RhoA/ROCK2 signalling is enhanced by PDGF-AA in fibro-adipogenic progenitor cells: implications for Duchenne muscular dystrophy

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Abstract

Background The lack of dystrophin expression in Duchenne muscular dystrophy (DMD) induces muscle fibre and replacement by fibro-adipose tissue. Although the role of some growth factors in the process of fibrogenesis has been studied, pathways activated by PDGF-AA have not been described so far. Our aim was to study the molecular role of PDGF-AA in the fibrotic process of DMD.

Methods Skeletal muscle fibro-adipogenic progenitor cells (FAPs) from three DMD treated with PDGF-AA at 50 ng/mL were analysed by quantitative mass spectrometry-based proteomics. Western-blot, immunofluorescence, and G-LISA were used to confirm the mass spectrometry results. We evaluated the effects of PDGF-AA on the activation of RhoA pathway using two inhibitors, C3-exoenzyme and fasudil. Cell proliferation and migration were determined by BrdU and migration assay. Actin reorganization and collagen synthesis were measured by phalloidin staining and Sircol assay, respectively. In an in vivo proof of concept study, we treated dba/2J-mdx mice with fasudil for 6 weeks. Muscle strength was assessed with the grip strength. Immunofluorescence and flow cytometry analyses were used to study fibrotic and inflammatory markers in muscle tissue.

Results Mass spectrometry revealed that RhoA pathway proteins were up-regulated in treated compared with non-treated DMD FAPs (n = 3, mean age = 8 ± 1.15 years old). Validation of proteomic data showed that Arhgef2 expression was significantly increased in DMD muscles compared with healthy controls by a 7.7-fold increase (n = 2, mean age = 8 ± 1.14 years old). In vitro studies showed that RhoA/ROCK2 pathway was significantly activated by PDGF-AA (n = 3, 1.88-fold increase, P < 0.01) and both C3-exoenzyme and fasudil blocked that activation (n = 3, P < 0.05 and P < 0.001, respectively). The activation of RhoA pathway by PDGF-AA promoted a significant increase in proliferation and migration of FAPs (n = 3, P < 0.001), while C3-exoenzyme and fasudil inhibited FAPs proliferation at 72 h and migration at 48 and 72 h (n = 3, P < 0.001). In vivo studies showed that fasudil improved muscle function (n = 5 non-treated dba/2J-mdx and n = 6 treated dba/2J-mdx, 1.76-fold increase, P < 0.013), and histological studies demonstrated a 23% reduction of collagen-I expression area (n = 5 non-treated dba/2J-mdx and n = 6 treated dba/2J-mdx, P < 0.01).

Conclusions Our results suggest that PDGF-AA promotes the activation of RhoA pathway in FAPs from DMD patients. This pathway could be involved in FAPs activation promoting its proliferation, migration, and actin reorganization, which represents the beginning of the fibrotic process. The inhibition of RhoA pathway could be considered as a potential therapeutic target for muscle fibrosis in patients with muscular dystrophies.

Keywords Duchenne muscular dystrophy; Fibro-adipogenic precursor cells; Platelet-derived growth factor; Muscular dystrophies; Fibrosis
Introduction

Duchenne muscle dystrophy (DMD) is a genetic disease produced by mutations in the DMD gene that leads to a fragile muscle membrane susceptible to be damaged during muscle contraction. Muscle membrane fragility is the responsible of chronic injury resulting on continuous cycles of necrosis and regeneration of muscle fibres, persistent inflammation and eventually loss of muscle fibres and their replacement by fat and fibrous tissue leading to permanent weakness and disability.

Fibro-adipogenic precursor cells (FAPs) are mesenchymal muscle-resident stem cells characterized by the expression of platelet-derived growth factor receptor alpha (PDGFRα). FAPs are the main responsible of the fibrotic and fatty tissue expansion in muscular dystrophies. FAPs are activated upon acute injury, proliferate, and release components of the extracellular matrix (ECM) to serve as scaffold where the new regenerated myofibres are displayed. After muscle regeneration, excessive FAPs are cleared by apoptosis mediated by tumour necrosis factor alpha (TNF-α). However, in dystrophic muscles, continuous release of growth factors by M2 macrophages permanently activates FAPs proliferation, that release collagen-I fibrotic tissue. The molecular pathways driving FAPs in muscular dystrophies are just started to be understood. It is well-known that TGF-β promotes FAP proliferation, inhibits TNF-α mediated FAP apoptosis, and drives FAPs to differentiate into fibroblasts.

Apart from TGF-β, the family of platelet-derived growth factors (PDGFs) has been involved in the regeneration and degeneration process. On the one hand, PDGF-BB has been shown to activate satellite cell proliferation and chemotaxis and PDGFRβ is highly expressed in dystrophic muscle compared with healthy muscle probably influencing muscle regeneration. On the other hand, PDGF-AA has been involved in the fibrotic process of several tissues including the skin, lungs, or liver. PDGF-AA binds to PDGFRα, a tyrosin kinase receptor, inducing receptor dimerization and autophosphorylation of the intracellular domain. Autophosphorylation of PDGFRα triggers signalling pathways such as Ras-MAPK, PI3K, or PLC-γ, which are involved in different cellular responses including proliferation, cell differentiation, apoptosis inhibition, mobilization of intracellular calcium, or cell motility.

