Original Paper

Control of Muscle Fibro-Adipogenic Progenitors by Myogenic Lineage is Altered in Aging and Duchenne Muscular Dystrophy

Claudine Moratal[a,b]   Nicole Arrighi[a]   Claude A. Dechesne[a]   Christian Dani[a]

[a]Université Côte d'Azur, CNRS, Inserm, iBV, Nice, France, [b]Université Côte d'Azur, Inserm, C3M, Nice, France

Key Words
Adipocytes • Myofibroblasts • Muscle progenitors • Myopathies

Abstract
Background/Aims: Fibro-adipogenic progenitors (FAPs), a muscle-resident stem cell population, have recently emerged as important actors of muscle regeneration by interacting with myogenic progenitors (MPs) to promote the formation of new muscle fibers. However, FAPs are also considered as main contributors of intramuscular fibrotic and fat depositions, resulting in a poor quality of muscles and a defective regeneration in aging and Duchenne Muscular Dystrophy disease (DMD). Therefore, the understanding of the control of FAP fate is an important aspect of muscle repair and homeostasis, but little is known in humans. We wondered the extent to which human FAP proliferation, adipogenesis and fibrogenesis can be regulated by human myogenic progenitors (MPs) in physiological and pathological contexts. ***Methods:*** FAPs and MPs were isolated from skeletal muscles of healthy young or old donors and DMD patients. FAP/MP contact co-cultures and conditioned-media from undifferentiated MPs or differentiated myotubes were assessed on both proliferation and fibro-adipogenic differentiation of FAPs. ***Results:*** We showed that soluble molecules released by MPs activate the phosphoinositide 3-kinase (PI3Kinase)/Akt pathway in FAPs, resulting in the stimulation of FAP proliferation. FAP differentiation was regulated by MP-derived myotubes through the secretion of pro-fibrogenic factors and anti-adipogenic factors. Importantly, the regulation of FAP adipogenic and fibrogenic fates by myotubes was found to be mediated by Smad2 phosphorylation and the gene expression of glioma-associated oncogene homolog 1 (*GLI1*). Surprisingly, the regulations of proliferation and differentiation were disrupted for FAPs and MPs derived from aged individuals and patients with DMD. ***Conclusion:*** Our results highlight a novel crosstalk between FAPs and the myogenic lineage in humans that could be crucial in the formation of adipocyte and myofibroblast accumulation in dystrophic and aged skeletal muscle.

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Claudine Moratal   C3M, Centre Méditerranéen de Médecine Moléculaire, INSERM U1065, Bâtiment universitaire ARCHIMED, 151, route Saint Antoine de Ginestière, BP 2 3194, 06204 Nice CEDEX 3 (France)
Tel. +33 489 06 43 46, Fax +33 489 06 42 21, E-Mail cmoratal@unice.fr

Introduction

Adult skeletal muscle possesses a powerful regenerative capacity that depends on satellite cells which give rise to myogenic progenitors (MPs). Satellite cells reside beneath the basal lamina, closely juxtaposed to the muscle fibers [1]. After injury, quiescent satellite cells become activated, proliferate, and give rise to myoblasts which fuse to existing damaged fibers for repair or among themselves for new myofiber formation. Muscle regeneration is a complex phenomenon that requires a close collaboration between satellite cells and populations such as bone-marrow or muscle-derived stem cells, mesoangioblasts, pericytes and immune cells [2–6].

In the last past decade, a population of fibro-adipogenic progenitors (FAPs) localized between skeletal muscle fibers has been identified in mice [7, 8] and in humans by us and other groups [9–14]. When isolated from human muscle, FAPs have an intrinsic adipogenic potential manifested in culture by exposure to adipogenic conditions [10–14] and can also differentiate into fibrogenic cells in response to transforming growth factor β1 (TGFβ1) [13, 15]. FAPs are considered as a determinant source of intramuscular adipocyte deposition and fibrosis [16, 17]. Fibro-adipogenic depositions within muscles are encountered in a variety of physiopathological conditions resulting in significant muscle weakness and decreased passive range of motion [18]. In particular, such depositions can be found in aged muscles [19, 20] and are a hallmark of Duchenne muscular dystrophy (DMD) [21].

The knowledge of FAP roles in tissue regeneration is largely based on data obtained in mice. FAPs expand during regeneration at the same time as MPs and secrete paracrine factors that support myoblast differentiation [7, 22]. The regulation of FAP proliferation and differentiation just begins to be unveiled. During normal repair, FAP expansion is transient and followed by a rapid reduction in their number [7]. This is controlled by macrophages that tightly regulate the balance of pro-apoptotic and pro-survival signals driven by tumor necrosis factor α (TNFα) and TGFβ1, respectively [23]. These signals appear in two sequential separate waves in the case of successful regeneration; however, in chronic conditions, such as in dystrophic muscle, this sequence is disrupted, leading to timely overlap of TNFα and TGFβ1 contradictory signaling [23, 24]. Also, FAP adipogenesis is inhibited by satellite cell-derived muscle fibers [8].

In humans, a pro-mitotic role of platelet-derived growth factor-AA [15] and IL-15 [25] on FAP proliferation was demonstrated, and an anti-adipogenic action of IL-15 [25], Hedgehog signaling [26], TNFα and TGFβ family members [13] was shown on FAP differentiation. While these studies demonstrate a regulation of FAPs by molecules secreted in regenerating muscles, a direct link between myogenic lineage and FAPs remains to be established in a physiological context and in DMD and aging environment in which FAPs number is disrupted [15, 27]. Our hypothesis is that, in a non-pathogenic context, MPs control FAPs to prevent the development of adipose and fibrotic deposits that are observed in skeletal muscles of DMD patients and aged donors. Thus, this study aimed to investigate the molecular mechanisms involved in the *in vitro* regulation of FAP proliferation and differentiation by MP lineage isolated from skeletal muscles of healthy young or aged donors and DMD patients.

Materials and Methods

Reagents and antibodies

Cell culture media, serum, buffer, and trypsin were purchased from Lonza (Verviers, Belgique) and cell culture reagents from Sigma-Aldrich Chimie (Saint-Quentin Fallavier, France).

Fluorochrome-conjugated antibodies for flow cytometric analysis were purchased from BD Biosciences (San Diego, USA) (CD56-APC 345812; CD140a-PE 556002).

Monoclonal antibodies for immunofluorescence analysis were purchased as indicated: anti-αSMA (A 5228) from Sigma-Aldrich Chimie, Alexa Fluor 594 goat anti-mouse IgG from Molecular Probes (Eugene, USA).

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Antibodies for immunoblot analysis were purchased from Cell Signaling (Ozyme, St Quentin en Yvelines, France): anti-phospho-Akt (Thr308, 9275), anti-Akt (9272), anti-phospho-p44/42 (Thr202/Tyr 204, 9106), anti-p44/42 (9102), anti-phospho-Smad2 (Ser465/467, 3101), anti-Smad2/3 (3102). Anti-β-tubulin antibody was purchased from Sigma-Aldrich.

