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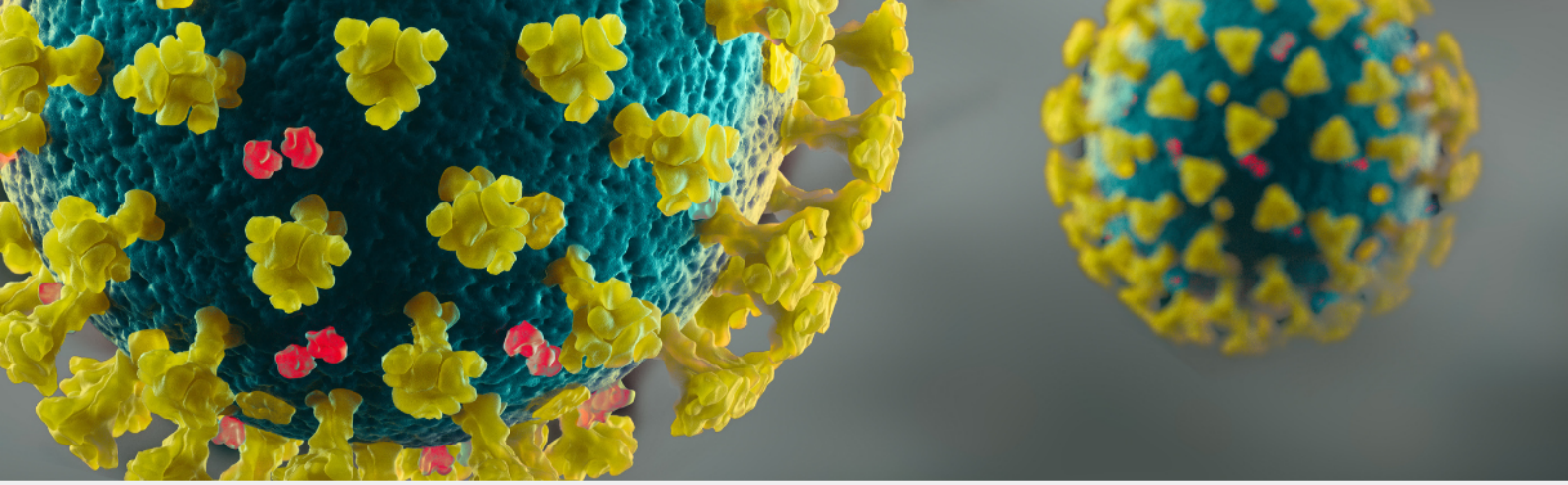

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METHODS

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Isolation of human fibroadipogenic progenitors and satellite cells from frozen muscle biopsies

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Abstract

Skeletal muscle contains multiple cell types that work together to maintain tissue homeostasis. Among these, satellite cells (SC) and fibroadipogenic progenitors cells (FAPs) are the two main stem cell pools. Studies of these cells using animal models have shown the importance of interactions between these cells in repair of healthy muscle, and degeneration of dystrophic muscle. Due to the unavailability of fresh patient muscle biopsies, similar analysis of interactions between human FAPs and SCs is limited especially among the muscular dystrophy patients. To address this issue here we describe a method that allows the use of frozen human skeletal muscle biopsies to simultaneously isolate and grow SCs and FAPs from healthy or dystrophic patients. We show that while the purified SCs differentiate into mature myotubes, purified FAPs can differentiate into adipocytes or fibroblasts demonstrating their multipotency. We find that these FAPs can be immortalized and the immortalized

Abbreviations: bFGF, basic fibroblast growth factor; CDK4, cyclin dependent kinase 4; DMD, Duchenne muscular dystrophy; DMEM, Dulbecco's modified eagle medium; DMSO, dimethyl sulfoxide; FABP4, fatty acid-binding protein 4; FACS, fluorescence-activated cell sorting; FAP, fibroadipogenic progenitors; FBS, fetal bovine serum; FMO, fluorescence minus one; hTERT, human telomerase reverse transcriptase; ICW, in-cell western; iDMD, immortalized DMD FAPs; iFAP, immortalized FAPs; IL-10, interleukin-10; IL-13, interleukin-13; IL-4, interleukin-4; IL-6, interleukin-6; M199, medium 199; MD, muscular dystrophy; MOI, multiplicity of infection; MyHC, myosin heavy chain; Nm, nanometer; OR, Oil-Red; PBS, phosphate buffered saline; PDGFRα, platelet derived growth factor receptor alpha; SC, satellite cell; SD, standard deviation; SEM, standard error of the mean; TGFβ, transforming growth factor β.

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FAPs (iFAPs) retain their multipotency. These approaches open the door for carrying out personalized analysis of patient FAPs and interactions with the SCs that lead to muscle loss.

KEYWORDS

Duchenne muscular dystrophy, fibroadipogenic progenitors cells, muscle explant muscle biopsies, satellite cells

1 | INTRODUCTION

Muscle regeneration and degeneration are complex processes that have been only partially characterized at the cellular level in human. A number of different cells in the muscle, including muscle-resident stem cells such as satellite cells (SC) and fibroadipogenic progenitors cells (FAPs), interact among them, with the extracellular matrix and with the lymphocytes and macrophages that infiltrate the muscle during injury.[1] The microscopic environment where all these interactions take place is commonly known as the cell niche, which is dynamic and contributes to both the regenerative and the degenerative process of injured muscles.[2,3] For example, after an acute muscle damage, muscle fibers release cytokines that promote muscle infiltration by inflammatory cells activating SC and FAPs, while inflammatory cells secrete interleukin-4 and interleukin-13 that promote FAP proliferation and maintain a pro-regenerative microenvironment that fosters SC differentiation through interleukin-6, follistatin, and interleukin-10.[1,4] This interaction between SCs, FAPs, and inflammatory cells is crucial for normal muscle regeneration after acute injury. During chronic muscle damage, as is the case of muscular dystrophies (MD), muscle fibers are progressively lost and there is a persistent infiltration of muscles by M2 macrophages which secrete transforming growth factor β (TGFβ) leading to a persistent accumulation of FAPs which differentiate into fibrocytes and adipocytes contributing to replacement of muscle fibers by fibrotic and fat tissue.[5] The description of the underlying cellular interactions between FAPs, SCs and inflammatory cells has been described using cells isolated from different mouse models.[6-9]