Although the role of PDGF-AA has been widely studied in fibrotic processes, its role in driving muscle fibrosis in muscular diseases is not completely known. We have previously demonstrated that PDGF-AA activates fibroblast proliferation and migration and increases the release of components of the extracellular matrix. Treatment of murine models of DMD with tyrosine kinase inhibitors blocking the PDGFRα such as imatinib, crenolanib, and nintedanib, decreased muscle fibrosis and improved muscle function. Moreover, previous studies have shown that PDGF-AA expression is higher in dystrophic compared with healthy muscles and that PDGF-AA serum levels are increased in DMD patients correlating with several muscle function tests. However, the pathways activated by PDGF-AA in FAPs in muscular dystrophies have not been described so far. This knowledge could help us to identify new targets for therapies aiming to reduce fibrosis in DMD and slow down the on-going degenerative process. In this study, we have used mass spectrometry to identify Rho-kinase as one of the pathways activated by PDGF-AA in FAPs isolated from human skeletal muscles. These results lead us to test fasudil, a pan-Rho-kinase inhibitor, in a murine model of DMD observing a reduction of muscle fibrosis and consequently an improvement on muscle strength.

Material and methods

Cell culture

We used muscle biopsies from three DMD patients seen at Hospital de la Santa Creu i Sant Pau (HSCSP) in Barcelona (Table 1). The Ethics Committee of HSCSP approved the study, and all participants signed an informed consent form. All study procedures were performed in accordance with Spanish regulations.
Muscle explants were cultured and FAPs were isolated as described in the supporting information. FAPs were treated with PDGF-AA at 50 ng/mL each day for 4 days (Figure S1E). To test the activation of the RhoA pathway with PDGF-AA, the pathway was first inhibited with fasudil 50 μM (Bio-Techne, Minnesota, USA) or C3-exoenzyme 2 μg/mL (Cyto-skeleton, Denver, USA) for 15 h and then activated with PDGF-AA at 50 ng/mL for 20 min and proceed to the G-LISA or IF assay of myosin light chain phosphorylation (p-MLC). To test the functional effect of the PDGF-AA pathway and its inhibition, C3-exoenzyme at 2 μg/mL or fasudil at 50 μM was first added to the culture and then PDGF-AA was added 4 h later at 50 ng/mL. This inhibition/induction cycle was repeated for as many days as the test lasted.

Quantitative proteomics

The protein samples were extracted and analysed using a Lumos Orbitrap mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) coupled to an EASY-nLC 1000 [Thermo Fisher Scientific (Proxeon), Odense, Denmark]. The samples were analysed with the MaxQuant software (version 1.6.1.0) through the human Swissprot database (see supporting information).

Cell viability

The effect of each treatment in FAPs viability was measured with the PrestoBlue reagent (Invitrogen) following manufacturer’s instructions. Fluorescence was measured at wavelengths 570 nm excitation and 600 nm emission using a microplate reader (INFINITE M1000 PRO, Tecan Trading AG, Switzerland).

G-LISA, ROCK activity, Sircol assay, and cytokine array

The RhoA-GTP protein was analysed by a colorimetric assay using the G-LISA assay (Cytoskeleton). The enzymatic activity of ROCK was assessed by a colorimetric method by using a ROCK activity assay (Abcam) in protein extract from quadriceps. Total soluble collagen released by FAPs was measured using Sircol kit (Biocolor, UK). Mouse cytokines were determined using the Proteome Profiler Cytokine Array Panel A (R&D Systems, USA) (see supporting information).

Proliferation and migration assays

The proliferation assay was carried out following the manufacturer’s instructions (Roche, Indianapolis, IN) at 24 and 72 h. The migration assay was performed using 24-well plates with inserts (Ibdi, Munich, Germany) at 48 and 72 h (see supporting information).

Western blot

Cells corresponding to each condition or muscle tissue were lysed in RIPA buffer (Sigma-Aldrich) with protease and phosphatase inhibitors (Roche, Basel, Switzerland), and the specific bands corresponding to the proteins of interest were visualized with the Odyssey infrared detection system (Li-Cor) together with the Image Studio software (Li-Cor). Total protein bands were measured with the Revert 700 kit (Li-Cor) (Figure S1F). The primary and secondary antibodies used in the study are listed in Table 1 (see supporting information).

Histology and immunofluorescence

Cultured FAPs were fixed with ethanol and incubated with blocking solution (Santa Cruz Biotech, Dallas, TX, USA). Frozen muscle sections were obtained with a cryostat (Leica Microsystems, Wetzlar, Germany), fixed with acetone and incubated with blocking solution (Santa Cruz Biotech). The primary and secondary antibodies used in the study are listed in Table 2.

Images were obtained with an Olympus BX51 microscope coupled to an Olympus DP72 camera. ImageJ software was used to quantify the intensity of individual cells and the area of positive staining in muscle tissue. A minimum of five independent fields per staining were quantified.25

Mouse model

Animal procedures were performed according to the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and were approved by the Ethical Committee of the Universitat Autònoma de Barcelona. The animals used in this study were all 7-week-old males. Six dba/2J-mdx males were treated with fasudil (LC Laboratories) that was diluted in water and administered at 100 mg/kg/day orally for 6 weeks by gavage, according to previous studies.26–30 Five untreated dba/2J-mdx males were used as controls and six dba/2J males were used as healthy controls.