Human recombinant TGFβ1 (100-21) was purchased from Peprotech (Neuilly sur Seine, France) and used at 5 ng/ml. Specific inhibitors wortmannin (W1628) and SB431542 (S4317) were purchased from Sigma-Aldrich Chimie and used at 200 nM and 5 μM, respectively. Cyclopamine was purchased from Calbiochem (Merck Millipore, Fontenay sous Bois, France) and used at 5 μM. U0126 was purchased from Promega (Charbonnière-les-Bains, France) and used at 10 μm.

Table 1. Characteristics of healthy young or aged donors and DMD patients. ND: not determined.

Name	Gender	Age (year)	Muscle origin	Use (figure number)
healthy and young donors				
1	male	8	paravertebral	1A, 1C, 2F, 3B, 3D, 3E, 3F, 4A, 4B, 4C, 4D, 5D, 6C
2	male	1	paravertebral	3B, 3D, 3E, 3F, 4A, 4B, 4C, 4D, 5D
3	male	4	nevus left shoulder	3B, 3D
4	male	1	gluteus maximus	1A, 3B, 3D
5	male	17	gluteus maximus	1A, 1B, 1C, 2F, 3B, 3D, 3E, 3F, 4A, 4B, 4C, 4D, 5D, 6C
6	male	<10	ND	1A, 1C, 3B, 3D, 3E, 3F
7	female	10	quadriceps	1A
8	male	<10	ND	1B
9	male	8	abdominal wall	1B
10	male	19	paravertebral	6B
11	female	17	paravertebral	2E, 2F, 6B, 6C
healthy and aged donors				
12	male	48	ND	2A
13	male	>30	ND	2A, 2B
14	female	43	ND	2A, 2B
15	male	51	ND	2A, 2B, 5B
16	male	68	ND	5D, 5B
17	male	29	ND	5D, 5B
18	male	34	ND	5B
DMD donors				
19	male	16	paravertebral	2C, 2E, 6A, 2F
20	male	15	paravertebral	2C, 6A, 2F
21	male	16	paravertebral	2E, 2D, 6A, 6B, 6C, 2F
22	male	16	gluteus maximus	2C, 2E, 6A, 6B, 6C
23	male	15	paravertebral	2C, 2D
24	male	11	tensor fasciae latae	2C, 2D, 6A, 6B, 6C
25	male	14	paravertebral	2D
26	male	15	deltoid	2C, 2E, 6A

Skeletal muscle progenitor isolation

Tissue samples were obtained as *res nullius* from surgeries or diagnostic biopsies performed in local hospitals. All protocols for healthy young donors aged from 1 to 19 years and for healthy aged donors from 29 to 68 years were approved by the Centre Hospitalier Universitaire de Nice Review Board, according to the rules of the French Regulatory Health Authorities and with the informed consent of the parents for young donors. Samples were placed in F10 medium and transferred to the laboratory. DMD frozen samples from donors aged 11 to 16 years were provided by the Myobank of the Institut de Myologie, AFM-Téléthon. Characteristics of healthy and DMD muscles are reported in Table 1. Skeletal muscle cells were isolated by a standard method [14, 28]. Briefly, healthy or DMD skeletal muscles were minced into 1 mm^3 fragments and digested at 37°C, first using Liberase (Roche Diagnostics, Meylan, France) for 1 hour and then using 0.25% trypsin-EDTA for 20 minutes. The enzymatic reaction was stopped by adding 10% fetal bovine serum. The suspension was homogenized, and finally cells were pelleted by centrifugation and platted in growth culture medium.

Human skeletal muscle FAP and MP isolation

FAPs and MPs were isolated by a standard method [13, 14]. Adherent cells derived from the digestion and amplification of healthy and DMD skeletal muscle biopsies were sorted by flow cytometry with the BD FACSARIA II sorter equipped with 4 lasers and the FACSDiva software (Becton, Dickinson and Company, Franklin Lakes, USA). Briefly, about 2 000 000 cells were labelled with anti-CD56-APC and CD140a-PE (PDGFRα) antibodies or the relevant isotype control in PBS 1X, EDTA 2mM and 10% FBS for 30 minutes at 4°C. The labelled-cell suspension was filtered through 40-μm cell strainers, pelleted by centrifugation and re-suspended in PBS/EDTA/Fetal bovine serum for isolation by the flow cytometer. For CD140a-PE, fluorescence was excited with the 561-nm laser and measured with a 586/15 bandpass filter. For CD56-APC, fluorescence was excited with the 633-nm laser and measured with a 670/14 bandpass filter. Unlike

MPs, FAPs are negative for myogenic marker CD56 and positive for PDGFRα (Supplementary Fig. 1A – for all supplemental material see www.cellphysiolbiochem.com).

Growth and differentiation of FAPs and MPs

The growth culture medium was Ham's F10 medium supplemented with 20% FBS, 10 mM Hepes, 10^{-6} M dexamethasone, 2.5 ng/ml basic fibroblast growth factor (FGF2), 100 U/ml penicillin, and 100 mg/ml streptomycin. Myogenic and adipogenic differentiations were induced by switching to differentiation medium (DM): Ham's F10/F12/low-glucose DMEM with 2mM glutamine (2v/1v/1v) supplemented with 1% horse serum (Gibco, Thermo Fisher Scientific, Villebon sur Yvette, France), 500 nM dexamethasone, 50 μM 1-methyl-3-isobutylmethyl-xanthine (MIX), 7.5 μg/ml insulin, 7.5 μg/ml transferrin, 0.1 nM triiodothyronine, and 50 nM rosiglitazone (PPARγ agonist). Three days later, cells were placed in the same medium lacking MIX and dexamethasone. This differentiation medium was replaced every 3 days and cells were collected after 10 days of differentiation to analyze fibrogenesis and adipogenesis, or 7 days for myogenesis. Fibrogenic differentiation was obtained with the above DM complemented with 5ng/ml TGFβ1 for 5 days.

Validation of FAP and MP differentiation

In the dual myogenic-adipogenic culture medium, FAPs differentiated only into adipocytes containing lipid droplets and MPs only into multinucleated myotubes (Supplementary Fig. 1B). Accordingly, the adipogenic markers fatty acid binding protein 4 (*FABP4*)*, Adipsin* and perilipin 1 (*PLIN1*) were specifically expressed in FAP differentiating cultures and the myogenic markers muscle creatine kinase (*MCK*), myogenin (*MYOG*) and δ-sarcoglycan (*SGCD*) were specifically expressed in MP differentiating cultures (Supplementary Fig. 1D). In a fibrogenic culture medium, only FAPs differentiated into alpha smooth muscle actin (αSMA)-positive myofibroblasts with stress fibers (Supplementary Fig. 1C) and the TGFβ1 induction of the fibrogenic markers collagen type I alpha 1 chain (*COL1A1*)*,* fibronectin 1 (*FN1*) and *aSMA* was clearly stronger in FAPs than MPs (Supplementary Fig. 1E). In the next experiments, MP interactions on FAPs were assessed with FAPs and MPs extracted from the same human donor and a collection of donors was used.

For co-cultures experiments, 100 000 FAPs and 100 000 MPs were seeded in a well of a 12-well plate. One day later the growth culture medium was replaced by the differentiation medium. Data were compared to mono-cultures conducted in the same conditions.