While isolation of human cells from muscle is feasible,[6,9,10] studying pathological cell interactions in dystrophic muscle is challenging mainly due to the limited access to fresh muscle biopsies from patients with genetically confirmed diagnosis. Muscle biopsies are typically performed as part of the diagnostic process of patients. Once obtained, muscle biopsies are flash-frozen in liquid nitrogen or at −80°C for structural analysis and protein quantification. This approach excludes the possibility of isolating live cells for cellular studies. However, under specific conditions it is possible to store muscle biopsies that allow isolation of live SCs.[11] This approach would potentially enable the isolation of other cell types apart of SCs such as FAPs from the same patient and therefore allowing the study of the cell interactions leading to a better understanding of the degenerative process on each muscle disease and on each individual condition. Thus, it can enhance the development of new treatments for patients with muscle diseases and facilitate testing personalized based-mechanism therapies targeting single or both cells and cellular interactome. Although FAPs have been isolated from fresh human muscles,[6,10] studies using FAPs from patients with a genetically confirmed muscular diseases are lacking because of the unavailability of fresh muscle tissue. Our study aims to address this issue by describing a new approach that allows the simultaneous isolation of functional SCs and FAPs from frozen human muscle biopsies and the generation of immortalized FAPS (iFAPs) which retain their multipotency and efficiently differentiate into fibrogenic and adipogenic lineages.

2 | METHODS

2.1 | Muscle samples

Frozen muscle biopsies from 15 healthy controls and from 1 Duchenne muscular dystrophy (DMD) patient were included in the study. Control samples were obtained during conventional surgeries in collaboration with the Orthopaedic Surgery and Plastic Surgery Departments at Hospital de la Santa Creu I Sant Pau (Barcelona) and patient samples were obtained as part of the diagnostic process. We included muscles of 5 men and 10 women with a mean age of 44 ± 19 years. Muscle fragments included in this study were frozen during 2.2 ± 2.7 years (Table 1). DMD muscle biopsy was obtained from 8-year patient with exon 18-44 deletion in the dystrophin gene. The DMD muscle biopsy was obtained from the right biceps which was not weak at clinical examination.

2.2 | Human skeletal muscle explants culture

Fresh muscle biopsies were washed and minced into 2-3 mm fragments in Hanks' balanced salt solution

TABLE 1 Frozen muscle samples from healthy controls included in this study

Sample	Gender	Age	Muscle	Years frozen	%FAP	%SC
1	F	35	Triceps	2	86	10
2	F	32	Triceps	2	99	0.1
3	M	12	Ticeps	1	56	34
4	F	57	Pectoralis	1	89	4
5	F	47	Pectoralis	1	87	5
6	F	45	Pectoralis	0.5	90	3
7	M	34	Gastrocnemius	2	75	15
8	F	65	Pectoralis	1 month	88	2
9	F	15	Vastus lateralis	2 months	56	27
10	F	54	Biceps	1 week	93	4
11	M	67	Quadriceps	10	90	10
12	M	54	Triceps	4	77	15
13	F	67	Vastus lateralis	3	99	0
14	M	20	Quadriceps	6	98	0
15	F	59	Pectoral	1	88	9

Note: Muscle explants were cultured in medium I and the proportion of FAP and Satellite cells (SC) cells were determined by platelet derived growth factor receptor alpha and CD56 using flow cytometry.

Abbreviations: FAP, fibroadipogenic progenitors; SC, satellite cells.

with 1% penicillin–streptomycin (PS) (Lonza, Basilea, Switzerland). Muscle fragments were placed in a dish with conditioned media containing M199 (Lonza), 35% fetal bovine serum (FBS) (ThermoFisher [Gibco], Waltham, MA), and 1% PS (Lonza) and incubated for 24 hours at 37℃ and 5% CO_2. Then, five to ten muscle fragments were placed in a cryotube containing 1 mL of freezing medium consisting of Dulbecco's modified eagle medium (DMEM), 10% FBS and 8% dimethyl Sulfoxide (DMSO).[11] These specimens were then put into cryofreezers at −80℃ and transferred to liquid nitrogen after 24 hours. Cryotubes were thawed at 37℃ and rapidly transferred to coated dishes with 1.5% gelatine plus an equal volume of human plasma. We added several drops of proliferation medium to each fragment and incubated it for 30 minutes at 37℃ and 5% CO_2 to increase the attachment of muscle fragments to the matrix. Then, proliferation medium was added until cover the muscle explants in the dish. We cultured muscle explants with two different proliferation media. The medium I consisted of three parts of DMEM-GlutaMAX (Gibco) and one part of M-199 (Lonza), with 10% FBS (Gibco), 25 ng/mL of basic fibroblast growth factor (bFGF), 10 ng/mL of epidermal growth factor (Peprotech, Rocky Hill, NJ), and 1% PS (Lonza). Medium II consisted of DMEM-GlutaMAX supplemented with 20% FBS, 1% PS and 2.5 ng/mL bFGF. After 5-7 days, cells started to sprout from muscle explants and when the number of sprouting cells was enough, approximately after 1 week but it is very variable, explants were removed and re-seeded if needed. The attached cells were trypsinized and expanded or resuspended in fluorescence-activated cell sorting (FACS) buffer (PBS with 2% FBS) for flow cytometry analysis.