Grip strength test

Force was measured using a grip strength meter. Mice were initially held by the tail above the grid. Once mice grasped the grid, they were pulled out until release. The procedure was repeated 5 times, and the mean of the three highest values was used. Mean result was normalized.
to the body weight of the animal and is presented as grip force/weight (N/g).

**Flow cytometry**

Single cell suspension from cell culture, quadriceps, and tibial anterior were obtained for flow cytometry. Cultured human muscle derived cells were labelled using anti-PDGFRα followed by streptavidine-PECy5 and anti-CD56. Cells obtained from mice were labelled using anti-sca1, anti-CD45, anti-F4/80, anti-CD163, and a viability marker (Table 1). Samples were acquired with the MACSQuant Analyzer 10 flow cytometer (MiltenyiBiotec). Doublet cells were excluded using Forward scatter area and height. Compensations were adjusted according to the single stained controls. Total viable FAPS (Viable, Ter119-/CD45/CD31/C0/integrin-α7/C0/sca1+) and macrophages (Viable, CD45 + F4/80+) counts were quantified using MACSQuantify software and normalized according to grams of muscle tissue. FlowJo v10 software was used for data analysis and data elaboration.

**Statistical analysis**

Results are expressed as mean ± standard error of means (SEM). Differences among the groups were analysed using One-way ANOVA Test. When ANOVA revealed significant differences, the Tukey post hoc test was performed. The significance level was set at $P < 0.05$. The statistical analyses were

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| Target          | Host   | Dilution | Application | Manufacturer   |
|-----------------|--------|----------|-------------|----------------|
| ArHGEF2         | Rabbit | 1/100    | WB          | Sigma-Aldrich  |
| RhøA            | Rabbit | 1/100    | WB          | Cell Signalling|
| TE-7            | Mouse  | 1/100    | IF          | Merck          |
| Desmin          | Mouse  | 1/50     | IF          | Novocastra     |
| p-MLC2          | Mouse  | 1/100    | IF          | Cell Signalling|
| PDGFRα          | Goat   | 1/50     | IF          | ProteinTech    |
| LIMK2           | Rabbit | 1/100    | IF          | Merck Millipore|
| PDGFA           | Rabbit | 1/50     | IF          | Cell Signalling|
| MLC2            | Rabbit | 1/50     | IF          | Cell Signalling|
| F4/80           | Rabbit | 1/200    | IF          | Cell Signalling|
| CD3             | Rabbit | 1/50     | IF          | Abcam          |
| Ly6g + Ly6c     | Rat    | 1/50     | IF          | Abcam          |
| CD19            | Rabbit | 1/50     | IF          | Abcam          |
| Wheatgerm agglutinin-488* | N/A   | 1/100    | IF          | Invitrogen     |
| Hoesch 33342*   | N/A    | 1/1000   | IF          | Invitrogen     |
| Rhodamine Phalloidin* | N/A   | 1/1000   | IF          | Abcam          |

| Target          | Host   | Dilution | Application | Manufacturer |
|-----------------|--------|----------|-------------|--------------|
| CD140a-PE       | Recombinant | 1/200  | IF          | Miltenyi Biotec |
| CD56            | Ms     | N/A°     | FC          | Miltenyi Biotec |
| PDGFRα-biot     | Goat   | 1/75     | FC          | R&D System   |
| CD56-PE         | Ms     | 1/20     | FC          | Miltenyi Biotec |
| LIVE/DEAD°      | N/A    | 1/1000   | FC          | Thermo Fisher Scientific |
| F4/80           | Rat    | N/A°     | FC          | Bio Legend   |
| CD45            | Rat    | N/A°     | FC          | eBioscience  |
| Integrin-α7     | Rat    | N/A°     | FC          | Miltenyi Biotec |
| Ter119          | Rat    | N/A°     | FC          | eBioscience  |
| CD31            | Rat    | N/A°     | FC          | Thermo Fisher BD |
| Sca1            | Rat    | N/A°     | FC          | BD           |
| CD163           | Rat    | N/A°     | FC          | eBioscience  |
| CD206           | Rat    | N/A°     | FC          | Bio Legend   |
| CD80            | REA    | N/A°     | FC          | Miltenyi Biotec |
| HLA-DR          | Rat    | N/A°     | FC          | Miltenyi Biotec |

| Secondary antibody | Dilution | Conjugation | Application | Manufacturer |
|--------------------|----------|-------------|-------------|--------------|
| Donkey anti-goat   | 1/500    | Alexa594    | IF          | Invitrogen   |
| anti-mouse IgG     | 1/500    | Alexa594    | IF          | Invitrogen   |
| Donkey anti-rabbit | 1/500    | Alexa647    | IF          | Invitrogen   |
| anti-mouse IgG     | 1/500    | Alexa594    | IF          | Invitrogen   |
| Goat anti-mouse    | 1/500    | Alexa488    | IF          | Invitrogen   |
| Goat anti-rabbit   | 1/500    | Alexa488    | IF          | Invitrogen   |
| Goat anti-rat      | 1/500    | Alexa594    | IF          | Invitrogen   |
| Goat anti-rabbit   | 1/7500   | IRDye 800   | WB          | LICOR        |
| Goat anti-rabbit biotinylated | 1/2000 | N/A | WB | Invitrogen |
| Streptavidin 800   | 1/2000   | IRDye 800   | WB          | LICOR        |
| Streptavidin PE-Cy5| 1/200   | PE-Cy5      | FC          | Biolegend    |

*It is not an antibody.