Production of conditioned-media

To generate conditioned-medium from proliferating-MPs (MP CM) used in proliferation experiments, CD56+ cells were cultured in the growth medium and at 60-80 % of confluence treated with fresh growth medium for 1 day. To generate conditioned-medium from undifferentiated MPs (MP CM) used in differentiation experiments, CD56+ cells were cultured in the growth medium and at 80 % of confluence treated with cytokines-free differentiation medium containing 1 % horse serum for 1 day. To generate conditioned-medium from differentiated myotubes (Myotube CM) used for differentiation experiments, confluent CD56+ cells were differentiated with the differentiation medium and at day 6 treated with cytokines-free differentiation medium for 1 day. At the same time, control CM (Ctrl CM) was obtained by incubating growth or differentiation medium without cells in the incubator and collected 24 hours later. After collecting, all these conditioned-media were spined at 2000 rpm for 10 minutes, then stocked at -20°C. They were used without dilution. Dexamethasone and FGF2 were added before freezing in all conditioned-media employed in proliferation experiments. The cocktail of myogenic-adipogenic cytokines was added before freezing in all conditioned-media employed in differentiation experiments.

MTT assays

Cells were seeded at 1500 cells/well in a 96-well plate in the presence of adequate conditioned-medium. Cellular viability was measured by adding 10 μl of 5 mg/ml thiazolyl blue tetrazolium bromide (MTT, Sigma-Aldrich) in wells and the plate was incubated at 37°C for 4 hours in culture hood. Then, the medium was removed and MTT crystals were dissolved in 150 μl of DMSO/isopropanol (v/v). The plate was incubated at room temperature for 10 minutes with gently agitation, and absorbance was read at 570 nm, and 620 nm which is the reference filter. Each condition was done in triplicate.

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Immunofluorescence analysis

Cells plated on slides were fixed with Histofix 4% (Carl Roth, Lauterbourg, France) for 10 minutes, permeabilized and saturated with 0.1 % Triton X-100 / 3% bovine serum albumin for 30 minutes at room temperature, and sequentially incubated with primary antibody (Sigma-Aldrich) overnight at 4°C then with secondary antibody for 45 minutes at room temperature. PBS wash was performed three times between all steps. Cells were finally mounted in Mowiol containing DAPI and visualized with an Axiovert microscope (Carl Zeiss, Le Pecq, France) under oil immersion and pictures were captured and treated with AxioVision software (Carl Zeiss).

RNA extraction and Reverse Transcription quantitative Polymerase Chain Reaction

Total RNA was extracted using TRI-reagent (Euromedex, Souffelweyersheim, France). The protocols of RNA extraction and quantitative RT-PCR were previously mentioned [13]. The housekipping gene TATA box-binding protein (*TBP*) was used as reference. The 5'–3' sequences of forward and reverse primers were, respectively,

ACGCCAGCTTCGGAGAGTTC and CAAACCGCTTGGGATTATATTCG for *TBP*,
ATGGGATGGAAAATCAACCA and TGCTTGCTAAATCAGGGAAAA for *FABP4*,
AGGGTCACCCAAGCAACAAAG and TACGTGGCCCATGCTGATCT for *adipsin*,
ACCATCTCCACCCGCCTC and GATGGGAACGCTGATGCTGT for *PLIN1*,
ACCTGCGTGTACCCCACTCA and CCGCCATACTCGAACTGGAA for *COL1A1*,
CTGGCCGAAAATACATTGTAAA and CCACAGTCGGGTCAGGAG for *FN1*,
TGCCTGCATGGGCAAGTGA and CTGGGCAGCGGAAACG for *αSMA*,
CTGCGTGGCTGGTGATGAG and CAGGTCGTCTCCACCCTTGA for *MCK*,
CAGCTCCCTCAACCAGGAG and GCTGTGAGAGCTGCATTCG for *MYOG*,
CGGGAGTGTTGAGTGAAGGG and CTCCGGTGGTGAGTGTACTG for *SGCD*,
TGCAAAGCCTTCAGCAATG and TTTTCGCAGCGAGCTAGGAT for *GLI1*.

Immunoblot analysis

Cells were lysed in RIPA buffer consisting of 50 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 5 mM NaF, 2.5 mM $NA_4P_2O_7$, 1% NP40, 2 mM sodium vanadate and protease inhibitor cocktail (Roche Diagnostics, Meylan, France). The protein content was determined according the BCA method (Pierce BCA Protein Assay Kit, Thermoscientific, Illkirch Graffenstaden, France #23227). Cell lysates were centrifuged at 13000 g for 10 minutes at 4°C and the supernatants were recovered.

10 µg of proteins were resolved by 7.5% SDS-PAGE under reducing conditions and transferred to Immobilon-P membranes (Millipore Corporation, Bedford, USA). The membranes were probed with the bound primary antibody that was detected by horseradish peroxidase-conjugated secondary antibody (Promega, Charbonnières les Bains, France) and visualized with an electrochemical luminescence detection kit (Bio-Rad, Marnes la Coquette, France). The band intensity was measured using the Quantity One software (Bio-Rad).

Statistical analysis

Differences between data groups were evaluated for significance using the two-tailed unpaired Student's t-test. A P-value < 0.05 was considered significant. The data are presented as mean ± S.E. of the mean of at least three independent muscle biopsies in duplicate or triplicate. Muscle biopsy samples were randomly chosen in our collection.

Results

MPs stimulate FAP proliferation via the PI3K/Akt signaling pathway

The proliferation of FAPs was found to be accelerated in the presence of MP conditioned medium (MP CM); this effect was soon as 24 hours after the addition of conditioned medium and was observed for two more days (Fig. 1A). FAP-induced proliferation was associated with a two-fold increase of Akt phosphorylation (Fig. 1B). Addition of the phosphoinositide 3-kinase (PI3Kinase) inhibitor wortmannin alleviated the pro-proliferative action of MP

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conditioned medium on FAPs (Fig. 1C) without induction of cell mortality (data not shown). No significant variation of extracellular signal-regulated kinases 1/2 (ERK1/2) phosphorylation, another important regulation pathway of cell proliferation, was observed in the same conditions (Supplementary Fig. 2). Taken together, these results show that MP-secreted factors can stimulate FAP proliferation via an over-activation of PI3K/Akt pathway.

The regulation of FAP proliferation by MPs is altered with aging and DMD

We checked whether the regulation of FAP proliferation by MPs was modified with aging and dystrophic environment. Unlike young progenitors (Fig. 1A), no stimulation of aged FAP proliferation by conditioned medium from aged MPs was observed (Fig. 2A). Consistently, this was not associated with a stimulation of Akt phosphorylation (Fig. 2B). To note, the proliferation capacity of aged FAPs was similar to young FAPs (Supplementary Fig. 3A). These results indicate that MPs do not stimulate PI3Kinase/Akt pathway resulting in the absence of stimulation of FAP proliferation for progenitors from aged donors.