2.3 | Flow cytometry and cell sorting

Cells from muscle explants were trypsinized, washed, and stained with the following antibodies for 30 minutes at 4℃: anti-PDGFRα-biotin at 2.5 μg/mL (Cat. BAF322, R&D Systems, Minneapolis, MN), CD56-FITC (Cat. 130-114-549), CD90-APC (Cat. 130-114-903), CD105-APC-Vio770 (Cat. 130-112-326), and CD166-PE-Vio770 (Cat. 130-106-620) (at the recommended dilutions, all from Miltenyi Biotec, Bergisch Gladbach, Germany). After washing steps, we incubated streptavidin PE-Cy5 (Cat. 405202; 1:200; Biolegend, San Diego, CA) for 30 minutes at 4℃. Stained cells were analyzed using a cytometer MACSQuant 10 (Miltenyi Biotec) or sorted on a FACSAria using FACSDiva software (Becton Dickinson, Ashland, OR). Doublet cells were excluded using forward scatter area and height and fluorescence minus one (FMO) controls were used to determine positivity. Compensations were adjusted according to the single stained controls. PDGFRα+/CD56− fraction was defined as FAPs and the PDGFRα−/CD56+ fraction as SC. FlowJo v10 software was used for data analysis and data elaboration (Becton Dickinson).

2.4 | In vitro differentiation assays

Purified FAPs were subcultured with the medium I and purified SC were subcultured with Skeletal Muscle Cell Growth Medium (PromoCell, Heidelberg, Germany). FAPs and SC were induced to fibrogenic differentiation culturing them for 3 days with DMEM-GlutaMAX with 10% FBS with or without 5 ng/mL TGFβ (R&D Systems). For adipogenic differentiation, FAPs and SC were cultured for 14 days with StemPro adipogenesis differentiation kit (Gibco). Myogenic differentiation was induced by culturing confluent cells with DMEM-GlutaMAX with 2% FBS for 9 days. At the indicated time points, cells were harvested for gene expression analysis and/or cell staining. SC from digestion were isolated using an enzymatic disgregation with 0.2% collagenase and Dispase II 2.4U/mL, expanded in vitro and sorted as described by CD56.

2.5 | Cell staining

We analyzed the expression of collagen I in the cell cultures exposed to fibrogenic differentiation by quantitative in-cell western (ICW) assays in microplates. Muscle differentiation of expanded SCs clones was also assessed by ICW by myosin heavy chain staining (MF-20). Wells were rinsed with PBS and fixed with cold 4% PFA for 10 minutes. After washing steps and blocking with casein solution for 30 minutes (ThermoFisher), we incubated goat anti-collagen I (1:200; Cat. 1310-01; Southern Biotech, Birmingham, AL) for 1 hour. After washing steps, we incubated for 1-hour donkey anti-goat IRDye800 or donkey anti-mouse IRDye800 secondary antibody (1:1000; Li-COR, Lincoln, NE) and CellTag IRDye 700 (Li-COR) following the manufacturer's instructions. After washing with PBS, the fluorescent signal of collagen I was measured in the 800 nm channel by an Odyssey Imaging system (Li-COR), and normalized by cell number measured in the 700 nm channel.

In adipogenic culture conditions, we analyzed the percentage of cells that differentiated to adipocyte by FABP4 immunofluorescence. After fixation and incubation with Ultracruz blocking solution (Santa Cruz Biotechnology, Dallas, TX), we incubated the primary antibodies goat anti-FABP4 (Cat. AF3150; 1:100; R&D systems) and rabbit anti-perilipin A/B (Cat. P1873-200UL; 1:100; Sigma-Aldrich, St. Louis, MO) for 1 hour. After washing steps, we incubated for 1 hour donkey anti-goat Alexa488 and donkey anti-rabbit Alexa594 (1:200; ThermoFisher). Oil Red staining was performed using the Lipid Staining kit (Promocell) following the manufacturer's protocols. When indicated, cells were stained with mouse anti-desmin (1:100; Leica, Wetzlar, Germany), goat anti-PDGFRα (Cat. AF1062; 1:100; R&D Systems), mouse anti-Pax7 (DSHB; 1:100), mouse anti-MyoD (Cat. M11-41017; 1:100; Invitrogen), mouse anti-myogenin (Cat. 556358; 1:100; BD) or with mouse anti-myosin heavy chain (MF-20; 1:100; R&D systems) followed by the incubation of the secondary antibodies donkey anti-mouse Alexa488 and donkey anti-goat Alexa594 as described above. Pictures of random fields and of replicates were obtained by using an Olympus BX51 microscope coupled to a DP72 camera. Quantitative analysis was performed using ImageJ software (National Institute of Health).[12]

2.6 | Real-time PCR

We performed gene expression assays using the Taqman Fast Advanced Cells-to-CT kit (ThermoFisher) following the manufacturer's instructions. Briefly, cells were washed with PBS and incubated with lysis solution for 5 minutes. Sample lysate was mixed with the RT Master mix and run the reaction to obtain cDNA. qPCR was performed by triplicate using the Fast Advanced Master Mix and the 7900 HT Fast Real-Time PCR System (ThermoFisher). The mRNA-specific probes were: COL1A1 (Hs00164004_m1), ADIPOQ (Hs00605917_m1), PAX7 (Hs00242962_m1), MYOD1 (Hs00159528_m1), MYOG (Hs01072232_m1), MYH1 (Hs00428600_m1), and GAPDH (Hs99999905_m1) (ThermoFisher). Relative quantification was performed using the comparative Ct method.

2.7 | FAP immortalization

Human FAPs were transduced with a retroviral vector containing a sequence encoding the catalytic subunit of human telomerase reverse transcriptase (hTERT) alone or with both hTERT and cyclin-dependent kinase 4 (Cdk4) as described previously.[13] cDNAs were cloned into different lentiviral vectors containing puromycin and neomycin selection markers, respectively. FAPs were transduced with lentiviral vectors with a MOI of 5 in the presence of 4 μg/mL of polybrene (Sigma-Aldrich). Transduced cell cultures were selected with puromycin (0.2 μg/mL) and neomycin (0.3 mg/mL) for 10 days. To assess the most efficient method of immortalization, we followed the number of cell divisions during 2 months.

2.8 | Statistics

Statistical comparisons between non-induced and induced conditions were performed using the ratio paired *t* test. Statistical analyses and graphic representations were performed with GraphPad Prism Software 8 (La Jolla, CA, USA).

2.9 | Study approval

The human studies described here were approved by the institutional review board of the Hospital de la Santa Creu i Sant Pau. Informed consent for the donation of muscles to our sample collection was received prior to the inclusion to the study.