*Volume per test following manufacturer’s instructions.

FC, flow cytometry; IHQ, immunohistochemistry; MACS, Magnetic activated cell sorting; Ms, mouse; N/A, not applicable.
calculated using GraphPad Prism version 7 for Windows (San Diego, CA: GraphPad Software, Inc).

**Results**

**PDGF-AA induces activation of Rho-A pathway in human muscle FAPs**

We first confirmed that the number of FAPs was increased in DMD muscle biopsies \((n = 2)\) compared with healthy controls \((n = 2)\) (Figure S1A,B). Then, we isolated FAPs from muscle biopsies of three DMD patients and treated them with 50 ng/mL of PDGF-AA for 4 days. To analyse changes in the protein profile, we used quantitative proteomic analysis with mass spectrometry comparing PDGF-AA treated FAPs and non-treated FAPs. We observed that 1890 proteins were differentially expressed (Volcano plot in Figure 1A). We built a proteomap of the up-regulated proteins according to the functional gene classification [Kyoto Encyclopedia Genes and Genomes (KEGG)] (Figure 1B). We observed an increase in the expression of proteins involved in various signalling pathways (MAPK, PI3K-Akt, FoxO, or Hedgehog), cell metabolism, and genetic information processing (Figure 1C,D). Among the different protein groups that were up-regulated, we were interested in those involved in cell functions related to fibrosis such as cytoskeletal rearrangement, cell adhesion, and rearrangement of actin filaments. Proteins related to these cellular functions, such as proteins of the Ras homologue gene pathway (Rho), were increased in FAPs treated with PDGF-AA as displayed in a heatmap showing the differences between DMD-FAPs untreated and treated with PDGF-AA (Figure 1E). Among the different proteins of this

![Figure 1](image-url) Proteome analysis of PDGF-AA treated DMD FAPs. (A) Volcano plot representing the total proteome. Data are presented as the protein abundance changes of PDGF-AA treated FAPs relative to untreated FAPs. Significantly differentially expressed proteins (Student’s t-test, \(P \leq 0.05\)) are highlighted in blue. (B–D) Proteomap of up-regulated proteins after PDGF-AA treatment. The proteomic map visualizes the composition of the proteomes in terms of abundance and function of the proteins. Each protein is represented by a polygon, and the area of each polygon reflects the abundance of the proteins (calculated with fold-change). Functionally related proteins appear in adjacent regions. The three panels represent three hierarchy levels, being the first one the individual protein detected (B), the second one shows proteins grouped into pathways (C), and the last one shows the functional category associated to a group of proteins (D). (E) Up-regulated proteins shown in proteomaps were also represented in heatmap showing the results of Reactome database.
family, we focused on the Ras homologue gene family member A (RhoA) pathway because it has been involved in fibroblast activation and fibrosis in several other pathologies.31

C3 exoenzyme and Fasudil treatment block Rho-kinase pathway activation mediated by PDGF-AA

We validated mass spectrometry results in two different experiments. First, we studied the expression of guanine nucleotide exchange factor Rho 2 (ArhGEF2), which is the effector protein in the Rho-kinase pathway (Figure 2A), in DMD muscle samples and aged and sex matched controls (loading control in Figure S1E). We observed a 7.7-fold increase in the expression of ArhGEF2 in DMD muscles compared with control muscles (n = 2) (Figure 2B). In a second step we analysed if the addition of PDGF-AA to DMD-FAPs in culture induced a significant increase in RhoA bound to guanosine triphosphate (GTP) (RhoA-GTP) compared with untreated FAPs (c−). To further confirm whether the increase in RhoA-GTP was mediated via activation of RhoA pathway, the effect of pretreatment with the exoenzyme C3 (C3) was also analysed. Exoenzyme C3 is an ADP ribosyl transferase that selectively ribosylates RhoA proteins at asparagine residue 41, rendering it inactive. As expected, PDGF-AA treatment induced a significant 1.88-fold increase in the RhoA-GTP levels (n = 3, SEM = 0.206, P < 0.01), while pretreatment with C3 blocked the effect induced by PDGF-AA (n = 3, SEM = 0.07, P < 0.05) (Figure 2C). Treatment with PDGF-AA, C3, or fasudil did not affect cell viability at any time tested (Figure S1H).