As with aged progenitors, DMD MPs did not have a stimulatory effect on DMD FAP proliferation, in conditioned medium experiments (Fig. 2C). Surprisingly, the significant increase of Akt phosphorylation was maintained in DMD FAPs cultured with conditioned medium from DMD MPs (Fig. 2D), as observed with healthy progenitors (Fig. 1B). Interestingly, the stimulatory effect of DMD FAP proliferation was rescued with healthy MPs (Fig. 2E), while DMD MPs did not increase the proliferation of healthy FAPs (Fig. 2F). Moreover, DMD FAP proliferation rate was similar to healthy FAPs (Supplementary Fig. 3B). Thus, we can conclude that factors secreted by MPs are modified in a dystrophic environment resulting in alteration of FAP proliferation regulation.
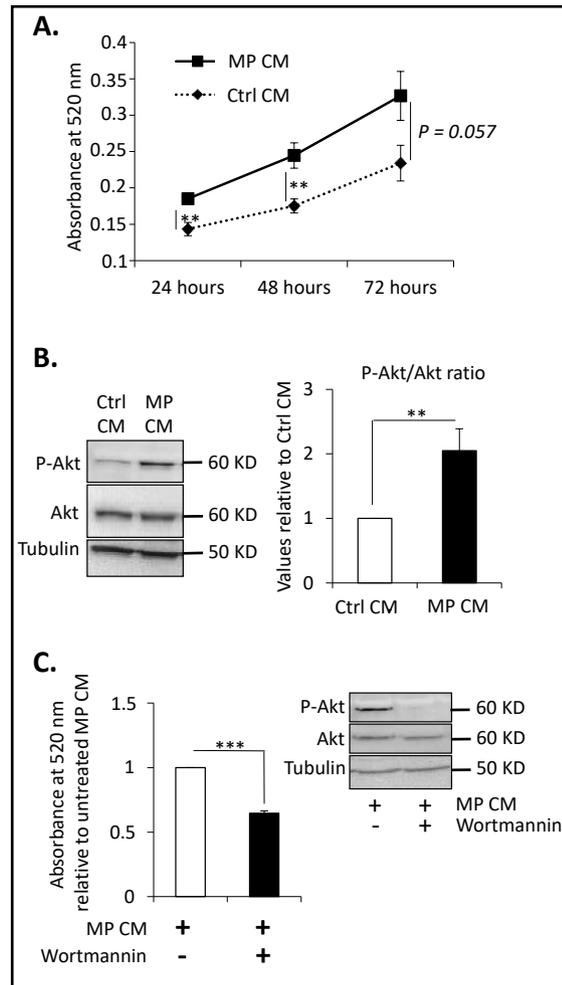
Fig. 1. The MP-dependent stimulation of FAP proliferation is mediated by Akt signaling. FAPs proliferated in the presence of conditioned medium (CM) from MPs (MP CM) compared to control conditioned medium (Ctrl CM). (A) Proliferation of FAPs was measured by MTT assays 24 hours, 48 hours and 72 hours after plating (n=5 donors). (B) Confluent FAPs were treated with Ctrl CM or MP CM and total proteins were extracted 15 minutes later. Protein expression of phosphorylated Akt (P-Akt), total Akt (Akt) and tubulin was assessed by western blot (left panel). P-Akt band intensity was quantified and normalized to total Akt signals (right panel) (n=3 donors). (C) FAPs proliferated in the presence of MP CM with or without wortmannin. Proliferation of FAPs was measured by MTT assays 48 hours after plating. Protein levels of P-Akt, total Akt and tubulin was assessed by western-blot. Representative immunoblot is shown (n=3 donors). *** P<0.001; ** P<0.01; p values close to significance are indicated.

Fig. 2. The MP-dependent regulation of FAP proliferation is altered in aging and DMD progenitors. (A and B) Aged FAPs proliferated with control conditioned medium (Ctrl CM) or conditioned medium from aged MPs (Aged MP CM). (A) Proliferation was measured by MTT assays 24 hours, 48 hours and 72 hours after plating. (n=4 aged donors). (B) 15 minutes after addition of the conditioned medium, total proteins were extracted; expression levels of phosphorylated Akt, total Akt and tubulin were assessed by western blot. Representative immunoblot is shown (n=3 aged donors). (C and D) DMD FAPs proliferated with control conditioned medium (Ctrl CM) or conditioned medium from DMD MPs (DMD MPs CM). (C) Proliferation was measured by MTT assays 24 hours, 48 hours

and 72 hours after plating. (n=6 DMD donors). (D) 15 minutes after addition of the conditioned medium, total proteins were extracted; expression levels of phosphorylated Akt, total Akt and tubulin were assessed by western-blot. Representative immunoblot is shown (n=3 DMD donors). (E) DMD FAPs proliferated with control conditioned medium (Ctrl CM) or conditioned medium from Healthy MPs (Healthy MP CM). Proliferation was measured by MTT assays 24 hours, 48 hours and 72 hours after plating. (n=3 DMD and n=1 healthy donor). (F) Healthy FAPs proliferated with control conditioned medium (Ctrl CM) or conditioned medium from DMD MPs (DMD MP CM). Proliferation was measured by MTT assays 24 hours, 48 hours and 72 hours after plating (n=3 DMD and n=3 healthy donors). ns= non-significant. * P < 0.05.
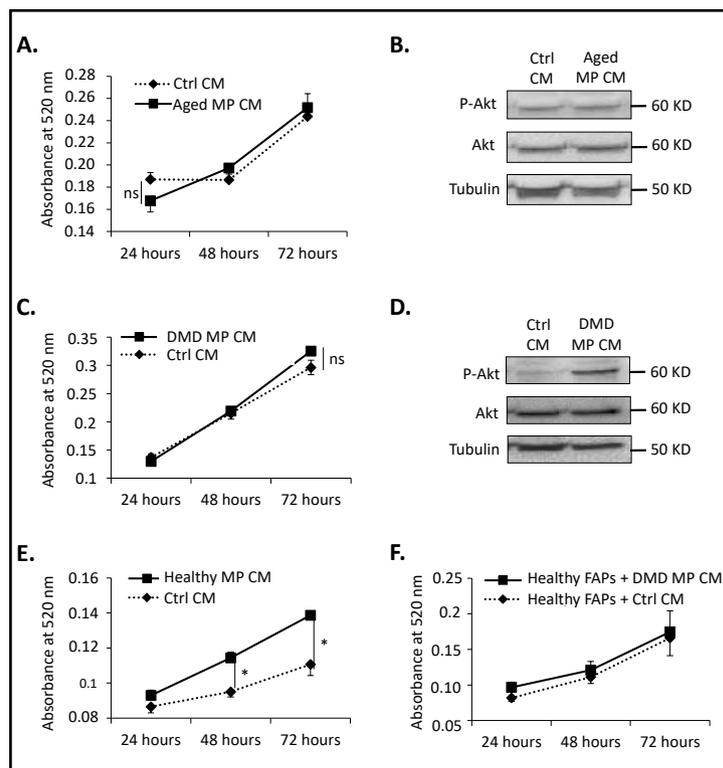
Molecules secreted by myotubes inhibit adipogenesis and stimulate fibrogenesis of FAPs

Adipogenic and fibrogenic potentials of FAPs derived from healthy young donors were investigated in FAP/MP co-cultures with direct cell contact. In these conditions, FAP adipogenesis was abolished as indicated by the decreased number of mature adipocytes (Fig. 3A) and inhibition of mRNA expression of the specific adipogenic markers fatty acid binding protein 4 (*FABP4*) and perilipin 1 (*PLIN1*) (Fig. 3B). Conversely, FAP fibrogenesis was clearly increased. Alpha smooth muscle actin (αSMA)-positive myofibroblasts appeared in FAP/MP co-cultures (Fig. 3C) and expression of the specific fibrogenic mRNAs collagen type I alpha 1 chain (*COL1A1*) and fibronectin 1 (*FN1*) was noticeably higher in co-cultures than in FAP and MP mono-cultures (Fig. 3D).