3 | RESULTS

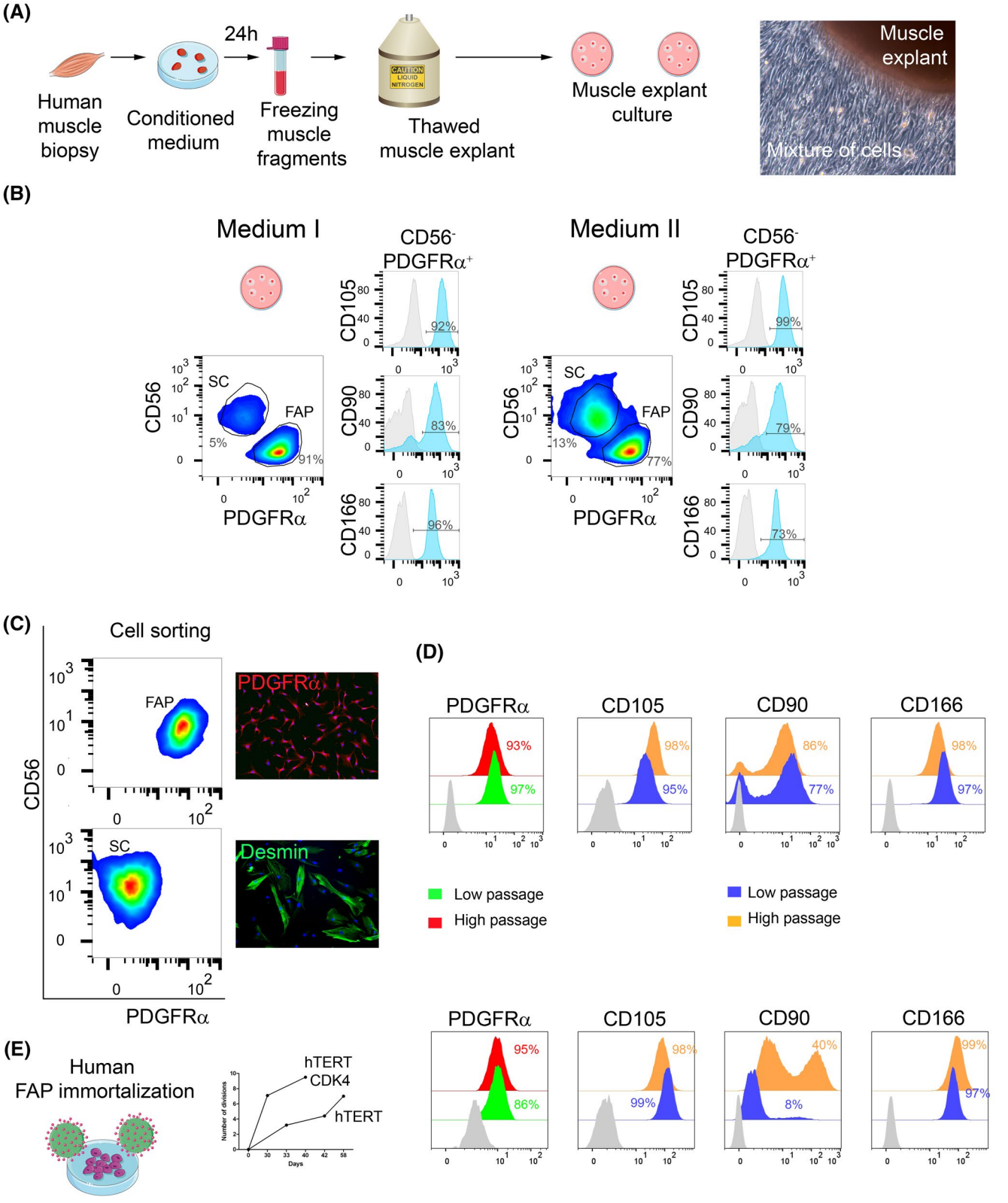
3.1 | Human SC and FAPs can be isolated from frozen muscle biopsies

To assess the utility of frozen muscle biopsies for SCs and FAP isolation, we thawed skeletal muscle fragments that were frozen in DMEM plus 10% FBS and 8% DMSO after culturing them in the conditioned medium for 24 hours. We included samples from five different healthy individuals that were frozen for 1 week and up to 10 years ago from (Table 1). We cultured these muscle explants in two different growth media (Figure 1A). Flow cytometry analysis of the mixture of cells that migrated from these explants identified cells expressing PDGFRα$^+$, which is a well-known marker of FAPS, in medium I and II (91% ± 5% and 77% ± 17%; n = 5; mean ± SD) and also cells expressing CD56$^+$, a marker of SC cells, in both cell media (5% ± 4% and 13% ± 40%; Figure 1B). To further characterize the phenotype of the PDGFRα$^+$ fraction (from now on FAP cells) in the different cell culture conditions, we analyzed the expression of the mesenchymal markers CD105, CD90 and CD166[14] previously described in human FAPs isolated from fresh tissue.[10] In both cell culture conditions, most of the FAPs expressed CD105 (92% ± 14% and 99% ± 1%) and CD166 (96% ± 5% and 73% ± 11%). A vast majority of FAPs were also positive for CD90 (83% ± 19% and 79% ± 23%) although a short fraction were negative in both culture conditions (Figure 1B). We decided to continue characterizing FAPs isolated just from the medium I because cell markers where similar between cells obtained in both culture conditions and medium I is the media most commonly used for human FAP culture. To further examine the utility of our approach to isolate both FAPs and SCs from the same frozen biopsy, we repeated the process in ten additional muscle biopsies. Flow cytometry analysis confirmed our previous results demonstrating that this cell culture approach enables the growth of both SC and FAPs from fragments of skeletal muscle (FAPs: 84.7% ± 13%; SC: 9% ± 10%; n = 15; mean ± SD; Table 1).