Fasudil is a commercially available vasodilator used to treat cerebral vasospasm but also useful for pulmonary hyper-

![Figure 2](https://example.com/figure2.png)

**Figure 2** RhoA pathway is up-regulated in DMD and PDGF-AA activates it. (A) Molecular pathway of RhoA/ROCK2 signalling downstream of ArhGEF2 which catalyses the replacement of Rhoa-GDP to RhoA-GTP, controlling the RhoA activation. RhoA bound to GTP activates the rho-associated coiled coil-forming protein kinase (ROCK), a serine/threonine kinase that regulates actin filament remodelling trough light chain myosin (MLC) phosphorylation. (B) Quantification of WB bands of ArhGEF2 relative to total protein detection and representative bands of ArhGEF2 expression in muscle tissue obtained from healthy controls (n = 2) and DMD patients (n = 2). Total protein detection was used as loading control signal (Figure S1D). (C) Optical density at 490 nm indicating RhoA activity relative to total RhoA protein detected by WB. (D) Representative images of p-MLC staining using fluorescence microscopy. Scale bar: 50 μm. (E) Quantification of p-MLC intensity of each cell after different treatments. (F) Representative images FAPs stained with phalloidin after different treatments. Scale bar: 50 μm. C−: Untreated FAPs. Data are represented as the mean of three replicates ± standard error of the mean. Results were statistically analysed using one-way ANOVA followed by Tukey post hoc test. Statistical significance was set at P < 0.05. **P < 0.01; ***P < 0.001.
tension. Fasudil inhibits RhoA pathway by blocking the Rho-associated protein kinase (ROCK) which phosphorylates myosin light chain 2 (p-MLC2). Treatment of DMD-FAPs with PDGF-AA induced a statistically significant 2.3-fold increase in MLC2 phosphorylation \((n = 3, \text{SEM} = 0.33, P < 0.001)\), which was reversed by pretreatment with C3-exoenzyme \((n = 3, \text{SEM} = 0.11, P < 0.001)\) and fasudil \((n = 3, \text{SEM} = 0.09, P < 0.001)\), as shown in Figure 2D,E. Last step on the Rho-kinase pathway is the polymerization of F-actin filament mediated by MLC2 phosphorylation. Treatment of FAPs with PDGF-AA induced polymerization on intracellular actin filaments, which was impinged by pretreatment with C3 or fasudil (Figure 2F).

**Fasudil blocks PDGF-AA mediated increase in proliferation, migration and expression of collagen-I by human FAPs in vitro**

We studied the effect of PDGF-AA, C3-exoenzyme, and fasudil in proliferation, migration, and collagen-I production *in vitro*. Addition of PDGF-AA to the culture medium significantly increased FAPs proliferation at both 48 \((n = 3, \text{SEM} = 0.02, P < 0.001)\) and 72 h \((n = 3, \text{SEM} = 0.05, P < 0.001)\) (3.6-fold and 6-fold increase, respectively) compared with untreated FAPs. In contrast, treatment of FAPs with C3-exoenzyme \((n = 3, \text{SEM} = 0.01, P < 0.001)\) and fasudil \((n = 3, \text{SEM} = 0.02, P < 0.001)\) prior to stimulation with PDGF-AA lead to a significant decrease in proliferation rate (3.27-fold and 2.6-fold decrease, respectively) compared with FAPs treated with PDGF-AA at 72 h (Figure 3A). All treatment doses and the different times of cell exposure were tested for cell viability. PrestoBlue data showed that neither C3-exoenzyme nor fasudil had any effect on cell viability (Figure S1H).

Treatment with PDGF-AA significantly enhanced FAPs migration *in vitro* compared with untreated FAPs at both 48 h \((n = 3, \text{SEM} = 0.51, P < 0.001)\) and 72 h \((n = 3, \text{SEM} = 0.66, P < 0.001)\) (2.48-fold and a 3.37-fold increase, respectively). This effect was significantly blocked when C3-exoenzyme and fasudil were added to the medium (Figure 3B,C) either at 48 or 72 h. C3-exoenzyme reduced migration rate 2.1-fold.

![Figure 3](https://www.plateletseries.org/images/Platelet-Derived%20Growth%20Factor%20AA%20enhances%20RhoA/ROCK2%20signalling%201379.png)

**Figure 3** Effects of C3, fasudil, and PDGF-AA on FAPs from DMD patients. (A) FAP proliferation was analysed after 48 and 72 h. (B) FAP migration was analysed after 48 and 72 h. (C) Representative images of migration assay at both 48 and 72 h. Scale bar: 200 μm. (D) The amount of collagen delivered to the supernatant was analysed after 4 days treatment. C:- Untreated FAPs. Data are represented as the mean of three replicates ± standard error of the mean. Results were statistically analysed using one-way ANOVA followed by Tukey post hoc test. Statistical significance was set at \(P < 0.05\). \(*P < 0.05; **P < 0.01; ***P < 0.001.\)
at 48 h (n = 3, SEM = 0.47, P < 0.001) and 3-fold at 72 h (n = 3, SEM = 0.25, P < 0.001). Fasudil reduced migration rate 2.43-fold at 48 h (n = 3, SEM = 0.41, P < 0.001) and 4-fold at 72 h (n = 3, SEM = 0.22, P < 0.001).

PDGF-AA treatment also increased non-significantly the release of collagen-I to the culture medium an effect that was reversed by the addition of C3-exoenzyme and fasudil (Figure 3D). C3-exoenzyme significantly reduced the release of collagen-I 1.7-fold (n = 3, SEM = 0.33, P < 0.05).