Under the dual myogenic-adipogenic culture conditions, FAP/MP co-cultures rapidly contained a mix of MPs and myotubes. To define their respective roles, conditioned media from MPs (MP CM) or from differentiated MPs (Myotube CM) were added to FAP mono-cultures. Expression of adipogenic markers in FAP cultures was significantly decreased by conditioned medium from myotubes but not by conditioned medium from MPs (Fig. 3E). Similarly, only conditioned medium from myotubes and not from MPs stimulated significant expression of fibrogenic markers in FAPs (Fig. 3F).

These data show that myotubes secrete molecules that regulate the FAP fate by inhibiting adipogenesis and stimulating fibrogenesis.
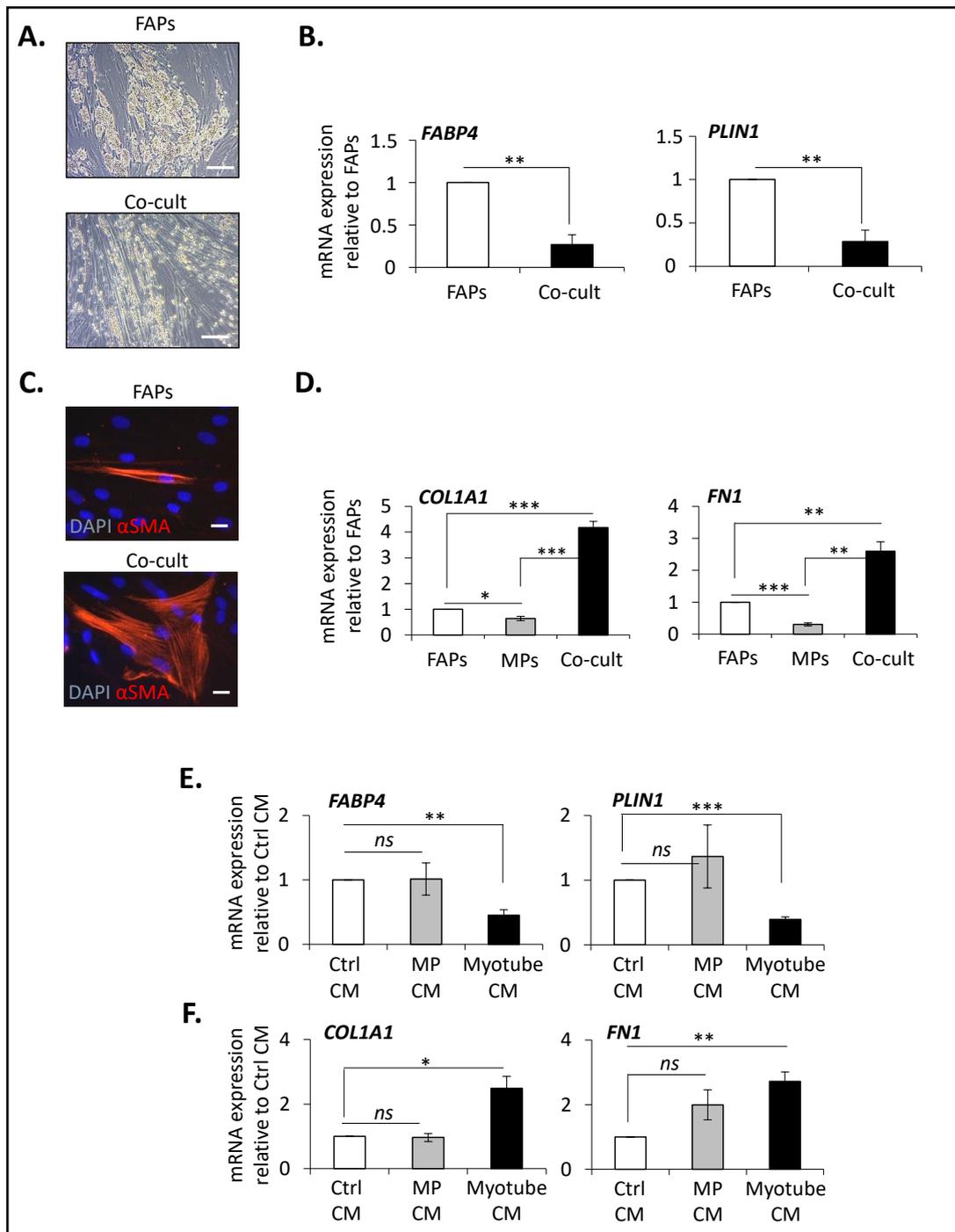
Fig. 3. Myotubes inhibit adipogenesis and stimulate fibrogenesis of FAPs. (A to D) For co-culture assays, FAPs were cultured alone (FAPs) or with MPs (Co-cult) for 10 days in differentiation medium. (A) FAP-derived adipocytes were visualized with phase-contrast microscopy (scale bar: 20 μm). (C) FAP-derived myofibroblasts were visualized by αSMA immuno-labelling. Nuclei were labelled with DAPI (scale bar: 5 μm). Expression of the adipogenic genes FABP4 and PLIN1 (B) and expression of the fibrogenic genes COL1A1 and FN1 (D) were measured by quantitative Q-PCR (n=6 donors). (E and F) For conditioned medium experiments, FAPs were cultured in the presence of conditioned medium from control (Ctrl CM), MPs (MP CM) or differentiated myotubes (Myotube CM) for 10 days. Expression of adipogenic genes (E) and fibrogenic genes (F) was measured by quantitative Q-PCR (n=4 donors). *** P<0.001; ** P<0.01; * P<0.05; ns= non-significant.

Myotubes regulate FAP differentiation via stimulation of the ALK/Smad2 signaling pathway

We recently showed that adipogenesis is negatively regulated in FAPs at the expense of fibrogenesis by factors from the TGFβ family [14]. We wondered whether this pathway is implicated in the regulation of FAP fibro-adipogenesis by myotubes from healthy young donors.

SB431542 is a specific inhibitor of TGFβ superfamily type I activin receptor-like kinase (ALK) receptors ALK4, ALK5, and ALK7. Remarkably, the expression of adipogenic markers was increased and the expression of fibrogenic markers was even decreased (Fig. 4A) in co-cultures treated with SB431542 compared to untreated co-cultures.

As expected, we found that phosphorylation of Smad2, a downstream effector of ALK activation, was activated in FAPs cultured with myotube conditioned medium (Fig. 4B).