We purified SCs and FAPs by FACS from three healthy controls and expanded them in vitro for further analysis. The purity of the cultured SCs and FAPs was confirmed by immunostaning of desmin and PDGFRα (Figure 1C). We cultured explant-derived FAP cells for multiple passages to test if they retained the expression of PDGFRα considering low passage between 1 and 4 and high passages between 5 and 9 passages. We observed that the expression levels of PDGFRα were stable during expansion of cells (Figure 1D). Similarly, the percentage of positivity for the mesenchymal markers was not modified during cell passages: CD105: 97% ± 2% versus 94% ± 4%, CD90: 72% ± 18% versus 86% ± 11%, CD166: 94% ± 3% versus 86% ± 13% (mean ± SD; low passages vs. high passages; n = 3). The use of frozen muscle fragments for obtaining human FAP cells opens the door to multiple new research projects such as high throughput screening of drugs able to counteract or modulate the differentiation capacity of FAPs. In fact, drugs able to modulate FAP cells function are being tested both in animal models and in patients with MD.[15-21] However, one of the limitations of primary cell lines is their limited ability to proliferate as these cells enter into senescence quickly. To address this limitation we attempted to immortalize human FAPs as is commonly done in SCs.[22] We tested two different strategies to generate iFAPs (iFAPs): (1) transduction of hTERT alone, as has been used for the immortalization of skin fibroblast cells[23] and (2) transduction of both hTERT and CDK4, as has been used for the immortalization of SCs from patients with MD.[13,24] Cells transduced with hTERT had low proliferation rate, undergoing seven divisions in almost 2 months, while in contrast, cells transduced with hTERT and CDK4 underwent seven divisions in just 1 month (Figure 1E). We further characterized iFAPs generated by hTERT and CDK4 transduction. Flow cytometry showed that after transduction, equivalent percentage of the iFAPs kept the expression of PDGFRα which was not modified after cell passages (Figure 1E). CD105 and CD166 expression levels remained stable in iFAPs, but in the case of CD90 we observed a prominent reduction of its expression that was partially recovered after passaging (60% low vs. 40% high expression; Figure 1E).

3.2 | Purified human FAPs and SCs maintain their differentiation capacity

Once we generated stable primary and immortalized human FAP and SC cells from the same muscle biopsy, the next step was to assess their differentiation potential. FAPs and iFAPs, but not SC, cultured in adipogenic medium significantly differentiated into adipocytes (Figure 2A-C), as indicated by the increased expression of adiponectin gene (ADIPOQ) (Figure 2A,B), which is specifically expressed in mature adipocytes.[25] ADIPOQ was not detected neither in FAPs nor iFAPs at baseline, but was upregulated in both primary FAPs and in iFAPs after culture in adipogenic medium (Figure 2A,B). Differentiated FAPs and iFAPs also expressed the late adipogenic differentiation markers FABP4 and perilipin and produced fat as was demonstrated by oild red



staining (Figure 2A,B). Quantification studies of FABP4+ cells showed a variable degree of adipogenic differentiation (38% ± 29%) of FAPs isolated from different healthy individuals. The proportion of iFAPs differentiated into adipocytes was 42% ± 15% (Figure 2B). Lipid droplets were in general bigger in adipocytes obtained from FAPs than in adipocytes obtained from iFAPs (Figure 2A,B). To assess fibrogenic differentiation of these cells, we treated FAPs, iFAPs and SCs with TGFβ and we quantified collagen-I expression by in-cell western-blot (ICW) and by qPCR. Both FAPs and iFAPs

FIGURE 1 Simultaneous isolation of human satellite cells (SC) and FAPs from frozen muscle fragments. Fresh muscle biopsies were cultured for 24 hours in conditioned medium and after 24 hours frozen and stored in liquid nitrogen. After thawing, muscle fragments were cultured as muscle explants and after 7-10 days, the mixture of cells sprouting out from muscle explant were analyzed (A). Muscle explants cultured in two different media were analyzed by flow cytometry for the presence of SC CD56+ and FAPs PDGFRα+ cells. Both cell culture conditions showed that the mixture of cells contained SC and FAPs which expressed the mesenchymal markers CD105, CD90 and CD166. Grey histograms correspond to fluorescence minus one (FMO) controls and cell counts were normalized to mode. Representative data from five different healthy controls is shown (B). SC and FAPs were sorted and verified by flow cytometry and immunofluorescence (C). Purified FAPs were cultured for multiple passages and platelet derived growth factor receptor alpha (PDGFRα) and mesenchymal markers expression levels were compared. Representative data from additional 10 different healthy controls is shown (D). FAPs from a healthy donor were transduced with hTERT alone or with CDK4 for their immortalization (iFAP). The number of cell divisions after the different transduction strategies was compared. PDGFRα and mesenchymal markers expression levels were compared in iFAPs after multiple passages. Grey histograms correspond to FMO control (E)

showed collagen-I expression at baseline which increased significantly in response to TGFβ-treatment (Figure 2D,E). In contrast to FAPs, SCs showed no production of collagen-I at baseline or after being cultured with TGFβ (Figure 2D). The myogenic potential of SCs isolated from muscle explants was confirmed by the staining of the myogenic transcription factors Pax7, MyoD and Myogenin (Figure 2F). Moreover, clonogenic assays supported the self-renewal property of SCs obtained as 52% of the clones were able to proliferate and differentiate (Supplemental Figure S1). We compared the myogenic potential of SCs obtained from muscle explants and SCs obtained from enzymatic muscle digestion. We assessed the expression of the aforementioned myogenic transcription factors and of the marker of myotube differentiation myosin heavy chain during 9 days while cells were cultured in a differentiation medium (Figure 2G). At day 3, we observed an upregulation of the expression of the early myogenic transcription factors Pax7 and MyoD that decreased at day 6 and 9. In the case of Myogenin, which is expressed later during myogenic differentiation, expression levels sharply increase at day 3 and then plateaued during day 6 and 9. Myosin heavy chain expression levels progressively increased during the follow-up peaking up at day 9 (Figure 2G). A similar profile of expression was observed between SCs obtained from explants and SCs obtained from muscle digestion. Although the magnitude of the upregulation of MyHC in SCs from digestion was less pronounced, mature myotubes expressing MyHC in both SC types were observed while no myotubes were observed when FAPs or iFAPs were cultured in myogenic conditions (Figure 2H).