Fasudil treatment improves muscle function and reduces muscle fibrosis in the dba/2J-mdx murine model of DMD

Based in our in vitro results, we decided to test if fasudil had an antifibrotic effect in vivo in the dba/2J-mdx murine model of DMD. We first confirmed that PDGF-AA levels were increased in muscle samples of dba/2J-mdx mice compared with healthy WT controls (Figure S2A) as happens in DMD patients. Based on these results, we treated six dba/2J-mdx mice of 7 weeks old with fasudil at a dose of 100 mg/kg/day orally for 6 weeks. Mice did not experience any change in their behaviour during the treatment period suggesting that there were not significant adverse effects. However, we observed a decrease in weight when compared treated (n = 6, SEM = 0.94) and untreated mice (n = 5, n = 0.60) (Figure S2B) although these differences did not reach statistical significance. We confirmed that fasudil inhibited RhoA pathway by analysing different downstream effector targets of RhoA, such as ROCK2, LIM domain kinase 2 (LIMK2), and Formin Homology 2 Domain Containing 1 (FHOD1), being the two later proteins involved in the regulation of actin filament dynamics (Figure S3A). We also quantified the enzymatic activity of ROCK, which was decreased in quadriceps in treated mice (n = 6, SEM = 0.12) compared with non-treated mice (n = 5, SEM = 0.14), although these changes were non-significant (Figure S3B). However, the protein expression of LIMK2 and MLC2 was significantly reduced in treated (n = 6, SEM = 0.45 and n = 6, SEM = 0.05, respectively) compared with untreated mice (n = 5, SEM = 0.97 and n = 5, SEM = 0.64, respectively) (Figure S3C–F). We analysed muscle strength using forelimb grip-strength after 6 weeks of treatment and compared the results obtained in non-treated dba/2J-mdx and healthy control mice. We observed a significant 1.76-fold increase in forelimb strength in treated mice (n = 6, SEM = 1.63) compared with non-treated mice (n = 5, SEM = 2.29, P < 0.01) (Figure 4A). Interestingly, differences between treated mice and WT mice did not reach significance. We also analysed the effect of fasudil on quadriceps histology. Fasudil induced a significant 23% reduction in collagen-I area in the quadriceps of treated mice (n = 6, SEM = 1.63, P < 0.05) (Figure 4B,D). We detected a significant increase in the myofibre size of treated mice (n = 6, SEM = 0.60) compared with non-treated (n = 5, SEM = 0.64, P < 0.001) (Figure 4C). Additionally, we observed a 42% decrease in the PDGFRα area as a measure of the number of FAPs present in the quadriceps of treated mice (n = 6, SEM = 0.38) compared with non-treated mice (n = 5, SEM = 0.95), although these differences did not reach significance (Figure 4E,F).

Effect of fasudil treatment in inflammation

Quantification of macrophages was performed by analysing the area of the quadriceps stained with F4/80 maker. Fasudil induced a 74% reduction in treated mice (n = 6, SEM = 0.15) when compared with untreated mice (n = 5, SEM = 0.73) (Figure 4G). Those results were in accordance with flow cytometry analysis which showed a 18.3% decrease in the number of F4/80 positive cells in treated mice (n = 6, SEM = 33.49) compared with non-treated mice (n = 5, SEM = 38.92) (Figure 4H). Indeed, we observed a trend towards a reduction in the expression of profibrotic M2 markers, such as CD163 and CD206, in macrophages obtained from fasudil treated mice. In detail, we observed a 31% reduction in the expression levels of CD163 marker in macrophages obtained from skeletal muscles of treated mice (n = 6, SEM = 1.10) although there was only a 3% lower number of CD163+ cells compared with non-treated mice (n = 5, SEM = 2.58). Similarly, we observed a 18% reduction in the expression levels of CD206 marker in macrophages obtained from treated mice (n = 6, SEM = 1.43) although there was only a 10% lower number of CD206+ cells compared with non-treated mice (n = 5, SEM = 5.91) (Figure 4I). We did not observe any difference either in the expression levels or in the number of CD209 cells which also identify M2 macrophages. We did not observe changes in the percentage of positive cells or in the expression of CD80 and DR, two well-known M1 markers, in the macrophages obtained from skeletal muscles of treated mice compared with non-treated mice (Figure S2C).

We also studied the population of T cells, B cells, and neutrophils and observed a trend towards a reduction in neutrophils population in treated mice (n = 6, SEM = 0.72) compared with non-treated (n = 5, SEM = 0.74), while there were no changes in the number of T and B cells (Figure S4A–D). Finally, we studied if fasudil treatment modified the expression of several cytokines involved in the inflammatory process in muscular dystrophies. As shown in a heatmap in Figure S4E, we observed a reduction in TNFα, IL-4, IL-17, CXCL1, CXCL2, CXCL10, CCL2, CCL3, or CCL17 among others in fasudil treated mice when compared with non-treated mice.

Discussion

In this study, we have investigated the molecular pathways activated by PDGF-AA in skeletal muscles of patients with...
DMD driving to muscle fibrosis. We have observed that PDGF-AA activates RhoA/ROCK2 pathway which can be effectively blocked by fasudil, a well-known Rho-kinase inhibitor that reduces FAPs activation in vitro. We performed a proof-of-concept preclinical study to validate these findings observing a reduction in muscle fibrosis in the murine model.