Fig. 4. Regulation of FAP fate by myotubes requires activation of ALK/Smad2 pathway and GLI1 expression in FAPs. (A) FAPs were cultured with MPs (Co-cult) for 10 days in differentiation medium with or without SB431542 that is a specific inhibitor of ALK4, ALK5, and ALK7. Expression of FABP4, PLIN1, COL1A1 and FN1 was measured by quantitative Q-PCR (n=3 donors). (B) FAPs were cultured with Ctrl CM or myotube CM for 1 hour, then total proteins were extracted. Expression levels of phosphorylated Smad2 (P-Smad2), total Smad2 and tubulin were assessed by western-blot. Representative immunoblot is shown (n=3 donors). (C) FAPs were cultured with MPs (Co-cult) for 10 days in differentiation medium with or without cyclopamine that is a specific inhibitor of GLI1 expression. Expression of FABP4, PLIN1, COL1A1 and FN1 was measured by quantitative Q-PCR (n=3 donors). (D) FAPs were cultured with Ctrl CM or myotube CM for 10 days in differentiation medium. Expression of GLI1 was measured by quantitative Q-PCR (n=3 donors). *** P<0.001; ** P<0.01; * P<0.05; p values close to significance are indicated.

Therefore, ALK/Smad2 signaling pathway within FAPs is an essential mediator of the regulation of FAP adipogenic and fibrogenic differentiations by MP-derived myotubes.

Myotubes regulate FAP differentiation via the stimulation of GLI1 expression

GLI1 is a downstream effector of Hedgehog signaling that plays a crucial role in the adipogenesis of mesenchymal stem cells [29] and in the regulation of intramuscular fibrosis in response to injury [30]. We assessed the importance of this signaling in the control of FAP fibro-adipogenesis by myotubes in progenitors derived from healthy young donors.

Cyclopamine is an inhibitor upstream of the Hedgehog pathway by directly inhibiting the Smoothened receptor that induces a drastic inhibition of *GLI1* expression. Cyclopamine had no effect on autonomous adipogenesis and fibrogenesis of FAPs (data not shown). Interestingly, cyclopamine alleviated the inhibition of adipogenesis and reduced the stimulation of fibrogenesis in FAP/MP co-cultures, with an increase of expression of the adipogenic markers *FABP4* and *PLIN1* and a decrease expression of fibrogenic markers *COL1A1* and *FN1* (Fig. 4C). Consistent with this, *GLI1* expression was increased in FAPs cultured with myotube conditioned medium (Fig. 4D).

These results indicate that *GLI1* expression is essential for the regulation of FAP differentiations by myotubes for healthy young progenitors.

The regulation of FAP differentiation by myotubes is altered with aging and DMD

Firstly, we found that differentiation potentials of aged FAPs were higher than young FAPs as shown by the high decrease in expression of *FABP4* (Supplementary Fig. 3C) and *COL1A1* (Supplementary Fig. 3D) in aged FAPs in comparison to young progenitors. Secondly, we inspected whether aging or DMD could interfere with the myotube-dependent control of FAP differentiation. Unlike young progenitors (Fig. 3A and B), FAP adipogenesis was not inhibited in aged co-cultures. Neither phenotype modification (Fig. 5A) nor decrease in *FABP4* expression were observed (Fig. 5B). Regarding the fibrogenic differentiation, specific marker (*COL1A1* and *FN1*) expressions were higher in aged co-cultures than in aged FAP mono-cultures, but this might be due to the high expression of fibrogenic mRNAs by aged MPs (Fig. 5B). To get more insights into the effects of aging, young MPs instead of aged MPs, were co-cultured with aged FAPs. The inhibition of aged FAP adipogenesis was restored according to the important reduction of adipocyte amount (Fig. 5C) and the decrease of *FABP4* expression (Fig. 5D). In parallel, a strong rise of fibrogenesis was obtained, as seen with the increased expression of *COL1A1* and *FN1* concomitantly with a low level of expression in both FAP and MP mono-cultures (Fig. 5D). These results show that aging causes an alteration in the intrinsic fibro-adipogenic potential of aged FAPs and in control of FAP differentiation by the myogenic lineage.

As with healthy progenitors (Fig. 3A and B) and in contrast with aged progenitors (Fig. 5A and 5B), DMD MPs inhibited DMD FAP adipogenesis. This was observed in DMD FAP/MP co-cultures, with the decrease of *FABP4* expression (Fig. 6A), as well as in cultures of DMD FAPs submitted to conditioned medium from DMD myotubes with the high decrease expression of *PLIN1* (Supplementary Fig. 4A). Unlike healthy progenitors (Fig. 4C and D), DMD MPs did not significantly stimulate DMD FAP fibrogenesis. As shown in Fig. 6A, expression of *COL1A1* and *FN1* was not significantly increased in co-cultures; expression of *FN1* was higher in co-cultures than in DMD FAP mono-cultures but were not significantly different from the expression in DMD MP mono-cultures. Accordingly, DMD myotube conditioned medium did not stimulate *COL1A1* expression in FAPs (Supplementary Fig. 4A). To further characterize the impacts of DMD on regulation of FAP differentiation, DMD MPs were replaced by healthy age-matching MPs. The inhibition of DMD FAP adipogenesis was maintained as shown by the high decrease in expression of *FABP4* in DMD FAP/healthy MP co-cultures (Fig. 6B). Unlike results obtained with DMD MPs, DMD FAP fibrogenesis was stimulated in co-cultures with healthy MPs. The stimulation of *COL1A1* and *FN1* expressions was two-times higher in DMD FAP/healthy MP co-cultures (Fig. 6B) than in DMD FAP/DMD MP co-cultures (Fig. 6A). Consistently, an increase in the *COL1A1* expression was similarly found in DMD FAPs
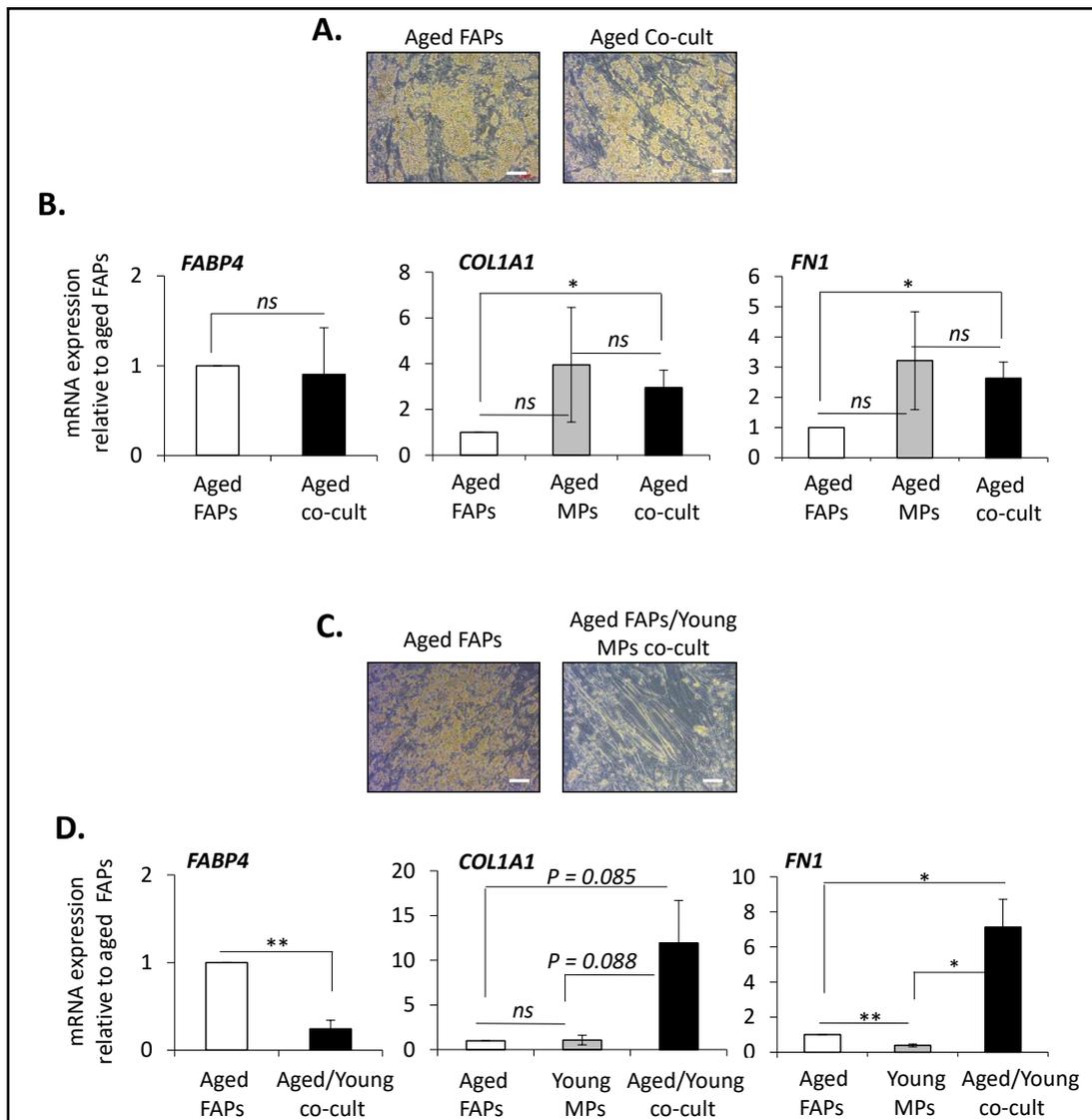
Fig. 5. The myotube-dependent regulation of FAP differentiation is altered in aging. Aged FAPs were cultured alone or with aged MPs (Aged co-cult) (A and B) or with Young MPs (Aged/Young co-cult) (C and D) for 10 days in differentiation medium. (A and C) Adipocytes derived from aged FAPs were visualized by phase-contrast microscopy (scale bar: 5 μm). (B and D) Expression of FABP4, COL1A1 and FN1 was measured by quantitative Q-PCR (n=4 aged donors and n=3 young donors). ** P<0.01; * P<0.05; ns= non-significant; p values close to significance are indicated.

