8%            | 98.0%      |
| Reads Mapped Confidently to Transcriptome | 63.1%   | 66.3%      |
| Reads Mapped Confidently to Exonic Regions | 67.0%   | 70.3%      |
| Reads Mapped Confidently to Intronic Regions | 17.4%   | 15.3%      |
| Reads Mapped Confidently to Intergenic Regions | 3.6%     | 3.4%       |
| Reads Mapped to Antisense to Gene | 4.7%     | 4.9%       |
| Sequencing Saturation          | 79.0%            | 54.8%      |
| Fraction Reads in Cells        | 21,100           | 22,238     |
SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Flow cytometry

Cells were treated with Accutase for 20 min at 37°C, washed with FACS buffer, and stained for HNK1-PE, CD45-PE, CD31-PE, CD56-APC, CXCR4-PE-Cy7, and CD29-FITC (eBioscience). HNK1−CD45−CD31− cells [to select against neuronal cells (HNK1, Human Natural Killer-1) (Choi et al., 2016; Hicks et al., 2018), hematopoietic cells (CD45), and endothelial cells (CD31)], co-expressing CD29, CXCR4, and intermediate CD56, markers present on human PAX7+ cells (Garcia et al., 2018; Garcia et al., 2017; Xu et al., 2015) were sorted with a FACS Aria III (BD Biosciences) and Sytox Blue (Life Technologies) was used as a viability marker. Alternatively, cells were permeabilized and fixed (Fix/Perm Buffer Set, BioLegend). Fixed cells were first incubated with primary antibodies (PAX7 (DSHB), CD29 (BD Biosciences) and CD56 (BD Biosciences) following by secondary antibodies (Alexa350-conjugated goat anti-mouse IgG, Alexa488-conjugated goat anti-rat IgG and Alexa546-conjugated donkey anti-goat IgG, Life Technologies).

Cell Immunostaining and NSG Tibialis Anterior Analysis
Collected tibialis anterior frozen cross sections were fixed in 4% PFA for 10 min at room temperature, washed with PBST (PBS with 0.1% Tween-20 (Sigma-Aldrich) and blocked in PBS with 10% goat serum for 1hr at room temperature. Slides were then incubated 4hrs at room temperature with the following: mouse monoclonal anti-human DYSTROPHIN (DSHB), mouse monoclonal IgG1 anti-PAX7 (DSHB), rabbit polyclonal anti-Laminin (Sigma-Aldrich), mouse monoclonal IgG2 anti-human SPECTRIN (Leica Microsystems), and mouse monoclonal IgG2b anti-human LAMIN A/C (Vector Laboratories). After PBST wash, slides were incubated with the following secondary antibodies: Alexa Fluor 555 goat anti-mouse IgG, Alexa Fluor 594 goat anti-mouse IgG1, Alexa Fluor 488 goat anti-mouse IgG2b, and Alexa Fluor 647 goat anti-rabbit (Life Technologies). Finally, sections were mounted with VECTASHIELD mounting media with DAPI (Vector Laboratories). All samples were examined using a Leica upright or DMi8 Leica microscope. Sections with the most human fibers were used for human DYSTROPHIN and PAX7 quantification for each condition.

Single cell RNA Sequencing and Analysis

45,000 (FOP) and 30,000 (control) HNK1–CD45–CD31–CXCR4+CD29+CD56<sup>dim</sup> cells isolated from the iMPC differentiations were loaded onto one well of a 10X chip to produce Gel Bead-in-Emulsions (GEMs). GEMs underwent reverse transcription to barcode RNA before cleanup and cDNA amplification. Libraries were prepared with the Chromium Single Cell 3' Reagent Version 2 Kit. Each sample was sequenced on 1 lane of the NovaSeq 6000 S4. Sequencing reads were processed with Cell Ranger version 2.0.0. using the human reference transcriptome GRCh38. The estimated number of cells, mean reads per cell, median genes per cells, median UMI (Unique Molecular Identifier) counts per cells as well as other quality control information are summarized in Figure 4-Source Data 1. Gene-barcoded matrices were analyzed with the R package Seurat v3.1.5 (Satija, Farrell, Gennert, Schier, & Regev, 2015; Stuart et al., 2019; Team, 2014; Zheng et
Gene core matrices from single cell RNA sequencing of primary human satellite cells isolated from a vastus muscle (Barruet et al., 2020) was used when comparing the transcriptional profile of hiPS-derived HNK1^CD45^-CD31^-CXCR4^+CD29^+CD56^{dim} cells. For the comparison with primary Hu-MuSCs, hiPSC-derived cell sequencing reads were re-aligned using the human reference transcriptome hg19. Cells with fewer than 500 genes, greater than 5000 genes and genes expressed in fewer than 5 cells were not included in the downstream analyses. Cells with more than 10% mitochondrial counts were filtered out. Samples were normalized with NormalizeData using default settings. The FindVariableFeatures function was used to determine subset of feature that exhibit high cell-to-cell variation in each dataset based on a variance stabilizing transformation (“vst”). We used the default setting returning 2,000 feature per dataset. These were used for downstream analysis. In the case of the merged data analysis samples were combined utilizing the FindIntegrationAnchors function with the ‘dimensionality’ set at 30. Then, we ran these “anchors” to the IntegratData function for batch correction for all cells enabling them to be jointly analyzed. The resulting outputs were scaled mitochondrial contamination regressed out with the ScaleData function. In addition, while we didn’t regress out heterogeneity associated with cell cycle stage since it is an important factor in determining the state of quiescence of our sorted human muscle stem cells, we regressed out differences between G2/M and S cell cycle stage. PCA was performed with RunPCA, and significant PCs determined based on the Scree plot utilizing the function PCElbowPlot. The resolution parameter in FindClusters was adjusted to 0.5. Clusters were visualized by UMAP with Seurat’s RunUMAP function. We performed differential gene-expression utilizing Seurat v3’s FindMarkers function with default settings which utilizes the Wilcoxon rank-sum test to calculate adjusted p values for multiple comparisons. We used the CellCycleScoring function to assign score based on the expression of G2/M and S phase markers (Regev et al., 2017). Myogenic cells were further analyzed by sub-clustering using the subset function. We then use the FindNeighbors (dims=15) and FindClusters (resolution = 0.4) functions
on the myogenic cell subset of the merged hiPSCs samples only to identify sub-clusters corresponding to different myogenic states. To order the cells in pseudotime based on their transcriptional similarity we used Monocle 2.12. Variable genes from Seurat analysis were used as input and clusters were projected onto the minimum spanning tree after ordering. Gene expression patterns were plotted with `plot_genes_branched_heatmap`, `plot_genes_branched_pseudotime`, and `plot_multiple_branches_pseudotime`. The BEAM (branch expression analysis modeling) function was used to score gene significance in a branch-dependent manner. Cells were re-ordered using the `orderCells` function to set branch A (myocytes) in Figure 5F as the “root-state”. This allowed us to determine genes that were significantly branch dependent in branch B (mainly Hu-MuSCs) vs branch C (hiPS-derived cells) using the BEAM analysis.