**Figure 4** Effect of fasudil treatment in the dba/2J-mdx mice. (A) Grip strength test after 6 weeks treatment with fasudil in dba-2J WT mice, untreated dba/2J-mdx, and fasudil treated dba/2J-mdx. Results are relative to weight of animals. (B) Quantification of collagen I expression area in quadriceps. Scale bar: 200 μm. (C) Quantification of muscle fibre size mean and frequency in dba-2J WT mice, untreated dba/2J-mdx, and fasudil treated dba/2J-mdx. (D) Representative immunofluorescence images of collagen I expression in muscle samples. Scale bar: 200 μm. (E) Quantification of PDGFRα+ expression area in quadriceps. (F) Representative immunofluorescence images of PDGFRα+ (yellow arrows), F4/80 (white arrows) expression in muscle samples. ECM is stained with wheat germ agglutinin (WGA) in green. Scale bar: 200 μm. (G) Quantification of macrophages expression area in quadriceps. (H) Representative immunofluorescence images of collagen I expression in muscle samples. Scale bar: 200 μm. (I) Quantification of results obtained with flow cytometry to analyse the F4/80 macrophages (Mo) accumulated in quadriceps and tibial anterior obtained from mice. Data are represented as counts of F4/80 relative to the weight of quadriceps and tibial anterior. (J) Percentage of total macrophages and mean fluorescence intensity (MFI) of CD163 and CD206 markers were analysed by flow cytometry. Data are represented as the mean of the groups analysed ± standard error of the mean. Results were statistically analysed using one-way ANOVA followed by Tukey post hoc test. Statistical significance was set at \( P < 0.05 \). \* \( P < 0.05 \); \** \( P < 0.01 \); *** \( P < 0.001 \).
of DMD. Our results expand the molecular pathways involved in the process of muscle fibrosis in DMD and support further investigations in the efficacy of Rho-kinase inhibitors as a treatment of DMD in particular and muscular dystrophies in general.

Absence of dystrophin in DMD produces sarcolemma instability leading to expansion of fibrotic and fat tissue. Several growth factors have been related to the fibrosis expansion; however, the role of PDGF-AA has not been fully studied. Although previous studies found that the signalling through PDGFRα is persistent in DMD leading to fibrosis and hindering repair, the molecular consequences of persistent exposure of FAPs to PDGF-AA have not been addressed. To study the molecular changes that occurs in DMD, we treated FAPs from DMD with PDGF-AA and observed an up-regulation of several proteins belonging to the RhoA/ROCK2 pathway. The RhoA/ROCK2 signalling pathway is well known for regulating actin cytoskeleton organization and cellular dynamics in several cell types. RhoA acts through two molecular conformations: it is inactive when bound to a guanosine diphosphate (GDP) and active when bound to GTP. ROCK2, the Rho downstream effector molecule, is a serine/threonine kinase protein that regulates actin filament remodelling by phosphorylating numerous downstream target proteins, including the myosin binding subunit of myosin light chain 2 (MLC2) phosphatase. Actin reorganization triggered by RhoA/ROCK2 signalling pathway drive fibroblasts activation in different pathologic conditions such as cardiac fibrogenesis, tumour-cell growth or pulmonary artery hypertension. Actin polymerization regulates cell polarization, organization of adhesion structures, and the generation of the force essential for cell migration. These events allow cells to migrate into the injured site where they proliferate and release components of the ECM to regenerate the damaged tissue. However, an excessive activation of fibroblasts increases the ECM deposition leading to accumulated fibrogenesis. Although the rearrangement of actin produced in fibroblast activation has not been characterized in FAPs, previous studies suggest that fibroblasts and FAPs are phenotypically and biochemically equivalent supporting that Rho-kinase activation could have a similar effect in both cells. We demonstrated that RhoA/ROCK2 pathway is increased in muscles of DMD patients when compared with age and sex matched healthy controls subjects. Therefore, we decided to analyse the effect of PDGF-AA on RhoA/ROCK2 signalling pathway in FAPs isolated from DMD patients. First, we confirmed that RhoA/ROCK2 pathway can be modulated by PDGF-AA because C3-exoenzyme and fasudil significantly reduced the signalling pathway activation. Our results show that PDGF-AA activated RhoA/ROCK2 pathway resulting in an increase of actin filament polymerization, proliferation, and migration and that both C3 and fasudil attenuated these effects. Although PDGF-AA induced a higher proliferation rate at 48 and 72 h, the blocking effect of RhoA/ROCK2 inhibitors was only observed at 72 h. The lack of proliferation inhibition at 48 h after C3-exoenzyme or fasudil treatment could be due to other signalling pathways activated after PDGF-AA treatment. PDGF-AA is a mitogen growth factor that not only activates RhoA pathway but also triggers other signalling pathways, such as MAPK, PLC, or PI3K. While these molecular pathways are mainly involved in cell proliferation, RhoA/ROCK2 is involved in actin remodelling.