differentiated in healthy myotube conditioned medium (Supplementary Fig. 4B). On the other hand, we assessed the effects of DMD MPs on differentiation of healthy FAPs in co-culture (Fig. 6C). Adipogenesis was not decreased as shown by the similar expression of *FABP4* in co-cultures and healthy FAP mono-culture. Fibrogenesis was not stimulated in co-cultures since *COL1A1* expression was not significantly different from healthy FAP mono-culture and *FN1* expression was similar to DMD MP mono-culture. To note, FAP differentiation into adipocytes [14] and myofibroblasts (Supplementary Fig. 3D) was conserved in FAPs isolated from skeletal muscles of DMD patients. Surprisingly, these results indicate that the control of FAP fibrogenesis by myotubes is abolished while the inhibition of adipogenesis is conserved in DMD progenitors. Interestingly, the deficiency in the regulation of DMD FAP fibrogenesis implicates an alteration of myotubes-secreted factors induced by the dystrophic environment.
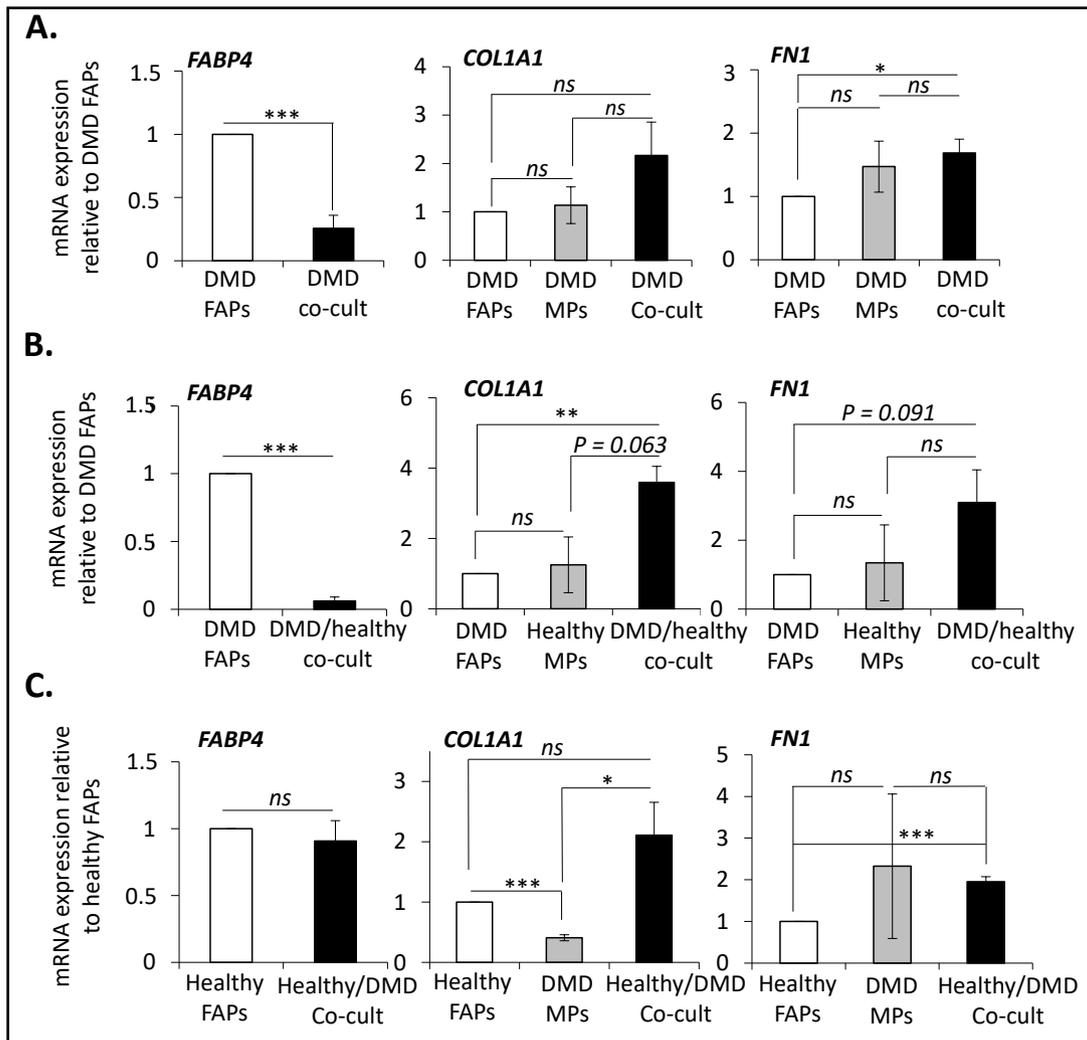
Fig. 6. The myotube-dependent regulation of FAP differentiation is altered in DMD. DMD FAPs were cultured alone or with DMD MPs (DMD co-cult) (A) or with Healthy MPs (DMD/Healthy co-cult) (B) for 10 days in differentiation medium. (C) Healthy FAPs were cultured alone or with DMD MPs (Healthy/DMD co-cult) for 10 days in differentiation medium. Expression of FABP4, COL1A1 and FN1 was measured by quantitative Q-PCR (n=6 DMD donors and n=3 healthy donors). *** P<0.001; ** P<0.01; * P<0.05; ns= non-significant; p values closed to significance are indicated.