3.3 | FAPs and SCs can be purified from frozen biopsies of muscular dystrophy patients

In view of the potential of our approach to isolate matched FAPs and SCs from frozen muscle biopsies from healthy donors, we explored if these cells could also be obtained from frozen muscle biopsies of patients with MD. We used a frozen muscle biopsy of a DMD patient. Flow cytometry analysis of the cells growing from the cultured explants identified both FAPs and SC. Cell sorting and posterior immunolabeling with PDGFRα and desmin staining confirmed their identity (Figure 3A). We analyzed the stability of PDGFRα and the mesenchymal markers during the subculture of FAPs from DMD. PDGFRα, CD105, and CD166 expression was consistently detected during cell passages (Figure 3B, upper panel). At low passages, CD90 expression was similar to that of healthy controls but its expression was increased progressively through the passaging (Figure 3B, upper panel). Having confirmed successful isolation of FAPs and SCs from the patient biopsy, next we immortalized the FAPs (iDMD) using the hTERT and CDK4 transduction strategy. Flow cytometry demonstrated a similar profile of PDGFRα, CD105, and CD166 expression in iDMD compared to non- iFAPs. However, in iDMD we observed changes in the CD90 expression profile similar to what we observed in FAPs from healthy controls: 60% of FAP iDMD cells had high CD90 expression while the 40% of the cells expressed CD90 at low levels and became negative after passages (Figure 3B, down panel). High CD90 expression levels have been related to an undifferentiated status of mesenchymal cells. In fact, a decrease in CD90 level can be correlated with the temporal lineage commitment in vitro[26] and therefore we could speculate that the decrease of CD90 expression in iFAPs may reflect a reduction of multilineage capacity although our in vitro experiments do not reveal such a possible explanation. We then performed differentiation assays with primary DMD FAPs and the immortalized iDMD FAPs. Adipogenic induction showed that both FAP cells differentiated into adipocytes being positive for perilipin and FABP4 and accumulating lipid droplets as was shown using oil red staining (Figure 3C). The differentiation index was similar between primary and immortalized cells in DMD: 57% ± 8% and 53% ± 14% of cells differentiated into adipocytes, respectively (Figure 3C). Gene expression analysis revealed undetectable adiponectin levels at baseline, which was significantly upregulated in adipogenic culture conditions in both primary and immortalized DMD FAPs (Figure 3B). Similarly, treatment with TGFβ-induced DMD FAPs and iDMD FAPs to adopt a fibrogenic fate with a significant increase in their production of collagen-I (Figure 3D). As observed in healthy controls,