Because the Rho/ROCK-mediated pathway interacts with other signalling pathways known to contribute to fibrosis, we tested whether fasudil reduces fibrosis in the dba/2J-mdx mice. Fasudil is a selective inhibitor of ROCK1 and ROCK2 isoforms that has been used in clinic as a first-generation selective Rho/ROCK inhibitor. Fasudil has been tested in different diseases such as pulmonary hypertension, amyotrophic lateral sclerosis, or cardiovascular disease. We observed that dba/2J-mdx mice treated with fasudil had better performance on grip strength test than non-treated mice. These results were associated to a decrease in the amount of collagen-I and to a tendency towards a decreased number of FAP cells in the skeletal muscles of the treated animals compared with the non-treated ones. These results are in accordance with previous studies using fasudil as an antifibrotic agent as it has been shown that fasudil reduces collagen area, number of infiltrating macrophages and production of TGF-β1 and CTGF in renal interstitial fibrosis induced by unilateral ureteral obstruction and in bleomycin-induced pulmonary fibrosis in mice.

Because the inflammatory response is another characteristic feature of DMD muscles, we also analysed inflammatory cells profile after treatment. Fasudil induced a trend towards a reduction in the number of neutrophils and macrophages, particularly in the number of CD163 and CD206 positive macrophages when blocking this pathway with fasudil. The changes that we have observed in the number of infiltrating inflammatory cells and in the reduction of the cytokines levels could be explained either by a direct effect of fasudil on inflammatory cells or by an indirect effect of the reduced fibrosis content of the muscle. It is well known that continuous muscle damage perpetuates the inflammatory response in muscular dystrophies, and therefore, treatments improving the general architecture of the muscles reducing muscle degeneration can have an indirect effect reducing inflammation. For example, the attenuation of the fibrotic process in the muscle may improve muscle regeneration in treated animals reducing the number of degenerating fibres and preventing macrophages and neutrophils to infiltrate the injured muscle. Indeed, the increased fibre size observed in treated mice could be due to a better re-

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DOI: 10.1002/jcsm.12923
generation of the muscle together with a less affected muscle architecture. In summary, treatment with fasudil on dba-2J mdx mice improved grip-strength test, reduced expression of collagen-I and resulted in a trend towards a decreased number of PDGFRα+ cells on skeletal muscles, confirming our results in vitro. Our results suggest that these effects are mainly explained by the effect that RhoA/ROCK2 inhibition has on fibrosis deposition.

Although ROCK is expressed in different cell types, side effects produced by fasudil does not include major safety concerns. Fasudil targets the ATP-dependent kinase domain of either ROCK1 and ROCK2 with equal potency and without selective effects. It is worth noting that ROCK2 is the isoform detected in our proteomic analysis, and previous studies have shown that ROCK2 is the predominant isoform in skeletal muscle.

Muscle fibrosis is an irreversible process that takes place in muscular dystrophies and should be prevented before the accumulation of ECM disturbs muscle architecture and function. Because FAP activation and ECM remodelling start early during skeletal muscle degeneration, inhibiting that the initial cellular changes that occur on FAPs could prevent the increase of ECM deposition. Therapies focused on targeting actin rearrangement to prevent FAP migration into the injury site may help to slow down the progression of fibrosis. Coupling anti-fibrotic therapies with cell therapy or gene therapy could also lead to a better outcome of these experimental therapies. Then, by inhibiting the ROCK2 signalling in FAPs, we are targeting the first stages of fibrosis which could be further studied as a potential treatment to reduced muscle fibrosis in DMD.

In summary, this study demonstrates that PDGF-AA induces RhoA/ROCK2 pathway signalling in DMD-FAPs leading to their proliferation, migration, and actin reorganization. Treatment with fasudil, a well-known ROCK inhibitor, blocks the effect of PDGF-AA on cells in vitro and reduces muscle fibrosis increasing muscle strength in a DMD murine model. Our results open the door to new studies further characterizing the efficacy of more specific ROCK2 inhibitors as potential new treatments for DMD.

**Acknowledgements**

This study has been funded by Instituto de Salud Carlos III-FEDER through the project ‘PI18/01525′ (Co-funded by European Union ERDF), by AFM (France) through the project 22525, by Fundación Isabel Gemio, by Academy of Medical Sciences Professorship Scheme to JDM (APRA/1007) and by a MRC grant to JDM (MR/W019086/1).

**Conflict of interest**

Esther Fernández-Simón, Xavier Suárez-Calvet, Ana Carrasco-Rozas, Patricia Piñol-Jurado, Susana López-Fernández, Joan Josep Bech Serra, Carolina de la Torre, Noemí de Luna, Eduard Gallardo, and Jordi-Díaz-Manera declare that they have no competing interests.

**Ethical guidelines statement**

The study was approved by The Ethics Committee of Hospital de la Santa Creu i Sant Pau (HSCSP) in Barcelona. Patient and controls signed an informed consent to use their muscle biopsy for research purposes. Animal procedures were performed according to the ethical standards laid down in the 1964 Declaration of Helsinki, National Institutes of Health Guidelines for the Care, and Use of Laboratory Animals and were approved by the Ethical Committee of the Universitat Autònoma de Barcelona. The authors of this manuscript certify that they comply with the ethical guidelines for authorship and publishing in the *Journal of Cachexia, Sarcopenia and Muscle*.4

**Online supplementary material**

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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