Thus, aging and DMD have a different impact on the potential of myotube to regulate FAP differentiation.

Discussion

In response to injury, skeletal muscle undergoes a highly orchestrated degenerative and regenerative process for which satellite cells are central actors as myogenic progenitors. FAPs have recently emerged as other important actors of regeneration in healthy and dystrophic mouse muscles. In addition to cells from the immediate niche surrounding satellite cells, the interstitial cells FAPs have the potential to regulate satellite cell fate and thus contribute to muscle regeneration [3, 5]. These findings stress the importance of studying the FAP/MP interactions in humans. Here, we focused on the *in vitro* regulation of FAPs by MPs isolated from human muscles.

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Firstly, we showed that MPs are actors of a positive regulation of FAP proliferation mediated by the Akt phosphorylation. This is in accordance with previous data showing that activation of PDGFRα signaling inducing PI3K-Akt pathway stimulates proliferation of FAPs [15, 31, 32]. While platelet-derived growth factor-AA could be a likely factor controlling FAP proliferation [15, 32], further investigations are needed to identify factors activating PI3K-Akt pathway in FAPs submitted to MP secretome.

Another incidence of MP lineage on FAPs is the regulation of FAP differentiation. Myotubes, but not MPs, inhibited adipogenesis of FAPs as reported in mice [8]. Another finding is the profibrogenic role of human myotubes on FAPs, which has not been documented in mice. This other function of myotubes is of special interest and emphasis the importance of the coordination between FAPs and myotubes for muscle regeneration. Thus, myotube-secreted factors control a differentiation balance between fibrogenesis and adipogenesis of FAPs.

Mechanistically, we showed that myotube-secreted factors highly phosphorylated Smad2 in FAPs, and this phosphorylation is mediated by ALK4/5/7 receptor activity. Among canonical members of the TGFβ superfamily, TGF-β1 and activin A are known to exert anti-adipogenic [33–35] and pro-fibrogenic effects [36, 37], through Smad2 pathway activation [38]. In accordance, we previously found that TGFβ1 and activin A inhibit adipogenesis in FAPs [13]. We have tested the involvement of these two factors in FAP regulations by myotubes. We disproved this hypothesis since the immuno-blocking of TGFβ and/or activin A did not counteract the effects of myotube conditioned media on FAPs fibro-adipogenesis (data not shown). Furthermore, no secretion of TGFβ1 by myogenic lineage was detected by Elisa assay. Members of the TGFβ family responsible to the effects on FAP differentiation remains to be identified.

In addition to ALK/Smad2 signaling, we found that the transcription factor *GLI1,* a final transcriptional activator of Hedgehog signaling [39, 40], is a central factor for the regulation of FAP differentiation by myotube-secreted factors. Indeed, myotubes secreted factors that promote *GLI1* expression in FAPs resulting in the inhibition and the stimulation of FAP adipogenesis and fibrogenesis respectively. This prominence of *GLI1* is in agreement with recently published results showing that activating Hedgehog signaling in FAPs blocked injury-induced intramuscular adipogenesis [26]. Interestingly, *GLI1* was also reported to be an effector of functional Smad pathway [41, 42]. This indicates a potential cooperation between ALK/Smad2 pathway and *GLI1* expression in the regulation of FAP fate by myotubes, as already proposed in cancer [43].

As the intrinsic differentiation potential of FAPs, FAP regulation by MP lineage is altered with aging. MPs did not stimulate Akt phosphorylation nor consequently FAP proliferation anymore, and myotubes stopped to regulate the fibro-adipogenic potential of FAPs. This was consistent with recent results indicating that aging disturbed the myogenic support of FAPs to MPs [44]. We showed that the aging of MPs is responsible for this lack of regulation. Such results are in accordance with the functional decline of satellite cells described in sarcopenic muscles [45, 46]. Further experiments are necessary to demonstrate that aged MPs could be incompetent to stimulate fibrogenesis and inhibit adipogenesis of young FAPs. The age effect we observed fits the accumulation of adipose tissue in aged muscles at the expenses of muscle regeneration [47, 48].

Similarly to aging, DMD altered the above model of FAP regulation in young donors. Interestingly, these deleterious effects can be reversed by signals from healthy MPs or myotubes suggesting that DMD altered their molecule secretion. The lack of the sub-membrane protein dystrophin, which is only expressed in the myogenic lineage, could lead to modifications of factors secreted by MPs and myotubes. Indirect effects to the lack of dystrophin should also be considered since the DMD MPs are submitted for years to chronic inflammatory regenerative/degenerative conditions, which have the potential to modify MP intrinsic properties. Interestingly, the anti-adipogenic effect on DMD FAPs by myotubes and the absence of positive control of DMD FAP proliferation and fibrogenesis by MPs may contribute to tentatively limit the development of intramuscular fat and fibrotic infiltrations.

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While myotubes would have a protective role against muscle adipocyte and myofibroblast accumulations, other cells that have infiltrated DMD muscles could be the source of the formation of fat and fibrotic deposits such as macrophages [14, 49].

Conclusion

In summary, we described human specific features and uncovered the role of MPs and differentiated MPs for the regulation of FAP proliferation, adipogenesis and fibrogenesis. Importantly, we also found that the incidence of FAP/MP interactions is age dependent and largely modified with DMD progenitors. Finally, we identified signaling pathways essential to mediate the regulations of FAPs by MPs and MP-derived myotubes. The identification of Hedgehog and TGFβ signaling pathways as important mediators provide molecular bases to evaluate the control of FAPs by MPs as clinical tools to improve muscle repair in healthy, aging and dystrophic muscles.

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Author Contributions
Christian Dani (CD), Claude A Dechesne (CAD) and Claudine Moratal (CM) designed this project. CM, Nicole Arrighi (NA) and CAD were involved in acquisition, analysis and interpretation of data. CM and CAD wrote the article. NA and CD participated to the revising of this article.

Disclosure Statement

The authors have no conflicts of interest to declare.

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