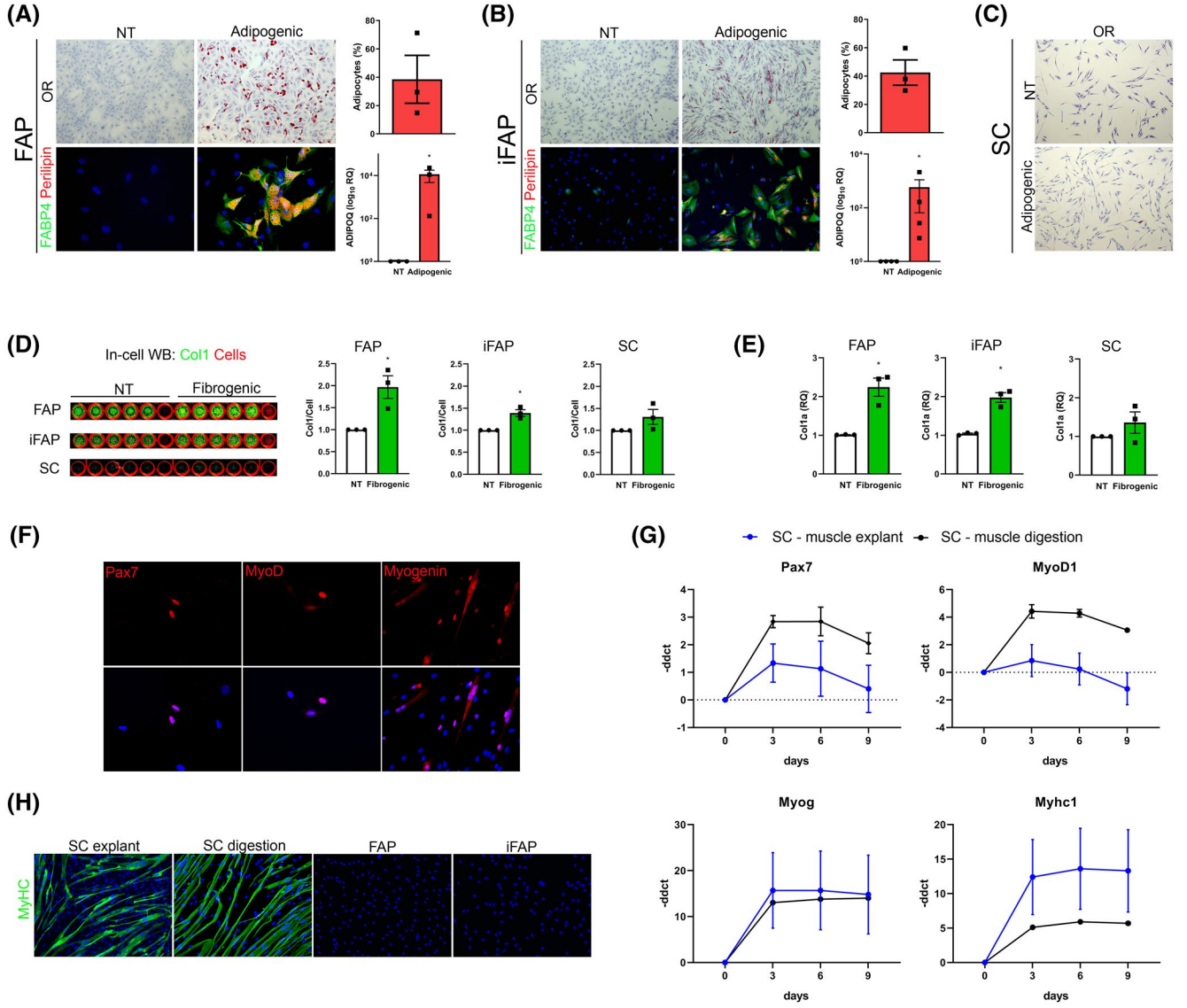


FIGURE 2 Differentiation of FAPs, satellite cells (SCs), and immortalized FAPs (iFAPs) obtained from frozen muscle explants. FAPs, iFAPs and SCs from healthy donors were cultured with adipogenic medium. Oil-red staining (OR) and fatty acid-binding protein 4 (FABP4) and perilipin co-staining showed that FAPs (A) and iFAPs (B) differentiated to adipocytes while SC did not (C). The percentage of adipocytes was determined by the number of FABP4-positive cells. A minimum of four random fields in each of the three healthy controls were analyzed. Adiponectin (ADIPOQ) gene expression was significantly increased in FAPs ($P = .037$) (A) and in iFAPS ($P = .035$) (B). Analysis of collagen I by ICW on FAPs, iFAPs and SC cultured in fibrogenic medium showed a significant increase in FAPs ($P = .041$) and iFAPs ($P = .026$) of collagen I in fibrogenic conditions, while SC did not ($P = .193$) (D). qPCR gene expression analysis showed a significant increase of collagen I in FAPs ($P = .019$), iFAPs ($P = .011$) but not in SC ($P = .37$) (E). Immunolabeling of the myogenic transcription factors Pax7, MyoD and myogenin in SC isolated from muscle explants (F). Gene expression analysis of Pax7, MyoD1, Myogenin and myhc1 at 3, 6 and 9 days of differentiating SC isolated from muscle explants or from muscle digestion (G). Confluent culture with low-serum conditions and MyHC staining was performed to identify mature myotubes. Both SC from explants or from digestion showed the presence of MyHC-expressing myotubes while FAPs and iFAPs did not (H). Data from FAPs from 3 healthy donors is shown. In experiments with iFAPs, technical replicates are shown. In ICW, data from five replicates is shown and was normalized to the number of cells. The last well of each condition corresponds to the negative control for collagen I staining. qPCR data was normalized to GAPDH endogenous control. Data from SC-explants from 3 healthy donors are shown while technical replicates from one individual is shown in SC from digestion. Standard error of the mean is shown. ICW, in-cell western-blot; MyHC, myosin heavy chain; NT, non-treated. Statistical comparisons between non-induced and induced conditions were performed using ratio paired *t* test.
$^*P \leq .05$

the SC cell fraction obtained from the DMD patient did not differentiate into adipocytes and did not express collagen-I in response to TGFβ (Figure 3E). However, myotubes expressing MHC were only obtained after culturing DMD SCs, and not DMD FAPs or iDMD FAPs for 9 days in low-serum culture medium (Figure 3D). In summary, although

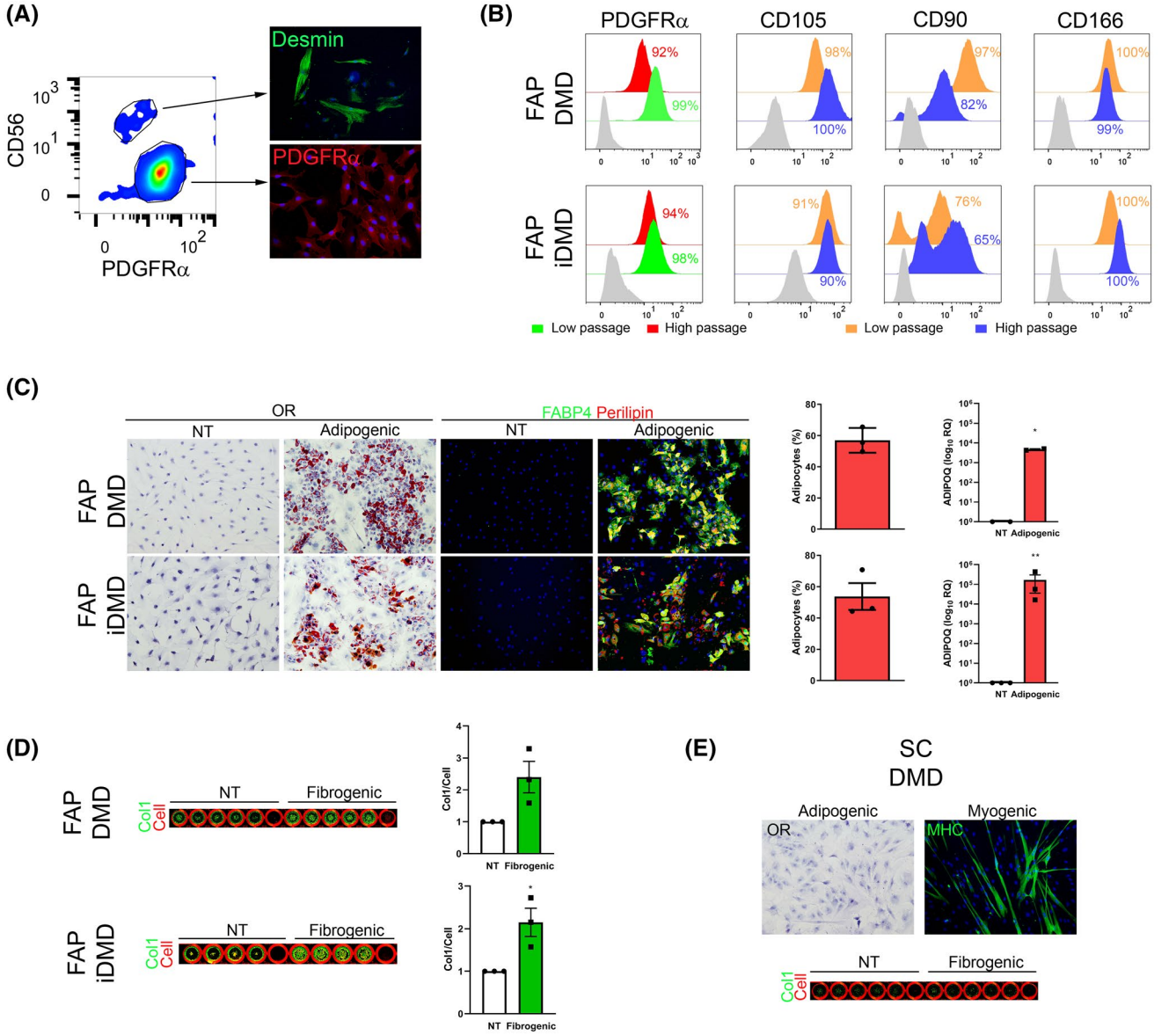


FIGURE 3 Isolation, immortalization and differentiation of FAPs from frozen Duchenne muscular dystrophy (DMD) muscle explant. Frozen muscle explants from a DMD patient were thawed and cultured to allow cells to sprout out from the explant. These cells were analyzed by flow cytometry. CD56+ cells were identified as satellite cells (SCs) and PDGFRα+ as FAPs. Immunofluorescence confirmed the identity of both cell types by the staining of desmin and PDGFRα (A). The expression levels of PDGFRα and the mesenchymal markers CD105, CD90, and CD166 were assessed by flow cytometry in DMD and in iDMD (immortalized) FAP cells during multiple passages. Grey histograms correspond to fluorescence minus one (FMO) controls and cell counts were normalized to mode (B). Adipogenic induction in DMD and iDMD FAP cells promote the presence of lipid droplets observed in the OR staining. Perilipin and FABP4 stainings demonstrated their adipogenic fate, and the percentage of adipocytes was calculated based on FABP4-positive cells. Gene expression analysis demonstrated a significant upregulation of adiponectin (ADIPOQ) in DMD ($P = .007$) and in iDMD ($P = .007$) FAPs (C). ICW showing a significant overexpression of collagen I after culturing DMD ($P = .05$) and iDMD ($P = .04$) FAPs with transforming growth factor β (TGFβ) (D). SC fraction from a DMD patient was exposed to adipogenic, myogenic, and fibrogenic medium. OR staining showed the absence of adipocytes, MyHC staining showed the presence of myotubes and ICW showed no effect of TGFβ on collagen I expression (E).; ICW, in-cell western-blot; MyHC, myosin-heavy chain; NT, non-treated; OR, oil-Red. Statistical comparisons between non-induced and induced conditions were performed using ratio paired *t* test $*P \leq .05$; $**P \leq .01$

we only tested one sample from a DMD patient, these results support that FAPs can be obtained from frozen DMD muscle biopsies, opening the door to further studies characterizing FAPs obtained from patients with DMD and other MD and studying their interactions with SCs obtained from the same patient.

4 | DISCUSSION

One of the main limitations for the study of the mechanism leading to muscle degeneration in humans is the lack of available tissue for the isolation of cells. The approach that we describe here allows freezing muscle in optimum conditions for cell culture enabling the posterior isolation of FAPs and SCs concomitantly from the same patient. We have extensively applied our protocol to healthy muscle biopsies and verified our approach in a muscle sample obtained from a DMD patient. We cannot obtain any conclusions from a biological point of view in the sample from a DMD patient we tested in terms of the ratio of FAPs/SCs obtained or the differentiation potential of FAPs but this experiment supports that our protocol can be applied to samples from patients with muscle diseases. Muscle biopsies are commonly performed for diagnostic purposes and therefore, the diagnosis is not known when the muscle biopsy is obtained limiting the inclusion of these fresh muscle samples in any predesigned research study. Moreover, the popularization of next generation sequencing as standard for diagnostic in many countries has reduced the number of biopsies performed. Obtaining a new muscle biopsy from a patient with a confirmed genetic disease is ethically questionable, as the procedure is invasive and painful for patients. Additionally, MD are rare diseases and the inclusion of several muscle biopsies in a short period of time for research studies is commonly not feasible. We have demonstrated that FAP cells can be isolated from frozen muscle and can be expanded in vitro maintaining the expression of cell specific markers without limiting their differentiation potential. FAPs can undergo adipogenic or fibrogenic fate using specific culture conditions and SCs differentiate producing mature myotubes. The standard use of the approach used here by worldwide biobanks and specialized research laboratories would enable long-term storage of muscles and the design of experiments based in the obtention of SCs, FAPs and other muscle cells from the same patient, to better understand the pathomechanims of these diseases. In this sense, the knowledge on the physiopathology of muscular diseases has experienced an authentic revolution thanks to new techniques such as single cell RNA sequencing or proteomics characterization of muscle cells obtained from animal models. In recent years several subpopulations of muscle resident cells have been identified as the most prevalent at different stages of disease, remarking the heterogeneity of cells participating in the process of muscle degeneration and suggesting that this is a highly dynamic process.[27-29] However, there is a clear gap of knowledge in humans, as there is only few data coming from a minority of studies done with cells from healthy donors.[30,31] Our approach could enhance basic research in human samples revealing what are the subpopulations of muscle resident cells more prevalent in patients at different stages of the disease, what are their main biological properties and how the cells interact among them. Such possibilities enhance the impact of the approach described here for studying human skeletal muscle biology under healthy and pathologic conditions, leading to translational impact on the discovery of new mechanism and subconsequently to the development of new therapies for these disorders.

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CONFLICTS OF INTEREST

The authors have declared that no conflict of interest exists.

AUTHOR CONTRIBUTIONS

X. Suárez-Calvet, JK. Jaiswal, and J Díaz-Manera designed research. X. Suárez-Calvet, E. Fernández-Simón, P. Piñol-Jurado, J. Alonso-Pérez, A. Carrasco-Rozas, and C. Lleixà performed research. A. Bigot and V. Mouly performed immortalization experiments. X. Suárez-Calvet, E. Fernández-Simón, and J. Díaz-Manera analyzed data. S. López-Fernández, G. Pons, L. Soria, I. Illa, and J. Díaz-Manera provided human samples. X. Suárez-Calvet, E. Gallardo, and J Díaz-Manera obtained funding. X. Suárez-Calvet and J. Díaz-Manera wrote the paper with contributions from JK. Jaiswal and E. Gallardo.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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